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Alternative lengthening of telomeres in hTERT-inhibited laryngeal cancer cells

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In most human malignancies, telomere homeostasis is maintained by the reactivation of telomerase. While inhibiting telomerase provides a novel approach to the treatment of many cancers, telomere maintenance can occur in the absence of telomerase activity by the alternative lengthening of telomeres (ALT) mechanism. Therefore, it must be determined if inhibiting telomerase selects for cancer cells that activate ALT. Here, we report that Hep-2 cells that survived anti-telomerase treatments showed sustained proliferation in culture with down-regulated human telomerase reverse transcriptase (hTERT) expression and significantly enhanced levels of ALT-specific promyelocytic leukemia (PML) bodies. Analysis of the telomere lengthening kinetics also demonstrated elevated telomeric sister-chromatid exchange (T-SCE) in surviving Hep-2 cells, consistent with their long and heterogeneous telomeres. Similar to ALT cells, the surviving cells showed evidence of ALT telomere homeostasis. Furthermore, proteomic analysis identified several proteins differentially expressed between the untreated Hep-2 cells and surviving cells that may provide new insight for understanding these two telomere maintenance mechanisms. Thus, the findings in this study may help to improve telomerase-based therapy for cancer. (*Cancer Sci* 2010; 101: 1769–1776)

Telomere length is associated with the proliferative capacity of cells. Whereas normal cells with reduced proliferative capacity exhibit low telomerase activity and shortened telomeres, cancer cells maintain telomere length primarily by reactivation of telomerase.[1] The telomerase enzyme which adds specific DNA sequences to the ends of chromosomes is composed of three elements: human telomerase reverse transcriptase (hTERT), telomerase RNA, and dyskerin.[2] While the majority of tumors show high telomerase activity, some cancer cells may utilize the alternative lengthening of telomeres (ALT) mechanism.[3,4] The ALT-mediated elongation of telomeres has been associated with homologous recombination events between sister chromatids at telomeres, known as telomeric sister-chromatid exchange (T-SCE).[5] Alternative lengthening of telomeres (ALT) cells are usually characterized by a remarkable heterogeneous telomere length within a given cell and the presence of promyelocytic leukemia (PML) nuclear bodies that contain telomeric DNA and telomere-binding proteins.[6,7] While these ALT-associated PML bodies (APBs) may be used to identify tumors which use the ALT pathway, their function is still unknown.

It has been hypothesized that cancer cells may activate the ALT pathway when telomerase is inhibited or otherwise rendered non-functional.[8] For example, when cells with active ALT were fused with normal somatic cells or telomerase-positive cancer cells, the ALT activity was suppressed, suggesting that it is normally repressed.[9] Another study demonstrated that human fibroblasts with spontaneous inactivation of telomerase had sustained proliferation *in vitro*, although these cells did not express APBs or telomere length patterns typical of ALT.[10] When treated with the demethylating agent 5-aza-20-deoxycytidine, these cells had reactivated telomerase. Still, other studies suggest that ALT and telomerase can occur together in human cancer cells.[9] Therefore, the environmental conditions which trigger or maintain the ALT pathway are yet to be precisely defined.

In a previous study, we reported that some laryngeal cancer cells could still replicate after RNAi-hTERT-based anti-telomerase treatment,[11] but whether the survival of these cells depended on the ALT pathway upon inhibition of telomerase was not clear. In the present study, we collected cells surviving RNAi-hTERT treatment and analyzed their hTERT levels, telomerase activity, and APB expression in order to explore the telomere maintenance mechanisms (TMM) in laryngeal squamous carcinoma (Hep-2) cells when telomerase is inhibited. We also evaluated differences in invasive ability, tumorigenicity, telomere length, and T-SCE between Hep-2 cells and the surviving cells. We further performed a proteomic survey of proteins differentially expressed between the two cell lines by two-dimensional gel electrophoresis (2-DE) and electrospray ionization tandem mass spectrometry (ESI-MS/MS) and validated the differentially expressed proteins by immunoblot analysis. These ALT-specific proteins may be candidates for further studies as diagnostic, prognostic, or therapeutic tools for cancer.

Materials and Methods

Cell lines and culture conditions. The human laryngeal cancer cell line Hep-2 was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and grown in RPMI-1640 media supplemented with 10% fetal calf serum (FCS; Hyclone, Logan, UT, USA) and 100 units/mL penicillin/streptomycin and maintained at 37°C in 5% carbon dioxide.

RNAi plasmid construction and transfection. A short hairpin RNA (shRNA) sequence was designed consisting of a 19-nucleotide target sequence of hTERT (GenBank AB085628), and the corresponding cDNA was subcloned into the pEGFP-C1 vector.[12] Plasmids were transfected into Hep-2 cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), and the cells were harvested 48 h after transfection.

Flow cytometry analysis and cell sorting. Hep-2 cells transfected with the GFP-expressing plasmids were harvested by trypsinization, resulting in a single cell suspension, and analyzed on a FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). The sorted GFP-positive cells were subcultivated for studies to evaluate the cell viability and to determine their mechanism of telomere elongation.

Evaluation of cell viability. The viability of surviving cells and untreated Hep-2 cells were measured by a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium (MTT) assay. Briefly,

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cells were plated at a density of 2×10^3/well in 96-well plates and incubated for 0, 3, 6, 9, 12, 15, and 18 days in complete culture medium containing 0.5 mg/mL MTT (Sigma, St. Louis, MO, USA). Four hours later, the medium was replaced with 100 μL dimethylsulfoxide (DMSO) (Sigma) and vortexed for 10 min to dissolve the crystals. Absorbance of each well was determined by enzyme-linked ELISA at 490 nm of wavelength with subtraction of the baseline reading. Each time point was repeated three times, and the mean and SE were calculated.

Measurements of telomerase activity. To determine telomerase activity, we used the telomeric repeat amplification protocol in the Trapeze Telomerase Detection kit (Millipore, Billerica, MA, USA). Cell extracts heated to 80°C for 10 min were used as a negative control, and cell extracts from telomerase-active 293 cells were used as a positive control. These experiments were performed in triplicate.

Western blotting. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was performed using standard techniques. For evaluating the effect of hTERT knockdown by RNAi, the anti-hTERT polyclonal antibody (H-231; Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used to detect the protein in Hep-2 cells or surviving cells. A polyclonal anti-actin antibody (Santa Cruz Biotechnology) was used as an internal loading control.

For confirming the identity of proteins differentially expressed between Hep-2 cells and surviving cells in 2-DE analysis, cell nuclear proteins were extracted (Nuclear Protein Extraction kit; Takara, Otsu, Shiga, Japan) and analyzed by SDS-PAGE. The primary antibodies against the following proteins were used: telomerase reverse transcriptase (TERT), protein polybromo-1 (PB1), growth arrest-specific protein 8 (GAS8), nibrin (NBS1), PML, and structural maintenance of chromosomes protein 1β (SMC1β) (Santa Cruz Biotechnology); synaptonemal complex central element protein 1 (SYCE1), sarcoma antigen 1 (SAGE1), and NEDD4-binding protein 2 (N4BP2) (Millipore); and β-actin (Invitrogen, Carlsbad, CA, USA).

Immunofluorescence. To determine if the surviving cells had changed their telomere maintenance mechanisms to the ALT pathway, we investigated whether they had elevated levels of ALT-specific PML bodies (a protein marker which characterizes cells in which the ALT mechanism is active) compared with the untreated cell line. Accordingly, we stained the cells for immunofluorescent detection using the N-19 anti-PML body substrate (1:200 dilution; Santa Cruz Biotechnology) and the Cy3-conjugated antirabbit secondary antibody (1:300 dilution; Sigma).

Transwell migration assays. Transwells (8.0-μm membrane pores; Costar, Cambridge, MA, USA) were coated with 2.5 μg/mL collagen overnight at 4°C. Hep-2 cells and surviving Hep-2 cells (2×10^5) in 200 μL medium with 5% FCS were added to the top chamber. The bottom chamber was supplemented with platelet-derived growth factor (30 ng/mL) in medium containing 10% FCS. Cells were allowed to migrate for 24 h at 37°C. After the incubation period, non-migrating cells were removed with cotton-tipped applicators from the upper side of the Transwells. The Transwell filter membranes were then fixed in 4% paraformaldehyde and stained with Giemsa stain (Amresco, Solon, OH, USA). The migrated cells were quantified by counting the number of cells in eight random microscopic fields per filter at a magnification of ×200. Statistical significance was determined by Student's two-tailed *t*-test. The limit of statistical significance was $P < 0.05$.

***In vivo* tumor formation.** Athymic nude mice (6–8 weeks old, 18–22 g weight, Institute of Zoology, Chinese Academy of Sciences) were maintained and used in accordance with the United Kingdom Coordinating Committee on Cancer Research Guidelines.[13] To induce tumors *in vivo*, 5×10^6 surviving Hep-2 cells or Hep-2 cells were washed in PBS, resuspended in 200 μL, and injected into each flank. There were no signs of cell death, and the cells were growing at the same proliferation rate at the time of injection. Mice tumor xenografts were resected 42 days post inoculation. The tumor volumes were calculated from the following formula: tumor volume = (width^2 × length)/2.

Flow cytometry fluorescent *in situ* hybridization (flow-FISH). For analyses of telomere lengths, nuclei were isolated by resuspending 3×10^5 cells in 2% Triton X-100/0.1 M citric acid buffer, and vortexing, incubating for 10 min at room temperature (RT), and vortexing again. After a single washing step with PBS, samples were directly subjected to denaturation/hybridization. The hybridization mixture contained 75% formamide, 1% BSA, 20 mM Tris HCl pH 7.l, 20 mM NaCl, and 50 nM FITC-conjugated $(CCCTAA)_3$ peptide nucleic acid (PNA) probe (Dako, Carpinteria, CA, USA). After 10 min' denaturation on a thermo block at 80°C, the samples were allowed to hybridize at RT overnight. Washing steps were performed in PBS by incubation on a heat block at 40°C for 10 min and then 5 min' centrifugation at 700*g* for cells. Samples were resuspended in 200 mL DNA staining solution containing 50 ng/mL propidium iodide (PI), 0.1% BSA, and 10 mg/mL RNaseA (DNAse free) in PBS. For DNA counterstaining, cells were resuspended in 500 μL staining solution (PBS, 10 U/mL RNase A, 0.1% BSA, 0.1 μg/mL PI) for 2 h before acquisition on a FACScan (Becton Dickinson, Franklin Lakes, NJ, USA) flow cytometer. Mean telomere fluorescence of cells was analyzed with CellQuest software (Becton Dickinson).

Chromosome orientation fluorescence *in situ* hybridization (CO-FISH). For measuring telomere length kinetics, chromosome orientation analysis (CO-FISH) was performed following the basic protocol established by Bailey *et al.*[14] with several modifications. Cells were subcultured in the presence of 5′-bromo-2′-deoxyuridine (BrdU; Sigma) at a final concentration of 1×10^{-5} M, and then allowed to divide once at 37°C overnight. Subsequently, cells were arrested in metaphase by colcemid (0.1 μg/mL), treated with 0.075 M KCl for 15 min at 37°C, and fixed and stored in methanol-acetic acid fixative (3:1). Cell suspensions were dropped onto slides and dried overnight at RT. After fixation in 4% formaldehyde in PBS for 5 min, the slides were washed in PBS and treated with pepsin at 1 mg/mL for 15 min at 37°C at pH 2.0. After a brief rinse in PBS, the slides were fixed again with formaldehyde and repeatedly washed. To denature the DNA, the slides were heated for 3 min at 80°C in 70% formamide/2 × SSC, then rapidly cooled to −20°C in 70% ethanol, dehydrated with ethanol, and air-dried. A hybridization mixture containing 70% formamide, 0.3 μg/mL FITC-$(CCCTAA)_3$ peptide nucleic acid (PNA) probe (Dako), and 1% (w/v) blocking reagent (Boehringer-Ingelheim, Ingelheim, Germany) in 10 mM Tris pH 7.2 was added to the slide. After hybridization for 24 h at 25°C, the slides were washed at RT with 70% formamide/10 mM Tris pH 7.2 (2 × 15 min) and with Tris-buffered saline (TBS: 150 mM NaCl, 50 mM Tris pH 7.5) containing 0.05% Tween-20 (3 × 5 min). After counterstaining with PI (0.1 μg/mL), the slides were mounted with anti-fade solution (Dabco; Sigma). Images were captured on an Olympus BH-2 microscope with a CoolSNAP cooled CCD camera using RSImage software (Roper Scientific, Tucson, AZ, USA).

Sample preparation for 2-DE. Briefly, monolayer cultures of the two cell lines were harvested and washed with cold PBS. Nuclear proteins were then extracted in the extraction buffer supplied with the Nuclear Protein Extraction kit (Takara). The concentrated nuclear proteins was deposited in precipitation solution (50% acetone/50% ethanol/0.1% acetic acid, sample volume:precipitation solution volume = 1:5) for at least 12 h at −20°C. The pellets were washed with 100% acetone and 70% ethanol, then re-dissolved in 6 mM guanidine-HCl/100 mM Tris pH 8.3. The protein concentrations were measured by the Bio-Rad protein assay kit.

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Two-dimensional gel electrophoresis (2-DE) gel analysis of differentially expressed proteins. The protein composition was analyzed by 2-DE using an IPGphor Instrument. For the first dimensional isoelectric focusing (IEF), 300 μg protein (in 250 uL of 7 M urea, 2% CHAPS, and 2.5 μL IPG buffer) was loaded on a 13-cm IPG strip (pH range 3–10) using the following focusing conditions: 30 V for 12 h, 200 V for 1 h, 500 V for 2 h, then from 100 to 8000 V for 1 h, and 500 V for 3 h. Electrophoretic separation (second dimension) was performed in a 16-cm 12% acrylamide gel. Coomassie Brilliant Blue G-250 was used for protein staining.

Gel scanning and image analysis. The stained 2-DE gels were scanned with MagicScan software on Imagescanner (maximum resolution: 9600 × 9600 dpi) (Amersham Biosciences, Uppsala, Sweden), and analyzed using the PDQuest system (Bio-Rad Laboratories, Hercules, CA, USA). Spot intensities were quantified by calculation of spot volume after normalization of the image using the total spot volume normalization method multiplied by the total area of all the spots. The change index was defined as the ratio between spot volumes in surviving Hep-2 cells to untreated Hep-2 cells. Proteins were considered differentially expressed between the two cell lines when the spot intensity varied between the two cell lines. Significant differences in protein expression levels were determined by Student's *t*-test with a set value of $P < 0.05$.

ESI-MS/MS analysis. Protein spots were excised from the gel and destained with 100 mM ammonium bicarbonate/acetonitrile (1:1). Then protein spots were alkylated by the standard protocol in which 50 μL of 10 mM DTT in 50 mM ammonium bicarbonate was first added and incubated for 1 h at 65°C. Subsequently, 50 μL of 55 mM iodoacetamide in 50 mM ammonium bicarbonate was added, and the fractions were incubated in the dark at RT for 45 min. All excised and dried gel spots were digested by adding 10 μL trypsin digest solution to each gel piece and incubated overnight at 37°C. The digested peptides were extracted by 40 μL 60% ACN/5% formic acid. Tryptic peptides were concentrated by vacuum centrifugation before mass spectrometric analysis. MS-MS analyses were conducted on a Q-tof tandem mass spectrometer (Applied Biosystems, Carlsbad, CA, USA). Positive ion mode ESI-MS was used for the analysis, and the optimized parameters of the TurboIonSpray were voltage (IS) 2200 V and declustering potential 60 V. The mass range chosen was from 400 to 1600 m/z. The ion source gas I (GSI), gas II (GSII), curtain gas (CUR), and the temperatures of GSII were set at 40, 5, 30, and 175°C, respectively.

Results

Celluar characterization and telomerase activity in surviving Hep-2 cells with hTERT reduced by RNAi. Following transfection of Hep-2 cells with an shRNA vector specifically targeting mRNA transcripts of the telomerase catalytic subunit hTERT, we sorted the cells based on expression of the GFP marker also expressed by the vector. The percentage of GFP-positive cells in culture was determined by flow cytometry, as seen in Figure 1(a). By analyzing the growth curve after sorting, the proliferation rates of the surviving cells were significantly lower than that of control Hep-2 cells (Fig. 1b, $P < 0.05$). Control Hep-2 cells showed normal proliferation and growth, while the RNAi-hTERT treated cells grew slowly initially, accompanied by a large number of cell senescence and apoptosis. After 15 days, the replication of the hTERT knockdown cells, although still reduced compared with the control cells, began to stabilize with each passage and became the surviving cell line with sustained growth.

Western blot analysis on the surviving cell population, which doubled up to 50 times, showed that the hTERT protein expression was significantly reduced (χ^2-test: $P < 0.01$; Fig. 2). To analyze the telomerase status in these surviving cells, we performed telomerase activity (TRAP) assays at cell doublings of 10, 30, and 50 times in culture. The telomerase activity was significantly inhibited in the surviving cells compared to the untreated cell line (Fig. 3a). Even after 50 doublings of the cell population, the surviving cells continued to show telomerase inhibition, suggesting that no detectable increase in telomerase activity occurred during telomere shortening.

Promyelocytic leukemia (PML) bodies are characteristic of cells utilizing an ALT mechanism. Significantly enhanced levels of ALT-specific PML bodies were found by immunofluorescence staining of the surviving Hep-2 cells (Fig. 3b), suggesting activation of the ALT mechanism of telomere stabilization. Combined with our telomerase activity analyses, these results suggest that the surviving Hep-2 cells lacked telomerase but remained capable of proliferation via ALT as one of the mechanisms of telomere stabilization.

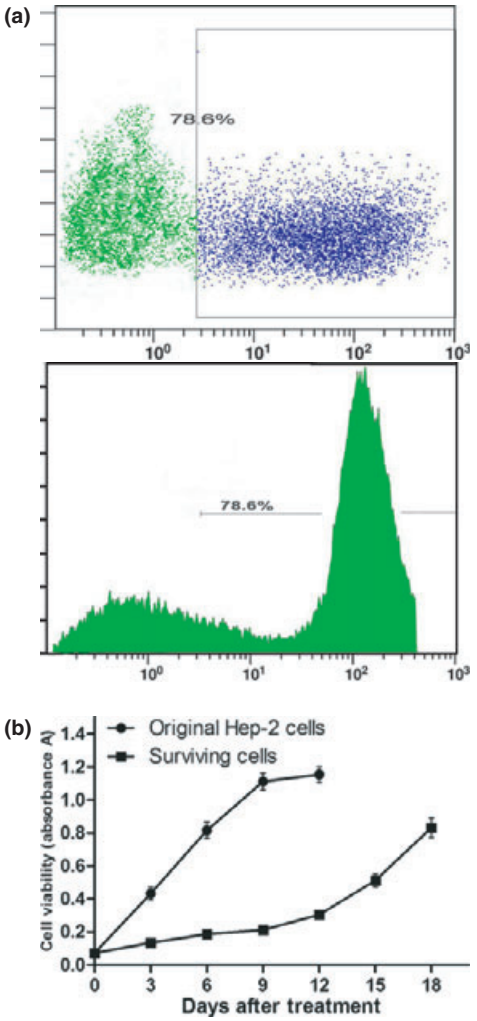

Fig. 1. (a) Detection of cell transfection efficiency by flow cytometry. The percentage of GFP-positive cells in culture was 78.6% as shown. (b) Growth curves of untreated Hep-2 cells and surviving cells were analyzed by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium (MTT) assay. Data are represented as mean ± SD. After 9 days of culture, the untreated Hep-2 cells overgrew in 96-well plates and were no longer proliferating because of overcrowding. Meanwhile, the growth rate of the surviving cells was significantly lower than that of untreated cells within the first 12 days ($P < 0.01$). At around the 15th day, cell proliferation accelerated. Although the proliferation capacity was still low compared with that of control cells, the cells could grow steadily at this time.

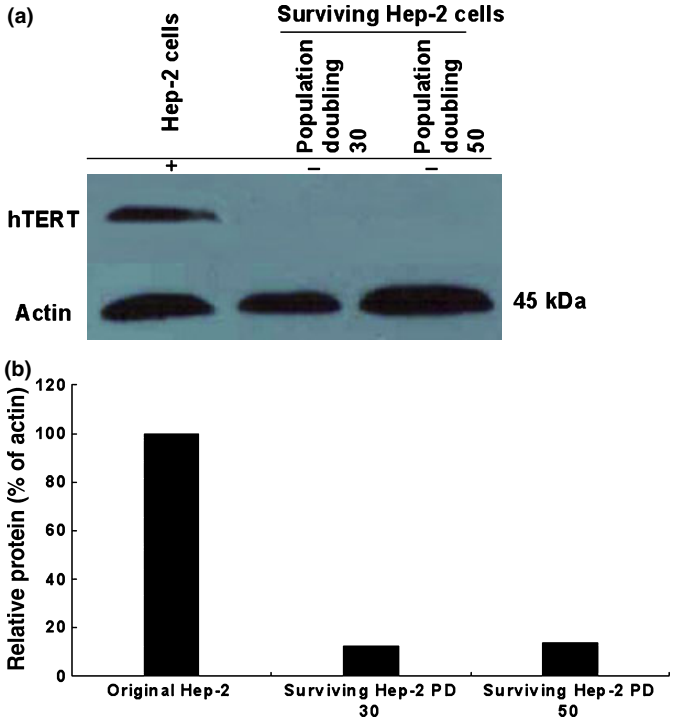

Fig. 2. Expression of human telomerase reverse transcriptase (hTERT) in cell lines. (+) Indicates cells with telomerase expression and (–) indicates cells without telomerase expression. No hTERT protein expression was detected in surviving Hep-2 cells. (a) Western blot detecting hTERT protein. (b) Ratio of hTERT to β-actin in Hep-2 cells and surviving Hep-2 cells. Human telomerase reverse transcriptase (hTERT) was significantly reduced in surviving cells (χ^2-test, $P < 0.01$).

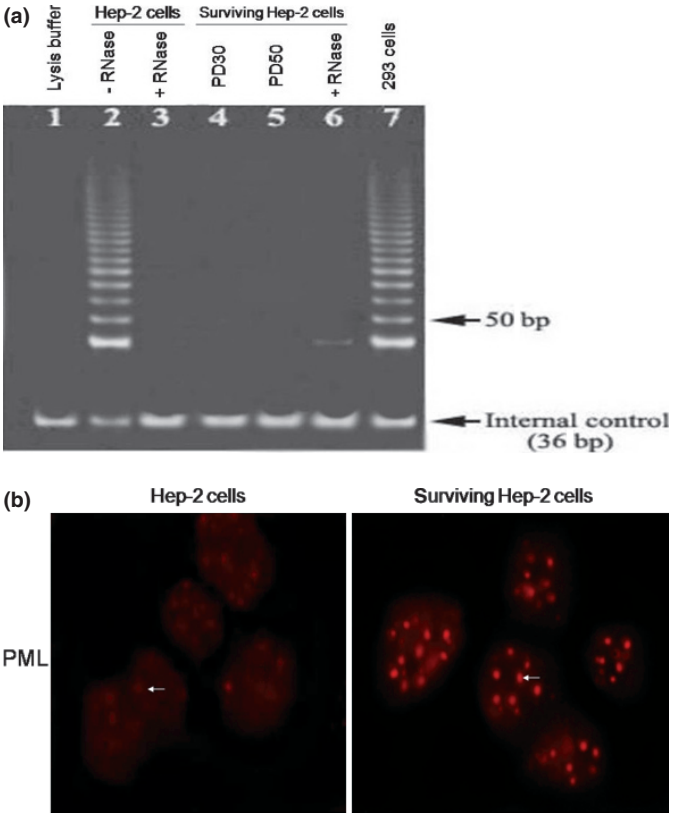

Fig. 3. (a) Telomerase activity in the untreated Hep-2 and surviving Hep-2 cells. Telomerase activity is indicated by a 6-bp ladder on a polyacrylamide gel. The lysis buffer containing no cell extract and RNase (+) samples served as negative controls. Telomerase-positive 293 cells (lane 7) were a positive control. (b) Immunofluorescence staining of promyelocytic leukemia (PML) bodies. Elevated levels of alternative lengthening of telomeres (ALT)-specific PML bodies in the surviving Hep-2 cells are seen (arrows) compared to the untreated Hep-2 cell line.

Changes in telomere length and dynamics in surviving Hep-2 cells. In the flow-FISH assay, labeling by the PNA probe-specific signal corresponded to the relative telomere length. Using this technique, we found that the mean telomere-specific fluorescence in the untreated Hep-2 cells (in the G0/G1 phase cells, Fig. 4a) was significantly less than in the surviving cells ($P < 0.05$, Fig. 4b), indicating that the surviving cells have long telomere lengths compared with the average telomere length in the untreated Hep-2 cell line. Since ALT cell lines generally show great telomere length heterogeneity and significantly longer telomeres than non-ALT cell lines, the presence of unusually long telomeres in surviving Hep-2 cells suggest that these cells were likely to utilize ALT mechanisms.

To examine the telomere dynamics in surviving Hep-2 cells, we performed CO-FISH experiments. Alternative lengthening of telomeres (ALT) cells feature a high rate of post-replicative exchange between telomeres of sister-chromatids (T-SCE).[15] Therefore, we tested whether surviving Hep-2 cells had an activated T-SCE pathway. As shown in Figure 5(a) (arrows) and summarized in Figure 5(b), T-SCE was greatly increased in surviving cells over that in untreated Hep-2 cells ($P < 0.05$). These observations highly suggest that the surviving Hep-2 cells proliferated by the utilizing the ALT pathway.

Proteomic identification of proteins differentially expressed between untreated and surviving Hep-2 cells. To identify the cell nuclear proteins associated with ALT in surviving Hep-2 cells, we compared proteomic profiles of the untreated and surviving Hep-2 cell lines, and two representative 2-DE maps are shown in Figure 6(a). For a reliable analysis of protein expression, each cell line was run in triplicate 2-DE gels. The attained 2-DE patterns of each cell line were highly reproducible and well resolved, with each map displaying about 100 protein spots. Close-up images of certain regions of the gels showing differentially expressed proteins between Hep-2 cells and surviving Hep-2 cells are shown in Figure 6(b). Image analysis revealed that four spots (1, 2, 3, 4) were down-regulated ($P < 0.05$), and seven spots (5, 6, 7, 8, 9, 10, 11) were up-regulated ($P < 0.05$) in the surviving Hep-2 cells.

The 11 differentially expressed protein spots, marked with the numbers and arrows in Figure 6(a), were excised from the Coomassie Brilliant Blue-stained gels, *in situ* digested with trypsin, and analyzed by ESI-Q-TOF-MS. High-quality peptide mass fingerprints and peptide sequence tags were obtained, and all of the nine differentially expressed proteins were identified. The annotations of all the identified proteins are summarized in Table 1. In this study, hTERT protein with different pIs were found in spot 2, and PML was found in spot 6, which could possibly be due to post-translational modifications such as phosphorylation. Based on information on protein functions from the NCBI databases, the nine identified proteins associated with TMM were categorized into two protein groups related to telomerase function (TERT and PB1) and the ALT pathway (SYCE1, PML, GAS8, N4BP2, SMC1β, NBS1, SAGE1).

The presence of seven differentially expressed proteins from normal and surviving Hep-2 cells were verified by western blot analysis. As shown in Figure 7, in surviving Hep-2 cells, two proteins were visualized (PML and NBS1). The presence of SAGE1, GAS8, and SYCE1 in surviving Hep-2 cells was also

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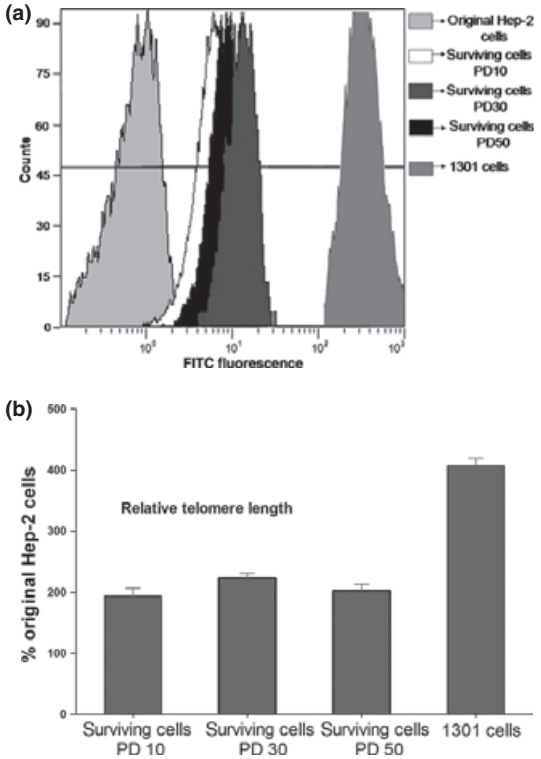

Fig. 4. Telomere length assessment by flow-FISH. (a) Relative telomere length of Hep-2 cells and surviving Hep-2 cells at after 10, 30, and 50 population doublings (PD10, PD30, and PD50, respectively) were determined after hybridization with a PNA probe and monitoring by flow cytometry. A total of 1301 cells with known long telomeres were also analyzed as a positive control. (b) The telomere lengths were quantified and graphed for the indicated cell populations as a percentage of that found in the untreated Hep-2 cells. Error bars represent standard deviations of triplicate samples. The telomere length was significantly increased in surviving Hep-2 cells *versus* untreated Hep-2 cells.

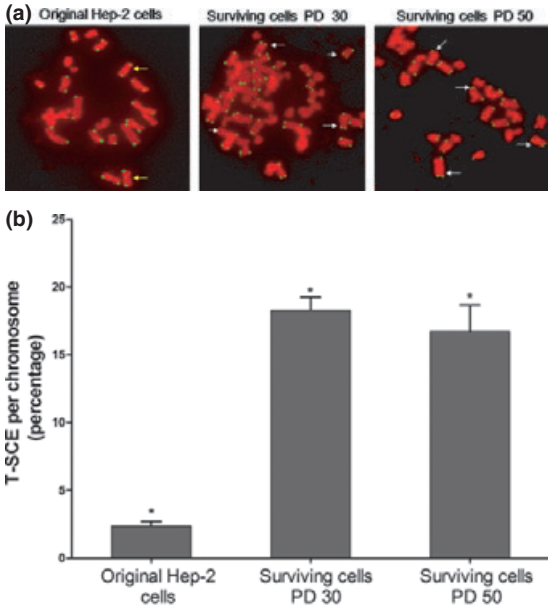

Fig. 5. Analysis of telomere lengths by chromosome orientation (CO)-FISH. Metaphase spreads were prepared and telomeric repeats were detected by CO-FISH. The lengths of at least 300 individual telomeres derived from at least two different metaphases were analyzed. (a) Representative metaphase images derived from Hep-2 cells and the alternative lengthening of telomeres (ALT)-positive surviving Hep-2 cells. The intense signals at the end of chromosomes (yellow arrows) are the telomere restriction fragments. A sister-chromatid exchange within telomeric DNA (T-SCE, white arrows) led to three telomeric signals per chromosome. (b) Increased T-SCE were found in surviving Hep-2 cells.

verified. Both TERT and SMC1β were produced and expressed by Hep-2 cells, while neither protein was detected in surviving Hep-2 cells.

Evaluation of invasion and tumorigenic abilities of surviving Hep-2 cells. We further evaluated the invasive abilities of Hep-2 cells and surviving Hep-2 cells using a Transwell migration assay. After 24 h of migration, surviving Hep-2 cells exhibited a reduction in migration when compared to Hep-2 cells (Fig. 8), indicating that the invasive potential of surviving cells was significantly decreased after alteration of TMM ($P < 0.05$).

The tumorigenicity of these two cell populations was also evaluated in a xenograft mouse model (Fig. 9). Surviving Hep-2 cells which survived telomerase inactivation were injected into five mice (test group). Control groups received either injections with the Hep-2 cell line or injection with cells transfected with control siRNA. Assessment of tumor volume was performed after 35 days. The average tumor volume for the Hep-2 cells was 1796.4 ± 189.6 mm^3. However, surviving cells showing ALT formed smaller tumors with an average volume of 1025.7 ± 97.5 mm^3. Specifically, the number of growing tumors originating from surviving Hep-2 cells was significantly lower than that observed for Hep-2 cells ($P < 0.05$).

Discussion

Although in most cells telomerase inhibition triggers apoptosis and induces cellular senescence,[16] some cells continue to proliferate without detectable telomerase activity. In this study, we demonstrated that telomere elongation can occur even with inhibition of telomerase activity. Telomerase activity decreased substantially in Hep-2 cells with the down-regulation of hTERT expression. However, even after telomerase was inactivated in the surviving Hep-2 cells, they continued to proliferate although slowly. After the population doubled 50 times, the cells still exhibited no telomerase activity, low levels of hTERT expression, and increased APBs, suggesting that they had another mechanism to maintain telomere length. These cells were likely utilizing the ALT mechanism which was either acquired following anti-telomerase treatment, or alternatively, these surviving cells already had an active ALT pathway which allowed them to survive the telomerase inhibition. Determining if either or both of these possibilities are true would require further research.

Because the surviving Hep-2 cells are immortal and lack detectable telomerase, detection of other the markers such as APBs and T-SCE could be used to screen these cells for the ALT phenotype.[17,18] The ALT mechanism is thought to rely on DNA intertelomeric recombination, and T-SCE may be responsible for the observed rapid lengthening and shortening of telomeres that contribute to the heterogeneity in the telomere length.[19,20] Increased T-SCE is known to be an associated feature of ALT and is one of a variety of mechanisms that could be responsible for ALT telomere lengthening. In our study, FISH analyses of metaphase chromosomes revealed elevated levels of T-SCE in the surviving Hep-2 cells, suggesting that these cells used T-SCE to amplify telomere ends. The detection of T-SCE was also consistent with long and heterogeneous telomeres in the surviving Hep-2 cells, and confirmed that these cells utilize one of the ALT TMM.

Since ALT telomere elongation occurred in surviving Hep-2 cells when telomerase was inhibited, it is important to determine

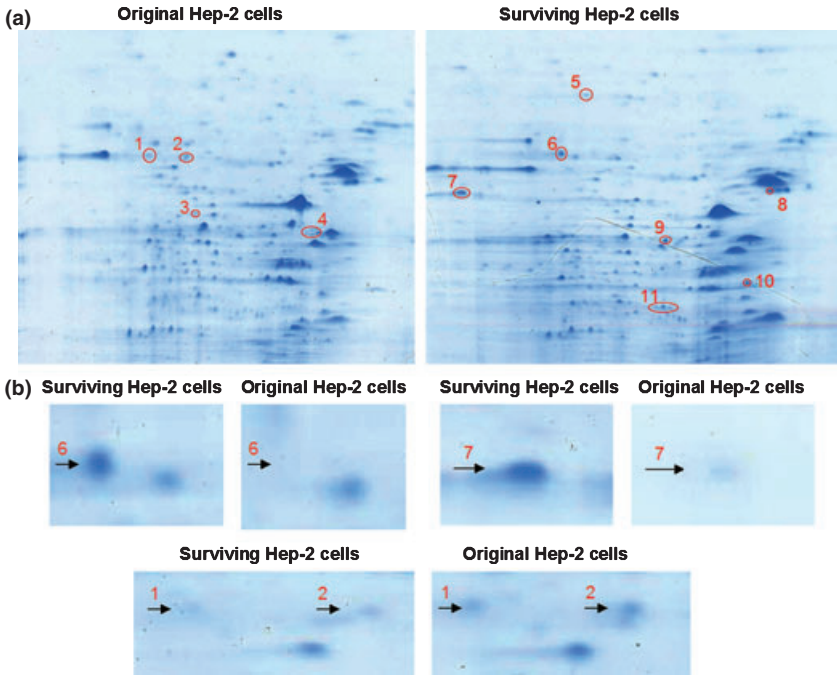

Fig. 6. Two-dimensional gel electrophoresis (2-DE) maps. (a) Representative 2-DE maps of untreated Hep-2 cells and surviving Hep-2 cells. Eleven differentially expressed protein spots marked with red circles were identified using electrospray quadrupole time-of-flight tandem mass spectrometry (ESI-Q-TOF-MS) (see Table 1). (b) Close-up images of some of the differentially expressed protein spots between the two cell lines.

Table 1. Differentially expressed proteins identified by ESI-Q-TOF-MS

Spot	Protein name	Mass	Score	Query	ppm	Peptide	Function
1	PB1	194201	62	826	80.1	R.VASVFANADKGDDEK.N	Transcription and translation
2	TERT	57880	74	881	182	K.LLLEELNVFR.G	Part of telomerase
3	Unknown polypeptide	–	–	–	–	–	–
4	SMC1β	145161	63	528	199	R.YKELKEQVR.K	Stable chromosome
5	SYCE1	40098	52	895	109	K.IEDLMEMVQK.L	Chaperone
6	PML	99204	82	576	171	R.SAMAAVLAMR.D	Chromosome repair
7	GAS8	56469	57	935	153	K.MLRDELDLRR.K	Metabolism
8	N4BP2	200568	48	814	163	K.TEILNPTPAMAK.S	Chaperone
9	Unknown polypeptide	–	–	–	–	–	–
10	NBS1	32746	94	921	64.9	K.VDNDENEHQLSLR.T	Chromosome repair
11	SAGE1	99680	49	1059	166	K.KVVLIQQLEK.A	Antigen

Differentially labeled proteins from 2-DE analysis were identified by ESI-Q-TOF-MS. The name, predicted mass, score from Mascot searches, query (from gels), ppm (from the database), peptide, and the function are shown for each protein. GAS8, growth arrest-specific protein 8; N4BP2, NEDD4-binding protein 2; NBS1, nibrin; PB1, protein polybromo-1; PML, promyelocytic leukemia; SAGE1, sarcoma antigen 1; SMC1β, structural maintenance of chromosomes protein 1β; SYCE1, synaptonemal complex central element protein 1; TERT, telomerase reverse transcriptase.

the key proteins responsible for maintaining the telomere stabilization and elongation in both the telomerase and ALT mechanisms. Telomere dynamics in tumor cells is associated with some important nuclear proteins, such as telomerase in telomerase activity and APBs in ALT. ALT-associated PML bodies (APBs) are important hallmarks of ALT that are involved in recombination and DNA repair, as well as DNA damage response and apoptosis.[21,22] As a reliable cell proteome model, seven differentially expressed nuclear proteins between the two cell lines were identified by ESI-Q-TOF-MS and Western blot. ALT-associated PML bodies (APBs) which are similar to PML bodies contain many proteins. It was hypothesized that they could be both storage locations and functional platforms where ALT occurs,[23] although the content and the function of the APBs are still unclear. As mentioned previously, the difference between untreated Hep-2 cells and surviving Hep-2 cells was the alteration of TMM; therefore, some or all of the seven differentially expressed nucleus proteins found here likely play a role in telomerase mechanism or ALT mechanism. In Hep-2 cells, TERT is already known to be associated with the telomerase function, but we hypothesized that the other up-regulated protein (SMC1β) may be a novel protein associated with telomerase function or interacting with telomerase. Similarly, in surviving Hep-2 cells, the PML and NBS1 proteins are known constitutive proteins of nuclear body APBs,[24–26] while SAGE1, GAS8, and SYCE1 may also be APB proteins that play a direct role in ALT. Further characterization of these differentially expressed proteins may provide new clues to understanding the relationship between these two TMMs.

Our data also showed differences in the behavior of these tumor cells upon telomerase inhibition and alteration of the

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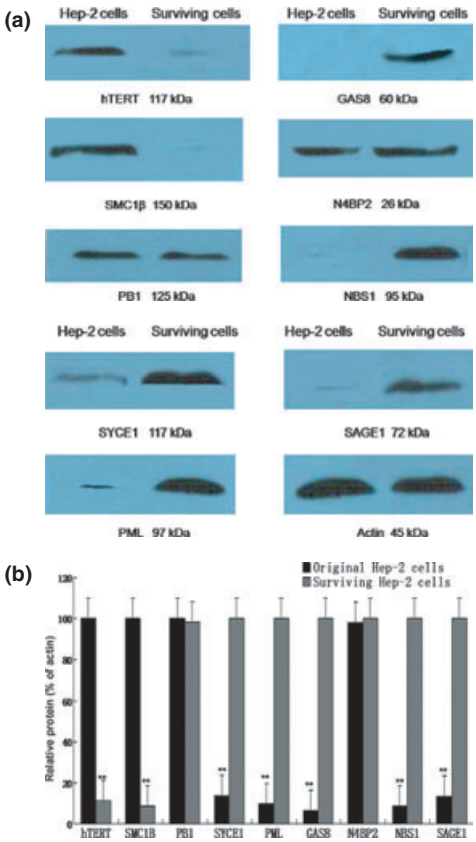

Fig. 7. Western blot verification of differentially expressed proteins differentially expressed in two-dimensional gel electrophoresis (2-DE) analysis from untreated Hep-2 cells and surviving Hep-2 cells. (a) Cell nuclear proteins were analyzed by Western blotting with antibodies against telomerase reverse transcriptase (TERT), synaptonemal complex central element protein 1 (SYCE1), nibrin (NBS1), promyelocytic leukemia (PML), NEDD4-binding protein 2 (N4BP2), sarcoma antigen 1 (SAGE1), protein polybromo-1 (PB1), growth arrest-specific protein 8 (GAS8), and structural maintenance of chromosomes protein 1β (SMC1β). (b) Expression of each protein was estimated by densitometry of the immunoblots and presented as a ratio to the loading control (actin). Error bars represent SDs of means from three independent experiments.

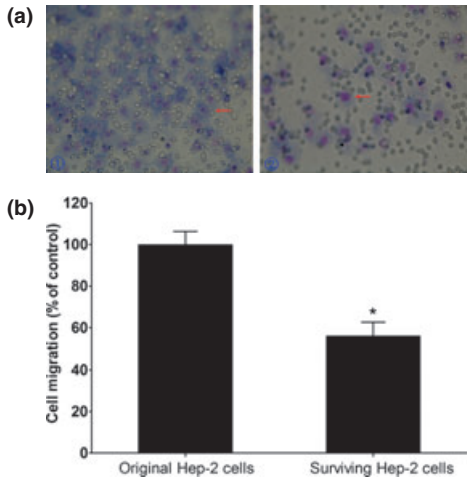

Fig. 8. *In vitro* cell migration analysis of (a1) Hep-2 cells or (a2) surviving Hep-2 cells placed on the top chamber and incubated for 24 h. Original magnification: ×200. The red arrow indicates the migrated cells. (b) The invasive potential of surviving Hep-2 cells was significantly weaker than Hep-2 cells ($P < 0.05$).

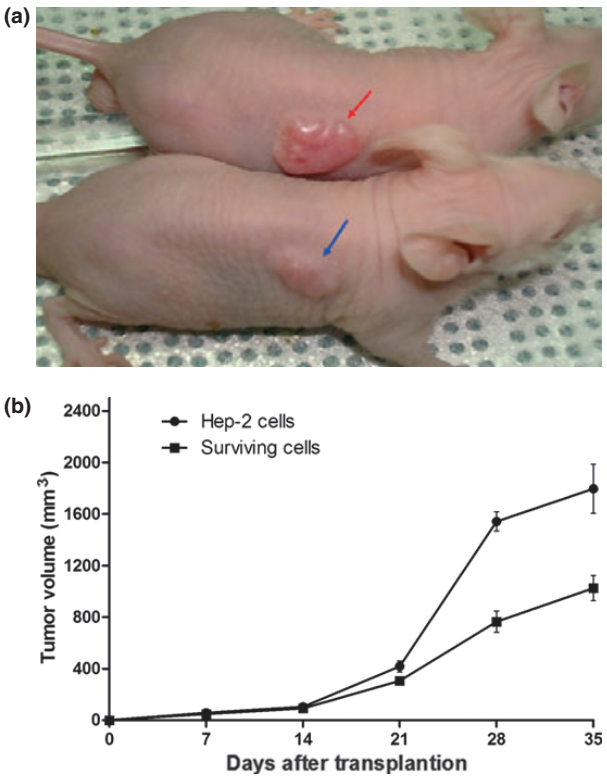

Fig. 9. Tumor formation in nude mice. Tumor sizes were measured by calipers every 7 days throughout the experiment. The data is represented as mean ± SD ($n = 5$) values for a representative experiment. (a) After 35 days, Hep-2 cells formed tumors (1796.4 ± 189.6 mm^3; red arrow), but mice injected with surviving Hep-2 cells showed smaller tumors (1025 ± 97.5 mm^3; blue arrow). (b) Tumor growth curve of xenograft tumors. Tumor growth for the surviving Hep-2 cells was slower, and the tumor sizes were obviously smaller than that of the Hep-2 cell group ($P < 0.05$).

TMM. The invasive potential of the surviving cells was significantly decreased; that is, significantly delayed development of visible tumors and markedly slowed increase in tumor volumes were observed in mice inoculated with surviving Hep-2 cells. Thus, we showed that the surviving Hep-2 cells that used ALT were significantly less tumorigenic than the telomerase-positive Hep-2 cells. We believe that this is not necessarily the essential difference between ALT cells and telomerase-positive cells. We speculate that this result which is consistent with that of the cell proliferation assay might be caused by the disappearance of telomerase activity and the loss of steady-state conditions of telomerase regulated cells. Although surviving cells may have used an ALT mechanism to elongate telomeres, this mechanism of telomere elongation leads to instability of the cells and, therefore, they would become less invasive and less tumorigenic.

Although these surviving Hep-2 cells may not be as robust as their telomerase-positive counterparts, our observation has direct implications for tumor treatments involving telomerase inhibitors. The ALT pathway in tumors requires further exploration to develop therapeutic targets that work in conjunction with current anti-telomerase treatments to increase efficacy.[27]

Our study confirmed that ALT telomere elongation is present following telomerase inhibition, although the precise mechanism of ALT in cancer remains unexplained. Elucidation of

this TMM could be crucial for the development of anticancer treatments that broadly target tumors with alternative mechanisms of survival.

Acknowledgments

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Abbreviations

GAS8	growth arrest-specific protein 8
N4BP2	NEDD4-binding protein 2
NBS1	nibrin
PB1	protein polybromo-1
PML	promyelocytic leukemia
SAGE1	sarcoma antigen 1
SMC1β	structural maintenance of chromosomes protein 1β
SYCE1	synaptonemal complex central element protein 1
TERT	telomerase reverse transcriptase

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