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Multiple Pathways for the Regulation of Telomerase Activity

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The ends of vertebrate chromosomes are composed of large tracts of a repeated sequence, TTAGGG, which are known as telomeres. Normal somatic cells progressively lose telomeric repeats with each successive cell division due to incomplete replication. Immortal and cancer cells compensate for telomeric loss by expressing the enzyme telomerase, an RNA-dependent DNA polymerase that maintains telomere length. Telomerase activity has been detected in almost 90% of all human cancers. Telomerase activity is generally absent in normal somatic tissues but is detected in adult testes, activated lymphocytes, and lower levels are expressed in proliferative cells of renewal tissues. Telomerase activity is downregulated in cells that exit the cell cycle via either terminal differentiation or (reversible) quiescence. Inhibition of telomerase activity in tumour cells may provide an effective way to treat cancer by potentially reducing the recurrence of tumours due to occult micrometastases. An understanding of the pathways involved in telomerase regulation will be important for determining the most practical means of inhibiting its activity. © **1997 Elsevier Science Ltd. All rights reserved.**

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INTRODUCTION

TELOMERES ARE specialised heterochromatic structures at the ends of vertebrate chromosomes that have been implicated in stabilising and protecting the chromosomes, anchoring chromosomes within the nucleus, and assisting the replication of linear DNA [1, 2]. Telomeric repeats are lost with each cell division because DNA polymerases cannot replicate the very end of a linear DNA molecule [3, 4] because lagging strand synthesis proceeds as a series of discrete events (i.e. the formation of Okazaki fragments), each requiring an RNA primer [4]. Since there is no DNA beyond the 3′ end of the chromosome to which this primer can anneal, DNA polymerase cannot fill in the gap between the final Okazaki fragment and the end of the chromosome (the "end replication problem"). In the absence of a molecular mechanism to compensate for the "end replication problem", the incompletely replicated telomeres are inherited by daughter cells and the process repeats itself in subsequent cell divisions, resulting in progressive telomere shortening. Telomere shortening has been proposed as a regulatory mechanism that controls the number of times a cell can divide before undergoing cellular senescence [1, 2].

Cellular senescence in human cells can be divided into two components: Mortality Stage 1 (M1) and Mortality Stage 2 (M2) [5–7]. M1 occurs after significant telomere shortening and is controlled by the normal functions of the tumour suppressor proteins p53 and pRb (or a pRb-like function), which are proposed to signal a growth checkpoint allowing cells to arrest in a G0 or G1-like state [5–8]. Viral oncoproteins that can sequester or degrade these proteins have the ability to allow cells to overcome M1, leading to an extended lifespan until a second growth checkpoint, M2, is reached [5–8]. M2 occurs when few, if any, telomeric repeats remain at the ends of the chromosomes and may represent the critical shortening of telomeres [9, 10]. Only a rare event will allow cells to overcome M2, stabilise the telomeres and become immortal [9, 10]. This rare event is most often associated with the upregulation or reactivation of telomerase [11], the ribonucleoprotein responsible for the synthesis of the telomeric repeats at the ends of the chromosomes. Telomerase activity has been found in germline tissues, immortal cells, cancer cells and the proliferative cells of renewal tissues including activated lymphocytes ([11–24] and see the article in this Special Issue by K.-F. Norrback and G. Roos, pages 774–780). Numerous lines of evidence suggest that detection of telomerase activity could be an important marker for the diagnosis of human tumours and that telomerase may be an important therapeutic target for the treatment of many different forms of cancer.

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The objective of this review is to explain some of the refinements of the telomere/telomerase hypothesis of ageing and cancer that are necessary for understanding the molecular mechanisms regulating human telomerase. A summary of the main points is illustrated in Figure 1. After embryogenesis, germline cells express telomerase activity, but this activity is absent in most somatic cells. Both dividing lymphocytes and proliferative cells from renewal tissues have telomerase activity, yet the cells continue to lose telomeric repeats, although presumably at a reduced rate compared to normal somatic cells devoid of telomerase activity. Dividing, telomerase-negative somatic cells continue to lose telomeric repeats until they undergo cellular senescence [1, 2] or reach a point where they are capable of immortalisation. In general, the cells that become immortal take a telomerase-positive pathway, but in some *in vitro* models of cellular immortalisation, a telomerase-negative pathway is observed. In the telomerase-negative pathway, very long and heterogeneous telomere lengths are found in the absence of detectable telomerase activity (Figure 1) [11, 25, 26]. In lymphocytes that exit the cell cycle by becoming quiescent, telomerase activity is virtually undetectable. Stem cells from renewal tissues also retain the ability to regulate the expression of telomerase (Figure 1). Most primary human tumours contain telomerase activity, which either represents the reactivation of telomerase (presumably due to the inactivation of a telomerase repression pathway), the persistence of telomerase activity in a stem cell-derived cancer or the upregulation of telomerase activity in a stem cell-derived cancer to levels sufficient to maintain telomere lengths (Figure 1). These telomerase-competent tumour cells nonetheless contain a regulated expression of telomerase activity, such that the cells are capable of downregulating telomerase activity upon exit from the cell cycle through processes such as differentiation or quiescence.

THE CELL CYCLE AND TELOMERASE ACTIVITY

Telomerase activity has been detected at each stage of the cell cycle in several model systems. While initial studies showed telomerase activity during both the S and M phases of the cell cycle in *Xenopus* oocytes (which lack G1 or G2 phases) [27], more recent data show that the levels of telomerase activity remain constant during G1, S and G2/M phases of the cell cycle [28]. By treating cells with cycloheximide, a potent inhibitor of protein synthesis, a half-life of 24 h for telomerase has been determined, consistent with the lack of cell cycle regulation of the enzyme ([28]; unpublished observations).

The expression of telomerase appears to be, at least in part, independent of DNA synthesis [28, 29]. Many telo-

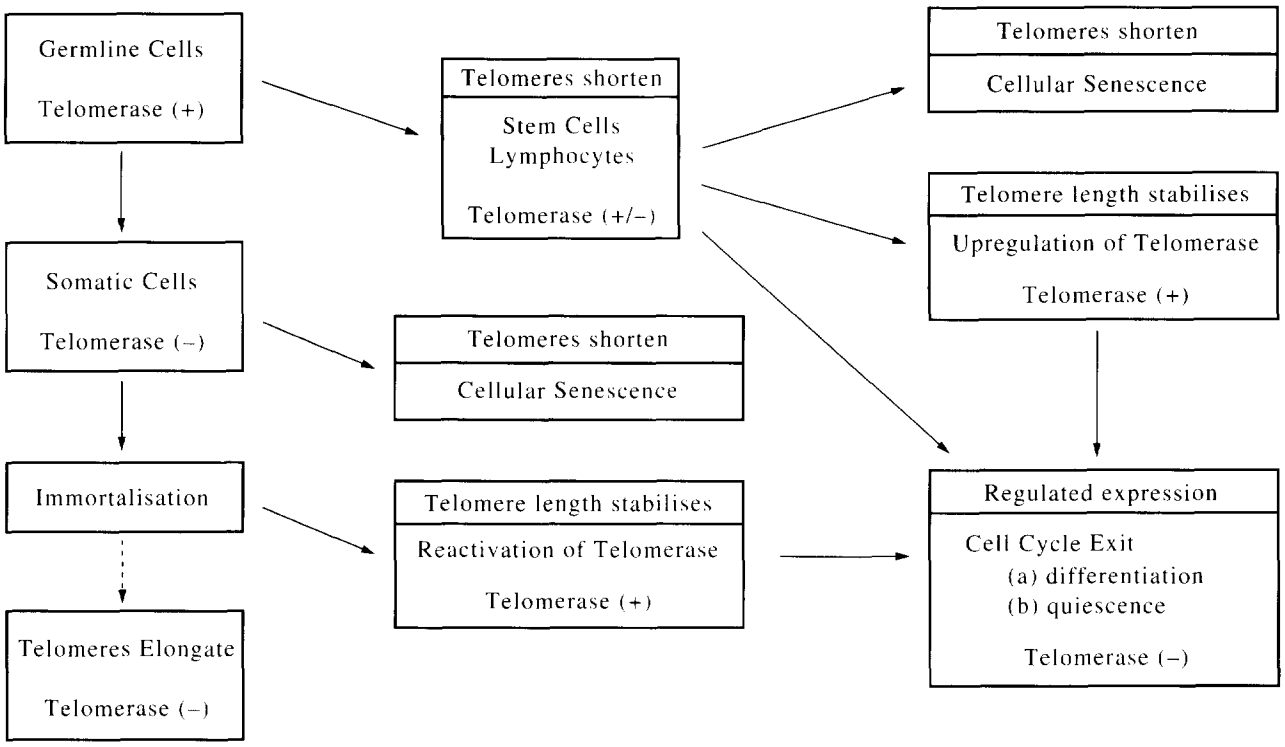

Figure 1. Pathways for the expression and control of telomerase activity. While telomerase activity is present in both fetal and adult testes, telomerase activity is detected in fetal but not adult ovaries [11, 20]. Since oocytes in the adult are generally held in a quiescent state in meiosis, this is a likely explanation for the lack of detectable telomerase activity in the adult ovary. Even though telomerase activity is detected in activated lymphocytes and stem cells of renewal tissues, their telomeres shorten throughout their lifespan. Cancers that arise from these tissues may be mortal (e.g. leukaemias in which defects in apoptosis lead to overgrowth of tumour cells or where a rapid accumulation of particular mutations occurs before the cells have exhausted their proliferative capacity), or immortal through upregulation of telomerase to levels sufficient to prevent further telomere shortening. Telomerase-negative somatic cells that escape cellular senescence undergo a series of mutations that ultimately results in an immortalisation event, which is most often associated with the reactivation/depression of telomerase activity. Even after somatic cells have inactivated the telomerase repression pathway and become immortal, they retain the ability to regulate expression of telomerase activity through differentiation or quiescent pathways, as do other telomerase-competent cells such as lymphocytes and stem cells. Telomerase (−), no detectable telomerase activity; telomerase (±), low or transient expression of telomerase activity; telomerase (+), higher levels of telomerase activity.

merase-positive cells repress telomerase activity when they become quiescent. In IDH4 cells, the expression of SV40 T antigen can be downregulated, which allows cells to go into an induced senescence phenotype concomitant with downregulation of telomerase activity. The re-entry of the cells into the cell cycle following T antigen induction is accompanied by the re-expression of telomerase activity prior to the onset of the first S phase [28]. Similarly, Buckovich and Greider [29] have shown that treatment of T cells with compounds that prevent the entry of cells into S phase does not block the upregulation of telomerase after activation of T cell proliferation. In addition, they were able to show that induction of telomerase activity is dependent on signal transduction pathways involving the T cell receptor and cdk2 kinase activity [29]. These data indicate that telomerase activity and DNA replication may be regulated independently.

LYMPHOCYTES AND STEM CELLS

Normal quiescent peripheral blood T and B cells express very low levels of telomerase activity (1–2% of immortalised cells), but when activated to divide, they exhibit a significant increase in telomerase activity levels [12, 13, 15, 18]. Interestingly, the telomeres in these cells shorten during long-term *in vitro* culture and as a function of age *in vivo* [30, 31]. Upon mitogen stimulation, telomerase activity in T cells initially increases 500–1000-fold followed by a gradual decrease after 5–10 population doublings [32]. However, the levels of the human telomerase RNA component (hTR) increase only approximately 20-fold after mitogen stimulation [32]. The T cells appear to maintain their telomere lengths for at least the initial 5–10 population doublings followed by a gradual decrease with successive population doublings [32]. Since the expression of telomerase activity in T cells also declines, this may in part explain the telomere shortening associated with increased population doublings.

While the reason for telomerase activity in lymphocytes is speculative, it may be that the repeated expansion of individual clones during antigen exposure throughout their lifespan requires telomerase to slow down the rate of telomere erosion that normally occurs in normal somatic cells without telomerase activity. Because T and B cells are transiently activated in response to mitogen stimulation *in vivo*, the transient expression of telomerase activity may allow only short-term telomere maintenance. The upregulation or reactivation of telomerase activity in T and B cells after mitogen stimulation indicates that there exists regulated expression of telomerase that correlates with re-entry of the cells into the proliferative compartment.

PROLIFERATION AND TELOMERASE ACTIVITY

The non-proliferative status of cells can be classified into at least three major groups: (1) growth arrest; (2) quiescence; and (3) terminal differentiation. Growth arrest is similar to quiescence in that both are reversible events resulting in an interruption in cell division. However, cells can become growth arrested at any stage of the cell cycle without exiting the cell cycle. Cells undergoing quiescence exit the cell cycle, generally referred to as a G0 arrest. The process of terminal differentiation involves a complex series of cellular changes that often results in irreversible growth arrest (and cell cycle exit) while the cells remain viable.

We and others have demonstrated that telomerase activity is repressed during terminal differentiation in HL60 human promyelocytic leukaemia cells and C2C12 mouse myoblasts [28, 33–46]. The postmitotic state is irreversible following differentiation in both of these lineages and should be contrasted with a reversible downregulation of telomerase activity accompanying the reversible induction of quiescence [28]. When NIH3T3 cells are held confluent for 2–3 weeks in culture, the cells become contact inhibited and cease cellular division. In these confluent cultures of NIH3T3 cells, telomerase activity is greatly reduced (20–30-fold) and the process is reversible when the cells are passaged [28]. In another model system, the differentiation of C2C12 myoblasts can be blocked by growing cells in the presence of BrdU (bromodeoxyuridine). When BrdU-blocked myoblasts are stimulated to differentiate by serum-deprivation, they fail to form myotubes but instead become quiescent. Again, telomerase activity is decreased, presumably due to an exit from the cell cycle, and the process of telomerase repression is reversed upon serum stimulation [28]. In a third system, IDH4 cells, which were immortalised using the dexamethasone-inducible expression of SV40 T antigen, revert to a state that appears similar to cellular senescence when dexamethasone is removed from the medium and T antigen levels decrease [7]. In these experiments, telomerase activity declined after the cells were held in the senescence-like state for 14 days, and when dexamethasone was added back to the medium, telomerase activity was detectable within 3 h and returned to steady-state levels by 24–36 h [28].

In these examples of induced quiescence, telomerase activity is significantly decreased when compared to dividing telomerase-positive cells. In addition, re-expression of telomerase activity occurs prior to the onset of DNA synthesis [28, 29]. The rate of the loss of telomerase activity is much faster in terminally differentiated HL60 cells than in those cell types that are induced to undergo quiescence and is faster than the approximately 24 h half-life observed in cycloheximide treated cells [28]. This suggests that an active degradation of telomerase may be taking place during terminal differentiation. These results indicate that the repression of telomerase activity in postmitotic cells may reflect mechanisms specific to particular differentiation lineages as well as general mechanisms accompanying quiescence.

Recent evidence has suggested that different tumour types from similar tissues have differing levels of telomerase activity [22, 23]. Because many tumour tissues are highly heterogeneous, the levels of telomerase activity may reflect the percentage of tumour tissue in a background of normal cells. However, since telomerase-competent cells have mechanisms for regulating the levels of telomerase activity, the amount of telomerase activity in some tumours may also reflect the percentage of quiescent/differentiated cells. Thus, those tumours with a higher percentage of cells in the proliferative pool are likely to have more telomerase activity than those tumours that contain differentiated or quiescent cells.

The use of differentiation therapy in the treatment of cancer has been investigated [37]. Because cancer is a multistep process and tumours are inherently heterogeneous, not all of the cells from each tumour will react similarly to a single therapeutic regimen. Therefore, complete remission in

patients is rare since a small population of cells frequently does not respond to differentiation therapy, and their continued proliferation eventually leads to relapse. The cancer cells that do not respond to differentiation therapy may take months or years to regrow to the point where the tumour is clinically detectable [37]. Continuous monitoring of telomerase activity in patients undergoing chemotherapy may help in the early detection of chemotherapy failure.

REPRESSION PATHWAYS OF TELOMERASE ACTIVITY

The regulation of telomerase activity in telomerase-competent cells is likely to be complex and the following discussion only considers three pathways: (1) the repression of telomerase activity during development; (2) repression of telomerase activity as a result of a non-proliferative state or cell cycle exit; and (3) regulation of telomerase action during cell cycle progression.

While most tissues contain telomerase activity during embryonic development, most become telomerase-negative after the neonatal period, suggesting the repression of telomerase activity [20]. In addition, when cell hybrids of telomerase-positive immortal cells and telomerase-negative mortal cells are made, telomerase activity becomes greatly reduced or undetectable in most of the resultant hybrids even though the hybrid clones continue to proliferate [25, 38]. Another line of evidence suggests that a locus for a repressor of telomerase activity maps to the short arm of chromosome 3 [39]. The 3p21 region is frequently deleted in many small cell and non-small cell lung carcinomas and has been proposed to contain loci for potential tumour suppressor genes [40–43]. Telomerase activity is often detected in preneoplasia of the lung at approximately the same time as loss of heterozygosity at the 3p locus is detected (unpublished observations). In addition, introduction of a normal chromosome 3 via microcell-mediated chromosome transfer into a tumour cell line with telomerase resulted in an inhibition of telomerase activity, gradual telomere erosion and the eventual cessation of cell proliferation after 100 days of continuous culture [39]. Taken together, these data indicate that there exists a means for cells to repress telomerase activity in the presence of cell proliferation and that this mechanism for developmentally regulated repression is likely to be mutated in many immortal cells.

In spite of presumed mutations in the pathway for the repression of telomerase, most telomerase-competent cells examined to date are nonetheless able to repress telomerase and become telomerase-silent when they become quiescent. This strongly suggests that the mechanism for repressing telomerase activity in quiescent cells is independent of the mechanism by which it is silenced during development. A third level of regulation is suggested by the lack of variation of telomerase activity during the cell cycle. While telomerase activity is detectable throughout the cell cycle, it is unlikely that it is continuously acting upon the telomeres. Clearly, there exist many possibilities for controlling when telomerase actually elongates telomeres (e.g. post-translational modification of the catalytic or other components of the holoenzyme, or changes of telomeric binding proteins that may regulate accessibility of the 3′ overhang [44]) and much more needs to be learned before all the complexities are adequately explained [45].

CANCER THERAPY USING TELOMERASE INHIBITORS

The current regimen of cancer therapy for patients with advanced cancer generally includes tumour resection followed by intensive chemotherapy and/or radiation therapy. In some instances, focused radiation therapy is used to reduce the tumour size prior to resection. Since telomerase activity is detected in almost all advanced tumours, the use of inhibitors of telomerase may provide an effective cancer therapy, possibly with reduced side-effects ([46] and see the article in this Special Issue by N.W. Kim, pages 781–786). Normal somatic cells that lack telomerase expression should be largely unaffected by antitelomerase therapy. Antitelomerase therapies are likely to be used in conjunction with tumour reduction via surgery and perhaps followed by conventional chemotherapies and/or radiation. Telomerase inhibitors may be most effective when directed toward the small numbers of telomerase-positive cancer cells in adjacent tissues not removed during tumour resection thereby reducing the risk of relapse. Telomerase inhibitors would be predicted to lead to progressive telomere shortening in the cancer cells, eventually leading to growth arrest and/or cellular senescence.

Because telomerase utilises an RNA component to add telomeric repeats to the ends of chromosomes, one target for inhibiting telomerase activity may be physical blockage of the RNA template [47]. *In vitro* inhibition of telomerase activity has been achieved using very high concentrations of DNA oligonucleotides [48]. Recently, the use of a modified oligonucleotide known as a peptide nucleic acid (PNA) has allowed effective inhibition of telomerase activity *in vitro* [47]. PNAs are sequence-specific and inhibit telomerase activity at significantly reduced concentrations compared to standard DNA oligonucleotides [47–49]. Because PNAs hybridise to RNA molecules more efficiently than normal DNA oligonucleotides and are resistant to degradation by nucleases or proteases, PNAs provide a potential avenue for directed inhibition of telomerase activity and a possible future approach in treating cancer patients with antitelomerase molecules [47]. Another potential target for inhibition is the telomerase nucleotide binding site, which appears to be preferentially inhibited by agents such as dideoxyguanine (ddG) and azidothymidine (AZT) [50, 51]. Although specific inhibitors have not yet been identified, the anchor site by which telomerase maintains its association with DNA is an additional potential drug target (reviewed in [52]).

The primary side-effect of telomerase inhibition therapy might be on telomerase-positive male germline cells, activated lymphocytes and other proliferative cells of renewal tissues. Cells from such tissues generally have much longer telomeres than most tumour cell populations, and antitelomerase treatment for tumours could be designed to end prior to telomere depletion in these cell types. In addition, the most primitive stem cell populations only rarely divide and thus should shorten their telomeres at a much slower rate than telomerase-inhibited proliferating cancer cells. Because lymphocytes and stem cells proliferate only transiently and telomerase activity is negligible in the absence of cell division in these cell types, they may be less affected than dividing tumour cells. After the cancer cells have shortened their telomeres and stopped proliferating and/or die, antitelomerase therapy could be discontinued, and telomerase activity in the germline and stem cells would be

restored. Thus, antitelomerase therapy is likely to eliminate the proliferative potential of cancer cells before the telomere lengths in lymphocytes and stem cells shorten sufficiently to disrupt their function (reviewed in [46]).

CONCLUSION

Telomerase activity has become a new marker for the detection of cancer and is potentially a novel target for cancer therapy. Understanding the regulatory mechanisms controlling the expression and activity of telomerase may provide insights leading to more effective anticancer therapies. There appear to be multiple pathways by which telomerase is regulated. Downregulation of telomerase activity may be due to a developmental repression of telomerase or it may reflect the proliferative status of telomerase-competent cells. Since similar levels of telomerase activity are detected at each stage of the cell cycle, the action of telomerase on the telomeres may be regulated during cell cycle progression by telomeric binding proteins or other mechanisms. Knowledge of the regulation of telomerase activity may translate into therapies designed to inhibit telomerase activity. Several molecules have been identified that have shown promise in the inhibition of telomerase activity in *in vitro* systems, and the development and testing of molecules that act similarly *in vivo* is eagerly awaited.

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