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# Evidence for Multiple Pathways to Cellular Senescence

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## Abstract

**Normal cells in culture generally senesce whereas tumor-derived cells are often, but not without exception, immortal and grow indefinitely. For cells to escape the senescence program, normal genes must be lost or inactivated as shown by somatic cell genetic studies. For example, the introduction of specific chromosomes by microcell-mediated chromosome transfer has been shown to induce senescence of human and rodent tumor cell lines, and the mapping of over ten senescence genes has been achieved by this method. In this study, we observed that two different normal chromosomes induce senescence in the same human endometrial carcinoma cell line, which suggests that multiple pathways to senescence are inactivated in this cell line. This hypothesis has implications for the mechanisms of cellular senescence and its role in carcinogenesis. Furthermore, this hypothesis can explain why not all tumor-derived cells are immortal.**

## Introduction

Normal cells *in vitro* and *in vivo* have a limited life span whereas tumor cells can often grow indefinitely (1, 2). At the end of their proliferative life span in culture, normal cells exhibit morphological changes, enlarge in size, and cease proliferation, a process termed cellular senescence (1–3). Tumor cells often have escaped senescence and are immortal (4). Escape from cellular senescence predisposes a cell to neoplastic conversions (5–7); however, not all tumor cells are immortal (4). We have proposed the hypothesis that cellular senescence is controlled by genes that are activated or with functions that become manifest at the end of the proliferative life span of the cell (3, 8). According to this hypothesis, a family of senescence genes exists and immortalization occurs due to defects in these genes that allow cells to escape the program of senescence. Immortalization relieves one constraint on tumor cell growth, which may be a necessary malignant progression. A genetic basis for cellular senescence is suggested from studies of somatic cell genetics. Hybrids of normal cells and tumor cells generally senesce, which indicates that normal cells can correct defects in the senescence program of tumor cells (9–11). Pereira-Smith and Smith (12) further showed that hybrids between certain immortal cells senesce. This finding implies that one immortal cell can complement the defects in another immortal cell, resulting in senescence. Four complementation groups were determined from fusion experiments with 31 cell lines (12). These experiments were interpreted to indicate that only a few genes, perhaps four, are involved in escape from senescence.

If this hypothesis is correct, then it should be possible to map genes involved in cellular senescence to specific chromosomes. Using microcell-mediated chromosome transfer, we showed that human chromosome 1 could confer senescence when introduced into an immortal hamster cell line (5) and a human endometrial cell line (13). Hensler *et al.* (14) observed that chromosome 1 also induces senescence of cell lines belonging to complementation group C as defined by Pereira-Smith and Smith (12). Ning *et al.* (15) reported that chromosome 4 induces senescence in HeLa cells and other cell lines belonging to complementation group B as defined by Pereira-Smith and Smith (12). Senescence genes on chromosomes 6[3] (16), 7 (17), 9,[4] 11 (18), and the X chromosome (19) also have been mapped. These data suggest that more than four senescence genes exist.

An important question is whether a single pathway with multiple genes is involved in controlling the cellular senescence program or whether multiple pathways exist (Fig. 1). According to the single pathway model for senescence, a defect in a single gene could allow a cell to escape this program and become immortal. Alternatively, if multiple pathways leading to the senescent phenotype exist, immortal cells would arise only after mutations or inactivation of genes controlling each of multiple pathways. The complementation studies do not provide a distinction between these possibilities. In the current study, we observed that in addition to chromosome 1, which induces senescence of the HHUA endometrial carcinoma cell line when introduced by microcell-mediated chromosome transfer, another chromosome (chromosome 18) also induced senescence of these cells. These findings provide genetic evidence for multiple pathways to senescence.

## Materials and Methods

The HHUA cell line was established from a well-differentiated endometrial adenocarcinoma of a Japanese female (13). The karyotypic analyzes showed 73% of the cells to be apparently normal; the remaining cells had a 46,XX,4q+ karyotype (13). The cells were tumorigenic, forming progressively growing tumors within 3 to 5 weeks in 100% of injected sites following inoculation with $1 \times 10^7$ cells. These cell lines were maintained on DMEM[5] supplemented with 10% fetal bovine serum.

Microcells were prepared from mouse A9 cells containing single human chromosomes as described previously (8, 13). Briefly, A9 cell clones containing a single human chromosome were incubated with 0.03 μg/ml Colcemid in DMEM supplemented with 20% fetal bovine serum for 48 h. The microcells, which were obtained by the treatment with cytochalasin B and the centrifugation, were fused with HHUA cells in a 47% polyethylene glycol (*M*W 1450) solution for 1 min. Isolation of microcell-hybrid clones were performed 21 to 60 days after the cultivation of the cells with DMEM containing 400 μg/ml G418.

Chromosomes in HHUA cells and their microcell hybrids were analyzed by the quinacrine mustard staining method. Karyotypes from 10 to 20 well-banded metaphases were analyzed for each clone. For calculation of cell population doubling times, cells from the parental HHUA cells and their microcell hybrids were plated in triplicate in DMEM containing 10% calf serum on 24-well dishes at a density of $5 \times 10^4$ cells/dish. The cells were

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[3] S. Kodama *et al.*, submitted for publication.

[4] R. Newbold, personal communication.

[5] The abbreviation used is: DMEM, Dulbecco's modified Eagle's medium.

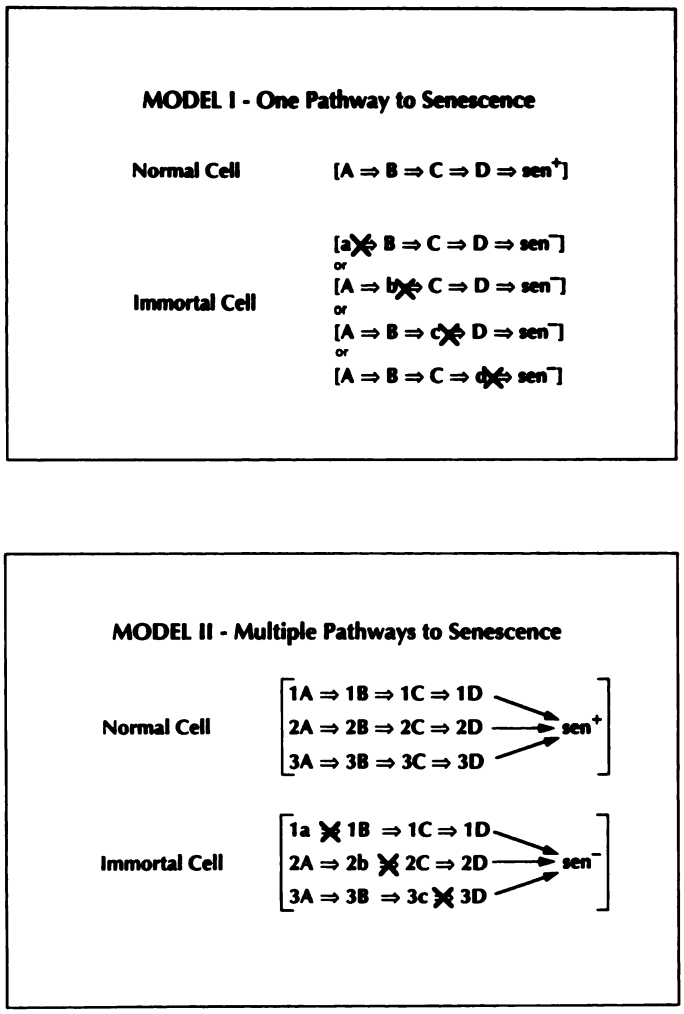


Fig. 1. Alternative models for cellular senescence.

cultured in media containing 10% calf serum. Media were changed at 2-day intervals, and the number of cells were counted during the following 1 to 10 days.

## Results

Using a mouse A9-human chromosome library consisting of cell clones with an individual pSV2neo-tagged human chromosome, different chromosomes were independently transferred to HHUA cells via microcell fusion and selected for G418-resistant cells. When chromosome 1 was introduced into HHUA cells, 70 of 92 clones (76%) senesced, which confirmed and extended our previous experiments with the HHUA cell line (13). Surprisingly, 31 of 97 clones (32%) with an introduced chromosome 18 also senesced. However, among 59 clones selected after transfer of either chromosome 6, 9, 11, or 19, none senesced.

The HHUA microcell hybrids with chromosome 1 or 18 exhibited two types of morphology; some of the cells consisted of cells with polygonal cells similar to the parental cells and the other clones were composed of flat, senescent-like cells (Fig. 2). The cells with a flat morphology also had a decreased growth rate; their doubling time was 46–52 h *versus* 24–30 h. Many of these clones ceased proliferating and senesced (Table 1). The clones that did not senesce were overgrown by a subpopulation of cells that reexhibited the parental cell morphology and growth rate. As shown in Table 2, the doubling times of the nonmorphologically altered clones were the same as the parental cells or microcell hybrids with other chromosomes.

Karyotypic analysis was performed on the microcell hybrids. The karyotype of the parental HHUA cells was 46,XX,4q+ (13). Karyotypic analysis of the flat, senescent-like cells showed an extra intact copy of chromosome 1 in 7 of 8 hybrid clones. In contrast, none of the 8 clones with increased growth and normal morphology had an intact copy of the introduced chromosome. Likewise, senescent clones resulting from microcell transfer of chromosome 18 had an intact, extra copy of chromosome 18 in 5 of 5 clones, whereas all 6 of the nonsenescent clones did not (Table 2). The microcell hybrids that were not growth suppressed after transfer of chromosome 6, 9, 11, or 19 retained intact copies of the introduced chromosomes in 31 of 39 clones analyzed (Table 2).

The present study does not permit definition of the regions on chromosomes 1 and 18 involved in the induction of cellular senescence. Previous studies have mapped the location of senescence genes on chromosome 1 to two regions, 1p25 and 1p42 (8, 14).[6] The regional mapping of the gene on chromosome 18 will require additional studies and isolation of more hybrids that escape senescence.

## Discussion

Senescence genes have now been mapped to chromosomes 1, 4, 6, 7, 9, 11, 18, and X (8, 13–19). In addition, the *Rb* and *p53* tumor suppressor genes can be considered as putative senescence genes. Operationally, these genes fit the criteria for senescence genes in certain contexts. Down-regulation of their expression by antisense methods results in extension of the life span of normal human cells (20), and reintroduction of the genes into certain immortal cells causes cessation of growth and morphological changes similar to senescent cells (21, 22).

As illustrated in Fig. 1, the senescence program could be activated by a single pathway and immortal cells could arise due to mutations in any of the genes that encode proteins required in this pathway. An alternative hypothesis is that the senescence program is activated by multiple independent pathways. We observed that introduction of either of two different chromosomes induces senescence in the same immortal cell line. This finding implies that multiple pathways of senescence exist and that immortal cells arise due to defects or mutations in genes in each of the pathways. Mutations that affect only a single pathway would not result in cells that were immortal, but the cells might have an extended life span. For example, SV40 infection (which inactivates Rb and p53 proteins) results in extended life span but not immortalization of infected cells (23, 24). Additional genetic changes, possibly loss of chromosome 6, are required for immortalization of SV40-infected human cells (25, 26). Reintroduction of a normal chromosome 6 results in senescence of SV40 immortal cells (16).[6] Antisense down-regulation of Rb and p53 mRNAs also results in extension of the life span of human cells without immortalization (20). Based on the results with SV40 infection, the most likely number of pathways for human fibroblast immortalization is three, one involving *Rb*, one involving *p53*, and one involving an unknown gene, possibly on chromosome 6 (25). The multiple pathways to a senescence hypothesis is consistent with the multistep nature of immortalization observed in chemically induced immortalization (4, 27, 28). Furthermore, the inability to assign certain immortal cells to a single complementation group is also explained by this hypothesis (29, 30).

[6] P. A. Futreal, L. A. Annab, and J. C. Barrett. Mapping of a senescence inducer (*SI*) locus on human chromosome 1q and generation of an ordered STS map of the 1q25–qter region, submitted for publication.

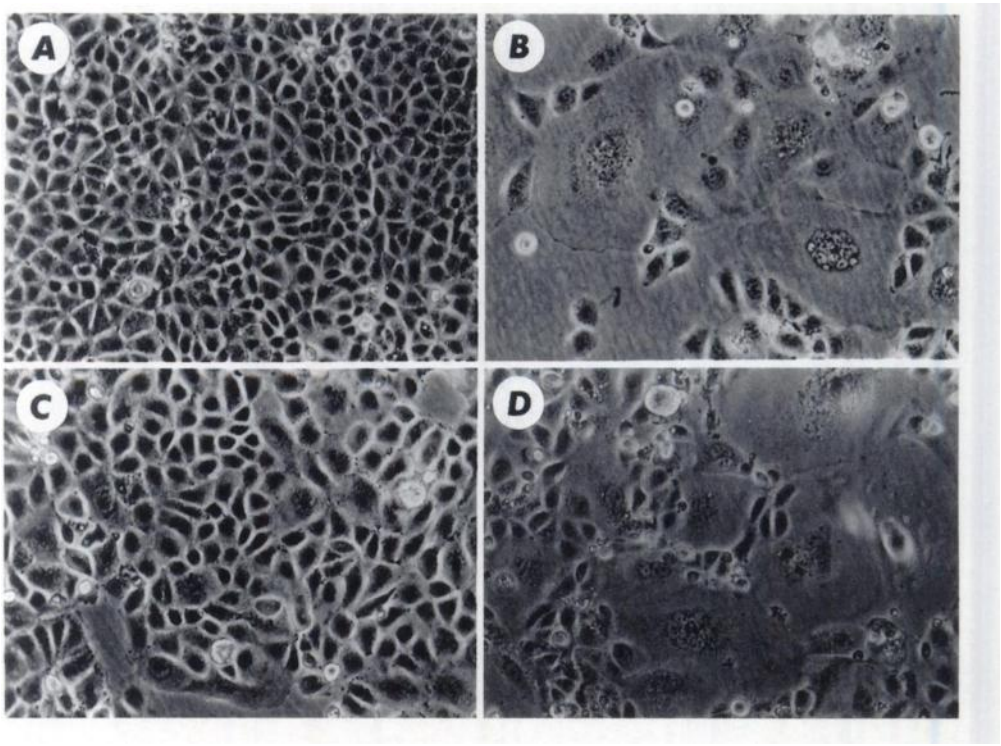


Fig. 2. *A*, HHUA parental cells with polygonal morphology; *B*, HHUA microcell hybrids with the introduction of a chromosome 1, showing flat morphology, vast cytoplasm, and multinuclei; *C*, HHUA microcell hybrids, following the transfer of chromosome 18, with the morphology similar to that of parental HHUA cells. *D*, HHUA senesced microcell hybrids, following the transfer of chromosome 18, with flat morphology.

An important aspect of the hypothesis of multiple pathways to cellular senescence is that it explains why many tumor-derived cells are not immortal. Hayflick (1) has shown that cells from adults can be grown in culture for 14 to 29 population doublings. If all the changes necessary for tumorigenic conversion were to accumulate in an adult cell without loss or gain of life span potential (which may be unlikely), then this cell could grow to form a tumor of only 16,354 cells (14 doublings or $2^{14}$ cells) or a maximum of $5.4 \times 10^8$ cells (29 doublings or $2^{29}$ cells). It is estimated that a tumor formed after 30 cell doublings would be approximately 1 $cm^3$ in size (31). Interestingly, Paraskeva *et al.* (32, 33) have shown that colon adenomas <1 $cm^3$ in size are rarely capable of indefinite growth *in vitro* whereas cells from adenomas >1 $cm^3$ are often immortal, which supports the hypothesis that escape from senescence is a requirement for tumor growth beyond a certain size or cell number. A tumor of <1 $cm^3$ would not generally be lethal. For the tumor to expand, an extension of the life span would be necessary. Extension of the life span from 30 to 40 population doublings would yield a 1-kg tumor and an additional life span extension by 10 more population doublings would yield a 1000-kg tumor (32), which would very certainly be lethal. Thus, an important event in malignant progression is extension of cellular life span, which allows tumor growth and increases the likelihood of additional, critical mutations including further changes necessary for immortalization. According to the multiple pathways model, mutations of senescence genes in one pathway may result in an extended life span of a cancer cell without immortalization. This hypothesis may explain why tumor cells derived from malignant cancers are not always immortal when cultured *in vitro*.

Table 1 *Introduction of various human chromosomes into HHUA endometrial carcinoma cell line*

| Chromosome introduced | Total no. of colonies isolated | No. of colonies that senesced | % of senescent clones |
|---|---|---|---|
| 1 | 92 | 70 | 76 |
| 6 | 19 | 0 | 0 |
| 9 | 11 | 0 | 0 |
| 11 | 14 | 0 | 0 |
| 18 | 97 | 31 | 32 |
| 19 | 15 | 0 | 0 |

Table 2 *Comparison of karyotype, morphology, and growth of microcell hybrids*

| Introduced chromosome | Senescent morphology | No. of clones with intact introduced chromosome/no. of clones analyzed | Doubling time[a] (h) |
|---|---|---|---|
| 1 | + | 7/8 | 63–80 |
| 1 | – | 0/8 | 30–31 |
| 6 | – | 6/9 | 24–30 |
| 9 | – | 9/11 | 24–30 |
| 11 | – | 11/14 | 24–30 |
| 18 | + | 5/5 | 37–59 |
| 18 | – | 0/6 | 25–31 |
| 19 | – | 5/5 | 24–30 |

[a] The doubling time for the parental cells was 24–30 h.

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