MINIREVIEW

Replicative Senescence and Cell Immortality: The Role of Telomeres and Telomerase (44075)

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Abstract. **Telomere shortening is correlated with cell senescence *in vitro* and cell aging *in vivo*. The telomere hypothesis suggests that telomere length serves as a mitotic clock for timing cellular replicative life span. Expression of telomerase stabilizes telomere length and allows for continual replication, or cell immortality. This article reviews recent evidences for the role of telomere length and telomerase in the regulation of cellular replicative life span. The therapeutic potential of manipulating telomerase expression and telomere length is also discussed.** [P.S.E.B.M. 1997, Vol 214]

Hayflick and Moorhead (1) have observed that normal human fibroblasts grow for only a limited number of cell divisions in culture before they become senescent. The finite replicative life span *in vitro,* or the Hayflick limit, has since been demonstrated for many other somatic cell types such as keratinocytes, endothelial cells, and lymphocytes (2) and generally ranges between 50 and 100 population doublings. Senescence at the Hayflick limit is characterized by the withdrawal from cell cycle, chromosomal instability, and various morphological and biochemical changes. Furthermore, an altered pattern of gene expression has been reported between young and senescent cells (2–6). Notably, these post-mitotic senescent cells remain metabolically viable for extended periods of time provided that appropriate growth conditions are maintained (7).

Accumulating evidences suggest that *in vitro* replicative senescence has biological significance in *in vivo* aging. The cellular replicative life span decreases with increasing age of the donors, presumably reflecting an increased number of cell divisions occurring with age (8). Cells derived from different species have a Hayflick limit that is correlated with the species longevity (9, 10). Furthermore, cells from patients with accelerated aging syndromes (e.g., progeria, Werner's syndrome, and Trisomy 21) have a significantly reduced replicative capacity compared with those from age-matched controls (11, 12). These results suggest the presence of a genetic mitotic clock which counts the number of cell divisions rather than chronological or metabolic age (13). Perturbations in this clock may contribute to the pathologies associated with certain diseases. In addition, a similar pattern of gene expression has been observed in senescent cells and cells aged *in vivo* (4, 14, 15), suggesting the presence of senescent cells in aging tissues. The pattern of gene expression from these senescent cells may contribute to a variety of chronic diseases of the elderly.

The molecular mechanism(s) for regulating replicative senescence is still unknown. However, the onset of senescence can be delayed and in rare cases, cells may eventually escape from cell cycle control completely and become immortalized. A two-phase cell cycle checkpoint model has been proposed to explain this phenomenon (16) (Fig. 1). At mortality phase 1 (M1), or the Hayflick limit, cells are signaled to withdraw from cell cycle and enter replicative senescence. *In vitro* transformation with viral oncogenes (e.g., SV40 large T antigen, adenovirus E1A, and HPV E6 and

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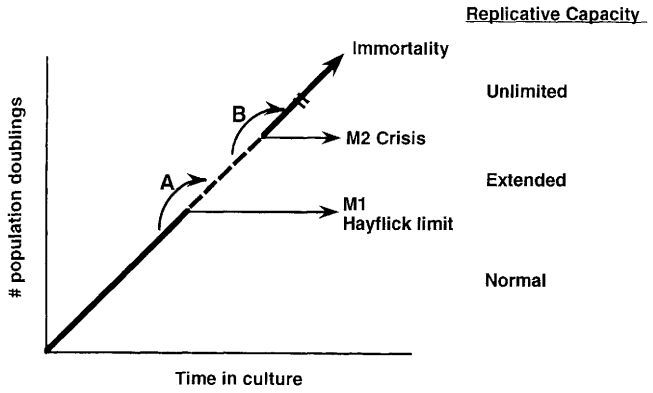

Figure 1. M1 and M2 cell cycle checkpoints. At the mortality phase 1 checkpoint (M1 or Hayflick limit), normal human somatic cells enter into replicative senescence and stop dividing. Mutations or transformation events (A) allow cells to escape from M1 and acquire an extended life span. These cells eventually undergo crisis at mortality phase 2 (M2) and rare mutational events (B) allow a few clones to escape the M2 checkpoint and become immortalized.

E7), presumably mediated by tumor suppressor genes such as p53 and RB, allows cells to bypass M1 and acquire an extended life span (16,17). The extended life span is not unlimited, as it eventually ends in mortality phase 2 (M2), or crisis, which is usually associated with unstable chromosomes and significant cell death. Rarely, immortal clones will emerge from crisis, presumably as a result of somatic mutation(s).

Telomeres and Telomerase

Eukaryotic chromosome ends are capped by telomeres, which in the vertebrates consist of repeated sequences of TTAGGG. Telomeres have been demonstrated to be important in maintaining chromosomal stability and may also be involved in attachment of chromosomes to the nuclear matrix (18, 19).

Experimentally, telomeres can be visualized by fluorescent *in situ* hybridization (FISH) using a telomeric sequence specific probe (20, 21). For more quantitative assessment of telomere length, the standard assay has been the measurement of terminal restriction fragment (TRF) sizes using Southern analysis (22–24). TRFs typically contain a subtelomeric region in addition to the telomeric region containing the terminal TTAGGG repeats. The length of telomeric and subtelomeric sequences apparently varies among different chromosomes within the same cell or even among the same chromosome in different cells. Thus, hybridization of the TRFs using telomeric probe generates a smear from which a mean value (mean TRF length) can be calculated.

Telomerase is a ribonucleoprotein complex capable of synthesizing the telomeric repeat sequence *de novo* using its RNA component as a template (Fig. 2). The enzyme activity was first identified in Tetrahymena and later in other species, including ciliates, yeast and human (26–30). The conventional assay for telomerase activity detects the incorporation of radioactive nucleotides into TTAGGG repeats at the 3′ end of a single strand DNA oligonucleotide substrate.

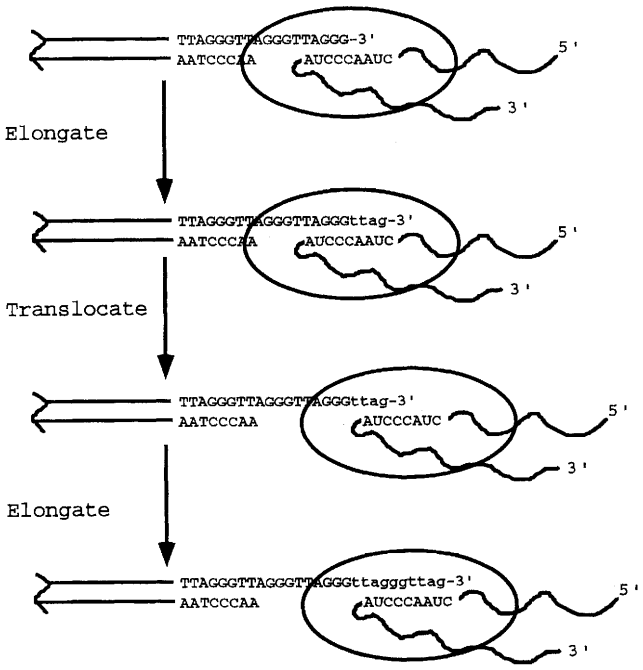

Figure 2. Model of processive telomerase action. The putative template domain of the RNA component of human telomerase is shown aligned against an arbitrary 3′ end of a human telomere. This primer-template configuration allows extension of the telomere (lowercase letters) in the first round of elongation until the extended product reaches the 5′ end of the template domain. Translocation then moves the extended DNA back one repeat relative to the template domain, positioning it for another round of elongation in which a full repeat (ggttag) is added to the 3′ end of the chromosome. (Adapted from Ref. 25.)

Recently, a PCR-based telomerase assay, Telomeric Repeat Amplification Protocol (TRAP), has been developed, and its increased sensitivity allows the detection of low levels of telomerase even down to a single cell level (31, 32).

The Telomere Hypothesis

Olovnikov (33) and Watson (34) independently suggested that in the absence of special mechanisms, there would be incomplete replication of linear chromosomes because DNA polymerase requires a labile primer to initiate DNA synthesis. This creates the end replication problem which predicts that the 5′ end of the daughter strands will shorten at each cell division (Fig. 3). In the absence of any compensating mechanisms, then, there would be a net loss of terminal chromosomal DNA as a function of replicative aging. In most immortal eukaryotic cells, telomerase functions as the compensating mechanism to maintain telomere length.

The telomere hypothesis of cell aging and immortalization (Fig. 4) suggests that telomere shortening in the absence of telomerase is the mitotic clock for replicative senescence in normal somatic cells (24, 37). The number of cell divisions is registered by the gradual loss of telomeric sequences. As the telomere(s) shortens to a critical length, signals are sent to the cell to exit from cell cycle (M1). The

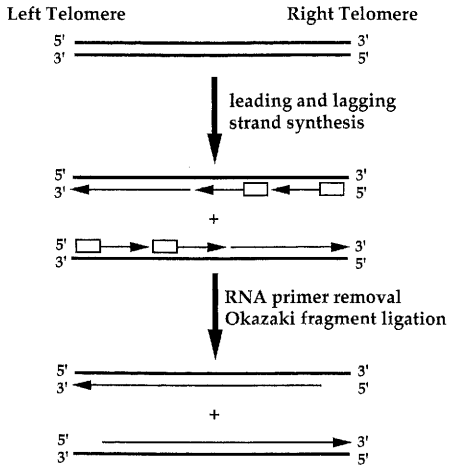

Figure 3. The end-replication problem. Replication of a linear parent duplex (heavy lines) is shown. Lagging strand synthesis is discontinuous, consisting of Okazaki fragments which initiate with a labile RNA primer (box). After RNA primer removal and Okazaki fragment extension and ligation, the most 5′ Okazaki fragment will remain incomplete since the RNA primer cannot be replaced. If the 5′-terminal Okazaki fragment does not initiate directly opposite the 3′ end of the template DNA, there will be additional bases incompletely replicated. (Adapted from Ref. 35.)

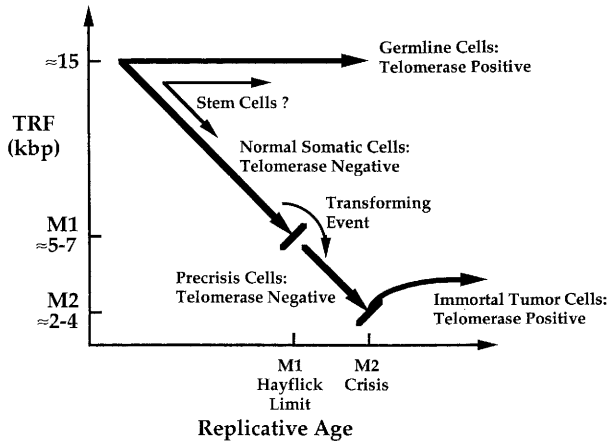

Figure 4. The telomere hypothesis of cell aging and immortalization. Telomerase is active in the germ line, maintaining long stable telomeres, but is repressed in most normal somatic cells, resulting in telomere loss in dividing cells. At M1 (the Hayflick limit), there is presumed critical telomere loss on one or perhaps a few chromosomes signaling irreversible cell cycle arrest. Telomerase activity and telomere length are not known for true somatic stem cells. Transformation events may allow somatic cells to bypass M1 without activating telomerase. When telomeres become critically short on a large number of chromosomes, cells enter crisis (M2). Rare clones that activate telomerase escape M2, stabilize chromosomes, and acquire an indefinite growth capacity. (Adapted from Ref. 36.)

M1 cell cycle checkpoint can be bypassed (e.g., by viral transformation), and cells continue to divide with further decrease in telomere length. At crisis (M2), telomerase is reactivated, possibly as a result of loss of chromosomal integrity. This allows the cells to maintain stable telomeres, bypass crisis, and acquire unlimited replicative capacity.

Telomere Dynamics and Cell Immortality

In the telomere hypothesis, telomere length serves as a biomarker and possible causal determinant of replicative capacity. Experimental evidence demonstrates that telomere shortening is correlated with cell replication and a common telomere length is associated with replicative senescence. A decrease in the mean TRF lengths has been observed with increasing number of population doublings *in vitro* and with aging *in vivo*. This is true for many different cell types, including fibroblasts, keratinocytes, peripheral blood leukocytes, mucosal epithelial cells, and candidate hematopoietic stem cells (12, 24, 38–41). In general, mean TRF lengths for normal human somatic cells range from ~5 to 11 kb, and this value decreases at an average rate of 30–200 bp/cell doubling *in vitro* and 10–50 bp/year *in vivo*. Indeed, this telomere shortening is largely dependent on cell division both *in vitro* and *in vivo* (42), implicating that *in vivo* aging of tissues is associated with a gradual exhaustion of cell replicative capacity. Significantly, the initial mean TRF length of a cell population is found to be a better predictor of the remaining replicative capacity than donor age (40), and there appears to be a common TRF of about 5–8 kb in senescent cultures and in cells obtained from centenarians (12, 24, 43).

In syndromes characterized by premature or accelerated aging of various tissues such as Hutchinson-Gilford progeria and Down's syndrome, mean TRF length in fibroblasts or lymphocytes is shown to be shorter than that of age-matched controls (40) or to decrease at a faster rate (12), respectively. Agents that cause DNA damage (e.g., oxidative damage) have been shown to induce cell cycle arrest similar to replicative senescence (44). A link between rapid telomere loss and experimentally induced senescence has been reported recently by von Zglinicki *et al.* (45). Mild oxidative stress induces a "senescent" state in fibroblasts and the telomere length in these growth arrested cells approximates that in cells at normal senescence. At the same time, the apparent rate of telomere loss is calculated to increase more than 5-fold (from 90 bp/doubling to 500 bp/doubling) in cultures under hyperoxia. Thus, regardless of how telomeres are lost, the senescence state appears to be associated with a critically short telomere length.

Conversely, a stably maintained telomere length has been highly correlated with the immortal phenotype. Consistent with the M1/M2 model, normal somatic cells such as human embryonic kidney cells show a decrease in mean TRF length with *in vitro* replication (46). Transformation of these cells with SV40 large T antigen or transformation of B lymphocytes with EBV extended cellular lifespan while their mean TRF lengths continue to decrease. The appearance of immortal clones following crisis in these cells is associated with stabilized, albeit much shorter, telomere length (46). Similarly, reduced telomere lengths are found in many tumor derived tissues and immortal transformed cells (46–51), and, for some immortal cell lines, stable telomeres are shown to be maintained with continued replication (48, 52). Furthermore, in the essentially immortal germ

line tissues such as testes, the telomeres remain long and stable with *in vivo* age (40).

Several recent reports have described abnormally/extremely long mean TRF lengths (>20 kb) associated with certain tumors and transformed cells (53–57). In addition, the telomere dynamics of specific chromosomes have been shown to fluctuate substantially with cell replication (55). The biological implications of these results are not clear at this point. However, it should be emphasized that TRF measurements include both TTAGGG and non-TTAGGG repeats at and close to the telomeric region and it is possible that the size of the most distal block of TTAGGG sequence may be of critical importance. A more detailed analysis of telomere structure and telomere dynamics at the single cell level would be required to further our understanding of the role of telomeres in regulating cell immortality.

Telomerase and Immortality

The presence of telomerase to maintain stable telomere length, according to the telomere hypothesis, would be necessary for maintaining the immortal phenotype. This has been substantiated by the wealth of evidence correlating telomerase expression with cell immortality. Simple eukaryotes such as Tetrahymena, ciliates, and yeast have unlimited replicative capacity, and they express detectable telomerase activity (58). In humans, germ line cells from the reproductive tissues such as testes are essentially immortal and have long and stable telomeres with increasing age (40). Telomerase activity has been detected in both testes and ovaries (31, 32). In addition, telomerase activity has been found in association with most immortal cells and tumor tissues (Table I), whereas no or a very low level of telomerase is present in normal cells and tissues. Specifically, telomerase activity appears to be more frequently detected in late stage tumors when the temporal pattern of telomerase expression is analyzed in hematological tumors, gastric cancer, and colorectal carcinoma (63, 69, 71). This is consistent with a two-stage tumor progression model in which cells initially escape from the normal growth control mechanisms but are not yet immortal. With subsequent changes/mutations, the cancer cells become immortalized and telomerase is activated. More thorough analysis of telomerase expression in individual tumor types at different stages of cancer development and correlation of the clinical outcome would provide insights into the role of telomerase in tumor progression and potential use of telomerase as a diagnostic/prognostic marker for cancer.

Bryan *et al.* (56) and Rogan *et al.* (57) have recently reported the absence of detectable telomerase activity in several immortal cell lines. All of these cells, however, exhibited abnormally long telomeres with mean TRF lengths of up to 50 kb, and in at least one cell line there was no detectable change in telomere length over 100 population doublings. However, it should be noted that the resolution on pulsed-field gel electrophoresis may not be sensitive enough to detect small changes in TRF sizes given that the estimated loss of TRF length can be as low as ~50 bp per cell division. Notwithstanding, the lack of telomerase in these cells suggests that rare alternative mechanisms exist (e.g., by recombination) that would allow for the generation of extremely long TRF, which is apparently necessary and sufficient for the maintenance of the immortal phenotype.

The requirement for telomerase in maintaining cell immortality has been clearly demonstrated in Tetrahymena and yeast, in which the RNA component of telomerase has been cloned (25, 72). Mutations introduced into the telomerase RNA component leads to telomere instability and a senescent phenotype, indicating that telomere length maintenance *via* a functional telomerase is necessary for cell viability and the immortal phenotype (72–74).

The telomerase RNA component for mouse and humans has recently been cloned (75, 76). The biological function of telomerase in human cells was investigated by expressing an antisense construct to the human telomerase RNA in the immortal HeLa cells. Similar initial growth rates in both the antisense transfected cells and the control cells suggest that there is no acute cytotoxicity from the antisense construct. However, cell death in the antisense transfected cultures occurs quite abruptly after about 20 cell divisions and is associated with significantly reduced mean TRF length compared with that in the controls. Thus, for the first time inhibition of a mammalian telomerase has been shown to cause telomere shortening and cell death in an otherwise immortal cell population.

In most strains of mice, changes in telomere length are difficult to measure due to the large size of the TRFs and the presence of other $(TTAGGG)_n$-containing fragments. In addition, telomerase activity has been detected in many normal mouse tissues, such as liver, kidney, spleen, and mammary tissues (77, 78). The more ubiquitous telomerase expression may explain why rodent cells can be more readily transformed *in vitro* and more susceptible to cancer *in vivo* (59). Similar to the human system, however, telomerase activity is found to be elevated in murine tumor tissues as compared with adjacent normal tissues (78), suggesting that a quantitative correlation exists with cell immortality. With the recent cloning of the mouse telomerase RNA component (75), it is now possible to generate telomerase "knock-out" mice and examine the effect of null telomerase expression on replicative potential and the frequency of cancer incidence in an *in vivo* model.

Telomerase Regulation in Normal Cells

As mentioned above, most normal human somatic tissues and cell strains do not express detectable telomerase activity. With increasing sensitivity afforded by the TRAP assay, low levels of telomerase activity have been detected in various fetal tissues and in normal bone marrow cells (32, 60, 63–65). Since bone marrow contains the hematopoietic stem cells which might have self-renewing capability and extensive replicative potential, telomerase expression in the bone marrow may be due to the rare stem cells in this tissue.

Table I. Telomerase and Telomere Dynamics in Human Cells and Tissues

Source	Phenotype	Tissue origin	Telomere dynamics	Telomerase activity[a]	References
Cultured cells	Mortal (dividing)	Various (skin, lung, vascular, hemopoietic, ovary)	Shorten or n.t.[b]	0/25	12, 24, 40, 48, 35
		Hematopoietic/blood	n.t.	7/7[c]	59, 60
	Mortal (nondividing)	Connective (skin, lung)	Stable	0/3	42
	Mortal (extended life span)	Emb. kidney	Shorten		
		Connective	Shorten	0/5	46, 48
		Blood	Shorten		
	Immortal (transformed lines)[d]	Various (lung, kidney, prostate, retina)	Stable or n.t.	8/10	31, 46, 48, 61
		Fibroblasts	Extra long or n.t.	20/35	56
	Immortal (tumor lines)	Various (14 different tissue origins)	Stable or n.t.	94/94	31, 48, 62
Normal tissues	Immortal[e]	Testes	Stable	2/2	32, 40
	Mortal[f]	Connective	Shorten		24, 31, 40
		Epidermis	Shorten[g]		39[g]
		Blood	Shorten	111/135	12, 38, 46, 62–65
		Bone marrow	Shorten or n.t.	12/15[c]	60, 63–65
		Vascular intima	Shorten	0/>60	31
		Brain	Stable		31, 42
		Others (breast, prostate, uterus, intestine, kidney, liver, lung, muscle, spleen)	n.t.		31
Tumor tissues	?	Breast	n.t.		31
		Prim. node neg.		1/4	
		Ductal node pos.		14/15	
		Ovarian carcinoma	Stable	7/7	62
		Prostate	n.t.		31
		BPH		1/10	
		PIN3		3/5	
		Adenocarcinoma		2/2	
		Neuroblastoma	Shorten or elongated	99/105	31, 66
		Head and neck	n.t.	14/16	31
		Colon			31, 38
		Polyp	n.t.	0/1	
		Tubular aadenoma	Shorten or n.t.	0/1	
		Carcinoma	Shorten or n.t.	8/8	
		Uterine	n.t.		31
		Fibroids		0/11	
		Sarcoma		3/3	
		Bone	Shorten or extra long	5/5	49
		Gastric cancer	Shorten	56/66	67
		Hepatic	n.t.	28/33	68
		Renal	n.t.	40/55	69

Note. Modified and updated, with permission, from Ref. 70.
[a] Number of positive samples/number of total samples tested.
[b] n.t., not tested.
[c] Weak telomerase activity was detected with the PCR based TRAP assay.
[d] Sublines of T-antigen transformed and immortalized cells may become telomerase negative, perhaps through genetic instability; it is not known whether these subclones have an indefinite replicative capacity (31).
[e] The germline lineage is immortal, even if specific cell types may not be.
[f] The question of whether normal tissues contain a rare immortal stem cell has not been addressed in these studies.
[g] Prowse KR, Harley CB, unpublished data.

Subfractionation of bone marrow hematopoietic cells into primitive stem cells and other progenitors suggests that telomerase expression is present in stem cells and in progenitors (60, 65). Furthermore, the level of telomerase activity appears to be regulated depending on the cell population and the presence of mitogenic stimulation (60). Similarly, telomerase activity is detectable in resting peripheral blood lymphocytes, and the level is increased upon T-cell activation *in vitro* (63–65). By contrast, previous studies have demonstrated telomere loss in hematopoietic stem cells and peripheral blood lymphocytes (12, 41), which suggests that these cells still have a finite replicative life span (79). It is possible that the level of telomerase detected in these cells is too low to maintain telomere length or that its presence has already contributed to a slowing of the rate of telomere loss. Alternatively, telomerase may be present in a subset of cells whose telomere length is maintained but not readily discerned when the analysis is performed en masse.

Development of *in situ* assays for telomerase expression and telomere length measurement would help to resolve this issue.

It should be noted that, to date, telomerase activity has been detected only in normal somatic cells that have the potential for a high turnover rate *in vivo* (i.e., fetal cells, adult hematopoietic cells, and peripheral blood lymphocytes). More recently, telomerase activity has also been reported in the epidermis of normal skin and in the crypts of the intestine (80, 81). The transient induction of telomerase in the hematopoietic cells and lymphocytes in response to mitogenic stimulation suggests that telomerase expression may be important in conferring added replicative potential during clonal expansion.

Therapeutic Opportunities

Understanding the regulation of telomerase in normal and immortal cells may lead to alternative therapeutic strategies in age-related diseases including cancer. The association of telomerase expression with immortal cells has led to the development of telomerase inhibition as a novel anticancer therapy (70). The studies reviewed here suggest that inhibition of telomerase function and experimental induction of telomere shortening can reverse cell immortality and impair cell viability (45, 76). Since telomerase inhibition acts by restoring the normal telomere shortening associated with cell division in normal somatic cells, tumors with a short initial telomere length would be predicted to respond most rapidly. Alternatively, therapeutic efficacy may be improved by combining telomerase inhibition with cytoreductive agents to reduce the initial tumor load. The impact of telomerase inhibition on the immune system is a potential concern since low levels of telomerase activity are found in hematopoietic cells and lymphocytes. However, given that telomere lengths in these cells do shorten *in vivo*, inhibition of telomerase activity may not have a deleterious effect on normal functioning of the immune system.

Conversely, the tight association of a critically short telomere length with the senescent state suggests a target for therapeutic intervention. The existence of senescent cells *in vivo* and their accumulation with age (4, 15, 43) may contribute to age-related diseases. Extending telomere lengths or slowing telomere loss in normal somatic cells (e.g., by activating telomerase expression *in vivo* or *ex vivo*) may increase the replicative potential of the cells. This may in turn delay the onset of senescence and some of the pathological symptoms associated with age-related diseases.

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