Human Telomerase Reverse Transcriptase Immortalizes Bovine Lens Epithelial Cells and Suppresses Differentiation through Regulation of the ERK Signaling Pathway*

Juan Wang¶¶, Hao Feng§§, Xiao-Qin Huang§§, Hua Xiang†††, Ying-Wei Mao‡‡, Jin-Ping Liu§§, Qin Yan, Wen-Bin Liu, Yan Liu, Mi Deng, Lili Gong, Shuming Sun, Chen Luo, Shao-Jun Liu, Xuan-Jie Zhang, Yun Liu, and David Wan-Cheng Li¶¶

From the College of Life Sciences, Hunan Normal University, Changsha, Hunan, China 410081, Department of Molecular Biology, University of Medicine and Dentistry of New Jersey, Stratford, New Jersey 08084, and Hormel Institute, University of Minnesota, Austin, Minnesota 55912

Telomerase is a specialized reverse transcriptase that extends telomeres of eukaryotic chromosomes. The functional telomerase complex contains a telomerase reverse transcriptase (hTERT) catalytic subunit functionally compatible with a telomerase template RNA from rabbit. In this study, we show that hTERT is also functionally compatible with a telomerase template RNA from bovine. Introduction of hTERT into bovine lens epithelial cells (BLECs) provides the transfected cells telomerase activity. The expressed hTERT in BLECs supports normal growth of the transfected cells for 108 population doublings so far, and these cells are still extremely healthy in both morphology and growth. In contrast, the vector-transfected cells display growth crisis after 20 population doublings. These cells run into cellular senescence due to shortening of the telomeres and also commit differentiation as indicated by the accumulation of the differentiation markers, β-crystallin and filensin. hTERT prevents the occurrence of both events. By synthesizing new telomere, hTERT prevents replicative senescence, and through regulation of MEK/ERK, protein kinase C, and protein kinase A and eventual suppression of the MEK/ERK signaling pathway, hTERT inhibits differentiation of BLECs. Our finding that hTERT can suppress RAS/RAF/MEK/ERK signaling pathway to prevent differentiation provides a novel mechanism to explain how hTERT regulates cell differentiation.

Telomeres are specialized structures present at the ends of eukaryotic chromosomes consisting of tandem arrays of highly conserved hexameric TTAGGG repeats in vertebrates (1–2), that are associated with binding proteins such as TTAGGG repeat-binding factors 1 (TRF1) and 2 (TRF2) to form a T-loop structure (3). Telomeres have been implicated in stabilizing and protecting linear chromosomes from exonucleolytic degradation, preventing chromosome-to-chromosome fusions and recombination, anchoring chromosomes within the nucleus, and assisting the replication of linear DNA (1–2).

Telomerase, initially identified in Tetrahymena (4), is a specialized DNA polymerase that adds telomeric sequences onto chromosome ends and provides a mechanism to balance the loss of repeats from chromosome ends during cell division (2). Structurally, telomerase is a ribonucleoprotein enzyme complex that contains a catalytic protein subunit and an essential RNA component (2, 5–8). The catalytic protein subunit contains the telomerase reverse transcriptase activity. In humans, the telomerase reverse transcriptase (hTERT)1 catalytic subunit is a 127-kDa protein (9–12). The encoding sequence for this catalytic subunit has been cloned in 32 different species (9–14). Apparently, telomerase from these different sources shares both sequence and functional similarity with the reverse transcriptase (15). Recently, the gene encoding the telomerase template RNA has been cloned in 32 different species and the topological structures of these different telomerase RNAs are well conserved (16).

Both in vitro and in vivo reconstitution studies in a number of settings have revealed that hTERT can form a functional complex with human template RNA (hTR) (17–20). Introduction of the hTERT catalytic subunit into telomerase-negative normal human cell types enabled these cells to obtain elongated telomeres and an extended lifespan (21–27). Whether hTERT is functionally compatible with a template RNA from

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These authors have equally contributed to this work.

¶ Present address: Division of Endocrinology, Diabetes, and Metabolism, Dept. of Medicine and Genetics and The Penn Diabetes Center, University of Pennsylvania, Philadelphia, PA 19104.

§ Present address: State Key Laboratory of Microbial Resources and Center for Molecular Microbiology, Institute of Microbiology, Chinese Academy of Sciences, Beijing 100080, China.

¶¶ Present address: Dept. of Pharmacology, University of Michigan, Ann Arbor, MI 48109.

To whom correspondence should be addressed: The Hormel Institute, University of Minnesota, 801 16th Ave., N., E., Austin, MN 55912. Tel.: 507-437-9636; Fax: 507-437-9606; E-mail: dwcli@hi.umn.edu.
hTERT Regulates Differentiation via ERK

other vertebrates remained unknown until our recent study (28) in which we demonstrate that hTERT can form a functional telomerase complex with a rabbit template RNA. In the present study, we further demonstrate this functional compatibility between hTERT and a template RNA from bovine. Expression of hTERT in bovine lens epithelial cells (BLEC) results in elongated telomeres and extended lifespan.

Besides telomere maintenance, our previous studies have demonstrated that telomerase has other functions beyond telomere synthesis. First, overexpression of hTERT in rabbit lens epithelial cells not only yields telomerase activity but also distinctly suppresses camptothecin-induced apoptosis. The suppression of induced apoptosis by hTERT is associated with its repression of expression of the apoptotic genes including p53 and Bcl-2 but not linked to telomere synthesis (28). Second, when introduced into human lens epithelial cells, regardless of the presence or absence of the endogenous telomerase activity, hTERT regulates the retinoblastoma (Rb)/E2F pathway to accelerate the cell growth rate (29).

In the present study, we report that hTERT displays additional functions beyond telomere synthesis. Here we demonstrate that hTERT introduced into BLECs can suppress differentiation. Furthermore, we provide the first evidence that hTERT can regulate the RAS/RAF/MEK/ERK pathway to mediate the suppression of lens epithelial cell differentiation. Thus, our studies provide evidence that hTERT regulates both proliferation and differentiation in eukaryotes.

**EXPERIMENTAL PROCEDURES**

Establishment of Stable Expression Cell Lines—The hTERT expression construct, pCI-hTERT, was kindly provided by Dr. Robert Weinberg (30). The parallel control vector pCI-neo was purchased from Promega. The pCI-neo and pCI-hTERT were introduced into BLECs derived from primary cultures using electroporation with a BTX Electro Cell Manipulator as described previously (28–29). Selection of individual stable expression cell clone was conducted in the corresponding medium containing 600 μg/ml G418 (Invitrogen) for 6 weeks.

RNA Preparation and Semiquantitative RT-PCR—Total cellular RNAs were extracted from 100% confluent cultures using TRIzol reagent (Invitrogen) as previously described (31, 32), and 2 μg of total RNAs were used for reverse transcription in a 20-μl reaction with Superscript II (reverse transcriptase from Invitrogen) according to the manufacturer’s protocol. 2 μl of the reverse transcripts were used for PCR. To determine the expression of hTERT, semiquantitative RT-PCR was conducted using β-actin as an internal control. The primers used in this study were as follows: 5′-GTTGATGTAACACTGGATAG-3′ (forward) and 5′-CTTCTTCCTTCGATCCCTTG-3′ (reverse) for hTERT. The mixture was incubated for 1 h at 37 °C and then protease K was added to a final concentration of 100 μg/ml. After overnight incubation at 37 °C, phenol extraction was performed for three times. The DNA was precipitated, washed, and resuspended in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA. The genomic DNA was digested with restriction enzyme HindIII and SalI (Promega) at 37 °C overnight and then precipitated with 2.5 volumes of 100% ethanol in the presence of 20 μg of E. coli tRNA as carrier. The DNA fragments were resolved by 0.8% agarose gel electrophoresis at 100V for 4 h. After depuration by soaking in 0.1 M sodium citrate, pH 3.0, for 30 min and denaturation by heating at 65 °C for 20 min, the DNA fragments were neutralized with 1.5 M NaCl, 0.5 M Tris-HCl, pH 7.0, and then transferred onto a nitrocellulose membrane in 10 × SSC (1.5 M NaCl, 0.15 M sodium citrate). The membrane was pre-blot and hybridized overnight at 40 °C in the prehybridization buffer and then hybridized with a [γ-32P]ATP-labeled oligonucleotide (TTAGGG)n probe, in the same buffer at 40 °C for 3 h and washed in moderate stringency condition three times. DNA fragments were visualized by autoradiography.

Protein Preparation and Western Blot Analysis—Western blot analysis was conducted as we previously described (37–39). Total protein was extracted from BLECs, pCI-BLECs, and pCI-hTERT-BLECs using 300 μl of extraction buffer (1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 9.1 mM Na2HPO4, 1.7 mM NaH2PO4, 150 mM NaCl, 100 μg/ml, 0.1% aprotinin with pH adjusted to 7.4). The protein concentration was determined as described previously (40). 50 or 100 μg of total protein from each sample were resolved by 10% SDS-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes (Invitrogen). The protein blots were blocked with 5% milk in Tris-NaCl buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl) overnight at 4 °C and then incubated for 1 h with anti-hTERT (Calbiochem.), anti-PKA, anti-PKC, anti-ERK, anti-β-catenin antibodies, and antibodies (Santa Cruz Biotechnology), anti-PKC and anti-PAK antibodies (Pharmingen), anti-β-crystallin antibody (StressGen), and anti-filensin antibody (Sigma) at a dilution of 1:500–2000 (μg/ml) in 5% milk prepared in Tris-NaCl buffer. The secondary antibody was anti-mouse IgG, anti-rabbit IgG, or anti-goat IgG (determined by primary antibodies) at a dilution of 1:1000–2000 (Amersham Biosciences). Immunoreactivity was detected with an enhanced chemiluminescence detection kit (ECL, Amersham Biosciences) according to the company’s instructions.

Overexpression of PAK, PCK, and MEK and Inhibition of PAK, PCK, and RAF/MEK/ERK Signaling Pathways with Pharmacological Inhibitor or Dominant Negative Mutants—The expression vectors of pCMV-PKAα and pCMV-MEK were obtained from the Clontech (Palo Alto, CA). The PCKα expression vector, pCMV-PCKα, and its dominant negative mutant (DNM), pCMV-DNM PCKα, were kind gifts of Dr. Zigang Dong (University of Minnesota). The PAK-specific inhibitor, H-89 (K1 = 48 nM for PAK), was purchased from Calbiochem. Both vector- and hTERT-transfected BLECs were treated with 250 nM H-89 for 24 h before the treated cells were harvested for analysis. H-89 does not affect the proliferation of the BLECs, and RAS dominant negative mutants (41, 42) were obtained from the Clontech. Both overexpression and DNM constructs were amplified in DH-5α and purified using the Plasmid Maxiprep kit from Bio-Rad (catalog number 732-6130). Transfection of pCI-BLECs and pCI-hTERT-BLECs was conducted with Lipofectamine 2000 from Invitrogen according to the company instruction manual. Expression levels of each construct in the transfected cells were monitored with Western blot analysis.
Cell Growth Assay—To determine the cell growth rate, an equal number of cells from different cell lines were seeded in 6-well plates at day 0. The medium was replaced every 2 days, and the cells in each well were collected by trypsinization and counted using the hemocytometer in triplicate in an interval of 24 h for 5 days.

Senescence-associated-β-Galactosidase (SA-β-Gal) Assay—SA-β-Gal assay was used to determine cellular senescence. Senescent cells express a higher level of lysosomal β-galactosidase activity detectable in the presence of X-gal at pH 6.0, which forms a local blue precipitate upon cleavage (43). 3 × 10^4 cells of vector-transfected cells (pCI-BLEC) and two clones of hTERT-transfected cells (pCI-hTERT-BLEC-C1 and pCI-hTERT-BLEC-C2) at 10 and 20 PDs were plated in slide culture chambers and allowed a total of 48 h of growth. The media were then removed, and the cells in each chamber were washed with PBS twice followed by fixation with 3% formaldehyde for 5 min. After fixation, the cells in each chamber were washed with PBS twice and then incubated for 12 h with fresh SA-β-Gal staining solution (1 mg/ml X-gal, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM MgCl₂ in PBS) (43). For quantitation of senescent cells in each sample, a total of 200 cells were counted in each field and six fields in three slide chambers were examined. The results were shown in Table I.

Quantitation of the Total Protein and Kinase Activity Levels—For quantitative comparison, some of the x-ray films were analyzed with the automated digitizing system from the Silk Scientific Corporation. A quantitative comparison, some of the x-ray films were analyzed with the automated digitizing system from the Silk Scientific Corporation.

RESULTS

The Establishment of the hTERT-Expression Bovine Lens Epithelial Cell Line—We have previously demonstrated that hTERT is functionally compatible with the telomerase RNA from rabbit (28). To further confirm the functional compatibility between hTERT and other vertebrate TR, we have introduced the hTERT into BLEC derived from primary cultures. The primary cultures of BLECs were established from intact bovine lens organ as described under “Experimental Procedures.” These cells lack detectable telomerase activity as demonstrated by TRAP assay (Fig. 1C, left lane). After introduction of either the vector, pCI-neo, or the hTERT expression construct, pCI-hTERT, into BLECs, the cells underwent a 6-week selection in the presence of 600 μg/ml G418. Thereafter, the stable clones either carrying only the vector or expressing hTERT were obtained. Fig. 1A showed the morphology of a 100% confluent culture of one of the hTERT expression lines (pCI-hTERT-BLEC-C1). RT-PCR was used to detect the mRNA expression of hTERT in this line and another line (pCI-hTERT-BLEC-C2). As shown in Fig. 1B, top panel, the 309-bp hTERT DNA band amplified from hTERT mRNA was detected only in the two clones of hTERT-transfected BLECs but not in vector-transfected cells. The expression of hTERT was further confirmed by Western blot analysis as shown in Fig. 1B, bottom panel. The successful expression of the hTERT in BLECs does not necessarily imply that it is functional, because the telomerase requires the correct template RNA for the execution of its function. Therefore, telomerase activity was analyzed for the vector-transfected cells and the two clones of hTERT-transfected cells by TRAP assay. As shown in Fig. 1C, telomerase activity was only detected in the two clones expressing hTERT. Thus, hTERT-expression stable lines (pCI-hTERT-BLEC-C1 and pCI-hTERT-BLEC-C2) were established.

The Overexpressed hTERT Protein Is Functionally Compatible with the Bovine TR—The presence of telomerase activity in hTERT-transfected cells but not in vector-transfected cells suggests that hTERT is functionally compatible with the telomerase template RNA from bovine. To further confirm the direct interaction between hTERT and bTR, we performed immunoprecipitation-linked RT-PCR. Firstly, we precipitated the cell extracts from both pCI-hTERT-BLEC and pCI-BLEC cells with an anti-hTERT antibody. The precipitated complexes then were used for RNA extraction, and the extracted RNA samples were processed for RT-PCR analysis. As shown in Fig. 2B, a predicted DNA band of 296 bp was detected in the RT-PCR reaction with the immunoprecipitated RNA from the pCI-hTERT-BLEC cell line but not in the RT-PCR reaction with the

![Image](https://example.com/image.png)

**Fig. 1. Establishment of the stable hTERT-expression BLEC line.** The eyeballs were removed from 2-year-old bovines within 2 h of sacrifice, and the lenses were carefully dissected. The anterior lens capsule with adherent lens epithelium was removed and cultured in 60-mm Petri dishes in Dulbecco’s modified Eagle’s minimal essential medium (DMEM) with 10% fetal bovine serum and 50 units/ml penicillin and 50 μg/ml streptomycin. After 100% confluence, the primary cultures were passage twice before used for transfection. Transfections were conducted using electroporation (28–29). 36 h after transfection, G418 (600 μg/ml) was added into the culture medium for selection. After a 6-week selection, the individual stable clones were obtained. A, the 100% confluent culture from one of the stable lines of hTERT-transfected BLEC cells (pCI-hTERT-BLEC-C1). Bar, 5 μm. B, top panel, RT-PCR to detect the mRNA expression from the exogenous hTERT. Total RNA was extracted with TRIzol reagent from the vector-transfected BLECs (pCI-BLEC) and two clones of hTERT-transfected BLECs (pCI-hTERT-BLEC-C1 and pCI-hTERT-BLEC-C2), and RT-PCR was conducted as described under “Experimental Procedures.” Note that only the hTERT-transfected BLECs (pCI-hTERT-BLEC-C1 and pCI-hTERT-BLEC-C2) contain the hTERT mRNA as reflected by an amplified DNA band of 309 bp. The top panel is the internal control band of 540 bp amplified from the β-actin mRNA. Middle and bottom panels, Western blot analysis of hTERT protein (middle panel) and β-actin (bottom panel) in vector-transfected cells (pCI-BLEC) and hTERT-transfected cells (pCI-hTERT-BLEC-C1 and pCI-hTERT-BLEC-C2). 100 μg of total proteins extracted from vector-transfected cells (pCI-BLEC) and hTERT-transfected cells (pCI-hTERT-BLEC-C1 and pCI-hTERT-BLEC-C2) were resolved in 6% SDS-PAGE and analyzed with antibodies against human TERT and β-actin as described under “Experimental Procedures.” Note that only the hTERT-transfected cells (pCI-hTERT-BLEC-C1 and pCI-hTERT-BLEC-C2) contain the hTERT protein (middle panel). C, demonstration that the hTERT-transfected cells display telomerase activity. Telomerase activity in parental BLEC (lane 1 from the left), pCI-BLEC (lane 2 from the left), and two clones of pCI-hTERT-BLEC cells (lanes 3 and 4 from the left) was determined with the TRAP assay as described under “Experimental Procedures.”
transfected BLEC cells were larger in size and displayed a procedure to detect senescent cells. As shown in Fig. 4, the vector-transfected BLEC cells showed no such changes andistics of senescent cells. In contrast, the two clones of hTERT-treated with RNase (10 kb/reaction) for 1 h before extraction of the template RNA.

We predicted that the extremely slow growth rate of the pCI-hTERT-BLEC cells. Treatment of the precipitated samples with RNase led to degradation of the bTR, and thus, the 296 bp band was not detected (Fig. 2C). Together, these results confirm that hTERT interacts with bTR and that this interacting complex is functional.

**hTERT Supports Normal Growth of pCI-hTERT-BLECs—**The BLECs, vector-transfected cells (pCI-BLEC), and the two clones of hTERT-transfected cells (pCI-hTERT-BLEC-C1 and pCI-hTERT-BLEC-C2) were cultured continuously. After 20 PDs, both the parental and the vector-transfected BLECs displayed extremely slow growth (Fig. 3). In contrast, the two clones of hTERT-transfected BLECs maintained normal growth rates (Fig. 3). So far, the two clones of hTERT-transfected BLECs (pCI-hTERT-BLEC-C1 and pCI-hTERT-BLEC-C2) have been passaged for 108 and 88 PDs, respectively. They are still in normal growth condition (Fig. 3).

**hTERT Prevents Cellular Senescence in pCI-hTERT-BLECs—**We predicted that the extremely slow growth rate of the pCI-BLEC cells may be due to cellular senescence. To test this possibility, we performed the SA-β-Gal assay, a standard procedure to detect senescent cells. As shown in Fig. 4, the vector-transfected BLEC cells were larger in size and displayed a flattened morphology and ~60% of these cells were β-galactosidase-positive (Table I). All of these are typical characteristics of senescent cells. In contrast, the two clones of hTERT-transfected BLEC cells showed no such changes and ~5% β-galactosidase-positive cells were found (Table I). These results showed that hTERT could prevent cellular senescence in the hTERT-transfected clones of BLECs.

**hTERT Supports Normal Growth of pCI-hTERT-BLEC Cells by Synthesizing Telomeres—**To explore the possible mechanism by which hTERT prevents cellular senescence, we examined telomere lengths in vector-transfected cells (pCI-BLEC) and two clones of hTERT-transfected cells (pCI-hTERT-BLEC-C1 and pCI-hTERT-BLEC-C2) at 20 PDs. As shown in Fig. 5, the two clones of hTERT-transfected BLECs displayed a maximal telomere length of 12 kb. In contrast, the vector-transfected BLEC cells had a maximal telomere length of 10 kb, which was 2 kb shorter than that of hTERT-transfected BLEC cells. These results indicate that hTERT introduced into BLECs can synthesizenew telomere, which helps to prevent cellular senescence.

**hTERT Regulates Differentiation via ERK**

Cells—The in vivo lens epithelial cells undergo differentiation after limited divisions at the germinal zone (44–47). The differentiated cells express differentiation markers such as β-crystallin and filensin (44–48). Both the parental BLECs and the vector-transfected BLECs after 20 PDs displayed extremely slow growth rate. It is possible that these cells may commit differentiation. To test this possibility, we have examined the expression of β-crystallin and filensin at 10 and 20 PDs using Western blot analysis. As shown in Fig. 6A, β-crystallin and filensin were expressed at significant levels at 10 PDs and substantially up-regulated at 20 PDs in the parental and the vector-transfected BLECs. In contrast, in the two clones of hTERT-transfected BLEC cells, the expression of both β-crystallin and filensin was not detected at either 10 or 20 PDs. Inhibition of telomerase activity led to the expression of β-crystallin and filensin, suggesting the induction of differentiation. These results demonstrated that hTERT expressed in BLECs is able to suppress differentiation.

**hTERT Represses Activation of ERK1/2 and MEK1/2**—Previous studies (48–58) have shown that MAPK, especially ERK1/2 kinases, are involved in the regulation of lens cell differentiation. To examine the possible mechanism by which hTERT prevents lens cell differentiation, we have examined the expression and activity of the ERK1/2 in parental BLECs, vector-transfected cells, and the two clones of hTERT-transfected BLECs at 10 and 20 PDs using Western blot analysis. As shown in Fig. 6B, the ERK1 activity was only present in the parental and vector-transfected cells at both 10 and 20 PDs. The ERK2 activity was ~20-fold higher in the parental and the vector-transfected cells than that in the two clones of hTERT-transfected cells. Inhibition of telomerase activity in hTERT-transfected cells resulted in the recovery of the activities of ERK1/2. These results suggest that hTERT-mediated suppression of differentiation is associated with its repression of ERK1/2 activation.

Because ERK1/2 is activated by MEK1/2 (59, 60), we next examined the expression and activity of MEK1/2 in the parental BLECs, the vector-transfected BLECs, and the two clones of hTERT-transfected BLECs again at 10 and 20 PDs with Western blot analysis. As shown in Fig. 6C, the expression of total
MEK1/2 was down-regulated in both clones of hTERT-transfected BLECs (pCI-hTERT-BLEC-C1 and pCI-hTERT-BLEC-C2) by 4–8-fold at 10 and 20 PDs compared with that in the parental BLECs and vector-transfected pCI-BLEC cells. The MEK activity was barely detectable in both clones of hTERT-transfected cells. In contrast, MEK activity was easily detected in both the parental BLECs and the vector-transfected BLECs at either 10 or 20 PDs. Inhibition of telomerase activity led to elevated expression of MEK1/2 and also enhanced activity of these two kinases. Together, these results suggest that hTERT suppresses ERK1/2 through down-regulation of MEK1/2 expression and activity.

**Suppression of the RAS/RAF/MEK/ERK Signaling Pathway Is Necessary for hTERT to Prevent Differentiation of BLECs—**

To demonstrate that hTERT-mediated suppression of the MEK/ERK signaling pathway is necessary for hTERT to prevent differentiation of BLECs, we have introduced the dominant negative mutant RAF and RAS, which are known to interfere with the activation of the MEK/ERK kinases (41, 42), into both vector-transfected cells (pCI-BLEC) and hTERT-transfected cells (pCI-hTERT-BLEC-C1). As shown in Fig. 7, these mutants not only displayed interference with the activation of MEK/ERK kinases but also greatly attenuated the expression of β-crystallin and filensin in both vector- and hTERT-transfected BLECs. To further confirm that hTERT suppresses differentiation through MEK/ERK pathway, we overexpressed...
MEK in both vector- and hTERT-transfected cells. Previous studies have shown that overexpression of MEK activates ERK1/2 (61–63). Overexpression of MEK1 in both vector- and hTERT-transfected cells not only activates ERK1/2 but also promotes differentiation as reflected by the expression of β-crystallin and filensin (Fig. 8). However, the overexpression of MEK1 did not affect the senescence cell levels of the vector- and hTERT-transfected cells (Table I). Thus, the overexpression of MEK only overrides the hTERT suppression of differentiation. Together, our results showed that hTERT prevents differentiation of BLECs through suppression of the RAS/RAF/MEK/ERK signaling pathway.

hTERT Modulates Expression of PKAα and PKCα, Which Enhances Suppression of MAPK and Differentiation—Because PKA and PKC are involved in the regulation of MAPK (63, 64), we next examined whether hTERT also regulates these kinases. Western blot analysis revealed that hTERT modulated their expression. As shown in Fig. 9, the expression of the catalytic subunit of PKA (PKAα) was up-regulated ~2-fold in the hTERT-transfected cells than that in vector-transfected cells. In contrast, the expression of PKCα was down-regulated in hTERT-transfected cells compared to vector-transfected cells. These results suggest that hTERT modulates the expression of PKAα and PKCα, which may contribute to the suppression of MAPK and differentiation in BLECs.
cells. In contrast, the expression of the catalytic subunit of PKC (PKCα) was down-regulated 4-fold in hTERT-transfected cells than that in vector-transfected cells. Inhibition of the telomerase activity in hTERT expression cells largely reversed the expression patterns of these two kinases to those found in the vector-transfected cells. These results suggest that hTERT may modulate additional upstream kinases to enhance the suppression of the RAS/RAF/MEK/ERK signaling pathway.

To confirm that PKCα could negatively regulate ERK1/2 and differentiation in BLECs, we overexpressed PKCα or inhibited the activity of this kinase in both vector- and hTERT-transfected BLECs. As shown in Fig. 10, overexpression of PKCα substantially attenuated ERK1/2 activation and largely inhibited the expression of the two differentiation markers, β-cristallin and filensin, in vector-transfected cells. In hTERT-transfected cells, because PKCα was expressed in a high level and hTERT suppressed both ERK1/2 activation and differentiation, the effect of overexpressing PKA was masked. On the other hand, inhibition of PKA activity by H-89, a specific PKA inhibitor, lead to the activation of ERK1/2 and expression of the two differentiation markers in the hTERT-transfected cells. These results show that PKA can negatively modulate MAPK and differentiation in BLECs and that hTERT modulates PKA to enhance suppression of ERK1/2 and differentiation.

To demonstrate that PKCα can positively modulate MAPKs and promote differentiation, we also overexpressed PKCα or blocked its activity by DNMs. As shown in Fig. 11, overexpression of PKCα in hTERT-transfected BLECs induced the activation of ERK1/2 and expression of β-cristallin and filensin. In contrast, the inhibition of PKC activity by DNMs blocked the activation of ERK1/2 and expression of the differentiation markers in the vector-transfected cells. Such results indicate that hTERT can negatively regulate PKC to enhance differentiation of BLECs through repression of the MEK/ERK signaling pathway.

**DISCUSSION**

In the present study, we have demonstrated the following: 1) hTERT is functionally compatible with a telomerase template RNA from bovine. 2) hTERT when expressed in bovine lens epithelial cells elongates telomeres of the transfected cells to extend proliferative lifespan and to prevent replicative senescence. 3) hTERT prevents differentiation of the transfected cells. 4) hTERT also suppresses the RAS/RAF/MEK/ERK pathway.
way, and suppression of this pathway is necessary for hTERT to prevent differentiation of BLECs, and 5) hTERT modulates the expression of PKA and PKC to enhance the suppression of the RAS/RAF/MEK/ERK pathway and thus the inhibition of differentiation (Fig. 12).

**hTERT Can Function with the Bovine Template RNA**—The minimal function core of telomerase contains a reverse transcriptase catalytic subunit (TERT) and a TR (2). Previous studies have shown that human telomerase activity can be reconstituted from these two components either in vitro or in vivo. The first system was reconstituted with partially purified micrococcal nuclease-treated 293 cell extracts and recombiantly synthesized human template RNA (17). Later, reconstitution was conducted with in vitro transcribed and translated hTERT and either co-synthesized hTR or isolated hTR in the presence of rabbit reticulocyte lysate (18, 19). More recently, reconstitution was accomplished with a glutathione S-transferase-hTERT fusion protein and hTR co-expressed in yeast (20). In all of these cases, hTERT uses hTR as a functional template. The hTERT has also been expressed in a variety of human cell lines, and the expressed hTERT is functional (21–27). This is not surprising, because in these cases, the hTERT again uses the endogenous human template RNA. Whether the hTERT can use a template RNA from other species had not previously been demonstrated until our recent study (28) in which we showed that hTERT is functionally compatible with a rabbit template RNA. In this study, hTERT was introduced into bovine lens epithelial cells, which lacks detectable telomerase activity. TRAP assay showed that the exogenous hTERT is functionally compatible with the endogenous bovine TR. Immunoprecipitation-linked RT-PCR confirmed that the expressed hTERT indeed forms a complex with bTR. From these results and our previous studies, we conclude that hTERT can use non-human TR to form a functional enzyme. Such compatibility largely depends on the conservation of the template RNA (65). In this regard, it is interesting to mention that replacement of the endogenous yeast TR with human TR does not impair yeast growth (66). Whether hTERT can replace endogenous yeast TERT remains to be elucidated.

**Fig. 9.** hTERT regulates PKA and PKC. The parental BLECs, the vector-transfected cells (pCI-BLEC), and the hTERT-transfected cells (pCI-hTERT-BLEC-C1) were grown in DMEM at 18 PDs to 100% confluence. Inhibition of hTERT activity was conducted as previously described (29). Different cell samples were harvested for extraction of total proteins. 100 μg of total proteins from each sample were resolved in 10% SDS-PAGE and analyzed with antibodies against PKAα, PKCα, and β-actin as described under “Experimental Procedures.” Note that, in hTERT-expression cells, the expression of PKAα was up-regulated 2-fold and that of PKCα was down-regulated 4-fold in comparison with the expression of the same kinases in parental BLECs and vector-transfected BLECs. Inhibition of telomerase activity leads to significant recovery of the expression patterns of the two kinases as those found in the parental and the vector-transfected cells.

**Fig. 10.** Demonstration that PKA negatively regulates MAPK activity and differentiation in BLECs. Both vector-transfected cells (pCI-BLEC) and hTERT-transfected cells (pCI-hTERT-BLEC-C1) at 18 PDs were further transfected with pCMV vector (lanes 1 and 2 from the left) or with the PKAα expression vector, pCMV-PKAα (lanes 3 and 4 from the left), or treated with 250 nM H-89 for 48 h (lanes 5 and 6 from the left). The double transfected cells were cultured in DMEM in the presence of 800 μg/ml G418 for 48 h before they were harvested for extraction of total proteins. 100 μg of total proteins from each sample were resolved in 10% SDS-PAGE and analyzed with antibodies against total ERK1/2 (T-ERK1/2 and 2), or phospho-ERK1/2 (p-ERK1 and p-ERK2, activated form), PKAα, β-crystallin, filensin, or β-actin, as described under “Experimental Procedures.” Note that, in vector-transfected cells, overexpression of PKAα substantially suppresses the activation of ERK1/2 and expression of β-crystallin and filensin. In contrast, inhibition of PKA activity in hTERT-transfected cells induces the activation of ERK1/2 and differentiation of BLECs as reflected by the expression of β-crystallin and filensin. These results support that hTERT-mediated positive regulation of PKAα enhances its suppression of the MEK/ERK signaling pathway and BLEC differentiation.

**hTERT Prevents Cellular Senescence through Maintenance of Telomere Length**—Cellular or replicative senescence is a process of terminal growth cessation and morphological change displayed by normal cells after they have undergone a finite number of population doublings in vitro (67). Cells undergoing replicative senescence remain adherent to the growth surface and metabolically active for an extended period of time following cessation of proliferation (68). The proliferative potential of a given cell population in culture is directly correlated with the number of prior cell doublings in vitro (69). Two theories (70, 71) have been proposed regarding the senescence basis: the error/genetic damage and the programmed control. In the programmed control theory, it is considered that a genetic program becomes activated or manifested at the end of the proliferative lifespan of a normal cell, causing the characteristic morphological changes and growth arrest (70, 71). Telomere length and telomerase activity can be part of the genetic program. In the absence of telomere elongation, telomeres shorten with each cell division (72). In each round of chromosome replication, telomeres typically lose —150 base pairs of nucleotide sequence at the 5’ end of a DNA molecule. Thus, an
hTERT Regulates Differentiation via ERK

Fig. 11. Demonstration that PKC positively regulate MAPK activity and differentiation in BLECs. Both vector-transfected cells (pCI-BLEC) and hTERT-transfected cells (pCI-hTERT-BLEC-C1) at 18 PDs were further transfected with pCMV vector (lanes 1 and 2 from the left) or with the PKCα expression vector, pCMV-PKCα (lanes 3 and 4 from the left), or with the dominant negative mutant PKCα construct, pCMV-DNM PKCα (lanes 5 and 6 from the left), and cultured in DMEM in the presence of 600 μg/ml G418 for 48 h before they were harvested for extraction of total proteins. 100 μg of total proteins from each sample were resolved in 10% SDS-PAGE and analyzed with antibodies against total ERK1/2 (analyzed with antibodies against total PKCα and also induces the expression of the MEK/ERK signaling pathway and BLEC differentiation.

Fig. 12. Diagram to show a novel mechanism for hTERT to regulate BLEC differentiation. hTERT, probably by repressing the DNA-damage response, suppresses the upstream signaling components, RAS and RAF-1, leading to down-regulation of expression and activity of the downstream kinases, MEK1/2, which then suppresses ERK1/2. Suppression of ERK1/2 results in the changes in the transcriptional activities of multiple transcription factors such as MafA. Such changes lead to suppression of expression of the differentiation related genes and prevention of differentiation by hTERT. Besides, hTERT can also positively regulate the expression of PKA and negatively control the expression of PKC to further enhance the suppression of the RAS/RAF/MEK/ERK signaling pathway and to prevent differentiation. It may be possible that hTERT-mediated regulation of PKA and PKC could directly contribute to the prevention of differentiation through other targets. Upward arrow refers to up-regulation, and downward arrow on the side of the signaling components represents down-regulation.

Increasing cell division number is usually accompanied by declining telomere length. Such DNA replication-dependent loss of telomere length seems to be a mitotic clock for counting the number of cell divisions and signaling cellular senescence (73). In our present study, we have shown that the telomere length becomes shortened during division of bovine lens epithelial cells. If the decreased telomere length is not compensated with new telomere synthesis as found in the case of vector-transfected BLECs, the cells then run into senescence (Fig. 4).

Telomerase activity is present in germ cells and in most tumor cells but absent in most somatic cells (33). Like most somatic cells, the lens epithelial cells isolated from adult bovine lenses lack detectable telomerase activity (Fig. 1C). These cells when cultured under in vitro conditions can only be passaged for a limited number of generations (∼20 PDs). The introduction of the hTERT into these lens epithelial cells confers the transfected cells with telomerase activity (Fig. 1C). Moreover, the two clones of hTERT-transfected cells (pCI-hTERT-BLEC-C1 and pCI-hTERT-BLEC-C2) have been passaged for >108 and 88 PDs, respectively, and they are still growing normally (Fig. 3). However, the vector-transfected cells display extremely slow growth rate after 20 PDs (Fig. 3). The vector-transfected cells display extremely slow growth rate after 20 PDs (Fig. 3). SA-β-Gal analysis of both the vector- and the hTERT-transfected BLEC cells after 20 PDs revealed that ~60% vector-transfected BLECs have undergone cellular senescence (Fig. 4 and Table I). Thus, hTERT prevents cellular senescence of the BLEC cells with telomerase activity. The presence of telomerase activity allows hTERT expression cells to carry out new synthesis of telomeres as reflected by the fact that the telomeres in hTERT-transfected cells are ~2 kb longer than those in the vector-transfected cells. Our results are consistent with numerous earlier studies where the hTERT has been overexpressed in a variety of human cell lines (21–27). In all of the cases, the hTERT-transfected cells have extended telomere length and lifespan to variable degrees.
hTERT Prevents Cell Differentiation through Regulation of the RAF/MEK/ERK Pathway—It is now becoming more clear that telomerase, besides synthesis of telomere, also bears other functions (74). One of such functions is its involvement in regulation of differentiation. Earlier studies have shown that telomerase activity decreases as cells differentiate during brain development (75, 76), suggesting a possible role for this enzyme in the differentiation process. The treatment of cells with telomerase antisense RNA (77) can induce differentiation, indicating that telomerase activity may execute control over the proliferation/differentiation decision. Similar observations have been reported in PC12 cells (77, 78). In this study, we demonstrated that both parental and vector-transfected BLECs after 10 PDs commit differentiation as reflected by exclusive expression of two differentiation markers, β-crystallin and filensin. When most of these cells become senescent, the differentiation is further enhanced as reflected by the elevated expression of the differentiation markers (Fig. 6). In contrast, in hTERT-expression cells, the expression of β-crystallin and filensin was undetectable, suggesting that hTERT suppresses differentiation of the transfected cells. To further confirm the anti-differentiation function of hTERT, telomerase activity in the pCI-hTERT-BLECs was inhibited and the expression of the differentiation markers β-crystallin and filensin was examined. When telomerase activity is inhibited, the hTERT-expression cells are forced to enter differentiation status as reflected by the expression of β-crystallin and filensin. Thus, hTERT can suppress differentiation of lens epithelial cells.

What is the possible mechanism by which hTERT prevents differentiation of BLECs? Recent studies (48–58) have revealed that the MAPks, especially ERK1/2, mediate lens differentiation by different factors. For example, fibroblast growth factor-induced cell proliferation and differentiation require participation of ERK MAPks. ERK activity is found to be necessary and sufficient to induce cell proliferation and differentiation in rat lens epithelial transplants (48). In transgenic mice overexpressing a truncated fibroblast growth factor receptor R3, down-regulation of ERK1/2 phosphorylation (activity) is associated with delayed lens fiber cell differentiation (51). Based on these observations, we hypothesize that hTERT may prevent differentiation of BLECs by regulating MAPK pathway. Indeed, when the activity and expression of ERK1/2 and MEK1/2 in both vector- and hTERT-transfected cells were analyzed with Western blot, it was found that activation of ERK1 was completely suppressed and ERK2 activity was substantially down-regulated in hTERT expression cells compared with those in vector-transfected cells. Comparative analysis of the upstream activation kinases, MEK1/2, revealed that, in hTERT expression cells, both expression and activity of MEK1/2 are significantly down-regulated in hTERT-expression cells than they are in the vector-transfected cells or in the parental BLECs undergoing similar PDs (both 10 and 20 PDs). Thus, the suppression of BLEC differentiation by hTERT is associated with the down-regulation of MEK/ERK expression and activation. To determine that hTERT-mediated repression of MEK/ERK pathway leads to suppression of differentiation of BLECs, dominant negative mutant RAF-1 (blocking activation of MEK1/2) (42) and dominant negative RAS (interfering with activation of RAF-1) (41) were introduced into both vector- and hTERT-transfected cells. These mutants greatly attenuate the activation of MEK1/2 and ERK1/2 and substantially suppress the expression of β-crystallin and filensin in vector-transfected cells (Fig. 7). On the other hand, overexpression of MEK in the hTERT-expression cells induces both activation of ERK1/2 and also differentiation (Fig. 8). However, overexpression of MEK does not seem to interfere with the senescence process of the vector- and hTERT-transfected cells (Table I). Clearly, the enforced activation of ERK1/2 overrides the suppression of differentiation by hTERT. Thus, our results demonstrate that hTERT prevents differentiation of BLECs through suppression of the RAS/RAF/MEK/ERK signaling pathway.

How could the RAS/RAF/MEK/ERK signaling pathway be activated in vector-transfected BLECs but not in hTERT-transfected BLECs? It has been shown that telomere shortening as found in vector-transfected BLECs could trigger a response, which bears the hallmark of DNA damage response (79, 80). In either case, the signal is transmitted through ataxia telangiectasia mutated (ATM) and ataxia telangiectasia and Rad3-related protein (ATR) kinases, which activate the tumor suppressor, p53 (79–84). The activation of p53 or constitutive expression of p53 has been shown to activate the RAS/RAF/MEK/ERK signaling pathway through activators upstream RAS (85). A recent study (86) suggests that, in response to DNA damage, activated p53 induces the expression of growth factors such as heparin-binding epidermal growth factor-like growth factor—like growth factor, which then activates the RAS/RAF/MEK/ERK signaling pathway. In hTERT-transfected BLECs, hTERT prevents telomere shortening (Fig. 5) and thus can suppress p53-dependent activation of the RAS/RAF/MEK/ERK signaling pathway. Furthermore, hTERT may also suppress the RAS/RAF/MEK/ERK signaling pathway through modulation of PKA and PKC as discussed below.

In this study, we have shown that hTERT also exerts positive control on PKAα expression (Fig. 9). The hTERT-mediated positive regulation of PKAα can enhance the suppression of ERK signaling pathway and prevention of differentiation as demonstrated from two experiments: overexpression of PKAα and inhibition of PKAα activity in vector- and hTERT-transfected BLECs. Overexpression of PKAα substantially inhibits activation of ERK1/2 and largely prevents the expression of the two differentiation markers, β-crystallin and filensin, in vector-transfected BLECs (Fig. 10). In contrast, the inhibition of PKAα activity in hTERT-transfected BLECs induces substantial activation of ERK1/2 and promotes clear expression of β-crystallin and filensin (Fig. 10). Our observations that PKAα negatively regulates MAPks in BLECs are consistent with a previous study (61) where PKA has been shown to negatively regulate the RAS/RAF/MEK/ERK pathway through RAF-1. Similarly, hTERT-mediated negative regulation on PKCα expression leads to further suppression of ERK signaling pathway and prevention of differentiation. This is also derived from the results of another two experiments: PKCa overexpression and inhibition in vector- and hTERT-transfected BLECs (Figs. 9 and 11). Our results that overexpression of PKCa promotes activation of ERK1/2, whereas inhibition of PKC activity by DNPKC suppresses the ERK1/2 activation, are consistent with the earlier studies in which PKC was found to positively modulate the ERK signaling pathway at multiple sites including RAS (87, 88), RAF-1 (89), and other downstream kinases (90–92). Overexpression of PKCa promotes differentiation not only in BLECs as shown in the present study but also in other lens epithelial cells where PKC is found to promote lens cell differentiation through modulation of multiple targets such as intermediate filament, gap-junction, and intrinsic membrane proteins (58, 93–96). Taken together, hTERT suppresses the RAS/RAF/MEK/ERK signaling pathway by repressing DNA damage response and also by differentially modulating the upstream kinases, PKA and PKC.

How could suppression of ERKs lead to prevention of differentiation? It is well known that the ERKs can phosphorylate multiple transcription factors (59–60, 92). Phosphorylation of these transcription factors has profound effects on their func-
H9252 tion of differentiation-related genes, such as the ones encoding H9252
results in changed transcriptional activities of multiple tran-

sion at Ser-65 by ERK2 kinase enhances its transcriptional activ-

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