Proc. Natl. Acad. Sci. USA
Vol. 95, pp. 9078–9081, August 1998

Review

Can ends justify the means?: Telomeres and the mechanisms of replicative senescence and immortalization in mammalian cells

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Contributed by George Klein, June 15, 1998

ABSTRACT **Finite replicative lifespan, or senescence, of mammalian cells in culture is a phenomenon that has generated much curiosity since its description. The obvious significance of senescence to organismal aging and the development of cancer has engendered a long-lasting and lively debate about its mechanisms. Recent discoveries concerning the phenotypes of telomerase knockout mice, the consequences of telomerase reexpression in somatic cells, and genes that regulate senescence have provided striking molecular insights but also have uncovered important new questions. The objective of this review is to reconcile old observations with new molecular details and to focus attention on the key remaining puzzles.**

A Historical Perspective of Senescence. Although mechanisms may differ, some form of replicative limit is probably operative in most living cells (1). Finite replicative lifespan in mammalian cells is evidenced, quite simply, by a decline and eventual complete cessation of cell division (2). This phenotype is open to the obvious criticism of inadequate culture conditions. Although in some cases it has indeed been possible to significantly extend the apparent lifespan of cultures by manipulating the medium (3), it now is accepted generally that gross artifacts caused by culture conditions have been addressed adequately. Three classical observations usually are cited to argue that *in vitro* replicative senescence is a phenomenon with biological significance: (*i*) the correlation of *in vitro* lifespan with the age of the donor (4, 5, 6); (*ii*) the correlation of *in vitro* lifespan with the average life expectancy of the species (7, 8); and (*iii*) the reduced *in vitro* lifespan of cells from patients afflicted with premature aging syndromes (9, 10).

Two major theories have been used to explain limited replicative capacity. The first hypothesis invokes the gradual accumulation of mutations (11), and the second invokes the existence of a molecular clock (or clocks) that can keep track of cell divisions (12–14). The second theory now is believed generally to be true (15, 16).

How is senescence different from quiescence, the normal physiological withdrawal from the cell cycle that is displayed by almost all cells? Quiescence is defined as a reversible process, such that stimulation with proper growth factors or other stimuli will result in resumption of proliferation. Senescence, on the other hand, is irreversible (17). This, then, makes it necessary to differentiate senescence from terminal differentiation. This may not be easy; in general, one would like to observe the absence of features characteristic of terminally differentiated, postmitotic cells (18, 19). In fact, senescence has been likened by some to a quasi-differentiation process (15, 20) because of the observation that senescent cultures can be maintained in a viable, albeit non replicating, state for very long periods of time (21).

We all know that senescence can be overcome, because many cell lines in common use are quite obviously immortal. Rodent cells can overcome senescence spontaneously (22). The relatively low frequency of this event, and the fact that it can be stimulated by mutagens, has led to the hypothesis that it is mutational in nature (23). This theory is supported by the existence of genes that can cause immortalization, such as the simian virus 40 and polyoma virus large T antigen genes and the adenovirus E1A gene (24, 25). These genes, when introduced into rodent cells, are sufficient to cause immortalization in what appears to be a single step (26, 27). Even deregulated expression of cellular genes, such as c-Myc, can, in some cases, display this property (28).

At the cellular level, senescence is genetically a dominant trait. In both rodent and human cell fusion studies, the senescent phenotype is dominant over either the presenescent or the immortal phenotype (29, 30). The observation that microinjection of mRNA from senescent cells can cause growth inhibition is consistent with the existence of a negatively acting effector(s) whose up-regulation triggers senescence (31). Recent data from knockout mice indicate that the elimination of a variety of negatively acting effectors can result in apparent one-step immortalization. To date, embryo fibroblasts derived from homozygously deleted p53, $p16^{INK4a}$, $p19^{ARF}$, and $p21^{CIP1}$ mouse embryos continued to divide indefinitely without any apparent replicative decline (32–35). It is assumed widely that viral oncogenes such as SV40 large T antigen or adenovirus E1A immortalize cells by eliminating the effects of activity of negatively acting cellular effectors.

In contrast, normal human cells never have been observed to immortalize spontaneously. In spite of numerous attempts, senescent cultures never give rise to subpopulations that resume proliferation (36). Treatment with mutagens has been shown to give rise sporadically to immortalized derivatives, but the frequency of these events is significantly lower than in rodent cells (37, 38). Typically, several treatments with mutagens over an extended period of time are required. These observations have given rise to the notion that, although few (maybe only one) mutations are required to immortalize a rodent cell, significantly more are required to immortalize a human cell. Chicken, bovine, and horse cells are further examples of cells that rarely, if ever, immortalize spontaneously whereas hamster and rat are examples of cells that do (8, 39–42).

Bypass of Senescence and the Phenomenon of Crisis. Consistent with these observations, the introduction of positively acting effectors into human cells, such as the SV40 large T antigen, adenovirus E1A, human papilloma virus E6 or E7, and c-Myc, or the elimination of negatively acting effectors such as p21, does not cause immortalization. What these interventions accomplish is extension of *in vitro* lifespan (25,

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43). This lifespan extension is readily apparent and quantifiable, given good tissue culture technique. The reports of the extent of lifespan extension are somewhat variable but typically are clustered at 20 to 30 extra population doublings for fibroblasts (44–46) and up to 60 to 100 doublings for some other cell types (47–49).

What happens at the end of the extended lifespan? Another proliferative decline is observed, but this decline is distinct from the decline that was defined as senescence in normal (nontreated) cultures (43). The main distinction is that cells in senescence are truly nondividing; this can be observed by labeling cultures with tritium or BrdU for relatively long periods of time (2 days). Under these conditions, only a very small fraction of the senescent nuclei (<5%) incorporate label. In contrast, when cells at the end of the extended lifespan phase are treated in this way, the labeling index is typically quite high (>30%). This is because in these cultures the apparent absence of macroscopic proliferation is in fact the result of ongoing cell division combined with ongoing cell death. Another criterion often used is that senescent cells can be maintained in a viable, nonproliferative state for very long periods of time (4–6 months; up to 2 years has been reported) provided they are fed regularly (21, 50) whereas cultures at the end of the extended lifespan phase decline and die within a window of 4–6 weeks.

The proliferative decline of cultures at the end of the extended lifespan phase of human cells has been called crisis, and this is the term I will use here. Use of this word is ambiguous, because it also has been applied to rodent cells, which do not display the two-step proliferative decline characteristic of human cells. To distinguish more clearly between senescence and crisis, it has been proposed (51) to redefine the former as mortality stage I (M1) and the latter as mortality stage II (M2), but this terminology has not gained universal acceptance.

Spontaneously immortalized cells can be obtained from human cultures undergoing crisis at a frequency of 10^{-7} to 10^{-5} (46, 52). The immortal phenotype depends on the continuous expression of the senescence-bypassing function, such as SV40 large T antigen (53) or HPV E6 and E7 (54). For example, inactivation of a temperature-sensitive large T antigen by a temperature shift-up results in immediate growth arrest. Rodent cells that underwent a single-step immortalization event because of expression of SV40 T antigen are likewise dependent on its continuous presence for proliferation (26). The phenomenon of senescence bypass, extended lifespan, crisis, and, finally, immortalization also is observed in human B cells undergoing immortalization with Epstain–Barr virus (55, 56).

The Molecular Clock of Aging. Older observations that correlated entry into senescence with elapsed cell divisions rather than chronological time led to proposals for the existence of a molecular clock that keeps track of cell divisions (57). The "running down" of this clock was hypothesized to generate a signal that triggers the senescence program. The expression of, for example, SV40 large T antigen then could prevent senescence by overriding the signal from the clock, or, more likely, by interfering with the senescence machinery itself (58). It is amazing that, in spite of very long periods of apparent "immortality," the senescent program remains intact and, on the removal of the overriding agent, is capable of establishing rapid growth arrest (53, 54).

The currently prevailing hypothesis for the nature of this molecular clock is the attrition of telomeres (14, 59). Germ cells, and some key stem cells, are known to express telomerase catalytic activity whereas the majority of somatic cells lack it (60–62). Murine embryonic stem cells express telomerase and are functionally immortal, and elimination of telomerase eventually results in loss of proliferation (63). The estimation of telomere shortening per one cell generation of 50–100 bp correlates well with the observed telomere lengths of 18–25 kbp and 8–10 kbp in young and senescent human fibroblasts, respectively (48, 55, 64, 65). An important observation is that senescent cells still possess appreciable telomeres and that telomeres continue to shorten if cells are driven into the extended lifespan phase (48, 66). Cells in crisis display average telomere lengths in the 3–4 kbp range, but, because of presumed heterogeneity in the attrition process, most cells probably contain at least one chromosome with a critically shortened, or even absent, telomere. In fact, by using quantitative fluorescence *in situ* hybridization analysis, individual chromosomes with telomere lengths of 1 kb and less have been demonstrated in crisis cells (67). Therefore, the observed karyotypic instability characteristic of cells in crisis is consistent with ongoing fusion-bridge cycles first observed by McClintock in maize (68).

The linguistic definition of "senescence" is "the state of being, or the process of becoming, old," and therefore this term has been used often to describe any sort of age-related, irreversible proliferative decline. In light of new molecular data, I prefer to use "senescence" in a more restricted, mechanistic sense, to designate the response triggered in normal cells. I think of cellular senescence as an active, genetically programmed process that responds to an inductive signal: in this case, perhaps telomere shortening. How this signal is generated in a senescent cell that maintains, on average, 8- to 10-kbp telomere lengths is not well understood (69). It has been shown, by using quantitative fluorescence *in situ* hybridization analysis, that senescent human cells contain individual chromosomes with telomeres of significantly shorter length than the average of the population. These very short telomeres may be important for the triggering of senescence by generating a DNA damage-like signal (70, 71). It has been suggested that telomeres that have reached a critical minimal length are impaired in their ability to recruit the telomere-binding protein TRF2, resulting in chromosome end-to-end fusions (72). An alternative hypothesis invokes the derepression of growth regulatory genes in subtelomeric regions as a result of telomere shortening (73), an idea that has precedent in yeast (74). Notwithstanding the mechanism, it can be argued that the ensuing growth arrest has the obvious advantage of preventing the cell from becoming grossly genetically unstable. In contrast, I think of crisis as an unphysiological phenomenon caused by a bypass of senescence and leading eventually to the catastrophic breakdown of chromosome stability caused by critical telomere shortening on many chromosomes.

Using the above definitions, I then propose that immortalization of human cells requires a bypass of both senescence and crisis whereas in rodent cells crisis does not exist and lifespan is limited only by senescence. This explains why interventions that cause one-step immortalization of rodent cells result only in lifespan extension in human cells. Furthermore, the imposition of senescence would involve common mechanisms, namely, the p53 and/or Rb pathways (or components thereof), which is a comforting notion from the perspective of evolutionary conservation of important regulatory mechanisms. Although p21 has been shown to be a major determinant of senescence in human cells (66), a role for p16 has not been ruled out—in fact, much circumstantial evidence points to some role for p16 in limiting growth (75). The individual roles of $p16^{INK4a}$ and $p19^{ARF}$ in immortalization (33, 34), especially with respect to human cells, remain to be established.

If crisis does not exist in rodent cells and bypass of senescence is sufficient for immortalization, how does telomerase become expressed in somatic cells? The bottom line seems to be that telomerase is not regulated very strictly in rodent cells and tissues. A variety of rodent tissues have been shown to express telomerase, and telomerase-negative primary cultures often become telomerase-positive over time, often even before reaching senescence (76–78). In contrast, telomerase appears to be regulated very stringently in human cells. Therefore, telomerase activation could occur in rodent cells undergoing

immortalization either before or after the senescence bypassing event and could easily occur in a subtle and gradual fashion such that no clearly apparent downturn in proliferative capacity of the bulk culture would be observed. In other words, one-step immortalization may in fact require two steps: the obvious step of senescence bypass and a very subtle step, at least in rodent cultures, of telomerase activation.

Another interesting conundrum is the existence in *Mus musculus* of very long telomeres (20 to 60 kbp, mean length 30 kbp). If telomere shortening is the molecular clock that triggers senescence, one would predict that these cells would have a very long *in vitro* lifespan. In reality, the opposite is observed: The cultures senesce after relatively few generations, and senescent cells contain imperceptibly shortened telomeres (79). In a more recent study, a shortening of 4 kbp at senescence was reported (80). How can such minimal telomere shortening trigger senescence? What could be the nature of the molecular mechanism that would signal proliferation at an average telomere length of 30 kbp and senescence at a length of 26 kbp? In *Mus spretus*, telomere lengths are in the range of human telomeres, yet *M. spretus* and *M. musculus* cells have the same *in vitro* lifespan (81). In this regard, it is of interest that *M. musculus* cells appear to have large interchromosomal variations in telomere lengths, such that, in spite of the long average telomere length, each cell may contain a few telomeres with lengths in the range of 10 kbp (80). These minority short telomeres may be important in regulating the replicative lifespan.

The Telomerase Knockout Mouse. The telomerase knockout mouse is viable for at least six organismal generations (82), although the average telomere length declines with each generation. Given the very long telomeres in the embryonic stem cells in which the knockout was performed, it is not unreasonable that it took several organismal generations to observe gross phenotypes caused by critical telomere shortening. With respect to senescence, the *in vitro* lifespan of embryo fibroblasts did not vary from generation to generation and was in fact similar to the *in vitro* lifespan of cells recovered from normal animals. How could an average telomere length of, for example, 25 kbp in an old cell from a first- or second-generation mouse signal senescence whereas a shorter telomere length of, for example, 15 kbp in a young cell from a later-generation mouse signal proliferation? If a minority population of chromosomes with short telomeres is responsible for generating the senescence signal, one would have to assume that these chromosomes, and their frequency distribution, would not be affected by absence of telomerase activity for several generations.

Expression of Telomerase in Presenescent Human Cells. Expression of the telomerase catalytic subunit in presenescent human cells has been reported to extend significantly the lifespan of the cultures (83, 84). In these experiments, the expression of telomerase enzymatic activity, the maintenance of telomere length, and the delay of senescence was demonstrated clearly. Therefore, at least in human cells, telomere length appears to be linked critically to the triggering of senescence. Although it remains to be rigorously demonstrated, this result strongly implies that activation of telomerase can result in one-step immortalization.

Perspectives. From a historical perspective, the apparent one-step immortalization caused by telomerase expression is a very unexpected and perplexing finding. If telomerase activation is sufficient for immortalization, why has it been impossible to isolate spontaneously immortalized human cell lines? If telomerase activation, and concomitant immortalization, occurs in SV40 large T-expressing cells at a frequency of 10^{-5} to 10^{-7}, why doesn't it happen in normal cells? Why do some postcrisis SV40 large T-expressing cells, which contain abundant telomerase activity and long telomeres, stop dividing immediately after withdrawal of large T antigen? Why do mouse cells that have activated telomerase before senescence still senesce apparently on time? A similar phenomenon also has been observed in human keratinocytes and mammary epithelial cells (85). Why has telomerase never been picked up as an oncogene or a gene conferring a predisposition to malignancy?

Only further research can answer these questions fully, but certain possibilities immediately come to mind. Some of the older experiments simply may be wrong. It may be necessary to express telomerase activity beyond a certain threshold to achieve one-step immortalization. A corollary of this hypothesis is that spontaneous activation mechanisms only rarely (or never) can result in the necessary threshold levels and that expression of SV40 large T (and other viral oncogenes) may either predispose or augment the expression mechanism. Another possibility is that the long-lived, telomerase-expressing clones (83) picked up, unbeknownst to the investigators, a secondary mutation in a negative growth regulator such as p53, p16, $p19^{ARF}$, or p21.

Definition of one-step immortalization involves operationally the observation that normal cultures undergo a replicative decline characterized by very slow, or even negative, growth. The immortalization process is evidenced by a gradual resumption of growth as one or several immortalized clones eventually take over. One-step immortalization is assumed to have taken place if no apparent downturn in the growth rate is evident in the cultures. However, experience with rodent cultures that immortalize quite readily has shown that such a replicative decline may be apparent only under some, very carefully controlled, passaging regimens (22). Therefore, whether historical or contemporary literature is considered, it is a good idea to keep in mind that either mutational or epigenetic phenomena can occur in cultures subjected to long-term serial subculture and that these changes may not reveal themselves on a macroscopic scale in the growth rate or passage history of the culture. For example, dominant-defective p53 mutations (86) and the loss of p16 expression because of promoter methylation (87–90) are two occurrences frequently encountered in human tumors and could certainly contribute to immortalization processes.

In my opinion, the key outstanding question is the linkage of telomere shortening to the triggering of senescence. The mechanisms by which senescence, once triggered, actually is imposed are coming rapidly into focus. Is telomere attrition the key molecular clock? Is it possible that another clock, perhaps parallel, or perhaps connected in some way to the telomere clock, may exist? It is clear that at least some genetic components are shared and that the rodent system is clearly more permissive to immortalization, but, at face value, the results of the telomerase knockout mouse (82) and telomerase expression in presenescent human cells (83) run counter to each other. Does this mean that the rodent and human mechanisms are fundamentally different? This is an unsettling notion from the perspective of evolutionary conservation of important regulatory mechanisms. I expect that, once we get a handle on these issues, the two really big questions (namely, does senescence limit organismal lifespan? and is telomerase expression necessary for cancer progression *in vivo*?) quickly will become approachable.

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