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INSIGHTS FROM MODEL SYSTEMS
The Genetics of Cellular Senescence

Nathalie G. Bérubé,[1] James R. Smith,[1,2] and Olivia M. Pereira-Smith[1,2]

[1]Roy M. and Phillis Gough Huffington Center on Aging, Division of Molecular Virology, and [2]Departments of Cell Biology and Medicine, Baylor College of Medicine, Houston

The Phenomenon of Cellular Aging

Human aging is a highly complex process, resulting from a number of small changes that differ from tissue to tissue. Several major obstacles have interfered with the study of aging in whole organisms. These include the genetic heterogeneity between individuals and the difficulty in distinguishing the consequences of normal aging from the effects of diseases that occur throughout life (see Schächter 1998 [in this issue]). For these reasons, human cells grown in culture offer a simplified and attractive model with which to study cellular processes involved in aging. More than 30 years ago, Hayflick and Moorhead reported that diploid fibroblasts undergo a finite number of cell divisions, after which they stop proliferating. This phenomenon was equated with normal cellular aging and was termed "replicative senescence" (Hayflick 1965). Various other cell types have since been found to undergo replicative senescence, including epidermal keratinocytes, smooth-muscle cells, lens epithelial cells, glial cells, endothelial cells, melanocytes, T lymphocytes (see Effros 1998 [in this issue]), and adrenocortical cells.

What defines replicative senescence? Cells having completed a finite number of divisions in culture become irreversibly growth arrested in the G1 stage of the cell cycle. A distinctive feature of senescent cells is that they persist in this state from months to as long as several years, remaining metabolically active but incapable of DNA synthesis (Matsumura et al. 1979). This block in cell-cycle progression in senescent cells is irreversible and is not associated with programmed cell death. Senescent cells also undergo morphological changes that include enlargement and flattening of the cells and an unexplained expression of a β-galactosidase activity at pH 6 (Dimri et al. 1995). These criteria are few, in part because many changes that occur in senescence are also seen in the reversible quiescent state that some young cells undergo in the absence of growth factors.

The role of replicative senescence in aging of the organism remains controversial, but correlative evidence suggests that it is an in vitro manifestation of an in vivo phenomenon. First, the replicative capacity of cells cultured from old donors is less than that of young donors, suggesting that cells keep track of the number of cell doublings that they have undergone in vivo. Second, the replicative capacity of cells is roughly proportional to the maximum life span of the animals from which they originated. A third line of evidence comes from the study of diseases, such as Werner syndrome (WS), that mimic premature aging. Fibroblasts obtained from WS patients senesce much earlier than normal age-matched controls (Norwood et al. 1979). The WS gene (*WRN*) has recently been cloned and displays homology to some DNA helicases, although all mutations thus far identified map outside the helicase region, suggesting other possible functions for this protein (Yu et al. 1996).

Two predominant models have been suggested to explain the loss of proliferation in senescence. The first proposes that cellular aging results from the accumulation of errors that occur, perhaps, as a result of either impaired DNA repair mechanisms or inadequate free radical–scavenging abilities. Thus, the cell is seen as passive in the aging process, although it may be able to regulate the process by repairing or removing damaged cellular components. The second model proposes that cells age according to an intrinsic genetic program and, hence, suggests that cells are active participants in their own aging. The majority of the data described below implicate genetic factors in the activation of cellular senescence.

Cellular Senescence Is Genetically Dominant: Somatic Cell–Hybrid Studies

We have exploited clonal cell strains with unusually long life spans, in order to study the genetics of proliferative potential in somatic cells (Smith and Pereira-Smith 1996). We found that fusions between young and old cells yield hybrids with life spans similar to that of the old parental cells, suggesting that the senescent phe-

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Address for correspondence and reprints: Dr. Nathalie G. Bérubé, Huffington Center on Aging, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030-3498. E-mail: nberybe@bcm.tmc.edu

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notype is dominant. Moreover, fusion between old cell populations does not produce hybrids with a life span greater than that of either parental cell line, providing further evidence for dominant-negative regulatory mechanisms causing senescence. Confirmation of the dominance of the senescent phenotype came from the fusion of various immortal and normal (mortal) human fibroblasts, resulting in hybrids with a limited doubling potential. The same outcome was observed when different immortal cell lines were fused with highly differentiated cell types, such as normal human T cells and endothelial cells (Pereira-Smith et al. 1990). Together, these data support a genetic basis for cell aging and indicate that immortal cells arise because of recessive mutations that allow them to escape senescence. Furthermore, it suggests that senescence is controlled by a common mechanism(s) in very different cell types.

Pathways Leading to Senescence: A Genetic Approach

To establish how many distinct genes play a part in cellular senescence, a series of immortal human cell lines were fused with each other. If they possessed the same recessive defects, the resulting hybrids would be expected to be immortal, and the cell lines would be assigned to the same complementation group for indefinite division. However, fusion of immortal cells with different recessive mutations would result in a limited proliferative potential, thus corresponding to different complementation groups. By means of this approach, >40 different immortal cell lines have been assigned to four complementation groups for indefinite division, groups designated "A"–"D" (Smith and Pereira-Smith 1996), indicating that at least four genes are required for the induction of senescence.

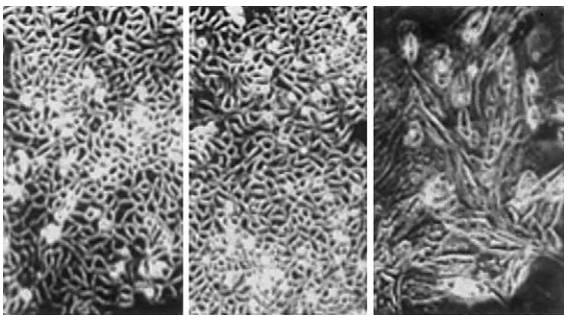
The demonstration of different complementation groups provided the tools for a more focused analysis of the genetic pathways of cell aging. One approach was to identify specific chromosomes encoding senescence-inducing genes. By definition, these genes should be capable of complementing the immortal phenotype of cell lines assigned to the same group, but not of cells of the other groups. Microcell-mediated chromosome transfer allowed the introduction of single human chromosomes into immortal human cell lines assigned to the different complementation groups (Smith and Pereira-Smith 1996). Three candidate chromosomes were initially tested because of their prior association to tumor-suppressive mechanisms. Whereas chromosome 11 demonstrated no senescence-inducing effects, chromosomes 1 and 4 reversed the immortal phenotype in complementation groups C and B cell lines, respectively (Smith and Pereira-Smith 1996). This effect was group specific in that either the introduction of human chromosome 4 into cell lines assigned to complementation groups A, C, or D, or the introduction of chromosome 1 into groups A, B, or D had no effect on proliferation. Ogata et al. (1993) reported that human chromosome 7 suppressed proliferation of two human transformed fibroblast cell lines, SUSM-1 (complementation group D) and KMST-6 and followed with a complete analysis of cell lines assigned to all the groups (Ogata et al. 1995). Together, these results suggested that chromosomes 1, 4, and 7 encode senescence genes inactivated in cell lines of complementation groups C, B, and D, respectively. A chromosome able to complement the group A defect has not yet been identified by such genetic analysis, although chromosome 6 has been found to induce senescence in one cell line assigned to this group (Sandhu et al. 1994). Other chromosomes, including 2, 3, 10, and X, were also shown to induce senescence in various cell lines (see review by Oshimura and Barrett [1997]), but the specificity of their effects has not been shown. The strength of using cell lines already assigned to complementation groups is that it allows one to test whether multiple cell lines within a group respond to the introduction of a particular chromosome by losing proliferation but without having any effect on cell lines assigned to the other groups. This criterion is essential to establish that the chromosome is complementing a specific genetic lesion rather than merely suppressing its effects.

Once a chromosome encoding a senescence gene has been assigned to a complementation group, a smaller region must be identified that causes the same phenotype. This can be achieved in multiple ways. One approach is to obtain naturally occurring immortal clones on continuous subculture of microcell hybrids and to analyze the revertant clones for common deletions of a chromosomal region. This approach was successful in the identification of two candidate regions, at 1q25 and 1q41-42, for a senescence gene on chromosome 1 (Karlsson et al. 1996; Vojta et al. 1996). Alternatively, radiation can be used effectively to generate radiation-hybrid panels containing different fragments of the tagged chromosome. This step is crucial to increase the resolution to a few megabases of DNA so that a genomic contig can be assembled that covers the entire region of interest.

During the past few years, we have focussed our study on identification of the senescence gene encoded on human chromosome 4 (see sidebar). Fortunately, a smaller fragment of the q arm was isolated in a mouse A9 background (A9+4), without the need to establish a radiation-hybrid panel. This small chromosomal fragment was shown to induce a senescent phenotype when transferred into group B cell lines, reproducing the earlier results observed with the entire chromosome. This effect was specific to group B cell lines in that no effect on proliferation was observed in the cell lines of groups A, C, and D. A bacterial artificial chromosome (BAC) con-

Four ways to achieve mortality

Cellular senescence is the terminally nondividing state that all normal cells enter after a limited number of divisions. Cells that fail to undergo senescence—that is, immortal cells, such as the human cell line HeLa—are convenient tools in cell biology. Virtually all spontaneously arising immortal cell lines are karyotypically abnormal. When these cells are fused with normal cells that can undergo senescence, the hybrid cells produced regain normal patterns of growth control, and they eventually stop dividing. This suggests that immortal cells are defective or lacking in expression of senescence-related genes and that this defect is complemented by fusion with the normal cell. As described in the main text, we have used a similar somatic-cell genetic approach to define four "complementation groups," corresponding to at least four different genetic defects that lead to indefinite division. When cell lines from different complementation groups are fused, the two genomes complement each other's defects, allowing the hybrid to senesce after some number of divisions. All of the immortal human cell lines that we have tested fall into one or another of these groups. In principle, each of the four genetic lesions could be complemented not just by whole-cell fusion but also by introduction of individual chromosomes or even defined genes; this provides a ready strategy for cloning of senescence genes by complementation.



Senescent cells are defined by their inability to proliferate, which can be a tedious phenotype to determine, but, generally, they can be identified at a glance by their morphology. Senescent cells become large and flat as they exit the cell cycle. Depicted above are cultures of HeLa cells in the presence or absence of a rescuing chromosome. The left panel is a phase micrograph of these immortal cells as they normally grow on tissue-culture plastic: small and tightly packed, like tiles in a mosaic. The middle panel shows HeLa cells with an extra copy of chromosome 11, which fails to rescue the defect in these cells, so they appear and divide as before. However, cells that carry an extra, wild-type copy of chromosome 4 can exit the cell cycle and persist for long periods without dividing. The unusual morphology of these senescent cells is seen in the right panel. This finding was a key step toward the cloning of the senescence gene that is defective in HeLa cells, because it directed our attention to chromosome 4. We have now identified this gene, which we name "*MORF4*."

tig was constructed that covered the region of interest, initially estimated to measure 500–800 kb of DNA (M. J. Bertram, N. J. Bérubé, X. Hang-Swanson, Q. Ran, Y. Ning, unpublished data). By means of this genomic DNA in standard cDNA selection strategies, five cDNAs were identified that mapped to the region encoding the senescence gene. The final step consisted of the transfer of these candidate genes into group B immortal cell lines, to assess their capacity to induce senescence. This was observed for one of the genes, which we named "MORF4" (for mortality factor 4 [M. J. Bertram, N. J. Bérubé, X. Hang-Swanson, Q. Ran, Y. Ning, unpublished data]). Further analysis of this gene and its protein product are underway and should provide insights into the mechanisms of cellular aging. This analysis also demonstrates the efficacy of a genetic approach for the identification of senescence genes.

The Tumor-Suppressor Connection

The loss of growth potential observed in senescent cells is reminiscent of tumor-suppressive mechanisms. In fact, many well-characterized tumor suppressors—such as pRb, p53, p21, p16, and, more recently, p33ING1—have now been linked to the onset of cellular senescence. Although they may initially exert a protective effect on cells by limiting their growth potential, the random loss of these genes that is incurred by old age may lead to increased proliferation, and, eventually, to cancer development.

Two of the well-known tumor-suppressor genes, *P53* and *RB*, participate in replicative senescence. Decreasing or eliminating the activities of either of these gene products generally has minor effects on the proliferation potential of normal human fibroblasts. However, inactivating both simultaneously can cause significant (>20%) increases in the number of population doublings achieved. Dual inactivation has been accomplished by expression of SV40 T antigen, by expression of the human papilloma-virus proteins E6 and E7, and by use of antisense oligonucleotides to p53 and Rb RNA (reviewed in Campisi 1997). In one case, inactivation of p53 by the introduction of a dominant-negative form of p53 resulted in an extension of replicative life span, of ~16 population doublings (Bond et al. 1995). However, it is not known what other events, in addition to inactivation of p53, might have resulted from the expression of the dominant-negative p53 molecule.

A plausible role for Rb in cellular senescence can be inferred from the increased expression of the cyclin dependent kinase (CDK) inhibitors p21 and p16. Hypophosphorylated Rb acts directly and indirectly as a transcriptional repressor. The indirect action of Rb is due, at least in part, to the sequestration and inactivation of the transcription factor E2F. Active E2F is necessary for

the induction of several genes required for the initiation and maintenance of the S phase; examples include the genes for dihydrofolate reductase, cyclin A, thymidine kinase, and DNA polymerase alpha (Campisi 1997).

A role for p53 is less evident. Although p53 activity has been reported to increase with in vitro aging (Atadja et al. 1995), the evidence is not consistent with results from other studies. Although a basal level of p53 appears to be required for normal replicative senescence, it does not appear to be responsible for the increase in p21 and p16 CDK inhibitors, which has been discussed above.

ING1 has been identified as a new potential player in cell senescence. It was first cloned as a tumor-suppressor gene encoded on 13p33-34 (Garkavtsev et al. 1997). Suppression of ING1 mRNA in normal cells induces a tumorigenic phenotype, whereas its overexpression leads to a G1 arrest akin to senescence (Garkavtsev et al. 1996). Confirming its role in senescence are the fact that (*a*) the protein product p33ING1 increases several fold as the cells age in culture and (*b*) expression of antisense ING1 RNA in presenescent fibroblasts results in extension of life span (Garkavtsev and Riabowol 1997). It is not yet clear how all these gene changes interact or contribute to senescence.

The Telomere Hypothesis

Cells undergo a limited number of divisions in culture, implying the use of a counting mechanism for cell division. Various data demonstrate that the number of population doublings, not time in culture, is the basis of senescence. Several hypotheses might explain this phenomenon, but the most attractive at this time is derived from the observation that telomeres shorten as the cells age in culture, because of incomplete replication of chromosome ends at each cell division. Human telomeres consist of TTAGGG-sequence tandem repeats; it is proposed that cells can sense the length of these hexanucleotide tracts and, hence, can "count" how many divisions have occurred (Shay and Wright 1991; Harley and Villeponteau 1995; Levy et al. 1992). Supporting this hypothesis is the fact that most somatic cells (except for germ cells and certain stem cells) lack the enzyme telomerase, which is responsible for the maintenance of telomeric repeats, whereas most immortal cells have reactivated this enzyme (reviewed in Holt et al. 1997). By the addition of telomeric repeats to chromosomal ends, telomerase is thought to stabilize the chromosomes and to prevent chromosomal degradation, fusions, and translocations resulting from excessive telomere shortening (Greider 1990). A more comprehensive description of telomerase biochemistry and mode of action has been described by Morin (1997).

Several possible mechanisms by which shortened telomeres might trigger senescence have been hypothesized. One possibility is that telomeres bind transcription factors that are released on loss of telomeric repeats and that are free to either activate senescence-inducing genes or inactivate genes necessary for cell-cycle progression. Another possibility is that telomere shortening somehow induces DNA damage responses leading to cell-cycle arrest. Alternatively, a gene-silencing mechanism has been proposed, whereby telomeres maintain the surrounding chromatin in a heterochromatic structure that disappears on the loss of the telomere. Consequently, genes in that region become expressed and are able to induce a senescent phenotype. These possibilities have been discussed in more detail in a recent review (Campisi 1997).

There are limitations to the telomerase model, because it does not readily extrapolate to other organisms. For example, mice have very long telomeres, and telomerase is present in their somatic cells. Mice lacking an essential subunit of telomerase are still able to undergo malignant transformation (Blasco et al. 1997). New data in human models also cannot easily be reconciled with the telomerase hypothesis. Thus, telomerase-negative immortal human cell lines have been reported (Bryan and Reddel 1997), as have telomerase-positive normal somatic cells with telomeres that continue to shorten with rounds of replication. Additionally, telomerase activity in somatic-cell hybrids does not correlate with their ability to undergo senescence or proliferate, suggesting that telomerase expression is not strictly necessary for immortalization and that its absence is not strictly sufficient for the induction of senescence. However, it recently has been shown that the transfection of the telomerase reverse-transcriptase–subunit gene (hTRT) in cultured fibroblasts extended their life span, further strengthening a role for telomerase in human cellular senescence (Bodnar et al. 1998). At the time of publication of the study's results, the number of extra doublings induced by hTRT was ~30 population doublings, which is the same as that observed in the inactivation of Rb and p53. It will be of interest to know both how many more population doublings these transfected fibroblasts will achieve and whether the ploidy of the cells will remain normal.

Conclusions

The study of in vitro cellular senescence has proved useful as a model to establish a genetic basis for human aging and continues to be used to resolve the molecular details of the gene pathways involved. The implications that mechanisms of tumor suppression have for cell senescence are becoming obvious and could also explain the phenomenon of reduced growth potential, which occurs in some tissues and organ systems with age. Ex-

amples include a decline in immune response with aging, resulting from a decreased proliferative response of T cells to antigen, and the reduced proliferation of osteoblasts, perhaps leading to osteoporosis. The identification of the genes involved in senescence will allow us to determine whether such changes accompanying human aging are in fact due to programmed senescence pathways at the cellular level.

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