

Telomeres and Telomerase: From Discovery to Clinical Trials

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Telomeres are the ends of linear chromosomes. They cannot be fully replicated by standard polymerases and are maintained by the ribonucleoprotein telomerase. Telomeres and telomerase stand at a junction of critical processes underlying chromosome integrity, cancer, and aging, and their importance was recognized by the 2009 Nobel Prize in Physiology or Medicine to Elizabeth Blackburn, Jack Szostak, and Carol Greider. Where will the field go now? What are the prospects for antitelomerase agents as drugs? Nearly 30 years after Szostak and Blackburn's pioneering manuscript on telomere ends, the challenges of discovery remain.

Cells are packed with nucleic acids: chromosomal DNA, mRNA, tRNA, ribosomal RNA, and the rapidly expanding world of other functional RNAs. Each nucleic acid has a unique chemical personality and the potential for selective recognition by designed agents to control almost any biological process. The 2009 Nobel Prize in Physiology or Medicine was awarded for the discovery of one of the most elegant nucleic acid systems inside cells—telomeres and telomerase.

The End Replication Problem

The ends of linear chromosomes pose a biological problem. During replication the lagging strand cannot be fully copied by standard polymerases. Olovnikov (1973) and Watson (1972) first pointed out the implications of this “end replication problem.” Lacking a means to replicate chromosome ends, chromosomes would shorten with each cell doubling, eventually reaching a critical point leading to cell senescence or death.

Hayflick had previously noted that most cultured cells could survive only a limited number of cell divisions and suggested that finite cellular lifespans might explain why physiologic function breaks down as an organism ages (Hayflick and Moorhead, 1961). It seemed logical that progressive shortening of chromosome ends, telomeres, might explain the “Hayflick limit.” However, as one question was answered, another arose: how do organisms survive from one generation to the next? Telomeres are long enough that a given organism might not suffer the catastrophic consequences of chromosome shortening, but eventually the

erosion would take its toll and make life impossible for the next generation. Both Olovnikov and Watson suggested that physiologic systems that maintain telomere length must exist.

Discovery of Telomeres and Telomerase

The experimental answer to the end replication problem began to be revealed when then-postdoctoral fellow Elizabeth Blackburn and Joseph Gall noted that chromosome ends from *Tetrahymena thermophila* contain the six base sequence TTGGGG repeated 20–70 times (Blackburn and Gall, 1978). Choosing *Tetrahymena* as a model organism was a key factor in the success of this and subsequent studies because it contains thousands of chromosomes, providing an abundance of telomeric material to analyze.

In 1981, Blackburn, by then an independent investigator, collaborated with Jack Szostak to demonstrate that telomeric function could be transferred from one organism (*Tetrahymena*) to another (*Saccharomyces cerevisiae*) (Szostak and Blackburn, 1982). This result showed that some feature of the telomeric sequence could preserve function from one organism to the next. Then, in 1985, Blackburn and Carol Greider identified an enzymatic activity capable of extending telomeric sequences (Greider and Blackburn, 1985). Soon thereafter they identified the “terminal transferase” activity as belonging to a ribonucleoprotein with essential RNA and protein components and named it “telomerase” (Greider and Blackburn, 1987). The RNA

component was found to be complementary to the sequence of the telomeric repeat, suggesting that it was acting as a template for repeat addition (Greider and Blackburn, 1989).

Telomerase and Cancer

Soon after these discoveries, telomerase research moved up the evolutionary ladder as it was established that humans also have telomeres and that they consist of the repeated sequence TTAGGG (Moyzis et al., 1988). In 1989, Gregg Morin identified telomerase activity in human cells (Morin, 1989). His finding was no small achievement, because human cells have far fewer telomeres than *Tetrahymena* and much lower levels of telomerase. Morin hypothesized that immortal cultured human cell lines would express telomerase, and developed methods for purifying telomerase activity. Like the *Tetrahymena* enzyme, the human enzyme appeared to be a ribonucleoprotein. In a tantalizing glimpse of the explosion of research activity soon to follow, telomerase activity was identified in ovarian tumor cells, but not in isogenic nonmalignant cells, suggesting that telomerase reactivation might be linked to cancer cell proliferation (Counter et al., 1994).

In spite of the obvious importance of telomerase, only a few manuscripts appeared before 1995 (Figure 1). The major reason for this was that levels of human telomerase are so low that much effort was needed to obtain sufficient activity for a handful of assays. Then, in 1994, Shay, Wright, and coworkers developed a simple PCR-based assay, the telomeric repeat amplification protocol

(TRAP), that greatly increased the ability to detect telomerase activity (Kim et al., 1994). An example of using TRAP to measure the effectiveness of an inhibitor of telomerase activity is shown in Figure 2A. With TRAP, only a few cells were needed to quantitate activity. It was now possible to ask, on a massive scale, how telomerase activity correlated with the occurrence of human cancer.

Shay and colleagues used TRAP to show that telomerase activity could be detected in almost all immortal cell lines and cancer biopsies, but not in mortal cells or normal tissues (Kim et al., 1994). In one of the first examples of the power of telomerase measurement for clinical investigations, TRAP revealed that neuroblastoma samples lacking telomerase activity were associated with a favorable clinical prognosis (Hiyama et al., 1995), demonstrating the potential for telomerase activity to be used as a clinical marker.

The impact of the combination of basic science and technical breakthroughs is readily apparent, with publications mentioning “telomerase” increasing dramatically during the mid-1990s (Figure 1). Telomerase activity had been observed in greater than 90% of patient samples from a wide range of different cancers (Shay et al., 2001). Telomerase activity was also observed in proliferative stem cells and germ cells (Mantell and Greider, 1994; Broccoli et al., 1995), consistent with the requirement that these cells maintain chromosome length over many cell divisions or, in the case of germ cells, from one generation of an organism to the next.

Telomerase: A Target for Inhibitor Development

Unlike normal cells, cancerous cells must divide indefinitely to populate a mass sufficiently large and aggressive to threaten a patient. In the absence of a mechanism for fully replicating telomeres, cancer would be self-limiting or would require development of an alternate mechanism for maintaining the integrity of their chromosomes. The observation of telomerase

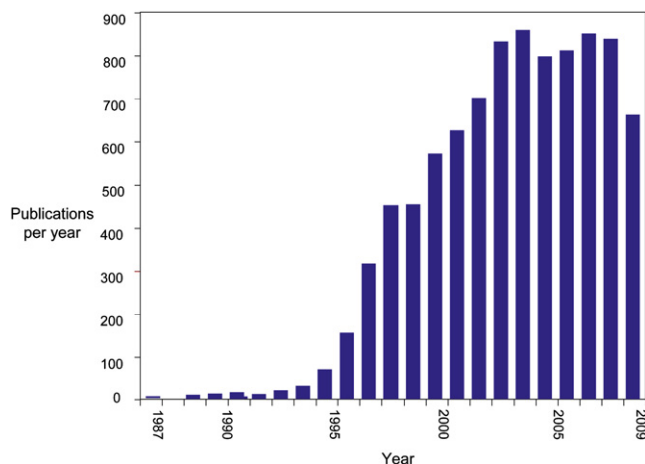


Figure 1. The Number of Publications in PubMed Citing the Word “Telomerase” and the Year in which They Were Published
Data from 2009 are through October 20, 2009.

activity in cancerous tissues provided an explanation for how cancer cells can proliferate indefinitely.

Telomerase activity is not always required for cellular immortality. A relatively small number of immortal cell lines have been identified that do not express telomerase activity but maintain telomere length by alternative pathways that depend on recombination (Bryan et al., 1995; Cesare and Reddel, 2008). The existence of these cell lines suggests that some cancer cells might not require telomerase activity, but reactivation of telomerase appears to be much more common.

The belief that telomerase activity was critically important for sustained cancer cell proliferation led to the hypothesis that agents that inhibit telomerase activity might cause the telomeres of cancer cells to erode and limit their proliferation. Successful antitelomerase drugs would act by a mechanism different from existing cancer drugs, potentially providing a valuable new tool for treating cancer (Shay and Keith, 2008; Harley, 2008).

This hypothesis also predicted that antitelomerase agents would not kill cells immediately. Instead, they would cause steady telomere shortening until a critical point was reached. In contrast to most existing drugs, where effects are noted soon after administration, the antiproliferative effects of antitelomerase drugs might occur after weeks or months of continuous treatment. The potential for a lag between initiating treatment and

observing efficacy is a central consideration for understanding the challenges of telomerase as an anticancer target.

The expected lag between initial dosing and decreased cell proliferation leads to the expectation that telomerase inhibitors would not be useful drugs when used to treat primary tumors because the tumor might become dangerously advanced before drug-induced telomere shortening could reduce cancer cell proliferation. Instead, it is more likely that telomerase inhibitors would be used to prevent cancer recurrence or treat secondary cancers that arise after treatment with existing

drugs. It is also unlikely that telomerase inhibitors would be used alone. Instead, they will probably be an adjuvant therapy that compounds the effects of antiproliferative agents that act through other mechanisms.

Biological studies provided support for this hypothesis that inhibiting telomerase would have an antiproliferative effect. Expression of a mutated inactive protein component of telomerase was used to reduce telomerase activity in cells (Hahn et al., 1999; Zhang et al., 1999). As predicted, in the absence of telomerase activity, telomeres grew progressively shorter and cell proliferation was decreased. The effects of telomerase activity were also examined in knockout mice lacking telomerase RNA ($mTR^{-/-}$). Mice have relatively long telomeres, and for six generations few phenotypes were observed (Blasco et al., 1997). Eventually, however, more severe phenotypes were observed and succeeding generations became less viable (Lee et al., 1998).

Efforts to develop synthetic telomerase inhibitors encountered several obstacles: (1) purified human telomerase was not available in large quantities, making screening for inhibitors more cumbersome; (2) until relatively recently (Gillis et al., 2008; Sekaran et al., 2009), no high-resolution structural information was available, complicating structure-based design strategies; (3) telomerase is a polymerase, and inhibitors would need to be selective for its inhibition relative to other cellular polymerases; and (4) as

mentioned above, telomere shortening was expected to yield delayed antiproliferative effects. Observation of the key endpoint, cell death, would be delayed, forcing assays to be carried out over weeks or months. As a consequence, two or three months (or more) might be required to evaluate a compound and decide how to proceed with the next generation of compounds.

Preclinical and Clinical Development of Telomerase Inhibitors

One strategy for blocking telomerase focuses on their substrate: telomeres. Telomeres are G-rich sequences, TTAGGG in mammals. Structural studies have shown that these sequences form four-stranded G quadruplexes (Henderson et al., 1987; Parkinson et al., 2002). Such quadruplex structures would need to unfold before telomerase could recognize telomere ends, leading to the hypothesis that small molecules promoting the formation of quadruplexes would block binding to telomerase.

Many different quadruplex-stabilizing agents have been identified (Sun et al., 1997), and several have been shown to have antiproliferative effects (Read et al., 2001; Riou et al., 2002). However, G-rich sequences exist elsewhere in the genome, and binding to these sites can cause unexpected effects (Siddiqui-Jain et al., 2002). Another obstacle is the inherent difficulty of designing a planar compound with sufficiently selective intercalation into G quadruplexes relative to the vast amounts of duplex chromosomal DNA. Advances in the field have slowed over the past five years, indicating that the obstacles confronting the development of potent, highly selective G-quadruplex-interacting agents have yet to be overcome.

Another strategy is to use oligonucleotides that are complementary to the RNA component of telomerase (Figure 2). To help elongate telomeres, this RNA

component must be accessible to binding by telomere ends. It is logical that it should also be accessible to binding by synthetic oligonucleotides and that such binding should block interactions with telomeres and inhibit telomerase function. Two oligonucleotides are approved drugs and other promising candidates are advancing through clinical trials (Corey, 2007), suggesting that nucleic acids are a feasible option for clinical development of antitelomerase agents.

In 1996, my laboratory showed that single-stranded peptide nucleic acid (PNA) oligomers (Figure 3) that are complementary to the template regions of the telomerase RNA subunit could inhibit telomerase activity in cancer cell extracts (Norton et al., 1996). Inhibition was both potent and sequence specific. Rather than function like a standard antisense

oligomer to degrade RNA or block translation, the oligomer acts like an enzyme inhibitor to block the telomerase active site. It is likely that the effectiveness of anti-telomerase oligomers is due to their simple and direct mechanism of action.

We observed that PNAs, 2'-O-methyl, 2'-methoxyethyl, and locked nucleic acid (LNA) oligomers could be introduced into cells and inhibit intracellular telomerase (Herbert et al., 1999; Shamma et al., 1999; Chen et al., 2002, 2003; Elayadi et al., 2002). The oligonucleotides caused telomeres to gradually shorten (Figure 2B). Effects were observed most rapidly in cells with short telomeres and more slowly in cells with longer telomeres. 2'-O-methoxyethyl oligomers slowed tumor growth both in xenograft tumors (Figure 2C) (Chen et al., 2003) and in an orthotopic intraosseous prostate cancer model upon systemic administration (Li et al., 2009).

The potency and selectivity of oligonucleotides as telomerase inhibitors encouraged their development as drugs. Similar to the development of any oligonucleotide

drug, the challenge was to identify oligonucleotide chemistry with optimal pharmacological properties. Geron Corporation chose to focus on the development of oligomers containing phosphothioamide backbones and, working with the Shay laboratory, demonstrated potent inhibition of telomerase (Herbert et al., 2002). Administration of antitelomerase phosphoramidate oligonucleotides in a mouse xenograft model of prostate cancer suppressed tumor growth (Asai et al., 2003).

Most researchers working with therapeutic oligonucleotides had assumed that they could not efficiently enter cultured cells in the absence of transfection reagent. Antitelomerase phosphoramidate oligomers, however, were potent inhibitors in the absence of transfection reagent (Herbert et al., 2002). This

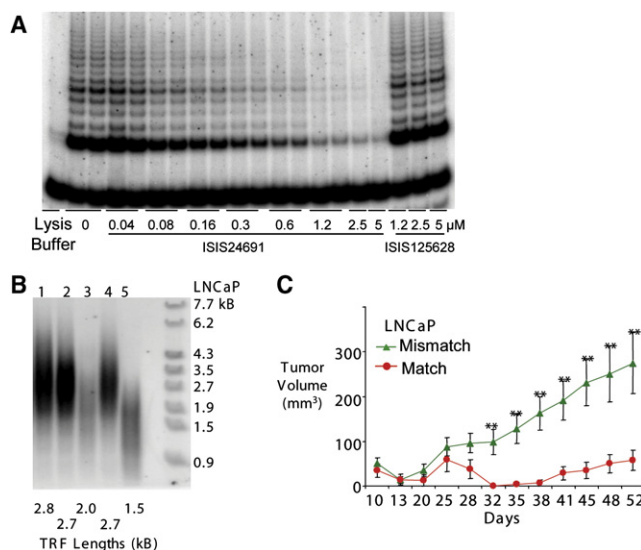


Figure 2. Typical Data from Assays of Telomerase Activity Demonstrating the Effects of Inhibiting Telomerase

(A) TRAP assay showing inhibition of telomerase activity in C4(2)b cells treated with complementary 2'-methoxyethyl RNA inhibitor ISIS24691 or noncomplementary oligomer ISIS125628 (Canales et al., 2006). Concentration of oligomer is μ M, and compound was delivered into cells in the absence of transfection reagent. Measurements were in duplicate or quadruplicate. The band at the bottom of the gel is the internal amplification standard used for quantitation.

(B) Telomere shortening in LNCaP cells measured by the telomere restriction fragment (TRF) assay. Lane 1: no oligonucleotide added; cells were harvested after three passages. Lane 2: no oligonucleotide added; cells were harvested after 17 passages. Lane 3: match oligonucleotide ISIS24691 added for 55 days. Lane 4: mismatch oligonucleotide ISIS125628 added for 55 days. Lane 5: match oligonucleotide ISIS24691 added for 70 days. Size markers are shown on the right (Chen et al., 2003). Signal is weaker in the lanes showing telomere shortening because shortened telomeres have less DNA available for probe hybridization.

(C) Effect of telomerase inhibition on growth of xenograft tumors in mice (Chen et al., 2003). LNCaP cells had been treated with match or mismatch oligonucleotide for 21 days prior to implantation. * $p < 0.05$; ** $p < 0.001$.

observation suggested that phosphoramidate chemistry might confer advantages for cell uptake in vivo, a critical consideration for further development. Excellent cell uptake was also shown by anti-telomerase 2'-methoxyethyl oligonucleotides (Chen et al., 2002). Potent activity in the absence of transfection agent by two different oligomer chemistries reemphasizes the fact that telomerase is not a typical cellular RNA target and may have advantages for recognition by oligonucleotides that will facilitate progress in the clinic.

Examination of chemical modifications to improve anti-telomerase thiophosphoramidate oligomers continued, and it was discovered that addition of a lipid group further increased potency inside cells (Herbert et al., 2005). Lipidated oligonucleotide GRN163L was shown to reduce tumor growth in several animal studies (Dikmen et al., 2005; Harley, 2008). GRN163L is now being tested in phase I and phase I/II clinical trials for chronic lymphoproliferative disease, solid tumor malignancies, non-small-cell lung cancer, multiple myeloma, and breast cancer (<http://www.geron.com>). Initial reports suggest that GRN163, the nonlipidated parent oligonucleotide, is well tolerated by patients (Harley, 2008), although efficacy data have yet to be reported. Recently disclosed phase I results suggest that lipidated GRN163L is also well tolerated by patients, with some observation of telomerase inhibition in tissue (<http://www.geron.com>).

Most of these trials examine administration of GRN163L in addition to standard small-molecule or antibody chemotherapeutic agents. Because of the potential lag between the onset of treatment and critical telomere shortening, inhibition of telomerase alone may not be sufficient. Combination treatments will also be necessary for most trials because of the necessity of providing the best existing care to patients partici-

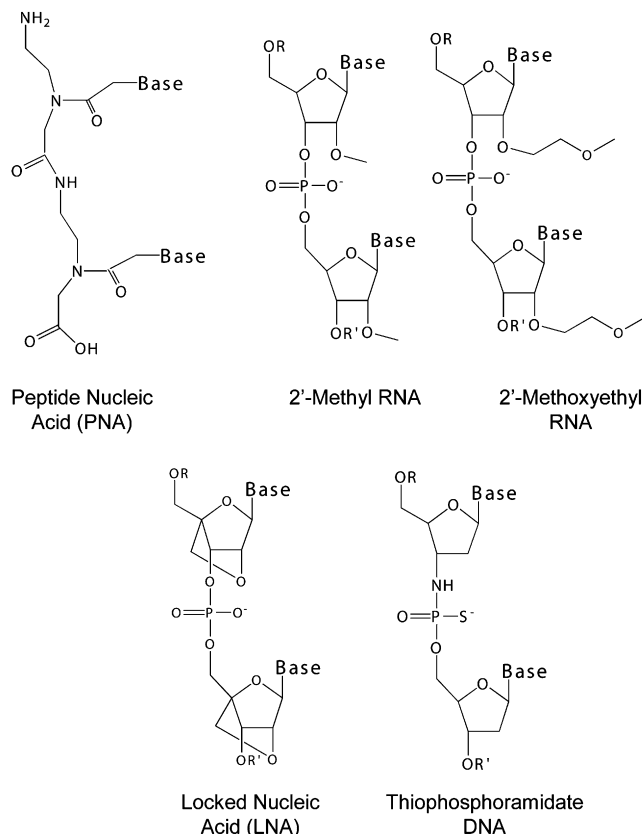


Figure 3. Structural Features of PNA and Chemically Modified Oligonucleotides Used to Inhibit Human Telomerase

pating in the trial. Because the mechanisms of action of telomerase inhibitors and standard antiproliferative agents differ, it is possible that they might yield synergistic effects (Shay and Keith, 2008).

Three Decades of Progress

The science of telomerase and telomeres has come a long way since Elizabeth Blackburn first began experiments investigating chromosomal ends. The topic has drawn in researchers from many disciplines, including chemistry, structural biology, cell biology, aging, and cancer biology. This perspective has focused on the connection between telomerase, cancer, and cancer therapy, but telomerase/telomeres also play a role in cellular aging (Aubert and Lansdorp, 2008), and recent work suggests that telomerase may play roles that go beyond telomere maintenance (Cong and Shay, 2008; Maida et al., 2009). Similarly, the discovery of noncoding transcription at telomeres (Luke and Lingner, 2009;

Schoeftner and Blasco, 2009) suggests that they may be even more complex than had been originally imagined. It is likely that the next 30 years will yield a steady stream of new discoveries that profoundly affect our understanding of cells and provide opportunities for developing novel therapeutics.

Chemistry & Biology invites your comments on this topic. Please write to the editors at chembiol@cell.com.

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