Telomerase Reverse Transcriptase (TERT) Is A Novel Target Of Wnt/β-Catenin Pathway In Human Cancer*

Yong Zhang1, LingLing Toh1,2, Peishan Lau1 and Xueying Wang1

1Department of Biochemistry, Yong Loo Lin School of medicine, 8 Medical Drive, National University of Singapore, 117597, Singapore.

2Institute of Molecular and Cell Biology, 61 Biopolis Drive, Proteos, 138673, Singapore.

*Running title: hTERT is a Wnt pathway target gene

To whom correspondence should be addressed: Xueying Wang, Assistant Professor, Department of Biochemistry, Yong Loo Lin School of Medicine; National University of Singapore, 10 Kent Ridge Crescent, Blk MD4A #02-04, Singapore 119260, Tel.: (65) 66012360; Fax: (65) 67791453; E-mail: bchwxy@nus.edu.sg

Keywords: Carcinogenesis; Wnt/β-catenin pathway; Telomerase regulation

Background: Telomerase up-regulation is found in about 90% 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 Wnt/β-catenin pathway in telomerase regulation provides better insight into understanding the role of Wnt pathway in carcinogenesis.

Summary

Telomerase activation plays a critical role in human carcinogenesis through the maintenance of telomeres, but the activation mechanism during carcinogenesis remains unclear. The telomerase reverse transcriptase (hTERT) promoter has been shown to promote hTERT gene expression selectively in tumor cells but not in normal cells. Deregulated Wnt/β-catenin signaling pathway is reported to be associated with human carcinogenesis. However, little is known about whether Wnt/β-catenin pathway is involved in activating hTERT transcription and inducing telomerase activity (TA). In this study, we report hTERT is a novel target of the Wnt/β-catenin pathway. Transient activation of Wnt/β-catenin pathway either by transfection of a constitutively active form of β-catenin or by LiCl, or by Wnt-3a conditioned medium treatment induced the 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 LEF/TCF family, only TCF4 showed more effective stimulation on 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 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.

Introduction

Telomeres are highly specialized structures at chromosome ends that are essential for genome stability (1). Telomerase is a ribonucleoprotein
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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). High level of telomerase activity (TA) is detected in about 90% of human cancer specimens, whereas most somatic cells do not display TA or just 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 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, while 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 the hTERT should be essential for elucidating molecular mechanisms of telomerase regulation, cellular senescence, immortalization, and carcinogenesis in humans. Many factors have been implicated in direct or indirect regulation of the hTERT in cancer and normal cells, including cellular transcriptional activators (like c-Myc, Sp1, HIF-1, 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 and colleagues reported that inhibition of PinX1 can contribute to carcinogenesis by activating telomerase and inducing chromosome instability. (24). However, it remains largely unknown how hTERT is inactivated during development and how it is reactivated during carcinogenesis. Given that most cancer cells exhibit telomerase activity, we hypothesized that certain cancer-specifically activated signaling pathway plays critical roles in telomerase activation. In this study, by using qTRAP (Telomere Repeat Amplification Protocol), a real-time PCR–based version of the TRAP (5), we sought to identify novel TA inhibitors from well-known Wnt, EGFR, and JAK/STAT pathway inhibitor libraries. Moreover, this screen will unveil novel signaling pathways implicated in telomerase regulation. We have identified one of Wnt signaling pathway inhibitors-FH535 (β-catenin/TCF complexes inhibitor), which could significantly inhibit TA in all cell lines used in this study, suggesting that 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 (GSK3β), 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 LEF (lymphoid-enhancing factor)/TCF (T-cell factor) transcription factors. In the presence of Wnt signaling, β-catenin is uncoupled from the degradation complex and
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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 Wnt/β-catenin pathway in regulating telomerase gene expression has not been elucidated yet. 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. HCT116, LS174T and 293T cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM, Gibco, Invitrogen) while AGS was cultured in Roswell Park Memorial Institute-1640 (RPMI-1640, Sigma-Aldrich). Both medium contain 10% heat inactivated fetal bovine serum. (FBS, Gibco, Invitrogen), and 1% penicillin-streptomycin mixtures (Gibco, Invitrogen). MCF10A was cultured in DMEM/F12 with 15 mM HEPES buffer, 5% horse serum, 10 µg/ml insulin, 20 ng/ml EGF, 100 ng/ml choleratoxin, and 1% penicillin-streptomycin mixtures (Gibco, Invitrogen). MCF10A was cultured in DMEM/F12 with 15 mM HEPES buffer, 5% horse serum, 10 µg/ml insulin, 20 ng/ml EGF, 100 ng/ml choleratoxin, and 1% penicillin-streptomycin mixtures (Gibco, Invitrogen). MCF10A was cultured in DMEM/F12 with 15 mM HEPES buffer, 5% horse serum, 10 µg/ml insulin, 20 ng/ml EGF, 100 ng/ml choleratoxin, and 1% penicillin-streptomycin mixtures (Gibco, Invitrogen). MCF10A was cultured in DMEM/F12 with 15 mM HEPES buffer, 5% horse serum, 10 µg/ml insulin, 20 ng/ml EGF, 100 ng/ml choleratoxin, and 1% penicillin-streptomycin mixtures (Gibco, Invitrogen). MCF10A was cultured in DMEM/F12 with 15 mM HEPES buffer, 5% horse serum, 10 µg/ml insulin, 20 ng/ml EGF, 100 ng/ml choleratoxin, and 1% penicillin-streptomycin mixtures (Gibco, Invitrogen). MCF10A was cultured in DMEM/F12 with 15 mM HEPES buffer, 5% horse serum, 10 µg/ml insulin, 20 ng/ml EGF, 100 ng/ml choleratoxin, and 1% penicillin-streptomycin mixtures (Gibco, Invitrogen). MCF10A was cultured in DMEM/F12 with 15 mM HEPES buffer, 5% horse serum, 10 µg/ml insulin, 20 ng/ml EGF, 100 ng/ml choleratoxin, and 1% penicillin-streptomycin mixtures (Gibco, Invitrogen). MCF10A was cultured in DMEM/F12 with 15 mM HEPES buffer, 5% horse serum, 10 µg/ml insulin, 20 ng/ml EGF, 100 ng/ml choleratoxin, and 1% penicillin-streptomycin mixtures (Gibco, Invitrogen). MCF10A was cultured in DMEM/F12 with 15 mM HEPES buffer, 5% horse serum, 10 µg/ml insulin, 20 ng/ml EGF, 100 ng/ml choleratoxin, and 1% penicillin-streptomycin mixtures (Gibco, Invitrogen). MCF10A was cultured in DMEM/F12 with 15 mM HEPES buffer, 5% horse serum, 10 µg/ml insulin, 20 ng/ml EGF, 100 ng/ml choleratoxin, and 1% penicillin-streptomycin mixtures (Gibco, Invitrogen). MCF10A was cultured in DMEM/F12 with 15 mM HEPES buffer, 5% horse serum, 10 µg/ml insulin, 20 ng/ml EGF, 100 ng/ml choleratoxin, and 1% penicillin-streptomycin mixtures (Gibco, Invitrogen). MCF10A was cultured in DMEM/F12 with 15 mM HEPES buffer, 5% horse serum, 10 µg/ml insulin, 20 ng/ml EGF, 100 ng/ml choleratoxin, and 1% penicillin-streptomycin mixtures (Gibco, Invitrogen). MCF10A was cultured in DMEM/F12 with 15 mM HEPES buffer, 5% horse serum, 10 µg/ml insulin, 20 ng/ml EGF, 100 ng/ml choleratoxin, and 1% penicillin-streptomycin mixtures (Gibco, Invitrogen). MCF10A was cultured in DMEM/F12 with 15 mM HEPES buffer, 5% horse serum, 10 µg/ml insulin, 20 ng/ml EGF, 100 ng/ml choleratoxin, and 1% penicillin-streptomycin mixtures (Gibco, Invitrogen). MCF10A was cultured in DMEM/F12 with 15 mM HEPES buffer, 5% horse serum, 10 µg/ml insulin, 20 ng/ml EGF, 100 ng/ml choleratoxin, and 1% penicillin-streptomycin mixtures (Gibco, Invitrogen).
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and activity expressed as Relative TA relative to the control.

**Real-time PCR**
Reverse transcription was performed using the Promega RT-PCR kit and oligo dT primer as per manufacturer's protocol (Promega). Real-time PCR was performed using Brilliant SYBR Green qPCR Master Mix on the Rotor-Gene 6000 (Qiagen). The following primers were used for real-time PCR: 5'-CATGAGGTGGTAGCCTGGAT-3' and 5'-ACCACTGTGCCCTGTCTTCT-3' (human *DKCI*), 5'-GCGAAGAGTTGGGCCTCTGCT-3' and 5'-CATGTGTGAGCCGAGTCCTG-3' (human *TER*), 5'-ATGCCACAGTTCGTGGCTCA-3' and 5'-ATCCCTTGGCAGTGAGTA-3' (human *TERT*), 5'-GTGGAGCCCTGACCTGGTCCTG-3' and 5'-GGAGGAGTGGGTGTCGCTGT-3' (human *GAPDH*). Data were analyzed using the ∆∆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 µg/ml puromycin or 100 µg/ml hygromycin (AG Scientific, Inc.) selection 24 hour 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 DNeasy Blood & Tissue Kit (Qiagen). Telomere length analysis was carried out using a non-radioactive TeloTAGGG Telomere Length Assay (Roche) as described by the manufacturer. Approximately 1 µg DNA of each sample was digested with Hinf I/Rsal enzyme mix and separated by gel electrophoresis. DNA fragments were transferred to nylon membrane (Amersham, GE) by southern transfer and hybridized to digoxigenin (DIG-labeled probe), specific for telomeric repeats. A DIG-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 (Amersham, GE). Mean telomeric repeat binding factor (TRF) lengths were determined by comparing to the molecular weight standard.

**Generation of Mutants 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 (National Cancer Institute). The putative TBE (5'-TGCAAAAG-3') contained in *hTERT* promoter (between -659 bp and -653 bp) was mutated to 5'-TGCGGAG-3' (Mut1) and 5'-TGCAAGA-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 CCT GAG AAC CTG CGG AGA GAA ATG ACG GGC C -3' and 5'-GGC CCG TCA TTT CTC T CC GCA GGT TCT CAG GCG G -3' (Mut1), 5'-GCC GCC TGA GAA CCT GCA A GA AGA AAT GAC GGG CC -3' and 5'-GGC CCG TCA TTT CTT CT GCA GGT TCT CAG GCG G -3' (Mut2), 5'-GCC GCC TCA TTT CTT CT GCA GGT TCT CAG GCG G -3' (Truncate).

**Transient Transfections and Luciferase Reporter Assays**
All transient transfections were performed using Lipofectamine 2000 (Invitrogen, Lakewood, NJ) according to the manufacturer’s recommendations. Respective amounts of cells (2 × 10^5/ml for MCF7, 4 × 10^5/ml for HCT116 and 293T cells) were seeded in 24-well plate. 1µg of plasmid encoding the following proteins (ΔN β-catenin, ΔN TCF4, LEF1, TCF1, TCF3, TCF4, TAK1, TAB) or PCDNA control were transfected into cells. 2 days after transfection, cells were harvested and analyzed by real-time PCR and qTRAP. In 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 Dual-luciferase Reporter Assay System (Promega). To examine the effect of activated Wnt pathway on 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 hTERT promoter. All firefly luciferase readings were normalized with the Renilla luciferase readings. Reporter assays were conducted in duplicates and repeated 3 times.

**Electrophoretic Mobility Shift Assay (EMSA)**
Sense and antisense oligonucleotides containing TCF4 binding sites was labeled by Biotin 3’ End DNA Labeling Kit (Thermo Scientific). The binding reactions contain 10μg of crude 293T nuclear extract, 1μg of double-stranded polydeoxyinosinic-deoxycytidylic acid (poly[dl-dC]-poly[dl-dC]), and 10 fmol of biotin-labeled probe in 20 μl of EMSA buffer (25 mm HEPES, pH 7.9, 62.5 mm KCl, 0.05% Nonidet P-40, 2 mm MgCl2, 8% Ficoll (400), 500 μg/ml bovine serum albumin, protease inhibitors (0.5 mm PMSF, 5 μg/ml each of antipain, leupeptin, aprotinin, chymostatin, and pepstatin A, and 3.75 μl of H2O). 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 super-shift analysis. The reaction was loaded on 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 peroxide conjugate and chemiluminescent substrate.

**Chromatin Immunoprecipitation (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 5x107 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). ChIP enriched DNA was quantified by quantitative real-time PCR, and primer sets were designed flanking the putative TBE: Forward: 5’-CCCGTGAGGTGATTAAACAGAT -3’, Reverse: 5’-GGACGTGAAGGGGGAC -3’ (hTERT 1). Forward: 5’-CCCCGTGGTGATTAAACAG -3’, Reverse: 5’-GGAGTGCTCCCTTGCAACAC -3’ (hTERT 2). Forward: 5’-CTCCATTTCCACCCCTTCT -3’, Reverse: 5’-ACUTGGGTCTCCTTGACACAG -3’ (hTERT 3). Forward: 5’-AGGCCGTGGTGGCTGGTGAG -3’, Reverse: 5’-CCGTATTCCCTTTCGAG -3’ (hTERT 4). Forward: 5’-TTGCTCATGGTGGGGAC -3’, Reverse: 5’-GACGAACCCAGGGCGCAT -3’ (hTERT 5). Data are expressed as percentage of input.

**Search for Consensus TCF/LEF site**
TCF binding sites (A/T)(A/T)CAAAG in hTERT promoter was identified using an in silico tool named TFExplorer. TFExplorer is database of regulatory elements in the genomic sequences of human, mouse, and rat. We obtained a TCF4 binding sequences 653 bp upstream of the transcription start site of 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 (PDL) in the presence or absence of LiCl, or Wnt-3a CM. Cell staining was performed using a kit from US Biological. After removing growth medium from the cells, the cells were washed with PBS and fixed with the manufactures fixative solution for 15 minutes 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 were presented as mean ± SD of data obtained and were analyzed using either one-way Anova.
or Student’s T test, and \( p < 0.05 \) was considered statistically significant.

**Result**

**Identification of signaling pathway inhibitors that preferentially inhibit TA**

To identify telomerase inhibitors from the InhibitorSelect™ Inhibitor Library, consisting of Wnt, EGFR and JAK/STAT inhibitors libraries, we modified the existing qTRAP to suit the screen (36). TA was quantitated using qTRAP and all data obtained are 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 that have shown to be able to reduce TA by at least 50% in the initial screen will be considered as a potent telomerase inhibitor 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. Screen result showed four Wnt pathway inhibitors (B: Casein Kinase II Inhibitor III, TBCA ; C: β-catenin/TCF Complexes Inhibitor, FH535; F: protein kinase A H-89, Dihydrochloride; K: TAK1 inhibitor, (5Z)-7-Oxozeaenol, Supplementary Table S1) were able to inhibit TA by at least 50% in the initial screen will be considered as a potent telomerase inhibitor 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. Screen result showed four Wnt pathway inhibitors (B: Casein Kinase II Inhibitor III, TBCA ; C: β-catenin/TCF Complexes Inhibitor, FH535; F: protein kinase A H-89, Dihydrochloride; K: TAK1 inhibitor, (5Z)-7-Oxozeaenol, Supplementary Table S1) were able to inhibit TA by at least 50% compared to vehicle controls after 2 days of treatment in HCT116 cells (Fig. 1B, left panel). To further confirm the result and avoid cell-type specificity, the same screen was performed with LS174T cells (Fig. 1B, right panel). We also found that FH535 could efficiently reduce TA by 50%, while other hits did not show inhibitory effect in LS174T cells. Moreover, FH535 also demonstrated 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 Wnt pathway, 293T, HCT116, and MCF7 cells were exposed to Wnt-3a conditioned medium (CM) or control medium for 48 hours. 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-fold, 1.41-fold, and 1.34-fold in 293T, HCT116, and MCF7 cells, respectively, following Wnt-3a CM treatment (Fig. 2A, left panel). Consistent with this result, we found that Wnt-3a CM treatment could largely elevate the hTERT mRNA level (Fig. 2A, right panel), suggesting 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-3β, hence increases and stabilize β-catenin protein levels (38). As shown in Fig. 2B, 15mM LiCl of treatment for 24 hours in multiple human cell lines we have tested efficiently elevated TA (left panel), as well as hTERT mRNA level (right panel).

To further confirm these results, 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 panel) or LiCl (right panel) 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/TCFs family transcriptional factors. When activated by TAK1, NLK can directly phosphorylate LEF/TCFs to prevent the β-catenin-LEF/TCFs 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/TCFs complex in hTERT gene regulation, 293T cells were treated with Wnt-3a CM to stimulate the accumulation of β-catenin in 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 exogenous TAK1/TAB could reduce hTERT expression in all cell lines tested (Fig. 2E). In conclusion, these data indicate that the expression of hTERT gene can be regulated by Wnt/β-catenin pathway in human cancer cells.

Knockdown of β-catenin, or its overexpression dramatically affect 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 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 (Supplementary 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, luciferase reporter assay results showed that repressing endogenous β-catenin expression could significantly inhibit hTERT promoter activity in 293T, HCT116, MCF7 cells (Fig. 3C).

The activation of hTERT is required for the maintenance of telomere length in cancer cells. To better understand the biologic 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, 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, the silencing β-catenin gene in 293T cells had no effect on telomere length maintenance. It is possible that β-catenin knockdown may take 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 (ΔN β-catenin) as described (41) and examined the β-catenin dependency 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 panel) and subsequently elevated TA (right panel). 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 (Supplementary Fig. S2). Compared with the controls, real-time
PCR analysis showed that hTERT gene expression was elevated by 1.7-fold and 6.7-fold in stable β-catenin overexpression HCT116 and MCF10A cell lines respectively (Fig. 3F, upper panel). In consistent with this result, TA was correspondingly enhanced in both cell lines (Fig. 3F, lower panel). Though 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 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 upregulate promoter activity of hTERT

LEF/TCFs are sequence-specific DNA binding transcription factors that bind to Wnt target genes promoter and repress gene transcription. Upon β-catenin binding to LEF/TCFs, the complex will activate target genes 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/TCF4 complexes in telomerase regulation, we sought to identify which LEF/TCFs member can bind to the hTERT promoter and synergistically activate hTERT expression with β-catenin. To this end, we transiently co-transfected ΔN β-catenin construct alone or with different LEF/TCFs members and 949bp 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 ΔN β-catenin alone, the co-transfection of ΔN β-catenin and TCF4 could significantly activate hTERT promoter in 293T, HCT116 and MCF7, while ΔN 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 ΔN β-catenin/TCF3/4 complexes on hTERT regulation. The result demonstrated that exogenous β-catenin/TCF4 complex could slightly elevate hTERT expression and TA compared with ΔN β-catenin alone or ΔN β-catenin/TCF3 (Fig. 4 B and C). To further investigate whether hTERT is a transcriptional target of β-catenin/TCF4 complex and whether activation of the hTERT promoter is due to synergistic effect of β-catenin and TCF4, we performed luciferase reporter assay utilizing the dominant negative form of TCF4 (ΔN TCF4), which is known to diminish the transcriptional activity of TCF-responsive promoters (43). We observed the stimulation of hTERT promoter upon ΔN β-catenin overexpression was significantly suppressed by co-overexpression of ΔN TCF4 (Fig. 4D). In addition, ectopic expression of ΔN TCF4 could inhibit ΔN β-catenin induced hTERT expression (Fig. 4E, left panel), and hence TA (Fig. 4E, right panel). 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 transcriptional complex with β-catenin.

Characterization of the distal TCF4 binding element (TBE) in the human hTERT promoter

We next sought for evidence of functional binding of β-catenin/TCF4 complex on hTERT promoter. To this end, luciferase reporter assay was carried out to examine the effect of the β-catenin/TCF4 complex on various length of the hTERT promoter (88, 385, and 949 bp) in multiple cell lines. As shown in Fig. 5A, the 949 bp promoter could induce relative higher luciferase activity when compared to 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 the 88 and 385 bp promoter. As expected, we found co-transfection of TAK1/TAB significantly reduced luciferase activity with 949 bp hTERT promoter, while had no effect on 88 or 385 bp hTERT promoter (Fig. 5 C). These results imply that β-catenin/TCF4 can directly
hTERT is a Wnt pathway target gene

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). Searching result revealed the presence of a putative consensus TBE between position -659bp and -653bp (5’-TGCAAAG-3’) upstream of transcription start site of hTERT (Fig. 5D), that showed a high degree of homology to the core consensus sequence (CTTG 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. 5 E) by site-directed mutagenesis and constructed a shorter hTERT promoter (652bp) 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 to the wild-type promoter in all the three cell lines (Fig. 5E). And the deletion of the TBE (Truncate) significantly repressed the hTERT promoter activity by ~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 hTERT promoter

To verify whether TCF4 could bind to the putative distal TBE, we carried out electrophoretic mobility shift assay (EMSA) with a probe containing the potential TBE. As shown in Fig. 6 A, 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), while normal 293T nuclear extracts could not form visible complex with biotin-labeled wild-type TBE probe (Fig. 6 A, lane 2). The specificity of the interaction between β-catenin/TCF4 and wild-type TBE probe was confirmed when a mutant probe (Mut 1), with two nucleotide substitution in the core of TBE, dramatically decreased complexes formation (Fig. 6 A, lane 6), which is consistent with 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 nuclear extract (Fig. 6 A, lane 4), but the addition of 500-fold cold mutant probe was unable to inhibit formation of the specific complexes (Fig. 6 A, 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. 6 B, 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 supershifted band (Fig. 6 B, lane 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 (Supplementary Fig. S3). Taken together, the putative TBE between -659bp and -653bp 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 chromatin immunoprecipitation (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 glyceraldehydes-3-phosphate dehydrogenase (GAPDH) coding region as a negative control. As shown in Fig. 6 C (left panel), 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. 6 C (right panel), anti β-catenin antibody was able to specifically precipitate chromatin-DNA
complexes containing hTERT promoter fragment. In addition, LiCl treatment enhanced binding of β-catenin on the TBE of 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, while 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 cell) compared to the control (Fig. 7A 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 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 PDL (20 PD) 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 active Wnt/β-catenin pathway may lead to extend somatic cells life-span via reactivating telomerase (Fig. 7 C). 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 define telomerase regulation in cancer cells, it remains unclear how cancer cells gain 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 Wnt pathway is frequently deregulated, resulting in the activation of target genes whose dysregulation has significant consequences on 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 the β-catenin associated carcinogenesis. Recently,
Park et al. showed that hTERT plays an essential role in Wnt/β-catenin signaling pathway by serving as a cofactor in a β-catenin transcriptional complex (51). However, whether 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 are a powerful approach for discovering and detecting signaling pathways which may be involved in telomerase regulation. Using qTRAP assay, we clearly detected a β-catenin/TCF complexes inhibitor, FH535, which could effectively inhibit TA in gastric cancer, breast cancer, and colorectal cancer cells, suggesting Wnt pathway is widely implicated in telomerase regulation in human cancers.

To assess the function of 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 lead 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 (TERT); a telomerase RNA (TER) 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 (hTER) in vivo (52), the mutation of dyskerin is associated with dyskeratosis congenital, a human stem cell disorder (53). So 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 hTER and DKC1 expressions in human cancer cells (HCT116 and MCF7) and normal stable cell lines (293T and MCF10A) (Supplementary Fig. S4). Taken together, these results suggest that 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 (APC), 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 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, hTER and DKC1 expressions and repressing TA in human ES cell lines hES3(Supplementary Fig. 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 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 activated Wnt pathway could increase hTERT expression and reanimate telomerase, leading to extended life-span in BJ cells. Somatic cells do not display TA or just 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 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), while silencing β-catenin just moderately repressed telomerase genes expression in human ES cells and MCF10A, in which Wnt pathway is tightly regulated. It seems like, in Wnt pathway deregulated cells, Wnt pathway plays a predominant role in telomerase regulation. Activated 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). LEF/TCF transcription factor family has four major members: LEF1, TCF1, TCF3, and TCF4, which recognize the consensus sequence 5′-CTTTGWW-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 LEF/TCF transcription factor and β-catenin (62). In this study, our data suggest that 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 β-catenin/TCF4, which could stimulate hTERT expression in 293T, HCT116, and MCF7 cells. Furthermore, β-catenin induced hTERT transcription was suppressed by dominant negative form of TCF4. Though LEF/TCFs share remarkable ~95-99% amino-acid sequence conservation in HMG domain and bind to the consensus sequence 5′-CTTTGWW-3′, numerous genetic evidences show 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 TCF4 binding element (TBE) in human hTERT promoter between position -659 and -653 (5′-TGCAAAG-3′) upstream of transcription start site of hTERT. Luciferase reporter assay showed that mutations in TBE could significantly repress β-catenin/TCF4 mediated activation of the hTERT promoter, while 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 factors binding site usually exist in the sequence of proximal promoter of target genes, for example, known hTERT activators, such as c-Myc, Sp-1, and AP-1 binding sites mostly locate between position -200bp and transcription start site (Fig. 5D) (47). Our finding is supported by a previous study which suggests that most of 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, ChIP result demonstrated that activated Wnt signaling could enhance β-catenin binding on the TBE of 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 the evidence that Wnt/β-catenin pathway regulates the expression of hTERT. hTERT has been shown to serve as a cofactor in β-catenin transcriptional complex (51), we are curious that 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 (Supplementary Fig. 7). However, our data showed that overexpression of hTERT had no effect on expression of Wnt pathway target genes, c-MYC and NOS2 (Supplementary Fig. 8), suggesting 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 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. Since high TA is most common in human cancers, understanding the role of β-catenin/TCF4 complex in telomerase regulation is of interest and may lead to new therapeutic perspectives.

References

hTERT is a Wnt pathway target gene

Acknowledgments

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Conflict of interest

The authors declare that they have no competing interests.

Figure Legends

Figure 1. Screening telomerase inhibitor from well-known signaling pathway inhibitor libraries

A. STAT III/V inhibitors inhibit TA in HCT116 cells. 24 hours before drug treatment, 4 × 10^5 HCT116 cells were seeded into 12-well plates. The seeding density was chosen to obtain the optimum 60% confluency prior to drug treatment. Cells were treated with inhibitors or DMSO (vehicle control) and incubated for 48 hours. 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 names A-O were listed in Supplementary Table S1. 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 was normalized against DMSO treatment. Data were the average of three independent experiments. (*) p<0.05.

Figure 2. Activated Wnt/β-catenin directly affects hTERT gene expression and TA

A. 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 hours or 15mM LiCl for 24 hours (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 hours after transfection, cells were treated with Wnt-3a CM (left) or 15mM LiCl (right), respectively. Wnt-3a CM or 15mM LiCl could significantly activate \( hTERT \) promoter compared to controls.

D. Wnt pathway negative regulator TAK1 represses \( hTERT \) promoter activities. 293T (Wnt-3a CM treated), HCT116, and MCF7 cells were co-transfected with 949bp \( hTERT \) promoter driven reporter construct and indicated expression constructs for TAK1 and TAB (D). In another assay, after transfection with TAK1, cells were subjected for real-time PCR to measure \( hTERT \) expression (E). Empty expression vector pCDNA was used as a control. Expression of TAK1/TAB abolished \( hTERT \) promoter activity compared to controls. Relative luciferase activity was standardized to Renilla luciferase activities. Data were the average of three independent experiments. (*\( p<0.05 \)).

**Figure 3. Effects of \( \beta\text{-catenin} \) knockdown on the \( hTERT \) gene and TA.**

A. Real-time PCR analysis of \( hTERT \) expression in \( \beta\text{-catenin} \) knockdown stable 293T (Wnt-3a CM treated), HCT116, MCF7 and MCF10A cells. \( hTERT \) mRNA level were normalized by comparison to GAPDH as internal controls. Fold change is calculated relative to the control.

B. TRAP was carried out to measure TA in \( \beta\text{-catenin} \) knockdown stable cells and control cells.

C. 949bp \( hTERT \) promoter driven luciferase reporter construct was transiently transfected into \( \beta\text{-catenin} \) knockdown stable cells: 293T (Wnt-3a CM treated), HCT116, MCF7. 24 hours after transfection, cells were collected for luciferase activity measurement. \( \beta\text{-catenin} \) knockdown could significantly inhibit \( hTERT \) promoter activity compared to controls. Relative luciferase activity was standardized to Renilla luciferase activities. Data were the average of three independent experiments. (*\( p<0.05 \)).

D. Telomere length was measured by Southern blotting in \( \beta\text{-catenin} \) knockdown stable cells and control cells. Table beside the Southern blot indicates the mean telomere length measured.

E. Expression construct containing constitutively active form of \( \beta\text{-catenin} \) (\( \Delta N \) \( \beta\text{-catenin} \)) was transiently transfected into 293T, HCT116, and MCF7 cells. 48 hours after transfection, cells were harvested and subjected for 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 were the average of three independent experiments. (*\( p<0.05 \)).

F. \( \beta\text{-catenin} \) overexpression (OE) stable HCT116 and MCF10A cells line were obtained as described under “Experimental procedures.” These stable cells were harvested and subjected for real-time PCR or qTRAP to measure \( hTERT \) mRNA expression level (upper) or TA (lower). Empty retroviral expression vector was used as a control.

G. Telomere length was measured by Southern blotting in \( \beta\text{-catenin} \) overexpression (OE) stable HCT116 and MCF10A cells line. Table beside the Southern blot indicates the mean telomere length measured.

**Figure 4. \( \beta\text{-catenin}/TCF4 \) specifically upregulate promoter activity of \( hTERT \)**

A. 293T, HCT116, and MCF7 cells were co-transfected with 949bp \( hTERT \) promoter driven reporter construct, indicated expression construct for \( \Delta N \) \( \beta\text{-catenin} \), and/or LEF1, TCF1, TCF3, TCF4 expression constructs. pGL3.0 reporter plasmid was used as a control. Co-transfection of \( \beta\text{-catenin} \) and TCF4 specifically activates human \( hTERT \) promoter.

B. 293T, HCT116, and MCF7 cells were transfected with expression construct for \( \Delta N \) \( \beta\text{-catenin} \) alone, or with TCF3 or TCF4 expression constructs, respectively. Empty expression vector pCDNA was used as a control. 48 hours after transfection, cells were harvested and subjected for real-time PCR to measure \( hTERT \) mRNA. Co-transfection of \( \beta\text{-catenin} \) and TCF4 could more efficiently increase \( hTERT \) expression.
C. 293T, HCT116, and MCF7 cells were transfected with expression construct for ΔN β-catenin alone, or with TCF3 or TCF4 expression constructs, respectively. Empty expression vector pCDNA was used as a control. 48 hours 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 949bp hTERT promoter driven reporter construct, indicated expression construct for ΔN β-catenin, TCF4 or ΔN TCF4 expression constructs. pGL3.0 reporter plasmid was used as a control. Co-transfection of ΔN TCF4 significantly inhibit human 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 ΔN β-catenin and ΔN TCF4. pcDNA plasmid was used as a control. Co-transfection of ΔN TCF4 was able to reduced β-catenin induced hTERT expression (left) and TA (right). Data were the average of three independent experiments. (*p<0.05).

Figure 5. Identification of TCF4 binding element (TBE) in 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 responding 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 line, respectively, and the E box (c-Myc binding site) consensus motif is in Italics. The putative TBE consensus motif is boxed. The initiating ATG codon is shown in bold.
E. TBE is important for β-catenin/TCF4 function on hTERT promoter. Schematic representation of various luciferase reporter plasmids of hTERT promoter. The putative TCF4 binding element is located between -659bp and -653bp from the transcription initiation site. Construct WT contains the wild-type binding element; Mut1 has a 2-bp substitution (AA→GG, underlined); Mut2 has another 2-bp substitution (AG→GA, underlined); Truncate (Trn) only consist of the 652bp 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 were the average of three independent experiments. (*p<0.05).

Figure 6. Binding of TCF-4 and β-catenin to putative TCF4 binding element (TBE) in 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 µg of 293T nuclear extracts or 293T nuclear extracts with β-catenin/TCF4 overexpression for 30 min. Competition experiments were performed by preincubating with 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 µg 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 hTERT promoter in vivo. 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 as a positive control.

Figure 7. Wnt/β-catenin signaling pathway is involved in telomerase reactivation in somatic cells

A. 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 Wnt pathway could extend life-span in BJ cells. BJ cells were treated with LiCl or Wnt-3a CM for 20 PD and the morphological changes were monitored under a microscope. Blue coloration in cells indicates senescence, indicated by arrows. The bar represents 251µm.

D. Bars represent the percentage of β-galactosidase positive cells. Data are mean ± S.D. from 5 images each.
Figure 2

A

B

C

pGL3-949bp

Luc

20
D

293T

HCT116

MCF7

Relative Luciferase Activity

pGL3 pGL3-949bp

Relative Luciferase Activity

pGL3 pGL3-949bp

Relative Luciferase Activity

pGL3 pGL3-949bp

E

293T HCT116 MCF7

Relative hTERT mRNA Level

pCDNA TAK1/TAB

pCDNA TAK1/TAB

pCDNA TAK1/TAB

by guest on October 5, 2017 http://www.jbc.org/ Downloaded from
Figure 3

A

B

C
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Figure 4

A: Bar graphs showing Relative Luciferase Activity for different cell lines (293T, HCT116, MCF7) for different constructs (pGL3.0 and pGL3.0-949bp).

B: Bar graphs showing Relative hTERT mRNA Level for different cell lines (293T, HCT116, MCF7) for different constructs.

C: Bar graphs showing Relative TA for different cell lines (293T, HCT116, MCF7) for different constructs.

D: Bar graphs showing Relative Luciferase Activity for different cell lines (293T, HCT116, MCF7) for different constructs.
E

**Relative hTERT mRNA Levels**

- **ΔN β-catenin + pcDNA**
- **ΔN β-catenin + ΔN TCF4**

**Relative TA**

- **ΔN β-catenin + pcDNA**
- **ΔN β-catenin + ΔN TCF4**

* indicates statistical significance.
**E**

WT

```
-949 TBE-659
-659 TCAAAG-653 +1 Luc
```

Mut1

```
-949 TBE-659
-659 TCAGG-653 +1 Luc
```

Mut2

```
-949 TBE-659
-659 TCAAGA-653 +1 Luc
```

Truncate (Trn)

```
-652 +1 Luc
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---

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### Figure 6

#### A

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**WT**: ctgagaacctgcaagagaaatgac

**Mut1**: ctgagaacctgcaagagaaatgac

**TBE**

#### B

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<th>Biotin labeled WT probe</th>
<th>Anti TCF4 antibody</th>
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**293T (β-catenin/TCF4) NE**

**Biotin labeled WT probe**

**Anti TCF4 antibody**

**Anti c-Myc antibody**

**Normal Mouse IgG**

#### C

**ChIP: TCF4**

**ChIP: β-catenin**

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Figure 7

A

B

C

D

![Graph A](image)

![Graph B](image)

![Graph C](image)

![Graph D](image)
Telomerase reverse transcriptase (TERT) is a novel target of Wnt/β-catenin pathway in human cancer
Yong Zhang, LingLing Toh, Peishan Lau and Xueying Wang

J. Biol. Chem. published online July 31, 2012

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