fenses together with the MLS extension by the overexpression of Cu,Zn–superoxide dismutase and catalase in transgenic *Drosophila* implicate specific genes in the control of oxidative stress and aging. The extension of MLS by experimental regimens such as CR in mammals and hypometabolic states in poikilotherms, which decrease the rates of ROM generation, point toward the involvement of environmental-genetic interactions in the governance of longevity. The recent finding that the life-span extension of Clk mutants of *Caenorhabditis elegans* is associated with a hypometabolic phenotype is consistent with this concept (58). Although the present discussion has focused on the damaging effects of ROMs, it has been extensively documented that ROMs also modulate gene expression (59), which further broadens their possible role in the aging process.

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Replicative Senescence: Implications for in Vivo Aging and Tumor Suppression

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Normal cells have limited proliferative potential in culture, a fact that has been the basis of their use as a model for replicative senescence for many years. Recent molecular analyses have identified numerous changes in gene expression that occur as cells become senescent, and the results indicate that multiple levels of control contribute to the irreversible growth arrest. These include repression of growth stimulatory genes, overexpression of growth inhibitory genes, and interference with downstream pathways. Studies with cell types other than fibroblasts will better define the role of cell senescence in the aging process and in tumorigenesis.

A variety of human and other animal cell types can now be grown in the laboratory, and the majority of these cell cultures have a finite ability to proliferate. After accruing a number of population doublings, they enter the terminally nondividing state referred to as replicative senescence. This phenomenon was first described for normal human fibroblasts (*1*) but has now been observed for a variety of other types of human cells (*2*, *3*), as well as fibroblasts from other species (*4*). Cell cultures do not die after

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entering senescence but remain viable for years if maintained with weekly changes of culture medium (5). Senescence is determined by the number of times the cells divide, not by calendar time (6). Senescent cells accumulate gradually in culture, and very young cultures of cells always contain a small percentage of senescent cells. This percentage increases through the life-span of the culture until all the cells become senescent (7).

Cells can withdraw from the cell cycle and become nondividing for a number of reasons that do not necessarily involve replicative senescence, such as DNA damage or terminal differentiation. For example, melanocytes can go through many rounds of division to replicative senescence. However, if young cultures of melanocytes are exposed to cyclic AMP (adenosine 3′,5′-monophosphate)–inducing agents, the cells achieve only four to six additional rounds of division before they become nondividing and express large amounts of melanin. This state has been described as terminal differention. Analyses of senescent and terminally differentiated melanocytes have revealed common, as well as distinct, alterations in gene expression. Both cell types are unable to phosphorylate the ERK2 protein and contain elevated concentrations of the cyclin-dependent kinase (CDK) inhibitor p21, but only terminally differentiated cells express large amounts of the CDK inhibitor p27 and the melanocyte-specific transcription factor MITF (3). These results indicate that the different states of loss of division are not the same. When human fibroblasts are treated with high concentrations of agents such as hydrogen peroxide or ceramide, they stop dividing and become enlarged similar to senescent cells. This has been interpreted as the induction of senescence (8). However, until changes in gene expression and activity in cells treated with such agents have been compared with those observed in senescent cells, the relation to replicative senescence remains unknown.

The stringency with which cells maintain finite proliferative potential varies with species. There are no confirmed reports of human or chick fibroblasts from normal donors spontaneously immortalizing. In contrast, fibroblasts of rodents, particularly mice, spontaneously immortalize at a high frequency (9). Although human cells can be immortalized by DNA tumor viruses, and by repeated treatment with ionizing radiation or carcinogens, this occurs at a very low frequency when compared with rodent cells (10). Because cellular aging is dominant over cellular immortalization (see below), this difference in immortalization rate may be related to the frequency with which recessive genetic events occur in the different species.

The cell senescence phenomenon can be delayed, but not reversed, by the expression of DNA tumor viral genes [such as simian virus 40 (SV40) T antigen], by treatment with antisense oligonucleotides to the tumor supressor genes *p53* and *RB1*, or by expression of a dominant-negative *p53* mutant (11). The treated cells will proliferate for 10 to 20 more doublings than control cells, but they eventually enter senescence, or in the case of SV40-transformed cells, crisis. However, these manipulations must be performed before the cell culture becomes senescent, because senescent cells cannot be induced to divide (12). SV40 T antigen will induce senescent cells to initiate DNA synthesis, as measured by tritiated thymidine uptake; however, the cells fail to enter mitosis (13). These data indicate that senescent cells are truly terminally nondividing. The fact that cell cultures derived from human tumors often can divide indefinitely suggests that escape from senescence does occur in vivo but, on the basis of the above facts, must be a rare event. It is for this reason that cellular senescence has been proposed as a tumor suppressor mechanism (14).

The Relation of in Vitro to in Vivo Cellular Aging

A major question has been whether the study of in vitro cellular aging can help elucidate the basic processes of aging in vivo. Many investigations have established a link between aging in vivo and the proliferative potential of cells in culture. The growth capability of fibroblasts (15), vascular endothelial and smooth muscle cells, T lymphocytes, and a variety of epithelial cell types (2) has been shown to decrease with increasing donor age. Cells from patients with Werner syndrome, a premature aging disorder, achieve fewer divisions before becoming senescent than cells from normal individuals of the same age (15, 16). The proliferative capacity of fibroblast cells is also directly related to the maximum life-span of the species from which they are derived (3, 17). Cells from the longest lived species tested, the Galapagos tortoise (maximum life-span of more than 100 years), achieved up to 130 population doublings before senescence, whereas those from the mouse (maximum life-span of 3 years) could undergo only 10 or fewer doublings before becoming senescent or immortalizing in culture.

Recently, a clear demonstration that cell senescence does indeed occur in vivo was reported. The marker used was a novel β-galactosidase activity that can be detected at pH 6.0 in senescent cells, but is absent in proliferating young cells and, more importantly, absent in young cells made reversibly nondividing by removal of serum growth factors. This discrimination between reversibly arrested, quiescent cells and senescent, terminally nondividing cells had not previously been possible. This biological marker for cell senescence allowed Dimri *et al.* (18) to demonstrate that it was expressed not only by cells that had become senescent in vitro, but also by senescent fibroblasts and keratinocytes in skin in vivo. The number of positive cells was found to increase strikingly with increasing age of the donor. Although some cell types such as melanocytes and cells in the hair follicle express this gene constitutively, it should be possible to use this marker to determine the role of cellular senescence in various age-related disorders.

Genetic Control of Senescence

A number of studies have demonstrated that the replicative senescence phenotype is dominant in fusions of normal with immortal cells (19). This finding suggested a genetic basis for senescence and indicated that immortality results from recessive defects in senescence-related genes. If multiple genes were involved in causing senescence, one would predict that in fusions of different immortal cell lines immortal hybrids would sometimes be obtained, indicating that the two parental cell lines had the same genetic defect. These lines would then be assigned to the same complementation group for indefinite division. In other fusions of immortal cell lines, hybrids that exhibited replicative senescence would be obtained, indicating that the parental cell lines had different genetic defects that were complemented in the hybrid. The cell lines could then be assigned to different complementation groups for indefinite division. Such an analysis has been used to assign 40 different immortal human cell lines to four complementation groups (20, 21), indicating that at least four genes or gene pathways contribute to senescence.

Independent confirmation of the complementation groups has come from studies of the cellular staining pattern of mortalin, a protein similar to heat shock 70-kD protein found in both normal and immortal cells (22). Immunostaining with antibodies to this protein reveals patterns that distinguish normal from immortal cells and identifies the complementation group to which an immortal cell has been assigned by cell fusion analysis (23). The molecular basis for this intriguing result is currently unknown.

Microcell-mediated chromosome transfer experiments, in which a single normal human chromosome is introduced into im-

mortal human cells to determine the effect on proliferation, have led to the inference that chromosomes 1, 4, 6, 7, 11, 18, and X are involved in senescence (*24, 25*). However, only three of these studies have applied the stringent genetic test used to define the complementation groups; that is, the chromosome should induce senescence in multiple cell lines assigned to one complementation group and have no effect on cell lines assigned to the other groups. By the latter analysis, chromosomes 1 (group C), 4 (group B), and 7 (group D) have been shown to carry cell senescence–related genes (*25*). The other chromosomes were transferred into single immortal cell lines that had not been assigned to a complementation group in the majority of cases. Thus, the reason for the loss of cell division observed is much less clear and could be the result of gene dosage effects on these aneuploid cell lines or other more complex phenomena.

Molecular Changes During Cellular Aging

Molecular analyses have identified many changes in gene expression and the activity of gene products during cellular aging. Because senescent cells appear to be blocked in the G_1 phase of the cell cycle and cannot enter S phase, one approach has been to analyze the genes involved in the G_1 cell cycle in young quiescent cells and senescent cells after serum stimulation. This has led to the identification of a number of differences between young and senescent cells (Fig. 1) (*26, 27*). Differential screening of RNAs from young and senescent cells has also identified differences in the expression of genes ranging from those encoding extracellular matrix components and metabolic enzymes to cell cycle regulatory genes and several unknown genes (*28*). Additionally, senescent fibroblasts have reduced transcription factor–binding activities (*29*); for example, AP1 (activator protein factor 1), CREBP (cyclic AMP response element–binding protein), ID1 (inhibitor of DNA binding 1), and ID2.

However, a major question is, which changes in gene expression cause cellular senescence and which result from the senescent phenotype? Of the gene products listed in Fig. 1, c-FOS is potentially involved in arrest of early G_1 events in fibroblasts (*30*) because the induction of c-FOS is important for proliferation. However, this repression of c-FOS is not observed in senescent melanocytes or fibroblasts from Werner syndrome patients, indicating that it is expressed in a cell type–dependent manner (*31*).

The CDK inhibitor genes encoding p21 and p16, which are overexpressed in senescent cells, are additional candidate causal genes (*32*). They could act to inhibit G_1 cyclin-CDK complexes, thereby preventing phosphorylation of the retinoblastoma tumor suppressor protein (RB1), which is known to be hypophosphorylated in senescent cells (*33*). This in turn would result in sequestration of E2F transcription factors by hypophosphorylated RB1 and therefore lack of activation of many genes [for example, those encoding cyclin A (Cyc A), CDC2, dihydrofolate reductase, thymidine kinase, thymidylate synthase, DNA polymerase α, and ribonucleotide reductase], which are transactivated by the E2F family of transcription factors or have an E2F consensus sequence in their promoter. However, adrenocortical cells express high levels of p21 from the time they are placed in culture and throughout their replicative life-span to senescence (*34*), and the mechanism by which these cells continue to divide is presently unknown.

The indication from these multiple and varied molecular changes that occur in senescent cells is that these cells have built layers of growth regulation, so that if one pathway should become inactive, a back-up mechanism is in place. It is therefore not surprising that introduction of single genes such as c-*fos* and *CDC2* by microinjection fails to induce senescent cells to enter the cell cycle (*27, 35*).

Telomeres and Telomerase

The idea that incomplete replication of chromosome ends could account for the gradual loss of proliferation potential in cellular aging was first proposed in 1973 (*36*). Telomeres are specialized structures comprising repeat sequences, which protect the chromosome ends from degradation and fusion with other chromosome ends (*37*). The first evidence that telomere loss occurred during cell aging was provided by Harley *et al.* (*38, 39*) from an analysis of cultured human fibroblasts, in which the mean length of the terminal restriction fragment (TRF) was found to decrease in a replication-dependent manner. The decrease also correlated with in vivo aging, in that TRF length in older donors was less than that in younger donors in fibroblast cells and peripheral blood lymphocytes (*40*). In contrast, telomere length did not decrease in cells immortalized in vitro, in tumor cells, or in germline cells. These cells express the enzyme telomerase, which adds essential telomeric sequences to maintain the ends of chromosomes (*41*). Such results led to the proposal that during successive rounds of DNA replication, progressive loss of telomeric sequences occurs in normal somatic cells until a critically short telomere length is sensed as DNA damage and causes cells to exit the cell cycle. Immortal cells need a mechanism to stabilize chromosome ends and therefore expression of telomerase is required for immortalization (*39*). This modified telomere hypothesis of aging was particularly attractive because it provided a molecular mechanism for counting cell divisions in the normal somatic cell.

However, additional studies of telomeres and telomerase in cell senescence have revealed a more complex picture than that proposed in the hypothesis. Telomerase-negative immortal cell lines have been identified (*42, 43*), and treatment of im-

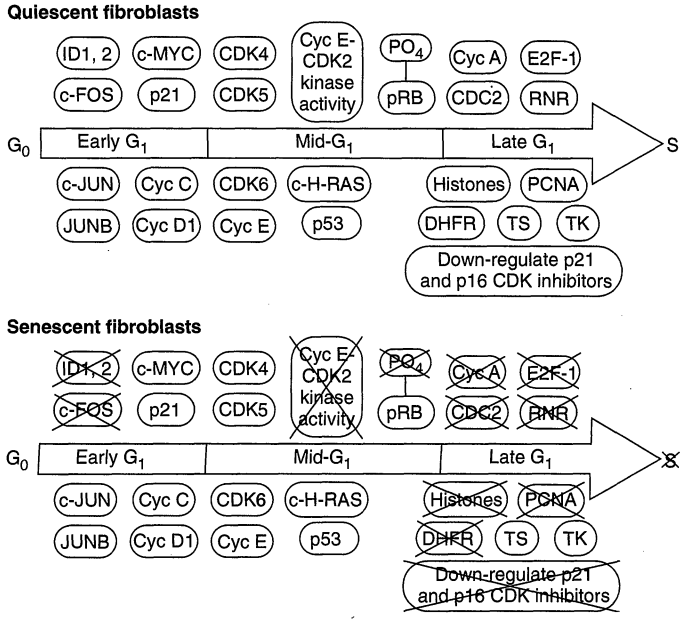

Fig. 1. Comparison of mitogen induction on the expression of cell cycle regulatory genes in quiescent early passage and senescent normal human diploid fibroblasts. Cyc, cyclin; CDK, cyclin-dependent kinase; PO_4-pRB, phosphorylated form of retinoblastoma suceptibility gene product; PCNA, proliferating cell nuclear antigen; RNR, ribonucleotide reductase; DHFR, dihydrofolate reductase; TS, thymidylate synthase; TK, thymidine kinase. [Modified from (*26*)]

mortal human B and T cell lines with chemical inhibitors of reverse transcriptase reduces telomerase activity to levels in pre-immortal cells, with no effect on the immortal phenotype (*44*). On the other hand, some normal somatic cells have been found to express telomerase, although their telomeres continue to shorten with rounds of replication (*45*). Telomerase activity in various somatic cell hybrids does not correlate with their ability to undergo senescence or proliferate, that is, some senescent hybrids continue to express telomerase (*43*). These data suggest that telomerase activity alone does not maintain telomere length. They also indicate that telomerase-independent mechanisms to stabilize chromosome ends must exist.

Holliday (*46*) recently summarized additional experimental observations that are not easily reconciled with the telomere hypothesis, including the fact that the two daughter cells from one cell division can have very different proliferative potential, differing by as many as 30 population doublings, and that very different experimental manipulations can significantly increase the life-span of human fibroblasts. Nonetheless, the telomere hypothesis is presently the most viable hypothesis as a molecular basis for a mitotic clock. Additional experiments must be performed, however, to provide critical tests of the hypothesis and to examine in more detail other proposed mechanisms for a mitotic clock, such as the Werner syndrome gene and DNA methylation changes (*47*).

Tumor Suppression Compared with Aging

The term tumor suppressor describes genes that either cause loss of proliferation after they are overexpressed in tumor cell lines or those that are frequently lost or mutated in tumor tissue. *RB1* and *p53* are the best examples of such genes. However, cell senescence genes can act dominantly to control the growth of immortal cells in which both *p53* and *RB1* are lost or inactive. For example, when one fuses different SV40-transformed cell lines in which *RB1* and *p53* have been inactivated, the hybrids cease proliferation if the parental lines are from different complementation groups, indicating that the parental lines have recessive defects in different cell senescence genes. This suggests that human cells have developed a hierarchy of tumor suppressor genes, which has led to the proposal that cell senescence evolved as a mechanism for tumor suppression (*14*), and that aging may be an indirect effect of this activity.

However, there are examples of changes in proliferation that have a major effect on aging. The decline in immune response that is observed with increasing age clearly stems from a decreased proliferative response of T lymphocytes to antigen (*48*). The precise molecular mechanisms responsible for this loss are not known but could well involve some of those related to replicative senescence. Once the genes that cause senescence are identified, these questions can be directly addressed. In the case of the fibroblast cell, which has very specific functions in the skin, the increase in the number of senescent cells with advancing age could have a significant effect on aging skin. Gene expression changes quite significantly in senescent fibroblasts. They increase collagenase and TIMP1 secretion as well as expression of fibronectin (*49*). These changes could well contribute to the thinning and loss of elasticity in aging skin. However, these ideas remain somewhat speculative and will require additional experimentation to prove.

Current Perspectives and Future Directions

In past years, the model systems used for the study of cellular senescence have expanded to include a variety of cell types in addition to fibroblasts. Results with these new systems have begun to help define the senescence-related changes that may be involved in causing the senescent phenotype. Thus, the dominance of senescence in cell hybrids is observed when fibroblasts, endothelial cells, or T lymphocytes are fused with immortal cells, indicating that these pathways may be common to many or all cell types (*50*). In contrast, differences in the repression or expression of specific genes are observed as more cell types are analyzed. Future studies should help dissect the various pathways and key changes in gene expression used by different cells to establish senescence. The identification of the genes involved in the complementation groups should further our understanding of the molecular mechanisms of senescence. The identification of the effects of mutation of the helicase cloned as the Werner syndrome gene (*51*), as well as the use of the β-galactosidase marker, should help clarify the relation between in vitro and in vivo aging. The recent identification of a telomeric binding protein that is required for chromosome maintenance, in addition to telomerase (*52*), suggests that other proteins will be identified. These data should help in the design of experiments to critically test the telomere hypothesis of aging. Finally, all these studies should help elucidate the interrelation of cell cycle control, tumor suppression, differentiation, and senescence.

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Menopause: The Aging of Multiple Pacemakers

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Menopause signals the permanent end of menstrual cyclicity in a woman's life. Its impact reaches far beyond just the reproductive system. An understanding of the factors that interact and govern the process of aging in the reproductive system will help us to develop strategies for alleviating the negative aspects of menopause and may help us to better comprehend the process of biological aging.

Current interest in the natural decline of fertility in middle-aged women and the onset of menopause is a result of three converging areas of understanding. First, the average life-span of humans has increased during the past 100 years. Thus, an increasing proportion and a larger total number of women will live a larger fraction of their lives in the postmenopausal state. Currently, 35 million American women are postmenopausal and over a million enter menopause each year (*1*). The dramatic and fairly rapid endocrine changes brought about by menopause have biological, societal, and cultural implications that profoundly influence the latter half of a woman's life. Therefore, a better understanding of the consequences of prolonged exposure to low estrogen levels, as occurs in menopause, is increasingly important to our society.

Second, over the past decade the perception that estrogen is merely a female reproductive hormone that influences predominantly classical reproductive tissues, such as the hypothalamus, anterior pituitary, mammary glands, uterus, and vagina, has changed considerably. We now are beginning to appreciate the fact that estrogen also affects a number of other functions, including urinary continence (*2*), nutrient absorption and metabolism (*3*), bone and mineral metabolism (*4*), blood pressure and cardiovascular function (*5*), memory and cognition (*6*), organization and expression of daily rhythms (*7*), and the progression of age-related diseases (*8, 9*). Thus, the cessation of menses and the resulting hypoestrogenicity affect multiple physiological systems and can lead to significant morbidity in later life. Finally, because menopause occurs relatively early during the life-span of many mammalian species, the female reproductive system serves as an excellent model system in which to study the aging process in the absence of confounding pathological changes that complicate many gerontological studies.

By the time a woman is 65 years old, the ovary is virtually devoid of follicles (*10, 11*) and is no longer the primary site of estradiol or progesterone synthesis (*12*). Also, inhibin, a glycoprotein that is synthesized in granulosa and luteal cells of the ovary and that selectively suppresses follicle-stimulating hormone (FSH) secretion, becomes undetectable in the blood (*13*). In response, the anterior pituitary gland secretes copious amounts of both of the gonadotropins FSH and luteinizing hormone (LH). Amounts of FSH increase by the time women are 45 to 50 years old, while they are still menstruating, whereas amounts of LH increase later, when women are postmenopausal. In addition, concentrations of the hypothalamic releasing hormone, gonadotropin-releasing hormone (GnRH), in the mediobasal hypothalamus are low, perhaps because of prolonged high levels of release and decreased synthesis (*14*).

For many years, the prevailing view was that menopause resulted from an exhaustion of ovarian follicles. More recently, the relative contribution of the ovaries and the hypothalamic-pituitary unit in the menopausal transition has been intensively debated. One view maintains that the impending exhaustion of the pool of growing ovarian follicles triggers the menopausal transition: the hypothalamic-pituitary changes that accompany menopause are a consequence of compromised ovarian function. The alternative perspective is that age-related changes in the central

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