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Review Article

Telomerase reverse transcriptase moonlights: Therapeutic targets beyond telomerase

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Telomeres, the repetitive sequences at chromosomal ends, protect intact chromosomes. Telomeres progressively shorten through successive rounds of cell divisions, and critically shortened telomeres trigger senescence and apoptosis. The enzyme that elongates telomeres and maintains their structure is known as telomerase. The catalytic subunit of this enzyme (telomerase reverse transcriptase [TERT]) is expressed at a high level in malignant cells, but at a very low level in normal cells. Although telomerase activity was long believed to be the only function of TERT, emerging evidence indicates that TERT plays roles beyond telomeres. For example, TERT contributes to stem cell maintenance and cell reprogramming processes in a manner independent of its canonical function. Even some types of splice variants that lack the telomerase catalytic domains exhibit the functions in a manner that does not depend on telomerase activity. We recently demonstrated that the RNA-dependent RNA polymerase (RdRP) activity of TERT is involved in regulation of gene silencing and heterochromatic transcription. Moreover, TERT RdRP activity is mediated by a newly identified complex, distinct from the authentic telomerase complex, that plays a role in cancer stem cells in a telomere maintenance independent manner. TERT has attracted interest as a molecular target for anticancer treatment, but previous efforts aimed at developing novel therapeutic strategies focused only on the canonical function of TERT. However, accumulating evidence about the non-canonical functions of TERT led us to speculate that the functions other than telomerase might be therapeutic targets as well. In this review, we discuss the non-canonical functions of TERT and their potential applications for anticancer treatment.

History of Telomerase Reverse Transcriptase Research

Human telomerase was identified at the end of the 1980s.[1] In the 1990s, rapid progress in this field revealed the biological significance of this enzyme, especially in cancers. The minimum essential components of telomerase are the catalytic subunit, telomerase reverse transcriptase (TERT), and a non-coding RNA (*TERC*);[2] TERT reverse transcribes telomere DNA using *TERC* as the template. Development of the telomeric repeat amplification protocol (TRAP),[3] a PCR-based assay for assessing telomerase activity, and cloning of human TERT[4] and *TERC*[5] paved the way for investigations of expression patterns of telomerase and its components in both cell lines and clinical samples. These studies revealed that telomerase is activated in malignant cells,[3,4] and that telomerase activation in cancers is closely related to acquired expression of TERT.[4] The significance of TERT in tumor biology has led to many efforts to develop anticancer therapies that target telomerase. In addition, identification of the *TERT* promoter region and subsequent studies on its transcriptional regulation have also led to the development of tumor-specific gene expression systems using *TERT* promoter activity.[6,7] However, despite tremendous efforts over the past two decades, the results of anti-neoplastic strategies targeting authentic telomerase function(s) have been disappointing, and no such approach has attained clinical approval.

Recent genome-wide studies of clinical samples have once again highlighted the importance of TERT in cancers. These studies have revealed that cancer-associated single-nucleotide polymorphisms (SNP) in the *TERT* gene,[8] as well as frequent *TERT* promoter mutations in some types of tumors, including melanoma, malignant glioma, hepatocellular carcinoma and urothelial carcinoma, upregulate transcription of *TERT* (Fig. 1).[9–15] These findings demonstrate that *TERT* is one of the most clinically important driver genes in many types of cancers, and suggest that tumors with high levels of TERT would be the optimal systems in which to validate the potential utility of TERT-based anticancer strategies. Although most previous research on TERT has focused on its telomerase activity, recent studies suggest that TERT has functions unrelated to telomere maintenance. To date, many groups have engaged in the development of telomerase inhibitors for use in

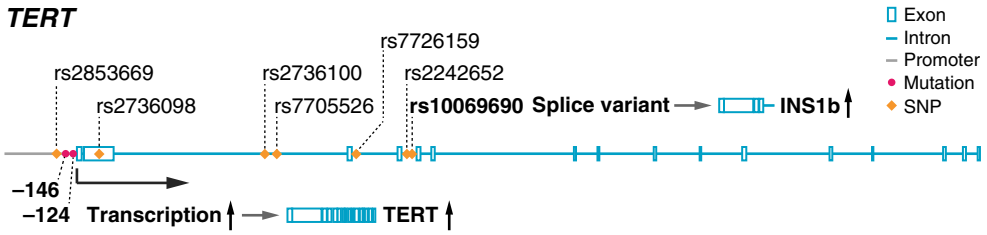

Fig. 1. Mutations and cancer-associated single-nucleotide polymorphisms (SNP) at the *TERT* locus. *TERT* promoter mutations located at −124 bp and −146 bp from ATG translation start site generate new binding motifs for Ets transcriptional factors and upregulate *TERT* expression. Cancer-associated SNP are mapped on the *TERT* gene. rs10069690 gives rise to aberrant splicing.

anticancer therapies, based on the notion that telomerase inhibition will lead to telomere shortening and eventual cell death. In contrast, we suggest that inhibitors targeting the non-canonical function(s) of TERT would facilitate the development of novel cancer treatments. In this review, we focus on the non-canonical functions of TERT in tumor biology, which represent new and promising molecular targets for cancer therapy.

Splice Variants of Telomerase Reverse Transcriptase without Telomerase Activity

Human *TERT* is encoded by 16 exons: the RNA-binding domain (RBD) is located in exons 2–4, and reverse transcriptase (RT) motifs are located in exons 4–11. To date, more than 20 types of differently spliced variants of human *TERT* have been reported, and emerging evidence reveals that some spliced variants lacking RT activity have functions in a manner independent from telomerase activity.

Genome-wide association studies (GWAS) identified numerous cancer-associated SNP in human *TERT* (Fig. 1). One of these, rs10069690, is located in intron 4 and has a major (G) and minor (A) allele.[16] The A allele has been studied as a candidate causal variant in a variety of malignancies.[16,17] Killedar *et al.*[17] report that the A allele creates an alternative splice donor site in intron 4, leading to expression of an alternatively spliced variant (INS1b). INS1b has a premature stop codon in the retained intron 4, resulting in truncation of the RT motifs. INS1b interferes with telomerase activity of full-length TERT through competitive binding to *TERC*, thus acting in a dominant-negative manner. Consequently, high levels of INS1b expression result in telomere shortening and elevation of the telomere damage response.

β-deletion is a variant that lacks exon 7 and 8. Exon 6 of β-deletion is directly fused to exon 9, resulting in a frameshift that creates a premature termination codon in exon 10.[18] β-deletion mRNA is expressed in a variety of cell types, including both normal and malignant cells.[19] The protein translated from β-deletion mRNA does not possess telomerase activity, although it retains the RBD and efficiently binds *TERC*; as expected, the β-deletion can act as a dominant-negative inhibitor of telomerase.[20] Listerman *et al.*[20] quantitated β-deletion mRNA in a series of human breast cell lines (45 breast cancer and five non-malignant breast cell lines) and found β-deletion transcripts in all cells tested, with an abundance in the range of 21–79% relative total TERT mRNA. The levels of β-deletion mRNA were correlated negatively with telomerase activity, but were not correlated with telomere length. These results indicate that telomere length is not regulated by the TERT splice variants in these cell lines; instead, the β-deletion protein protects breast cancer cell lines from cisplatin-induced apoptosis. Regarding the anti-apoptotic mechanisms of this variant, the authors discussed possible interactions between the β-deletion and apoptotic pathways at the level of the mitochondria and/or DNA repair systems.

Another variant, Δ4–13, maintains the original reading frame but contains a deletion of exons 4–13, and, therefore, lacks telomerase activity.[21] Δ4–13 is expressed in both normal and malignant cells.[21] Forced expression of Δ4–13 in telomerase-positive or telomerase-negative malignant cell lines promotes cell proliferation, whereas suppression of Δ4–13 decreases proliferation of telomerase-negative malignant cells and telomerase-negative fibroblasts.[21] These results indicate that the Δ4–13 variant has a telomerase-independent function related to proliferation. Activation of the Wnt signaling pathway by the Δ4–13 variant has been proposed as an underlying mechanism of this function.[21]

Wild-type TERT and its variants are expressed simultaneously, and maintain the balance between the canonical and non-canonical functions of TERT. Alterations in expression patterns of wild-type and variant TERT in tumor tissues have been reported in breast,[22] lung[23] and thyroid[24] tumors. Ongoing structural and functional analyses are clarifying the specific characteristics of each variant. Some of the variant-specific characteristics represent potential targets for the development of new drugs that specifically inhibit wild-type or variant TERT in order to restore the healthy balance between protein functions.

Non-Canonical Functions of Telomerase Reverse Transcriptase in Stem Cells and Cancers

Normal stem cells and cancer stem cells (also called "tumor-initiating cells") share several characteristics: both have self-renewal and differentiation capacity, and telomerase activity sustains their prolonged life spans. Acquired TERT expression and telomerase activity have also been observed in induced pluripotent stem (iPS) cells generated by reprogramming human somatic cells.[25] Although it is certain that telomere maintenance by TERT is critical for the stem cell phenotype, recent evidence indicates that TERT also has non-canonical functions in stem cells. Kinoshita *et al.* report the generation of iPS cells from fibroblasts of TERT-knockout (KO) mice. Although the efficiency of reprogramming was lower in TERT-KO fibroblasts than in wild-type fibroblasts, it was restored by the introduction of an enzymatically inactive mutant of TERT.[26] These findings suggest that TERT functions independent from telomerase (RT) activity are involved in the reprogramming process. Moreover, in human cancer cell lines, we found that TERT physically interacts with BRG1, a SWI/SNF-related chromatin remodeling protein, and nucleostemin, a nucleolar GTP-binding protein; the TERT-BRG1-nucleostemin (TBN) complex contributes to maintenance of

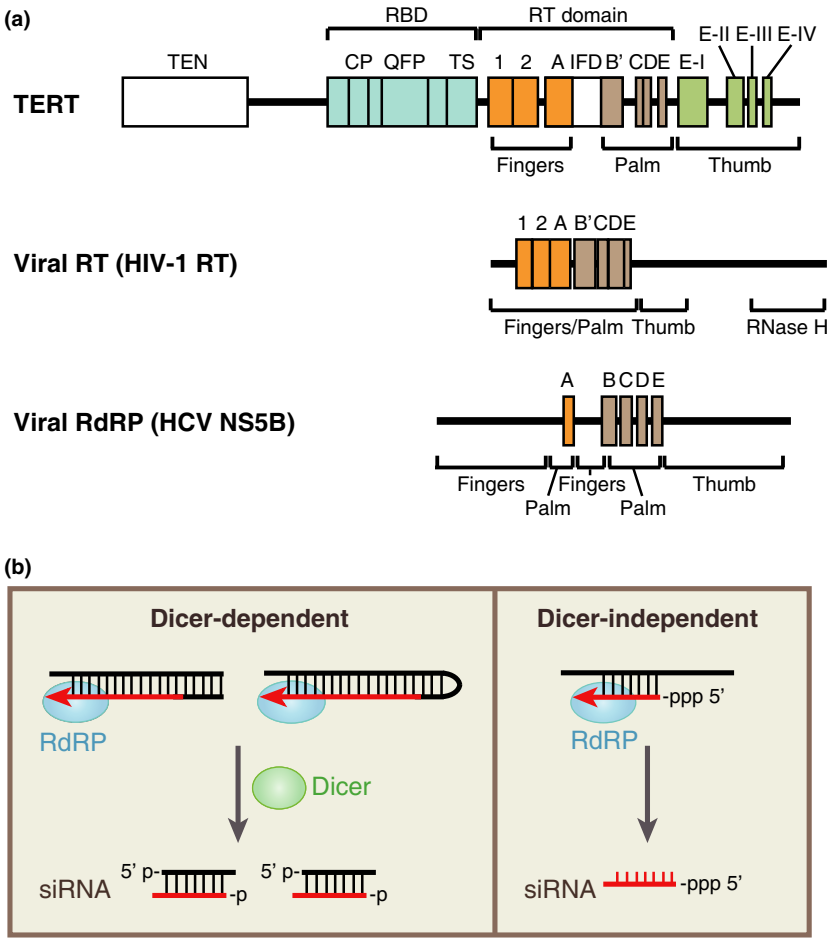

Fig. 2. Structures and functional modes of RNA-dependent RNA polymerase (RdRP). (a) Structure of human TERT, viral reverse transcriptase (HIV-1 RT) and viral RdRP (HCV NS5B). Human TERT has the RNA-binding domain (RBD) and the catalytic reverse transcriptase (RT) domain. The RBD contains the telomere-specific motifs CP, QFP and TS. The RT domain consists of seven evolutionarily conserved motifs (1, 2, A, B′, C, D, and E) and the insertion in fingers domain (IFD). The motifs (A, B′ or B, C, D and E) and the "right-hand" structure are shared across all three polymerases. TEN, telomerase essential N-terminal domain. (b) Dicer-dependent and -independent generation of siRNA by RdRP. In Dicer-dependent siRNA synthesis, long double-stranded RNAs synthesized by RdRP are cleaved into siRNAs by Dicer. In another mode, RdRP can directly synthesize siRNAs *de novo*, independently of Dicer.

tumor-initiating cell phenotypes in a manner independent of the telomerase enzyme complex.[27]

Telomerase reverse transcriptase overexpression in transgenic mice promotes proliferation of epidermal stem cells.[28,29] This effect is independent of *TERC*[28] and telomere elongation,[29] suggesting that non-canonical functions of TERT are involved in stem cell biology. An analysis showed that acute induction of TERT in mouse skin activates a transcriptional program very similar to those regulated by Wnt and Myc, two pathways essential for stem cell as well as tumor biology.[30] The same group showed that TERT directly regulates expression of Wnt/β-catenin target genes, including *Myc*, through physical association with their promoters in complex with BRG1.[31] Another study showed that in human gastric cancer cells, TERT and β-catenin physically interact, and TERT mediates transcriptional induction of β-catenin target genes;[32] although physical association between TERT, BRG1 and β-catenin, and downstream effects of TERT on Wnt target genes remain somewhat controversial.[33] c-Myc, a Wnt/β-catenin target gene, upregulates TERT expression by direct binding to the *TERT* promoter. Intriguingly, β-catenin itself activates transcription of *TERT* in cooperation with KLF4 in both a mouse intestinal tumor model and human cancer cell lines.[34] In addition, TERT stabilizes MYC in cancer cells and contributes to either activation or repression of MYC target genes.[35,36] The interdependence between TERT and these signaling pathways suggests that molecules that interfere with the activation of the Wnt/β-catenin pathway and/or MYC by TERT could serve as anticancer drugs.

Novel Enzymatic Activity of Telomerase Reverse Transcriptase

Telomerase reverse transcriptase elongates telomeres through its RNA-dependent DNA polymerase (i.e. reverse transcriptase) activity. Since the discovery of TERT protein, DNA polymerase activity was believed to be its only enzymatic activity. Recently, however, our group identified another polymerase activity of TERT, RNA-dependent RNA polymerase (RdRP).[37] RdRP catalyzes synthesis of an RNA strand complementary to a template RNA. RdRP was first identified in RNA viruses; viral RdRPs replicate and transcribe RNA genomes during the viral life cycle. RdRPs have been identified in eukaryotes, including plants, fungi and nematodes, but until recently it remained unclear whether mammals have RdRPs.

RNA silencing is a sequence-specific gene-regulatory system involved in a variety of physiological and pathological molecular processes, and double-stranded RNA (dsRNA) synthesis by eukaryotic cellular RdRPs is a fundamental step in this conserved mechanism. The identification of RdRPs as part of the RNA interference machinery in model organisms has intensified the debate about mammalian RdRPs, supported by findings that mammals produce dsRNA that is diced into small interfering RNAs (siRNAs).[38,39] Viral and cellular RdRPs bear little sequence and structural similarity. The crystal structures of viral RdRPs are similar to those of retroviral reverse transcriptases; they have a characteristic closed "right-hand" structure, including thumb, palm and fingers domains.[40] By contrast, cellular RdRPs are "double-barrel" polymerases with

the catalytic double-psi β-barrel (DPBB) domain.[41] Although mammalian homologs of cellular RdRPs have not been identified, phylogenetic and structural analyses revealed that TERT is a right-hand-shaped polymerase that is closely related to RdRPs of RNA viruses as well as retroviral reverse transcriptases (Fig. 2a).[42,43] Therefore, it seems reasonable that TERT would possess RdRP activity as well as reverse transcriptase activity.

Double-stranded RNA synthesis by RdRP induces transcriptional and post-transcriptional gene silencing (PTGS) in eukaryotic cells. In PTGS, dsRNA formed by RdRP is processed into siRNAs in a Dicer-dependent or Dicer-independent manner (Fig. 2b). These siRNAs bind to target mRNAs with complementary sequences and decrease target expression by promoting cleavage or inhibiting translation of their targets. Induction of PTGS from an endogenous non-coding RNA by human TERT has been demonstrated in cancer cells.[37] Dicer-independent siRNA generation occurs in model organisms, in which RdRP synthesizes siRNAs *de novo* (Fig. 2b).[44,45] Because viral RdRPs synthesize RNAs *de novo* (primer-independent manner) as well as in a primer-dependent manner, it is likely that TERT has the potential to perform primer-independent as well as primer-dependent RNA synthesis.[37] If so, TERT could regulate RNA silencing in human cancer cells in a Dicer-dependent and Dicer-independent manner.

In the fission yeast *Schizosaccharomyces pombe*, RdRP mediates pericentromeric heterochromatin formation. The RdRP generates dsRNA, which is processed into siRNAs, using nascent transcripts from the pericentromeric region as templates. The resultant siRNA guides the RNA-induced transcriptional silencing (RITS) complex to the region, and recruits a protein complex that mediates histone H3K9 methylation and heterochromatin formation.[46] During this process, RdRP interacts with a helicase and a nucleotidyltransferase to form the RNA-dependent RNA polymerase complex (RDRC). An RDRC-like complex is also found in *Caenorhabditis elegans*.[47] Loss of components of RDRC results in derepression of centromeric transcription,[46] and chromosomal mis-segregation in mitosis.[46,47] Therefore, RdRP is important for proper chromosomal segregation and mitotic progression in these model organisms. Human TERT also interacts with the helicase BRG1 and nucleostemin[27] to form the TBN complex, which, like RDRC complex, exerts RdRP activity.[48] Intriguingly, both expression and association of the TBN complex is enriched in mitotic cells, and it silences heterochromatic transcription from centromeres and transposons.[48] Moreover, suppression of the TBN complex increases the transcripts from heterochromatic regions and the proportions of binucleate cells, and cells arrested in mitosis, indicating that the TBN complex regulates mitotic progression via maintenance of heterochromatic status[48] (Fig. 3) by a similar mechanism found in model organisms. Because the TBN complex is directly involved in RdRP activity as well as maintenance of cancer stem cell traits, as described above,[27] we speculate that the TBN complex might be important for cancer stem cell maintenance by using its RdRP activity, with the detailed mechanism yet to be elucidated. Because cancer stem cells are intimately involved in tumor recurrence, metastasis and drug resistance, an anticancer strategy targeting the TBN complex, and, thus, its RdRP activity, might induce dysfunction of cancer stem cells and lead to complete tumor regression.

Canonical and Non-Canonical Functions of Telomerase Reverse Transcriptase as Targets of Cancer Therapy

Telomerase is an attractive molecular target for cancer therapy because it is expressed at high levels in most cancers but at very low levels in normal somatic cells, and is indispensable for immortality. Various approaches have been proposed to inhibit telomerase function, and some of them are in clinical trials (Table 1). BIBR1532 is a candidate small molecule that selectively interferes with the processivity of telomerase;[49] specifically, it prevents telomerase from forming long TTAGGG repeat-products.[50] Telomerase inhibition and telomere shortening upon BIBR1532 treatment have been confirmed in malignant cell lines.[51–54] Despite a considerable number of studies demonstrating the anticancer effects of BIBR1532 both *in vitro* and *in vivo*, the compound is still in preclinical evaluation. GRN163L (imetelstat) is a lipid-modified 13-mer oligonucleotide complementary to the template region of *TERC*.[55] GRN163L interacts with *TERC* and prevents telomerase from accessing telomeres, resulting in telomere shortening as well as telomerase inhibition, in many types of cancers.[56] GRN163L treatment also induces telomere length-independent effects, including reduction of adhesion properties,[57,58] colony formation capacity,[59–61] invasive potential[60] and tumorigenicity,[61,62] as well as sensitization to anticancer drugs.[57,63] A number of phase I/II clinical trials involving GRN163L are ongoing. Another compound, telomestatin, is a G-quadruplex ligand that interacts and stabilizes

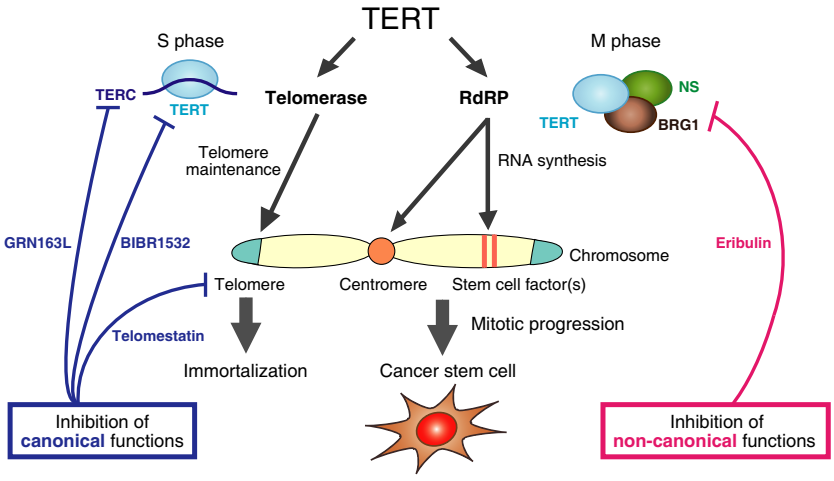

Fig. 3. Dual polymerase activities of telomerase reverse transcriptase (TERT) as targets of anticancer therapies. TERT exerts dual polymerase activities: telomerase and RNA-dependent RNA polymerase (RdRP). As telomerase, TERT maintains telomere structure and contributes to cellular immortalization. By contrast, the RdRP activity of TERT mediates RNA synthesis and heterochromatin maintenance, and regulates mitotic progression and cancer stem cell traits. Both the telomerase activity and the RdRP activity of TERT are promising molecular targets for anticancer therapies.

Table 1. Telomerase-targeting drugs in development

Product	Target	Clinical trials	
BIBR1532	TERT	–	
GRN163L	TERC	Phase I	
		Chronic lymphoproliferative disease, single agent	NCT00124189
		Breast cancer, combination with trastuzumab	NCT01265927
		Solid tumors and lymphoma (young patients), single agent	NCT01273090
		Phase II	
		Non-small cell lung cancer, combination with bevacizumab	NCT01137968
		Multiple myeloma, single agent	NCT01242930
		Breast cancer, combination with paclitaxel (with or without bevacizumab)	NCT01256762
		Brain tumors (young patients), single agent	NCT01836549
Telomestatin	G-quadruplex	–	

– No clinical trails have been performed.

G-quadruplex structures at the 3′ single-stranded overhang of telomeres.[64] Telomestatin blocks access of telomerase and telomere-binding proteins to telomeres, and induces uncapping and shortening of telomeres, leading to growth arrest and apoptosis of cancer cells.[65,66] Telomerase inhibition and subsequent telomere shortening leads to senescence and/or apoptosis of cancer cells, although there is a lag period between initiation of telomerase inhibition and growth arrest that is primarily determined by the original telomere length. However, all telomerase-inhibition therapies share a fundamental vulnerability: telomerase inhibition in cancer cells gives rise to acquisition of alternative lengthening of telomeres (ALT), a homologous recombination-based telomere elongation mechanism, leading to restoration of telomere length and escape from telomerase-targeting anticancer therapy.[67–69]

Recent evidence demonstrating TERT functions other than telomere maintenance in tumor biology suggests that non-canonical functions of this protein could be novel therapeutic targets. Based on this idea, we looked for compounds that inhibit the RdRP activity of TERT. Eribulin mesylate (eribulin), an anticancer drug approved for the treatment of breast cancer and designated as an orphan drug for soft-tissue sarcoma, blocks the elongation of microtubules and induces G2/M arrest and apoptosis in cancer cells. We confirmed that eribulin specifically inhibits the RdRP activity, but not the telomerase activity, of TERT *in vitro* (Fig. 3).[70] The *ex vivo* studies using a series of ovarian cancer cell lines revealed that eribulin-sensitive ovarian cancer cell lines expressed higher levels of TERT compared to eribulin-resistant cell lines, and suppression of TERT protein expression reduced sensitivity to eribulin.[70] In addition, eribulin-sensitive cell lines have enhanced cancer stem cell-like traits; that is, characteristics associated with TERT.[70] Therefore, the anticancer effect of eribulin is likely due in part to dysfunction of TERT.

Conclusions

Emerging evidence demonstrates that TERT is a multifunctional protein: it elongates telomeres as the canonical telomerase catalytic enzyme, and further modulates gene expression, mitotic progression and stemness through non-canonical functions in cooperation with telomerase-independent molecules. We speculate that the nearly ubiquitous expression of TERT in cancer cells is driven not only by the need for telomere maintenance, but also by the protein's effects beyond telomere maintenance. Although tremendous effort has been expended on the development of telomerase inhibitors, with the goal of complete cure from cancer, no such therapy has yet succeeded, in part due to the lack of attention to the non-canonical functions of the key protein TERT. Therefore, the novel functions of TERT represent promising targets for achieving breakthroughs in anticancer treatments targeting TERT, which would be of benefit to many patients with malignant disease.

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Disclosure Statement

The authors have no conflict of interest to declare.

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