Bone Morphogenetic Protein-7 Inhibits Telomerase Activity, Telomere Maintenance, and Cervical Tumor Growth

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Abstract
Telomere maintenance is critical in tumor cell immortalization. Here, we report that the cytokine bone morphogenetic protein-7 (BMP7) inhibits telomerase activity that is required for telomere maintenance in cervical cancer cells. Application of human recombinant BMP7 triggers a repression of the human telomerase reverse transcriptase (hTERT) gene, shortening of telomeres, and hTERT repression–dependent cervical cancer cell death. Continuous treatment of mouse xenograft tumors with BMP7, or silencing the hTERT gene, results in sustained inhibition of telomerase activity, shortening of telomeres, and tumor growth arrest. Overexpression of hTERT lengths telomeres and blocks BMP7-induced tumor growth arrest. Thus, BMP7 negatively regulates telomere maintenance, inducing cervical tumor growth arrest by a mechanism of inducing hTERT gene repression. [Cancer Res 2008;68(22):9157–66]

Introduction
Bone morphogenetic proteins (BMPs) operate through autocrine and paracrine mechanisms to regulate cell proliferation, differentiation, and apoptosis during development (1, 2). Similar to other transforming growth factor-β (TGF-β) family members, BMPs bind cell membrane type I and II receptors of serine/threonine kinases eliciting intracellular signaling via Smad1, Smad5, Smad8 and Smad9 proteins (3, 4), and the Lim kinase (5, 6), and mitogen-activated protein kinase pathways (7, 8). BMPs are present in cancers including breast, prostate, and colon cancers (9–11) where BMPs are implicated in regulating cancer cell proliferation toward immortalization (8, 12–14). BMP7 (osteogenic protein-1) is a potent inducer of cell differentiation required for vertebrate development, deletion of which is postnatal-lethal with multiple defects in differentiated tissues (15). Recent studies show that BMP7 maintains epithelial and endothelial phenotypes against epithelial-mesenchymal transition (16, 17). BMP7 inhibits cancer cell proliferation causing apoptosis of myeloma cells (12) and prostate cancer cells (13, 18), and inhibits breast (19) and prostate (16) cancer metastasis. However, apart from BMP7 antagonizing TGF-β–mediated proinvasive and protumorigenic effects (16, 17, 19), the exact mechanisms in gene expression underlying these inhibitory actions of BMP7 in cancer remain unknown.

Cancer cells hold unlimited proliferative potential as the cells maintain their telomeres (ends of chromosomes) when they divide. Most cancer cells maintain telomeres by mechanisms requiring the ribonucleoprotein complex telomerase (20). Telomerase is thus crucial to cancer cell proliferation toward immortalization. Containing human telomerase reverse transcriptase (hTERT), human RNA template component, and its binding protein dyskerin, telomerase catalyzes telomeric DNA synthesis and protects telomere ends (21–23). As the catalyst of telomerase complex, hTERT is rate limiting and expressed specifically in most cancers to maintain short telomeres. Ectopic expression of hTERT lengthens telomeric DNA, stabilizes telomeres, and mediates cell immortalization (24, 25). Inhibition of hTERT by gene silencing, dominant-negative gene expression, or substrate competitive inhibitors accelerates telomere shortening and thereby triggers cancer cell senescence and apoptosis in cell cultures and tumors (24, 25).

To date, little is known of how hTERT is regulated in cells by extracellular cues. Considerable evidence indicates that when normal cells differentiate in most tissues and organs, hTERT gene expression becomes repressed, conferring a limited length of telomeres and therefore proliferative potential on cells (26, 27). Repression of the hTERT gene occurs at the gene transcription level under a concerted regulation by a number of transcription factors and repressors (28–30). We hypothesize that extracellular factors regulating cell differentiation exert an effect on hTERT gene expression, telomerase activity, and telomere maintenance through intracellular signaling. Studies from our laboratory and others show that TGF-β can induce an hTERT gene repression (30–35). In the present study, we have examined effects of the BMP family on telomerase activity and telomere maintenance in cervical cancer cells. We show that BMP7 induces sustained telomerase inhibition and telomere shortening in vitro and in vivo, resulting in cancer cell apoptosis by a mechanism involving the repression of the hTERT gene and telomerase activity.

Materials and Methods
Cytokines, gene expression plasmids, and antibodies. Human recombinant BMP 2, 4, 5, 6, and 7 were from R & D systems. Plasmid pEGFP-C1, pEGFP-C1-hTERT, and pEGFP-C1-hTERT shRNA were produced in this laboratory. For hTERT shRNA, the oligonucleotide (5′-AATT-CAAAAAAGGTCTTTCTACCAGAGGTGCTTCTCTTGAAATCATCTCTGG-¶-3′) was annealed and cloned to the EcoRI site downstream of the U6 promoter. All plasmids were verified by DNA sequencing. The primary antibodies of mouse anti-c-myc, mouse anti-p53, and mouse anti-p21 were from Santa Cruz Biotechnology, rabbit anti-p16 were from Cell Signaling Technology, mouse anti-actin were from Chemicon, and horseradish peroxidase–coupled secondary antibodies were from Dako.

Cell culture and treatment. Human cervical cancer HeLa cells were grown in 5% CO2 atmosphere at 37°C in DMEM (Invitrogen) containing 10% fetal bovine serum (FBS) in 6-well plastic plates or 10-cm dishes (Nunc). Recombinant BMP proteins at concentrations of 0.1, 0.3, 3, 10, and 30 ng/mL were added to cell cultures for various periods of time as indicated in individual experiments, in which the serum concentration was 0.5% in the cell culture medium. Cells were lysed in ice cold CAHPS lysis buffer (0.5% 3-[[(Cholamidopropyl)dimethylammonio]-1-propanesulfonate, 10 mmol/L Tris (pH 7.5), 1 mmol/L MgCl2, 1 mmol/L EGTA, 0.1 mmol/L benzamidine, 5 mmol/L β-mercaptoethanol, and 10% glycerol), in the

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presence of complete mini protease inhibitors (Roche Diagnostic). Clarified cell lysates were normalized for total protein concentrations by the Bradford protein assay (Bio-Rad). To reverse BMP7 inhibition of telomerase, green fluorescent protein (GFP)-hTERT was expressed by transfection with pEGFP-C2-hTERT plasmid, with pEGFP-C2 plasmid as control. Cell transfection was conducted using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instruction. After 24 h of transfection, cells positive for GFP were isolated by fluorescence-activated cell sorting, cultured, and incubated with BMP2, 4, 5, 6, or 7 for different periods of time and analyzed in Western blotting, semi quantitative reverse transcription-PCR (RT-PCR) for gene expressions, telomerase activity assay, and cell death analysis, as indicated in individual experiments.

**FACS.** GFP-transfected cells were sorted using FACSaria flow cytometer (BD Biosciences). The GFP-positive cells were reseeded into 6-well plates at a density of 0.2 × 10^6 cells/mL in DMEM plus 10% FBS including Gentamicin antibiotics (Pfizer). For apoptosis analysis, after treatment of cells as indicated in individual experiments, cells were dislodged from tissue culture plates using 0.5M EDTA for 5 min at 37°C and incubated with annexin-V-FLUOS conjugate (Roche Diagnostic) and propidium iodide (PI; Sigma) for 15 min at room temperature in an incubation buffer that facilitates binding per the manufacturers’ instructions. The cells were then analyzed using FL-2 and FL-3 channel, respectively. Percentages of stained apoptotic cells were determined using a FASCalkarib flow cytometer (BD Biosciences). An acquisition gate was set to include ~ 20,000 of the centrally located cells for each sample acquisition using linear forward scatter versus linear side scatter. This acquisition strategy resulted in ~ 40,000 ungated events being included for each sample analysis. Dot-plot integration was determined from the background fluorescence using unstained HeLa cells. This integration cursor placement remained unchanged when stained HeLa cells were analyzed.

**Preparation of protein extracts and Western blotting.** These procedures were described previously (30). Briefly, tumor cells were treated by CHAP lysis buffer, including protein inhibitors (Roche Diagnostic), at 4°C and homogenized immediately. The lysates were incubated for 10 min on ice and then centrifuged for 45 min at 5,000 × rpm, then centrifuged again for 30 min at 13,000 rpm. The supernatants were collected and stored at −80°C. Extracted proteins (~ 25 μg) in SDS sample buffer were boiled and electrophoresed on a 10% SDS-polyacrylamide gel and electroblotted onto a pre-wet nitrocellulose membrane (Bio-Rad Laboratories). The blotted nitrocellulose membrane was blocked in PBS containing 5% skim milk and 0.02% Tween 20, and probed with antibodies as indicated in individual experiments at 4°C overnight. The blots were developed using enhanced chemiluminescence Western blotting detection system (Amersham Biosciences).

**RNA isolation and gene expression analysis.** As previously described (30), total RNA was extracted by TRIzol reagent (Invitrogen) according to the manufacturer's instructions. The RNA (1 μg) was transcribed by oligo(dT) and ThermoScript Reverse Transcriptase (Invitrogen) in a volume of total 21 μL. Contaminants were removed from the samples by the Clone H treatment, following the manufacturer's instruction (Invitrogen). Linear amplification for semiquantitative PCR was performed using ThermoScript RT-PCR kit following the manufacturer's instruction (Invitrogen), for 25 to 35 cycles of 30 s at 72°C as the optimized annealing temperature and 64°C extension. Primers specific for different genes were as follows: hTERT (5'-CCACCTTGACAAAGTACG-3') and (5'-GGCTCAGACTGGCTTCAT-3'), and Actin (5'GCTGTCGTCGACAGAACGGCT-3') and (5'-CAGATGATCCTGCTCTTCC-3'). Actin was used as control. PCR products were mixes with 6 × loading dye (30% glycerol, 0.5 μL/EDTA, and Bromophenol Blue) and run on 1.5% agarose gel, visualized in the presence of ethidium bromide, photographed in a gel 1000 UV documentation system (Bio-Rad), and analyzed by densitometry.

**Telomerase activity assay.** Telomerase activity was determined by TRAP as described previously (36). Briefly, cells treated with different reagents were washed and lysed by detaching and passing the cells though a 261/2G needle attached to a 1-mL syringe in prechilled TRAP lysis buffer. Equal amounts of telomeric protein extract (0.4 μg) were incubated with telomeric DNA substrate and deoxynucleotide triphosphate, and newly synthesized telomeric DNAs were observed after PCR using specific telomeric DNA primers and [α-32P]ATP (Amersham Biosciences), polyacrylamide slab gel electrophoresis, and autoradiography. As an internal control of the PCR and loading, the primers NT (ATCCGTCCTC-GGCTTTTTT) and TSNT (AATCGCTGACAGACAGTTAAAGGGCG-GAAGCGAT) were included in the reaction.

**Telomere length analysis.** For telomeres in cultured cells, metaphase spreads from cycling HeLa cells that were treated with or without BMP7 (30 ng/mL, 15 h) thrice per week for 2 wk were generated using standard laboratory protocols. The slides of metaphase cells or tumor sections were fixed in 4% formaldehyde before treatment with acidified 1% pepsin solution, and hybridized with the probe solution [0.3 μg/mL Cy5-conjugated [CCCTAA], PNA probe (Panagene), 70% formamide, 20 mmol/L Tris-HCl (pH 7.0), 1% bovine serum albumin (BSA)]. Washing was conducted in PBS/Tween 20 with one high stringent wash at 57°C. DNA was counterstained with 4,6-diamidino-2-phenylindole, visualized, and captured using Nikon Eclipse TE2000 microscope, Plan Fluor ×40 objective, DS-5MC CCD camera, and NIS-Elements F 2.20 software (Nikon). Telomere images were captured with a Plan Fluor ×100 oil emersion objective, and individual telomere fluorescence was integrated using spot IOD analysis in the TFL-Telo 2.2 program (gift from Dr. Peter Lansdorp, Vancouver, Canada; ref. 37). Images from at least 13 metaphase spreads from each data point were quantified before assembly of data in a standard spreadsheet program. At least 50 cell nuclei from each condition were analyzed.

**Tumor cell growth analysis in soft agar.** Tumor cell colonies were grown in soft agar to assess the effects of BMP7 on tumor growth in vitro. Two milliliters of 2% DMEM containing 20% FBS were used to make 0.5% agar (base layer) with or without BMP7 in a 35-mm culture dish or 6-well plate. On top of the base layer, a mixture of serum supplemented medium and 0.35% agar (2 mL) containing 2,500 HeLa cells in the presence or absence of BMP7 (100 ng/mL or as indicated; top layer) was added. The dishes were kept in tissue culture incubator at 37°C and 5% CO2 for 14 d to allow for colony formation and growth. All assays were performed in triplicates. The colony assay results were photographed or scanned after the plates were stained with methylthiazolyltetrazolium (MTT).

**Tumor xenografting and treatment procedure.** HeLa cells were xenografted in female nude mice (BALB/c Nude; Animal Resources Centre ARC). Cultured monolayer cells were detached by trypsinization, washed in PBS, and counted. Approximately 10 × 10^6 cells were resuspended in 0.1 mL PBS and inoculated s.c. in the flank of the mice. Twenty-four hours after inoculation, BMPs and other reagents prescribed as indicated in individual experiments were administered into the xenografts on every second day. The development of xenograft tumors was measured with Vernier callipers after 3 weeks. The mice were maintained under specific pathogen-free conditions at constant temperature (~ 22°C) and humidity (~ 40%). Sterilized food and water were given ad libitum.

**Tumor tissue sectioning and staining.** Freshly dissected tumor samples were embedded in orthocene carmaloy transfere compound and frozen using isopentane cooled with liquid nitrogen. Five-micrometer fresh-frozen sections were prepared and used to stain for the Ki67 antigen, a marker of cell proliferation. Sections were fixed in 4% paraformaldehyde/10% sucrose, washed once in PBS, and blocked with 5% BSA (Fraction V; Sigma) for 30 min. Sections were incubated with polyclonal rabbit anti-Ki67 antibodies (ab15580; Abcam) at 4°C overnight at a 1:400 dilution. Primary antibody was detected with Cy3-conjugated goat anti-rabbit antibodies (Chemicon) at 90 min at a 1:2000 dilution. Nuclei were stained with 0.5 μg/mL Hoechst 33258 (Sigma). The staining was visualized by fluorescence microscopy. Five random images within the tumor area were analyzed in 3 sections, ~ 5 μm apart, for each treatment group.

**Statistical analysis.** Data were analyzed using Student’s t tests and a P value of <0.05 was considered statistically significant.
Results

BMP7 induces telomerase inhibition and telomere shortening in cultured cancer cells. To investigate a potential role of the BMP family in telomerase activity, we examined effects of BMP2, BMP4, BMP5, BMP6, and BMP7 by spiking the medium of cancer cell cultures with purified recombinant human cytokines. Incubation of human cervical cancer HeLa cells with different concentrations of BMP7 for 48 hours resulted in significant inhibition of telomerase activity (Fig. 1A). The maximal inhibition was ~85% inhibition of telomerase activity, achieved with the median inhibitory concentration (IC50) of BMP7 of 8 ± 0.6 ng/mL and the maximal inhibitory concentration of 35 ± 1.4 ng/mL (n = 3; Fig. 1A). Incubations of the cells with BMP2, BMP4, BMP5, or BMP6 showed no significant inhibitory effect on telomerase activity (data not shown). In the time course studies, BMP7 (10 ng/mL) induced telomerase inhibition in 24 hours of the treatment for ~3 days (Fig. 1B). To determine the inhibitory effect on telomerase in another cancer cell type, we tested human breast cancer PMC42 cells with different concentrations of BMP7. As shown in Fig. 1C, BMP7 induced significant inhibition of telomerase activity in a dose-dependent manner in PMC42 cells.

To verify that the inhibition of telomerase activity in HeLa and breast cancer cells was at the levels of gene expression of hTERT, we measured hTERT mRNA by semiquantitative RT-PCR. As shown in Fig. 1D, BMP7 induced a significant down-regulation of hTERT gene expression in both HeLa and PMC42 cells. In addition, we noted that BMP7 also induced a significant down-regulation of c-myc gene expression (Fig. 1D). The inhibition of c-myc gene expression was ~80% of control, and the inhibition of hTERT gene expression was ~70% of the control (Fig. 1D).

To determine the effect of BMP7 on telomeres, we treated HeLa cells with 30 ng/mL of BMP7 for 15 hours each time on every second day for 2 weeks. The pulsatile treatments of cultured HeLa cells with BMP7 resulted in a sustained telomerase inhibition and significant shortening of telomeres as measured by quantitative fluorescence in situ hybridization (Q-FISH), using a specific telomeric DNA probe in cell metaphase spreads. As shown in Fig. 2A, BMP7 treatments of cultured HeLa cells caused a marked shift of the telomere fluorescence peak compared with the control. The size of telomeres in the control cells was ranged with a major peak of >100 frequencies between ~300 and ~1,200 fluorescence unit. In
contrast, the telomeres in the BMP7-treated cells were shown as a major peak with a relatively narrow range exhibiting between 100 and 1,100 fluorescence unit. The highest frequency of telomere fluorescence in control cells occurred at 800 to 900 fluorescence units, whereas the highest frequency of telomere fluorescence in BMP7-treated cells occurred at 500 to 600 fluorescence units showing significantly shortened telomeres compared with the control (Fig. 2A). Analyzing the mean telomere length in control and BMP7-treated cells showed that the mean telomere length in the BMP7-treated group could be ~25% shorter than that in the controls (Fig. 2B). The fluorescence micrographs in Fig. 2C showed typical images of telomere fluorescence (yellow dots) in different sizes at the ends of chromosomes (blue) between control (left) and BMP7-treated (right) HeLa cells.

**BMP7 induces cancer cell death by a mechanism largely dependent on hTERT gene repression.** To determine the functional consequence of BMP7-induced telomerase inhibition and telomere shortening, we examined the changes in cell number and apoptotic death in the cell cultures treated with or without different cytokines over a time course of several days. As shown in Fig. 3A, whereas the cells underwent population doubling in the control group, a single-dose treatment of HeLa cells with BMP7 (10 ng/mL) resulted in a marked reduction of total cell number. The reduction of cell number in the cell cultures treated with BMP7 was ~50% on each day of the controls treated with diluent, BMP2, BMP4, BMP5, or BMP6, showing a complete arrest of cell population doubling by the presence of BMP7 (Fig. 3A). Treatment of the cells with different concentrations of BMP7 for 24 hours showed a concentration-dependent reduction of cell numbers (data not shown). To determine if BMP7 induces cancer cell apoptosis, we examined HeLa treated with or without BMP7 for the apoptotic markers of Annexin V and PI staining by FACS. As shown in Fig. 3B, a single dose of BMP7 treatment resulted in a significantly increase in HeLa cell apoptosis compared with controls (7% versus 17%).

To investigate if BMP7-induced cell death is a consequence of hTERT gene repression and telomerase inhibition, we analyzed the effect of hTERT gene expression on BMP7-induced cell death in HeLa cell cultures. Underexpression of hTERT with hTERT shRNA for 48 hours mimicked the cell killing effect of BMP7 (Fig. 3B), whereas overexpression of hTERT for 24 hours before BMP7 treatment inhibited subsequent BMP7-induced cell death (Fig. 3B), suggesting that the proapoptotic effect of BMP7 is mediated in a significant part by hTERT repression. To further attest the role of hTERT in the BMP7-induced cell death, we transfected HeLa cells with GFP-hTERT, GFP-hTERT shRNA, or GFP-only gene expression plasmids, then isolated the GFP-positive transformants by FACS and treated the cells with BMP7 (10 ng/mL) for 24 hours. In these...
homogenously transfected cell cultures, we found that the expression of GFP-hTERT significantly decreased the levels of cell death induced by BMP7 (from 10-22% versus from 10-17%; Fig. 3C), corroborating that hTERT repression is required to a significant degree in BMP7-induced cell death. Consistently, down-regulation of hTERT with GFP-hTERT shRNA increased cell death above basal levels (Fig. 3C). Consistent with a significant role of telomerase inhibition in mediating BMP7-induced apoptosis, BMP7 did not induce significant cell death in the telomerase-negative GM847 and Saos2 cell cultures (Fig. 3D). Verification of BMP receptors by Western blotting with specific antibodies confirmed the presence of both BMPR1A and BMPR2 receptors in Saos2 and HeLa cell lines, although BMPR1A was not detectable in GM847 cells (data not shown).

BMP7 inhibits tumor growth with hTERT gene repression and telomere shortening in mouse xenograft tumors. To investigate if BMP7 inhibits tumor growth, we examined the effect of BMP7 on tumor cell anchorage-independent growth in soft agar assays. As shown in Fig. 4A (left), numerous tumor colonies grew from the HeLa cell suspension under basal conditions. In the presence of BMP7, however, there was a dramatic inhibition of tumor colony formation and growth, resulting in <15% colonies of the control (Fig. 4A). The inhibition was concentration dependent, with the IC50 and maximal inhibition concentrations being 4 and 20 ng/mL, respectively (Fig. 4A, right). To attest the effect of BMP7 on tumor growth in vivo, we exploited the xenograft tumor model in immune-deficient nude mice and carried out intratumor injection of different concentrations of BMP7 on every second day for 2 weeks. BMP7 markedly inhibited tumor growth compared with the controls (Fig. 4B). Significant tumor growth inhibition was observed after the first one or two injections of 10 ng/mL of BMP7, with maximal inhibition observed after three injections (Fig. 4B). Inhibition was concentration dependent, with the IC50 being ~10 ng/mL and maximal inhibitory concentration of 30 ng/mL of BMP7 (Fig. 4C). The effective concentrations were comparable with that inducing telomerase inhibition (Fig. 1A) and inhibiting thymidine incorporation in human myeloma cells (12). In 2 weeks of the treatment, BMP7 induced a maximal tumor growth inhibition by ~75% of the controls (Fig. 4C), whereas no significant inhibitory effect was observed on the tumors receiving BMP2, BMP4, BMP5, or BMP6 under the same experimental conditions (Fig. 4B).

To determine the intermediate role of hTERT gene repression in the BMP7-induced tumor growth inhibition, we carried out underexpression and overexpression of hTERT in the xenograft
Figure 4. BMP7 inhibition of tumor growth. A, micrograph of BMP7 suppression of tumor growth in soft agars. HeLa cells ($3 \times 10^7$) in 0.35% soft agar suspension were incubated with or without BMP7 at 10 ng/mL (left) or different concentrations (right) for 7 d. Tumor colonies were observed after staining with MTT. Columns, mean from four different determinations; bars, SD. B, effects of different BMPs on xenograft tumor growth in mice. HeLa cells ($3 \times 10^7$) were inoculated in nude mice s.c. Different BMPs (10 ng/mL in 50 µL PBS) were injected in each xenograft 24 h after inoculation and then on every second day for 2 wk. Tumor growth was measured before each injection. Columns, mean from multiple experiments (22 animals in PBS groups, 24 animals in BMP7-treated groups, and 4 to 8 animals in groups receiving other BMPs); bars, SD. *, significant difference from PBS control with $P < 0.01$. C, dose-dependent effect of BMP7 on xenograft tumor growth in mice. BMP7 of different doses was administered as indicated for every second day for 2 wk. Columns, mean from four animals in each group; bars, SD. D, expression of hTERT reverses BMP7-induced tumor growth inhibition. Empty plasmid, hTERT shRNA expression plasmid, or hTERT wild-type gene expression plasmid was intratumor injected 1 d after tumor inoculation and continued on every second day for 2 wk. One day after injections of empty plasmid and hTERT wild-type gene expression plasmid, and 2 d after HeLa cell inoculation, BMP7 (10 ng/mL) was administered, which was continued on every second day for 2 wk. Columns, mean from four animals in each treatment group; bars, SD. *, significant differences from that in PBS control.
and determined the effect of BMP7 in different backgrounds of \( hTERT \) gene expression on tumor growth. Significant inhibitory effect on tumor growth was observed in the animals treated by intratumor injection of the \( hTERT \) shRNA expression plasmid (Fig. 4D). Overexpression of wild-type \( hTERT \) has no significant effect on tumor growth in the cohort receiving intratumor injection of \( hTERT \) expression plasmid. However, overexpression of \( hTERT \) significantly compromised the BMP7-induced inhibition of tumor growth, reversing tumor growth inhibition from 25% back to 70% to 80% of the control after 2 weeks of treatments (Fig. 4D). Examinations of telomerase activity in the tumors treated with or without BMP7 showed that telomerase activity was almost completely inhibited in the tumor tissues treated with BMP7 for 2 weeks, in contrast to the controls and other groups receiving treatments with BMP2, BMP4, BMP5, or BMP6 (Fig. 5A and B). To determine altered gene expressions, we found that BMP7 treatment rendered a marked inhibition of the endogenous \( hTERT \) gene expression after 2 weeks of treatment (Fig. 5C and D). In addition, BMP7 treatment also induced a significant inhibition of the oncogene \( c-myc \) gene expression (Fig. 5C and D), suggesting that BMP7 induces the repression of the \( hTERT \) gene through a transcription-dependent mechanism involving \( c-myc \) gene repression (30, 33). Furthermore, corresponding with a BMP7-triggered deregulation of telomeres and its associated DNA damage response, we observed significant increases in the levels of p53 and p16 tumor suppressors in the tumors treated with BMP7 (Fig. 5C and D). The increases of p53 and p16 were significant compared with the control, reflecting an activation of the cell cycle checkpoints and arrest of the cell cycle (Fig. 5D).

Examination of telomeres in the tumor tissues showed that the average telomere length in the tumors treated with BMP7 for 2 weeks was significantly shorter than that in the controls (Fig. 6A and B). The shortening of telomeres induced by the pulsatile treatment with BMP7 for 2 weeks was 25% to 30% of the controls. On the contrary, overexpression of \( hTERT \) resulted in lengthened telomeres to ~130% of the control (Fig. 6A and B) in association with a significant reversal of BMP7-induced tumor growth arrest (Fig. 4D). Consistent with a functional consequence of telomere shortening, we observed a significant decrease of cell proliferation as indicated by Ki67 staining in BMP7-treated tumors. As shown in Fig. 6C and D, BMP7 elicited an inhibition of cell proliferation by ~60% as estimated in Ki67 staining on tumor tissue sections. These \textit{in vivo} findings from the xenograft tumors mirrored the findings from cell culture studies, demonstrating that BMP7 triggered an intracellular signaling pathway leading to repressed gene expressions of \( c-myc \) and \( hTERT \), increased gene expressions of cell cycle checkpoints, inhibition of telomerase activity, shortening of telomeres, and cancer cell apoptosis.

**Discussion**

The maintenance of telomeres is critical for cancer cell proliferation particularly as telomeres are already very short in cancer cells (21, 24). Inhibition of telomere maintenance offers an important mechanism to inhibit cancer cell proliferation. In this study, we show that the cytokine BMP7 triggers an inhibition of telomere maintenance and cervical cancer cell growth arrest \textit{in vitro} and in tumor xenographs. A single application of recombinant BMP7 produces a significant inhibition of telomerase activity critical in telomere maintenance in cultured cervical cancer HeLa cells. Continuous applications of BMP7 on every second day for 2 weeks in HeLa cell cultures or xenograft tumors

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**Figure 5.** BMP7 induces telomerase inhibition, \( hTERT \) gene repression, and p53 and p16 gene activation in mouse xenograft tumors. A. effects of BMPs on telomerase activity in tumors. Tumors were treated with or without different BMPs (10 ng/mL) on every second day for 2 wk. Telomerase activity in the tumors was measured by TRAP. B, telomerase activity quantified by densitometry. Columns, mean (\( n = 3 \)); bars, SD. Telomere DNA and internal control are indicated. C, altered gene expressions of \( hTERT \), \( c-myc \), and p53 and p16 in the xenograft tumors treated with BMP7. Quantitative data of gene expressions of \( c-myc \), \( hTERT \), p53, p16, and actin in tumors treated with and without BMP7 as indicated. Data were obtained by densitometry; columns, mean from three determinations; bars, SD. D, semiquantitative RT-PCR determination of \( hTERT \) and \( c-myc \), and Western blotting for p53 and p16. The results are representative of multiple experiments.
involving repression of the hTERT expression in the tumor cells. We identify that the effect of telomerase activity by modifying specific programs of gene expression pattern of decreased hTERT and c-myc, and increased p16 and p53. Furthermore, BMP7 induces inhibition of cancer cell anchorage–independent growth in soft agar and xenograft tumor growth. These findings suggest that telomere homeostasis is subject to regulation by extracellular cytokines in which BMP7 plays a significant role in eliciting a negative regulation of telomere maintenance and cell proliferative capacity in cancer. Furthermore, our data also suggest that BMP7 has a potential utility in anticancer effect by targeting cancer cell telomere maintenance.

The specificity of BMP7-induced inhibition of telomerase activity, telomere maintenance and cancer cell proliferation was shown by testing different BMPs. The dose-dependent effect of BMP7, with an effective concentration within a physiologically relevant range (11, 38, 39), supports a specific effect of BMP7 mediated by BMP7 receptors on tumor cell surface. Time course studies showing that BMP7 inhibition of telomerase activity occurs in 24 hours of treatment further suggest that BMP7 inhibits telomerase activity by modifying specific programs of gene expression in the tumor cells. We identify that the effect of BMP7 on cancer cell apoptosis is mediated by a mechanism involving repression of the hTERT gene and inhibition of telomerase activity. Underexpression of hTERT mimics the cell killing effect of BMP7, whereas overexpression of hTERT significantly inhibits the BMP7-induced cell death. Although future studies are required to investigate the mechanisms underlying BMP7-induced hTERT gene repression and telomere shortening, the findings that c-myc is down-regulated in association with hTERT down-regulation suggest that repression of c-myc contributes to the repression of the hTERT gene induced by BMP7.

Previous studies show that BMP7 induces apoptosis of myeloma cells (12) and prostate cancer cells (13, 18). Consistently, our studies show that BMP7 also induces cervical cancer cell apoptosis. In addition, we show for the first time that BMP7 inhibits cervical tumor growth in mice. Our in vivo findings in cervical cancer cells are consistent with recent studies showing BMP7 antitumor effect in breast cancer (19) and prostate cancer (19). Furthermore, we have found that BMP7 inhibits telomerase activity and induces telomere shortening as a fundamental mechanism in BMP7-induced cell death. In line with the recent findings that BMP7 maintains epithelial cell phenotypes, and inhibits epithelial-mesenchymal transition and cancer cell metastasis (16, 17, 19), our data suggest that BMP7 serves a function to inhibit cancer cell proliferation by inducing intracellular signaling to counteract mitogenic stimulation of telomerase activity by factors such as c-myc. Endogenous BMP7 produced in cancer cells may thus be a protective mechanism against oncogenic development, and reinforcement with exogenous recombinant BMP7 produces an imbalance in favor of BMP7 predominance by counterbalancing the actions of oncogenic growth factors and cytokines in extracellular microenvironment of cancer. BMP7-induced inhibition of telomerase activity and shortening of telomeres may therefore represent a powerful mechanism to intercept the process of immortalization of neoplastic cells that use telomerase to maintain telomeres. To the cancer cells that adopt alternative mechanisms to maintain telomeres, BMP7 seems ineffective as we show that BMP7 does...
not induce significant cell death of Saos2 cells that express BMP7 receptors and are telomerase negative. Because BMP7 inhibition of prostate cancer cell proliferation is also dependent on the subtypes of derived cell lines (18), it would be interesting to determine if cells that respond differently to BMP7 without undergoing apoptosis assume a different mechanism to stabilize telomeres during continuous proliferation. However, our findings that constant presence of high levels of BMP7 results in sustained telomere inhibition and telomere shortening illustrate a novel model in cytokine therapeutic intervention of cancer development through targeting telomerase maintenance of telomeres. It is currently thought that deregulation of telomeres instigates rapid cell death via mechanisms including telomere decapping and DNA damage response. Our observation that BMP7 induces telomere inhibition in association with telomere arrest (42, 43). In support of telomere DNA damage response, we show that BMP7 induces increased p16 and p53 cell cycle checkpoint activities. Critical in tumor cell aging and death (44, 45), increased p16 and p53 activities have previously been shown to be involved in coupling telomere deregulation with cell senescent and apoptotic response (46, 47). Thus, BMP7-induced cancer cell death may be prompted by a series of interchanges including telomere inhibition, telomere shortening, telomere-associated DNA damage responses, and subsequent cell senescence and apoptosis (48–50). In summary, our data show a novel mechanism of BMP7-negative regulation of telomere maintenance and cell proliferation in cancer by a mechanism of hTERT gene repression and telomerase inhibition in vitro and in vivo. Our data suggest that telomeres undergo continuous remodeling, which can be reprogramming by defined combinations of extracellular factors, with BMP7 serving as a major negative regulator. Further studies should unveil a complete picture of extracellular regulation of telomere remodeling by various factors in cancer and other highly proliferative cells such as stem cells.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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