Senescence: an antiviral defense that is tumor suppressive?

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Senescence phenotype in vitro

In addition to the large flattened morphology, senescent cells in vitro have a number of other phenotypic features. Although metabolically active, they are growth arrested (usually in G1 phase of the cell cycle) and, provided key tumor suppressors are not disrupted, do not re-enter the cell cycle even in response to maximal growth factor stimulation (5,7). Levels of the p53-responsive p21(CIP1) and plasminogen activator inhibitor-1 (PAI-1) proteins and miR-34-33 of microRNAs and of p16INK4a are elevated in these cells (8–11). Senescent cells have β-galactosidase activity at pH 6 (referred to as senescence-associated SAβ-galactosidase activity; although this is not a completely specific senescence biomarker), which can be detected histochemically (12,13). They also have an increased number of telomere dysfunction-induced foci (TIFs), consisting of DNA damage response proteins such as phosphorylated histone H2AX, tumor suppressor p53-binding protein 1 (53BP1), mediator of DNA damage checkpoint protein 1 (MDC1) and nibrin localized at telomeres, and have activated forms of the DNA damage checkpoint kinases, CHK1 and CHK2 (14).

Distinct structures, referred to as senescence-associated heterochromatin foci (SAHF), accumulate in the nuclei of senescent human fibroblasts (15). These can be visualized as punctate foci after staining DNA with 4′,6-diamidino-2-phenylindole. Although not all cell types exhibit nuclear heterochromatinization to the same extent as some human fibroblast strains, some degree of heterochromatinization appears to be a general feature of senescence in a variety of cell types from several species (16). The foci contain heterochromatin histone posttranslational modifications, such as methylated lysine 9 of histone H3 (H3K9Me), and associated proteins, including heterochromatin protein 1 (H1P1) and histone variant macroH2A, and also high mobility group A (HMGA) proteins (16). Formation of SAHF depends on an intact retnoblastoma (Rb) tumor suppressor pathway (15), and proteins such as the histone repressor A (HIRA)/antisilencing factor 1a (ASF1a)/ubinuclein 1 (UBN1) complex (17), and appears to contribute to the permanence of the cell cycle arrest and other changes characteristic of senescence (15,18).

Occurrence of senescence in vivo

Lack of suitable markers has made it difficult to assess the occurrence and importance of senescence in vivo. It has been reported that in skin samples from human donors, there was an age-dependent increase in SA-β-galactosidase in dermal fibroblasts and epidermal keratinocytes (12), although this result was not replicated by others (13). Increased SA-β-galactosidase has been seen in aging human corneal endothelium (19) and articular chondrocytes (20), but significant activity was not found in esophageal epithelium at any age (21). TIFs have been observed to increase exponentially with age in baboon dermal fibroblasts (22) but not in skeletal muscle (23). There was some evidence for an age-related increase of heterochromatinization and of p16INK4a expression in the nuclei of baboon dermal fibroblasts (22).

Although there are substantial differences between the senescence mechanisms of human and mouse cells (24), in vivo carcinogenesis studies in mice also provide evidence for the importance of senescence. In KRAS transgenic mice, adenomas had increased HPI-γ expression (possibly indicating SAHFeformation) and p16INK4a expression and SA-β-galactosidase activity, whereas malignant adenocarcinomas did not (25). It was observed that acute phagocytosis and tension homolog (PTEN) inactivation in mice caused p53-dependent cellular senescence both in vitro and in vivo and that, in the presence of functional p53, prostate tumors occurred only after a long latency (26). A study of neuroblastoma RAS viral oncogene homolog (NRAS)-induced lymphomagenesis in mice provided evidence that H3K9Me-mediated senescence is a tumor suppressor mechanism in vivo (27). The relevance of senescence to tumor suppression in humans in vivo is suggested by

Introduction

Cellular senescence (1) refers to a complex set of changes that include permanent withdrawal from the cell cycle and cytoskeletal alterations that usually result in the cell becoming large and flat. Senescent cells are resistant to apoptosis (2) and may remain alive in vitro for many years. The limited proliferative potential and senescence of normal human cells was first described for fibroblasts by Hayflick et al. (3). It has subsequently been found that other cell types, including epidermal keratinocytes, vascular smooth muscle cells, T-lymphocytes, glial cells, adrenocortical cells, endothelial cells and melanocytes, also become senescent (4,5). The maximum number of population doublings for a given cell type is sometimes referred to as the Hayflick limit or number. Conceptually, the Hayflick limit is equivalent to the maximum proliferative potential of a cell population, but in practice, the limit that is observed is determined by factors extrinsic to the cell, such as the suitability of the cell culture conditions.

Although cellular senescence in vitro has been used extensively as a model system for studying organismal aging, it seems most likely that cellular senescence is only one of the factors that contributes to aging in vivo. The relationship between the Hayflick number and the age of the cells’ donor has been controversial for several decades. A recent meta-analysis of the published studies on this subject concluded that, although the replicative capacity of fibroblasts from individuals with accelerated aging syndromes was consistently lower than that of age-matched controls, replicative capacity did not correlate with the presence of age-related pathologies or with chronological age (6).

Abbreviations: ALT, alternative lengthening of telomeres; PML, promyelocytic leukemia; Rb, retinoblastoma; RNP, ribonucleoprotein; SA, senescence-associated; SAHF, senescence-associated heterochromatin foci; TERC, telomerase RNA Component; TERT, telomerase reverse transcriptase; TMM, telomere length maintenance mechanism.
observation of cellular senescence in early-stage prostate cancer (26) and in nevi (benign skin moles) (28).

**Immune response to senescent cells**

Recent studies indicate that senescent cells upregulate a number of ligands that stimulate immune responses, possibly as a result of DNA damage response signaling (29), including intercellular adhesion molecule 1 (ICAM1), and receptor natural killer group 2, member D (NKG2D) ligands such as MHC class I polypeptide-related sequence A (MICA) and UL16 binding protein 2 (ULBP2) (30,31). The results suggest that senescent cells may be cleared by an innate immune response, but the extent to which this may contribute to prevention of age-related pathologies and cancer is currently not clear.

**Evidence that senescence is tumor suppressive**

Although unlimited proliferative capacity (i.e. immortalization) is often regarded as an essentially universal characteristic of cancer (32), it is not immediately obvious why this should be so. Normal fibroblasts from adult humans often divide for 40–50 population doublings, which would be enough to form a very large tumor. As proposed previously, the reasons that may explain why normal proliferative capacity is usually not sufficient to support tumorigenesis are that (i) many tumors have a high cellular death rate due to periods of inadequate vascularization and (ii) the requirement for moderately large numbers of genetic and epigenetic changes means that many rounds of clonal evolution need to occur (1). The result is that many more cell divisions are required for tumorigenesis than is possible unless cells breach the senescence proliferation barrier and become immortalized. Tumors that arise in cells that have a large proliferative capacity, where the cell death rate is not high or where the number of molecular genetic changes required is relatively small, may not need to contain an immortalized population (1). In other more common circumstances, however, the barriers to unlimited cellular proliferation provided by senescence and related terminal proliferative arrest states (described below) are a potent protection against carcinogenesis, and the evidence for this has been discussed in many review articles (1,33–38). In the context of the antitumorigenic effects of senescence, it is relevant to note the evidence that senescence may be an important determinant of the response of tumors to cytotoxic chemotherapy (39).

**Protumorigenic aspects of senescence**

A very interesting aspect of senescence is the development of a senescence-associated secretory phenotype (SASP) (40). This results, at least in part, from persistent DNA damage response signaling (41). Some of the chemokine and cytokine factors secreted, including interleukin-6 and interleukin-8, are associated with wound healing and are proinflammatory and also reinforce the senescence phenotype in an autocrine or paracrine manner, which may involve generation of reactive oxygen species, DNA damage and p53 activation (42,43). The SASP of senescent human fibroblasts can impair the differentiation of mouse and human immortalized breast epithelial cell lines (44) and promote cell invasion (45) and tumor growth (44). These properties of senescent cells suggest that they may contribute to age-related tissue pathologies and cancer.

In addition, there is now a substantial body of evidence that replicative senescence in human cells may result from the telomere shortening that accompanies normal cell division (see below). Telomere shortening, especially in the context of p53 inactivation, may result in genomic instability and secondary genetic changes that facilitate carcinogenesis (46).

**Hypothesis: senescence was selected for as an antiviral mechanism**

There seems to be strong evidence that senescence functions as a tumor suppressor mechanism overall (47), but there is also evidence for antagonistic pleiotropy (e.g. telomere shortening and the SASP may both be protumorigenic). This has been explained on the basis that the selection pressure for a tumor suppression mechanism is to prevent disease prior to completion of reproduction and that any deleterious effects that the mechanism may have after the reproductive years are of little or no consequence to the species’ survival (48). An alternative explanation that may account for all known aspects of the senescence program is that the primary selection pressure for senescence was as an antiviral mechanism. This would fit with the observations that senescence can be induced by inappropriate stimulation to proliferate (49), that prolonged signaling by cytokines such as β-interferon in response to viral infection can cause senescence (50) and that senescent cells secrete cytokines that have the ability to induce the senescence of neighboring cells (42), which would have a high probability of being infected because of their proximity, and to elicit an innate immune response (29). It also fits with the observation that many viruses have developed mechanisms to overcome senescence (51–54) and to target promyeloctytic leukemia (PML) bodies (55), which are involved in senescence (56), together with mechanisms to evade other antiviral defenses such as apoptosis and immune responses. The ability of senescence to suppress tumorigenesis may be a secondary benefit.

**Additional barriers to unlimited proliferation**

Bypass of senescence is not sufficient for immortalization because senescence is not the only barrier to unlimited cellular proliferation. If genetic or epigenetic changes occur that allow cells to bypass senescence, there are other forms of terminal proliferation arrest that may subsequently occur.

**Crisis**

Culture crisis is a well-known but still poorly understood terminal proliferation arrest state that was first described in simian virus 40 (SV40)-infected human fibroblasts (57). SV40 early region genes encode oncoproteins that cause functional inactivation of the p53 and Rb family of tumor suppressor proteins (53), and this temporarily bypasses senescence and allows the cell population to continue dividing despite continuing telomere shortening (58). The explanation appears to be that the viral oncoproteins disrupt the signaling mechanism whereby excessive telomere shortening results in growth arrest. After this finite ‘life span extension’, the cell population eventually ceases expanding and enters crisis, also referred to as mortality stage 2 (M2) to distinguish this arrest state from senescence or mortality stage 1 (M1) (59). Crisis usually results from the cell death rate being matched or exceeded by the proliferation rate so that there is no further increase, and often a substantial decrease, in cell numbers (57). Some of the cells, however, may stop expressing the viral oncoproteins and survive long term in a viable growth-arrested state that resembles senescence (60) and may persist for at least 9 months in some cultures (Duncan,E.L., and Reddel,R.R., unpublished data). At a frequency of ~1 × 10⁻⁷ (61), cells may escape from crisis and form an immortalized cell population, presumably via genetic or epigenetic alterations that have not yet been identified, but which always result in the activation of a telomere length maintenance mechanism (TMM) (62). This very tight correlation between escape from crisis and TMM activation strongly supports the hypothesis that crisis results from failure of telomere maintenance. A similar sequence of events has been observed when cells escape temporarily from senescence due to disruption of the p53 and pRb/p16INK4a pathways by non-viral means (63,64).

**Intermediate barriers to unlimited proliferation**

The first evidence for separate and additive contributions of functional inactivation of the p53 and pRb/p16INK4a pathways to culture life span extension came from studies of fibroblasts with an inherited p53 mutation (one allele was essentially null) (63). Spontaneous inactivation of the wild-type (wt) allele resulted in culture life span extension and then spontaneous loss of both p16INK4a alleles resulted in further proliferation prior to TMM activation (63). Other cultures were also identified in which loss of p16INK4a function contributed to culture life span...
span extension (65). Under standard culture conditions, human breast epithelial cells have an unusual pattern of growth: the cultures stop proliferating after a small number of population doublings, until a small subpopulation ceases expression of p16<sup>INK4a</sup>, usually via methylation of its promoter (66), and enters a prolonged phase of rapid proliferation prior to a senescence-like growth arrest from which spontaneous escape has not been observed (67). These and related findings have led to the proposal that there are proliferative barriers to immortalization other than crisis and that they can be signaled separately by the p53 and pRb/p16<sup>INK4a</sup> pathways (68).

Central roles of p53 and pRb/p16<sup>INK4a</sup>

As will be evident from the preceding description, the p53 and pRb/p16<sup>INK4a</sup> pathways have central roles in preventing unlimited cellular proliferation. Genes involved in senescence have been reviewed recently (69), and most of the relevant genes encode proteins that modulate, are activated in or feed into the p53 and/or pRb/p16<sup>INK4a</sup> pathways or are downstream effectors. As might be expected from the profound morphological changes that occur at senescence, some of the genes relate to the cytoskeleton. Consistent with the evidence referred to above that extracellular factors are involved in senescence and that in at least some cases this is dependent on generation of reactive oxygen species, interferon-related, insulin growth factor-related, mitogen-activated protein (MAP) kinase and oxidative stress pathway factors also have substantial roles (69).

p53 promotes senescence by transactivating genes that inhibit proliferation, including the p21<sup>CIP1</sup> cyclin-dependent kinase (CDK) inhibitor (70,71) and the miR-34 class of microRNAs (11). Additional insights into the role of p53 have been provided by a recent study of p53 isoforms: in normal human fibroblasts, an increase in p53β and a decrease in Δ133p53 was causally associated with replicative senescence but not with oncogene-induced premature senescence (72). At replicative senescence, fibroblasts also expressed increased levels of a p53-induced miRNA, miR-34a. Overexpression of Δ133p53 repressed miR-34a and extended the replicative life span, whereas small interfering RNA-mediated knockdown of endogenous Δ133p53 induced cellular senescence via upregulation of p21<sup>CIP1</sup> and other p53 transcriptional target gene products such as PAI-1, insulin-like growth factor-binding protein-7 (IGFBP-7) and matrix metalloproteinase 3 (MMP3). The in vivo relevance of these observations was suggested by the finding that increased p53β and decreased Δ133p53 occurred in colon adenomas with senescent phenotypes (72).

Overexpression of p53 in transgenic mice can result in accelerated aging and an increase in the proportion of senescent cells in tissues in vivo (73,74). This suggests that cellular senescence may contribute to organismal aging. Interestingly, although p53 can also cause increased apoptosis in response to DNA damage, lymphoid tissues of the p53-hypermorphic mice exhibited reduced apoptotic activity and increased induction of senescence in response to ionizing radiation, leading to the suggestion that a potential mechanism for accelerated aging in the presence of increased p53 levels is failure to remove damaged or dysfunctional cells through apoptosis (74). An alternative explanation would be that an overall increase in removal of stem and progenitor cells via apoptosis and senescence results in a premature failure of regenerative capacity in proliferative tissue compartments.

Like the p53 isoform, Δ133p53, the p53 family member, p63, may also have an opposing role to full-length p53 on senescence: in mice, both germ line and somatically induced p63 deficiency activated widespread cellular senescence with increased expression of SA-β-galactosidase and 16<sup>INK4a</sup> (75). p63 deficiency was also shown to be associated with accelerated aging, suggesting a causal link between cellular senescence and organismal aging (75). The action of p63 is opposed by its dominant-negative ΔNp63α isoform, which induces cellular senescence and accelerates aging when overexpressed in transgenic mice (76).

In contrast to p53, the mechanism of p16<sup>INK4a</sup>-promoted senescence involves inhibiting cyclin-dependent kinases 2 and 4, thereby preventing pRB phosphorylation and allowing pRB to promote a transcriptionally repressive heterochromatin environment that silences proliferation-associated E2F transcription factor target genes (15). p16<sup>INK4a</sup> therefore co-operates with proteins, such as HP1, HMG A, HIRA, ASFIa, UBN1 and histone variant macroH2A, to promote SAHF formation (15–18,77), and is very important for the long-term stability of the senescent phenotype. The pRb/p16<sup>INK4a</sup> pathway can be inactivated by the well-known mechanisms of mutation or deletion of the Rb gene, oncoproteins that inactivate pRb, proteins such as inhibitor of DNA binding 1 (ID1) that cause transcriptional repression of p16<sup>INK4a</sup> and deletion, mutation or epigenetic silencing by promoter DNA methylation of the p16<sup>INK4a</sup> component of the cyclin-dependent kinase inhibitor 2A (CDKN2A) locus (78,79). In view of the proposal that cellular senescence originated as an antiviral defense, it is of interest that it has recently been reported that the Hbx protein of hepatitis B virus can overcome premature senescence, in part by inducing methylation of the p16<sup>INK4a</sup> promoter (80).

Although the p53 and pRb/p16<sup>INK4a</sup> pathways act in parallel and inactivation of these pathways has additive effects on life span extension, there are some areas of overlap. For example, the p53-responsive p21<sup>CIP1</sup> protein is a CDK inhibitor that prevents hyperphosphorylation of pRB. The pathways also cross-regulate each other (48). In an arrangement that is apparently unique in mammalian biology, the CDKN2A locus encodes both p16<sup>INK4a</sup> and, in a different reading frame, an alternative reading frame (ARF) protein product which is a p53 activator. Polycomb proteins such as Bmi-1 (81) and chromobox homolog 7 (CBX7) (82) are able to downregulate expression of both ARF and p16<sup>INK4a</sup>. It has been shown that ARF and p16<sup>INK4a</sup> can both be repressed when replication protein Cdc6 binds to a region of the locus that is both a transcriptional regulatory element and a replication origin and recruits histone deacetylases that heterochromatinize and repress the locus (83).

What triggers senescence?

Although senescence may have originated to deal with acute stresses, especially resulting from viral infection, accumulation of macromolecular changes may eventually trigger the same programmed response. According to the telomere hypothesis of senescence, the telomere shortening that normally accompanies the replication of somatic cells acts as a mitotic counting mechanism, or ‘clock’, that eventually results in their permanent exit from the cell cycle (84). It was subsequently proposed that there may be more than one clock mechanism and that there may also be non-clock mechanisms that mostly result in acute induction of senescence (68). Because the putative mitotic clocks count cell cycles, the senescent that eventually ensues is referred to as replicative senescence.

Non-clock triggers (premature senescence)

A number of acute stimuli can result in senescence prior to the population doubling level at which replicative senescence would otherwise have occurred. The resulting senescence is therefore referred to as ‘premature’ (85). Premature senescence can be caused by excessive oncogenic stimulation, either by the loss of tumor suppressor genes such as PTEN (26) or by activation of oncogenes such as mitogen-activated protein kinase or extracellular signal-regulated kinase (MEK), rat sarcoma virus (RAS), and V-raf murine sarcoma viral oncogene homolog B1 (BRAF) (28,86). Two main signaling pathways are involved in oncogene-induced senescence: p16<sup>INK4a</sup>pRb and ARF/p53 (49). Induction of senescence by these oncogenic stimuli is dependent upon an interleukin-6-mediated inflammatory response (42), which is consistent with the proposal that oncogene-induced senescence originated as a defense against viruses that provide potent mitogenic signals. There may be some co-operation between telomere shortening and mitogenic stimulation in senescence induction; it was found that replicative senescence signaling operates at a low level in cells with shortened telomeres but only becomes fully activated when cells are stimulated to enter the cell cycle (87).

Various forms of macromolecular damage can also trigger senescence. These include cytotoxic drugs that act by causing DNA
damage; a recent study demonstrated that senescence is a key determinant of the in vivo response to cytotoxic chemotherapy in a transgenic mouse model of B-cell lymphoma (39). Senescence can be induced by treatment with sublethal levels of H$_2$O$_2$ (88) or by reagents such as bromoecdysone (89), interferon-γ that induces reactive oxygen species and causes DNA damage (90), insulin-like growth factor-binding protein 5 (91) and others that have been reviewed previously (68). Some of these stresses might act via the telomere dysfunction-induced senescence pathway by causing telomere shortening or by causing telomere dysfunction via a more subtle mechanism. Other reagents may trigger senescence via telomere-independent signaling pathways (68).

**Clocks (replicative senescence)**

It has been proposed that progressive accumulation of epigenetic changes may act as a mitotic clock. These may be the progressive loss of DNA methylation that has been demonstrated to occur with increasing cell divisions (92) or progressive decrease in stability of heterochromatin domains (93). However, telomere shortening has attracted most interest to date as the replication counting mechanism.

Replication-driven telomere shortening was predicted on theoretical grounds (84,94) and confirmed experimentally a couple of decades later (95). Several factors contribute to the telomere shortening that accompanies replication of normal somatic cells. First, inability to fill in the gap left by degradation of the RNA primer closest to the terminus of the sister chromatid replicated by lagging strand DNA synthesis is referred to as the end-replication problem (96). Second, processing of telomeres by a putative exonuclease (97) increases the length of the single-stranded telomeric overhang and, like the end-replication problem, decreases the length of the template for the next round of DNA synthesis. It has also been proposed that there may be sudden telomere shortening events to account for the apparently stochastic nature of the onset of senescence (98). It was initially demonstrated that telomere shortening occurs in fibroblasts in vitro (95), and since then, it has been shown in many types of human cells in vivo, including lymphocytes, vascular endothelial cells, hepatocytes, renal, intestinal and lung epithelial cells and myocytes (99).

The observation that senescent cells accumulate TIFs, i.e. foci of DNA damage checkpoint factors and repair proteins at telomeres, suggests that telomere shortening results in recognition of telomeres as DNA double-strand breaks and activation of a cell cycle checkpoint (14). The proteins involved in signaling cell cycle arrest in response to telomere shortening were found to include ataxia telangiectasia mutated (ATM), p53 and p21 (99) but not p16 (87) (100). Interestingly, when telomere dysfunction was induced by acute disruption of telomere-binding protein 5 (91) and others that have been reviewed previously (68). Some of these stresses might act via the telomere dysfunction-induced senescence pathway by causing telomere shortening or by causing telomere dysfunction via a more subtle mechanism. Other reagents may trigger senescence via telomere-independent signaling pathways (68).

Telomerase is a ribonucleoprotein (RNP) complex that reverse transcribes telomeric DNA sequence from an RNA template molecule (referred to as the telomerase RNA component; the RNA is abbreviated as TR, TER, or TERC, and the gene symbol is TERC) that is part of the complex. The reverse transcriptase catalytic activity is provided by the telomerase reverse transcriptase (TERT) protein, and the active enzyme complex also contains an RNA-binding protein, dyskerin (encoded by the DKC1 gene). TERT, TERC and dyskerin were identified in active endogenous telomerase purified from human cells, and based on the molecular weights of these components and of the enzyme complex, it was proposed that the active enzyme contains two molecules of each component (102). The assembly of this complex involves Cajal bodies, which are nuclear RNP processing sites, and proteins that are very likely to be required for telomerase activity. A protein, TCAB1 (telomerase Cajal body protein 1), that localizes to Cajal bodies and associates with small Cajal body RNAs that are involved in modifying splicing RNAs also associates with active telomerase (103). Two adenosine triphosphatases, pontin and reptin, were found to be involved in telomerase RNP assembly and to associate with telomerase components in a cell cycle-dependent manner but not with the most active form of the enzyme (104). Nuclear assembly factor 1 homolog (NAF1) is another protein that is required for assembly of telomerase and that does not remain associated with the mature active complex; NAF1 may be involved early in telomerase biogenesis and appears to dissociate before trafficking of the complex to Cajal bodies (105). Other proteins, including nucleolar protein 10 (NOP10), nucleolar protein family A member 2 (NHP2) and member 1 (GAR1), remain associated with RNP that is more mature and may be involved in its trafficking, stabilization or regulation (106,107).

Many types of normal somatic cells have low or undetectable levels of telomerase activity or they have levels of activity that slow down but do not prevent telomere shortening (108). It has been found that telomerase activity can be upregulated in normal somatic cells to levels that prevent telomere shortening, by transducing them with expression constructs containing TERT under the control of a heterologous promoter (109,110). It was therefore concluded that TERT levels are limiting, that other telomerase components must be expressed constitutively and that the level of telomerase activity within a cell is regulated primarily by control of TERT levels. However, it has subsequently become clear that TERC levels are also limiting for telomerase activity (111), and the reason it took so long to recognize this is that TERC levels often increase in cells overexpressing exogenous TERT (112). This results in part from stabilization of the RNA by overexpressed TERT and also from mechanisms that are not yet understood (112).

Consistent with other evidence that expression levels of TERC are limiting for telomerase activity, studies of mice and humans have shown that the TERC gene is haploinsufficient. Evidence for TERC haploinsufficiency has been obtained from gene targeting studies in mice (113) and from studies of humans with increased telomere shortening (108). There is a rapidly increasing body of knowledge regarding genetic syndromes, typified by dyskeratosis congenita (DC), in which excessively shortened telomeres are associated with premature decrease in proliferative capacity of tissues that are normally characterized by extensive cellular proliferation such as the skin, bone marrow and gastrointestinal tract (114). These may result from mutations in the genes encoding the core components of active telomerase (TERT, TERC and DKC1), proteins involved in telomerase biogenesis (NOP10 and NHP2) or telomere binding and protection (TIN2) (Figure 1), but in many cases, the affected genes have not yet been
increased copy numbers of the TERT genes (115), by mechanisms that are largely unknown, but include isolation of telomeric chromatin (137), and with the high levels of t-circles in ALT cells (133). There is also evidence that telomere trimming occurs in the germ-line and in some rapidly proliferative somatic cells (Pickett,H.A. et al., unpublished data). Telomere length can therefore be considered to be affected by at least three types of processes (Figure 2): (a) the steady attrition that accompanies normal DNA replication; (b) lengthening by telomerase or ALT; and (c) trimming of over-lengthened telomeres. Whereas telomere trimming rather than the ALT mechanism itself is most likely responsible for t-circles, ALT-associated PML bodies, and telomere length heterogeneity, elevated levels of C-circles appear to be closely linked to ALT activity. There was a quantitative relationship between level of activity and level of C-circles, and two telomerase-negative cell lines that are variant with regard to other characteristics usually associated with ALT were nevertheless positive in the C-circle assay (125).

To demonstrate telomere length maintenance in telomerase-negative tumors in humans in vivo would require multiple biopsies over time, which would very rarely be clinically appropriate. However, it has been shown that some telomerase-negative cell lines derived from human tumors are able to maintain their telomere lengths. Moreover, it has been demonstrated that some telomerase-negative tumor samples have a telomere phenotype that is very similar to that of cell lines that use ALT, including very heterogeneous telomere lengths (138), and PML bodies containing telomeric chromatin (132,139), and it is a reasonable assumption that these tumors most likely utilize an ALT mechanism. The types of tumors where ALT is common tend to be of mesenchymal origin. Interestingly, the prognosis for patients with ALT-positive tumors may be no different (144,145), better (139,141), or worse (143) than those with ALT-negative tumors. Although some epithelial tumors, such as breast carcinomas utilize ALT (138,146), tumor types where ALT is common tend to be of mesenchymal origin. Interestingly, the messenger RNA expression pattern of ALT liposarcomas clustered with that of mesenchymal stem cells rather than that of telomerase-positive tumors (147), but similar analyses have not been done for other types of ALT tumors.

Alternative lengthening of telomeres

Some immortalized human cell lines prevent telomere shortening by a mechanism that does not involve telomerase, and which is therefore referred to as ALT (122). Although it cannot be assumed that there is only one ALT mechanism, the evidence to date indicates that ALT involves the synthesis of new telomeric DNA using existing telomeric DNA as a template via homologous recombination and DNA replication. The template DNA may be on another chromosome (123), or within the same telomere (124), or possibly extrachromosomal (125). An alternative hypothesis regarding the nature of the ALT mechanism is that it involves unequal telomeric sister chromatid exchanges (SCE) (126), although this could only result in immortalization of the population if there is a mechanism for segregating the lengthened telomeres into one of the two daughter cells (127).

An increase in telomeric SCE frequency (126,128) is one of several characteristics of cells that use the ALT mechanism. These include telomeres of very heterogeneous length, ranging from very short to very long (129), extrachromosomal telomeric DNA (130,131), telomeric chromatin localized within PML bodies (132), abundant double-stranded circles of telomeric DNA (t-circles) (133), an increased rate of telomere length fluctuation (134), an increased level of DNA damage response foci at telomeres (135) and levels of partly double-stranded C-rich telomeric circles (C-circles) that are on average 750-fold higher than in telomerase-positive and TMM-negative cell cultures (125).

Some of the features of the telomere phenotype in ALT cells may not be directly related to the mechanism itself. When telomerase-positive human cancer cell lines were transduced with a TERC expression construct, their telomere lengths increased for many population doublings and then reached a plateau that coincided with the accumulation of double-stranded circular telomeric DNA (t-circles), localization of telomeric DNA in PML bodies, and the generation of telomere length heterogeneity (136). These data showed that there is a mechanism that limits excessive telomere lengthening by trimming telomeric DNA, most likely by resolution of t-loops to form t-circles. Presumably, telomeres that are over-lengthened by ALT may also be trimmed by the same mechanism, which would be consistent both with the rapid shortening events observed in a telomerase-negative cell line (137), and with the high levels of t-circles in ALT cells (133). There is also evidence that telomere trimming occurs in the germ-line and in some rapidly proliferative somatic cells (Pickett,H.A. et al., unpublished data). Telomere length can therefore be considered to be affected by at least three types of processes (Figure 2): (a) the steady attrition that accompanies normal DNA replication; (b) lengthening by telomerase or ALT; and (c) trimming of over-lengthened telomeres. Whereas telomere trimming rather than the ALT mechanism itself is most likely responsible for t-circles, ALT-associated PML bodies, and telomere length heterogeneity, elevated levels of C-circles appear to be closely linked to ALT activity. There was a quantitative relationship between level of activity and level of C-circles, and two telomerase-negative cell lines that are variant with regard to other characteristics usually associated with ALT were nevertheless positive in the C-circle assay (125).
Some tumors are positive for both ALT and telomerase activity (138,141,145). It is currently not possible to determine whether both mechanisms are active in the same tumor cells, or whether this results from intratumoral heterogeneity. It has been shown that intratumoral heterogeneity with regard to TMM occurred within some osteosarcomas (139), but minimal intratumoral heterogeneity was found in liposarcomas (143). However, both mechanisms can coexist in the same cell when telomerase activity is artificially induced in ALT cells by transduction with exogenous TERT (148).

As might be expected if ALT involves homologous recombination, the MRE11/RAD50/NBS1 (MRN) complex of homologous recombination proteins has been shown to be required for ALT (149,150), and inhibition of their function resulted in telomere shortening at a rate within the range reported for normal fibroblasts that lack a TMM (149). Inhibition of the SMCS/6 complex that is involved in repair of DNA damage also resulted in telomere shortening in ALT cells (151). Other proteins that are potentially involved in the ALT mechanism have not yet been shown to prevent telomere shortening.

Summary
The relationships among telomere shortening, cellular senescence, aging and cancer are complex. It is proposed here that senescence is primarily an antiviral response that has subsequently been utilized as a tumor suppressor mechanism. The tumor suppressor function occurs both in response to aberrant oncogenic signaling or macromolecular damage (premature senescence or oncogene-induced senescence) and in response to telomeric DNA damage signals that accumulate and exceed a threshold level as a result of continued replicative senescence. This link between telomere shortening and replicative senescence seems clear, and presumably, the exquisite controls over telomerase and ALT in normal tissues modulate the rate of telomere shortening to allow sufficient cellular proliferation within a normal lifetime, while preventing cellular immortalization and therefore tumorigenesis. Presumably, it is the result decline in the proliferative capacity of tissue stem cells that is most relevant to aging; this is exacerbated in the telomere length deficiency syndromes. However, the tumor suppressive function of limited proliferative capacity is counteracted by at least two factors. Senescent cells secrete molecules that are proinflammatory and create limited proliferative capacity is counteracted by at least two factors. Senescent cells secrete molecules that are proinflammatory and create

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Senescence and cancer


