Neoplastic Progression of Syrian Hamster Embryo Cells in Culture (43511E)

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The transformation of a normal cell into a malignant cell is generally recognized as a multistep process involving genetic alterations in multiple genes in a progressive, stepwise manner (1, 2). The progression of cells toward malignancy can be studied in cell culture models of neoplasia. The advantage of this approach is that it allows one to select and isolate cells at different stages of the process for biochemical, molecular, and cellular studies.

One of the best-characterized cell culture models of neoplastic progression utilizes the Syrian hamster embryo cells, which have a low frequency of spontaneous transformation but can be neoplastically transformed by treatment with chemical carcinogens (3). It was shown several years ago that the transformation of the cells in culture is a multistep, progressive process (4), and the cellular, biochemical, and molecular changes of cells at different stages have been described (2, 3, 5–8).

The most common pathway of neoplastic development when these cells are exposed to chemical carcinogens is depicted in Figure 1. In this model, at least four genetic changes, which include activation of oncogenes and inactivation of tumor suppressor genes, are required for neoplastic conversion of the cells. In this regard, this *in vitro* model parallels the *in vivo* model of colorectal cancer described by Fearon and Vogelstein (1). The earliest phenotypic alteration induced in Syrian hamster embryo cells by chemical carcinogens is a morphologic change; carcinogen treatment induces morphologically transformed colonies with a disorganized, crisscrossed pattern of cell growth. Most of the morphologically transformed colonies still have a limited life span, characteristic of the normal cells, and senesce after additional growth. Some of the colonies escape senescence, which possibly requires additional genetic changes. The molecular basis for morphologic transformation remains unidentified. After transfection of the cells with the viral Ha-*ras* oncogene, the cells exhibit morphologic changes characteristic of chemically induced, morphologically transformed cells, but v-Ha-*ras*-transfected cells senesce and do not escape cellular senescence (9). Furthermore, chemically induced immortal cells at early passages do not have activated Ha-*ras* genes (10). A characteristic karyotypic alteration in early, immortal cell lines is the gain of specific chromosomes, particularly trisomy 11 (11). This may represent an amplification of an unknown immortalizing oncogene in these cells. Mutated forms of the P53 gene effectively immortalize the cells after transfection, but the chemically induced immortal hamster cells do not have mutations in this gene (12).

The process of immortalization in these cells as well in other human and rodent cells appears to be a multistep process (3). In addition to activation of oncogenes, a key event is loss or inactivation of normal genes involved in cellular senescence. These senescence genes will be discussed in more detail later.

Carcinogen-induced immortal cell lines must undergo at least two additional genetic events in order to become tumorigenic. These events involve loss or inactivation of a tumor suppressor gene and activation of an oncogene (5). The oncogenes activated in the model in the latter stages of neoplastic progression include the Ha-*ras* proto-oncogene as well as non-*ras* genes (10). The tumor suppressor genes inactivated in this model have not been identified; however, the RB and P53 genes do not appear to be involved (12, 13).

One of the unique features of this model is the isolation of cells that either have lost or have retained tumor suppressor gene function (termed sup^- and sup^+ cells) when assayed by the ability of the subclones to suppress tumorigenicity of tumor cells in cell-cell hybrids (5, 6). The use of these sup^+ and sup^- cell lines to study the role of a tumor suppressor gene in nontumorigenic cells is discussed elsewhere (5, 6, 14).

Chemical carcinogens can influence both the escape from cellular senescence (immortalization) as well as the neoplastic conversion of immortal cell lines (3, 15). The sequence of events in neoplastic progression

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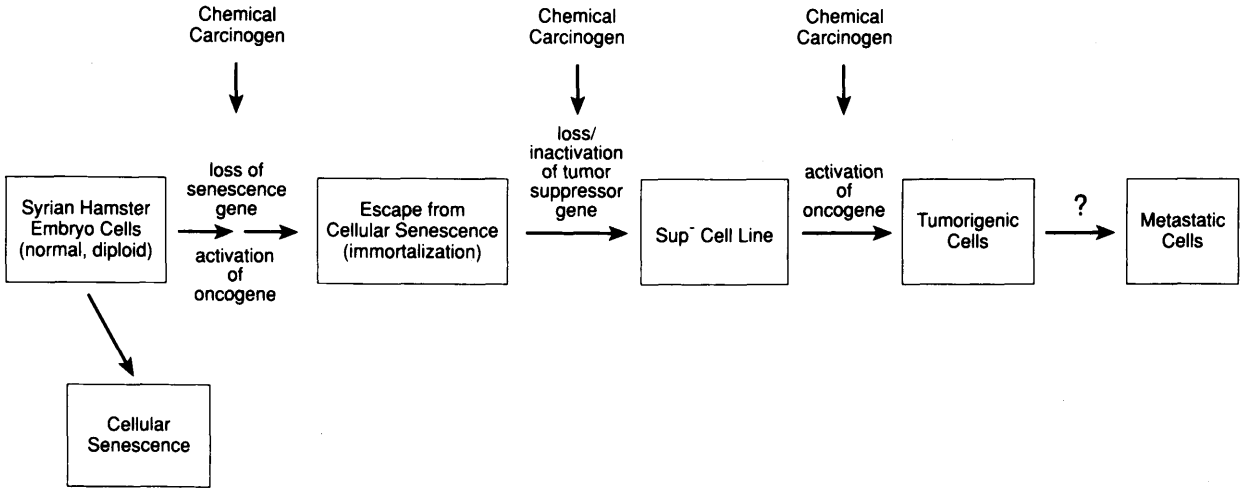


Figure 1. Cell proliferation versus cell death in normal and tumor cells.

can be viable (3). Escape from cellular senescence (immortalization) is generally an early event in *in vitro* models of carcinogenesis because growth *in vitro* is highly selective for this change. This may be a later event in the carcinogenic process *in vivo*, although in some *in vivo* models, it is also an early event (3, 16). Since immortalization is a key event for *in vitro* models of cancer and the mechanisms of the change can be studied best with *in vitro* models, the remainder of this review will focus on the mechanisms and significance of this event in neoplastic development.

Cellular Senescence and Cancer

Both human and rodent cells in culture can be grown for only a limited number of cell divisions, after which they exhibit morphologic changes and cease proliferation, a process termed cellular senescence of cellular aging (17, 18). For example, embryonic human fibroblasts can be grown for 50 to 60 population doublings before senescence. The failure of the cells to grow beyond this limit is an inherent property of the cells that cannot be explained simply by inadequate media components (17). The key determinant in the life span of cells in culture is the number of cell doublings, not the length of time in culture (17). Normal cells transplanted serially *in vivo* also exhibit a finite life span, which suggests that cellular senescence is not a cell culture artifact (19).

Escape from cellular senescence is an important step in neoplastic progression of human and rodent cancers (3). Many, but not all, tumor cells can be grown indefinitely in culture and, therefore, have escaped senescence and are termed immortal. It is not clear whether the failure of some tumor cells to grow in culture is a technical artifact or an indication that escape from senescence is not required for these cancers. Many of these tumors cannot be maintained *in vivo* in nude mice, which indicates that only a small growth fraction of cells exists in the tumor and may explain the inability of some types of tumor cells to grow *in vitro*. Improvements in cell culture techniques have led to the establishment of cell lines from most tumor types, which suggests that it is possible to obtain immortal cell lines if the culture conditions are optimal. However, some cancers when detected *in vivo* may not have progressed to this stage.

Because cellular senescence limits the growth of cells, it is reasonable that senescence might be one mechanism by which tumor suppressor genes operate. Hayflick (17) has shown that cells from adults can be grown in culture for 14 to 29 populations doublings. If all the changes necessary for tumorigenic conversion were to accumulate in an adult cell without loss or gain of life span potential, then this cell could grow to form a tumor of 16,384 cells (14 doublings or 2^{14} cells) to 5.4×10^8 cells (29 doublings or 2^{29} cells). It is estimated that a tumor formed after 30 doublings would be approximately 1 cm^2 in size. Interestingly, Paraskeva and co-workers (20–22) have shown that colon adenomas of <1 cm^2 in size are rarely capable of indefinite growth *in vitro*, whereas cells from adenomas of >1 cm^2 are often immortal, which suggests that escape from senescence is a requirement for tumor growth beyond a certain size or cell number and is consistent with the hypothesis that cell senescence is a constraint on tumor growth.

Since no property of cancer cells is universal, it is not necessary to demonstrate that escape from senescence has occurred in every cancer. However, in those cancers where this change is evident, it is probably a critical change based on the following additional lines of evidence (3). The observation that treatment of normal cells with diverse carcinogenic agents, including chemical carcinogens, radiation, viruses, and oncogenes, allows cells to escape senescence indicates that this change is important in cancer induction. While immortality is not sufficient for neoplastic transformation, most immortal cells have an increased propensity

for spontaneous, carcinogen-induced or oncogene-induced neoplastic progression (3). Therefore, escape from senescence is a preneoplastic change that predisposes a cell to neoplastic conversion. It is clear that immortal cells are further along the multistep pathway to neoplasia than normal cells. Because cellular senescence limits the growth of cells, it is reasonable that senescence might be one mechanism by which tumor suppressor genes operate (3, 23).

Two major theories of cellular senescence have been debated for many years (24). One is the error catastrophe or damage model, which proposes that the random accumulation of damage or mutations in DNA, RNA, or protein leads to the loss of proliferative capacity. The experimental evidence supporting the error accumulation hypothesis has been criticized (24). A second hypothesis is that senescence is a genetically programmed process. Strong experimental support for a genetic basis for senescence is provided by studies of Pereira-Smith and Smith (25) and by Sugawara *et al.* (7), which are discussed below.

It is possible to fuse cells of different origins and to select for the hybrid cells using biochemical markers for drug sensitivity or resistance that differ in the parental cells. When cells with a finite life span are fused to immortal cells with an indefinite life span, the majority of these hybrids senesce (5, 25). Even hybridization of two different immortal human cell lines with each other can result in senescence, which indicates that different complementation groups exist for the senescence function lost in these cells. By fusing different immortal human cell lines with each other, Pereira-Smith and Smith (25) have established four complementation groups, suggesting that loss or inactivation of one of multiple genes allows escape from senescence. If this hypothesis is correct, it should be possible to map the genes involved in cellular senescence, and recent findings with hamster and human interspecies hybrids and microcell-mediated chromosome transfer experiments have mapped putative senescence genes to specific human chromosomes (7, 26, 27).

When normal human lung fibroblasts (MRC-5), which have a finite life span, are fused to immortal hamster cells, the hybrids that form exhibit a finite lifespan (Fig. 2), which is characteristic of the normal human cells (7). At the end of this life span, the cells display signs of cellular senescence, which are indistinguishable from those exhibited by the parental human cells at the end of their life span. When MRC-5 cells, which have a life span of 60 population doublings, are used at a population doubling level of 40, the human-hamster hybrids grow for approximately 20 population doublings, i.e., the remaining life span of the parental human cells. Because the cell hybrids grow extensively before dying, the cessation of growth is not d e to a toxic effect of the fusion protocol. Furthermore, when earlier passage MRC-5 cells are used (a population

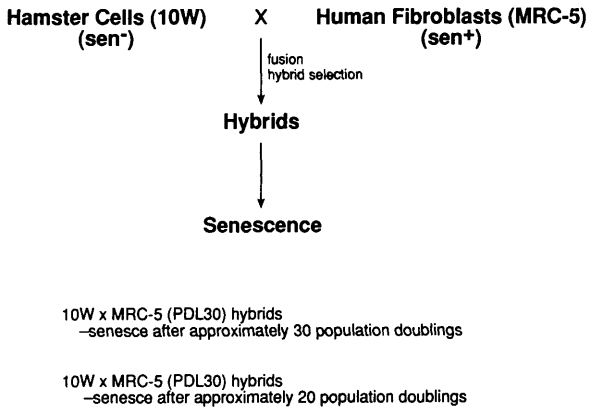
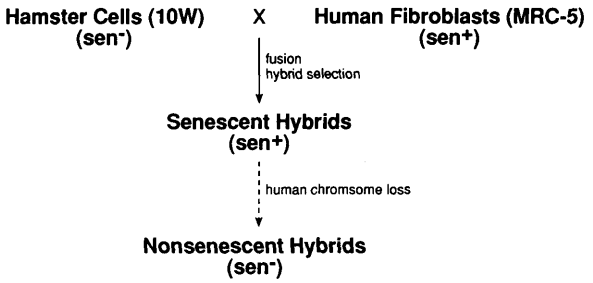


Figure 2. The senescent phenotype is dominant in cell hybrids. When immortal cells are fused with normal cells, the resultant hybrid cells senesce. The life span of the hybrid is determined by the population doubling level (PDL) of the normal parental cell. The hybrids achieve approximately the life span remaining of the normal cell.



Most hybrids between normal human cells and immortal hamster cells senesce, but some escape senescence following loss of human chromosomes. Both copies of chromosome 1 are lost in all nonsenecent hybrids.

Figure 3. Nonsenescent hybrids lose specific chromosomes. Examination of the nonrandom loss of human chromosomes in hamster-human hybrids allows the identification of chromosomes necessary for senescence (Sugawara *et al.* [7]).

doubling level of 30), the hybrids grow longer, for up to 30 population doublings, again achieving the life span of the parental cells (Fig. 2). Therefore, the senescence of the hybrids is an active process dictated by the senescence program of the normal human cells. The limited life span of the hybrids indicates that cellular senescence is dominant in these hamster-human hybrids. A similar conclusion was drawn from studies of intraspecies hybrids, i.e., human-human (25) and hamster-hamster (5).

Although the majority of the hamster-human hybrids senesce, some of the hybrids ultimately escape senescence due to outgrowth of a few cells in the senescent population (Fig. 3). Because human chromosomes are usually lost in interspecies hybrids, the possibility exists that escape from senescence is due to loss of an essential chromosome or chromosomes, which allow the hybrids to escape senescence. The human-hamster hybrid clones that escaped senescence had lost both copies of human chromosome 1, whereas all other human chromosomes were present in one or

two copies in at least one of the immortal hybrids (7). This suggested that a gene or genes on this chromosome may be necessary for cellular senescence.

This hypothesis was confirmed by two additional experimental approaches. One involved interspecies cell hybrids, with human cells carrying a t(1;X) chromosome translocation. These experiments showed that applying selective pressure for the long arm of chromosome 1 increased the percentage of senescent hybrids. The second approach used microcell-mediated chromosome transfers (Figs. 4 and 5) to introduce chromosome 1 into the immortal cell line (7). This chromosome, but not another control chromosome (chromosome 11) induces senescence in hamster 10W cells (7). Chromosome 1 induces senescence in some cell lines but not others (28).

By the same technique of microcell-mediated chromosome transfer, Klein *et al.* (26) have mapped another senescence gene to chromosome X, and Ning *et al.* (27) have mapped a senescence gene for HeLa cells to chromosome 4. Thus, three senescence genes have now been mapped. In addition, several unpublished studies indicate the presence of senescent genes on other chromosomes (Table I).

These results have led us to propose the following hypothesis: Cellular senescence is controlled by genes that are activated or whose functions become manifested at the end of the life span of the cell. Defects in the function of these gene products allow cells to escape the program of senescence and become immortal. Immortalization relieves one constraint on tumor cell growth, allowing malignant progression.

According to this hypothesis, a family of senescence genes exists. Immortalization occurs due to a defect in any one of these genes. This explains the complementation studies of Pereira-Smith and Smith (25), which showed that different immortal cell lines could complement the defect in other immortal cells in hybrids that senesce after cell-cell fusion. This also explains our results that introduction of a specific human chromosome causes senescence in some cell lines but not others. It appears that senescence gene mutations are random. The senescence gene altered in a specific immortal cell line does not correlate with the tumor histology, activated oncogenes, or inactivated tumor suppressor genes in the cell. Current efforts are focused on the cloning of these genes and on understanding how they operate to arrest cell growth.

The RB protein, which is involved in retinoblastoma and in several other malignancies, is differentially phosphorylated during the cell cycle (29). The RB protein is unphosphorylated in the G_1/G_0 compartment of the cell cycle and is phosphorylated as cells enter the S phase. The protein is increasingly phosphorylated as the cells progress through G_2 and M and is again primarily in the unphosphorylated form as cells re-

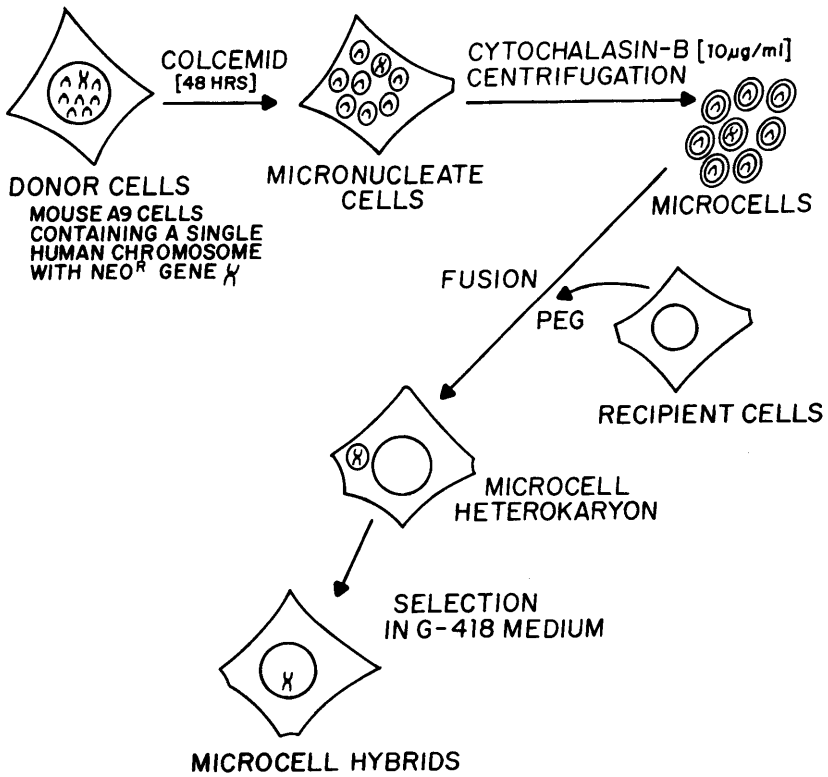


Figure 4. Microcell-mediated chromosome transfer. Individual human chromosomes can be transferred into recipient cells of interest by this technique. Sugawara *et al.* (7) confirmed that human chromosome 1 induces senescence of the hamster cell line 10W. Other authors have mapped senescence genes to other chromosomes by this approach.

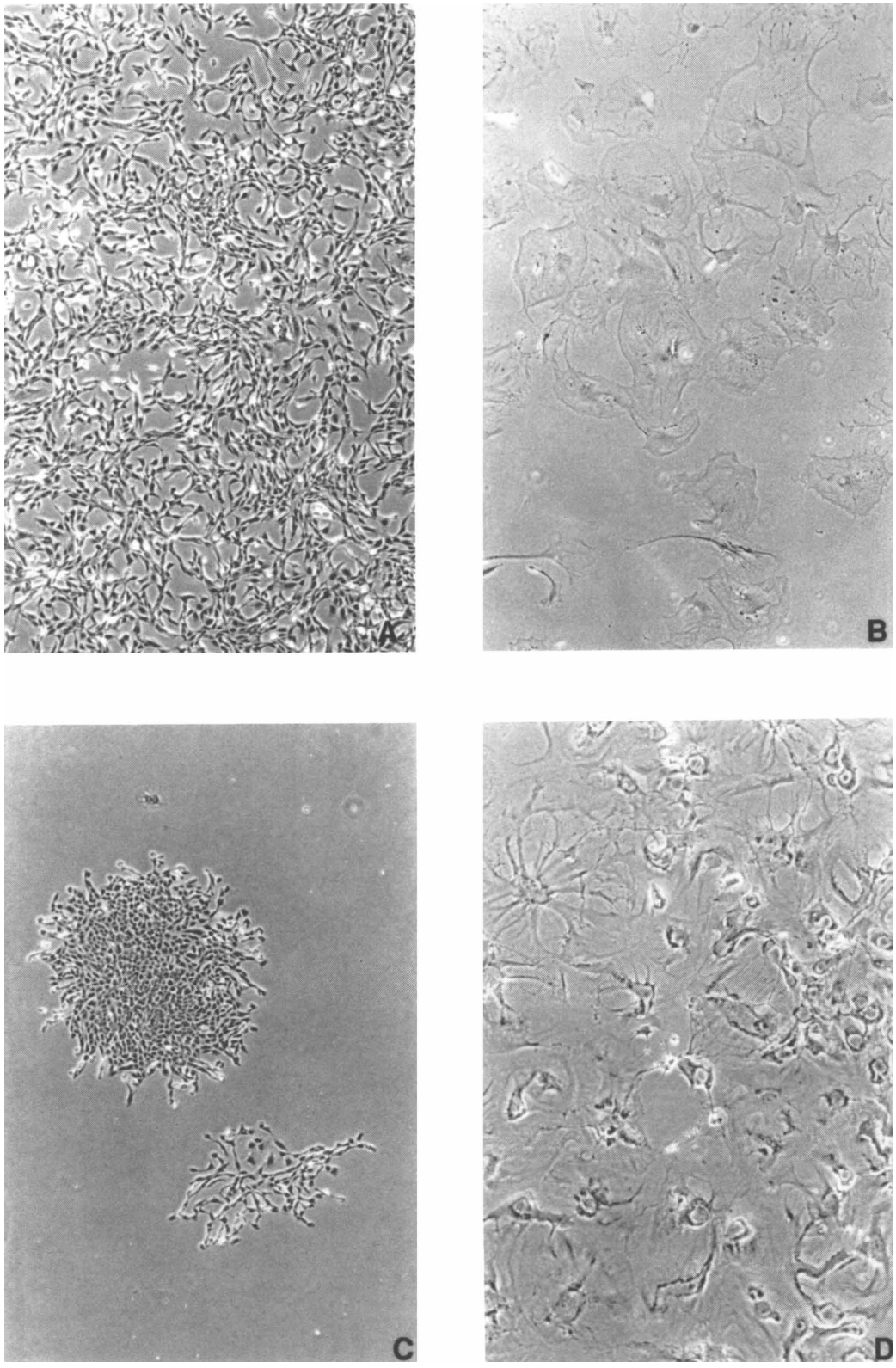


Figure 5. Senescence after reintroduction of normal chromosome. The (A) 10W cell line is immortal. When normal human chromosome 1 is introduced into the cells by microcell-mediated chromosome transfer, the cells (D) stop growing and display signs of senescence that are typical of (B) normal hamster cells at senescence. (C) 10W cells with deleted portions of chromosome 1q continue to grow.

Table I. Mapping of Putative Senescence Genes

Chromosome localization of *Sen*$^+$ gene	Cell line(s)	Reference
1	Syrian hamster 10W	Sugawara *et al.* (7)
	Human endometrial	Yamada *et al.* (28)
4	Cervical carcinoma (HeLa)	Ning *et al.* (27)
X	Chinese hamster (Ni-2)	Klein *et al.* (26)
6	SV40 immortalized human fibroblasts	H. Ozer *et al.*, unpublished
9	Human leukemia (K562)	B. Porterfield *et al.*, unpublished
	Human melanoma (H3294T)	
11	Human bladder carcinoma (H-2)	M. Oshimura *et al.*, unpublished

enter G_1. Also, the RB protein is unphosphorylated in cells induced to differentiation (29). These data suggest that the unphosphorylated form of the protein is growth inhibitory and that the tumor suppressor function of the RB protein may be linked to cell cycle control and differentiation.

The product of the retinoblastoma tumor suppressor gene may be a key regulator of cellular senescence as well (13, 30). Senescent cells express levels of RB protein comparable to young cells; however, only the unphosphorylated form of RB is observed. Quiescent cells, maintained in media containing low serum, exhibit only the unphosphorylated form of the RB protein. When the cells are stimulated with serum, phosphorylation of the RB protein is observed between 10 and 20 hr after stimulation, which corresponds with the time course for stimulation of DNA synthesis. However, when senescent cells are stimulated with serum, the RB protein remains unphosphorylated (30, 31). This result indicates that senescent cells are blocked in their ability to phosphorylate the RB protein in response to normal growth stimuli (Fig. 6).

This finding implicates upstream modifiers of RB phosphorylation as possible crucial regulatory elements in mediating cellular senescence, with the end result being a block to proliferation caused by the presence of unphosphorylated RB protein acting on its own or through other effector molecules. Down-regulation of an RB kinase activity in senescent cells and/or up-regulation of an RB phosphatase are possible mechanisms for the alterations of RB phosphorylation in senescent cells. Recent studies indicate that the $p34^{cdc2}$ kinase, which is a candidate RB kinase, is down-regulated in senescent human and hamster cells (32, 33). However, transfection of human $p34^{cdc2}$ under the control of a constitutive promoter into hamster cells fails to allow the cells to enter into S phase, even when the protein is synthesized (32). The $p34^{cdc2}$ kinase is the catalytic subunit of a serine/threonine kinase, which depends on interactions with members of a family of proteins called cyclins for activation (34). In growing cells, cyclins are synthesized in a periodic pattern and then rapidly degraded. Some cyclins function in G_1, while others are involved in cell cycle progression during the S phase, G_2 phase, and mitosis (35). Thus, the block to entry into DNA synthesis in senescent cells may also be due to the down-regulation of cyclin proteins and/or cdc2-like proteins (30; C. A. Afshari and J. C. Barrett, unpublished). Further studies of cell cycle control proteins should yield insights into negative growth control in cellular senescence.

In summary, a key event in neoplastic progression *in vitro* or *in vivo* is the loss or inactivation of senescent genes, which are a subclass of tumor suppressor genes.

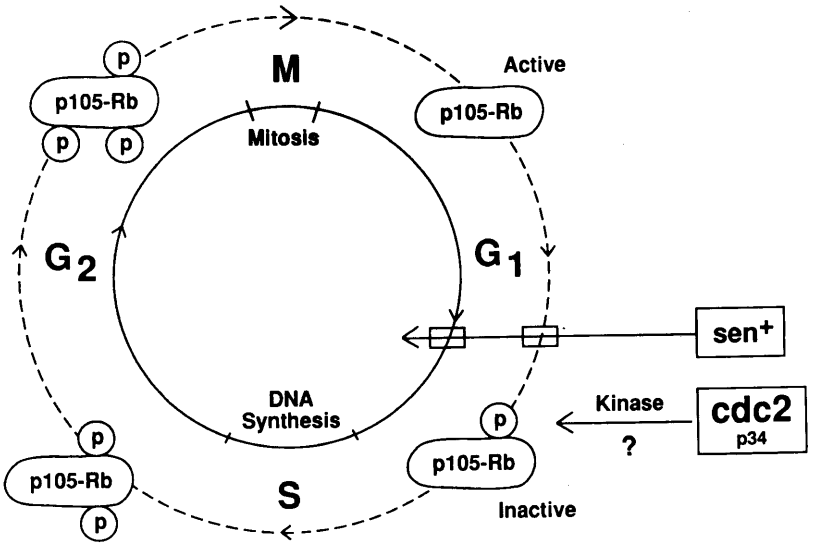


Figure 6. RB and cellular senescence. The block to DNA synthesis in senescent cells correlates with the failure to phosphorylate the RB cell cycle control protein. The kinases and/or phosphatases controlling this phosphorylation are probably important in cellular senescence.

These genes have mapped to different chromosomal regions and current efforts to clone these genes are in progress. These genes are normally activated or their functions become manifest at the end of the life span of a normal cell, which results in a block to DNA synthesis due to a down-regulation of cell cycle control proteins, including cdc2, cdk2, and cyclins. Inactivation or loss of senescent genes in immortal cells allows escape from senescence. This program can be reinitiated in tumor cells by introduction of specific chromosomes from normal cells.

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