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The biological clock that measures the mitotic life-span of mouse embryo fibroblasts continues to function in the presence of simian virus 40 large tumor antigen

ZEBBIE IKRAM*, TRISHA NORTON†, AND PARMJIT S. JAT*‡

*Ludwig Institute for Cancer Research, Courtauld Building, 91 Riding House Street, London W1P 8BT, United Kingdom; and †National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA, United Kingdom

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ABSTRACT **Normal mammalian fibroblasts cultured *in vitro* undergo a limited number of divisions before entering a senescent phase in which they can be maintained for long periods but cannot be induced to divide. In rodent fibroblasts senescence can be prevented by expression of simian virus 40 large tumor antigen (T antigen). Cells expressing T antigen can proliferate indefinitely; however, such cells are absolutely dependent upon continued expression of T antigen for maintenance of growth; inactivation of T antigen results in a rapid and irreversible entry into a postmitotic state. To determine when, after the initial expression of T antigen, fibroblasts become dependent upon it for continued growth, we serially cultivated embryonic fibroblasts prepared from *H-2K^b-tsA58* transgenic mice. We show that these fibroblasts become dependent upon T antigen for maintenance of proliferation only when their normal mitotic life-span has elapsed and that the biological clock that limits the mitotic potential continues to function normally, even in cells expressing this immortalizing gene. Our results suggest that random accumulation of cellular damage is unlikely to be the factor that limits fibroblast division but support the hypothesis that senescence is regulated via a genetic program.**

Normal mammalian fibroblasts cultured *in vitro* undergo a limited number of divisions before entering a senescent phase in which they can be maintained for long periods but cannot be induced to divide (1–6). The molecular basis for the determination of the finite mitotic life-span is not known, nor is it understood how the measurement takes place (5–9). However, it has been suggested that the proliferative potential is limited either by random accumulation of cellular damage (6, 10, 11) or by a genetic program involving a biological clock that measures the finite life-span so that, upon its completion, fibroblasts cease dividing and undergo terminal differentiation into a postmitotic state (4–6, 12, 13).

Even though the actual cause of fibroblast senescence is not known, it can be overcome. Continuous passage of senescent cultures under appropriate conditions often leads to the outgrowth of variant populations of cells with an infinite proliferative potential, a process that has been termed immortalization (2). In rodent cells spontaneous immortalization is a rare but measurable event and occurs by the selection of rare cells that have spontaneously overcome the finite life-span. Senescence can also be overcome by expression of any member of the family of viral and cellular immortalizing genes (14, 15).

One viral gene that has been shown to be capable of immortalizing rodent cells is the simian virus 40 (SV40) gene encoding the large tumor antigen (T antigen, refs. 14–18). Cells immortalized with the SV40 T antigen proliferate indefinitely but are absolutely dependent upon it for maintenance of growth; thus, inactivation of T antigen in cell lines conditionally immortalized by using the thermolabile *tsA58* T antigen (19) results in a rapid and irreversible cessation of growth (20). It is possible that introduction of T antigen immediately abrogates the mechanisms that normally limit fibroblast division so that its inactivation would result in growth arrest. Another possibility is that embryonic fibroblasts only become dependent upon the T antigen when the normal life-span has elapsed; thus, inactivation of T antigen prior to this point would not result in the cells ceasing growth, whereas inactivation of T antigen after this point would result in cells undergoing senescence. So far it has not been possible to distinguish unequivocally between these two hypotheses because such experiments require cultures of embryonic fibroblasts in which the expression of T antigen can be modulated in every cell from the moment of isolation from the animal, so that T antigen can be inactivated after different periods of times in culture to determine when the cells become dependent upon it for continued growth.

It has now become possible to address this question because of the recent development of *H-2K^b-tsA58* transgenic mice (21). These mice harbor the thermolabile *tsA58* T antigen (19) under the control of the γ interferon (INF-γ)-inducible *H-2K^b* promoter (22–24). These animals allow preparation of fibroblast populations in which the expression of T antigen can be induced or suppressed in every cell by manipulating the growth conditions (21). Skin fibroblasts prepared from these mice readily yield immortal cultures when grown at 33°C in the presence of INF-γ (33°C INF-γ$^+$ conditions), whereas those from nontransgenic mice cease dividing (21). Subsequent transfer of these immortal cultures to 39.5°C in the absence of INF-γ (39.5°C INF-γ$^-$ conditions) to inactivate the T antigen or removal of INF-γ at 33°C (33°C INF-γ$^-$ conditions) to reduce the level of T antigen results in complete cessation of growth (21).

By using the *H-2K^b-tsA58* transgenic mice as a source of embryonic fibroblasts, we have found that embryonic fibroblasts become dependent upon the T antigen for growth only when the normal mitotic life-span has elapsed and that the measurement of the mitotic life-span continues normally in cells expressing the immortalizing gene.

MATERIALS AND METHODS

Fibroblast cultures were prepared from 12/13-day-old embryos from transgenic homozygous males × normal females (TMEFs) and normal males × normal females (NMEFs). The

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Abbreviations: SV40, simian virus 40; T antigen, large tumor antigen; INF-γ, γ interferon; 33°C INF-γ$^-$ or 33°C INF-γ$^+$, etc., culture conditions of 33°C in the absence or presence of INF-γ; TMEFs, transgenic homozygous males × normal females; NMEFs, normal males × normal females.

‡To whom reprint requests should be addressed.

embryos were isolated, heads and tails were removed, and the remaining tissues were disaggregated by fine mincing followed by treatment with trypsin to produce a cell suspension. Cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% (vol/vol) heat-inactivated fetal calf serum, 100 units of penicillin, 100 μg of streptomycin, 2 mM glutamine, and 10 units of recombinant murine INF-γ per ml when appropriate. All of the media and the various additives were obtained from GIBCO/BRL.

Cells were plated at a density of 1×10^6 cells per 10-cm dish in triplicate under the different conditions. The day of the first plating was considered to be passage 1; this was not taken as the starting point of the experiment because of the very low initial plating efficiency and the highly heterogeneous nature of the culture at this stage. Passage 2 was taken as the starting point for the growth experiments; by this time there had already been considerable selective growth of fibroblast-like cells in the culture conditions utilized, especially at 37°C and 39.5°C. Cultures were then serially cultivated by using a strict passaging regime until cell division ceased (2). This involved plating 10^6 cells per 10-cm dish and passaging the cultures every 4 days. Mice were sacrificed in accordance with Home Office Regulations.

RESULTS

TMEFs Grown in the Absence of INF-γ at 33°C and 39.5°C Undergo a Finite Number of Divisions. TMEFs and NMEFs were prepared and serially cultivated by using the strict passaging regime described in *Materials and Methods*. This regime was used because initial experiments showed that it allowed NMEFs to be serially cultivated for five to six passages at 33°C. Since cultures varied somewhat in their growth profiles from experiment to experiment, we present data from three independent series of experiments for growth of both NMEFs and TMEFs cultured under different conditions to demonstrate the reproducibility of the basic phenomenon under investigation. Fig. 1 shows the growth ratios obtained at each passage for the different cultures until they ceased dividing; this point is indicated by a growth ratio of <1. A sum of the growth ratios at every passage for any particular culture yields the cumulative fold increase in cell number undergone by that culture and is a measure of the proliferative potential under those conditions.

Table 1. Growth potential of primary mouse embryo fibroblasts cultured under different conditions

Cells	Conditions	Exp. 1	Exp. 2	Exp. 3	Average fold increase
TMEFs	33°C INF-γ$^+$	Immortal	Immortal	Immortal	NA
	33°C INF-γ$^-$	7.2	5.5	8.2	7.0
	39.5°C INF-γ$^-$	13.8	21.6	22.4	19.3
	37°C INF-γ$^-$	—	—	39.7	39.7
NMEFs	33°C INF-γ$^+$	8.1	11.9	13.7	11.2
	33°C INF-γ$^-$	6.9	1.7	8.1	5.6
	39.5°C INF-γ$^-$	10.4	11.0	20.2	13.9
	37°C INF-γ$^-$	—	—	49.3	49.3

Data shown represents the sum of growth ratios obtained for TMEFs and NMEFs cultured under different conditions as shown in Fig. 1. The average fold increase for the NMEFs at 39.5°C is lower than that for equivalent cultures of TMEFs and is due to the NMEFs ceasing division much earlier than TMEFs in experiment 2. In fact, in this experiment the NMEF cultures grown under 33°C INF-γ$^-$ conditions also grew less than the TMEF cultures grown under 33°C INF-γ$^-$ conditions, whereas in other experiments TMEFs and NMEFs exhibited a very similar growth potential. NA, not applicable.

The results presented in Fig. 1 and Table 1 show that NMEFs ceased dividing under all of the different conditions but after undergoing different numbers of divisions. Fibroblasts grown in the absence of INF-γ (INF$^-$) consistently underwent more divisions when cultured at 39.5°C than at 33°C: these INF$^-$ cultures at 39.5°C exhibited a 13.9-fold increase in cell number, whereas those maintained at 33°C yielded only a 5.6-fold increase. As this result was unexpected, we also passaged cultures at 37°C in the absence of INF-γ and observed a 49.3-fold increase in cell number before the cultures became senescent; together these results suggest that the mitotic life-span was also dependent upon the growth temperature. Cultures grown in the presence of INF-γ consistently grew better than those grown in its absence; they

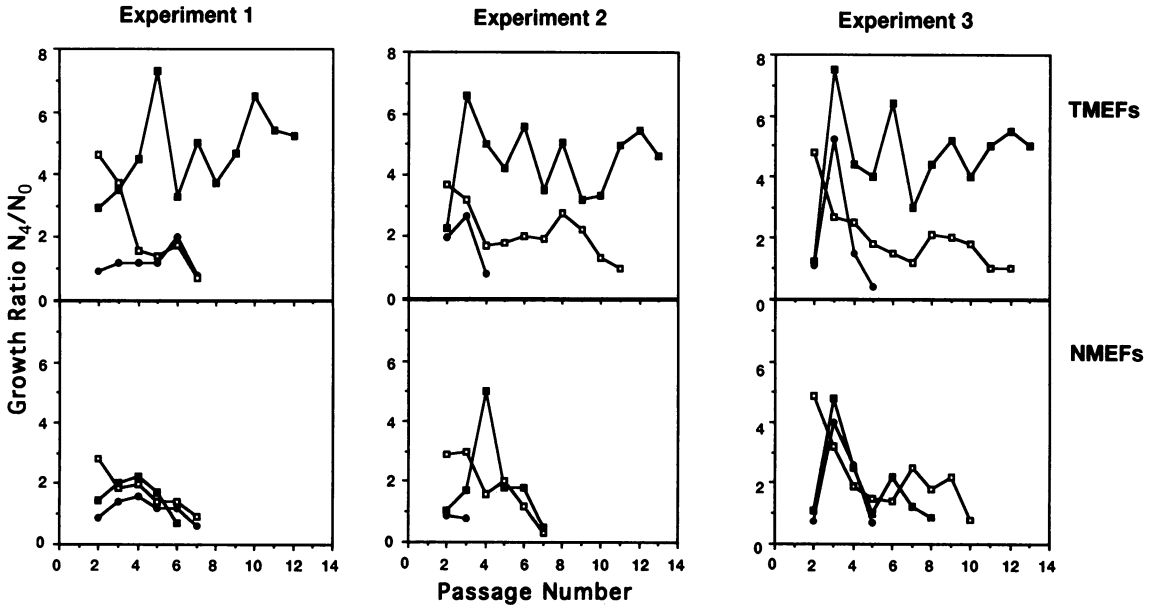

FIG. 1. Growth of primary mouse embryo fibroblasts under different culture conditions: 33°C + 10 units of INF-γ per ml (■), 33°C INF-γ$^-$ (●), and 39.5°C INF-γ$^-$ (□). Cells were trypsinized and counted every 4 days. Growth ratios were calculated by dividing the total number of cells obtained every fourth day (N_4) by the number of cells originally plated (N_0). Cells were considered to have stopped growing when the growth ratio fell below 1, at which point they were no longer plated.

underwent an 11.2-fold increase in cell number in contrast to 5.6 in its absence. TMEFs also divided for a finite period when grown under 33°C INF-γ^- or 39.5°C INF-γ^- conditions, where the level of T antigen is not sufficient for immortalization. The growth of the cultures varied between experiments, but the results within each experiment were very similar for NMEFs and TMEFs. As with NMEFs, TMEFs cultured without INF-γ underwent more population doublings (*i*) at 39.5°C (19.3-fold increase in cell number) than at 33°C (7.0-fold increase) and (*ii*) at 37°C (39.7 fold increase) than at 39.5°C, consistent with the observation that the finite proliferative potential was dependent upon the growth temperature. In contrast, TMEFs grown with INF-γ at 33°C (conditions that result in an elevated level of functional T antigen) could be passaged indefinitely; in these cultures there was a small but reproducible decline in the growth rate after the initial rapid growth period, after which the growth rate remained relatively constant. NMEFs grown under these 33°C INF-γ^+ conditions did not yield immortal cultures; thus, the continued proliferation of TMEFs under these conditions was due to the activation of the transgene by INF-γ rather than any other activity of INF-γ.

Inactivation of T Antigen in TMEFs Does Not Result in Loss of Proliferative Potential While NMEFs Are Still Dividing. Once we had shown that TMEFs grown in the absence of INF-γ behaved like NMEFs, we went on to determine when cells became dependent upon T antigen for proliferation and whether growth in the presence of functional T antigen, followed by its inactivation, would affect the finite life-span of these cells. This involved shifting TMEFs cultured under 33°C INF-γ^+ and 33°C INF-γ^- conditions to 39.5°C INF-γ^- conditions and serially passaging them under the new conditions until they stopped dividing. NMEFs cultured under 33°C INF-γ^+ and 33°C INF-γ^- conditions were also shifted to 39.5°C INF-γ^- and passaged until they too ceased to divide because data obtained from these cultures should indicate how far the fibroblasts have progressed along their normal finite life-span under each particular growth condition. The results for three experiments in Fig. 2 show the total fold increase in cell number undergone by each culture of fibroblasts after the shift to the new conditions (39.5°C INF-γ^-) and before senescence sets in; an average of the data for these experiments is presented in Table 2.

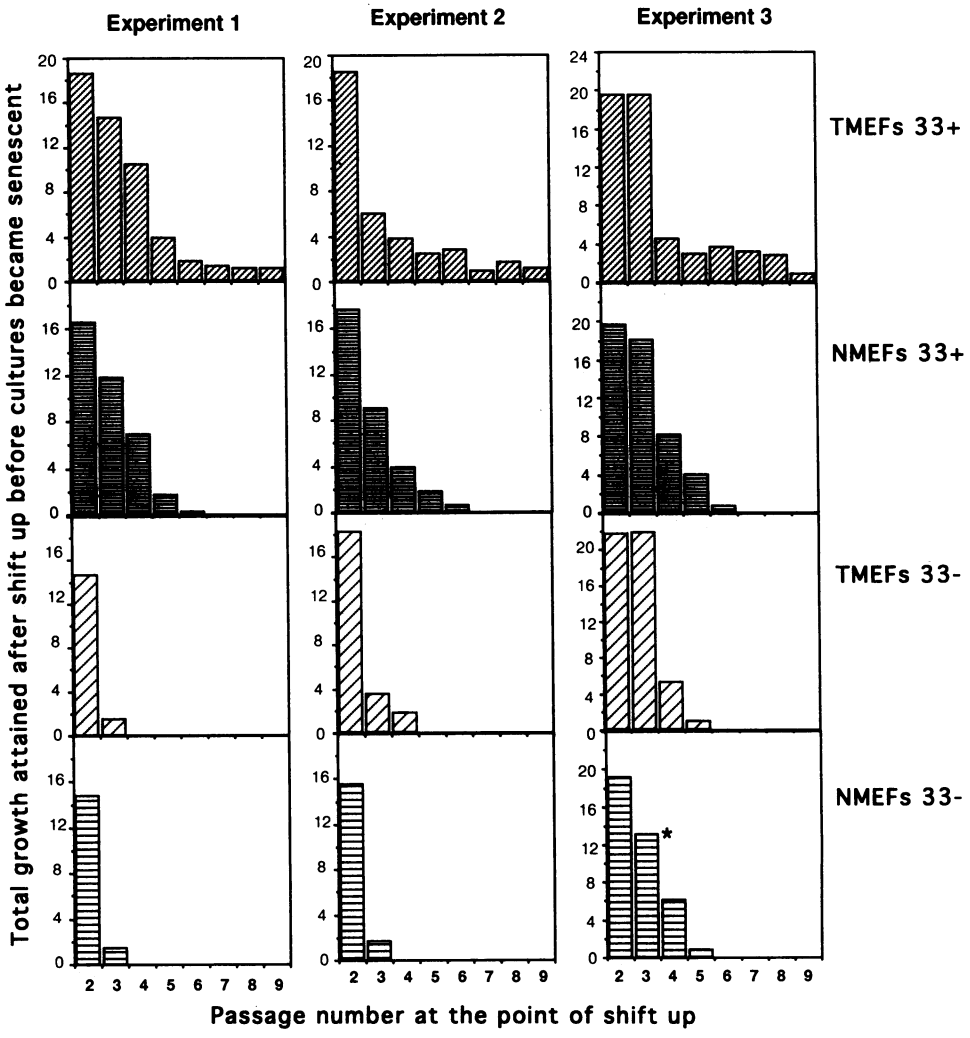

FIG. 2. Fibroblast growth upon shift to 39.5°C. This data is for the corresponding experiments shown in Fig. 1. In addition to serial passaging, representative cell populations were shifted from culture under 33°C INF-γ^+ (33^+) and 33°C INF-γ^- (33^-) conditions to 39.5°C INF-γ^- and passaged until they ceased to divide. The data represent the total growth calculated as the sum of the growth ratios (see Fig. 1) achieved by each culture before becoming senescent. The asterisk in NMEFs 33^- of experiment 3 identifies a culture that had essentially become stationary when it underwent spontaneous immortalization and the growth rate started to increase; it was subsequently serially cultivated for a further 20 passages without any loss of proliferative potential. The data presented corresponds to the growth achieved prior to the increase in growth rate. Interestingly we have also observed spontaneous immortalization in TMEFs that could not have been caused by reversion of T antigen because it was not detectable in these cultures. A low frequency of spontaneous immortalization has been observed previously for cultures of rodent fibroblasts (2).

As long as 33°C INF-γ^+ cultures of TMEFs were shifted to the nonpermissive temperature before the parallel 33°C INF-γ^+ and 33°C INF-γ^- cultures of NMEFs and 33°C INF-γ^- cultures of TMEFs had stopped proliferating, the cells continued to divide but eventually stopped, whereas cultures shifted after this point did not show any significant increase in cell number. Cultures from all conditions shifted at passage 2 to 39.5°C INF-γ^- conditions continued to proliferate, whereas TMEFs cultures shifted to 39.5°C INF-γ^- after six or more passages exhibited very little growth. Cells shifted at passage 2 to the new 39.5°C INF-γ^- culture conditions divided more than those maintained for one more passage at 33°C INF-γ^+ before the shift; passage 2 cultures underwent 18.9-fold increase in cell number, whereas passage 3 cultures underwent a 13.3-fold increase. Cells shifted to the new culture conditions at passage 4 divided even less before ceasing proliferation (Fig. 2 and Table 2). Since it was not possible to determine exactly how many divisions had occurred prior to the shift, these results were compared with those for the TMEFs grown under 33°C INF-γ^- and NMEFs grown under 33°C INF-γ^+ and 33°C INF-γ^- conditions. The results presented in Fig. 2 and Table 2 show that for each of the original growth conditions, TMEFs and NMEFs shifted at different passages to the 39.5°C INF-γ^- conditions underwent approximately the same fold increase in cell number before becoming senescent. Even though the increases in cell number that occurred upon shift to the 39.5°C INF-γ^- culture conditions varied slightly between the different experiments and depended upon whether the cultures had been grown originally with or without INF-γ, the results were consistent within each experiment (Fig. 2). Moreover, since growth at 33°C in the presence of T antigen reduced the cumulative fold increase in cell number that occurred at 39.5°C in the absence of INF-γ prior to entry into senescence, and this reduction was similar for parallel cultures of NMEFs, the results show that the biological clock that limits the mitotic potential of fibroblasts continues to count normally in cells expressing T antigen.

DISCUSSION

The finite proliferative life-span of rodent embryonic fibroblasts can be overcome by either cellular mutation or exogenous introduction of an immortalizing gene (2, 14, 15, 25). Such cells can proliferate indefinitely but are absolutely dependent upon the immortalizing gene for continued growth. Here we have shown that expression of T antigen in primary mouse embryo fibroblasts does not immediately induce them to become dependent upon it for growth but was only required to maintain the proliferative state once the normal mitotic life-span had elapsed. The results also show that the biological clock that limits the mitotic potential continues to function normally in the presence of T antigen.

Table 2. Average total growth attained after shift of culture conditions to 39.5°C INF-γ^- before ceasing proliferation

Passage no. at point of shift	TMEFs		NMEFs	
	33°C INF-γ^+	33°C INF-γ^-	33°C INF-γ^+	33°C INF-γ^-
2	18.9	18.2	17.9	16.4
3	13.3	8.9	13.0	5.2
4	6.2	2.4	6.3	6.0
5	3.0		2.2	0.8
6	2.7		1.0	
7	1.7		0.5	

Data shown is an average of the three experiments in Fig. 2 and represents the average cumulative fold increase in cell number achieved by the different cultures after shift from the culture conditions shown to 39.5°C INF-γ^- before ceasing proliferation.

The mechanism that limits the proliferative potential of normal cells is not known, but it has been suggested that it may be linked to the random accumulation of cellular damage (4, 6, 10, 11). This hypothesis suggests that as cells divide *in vitro* they accumulate mutations, karyotypic changes, and other forms of DNA damage (such as loss of DNA methylation) and that these lead to changes in the expression of positive and negative regulators of cell growth or to a predisposition to karyotypic instability, resulting in loss of proliferative potential (5, 26). Recently, it has been proposed that the processive loss of telomeric DNA and other essential sequences from the ends of chromosomes determines the finite proliferative potential (27), but a causal relationship between telomere length and the finite proliferative potential has not yet been demonstrated.

None of the above hypotheses for the regulation of the finite mitotic life-span appears to be consistent with our data. Our experiments show that the measurement of the life-span continues normally in the presence of the T antigen. If the accumulation of mutations, karyotypic changes, and other forms of DNA damage were occurring normally, then they should have resulted in cells undergoing senescence even in the presence of T antigen. Since the T antigen allows rodent fibroblasts to proliferate indefinitely, it suggests that if such changes are the molecular basis of a finite life-span, then either they can all be overridden by T antigen or they alone are not sufficient for the loss of the finite life-span.

Others have hypothesized that senescence is regulated via a genetic program, corresponds to terminal differentiation, and is a manifestation of aging at the cellular level (4–6, 12, 13). A detailed analysis of the molecular basis of senescence has so far been hampered severely by the lack of suitable systems and the asynchrony of the process. Nevertheless, a number of genes that may be involved in regulating senescence have been identified, although none of them has yet been shown to directly induce senescence (28–31). Negative growth regulatory genes have also been proposed to be involved in regulating senescence (32). Senescent cells have been found to contain the underphosphorylated form of the retinoblastoma protein, the form suggested to be an inhibitor of progression through the cell cycle (33). This failure to phosphorylate the retinoblastoma protein may be due to the absence of an appropriate member of the family of cyclin-dependent kinases or an appropriate cyclin protein (34). This has not yet been clarified, nor has it been determined whether other known tumor suppressor proteins such as p53 or other as-yet-unknown growth-suppressing genes are involved in regulating senescence. Our data are consistent with the genetic-program hypothesis and suggest that the biological counting mechanism is temperature dependent and continues to function in the presence of T antigen, resulting in cells that are committed to becoming senescent but are blocked from undergoing this transition by its presence.

Our results show that regardless of the mechanism(s) of measurement—whether it is via telomere shortening, DNA demethylation, karyotypic changes, dephosphorylation of retinoblastoma protein, or a genetic program—the changes occur normally in the presence of T antigen but do not result in the cells undergoing senescence. Even though our data supports the notion that senescence is regulated by a genetic program, it does not distinguish between the following possibilities: (*i*) fibroblasts continue to divide under their own genetic program until their normal life-span has elapsed, at which point the T antigen is required to maintain proliferation; or (*ii*) T antigen plays a role in cell division as soon as it is introduced, but the normal growth program is still operative until the end of the finite life-span, so that if the T antigen were inactivated, the cell would default to the normal growth program. This regulation of fibroblast senescence may be very similar to the regulation of oligodendrocyte

differentiation because it has been found that O-2A progenitors cultured in the presence of platelet-derived growth factor and basic fibroblast growth factor (which stimulate continuous division while preventing differentiation into oligodendrocytes) also measure the O-2A progenitor life-span (35). Thus, conditional immortalization by growth factor cooperativity may involve mechanisms that are similar to immortalization by T antigen. Moreover, it recently has been suggested that in Werner syndrome, a rare autosomal recessive disorder characterized by premature aging, the gene responsible may be a "counting" gene that controls the number of divisions that the cells undergo before terminal differentiation (36).

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