Telomerase underpins stem cell renewal and proliferation and is required for most neoplasia. Recent studies suggest that hormones and growth factors play physiological roles in regulating telomerase activity. In this report we show a rapid repression of the telomerase reverse transcriptase (TERT) gene by transforming growth factor β (TGF-β) in normal and neoplastic cells by a mechanism depending on the intracellular signaling protein Smad3. In human breast cancer cells TGF-β induces rapid entry of Smad3 into the nucleus where it binds to the TERT gene promoter and represses TERT gene transcription. Silencing Smad3 gene expression or genetically deleting the Smad3 gene disrupts TGF-β repression of TERT gene expression. Expression of the Smad3 antagonist, Smad7, also interrupts TGF-β-mediated Smad3-induced repression of the TERT gene. Mutational analysis identified the Smad3 site on the TERT promoter, mediating TERT repression. In response to TGF-β, Smad3 binds to c-Myc; knocking down c-Myc, Smad3 does not bind to the TERT gene, suggesting that c-Myc recruits Smad3 to the TERT promoter. Thus, TGF-β negatively regulates telomerase activity via Smad3 interactions with c-Myc and the TERT gene promoter. Modifying the interaction between Smad3 and TERT gene may, thus, lead to novel strategies to regulate telomerase.

Telomerase reverse transcriptase (TERT) \(^4\) is required to regulate the structures of chromosomal ends (telomeres) for continuous cell division during embryonic development, stem cell renewal and proliferation, and cancer cell immortalization. TERT interacts with telomere DNA and telomere-binding protein, catalyzing telomeric DNA reverse transcription and telomere end capping (1–6). In the absence of TERT, telomeres undergo shortening for about 150 base pairs and rearrangement of telomere structure in each cell cycle. Short telomeres or uncapped telomere ends subsequently trigger cell senescence or apoptosis. Although TERT is expressed and telomerase is active during embryro development, TERT is down-regulated, and telomerase activity becomes suppressed during cell differentiation to mature somatic cells. This occurs in association with a gradual loss of cell proliferative potential. Although the role(s) of TERT repression in cell differentiation remains to be established (7–10), expression of TERT mobilizes stem cells from cell renewal to proliferation (11, 12) and extends cell proliferative lifespan toward immortality under certain conditions (13–15). Reactivated in most immortal cell lines and cancers, TERT has been a frequent target for inhibiting tumor cell proliferation (16).

Transcriptional activation of the TERT gene is, thus, a critical, initial rate-limiting step in TERT function and telomerase activity. Reflecting its multifactorial regulation, the TERT gene promoter in man has multiple sites for transcriptional regulation. There are two typical E-boxes and several GC-boxes for the transcription factors c-Myc/max and Sp1, respectively (17–20). Expression of N-Myc or c-Myc entrains a direct binding of Myc to the E-box (18, 21) and induction of TERT gene transcription followed by cell proliferation (22, 23). Another E box-binding protein (upstream stimulatory factor) also up-regulates TERT promoter activity, with binding negatively regulated by the N-terminal-truncated form upstream stimulatory factor 2, as an inhibitory competitor whose levels are increased in telomerase-negative cells (24). Little is known of the mechanisms whereby the TERT gene is repressed during cell development and differentiation. Recent studies show that transcription factor activator protein 1 (AP-1) is involved in repressing TERT gene transcription; combinations of c-Fos and c-Jun or c-Fos and JunD suppress TERT gene activation by binding to two AP-1 sites in the TERT gene promoter, suggesting a broad involvement of AP-1 in the regulation of telomerase in cell proliferation, differentiation, carcinogenesis, and apoptosis (25).

Transforming growth factor β (TGF-β) is a secreted autocrine or paracrine growth inhibitor that restricts proliferation and promotes differentiation of diverse cell types including epithelial, endothelial, and hematopoietic cells. It, thus, plays important roles during development and in pathophysiology (26–29). TGF-β exerts its biological effects through specific
in intracellular effector molecules called Smads that are phosphorylated by type I and type II transmembrane serine/threonine kinase receptors; phosphorylated Smad proteins such as Smad3 enter the cell nucleus to positively or negatively regulate gene expression by binding to DNA and interacting with DNA sequence-specific transcription factors (30–32). Recent studies suggest that TGF-β limits cell proliferation and induces cell senescence (33–35), which is regulated by telomeres and telomerase (36, 37). In contrast to epidermal growth factor, that stimulates telomerase (38, 39), TGF-β inversely correlates with telomerase activity (40). Interrupting TGF-β autocrine correlates increases telomerase activity in human breast cancer MCF-7 cells, whereas restoring autocrine TGF-β activity in human colon carcinoma HCT116 cells decreases telomerase activity (40). The available evidence suggests that TGF-β elicits inhibition of telomerase by suppression of the proto-oncogene c-Myc (40–42) or partially via SIP1, a transcriptional target of the TGF-β pathway (43).

The present study was undertaken to characterize the actions of TGF-β in the regulation of telomerase in human breast cancer cells. We show that TGF-β induces a rapid repression of TERT gene transcription in various cell lines and normal vascular smooth muscle cells. We show that repression requires the TGF-β signaling transducer protein Smad3, as demonstrated by overexpression of antagonistic Smad7, by silencing Smad3 gene expression and by genetic deletion of the Smad3 gene. In response to TGF-β, Smad3 directly binds to the TERT gene, as demonstrated by in vitro gel shift assay and intact cell chromatin immunoprecipitation. Mutation of the TERT promoter Smad3 binding site abrogates the binding of Smad3 and inhibits TGF-β-induced repression of TERT gene promoter activity. Furthermore, Smad3 interacts with c-Myc in response to TGF-β, and silencing c-Myc gene expression abrogates the binding of Smad3 to the TERT gene. These findings suggest a novel mode of rapid inhibition of TERT and telomerase activity in both normal and neoplastic cells. They show for the first time that Smad3 directly represses TERT gene expression in human cells and that this repression involves Smad3 interactions with c-Myc and TERT gene promoter DNA.

**EXPERIMENTAL PROCEDURES**

**Cytokines, siRNA, Expression Plasmids, and Antibodies**—Human TGF-β1 was from R&D Systems (Minneapolis, MN). Smad3, c-Myc, and relevant control siRNAs were from Cellogenetics. The pcDNA3 M2-tagged Smad3 gene expression plasmid and the p(CAGA)13 Luc TGF-β-inducible luciferase reporter construct were gifts from Dr. Hong-Jian Zhu (Ludwig Institute for Cancer Research, Melbourne, Australia) (44). Mouse Smad7 cDNA with a FLAG tag (m2) at its N terminus in pcDNA3 was from Dr. Peter ten Dijke (Ludwig Institute for Cancer Research, Uppsala, Sweden) (45). The plasmid human TERT (hTERT) promoter p330-Luc, containing a 330-bp fragment of the human TERT gene promoter, was provided by Dr. Silvia Bacchetti (Cancer Research Group, Dept. of Pathology and Molecular Medicine, McMaster University) (17). The Smad3 consensus sites (−284 to −281, 5′-GGCT-3′ and −262 to −259, 5′-CAGA-3′) in the plasmid hTERT promoter p330-Luc were changed into 5′-TTT-3′ by the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). The primers used were: 5′-CGT GAG CCG GCC AGG CCG TTA AAC CAG-3′ and 5′-GCC GCC GAA TCC ACT GTT TTA ACG GCC TGG-3′; CCC AGT GGA TTC GCG GGC CAT TTT TGC CCA GCA GAC-3′ and 5′-GAA GCG CCG TCC TGG GCA AAA ATG CCC GC-3′. All expression plasmids, wild types, and mutants were verified by DNA sequencing before use. Mouse anti-Smad3 antibodies, mouse anti-c-Myc antibodies, and rabbit anti-phosphorylated Smad2/3 and TERT were from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse antibodies to Smad2 were from Transduction Laboratories (Lexington, KY). Rabbit anti-Smad3 antibodies were from Zymed Laboratories Inc.. Antibodies against FLAG M2 were from Sigma. Antibodies against β-actin and β-tubulin were from Chemicon. Horseradish peroxidase-coupled secondary antibodies were from Bio-Rad.

**Cell Culture, Transfection, and Isolation**—The breast cancer epithelial line MCF-7, the normal rat kidney tubular epithelial cell line NRK52E, the normal rat Wistar-Kyoto vascular smooth muscle cell line, spontaneous hypertensive rat smooth muscle cell line (46, 47), mouse Smad2- and Smad3-deficient (Smad2 KO, Smad3 KO) and wild type (Smad2 WT, Smad3 WT) fibroblasts were grown in a 5% CO2 atmosphere at 37 °C in Dulbecco’s modified Eagle’s medium (Invitrogen) containing 0.5% fetal bovine serum in 6-well plastic plates, 10-cm dishes, or 8-chamber glass slides (Nunc, Naperville, CT). Recombinant human TGF-β1 at concentrations of 0, 0.25, 1, or 4 ng/ml was added into the cell culture for 15 min and 1, 2, 6, 15, and 24 h or as indicated in individual experiments. Cells were lysed in ice-cold lysis buffer (0.5% Triton X-100, 120 mM NaCl, 40 mM Tris-HCl, pH 7.4, containing 10 mM sodium pyrophosphate, 2 mM EGTA, 2 mM EDTA, 10 mM NaF, 10 μg/ml leupeptin, 5 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, 2 mM sodium vanadate). Clarified cell lysates were normalized for total protein concentration by the Bradford protein assay (Bio-Rad). To determine the effect of Smad3 on TGF-β-mediated TERT gene suppression, Myc-tagged Smad3 was overexpressed by transfecting cultured cells with pcDNA3-Myc-Smad3 with empty plasmids as controls. To block TGF-β activity, a FLAG M2-tagged Smad7-expressing vector (pcDNA3 m2Smad7) and a control plasmid pcDNA3 were used. The transfection was conducted using Lipofectamine (Invitrogen) according to the manufacturer’s instruction. After a 24-h transfection, cells were rested with serum-free medium for 24 h and then were stimulated with TGF-β1 for different times for Western blotting and semiquantitative RT-PCR to detect gene expressions as indicated in individual experiments.

**RNA Interference**—MCF-7 cells were cultured to 30% confluence. For each well in a 24-well transfection, 1 μl of a 40 μM stock of Smad3 siRNA, c-Myc siRNA, or appropriate negative controls siRNAs (Cellogenetics) was diluted into 41.5 μl of Opti-MEM I reduced serum medium, and 2 μl of Oligofectamine reagent (Invitrogen) was diluted into Opti-MEM® I reduced serum medium to a final volume of 7.5 μl. The diluted Oligofectamine™ reagent was added to the diluted siRNA, mixed gently, and incubated at room temperature for 15 min; 200 μl medium was then added to each well containing cells. Fifty μl of the above complex was overlaid onto the cells and
mixed gently. Cells were incubated for 4 h at 37 °C in a CO₂ incubator, and after incubation, 125 µl of 30% serum growth medium was added to the transfection mixture. Cell extracts were assayed by Western blot for Smad3 at 72 h post-transfection.

**RNA Isolation and Gene Expression Analyses**—Total RNA was isolated with oligo-dT primers and the High Pure RNA Isolation kit according to the manufacturer’s instructions (Roche Applied Science). Total RNA (2 µg) was used to synthesize cDNA with Moloney murine leukemia virus reverse transcriptase (Roche Diagnostics) according to the manufacturer’s instructions; contaminating DNA was removed by treating the samples with RNase-free DNase. The PCR mix was then set up in an enclosed environment distant from the one in which RNA extraction was carried out. Linear amplification for semiquantitative PCR was performed using a ThermoScript RT-PCR kit following the manufacturer’s instructions (Invitrogen) for 25–40 cycles of 30 s at 90 °C as the optimized annealing temperature and 72 °C extension. Primers specific for different genes were: human TERT (5′-CCACCTTGACAAAGTAC-3′ and 5′-GGTCAAGCTTCTCAACT-3′), human GAPDH (5′-GAGGACCCCTCCTGCTG-3′ and 5′-GATGGTATATGACAAAGTGT-3′), rat TERT (5′-GTGTGGCTTCCTACTGCGCAGCC-3′ and 5′-CACGCTGGTGCTGAAGGCCATG-3′), mouse TERT (5′-CTCTCCTGCTGGCGAGCCGATAC-3′ and 5′-CCTCTGTATAGCACCTCAAAG-3′), and mouse GAPDH (5′-CATGACAACTTTGGCAATT-3′ and 5′-GAGAGACCCTCACTGCTG-3′).

Probes were subjected to PCR for the housekeeping gene GAPDH as a positive control and as the internal standard. PCR products were resolved on 1.5% agarose gels in 1× Tris-acetate-EDTA, pH 8.0, containing a 1:50 dilution of a protease inhibitor cocktail. The PCR mix was then incubated with TGF-β1 (1 ng/ml) for different periods of time, fixed with 4% paraformaldehyde in PBS for 10 min, and blocked with 5% BSA in PBS for 30 min at room temperature. Cells were then incubated with Smad3 or TERT primary antibodies at RT for 1 h and washed 3 times with PBS, 0.01% Triton for 5 min each. Cells were then incubated for 1 h with fluorescein isothiocyanate-conjugated anti-rabbit secondary antibodies. For staining cell nuclei, cells were incubated with 50 ng/ml Hoechst (Sigma) for 10 min at RT. Slides were then washed with PBS-Triton, mounted in anti-fade medium (Bio-Rad), and analyzed by fluorescence microscopy (Leica Instruments). For immunocytochemical analysis, cells were cultured on 8-chamber glass slides in the presence or absence of TGF-β1, fixed in 2% paraformaldehyde, and preincubated with 10% fetal calf serum. The blot was then developed using the ECL (Amersham Biosciences) to produce a chemiluminescence signal, which was captured on x-ray film. For TERT, proteins were resolved on 8% SDS-PAGE, transferred to Immobilon-FL membranes (Millipore), and probed with specific antibodies raised in rabbits (49) or purchased from Santa Cruz Biotechnology. Western blots were probed with goat anti-mouse or anti-rabbit secondary antibodies conjugated to Alexa Fluor 680 (Molecular Probes) or IRdye 800 (Rockland Immunocytoscalic). Blotted proteins were detected and quantified by the Odyssey infrared imaging system (LI-COR).

**Immunofluorescence Microscopy**—MCF-7 cells seeded in chamber slides (Lab-Tek II, Nalge Nunc International) at 60–80% confluence were treated with TGF-β1 (1 ng/ml) for 15 h after TGF-β1 treatment. The dual luciferase assay (Promega) was performed as described in the user manual. Different doses of TGF-β1 were added 24 h after transfection, and luciferase activity was measured 15 h after TGF-β1 treatment. The dual luciferase assay (ML 3000 Microtiter Plate Luminometer) was performed according to the manufacturer’s instructions.

**Luciferase Reporter Assay**—The p(CAGA)₁₄Luc TGF-β-inducible luciferase reporter construct (0.5 µg) was transfected into MCF-7 cells or human colon cancer HCT116 cells to determine TGF-β and Smad3 signaling. To determine the regulation of human TERT promoter activity by TGF-β-induced Smad3 signaling, cells were transfected with 0.5 µg of hTERT promoter p330-Luc (wild type), p330-mutated (281–284, p330-mutated 259–262 (GenBank™ accession AB018788), and 0.125 µg of co-transfected Renilla luciferase control reporter (pRL-TK). All transfections were carried out using LipofectAMINE2000 (DNA:LipofectAMINE2000 = 1:3) as described in the user manual. Different doses of TGF-β1 were added 24 h after transfection, and luciferase activity was measured 15 h after TGF-β1 treatment. The dual luciferase assay (ML 3000 Microtiter Plate Luminometer) was performed according to the manufacturer’s instructions.
Electrophoretic Mobility Shift Assays—Four μg of MCF-7 cell nuclear extract was incubated with 10,000 cpm of double-stranded Smad3 wild type oligonucleotide probe (WT 5′-CCGGCGGCAACAGGAGGCAGAGGAC 3′ (the AGAC box is underlined)), its mutant oligonucleotide (mutant, 5′-CCGGCGGCA-TATACGCCGAGGAC 3′), Smad3 wild type 2 oligonucleotide probe (WT2, 5′-CGGGCGCCCGAGGCCCCGGGT-3′), or Myc wild type oligonucleotide probe (Myc WT, 5′-CGCGGCTCCAC-GTGGCCCGAGGGA-3′). Probes were labeled with [γ-32P]dATP (Amersham Biosciences). Incubations were performed for 30 min at room temperature in the presence of 2 μg of poly-(dl-dC) as nonspecific competitor and 10 mM Tris-HCl, pH 7.5, containing 100 mM NaCl, 1 mM EDTA, 5 mM dithiothreitol, 4% glycerol, and 100 μg/ml nuclease-free bovine serum albumin. For competition studies, unlabeled wild-type Smad3 (wild type 1) oligonucleotide was added to the binding reaction 30 min before the addition of the radiolabeled probe. Specific Smad3 antibody (0.5 μl, Santa Cruz Biotechnology) was incubated with nuclear extracts for 20 min on ice before the binding reaction. All incubation mixtures were subjected to electrophoresis on native 5% (w/v) polyacrylamide gels, which were subsequently dried and subjected to autoradiography.

Chromatin Immunoprecipitation (ChIP) Assays—The ChIP assays were performed using the ChIP assay kit according to manufacturer’s instructions (Upstate Biotechnology). Briefly, MCF-7 cells were fixed with formaldehyde (final concentration 1% v/v) in serum-free Dulbecco’s modified Eagle’s medium at 37 °C for 10 min after TGF-β1 stimulation for 1 h. Cells were washed twice with ice-cold PBS containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, and 1 μg/ml peptatin A), pelleted for 4 min at 2000 rpm at 4 °C, resuspended in 200 μl of SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris, pH 8.1), and incubated for 10 min on ice. After sonication, lysates were centrifuged at 10,000 rpm at 4 °C, and the supernatant was transferred to a new 2-ml microcentrifuge tube. The sonicated cell supernatants were diluted 10-fold in ChIP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 167 mM NaCl, 16.7 mM Tris-HCl, pH 8.1), the rabbit anti-Smad3 antibodies were added to the 2-ml supernatant fraction, and the mixture was incubated overnight at 4 °C with rotation followed by the addition of 60 μl of protein A-agarose/salmon sperm DNA (50% slurry) for 1 h at 4 °C with rotation.

The protein-DNA complex on protein A-agarose was pelleted at 1000 rpm at 4 °C for 1 min and washed for 3–5 min on a rotating platform with 1 ml of each buffer listed in order (a) low salt immune complex wash buffer (SDS, 1% Triton X-100, 2 mM EDTA, 150 mM NaCl, 20 mM Tris-HCl, pH 8.1), (b) high salt immune complex wash buffer (SDS, 1% Triton X-100, 2 mM EDTA, 500 mM NaCl, 20 mM Tris-HCl, pH 8.1), and (c) LiCl immune complex wash buffer (0.25 mM LiCl, 1% Igepal-C-630, 1% deoxycholic acid (sodium salt), 1 mM EDTA, 10 mM Tris, pH 8.1, and Tris-EDTA buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). The protein-DNA complex was eluted by adding 250 μl of elution buffer (1% SDS, 0.1 M NaHCO3) to the pelleted protein A-agarose-antibody-DNA complex. 5 mM NaCl (20 μl) was added to the eluate followed by heating at 65 °C for 4 h to reverse Smad3-DNA cross-links. Samples were extracted twice with phenol/chloroform and precipitated overnight with ethanol.

DNA fragments were recovered by centrifugation, resuspended in double distilled H2O, and used for PCR amplification of the hTERT gene promoter DNA. The primers for hTERT PCR were 5′-GGC CGG GCT CCC AGT GGA TTC-3′ and 5′-CAG CGG GGA CGG CGC GGC ATC G-3′. The primers for rat TERT PCR were 5′-AAG CCT GGT TGG GAA AAA CT-3′ and 5′-AGT GGT TGG CGG AAG TGT AG-3′ for a 250-bp TERT promoter DNA.

Telomerase Activity Assay—A telomeric repeat amplification protocol, performed essentially as described previously (49), was employed to determine telomerase activity. Briefly, cells treated with different reagents were washed and lysed by detaching and passing the cells through a 26Gx1/2 needle attached to a 1 ml syringe in prechilled telomeric repeat amplification protocol lysis buffer (0.5% APS, 10 mM Tris-HCl, pH 7.5, 1 mM MgCl2, 63 mM KCl, 0.05% Tween 20, 1 mM EDTA, 10% glycerol, 5 mM β-mercaptoethanol and mixture protease inhibitors). Nuclei were isolated by centrifugation, and protein content was determined. Equal amounts of nuclear telomerase extract (0.4 μg) were incubated with telomeric DNA substrate and dNTP, and de novo synthesized telomeres with or without phenol and chloroform extraction were amplified by PCR using specific telomeric DNA primers in the presence of [α-32P]ATP (Amersham Biosciences) and TaqDNA polymerase. The resulting 32P-labeled telomeres were resolved by polyacrylamide slab gel electrophoresis followed by autoradiography. Quantitative analysis of telomerase activity was performed by counting 32P activity in de novo-synthesized telomeric DNA in a β-counter as described previously (50). To monitor nonspecific PCR effects, additional primers were included: NT (ATCGCTTCT-CGGCCTTCT) and TSNT (AATCCGTCAGCAGATTA-AAGGCGGCAGAGCAT). Negative controls treated with either RNase A or alkaline phosphatase to inactivate telomerase were included in each experiment.

RESULTS

TGF-β Suppression of hTERT Gene Expression and Telomerase Activity in Normal, Highly Proliferative, and Cancerous Cells—TGF-β has an inhibitory effect on cell proliferation in normal development, acting as a tumor suppressor (51, 52). To determine whether the growth inhibitory effect of TGF-β is mediated by regulating telomerase activity, we determined the concentration- and time-dependent effect of TGF-β on telomerase activity and gene expression of hTERT in MCF-7 breast cancer cells. Previous work has shown that in human MCF-7 breast cancer cells, specific inhibition of the autocrine actions of TGF-β increases telomerase activity, and the addition of TGF-β is associated with hTERT gene suppression in MCF-7 cells (40, 43). In the present study incubation of MCF-7 cells with increasing concentrations of TGF-β for 15 h resulted in a concentration-dependent down-regulation of telomerase activity and hTERT protein in the nucleus (Fig. 1). Likewise, hTERT mRNA was reduced by TGF-β in a dose-dependent manner (Fig. 2A). Inhibition occurred at physiologically relevant concentrations with half-maximal inhibition at ~1 ng/ml. Thus, TGF-β is a potent negative regulator of telomerase, presumably through a specific ligand-receptor interaction at cell surface.
TGF-β inhibition of hTERT gene expression occurred in 2–6 h, with complete inhibition achieved by 12 h of administration of TGF-β in cultured MCF-7 cells (Figs. 1, B and C, and 2B). Different degrees of sensitivity for the time course of inhibition were observed in normal renal epithelial NRK52E cells, vascular smooth muscle Wistar-Kyoto cells, and spontaneously hypertensive vascular smooth muscle cells of rats (Fig. 2). In breast and kidney epithelial cells, inhibition occurred within 2 h of incubation with 4 ng/ml of TGF-β, whereas in vascular smooth muscle cells TGF-β did not induce significant reduction of hTERT mRNA until 6 h of treatment with 4 ng/ml of TGF-β (Fig. 2B). Because hTERT repression may be mediated by TGF-β-induced c-Myc down-regulation, we examined the levels of c-Myc in these cells after TGF-β treatment for different times (Fig. 2C). TGF-β1 induced c-Myc down-regulation after 12–24 h of treatment with TGF-β, which was after the initiation of hTERT repression (Fig. 2C). The relatively belated inhibition of c-Myc by TGF-β is consistent with the findings in a similar study using a rat fibroblast model recently in which TGF-β induces c-Myc repression in 48 h of TGF-β treatment (42). Thus, the molecular mechanism underlying the rapid repression of hTERT induced by TGF-β1 is still unclear, although c-Myc down-regulation may be involved in the sustained phase of repression of the hTERT gene. Nevertheless, a time course of 2–6 h of TGF-β treatment suggests a direct action of TGF-β signaling protein(s) on TERT gene expression.

**Smad3 Signaling Is Required for TGF-β Repression of hTERT Gene**

The TGF-β signaling protein, Smad3, has been shown to be involved in many TGF-β inhibitory activities (53). To explore the role(s) of Smad3 in TGF-β inhibition of hTERT gene expression, we examined Smad3 phosphorylation, intranuclear trafficking, and transcriptional activity during TGF-β suppression of hTERT in human MCF-7 breast cancer cells. In line with the inhibition of hTERT gene expression, Smad3 protein was increased significantly in the nucleus 30 min after TGF-β treatment, concomitant with its phosphorylation (Fig. 3A). Phosphorylation of Smad3 was also evaluated by Western blotting using anti-phospho-Smad3 antibodies (Fig. 4A). Endogenous Smad3 (along with Smad2) was phosphorylated within 30 min of TGF-β treatment (Fig. 4A), before Smad3 migration into the nucleus (Fig. 3A). We next determined Smad3 transcriptional activity using a Smad3 response element consisting of repeated CAGA boxes placed upstream of a luciferase gene. TGF-β stimulation of cultured MCF-7 cells transfected with the CAGA box promoter led to significant increases in Smad3 transcriptional activity in a TGF-β-concentration-dependent manner.
Regulation of hTERT Gene by Smad3

FIGURE 2. TGF-β down-regulates the TERT gene in smooth muscle cells, kidney epithelial cells, and breast cancer cells. A, human breast cancer cells (MCF-7), rat vascular smooth muscle cells (Wistar-Kyoto (WKY)), spontaneously hypertensive rat (SHR) smooth muscle cells, and rat kidney epithelial cells (NRK52E) were stimulated with different concentrations of TGF-β1 for 12 h. TERT mRNA was determined by RT-PCR as described under “Experimental Procedures.” B, time course of TGF-β1 inhibition of TERT. Cells were treated with (lanes 3, 5, and 7) or without (lanes 1, 2, 4, and 6) TGF-β1 (4 ng/ml) for the time indicated followed by determination of TERT mRNA by RT-PCR with specific primers. The gene expression levels of GAPDH in different cell types are shown as controls. C, time course of TGF-β1 down-regulation of c-Myc. Cells were treated with TGF-β1 (4 ng/ml) for the periods as indicated, which was followed by determination of c-Myc gene expression by Western blotting with specific antibodies. Controls for loading proteins were measured with anti-actin antibodies. Data are from representative experiments repeated at least three times.

The synergistic inhibitory effect between TGF-β and exogenous Smad3 on the hTERT promoter activity. The lack of additive or synergistic inhibitory effect between TGF-β and Smad3 on the hTERT promoter suggests that Smad3 is the primary signaling molecule that mediates TGF-β suppression of the hTERT gene. To further attest the repressor effect of Smad3 on the hTERT gene, we expressed Smad3 in human colon cancer HCT116 cells in which the type II TGF-β receptor is mutated (40). Although TGF-β failed to repress the hTERT gene as expected, expression of Smad3 still inhibited the hTERT gene promoter activity (Fig. 3D), confirming that Smad3 is an hTERT gene repressor.

To establish an essential requirement of Smad3 in mediating TGF-β-induced hTERT gene expression, we determined the effects of Smad7 (an antagonist Smad) on TGF-β-induced hTERT gene suppression. We also determined the effects of knocking down Smad3 gene expression and the genetic deletion of the Smad3 gene on TGF-β-induced hTERT gene suppression. As shown in Fig. 4A, stimulation of MCF-7 cells with TGF-β (4 ng/ml) resulted in up-regulation of protein phosphorylation of Smad3 and down-regulation of hTERT gene, with these effects abolished when antagonist Smad7 was expressed in MCF-7 cells. The failure of TGF-β to induce Smad protein phosphorylation and hTERT gene repression in the presence of Smad7 suggests that Smad3 activation is required for TGF-β-induced hTERT repression. To determine the specificity of Smad3 action and to exclude involvement of Smad2 in hTERT gene expression, three mouse fibroblast cell lines, wild type Smad3, Smad3 knock-out, and Smad2 knock-out, were examined for the effects of TGF-β on hTERT gene expression. Although TGF-β inhibition of hTERT gene expression remained unchanged in both Smad3 wild-type and Smad2-deficient cells, deficiency of Smad3 abolished TGF-β-mediated hTERT gene repression (Fig. 4B). Furthermore, temporarily knocking down Smad3 gene expression with Smad3 siRNA in human MCF-7 breast cancer cells also eliminated the effect of TGF-β on hTERT gene suppression (Fig. 4C). These results together suggest that Smad3 phosphorylation and nuclear migration mediate TGF-β-induced hTERT suppression, which is reversible by expression of Smad7.

Identification of Smad3 Interaction and Binding Site on the hTERT Gene That Represses hTERT Gene Transcription in Response to TGF-β Signaling—It is noteworthy that incubation of MCF-7 cells with TGF-β for several hours did not alter the level of c-Myc gene expression and that silencing Smad3 similarly had no effect on c-Myc gene expression (Fig. 4C). These data together with the time course of 4–6 h for TGF-β-mediated TERT repression suggest that Smad3 plays a direct role in mediating TGF-β-induced hTERT gene repression. The finding that expression of Smad3 markedly represses the activity of the hTERT promoter (hTERT1+3 to −330) suggest that Smad3 acts at a site within this 330-bp region. Inspection of the hTERT promoter suggests several putative Smad3 binding sites, including two non-canonical CAGA boxes, at positions −281 to −284 and −259 to −262 relative to the translation start codon (Fig. 5A). Mutagenesis studies of the hTERT promoter with a luciferase reporter suggest a specific repression by Smad3 after phosphorylation of Smad3 and down-regulation of hTERT gene, with these effects abolished when antagonist Smad7 was expressed.
Regulation of hTERT Gene by Smad3

reporter assay showed TGF-β-induced suppression of the hTERT promoter activity in both the wild type hTERT promoter and the hTERT<sup>281–284mut</sup> mutated promoter. This was specific to Smad3 in that silencing Smad3 eliminated the TGF-β-induced down-regulation (Fig. 5B). However, mutation of the CAGA box at −259–262 of the hTERT promoter disabled TGF-β down-regulation of the hTERT gene and resulted in increases in the basal activity of the hTERT gene promoter (Fig. 5B), suggesting that the hTERT promoter −259–262 sequence provides the binding site for Smad3 that mediates Smad3 repression of the hTERT promoter gene transcription. This identification of the CAGA box at −262CAGA−259 is in contrast to a recent study suggesting Smad3 binding site(s) in a region from −748 to −729 in the rat TERT gene promoter (42). This discrepancy is consistent with the hypothesis that the TERT genes are differentially controlled between different species.

To further address the hypothesis that Smad3 directly interacts with the hTERT gene in response to TGF-β stimulation, we performed in vitro electrophoresis gel mobility shift assays with MCF-7 cell nuclear proteins and a 32P-labeled hTERT promoter DNA probe containing the −262CAGA−259 sequence. As shown in Fig. 6, nuclear protein extracts from untreated MCF-7 cells showed little Smad3 binding activity to the hTERT DNA probe containing the −262CAGA−259 sequence, whereas after TGF-β stimulation, significantly increased binding activity to the hTERT DNA probe was observed (left panel of Fig. 6A). The increased binding occurred from 30 to 120 min after administration of TGF-β. This was not observed when the hTERT DNA probe was replaced with another labeled probe derived from hTERT<sup>341–321</sup> containing the putative −332CAGA−329 box (right panel of Fig. 6A), suggesting specific binding to −262CAGA−259. To further establish the binding specificity of Smad3 protein to the hTERT promoter, we determined the effects of competitive inhibition by nonradioactive hTERT promoter DNA and Smad3 monoclonal antibodies. As shown in Fig. 6, B and C, the binding activity to the hTERT DNA probe was dependent on TGF-β. In the presence of an excess amount of nonradioisotope-labeled hTERT promoter, DNA binding was completely inhibited, evidence for inhibition of specific binding to hTERT DNA. In the presence of Smad3 monoclonal antibodies, the binding to the hTERT DNA probe was also inhibited significantly (Fig. 6, B and C), similarly demonstrating that Smad3 is involved in direct binding to the hTERT promoter. Consistent with this, mutation of the −262CAGA−259 sequence in the Smad3 binding nucleotide probe resulted in no binding (lane 3
Regulation of hTERT Gene by Smad3

Figure 4. Smad3 protein phosphorylation and gene expression are required for TGF-β-induced suppression of hTERT. A, Smad3 inhibits TGF-β1-induced phosphorylation of Smad3 and prevents human TGF-β-mediated hTERT gene repression in MCF-7 cells. Cells were transfected with pcDNA3-M2 empty vector or Smad7 for 24 h and monitored by Western blotting before incubation with or without TGF-β1 (4 ng/ml, 15 h). Phosphorylated Smad3 was detected by Western blotting using specific antibodies. Telomerase hTERT mRNA and human GAPDH were examined by RT-PCR with specific primers. B, effect of TGF-β1 on TERT gene expression in mouse embryonic fibroblasts with Smad3 and Smad2 gene deletions. Wild type (WT) Smad3 knock-out and Smad2 knock-out fibroblasts were treated with or without TGF-β1 (4 ng/ml) for 15 h as indicated and subjected to measurement of TERT mRNA by RT-PCR (top panel) and real-time PCR (bottom panel; results are the mean ± S.D. of three determinations). C, effect of silencing the Smad3 gene by specific siRNA on TGF-β suppression of hTERT in human breast cancer cells. MCF-7 cells were treated with specific or control siRNA by gene transfection for 48 h followed by treatment with TGF-β1 for 18 h. Cells were lysed, and cellular protein and RNA extracts were assayed for gene expressions of Smad proteins and c-Myc by immunoblotting and of TERT by semi quantitative RT-PCR.

Figure 5. Identification of Smad3 binding site in the hTERT gene promoter. A, schematic drawing of two putative Smad3 binding sites within the hTERT promoter and the mutations of these sites made for in vitro and intact cell analyses of the action of Smad3. B, mutation of the hTERT gene promoter increases basal hTERT promoter activity and inhibits TGF-β repression of promoter activity. MCF-7 cells were transfected with or without Smad3 siRNA as indicated. After 48 h of transfection with siRNA, the cells were then transfected with hTERT promoter p330-Luc (wild type (WT)), p330-mutated 1, or p330-mutated 2 together with pRL-TK. After a further 24 h, MCF-7 cells were treated with (light bars) or without (dark bars) TGF-β1 for 24 h followed by dual luciferase assay to detect hTERT promoter activity.

of Fig. 6C). As positive controls for specificity, we also determined the binding of the 32P-labeled hTERT promoter E-box and found high levels of binding of c-Myc and perhaps upstream stimulatory factor to the E-box, with binding not altered by TGF-β stimulation (Fig. 6D), consistent with unaltered gene expression of c-Myc after TGF-β treatment for up to 15 h (Fig. 4C).

To determine Smad3 binding to the endogenous hTERT gene in cultured MCF-7 cells, we carried out ChIP analysis using specific anti-Smad3 monoclonal antibodies. Negative and positive controls for antibodies included diluent, normal IgG, and c-Myc monoclonal antibodies. Controls for nonspecific precipitation included detection of GAPDH in the presence or absence of specific siRNA to Smad3 or c-Myc. As shown in Fig. 7A, the hTERT gene was significantly detected in either cell lysate input with GAPDH before precipitation or immunoprecipitates of c-Myc, confirming c-Myc binding to the hTERT gene as reported previously (23, 54). Consistent with the data shown in Figs. 4C and 6D, co-precipitation of c-Myc with the hTERT gene was not significantly altered after TGF-β treatment (Fig. 7A). In contrast, whereas the hTERT gene was not detected in Smad3 without TGF-β treatment, significant levels of the hTERT gene fragment were found in Smad3 immunoprecipitates after treatment with TGF-β, thereby demonstrating that Smad3 binds to the hTERT gene in response to TGF-β. The specific immunoprecipitation of a complex between Smad3 and hTERT gene was verified in that hTERT gene precipitation became undetectable once Smad3 was silenced by RNA interference (Fig. 7B). To determine that Smad3 binds to the hTERT gene promoter in other cell types, we immunoprecipitated Smad3 from TGF-β-responsive NRK52E cells and TGF-β-insensitive HCT116 cells treated with TGF-β1 and observed that Smad3 bound the hTERT promoter DNA in both TGF-β-sensitive and -insensitive cells (Fig. 7, C and D).

Smad3 Interacts with c-Myc, Which Is Required for TGF-β Repression of hTERT Gene Transcription—Experiments to confirm specific precipitation between c-Myc and the hTERT gene using c-Myc siRNA led to an unexpected finding in terms of the involvement of c-Myc in mediating Smad3 binding to the hTERT gene. Treatment with c-Myc siRNA knocked down c-Myc and prevented co-precipitation of c-Myc and the hTERT gene with anti-Myc specific antibodies. However, silencing c-Myc also compromised the binding of Smad3 to the hTERT promoter (Fig. 8A), suggesting that Smad3 binding to the hTERT gene requires c-Myc.
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To determine whether c-Myc might affect TGF-β signaling and be required for TGF-β suppression of hTERT gene, we assessed the levels of Smad3, Smad2, and hTERT gene expression as a function of silencing c-Myc gene. Although having no effect on the gene expression of Smad3 and Smad2, silencing c-Myc induced not only a decrease in hTERT gene expression but also a failure of TGF-β-induced down-regulation of hTERT gene expression (Fig. 8, B and C). Down-regulation of c-Myc to 70–80% that of normal levels resulted in reduction of hTERT gene expression to ~50–60%, similar to that mediated by TGF-β repression. When TGF-β was applied to cells with down-regulated c-Myc, no additive inhibition of hTERT gene expression was observed (Fig. 8C). These data suggest that TGF-β employs a c-Myc-dependent mechanism to suppress hTERT gene expression and that c-Myc is required for not only TGF-β-induced hTERT down-regulation (Fig. 8, B and C) but also Smad3 binding to the hTERT gene promoter DNA (Fig. 8A).

Because the Smad3 binding site 259–262CAGA–259 is adjacent to a c-Myc E-box binding site 235–246CACGTG–235 (17) in the hTERT promoter and given that deprivation of c-Myc blocked Smad3 binding to the hTERT gene, we hypothesized that in MCF-7 cells Smad3 and c-Myc interact directly to inhibit c-Myc transcriptional activation of the hTERT gene and that their interaction recruits Smad3 to the hTERT gene promoter for repression. To test this hypothesis, we determined if Smad3 binds to c-Myc in response to TGF-β treatment of MCF-7 cells by co-immunoprecipitation. Immunoprecipitation of c-Myc showed low co-immunoprecipitation of Smad3 in the presence of TGF-β stimulation, but after stimulation of the cells by TGF-β (4 ng/ml) for various periods of time, significant levels of Smad3 were detected in the c-Myc immunoprecipitates, with Smad3 detectable 15 min after TGF-β stimulation (Fig. 9A). Immunoprecipitation of Smad3 also co-precipitated c-Myc but only if the cells were stimulated by TGF-β (Fig. 9B). Thus, the TGF-β-induced interaction between Smad3 and c-Myc is associated with TGF-β-induced Smad3 binding to, and suppression of the hTERT gene promoter, suggesting a novel protein-DNA complex involving Smad3, c-Myc, and the hTERT gene promoter that initiates TGF-β-induced repression of the hTERT gene. Together with the requirement for both Smad3 and c-Myc in TGF-β suppression of the hTERT gene, these data suggest that specific temporal and spatial interactions between Smad3, c-Myc, and hTERT gene promoter in response to TGF-β are responsible for TGF-β-induced hTERT gene suppression in human breast cancer cells.

DISCUSSION

hTERT gene expression is the first step in telomerase activation for continuous stem cell renewal and proliferation. Repression of the hTERT gene occurs during cell differentiation, and de-repression takes place in tumorigenesis in most cancers by mechanisms...
that remain largely unexplored (7–10). As a pleiotropic autocrine and paracrine cytokine in a variety of tissues, TGF-β has a common effect of inhibiting cell proliferation in epithelial, endothelial, and hematopoietic cell types. This TGF-β checkpoint is implicated in mediating cell senescence (33–35), failure of which is a hallmark of many cancer cells (51). Recent studies suggest that TGF-β and hTERT form an important regulatory system in which TGF-β instigates hTERT down-regulation to inhibit cell proliferative potential (40, 41, 43) in addition to interactions with other growth control genes such as p21WAF1/Cip1, p15INK4b, Cdc25A, cyclin-dependent kinase, mitogen-activated protein kinase, and Akt (21, 30, 55–57). In characterizing the complex signaling pathways from TGF-β to the hTERT gene, the present study has for the first time revealed that Smad3 is phosphorylated in response to TGF-β, upon which it shuttles into the cell nucleus and interacts with the hTERT gene transcription factor c-Myc and with a specific site on the hTERT promoter, leading to repression of hTERT gene expression. Thus, TGF-β may induce telomerase inhibition through multiple mechanisms including a rapid repression of the hTERT gene mediated by direct actions of Smad3 on the hTERT gene promoter followed by a sustained inhibition mediated by a transcriptional withdrawal of c-Myc (Fig. 10).

We have shown that TGF-β rapidly down-regulates telomerase activity after 2–6 h in cell cultures of normal smooth muscle cells, highly proliferative smooth muscle cells, immortal kidney epithelial cells, and human breast cancer cells. This finding provides an important connection between TGF-β and cell senescence in that TGF-β induces cell senescence in several cell types and models (33–35). For example, when human skin diploid fibroblasts are exposed to UV, they suffer premature senescence with increased TGF-β signaling; removal of TGF-β or targeting TGF-β receptors using specific neutralizing antibodies markedly alleviates UV-induced cellular senescence (35). In mouse models, TGF-β similarly represses hTERT gene and telomerase activity (42); overexpression of Smad3, but not Smad2 or Smad4, induces mouse keratinocyte senescence, whereas deletion of Smad3 delays keratinocyte senescence induced by v-RasHA (34). The telomerase connection between TGF-β and cell senescence is further underlined by the finding that activation of endogenous telomerase or ectopic expression of hTERT induces resistance to TGF-β-induced senescence of human mammary epithelial cells (36, 58). In addition, recent studies suggest that TGF-β signaling may be feedback-controlled by telomere maintenance with short telomeres activating Smurf2 that inhibits TGF-β signaling (37).

Central to TGF-β regulation of telomerase activity, Smad3 is shown for the first time to be not only required but also to act directly to repress the hTERT gene in human cells. Using specific gene silencing and expression to target Smad3, the present study shows that Smad3 responds to TGF-β stimulation by phosphorylation, migration into breast cancer cell nucleus, binding to hTERT gene promoter, and repression of hTERT expression. The direct binding of Smad3 to the hTERT gene promoter DNA is consistent with a recent study in rat fibroblasts (42). The data that expression of Smad7 prevents Smad3 phosphorylation and down-regulation of telomerase are consistent with the notion that Smad3 plays a physiological role in regulating the hTERT gene, which is balanced and reversible by intracellular antagonist Smad proteins. Consistent with Smad3 as the predominant signaling molecule mediating TGF-β regulation of the hTERT gene are the data that Smad3 overexpression mimics but is not additive for TGF-β suppression of hTERT promoter activity. Mediating the relatively rapid sup-
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**FIGURE 8. Effects of c-Myc on Smad3 binding to hTERT promoter DNA on TGF-β suppression of the hTERT gene.** A, co-precipitation of Smad3 and the hTERT gene promoter is inhibited by silencing c-Myc. MCF-7 cells with normal (lanes 1, 2, 5, 6, 9, 10, 13, and 14) or silenced c-Myc gene expression (lanes 3, 4, 7, 8, 11, 12, 15, and 16) were treated with (even lane numbers, 4 ng/ml, 15 h) or without TGF-β1 (odd lane numbers) followed by ChIP using control or specific antibodies (Ab). NS, normal serum. The DNA hTERT promoter and human GAPDH gene were detected by PCR using specific primers. B and C, effect of c-Myc knockdown on TGF-β-mediated suppression of hTERT in human breast cancer cells. MCF-7 cells were transfected with specific c-Myc siRNA or control siRNA for 48 h followed by treatment with or with TGF-β1 for 15 h. Cells were lysed, and cellular protein and RNA extracts were assayed for gene expression as indicated by immunoblotting or RT-PCR (panel B). Panel C shows densitometry quantitative analysis of hTERT suppression as the mean ± S.D. from three similar experiments.

expression of the hTERT gene after 2–6 h of TGF-β treatment, Smad3 binds the hTERT promoter directly as demonstrated by in vitro binding analysis and intact cell chromatin immunoprecipitation assays. This finding of direct binding by Smad3 to the hTERT promoter is consistent with recent findings in a rat model, in which Smad3 binds directly to a Smad binding site contained in the sequence of rat TERT DNA (42). In contrast with the Smad3 binding site in rat, we have found that the transcriptional repression activity of Smad3 lies within the 330 bp of the hTERT promoter relative to the transcription start site of rat TERT (42). In contrast with the Smad3 binding site in rat, we have identified the transcriptional repression activity of Smad3 lies within the 330 bp of the hTERT promoter relative to the transcription start site of human TERT.

Structure-function analyses of the hTERT promoter activity of the 330-bp fragment and in vitro Smad3 binding studies using various mutants allowed us to identify the −262CAGA−259 box as responsible in mediating Smad3 binding and repression of hTERT gene promoter activity during TGF-β stimulation.

Consistent with previous findings that expression of the TGF-β type II receptor allows telomerase down-regulation through autocrine signaling of TGF-β (40), we found that mutation of the Smad3 binding site (−262CAGA−259) in the hTERT promoter increases hTERT gene promoter transcriptional activity in the absence of exogenous TGF-β (Fig. 5), suggesting that the TGF-β and Smad3 signaling pathway plays an important role in repressing the hTERT gene without exogenously applied TGF-β. In addition, the data that silencing Smad3 further promotes the elevation of hTERT promoter activity induced by mutation of the Smad3 binding site suggest that in addition to interacting with hTERT promoter DNA, Smad3 may also regulate another factor(s) that is involved in regulating hTERT gene activity. These factors might be SIP1 (43), AP1 (25, 59), and/or c-Myc (see below). Nonetheless, engineering the Smad3 binding site in the hTERT promoter to enhance the Smad3 effect on hTERT promoter repression may provide a new strategy to target telomerase in anti-cancer therapy. Although Smad3 null mice exhibit variable frequencies of spontaneous colon cancer (60, 61), studies have shown that mice lacking Smad3 have impaired mucosal immunity and accelerated wound healing (62, 63). Furthermore, whereas loss of Smad3 alone is insufficient to induce leukemia, additional loss of the p27Kip1 cyclin-dependent kinase inhibitor that is frequently altered in human T-cell acute lymphoblastic leukemia causes leukemia to develop in Smad3-deficient mice (64). Finally, despite normal levels of Smad3 mRNA, deficiency of Smad3 protein is a specific feature in human acute lymphoblastic leukemia in children (64).

Remaining to be determined are the detailed mechanisms of temporal and spatial interactions between Smad3 and the hTERT gene in response to TGF-β and how these interactions are regulated. In analyzing the role of c-Myc, however, we
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A

FIGURE 10. Model of the mechanisms by which TGF-β induces hTERT gene suppression. A three-input signaling mediates TGF-β suppression of the hTERT gene. Suppression is mediated by Smad3 repression of hTERT gene transcription, relief of Myc transcriptional stimulation, and SIP1 repression by different Smad repressor and activator complexes as indicated. SIP1 is partially required for TGF-β suppression of hTERT (43). Stimulation of hTERT gene transcription (23), c-Myc is down-regulated by a Smad repressor complex involving E2F4/5 and p107 (60). B, schematic explaining how telomerase inhibition TGF-β occurs through interactions of Smad3, c-Myc, and hTERT gene. In the absence of TGF-β signaling, nonphosphorylated Smad3 exists in the cytoplasm, and hTERT gene transcription is driven by c-Myc. TGF-β stimulation results in Smad3 phosphorylation, Smad3 nuclear entry, and binding to c-Myc, leading to hTERT gene inactivation. By interacting with c-Myc, Smad3 is recruited to bind the hTERT gene promoter, resulting in further repression of the hTERT gene.

found that treatment of MCF-7 cells with TGF-β for several hours caused no discernible changes in c-Myc gene expression or in c-Myc binding to the hTERT gene promoter. Surprisingly, however, silencing c-Myc blocks Smad3 binding to the hTERT promoter induced by TGF-β, whereas knocking down Smad3 had no effect on c-Myc binding to hTERT promoter DNA. In addition, silencing c-Myc also prevented TGF-β from inducing inhibition of hTERT gene transcription, a finding consistent with an essential requirement for c-Myc in Smad3 binding and repressing the hTERT gene promoter. Furthermore, TGF-β fails to down-regulate hTERT gene expression when c-Myc is down-regulated. These findings suggest that TGF-β elicits Smad3 interactions with c-Myc in regulating hTERT gene expression. To explore if Smad3 might directly interact with c-Myc and cooperatively regulate the hTERT gene, we determined if Smad3 and c-Myc bind directly to each other. Indeed, immunoprecipitation of c-Myc co-precipitated Smad3 and immunoprecipitating Smad3 co-precipitated c-Myc. Thus, in addition to binding to the CAGA box of the hTERT gene promoter, Smad3 also forms a complex with c-Myc to regulate the hTERT gene in response to TGF-β, although the binding of Smad3 to c-Myc does not cause c-Myc dissociation from the hTERT promoter (Figs. 6 and 7). Consistently, previous studies have shown that Smad3 and c-Myc bind to each other directly in regulating the TGF-β-induced cyclin-dependent inhibitor p15Ink4b gene (65). It is possible that Smad3 forms complexes with c-Myc to disable c-Myc function and that each may recruit the other to their binding sites at specific gene promoters and, thus, regulate each other’s function (65). Thus, we propose a mechanism of TGF-β-induced hTERT gene repression involving Smad3 cis and trans actions on the hTERT gene and also c-Myc transcription-dependent repression (Fig. 10). In this model is a 3-step regulation; 1) the binding of Smad3 to c-Myc recruits Smad3 to the hTERT gene, and this initiates the inhibition of c-Myc in recruiting the transcriptosome for hTERT gene transcription, 2) subsequently, Smad3 physically binds to the CAGA box of hTERT gene promoter DNA to further prevent c-Myc activity on hTERT gene transcription, 3) given the TGF-β induced down-regulation of c-Myc in the later phase of hTERT gene repression (Fig. 2), transcriptional repression of the c-Myc gene sustains the repression of the hTERT gene in response to TGF-β signaling (Fig. 10).

In summary, we have identified a direct transcriptional inhibitory pathway involving extracellular TGF-β signaling to the hTERT gene in human breast cancer cells. This mechanism involves Smad3 phosphorylation, nuclear migration, interaction with c-Myc, and binding to the hTERT gene promoter to repress hTERT gene transcription and inhibit telomerase activity. Additional studies are also required to target the interface between Smad3 and hTERT promoter DNA to develop reagents as novel modalities for aging and malignant cells.

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