## Title

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# An Investigation of the Effects of the Core Protein Telomerase Reverse Transcriptase on Wnt Signaling in Breast Cancer Cells

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**Telomerase canonically maintains telomeres, but recent reports have suggested that the core protein mammalian telomerase reverse transcriptase (TERT) component, together with the chromatin remodeling factor BRG1 and β-catenin, may also bind to and promote expression of Wnt target genes. However, this proposed noncanonical role of TERT in Wnt signaling has been controversial. Here, we investigated the effects of human TERT (hTERT) on Wnt signaling in human breast cancer lines and HeLa cells. We failed to find evidence for physical association of hTERT with BRG1 or β-catenin; instead, we present evidence that anti-FLAG antibody cross-reactivity properties may explain the previously reported interaction of hTERT with β-catenin. Furthermore, altering hTERT levels in four different breast cancer cell lines caused minimal and discordant effects on Wnt target and Wnt pathway gene expression. Although hTERT's role in Wnt signaling was addressed only indirectly, no significant representation of Wnt target genes was detected in chromatin immunoprecipitation-sequencing (ChIP-seq) and chromatin isolation by RNA purification and sequencing (ChIRP-seq) loci cooccupied in HeLa S3 cells by both BRG1 and hTR. In summary, our evidence fails to support the idea of a biologically consistent hTERT interaction with the Wnt pathway in human breast cancer cells, and any detectable influence of hTERT depended on cell type and experimental system.**

The mammalian telomerase ribonucleoprotein complex adds TTAGGG repeats to telomeres, the ends of linear chromosomes. The core human telomerase contains the catalytic reverse transcriptase protein component (hTERT) and the telomerase RNA (called hTR, hTER, or hTERC) that provides the template for telomeric DNA synthesis (1). In most human somatic cells, telomerase expression is very low. In contrast, telomerase expression is upregulated in many human cancer cells and stem cells (2). In human cancer cells, the degree of telomerase expression seems higher than would appear necessary solely for maintaining telomere length. In fact, many studies suggest telomere-independent roles for telomerase. We and others have shown that overexpression of TERT protects cells in culture from apoptosis independently of the telomere-lengthening properties of telomerase (3–5). Furthermore, overexpression of mouse and human TERT promotes cell proliferation in stem, normal, and cancer cell lines (6–11). Experiments employing overexpression or reduced expression of hTERT in cells in culture have suggested roles for hTERT in controlling expression of growth factor response and other genes (9, 12). Gene expression changes have been reported to occur as soon as 1 week after ectopic hTERT overexpression (9). Taken together, these results strongly suggest nontelomeric roles for telomerase; however, the mechanisms by which telomerase might protect against apoptosis and promote proliferation remain largely unknown.

Some previous studies have linked TERT expression and Wnt/β-catenin signaling, here referred to as Wnt signaling (13–15). The Wnt signaling pathway plays a central role in development, stem cell renewal, and cancer. In the absence of Wnt signaling, cytoplasmic β-catenin is bound by destruction complex proteins, including AXIN, adenomatous polyposis coli (APC), and glycogen synthase kinase 3 beta (GSK3B). Consequently, β-catenin is phosphorylated and degraded by the ubiquitin-proteasome pathway. When secreted Wnt proteins bind to Frizzled and low-density lipoprotein receptor-related proteins (LRPs) at the plasma membrane, a signal is transduced to destabilize the β-catenin destruction complex. β-Catenin can then translocate to the nucleus, where it complexes with T-cell factor/lymphoid enhancer factor (TCF/LEF) transcription factors to promote target gene transcription (16). The Wnt pathway has been previously shown to upregulate telomerase in mouse mammary tumors and human cells (17, 18). Furthermore, β-catenin may contribute to telomerase upregulation in stem and cancer cells by directly regulating TERT expression via binding to the TERT promoter in complex with Klf4, as previously reported in a study of mouse adult stem cells and human carcinoma lines NTera2 and SW480 (15).

Reciprocally, Park et al. previously suggested that TERT expression promotes Wnt signaling (13). In that study, $TERT^{-/-}$ knockout mice in the first generation were reported to have developmental defects such as homeotic transformations of the vertebrae. Such defects, occurring before the onset of significant telomere shortening, resembled effects of aberrant Wnt signaling. Those authors additionally reported protein-protein interactions between hTERT and the chromatin remodeling factor BRG1 and between hTERT and β-catenin. It was also reported that TERT overexpression upregulated expression of a Wnt luciferase reporter in $TERT^{-/-}$ and $TR^{-/-}$ mouse embryonic fibroblasts (MEFs) and human fibroblast (BJ) cells and that, in SW-13 and HeLa cancer cells, TERT overexpression hyperactivated a Wnt signaling reporter in a BRG1-dependent manner (13). Consistent with these results, Hrdlicková et al. reported increased proliferation and a slight but significant increase in Wnt reporter activation

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upon overexpression of either hTERT or a catalytically incompetent hTERT splice variant, in both U2OS (telomerase-deficient) and HeLa (telomerase-positive) cell lines (19). BRG1 has been reported to bind to β-catenin and to promote β-catenin target gene expression (20, 21). Because many growth-promoting genes are β-catenin targets and because Wnt signaling plays an important role in self-renewal, proliferation, and survival, these reports suggested that TERT, in concert with BRG1, might promote cell proliferation via Wnt signaling.

An influence of TERT on Wnt signaling has not been consistently reproduced in other experimental settings. Strong et al. did not detect homeotic transformations or diminished Wnt reporter activity in $TERT^{-/-}$ knockout mice or mouse embryonic fibroblasts (MEFs) derived from these mice (22). The discrepancies between the two mouse $TERT^{-/-}$ knockout studies could have been due to slightly different experimental conditions, such as different mouse backgrounds and/or Wnt signaling activators (13, 22). Alternatively, the TERT overexpression system that identified a Wnt pathway interaction (13) may not produce a biologically relevant phenotype. While Strong et al. disputed a TERT/Wnt signaling interaction in mice and MEFs (22), they did not address a possible TERT/Wnt signaling interaction in human cancer cells.

The primary aim of the present study was to determine whether hTERT promotes or otherwise affects Wnt signaling in cultured human breast cancer cells. Wnt signaling is often dysregulated in breast cancer (23). Furthermore, either TERT overexpression or Wnt activation leads to mammary tumorigenesis in mice (23, 24). We therefore focused on breast cancer cell lines to further study the potential for biologically relevant TERT/Wnt signaling interactions.

## MATERIALS AND METHODS

**Cell lines.** HeLa cells were purchased from the American Type Culture Collection (ATCC) and grown in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin, and 1% GlutaMax (Invitrogen). HCC3153, HCC1806, SUM149PT, and MCF10A cells were obtained from the laboratory of Joe W. Gray, Oregon Health and Sciences University. HCC3153 and HCC1806 cells were grown in RPMI medium with 10% FBS, 1% penicillin-streptomycin, and 1% GlutaMax (Invitrogen). SUM149PT cells were grown in Ham's F-12 medium with 5% FBS, 0.01 mg/ml insulin, 500 ng/ml hydrocortisone, and 1% penicillin-streptomycin. MCF10A cells were grown in DMEM–F-12 with 5% horse serum, 20 ng/ml epidermal growth factor, 100 ng/ml cholera toxin, 0.01 mg/ml insulin, 500 ng/ml hydrocortisone, and 1% penicillin-streptomycin. All cell lines were grown at 37°C with 5% $CO_2$.

**Light microscopy.** The HCC3153, HCC1806, SUM149PT, and MCF10A cell lines were grown on chamber slides (Lab-Tek II 154526) and treated with 25 mM LiCl or 200 ng/ml Wnt3a (5036-WN-010/CF; R&D Systems) for 4 h. Cells were fixed in 2% paraformaldehyde–phosphate-buffered saline (PBS) and permeabilized with 0.5% NP-40–PBS. Immunostaining was performed with anti-β-catenin antibody clone 14 (BD Biosciences) followed by secondary Alexa Fluor 488 (Molecular Probes). DNA was visualized with 4′,6-diamidino-2-phenylindole (DAPI; Invitrogen). Images were acquired in 0.5 -μm increments using a Deltavision RT deconvolution microscope (Applied Precision) with a 100×/1.40 N PlanApo objective (Olympus). Images were deconvolved, Z-projected in Softworx (Applied Precision), and then adjusted for brightness and contrast in FIJI (25).

**cDNA generation and qPCR.** Total RNA was extracted with a Qiagen RNeasy minikit from cells treated with 25 mM LiCl, 200 ng/ml Wnt3a, or PBS for 4 h. cDNA synthesis was performed using 2 μg RNA, random hexamers, and SuperScript III (Invitrogen). cDNA was amplified in 10-μl reaction mixtures containing LightCycler 480 DNA SYBR green I Master (Roche Applied Science) and a 0.5 to 1 μmol/liter final concentration of each primer using a Light Cycler 480 instrument (Roche Applied Science). The cycling conditions were 95°C for 5 min, 50 cycles of 95°C for 10 s and 60°C for 20 s, and 72°C for 20 s. A melting curve (65 to 98°C) was generated at the end of each run. Relative expression levels were determined by the $2^{-\Delta\Delta CT}$ method (26) and were normalized to GAPDH (glyceraldehyde-3-phosphate dehydrogenase). AXIN2 forward (5′-CATGTTCGTC ATGGGTGTGAACCA-3′) and AXIN2 reverse (5′-TGGCTGGTGCAAA GACATAG-3′) and GAPDH forward (5′-CATGTTCGTCATGGGTGTG AACCA-3′) and GAPDH reverse (5′-ATGGCATGGACTGTGGTCATG AGT-3′) primers were used. For Wnt target gene expression analysis, cell lines were transduced with control or wild-type hTERT lentivirus pHR′cytomegalovirus (CMV)-hTERT-internal ribosome entry site (IRES)-PURO and selected with 1 μg (HCC1806 and SUM149PT) or 2 μg (HCC3153) puromycin for 3 days and allowed to recover for 1 to 2 days. Cells were treated with 25 mM LiCl for 6 h, following total RNA extraction using an RNeasy kit (Qiagen) and cDNA generation using an $RT^2$ First Strand kit (Qiagen) according to the manufacturer's instructions. A total of 84 Wnt target genes were measured using quantitative PCR (qPCR) human Wnt signaling target arrays (PAHS-243G; SABiosciences) according to the manufacturer's instructions. A minimum cutoff of a 2.5-fold change compared to control results was used to determine significant gene changes.

**Wnt luciferase reporter assays.** The M50 Super TOPFLash and M51 Super FOPFlash luciferase reporter vectors were obtained from Addgene (27). pRL-TK *Renilla* luciferase was used as an internal control (Invitrogen). The lentivirus plasmids pBARL (β-catenin activated reporter luciferase) and pfuBARL (mutated pBARL) and pSL9/rLuc (*Renilla* luciferase) were obtained from the laboratory of Randall Moon, University of Washington (28). Cells were seeded on 96-well microplates (655083; Greiner Bio-One), and each well was transiently transfected with 0.5 pg pRL-TK control along with either 50 pg SUPER TOPFLash or 50 pg mutated FOPFLash and with either 10 pg empty vector or 10 pg pcDNA3-FLAG-hTERT using X-tremeGene HP (Roche) and treated or not treated with 25 mM LiCL for 24 h, followed by cell lysis with passive lysis buffer (Promega) for 10 min and analysis using a dual-luciferase reporter assay system (Promega). Firefly and *Renilla* luciferases were read with a Veritas Microplate Luminometer (Turner Biosystems). Lentivirus production and transduction were carried out as previously described (28). Stable cell lines expressing reporter *Renilla* luciferase, pBARL, or pfuBARL lentivirus were generated as reported previously (28), using the same titer of lentivirus in all cell lines. Then, cell lines were transduced with either control or hTERT lentivirus (28), selected with puromycin, treated with 25 mM LiCl for 24 h, and analyzed as described above. Background luciferase readings were subtracted, and firefly luciferase values were normalized to *Renilla* luciferase.

**RNA interference (RNAi).** Lentivirus expressing short hairpin RNAs (shRNAs) against β-catenin (5′-CCGGAGGTGCTATCTGTCTGCTCT ACTCGAGTAGAGCAGACAGATAGCACCTTTTTT-3′; 29), hTERT (5′-GGAGACCACGTTTCAAAAGTCTCTTGAACTTTTGAAACGTGG TCTCC-3′), and scramble shRNA (5′-GTTCTACAACGTAACGAGGTT TCTCTTGAAAACCTCGTTACGTTGTAGAAC-3′; 30) was generated as described previously (30). Cells were transduced with shRNA and control vector lentivirus and were selected with 1 μg/ml puromycin for 3 days and then expanded.

**IP and Western blotting.** HeLa cells were transfected with pcDNA3 constructs containing wild-type hTERT with one N-terminal FLAG tag using Lipofectamine 2000 (Invitrogen) for 18 h, followed by treatment with 25 mM LiCl for 6 h. The following antibodies were used: anti-FLAG (M2; Sigma F3165 and F1804), anti-BRG1 (H-88; Santa Cruz), anti-β-catenin (clone 14) (610153; Transduction Laboratories), and anti-GAPDH (MAB374; EMD Millipore). Cell lysis and immunoprecipitation

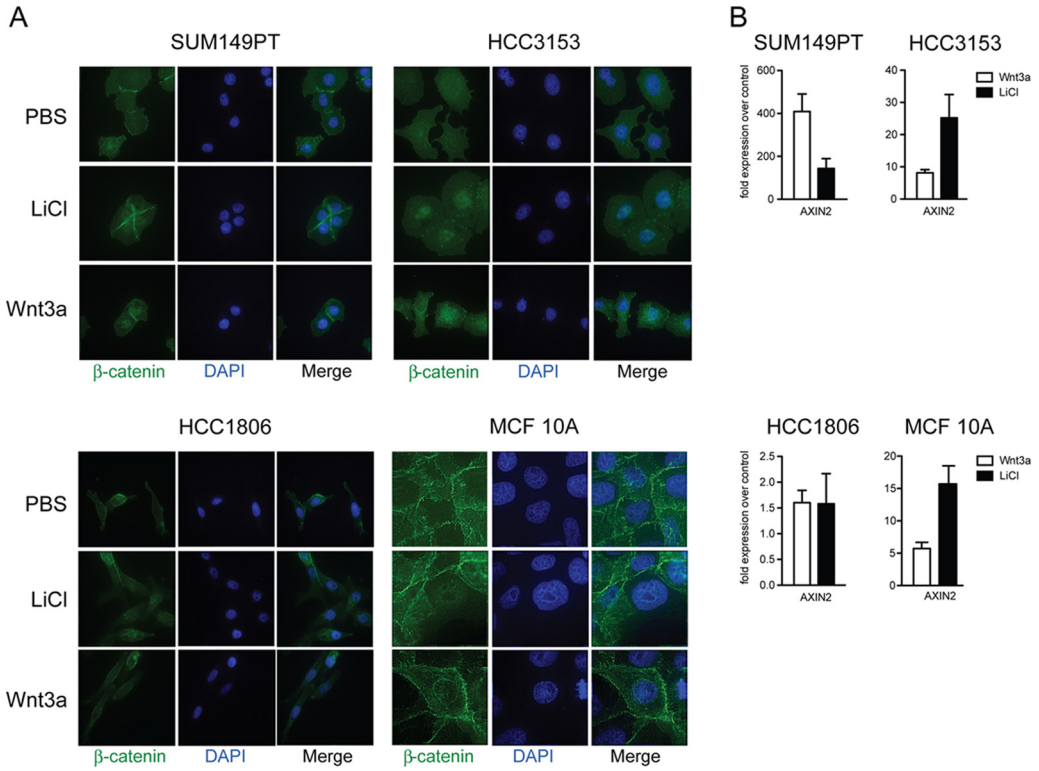


**FIG 1** Endogenous Wnt/β-catenin target gene induction varies in breast cancer cell lines. (A) Cell lines SUM149PT (high telomerase), HCC3153 (medium telomerase), HCC1806 (high telomerase), and MCF10A (low telomerase) were treated with PBS, 25 mM LiCl, or 200 ng/ml Wnt3a for 4 h prior to staining for β-catenin (green) and DAPI (blue). (B) The increase in Wnt/β-catenin target gene AXIN2 mRNA expression over that of PBS control-treated cells was measured by qRT-PCR following activation with Wnt3a or LiCl for 4 h.

(IP) procedures and immunoblotting were done as described previously (13), using Western Lightning Plus ECL (PerkinElmer) for detection of horseradish peroxidase-conjugated secondary antibodies and Gamma-Bind G Sepharose (GE Healthcare Life Sciences) for preclearing and IP.

**Bioinformatics analysis.** We determined the union of published BRG1- and hTR-enriched regions identified by chromatin immunoprecipitation-sequencing (ChIP-seq) and chromatin isolation by RNA purification and sequencing (ChIRP-seq), respectively, in HeLa S3 cells (31, 32) and merged any united regions that were separated by ≤100 bp using the ChIPPeakAnno R package (33). Enriched gene sets were obtained through use of the Genomic Regions Enrichment of Annotations Tool (GREAT) (34) on all 145 genomic regions. Gene ontology (GO) terms were identified using DAVID (35, 36).

## RESULTS

**Endogenous Wnt signaling competency varies among the basal breast cancer cell lines SUM149PT, HCC1806, HCC3153, and MCF10A.** One immortalized breast cell line with low telomerase activity and three basal breast cancer cell lines with midrange to high levels of telomerase activity (MCF10A [lower telomerase activity level], HCC3153 [midrange telomerase activity level], and SUM149PT and HCC1806 [higher telomerase activity levels]) were selected (4). First, to determine the extent of Wnt signaling in the breast cancer cell lines, we induced Wnt signaling with Wnt3a or LiCl. Wnt3a activates the pathway at the cell surface receptor level and specifically induces β-catenin signaling by binding to Frizzled and LRP receptors (37). LiCl pharmacologically inhibits GSK3B kinase activity in the cytoplasm, thus leaving β-catenin unphosphorylated and stabilized (38).

Under control conditions, both SUM149PT and HCC3153 cells showed diffuse cytoplasmic and nuclear β-catenin staining, suggesting that they may have had dysregulated Wnt signaling, which is found frequently in breast cancers (39). Upon Wnt3a or LiCl treatment, SUM149PT and HCC3153 cells showed stronger nuclear localization of β-catenin (Fig. 1A). In accordance with the increased nuclear localization of β-catenin in those cells, both treatments increased the expression of the endogenous AXIN2 β-catenin target gene (Fig. 1B). In contrast, in HCC1806 cells, β-catenin was largely membrane bound and remained so even after LiCl or Wnt3a treatment (Fig. 1A). Consistent with these findings, HCC1806 cells did not significantly upregulate AXIN2 after Wnt signaling induction (Fig. 1B). In MCF10A cells, β-catenin was also largely membrane bound but showed weak nuclear localization after LiCl or Wnt3A treatment (Fig. 1A), and AXIN2 was moderately upregulated (Fig. 1B). These results suggest that HCC1806 cells are not competent for Wnt signaling induction by LiCl or Wnt3A. We conclude that Wnt signaling can be activated in SUM149PT and HCC3153 lines, and somewhat less in MCF10A cells, but at most minimally in HCC1806 cells.

**hTERT overexpression has minimal and nonconcordant effects on Wnt signaling reporters in breast cancer cell lines.** Having established that HCC3153, SUM149PT, and MCF10A but not HCC1806 cancer cells can strongly to moderately activate Wnt signaling, we tested whether hTERT overexpression modulated Wnt signaling reporter genes in these lines, as has been reported for MEFs, HeLa cells, and U2OS cells (13, 19). For independent

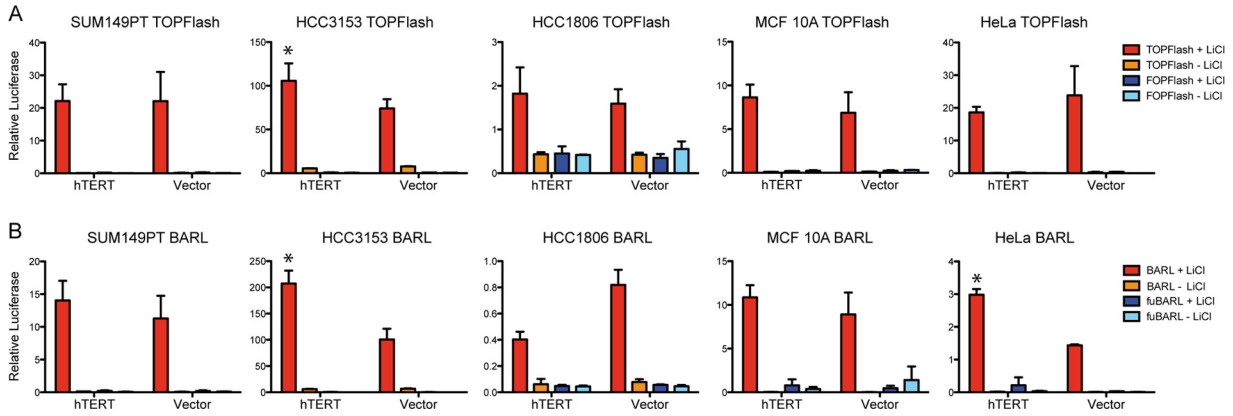


**FIG 2** Effect of hTERT overexpression on two Wnt/β-catenin reporters. (A) SUM149PT, HCC3153, HCC1806, MCF10A, and HeLa cells were transiently transfected with pRL-TK *Renilla* luciferase vector (internal control), hTERT or vector, and M50 SuperTOPFlash or M51 SuperFOPFlash reporter vectors and treated or not treated with 25 mM LiCl for 24 h prior to luciferase measurement. *, $P < 0.05$. (B) Cell lines were transduced with pSL9/rLuc, pBARL, or pfuBARL and either hTERT or vector control lentivirus and selected for stable expression prior to LiCl treatment and luciferase measurement.

verification, we employed two different Wnt signaling reporter construct systems with multimerized TCF/LEF binding sites driving luciferase expression: the M50 Super TOPFlash reporter and its corresponding control M51 Super FOPFlash Wnt reporter (27) and the BARL (β-catenin activated reporter luciferase)/fuBARL (control) system (28). While TOPFlash contains 7 TCF/LEF binding sites and was transiently expressed via plasmid transfection, BARL contains 12 TCF/LEF binding sites and was stably integrated. Vector or hTERT plasmids were cotransfected together with TOPFlash/FOPFlash in SUM149PT, HCC5313, HCC1806, MCF10A, and HeLa cells, followed by LiCl treatment (Fig. 2A). As expected, LiCl treatment strongly increased luciferase activity in the vector-transfected TOPFlash SUM149PT, HCC3153, MCF10A, and HeLa cells. LiCl treatment induced the luciferase activity in HCC1806 cells only weakly, with maximum luciferase expression being 5- to 100-fold lower than in the other four cell lines, consistent with our observations (Fig. 1), indicating that HCC1806 cells are severely impaired in Wnt signaling. Furthermore, only HCC3153 cells exhibited a statistically significant but mild (~1.4-fold) increase in relative luciferase activity over that of the vector control, while the relative luciferase activity did not change significantly compared to that of vector controls in SUM149PT, HCC1806, MCF10A, or HeLa cells. In the pBARL cells, overexpressing hTERT increased luciferase activity over that of the vector control cells only in HCC3153 cells (by ~2-fold) (Fig. 2B). Overexpression of hTERT did not detectably change the luciferase activity in SUM149PT, HCC1806, or MCF10A cell lines expressing pBARL. In HeLa cells that stably expressed the TCF/LEF mutant (control) binding site construct, fuBARL, with hTERT overexpression, we observed an ~3-fold increase in luciferase activity over that of control cells. However, in HeLa cells stably expressing BARL, there was an only ~2-fold increase in luciferase activity in hTERT-overexpressing cells over that of control cells (Fig. 2B). Hence, hTERT overexpression activated luciferase expression regardless of the presence or absence of a functional TCF/LEF promoter in HeLa cells. Because hTERT overexpression led to mild hyperactivation of both Wnt signaling reporters only in HCC3153 cells and not in the four other cell lines, we conclude that Wnt reporter hyperactivation through hTERT is dependent on the context. Hence, hTERT does not hyperactivate Wnt reporters universally but instead does so in a cell line- and context-dependent manner.

**Lack of evidence for hTERT interaction with β-catenin or BRG1 in HeLa cells.** Since Park et al. (13) reported that FLAG-hTERT in HeLa cells coimmunoprecipitated (co-IP) with BRG1, a protein previously reported to interact with β-catenin (13, 20), we also investigated hTERT/Wnt pathway interactions using HeLa cells. To independently verify the previously published results (13), we transiently overexpressed FLAG-hTERT in LiCl-treated HeLa cells and tested whether BRG1 or β-catenin interacted with FLAG-hTERT by coimmunoprecipitation (co-IP). Interestingly, using the same buffers as described by Park et al. (13) and the non-affinity-isolated version of anti-FLAG antibody M2 (F3165; Sigma) for IP, we observed a strong band migrating slightly slower than the β-catenin band in Western blots (M2 lanes in Fig. 3A) when the FLAG-IP Western blot was stained with the β-catenin antibody. Importantly, we also observed the same band in similar quantities independently of whether FLAG-hTERT was expressed in the HeLa cells (M2 lanes in Fig. 3A) and using a variety of washing procedures in the IP and Western blot experiments. We extended these experiments using the affinity-isolated anti-FLAG M2 antibody (F1804; Sigma), which, while it did not enrich for this background band, instead detected another band of the expected size for β-catenin at low levels (1.7-fold over IgG control IP results) that again were identical regardless of whether FLAG-hTERT was expressed (Fig. 3B). We verified the identity of this cross-reacting coimmunoprecipitated protein band as β-catenin by RNAi: reducing β-catenin expression produced corresponding reductions in the intensity of the band pulled down by the affinity-purified M2 antibody co-IP experiments (Fig. 3C). Thus, we did not detect a significant or specific interaction between hTERT and β-catenin in HeLa cells above the background signals caused by anti-FLAG antibody cross-reactivity. Using the same antibodies and IP buffers as Park et al. (13), we were also unable to detect an interaction between overexpressed FLAG-hTERT and endogenous BRG1, despite obtaining high signals corresponding to FLAG-hTERT itself with the FLAG antibodies used (Fig. 3B). In addition, we were able to detect only a weak interaction, at best, between endogenous levels of BRG1 and β-catenin; such an interaction has previously been reported only in a BRG1

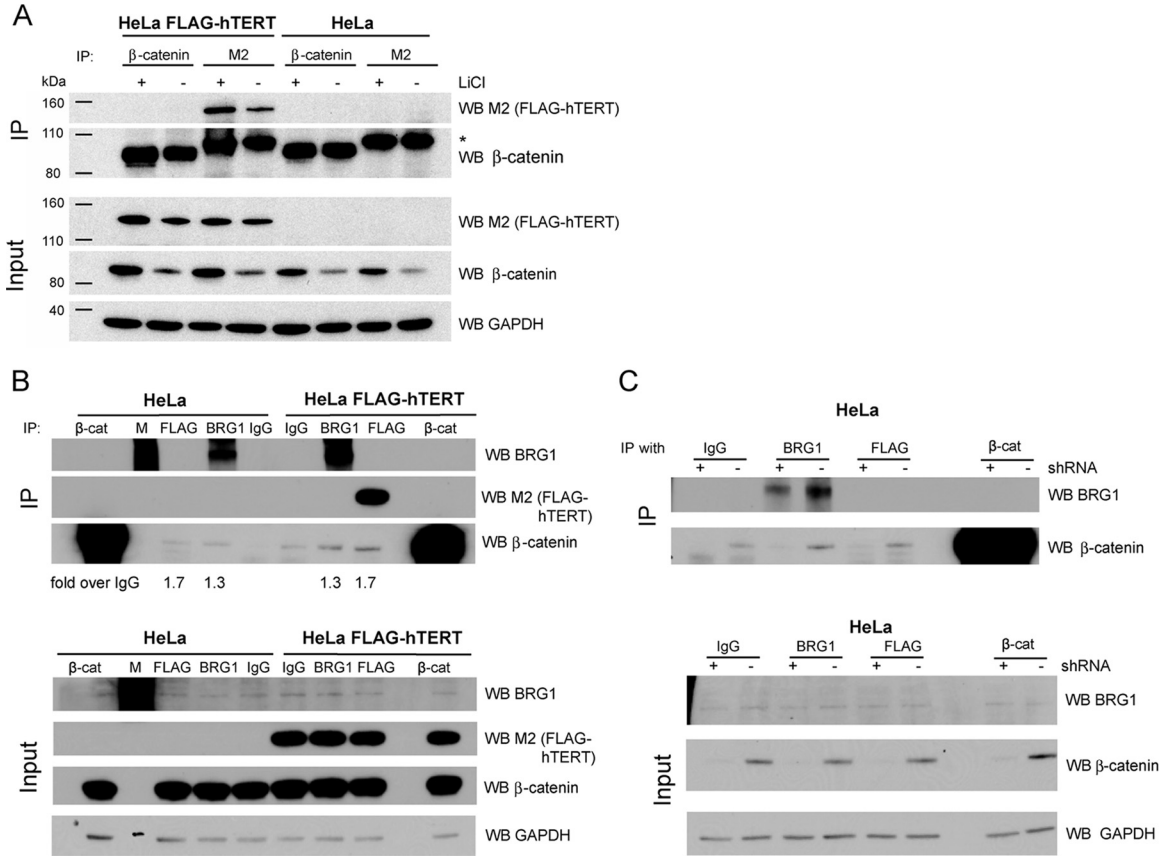


**FIG 3** hTERT does not interact with β-catenin or BRG1. (A) Anti-β-catenin antibody cross-reacts with anti-FLAG immunoprecipitate. HeLa cells were either transfected with pcDNA3-FLAG-hTERT for 16 h (left) or left untransfected (right), followed by treatment with 25 mM LiCl or no LiCl treatment. Precipitates from anti-β-catenin (clone 14) or anti-FLAG (M2 antibody F3165, Sigma) IP were subjected to Western blotting (WB) with anti-β-catenin (clone 14) or anti-FLAG antibodies. The asterisk indicates a slower-migrating background band. (B) Top: Western blot of HeLa cells transfected with FLAG-hTERT (right hand side) or left untransfected (left hand side) following IP with specific antibodies. M indicates size marker lane. Bottom: Western blot of input samples from experiment. (C) Top: Western blot of HeLa cells treated with or without β-catenin shRNA following IP with specific antibodies. Bottom: Western blot of input samples from the experiment.

overexpression context (20). We conclude, first, that the endogenous expression levels of BRG1 in HeLa cells were too low to detect strong interactions with β-catenin in our experiments, second, that M2 anti-FLAG antibody cross-reacts with a protein with a gel mobility close to that of β-catenin, and third, that the interaction of FLAG-hTERT with BRG1 or with β-catenin was not significantly above background IP levels.

**BRG1 and hTR do not colocalize at Wnt target genes.** In human cells, the hTERT protein and the telomerase RNA hTR, together with additional proteins, assemble to form the telomerase ribonucleoprotein complex, although it has not been determined what fractions of the total levels of hTERT and hTR exist in these complexes. Previously, Chu et al. (31) used whole-genome chromatin isolation by RNA purification (ChIRP) in HeLa S3 cells to detect hTR associated with chromatin at 2,198 genomic locations in HeLa S3 cells. Those authors additionally reported that the hTR-bound peaks they had identified were significantly enriched at loci of genes in the "Wnt receptor signaling pathway" gene ontology (GO) term and on this basis proposed that hTR in complex with hTERT cooccupies Wnt target genes (31). Given the evidence described above that a previously reported interaction between hTERT and β-catenin protein can be explained by cross-reactivity of anti-FLAG antibody rather than a bona fide interaction, we used a bioinformatics approach to reexamine any potential connection between the published genomic localizations of BRG1 cross-linked sites, telomerase RNA cross-linked sites, and Wnt signaling genes. To identify loci on the HeLa S3 genome enriched for localization sites of both BRG1 and hTR (with hTR inferred to likely be in complex with hTERT, as was described previously [31]), we merged the published BRG1-enriched localization sites identified by ChIP-seq in HeLa S3 cells (32) and the published hTR-enriched localization sites (31). We applied the criterion that they were separated by ≤100 bp, as the same criterion was previously used to determine cooccupancy of BRG1 with other members of the SWI/SNF complex at genomic loci (32). Using this criterion, 217 genes in the vicinity of the merged BRG1/hTR-enriched loci were identified. However, while "Positive regulation of apoptosis" was identified as a highly significant GO term among these 217 genes, Wnt signaling was not identified as a significant GO term (Table 1). Of the 217 genes, only MYC is known to be a target gene of Wnt signaling. Thus, this analysis in HeLa S3 cells (applying the criterion of peak separation no greater than 100 bp) failed to verify any significant cooccupancy by hTR and BRG1 of Wnt target genes or their nearby controlling regions

**TABLE 1** Significant GO terms for genes close to BRG1 and hTR co-occupied loci in HeLa S3 genome

| Gene | GO term | *P* value |
|---|---|---|
| GO:0043065 | Positive regulation of apoptosis | 0.005 |
| GO:0006928 | Cell motion | 0.025 |
| GO:0051693 | Actin filament capping | 0.027 |
| GO:0007028 | Cytoplasm organization | 0.035 |
| GO:0006917 | Induction of apoptosis | 0.035 |
| GO:0048146 | Positive regulation of fibroblast proliferation | 0.037 |
| GO:0030834 | Regulation of actin filament depolymerization | 0.040 |
| GO:0033043 | Regulation of organelle organization | 0.043 |
| GO:0032272 | Negative regulation of protein polymerization | 0.045 |
| GO:0032271 | Regulation of protein polymerization | 0.046 |

except for MYC. This analysis does not directly address hTERT and BRG1 cooccupancy on chromatin genomic loci. However, because some fractions of hTERT and hTR exist as telomerase complexes in HeLa S3 cells, colocalization of hTR with BRG1 at Wnt signaling gene loci might be predicted if hTERT protein and BRG1 protein interact, as suggested previously (31). Our negative finding for hTR and BRG1 cooccupancy at Wnt signaling gene loci thus does not support, but by itself does not refute, the possibility of interaction between hTERT and BRG1.

**Effects of hTERT overexpression on Wnt signaling target gene expression in cell lines do not reflect cellular Wnt signaling competency.** While our bioinformatics data analysis did not identify a significant overlap of Wnt pathway genes with genomic loci cross-linkable to hTR and BRG1 in HeLa cells, this analysis did not exclude the possibility that TERT, possibly not bound to hTR, still promotes Wnt signaling. To test directly whether hTERT modulates endogenous Wnt signaling target gene expression in breast cancer cells, we stably overexpressed hTERT in the high-Wnt-signaling SUM149PT and HCC3153 cells and, as a control, in the Wnt-signaling-impaired HCC1806 cells. We then measured the mRNA levels of 84 endogenous Wnt downstream target genes supplied as arrays for quantitative RT-PCR (qRT-PCR) (see Materials and Methods). First, with hTERT overexpression (Fig. 4C), the Wnt-signaling-competent SUM149PT cells showed a modest overall trend to greater expression of the Wnt target genes as a group compared with vector controls; however, this trend was no greater than that seen for the Wnt-signaling-impaired HCC1806

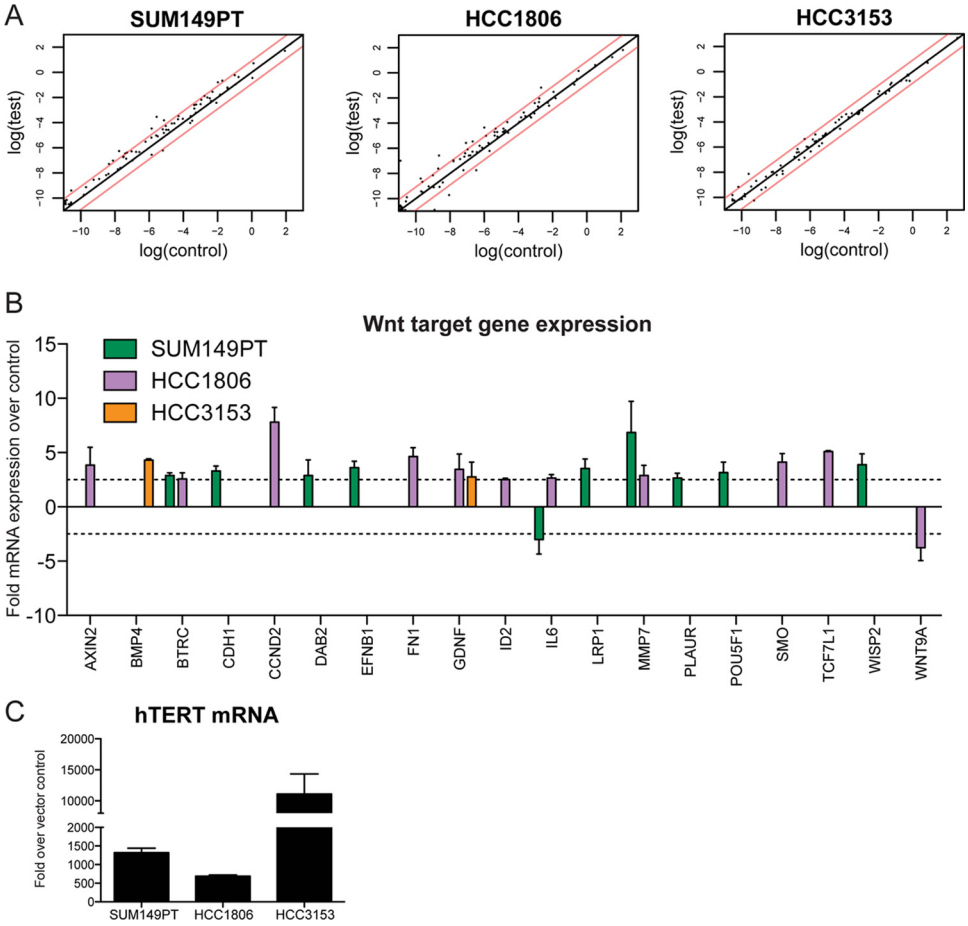


**FIG 4** Effect of hTERT overexpression on Wnt target gene expression in breast cancer cell lines. SABiosciences qPCR arrays were used to measure the effect of hTERT overexpression on the mRNA expression of endogenous Wnt target genes after treatment with 25 mM LiCl for 6 h. (A) Scatter plots of log-transformed relative expression levels of each gene. Red lines indicate a 2.5-fold change in gene expression. (B) Wnt target genes that changed ±2.5-fold compared to the control results (indicated by dotted line) upon hTERT overexpression in SUM149PT, HCC1806, and HCC3153 cells. (C) hTERT mRNA expression relative to GAPDH mRNA and vector control expression in SUM149PT, HCC1806, and HCC3153. Bars represent means of the results of 3 (SUM149PT and HCC1806) and 2 (HCC3153) biological replicates. Error bars indicate standard deviations (SD).

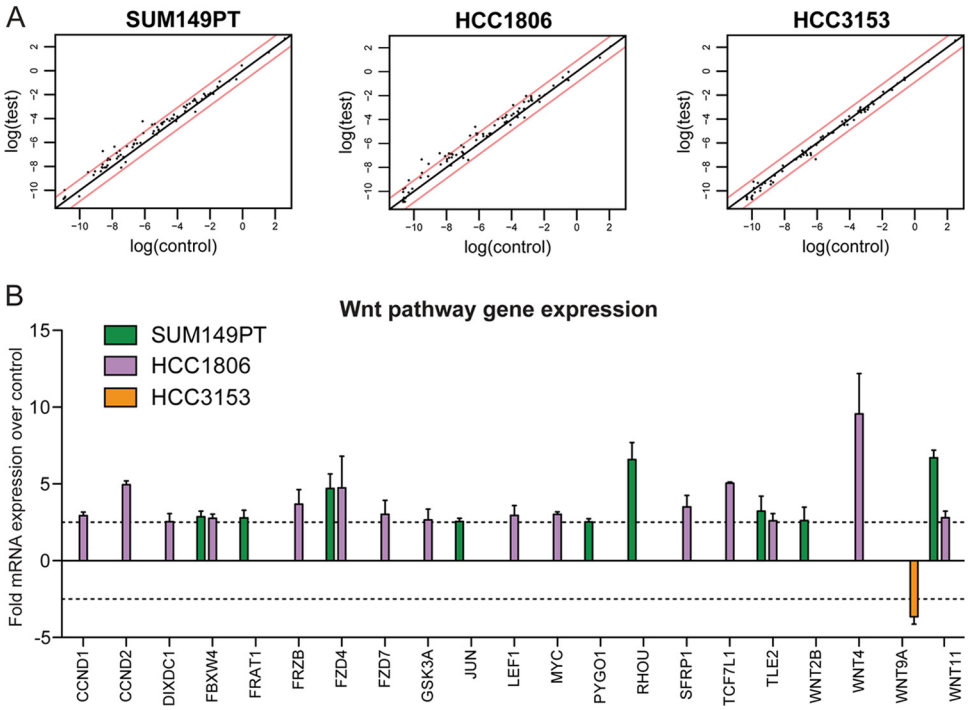


**FIG 5** Effect of hTERT overexpression on Wnt pathway gene expression in breast cancer cell lines. SABiosciences qPCR arrays were used to measure the effect of hTERT overexpression on the mRNA expression of endogenous Wnt pathway genes after treatment with 25 mM LiCl for 6 h, using the same RNA samples as described for Fig. 4. (A) Scatter plots of log-transformed relative expression levels of each gene. Red lines indicate a 2.5-fold change in gene expression. (B) Wnt pathway genes whose expression changed ±2.5-fold compared to the control results (indicated by dotted line) upon hTERT overexpression in SUM149PT, HCC1806, and HCC3153 cells. Bars represent means of 3 biological replicates. Error bars indicate SD.

cells and, furthermore, was not found for the Wnt-signaling-competent HCC3153 cells (Fig. 4A). In addition, the genes that were significantly (at least 2.5-fold) changed by hTERT overexpression differed between the cell lines (Fig. 4B). Among the 84 Wnt target genes analyzed, 10 genes were upregulated and 1 gene was downregulated in the Wnt-signaling-impaired HCC1806 cells, while 9 genes were upregulated and 1 gene was downregulated in SUM149PT cells, which can strongly activate Wnt signaling (Fig. 4B). In HCC3153 cells, which can also strongly activate Wnt signaling, only 2 target genes were upregulated by at least 2.5-fold in response to hTERT overexpression. Only three genes (the BTRC, GDNF, and MMP7 genes) were upregulated at least 2.5-fold in any two of the cell lines, and for all three of those genes, one of the cell lines was always the Wnt-signaling-impaired HCC1806 cell line. Furthermore, in SUM149PT and HCC1806 cells, IL-6 expression responded to hTERT overexpression in opposite directions.

We conclude that hTERT overexpression affects expression of some Wnt target genes, but without a discernible pattern, in 3 different breast cancer cell lines with different Wnt signaling capacities and sometimes in discordant directions between cell lines. Since hTERT overexpression also produced comparable magnitudes of effects on gene expression in the control HCC1806 line, which has severely diminished Wnt signaling, it is unlikely that hTERT changes gene expression in concert with β-catenin function.

We also measured the mRNA levels of a panel of 84 endogenous Wnt signaling pathway genes. As with the Wnt target gene panel, upon hTERT overexpression, the Wnt-signaling-competent SUM149PT cells showed a small trend to greater expression of the Wnt pathway genes as a group compared with vector controls; however, again, this trend was no greater than that seen for Wnt-signaling-impaired HCC1806 cells (Fig. 5A). Furthermore, hTERT overexpression caused the Wnt-signaling-competent HCC3153 cells to show slightly decreased, rather than increased, expression of the Wnt pathway genes as a group. Again, among the three cell lines, hTERT overexpression caused specific Wnt pathway genes to be up- or downregulated (at least 2.5-fold) in a cell line-specific manner (Fig. 5B). Additionally arguing against the idea of hTERT expression promoting Wnt signaling, knockdown of endogenous levels of hTERT (as measured by reduction of telomerase activity in Fig. 6B) in SUM149PT and HCC1806 cells resulted in a corresponding decrease in gene expression of only one (Wnt11) of those genes that we found to be significantly upregulated by hTERT overexpression; furthermore, this effect occurred only in the Wnt-signaling-impaired HCC1806 cells (Fig. 6A).

In summary, the magnitudes of the effects of increases or decreases in hTERT expression on gene expression were similar regardless of functional Wnt signaling. Furthermore, hTERT expression alterations in the cell lines examined resulted in patterns predicted to have discordant and counteractive effects on Wnt target and pathway gene expression. Thus, hTERT is unlikely to universally promote the Wnt pathway in human breast cancer cell lines.

## DISCUSSION

While the role of telomerase in protecting and maintaining telomeres can explain the upregulation of telomerase in stem cells and tumors, it does not exclude the possibility of other, nontelomeric roles for telomerase. Recently, it was reported that hTERT inter-

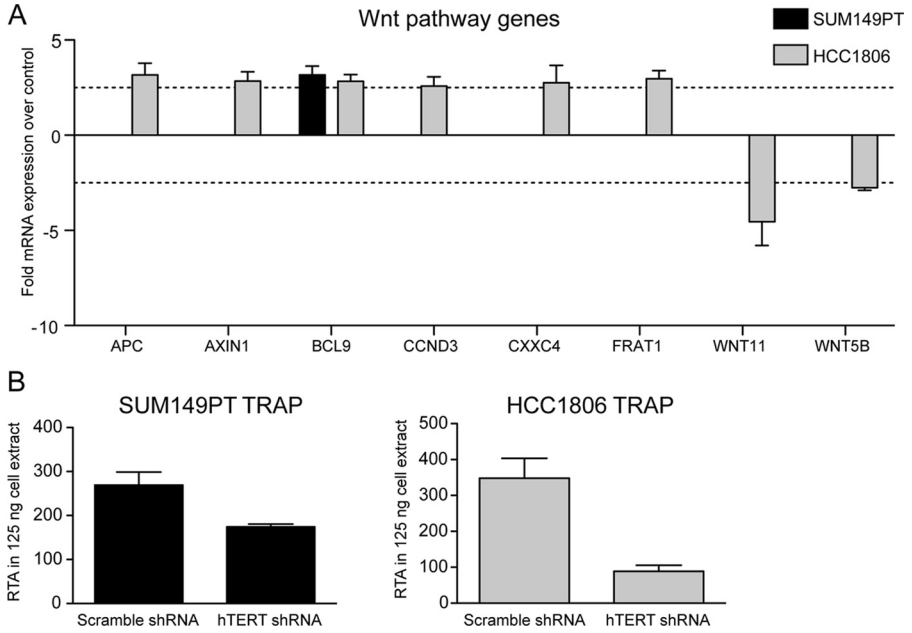


**FIG 6** Effect of hTERT knockdown on Wnt pathway gene expression in SUM149PT and HCC1806 cells. SABiosciences qPCR arrays were used to measure the effect of hTERT knockdown on mRNA expression of endogenous Wnt pathway genes. (A) Wnt pathway genes that changed ±2.5-fold compared to control results (indicated by dotted line) upon hTERT knockdown in cells treated with 200 ng/ml Wnt3a for 6 h. (B) In the same cells as were used for the analysis described for panel A, relative telomerase activity (RTA) as measured by the TRAP assay was reduced after shRNA-mediated hTERT knockdown. Bars represent means of the results of 2 (SUM149PT) and 3 (HCC1806) biological replicates. Error bars indicate SD.

acts with BRG1 and promotes Wnt signaling (13). In the present study, we set out to determine whether hTERT promotes Wnt signaling in human breast cancer cells. We tested this using different biochemical and molecular biology approaches. Using three human basal breast cancer cell lines, the MCF10A breast cell line, and HeLa cells, we failed to find evidence consistent with the hypothesis that hTERT promotes Wnt signaling in a biologically significant way in these cells. While our results were not aimed at addressing the issue of whether there is any interaction between TERT and Wnt signaling in the mouse system, they add some new information for the context of such investigations.

Using both transient and stably integrated Wnt reporter systems, we found that hTERT hyperactivated the reporters in a TCF/LEF site-dependent manner only mildly in only one (HCC3153) of the five cell lines tested. Although we observed a 2-fold increase in luciferase activity upon hTERT overexpression in HeLa cells using BARL, we believe this to be an insignificant result since in the control experiment, overexpression of hTERT together with the mutant control TCF/LEF fuBARL led to a 3-fold increase in luciferase activity. This result renders it unlikely that luciferase expression in HeLa cells is dependent on hTERT promoting a functional TCF/LEF promoter. One possible partial explanation for the apparent discrepancy between our study and previous reports that hTERT overexpression increases Wnt reporter activity in HeLa cells (13, 19) is the number of TCF/LEF sites in the different reporters used. Park et al. utilized a transiently expressed Mega TOPFlash with 14 TCF/LEF binding sites (13) and observed a 2-fold increase in luciferase activity upon hTERT overexpression. Hrdlicková et al. and the present study used SuperTOPFlash with 7 TCF/LEF binding sites (27) and observed a small but statistically significant ~1.5-fold increase in HeLa cells (19) and a ~1.4-fold increase in HCC3153 cells (this study). Mouse Wnt reporters in MEFs have also yielded various results. MegaTOPFlash hyperactivation (6-fold) was reported with mTERT overexpression in $TERT^{-/-}$ MEFs using LiCl to activate Wnt signaling (13), while Strong et al. were unable to detect a reciprocal decrease in SuperTOPFlash activity in $TERT^{-/-}$ MEFs using Wnt3a activation of Wnt signaling (22). We conclude that the effect of hTERT on Wnt reporters is small but not universal in cancer cells; rather, it depends on the cell type and context and on the reporter system used.

Since hTERT hyperactivated Wnt/b-catenin reporters only mildly, and in only one of the five human cancer cell lines tested, we reexamined the possible biochemical links between hTERT and Wnt signaling that were previously reported to be mediated by BRG1 (13). We failed to detect a significant protein-protein interaction between our overexpressed FLAG-hTERT and endogenous BRG1 in HeLa cells, using the same buffers and antibodies as described in Park et al. Since an interaction with overexpressed and endogenous hTERT and BRG1 was reported in another study in HeLa and 293T cells (40), this discrepancy between the results may be due to variations in BRG1 expression in different HeLa strains. Additional considerations that may underly the discrepancy between those two reports and our results include possible cross-reactivities of antibodies; in those reports (13, 40), various controls to rule out such cross-reactivities were not shown.

We were unable to detect an interaction between FLAG-hTERT and β-catenin (hypothesized to be mediated by BRG1) using an affinity-purified anti-FLAG M2 antibody for IP. Instead, we detected cross-reactivity between the immunoprecipitates from non-affinity-purified anti-FLAG M2 antibody IPs and the β-catenin antibody, even (i) in the absence of FLAG-tagged hTERT and (ii) when β-catenin was not stabilized with LiCl. Thus, we cannot exclude the possibility that the cross-reactivity

we observed with the M2 antibody provides an explanation for the conclusion reached by Park et al. (13) that FLAG-hTERT interacts biochemically with β-catenin. Furthermore, we detected only a very weak interaction between endogenously expressed BRG1 and β-catenin. To our knowledge, the only published evidence of a protein-protein interaction between BRG1 and β-catenin was reported only under conditions where BRG1 was overexpressed in 293T and DK11 cells (20). Furthermore, native BRG1 did not bind to glutathione *S*-transferase (GST)-tagged β-catenin *in vitro* (41). Our negative findings are thus consistent with the possibility that BRG1 is associated with β-catenin only transiently, which may hinder the detection of an interaction using immunoprecipitation. Alternatively, BRG1 may not be expressed at high enough levels under normal circumstances to allow detection of its interaction with hTERT or β-catenin.

A model was previously proposed by which the telomerase complex interacts with BRG1 to promote β-catenin gene expression (13, 31). If hTERT interacts with BRG1 to directly bind to β-catenin target genes, it is reasonable to postulate that the telomerase ribonucleoprotein complex (as monitored by hTR cross-linking to chromatin [31]) would be colocalized with at least some genomic positions together with BRG1. However, in our reanalysis of published data to seek simultaneous enrichment of hTR and BRG1, Wnt signaling was not among the significant GO terms for the 217 genes that we identified in the vicinity of the overlap between BRG1- and hTR-enriched genomic sites. Among these 217 genes, only one Wnt target gene, MYC, was identified as an hTR- and BRG1-enriched cross-linked locus. Since the enrichment peaks of hTR (mean peak size = 761 bp) and BRG1 (mean peak size = 1,245 bp) were analyzed and reported by different groups using slightly different background corrections, this does not rule out the possibility that hTR and BRG1 cooccupy sites in the genome further apart than the 100-bp spacing between the hTR- and BRG1-enriched regions that we allowed for in our analysis. However, given the report that hTERT and BRG1 interact directly, it was reasonable to investigate whether BRG1 and hTR are localized at the same genomic sites, as has been reported for BRG1 and other members of the SWI/SNF complex (32). Merged regions encompassing BRG1 and hTR peaks with ≤100 bp of separation span a distance of 1,700 bp on average, a distance that can incorporate ~11 to 12 nucleosomes. By this criterion, we were able to detect cooccupancy of BRG1 and hTR (potentially in a complex with hTERT) in HeLa S3 cells at only one Wnt target gene, the MYC gene. However, this analysis does not exclude the possibility that hTR is localized at Wnt genes that are not bound by BRG1 and does not address directly whether hTERT is bound to BRG1-bound sites.

We found that hTERT overexpression and depletion did change the expression of some Wnt pathway or target genes but did so independently of whether or not the cell line was strongly activating Wnt signaling and not in directions predicted to have concordant effects on Wnt signaling. These data suggest that while hTERT seems to affect gene expression, these changes are not necessarily β-catenin signaling related. Interestingly, even though overexpression of hTERT in human mammary epithelial cells (hMECs) provided a proliferative advantage under low-mitogen conditions and led to gene expression changes (9), another study failed to find upregulation of Wnt target gene AXIN2 after hTERT overexpression in hMECs (6). Furthermore, hTERT has been reported to bind to and regulate NFKB1 target genes directly (42). Since many Wnt signaling target genes are also regulated by other pathways, such as the transforming growth factor beta (TGF-β) pathway (43), we propose that hTERT may promote the expression of certain genes but not necessarily by acting solely in Wnt signaling. MYC, which emerged as the strongest candidate regulated by BRG1 in combination with hTERT/hTR, is, for example, an important transcriptional activator for cell growth and proliferation and is downstream of other receptor signal transduction pathways such as receptor tyrosine kinase and T cell receptor pathways (44).

In summary, we consistently failed to find evidence that hTERT expression promotes Wnt signaling in three human breast cancer lines, an immortalized human breast cell line, and HeLa cells. Our results conflict with previous studies reporting such an effect of TERT in HeLa cells and also suggest that any effect of TERT on Wnt signaling may be context and cell line specific. Studies to further investigate the mechanisms by which hTERT promotes proliferation, inhibits apoptosis, and alters gene expression will provide insights into the noncanonical roles of telomerase.

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