

Human Telomerase Reverse Transcriptase (*hTERT*) Is a Novel Target of the Wnt/ β -Catenin Pathway in Human Cancer*[§]

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Background: Telomerase up-regulation is found in about 90% of human cancer specimens and contributes actively to carcinogenesis.

Results: β -Catenin·TCF4 plays an important role in telomerase activation.

Conclusion: *hTERT* is a direct transcriptional target of the Wnt/ β -catenin pathway.

Significance: Identifying the function of the Wnt/ β -catenin pathway in telomerase regulation provides better insight into the role of Wnt pathway in carcinogenesis.

Telomerase activation plays a critical role in human carcinogenesis through the maintenance of telomeres, but the activation mechanism during carcinogenesis remains unclear. The human telomerase reverse transcriptase (*hTERT*) promoter has been shown to promote *hTERT* gene expression selectively in tumor cells but not in normal cells. Deregulation of the Wnt/ β -catenin signaling pathway is reported to be associated with human carcinogenesis. However, little is known about whether the Wnt/ β -catenin pathway is involved in activating *hTERT* transcription and inducing telomerase activity (TA). In this study, we report that *hTERT* is a novel target of the Wnt/ β -catenin pathway. Transient activation of the Wnt/ β -catenin pathway either by transfection of a constitutively active form of β -catenin or by LiCl or Wnt-3a conditioned medium treatment induced *hTERT* mRNA expression and elevated TA in different cell lines. Furthermore, we found that silencing endogenous β -catenin expression by β -catenin gene-specific shRNA effectively decreased *hTERT* expression, suppressed TA, and accelerated telomere shortening. Of the four members of the lymphoid-enhancing factor (LEF)/T-cell factor (TCF) family, only TCF4 showed more effective stimulation on the *hTERT* promoter. Ectopic expression of a dominant negative form of TCF4 inhibited *hTERT* expression in cancer cells. Through promoter mapping, electrophoretic mobility shift assay, and chromatin immunoprecipitation assay, we found that *hTERT* is a direct target of β -catenin·TCF4-mediated transcription and that the TCF4 binding site at the *hTERT* promoter is critical for β -catenin·TCF4-dependent expression regulation. Given the pivotal role of telomerase in carcinogenesis, these results may offer insight into the regulation of telomerase in human cancer.

Telomeres are highly specialized structures at chromosome ends that are essential for genome stability (1). Telomerase is a

ribonucleoprotein that catalyzes *de novo* synthesis of repetitive telomeric DNA after cell division and maintains chromosomal stability, leading to cellular immortalization (2, 3). Telomere dysfunction and telomerase activation have been implicated in human cancer progression (4). A high level of telomerase activity (TA)² is detected in about 90% of human cancer specimens, whereas most somatic cells do not display TA or express it only at very low levels in a cell cycle-dependent manner (5, 6). Overexpression of telomerase can stabilize telomeres in normal human cells and extend their replicative life span by at least 20 doublings (7). Conversely, inhibition of TA in cancer cells leads to reduction in telomere length and death of tumor cells (8). These findings establish an important role of telomerase-mediated telomere maintenance in human cells and suggest that telomerase up-regulation may contribute actively to cellular immortalization and carcinogenesis (9). Therefore, telomerase can be considered as a prime target for cancer diagnosis, and telomerase repression may be a tumor-suppressive mechanism (10, 11).

The expression level of human telomerase reverse transcriptase (*hTERT*), a catalytic subunit bearing the enzymatic activity of telomerase, is the rate-limiting determinant of human TA and is thought to be a sensitive indicator of telomerase function and activity, whereas the other subunits are constitutively expressed both in normal and cancer cells (12–14). Therefore, there is no doubt that *hTERT* expression plays a key role in cancer-specific telomerase activation. Numerous studies suggest that TA and the expression of telomerase components are regulated at multiple levels, including transcription and post-transcription, accurate assembly, and proper localization; however, *hTERT* expression level is considered primarily under transcriptional control (15–17). Thus, investigation of transcriptional regulation of *hTERT* should be essential for elucidating molecular mechanisms of telomerase regulation, cellular senescence, immortalization, and carcinogenesis in humans.

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[§] This article contains supplemental Table S1 and Figs. S1–S8.

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² The abbreviations used are: TA, telomerase activity; TRAP, telomere repeat amplification protocol; qTRAP, real-time PCR-based version of TRAP; LEF, lymphoid-enhancing factor; TCF, T-cell factor; CM, culture medium; ES, embryonic stem; HMG, high mobility group.

Many factors have been implicated in direct or indirect regulation of *hTERT* in cancer and normal cells, including cellular transcriptional activators (like *c-Myc*, Sp1, HIF-1, and AP2) (18–21) as well as the repressors, most of which comprise tumor suppressor gene products, such as p53 and WT1 (22, 23). Recently, Zhou *et al.* (24) reported that inhibition of PinX1 can contribute to carcinogenesis by activating telomerase and inducing chromosome instability. However, it remains largely unknown how *hTERT* is inactivated during development and how it is reactivated during carcinogenesis. Given that most cancer cells exhibit high TA, we hypothesized that certain cancer-specifically activated signaling pathways play critical roles in telomerase activation. In this study, by using qTRAP, a real-time PCR-based version of the telomere repeat amplification protocol (TRAP) (5), we sought to identify novel TA inhibitors from well known Wnt, epidermal growth factor receptor, and JAK/STAT pathway inhibitor libraries. Moreover, this screen will unveil novel signaling pathways implicated in telomerase regulation. We have identified one of the Wnt signaling pathway inhibitors, FH535 (β -catenin-TCF complex inhibitor), which could significantly inhibit TA in all cell lines used in this study, suggesting that the Wnt pathway may play a critical role in telomerase regulation in cancer cells.

Signaling by the Wnt family is one of the fundamental mechanisms that regulate cell proliferation, cell polarity, and cell fate determination during embryonic development and tissue homeostasis (25). As a result, abnormalities in Wnt signaling are reported to promote cancer development (26, 27). β -catenin, a central effector of the Wnt pathway, is involved in diverse cellular processes, including cell adhesion, growth, differentiation, and transcription of Wnt-responsive genes (28, 29). In the absence of the Wnt signaling, β -catenin is tightly regulated by a multiprotein degradation complex, in which β -catenin is phosphorylated by glycogen synthase kinase-3 (GSK3 β), leading to its degradation via the ubiquitin-proteasome pathway (30). This continual elimination of β -catenin prevents it from reaching the nucleus, and Wnt target genes are thereby repressed by the DNA-bound lymphoid-enhancing factor (LEF)/T-cell factor (TCF) transcription factors. In the presence of Wnt signaling, β -catenin is uncoupled from the degradation complex and translocates to the nucleus to form complexes with LEF/TCF, thus activating Wnt target gene expression. Deregulation of β -catenin leads to the formation of β -catenin-TCF complexes and altered expression of oncogenes, such as *c-MYC* (31), cyclin D1 (32), and *NOS2* (33), which can then contribute to the development of cancer (34). However, the involvement of the Wnt/ β -catenin pathway in regulating telomerase gene expression has not yet been elucidated.

Here, we demonstrate for the first time that the *hTERT* gene is a novel target of the Wnt pathway. We employed a combination of electrophoretic mobility shift assay (EMSA), chromatin immunoprecipitation (ChIP), and luciferase reporter gene assays that led to the identification of *hTERT* as a direct transcriptional target of the β -catenin-TCF4 complex. Moreover, knockdown of β -catenin by shRNA largely repressed *hTERT* gene expression and TA in cancer cells. Our findings highlight the significance of the Wnt/ β -catenin pathway in telomerase

regulation and aid in the advancement of our understanding of the role of the canonical Wnt pathway in carcinogenesis.

EXPERIMENTAL PROCEDURES

Cell Lines—AGS, MCF7, 293T, MCF10A, HCT116, and LS174T were used in this study. HTC116, LS174T, and 293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen), whereas AGS was cultured in Roswell Park Memorial Institute-1640 (RPMI 1640; Sigma-Aldrich). Both media contain 10% heat-inactivated fetal bovine serum (FBS; Invitrogen) and 1% penicillin/streptomycin mixtures (Invitrogen). MCF10A was cultured in DMEM/F-12 with 15 mM HEPES buffer, 5% horse serum, 10 μ g/ml insulin, 20 ng/ml EGF, 100 ng/ml cholera toxin, and 1% penicillin/streptomycin mixtures.

L-cells (control) and Wnt-3a-expressing L-cells (ATCC) were maintained in DMEM supplemented with 10% FBS. Wnt-3a-expressing L-cells were additionally supplemented with 400 μ g/ml G418. For the preparation of L-control medium and Wnt-3a conditioned medium (CM), CM was collected from cultured parental L-cells or Wnt-3a-expressing L-cells and was diluted to 50% in serum-free DMEM. All cells were cultured in an incubator with 5% CO₂ and 37 °C.

Inhibitor Library—The InhibitorSelectTM inhibitor library, consisting of well defined inhibitors from Wnt, epidermal growth factor receptor, and JAK/STAT signaling pathway inhibitor libraries, was purchased from Merck. Wnt pathway inhibitors A–O are listed in supplemental Table S1.

qTRAP—qTRAP is a real-time PCR-based method that measures the ability of telomerase to add telomeric repeats to a substrate. The real-time PCR-based version of the TRAP assay allows the estimation of TA in real time via fluorescence measurements. The qTRAP assay was modified from a conventional TRAP assay for use on the Rotor-Gene 6000 system (Qiagen) as described previously (35). Briefly, cells were treated with various signaling pathway inhibitors, and samples were lysed in 0.5% (v/v) CHAPS buffer (pH 7.5) supplemented with 10 mM Tris-HCl, 1 mM MgCl₂, 1 mM EGTA, 0.1 mM benzamidine, 5 mM 2-mercaptoethanol, and 10% glycerol for 30 min on ice. Following lysis, the samples were centrifuged for 20 min at 12,000 \times g at 4 °C to remove cell debris. The telomerase reaction was carried out in 1 \times TRAP buffer (20 mM Tris-HCl, pH 8.3, 1.5 mM MgCl₂, 63 mM KCl, 0.05% Tween 20, 1 mM EGTA, 0.1 mg/ml BSA) and 50 μ M each of the four dNTPs and 80 ng/ μ l TS primer (5'-AAT CCG TCG AGC AGA GTT-3') and 2 μ g (protein amount) of cell lysate in a total volume of 10 μ l for 30 min at 30 °C and was stopped by incubation at 94 °C for 10 min. The qTRAP was subsequently carried out by adding 10 μ l of the following 2 \times PCR mixture (2 \times TRAP buffer, 1 mg/ μ l BSA, 40 ng/ μ l ACX primer (5'-GCG CGG CTT ACC CTT ACC CTT ACC CTA ACC-3'), 15% glycerol, 1:10,000 SYBR Green, 0.08 unit/ μ l *Taq* polymerase). The PCR conditions used were as follows: 10-min incubation at 94 °C and 40 cycles of PCR at 94 °C for 30 s and 60 °C for 90 s. All of the samples were quantified using the Rotor-Gene quantification software and then compared with the standard curve generated using 293T cells and activity expressed as relative TA, relative to the control.

hTERT Is a Wnt Pathway Target Gene

Real-time PCR—Reverse transcription was performed using the Promega RT-PCR kit and oligo(dT) primer as per the manufacturer's protocol (Promega). Real-time PCR was performed using Brilliant SYBR Green qPCR Master Mix on the Rotor-Gene 6000 system (Qiagen). The following primers were used for real-time PCR: 5'-CATGAGGTGGTAGCCTGGAT-3' and 5'-ACCACTGTGCCCTGTCTTCT-3' (human *DKC1*), 5'-GCGAAGAGTTGGGCTCTGTC-3' and 5'-CATGTGTG-AGCCGAGTCCTG-3' (human telomerase RNA), 5'-ATGC-GACAGTTCGTGGCTCA-3' and 5'-ATCCCCTGGCACTG-GACGTA-3' (*hTERT*), and 5'-GTGGACCTGACCTGCCG-TCT-3' and 5'-GGAGGAGTGGGTGTCGCTGT-3' (human glyceraldehydes-3-phosphate dehydrogenase (*GAPDH*)). Data were analyzed using the $\Delta\Delta CT$ method.

Generating Stable Cell Line—Lentiviral pLKO.1 short hairpin RNA (shRNA) against human β -catenin and control scramble shRNA lentiviral particles (Sigma) and retroviral vector pSUPER-retro containing human β -catenin sequence, retroviral vector pBabe-*hTERT*-hygro, were used to generate lentivirus and retrovirus in 293T or phoenix cells. The viruses were then used to infect cells, followed by 2 $\mu\text{g}/\text{ml}$ puromycin or 100 $\mu\text{g}/\text{ml}$ hygromycin (AG Scientific, Inc.) selection 24 h after infection. After 14 days of selection, stable pools of puromycin- or hygromycin-resistant cells were obtained and further expanded.

Telomere Length Assay—DNA was extracted from the cells using the DNeasy blood and tissue kit (Qiagen). Telomere length analysis was carried out using a non-radioactive TeloTAGGG telomere length assay (Roche Applied Science) as described by the manufacturer. Approximately 1 μg of DNA of each sample was digested with *HinfI*/*RsaI* enzyme mix and separated by gel electrophoresis. DNA fragments were transferred to a nylon membrane (GE Healthcare) by Southern transfer and hybridized to digoxigenin (digoxigenin-labeled probe), specific for telomeric repeats. A digoxigenin-specific antibody conjugated to alkaline phosphatase was used to incubate the membrane, and the probe was visualized by chemiluminescence detection and subsequent exposure to x-ray film (GE Healthcare). Mean telomeric repeat binding factor lengths were determined by comparison with the molecular weight standard.

Generation of Mutant Promoter Reporter Vectors—Luciferase vector pGL3-88bp, pGL3-385bp, and pGL3-949bp driven by various length of wild-type *hTERT* promoter were kind gifts of Prof. Horikawa, Izumi (NCI, National Institutes of Health). The putative TCF4 binding element (TBE) (5'-TGCAAAG-3') contained in the *hTERT* promoter (between -659 and -653 bp) was mutated to 5'-TGCGGAG-3' (Mut1) and 5'-TGC-AAGA-3' (Mut2) using site-directed mutagenesis according to the manufacturer's recommendations (QuikChange™ site-directed mutagenesis kit, Stratagene). The mutations were confirmed by sequencing. The following primers were used for generating the mutants: 5'-CCG CCT GAG AAC CTG CCG AGA GAA ATG ACG GGC C-3' and 5'-GGC CCG TCA TTT CTC TCC GCA GGT TCT CAG GCG G-3' (Mut1), 5'-GCC GCC TGA GAA CCT GCA AGA AGA AAT GAC GGG CC-3' and 5'-GGC CCG TCA TTT CTT CTT GCA GGT TCT CAG GCG GC-3' (Mut2), and 5'-GCG AGA TCT GCG AGA AAT

GAC GGG CCT GTG TCA AGG-3' and 5'-GGC CAG GGC TTC CCA CGT GCG CAG TAT CCA TGG TAT-3' (Truncate).

Transient Transfections and Luciferase Reporter Assays—All transient transfections were performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's recommendations. Respective amounts of cells ($2 \times 10^5/\text{ml}$ for MCF7, $4 \times 10^5/\text{ml}$ for HCT116 and 293T cells) were seeded in a 24-well plate. 1 μg of plasmid encoding the protein ΔN β -catenin, ΔN TCF4, LEF1, TCF1, TCF3, TCF4, TAK1, or TAB or pCDNA control were transfected into cells. 2 days after transfection, cells were harvested and analyzed by real-time PCR and qTRAP. In a luciferase reporter assay, wild-type pGL3-88bp, pGL3-385bp and pGL3-949bp or mutant reporter plasmids were transfected into cells. After 24 h, cells were harvested, and whole cell lysates were used to measure luciferase activities using the Dual-Luciferase reporter assay system (Promega). To examine the effect of the activated Wnt pathway on the *hTERT* promoter, cells were treated with Wnt-3a CM or LiCl after transfection. In another assay, pGL3-88bp, pGL3-385bp, and pGL3-949bp reporter plasmids were co-transfected with TAK1 and TAB expression vector to study TAK1 negative regulation on the *hTERT* promoter. All firefly luciferase readings were normalized with the *Renilla* luciferase readings. Reporter assays were conducted in duplicates and repeated three times.

EMSA—Sense and antisense oligonucleotides containing TCF4 binding sites were labeled by the Biotin 3' End DNA labeling kit (Thermo Scientific). The binding reactions contain 10 μg of crude 293T nuclear extract, 1 μg of double-stranded poly(dI-dC)-poly(dI-dC), and 10 fmol of biotin-labeled probe in 20 μl of 1 \times EMSA buffer (25 mM HEPES, pH 7.9, 62.5 mM KCl, 0.05% Nonidet P-40, 2 mM MgCl_2 , 8% Ficoll 400, 500 $\mu\text{g}/\text{ml}$ bovine serum albumin, and protease inhibitors (0.5 mM PMSF, 5 $\mu\text{g}/\text{ml}$ each of antipain, leupeptin, aprotinin, chymostatin, and pepstatin A), and 3.75 μl of H_2O). The incubation was carried out for 30 min at room temperature. Unlabeled probes in 500-fold molar excess were included at the time of reaction for competitive binding assays. Anti-TCF4 and c-Myc antibodies were used for supershift analysis. 20 μl of the reaction was loaded onto a 6% non-denaturing polyacrylamide gel and transferred onto a nylon membrane. DNA was cross-linked to the membrane under UV light. The biotin-end-labeled DNA was detected using the streptavidin-horseradish peroxidase conjugate and chemiluminescent substrate.

ChIP Assays—The assays for ChIP were performed using reagents commercially obtained from Upstate Biotechnology and conducted according to the manufacturer's instructions. Briefly, formaldehyde-cross-linked chromatin was isolated from 5×10^7 of HCT116 cells and sonicated into 500–1000-bp fragments. Immunoprecipitation was performed with anti-TCF4, anti- β -catenin (Millipore), and normal mouse IgG (Santa Cruz Biotechnology, Inc.). ChIP-enriched DNA was quantified by quantitative real-time PCR, and primer sets were designed flanking the putative TBE: *hTERT* 1, forward (5'-CCG-GTGGGTGATTAACAGAT-3') and reverse (5'-GGACGTG-AAGGGGAGGAC-3'); *hTERT* 2, forward (5'-CCCCGGTGG-GTGATTAACAG-3') and reverse (5'-GGAGTGCCTCCCT-GCAACAC-3'); *hTERT* 3, forward (5'-CTCCATTTCCCACC-

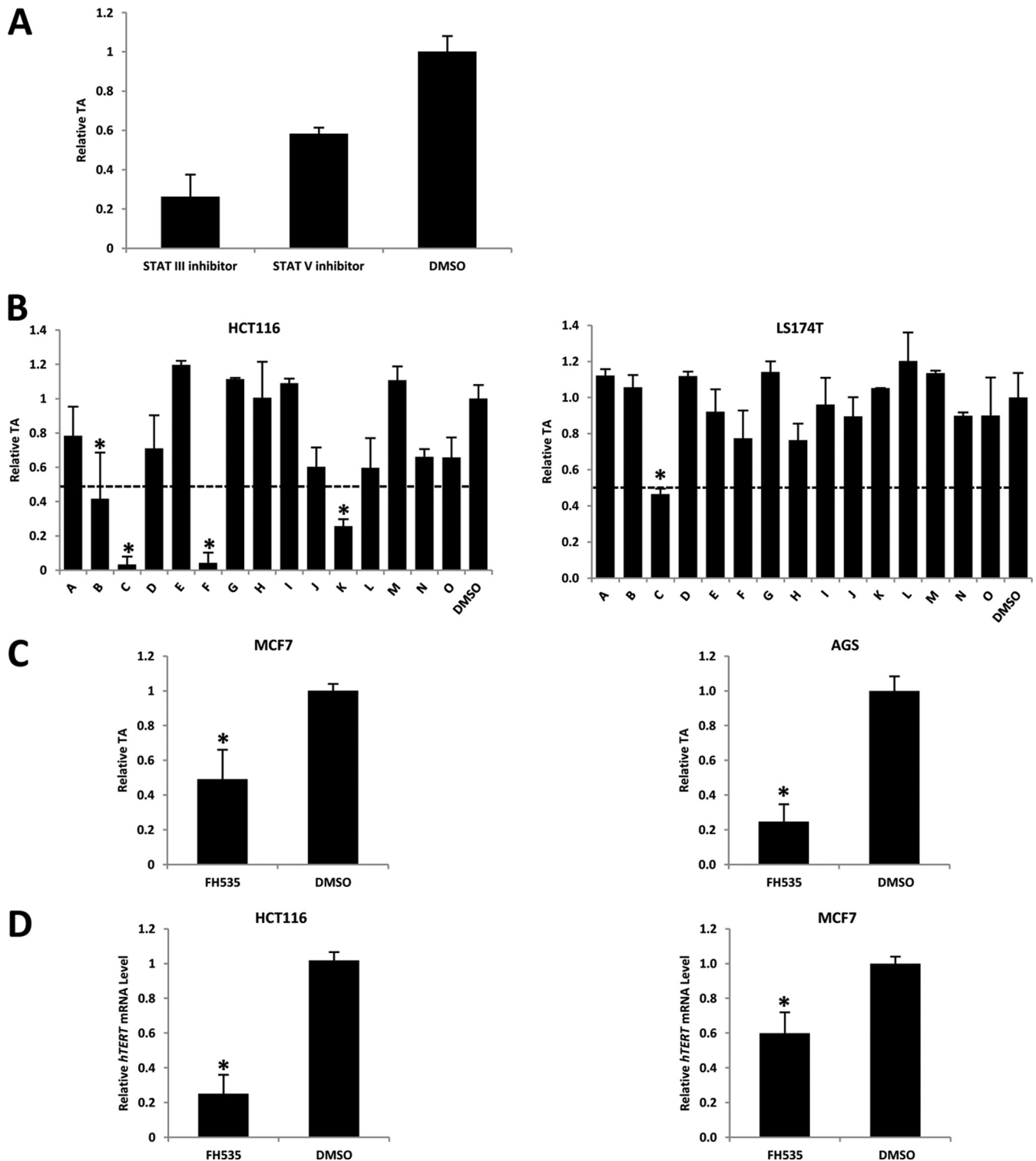
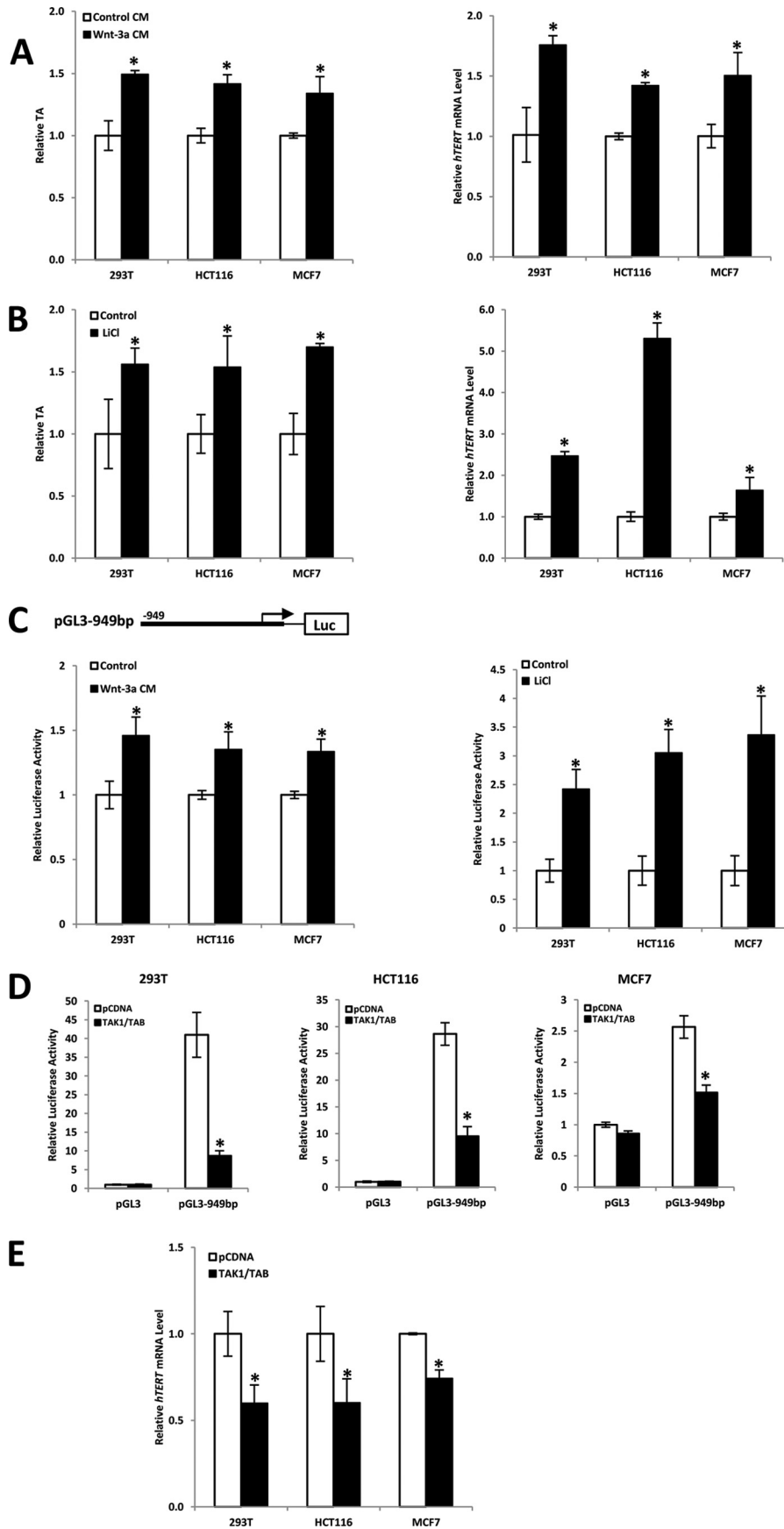


FIGURE 1. Screening telomerase inhibitor from well known signaling pathway inhibitor libraries. A, STAT III/V inhibitors inhibit TA in HCT116 cells. 24 h before drug treatment, 4×10^5 HCT116 cells were seeded into 12-well plates. The seeding density was chosen to obtain the optimum 60% confluence prior to drug treatment. Cells were treated with inhibitors or DMSO (vehicle control) and incubated for 48 h. After treatment, cells were harvested for qTRAP to measure TA. B, effect of Wnt pathway inhibitors on TA in HCT116 and LS174T cells. FH535, inhibitor C, is the most effective TA inhibitor. Inhibitors named A–O are listed in supplemental Table S1. The dotted line marks 50% TA inhibition, which was normalized with DMSO control. C, FH535 inhibitory effect on TA is validated in MCF7 and AGS cell lines. D, FH535 treatment leads to reduction of *hTERT* expression. Data were normalized against DMSO treatment. Data are the average of three independent experiments. *, $p < 0.05$. Error bars, S.D.

CTTTCT-3') and reverse (5'-ACTTGGGCTCCTTGACA-CAG-3'); *hTERT* 4, forward (5'-AGGCCGTTGTGGCTGGT-GTGAG-3') and reverse (5'-CCGTCATTTCTCTTTGCA-

GGT-3'); *hTERT* 5, forward (5'-TTGCTCATGGTGGGGA-CCC-3') and reverse (5'-GACGAACCCGAGGACGCAT-3'). Data are expressed as percentage of input.

hTERT Is a Wnt Pathway Target Gene



Search for Consensus TCF/LEF Site—TCF binding sites (A/T)(A/T)CAAAG in the *hTERT* promoter were identified using an *in silico* tool named TFEplorer. TFEplorer is a database of regulatory elements in the genomic sequences of humans, mice, and rats. We obtained a TCF4 binding sequence 653 bp upstream of the transcription start site of the *hTERT* promoter from the human genome annotation files provided by NCBI.

Senescence-associated β -Galactosidase Staining—BJ fibroblast cells were cultured to the same population doubling level in the presence or absence of LiCl or Wnt-3a CM. Cell staining was performed using a kit from U.S. Biological. After removing growth medium from the cells, the cells were washed with PBS and fixed with the manufacturer's fixative solution for 15 min at room temperature. The cells were then washed twice with PBS and stained with X-gal staining solution overnight at 37 °C following the manufacturer's protocol.

Statistical Analysis—All experiments were independently performed at least three times, with similar results. All data are presented as mean \pm S.D. of data obtained and were analyzed using either one-way analysis of variance or Student's *t* test, and $p < 0.05$ was considered statistically significant.

RESULTS

Identification of Signaling Pathway Inhibitors That Preferentially Inhibit TA—To identify telomerase inhibitors from the InhibitorSelect™ inhibitor library, consisting of Wnt, epidermal growth factor receptor, and JAK/STAT inhibitor libraries, we modified the existing qTRAP to suit the screen (36). TA was quantitated using qTRAP, and all data obtained were normalized against the vehicle control, DMSO. The concentration of each inhibitor was chosen based on its half-maximal inhibitory concentration (IC_{50}). Screen and validation were carried out in a wide range of cancer cell lines (gastric cancer, AGS; breast cancer, MCF7; colorectal cancer, HCT116 and LS174T) that have similarly reactivated telomerase enzymatic activity but differ in genetic backgrounds. Inhibitors shown to be able to reduce TA by at least 50% in the initial screen were considered as potent telomerase inhibitors and further validated. It has been shown that STAT III and V are implicated in transcriptional activation of the *hTERT* promoter (36, 37); thus, their respective inhibitors served as positive controls in our screen platform. As shown in Fig. 1A, STAT III and V inhibitors were able to significantly reduce TA in HCT116 cells by 60 and 40% respectively, suggesting that the screen platform is workable.

The screen result showed that four Wnt pathway inhibitors (B, casein kinase II inhibitor III, TBCA; C, β -catenin·TCF complex inhibitor, FH535; F, protein kinase A H-89, dihydrochloride; K, TAK1 inhibitor, (5Z)-7-oxozeaenol; supplemental

Table S1) were able to inhibit TA by at least 50% compared with vehicle controls after 2 days of treatment in HCT116 cells (Fig. 1B, left). To further confirm the result and avoid cell type specificity, the same screen was performed with LS174T cells (Fig. 1B, right). We also found that FH535 could efficiently reduce TA by 50%, whereas other hits did not show an inhibitory effect in LS174T cells. Moreover, FH535 also demonstrated a strong inhibitory effect on TA in MCF7 and AGS cells (Fig. 1C). Our data showed that FH535 treatment led to significant reduction in *hTERT* expression (Fig. 1D), suggesting that the inhibition of TA was through inhibition of the telomerase component, *hTERT* transcription. Taken together, these results promoted us to hypothesize that *hTERT* is a novel Wnt target gene, and β -catenin·TCF complexes may play a critical role in telomerase regulation in cancer cells.

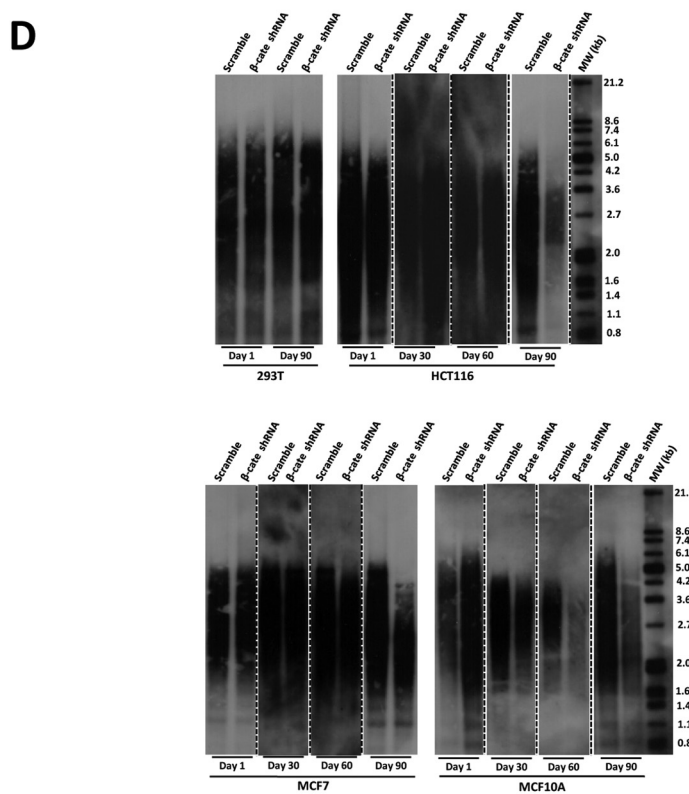
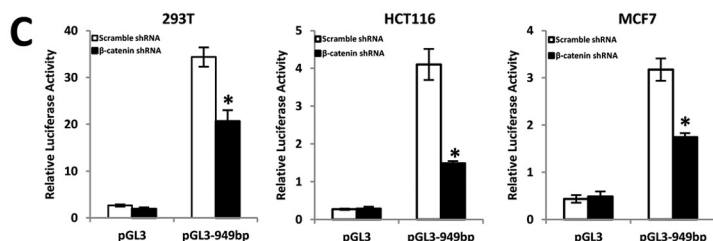
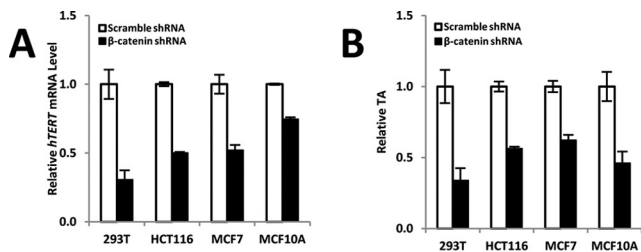
***hTERT* Is Up-regulated by Activated Wnt/ β -Catenin Signaling**—To identify whether the *hTERT* gene is regulated by the Wnt pathway, 293T, HCT116, and MCF7 cells were exposed to Wnt-3a CM or control medium for 48 h. TA and *hTERT* gene transcription were measured by qTRAP and real-time PCR, respectively. Compared with controls, we found that TA was increased 1.5-, 1.41-, and 1.34-fold in 293T, HCT116, and MCF7 cells, respectively, following Wnt-3a CM treatment (Fig. 2A, left). Consistent with this result, we found that Wnt-3a CM treatment could largely elevate the *hTERT* mRNA level (Fig. 2A, right), suggesting that the activated Wnt pathway is able to stimulate *hTERT* expression and hence enhance TA. To support this result, we examined the effect of LiCl on the expression of the *hTERT* gene. LiCl, a small molecular activator of Wnt signaling that works by inhibiting GSK-3 β , hence increases and stabilizes β -catenin protein levels (38). As shown in Fig. 2B, 15 mM LiCl treatment for 24 h in multiple human cell lines that we have tested efficiently elevated TA (left) as well as *hTERT* mRNA level (right).

To further confirm these results, a luciferase reporter assay was carried out to examine the effect of Wnt signaling on activation of the *hTERT* gene promoter. A luciferase reporter construct containing 0.95 kb upstream of the *hTERT* gene transcription initiation site (39) was transiently transfected into 293T, HCT116, and MCF7 cells. As shown in Fig. 2C, Wnt-3a CM (left) or LiCl (right) treatment significantly enhanced *hTERT* gene promoter activity as compared with controls.

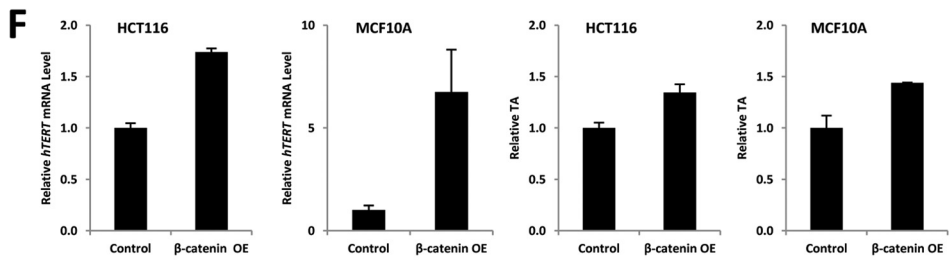
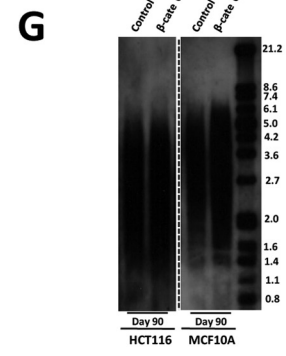
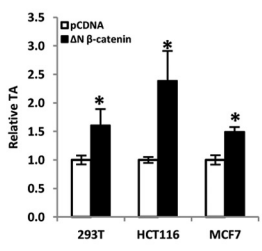
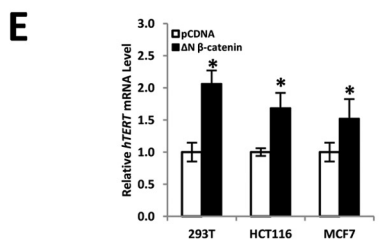
Additionally, the involvement of β -catenin·TCF complexes in *hTERT* gene transactivation is suggested by the inhibition of *hTERT* promoter activation using TAK1 (transforming growth factor- β -activated kinase 1). The TAK1-NLK (Nemo-like kinase) pathway plays a negative role in the canonical Wnt/ β -catenin signaling through regulating the LEF/TCF family tran-

FIGURE 2. Activated Wnt/ β -catenin directly affects *hTERT* gene expression and TA. A and B, Wnt-3a and LiCl stimulation of 293T, HCT116, and MCF7 cells increases *hTERT* gene expression and TA. Cells were treated with Wnt-3a CM or control CM (A) for 48 h or 15 mM LiCl for 24 h (B), respectively, and assayed for real-time PCR and qTRAP to measure TA (left) and *hTERT* expression level (right). C, activated Wnt pathway elevates *hTERT* promoter activities. A luciferase reporter construct, containing 0.95 kb upstream of the *hTERT* gene transcription initiation site, was transiently transfected into 293T, HCT116, and MCF7 cells. 24 h after transfection, cells were treated with Wnt-3a CM (left) or 15 mM LiCl (right), respectively. Wnt-3a CM or 15 mM LiCl could significantly activate *hTERT* promoter compared with controls. D and E, Wnt pathway negative regulator TAK1 represses *hTERT* promoter activities. 293T (Wnt-3a CM-treated), HCT116, and MCF7 cells were co-transfected with a 949-bp *hTERT* promoter-driven reporter construct and the indicated expression constructs for TAK1 and TAB (D). In another assay, after transfection with TAK1, cells were subjected to real-time PCR to measure *hTERT* expression (E). Empty expression vector pCDNA was used as a control. Expression of TAK1/TAB abolished *hTERT* promoter activities compared with control. Relative luciferase activity was standardized to *Renilla* luciferase activities. Data are the average of three independent experiments. *, $p < 0.05$. Error bars, S.D.

hTERT Is a Wnt Pathway Target Gene



Samples	Mean Telomere length (kb)
293T Scramble(Day1)	2.7
293T β-cate shRNA (Day1)	2.7
293T Scramble(Day 90)	2.7
293T β-cate shRNA (Day 90)	2.7
HCT116 Scramble(Day1)	2.2
HCT116 β-cate shRNA (Day1)	2.2
HCT116 Scramble(Day30)	2.2
HCT116 β-cate shRNA (Day30)	2.2
HCT116 Scramble(Day60)	2.2
HCT116 β-cate shRNA (Day60)	2.1
HCT116 Scramble(Day 90)	2.2
HCT116 β-cate shRNA (Day 90)	2.0
MCF7 Scramble(Day1)	2.7
MCF7 β-cate shRNA (Day1)	2.7
MCF7 Scramble(Day30)	2.7
MCF7 β-cate shRNA (Day30)	2.6
MCF7 Scramble(Day60)	2.7
MCF7 β-cate shRNA (Day60)	2.5
MCF7 Scramble(Day 90)	2.7
MCF7 β-cate shRNA (Day 90)	2.3
MCF10A Scramble(Day1)	3.0
MCF10A β-cate shRNA (Day1)	3.0
MCF10A Scramble(Day30)	3.0
MCF10A β-cate shRNA (Day30)	3.0
MCF10A Scramble(Day60)	3.0
MCF10A β-cate shRNA (Day60)	2.6
MCF10A Scramble(Day 90)	3.0
MCF10A β-cate shRNA (Day 90)	2.6



Samples	Mean Telomere length (kb)
HCT116 Control(Day 90)	2.2
HCT116 β-cate OE (Day 90)	2.2
MCF10A Control(Day 90)	3.0
MCF10A β-cate OE (Day 90)	3.0

scriptional factors. When activated by TAK1, NLK can directly phosphorylate LEF/TCFs to prevent the β -catenin·LEF/TCF complex from binding to DNA (40). It is known that β -catenin is able to accumulate in the nucleus of cancer cells but not in 293T cells. To check the biological function of β -catenin·LEF/TCF complex in *hTERT* gene regulation, 293T cells were treated with Wnt-3a CM to stimulate the accumulation of β -catenin in the nucleus. Luciferase reporter assay results demonstrated that transactivation of the *hTERT* promoter by endogenous β -catenin·TCF complexes was strongly repressed by co-transfection of TAK1/TAB (TAK1-binding protein) compared with the empty vector pCDNA3 (Fig. 2D). Consistent with this result, our data demonstrated that exogenous TAK1/TAB could reduce *hTERT* expression in all cell lines tested (Fig. 2E). In conclusion, these data indicate that the expression of the *hTERT* gene can be regulated by Wnt/ β -catenin pathway in human cancer cells.

Knockdown of β -Catenin or Its Overexpression Dramatically Affects *hTERT* Expression and Telomerase Activity—To study the possible role of β -catenin·TCF complexes in telomerase regulation, we examined whether endogenous β -catenin·TCF signaling activates the endogenous *hTERT* gene. Here, we focused on β -catenin, which is a central player in the Wnt signaling pathway. We established stable β -catenin knockdown in 293T, HCT116, MCF7, and MCF10A cell lines with lentiviral shRNA against β -catenin. These β -catenin knockdown cell lines were used to examine the effect of β -catenin on telomerase regulation. Western blot results showed that β -catenin protein expression levels were significantly decreased in the β -catenin knockdown stable cell lines (supplemental Fig. S1). Analysis of *hTERT* expression in such stable cell lines, where β -catenin levels were suppressed, revealed a major decrease in *hTERT* mRNA level (Fig. 3A). In addition, qTRAP assay results indicated that silencing the β -catenin gene markedly reduced TA (Fig. 3B), which is consistent with real-time PCR results in Fig. 3A. Moreover, the luciferase reporter assay results showed that repressing endogenous β -catenin expression could significantly inhibit *hTERT* promoter activity in 293T, HCT116, and MCF7 cells (Fig. 3C).

The activation of *hTERT* is required for the maintenance of telomere length in cancer cells. To better understand the biological function of β -catenin in regulating activation of *hTERT*, we determined the effects of inhibition of β -catenin expression on telomere length in stable β -catenin knockdown cells. We continuously cultured stable β -catenin knockdown cells over 90 days through ~30 cell passages. Cells were collected at day 1,

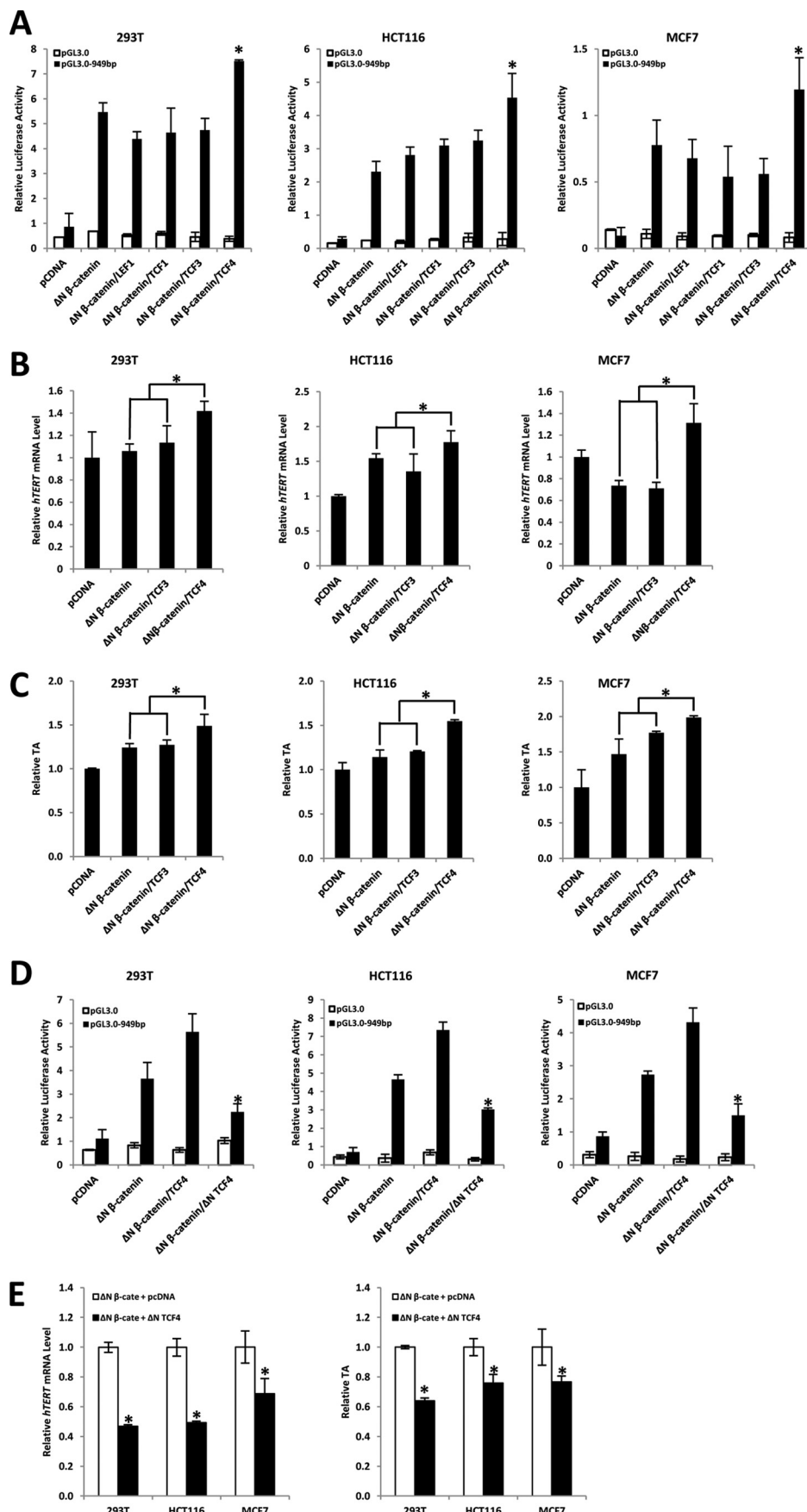
day 30, day 60, and day 90, and the effect of β -catenin on telomere length was determined by Southern blotting. Telomere signals appeared as a broad smear of densities. As shown in Fig. 3D, β -catenin knockdown effectively induced significant telomere shortening in HCT116, MCF7, and MCF10A cells compared with stable cells infected with scramble shRNA lentivirus. However, silencing β -catenin gene in 293T cells had no effect on telomere length maintenance. It is possible that β -catenin knockdown may take a longer time to affect the telomere length in 293T cells.

Next, we examined whether the expression of *hTERT* is regulated in a β -catenin-dependent manner. We exogenously provided 293T, HCT116, and MCF7 cells with a constitutively active form of β -catenin (Δ N β -catenin) as described (41) and examined the β -catenin dependence of the *hTERT* gene expression and TA by real-time PCR or qTRAP analysis, respectively. As shown in Fig. 3E, ectopic expression of the stable active β -catenin form enhanced transcriptional activation of the *hTERT* gene (left) and subsequently elevated TA (right). To further confirm this result, we established stable β -catenin overexpression in HCT116 and MCF10A cell lines with retroviral expression vector. Real-time PCR results demonstrated that β -catenin expression levels were significantly enhanced in the β -catenin overexpression stable cell lines (supplemental Fig. S2). Compared with the controls, real-time PCR analysis showed that *hTERT* gene expression was elevated by 1.7- and 6.7-fold in stable β -catenin overexpression HCT116 and MCF10A cell lines, respectively (Fig. 3F, top). Consistent with this result, TA was correspondingly enhanced in both cell lines (Fig. 3F, bottom). Although *hTERT* gene expression and TA were increased in β -catenin overexpression stable cell lines, we did not find obvious telomere length extension (Fig. 3G). It could be that β -catenin overexpression may take a longer time to affect the telomere length or that immortalized cells just need telomerase to maintain telomere length rather than extend telomere length. Overall, these results suggest that β -catenin plays an important role in regulating the expression of *hTERT*.

β -Catenin and TCF4 Up-regulate Promoter Activity of *hTERT*—LEF/TCFs are sequence-specific DNA binding transcription factors that bind to Wnt target gene promoters and repress gene transcription. Upon β -catenin binding to LEF/TCFs, the complex will activate target gene transcription (42). Higher organisms have four family members: LEF-1, TCF-1, TCF-3, and TCF-4. To further evaluate the function of β -catenin·TCF complexes in telomerase regulation, we sought

FIGURE 3. Effects of β -catenin knockdown on the *hTERT* gene and TA. A, real-time PCR analysis of *hTERT* expression in β -catenin knockdown stable 293T (Wnt-3a CM-treated), HCT116, MCF7, and MCF10A cells. *hTERT* mRNA levels were normalized by comparison with GAPDH as internal controls. -Fold change was calculated relative to the control. B, TRAP was carried out to measure TA in β -catenin knockdown stable cells and control cells. C, 949-bp *hTERT* promoter-driven luciferase reporter construct was transiently transfected into β -catenin knockdown stable cells: 293T (Wnt-3a CM-treated), HCT116, and MCF7. 24 h after transfection, cells were collected for luciferase activity measurement. β -Catenin knockdown could significantly inhibit *hTERT* promoter activity compared with controls. Relative luciferase activity was standardized to *Renilla* luciferase activities. Data are the average of three independent experiments. *, $p < 0.05$. D, telomere length was measured by Southern blotting in β -catenin knockdown stable cells and control cells. The table beside the Southern blot indicates the mean telomere length measured. E, expression construct containing the constitutively active form of β -catenin (Δ N β -catenin) was transiently transfected into 293T, HCT116, and MCF7 cells. 48 h after transfection, cells were harvested and subjected to real-time PCR or qTRAP to measure *hTERT* mRNA expression level (left) or TA (right). Empty expression vector pCDNA was used as a control. Data are the average of three independent experiments. *, $p < 0.05$. F, β -catenin overexpression (OE) stable HCT116 and MCF10A cell lines were obtained as described under "Experimental Procedures." These stable cells were harvested and subjected to real-time PCR or qTRAP to measure *hTERT* mRNA expression level (top) or TA (bottom). Empty retroviral expression vector was used as a control. G, telomere length was measured by Southern blotting in β -catenin overexpression stable HCT116 and MCF10A cell lines. The table beside the Southern blot indicates the mean telomere length measured. Error bars, S.D.

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to identify which LEF/TCF member can bind to the *hTERT* promoter and synergistically activate *hTERT* expression with β -catenin. To this end, we transiently co-transfected the Δ N β -catenin construct alone or with different LEF/TCF members and a 949-bp *hTERT* promoter-driven luciferase construct into 293T, HCT116, and MCF7 cells to analyze the effect on promoter activity of *hTERT*. As shown in Fig. 4A, compared with Δ N β -catenin alone, the co-transfection of Δ N β -catenin and TCF4 could significantly activate the *hTERT* promoter in 293T, HCT116, and MCF7, whereas Δ N β -catenin·TCF1/3 or LEF1 complexes had no effect on activating the *hTERT* promoter. To further confirm this result, we carried out real-time PCR to examine the effect of Δ N β -catenin·TCF3/4 complexes on *hTERT* regulation. The result demonstrated that the exogenous β -catenin·TCF4 complex could slightly elevate *hTERT* expression and TA compared with Δ N β -catenin alone or Δ N β -catenin·TCF3 (Fig. 4, B and C). To further investigate whether *hTERT* is a transcriptional target of the β -catenin·TCF4 complex and whether activation of the *hTERT* promoter is due to a synergistic effect of β -catenin and TCF4, we performed a luciferase reporter assay utilizing the dominant negative form of TCF4 (Δ N TCF4), which is known to diminish the transcriptional activity of TCF-responsive promoters (43). We observed that the stimulation of the *hTERT* promoter upon Δ N β -catenin overexpression was significantly suppressed by co-overexpression of Δ N TCF4 (Fig. 4D). In addition, ectopic expression of Δ N TCF4 could inhibit Δ N β -catenin-induced *hTERT* expression (Fig. 4E, left) and hence TA (Fig. 4E, right). Thus, β -catenin regulation on *hTERT* occurs via a TCF4-dependent pathway. These data imply that TCF4 may bind to *hTERT* promoter and activate *hTERT* gene transcription upon forming a transcriptional complex with β -catenin.

Characterization of the Distal TBE in the *hTERT* Promoter—We next sought evidence of functional binding of the β -catenin·TCF4 complex on the *hTERT* promoter. To this end, a luciferase reporter assay was carried out to examine the effect of the β -catenin·TCF4 complex on various lengths of the *hTERT* promoter (88, 385, and 949 bp) (Fig. 5A) in multiple cell lines. As shown in Fig. 5B, the 949-bp promoter could induce relatively higher luciferase activity when compared with the 88- or 385-bp *hTERT* promoter in the presence of β -catenin·TCF4 in 293T, HCT116, and MCF10A. The TAK1-NLK pathway phosphorylates LEF/TCFs and, hence, inhibits β -catenin-dependent transcription. We therefore proposed that if β -catenin·TCF4 can directly regulate *hTERT* gene expression at the 949-bp promoter, co-transfection of TAK1/TAB will only reduce the 949-bp length *hTERT* promoter activity but not

that of the 88- and 385-bp promoters. As expected, we found that co-transfection of TAK1/TAB significantly reduced luciferase activity with the 949-bp *hTERT* promoter, whereas it had no effect on the 88- or 385-bp *hTERT* promoter (Fig. 5C). These results imply that β -catenin·TCF4 can directly regulate *hTERT* expression, and one or more distal TCF4 binding sites exist between position -386 and -949 bp in the *hTERT* promoter.

To further investigate the functional interaction between β -catenin·TCF4 signaling and *hTERT* gene expression, we analyzed the transcriptional control sequences of the *hTERT* gene for TCF protein binding sites by using TESS (Transcription Element Search System). The search result revealed the presence of a putative consensus TBE between positions -659 and -653 bp (5'-TGCAAAG-3') upstream of the transcription start site of *hTERT* (Fig. 5D) that showed a high degree of homology to the core consensus sequence (CTTTG or CAAAG) for TCF4 binding (44). To test whether the putative TCF4 binding site could affect the promoter activity of *hTERT*, we introduced mutations in the TBE (Fig. 5E) by site-directed mutagenesis and constructed a shorter *hTERT* promoter (652 bp) without the TBE. We transiently co-transfected various mutant luciferase reporter constructs with β -catenin·TCF4 expression constructs into 293T, HCT116, and MCF7 cells. Introduction of the mutations into the putative TBE reduced the stimulatory effect of β -catenin·TCF4 by more than 50% compared with the wild-type promoter in all of the three cell lines (Fig. 5E), and the deletion of the TBE (Truncate) significantly repressed the *hTERT* promoter activity by $\sim 80\%$ (Fig. 5E). These results suggest that the putative TBE motif is responsible for the β -catenin·TCF4-dependent promoter activity of *hTERT*.

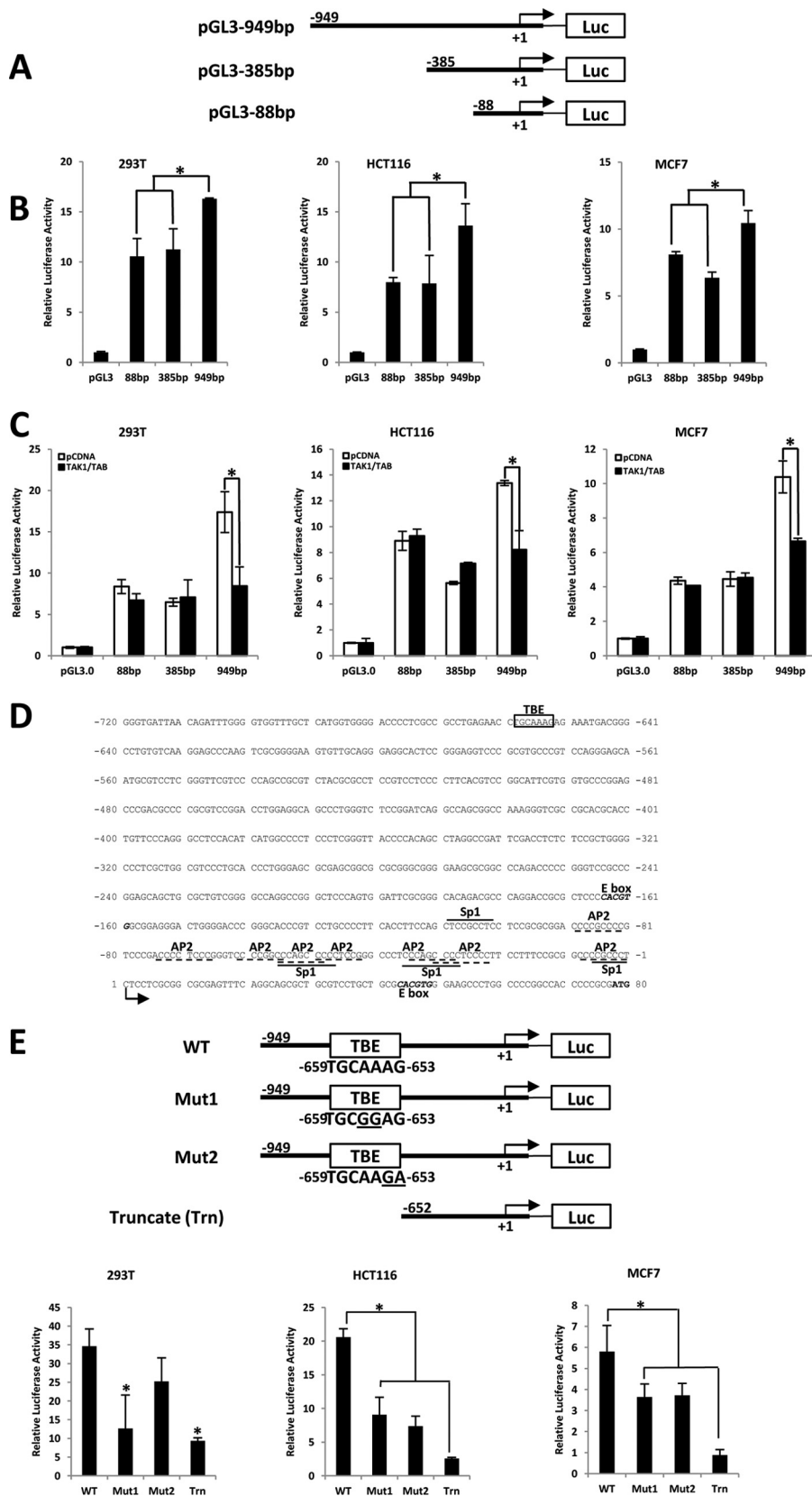
TCF4 Directly Binds to the TBE in the *hTERT* Promoter—To verify whether TCF4 could bind to the putative distal TBE, we carried out an EMSA with a probe containing the potential TBE. As shown in Fig. 6A, complexes were formed between the biotin-labeled wild-type TBE probe and proteins in 293T nuclear extracts with β -catenin·TCF4 overexpression (Fig. 6A, lane 3), whereas normal 293T nuclear extracts could not form a visible complex with biotin-labeled wild-type TBE probe (Fig. 6A, lane 2). The specificity of the interaction between β -catenin·TCF4 and wild-type TBE probe was confirmed when a mutant probe (Mut1), with a two-nucleotide substitution in the core of TBE, dramatically decreased complex formation (Fig. 6A, lane 6), which is consistent with the luciferase reporter assay results (Fig. 5E). The addition of 500-fold competitor (unlabeled wild-type TBE probe) could abrogate the complex formation between the biotin-labeled probe and the

FIGURE 4. β -Catenin·TCF4 specifically up-regulates promoter activity of *hTERT*. A, 293T, HCT116, and MCF7 cells were co-transfected with a 949-bp *hTERT* promoter-driven reporter construct, the indicated expression construct for Δ N β -catenin, and/or LEF1, TCF1, TCF3, and TCF4 expression constructs. pGL3.0 reporter plasmid was used as a control. Co-transfection of β -catenin and TCF4 specifically activates the *hTERT* promoter. B, 293T, HCT116, and MCF7 cells were transfected with the expression construct for Δ N β -catenin alone or with TCF3 or TCF4 expression constructs, respectively. Empty expression vector pCDNA was used as a control. 48 h after transfection, cells were harvested and subjected to real-time PCR to measure *hTERT* mRNA. Co-transfection of β -catenin and TCF4 could more efficiently increase *hTERT* expression. C, 293T, HCT116, and MCF7 cells were transfected with expression construct for Δ N β -catenin alone or with TCF3 or TCF4 expression constructs, respectively. Empty expression vector pCDNA was used as a control. 48 h after transfection, cells were harvested and subjected for qTRAP to measure TA. Co-transfection of β -catenin and TCF4 could more efficiently elevate TA. D, 293T, HCT116, and MCF7 cells were co-transfected with a 949-bp *hTERT* promoter-driven reporter construct or the indicated expression constructs for Δ N β -catenin, TCF4, or Δ N TCF4. pGL3.0 reporter plasmid was used as a control. Co-transfection of Δ N TCF4 significantly inhibited *hTERT* promoter activation. Relative luciferase activity was standardized to *Renilla* luciferase activities. E, 293T, HCT116, and MCF7 cells were co-transfected with expression construct for Δ N β -catenin and Δ N TCF4. pCDNA plasmid was used as a control. Co-transfection of Δ N TCF4 was able to reduce β -catenin-induced *hTERT* expression (left) and TA (right). Data are the average of three independent experiments. *, $p < 0.05$. Error bars, S.D.

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nuclear extract (Fig. 6A, lane 4), but the addition of 500-fold cold mutant probe was unable to inhibit formation of the specific complexes (Fig. 6A, lane 5), validating that the binding of

β -catenin-TCF4 to the labeled probe was specific. Moreover, the addition of anti-TCF4 antibody could result in formation of the supershifted band between nuclear proteins and TBE probe



(Fig. 6B, lane 4), providing evidence that TCF4 is one of the transcription factors that binds to the TBE in the *hTERT* promoter. Neither normal IgG nor anti-c-Myc antibody could result in formation of a supershifted band (Fig. 6B, lanes 2 and 3). We also examined whether TCF3 could bind to TBE. The EMSA data showed that TCF3 is unable to form complexes with TBE probe (supplemental Fig. S3). Taken together, the putative TBE between -659 and -653 bp in the *hTERT* promoter is indeed a TCF4 binding element. EMSAs provided direct evidence for the physical interaction between TCF4 and the TBE probes *in vitro*. To show the *in vivo* occupancy of TCF4 on the *hTERT* promoter, we performed ChIP with TCF4 antibody in HCT116 cells. The quality of the ChIP DNA was confirmed using the *SP5* promoter, a well known Wnt/ β -catenin target gene, as a positive control, and a region in the *GAPDH* coding region as a negative control. As shown in Fig. 6C (left), our data showed a specific enrichment of the *hTERT* promoter in TCF4 ChIP DNA using five pairs of *hTERT* primers, implying that TCF4 is likely to directly interact with the *hTERT* promoter. To further examine whether *hTERT* is directly regulated by β -catenin-TCF4, another ChIP assay was performed using anti- β -catenin antibody. As shown in Fig. 6C (right), anti- β -catenin antibody was able to specifically precipitate chromatin-DNA complexes containing an *hTERT* promoter fragment. In addition, LiCl treatment enhanced binding of β -catenin on the TBE of the *hTERT* promoter. Collectively, our data confirmed that endogenous TCF4 and β -catenin bound to *hTERT* TBE *in vivo*, consistent with the *in vitro* results in Fig. 6, A and B.

Wnt/ β -Catenin Pathway Is Involved in Regulation of hTERT in Somatic Cells—Association of deregulated Wnt/ β -catenin signaling with cancer has been well documented, whereas the Wnt pathway is tightly regulated in normal somatic cells. We questioned whether activation of Wnt/ β -catenin signaling could stimulate *hTERT* transcription and even reactivate telomerase in somatic cells. Very interestingly, we found LiCl or Wnt-3a CM treatment could significantly increase *hTERT* gene expression and hence reactivate telomerase in BJ cells (human normal fibroblast cells) compared with the control (Fig. 7, A and B).

Human somatic cells gradually lose telomeric sequences as a result of incomplete replication (45). Telomere shortening can induce replicative senescence, which may play an important role in suppression of cancer emergence, although inheriting shorter telomeres probably does not protect against cancer (46). To check whether activation of Wnt/ β -catenin signaling

could affect cellular senescence via reactivating telomerase, we performed a β -galactosidase assay to determine the senescence status of BJ cells in the presence or absence of LiCl and Wnt-3a CM. Cells were cultured to the same population doubling level (20 population doublings) and stained. Morphologic examination of the cells without activating Wnt/ β -catenin pathway showed an increased proportion of flat and giant cells with phenotypic characteristics of senescence and overexpression of β -galactosidase activity, suggesting that the active Wnt/ β -catenin pathway may lead to extension of the life span of somatic cells via reactivating telomerase (Fig. 7C). Taken together, these results lead us to hypothesize that Wnt/ β -catenin plays a critical role in telomerase reactivation in carcinogenesis. However, further mechanistic studies are necessary to explain the phenomenon.

DISCUSSION

In this study, we showed for the first time that *hTERT* is a direct transcriptional target of the Wnt/ β -catenin signaling pathway.

Previous studies have demonstrated that *hTERT* expression is highly specific to cancer cells and tightly associated with telomerase activity; therefore, it is the main protein that holds the key to one of the important hallmarks of cancer, the infinite proliferative capacity (14, 47–49). Despite the tremendous attention to defining telomerase regulation in cancer cells, it remains unclear how cancer cells gain the ability to reactivate telomerase and whether genetic variations affect *hTERT* expression in cancer cells. The almost universal presence of telomerase in human cancers and cancer stem cells, together with its near absence in most normal tissues make telomerase an attractive therapeutic target. Consequently, it is rather reasonable to hypothesize that the tumor-specific activation of the *hTERT* promoter may be regulated by various cellular factors, such as transcription factors and effectors, which are differentially activated in tumor cells or repressed in normal cells. These tumor-specific cellular factors can either specifically bind to the *hTERT* promoter or interact with its effectors to differentially regulate *hTERT* transcription. A large class of oncogenes can be assigned to the DNA-binding proteins, the function of which is controlled not just by their abundance or expression level but mainly at the level of their activity in terms of their interactions with DNA and protein targets.

Evidence to date indicates that the activity of the Wnt pathway is frequently deregulated, resulting in the activation of tar-

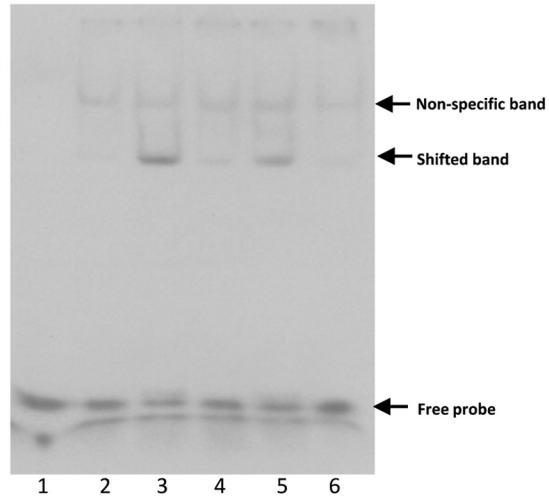
FIGURE 5. Identification of TBE in the *hTERT* promoter region. A, schematic representation of various luciferase reporter plasmids of *hTERT* promoter (88, 385, and 949 bp). B, 949-bp *hTERT* promoter is most responsive to β -catenin-TCF4. 293T, HCT116, and MCF7 cells were transiently transfected with various luciferase reporter constructs containing 88, 385, and 949 bp (upstream of the transcriptional start site) of the *hTERT* promoter and β -catenin-TCF4 expression constructs. C, TAK1/TAB reduces promoter activity of the *hTERT* by β -catenin-TCF4. 293T, HCT116, and MCF7 cells were co-transfected with the indicated various *hTERT* promoter luciferase reporters and β -catenin-TCF4 expression constructs. TAK1/TAB overexpression leads to a significant reduction in 949-bp *hTERT* promoter activity only. D, the sequence of the distal and proximal promoter of *hTERT*. Putative protein binding sites for various transcription factors in the first 181 bp are indicated. The +1 indicates the first nucleotide of the *hTERT* mRNA. Consensus motifs for Sp1 and AP2 are underlined by solid and broken lines, respectively, and the E box (c-Myc binding site) consensus motif is in *italic type*. The putative TBE consensus motif is boxed. The initiating ATG codon is shown in *boldface type*. E, TBE is important for β -catenin-TCF4 function on the *hTERT* promoter. Schematic representation of various luciferase reporter plasmids of the *hTERT* promoter. The putative TCF4 binding element is located between -659 and -653 bp from the transcriptional initiation site. Construct WT contains the wild-type binding element; Mut1 has a 2-bp substitution (AA \rightarrow GG, underlined); Mut2 has another 2-bp substitution (AG \rightarrow GA, underlined); and Truncate (Trn) only consists of the 652-bp sequence upstream of the transcriptional start site of the *hTERT* promoter, which does not contain the putative TBE. 293T, HCT116, and MCF7 cells were transiently transfected with various luciferase reporter constructs (WT, Mut1, Mut2, and Truncate) and β -catenin-TCF4 expression constructs. The promoter reporter activity was normalized with *Renilla* luciferase reporter. Data are the average of three independent experiments. *, $p < 0.05$. Error bars, S.D.

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A

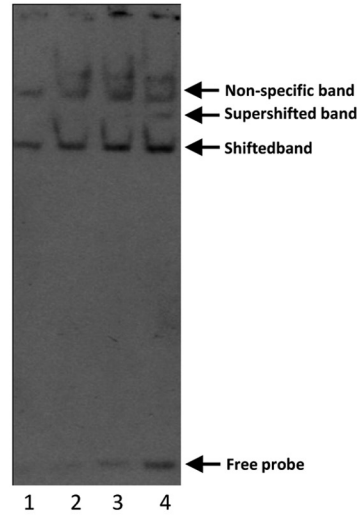
293T NE	-	+	-	-	-	-
293T (β-cate/TCF4) NE	-	-	+	+	+	+
Biotin labeled WT probe	+	+	+	+	+	-
Biotin labeled Mut1 probe	-	-	-	-	-	+
500x competitor	-	-	-	+	-	-
500x mutant competitor	-	-	-	-	+	-

WT: ctgagaacctgcaaaagagaaatgac
TBE
Mut1: ctgagaacctgaggagagaaatgac
M TBE

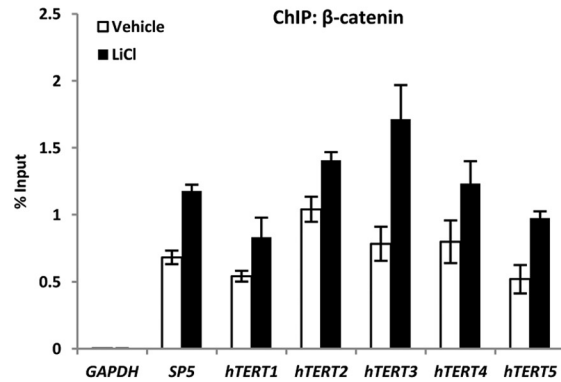
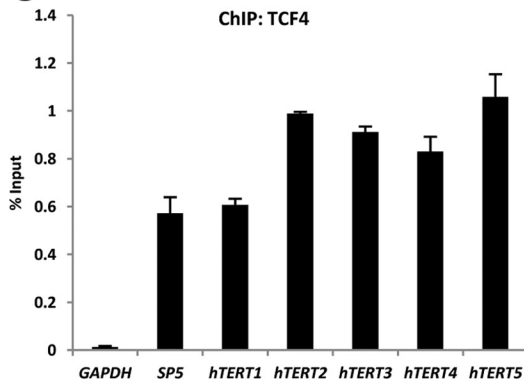


B

293T (β-cate/TCF4)NE	+	+	+	+
Biotin labeled WT probe	+	+	+	+
Anti TCF4 antibody	-	-	-	+
Anti c-Myc antibody	-	-	+	-
Normal Mouse IgG	-	+	-	-



C



get genes whose dysregulation has significant effects on the development and progression of cancer, but the molecular details remain unclear. Elevated levels of β -catenin, the central player of the canonical Wnt pathway, have been observed in most common forms of human malignancies (50). Therefore, identifying the target genes of Wnt signaling is important for understanding β -catenin-associated carcinogenesis. Recently, Park *et al.* (51) showed that *hTERT* plays an essential role in the Wnt/ β -catenin signaling pathway by serving as a cofactor in a β -catenin transcriptional complex. However, whether the Wnt/ β -catenin signaling pathway is involved in telomerase regulation was not investigated.

In this study, we have demonstrated that the real-time PCR-based qTRAP assay coupled with well defined signaling pathway inhibitor libraries is a powerful approach for discovering and detecting signaling pathways that may be involved in telomerase regulation. Using the qTRAP assay, we clearly detected a β -catenin·TCF complex inhibitor, FH535, which could effectively inhibit TA in gastric cancer, breast cancer, and colorectal cancer cells, suggesting that the Wnt pathway is widely implicated in telomerase regulation in human cancers.

To assess the function of the Wnt pathway on telomerase activation, Wnt-3a CM and LiCl were used to activate the Wnt pathway in 293T, HCT116, and MCF7 cells. We found that the activated Wnt pathway is indeed able to up-regulate *hTERT* expression and increase TA. Conversely, the Wnt pathway negative regulatory factor TAK1 could eliminate *hTERT* promoter activation. These results led us to postulate that the activated *hTERT* promoter in those tumor cells was due to an increase in the activated form of β -catenin. The results from β -catenin knockdown and overexpression experiments confirmed this notion and illustrate the critical role of β -catenin in telomerase regulation. Moreover, expression of the constitutively activated form of β -catenin could stimulate *hTERT* expression. On the other hand, silencing β -catenin gene expression resulted in down-regulation of *hTERT* expression and significant reduction of TA, which consequently led to telomere length shortening in multiple cells tested. The data from β -catenin shRNA knockdown experiments further confirmed the role of β -catenin in regulating TA and altering telomere length. Telomerase is a unique reverse transcriptase (RT) that contains a catalytic protein subunit, the telomerase RT, a telomerase RNA, and species-specific accessory proteins. Telomerase accessory protein components are known to play important roles in regulating telomerase biogenesis, subcellular localization, and function *in vivo*. For example, dyskerin is required for the stability and accumulation of human telomerase RNA *in vivo* (52), and the mutation of dyskerin is associated with dyskeratosis congenital, a human stem cell disorder (53). Thus, it would be very interesting to examine whether other telomerase

component genes are also under the Wnt pathway regulation. Our data demonstrated that β -catenin knockdown also decreased human telomerase RNA and *DKC1* expressions in human cancer cells (HCT116 and MCF7) and normal stable cell lines (293T and MCF10A) (supplemental Fig. S4). Taken together, these results suggest that the Wnt/ β -catenin pathway may play more important roles in regulating the whole telomerase holoenzyme level *in vivo*.

It is known that human embryonic stem (ES) cells also present high TA, which is essential for the cells' self-renewal characteristics. Previous studies demonstrated that modulation of Wnt signaling by controlling the dose of adenomatous polyposis coli or by treatment with a GSK3- β inhibitor can enhance self-renewal of both mouse and human ES cells (54, 55). These results led us to question whether the Wnt pathway is also involved in telomerase regulation in human ES cells. In the present study, we found that silencing β -catenin was capable of inhibiting *hTERT*, telomerase RNA, and *DKC1* expressions and repressing TA in human ES cell line hES3 (supplemental Figs. S5 and S6), suggesting that the Wnt pathway may play a pivotal role in telomerase regulation not only in human cancer cells but also in ES cells. This is supported by the fact that the β -catenin·TCF4 complex drives the same genetic program in colorectal cancer cells as in crypt stem and progenitor cells (56). Moreover, we demonstrated that the activated Wnt pathway could increase *hTERT* expression and reactivate telomerase, leading to an extended life span in BJ cells. Somatic cells do not display TA, or they express it only at very low levels in a cell cycle-dependent manner (5, 6). However, telomerase is reactivated and TA is largely increased during carcinogenesis and reprogramming from somatic cells to iPS (induced pluripotent stem) cells. The molecular mechanism of how telomerase is reactivated still remains unclear. Our data may suggest that the Wnt pathway could be involved in telomerase reactivation in carcinogenesis and reprogramming. However, further studies are required. Moreover, other signaling cascades can also impinge on the expression of telomerase (10). For instance, STAT III selectively stimulates *hTERT* expression through the JAK/STAT signaling pathway (37). Therefore, several signaling cascades can contribute to the control of the *hTERT* gene expression, which might be used differentially in distinct types of tumors. Our findings indicated that β -catenin knockdown significantly inhibited telomerase genes expression in Wnt pathway deregulated cells (*i.e.* HCT116), whereas silencing β -catenin just moderately repressed telomerase gene expression in human ES cells and MCF10A, in which Wnt pathway is tightly regulated. It seems like, in Wnt pathway deregulated cells, the Wnt pathway plays a predominant role in telomerase regulation.

FIGURE 6. Binding of TCF-4 and β -catenin to a putative TBE in the *hTERT* promoter. A, TBE is important and is the minimal sequence for TCF4 binding. Biotin-labeled wild-type TBE probe (lanes 1–5) and mutant TBE probe (lane 6) were incubated with 10 μ g of 293T nuclear extracts or 293T nuclear extracts with β -catenin·TCF4 overexpression for 30 min. Competition experiments were performed by preincubating with a 500-fold molar excess of the unlabeled TBE probes (competitor; lane 4), or 500-fold molar excess of the unlabeled mutant TBE (mutant competitor; lane 5). The arrow indicates the position of specific transcription factor complexes. B, biotin-labeled TBE (lanes 1–4) were incubated with 10 μ g of 293T cell nuclear extracts with β -catenin·TCF4 overexpression for 30 min. Antibody binding experiments were carried out following the vendor's instruction with anti-TCF4 antibody (lane 4), normal mouse IgG as control (lane 2), or anti-c-Myc antibody (lane 3). The arrows indicate the position of shifted and supershifted bands. C, TCF4 binds to the *hTERT* promoter *in vivo*. A ChIP assay was performed using anti-TCF4 or β -catenin antibodies and analyzed by real-time PCR. GAPDH was used as a negative control, and *SP5* was used as a positive control. Error bars, S.D.

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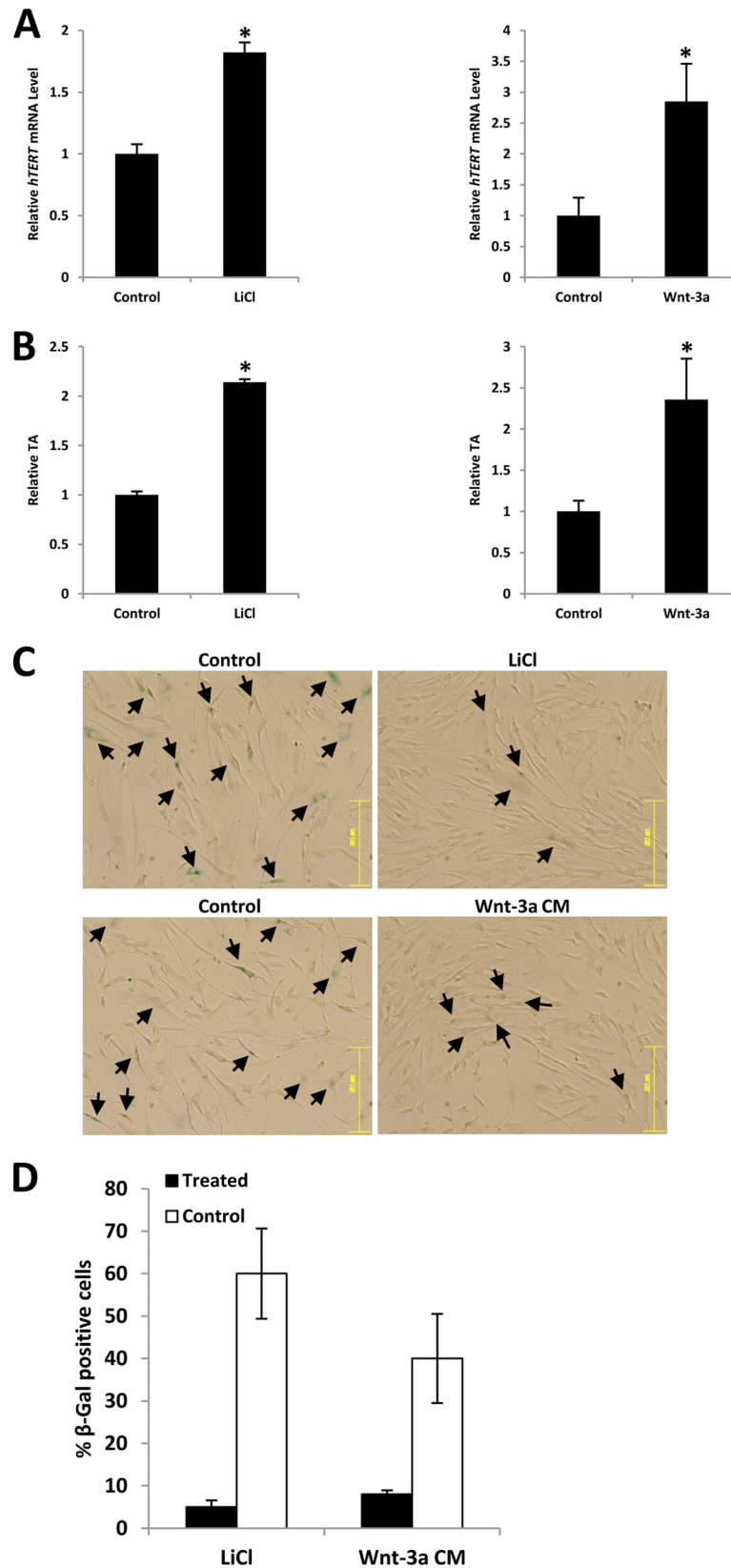


FIGURE 7. The Wnt/ β -catenin signaling pathway is involved in telomerase reactivation in somatic cells. A and B, LiCl or Wnt-3a CM stimulation of BJ cells increases *hTERT* gene expression and reactivates telomerase. BJ cells were treated with LiCl or Wnt-3a and subjected to real-time PCR or TRAP to measure *hTERT* expression level (A) or TA (B), respectively. C, activation of the Wnt pathway could extend the life span of BJ cells. BJ cells were treated with LiCl or Wnt-3a CM for 20 population doublings, and the morphological changes were monitored under a microscope. Blue coloration in cells indicates senescence, indicated by arrows. Bar, 251 μ m. D, bars represent the percentage of β -galactosidase-positive cells. Data are mean \pm S.D. (error bars) from five images each.

Activation of the Wnt pathway leads to the accumulation of stabilized cytosolic β -catenin, which then translocates to the nucleus to form complexes with members of the DNA-bound LEF/TCF family of proteins and activates Wnt target gene expression. Within the functional complex, LEF/TCF contributes the DNA binding, and β -catenin confers the transcription activation (57). The LEF/TCF transcription factor family has four major members, LEF1, TCF1, TCF3, and TCF4, which recognize the consensus sequence 5'-CTTTGW-3' (or, in reverse orientation, 5'-WWCAAAG-3') through the high mobility group (HMG) domain (58–61). DNA binding by LEF/TCF alone is not sufficient to cause transcription activation. Promoter activation is accomplished only after a functional bipartite transcriptional factor is created through complex formation between the LEF/TCF transcription factor and β -catenin (62). In this study, our data suggest that the β -catenin·TCF4 complex, but not β -catenin·TCF1/3 or LEF1, is an effective activator of *hTERT* expression in immortalized cells. This observation was extended using overexpression of β -catenin·TCF4, which could stimulate *hTERT* expression in 293T, HCT116, and MCF7 cells. Furthermore, β -catenin-induced *hTERT* transcription was suppressed by the dominant negative form of TCF4. Although LEF/TCFs share remarkable ~95–99% amino acid sequence conservation in the HMG domain and bind to the consensus sequence 5'-CTTTGW-3', much genetic evidence shows that LEF/TCFs play diverse roles in cell growth, development, and differentiation (42), suggesting that the complexity of LEF/TCF action must be due to context-dependent actions and differential recognition of endogenous target genes.

In the present study, we have identified one TBE in the *hTERT* promoter between positions –659 and –653 (5'-TGC-AAAG-3') upstream of transcription start site of *hTERT*. The luciferase reporter assay showed that mutations in TBE could significantly repress β -catenin·TCF4-mediated activation of the *hTERT* promoter, whereas deletion of TBE almost abolished the effect of β -catenin·TCF4 complex on *hTERT*, implying that the TBE is essential for *hTERT* promoter activities. Transcriptional factor binding sites usually exist in the sequence of the proximal promoter of target genes; for example, known *hTERT* activators, such as c-Myc, Sp1, and AP-1 binding sites, mostly locate between position –200 bp and the transcription start site (Fig. 5D) (47). Our finding is supported by a previous study that suggests that most of the TBEs in β -catenin·TCF4 target genes are located at large distances from transcription start sites (63). Finally, we provide evidence that *hTERT* is a direct transcriptional target of the β -catenin·TCF4 complex by showing that TCF4 physically occupies the TBE *in vitro* and *in vivo* using EMSA and ChIP assays. Moreover, the ChIP result demonstrated that activated Wnt signaling could enhance β -catenin binding on the TBE of the *hTERT* promoter, suggesting the critical role of Wnt signaling in *hTERT* regulation. The identification of TBE in the *hTERT* promoter will provide valuable information for the understanding of *hTERT* expression in human cancer cells.

The present study offers evidence that the Wnt/ β -catenin pathway regulates the expression of *hTERT*. *hTERT* has been shown to serve as a cofactor in the β -catenin transcriptional

complex (51), so we are curious whether *hTERT* drives a positive feedback loop to reinforce the Wnt/ β -catenin signaling. To this end, we established stable *hTERT* overexpression in 293T, HCT116, MCF7, and MCF10A cell lines (supplemental Fig. 7). However, our data showed that overexpression of *hTERT* had no effect on expression of Wnt pathway target genes, *c-MYC* and *NOS2* (supplemental Fig. 8), suggesting that *hTERT* cannot form a positive feedback loop with Wnt/ β -catenin signaling in these cell lines we studied.

In conclusion, we show that *hTERT* is a novel target gene of the Wnt pathway and that the *hTERT* TBE is the critical transcriptional element controlling *hTERT* expression in cancer cells. Our results demonstrate that the β -catenin·TCF4 complex is capable of activating the *hTERT* promoter and increasing TA. Furthermore, our findings shed light on the biological roles of β -catenin in regulating activation of the *hTERT* promoter and facilitate our understanding of the telomerase regulation in various human cancers. Because high TA is most common in human cancers, understanding the role of the β -catenin·TCF4 complex in telomerase regulation is of interest and may lead to new therapeutic perspectives.

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