

## MECHANISM OF IMMORTALIZATION

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### ABSTRACT

**Model systems implementing various approaches to immortalize cells have led toward further understanding of replicative senescence and carcinogenesis. Human diploid cells have a limited life span, termed replicative senescence. Because cells are terminally growth arrested during replicative senescence, it has been suggested that it acts as a tumor suppression mechanism as tumor cells exhibit an indefinite life span and are immortal. The generation of immortal cell lines, by the introduction of SV40 and human papillomavirus (HPV) sequences into cells, has provided invaluable tools to dissect the mechanisms of immortalization. We have developed matched sets of nonimmortal and immortal SV40 cell lines which have been useful in the identification of novel growth suppressor genes (SEN6) as well as providing a model system for the study of processes such as cellular aging, apoptosis, and telomere stabilization. Thus, their continued use is anticipated to lead to insights into other processes, which are effected by the altered expression of oncogenes and growth suppressors.**

### INTRODUCTION

Diploid mammalian cells have a limited life span in culture (1). This phenomenon has been termed replicative senescence because cells during this stage of development exhibit an irreversible growth arrest. The cell types and eukaryotic species which display replicative senescence are highly diverse. Human diploid fibroblasts (HF) have been most extensively used as a mammalian model system for study *in vitro*. Replicative senescence is characterized by growth arrest in the G1 state of the cell cycle (2). Senescent cells remain viable for an indeterminate period *in vitro* but cannot be induced to undergo normal cell cycle stages with replenished serum. The number of generations that various cell types can be maintained in culture prior to the onset of senescence may be different. For example, primary human newborn keratinocyte and fibroblast cultures typically senesce at approximately 45-50 generations (3) and 50-70 generations (4), respectively. Although the ability to overcome senescence and become spon-

taneously "immortal" occurs with rodent cells, spontaneous immortalization has not been observed for normal human diploid fibroblasts. Thus senescence has several potential implications for cellular regulatory processes. The most prominent is as a tumor suppressor mechanism. The induction of immortalization in human fibroblasts requires several mutational events. Furthermore, the overexpression of the activated *ras* oncogene can induce a senescence-like phenotype in normal human fibroblasts (5). A second implication is based on the observation that senescent cells have altered expression of genes. An example of such is the increased expression of collagenase in senescent fibroblasts, which may affect the extracellular matrix, potentially changing the surrounding tissue integrity in older individuals (6). Finally, both transformed and normal cells have the ability to undergo apoptosis or programmed cell death. It has been observed that senescent fibroblasts do not respond to certain apoptotic signals, such as serum starvation (7), but may be responsive to others (8). These observations have far-reaching implications because it is clear that the ability to respond to stress and mutagenic insults diminish with age. Therefore, the decision to undergo apoptosis in the case of a particular signal may be critical for senescent cells. It has been proposed that the responsiveness of senescent cells to apoptotic signals may require a forced reentry into the cell cycle (8). Therefore, the failure of cells in a growth arrested state to respond to certain apoptotic cues would not only affect its action as a tumor suppressor mechanism, but would also cause the cells to accumulate. In any event, replicative senescence provides an important experimental model for cellular aging and for those factors which can influence the escape of senescence or induce immortalization.

One experimental system that explores this issue involves SV40-mediated immortalization. The DNA tumor virus, SV40, can induce immortalization in rodent and human cells. The frequencies of SV-40 induced immortalization approximate 100% in rodent cells, whereas in human cells the occurrence is dramatically lower ( $10^{-8}$  to  $10^{-5}$ ) (9,10). These observations, in addition to the fact that human fibroblasts never spontaneously immortalize, suggest that evaluating the differences between the responsiveness of the two species to factors which induce immortalization may provide further insights into the mechanisms involved. Stable introduction of SV40

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results in the appearance of the transformed phenotype of cells expressing viral T antigens and the extension of their life spans. The continued proliferation of SV40-transformed cells is dependent on continuous large T antigen function (10, 11). These cells ultimately undergo cell death or "crisis" which is distinct from senescence. Immortal derivatives can be obtained from these cultures at low frequencies as discussed above for human cultures. As such, investigators are provided the opportunity to develop genetically matched nonimmortal SV40-transformed fibroblasts (SV/HF) and immortal clonal and nonclonal sublines. In this regard, we and others have exploited this system to identify putative tumor suppressor(s) and other functions involved in immortalization.

### **SV40 TRANSFORMATION AND THE EXTENSION OF LIFE SPAN**

The expression of the SV40 genome in multiple cell types extends the life span of cells and increases the likelihood that immortal derivatives will be obtained. These effects are attributed to the SV40 large T antigen (10, 11). Large T antigen has many effects on cellular gene expression, including induction of cellular DNA synthesis in not only actively dividing cells but also quiescent and senescent cells. The extension of life span is attributed largely to large T antigen forming complexes with pRB-1 and p53 (12). Once large T antigen binds to pRB-1, the transcription factor E2F-1 is released from pRB-1 (13). The subunit composition of cyclin kinase complexes are also rearranged in SV40 transformed cells. Although large T antigen is necessary, it is not sufficient, for immortalization (14). Both SV40 large T and the small t antigen are required for a transformation phenotype; however, large T antigen alone can extend the life span of fibroblasts and ultimately result in immortalization. The expression of a conditional, temperature-dependant large T antigen (e.g., SVtsA58) in cells at the permissive temperature (35°C) leads to an extended life span (14). When these cultures are shifted to a non-permissive temperature (39°C), large T function becomes inactivated and proliferation ceases. It appears that these cells resume the senescence program (i.e., return to the number of cell divisions before and subsequent to SV40 transformation) since they proliferate for a limited time, followed by arrest in a senescence-like phenotype. If the SV40 transformed cells have exceeded their normal life span or are immortal (10), they will growth arrest and/or undergo cell death within one or a few generations upon temperature shift. The initial extension of life span by large T antigen is also limited. The population of cells appears to slow in growth and the great majority of cells die after 20-30 additional generations. This terminal state is called "crisis" and should not be confused with senescence, as cells in crisis detach from culture dishes en masse and undergo apoptosis. Although large T antigen is necessary for the extension of life span, the low frequencies at which

immortal clones can be isolated have led to the proposal of a two stage model by us (10) and others (11); namely, that additional cellular events must occur following SV40 transformation to allow cells to become immortal. We propose that these additional events necessitate the inactivation of a cellular gene, *Sen 6*, which will be discussed subsequently.

It is not clear what is responsible for crisis, which has been best studied in human fibroblasts. The mechanism may be directly related to large T function. Large T antigen has been observed to promote apoptosis when transfected into immortal cervical carcinoma cells (15). The apoptotic phenotype is associated with the overexpression of the transcription factor c-jun and may produce chronic apoptotic effects involving up to 20% of cells. We have found that large T antigen does not block some forms of apoptosis such as myc-induced apoptosis in rat cells (16). Since large T antigen binds to p53 and blocks p53-dependent pathways, it is interesting to speculate that the apoptotic events associated with crisis may be p53-independent. Alternatively, a large T-inducible survival factor (e.g., IGF-1 or IGF-1 receptor) may have altered or decreased activity (17). In order for this type of phenomenon to occur, a change in large T function would have to be operable. No such change in large T function has been observed. One might also speculate that immortal cells, which have survived crisis, have a different genetic repertoire in response to apoptosis. We have recently identified genes which are underexpressed in immortal SV/HF. One of these includes a serine/threonine (PP2C) phosphatase that is involved in signal transduction pathways (18).

### **TELOMERES AND TELOMERASE**

Processes which regulate telomere length may offer mechanisms to explain the large T-independent apoptotic signal or senescence in HF. The rate of telomere loss on human chromosomes is approximately 40-50 bp per cell generation (19). This decrease correlates with the absence of detectable telomerase activity in somatic cells, whereas in germline cells and some stem cells in which telomeres are comparably longer telomerase is present. Thus, telomere attrition may provide a causal mechanism for replicative senescence and constitutes an important biological clock. This model is supported by numerous reports of telomerase activation in many human tumours. In any event, telomere stabilization, regardless of telomerase activation, appears to be a necessary component for continued cellular proliferation.

Recent investigations have tested the telomere hypothesis of aging. Both the catalytic subunit (hTERT) and RNA moiety of telomerase have been cloned and sequenced (20). Overexpression of the catalytic subunit of telomerase has been reported to significantly extend the life span of human cells in culture (20), possibly indefinitely. In addition, introduction of wild-type hTERT into pre-crisis SV/HF will markedly increase the fre-

quency of immortalization (21). Thus, telomere maintenance plays a dynamic role for both replicative senescence and immortalization. Studies with the human papillomavirus 16 (HPV16) in human epithelial cells demonstrate that immortalization requires both the inactivation of the p16<sup>INK4a</sup>/pRb pathway and the expression of active telomerase (22). Introduction of hTERT into normal human keratinocytes or mammary epithelial cells does not extend lifespan extensively in contrast to parallel studies in HF. On the other hand, a combination of the HPV gene E7 (which inactivates pRB) and hTERT can immortalize these cells. Surprisingly, inactivation of p53 or p14<sup>ARF</sup> (i.e. the human homolog of mouse p19<sup>ARF</sup>) was not sufficient to immortalize epithelial cells. Human papilloma virus E6 (which inactivates p53) can also induce the expression of hTERT but the level appears not to be sufficient to cause immortalization in epithelial cells. Similarly, E7 alone, which inactivates pRB and down regulates p16<sup>INK4a</sup>, cannot induce immortalization. Thus, both the expression of active telomerase and the down regulation of p16<sup>INK4a</sup>/pRb pathway are required in some, but not necessarily all, cells.

There is still controversy as to the universality of telomerase activation in immortalization. Firstly, not all human tumors or immortal SV/HF display detectable telomerase activity (23, 24). Therefore, other telomere stabilization mechanisms must be operable, such as telomere recombination or telomere binding proteins. Secondly, rodents have longer telomeres than humans but age faster. In mouse knockout studies, in which the RNA component has been inactivated, mice are viable even in the presence of age-related decline of telomeric sequences (25). Third, while rodent cells spontaneously immortalize in culture, this rarely occurs for human cells. One would predict that if a one-step mutational event could activate telomerase, it would lead to immortalization of human cells, and higher spontaneous immortalization rates would be operant for human cells. Yet this does not occur. Such paradoxes have elicited questions as to the exact role of telomerase during immortalization (26).

### SEN6, A NOVEL GROWTH SUPPRESSOR GENE

Invoking a two-stage model for immortalization as described earlier suggests that inactivation of a cellular growth suppressor gene occurs in the second step following SV40 transformation. There is much genetic evidence to support this view (27). Early studies have shown in cell hybrids between immortal and normal cells, that the hybrid displays a limited life span consistent with immortalization being a recessive phenotype. Also complementation resulted when immortal cell lines from various cell types were fused. The hybrids also exhibited limited growth and a limited number of genetic loci were involved with only four complementation groups identified. We have used chromosomal transfer and chromosomal deletion analysis to identify putative growth suppressor genes involved in SV40-mediated immortal-

ization. Introduction of human chromosome 6 or 6q into immortal SV/HF suppresses growth (28).

Mapping studies employing 12 independent SV/HF cell lines from three different laboratories have defined a minimal deleted region at 6q26 to 6q27 (29). More recent data (unpublished) have further localized this gene at or near AF6 (MLLT4) in 6q27. We have termed this putative growth suppressor gene on the long arm of chromosome 6 as *SEN6*. Currently, we are testing this region for growth suppressor activity using retrofitted YAC contigs. Ongoing studies are also consistent with the possibility that AF6 could be the actual growth suppressor. Not only is it located at 6q27, but one allele is disrupted in one immortal SV/HF and genetic evidence supports a mutation in the other allele. In DNA-mediated gene transfer into immortal SV/HF, constructs overexpressing AF6 cDNA inhibit colony formation. AF6 has several interesting features in that it is localized in tight junctions in rat epithelial cells (30), has domains which can bind to *ras* as well as actin (29) and has a PDZ domain, implicated in protein-protein interactions including with EphB3, a member of the membrane receptor protein tyrosine kinase family (31).

### CONCLUDING REMARKS

Immortalization by SV40 (and HPV) involves a myriad of processes. It includes inactivation of at least three growth suppressors, pRB, p53, and *SEN6*. For immortalization to proceed, telomeres need to be maintained whether be it through telomerase induction or telomerase independent mechanisms. SV/HF also provide a means to determine the relationship between apoptosis and replicative senescence. It is not clear how senescent cells die; therefore, comparisons of SV/HF to genetically-matched normal cell strains will be invaluable tools toward this end. Finally, study of immortalization should continue to shed light on key issues associated with human carcinogenesis.

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