

Genistein Represses Telomerase Activity via Both Transcriptional and Posttranslational Mechanisms in Human Prostate Cancer Cells

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Abstract

Genistein, the most abundant isoflavone present in soybean has antiproliferative effects on a variety of cancer cells, including prostate cancer. However, the molecular mechanism of antiproliferative effects of genistein is not entirely understood. Because the activation of telomerase is crucial for cells to gain immortality and proliferation ability, we examined the role of genistein in the regulation of telomerase activity in prostate cancer cells. Here, we show that genistein-induced inhibition in cell proliferation is associated with a reduction in telomerase activity. Using reverse transcriptase-PCR and hTERT promoter activity assays, we showed that genistein decreased hTERT expression and transcriptional activity dose-dependently. Using various deleted hTERT promoter constructs, we defined that the hTERT core promoter is enough to observe the genistein-induced repression of hTERT transcriptional activity. Because c-Myc is involved in transcriptional regulation of hTERT, c-Myc expression was examined. A dose-dependent decrease in c-Myc message and proteins was observed with genistein treatment. These results indicate that genistein represses hTERT transcriptional activity via the down-regulation of c-Myc expression. However, genistein-induced repression of hTERT transcriptional activity was not blocked by the mutation of c-Myc at the hTERT promoter, suggesting that additional factors are involved in genistein-dependent repression of telomerase activity. Interestingly, we observed that genistein down-regulates the activation of Akt thereby phosphorylation of hTERT and inhibits its translocation to the nucleus. These results show for the first time that genistein represses telomerase activity in prostate cancer cells not only by repressing hTERT transcriptional activity via c-Myc but also by posttranslational modification of hTERT via Akt. (Cancer Res 2006; 66(4): 2107-15)

Introduction

Telomerase is a ribonucleoprotein enzyme with specialized reverse transcriptase activity that catalyzes the synthesis and extension of telomeric DNA (1, 2). This enzyme is present in germ line cells, cancer-derived cell lines, and spontaneously immortalized cells in culture and is activated in 85% to 90% malignant tumors but usually absent in normal somatic cells, which results in

the progressive loss of telomeres with each cell division (3). Cells require a mechanism to maintain telomere stability to overcome replicative senescence, and telomerase activation may therefore be a rate-limiting step in cellular immortality and oncogenesis (4).

The telomerase complex is composed of telomerase reverse transcriptase (TERT; ref. 5), telomerase RNA (6), telomerase-associated proteins (TEP1; ref. 7), and chaperone proteins (p23 and Hsp90; ref. 8). Telomerase RNA, TEP1, p23, and Hsp90 are expressed in a wide variety of cells, irrespective of the presence or absence of telomerase activity. On the other hand, a strong correlation is observed between hTERT mRNA expression and telomerase activity in a variety of epithelial cancers, including cervical (9), breast, colon (10, 11), ovarian (12), and renal (13) carcinomas, indicating that hTERT may be mostly transcriptionally regulated (14). Transcription factors that are involved in up-regulation or down-regulation of hTERT transcriptional activity have been identified by various laboratories (15–23). Recent evidence shows that telomerase modulates expression of growth-controlling genes and enhances cell proliferation (24, 25).

In addition to its transcriptional regulation, telomerase activity can also be regulated by posttranslational modifications. It has been shown that Akt kinase enhances human telomerase activity through phosphorylation of the hTERT subunit at the region surrounding Ser⁸²⁴ (26). In addition, protein kinase C (PKC) has been involved in the phosphorylation of hTERT (27). Another possible mechanism for posttranslational modulation of telomerase activity is via the interaction of hTERT with accessory proteins. It has been shown that 14-3-3 proteins and nuclear factor- κ B (NF- κ B) could act as posttranslational modifiers of telomerase by controlling the intracellular localization of hTERT (28, 29). Therefore, factors that are involved in the regulation of telomerase activity have generated considerable interest in recent years. Such factors have significant importance in understanding and manipulating cell growth in neoplasia. Based on the anticancer effects of various phytoestrogens, we believe that genistein could be such factor.

Genistein (4,5,7-trihydroxyisoflavone), the most abundant isoflavonic compound of soybeans, has been suggested as the active agent underlying the putative anticancer effect of soy (30). The mechanisms by which genistein inhibit growth are not entirely understood. However, it is becoming clear that genistein exerts multiple effects on cancer cell growth. A number of studies have reported that genistein suppresses prostate cancer cell proliferation (31–34). In other systems, genistein inhibited neovascularization or angiogenesis (34, 35), protein tyrosine kinases (36), topoisomerase II (37), oncogene product activity (38), and prostaglandin synthesis (39). In 2004, we reported our initial results at the 86th annual meeting of the Endocrine Society that genistein represses telomerase activity in androgen-independent prostate

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cancer cell, DU-145 (40). In the course of this study, one report seemed documenting the role of genistein in the inhibition of telomerase activity in another prostate cancer cell line, LNCaP (41). It has been proposed that the genistein-induced decrease in telomerase activity is due to the transcriptional repression of hTERT.

The present study was undertaken to obtain an in-depth understanding of the molecular mechanism(s) underlying genistein-induced repression of telomerase activity in prostate cancer cells. Using androgen-independent prostate cancer cell lines, DU-145 and PC-3, we showed that genistein inhibits telomerase activity by repressing hTERT transcriptional activity, as well as posttranslational modification of hTERT. This study suggests for the first time that genistein controls multiple events to regulate telomerase activity in prostate cancer cells. Transcriptional down-regulation of hTERT via c-Myc and posttranslational modification of hTERT protein via the deactivation of Akt are two major events in genistein-induced repression of telomerase activity.

Materials and Methods

Cell line and cell growth assay. DU-145 and PC-3 cells (American Type Culture Collection, Manassas, VA) were grown in IMEM without phenol red (Biofluids, Rockville, MD) supplemented with 10% fetal bovine serum (Quality Biologicals, Gaithersburg, MD), 2 mmol/L glutamine, 100 units/mL penicillin G sodium, and 100 µg/mL streptomycin sulfate (Sigma, St. Louis, MO) in the presence of 5% CO₂ at 37°C.

For the cell growth experiment, 24 hours after seeding (2×10^4 per well), the attached DU-145 and PC-3 cells were treated with 0, 10, 25, 50, and 100 µmol/L genistein for 1, 2, and 3 days. Genistein containing media were replenished every 24 hours for the duration of the experiment. Control cells received equal amounts of ethyl alcohol (vehicle) in the media. After treatment, cells were washed with $1 \times$ PBS, trypsinized, and resuspended in growth medium. Trypan blue (0.4%) was added to the cell suspension, and both live and dead cells were counted using a hemacytometer.

Immunofluorescence staining. DU-145 cells were plated on ECL-coated (Upstate, Lake Placid, NY) Lab-Tek chamber slide. After 24 hours, cells were treated with 0 and 50 µmol/L genistein for 3 days. Cells were fixed in chilled methanol at -20°C for 30 minutes and then rehydrated and stained with hTERT antibody (Novus Biologicals, Inc., Littleton, CO) for ~18 hours at 4°C followed by incubation with Texas Red-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) for 30 minutes and counterstained with 4',6-diamidino-2-phenylindole (1:500) solution for 10 minutes. Slides were washed, mounted and viewed under a fluorescence microscope (Zeiss AxioPlan2 Imaging System, Jena, Germany).

Preparation of nuclear extracts and immunoprecipitation. The preparation of nuclear extracts was done using the nuclear extraction kit (Active Motif, Carlsbad, CA) according to the manufacturer's instructions. Briefly, cell pellets were resuspended in hypotonic buffer and detergent (provided with the kit) and vortexed for 10 seconds at high speed. The suspension was centrifuged at $14,000 \times g$ at 4°C . The supernatant was used as the cytoplasmic fraction. Then, nuclear pellet from 1×10^8 cells treated with or without genistein was resuspended in lysis buffer [50 mmol/L Tris (pH 7.4) containing 150 mmol/L NaCl, 1 mmol/L EDTA, 0.5% NP40, 15% glycerol, 1 mmol/L NaF, 1 mmol/L Na₃VO₄, 1 mmol/L phenylmethylsulfonyl fluoride, 5 µg/mL soybean trypsin inhibitor, 5 µg/mL leupeptin, and 1.75 µg/mL benzamidine]. Samples were centrifuged ($12,000 \times g$, 15 minutes) at 4°C , and supernatants were immunoprecipitated with anti-hTERT (1:50; Novocastra Laboratories, Newcastle upon Tyne, United Kingdom) overnight at 4°C . Immune complexes were collected by using protein A/G-Sepharose beads (Santa Cruz Biotechnology, Santa Cruz, CA), washed, and eluted in sample buffer. Samples (equivalent to 1×10^7 cells) were run on 8% SDS-PAGE gels, transferred to nitrocellulose membranes, and probed with phospho-serine antibody (1:1,000; Cell Signaling, Beverly, MA). Blots were developed by using Western Blotting

Luminol Reagent (Santa Cruz Biotechnology), and images were captured with the Fuji LAS-1000 imager (Tokyo, Japan).

Western blot analysis. For regular Western blot analysis, protein extracts were prepared from DU-145 and PC-3 cells treated with or without various concentrations of genistein according to our previously published methods (42). Membranes were probed with total Akt, phospho-Akt Ser⁴⁷³ and Thr³⁰⁸, and poly(ADP-ribose) polymerase (PARP) antibodies (Cell Signaling) separately, then each membrane was stripped and reprobed with β-actin antibody (Sigma) to ensure for equal loading. Molecular weight markers (Invitrogen, Carlsbad, CA) were run on each gel to confirm the molecular size of the immunoreactive proteins.

Caspase-3/7 activity assay. DU-145 and PC-3 cells were plated in triplicates. After 24 hours, cells were treated with various concentrations of genistein (0, 10, 25, 50, 100 µmol/L) or with 10 ng/mL paclitaxel for 24 hours (as positive control), and then caspase activity was measured by a microplate luminometer (Harta Instruments, Inc., Gaithersburg, MD) using the Caspase-Glo 3/7 assay kit (Promega, Madison, WI) according to the manufacturer's protocol.

Telomeric repeat amplification protocol assay. A cellular extract was prepared as described by Banerjee et al. (43), and the telomeric repeat amplification protocol (TRAP) assay was done according to the method of Kim et al. (3) with some modifications. Detection of telomerase activity in cell extracts was done in a two-step process: (a) telomerase-mediated extension of an oligonucleotides (TS, 5'-AATCCGTCGAGCAGAGTT-3') and (b) PCR amplification of the resultant product with forward (TS) and reverse (ACX, 5'-GCGCGGCTTACCCTTACCCTTACCCTAACCC-3') primers. In addition, as internal control, NT, 5'-ATCGCTTCTCGGCCTTTT-3'; TSNT, 5'-AATCCGTCGAGCAGAGTTAAAAGCCGAGAAGCGAT-3' primers were used. Telomeric DNA products were separated by 10% nondenaturing PAGE in $0.5 \times$ Tris-borate-EDTA buffer (pH 8.3) at 300 V for 2.5 hours. Gels were then stained with SYBR Green I, and image was captured with Fuji LAS-1000 imager. TRAP products were quantified by densitometric analysis using NIH Image J program (NIH, Bethesda, MD).

Isolation of total RNA and reverse transcriptase-PCR. DU-145 and PC-3 cells were plated and cultured as described above. RNA was extracted with TRIzol solution as suggested by the manufacturer (Invitrogen). Genes of interest were amplified using 1 µg of total RNA reverse transcribed to cDNA using Superscript II kit (Invitrogen) with random hexamers. Human-specific primers were designed by us using the Primer Quest program and purchased from IDT (Coralville, IA). hTERT-F, 5'-CCGAAGAGTGTCTGGAGCAA-3'; hTERT-R, 5'-GGATGAAGCCGAGTCTGGA-3'; TEPI-F, 5'-TCAAGCAAACCTGAATCTGAG-3'; TEPI-R, 5'-CCCAGTGAATCTTTCTACGC-3'; and glyceraldehyde-3-phosphate dehydrogenase (GAPDH)-F, 5'-CCACCATGGCAAAT TCCATGGCA-3'; GAPDH-R, 5'-TCTAGACGGCAGGT-CAGGTCCACC-3'. PCR reaction was initiated at 94°C for 2 minutes followed by 28 cycles of 94°C for 1 minute, 1-minute annealing temperature, 72°C for 1 minute followed by final extension at 72°C for 5 minutes. Annealing temperatures for hTERT, TEPI, and GAPDH were 58°C , 55°C , and 60°C , respectively. After amplification, PCR products were separated on 1.5% agarose gels and visualized by ethidium bromide fluorescence using the Fuji LAS-1000 imager. Images were captured and imported to Photoshop.

Luciferase assay. DU-145 and PC-3 cells were transfected with pGL3-vector containing either 3.3-kb hTERT promoter-luciferase construct or its deleted constructs along with the c-myc sense or antisense expression vectors using GeneJammer transfection reagent (Stratagene, La Jolla, CA) in the presence of complete growth medium. After 48 hours of transfection, cells were treated with various concentrations of genistein for another 48 hours. Luciferase activity was measured in cell lysates by a microplate luminometer using the Dual Luciferase assay kit (Promega) according to the manufacturer's protocol. Luciferase activity was normalized by *Renilla* luciferase activity by cotransfection of pRL-TK plasmid. Each experiment was repeated thrice in triplicates.

Statistical analyses. Data from the cell count, caspase-3/7 activity, TRAP, and luciferase assays were derived from at least three independent experiments. For quantification of Western blots and reverse transcriptase-PCR (RT-PCR) products, Image J program was used. Statistical analyses

were conducted using the Prism3 GraphPad software, and values were presented as mean \pm SE. Significance level was calculated using the one-way ANOVA followed by the Dunnett post-test, with an assigned confidence interval of 95%. $P < 0.05$ was considered significant.

Results

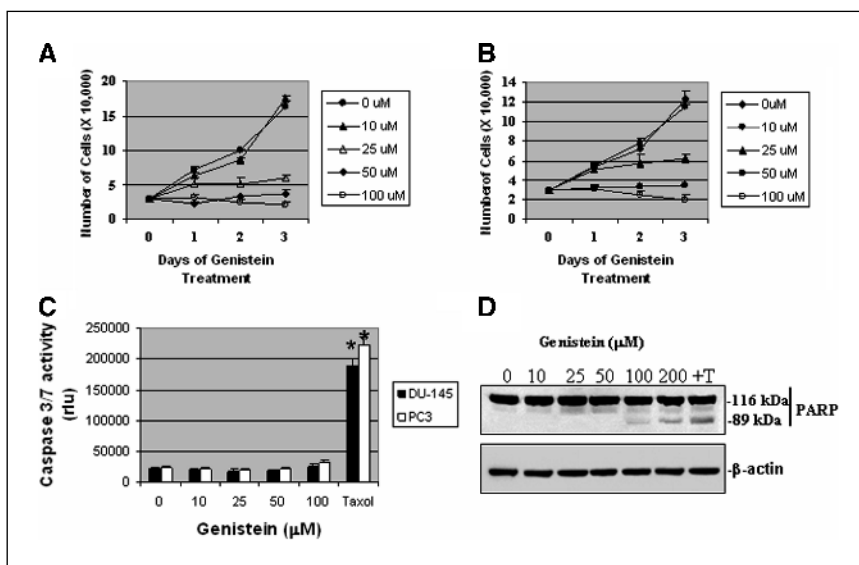
Genistein inhibits growth of human prostate cancer cells without inducing cell death. Although it is known from previous published reports that genistein has antiproliferative effect on prostate cancer cells, to initiate our study, it was necessary to determine the dose that would cause growth arrest but will not induce cell death. Using DU-145 and PC-3, we observed a dose-dependent inhibition of growth with genistein treatment (Fig. 1A and B). Significant inhibition of growth (50%) was observed with as little as 25 $\mu\text{mol/L}$ genistein ($P < 0.01$), and 50 $\mu\text{mol/L}$ ceased cell growth completely without induction of cell death, whereas 100 $\mu\text{mol/L}$ caused a slight but nonsignificant increase in cell death. These results show that 50 $\mu\text{mol/L}$ genistein is capable of inhibiting cell growth completely without inducing cell death. Because, trypan blue staining is a less sensitive method of detecting cell death, we examined the caspase-3/7 activity after 3 days of treatment with various concentrations of genistein. In both DU-145 and PC-3 cells, up to 50 $\mu\text{mol/L}$ genistein did not induce caspase activity over the control level. A slight but nonsignificant increase in caspase activity was observed with 100 to 200 $\mu\text{mol/L}$ genistein treatments, whereas paclitaxel treatment for 24 hours increased caspase activity significantly by 10-fold (Fig. 1C). Similarly, PARP cleavage was not observed in DU-145 or PC-3 (data not shown) cells with 50 $\mu\text{mol/L}$ (Fig. 1D), but 200 $\mu\text{mol/L}$ genistein resulted a slight increase in PARP cleavage, indicating an induction of apoptotic cell death with higher doses of genistein. As expected, paclitaxel treatment for 24 hours caused an increase in PARP cleavage (Fig. 1D). These results suggest that genistein inhibits growth of prostate cancer cells not due to an increase in cell death; rather, genistein is involved in regulating the expression of genes that are involved in cell proliferation. Because telomerase provides indefinite replicative potential of cancer cells and acts as a growth-promoting factor (24, 25), we examined whether telomerase activity was repressed by the treatment of genistein in prostate cancer cells.

Genistein represses telomerase activity in prostate cancer cells. Because genistein has antiproliferative effect on cancer cells, and the majority of cancer cells expresses high telomerase activity that provide the cancer cell's ability to survive and proliferate, we examined whether genistein represses telomerase activity in prostate cancer cells. Using the TRAP assay, we observed that genistein repressed telomerase activity in both DU-145 and PC-3 cells dose-dependently (Fig. 2A and B). Genistein (10 $\mu\text{mol/L}$) repressed telomerase activity in DU-145 cells significantly ($P < 0.05$; Fig. 2C), but such repression was not observed in PC-3 cells at this concentration (Fig. 2D). However, 25 to 100 $\mu\text{mol/L}$ genistein repressed telomerase activity ($\sim 50\%$) in both DU-145 and PC-3 cells (Fig. 2C and D). This concentration of genistein did not induce cell death in these prostate cancer cells. This result suggests that genistein has an inhibitory effect on telomerase activity in prostate cancer cells.

Genistein down-regulates hTERT expression but not telomerase-associated protein TEPI. Expression of the hTERT is tightly regulated with telomerase activity in various cancer cells (9–13); therefore, it is important to examine whether the inhibitory effect of genistein on telomerase activity in prostate cancer cells is due to the down-regulation of hTERT expression. We observed that genistein treatment for 3 days decreased hTERT expression in DU-145 (Fig. 3A) and PC-3 (Fig. 3B) dose-dependently. However, the expression of telomerase-associated protein TEPI did not change (Fig. 3A and B). With increasing concentrations of genistein, hTERT message levels were decreased dose-dependently and significantly ($P < 0.01$; Fig. 3C and D), and 50 $\mu\text{mol/L}$ genistein decreased $>50\%$ of hTERT message. These results suggest that repression of telomerase activity in DU-145 and PC-3 cells with genistein treatment is due to the genistein-dependent down-regulation of hTERT expression.

Genistein represses hTERT transcriptional activity in prostate cancer cells, and the minimal core promoter is enough for genistein-induced repression of hTERT. Because the expression of hTERT correlates with the telomerase activity, to understand the molecular mechanism of genistein-induced repression of telomerase activity, we examined the hTERT promoter activity using full-length and various deleted hTERT promoter constructs (21, 44) in prostate cancer cells. Luciferase reporter

Figure 1. Genistein-induced growth inhibition in prostate cancer cells is not due to an induction of apoptotic cell death. DU-145 (A) and PC-3 (B) cells were plated in triplicate wells and exposed to various concentrations of genistein (0, 10, 25, 50, and 100 $\mu\text{mol/L}$) for 1, 2, and 3 days. After genistein treatment, viable cells (as assessed by trypan blue exclusion dye assay) were counted using a hemacytometer. C, DU-145 and PC3 cells were seeded in triplicate plates and treated with 0, 10, 25, 50, and 100 $\mu\text{mol/L}$ genistein or 10 ng/mL paclitaxel for 3 days. After treatment, caspase activity was measured by Caspase-Glo 3/7 assay. Arbitrary relative light units (rlu) compared with the background (with no cells). D, Western blot showing PARP cleavage after various doses of genistein treatments on DU-145 cells for 3 days. Paclitaxel-treated DU-145 cell extract was used as positive control. Protein lysates (50 μg) were resolved on 12% SDS-PAGE, transferred onto nitrocellulose membrane, and probed with anti-PARP antibody. Membranes were re-probed with β -actin antibody to ensure for equal loading. All from at least three independent experiments. Points, mean; bars, SE. *, $P < 0.01$, significantly different from control.



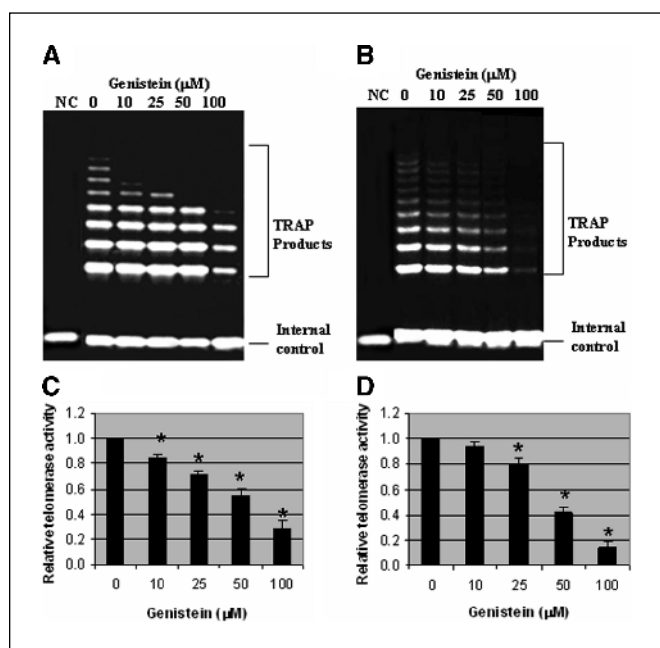


Figure 2. Genistein represses telomerase activity in prostate cancer cells. *A* and *B*, effect of genistein on the level of telomerase activity in DU-145 and PC-3 cells, respectively. Twenty-four hours after plating, cells were exposed to various concentrations of genistein (0, 10, 25, 50, and 100 μmol/L) for 3 days. Cell pellets were collected and subjected to TRAP assay. *NC*, negative control using lysis buffer only. *C* and *D*, quantitative estimations of telomerase activity in DU-145 (*C*) and PC-3 (*D*) cells determined by densitometric measurements of TRAP products from three independent experiments. *Columns*, mean; *bars*, SE. *, $P < 0.01$, significantly different from control.

plasmids containing the full-length (3.3 kb) hTERT 5' regulatory region (pGL3-3328) and basic vector (pGL3-basic) were transiently transfected into DU-145 and PC-3 cells. Transfection of pGL3-3328 induced hTERT promoter activity 45-fold in DU-145 cells and 20-fold in PC-3 cells compared with the basic vector-transfected cells (Fig. 4A). However, genistein treatment decreased hTERT transcriptional activity dose-dependently in both DU-145 and PC-3 cells (Fig. 4A). These results suggest that genistein represses hTERT transcriptional activity in prostate cancer cells. To further delineate the region of hTERT promoter responsible for genistein-dependent repression of hTERT promoter activity, various deleted fragments of hTERT promoter were examined. Genistein (50 μmol/L) repressed promoter activity of various deleted fragments of hTERT promoter significantly ($P < 0.001$) in DU-145 cells and in PC-3 cells (only 181-Luc is shown; Fig. 4B). These results suggest that the core hTERT promoter (181-Luc) is enough to obtain genistein-dependent repression of hTERT transcriptional activity.

Genistein down-regulates c-Myc expression in prostate cancer cells. The core promoter region (–181bp) of hTERT has two c-Myc binding sites, and it has been shown previously that c-Myc regulates telomerase activity (15, 45–47). Because c-Myc is involved in telomerase regulation, we were interested in investigating whether genistein alters c-Myc expression in prostate cancer cells. Using RT-PCR assay, we observed that genistein treatment decreased c-Myc expression dose-dependently and significantly ($P < 0.01$; Fig. 5A), and >50% of c-Myc message was decreased with 50 μmol/L genistein. Similarly, c-Myc protein was also decreased dose-dependently and significantly ($P < 0.01$) with various concentrations of genistein treatments (Fig. 5B). These results show that genistein down-regulates c-Myc expression, and this

could be a potential mechanism by which telomerase activity is repressed in prostate cancer cells by genistein.

Genistein down-regulates c-Myc transcriptional activity in prostate cancer cells. Because c-Myc expression is down-regulated by genistein, we next investigated whether c-Myc transcriptional activity is down-regulated in these cells. Transient transfection of p-Myc-TA-luciferase construct into DU-145 cells increased c-Myc transcriptional activity 6-fold over the control vector (TA-luciferase) transfected cells (Fig. 5C). Fifty micromolar genistein treatment decreased c-Myc transcriptional activity significantly (~6-fold; $P < 0.001$). Similarly, genistein down-regulated c-Myc transcriptional activity (c-Myc minimal promoter; XNM-luciferase) significantly (>50%; $P < 0.01$; Fig. 5C). These results collectively suggest that genistein down-regulates the c-Myc transcriptional activity and could be a cause of repressed telomerase activity in prostate cancer cells.

c-Myc regulates hTERT transcriptional activity in DU-145 prostate cancer cells, but c-Myc alone is not involved in genistein-induced repression of telomerase activity. Although our previous studies (Fig. 5A-C) show that genistein down-regulates c-Myc expression and transcriptional activity, it does not imply that c-Myc alone is involved in the regulation of telomerase activity in prostate cancer cells. In addition, it has been not evaluated whether genistein can repress telomerase activity in prostate cancer cells without the involvement of c-Myc. To test this, pGL3-181 hTERT promoter with or without sense or antisense c-Myc expression vectors were transfected in DU-145 cells. Transfection of pGL3-181 hTERT alone gave a 60-fold induction of luciferase activity compared with pGL3-basic (Fig. 5D). Addition of c-Myc expression vector increased hTERT transcriptional activity significantly by 2-fold ($P < 0.01$) compared with the levels of pGL3-181 hTERT promoter. When antisense c-Myc expression vector was expressed along with pGL3-181-hTERT-promoter, hTERT transcriptional activity was significantly reduced by 3-fold

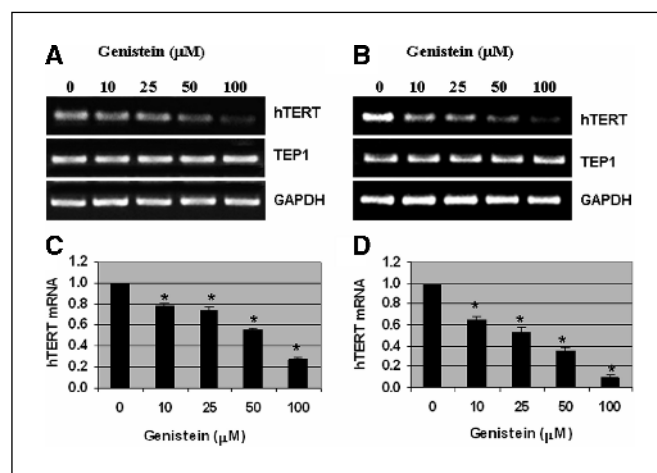
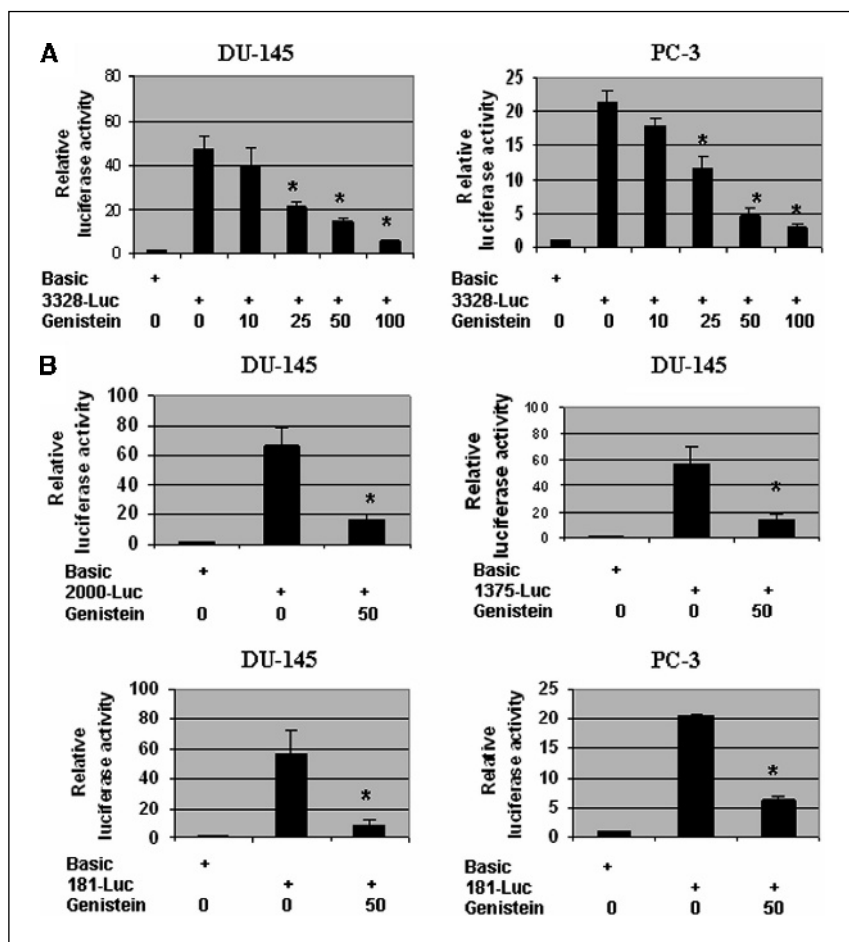


Figure 3. Genistein down-regulates hTERT expression but not telomerase-associated protein (TEP1) in prostate cancer cells. *A* and *B*, after 24 hours of plating, DU-145 (*A*) and PC-3 (*B*) cells were exposed to various concentrations of genistein (0, 10, 25, 50, and 100 μmol/L). After 3 days of treatment, RNA was extracted, and RT-PCR assays were done to detect hTERT, TEP1, and GAPDH mRNAs. Representative photograph from an experiment that was repeated thrice. *C* and *D*, quantitative estimations of hTERT mRNA in DU-145 (*C*) and PC3 (*D*) cells were determined by densitometric measurements of RT-PCR gels from three independent experiments after normalization with GAPDH. *Columns*, mean; *bars*, SE. *, $P < 0.01$, significantly different from control.

Figure 4. Genistein represses hTERT transcriptional activity in prostate cancer cells. **A**, DU-145 and PC-3 cells were transfected with 200 ng of full-length hTERT promoter luciferase plasmids (pGL3-3328-Luc) and 10 ng of *Renilla* luciferase (pRL-TK-Luc) plasmids for 48 hours in normal growth media and then exposed to various concentrations of genistein (0, 10, 25, 50, and 100 $\mu\text{mol/L}$) for another 48 hours. Genistein-containing medium was replenished every 24 hours. After treatment, cells were harvested, and luciferase assays were done. Relative luciferase activity after normalization with *Renilla* luciferase activity. Luciferase activity in control samples transfected with basic plasmid (pGL3-basic) was normalized to 1.0. **B**, DU-145 and PC-3 cells were transfected with 200 ng of various 5'-deleted hTERT promoters (pGL3-2000-Luc, pGL3-1175-Luc, and pGL3-181-Luc) for 48 hours and then treated with or without 50 $\mu\text{mol/L}$ genistein for 48 hours. Relative luciferase activity was determined as mentioned above. *Columns*, mean of three independent experiments with triplicates samples; *bars*, SE. *, $P < 0.01$, significantly different from control.



($P < 0.001$; Fig. 5D). These results suggest c-Myc has the ability to regulate transcription of hTERT in prostate cancer cells. To address our second question, we transfected the c-Myc-mutated core hTERT promoter (pGL3-181-Myc- MT1+2-Luc; ref. 21) and then treated the cells with or without genistein. In both DU-145 and PC-3 cells, c-Myc-mutated hTERT core promoter failed to block genistein-induced repression of hTERT transcriptional activities completely (Fig. 5D). These results suggest that the down-regulation of c-Myc is not the only reason for genistein-induced repression of telomerase activity. However, the largest effect of genistein seems to be mediated via c-Myc.

Genistein deactivates AKT in prostate cancer cells. It has been shown that Akt enhances human telomerase activity through phosphorylation of hTERT (26); therefore, we investigated whether the repression of telomerase activity by genistein was due to the deactivation of Akt. As we see in Fig. 6, total Akt level did not change with various doses of genistein treatment of DU-145 cells, whereas phospho-Akt Ser⁴⁷³ and Thr³⁰⁸ residues were decreased dose-dependently (Fig. 6A). Quantitatively, both phospho-Akt Ser⁴⁷³ and Thr³⁰⁸ residues were decreased significantly ($P < 0.01$) with as little as 10 $\mu\text{mol/L}$, and with 50 $\mu\text{mol/L}$ genistein, >50% of phospho-Akt Ser⁴⁷³ and 40% of phospho-Akt Thr³⁰⁸ were decreased. These results suggest that genistein deactivates Akt kinase by preventing phosphorylation of Ser⁴⁷³ and Thr³⁰⁸ residues.

Genistein prevents phosphorylation of hTERT and thereby inhibits translocation to the nucleus in prostate cancer cells. Because activated Akt phosphorylates hTERT, and because it is

necessary for full telomerase activity, we examined whether phosphorylation of hTERT is inhibited by the genistein treatment. Immunoprecipitation immunoblot analyses show that 50 $\mu\text{mol/L}$ genistein decreased the phospho-hTERT in DU-145 and PC-3 cells (Fig. 6B). More than 2-fold decrease in phospho-hTERT was observed in genistein-treated DU-145 and PC-3 cells compared with the vehicle-treated cells. Because phosphorylation of hTERT is necessary for its nuclear translocation, we subsequently examined the localization of hTERT protein in DU-145 cells with or without genistein treatments. As we see in Fig. 6C, hTERT protein is localized predominantly in the nucleus, with negligible staining in the cytoplasm. The treatments of 50 $\mu\text{mol/L}$ genistein decreased overall hTERT protein staining intensity accompanied by a complete loss of nuclear but a slight increase in cytoplasmic staining. These results suggest that genistein decreased phosphorylation of hTERT protein and possibly thereby inhibited its translocation to the nucleus.

Discussion

In the present study, we investigated the molecular mechanism of genistein-induced repression of telomerase activity in prostate cancer cells. Using two human prostate cancer cell lines, DU-145 and PC-3, we showed that genistein dose-dependently (10-100 $\mu\text{mol/L}$) represses telomerase activity. We show that genistein-induced repression of telomerase activity is not only due to the transcriptional inhibition of hTERT but also due to the posttranslational modification of hTERT protein.

It is becoming increasingly obvious that the regulation of telomerase is highly complex and multifactorial in mammalian cells. It involves expression of the telomerase gene, association of telomerase-associated proteins (Hsp90 and p23), posttranslational protein-protein interactions, and protein phosphorylation. We focused our initial study by examining the effect of genistein at the transcriptional regulation of hTERT, because large numbers of published reports suggest that the expression of hTERT is tightly correlated with the telomerase activity (9–13). We observed a dose-dependent decrease in hTERT mRNA in both DU-145 and PC-3 cells with increasing concentrations of genistein (Fig. 3), and the decrease in hTERT expression was also associated with a decrease in telomerase activity (Fig. 2). These results are in agreement with a recently published report in LNCaP cells, where hTERT message level also decreased after genistein treatment (41). Although we observed a dose-dependent decrease in hTERT message, we did not observe a significant induction in cell death. We believe that the induction of apoptosis or the activation of caspase-3 (48) requires higher genistein concentration ($\geq 100 \mu\text{mol/L}$). Moreover, we did not observe any change in telomerase-associated protein TEPI, suggesting that the repressive effect of genistein on hTERT is specific. However, TEPI is not the only telomerase-

associated protein. Other chaperone proteins, such as Hsp90, Hsp70, and p23, are important for telomerase activity by their functional association with telomerase (8). Expression of these chaperone proteins is up-regulated during malignant transformation or in cancer tissues compared with the noncancerous tissues (49). It is possible that genistein might alter the expression of these chaperone proteins and inhibit telomerase assemblage that is necessary for full telomerase activity. Therefore, future experiments would be necessary to determine whether genistein alters the expression of telomerase-associated chaperone proteins and thereby alters telomerase assemblage.

Using full-length hTERT promoter construct, we observed that genistein dose-dependently (10–100 $\mu\text{mol/L}$) repressed the hTERT transcriptional activity in both DU-145 and PC-3 cells (Fig. 4). These results corroborate with the earlier published result in LNCaP cells (41). However, the earlier published report has not identified a specific region of hTERT promoter that is responsible for genistein-induced repression. Using various deleted hTERT promoter constructs (pGL3-2000-Luc, pGL3-1375-Luc, and pGL3-181-Luc), we showed that the hTERT core promoter (pGL3-181-Luc) region is enough to obtain genistein-induced repression of hTERT transcriptional activity (Fig. 4). Therefore, transcription

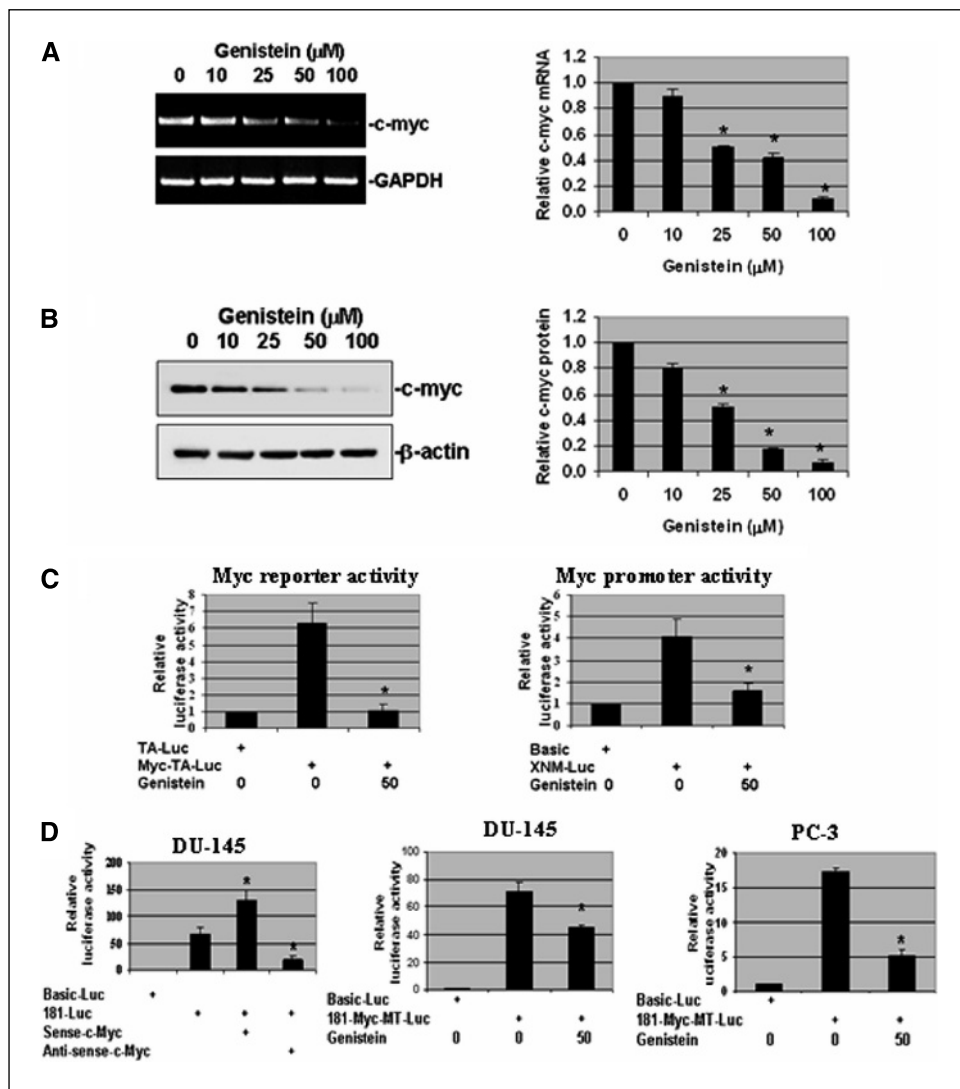
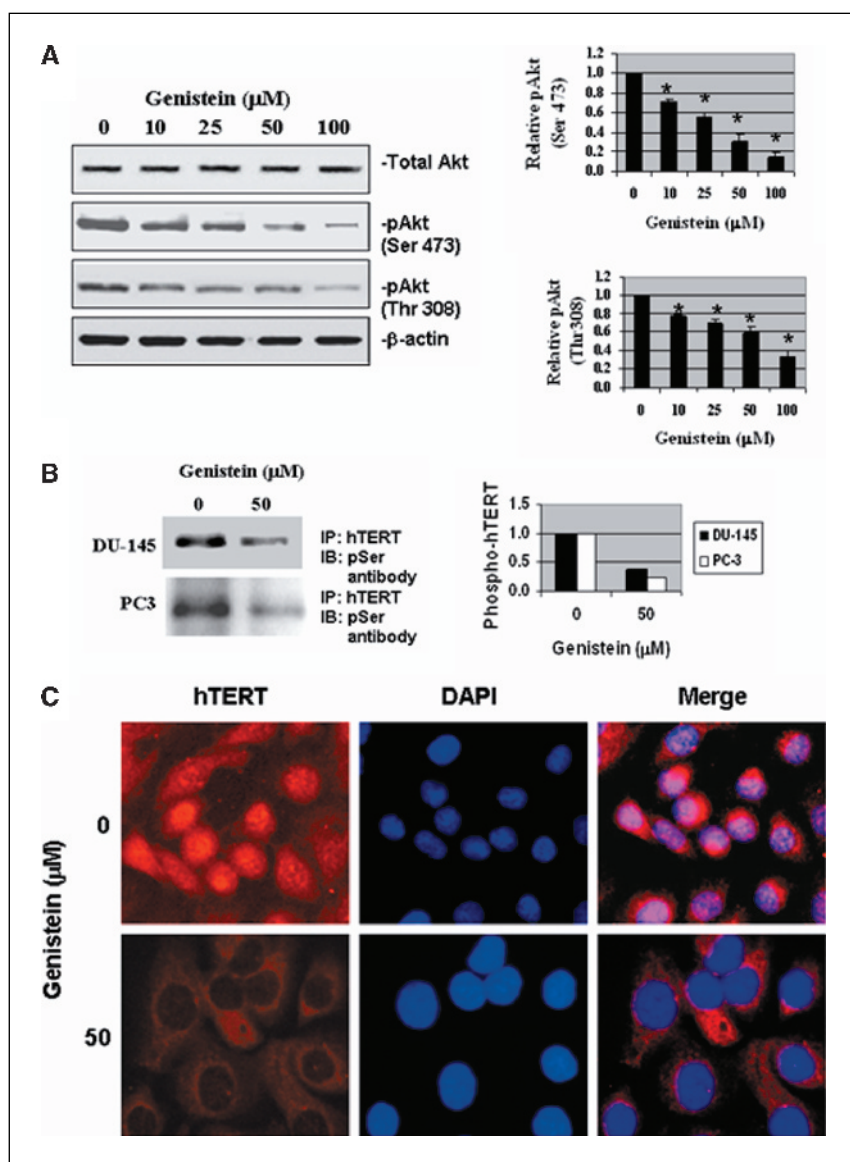


Figure 5. Genistein down-regulates c-Myc expression and transcriptional activity in prostate cancer cells. **A**, after 24 hours of plating, DU-145 cells were exposed to various concentrations of genistein (0, 10, 25, 50, and 100 $\mu\text{mol/L}$). After 3 days of treatment, RNA and protein were extracted for RT-PCR and Western blot analyses of c-Myc. **A**, representative photograph from a RT-PCR experiment that was repeated thrice. Relative levels of c-Myc mRNA in genistein-treated or untreated DU-145 cells (*right*). **B**, Western blot analysis of c-Myc protein with various concentrations of genistein treatments of DU-145 cells for 3 days. Protein lysates (50 μg) were resolved on 12% SDS-PAGE, transferred onto nitrocellulose membrane, and probed with anti-c-myc antibody. Membranes were reprobbed with β -actin antibody to ensure for equal loading. Relative levels of c-Myc protein in genistein-treated or untreated DU-145 cells (*right*). **C**, to examine genistein-induced down-regulation of c-Myc transcriptional activity, DU-145 cells were transfected with 200 ng of Myc reporter (Myc-TA-Luc) or c-Myc core promoter (XNM-Luc) luciferase plasmids along with 10 ng of *Renilla* luciferase 48 hours and then treated with or without 50 $\mu\text{mol/L}$ of genistein for another 48 hours. Relative luciferase activity was determined after normalization with *Renilla* luciferase. **D**, to evaluate the role of c-Myc in the regulation of hTERT transcriptional activity in prostate cancer cells, however, DU-145 cells were transfected with 200 ng hTERT core promoter (pGL3-181-Luc) with 100 ng sense or antisense c-Myc expression vectors for 48 hours. In two separate studies, DU-145 and PC-3 cells were transfected with 200 ng of mutated c-Myc hTERT promoter (pGL3-181-Myc-MT 1+2-Luc) for 48 hours and then treated with or without genistein (50 $\mu\text{mol/L}$) for another 48 hours. Relative luciferase activities were determined after normalization with *Renilla* luciferase. *Columns*, mean of three independent experiments with triplicates; *bars*, SE. *, $P < 0.001$, significantly different from control.

Figure 6. Genistein inhibits phosphorylation of Akt and thereby prevents translocation to the nucleus in prostate cancer cells. **A**, 50 $\mu\text{mol/L}$ protein lysates from DU-145 cells treated with various concentrations of genistein (0, 10, 25, 50, and 100 $\mu\text{mol/L}$) for 3 days were resolved on 12% SDS-PAGE, and immunoblots were probed with antibodies to total Akt, phospho-Akt Ser⁴⁷³, and phospho-Akt Thr³⁰⁸. All immunoblots were reprobed with β -actin antibodies to ensure equal loading. Representative photograph from an experiment that was repeated thrice. Quantitative analyses of relative levels of phospho-Akt Ser⁴⁷³ and phospho-Akt Thr³⁰⁸ (right). Columns, mean from three independent immunoblots; bars, SE. *, $P < 0.01$, significantly different from levels in untreated cells. **B**, genistein dephosphorylates hTERT in prostate cancer cells. DU-145 and PC-3 cells were treated with or without 50 $\mu\text{mol/L}$ genistein for 3 days, and then cells were harvested, and nuclear fractions were immunoprecipitated with hTERT antibody. Immunoprecipitated proteins were resolved on 8% SDS-PAGE, and immunoblots were probed with phospho-serine antibody. Quantitative analyses of relative levels of phospho-hTERT in the nucleus (right). Columns, mean of two independent experiments. **C**, DU-145 cells were plated on chamber slides and treated with or without 50 $\mu\text{mol/L}$ genistein for 3 days then fixed in methanol, incubated with hTERT antibody, and counterstained with 4',6-diamidino-2-phenylindole. Slides were then mounted and examined using a fluorescence microscope. Photographs were taken at the same magnification ($\times 20$) and then transported to Photoshop.



factors that are present at the core promoter region might be involved in genistein-induced repression of telomerase activity. There are two *c-myc*, five Sp1, one Ets, and two Inr binding sites present at the hTERT core promoter (22). Out of all these transcription factors, the role of c-Myc in the regulation of hTERT transcription have been studied extensively (15, 45–47). It has been shown that c-Myc binds to the E-boxes at the core promoter region of hTERT and activates hTERT transcription (15, 46). We observed that c-Myc regulates hTERT transcriptional activity in prostate cancer cells (Fig. 5). This result support the concept that c-Myc is a major regulator of hTERT transcription.

Based on the paramount importance of c-Myc in hTERT transcriptional activation, we examined the expression of c-Myc in genistein-treated or untreated DU-145 cells. We observed that similar to hTERT, c-Myc mRNA and protein levels were also decreased dose-dependently with genistein treatment (Fig. 5), suggesting that the repression of telomerase activity in prostate cancer cells could be via the decreased expression of c-Myc. These results are in agreement with some previous reports where c-Myc

protein and/or mRNA levels were decreased in mouse melanoma and in human colon cancer cell lines (50, 51) as well as in androgen-responsive prostate cancer cells, LNCaP (41). To understand the precise role of c-Myc in genistein-induced repression of telomerase activity in prostate cancer cells, we transfected c-Myc mutated hTERT core promoter constructs in DU-145 and PC-3 cells and then treated with genistein. We observed that the repressive activity of genistein prevailed even after mutation of c-Myc from the hTERT core promoter, suggesting that c-Myc is not solely involved in the inhibition of telomerase activity in prostate cancer cells by genistein as suggested by Ouchi et al. (41). Transcription factors that up-regulate telomerase activity, such as Ets or SP1, could also be involved in genistein-induced repression of telomerase activity. Although it is speculative at this time, future experiments would be necessary to confirm the role of Ets or SP1 in genistein-induced repression of telomerase activity.

In addition to the transcriptional regulation of hTERT, posttranslational modifications are also equally important for full

telomerase activity. hTERT is a phosphoprotein, and its activity is modulated by a complex set of protein kinases, providing additional control on telomerase regulation. It has been shown that protein kinase B/Akt is involved in phosphorylation of hTERT protein and the up-regulation of telomerase activity (26). Telomerase activity in human breast cancer cells is significantly inhibited by the treatment with protein phosphatase 2A (52). Treatment of human melanoma cells (SK-MEL 28) with the protein phosphatase inhibitor okadaic acid enhances both hTERT peptide phosphorylation and telomerase activity (26). However, when these melanoma cells were treated with the phosphatidylinositol 3-kinase inhibitor Wortmannin, hTERT peptide phosphorylation and telomerase activity were down-regulated. In this study, Kang et al. showed that the serine residue at position 824 of hTERT was phosphorylated by the Akt (26). In the present study, we observed that genistein treatment down-regulated phospho-Akt Ser⁴⁷³ and Thr³⁰⁸ dose-dependently in DU-145 cells without affecting the total Akt (Fig. 6). Moreover, phosphorylation of hTERT was also decreased significantly after genistein treatment. These results clearly suggest that genistein acts at the posttranslational level to down-regulate phosphorylation of hTERT via the Akt pathway. Deactivation of Akt by genistein treatment has been shown by earlier study (53). Beside Akt, PKC, another serine/threonine kinase, is also known to be involved in phosphorylation of hTERT and thereby telomerase activity (27). It is possible that in addition to the deactivation of Akt, genistein might also inhibit PKC and thereby suppress telomerase activity. Future studies will be required to determine the whether PKC is involved in genistein-induced dephosphorylation of hTERT.

It is now well established that cellular localization of hTERT could be a potential mechanism of telomerase activation through hTERT phosphorylation linked to nuclear localization (54). Nuclear translocation of hTERT from a presumably nonfunctional cytosolic location to a physiologically relevant nuclear compartment may be one mechanism of the regulation of the telomerase function in cells. It was previously reported that 14-3-3 proteins binds to and increases nuclear localization of hTERT (28). Similarly, NF- κ B p65 is also a posttranslational modifier of telomerase, which is involved in the intracellular localization of hTERT (29). Using immunofluorescent staining, we showed that genistein treatment decreased the nuclear staining for hTERT, and the residual staining was observed in the cytoplasm (Fig. 6), suggesting that genistein decreased the phosphorylation of hTERT and thereby was unable to bind its nuclear translocator or decreased phosphorylation of hTERT forced them to translocate from the

nucleus to the cytoplasm. It is also possible that genistein might down-regulate NF- κ B and/or 14-3-3 protein levels and thereby decrease their association with hTERT and eventually inhibits translocation to the nucleus. In fact, it has been shown that genistein decreases NF- κ B activation and protein expression in prostate cancer cells (55).

The question that might arise is how genistein-induced reduction of telomerase activity would be linked to the growth inhibition of cancer cells. We believe that in addition to telomere length regulation, telomerase acts as a growth-promoting factor and modulates the expression of various growth-promoting factors (basic fibroblast growth factor and epidermal growth factor receptor; 24, 25). In fact, some of our preliminary results indicate that number of growth-promoting factors and cell cycle regulatory components are regulated by hTERT. Therefore, it is possible that reduction of TERT expression by genistein will inhibit growth-promoting and cell cycle regulatory factors that will eventually control growth of cancer cells. Our preliminary result also indicates that genistein treatment enhances PTEN expression in DU-145 cells (data not shown) that could ultimately deactivate Akt. Deactivation of Akt will increase GSK3 α/β activity and eventually degrade c-Myc protein. Future studies will be necessary to define the precise molecular mechanism by which genistein deactivates Akt and down regulates c-Myc.

In summary, this study shows for the first time that genistein-induced repression of telomerase activity is multifactorial. Down-regulation of hTERT transcriptional activity as well as the posttranslational modifications of hTERT protein is involved in genistein-induced repression of telomerase activity in prostate cancer cells. Although the mechanisms of genistein-induced down-regulation of *c-myc* expression and deactivation of Akt are elusive at this point, this study clearly suggests that genistein could be a potential therapeutic agent to repress telomerase activity and for the treatment of various malignancies, including prostate cancer.

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References

- Greider CW, Blackburn EH. Identification of a specific telomere terminal transferase activity in *Tetrahymena* extracts. *Cell* 1985;43:405-13.
- Greider CW, Blackburn EH. A telomeric sequence in the RNA of *Tetrahymena* telomerase required for telomere repeat synthesis. *Nature* 1989;337:331-7.
- Kim NW, Piatyszek MA, Prowse KR, et al. Specific association of human telomerase activity with immortal cells and cancer. *Science* 1994;266:2011-5.
- Harley CB, Villeponteau B. Telomeres and telomerase in aging and cancer. *Curr Opin Genet Dev* 1995;5:249-55.
- Kilian A, Bowtell DD, Abud HE, et al. Isolation of a candidate human telomerase catalytic subunit gene, which reveals complex splicing patterns in different cell types. *Hum Mol Genet* 1997;6:2011-9.
- Feng J, Funk WD, Wang SS, et al. The RNA component of human telomerase. *Science* 1995;269:1236-41.
- Harrington L, McPhail T, Mar V, et al. A mammalian telomerase-associated protein. *Science* 1997;275:973-7.
- Holt SE, Aisner DL, Baur J, et al. Functional requirement of p23 and Hsp90 in telomerase complexes. *Genes Dev* 1999;13:817-26.
- Nakano K, Watney E, McDougall JK. Telomerase activity and expression of telomerase RNA component and telomerase catalytic subunit gene in cervical cancer. *Am J Pathol* 1998;153:857-64.
- Boldrini L, Faviana P, Gisfredi S, et al. Evaluation of telomerase in the development and progression of colon cancer. *Int J Mol Med* 2002;10:589-92.
- Boldrini L, Faviana P, Gisfredi S, et al. Evaluation of telomerase mRNA (hTERT) in colon cancer. *Int J Oncol* 2002;21:493-7.
- Park TW, Riethdorf S, Riethdorf L, Loning T, Janicke F. Differential telomerase activity, expression of the telomerase catalytic sub-unit and telomerase-RNA in ovarian tumors. *Int J Cancer* 1999;84:426-31.
- Kanaya T, Kyo S, Takakura M, Ito H, Namiki M, Inoue M. hTERT is a critical determinant of telomerase activity in renal-cell carcinoma. *Int J Cancer* 1998;78:539-43.
- Ducrest AL, Szutorisz H, Lingner J, Nabholz M. Regulation of the human telomerase reverse transcriptase gene. *Oncogene* 2002;21:541-52.
- Wu KJ, Grandori C, Amacker M, et al. Direct activation of TERT transcription by c-MYC. *Nat Genet* 1999;21:220-4.

16. Oh S, Song Y, Yim J, Kim TK. The Wilms' tumor 1 tumor suppressor gene represses transcription of the human telomerase reverse transcriptase gene. *J Biol Chem* 1999;274:37473-8.
17. Kyo S, Takakura M, Taira T, et al. Sp1 cooperates with c-Myc to activate transcription of the human telomerase reverse transcriptase gene (hTERT). *Nucleic Acids Res* 2000;28:669-77.
18. Yin L, Hubbard AK, Giardina C. NF-kappa B regulates transcription of the mouse telomerase catalytic subunit. *J Biol Chem* 2000;275:36671-5.
19. Fujimoto K, Kyo S, Takakura M, et al. Identification and characterization of negative regulatory elements of the human telomerase catalytic subunit (hTERT) gene promoter: possible role of MZF-2 in transcriptional repression of hTERT. *Nucleic Acids Res* 2000;28:2557-62.
20. Crowe DL, Nguyen DC, Tsang KJ, Kyo S. E2F-1 represses transcription of the human telomerase reverse transcriptase gene. *Nucleic Acids Res* 2001;29:2789-94.
21. Maida Y, Kyo S, Kanaya T, et al. Direct activation of telomerase by EGF through Ets-mediated transactivation of TERT via MAP kinase signaling pathway. *Oncogene* 2002;21:4071-9.
22. Li H, Lee TH, Avraham H. A novel tricomplex of BRCAl, Nmi, and c-Myc inhibits c-Myc-induced human telomerase reverse transcriptase gene (hTERT) promoter activity in breast cancer. *J Biol Chem* 2002;277:20965-73.
23. Nishi H, Nakada T, Kyo S, Inoue M, Shay JW, Isaka K. Hypoxia-inducible factor 1 mediates upregulation of telomerase (hTERT). *Mol Cell Biol* 2004;24:6076-83.
24. Smith LL, Collier HA, Roberts JM. Telomerase modulates expression of growth-controlling genes and enhances cell proliferation. *Nat Cell Biol* 2003;5:474-9.
25. Gorbunova V, Seluanov A. Telomerase as a growth-promoting factor. *Cell Cycle* 2003;2:534-7.
26. Kang SS, Kwon T, Kwon DY, Do SI. Akt protein kinase enhances human telomerase activity through phosphorylation of telomerase reverse transcriptase subunit. *J Biol Chem* 1999;274:13085-90.
27. Kim YW, Hur SY, Kim TE, et al. Protein kinase C modulates telomerase activity in human cervical cancer cells. *Exp Mol Med* 2001;33:156-63.
28. Seimiya H, Sawada H, Muramatsu Y, et al. Involvement of 14-3-3 proteins in nuclear localization of telomerase. *EMBO J* 2000;19:2652-61.
29. Akiyama M, Hideshima T, Hayashi T, et al. Nuclear factor-kappaB p65 mediates tumor necrosis factor alpha-induced nuclear translocation of telomerase reverse transcriptase protein. *Cancer Res* 2003;63:18-21.
30. Barnes S. Effect of genistein on *in vitro* and *in vivo* models of cancer. *J Nutr* 1995;125:777-83S.
31. Peterson G, Barnes S. Genistein and biochanin A inhibit the growth of human prostate cancer cells but not epidermal growth factor receptor tyrosine autophosphorylation. *Prostate* 1993;22:335-45.
32. Naik HR, Lehr JE, Pienta KJ. An *in vitro* and *in vivo* study of antitumor effects of genistein on hormone refractory prostate cancer. *Anticancer Res* 1994;14:2617-9.
33. Santibanez JF, Navarro A, Martinez J. Genistein inhibits proliferation and *in vitro* invasive potential of human prostatic cancer cell lines. *Anticancer Res* 1997;17:1199-204.
34. Zhou JR, Gugger ET, Tanaka T, et al. Soybean phytochemicals inhibit the growth of transplantable human prostate carcinoma and tumor angiogenesis in mice. *J Nutr* 1999;129:1628-35.
35. Fotsis T, Pepper M, Adlercreutz H, et al. Genistein, a dietary-derived inhibitor of *in vitro* angiogenesis. *Proc Natl Acad Sci U S A* 1993;90:2690-4.
36. Akiyama T, Ishida J, Nakagawa S, et al. Genistein, a specific inhibitor of tyrosine-specific protein kinases. *J Biol Chem* 1987;262:5592-5.
37. Okura A, Arakawa H, Oka H, Yoshinari T, Monden Y. Effect of genistein on topoisomerase activity and on the growth of [Val12]Ha-ras-transformed NIH 3T3 cells. *Biochem Biophys Res Commun* 1988;157:183-9.
38. Zwiller J, Sassone-Corsi P, Kakazu K, Boynton AL. Inhibition of PDGF-induced c-jun and c-fos expression by a tyrosine protein kinase inhibitor. *Oncogene* 1991;6:219-21.
39. Coyne DW, Morrison AR. Effect of the tyrosine kinase inhibitor, genistein, on interleukin-1 stimulated PGE2 production in mesangial cells. *Biochem Biophys Res Commun* 1990;173:718-24.
40. Jagadeesh S, Banerjee PP. Genistein suppresses growth of androgen-independent human prostate cancer cells by inhibiting telomerase activity. New Orleans (Louisiana): In 86th Annual Meeting of Endocrine Society; 2004. p. 373.
41. Ouchi H, Ishiguro H, Ikeda N, Hori M, Kubota Y, Uemura H. Genistein induces cell growth inhibition in prostate cancer through the suppression of telomerase activity. *Int J Urol* 2005;12:73-80.
42. Banerjee S, Banerjee PP, Zirkin BR, Brown TR. Regional expression of transforming growth factor-alpha in rat ventral prostate during postnatal development, after androgen ablation, and after androgen replacement. *Endocrinology* 1998;139:3005-13.
43. Banerjee PP, Banerjee S, Zirkin BR, Brown TR. Lobe-specific telomerase activity in the intact adult brown Norway rat prostate and its regional distribution within the prostatic ducts. *Endocrinology* 1998;139:513-9.
44. Kyo S, Takakura M, Kanaya T, et al. Estrogen activates telomerase. *Cancer Res* 1999;59:5917-21.
45. Wang J, Xie LY, Allan S, Beach D, Hannon GJ. Myc activates telomerase. *Genes Dev* 1998;12:1769-74.
46. Greenberg RA, O'Hagan RC, Deng H, et al. Telomerase reverse transcriptase gene is a direct target of c-Myc but is not functionally equivalent in cellular transformation. *Oncogene* 1999;18:1219-26.
47. Park NH, Guo W, Kim HR, Kang MK, Park NH. c-Myc and Sp1/3 are required for transactivation of hamster telomerase catalytic subunit gene promoter. *Int J Oncol* 2001;19:755-61.
48. Kumi-Diaka J, Sanderson NA, Hall A. The mediating role of caspase-3 protease in the intracellular mechanism of genistein-induced apoptosis in human prostatic carcinoma cell lines, DU145 and LNCaP. *Biol Cell* 2000;92:595-604.
49. Akalin A, Elmore LW, Forsythe HL, et al. A novel mechanism for chaperone-mediated telomerase regulation during prostate cancer progression. *Cancer Res* 2001;61:4791-6.
50. Heruth DP, Wetmore LA, Leyva A, Rothberg PG. Influence of protein tyrosine phosphorylation on the expression of the c-myc oncogene in cancer of the large bowel. *J Cell Biochem* 1995;58:83-94.
51. Yan CH, Chen XG, Li Y, Han R. Effects of genistein, a soybean-derived isoflavone, on proliferation and differentiation of B16-BL6 mouse melanoma cells. *J Asian Nat Prod Res* 1999;1:285-99.
52. Li H, Zhao LL, Funder JW, Liu JP. Protein phosphatase 2A inhibits nuclear telomerase activity in human breast cancer cells. *J Biol Chem* 1997;272:16729-32.
53. Park SS, Kim YN, Jeon YK, et al. Genistein-induced apoptosis via Akt signaling pathway in anaplastic large-cell lymphoma. *Cancer Chemother Pharmacol* 2005;56:271-8.
54. Liu K, Hodes RJ, Weng NP. Cutting edge: telomerase activation in human T lymphocytes does not require increase in telomerase reverse transcriptase (hTERT) protein but is associated with hTERT phosphorylation and nuclear translocation. *J Immunol* 2001;166:4826-30.
55. Davis JN, Kucuk O, Sarkar FH. Genistein inhibits NF-kappa B activation in prostate cancer cells. *Nutr Cancer* 1999;35:167-74.