Telomerase: diagnostics, cancer therapeutics and tissue engineering

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The enzyme telomerase has a key role in controlling the lifespan of human cells. It is absent from most somatic tissues but is reactivated in more than 85% of cancers, making the enzyme ideal as a marker of cancer cells and as a therapeutic target. In the context of normal human cells, the enzyme can extend cellular lifespan without causing cancer-associated changes or altering phenotypic properties. This capability could solve a major obstacle in the use of normal human cells for tissue engineering, that is, the induction of cellular senescence.

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▼ Normal human cells have a finite lifespan and undergo senescence after a limited number of cell divisions[1]. At senescence, the cells are metabolically active but no longer divide. Similar to terminal differentiation, entry into senescence is accompanied by changes in morphology and gene expression. At least two forms of senescence exist that control the lifespan of human cells in culture, termed M1 and M2 (for Mortality stages 1 and 2). The onset of M1 and/or M2 is controlled by the progressive shortening of telomeres that occurs each time normal human cells divide[2–4].

Human telomeres are made of a simple double-stranded-DNA sequence, TTAGGG, repeated over thousands of base pairs[5]. They are essential structures that cap and protect the ends of chromosomes against degradation, inappropriate recombination and interchromosomal fusions. DNA polymerase-α is incapable of completing the replication of the ends of linear chromosomes; as a consequence, gaps of unreplicated DNA are created at the end of telomeres each time normal human cells divide. By a mechanism that has yet to be uncovered, M1 is induced when the size of the shortest telomeres has declined below a certain threshold[2]. Entry into M1 involves the activation of the tumor suppressors p53 and pRB (Ref. 6). Viral oncogenes that can block both suppressors, such as the SV40 large T-antigen, can greatly extend the lifespan of human cells beyond M1, but cells expressing the oncogene are not yet immortal as telomeres continue to shorten with divisions. Terminal telomere shortening eventually leads to M2, a state of crisis characterized by massive cell death[7]. Rare clones can sometimes emerge from crisis (at a frequency of 10^{-7}) that have gained the capacity to maintain their telomeres[8].

Telomerase is an enzyme that can prevent the erosion of telomeres and the induction of M1 and M2 (Refs 3,4,7). Telomerase acts by synthesizing and adding telomeric repeats to the ends of telomeres. It is composed of at least two essential components: the protein hTERT (human telomerase reverse transcriptase) and the small nuclear RNA hTR (human telomerase RNA)[9–11]. The latter contains a short sequence (5′-CUAACCCUAAC-3′), which serves as a template for the synthesis of telomeric repeats, whereas hTERT provides catalytic activity[9–11]. The enzyme is a reverse transcriptase that uses hTR as a template, and the ends of telomeres as primers (Fig. 1). The activity of telomerase is absent from most human tissues with the exception of certain germline cells and rare stem cells of renewal tissues, including skin, blood and the digestive system[12–15]. As a consequence, telomeres are lost throughout life in many cellular systems as cells turnover and are replaced. This phenomenon has been experimentally verified in lymphocytes, skin fibroblasts, endothelial cells, osteoblasts and myoblasts[2,16–19]. Although the enzyme is present in many renewal tissues, these tissues still display a gradual shortening of telomeres with age, implying that the level of telomerase activity present is insufficient to maintain telomere length. Finally, other studies have shown that the presence of active telomerase is determined by the expression of hTERT and not by the level of hTR, which abounds in all

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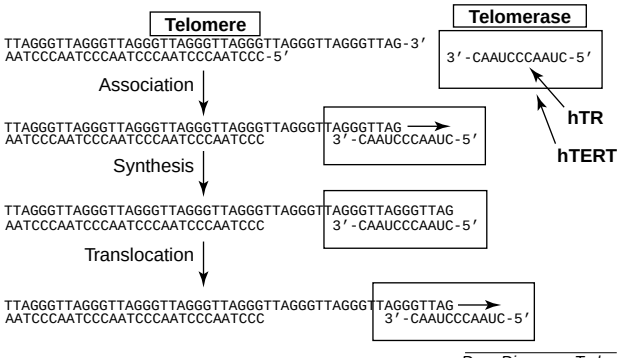

Figure 1. The biochemical activity of telomerase. The enzyme telomerase is composed of two essential subunits: human telomerase reverse transcriptase (hTERT) and human telomerase RNA (hTR). The RNA hTR contains a short sequence, complementary to that of the telomeric repeats (5′-CUAACCCUAAC-3′), which the enzyme uses as a template. As the enzyme associates with the telomeres, this sequence hybridizes with the 3′ overhang that caps all telomeres. The protein hTERT, acting as a reverse transcriptase, then uses the telomere as a primer and hTR as a template to synthesize a six base telomeric repeat. The enzyme is highly processive and can add many repeats to the same DNA substrate through cycles of synthesis and translocation.

cells[10,11]. We, and others, have shown that the expression of an hTERT cDNA in telomerase-negative human cells can reconstitute telomerase activity, prevent telomere shortening and overcome senescence[3,4,19–21].

The role of telomerase in cancer progression

The activity of telomerase is absent from most normal human tissues but is detected in >85% of cancers[22,23]. This observation implies a role for telomerase in cancer progression and suggests that M1 and M2 are important tumor-suppressive mechanisms. Carcinogenesis is a multistep process that necessitates successive alterations in a limited number of genes (4–7 genes)[24]. Each step generally involves the alteration of a gene, selection of the mutant cells and clonal expansion of these cells that favors the occurrence of the next mutation. By imposing limits on clonal expansion, M1 and M2 could halt the progression of individual clones through the many steps of carcinogenesis. According to this model, the role of this enzyme in cancer progression would simply be to allow carcinogenesis to proceed without being blocked by the induction of senescence or crisis. Telomerase would also give the transformed cells the extra doublings needed for tumor growth and metastasis. This model is supported by the observation that exogenous hTERT appears to immortalize without causing cancer-associated changes or changing phenotypic properties[20,25,26]. Human cells immortalized with hTERT alone are diploid, contact-inhibited and anchorage-dependent. They also display no changes in growth factor requirements and possess functional cell-cycle checkpoints.

By contrast, conventional approaches for establishing cell lines, such as the cultivation of cancer samples or the introduction of viral oncogenes, invariably produce lines of cells carrying changes that are normally associated with cancers. Although these observations suggest that transformation (uncontrolled and/or deregulated proliferation) and immortalization (the capacity to divide beyond senescence) are independent mechanisms, recent studies hint to subtle interactions between the two processes. Primary human cells can be transformed to grow in soft agar and to form tumors in nude mice given a cocktail of transgenes that include hTERT, oncogenic Ras and the T-antigens of SV40 (Refs 27,28). A relevant observation is that the omission of hTERT blocks the capacity of these cells to transform. Conversely, the inhibition of endogenous telomerase using a dominant-negative mutant of hTERT can prevent cancer cells from forming tumors in nude mice[29,30]. Finally, one recent publication suggests that the expression of telomerase might cause defects in transforming growth factor (TGF)-β signaling[31]. The exact mechanisms responsible for this apparent interplay between immortality and transformation are yet to be understood.

Telomerase activity for the detection of cancer cells

In 1994, the sensitive TRAP assay (Telomeric Repeat Amplification Protocol) was described for the detection of telomerase activity in biological samples[22,23,32]. The assay uses an oligonucleotidic substrate that mimics the ends of telomeres. The substrate is incubated in cell extracts and the products of the telomerase reaction are then amplified by PCR. Using this assay, Kim and coworkers demonstrated that the activity of telomerase was specifically associated with the presence of cancer cells[22]. Since then, >1000 studies have used the assay to compare cancer samples with healthy tissues. In many studies, the activity of telomerase was detectable within the tumors but was absent from the surrounding healthy tissues. In 1997, a comprehensive compilation of all published data indicated that the activity was abnormally expressed in >85% of cancers, irrespective of tumor type[23]. With this finding came the realization that the abnormal expression of telomerase is the most universal marker of cancer cells known to date. In certain tumor types, increased telomerase activity correlates with poor prognosis, whereas in other cases it can distinguish between benign and malignant lesions.

The TRAP assay is versatile and highly sensitive. It can detect a single cancer cell mixed with thousands of normal human cells and can be used to reveal the presence of

cancer cells in almost any clinical specimen including biopsies, frozen sections, fine needle aspirates, brushes, washes and biological fluids (e.g. urine, pancreatic juice or blood)[22,32]. Modifications have also been made to adapt the original assay to the clinical environment, reduce cost and facilitate the analysis of large numbers of samples[33]. Other alternatives have also been explored that are based on the detection of either the hTERT mRNA (Ref. 34) or the hTERT protein[35].

The value of the TRAP assay for the detection of malignant cells and for the diagnosis of cancers has now been established for many of the major cancer-growth sites, including the prostate[36], lung[37], pancreas[38], bladder[39] and breast[40]. In one large study, the TRAP assay was compared with the current technology for the diagnosis of breast cancer. After testing fine-needle aspirates from 617 patients with palpable tumors, the study found that the sensitivity (86% versus 70%) and specificity (93% versus 72%) of the TRAP assay were both higher than that of conventional histopathology[40]. Similar findings have been reported for other tumor types[37–39].

In certain tumor types, the upregulation of telomerase appears to be a late event, in which case the marker has been found to have prognostic values. One such example is the case of the ordinary meningioma[41]. Remissions are most often achieved by surgical resection, but the cancer sometimes recurs. Interestingly, one study found that, although most of these cancers are negative for telomerase activity, those that relapse tend to express the enzyme at the time of diagnosis[41]. Similar findings have been described for the prognosis of neuroblastomas and of gastric and colorectal cancers[42–44]. Finally, because the sensitivity of the TRAP assay is sufficient for the detection of minute metastasis that have been left behind following cancer treatments, the assay might also be useful for assessing the success of therapeutic regimens[45].

Telomerase as a therapeutic target for the treatment of cancers

It has been hypothesized that tumor cells must maintain their telomeres to sustain proliferation beyond the limits of senescence. Most cancer cells do so by reactivating telomerase. The notion that telomerase is required for the malignancy and the long-term growth of cancer cells is supported by direct experimental data. In 1995, the hTR gene was cloned and its sequence published[9]. A few years later, the sequence of the hTERT cDNA was made available through the efforts of many independent groups[10,11]. Based on the sequences of hTR and hTERT, mutants were designed for the purpose of inhibiting the activity of telomerase and testing the hypothesis that telomerase is required for the unlimited lifespan of cancer cells. Among the mutant molecules designed were an anti-sense RNA against hTR (Ref. 9), mutants of hTERT with dominant-negative activity[29,30] and ribozymes directed against the hTERT mRNA (Ref. 46). After a limited number of doublings, cancer cells lacking the activity would eventually experience a non-proliferative state similar to senescence or crisis. This requirement for active telomerase, and the fact that it is reactivated in most cancers, made the enzyme an ideal target for the development of novel therapeutic approaches.

When considering telomerase inhibitors as anticancer agents, at least two predictions must be taken into account. First, a delay is expected between the inhibition of telomerase and the time when telomeres have become sufficiently short to affect either survival or proliferation. In theory, the length of this lag phase should depend on the initial telomere size and on the rate of shortening, which both vary depending on cell types. Because of this delay, inhibitors of telomerase are unlikely to be effective as a first line of defense against cancers. Instead, the inhibitors would be most useful as an adjuvant to conventional therapy and are likely to be administered during, or following, conventional treatments to prevent the growth of residual micrometastasis. Residual tumor cells are likely to require many rounds of cell divisions before giving rise to malignancies, a process that could be blocked by telomerase inhibitors.

A second prediction to consider is that inhibitors of telomerase are likely to affect other normal tissues that are maintained by telomerase-positive stem cells. Such stem cells have been detected in the hematopoietic system and the basal layers of the skin and intestinal crypts[13–15]. The effects that the inhibitors might have on these normal cells are difficult to predict. However, because cancer cells are likely to have shorter telomeres than stem cells, and because the deepest stem cells only divide occasionally, inhibitors are likely to have less pronounced effects on these normal cells. Unfortunately, most animal models that are currently in use for evaluating the safety of new therapeutic agents are species with very large telomeres[47], making these models inadequate for predicting the toxicity of telomerase inhibitors on normal tissues. The evaluation of the real risks associated with these new therapeutic agents might have to await the results of the first clinical studies.

At least three components of the telomerase complex have been targeted for the purpose of inhibiting the enzyme telomerase: the hTERT subunit, the RNA hTR and the substrate of the enzyme, the telomeres. Many molecules with inhibitory activity have been described in the literature. Some were the results of rational drug design, whereas others were discovered through the systematic screening

of pharmaceutical compounds. A prototypical inhibitor should display a number of essential properties, which include: (1) the therapeutic agents should inhibit the enzyme at nanomolar concentrations; (2) a continuous exposure to the inhibitor should cause the telomeres to shorten gradually; (3) chronic exposure to the inhibitor should lead to senescence or crisis; (4) the inhibitor should display low toxicity and fail to influence growth rates until the telomeres have shortened; and (5) the inhibitor should be a small molecule that can penetrate a tumor deep enough to reach all cancer cells. Here we describe the different molecules that display many of these properties.

The antisense approach

The first therapeutic agents with inhibitory activity to be generated through rational drug design were antisense oligonucleotides directed towards the template region of hTR. This portion of hTR is believed to be accessible at the surface of the enzymatic complex where it interacts with the telomeres. Two major obstacles have to be overcome to ensure the efficient delivery of oligonucleotides to cells. First, the molecules must be able to penetrate lipid barriers and gain access to the nucleus. Oligonucleotides can cross the plasma membrane by an endocytic mechanism. Although the efficiency of this process is remarkably low, transfection reagents can be used to facilitate the transfer. Once inside the cells, a second obstacle is the rapid degradation of the transferred molecules by both exo- and endonucleases. This last obstacle can be alleviated by chemical modifications aimed at protecting the molecules from such degradation.

The feasibility of using an antisense approach to inhibit telomerase was first demonstrated with the publication of the sequence of hTR (Ref. 9). In this publication, HeLa cells were transfected with vectors expressing an antisense RNA corresponding to the first 185 nucleotides of hTR. After selecting for plasmid integration, the majority of surviving clones exhibited a finite lifespan and entered crisis after 23–26 doublings, at which time they displayed reductions in both telomere size and telomerase activity. Because of the accessibility of the template region, most studies published thereafter have more specifically targeted this shorter region of 11 nucleotides. Two approaches have been particularly successful in reducing the size of telomeres in cancer cell lines: (1) the use of chimeric 2′-*O*-methyl RNA, and (2) the use of peptide nucleic acids (PNA). The first protects the antisense DNA from nucleases by capping the sequence with modified RNA bases and the second are analogs of DNA, in which the pentose-phosphate backbone is replaced by an oligomer of *N*-(2-aminoethyl)glycine, making them resistant to nuclease degradation. Other chemical modifications have also been exploited but with mixed results; they either failed to show sequence specificity or the expected delay that would be required for the telomeres to shorten[48,49].

The sequence and length of chimeric 2′-*O*-methyl RNA and of PNAs have been optimized for telomerase inhibition to yield EC_{50} in the nanomolar range[50]. In one study, a series of 13-mer PNAs hybridizing from nucleotide 36–67 of hTR were tested in cell lysates containing telomerase, with the most active oligomer displaying an EC_{50} value of just 1 nM (Ref. 50). In a follow-up study, the same group repetitively electroporated an optimized PNA into an immortal cell line and were able to show a progressive shortening of the telomeres with the cells entering crisis after an initial period of growth[51]. Other studies have indicated that the transfection of 2′-*O*-methyl RNA oligomer could produce similar outcomes. In one study, an optimized 2′-*O*-methyl RNA oligomer (EC_{50} = 10 nM) was repetitively transfected into an immortalized human-breast epithelial cell-line over a period of 120 days[52]. Again, the oligomer inhibited telomerase activity and caused a progressive shortening of telomeres that led to crisis. Clearly, one of the most pressing challenges in using oligomers for cancer treatment is the delivery problem. Recent advances in oligomer chemistry and the development of novel transfection techniques could overcome this important obstacle to their clinical use[53].

Small drug molecules

A second group of inhibitors, obtained through rational drug design, is represented by the G-quartet stabilizers. Human telomeres end with a short 3′ overhang of 50–200 nucleotides corresponding to the G-rich strand of the TTAGGG repeat. *In vitro*, this structure can fold into G-quartets, a DNA conformation known to inhibit telomerase activity. Molecules that can promote and/or stabilize the formation of G-quartets could potentially block the action of telomerase on telomeres. Many small molecules have been described in the literature that both stabilize G-quartets and inhibit telomerase. These include derivatives of porphyrins, acridines, perylenes, dibenzophenanthroline, diamidoanthraquinones and of fluorenone-based compounds[54–57]. Although many of these compounds inhibit the enzyme with EC_{50} values in the nanomolar range, their specificity and efficacy in targeting cancer cells have not yet been described. One potential problem associated with these inhibitors is that they might also interfere with the proper functioning of telomeres in cells lacking telomerase. The lack of specificity of some of these inhibitors towards cancer cells supports this notion[58].

A third group of inhibitors is represented by the reverse transcriptase inhibitors. The enzyme hTERT acts as a reverse

transcriptase and its amino acid sequence shares seven motifs with this class of enzymes. The translational efforts in finding successful treatment for the AIDS epidemic have produced numerous compounds with inhibitory activity against reverse transcriptases. Not surprisingly, some of these molecules and related compounds were also found to inhibit telomerase. The prototypical and most extensively studied compound of this class is the 3′-azido-3′-deoxythymidine (AZT). A major problem associated with the use of AZT and related compounds, is the high EC_{50} value required for telomerase inhibition (0.10–1.75 mM)[59–61]. A second problem is that most studies have either failed to document the shortening of telomeres or failed to observe evidence of senescence.

Finally, the systematic screening of small drug molecules has identified many compounds with inhibitory activity against telomerase. Among the many candidates identified, most were reported to inhibit the enzyme with EC_{50} values that were equal to, or higher than, the micromolar[62–64]. One notable exception is telomestatin, a polyoxazole ring produced by *Streptomyces anulatus* 3533-SV4. This compound displays an EC_{50} value of only 5 nM (Ref. 65). However, the effects of telomestatin on living cancer cells have not yet been documented. The systematic screening of chemical libraries could yield many new promising compounds with inhibitory activity against telomerase.

The use of exogenous hTERT for tissue engineering

Although the expression of telomerase in transformed cells promotes tumor growth, its use in normal human cells could facilitate the development of cell-based therapies and the engineering of artificial tissue. The two approaches share the promise of new treatments for chronic illnesses and age-related diseases. In cell-based therapies aimed at correcting a genetic defect, cells would be taken from a patient, grown in culture, genetically manipulated and expanded until sufficient numbers of cells are available for transplantation. A major obstacle to the use of these approaches is the limited lifespan of normal human cells. The expression of exogenous hTERT could circumvent this limitation by extending the lifespan of the patient's cells. Conventional methods for generating immortal lines of human cells (such as cultivation of tumor cells, the transfer of viral oncogenes or the use of carcinogens) invariably give rise to cells that exhibit significant alterations in physiological and biological properties. Most notably, these cells often display cancer-associated changes that might include aneuploidy, spontaneous hypermutability, loss of contact inhibition and alterations in cell-cycle checkpoints. Although indirect evidence exists suggesting that hTERT might have transforming capability beyond its capacity to immortalize, hTERT-immortalized cells tend to display far less alterations than those immortalized using conventional methods[20,25,26]. Thus, in the context of cell-based therapy, hTERT would be ideal for solving the obstacles imposed by the early senescence of limited material.

However, it would also be inappropriate to assume that the use of hTERT-immortalized cells in transplants and engineered tissues would be without risks. In the advent that rare precancerous cells are present within the transplant, the constitutive expression of hTERT would exacerbate the risks associated with these rogue cells by providing them with immortality. To limit the risks of cancers associated with the implantation of hTERT-immortalized cells, fail-safe mechanisms and quality-control procedures would need to be implemented. First, a homogenous population of cells corresponding to a single clone would certainly be used, which would first be characterized in terms of differentiated phenotype, growth properties (e.g. contact inhibition, growth in soft agar, growth factor requirements), karyotype and cell-cycle controls. Second, the transferred cells could be engineered to express a suicide gene, such as that encoding the thymidine kinase of the herpes simplex virus[66]. In the advent of an adverse event, the tagged cells could be purged from the recipient with the administration of the drug ganciclovir, a substrate metabolized by the kinase into a cytotoxic product. Third, cellular lifespan could be extended using an hTERT cDNA flanked by LoxP sites (ATAACTTCGTATAGCATACATTATACGAAGTTAT), which could later be excised by the forced expression of Cre, a recombinase of the bacteriophage P1. The enzyme Cre can recognize and recombine adjacent LoxP sites in a reaction that deletes all sequence positioned between the two sites. In a recent study, the feasibility of this approach for the transient expression of hTERT has been demonstrated[67]. The authors show that the expression of exogenous hTERT for a period of three weeks, followed by its excision, suffices in extending the cellular lifespan of normal human fibroblasts by 50% and that the post-excision cells did regain their mortal phenotype.

The list of human cell types that can be immortalized with hTERT alone grows every year. So far, this list includes fibroblasts, retinal pigmented epithelial cells, endothelial cells, mammary epithelial cells, keratinocytes and osteoblasts[4,19–21]. However, it should be noted that not all cell types can be immortalized using hTERT alone. First, the enzyme telomerase does not appear to alter the phenotypic properties of cells, such that post-mitotic terminally differentiated cells are unlikely to be rescued by the enzyme. Second, certain cell types experience, in culture, additional forms of senescence, which are independent of telomere length. When human keratinocytes and mammary epithelial cells

are cultivated on plastic dishes, the cells eventually arrest after a limited number of doublings. This growth arrest is accompanied by an increased level of $p16^{INK4a}$ and cannot be bypassed by hTERT alone[68]. However, when the same cells are expanded over a feeder layer, this form of premature senescence can be averted and the cells immortalized with hTERT alone[21]. Telomere-independent forms of senescence have also been documented in other species, such as rodents, where telomeres are long and telomerase is expressed in most somatic cells. Again, with the use of optimized tissue-culture conditions, premature forms of senescence can be prevented and the rodent cells immortalized[69,70]. Although they probably represent tissue-culture artifacts, these premature forms of senescence still represent a major obstacle to the establishment of cell lines. Most culture media that are currently in use have been optimized for short-term growth. As we optimize culture conditions for the long-term growth of hTERT-transfected cells, many more lines of normal human cells should become available for tissue engineering.

Conclusion

The enzyme telomerase is thought to control the lifespan of human cells, both *de novo* and *in vitro*. Its repression during early embryogenesis gives human cells a finite lifespan, forcing them into senescence after a limited number of doublings. The evolutionary advantage provided by this repression, a process not shared by rodents and other short-lived species, is to impose an additional obstacle to the clonal evolution of cancer cells. In most cases of cancers, this obstacle is eventually bypassed by the reactivation of telomerase, making the enzymatic activity the best-known marker of cancer cells. As the technology for the detection of this new marker improves and becomes cost effective, these recent advances will soon move from the research arena to clinical laboratories, thus revolutionizing our current approaches for the detection, diagnosis and prognosis of cancers.

With the discovery of new therapeutic agents and approaches aimed at controlling the activity of telomerase, new avenues will be made possible that will have high impact on the treatment of cancers, the engineering of human tissue and the treatment of age-related disease. In the context of normal human cells, exogenous telomerase appears to represent the safest route for extending cellular lifespan. This approach could greatly facilitate the implementation of cell-based therapies, the engineering of tissue and the treatment of certain age-related diseases. In the context of cancer cells, the presence of the enzyme exacerbates the malignancy of transformed cells by giving them an unlimited capacity for continuous growth and expansion. By reverting the immortal phenotype of cancer cells, inhibitors of telomerase could block the spread of residual disease and improve the outcome of current cancer therapies.

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