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Tiptoeing to chromosome tips: facts, promises and perils of today's human telomere biology

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The past decade has witnessed an explosion of knowledge concerning the structure and function of chromosome terminal structures—telomeres. Today's telomere research has advanced from a pure descriptive approach of DNA and protein components to an elementary understanding of telomere metabolism, and now to promising applications in medicine. These applications include 'passive' ones, among which the use of analysis of telomeres and telomerase (a cellular reverse transcriptase that synthesizes telomeres) for cancer diagnostics is the best known. The 'active' applications involve targeted downregulation or upregulation of telomere synthesis, either to mortalize immortal cancer cells, or to rejuvenate mortal somatic cells and tissues for cellular transplantations, respectively. This article reviews the basic data on structure and function of human telomeres and telomerase, as well as both passive and active applications of human telomere biology.

Keywords: human telomere; telomerase regulation; cancer; inhibition; cell engineering; tissue engineering

1. HUMAN TELOMERE STRUCTURE AND FUNCTION

Telomeres are indispensable functional domains of eukaryotic chromosomes that form their termini. As the human genome is packed in 46 linear chromosomes, each having two telomeres, there are 92 telomeres in a normal diploid human cell. Biochemically, telomeres are composed of DNA and protein components. The DNA component of human and all the other known mammalian telomeres is formed by a simple tandem repeat $[TTAGGG]_n$. Owing to the end-replication problem of linear chromosomes (Olovnikov 1973) telomeric sequences of the lagging DNA strand become shorter each time the DNA replicates. While some telomere shortening is tolerated (telomeres do not contain genes), their loss below a critically short length stops cell division and the cell enters a state known as senescence, characterized by changes in protein expression patterns and growth arrest. Although the role of telomeres as the replication counting mechanism was suggested about 30 years ago, direct evidence of involvement of telomeres in cellular ageing has been published only recently: it was shown that the introduction of a gene coding for the catalytic component of telomerase, the enzyme complex synthesizing telomeres (see below), resulted in extension of telomere lengths and unlimited cell division potential, the apparent consequence of a bypass of cellular ageing (Bodnar *et al.* 1998). In normal cells, the proliferative block due to telomere shortening may occur in two stages. The first stage, M1 (mortality stage 1) occurs when there are still several kilobases of telomeric DNA left on most chromosomes and may be induced by a DNA damage signal produced by particularly short telomeres on a single or a few of the 92 telomeres; the growth arrest is caused by the tumour suppressor genes *p16*/*pRB* and *p53*. In the case of a block of *p53* and *p16*/*pRB* action, the cell continues dividing with consequent shortening of telomeres till the onset of M2 (mortality stage 2). At this stage, telomeres lose their end-protective function owing to their overall critical shortening and they are recognized as unrepaired chromosome breaks. Subsequent end-degradation and attempts to repair short telomeres then result in chromosome fusions, translocations and extensive genomic instability.

At this stage, immortalization may sporadically occur. Arising immortal cells show in most cases expression and reactivation of telomerase or, in extremely rare cases, an alternative (telomerase-independent) lengthening of telomeres (ALT) (see below).

The function of telomeres is ensured by their DNA component and associated telomere-binding proteins, forming together the functional nucleoprotein structure of the chromosome end. The junction region between the telomere and the rest of the chromosome is termed the 'telomere-associated sequence' and can be regarded as the most distal part of the subtelomere. Subtelomeric sequence structure appears to vary widely, mainly as a result of large differences in subtelomeric repeat sequence abundance and organization at individual telomeres. Many human subtelomeric regions appear to be gene-rich, containing both known and unknown expressed genes.

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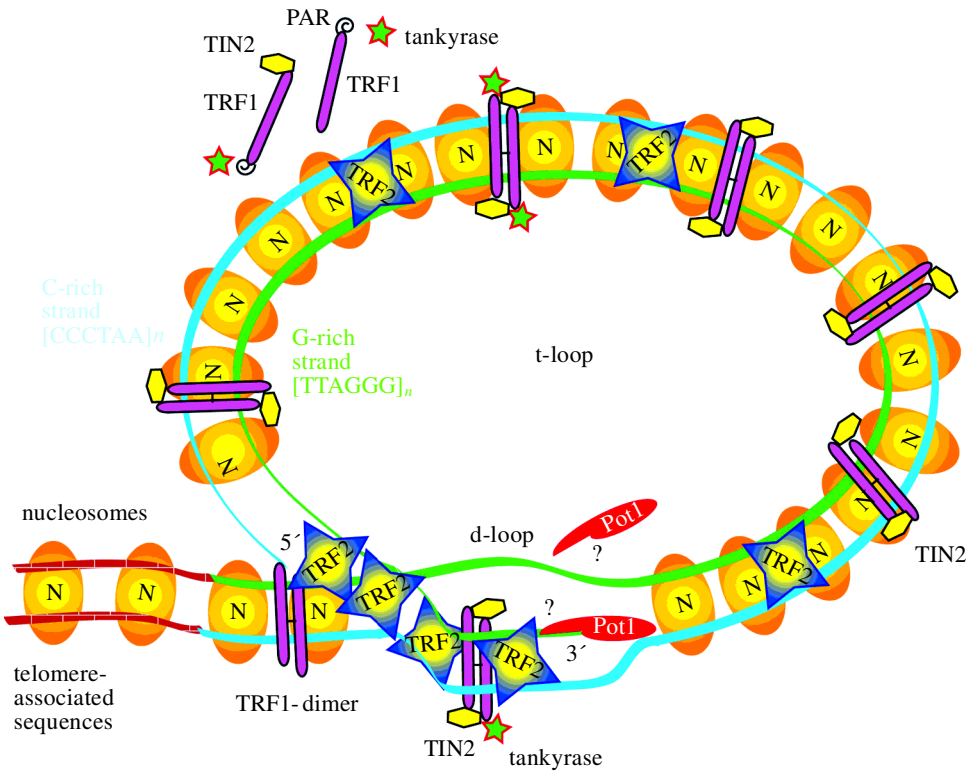

Figure 1. A schematic view of the human telomere. Most of the double-stranded telomeric DNA is packed with histone octamers in nucleosomes. A duplex part of the telomere folds back on itself forming a large telomere loop (t-loop) and the 3′ G-strand extension invades the duplex telomeric repeats and forms a displacement loop (d-loop). The telomere DNA-binding proteins TRF1, TRF2 and Pot1 are required for normal telomere function (see § 1 and table 1 for details). The binding of TRF1 to telomeric DNA may be modulated either negatively by its poly-ADP-ribosylation (PAR) mediated by tankyrase, or positively by associating with TIN2.

This indicates that human subtelomeric regions are not simply buffers of non-functional 'junk DNA' next to the molecular telomere, but are instead functional parts of the expressed genome (Riethman *et al.* 2001). Moreover, subtelomeres may occasionally serve as a backup system for chromosome capping in case of telomere loss. So far, examples of the latter function have been found in some insect and plant species (for a review, see Biessmann & Mason 1997).

The major part of human telomeres (up to 15 kb) is formed by double-stranded telomeric DNA of $[T\,TAGGG]_n$ sequence (see figure 1). Imperfect (degenerated) telomeric repeats occur at the boundary with the subtelomere. Most of the double-stranded telomeric DNA is packed with histone octamers in nucleosomes (Tommerup *et al.* 1994), whereas a non-nucleosomal structure termed the telosome (Wright *et al.* 1992) may be present only at telomere ends (Lingner & Cech 1998). Studies of telomeric chromatin have shown its specific features: on the one hand a lack of nucleosome positioning, and on the other hand a specific and very short nucleosome spacing as revealed by micrococcal nuclease digestion (Fajkus *et al.* 1995; Makarov *et al.* 1993; Rossetti *et al.* 1998). On the basis of these properties and on accumulated experimental data, we proposed a model for a columnar packing of nucleosomes in telomeric chromatin in which the DNA is continuously wound in a parallel manner around the stacked histone cores (Fajkus & Trifonov 2001).

The very end of the telomeric G-rich strand forms a 3′ overhang (further termed the 'G-overhang') on the chromosome terminus. In general, these overhangs represent substrates for telomerase and, consequently, their presence and microstructure may be of key importance for certain levels of regulation of telomere maintenance. Current models for telomere function assume that G-overhangs are found on both chromosome ends and that their association with specific end-binding proteins is critical in allowing cells to distinguish natural ends from double-strand breaks (Griffith *et al.* 1999; Van Steensel *et al.* 1998). A different interpretation of the data is that G-overhangs are simply a by-product of the DNA replication mechanism that must be hidden to prevent chromosome instability or cell-cycle arrest. This idea comes from our observations in plants, where only about half of the telomeres showed the presence of G-overhangs (Riha *et al.* 2000) and is supported by the finding that accumulation of single-stranded G-telomeric DNA triggers p53-dependent cell-cycle arrest (Saretzki *et al.* 1999). Recent studies (Wright *et al.* 1997, 1999) indicate that human chromosome termini are indeed asymmetric. Although the length of the G-overhang may be of great interest, its structure seems to be even more important. Although the single-strand G-rich telomeric strand is a favourite subject of *in vitro* studies on quadruplex (G4) DNA, the existence of this kind of structure has not yet been proved directly *in vivo* or *in situ*, although its existence could be assumed from the occurrence of specific proteins or antibodies

recognizing this structure (Brown *et al.* 1995, 1998; Fang & Cech 1993; Muniyappa *et al.* 2000). The current alternative to a G4 structure is a t-loop (telomeric loop) structure in which a duplex part of the telomere folds back on itself forming a lariat structure, and the 3′ G-strand extension invades the duplex telomeric repeats and forms a d-loop (displacement loop) (Griffith *et al.* 1999). In both G4 and t-loop structures, the G-overhang is masked and made inaccessible to telomerase, a cellular reverse transcriptase that synthesizes telomeres (see § 2). In the formation of either of these structures, or maybe some as yet unknown structure of the G-overhang, end-specific telomere-binding proteins probably participate in analogy to the situation in *Oxytricha*, *Euplotes* or *Xenopus* (Cardenas *et al.* 1993; Price 1990; Price & Cech 1989). Such proteins have recently been identified also in fission yeast and humans. These Pot1 (protection of telomeres) proteins each bind the G-rich strand of their own telomeric repeat sequence, consistent with a direct role in protecting chromosome ends. Deletion of the fission yeast *pot1*$^{+}$ gene has an immediate effect on chromosome stability, causing rapid loss of telomeric DNA and chromosome circularization (Baumann & Cech 2001).

Two previously characterized human telomere-binding proteins, TRF1 and TRF2 (Broccoli *et al.* 1997), have been implicated in the formation of the t-loop structure (Griffith *et al.* 1999; Smogorzewska *et al.* 2000). According to their DNA binding domains, both proteins belong to a group of Myb-like proteins. Protein TRF1 binds the double-strand part of the telomere and may participate in bending telomeric chromatin so that a lariat t-loop structure can be formed. TRF1 functions as a telomerase repressor. Its affinity to a telomere may be abolished by poly-ADP-ribosylation by 'tankyrase' (TRF1-interacting, ANKYrin-related ADP-ribose polymerase), thus making telomeric DNA accessible to telomerase (Smith & De Lange 2000; Smith *et al.* 1998). Protein TIN2 (TRF1-interacting nuclear protein 2) has the opposite effect (Kim *et al.* 1999). The second Myb-like protein, TRF2, participates somehow in maintenance of single-strand overhangs whose absence results in chromosome fusions (Van Steensel *et al.* 1998). In t-loop-reconstitution experiments, TRF2 binds the d-loop at the junction of the lariat (Griffith *et al.* 1999).

Besides these proteins, some others previously known as DNA recombination and repair factors (Rad50, Rad51, Rad52 and Ku70/Ku86 heterodimer) were identified as telomere-binding proteins (Hsu *et al.* 1999, 2000; Laroche *et al.* 1998; Samper *et al.* 2000; Song *et al.* 2000). Their role in the biology of human telomeres is not clear yet, but their involvement in telomerase-independent, alternative lengthening of telomeres (ALT) is generally anticipated. In *Saccharomyces cerevisiae*, two telomerase-independent mechanisms can overcome the senescence resulting from the loss of telomerase activity. Type I survival is characterized by amplification of the subtelomeric Y′ elements with a short telomere repeat tract at the terminus whereas type II survivors arise through the abrupt addition of long tracts of telomere repeats. Both mechanisms are dependent on RAD52 and on either RAD50 or RAD51. The telomere elongation pathway in yeast (type II) is dependent on SGS1, the yeast homologue of the gene products of Werner's (WRN) and Bloom's (BLM) syndromes (Huang *et al.* 2001; Chen *et al.* 2001; Le *et al.* 1999).

In human cells, telomerase-independent telomere maintenance (ALT) was observed in a minor fraction of telomerase-negative *in vitro* immortalized and tumour-derived cell lines (Bryan *et al.* 1997). Molecular analysis of these cells showed an extreme heterogeneity in telomere length and differences in the dynamics of telomere maintenance compared with telomerase-positive cell lines. The nature of the ALT mechanism(s) is currently unknown, but studies of telomere dynamics in an ALT cell line containing a marker just proximal to the telomeric sequences showed gradual shortening of the telomere followed by rapid elongation, consistent with a non-reciprocal recombinational mechanism similar to that found in telomerase-defective mutant yeast strains (Reddel *et al.* 1997). Fusion of ALT cells to telomerase-positive immortal cells resulted in hybrids that appeared immortal and also exhibited repression of the ALT telomere phenotype. In these hybrids, which were all telomerase positive, an initial rapid loss of most long telomeres was observed, followed either by gradual loss of the remaining long telomeres at a rate similar to the rate of telomere shortening in normal telomerase-negative cells, or by maintenance of shortened telomeres (Perrem *et al.* 1999). In contrast, expression of exogenous *hTERT* in the ALT cell line GM847 resulted in lengthening of the shortest telomeres, thus showing that induced telomerase is active at the telomere. However, rapid fluctuation in telomere length still occurred in the GM847/*hTERT* cells after more than 100 population doublings. Very long telomeres and ALT-associated promyelocytic leukaemia (PML) bodies continued to be generated, indicating that telomerase activity induced by exogenous *hTERT* did not abolish the ALT mechanism. These data indicate that the telomerase-positive cells contain a factor that represses the ALT mechanism but that this factor is unlikely to be telomerase. The transfection data also indicate that ALT and telomerase can coexist in the same cells (Perrem *et al.* 2001). However, in a similar study using the simian virus 40 (SV40) large T-antigen-immortalized human lung fibroblast cell line VA13, which uses ALT, a significant functional inhibition of ALT upon telomerase expression has been observed (Ford *et al.* 2001). Furthermore, expression of mutant telomerase (synthesizing mutated telomere sequence) in immortal telomerase-negative human cells results in cell-cycle deregulation, nuclear and chromosomal abnormalities and rapid loss of viability. These results suggest that even very limited synthesis of mutated sequences can affect telomere structure in human cells, and that the toxicity of mutant telomerases is due to telomere malfunction (Guiducci *et al.* 2001).

2. HUMAN TELOMERASE AND ITS REGULATION

The primary mechanism of telomere synthesis in human is telomerase (originally termed 'telomere-terminal transferase') (Greider & Blackburn 1985). Telomerase is found in foetal tissues, adult germ cells and tumour cells, and lower telomerase activities can also be detected in self-renewing tissues of a high proliferative capacity. Telomerase activity is regulated during development and in most somatic cells is repressed to an almost undetectable level.

The lack of telomerase in somatic cells results in their ageing, which then contributes to ageing of tissues, organs and the body (Shay & Wright 2001; Shay *et al.* 2001). It is therefore no surprise that telomerase has become a hot topic in cancer and ageing research.

Telomerase is an enzyme complex made of a catalytic subunit with reverse transcriptase activity, and an RNA subunit that serves as a template for telomere sequence synthesis. The whole RNA subunit, hTR, is 451 nucleotides long and lacks polyadenylation. Its template region is formed by an 11-nucleotide sequence 5′-CUAACC CUAAC-3′ complementary to one complete and one incomplete repeat unit of G-strand telomeric sequence. The gene coding for hTR is located on the long arm of chromosome 3 (3q26.3) and its promoter region comprises CpG islands (Soder *et al.* 1997). Several transcription factors that modulate the expression of *hTR* have been found. NF-Y binding to the CCAAT region of the *hTR* promoter is essential for promoter activity whereas Sp1 and the retinoblastoma protein (pRb) are activators of the promoter and Sp3 is a potent repressor. These factors appear to act in a species-specific manner (Zhao *et al.* 2000). In cell lines exhibiting ALT and total absence of *hTR* expression, the promoter of the *hTR* gene is methylated, and treatment with 5-azacytidine in combination with trichostatin A resulted in partial demethylation of the promoter and expression of the gene (Hoare *et al.* 2001). Another example of *hTR* regulation is that imposed by dyskerin. Dyskerin is the protein whose absence or defect results in dyskeratosis congenita, a rare inherited disorder whose symptoms include fragile bones, the absence of hair, progressive nail dystrophy, underdeveloped testes, precancerous cells in mucous membranes, abnormalities of the gastrointestinal tract, pulmonary fibrosis, immune dysfunction and increased risk of skin cancer. It has been reported recently that this disorder may be caused by deficiency of telomerase and abnormal shortening of telomeres (Mitchell *et al.* 1999). Since normal dyskerin is associated with hTR as a component of the telomerase ribonucleoprotein (RNP) complex, it may be important for the biogenesis, processing or turnover of the telomerase RNP.

The catalytic activity of telomerase reverse transcriptase resides in its protein component hTERT whose amino acid sequence comprises a telomerase-specific motif (the t-motif) and seven further motifs conserved in reverse transcriptases of retrotransposable elements. The *hTERT* gene (of 40 kb length) has been mapped to the very distal part of chromosome 5p (5p15.33; Bryce *et al.* 2000). Its coding sequence is partitioned into 16 exons and is translated to the 127 kDa protein of 1132 amino acid residues (Meyerson *et al.* 1997). In most normal somatic cells, the *hTERT* gene is repressed and is therefore regarded as the limiting component of the telomerase holoenzyme. The principles of regulation of *hTERT* are the focus of intense study (see table 1).

At the transcriptional level, the *hTERT* promoter may be methylated in its CpG island. Consequent histone deacetylation (Cong & Bacchetti 2000) could repress transcription; however, the results obtained in cell lines are ambiguous (Devereux *et al.* 1999). The promoter of *hTERT* contains binding sites for several transcription factors; a positive regulatory role has been proved in the case of c-Myc and Sp1 proteins, which cooperate in transcription activation of *hTERT* (Kyo *et al.* 2000), and oestrogen (Kyo *et al.* 1999), while myeloid-specific zinc finger protein 2 (MZF-2) and Mad are possible negative regulators of expression (Fujimoto *et al.* 2000; Oh *et al.* 2000). The observed opposite effects of Myc and Mad proteins on *hTERT* expression correspond to replacement of Myc–Max complex by Mad/Max during the process of differentiation and during inhibition of cell growth (Henriksson & Luscher 1996). In recent reports, there are a number of other factors influencing expression of *hTERT* indirectly; interferon-α (IFN-α) downregulates *hTERT* in a cell-cycle dependent manner (possibly via downregulation of c-Myc) (Akiyama *et al.* 1999) whereas the p53 tumour suppressor protein inhibits *hTERT* expression via inhibition of Sp1 binding to the *hTERT* promoter by forming a p53–Sp1 complex (Kanaya *et al.* 2000; Xu *et al.* 2000).

In addition to these examples of *hTERT* regulation at different levels, a search for telomerase repressor genes in individual human chromosomes has been performed using microcell-mediated chromosome transfer. Two putative 'mortality genes' that suppressed telomerase activity at the *hTERT* transcription level and whose expression results in telomere shortening have been found in chromosome 3 (Horikawa *et al.* 1998; Tanaka *et al.* 1998), chromosome 7 (Nakabayashi *et al.* 1999) and chromosome 10 (Nishimoto *et al.* 2001).

Although the primary level of telomerase activity depends on transcriptional control of *hTERT*, another mechanism of *hTERT* regulation has been found recently at the level of its mRNA splicing. Primary *hTERT* transcripts can be spliced into six variants, only one of these being a precursor of functional telomerase, reflected by frequent findings of *hTERT* mRNA even in samples lacking telomerase activity. Moreover, one of the 'deletion' splicing variants (hTERTα), which is missing conserved residues from the catalytic core of the protein, inhibits telomerase activity (Colgin *et al.* 2000; Yi *et al.* 2000).

A post-transcriptional mechanism is also responsible for upregulation of telomerase activity in neural precursor cells by fibroblast growth factor 2 (FGF-2) (Haik *et al.* 2000).

At the post-translational level it appears that phosphorylation of hTERT is essential for its activity (Kharbanda *et al.* 2000) and selective dephosphorylation by protein phosphatase 2A (PP2A) inhibits telomerase activity (Li *et al.* 1997). This inhibition may be reversed by protein phosphorylation. In accordance with this finding, overexpression of the cyclin-dependent kinase inhibitors p16INK4A (*CDKN2A*) and p15INK4B (*CDKN2B*) inhibits telomerase activity (Fuxe *et al.* 2000).

In addition to the hTR and hTERT subunits, which are both essential and sufficient for telomerase action *in vitro*, further proteins were identified that participate in the telomerase RNP complex. Telomerase-associated protein 1 (TEP1) physically associates with both hTR and hTERT and contributes to RNP complex stability. However, no obvious phenotype was observed in mice mutants lacking mTep1 (Kickhoefer *et al.* 2001). The molecular chaperones p23 and hsp90 physically and functionally associate with hTERT and blockade of this interaction inhibits assembly of active telomerase *in vitro* (Forsythe *et al.* 2001; Holt *et al.* 1999).

Table 1. Examples of telomere maintenance regulation.

subject of regulation	name of the factor	mechanism/result	reference
hTR gene promoter	NF-Y	activation of transcription	Zhao *et al.* (2000)
	Sp1	activation of transcription	Zhao *et al.* (2000)
	pRB	activation of transcription	Zhao *et al.* (2000)
	Sp3	repressor of transcription	Zhao *et al.* (2000)
	CpG methylation	repressor of transcription	Hoare *et al.* (2001)
hTR RNA	dyskerin	telomerase RNP processing?	Mitchell *et al.* (1999)
hTERT gene promoter	CpG methylation, histone deacetylation	repression of transcription	Cong & Bacchetti (2000) and Devereux *et al.* (1999)
	MZF-2	repression of transcription	Fujimoto *et al.* (2000)
	oestrogen	activation of transcription	Kyo *et al.* (1999) and Misiti *et al.* (2000)
	c-Myc	activation of transcription	Kyo *et al.* (2000)
	Sp1	activation of transcription	Kyo *et al.* (2000)
	Mad	repression of transcription (competition with c-Myc)	Henriksson & Luscher (1996) and Oh *et al.* (2000)
	IFN-α	repression via downregulation of c-Myc	Akiyama *et al.* (1999)
	p53	repression via Sp1 binding	Kanaya *et al.* (2000) and Xu *et al.* (2000)
	mortality genes at chromosomes 3 and 7	repression of *hTERT* transcription	Horikawa *et al.* (1998), Tanaka *et al.* (1998) and Nakabayashi *et al.* (1999)
hTERT pre-mRNA	hTERTα	alternative splicing variant/activity inhibition	Colgin *et al.* (2000) and Yi *et al.* (2000)
hTERT mRNA	FGF-2	downregulation of *hTERT* mRNA	Haik *et al.* (2000)
hTERT protein	c-Abl tyrosine kinase	*hTERT* phosphorylation/telomerase activation	Kharbanda *et al.* (2000)
	PP2A	*hTERT* dephosphorylation/telomerase inhibition	Li *et al.* (1997)
	$p16^{INK4A}$, $p15^{INK4B}$	inhibition of cyclin-dependent kinase/inhibition of telomerase	Fuxe *et al.* (2000)
	p23/hsp90	*hTERT* association/required for active telomerase assembly	Forsythe *et al.* (2001) and Holt *et al.* (1999)
hTERT + *hTR*	TEP1	required for telomerase RNP assembly and hTR stability	Kickhoefer *et al.* (2001)
telomeric DNA	TRF1	double-stranded telomeric DNA binding and bending/repressor of telomere lengthening	Broccoli *et al.* (1997)
	TRF2	protection of G-overhangs, promotes t-loop formation	Broccoli *et al.* (1997) and Griffith *et al.* (1999)
	Pot1	single-stranded telomeric DNA binding, protection of G-overhangs	Baumann & Cech (2001)
	tankyrase	poly-ADP-ribosylation of TRF1/repressor of TRF1 binding to telomere, enhances accessibility of telomeric DNA to telomerase	Smith & De Lange (2000) and Smith *et al.* (1998)
TRF1	TIN2	TRF1-interaction/opposite effect to tankyrase	Kim *et al.* (1999)

Besides the number of possibilities of telomerase regulation at the level of telomerase components and telomerase-associated proteins, one should not forget about the regulation of telomere lengths by telomeres themselves which is performed at the level of formation of alternative secondary structures of DNA (e.g. t-loop and G4) and interdependent changes in interactions with telomerase and telomere-binding proteins.

3. TELOMERASE IN AGEING, PROLIFERATION, DIFFERENTIATION AND CANCER

At present, not only has the role of replicative telomere loss as a central timing mechanism for cellular ageing been proved but also the possibility of resetting this mechanism has been demonstrated (for a review, see Fossel 1998). For example, it has been shown that telomeres are shorter in somatic than in germline cells and that they shorten in somatic cells with the individual's age. Children born with progeria (early ageing syndrome) have shortened telomeres compared with age-matched controls. Introduction of a construct encoding and expressing the human telomerase catalytic subunit results in elongation of telomeres and extension of proliferative capacity of cultured cells without their conversion to a malignant phenotype (Bodnar *et al.* 1998).

Telomerase activity and the levels of telomerase subunits hTR and hTERT are associated with cell proliferation in cultured and tumour cells. In those cells that express telomerase, a tight correlation between telomerase level and cell growth was found, e.g. in human tumours *in situ* hybridization assays showed that the levels of the telomerase RNA component correlated with the proliferative marker MIB-1 (for a review, see Greider 1998). Contrary to original expectations, telomerase has also been found in a number of proliferating normal somatic cells, e.g. mitogenic stimulation of lymphocytes caused upregulation of telomerase (Holt *et al.* 1996). Telomerase was also detected in the proliferative basal layer of skin, and in epithelial and endothelial cell lines growing in culture. Endometrial tissue and the proliferative zone of intestinal crypts are also telomerase positive. However, there are numerous reports that some primary cell types, such as fibroblasts, mammary epithelium and embryonic kidney cells, do not express telomerase activity even when they are proliferating (Greider 1998). Irrespective of the telomerase detected in some proliferative renewal tissues, telomeres are shorter in older individuals compared with younger ones, and thus the telomerase detected is not sufficient to completely maintain telomeres. Downregulation of telomerase occurs during cell differentiation, including in various cell lines induced to differentiate, demonstrating an indirect relationship between telomerase levels in the processes of proliferation and differentiation. The decrease of telomerase activity has been shown in various cell lines induced to differentiation (Albanell *et al.* 1996).

Conflicting reports have appeared concerning the cell-cycle regulation of telomerase activity and its possible repression during quiescence and cell differentiation. Therefore, these issues have been re-examined to uncover the basis for the discrepancies (Holt *et al.* 1997). Variations in extracted telomerase activity during the cell cycle were not observed in cells sorted on the basis of DNA content. Variations were observed in cells synchronized using some biochemical cell-cycle inhibitors of evident cellular toxicity. A progressive decline in telomerase activity is observed in cells whose growth rate is reduced from seven to eight population doublings per week to one to two doublings per week. Telomerase is largely absent from cells that truly exit the cell cycle and do not divide over the seven-day period. In the immortal cultured cell lines examined, extracted telomerase activity does not change significantly during progression through the stages of the cell cycle. Telomerase activity generally correlates with growth rate and is repressed in cells that exit the cell cycle and become quiescent.

The main role of telomerase in cancer cells is to assure their immortality, a process necessary to accumulate mutations required for cells to become malignant (Holt *et al.* 1996). The telomere lengthening reverses replicative senescence and increases the risk of cancer because it allows transformed cells to continue dividing and to achieve malignant characteristics (Fossel 1998). Telomerase reactivation or upregulation may be caused by mutations in the telomerase repression pathways (Shay & Wright 2001). Telomerase activity in cancer cells correlates with the stabilization of telomere length and cellular immortalization. Thus, telomerase is an important mechanism almost universally required to maintain telomere stability as a critical step in cancer progression. However, it has been established that although telomerase expression is a hallmark of cancer, ectopic expression of telomerase in normal human fibroblasts, which is sufficient for their immortalization, does not result in changes typically associated with malignant transformation (Morales *et al.* 1999).

It should be mentioned here that reports differ as to whether reconstitution of telomerase activity alone is sufficient for immortalization of different types of human somatic cells or whether additional activities encoded by other 'immortalizing' genes are also required. For example, it has been known that the *E6* and *E7* oncogenes of human papillomavirus type 16 (HPV-16) are sufficient for the immortalization of human genital keratinocytes *in vitro*. The products of these viral genes associate with p53 and pRb tumour suppressor proteins, respectively, and interfere with their normal growth-regulatory functions. Recently, the HPV-16 E6 protein (but not the E7 protein) has been shown to increase the telomerase enzyme activity in primary epithelial cells by inducing *hTERT* gene transcription (Veldman *et al.* 2001). Nevertheless, in addition to upregulation of telomerase by E6, E7 is required. Correspondingly, exogenous expression of *hTERT* in human foreskin keratinocytes and human mammary epithelial cells was found to be insufficient for their immortalization. These cell types can overcome senescence by co-expression of *hTERT* and *HPV E7* or by expression of *hTERT* and loss of *p16*(*INK4a*) expression, indicating that the retinoblastoma (Rb) pathway, along with a telomere maintenance pathway, plays a role in determining the lifespan of epithelial cells (Farwell *et al.* 2000).

In analogy with these results, it has been shown that ectopic expression of either the catalytic subunit of human telomerase (hTERT) or a temperature-sensitive mutant (U19tsA58) of SV40 large-tumour antigen alone was not sufficient for immortalization of freshly isolated normal

adult human mammary fibroblasts and endothelial cells. However, a combination of both genes resulted in the efficient generation of immortal cell lines. Temperature-shift experiments revealed that maintenance of the immortalized state depended on continued expression of functional U19tsA58 large-tumour antigen, with hTERT alone unable to maintain growth at non-permissive temperatures for U19tsA58 large-tumour antigen (O'Hare *et al.* 2001).

4. CLINICAL ASPECTS OF TELOMERASE: DIAGNOSTIC AND PROGNOSTIC VALUE OF TELOMERASE ACTIVITY AND EXPRESSION OF *hTR* AND *hTERT* IN CANCER CELLS

Owing to the prevalent expression of telomerase in human malignant tumours, and its potential use not only as a diagnostic and prognostic tool but also as a potential target for therapy, telomerase activity and both telomerase components, i.e. *hTR* and *hTERT*, have been the focus of intensive studies in many tumours. Qualitative assays for telomerase activity were replaced by a modified telomere repeat amplification protocol (TRAP) assay for semi-quantitative determination of telomerase activity levels (Kim *et al.* 1994; Wright *et al.* 1995). In the first step of this assay, telomerase adds telomeric repeats to the 3′-end of a synthetic primer of nontelomeric sequence and the extended products are subsequently amplified by PCR. The amplicons are either analysed by polyacrylamide gel electrophoresis with fluorescent or isotopic detection or quantified by enzyme immunoassay (TRAPeze ELISA detection kit, Oncor, USA; *T*elo*TAGGG* Telomerase PCR ELISA PLUS, Roche, Germany; Telomere Length and TRAP assay kits, BD PharMingen, USA). Both telomerase subunits (hTR and hTERT) can be quantified by LightCycler *T*eloTAGGG hTERT and/or hTR Quantification Kits (Roche, Germany).

Over the past six years, screening of most types of human cancers has shown a strong association between telomerase activity and malignancy. Telomerase was detected in almost all advanced tumours of over 30 types that have been studied. A detailed survey of telomerase activity in human cancer was published in 1997 (Shay & Bacchetti 1997). Positive telomerase estimated with the TRAP assay was found in 86% of head and neck cancers (number of positive/number of tested cases: 112/130), in 81% of lung cancers (113/140), in 87% of gastrointestinal cancers (195/223), in 95% of pancreatic cancers (41/43), in 86% of hepatic tumours (149/173), in 88% of breast cancers (300/339), in 96% of female reproductive tract tumours (49/51), in 90% of male reproductive tract tumours (52/58), in 89% of kidney and urinary tract tumours (273/306), in 69% of neural malignant tissues (199/290), in 92% of skin cancers (94/102) and in 73% of haematological malignancies (143/194). However, positive telomerase activity was also detected in 10% of adjacent tissues (77/749), in 26% of benign and other non-malignant lesions (69/262), and in 5% of normal tissues (12/224) (Shay & Bacchetti 1997). These values were confirmed in an extended group of tumours in the year 2000 (table 2).

Table 2. Telomerase activity in human normal tissues and malignant tumours (according to Shay & Wright (1999)).

tissue	no. of positive/no. tested	positive (%)
normal tissue or adjacent to tumour	367/2350	16
preinvasive	410/1391	29
malignant tumours		
respiratory	431/541	80
digestive	1136/1330	85
reproductive	709/801	89
breast	777/896	87
urinary	392/443	88
haematological	117/157	75
total malignant	3562/4168	85

(a) *Head and neck squamous cell carcinomas*

Telomerase was detected in up to 86% of head and neck squamous cell carcinoma tissues and also in hyperplastic squamous epithelium, but not in normal squamous mucosa. However, telomerase activity was found in 58% of histologically tumour-free resection margins (Koscielny *et al.* 2000).

(b) *Lung carcinomas*

Almost all lung carcinomas of both types (small cell and non-small cell) express telomerase activity (Shay & Bacchetti 1997). Low-level activity was shown in normal bronchial epithelium. Telomerase activity was detected in 93% (107 of 115) lung carcinomas, but not in any adjacent non-cancerous tissues, and was significantly higher in small cell carcinoma than in any other histological type. The overall survival rate was significantly lower in the high telomerase group (Kumaki *et al.* 2001). Immunohistochemical staining of hTERT revealed this protein in the nuclei of cancer cells, while mRNA for hTERT and hTR were mainly detected in their cytoplasm (Kumaki *et al.* 2001). The concordance between telomerase activity in lung cancer tissue and *hTERT* expression was 77% (Arinaga *et al.* 2000).

(c) *Breast cancer*

Determination of telomerase activity in breast cancer can be a diagnostic tool, and mean telomerase levels show a correlation with the severity of histopathological changes: telomerase was positive in 14% of benign breast diseases, in 92% of carcinoma *in situ* lesions and in 94% of invasive breast cancers (Yashima *et al.* 1998*b*). Careful handling of initially negative carcinoma samples revealed that the true values of positive tissues may be even higher—*ca.* 95% for breast cancer (Carey *et al.* 1998). Detection of telomerase activity in preoperative specimens, such as fine needle biopsies, may improve diagnostic accuracy (reviewed in Herbert *et al.* 2001). Some studies evaluating prognosis based on this parameter reported increased values of telomerase activity associated with decreased disease-free survival (Clark *et al.* 1997; Herbert *et al.* 2001; Simickova *et al.* 2001). However, conclusions concerning the prognostic significance of telomerase activity in breast cancer are still incomplete.

Molecular detection of telomerase-positive circulating epithelial cells appears to be a sensitive, specific and non-invasive approach for detecting circulating cancer cells in patients with metastatic breast cancer (Soria *et al.* 1999). Quantitation of *hTERT* gene expression in breast cancer tissue by use of real-time PCR revealed statistical correlations between high hTERT mRNA levels and histopathological grade, and negative oestrogen and progesterone receptors (Bieche *et al.* 2000).

(d) *Colorectal and gastric carcinomas*

These carcinomas are characterized by high levels of telomerase activity (Shay & Bacchetti 1997), but normal tissues of this region are usually negative or weakly positive. In an extensive study of 100 primary colorectal cancer specimens, the prognosis of the patients with high telomerase activity (more than 50-fold more than in non-cancerous mucosa) was significantly worse than that for patients with moderate and low telomerase activity. Among the 87 patients with curative surgery, disease-free survival rate of those with high telomerase activity was also significantly poorer (Tatsumoto *et al.* 2000). Immunohistochemical localization of hTERT protein in tumour and non-tumour colorectal tissues, using antibodies raised against a partial peptide of hTERT, revealed expression of this protein in normal mucosa and did not always result in significant expression of telomerase activity (Tahara *et al.* 1999). Gastric cancer with high telomerase activity tended to have deeper invasion, lymph node metastasis, liver metastasis and peritoneal dissemination (Kakeji *et al.* 2001).

(e) *Hepatocellular and pancreatic carcinomas*

Most hepatocellular and pancreatic carcinomas showed telomerase activity (Shay & Bacchetti 1997) but cirrhotic liver tissues and benign pancreatic lesions were telomerase negative. Survival of patients seemed to be better in the absence of telomerase expression. Expression of *hTR* was detected in 78% of cases of hepatocellular carcinoma tested and in 33% of adjacent non-cancerous tissue (Ferlicot *et al.* 1999). *In situ* hybridization for hTR has been successfully used to differentiate normal ductal epithelium from adenocarcinoma in endoscopic brushings (Morales *et al.* 1998).

(f) *Gynaecological cancers*

In all gynaecological cancers, telomerase activity of the cancerous tissues was significantly higher than that of the non-cancerous lesions (Sakamoto *et al.* 2000). Telomerase activity in ovarian cancer increased with stage progression; clear cell adenocarcinomas showed significantly lower activity (Sakamoto *et al.* 2000). In endometrial carcinomas (Ebina *et al.* 1999), telomerase correlated significantly with advanced stage and pelvic node metastases. Messenger RNA expression of hTERT was closely associated with telomerase activity in ovarian carcinomas (Park *et al.* 1999). Increased frequency of telomerase activation in cervical cancer was correlated with increased severity of histopathological changes, but not with human papillomavirus infection (Yashima *et al.* 1998*a*). However, semiquantitative *hTERT* mRNA levels as well hTR levels were related to telomerase activity and the presence of an oncogenic type of papillomavirus (Wisman *et al.* 2000). Interesting results were obtained by the study of normal and neoplastic human models (ovary, endometrial and myometrial tissues) where the lack of telomerase activity in several non-malignant tissues, expressing hTERT containing complete critical reverse transcriptase motifs, suggests that there are further mechanisms for suppressing telomerase activity by translational or post-translational modifications (Ulaner *et al.* 1998).

(g) *Prostatic hyperplasy and prostatic cancer*

Telomerase activity in fine needle biopsies seems to be of value for distinguishing benign prostatic hyperplasy from prostatic cancer (Shay & Bacchetti 1997). In high grade prostatic intraepithelial neoplasia (PIN) telomerase activity is present in a low percentage of high-grade PIN foci (Koeneman *et al.* 1998).

(h) *Others*

In melanomas, clinicopathological correlations showed association between high telomerase activity and early metastatic spread (Rudolph *et al.* 2000; Ramirez *et al.* 1999). In renal cell carcinoma, significant correlations were found between the telomerase activity and tumour grade (Fujioka *et al.* 2000; Mehle *et al.* 1996). The results of the measurement of telomerase activity from voided urine may help provide earlier diagnosis of bladder cancer and earlier postoperative indication of recurrence (Cheng *et al.* 2000). However, limitations of such measurement for the diagnosis of urothelial cancer should be considered (Arai *et al.* 2000). Clinical trials are needed to evaluate the utility of telomerase in easily obtained cells from other body cavities and effusion samples.

Preliminary results showed that suppression of telomerase activity after cytotoxic drug treatment *in vitro* could be an indicator of drug-induced cytotoxicity in chemosensitivity testing (Faraoni *et al.* 1999).

In conclusion, telomerase activity seems to be a useful indicator of malignant potential of tissues (Shay & Bacchetti 1997). However, a key question as yet unresolved is whether the detection of telomerase activity will allow discrimination between slowly and rapidly progressing malignancies. Telomerase could be also a promising marker of minimal residual disease. Various investigations correlating telomerase activity with known prognostic factors have yielded conflicting results. However, with increasing accumulation of data on telomerase activity and hTERT status in comparison with clinicopathological data, it seems possible that the determination of these parameters may help in the stratification of risk of individual patients.

5. ANTI-TELOMERASE THERAPY OF CANCER

(a) *Promises and perils of telomerase inhibition*

It follows from the above discussion that telomerase is an almost universal marker and immortalization factor of tumour cells. Consequently, it has become a promising candidate target for new cancer therapies, which could meet the criteria of the desired therapeutic effect with minimal side effects. Nevertheless, the fact that telomerase activity is not restricted only to tumour cells, but is essential for stem cells, self-renewing tissues and germinal cells, raises the question of potential adverse effects of anti-telomerase treatment. On the other hand, similar effects

on stem cells and proliferative descendents are present in all standard treatments.

Fortunately, stem cells typically possess much longer telomeres than cancer cells, which may provide a sufficient window for application of anti-telomerase therapy. Moreover, stem cells proliferate intermittently and require minimum telomerase activity during their quiescent period.

Another potential problem of anti-telomerase therapy is a delay in efficacy; sufficient shortening of telomeres to cause cells to stop dividing takes a period of time and therefore anti-telomerase agents would need to target small numbers of cells, not large bulk tumours for which preceding surgery or chemotherapy may be necessary.

Probably the last possible problem known to date is the existence of telomerase-independent ALT cells (see above) in a minor fraction of tumours, which would thus be resistant to anti-telomerase therapy; development of agents which inhibit ALT is therefore desirable for a complete block of telomere maintenance.

Currently many papers have been published on drugs with telomerase activity inhibitory effects, but to conclude that any anti-telomerase compound acts purely through a telomere–telomerase pathway, certain criteria must be met (White *et al.* 2001).

(i) Initially after administration of inhibitors, only telomerase activity, but not cell growth rate, should be affected (the so-called 'lag phase').
(ii) Progressive telomere shortening should be observed after addition of inhibitors.
(iii) Administration of the inhibitor after sufficient telomere shortening should cause growth arrest or initiation of apoptosis.
(iv) The time necessary to affect growth rates should vary depending on initial telomere lengths.
(v) Chemically related molecules that do not inhibit telomerase activity should not cause decreased cell proliferation or telomere shortening.

(b) *Telomerase inhibition strategies*

The available data on structure and function of the telomerase–telomere complex offer a number of possible targets for inhibitors of telomere maintenance. Here we review possible sites and mechanisms of anti-telomerase strategies (see table 3).

(i) *Targeting the catalytic subunit hTERT*

In the data reviewed above, hTERT is the limiting telomerase component and thus the target of first choice. A number of strategies are being investigated:

Reverse transcriptase inhibitors (RTIs)

This approach makes use of inhibiting the reverse transcriptase activity of hTERT using mainly agents that are applied in human immunodeficiency virus (HIV) therapy. In an early report on this subject, Strahl & Blackburn (1996) treated two immortalized cell lines (B and T cells) with a range of inhibitors of retroviral reverse transcriptase. Among RTIs, only dideoxyguanosine (ddGTP) and 3′-azido-3′-deoxythymidine (AZT) inhibited telomerase activity *in vitro*. Passaging of these cell lines with ddGTP caused progressive shortening of telomeres, but after several weeks the telomeres became stabilized at their short length. No effect on population doubling rates or morphology, or growth arrest, was observed after prolonged passaging. Several other groups published results of tests of RTIs, predominantly with AZT, and although they found telomerase inhibition and affected growth rates they were not able to see either telomere shortening, morphological changes or growth arrest (Gomez *et al.* 1998; Murakami *et al.* 1998; Yegorov *et al.* 1996). Since these RTI-affected cells did not have a lag phase (described above), their action is probably not limited to inhibition of telomerase.

Dominant negative hTERT (DN-hTERT)

The principle of this method is simple: a construct of mutant *hTERT* cDNA is prepared, which produces a protein lacking catalytic activity but still exerting binding properties for the RNA subunit of telomerase, which consequently acts as a dominant negative version of *hTERT* and inhibits telomerase activity. This strategy was published by Hahn *et al.* (1999) who generated a construct of mutant *hTERT* cDNA with replacement of two amino acids (positions 710, 711) and transfected it into certain human cancer cell lines. Cells with ectopic expression of DN-*hTERT* showed gradual telomere shortening and loss of telomerase activity, and finally an influence on replicative potential of the transfected cells was observed with cell-cycle arrest and induction of apoptosis. In this work, *in vivo* effects on tumorigenicity were studied in athymic mice inoculated with various tumour cell lines transfected with DN-*hTERT*; no tumours were observed. An immortal cell line GM 847, which possesses an ALT mechanism of telomere maintenance, was also transfected with DN-*hTERT*; clones remained negative for telomerase activity and showed no change in telomere dynamics and cell growth. On the other hand, GM 847 clones infected with a wild-type *hTERT* construct recovered telomerase activity but kept heterogeneous telomere lengths, a marker of ALT; as mentioned above, a different result has been obtained in recent work showing that wild-type *hTERT* expression resulted in loss of ALT phenotype (Ford *et al.* 2001). Using a similar approach, telomerase inhibition in cells with short telomeres was observed, which led to chromosomal damage and apoptotic cell death (Zhang *et al.* 1999).

Immunotherapy using hTERT as a tumour-specific antigen

Recently published work has opened possibilities of using hTERT-mediated anti-tumour cytotoxic T cell responses. Telomerase, which is activated in 85–90% of tumours, could be a good candidate for a universal tumour-associated antigen usable in immunotherapy of tumours. The amino acid sequence of hTERT has been screened for 9 mer peptide sequences containing known binding motifs for the HLA-A2.1 molecule of the HLA-A2 allele. This MHC class I allele is expressed in *ca.* 50% of the population. A peptide derived from hTERT, I540, was identified that possesses binding properties to HLA-A2.1 antigen. Autologous dendritic cells were primed with this peptide, which generated $CD8^+$ cytotoxic T lymphocytes (CTLs) that lysed $hTERT^+$ tumours of multiple histologies whereas neither telomerase-negative nor

Table 3. Overview of telomerase inhibition strategies.
(Abbreviation: HHR, hammerhead ribozymes.)

target	mechanism	reference
hTERT	RT inhibitors	Strahl & Blackburn (1996), Murakami *et al.* (1998), Gomez *et al.* (1998) and Yegorov *et al.* (1996)
	immunotherapy	Vonderheide *et al.* (1999) and Minev *et al.* (2000)
	dominant negative *hTERT*	Hahn *et al.* (1999) and Zhang *et al.* (1999)
hTERT promoter	*hTERT* promoter driven suicide gene expression	Koga *et al.* (2000), Komata *et al.* (2001), Gu *et al.* (2000) and Abdul-Ghani *et al.* (2000)
hTER	HHR	Kanazawa *et al.* (1996), Yokoyama *et al.* (1998) and Folini *et al.* (2000*a*)
	2′-*O*-methyl modified HHR	Wan *et al.* (1998)
	antisense RNA transfection	Feng *et al.* (1995), Kondo *et al.* (1998*a*,*b*), Naka *et al.* (1999), Yamaguchi *et al.* (1999) and Bisoffi *et al.* (1998)
	modified antisense RNA	Pitts & Corey (1998) and Herbert *et al.* (1999)
	antisense oligodeoxynucleotides	Glukhov *et al.* (1998)
	PNA	Hamilton *et al.* (1999), Ray & Norden (2000), Norton *et al.* (1996), Shammas *et al.* (1999) and Herbert *et al.* (1999)
	2′-5′-oligoadenylate oligonucleotides	Kondo *et al.* (1998*a*, 2000) and Kushner *et al.* (2000)
	phosphorothioate oligonucleotides	Mata *et al.* (1997), Ohnuma *et al.* (1997) and Catapano *et al.* (1999)
telomeres	G-quadruplex interacting agents	Mergny *et al.* (1999), Kerwin (2000), Raymond *et al.* (2000), Perry *et al.* (1998*a*,*b*), Fedoroff *et al.* (1998), Harrison *et al.* (1999), Perry *et al.* (1999), Izbicka *et al.* (1999) and Neidle & Kelland (1999)
	telomeric DNA interacting agents	Raymond *et al.* (2000) and Mergny *et al.* (1999)
undetermined	small molecules inhibitors	Naasani *et al.* (1998, 1999), Tabata *et al.* (1999) and Wu *et al.* (1999)

HLA-A2.1-negative cell lines showed a CTL response (Vonderheide *et al.* 1999).

These results were confirmed and expanded by another group (Minev *et al.* 2000) using two hTERT peptides (p540 and p865) selected using the procedure described above. The CTLs of cancer patients specifically lysed a variety of HLA-A2$^{(+)}$ cancer cell lines, demonstrating immunological recognition of endogenously processed hTERT peptides. *In vivo* immunization of HLA-A2.1 transgenic mice generated a specific CTL response against both hTERT peptides. On the basis of the induction of CTL responses *in vitro* and *in vivo*, and the susceptibility to lysis of tumour cells of various origins by hTERT CTL, hTERT peptides were suggested as a universal cancer vaccine for humans.

A potential problem of hTERT-based vaccination could be induction of an autoimmune response, e.g. against stem and germinal cells. In the above work a CTL-specific response to CD34^{+} cells, as a representative of haemopoietic cells, was not generated. The advantage of this approach compared with most other strategies is the absence of a lag period. There are currently clinical trials ongoing in this area which should answer these questions (J. W. Shay, personal communication).

(ii) *Targeting the hTERT promoter*

The specificity of *hTERT* expression in cancer cells may be used for selective targeting of tumour cells. For turning telomerase on or off, the promoter region of the *hTERT* is responsible and can therefore be used to induce tumour-specific transgene expression in cancer gene therapy. Thus, a *hTERT* promoter-driven *caspase-8* expression viral vector (*hTERT*/*caspase-8*) results in apoptosis that is restricted to a wide range of telomerase-positive tumour cells but is not seen in normal fibroblast cells without telomerase activity. Moreover, treatment of subcutaneous tumours in nude mice with this construct inhibits tumour growth significantly because of induction of apoptosis (Koga *et al.* 2000). In another recent study, an expression vector was constructed consisting of the constitutively active *caspase-6* (*rev-caspase-6*) under the *hTERT* promoter (*hTERT*/*rev-caspase-6*). The *rev-caspase-6* gene induces apoptosis independent of the initiator caspases. The *hTERT*/*rev-caspase-6* construct induced apoptosis in *hTERT*-positive malignant glioma cells, but not in *hTERT*-negative astrocytes, fibroblasts and ALT cells. In addition, the growth of tumours in nude mice was significantly suppressed by the treatment with *hTERT*/*rev-caspase-6* construct (Komata *et al.* 2001). Using a binary adenoviral system that can induce *Bax* gene expression, another group has shown that induction of *Bax* expression via the *hTERT* promoter elicited tumour-specific apoptosis *in vitro*, suppressed tumour growth in nude mice, and prevented the toxicity of the *Bax* gene *in vitro* and *in vivo* (Gu *et al.* 2000). Both *hTR* and *hTERT* promoters have been used to control the expression of a cytotoxic gene—the diphtheria toxin A-chain (*DT-A*) gene—in bladder tumour cells, resulting in the selective destruction of this cell population. Inhibition of protein synthesis occurred in transfected bladder and hepatocellular carcinoma cells (Abdul-Ghani *et al.* 2000). A number of variants of this approach are being investigated by scientists in Geron Corporation (USA). In one of these, the *hTERT* promoter was used to turn on expression of the thymidine kinase (TK) suicide gene, which promotes the death of cells that express telomerase by making them sensitive to

the drug gancyclovir. In another variant, the *hTERT* promoter drives expression of the genes that are required for the adenovirus to replicate so that when cells are infected, the virus replicates and causes lysis (rupture and death) of the tumour cells, but remains inert in normal cells (see also http://www.geron.com). In all these approaches, the problems of killing all telomerase-expressing cells in a person must be carefully considered.

(iii) *Targeting the RNA subunit: hTR*

Hammerhead ribozymes

These are small molecules which exert specific endoribonuclease activity; this activity is located in the catalytic core domain, while the specific recognition of the target RNA sequences is mediated by the rest of the molecule. Telomerase inhibition using several ribozyme constructs targeting different *hTR* domains was reported *in vitro* (Kanazawa *et al.* 1996). After introduction into an endometrial cancer cell line, reduction of telomerase activity *in vivo* and consequent telomere shortening was achieved in the case of a ribozyme targeting the template domain of *hTR*, but the proliferation rate was not affected by this approach (Yokoyama *et al.* 1998). Similarly, both *in vivo* and *in vitro* inhibition of telomerase in human melanoma cells was obtained by a hammerhead ribozyme (HHR) that targeted *hTR* by another group (Folini *et al.* 2000*a*). This inhibition was both concentration and time dependent. Furthermore, reduced telomerase RNA level, significant elongation of population doubling time but no telomere shortening was observed. Susceptibility of human melanoma cells to a variety of anticancer agents (platinum compounds, taxanes, topoisomerase I inhibitors) was not affected by this treatment (Folini *et al.* 2000*b*). A 2′-*O*-methyl-modified HHR has been designed (Wan *et al.* 1998) to enhance its biological activity; this showed a dose-dependent inhibition of telomerase activity, but no effect on telomere dynamics could be observed.

Antisense strategies

Antisense (AS) molecules bind their complementary DNA or RNA sequence, thus preventing transcription or translation of the targeted gene. Their action can proceed in an active or passive manner; the passive mechanism involves only competitive binding of AS molecules to the target sequence, whereas in the active mechanism RNase degradation of the target RNA in an RNA–AS complex takes place. Both natural and chemically modified antisense molecules may be used for the AS strategy. Besides the possibility of direct transfection of AS molecules into cells, antisense RNA may be generated by transcription (constitutive or inducible) inside cells that have been transformed with a recombinant vector coding for AS–RNA. The latter approach has been applied to functionally prove the identity of the cloned RNA component of human telomerase (Feng *et al.* 1995). Introduction of an AS *hTR* expression construct (against the first 185 nucleotides) into HeLa cells resulted in crisis after 23–26 doublings, and telomere shortening was observed compared with control clones transformed by the vector alone. Using the same AS *hTR* in a malignant glioma cell line, a reduction of telomerase activity after 15 cell doublings has been reported (Kondo *et al.* 1998*b*). After 30 cell doublings, morphological analysis showed apoptotic cell death in 40% of cells. This group further reported synergistic effects of AS *hTR* and cisplatin in U251-MG cells that are cisplatin resistant (Kondo *et al.* 1998*c*). An AS *hTR* has been used also in gastric cancer cell lines (Naka *et al.* 1999) and glioma cell lines (Yamaguchi *et al.* 1999) with similar findings. When a replication-deficient retrovirus expressing a 48-nucleotide RNA sequence containing six repeats of the sequence complementary to the template region of *hTR* was used for transduction of mouse PA317 and human HeLa cells, giant senescent-like cells emerged shortly after cloning; however, the fraction of giant cells varied from 100% at the fifth population doubling (PD) in one culture to 2–6% at 50 PD in others, showing that the effect of inhibition on individual cells is highly variable (Bisoffi *et al.* 1998).

The other approach—direct transfection of cells by synthetic AS *hTR* molecules—was initially performed using unmodified AS oligodeoxynucleotides. Telomerase inhibition and subsequent telomere shortening have been demonstrated in tumour cell lines (Glukhov *et al.* 1998). Nevertheless, in current studies various modifications of synthetic oligodeoxynucleotides, e.g. 2′-*O*-methyl-RNA, 2′-5′-oligoadenylate (2-5A), phosphoramidate or phosphorothioate (PS) oligodeoxynucleotides or peptide–nucleic acids (PNAs) are preferred; these modifications may facilitate transfer across the cell membrane, increase resistance of AS drugs to cellular nucleases and strengthen binding of the AS molecule to its target.

Synthetic 2′-O-methyl-RNA oligonucleotides

Synthetic oligonucleotides complementary to the *hTR* template sequence were studied with the human prostate cancer cell line DU-145 (Pitts & Corey 1998). Telomerase inhibition was observed in response to treatment with 6–9 mers and a 13 mer of 2′-*O*-methyl oligonucleotides and the most potent inhibitor was a 13 mer containing two nucleotides with phosphorothioate bonds at both the 3′ and the 5′ ends. 2′-*O*-methyl RNA has been tested also on immortalized human breast cells (Herbert *et al.* 1999); 13 mer complementary to the template region of *hTR* were used in both match and mismatch manners. Over a 120-day period the cells demonstrated telomerase activity inhibition, and after a lag-phase telomere shortening with subsequent apoptosis was observed. Control cells and the mismatch control cells did not show changes in telomere dynamics. No revertants were observed, indicating that the engagement of the ALT pathway is not very likely in human tumours treated with telomerase inhibitors.

The reason for using 2-5A DNA oligonucleotides is that modification by linking to 2-5A recruits ribonuclease L (RNaseL) with ribonuclease H (RNaseH) upon hybridization to the target sequence. In malignant glioma cells, a 19 mer 2-5A-oligonucleotide against a non-template region of *hTR* caused *hTR* to become undetectable by reverse-transcription-mediated PCR in only 5 hours and, within 14 days of treatment, 79% of the cells underwent apoptosis (Kondo *et al.* 1998*a*). In human ovarian cancer cell lines, growth arrest, activation of caspase and apoptosis were observed after only 7 days of treatment with 2–5A AS oligodeoxynucleotide (Kushner *et al.* 2000). Moreover, *in vivo* experiments in severe combined immunodeficiency (SCID) mice have been performed using human prostate cancer xenografts and 2–5A AS

19 mer oligodeoxynucleotides injected directly into the tumour. Within 7 days, a significant reduction of tumour progression was observed compared with the control (Kondo *et al.* 2000).

Phosphorothioate oligodeoxynucleotides

These oligonucleotides, which mimic the human telomeric repeat sequence were used in a Burkitt's lymphoma cell line (OMA-BL1); telomerase inhibition, lengthening of doubling time and final induction of apoptosis were observed (Mata *et al.* 1997). To demonstrate an *in vivo* effect, the 6 mer PS-DNA oligonucleotide TTAGGG was injected into BALB/c mice with human tumour xenografts and caused a significant dose-dependent decrease in tumour volume. In contrast, another group observed no growth inhibitory effect of 6 mer and 12 mer PS oligodeoxynucleotides on different cancer cell lines although the 18 mer and 24 mer displayed strong growth inhibition (Ohnuma *et al.* 1997). A new approach in the PS oligodeoxynucleotide field is represented by phosphorothioate foldback triplex-forming oligonucleotides (FTFOs), of which one domain forms a duplex with a target site in *hTR*, while the second domain, linked by a dinucleotide loop, is designed to bind to the duplex, thus forming a triple-helical complex. The telomerase inhibitory effect was 10–50-fold higher than that of a conventional oligodeoxynucleotide (Catapano *et al.* 1999).

Peptide nucleic acid analogues

These peptide nucleic acid (PNA) analogues show higher stability and more efficient transfer through the cell membrane. In PNAs, the pentose phosphate backbone is replaced by an *N*-(2-aminoethyl) glycine oligomer that possesses specific and high-affinity binding to the target nucleotide sequences and resistance to degradation (Hamilton *et al.* 1999; Ray & Norden 2000). *In vitro* studies show that PNA could inhibit both transcription and translation of targeted genes. Targeting of *hTR* by PNA in cultured cells resulted in telomerase inhibition (Norton *et al.* 1996) and, in further experiments, PNA treatment of cell lines immortalized by SV40-transfection caused inhibition of telomerase activity, telomere shortening after a lag-phase and finally a growth arrest (Shammas *et al.* 1999). It is worth noting that telomere shortening is reversible: if the inhibitor is removed, telomeres regain their initial lengths and this may be an important advantage of this strategy by suppressing adverse effects on germinal and stem cells after complete removal of tumour cells (Herbert *et al.* 1999).

The good prospect of AS PNA and AS oligodeoxynucleotide strategies is enhanced by using them in combination with an efficient method of delivery into the cells such as lipofection or electroporation (Hamilton *et al.* 1999; Shammas *et al.* 1999).

(iv) *Targeting the telomeres*

G-quadruplex interacting agents

The G-overhang, the natural substrate of telomerase, cannot be accessed by the enzyme when folded into G-quadruplexes and, consequently, agents stabilizing these structures act as potent inhibitors of telomerase (Kerwin 2000; Mergny *et al.* 1999). A number of G-quadruplex stabilizing compounds have been described, including 2,6-diamidoanthraquinones (Perry *et al.* 1998*a*,*b*), porphyrin derivatives (Fedoroff *et al.* 1998), acridines (Harrison *et al.* 1999) and fluorenones (Perry *et al.* 1999). These drugs cause telomerase inhibition and growth arrest, but corresponding telomere shortening and the predicted lag phase have not been demonstrated (Neidle & Kelland 1999). For example, MCF-7 cells treated with cationic porphyrins show growth arrest only after 15 days (ten population doubling times) (Izbicka *et al.* 1999). These results indicate that the effect of G-quadruplex stabilizers is not limited to telomerase inhibition and that other effects, such as unspecific binding to DNA and RNA, may be involved.

The other known folded G-overhang structure—the t-loop (see § 1)—also masks G-overhangs from telomerase and may thus be a perspective for specific telomerase inhibition. Unfortunately, no t-loop-specific agents are available so far.

Telomeric DNA/G-rich DNA-interacting agents

A number of drugs are reported to interact with G-rich sequences of DNA, including telomeric sequences. These include known anticancer drugs (cisplatin, etoposide, etc.), DNA gyrase inhibitors (ofloxacin, levofloxacin), and novel alkaloids or their semi-synthetic analogues (ET-743, MGI 114) with potent anticancer effects (Raymond *et al.* 2000; Mergny *et al.* 1999). The observed effects of these drugs, such as telomere shortening and decreased telomerase activity, are unlikely to be due to specific and selective inhibition of telomerase, and probably just arise from one of their mechanisms of action.

The above list of strategies for telomerase inhibition is far from being complete. There is a growing heterogeneous group of 'small molecule inhibitors' of telomerase of which some are G-quadruplex interacting agents, but in most cases their mechanism of action is not known, for example: tea catechins have been demonstrated to be potent inhibitors of telomerase activity; and cells treated with epigallocatechin gallate, epigallocatechin, epicachetin gallate and epicachetin showed decreased levels of telomerase activity, shortening of telomeres and subsequent crisis (Naasani *et al.* 1998). Diazaphilonic acid, a new fermentation metabolite from *Talaromyces flavus*, has been reported to be an inhibitor of telomerase activity in a dose-dependent manner (Tabata *et al.* 1999). Similarly, the naturally occurring alkaloid barberine has been shown to possess anti-telomerase activity (Wu *et al.* 1999).

A number of telomerase inhibitors have been found by exploiting the database of the disease-oriented screening program (DOS) using COMPARE analysis (Naasani *et al.* 1999). In primary and arbitrary screening, the alkaloid berberine was identified as a moderate inhibitor with 50% inhibition at *ca.* 35 μM. Using this alkaloid as a seed compound in COMPARE resulted in the identification of other berberine-like compounds among which MKT077, a rhodacyanine derivative currently under clinical trials, showed a potent inhibitory effect with 50% inhibition at *ca.* 5 μM. Using MKT077 as an upgraded seed for a new round of COMPARE analysis, rhodacyanine FJ5002, a close derivative of MKT077, was identified as the most potent telomerase inhibitor with 50% inhibition at *ca.* 2 μM. Long-term cultivation of U937, a human leukaemia cell line, with subacute concentrations of FJ5002 resulted in population-doubling dependent changes characterized

by progressive telomere erosion, increased chromosome abnormalities and senescence/crisis-like features. These results indicated that FJ5002 may be a genuine and effective anti-telomerase agent.

Established anticancer agents have been the subject of numerous studies to assess their telomerase inhibitory properties, but in most cases, e.g. cisplatin, doxorubicin, daunorubicin and 5-fluorouracil, controversial results were obtained by different groups (Perry & Jenkins 1999). Some other compounds, such as staurosporine or curcumin, are under evaluation.

It should be noted that besides the strategies reviewed above, it could well be that cellular factors involved in natural telomerase regulation would provide probably the optimal agents for anti-telomerase-based therapy of cancer.

6. CELL AND TISSUE ENGINEERING

Although it might seem from the above text that telomerase is always an enemy to fight against, restoration of telomerase activity may be beneficial in certain medical applications. The demonstration that ectopic expression of *hTERT* is sufficient to restore telomerase activity and extend lifespan (Bodnar *et al.* 1998) makes it possible to immortalize normal cells of various tissues while taking advantage of the fact that immortalization via introduction of an *hTERT*-expressing construct does not result in neoplastic transformation of the cell. This enables researchers to develop better cellular models of human diseases (Ouellette *et al.* 2000*b*) and to produce unlimited quantities of normal human cells of virtually any tissue type. The possibility of rejuvenation of patient's or donor cells may be extremely useful in the field of gene therapy or transplantation, and currently remarkable progress can be seen in this promising field.

During limited expression of *hTERT* short telomeres were stable in length, in contrast with long telomeres that shorten, suggesting that there is a preferential recruitment of hTERT to shorter telomeres in cells (Ouellette *et al.* 2000*a*). Important steps towards the safety of using *hTERT* in tissue engineering have been made. As the first step, the absence of neoplastic transformation as a consequence of transfecting cells with *hTERT* was reported (Jiang *et al.* 1999; Morales *et al.* 1999). The *in vitro* growth requirements, cell-cycle checkpoints and other characteristics were similar to those of untransfected controls and these cells also did not cause tumours in immunosuppressed mice. Thus telomerase alone is not sufficient for malignant transformation of transfected cells. Development of the Cre-lox recombination system for transient expression of telomerase activity may represent a further step (Steinert *et al.* 2000); a short-term expression of *hTERT* in human fibroblasts using this system is sufficient to maintain critically shortened telomeres, which results in a 50% increase in lifespan.

Recently the first use of *hTERT* expression in experimental xenotransplantation has been performed (Thomas *et al.* 2000). Primary bovine adrenocortical cells were co-transfected with plasmids encoding hTERT, SV40 T antigen, neo and green fluorescent protein. The transfected clones did not undergo loss of telomeric DNA, showed telomerase activity and appeared to be immortalized. Two clones were transplanted beneath the kidney capsule of SCID mice and the animals that received cell transplants survived indefinitely despite adrenalectomy. The mouse glucocorticoid, corticosterone, was replaced by the bovine glucocorticoid, cortisol, in the plasma of these animals. The tissue formed from the transplanted cells resembled that formed by transplantation of cells that were not genetically modified and was similar to normal bovine adrenal cortex. The proliferation rate in tissues formed from these clones was low and there were no indications of malignant transformation.

These results document the state of the art of cell and tissue engineering technology based on introduction of the *hTERT* gene. Future prospects include production of desired and commercially important proteins, slowing down senescence of specific tissues, and rejuvenation of haemopoietic stem cells for either improving bone marrow transplants or enhancing general immunity for older patients. This technology may be further used to increase the proliferative capacity of cells in chronic skin ulcers, to immortalize chondrocytes to repair knee damage, to produce osteoprogenitor cells for bone grafts and endothelial cells for the generation of tissue-engineered blood vessels. Extending the lifespan of muscle satellite cells would be important for cure of Duchenne muscular dystrophy in combination with gene therapy of these cells. Similarly, 'telomerized' retinal cells could be used for treatment of age-related macular degeneration. The advantage of these approaches is that the patient's own cells would be used and immune rejection could be avoided (Shay & Wright 2001).

The realization of the perspectives presented in this review on the use of telomerase for cell and tissue engineering, as well as those of anti-telomerase-based anticancer therapy, will depend on future progress in our detailed understanding of the biology of telomeres and the initiation of clinical trials to test their efficacy. Although much remains to be done, the progress in telomere biology achieved in the past decade has been extraordinary and promises to fulfil the expectations of professionals and the public in the near future.

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