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The Biology of Replicative Senescence

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Most cells cannot divide indefinitely due to a process termed cellular or replicative senescence. Replicative senescence appears to be a fundamental feature of somatic cells, with the exception of most tumour cells and possibly certain stem cells. How do cells sense the number of divisions they have completed? Although it has not yet been critically tested, the telomere shortening hypothesis is currently perhaps the best explanation for a cell division 'counting' mechanism. Why do cells irreversibly cease proliferation after completing a finite number of divisions? It is now known that replicative senescence alters the expression of a few crucial growth-regulatory genes. It is not known how these changes in growth-regulatory gene expression are related to telomere shortening in higher eukaryotes. However, lower eukaryotes have provided several plausible mechanisms. Finally, what are the physiological consequences of replicative senescence? Several lines of evidence suggest that, at least in human cells, replicative senescence is a powerful tumour suppressive mechanism. There is also indirect evidence that replicative senescence contributes to ageing. Taken together, current findings suggest that, at least in mammals, replicative senescence may have evolved to curtail tumorigenesis, but may also have the unselected effect of contributing to age-related pathologies, including cancer.

Key words: replicative senescence, cancer, ageing, somatic cells, tumour suppression

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INTRODUCTION

MOST HIGHER eukaryotic cells that can divide *in vivo* cannot do so indefinitely. Thus, normal cells are said to have a finite replicative life span. The process that limits the proliferative potential of cells has been termed cellular or replica tive senescence. Replicative senescence was first described formally more than 30 years ago, for human fibroblasts in culture [1]. Since then, many cell types from a variety of animal species have been shown to have a finite replicative life span (Figure 1). Most studies of replicative senescence have used cells grown in culture. However, a limited number of studies using cells passaged in intact animals, or cells identified in intact tissues, strongly suggest that many cells undergo replicative senescence *in vivo* (reviewed in [2–4]).

Do all dividing cells senesce? Studies of simple, single-cell organisms, such as *Saccharomyces cerevisiae*, suggest that replicative senescence may be a very primitive phenotype [5]. Nonetheless, here I focus on mammalian, and principally human, cells. Replicative senescence is especially stringent in human cells which, unlike many rodent cells, rarely spontaneously immortalise (that is, spontaneously fail to senesce) [6–8]. Most proliferatively competent (that is, nonterminally differentiated) mammalian cells senesce. There are, however, three notable exceptions. First, the germ-line is capable of continuous replication. Thus, somatic cells acquire a finite replicative life span sometime during embryonic development. Very little is known about when, where or how this occurs during development. Second, some stem cells may, even in adult organisms, replicate continuously. This possibility has not yet been critically tested, and thus remains an interesting speculation, particularly for human cells. Third, many malignant tumours appear to have cells with an infinite, or immortal, replicative life span. This finding, and results discussed below, suggest that replicative senescence is a tumour suppressive mechanism, and that tumour cells often acquire mutations that allow them to overcome the proliferative constraints it imposes [6].

THE SENESCENT PHENOTYPE

The number of divisions that normal cells complete before they senesce depends on the species, age and genetic background of the donor, as well as the particular cell type (reviewed in [2, 3]). This number can be fairly large, for example, 60–80 doublings for fetal or neonatal human cells. The mechanism by which cells sense the number of divisions they have completed appears to depend, at least in part, on the length of their telomeres. However, virtually nothing is known in mammalian cells about why telomeres

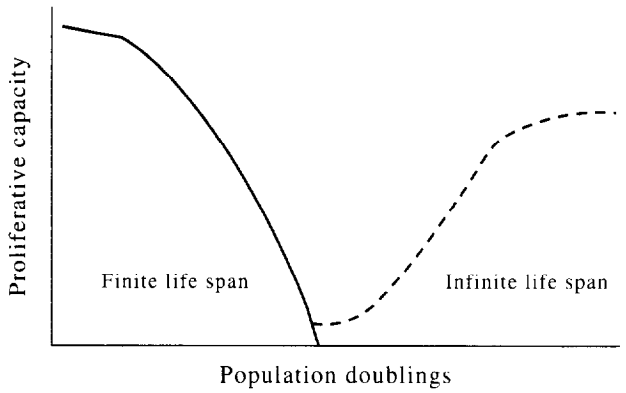

Figure 1. Replicative senescence. The proliferative capacity of most normal somatic mammalian cell populations declines more or less exponentially with each doubling. Such cells are said to have a finite replicative life span, and the process that limits the capacity for cell division has been termed cellular or replicative senescence. Replicative senescence is particularly stringent in human cells. However, oncogenic viruses or chemical or physical carcinogens may permit some human cells to escape replicative senescence and acquire an immortal or indefinite replicative life span. Cells from many rodent species may spontaneously escape senescence. In both cases, escape from senescence is a rare event, occurring with frequencies that range from 1 in 10^{-5} to 1 in 10^{-7}.

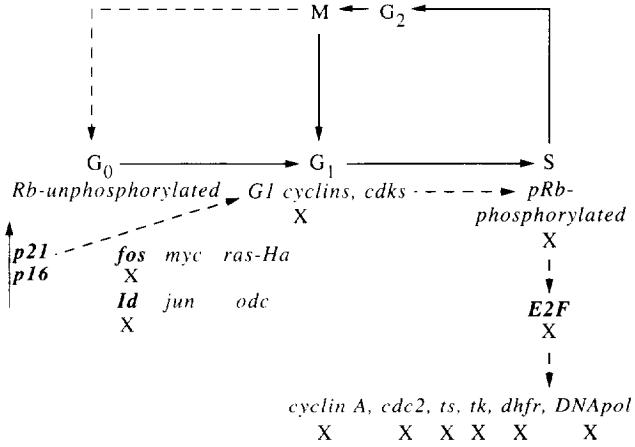

Figure 2. Blocks to cell cycle progression in senescent cells. Mitogens induce a number of growth-related genes in senescent cells. However, at least three transcriptional regulators (c-fos, Id and E2F), whose activities are important for cell proliferation, are repressed in senescent cells (indicated by an X). The repression of E2F is very likely due to the overexpression of p21, which inhibits the activity of cyclin-dependent protein kinases (CDKs). Because CDKs are responsible for phosphorylating the pRb protein, pRb remains in its growth suppressive or underphosphorylated form in senescent cells. Underphosphorylated pRb, in turn, represses E2F activity, which prevents the expression of a host of genes that are necessary or permissive for the initiation of DNA synthesis.

should influence cell proliferation or other cell phenotypes (discussed below) so strongly. Thus, before discussing the role of telomeres in replicative senescence, its genetics and the phenotypic changes that it causes will be briefly discussed.

Genetics

The finite replicative life span of cells is a dominant phenotype. This conclusion is based primarily on a large number of somatic cell fusion studies in which proliferating normal cells were fused to immortal tumour-derived cells. In the majority of cases, the hybrid cells proliferated for some time, but eventually they senesced (see [2, 3] for review). Moreover, fusions between different immortal human tumour cells often, but not always, as discussed below, produced hybrid cells that senesced [9]. Thus, replicative senescence appears to be genetically dominant, and replicative immortality appears to be recessive.

The ability of some, but not all, fusion pairs of immortal human cells to produce hybrid cells that are *not* immortal has led to the identification of four complementation groups for replicative immortality [10]. To date, three of these complementation groups have been assigned to specific human chromosomes. Thus, normal human chromosomes 4, 1 and 7 have been shown to reverse immortality (i.e. induce senescence) in multiple cell lines assigned to complementation groups B, C and D, respectively [11–13]. The genes of interest that reside on these chromosomes have not yet been identified. Taken together, these data suggest that at least four dominant-acting genetic loci act to limit the replicative potential of normal human cells.

Growth arrest

Upon completing a finite number of divisions, cell growth (used here interchangeably with proliferation) is arrested with a G1 DNA content (Figure 2). Once thus arrested, they cannot be stimulated to enter the S phase of the cell cycle by any known combination of physiological mitogens. This growth arrest, like the finite replicative life span, is a dominant trait in somatic cell hybrids (reviewed in [2, 3]). These findings suggest that once cells undergo replicative senescence they express one or more inhibitors of cell cycle progression that can act in a transdominant manner.

The growth arrest associated with cellular senescence has been studied most extensively in cultured human fibroblasts [14]. It is clear from these studies that many genes, including at least three proto-oncogenes, remain mitogen-inducible in senescent cells [15, 16] (Figure 2). Thus, senescent cells do not fail to proliferate due to a *general* breakdown of growth factor signal transduction. Rather, it is now known that replicative senescence causes the selective repression of a few positive-acting, growth regulatory genes whose expression has been shown to be important for G1 progression and DNA synthesis. In fibroblasts, these genes include the *C-FOS* proto-oncogene [16], the helix-loop-helix *ID-1* and *ID-2* genes [17], and the E2F-1 [18] and E2F-5 [19] components of the E2F transcription factor. In addition, the retinoblastoma tumour suppressor protein (pRb) remains in its growth suppressive form (that is, remains constitutively underphosphorylated) [20]. Thus, the immediate cause for the growth arrest of senescent cells appears to be a deficiency in a few, key positive-acting growth regulators, including c-fos, Id and E2F activity, and phosphorylated pRb (Figure 2).

In addition to these deficiencies in positive growth-regulators, senescent human fibroblasts overexpress two negative growth regulators: the p21 and p16 inhibitors of cyclin-dependent protein kinases (*CDKs*) [21, 22]. These high levels of p21 and p16 are very likely responsible for the accumulation of inactive CDK complexes [23], and thus the constitutive underphosphorylation of pRb [20] in senescent cells. Furthermore, because p21 can inhibit E2F by both

pRb-dependent and pRb-independent mechanisms [24], the high level of p21 may well be also responsible for the lack of E2F activity in senescent cells [18, 19] (Figure 2).

p16 and p21 are obvious candidates for the dominant inhibitors of cell proliferation that are expressed by senescent cells. The mechanisms responsible for the senescence-associated increase in p16 and p21 mRNA and protein are not known. In the case of p21, the large increase in mRNA does not appear to be due to an increase in transcription (M. Nakanishi, G. Dimri, J. Campisi and J. Smith, Baylor College of Medicine and Berkeley National Laboratory, U.S.A.). It is unlikely, however, that p16 and p21 are the only growth inhibitors expressed by senescent cells. For example, indirect evidence suggests that senescent cells express at least one additional growth inhibitor, which may belong to a family of basic helix-loop-helix transcription factors [25]. Moreover, the upstream regulators of p16 and p21 expression in senescent cells, which have not yet been identified, may induce additional growth-inhibitory genes in the cells.

Other phenotypic changes

The arrest of cell proliferation is clearly the most important feature of replicative senescence with regard to its role in tumour suppression. However, in addition to this essentially irreversible arrest of cell proliferation, senescent cells display two other striking phenotypic changes.

First, senescent cells acquire resistance to apoptotic stimuli [26]. The mechanism by which senescent cells resist apoptotic death is not well understood. Nonetheless, the fact that senescent cells are in fact resistant to apoptosis emphasises the distinction, often blurred in the literature, between cell death and cell senescence [27].

Second, senescent cells show sometimes striking changes in differentiated functions. The functions that are altered by senescence depend on the cell type. For example, senescent human fibroblasts and endothelial cells overexpress the inflammatory cytokine interleukin-1α [28], senescent endothelial cells overexpress the cell-specific adhesion molecule I-CAM [29], and senescent mammary epithelial cells overexpress the β isoform of the retinoic acid receptor [30]. Very little is known about the mechanisms that alter differentiated gene expression in senescent cells. However, as is the case with growth and differentiation in many cells, the senescence-associated changes in differentiation appear to be tightly linked to growth arrest.

The altered differentiation associated with replicative senescence can have rather profound consequences for cell and, at least in principle, tissue function. For example, presenescent dermal fibroblasts express low levels of collagenase and stromelysin, metalloproteases that degrade extracellular matrix proteins. They also express high levels of the metalloprotease inhibitors TIMP-1 and TIMP-3 (tissue inhibitor of metalloproteinases 1 and 3). Upon senescence, metalloproteinase expression rises and TIMP expression falls [31–33]. Thus, in dermal fibroblasts, replicative senescence entails a clear switch from a matrix-producing phenotype to a matrix-degrading phenotype. There are now a number of examples in which senescent cells elaborate cytokines, extracellular matrix-modifying enzymes, or other molecules that can have far-reaching effects on the micro-environment of neighbouring cells.

PHYSIOLOGICAL SIGNIFICANCE: CANCER AND AGEING

A priori, a process that limits cell proliferation is a candidate tumour suppressive mechanism. Thus, the idea that replicative senescence, which entails an irreversible growth arrest, curtails tumorigenesis is intuitively appealing. In addition, there are substantial cell and molecular biological data (discussed below) to support this idea. By contrast, the senescence-associated changes in differentiation may have little to do with tumour suppression *per se*. Changes in differentiated gene expression are often coupled to changes in growth state or potential. Thus, altered differentiation may be a by-product of the senescence-associated growth arrest. However, it may not be without physiological consequence.

Altered differentiation, more than arrested growth, may be important for the role of replicative senescence in ageing [3, 4]. The idea that cell senescence has a role in both tumour suppression and ageing may seem contradictory. However, evolutionary theories suggest that some traits that were selected to optimise health during the period of reproductive fitness can have unselected, deleterious postreproductive effects (see [34] for review). Thus, replicative senescence, by curtailing cell proliferation, may contribute to the relative freedom from cancer that is seen during the first half of life. Later in life, it may be deleterious because senescent cells accumulate in aged tissue [35], where their altered differentiation may compromise tissue function and integrity [3, 4, 36]. For example, the age-dependent loss of dermal collagen may be due to senescent fibroblasts, which accumulate in aged skin [35] and express an altered balance of collagenase and TIMPs [31–33]. An additional, but highly speculative, possibility is that senescent cells may actually contribute to carcinogenesis late in life by altering the micro-environment, which is paramount for maintaining normal cell growth and differentiation [36].

TUMOUR SUPPRESSION

What is the evidence that replicative senescence is a tumour suppressive mechanism? Firstly, cells that have a finite replicative life span are orders of magnitude less likely to form tumours than replicatively immortal cells. Moreover, many tumours contain cells that have an increased division potential or are immortal (see [6–8, 37] for review). There is no evidence that immortality is required for tumorigenesis (although it may be for metastasis). Rather, there appears to be a selection for cells with an increased replicative potential in the development of cancer. Immortality, of course, permits the extensive cell division that is needed to acquire the many mutations that are the hallmark of malignant tumours.

Secondly, a number of well-recognised oncogenes appear to act, at least in part, by allowing cells to escape replicative senescence (see [3, 6, 8] for review). These include mutated or deregulated cellular genes such as *TP53* or *C-MYC*, as well as the oncogenes of certain viruses that are implicated in some human cancers (for example, the human papilloma virus genes *E6* or *E7*, or the SV40 virus large T antigen gene). Thus, mutations that lead to tumorigenesis, or the strategies of oncogenic viruses, engage mechanisms that overcome replicative senescence.

Thirdly, among the genes that are essential for establishing and maintaining replicative senescence, are two well-recognised tumour suppressors: the *TP53* and *RB* genes.

p53 and/or pRb inactivation is the primary means by which viral oncoproteins extend the replicative life span of cells (see [3, 6, 8] for review). Moreover, inactivation of *TP53* or *RB* by antisense oligonucleotides also extends the replicative life span of human fibroblasts [38]. In the absence of either *TP53* or *RB*, human fibroblasts continue to proliferate beyond the population-doubling level at which they would normally senesce, but eventually they do senesce. In the absence of both *TP53* and *RB*, human fibroblasts become genomically unstable and either die or mutate to an immortal phenotype [3, 6–8, 36, 37]. There is a striking and extremely interesting distinction between human fibroblasts and certain epithelial cells in this regard. Inactivation of only *TP53* or *RB*, for example, by the papilloma virus oncoproteins E6 or E7, is not sufficient to immortalise human fibroblasts. However, E6 or E7 alone will immortalise certain epithelial cells (for example, mammary epithelial cells) albeit at a very low frequency [39, 40]. Thus, *TP53* and *RB* are essential for the normal replicative senescence of cells. Needless to say, *TP53* and *RB* are together the most commonly lost functions in human cancers.

THE ROLE OF TELOMERES AND TELOMERASE

Telomere shortening as the cell division counting mechanism

How do cells sense the number of divisions through which they have gone? As noted above, this number can easily exceed 50 cell cycles. Moreover, for a given culture, the number of doublings at which the entire cell population senesces is fairly reproducible. At present, telomere shortening is perhaps the most viable explanation for a cell division 'counting' mechanism [8, 37, 41].

As discussed elsewhere in this Special Issue, telomeres are the ends of linear chromosomes, consisting of the repetitive sequence TTAGGG in humans and other vertebrates, and specialised proteins. The telomeric sequence and its binding proteins form a distinctive structure [42] which prevents chromosome fusions, translocation and non-dysjunctions. Thus, telomeres are essential for maintaining the stability of eukaryotic genomes [43]. Because DNA polymerases are unidirectional and require a labile primer, each round of replication leaves some 3′ bases at the telomere unreplicated [8, 37, 41, 43]. Telomerase, a multimeric enzyme that adds telomeric repeats to chromosome ends *de novo* [44], is not expressed by most normal somatic cells. Thus, for most cells, the telomeres shorten with each cell cycle.

Telomere length is generally estimated from the terminal restriction fragment (TRF), which contains the telomeric TTAGGG tract plus telomere-like and non-telomeric sequences [45]. As expected, senescent human cells have shorter TRFs than their presenescent counterparts [46]. How much shorter? Germ cells, which express telomerase, have mean TRFs of 10–15 kb [45]. However, presenescent cultures from fetal or neonatal tissue generally have mean TRFs of 8–10 kb; TRFs in cells from older donors may be even shorter [46–48]. The mean TRF decreases by approximately 50 bp per doubling for cultured human fibroblasts [41, 49]. However, regardless of the starting length, the mean TRF declines to approximately 6 kb at senescence. Thus, initial mean TRF length is a good predictor of the replicative life span of cells [49]. Because TRF lengths vary substantially within a cell population, it has been proposed that senescence occurs when one or more TRF reaches a critical length [50]. This length is likely to be somewhat less than 6 kb, with perhaps most or all of the telomeric TTAGGG tract being lost from the critical chromosome.

Inactivation of *TP53* or *RB* allows cells to continue to proliferate, despite critically short TRFs [8, 38–40]. It is not known how these tumour suppressor proteins act in this capacity, but presumably they prevent cells from sensing, or allow cells to bypass, the signal(s) generated by a short telomere. As noted earlier, the majority of cells lacking functional p53 or pRb proteins do eventually senesce. There may be a minimal telomere length (TRF of approximately 2 kb) below which cell viability may be compromised. Thus, in the absence of telomerase or other mechanisms for maintaining telomere length (discussed below), cells that fail to senesce may be destined to die.

The idea that telomere shortening is the 'counting' mechanism that drives replicative senescence is attractive because it provides a plausible scheme, based on the known biochemistry of DNA replication, by which cells sense the number of completed divisions. It does not predict how a critically short telomere induces the senescent phenotype, nor does it necessarily predict that telomerase-expressing cells should not senesce. To date, however, it has not been possible to test critically this hypothesis by, for example, preventing telomere shortening in proliferating normal cells. However, certain oligonucleotides have been found to elongate the telomeres of immortal cells with relatively short telomeres; when fused to normal cells, these telomere-elongated immortal cells give rise to hybrids with a greater division potential than hybrids between normal and untreated immortal cells [51]. Because these oligonucleotides have no effect on the telomeres of normal cells, it remains to be shown that manipulation of telomere length alters the onset of normal cell senescence. Thus, despite its teleological appeal and an increasing body of indirect and correlative evidence, the telomere-shortening hypothesis of replicative senescence awaits critical testing.

The role of telomerase

As noted earlier, three cell types do not appear to undergo replicative senescence: the germ-line, certain stem cells, and many malignant tumour cells. These cells differ from most other cell types in that they express telomerase [52–58]. Thus, telomerase is undoubtedly important for unrestricted cell proliferation. However, telomerase is neither sufficient nor necessary for an unlimited or immortal replicative life span.

Firstly, normal human T-cells transiently express telomerase activity upon activation. Nonetheless, the telomeres of these cells shorten and the cells do senesce [59–62]. T-cells may not be unusual among normal somatic cells in that they express telomerase when stimulated to proliferate. Telomerase is also expressed, albeit at a low level, by cells in the proliferating basal layer of human skin [63]. However, the results in T-cells demonstrate clearly that telomerase activity *per se* can be insufficient to prevent telomere shortening and replicate senescence. The reason for this insufficiency is not known, although there are several possibilities. For example, the level of telomerase expressed by T-cells may be quantitatively inadequate to keep pace with the telomere shortening that occurs with each cell division. Alternatively, the enzyme or the telomeres may be modified such that access to the telomere or telomerase activity may be retarded or inhibited.

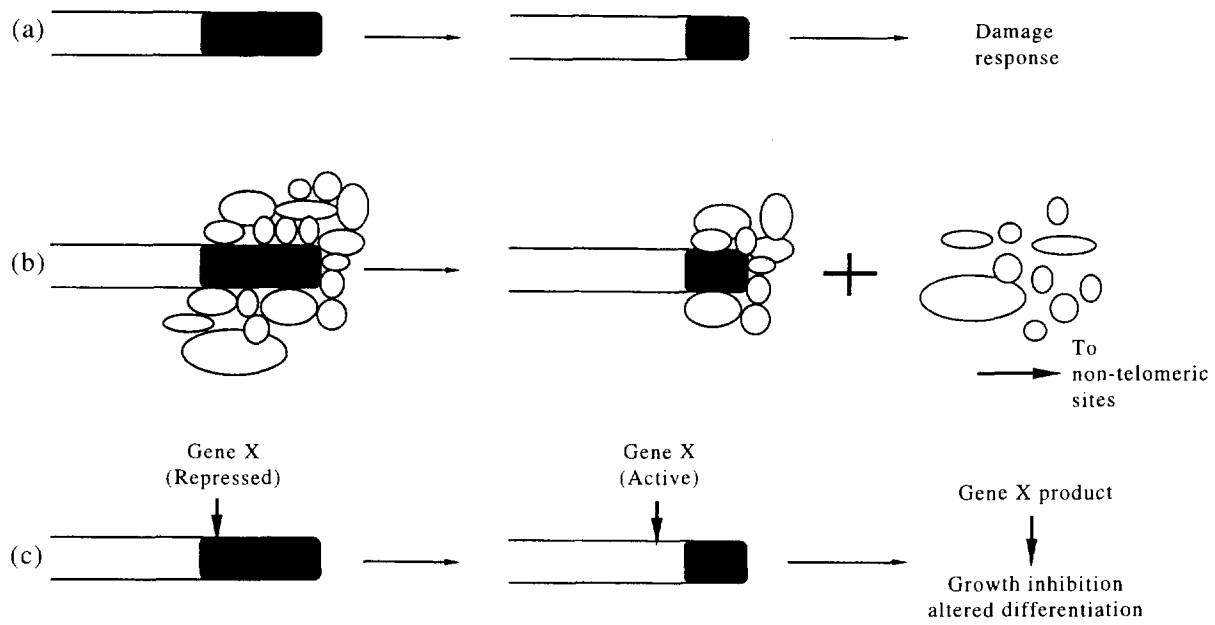

Figure 3. Three models by which a short telomere might induce the senescent phenotype. Chromosome ends are depicted, with the telomeric and immediate subtelomeric portions shown in black. (a) A shortened telomere resembles a double-strand DNA break, and thus triggers a DNA-damage response. (b) Telomeres sequester proteins that can bind to intragenomic sites, where they may activate or repress gene expression. As the telomere shortens, these proteins are freed. They may then induce or repress genes, such that growth is inhibited and differentiation is altered. (c) The field of heterochromatin surrounding the telomere shrinks as the telomere shortens. Genes within the heterochromatic region are repressed, and this repression is relieved upon telomere shortening. One or more of such genes may encode proteins that can arrest growth and/or alter differentiation.

Secondly, telomerase is not essential for maintaining telomere length or an unlimited division potential. Some human tumour cells do not express detectable telomerase activity, despite an immortal replicative life span [56, 64]. Such cells have frequently long or very heterogeneous telomeres. Moreover, some telomerase-expressing cells maintain long telomeres and an immortal phenotype even when telomerase is inhibited by reverse transcriptase inhibitors [65]. Thus, although many, if not most, tumour cells express telomerase [56], it is clear that both telomere length and an indefinite cell division can be maintained by a telomerase-independent mechanism.

Telomere shortening and the senescent phenotype

How might critically short telomeres induce the senescent phenotype? Currently, there is only speculation in mammalian cells. However, several hypotheses, based largely on experiments in lower eukaryotes, have been proposed. Of these, three are discussed here (Figure 3).

(a) A short telomere may signal a DNA damage response [14, 37, 66]. This hypothesis suggests that a critically short telomere triggers a DNA damage checkpoint from which cells cannot recover. In yeast, elimination of a telomere (from a dispensable chromosome) causes cell-cycle arrest that depends on a DNA damage–response pathway [67]. One problem with this hypothesis is that this growth arrest in yeast is transient, whereas the growth arrest of senescent mammalian cells is essentially irreversible. However, some forms of damage to mammalian cells (e.g. radiation, hydrogen peroxide) can induce permanent cell cycle arrest, although in these cases it is not known whether DNA is the critically damaged target. Moreover, the p53 protein level, which increases in the mammalian DNA damage response, is not elevated in senescent cells [68, 69]. In addition, if senescence is a damage response, immortalisation must overcome the damage-induced arrest and allow repair. However, cells reversibly immortalised by a conditional SV40 T antigen return to a growth-arrested state resembling senescence when the T antigen is inactive [66, 70, 71]. Finally, it is difficult to explain the altered differentiation of senescent cells as a simple DNA-damage response.

(b) The telomeres may bind or sequester transcription factors which can activate or repress a variety of genes. As the telomeres shorten, these factors would be available to bind intragenomic target sites, where they could act to repress or activate genes important for cell cycle progression and maintenance of differentiated function. There is a precedent for this idea in yeast, where the telomere-associated Rap1 protein has been shown to sequester silencing factors that can act at non-telomeric sites when they are not sequestered [72]. Although there is no structural homologue to Rap1 in mammalian cells, there may well be functional homologues. Although this hypothesis can in principal explain the complexity of the senescent phenotype, there is currently no molecular data to support it in mammalian cells.

(c) The heterochromatic structure of DNA near the telomere may repress regulatory genes (for example, transcription factors) that, in turn, act to arrest the growth and alter the differentiation of cells; as telomeres shorten, this field of heterochromatin should diminish, thereby derepressing these putative regulators [37, 66, 73, 74]. Again, there is strong precedent for the silen-

cing of loci near telomeres in yeast [75, 76], although far less evidence for such effects in mammalian cells. A related hypothesis suggests that loss of gene silencing or changes in chromatin structure independent of telomere length may induce the senescent phenotype [77]. In the yeast *Saccharomyces cerevisiae*, individual cells have a finite replicative life span, but the telomeres do not shorten during the life span [5]. Despite a constant telomere length, there is nonetheless a loss of silencing near at least one, but not all, telomeres [78]. Moreover, loss of silencing at non-telomeric loci also appears to occur during replicative ageing in yeast [79]. Whether and how the senescence of yeast, in which the cell is the organism, reflects the replicative senescence of individual mammalian cells is not yet clear. The idea that chromatin structure links telomere shortening with replicative senescence in mammalian cells awaits critical testing.

CONCLUSION

Replicative senescence prevents cells from dividing indefinitely, and appears to be a powerful, albeit imperfect, tumour suppressive mechanism. This process very likely also has the unselected evolutionary consequence of contributing to ageing. As such, the growth arrest associated with senescence may be the selected phenotype that suppresses tumorigenesis, whereas the altered differentiation of senescent cells may be the linked but unselected phenotype that contributes to ageing. Very little is known at a molecular level about how the growth arrest is linked to the altered differentiation. Nonetheless, there is strong circumstantial evidence that the length of the telomeres plays an important role in timing and establishing the senescent state, and that the cell's response to a critically short telomere is mediated by the *TP53* and *RB* tumour suppressor genes.

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