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Alternatively Spliced Telomerase Reverse Transcriptase Variants Lacking Telomerase Activity Stimulate Cell Proliferation

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Eight human and six chicken novel alternatively spliced (AS) variants of telomerase reverse transcriptase (TERT) were identified, including a human variant (Δ4-13) containing an in-frame deletion which removed exons 4 through 13, encoding the catalytic domain of telomerase. This variant was expressed in telomerase-negative normal cells and tissues as well as in transformed telomerase-positive cell lines and cells which employ an alternative method to maintain telomere length. The overexpression of the Δ4-13 variant significantly elevated the proliferation rates of several cell types without enhancing telomerase activity, while decreasing the endogenous expression of this variant by use of small interfering RNA (siRNA) technology reduced cell proliferation. The expression of the Δ4-13 variant stimulated Wnt signaling. In chicken cells, AS TERT variants containing internal deletions or insertions that eliminated or reduced telomerase activity also enhanced cell proliferation. This is the first report that naturally occurring AS TERT variants which lack telomerase activity stimulate cell proliferation.

Telomerase is a ribonucleoprotein complex with reverse transcriptase (RT) enzymatic activity which is responsible for adding telomeric repeats (TTAGGG) to the ends of chromosomes (23, 24). Telomerase plays a major role in protecting telomeres from erosion that results from DNA replication and oxidative damage (48, 71). In addition to this canonical function, there is an accumulating body of evidence which indicates that telomerase has additional activities (50). These noncanonical activities include stimulation of cell proliferation, protection against oxidative damage and apoptosis, modulation of global gene expression, activation of stem cells, and tumor promotion (8, 14). Telomerase activity is downregulated in adult tissues of most vertebrates. This downregulation is thought to reduce the probability of tumor development (17). Telomerase is activated during the immune response and wound healing and is reactivated in approximately 85% of human tumors (4, 19, 21, 35, 58).

Telomerase is a multisubunit enzymatic complex consisting of the telomerase reverse transcriptase (TERT), an RNA component (TR) which acts as a template, and other associated proteins (6). Telomerase activity is tightly regulated, and transcriptional regulation of TERT plays the key role in telomerase regulation (74). The human TERT (hTERT) gene is expressed principally as alternatively spliced (AS) forms in both normal and tumor cells (65–67). Six AS variants (Δα, Δβ, and INS1 to -4) were discovered in the process of cloning the hTERT gene, and subsequently, hTERT-Δγ was also identified (27, 34, 73). Later, Sæbøe-Larssen and coworkers described six additional AS variants of human TERT, bringing the total to 13 (52). The other species in which alternative splicing of TERT has been characterized extensively is the chicken. Thirty-one chicken AS TERT variants have previously been identified in various cell lines and tissues (2, 11, 31). Individual alternative splicing events can occur in various combinations in a single TERT transcript (2, 11, 13, 27, 31).

The functions of only two human AS variants, Δα and Δβ, have been analyzed by overexpression in several cell lines (13, 77). The Δα variant, which has the first 36 bp of exon 6 deleted without disrupting the open reading frame (ORF), lacks telomerase activity and has been proposed to function as a dominant-negative mutant (13, 77). The Δβ variant has exons 7 and 8 deleted, resulting in the introduction of a premature termination codon (PTC) (77).

This report describes the identification of eight additional hTERT and six additional chicken TERT (chTERT) AS variants. A human variant maintaining the original TERT ORF with a deletion of exons 4 through 13 (Δ4-13) is expressed in normal human cells and tissues and in cell lines which use alternative lengthening of telomeres (ALT) to maintain telomere length, as well as in telomerase-positive cell lines. Exons 4 through 13 encode the telomerase reverse transcriptase domain and a segment of the C-terminal (CT) domain. Though this variant lacks telomerase activity, it retains the ability of TERT to stimulate cell proliferation. The Δ4-13 variant activates Wnt signaling. Similarly, two chicken variants, A and D, which lack or have very low telomerase activity and maintain an original TERT ORF, stimulate cell proliferation.

MATERIALS AND METHODS

Cloning. The AS variants of TERT were amplified by RT-PCR and cloned into pGEM-T Easy (Promega, Madison, WI) or into pCR2.1-TOPO vectors (Invitrogen, Carlsbad, CA). The sequence of hTERT AS variant Δ2p is provided in the supplemental material.

Nomenclature of AS TERT variants. The basal form of the TERT transcript, which is properly spliced using all 16 exons and yields an enzymatically active protein, is designated full-length TERT (6). Transcripts that diverge from this are considered alternatively spliced forms and are designated based on the alternative splicing event or events which they contain, for example, Δα, ΔαΔβ, A, BD, etc. (Fig. 1). A Δ sign was added to the designations of α, β, and γ variants to avoid possible confusion

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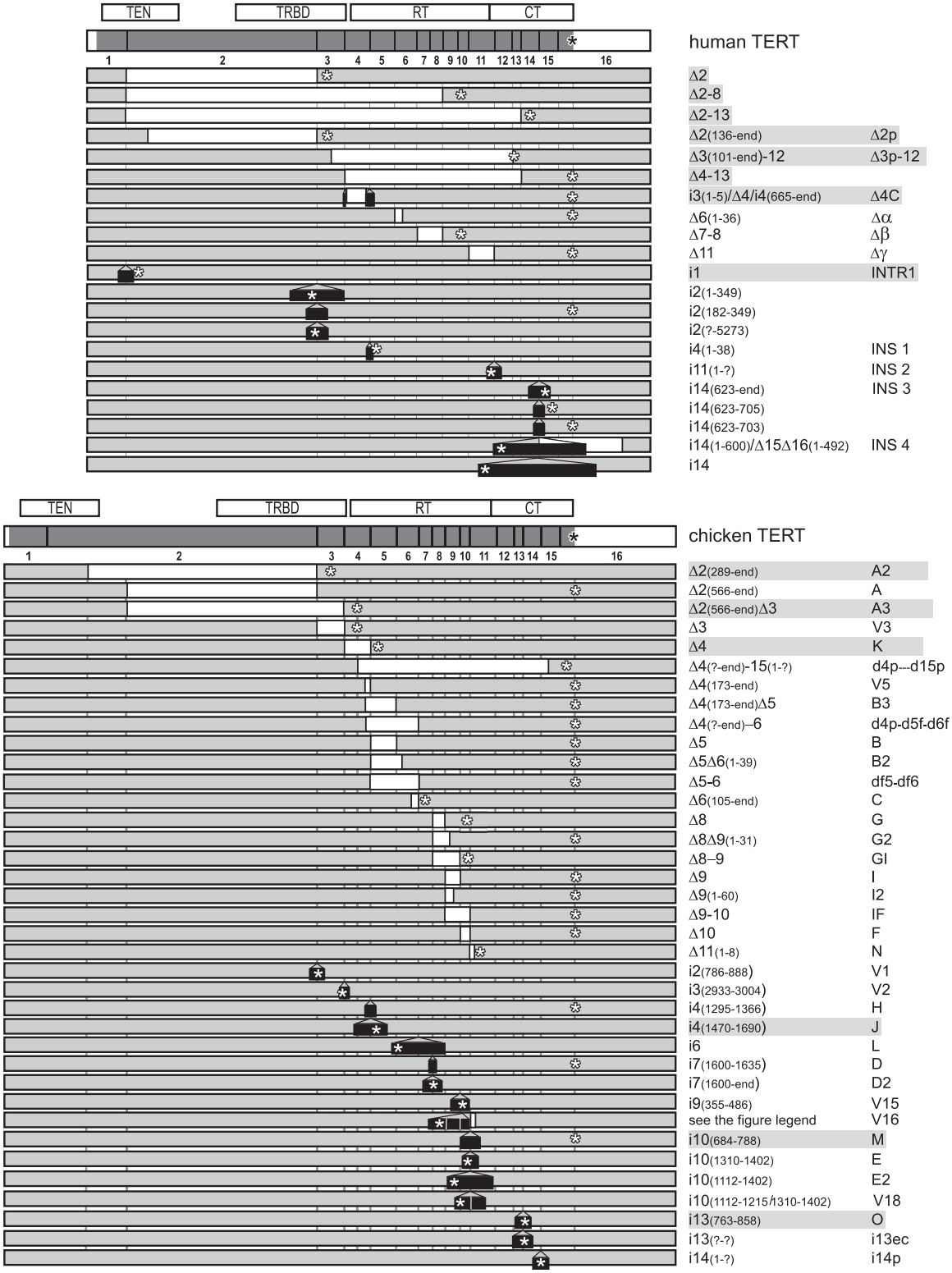

FIG 1 Human and chicken AS TERT variants. The structure of full-length human or chicken TERT mRNA is shown at the top of each set of single-event AS variants. The ORFs in full-length TERT are shaded in dark gray. The positions of the regions encoded by separate exons and of functional domains (TEN, telomerase essential N-terminal domain; TRBD, telomerase RNA-binding domain; RT, reverse transcriptase domain; and CT, C-terminal domain) are indicated. The full-length TERTs and AS variants are drawn to their relative sizes. Asterisks identify the locations of stop codons. Deletions (white rectangles) and insertions (black rectangles) resulting from splicing events are indicated in the sets of AS variants. The descriptive names ("Δ" indicates deletion of exon-derived sequences, and "i" indicates insertion of intron-derived sequences) and abbreviated names of the TERT AS variants are shown in the right margin. The descriptive name of the V16 variant is i9(355-486/926-988/1091-1162)/Δ11(1-3). Variants V16 and V18 likely arise through combinations of several single AS events. Names of the AS forms newly described in this report are shaded.

between the variant and the region of full-length TERT deleted in the variant. PCR-based detection of TERT variants is limited to a specific region of the TERT gene, and this analysis determines only if the transcript does not have any alternative splicing events in a specific area. Such a transcript is designated by a superscript minus sign following the designation of a missing splicing event (for example, $\Delta\alpha^-$, $\Delta\alpha^-\Delta\beta^-$, etc.) or is designated wild-type TERT.

Expression of TERT AS variants. The hTERT AS variants in cell cultures were expressed from the pBabe retroviral expression vector (42). Four plasmids were used: pBabe-hTERT, pBabe, pBabe-hTERTΔ4-13, and pBabe-GFP. pBabe-hTERT was obtained from W. E. Wright. This plasmid, containing the hTERT coding sequence in modified pBabe-puro, is referred to as pBabehTERTloxp in the original article (61). The pBabe control vector plasmid was constructed by deletion of the EcoRI-flanked hTERT-containing region from pBabe-hTERT. pBabe-hTERT-Δ4-13, encoding the hTERT Δ4-13 AS variant, was constructed by deletion of the region containing exons 4 to 13. pBabe-GFP was obtained from Cell Biolabs (San Diego, CA).

Human cells susceptible to infection by mouse ecotropic viruses were generated by infection with amphotropic WZL/neo/Eco viruses rescued by the transfection of an amphotropic packaging cell line (Plat-A) with the pWZL/neo/Eco plasmid. This plasmid, expressing the mouse ecotropic receptor and neomycin resistance, was obtained from S. W. Lowe (60).

chTERT variants were expressed in cell cultures by use of a pREV-T-based retroviral expression system. Five plasmids were employed: pREV-chTERT, pREV-chTERT-A, pREV-chTERT-B, pREV-chTERT-D, and pCSV11S3. pREV-chTERT, expressing full-length chicken TERT, was described previously (as pREV-TERT) (31). pREV-chTERT-A, pREV-chTERT-B, and pREV-chTERT-D, expressing chTERT AS variants A, B, and D, were constructed by the replacement of internal regions of chTERT by the corresponding regions from TERT AS variants. pREV-chTERT-A^r expressed chTERT variant A, but the construct was modified in such a way that it is not recognized by the small interfering RNA (siRNA) siA (see Table S1 in the supplemental material). The nucleotide sequence TTCAAGCAGGTAATCACTCTG, which is targeted by siA, was replaced by TCCAGGCCGGGAACCATAGCG (changes are underlined), which codes for the same amino acids. pCSV11S3 contains an infectious genomic clone of chicken syncytial virus (CSV) (44). Virus stocks of REV-chTERT, REV-chTERT-A, REV-chTERT-B, and REV-chTERT-D were generated by cotransfection of pCSV11S3 with the respective plasmids (31).

RNA sources. Total RNAs from the Namalwa cell line and normal human tissue were obtained from a FirstChoice human total RNA survey panel (Ambion, Austin, TX). These samples were stringently treated with a DNase (Ambion). Total RNAs from chicken embryonic cells and the BJ, WI38, Saos2, U2OS, HeLa, and chicken cell lines were isolated using Tri-Reagent and treated with recombinant DNase I (Ambion).

Cell lines, viruses, and tissue culture. BJ mortal embryonic fibroblasts were a kind gift of W. E. Wright. WI38 mortal embryonic fibroblasts, Saos2 osteosarcoma cells, and HeLa cells were obtained from the American Type Culture Collection (Manassas, VA). The U2OS osteosarcoma and Namalwa cell lines were a kind gift from P. Tucker. Saos2, U2OS, and HeLa cells overexpressing the Δ4-13 hTERT variant or Babe vector were established by the transfection of parental cells with pBabe-hTERT-Δ4-13 or pBabe followed by selection with puromycin. DF1 is an immortalized chicken fibroblast cell line with a weak transformation phenotype (26, 56). The mortal 26TS cell line was established from a sarcoma derived from a chicken infected with retroviruses expressing c-*rel* containing oncogenic mutations (c-*rel* gi) (29). In early passages, 26TS cells had significant telomerase activity that was lost in the more advanced passages used for the experiments in this study. Chicken embryonic fibroblasts (CEFs) were prepared from 10-day-old embryos from pathogen-free White Leghorn chickens (Charles River SPAFAS, North Franklin, CT). Secondary cultures of CEFs were used for transfection of plasmid DNA by use of Metafectene Easy (Biontex, San Diego, CA). Viruses were harvested between 5 and 7 days after transfection.

The adherent human cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) (Atlanta Biologicals, Norcross, GA). The lymphoid cell line Namalwa was cultured with RPMI medium with 10% FCS. Chicken DF1 cells and CEFs were cultured with DMEM supplemented with 5% FCS and 5% chicken serum (Invitrogen). All cells were cultivated with the addition of 100 U of penicillin and 50 μg of streptomycin per ml of culture medium.

Packaging cell lines Platinum-E (Plat-E) and Platinum-A (Plat-A) (Cell Biolabs) were cultured in DMEM supplemented with 10% FCS and with 1 μg puromycin, 10 μg blasticidin, 100 U of penicillin, and 50 μg of streptomycin per ml. Exponentially growing cells were transfected with appropriate plasmid DNA by use of Fugene HD (Roche, Indianapolis, IN), and viruses were harvested 2 to 5 days after transfection. Babe retroviral particles which recognized the mCAT-1 receptor were harvested from the packaging cell line Plat-E. The mCAT-1 protein is the natural receptor for mouse type C ecotropic retroviruses (1). In order to introduce the Babe/mCAT-1 retroviruses into HeLa cells, the cells were first infected with retroviruses expressing the mCAT-1 protein, which were harvested from the packaging cell line Plat-A after transfection with pWZL/neo/Eco plasmid. HeLa cells infected by WZL/neo/Eco virus expressing mCAT-1 were selected using neomycin resistance and then infected with Babe retroviruses.

Cell proliferation assays. The proliferation rate of cells was determined by seeding cells expressing the various constructs or treated with siRNAs, or control cells, on 12-well or 6-well plates (4×10^4 to 8×10^4 cells per ml, depending on the cell type) and then counting them, after 48 to 72 h, with a Scepter automated cell counter (Millipore, Billerica, MA) or a hemocytometer.

Cell cycle analysis. Cell cycle analysis was performed as described previously (28). Briefly, cells were washed and fixed in 70% ethanol and then stored at 4°C before analysis. Cells were pelleted, resuspended in phosphate-buffered saline (PBS)–EDTA, and digested with RNase A. DNA was then stained by addition of propidium iodide, and dye incorporation was measured with a FACSCalibur flow cytometer (BD Immunocytometry Systems, San Jose, CA). Data were analyzed by FlowJo software using Watson pragmatic and Dean-Jett-Fox mathematical models.

RNA interference. Silencer Select siRNAs (see Table S1 in the supplemental material) and a negative control (NC1) were transfected into exponentially growing cells by use of siPORT NeoFX transfection agent or siPORT amine transfection reagent (Ambion). The levels of TERT mRNA and telomerase activity were analyzed 24 to 48 h later. RNA was isolated with an RNAqueous-Micro kit (Ambion), and the expression level of TERT mRNA was determined by RT-PCR.

Reporter assays. Firefly luciferase-based reporter plasmids pSuper 8× TOPFlash (with 7 TCF/LEF binding sites) and pSuper 8× FOPFlash (with 6 mutated TCF/LEF binding sites) were obtained from Addgene (69). The *Renilla* luciferase-based control reporter plasmid pRL-TK and the pEGFP-N1 plasmid (Promega), expressing green fluorescent protein (GFP), were cotransfected. Transfections were monitored visually using a fluorescence microscope. Cells were harvested in passive lysis buffer, and luciferase activity was measured by using a dual-luciferase reporter assay system (Promega).

Western blot analysis. Nuclear lysates were prepared and Western blot analysis was performed as described previously (31, 43), with the following modifications. The cell nuclei were isolated and lysed in buffers supplemented with Complete protease inhibitor cocktail (Roche), 2 mM phenylmethylsulfonyl fluoride (PMSF), and aprotinin (Sigma-Aldrich, St. Louis, MO). Proteins were separated in NuPAGE 4 to 12% Bis-Tris gels and electroblotted onto an Invitrolon polyvinylidene difluoride (PVDF) membrane (Invitrogen). Blotted proteins were visualized with SuperSignal West Dura chemiluminescent substrate (Thermo Fisher Scientific). Human TERT protein isoforms were detected by a rabbit monoclonal antibody to the hTERT C terminus (clone Y182; Novus Biologicals, Little-

ton, CO). Donkey anti-rabbit IgG(H+L) coupled with horseradish peroxidase (Jackson ImmunoResearch Laboratories, West Grove, PA) was used as the secondary antibody.

TRAP. The level of telomerase activity was evaluated using a telomerase repeat amplification protocol (TRAP) assay (36). Whole-cell extracts were prepared with 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS) buffer, and the concentration of total protein was measured by Bradford assay (35). Equivalent amounts of protein extracts were used to extend unlabeled TS primer at 37°C for 45 min. Aliquots of the extension reaction mixture were then subjected to PCR with Cy5-labeled TS primer and unlabeled ACX primer, using either Advantage cDNA polymerase mixture (BD Biosciences Clontech, Mountain View, CA) or KAPA2G Robust polymerase (Kapa Biosystems, Woburn, MA). The TRAP PCR products were separated in 7.5% acrylamide gels, and gel images were captured using a Typhoon Trio imager in fluorescence mode (GE Healthcare, Waukesha, WI).

Identification of TERT and its AS variants. First-strand cDNA synthesis was performed with total RNA (500 ng to 3 μg), using random hexamer primers and ThermoScript RT (Invitrogen) (28). For detection of TERT, its AS variants, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) by RT-PCR, the first-strand synthesis reaction mix was used together with 2.5 U Herculase Hotstart DNA polymerase (Agilent Technologies, Santa Clara, CA) or KAPA2G Robust polymerase (Kapa Biosystems), using the appropriate primers (see Table S2 in the supplemental material). The optimal conditions for each primer set were determined and are shown in Table S3.

Nucleotide sequence accession numbers. The sequences of the AS variants of TERT were submitted to GenBank under accession numbers DQ681296 to DQ681298, DQ681311, DQ681312, and JF896279 to JF896286.

RESULTS

Identification and characterization of novel alternatively spliced human and chicken TERT variants. Telomerase activity in humans and chickens is regulated similarly. Telomerase is downregulated in adult organs but activated during the immune response, regeneration, and carcinogenesis. Both the human and chicken TERT genes are also expressed principally as AS isoforms. Thirty-one AS variants of chTERT have been described previously (2, 11, 31) (Fig. 1). In contrast, only 13 AS hTERT variants have been identified (27, 34, 52, 73). These AS hTERT variants include variants containing three small deletions in the RT domain (a part of exon 6 and exons 7, 8, and 11) and 10 insertions involving complete or partial sequences of introns 2, 4, 11, and 14. Based on the number and nature of the AS variants expressed in avian cells, we hypothesized that additional AS TERT isoforms would be expressed in human cells.

Eight novel AS hTERT variants were identified in a screen of telomerase-positive and -negative cell lines by use of primers against the first exon in combination with primers targeting exons 3 through 16 (Fig. 1). Four of these AS variants, lacking either a part of exon 2, all of exon 2, exons 2 through 8, or exons 2 through 13 (Δ2p, Δ2, Δ2-8, and Δ2-13), contain PTCs. Two other variants retain the second exon but lack the RT domain. One of these two variants contains a deletion encompassing exons 4 through 13 (Δ4-13) and retains the original TERT ORF. The second variant lacks a part of exon 3 and all of exons 4 to 12 (Δ3p-12), which introduces a PTC. These variants lack regions of TERT where the previously described Δα and Δβ splicing events occur. One additional variant (Δ4C) lacks the 4th exon and contains an insertion of parts of the 3rd and 4th introns. This variant maintains the original ORF. Finally, a variant (INTR1) which retains the first intron and contains a PTC was also identified.

We also identified several AS variants of chicken TERT which were not previously reported. They include variants containing a partial deletion of exon 2, partial deletion of exon 2 plus deletion of all of exon 3, deletion of exon 4, and three insertions containing sequences derived from introns 4, 10, and 13 (A2, A3, K, J, M, and O). All of these variants, with the exception of the variant with a partial insertion of intron 10 (variant M), contain PTCs.

Several AS TERT variants have been cloned from plants and different vertebrate species (63). However, only the human and chicken TERT AS variants have been studied extensively, and this allows a comparison of these TERT isoforms in these two vertebrates (2, 11, 31, 52) (Fig. 1; see Table S4 in the supplemental material). Currently, 21 human and 37 chicken TERT AS variants have been identified. Approximately one-third of the variants in both species retain the original ORF (29% in humans and 41% in chickens). In hTERT, splicing events which result in deletions and insertions were detected in roughly equal numbers (43% and 48%, respectively). In contrast, slightly more deletions (57%) than insertions (41%) were identified in chTERT. Despite the large number of different TERT variants in both species, there is not a single variant which is identical between these two species.

hTERT RNA is detected in both telomerase-negative and -positive cell lines. To determine the expression pattern of the novel human AS TERT variants, several cell lines expressing different levels of telomerase activity were analyzed. BJ and WI38 fibroblasts are normal diploid fibroblasts (7, 16). The osteosarcoma U2OS cell line maintains telomere length by a telomerase-independent mechanism referred to as alternative lengthening of telomeres (ALT) (15). HeLa cells were derived from an adenocarcinoma (57). Namalwa is a cell line derived from a B-cell Burkitt lymphoma (37). These cell lines differ in telomerase activity as measured by the TRAP assay (Fig. 2A). Telomerase activity was undetectable in BJ, WI38, and U2OS cells, but the other two cell lines were telomerase positive (9, 10). HeLa cells expressed lower levels of telomerase activity than those expressed by the Namalwa cell line.

The steady-state levels of TERT mRNA in these cell lines were determined by RT-PCR using primers targeting a region in the 1st exon, an area which has not been shown to be involved in any alternative splicing events (Fig. 2B). All cell lines, including the telomerase-negative BJ and WI38 cells, expressed TERT mRNA. The lowest levels of TERT mRNA were expressed in BJ and WI38 cells, followed by HeLa cells, and the highest levels were detected in the Namalwa cell line.

A panel of several human tissues was also analyzed (spleen, thymus, lung, heart, brain, ovary, testis, and placenta). The tissues examined were expected to express widely different levels of telomerase activity. The total steady-state levels of TERT transcripts were first analyzed by RT-PCR as described above (Fig. 2B). The tissues with the highest levels of TERT were the testis, thymus, and placenta, with levels of TERT comparable to those in transformed HeLa cells. Intermediate levels of TERT were detected in spleen and lung tissues, with levels corresponding to those in BJ and WI38 cells. The ovary, heart, and brain expressed very low levels of TERT. Collectively, these results indicate that TERT mRNA is present in all cells proliferating in culture, even when they do not have telomerase activity. There were significant differences in the levels of TERT mRNA in the various tissues and organs obtained from adult donors, and in some of them, TERT mRNA was present at lower levels than those in tissue culture cell lines.

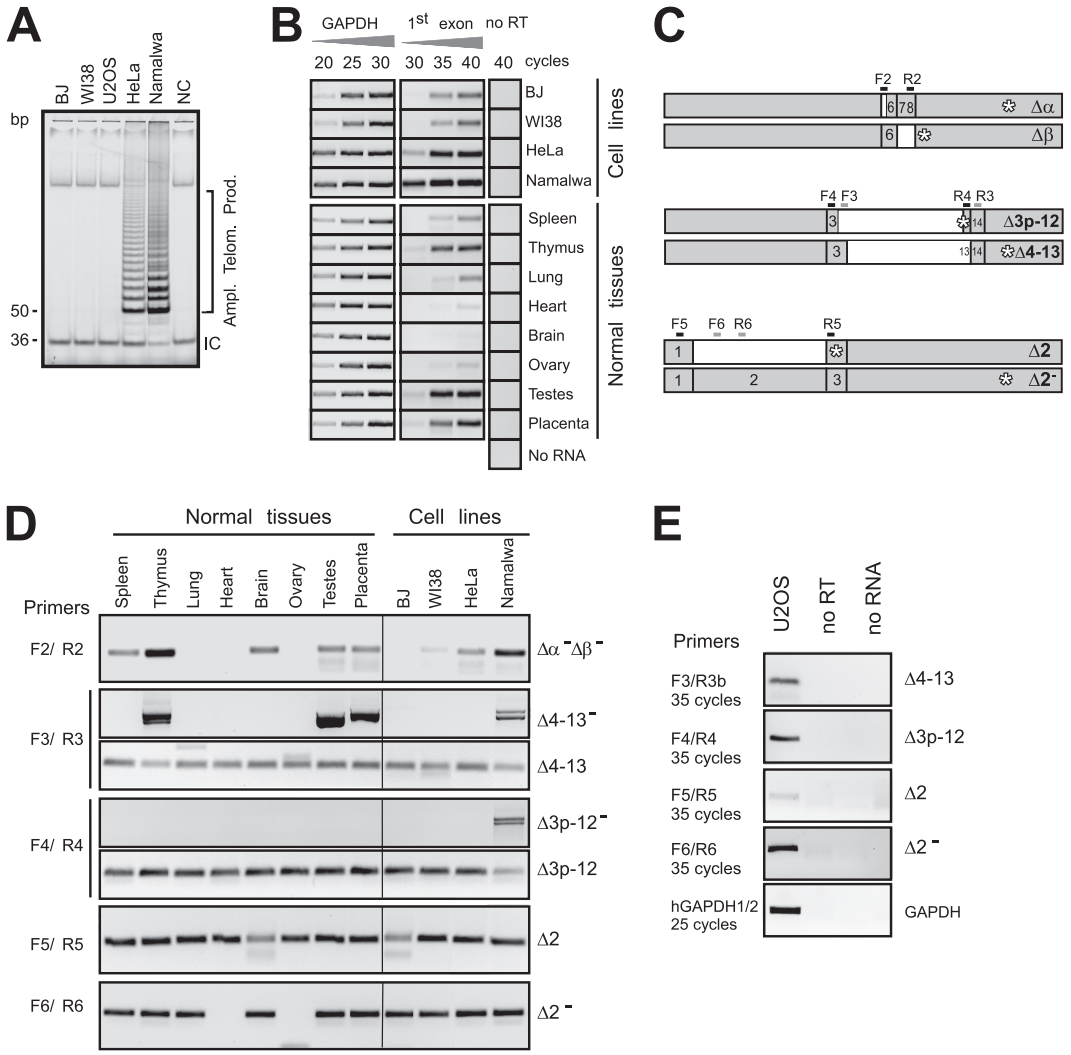

FIG 2 Expression of AS variants of TERT in human cell lines and tissues. Telomerase activities and levels of mRNA of wild-type and AS variants of TERT were determined. RNA samples from normal human tissues were pooled for several healthy adult donors of different ages, sexes, and origins. The PCR data represent typical results from three independent assays performed with the same pair of primers. (A) Telomerase activity was determined by TRAP assay. Molecular sizes are indicated in the left margin, and the positions of the amplified telomerase products (6-bp ladder starting with a 50-bp band) and a 36-bp PCR internal control (IC) are shown in the right margin. Extraction buffer served as the negative control (NC). (B) Semiquantitative RT-PCR using primers F25 and R234, which recognize sequences in the first exon, was employed to determine levels of total TERT mRNA (239 bp). The level of GAPDH served as an input control (355 bp). The PCR conditions are described in Tables S2 and S3 in the supplemental material. The number of cycles is indicated, and gray triangles represent increasing numbers of PCR cycles. Control cDNA reactions were performed without reverse transcriptase (no RT) or without RNA (no RNA). (C) Schematic representation of the PCR strategy. Small boxes represent primers used for RT-PCR. (D) The expression of AS variants in different normal human tissues and human cell lines was determined by RT-PCR. Expression of $\Delta\alpha^-/\Delta\beta^-$ (281 bp), Δ4-13$^-$ (1,411 bp), Δ4-13 (147 bp), Δ3p-12$^-$ (1,433 bp), Δ3p-12 (134 bp), Δ2 (199 bp), and Δ2$^-$ (262 bp) is shown. The Δ4-13$^-$ and Δ4-13 variants and the Δ3p-12$^-$ and Δ3p-12 variants are each products of one PCR run on the same gel. cDNA was made from 500 ng of total RNA, and PCR products were amplified for 40 cycles. PCR was performed using 1/20 of the cDNA reaction mix, using Herculase Hotstart DNA polymerase. (E) The expression of AS variants in U2OS osteosarcoma cells was determined by RT-PCR. cDNA was made from 3 μg of total RNA. Control cDNA reactions were performed without reverse transcriptase (no RT) and without RNA (no RNA). PCR products were amplified for 35 cycles. PCR was performed using KAPA2G Robust polymerase, using the indicated primers (see Table S2 in the supplemental material). The expression of the $\Delta\alpha^-/\Delta\beta^-$ variant was not detected in these cells (data not shown).

Telomerase-negative cells express novel AS forms. The most frequently occurring AS variants in human cells are the $\Delta\beta$ and $\Delta\alpha$ isoforms, which both lack telomerase activity (34). To estimate the levels of transcripts without the α and β deletions, we used a PCR strategy to detect "wild-type" TERT ($\Delta\alpha^-\Delta\beta^-$) (using primers F2 and R2). These TERT transcripts, which could produce telomerase activity, were expressed in the spleen, thymus, brain, testis, and placenta and in the telomerase-positive cell lines HeLa and Namalwa (Fig. 2D). AS variants with internal deletions (Δ3p-12 and Δ4-13) lack the sequences in which the $\Delta\beta$ and $\Delta\alpha$ events occur. When primers recognizing sequences in the 3rd, 13th, and 14th exons (F3, F4, R3, and R4) were employed, these novel AS TERT forms were detected in all tissues tested and in BJ, WI38, and HeLa cells. Low levels of these variants were detected in Namalwa cells. In the thymus, testis, placenta, and Namalwa cells, at least two other transcripts lacking the Δ4-13 sequences were also

detected, the shortest of which presumably contains the Δβ deletion (deletion of 182 bp) (34). These results indicate that the middle portion of hTERT mRNA can be spliced in two different ways.

We also identified several novel alternative splicing events in hTERT in which the second exon is deleted either alone or in combination with other sequences (Fig. 1). The deletion of the second exon (Δ2), which introduces a PTC immediately after the AS junction, often occurs together with other AS events in a single TERT molecule (data not shown). If the Δ2 splicing event is combined with any of the potentially protein-encoding AS TERT variants, such as the Δα, Δγ, or Δ4-13 variant, the resulting TERT mRNA acquires a PTC and could be targeted for degradation by nonsense-mediated decay (NMD). To determine the frequency of this event, the expression of the Δ2 AS variant was determined in a panel of human tissues and cell lines (Fig. 2D). The Δ2 variant was present in virtually all samples tested. Therefore, we also determined whether TERT transcripts which include the second exon ($\Delta2^-$) and may produce either full-length TERT or variants that can be translated were expressed. The steady-state levels of the $\Delta2^-$ TERT transcripts were detected in all tissues except for the heart and ovary. In conclusion, while the Δ2 AS TERT variant is expressed in human cells, significant amounts of $\Delta2^-$ TERT transcripts with an uninterrupted second exon may be combined with other in-frame spliced TERT sequences to yield full-length and AS TERT proteins.

The Δ3p-12, Δ4-13, and Δ2 AS variants were detected in normal tissues and cells (BJ and WI38 cells) and in transformed telomerase-positive cell lines (HeLa and Namalwa). To determine whether these AS variants are also expressed in transformed cells which use ALT, we analyzed the U2OS osteosarcoma cell line (Fig. 2E). This transformed cell line does not express telomerase activity (9) (Fig. 2A). The AS variants (Δ3p-12, Δ4-13, and Δ2) and TERT transcripts containing the second exon ($\Delta2^-$) were expressed in these cells. However, the transcript potentially encoding full-length TERT ($\Delta\alpha^-\Delta\beta^-$) was not detected (data not shown). In conclusion, these results demonstrate that several AS variants are expressed in telomerase-positive as well as telomerase-negative cells.

The Δ4-13 variant enhances cell proliferation. Since the Δ4-13 variant is expressed in both telomerase-negative and -positive dividing cells in culture, we evaluated whether this variant may be involved in cell proliferation, one of the noncanonical functions of telomerase. Saos2 and U2OS cells stably expressing the Δ4-13 variant or Babe vector alone were established (Fig. 3A). Western blot analysis demonstrated the increased expression of the Δ4-13 protein in each of these cultures. Telomerase activity remained undetectable in the ALT cell lines Saos2 and U2OS expressing the Δ4-13 variant. The proliferation of these cell lines was compared to the proliferation of the parental cell lines expressing the vector alone. In both cell lines, the overexpression of Δ4-13 resulted in an increase in cell proliferation.

The proliferation of HeLa cells infected for a period of 10 days with a retrovirus expressing the Δ4-13 variant was also determined. HeLa cells which were selected to stably express the mCAT-1 receptor required for Babe retrovirus entry were infected with a Babe retrovirus expressing the Δ4-13 AS variant or left uninfected. The use of a Babe retrovirus expressing GFP in parallel experiments confirmed that all cells in these cultures were infected (data not shown). Western blot analyses confirmed that the Δ4-13 variant was expressed (Fig. 3B). Furthermore, telomerase activity did not increase in HeLa cells expressing the Δ4-13 variant. The proliferation rate was determined 10 days after infection of these cultures. Consistently, HeLa cells expressing the Δ4-13 variant showed a significantly enhanced rate of cell proliferation (50%) relative to that of control cells. These results confirmed that an increase in the expression of the Δ4-13 variant above endogenous levels normally found in transformed cell lines stimulates cell proliferation.

To demonstrate that endogenous levels of the Δ4-13 variant in human cells also enhance proliferative activity, siRNA technology was employed to specifically reduce the levels of the Δ4-13 transcript. The U2OS osteosarcoma cell line was used in these experiments because this cell line expresses the Δ4-13 variant and fails to express telomerase activity as determined by the TRAP assay (Fig. 2A). Two siRNAs (siΔ4-13 and siex3b) that specifically target the AS Δ4-13 variant were employed (Fig. 4A). siΔ4-13 targets only the Δ4-13 AS variant, because it was designed to recognize the junction between exons 3 and 14. The second siRNA (siex3b) recognizes a region in the 3rd exon, absent in the PTC-containing Δ3p-12 AS variant, which is expressed at levels similar to those of the Δ4-13 transcript in U2OS cells. Both siRNAs decreased the level of the AS Δ4-13 variant in U2OS cells without altering telomerase activity (Fig. 4B). We also analyzed the effects of expression of these siRNAs on the expression of the Δp3-12 and Δ2 AS variants. The Δp3-12 isoform is not targeted by these siRNAs and cannot exist together with the Δ4-13 variant. The expression of this AS variant did not change with siRNA treatment. The Δ2 variant was present at low levels in U2OS cells and could be present with the Δ4-13 alternative splicing event. Its levels increased slightly after treatment with siRNAs. However, since this variant contains a premature termination codon, its mRNA should be degraded during the first round of translation, so it is unlikely that the slight increase in expression was responsible for a decrease in cell proliferation. Finally, we analyzed the levels of $\Delta2^-$ TERT transcripts (containing the second exon). Control cells contained significant levels of TERT transcripts containing the second exon, and the levels of these transcripts decreased after treatment with both siRNAs. These results suggest that the majority of the Δ4-13 AS TERT in control cells has the second exon, and treatment of these cells with siRNA eliminates or reduces the frequency of these forms. Cell proliferation was significantly decreased (40 to 50%) by siRNAs that target the AS Δ4-13 variant, while the negative control (NC1) siRNA alone had no influence on proliferation (Fig. 4C). The siRNA treatments did not result in increased cell death. The transfection of both siRNAs, however, led to changes in the proportions of cells found in different stages of the cell cycle. The analysis of DNA content indicated that cells treated by siRNA had a reduced number of cells in S phase (by 1/3 to 1/2), resulting in a corresponding increase in the proportion of cells in G_1/G_0 phase (Table 1). A decrease of cell proliferation to an extent similar to that in U2OS cells was also observed in telomerase-negative normal BJ and WI38 fibroblasts and another ALT osteosarcoma cell line, Saos2 (data not shown). The results from these overexpression and knockdown experiments indicate that the Δ4-13 AS variant possesses the proliferative function of TERT in the absence of telomerase activity.

The Δ4-13 AS variant functions as an enhancer of Wnt signaling. The activation of the Wnt pathway has been correlated with the ability of the human TERT protein to stimulate cell proliferation (49). Both enzymatically active and inactive TERT pro-

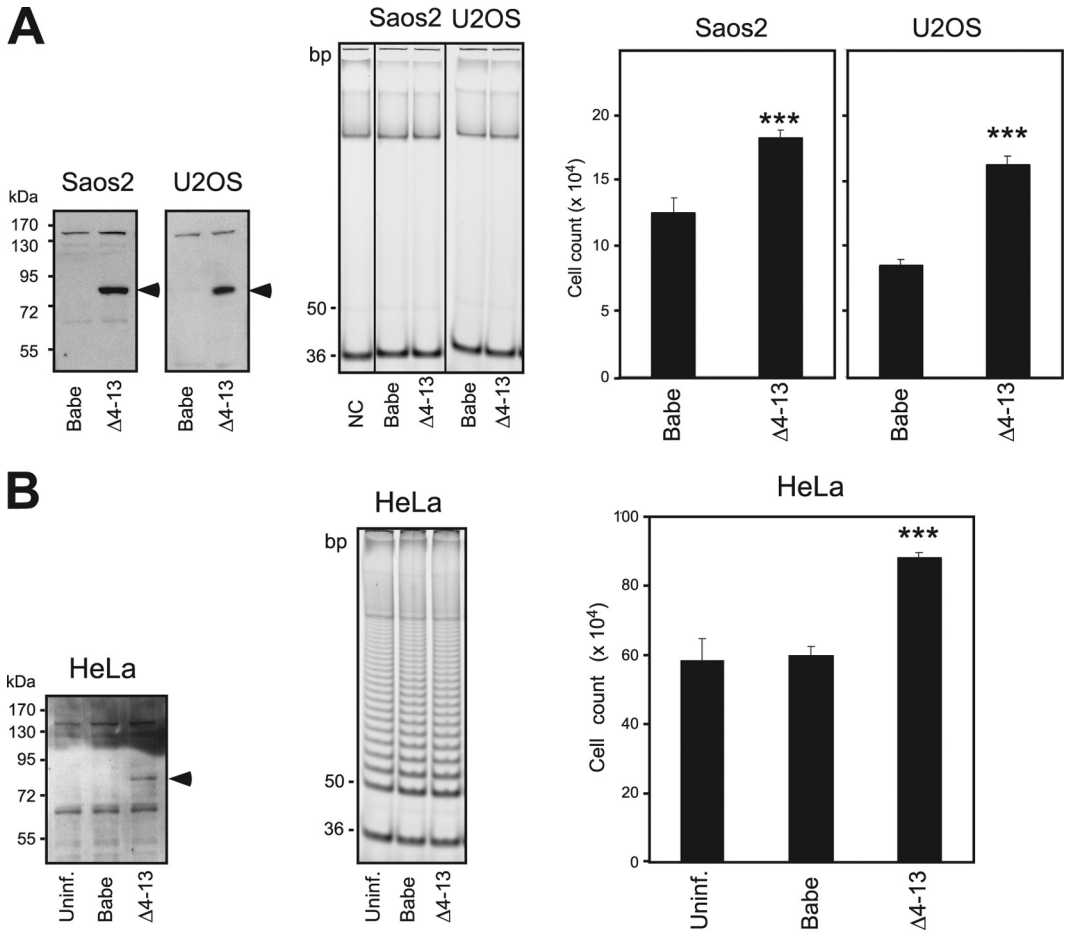

FIG 3 Overexpression of the Δ4-13 AS hTERT variant stimulates cell proliferation. (A) Saos2 and U2OS cells stably expressing the Δ4-13 protein or vector (Babe) were established by puromycin selection. Western blot analyses confirmed the overexpression of the 80-kDa Δ4-13 protein (arrowheads). The band with slower migration (>130 kDa) does not represent full-length TERT but is a cross-reacting protein. Telomerase activity was measured by TRAP assay as described in the legend to Fig. 2A. Cell proliferation was determined by plating cells (4×10^4 cells per ml) and counting them after 2 days. Means and standard errors were calculated from four independent experiments for each cell line. Statistically significant differences relative to negative controls are indicated. *P* values for differences were determined by two-tailed Student's *t* test (*, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$). (B) HeLa cells expressing the mCAT-1 receptor were infected with Babe retroviruses expressing Δ4-13 or with empty vector alone (Babe), or they were left uninfected. Western blot, TRAP, and proliferation analyses were performed 10 days after infection. The analyses were done as described above.

teins were able to enhance the activity of a Wnt signaling reporter construct in cells stimulated by the inactivation of the β-catenin-phosphorylating kinase glycogen synthase kinase 3 (GSK-3) by LiCl treatment (49). To test if the human TERT Δ4-13 variant had preserved the Wnt-stimulatory activities of full-length TERT, we cotransfected the Wnt signaling reporter Super 8× TOPFlash with plasmids expressing the Δ4-13 protein or full-length TERT or with a vector control. One day later, these cells were treated with LiCl, and the reporter activity was determined after an additional 24 h. In both telomerase-negative U2OS cells and telomerase-positive HeLa cells, the Δ4-13 variant significantly enhanced the LiCl induction of Wnt signaling (Fig. 5A). The enhancement was only half as strong as the effect of full-length TERT. Wnt signaling was also enhanced in LiCl-treated HeLa cells stably expressing the Δ4-13 variant (Fig. 5B). These results suggest that the Wnt pathway is involved in the stimulation of cell proliferation by the Δ4-13 variant.

Chicken AS TERT variants enhance cell proliferation. Chicken cells also express several AS TERT variants which retain the original ORFs (Fig. 1). While all of these AS variants retain an intact TEN domain and a segment of the CT domain, similar to the human Δ4-13 AS variant, they are structurally different. Experiments were performed to determine whether the most frequently expressed chicken AS TERT variants, A, B, and D, also contribute to cell proliferation. Variant A lacks most of the 2nd exon of TERT, variant B lacks the 5th exon, and the D variant has a small (36-nucleotide [nt]) insertion between the 7th and 8th exons. These variants and full-length chicken TERT were expressed from the REV-A-based retroviral vector. The experiments were performed with primary CEFs and the immortal DF1 fibroblast cell line. DF1 cells use the ALT method to maintain telomere length (26, 47, 56). Primary CEFs cultured for 5 days and DF1 cells express very-low-level or no telomerase activity. These cultures were infected with retroviruses expressing chTERT or an AS variant (A, B, or D), their expression was verified by RT-PCR, and telomerase activity was determined 2 days after infection (Fig. 6A and B). The exogenous expression of full-length TERT significantly increased telomerase activity, while TERT-D only moder-

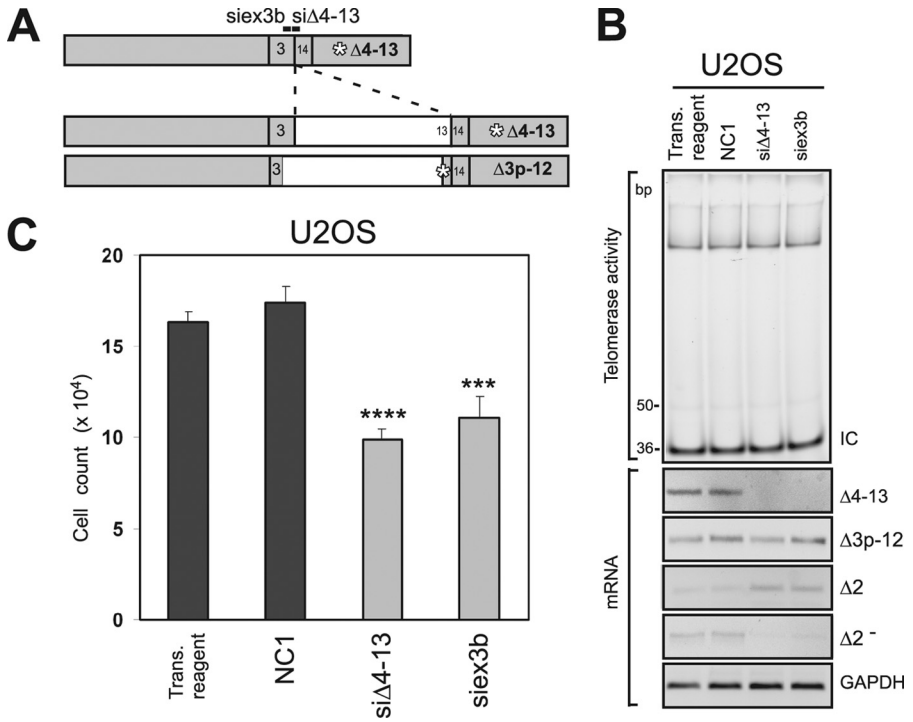

FIG 4 Suppression of AS hTERT variant Δ4-13 expression correlates with a reduction in cell proliferation. U2OS cells were transfected with siRNAs (1 nM) targeting the Δ4-13 variant (siΔ4-13 and siex3b), a negative-control siRNA (NC1), or transfection reagent only. (A) Schematic representation of the siRNA strategy. siRNA siΔ4-13 targets the junction between the 3rd and 14th exons. siRNA siex3b targets sequences which are present in the Δ4-13 variant as well as in full-length TERT but are deleted in the Δ3p-12 variant. (B) Telomerase activity was determined 48 h after siRNA transfection, as described in the legend to Fig. 2A. The levels of the Δ4-13, Δ3p-12, Δ2, and Δ2$^-$ AS TERT variants and GAPDH mRNA were analyzed by RT-PCR 48 h after transfection, as described in the legend to Fig. 2E. (C) Cell proliferation was determined 3 days after transfection, as described in the legend to Fig. 3. The percentage of dead cells determined by trypan blue dye exclusion was between 3 and 5% in all cultures.

ately enhanced the level of telomerase activity. Cells expressing the TERT variants A and B remained telomerase negative. In DF1 cells, the pattern of telomerase activity was similar to that of CEFs. The proliferation of cells expressing these variants was determined (Fig. 6C). The ectopic expression of TERT-A, TERT-D, and TERT enhanced cell proliferation (45 to 70%) in CEFs. Variants A and D also significantly increased proliferation of DF1 cells (50 to 70%), while the stimulatory effect of full-length TERT was only modest in these cells (20%). The overexpression of the TERT-B variant resulted in a slight (though not statistically significant) decrease of cell proliferation. These results suggest that like the human AS TERT Δ4-13 variant, the chicken A variant enhances cell proliferation in the absence of telomerase activity. The D variant, which retains very low levels of telomerase activity, also enhances cell proliferation.

TABLE 1 Cell cycle analysis of U2OS cells treated with anti-Δ4-13 siRNAs[a]

Reagent[b]	% of cells in cell cycle phase[c]			Ratio of cell percentages[d]	
	$G_1 + G_0$	S	$G_2 + M$	$G_1 + G_0$ to S	S to $G_2 + M$
Amine	80/80	16/15	4/5	4.9/5.5	4.0/2.7
NC1	80/76	19/16	4/8	4.2/4.7	4.9/2.0
siΔ4-13	86/84	13/12	5/4	6.8/7.2	2.4/2.6
siex3b	89/86	11/9	5/5	8.1/9.2	2.4/1.9

[a] For cell cycle analysis, cells were fixed, RNase treated, stained with propidium iodide, and analyzed by FACSCalibur flow cytometry.
[b] U2OS cells were treated with a transfection reagent control (amine), a negative-control siRNA (NC1), or two siRNAs (siΔ4-13 and siex3b) specific to the Δ4-13 AS TERT variant.
[c] Percentages of cells in different stages of the cell cycle were determined by FlowJo software using two models: the Watson pragmatic model (number before the slash) and the Dean-Jett-Fox model (number after the slash).
[d] Ratios of percentages of cells in $G_1 + G_0$ phases (or S phase) to percentages of cells in S phase (or $G_2 + M$ phases).

To confirm that the chicken variants enhance cell proliferation upon expression at endogenous levels, siRNA technology was employed (Fig. 7). CEFs and DF1 cells, as well as the fibroblast cell line 26TS, which has a more transformed phenotype, were used for this analysis. siRNAs targeting sequences specific for variants A, B, and D were transfected into CEFs and DF1 and 26TS cells (Fig. 7A). siRNA-A and -B were designed against sequences at the junctions created after deletion of part of the second exon and the fifth exon, respectively. siRNA-D targeted the region which was acquired by inclusion of a part of intron 7 in AS variant D. Reductions in the levels of mRNAs of the A, B, and D variants were confirmed by RT-PCR (Fig. 7B). Sequences of all of these variants can exist in the same mRNA transcript (for example, ABD, AB, BD, and AD variants); therefore, these siRNAs may decrease the levels of not only the AS variants containing the targeted single splicing event but also other AS variants. Cell proliferation and telomerase activity were determined 48 h after transfection (Fig. 7C and D). The proliferation of all cell types was inhibited 30 to 55% as a result of the reduction of the endogenous levels of these AS TERT variants. An increase in cell death was not observed. The

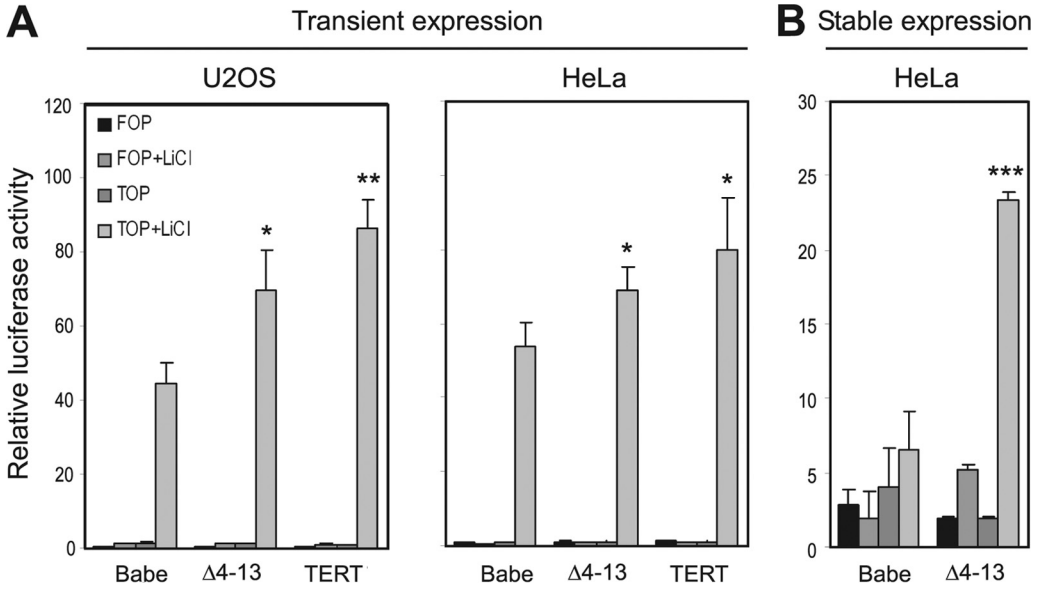

FIG 5 The AS hTERT variant Δ4-13 functions as an enhancer of Wnt signaling. (A) Transient expression of the Δ4-13 variant and full-length TERT (TERT) increases stimulation of Wnt signaling following LiCl treatment. U2OS and HeLa cells were cotransfected with the reporter plasmid Super 8× FOPFlash (FOP) or Super 8× TOPFlash (TOP), the pRL-TK coreporter, and plasmids expressing TERT proteins (pBabe-Δ4-13, pBabe-TERT, and vector control pBabe) and treated with 25 mM LiCl 1 day later. After an additional 24 h, cells were harvested and luciferase activity determined. The firefly luciferase fluorescence intensity of the reporters was normalized to the cotransfected coreporter *Renilla* luciferase activity. (B) HeLa cells stably expressing the Δ4-13 TERT variant are more responsive to stimulation of the Wnt pathway. Two cell strains, one stably expressing the pBabe vector control (Babe) and the other expressing Δ4-13 TERT (Δ4-13), were sequentially transfected with reporter plasmids and treated with LiCl, and luciferase activity was determined as in the transient-transfection experiments described for panel A. Means and standard deviations in both panels A and B were calculated from three independent experiments for each cell line. Statistically significant differences of LiCl-treated cultures transfected with Super 8× TOPFlash relative to the Babe vector control are indicated (*, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$).

greatest effect on proliferation was detected in CEFs, where proliferation was reduced by 50 to 55%. Reduction in the endogenous level of variant B had the least effect on cell proliferation. The negative effect of both overexpression and depletion of variant B suggests that its endogenous level is in the optimal range and that cell proliferation is not enhanced by increasing its expression. The results of the TRAP assay confirmed that the siRNAs specifically targeting chicken AS TERT variants did so without reducing telomerase activity. On the contrary, reducing the levels of variant B in CEFs and DF1 cells and of variant A in DF1 cells resulted in increases in telomerase activity (Fig. 7D). The decrease in the level of variant D in CEFs also increased telomerase activity, suggesting that despite its modest enzymatic activity, the overall effect of this variant is also inhibitory. These observations suggest that the AS TERT variants normally inhibit endogenous telomerase activity in cells. No changes in telomerase activity were observed in transformed 26TS cells.

To confirm the specificity of the siRNA effect, experiments were performed to demonstrate that the expression of the most ubiquitous chicken AS variant (variant A) is able to rescue the negative effect of the reduction of endogenous levels of this variant on cell proliferation (Fig. 7D). DF1, an ALT cell line, was infected with a retrovirus vector expressing a variant A sequence which was mutated to prevent its recognition by siRNA targeting wild-type variant A, as well as with the control helper virus. Two days after infection, cells were transfected with the siRNA against AS variant A and with controls. The results demonstrate that all cells overexpressing AS variant A proliferated more than control cells, indicating that the expression of AS variant A is able to compensate for the decrease of proliferation by siRNA targeting variant A. In conclusion, these results confirm that the chicken variants possess extratelomeric functions, similar to the human Δ4-13 variant, even though they are not structurally identical.

DISCUSSION

Evolution of AS variants. In many species of Metazoa, TERT transcripts are alternatively spliced. Several AS TERT variants have been reported in a number of species, including human, rat, dog, chicken, zebrafish, and plants (63). In the case of humans and chickens, which have been studied extensively, 21 and 37 single-event AS TERT variants have been identified (see references 2, 11, 27, 31, 52, and 73 and the data described here). All of the regions within the TERT gene are involved in alternative splicing in both species, and deletions involving every exon except the first exon have been described. Interestingly, none of the human and chicken AS variants are identical, suggesting that AS TERT variants evolved independently in humans and chickens. There are two possible explanations for this striking difference in AS variant repertoire between these two species. First, splice sites may have evolved slowly, allowing the disappearance of some AS variants and the simultaneous appearance of others, thereby maintaining the number of AS TERT variants constant in different species. The second possibility is that the number of AS TERT forms was drastically reduced in some species as a result of the elimination or modification of splice sites. In new species that originated from such predecessors, novel AS variants would have evolved by the introduction of new splice sites creating structurally different AS variants with similar functions. We recently exhaustively analyzed

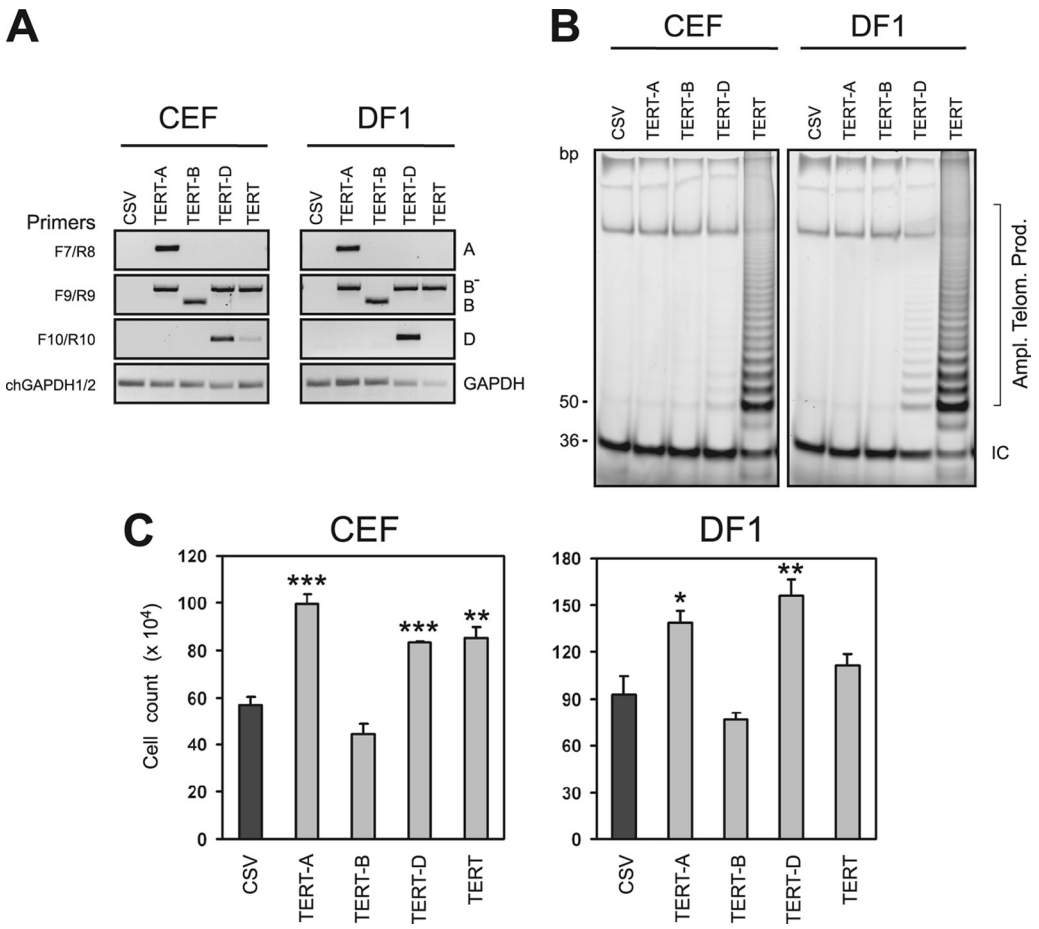

FIG 6 The ectopic expression of chicken AS TERT variants A and D stimulates cell proliferation. CEFs and DF1 cells were infected with retroviruses expressing full-length chicken TERT, one of its AS variants (A, B, or D), or a CSV empty vector. Cells were harvested for RNA, TRAP, and proliferation analyses 48 h after infection. (A) The overexpression of TERT and its AS variants A, B, and D was confirmed by RT-PCR. (B) TRAP assays were performed as described in the legend to Fig. 2. Data for the heat-treated control for this experiment are provided in the supplemental material. (C) Cell proliferation was determined as described in the legend to Fig. 3.

alternative splicing of TERT in the platypus and found only a few AS variants (30). Therefore, the second scenario is more likely, as some vertebrate species express only a limited number of AS variants.

Although human and chicken AS TERT variants are not identical, it is plausible that some of these variants may serve similar functions. There are structural and functional similarities between the human Δ4-13 variant and the chicken A variant. These variants lack very large though not overlapping fragments of the central region of TERT (1,263 and 1,359 bp, respectively), which encodes the domains essential for catalytic function (75). In the chicken A variant, most of the TR binding domain (TRBD) is lost, while in the human Δ4-13 isoform, the entire catalytic (RT) domain has been deleted. The Δ4-13 and chicken A variants do not encode telomerase activity, but both stimulate cell proliferation. Recently, we identified a TERT isoform similar to the chicken TERT A variant in the platypus (30). The conservation of AS TERT forms with deletions in the central region further suggests that their function has been retained in evolution.

Function of AS TERT variants. AS variants can be divided into two groups—the first group contains PTCs, while the second group of transcripts retains the ORFs. The RNAs of AS variants with PTCs are predicted to be degraded by NMD (40). Nevertheless, two-thirds of the human and chicken AS TERT variants are spliced out of frame, and their RNA levels are similar to those of AS variants with the original ORFs, suggesting that at least some of these TERT AS variants escape NMD and may provide an important, though unrecognized, function. The AS variants with the original ORFs can be translated and contribute to proteome diversification (45). A frequent AS hTERT variant with a small deletion in the reverse transcriptase domain (Δα) lacks telomerase activity and functions as a dominant-negative mutant (13, 77). The results presented in this report indicate another extratelomeric function of AS TERT variants. Both human and chicken AS TERT variants which contain extensive internal deletions possess proliferative function in the absence of telomerase activity. These results are in agreement with findings that full-length TERT enhances cell proliferation independent of its telomerase activity (12, 55) and demonstrate that naturally occurring AS TERT variants provide this function.

In both species, several other variants with ORFs were identified (Fig. 1), such as the human variants Δγ, Δ4C, i2(182-349), and i14(623-703). The structure of the majority of these AS variants suggests either that they lack telomerase activity or that the activity is

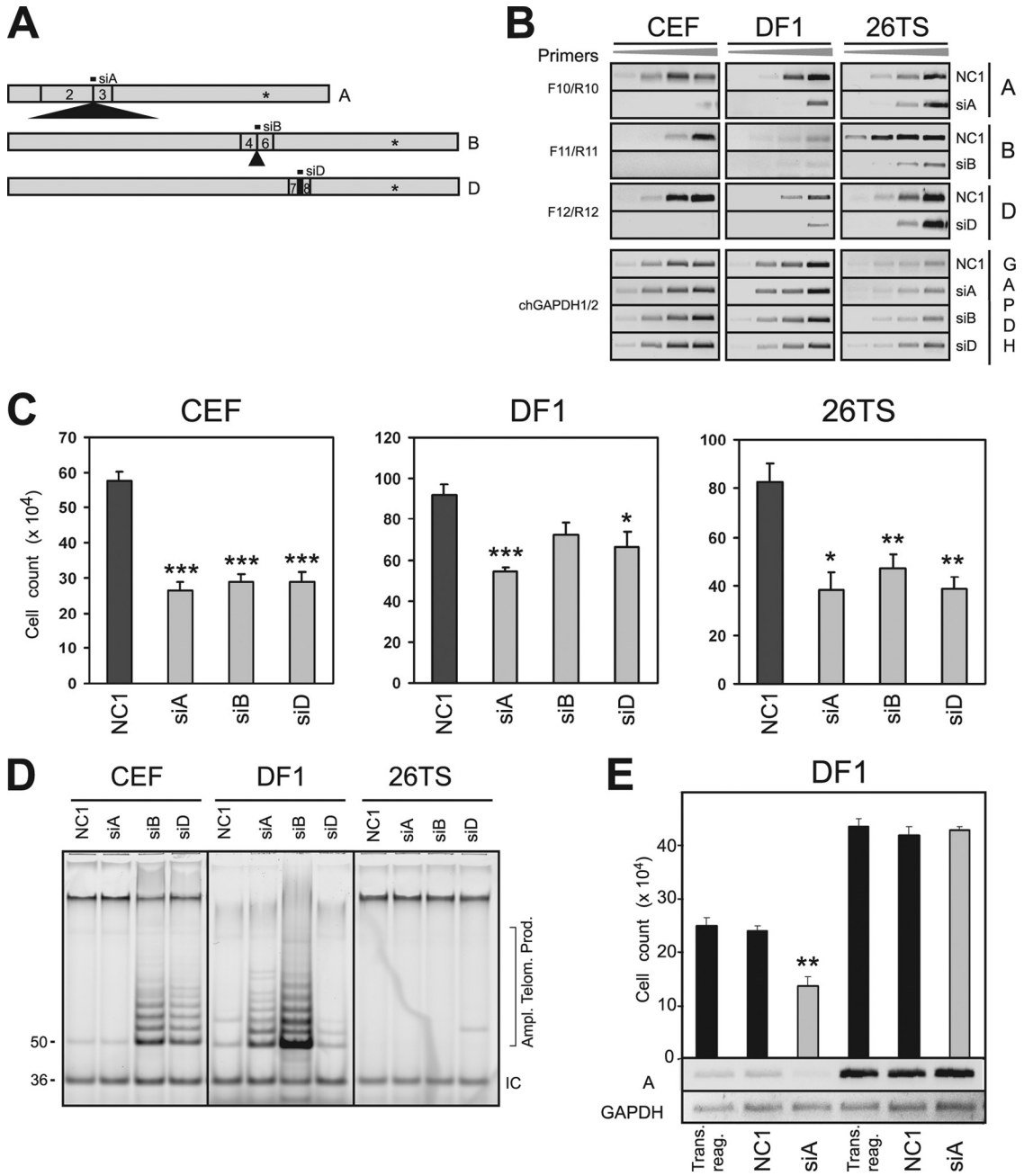

FIG 7 Inhibition of chicken AS TERT variants correlates with a reduction in cell proliferation. CEF, DF1, and 26TS cells were transfected with siRNAs targeting chicken AS TERT variants or with negative-control siRNA (NC1). (A) Schematic representation of the siRNA strategy. siA and siB siRNAs targeted sequences at the novel junctions where deletions occurred. siD siRNA targeted unique sequences found within the cassette insertion. (B) RNAs were harvested at 24 h. The levels of the A, B, and D variants and GAPDH mRNA were analyzed by RT-PCR. (C) Cell proliferation was determined as described in the legend to Fig. 3. The percentage of dead cells determined by trypan blue dye exclusion was 3% for CEF and DF1 cultures and 6% for cultures of 26TS cells. (D) Telomerase activity was determined 24 h after siRNA transfection, as described in the legend to Fig. 2. (E) DF1 cells were infected with REV-TERT-A^r (lanes 3 to 6) or the CSV helper control (lanes 1 to 3). Two days after infection, cells were transfected with siRNA siA or with the negative control, and the number of cells was determined 48 h after transfection, as described in the legend to Fig. 2. The overexpression of REV-TERT-A^r and the reduction of endogenous levels of AS variant A were determined as described above and in the legend to Fig. 6.

severely reduced, though they still may possess extratelomeric functions. In cells, AS variants retaining an original TERT ORF are expressed together, and often simultaneously with different levels of full-length TERT, which possesses telomerase activity. Therefore, it is likely that their relative levels and their interactions (cooperation and competition) influence the final biological outcome. The expression of some TERT AS variants is ubiquitous, while others are cell type specific (27, 34, 52). Therefore, the large number of AS TERT variants with extratelomeric functions may permit a differential regulation of telomerase functions during cell differentiation.

Mechanism of enhanced cell proliferation by TERT AS variants. The stimulation of proliferation by TERT is independent of

telomerase activity. In mice lacking the TR gene, the structural element that provides the template for telomere extension, the conditional expression of TERT was able to promote the growth of cells in hair follicles (12, 55). TERT interacts with BRG1, a chromatin remodeling protein, and physically occupies promoters of Wnt-dependent genes, suggesting that TERT may function as a direct transcriptional regulator of gene expression. These activities do not require a catalytic telomerase function (49). Interestingly, the Δ4-13 TERT AS variant was capable of enhancing the activity of a Wnt signaling reporter in cells stimulated by LiCl. However, this enhancement was only half as strong as the effect of full-length TERT, indicating that these activities are negatively influenced by the large internal deletion and do not directly correlate with the strength of the proliferation effect. These results suggest that while the Wnt pathway may be involved in the stimulation of cell proliferation by the Δ4-13 variant, the activation of additional pathways controlling cell division is likely to play an important role.

The decrease in the proportion of cells found in the S phase of the cell cycle and the increase in cell number in G_1/G_0 after treatment with siRNA against the Δ4-13 variant suggest that this variant may function by facilitating cell transit through the G_1/S checkpoint. This observation is consistent with reports of increased cell growth through the release of cells from the G_1/S checkpoint for human cytotrophoblast and lens epithelial cells expressing an exogenous *TERT* gene (72, 76). These results suggest that some catalytically inactive AS variants may preserve the cell cycle regulatory functions of full-length TERT.

It is not clear which regions are responsible for the proliferation activity of TERT. Our results suggest that the TEN domain and a segment of the CT domain, which are involved in DNA binding, are likely required for proliferative activity, as they are retained in both the human Δ4-13 variant and the chicken A AS TERT variant. In contrast, the RT and TRBD regions, which are deleted in these variants, are dispensable for the telomerase proliferative activity. It is not known whether a single variant with both deletions combined would still possess proliferative activity, because there may be a specific length requirement for the linker between the TEN and CT domains to retain this function of TERT. The extended deletions in the Δ4-13 and chicken A variants may cause the differences in biological characteristics between these AS variants and variants with only a small deletion in the RT domain. The Δα AS variant, with a small deletion, does not enhance cell proliferation and strongly inhibits telomerase activity (13, 77). The extended deletions likely result in modifications of the tertiary structure that decrease the inhibitory effect of this variant on full-length TERT and ultimately unmask its cell proliferation function. The mechanism involved in the stimulation of cell proliferation by the Δ4-13 variant is not species specific, as the ectopic expression of a chicken construct which structurally corresponds to the human Δ4-13 variant stimulated proliferation of chicken fibroblasts (data not shown).

Role of AS TERT variants in human disease. Telomerase activity is downregulated during cell differentiation, likely as a protection against cellular transformation and tumor development (20, 22, 33, 35, 51, 64, 70). However, since TERT also possesses extratelomeric cellular functions which might play an important role in normal cells, the complete reduction of TERT transcription would eliminate these functions. The severe repression of TERT transcription might have a negative effect on cell proliferation and lead to premature cell senescence. We hypothesize that the AS TERT variants that lack telomerase activity may solve this problem by providing extratelomeric functions without increasing the probability of cell transformation. The deregulation of the expression of AS TERT variants would likely have several consequences for human health.

The telomerase-negative osteosarcoma ALT cell line U2OS expresses the Δ4-13 variant. Several cancer types, especially those of mesenchymal origin (such as osteosarcoma, sarcoma, and liposarcoma), use ALT for maintenance of telomere length (25, 54, 68). These tumors may or may not retain telomerase activity. In some ALT cell lines lacking telomerase activity, the failure to express the Δα, Δβ, ΔαΔβ, and $\Delta\alpha^{-}\Delta\beta^{-}$ (wild type in the RT region) variants correlates with specific chromatin modification of the hTERT promoter (3). This is consistent with the fact that alternative splicing is regulated by histone modification (41). It is plausible that chromatin remodeling contributes to the changes in the alternative splicing pattern of hTERT in human telomerase-negative ALT tumors, resulting in the preferential splicing of hTERT to the AS Δ4-13 variant. It has been reported that telomerase also contributes to tumorigenesis by a telomere length-independent mechanism (5, 62). Though the Δ4-13 AS TERT variant may not provide cells with the indefinite growth potential necessary for tumor development seen with full-length TERT, it may still promote the growth of cancer cells. Moreover, the Δ4-13 variant is also coexpressed together with full-length TERT in transformed cells and therefore may contribute to the proliferation ability of certain cancers. The screening for TERT AS variants and their intentional targeting in addition to that of wild-type TERT may improve the treatment for certain types of cancer.

Reduction of telomerase activity is associated with the onset of diseases characteristic of replicative senescence (for a review, see references 18, 53, and 59). The induction of telomerase activity in late-generation mice resulted in the reversion of this senescence (32). Though the phenotype of proliferative senescence is associated principally with telomere attrition, TERT also promotes cellular and organismal survival independent of telomerase activity (38, 39, 46). Therefore, telomerase noncanonical functions associated with expression of AS TERT variants may play a role in protection against organ deterioration, with a reduced risk of cancer development compared to that with the expression of full-length TERT.

In conclusion, the alternative splicing of TERT mRNA transcripts is a mechanism which decreases the level of telomerase activity, but AS TERT variants may provide at least some of the noncanonical functions of TERT.

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