PREFACE

From the fertilized egg in the beginning of development, and throughout adult life until the last breath is taken, cells in all tissues are in a dynamic state as they may divide, carry out their functions and adapt to environmental demands. In some tissues, cells have only a relatively short lifespan during which they perform their functions. It is critical that these cells die and then are removed from the tissue without adversely affecting neighboring cells. On the other hand, cells in other tissues, such as neurons, must remain viable and in communication with their neighbors throughout life in order for that tissue to carryout its various functions. While the molecular and cellular mechanisms that modulate cell division, differentiated functions and death are complex, emerging data suggest a particularly important role for the telomerase enzyme in development and aging. Accordingly, telomerase is also increasingly implicated in the pathogenesis of a variety of age-related diseases ranging from cancer to neurodegenerative disorders.

The present volume of *Advances in Cell. Aging and Gerontology* critically reviews the rapidly advancing area of telomerase research with a focus at the molecular and cellular levels. The clearly established function of telomerase is to maintain chromosome ends during successive rounds of cell division by adding a six base DNA repeat on to the telomeric ends of chromosomes. As presented in the chapters of this volume, the mechanisms that regulate telomerase expression and activity are complex. Moreover, emerging data suggest additional roles for telomerase in the regulation of cell differentiation and survival. This volume begins with an Introductory Forward by Jerry Shay and Woody Wright that raises fundamental issues concerning the roles of telomerase in aging, and the interrelationships between genomic instability, cell proliferation, aging and cancer.

The next two chapters by Klapper and co-workers and Liu detail the complex molecular interactions required for telomerase reverse transcriptase activity, and how this machinery is regulated at the transcriptional and post-transcriptional levels. An increasing number of proteins that interact with either the catalytic subunit of telomerase (TERT) or telomeres, and their relationship to DNA remodeling and chromosome maintenance are presented. Interestingly, a number of second messenger pathways are being identified that can modulate telomerase activity and/or TERT expression, some of which have interesting implications for development, aging and disease. The mechanisms whereby telomerase regulates the cell cycle on the one hand, and may be modulated by the cell cycle on the other hand, are detailed by Tej Pandita in a chapter on Telomerase and the Cell. Cycle. Lynne Elmore and Shawn Holt provide a more detailed consideration of the role of telomerase in regulation of cell proliferation and cancer with a focus on the transition to immortality. Martha Stampfer and Paul Yaswen present an intriguing hypothesis for the mechanisms controlling the transformation of cells and reactivation of telomerase during this process.

A mode of programmed cell death called apoptosis is increasingly recognized as playing pivotal roles in normal development and in many different disease conditions.

Recent evidence that telomerase, and TERT in particular, play important roles in preventing apoptosis is presented in a chapter written by my colleagues and me at the National Institute on Aging. By suppressing apoptotic biochemical cascades, TERT may play important roles in modulating developmental cell death and cell death in pathological conditions such as neurodegenerative disorders and ischemic injury. A better understanding of the anti-apoptotic function of TERT may lead to novel therapeutic strategies for disorders that involve abnormal apoptosis. As is the case in most tissues, telomerase is expressed during early development of the nervous system. In an additional chapter, my colleagues and I consider the evidence suggesting that telomerase plays important roles in regulating the proliferation, differentiation and survival of cells during brain development.

Maria Blasco has been a leader in the development and characterization of telomerase knockout mice. She contributes a chapter to the present volume detailing the phenotypic alterations in mice lacking the RNA component of telomerase. These mice are providing an invaluable model for understanding the roles of telomerase in cancer and aging, and are also likely to contribute greatly to our understanding of a variety of age-related diseases. Tissue-specific abnormalities in these mice suggest complex roles for telomerase in maintenance of organ function and in responses to environmental challenges.

In the final chapter, Len Hayflick provides a provocative commentary presenting his views of the history of telomerase research in relation to aging, and considers the future directions in this field. It is expected that this quite comprehensive volume will provide a valuable resource for graduate students and postdocs in the telomerase field and for established investigators in other fields who are beginning to study telomerase in their particular research program. With an increasing number of proteins being brought into the fold of telomerase research (e.g., DNA damage and repair response proteins, heat-shock proteins, and proteins in various signal transduction cascades) many new scientists are beginning to study this enzyme from novel vantage points. I expect that many surprises will emerge from these studies in the not too distant future.

Mark P. Mattson

CHAPTER 1

FORWARD: AGING AND CANCER: ARE TELOMERES AND TELOMERASE THE CONNECTION?

JERRY W. SHAY and WOODRING E. WRIGHT

Table of contents

This volume of "Advances in Cell Aging and Gerontology" focuses on the role of telomerase as it relates to aging and cancer. Since most aspects of this rapidly expanding field are reviewed in the accompanying articles, the intent of this introductory chapter is to provide a brief background and some terminology in the field as well as to give a perspective of where the telomerase field has progressed, some recent misconceptions that have arisen during the previous few years, and what we believe will occur in the future.

Telomerase, Aging and Disease. Ed. by M. P. Mattson and T. K. Pandita. 1 – 13

1. Introduction/background

1.1. Telomeres

Telomeres are the repetitive DNA sequences at the end of all linear chromosomes [1, 2]. In humans there are 46 chromosomes and thus 92 telomere ends. Each telomere contains thousands of repeats of the six nucleotide sequence, TTAGGG [3, 4]. The mechanisms of DNA replication in linear chromosomes is different for each of the two strands (called leading and lagging strands). The lagging strand is made as a series of discrete fragments, each requiring a new RNA primer to initiate synthesis. The DNA between the last RNA priming event and the end of the chromosome cannot be replicated because there is no DNA beyond the end to which the next RNA primer can anneal, thus this gap cannot be filled in (this is referred to as the "end replication problem") [5]. Since one strand cannot copy its end, telomere shortening occurs during progressive cell divisions. The shortened telomeres are inherited by daughter cells and the process repeats itself in subsequent divisions [6].

1.2. Replicative aging

In contrast to tumor cells, which can divide forever (are "immortal"), normal human cells have a limited capacity to proliferate (are "mortal") [7]. In general, cells cultured from a fetus divide more times in culture than those from a child, which in turn divide more times than those from an adult. The length of the telomeres decreases both as a function of donor age and with the number of times a cell has divided in culture [8]. There appear to be two mechanisms responsible for the proliferative failure of normal cells [9, 10]. The first, M1 (Mortality stage 1), occurs when there are still at least several thousand base pairs of telomeric sequences left at the end of most of the chromosomes. M1 may be induced by a DNA damage signal produced by one or a few of the 92 telomeres that have particularly short telomeres [11]. The M1 mechanism causes a growth arrest mediated primarily by the tumor suppressor genes p53 [12]. There is also some evidence for p16/pRB function in cellular senescence but this may be due in part to inadequate culture conditions (described in a later Section). However, if the actions of p53 and p16/pRB are blocked, either by mutation or by binding to viral oncoproteins, then cells can continue to divide and telomeres progressively shorten until the M2 (Mortality stage 2) mechanism is induced. M2 represents the physiological result of critically short telomeres when cells are no longer able to protect the ends of the chromosomes, so that end-degradation and end-to-end fusion occurs causing genomic instability and cell death. In cultured cells, a focus of immortal cells occasionally arises. In most cases, these cells have reactivated the expression of telomerase, which is able to repair and maintain the telomeres.

1.3. Telomerase

Telomere terminal transferase or telomerase (TEE-LÓM-ER-ACE) is a ribonucleoprotein reverse transcriptase enzyme (composed of both RNA and proteins) [13-21].

It uses its internal RNA component (complementary to the telomeric single stranded overhang) as a template in order to synthesize telomeric DNA (TTAGGG)n, directly onto the ends of chromosomes. After adding six bases, the enzyme pauses while it repositions (translocates) the template RNA for the synthesis of the next six base pair repeat (e.g. telomerase is processive). This extension of the 3' DNA template eventually permits additional replication of the 5' end of the lagging strand, thus compensating for the end-replication problem. The enzyme is expressed in embryonic cells and in adult male germline cells [22], but is undetectable in most normal somatic cells except for proliferative cells of renewal tissues (e.g. hematopoietic stem cells, activated lymphocytes, basal cells of the epidermis, proliferative endometrium, and intestinal crypt cells). In normal somatic cells, even including stem-like cells expressing telomerase, progressive telomere shortening is observed, eventually leading to greatly shortened telomeres and to a limited ability to continue to divide.

1.4. Preventing cellular aging

While there have been many studies indicating a correlation between telomere shortening and proliferative failure of human cells, the proof that it is causal was demonstrated for the first time about three years ago [23]. Introduction of the telomerase catalytic protein component (hTERT) into normal human cells without detectable telomerase results in restoration of telomerase activity and telomere maintenance or elongation [23-26]. Normal human cells stably expressing transfected telomerase are functionally immortal and have divided hundreds of times, providing direct evidence that telomere shortening controls cellular aging. The cells with introduced telomerase maintain mostly a normal chromosome complement and continue to grow in a normal manner. These observations provide direct evidence for the hypothesis that telomere length determines the proliferative capacity of human cells.

1.5. Replicative aging and cancer

Cellular senescence may have evolved, in part, to protect long-lived organisms, such as humans, against the early development of cancer. Cancer cells must acquire many mutations before they become malignant, and replicative aging inhibits this progression by halting cell division before more than a few mutations have accumulated. The telomere-telomerase hypothesis of aging and cancer is strengthened by the finding that most human tumors have upregulated or reactivated telomerase activity [27-54]. In contrast to normal cells, tumor cells show no net loss of average telomere length with cell division (Figure 1), suggesting that telomere maintenance may be required for cells to escape from replicative senescence and proliferate indefinitely. Most, but not necessarily all, malignant tumors [27, 29] may need telomerase to sustain their growth. Immortalization may occur through a mutation of a gene (or genes) in the telomerase repression pathway. Thus, upregulation or reactivation of telomerase activity may be a rate-limiting step required for the continuing proliferation of advanced cancers. There is experimental evidence from hundreds of independent laboratories that telomerase activity is present in approximately 90 percent of all human tumors but not

in tissues adjacent to the tumors [27-54]. Thus, clinical telomerase research is currently focused on the development of methods for the accurate diagnosis of cancer and on novel anti-telomerase cancer therapeutics.

1.6. Can telomerase be used as a product to extend cell lifespan?

The ability to immortalize human cells and retain normal behavior holds promise in several areas of biopharmaceutical research including drug development, screening and toxicology testing [55]. The development of better cellular models of human disease and production of human products are among the immediate applications of this new advance. This technology has the potential to produce unlimited quantities of normal human cells of virtually any tissue type and may have its most immediate translational applications in the area of transplantation medicine. In the future it may be possible to take a persons own cells, manipulate and rejuvenate them without using up their lifespan and then give them back to the patient. For example, genetic engineering of telomerase-immortalized cells could lead to the development of cell-based therapies for certain genetic disorders such as muscular dystrophy.

2. Recent misconceptions in the cell immortalization and telomerase field

There have been a number of papers published during the past two years that are accurate in regards to the observations reported but that have been misinterpreted by many scientists and science journalists and have thus led to some confusion in the telomerase and cell immortalization fields. The subjects listed in Table 1 will be discussed individually.

Table 1.

• Is telomerase insufficient to immortalize human keratinocytes and breast epithelial cells?
• Is there a difference in the frequency of immortalization between rodent and human cells?
• Is immortalization by telomerase risky?
• Can human cells be transformed by alterations in three cellular pathways?

2.1. Is telomerase insufficient to immortalize human keratinocytes and breast epithelial cells?

Replicative aging is one of the basic mechanisms that long-lived organisms have evolved for limiting the number of oncogenic mutations that accumulate before growth arrest intervenes. Telomere shortening has been shown to be one mechanism capable of counting cell divisions. The apparent failure of telomerase to immortalize a variety of epithelial cell types has led many authors to propose that the p16/RB pathway

may represent a telomere-independent mechanism for counting cell divisions during replicative aging [56-61]. We have re-examined the evidence for these claims and the results demonstrate that the p16-mediated growth arrest observed with at least two different epithelial cell types (skin keratinocytes and HMECs) is a consequence of the particular tissue culture conditions used, and can be prevented by growing the cells on appropriate feeder layers [62]. Keratinocytes immortalized with telomerase on feeder layers can still exhibit a p16-mediated growth arrest after being transferred to plastic culture dishes. Rare cells that eventually escape this checkpoint on plastic and resume growth no longer express p16, consistent with the previous demonstrations that inactivation of the INK4a locus is required for extended growth of these cells in chemically defined media on plastic substrates. However, since these cells are already immortal when grown on feeder layers, it is inappropriate to describe this change as required for immortalization. Instead, p16 inactivation should be characterized as an event necessary for proliferation in an inadequate culture environment. We thus conclude that the published evidence does not support the presence of telomere-independent mechanisms of replicative aging.

It is unambiguous that $p16^{Ink4A}$ is an important tumor-suppressor involved in cell cycle regulation. The demonstration that the p16-mediated growth arrest found in some cultured cells represents a response to inadequate culture conditions rather than a telomere-independent mechanism of replicative aging focuses attention on its role in mediating stress theoretically induced by a variety of factors, rather than in counting cell divisions [63, 64]. This will permit more effective analyses of telomeres in replicative aging, their role in tumor prevention and the possibilities of manipulating telomere biology for the treatment of cancer.

The cell cycle is regulated by positive growth factors that induce progression through the cell cycle, as well as negative regulators that can block this progression once a threshold level of inhibition is reached. As is illustrated in Figure 2 and indicated by the dotted line, there is a threshold for growth arrest by negative regulators. All tissue culture conditions probably induce a certain level of damage or stress responses to this non-physiological environment. If the level of stress plateaus below the threshold value, then cells can proliferate until additional checkpoint activities, induced by telomere shortening, intervene and cause the growth arrest of cellular senescence. However, if the culture conditions (e.g. plastic *versus* feeder layers) cause the stress response to progressively increase, then growth arrest can occur before the effects of replicative aging can be seen. When the cells are maintained in a culture environment that does not exceed the threshold for growth arrest until telomeres become too short, then introduction of hTERT can maintain the cells below this growth arrest threshold resulting in cell immortalization.

2.2. Is there a difference in the frequency of immortalization between rodent and human cells?

The confusion between replicative aging and inadequate culture conditions illustrated above for human epithelial cells is similar to what is found for mouse embryo fibroblasts

[65, 66]. The growth arrest of mouse fibroblasts that occurs after only a few divisions in cell culture is telomere-independent, and we believe it has been inappropriately referred to as replicative or cellular senescence. Since this growth arrest occurs in rodent cells when there are still very long telomeres and at the normal time in cells from mice lacking the RNA component of telomerase [67] whose telomeres are shorter than those in wild-type mice, we believe this phenomenon should not be compared to telomere-based replicative senescence that occurs in human cells grown under the appropriate conditions. There is no experimental evidence that the replicative growth arrest experienced by primary rodent fibroblasts in culture depends upon a biologic clock that measures generation number. The observation that the transcriptional activity of p53 is induced 10- to 40- fold within the first three passages after mouse embryo fibroblasts are put in culture [68] suggests that the culture environment is producing DNA damage in these cells, as does the rapid growth arrest that occurs in culture using cells from mice with deficiencies in many DNA-repair factors [69-73].

Human fibroblasts have a more extended proliferative potential than their mouse counterparts in culture, yet essentially never become adapted to continuous growth as immortal cell lines ("establishment"). Human cells are more resistant than rodent cells to oncogene-mediated transformation and are more chromosomally stable. Despite these obvious differences, many reports have linked conclusions drawn from studies in these different cell systems, equating senescence in mouse and human cells. Sherr and DePinho [65] recently reviewed the idea that a growth failure of cultured cells results from two sources of signals, either of which can induce the expression of a common set of inhibitors of the cell division cycle. One source that may trigger a cellular growth arrest is extrinsic and stems from inadequate conditions cells experience when they are explanted into culture. The second class is intrinsic and depends upon cell cycle cellular machinery that monitors the integrity of telomeres. Mouse cells are more sensitive to a variety of stresses of the culture environment because these shorter-lived animals have not evolved as effective damage protection and repair machinery as longer-lived humans [74]. It is likely that culture conditions that are adequate for human fibroblasts do not support the long-term proliferation of mouse fibroblasts. However, mouse cells can overcome this early growth arrest by inactivating the ARF pathway [65, 75]. We believe this is one of the reasons that murine cells "immortalize" with a much higher frequency than comparable human cells (Table 2).

Table 2. Of mice and men

• Normal mouse cells spontaneously immortalize with high frequency
• Normal human cells do not spontaneously immortalize
• Laboratory mice are 350 times smaller yet get cancer much more frequently, per animal per year, than humans
• The shortest mouse telomere is longer than the longest human telomere
• Telomerase is repressed less effectively in mice

Finally, it is entirely possible that telomere-based replicative senescence does not exist in the mouse and only evolved in larger and longer-lived organisms as an additional restraint against tumor formation [66].

2.3. Is immortalization by telomerase risky?

Introduction of telomerase into normal cells has been shown to result in maintenance of telomeres and extension of lifespan in several types of fibroblasts, epithelial and endothelial cells [23-26, 76, 77]. Since telomerase is expressed in almost all cancer cells, it is legitimate to ask if introduction of hTERT into normal cells results in an increased risk of cancer progression. As illustrated in Table 3, it has been shown that the properties of hTERT-expressing cells are essentially identical to matched normal cells except in the ability of the cells to divide indefinitely [78, 79].

Table 3. Properties of normal, cancer and hTERT-expressing fibroblasts in culture

Characteristics	Normal	Cancer	hTERT
Contact inhibition of growth	present	absent	present
Growth factor requirements	high	low	high
Anchorage-dependence	present	absent	present
Cell. cycle checkpoints	intact	absent	intact
Karyotypic profile	normal	abnormal	normal
Proliferative life span	finite	indefinite	indefinite

It has been shown that hTERT expressing cells have similar growth rates to normal cells; that p53, p21, p16 and pRB protein levels are unchanged; DNA damage checkpoints are intact (p53 is induced after UV-B and gamma irradiation); oncogene-induced checkpoints are intact (e.g. ras induced "premature senescence" still occurs); and "telomerized" cells do not make tumors in nude mice. Thus, telomerase by itself does not cause the acquisition of any abnormal growth characteristics, but only provides the cells with unlimited divisions. Cells almost certainly would have to accumulate many additional alterations to become cancerous.

In this regard, it was recently reported that human mammary epithelial cells (HMEC) established in cell culture and expressing hTERT upregulated c-myc and expressed their endogenous hTERT when the hTERT expression vector was removed after 135 population doublings [80]. While the cells were still karyotypically normal and nontumorigenic, this was interpreted to indicate that the cells were one additional step closer to cancer (since c-myc is upregulated in most cancers) and that expressing telomerase in cells could potentially be risky. However, it is important to note that because the HMECs were grown on a plastic substrate in culture, they had almost certainly inactivated p16 [61, 62]. Normally p16 would sense cellular stresses and DNA damage and prevent abnormal cells from progressing through the cell cycle. Given that the culture conditions were inadequate, there would be a strong selection pressure for

cells that had activated growth-promoting factors such as c-myc that might help cells proliferate better. The absence of p16 is likely to have contributed to the survival of cells with elevated c-myc. Thus, the most likely explanation for the increased c-myc expression in the HMECs in this study was growing them under inadequate culture conditions that produced multiple abnormalities. In similar experiments conducted in our laboratory, hTERT was introduced into normal fibroblasts for only a short period of time, and after hTERT was excised and there was no activation of the endogenous telomerase [81]. The cells had elongated their telomeres and after removal of hTERT the telomeres began to shorten and eventually the cells stopped proliferating and underwent normal senescence (without any increase in c-myc). Our cells had normal p16, and were cultured under conditions that appear excellent for fibroblasts, so this could explain the difference between our study and the ones conducted on HMECs. Thus, while we agree it is entirely appropriate to be cautious, we also believe that putting hTERT into cells that can be well characterized would not increase the risk of cancer very much and may be worth the risk in certain cases.

2.4. Can human cells be transformed by alterations in three cellular pathways?

The research of many scientists over the previous decades has clearly documented that there are several parallel paths to tumorigenesis [82, 83]. While the exact sequence of alterations that must occur within the putative cancer cell are not fixed, there are general paths leading to tumorigenesis (Table 4) that almost all developing cancer cells must follow. Losing tumor suppressor functions such as those of p53 and p16/pRB, activation of ras signaling pathways, producing insulin-like growth survival/anti-apoptosis factors, inducing angiogenesis by producing VEGF, losing the ability to undergo normal differentiation, upregulating or turning on telomerase to maintain the telomeres, and inactivating E-cadherin to produce cell invasion through the stromal compartment are general pathways that are often affected within the putative cancer cell [82]. However, there are also extrinsic cellular factors that occur that provide a favorable environment for the growth of the cancer cell. In addition to interactions between cancer cells, the concept of a heterotypic tumor environment must incorporate the idea that inflammary cells producing proteases (Hanahan, personal communication), endothelial cells migrating towards the tumor bed, and stromal fibroblasts participating in cell-cell interactions within the tumor environment are central to cancer progression.

Table 4. Paths to tumorigenesis

- Self sufficiency in growth signals
- Insensititivy to antigrowth signals
- Loss of differentiation capacity
- Evading apoptosis
- Sustained angiogenesis
- Limitless replicative potential

Many previous attempts to convert normal human cells to tumorigenic cells have failed using protocols that efficiently convert murine cells. In an attempt to begin defining specific genetic alterations to convert a normal human cell to a tumor cell, it was reported that the ectopic expression of an hTERT cDNA in combination with two oncogenes (SV40 large T-antigen and an oncogenic allele of H-*ras*), resulted in direct tumor conversion of normal fibroblasts and epithelial cells [84]. We had conducted similar experiments using the same normal fibroblasts ectopically expressing an hTERT cDNA, HPV 16 E6/E7, plus oncogenic H-*ras* and our cells did not grow in soft agar or induce tumor formation [79]. HPV16 E6/E7, similarly to SV40 large T-antigen, abrogates p53 and p16/Rb pathways so it was not initially obvious why there was this very different result. Clearly HPV and SV40 oncoproteins could "take care of" different cellular functions. However, as it turns out, the explanation may be very simple. The original report apparently was using a vector that was expressing not only SV40 large T-antigen but also small t-antigen. Small t-antigen inhibits protein phosphatase 2A activity [85] and its presence was required for the effect (Hahn and Weinberg, personal communication). In addition it appears that extraordinary high (10-fold higher than in tumor cells) levels of ras are required. The bottom line is that the steps leading to tumorigenesis are quite complex and not yet fully understood. The key is to understand the specific genes altered in the multiple cellular pathways as outlined in Table 4.

To begin to address the question of how many "hits" does it take to transform a normal cell to a tumor phenotype, there was an interesting animal study in which bovine adrenal cells were "telomerized" with hTERT and also transfected with the viral oncoprotein SV40 large T-antigen. T-antigen takes over cell growth control (by inactivating at a minimum both the p53 and p16/Rb pathways) and hTERT immortalized the bovine cells [76]. This study showed that these hTERT, T-antigen expressing bovine adrenal cells could be placed in the kidneys of immunosuppressed scid mice. The scid mice were also adrenalectomized (so that they did not produce glucocorticoids). Such mice die within 25-days after adrenalectomy as glucocortocoids are essential for life. However, the introduced bovine cells intermingled with stromal cells of the scid mice, differentiated, and produced bovine cortisol which rescued the animals from dying. This indicates that hTERT and T-antigen expressed in cells and positioned in the correct environment are not tumor cells, but behave normally and differentiate and produce differentiated functions. Although great caution will clearly be needed if oncogenes such as T-antigen are combined with telomerase, these results emphasize how difficult it may be to make even immortal cells behave malignantly.

3. Summary and future directions

In this introductory chapter, we have reviewed the evidence that escape from senescence and crisis are important events in the life history of human cancer cells, and that the presence of telomerase activity *per se* can reflect an immortal state, but does not directly imply an oncogenically transformed one [82]. In addition, we have shown that several misconceptions about cellular senescence have resulted from signals that can occur

after explanting murine and human cells from their *in vivo* location to one growing in the tissue culture environment. While presently we believe that the sole function of telomerase is to maintain telomere stability, it is possible that TERT expression might provide other as yet unknown functions. Finally, whether cell senescence has anything to do with organismal aging remains a major unresolved issue that will require additional research.

Knowledge of telomerase and telomere biology is important and likely to have a major impact on human health in the future. While the field is still young in regards to our molecular understandings, the chapters in this volume of "Advances in Cell. Aging and Gerontology" begin to explore some of the specific details that will be required for clinical exploitations. A central goal is to improve human health span and we believe knowledge of telomeres and telomerase may help in this regard. Inhibition of telomerase for cancer therapeutics or as a chemoprevention approach are exciting new areas of cancer research [86-87] and early clinical trials are likely to be initiated in the near future. Telomerase diagnostics [45] for detecting cancer at an early stage is another important area of research and may also serve to improve prognostics and detection of residual disease in combination with standard pathology. The use of telomerase for tissue engineering is a very new field [55] and additional studies will determine in which chronic disease settings rapid advances will be made.

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CHAPTER 2

ASSEMBLY AND FUNCTION OF THE TELOMERASE ENZYME COMPLEX

WOLFRAM KLAPPER, REZA PARWARESCH and GUIDO KRUPP

Table of contents

1. Introduction

Telomerases are ribonucleoprotein complexes that synthesize telomeric repeats on the chromosome end, the telomere. They are reverse transcriptases because they copy a built-in template which is part of the integral RNA component of the enzyme and functions like a blueprint for new telomeric repeats. Telomerase activity was first detected by Blackburn and Greider in extracts of the ciliate *Tetrahymena thermophila* [1]. Telomerase has a very low cellular abundancy in higher eukaryotes and still today, most data about telomerase comes from research with unicellular eukaryotes like the ciliates *Tetrahymena* or *Euplotes* with their much higher enzyme levels or yeasts using highly developed genetic techniques (Figure 1). In the last few years our knowledge

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about human telomerase expanded enormously, starting with the introduction of the PCR based telomerase activity assay "TRAP" [2] and continuing with two milestones in telomerase research: the identification of the two core components of the human enzyme the RNA component hTR [3] and the reverse transcriptase hTERT [4-7].

This chapter describes the components and functions of the telomerase enzyme complex. The chapter will focus on the description of the human enzyme but also considers telomerases from other species.

2. The telomerase enzyme complex

The activity of telomerase detected by *in vitro* activity assays is sensitive to both, proteinase and RNase digestion; thus telomerases are ribonucleoprotein enzymes that consist of protein and RNA components [1]. Telomerases synthezise multiple telomeric repeats on short single stranded DNA oligonucleotides *in vitro* or on chromosome ends *in vivo*. A blueprint for the new DNA repeats is carried on the intregral RNA component. Because telomerase synthezises DNA from an RNA template it is a reverse transcriptase [8]. The *in vivo* substrate of telomerase is the chromosome end, the telomere, which is composed of thousands of tandem telomeric repeats and specific binding proteins (for review see [9-11]). Consequently, telomerase is a nuclear RNP, although similar levels of active enzyme can be detected in nuclear as well as in cytoplasmic extracts of human cells [12]. The level of abundancy of the human enzyme is very low with estimated 3000-5000 copies of hTR and 30 copies of hTERT protein per cell of *in vitro* growing tumor cell lines, the richest source of human telomerase [13, 14]. Glycerol gradient fractionation of the human enzyme complex from nuclear extracts revealed a size of ~1000 kDa for the native enzyme and ~550 kDa for affinity purified telomerase [13]. Changes in the enzyme size during successive affinity purification steps was also observed with telomerases from other species and may be caused by the loss of loosely associated factors during purification [15]. Telomerases from ciliates differ strikingly in size, depending on the growth state of the organisms or the intra-cellular localization, e.g. macronucleus *versus* micronucleus [15]. Whether human telomerase displays similar growth state or cell type dependent changes is unknown.

Two components of the enzyme, hTR (the RNA component) and hTERT (the catalytic subunit) are considered as the core components. The *in vitro* transcribed hTR and recombinant hTERT alone could be assembled into an active enzyme, as detected by the common *in vitro* activity assays [16-18]. Although both, hTR and hTERT, are considered as core components, only hTERT seems to correlate with telomerase activity *in vivo*. Except for a few known exceptions, telomerase activity measured by the *in vitro* assay is closely correlated with the presence of hTERT protein [19]. On the other hand, the RNA component hTR is expressed in several tissues and cells lacking telomerase activity [3]. Thus *in vivo* hTERT seems to be the limiting factor of an active telomerase enzyme (for review see [20]).

2.1. hTR — the RNA component

Telomerase is a unique reverse transcriptase, opposed to other RTs, it contains an RNA subunit (hTR) that is a permanently bound core component of the enzyme. A short section of its sequence functions as a built-in template for the step-wise synthesis of repetitive DNA, forming the telomeric sequence of telomeres. The functional importance of telomerase RNA was shown by nuclease inactivation experiments which destroyed the RNA, and the successful recovery of enzyme activity by readdition of *in vitro* transcribed telomerase RNA [21]. Human and mouse telomerase RNA are most likely transcribed by RNA polymerase II because the promotor region contains elements typically arranged like mRNA promotors: a TATA box-like element and a consensus CCAAT box [22]. In contrast, the ciliate RNA promotor region resembles the RNA polymerase III promotor for U6 small nuclear RNA (snRNA). A possible explanation of the different polymerase usage might be the greater flexibility of expression with RNA polymerase II in vertebrates *versus* the required higher levels obtained with RNA polymerase III in ciliates [22]. Yeast telomerase RNA component has recently been shown to contain a 5'-2, 2, 7-trimethylguanosine (TMG) cap and a binding site for Sm proteins, both hallmarks of small nuclear ribonucleoprotein particles (snRNPs) that are involved in nuclear messenger RNA splicing [23]. Association of the human RNA component with snRNPs could not be demonstrated [23, 24]. Interestingly hTR has recently been demonstrated to be linked to small nucleolar RNAs which are involved with rRNA maturation (see below) [25].

2.1.1. *Structural model for the phylogenetically conserved vertebrate telomerase RNA core*

In all vertebrates, the telomeric repeat is the hexamer 5'-GGTTAG-3' and the RNA segment which encodes this sequence is termed the "template region". Based on the recent compilation of 35 RNA sequences, a minimum consensus vertebrate telomerase RNA sequence was derived and phylogenetic comparisons allowed the proposal of a conserved secondary structure [26]. Four structural domains are well conserved: the CR4/CR5 region, the pseudo-knot, the H/ACA box and the CR7 region (see Figure 2). The generalized structure will help in elucidating details of RNA-protein interactions and telomerase enzyme functions.

2.1.2. *Subunits in a minimal human telomerase enzyme*

The minimal set of enzyme subunits could be determined by *in vitro* reconstitution of a functional human telomerase enzyme. The highly purified telomerase RNA component is available by *in vitro* transcription whereas, initially, the expression of recombinant hTERT protein was limited to coupled *in vitro* transcription/translation in rabbit reticulocyte lysate [16, 17]. The *in situ* hTERT protein synthesis in the presence of telomerase RNA resulted in telomerase activity; however, these experiments could not rule out an important functional contribution of one or several of the many mammalian cellular components present in the rabbit cell lysate [16, 17]. Recently, recombinant

hTERT protein could be expressed and recovered from baculovirus transfected insect cells [18]. Also this highly enriched hTERT protein could be combined with hTR to obtain telomerase activity [18]. This defines hTERT protein and hTR RNA as the minimal subunits required for telomerase enzyme activity *in vitro*. But as discussed below, a fully functional telomerase *in vivo* requires more than this core enzyme.

A plasmid in which the 3' end of the hTERT coding sequence was extended by the addition of the sequence encoding hTR was generated. Expression of the hTERT-hTR cis construct *in vitro* (in rabbit reticulocyte lysate) and *in vivo* (in the yeast *Saccharomyces cerevisiae*) produced active human telomerase [27].

The ability of *in vitro* reconstitution of a telomerase core enzyme was further used to define the minimal functionally important regions of hTERT protein (see 2.2.1.) and of hTR RNA. Already in the first experiments with nuclease inactivated human telomerase and *in vitro* transcribed complete hTR and of hTR segments, the minimal functionally important region was mapped between nucleotides 44-203 [21]. More detailed analyses with *in vitro* produced hTR and hTERT subunits revealed that a fragment spanning nucleotides 10-159 (see arrow in Figure 2) was sufficient to support telomerase activity [28, 29].

2.1.3. *Larger telomerase RNA is required* in vivo

Although not a single complete conserved RNA structural domain is present in this minimal functional RNA segment determined *in vitro*, they can be important *in vivo*. The 3'-terminal H/ACA domain is known from the H/ACA small nuceolar RNAs (snoRNAs) involved in pseudouridylation of pre-rRNAs. In telomerase RNA, this domain is possibly important for proper 3'-end processing, *in vivo* RNA stability, and localization of the telomerase RNA to the nucleolus [26]. Recent evidence suggests binding of this domain to H/ACA proteins present in the RNP's formed by snoRNAs [30]. Interestingly, one of them could be the human homologue of yeast Cbf5p, known as dyskerin [30, 31]. This potential inclusion of vertebrate telomerase RNA in the snoRNP assembly pathway — and the absence of a conserved Sm sequence — are in remarkable contrast to the previous data with yeast telomerase RNA [23] which contains an Sm protein-binding motif with a possible inclusion in the nuclear snRNP assembly pathway.

2.1.4. *Changes in the template region*

Whereas the location of the single-stranded template region of vertebrate telomerase RNA component within the model is well conserved (Figure 2) and always contains the minimal octamer sequence 5'-CUAACCCU-3', species-specific short additions with potential coding function are also present. So far, *in vivo* experiments with mutations in the template region of telomerase RNA were only reported for the yeast *Saccharomyces cerevisiae*. It was shown that these mutations resulted not only in changes of the telomeric repeat sequence, but they grossly affected telomere length regulation, possibly mediated by altered binding of the thus mutated telomeric DNA to telomere binding factors, like the yeast Rap1p which possibly combines functions of human TRF1 and

TRF2 [32]. Altering the telomere repeat sequence by mutating the RNA template region will, also in human cells, be a valuable model to elucidate the structural function of telomeric DNA and telomere binding proteins in telomeres *in vivo*.

2.2. hTERT — the catalytic subunit of human telomerase

2.2.1. *The hTERT protein — a reverse transcriptase*

Telomerase is a reverse-transcriptase-like enzyme [33]. Its catalytic subunit hTERT is a mainly basic protein with 1132 amino acids (isoelectric point calculated as 11.3) and a molecular mass of 127 kDa in SDS-PAGE. The hTERT gene has been cloned by four independent groups and the now generally accepted gene name hTERT (human telomerase reverse transcriptase) has been previously referred to as hTRT [4], hEST2 [5], hTCS1 [7] and TP2 [6]. The identification of the human protein resulted from two independent experimental approaches for the cloning of telomerase components (Figure 1). A genetic screen in the yeast *Saccharomyces cerevisiae* identified the gene *est2* that is essential for telomere maintenance and telomerase activity [34]. Biochemical purification of *Euplotes aediculatus* extracts pulled out the proteins p123 and p43 [35] which copurified with the RNA component and the activity of the enzyme. EST2 and p123 share 20% sequence homology, and additionally contain a domain that can be found in all other known reverse transcriptases [36]. The human protein was subsequently identified by its similarity to the yeast and the *Euplotes* protein. Based on sequence homology of the RT domain, telomerases seem more closely related to RTs associated with msDNA (multicopy single-stranded), group II introns, and non-LTR (long terminal repeat) retrotransposons than to the LTR-retrotransposons and retroviral RTs [4]. The area of high similarity of hTERT to other RTs is found in the assumed catalytic region of the protein and includes the motifs 1, 2, A, B', C, D, E that have been defined as characteristic for the active site of RTs (Figure 3) [37]. The tertiary structure of hTERT has not been analyzed yet, presumably because of difficulties in expression or purification of large protein amounts [18]. The crystal structure of the HIV1-RT p66 subunit has been determined [38]. Sequence similarities between both types of reverse transcriptase suggest a similar structure of the domain with the active site.

Single amino acid exchanges of three invariant aspartate residues within motif A (D712) or motif C (D868 or D869) abolish the activity of RTs and as well the activity of telomerase [17, 19]. But besides similarities there are also distinct sequence differences between telomerases and other RTs. The distance between the motif A and B' is much bigger in telomerases and an additional motif can be found in telomerases, the T motif, that is absent in other RTs and seems important for telomerase function [17]. Because the region with the motifs A and B' in the HIV RT interacts with its template, the larger distance between these motifs in all telomerases was speculated to be the cause for a major mechanistic difference between telomerases and RTs: Telomerase synthezises multiple copies of its short built-in template, whereas RTs produce single long stretches from exogeneous RNA templates [39]. Future experiments with recombinant protein with altered sequences will give insigths into the mechanistic process that distinguishes telomerases from RTs. So far, most

experiments that determine the function of certain regions of the TERT protein have been carried out with yeast or *Tetrahymena* TERT [40, 41]. A question in the analysis of deletions or mutational alterations in the TERT protein is if there are differences of *in vitro* and *in vivo* functions. Indeed, some deletion variants produced by Beattie et al. [41] were inactive when co-expressed in a reticulozyte expression system but active if expressed in 293T cells. Possible explanations for this observation are the absence of factors in the reticulocyte system that help to assemble the active enzyme in 293T cells. These experiments also showed that deleting the N-terminal 300 amino acids did produce an enzyme that synthezised shorter products than the native enzyme. Deletions at the C-terminal end resulted in an inactive enzyme, although the proteins were still able to bind the RNA component and hTEP1 [41]. Again, this is a striking difference with the HIV RT catalytic subunit, where the C-terminal end up to the RT-domain can be deleted without affecting the polymerization ability of the enzyme.

2.2.2. *Transcriptional regulation*

The promotor region of hTERT has been cloned and includes several binding sites for a number of transcription factors [42-45]. Transcription of hTERT is enhanced by the transcription factor c-myc in cooperation with Sp1 [46], estrogen [47, 48] and presumably NF-kB [49]. Repression of transcription is induced by Mad [50, 51], MZF-2 [52], Wilms' tumor 1 suppressor gene [53] and p53 [54, 55]. Progesterone seems to have a time dependant positive and negative regulating function on the hTERT transcription. Inhibition of histone deacetylases by trichostatin A in telomerase-negative cells resulted in activation of telomerase activity and up-regulation of hTERT mRNA [56]. DNA methylation may contribute to the regulation of the hTERT gene, but CpG island methylation in the hTERT promotor region seems not responsible for the repression of hTERT expression in most telomerase-negative cells [57, 58].

2.2.3. *Posttranscriptional and posttranslational changes*

The hTERT mRNA has been shown to be alternatively spliced during human embryonic development and the disappearance of the full length transcript correlates with the downregulation of telomerase activity [59]. Hence, at least in humans, alternative splicing of hTERT could be a major mechanism of developmental telomerase regulation. Alternatively spliced hTERT mRNA variants which lack some of the critical RT motifs have been demonstrated in uterine tissue [60] and mesotheliomas [61].

The hTERT protein is a substrate of the c-Abl tyrosine kinase. Phosphorylation of hTERT by c-Abl is induced by ionizing radiation of cells and inhibits telomerase activity most likely due to hTERT phosphorylation [62]. Interestingly mouse cells deficient for c-Abl do show telomere lengthening [62]. hTERT is also phosphorylated by Akt protein kinase, a down-stream effector of the posphatidylinositol 3-OH kinase (PI3K) signaling pathway. Opposed to the effect of c-Abl, phosphorylation by Akt increases telomerase activity [63]. Protein phosphorylation of hTERT is further carried out by protein kinase C alpha. The stimulative effect of PKCalpha on telomerase

activity can reverse the inhibitory effect of protein phospatase 2A (PP2A) and it is speculated that both enzymes act inversely on the same phosphorylation sites which have not been determined yet [64, 65].

hTERT possesses several more motifs e.g. a Poly(ADP-ribose) binding site [66] but their functional importance is still unknown (Table 1). However, all regulatory mechanisms described here modulate the hTERT protein or the enzyme activity measured by the *in vitro* TRAP assay. Additionally the access and action of telomerase on the telomere is a second level of telomerase regulation [67-69]. Even if active telomerase can be detected by the *in vitro* assay, the biological function can be impaired e.g. if the telomere binding protein TRF1 is overexpressed [70]. The interaction of these two different levels of telomerase regulation are not completely understood yet.

2.2.4. *Cellular localization*

Telomerase acts on the chromosome end to synthezise new telomeric repeats. Consequently telomerase and its catalytic subunit hTERT is expected to localize mainly in the nucleus. Surprisingly a significant amount of active enzyme and of hTERT can be detected in the cytoplasm [12, 71]. The cytoplasmatic localization is speculated to be due to alternative functions of the enzyme or its subunits e.g. in the regulation of apoptotic pathways [72].

The hTERT protein has been demonstrated to possess a 14-3-3 protein binding site and its nuclear transport is mediated by 14-3-3 proteins. hTERT also binds CRM/exportin1 which transfers hTERT from the nucleus into the cytoplasm. It seems that binding of 14-3-3 protein does functionally suppress the effect of CRM/exportin1 binding to the nuclear export signal and the cytoplasmatic localization [71] (Table 1).

2.3. Telomerase associated proteins

Two components constitute the core of the human telomerase enzyme complex: hTERT and hTR. Both components are sufficient and necessary to reconstitite an active enzyme measured by the *in vitro* assay. This means, that recombinant hTERT together with the *in vitro* transcribed RNA component hTR give an active enzyme in reconstitution assays [18]. No other component described below can compensate for one of the two core components nor alter the *in vitro* activity in a way that can be detected by the conventinal telomerase assays. However, the native human telomerase enzyme complex, when biochemically characterized is estimated to have a molecular mass of 1000 kDa [13, 73]. The two core components combined only make up ~300 kDa (127 kDa of hTERT and 150 kDa of hTR), therefore the enzyme complex must contain additional subunits. Furthermore, properties known for telomerases like a 3'-5'exonuclease activity that can be demonstrated by *in vitro* experiments with native enzyme still lack any protein correlate that might carry out this function [74, 75]. When the native enzyme is biochemically purified by successive affinity purification steps, a change in the size of the active enzyme was detected [13]. This phenomenon can be explained by the loss of loosely associated components during the purification steps. Unfortunately telomerase is an enzyme with a very low

Table 1. Posttranslational changes and protein interaction sites of hTERT. Known sequences and the functions of the interactions are described.

	Sequence	Function	Reference
c-Abl binding site	PSTSRPPRP (amino acids 308-316)	inhibition of telomerase activity by phosphorylation at an unknown site	[62]
Akt phosphorylation site	GARRRGGSAS (amino acids 220-229) AVRIRGKSYV (amino acids 817-826)	increase of telomerase activity by phosphorylation	[63]
protein kinase Cα phosphorylation site	unknown	increase of telomerase activity by phosphorylation	[65]
protein phosphatase 2A dephosphorylation site	unknown	decrease of telomerase activity by dephosphorylation	[64]
poy(ADP-ribose) binding site	RGFKAGRNMRRKLFGVLRLKCH (amino acids 962-983)	unknown	[66]
nuclear export signal (NES)	NMRRKLFGVLRLKC (amino acids 969-981)	nuclear export by binding of CRM/exportin1	[71]
14-3-3 protein binding site	unknown	nuclear localization by binding of 14-3-3 protein	[71]

cellular abundancy even in immortalized tumor cell lines (30 copies per cell [14]), the richest source of human telomerase known and biochemical purification of the human enzyme complex to a purity that allows the protein sequencing of enzyme components is very difficult. Consequently, most of the human enzyme components have been discovered by sequence homology search of known components from other species or genetic tools like the yeast-two-hybrid-screen (Figure 1).

So far, besides hTERT and hTR, no other enzyme component has been discovered that is exclusively found in the telomerase complex. The proteins listed in Table 2 as telomerase associated proteins also play importent roles in the assembly of other protein complexes as chaperones (e.g. hsp90 and p23) or are involved in other functional processes like cellular localization of proteins (14-3-3 and CRM) or the maturation of small nucleolar RNA (dyskerin) (Table 2). Thus, although their precise function in the telomerase enzyme complex still has to be determined, these proteins will be termed telomerase associated proteins (TAP) and two of them will be discussed in more detail.

The function of the human telomerase associated protein hTEP1 is still unclear, although it is one of the longest known TAPs. The protein has been shown to be associated with hTERT and hTR but seems not necessary for proper telomerase function *in vitro* as well as *in vivo*, as demonstrated by a knock-out mouse [76]. This protein may play a role in the assembly of ribonucleoproteins and other complexes because it was detected in "vaults" [77]. hTEP1 has been discovered by its homology to the *Tetrahymena thermophila* protein p80 [78] which had been demonstrated to copurify with active telomerase enzyme in affinity chromatography [79]. Interestingly the *Tetrahymena* homolog p80 seems necessary for proper telomere function *in vivo*, although p80 like its human counterpart hTEP1 does not influence telomerase activity in the reconstitution assay in-vitro [80]. These experiments in *Tetrahymena* demonstrated for the first time that p80 is functionally involved in the telomerase/telomere biology *in vivo*. The precise mechanism and whether this function is conserved in humans has still to be determined.

Dyskerin is a potential pseudouridin synthetase that is mutated in the syndrom dyskeratosis congenita [81]. Recently, this protein was identified as a TAP because of its ability to bind H/ACA motif containing snoRNAs and the telomerase RNA component hTR which contains such a sequence motif [31]. Dyskerin binds to hTR and a dysfuntional dyskerin, like in the disease dyskeratosis congenita, leads to lower levels of hTR and lower levels of telomerase activity [31]. Dyskerin is speculated to be involved in the maturation of a fully functional hTR, possibly by RNA maturation and hTR accumulation. The resulting defect in telomere maintenance in dyskerin deficient cells is very likely to be the cause of the disease. It is not clear if dyskerin is physically associated with the mature telomerase enzyme complex, but it seems unlikely.

3. Telomerase biochemistry

The "direct assay" for telomerase activity requires high activity levels and lacks sufficient sensitivity to examine e.g. cancer tissues. The "TRAP" assay for telomerase activity includes a PCR-mediated amplification of the telomerase products and thus it

Table 2. Components of the telomerase enzyme complex. All components of the human telomerase complex are shown. The table distinguishes between core components and telomerase associated proteins (TAP) as described in the text. Functions in the telomerase complex and known other cellular functions are indicated.

Core components

Component	Size	Telomerase function	Other functions	Reference
hTR	451 nt	template	none	[3]
hTERT	127 kDa	catalytic subunit, reverse transcriptase	none	[4-7]

Human telomerase associated proteins (TAPs)

Component	Size	Telomerase function	Other functions	Reference
hTEP1	240 kDa	hTR binding protein	present in vaults, function in there unknown	[6]
dyskerin	25 kDa	maturation of RNA component	modification by and processing of snoRNA	[31]
hsp90	90 kDa	molecular chaperone	mediates proper folding, stabilization and localization of numerous proteins	[100]
p23	23 kDa	molecular chaperone	mediates proper folding, stabilization and localization of numerous proteins	[100]
hStau	55 kDa	hTR binding protein	binding of bicoid RNA	[101]
L22	15 kDa	hTR binding protein	ribosome associated protein, binding of Ebstein-Barr virus small RNA (EBER1)	[101]
14-3-3 proteins	29-33 kDa	nuclear localization	interaction with cellular signaling molecules like Raf-1, Cbl, Bad, IGF-1, Molecular chaperones and regulators of cellular localization of several proteins	[71]
CRM/exportin 1	112 kDa	nuclear export	nuclear export factor for numerous proteins	[71]
hnRNP C1	42 kDa	hTR binding protein	component of the 40S-hnRNP particle that is involved in pre-mRNA splicing and regulation of mRNA turnover	[24]
hnRNP C2	45 kDa	hTR binding protein	component of the 40S-hnRNP particle that is involved in pre-mRNA splicing and regulation of mRNA turnover	[24]

is significantly more sensitive, experimentally more convenient and allows large scale screening programs for telomerase activity [2, 82]. On the other hand, the TRAP assay prevents a direct biochemical characterization of the telomerase reaction. Until recently, no highly enriched human telomerase enzyme (or from other vertebrates) was available for detailed biochemical studies. The ability to express hTERT in an insect cell system (baculovirus) is a major step in producing sufficient amounts of recombinant hTERT [18]. Most data on telomerase biochemistry are derived from ciliates (for review see [83]), but large scale production of recombinant human protein will be a valuable tool to study telomerase biochemistry *in vitro*.

3.1. Telomerase inhibitors

Due to the enourmous impact expected from specific telomerase inhibitors on cancer treatment, a vast number of substances have been tested. The PCR-assay permitted the characterization of some low molecular weight telomerase inhibitors. The results have to be evaluated critically because not all studies distinguish carefully between inhibition of telomerase and Taq DNA polymerase in the PCR-based TRAP assay, although a reliable high-throughput assay for inhibitor screening has been described [84]. Substances that have been examined in detail include the nucleotide analogues already known as competitive inhibitors of viral reverse transcriptase [85-88] and several compounds which might act by stabilizing the unique G-quartet structures formed by the G-rich sequences of telomeric DNAs [72, 89-91].

Telomerase contains an RNA component that in addition to the mRNA of hTERT is a promissing target for antisense approaches. Antisense inhibitors for these targets have been described and demonstrated to function in cell culture systems [92]. They will be very helpful in elucidating effects of telomerase inhibition on the cellular level, but practical problems in their administration to animals and patients might favor a screen for low molecular weight inhibitors.

3.2. Primer binding and processivity

With the direct assays for human telomerase activity it could be shown that the enzyme is processive and adds up to ca. 70 hexamer reapeats on the primer substrate [12]. More detailed studies were reported only for lower eukaryotes. With telomerase from the ciliate *Tetrahymena*, it was observed that many repeats (several hundred) are added to a single primer molecule before the product dissociates from the telomerase enzyme [93]. The template region in telomerase RNA codes for the addition of a single telomeric repeat. Therefore, the high processivity requires dissociation and relocation of the 3'-end, but the primer has to remain bound to the enzyme, possibly via interaction with an upstream segment, the "anchor region". Anchor binding was confirmed by studies of primer interaction with telomerases from the ciliates *Euplotes* by photo-cross-linking [94] and *Tetrahymena* by interference footprinting [95] and by demonstrating binding of telomeric DNA by the isolated recombinant telomerase protein p95 [96]. Anchor binding seems to involve also the sugar moiety since replacement of the normal deoxyribonucleotides in the primer by ribonucleotides resulted in reduced binding by

p95 of *Tetrahymena* telomerase [79]. Furthermore, primer binding by yeast telomerase was affected by sequence changes in a region 20 nucleotides distant from the primer 3'-end [97].

Interestingly, endogeneous telomerase enzyme from *Tetrahymena* has a much higher processivity than recombinant enzyme produced by coupled transcription/translation of RNA component and catalytic protein TERT in rabbit reticulocyte lysate [96]. This complex lacks the *Tetrahymena* telomerase proteins p80 and p95 which may compromise anchor binding. Recently, it was revealed with the "minimal" recombinant *Tetrahymena* telomerase, that processivity could be stimulated by binding of a guanosine nucleotide moiety (7-deaza-GTP and dGMP were sufficient, whereas rGTP, dITP failed to stimulate). Binding of this nucleotide seems distinct from the template region and could promote a conformational change within the recombinant minimal enzyme [98]. In line with these findings, it was reported that the "non-essential" *Tetrahymena* proteins p80 and p95 are required *in vivo* for proper telomere length maintenance and genome stability [80].

This may indicate functional roles of the anticipated auxilliary factors present in the native human telomerase holoenzyme with its molecular mass of >1000 kDa [13, 99]. Although their contribution may not be essential for enzyme activity and not easily revealed *in vitro*. Furthermore, they may not be absolutely specific, and thus other related proteins may replace their function *in vivo*.

4. Perspective

hTERT, the catalytic subunit of human telomerase, contains evolutionary highly conserved regions like the T motif and variable regions like the C- and N-terminal ends. The impact of these regions on the function of the enzyme will have to be determined by site-directed mutagenesis and the generation of recombinant human protein.

The human telomerase enzyme complex is assumed to be a multiple subunit enzyme. Although basic structural features of the enzyme are evolutionary conserved e.g. the RT motifs in the catalytic subunit, there are still interspecies differences in our knowledge of the enzyme assembly. For example, p95, one of the first two telomerase associated proteins known from biochemically purified *Tetrahymena thermophila* extracts, still lacks any homolog in yeast, mouse or humans [79] and the function of the protein is barely understood [80]. Table 3 summarizes telomerase associated proteins from model organisms and indicates their known human homologoues. In addition to the interspecies comparison of known components, a lack of knowledge about subunits of the human enzyme is revealed by functional experiments. Besides its polymerase features, telomerase does possess exonuclease activity [74, 75]. A component of the enzyme which specifically carries out this function is not yet known.

Besides the search for additional enzyme components, future reseach will focus on biochemical properties of the subunits, especially the core components hTR and hTERT. Most of the three-dimensional features of hTERT and also of hTR are based on indirect evidence, like comparison with similar proteins in the case of hTERT or computer-based calculation and phylogenetic conservation of structures in the case

Table 3. Telomerase components from several species. Telomerase components that have been specifically associated with telomerases from model organisms and their known human homologs are shown.

	Tetrahymena thermophila	*Euplotes aediculatus*	*Saccharomyces cerevisiae*	*Caenorhabditis elegans*	Mouse	Human
Catalytic subunit (reverse transcriptase)	Tt-TERT	p123	EST2	(ce-TERT)	mTERT	hTERT
RNA component	*Tetrahymena* RNA component	*Euplotes* RNA component	TLC1	?	mTR	hTR
Telomerase associated	p80	?	?	?	mTEP1	hTEP1
proteins from selected	p95	?	?	?	?	?
species and their	?	p43	?	?	?	?
human homologs	?	?	EST1	?	?	?
	?	?	EST3	?	?	?
	?	?	EST4	?	?	?

of hTR. An important future goal of telomerase research will be the determination of the three-dimensional structure of the core components by the use of recombinant proteins and *in vitro* expressed RNA. These data will have a major impact on telomerase research in general and specifically in the search for telomerase inhibitors for cancer therapy.

5. References

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CHAPTER 3

MOLECULAR MECHANISMS REGULATING TELOMERASE ACTIVITY

JUN-PING LIU

Table of contents

Telomerase, Aging and Disease. Ed. by M. P. Mattson and T. K. Pandita. 33 – 59

1. Introduction

Telomerase, as a key determinant of telomere homeostasis, is subject to tight regulation during tissue development, organ formation, cellular aging and tumorigenesis. It is activated ubiquitously in the human embryo, down regulated with somatic tissue maturation and organ establishment shortly before (or just after) birth, and reactivated in cell renewal, tissue repair, organ regeneration and tumor development. The activity of telomerase entrains *de novo* synthesis of telomeric DNA. Acting at the single-strand overhang of 3' GT-rich telomeric DNA, telomerase polymerizes deoxynucleoside triphosphates from the 5' to 3' direction. Thus, telomerase activity constitutes an important mechanism in the maintenance and elongation of the telomere, an element important in safeguarding genomic stability by protecting against DNA exonuclease degradation and preventing chromosome fusion [1-6].

By maintaining telomere structure and genomic integrity [7-9] telomerase is involved in regulating many cellular processes. These include cell proliferation, senescence, renascence, immortalization and apoptosis, which are important in the genesis, development, normal functioning, regeneration and degeneration of human tissues and organs. Whereas activation of telomerase is associated with activation of immune cells, revitalization of bone marrow blood cells, reproliferation of senescent cells, immortalization of neoplastic cells and cancer development, inhibition of telomerase is implicated in apoptosis, premature aging and degenerative diseases [9-15]. The versatility of telomerase cellular functions in various cell types at various stages spanning birth to death promises the probability of multifactorial regulation of telomerase, in a time-dependent and cell-type specific fashion.

Although the mechanisms of telomerase regulation are incompletely understood, multiple pathways in the regulation of telomerase have begun to emerge from a variety of studies ranging from molecular cloning, genetic analysis and gene expression to protein modification, trafficking and subcellular localization. Telomerase activity is switched on and off, or modulated up and down, in line with tissue development and homeostasis under physiological conditions. In many abnormal circumstances, however, telomerase escapes from normal control mechanisms in response to factors intimately involved in various disease conditions. Centered on gene transcription and post-translational protein modifications and interactions, such factors include transcriptional factors, repressors, signaling molecules and products of oncogenes and tumor suppressors. They activate or inactivate telomerase at various levels, by influencing gene expression, protein structures, trafficking, and assembly and disassembly of telomerase components.

This chapter sets out to summarize the current status of telomerase regulation, and to provide a general outline with a number of working hypotheses, to stimulate further study of molecular mechanisms controlling telomerase activity. A fuller understanding of the mechanisms regulating telomerase activity will in turn allow manipulation of telomerase for prophylactic and therapeutic ends. As our knowledge increases, telomerase may thus become an increasingly attractive target for drug development, particularly in the treatment of cancer and other proliferative disorders, and in the fields of aging and degenerative disease.

2. Structural considerations in telomerase regulation

Consistent with multifactorial regulation, telomerase has a complex structure comprising at least three components: the catalytic subunit telomerase reverse transcriptase (TERT), telomerase-associated protein 1 (TEP1) and telomerase RNA subunit (TR). This overall structure is conserved from unicellular eukaryotes to mammals. TERT was firstly purified as a 120 kDa protein in association with telomerase activity and a 43 kDa La motif RNA-binding protein from the ciliate *Euplotes aediculatus* [16, 17], then identified to be homologous with the yeast protein Est2p [18]. The *EST* (ever shorter telomeres) gene in yeast was initially discovered by genetic analysis of a mutation causing massive loss of telomeric DNA and cellular senescence [19]. On the basis of the integral function of Est2p in telomerase activity [18, 20] and its homology with *Euplotes* p123 [18], TERT has been characterized structurally by independent laboratories in human [21-25], mouse [26], and the ciliates *Tetrahymena thermophila* and *Oxytricha trifallax* [27].

The human TERT (hTERT) is a polypeptide of 1132 amino acid residues with a molecular mass of 127 kDa and a pI of 11.3 (Figure 1). Sequence analysis and comparison with p123, Est2p and Trt1p (a telomerase catalyst in the fission yeast *Schizosaccharomyces pombe*) indicate that hTERT shares significant amino acid sequence similarity to the telomerase catalytic subunits from lower eukaryotes (46-49%) and mouse (90%) in various conserved regions [27]. All these telomerase catalytic subunits possess a unique conserved region called the T-motif that is centrally located and N-terminal to the seven-conserved reverse transcriptase motifs (Figure 1). Deletion of the T-motif (^{560}FFYVTE^{565}) or single amino acid mutation (such as F561A) markedly reduces telomerase activity [28] and TR binding [29], suggesting a critical function of the T-motif for interacting with TR [29]. In addition, another telomerase specific structure, the T2 motif, has recently been identified in the extended N-terminal region [30]. Selective mutations of single amino acid residues conserved in the T2 motif of TERT (F158A, Q168A or Q168N) of *Tetrahymena* [30], or in the reverse transcriptase motif 1 (K626A), motif 2 (R631A), motif A (D712A or Y717A) or motif C (D868A, D869A) [25, 28, 31], also abolish telomerase activity. However, while selective mutation in the reverse transcriptase motifs impairs TR template use [30], it has little effect on the binding of TR [29]. These findings therefore suggest that the telomerase specific T and T2 motifs play essential roles in binding to TR with specificity and stability, whereas the reverse transcriptase motifs contribute to the formation of a catalytic active site in the telomerase complex which reversibly interacts with the template [29, 30]. From the crystal structure of p66 subunit in HIV-1 reverse transcriptase, the active site of telomerase may be contained in a TERT quaternary structure similar to a right hand cupped with the RNA template sequence a mosaic in the cleft of thumb, palm and fingers [21].

Compared with the retroviral and retrotransposon consensus sequences, the reverse transcriptase motifs of TERT are significantly larger with long inter-motif sequences, a conserved arginine residue in motif 1 and an aromatic residue (tyrosine or phenylalanine) downstream of the two critical aspartates in motif C [21]. These structures suggest that the active site of TERT may be flexible in interacting with the RNA template and substrate DNA, possibly conferring its unique ability to translocate many times to

the beginning of its template region and thus synthesize multiple DNA repeats on a single primer [32]. In addition, unlike retroviral reverse transcriptases, TERT has an extended N-terminal region upstream of T2 and between the T2 and T motifs, plus a longer C-terminal tail. Mutagenesis studies suggest that the N-terminal extension of *Tetrahymena* TERT allows high affinity RNA binding and 5' template definition [30]. The N-terminal region between the T2 and T motifs of hTERT is proline- and arginine-rich, and contains a putative SH3-binding motif and multiple potential serine/threonine phosphorylation sites [33-36]. It is possible that this proline-rich basic domain of hTERT may also provide an interface for interactions with other signaling molecules, controlling hTERT interactions with hTR and catalytic activity. In terms of the C-terminal extension, tagging with HA epitopes inhibits telomere and cell lifespan extension without affecting telomerase activity *in vitro* [37-39], and targeted amino acid mutations (T1030A, S1037A and S1041A) eliminate hTERT nuclear localization [40]. These findings suggest that the C-terminal region is involved in regulating TERT telomeric DNA targeting. TERT may thus have evolved to incorporate specialized structures to respond to cellular signaling and meeting additional needs in order to convene its reverse transcriptional activity in telomere homeostasis.

Interacting with both TERT and TR [23, 31, 41-44], TEP1 was identified and cloned by sequence homology with its analogue p80 that co-purified with telomerase activity and a p95 protein in *Tetrahymena thermophila* [41]. TEP1 is considerably larger than p80 in man, with 2627 predicted amino acid residues that produce a modular structure of several domains including a large number of WD-40 repeats implicated in protein-protein interactions. Like *Tetrahymena* p80, human TEP1 (hTEP1) binds to human telomerase RNA (hTR) [44], suggesting an involvement of the p80 homology domain. Furthermore, hTEP1 co-immunoprecipitates with hTERT [23, 36, 44]. Interestingly, deletion of TEPI in mouse does not affect telomerase activity [45]. Thus, whether or not TEP1 may play a role in processing and coordinating the tertiary and quaternary structures of telomerase components, and/or serve as a scaffold in recruiting telomerase regulatory factorsin human requires further study.

Recently, three other proteins, dyskerin [13], hStau and L22 [46], have been identified to interact with TR in human cells. Dyskerin with putative pseudouridine synthase activity binds to the H/ACA RNA motif of TR [13]. Mutation of dyskerin is associated with decreased TR, reduced telomerase activity and compromised telomere maintenance, which may limit the proliferative capacity of human somatic cells in epithelia and blood [13]. On the other hand, hStau and L22 co-immunoprecipitate with TR, hTERT and telomerase activity, suggesting that both proteins are associated with the telomerase complex. However, hStau and L22 are more abundant than TERT and located in both nucleolus and cytoplasm; neither protein is associated with the other, or with hTEP1, suggesting that there may be different complexes of telomerase with different components under different conditions. It is also possible that hStau and L22 interact with other ribonucleoprotein complexes as well, and by binding to TR may play a role in TR processing, telomerase assembly, or localization *in vivo* [46].

Although multiple proteins are found within the large ribonucleoprotein complex of telomerase, only a single TR is found to operate as telomeric DNA template in a given species [3, 4, 47-49]. This RNA moiety of telomerase has been characterized in

a variety of species including ciliates, yeast and mammals and is indispensable for the enzymatic function of telomerase [50]. Ciliate TR is 150-190 nucleotides transcribed by RNA polymerase III, whereas the TR from budding yeasts is ~1, 300 nucleotides transcribed by polymerase II [51]. Mutagenesis in TR templates from these species suggests that bases substituted in the template RNA are usually copied into the telomeric DNA products. Complete replacement of the nine base *Tetrahymena* TR template with non-telomeric sequences does not ablate telomerase polymerization activity, and these nucleotides from non-telomeric templates are faithfully copied onto telomeric primers, reflecting correct dNTP selection and insertion probably in turn reflecting steric constraints [52]. However, template mutations also cause specific changes in telomerase properties, including impaired enzyme processivity and translocation [52]; a specific triplet substitution in the yeast *S.cerevisiae* TR template induces complete loss of telomerase activity [53]. Interspecies substitution of telomerase RNA with an identical template base sequence from the ciliate *Glaucoma chattoni* into *Tetrahymena thermophila* cells produces a functional but aberrant telomerase [54]. These results suggest that specific template RNA nucleotides play an active role in enzymatic activity, beyond providing a template for nucleotide assembly, through base-specific interactions with other residues within the telomerase complex [52].

Human telomerase RNA (hTR) is transcribed by pol II [55] and is 445 nucleotides long with an eleven nucleotide template sequence (5'-CUAACCCUAAC-3') coding for the telomere repeats of (TTAGGG)n [56]. Its larger size relative to ciliate TRs is due to the presence of an extra domain resembling a box H/ACA small nucleolar RNA (snoRNA), which is essential for hTR accumulation, 3'-end processing and telomerase activity [57]. *In vitro* mutagenesis studies also suggest that the region required for minimal function is located between residues 44-203, with mutations between residues 170-179, 180-189 or 190-199 inhibiting both templating function and telomerase activity [31, 58]. Thus, besides serving as a template for reverse transcription in telomeric DNA elongation, hTR is also involved in the enzyme active site, probably by specific nucleotides interacting with structural components of the DNA substrate primer and protein subunits [59]. Removal or down-regulation of the RNA subunit leads to inhibition of telomerase, erosion of telomeres, compromise of growth capacity of highly proliferative embryonic stem cells [60], testicular cells and haematopoietic cells [61] in the mouse, and death of both cultured HeLa cells [47] and malignant human glioma cells [62]. Thus, telomerase shows structural and functional specialization of multiple components and of multiple motifs in each component. These structures may be used in executing telomerase elongation of telomeric DNA and subject to molecular regulation. It may therefore be possible to selectively inhibit telomerase by targeting these different motifs within the components with specificity and acceptable toxicity.

3. Telomerase catalytic subunit (TERT) gene expression

Many lines of evidence indicate that telomerase is reversibly regulated, and that this regulation in many cases involves differential gene expression. For example, telomerase activity increases during neurogenesis and decreases as the cortex matures [63, 64].

Deletion of TR in mouse results in defects in telomere function and in brain neural tube closure [65]. Resting lymphocytes express little telomerase activity, stimulation of specific antigen receptors on the cell plasma membrane markedly increases telomerase activity and telomere length and removal of telomerase compromises clonal lymphocyte expansion [66-68]. Lengthy high-level exposure of normal human skin to the sun results in increased telomerase activation [68]. Bombarding Chinese hamster cells with UV [70], human haematopoietic cells with γ-rays [71], immortalized human bronchial epithelial cells with radon stimulated α particles [72], or human carcinoma cell lines with X-rays [73] induces activation of telomerase. Approximately 85% of human cancers express telomerase activity, while no telomerase activity is present in most normal human somatic cells [74, 75]. Activated telomerase in cancer cells is repressed when the cells exit the cell cycle and become quiescent [76-78]. Treatment with an antagonist of growth hormone-releasing hormone dramatically decreases telomerase activity in xenografted U-87MG human glioblastoma cells [79].

The activation, inactivation, and up- and down-regulation of telomerase have been suggested to be highly related to hTERT gene expression. The expression of TERT is normally repressed in most human somatic tissues after birth [21, 22, 80, 81], but becomes activated in most tumors and immortal cell lines tested to date [21, 22, 82-84]. This pattern of hTERT expression is in marked contrast to those of hTR and hTEP1, which are widely expressed in normal human tissues [43, 44, 47, 49, 83-85]. Ectopic expression of hTERT in telomerase-negative cells is sufficient to reconstitute telomerase activity [25, 28, 37, 86-88], to elongate telomeres [86, 87], and to extend some cellular lifespans [37, 86, 87]. Deletion of TERT in mouse embryonic stem cells induces genomic instability, aneuploidy and telomeric fusion [88]. Thus, correlating with telomerase activity and cellular function, *de novo* activation of hTERT gene transcription in neoplastic and immortalized cells appears to be a dominant, rate-limiting step in telomerase activation, potentially regulated by changes in the levels and functions of multiple transcription factors, repressors, oncogenes and tumor suppressor gene products (Figure 2).

3.1. The organization of the hTERT gene

With the use of hTERT cDNA probes, the hTERT gene has been cloned from human genomic libraries and shown to comprise more than 35 kb DNA at chromosome 5p15.33 (gdbwww.gdb.org) to code for an RNA including 16 exons and 15 introns [90, 91]. This intron-exon arrangement for hTERT is comparable to that for *Tetrahymena thermophila* TERT (18 introns) and *Schizosaccharomyces pombe* TERT (15 introns), but dissimilar to the TERT genes in *Oxytricha trifallax*, *Euplotes aediculatus* and *Saccharomyces cerevisiae* which contain no introns [27]. Although the significance of these differences in TERT gene structure between eukaryotes is still an open issue, the coding of hTERT sequences in 16 exons separated by 15 introns is consistent with the presence of various hTERT isoforms [24], and with the possibility of complex regulation of hTERT gene expression and function in mammals. Examination of the hTERT exon/intron junctions suggests that all hTERT variants with insertions and/or deletions described to date derive from alternative splice consensus sequences (GT/AG)

[90, 91]. The mechanism underlying these alternative-splicing events for various hTERT messages is still unknown.

By unidirectional deletion of the 5' flanking region of the hTERT gene and expression of these structures in a luciferase reporter assay, the promoter for hTERT gene transcription and the regulatory elements in mediating the promoter activity have been shown to lie within a region extending to 330 bp upstream of the translation start site (-330) to the second exon of the gene (+228) [90], with a region of 59 bp (-208 to -150) required for maximal promoter activity [92]. It cannot be ruled out, though, that regions further upstream of -330 contain additional promoter and enhancer activity *in vivo*. The promoter of the hTERT gene is GC-rich, forming a dense 5'-regulatory CpG island that is often found in the vicinity of the promoters of many genes as potential targets for repression via DNA methylation [93]. CpG islands are normally methylation free in animal cells, whereas *de novo* methylation of cytosine residues in 5' regulatory CpG islands in postembryonic cells can lead to transcriptional repression [93]. Interestingly, *de novo* methylation of CpG islands is associated with aging and tumorigenesis, and has been found to be a mechanism of transcriptional repression of several tumor suppressor genes including Rb and $p16^{INK4\alpha}$ which may be inhibitory to telomerase (see below).

Whether or not methylation of the CpG island in the hTERT gene may be involved in hTERT gene repression has recently been explored. The data show that the hTERT CpG island is not methylated in primary tissues and cultured cells, suggesting that CpG island methylation is not responsible for hTERT repression in telomerase-negative cells [94, 95]. In many tumors, however, the CpG island is methylated. Although the levels of methylation do not correlate with the levels of hTERT gene expression, demethylation of DNA with 5-azacytidine in two cell lines induced hTERT gene expression [95]. These findings therefore suggest that cytosine methylation is not the primary mechanism of hTERT repression in normal telomerase-negative cells, but is involved in silencing hTERT gene expression in tumors, probably as a negative feedback regulatory mechanism of telomerase activation. The mechanisms of DNA methylation-induced gene repression are not fully understood, but it is generally held that DNA methylation inhibits transcription by interfering with transcription initiation, and mediating the recruitment of other molecules including transcriptional repressors and histone deacetylases [93]. Consistently, pharmacological inhibition of histone deacetylation by inhibiting histone deacetylases with Trichostatin A (TSA) induces activation of hTERT promoter activity and activation of telomerase in telomerase negative cells [96]. Thus, histone deacetylation is involved in silencing hTERT gene transcription in human cells, which may induce more compact nucleosomal packing and chromatin condensation-mediated exclusion of transcriptional factors and/or recruitment of transcriptional repressors [96]. Together, these data suggest that DNA demethylation and methylation, probably in association with histone acetylation and deacetylation, is an important mechanism in the potentiation and depotentiation of hTERT gene responses to transcription factors, by inducing DNA decondensation and condensation, respectively.

The hTERT promoter features no TATA or CAAT boxes. By the CapSite Hunting method, the transcription start site has been shown to be within 78 base pairs upstream

of the translational start site [82, 92]. Expression of the hTERT gene promoter in various types of cells shows that minimal telomerase gene promoter activity is present in normal primary cells, whereas high levels of activity are detected in telomerase positive embryonic cells, immortalized cell lines and cancer cells [82, 90, 92]. These findings of promoter activation coinciding with telomerase activity and cell immortalization suggest that the regulation of telomerase activity is primarily at the transcriptional level, reinforcing the hypothesis of primarily transcriptional mechanisms for repression and activation of the enzyme [21, 22, 24].

Several transcription factor and repressor binding sites have been identified in the hTERT promoter (Figure 2). Notable among these are two E-boxes for the Myc oncogene, several GC-boxes as Sp1 binding sites, and the *cis*-regulatory elements that may recruit AP1, AP2, AP4, ATF, CREB, ER, PR, GC, IK2, MYOD, NF1, T3Rα and USF [90, 92, 97, 98]. Thus, apart from having a 5'-regulatory CpG island and possible regulatory structures for DNA methylation and histone deacetylation, the TERT 5' flanking region provides a platform for transcription regulatory proteins to bind both directly and indirectly, placing the TERT gene squarely as a transcriptional target under the sway of multiple transcriptional factors.

3.2. Transcriptional activation of hTERT gene

The hTERT promoter is inactive in normal human somatic cells, but becomes activated during cell immortalization [90]. Sequence analysis has shown that the hTERT promoter contains binding sites for multiple transcription factors, suggesting that hTERT expression may be subject to multiple levels of control and regulated by different factors through cooperative interactions (Figure 2) [90, 91].

3.2.1. *c-Myc*

The presence of two E-boxes is consistent with the findings that Myc induces hTERT gene expression and that this is mediated by the evolutionarily conserved E-box (CACGTG) [82, 92, 97-101]. Expression of hTERT in both normal human mammary epithelial cells and normal human diploid fibroblasts is stimulated by c-Myc [99], an effect attributed to direct interaction of c-Myc with the hTERT promoter [92, 97, 100]. Treatment of human leukemic cells with antisense pentadecadeoxynucleotides against c-Myc mRNA leads to inhibition of telomerase activity [102]. Furthermore, the c-Myc-induced increase in hTERT transcription may occur through local retrieval of c-Myc to the hTERT gene promoter, since inhibition of protein synthesis by cycloheximide does not block c-Myc activity in hTERT gene transcription [103].

These findings of c-Myc activation of hTERT gene transcription are consistent with the oncogenic functions and deregulation of c-Myc in various human tumors. c-Myc induces accelerated passage through G1 of the cell cycle, rendering primary cells independent of growth factors and thus immortalized [104, 105]. Although overexpression of c-Myc inhibits differentiation and elicits transformation of primary cells in cooperation with activated ras, c-Myc also induces apoptosis. The multifaceted functions of c-Myc are believed to involve complex regulation of c-Myc action as a transcription factor by its

binding proteins including Max, TFII.1, YY1 and Mitz, and by the sequences adjacent to and flanking the E-box of particular targeted genes [104, 105]. The complexity of c-Myc activation of hTERT gene expression is reflected by the findings that c-Myc up-regulates telomerase activity but fails to overcome telomerase down-regulation induced by herbimycin A in human leukemia cells [106]. In addition, up-regulation of telomerase activity by c-Myc can be partially inhibited by mutating the Sp1 binding site of the GC-box [107], and completely eliminated by the Mad transcriptional repressor (see below) (Figure 2) [103]. Thus, c-Myc may be a primary transcription factor that may also interact with other transcription factors (such as Sp1) and repressors (such as Mad) in the regulation of hTERT gene transcription.

3.2.2. *Sp1*

Sp1 is a general transcription factor that binds to the GC-boxes in promoters, enhancers and locus control regions to activate housekeeping, tissue-specific and cell cycle-regulated genes, and to prevent methylation of CpG islands [108]. Structure and activity analysis of the hTERT promoter shows that both the E-box and the GC-box are important for initiation of hTERT gene transcription, with deletion of either element repressing hTERT gene promoter activity [107]. While Sp1 expression parallels that of c-Myc and hTERT, and cellular immortalization, overexpression of Sp1 results in significant increases in hTERT gene transcriptional activity [107]. Moreover, co-transfection of hTERT promoter with Sp1 in Sp1-negative cells also leads to activation of hTERT gene transcription blocked by expression of wild type p53 (see below) (Figure 2) [109]. Since Sp1 is ubiquitously expressed and implicated in activating genes involved in various cellular processes including cell cycle regulation, chromatin remodeling and the propagation of methylation-free islands [110] and since Sp1 mutation retards c-Myc stimulated hTERT gene transcriptional activity [107], it might be possible that Sp1 plays a constitutive priming or permissive role in regulating the sensitivity of hTERT gene transcription to c-Myc. Since Sp1 is expressed in the same cells and shares indistinguishable DNA-binding specificity with Sp3 which acts as a repressor [108], it would also be of interest to determine possible interactions of these proteins in the regulation of hTERT gene transcription.

3.2.3. *Steroid hormones*

The sex steroid hormones (estrogen and progesterone) regulate hTERT gene expression via classical hormone receptors. An estrogen response element (ERE) is present in the hTERT promoter, and 17β-estradiol (E2) treatment of ER positive cells for 3 hours is followed by binding of the estrogen receptor ERα to the ERE region, a significant increase in hTERT promoter activity and activation of telomerase in telomerase-negative human ovary epithelial cells [111]. Similarly, in ER- and telomerase-positive human breast cancer cells, E2 treatment also up-regulates hTERT gene expression and telomerase activity [112]. In addition to the direct action on the hTERT promoter, estrogen and progesterone also regulate telomerase activity indirectly via distinct signaling pathways. E2 stimulates c-Myc gene expression and induces an additive c-Myc

interaction with the E-box of hTERT promoter in human breast cancer cells, suggesting that estrogen may activate telomerase gene expression via both direct and indirect interactions with the hTERT promoter [112]. Moreover, the anti-estrogen tamoxifen inhibits telomerase activity at a concentration of 10^{-8} M in cultured MCF-7 and MDA-MB-231 breast cancer cells [113]. In contrast, progesterone modulates hTERT gene expression through activating mitogen-activated protein kinase [114]. Progesterone treatment of breast and endometrial cancer cell lines positive in progesterone receptor induces significant increases in hTERT mRNA expression within 3 hours, which is followed by inhibition after 12 hours by a mechanism involving $p21^{WAF1/Cip1}$ (a universal cyclin-dependent kinase inhibitor) and mitogen-activated protein kinase signaling pathways [114].

3.2.4. *Others*

Up-regulation of telomerase has also been reported to be associated with introduction of the human papillomavirus (HPV) type-16 E6 protein in early-passage human keratinocytes and mammary epithelial cells [115, 116], with expression of the oncogenes SV40 or v-Ki-ras in human prostate epithelial, prostate endothelial or umbilical vein fibroblast cells [117, 118], and with overexpression of Bcl-2 in human cancer cells [119] and rat pheochromocytoma cells [120]; the mechanisms of telomerase up-regulation by these proteins remain to be explored.

3.3. Transcriptional repression of hTERT gene

The identification of telomerase gene repressors has been of central interest in our investigation of how to rein in telomerase activity in cancer. A number of studies have shown that hTERT repression activity is encoded in chromosome 3 (but not chromosomes 7, 8, 11, 12 or 20) [121-125]. Introduction of chromosome 3 into telomerase positive cell lines, human renal carcinoma [122, 123] or breast cancer cells [124], induces repression of hTERT expression, down-regulation of telomerase activity, up-regulation of telomere shortening and cessation of cell growth. The putative telomerase repressor gene has been further mapped to chromosome region 3p14.2-p21.3 [123, 126]. Also consistent with the presence of telomerase repressor(s) is the finding that hTERT gene expression is repressed upon cell becoming quiescent [76-78] or differentiated by 12-*O*-tetradecanoylphorbol-13-acetate (TPA) [127, 128] or nerve growth factor [120].

3.3.1. *Mad*

Mad (for Max hybridization) has recently been suggested to be a hTERT repressor by independent studies [103, 107, 128]. Oh et al. co-transfected 293T cells (an immortalized human kidney cell line expressing the SV40 large T antigen) with a luciferase reporter gene under the control of a 3396 bp hTERT promoter and variously pooled fractions of expression library plasmids containing the SV40 origin of replication [103]. This expression cloning approach effectively pins an inhibitory activity of hTERT

promoter on ectopic Mad [103]. Transient overexpression of Mad1 results in consistent and significant decreases in hTERT promoter activity. This inhibition of hTERT gene expression by Mad is dependent upon the integrity of the evolutionarily conserved E-box, as deletion or mutation of the E-box significantly alleviates the repression of the hTERT promoter activity [103, 128]. Consistent with the transcriptional silencing of hTERT by Mad, there is an increased expression of Mad in human tumors and decreased expression of Mad in normal somatic tissues and non-transformed cells, or during differentiation of immature cells, in parallel with hTERT gene expression [103, 128]. Thus, Mad may operate as an important hTERT gene transcription repressor.

Since both c-Myc and Mad can form heterodimers with Max that bind to E-boxes, Oh et al. examined the effects of c-Myc/Max and Mad/Max on hTERT promoter activity in both mortal and immortal cells by co-transfection of c-Myc and Mad [103]. While Mad significantly represses hTERT promoter activity in immortal cells, co-transfection with c-Myc competitively inhibits the effect of Mad in a concentration-dependent manner. Conversely, c-Myc activates hTERT promoter activity in mortal cells, activation which is inhibited by Mad in a concentration-dependent fashion [103]. Furthermore, activation of endogenous hTERT expression by inducible c-Myc is also repressed by ectopic expression of Mad [103]. These observations suggest that Mad may sequester Max from c-Myc, thereby conferring a negative regulation *in trans*, and that an imbalance between c-Myc and Mad may play a role in determining the ultimate activation or inhibition of hTERT gene transcription (Figure 2).

In addition to binding to Max in competition with c-Myc/Max for the E-box of hTERT gene promoter, Mad has also been suggested to actively repress its target genes by recruiting the mSin3 repressor and histone deacetylase complexes to the promoter [104]. With a Mad1 mutant lacking the mSin3 interaction domain (SID), Gunes et al. show that in the absence of SID, Mad1 repressor activity is abolished and the hTERT promoter activity elevated, reflecting a possible displacement by the mutant of endogenous repression activity of Mad1, thereby suggesting that Mad1 repression of the hTERT promoter involves an active repression mechanism rather than simple competition with c-Myc [128]. Consistently, Cong and Bacchetti have recently provided evidence that inhibition of histone deacetylases by TSA activates telomerase in telomerase-negative cells and Mad repression of the hTERT promoter requires histone deacetylase activity, further suggesting a possible mechanism of chromatin condensation by Mad recruited histone deacetylases in the repression of hTERT gene expression [96]. Thus, histone deacetylation may be a major mode of transcriptional silencing of hTERT gene by the Mad proteins, given their overlapping binding sites with those for c-Myc within the hTERT gene promoter.

3.3.2. *p53*

In addition to Mad, recent studies have shown that the tumor suppressor protein p53 is involved in suppressing hTERT gene expression. Introduction of wild type p53 into tumor cells by recombinant adenovirus leads to a significant depression of hTERT mRNA and telomerase activity [129, 130]. Activation of endogenous p53 also significantly represses hTERT promoter activity [109]. Mechanistic studies show

that p53 repression of hTERT gene expression is mediated by direct interaction of p53 with Sp1, independent of p53 transcriptional effect on $p21^{WAF1/Cip1}$ [109, 130]. Full-length wild type p53 forms a complex with Sp1 and blocks Sp1 binding to the hTERT promoter [109]; mutations of Sp1 binding sites inhibit p53-induced repression of hTERT transcriptional activity [109, 130]. In addition, although $p21^{WAF1/Cip1}$ gene expression is not required for p53-produced inhibition of hTERT gene transcription [109], overexpression of $p21^{WAF1/Cip1}$ induces suppression of telomerase activity in human immortalized keratinocytes [131] and human glioma cell lines [132]. The function of $p21^{WAF1/Cip1}$ in reducing telomerase activity may be mediated by down-regulation of hTR gene expression [131]. Thus, both hTERT and hTR may be subject to regulation by p53 signaling pathways at the level of gene expression. Furthermore, given that p53 also inhibits telomerase activity directly by protein-protein interaction (see below), these data are consistent with the notion that p53 may regulate telomerase activity via multiple mechanisms, both directly and indirectly.

3.3.3. *Others*

Another tumor suppressor involved in telomerase expression is the Wilms tumor supressor gene [133]. Using the expression cloning approach, the Wilms tumor 1 suppressor gene product (WT1) is found to bind the hTERT promoter DNA and repress hTERT promoter activity in 293 kidney cells. Stepwise 5' deletion of the hTERT promoter identifies a WT1 binding site between -307 and -423, and alteration of the binding site significantly derepresses transcription from an isolated hTERT promoter by inhibiting WT1 interaction with DNA [133]. Reestablishment of expression of the retinoblastoma protein (Rb) in $Rb^-/p53^-$ tumor cells is associated with telomerase inhibition and cellular senescence [134]. Overexpression of full length Rb in human squamous carcinoma cell lines also results in down-regulation of telomerase activity [135]. Consistently with this, inhibition of $p16^{INK4a}$ which inhibits Rb phosphorylation and inactivation is associated with telomerase activation in human keratinocytes [116, 136, 137]. These data suggest that the Wilms and the Rb tumor suppressors are both involved in negative regulation of telomerase activity at the levels of gene expression under particular conditions.

In searching for other negative regulatory elements in the hTERT promoter, Fujimoto et al. reported a 400 bp silencer for the hTERT gene between -776 and -378 bp upstream of the proximal core promoter, and multiple binding motifs for myeloid-specific zinc finger protein 2 (MZF-2) within the silencer [138]. Gel shift assays show that MZF-2 proteins specifically bind these sites. Overexpression of MZF-2 in cells leads to down-regulation of hTERT transcription and telomerase activity, whereas mutations of these sites induce significant activation of hTERT transcription [138]. Moreover, retinoic acid has also been reported to down-regulate hTERT gene expression and telomerase activity in some cancer cells by unknown mechanisms [139-141]. Since telomerase is activated in most cancers, it is possible that other repressors which remain to be identified are also involved in the repression of hTERT gene.

Thus, telomerase activity is regulated at the transcriptional level by multiple transcription factors, repressors and silencers, which act on the activity of hTERT

gene promoter and the timing of hTERT gene transcription. Given the numbers and complexity of these transcription factors, repressors and silencers that have hitherto been found, it is probable that these molecules operate in a coordinated manner to regulate hTERT gene transcription during cell development, aging and tumorigenesis. Mutations of the hTERT gene, particularly at the sites of action of these nuclear factors, may result in altered structures of the transcriptional machinery, and altered and/or disordered protein-protein and protein-DNA interactions, ultimately leading to an unbalanced regulatory activity between transcriptional factors and repressors skewing towards aberrant gene transcription activation or suppression. In addition, altered expression of the genes coding for these nuclear regulatory factors, structural modifications of the proteins, and their availability to the functional sites in the hTERT gene promoter, may be regulated by multiple signaling pathways in response to extracellular environmental changes under various conditions, and thus may contribute additional layers of complexity to the regulation of hTERT gene transcription.

3.4. Alternative splicing of TERT mRNA

Adding to the complex regulation of hTERT gene transcription, several alternative splicing variants of hTERT mRNA have been shown in various tissues and cell lines [24, 81, 91, 142, 143]. These hTERT variants include hTERTα with an in-frame deletion of 36 bp (bases 2186-2221) from the reverse transcriptase domain A, hTERTβ with a 182-nucleotide deletion (bases 2342-2524) resulting in a reading frameshift with premature stop codon, and another variant encoding an alternative C-terminus [24, 142]. Since none of these intra-mRNA deletion events produces active telomerase, the alternative splicing variants may compete with or substitute for full-length hTERT to modulate telomerase activity during tissue and organ development in man [28, 143]. For instance, in 20 ovarian serous papillary adenocarcinomas, two of three telomerase-negative tissues showed expression of hTERTβ [143]. Human tissues expressing hTERT message with complete reverse transcriptase motifs also express one or more spliced variants of hTERT message; normal adult ovarian stroma express both hTERT and hTERTb without detectable telomerase activity [143]. Alternative splicing of hTERT mRNA may thus constitute one means of regulating telomerase activity during development and tumorigenesis. What remain to be determined are the mechanisms of action of the splice variant proteins, and whether or not the telomerase activity-dead hTERT isoforms have other cellular functions.

4. Post-translational modification: protein phosphorylation and dephosphorylation

Considerable evidence suggests that telomerase activity is also controlled by post-translational mechanisms [144, 145]. For example, the decline of telomerase activity in confluent NIH 3T3 fibroblasts has been shown not to reflect significant down-regulation of hTERT gene expression [26]. Similarly, up-regulation of telomerase activity in various normal or neoplastic cell types is independent of concomitant changes in TERT

or TR gene expression [63, 146]. On the other hand, levels of expression of hTERT mRNA appear incompatible with the levels of telomerase activity in multiple subsets of human lymphocytes from thymus, tonsil and peripheral blood [147]. Furthermore, the majority of normal ovarian tissues and uterine leiomyomas express both hTERT and hTR but display no telomerase activity [143]. Thus, under certain conditions, telomerase activity is controlled beyond hTERT gene expression; the possible mechanisms underlying the dissociation of hTERT gene expression from telomerase activity may involve post-translational protein modification and/or protein-protein interactions (Figure 3).

4.1. Protein kinase C and protein phosphatase 2A

Post-translational protein modifications include serine/threonine/tyrosine phosphorylation, proline/lysine hydroxylation, asparagine/serine/threonine glycosylation, cysteine palmitoylation and N-terminal glycine myristoylation. Since protein phosphorylation is the post-translational mechanism most commonly seen in reversible control of protein structure and function [148], the roles of protein phosphorylation in telomerase activity have been extensively studied by several laboratories. First, in cultured human breast cancer cells (or with partially purified telomerase from affinity chromatography), dephosphorylation by protein phosphatase 2A (PP2A) inhibits telomerase activity [144]. Likewise, in human melanoma cell lysates, telomerase is also inhibited by PP2A [35]. The inhibition appears to be mediated by protein dephosphorylation, in that the non-specific protein phosphatase alkaline phosphatase mimics, and the PP2A inhibitor okadaic acid inhibits, the effect; the other two major protein phosphatases (protein phosphatase 1, protein phosphatase 2B) are without effect. These findings indicate that dephosphorylation locks telomerase into an inactive conformation, with protein phosphorylation able to restore telomerase activity [144]. An allosteric mechanism has thus been proposed in which telomerase holoenzyme exists in two different configurations, which can be switched on and off by reversible phosphorylation and dephosphorylation (Figure 3 and 4) [145].

On affinity chromatography using peptide sequences derived from hTERT and hTEP1, no binding of cdc2 kinase, casein kinase IIα and ERK was seen. In contrast, significant binding of protein kinase Cα (PKCα), but not PKCβ1, PKCε, or PKCζ, was detected, suggesting the possible involvement of PKC in regulating telomerase structure and function [36]. Immunoprecipitation studies show that both hTERT and hTEP1 are phosphoproteins, dephosphorylated by PP2A and re-phosphorylated by PKCα [36]. Furthermore, analysis of telomerase activity shows that purified recombinant PKCα markedly stimulates basal and PP2A-inhibited telomerase activity in an ATP-dependent manner, suggesting that PKC-induced phosphorylation is involved in telomerase activation during the assembly of telomerase holoenzyme into a functionally active configuration [35, 36]. In addition, while cdc2 kinase and DNA-dependent protein kinase have no effect, PKCδ, PKCε and PKCζ are each capable of reactivating telomerase post PP2A dephosphorylation. Given that PKC has multiple isoforms expressed in various cell types with more than 100 substrates identified [149], it is still unclear whether or not different protein kinase C isoforms might play particular roles in the regulation of telomerase activity in different types of cell under distinct conditions [36].

In cultured peripheral blood mononuclear cells, the PKC activator phorbol myristate acetate (PMA) stimulates telomerase activity, and the increased telomerase activity during T cell activation is inhibited by the PKC inhibitor bisindolylmaleimide [150]. Similarly, the PKC inhibitor bisindolylmaleimide I also inhibits telomerase activity in cultured nasopharyngeal cancer cells [151]. Given these reports, and the finding that okadaic acid stimulates [144] and bisindolylmaleimide I inhibits (unpublished observation) telomerase activity in cultured human breast cancer cells, it is possible that PKC and PP2A are more generally involved in reciprocally controlling telomerase activity through protein phosphorylation and dephosphorylation in neoplastic cells. Such a reciprocal effect of PKCα and PP2A on telomerase activity in human breast cancer cells is highly consistent with the findings that PKCα activity is markedly elevated [152-155] and PP2A inhibited [155-157] in the nucleus of human breast cancer cells, in response to various oncogene products and tumor promoting compounds. In turn, this would be consistent with the notion that a balance between PKC activation [149, 158] and PP2A inhibition [155-157] plays an important part in tumorigenesis (Figure 4) [145].

4.2. Protein kinase B (Akt)

Studies have also shown that besides PKC, protein kinase B (PKB or Akt) is involved in up-regulating telomerase activity [35]. *In vitro*, PKB phosphorylates the hTERT peptide ^{817}AVRIRGKSYV^{826} and stimulates telomerase activity. Treatment of human melanoma cells with okadaic acid stimulates both hTERT peptide phosphorylation and telomerase activity, whereas treatment of the cells with PI3 kinase inhibitor Wortmannin inhibits phosphorylation and telomerase activity. Thus, the pathways of PKCα and PKB converge on the same output to promote telomerase activation. However, the actions of PKCα and PKB may be on different sites and mediate different signal transduction, representing responses to different extracellular stimuli. For example, the serine residue at 824 of hTERT may be phosphorylated by PKB in human cancer cells in response to growth factor activation of the PI3 kinase pathway [35]. Since PKB phosphorylates Bad, inducing Bad association with 14-3-3, and thus preventing Bad participation in apoptosis to allow cell survival, PKB may thus act on multiple molecular targets including telomerase in the processes of apoptosis, cell survival and proliferation during aging and tumorigenesis. Although PKB and PKCα signaling pathways may interact under particular conditions, it is yet to be shown whether or not both mechanisms are required in telomerase activation in the same cell, and whether or not the two mechanisms are inter-dependent and/or synergistic in telomerase activation and regulation in cancer cells (Figure 4).

4.3. Tyrosine kinase c-Abl

Recently, Kharbanda et al. have shown that the ubiquitously expressed tyrosine kinase c-Abl associates with hTERT leading to hTERT tyrosine phosphorylation and telomerase inhibition [34]. They suggest that c-Abl through its SH3 domain interacts with the proline-rich motif (^{308}PSTSRPPRP^{316}) of hTERT and induces tyrosine phosphorylation

in response to ionizing radiation in human breast cancer cells. Transient overexpression of c-Abl in human 293 cells or mouse embryo fibroblasts (MEF) also entrains telomerase inhibition, whereas targeted deletion of c-Abl in MEF is associated with telomere lengthening, reflecting telomerase activation or up-regulation [34]. Given that c-Abl is also associated with and activated by DNA-dependent protein kinase and ATM kinase which are involved in the DNA damage response, cell cycle arrest and pro-apoptotic activity, the finding that c-Abl directly regulates telomerase activity suggests a novel signaling pathway whereby DNA damage may inhibit cell proliferative lifespan through DNA-dependent protein kinase/c-Abl/TERT (Figure 4). This assumption is also supported by recent findings that deficiency in DNA-dependent protein kinase is associated with longer telomeres [159]. Thus, it is possible that c-Abl induces cell cycle arrest, cellular senescence and apoptosis through a mechanism targeting multiple components including not only p53 and its homologue p73 [160-162] but also hTERT. Additional studies are required to determine the kinetics of these protein-protein interactions, and to test the hypothesis that these proteins might form a complex under certain conditions, such as during development or anti-tumor radio/chemotherapy.

Taken together, these studies indicate that telomerase activity is subject to regulation by protein phosphorylation. Phosphorylation at serine/threonine residues of hTERT by protein kinase C and protein kinase B mediates positive signaling to activate telomerase, whereas phosphorylation at a tyrosine residue of hTERT by c-Abl proffers a negative signal to inactivate telomerase (Figure 4). The exact sites of *in vivo* phosphorylation are yet to be determined; their identification may reveal distinct structural configurations of telomerase and provide potential sites and mechanisms for therapeutic targeting. While protein phosphatase 2A switches off serine/threonine phosphorylation of hTERT and telomerase activity, the tyrosine phosphatase that reverses c-Abl-mediated tyrosine phosphorylation (and may thus be involved in telomerase activation during the cellular immortalization of tumorigenesis) remains unknown.

5. Intracellular trafficking

Intranuclear localization of telomerase is an essential premise in the regulation of telomere maintenance by telomerase. Given the potential size of the telomerase holoenzyme [163], a prerequisite for assembly and activation of the complex is the regulation of telomerase protein trafficking into the nucleus and its recruitment to the telomeric DNA substrate. It is envisaged that hTERT initially traverses nuclear pores and is then recruited to telomeres, where it forms a ribonucleoprotein holoenzyme with hTR to allow telomere elongating activity. It is also possible that the telomerase holoenzyme undergoes an assembly, disassembly and reassembly cycle *in situ*, with hTERT shuttling in and out the nucleus as a function of cell division cycle. However, little was known of these protein trafficking processes until recent studies providing evidence for the nuclear export of hTERT controlled by 14-3-3 proteins [40, 164].

5.1. 14-3-3 proteins

14-3-3 proteins are ubiquitous molecules that exist as a dimer with an amphipathic groove forming a pocket for an amphipathic helix including a phosphorylated serine residue common to many phosphoproteins (consensus motif: RSXpSXP) [165-167]. In searching for hTERT interacting proteins, Seimiya et al. used the hTERT C-terminal region of 129 amino acids and yeast two-hybrid screening of HeLa cell and human testis libraries. They found that 14-3-3 proteins interact with the hTERT C-terminal region and thereby in a position to regulate hTERT nuclear trafficking in human foreskin fibroblasts and 293T cells (human embryonic kidney cells positive for Ad5 E1 and SV-40 large T antigen) [40]. The C-terminal region of 14-3-3 binds to a C-terminal serine/threonine clustering α helix of hTERT ($hTERT^{1030-1047}$), binding which allows hTERT nuclear localization. Although the molecular interaction between 14-3-3 and hTERT appears to be independent of phosphorylation, alanine substitution mutagenesis of the three serine/threonine residues in the hTERT α helix to prevent 14-3-3 binding inhibits hTERT nuclear localization [40]. These studies thus provide evidence for a mechanism of hTERT nuclear export negatively regulated by 14-3-3, implying an important balance between nuclear import and export of hTERT in telomerase function (Figure 5).

5.2. Nuclear export signal (NES) and CRM1/exportin 1

Structure/function analysis suggests that TERT contains a nuclear export signal (NES) represented by a leucine-rich sequence ($hTERT^{970-981}$) with conserved spacing and hydrophobicity, and N-terminal to the 14-3-3 binding site. Mutation of the three conserved leucine residues to alanine in the putative NES of hTERT abolishes cytoplasmic accumulation of hTERT produced by mutations in the 14-3-3 binding site of hTERT or by N-terminal truncation of the 14-3-3θ isoform [40]. NES provides the binding site for CRM1 (chromosome region maintenance 1) or exportin 1, a key protein in nuclear export, and inhibition of CRM1/exportin 1 with leptomycin B has been shown to inhibit hTERT export [40]. These data therefore suggest a key role for 14-3-3 proteins in hTERT nuclear localization by regulating hTERT interaction with CRM1. The C-terminal tail to tail interaction between 14-3-3 and hTERT might place the N-terminal "head" of 14-3-3 in a position to mask the NES, thereby directly preventing hTERT interaction with CRM1/exportin 1 and thus nuclear export, whereas deletion of the N-terminal portion of 14-3-3 might disable 14-3-3 interaction with NES, reflected in hTERT cytoplasmic localization [40]. What is still unclear is the stoichiometry of the 14-3-3 interaction with hTERT, since 14-3-3 commonly exists as a dimer with the two monomers binding in inverted orientation [167]. It is therefore also possible that 14-3-3 binds to the C-terminal region of hTERT in such a way that it alters the conformation of hTERT and thus indirectly inhibits CRM1/exportin 1 access to NES (Figure 5). This possibility is consistent with the findings that C-terminal tagging of hTERT renders hTERT ineffective in telomere elongation in cells [37, 38]. These findings have provided a testable hypothesis for NES-mediated interaction of hTERT with CRM1/exportin 1, and provide a new basis

for studying NES and NLS (nuclear localization signal) structure and function in hTERT intra- and extra-nuclear transportation.

5.3. Cellular phenotypes of 14-3-3 expression in telomerase function

Studies by Pandita and colleagues have shown that inactivation of 14-3-3σ isoform gene in human colorectal immortalized cell line (HCT116) induces aberrant telomere behavior and increased chromosomal instability in response to ionizing radiation [168]. The telomere aberrations in 14-3-3 null HCT116 cells assessed at metaphase include a clear reduction in the extent of G-strand overhang, loss of telomeric repeat sequences, and an altered telomere nuclear matrix relationship, in association with increased frequencies of chromosome end-to-end fusion and terminal non-reciprocal translocation [168]. While the cellular levels of telomerase activity were not altered by eliminating 14-3-3σ [168] or dominant negative overexpression of 14-3-3θ [40], these 14-3-3σ mediated chromosomal abnormalities have been imputed to a defect of cell cycle G2 checkpoint repair before entering M phase. This interpretation is based on the finding that the chromosomal aberrations occur preferentially in G2 after gamma irradiation [168], and accordingly 14-3-3σ has been implicated as promoting G2 arrest following DNA damage by sequestering cyclin B1-CDK1 complexes outside the nucleus [169, 170]. However, it may also be possible that while G2 checkpoint is faulty and fails to arrest dividing cells with DNA damage for repair, there may exist a parallel increase in telomere shortening as a consequence of the loss of nuclear hTERT in 14-3-3σ null cells. Removal of 14-3-3 may thus trigger more than one subcellular malfunction [167], and the double default of 14-3-3 at the G2 checkpoint [168] and in hTERT transport [40] may together account for by the chromosomal disorders observed in 14-3-3σ null human colorectal cells.

In human keratinocytes, however, inhibition of the single protein 14-3-3σ induces telomerase activation, telomere elongation and cellular immortalization, a phenotype contrasting to that seen in colorectal cells [137]. By retroviral-mediated 14-3-3σ antisense DNA transfection, Dellambra et al. show that the primary human keratinocytes have a marked decrease in immunoreactive 14-3-3σ which is otherwise elevated in control cells toward replicative senescence. The antisense-induced decrease of 14-3-3σ is concurrently accompanied with up-regulation of telomerase activity, elongation of telomeres, inactivation of $p16^{INK4a}$ and immortalization of the primarily cultured cells. The genotypes and phenotypes can be reversed to 14-3-3σ down-regulated cells upon the reconstitution of 14-3-3σ [137]. Thus, down-regulation of 14-3-3σ shows no negative effect on hTERT nuclear localization and telomerase maintenance of telomeres in primary human keratinocytes. These apparently paradoxical findings in human keratinocytes [137], colorectal cells [168], and embryonic kidney cells and foreskin fibroblasts [40] suggest a complexity of function for the 14-3-3 proteins. It might be possible that different 14-3-3 isoform(s) are involved in hTERT nuclear localization in keratinocytes, and/or the interaction between 14-3-3 and hTERT might be up regulated with increased hTERT gene expression. The inverse relationship between telomerase and $p16^{INK4}$ is consistent with previous findings in which the levels of $p16^{INK4}$ and Rb are negatively correlated with telomerase activity [116, 134-136]. Thus, increased

expression of hTERT might circumvent or compensate for the loss of nuclear hTERT due to impaired hTERT nuclear retention induced by 14-3-3σ down-regulation in keratinocytes. These hypotheses require further investigation.

6. Intra- and inter-molecular interactions

6.1. Telomerase-inhibitory polypeptides

Following protein translation and post-translational protein modification, the assembly, maintenance and disassembly of functionally active telomerase holoenzyme are likely to require continuous interactions with other proteins in telomerase-positive cells. Although little is known of how proteins interact within the telomerase holoenzyme, it is noteworthy that while hTEP1 binds to hTERT and the complex exhibits telomerase activity [23, 36], a peptide from the N-terminal region of hTEP1 specifically inhibits telomerase activity *in vitro* [171]. This hTEP1 peptide (aa 385-399), termed telomerase inhibitory polypeptide 1 or TEIPP1, binds intact full-length hTEP1 on affinity chromatography, suggesting the possibility of potential hTEP1 oligomer formation *in vivo* [171]. The possibility of hTEP1 oligomerization is also supported by immunoprecipitation experiments in which hTEP1 sometimes shows a molecular weight of greater than 1000 kDa (unpublished data). These observations are thus consistent with the view that hTEP1 is a regulatory subunit of telomerase, potentially serving as a scaffold for recruiting and organizing hTR, hTERT and other potential regulatory factors.

In addition, of five synthetic peptides derived from hTERT, the peptide ^{641}GARTFRREKRAERLTSRVK^{659} showed significant inhibitory effect on telomerase activity in a concentration-dependent manner [172]. The inhibition was gradually reversed by increasing amounts of telomerase extracts, with increasing concentrations of telomerase substrate DNA having no effect, suggesting that interaction may occur within and/or between individual telomerase protein components within the telomerase complex, but not at the interface between telomerase and its substrate DNA [172]. This peptide, referred as TEIPP2, may thus act to mimic an endogenous element of telomerase with a negative signal in the intra-molecular regulation of telomerase activity [172]. Furthermore, TEIPP2 enhanced TEIPP1 inhibition of telomerase, an additive effect distinct sites of action.

6.2. Tumor suppressor protein p53

Since hTEP1 may potentially serve as a scaffold for telomerase regulatory factors, we have attempted to identify telomerase interactive proteins by affinity chromatography with synthetic peptides derived from the sequences of hTEP1, and have thus screened nuclear proteins from human breast cancer cells for binding [171]. Several proteins have been found, among them being a 53-kDa species that turned to be on immunological criteria the tumor suppressor protein p53 [171]. Further studies show that both endogenous nuclear and recombinant p53 proteins co-immunoprecipitate with hTEP1, and that recombinant p53 not only binds to the hTEP1 peptide affinity column

specifically, but also inhibits telomerase activity *in vitro*. An intact C-terminus of p53 is essential for inhibition. The inhibitory effect of p53 on telomerase is abrogated by TEIPP1, further suggesting a direct interaction between p53 and hTEP1. Furthermore, by confocal microscopy, p53 and hTERT share a similar subcellular distribution pattern throughout the cell cycle in human breast cancer cells (unpublished data), implicating a possible interaction *in vivo*. These data suggest that telomerase may be a downstream target of p53, with its activity being regulated by the tumor suppressor. This conclusion is consistent with the findings that p53 specific mutations are involved in telomerase activation in sun-exposed human skin [69], and that expression of a p53 mutant in human mammary epithelial cells is occasionally associated with telomerase activation [173]. Given that overexpression of wild type p53 induces inhibition of hTERT gene expression and telomerase activity [109, 129, 130], it is possible that p53 plays an obligatory role in the negative regulation of telomerase first by preventing telomerase activation through inhibiting hTERT gene expression, and then by direct protein interaction with the enzyme, ensuring an inhibitory outcome. It is thus tempting to speculate that p53 plays a guardian role in cellular senescence [174-177] at least in part through controlling telomerase activity. Down-regulation of p53 or interference of its interaction with telomerase may favor telomerase activation and cell immortalization *in vivo* during tumorigenesis.

6.3. Heat shock proteins

Using yeast two-hybrid screening with the amino terminus of hTERT (amino acids 1-195), Holt and colleagues have shown that the molecular chaperone p23 is associated with hTERT [178]. In addition, Hsp90 also binds hTERT as shown by co-immunoprecipitation studies. Antibodies against either p23 or Hsp90 co-immunoprecipitate hTERT independently of hTR from an *in vitro* reconstitution system and telomerase activity from human fibrosarcoma cell extracts. The co-precipitation is specific as irrelevant antibodies produce no telomerase activity, and the telomerase activity precipitated by antibodies against p23 and Hsp90 can be eliminated by premixing the cell lysates with the relevant antigens [178]. Furthermore, binding of p23 and Hsp90 is essential in telomerase holoenzyme assembly and activity, as immunological removal of p23 inhibits telomerase activity. The inhibition is reversed by exogenous p23. This function of molecular chaperones in telomerase protein folding is also fostered by the data that purified recombinant p23 and Hsp90 sufficiently substitute for the requirement of cell lysates in telomerase assembly [178]. Consistently, geldanamycin, the Hsp90 inhibitor, inhibits p23 binding to hTERT and prevents telomerase assembly if applied prior to telomerase reconstitution *in vitro* or prior to telomerase induction in intact cells [178]. Thus, telomerase activation may be an ATP-dependent process of *de novo* holoenzyme assembly that requires Hsp90 and subsequent recruitment of p23 for elaboration of a functionally active conformation [178].

6.4. Other telomeric proteins

When potential telomere and telomerase regulatory factors have been isolated by molecular genetic approaches, various studies have implicated these proteins as playing important roles in the action of telomerase on telomeric DNA. The telomeric proteins include human duplex telomeric DNA-binding proteins TRF1 [179, 180], TRF2 [2, 180, 181], hRap1p [182], mammalian heterogeneous nuclear ribonucleoprotein A1 (hnNRP A1) [183, 184], yeast duplex telomeric DNA-binding proteins Rap1p [185], Taz1p [186], yeast single strand telomeric DNA binding proteins Cdc13p [187], and Est1p [188]. Considerable evidence suggests that these telomere proteins regulate telomerase access to telomeres, and the regulation is likely to take place at the interface between telomerase and the substrate telomeric DNA. This involves positive recruitment of telomerase and negative feedback control to limit telomerase accessibility to telomeres.

The yeast proteins Est1p and Cdc13p are the positive regulators of telomerase access to telomeres [189, 190]. Cdc13p may recruit Est1p first and then telomerase in sequence. Cdc13p is tightly associated with telomeric chromatin, but loosely with telomerase [189, 190]. In contrast, Est1p is tightly associated with telomerase [188, 191, 192], probably by binding to the telomerase RNA subunit [188]. In addition, Cdc13p and Est1p bind to each other and the complex can be immunoprecipitated [193]. Removal or mutations of the genes coding for Est1p and Cdc13p have no impact on telomerase catalytic activity, but render telomerase ineffective on telomere elongation [189]. In the absence of Est1p, moreover, a fusion between Cdc13p and telomerase catalytic subunit reverses the phenotype of telomere lengthening defect induced by Est1p deficiency [190]. Thus, the single strand telomere-binding proteins Cdc13p and Est1p may be viewed as docking and bridging proteins playing an important role in recruiting telomerase onto telomeric DNA substrate in yeast.

In contrast with the positive regulation of telomerase interaction with telomeres by single-strand telomeric DNA binding proteins, the double stranded telomere-binding proteins *in cis* negatively inhibit telomerase access to telomeres. In yeast, elongation of telomeres gradually and progressively inhibits telomerase elongation of telomeric DNA, while telomere shortening in the absence of telomerase activity remains at a constant rate independent of telomere length [194]. This inhibition of telomerase by telomeres is thought to reflect the cumulative effect of each individual Rap1p molecule assembled onto telomeric DNA during telomere elongation [194]. In addition, it is possible that the continuously elongated telomere entails continuous structural changes that regulate telomerase accessibility, and that the primary structure of *de novo* synthesized telomeres may subsequently be further regulated by the binding of telomeric proteins. The binding of Rap1p, through its two Myb-like domains to duplex telomeric DNA, is associated with telomere shortening in proportion to the number of bound Rap1p molecules [185, 195]. Disruption of the Taz1 gene (an ortholog of human TRF in yeast) causes an increase in telomere length [186]. Thus, these data suggest that both Rap1p and Taz1 bound to double strand telomeric DNA negatively regulates telomerase access to its substrate DNA by regulating telomere structure in yeast [195].

In human telomerase-positive tumor cells, overexpression of double strand telomeric DNA-binding proteins TRF1 and TRF2 results in gradual and consecutive telomere

shortening, whereas removal of TRF1 from telomeres leads to lengthening. This suggests that both TRF1 and TRF2 inhibit telomerase activity *in cis* [2, 179]. However, loss of TRF2 function also leads to telomere 3' G-strand overhang loss and telomere-telomere fusion, suggesting that TRF2 protects the G-strand overhangs from being degraded (Figure 6). Intriguingly, recent studies have also shown that TRF1 operates *in trans* to mediate intercalation of telomere repeats into non-telomeric DNA [196]. In addition, a novel type of cellular promyelocytic leukemia (PML) body has been found containing telomeric DNA and telomere binding proteins including TRF1 [197, 198]. Thus, TRF1 and TRF2 may be important molecules not only in maintaining and regulating telomere structures, and in regulating the function of telomerase, but also in DNA repair and signaling.

By binding to TRF1 and catalyzing TRF1 ADP ribosylation at telomeres, tankyrase, a poly(ADP-ribose) polymerase, induces the dissociation of tankyrase and TRF1 complex from telomeres [199]. This regulatory mechanism of TRF1 dissociation may thereby counteract TRF1 inhibition of telomerase access and allow telomerase extension of telomeres in intact cells [199, 200]. By interacting with TRF2, on the other hand, hRap1p (an ortholog of the yeast Rap1p) also extends telomeres in the human fibrosarcoma cell line HTC75 [182]. Since human double-stranded telomere DNA bends to form a lariat-like telomere loop (t-loop) with its 3' G-rich single-strand overhang invading the double-stranded telomere as a displacement loop (d-loop) [1], it is possible that the binding of TRF1 and TRF2 to telomeres determines the loop-like configurations of telomeres preventing telomerase access to telomeres (Figure 6). In contrast, the binding of the large molecular mass of tankyrase to TRF1, dissociation of both proteins from telomeres, and the sequestration of TRF2 by hRap1p together act to open up the telomere loops allowing telomerase access and telomere homeostasis (Figure 6). Thus, it is likely that telomeric proteins at different layers potentially regulate telomere tertiary and quaternary structures to confer telomere plasticity with sensitivity to telomerase bindings and other telomeric proteins through conformational changes.

7. Concluding remarks

Telomerase activity constitutes an important mechanism in stabilizing the structure of telomeres and thus the genome. Activation of telomerase requires *de novo* transcription of TERT gene, appropriate splicing of TERT mRNA, and post-translational modification and incorporation of full-length TERT protein into the holoenzyme. Regulation thus can occur at multiple levels, sites and stages, each involving complex interactions and cooperation. The result is the precise temporal and spatial pattern of telomerase activity throughout cell proliferative cycles during development and oncogenesis.

The regulation of TERT gene expression is likely to be primarily at the promoter level; oncogenes and tumor suppressor genes including the c-Myc/Max/Mad system have been shown to regulate hTERT gene expression. Post-translation, hTERT is imported into the nucleus and interacts with 14-3-3 proteins thus avoiding being exported by CRM1. In the presence of heat shock proteins, telomerase undergoes appropriate assembly and activation with serine/threonine phosphorylation by PKC and

PKB. Telomerase activity is inhibited by hTERT gene down-regulation, intra-molecular peptide interactions, the tumor suppressor p53, specific dephosphorylation by PP2A and tyrosine phosphorylation by c-Abl. Given the dominant role that telomerase plays in cell survival and proliferation, and its common occurrence in human cancers, understanding telomerase regulatory mechanisms may open a new insightful molecular window to throw a light on the molecular mechanisms of cellular aging and oncogenesis. The combination of structural, genetic, biochemical, and biophysical data should soon give us a fuller understanding of the exquisite details in the regulation of the telomerase system.

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CHAPTER 4

TELOMERASE AND THE CELL CYCLE

TEJ KRISHAN PANDITA

Table of contents

1. Introduction

Telomerase is a ribonucleoprotein complex that elongates telomeres. Biochemical and genetic studies have established an association between telomere maintenance and extended life-span through telomerase expression. Telomerase activity is present in unicellular organisms and germ cells, both situations where it is expected to play a role for indefinite cycling and protection from shortening of the telomeres. Telomeres contain distinctive repeats of guanine-rich sequences that are replicated by DNA-dependent DNA polymerases and by telomerase dependent synthesis of telomeric DNA from an RNA template. Telomerase consists of proteins complexed with an essential telomerase

Telomerase, Aging and Disease. Ed. by M. P. Mattson and T. K. Pandita. 61 — 88

RNA that is an intrinsic part of the enzymatic active complex. The demonstration that expression of the telomerase catalytic subunit TERT and activation of telomerase activity can extend the life span of normal human cells has suggested that decreases in telomere length contribute to senescence. It has been suggested that the telomere-telomerase system represents an adaptation of organisms with prolonged lifespan to avoid malignant tumors, at the expense of the cellular dysfunction associated with the aged phenotype. An important recent discovery has been the observation that about 85% of tumors are positive for telomerase activity.

Telomerase activity in mammals is ubiquitous in embryonic tissues but downregulated in most of the somatic tissues. Telomerase activation is controlled by cellular proliferation, but it is an early step in the development of many tumors. Normal human somatic cells have a finite life span *in vivo* as well as *in vitro* and retire into senescence after a limited number of cell divisions. Cellular senescence is triggered by the activation of two interdependent mechanisms which involve irreversible cell cycle exit and this is indicated by a critical shortening of telomeres. The loss or shortening of telomeres in telomerase-negative somatic cells has been linked with genomic instability and carcinogenesis. Intense research is ongoing to determine the link between cell cycle checkpoint genes and telomerase activity.

The challenge at present is to determine whether shortening of somatic telomeres really constitutes a mutator suppressor mechanism *in vivo* and to evaluate how telomerase may contribute to cancer development. To address these questions, we need to better understand human cell growth patterns and the developmental control of the gene or genes that suppress or activate telomerase activity. Recent research has led to characterization of many molecular and biochemical events that control the transition from cells with and without telomerase activity for maintaining telomeres. Two common regulatory themes have emerged for telomerase function. First, how the components of telomerase are regulated at the transcriptional or at the posttranscriptional level. Second, how the telomere binding factors influence the accessibility of telomerase for the elongation of the telomeres.

In this chapter, I summarize information about the relationship between regulation of telomerase and the cell cycle regulatory factors. The information about the regulation of the telomerase through the cell cycle may prove helpful in designing the therapeutic agents either for its activation in cells where its expression can overcome the senescence and prolong the life of post mitotic cells or inhibition of telomerase where it is essential for the proliferation and provide a therapeutic advantage to kill the tumor cells.

2. Telomerase

Eukaryotic DNA polymerases are unable to synthesize in the 3'-5' direction or start *de novo*, leading to the problem that extreme terminal sequences will not be replicated. This could lead to loss of the 5' end of one daughter DNA strand (Figure 1). To counter this problem, a special type of DNA polymerase related to reverse transcriptase and called telomerase is found in eukaryotes. Telomerase activity was first reported *in vitro* in *Tetrahymena* by a biochemical assay in which a single strand telomeric primer

d(TTGGGG)4 oligonucleotide was used as substrate for the addition of dTTP and [^{32}P]dGTP [1]. These synthetic oligonucleotides are thought to mimic the G-strand of natural telomeres which have a single-stranded overhang. The products synthesized by *Tetrahymena* telomerase are hundreds of nucleotides long and have a repeated 6-base pattern, d(TTGGGG)n which is added one nucleotide at a time onto telomeric primer 3' ends. Telomerase also directs the incorporation of dideoxynucleotides that lead to chain termination. The initial characterization of *Tetrahymena* telomerase activity showed that elongation is specific for oligonucleotide primers that consist of telomere repeat sequences which was evident from the fact that oligonucleotides consisting of telomere repeats from other species are also elongated efficiently by *Tetrahymena* telomerase. These observations suggested that input primer does not determine the sequences synthesized by telomerase. Further, it became apparent that telomerase activity might be highly conserved. The processivity of the telomerase suggested that it has a two-site binding mechanism to allow processivity. Since then several groups have made an attempt to determine how such binding factors are regulated. The essential factor that influences the correct synthesis of telomeric repeats without a DNA strand was characterized by Greider and Blackburn [2]. It was found that telomerase contains an essential RNA component. This RNA contained the sequence that serves as a template for telomere repeat addition. Based on the sequence complementarity of RNA moiety and telomeric DNA, it has been suggested that telomerase binds to the chromosome ends and the telomere is then elongated by the addition of TTG at the 3' end. The RNA component has been cloned. Alteration of a single-nucleotide in the CAACCCCAA region of RNA gene of *Tetrahymena* telomerase lead to change in telomere lengths. These observations confirmed that the telomerase is a reverse transcriptase with an RNA moiety acting as a template and is responsible for the synthesis of telomeres. Telomerase is capable to extend the 3' end of the G-rich strand of the telomeric repeats, and continue lagging-C-rich-strand synthesis to complete the replication of chromosomal ends, thus compensating for the shortening that otherwise occurs. Moyzis et al., [3] demonstrated that the vertebrate telomeric DNA repeat sequence is TTAGGG and Morin [4] demonstrated that HeLa cells have telomerase activity that could ligate (TTAGGG)n oligonucletides *in vitro*. Realizing the role of telomerase in maintaining telomeres, telomerase activity has been examined in a variety of organisms. Telomerase activity has been identified in the Euplotes, Oxytricha, tissues of human, mouse, rat, Xenopus, Chinese hamster etc. Telomerase can maintain the telomeres in immortalized and germ line cells, with some exceptions where telomeres in immortal cells might be maintained by alternative mechanisms that are independent of telomerase.

2.1 Telomerase components

Telomerase is composed of RNA moiety and several proteins of which telomerase reverse transcriptase (TERT) was first identified in the yeast *Saccharomyces cerevisiae* (EST2) and *Schizosaccharomyces pombi* (tri1+) and the ciliate *Euplote saidiculatue* (p123) and subsequently in humans (hTERT). In yeast, several proteins that are required for telomerase activity *in vivo* are known to directly or indirectly interact with RNA component of the telomerase; e.g., direct or indirect interaction of the TLC1 RNA to

Est2p, the reverse transcriptase subunit of telomerase [5]; Est1p which binds TLC1 RNA [6] and Cdc13P, which binds single stranded telomeric DNA and may recruit telomerase to the chromosome end through interaction with Est1p [7]. In mammalian systems several proteins have been identified that interact with RNA component of the telomerase; however the only protein that has been found to be required for the telomerase activity is TERT. In addition to the RNA and TERT components, telomerase complex contains several other proteins. In humans, the proteins hsp 90 and p23 and three telomerase RNA binding proteins, dysterin, L22 and hStau are each associated with telomerase activity in cell extract [8-10]. Another protein that interacts with telomerase RNA is p80 in *Tetrahymena thermophila* and its mammalian homolog TEP1 in humans [11]. Even though Tep1 is mammalian telomerase RNA and the telomerase catalytic subunit TERT associated protein, mTep1-deficient mice show no significant alteration in telomerase activity or telomere length. TEP1 is expressed in many tissues including those that are negative for telomerase activity. These and other reports suggest that telomerase-associated proteins like TEP1 may not be required for the telomerase activity, but may be important for other cellular functions. The telomerase enzyme has been partially characterized and was shown *in vitro* to be able to polymerize TTAGGG sequences. Telomerase recognizes the G-rich strand of an existing telomere repeat sequence and, in the absence of a complementary DNA strand, synthesizes a new copy of the repeat using its internal RNA as a template. The RNA component and some of the associated protein components of telomerase have been cloned from different yeast species, human and mouse.

2.2. Telomerase activity during development

The development and maintenance of metazoans require precise control of cell division. During development, mitogenic signals function within particular proliferative domains and are opposed by antimitogenic signals that halt cell proliferation (Figure 2). Inhibition of cell proliferation is accompanied by replicative senescence, a phenomenon observed in explanted mammalian cells, where they undergo a limited number of cell divisions and then become permanently arrested [12]. At senescence, cells are viable and metabolically active but no longer divide. Since telomere shortening has been proposed as a regulatory mechanism that controls the replicative capacity of eukaryotic cells, this arrest in cell division is associated with critically shortened telomeres [13]. It is important to remember that the term, *in vitro* replicative senescence, refers to the point at which primary cells, when grown in culture, enter a phase of irreversible cell cycle arrest. The probability that a human cell will enter into the number of population doublings that a telomerase-negative human cell can undergo is determined by telomere shortening [14, 15]. The gradual loss of about 20 to 100 bp of telomere length during each round of replication has been proposed to explain the loss of the telomeric sequences from chromosomes in cultured human fibroblasts. Ectopic expression of the catalytic unit of telomerase (hTERT) into human cells halts the telomere loss and subsequently overcomes the proliferation block, even in cells that are deficient in the ATM (ataxia telangiectasia mutated) signaling pathways [16, 17].

During adult life, telomeres progressively shorten in somatic cells. Suppression of such shortening is accomplished by telomerase. Telomerase activity decreases dramatically in association with growth arrest and cell differentiation, and increases in many tumors, suggesting a role for telomerase in preventing cellular senescence [18-22].

Telomerase activity in human germline, somatic tissues and cells during development has been detected in fetal, newborn, and adult testes and ovaries, but not in mature spermatozoa or oocytes. It has been suggested that telomerase activity remains high in germ cells to ensure normal chromosome length in offspring and decrease in embryo cells as they differentiate [18, 23]. Telomerase activity is present in pachytene spermatocytes and round spermatids of the rat but telomerase activity has not been detected in ejaculated human or rat spermatozoa [24, 25]. Blastocysts express high levels of telomerase activity as do most human somatic tissues at 16-20 weeks of development with the exception of human brain tissue. Telomerase activity could no longer be detected in the somatic tissues examined from the neonatal period onward. Neonatal human somatic tissue have very little or no detectable telomerase activity. However, when fetal tissues are explanted into primary cell culture, they show a dramatic decline in telomerase activity which becomes undetectable after the first passage *in vitro*. Betts and King [26] reported that telomerase activity decreases during oocyte maturation and subsequent development to the 8-cell stage but it significantly increases by about 40 fold at the morula and blastocyst stages during bovine development. Elucidation of the regulatory pathways involved in the repression of telomerase activity during development may lead to the ability to manipulate telomerase levels and explore the consequences both for cellular aging and for the survival of cancer cells.

Normal human somatic cells have a finite life span *in vitro* and retire into senescence after a predictable number of divisions. Cellular senescence is triggered by the activation of two interdependent mechanisms. One induces irreversible cell cycle exit involving activation of two tumor suppressor genes, p53 and pRb, at the proper time point which is triggered by a critical shortening of chromosomal ends due to the end-replication problem of DNA synthesis. A second occurs when telomeres are so short that massive end-fusion events prevent further replication. The development of a malignant cancer cell is only possible when both mechanisms are circumvented. While the hTR subunit of telomerase is present in almost all human cells, this is not the case for a telomerase reverse transcriptase protein (hTERT) which is limited to germ cells and certain stem cells. Thus, telomerase activity has been shown to correlate more closely with the expression status of the telomerase catalytic subunit gene TERT. The human TERT is a polypeptide with a molecular weight of 127 kDa having a unique conserved region called the T-motif.

2.2.1. *TERT expression*

Human telomerase is expressed in germ tissues and in the majority of primary tumors. Self-renewing tissues and some pre-cancerous tissues also have weak telomerase activity. Yet, neither the exact location and frequency of telomerase-positive cells nor the changes in telomerase expression during differentiation or carcinogenesis of individual cells are known. Recent studies have revealed the expression of hTERT

protein in tumor and non-tumor colorectal tissues by Western blotting and tissue sections by immunohistochemistry [27]. Though telomerase activity and hTERT expression at both mRNA and protein levels have been found generally higher in tumor part than in non-tumor cells, these two do not always correlate; expression of hTERT did not always give rise to high telomerase activity. Colonic carcinoma cell nuclei are stained with anti-hTERT antibodies but not with antigen-preabsorbed antibodies. In normal mucosa, hTERT protein is expressed, though weaker than in carcinomas, in all colonic crypt epithelial cells except those at the tip; the expressing-cell distribution is much wider than that of Ki-67 positive cells which are located at the bottom of the crypt. Isolated crypts contain a significant level of hTERT protein revealed by Western blotting, while having very weak telomerase activity. Telomerase activity has been detected in epithelial cells only at the bottom half of the crypt. Specific hTERT-staining is positive in tissue lymphocytes but negative in almost all other stromal cells. It is of interest to see whether a significant level of hTERT expression with low telomerase activity is characteristic of physiologically regenerating tissues containing stem cells. *In situ* detection of the hTERT protein will permit further analysis of cancer diagnosis and stem cell differentiation.

The mouse TERT (mTERT) is widely expressed at low levels in adult tissues, with greatest abundance during embryogenesis and in adult thymus and intestine [28]. mRNA levels of the mTERT component are regulated during both differentiation and proliferation, while mTR levels remain constant throughout both processes. Comparison of mTERT and mTR levels to telomerase activity indicates that mTERT expression is more tightly linked to the regulation of telomerase activity during these processes than is mTR. In contrast to the situation in human cell cultures, mTERT transcript levels are present at readily detectable levels in primary cultured cells and are not upregulated following crisis. The widespread expression of mTERT in primary cells and mouse tissues could explain the increased frequency of spontaneous immortalization of mouse cells in culture and tumorigenesis *in vivo*.

2.2.1.1. *Regulation of hTERT*

Available evidence suggests that regulation of telomerase activity primarily depends on transcriptional control of hTERT. This is based upon the fact that the introduction of the hTERT cDNA is sufficient to produce telomerase activity and immortalize normal human cells, suggesting that the repression of telomerase activity is transcriptional. However, several human tissues as well as some normal cell strains have been shown to express low levels of hTERT mRNA even though they lack telomerase activity. The hTERT transcript has been shown to have at least six alternate splicing sites (four insertion sites and two deletion sites), and variants containing both or either of the deletion sites are present during development and in several cancer cell lines [29]. Several of the deletion variants have been detected in normal and developing human tissues. One deletion (beta site) and all four insertions have been shown to cause premature translation terminations, whereas the other deletion (alpha site) of 36 bp lies within reverse transcriptase (RT) motif A, suggesting that this deletion variant may be a candidate as a dominant-negative inhibitor of telomerase. Yi et al [29] have cloned three alternately spliced hTERT variants that contain the alpha, beta or both alpha and beta

deletion sites. These alternate splicing variants did not reconstitute telomerase activity in fibroblasts. However, hTERT alpha inhibits telomerase activities in telomerase-positive cells, causes telomere shortening and eventually cell death. This alternately spliced dominant-negative variant may be important in understanding telomerase regulation during development, differentiation and in cancer progression.

2.2.2. *hTR expression*

With the cloning of human and mouse telomerase RNA component, it has been possible to determine its expression, regulation and function during development. Although there are subtle differences in the biochemistry of telomerase between species, there are large number of similarities in the region of the RNA complementary to the telomeric DNA sequences which might allow the telomerase to recognize the higher-order structure of telomeres. It is easy to appreciate the evolutionary logic of programming somatic cells with mitotic clock for senescence and why selective pressure for immortalization in the development of cancerous clones should exist. There could well be situations where during the development telomerase is genuinely activated. Insight into how the telomerase gene itself is positively and negatively regulated in normal cells during the process of development would be extremely useful.

The integral RNA component of human telomerase (hTR) is 451 bases, with a 3' region resembling the box H/ACA family of small nucleolar RNAs (snoRNAs). The H/ACA motif of hTR is not required for *in vitro* assembly of telomerase, but it is essential for proper 3'-end processing, stability, and nucleolar targeting *in vivo* [10, 30, 31]. The 5'- end of hTR contains the template used for the addition of telomeric sequences to the end of the chromosomes and can form a pseudoknot that is likely to be important for telomerase function [30, 32]. The 5'- end of hTR also contains a 6 base U-rich tract required for a direct interaction with heterogeneous ribonucleoproteins (hnRNP) C1 and C2 [33]. While several regions of hTR interact with the catalytic protein component of telomerase [34-36], it is yet to be established whether these interactions are mediated by direct contacts, auxiliary proteins or both. In the recent past, several auxiliary proteins have been identified that associate with the human telomerase [8-11, 37-42]. The first such protein identified was TEP1 [11, 39]. The snoRNA binding proteins like dyskerin and hGAR1 bind the snoRNA motif at the 3' end of hTR [10, 40]. Members of the hnRNP family of RNA biding proteins interact with telomeric DNA as well as telomerase [33, 41, 42]. The chaperone proteins p23/hsp90 are involved in the assembly of telomerase activity [8]. It has been found that La autoantigen is important for the assembly of other RNA particles [43-45], the maturation of tRNAs [46] and interaction directly with the human telomerase RNP. The levels of La autoantigen expression influence telomere length in a telomerase RNP dependent fashion [43].

2.2.2.1. *Regulation of hTR*

The template RNA (hTR or hTERC) is widely expressed in many cell types and to understand the mechanism for its regulation is of immense interest. The immortal phenotype of most human cancers is attributable to telomerase expression. However, a

number of immortal cell lines and tumors achieve telomere maintenance in the absence of telomerase via alternative mechanisms known as ALT (alternative lengthening of telomeres). In several ALT cell lines the promoter of the hTR gene is methylated and is associated with a total absence of hTR expression in several cell lines. Treatment of cells with 5-azacytidine in combination with trichostatin A, results in partial demethylation of the hTERC promoter and expression of the gene. Partial methylation has been detected in tumors (5%) and in immortal cell lines (27%). Cell lines with partial methylation express hTR. Only in ALT cell lines does there appear to be a strong correlation between hTR promoter hypermethylation and lack of hTR expression [47].

3. Telomerase and cellular metabolism

Telomerase synthesizes telomere repeats onto chromosome ends to overcome the loss of sequences during normal replication. Telomeres shorten in most of the somatic cells because of the lack of the telomerase activity, however, in germ and some stem cells, telomeres are maintained as these cells have telomerase activity. Telomere attrition has been proposed as a signal that may determine the replicative life span of cells in culture [48]. It has been demonstrated that during the process of immortalization of human cells *in vitro*, telomerase is activated after crisis. In support of this, Counter et al., [49] demonstrated that primary cells transfected with T-antigen initially show telomere shortening and no telomerase activity; subsequently cells go through crisis during which most of the cells die, but those cells that continue to grow have telomerase activity and relatively long telomeres. In such situations, it is believed that activation of the telomerase occurs by introduction of the T-antigen. This is in contrast to the situation when human primary cells are transfected with hTERT the telomerase activity is seen immediately. This rules out the possibility that short telomere or telomere attrition is the cause for generation of the signal for the transcriptional activation of hTERT.

Several human tissues lack detectable telomerase activity, but some tissues do have telomerase activity. It is not clear what regulates telomerase activity in different cell types. Furthermore, telomerase activity is present in several normal tissues from both mouse and Xenopus [23, 50]. Telomerase activity is detected in human tumors and not in the adjacent tissues, suggesting that telomerase may be tightly regulated in humans, which may be the reason for inability of human cells to immortalize spontaneously in culture. In contrast, in mouse and some other species, telomerase activity is found in normal tissues and thus such cells immortalize very frequently in culture as compared to the human cells.

Ectopic hTERT expression has been demonstrated to prevent replicative senescence in several normal cell types including fibroblasts and epithelial cells [16, 51, 52]. Recent studies demonstrated that TERT exerts its anti-apoptotic action at an early stage of the cell death process prior to mitochondrial dysfunction and caspase activation [53]. Immortalization of human keratinocytes and mammary epithelial cells in culture was also achieved by hTERT expression in conjunction with the cell culture induced loss of p16-dependent cell cycle control, although these data are subject to other interpretations [54, 55]. These observations support the hypothesis that replicative senescence in

humans results from inactivation of telomerase and short telomeres.

Telomerase may not be necessary in nonproliferating cells as telomeres do not continue to shorten in the absence of cell division; quiescent and differentiated cells may have alternative means of regulating the expression and activity of telomerase to compensate for the lack of cellular proliferation [56-58]. It is possible that quiescent cells downregulate the expression of telomerase by repressing transcription as the kinetics of the loss of activity are not significantly different from the half-life studies [59]. The half life data suggest that telomerase is a highly stable molecule, although differentiating cells appear to down-regulate telomerase within the first 24 hr of being stimulated to differentiation. The regulation of telomerase activity in differentiating cells may be either through the direct physical interaction of telomerase with regulatory proteins or degradation of the RNA or protein components.

Several studies indicate that decline in physiological function during aging may be the result of telomere-dependent *in vivo* replicative senescence among certain cell types. This is evident from the fact that wound healing is less efficient in older animals [60] and age related changes in collagen synthesis have been reported in fibroblasts [61]. A small number of genes show consistent age-related changes in their expression both in fibroblasts [62] and in tissues [63, 64]. Interestingly the patterns of gene expression in immortalized hepatoma cells is similar to young but not old hepatocytes [65], which are quiescent telomerase-negative cells with defective telomere status. Several investigations are under way to determine whether alteration in telomere size or structure influences the gene expression in human systems. It was already demonstrated in yeast that non-telomeric DNA created by enzymatic cleavage leads to genomic instability and cell cycle arrest [66], and yeast telomeres can exert a position effect on recombination between internal tracts of telomeric DNA [67]. It is clear in the yeast that alteration in the telomere chromatin could influence the expression of genes in the subtelomeric region. Such a phenomenon has yet to be demonstrated in mammals and it is not known whether telomeres exert a position effect on the gene expression. However, some are of the opinion that change in gene expression in mammals could be the result of age-related changes in transcription factor composition [68], possibly dependent on changes in the redox state of the cell [69, 70]. Recent studies have shown that serum stimulated cell growth in culture can exhibit age-related changes in gene expression, which can be correlated with known changes occurring during organismal aging. Since human fibroblasts lack detectable levels of telomerase activity and may not be able to maintain the telomeres, it is reasonable to believe that telomerase has a significant role to play in cellular metabolism during organismal development.

3.1. Telomerase activity and cell cycle checkpoints

Genetic lesions that disable key regulators of G1 phase progression in mammalian cells are present in most human cancers and such cells are mostly positive for telomerase activity. Mitogen-dependent cdk4 and cdk6 phosphorylate retinoblastoma (Rb) protein, that helps to cancel Rb's growth-inhibitory effects and enable E2F transcription factors to activate genes required for entry into the S phase, and may be involved in the upregulation of the telomerase or the activation of the telomerase. Disruption of the

Rb pathway by overexpression of cyclin D-dependent kinases or through loss of p16 (INK4a), an inhibitor of the cyclin D-dependent kinases may be linked with the regulatory role of telomerase function. Evidence is accumulating that the reduction in levels of p27(Kip1) and increased expression of cyclin E correlate with the telomerase activity. Whether there is any correlation between the abnormal growth signal and the activation of the telomerase is yet an open area of investigation. It will be worthwhile to test whether ARF tumor suppressor, encoded by an alternative reading frame of the INK4a-ARF locus, senses mitogenic current flowing through the Rb pathway and is induced by abnormal growth promoting signals that could be linked with the regulation of telomerase either at the transcriptional or post translational level.

There is evidence that telomerase activity is enhanced when cells are challenged with DNA damaging agents, however, it is known that ARF is not directly activated by signals that damage DNA. Inactivation of ARF not only dampens the p53 response to abnormal mitogenic signals but also renders tumor cells resistant to treatment by cytotoxic agents and such tumor cells have been found to have relatively higher telomerase activity, indicating that inactivation of ARF might play a role in the function of telomerase.

The most compelling data that supports the view that telomere loss eventually restrains the proliferation of human cells arose from studies of human tumors and immortal cells. Telomere shortening has been believed to restrict the number of cell divisions and this restraint is overcome by immortalization which is accompanied by the activation of telomerase or by an alternative mechanism in order to maintain the telomeres. Telomere loss and activation of telomerase is as frequent an event in tumors as is mutations in the Rb and p53 pathways. It has been suggested that telomerase activation is not simply the consequences of rapid proliferation because of the fact that stem cells have much lower telomerase activity than tumors, and many proliferating cells e.g. fibroblasts lack telomerase.

Several classes of human cells are now known to count divisions by monitoring the progressive attrition of telomeres, leading to the activation of a p53-p21WAF-dependent G1 checkpoint. Ectopic expression of hTERT has been shown to prevent senescence in several cell types and offers the potential for interventions in the aging process based on tissue engineering, gene therapy or homeografts. However, this telomere-driven senescence mechanism seems to be absent from rodents, which use telomere-independent means (perhaps based upon p14arf) to count divisions. Similar senescence pathways are now being reported in humans, and this, coupled with the demonstration of tissue-specific telomeric loss rates, has the potential to render strategies based on the use of telomerase dependent on the characteristics of the target tissue.

3.1.1. *hTERT expression and cell cycle checkpoints*

Ectopic expression of the human telomerase reverse transcriptase (hTERT) a subunit of telomerase, can immortalize normal human as well as A-T fibroblasts [16, 17, 51]. Several groups have shown that the p53-dependent G1 checkpoint in response to ionizing radiation was intact in normal human fibroblasts expressing hTERT [17, 71, 72]. Further it is investigated whether expression of hTERT could correct the cell cycle

checkpoint defects. Within this context, A-T cells which are defective in DNA damage induced cell cycle checkpoints [73-75] were immortalized with hTERT. Wood et al., [17] determined whether telomerase activity affects the DNA damage induced cell cycle response in A-T fibroblasts and found ionizing radiation treated normal fibroblasts expressing hTERT showed a significant decrease in S phase entry indicating G1 arrest, while there was not a significant decrease in S phase entry in irradiated A-T cells. The loss of the G1 checkpoint seen was similar to the parental without hTERT indicating that this phenotype was preserved in hTERT immortalized A-T cells. The level of telomerase activity in fibroblasts derived from A-T patients with ectopic expression of hTERT were two to three fold higher that of normal fibroblasts with ectopically expressing hTERT.

In addition to the G1 checkpoint, ionizing radiation causes a transient inhibition of DNA replication. Cells from A-T individuals and Atm deficient mouse fibroblasts exhibit radioresistant DNA synthesis (RDS) [76, 77]. Normal fibroblasts expressing hTERT showed an inhibition in DNA synthesis similar to their parental cells following ionizing radiation exposure [17]. In contrast, both the parental and hTERT+A-T cells showed radioresistant DNA synthesis. These results indicated that hTERT expression extends the proliferative life span of A-T cells without altering the fundamental phenotype, as characterized by loss of ionizing radiation-induced cell cycle checkpoints. These studies suggested that hTERT does not influence the damage response cell cycle checkpoints as the cells expressing hTERT have an intact p53-dependent G1 checkpoint in response to ionizing radiation.

3.2. Telomerase activity and genomic stability

Telomerase expression in mammals is ubiquitous in germ cells, embryonic tissues and downregulated in most of the somatic tissues. Telomerase activation is controlled by cellular proliferation. Telomerase is able to elongate DNA termini that are not complementary to its RNA template sequence and is implicated in the healing of chromosome breaks by direct addition of (TTAGGG)n repeats to stabilize the ends. The question whether telomerase activity influences genomic stability, was addressed by Vaziri et al., [78] by analyzing chromosomal aberrations employing spectral karyotyping in isogenic cells with and without hTERT expression. This analysis used colored fluorescent chromosome-specific paints that provide a complete analysis of the human chromosomal complement. Thus, chromosomal rearrangements can be identified by the juxtaposition of different colors along a single chromosome. The frequency of aberrations found in cells with hTERT were comparable to parental cells without hTERT. Furthermore, Wood et al. [17] investigated the DNA and chromosome damage and radiation sensitivity in isogenic cells with and without hTERT expression. Neither the G1 nor G2 type of chromosome damage was different among cells with and without hTERT, suggesting that hTERT does not seem to have any specific influence on the repair of the chromosome damage [17]. The levels of chromosome aberrations in A-T cell lines were higher than the normal cells, indicating the defective chromosome repair is not corrected in A-T cells by the ectopic expression of hTERT. DNA strand break rejoining activity was also identical in cells with and without hTERT expression.

Although one cannot rule out the possibility that telomerase is involved in the addition of (TTAGGG)n repeats to sites of double-strand breaks, one cannot detect a measurable physiological difference between the isogenic cells with and without telomerase in response to ionizing radiation. These studies suggested that hTERT expression has minimum influence on DNA repair or the cell cycle check point functions.

3.3. Telomerase activity and cell survival

Although activation of telomerase by ectopic expression of hTERT had minimal influence on DNA repair and cell cycle checkpoints, Wood et al., [17] and Vaziri et al., [78] addressed the question whether expression of hTERT influences cell survival after treatment with ionizing radiation. The presence of hTERT slightly improved the survival of some cell lines. This probably reflects the fact that the such cells were assayed only 8-10 doublings prior to senescence, when clonogenicity in the absence of treatment is already compromised in the hTERT- control cells. The mild change in clonogenicity in the irradiated hTERT-expressing cells probably reflects the reduction of the signal from too-short telomeres rather than any fundamental change in the damage-response pathway. These results demonstrate that expression of telomerase does not significantly influence the radiosensitivity of A-T patient fibroblasts.

Several investigators have made an attempt to determine whether telomerase activity correlates with apoptosis or cell growth or survival. Zhang [79] reported that telomerase activity is not related to apoptosis in leukemia cell lines. However, Kanaya et al., [80] demonstrated that adenovirus expression of p53 represses telomerase activity through downregulation of human telomerase reverse transcriptase transcription. It has been shown that retinoic acid extends the *in vitro* life span of normal human oral keratinocytes by decreasing p16(INK4A) expression and maintaining telomerase activity [81]. Inhibition of telomerase limits the growth of human cancer cells [82]. Sawant et al. [83] found ionizing radiation treatment decreases telomerase activity in a dose-dependent manner, which correlated with cell death in *in vitro* tests as well as during tumor regression in nude mice.

4. Telomerase and cell cycle regulators

Extension of telomeric DNA by telomerase is coupled to DNA replication. Several investigations have established a link between activity of DNA polymerase function and telomere length. Carson and Hartwell [84] revealed a connection between a temperature-sensitive DNA polymerase alpha subunit mutant of yeast, which at the semipermissive temperature had telomeres that slowly lengthened. Adams and Holm [85], demonstrated a mutation in the large subunit of replication factor C also lengthened telomeres. Subsequently, Marcand et al., [86] reported that the abruptly shortened telomere becomes elongated in cells during late S phase, a process also dependent on ongoing DNA replication of the telomeric DNA. Processes that act on the telomeric DNA include its replication by a combination of DNA-template DNA replication and elongation of G-overhang (single stranded DNA) by telomerase. Most of the double

stranded telomeric DNA is replicated like the rest of the bulk chromosomal DNA. In yeast, bulk telomeric DNA is copied late in S phase [87]. The late-replicating property is governed by the presence of terminally located cis-acting telomeric repeats, and an epigenetic state that is set in early G1 phase of the cell cycle [88].

Although the processing of the increase in the size of the single-stranded 3' overhang of telomeric DNA takes place in yeast in late S phase, but the deletion of an essential telomerase component does not effect the increase of the single-stranded 3' overhang. Thus there are some other active processes ongoing to maintain the 3' overhang of the telomeres even in the absence of telomerase. In most human cancer cells and immortalized cells the maintenance of telomeres is linked to the presence of telomerase. Most of these cell types do have 3' overhangs. About 10% of tumor cell lines and 30% of spontaneously immortalized human fibroblast cell lines lack detectable telomerase activity as determined by *in vitro* analysis of their cell extracts [18, 57, 89-92].

Different reports have appeared concerning the cell cycle regulation of telomerase activity. Some studies have suggested that telomerase activity is regulated at each stage of the cell cycle [93], yet others have found that telomerase activity does not vary significantly at the different stages of the cell cycle [59, 94]. There is also a controversy whether the quiescent cells have telomerase activity. A situation where cells are pushed to the stage of terminal differentiation by the agents like retinoic acid also show downregulation of the telomerase activity. However it is not yet clear how telomerase activity is switched off in such cellular systems. Since telomerase has been implicated in the maintenance of telomere length, it is assumed that telomere elongation occurs during S phase of the cell cycle. Most of the replication enzymes are active in S phase. Whether telomerase is active only during S phase and not in the other phases several laboratories have attempted to address this issue. In the ciliate *Tetrahymena,* alteration of a single nucleotide in the T_4G_2 telomeric repeat sequence disrupts cell division and causes cellular senescence [94]. Cell-cycle dependent telomerase activity has been reported in yeast as single-stranded telomeric DNA appears on telomeres specifically during S phase [87]. It has been suggested that telomere replication may serve as a checkpoint for completion of the S phase [95]. In the micronucleus of the ciliate *Oxytricha nova*, the telomeres are bound to a heterochromatic telomere protein that is phosphorylated by cyclin dependent kinases [96]. Further progression through the M phase involves phosphorylation of a set of proteins including histone H1, nuclear lamins, caldesomon and vimentins. It is possible that Cdc2/cyclin A and Cdc2/cyclin B kinases may be required for the regulation of telomerase activity. However this assumptions is not yet supported by the experimental evidence as it has been demonstrated that extracts from S-phase cells of Xenopus with no Cdc2 kinase activity and M-phase extracts with high Cdc2 kinase activity both have identical levels of telomerase activity per unit of protein as determined by *in vitro* assay [50]. A similar situation was found when telomeras activity was compared in S- and M phase cells of HeLa cells or NIH3T3 cells [97]. However, telomerase activity in lymphocytes was present in S-phase cells but not in G_0-phase cells [97]. Furthermore, in quiescent mouse cells arrested by either serum deprivation or growth to confluency, telomerase activity was reported to be similar to those in exponentially growing cells [50]. Holt et al., [59] found no variation in telomerase activity during the cell cycle, in studies carried

out on FACS sorted cells. Furthermore, a decline in telomerase activity was observed in cells whose growth rate was reduced from seven to eight population doublings per week to one to two doublings per week. Thus, telomerase activity correlates with growth rate and is repressed in cells that exit the cell cycle and become quiescent. A different situation was reported by Zhu et al., [93] who reported that highest levels of telomerase activity were detected in S-phase cells. They found an increase of 2 to 3 fold in telomerase activity during S phase cells in that were synchronized by treatment with thymidine/aphidicolin or hydroxyurea. Surprisingly they also reported cells arrested at G2/M phase of the cell cycle were almost devoid of telomerase activity. This group of investigators attempted to demonstrate that cell cycle blockers (e.g., transforming growth factor 1 and various cytotoxic agents), also caused inhibition of telomerase activity. However it is not clear how the cell cycle blockers could down regulate telomerase activity. It is possible that if the cell cycle regulators are protein modifiers like kinases or acetyltransferases, the presence or absence of such factors could effect the stability and the enzymatic function of telomerase. But the most probable factor seems to be whether the cells are differentiated or physiologically dead such that the telomerase complex is degraded. It will be of great interest if any of the cell cycle regulators interact with the component of telomerase and thus influence its enzymatic role, thus a new area of research will emerge for the identification of new targets for telomerase inhibition.

4.1. Telomerase activity throughout the cell cycle

In any somatic dividing mammalian cell, the replication of the entire genome and the division of the cell into genetically identical daughter cells can be broken down into two components: a stationary phase and a kinetic phase. Every cell has a stationary phase and the duration of this phase depends upon the differentiation status of the cell. If the stationary phase is dominant then the cells exit from the kinetic phase. In general a proliferating somatic cell has four distinct cell cycle phases (G1, S G2 and M). In proliferating cells, molecular events in G1 phase prepare the cell for the synthesis of new DNA. Now the question arises whether the components of telomerase are also synthesized during this phase and how they are constituted.

The regulation of the telomerase activity can be divided into two states: an assembly state and a replication state. The former state allows the assembly of the RNA component with the proteins, but this assembly is not sufficient to initiate the synthesis of the telomeres until other critical factors are supplied such as G1/S cyclin/cdk activity. The latter state allows telomere synthesis from preassembled origin of replication complex. The loss of the cyclin/cdk activity at the end of mitosis has shown very little effect on telomerase activity.

One way to look for the regulation of the components of telomerase is to find a link between the factors that arrest cells in Go phase. This phase of the cell cycle is achieved when a cell does not traverse G1. *In vivo*, lymphocytes or epithelial cells which are normally arrested in a quiescent phase can be induced into proliferative phase by agents like mitogenic factors. These cells in Go phase do not show any telomerase activity. *In vitro* it is clear that the complete transit across G1 phase is governed by

the presence of extracellular growth factors that allow the cell to bypass the restriction point. Biochemical events that are concurrent with passage through this restriction point include the hyperphosphorylation of Rb protein, the functional activation of E2F-1, loss of several cyclin-dependent kinase inhibitors and subsequent accumulation of a certain threshold level of cyclin/cdk activity. It has been demonstrated in the past that forced Rb gene expression causes cells to arrest in G1 phase Rb protein becomes progressively hyperphosphorylated as cells pass from G1 into S phase [98, 99]. Although Rb phosphorylation has been demonstrated to be important in regulation of cell cycle control, it may not be the sole pathway to control the restriction point because cells without Rb can have intact G1/S restriction points. Several investigations are aimed at finding a link between the expression of the component of the telomerase or the activation of the telomerase and the phosphorylation status of the Rb protein.

Several proteins that interact with Rb protein have been identified, however, as yet it is not known that the telomerase complex or the components of telomerase interact with Rb. One of the proteins that interacts with the Rb is E2F-1 and is central to how Rb prevents cell proliferation. E2F-1 is particularly important for regulating the G1 to S transition. As a transcriptional factor E2F-1 activates the expression of a variety of genes, many of which encode transcription factors or proteins that are involved in DNA synthesis.

4.1.1. *Proliferation status and telomerase activity*

The major task of the cell division cycle is to have error free DNA replication in order to segregate the replicated DNA equally to daughter cells during mitosis or meiosis stage 2. Cell cycle transitions are controlled by holoenzymes that contain catalytic (cdk) and regulatory subunits (cyclin). These most likely exist as higher order complexes that include additional proteins. Early embryonic cell cycles exhibit rapidly alternating S and M phases without gap phases between them. Whether the reduced gap phases seen in somatic cell cycle has any link with cellular differentiation is not yet known. It is surprising that many of the cell cycle regulatory genes that are essential for cell cycle control are proteins whose inactivation does not seem to have any influence on telomerase activity. Although there may not be a direct correlation between the cell cycle regulatory elements, it is believed that cells which use Myc or loss of RB/p53 to circumvent senescence will eventually face frequent genome instability partly due to the loss of the chromosome ends. Such cells require a mechanism to maintain the telomeres. In this context, whether telomerase is an oncogene or a tumor suppressor has been addressed by several groups. Wood et al., [17] examined the effect of hTERT expression on proliferation markers and tumorigenesis in fibroblasts derived from ataxia telangiectasia (A-T) and normal individuals. Since biochemical studies have established an association between telomere maintenance by hTERT and cellular immortalization. In most transformed cell populations, upregulation of telomerase is required for immortality, and telomerase activity is detected in most human tumors. Wood et al. [17] found that there were no significant differences in the expression of PCNA, an accessory factor of DNA polymerase that reflects the proliferative activity of the cells. However, there was a significant increase in protein levels for ER, PgR,

HSP and ErbB-3 both in A-T and normal fibroblasts with ectopic expression of hTERT compared to fibroblasts without hTERT expression, suggesting upregulation of these genes might be required for immortalization, but not for the tumorigenic transformation. The levels of tumor suppressor genes like Tsg101 and ErbB-2 were similar in A-T and control cells with and without ectopic expression of hTERT gene. Furthermore, no tumors were formed by injecting hTERT+A-T cells or hTERT + normal cells in Nu/Nu mice. The current data does not support the possibility that telomerase is an oncogene or a tumor suppressor. The evidence for such conclusions have come from several different groups as well. However, there is evidence that some of the oncogenes interact with hTERT and thus regulate telomerase activity.

4.1.1.1. *Rb protein*

Bypass of senescence can be accomplished by inactivating the function of genes involved in tumor suppressor pathways (Figure 2). For example retinoblastoma protein (Rb) regulates the G1/S transition in the cell cycle [100, 101]. Rb interacts with a family of cell cycle translational factors known as E2F. Inactivation of Rb/p16 and p53 allows continued proliferation which may be accompanied by further telomere loss [21, 49]. In such situations stabilization of telomeric repeats may be prerequisite for tumorigenesis. It has been proposed that oncogenes might have a role to play in maintaining the telomeres and thus these may be involved in the activation of the components of telomerase. Within this context several laboratories have made an attempt to determine whether expression or inactivation of such oncogenes could activate telomerase.

The expression of hTERT can prolong the life span of the several different cell types. Some of these cells become immortalized where as some of the cells need additional genetic alterations for the immortalization. Kiyono et al. [55] found that ectopic expression of hTERT in some primary epithelial cells is not sufficient to immortalize. They reported that expression of hTERT is a primary event in the process of immortalization which is followed by a second step involving the inactivation of the Rb/p16 pathway. This suggests that Rb/p16 may be indirectly involved in the regulation of the telomerase and this regulation may be at the post transcriptional levels.

The speculation that Rb/p16 may be involved in the regulation of telomerase came from the studies on the human primary keratinocytes and fibroblasts. Either human papilloma virus (HPV) E6, which targets p53 for degradation or E7 which inactivates Rb protein, can extend the lifespan of cells. E6 plus E7 both together are more efficient in immortalization of cells. E7 expression resulted in high levels of p16 [55] and reduced Rb, although some phosphorylated Rb remained. Kiyono et al., [55] and others are of the opinion that inactivation of the Rb/p16 pathway is critical for immortalization of epithelial cells. Since most of the immortalized cells have been reported to have telomerase activity, it seems that E7 or E6 cooperates with hTERT protein. This seems to be consistent with situations where E7 proteins are mutated and thus are impaired in Rb binding, such cells do not immortalize. However, inactivation of Rb protein does not seem to be essential for the immortalization of the cells as there are several immortalized cell types that have intact Rb function.

4.1.1.2. *p53 protein*

The direct test of whether p53 is involved in the regulation or activation of telomerase came from studies of Kiyono et al., [55] when they demonstrated that 16E6-8S9A10T (HPV), which does not target p53 for degradation but was able to induce telomerase activity, however 16E6Δ146-151, which lacks the hDLG-binding motif induces telomerase. Further, 16E6Δ146-151 which eliminated p53 did not induce telomerase activity. These studies indicate that oncoproteins are inducers of the telomerase activity without the involvement of p53 protein. It is consistent with the view that telomerase may be required during certain stages of development and this expression may be regulated by oncoproteins which are expressed during the development.

4.1.1.3. *c-Myc protein*

Several oncogenes e.g., mdm-2, E7, E6, activated Ras (V12), cyclin D1 cdc24A and cdc25C that were ectopically expressed in human mammary epithelial cells (HMEC) failed to induce telomerase activity. Besides, these genes, expression of dominant -negative p53 allele or introduction of E6 failed to activate telomerase. However, introduction of c-Myc expression was able to stimulate telomerase activity in HMCEs, IMR-90 or WI-38 cells. The c-myc gene encodes a nuclear protein whose expression is closely associated with the proliferative state of many mesenthymal cells. It induces a quick passage through G1 of the cell cycle in order to make cells independent of growth factors and cellular proliferation. It is activated during oncogenesis by proviral insertion, chromosomal translocation and gene amplification. The levels of c-myc are controlled by the action of growth factors. The constitutive expression of c-myc in certain cell types relieves dependence on the growth factors dependent entry into cell cycle. Constitutive expression of c-myc is also able to block the differentiation of several cell types, particularly the cells from hemopoietic lineage. These observations suggest that c-myc plays role in the regulatory network that control cellular proliferation and differentiation. How c-myc affects the cellular growth-regulatory network in the process of immortalization has been the focus of several research groups (Figure 3). Kinoshita et al., [102] have found that E6 can activate the Myc promoter. Wang et al., [103] addressed the question whether E6 regulated the telomerase activity through an effect on Myc expression. Surprisingly, expression of E6 in HMECs induced Myc to levels similar to transduction of HMECs with a Myc retrovirus. Interestingly, E6-induced alterations in Myc protein did not reflect changes in the abundance of Myc mRNA. These observations suggest that Myc expression must be controlled post-transcriptionally by E6 in HMECs. The situation in IMR-90 cells was different where Myc levels remained unaltered following expression of E6 and thus it was incapable of activating telomerase. This suggested that E6 may regulate telomerase by other mechanisms, and therefore there may be cell type specific inhibitors or activators in which E6 regulates telomerase in HMECs by altering the levels of Myc.

4.1.1.4. *c-Abl protein*

The c-Abl proto-oncogene encodes a protein tyrosine kinase that is distributed in the nucleus and the cytoplasm of proliferating cells. In the nucleus, c-Abl protein is negatively regulated by the retinoblastoma protein (Rb) and positively regulated by DNA damage signals. The Abl protein contains the characteristic protein kinase domain

and the Src homology domains SH3. SH2 [104]. c-Abl has a nuclear localization signal [105] and a nuclear export signal, and also interacts with the cytoskeleton because c-Abl contains binding sites for actins. Most interestingly, c-Abl is associated with the chromatin [106] because it contains three HMG-like boxes (HLBs) that preferentially bind to A/T duplex and distort DNA structure [107, 108]. The c-Abl tyrosine kinase is essential to the proper development of a mouse, because c-Abl-knockout mice exhibit embryonic and neonatal lethality which can be rescued by the transgenic expression of c-Abl cDNA [109].

The ubiquitously expressed c-Abl protein tyrosine kinase is tightly regulated in cells [110, 111]. c-Abl is activated by ATM in cells exposed to ionizing radiation and other DNA-damaging agents [112]. c-Abl-deficient cells are resistant to DNA-damage-induced apoptosis [113]. Perusal of the literature indicates that c-Abl confers growth arrest and proapoptotic responses to DNA damage by mechanisms that depend partly on p53 and its homolog p73 [104]. It also functions as an upstream effector of the Jun N-terminal kinase/stress-activated protein kinase and p38 mitogen-activated protein kinase pathways [104]. Since c-Abl is activated by DNA double strand breaks [114] and several proteins involved in the repair of DNA damage also function in telomere control [115-117]. It was expected that c-Abl may interact with the telomere maintaining players. Kharbanda et al. [118] found that c-Abl associates with the catalytic unit of hTERT in human cell lines. They also found that endogenous hTERT is detectable in anti-c-Abl immunoprecipitates from MCF-7 cells stably overexpressing a kinase-inactive form of c-Abl, c-Abl(K-R) and this further confirmed that the association of c-Abl with hTERT was independent of the c-Abl kinase function. Although the binding of c-Abl with hTERT is independent of c-Abl kinase function, when lysates from 293T cells transfected with HA-hTERT are incubated with glutathione-S-transferase (GST) fusion proteins containing c-Abl (GST-c-Abl) or the Src homology domain of c-Abl (GST-Abl SH3), hTERT binds to GST-c-Abl and GST-Abl SH3; however there was no detectable binding of hTERT to a GST-Grb2 fusion protein that contained the amino-terminal SH3 domain. Direct interaction of hTERT (amino acids 308-316) and c-Abl SH3 has been established [118]. Since c-Abl is a tyrosine kinase, it has been shown that ionizing radiation induces tyrosine phosphorylation of hTERT by a c-Abl-dependent mechanism. The functional significance of the interaction between c-Abl and hTERT was investigated by assay of telomerase activity. The telomerase activity was inhibited in cells expressing wild type c-Abl compared to cells expressing mutant version of c-Abl (K-R) [118].

The role of regulation of telomerase activity by c-Abl has been further complemented by examining the early-passage MEFs deficient in c-Abl. Such cells have relatively high telomerase activity as well as long telomeres [118]. These studies seem to be consistent with yeast, wherein the Rap1p protein binds to telomeric DNA and negatively regulates telomere length [7]. The function of Rap1p in telomere regulation is mediated by Rap1-interacting factors, known as Rif1 and Rif2 [7]. Telomere repeat-binding proteins implicated in regulation of telomere length have been identified in *Schizosaccharomyces pombe* (Taz1p) [119], in human cells (hTRF1) [120] and in Chinese hamster cells (chTRF1) [121]. Such genes negatively regulate telomerase activity as the target site by limiting the access of telomerase for the extension of G-over hang and thus

maintaining the length of the telomeres. However, c-Abl regulates telomerase activity by phosphorylation of hTERT and thus negatively regulates telomere length.

4.1.1.5. *14-3-3 proteins*

The 14-3-3 family of proteins play a key regulatory role in signal transduction, checkpoint control, apoptotic, and nutrient-sensing pathways. The 14-3-3 proteins appear to modulate the activity of a large variety of functional proteins and enzymes, many of which are involved in control of cell cycle, cell death, and mitogenesis [122, 123]. The 14-3-3 proteins are thought to function as adaptor proteins that allow interaction between signaling proteins that do not associate directly with each other [122]. 14-3-3 proteins act by binding to partner proteins, and this binding often leads to the altered subcellular localization of the partner and thus promote the cytoplasmic localization of binding partners, which include cell cycle regulatory phosphatase, Cdc25c as well as catalytic subunit of telomerase (TERT). 14-3-3 proteins are highly acidic dimeric intracellular proteins that chiefly bind to phosphoserine motifs. Some well described 14-3-3 binding partners include the protein kinases Raf-1, KSR-1, Ask1, MEKK1, Bcr, calcium/calmodium kinase, protein kinase C, the protein phosphatase Cdc25C, c-Cbl, BAD, A20, transcriptional factors like FKKHRL1, MSN2, MSN4, glucocorticoid receptor (GR), Tlx-2, NF-AT and the telomerase catalytic subunit, TERT [124]. One of the isoforms of 14-3-3 protein is 14-3-3σ gene, which was originally identified as an epithelial-specific marker, HME1, which was downregulated in a few breast cancer cell lines but not in cancer cell lines derived from other tissue types [125]. Recent data indicate that the expression of 14-3-3σ is lost in 94% of breast tumors [126]. At the functional level, the 14-3-3σ protein has been implicated in the G2 checkpoint [127]. For instance, its association with different kinases in the cytosol and on the nuclear membrane may contribute to kinase activation during intracellular signaling [128], and the protein appears to sequester the mitotic initiation complex, cdc2-cyclinB1, in the cytoplasm after DNA-damage. The latter prevents cdc2-cyclin B1 from entering the nucleus, where the protein complex could normally initiate mitosis. Thus, 14-3-3σ has been implicated in maintaining a post-DNA-damage G2-arrest, thereby allowing for DNA repair [129]. Such cell cycle checkpoints are considered to be the guardians of genome integrity, with their abrogation contributing to reduce genomic stability.

14-3-3σ is highly specific for stratified epithelia [130, 131]. Dellambra et al., [131] reported that dowregulation of 14-3-3σ is accompanied by the maintenance of telomerase activity and by a strong downregulation of the p16INK4a tumor suppressor gene. Interestingly inactivation of 14-3-3σ in keratinocytes leads to maintenance of telomerase activity [131]. This maintenance is accompanied by the downregulation of the p16INK4a tumor suppressor gene in keratinocytes. 14-3-3σ can also act as a p53-regulated inhibitor of the G2/M progression phase of the cell cycle after DNA damage. There seems to be consensus that cell cycle, senescence, and cell differentiation can be regulated by common molecular pathways which, however, can act independently.

Another cell cycle regulatory gene that may have some influence on the regulation of the telomerase activity is Id2. Id proteins inhibit the functions of transcription factors in a dominant-negative manner by suppressing their heterodimerization partners through

the HLH domains. Members of the Id family also promote cell proliferation, implying a role in the control of cell differentiation. This is based upon the fact that cell-cycle progression induced by Myc oncoproteins requires inactivation of Rb by Id. This is supported by the fact that in neuroblastoma, an embryonal tumor derived from the neural crest, Id2 is overexpressed in cells carrying copies of the n-myc gene. The overexpression of Id2 results from transcriptional activation by oncoproteins of the Myc family. Cell-cycle progression induced by Myc oncoproteins require inactivation of Rb by Id2 [132]. Thus a dual connection links Id2 and Rb: during normal cell-cycle, Rb prohibits the action Id2 on its natural targets, but oncogenic activation of the Myc-Id2 transcriptional pathway overrides the tumor-suppressor function of Rb.

The other gene product that has been reported to influence telomerase activity is PTEN. Restoration of wild-type PTEN expression leads to apoptosis, induces differentiation, and reduces telomerase activity in human glioma cells [133].

4.2. Telomerase activity and cell signaling proteins

As described earlier, several laboratories are attempting to determine whether telomerase a tumor suppressor or an oncogene. With this regard ectopic expression of hTERT has been demonstrated to prevent replicative senescence in several normal cell types including fibroblasts and epithelial cells [16, 51, 103]. Recently it has been demonstrated that TERT may exert its anti-apoptotic action at early stage of the cell death process prior to mitochondrial dysfunction and caspase activation [53]. Ectopic expression of hTERT in human keratinocytes and mammary epithelial cells has been reported to induce loss of p16-dependent cell cycle control and thus to immortalization, however, this data is subjected to other interpretations [54, 55]. These studies support the hypothesis that replicative senescence results from telomere shortening. It has been proposed that telomere shortening during replicative aging of some cells finally generates antiproliferative signals which accumulate p53 protein accompanied by the G1 arrest, frequently observed in senescent cells. However in the presence of T antigen or E6, known to bind p53 protein, telomere shortening signals is bypassed, leading to extension of the life span, which may or may not be accompanied by the genomic instability. One way to prevent the antiproliferative signal is to maintain the telomere length equilibrium. It is possible that expression of telomerase prevents the antiproliferative signal generated by telomere shortening at senescence. Vaziri et al. [78] suggested that prevention of the p53-mediated antiproliferative signal in response to telomere shorting allows cells to divide further. The extension of the life span by SV40 large T antigen, however, relies on inactivation of p53 and pRb proteins, and telomere shortening continues to persist until crisis. This suggested that extended growth of cell by forced telomerase expression may thus not interfere with the p53-dependent signaling pathway, and therefore cells immortalized with ectopic expression of hTERT retain the genomic integrity. Furthermore, direct analysis was made by Wood et al. [17] to determine whether expression of hTERT influences the ATM (ataxia telangiectasia mutant gene) functions, which has been implicated in cell signaling.

4.2.1. *ATM protein*

Replicative senescence can be accelerated by specific mutations that cause some human diseases. Among them is Ataxia-telangiectasia (A-T) which is a rare autosomal recessive disorder characterized by progressive cerebellar degeneration, premature aging, growth retardation, specific immunodeficiencies, genomic instability and gonadal atrophy [134-136]. A-T patients have an increased sensitivity to ionizing radiation and an elevated incidence of cancer. Cells derived from A-T patients show an increase in chromosome end-to-end associations, suggesting defects with their telomeres [137-139]. The gene mutated in A-T, ATM, encodes a protein kinase that is distantly related to the yeast MEC1 and TEL1 proteins that function in maintaining the integrity of telomeres [140]. ATM has been shown to activate throughout the cell cycle and phosphorylate a number of nuclear proteins, including the nuclear c-Abl tyrosine kinase [141-143], the tumor suppressor protein p53 [144, 145], the breast cancer susceptibility gene product BRCA1 [146], the human checkpoint kinase hCds1/chk2 [147-150], and the Nijmegen Breakage Syndrome gene product NBS1 [151]. This network of ATM-regulated phosphorylation events regulate cell cycle checkpoints and contribute to the processing of DNA double strand breaks following ionizing radiation [104; 152]. To determine precise mechanism by which ATM may regulate the structure and function of telomeres is currently under intensive investigation. The downstream targets like c-Abl has been shown to negatively regulate the telomerase activity. Lymphoblastoid cells derived from A-T individuals have been shown to have relatively higher telomerase activity as compared to the normal controls [137]. Recent studies have demonstrate that expression of the catalytic subunit of telomerase extends the lifespan of fibroblasts derived from A-T individuals without changing their phenotypic properties [17]. The parental A-T primary fibroblasts exhibited the hallmark characteristics of senescence quite early during culture including the increase in size, appearance of SA-Gal staining and cessation of DNA replication. Though expression of hTERT extended the lifespan of A-T cells, however occasional appearance of SA-Gal cells was still observed. DNA damaging agents (ionizing radiation, bleomycin) are known to induce SA-Gal positivity [153]. It has been suggested that the occasional SA-β-Gal (+) cell in the hTERT cells reflects the consequences of DNA damage (chromosome end-to-end associations) events in the A-T cells. The presence of end-to-end chromosome associations suggested that such cells could have still altered telomere nuclear matrix interactions as has been reported previously [139], and such altered telomere interactions may influence the senescence signaling mechanism. Alternatively, though hTERT lengthened the mean telomere length in A-T cells, it may be possible that a separate triggering event occurs in conjunction with the loss of ATM expression leading to the low level but continual entry into senescence of the hTERT+ A-T cell population. These hTERT+ A-T cells continue to grow slowly as compared to normal human fibroblasts expressing hTERT. These studies suggested that though hTERT can extend the lifespan, the overall growth properties of the cells are not affected. These studies also suggested that the additional loss of ATM signaling with telomerase expression is still not sufficient to transform cells and that further genetic changes are necessary for malignant transformation. Cells with introduced telomerase are not cancer cells

since they have not accumulated the other changes needed to become cancerous. This indicates that telomerase-induced telomere length manipulations may have utility for tissue engineering and for dissecting the molecular mechanisms underlying genetic diseases including cancer.

5. Prospects

Information gathered to date suggests that telomerase is neither a tumor suppressor nor oncogene. What then is the role of telomerase in carcinogenesis and what turns telomerase on and off *in vivo*? Is there any correlation with the cell cycle activation of telomerase? Although evidence is accumulating that activation of telomerase has no influence on the cell cycle check point genes nor the other way around, its activation certainly breaks the barriers of limited replicative life span. Because of the fact that in normal human cells, insufficient telomerase activity and a finite store of telomeric DNA limit the number of divisions a cell can undergo before critical shortening of telomeres which signals entry to replicative senescence, which is dictated by a finite capacity for cell division. Where then lies the fountain of youth?

The current major focus is to extend human life span and to improve the quality of life. Jennings et al., [154] proposed that telomeres in addition to their role in counting cell division, telomeres through their GGG sequences are also important monitors of oxidative damage over the life span of cell. Introduction of telomerase could have potential relevance in the event that in human subject organ damage occurs from accumulation of senescent cells, which at the end of their replicative life span are resistant to apoptosis. The accumulation of such cells in an organ may lead to decline in organ function and contribute to disease. Indeed, senescent cells display altered differentiated functions which may be detrimental to tissues: depending on the cell type, there may be over or under expression of different proteins. Such changes from matrix-producing to matrix-degrading functions of the cell may contribute to decline in organ function and thus lead to disease. Introduction of telomerase catalytic unit into human cells under a proper control may lead to therapeutic benefit for individuals with organ damage such as the skin atrophy, atherogenesis or macular degeneration. There have been concerns about applying the results of cell cultivation studies *in vitro* to cellular events in aging human organs, but it is possible that a controlled transient expression of telomerase may prove to be a fountain to maintain relative youth.

Some human cells are now known to count divisions by monitoring the progressive attrition of telomeres, leading to the activation of a p53-p21 waf-dependent G1 checkpoint. Ectopic expression of telomerase has been shown to prevent senescence in several cell types and thus offers the potential for interventions in the aging process based on tissue engineering, gene therapy or homeografts.

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7. References

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CHAPTER 5

CELL. PROLIFERATION, TELOMERASE, AND CANCER

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Table of contents

Summary

The close association of telomerase activity with human cancer indicates its importance in the immortalization and transformation processes. Telomerase is associated with over 85% of all human malignancies, making it currently one of the most prevalent molecular markers. The primary role of telomerase in immortal and cancer cells is to provide the cell with telomeric stability and prevent telomere shortening-induced growth arrest.

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Telomerase activity has been tightly correlated with cell proliferation in that telomerase is either down-regulated in cells that exit the cell cycle or activated upon re-entry into the cell cycle. Clearly, the association of cancer and telomerase suggests the possibility of telomerase inhibitory therapy, where inhibition would result in apoptosis or triggering either cellular senescence or differentiation. Therefore, repression of telomerase may provide a novel and specific adjuvant therapy for human malignancies. This enzyme, however, may also have important functions in the regenerative and repair processes, as well as be implicated in tumor suppression and vertebrate cloning. Telomerase is a multifaceted enzyme whose only known function is to maintain or elongate telomere lengths, a result that has far reaching effects on a variety of cellular pathways.

1. Introduction

Normal somatic human cells have a finite proliferative life span known as the Hayflick limit or cellular senescence [1]. This limited proliferative capacity has been shown to be the result of incomplete DNA replication at the end of chromosomes. Vertebrate chromosome ends are comprised of a highly repetitive sequence, TTAGGG, known as the telomere [2]. Without appropriate mechanisms to counteract the inadequate replication at the ends of linear chromosomes, telomeres shorten with successive rounds of DNA synthesis or every time a cell divides. This "end replication problem", first proposed by Olovnikoff [3] and then by Watson [4], is defined as the inability of conventional DNA polymerases to replicate to the end of a linear molecule. Harley and coworkers [5], followed independently by Hastie et al. [6] and Lindsey et al. [7], proposed that the end replication problem is directly responsible for the onset of replicative senescence. That is, the progressive loss of telomeric DNA results in the eventual induction of the senescence program (The Telomere Hypothesis) presumably because shortened telomeres may be recognized as damaged DNA. Shortened, "damaged" telomeres then signal a DNA damage response that results in the induction of the senescence phenotype involving the tumor suppressor p53 and other repair enzymes.

1.1. Proof of the telomere hypothesis

The cloning of the gene for the catalytic subunit of human telomerase, hTERT, proved to be one of the critically important pieces necessary for proving the validity of the telomere hypothesis [8-11]. Telomerase activity is absent in most somatic tissues, yet the RNA template, hTR, is ubiquitously expressed while hTERT appears to be the limiting factor for telomerase expression in normal cells [8]. If the telomere hypothesis is correct, expression of hTERT should allow for telomerase activation and telomere maintenance, which would prevent cellular senescence imposed by telomere shortening. Fortunately, this proved to be the case: ectopic expression of hTERT in fibroblasts and epithelial cells provided telomerase activity, maintained telomeres, and extended life span [12, 13]. This was the first direct experimental evidence demonstrating that telomere shortening was one of the critical components triggering the onset of cellular senescence. Subsequent experiments revealed that these cells with exogenous telomerase

maintained normal cellular characteristics without transformation, unlike what would have been expected if telomerase alone were oncogenic [14, 15]. Thus, telomerase expression in normal cells prevents replicative senescence by maintaining telomere length without progressing toward an immortal or transformed phenotype (Figure 1) (discussed in more detail below).

1.2. Immortalization and the role of tumor suppressors

The direct involvement of p53 and pRB in senescence has been shown using viral oncogene expression in normal cells, namely SV40 large T antigen and human papillomavirus E6 and/or E7 [16-18]. Expression of these oncogenes in normal fibroblasts or epithelial cells inactivates p53 and pRB functions and allows cells to bypass the senescence checkpoint, whereby telomeres continue to shorten. In this extended life-span phase, a miniscule subset of cells acquires the additional mutations necessary to overcome crisis and eventually immortalize. Those rare cells that are capable of immortalizing require a telomere maintenance mechanism, which most of the time is accomplished by activating telomerase activity [19].

Telomerase, a ribonuclear protein minimally consisting of an RNA template (hTR) and a protein component (hTERT), is found in the vast majority of human cancers and almost all human tissue culture-immortalized cells [20-22]. Alternative mechanisms for maintaining telomere length have been described (termed ALT pathway, alternative lengthening of telomeres) in some *in vitro*-immortalized cells [23, 24], but while the ALT pathway remains a viable immortalization option, it is rarely documented in primary human cancer. Even so, the activation of telomerase as cells progress remains the primary telomere maintenance pathway associated with immortalization and human tumors. A wide variety of human cancers express telomerase activity, including breast, brain, prostate, lung, liver, skin, and kidney, as well as many hematological malignancies [reviewed in 22] (Table 1). In fact, telomerase is one of the most prominent molecular cancer markers known, and because of this close association with most human malignancies, telomerase has great potential as a diagnostic aid and therapeutic target (discussed below).

Table 1. Association of telomerase with cancer*

Pathology	Frequency	Examples of Cell and Cancer Types
Normal	1-2%	Cells from renewal tissue, activated lymphocytes
Benign	25-30%	Fibroadenoma/fibrocystic disease, benign prostatic hyperplasia, hepatitis, cirrhosis, colorectal adenoma, leiomyoma
Malignant	84-85%	Breast, lung, prostate, liver, pancreas, colon, head and neck, bladder, brain, skin, lymphoma, leukemia, ovarian, cervical
Advanced	>95%	Breast, lung, liver, prostate, skin, bladder
"Cleared" Margins	5-7%	Lung, breast, brain, pancreas, renal

*taken from Shay and Bacchetti, 1997

2. The role of telomerase and cell proliferation

2.1. Telomerase and the cell cycle

Telomerase is an RNA-dependent DNA polymerase that functions to lengthen the 3' overhang at the telomere termini, providing additional DNA at the chromosome end thus allowing conventional polymerases to replicate further out on the linear chromosome. As a consequence of the end replication problem, telomeres will only shorten in normal somatic cells (telomerase negative) that have gone through a complete S phase (DNA synthesis) of the cell cycle. This suggests that cells that do not go through DNA synthesis or cell division will not experience telomere shortening. In other words, non-dividing cells have no need for a telomere maintenance mechanism; therefore telomerase would be unnecessary. In addition, in order for telomere maintenance to occur in dividing cells, it is possible that telomerase needs to be active in maintaining telomere length either prior to or early in S phase. However, extracted telomerase activity is observed at similar levels in every stage of the cell cycle [25]. But these experiments provides little insight regarding the ability of telomerase to maintain telomere lengths at each stage of the cell cycle. Additional, as yet undiscovered, regulatory mechanisms are likely responsible for modulating the accessibility and interaction of telomerase with the telomere.

2.2. Proliferation states

As discussed previously [25, 26], distinct subclasses of non-proliferative states exist for cells (Table 2). Two of these are irreversible and involve a defined change in gene expression: terminal differentiation and cellular senescence. While both processes entail irreversible growth arrest and resistance to apoptosis [27], other molecular alterations are only observed in senescent cells. For example, senescence typically involves a series of events triggered by telomere shortening and an increase in tumor suppressor function [27]. The prevailing dogma surrounding senescence suggests that a small fraction of shortened telomeres within a given cell signals a DNA damage response, which triggers an upregulation of tumor suppressor proteins and the subsequent growth arrest. However, distinct from the typical DNA damage-induced growth arrest (i.e., repair of double strand breaks with resumption of cell cycling) [28], damage recognized by the tumor suppressor proteins in a near-senescent cell is irreparable and leads to inactivation of cell cycle progression. Other non-proliferative states, most notably, quiescence and stress-induced growth arrest, exist as reversible phenomenon [25, 29]. Quiescence is characterized by a reversible exit from the cell cycle (G_0 state) that typically results following the removal of critical peptide growth factors or by elimination of mitogenic stimulation. A classic *in vivo* example of quiescence is the deactivation of lymphocytes [30]. Reversible, growth arrested states have also been described where cells have not exited the cell cycle. This unique non-dividing state can be triggered following certain drug treatments, DNA damage, or other stress-inducing signals [29].

Table 2. Defined non-proliferative states

State	Description
Terminal Differentiation	An irreversible exit from the cell cycle(permanent G_0 state)
Senescence	Age-related, telomere-dependent, irreversible exit from the cell cycle (permanent G_0 state)
Quiescence	A reversible exit from the cell cycle (G_0 state; typically induced by an absence of critical mitogenic signals)
Growth-Arrested	Non-dividing cells within the cell cycle (stress-induced arrest)

2.3. Telomerase and proliferation

In many tumor-derived and *in vitro*-immortalized cell lines, telomerase activity and cellular proliferation have been tightly linked. Numerous reports have shown that telomerase is down-regulated as cells undergo terminal differentiation [25, 31-33], senescence [25], and quiescence [25, 34, 35], as well as under certain growth arrested conditions (i.e., following drug treatment or DNA damage) where the cells remain in the cell cycle but are not dividing [29]. As discussed above, non-dividing cells would not require a telomere maintenance function, as they do not shorten their telomeres, resulting in a down-regulation of telomerase activity. In fact, activation of differentiation pathways causes a gradual decline in telomerase activity, an event that is irreversible [25, 31-33]. This down regulation also holds true during development and tissue differentiation appears to cause a down-regulation of telomerase activity [36]. Also, cells that undergo a quiescent state, down-regulate telomerase activity, but in a reversible manner. That is, if stimulated by growth factors or mitogens to re-enter the cell cycle and undergo cell division, telomerase is upregulated, and interestingly, expression of telomerase activity is not dependent upon DNA synthesis, as telomerase is activated prior to the onset of S phase [25, 34].

2.3.1. *Telomerase half-life*

The timing of telomerase down-regulation varies between the different non-proliferative states and may actually correlate with reversibility. For example, induction of differentiation in the promyelocytic leukemia cells, HL60, results in an immediate down-regulation of hTERT, followed by loss of telomerase activity within 24-48 hours [8, 25]. In contrast, tumor-derived cells that undergo reversible quiescence via growth factor removal also down-regulate telomerase but do so in more of a gradual manner. Interestingly, cells subjected to decreasing amounts of serum or growth stimulatory factor divide much more slowly and down-regulate telomerase activity accordingly as well. Therefore, a linear relationship appears to exist between telomerase activity and the rate of cellular proliferation: the slower a cell divides, the less telomerase it requires. In some of our half-life studies, activity is expressed in some cell lines without serum

or growth factor for well over 24 hours and at times for more than 7-10 days [25, 35]. Highly consistent with these studies is our recent data in MCF-7 cells indicating that hTERT is immediately repressed following acute adriamycin exposure, while telomerase activity persists for more than 7 days [37]. Thus, the half-life of the telomerase enzyme is quite long (well over the obligatory 24 hours) and the regulation of hTERT transactivation appears dependent on the mode of growth arrest. Of course, there are studies that have looked at repression of telomerase activity in tumor-derived cells after treatment with cell cycle inhibitory compounds, where they show no change in activity after 3 days of treatment for most drugs that arrest cells in the G1 phase [38]. Based on some of our kinetic studies, a decline in activity would be expected after 7-10 days of treatment. In some cases, chronic drug treatment resulted in an immediate, dramatic decline in activity [38], which was later shown to be due to cellular toxicity rather than exit from the cell cycle [35].

2.3.2. *Telomerase and tumor suppressors*

There are many studies that appear to show an effect on telomerase activity after ectopic expression of certain proteins, including tumor suppressors and oncogenes [39-44]. Most often, the repression of telomerase is an indirect effect of the over-expression of the protein of interest rather than a direct effect on hTERT or hTR transcription or telomerase activity. For instance, when p21 or pRB are over-expressed in cancer cells, the cells will most often exit the cell cycle, which will indirectly result in the repression of telomerase activity [39, 40]. This telomerase reduction is probably not directly related to hTERT transcription or telomerase function; rather it is likely a byproduct of the decline in the rate of proliferation or the cell cycle exit. A notable exception is p53-induced suppression of telomerase activity. Here, formation of p53-Sp1 complexes appears to interfere with the binding of Sp1 to the hTERT promoter, indirectly resulting in transcriptional downregulation of hTERT [42-44]. In contrast, overexpression of c-myc results in the activation of hTERT transcription and telomerase activity [41, 43, 45, 46]. Like p53, this may be an indirect transcriptional effect possibly involving Sp1.

2.4. The telomerase knockout mouse and proliferation

The RNA component of mouse telomerase (mTR) was homologously deleted from mouse embryonic stem cells in order to test the importance of telomeres and telomerase in senescence and cancer [47, 48]. It is critical to realize that unlike human systems, somatic cells in laboratory strains of mouse have telomerase activity and inordinately long telomere lengths, averaging 40-60 kb (compared to humans at 15-18 kb) [49]. Although telomeres shortened an average of 5000 bp per generation, no substantial phenotype was observed in these knockout mice after 5 generations [47], most likely due to their extremely long telomeres. However, the sixth generation mice (G6) were found to have impaired proliferation in stem cells from germ line and renewal tissues and the G6 females proved to be reproductively incompetent [48]. The hematopoietic system of self-renewing cells showed reduced proliferation ability without changes in

the anatomy of bone marrow, thymus or spleen. Taken together, these results indicate that telomere loss negatively affects the proliferation and self-renewal potential of regenerative cells and tissues in the mouse. In addition, these data also establish the fundamental requirement for telomere maintenance in highly proliferative cells and tissues of the mouse [26].

2.5. Telomerase and vertebrate cloning

Given their importance during development, telomerase and telomere maintenance would be expected to be a required element of the organismal cloning process. Many types of vertebrates have been cloned, including sheep, pigs, cattle, and goats [50-53]. In each case, the efficiency is extremely low, approaching the proverbial "needle in a haystack". When cloning these agriculturally important species, it seems necessary to reprogram the cells, whether differentiated or not, to start cell division. But not just any kind of proliferation; this type of cell division requires a telomere maintenance function, most likely provided by active telomerase. To achieve a successful clone, the progenitor cells must be capable of thousands and thousands of cell divisions without limits on cell growth imposed by gradually shortening telomeres. To this end, it is likely that these "cloning" scientists were fortunate enough, for whatever reason, to use the nucleus from a cell that was able to reprogram itself to derepress telomerase early during the developmental process to provide telomere maintenance. In some cases, it may have been a stem cell that was used to make the cloned animal, which would already have the ability to regulate telomerase activity and maintain telomere lengths. Yet, even with the use of a differentiated cell as was the case for porcine cloning [53], the single cloned animal that was made (appropriately named Xena) must have come from a cell with de-differentiating capabilities, which likely included telomerase activation. Thus, telomere maintenance provided by telomerase is most likely a necessary element for cloning of vertebrate species.

3. Telomerase in the detection and treatment of human cancer

3.1. Detection of cancer using telomerase

With the tight correlation between telomerase expression and cancer (Table 1), early detection of telomerase could aid surgical and cyto-pathologists in their search for less qualitative and more quantitative approaches to diagnosing borderline cases. In addition, certain lesions are typically diagnosed as pathologically benign, yet occasionally progress to a more malignant disease. In those instances, telomerase detection may be useful for pathologists to identify the atypical benign disease with malignant potential, which in turn, would allow for more aggressive therapy earlier increasing the likelihood for a disease-free recovery. One prime example of early diagnosis leading to a better prognosis comes from cases of bladder cancer, where over half of the early stage cases are missed due to ineffectual screening procedures. Using less invasive procedures like bladder washes or voided urine rather than biopsy, detection of telomerase identifies

nearly 90% of the missed bladder cancers [54-57], suggesting its utility in screening and diagnosis to aid pathologist with difficult cases.

3.2. Telomerase inhibition and cancer treatment

Since telomerase is necessary for immortalization and critical for continuous cancer cell proliferation, it has been postulated that inhibiting telomerase will specifically and effectively stop tumor growth. There are many avenues to pursue in terms of telomerase down-regulation as a therapy or as a marker for recurrence of disease. Inducing proliferative arrest, whether it be differentiation, senescence, or quiescence, may be critically important for treating many types of cancer, including lymphoma and leukemia where tumor resection is more problematic. Monitoring telomerase activation in these arrested cells after treatment would be useful in tracking resistance or recurrence of disease, which may become more aggressive and/or metastatic.

Many groups have been able to block telomerase in immortal and tumor-derived cells using a number of different methods. Repression of telomerase was first accomplished using microcell-mediated fusion of chromosome 3p into renal cell carcinoma cells, which resulted in gradual telomere shortening and reestablishment of cellular senescence [58]. This was followed closely be antisense expression of the integral RNA component of telomerase, hTR [59], to repress telomerase, as well as *in vitro* studies using small molecules directed at hTR, known as peptide nucleic acids (PNAs) [60]. In most of the initial experiments, inhibiting telomerase caused gradual telomere shortening and eventually, after a few weeks of culture, cessation of growth due to reactivation of the senescence program. However, more recent data utilizing dominant-negative mutants or anti-sense of the catalytic subunit of human telomerase, hTERT, caused a repression of telomerase and a subsequent apoptotic response [61-65]. The basis for such a dramatic difference in the response to varying telomerase repression pathways remains unknown. One possibility may be that the overexpression of a dominant-negative hTERT would essentially "soak up" the binding of accessory proteins and prevent the wild-type hTERT from associating. The only known proteins to functionally associate with telomerase and modulate its activity through assembly are the hsp90 chaperone complex of proteins [66]. The hsp90, hsp70, p23 and other proteins in the complex are responsible for inducing the proper conformation of telomerase during assembly. This set of proteins, and other telomerase-associated proteins as they become known, represents yet another target for specific telomerase inhibition. By blocking the interaction of modifying or associated proteins with telomerase, telomerase function in cancer cells will be potentially disrupted without affecting any of the surrounding normal cells.

3.3. Alternative consequences of telomerase inhibition

There has been concern as to whether inhibition of telomerase will trigger an ALT pathway for maintaining telomeres [23, 24], thus theoretically reducing the efficacy of this anti-cancer therapy. As discussed above, there have been a number of studies performed that directly inhibit telomerase in tumor-derived cell lines and the results are either reappearance of the senescence pathway [58], activation of apoptosis [61-65],

or induction of differentiation [61]. This suggests that tumor-derived cancer cells are committed to a telomerase-dependent immortalization pathway, remaining as such rather than relying on an alternate telomere maintenance pathway. Therefore, while it seems plausible in some *in vitro* models of immortalization, the ALT pathway appears to be less relevant when discussing telomerase inhibition and cancer therapy.

Realistically speaking, it is highly unlikely that telomerase alone will be the "magic bullet". In the case of resectable solid tumors, inhibition of telomerase will not replace the need to surgically reduce the tumor burden. However, after tumor debulking, telomerase inhibition may provide an effective means of preventing spread and recurrence of disease. We believe that telomerase inhibitors, if specific, would certainly be an attractive adjuvant therapy option following surgery and/or together with more conventional chemotherapeutic agents. Noteworthy, data indicate that inhibition of telomerase increases the sensitivity of cancer cells to cisplatin-induced apoptosis, suggesting that blocking telomerase may chemosensitize tumors that are typically resistant to conventional therapy [62].

When discussing telomerase inhibitors for cancer treatment, consideration must also be given to the effect of inhibition on "normal" cells in the body that express telomerase activity, such as lymphocytes, germ cells, and stem cells [67, 68]. Importantly, progenitor cells and germ cells, unlike cancer cells, divide much less frequently and therefore have longer, more stable telomeres. We expect anti-telomerase therapies to only be necessary for short periods of time (i.e., long enough to shorten cancer cell telomeres to a senescence-or apoptotic-inducing length without adversely affecting the viability or proliferative potential of immune, germ, or stem cells).

4. Tumor suppression using telomerase?

4.1. General principles

In this chapter, there are two seemingly contradictory statements that have yet to be addressed, namely that telomerase is associated with nearly 90% of malignant human cancer and that telomerase expression in normal cells can prevent cellular aging. This seems paradoxical in that telomerase prevents aging but is also associated with cancer. However, after careful thought, we have come to the conclusion that these roles are mutually exclusive. As we have discussed previously, ectopic expression of telomerase prior to senescence leads to prevention of the senescence block while all the normal "senescence-related" checkpoints remain completely intact [69]. Cells that are capable of bypassing senescence must inactivate these checkpoints to overcome this block, creating an atmosphere of genomic instability combined with increased frequencies of mutation. At this point, the cells are rapidly approaching crisis while continuing to shorten their telomeres. It is only the rare cell that is capable of overcoming crisis, turning on telomerase endogenously, and immortalizing (Figure 1). This is not the case for the ectopic telomerase-expressing cells as they never bypass crisis (they merely postpone it) and appear to maintain their karyotypic and genomic stability indefinitely, as we have previously proposed [69]. Extended life-span or "telomerized"

cells, continuously divide in a normal fashion without changes in karyotype, growth characteristics, or tumor suppressor function [14, 15]. The extended life-span cells have essentially identical characteristics to young normal cells, except in their ability maintain telomeres and continuously divide without senescence (Figure 1).

4.2. Redefining immortalization

Since "telomerized" cells have distinct features from those that have bypassed both senescence and crisis, we feel it is important to clarify and redefine certain nomenclature. Immortality has been previously defined as indefinite proliferative capacity. With this definition, telomerase-expressing normal cells that continuously divide would be classified as immortal. However, the classical definition is actually much more narrowly defined. Specifically, cells that progress to immortality bypass cellular senescence through inactivation of tumor suppressor proteins, continue to shorten telomeres, thus creating an atmosphere of genomic instability, acquire additional mutations during their extended life span, and overcome crisis. Most often, these immortalized cells have reactivated endogenous telomerase for stabilization of telomere lengths. In contrast, the telomerase-expressing normal cells, do not bypass senescence, but rather prevent senescence altogether in a genomically stable environment. Semantics aside, these extended life span cells are clearly distinct from immortalized or tumor-derived cell lines, with the notable exception of telomere maintenance by telomerase. One could argue that "telomerized" cells are more like pre-senescent normal cells in many respects.

4.3. Evidence for telomerase as a tumor suppressor

We further propose that if telomerase is expressed prior to the molecular changes that lead to classical immortalization, not only is senescence prevented but classical immortalization may be as well. As we have suggested [69], telomerase expression in normal cells may act with tumor suppressor-like abilities by maintaining telomere length and genomic integrity. There is ample evidence to suggest that telomerase is able to prevent some of the instability/mutation events that occur in normal cells predisposed to immortalization. Interestingly, some of the first studies with telomerase demonstrated its ability to participate in the chromosome healing process, which would suggest that telomerase is capable of participating in DNA repair [70, 71]. Kiyono and colleagues [72] have shown that telomerase expression blocks p16 methylation in normal mammary epithelial cells (HMEs), a functional step required for HME cells to spontaneously inactivate the pRB arm of cellular senescence, a stage known as M0 [72, 73]. In addition, we and others have shown that the telomere maintenance function of telomerase may also prevent some of the karyotypic changes associated with age and/or oncogene expression [14, 15]. Evidence is also accumulating to suggest that telomerase expression is able to increase the ability of cancer cells to avoid programmed cell death or apoptosis [74, 75]. Moreover, we have demonstrated that ectopic telomerase-expressing normal cells also have an increased resistance to apoptosis [75], suggesting that maintenance of genomic stability through telomere stabilization aids in the prevention of programmed cell death. Our more recent data indicate that exogenous telomerase expression is

able to extend the *in vitro* life-span of Li Fraumeni HME cells while importantly conferring genomic stability and preventing spontaneous immortalization [unpublished data]. Collectively, these data strongly suggest that telomerase can indeed function as a tumor suppressor.

Replicative senescence is generally thought of as a potential tumor suppressive cellular function and those cells that bypass it are more likely to progress to a cancer-like state. If the telomerase expressing, extended life-span cells prevent senescence, then telomerase expression in normal cells may be tumor suppressive rather than oncogenic (Figure 1). So, our model suggests that by preventing cellular senescence, progression to cancer will also be prevented. There are some obvious exceptions to this, for example, leukemias and cancers arising from stem cells which are derived from telomerase expressing cells. However, there are still many other types of epithelial cell tumors where introduction of telomerase, at activity levels where telomere lengths are maintained, may provide a protective function. Obviously, additional studies are necessary to support this hypothesis; nevertheless, it remains an intriguing possibility.

5. Final thoughts

While cellular proliferation is a critical component in telomerase activation, the precise mechanisms for the down-regulation of telomerase in non-dividing cells remain elusive. Some groups claim that telomerase is regulated by c-myc and/or p53 at the transcriptional level (hTERT), yet mounting evidence suggests that some regulation of telomerase may also be post-translational (e.g. phosphorylation, proteolysis or assembly). The developmental differentiation pathways that result in telomerase repression may prove to be some of the most informative ways to block enzyme function. If one is able to shut down telomerase in cancer cells by reactivating the pathways observed during development, adjuvant anti-telomerase therapies may become a reality. The flip side of that coin is that if we can determine how to turn on telomerase in normal cells, we may be able to prevent cellular aging, increase proliferative capacity, and perhaps prevent oncogenesis. Telomerase function clearly plays an important role in a variety of biological systems, including aging, cancer, regeneration, and cloning by providing proliferative potential indirectly via telomere maintenance. Unfolding the regulation of telomerase as it relates to cellular proliferation as well as other potential functions, such as tumor suppression and DNA repair, will be a tremendous and exciting challenge for scientists of the 21st century.

6. References

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CHAPTER 6

IMMORTAL TRANSFORMATION AND TELOMERASE REACTIVATION OF HUMAN MAMMARY EPITHELIAL CELLS IN CULTURE

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Table of contents

Telomerase, Aging and Disease. Ed. by M. P. Mattson and T. K. Pandita. 103 — 130

1. Introduction

High levels of telomerase activity are one of the most consistent distinguishing features of cancer tissues and tumor-derived immortal cell lines when compared to normal human somatic tissues and finite lifespan cells. The attainment of unlimited proliferative potential conferred by telomerase activity is thought to be critical for malignant progression because tumor cells need to undergo sufficient cell divisions to accumulate the multiple errors necessary for invasive and metastatic potential. In the absence of high telomerase levels, replicative senescence halts growth before such error accumulation is possible. The cellular senescence normally observed in somatic cells from long-lived species such as humans may therefore have developed as a tumor suppressor mechanism.

The studies to be described in this chapter concern the mechanisms by which normal, finite lifespan human mammary epithelial cells (HMEC) overcome replicative senescence and activate telomerase activity. We suggest that these steps may be key rate-limiting events in human breast carcinogenesis. Figure 1 summarizes our current model for the processes involved in HMEC growth and immortalization. In our HMEC culture system, attaining an indefinite proliferative potential requires cells first to overcome two senescence barriers. The first barrier can be overcome by alterations in the pathways governing RB. In culture, the most common means by which this is effected is the downregulation of the cyclin dependent kinase inhibitor (CKI) $p16^{INK4a}$. The second barrier appears to be regulated by telomere length, and is extremely stringent. It involves generation of abnormal metaphases and mitotic catastrophes. The means by which the second barrier is overcome have not yet been fully defined. Loss of p53 function appears to contribute to this process, but is not required or sufficient. Likely, more than one error is necessary. However, even after overcoming both senescence barriers, the resultant cells with indefinite proliferative *potential* must still progress through further changes. In HMEC that retain p53 function, cells proceed through a gradual epigenetic process, which we have termed conversion, in order to reactivate telomerase and attain uniform good growth. Conversion appears to be triggered by the critically short telomeres that occur in p53(+) HMEC that maintain proliferation after overcoming stringent senescence. In HMEC immortalized with loss of p53 function, the conversion process is greatly accelerated, and some aspects are not expressed. As a result, uniformly good-growing telomerase-expressing cells appear much more rapidly.

Our observations of cultured HMEC will be discussed in the context of what is known about human breast carcinogenesis *in vivo*. We propose that attainment of short telomeres may play an essential role in human carcinoma progression. Additionally, we describe how different methods of producing immortal HMEC can yield cell lines with significantly different histories and phenotypes. These methods may vary in the extent to which they model human malignant progression *in vivo*.

2. Growth of finite lifespan HMEC *in vitro* (see Figure 2 for schematic chart)

2.1. Growth properties of finite lifespan HMEC

Large quantities of finite lifespan HMEC are readily obtained as discard material of the commonly performed surgery for reduction mammoplasty. Epithelial and isogenic fibroblast cells can be isolated and stored frozen for repeated experiments with cells from the same individual [1, 2]. Our laboratory has introduced two types of culture media for long-term growth of cultured HMEC. The earliest studies used MM medium, which contains serum, growth factors, and conditioned media from other human cell lines [1, 3]. MM supports active HMEC growth for 3-5 passages, or ~15-25 population doublings [4]. As the population becomes non-proliferative, larger, flatter senescence-associated β–galactosidase (SA-β–gal) positive cells predominate. Cultures that maintain growth beyond passage 5 display uneven proliferation. Pockets of small, actively growing cells may appear, but these cells soon become larger and less proliferative.

MM-grown cultures show a mixed morphology and cell composition. Cells with both basal (keratin 5/14 positive, α-actin positive, keratin 19 negative) and luminal (keratin 8/18/19 positive, polymorphic epithelial mucin positive) markers are initially present [5]; however, the keratin 19 positive cells show little proliferative potential in culture. As the population becomes non-proliferative, expression of the CKI p16 increases sharply [6]. The mean TRF (terminal restriction fragment) length of the largely growth-arrested population is ~6-8 kb [7, 8].

In collaboration with Richard Ham's laboratory, a serum-free medium, MCDB 170, was developed for HMEC. MCDB 170 supports a mixed cell population in primary cultures, but cells that maintain proliferation express a predominately basal phenotype [5]. Active division of cobblestone-appearing cells occurs for 2-3 passages. The cells then gradually become larger and flatter, with expression of SA-β-gal and reduced proliferative capacity. High levels of p16 are also present in this growth-arrested population, and the mean TRF is ~6-7 kb [6, 7].

In MCDB 170, a small subpopulation of cells is able to spontaneously overcome this p16-associated growth arrest, which we originally termed selection [9]. After 1-3 weeks with no visible mitotic activity, pockets of actively proliferating cobblestone-appearing cells appear. These cells no longer express p16 and show specific methylation of the p16 promoter [6]; they also contain wild-type p53 with a longer half-life [10]. We have called the process associated with p16 down-regulation, self-selection, and the p16(-) cells, post-selection HMEC [9]. Although selection was later referred to as M0 in the context of viral oncogene transformation [11], we believe it most closely resembles the previously described M1 senescence block (see below) [8, 12]. The absence of spontaneous p16 downregulation in MM vs. MCDB 170-grown HMEC indicates the influence of culture conditions on this process. We have also noted that even in MCDB 170, HMEC from some individual specimen donors required a cAMP stimulator to generate p16(-) post-selection cells. Epigenetic silencing of p16 has been associated with many human cancers, and *de novo* methylation of the p16 promoter has been found in approximately a third of primary breast tumors and breast cell lines [13].

Depending upon the individual specimen, post-selection cells may proliferate for an additional 30-70 population doublings before reaching a second block to further growth. Growth slows around 3 passages prior to the passage at which no further net gains in cell number are observed, with increasing numbers of SA-β-gal positive cells displaying a larger, vacuolated, but still cobblestone epithelial morphology. This block is extremely stringent, as we have never observed any HMEC spontaneously able to overcome this block and maintain growth, although many cultures from over 20 individuals have reached this block. The mean TRF of this growth-arrested population is ~4-5 kb [14].

The finite lifespan HMEC cultures have been examined for telomerase activity using the TRAP assay. We have never observed any activity in the post-selection HMEC [14]. However, in MM-grown HMEC from some individuals, a low level of telomerase activity has been detected [7]. Similar results have been reported from other laboratories [15, 16].

2.2. Senescence of finite lifespan HMEC

Recent studies in Thea Tlsty's laboratory have shed new light on the nature of the two growth arrest barriers encountered by the HMEC (see Figure 1 for a schematic outline of overall HMEC growth, senescence, and immortalization) [8]. Experiments were performed directly comparing isogenic human mammary epithelial and fibroblast cells. Both cell types showed an initial growth arrest associated with high levels of p16 and SA-β-gal expression. In growth-arrested cultures, there was little ongoing DNA synthesis, with fewer than 2% of cells incorporating bromodeoxyuridine during a four-hour pulse, little cell death, with only 1% Annexin-V staining, and retention of normal diploid karyotypes. The arrested cells displayed a FACS DNA profile consistent with G0/G1 arrest, with a 2N to 4N ratio of ≥4. When directly compared, both cell types arrested with a mean TRF of ~6 kb. The fibroblasts differed from the epithelial cells in displaying a larger number of population doublings (~40) before reaching this growth arrest, and never showed spontaneous escape from this arrest. In fibroblasts, this arrest has been previously termed senescence or M1. Given the overall similarities between the two cell types at this growth arrest, we suggest that the selection block in HMEC is most equivalent to what has been called senescence in human fibroblasts [8]. Whether or not a short telomere length is critical for triggering this block has not been definitively determined for HMEC. The nature of the culture conditions, as well as possibly other unknown variables, clearly have an influence on both how many population doublings occur before this block is engaged, and whether some HMEC can overcome this block.

The growth arrest encountered by the p16(-) post-selection HMEC population is notable for its extreme stringency in culture. Cytogenetic analysis of post-selection HMEC showed gross chromosomal abnormalities in metaphase spreads beginning 10-20 population doublings before the final passage. The abnormalities included abundant telomeric association and chromosome fusion and breakage events, polyploidy and aneuploidy. This onset of genomic aberrations coincided with the slow-down in proliferation rate. At the point of no net proliferation, 100% of metaphases exhibited structural abnormalities. Further analysis indicated that the post-selection HMEC at

the final passage showed ~16% bromodeoxyuridine incorporation, ~20% staining with Annexin-V, a 2N to 4N ratio of ~1, and a substantial polyploid subpopulation. There was no net increase in cell number since continued DNA synthesis was associated with mitotic catastrophes and resulted in cell death or non-proliferative multi-nucleated cells. This second senescence barrier has been termed agonescence [17].

On gross inspection, HMEC at agonescence exhibit long-term viability. Metabolically active cultures have been maintained for over a year. This overall viability contributed to the assumption that this second growth arrest was similar to the previously reported viable senescence block in fibroblasts, and unlike the viral oncogene-induced crisis or M2 stage [18]. However, the more recent studies offer a new perspective. *In vitro* model systems of crisis have utilized cells lacking functional RB and/or p53, e.g., cells exposed to SV40 LgT or Human Papilloma Virus (HPV)16 E6/E7, or derived from Li-Fraumeni patients. *In vivo*, normal cells would retain p53 and RB function upon reaching senescence barriers, as do our HMEC cultures at agonescence. Unlike cells at crisis, the HMEC at agonescence do not spontaneously transform to immortality in culture. Similar to crisis, there are widespread chromosomal aberrations, as well as some ongoing DNA incorporation. However, the percentage of cells showing DNA incorporation is significantly lower than in crisis [19], and most cells remain arrested in G1 or G2. The retention of wild type p53 may constitute the difference between the largely viable agonescence observed in post-selection HMEC, and the crisis seen in cells lacking functional p53. The nature of the agonescence block can account for the observed stringent senescence in cultured human epithelial cells; cells which fail to maintain a G1 or G2 arrest at agonescence will eventually die or become non-proliferative via mitotic catastrophe. Unlike an arrest based upon blocking cell cycle progression (e.g., elevated levels of CKIs), the agonescence barrier involving structural failures at mitosis can not be readily overcome and is irreversible. This block also appears to be tightly correlated with telomere length, and thus may serve as a telomere-based clock for limiting cell division.

2.3. Extended life cultures following exposure to the chemical carcinogen benzo(a)pyrene

With the goal of developing a series of progressively transformed HMEC from one individual, primary cultures of MM-grown cells from specimen 184 were exposed to the chemical carcinogen benzo(a)pyrene [4, 20]. We chose to use a chemical carcinogen to induce transformation because we wished to develop a model system that might as closely as possible model human breast carcinogenesis *in vivo*. Chemical carcinogens were known to induce mammary carcinomas in rodents, benzo(a)pyrene is an abundant pollutant in the environment, and we first ascertained that HMEC could metabolize the pro-carcinogen, benzo(a)pyrene, to its active carcinogenic form [21, 22].

MM-grown cells normally cease active growth by passage 5, with high expression of p16, and without spontaneous down-regulation of p16. In the benzo(a)pyrene-treated cultures, there were numerous instances in which growth was extended. These extended life (EL) cells showed heterogeneity with respect to morphology and growth potential. Subsequent testing indicated that all EL cultures examined did not express p16 and

contained a stable form of p53. In only one case tested, EL 184Aa, was the absence of p16 expression due to a detectable mutation. These data provide additional evidence supporting the role of p16 in mediating the first senescence barrier and implicating it as a possible target of carcinogen-induced changes.

In almost all cases, the EL cultures subsequently ceased proliferation. Mean TRF at this growth arrest was ~4-5 kb, similar to the value of post-senescent HMEC at agonescence. Although we have not yet examined the karyotypes of growth-arrested EL cultures, it is most likely that they too are aberrant. However, unlike the post-selection cells, these cultures have been exposed to a carcinogen, and may harbor unknown derangements in addition to their down-regulation of p16. Consequently, they also differ from post-selection cells in their ability to give rise, very rarely, to immortally transformed lines, and in their increased ability to immortalize when exposed to further immortalization promoting agents (see Section 7).

3. Conversion of conditionally immortal p53(+) HMEC lines (see Figure 3A for schematic chart)

3.1. Telomerase reactivation and mean TRF length stabilization of carcinogen-immortalized p53(+) HMEC

We have observed three instances where EL cultures gave rise to HMEC lines of indefinite lifespan without any further treatment [2, 23]. Two lines, 184A1 and 184AA4, were derived from the EL 184Aa culture and were first detected at passages 9 and 14 respectively. One line, 184B5, appeared in the EL 184Be culture at passage 6. Karyotypic analysis indicated distinct clonal origins, and a very low level of genomic instability, in both 184A1 and 184B5 [24]. 184AA4 exhibited numerous abnormal karyotypes when first assayed; however later passages contained fewer abnormalities [23]. Like most breast tumor cells, none of these lines has a known defect in the expression or phosphorylation of RB, or in the sequence of p53 [10, 25]. Similar to their finite lifespan EL precursors, these lines lack expression of p16 and contain a stable form of the p53 protein. None of the lines displays sustained anchorage-independent growth, growth factor independence, or tumorigenicity. These lines allow examination of the process of human cell immortalization without the possible confounding factors of malignant transformation, genomic instability, or viral oncogene exposure.

Although mass cultures of 184A1 and 184B5 consistently displayed an indefinite lifespan, we were initially puzzled to note that many individual cells in these immortal lines lost proliferative potential during early passage culture. Careful examination of these p53(+) lines then uncovered the process of conversion, a previously undescribed, presumably epigenetic step in the transformation of HMEC [14]. Early passage cells of the immortal, p53(+) 184A1, 184AA4, and 184B5 lines show no telomerase activity and have continued telomere erosion with passage (Figure 4A,B). When the mean TRF declined to $\leq$ 2.5 kb, low levels of telomerase were first detectable. Telomerase levels gradually increased thereafter, and the mean TRF stabilized at 3-7 kb. During the prolonged period when the mean TRF was $\leq$3 kb, and before telomere length was stabilized, growth in

culture was heterogeneous and slow (see Figure 4C). Since many individual cells did not maintain proliferation, we termed early passage cultures of these lines "conditionally immortal". As we have subsequently seen, several variables can influence the percentage of, and speed with which, conditionally immortal cells are able to manifest their pre-existing potential for uniformly good-growing indefinite proliferation. Conversion of telomerase(-) conditionally immortal HMEC to telomerase(+) uniformly good-growing fully immortal HMEC is reproducibly observed in both mass cultures and repeatedly subcloned populations of these early passage p53(+) HMEC lines. This observation, along with the very gradual nature of conversion, suggests an epigenetic rather than a mutational cause.

Our studies of the past few years have indicated that the conversion of telomerase(-) conditionally immortal HMEC to telomerase(+) fully immortal HMEC is accompanied by a variety of alterations in cellular biology, as discussed below.

3.2. Expression of $p57^{KIP2}$ in conditionally immortal p53(+) HMEC and loss of p57 expression in fully immortal HMEC

The presence of a slow growth phase in the conditionally immortal HMEC led us to examine these cells for expression of growth inhibitory molecules. Examination of the CKI p57 mRNA and protein found a tight association between p57 expression and slow growth [26]. p57 belongs to the CIP/KIP family of CKIs which also include p21 and p27. The p57 gene has been localized to chromosome 11p15.5, a region displaying frequent allelic loss in cancers of the breast, lung, and bladder [27]. LOH and microsatellite instability at 11p15 have been associated with rapid proliferation, DNA aneuploidy, and poor prognosis in primary breast tumors [28]. In epithelia *in vivo*, p57 is reported to be expressed in regions of differentiated cells, but not in regions of actively dividing cells, suggesting that p57 may be up-regulated when cells exit the cell cycle and start their differentiation programs. p57 is not detectable in most immortal cell lines. Our examination of p57 expression in our cultured HMEC has found the following [26]:

a) No p57 mRNA was detected in finite lifespan HMEC, including MM-grown and post-selection HMEC, and the EL cultures 184Aa and 185Be.
b) p57 was expressed in conditionally immortal p53(+) HMEC arrested in G0 by blockage of EGF receptor signal transduction. When the mean TRF was > 3 kb and the cells displayed good growth, p57 was down-regulated upon mitogenic stimulation and entry into G1. (Figure 5A).
c) When the mean TRF of conditionally immortal p53(+) HMEC declined to ≤ 3 kb, p57 expression remained high even after mitogenic stimulation and exit into G1 (Figure 5B). This failure to down-regulate p57 coincided exactly with the beginning of the slow, heterogeneous growth phase.
d) The level of p57 expression in G0 and cycling populations was gradually reduced as the conditionally immortal cells underwent conversion and gradually regained uniform good growth. Fully immortal HMEC lines expressed little or no detectable p57 in G0 or when cycling (Figure 5C).

We do not yet know what is responsible for the initial activation of p57 expression in G0-arrested conditionally immortal HMEC. Perhaps the events that enable cells to overcome agonescence give rise to p57 expression. Finite lifespan HMEC with mean TRF lengths equivalent to those in early passage 184A1 do not express p57, indicating that telomere length alone does not dictate p57 expression. We do know that the expression of p57 is dependent upon the expression of p53 (see Section 4 below).

The failure to down-regulate p57 after release from G0 appears tightly correlated with the telomere length of conditionally immortal HMEC, suggesting that development of extremely short telomeres (mean TRF ≤ 3 kb) causes changes resulting in increased steady state p57 levels. When the mean TRF of early passage pre-conversion 184A1 is kept > 3 kb by transduction with agents that provide or reactivate telomerase activity (e.g., hTERT, or inhibitors of p53 function), p57 is not expressed during G1, the level of p57 in G0-arrested cells declines, and the cell populations never undergo a prolonged period of slow heterogeneous growth [23, 26].

The loss of p57 expression as cells convert to fully immortal HMEC can involve both genetic and epigenetic mechanisms. The p57 gene is imprinted with preferential expression of the maternal allele [29]; loss of the maternal allele by itself can severely reduce p57 expression. The conditional immortal growth constraint exhibited by MCDB170-grown 184A1 during passages 16-20 is particularly severe, and the major (presumably maternal) p57 allele is frequently lost during this period. Deletion of the initially expressed p57 allele coincides with upregulation of the previously imprinted paternal p57 allele [26]. We do not know how expression of this remaining p57 allele is lost as 184A1 gains full immortality. Where the slow growth phase is not as severe, as in 184B5, and in 184A1 grown in the nutritionally richer MM medium, no p57 allele loss is detected. Preliminary results suggest that, in these cases, downregulation of p57 expression is accompanied by increasing methylation of the p57 genes.

Collectively, these results indicate that p57 is expressed by p53(+) HMEC that have recently overcome agonescence. The data suggest that p57 may play an important role in the observed slow heterogeneous growth of conditionally immortal HMEC when the mean TRF declines to ≤ 3 kb, thus inhibiting the conversion of conditionally immortal cells to the fully immortal state. The absence of p57 expression in almost all human tumor-derived cell lines is consistent with the hypothesis that downregulation of p57 is required for full immortalization. As discussed later, if a conversion process occurs during *in vivo* breast carcinogenesis, the expression of p57 in p53(+) cells could have a significant influence on cancer growth and progression.

3.3. TGFβ-induced growth inhibition in conditionally immortal HMEC and gradual gain of resistance during conversion to full immortality

Malignant progression in human carcinomas is commonly associated with acquiring the ability to maintain growth in TGFβ [30]. In some cases, this acquisition can be attributed to loss of functional TGFβ receptors or other mutations in the associated signal transduction pathways, but in most instances, including most breast cancers, no such mutations are detected. In contrast to carcinoma-derived cells, cells derived from normal human epithelial tissues are severely growth inhibited by TGFβ. We have not

observed any finite lifespan HMEC with the ability to maintain growth in the presence of TGFβ. However, all the fully immortal lines can maintain growth in TGFβ, although the rate of growth is generally somewhat slower in its presence. To emphasize this distinction in TGFβ growth responsiveness of finite lifespan vs. fully immortal HMEC, we have defined TGFβ resistance as the ability to maintain growth in TGFβ, even with reduced growth rates. Our fully immortal HMEC that maintain active growth in TGFβ have not lost the ability to recognize and respond to TGFβ. They maintain expression of TGFβ receptors and respond to TGFβ with increased protein synthesis and secretion, including induction of extracellular matrix associated proteins such as fibronectin and plasminogen activator inhibitor 1 [31]. In this manner, they resemble mesenchymal cells that can respond to TGFβ while maintaining active growth. Possibly, the metabolic demands of the greatly increased protein synthesis, as well as other aspects of the cells' on-going TGFβ responsiveness, may be indirectly affecting the fully immortal HMEC growth rate in presence of TGFβ.

Examination of conditionally immortal HMEC indicated an absence of any ability to maintain growth in the presence of TGFβ at the earliest passages. As the conversion process proceeded, and low levels of telomerase became detectable, there was a gradual increase in the number of cells with progressively better growth capacity in TGFβ. For example, in the 184A1 line (see Figures. 4C & 8B), only rare cells capable of maintaining very poor growth in TGFβ were present by around passage 28. By passage 44, ~75% of cells were capable of good growth in TGFβ. In general, uniform good growth in the presence of TGFβ was present around 10-20 passages after telomerase activity was first detectable. Subsequent experiments (see Section 5 for more details) have shown that expression of an exogenously introduced hTERT gene induces the gain of TGFβ resistance in these HMEC [32]. This induction of TGFβ resistance by hTERT expression may account for the tight correlation between telomerase activity and TGFβ resistance in isolated clones of conditionally immortal HMEC in the process of conversion [14].

Since our assays for TGFβ resistance allow us to examine the fate of individual cells, we could use this phenotype to acquire information about the heterogeneity of conditionally immortal HMEC. One of the more remarkable aspects of conversion is the degree to which heterogeneity is rapidly generated from repeatedly cloned populations [14]. Thus, single cell-derived colonies at passage 25 of a subclone derived at passage 20 from a clone derived at passage 15 of the clonally-derived line 184B5 varied from showing none to uniform good growth in TGFβ. By passage 38, this subclone displayed uniform good growth in TGFβ. Any mechanistic explanation for conversion must be able to account for this rapidly generated heterogeneity.

3.4. Other molecular changes associated with HMEC conversion

The conversion of HMEC to full immortality appears to be a complex set of interactive changes. Our laboratory has been engaged in defining what these changes are, and in determining the nature of the interactive processes. The following are some additional cellular phenotypes that change in the process of attaining full immortality:

a) Finite lifespan HMEC show reduced levels of the proto-oncogene c-myc mRNA and protein when they are arrested in G0 [33]. The fully immortal lines display equivalent levels of c-myc in G0-arrested and cycling cell populations. Early passage conditionally immortal HMEC behave like the finite lifespan HMEC, but then gradually show increased levels of c-myc in G0 as conversion progresses. The overall level of c-myc expression in the cycling population is also increased in fully immortal HMEC.

b) Finite lifespan cells have been reported to be growth inhibited when exposed to overexpressed H-Ras or Raf-1, whereas the growth of immortal cells is often enhanced under the same conditions [34, 35]. We have utilized a retroviral expression vector encoding the catalytic domain of human Raf-1 fused to the hormone-binding domain of the human estrogen receptor, to demonstrate that our p16(-) finite lifespan HMEC are also severely growth inhibited when oncogenic Raf-1 is induced with 4-hydroxy-tamoxifen [Olsen, Yaswen, & Stampfer, in preparation]. In contrast, the fully immortal lines transduced with oncogenic Raf-1 are able to maintain growth, even in the absence of signal transduction through the EGF receptor, and gain some anchorage independent growth. Assay of good-growing pre-conversion 184A1 indicated that these cells are still severely growth-inhibited by oncogenic Raf-1. As has been reported for other cell systems [36], hTERT-immortalized HMEC, which do not undergo conversion, are also growth inhibited by oncogenic Raf-1 [Olsen, Yaswen, & Stampfer, in preparation]. Thus, the change in response to oncogenic Raf-1 occurs not with the overcoming of agonescence, or by acquiring telomerase activity, but as a consequence of the conversion process to full immortality.

c) Actively growing finite lifespan HMEC do not express SA-β-gal, while cells arrested at the selection/M1/senescence and at the agonescence blocks, are all positive for SA-β-gal staining. Curiously, the good-growing pre-conversion conditionally immortal HMEC (mean TRF > 3 kb) remain positive for SA-β-gal. Conditionally immortal HMEC retain SA-β-gal staining as conversion begins, and then gradually lose expression of SA-β-gal as conversion proceeds [unpublished data]. Fully immortal HMEC are largely negative for SA-β-gal, though some positive staining cells are present.

Altogether, these studies indicate that major changes occur in cellular signal transduction pathways as HMEC transform from finite lifespan to full immortality. It is likely that further examination will uncover additional cellular changes. These differences between finite lifespan, conditionally immortal, and fully immortal HMEC could have implications for the design of therapeutic interventions in cancer. They also serve as a reminder that immortally transformed cells may not model normal finite lifespan cell behavior in key pathways.

3.5. Immortalization of HMEC with the putative breast cancer oncogene, ZNF217 (see Figure 3B for schematic chart)

More recent studies have used another potentially pathologically relevant means, the candidate oncogene ZNF217, to immortally transform finite lifespan HMEC [37].

ZNF217 was originally identified based on its location on chromosome 20q13.2, an amplicon common in breast cancers and associated with poor prognosis [38]. Extra copies of this chromosomal region occur in approximately 18% of breast tumors and 40% of breast cancer cell lines [39]. ZNF217 encodes a conserved member of the C2H2 Kruppel family of transcription factors, with a DNA-binding domain of eight C2H2 zinc fingers and a separate proline-rich domain. Members of the Kruppel family have been implicated in both neoplastic and developmental disorders [40].

We investigated the functional consequences of ZNF217 overexpression by transducing the gene into finite lifespan 184 HMEC and EL 184Aa. In five independent experiments, ZNF217-transduced cultures gave rise to immortalized cells. The ZNF217-transduced HMEC showed no initial growth advantage over the control cultures, but continued to grow beyond the point where the control population growth arrested at the agonescence barrier. Numerous foci of small, mitotic, SA-β-gal negative cells appeared among the SA-β-gal positive agonescent cells. Growth was at first slow and heterogeneous, but became faster and more uniform with continued passage. After ~5-15 passages, varying among experiments, most cells were SA-β-gal negative and grew well.

Telomerase activity was not initially detectable in the ZNF217-transduced 184 and 184Aa cultures that maintained growth past agonescence, and the mean TRF length continued to decrease (Figure 6). Telomerase activity was detectable within 10 passages and then gradually increased, and mean TRF length stabilized at ~4 kb. When assayed for growth in TGFβ, ZNF217-transduced 184 and 184Aa were initially completely growth-inhibited prior to and just after overcoming agonescence. With increasing passage, there was a very gradual increase in the number of cells with progressively better growth capacity in TGFβ. Assay for p57 showed some expression in G0-arrested and cycling populations in the earliest passages after overcoming agonescence, when growth was slow and heterogeneous, but none in the later good-growing cultures.

Southern analysis of retroviral integration sites in ZNF217-transduced HMEC growing past agonescence suggested that these cultures were rapidly overgrown by distinct clonal populations. To determine whether distinct chromosomal alterations might be conferring growth advantages on clones immortalized with ZNF217, DNA from three different immortalized cultures was used for quantitative measurement of DNA copy number using comparative genomic hybridization [39]. Analysis showed low level regional DNA-sequence copy number variations on chromosomes 1q and 8q common to all three cell lines. The region amplified on 8q included the c-myc oncogene. In addition, each line showed unique regions of high and low level DNA-sequence copy number variations. These sites of regional copy number variation, some of which have also been frequently observed in breast cancer cell lines and primary tumors [39], could contain genes that cooperate with ZNF217 in facilitating immortalization.

To determine whether loss of p53 function contributed to the immortalization of the ZNF217-transduced HMEC, p53 function was assayed by measuring p53 expression after exposure to the DNA damaging agent actinomycin D, and p53-dependent induction of GADD45 transcripts following UV irradiation. Induction of p53 similar to that in the finite lifespan cells was observed in all three ZNF217-transduced immortalized HMEC tested, and GADD45 mRNA levels were increased 4 hrs. after UV exposure in

both finite lifespan 184 and ZNF217 immortalized 184Aa. pRB was also present and underwent normal cycles of phosphorylation and dephosphorylation in these cells.

Thus, the ZNF217-immortalized HMEC showed many similarities to the immortal HMEC lines derived by carcinogen exposure. Alterations in p53 and/or RB were not obligatory for immortalization. Most significantly, these p53(+) immortal lines underwent a conversion process before attaining a fully immortal phenotype. There was a gradual reactivation of telomerase activity and an incremental gain of TGFβ resistance, and a transient expression of p57 associated with a period of slow heterogeneous growth.

3.6. Telomerase control in fully immortal HMEC

Telomerase-expressing cells, from unicellular organisms such as yeast to human tumor-derived immortal cell lines, have been shown to maintain control of telomere length within a set range through regulation of telomerase access/activity [41, 42, 43]. The short regulated telomere lengths observed in most human tumor-derived lines is consistent with a model of cells overcoming agonescence and undergoing conversion, followed by a mechanism to regulate the resulting short telomere length. Our immortal HMEC lines display a mean TRF around 4 kb, however analysis of individual clones has indicated a range of ~3-7 kb [14]. Additionally, we have observed that clones at the short end of this range may not express TRAP activity, associated with a slowdown in growth to a brief period (a few weeks) of total loss of proliferation, followed by a rapid re-expression of TRAP activity and a resumption of growth. We have not yet examined the mechanisms governing this behavior. It is likely that the telomeric ends of fully immortal HMEC have undergone an irreversible change into a conformation and set of telomere-associated proteins that allow assessment of telomere length in order to maintain stable, short telomeres.

4. Immortalization of HMEC associated with loss of functional p53 (see Figure 3A for schematic chart)

4.1. Derivation of p53(-/-) immortal HMEC lines

Many immortal and malignant human cells show loss of normal p53 function, however, 70-85% of human breast tumors contain wild-type p53 [44, 45], and our immortally transformed 184A1 and 184B5 HMEC lines also contain wild-type p53. We attempted to use expression selection of genetic suppressor elements (GSEs) to define alterations responsible for generating these lines. Retroviruses containing short random cDNA fragments were introduced into 184Aa and 184Be, the EL cultures that gave rise to 184A1 and 184B5. Our goal was to generate new lines with indefinite growth potential by inactivating potential tumor suppressor genes, and then identifying the inactivated genes. Although we were unsuccessful at the goal of defining the alterations responsible for generating the p53(+) lines, these experiments did provide us with two p53(-/-) immortally transformed HMEC lines, 184AA2 and 184AA3, derived from EL 184Aa [23]. Comparison of these two p53(-/-) lines with the three closely related p53(+) lines suggests a novel role for p53 loss in immortal and malignant transformation.

184Aa was infected with retroviruses at passage 12. 184AA2 appeared at passage 13 as tight patches of refractile cells with many mitoses, as well as larger flatter cells. It maintained good growth and a somewhat heterogeneous morphology thereafter. 184AA3 appeared at passage 14 as areas of small densely packed, grossly vacuolated and extremely slow-growing cells. By passages 16-18, mass cultures began a gradual increase in growth rate, and some colonies became less densely packed with fewer grossly vacuolated cells. When seeded at very low densities to permit visualization of the growth of individual colonies, these gradual changes in growth and morphology could be observed in single cell outgrowths. After a few additional passages, mass culture growth increased more rapidly, with uniform good growth attained by passages 20-24. Coincident with the better growth, 184AA3 cell morphology changed to more rounded and refractile, without the tightly packed colonial cell growth seen initially.

Since the one retroviral insertion in 184AA3 lacked detectable HMEC cDNA, we tested the possibility that immortalization resulted from insertional mutagenesis. Inverted PCR analysis showed that the virus had inserted into the p53 gene. Immunoblot analysis for p53 protein showed no p53 expression in either 184AA2 or 184AA3, suggesting that both p53 alleles were inactivated in both lines. Southern hybridization indicated viral integration into one p53 allele, and the absence of a normal second p53 allele in both lines; these HMEC lines are thus p53(-/-).

4.2. Attainment of full immortality in p53(-) HMEC

The good initial growth of 184AA2, and the more rapid attainment of good growth in 184AA3, compared to 184A1 and 184AA4, indicated that these p53(-/-) lines did not undergo a very gradual conversion process. Indeed, some telomerase activity was present in both 184AA2 and 184AA3 at the earliest passages that could be tested (Figure 4A) and the mean TRF length never declined below 3.5 kb (Figure 4B). In both p53(-/-) lines, with increasing passage there was a gradual increase in the number of cells with progressively better growth capacity in TGFβ (see Figure 8B), a result consistent with the induction of TGFβ resistance by the presence of hTERT.

Unlike the three p53(+) lines, no p57 mRNA expression was detected in either 184AA2 or 18AA3 at early or late passages, in G0-arrested or in cycling populations. Consistent with the absence of p57, neither line displayed a prolonged slow growth phase. Although some large vacuolated cells were initially present in 184AA2, it maintained good growth from the outset. Initial 184AA3 growth was very poor, but good uniform growth was quickly attained by passages 20-24. We do not know what is responsible for this initial short period of slow growth in newly emerged p53(-/-) lines with mean TRF > 3 kb and no p57 expression.

Both 184AA2 and 184AA3 displayed anchorage-independent growth when examined at passage 50. They were also capable of forming tumors in nude mice, although the tumors started to regress by ten days post-injection. Karyotypic analysis indicated that both p53(-/-) lines had both a high level of initial chromosomal derangements, as well as increased chromosomal complexity and instability with continued passage. Both lines were able to maintain growth after transduction with oncogenic Raf-1.

These data indicate that the behavior of early passages of both p53(-/-) lines significantly differed from that displayed by early passages of the three p53(+) lines with respect to the conversion process. The p53(-/-) lines showed rapid telomerase reactivation, mean TRF lengths which did not decrease below 3.5 kb, and early acquisition of uniform good growth potential. Thus, although the fully immortal p53(-/-) lines shared many similarities with the fully immortal p53(+) lines, they acquired these properties rapidly after overcoming agonescence. The p53(-/-) lines completely differed from the p53(+) lines in their total absence of both p57 expression and an extended period of poor heterogeneous growth, and in their expression of the malignancy-associated properties of anchorage-independent growth, tumorigenicity, and ongoing genomic instability.

4.3. The effect of functional inactivation of p53

The capacity of both p53(-/-) lines to rapidly attain full immortality, relative to the p53(+) lines, and to not express any p57, suggested that these properties were the result of the lack of p53 function. To directly test this possibility, we inactivated p53 function in early passage pre-conversion 184A1 by transduction with the p53-inactivating GSE, GSE22 [46].

184A1 transduced at passage 12 with GSE22 rapidly gained full immortality [23]. A moderate level of telomerase activity was present seven days after infection, and strong activity was present by passage 19. The mean TRF length in 184A1-GSE22 showed a modest decline from ~5 kb at passage 13 to a stabilized length of ~4 kb by passage 25, similar to the stabilized length in 184AA2 and 184AA3. The TRF signal never became faint nor declined below 4 kb. 184A1-GSE22 assayed up to passage 30 showed a gradual increase in the capacity to maintain growth in the presence of TGFβ. Analysis for p57 showed no p57 protein expression in cycling populations at passages 14-22, in contrast to the abundant p57 by passage 14 in the 184A1 populations infected with control virus. Very low levels of p57 compared to controls were seen in the G0-arrested cells. 184A1-GSE22 also did not have a prolonged slow growth phase. Good uniform growth was present by passage 25, although, as with the p53(-/-) lines, growth capacity progressively increased to that point.

These results indicate that (1) p53 may be acting to suppress telomerase activity in newly immortalized p53(+) HMEC lines; (2) p57 expression in conditionally immortal HMEC is dependent upon expression of functional p53. The absence of p57 expression in p53(-) HMEC lines may in turn be responsible for the absence of a prolonged conversion-associated slow growth phase. Altogether, these data indicate that an absence of functional p53 can directly accelerate the conversion process, and produce more aggressively growing cells more rapidly. *In vivo*, breast cancers that show p53 loss have a poorer prognosis [44, 45, 47]. If a conversion process occurs in breast cancer development *in vivo*, our results suggest a significant additional mechanism whereby p53 loss may contribute to more aggressive cancer progression.

5. Effect of hTERT on growth of finite lifespan and conditionally immortal HMEC (see Figures. 3C and 2B for schematic charts)

The identification of the human catalytic subunit of the telomerase complex, hTERT, has made it possible to experimentally activate cellular telomerase activity, since hTERT is the limiting factor for such activity in human cells. Exogenous introduction of hTERT has been reported to render some cells immortal, e,g. human retinal epithelium, BJ fibroblasts, and post-selection HMEC [48, 49]; however other cell types remained mortal, e.g, pre-selection HMEC and p16(+) human keratinocytes [49, 50]. Initial published reports on hTERT-immortalized human cells showed no alterations in growth control in response to serum deprivation, high cell density, specific pharmacological inhibitors, or oncogenic Ras, nor were gross chromosome instability, anchorage-independent growth or tumorigenicity reported [36, 51, 52]. These data suggested that hTERT-induced immortalization does not affect normal cell behavior. However, long-term culture of hTERT-transduced post-selection HMEC was reported to be associated with increased expression of c-myc [53]. Additionally, ectopic hTERT expression in conjunction with ectopic expression of the oncogenes SV40-T and H-ras, was able to malignantly transform normal human cells [54, 55].

We have examined the consequences of transducing hTERT into both pre-selection and post-selection finite lifespan HMEC, and into conditionally immortal 184A1 both pre-conversion and during conversion [32]. In particular, since conversion of conditionally immortal HMEC to full immortality was consistently associated with acquisition of the ability to maintain growth in the presence of TGFβ, we wanted to examine TGFβ responses. Our results indicate that the expression of exogenously introduced hTERT alone can be responsible for inducing resistance to TGFβ inhibition in HMEC lacking expression of p16.

hTERT was transduced into three different post-selection HMEC at differing passage levels. All hTERT-transduced cultures showed telomerase activity when examined one passage after infection, and the mean TRF rose rapidly to ~10-12 kb (Figure 7A). Cultures exposed to vector alone had no telomerase activity, showed continued telomere erosion, and senesced as expected. All the hTERT-exposed post-selection HMEC have maintained rapid continuous growth. Similar to other reports, the hTERT-transduced cells exhibited no anchorage-independent growth, and remained severely growth inhibited when exposed to oncogenic Raf-1 [Olsen, Yaswen & Stampfer, in preparation]. Assay for growth in TGFβ indicated that these post-selection HMEC rapidly gained TGFβ growth resistance (Figure 8A). We have also noted an increased expression of c-myc with continued passage (unpublished observations).

Pre-selection p16(+) HMEC transduced with hTERT at passage 3 did not become immortal nor acquire TGFβ resistance. Transduced and control cells senesced similarly around passage 5. However, one hTERT-transduced culture dish did give rise to a continuously growing population. With increasing passage, this population displayed decreasing levels of p16 expression. By passage 20, almost all cells were p16(-), and the earliest indications of the ability to maintain growth in TGFβ were detectable. Subsequently, there was a gradual increase in the number of cells with progressively better growth capacity in TGFβ. These results showing hTERT failure to immortalize

p16(+) human epithelial cells are similar to what has been reported for human keratinocytes [50].

hTERT transduction into good-growing, pre-conversion conditionally immortal 184A1 at passage 12 produced rapid telomere elongation (Figure 7B). Consistent with the prevention of telomere erosion to < 3 kb, hTERT transduction eliminated elevated p57 protein expression as well as the associated slow heterogeneous growth phase [26]. hTERT also conferred TGFβ resistance, well before it would have been acquired as part of the conversion process (Figure 8B). Similar to the kinetics observed in our five immortally transformed HMEC lines following endogenous reactivation of telomerase activity, TGFβ resistance was acquired gradually over 10-20 passages.

hTERT transduction of conditionally immortal 184A1 at passage 22, which had already begun the conversion process (mean TRF < 2.5 kb, poor heterogeneous growth, elevated p57 levels) also resulted in rapid telomere elongation (Figure 7C). However, in this case there was no significant effect relative to control cultures on the existing levels of p57, nor on growth capacity in the absence of TGFβ. hTERT again conferred TGFβ resistance gradually over 10-20 passages (Figure 8B). Thus, the acquisition of good growth in TGFβ following hTERT expression is distinct from attainment of good growth in the absence of TGFβ.

To determine what functions of the ectopic hTERT were required for induction of TGFβ resistance, two different hTERT mutants were introduced into post-selection 184 HMEC at passage 12. One mutant contains inactivating amino acid substitutions in the reverse transcriptase domain [56], and the other is a wild type hTERT with a carboxyl-terminal HA epitope tag that shows *in vitro*, but not *in vivo*, telomerase activity [57]. Neither mutant induced TGFβ resistance in recipient cells, indicating that in addition to being catalytically active, telomerase must be capable of telomere maintenance *in vivo* in order to confer TGFβ resistance.

The mechanism responsible for hTERT induction of TGFβ resistance remains to be elucidated. Our data indicate that there is no correlation between telomere length and TGFβ resistance. The incremental acquisition of TGFβ resistance in conditionally immortal HMEC suggests that the effect of hTERT is likely to be indirect, possibly involving cumulative changes in chromatin structure and/or soluble factors. hTERT expression might indirectly change the abundance, modification, and/or spatial arrangements of signalling molecules involved in TGFβ growth inhibition through altering telomere association with nuclear matrix, or affecting the activities of telomere-associated proteins. Although the hTERT-transduced HMEC were no longer sensitive to TGFβ-induced growth inhibition, like our other finite lifespan and immortal HMEC, they were still capable of responding to TGFβ with differentiated functions.

These studies provide another cause-and-effect link in our understanding of HMEC immortalization and the conversion process; the gradual acquisition of TGFβ resistance observed in the HMEC which have recently overcome agonescence is likely a consequence of their reactivation of telomerase activity. An obligate gain of TGFβ resistance as a result of telomerase reactivation could explain why this phenotype is common to carcinoma cells. However, the very gradual nature of both the conversion-induced telomerase reactivation, and the telomerase-induced TGFβ resistance, along with the potentially strong growth advantage provided by the loss of TGFβ inhibition

during carcinogenesis, could also promote selection of the observed mutation-associated mechanisms of TGFβ resistance.

The ability of hTERT to induce TGFβ resistance suggests that immortality could be more than a passive facilitator of malignant progression. However, although TGFβ resistance may be a tumor promoting property for immortal epithelial cells, it is not a malignant property *per se*, since normal mesenchymal cells may be TGFβ responsive but not growth inhibited by TGFβ. Furthermore, hTERT-induced immortalization did not produce other phenotypes characteristic of malignancy (e.g., anchorage-independent growth), or of the full immortality resulting from overcoming agonescence and undergoing conversion. Unlike our p53(+) fully immortal HMEC, hTERT-induced indefinite lifespan HMEC remained sensitive to oncogenic Raf-1 induced growth inhibition, did not express p57 nor the associated period of slow heterogeneous growth, and most importantly, never underwent an extended period with critically short telomeres. hTERT transduction may therefore generate the least deviant indefinite lifespan human cells. However, some changes in signal transduction do occur, such as the responsiveness to TGFβ and possible alterations in c-myc regulation. Consequently, cells immortalized by hTERT are not totally equivalent to normal finite lifespan HMEC.

Conversely, hTERT transduction may not provide the best model for understanding human carcinogenesis. Ectopic hTERT produces long telomeres [32, 54], while most human cancers and tumor-derived cell lines contain short telomeres [58, 59]. In addition, the hTERT immortalization process bypassed agonescence and conversion. We propose that the reactivation of telomerase during human carcinogenesis may involve an obligate stage of very short telomeres, as part of overcoming senescence and undergoing conversion. Overcoming agonescence requires cells to sustain as yet undefined error(s) that most likely occur only after very short telomeres and chromosomal aberrations have been generated. Conversion in p53(+) HMEC provides a prolonged period where cells possess extremely short telomeres, exhibit slow heterogeneous growth and high p57 levels, and undergo other changes in signal transduction pathways. *In vivo*, such conditions might favor generation and growth of rare aberrant cells that have acquired malignancy-promoting advantages such as anchorage-independent growth, growth factor independence, or angiogenesis. Furthermore, conditionally immortal cells, and cells with extremely short telomeres, might possess unique properties that are vulnerable to therapeutic interventions. Methods of HMEC immortalization that do not model overcoming senescence and undergoing conversion and do not produce cells with critically short telomeres, do not allow testing of therapeutic interventions that target these potentially rate-limiting steps in immortalization and tumorigenicity.

6. Effect of viral oncogenes on growth of finite lifespan and conditionally immortal HMEC

The virtual lack of spontaneous immortal transformation of normal human cells in culture, coupled with the rarity of carcinogen-induced *in vitro* transformation, has led to the use of viral oncogene exposure in order to achieve more reproducible and efficient immortalization. Exposure of HMEC to the SV40 LgT oncogene yields inefficient but

reproducible immortalization [18, 60]; while exposure to the high risk HPV E6 and E7 oncogenes provides reproducible and efficient immortalization [61, 62, 63]. Similarly, other human epithelial cell types have been readily transformed to immortality following exposure to HPV16 E6 and E7 [64, 65]. The HPV16 E6 oncogene alone is also capable of efficient immortalization of the p16(-) post-selection HMEC [7, 66]. These reports of viral oncogene-induced immortal transformation of human epithelial and fibroblast cells did not describe a gradual conversion process. It was difficult to reconcile the M1/senecence — M2/crisis model presented for this mode of immortalization [67], with what we were observing in our HMEC cultures. As discussed previously in this chapter, we are now aware that some of this difficulty was due to: (a) assuming that the viable agonescence block resembled M1/senescence; (b) the absence of any event obviously resembling crisis (associated with situations where p53 is non-functional) in the immortalization of our p53(+) HMEC.

To address the effect of viral oncogenes on the conversion process, we examined the consequences of exposing good-growing pre-conversion conditionally immortal 184A1 to SV40 LgT, HPV16 E6, or HPV16 E7 [7]. E6 immediately and efficiently converted 184A1 to telomerase(+) cells that maintained good uniform growth in the absence or presence of TGFβ (Figure 9A). This activity of E6 was not due simply to its ability to inactivate p53 because: (a) LgT, which also inactivates the p53 gene, did not produce immediate strong telomerase activity or uniform good growth, and (b) a mutant E6 which does not target p53 for degradation [68], also caused immediate conversion to full immortality.

The ability of HPV16 E6 to cause immediate conversion to full immortality may be due to its capacity to subvert many cellular functions at once. In addition to its ability to bind p53, HPV16 E6 has been demonstrated to bind at least six additional cellular proteins in *in vitro* and *in vivo* assays [69], only a few of which have been identified [70, 71]. E6 can reactivate telomerase activity, independent of p53 inactivation [15], and has also been reported to increase the level of c-myc protein expression [53]. One or more of these independent transforming functions may be related to its ability to confer immediate full immortality to conditionally immortal 184A1.

SV40 LgT and HPV16 E7 did not produce immediate conversion of 184A1 to full immortality. They did greatly accelerate aspects of the conversion process (reactivation of telomerase activity and attainment of uniform good growth) and also induced a rapid gain of resistance to TGFβ growth inhibition (Figure 9B&C). The inactivation of p53 could be responsible for some of these effects of SV40 LgT, as the data on telomerase reactivation and growth are similar to what we see in the p53(-/-) lines. The means by which HPV16 E7 accelerates conversion are unknown. It is possible that the rapid gain of TGFβ resistance could be related to the ability of these oncogenes to bind and inactivate the CKI p27 [72, 73], which is associated with TGFβ growth inhibition in our HMEC system and other cell types [25, 74]. The role of p57 in these viral oncogene-transduced 184A1 is not yet clear. Cycling cultures of HPV16-E7 transduced 184A1 cells expressed more p57 than parallel cultures infected with control virus (unpublished data). This finding suggests that this viral oncoprotein might also be able to bind and inactivate p57, or might alter the function of cell-cycle mediators downstream of p57, causing aberrant feedback regulation of p57.

Understanding how these viral oncogenes function may help elucidate the mechanisms of immortal transformation of human cells. However, systems of immortalization that employ viral oncogenes with pleiotrophic effects, some of which are still unknown, may not accurately model the gradual changes that occur during human carcinogenesis *in vivo*. Additionally, the common use of oncoproteins which inactivate p53 function has prevented understanding of the mechanisms by which p53(+) human cells overcome senescence, undergo conversion, and gain full immortality

7. An overall model of HMEC immortalization and telomerase reactivation

7.1. Alterations involved in immortalization of HMEC by different methods

Our laboratory has been developing HMEC culture systems in order to understand the normal processes controlling growth and differentiation in these cells, and how these normal processes are altered in the course of immortal and malignant transformation. The studies described in this chapter represent the information we have gained on this subject in the past 25 years. Many outstanding gaps remain in our knowledge of the mechanisms underlying HMEC immortalization *in vitro*. Nonetheless, the great strides of the past 10 years in elucidating the mechanisms and molecules involved in cell cycle progression, signal transduction networks, tumor suppression and oncogenesis, and the key role of telomerase in immortalization, have enabled us to begin to see the outlines of a coherent picture.

An important emerging concept in cell and molecular biology is the extent to which the many pathways of cellular communication form an interacting network, and the pivotal roles some molecules play as nodes of this network. RB and p53 serve as pivotal network nodes of pathways involving growth regulation, and monitoring and directing of cellular status. The biological consequences for the cell may be significantly different depending upon whether a pivotal molecule like RB or p53 is inactivated, or whether a smaller subset of the pathways they govern is altered. This point may be important with regard to human breast carcinogenesis *in vivo*, and the *in vitro* systems which try to model this process, since inactivation of the RB and p53 molecules themselves is not required nor usually occurs in HMEC immortalization *in vitro* or *in vivo*.

The first senescence barrier encountered by the HMEC *in vitro* appears to be governed by the RB pathway. Agents that inactivate RB e.g., HPV E7, are able to overcome this barrier [11, 66]. However, the large majority of human breast cancers retain wild type RB [75]. Around a third of breast cancers do contain a known alteration in the RB pathway — absence of p16 expression associated with hypermethylation of the p16 promoter [13]. Absence of p16 expression, either through mutation or associated with p16 promoter methylation, appears to be the favored route of overcoming this barrier in cultured HMEC in which RB has not been targeted by viral oncogenes. It is most likely that this *in vitro* phenomenon is modeling a pathway used *in vivo*. The mechanism by which p16(+) breast cancers overcome this barrier is unknown, but may involve amplification of cyclin D1 expression [76, 77], or alterations in other cyclins [78]. Loss of p16 expression through epigenetic means would not have the

same impact on a cell as total loss of RB function, as p16 mediates only a small portion of the information affecting RB. p16(-) post-selection HMEC remain responsive to other factors influencing whether or not growth is appropriate, e.g., RB remains unphosphorylated in the absence of required growth factor-induced signal transduction. Consequently, cells, including tumor cells, which lose p16 expression but retain normal RB would be expected to express a more normal phenotype. Immortalization of HMEC by methods that totally eliminate RB function may not model the state of a large percentage of human breast cancers. RB(+) cancers may resort to other errors as ways of evading conditions that would normally prevent growth.

An important question for which there is currently little definitive data is whether the senescence barrier associated with high p16 expression plays a role *in vivo*. Mutations and/or methylation of p16 genes in a wide variety of human cancers strongly suggest that there is selection against this growth regulator during malignant progression. However, comparatively little is known about the circumstances or stimuli that normally induce p16 expression *in vitro* or *in vivo*. Telomere attrition has been postulated as a key determinant of the p16-associated senescence/M1 block in both fibroblasts and epithelial cells. However, recent findings complicate this hypothesis: a) the mean TRF length is ~6-8 kb in the senescing HMEC at selection, while in post-selection HMEC mean TRF can decrease to $\leq$ 5 kb [7], and b) introduction of hTERT alone is insufficient to extend the lifespan of pre-selection HMEC, although it readily extends the lifespan of post-selection HMEC [32, 49]. These findings suggest that telomere shortening can not be solely responsible for the p16-associated growth arrest in the pre-selection cells. Nonetheless, there is some consistency in the mean TRF length at this block. It is possible that TRF length could influence the expression of p16, or other growth inhibitors which might be playing a role in the G1 arrest expressed by cells at this barrier. It remains to be determined whether the selection/M1/senescence barrier depends upon an intrinsic clock-like mechanism or whether p16 expression and growth arrest are primarily cumulative responses to the presence or absence of extrinsic factors. There may also be differences between the M1/senescence arrest experienced by fibroblast cells vs. selection in HMEC, for example in the role of the CKI, p21. Upregulation of p21 has been reported to play a role in fibroblast senescence [79, 80], but has not been reported in HMEC [8].

We do not currently know if p16(-) cells play a normal role in epithelial tissues. *In vivo*, epithelial cells may require much more extensive proliferative capacity than fibroblast cells. It is possible that down-regulation of p16 aids in this cell-type specific difference by providing a pool of cells able to proliferate beyond the barrier imposed by the selection/M1 block. However, the down-regulation of p16 in HMEC exposed to a carcinogen suggests that this process may occur under pathologic conditions. If p16 is normally down-regulated *in vivo*, then it would be beneficial to the organism for the p16(-) cells to have extremely vigilant error recognition and repair processes, and stringent barriers to immortalization, since p16(-) cells are one step closer to immortalization. Possibly, the enhanced stability of wild type p53 protein in the p16(-) cells may increase the efficiency of remaining surveillance mechanisms.

In contrast to the first senescence barrier, the second barrier to indefinite growth, agonescence, does appear to be largely or exclusively due to telomere attrition. Although

we have not been able to define the alterations required for overcoming agonescence, our existing data and ongoing experiments (Garbe, Yaswen & Stampfer, in preparation, see below) do provide some clues from which we venture the following hypotheses. We suggest that two to three alterations are required to overcome agonescence. In cultured HMEC under no selective pressures, the likelihood that all the necessary errors would occur in the same cell, even under the conditions at agonescence where widespread genomic errors are generated, is exceedingly small. Thus, the senescence arrest observed in cultured p16(-) HMEC is extremely stringent. However, if one error is already present, e.g., overexpressed ZNF217, or inactivated p53, the probability greatly increases that the required additional error(s) may be generated by the genomic instability produced by agonescence. EL cultures that gave rise to immortal lines, such as 184Aa, may harbor one predisposing error.

This hypothesis is based upon the data already presented as well as the following preliminary results:

(a) EL 184Aa is readily immortalized by transduction with c-myc or inactivation of p53 by the p53-inhibiting GSE22, whereas post-selection 184 HMEC are poorly, if at all immortalized by these methods. 184 HMEC infected with GSE22 do exhibit a brief extension of proliferative capacity, followed by large-scale cell death.

(b) 184 HMEC and EL 184Aa show a similar low capacity to be immortalized by ZNF217. Evidence of immortalization is first detected at agonescence, and the lines contain numerous chromosomal aberrations.

(c) GSE22 transduction leads to telomerase reactivation in p53(+) pre-conversion conditionally immortal 184A1 but not in finite lifespan 184 HMEC or EL 184Aa.

The p53 pathway appears to play a key role in overcoming agonescence. However, as with the case of RB, total loss of p53 is not required. It is possible that a subset of functions controlled by p53 is involved, and alterations in molecules responsible for these functions may be as effective as total p53 loss in overcoming agonescence. Such alterations would not have the same impact on a cell as total loss of p53 function. Similar to the situation with RB, cancer cells that immortalize while retaining normal p53 might be expected to express a more normal phenotype, and immortalization of HMEC by methods that totally eliminate p53 function may not model the state of a large percentage of human breast cancers. Our data indicate that whether or not p53 is lost as part of the immortalization process can have significant consequences for the cell. p53(-) immortal HMEC attained an aggressive growth potential much more rapidly than p53(+), and also evidenced ongoing genomic instability.

Our data generated from the p53(+) immortal HMEC initially suggested that the transformation to an indefinite lifespan was not coincident with the reactivation of telomerase activity. Our data with the p53(-/-) lines suggest a refinement of this model. Overcoming agonescence may involve induction of the *potential* to express telomerase activity. Where p53 remains present, it may inhibit telomerase activity, possibly through direct association with other proteins in the telomerase complex [81]. In this situation, telomerase reactivation is dependent upon telomere length. As the ongoing proliferation produces critically short telomeres, some as yet undefined change occurs when the TRF

reaches <2.5 kb, possibly in gene expression or in conformation of the telomeric ends, that gradually releases this p53-imposed inhibition. In the absence of p53, there is no requirement for telomeres to decline to <2.5 kb for telomerase reactivation. There is still a short conversion period for telomerase to be fully reactivated and uniform good growth to be attained. The more rapid telomerase reactivation stabilizes telomere length at ~4 kb. The nature of the events producing this more rapid, but still not immediate conversion, is currently unknown.

The ability of 184A1 to maintain growth with critically short telomeres, without generating widespread chromosomal aberrations, suggests that one of the alterations required to overcome agonescence is protection of short telomeric ends to permit continued proliferation with critically short telomeres. Changes in telomere-associated proteins and/or their conformation may be involved. It is also possible that a mechanical-tensile constraint is engendered by very short telomeres, contributing to mitotic failures. Alterations that relieve such a constraint might then be involved in overcoming agonescence. If the alterations that prevent mitotic failures occur prior to agonescence, as in 184A1, resulting immortal lines may be near diploid and stable. If they occur as cells enter agonescence, as is likely the situation for 184B5, or well into agonescence, as in 184AA4, the resulting lines will start out with chromosomal aberrations generated by the agonescence process rather than by immortalization. Once rearrangements and translocations are present, as exist in early 184B5 and 184AA4, further fusion-bridge-breakage cycles could give rise to the low level of genomic instability observed in these p53(+) lines. However, the widespread instability seen in the p53(-/-) lines is not present.

We have not yet been able to delineate all the cause-and-effect relationships among the changes that occur during conversion. It is also likely that further research will uncover additional alterations. At this point, it appears that:

(a) p53 positively controls expression of p57.
(b) Telomere length of < 3 kb, in the presence of p53, positively controls p57 expression after release from G0, which in turn leads to slow growth. We do not know what controls the down-regulation of this p57 expression as conversion proceeds.
(c) p53 negatively controls telomerase activity when the mean TRF is >2.5 kb.
(d) Telomerase activity induces acquisition of TGFβ resistance, and possibly changes in c-myc regulation,

The factors controlling the change in the effect of oncogenic Raf-1 expression during conversion, from being growth inhibitory to promoting malignancy, are not known.

The development of an extensive series of normal and transformed cells from one human epithelial cell type has allowed us to compare the effects of a variety of different agents. Our data indicate that different pathways of producing an indefinite lifespan give rise to cells with widely varying phenotypes. Immortal and tumorigenic HMEC may or may not: express RB, p16, or p53, be growth-inhibited or malignancy-promoted by oncogenic Raf, express p57 and undergo a prolonged period of slow heterogeneous growth, possess very short telomeres, or exhibit karyotypic aberrations or genomic instability. These many variables may have profound effects on the nature of the tumor

produced by such a cell, the progression of such a tumor *in vivo*, and how such a tumor may respond to therapeutic interventions. Our recent studies have particularly emphasized the importance of whether or not cells possess wild type p53. This one variable may distinguish between agonescence and crisis, and between very gradual or relatively rapid telomerase reactivation. Since most model systems of immortalization *in vitro* have obligately induced inactivation of p53 function, some of these important differences have been obscured. All the methods of immortalization that we have studied produce obligate alterations of some of the growth control processes found in normal finite lifespan cells. These data emphasize the importance of recognizing that normal human cells posses a finite lifespan and have wild type p53 and RB. Cells that are immortal and/or p53(-) and RB(-) are not normal, and their growth control processes may not accurately reflect the normal situation.

7.2. Potential role of conversion during *in vivo* carcinogenesis

There is currently no data clearly demonstrating that a conversion process occurs during *in vivo* human breast carcinogenesis. However, there are several ways in which the data we have obtained from our *in vitro* systems may model the development of human breast tumors *in vivo.*

(a) Telomerase activity is detectable early in breast cancer progression. It is seen in ~50% of ductal carcinomas *in situ*, and in most primary tumors [82, 83, 84, 85]. These data are consistent with a conversion process beginning early in breast carcinogenesis, in ductal carcinoma *in situ*, prior to acquisition of invasive and metastatic potential. Conditionally immortal cells undergoing conversion may express detectable telomerase activity, yet continue to exhibit slow non-uniform growth for an extended time. An extended period of conversion would provide a continuous pool of slowly dividing cells able to accumulate errors that both provide a selective growth advantage and promote malignant behavior.

(b) Many primary carcinomas (particularly those like breast that are mainly p53 wild type) exhibit an extended period of slow, heterogeneous growth prior to the appearance of more aggressive and metastatic tumors [86, 87]. A gradual conversion process, with its generation of extensive heterogeneity, could at least partially account for this heterogeneous growth and clonal selection. Conversion to full immortality might not even be necessary for metastasis; an extended period of conditional immortality could be sufficient. Our data indicate that conditionally immortal cells can undergo a very large number of population doublings before becoming fully immortal. We have also observed the rare stochastic emergence of more aggressively growing fully converted cells [14].

(c) p53(-) breast tumors have a poorer prognosis [44, 45, 47]. Where examined, loss of p53 occurs early in breast cancer, in ductal carcinoma *in situ*. These findings are consistent with a model in which p53 loss precedes and promotes full immortalization. The rapid conversion exhibited by the p53(-/-) immortal HMEC lines, with early telomerase reactivation, no p57 expression, and no prolonged slow growth phase, produces aggressively growing cells more rapidly. Our newly

uncovered role of p53 in retarding full immortalization could at least partially account for why p53(-) tumors have a poorer prognosis.

(d) Abnormal karyotypes are found in most human carcinomas. The generation of widespread chromosomal aberrations during agonescence could at least partially account for this finding, particularly for p53(+) tumors. Consistent with this hypothesis, karyotypic abnormalities are seen early in breast carcinogenesis, in ductal carcinoma *in situ* [88]. Pathways of immortalization that require generation of errors during agonescence might be expected to give rise to tumors that exhibit a greater degree of chromosomal aberrations and have a poorer prognosis.

(e) Human epithelial cancers show a steep age-dependent increase in incidence, and tumor tissues and tumor-derived cell lines express short, regulated telomere lengths. The need to undergo sufficient population doublings to reach and overcome senescence, and undergo conversion — processes that produce cells with short telomeres — could at least partially explain these findings. All of our immortal lines, other than those obtained from exogenous introduction of hTERT, express a mean TRF length of ~3-7 kb, similar to what is observed in most tumor derived lines.

(f) As discussed in Section 3.4, the common finding that tumor cells can maintain some growth in TGFβ even in the absence of detectable mutations in TGFβ pathways may be due to an obligate gain of TGFβ resistance following telomerase reactivation.

Although our HMEC model systems may provide a closer approximation of *in vivo* processes of carcinogenesis, it is important to recognize that they are also only partial approximations. They do not address the important issue of cell-cell, and cell-matrix interactions, and our immortally transformed cells lack certain phenotypes commonly found in breast cancer cells, such as expression of keratin 19 and the estrogen receptor.

8. Conclusions

Our data on immortal transformation of HMEC support a model whereby attaining full immortality by overcoming replicative senescence and undergoing conversion may be rate-limiting steps in cells' acquiring invasive and metastatic potential. Multiple alterations are necessary to overcome both the selection and agonescence barriers, and further limitations are imposed by conversion, particularly in p53(+) immortal cells. In contrast, we and others have shown that once HMEC have undergone immortal transformation and reactivated telomerase activity, further transformation to anchorage-independent growth and/or tumorigenicity is readily attained with exposure to one or two potential oncogenes e.g., SV40 LgT and H-Ras [89, 90], erbB2 [91, 92], or insulin receptor [93].

The model we have presented for telomerase reactivation and immortal transformation is only applicable where there is stringent imposition of replicative senescence, including stringent control of telomerase expression. Exogenous introduction of high

levels of hTERT, and immortalization via HPV16-E6/E7 do not accurately model what occurs as a result of overcoming senescence and undergoing conversion. Immortalization via other oncogenes, such as SV40-T antigens, also introduces multiple unknown cell-cycle deregulating functions, in addition to p53 inactivation, that may not occur during breast carcinogenesis *in vivo*. Short-lived animals, such as rodents, lack stringent barriers to indefinite growth potential. Telomerase activity is not strictly suppressed and immortal transformation is not rate-limiting. Consequently, other changes, which may be less rate-limiting or significant in human cells, may be more prominent in rodent cell systems.

The potential to translate discoveries seen *in vitro* into useful therapeutic approaches may require utilization of model systems that as accurately as possible model the biological process of intrinsic interest. Information gathered from the study of human cancer tissues and derived cells can provide the necessary guidance for evaluating the extent to which culture systems accurately model human carcinogenesis. In particular, the reactivation of telomerase activity appears to be the most crucial event necessary for attaining an indefinite lifespan, which in turn appears crucial for malignant progression. In our *in vitro* model systems, telomerase reactivation required overcoming agonescence and undergoing conversion — processes that entail the attainment of very short telomeres. This mode of telomerase reactivation may be critical for modeling *in vivo* carcinogenesis.

Further studies will be required to fill in some of the currently outstanding gaps in our knowledge of the mechanisms by which telomerase is reactivated, e.g., (a) how do very short telomeres induce the instability seen at agonescence; (b) what are the alterations involved in overcoming agonescence, including the suppression of chromosomal rearrangements and the acquisition of telomerase activity; (c) how does p53 suppress telomerase activity in conditionally immortal HMEC; (d) how do the critically short telomeres present in conditionally immortal HMEC alter gene expression and/or telomerase activity. The normal and transformed HMEC systems that we have developed may facilitate such studies.

9. References

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CHAPTER 7

TELOMERASE, DNA DAMAGE AND APOPTOSIS

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Table of contents

1. Introduction

DNA is the molecular template upon which the diversity of organisms, as well as their evolutionary stability and plasticity in response to environmental demands, is based. Every nucleotide needs to be in its proper place and, accordingly, cells have evolved a molecular machinery that ensures the precise duplication of the genome prior to cell division. Cells also possess highly refined mechanisms for repairing damaged DNA molecules, a vital capability in light of the considerable damage to DNA that occurs during the lifetime of most organisms. In humans many cells, for example neurons and cardiac myocytes, can live and function properly for more than 100 years despite their DNA being subjected to repeated attacks by free radicals and other damaging conditions. Nevertheless, excessive damage to DNA can trigger a form of programmed cell death called apoptosis, a stereotyped biochemical cascade involving proteins such as p53, Bcl-2 family members and caspases [1]. Dysregulation of apoptosis is thought to play central roles in the pathogenesis of numerous human diseases, particularly age-related diseases such as cancers, neurodegenerative disorders and diabetes. In the

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case of mitotic cells, the apoptotic response to DNA damage is beneficial as it eliminates a cell that harbors mutations that have the potential to transform it into a cancer cell. Indeed, it has become clear that many cancers are caused by cells not dying when they should [2]. On the other hand, postmitotic cells such as neurons cannot usually be replaced and their unwanted deaths are responsible for disorders ranging from Alzheimer's and Parkinson's diseases [3] to myocardial infarction [4] and muscular dystrophies [5].

Telomerase was initially described as an enzyme activity that adds repeats of a six-base DNA sequence (TTAGGG) to chromosome ends (telomeres) and thereby prevents their shortening during successive rounds of mitosis [6-7]. Telomerase activity is downregulated during the process of differentiation of somatic cells during development [8-9], and is also suppressed in tumor cells induced to cease dividing and differentiate [10-12]. Telomerase activity requires an RNA template (TR) and a protein called TERT that possesses reverse transcriptase activity. In addition, several telomere-associated proteins have been identified including: TRF1 (telomerase repeat-binding factor 1) which may inhibit telomerase activity and promote telomere shortening, and TRF-2 which may promote maintenance of telomeres, and TEP-1 which can modulate telomerase activity by interacting directly with TERT [13-14]. Telomerase can guard the genome against damage by preventing chromosome shortening, translocations and end-to-end fusions. Suppression of TERT expression or telomerase activity promotes apoptosis of tumor cells, and overexpression of TERT increases resistance of tumor cells to apoptosis[11-12]. Interestingly, recent findings described below suggest that telomerase exerts additional activities that can prevent cell death induced by DNA damage, as well as other stimuli that may not involve DNA damage.

2. DNA damage and repair mechanisms

DNA can be damaged in many ways including single-strand breaks, more severe double-strand breaks, and macromolecular rearrangements including translocations and end-to-end fusions of chromosomes. These kinds of damage can be inflicted by rather non-specific attack of the DNA strands by oxygen free radicals, by depletion or imbalances of DNA nucleotide precursor pools, or by more specific enzymatic reactions (Figure 1). In addition, many errors occur during the processes of DNA synthesis and chromosome movements in mitotic cells that are preparing to divide. Because of the dire consequences of DNA damage for the cell, organism and species, cells possess sophisticated systems for rapidly repairing damaged DNA and preserving its information content. The repair systems typically involve the coordinated efforts of multiple proteins that serve to identify the lesion, remove the defective molecules, and restore the molecular integrity of the DNA. Information concerning the functions of telomerase in DNA damage and repair has recently begun to accumulate, and it is therefore important for investigators in the telomerase field to understand mechanisms of DNA damage and repair, a heavily studied area of biomedical science.

2.1. DNA damage: enemy attacks and internal mistakes

How does the molecular integrity of DNA become compromised? One useful way of viewing this problem is to divide the mechanisms that cause DNA damage into those that involve attack of intact DNA strands by exogenous molecules such as free radicals or hydrolytic enzymes, and those that involve errors in the process of DNA replication. DNA damage can be caused by radiation, endogenous DNA-modifying enzymes (endonucleases), chemical toxins (both endogenous and exogenous) and mistakes during replication. One useful way to view DNA damage is to classify the damage as either single-strand breaks or double-strand breaks. A single-strand break leaves the other anti-parallel strand that provides physical integrity and nucleotide sequence content to diret the accurate repair of the defective strand. On the other hand, a double-strand break results in loss of both physical integrity and sequence information content of both strands such that the cell permanently loses information on that particular chromosome, even if it puts the two ends back together.

DNA strand breaks can be detected *in situ* using several techniques including the terminal dUTP nick-end labeling (TUNEL) protocol [15] and the "comet" assay [16]. Hydroxyl radical is generally considered to the major oxyradical that causes DNA damage in cells exposed to ionizing radiation or to conditions that elevate levels of hydrogen peroxide or ferrous iron (Fe^{2+} catalyzes the production of hydroxyl radical from hydrogen peroxide). Hydroxyl radical can induce either single- or double-strand breaks, with the number of double-strand breaks increasing in proportion to the concentration of hydroxyl radicals [17]. Peroxynitrite is another reactive oxygen molecule that can attack DNA [18]. Peroxynitrite is generated by the interaction of nitric oxide with superoxide anion radical, which is often stimulated by elevation of intracellular calcium levels (calcium activates nitric oxide synthase). HPLC-based protocols allow detection and quantification of specific oxidatively modified DNA bases, with 8-oxyguanine being the most widely used marker of hydroxyl radical attack on DNA [19]. Modifications of similar protocols can be used to detect DNA damaged by peroxynitrite[20].

DNA strand breaks can be induced by a variety of chemicals and, indeed, the ability of chemicals to induce DNA damage is strongly correlated with their ability to promote cancer formation [21]. Some DNA-damaging chemicals act directly on the DNA, whereas others act indirectly by inhibiting enzymes critical for maintenance of DNA integrity. An excellent example of the latter class of DNA-damaging agents are the topoisomerase inhibitors camptothecin (topoisomerase-1) and etoposide (topoisomerase-2) [22-23].

DNA damage can also result from aberrancies in one or more of the various enzymatic systems that control DNA replication and repair. Dysfunction of DNA polymerases, enzymes that unwind or cut DNA (helicases, topoisomerases and endonucleases), and proteins involved in regulating the dramatic macromolecular rearrangements of DNA that occur during DNA synthesis and cell division can lead to DNA damage of varying amounts. Mutations in several of these DNA-regulating proteins have been linked to defects in development and aging. For example, mutations in a DNA helicase cause a remarkable disorder called Werner's syndrome which is characterized by an acceleration of aging-like processes throughout the body [24]. Cockayne and Bloom's

syndromes also result from mutations in DNA helicases [25]. Mutations in topoisomerase-like genes may be responsible for Alport syndrome, an inherited nephropathy [26].

2.2. DNA protection and repair: high priority

In order to understand the functions of telomerase in protecting the genome and preventing DNA damage-induced death it is important to understand the various DNA protection and repair mechanisms that cells employ. Cells devote a great deal of their resources to protecting and repairing DNA and, although we will focus primarily on mechanisms operative in the immediate environment of the DNA, it should be recognized that indirect mechanisms for protecting DNA function throughout the cell. For example, let's consider how a cell reduces the likelihood of free radical-mediated damage to its DNA. Cells evolved in a highly oxidizing environment where their nucleic acids were surely exposed to repeated attacks by free radicals. Free radicals can be produced in different cell compartments, with mitochondria being the most prominent radical-generating organelle [27]. Cells possess very efficient pathways for rapidly removing reactive oxygen species. For example, the superoxide anion radical produced during mitochondrial respiration is rapidly converted to hydrogen peroxide via the action of manganese and copper/zinc superoxide dismutases, Hydrogen peroxide is then converted to water via the action of catalase and glutathione peroxidase. The activity of these antioxidant enzymes thereby prevent formation of DNA-damaging radicals such as hydroxyl radical and peroxynitrite. Other systems for preventing initial attacks on DNA by free radicals include thiols such as glutathione and free radical scavenging vitamins. Once DNA is damaged, a remakably extensive set of pathways are activated that are designed to repair the DNA and to protect the cell against further stress. The DNA damage stress response involves upregulation of families of genes that encode proteins that function in various cellular compartments. An extensively studied class of such stress proteins are the heat-shock proteins, which include proteins located in the nucleus, cytoplasm, mitochondria and endoplasmic reticulum. The literature on heat-shock proteins in DNA damage and cellular resistance to death is extensive [28-29] and will not be considered further here, except where direct links with telomerase are suggested.

DNA repair mechanisms can be divided into four types: mismatch repair, nucleotide excision repair, base excision repair and direct reversal (Figure 1). Mismatches can occur as the result of incorrect incorporation by DNA polymerases, by damage to nucleotides in the precursor pool, or by direct damage to the DNA. Mismatch repair involves recognition and correction of incorrectly paired oligonucleotides in DNA such that a large fragment of DNA from the mismatched strand is excised and new DNA synthesized. Several proteins involved in mismatch repair have been identified including MSH2, MSH3 and MSH6 [30], which may form one or more protein complexes that, by mechanisms not yet established, repair base to base and insertion/deletion mismatches. Defects in mismatch pair have been associated with several types of cancer [30-31] and mice lacking MSH2 have a greatly increased cancer susceptibility [32]. Nucleotide excision repair is coupled to transcription and repairs large lesions of transcribed genes. It can be divided into four steps: recognition, incision, gap-filling and structural repair. At least 11 different proteins, acting together in complex ways not yet fully understood,

play a role in nucleotide excision repair [33]. Mutations in genes encoding nucleotide excision repair proteins have been linked to several different inherited disorders including Cockayne syndrome, xeroderma pigmentosum and trichothiodystrophy (Lindahl et al., 1997). Xeroderma pigmentosum is characterized by greatly increased risk for skin cancer, whereas Cockayne syndrome and trichothiodystrophy are characterized by developmental abnormalities that are particularly pronounced in the nervous system and skeleton. Base excision repair involves removal of short stretches of DNA (typically less than 12 nucleotides) and is accomplished in four steps: removal of the damaged base by a glycosylase, cleavage of the phosphodiester backbone by an apyrimidinic/apurinic endonuclease, insertion of the complementary nucleotides by a polymerase with removal of the phosphodeoxyribose group, and ligation of the DNA backbone to restore the native sequence and structure [34]. Proteins involved in base excision repair include formamidopyrmidine-DNA glycosylase, 3-methyladenine DNA glycosylase and 8-oxoguanine DNA glycosylase [34-35]. After cleavage of the phosphodiester backbone, completion of repair is accomplished by DNA polymerase-β, which removes the deoxyribose phosphate and adds a nucleotide, followed by DNA ligase-I or DNA ligase-III which seals the nick. Direct repair of damaged DNA bases (fixing the base without removing it) is mediated by alkyltransferase proteins which remove alkyl groups from the O^6 position of guanine or the O^4 position of thymine. One such alkyltransferase is O^6-methylguanine-DNA methyltransferase [36]. Alterations in levels of the latter enzyme have been documented in many types of cancer including breast, ovarian, lung, colon and brain.

Two additional prominent DNA damage response pathways that appear to play particularly important roles in determining if, and by what mechanism (apoptosis or necrosis), a cell dies when DNA strand breaks occur involve poly(ADP-ribose) polymerase (PARP) and Ku proteins/DNA-dependent protein kinase. PARP is a predominately nuclear enzyme that catalyzes the transfer of successive molecules of ADP-ribose from NAD^+ to itself and other acceptor proteins. The catalytic activity is of PARP is dependent upon DNA strand breaks and is modified by autoribosylation. Studies that have employed chemical inhibitors of PARP and PARP-deficient mice, together with studies of protein substrates for PARP (e.g., DNA polymerases and ligases, topoisomerases, histones, DNA-dependent protein kinase and p53), strongly suggest major roles for PARP in DNA replication, recombination and repair [37-39]. PARP inhibitors can either promote death of tumor cells [40] or prevent death of postmitotic cells such as neurons[16]. It appears that PARP's function in DNA repair is central to its cytoprotective action. However, when the extent of DNA damage passes a threshold level such that a cell cannot effectively repair it, PARP becomes an inducer of cell death [41]. Under the latter conditions PARP is cleaved and inactivated by caspases, effectively shutting down DNA repair and facilitating activation of p53 and endonucleases, two PARP substrates that play important roles in the process of apoptosis. Interestingly, when caspases are inhibited in cells with extensive DNA damage, the mode of cell death can be shifted from apoptosis to necrosis and maintenance of PARP activity (due to lack of inactivation by caspases) plays a central role in such necrotic cell death by promoting massive depletion of cellular NAD^+ and ATP [16].

DNA double strand breaks are a prominent lesion induced by ionizing radiation and other free radical-mediated attacks on DNA. Three proteins that cooperatively repair such double strand breaks are Ku70, Ku80 and the 470 kDa catalytic subunit of DNA-dependent protein kinase (DNA-PKcs) [42]. Ku70 and Ku80 form a heterodimer that binds tightly to DNA ends and recruits DNA-PKcs [43-44]. Upon binding to Ku proteins and DNA, the protein kinase activity of DNA-PKcs increases resulting in the phosphorylation of several different DNA-associated and soluble protein substrates including Ku proteins, XRCC4 (a small protein that forms a tight complex with DNA ligase IV) and p53. Phosphorylation of p53 by DNA-PKcs weakens the interaction of p53 with its negative regulator MDM2 and also enhances p53 DNA binding. The critical role of the Ku proteins and DNA-PKcs in maintenance of DNA structure and function is evident from the phenotypes of mice lacking Ku70, Ku80 or DNA-PKcs (SCID mice) which each exhibit profound depletion of T and B lymphocytes, caused by defects in V(D)J. recombination, and predisposition to certain cancers [45-47].

3. DNA damage and age-related disease

DNA damage is implicated in the pathogenesis of many different age-related diseases including diabetes [48], arthritis [49], ischemic stroke [50] and myocardial infarction [51]. The two most prominent types of diseases in which DNA damage is clearly involved are cancers [52-53] and neurodegenerative disorders such as Alzheimer's and Parkinson's diseases [3, 54]. We will therefore briefly review the evidence supporting pivotal roles for DNA damage in the pathogenesis of cancers and Alzheimer's disease, and then focus the remainder of this article on how DNA damage can trigger apoptosis and how telomerase may intervene to suppress DNA damage and prevent apoptosis.

DNA damage is a major cause of cancer as is evident from the fact that many of the most prominent carcinogenic agents, including ionizing radiation and various toxic chemicals, are potent mutagens. Cancers most often result from cells with damaged DNA escaping elimination by cell cycle checkpoint and apoptotic control systems. A remarkable number of cancers are associated with mutations in p53, a key mediator of cell cycle arrest and apoptosis in cells with damaged DNA [55-56]. Mutations in other genes that encode proteins that control cell cycle checkpoint and/or apoptosis are also responsible for cancers including mutations in BRCA1 that cause breast cancer [57], mutations in mismatch repair genes that can cause colon cancer [58] and alterations in Bcl-2 which are associated with a variety of cancers including leukemias [59].

The evidence that DNA damage plays a role in the pathogenesis of Alzheimer's disease comes from studies of postmortem brain tissue from patients, and from experimental cell culture and animal models of this neurodegenerative disorder. Analyses of postmortem brain tissue from AD patients have demonstrated the presence of DNA damage including a greatly increased number of TUNEL-positive neurons [54, 60-61]and increased expression of several DNA damage-responsive genes including p53 [62] and GADD45 [63]. DNA repair capacity may also be compromised in Alzheimer's disease [64-65]. Exposure of cultured brain neurons to amyloid β-peptide, a neurotoxic protein that forms insoluble aggregates (plaques) in the brains of

Alzheimer's patients, can induce nuclear DNA damage and apoptosis [66]. Activation of p53 is required for amyloid-induced death of neurons, consistent with a DNA damage response [67]. Amyloid β-peptide induces oxidative stress in neurons including increased generation of superoxide anion radical, hydrogen peroxide, hydroxyl radical and peroxynitrite [68-70]. Overexpression of manganese superoxide dismutase, and treatment of cells with antioxidants (vitamin E, uric acid and reduced glutathione) can protect neurons against DNA damage and apoptosis [68, 69] demonstrating a requirement for oxyradical production in the death-promoting action of the amyloid peptide. Moreover, mutations in the presenilin-1 gene that cause early-onset inherited forms of Alzheimer's disease greatly enhance oxyradical production and increase the sensitivity of neurons to DNA damage-induced apoptosis when expressed in cultured neurons or knockin mice [71-72].

4. DNA damage and apoptosis

Apoptosis is a form of programmed cell death that plays important roles in sculpting the formation of many different organs and tissues during development, and in the maintenance of highly proliferative tissues in the adult. Aberrant regulation of apoptosis is increasingly recognized as a fundamental feature of many prominent human diseases including cancers and neurodegenerative disorders (where abnormalities in apoptosis may be primary), as well as cardiovascular disease, stroke and diabetes (where, by damaging heart, nerve and pancreatic cells) unwanted apoptosis contributes to the final outcome) [1, 3, 73]. Apoptosis can be induced by a variety of endogenous and exogenous signals including insufficient trophic support, overactivation of neurotransmitter and cytokine receptors, oxidative stress and exposure to toxins (Table 1).

Apoptosis is a cell death process involving caspase activation and lack of cell swelling with maintenance of organellar (mitochondrial and endoplasmic reticulum) integrity that occurs within a tissue in a "spotty" pattern such that dying cells are intermingled with healthy cells. This contrasts with necrosis, a form of cell death in which cellular organelles swell and the plasma membrane lyses resulting in massive death of groups of cells throughout a tissue. Mitochondria in cells undergoing apoptosis typically exhibit increased oxyradical production, opening of pores in their membranes and release of cytochrome c; these changes are central to the cell death process because agents such as manganese superoxide dismutase and cyclosporine A, that act directly on mitochondria to suppress oxidative stress and membrane pore formation also prevent cell death in experimental models [74]. The biochemical alterations that occur during the early stage of apoptosis induce mitochondrial alterations either directly or indirectly. The Bcl-2 family of proteins includes both antiapoptotic members such as Bcl-2 and Bcl-xL, and proapoptotic members such as Bax and Bad. Overexpression of Bcl-2 increases resistance of cells to death induced by many different insults including those that induce DNA damage [75]. Conversely, cells lacking Bax are protected against apoptosis. The mechanism by which Bcl-2 proteins control the cell death process appears to involve interactions among family members and association of the proteins with mitochondria resulting in alterations in ion movements across mitochondrial

membranes. Another protein that can regulate apoptosis at a premitochondrial stage is prostate apoptosis response-4 (Par-4). Par-4 is upregulated in prostate tumor cells undergoing apoptosis, and is now known to have an essential role in developmental and pathological death of many types of cells including neurons [76-77]. Par-4 levels rapidly increase in response to various apoptotic stimuli through enhanced translation of Par-4 mRNA. Par-4 induces apoptosis by interacting with other proteins via its leucine zipper domain; two proteins modulated by Par-4 that may play important roles in apoptosis are protein kinase Cζ and Bcl-2 [78].

Table 1. Examples of triggers of apoptosis in health and disease

Cell. Type	Trigger	Physiological or disease condition
Neurons	trophic withdrawal	developmental cell death
	glutamate	ischemia, seizures and neurodegeneraitve disorders
	amyloid peptides	Alzheimer's disease
	lipid peroxidation	neurodegenerative disorders
Epithelial cells	detachment from substrate	normal turnover
	ultraviolet light	cataracts, skin aging, cancer
Vascular endothelium	oxidized lipoproteins	atherosclerosis
	tumor necrosis factor	atherosclerosis
	amyloid	Alzheimer's, cerebrovascular disease
Monocytes	bacterial proteins	infection
Lymphocytes	Fas ligand	immune responses, cell turnover/selection
	glucocorticoids	stress response
Cardiac myocytes	trophic interactions	developmental cell death
	ischemia	myocardial infarction

Ref: 134-140

Caspases are cysteine proteases that play fundamental roles in coordinating the various structural and functional changes that occur in cells as they undergo apoptosis [79]. Some caspases are activated during the early phase of apoptosis; for example caspase-8 is activated in response to ligation of "death receptors" such as Fas and the TNF receptor. Upstream caspases can then activate "effector" caspases such as caspase-3 either directly or indirectly, and may thereby elicit apoptosis independently of mitochondrial alterations. Effector caspases are activated in response to mitochondrial changes and cytochrome c release; these caspases can then activate a DNAase that is

responsible for cleavage of DNA into oligonucleosome-size fragments. Caspases can also cleave a variety of substrate proteins that may coordinate the cell death process including enzymes such as poly-ADP-ribose polymerase and ATM kinase, ion channels, and cytoskeletal proteins such as actin and spectrin.

Three transcription factors that play prominent roles in apoptosis are p53, c-Myc and NF-κB [80-81, 124]. P53 promotes apoptosis by actions at a premitochondrial step; a large number of cancers involve p53 mutations that disrupt its death-promoting activity. In contrast to p53, both c-Myc and NF-κB prevent apoptosis, and activation of these transcription factors are associated with cancer formation.

5. Telomerase maintains DNA stability and prevents apoptosis

In mitotic somatic cells, telomeres shorten during successive cell divisions and ultimately reach a threshold length that triggers cell cycle arrest in the G1 phase (cellular senescence). In this way telomere shortening effectively limits the proliferative potential of cells, functions as a tumour suppressor mechanism and may contribute to the aging process [82-83]. Telomerase activity and TERT levels decrease during growth arrest and cellular senescence, and overexpression of TERT in cultured fibroblasts extends their lifespan [84], suggesting a pivotal role for telomerase in preventing cellular sensescence. Indeed, organisms such as fish and lobsters that undergo continuous growth of somatic tissues with negligible aging exhibit telomerase activity in all of their somatic cells throughout life [85-86]. Studies of mice lacking either the RNA component of telomerase [87] or TERT [88] support a role in suppressing cellular senescence, but also indicate that telomerase activity may not be essential for normal development of many tissues. Detailed reviews on telomere and telomerase research have recently appeared [83, 89]; the present article focuses on emerging evidence that telomerase functions to protect against DNA damage and inhibit apoptosis.

6. Telomerase, apoptosis and cancer

Many cancers are believed to result from cells not undergoing apoptosis when they should, which may result from either increased survival signals (such as constitutively active growth factor receptors, or increased NF-κB or Myc activity) or inactivating mutations in death-inducing proteins such as the tumour suppressor protein p53. The strong association of telomerase activity with cell immortalization and cancer [82, 90] suggested that telomerase might possess an anti-apoptotic function. Thus, cancer cells that are resistant to apoptosis have relatively high levels of telomerase activity, cell differentiation is associated with reduced telomerase activity and increased vulnerability to apoptosis, and levels of telomerase activity decrease in tumor cells undergoing apoptosis (but not in cells resistant to apoptosis) prior to their death [10-12]. Exposure of solid tumor cells to hypoxia results in an increase in telomerase activity which is correlated with an increase in activation of mitogen-activated protein (MAP) kinase and increased resistance to apoptosis [91]. Treatment of the cells with an

inhibitor of MAP kinase, and overexpression of a dominant negative MEK1, blocked hypoxia-induced activation of telomerase suggesting an important role for MAP kinase in regulation of telomerase activity and/or expression. Moreover, overexpression of Bcl-2 in cancer cells results in increased telomerase activity [12], and overexpresion of the tumor suppressor protein PTEN in cultured glioma cells results in a marked decrease in telomerase activity and increased spontaneous apoptosis [92]. Finally, TERT expression is induced by the transcription factor c-Myc, which is of considerable interest in understanding the anti-apoptotic mechanism of telomerase, because c-Myc is associated with cell immortalization and cancer [81]. Interestingly, c-Myc enhances cell proliferation and allows cells to grow in the absence of growth factors, but c-Myc can also promote apoptosis. The upregulation of TERT by c-Myc may therefore provide an explanation for why cancer cells expressing very high levels of c-Myc do not die.

Recent studies have established a cause-effect relationship between telomerase and resistance of cancer cells to apoptosis. The development of methods for inhibiting telomerase, in combination with the cloning of TERT and telomerase-associated proteins such as TRF1 and TRF2, has allowed direct tests of the role of telomerase in apoptosis. Treatment of cultured pheochromocytoma cells with agents that inhibit telomerase results in increased vulnerability of the cells to apoptosis induced by several different stimuli including trophic factor withdrawal and exposure to oxidative insults [12]. Telomerase inhibitors do not promote apoptosis in cells overexpressing Bcl-2, suggesting that telomerase acts to suppress an early step in the cell death pathway prior to the mitochondrial step at which Bcl-2 exerts its anti-apoptotic action. Treatment of cultured breast epithelial tumor cells with 2'-O-MeRNA and peptide-nucleic acid oligomers induces telomere shortening and apoptosis [93], and antisense oligonucleotides targeted against telomerase RNA induce apoptosis in ovarian cancer cells [94]. TERT appears to play a role in the anti-apoptotic action of telomerase because overexpression of TERT in pheochromocytoma cells results in decreased vulnerability to apoptosis (Figure 2), while suppression of TERT expression with antisense oligonucleotides promotes apoptosis [95-96]. In addition, expression of a non-functional catalytic subunit of telomerase in cultured tumor cells results in suppression of telomerase activity and cell death [97], and expression of a dominant-negative TERT mutant results in telomere length-dependent apoptosis [98]. Collectively, these findings suggest that at least some tumor cells are dependent upon telomerase activity for their survival.

7. Telomerase and apoptosis of mitotic and postmitotic somatic cells

Telomerase activity is present in cells throughout the body at very high levels during development, but is usually present at very low or undetectable levels in somatic cells of adults. The possibility that TERT and telomerase activity play roles in the death of somatic cells during development and in physiological and pathological settings in the adult has very recently gained support. The lifespan of vascular endothelial cells in culture can be extended by overexpression of TERT and this is associated with increased resistance to apoptosis [99]. In addition, overexpression of telomerase in normal diploid

cells with stable telomere lengths results in resistance to apoptosis induced by serum starvation and matrix deprivation [100]. Conversely, knockdown of TERT expression in developing neuron induces them to undergo apoptosis (Figure 3).

Studies of mice lacking either the RNA component of telomerase or TERT are proving valuable in understanding how telomerase activity and TERT modulate neuronal survival and death. Although mice lacking the RNA component of telomerase (TR-/-) exhibit no overt phenotypic abnormalities [87], they can be propogated for only a limited number of generations and then show decreased viability with age, which is associated with telomere shortening, reduced proliferation of B and T lymphocytes, and atrophy of the spleen and intestines [101-102]. Detailed analyses of fibroblasts cultured from successive generations of TR-/- mice reveal a major role for telomerase in preventing telomere shortening, and chromosome fusions and translocations [103]. Lymphocytes from TR-/- mice possess shortened telomeres, and the spleens of TR-/- mice have greatly reduced numbers of germinal centers [104], consistent with increased spontaneous apoptosis of lymphocytes. A major developmental alteration in TR-/- embryos is failure to close the neural tube which is strongly correlated with decreased telomere length [105], and may result from aberrant apoptosis of neural progenitor cells. TR-/- mice also exhibit abnormalities in liver regeneration and enhanced cirrhosis in response to chronic liver injury; these abnormalities are eliminated when telomerase RNA is restored [106]. Although data from TERT-deficient mice are only now being generated, initial findings suggest that, with respect to telomere maintenance, TERT-/- mice are similar to TR-/- mice [88].

A new and unexpected role for telomerase in postmitotic neurons in the developing brain was recently established [95, 105]. Measurements of telomerase activity, and TERT protein levels and cellular localization, revealed that TERT and telomerase activity are present at very high levels in neurons during embryonic and early postnatal development in rats and mice [107]. TERT and telomerase activity then rapidly decrease to undetectable levels in neurons by the second postnatal week; the time window during which the levels of TERT decrease corresponds to the time period when neurons undergo developmental cell death [107]. When TERT levels are decreased in cultured embryonic brain neurons using antisense technology, the neurons undergo apoptosis [95] (Figure 3). The mechanisms that regulate TERT expression during development of the nervous system are unknown, but the fact that TERT is present in postmitotic neurons suggests that its anti-apoptotic function may be independent of maintenance of chromosome ends. The temporal dissociation between the developmental decreases in telomerase activity (which occurs early in embryonic brain development) and TERT expression (which occurs postnatally) provides further evidence for a telomere maintenance-independent action of TERT in brain development [107].

8. How does telomerase suppress apoptosis?

In light of the established function of telomerase, one possibility is that telomerase suppresses DNA damage, or signals generated in response to DNA damage, by stabilizing chromosome ends. DNA damage is well-established as a trigger for apoptosis, and

telomerase can protect cells against apoptosis induced by agents known to cause DNA damage including oxidative insults and amyloid β-peptide [12, 96]. Studies of telomerase deficient mice have shown that activation of the DNA damage-sensitive protein p53 occurs in association with telomere shortening and leads to growth arrest and apoptosis [108]. P53 plays an important role in the phenotype resulting from telomerase deficiency because deletion of p53 significantly decreases the adverse effects of telomerase deficiency. In addition, induction of G-rich single stranded DNA fragments induces p53 dependent cell cycle arrest in fibroblasts and tumor cells, and reduced telomerase activity may promote cell cycle arrest by a similar mechanism [109]. Suppression of TRF2 function by overexpression of a dominant negative form of TRF2 induces apoptosis in HeLa cells [108]. However, suppression of TRF2 function does not induce apoptosis in all cell types examined. Thus, whereas immortalized B cells and an adenocarcinoma cell line are sensitve to the killing effect of TRF2 suppression, the fibrosarcoma cell line HT-1080 and the HDF cell strain IMR-90 are resistant. The resistant cell lines are either deficient in p53 function or lack an apoptotic response to DNA damage [110]. A critical role for p53 induction in apoptosis resulting from TRF2 inactivation is suggested by the demonstration that cells expressing dominant negative TRF2 exhibit increased p53 levels.

Further evidence that telomerase can suppress a DNA damage-induced apoptotic pathway comes from studies of the ataxia-telangiectasia mutated (ATM) kinase, mutations in which cause a disorder that involves increased apoptosis of neurons in the cerebellum. The ATM-p53 pathway may play a central role in the apoptotic pathway activated by DNA damage [111]. Activation of the ATM kinase, which acts upstream of p53, may be necessary for telomere dysfunction-induced apoptosis. Moreover, TERT interacts with the DNA damage-activated protein tyrosine kinase c-Abl resulting in phosphorylation of TERT and inhibition of its telomere maintenance activity [112]. Finally, a novel telomere-asssociated protein called tankyrase, with homology to the DNA damage-responsive protein poly (ADP- ribose) polymerase (PARP), was recently described [113], further strengthening the evidence that telomere-associated proteins may prevent apoptosis by suppressing a DNA damage response pathway. Interestingly, telomeres can be maintained in the absence of telomerase, and DNA damage response mechanisms may contribute to such telomere maintenance. Indeed, mice lacking PARP exhibit enhanced telomere shortening compared with wild-type mice, although telomerase activity is not altered [114]. When taken together with the compelling evidence that PARP functions in repair of DNA damage and a cell's decision to undergo apoptosis in response to DNA damage [16, 37-39], the structural and functional interelationships between TERT and PARP at telomeres strongly suggest a role for TERT in modulating DNA damage response and repair pathways.

Although suppression of a DNA damage response by protection of chromosome ends is an attractive hypothesis for the anti-apoptotic action of telomerase activity, accumulating data suggest that TERT may prevent apoptosis independently of its reverse transcriptase activity. Recent findings support a mechanism in which TERT interrupts the cell death process at a premitochondrial step (Figure 4). The increased vulnerability to apoptosis of embryonic neurons with low levels of TERT is associated with mitochondrial dysfunction and caspase activation[95-96], and treatment of

TERT-deficient neurons with caspase inhibitors or cyclosporin A, an agent that blocks the mitochondrial permeability transition, prevents cell death [95-96]. Conversely, overexpression of TERT in pheochromocytoma cells prevents mitochondrial dysfunction and caspase activation after exposure of the cells to apoptotic insults [12]. Although TERT is usually present at high levels in the nucleus, in many cells it is also present in the cytoplasm [95]. Because TERT acts at a premitochondrial step in the apoptotic cascade, it is possible that it may modify the function of cytoplasmic proteins such as Bcl-2 family members or Par-4, that are known to control apoptosis at that point in the cell death process. Thus, the available data suggest that TERT can suppress apoptosis by protecting telomeric DNA and by suppressing death signals at a premitochondrial step (Figure 5).

Even a superficial examination of proteins that interact with telomeres and/or telomerase strongly suggests mechanistic links between DNA damage, telomerase and apoptosis (Table 2). DNA damage-responsive proteins known to play major roles in DNA repair and apoptosis are associated with telomeres including Ku80 and PARP. Ku80 may associate with a stem-loop structure in TR and thereby recruit or activate telomerase at the telomere [115]. HSP90, a chaperone protein that plays critical roles in cellular stress responses and apoptosis [116] and is phosphorylated by DNA-dependent protein kinase [117], has been shown to bind TERT and enhance its catalytic activity [118]. The 14-3-3 protein, which is known to play important roles in suppressing apoptotic cascades by modulating activities of Bcl-2 family members [119], has been shown to bind TERT and may promote retention of TERT in the nucleus [120]. A final example is Rap-1 which can sequester proteins, including TERT, at telomeres [121-122]; Rap-1 has also been shown to modify the sensitivity of cells to apoptosis [123].

Table 2. Examples of telomere-associated proteins and their proposed functions

Protein	Established or proposed function
TERT	Telomerase reverse transcriptase; telomere length maintenance, cell proliferation, differentiation, survival
TR	Telomerase RNA component; template for synthesis of TTAGGG repeats
TRF1	Binds telomeres; may inhibit telomerase and promote telomere shortening
TRF2	Telomere maintenance
RAP1	Telomere maintenance
TEP1	Binds TERT and may modulate its enzymatic activity
Tankyrase	Poly (ADP ribose) polymerase that can ribosylate TRF1
PARP	Mediator of DNA damage-induced apoptosis
Ku80	Telomere-associated DNA repair protein
Dyskerin	Associates with TR; facilitates telomerase activity
HSP90	Chaperone that binds TERT and facilitates telomerase activity
P23	Chaperone that binds TERT and facilitates telomerase activity
L22	Associated with TR; function not established
Hstau	Associated with TR; function not established
14-3-3	Binds TERT; may function in retention of TERT in the nucleus

Finally, emerging data on the signals that regulate TERT expression reveal further links to DNA damage response and cell survival pathways (Table 3). NF-κB is a transcription factor that induces expression of an array of genes whose encoded proteins act to prevent apoptosis [124]. TERT expression can be induced by NF-κB [125], which suggests the possibility that TERT is upregulated in response to survival signals, such as growth factors, cytokines and stress responses. The contribution of such upregulation of TERT to the anti-apoptotic effect of NF-κB remains to be established. Estrogen, which is known to promote the survival of many cell types and is also linked to formation of certain types of cancer in women, can induce TERT expression in several types of cells [107, 126]. TERT expression is also subject to negative regulation. P53, a prominent DNA damage-responsive regulator of cell cycle checkpoint and apoptosis, can suppress transcription of TERT [127]. This action of p53 might contribute to the suppression of telomerase activity in cells undergoing p53-mediated apoptosis [12]. Another interesting negative regulator of TERT expression is MZF-2 [128], a transcription factor that may play an important role in differentiation of myeloid cells. As a final example, it was recently shown that the transcription factor Mad1 can inhibit TERT expression [129]. The ability of Mad1 to inhibit cell proliferation is consistent with suppression of TERT expression, whereas the ability of Mad1 to inhibit apoptosis would appear not to involve suppression of TERT expression[130].

Table 3. Examples of Transcriptional and Post-Transcriptional Regulation of TERT

Regulator	Effect on TERT expression or function	Reference
NF-κB	Increased transcription	125
Estrogen receptor	Increased transcription	126
Myc	Increased transcription	141
Sp1	Increased transcription	141
MZF-2	Increased transcription	128
P53	Suppresses transcription	127
Mad1	Suppresses transcription	129
Akt	Increases enzyme activity	142
PKC	Increases enzyme activity	143
Bcl-2	Increases activity	144

9. Telomerase as a target for disease prevention and treatment

The data described above suggest possible roles of telomerase activity, TERT, and other telomere-associated proteins in modulating apoptosis that occurs in both physiological settings and pathological states. Cancers and neurodegenerative disorders are two types of diseases for which the anti-apoptotic actions of telomerase have strong implications. The efficacy of telomerase inhibition in killing cancer cells has been

repeatedly demonstrated. Compounds that inhibit telomerase and promote death of cancer cells include: 3' -azido-2' 3' -dideoxythymidine (AZT), carbocyanine-based drugs, ribozymes, peptide nucleic acid and2'-O-MeRNA oligomers, and TTAGGG itself [12, 96, 131, 132]. In addition, TR and TERT antisense oligonucleotides have been used to kill tumor cells [95, 96, 133]. Because most somatic cells exhibit little or no telomerase activity, telomerase inhibitors would be predicted not to cause extensive damage to the non-cancerous tissue cells.

The strategy for neurodegenerative disorders is diametrically opposed to the cancer therapy strategy — the goal would be to increase expression of TERT and telomerase activity in neurons. A gene therapy approach involving overexpression of TERT proved effective in preventing neuronal death in experimental cell culture models relevant to the pathogenesis of Alzheimer's disease and stroke. A second approach would be to identify compounds that induce expression of TERT and telomerase activity in neurons. Studies indicate that telomerase is indeed subject to regulation by environmental signals (Table 3). For example, estrogens, and growth factors and cytokines that activate NF-κB, can induce TERT expression and telomerase activity in cells. The latter findings are intriguing because of data implicating estrogen in promoting some cancers. Finally, telomerase activity is induced in brain cells in response to neuronal injury (W. Fu and M. P. Mattson, unpublished data), suggesting a role for telomerase in cellular responses to tissue injury. In any case, the revelation that telomerase can prevent cell death in various experimental paradigms places telomere-associated proteins firmly in the field of apoptosis research and opens the door for future studies of telomerase in the pathogenesis of the multitude of human diseases that involve aberrant regulation of cell death.

10. Conclusion

The proper functioning of all cells, and the ability of cells and organisms to evolve through inheritance of genetic traits, relies on their ability to maintain the structural integrity of their DNA. This is not an easy task, because DNA is under constant attack by oxygen free radicals and enzymes that induce single- or double-strand breaks, and because even seemingly minor errors in the DNA replication process can have catastrophic consequences. A fascinating and complex molecular machinery has evolved that largely eliminates errors in DNA replication and can also repair DNA damaged as the result of attack by free radicals and other insults. The machinery includes mechanisms ranging from antioxidant and DNA repair enzymes to proteins that physically protect DNA. Excessive DNA damage can trigger a form of programmed cell death called apoptosis which involves a coordinated series of molecular interactions that include changes in mitochondria and activation of proteases called caspases. Apoptosis provides a mechanism that eliminates potentially cancerous cells from proliferative tissues, but may also result in the unwanted deaths of postmitotic cells such as neurons and myocytes. Accordingly, aberrant regulation of apoptosis is central to the pathogenesis of cancers, neurodegenerative disorders and ischemic tissue injury. Emerging findings suggest that major functions of telomerase include protecting nuclear

DNA from damage and suppressing apoptotic biochemical cascades. By adding repeats of a DNA sequence (TTAGGG) to the ends (telomeres) of chromosomes, telomerase reverse transcriptase (TERT) protects these sensitive regions of chromosomes and thereby prevents chromosome shortening and stabilizes the genome. In this way TERT can prevent cell cycle arrest and apoptosis and, indeed, telomerase activity is high in somatic cells during early development and in many cancers, settings in which cells often exhibit increased resistance to apoptosis. A role for telomerase in regulation of natural cell death is suggested by data showing that telomerase promotes survival of developing brain neurons. Suppression of telomerase activity and TERT expression promotes apoptosis, whereas overexpression of TERT prevents apoptosis by suppressing cell death triggered by DNA damage, oxidative stress and other stimuli. Therapeutic approaches to disorders ranging from cancer to Alzheimer's disease are being developed that target the anti-apoptotic action of telomerase.

11. References

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CHAPTER 8

THE TELOMERASE KNOCKOUT MOUSE

MARÍA A. BLASCO

Table of contents

Telomerase, Aging and Disease. Ed. by M. P. Mattson and T. K. Pandita. 151 — 165

1. Introduction

1.1. Telomeres and telomerase

Telomeres are the ends of eukaryotic chromosomes and consist of tandem repeats of a G-rich DNA sequence, which in all vertebrates is TTAGGG [1, 2]. There are proteins specifically bound to mammalian TTAGGG repeats; some of these telomeric proteins are found preferentially at the telomeres, such as TRF1, TRF2 or tankyrase [3-5], whereas others also have major roles in essential cellular processes, such as DNA repair (i.e., Ku, Mre11/Rad50/Nsb1) [6-9]. Telomeres protect chromosome ends from degradation, recombination and DNA repair activities. Loss of telomere function, due either to loss of telomeric sequences, or to mutation of telomeric proteins, results in end-to-end fusions and loss of cell viability [8, 10-12]. The current model is that telomeres can adopt two different conformations, (i) an "open" telomere that is accesible to telomerase and other celullar activities, and (ii) a "protected" telomere in which the G-strand overhang invades the double-stranded DNA, forming a loop structure denominated T-loop [13] (see Section 2.4 of this review).

What regulates the length of TTAGGG repeats in mammalian cells is not fully understood; however, the activity of telomerase, a cellular reverse transcriptase that synthesizes telomeric repeats *de novo*, is crucial in this process. Telomerase is composed of a catalytic subunit known as Tert (Telomerase Reverse Transcriptase) [14-20], an RNA molecule (Terc, Telomerase RNA Component) that is used as a template for addition of new telomeric repeats [21-24], and associated proteins [25-28].

1.2. Telomeres, telomerase and cancer

Telomerase activity is upregulated in the vast majority of human tumors as compared to normal somatic tissues, where it is undetectable or present at very low levels [29]. It has been shown that expression of the TERT catalytic subunit of telomerase in cultured human primary cells reconstitutes telomerase activity and allows immortal growth [30-33]. In addition, Tert-mediated telomerase reconstitution is able to cooperate with oncogenes in transforming cultured primary human cells into neoplastic cells [34]. These findings have opened up the possibility that telomerase upregulation, which occurs in more than 90% of all human tumors [29], may contribute actively to tumor growth [35]. Hence, telomerase inhibition may be an effective way to interrupt tumor growth.

1.3. Telomeres, telomerase and aging

In contrast to what is thought to occur in tumors, telomeric sequences are lost with each cell division in mortal cells and in most somatic tissues. This telomere shortening with increasing age has been proposed to limit the proliferative capacity of cells and contribute to the aging process. Telomerase activity reconstitution in adult somatic cells or tissues is thus envisioned as a potential approach for gene therapy in age-related diseases.

2. Construction and characterization of mice without telomerase

To address directly the potential role of telomerase in cancer and aging, three different telomerase-deficient mouse models have been generated to date. In two of the models, the gene encoding the telomerase RNA component Terc was eliminated from the mouse germ line [11, 36]; these mice lack telomerase activity although they still express the Tert subunit [20]. In a different knockout model, the gene coding for Tert was eliminated [37]. The phenotypes of the three telomerase knockout mice appear to be identical, suggesting that both the catalytic subunit and the telomerase RNA are essential for telomerase activity, and that they do not seem to have diferential roles in the cell. This review will focus on the most extensively studied mouse telomerase knockout model, the $Terc^{-/-}$ mice originally described by Blasco et al. [11].

2.1. Telomere shortening with increasing generations in the telomerase-deficient mouse

Since mouse telomeres are very long (40 Kb), it was possible to derive several $Terc^{-/-}$ mouse generations before loss of viability was observed in the colony. As expected from the absence of telomerase activity, $Terc^{-/-}$ mice show progressive telomere shortening with increasing mouse generations. Figure 1 shows telomeric quantitative FISH (fluorescence *in situ* hybridization) on metaphases from early and late generation $Terc^{-/-}$ primary mouse embryonic fibroblasts (MEF); the white arrows indicate critically short telomeres in late generation $Terc^{-/-}$ cells (Figure 1). Telomere shortening in $Terc^{-/-}$ mice is 3-5 Kb per mouse generation, which corresponds to 100-150 bp of telomeric loss per cell division, similar to that previously described for human cells. The number of mouse generations that can be generated in the absence of telomerase is directly proportional to the initial telomere length of the strain [38]. Whereas six generations were derived on the original mouse background, which showed an average telomere length of 40 Kb, only 3-4 generations were derived on the pure C57BL/6 background, with an average telomere length of 25 Kb [38]. These results indicate that telomerase activity is necessary and sufficient for long term maintenance of the species, and furthermore shows that telomerase activity is the only efficient pathway for maintaining telomeres in the organism (see below).

2.2. Chromosomal instability with increasing generations in the telomerase-deficient mouse

Study of the telomerase knockout mouse model has established that loss of telomeric TTAGGG repeats below a threshold length results in increased end-to-end chromosomal fusions and loss of viability (see Figure 1 for end-to-end fusions, indicated by a red arrow) [11, 39, 40]. Hence, a minimal length of TTAGGG repeats at the telomeres is required for proper telomere function. This length has been estimated to be around 1.5-3 kb in the case of mouse telomeres [40], similar to that previously described for human telomeres [29]. Telomere shortening is not the only way to lose telomere function, however; as mentioned above (Section 1.1), loss of telomere binding proteins such as TRF2 and Ku could render a similar end-to-end fusion phenotype in the absence of telomere shortening [8, 12]. In the case of TRF2 loss of function, the G-strand overhang at the telomere is proposed to be disrupted, hence afecting the formation of T-loops [12,13].

2.3. Cell growth and neoplastic transformation of cells derived from the telomerase knockout mouse: fibroblasts *versus* ES cells

As described above, late generation telomerase deficient mice show telomere shortening and increased numbers of end-to-end fusions. Several researchers have addressed the effects of these alterations in viability, immortalization, and neoplastic transformation ability of cells derived from these mice. In particular, mouse embryonic fibroblasts (MEF) and embryonic stem cells (ES) have been studied [11, 36, 40].

MEF

Telomerase-deficient MEF never showed a decreased growth rate with increasing population doublings (PD) in culture; even when reaching more than 600 PD in the case of first generation MEF and up to 300 PD in late generation $Terc^{-/-}$ MEF [40]. Furthermore, MEF derived from different-generation telomerase knockout mice immortalize spontaneously in culture at the same frequency as wild-type MEF. The MEF lacking telomerase can also be neoplastically transformed by oncogenes and are able to form tumors in nude mice [11]. These results suggest that, at least in cultured MEF, telomerase deficiency may be compensated by activation of alternative mechanisms to lengthen telomeres (ALT, alternative lengthening of telomeres) [41]. Indeed, a study using different passage number MEF from all telomerase knockout generations showed that once telomeres shorten to a critical length (which differed for the distinct chromosomes studied), they can be maintained at this length in the absence of telomerase activity [40]. These ALT cells generally show high chromosomal instability, which results from cell growth in the absence of telomerase activity. It is estimated that there is a 40-fold increase in chromosomal aberrations in cells growing without telomerase compared to wild-type cells [40]. Figure 2 shows chromosomal aberrations detected in wild-type and $Terc^{-/-}$ cells that have been passaged in culture for increasing population doublings. All together, these results illustrate the critical role of telomeres and telomerase in maintaining chromosomal stability.

ES cells

In contrast to cultured Terc$^{-/-}$ MEF, Terc$^{-/-}$ ES cells showed a decrease in viability after 450 PD in culture, suggesting that ES cells are more susceptible to telomere shortening and chromosomal instability than MEF [36]. ES cell clones were selected that were able to survive beyond 450 PD at a high growth rate. These Terc$^{-/-}$ ES cell survivors showed high chromosomal instability as well as telomere maintenance without telomerase, as described for late passage number MEF [42].

These results suggest that, at least in cultured cells, telomerase deficiency and telomere shortening to a critical length can be rescued by the activation of telomerase-independent telomere lengthening mechanisms which are thought to involve homologous recombination.

2.4. G-strand overhangs in the telomerase-deficient mouse

Telomeres are characterized by the ability to form a special structure termed T-loop, which is proposed to protect the telomere from DNA repair and recombination activities. Telomeric loops are maintained by the existence of a G-strand overhang at the end of the telomere and by telomeric proteins TRF1 and TRF2 [13]. Loss of TRF2 function due to a dominant negative mutation results in telomeric fusions which could be caused by loss of the G-strand overhang and consequent loss of the ability to form T-loops. It was postulated that the G-strand overhang may be originated by telomerase activity, since telomerase elongates the G-strand at the telomeres. The telomerase knockout models ruled out this possibility, however, since both Terc$^{-/-}$ and Tert$^{-/-}$ mice have normal G-strands at the telomeres, independent of generation number [37, 43].

3. Life without telomerase

3.1. Embryonic development

In both humans and mice, telomerase activity is abundantly expressed during embryonic development and is rapidly downregulated after birth, concurring with the fact that it is absent in most normal adult somatic tissues [24, 44]. High telomerase levels are postulated to be required during embryonic development to sustain the elevated proliferation that occurs in this process. In agreement with this, Terc$^{-/-}$ mice show increased embryonic mortality with increasing Terc$^{-/-}$ mouse generations [45]. In particular, Terc$^{-/-}$ mice of later generations show a larger percentage of neural tube defects (NTD), consisting of an open neural tube, indicating that the process most sensitive to telomere loss during embryonic development is neural tube formation and closure. Figure 3 shows images of late generation Terc$^{-/-}$ embryos, demonstrating the open neural tube at day 10.5 compared to an age-matched wild-type embryo. This phenotype concurs with the fact that Terc is most abundantly expressed in the central nervous system during the human embryonic development [46]. Neural tube defects, including spina bifida, anencephaly and congenital hydrocephalus are among

the major causes of infant mortality. It is possible that telomere shortening could be one of the multiple factors determining the occurrence of NTD in humans.

3.2. Fertility

The genetic baggage of a species must be transmitted intact from generation to generation; in humans and mice, telomerase activity is abundantly expressed in the germ line, in contrast to its absence from most adult somatic tissues [47]. Terc$^{-/-}$ mice from increasing generations show a progressive decrease in litter size, until no progreny are produced in late generation Terc$^{-/-}$ mouse intercrosses. This decrease in litter size is due to embryonic death as a consequence of a neural tube defect in these mice (see above), as well as to infertility. Fertility decreases with increasing generations in Terc$^{-/-}$ mice, eventually resulting in completely sterile Terc$^{-/-}$ males and females. The Terc$^{-/-}$ male infertility phenotype is well characterized in two different genetic backgrounds [38, 39]. The Terc$^{-/-}$ female infertility phenotype has been only partially studied [39].

Late generation Terc$^{-/-}$ males show testicular atrophy (a reduction of 60% in normal testicular weight after correction by total body weight), as well as a depletion of male germ cells. Histological analysis of late generation Terc$^{-/-}$ testes showed various abnormalities, including (i) depletion of germ cells in the epithelium, which shows only vacuolated Sertoli cells, and (ii) marked hyperplasia of Leydig cells. These lesions are similar to those found in patients affected by the "Sertoli Cell Only Syndrome" [48], and are also found in 40% of wild-type mice of 15-17 months of age [49], suggesting premature aging in late generation Terc$^{-/-}$ males. These lesions are the result of increased apoptosis and a decreased mitogenic index in the germ cells with increasing generation of Terc$^{-/-}$ males [39].

3.3. Decreased viability with increasing generations of mice deficient for telomerase activity

Late generation Terc$^{-/-}$ mice show increased mortality with age compared to wild-type littermates. Age at death depends on the genetic background (see Figure 4 for survival curves of late generation Terc$^{-/-}$ mice on two different genetic backgrounds). On the original mixed genetic background (C57Bl6/129Sv 50%/50%), which shows long, heterogeneous telomeres, there is decreased viability in Terc$^{-/-}$ mice older than 18 months compared to wild-type mice [50]. The fact that aging-associated phenotypes on this genetic background appeared only at very advanced ages suggested that telomere shortening does not trigger aging in mice [50]. Nonetheless, on a pure C57BL/6 background, which shows shorter, more homogeneous telomeres, 50% of late generation Terc$^{-/-}$ mice died at 5 months of age [38]. Death of these mice occurred within 48 hours of the detection of symptoms of poor health, such as reduced activity, lowered responsiveness to stimuli, bristly hair, dehydrated skin, a hunched position and small size [38]. Affected mice showed severe defects in some highly proliferative organs (see below). It is important to note, however, that 40% of the Terc$^{-/-}$ mice died suddenly, with no apparent signs of disease. These sudden death cases are currently under investigation and may be related to cardiovascular failure in these mice.

3.4. The digestive system

Late generation Terc$^{-/-}$ mice are significantly smaller than age-matched wild-type controls. This was particularly evident in late generation Terc$^{-/-}$ mice on a pure C57BL/6 background (Figure 5). A 20% reduction in body weight was observed in both C57BL/6 Terc$^{-/-}$ males and females older than 4 months [38]; this loss in body weight was not apparent on the mixed background until Terc$^{-/-}$ mice were 15-18 months old [50]. Reduced body size was coincidental with an increased number of deaths in the colony. The fact that this weight reduction is more apparent in adult mice suggests a relationship to a digestive system malfunction. Indeed, in all C57BL/6 Terc$^{-/-}$ mice showing signs of poor health, but not in age matched wild-type mice, histological analysis of the small intestine revealed various abnormalities, such as (i) atrophy of the villi and mucosal flattening in some areas, and (ii) epithelial and glandular hyperplasia in other areas. These histological alterations have been described as typical age-related lesions in C57BL/6/C3H hybrids over 2 years old [51]. The abnormal small intestine mucosa may result in reduced nutrient absorption, which could account for decreased body weight, dehydrated skin and hunched position. In accordance with this, lymphangiectasia and increased lymphocyte numbers, which are typical signs of the malabsorption syndrome, are also observed in the affected intestine. Ulcerations and initial stages of peritonitis are also detected in some areas of the intestine, which may result in rapid death of affected mice.

The small instestine epithelium is completely regenerated every 4-5 days [52]; hence, telomere shortening associated with cell division and aging may result in growth impairment of epithelial cells, triggering the alterations described [38].

3.5. Skin and hair

An increased incidence of hair graying and alopecia was detected in late generation Terc$^{-/-}$ mice as compared to wild-type littermates [50]; this phenotype was particularly evident on the C57BL/6 background, which has black hair (Figure 5 shows representative images of a wild-type and a late generation Terc$^{-/-}$ mouse on the C57BL/6 background; notice the hair graying, allopecia and smaller size of the Terc$^{-/-}$ mouse). Skin ulcerations were also detected in late generation Terc$^{-/-}$ mice at a higher frequency than in wild-type mice. These lesions were located predominantly at anatomical sites exposed to chronic mechanical stress, such as distal limbs, perineum, snout and throat area. Histologically, the lesions appeared as ulcerations with epidermal hyperplasia, hyperkeratosis and underlying dermal fibrosis [50], and are similar to those seen after chronic superficial trauma, particularly in debilitated elderly humans. These skin defects suggest decreased proliferative capacity of the skin in late generation mice. In this regard, old (>18 months) sixth generation mice are reported to show delayed wound healing compared to wild-type animals [50].

3.6. The liver

Histological analysis of the liver in Terc$^{-/-}$ mice on two different genetic backgrounds revealed no pathologies compared to wild-type mice [38, 50]. When late generation

Terc$^{-/-}$ mouse livers were challenged either by repetitive exposure to hepatotoxic substances (CCl_4) or by partial hepatectomy (surgical removal of two-thirds of the liver) [53], a marked difference was apparent between wild-type and Terc$^{-/-}$ mice. In wild-type mice, normal liver histology was restablished within one week of surgery, whereas late generation Terc$^{-/-}$ mice showed decreased organ weight compared to wild-types, and some of these mice died of postoperative complications [53]. The late generation Terc$^{-/-}$ mice that died were those with shorter telomeres, suggesting that the shorter telomeres of late generation Terc$^{-/-}$ mice impair the regenerative capacity of the liver [53]. From a clinical point of view, these results suggest that end-stage liver cirrhosis patients might benefit from telomerase-based gene therapy.

3.7. The immune system

A reduction in immune system reactivity with age is commonly known as immunosenescence, and is characterized by impairment of B and T lymphocyte function (diminished response to mitogens) coincident with decreased germinal center (GC) reaction [54]. Germinal centers are characterized by extensive clonal expansion and selection of B lymphocytes to generate the B memory cell pool. The decline in immune system reactivity in humans may be due to exhaustion of the proliferative potential of naive B cells as a consequence of telomere shortening with increasing age [55, 56]. Telomerase is activated when B cells enter the GC, and is subsequently downregulated when B cells differentiate to memory B cells [57-61]. Both diminished lymphocyte reactivity and impaired GC reaction have been described in late generation Terc$^{-/-}$ mice [38, 39, 62]. In particular, late generation Terc$^{-/-}$ mice, which are telomerase-deficient and have short telomeres, show a dramatic reduction in the number of GC formed following *in vivo* antigen immunization [62]. Furthermore, telomere length analysis of wild-type and Terc$^{-/-}$ mice showed that B cell telomeres were 5 Kb shorter in immunized first generation Terc$^{-/-}$ mice than in unimmunized controls, suggesting telomere shortening in the absence of telomerase upon immunization. In contrast, B cell telomeres were 5 Kb longer in immunized wild-type than in unimmunized control mice. These results indicate that the telomerase elongation detected in wild-type spleens following *in vivo* immunization is mediated by telomerase activity. Telomeres in B cells of immunized late generation Terc$^{-/-}$ mice were similar in length to wild-type telomeres. These late generation Terc$^{-/-}$ cells with long telomeres may derive from (i) a surviving subpopulation of late generation Terc$^{-/-}$ splenocytes that preserved long telomeres or (ii) activation of telomerase-independent telomere rescue mechanisms in late generation Terc$^{-/-}$ splenocytes. Either way, telomeres are crucial in sustaining cell proliferation during the GC reaction.

In these mice, the diminished proliferative capacity of late generation Terc$^{-/-}$ B and T lymphocytes to mitogen treatment is also a landmark of immunosenescence [38, 39, 62]. Late generation Terc$^{-/-}$ mice on the C57BL/6 background showed severe atrophy of the spleen, with reduced follicle numbers [38, 62]; this severe spleen phenotype was never observed in Terc$^{-/-}$ mice on the original mixed genetic background, in accordance to the milder phenotype of these mice [39].

3.8. The hematopoietic system

Initial peripheral blood counts in different generation Terc$^{-/-}$ mice on the mixed background revealed no significant differences compared to wild-type mice [39]. Nevertheless, study of late generation Terc$^{-/-}$ mice on a C57BL6 background revealed significant differences in lymphocyte and neotrophil numbers; lymphocyte numbers decreased significantly in late generation Terc$^{-/-}$ mice, whereas neutrophils were significantly increased [38]. The greater neutrophil numbers may be a compensatory mechanism of the immune system triggered by the decreased lymphocyte numbers. Similarly increased neutrophil numbers have been described in 27-month old wild-type animals [63]. No large differences were detected in total leukocyte numbers or in hematocrit between late generation Terc$^{-/-}$ mice and the wild-type controls.

Colony-forming unit (CFU) assays were performed in Terc$^{-/-}$ mice to determine whether the early progenitor cell compartment was compromised. These studies showed statistically significant decreases in the number of CFU-granulocyte, monocyte (CFU-GM), CFU-granulocyte, erythrocyte, monocyte, megakaryocyte (CFU-GEMM) and highly proliferative potential colony-forming cell (HPP-CFC) colonies [39]. These results indicate that the long term renewal of hematopoietic stem cells is decreased upon telomere loss.

3.9. Other organs

Initial histopathological studies of lung, brain, kidney, heart and blood vessels revealed no major differences between late generation Terc$^{-/-}$ mice and wild-type controls. Nonetheless, more detailed studies are currently underway which may reveal defects in these orgarns.

4. Cancer and telomerase

4.1. Spontaneous tumors in the telomerase-deficient mouse

Late generation Terc$^{-/-}$ mice show telomere shortening, increased chromosomal instability and severe proliferative defects in highly proliferative organs. One might thus predict decreased tumor incidence in these mice. In contrast, some authors have reported a moderately increased (5-fold) incidence of spontaneous tumors in late generation Terc$^{-/-}$ mice compared to age-matched wild-type controls [50]. Old late generation Terc$^{-/-}$ mice on the original mixed background developed lymphomas, with a lower incidence of teratocarcinomas, hepatomas, squamous cell carcinomas, and sarcomas. This increased tumor incidence was not detected in another colony of Terc$^{-/-}$ mice on the same genetic background, however, nor in late generation Terc$^{-/-}$ mice on a pure C57BL/6 background [38].

4.2. Cancer in mice deficient for telomerase and the INK4a/ARF tumor suppressor locus

To analyze the impact of short telomeres on tumorigenesis in these mice, mTerc$^{-/-}$ mice were crossed with mouse models that develop spontaneous tumors at a high incidence due to the fact that they carry mutations in tumor suppressor genes, such as mice deficient for the INK4a/ARF locus or for p53 (see below).

Telomere shortening in double (INK4a/ARF)$^{-/-}$/mTerc$^{-/-}$ mice decreased tumor incidence by 50% and increased survival of these animals. Cells derived from these mice also showed impaired colony formation, transformation and plating efficiencies. Reintroduction of the Terc gene in these cells restored their normal transformation potential [64]. These results are in contrast with the increased incidence of spontaneous tumors described in late generation mTerc$^{-/-}$ mice [50], and suggest that short telomeres result in decreased tumor cell growth. Other researchers have shown that telomere dysfunction, due to loss of a telomere binding protein, TRF2, results in increased apoptosis mediated by p53 and ATM proteins [65].

4.3. Cancer in mice deficient for telomerase and the p53 tumor suppressor

To examine the impact of short telomeres in tumorigenesis in the absence of the p53 tumor suppressor protein, Terc$^{-/-}$/p53$^{-/-}$ double knockout mice were generated and characterized [66]. Deletion of p53 significantly attenuated the adverse cellular and organismal effects of telomere dysfunction in the mTerc$^{-/-}$ mice, and these mice could be maintained for more generations than in the case of the single Terc$^{-/-}$ mutation [66]. A corresponding increase in tumor incidence and tolerance to chromosomal instability was observed in these mice, however, suggesting that p53 is one of the key regulators in the cellular response to short telomeres. These results indicate that p53 loss of function combined with telomere shortening may contribute to tumor progression.

Concurring with this, telomere attrition in ageing telomerase-deficient p53 mutant mice promotes development of epithelial cancers in the digestive tract [67]. It has been established that the ocurrence of these epithelial cancers is mediated by a process of fusion-bridge breakage, leading to the formation of non-reciprocal translocations [67], a classical cytogenetic feature of human carcinomas.

4.4. Carcinogen-induced tumors in the telomerase-deficient mouse

The increased tumor incidence associated to short telomeres has been observed in mice that lack the tumor suppressor protein p53. But what happens if p53 protein is wild-type? A recent study has addressed this question directly [68]. The authors used chemical carcinogenesis to induce skin papillomas in wild-type and different generation Terc$^{-/-}$ mice. The results indicate that late generation Terc$^{-/-}$ mice were resistant to carcinogens, in particular, the number of skin papilloma lesions in mTerc$^{-/-}$ mice was dramatically reduced compared to wild-type mice and all late generation mTerc$^{-/-}$ mouse papillomas disappeared after termination of the treatment.

These results suggest that short telomeres act as a tumor suppressor mechanism, preventing the appearance of tumors in the skin. This study also demonstrated that the absence of telomerase has a negative effect on papilloma formation, even in the presence of long telomeres, as shown by the decreased tumor incidence in early generation Terc$^{-/-}$ mice.

5. DNA repair and telomerase

5.1. Telomerase-deficient mice with short telomeres are hypersensitive to ionizing radiation

Double strand breaks (DSB) are generated by reactive byproducts of oxygen metabolism, exposure to ionizing radiation, and in V(D)J. recombination in lymphocytes. Efficient DSB DNA repair machinery eliminates these breaks, which might otherwise cause increased death or tumorigenesis. There is increasing evidence that short telomeres result in increased organismal sensitivity to ionizing radiation. Short telomeres render yeast and C. elegans more radiosensitive. Based on work in yeast, it has been proposed that short telomeres may directly impair the efficiency of DSB generated by ionizing radiation, since they may be the storage site for DNA repair proteins [69, 70], although this has not been formally addressed to date. Terc$^{-/-}$ mice with progressively shorter telomeres provide a unique system with which address (i) whether there is a causal relationship between short telomeres and radiation sensitivity in mammals, (ii) whether telomerase per se influences radiation sensitivity, even in the presence of long telomeres, and (iii) whether short telomeres result in defective DSB DNA repair in mammals.

Two independent studies using Terc$^{-/-}$ mice have indeed revealed that telomeres affect organismal sensitivity to ionizing radiation [71, 72]. Wong et al. [72] showed that late generation Terc$^{-/-}$ mice are less resistant to single radiation doses of gamma rays (7 Gy), and Goytisolo et al. [71] demonstrated that late generation mice are similarly radiosensitive using fractionated gamma ray doses (1.75 Gy per dose). This increased radiation sensitivity of Terc$^{-/-}$ mice with short telomeres has been associated to increased apoptosis as well as to increased chromosomal instability as a consequence of irradiation [71]. Intriguingly, no defect in the non-homologous end-joining DNA repair machinery was found in either study, suggesting that the major DNA repair pathaway in mammals is not affected in late generation Terc$^{-/-}$ mice. Similarly, the frequency of homologous recombination, measured as sister chromatid exchange frequency, was normal or slightly elevated in late generation Terc$^{-/-}$ mice [71]. All together, these results indicate that short telomeres are one of the biological determinants of radiation sensitivity, and that telomere dysfunction nonehteless does not affect the efficiency of the major NHEJ and HR recombination DNA repair pathways, invoking the existence of a direct effect of short telomeres on other repair pathways still to be determined.

5.2. The essential role of DNA repair proteins at the telomere

Work in yeast has shown that major DNA repair proteins such as Ku proteins, Mre11, and Sir proteins are essential components of the telomeres and that they move from the telomeres to the DSB upon induction of damage. The role of these DNA repair proteins at the telomere is unclear. Recent studies in mammals show that Ku proteins protect telomeres from end-to-end fusions independently of the length of TTAGGG repeats and of the integrity of the G-strand overhang [8, 73]. Furthermore, there is evidence that other members of DNA repair pathways, such as DNA-PK catalytic subunit, have a protective role at the telomere [73, 74].

6. Future studies

Knocking out telomerase activity from mice has provided a wealth of information on the role of telomerase and telomeres in such important aspects of human health as aging and cancer. There are a number of aspects of human aging, such as neurodegenative disorders and cardiovascular disease, that have not been addressed in telomerase knockout mice as yet. Future, more detailed studies of mice with short telomeres should try to address these issues carefully.

New mouse models overexpressing telomerase activity in the whole organism as well as in specific tissues should also be developed to address the role of telomerase activity in tumorigenesis. Only after a clear understanding of the role of telomerase in aging and cancer it will be possible to design telomerase-based therapies for these processes.

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8. References

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CHAPTER 9

TELOMERASE IN BRAIN DEVELOPMENT AND NEURODEGENERATIVE DISORDERS

MARK P. MATTSON, MAHENDRA RAO, WEIMING FU and WOLFRAM KLAPPER

Table of contents

1. Introduction

The human nervous system is the most highly evolved cellular signaling network, being responsible for the remarkably high level of intellectual function that engage most of our lives. Neurons are the fundamental cellular component of the nervous system, and form communication networks in which information is transferred between neurons at highly specialized structures called synapses. In addition, the nervous system contains glial cells; astrocytes provide trophic support to neurons, oligodendrocytes form myelin sheaths that surround axons of neurons, and microglia are phagocytic macrophage-like cells. The field of neuroscience research is largely based on understanding the functional complexity of the nervous system by identifying the molecular and cellular signaling mechanisms that control the development and integrated activities of nerve cell circuits. Biomedical neuroscience research aims to understand the biochemical cascades that result in neurological dysfunction and degeneration of nerve cells in the myriad of disorders that affect the nervous system. The most prominent of such disorders include Alzheimer's disease, Parkinson's disease, stroke, amyotrophic lateral sclerosis, and

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traumatic spinal cord and brain injuries. All cells in the nervous system have telomeres, and all cells in the nervous system appear capable of expressing telomerase, at least during embryonic and early postnatal development. The purpose of the present chapter is twofold. First, we will provide the reader with fundamental background information on mechanisms of development of the nervous system, and mechanisms of neurodegenerative disorders. Second, we will describe very recent findings suggesting important roles for telomerase in brain development, and the implications of telomerase actions in neural cells for the development of novel preventative and therapeutic approaches for neurodegenerative disorders.

2. Cellular signaling mechanisms that regulate development of the nervous system

As in other organ systems, the formation of the nervous system begins with proliferation of precurosor cells, the so-called stem cells [1]. The number of stem cells and the process of stem cell differentiation is carefully regulated to meet the demands of the developing brain or spinal cord, and complex signaling mechanisms have therefore evolved to maintain appropriate pools of undifferentiated precursor cells and differentiated progeny. There are several possible fates of a neural precursor cell (NPC) (Figure 1). It may remain quiescent and not enter the cell cycle (this may be important in sequestering a reserve pool of cells for use in times of stress or at later stages of development). It may undergo apoptosis and not contribute to further development (this may be the most common fate of a NPC in the adult brain where turnover of neurons and glia is very low). A NPC may undergo a symmetric cell division to self renew or undergo terminal differentiation, or it may undergo an asymetric cell division to generate both a differentiated progeny and a NPC. A dynamic balance between proliferation, survival and differentiation signals ensures that an appropriate balance between stem cells, precursor cells, and differentiated cells is maintained throughout development and adult life.

The intercellular signals that control development NPC and early development of the nervous system include diffusable messengers such as growth factors and cytokines, and cell adhesion molecules. Many different growth factors and cytokines have been identified that can promote the proliferation, differentiation and/or survival of one or more populations of NPC. For example, epidermal growth factor and basic fibroblast growth factor can maintain NPC in a proliferative state [2], while brain-derived neurotrophic factor (BDNF) can facilitate the survival and differentiation of NPC [3]. Insulin-like growth factor-1 is a survival and differentiation factor for several types of progenitor cells including precursors of neurons. Cell. adhesion proteins play important roles in regulating stem cell behaviors, with integrin receptors being a prominent example which transduce cell-cell and cell-extracellular matrix interactions into various cellular responses including changes in cell proliferation, differentiation and survival. Integrins are activated by binding to extracellular matrix proteins such as laminin, or to integrins on the surface of other cells, resulting in an intracellular signaling pathway involving PI3 kinase and Akt kinase. Integrin signaling promotes survival of many different cell

types including developing neurons [4]. Neural progenitor cells express several different integrins which appear to be differentially involved in the regulation of proliferation and cell migration/differentiation. For example, proliferation requires activation of $\alpha5\beta1$ integrins whereas migration requires activation of $\alpha6\beta1$ integrins [5].

One intriguing signaling pathway, initially discovered in the fruit fly *Drosophila*, involves a cell surface receptor called Notch. In flys, Notch is essential for the proper specification of many different cell fates during the processes of oogenesis, myogenesis, neurogenesis and wing and eye development [6]. Mice lacking Notch1 die during early embryonic development with major abnormalities including defects in somite formation, neurogenesis and hematopoiesis [7]. Notch is an integral membrane protein with a single membrane-spanning domain and is located primarily in the plasma membrane. The extracellular (N-terminal) domain contains a series of tandem epidermal growth factor-like repeats and three Lin/Notch repeats (LNR) that function in ligand binding and Notch activation; the major ligand for Notch1 in mammals is called Delta. The intracellular (C-terminal) region of Notch contains three ankyrin repeats which mediate interactions with other (cytoplasmic) proteins, a "PEST" sequence that regulates protein turnover, and a nuclear localization sequence. Activation of Notch by binding of ligand results in proteolytic cleavage of Notch at a site located at the cytoplasmic face of the membrane, resulting in release of a Notch C-terminal fragment (CTF). The CTF has been shown to translocate to the nucleus where it is thought to modulate transcription. Among a group of equipotent cells, those with higher levels of Notch will continue to divide, whereas those with lower levels of Notch will differentiate.

Fundamental players in programmed cell death pathways also have prominent roles in development of the nervous system. These include members of the Bcl-2 family of pro- and anti-apoptotic proteins [8] and members of the caspase family of cysteine proteases [9]. The expression and activity of these proteins is modulated by the kinds of intercellular signals described above. The ways in which Bcl-2 proteins and caspases promote or suppress apoptosis are described in more detail below.

Once neurons differentiate, the outgrowth of their axons and dendrites, the formation of synapses between the neurons, and the long-term maintenance of neurons and their connections, are regulated by an array of anterograde and retrograde signaling pathways. Neurotrophic factors such as nerve growth factor (NGF), basic fibroblast growth factor (bFGF), brain-derived neurotrophic factor (BDNF) and a secreted form of amyloid precursor protein (sAPP) have been shown to affect the outgrowth of axons and dendrites, and synaptogenesis in embryonic rat brain neurons in dissociated cell cultures [10, 11]. These neurotrophic factors have also been shown to enhance the survival of developing neurons in culture and *in vivo*. Interestingly, although neurotransmitters are traditionally viewed as serving the function of transducing electrical impulses between neurons at synapses, it has recently become very clear that neurotransmitters also regulate neurite outgrowth, synaptogenesis and cell survival during brain development [10]. Glutamate, the major excitatory neurotransmitter in the mammalian brain, plays a particularly important role in regulating dendrite outgrowth and synapse formation in the hippocamus [12]. Glutamate activates receptors linked to calcium influx through voltage-dependent and ligand-gated ion channels. Calcium regulates neurite outgrowth and synaptogenesis via transcription-dependent and postranslational mechanisms

involving changes in the cytoskeleton and cell adhesion molecules. Overactivation of glutamate receptors can induce neuronal apoptosis [13], and may thereby contribute to both programmed cell death during development and the pathogenesis of neurodegenerative disorders [10]. Another example of an important signaling mechanism regulating neuronal plasticity and survival during development of the nervous system is nitric oxide, a diffusable messenger that also plays important roles in learning and memory in the adult [14].

Although the adult brain consists primarily of postmitotic neurons and associated glial cells it also contains small populations of NPC that are concentrated in the subventricular zone and in the dentate gyrus of the hippocampus. These adult NPC can give rise to neurons or astrocytes, and their proliferation, differentiation and survival appear to be regulated by signaling mechanisms very similar to those operative during brain development [15]. Interestingly, NPC in the adult brain are responsive to activity in neuronal circuits, changes in cellular energy metabolism and various types of pathological insults including severe epileptic seizures, ischemia and trauma [3, 16].

3. The pathogenesis of neurodegenerative disorders

The nervous system is vulnerable to the aging process and, indeed, neurodegenerative disorders are major cause of disability and death in persons over the age of 65. Thus, the costs required to treat and care for stroke, Alzheimer's disease and Parkinson's disease patients is greater than the cost for those suffering from cardiovascular disease and cancer. Neurodegenerative disorders result from excessive death of one or more populations of neurons as the result of genetic and environmental factors superimposed upon the aging process (Figure 2). For example, death of hippocampal and cortical neurons is responsible for the symptoms of Alzheimer's disease, death of midbrain neurons that use the neurotransmitter dopamine underlies Parkinson's disease, Huntington's disease involves death of neurons in the striatum that control body movements, and death of lower motor neurons is responsible for amyotrophic lateral sclerosis.

Although the genetic and environmental factors that trigger neuronal death may be different in physiological and pathological settings (Table 1), many of the subsequent biochemical events that execute the cell death process are essentially the same. For example, mitochondria in cells undergoing apoptosis typically exhibit increased oxyradical production, opening of pores in their membranes and release of cytochrome c [17]; these changes are central to the cell death process because agents such as manganese superoxide dismutase and cyclosporine A, that act directly on mitochondria to suppress oxidative stress and membrane pore formation also prevent neuronal death in experimental models [18, 19]. Changes that occur during the early stage of apoptosis may induce mitochondrial dysfunction either directly or indirectly. Proteins of the Bcl-2 family play important roles in either inducing or suppressing mitochondrial alterations. For example, Bax and Bad promote mitochondrial permeability transition and release of cytochrome c, whereas Bcl-2 and Bcl-XL can prevent the changes [20]. Overexpression of Bcl-2 in cell cultures and in transgenic mice increases resistance of neurons to death induced by excitotoxic, metabolic and oxidative insults relevant to AD, stroke and other

disorders. Conversely, neurons lacking Bax are protected against apoptosis. The mechanism by which Bcl-2 proteins control the cell death process is not clear, but may involve interactions among family members and association of the proteins with mitochondria resulting in alterations in ion movements across mitochondrial membranes.

Table 1. Genetic and environmental factors that increase risk for age-related neurodegenerative disorders.

Disorder	Genetic factors	Environmental factors
Alzheimer's disease	Mutations in APP, presenilins ApoE-4, α2-M	Head trauma, high calorie intake, education
Parkinson's disease	Mutations in α-synuclein, parkin	Toxins, head trauma, high calorie intake
Huntington's disease	Mutation of huntingtin	Toxins?
ALS	Mutation of Cu/Zn-SOD	Toxins?
Stroke	Mutations in Notch-3, ApoE-4 LDL receptor	Calorie intake, smoking, lack of exercise

α2-M, α2-macroglobulin; ALS, amyotrophic lateral sclerosis; ApoE, apolipoprotein E; Cu/Zn-SOD, Cu/Zn- superoxide dismutase.

Two additonal types of proteins that can regulate the early stages of apoptosis are prostate apoptosis response-4 (Par-4) and caspases. Par-4 was discovered because it is upregulated in prostate tumor cells undergoing apoptosis, and has since been shown to have an essential role in developmental and pathological death of neurons [21, 22]. Levels of Par-4 protein can be rapidly increased in response to various apoptotic stimuli through enhanced translation of Par-4 mRNA. The death-promoting action of Par-4 requires interaction of Par-4 with one or more other proteins via a leucine zipper domain in the C-terminus of Par-4. Caspases are evolutionarily conserved cysteine proteases central to apoptosis of many cell types [23]. Some caspases are activated during the early phase of apoptosis, whereas other caspases are activated in response to mitochondrial changes and cytochrome c release. The latter caspases, which include caspase-3, can then activate a DNAse which is responsible for cleavage of DNA into oligonucleosome-size fragments. Caspases also cleave a variety of substrate proteins that may coordinate the cell death process including enzymes such as poly-ADP-ribose polymerase and ATM kinase, ion channels such as glutamate receptor subunits, and cytoskeletal proteins such as actin and spectrin.

Because it is essential for the proper function of the nervous system that neurons do not die, multiple signaling mechanisms have evolved that guard against neuronal death. As will be clear from our consideration of the roles of telomerase in the nervous system (below), an understanding of these signaling mechanisms will likely facilitate discovery of the mechanisms that regulate telomerase and, conversely, the mechanisms whereby telomerase modulates neural development. The dramatic symptoms of neurodegenerative disorders emphasize the importance of mechanisms that promote neuron survival and plasticity. There are several prominent anti-apoptotic signaling pathways including

those activated by neurotrophic factors and cytokines. Several different neurotrophic factors and cytokines use a survival pathway involving the transcription factor NF-κB which can protect neurons against death induced by diverse stimuli including trophic factor withdrawal and exposure to excitotoxic, oxidative and metabolic insults [24]. In addition to extracellular signal-mediated neuroprotection pathways, several intracellular signaling pathways have been identified that can protect neurons against apoptosis. For example, stress can induce the expression of neurotrophic factors and heat-shock proteins. The neurotrophic factors, in turn, act in an autocrine or paracrine manner to activate cell surface receptor-mediated kinase signaling pathways that ultimately induce expression of genes encoding survival-promoting proteins such as antioxidant enzymes. Heat-shock proteins act as "chaperones" for many different proteins, thereby maintaining protein stability; they may also interact directly with caspases, inhibiting their activation [25].

In continuing our prelude to a discussion of roles for telomerase in the nervous system, and possible therapeutic applications of manipulations of telomerase to neurodegenerative disorders, we briefly describe several of the most prominent neurodegenerative disorders and the current understanding of their pathogenesis Alzheimer's disease involves progressive impairment of cognition and emotional disturbances that result from degeneration of synapses and death of neurons in limbic structures such as hippocampus and amygdala, and associated regions of cerebral cortex. A prominent abnormalilty in Alzheimer's disease is accumulation of amyloid plaques formed by aggregates of amyloid β-peptide (Aβ) a 40-42 amino acid fragment generated by proteolytic processing of the amyloid precursor protein (APP) [26]. DNA damage, caspase activation, and alterations in expression of apoptosis-related genes such as Bcl-2 family members, Par-4 and DNA damage response genes have been documented in neurons associated with amyloid deposits in the brains of Alzheimer's patients [27, 28]. Studies of cell culture and animal models of Alzheimer's disease have shown that amyloid β-peptide can induce apoptosis of neurons directly and can greatly increase their vulnerability to death induced by conditions such as oxidative stress and reduced energy availability, that occur during aging. Amyloid β-peptide may promote cell death by inducing oxidative stress, mitochondrial dysfunction and DNA damage [26].

Mutations in three different genes, can cause early-onset inherited forms of Alzheimer's disease; one gene encodes APP, a second encodes presenilin-1, and a third encodes presenilin-2 [29, 30]. Mutations in each gene cause an increase in amyloid β-peptide production and accumulation in the brain. When mutant presenilin-1 protein is expressed in cultured cells and in transgenic and knockin mice, neurons become susceptible to death induced by a variety of insults including trophic factor withdrawal and exposure to amyloid β-peptide, glutamate and energy deprivation [29, 31, 32]. Mutant presenilin-1 causes a disturbance in cellular calcium homeostasis which appears to be a pivotal event in promoting DNA damage and degeneration of neurons [33].

Motor system disorders include Parkinson's disease, Huntington's disease and amyotrophic lateral sclerosis. Parkinson's patients have profound motor dysfunction as the result of degeneration of dopaminergic neurons in brain region called the substantia nigra. There is evidence that the dopaminergic neurons undergo apoptosis and that DNA damage is involved in the cell death process [34]. A prominent alteration

in Parkinson's disease is a deficit in mitochondrial complex I. Environmental and genetic factors may sensitize dopaminergic neurons to age-related increases in oxidative stress and energy deficits Environmental toxins including pesticides and others are implicated [35]. Mutations in α-synuclein, a component of the PD brain lesions called Lewy bodies, are responsible for a small percentage of Parkinson's disease cases, and expression of mutant α-synuclein in cultured cells promotes apoptosis [36].

Huntington's disease is caused by expansions of a trinucleotide (CAG) sequence in the huntingtin gene producing a protein containing increased polyglutamine repeats [37]. The mutant huntingtin protein may promote selective degeneration of striatal neurons by facilitating apoptosis as suggested by alterations in caspase activities in patients and in animal models of Huntington's disease [38]. Moreover, inhibition of caspase-1 was reported to slow disease progression in one transgenic mouse model [39]. Amyotrophic lateral sclerosis (ALS) results in progressive paralysis due to degeneration of motor neurons in the spinal cord. The neurodegenerative process involves increased oxidative stress, overactivation of glutamate receptors, cellular calcium overload and DNA damage [40]. Most cases of ALS are sporadic, but some cases are caused by mutations in the antioxidant enzyme Cu/Zn-superoxide dismutase; the mutations do not decrease antioxidant activity of the enzyme, but instead result in gain of an adverse proapoptotic activity which may involve increased peroxidase activity. The DNA damage in ALS is associated with increased mitochondrial localization of Bax and decreased association of Bcl-2 [41]. The ability of overexpression of Bcl-2 and administration of caspase inhibitors to delay motor neuron degeneration and death in Cu/Zn-SOD mutant mice suggests a major role for apoptosis in this disease [42, 43].

Neuronal degeneration resulting from acute brain insults such as ischemic stroke and traumatic injury may also manifest as apoptosis. Neurons in the brain region surrounding the central core of an ischemic stroke exhibit DNA damage and activation of the DNA damage-responsive proteins PARP and Ku80 [44]. In rodent stroke models, neurons in the ischemic penumbra exhibit morphological and molecular changes consistent with apoptosis including caspase activation, expression of pro-apoptotic genes and release of cytochrome c. Stabilization of mitochondrial function [18] and inhibition or knockout of caspases [45] results in reduced brain damage after a stroke. Traumatic injuries to the brain and spinal cord account for most deaths and permanent disabilities in people under the age of 40 years. Analyses of brains and spinal cords of patients that died after traumatic injuries have documented apoptosis-related changes in neurons including the presence of DNA strand breaks, caspase activation and increased Bax and p53 expression [46, 47]. Intraventricular administration of a caspase-3 inhibitor prior to injury reduces cell death and improves symptoms, and mice expressing a dominant negative inhibitor of caspase-1 exhibit reduced brain damage and free radical production after traumatic injury [49], suggesting a central role for caspases in a brain injury model [48],

4. Telomerase and development of the nervous system

Telomeres, the ends of chromosomes, consist of repeats of a six-base DNA sequence (TTAGGG) that preserve chromosome integrity and prevent end-to-end fusions [50, 51].

A reverse transcriptase called telomerase is responsible for adding the TTAGGG sequence to the chromosome ends. Telomerase activity requires a catalytic subunit (TERT) and an RNA template (TR). Several additional telomere-associated proteins have been identified that may modify telomerase activity or otherwise affect telomere stability. These include: TRF1 (telomerase repeat-binding factor 1) which may inhibit telomerase activity and promote telomere shortening and TRF-2 which may stabilize telomeres [52, 53]. Proteins involved in DNA repair such as PARP and Ku80 might also play a role in telomere maintenance and function [54, 55], and may thereby modulate cell proliferation and survival

In somatic cells telomerase activity and TERT levels decrease during growth arrest and cellular senescence [56], and overexpression of TERT in such cells can extend their lifespan [57, 58]. That telomerase can promote cell immortality is further suggested by the fact that telomerase activity is very high in most tumor cells [56], and by studies showing that organisms that undergo continuous growth with negligible aging exhibit telomerase activity in all of their somatic cells throughout life [59, 60]. Cancers can result from uncontrolled cell proliferation and/or from suppression of apoptosis [61]. Pharmacological inhibition of telomerase or treatment with antisense oligonucleotides can inhibit growth and survival of cancer cells [62. 63], suggesting a central role for telomerase in the immortal phenotype.

4.1 Evidence supporting roles for telomerase in neuronal proliferation, differentiation and survival

Telomerase is expressed in highly proliferative cells throughout the developing embryo and is then dramatically down-regulated as cells differentiate, and is not detectable in most somatic cells in the adult [64]. However, stem cells may retain telomerase activity [65]. Studies of cancer cells and somatic cells overexpressing TERT have shown that telomerase can confer upon cells an immortal phenotype, and recent studies have provided evidence that telomerase can promote cell proliferation by protecting telomeres and thereby preventing cell cycle arrest [51]. The brief history of research on telomerase in mammals has focussed on tumor cells and proliferative somatic tissues. In contrast, comparatively few studies have examined the roles of telomerase in the nervous system.

Levels of TR, TERT and telomerase reverse transcriptase activity are very high during the early period of brain development in rats and mice [64, 66]. When taken together with the observation that telomerase activity decreases in pheochromocytoma cells when they are induced to differentiate into a neuron-like phenotype in response to exposure to NGF [63], a role for telomerase in brain development is suggested. TERT and telomerase activity are widely expressed in neuronal precursor cells during embryonic development in rats and mice [66, 67]. A detailed study of telomerase activity and the expression of TERT, TRF1 and TRF2 in three different brain regions and the eye of mice at developmental stages ranging from embryonic day 13 (E13) to the adult revealed striking changes in telomerase activity and TERT expression [66]. Telomerase activity was very high throughout the brain and in the eye at E13, then declined markedly between E13 and E18, remained at a moderate level until postnatal

day 3 (P3) and thereafter declined to undetectable levels by P10 (Figure 3). Surprisingly, the sharp decrease in telomerase activity in the brain during embryonic development was not paralleled by a decrease in levels of TERT mRNA (Figure 4). The changes in telomerase activity and TERT expression during brain development are intriguing in that they are correlated with major developmental events including cell proliferation, differentiation and cell death. Thus, telomerase activity decreases as proliferation of neuroblasts decreases, which is also a time window when death of many newly generated neural cells may occur [68]. On the other hand, TERT levels decrease at a later stage of development in association with neuronal differentiation and synaptogenesis, a time when considerable programmed death of neurons also occurs. The dissociation between TERT levels and telomerase activity suggests that the enzyme activity is important for telomere maintenance in proliferating cells, while TERT may promote cell survival by a mechanism other than telomere maintenance. Consistent with an anti-apoptotic function of TERT in differentiated neurons are data showing that suppression of TERT expression in postmitotic embryonic hippocampal neurons can induce them to undergo apoptosis (Figures. 5 and 6). Level of mRNAs encoding TRF1 and TRF2 were essentially unchanged from E13 through adulthood [66], suggesting that these two proteins may not play active roles in controlling brain development (although permissive roles cannot be ruled out).

Although differentiated neurons and glial cells in the adult brain may not possess telomerase activity and express little or no TERT, there exist small populations of NPC (which are concentrated in the subventricular zone and dentate gyrus of the hippocampus) that are capable of dividing and differentiating into neurons or glial cells [69]. Such NPC taken from the adult rodent brain possess telomerase activity that can be maintained with continued propogation in cell culture [70]. NPC in primary cultures from embryonic mice express high levels of telomerase, which rapidly decreases upon their differentiation into neurons or glia (W. Fu and M. P. Mattson, unpublished data). Growth factors and the growth substrate may provide signals that regulate telomerase activity and/or TERT expression. As evidence, basic fibroblast growth factor causes an increase in telomerase activity and proliferation of the NPC without affecting TERT levels [71]. Upregulation of telomerase by neurotrophic factors might mediate the neuron survival-promoting actions of such growth factors during brain development [11], although this remains to be established. The possibility that neurotrophic factors can induce expression of TERT and/or telmoerase activity in differentiated neurons and glia in the adult brain is currently being examined.

4.2 Mechnasims whereby telomerase may regulate neural cell proliferation, differentiation and survival

The current view of the mechanism by which temolerase promotes cell proliferation is based upon the concept of a cell cycle checkpoint, a point where the cell must "decide" whether to divide or exit the cell cycle [72]. Several different proteins have been identified that play pivotal roles in the decision made at the checkpoint. These include members of the cyclin family of mitotic regulators, the ATM kinase, and the tumor suppressor protein p53. It is quite clear that shortening of telomeres

beyond a critical threshold length can trigger cell cycle arrest and, accordingly, telomerase maintains cells in the proliferative state by preventing telomere shortening. Although the mechanisms that control proliferation and cell cycle exit in NPC have not been studied with the same rigor as other cell types, it is likely that many of the same molecular mechanisms apply [73].

Because telomerase activity decreases as cells differentiate, a role for this enzyme in the differentiation process seems likely. One possibility is that telomerase promotes cell proliferation and that when telomerase activity decreases the cell exits the cycle and activates a differentiation program. In this way a decrease in telomerase activity would play only a permissive role in the processes of cell differentiation. A decrease in telomerase activity during brain development may thereby signal progenitor cells to exit the cell cycle and differentiate into neurons or glial cells. Such a mechanism has been clearly established in nonneural cells, and the available data demonstrating correlations between decreasing telomerase activity and differentiation of neural cell lines [63] and primary neurons [66, 74] support such a mechanism. However, it is unlikey that the shortening of telomeres itself is a critical trigger of neuronal differentiation because exposure of undifferentiated PC12 cells to nerve growth factor, which does not promote telomere shortening, causes a relatively rapid downgregulation of telomerase activity and differentition of the cells into a neuron-like phenotype [63]. Downregulation of telomerase activity by NGF suggests that telomerase is subject to regulation by specific intracellular signal transduction cascades that involve protein kinases and transcription factors. Because signals that induce cell differentiation also suppress telomerase actiivty it is possible that the decrease in telomerase activity is simply a consequence of the cell differentiation process and has no particular role in controlling the differentiation process. However, treatment of cells with telomerase antisense can induce differentiation [75], indicating that telomerase activity may in fact exert control on the proliferation/differentiation decision.

The mechanisms whereby telomerase activity and TERT prevent cell death likely overlap with the mechanisms that control cell proliferation and differentiation. Signals involved in "sensing" chromosome instability and DNA damage might be involved in both actions of telomerase. As described above, apoptosis is characterized by distinctive morphological features and biochemical cascades. The strong correlation of telomerase activity with immortal phenotypes suggests an anti-apoptotic action of telomerase. Consistent with this possibility, overexpression of Bcl-2 results in increased telomerase activity in cancer cells [63, 76], whereas overexpresion of the tumor suppressor protein PTEN decreases telomerase activity and increases spontaneous apoptosis [77]. The emerging physical and functional interrelationships of TERT with p53, c-Myc and NF-κB [24, 78, 79] further strengthen a major role for TERT in promoting cell survival. P53 is a protein that responds to DNA damage by engaging the apoptotic machinery; it does so by inducing Bax production and mitochondrial changes. In contrast, c-Myc and NF-κB prevent apoptosis by inducing the expression of genes that encode cell survival proteins including Mn-SOD, Bcl-2 and inhibitor of apoptosis proteins [24, 79]. Activation of the ATM kinase, which acts upstream of p53, may be necessary for telomere dysfunction-induced apoptosis and may also play a central role in the apoptotic pathway activated by DNA damage [80]. Suppression of TRF2 function can induce

apoptosis in some cell types, but not in cells that are deficient in p53 function or lack an apoptotic response to DNA damage [81]. TERT expression is induced by the transcription factor c-Myc, which is of considerable interest in understanding the anti-apoptotic mechanism of telomerase, because c-Myc is associated with cell immortalization and cancer [82]. Induction of TERT expression by c-Myc may therefore provide an explanation for why cancer cells expressing very high levels of c-Myc do not die. The TERT gene can also be induced by NF-κB [83], which is of interest from the perspective of both development of the nervous system and neurodegenerative disorders, because NF-κB is known to be an important mediator of the neuron survival-promoting actions of various neurotrophic factors and cytokines, and alterations in NF-κB activity have been linked to several neurodegenerative disorders including Alzheimer's disease and stroke [24].

Suppresion of TERT expression in cultured embryonic brain neurons using antisense technology, causes the neurons to undergo apoptosis [67; Figure 6], demonstrating a requirement for TERT in survival of these embryonic neurons. One mechanism whereby TERT might promote cell survival is by suppressing DNA damage, or signals generated in response to DNA damage [85]. DNA damage is well-established as a trigger for apoptosis, and telomerase can protect cells against apoptosis induced by agents known to induce DNA damage including oxidative insults and trophic factor deprivation [67. 84]. The functional relationships of telomerase with DNA damage response pathways involving the ATM kinase, p53, Ku proteins and PARP are consistent with telomere shortening being "sensed" as a type of DNA damage. Such DNA damage can either provide a trigger for apoptosis or can be repaired (by telomerase-mediated capping of telomeres) thus preventing apoptosis. In this way telomerase may play a role in determining which cells undergo programmed cell death during development of the nervous system.

The possibility that TERT and/or telomerase activity mediate survival-promoting actions of well-established signals such as neurotrophic factors merits consideration in light of evidence showing that neurotrophic factors such as FGF can induce TERT expression [71]. Another example comes from recent studies of the Akt kinase, a well-established mediator of survival signals activated by neurotrophic factors and other stimuli in neurons. It was recently reported that Akt phosphorylates TERT and enhances its enzymatic activity [86], suggesting a role for telomerase in the cell survival-promoting actions of Akt.

Although suppression of a DNA damage response is an attractive hypothesis for the anti-apoptotic action of telomerase activity, accumulating data suggest that TERT may prevent apoptosis independently of its reverse transcriptase activity. Indeed, recent findings support a mechanism in which TERT interrupts the cell death process at a premitochondrial step [67, 74]. The increased vulnerability to apoptosis of embryonic neurons with low levels of TERT is associated with mitochondrial dysfunction and caspase activation. Treatment of TERT-deficient neurons with caspase inhibitors or cyclosporin A, an agent that blocks the mitochondrial permeability transition, prevents cell death [67, M. P. Mattson, unpublished data). Overexpression of TERT in pheochromocytoma cells prevents mitochondrial dysfunction and caspase activation after exposure of the cells to apoptotic insults [63]. Although TERT is typically localized mainly in the nucleus, in many cells including embryonic neurons it is also

present in the cytoplasm [67; Figure 5]. Recent evidence suggests that TERT acts at a premitochondrial step in the apoptotic cascade by modifying the function of cytoplasmic proteins such as Bcl-2 family members or Par-4 that are known to control apoptosis at that point in the cell death process (M. P. Mattson, unpublished data).

Analyses of mice lacking the RNA component of telomerase (TR) are consistent with a role for telomerase in preventing apoptosis [for review see 87]. TR-/- mice can be propogated for only a limited number of generations and then show decreased viability with age, which is associated with telomere shortening, reduced proliferation of B and T lymphocytes, atrophy of the spleen and intestines, and impaired ability to regenerate damaged liver [88, 89]. Notably, from the perspective of the present chapters focus on telomerase in the nervous system, TR-/- mice show marked neural tube defects [90]. The failure of neural tube closure in these telomerase deficient mice established a role for telomerase in neural development *in vivo*, and is consistent with a role for telomerase in promoting the survival of NPC. Data from TERT-deficient mice suggest that TERT-/- mice are similar to TR-/- mice with respect to telomere maintenance and abnormalities in various organ systems including the nervous system [91].

Emerging data concerning transcriptional regulation of TERT expression further support developmental roles for telomerase. The promoter of TERT has been cloned, putative transcription regulatory sites identified, and in several cases the regulation of TERT expression by specific transcription factors has been demonstrated. The emerging picture is consistent with roles for TERT in regulating cell proliferation and survival. For example, TERT expression is induced by transcription factors such as NF-κB [83] and estrogen receptors [92], which are known to promote survival of a variety of cells including neurons. Conversely, TERT expression is suppressed by pro-apoptotic transcription factors such as p53 [93] and Mad [94].

5. Telomerase and neurodegenerative disorders

At the present time there is very little data available concerning the possible involvement of telomerase in the pathogenesis of age-related neurodegenerative disorders. However, recent studies of experimental cell culture models of neurodegenerative disorders are consistent with the possibility that telomerase can protect neurons against the environmental and genetic factors that promote neuronal degeneration. Suppression of TERT levels and function in embryonic mouse hippocampal neurons in culture using antisense technology and the telomerase inhibitor 3'-azido-2',3'-dideoxythymidine significantly increases their vulnerability to cell death induced by amyloid β-peptide [84]. The increased vulnerability of the neurons in which TERT levels were reduced was associated with increased levels of oxidative stress and mitochondrial dysfunction following exposure to amyloid β-peptide. Overexpression of TERT in cultured neural cells results in decreased vulnerability of the cells to amyloid β-peptide-induced apoptosis [84]. These findings demonstrate a neuroprotective function of TERT in an experimental model relevant to Alzheimer's disease.

In additional studies it was shown that suppression of TERT expression in cultured neurons increases their vulnerability to glutamate-induced death [66]. Because over-

activation of glutamate receptors is implicated in stroke, as well as neurodegenerative disorders such as Parkinson's disease and amyotrophic lateral sclerosis, the latter findings suggest the possiblity that stimulation of expression of telomerase in neurons might increase their resistance to such disorders.

Although telomerase activity is usually undetectable in the adult brain and spinal cord, and TERT expression is very low, no studies have yet evaluated telomerase activity or TERT expression in postmortem brain or spinal cord tissues from patients who died with a neurodegenerative disorder, There is ample precendence for a recapitulation of developmental mechanisms during neural cell responses to brain injury and neurodegenerative disorders [11], and the strong expression of telomerase during brain development leaves open the possibility that it can be reexpressed in pathological settings in the adult brain. Our very recent findings in studies of adult mice support this possibility. We have found that telomerase activity can be induced in cells in the adult brain in response to tissue injury and epileptic seizures. Whereas telomerase activity was not detected in tissue samples from the hippocampus and cerebral cortex of rats under basal conditions, it was present in tissue samples from the same brain regions of rats that had been administered a seizure-inducing excitotoxin called kainic acid (Fu et al., *Soc. Neurosci. Abstr.* (2000) 26, 1881). The cell type(s) in which telomerase activity appears in response to brain injury is not yet known, and the molecular mechanism responsible remains to be determined. Nevertheless, these findings suggest a role for telomerase in cellular responses to brain injury. Because the brain contains populations of neural stem cells that may express telomerase, it will be of considerable interest to determine the factors that regulate telomerase in these cells and, conversely, the roles of telomerase in regulating the proliferation, differentiation and survival of the neural stem cells (Figure 7). In this regard is of interest to note that several different environmental factors have been shown to affect the proliferation of neural stem cells and/or the survival of newly generated neurons or glia including: environmental enrichment [95, 96], dietary restriction [3] and physical exercise [97].

Telomerase activity, as well as TERT and other telomere-associated proteins, are now recognized as targets for drug discovery with potential applications in the treatment of cancers and neurodegenerative disorders. The goal would be to inhibit telomerase in tumor cells and to increase expression of TERT and telomerase activity in neurons. Preclinical studies support such approaches. For example, compounds that inhibit telomerase can suppress tumor growth [63, 98], and a gene therapy approach involving overexpression of TERT was effective in preventing neuronal death in experimental cell culture models relevant to the pathogenesis of Alzheimer's disease and stroke [63, 84]. A second approach would be to identify compounds that induce expression of TERT and telomerase activity in neurons. Studies indicate that telomerase is indeed subject to regulation by environmental signals such as neurotrophic factors and estrogen, and such data provide a starting point for further work in this area. In any case, the revelation that telomerase can prevent cell death in various experimental paradigms places telomere-associated proteins firmly in the field of apoptosis research and opens the door for future studies of telomerase in the pathogenesis of the multitude of human diseases that involve aberrant regulation of cell death.

6. Summary and future directions

During development of the nervous system, telomerase activity levels are high in neural progenitor cells, but then then decrease as the progenitor cells differentiate or undero apoptosis. The catalytic subunit of telomerase (TERT) remains at relatively high levels during the process of neuronal differentiation and then decreases sharply during the period when synapses form and programmed cell death occurs. TERT promotes survival of developing brain neurons. Suppression of telomerase activity and TERT expression promotes apoptosis of embryonic neurons, whereas overexpression of TERT prevents apoptosis by suppressing cell death at a premitochondrial step in the death cascade TERT may suppress DNA damage and/or apoptotic signals activated by damaged DNA. Interestingly, telomerase levels remain high in neural stem cell populations in the adult nervous system, and may contribute to their capability for self-renewal. Moreover, telomerase can be induced by injury to the nervous system, suggesting roles in adaptive responses of brain cells to injury. Recent studies of the transcriptional regulation of the TERT gene suggest that this enzyme may mediate the cell survival-promoting actions of diverse signals including estrogen, cytokines and neurotrophic factors. The elucidation of the functions of telomerase activity and TERT in neuronal differentiation and survival may lead to novel approaches for preventing neuronal death and promoting recovery of function in various neurodegenerative conditions.

There are strong correlations between telomerase activity and TERT expression and the proliferation, differentiation and survival of many types of cells. The mechanisms whereby telomerase controls these processes are beginning to be understood and include actions on signaling that regulate cell cycle arrest and apoptosis. Pressing areas for further studies include: 1) identifying proteins that interact with telomeres and TERT and establishing their roles in regulating cell proliferation and survival. 2) determining the specific mechanism whereby TERT prevents apoptosis. 3) identifying signaling pathways that regulate expression of TERT, TR and telomere-associated proteins. 4) elucidating roles for telomerase in aging and age-related disease.

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CHAPTER 10

THE ROLE OF TELOMERES AND TELOMERASE IN AGING AND LONGEVITY DETERMINATION

LEONARD HAYFLICK

Table of contents

1. Introduction

Present biological dogma is rooted in the belief that the changes that occur from conception in a developing organism are governed by the genome to form an increasingly complex hierarchy of molecular orderliness that results in the adult organism.

As has been observed frequently, the maintenance of the molecular orderliness achieved by the mature organism would seem to be a trivial accomplishment in light of the spectacular developmental miracles that preceded it. Yet, the appearance of what would seem to be a simpler mechanism for maintaining the adult organism indefinitely in the full vigor of adulthood does not occur. This failure results in the process called aging.

The once common notion that there are as many theories of aging as there are biogerontologists is no longer true. All theories of aging are derivative of one fundamental concept. Aging is simply an increase in molecular disorder that changes adult homeostasis and leads to an increased vulnerability to pathology and the likelihood of death.

Telomerase. Aging and Disease. Ed. by M. P. Mattson and T. K. Pandita. 185 – 198

Despite the burgeoning literature in which genes allegedly are involved in the aging of lower organisms, there is no evidence that genetics plays a direct role in the aging process.

The failure to demonstrate a direct role for genes in aging processes is based on the crucial distinction that must be made between two concepts, — aging and longevity determination. The failure to distinguish between these two concepts has led, and is continuing to lead, to much confusion.

2. Aging and longevity determination

Aging is a stochastic process that occurs after reproductive maturity and results from the increasing molecular disorder that continues to exceed the capacity for repair. Molecular disorder has multiple etiologies including damage by oxygen free radicals, cross-linking, loss of energy states necessary to maintain molecular fidelity, and an enormous number of other chance events Finch and Kirkwood, [1]. Age changes are not directly programmed by the genome because, like everything else in the universe, no formal instructions on how to age are necessary.

As for longevity determination, it is only indirectly determined by the genome for the following reasons.

Species survival depends on a sufficient number of members living long enough to reproduce and, if necessary, to raise progeny to independence. The verity of this premise is obvious because, if animals are unable to reach sexual maturity they will not reproduce and the species will vanish.

To insure survival to reproductive success, natural selection favors animals that have greater survival skills including increased physiological reserve in vital organs to avoid predators, disease, accidents and environmental extremes.

Greater, or redundant, physiological capacity increases the chances for animals to survive long enough to achieve reproductive success just as redundant vital systems in complex machines, better insures that they will achieve their goals. Once animals achieve reproductive success, the excess physiological capacity, like that engineered into a space vehicle, allows each to continue beyond the vital goal. Further survival of the animal beyond sexual maturation and the space craft beyond its primary mission is determined by the level of excess capacity present at the time each goal was reached [2, 3].

Because survival long beyond reproductive success has diminishing value for the survival of a species, responses to the forces of natural selection weaken. Energy is spent better guarantying reproductive success than it is for increasing individual longevity. However, after reproductive success an animal has the potential to survive for a period of time determined by the level of excess physiological capacity reached at sexual maturation. The potential for living beyond sexual maturation that was achieved during development by the acquisition of excess physiological capacity (longevity determination) is eventually overcome by increasing molecular disorder that outpaces the capacity for repair (aging) [2, 3]. This accelerating molecular disorder, or aging, increases the vulnerability of the animal or human to predation, accidents or disease.

Clearly, the developmental events that lead to the survival of animals to reproductive success are determined genetically. But, the survival of animals beyond sexual maturation is determined only indirectly by the genome. Survival beyond reproductive success is a potential that might be regarded as a period of "coasting" or "free-wheeling", where developmental processes have ended and the capacity to maintain vital systems declines.

3. Genes do not govern the processes of aging

Just as a blueprint is vital to manufacture a complex machine, but unnecessary to bring about the aging of that machine, the genome is necessary for biological development but unnecessary for the animal's aging. The animal and the machine fail as a result of increasingly irreparable molecular disorder, which in living systems increases vulnerability to pathology or disease and in inanimate objects increases their likelihood of failure in some vital component.

Another argument against the direct role of genes in programming the aging process is that even in inbred strains of animals, age changes in older animals are heterogeneous. Older animals do not age at the same rate or die on the same day, week or month. When the random events, characteristic of aging, are compared with the orderly, virtually lock-step, changes that occur during genetically driven embryogenesis and development in inbred animals the orderliness and precision of developmental changes stands out in stark contrast to the quantitative and qualitative disorder of age changes. The variability in the manifestations of aging differs from animal to animal but the variability in developmental changes differs trivially. Humans from conception to adulthood are virtually identical in respect to their biological development but from thirty onwards humans are a biologically heterogeneous group.

The aging of living things is not unlike the aging of everything else in the universe including the universe itself. The molecular disorder that defines aging, both in animate and inanimate objects, occurs passively by simple decrements in the energy necessary to maintain molecular order, other chance events occurring at the molecular level [1], or actively through, for example, the action of oxygen or its reactive species.

Finally, genes play no role in the aging process because it is unlikely that feral animals ever live long enough for putative genes that directly govern age changes to either evolve or to be selected for.

Clearly, there are genes whose modifications will increase survival skills or physiological reserve and thus indirectly increase the potential for greater longevity. Thus, the many studies now done in invertebrates, where evidence is given for genes that allegedly govern aging are, in fact, genes that govern longevity determination by acting directly on developmental processes or physiological capacity. The longevity of invertebrates is extremely malleable and can be influenced by such simple environmental conditions as light, temperature changes, food availability and life stages that resemble hibernation in higher animals. In invertebrates where genetic manipulation prolongs or even shortens life, the effects are on physiological capacity or developmental processes and not on the inexorable expression of molecular disorder that occurs after reproductive maturity.

Longevity determination in higher animals has been a profoundly neglected area of research. One class of animals that may provide some answers to the determination of longevity are those animals that do not reach a fixed size in adulthood and age slowly or not at all. If these animals do age, the process is either negligible or it occurs below the limits of detection. Animals of this class include some tortoises, many sport and cold-water deep-sea fish, some amphibians and the American lobster. Even telomerase expression, the hallmark of immortal cells has been found at extraordinary high levels in the cells of negligibly aging animals like the American lobster (*Homarus americanus*) and the rainbow trout (*Onchorhynchus mykiss*) [4, 5]. Whether these animals age at all, and the reasons for this, have been almost entirely neglected. They are not immortal because, like animals that do age, there is a constant threat of disease, predation and accidents [6]. The time is long overdue for more intense study of the phenomenon of negligible aging.

4. Aging in feral animals

Aging is a phenomenon peculiar to the human species and is an artifact of human civilization [2, 3, 7].

There is a good argument to be made for the belief that aging never occurs in feral animals because these animals rarely live long enough to experience the phenomenon. The same observation can be made for prehistoric humans who, like feral animals, also rarely lived long enough to experience aging. Natural selection could not select for a process like aging when few, if any, animals ever lived long enough to participate in the selection process.

If, through human intervention, feral animals are kept as pets or deposited in zoos and thus protected from predation, accidents and disease, age changes that may have never been experienced in the wild will now be unmasked. This longer survival is not the result of the expression of new genes. It is the result of human intervention that prevented what, in the wild, would have been certain death because the physiological decrements incurred after reproductive maturity would have quickly increased vulnerability to predation, disease and accidents.

Most animals do not die immediately on the day after reproductive success because it is prohibitively costly in energy to evolve a system that would cause an animal to die precisely on that day.

This is analogous to the manufacturer of a cheap watch who guarantees that his watches will function for one year. She will quickly fail in business if the one-year guarantee cannot be met. To achieve this goal the manufacturer makes certain that the materials used and the workmanship employed will result in a product that lasts at least one year. But, what the watchmaker and natural selection do not do is to engineer into the system an expensive mechanism that will insure that the watch fails on the 366th day or that causes the animal to die immediately after reproductive success. The high costs of including a sudden death mechanism will drive the cheap watchmaker out of business or cause the animal to expend energy on a process that has no survival value.

The class of animals generally referred to as "big bang animals" represented by the Pacific Salmon and the marsupial male rat, may appear to be an exception to this notion. However, it is more likely that the deaths that occur after reproductive success in these animals are the result of their unique expenditure of enormous amounts of energy that precedes mating. Furthermore, it is questionable whether the biological changes that precede their deaths are age changes. Indeed, there is no necessity for death to be preceded by age changes.

5. Aging is an artifact of human civilization

Engineers call the average period of time that they expect a mechanical device to survive, the "mean time to failure". For humans the mean time to failure for today's babies in developed countries is about 76 years and is equivalent to life expectation at birth.

Humans (or the animals we chose to protect) are the only species in which a large number of members experience aging. Furthermore, old humans or old animals are not essential for the survival of any species. Proof of this statement is that humans had a life expectation at birth of thirty years or less for more than 99.9 percent of the time that we have inhabited this planet [2]. Prehistoric human remains have never revealed individuals older than about fifty years of age. There appears to be no selective advantage favoring the survival of old animals or old humans.

Finally, members of exotic wild animal species, who for millions of years have not experienced aging, reveal age changes when protected by humans who keep them as pets or house them in zoos. It would be difficult to explain how evolution could have selected for a process like aging that could be made to appear in all members of a species after its expression was suppressed for millions of years.

Because humans, unlike feral animals, have learned how to escape the causes of death long after reproductive success, we have revealed a process that, teleologically, was never intended for us to experience. One might properly conclude, therefore, that aging is an artifact of civilization.

"Why do we age?" may be the wrong question. The right question is: "Why do we live as long as we do?".

6. Diseases and aging

The distinction that must be made between aging and disease is just as important as is the distinction that must be made between aging and longevity determination. Failure to recognize that age processes differ from pathology has not only blurred our understanding of aging but it has had, and still does have, profound political and economic consequences that have negatively impacted the field of biogerontology.

My concerns are based on what I believe to be the mistaken assumption that an understanding, or even the resolution, of age associated diseases will advance our knowledge of the fundamental processes of aging. This dubious assumption is a result of the failure

of many biogerontologists, to say nothing of the general public, to distinguish disease or pathology from the fundamental biological processes of aging. The distinction is central to an understanding of why the resolution of the leading causes of death in old age, — cardiovascular disease, stroke, cancer and even Alzheimer's Disease, will tell us little about the fundamental biology of age changes. The resolution of all three causes will only result in an increase of about fifteen years in human life expectation [8]. Then, aging, or the inexorable loss in physiological capacity that underlies the cause of these pathologies will be unmasked as the leading cause of death.

The resolution of age-associated diseases will advance our knowledge of aging processes to the same extent that the resolution of pediatric-associated diseases such as poliomyelitis, acute lymphocytic leukemia, Wilms' tumors and iron deficiency anemia advanced our knowledge of childhood development. Clearly, no advancement occurred at all.

Disease processes can be distinguished from age changes for at least four reasons. Unlike any disease, age changes (1) occur in every animal that reaches a fixed size in adulthood, (2) cross virtually all species barriers, (3) occur in all members of a species only after the age of reproductive success and (4) unlike any disease, aging occurs in animals removed from the wild and protected by humans even when that species has not experienced aging for thousands or even millions of years.

We would not know much about pathology or disease without first knowing what is normal or usual. In a similar way the etiology of age-associated diseases will not be well understood in the absence of a better understanding of why the biology of old cells makes pathology more likely than it does in young cells. Today, the study of age-associated diseases dominates the field and the study of fundamental aging processes comes in a very distant second.

Not all old cells compromise health or increase the likelihood of death. No one has ever died of wrinkled skin, gray hair, or the menopause.

The resolution of all causes of death currently written on the death certificates of those over age 65 will only result in an increase in life expectation at birth of about fifteen years. What needs to be better understood is that an understanding of why old cells are more vulnerable to pathology than are young cells does not put a fifteen-year limit on what is possible.

Today, the study of age-associated diseases and manipulating biological development in lower life forms dominates what many

believe to be aging research. It is not. One example of the former is that more than half the budget of the National Institute on Aging in the United States is spent on Alzheimer's disease research, yet motor vehicle accidents cause twice as many deaths [8] and from age 65 on, it is not even one of the five leading causes of death [8]. The likelihood of dying from Alzheimer's disease is 0.7%[3] and the complete resolution of this disease will add about 19 days onto average life expectation [8]. Nor will that accomplishment advance our knowledge of the fundamental biology of aging.

In the minds of the public, policy makers and many biomedical scientists, no one suffers or dies from aging. We suffer and die from the diseases associated with the aging process. Yet, it is age changes that are the etiology of, and increase the vulnerability to, everything that is written on the death certificates of the elderly.

No one over the age of, say, 75 has or will die from what is written on his or her death certificate. Death results from the inevitable increase in systemic molecular disorder that living long enough incurs. That disorder simply increases vulnerability to whatever was, or will be, written on death certificates. There are multiple pathologies in older people and the true cause of death is rarely known. Because there are few autopsies, and little research, the cause of most deaths in old age is still hidden in the proverbial black box.

More than 75% of all human deaths in developed countries now occur in those over the age of 75. If the causes of these deaths are resolved we will not become immortal but we will have revealed how death occurs in the absence of disease. What will be found is that the underlying cause of these deaths is the inexorable loss of physiological capacity that results from increasing molecular disorder in the cells of vital organs. This is the hallmark of aging and it will appear on all death certificates once the present leading causes are resolved.

There is no evidence to support the many outrageous claims of extraordinary increase in human life expectation that might occur in our lifetime or that of our children. Based on the United States Census Bureau Middle Series, life expectation in 2050 will be about 82 years for both sexes in the United States [9]. The United States Social Security Administration anticipates a life expectation of 78.1, 80.4 and 83.5 years for both sexes in 2066 based on three alternative assumptions about decreases in mortality rates [10].

The G7 industrialized countries project life expectation at birth in 2050 to range from a high of 83.5 in France to a low of 80.5 in the United States [11]. In a more recent analysis, in which it is assumed that the future decline in mortality rates will follow the exponential decline that has occurred during the last fifty years, it is forecasted that life expectation at birth in 2050 will vary from a high of 90.9 in Japan to a low of 82.9 in the United States [11].

7. Cytogerontology

In the last decade several significant findings have been made in cytogerontology at the molecular level that impact on our understanding of the genetics of longevity determination and on aging.

These observations have provided new insights at the molecular level into why normal cells have a limited capacity to replicate and what that might be telling us about aging and longevity determination. These new insights have generated enormous excitement because it appears that the first molecular counting mechanism has been discovered and it's discovery bridges aging, longevity determination and cancer.

This story has its' historical roots in the development of cell culture techniques at the turn of this century when, until 1960, it was believed that all cultured cells, whether derived from normal or cancerous tissue, had an unlimited capacity to replicate and to function. Consequently, aging was thought to have little to do with intracellular events and, as a result, research on aging focused on extracellular determinants.

In the early 1960's we overthrew this dogma after finding that cultured normal cells do have a finite replicative capacity and we interpreted this phenomenon to be

aging at the cellular level [12, 13]. We pointed out that there are two classes of cells, normal mortal cells and immortal cancer cells [12, 13]. This new understanding also provided the basis for the field of cell immortalization because without an appreciation that mortal, normal, cells existed the notion of cell immortalization would not have been possible. Since we made this distinction in 1961, interest in the process of immortalization of normal cells has attracted an enormous amount of attention (1, 2, 5-7). Our suggestion that the limited replicative capacity of cultured normal cells might be telling us something about aging and longevity determination launched the field of cytogerontology [2, 3, 14-16].

7.1. The idea of a counter

Three of our early observations led us to conclude that normal, mortal, human cells must contain a replication counting mechanism. First, was the reproducibility of our finding that human fibroblasts from different embryonic donors underwent a finite number of population doublings that spanned a narrow range between forty and sixty. Second, cells frozen at any population doubling level from one to fifty retained memory of that level until reconstitution so that the total number of population doublings traversed both before and after freezing totaled fifty [12, 13]. The ability of WI-38 to remember at what population doubling it is when frozen is as accurate today as it was when I first developed and froze the strain in 1962. After 39 years of cryopreservation, WI-38's memory has been retained without loss.

The third evidence for the existence of a counter resulted from our efforts in 1975 to determine the location of the putative counter. By employing enucleation and fusion techniques in which nuclei removed from old and young cultured cells were fused to opposite aged enucleated cytoplasts we concluded that the replicometer was located in the nucleus [17, 18].

The search for the counting mechanism remained virtually quiescent for the next fifteen years. Then, the remarkable convergence of data from several diverse fields of research with our own resulted in an explosion of fascinating information that has not only determined the location of the counting mechanism but has identified its' molecular structure. (For more detailed reviews see [19-26].)

7.2. The telomere replicometer

As we had suggested in 1965, cell mortality and immortality are inextricably linked to aging and cancer [13]. Consequently, the importance of identifying the putative counter would be difficult to exaggerate.

The counting mechanism should not be called a clock or chronometer because these devices measure the passage of time. The replicative limit of normal cells is directly related to the number of cell doublings, or more precisely, DNA replications. Thus, the putative mechanism should be more properly referred to as an event counter. Because a meter is a device that measures quantity, or counts events, this would justify the suggestion that the term "replicometer" be used in lieu of "clock".

In a 1938 lecture given by Hermann Muller [27] and followed by the work of Barbara McClintock [28], the tips of chromosomes were reported to contain discrete structures called telomeres. Although the precise role that these structures played was unclear, there was some evidence that telomeres prevented chromosomes from fusing to each other end to end and that they permitted the attachment of chromosome ends to the nuclear envelope.

In an entirely different area of biological inquiry it was observed, in the early 1970's, that the properties of DNA polymerase prevented it from fully replicating the linear ends of DNA [29-32]. This "end-replication problem" is the inability of DNA polymerase to completely replicate the 3' end of linear duplex DNA.

In the late 1960's, Alexey Olovnikov, who had just heard a lecture on our work, wondered how normal cells might have a limited capacity to replicate as he entered a Moscow subway station [31]. When the train stopped at the station he had a remarkable flash of insight. Olovnikov saw an analogy between the train, which represented the DNA polymerase, and the track that represented the DNA. If the train engine were imagined to be the polymerase that replicated the DNA track, the first segment of DNA would not be replicated because it was underneath the engine at the start. This was analogous to the "end-replication problem". Olovnikov realized that this repeated shortening of the DNA molecule at each round of DNA replication might explain our finding that normal cells can only replicate a specific number of times. James Watson independently arrived at a similar solution in 1972 [32].

Because the loss of DNA that contained vital genetic information at each division seemed unlikely, Olovnikov reasoned that telomeres might consist of repeated nucleotide sequences that did not contain genetic information but behaved much like a buffer. At each round of DNA replication the buffer would simply loose what portion of the DNA molecule was not copied (the telomeric ends) and thus protect downstream genes. The length of the buffer would determine the number of rounds possible for DNA replication.

Olovnikov's imaginative solution to the end replication problem, although published in 1971 in Russian [29] and in 1973 in English [30], languished in the literature for several years until laboratory reports emerged that have substantially supported his armchair speculations.

7.3. Telomere structure discovered

In 1978, Elizabeth Blackburn and Joseph Gall, working with the ciliated protozoan, Tetrahymena, found that telomeres consisted of a simple sequence of hexameric repeats of the nucleotides TTGGGG [33]. It was later found that the telomere repeat sequence in human cells was TTAGGG [34]. From slime molds to humans, telomeres consist of thousands of repeats of the highly conserved sequence TTAGGG [35].

In 1986 Howard Cooke suggested that telomere shortening might occur in human cells [36]. Later Calvin Harley, who had worked for several years with my system of senescent human cells, had a fortuitous discussion with Carol Greider and both decided to explore the possibility that the limited proliferative capacity of cultured normal cells might be explained by diminishing telomere length. They did, indeed, find that the mean

telomere length decreased by 2 to 3 kilobase pairs during the entire *in vitro* lifetime of several strains of cultured normal human diploid fibroblasts [37].

The decrease was found to be progressive and averaged fifty base pairs per population doubling [38]. The telomere shortening seen in aging cultured normal human fibroblasts also occurs in many other normal cultured and *in vivo* cell types.

Allsopp et al [39] reported that after analyzing the cultured normal fibroblasts from 31 human donors, aged from several months to 93 years, a striking correlation, valid over the entire age range, was found between replicative capacity and initial telomere length. Thus, cell strains with shorter telomeres underwent significantly fewer doublings than those with longer telomeres.

Telomere attrition appears to be the replicometer that determines the number of times that a normal cell is able to divide. Once a threshold number of telomeric $(TTAGGG)_n$ repeats is reached, presumably downstream events are triggered that signal the cessation of DNA replication. Wright and Shay [40] have offered an alternative explanation of how telomere shortening acts as a replicometer. Their telomere positional effect explanation of cell senescence is based on a novel two-stage model.

7.4. Achieving immorality

One essential question remains: How do transplantable tumors and immortal cell lines avoid telomere shortening that, when it occurs, would condemn them to lose division capacity?

The answer to this critical question also originated in studies with Tetrahymena by Greider and Blackburn [41], who discovered the ribonucleoprotein enzyme terminal transferase called telomerase. They found that telomeres are synthesized *de novo* by telomerase, a ribonucleoprotein enzyme that extends the 3′ end of telomeres, and thus elongates them. This ribonucleoprotein complex contains a reverse transcriptase and RNA template for the synthesis of the repeated sequence [42]. It was simultaneously reported that cancer cells have shorter telomeres than do adjacent normal cells [43-44] thus providing the first link for the role of telomeres in cancer biology.

Telomerase was later found to occur in extracts of immortal human cell lines [45, 6] and in about 90% of all human tumors studied [21, 26]. Subsequently, the telomerase RNA component [47] and the catalytic portion of the enzyme were cloned [48]. Telomerase is the only known reverse transcriptase that is necessary for normal cell activity.

Unlike normal mortal cultured cell strains, most immortal cell lines produce telomerase. Thus, the telomeres of most cell lines do not shorten with serial passage *in vitro*.

In recent years telomerase expression has been found in several classes of normal cells. These include fetal tissue, normal bone marrow stem cells, testes, peripheral blood lymphocytes, skin epidermis and intestinal crypt cells [21]. Prior to the 10th post-natal week in humans the RNA component of telomerase can be found in virtually every differentiating cell [49]. All of these normal cells have high turnover rates, or are in a continuously replicating pool of differentiating cells. The level of telomerase activity found in normal cell populations is, per cell, significantly less than that found in cancer cells [21].

The observation of telomere attrition as normal cells divide, provides the first direct evidence for the putative replicometer. This, in combination with the discovery of the enzyme telomerase, has gone very far in explaining why most normal somatic cells have a finite capacity to replicate *in vivo* and *in vitro* and how immortal cancer cells circumvent this inevitability.

In 1998 it was reported that normal, mortal, human cell strains could be immortalized with apparent retention of their normal properties by transfecting them with vectors encoding the human telomerase catalytic subunit [50]. Thus, the replicometer in normal cells can be purposefully caused to count indefinitely. This has provided direct evidence proving the role of telomere shortening in cell senescence and telomerase expression in cell immortality.

This discovery has profound theoretical and practical implications that include the immortalization of highly differentiated normal human cell types for the production of medically useful proteins. It also implies that the hundreds of biological changes that we [51] and others [52] have reported to herald the loss of replicative capacity in normal cells can be reversed. Exploitation of this insight could have enormous medical benefits because normal cells in aging individuals might be engineered to have their reduced or lost vital properties reversed or delayed.

Because exquisitely sensitive methods exist for the detection of telomerase in a single cell, this procedure is being exploited as a diagnostic tool to detect the presence of cancer cells in clinical specimens. Other researchers are exploring the possibility that telomerase inhibitors might be found that could be used therapeutically in the treatment of cancer [44, 53-55].

8. Telomere attrition as a longevity determinator

Telomere attrition may be better understood as a measure of longevity determination than it might be as a cause of age changes. After reproductive maturity in animals that age, the level of remaining physiological reserve determines longevity. The reserve does not renew at the same rate that it incurs losses because molecular disorder increases at a rate greater than does capacity for repair. As previously discussed, this increase in molecular disorder results in age changes and these increase vulnerability to predation, accidents or disease [3, 7].

As stated above, increasing molecular disorder and loss of cell function occurs in cultured normal cells resulting in hundreds of biological changes. Thus, the number of population doublings that a cell strain is capable of undergoing, and that is determined by telomere length, may be the *in vitro* expression of maximum potential longevity. The hundreds of molecular disorders that herald the approaching loss of replicative capacity, and diminution of telomere length, are age changes (Hayflick, 1980). When molecular disorder occurs in dividing cells *in vivo*, the hundreds of reported changes also may affect post-mitotic cells. Both cell types may then reveal increasing vulnerability to pathology that will lead to death of the individual well before species and cell maximum longevity is reached [2].

9. Ordinary mortals

Biological immortality has been a part of human thinking since the first humans realized their own mortality. Immortality, as a concept, appeared in writings at least 3, 500 years ago, was a major goal in alchemy, and in the last half of this millennium drove vast explorations in search of the fountain of youth [2]. In today's iteration, the pursuit of great longevity is driven by the belief that a particular diet, life style or concoction will achieve the desired result. Yet, the idea of biological immortality is an illusion [6].

The serious problems that could occur if immortality or extreme longevity was to become possible have been the theme of much of our literature, starting with Greek Mythology in which the Trojan Tithonus loves Eos, the Goddess of dawn. At her request, Zeus makes Tithonus immortal but, tragically, Eos neglects to also ask that he not age. Jonathan Swift rediscovered this theme in his immortal, but continuously aging, Struldbrugs. Despite the popular and glib belief that arresting or slowing the aging process is desirable, I am unaware of any way in which it might be done to the benefit of individuals or the larger society [2, 7].

At present, we know of no way in which the human aging process is likely to be slowed with the probable exception of caloric restriction. However, the results might also be interpreted to suggest that overeating diminishes longevity [2, 3, 7]. Although demonstrated in many species, including its' likely occurrence in non-human primates, it has yet to be demonstrated conclusively in humans [56]. Even if demonstrated, a near starvation diet is unlikely to be acceptable to most people whose quality of life is more important to them than is their quantity of life.

It is likely that a natural increase in the human life span is presently occurring but so slowly that our ability to detect it will only be made after millennia of careful record keeping. This belief is based on persuasive evidence in the fossil record that suggests that the life spans of most animals increase as evolution proceeds [2].

As some civilizations have, our society must learn that aging and youth should be valued equally if for no other reason than the youth in developed countries have an excellent chance of experiencing the phenomenon that they may now hold in low esteem. Then, the misplaced passion for cosmetic surgery, anti-aging nostrums and similar snake oil remedies touted to arrest aging will be recognized for what they truly are, — at best, a cover-up for an irreversible and inexorable process and, at worst, a delusion and waste of money by the uninformed.

If the main goal of our biomedical research enterprises is to resolve causes of death, then every old person becomes a testimony to those successes.

Biogerontologists have an obligation to emphasize that the goal of research on aging is not to increase human longevity regardless of the consequences but to increase longevity free from disability and functional dependence.

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FIGURE SECTION FOR CHAPTER 1

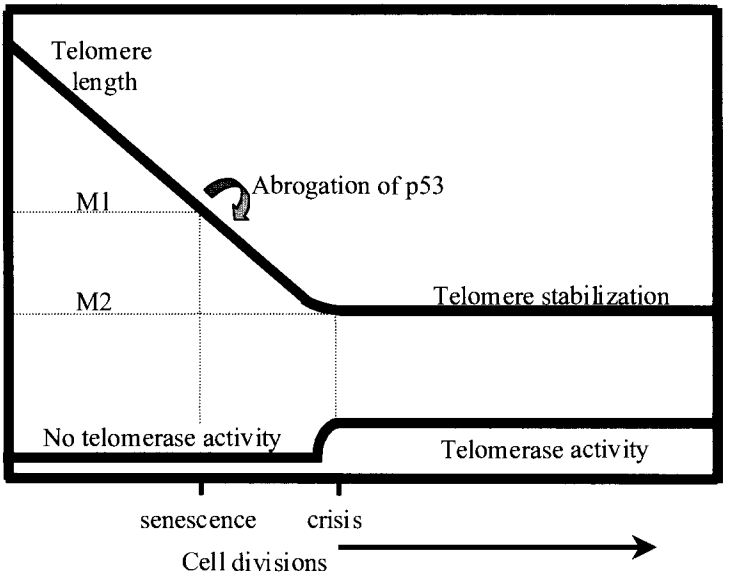


Figure 1. Activation of telomerase activity stabilizes telomere length.

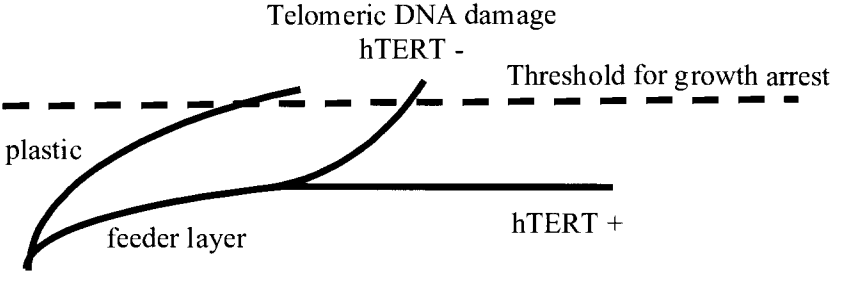


Figure 2. Telomeric DNA damage.

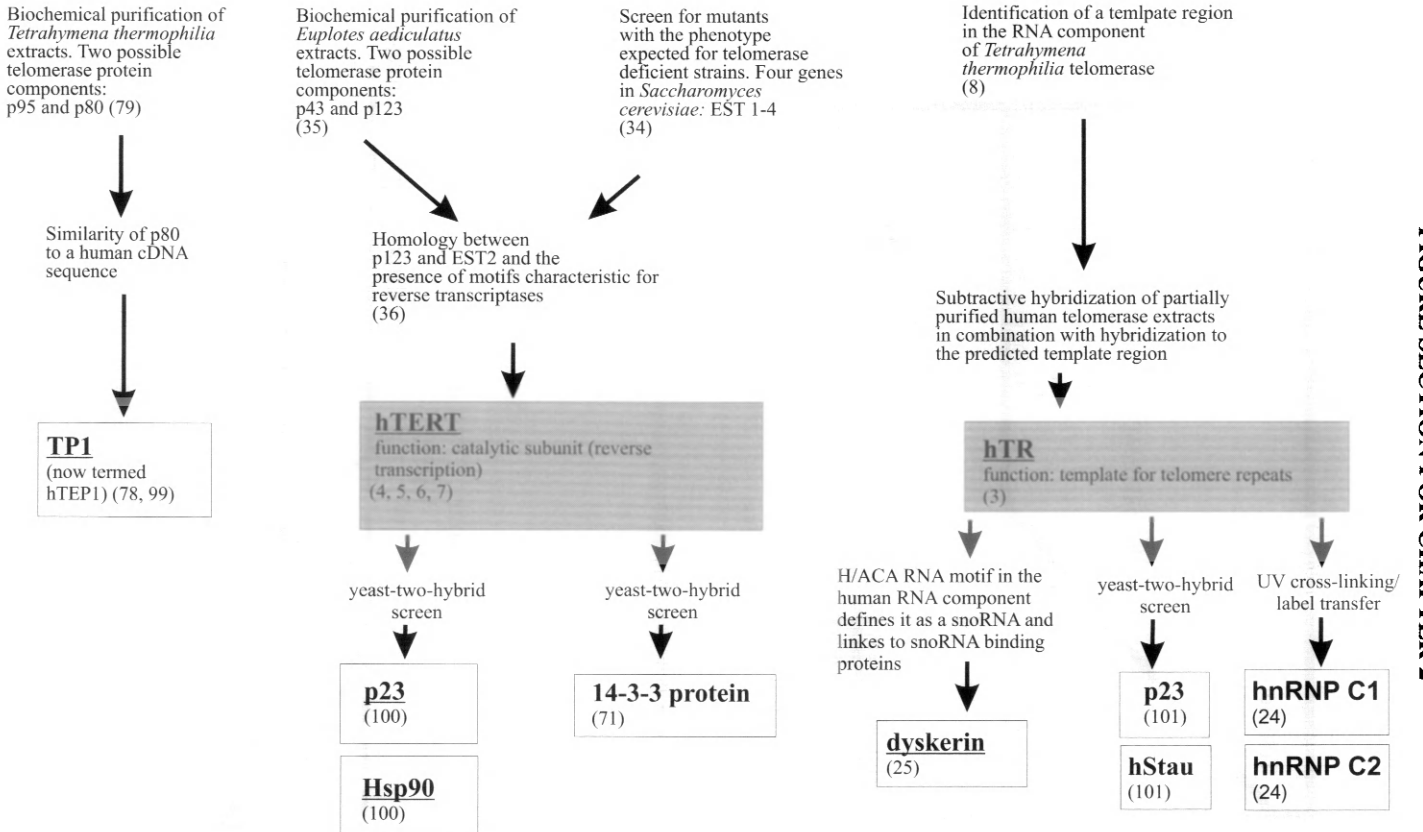


Figure 1. Discovery of human telomerase components. This overview briefly describes the methods used for the discovery of human telomerase enzyme components. hTERT and hTR, the core components are shown in grey shaded boxes, the telomerase associated proteins in open boxes.

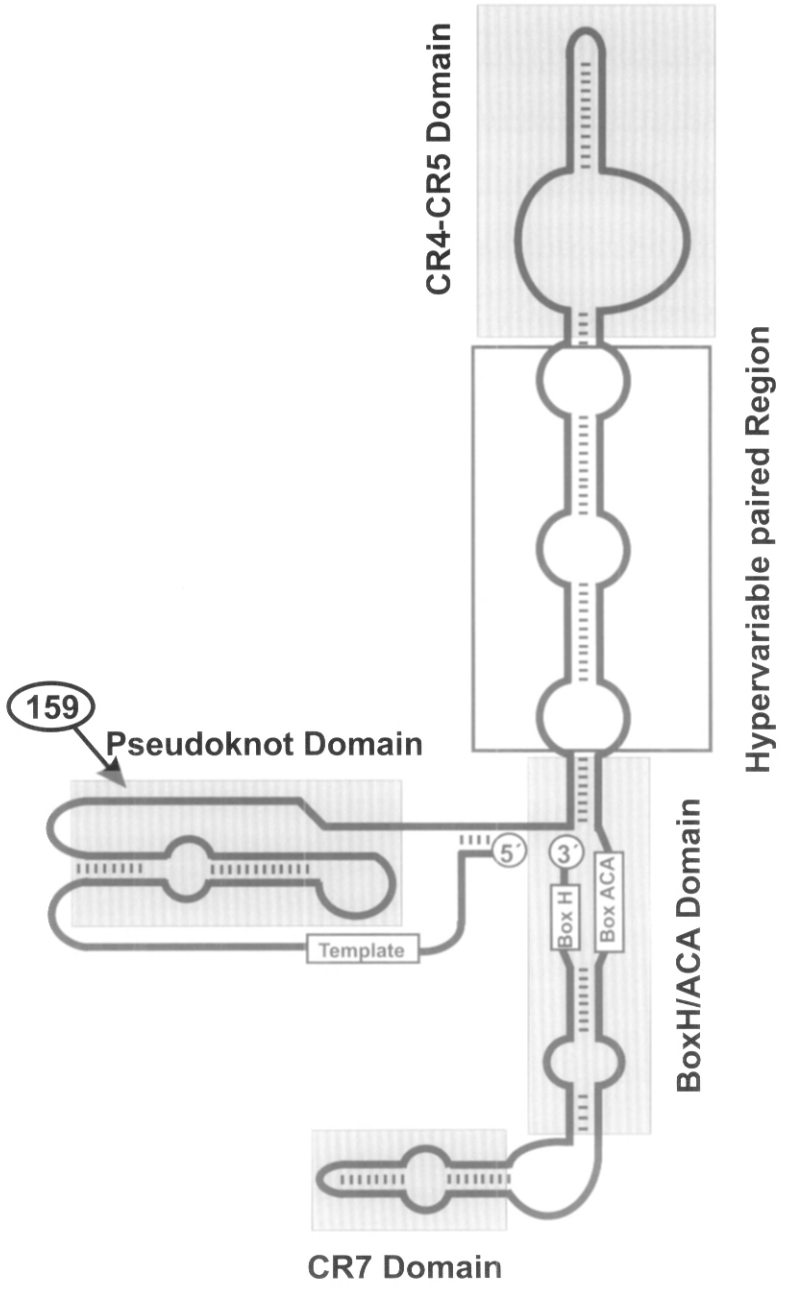


Figure 2. Minimum consensus structure of vertebrate telomerase RNA. A model for the consenus telomerase RNA for all vertebrates based on sequences of 35 vertebrate RNAs. Phylogenetic comparison suggested a secondary structure that includes conserved regions (shaded box) and a hypervariable region (open box) [26].

- [547]**WL**MSVYVVELLRS**FFY**V**TE**TTFQKNRLFF**YRK**SV**W**S**K**L**Q**SIG**I**RQHL**K**[594]

(motif T)

- [605]EVRQHREARPALLTSRL**R**FI**PK*P**DG[629]

(motif 1)

- [630]L**R***PIVNMDYVVGARTFRRE**K**RAERLTSRV[659]

(motif 2)

- [702]DPP**P**E**L**Y**F**VKV**D***VTGA**Y**D**T**IPQDRLTEVIASIIKP[736]

(motif A)

- [824]**K**S**Y**V**Q**CQ**GIPQ*G*SI**L**S**T**LLCSLC**Y**G**D**MENKLFA[856]

(motif B')

- [864]**LLRL**V**D***D***F**LLVTPHLTH[880]

(motif C)

- [881]AKT**F**LRTLVRGVPEYGCVV**N**LR**K***TVV[906]

(motif D)

- [926]HGLFP**W**C**G***LLL[936]

(motif E)

Figure 3. Reverse transcriptase motifs of hTERT and motif T. Motif 1, 2, A, B', C, D and E are conserved among reverse transcriptases and also telomerases. Motif T is conserved among telomerases only. The Position of the first and last amino acids are indicated. Amino acids shown in bold are highly conserved among telomerases. Asterics indicate amino acids where mutations severely decrease telomerase activity.

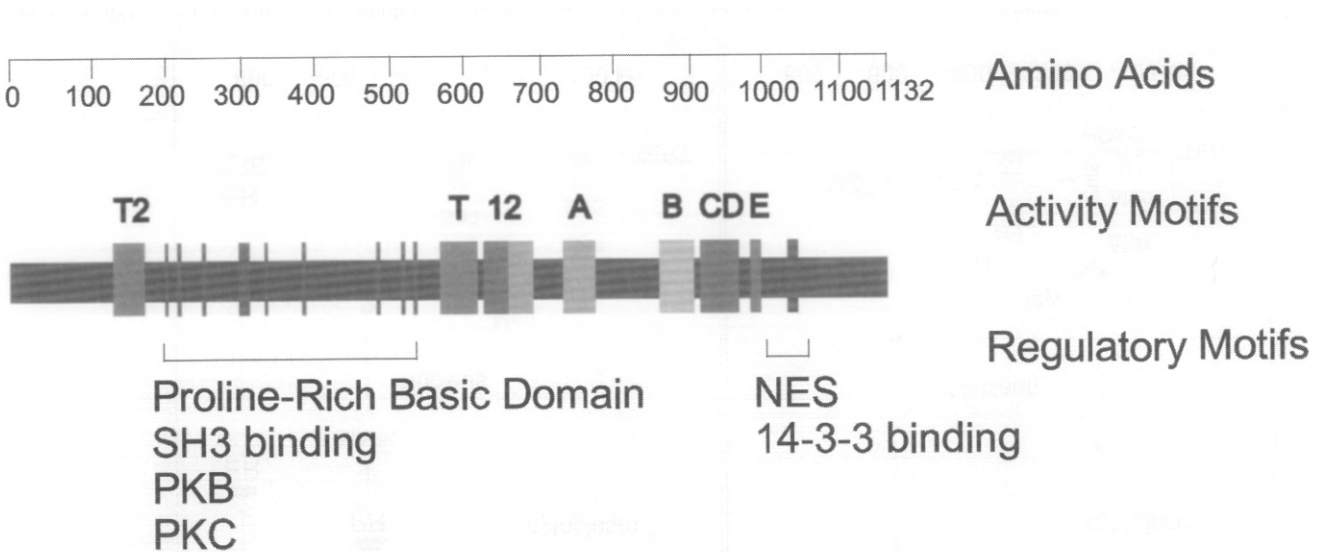


Figure 1. The molecular architecture of hTERT. The human telomerase catalytic subunit hTERT contains unique T and T2 motifs found in all TERT molecules of different animal species, and seven conserved reverse transcriptase motifs found in different reverse transcriptases. Between the T motifs is the proline-rich basic domain containing a putative SH3 binding motif flanked by multiple potential serine/threonine phosphorylation sites for PKB, PKC and casein kinase 2. Toward the C-terminus, there is a 14-3-3 binding motif downstream of a probable nuclear export signal (NES) for regulation of hTERT nuclear localization.

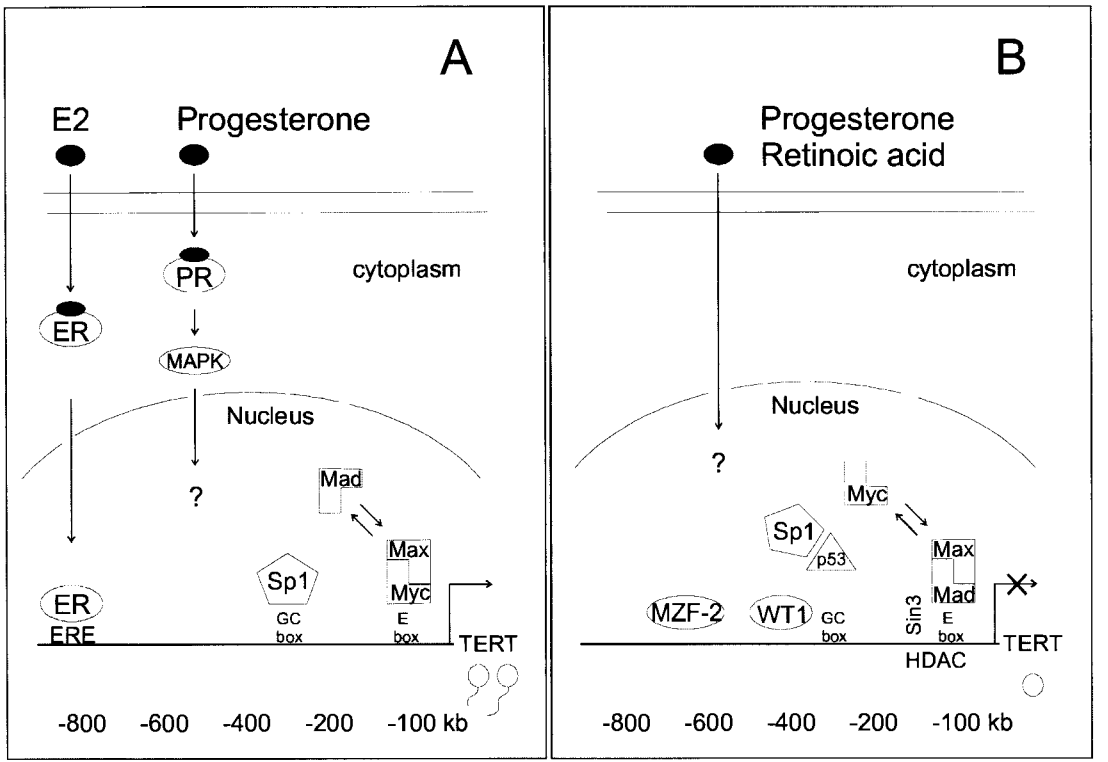


Figure 2. Multifactorial regulation of the hTERT gene by transcriptional factors and repressors. (A). Activation of hTERT gene. The binding of c-Myc as a heterodimer with Max to the E-box and Sp1 to the GC-box have been shown to be primarily responsible for hTERT gene transcription. In addition, estrogen also activates the hTERT gene in human ovarian epithelium and breast cancer cells, and progesterone stimulates the hTERT gene expression via activating the MAP kinase signaling pathway. (B). Repression of the hTERT gene. Mad competes with c-Myc for Max and inhibits hTERT gene transcription. The inhibition is also thought to involve Mad recruitment of mSin3 and histone deacetylase (HDAC). HDAC catalyzes histone deacetylation inducing DNA condensation; pharmacological inhibition of histone deacetylases with Trichostatin A (TSA) induces activation of hTERT promoter activity. Furthermore, the tumor suppressor p53 binds Sp1 blocking Sp1-induced hTERT gene activation. The tumor suppressors M2F1 and WT1 are also implicated in suppressing hTERT gene transcription.

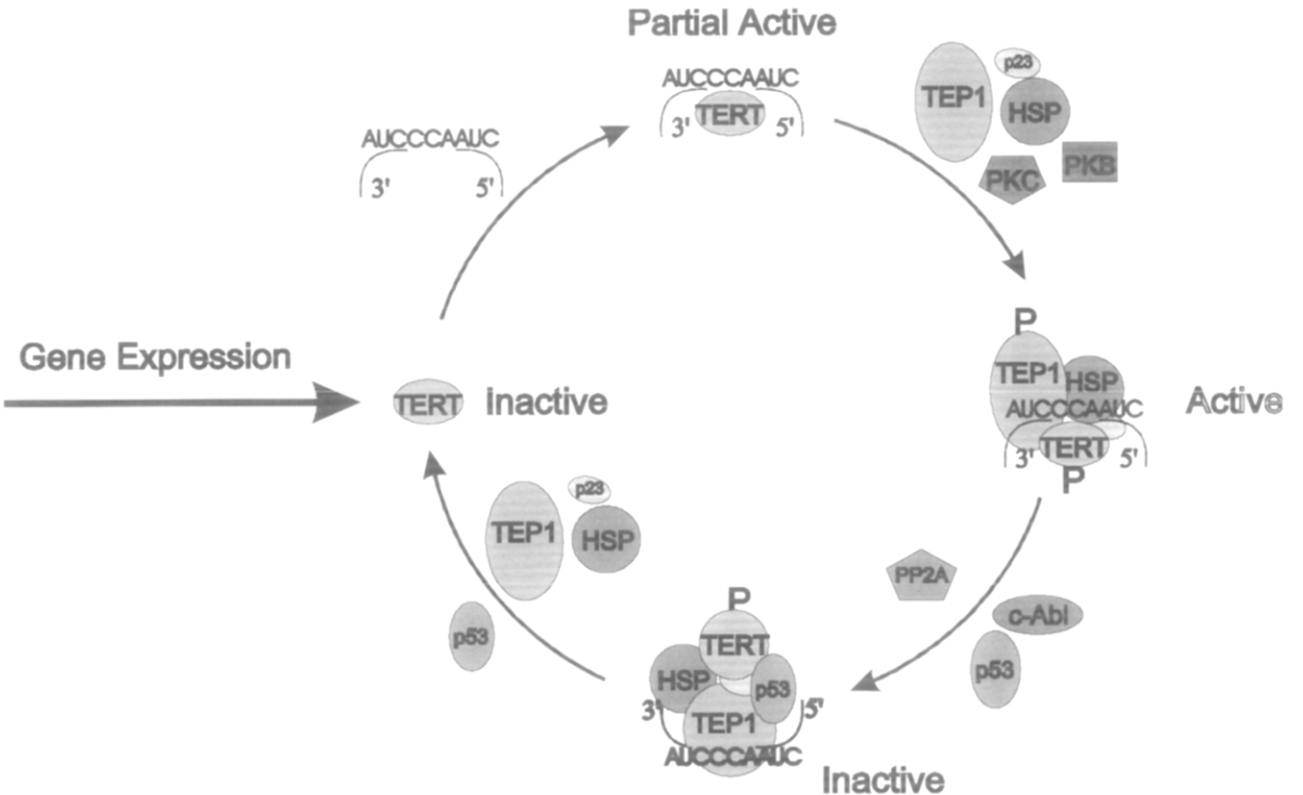


Figure 3. Schematic representation of telomerase assembly and disassembly cycle. Following *de novo* expression, hTERT binds hTR to form a minimal core structure expressing low telomerase activity. Although it does not regulate telomerase activity directly, hTEP1 is associated with hTERT and hTR, possibly contributing to telomerase holoenzyme assembly. The presence of Hsp 90 and p23 and phosphorylation by PKCα and PKB/Akt drastically increase telomerase activity reflecting a transition of telomerase from partially active to fully active configurations. Dephosphorylation by PP2A, tyrosine phosphorylation by c-Abl, and the presence of p53 significantly inhibit telomerase activity, a step representing telomerase deactivation or disassembly from an active complex to an inactive form.

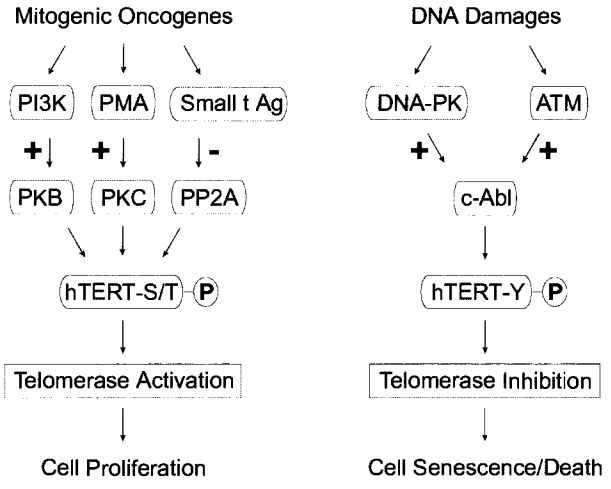


Figure 4. Signaling pathways of serine/threonine and tyrosine phosphorylation differentially regulate telomerase activity. Oncogenic molecules activate PKB/Akt, PKCα but inhibit PP2A, which together may lead to hTERT phosphorylation at serine/threonine residues and telomerase activation involved in cell proliferation. In contrast, DNA damage signals activate DNA-dependent and ATM protein kinases that activate c-Abl tyrosine kinase. The c-Abl tyrosine kinase may then induce hTERT tyrosine phosphorylation and telomerase inhibition in cellular senescence and apoptosis.

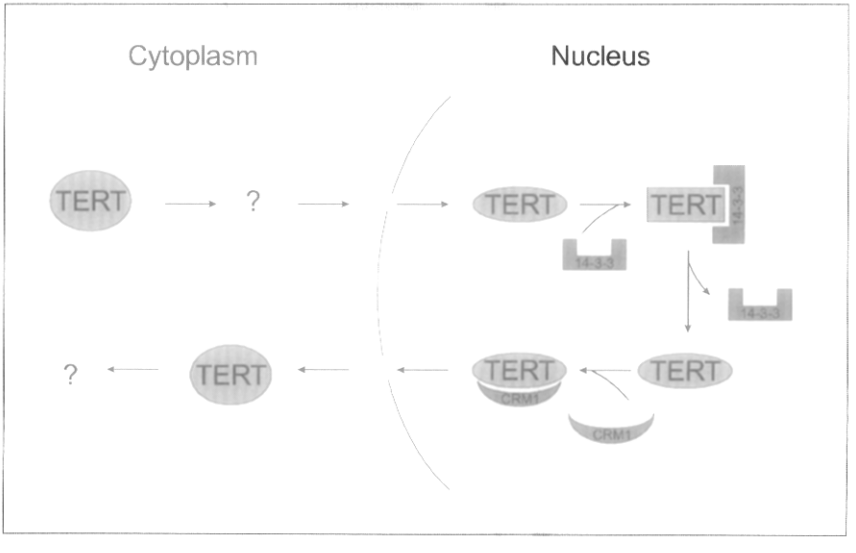


Figure 5. Model of hTERT nuclear trafficking. While little is known of the molecular process of intranuclear import and the fate of hTERT following nuclear export, recent studies suggest that the C-terminal region of 14-3-3 interacts with hTERT C-terminal region to prevent hTERT from interacting with CRM1/exportin 1 and being exported out of the nucleus.

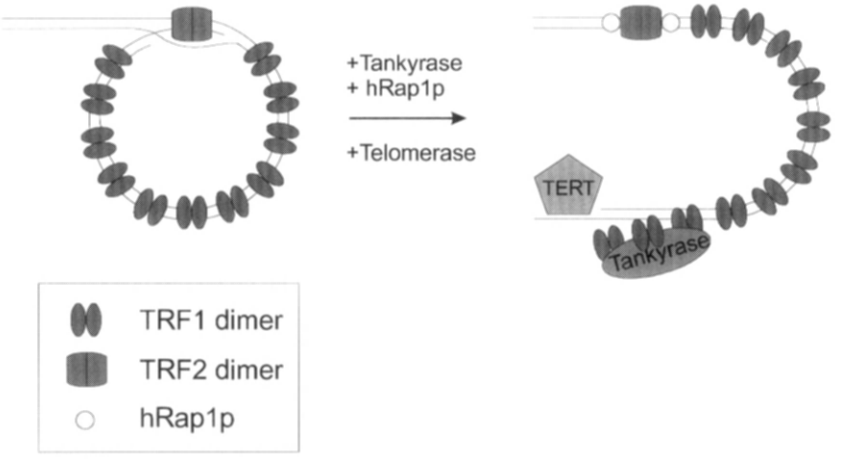


Figure 6. Model of molecular regulation of mammalian telomere structure in telomerase accessibility. Telomeres end in a large duplex loop (t-loop) with the 3' single strand invading the duplex region forming a small displacement loop (d-loop). TRF1 (56 kDa) and TRF2 (65-70 kDa) may play vital roles in the formation and/or stabilization of the telomere structures. In the presence of TRF1-binding protein tankyrase (142 kDa) and TRF2-binding protein hRap1p (47 kDa), however, telomerase elongates telomeres. Since tankyrase is a relatively larger protein with its poly(ADP-ribose) polymerase activity playing an important role in dissociating TRF1 from telomeres, it is possible that the "hijacking" of TRF1 by tankyrase and TRF2 by hRap1p opens up the t-loop allowing or steering telomerase access to its substrate for telomeric DNA elongation.

FIGURE SECTION FOR CHAPTER 4

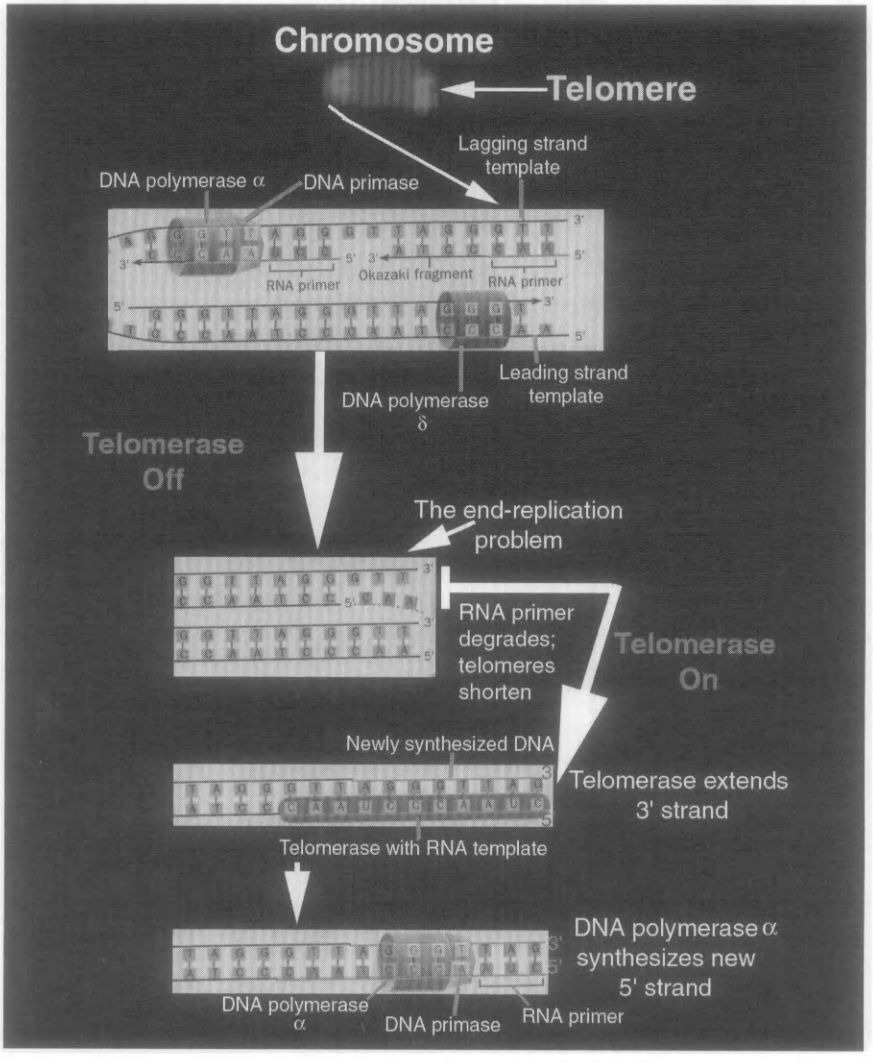


Figure 1. Model for telomerase action for maintenance of telomeres. In mammalian cells, DNA polymerase must synthesize an RNA primer to initiate the replication of each Okazaki fragment on the lagging DNA strand. On completion of DNA replication, the RNA primer degrades, leading telomeres to lose 50-200 nucleotides per cell division. In most of the immortal (normal or cancer) cells, telomerase recognizes the telomere substrate and the terminal TTAGGG repeat is base-paired with 11 nucleotide template sequence in the RNA component. The RNA is copied to the end of the template region. Translocation repositions the terminal 11 nucleotide sequence and exposes additional template sequence. Another round of template copying produces additional TTAGGG repeats. Telomerase then dissociates, allowing the DNA primase-DNA polymerase complex to fill in the complementary telomeric nucleotides.

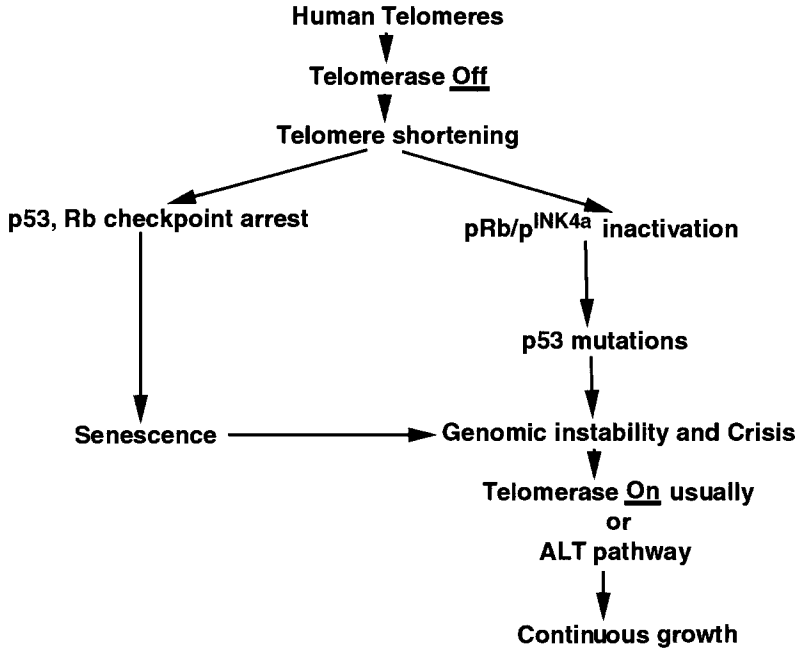


Figure 2. Telomeres are repetitive DNA sequences at the ends of linear chromosomes. Telomerase, a cellular reverse transcriptase, helps stabilize telomere length in human stem, reproductive and cancer cells by adding TTAGGG repeats onto the telomeres. Each time a telomerase-negative cell (telomerase off) divides, some telomeric sequences are lost (telomeres shorten). When telomeres are short, cells enter an irreversible growth arrest state called replicative senescence, which occurs by cell cycle checkpoint genes like (p53, Rb). In most instances cells become senescent before they can become cancerous, thus the growth arrest induced by short telomeres may be a potent anti-cancer mechanism. When pRB/pINK16 is inactivated, followed by p53 mutations, leads to genomic instability, followed by activation of telomerase or ALT pathway, required for the stability of telomeres. Since most cancers express telomerase, maintenance of telomere stability is likely to be required for the long-term viability of tumors.

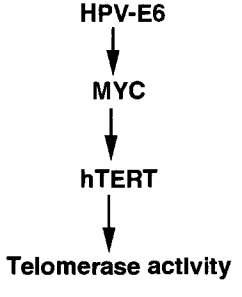


Figure 3. Figure showing activation of telomerase through cMYC expression. Introduction of HPV-E6 in some cell types leads to the activation of cMYC expression and then subsequently the telomerase activity. Introduction of cMYC expression also stimulates telomerase activity.

FIGURE SECTION FOR CHAPTER 5

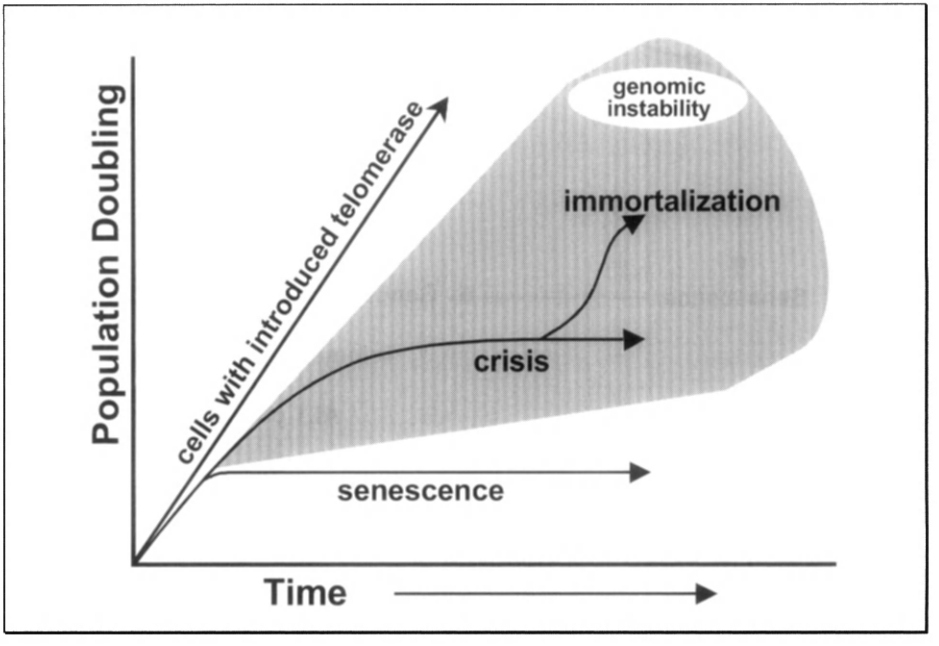


Figure 1. Modification of the M1/M2 model of cellular senescence. Most normal cells undergo cellular senescence after a certain number of cell divisions and progressively shortened telomeres, which has been tabbed as the M1/M2 hypothesis (19). Those cells that are capable of bypassing the senescence block, either spontaneously or through expression of viral oncogenes, have an extended life span with continued telomere shortening in the absence of tumor suppressor function. During that extended life span, the cells are in an atmosphere of genomic instability (indicated by the shaded area) allowing for increased frequencies of mutation and karyotypic changes. The modification of the original model relates to ectopic telomerase expressing cells, which have not bypassed senescence; they have merely postponed or prevented senescence altogether. These cells do not go through crisis, nor do they immortalize in the classical sense. They are genomically stable, functionally normal cells with an extended life span, stable karyotype, and maintained telomeres.

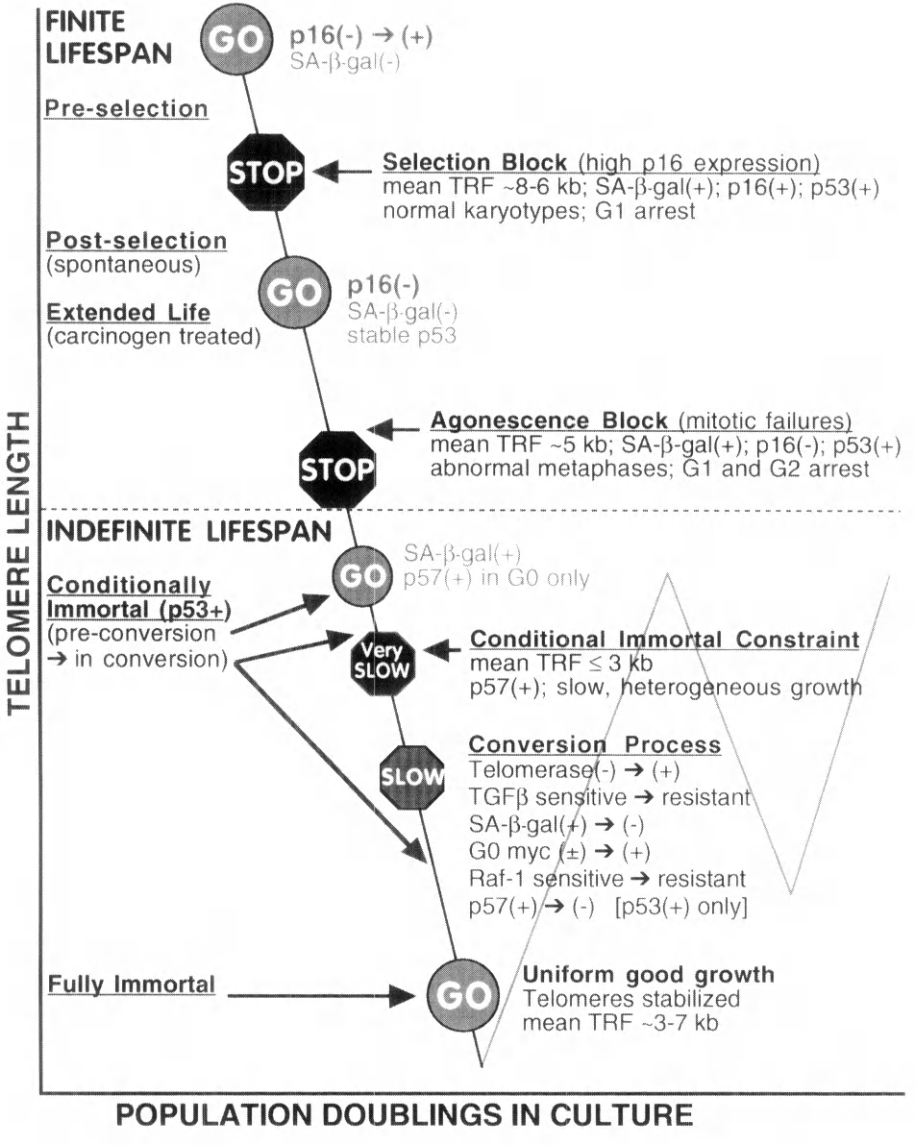


Figure 1. Model for steps involved in immortal transformation of cultured HMEC.

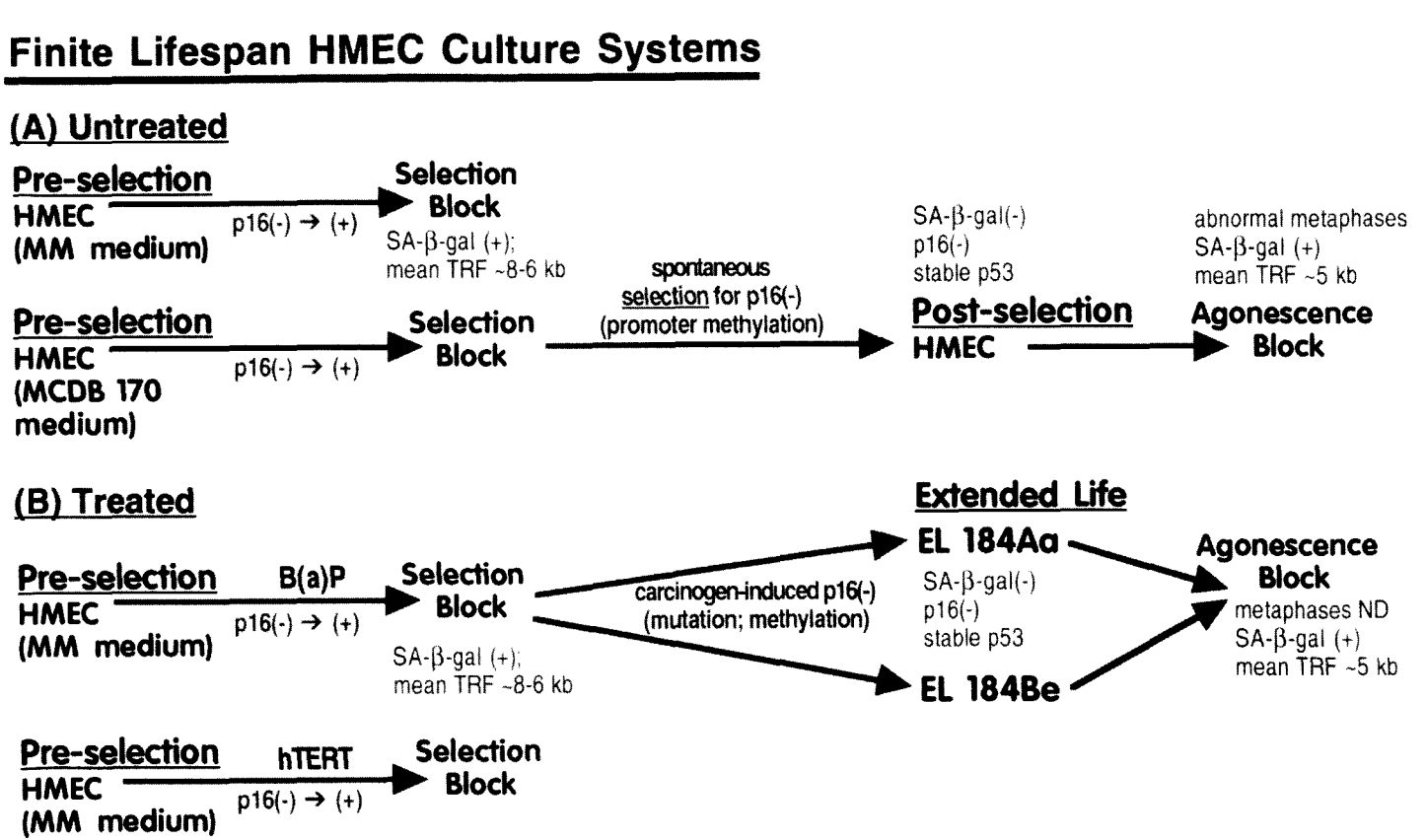


Figure 2. Schematic outline of growth and senescence of finite lifespan HMEC. (A) Untreated cultures; (B) cultures exposed to the chemical carcinogen benzo(a)pyrene or transduced with hTERT.

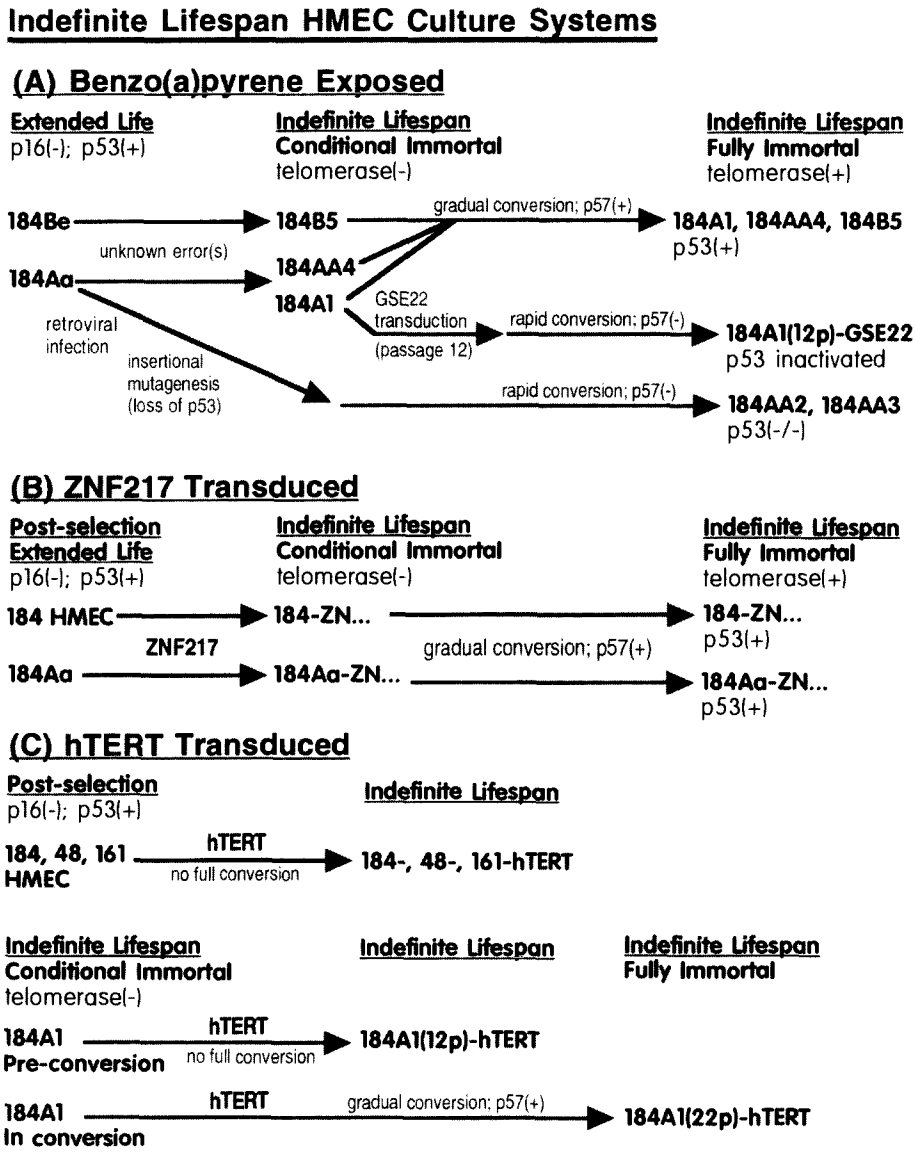


Figure 3. Schematic outline of the derivation of indefinite lifespan HMEC. A) Cultures exposed to the chemical carcinogen benzo(a)pyrene and/or the p53-inhibiting GSE, GSE22; (C) cultures transduced with the putative oncogene ZNF217; (B) cultures transduced with hTERT.

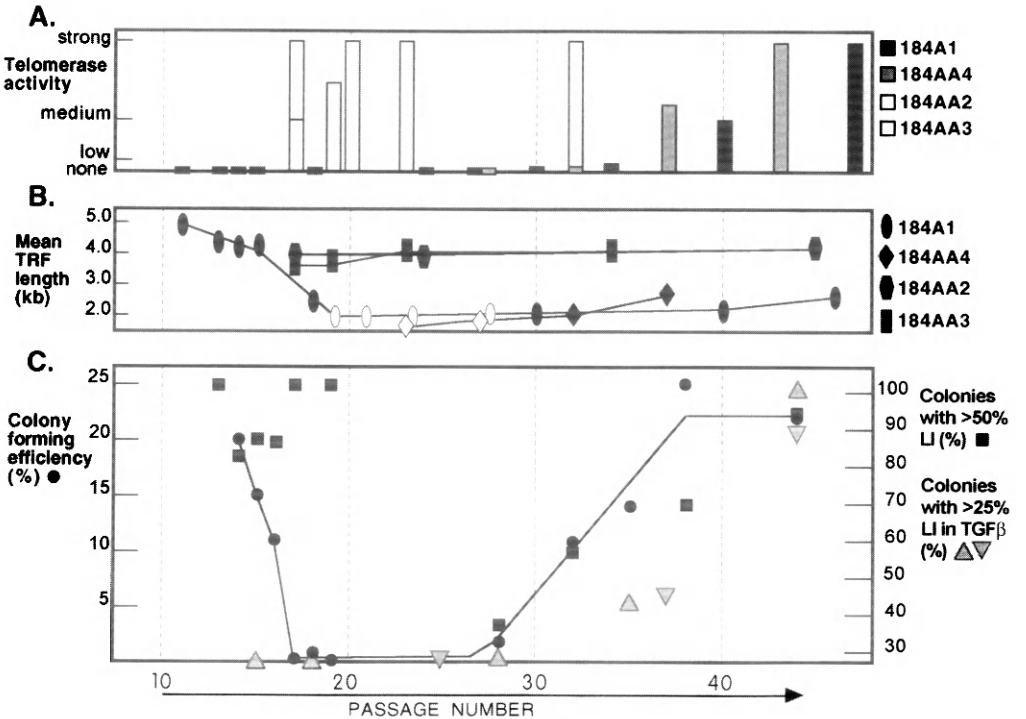


Figure 4. Telomerase activity, mean TRF length, and growth ± TGFβ, in immortal HMEC lines at different passage levels. (A) Telomerase activity in p53(+) lines 184A1 and 184AA4, and p53(-/-) lines 184AA2 and 184AA3, all derived from EL 184Aa. Telomerase activity was determined by TRAP assay as described [14]. The following categories were used to designate semi-quantitative values: none = no detectable telomerase products; low = ~10% of control 293 cells (1,000 cell equivalents); medium = 25-50% of control; strong = 75-100% of control. (B) Mean TRF length of 184A1, 184AA4, 184AA2 and 184AA3 determined as described [14]; lighter shaded symbols indicate a faint signal. (C) Growth assays in 184A1 (all data except inverted triangles) and 184AA4 (inverted triangles) determined as described [14]. Colony forming efficiency is based on colonies of > 50 cells counted 14-21 days post seeding; labeling index (LI) was determined in single cell derived colonies of >50 cells labeled for 24 hr. LI in TGFβ was determined by exposure to TGFβ for 10-14 days, prior to labeling, after the largest colonies contained 100-250 cells.

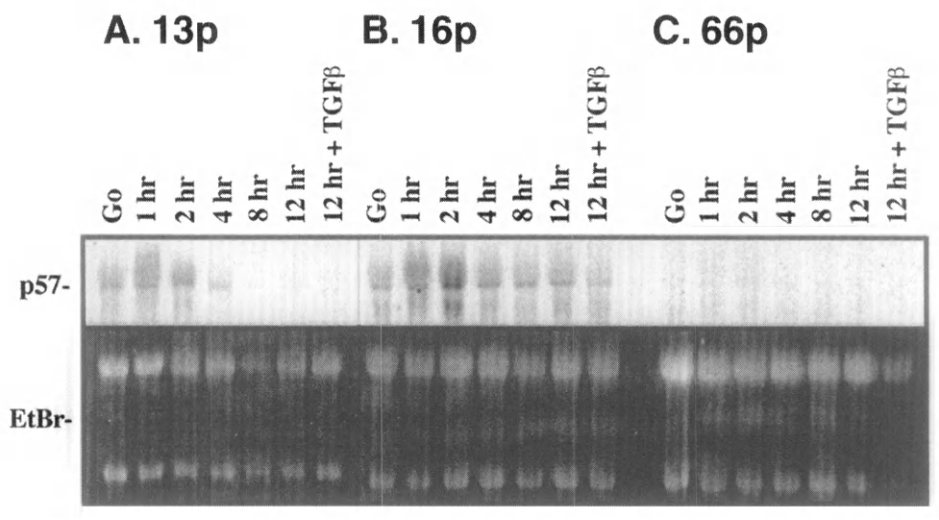


Figure 5. p57 mRNA abundance in synchronized (A) good-growing pre-conversion (passage 13) and (B) early conversion (passage 16) conditionally immortal 184A1, and (C) fully immortal (passage 66) 184A1. Total cellular RNA was prepared and analyzed as described [26]. Cells were growth-arrested in G0 by blockage of EGF receptor signal transduction, or refed with EGF for 0, 1, 2, 4, 8, 12, or 12 hrs. + TGFβ. 10μg of each sample was used for Northern blot analysis.

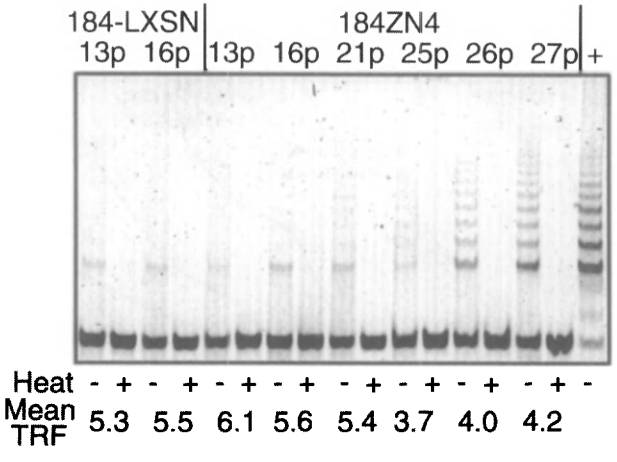


Figure 6. Telomerase activity and mean TRF length in post-selection finite lifespan 184 HMEC transduced with ZNF217 or LXSN vector alone at passage 10 [37]. Data show a gradual acquisition of telomerase activity and stabilization of telomere lengths in the ZNF217-transduced population.

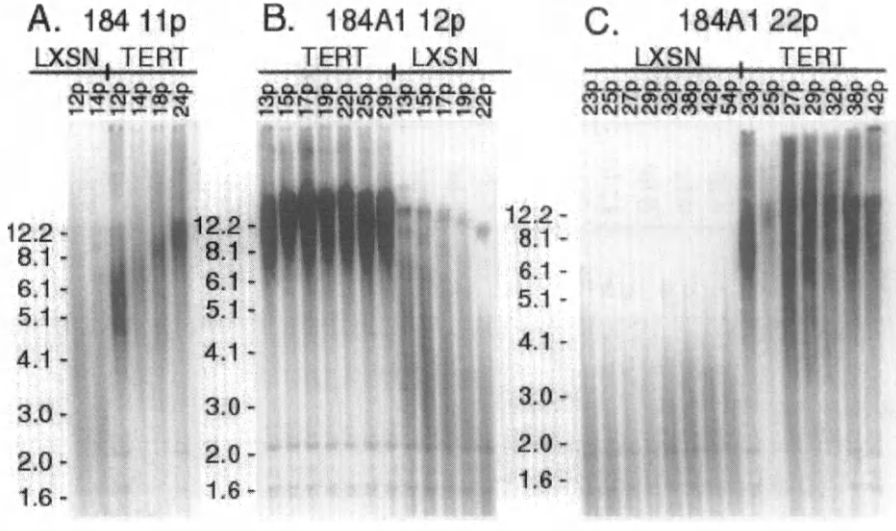


Figure 7. Ectopic hTERT causes telomeres to lengthen in normal and conditionally immortal HMEC, while telomeres become critically short in immortal HMEC undergoing conversion [32]. (A) Post-selection 184 HMEC transduced with hTERT or LXSN at passage 11. (B, C) Conditionally immortal 184A1 transduced with hTERT or LXSN at passages 12 or 22. hTERT-transduced populations show rapid telomere lengthening, while LXSN-transduced populations show the conversion-associated continued telomere erosion to a faint critically short mean TRF of ~2 kb, followed by telomere length stabilization.

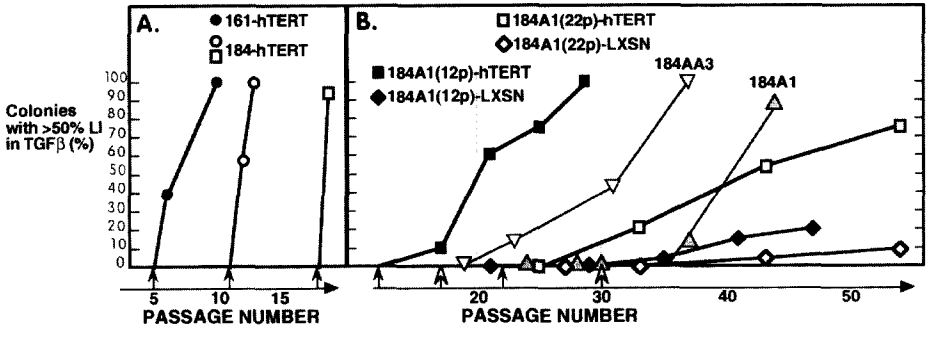


Figure 8. hTERT expression induces TGFβ resistance in (A) post-selection and (B) conditionally immortal HMEC [14, 23, 32]. Finite lifespan post-selection HMEC exhibit a rapid gain of TGFβ resistance. The hTERT-transduced conditionally immortal 184A1 and untreated 184A1, 184AA3, as well as 184AA4 and 184AA2 (not shown) exhibit a gradual gain of resistance, with similar kinetics, following telomerase expression. Closed arrows indicate passage of infection; open arrows indicate passage when telomerase activity was first detected in the uninfected lines.

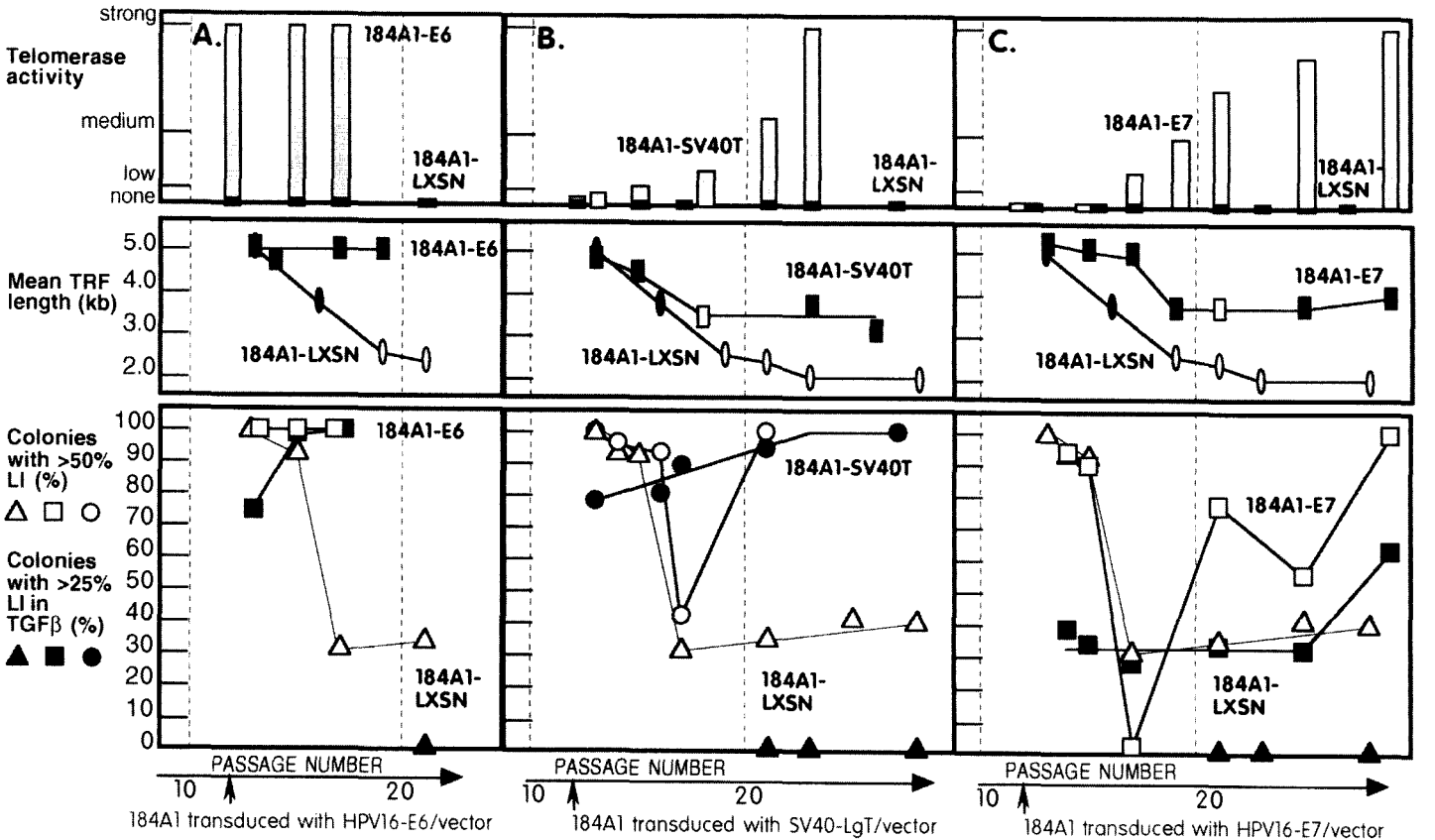


Figure 9. Effect of viral oncogenes (A) HPV16-E6; (B) SV40 LgT; (C) HPV16-E7, on telomerase activity, mean TRF length, and growth ± TGFβ of good-growing pre-conversion conditionally immortal 184A1 transduced at passage12. Methods as described for Figure 4 and references [7, 14].

DNA DAMAGE

Oxidative stress
- **hydroxyl radical**
- **superoxide**
- **peroxynitrite**

Toxins
- **alkylating agents**
- **nitrosamines**
- **mycotoxins**

Enzyme dysfunction
- **DNA polymerase**
- **topoisomerase**
- **helicase**
- **endonuclease**

DNA PROTECTION

Direct
- **histones**
- **telomeric DNA**

Indirect
- **antioxidants**
- **antioxidant enzymes**
- **heat-shock proteins**

DNA REPAIR

Mismatch
Nucleotide excision
Base excision
Direct reversal

PARP
p53
Telomerase?

Figure 1. Examples of mechanisms of DNA damage, protection and repair.

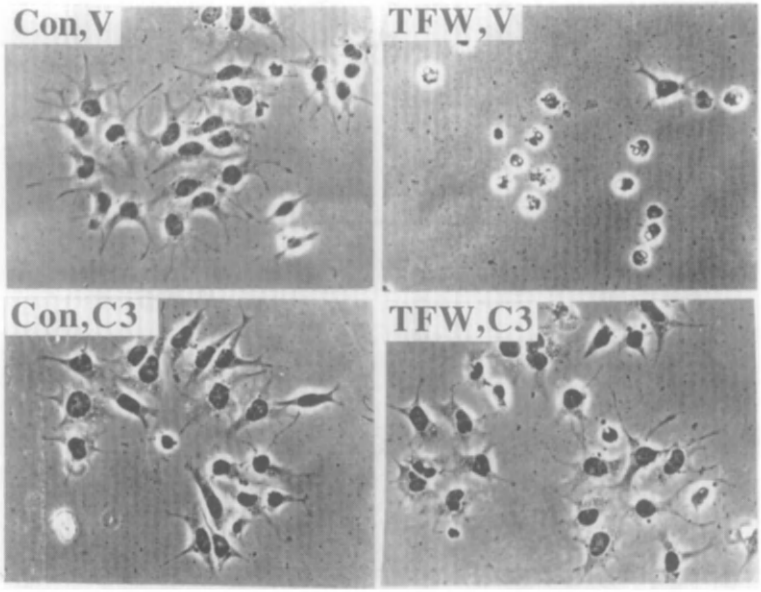
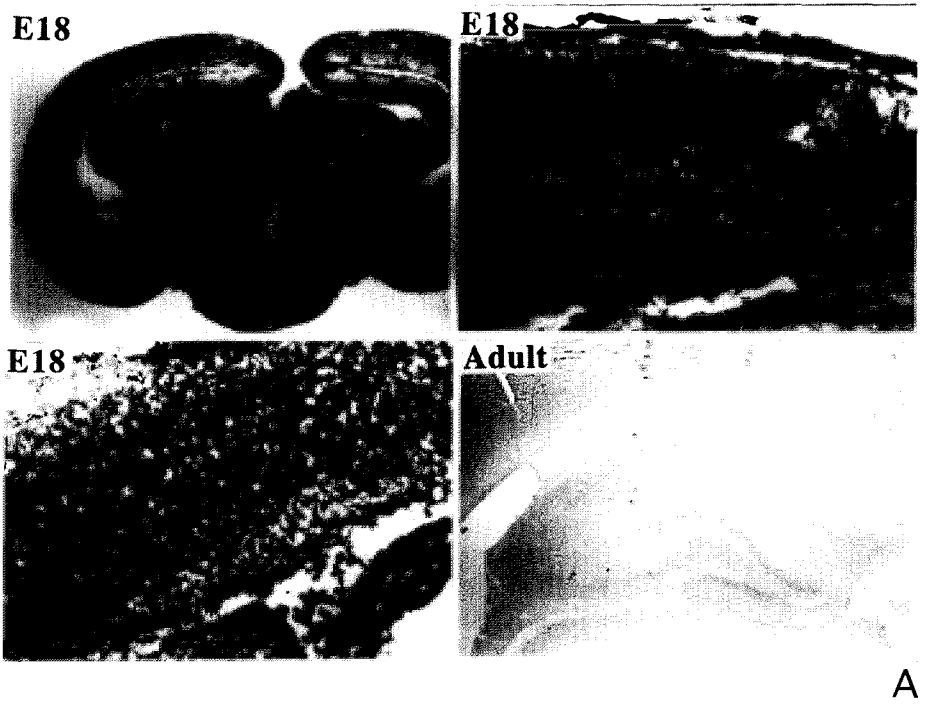


Figure 2. Cells overexpressing TERT exhibit increased resistance to apoptosis. Phase-contrast micrographs of control vector-transfected PC12 cells (V) and a clone of PC12 cells overexpressing TERT (C3) under basal culture conditions (Con) and 24 hours after trophic factor withdrawal (TFW). Note that the vast majority of control cells underwent apoptosis, while the cells overexpressing TERT were resistant to apoptosis.



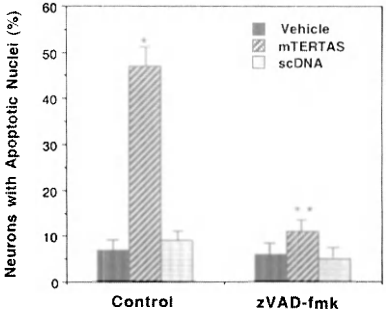


Figure 3. TERT is expressed in neurons in the developing brain and promotes their survival. (A) Sections of brains from an embryonic day 18 rat (upper) and an adult rat (lower) were immunostained with an antibody against TERT. Intense immunoreactivity is present in neuronal progenitor cells and differentiated neurons throughout the brain including the cerebral cortex, hippocampus (arrowhead). By comparison, TERT immunoreactivity is negligible in the adult brain. (B) Cultured mouse embryonic hippocampal neurons were exposed to saline (vehicle), TERT antisense DNA (mTERTAS) or scrambled control DNA (scDNA) in the absence or presence of the caspase inhibitor zVAD-fmk. Three days later the percentage of neurons undergoing apoptosis was determined. Values are the mean and SEM (n=4 cultures); *$p<0.001$ compared to corresponding control value for vehicle- and scDNA-treated cultures. **$p<0.001$ compare to control value for cultures treated with mTERTAS.

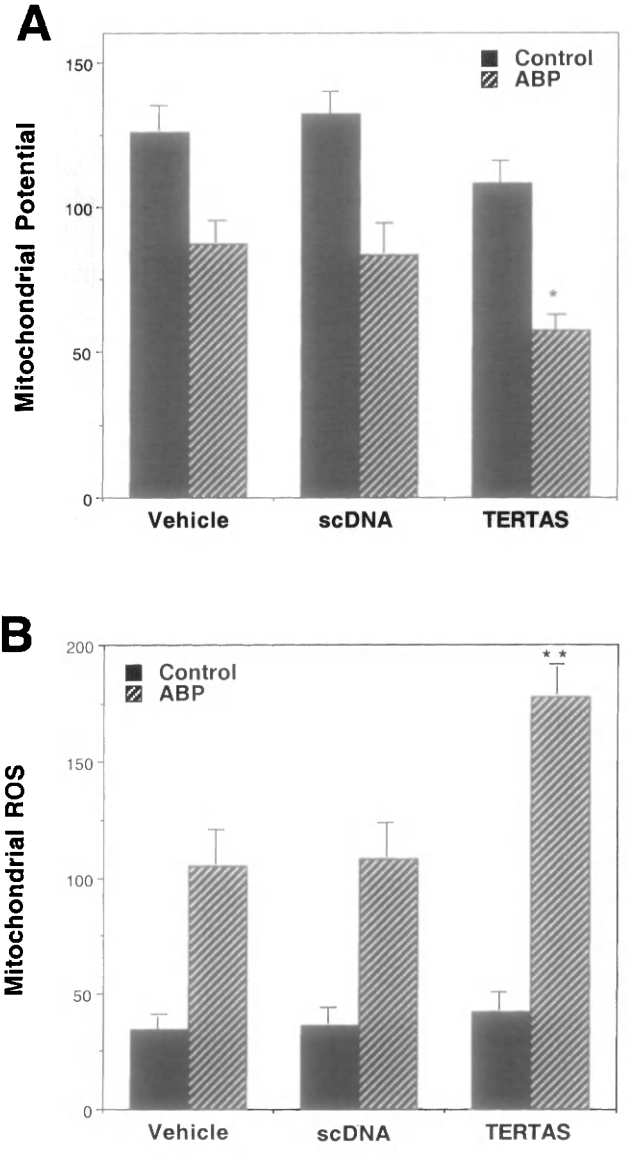


Figure 4. TERT reduces oxidative stress and stabilizes membrane potential in mitochondria of embryonic mouse hippocampal neurons exposed to amyloid b-peptide. Cultured neurons were incubated for 24 hours in the presence of vehicle (saline), TERT antisense DNA (TERTAS) or scrambled control DNA (scDNA). Cultures were then exposed to saline or amyloid β-peptide (ABP) for 8 hours and levels of rhodamine 123 fluorescence (A, a measure of mitochondrial membrane potential) or dihydrorhodamine fluorescence (B, a measure of mitochondrial hydroxyl and peroxynitrite levels) were quantified. Values are the mean and SD of determinations made in 4 cultures (40-55 neurons evaluated/culture). *p<0.05, **p<0.01 compared to corresponding values for cultures pretreated with vehicle or scDNA and then exposed to ABP.

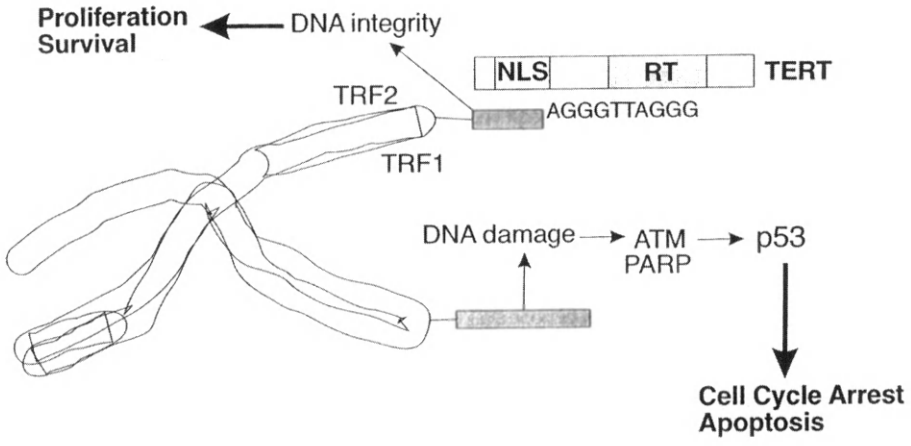


Figure 5. Working model for modulation of DNA damage-induced apoptosis by TERT. DNA damage activates PARP and the ATM kinase resulting in p53 upregulation and activation. p53 promotes apoptosis by upregulating Bax expression and facilitating its association with mitochondrial membranes resulting in mitochondrial membrane permeability changes, release of cytochrome c and caspase activation. TERT may suppress apoptosis by preventing DNA damage and by stabilizing mitochondrial function.

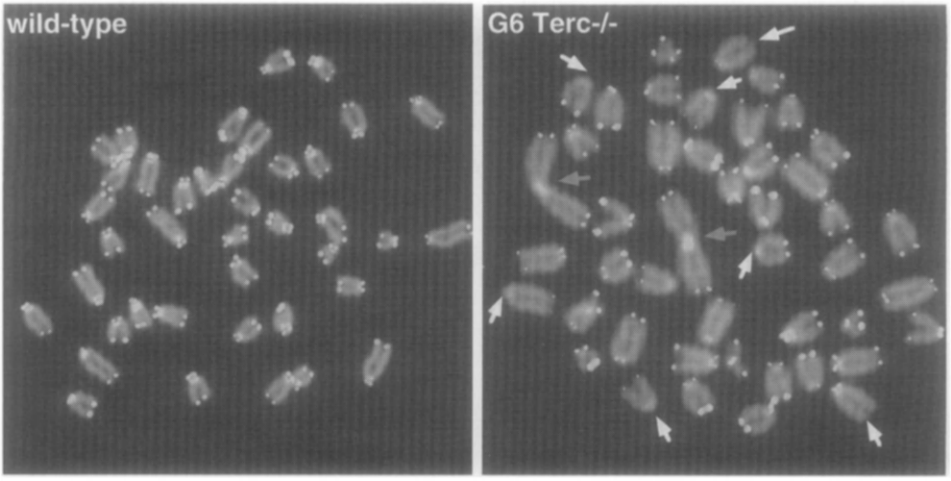


Figure 1. Telomere shortening and chromosomal instability in late generation Terc$^{-/-}$ primary cells. Representative telomeric FISH images of metaphases from wild-type and late generation Terc$^{-/-}$ MEF. Blue color: DAPI staining of chromosomes. Yellow dots: telomeric repeats. White arrows point to telomeres with undetectable TTAGGG repeats. Red arrows indicate end-to-end fusions. Note that the 2 end-to-end fusions in the Terc$^{-/-}$ metaphase lack telomeres at the fusion point. G6, sixth mouse generation.

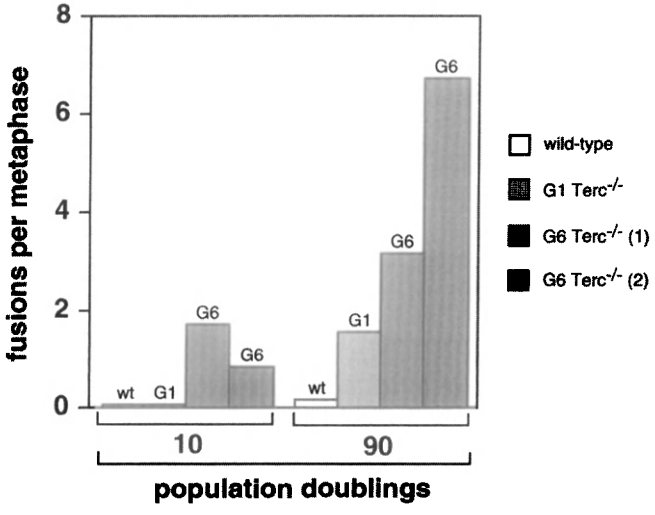


Figure 2. Chromosomal instability in late generation Terc$^{-/-}$ cells. Increased chromosomal aberrations in Terc$^{-/-}$ cells with increasing population doublings as compared with wild-type cells. G1, first generation; G2, second generation; G4, fourth generation and G6, sixth generation.

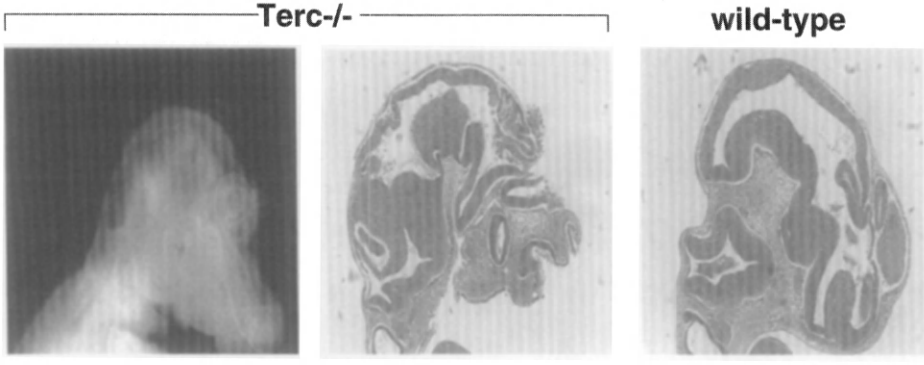


Figure 3. Neural tube defects in late generation Terc$^{-/-}$ mice. Images wild-type and late generation Terc$^{-/-}$ embryos at day 10.5 of embryonic development. The Terc$^{-/-}$ embryo shown has the neural tube opened at the forehead. The open neural tube can be also visualized with histological sections of the same embryo. As control, it is shown a similar section corresponding to the head of a wild-type embryo which shows the neural tube closed at day 10.5.

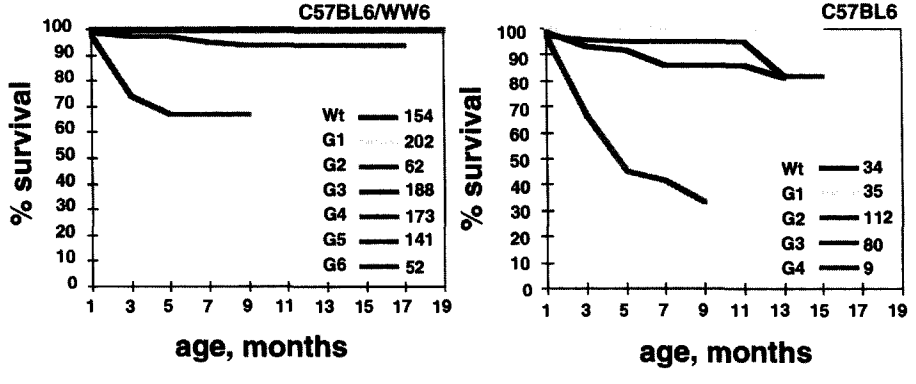


Figure 4. Decreased viability of late generation Terc$^{-/-}$ mice in two different genetic backgrounds. Late generation Terc$^{-/-}$ mice in two different genetic backgrounds (the original background, C57BL6/WW6, and a pure C57BL6 background) show decreased viability with age. This decreased viability was more dramatic in the late generation Terc$^{-/-}$ mice in the pure C57BL6 mice. 50% of the G4 Terc$^{-/-}$ mice died at 5 month of age.

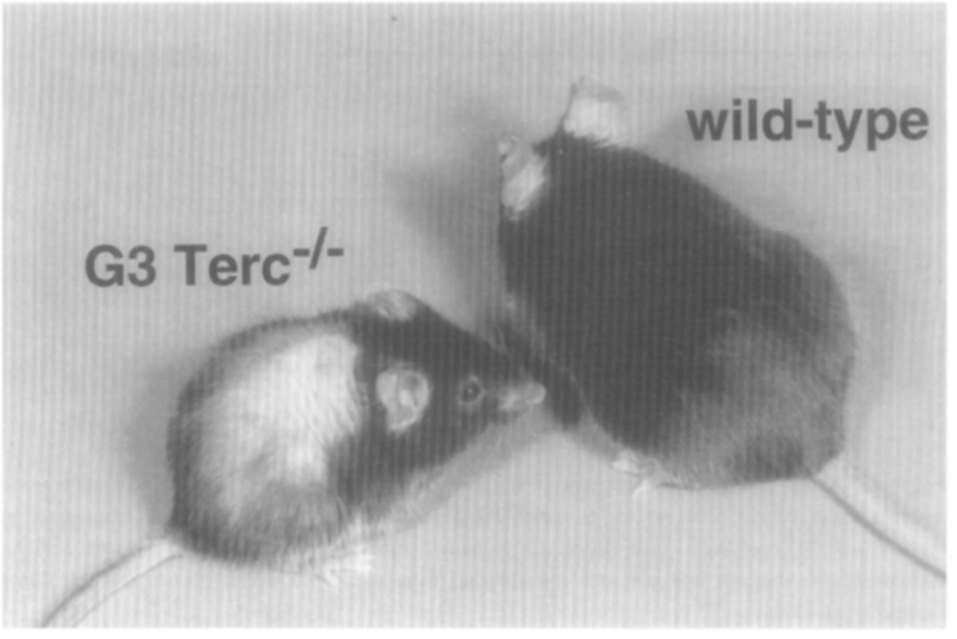


Figure 5. A diseased late generation Terc$^{-/-}$ mouse. Representative images of a wild-type mouse and a late generation C57Bl6 Terc$^{-/-}$ mouse showing signs of disease. Notice allopecia, hair graying, huntched position, small size of the Terc$^{-/-}$ mouse as compared with an age-matched wild-type.

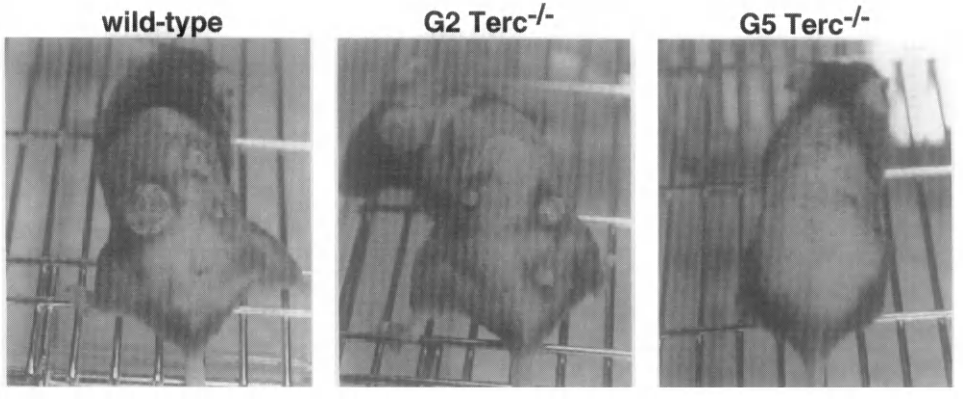


Figure 6. Reduced skin tumors in late generation Terc$^{-/-}$ mice. Reprentative images of skin papillomas in wild-type, early generation (G2, second generation) and late generation (G5, fifth generation) Terc$^{-/-}$ mice after skin carcinogenesis (DMBA + TPA treatment). Late generation Terc$^{-/-}$ mice we resistant to carcinogens, suggesting that short telomeres prevent tumor formation in the skin.

FIGURE SECTION FOR CHAPTER 9

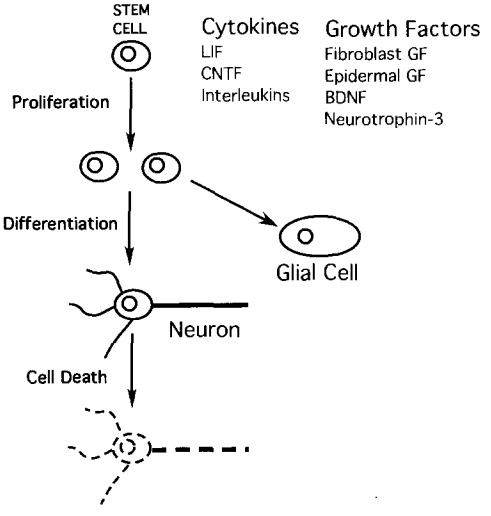


Figure 1. Mechanisms regulating the proliferation, differentiation and survival of neural progenitor cells. Progenitor cells can divide and differentiate into neurons or glia, and they may undergo apoptosis either prior to or after differentiation. Examples of signals known to control the processes of proliferation, differentiation and cell survival are listed.

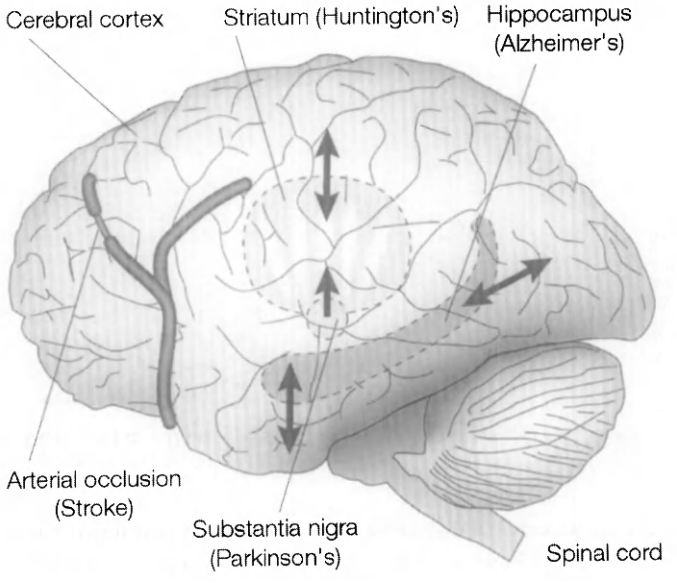


Figure 2. The most prominent neurodegenerative disorders and the regions of the nervous system most severely affected. See text for discussion.

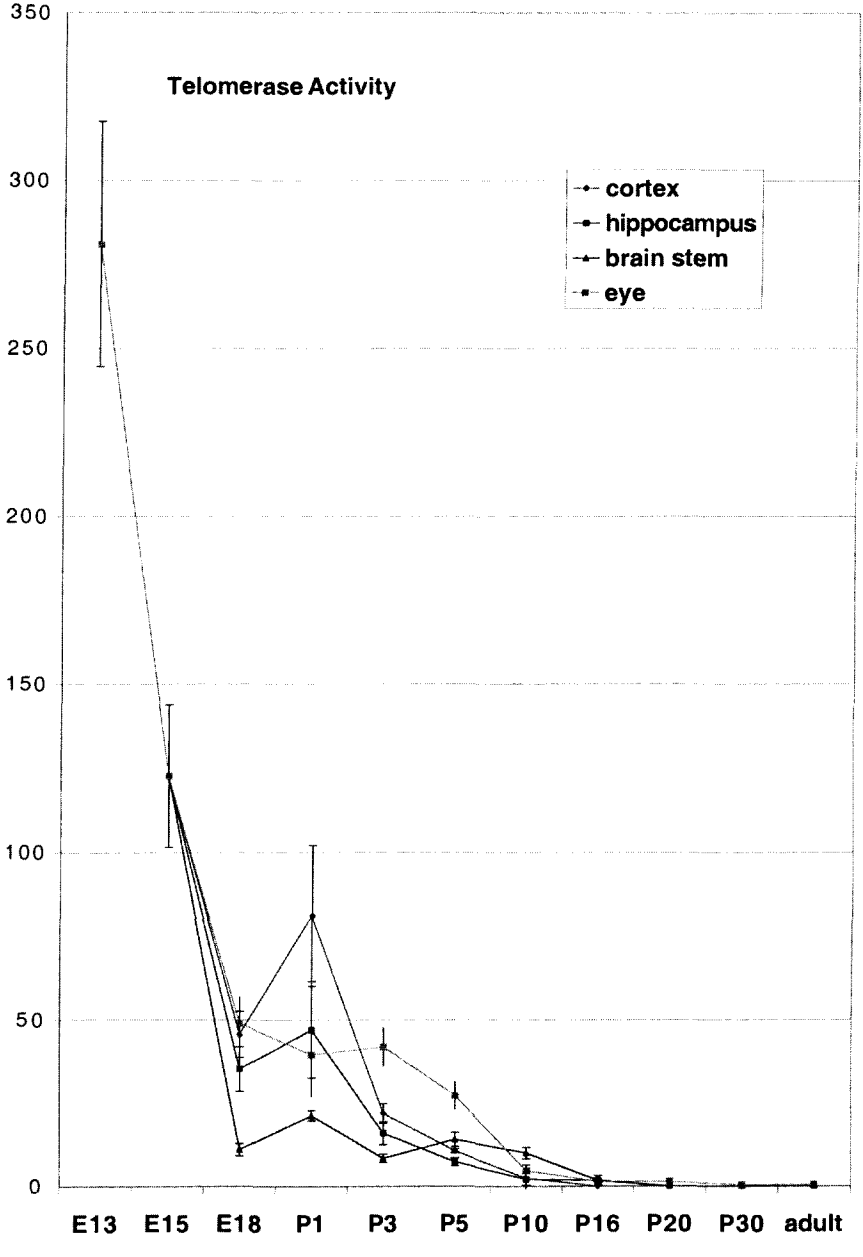


Figure 3. Levels of telomerase activity decrease rapidly during the early period of mouse brain development. Telomerase activity levels were quantified by a capillary electrophoresis-based TRAP protocol in tissue samples from the indicated brain regions and the eye of developing mice of the indicated embryonic (E) and postnatal (P) ages.

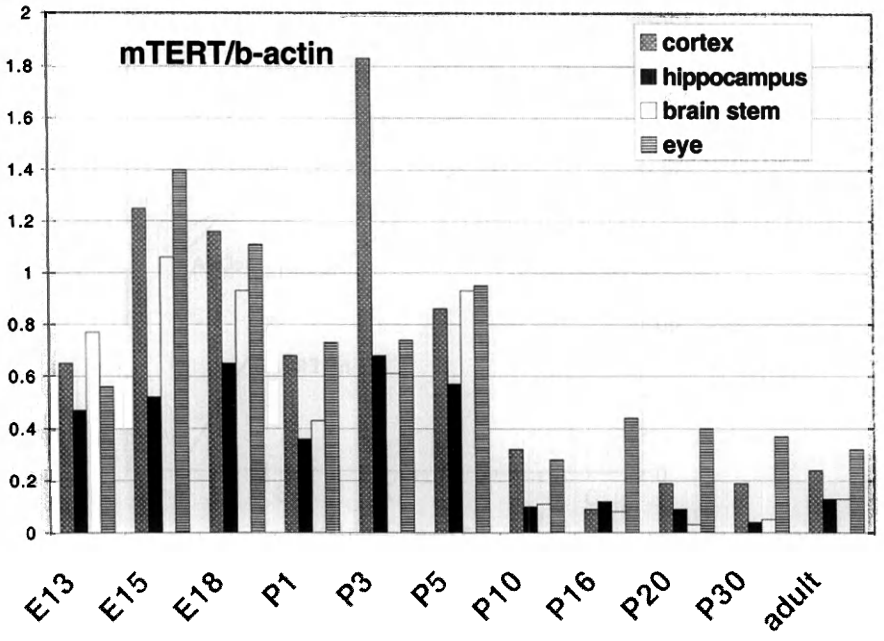


Figure 4. Developmental profile of expression of TERT in mouse brain. Levels of TERT mRNA (determined by RT-PCR analysis) were measured in cortical, hippocampal, brainstem and eye tissue from mice of the indicated embryonic (E) and postnatal (P) ages. Note that whereas telomerase activity declines sharply during embryonic development (Figure 3), TERT mRNA levels remain high well into the postnatal period.

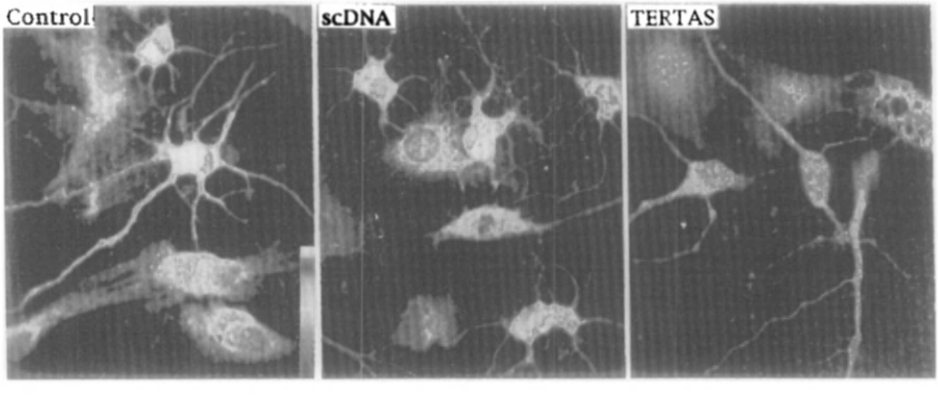


Figure 5. TERT is present at high levels in differentiated embryonic mouse hippocampal neurons in culture and is localized in both the nucleus and cytoplasmic compartments. Confocal laser scanning microscope images showing TERT immunoreactivity in neurons (cells with long, thin neurites) and astrocytes (flat cells). Levels of immunoreactivity are depicted in pseudocolor according to the scale bar shown. Cultures had been left untreated (Control) or had been treated for 24 hours with either scrambled control DNA (scDNA) or TERT antisense DNA (TERTAS). Note that levels of TERT immunoreactivity were greatly decreased in cells that had been treated with TERT antisense DNA.

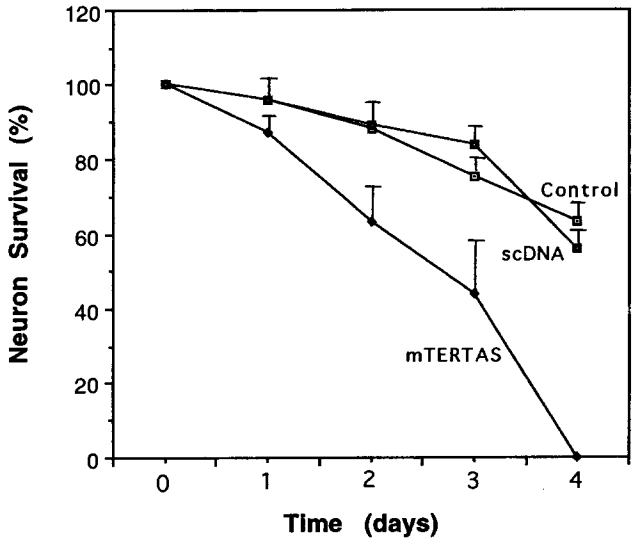


Figure 6. Suppression of TERT expression induces apoptosis of embryonic brain neurons in culture. Cultures of mouse hippocampal cells were exposed to saline (Control), 25 μM mTERT antisense DNA or 25 μM scrambled DNA (scDNA). The percentage of neurons remaining alive was determined at 1 day intervals.

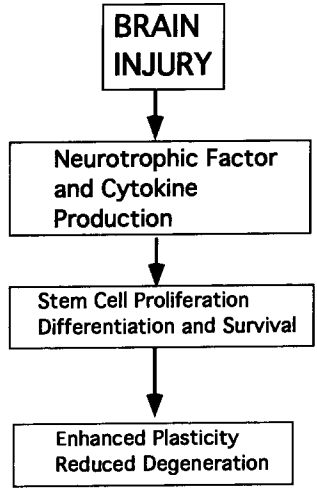


Figure 7. Possible roles for telomerase in cellular responses to brain injury. Brain injury (which may result from trauma, a stroke or chronic neurodegenerative disorders) results in production of trophic factors and cytokines which can affect the survival of neurons, and can also stimulate the proliferation, differentiation and/or survival of neural progenitor cells. TERT expression may be upregulated in neural cells in response to injury, and may in this way promote their proliferation and/or survival.

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