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CHAPTER 3

The Mechanisms of Telomere and Telomerase Regulation in Hematologic Malignancies

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Abstract: Human chromosome ends are capped by telomeric DNA composed of long arrays of (TTAGGG)n repeats. During each cell division, telomeric DNA is shortened by 50-100 bp. This attenuation of chromosome ends results in a loss of coding sequences and end-to-end chromosomal fusions, leading to chromosomal instability and aberrations. Therefore, telomere shortening is a critical event that acts as a mitotic clock to measure cellular life span. Telomerase, a ribonucleoprotein composed of a catalytic subunit (TERT), an RNA template (TERC), and the dyskerin protein, elongates telomeric sequences in germ cells and immature precursors such as hematopoietic stem cells. Telomerase is activated by multiple signaling pathways and its activity is regulated at the levels of gene transcription and translation, as well as post-translational modifications, trafficking, and assembly of the protein. Telomerase is upregulated in most cancer cells, including hematologic malignancies. Telomere shortening and telomerase activation are correlated with the prognosis and aggressiveness of various hematologic tumors. Therefore, targeting telomerase may be an attractive therapeutic strategy for hematologic malignancies. In fact, several strategies of telomerase inhibition, such as G-quadruplex-interacting agents, catalytic/reverse transcriptase inhibitors, oligonucleotides, and immunotherapy, have been developed as therapeutic methods.

Keywords: Acute leukemia, chronic leukemia, hematologic malignancies, malignant lymphoma, multiple myeloma, regulation mechanism, shelterin complex, signal transduction, telomerase, telomerase inhibitor, telomere.

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1. INTRODUCTION

In the 1930s, McClintock [1] and Muller [2] independently reported that the ends of chromosomes play a crucial role in protecting these structures against end-to-end fusion and breakdown during mitosis. Muller was the first to use the term "telomere", taken from the Greek words telos (meaning "end") and meros (meaning "part") to describe the special structure of chromosome ends. In the 1960s, Hayflick [3, 4] discovered that human fibroblasts stop dividing when cultured serially; this phenomenon is known as replicative senescence and the stage at which cell division stops is termed the Hayflick Limit, or M1. Subsequently, Watson [5] described the "end replication problem", which is caused by the inability of DNA polymerase to fill the gaps at the far ends of newly formed DNA strands during replication of a linear duplex. In 1973, Olovnikov [6] went on to suggest that chromosome-end shortening caused by an inability of somatic cells to compensate for chromosome loss would result in telomere shortening and hypothesized that the length of the DNA repeat sequence at chromosome ends might limit the number of times that a cell can replicate before entering senescence (known as the mitotic clock). In contrast to somatic cells, germline cells and stem cells are able to maintain the length of telomere ends *via* the elongating activity of telomerase, which was first discovered in *Tetrahymena thermophilia* extracts by Greider and Blackburn in 1985 [7]. When telomere ends reach a critical length, permanent growth arrest can be triggered by inducing cell cycle inhibitors such as p53; however, cells can continue to proliferate by inactivating p53, resulting in a further loss of telomere length and entry into a second proliferative lifespan crisis (M2), which is associated with genomic instability and cell apoptosis [8, 9]. Rare cells can escape from this crisis and become immortalized by activating telomerase or, in some cases, by utilizing an alternative telomere lengthening mechanism [10]. Telomerase activation occurs in almost 90% of cancerous cells [11]; therefore, telomeres and telomerase are believed to play an important role in the regulation of cellular senescence and carcinogenesis. Telomere stabilization and upregulation of telomerase have been implicated in the pathogenesis and clinical prognosis of hematologic malignancies [12-20]. Here, we discuss the roles of telomeres and telomerase in hematopoietic cells and their tumors, and consider potential strategies for clinical diagnosis and therapy.

2. STRUCTURE AND FUNCTION OF TELOMERES

About 50 years ago, Leonard Hayflick [3, 21] discovered that cultured normal human cells have a limited capacity for division, after which they become senescent. Hayflick and Moorhead [3, 21] cultured three separate populations of human fibroblasts simultaneously: one was derived from a male at population doubling level 14 (PDL14); another was derived from a female at PDL10; the third was a mixed population initially containing an equal number of male PDL14 and female PDL10 fibroblasts. When the unmixed male-derived PDL14 cell population stopped dividing, they discovered that the mixed population (which was cultured simultaneously) contained only female cells. Besides providing additional evidence that cells have a limited lifespan, this experiment demonstrated that older cells 'remembered' that they were old, even when surrounded by younger cells. Hayflick [22, 23] also made the important observation that cryo-preserved cells 'remembered' the number of times they had divided before they were frozen and stored. These experiments suggest that a counting mechanism is programmed into each cell, and that the cell stops dividing once this biological clock runs out. In commemoration of Hayflick's discovery that human cells have a finite lifespan, the term "Hayflick limit" is sometimes used to describe the maximum number of times that a cell can divide. Senescence, the withdrawal of a cell from the cell cycle after a certain number of divisions, can be triggered by shortened telomeres. Studies of replicative senescence have contributed greatly to our understanding of aging and created new opportunities in the area of regenerative medicine. Cancer cells evolve the ability to overcome senescence by exploiting mechanisms that maintain telomere length (*e.g.*, telomerase expression), thereby acquiring the ability to divide indefinitely.

2.1. Telomere Structure

2.1.1. Telomeric DNA

Telomeres are repetitive nucleotide sequences present at each end of eukaryotic chromosomes [24]. Telomeric DNA sequences are highly conserved in all well-characterized eukaryotic nuclear chromosomes, and are quite different from the terminal sequences of linear viral, extra-nuclear plasmid, or mitochondrial DNAs. Human telomeric DNA comprises 2-15 kb of a tandemly repeated sequence,

$(TTAGGG)_n$, which runs 5' to 3' toward the end of the chromosome [25] (Fig. **1A**). The evolutionary conservation of this repetitive DNA sequence implies that it is essential for cellular function [26]. Broken ends of chromosomes are unstable and can fuse end-to-end to form dicentric rings [27]. Such telomeric fusions may contribute to the chromosomal rearrangements frequently observed in human neoplasms. Telomeres prevent the ends of intact chromosomes from being mistakenly recognized as DNA breaks by the DNA replication machinery. Thus, telomeres protect chromosomes against degradation, fusion, and rearrangement during DNA replication. In addition, telomeres ensure that the chromosomes are positioned correctly within the nucleus prior to replication [28-30].

A

ORGANISM	SEQUENCE
Ciliates	
Tetrahymena	TTGGGG
Glaucoma	TTGGGG
Paramecium	TTGGGG/TTTGGG
Stylonychia	TTTTGGGG
Oxytricha	TTTTGGGG
Euplotes	TTTTGGGG
Slime Molds	
Physarum	TTAGGG
Didymium	TTAGGG
Dictyostelium	AG_{1-8}
Flagellates	
Trypanosomes	TTAGGG
Sporozoans	
Plasmodium	TTTAGGGG/TTCAGGG
Yeasts	
S. cerevisiae	TG_{1-3}
S. pombe	$T_{1-3}ACA_{0-1}G_{1-6}$
Plants	
Arabidopsis	TTTAGGG
Mammals	
H. Sapiens	TTAGGG

B

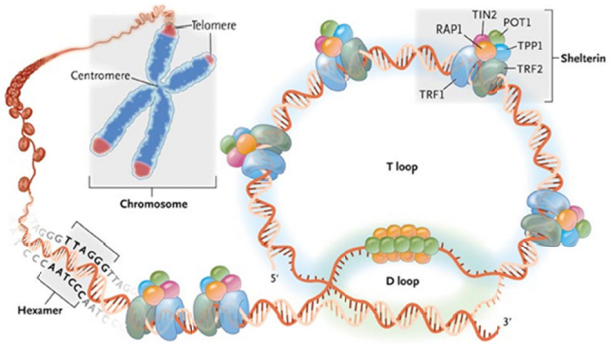


Figure 1: Sequences of telomeric repeats from a variety of eukaryotic cells. Telomeres consist of tandemly repeated G-rich sequences. One repeat of the G-rich strand is shown in Panel **A.** As shown in Panel **B**, telomeres are located at the ends of linear chromosomes. Telomeres comprise hundreds to thousands of tandem DNA repeat sequences: in humans, hexameric TTAGGG in the leading strand, and CCCTAA in the lagging strand. The protective proteins associated with telomeric DNA are collectively termed shelterins (TRF1, TRF2, TIN2, POT1, TPP1, and RAP1). The 3'end of the telomeric leading strand terminates in a single-stranded overhang, which folds back and invades the double-stranded telomeric helix, forming the T-loop (Adapted from [Ref. 312]).

2.1.2. The Mammalian Shelterin Complex

Telomere-associated proteins are important for maintaining telomere stability and regulating telomere length. Mammalian telomeres are associated with and

protected by the shelterin complex, which contains telomere-repeat-binding factor 1 (TRF1), TRF2, human repressor activator protein 1 (RAP1), TPP1 (also known as TINT1/PTOP/PIP1), protection of telomeres 1 (POT1), and TRF1-interacting nuclear protein 2 (TIN2) (Table **1**) [31-33]. TRF1 and TRF2 bind to double-stranded telomeric DNA and anchor shelterin along the telomere repeats [34-36].

Table 1: Components of the telomere complex

Components	Roles
Shelterin Complex	
Telomere-repeat-binding factor 1 (TRF1)	Binding to double-stranded telomeric DNA and folding telomere, resulating in prevention of the elongation of telomeres.
Telomere-repeat-binding factor 2 (TRF2)	Binding to double-stranded telomeric DNA and stabilizes telomere ends by mediating T-loop formation.
Repressor activator protein 1 (RAP1)	Interacts with TRF2 and enhances its affinity for telomeric 3' ends.
TRF1-interacting nuclear protein 2 (TIN2)	Serves as the hub of the complex linking TRF1 and TRF2 and Promotes T-loop formation.
Protection of telomeres 1 (POT1)	Interacts with TPP1 and protects single-stranded G-rich DNA overhangs from the DNA-damage response.
TINT1/PTOP/PIP1 (TPP1)	Recruits POT1 and modulates telomere function by negatively or positively regulating telomerase processivity.
Tankylase 1 (TANK1)	Poly (ADP-ribosyl)ates TRF1, resulting in its release from telomeric DNA and increased telomerase access to the telomere.

POT1 binds to the single-stranded G-rich DNA overhang [37-39]. TIN2 serves as the hub of the complex linking TRF1 and TRF2 [40, 41], and also recruits POT1 to the complex *via* TPP1 [38, 41, 42]. RAP1 associates with the telomere protein complex *via* its interaction with TRF2 [43, 44]. These telomere-specific protein complexes are found in many eukaryotes, indicating their importance for telomere function [45]. In humans, the shelterin proteins regulate telomere length and telomere capping. POT1 and TPP1 form a heterodimer and modulate telomere function by negatively regulating telomerase access to the 3'-overhang or by serving as a telomerase-processing factor during telomere extension [46-49]. Although TRF1 and TRF2 bind to double-stranded telomere DNA and are closely related to each other, the results of structural analyses indicate that they recruit different proteins to telomeres to facilitate distinct functions [50, 51]. TRF1 negatively controls telomere length *via* a length-dependent counting mechanism,

in which the interaction between POT1/TPP1 and TRF1 allows communication between double-stranded telomeres and telomerase [52]. On the other hand, TRF2 and POT1 are essential for the formation or regulation of the telomeric T-loop structure, which masks chromosome ends and prevents them from evoking a DNA damage response or undergoing recombination [53] (Fig. **1B**).

2.2. Telomeric Loss in Somatic Cells

Because DNA polymerase cannot completely replicate linear DNA molecules, early models predicted that terminal sequences would be lost from the chromosomes during each round of replication [5, 6, 54]. According to our current understanding of DNA replication, the polymerase that copies the DNA strands prior to each cell division absolutely requires a short RNA primer sequence to begin DNA polymerization in the 5' to 3' direction. After DNA polymerization, the RNA primers are degraded and replaced by DNA synthesized from an upstream 3' DNA end. However, the gaps resulting from degradation of the primers annealed to the extreme 3' end of each strand cannot be filled in this manner. Therefore, over multiple cycles, this replication strategy results in progressive shortening of chromosomes at both ends (Fig. **2A**). This loss of genetic material is predicted to contribute to senescence by limiting the proliferation of somatic cells, or by causing cell death [6]. Consistent with these predictions, the average length of the telomeres in human skin and blood cells *in vivo* decreases by 15-40 bp per year. In cell culture, the telomeres of fibroblasts, T cells, embryonic kidney cells, mammary epithelium, and cervical cells lose 50-200 bp from the TTAGGG repeats per population doubling [55-62].

2.3. Telomere Replication

In certain types of cells, such as germ cells and stem cells, telomere length must be preserved to prevent exit from the cell cycle and entry into senescence. Therefore, these cells express an enzyme called telomerase, a large dimeric ribonucleoprotein complex [7, 63] comprising a reverse transcriptase protein (TERT) and an RNA template (TERC, also known as TR) [64-67]. Both components are essential for telomerase activity. Telomerase utilizes a region within its RNA component as a template for synthesizing telomeric repeats. In other words, telomerase functions as a reverse transcriptase that adds telomeric repeats to the ends of chromosomes.

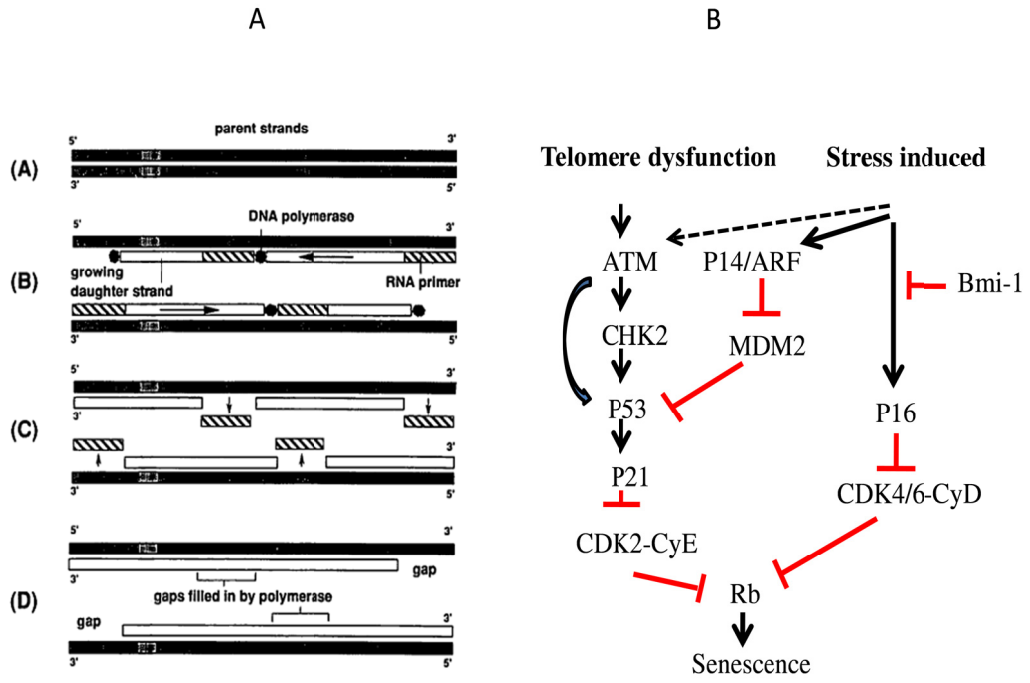


Figure 2: Schema showing DNA replication of a linear duplex. A: Telomere attrition. (A) Parent strands. **(B)** When the joined parent strands separate, RNA primers attach to them, enabling DNA polymerase to synthesize the daughter strands. **(C)** After DNA synthesis, the RNA primers are removed. **(D)** DNA polymerase then joins the new DNA fragments but cannot fill the gaps at the far ends (at the distal ends of the telomeres). Thus, in the absence of mechanisms to overcome this end-replication problem, the 5' end of the newly synthesized DNA in each duplex becomes shorter after each round of DNA replication (Modified from Ref. [25]). **B: Senescence pathways**. Senescence is primarily mediated by two pathways: p53/p21 and p16INK4A/pRb. As the telomeres progressively shorten over multiple rounds of division, they become uncapped. Either uncapping or DNA double-strand breaks activate ATM/ATR kinases. ATM phosphorylates CHK2, which then phosphorylates p53; ATM can also phosphorylate p53 directly. These phosphorylation events stabilize and activate p53, which in turn transcriptionally upregulates its target genes. Activation of these targets mediates the alternate fates induced by p53, including G1 arrest, senescence, and apoptosis.

Whereas TERC expression is relatively ubiquitous throughout embryonic and somatic tissues, TERT expression is tightly regulated and undetectable in most somatic cells [68]. Therefore, TERT expression is the rate-limiting step in telomerase activity. Although telomerase activity remains the most likely mechanism responsible for telomere elongation, other pathways, such as recombination and gene conversion, cannot be completely excluded [69-71].

2.4. Telomere Loss and Cellular Senescence

2.4.1. Transformation

Cultured human somatic cells undergo a finite number of divisions, the aforementioned Hayflick limit, before they senesce [55]. This limit is determined by telomere dynamics: the telomeres of somatic cells shorten at each division until they reach a length that prevents further cell division. Because the lengths of human telomeres are extremely heterogeneous, each cell may harbor one or more telomeric tracts that can no longer maintain telomere function. Induction of replicative senescence does not require that all telomeres within the cell be short; rather, senescence can be initiated by a small number of short telomeres [72, 73]. It is not clear, however, how short a telomere must become before it is recognized by the mechanisms that sense DNA damage. A telomere that still contains telomeric repeats, but is too short to form the protective T-loop structure, can trigger the initial damage signal. Although they arise infrequently, such uncapped chromosome ends may resemble a DNA strand break, and thus activate the p53 or retinoblastoma (Rb)-dependent cell cycle arrest pathways (Fig. **2B**) [3]. The p53 and pRb pathways are central to cellular senescence control. Telomere shortening, radiation, cytotoxic drugs and oncogene-induced DNA replication stress have all been shown to induce the DNA damage response, resulting in the activation of the checkpoint kinases ATM/ATR and CHK1/2. ATM can directly or indirectly phosphorylate p53 protein and leading to p53 accumulation. p53 can also be activated downstream of p14ARF, which binds to MDM2, preventing the degradation of p53. This stabilization and accumulation of p53 allows the activation of downstream genes such as p21, and the induction of cellular senescence. Another tumor suppressor p16INK4a, transcriptionally up-regulated in stressed cells, inhibits cyclin D-dependent kinases, thereby preventing the phosphorylation and inactivation of the retinoblastoma protein, pRb. This promotes the repressive association between pRb and transcriptional activators, preventing progression through the cell cycle [74-76]. But the mechanisms of this transcriptional regulation of p16INK4a and p14ARF in senescent human cells are not yet fully understood [77]. Alternatively, shortened telomeric tracts may lead to changes in the expression of subtelomeric senescence-related genes *via* the modulation of telomeric silencing [3, 78, 79]. This first mortality stage (Ml) can

be averted by mutations in the tumor suppressor genes p53 or Rb, suggesting that proteins encoded by these genes normally induce exit from the cell cycle at this point [80]. This notion was confirmed by Hara *et al*. [81], who showed that antisense p53 and Rb oligonucleotides act cooperatively to extend the replicative lifespans of human cells. Likewise, Ml can be bypassed by transfection of specific viral DNA sequences that are capable of overriding cellular growth control signals. For example, the expression of simian virus 40 (SV40) large tumor antigen allows fibroblasts and embryonic kidney cells to progress beyond Ml; human papillomavirus and adenovirus have much the same effect in other cell types [81-85]. In either case, the transformed cells continue to divide for as many as 50 divisions before they reach a second mortality stage (M2; also known as crisis), at which point the majority develop chromosomal abnormalities and die. M2 is not circumvented by viral transformation, and transformed cells do not survive M2. Progression beyond M2 is rare, apparently only occurring through mutational alterations in unidentified cellular gene(s). The M2 crisis probably represents the point at which nearly all chromosomes have lost most of their functional telomeres. Because the *cis*-acting elements that are required for the various functions of human telomeres remain unknown, it is difficult to gauge the point at which continued decline of telomere repeat will disable telomere function.

2.4.2. Immortalization

Cells can overcome the replication-induced M1 and M2 stages by activating a mechanism that elongates or stabilizes telomere lengths. Counter *et al*. [82] studied both telomere length and telomerase activity throughout the stages of immortalization in a primary cell line transformed with either SV40 or adenovirus DNA. They observed that telomere shortening continued through M2, often coinciding with the appearance of abnormal fused chromosomes; upon immortalization, however, even very short telomeres became stable. They then assayed telomerase activity in cell extracts, and found that telomere stabilization during immortalization coincided with the onset of telomerase activity [82]. These experiments solved the mystery surrounding the persistence of short telomeres in the presence of active telomerase, and confirmed that telomerase can be activated to stabilize the chromosome ends even after dramatic telomere loss.

2.5. Measurement of Telomere Length

The first technique that was developed to measure telomere length in mammalian cells was based on Southern blotting. In this method, the restriction enzymes *Hin*fI and *Rsa*I are used to digest genomic DNA at points outside, but not within, the TTAGGG repeats at the ends of human chromosomes. The resulting fragments are separated by electrophoresis, transferred to nylon membranes, and then hybridized with a [^{32}P]-labeled or biotin-conjugated $(TTAGGG)_7$ probe that detects terminal restriction fragments (TRFs), including all terminal repeats and the subtelomeric region [86]. The TRFs appear as a smear on a gel because both the size of the subtelomeric region and the number of TTAGGG repeats varies depending on the chromosome. Moreover, the mean TRF length varies among cells within a population. One disadvantage of this method is that conventional Southern blot analysis requires a relatively large sample of DNA.

Recent innovations in the technique involving fluorescence *in situ* hybridization (FISH) have enabled the study of telomere length in small numbers of cells. Quantitative fluorescence *in situ* hybridization (Q-FISH) can be used to characterize the dynamics of telomere length in dividing cells. In this method, telomere length is quantitated using Cy3-labelled peptide nucleic acid (PNA) probes that are specific for telomeric and centromeric sequences. Specifically, telomere length is determined by comparing the relative intensity of the fluorescent signals generated by the p- and q-telomeres of each individual chromosome with that of a reference centromeric signal on chromosome 2 [87, 88]. Digital images are recorded with a CCD camera attached to a fluorescence microscope, and analyzed quantitatively using the appropriate software. Q-FISH has been adapted for the flow cytometric analysis of cells in suspension (flow FISH) [89]. In this method, FISH (utilizing labeled PNA probes specific for telomeric repeats) is used in combination with flow cytometric fluorescence measurements to measure the average lengths of telomere repeats in cells. Flow FISH analysis can be performed using commercially available flow cytometers and, in contrast to other methods used to measure telomere length, it can be used to obtain multi-parameter information on the lengths of telomere repeats in thousands of individual cells. Flow FISH was used to demonstrate that the telomere length in granulocytes correlates with the response to

immunosuppressive therapy in patients with aplastic anemia [90], and with time to disease progression in patients with chronic myeloid leukemia [91].

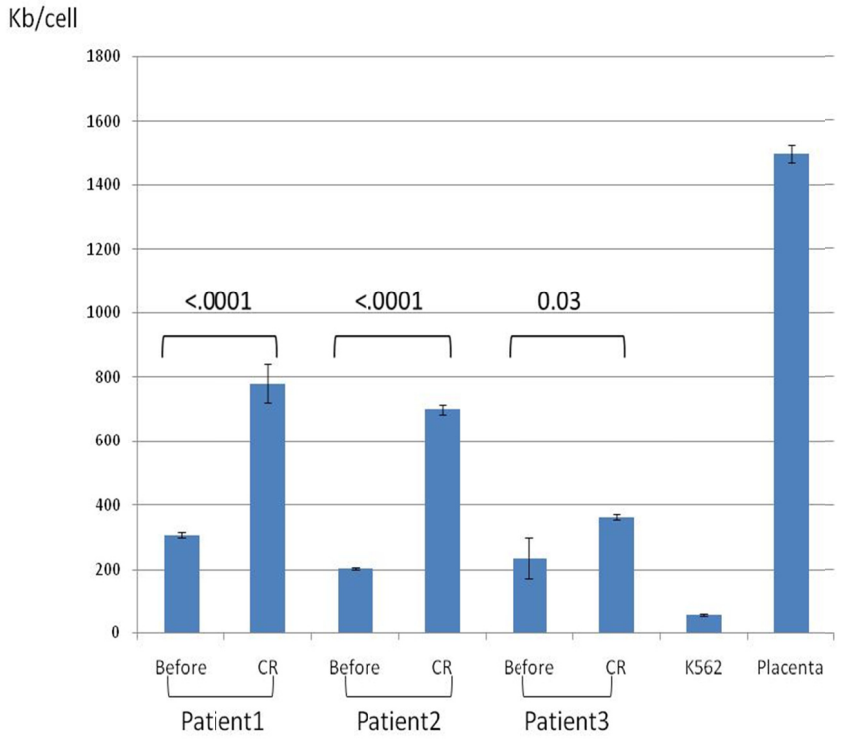


Figure 3: Measurement of telomere length by quantitative real-time PCR. Leukemic cells from three patients with acute myeloid leukemia showed reduced telomere lengths (3.2, 2.2, and 2.4kb); however, telomeres were relatively longer in peripheral blood mononuclear cells obtained from the same patients during complete remission (8.6, 7.6, and 4.1kb, respectively). The Y-axis shows total telomere size (kb/cell). The mean telomere length per cell is calculated by dividing total telomere size by 92. DNA from K562 cells was used as the leukemic cell control.

Two recently developed PCR-based methods enable measurements of average and chromosome-specific telomere length [92-94]. PCR-amplification of telomeric DNA is difficult because of the repetitiveness of the sequences. This problem was overcome by designing a set of primers that was not totally complementary to the telomeric sequence [92]. Using these primers, both relative and absolute measurements of telomere length have been obtained [95, 96]. Our group has developed and validated new primers and conditions for use in a quantitative real-time PCR (qRT-PCR) assay for measuring absolute telomere length. We used this

method to study telomeric DNA in blast cells obtained from three patients with acute myeloid leukemia and in peripheral blood mononuclear cells (PBMCs) obtained when the same patients entered remission. As shown in Fig. **3**, the telomeric DNA in the leukemic cells was actually shorter than that in mononuclear cells obtained from the same patients after they achieved complete remission. In the three patients examined, the average telomere lengths in leukemic cells were 3.2, 2.2, and 2.4 kb, whereas those measured in PBMCs during complete remission were 8.6, 7.6, and 4.1 kb, respectively (manuscript in preparation). This result is consistent with that of a previous report in which we used Southern blotting to make similar measurements [97], although the PCR-based method achieved higher resolution.

2.6. Telomere Length in Normal Controls

The mean telomere length in PBMCs from 119 normal individuals (age, 0-107 years) declined at a rate of 41±2.6 bp/year. This rate of TRF loss for PBMCs is close to that observed in peripheral blood leukocytes by both ourselves and Hastie *et al*. [56, 98, 99]. Separation of the data according to gender revealed that males lost telomeric DNA slightly faster than females (50 ± 4.2 bp/year *vs.* 40 ± 3.6 bp/year, respectively). The 18 centenarians (age, 99-107 years) in this population had a mean TRF length of 5.28 ± 0.4 kb. The TRF length in these long-lived individuals could be predicted by extrapolating the line for individuals aged 0-80 years, suggesting that the centenarians did not have a slower rate of telomere loss, but instead may have begun their lives with unusually long telomeres. The standard deviation of the mean TRF values for the centenarians (0.4 kb) was much smaller than that for other age groups (∼1 kb), suggesting that the centenarians were less genetically diverse than the younger populations from the standpoint of telomere length [60].

2.7. Shortening of Telomeres in Recipients of Hematopoietic Stem Cell Transplantation

Hematopoietic stem cell transplantation (HSCT) is used to treat various malignant and non-malignant diseases. Recovery of the hematopoietic system after HSCT requires hematopoietic stem cells (HSCs) to undergo numerous replication cycles. Although HSCs do possess telomerase activity, such enforced division may still

cause excessive shortening of the telomeres in the descendant cells. Our previous study analyzed telomere length in PBMCs isolated from 23 autologous HSCT patients (aged 4-61 years) and 46 allogeneic HSCT recipients (aged 6-52 years) to determine whether excessive shortening of telomeres is associated with HSCT [100]. After autologous HSCT, the mean telomere length in PBMCs was 9.7 kb (range 6.8 to 12.0 kb). A comparison between pre- and post-transplant PBMCs revealed that post-transplant telomeres were up to 1.9 kb shorter (mean ± s.d.: 0.64 ± 0.5 kb). Furthermore, the slopes of the regression lines differed between autologous HSCT patients and normal volunteers. After allogeneic HSCT, the mean telomere length of PBMCs was 9.3 kb (range 6.8 to 12.0 kb), and the telomeres in recipient PBMCs were up to 2.1 kb (0.60 ± 0.468 kb) shorter than those in donor PBMCs. The regression lines for allogeneic HSCT patients and normal volunteers were parallel. All patients were transplanted with more than 2.0×10^8 cells/kg. Telomere length did not correlate with the number of transplanted cells. In addition, there was no significant correlation between telomere length and the recovery of complete blood counts. However, three patients who had relatively short telomeres after HSCT (average telomere length, 6.8 kb) needed more time than the other patients to recover to a normal hematological state.

A recent report characterized the influence of mobilized and grafted cells on determining telomere length after HSCT [101]. Twenty patients received autografts collected after two sequential mobilization courses. Telomere length in the first collection was significantly longer than in the second. For autografts, the patients were divided into two groups: ten patients received HSCT from the first collection, and ten received HSCT from the second collection. Telomere length was also investigated before and after HSCT and in the grafts in ten cases of allogeneic HSCT. After transplantation, patients receiving autologous HSCT from the first collection had telomere lengths that were similar to those of the grafted cells (median: pre-HSCT, 7730 bp; post-HSCT, 7610 bp) and significantly longer than those of cells from the second collection. Likewise, patients who received autologous HSCT from the second collection had average telomere lengths of 7360 bp pre-HSCT *versus* 7120 post-HSCT; significantly shorter than those of the first collection. Taken together, these data suggest that most HSCT patients possess telomeres within the biological "safety range"; however, long-term

hematopoiesis and occurrence of possible hematopoietic disorders should be carefully monitored in patients with telomeres shorter than 7.0 kb after HSCT [102].

2.8. Telomeric DNA in Leukemic Blood Cells

Hematological malignancies are associated with progressive telomere shortening [12, 91, 103-105]. This shortening is thought to result from rapid clonal expansion, although oxidative damage or telomerase dysregulation may also contribute, at least in the early stages of some leukemias [106, 107]. The contribution of telomere shortening to the cytogenetic abnormalities commonly seen in leukemia is an active area of investigation, and many of these studies have been reviewed in detail by others [13, 108].

In a previous study, we measured the length of telomeric DNA in leukemic cells [97, 109]. Leukemic cells isolated from 12 patients with acute leukemia (seven with myeloid leukemia and five with lymphoid leukemia) exhibited shortening of telomeric DNA (range 2.7 to 6.4 kb). The average telomere lengths were 4.8 kb and 4.7 kb in patients with myeloid and lymphoid leukemia, respectively, whereas the telomere lengths in PBMCs obtained after the same patients had entered complete remission were 8.5 kb and 7.9 kb, respectively. When the same Southern blots were re-hybridized with Alu or alphoid sequences, which have important biological functions with regard to gene regulation and cell division, no significant changes in the size of the repetitive DNA sequences were observed, indicating that the DNA abnormalities in the leukemic cells were specific to telomeric regions.

Telomere sequences do not encode proteins, but they play an important role in maintaining chromosomal stability and activity [24, 25]. Translocations arising through the tandem fusion of telomeres were first observed at the chromosomal level in leukemic B cells [110]. Subsequently, telomeric fusions were reported in a case of pre-T-cell acute lymphoblastic leukemia [111] and in some solid tumors [112]. These studies suggested that the ends of the chromosomes in some tumor cells contain abnormalities. We demonstrated that telomere attrition was even present in leukemic cells with normal karyotypes [97]. These observations suggest

that telomeres are unstable in leukemia patients, and that sequences are lost during the course of tumorigenesis. In one study, telomere shortening was significantly more pronounced in patients with cytogenetic alterations than in patients with normal karyotypes [113], and patients with complex cytogenetic abnormalities had the shortest median telomere length. Furthermore, TERT was most highly over-expressed in patients with complex karyotypes, followed by patients with noncomplex karyotypes and patients without karyotypic changes. These findings suggest that with increasing telomere attrition, whether due to replication-dependent or -independent mechanisms, karyotypic abnormalities become more pronounced; consequently, telomerase upregulation becomes essential to prevent replicative senescence of the malignant clone.

What is the mechanism underlying the reduction in telomere length observed in leukemic cells? This telomere shortening may reflect the number of cell divisions required to generate leukemic cells in the absence of telomerase. In somatic cells, telomere loss may be due to incomplete DNA replication in the absence of telomerase. The loss of DNA at each division acts as a mitotic clock, ultimately resulting in cell cycle exit or cell death [82, 114]. Telomerase may be reactivated in somatic cells that are immortalized during tumorigenesis [8]; in these cells, *de novo* synthesis of telomeric DNA by telomerase counterbalances losses due to replication. In one study of telomere length in immortal cells, 300 sequential samples were collected from 40 patients during the course of acute promyelocytic leukemia, and their telomerase activity and TRF length were assessed. About 90% of the patients exhibited a significant reduction in telomere length relative to that in the control, and telomerase was activated in all patients [115]. Consistent with this, as noted above, our group previously showed that the average telomere length in leukemic cells was shorter than that in PBMCs isolated from patients during complete remission. Therefore, it is conceivable that telomerase reactivation is a late event in cellular transformation, and that cells with short telomeres can remain viable provided that they are stably maintained by telomerase.

Telomere loss may be a consequence of tumorigenesis, but it may also be a cause, *i.e.*, telomere shortening may play an active role in the development of malignancy. Assuming that repetitive $(TTAGGG)_n$ sequences are essential for

telomere function [116], partial loss of these sequences should eventually destabilize the chromosome ends. Chromosomes lacking terminal repeats may become less stable and more prone to the fusion-bridge-breakage cycle [117], leading to the formation of daughter cells containing partly deleted or duplicated chromosomes. Such alterations may also play a part allele loss of restriction fragment length polymorphisms, consistent with the notion that mutations in tumor suppressor genes must be homozygous before malignancy can occur [118, 119]. However, the specific effects of telomere reduction on tumorigenesis remain unclear, and will only be elucidated by future studies.

3. TELOMERASE REGULATION

Telomerase comprises an 1132 amino acid TERT component, a 445 nucleotide RNA component (TERC), and a number of associated proteins, such as dyskerin, p23, and heat shock protein 90 (HSP90), which are required for the stabilization and assembly of the enzyme. Telomeres form folded-cap structures comprised of T-loops and D-loops that are stabilized by telomere-binding proteins, including TRF1, TRF2, RAP1, TIN2, TPP1, and POT1 (known collectively as the shelterin complex). As described previously, these structures prevent recognition of chromosome ends as sites of DNA damage and restrict telomerase access, thereby ensuring telomere homeostasis (Fig. **4A**). Although TRF1 bends, loops and pairs telomeric DNA and inhibits access of telomerase, telomere attrition leads to poly(ADP-ribosyl)ation of TRF1 by tankylase 1 (TANK1), resulting in release of TRF1 from the telomere ends and subsequent increased telomerase access (Fig. **4B**). The telomerase complex associates with the single-stranded 3' overhang of the telomere end; five nucleotides of TERC, which contains the repeated sequence 5'-CUAACCCUAAC-3', bind to the 3' telomeric DNA and serve as an RNA template for reverse transcription by TERT. After DNA synthesis, the telomerase complex translocates without dissociating from the telomere and another round of telomeric synthesis is initiated. Therefore, both TERT and TERC are essential for telomerase activity. Whereas TERC is expressed in a wide range of cell types, TERT expression and activity is tightly regulated during cell proliferation and differentiation. Telomerase activity is downregulated in quiescent or differentiated cells, and is reactivated in proliferating cells induced by growth factors and cytokines, indicating that the enzyme complex is regulated in a cell cycle-

dependent manner [15, 18, 120, 121]. In fact, stimulation of the T-cell antigen receptor complex in human CD4 T-cells induces not only cell proliferation, but also upregulation of telomerase activity [122]. In addition, previous studies by our group demonstrated that stimulation of tumor cells from adult T-cell leukemia or natural killer (NK) cell lymphoma patients by interleukin 2 (IL-2) increases telomerase activity and promotes cell cycle progression [123, 124]. In human cells, the telomerase complex is formed, and has increased enzymatic activity, during the S-phase of the cell cycle [120, 125].

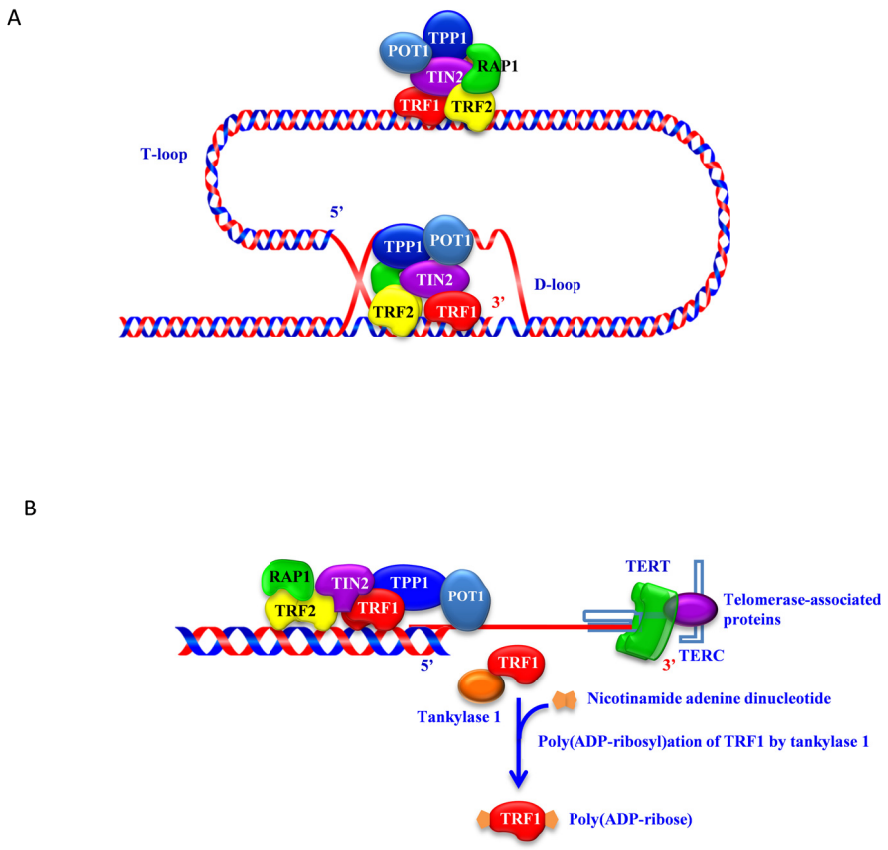


Figure 4: The structure of telomeres in human cells. (A) Telomeres at the ends of chromosomes are folded into a T-loop structure and are composed of DNA repeats (TTAGGG). A single-stranded overhang of the telomere end displaces a part of the DNA duplex and forms a D-loop. Telomere-binding proteins, including TRF1, TRF2, RAP1, TIN2, TPP1, and POT1, form the shelterin complex, which stabilizes the t-loop structure and prevents telomerase access. **(B)** TRF1 is poly(ADP-ribosyl)ated by tankylase 1, resulting in its release from the DNA duplex and increased telomerase access to the telomere (modified from [Ref. 20]).

Telomerase activity is regulated at the levels of gene transcription and translation, as well as *via* post-translational modifications, translocation, and assembly of the protein. Transcriptional regulation is the major mechanism of regulation of TERT and has been explored extensively [126]. The gene encoding TERT is located on the short arm of chromosome 5 (5p13.33), more than 2 Mb away from the telomere [127, 128], suggesting that expression of the *TERT* gene is unlikely to be affected by the progressive telomere shortening that occurs during cell division [9]. The *TERT* gene consists of 16 exons and 15 introns, and is over 40 kb in length [9]. The TERT core promoter, which is located 330 bp upstream of the transcription start site, lacks TATA and CAAT boxes, but contains GC-rich regions and binding sequences for various transcriptional activators and repressors [9, 126]. The transcriptional activators that target the TERT promoter include c-Myc, Sp1, Ets-1, nuclear factor of activated T-cells, nuclear factor kappa B (NF-κB), cAMP response element-binding protein, hypoxia inducible factor-1, and signal transducer and activator of transcription (STAT) proteins [20, 126]. On the other hand, the transcriptional repressors that negatively regulate telomerase expression include Wilms tumor 1 (WT1), myeloid zinc finger 2, p53, transcriptional activator 1, menin, Smad3, E2F1, PITX1, Mad1, and CCCTC-binding factor (CTCF) [126, 129-131]. c-Myc, an oncogene product containing a basic-helix-loop-helix-zipper domain that forms a heterodimer with its activation partner Max, acts as a critical regulator of TERT transcription by binding to two canonical E-boxes (5'-CACGTG-3') located 34 and 242 nucleotides upstream of the TERT transcription start site [11]. c-Myc also cooperates with the zinc finger transcription factor Sp1, which binds to GC-boxes located between the two E-boxes in the TERT promoter; therefore, the transcriptional function of Sp1 is dependent on c-Myc [132]. The tumor suppressor protein p53 prevents binding of Sp1 to the TERT promoter, and WT1 interacts with both the c-Myc promoter and the TERT core promoter region, resulting in suppression of TERT transcription [9, 126, 133]. In addition, activation of E2F1 through a positive feedback loop of c-Myc represses transcription of TERT and thus negatively regulates c-Myc-induced transcriptional activation of the gene [134].

Epigenetic modifications of DNA and histones also regulate TERT transcription. The TERT promoter contains a cluster of CpG sites, suggesting that it is

susceptible to regulation by changes in the DNA methylation status. Although methylation of CpG islands is generally considered to cause transcriptional silencing, there are some conflicting reports of the effects of methylation on TERT promoter activity. Global methylation of the CpG islands in the TERT promoter results in transcriptional repression [126]. By contrast, hypermethylation of a region upstream of the transcription start site of the *TERT* gene is crucial for its transcriptional activity in tumor cells, whereas hypomethylation at the core promoter region is required for transcription to proceed [129]. The transcription repressor CTCF is only able to bind to its recognition sequences in the first and second exons of TERT when these regions are demethylated; hence, hypermethylation prevents binding of CTCF and leads to transcriptional activation of TERT [135]. Histone modifications such as acetylation, methylation, phosphorylation, and ubiquitination are thought to alter chromatin remodeling and gene transcription [126, 129]. Histone deacetylase inhibitors activate the TERT promoter by recruiting Sp1 and mimic the induction of TERT mRNA expression upon T-cell antigen receptor stimulation by maintaining H3/H4 acetylation [136]. TERC expression is also regulated by histone deacetylation [137]. On the other hand, methylation of Lys4 of histone H3 (H3K4) is associated with increased TERT expression, while H3K9 methylation is associated with downregulation of TERT transcription [137]. Furthermore, phosphorylation of H3 by mitogen-activated protein kinase (MAPK) induces TERT expression and activation in normal and neoplastic T-cells [138].

TERT mRNA expression is also regulated by a splicing mechanism. TERT mRNA transcripts contain at least six splice sites, and ten splice variants have been identified to date [139, 140]. Alpha site deletion variants (α-/β+), which contain a 183 bp deletion, lack a reverse transcriptase motif and exert a dominant negative effect on normal transcripts [140, 141].

It is estimated that more than 1000 microRNAs (miRNAs or miRs) regulate at least one-third of all protein-coding genes, and some miRNAs play a critical role in tumorigenesis [142]. A number of miRNAs are also involved in the control of

TERT gene expression and translation [143-147]. For example, exogenous expression of miR-150 in NK/T-cell lymphoma cells in which native miR-150 is suppressed reduces telomerase activity by downregulating Akt kinase expression [145]. Conversely, miR-21 upregulates STAT3-mediated activation of TERT expression in glioblastoma cells [147].

Telomeres are thought to be heterochromatic and were therefore assumed to be transcriptionally silent. However, it was discovered recently that telomeres are actually transcribed into telomeric repeat-containing RNA (TERRA) [148, 149]. Mammalian TERRA molecules are large non-coding RNAs containing UUAGGG repeats that vary in size from 100-9000 nucleotides. TERRA is complementary to and forms a duplex with the template sequence of *TERC*, leading to the suppression of telomerase activity. Therefore, TERRA is implicated in negative feedback regulation of telomeres [141].

As a post-translational level, ubiquitination of the TERT protein may affect telomerase activity [141]. The half-life of the TERT protein (approximately 24 h) is shorter than that of TERC (approximately 5 days), suggesting that TERT is a key determinant of telomerase stability. Some ubiquitin ligases, such as the MKRN E3 ubiquitin ligase and C-terminus of HSC70-interacting protein, regulate TERT stability. Both of these ligases interact with TERT and mediate its polyubiquitination, leading to degradation of the protein. The enzymatic activity of TERT is also controlled at the post-translational level *via* phosphorylation and effects on protein folding. Akt kinase and protein kinase C (PKC) phosphorylate and activate TERT [150, 151]. By contrast, protein phosphatase 2A (PP2A), a serine/threonine-specific phosphatase, inhibits telomerase activity. Protein tyrosine kinases, such as Src and Abl kinases, also contribute to TERT regulation. The roles of these kinases and phosphatase in TERT regulation are discussed in more detail in the next section.

Telomerase biogenesis requires nuclear trafficking and assembly of TERT and TERC, each of which comprise multiple factors and form a complex (Table **2**).

Table 2: Components involved in telomerase assembly and trafficking

Components	Roles
TERC-Related	
NHP2/NPO10/dyskerin	Members of the pre-formed core trimer snoRNP complex required for stabilization, intracellular trafficking and function of TERC.
NAF1	Loads NHP2/NOP10/dyskerin core complex onto H/ACA domain of TERC via an interaction between NAF1 and the C-terminus of RNA polymerase II.
GAR1	Member of the H/ACA snoRNP family that binds to the core snoRNP complex via dyskerin by substituting NAF1; it is also required for correct trafficking of TERC.
TCAB1	RNA chaperon required for transporting TERC to the Cajal body and stabilizing dyskerin, resulting in an interaction between telomerase and telomere.
Coilin	Component of the Cajal body required for accumulation of telomerase to telomeres.
SMN	RNA assembly factor in the Cajal body that interacts with GAR1 and TERT and is required for proper assembly of the H/ACA snoRNP complex of TERC.
hnRNP A1	Associated with both TERC and telomeric DNA and protects single stranded telomeric ends against degradation via double-stranded DNA breaks.
Nopp140	Cajal body-related phosphoprotein that is associated with dyskerin and may play a role in trafficking of telomerase.
TERT-*Related*	
HSP90/p23	Molecular chaperone that binds to TERT and is required for the nuclear localization and stabilization of telomerase.
PinX1	Plays a role in trafficking of TERT to the telomere and inhibits telomerase via interacting with TRF1.
Nucleolin	Promotes translocation of TERT from the nucleolus to the nucleoplasm.
Pontin/Reptin	ATP-dependent helicase that is associated with TERT and dyskerin in cell cycle-dependent manner.

TERT and TERC are found in nucleoplasmic foci (TERT foci) and Cajal bodies (CBs), respectively. CBs are spherical sub-nuclear organelles that reside at the nucleolar periphery during the G1 phase and are implicated in RNA-related processes, including the maturation of small nuclear and nucleolar ribonucleoprotein (RNP) particles. As the cell cycle progresses to the S phase, TERT and TERC colocalize at nucleoli, where both components associate with CBs and form a telomerase complex that translocates to telomeres (Fig. **5**) [152]. Interaction of the HSP90-p23 complex with TERT is required for the nuclear localization of the protein and telomerase function [153]. Furthermore, transportation of TERT into the nucleus is also regulated by its phosphorylation.

Liu *et al*. [122] showed that TERT is phosphorylated and translocated from the cytoplasm to the nucleus during T-cell activation. Our own and other groups have demonstrated that activated TERT forms a complex with Akt, HSP90, mTOR, S6K, and NF-κB, which promotes its nuclear translocation [123, 124, 154, 155]. In the nucleus, PinX1 mediates transient localization of TERT in the nucleolus and binding of nucleolin promotes subsequent shuttling from the nucleolus to the nucleoplasm [150-157]. Export of TERT from the nucleus into the cytoplasm is also regulated by several factors. For example, the 14-3-3 signaling protein binds to TERT and inhibits its nuclear export through CRM1 (also known as exportin 1) [158]. Mammalian TERC contains a 3'-terminal domain resembling H/ACA small nucleolar RNAs that is required for binding to TERT and subsequent telomerase accumulation and function [159]. H/ACA small nucleolar RNAs comprise conserved structural elements known as the H box and ACA box and direct pseudouridylation of pre-rRNA. The H/ACA-RNP complex subunits dyskerin, NOP10, and NHP2 form a core trimer that associates with nuclear assembly factor 1 (NAF1). This interaction with NAF1 facilitates loading onto TERC transcripts and leads to the stabilization of the TERC-small nucleolar RNP complex, which is necessary for TERC trafficking and telomerase function [156, 160]. Substitution of GAR1 for NAF1 promotes the localization of TERC-H/ACA-RNPs at the nucleolus, prior to translocation of the complexes to CBs. Subsequently, telomere CB protein 1 (TCAB1) mediates translocation of TERC-H/ACA-RNPs to CBs, where CB-related proteins such as coilin, survival motor neuron (SMN), and probably Nopp140 (a 140 kDa nucleolar phosphoprotein) interact with TERC and may control telomerase trafficking and assembly [140, 161-167]. In CBs, TERC-H/ACA-RNPs form a complex with TERT through its telomerase RNA-binding domain, which contains a T motif that regulates the rate of template copying during catalysis [156]. In addition, the ATPases pontin and reptin promote the interaction of TERT with TERC-dyskerin, after which they dissociate from the complex to yield catalytically active telomerase [140, 156]. The oligonucleotide/oligosaccharide binding fold-containing telomeric protein TPP1 mediates the cell cycle-dependent recruitment of telomerase to telomeres [168]. Similarly, the heterogeneous nuclear RNP A1 associates with TERC and telomeric DNA simultaneously; resulting in protection of the single-stranded telomeric ends against degradation *via* double-stranded DNA breaks [169].

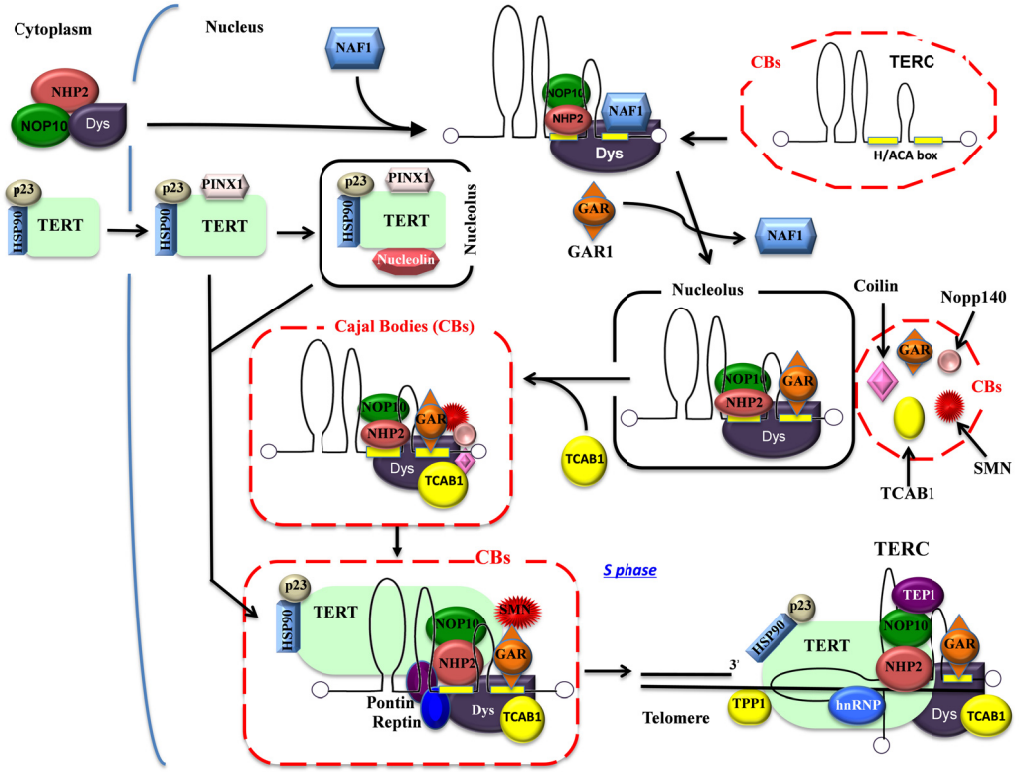


Figure 5: Biogenesis and assembly of the human telomerase enzyme. The pre-formed H/ACA core trimer complex comprising NHP2, NOP10, and dyskerin translocates to the nucleus and associates with NAF1, after which it is loaded onto TERC *via* an interaction between NAF1 and the C-terminal region of RNA polymerase II, thereby preventing exonuclease digestion of TERC. Substitution of GAR1 for NAF1 promotes the transition of the TERC-H/ACA RNP complex into the nucleolus. Binding of telomere CB protein 1 (TCAB1) to the CAB box on TERC facilitates the localization of TERC to Cajal bodies (CBs). In addition, CB-associated proteins, such as coilin, survival motor neuron (SMN), and Nopp140, may also contribute to the biogenesis of TERC. TERC binds to the telomerase RNA-binding domain of TERT to form active telomerase. TERT associated with the HSP90-p23 complex translocates to the nucleus. The ATPases pontin and reptin promote the interaction of TERT with TERC, which peaks during the S phase of the cell cycle. The heterogeneous nuclear RNP (hnRNP) A1 associates with both TERC and telomeric DNA and stabilizes the single-stranded telomeric ends. The telomeric protein TPP1 mediates the cell cycle-dependent recruitment of telomerase to telomeres.

4. THE ROLES OF SIGNALING PATHWAYS IN TELOMERASE REGULATION

The expression levels and enzymatic activity of TERT are regulated by multiple signaling pathways, including the Ras/Raf/MEK/MAPK, phosphatidyl inositol 3-

kinase (PI3K)/Akt/mTOR, Janus kinase (JAK)/STAT, phospholipase C/PKC, IKK/NF-κB, transforming growth factor β (TGF-β)/Smads, and Wnt/β-catenin pathways [20]. Disruption of these pathways not only contributes to carcinogenesis, but also induces telomerase activation in various tumors (Fig. **6**).

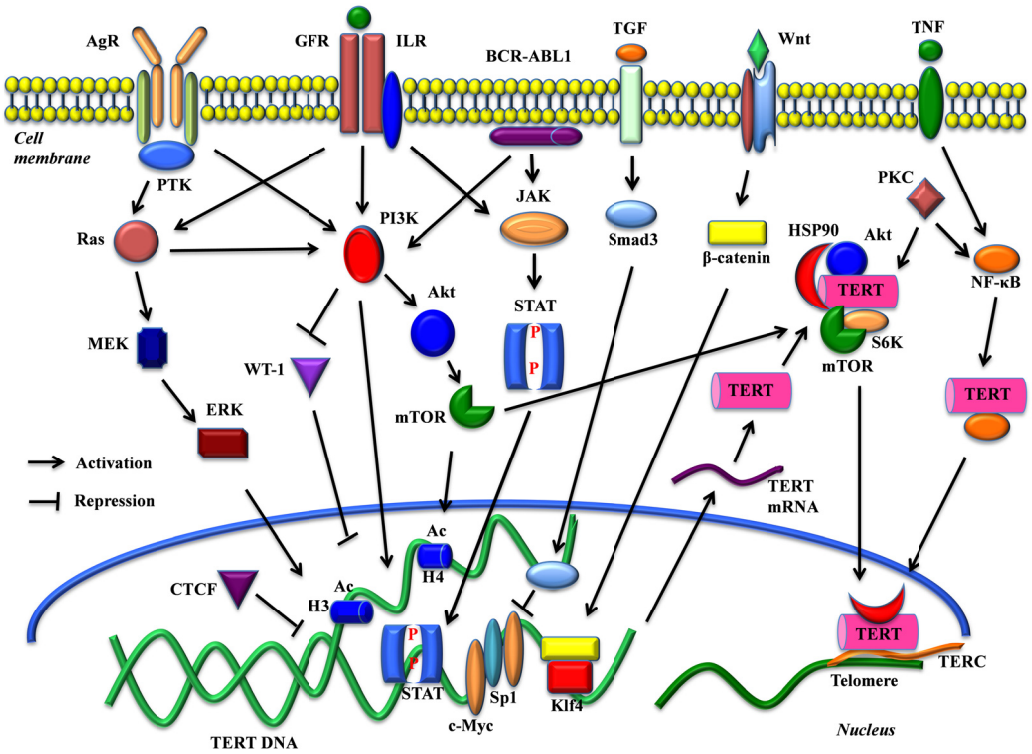


Figure 6: The regulation of TERT through multiple signal transduction pathways. TERT expression is regulated at the transcriptional and post-transcriptional levels. The Ras/RAF/MEK/ERK1/2, PI3K/Akt/mTOR, JAK/STAT, NFκB, PKC, TGFβ/Smads, and Wnt/β-catenin signaling pathways are implicated in the regulation of telomerase in response to growth factor, cytokine, and oncoprotein stimulation. These signaling pathways up-regulate TERT expression by recruiting critical transcription factors such as c-Myc and Sp1 to the TERT promoter. They also mediate the phosphorylation, activation and nuclear translocation of TERT by associating with several signaling proteins such as Akt, mTOR, and NFκB. There may also be some interplay between these signaling pathways (modified from Ref. 20). Ac, acetylation; AgR, antigen receptor; GFR, growth factor receptor; ILR, interleukin receptor; TNF, tumor necrosis factor receptor.

Ras, a small GTP-binding protein, plays a pivotal role in diverse physiological reactions and is mutated during oncogenic transformation [170]. Upon growth

factor stimulation, GTP-bound Ras sequentially activates its downstream effectors Raf, MEK, and MAPKs, particularly extracellular-regulated kinases 1 and 2 (ERK1/2). MAPK mediates epidermal growth factor-induced stimulation of Est-dependent TERT transcription, leading to telomerase activation [171]. Similarly, vascular endothelial growth factor upregulates telomerase activity in ovarian cancer cell lines *via* ERK1/2 and Sp1-mediated transcriptional activation of TERT [172]. MAPK is involved in the estrogen receptor β-mediated anti-apoptotic role of TERT in pancreatic cancer cells [173]. MAPK may also regulate TERT in lymphocytes; in concanavalin A-stimulated T-cells, phosphorylation of histone H3 at Ser10 by ERK1/2, followed by acetylation of H3 at Lys14, induces TERT expression [138]. ERK1/2 also mediates the activation of TERT induced by latent membrane protein-1 (LMP-1) in Epstein-Barr virus (EBV)-infected B-cells [174]. Furthermore, MAPKs other than ERK1/2, such as c-Jun N-terminal kinase and p38 MAPK, are associated with TERT expression [175-177]. Finally, the MAPK signaling pathway appears to play a role in the regulation of TERC transcription [141].

The PI3K/Akt/mTOR kinase cascade is one of the major pathways that regulates cell proliferation, growth, survival, metabolism, and autophagy; this pathway plays a pivotal role in tumorigenesis and hematopoietic malignancies [178]. PI3K generates PI-3,4,5-triphosphate, which recruits 3-phosphoinositide-dependent kinase 1 and Akt kinase to the cytoplasmic membrane. Akt kinase is activated *via* phosphorylation at its Thr308 and Ser473 residues by 3-phosphoinositide-dependent kinase 1 and mTORC2, respectively. Fully activated Akt kinase phosphorylates multiple downstream targets, including mTOR kinase, which then forms mTORC1 or mTOTC2 complexes. There is convincing evidence that the PI3K/Akt/mTOR pathway is involved in the regulation of TERT mRNA expression and post-transcriptional modification. The TERT protein contains two putative Akt kinase phosphorylation motifs (220-GARRRGGSAS-229 and 817-AVRIRGKSYV-826); therefore, it is likely that TERT activity is regulated through Akt-dependent phosphorylation at these sites, particularly Ser227 and Ser824 [150]. Indeed, a TERT synthetic peptide containing the Ser824 residue can act as a substrate for activated Akt kinase [150]. E2 estradiol activates TERT mRNA expression and telomerase activity *via* the PI3K/Akt cascade in ovarian

cancer cells and neuronal PC12 cells expressing the estrogen receptor [179, 180]. E2-induced phosphorylation of TERT mediated by the PI3K/Akt/NF-κB cascade accelerates the nuclear translocation of TERT [179]. Consistent with this finding, TERT contains a bipartite nuclear localization signal at amino acids 222-224 (222-RRR-224) and 236-240 (236-KRPRR-240), which is aligned in tandem [181]. Akt-mediated phosphorylation of TERT at Ser227 is required for nuclear translocation of TERT [181]. Akt-related proteins are also involved in TERT regulation. The tumor suppressor phosphatase and tensin homolog negatively regulates the PI3K/Akt pathway by dephosphorylating PI-3,4,5-triphosphate. DJ-1, a regulator that suppresses phosphatase and tensin homolog, activates TERT transcription in renal carcinoma cells by inducing the expression of phosphorylated Akt and c-Myc [182]. HSP90 is a chaperone protein that is necessary for TERT mRNA expression, nuclear translocation of TERT, and the formation of a functional telomerase complex [153, 183, 184]. Therefore, the physical interaction of Akt and HSP90 with TERT is essential for maintaining telomerase activity and thus protecting against cellular apoptosis [185]. Inhibition of mTOR, a major target of Akt kinase, downregulates TERT mRNA expression in endometrial cancer cells [186]. Furthermore, as mentioned above, our own and other groups have found that activated TERT forms functional complexes with Akt, HSP90, mTOR, and S6K, and that telomerase activity is inhibited by disruption of the complexes, suggesting a critical role of the PI3K/Akt/HSP90/mTOR pathway in the regulation of telomerase [123, 154].

The JAK-STAT signaling pathway has been implicated in the regulation of TERT in various tumors, including hematologic malignancies [20]. Cytokine and growth factor stimulations induce activation of JAK-STAT signaling in a variety of cells. Once activated by cytokines, JAK phosphorylates tyrosine residues of target receptors, itself, and members of the STAT family, including STAT1-4, STAT5A, STAT5B, and STAT6. Phosphorylated STAT proteins dimerize and translocate to the nucleus, where they function as transcriptional activators. STAT3 regulates the expression of TERT in a variety of human cancer and primary human cells. Chromatin immunoprecipitation assays of glioblastoma cells revealed that STAT3 binds directly to consensus binding sites in the TERT promoter, and telomerase activity is abolished by treatment of these cells with STAT3 siRNA [187]. Our

group has demonstrated that STAT3 and STAT5 are released from the TERT promoter during leukemic cell differentiation [188]. Moreover, STAT5 binds to the TERT promoter and activates telomerase in response to IL-2 stimulation of adult T-cell leukemia (ATL) cells [124].

Other signaling molecules, such as PKC, NF-κB, PP2A, Abl, Src, TGF-β and Wnt/β-catenin, also affect telomerase activity. Phorbol myristate acetate, a PKC activator, increases telomerase activity in T-cells, and the effect is abolished by treatment with a PKC inhibitor [189]. PKCα phosphorylates TERT and enhances telomerase activity both *in vitro* and in tumor cells [151]. The PKCβ, δ, ε, and ζ isoforms also upregulate TERT activity *via* phosphorylation- or transcription-dependent mechanisms [190, 191]. NF-κB is regulated by the suppressor IκB and the upstream activator IKK. PKCθ-activated NF-κB induces activation of TERT expression through a T-cell antigen receptor signaling pathway [192]. NF-κB also plays a critical role in the transcriptional regulation of TERT in human T-cells infected with lymphotropic virus type 1 (HTLV-1) and in the translocation of TERT from the cytoplasm to the nucleus in myeloma cells [155, 193]. In a study using breast cancer cell lines, PP2A abolished telomerase activity in nuclear extracts in a time- and concentration-dependent manner, and the inhibition of PP2A by okadaic acid reversed this effect [194]. A recent study demonstrated that the interaction of the catalytic subunit of PP2A and its scaffolding protein (PR65) with TERT is involved in the inhibitory effects of this kinase on telomerase activity [195]. The mechanism of telomerase inhibition by PP2A also appears to involve cytoplasmic accumulation of TERT and our group has demonstrated that DNA damage of retinoblastoma cells induced by doses of ionizing radiation above 10 Gy triggers PP2A activation, resulting in dephosphorylation of TERT and decreased telomerase activity [196]. In response to DNA damage, the SH3 domain of c-Abl binds to a proline-rich sequence (308-PSTSRPPRP-316) in TERT and phosphorylates the protein, leading to a reduction in telomerase activity [197]. Moreover, Src kinase phosphorylates TERT and promotes its export from the nucleus, while Shp-2 inhibits this process [140]. Recently, TERT has been shown to localize to mitochondria, where it protects cells against oxidative stress by potentiating antioxidant defense systems [198]. In human endothelial cells, Src kinase is activated by H_2O_2 and phosphorylates Tyr707 of

the mitochondrial TERT protein, resulting in downregulation of TERT [199]. TGF-β, which inhibits cell proliferation and promotes cellular differentiation, accelerates the nuclear import of Smad3, which is recruited to the TERT promoter following c-Myc interaction, resulting in repression of TERT transcription [200]. Recently, the Wnt/β-catenin signaling cascade has also been shown to regulate telomerase in embryonic stem cells and tumor cells. Interaction of β-catenin with the transcription factor Klf4 promotes its localization to the TERT promoter; the subsequent recruitment of histone methyltransferase affects chromatin modifications and upregulates TERT transcription [201].

5. TELOMERASE DEREGULATION IN HEMATOLOGIC MALIGNANCIES

5.1. Acute Leukemia and Myelodysplastic Syndrome

Acute myeloid leukemia (AML) is a hematologic malignancy with heterogeneous biological features, including the occurrence of distinct cytogenetic backgrounds during leukemogenesis. The development of leukemia requires at least two molecular changes: i) genetic aberrations characterized by specific chromosomal translocations, and ii) alterations in the expression levels of growth-related genes, such as those caused by mutations in the *FLT3* gene, which confer a growth advantage through the activation of various signaling pathways. Telomere and telomerase dysfunctions correlate with genomic instability and may be associated with hematological disease progression [19]. Telomeres are typically shorter in leukemic cells than normal cells [109]; in our study of 12 AML and ALL patients, the average telomere lengths in leukemic cells and normal peripheral blood mononuclear cells were 4.7-4.8 kb and 7.9-8.5 kb, respectively [97]. In another study, the age-adjusted telomere length in 137 untreated AML patients was significantly lower than that of matched controls, and patients with chromosomal aberrations had significantly shorter telomeres than patients with a normal karyotype [113]. The shortest telomeres were found in patients with complex aberrations, suggesting that telomere length is a prognostic marker of AML. When classified according to the French-American-British classification system, telomere lengths are reportedly shortest in the monocytic M5 subtype of AML. In addition, *FLT3* mutations appear to correlate with shorter telomere lengths. On the other hand, elevated telomerase activity has been found in approximately 75% of

patients with acute leukemia [12, 97]. The high levels of telomerase activity in AML patients return to normal upon disease remission, and increased telomerase levels are associated with poor prognosis [202]. Compared with AML patients, telomerase activity is lower and telomere length is shorter in ALL patients [19, 203]. However, in childhood, ALL patients have significantly higher levels of TERT mRNA expression than AML patients [204]. Furthermore, telomere shortening has been implicated in delayed hematopoietic reconstitution in leukemia patients undergoing allogeneic hematopoietic stem cell transplantation [100]. In any case, higher telomerase levels, shorter telomere lengths, and higher TERT levels are likely to be associated with poor prognosis [19, 204].

In myelodysplastic syndrome (MDS) patients, the level of telomerase activity is relatively low in bone marrow cells, but telomere lengths are shorter than those in normal control patients. Telomere shortening tends to be associated with specific chromosomal abnormalities and disease progression, indicating that short telomere lengths are a useful biomarker of MDS [12, 17, 205]. High risk MDS subtypes express high levels of the heterogeneous nuclear RNP B1, suggesting that this protein is related to leukemic transformation in these patients [206].

Recently, genetic mutations in telomerase components, including dyskerin, TERC, and TERT, have been reported to play a role in the pathogenesis of bone marrow failure syndromes and AML [207, 208]. A significant proportion of AML patients have mutations in the *TERT* gene, of which A1062T is the most common; these mutations can cause loss of telomerase activity and subsequent telomere shortening, and may predispose for hematologic malignancies, including AML [207].

A number of studies have examined the role of signaling pathways in the regulation of telomerase using AML cell lines or primary cells. DNA methylation inhibitors reduce TERT expression and telomerase activity, and the level of methylation in the TERT promoter differs in specific leukemia cell types. AML primary cells have significantly lower methylation levels at CpG islands in the *TERT* gene than control cells, suggesting that direct methylation of the TERT promoter is unlikely to occur in AML patients [209]. Akt kinase phosphorylates TERT and facilitates its translocation into the nucleus in AML cell lines [210].

The HSP90 co-chaperone p23 is over-expressed in bone marrow leukemia cells from childhood ALL patients [211]. Because p23 and HSP90 are required for telomerase activity, these findings suggest that p23 affects telomerase function in ALL patients. Furthermore, erythropoietin (EPO) induces *TERT* gene transcription *via* the JAK2/STAT5/c-Myc pathway and TERT protein phosphorylation *via* the PI3K/Akt pathway in EPO-responsive erythroleukemia cells [212], and EPO-induced transcriptional activation of TERT is repressed when c-Myc is downregulated *via* the TGF-β/Smad3 pathway [213]. Our group has demonstrated that AML patients have high expression levels of STAT3 and STAT5 and elevated telomerase activity [214]. Phosphorylation of STAT5 is associated with the expression of P-glycoprotein and TERT, suggesting that STAT5 is a transcriptional activator of these genes. Finally, telomerase is down-regulated during leukemic cell differentiation induced by agents such as vitamin D3, all-trans retinoic acid, and 12-O-tetradecanoylphorbol-13-acetate [188, 215]. Although PKC and Akt are both active in HL60 promyelocytic leukemia cells, transcriptional silencing of the *TERT* gene during cell differentiation renders these kinases incapable of activating telomerase in differentiated HL60 cells [215]. The mechanism of transcriptional silencing of TERT in differentiated cells involves the dissociation of STAT3 and STAT5, but not SP1, from the TERT promoter.

5.2. Chronic Myeloid Leukemia

Chronic myeloid leukemia (CML) is a clonal hematological stem cell disorder characterized by the presence of the BCR-ABL1 fusion gene. This fusion gene is generated by a reciprocal translocation [t(9;22)(q34;q11)] known as the Philadelphia chromosome, which produces the p190, p210, and p230 chimeric proteins [216]. CML is a good model for investigating the molecular mechanisms of leukemogenesis and drug resistance. Although telomere length and telomerase activity are altered in CML, the levels of these aberrations differ between the progressive chronic phase (CP), accelerated phase (AP), and blast phase (BP) of the disease. Telomeres in Philadelphia chromosome-positive cells are shorter than those in normal leukocytes, and age-adjusted telomere shortening correlates with the disease stage [91]. Telomeres in AP and BP patients are significantly shorter than those in CP patients, and telomere length correlates with the prognostic score

at diagnosis, suggesting a high turnover of leukemia cells [217]. Similarly, telomerase activity is higher in bone marrow leukemia cells from BP patients than those from CP patients, suggesting that it is a prognostic marker of shortened survival as well as rapid progression to the BP of the disease [12, 15, 19, 218]. The BCR-ABL1 chimeric proteins exhibit protein tyrosine kinase activities and play a central role in leukemogenesis by activating multiple downstream signaling pathways. Therefore, specific inhibitors of the BCR-ABL tyrosine kinases are ideal therapeutic agents for CML. Indeed, several protein tyrosine kinase inhibitors (TKIs), including imatinib, nilotinib, and dasatinib, have been shown to effectively eradicate leukemia cells and result in high molecular remission in CML CP patients [219]. TKIs such as imatinib inhibit TERT mRNA expression and thus telomerase activity in CML cell lines and primary cells [218, 220]. The inhibition of telomerase by imatinib is less effective in BP patients than CP patients, suggesting that increased telomerase activity is related to imatinib resistance [218]. Imatinib-induced telomerase inhibition is mediated in part through suppression of the PDK-1/Akt cascade [221]. Our group and others have found that STAT5 plays a crucial role in the regulation of *TERT* gene expression in CML cells [220, 222]; STAT5 activation induces upregulation of the *TERT* gene, as well as the gene encoding P-glycoprotein (*MDR1*), which is related to drug resistance, and knockdown of STAT5 recovers imatinib sensitivity [222]. These findings suggest that STAT5 confers imatinib resistance to leukemic cells *via* increased transcription of TERT and *MDR1*, and indicate that STAT5 is a promising therapeutic target for TKI-resistant CML patients. In addition, PKC and acetylation of multiple signaling molecules, such as histone H7, BCR-ABL, HSP90, p53, FAK kinase, and Rb protein, may play a role in the regulation of telomerase activity and the mechanism of imatinib resistance [223, 224].

5.3. Chronic Lymphocytic Leukemia

B-cell chronic lymphocytic leukemia (B-CLL) is a mature B-cell neoplasm characterized by a heterogeneous clinical course; the disease is divided into two subgroups according to the presence of somatic mutations in the gene encoding the immunoglobulin heavy chain variable (IGHV) protein [225]. B-CLL cells

have shorter telomeres than normal B-cells, and telomere length correlates with the survival rates of these patients [19]. In addition, although low telomerase activity is observed at the early-stages of B-CLL, elevated telomerase activity is associated with the advanced stages and poor prognosis [15]. Indeed, telomere lengths are inversely correlated with the levels of telomerase activity, and short telomeres/high levels of telomerase activity are independently associated with the rapid progression of B-CLL [226]. Compared with mutated *IGHV* cases, higher telomerase levels and shorter telomeres are frequently found in non-mutated *IGHV* cases, consistent with the poor prognosis of the latter group. Furthermore, telomere shortening appears to correlate with expression of telomere shelterin complex genes (*TRF1*, *TRF2* and *POT1*) and cytogenetic profiles [227]. A striking reduction in telomere length and shelterin gene expression occurs in B-CLL patients who have cytogenetic profiles associated with poor prognosis, such as multiple chromosomal aberrations and p53 or ATM gene deletions. B-cell antigen receptor (BCR) signaling also plays a pivotal role in the proliferation and survival of B-CLL cells [228, 229]. BCR signaling increases telomerase activity and promotes cell survival and proliferation in non-mutated B-CLL patients [230]. BCR-induced activation of telomerase is blocked by LY294002, a PI3K/Akt inhibitor, suggesting the involvement of the PI3K/Akt pathway in this activation. In addition to B-CLL, patients with T-cell prolymphocytic leukemia, a rare aggressive leukemia derived from post-thymic mature T-lymphocytes, have shorter telomeres and elevated telomerase activity [231].

5.4. Malignant Lymphoma

Malignant lymphomas include a variety of subtypes of B-cell and T/NK-cell neoplasms that originate from mature B-cells, T-cells, and NK-cells; these subtypes are grouped and defined according to the World Health Organization's classification system [232]. Malignant lymphomas often retain the biological features of their normal counterparts; however, shortened telomeres and/or elevated telomerase levels are observed in most type of lymphomas [14, 18, 19, 123, 233]. Furthermore, a recent study found that non-Hodgkin lymphoma patients have higher copy numbers of the *TERC* gene than patients in remission, suggesting the involvement of *TERC* amplification in lymphomagenesis [234].

5.4.1. B-Cell Lymphomas

Naïve B-cells, which have not encountered an antigen and reside in the interfollicular area in lymphoid tissues or migrate through the peripheral blood, and antigen-primed memory B-cells, which are long-lived and reside in the marginal zone of lymphoid tissues or recirculate through the peripheral blood, have barely detectable levels of telomerase activity [60, 235]. By contrast, antigen-activated germinal center (GC) B-cells have high levels of telomerase activity and longer telomere lengths than naïve B-cells [235, 236]. In addition, B-cell receptor ligation and cytokine stimulation upregulate telomerase activity in naïve and memory B-cells [237]. With the exception of some low-grade malignancies such as marginal zone B-cell lymphoma, most B-cell lymphomas have high levels of telomerase activity that are similar to those of reactive lymph nodes [238, 239]. Telomerase activity tends to be higher in high-grade lymphomas, in particular Burkitt lymphoma (BL), than other lymphomas [12, 19, 239]. The levels of telomerase activity in high-grade lymphomas are comparable to those in normal GC B-cells; therefore, the significance of high telomerase activity in B-cell lymphomas should be interpreted carefully.

A number of studies have examined telomere lengths in B-cell lymphomas. In a study of 123 mature B-cell lymphoproliferative disorder samples, the median telomeric terminal restriction fragment length was approximately 6 kb; the fragment lengths were greater in diffuse large B-cell lymphoma (DLBCL), BL, and follicular lymphoma (FL) than mantle cell lymphoma (MCL) [240]. In a study of 223 B-cell lymphomas/leukemias, the telomeres were shorter in non-GC-like DLBCL than FL, and were longest in GC-like DLBCL [241]. Notably, in a study of 44 patients with FL and nine patients with DLBCL secondary to FL, those with *BCL2* gene rearrangements had significantly longer telomeres than those without [242]. These findings indicate that telomeres are longer in GC-derived lymphomas than non-GC-derived lymphomas, although telomere shortening is observed in both of these B-cell lymphoma types. It should be noted that telomere length can vary substantially between patients with the same lymphoma type [243].

Mechanisms by which TERT is activated in B-cell lymphomas have been reported by a number of groups. The *TERT* gene, which is located at chromosome 5p13.33, is deregulated by chromosomal translocations in several B-cell tumors [244, 245]. Rearrangement of the TERT-cleft lip and plate transmembrane 1-like locus at chromosome 5p1 is associated with MCL and splenic marginal zone lymphomas, and tumors with breaks in this region display elevated TERT mRNA expression and telomerase activity, suggesting that TERT is involved in B-cell lymphomagenesis [244]. The LMP-1 oncoprotein, which is encoded by the *EBV* gene, transactivates the TERT promoter *via* an NF-κB-c-Myc-dependent mechanism; this upregulation of telomerase may contribute to lymphomagenesis in EBV-related lymphomas [15, 18, 246]. PAX5, a B-cell-specific transcription factor, also binds to the TERT promoter region and induces transcription of the gene [247]. Finally, latency-associated nuclear antigen, which is encoded by human herpes virus-8, transactivates the TERT promoter *via* a direct interaction with SP1 and induces TERT expression in BL-derived and primary effusion lymphoma-derived cell lines [18].

5.4.2. T/NK-Cell Lymphomas

Although elevated telomerase activity and expression levels of TERT mRNA are found in $CD4^+CD8^+$and $CD4^+CD8^-$ thymocytes, peripheral T-cells have barely detectable levels of telomerase activity [248]. Telomeres are shorter in naïve T-cells than memory T-cells, probably due to replication senescence [249]. However, TERT expression and telomerase activity can be upregulated by mitogens, cytokines, and stimulation of antigen receptors [121]. Indeed, anti-CD3 and anti-CD28 antibodies, phytohaemagglutinin, concanavalin A, IL-2, IL-15, and IL-17 upregulate TERT expression and telomerase activity in human T-cells [121, 250, 251].

Evidence of deregulation of telomeres and telomerase in T/NK-cell lymphomas is relatively limited. Anaplastic large cell lymphoma is associated with the presence of TERT mRNA variants; in a study of 38 anaplastic large cell lymphoma samples, TERT mRNAs were identified in over 90% of samples with ALK translocations but only approximately 60% of those without, suggesting an association of TERT expression with ALK translocation [252]. In another case,

high telomerase activity and telomere shortening were identified in skin-homing T-cells and peripheral blood lymphocytes from cutaneous T-cell lymphoma patients, even those with early stage disease [253].

ATL is an aggressive T-cell tumor caused by HTLV-1 that can be classified into four subtypes: smoldering, chronic, lymphoma, and acute [254, 255]. A study of 22 ATL patients and 13 asymptomatic HTLV-1 carriers revealed that telomerase activity is much higher in acute ATL patients than chronic ATL patients or HTLV-1 carriers, and telomere length is significantly shorter in acute and chronic ATL patients than HTLV-1 carriers and healthy volunteers [256]. Importantly, high telomerase activity and shortened telomere length are associated with poor prognosis. Tax, a protein encoded by the HTLV-1 genome, plays a central role in transforming $CD4^+$ T-lymphocytes [18] and IL-2 is required for the proliferation of HTLV-1-infected T-cells. There are conflicting reports of the effect of Tax on telomerase activity/expression. Gabet *et al*. [257] showed that Tax inhibits telomerase activity in transfected tumor cells by competing with c-Myc for binding to the E-box in the TERT promotor. Conversely, Sinha-Datta *et al*. [193] showed that Tax activates the TERT promoter through NF-κB-mediated activation of SP1 and c-Myc in IL-2-independent cells transformed by HTLV-1 and primary T-cells transduced by HTLV-1. This discrepancy may be explained by the evidence that Tax activates the TERT promoter in quiescent but not proliferating T-cell lines, indicating its association with the cell cycle status [258]. In acute phase ATL, T-cells expressing Tax can be eliminated by host immune surveillance due to Tax immunogenicity. Indeed, most ATL cells do not express Tax, despite having high telomerase activity. The HTLV-1 bZIP (HBZ) protein, which is encoded by the minus chain of the HTLV-1 genome and is frequently expressed in ATL cells, forms heterodimers with the transcription factor JunD and activates TERT transcription by binding to GC-rich SP1-recognition sites in the proximal region of the TERT promoter [259]. HBZ also acts as a suppressor of menin, the product of the tumor suppressor *MEN-1* gene that downregulates *TERT* gene transcription [260]. These actions of HBZ could be one explanation for Tax-independent elevated telomerase activity in ATL cells. Other pathways may also play a central role in upregulating telomerase activity in Tax-negative HTLV-1-infected cells or ATL cells [124, 261]. For example, in IL-2-dependent HTLV-1-

immortalized cell lines, IL-2-stimulated activation of PI3K induces cytoplasmic retention of the WT1 protein, a strong repressor of the TERT promoter, leading to increased TERT expression [261]. Furthermore, our group has demonstrated that STAT5 binds directly to the TERT promoter, and siRNA-mediated knockdown of STAT5 inhibits TERT transcription in IL-2-responsive Tax-negative ATL cells [124]. The JAK/STAT and JAK/PI3K/Akt/HSP90/ mTORC1 pathways are both involved in IL-2-induced activation of telomerase in ATL cells, suggesting that these signaling proteins may be promising molecular therapeutic targets for IL-2-dependent ATL.

The role of the deregulation of telomerase in NK-cell tumors has not been elucidated fully; however, our previous study demonstrated that the PI3K/Akt/mTOR pathway plays a critical role in regulating telomerase activity in NK-cell lymphoma cells [123]. In this study, telomerase activity in a human NK-cell line (NK-92), which requires IL-2 for proliferation, was increased after stimulation with IL-2, and the levels of TERT mRNA and protein correlated with telomerase activity. Inhibitors of PI3K prevented IL-2-induced telomerase activation and TERT expression. In addition, inhibition of HSP90 and/or mTORC1 prevented IL-2-induced telomerase activity and nuclear translocation of the TERT protein, but not TERT expression. Furthermore, TERT was shown to form a complex with Akt, HSP90, mTOR, and S6K in an IL-2-dependent manner. These findings indicate that IL-2 activates TERT at the transcriptional and post-translational levels in NK-cell tumors *via* the PI3K/Akt/HSP90/mTORC1 pathway.

5.5. Multiple Myeloma

Multiple myeloma (MM) is a clonal B-cell neoplasm characterized by the infiltration of atypical plasma cells into the bone marrow (BM) and associated with the presence of monoclonal protein in the serum or urine or both [262, 263]. MM constitutes approximately 10% to 15% of all hematologic malignancies and about 1% of all cancers [264]. Monoclonal gammopathy of undetermined significance (MGUS) progresses to MM at a rate of about 1% of cases per year, with an actuarial probability of malignant evolution of 30% at 25 years [264]. MM arises from a stepwise process of neoplastic progression with accumulation

of several genetic events that confer proliferative advantages and lead to the expansion of mutant plasma-cell clones [263]. Cells positive for CD138 isolated either at diagnosis or during relapse from 183 patients with MM had heterogeneous telomerase activity: compared with telomerase activity in the SK-N-SH neuroblastoma cell line used as a positive control, telomerase activity in patients with MM was less than 5% of control in 36% of patients, 5% to 100% of control in 51% of patients, and greater than 100% of control in 13% of patients [265]. Xu *et al*. [266] have reported that telomerase activity was increased in samples from 21 of 27 patients with MM and from all 4 patients with plasma cell leukemias but was not increased in samples from any of the 5 patients with MGUS. These results suggest that telomerase activation plays a role in the malignant transformation of MGUS to MM [266].

Analysis of telomere length in $CD138^+$ cells isolated from 115 patients with MM and 7 healthy donors showed a significant reduction in telomere length in MM [265]. The median telomere length was 5.3 kb (range, 3.0 to 15.0 kb). Telomeres were shorter than 4.0 kb in 25% of the patients. In contrast, healthy donors had long telomeres, ranging from 9.6 to 11.3 kb, with a median length of 10.6 kb. Telomere length is negatively correlated with telomerase activity in patients with MGUS or MM [264, 265]. Moreover, telomere length is negatively correlated with age and β_2-microglobulin titers [265]. Although interleukin 6 (IL-6) plays an important role in the pathogenesis of MM, the serum level of IL-6 does not correlate with telomerase activity but does negatively correlate with telomere length. Patients with MM who had high telomerase activity and short telomeres have a poor prognosis [264, 265]. The patients with telomerase activity less than 25% of that in neuroblastoma control cells and a telomere length greater than 5.5 kb had a 1-year survival rate of 82%; in contrast, patients with higher telomerase activity and shorter telomeres had a significantly lower 1-year survival rate of 63% (P=0.004). The 2-year survival rate was 81% in patients with telomerase activity less than 25% of that in control but was 52% in patients with telomerase activity higher than that in control. Shiratsuchi *et al*. [267] have also reported that telomerase activity was significantly increased in patients with MM who had a serum β2-microglobulin level greater than 6 mg/dL or had stage III disease (P=0.002). They also found that telomerase activity was significantly correlated

with Ki-67 positivity and the percentage of plasma cells in the BM [267]. Therefore, telomerase activity in MM might be a reliable marker of proliferative capacity and a useful prognostic factor.

Changes have also been reported in telomere maintenance genes, including TRF1, TRF2, and TANK1, in patients with MGUS and MM [264]. TRF1 serves as a negative length regulator that limits telomere elongation and stabilizes telomere length. TRF2 is a negative regulator of telomere length as well but also participates in T-loop formation and in capping and protecting the 3' single-strand overhang. Increased TRF2 expression induces telomere shortening, but its downregulation leads to telomere-telomere fusions, a finding that suggests that TRF2 plays a protective role in maintaining telomere structure and function. TANK1, on the other hand, is a positive regulator of telomere length, and its over-expression progressively elongates telomeres in human cells. Patients with MGUS show increased expression of TRF1 and decreased expression of TRF2 and TANK1 compared with those in patients with MM [264]. In MM, the rate of BM infiltration and the Ki-67-positivity index are positively correlated with the expression of TRF2, TANK1, and human TERT and are negatively correlated with telomere length [264]. In patients with MM, TRF1 expression is increased and is associated with decreased telomere length, regardless of TERT expression, suggesting the possible participation of TFR1 in the maintenance of short telomeres [264]. Positive correlations between TRF2 and TANK1, TRF2 and TERT, and TANK1 and TERT have been found in both patients with MGUS and patients with MM [264].

Recent studies demonstrate that various growth factors, including IL-6, insulin-like growth factor-1 (IGF-1), vascular endothelial growth factor, tumor necrosis factor α, transforming growth factor β, and stromal cell-derived factor 1α, play important roles in cell growth, survival, and migration in MM. Both IL-6 and IGF-1 are proliferative and survival factors for MM cells [268]. Both IL-6 and IGF-1 are produced at high concentrations in the BM microenvironment by osteoblasts, BM stromal cells, and bone endothelial cells. We have shown that IL-6 and IGF-1 increase telomerase activity in the MM.1S and U266 MM cell lines without alteration of TERT proteins [268]. These cytokines increase telomerase activity *via* TERT transcriptional regulation through NF-κB and *via* TERT

posttranscriptional regulation (phosphorylation) through the PI3K/Akt signaling pathway. Moreover, Hsp90 is a molecular chaperon, required to attain the specific conformation, stability, and proper functioning of telomerase [269]. The Hsp90 inhibitor 17-(allylamino)-17-demethoxygeldanamycin (17-AAG; also known as tanespimycin) suppresses IGF-1-induced telomerase activity through the dual regulation of TERT [269]. The nuclear localization of TERT is required for telomerase to elongate telomeres. Several proteins that regulate the nuclear localization of TERT have been identified. Our previous study in an MM cell line has shown that tumor necrosis factor α induces telomerase activity through TERT phosphorylation and that phosphorylated TERT bound to NF-κB is translocated from the cytoplasm to the nucleus [155].

Corticosteroids and conventional chemotherapy with such regimens as high-dose dexamethasone; melphalan and prednisolone; and vincristine, doxorubicin, and pulsed high-dose dexamethasone provide the basis for the therapeutic management of MM. Dexamethasone reduces telomerase activity by inhibiting TERT messenger RNA expression before apoptosis is induced [268]. Although both apoptosis and dexamethasone-induced downregulation of telomerase activity are abrogated by IGF-1 and IL-6, the protective effects of these cytokines against dexamethasone-induced downregulation of telomerase activity are blocked by both wortmannin and the specific IκB kinase inhibitor PS-1145 [264]. The proteasome inhibitor bortezomib is a 20S proteasome complex inhibitor that also inhibits NF-κB, thereby interfering with NF-κB-mediated cell survival, tumor growth, and angiogenesis [270]. Bortezomib is the first clinically used proteasome inhibitor and is effective against MM [270]. Bortezomib inhibits growth and decreases telomerase activity in ARP-1, CAG, U266, and RPMI8226 MM cell lines and in MM cells obtained from patients [270]. Telomerase inhibition is associated with downregulation of TERT transcription through decreased binding of SP-1 but not of Myc and NF-κB in ARP-1 and CAG cells [270]. Moreover, phosphorylation of TERT protein is inhibited by bortezomib in ARP-1 cells, and this inhibition may be related to PKC α, but not to Akt [270].

We have reported the effect of GRN163L, a palmitoyl (C16) lipid-attached N3'→P5' phosphoramidate oligonucleotide that targets the template RNA component (TERC) in MM cells [271, 272]. GRN163L inhibits telomerase

activity in MM.1S, U266, and RPMI8226 cell lines and in cells from patients with MM [271]. The exposure of MM cells to GRN163L effectively inhibits telomerase activity, reduces telomere length, and induces apoptotic cell death after 2 to 4 weeks [271, 272]. However, GRN163L inhibits the growth of MM.1S cells with short telomeres (2.5 kb) but not that of U266 cells with long telomeres (9.0 kb) [271]. Comprehensive and sequential gene-expression analysis has shown that progressive telomere shortening, leading to growth inhibition and cell death in MM.1S cells, is associated with upregulation of *p21*, *MAD2*, *replication factor C*, *Cdc27*, and *cyclin E binding protein* and with downregulation of *Cdc27* [271]. Moreover, this telomere shortening is also associated with increased expression of phosphorylated p53 (Ser15) and p21 protein [271]. In 2 murine models of human MM, GRN163L significantly reduced tumor cell growth and increased survival compared with those in untreated control mice [272]. The Hsp90 inhibitor 17-AAG enhances GRN163L-induced growth arrest in MM cell lines. Pretreatment with GRN163L for 1 week, before treatment with 17-AAG, led to complete growth arrest of MM cells within 4 days in, whereas cells not pretreated with GRN163L continued to grow [272]. These findings provide a rationale for combining GRN163L with other agents able to affect telomere shortening or genomic integrity to significantly enhance the killing of tumor cells.

6. TELOMERASE INHIBITORS

Telomerase-targeting therapy can have antitumor activity. A possible advantage of telomerase-targeting therapy is the safety margin based on the differences in telomerase activity and telomere length between tumor cells and normal cells, but a possible disadvantage is that the time lag between drug administration and clinical response can be long [273]. The clinical response to telomerase inhibition may be delayed owing to the time needed to achieve critical telomere shortening, which depends on telomere length and the potential kinetics of cell turnover [273]. Telomere DNA is shortened by approximately 100 bp with each cell division. The shortest telomeres in a cell will trigger a DNA damage checkpoint or genomic instability or both following telomerase inhibition [273]. The mechanism and clinical trials of telomerase inhibitors are demonstrated in Table **3**.

Table 3: Telomerase inhibitors

Compound	Mechanism	Clinical Trial
G-Quadruplex-Interacting Agent TMPyP4 SYUIQ-5 RHPS4 BRACO-19 Telomestatin	Stabilization of G-quadruplexes.	
Catalytic Inhibitor BIBR1532	Inhibition of active site in enzyme.	
Reverse Transcriptase Inhibitor Azidothymidine	Block of dNTP incorporation into DNA.	
Antisense Oligonucleotide Imetelstat (GRN163L)	hTR antagonist.	chronic lymphocytic leukemia (Phase I/II) multiple myeloma (Phase I) solid tumors or lymphoma (Phase I) breast cancer (Phase II) non-small-cell lung cancer (Phase II) essential thrombocythemia or polycythemia vera (Phase II).
Immunotherapy		acute myeloid leukemia (Phase II).
GRNVAC1	Immunological responses. Autologus dendritic cells transfected TERT or TERT-LAMP fusion mRNA.	
Telomelysin (OBP-401)	Telomerase-specific, replication-specific oncolytic adenovirus.	solid tumors including carcinomas, melanomas, and osteosarcomas (Phase I).
Vx-001	TERT cryptic peptide p572Y-580 and native TERT p572(R)-580.	solid tumors including breast, colorectal, ovarian, head and neck, pancreas, melanoma, hepatocellular, renal, prostate cancers, and non-small-cell lung cancer (Phase I/II).
GV1001	TERT peptide p611-626.	melanoma, non-small-cell lung cancer (Phase I/II) pancreatic cancer (Phase I/II/III).
GX301	four TERT peptides and two adjuvants.	Prostate cancer, renal cancer (Phase I/II).

6.1. G-Quadruplex-Interacting Agents

The 3' overhangs of human telomeres are rich in guanine (G) units and have been shown *in vitro* to form 4-stranded DNA structures termed G-quadruplexes. For

telomere sequences to be added by telomerase, the G-quadruplexes must be dissociated. Thus, drugs that stabilize G-quadruplexes might inhibit telomerase activity by preventing telomerase from using telomere sequences as a substrate. Although several classes of small molecule that interact with G-quadruplex DNA have been reported, in this review we focused on porphyrin derivatives (TMPyP4), cryptolepine derivatives (SYUIQ-5), pentacyclic acridine (RHPS4), trisubstituted acridine derivative (BRACO-19), and telomestatin.

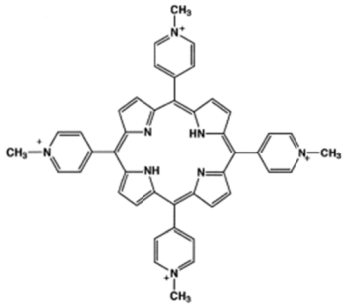
6.1.1 TMPyP4

The cationic porphyrin TMPyP4, 5,10,15,20-tetra-(*N*-methyl-4-pyridyl) porphyrin (Fig. **7**) can bind to and stabilize DNA G-quadruplexes. *In vitro* studies have shown G-quadruplex formation in the promoter or regulatory regions of important oncogenes, such as *c-myc*, *c-myb*, *c-fos*, and *c-abl*, and in the single-stranded G-rich overhang of telomeres [274, 275]. These G-quadruplexes affect essential cellular processes. Thus, G-quadruplex structures are a potential therapeutic target in hematologic malignancies. We have previously reported that TMPyP4 significantly inhibits the growth of K562 cells and TERT-transfected K562 cells [276]. Cell-cycle analysis showed decreases of cells in the G1 phase and increases of cells in the S and G2/M phases after 48 hours and cell death after 72 hours. Treatment with TMPyP4 decreases c-Myc protein expression, increases of $p21^{CIP1}$ and $p57^{KIP2}$ protein expression, and activates p38 MAPK, c-Jun N-terminal kinase, and ERK. Shammas *et al.* [277] have also reported that TMPyP4 is active against the U266, ARH77, and ARD MM cell lines. Telomerase activity in U266, ARH77, and ARD cells was inhibited by less than 10 μM of TMPyP4. Treatment with TMPyP4 had no effect on viability for the first 2 weeks but caused 75% to 90% of cells to die over the following 2 weeks.

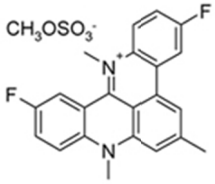
6.1.2. SYUIQ-5

The cryptolepine derivative SYUIQ-5 (*N*'-(10H-indolo (3,2-b) quinolin-11-yl)-*N,N*-dimethyl-propane-1,3-diamine) induces apoptosis in HL-60 and K562 leukemic cells and inhibits the *c-myc* gene promoter and telomerase activity through G-quadruplex interaction [278, 279]. SYUIQ-5 was found to have dose-dependent antitumor activity in a short-term culture, in which rapid and potent DNA damage responses, such as H2AX phosphorylation, were triggered through the dissociation of TRF2

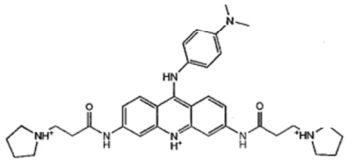
from telomeres [280]. Moreover, SYUIQ-5 induces the genes and proteins of cyclin-dependent kinase inhibitors, including p16, p21, and p27.



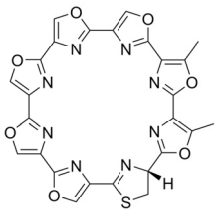
TMPyP4



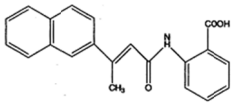
RHPS4



BRACO-19



Telomestatin



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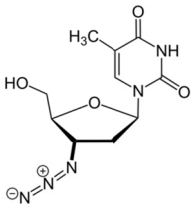

AZT

Figure 7: Structure of telomerase inhibitors. See text for details.

6.1.3. RHPS4

The pentacyclic acridine RHPS4 (3,11-difluoro-6,8,13-trimethyl-8*H*-quino[4,3,2-*kl*]acridinium methosulfate) (Fig. **7**), binds G-quadruplex DNA [281]. By stabilizing G-quadruplexes at telomere DNA, RHPS4 impairs fork progression

and telomere processing and induces telomere damage. The molecular response to telomere DNA damage induced by RHPS4 is the formation of several telomeric foci containing phosphorylated DNA damage-response factors, such as γ-H2AX, RAD17, and 53BP1. Ataxia telangiectasia mutated (ATM)- and Rad3-related ATM signaling is activated to repair RHPS4-induced telomere damage [282]. Overexpression of the telomere-binding protein TRF2 or POT1 antagonizes the antitumor activity of RHPS4 *in vitro* and *in vivo* [281]. Poly-adenosine diphosphate (ADP) ribose polymerase 1 (PARP1) is recruited and activated at telomeres upon G-quadruplexes and form several ADP-ribose polymers that are associated with TRF1 [283]. Both PARP inhibitors and PARP1-specific small interfering RNAs can prevent the repair of RHPS4-induced telomere DNA damage and lead to increases in chromosome abnormalities and, eventually, to the inhibition of tumor cell growth both *in vitro* and *in vivo* [283]. The addition of the topoisomerase I inhibitor SN-38, but not the topoisomerase II poison doxorubicin, to RHPS4 produces a synergistic antitumor effect [284]. Moreover, combination therapy with RHPS4 and camptothecins also produces a synergistic effect that inhibits tumor growth and increases the survival of mice [284].

6.1.4. BRACO-19

The 3,6,9-trisubstituted acridine compound BRACO-19, 9-[4-(*N,N*-dimethylamino)phenylamino]-3,6-bis(3-pyrrolodinopropionamido) acridine (Fig. **7**), has been developed as a ligand for stabilizing G-quadruplex structures [285]. Treatment with BRACO-19 inhibits cell growth with an increase of senescence-associated marker β-galactosidase in DU145 cells [286]. This growth inhibition is associated with upregulation of p21 and $p16^{INK4a}$. Moreover, treatment with BRACO-19 for 1 or 3 weeks causes end-to-end chromosomal fusions, which are consistent with telomere uncapping. Burger *et al.* [287] have reported that BRACO-19 inhibits telomerase activity through downregulation of TERT expression after 24 hours, resulting in telomere shortening and complete cessation of growth after 15 days in the UXF1138L human uterus carcinoma cell line. *In vivo* treatment with BRACO-19 (intraperitoneal administration at 2 mg/kg/day) inhibited tumor growth by 96% compared with vehicle controls, and the response was paralleled by loss of nuclear TERT protein expression and an increase in atypical mitoses in nude mice with UXF1138L cell xenografts. Gunaratnam *et al.*

[288] have reported that BRACO-19 can produce short-term cytotoxicity and long-term growth inhibition in cancer cell lines, such as MCF7, A549, DU145, HT-29, HGC-27, and A2780, but is significantly less active against normal fibroblast cell lines, such as WI-38 and IMR90. BRACO-19 inhibits telomerase activity and reduces telomere length in MCF7 cells. Moreover, BRACO-19 binds to telomeric single-stranded overhang DNA, consistent with G-quadruplex formation, and displaces single-stranded protein human POT1 from the overhang.

6.1.5. Telomestatin

Telomestatin (Fig. **7**) stabilizes G-quadruplex structures at the telomeric 3' overhangs (G-tails) of chromosome ends and inhibits telomerase elongation by telomerase. Telomestatin has a 70-fold higher selectivity for intramolecular G-quadruplex structures than for duplex DNA [289]. Telomestatin markedly reduces G-tails in cancer cell lines but not in normal fibroblasts and epithelial cells [289]. Telomestatin promptly induces cell death and is selectively effective against tumor cells [275]. In addition to inhibiting telomere elongation, telomestatin produces its anticancer effect by rapidly disrupting the capping function at the very ends of telomeres. In addition, treatment with telomestatin specifically dissociates TRF2 protein from telomeres [289]. Telomestatin that stabilizes G-quadruplex structures may affect T-loop formation as well as TRF2 binding [289]. TRF1 protein complexes in telomeres contain TIN2, TPP1, and POT1, whereas the TRF2 protein interacts with TIN2, RAP1, the Rad50/MRE11/NBS complex, and the DNA-PK, Ku70/Ku80 complex. Caspase-3 and poly-(ADP-ribose) polymerase is activated by telomestatin in U937 cells [286]. Moreover, activation of p38 MAPK and MKK3/6 was found. In U937 xenograft mouse, intraperitoneal administration of telomestatin decreased tumor telomerase activity and reduced the volume of tumors, which exhibited marked apoptosis [290].

6.2. Catalytic Inhibitors

6.2.1. BIBR1532

BIBR1532 (2-[(E)-3-naphtalen-2-yl-but-2-enoylamino]-benzoic acid) (Fig. **7**) is a small, highly selective telomerase inhibitor [291]. Short-term culture with BIBR1532 showed growth inhibition and cell death in the JVM13 leukemic cell

line and in primary cells from patients with AML or CLL [291]. The inhibitory effect was also noted in cells with long telomeres. The inhibitory effect was associated with decreased TRF2 and increased phosphorylated p53 (Ser15) but not with telomere shortening.

6.3. Reverse Transcriptase Inhibitors

6.3.1. Azidothymidine (AZT)

Reverse transcriptase inhibitors (RTIs) are incorporated into DNA and block chain elongation by the reverse transcriptase enzyme. Azidothymidine (Fig. **7**) can be incorporated into eukaryotic DNA in place of thymidine, although it has low affinity for DNA polymerases α, β, γ and high affinity for reverse transcriptase. Telomerase activity is reportedly inhibited by RTIs [292-294], because telomerase is a RNA-dependent DNA polymerase with the enzymatic activity of reverse transcriptase. The nucleoside analog dideoxyguanosine inhibits telomerase activity and causes progressive telomere shortening in cultures of immortalized human lymphoid cell lines, the JY616 B cell line, and the Jurkat E6-1 T-cell line [292]. Azidothymidine can induce progressive telomere shortening, but other RTIs, including arabinofuranyl-guanosine, dideoxyinosine (ddI), dideoxyadenosine (ddA), didehydrothymidine (d4T), and phosphonoformic acid, do not cause telomere shortening or decreased cell growth. Datta *et al.* [293] have reported that azidothymidine inhibits telomerase activity in HTLV I-infected cells, resulting in telomere shortening with increased expression of $p14^{ARF}$ and p53. Moreover, azidothymidine reactivates p53-dependent transcription and induces the accumulation of the cyclin-dependent kinase inhibitors $p21^{WAF}$ and $p27^{KIP}$ [294].

6.4. Oligonucleotide Targeting TERC

6.4.1. Imetelstat (GRN163L)

Imetelstat (GRN163L), a palmitoyl (C16) lipid-attached N3'→P5' phosphoramidate oligonucleotide, targets the template region of the human telomerase RNA (TERC) subunit. Imetelstat is now undergoing trials in patients with CLL, MM, solid tumors, or non-small-cell lung cancer (NSCLC). A phase I/II study to evaluate the safety and dose of imetelstat in patients with CLL is in progress. Moreover, phase I studies have been completed to evaluate the safety and dose of imetelstat administered weekly and of imetelstat and bortezomib

administered with or without dexamethasone, in patients with refractory or relapsed MM. In 8 of the 9 patients with MM treated with imetelstat, the number of circulating cancer stem cells decreased over 2 months [295]. A phase I study of imetelstat sodium in young patients with refractory or recurrent solid tumors or lymphoma is currently recruiting participants. Geron Corporation (Menlo Park, CA, USA) has announced that it was discontinuing a phase II study of imetelstat in 166 patients with breast cancer [295], because interim analysis of a randomized phase II study of imetelstat in combination with paclitaxel with or without bevacizumab in patients with metastatic human epidermal growth factor receptor 2-negative breast cancer has demonstrated no significant improvement in median progression-free survival (PFS). A randomized phase II study of imetelstat as maintenance therapy following platinum-based induction chemotherapy for advanced NSCLC has been performed [296]; patients (n=116) were randomly assigned 2:1 to imetelstat (9.4 mg/kg on days 1 and 8 of a 21-day cycle) or to observation [296]. The median number of imetelstat maintenance cycles was 3. In the overall analysis of PFS, no significant improvement was observed in the imetelstat arm [296]: median PFS was 2.8 months for imetelstat and 2.6 months for observation, and the 6-month overall survival rate was 80% for imetelstat and 72% for observation. Imetelstat was generally well tolerated, although rates of hematologic toxicity (predominantly neutropenia and thrombocytopenia) and nonhematologic toxicity (fatigue, nausea, vomiting) were increased in the imetelstat arm [296]. These data suggest that imetelstat has no clinically meaningful activity as a maintenance therapy in patients with NSCLC. A decrease in platelets has been noted as an adverse effect in earlier failed phase II trials of imetelstat in patients with breast and lung cancer [295, 296]. Although thrombocytopenia is an unwanted side effect when treating solid tumors, it is exactly the desired effect when treating some hematologic disorders, such as essential thrombocythemia and polycythemia vera [295]. A phase II study to evaluate the activity of imetelstat in patients with essential thrombocythemia or polycythemia vera is in progress.

6.5. Immunotherapy

Tumor-specific antigens have been identified for only a few cancers, most notably melanoma. Because most human tumor cells express telomerase, the polypeptide

component of telomerase is an attractive candidate for a broadly expressed, universal tumor-rejection antigen. Cancer immunotherapy is based on the protective role of the immune system against cancer, mainly *via* the ability of $CD8^+$ cytotoxic T lymphocytes (CTLs) to recognize and kill cancer cells. When recognizing tumor antigenic peptides on the surface of tumor cells in association with major histocompatibility complex class I molecules, CTLs may become activated and able to lyse the cancer cells expressing these antigens [297]. A major problem of cancer immunotherapy is that almost all human tumor-associated antigens are self-proteins, and, therefore, their specific T cells, mainly those with the highest affinity, are often tolerated [297]. Consequently, overcoming tumor-specific self-tolerance is a major goal in tumor immunotherapy [297].

6.5.1. GRNVAC1

GRNVAC1 is autologous vaccine product that uses dendritic cells transfected with the messenger RNA encoding TERT and a portion of the lysosome-associated membrane protein 1, matured, aliquoted, and cryopreserved [298]. A multicenter, open-label phase II study has been performed to evaluate the safety, feasibility, and efficacy of immunotherapy with GRNVAC1 in patients with AML in complete clinical remission [299]. GRNVAC1 was produced by means of patient-specific leukapheresis before or shortly after the completion of consolidation chemotherapy. Patients received injections of GRNVAC1 once weekly for 6 weeks, followed by 4 weeks of rest, and then received booster injections every other week for 12 weeks [299]. GRNVAC1 was administered to 21 patients, 19 in complete remission (CR) (16 in CR1 and 3 in CR2) and 2 in early relapse. The 19 patients received a median of 17 injections (range, 6 to 32 injections) of GRNVAC1. GRNVAC1 was well tolerated and produced no toxicities, except for thrombocytopenia, which developed in 1 patient after 6 injections. With a median follow-up of 10.5 months (range, 1.2 to 27.2 months), 4 of 19 patients relapsed and 7 patients continue to receive GRNVAC1. The disease-free survival (DFS) rate 12 months after the first GRNVAC1 injection was 79% for the 19 patients in CR. Moreover, the DFS rate was 75% for the 8 patients in the intermediate-risk group and 81% for the 11 patients in the high-risk group.

6.5.2. Telomelysin (OBP-401)

Telomelysin (OBP-401) is a telomerase-specific, replication-selective oncolytic adenovirus in which the TERT promoter element that drives expression of the *E1A* and *E1B* genes is linked with an internal ribosome entry site [300]. Treatment with telomelysin exerts a selective and efficient cytotoxic effect on various human cancers, including carcinomas, melanomas, and osteosarcomas, without damaging normal fibroblasts and mesenchymal cells [300]. In a phase I study [301] involving 16 patients, telomelysin was injected directly into advanced solid tumors and was well tolerated at all dose levels (1×10^{10}, 1×10^{11}, 1×10^{12} viral particles). One patient with a malignant melanoma had a partial response (PR), and 7 patients had stable disease 56 days after treatment, according to Response Evaluation Criteria in Solid Tumors.

6.5.3. Vx-001

Vx-001, an HLA-A*0201-restricted TERT-specific antitumor vaccine, is composed of the 9-mer cryptic $TERT_{572}$ peptide and its optimized variant $TERT_{572Y}$ [302, 303]. The $TERT_{572Y}$ (YLFFYRKSV) differs from the native $TERT_{572}$ at position 1, where a tyrosine has been substituted for an arginine [302, 303]. This substitution enhances the vaccine's affinity for HLA-A*0201, the most frequently expressed HLA allele, and increases its immunogenicity [302, 303]. In a phase I study of Vx-001 [303], 14 of 19 patients with advanced cancers completed the vaccination program, which involved 2 subcutaneous injections with escalated doses (2-6 mg) of the optimized $TERT_{572Y}$ peptide followed by 4 subcutaneous injections of the native $TERT_{572}$ peptide every 3 weeks. Peripheral blood $TERT_{572Y}$-specific $CD8^+$ lymphocytes were detected in 13 of 14 evaluable patients after 2 injections of the optimized $TERT_{572Y}$ peptide. No patients had CR or PR, but 4 patients showed stable disease for a median of 10.5 months. The vaccine produced no grade III or IV toxicity. Moreover, a phase II study was performed with a fixed peptide dose (2 mg) to evaluate the long-term safety and immunogenicity of Vx-001 in patients with various advanced solid tumors, including breast, colorectal, ovarian, head and neck, pancreas, melanoma, hepatocellular, renal, and prostate cancers [304]. Fifty-five patients expressing HLA-A*0201 were enrolled, and 34 patients (62%) completed the vaccination program consisting of 2 subcutaneous injections of the optimized $TERT_{572Y}$

peptide followed by 4 injections of the native $TERT_{572}$ peptide every 3 weeks. The second and sixth vaccinations induced a TERT-specific T-cell immune response in 55% and 70% of the patients. Disease control was achieved in 36% of cases and included 1 CR and 1 PR. The Vx-001 vaccine was well tolerated, and immunologically responsive patients had significantly better PFS and overall survival (5.2 months and 20 months) than did nonresponders (2.2 months, P=0.0001, and 10 months, P=0.041).

Subgroup analysis of 22 patients with advanced NSCLC showed that the vaccine-induced specific early immune response correlated with prolonged survival [305]. Twelve (54.5%) of the 22 patients completed the vaccine program. The patients with early immunological responses had a significantly better overall survival than did patients without immunological responses. The median overall survival was 30.0 months for responders and 4.1 months for nonresponders. A multicenter randomized phase IIb trial was designed to evaluate whether Vx-001 can prevent or delay tumor progression in patients with stage IV or recurrent stage I to III NSCLC whose disease had not progressed after 4 cycles of first-line platinum-based chemotherapy [306].

6.5.4. GV1001

GV1001, a peptide consisting of a 16-aa TERT sequence, contains binding motifs that allow highly promiscuous binding to a broad array of class II molecules and thus avoids the need for HLA testing [307]. Six patients with cutaneous T-cell lymphoma received vaccinations with GV1001 and granulocyte/macrophage colony-stimulating factor as an adjuvant [307]. None of the patients showed an objective clinical response to the vaccination, whereas 1 patient showed progressive disease. One of 6 patients showed a GV1001-specific T-cell response with a Th1 cytokine profile and expression of skin-homing receptors. This TERT-specific T-cell response was not associated with beneficial modulation of the tumor-infiltrating leukocytes [307]. Removal of regulatory T cells did not enhance responsiveness to GV1001 *in vitro* in any of the patients analyzed. These results suggest that the GV1001 vaccination is not effective in patients with cutaneous T-cell lymphoma. The TeloVac phase III trial tested the GV1001 vaccine in 1062 patients with advanced and metastatic pancreatic cancer who were recruited in 52

centers across the United Kingdom. There was no significant difference in overall survival between patients given the GV1001 vaccine and control patients given chemotherapy [308].

6.5.5. GX301

GX301 is a vaccine comprising 4 TERT peptides (peptide$_{540-548}$, peptide$_{611-626}$, peptide$_{672-686}$, and peptide$_{766-780}$) and 2 adjuvants (Montanide ISA-51 [Seppic, Puteaux, France] and imiquimod) [309]. The peptides are promiscuous and able to bind to HLA class I and II molecules. The adjuvants can induce efficient innate immune responses and strongly activate antigen-presenting cells. Moreover, Montanide ISA-51 produces a water-in-oil emulsion with the peptide solution that protects against protease clearance and favors uptake by phagocytes. In the clinical study, GX301 was administered by intradermally injecting 500 μg of each peptide dissolved in Montanide ISA-51 in the skin of the abdomen. Imiquimod was applied as a cream at the injection sites. An open-label, phase I/II clinical trial was performed to evaluate the safety, tolerability, and clinical response of immunotherapy with GX301 in patients with stage IV prostate or renal cancer. The schedule of vaccination included 8 administrations on days 1, 3, 5, 7, 14, 21, 35, and 63. Evidence of vaccine-specific immunological responses was detected in all 14 patients. Disease stabilization occurred in 4 patients. Prolonged PFS and overall survival were observed in 8 of 14 patients, who showed a full pattern of vaccine-specific immunological responses. No grade 3 or 4 adverse reaction was noted. Phase II/III trials in patients with early and late stage prostate or renal cancer are being planned.

7. CONCLUDING REMARKS

Telomeres and telomerase are major players in the control of senescence and carcinogenesis. Telomerase is regulated tightly during cell proliferation in a cell cycle-dependent manner. Because hematopoietic stem and progenitor cells continuously generate and provide mature blood cells to the periphery, telomerase is activated more frequently in these cells than other somatic cells. Subsequently, telomeres in hematopoietic cells are more susceptible to stress/ageing-induced

shortening, leading to a loss of telomeric function that may promote chromosomal instability and oncogenic changes. Several methods to measure telomere length, including terminal restriction fragment analysis, quantitative- and flow-fluorescence *in situ* hybridization, and single telomere length analysis, have been developed. Because telomeres comprise multiple repeated sequences, direct amplification of telomeric DNA by PCR is difficult. However, significant advances in quantitative real-time PCR assays and the use of new primers have enabled more accurate measurements of absolute telomere lengths. Most hematologic malignancies have high levels of telomerase activity and short telomeres, which are associated with chemotherapeutic resistance and poor prognosis. Furthermore, chromosomal aberrations involving telomerase genes may be involved in the tumorigenesis of hematologic malignancies. A number of promising therapeutic agents targeting telomerase have been developed. Current therapeutic strategies include the use of G-quadruplex-interacting molecules, TERT catalytic inhibitors, TERT reverse transcriptase inhibitors, TERT-targeting oligonucleotides, and immunotherapy.

Exogenous expression of specific transcription factors, including Oct4, c-Myc, Klf4, and Sox2, reprograms differentiated somatic cells such as fibroblasts into induced pluripotent stem (iPS) cells [310]. Because iPS cells resemble embryonic stem cells, they are capable of being rejuvenated. Intriguingly, telomere elongation accompanied by the upregulation of telomerase is comparable in iPS cells derived from young and old individuals. In addition, exogenous expression of the *TERT* gene and reprograming-associated transcription factors enhances the efficiency of iPS cell reprograming [311]. Therefore, telomerase may play an important role in iPS cell biology, suggesting that it could be an attractive target for regenerative biology as well as tumor therapy.

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CONFLICTS OF INTEREST

The authors confirm that this chapter contents have no conflict of interest.

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