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Aging clock: the watchmaker's masterpiece

F. Ishikawa

Tokyo Institute of Technology, Department of Life Science, 4259 Nagatsuta, Midori-ku, Yokohama 226-8501 (Japan), e-mail: fishikaw@bio.titech.ac.jp

Abstract. The phenomenon of cellular senescence has been known for almost four decades. Yet, until very recently, the molecular mechanisms that lead to senescence have been poorly understood. However, substantial progress has been made in the last few years toward identifying the pathways executing senescence. This r view focuses on two major advances in this field, the telomere aging clock theory and the cell cycle regulatory mechanisms in senescent cells. These recent studies indicate that cellular senescence is a highly elaborate and active process, which presumably works as an anti-oncogenic mechanism.

Key words. Senescence; telomere; telomerase; $p21^{CIP1}$; $p16^{INK4a}$; $p19^{ARF}$.

Cellular senescence

Normal somatic cells do not proliferate indefinitely. This observation, called Hayflick's limitation, was first documented with normal fibroblast cells derived from human tissues in the 1960s [1]. Typically, fibroblasts derived from human embryos stop growing after about 50–80 PD (population doublings, a unit roughly representing the number of cell divisions). Moreover, fibroblasts derived from older people undergo fewer cell divisions than those derived from younger people before ceasing proliferation (however, this notion has recently been criticized in one well-controlled study [2]). Based on these observations, Hayflick postulated that this phenomenon reflects the aging processes in vivo, and named it cellular senescence. Senescent cells are alive and have an active metabolism (they do not die), but they fail to respond to any growth stimulus. This is not because they lose growth factor receptors or the initial signal cascades activated by growth factors [3]. Fusion between young growing cells and old senescent cells resulted in the inhibition of DNA synthesis of the hybrid cells [4, 5]. Similarly, the enucleate cytoplasm of senescent cells showed growth-inhibitory effects when fused with young cells [6, 7]. These results indicate that senescence is not a passive phenomenon, such as one caused by an error accumulation catastrophe. Indeed, a large body of evidence has suggested the presence of active mechanisms leading to senescence phenotypes. When two immortal fibroblast cell lines are fused with each other, in some, but not in all, cases, the resultant fused cells become mortal, showing senescence [8]. Using this assay, human immortal fibroblast cell lines could be classified into several complementation groups in terms of their ability to induce senescence upon fusion. These results suggest that the senescence phenotype is dominant to immortality, and that there must be active and, probably more than one, independent mechanisms in normal somatic cells to induce senescence.

Phenotypes of senescent cells

Senescent cells exhibit a number of characteristic phenotypes, including overall appearance, cell cycle status, gene expression, and miscellaneous markers. Senescent cells are arrested in G1, and typically have a large flat cytoplasm, leading to reduced nucleocytoplasmic ratios [9]. The cells are alive and actively metabolizing, but lose the ability to respond to growth stimuli and to start DNA synthesis. Despite this inability, they are able to express several genes that are normally activated during the G1 to S transition by growth stimuli. These mRNAs include c-myc, c-Ha-ras, ornithine decarboxylase (ODC), and thymidine kinase (TK) [10, 11]. Interestingly, senescent cells fail to induce expression of the G1/S-specific c-fos or S-specific histone genes upon serum stimulation. c-fos and c-jun proteins comprise the

AP-1 transcription factor, and AP-1 activity has been reported to be low in senescent cells [12]. Finally, senescent cells have been observed to express a unique enzyme activity called senescence-associated β-galactosidase (SA-β-gal) [13]. Although the biological significance of this activity is not known, it has been widely used as a marker for senescent cells. While the significance of these phenotypes remains unclear, our understanding of how senescent cells are arrested in G1 and are unable to accomplish S phase has recently advanced greatly, as will be discussed below.

Telomeres, an aging clock

When human fibroblast cell cultures were stored in liquid nitrogen, and restarted after a period of storage, the cells showed the level of senescence expected from the number of cell passages, but not based on the amount of chronological time, including the storage period, that had elapsed [1]. This result indicated that there must be a molecular apparatus in cells that counts the number of cell divisions undergone. Once cells have undergone a critical number of divisions, this molecular apparatus activates a signal leading to the senescence phenotypes. Theoretically, this process can be divided into three components: (i) an aging clock that records the history of proliferation, (ii) aging signal cascades that are activated by the aging clock when the countdown reaches zero, and (iii) effector molecules that arrest the cell cycle upon the activation of the aging signal. A strong candidate for the aging clock has emerged from seemingly unrelated biological systems.

The chromosomal ends, called telomeres, contain the physical ends of linear genomic DNAs. The DNA ends at the telomeres have biological properties distinct from those of non-telomeric DNA ends: telomeres protect genomic DNA ends from end-to-end fusion and exonucleolytic digestion. Researchers in the early 1970s predicted that conventional DNA polymerase does not completely synthesize the very ends of the telomeres during their lagging-strand synthesis (end replication problem) [14, 15]. After human telomeric DNA was identified as consisting of tandem repeat arrays of TTAGGG, the telomere lengths of individuals of different ages were measured, and the results lent support to the aging clock prediction. The average telomere lengths of young human somatic cells were calculated to be about 10–20 kb, whereas those of older people were 5–10 kb [16, 17]. This observation has been interpreted as showing that the telomere lengths of somatic cells are reduced as individuals age due to the end replication problem. Therefore, telomere lengths appear to be a good candidate for a recorder counting the cumulative number of cell divisions [15, 18].

Normal somatic cells suffer from the end replication problem, but germ cells and cancer cells do not. This is because telomerase, a specialized DNA polymerase synthesizing the telomeric DNA, is active in these cells. Telomerase was first identified in the ciliate *Tetrahymena* [19], and was later found to be present in humans [20; reviewed in ref. 21]. Human telomerase is active only in limited types of cells, including the germ cells, activated lymphocytes, primitive progenitor cells, and most types of cancer cell [reviewed in ref. 22]. However, until recently, the molecular components of human telomerase were not known. Telomerase is a reverse transcriptase that synthesizes telomeric repeats with the catalytic subunit using its intrinsic RNA template [23, 24]. Human telomerase consists of the RNA template, hTR (human telomerase RNA) [25], the catalytic component, hTERT (human telomerase reverse transcriptase) [26, 27], and the associated protein, hTEP1 (human telomerase-associated protein 1) [28, 29]. hTR and hTEP1 are expressed almost ubiquitously, and their expression levels are unrelated to the level of telomerase activity. In contrast, hTERT expression levels are highly correlated with the level of activity [26, 27, 30]. Furthermore, expression of both TERT and hTR is sufficient to reconstitute telomerase activity in vitro and in telomerase-negative cells [30, 31], underscoring the critical role of TERT in regulating telomerase activity. Based on these results, hTERT-transfected normal human BJ fibroblast cells and RPE-340 retinal pigment epithelial cells were examined for their telomere lengths and proliferation capacity. The cells stably expressed telomerase activity, and the telomere lengths were not shortened despite cell growth in some cases. Importantly, in these cases, the cells continued to grow beyond the time when the mock-transfected cells entered senescence [32]. Detailed analyses of these apparently immortalized human fibroblast cells revealed that they contained normal karyotypes and did not show any indication of a transformed phenotype [33, 34]. These results clearly show that human fibroblast cells whose telomeres are artificially elongated do not show senescence, indicating that shortened telomeres actually induce senescence [reviewed in ref. 35]. However, telomerase induction has not always resulted in the extension of proliferation capacity beyond senescence in human fibroblast cells [36], human foreskin keratinocytes, and mammary epithelial cells [37]. Obviously, factors other than telomere lengths are involved in triggering senescence phenotypes (see below).

Telomeres are not just the simple physical ends of DNA. They contain telomere-specific and non-specific proteins to accomplish the various telomere functions. One human telomere protein that has been well studied is the telomeric DNA double-stranded DNA (TTAGGG repeats)-binding protein, TRF (telomere re-

peat factor) [38]. Two types of TRF protein, TRF1 and TRF2, are known [39]. Both contain myb-like DNA-binding domains, but the N-terminal regions are significantly different, TRF1 possesses an acidic region, whereas TRF2 has a basic region. Over-expression of the full-length TRF1 caused telomere length reduction, and a dominant-negative TRF1 led to telomere elongation in the telomerase-positive human immortal cell line HT1080. Thus, TRF1 seems to control the accessibility of telomerase in vivo [40]. TRF2 over-expression resulted in different phenotypes. Over-expression of the full-length TRF2 had minimal effect. Notably, however, dominant-negative TRF2 over-expression caused growth arrest in 4–8 days, a phenotype not seen with TRF1-over-expressing cells [41]. These cells were SA-β-gal positive and showed a flat cell morphology, reminiscent of senescent cells. These results suggested that the telomere aging clock transduces the aging signal by changing the quantity or composition of telomere binding proteins.

Effector molecules of senescence

Since the hallmark of senescence is cell cycle arrest in G1, a number of studies have been conducted to characterize cell-cycle-controlling molecules in senescent cells. Retrospectively, the first hint that p53 and Rb have critical roles in cellular senescence came from the observation that SV40 infection reinitiates DNA synthesis in senescent fibroblasts [42, 43]. Further study demonstrated that the T antigen encoded by SV40 is responsible for the extension of life span [44, 45]. The T antigen inactivates both the Rb and p53 proteins. Therefore, inhibition of the Rb and p53 proteins seems to bypass senescence. p53 is activated either quantitatively [46, 47] or qualitatively [10, 48–51] in senescent fibroblast cells. p53 is a pleiotropic protein, but one of its major functions is activating the expression of $p21^{CIP1}$, a general inhibitor of cyclin-dependent kinase (cdk), as a transcription factor. $p21^{CIP1}$ expression is consistently up-regulated in senescent human cells [52–54]. The Rb protein has a key role in driving the cell cycle from G1 to S phase. In G1, Rb is hypophosphorylated and bound with E2F, a transcription factor activating genes critical in the G1–S transition. However, E2F bound with hypophosphorylated Rb is inactive, since Rb tethers histone deacetylase that represses gene expression [55, 56]. Rb kinases, cyclin D/CDK4 and CDK6 complexes phosphorylate Rb and liberate E2F as an active form. Therefore, the cyclin D/CDK 4 and CDK6 complexes regulate the G1 to S transition through indirect activation of E2F. $p16^{INK4a}$ is a cyclin D/CDK4, CDK6-specific cdk inhibitor, whose up-regulation leads to G1 arrest [reviewed in ref. 57]. $p16^{INK4a}$ accumulates in senescent cells [54, 58]. Consequently, Rb phosphorylation levels are reduced in senescent cells [48, 59, 60]. Thus, both $p21^{CIP1}$ and $p16^{INK4a}$, each representing the p53 and Rb pathways, are activated in senescent cells. $p21^{CIP1}$ is almost ubiquitously expressed in a variety of tissues, but $p16^{INK4a}$ expression is limited to a small number of cell types, including senescent cells [61]. When the kinetics of $p21^{CIP1}$ and $p16^{INK4a}$ accumulation during the transition of young cells to senescence was analyzed, $p21^{CIP1}$ expression appeared to have increased initially, but later declined when senescent cells became established, whereas $p16^{INK4a}$ expression maintained high levels until senescence [61]. These results point to the more specific relationship between senescence and $p16^{INK4a}$, rather than $p21^{CIP1}$. In normal human fibroblast cells, ectopic expression of $p16^{INK4a}$ induced growth arrest and certain features characteristic of senescent cells, including positive β-galactosidase staining and up-regulation of plasminogen activator inhibitor-1 (PAI-1) expression, but $p21^{CIP1}$ induced these features to a much lesser extent [54]. This senescence-inducing activity of $p16^{INK4a}$ depends on functional Rb [62].

The relative contributions of the $p16^{INK4a}$-Rb and the p53-$p21^{CIP1}$ pathways to senescence seem to vary among different species and tissue types of senescent cells. Addition of an anti-sense Rb, but not p53 oligonucleotide to near-senescent human fibroblasts extended the life span by about ten PD [48]. Inclusion of both anti-p53 and Rb anti-sense RNAs produced an additive effect, extending the life span by about 20 PD. However, all the cells eventually underwent senescence. Addition of both (but not either alone) HPV16 E6 and E7, which inactivate p53 and Rb, respectively, restarted cell growth in senescent human fibroblasts [63]. However, in human mammary gland epithelial cells, p53 inactivation by dominant-negative p53 transfections alone bypassed senescence [64]. In contrast, E7 but not E6 extended to some extent the proliferation capacity in human foreskin keratinocytes [65]. Similarly, in uroepithelial cells, $p16^{INK4a}$ expression levels were elevated, and the ability to bypass senescence correlated with either the lack of expression of $p16^{INK4a}$ or Rb, but not with p53 status [66].

Another player in G1 arrest of senescent cells

Recently, $p19^{ARF}$ (alternative reading frame) has been shown to be produced from the *INK4a* locus using exon 1b, an alternative exon 1 present in the 5′-upstream region of that used by $p16^{INK4A}$. Both products of the *INK4a* locus, $p16^{INK4A}$ and $p19^{ARF}$, are present at negligible levels in mouse embryos and young mice, and are induced in older mice [61]. When embryos were

explanted in vitro, and mouse embryo fibroblasts (MEFs) were established, $p16^{INK4A}$ was immediately induced and further accumulated as MEFs were passaged and senesced, suggesting its pivotal role in cellular senescence.

Ectopic expression of $p19^{ARF}$ results in G1 or G2 arrest in mouse cells [67] in a p53-dependent manner [68]. $ARF^{-/-}$ mouse MEF cells, which are deleted for the $p19^{ARF}$-specific exon 1b but still express $p16^{INK4A}$, continue to grow without showing senescence, while normal mouse MEFs cease proliferation (senescence) at about 20 passages [68]. This result underscores the involvement of $p19^{ARF}$ in establishing senescence in MEFs. Immortal cell lines derived from wild-type mouse MEFs showed the loss of either both *ARF-INK4a* alleles or both p53 alleles [61]. When immortal cell lines spontaneously arising from $ARF^{+/-}$ MEFs were analyzed, the normal allele was found to have been replaced by the knockout allele lacking the $p19^{ARF}$ -specific exon 1b, but still expressing $p16^{INK4a}$, leading to $ARF^{-/-}$. These results imply that the inactivation of either p53 or $p19^{ARF}$ alone with the intact $p16^{INK4A}$ is sufficient to bypass senescence in MEFs. However, this does not exclude the possibility that $p16^{INK4A}$ has a role in senescence.

Other clocks triggering senescence

Several miscellaneous stimuli, other than the accumulated cell division number, have been previously reported to cause senescence. These stimuli include phosphoinositide 3-kinase inhibitors [69], DNA damage induced by chemotherapeutic reagents [70, 71], and psolaren cross-linking [72]. Little is known about the molecular signals leading to senescence-like phenotypes in these settings. Recently, however, oncogenic Ras has been shown to induce a premature senescence-like phenotype in primary human cells [73]. The molecular mechanisms of Ras have been extensively studied, thus providing a new approach to senescence biology. H-ras, which has a point mutation at codon 12 (glycine), has long been recognized as an oncogene transforming both human and murine cells. However, oncogenic ras alone does not render normal cells transformed, and another class of 'immortalizing oncogenes,' such as the over-expressed myc gene, is required for malignant transformation. These interations between ras and myc in tumorigenesis have been accepted as a 'two-step model' for the transformation of normal cells, and both myc and ras oncogenes have been recognized as producing mitogenic signals. However, Lowe and his colleagues found that ectopic expression of the oncogenic ras gene in normal human fibroblast cells resulted in growth arrest, instead of proliferation [73]. These cells showed several features reminiscent of senescence. The growth arrest was irreversible: once cells ceased dividing, they never resumed proliferation, even when ectopic expression of the oncogenic ras was stopped in an inducible system. The cells were large and flat, and were SA-β-gal positive. However, some features that are not shown by typical senescent cells were noted, for example, cells were arrested in both G1 and G2, not exclusively in G1. The Ras protein is known to relay its signal to a set of distinct downstream pathways, including the Ras-Raf-Mek-Erk (MAPK) pathway, PI3-kinase, and the Ral G protein family. Detailed analyses revealed that activated Raf and Mek also induce premature senescence, while other pathways did not. Therefore, constitutive activation of the Ras-Raf-Mek-Erk pathway can induce a senescence-like phenotype in normal human cells [74, 75]. These activities are dependent on both the $p19^{ARF}$-p53-p21 pathway and the $p16^{INK4a}$-Rb pathway, because the oncogenic ras induced proliferation instead of senescence in cells lacking p53 or $p16^{INK4a}$. Therefore, the outcome of Ras signaling is the opposite, senescence or proliferation, depending on the presence of p53 and Rb. One striking feature of the senescence induced by oncogenic ras is that the cells first proliferated for 2 days upon induction of oncogenic ras expression, but then ceased proliferation. Therefore, senescence is most likely a reactive phenotype to the initial cell growth induced by the activated Ras-Mek signaling. This cell response is mediated by p53 and Rb. In this sense, the senescence activated by Ras was proposed to represent a safeguard mechanism counteracting excessive cell growth and the development of tumor cells. Ras-activated senescence seems unrelated to the telomere aging clock. However, diverse signals such as those derived from excessive proliferation (ras-induced senescence), and accumulated cell division numbers (telomere-induced senescence), possibly constitute a single p53- and Rb-dependent pathway to induce the common phenomenon of senescence. Indeed, the idea that cellular senescence is a tumor-suppressive mechanism that limits the growth potential of transformed cells is not unprecedented. In the absence of senescence, early transformed cells will grow so extensively that telomeres will be reduced to an insufficient size. Chromosomes lacking functional telomeres are particularly unstable and will contribute to further transformation by producing abnormal chromosomes, such as those lacking other anti-oncogenes [76]. Thus, senescence can be viewed as a tumor-suppressive mechanism monitoring both proliferation speed (ras-induced senescence) and extent (telomere-induced senescence).

Future prospects

Significant progress has been achieved in the last decade in our understanding of the molecular mechanisms of senescence. In the near future, we will certainly come to understand more about how the aging clock, the watchmaker's masterpiece, operates. However, several key questions remain to be answered. First, it is not clear whether 'senescence phenotypes' in different systems represent a common biological phenomena. Obviously, diagnostic markers routinely used to demonstrate senescence, such as a flat and large cytoplasmic morphology and SA-β-gal activiy, are not ideal, because they do not provide any information about the underlying molecular events. Second, the extent to which cellular senescence might cause the senescence of individuals is totally unknown. In terms of medical application, any link between cellular senescence and pathological conditions in particular tissues and organs needs to be clarified [77]. Finally, one of the ultimate goals is to understand why we all have the aging clock, making senescence inevitable.

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