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Telomere Maintenance Mechanisms in Cancer: Clinical Implications

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Abstract: The presence of immortal cell populations with an up-regulated telomere maintenance mechanism (TMM) is an almost universal characteristic of cancers, whereas normal somatic cells are unable to prevent proliferation-associated telomere shortening and have a limited proliferative potential. TMMs and related aspects of telomere structure and function therefore appear to be ideal targets for the development of anticancer therapeutics. Such treatments would be targeted to a specific cancer-related molecular abnormality, and also be broad-spectrum in that they would be expected to be potentially applicable to most cancers. However, the telomere biology of normal and malignant human cells is a relatively young research field with large numbers of unanswered questions, so the optimal design of TMM-targeted therapeutic approaches remains unclear. This review outlines the opportunities and challenges presented by telomeres and TMMs for clinical management of cancer.

Keywords: Alternative lengthening of telomeres, cancer, diagnosis, prognosis, telomerase, telomeres, therapy.

INTRODUCTION

More than 50 years have elapsed since the discovery that normal human somatic cells have a finite proliferative capacity (subsequently termed the Hayflick limit), in contrast to the apparently unlimited growth potential – or immortality – of cell lines derived from tumors [1, 2]. This discovery suggested the very attractive possibility that cellular immortality may result from molecular abnormalities that clearly distinguish cancer cells from their normal counterparts, and may therefore represent an ideal opportunity for targeted anticancer therapies that have few if any side-effects on normal cells (reviewed in ref. [3]). The molecular alterations required for immortalization of human cells are complex. Two major tumor suppressor pathways, involving pRb and p53, play a role in activating the cellular senescence program at the end of the normal replicative life span, and loss of function of these pathways is usually required for immortalization of human cells (reviewed in [3]). A major determinant of cellular mortality is the telomere shortening that accompanies normal proliferation [4], so a key event in acquisition of cellular immortality is the up-regulation of a telomere length maintenance mechanism (TMM) [5]. This review is limited to the aspects of immortalization related to the TMMs, and the opportunities these present for clinical management of cancer.

1. TELOMERE STRUCTURE AND FUNCTION

1A. The Sequence and Structure of Telomeric DNA

Human telomeres contain repetitive DNA, predominantly tandem arrays of the 5'-TTAGGG-3' hexanucleotide [6], often termed the canonical telomeric sequence. The proximal end of human telomeres (i.e., the end closest to the centromere) also contains variant repeats such as TTGGGG, TGAGGG and TCAGGG [7, 8]. These variants are less common in the remainder (i.e., distal portion) of the telomere, which predominantly contains canonical repeats and hexanucleotides with base substitutions at positions 1 and 3, such as GTAGGG and TTCGGG [9].

The termini of telomeres consist of 25-200 nucleotides of single-stranded DNA - usually the G-rich strand (referred to as the "G-overhang"). It is presumed that most of the remainder of the telomere forms duplex DNA by Watson-Crick base pairing, but the G-rich strand is capable of Hoogsteen base pairing, which forms planar G-quartet structures that stack on each other to form ***G-quadruplexes***. Antibodies recognizing G-quadruplexes have demonstrated that these structures occur at human telomeres [10], and it is possible that they help protect the telomere against DNA repair.

Another form of higher-order structure that is proposed to help protect telomeres against DNA repair is the ***t-loop*** [11]. The telomere is able to fold back on itself, so that the single-stranded telomeric overhang can invade duplex telomeric DNA and anneal with the complementary strand to create a loop structure, a process which is facilitated by the TRF2 protein [12]. Visualization of t-loops by super-resolution light microscopy has demonstrated that the point of invasion can be located at almost any point along the duplex DNA, resulting in t-loops of highly variable sizes [13]. Formation of these circular DNA structures may be an important contributor to the protection of telomere ends from DNA repair.

1B. Telomere Binding Proteins

Telomeric DNA is bound by a protein complex, shelterin, which contains six proteins, TRF1, TRF2, TIN2, RAP1, TPP1 and POT1 (reviewed in ref. [14]). These proteins prevent telomeres being recognized by the cell as a DNA break and repaired by non-homologous end joining (NHEJ) or by homologous recombination (HR)-mediated repair. Repression of DNA repair at chromosome ends is essential for maintaining the organization of the genome into separate chromosomes, and failure of this repression results in genomic instability.

1C. Proliferation-Dependent Telomere Shortening

It has been observed that cell proliferation *in vitro* is accompanied by telomere shortening [15, 16] (Fig. **1**), which reflects the gradual overall decrease in telomere length in normal human somatic tissues with increasing age [17]. This is due in part to the inability of the normal processes of semi-conservative DNA replication to copy the termini of linear DNA molecules by lagging-strand synthesis [18, 19], referred to as the "end replication problem"). It is also partly due to enzymatic processes that generate or elongate the single-stranded overhang at telomeric termini [20, 21]. The template available for replicating telomeric DNA thus steadily decreases in length with each cell cycle.

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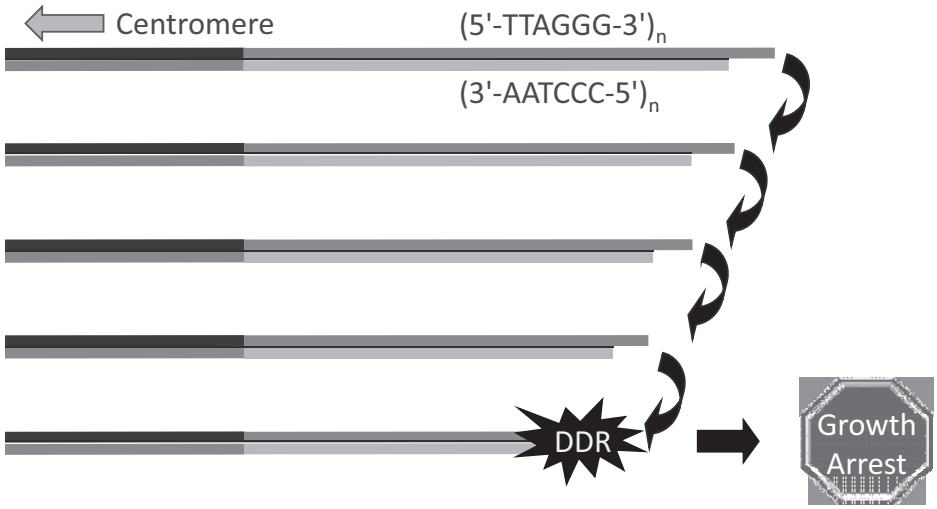

Fig. (1). Telomeres undergo gradual attrition during cellular proliferation. Telomeres (lighter bars; darker bars represent non-telomeric DNA) contain tandemly repeated arrays of the hexameric sequence, 5'-TTAGGG-3'. Telomeres are mostly double-stranded, but they terminate in a region of single-stranded (usually G-rich) DNA. In cultured human fibroblasts, telomeres shorten by approximately 50-150 base pairs per cell division. This ultimately results in a DNA damage response (DDR) focus, and, when a sufficient number of such foci accumulate, the cell undergoes permanent withdrawal from the cell cycle (i.e., becomes senescent).

1D. Telomere Capping and the Connection between Telomere Shortening and Senescence

A telomere that is fully protected, presumably by a combination of its higher-order DNA structure and its binding proteins, avoids eliciting a DNA damage response (DDR) or unwanted DNA repair, and is referred to as "capped". It has been proposed that the telomere needs to become temporarily uncapped to allow access of telomerase [22]. Telomeres can become fully uncapped by experimental disruption of specific shelterin components, resulting in an ATM- or ATR-dependent DDR and end-to-end fusion of chromosomes [23-25]. Uncapped telomeres are recognized by the co-localization of DDR proteins, such as phosphorylated histone H2AX (i.e., γH2AX) and chromosome ends, which are referred to as Telomere dysfunction-Induced Foci (TIFs) [23].

Replicating young human cells also exhibit a small number of telomeric DDR foci, and the number increases as the cells continue proliferating and their telomeres continue to shorten; in contrast to TIFs in cells with uncapped telomeres, these foci are not associated with end-to-end fusions. The cells finally arrest in G1 phase of the cell cycle and become senescent when the number of telomeric DDR foci reaches 4 or 5 [26]. If senescence is bypassed by loss of function of the p53 and pRb tumor suppressor pathways, continued proliferation is associated with further telomere shortening and further accumulation of telomeric DDR foci, until eventually there is widespread end-to-end fusion of chromosomes (indicating that many of the telomeres have become fully uncapped) and cell death - a state referred to as culture crisis [26]. Therefore, it was deduced that there must be a telomere conformation intermediate between fully capped and fully uncapped, which elicits a DDR but represses DNA repair, and that integrated signaling from 4 or 5 of these intermediate-state telomeres (IST) results in senescence [26] (Fig. **2**).

Telomere shortening is not the only stimulus for telomeres to adopt the IST conformation. When mitosis is prolonged excessively, ISTs are induced, and cells which eventually escape from mitosis undergo cell cycle arrest in the subsequent G1 phase, unless they lack wild-type p53 function, in which case they continue dividing and become aneuploid [27]. The signaling pathway for IST-induced G1 arrest involves ATM, but does not involve phosphorylation of CHK2, and is thus distinct from the genomic DDR [28]. In both the case of replication-associated telomere shortening and of prolonged mitosis, it is presumably advantageous for the organism if the cell is permitted to continue through the cell cycle until it reaches G1, at which point the senescence program can be activated (Fig. **2**). It will be very interesting to determine whether there are other abnormalities of cellular function where the same telomeric signaling pathway is used to allow the cell to continue through the cell cycle until it reaches G1 and becomes senescent, what upstream signaling events result in adoption of the IST conformation, and how the molecular players in an IST-associated DDR response differ from those at an uncapped telomere or a DDR elsewhere in the genome.

1E. Telomere Lengthening by Telomerase

Gradual attrition of chromosome ends would eventually result in loss of vital genomic material, so a mechanism for preventing this in the germ line is essential. The best known of the telomere lengthening mechanisms is telomerase, a ribonucleoprotein (RNP) enzyme that synthesizes new telomeric DNA to compensate for replication-associated telomere attrition by reverse transcribing a template region within its RNA moiety [29]. Telomerase mostly synthesizes the canonical repeat, but the presence throughout the distal telomere of repeats differing from this sequence at positions 1 and 3 indicates that telomerase sometimes substitutes bases at specific locations on its template [9]; the functional consequences of these substitutions remain to be determined.

Telomerase activity can be detected in human embryonic stem cells at levels which are sufficient to prevent telomere shortening [30]. Moreover, induced pluripotent stem cells are able to up-regulate telomerase levels sufficiently to elongate their telomeres compared to the normal cells from which they are derived [31]. In multipotent stem cells, such as those of the hematopoietic system, telomerase is under tight control and does not completely prevent telomere shortening [32]. Most somatic tissues have very low levels of telomerase and undergo telomere shortening throughout life. A rare exception to this is that CD19+CD27+ (memory) B-lymphocyte cells appear to have sufficient telomerase activity to undergo telomere lengthening during their ontogeny [33].

In contrast to normal somatic tissues, the great majority (~85%) of human cancers have detectable levels of telomerase [34], and in cancer-derived cell lines this appears to be sufficient to prevent telomere shortening. However, even in cell lines where telomerase

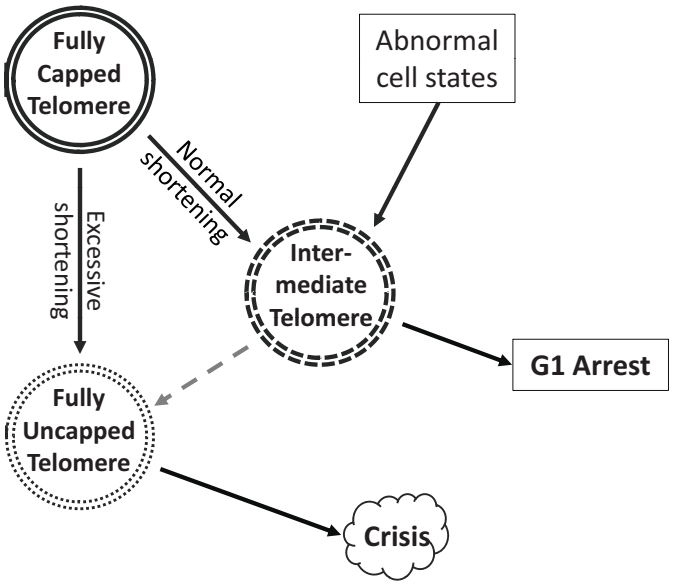

Fig. (2). Telomeres can adopt at least three different conformational states. The fully capped state inhibits both the DDR and DNA repair by non-homologous end-joining (NHEJ) which would result in end-to-end joining of chromosomes and therefore genomic instability. Normal telomere shortening results in an intermediate-state telomere which inhibits NHEJ but not DDR. When the threshold number of telomeric DDR foci is exceeded, the cell continues through mitosis and then arrests in G1 phase. An abnormal cell state, such as excessively slow transit through mitosis, also elicits intermediate-state telomeres (ISTs) by an unknown mechanism, with the result that the cell continues to proceed through mitosis until it reaches G1 and can then undergo an orderly exit from the cell cycle. Excessive telomere shortening results in fully uncapped telomeres which elicit both DDR and NHEJ and result in cell death; when most of the cells in a culture enter this state, this is referred to as "culture crisis".

has maintained telomere length for many hundreds of population doublings (PD), the number of active telomerase molecules has been estimated at 20-50 copies per cell [35], which means that there are fewer active telomerase molecules than there are telomere ends and that this is one of the least abundant molecules in the cell. This presents major challenges for studying this enzyme, e.g., for studying its intracellular location using antibody staining, and for obtaining sufficient enzyme for structural studies.

Telomerase is a complex molecule with several subunits, and additional associated proteins are involved in its biogenesis, all of which are potential targets for development of therapeutics that decrease telomerase activity. The molecules which are essential for its catalytic activity are the reverse transcriptase (***TERT***) and RNA (an H/ACA RNA which is variously referred to as ***TR***, ***TER*** or ***TERC***) moieties (Fig. **3**). Mass spectrometry of active human telomerase showed that it contains two molecules each of TERT, TR and a pseudouridylase H/ACA ribonucleoprotein protein, ***dyskerin*** (also named NAP57) [35]. It has been found that both TERT catalytic sites must be active in order for the complex to be active, implying that telomerase functions as a dimer, and this was supported by electron microscopy data at 23-Å resolution showing that the enzyme is bi-lobar [36]. Other proteins that associate with telomerase and are required for its assembly include two ATPases – ***reptin*** and ***pontin*** [37] – and three additional H/ACA RNP proteins – ***NOP10***, ***NHP2***, and ***GAR1*** (reviewed in [38]). A further protein that may be transiently involved in telomerase assembly is ***NAF1***, which initially binds dyskerin and escorts it to the nascent H/ACA RNA before being replaced by GAR1 to yield mature H/ACA RNPs in Cajal bodies and nucleoli [39].

The processes involved in relocation of telomerase from the nucleolus, where it is assembled [40], to its substrate, the telomere, also present a number of potential opportunities for therapeutic intervention. The processes, often collectively referred to as telomerase recruitment (reviewed in [41]), are as follows. The assembled telomerase travels to, and accumulates in Cajal Bodies in a process that is dependent on the RNA binding protein, TCAB1 [42], which then plays a direct role in the transport of telomerase to the telomere [43]. At the telomere, telomerase cannot act on its substrate unless the cis-acting inhibitory effects of TRF1 are relieved [44] and telomerase is able to interact with TPP1 and dock at the end of the telomeric single-stranded overhang [45]. The factors which control these mechanisms, as well as the processivity of telomerase (i.e., its ability to continue adding telomeric repeats to a telomere terminus), and the mechanisms whereby it relocates to other telomeres, are all incompletely understood at present.

1F. Mechanisms of Telomerase Up-Regulation in Cancer

Details of the mechanisms involved in the up-regulation of telomerase in cancer are continuing to emerge (Fig. **3**). Many studies have focussed on the role of transcriptional control of TERT expression or on phosphorylation-induced increases in TERT activity ([46-50]; reviewed in refs. [51-53]). Positively acting transcription factors and co-regulators include the MYC oncogene, the E6 protein of oncogenic human papillomaviruses, NF-κB and β-catenin. Up-regulated expression may also occur through loss of pRB, WT1, Menin and TGFβ pathway tumor suppressor function. Protein kinase Cα and AKT can phosphorylate TERT.

Mutations within the core promoter region of TERT are common, and these may increase TERT transcriptional activity by creating de novo consensus binding motifs for ETS transcription factors [54, 55]. TERT promoter mutations are most common in melanomas, bladder carcinomas, liposarcomas, hepatocellular carcinomas, squamous cell carcinomas of the tongue, medulloblastomas, and gliomas, especially primary glioblastoma multiforme [54-58]. TERT promoter mutations appear to be a useful urinary biomarker for early detection and monitoring of bladder cancer [59]. Another mechanism of up-regulation is translocation of the TERT gene to an immunoglobulin gene or other loci in B-cell neoplasms, which presumably contributes to the increased TERT transcription and telomerase activity observed in these tumors [60].

There is evidence that the levels of TERT and TR are both limiting for telomerase activity (reviewed in [61]), so it is reasonable to assume that up-regulation of telomerase requires an increase in both of these telomerase subunits. PAX8 is able to coordinately up-

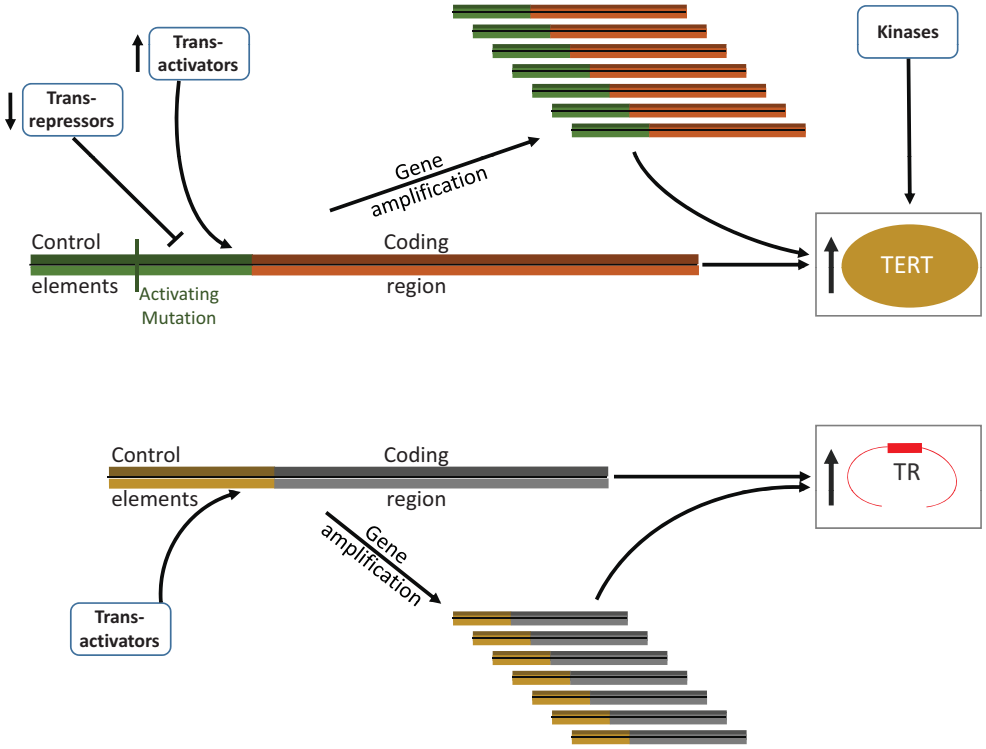

Fig. (3). Telomerase up-regulation in cancer cells can occur by a variety of mechanisms. The telomerase catalytic subunit (TERT) and the RNA template molecule (TR) are both present at very low copy number in normal cells, and it is likely that the expression of both must be increased in order to express sufficient telomerase to prevent telomere shortening in cancer cells. Increased transcriptional activation (for both TERT and TR), and loss of transcriptional repressors (for TERT) have been found in some cancer cells. Many telomerase-positive cancer cells have a mutation in the TERT gene promoter/enhancer region, which appears to result in increased trans-activation of this gene. The activity of TERT may also be increased by certain kinases. Increased copy number of TERT and/or TERC has been identified in many cancers.

regulate both TERT and TR expression, and the PAX8 expression level correlates with telomerase activity in gliomas [62]. The copy number of the TERT and TR genes is commonly increased in human cancers (reviewed in ref. [63]).

1G. Non-catalytic Functions of Telomerase

Evidence has been presented that telomerase has roles in cell proliferation, genome stability and protection against apoptosis in addition to its role in catalyzing synthesis of telomeric DNA. The TERT protein was found to have a mitochondrial targeting sequence in its N-terminus, and mitochondrial extracts were found to contain telomerase activity [64]. Although one study found that mitochondrial telomerase sensitized cells to oxidative stress and induction of apoptosis [64], other groups have reported that over-expression of TERT lowered mitochondrial production of reactive oxygen species, and protected mitochondria from oxidative stress, nuclei from DNA damage, and cells from apoptosis [65-68]. In another study, over-expression of the major splice variant of TERT (termed the β-deletion variant), which lacks the catalytic domain, protected breast cancer cells from cisplatin-induced apoptosis [69].

Another proposed non-catalytic role of TERT is induction of signaling via the Wnt pathway in mouse and human cells, by interacting with BRG1, a chromatin remodeling protein and by acting as a transcriptional co-factor in a complex with β-catenin [70, 71]. However, in different human cell lines, no association was found between TERT and BRG1 or β-catenin, and no consistent effects on Wnt pathway signaling were seen [72].

1H. Telomere Lengthening by ALT

Some immortalized cell lines maintain telomere length for hundreds of population doublings in the absence of telomerase activity, and it was therefore deduced that they must have an alternative lengthening of telomeres (ALT) mechanism [73, 74]. In contrast to telomerase, which uses an RNA template for de novo synthesis of telomeric DNA [29], ALT involves synthesis of new telomeric DNA from a DNA template via HR (reviewed in ref. [75] (Fig. **4**)). The template may be the telomere of another chromosome [76, 77] or another region of the same telomere via t-loop formation or sister telomere recombination [78]. Many molecular details of the ALT mechanism remain unknown, but it has been proposed that various HR proteins are involved [79-88].

ALT activity is detectable in normal mouse tissues [89], where it is clearly insufficient to maintain telomere length. In human cells where ALT activity is up-regulated sufficiently to maintain telomere length, there is a characteristic telomere phenotype. The telomeres are highly heterogeneous in length, but the average length (>17 kb) is about double that of most cells where telomere length is maintained by telomerase [73]. A large proportion of the telomeres in ALT cells evoke a DDR, and most likely are in an IST conformation [90]. There are substantial quantities of extra-chromosomal telomeric repeat (ECTR) DNA, which may be linear [91, 92], circular and double-stranded (t-circles) [93, 94], circular and at least partly single-stranded (referred to as C- or G-circles, if the C- or G-rich strand is intact, respectively) [95, 96], or high molecular weight and complex in structure [96]. C-circles can be detected as self-priming templates for rolling circle amplification by Φ29 polymerase [95, 97], and the C-circle level appears to correlate with the level of ALT activity [95].

Some of the ECTR DNA, as well as chromosomal telomeric DNA, is contained within PML nuclear bodies [98-100]. Because telomeric DNA and telomere binding proteins are not normally found within PML bodies, these structures are referred to as ALT-associated PML bodies (APBs). APBs can be used to determine whether tumors are ALT-positive [98, 101, 102]. Early studies

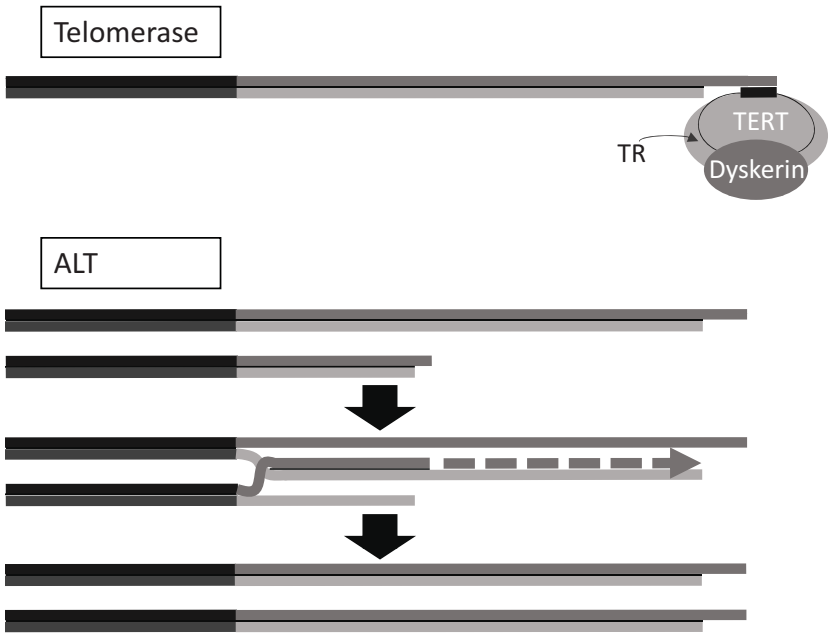

Fig. (4). Telomerase and ALT both result in de novo synthesis of telomeric DNA. Unlike telomerase, which reverse transcribes new telomeric sequence from the template region (black bar) of its RNA subunit, ALT involves formation of a DNA recombination intermediate and the use of a DNA template for synthesis of new telomeric sequence; see text for details.

showed that HR proteins, such as RPA, RAD51, RAD52, and NBS1, localize within APBs [98, 103] and many more APB components have subsequently been added to the list. Compared to other immortalized cells, there is a markedly increased level of recombination at telomeres [104, 105], but not at most other locations in the genome [104, 106]. However, a subset of minisatellite sequences undergo increased recombination for reasons that are not understood [107-109].

Types of tumors where ALT is very common include osteosarcomas, undifferentiated pleomorphic sarcomas, leiomyosarcomas, and grade 2 and 3 astrocytic brain tumors, but ALT is also common in some other types of soft tissue sarcomas, grade 4 astrocytic brain tumors (glioblastomas), and neuroblastomas, and occurs at low frequency in many types of carcinomas [101, 110-121] (reviewed in ref. [122]). A small proportion of ALT-positive tumors also have telomerase activity [110-113, 123]. The correlation between ALT and prognosis (discussed further in section 2B below) varies among different tumor types; for example, the presence of ALT in glioblastomas is associated with a better outcome, whereas in liposarcomas ALT is associated with a significantly worse outcome [112, 113, 117, 124].

1I. Mechanisms of ALT Up-Regulation in Cancer

Somatic cell hybridization analyses demonstrated that normal telomerase-negative human cells, as well as telomerase-positive immortalized cells, contain repressors of ALT activity [73, 125, 126]. The observation that the gene encoding one or other member of the ATRX/DAXX protein complex is mutated in many ALT-positive cancers and cell lines [120, 127-133] suggests that loss of ATRX/DAXX function is required for ALT to be activated.

Among many functions attributed to ATRX/DAXX, this complex is thought to be involved in chromatin remodeling, including telomeric deposition of the variant histone H3.3. Some pediatric glioblastomas have mutations both in H3.3 and ATRX [133], suggesting that the major selection pressure for loss of ATRX/DAXX is not related to its H3.3 function. There are other indications, however, that the architecture of telomeric chromatin is substantially altered in cells with up-regulated ALT activity (reviewed in ref. [134]). The telomeric DNA sequence becomes altered as variant repeats which are normally present in the most proximal portion of the telomere become spread throughout the telomere, presumably initiated when a proximal telomere is used as the template for ALT-mediated DNA synthesis [9, 77, 135]. Shelterin is therefore partially displaced from ALT telomeres by proteins such as the nuclear receptors, COUP-TF2 and TR4 [135]. Shelterin desaturation most likely contributes to the large number of telomeric DDR foci in ALT cells, which can be partially suppressed by over-expression of TRF2 [90], and would be expected to decrease the repression of HR at telomeres [136] and result in an increase in stalled replication forks within telomeres [137], thus stimulating telomeric recombination. For reasons that are not understood, telomeric chromatin appears to be less compacted in cells that utilize ALT compared to those that utilize telomerase, which may also favor interactions among telomeres [138].

1J. Telomere Trimming

In addition to the gradual telomere attrition that accompanies cellular proliferation, there is a more rapid telomere shortening process termed telomere trimming (reviewed in ref. [139]), which resembles telomere rapid deletion in yeast [140]. First observed in human cancer cell lines with telomeres that were over-lengthened by exogenous telomerase, telomere trimming appears to be a well-regulated mechanism for rapid correction of over-lengthening via excision of circularized telomeric DNA [141]. In cells with up-regulated telomerase activity, this would help maintain telomere length close to a set point [139]. Telomere trimming also occurs in cells of the male germ line and in normal lymphocytes following mitogen-stimulated up-regulation of telomerase activity [142]. Telomere length is therefore subject to multiple lengthening and shortening processes (Fig. **5**).

2. CLINICAL IMPLICATIONS OF TELOMERE MAINTENANCE MECHANISMS FOR CANCER

2A. Diagnosis

The standard method of detecting telomerase in tumors is the Telomere Repeat Amplification Protocol (TRAP) assay [143]; this is a highly sensitive, semi-quantitative assay in which telomerase present in tumor lysates extends an oligonucleotide substrate and

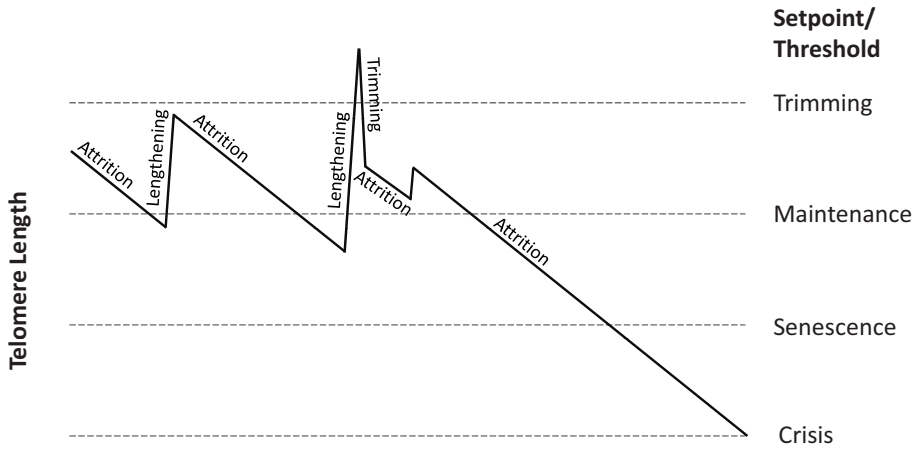

Fig. (5). Multiple factors contribute to telomere length dynamics in normal and cancer cells. Telomeres undergo proliferation-associated attrition, which may be counteracted by lengthening via telomerase and/or ALT. Over-lengthening may be corrected rapidly by telomere trimming. In immortalized cells, telomere length is maintained within a range around a maintenance set point, whereas in normal, mortal cells telomere length is not maintained overall and may decrease sufficiently to induce senescence. Cells in which tumor suppressor pathways, p53 and pRb, are inactivated may fail to enter senescence and their telomeres may continue shortening until they are no longer able to inhibit DNA repair by end-joining, at which point (crisis) they undergo cell death.

the products are detected by PCR. Tumor tissues may contain inhibitors of the TRAP assay which cause false-negative results unless the lysate is diluted appropriately. A possible alternative is to remove inhibitors from the sample by immunoprecipitation with an antibody against TERT prior to the TRAP assay [144]. Direct telomerase assays which have no PCR amplification step are quantitative, but are less sensitive by several orders of magnitude [145, 146]. In some studies, the presence of telomerase activity is inferred from TERT and/or TERC transcript levels measured by reverse transcription PCR, or by in situ hybridization in the case of TERC. Immunohistochemical detection of telomerase in tumor tissues is problematic, most likely due to the exceptionally low abundance of this molecule, even in cancer cells [35].

Use of telomerase detection for cancer diagnosis has been reviewed elsewhere (e.g., ref. [147]). Despite the potential promise of being able to diagnose cancer via detection of an enzyme activity which is up-regulated in 85% of all cancers [34], telomerase assays have not yet been adopted for routine cancer diagnosis. A systematic review and meta-analysis of the correlation between telomerase activity (measured by TRAP or telomerase component transcript levels) and histopathologic diagnosis in 2395 breast lesions found that 82% of breast cancers and 18% of benign lesions were positive for telomerase activity [148]. It is possible that a better correlation would be obtained if technical improvements in telomerase assays are able to decrease false negative and false positive results, if measurements of TERT mRNA levels distinguish between full-length transcripts and splice variants that do not encode catalytically active TERT protein, and if the presence of ALT is taken into account.

The most reliable methods for detecting ALT in tumor samples are assays for APBs [98, 101], C-circles [95], or telomere length [110]. APBs can be visualized in fresh-frozen tumors, cytology specimens, or formalin-fixed paraffin-embedded tumor samples after deparaffinization and an antigen retrieval step, by immunostaining for PML and either immunostaining for a shelterin protein or Fluorescence in situ Hybridization (FISH) with a telomeric probe. The amount of telomeric DNA in APBs is often much greater than at a single chromosome end, so ALT may also be detected through the presence of very bright foci after telomeric FISH [127]. C-circles can be quantitated in genomic DNA extracted from tumors by rolling circle amplification with Φ29DNA polymerase in the absence of any added primer, followed by detection of telomeric DNA [95]. Telomeres in ALT cells are typically very heterogeneous in length, but long on average, and this can be detected in good-quality genomic DNA from tumors by Southern blotting of terminal restriction fragments [110]. Alternatively, content of telomeric DNA may be measured in small samples of genomic DNA by quantitative PCR (qPCR), and this may be combined with qPCR detection of C-circles amplified by Φ29 DNA polymerase [97].

Reliable methods for detecting telomerase and ALT are likely to become an important pre-requisite for the use of treatments targeting one or other of these mechanisms. Moreover, in principle, a method for detecting both tumor-derived telomerase and markers of ALT activity in blood or other bodily fluids could be a simple screening test for cancer. It has been demonstrated that C-circles from ALT-positive osteosarcomas can be detected in blood samples [95], so it is possible that this could be used for surveillance in clinical situations where there is a high risk of developing an ALT-positive tumor, or to monitor response to treatment of an ALT-positive tumor. However, the presence of telomerase activity in normal leukocytes makes it difficult to use telomerase activity assays in an analogous manner, so use of TMM detection as the basis of a blood test for cancer would require some technical advances.

2B. Prognosis

In view of the technical difficulties in quantitating telomerase activity, it is perhaps not surprising that studies of the correlation between telomerase activity levels and patient outcome have yielded divergent results. Using breast cancer again as an example, several studies have shown that high levels of telomerase activity or subunit transcripts are associated with poor prognosis [149-153]. In contrast, a more recent study found that telomerase level was not associated with disease outcome overall, although an association may have been masked by the types of treatment [154]. Similarly, higher telomerase levels have been found to be associated with better [155] or worse [156] outcomes in stage II colorectal cancer, and worse outcomes in colorectal cancer overall [156, 157].

The correlation between ALT and prognosis varies among different tumor types. In osteosarcomas, a tumor type where ALT is the more common TMM, it is telomerase which appears to be the adverse prognostic indicator, based on the separate observations that progression-free and overall survival was worse in telomerase-positive tumors compared to telomerase-negative [158], absence of both telomerase and ALT was associated with better clinical outcome [111], and there was no significant difference between survival of patients with ALT and non-ALT tumors [101]. For malig-

nant fibrous histiocytomas, presence of ALT was associated with decreased survival, and this was not affected by co-existent telomerase activity [159], although a smaller study did not find a difference in survival between ALT and non-ALT tumors [101]. In liposarcomas, presence of ALT was more strongly associated with decreased survival than was telomerase activity [113, 124]. In contrast, presence of ALT in glioblastoma multiforme has been associated with better patient outcome [101, 112, 117], and telomerase was found to confer a worse outcome in pediatric high-grade gliomas [160].

Although the functional consequences of telomerase and ALT up-regulation are similar in that they both prevent overall telomere shortening in tumors, these TMMs differ in several regards which may account for their differential prognostic significance in various tumor types. As described above, the genetic and epigenetic events responsible for activation of these mechanisms differ (possibly in a manner that is specific for the cell of origin). Moreover, some of the functional consequences may differ: up-regulated telomerase may possibly have non-canonical functions (also as described above), and ALT has been associated with complex karyotypes and chromosomal instability which may drive tumor progression [113, 161, 162].

2C. Opportunities for TMM-Targeted Therapies

There are many aspects of telomere maintenance in cancers that present potential opportunities for developing targeted therapies. The most obvious of these are the catalytic events involved in de novo synthesis of telomeric DNA by telomerase or ALT, but other changes in cancer cells that are associated with activation of these mechanisms should also be considered as potential targets.

2C(i). Inhibition of Telomerase

The development of telomerase inhibitors has been the subject of many reviews, including refs. [163-174]. The telomerase-catalyzed telomere lengthening reaction proceeds via multiple steps, which include binding to its substrate, reverse transcription of telomeric DNA, and translocation of the enzyme on its extended substrate to continue the lengthening reaction (which is required for its processivity). All of these steps are potentially able to be inhibited, and inhibitors of the reverse transcription step could target either the template, as is the case for GRN163L (Imetelstat; a lipidated 13-mer oligonucleotide, which has entered clinical trials), or the catalytic subunit. A compound which has been used quite extensively for *in vitro* studies, BIBR1532, is a non-competitive telomerase inhibitor which appears to inhibit its processivity [175, 176].

Telomerase-mediated lengthening could potentially be inhibited by targeting other steps in the overall process, starting with expression of the subunits and their assembly into the active enzyme complex (Fig. **6**). The TERT gene is subject to alternative splicing with the majority of splice variants expected to result in catalytically inactive protein, if indeed they are translated [177], so modulation of splicing may also be a useful therapeutic strategy [178], as could selective targeting of TERT protein for proteasome-mediated degradation [179]. Mutations in two of the proteins involved in telomerase assembly, NHP2 and NOP10, result in telomere shortening *in vivo*, indicating that these proteins could potentially be targeted as a means of decreasing telomerase levels. In addition, telomerase must be transported to the telomere via processes that involve TCAB1 and Cajal bodies [43]. TCAB1 mutations are known to cause excessively short telomeres and the associated clinical manifestations of short telomere syndrome in humans [180]. This suggests that targeting of TCAB1 and other aspects of telomerase transport is likely to decrease telomerase activity in tumours, and that it therefore could be an effective therapeutic intervention.

Access of telomerase to its substrate is controlled by an incompletely-understood, and presumably intricately-regulated set of processes in which TIN2-anchored TPP1 is involved in recruiting telomerase [45, 181], and another shelterin component, TRF1, plays an inhibitory role [182]. The inhibitory role of TRF1 is relieved by tankyrase-induced poly ADP-ribosylation of TRF1 [183], which suggests that inhibitors of tankyrase may impede access of telomerase to telomeres. The telomeric transcript, TERRA, also appears to be involved in excluding telomerase from telomeres, and this can be alleviated by hnRNPA1 [184, 185], which is therefore another potential drug target. Finally, the number of active telomerase molecules in cancer cells appears to be smaller than the number of telomeres [35], which suggests that telomerase needs to undock

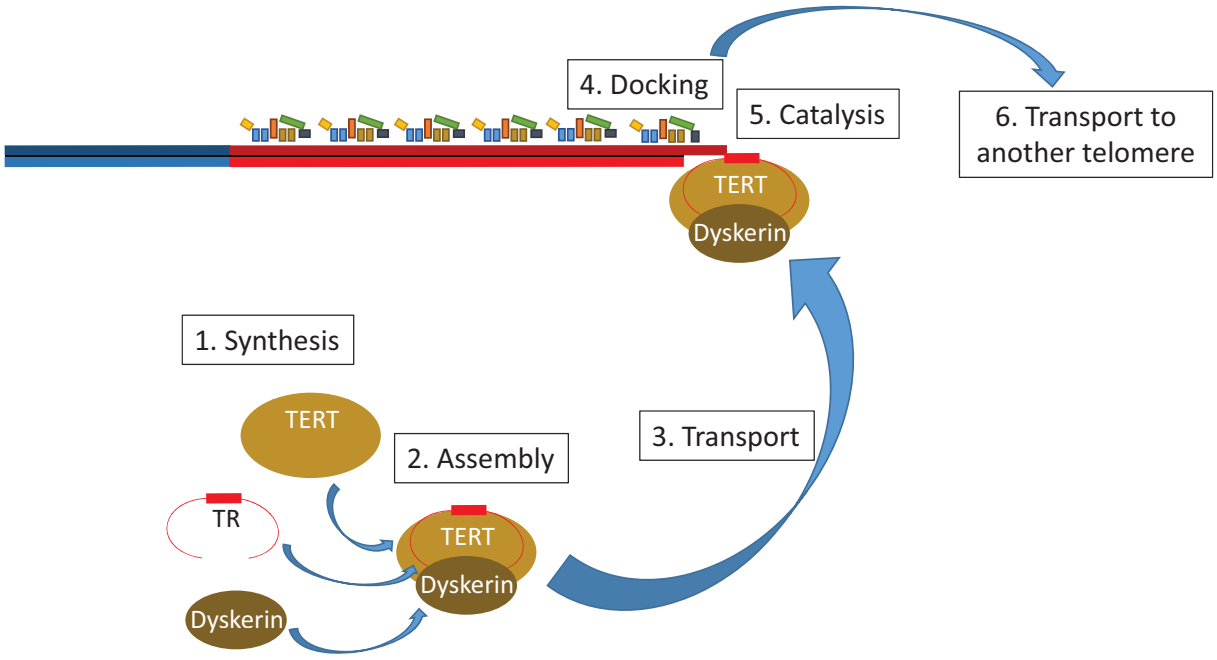

Fig. (6). The action of telomerase may be inhibited at multiple points in its "life cycle". These include synthesis of the individual components of active telomerase (TERT, dyskerin and TR), assembly into the enzyme complex, transport to a telomere, docking with the telomere, extending the telomere via its catalytic function, and movement to another telomere.

from one telomere and move to another telomere by a process which is essentially unknown and which is another potential target for anticancer therapeutics.

2C(ii). ALT Inhibitors

The ultimate steps in the ALT process involve HR and DNA synthesis, which are processes vital for repair and replication of DNA in normal cells. Although there may be a therapeutic window where cancer cells, especially those which have lost p53 function, are more vulnerable to inhibition of these functions than normal cells, the likelihood of toxic side-effects of such inhibition is high. Many molecular details of ALT remain to be discovered, however, and it is possible that key steps, e.g., those involved in the juxtaposition of telomeres that must be required for one telomere to use a non-homologous telomere as a copy template, involve molecules which are not expressed, or are expressed at much lower levels in normal somatic cells, and which therefore would be suitable targets for development of ALT inhibitors. Failing this, it still may be possible to exploit the causes and/or consequences of ALT up-regulation, as described below.

2C(iii). Immunotherapy

Peptides generated by degradation of hTERT are presented on the cell surface via the major histocompatibility complex (MHC) class I pathway; cytotoxic T-lymphocytes (CTL) can recognise these peptides and kill the cells that present them, which makes telomerase a potential target for the development of immunotherapies (reviewed in refs. [186, 187]). Cancer vaccines containing these peptides have been subjected to clinical trials and have shown acceptable safety and tolerability, and have generated strong immune responses in a substantial proportion of patients, as well as some encouraging effects on tumors [188-191]. If proteins are discovered that are expressed in ALT cells to a much greater extent than in normal somatic cells, it might also be possible to develop suitable immunotherapeutic approaches for tumors that depend on ALT.

2C(iv). Targeting Normal or Abnormal Telomere Sequence and Structure

It is possible to design drugs to bind to specific DNA structures, and given the G-rich nature of the telomeric sequence there have been extensive efforts to develop drugs that target telomeric G-quadruplex DNA (reviewed in ref. [192]). Somewhat paradoxically, there is evidence that such drugs cause telomere uncapping (reviewed in ref. [193]). Although it had been assumed that G-quadruplex stabilizing ligands would act as telomerase inhibitors, it has been found that ciliate telomerase is able to extend G-quadruplex DNA [194, 195], and it remains to be determined whether human telomerase also has this capability, and for which types of G-quadruplexes. Nevertheless, it remains a very interesting possibility that drugs targeting this, or some other aspect of telomeric DNA will have more adverse effects on cells that depend on an activated TMM than on normal somatic cells.

Cells with up-regulated telomerase are not known to contain abnormalities of telomere structure that could be targeted, but, as described above, the telomeres of cells immortalized by up-regulation of ALT contain abnormalities both of telomeric DNA sequence and of associated proteins (reviewed in ref. [134] (Fig. **7**)). Variant telomeric repeats are spread throughout the telomeres of ALT cells, there are proteins which are not normally telomeric present at the telomeres of ALT cells, and the telomeric chromatin appears to be less compacted than normal [9, 77, 135, 138, 196]. In principle, any of these abnormalities are potential targets for development of ALT-specific therapeutics. The very large numbers of DDR foci which are present at the telomeres of ALT cells [90] suggest that these cells may be very vulnerable to further perturbation of telomeric chromatin or conformation.

2C(v). Trans-Acting Factors

The genetic and epigenetic changes that result in telomerase or ALT being up-regulated in cancer cells through the gain or loss of trans-acting factors may result in therapeutically exploitable differences from normal cells. For example, if tumor cells up-regulate TERT by increased expression of trans-activating, or decreased expression of trans-repressing transcription factors that act on TERT gene enhancer elements, it may be possible to kill those cells by transducing them with transgenes which are driven by the relevant TERT enhancers. Examples of transgenes that may serve this purpose include those encoding: a prodrug-activating enzyme which can convert a systemically administered non-toxic drug precursor into an actively toxic form; a protein which causes cell death; or an immune-stimulatory factor which stimulates or enhances an anti-cancer immune response (reviewed in ref. [197]). Similarly, oncolytic viruses could be modified by incorporation of TERT enhancer elements so that they replicate selectively in cancer cells. As an example, an adenovirus (designated Telomelysin, OBP-301) has been constructed in which the E1A and E1B genes are driven by a TERT promoter/enhancer so that it will replicate selectively in cancer cells and thereby cause viral cytotoxicity to those cells [198]. The success of this type of approach will depend on the extent to which telomerase is up-regulated by alteration of TERT trans-acting factors, rather than the other mechanisms for up-

Fig. (7). Telomeric chromatin is altered in ALT cells. Variant repeat DNA sequences which are normally only abundant in the proximal region of the telomere (i.e., the portion closest to the centromere) become spread throughout the remainder of the telomere. This results in increased binding of other proteins such as nuclear receptors, and a relative decrease in the number of shelterin molecules.

regulating telomerase discussed above, such as TERT promoter mutations or TERT and/or TERC gene amplification.

Activation of ALT in tumors is commonly associated with loss of expression of the ATRX or DAXX genes, which encode proteins with multiple proposed functions that include chromatin remodeling, especially at the telomere, and resistance to various cellular stresses [120, 127-133]. It may therefore be possible to devise synthetic lethal approaches to killing ATRX- or DAXX-null ALT cells using cellular stressors for which ATRX and DAXX normally play a protective role.

2D. Challenges for TMM-Targeted Therapies

2D(i). Length of Time to Act

The normal rate of telomere attrition in the absence of a TMM is usually in the range of 50 – 150 base pairs per cell division, so it is expected that there will be a long lag time before TMM inhibitors cause cellular senescence or cell death. A possible exception to this might be in cells where one or more telomeres are already critically short. However, although the telomeres of almost every individual cell within an ALT cell line population range in length from very long to extremely short, when ALT is inhibited by genetic manipulation there are cells that are able to continue dividing for a further 60 – 80 population doublings [80]. This suggests that TMM inhibitors will be more useful for prevention of tumor recurrence than as front-line therapies, and that it will be important to learn how to use TMM inhibitors in combination with treatments that cause rapid tumor debulking. *In vitro* evidence supports the concept that TMM inhibitors will enhance the response to existing anticancer drugs [199], and that they may delay or prevent recurrence of treated tumors by depleting cancer stem cells (reviewed in ref. [171]).

In contrast, drugs or oncolytic viruses that exploit a vulnerability caused by up-regulation of a TMM may act much more rapidly. Moreover, the finding that mammalian cells undergo a well-controlled version of telomere rapid deletion (reviewed in ref. [139]), raises the possibility that a detailed understanding of how this process is regulated may lead to the ability to selectively induce it in cancer cells, which may help overcome the lag time before TMM inhibitors start to have a clinically useful effect.

2D(ii). Potential Side-Effects

Invaluable insights into telomere biology and the potential side-effects of TMM inhibitors have been provided by inherited mutations that result in excessive telomere shortening (reviewed in refs. [200-202]). There are now nine genes associated with short telomere syndromes, with more remaining to be identified. These genes encode telomerase protein and RNA subunits (TERT, DKC1, TERC), proteins involved in telomerase biogenesis (NHP2, NOP10), transport of telomerase (TCAB1), telomere binding (TINF2), and telomere processing (CTC1), and a helicase with telomeric functions (RTEL1). There is a wide spectrum of clinical manifestations, and common causes of mortality include bone marrow insufficiency and pulmonary fibrosis. Studies of these syndromes have revealed that TERT and TERC are haploinsufficient, and that a 50% reduction in telomerase activity can cause severe disease. Moreover, the clinical manifestations in family members inheriting these mutations have an earlier onset and tend to be more severe in successive generations, indicating that telomerase activity is important for maintaining telomere length in the human germ line.

These data suggest that a sustained pharmacological inhibition of telomerase activity (perhaps over a period of several years) could lead to substantial side-effects in tissues such as the bone marrow and lungs, and in the germ line. It is therefore of interest that reversible anemia, neutropenia and thrombocytopenia were noted in Phase I trials of a telomerase inhibitor, but it is not clear to what extent these toxicities were due to concurrent treatment with standard chemotherapy [171]. Although there is evidence that ALT activity also occurs in normal mammalian somatic cells [89], as yet there are no genetic or other data which provide insights into the likely side-effects of inhibiting ALT.

2D(iii). Drug Resistance

Some tumors have evidence of both telomerase and ALT activity [110-113, 159]. It is not yet known whether both TMMs may be present within individual cancer cells, or whether there are subpopulations within the tumors that use one or other TMM. However, *in vitro* studies in which exogenous telomerase was expressed in ALT cells have demonstrated that both TMMs can co-exist within an individual cell [203-207]. It might be expected that use of a single TMM inhibitor to treat such tumors would be ineffective, leading either to continued telomere maintenance via the other TMM in the case of cancers with dual-positive cells, or to selection for the overgrowth of cells using the other TMM in the case of tumors with mixed subpopulations. Moreover, in tumors that are dependent on a single TMM, inhibition of that TMM exerts a strong selection pressure for the activation of the other [208]. It might therefore be anticipated that successful treatment of cancer by targeting TMMs will require both telomerase and ALT inhibitors for dual-TMM tumors, and in single-TMM tumors will require either the use of both telomerase and ALT inhibitors upfront to prevent the emergence of drug-resistance, or initial treatment with the relevant TMM inhibitor, keeping inhibitors of the other TMM in reserve to treat drug-resistant cells if and when they emerge (Fig. **8**).

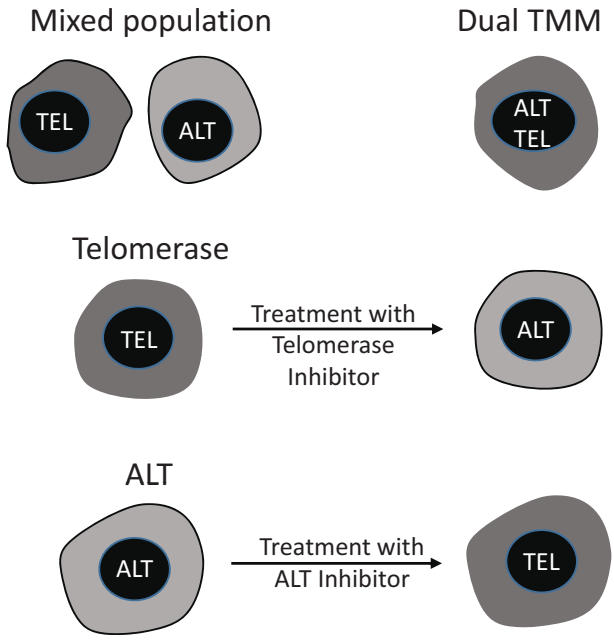

Fig. (8). Telomerase (TEL) and ALT may both need to be targeted for cancer therapy, either simultaneously or sequentially. A significant minority of cancers have both telomerase and ALT activity, either because they contain a mixture of cells with either TMM or, perhaps, because they contain cells which have both TMMs. Treatment of telomerase-positive cancers with potent telomerase inhibitors may be expected to exert a strong selection pressure for the cells to activate ALT, and treatment of ALT cancers with ALT inhibitors will potentially select for activation of telomerase.

2D(iv). Are there Cancers Without a TMM?

Although immortalization is regarded as an essentially universal feature of cancer [209, 210], there may be circumstances where activation of a TMM and acquisition of limitless growth potential is not required for oncogenesis [3]. It has been speculated that these circumstances may include a tumor arising in cells that start out with long telomeres, lack of the usual adverse tumor factors (e.g.,

poor oxygenation) which result in a low cell surviving fraction, and when there is an unusually small requirement for clonal evolution within the tumor (e.g., due to inherited mutations, or a virus that supplies the equivalent of multiple oncogenic mutations) [3]. There are a number of tumor types where TMM analyses have identified a subset of tumors which have neither telomerase activity nor evidence of ALT (e.g., refs. [111-113, 211]), and it is currently unclear whether this is due to a false-negative TMM assay (e.g., due incomplete correlation between ALT and markers such as APBs [109] or due to the presence in tumor tissues of as-yet unidentified inhibitors of the standard telomerase assay), or to the presence of another ALT mechanism, or whether these tumors do not have any TMM. This is an important question to address, because therapeutics that target a TMM are not expected to be effective against TMM-negative tumors.

CONCLUSION

The almost universal dependence of cancer cells on a TMM for immortalization, and the lack of cellular immortalization in normal somatic cells, suggests that the TMMs may be ideal targets for development of anti-cancer therapeutics. However, evidence is emerging from genetic studies that telomerase has an important role in the continued proliferation of a number of normal tissue compartments, and it needs to be investigated whether the same applies to ALT. This may indicate the likely toxicities of sustained use of TMM inhibitors. An alternative treatment strategy may be to target vulnerabilities that are created in cancer cells because of the genetic or epigenetic changes required to up-regulate the TMM, or because of the consequences of the up-regulated TMM. In addition, it will be important to determine the most effective ways to use TMM-targeted therapies in combination with other types of treatments, especially with regard to timing and duration of treatment.

Although good progress has been made to date in understanding telomere structure and function in normal and cancer cells, there are many unanswered questions. It is critically important that telomere biology continues to be studied so that attempts to develop therapies targeting cancer cell telomeres are rationally based and proceed more rapidly than would otherwise be possible.

CONFLICTS OF INTEREST

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