The role of senescence and immortalization in carcinogenesis

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Normal somatic cells are able to divide only a limited number of times before they become senescent. The occurrence of intratumoral cell death and the need for clonal evolution mean that many more cell divisions are required for tumorigenesis than is possible unless cells breach the senescence proliferation barrier and become immortalized. Senescence may therefore be a major tumor suppressor mechanism. During the past decade the study of senescence and immortalization has entered the mainstream of cancer research. A major reason for the current interest in this subject is the observation that most cancers have an activated telomere maintenance mechanism, a marker of immortalization. It has also been found that some of the most common genetic changes known to occur in cancer have a key role in the immortalization process.

Introduction

Normal mammalian somatic cells proliferate a limited number of times in vitro (1, 2), with the maximum number often being referred to as the ‘Hayflick limit’. When this limit is reached the cells undergo an array of biochemical and morphological changes suggestive of ageing, so the process is referred to as senescence. Typically the cells become enlarged and express a pH-dependent galactosidase activity (3). The altered pattern of gene expression in senescent cells may be determined partly by cell lineage (4). Senescent cells may remain metabolically active for a long period of time, even though they have permanently ceased proliferating (reviewed in refs 5–8).

It has been suggested that senescence forms a barrier against tumorigenesis (9) and that acquisition of the ability to proliferate an unlimited number of times (immortalization) is an essential step in the malignant transformation of normal cells. Two main arguments have been advanced against this suggestion. The first is based on the observation that it is very difficult to obtain immortalized cell lines from many cancers. The second argument is based on simple calculations showing that the Hayflick limit is compatible with the formation of very large tumors.

In this article, the evidence that immortalization has an important role in carcinogenesis is reviewed. The evidence includes the observation that most cancers have an activated telomere maintenance mechanism (usually, but not always, telomerase enzymatic activity), which is a marker of immortalization. It also includes the finding that some of the most common known genetic changes in cancer, inactivation of p53 and of the Rb/p16INK4a pathway, have a key role in the immortalization process. Finally, a consideration of aspects of tumor biology including the multistep nature of tumorigenesis suggests that the number of cell divisions required for the development of tumors is much greater than the Hayflick limit.

Telomere maintenance, immortalization and cancer

Hayflick’s finding that senescence is a function of the cumulative number of cell division cycles (1, 2) has been interpreted to mean that there is a cell division cycle counting mechanism, or ‘clock’, in normal somatic cells. According to the telomere hypothesis of senescence, the clock mechanism is the progressive telomere shortening that occurs with cell division (10). Telomeres form protective caps that prevent the ends of chromosomes being recognized as double-strand breaks and prevent end-to-end telomere fusion events. They contain repetitive DNA (in all vertebrates the repeat unit is a hexanucleotide, TTAGGG), to which proteins bind specifically. Before any details of telomere structure were known, Olovnikov hypothesized that DNA is lost from the ends of chromosomes each time a cell divides and that this acts as the counting mechanism which determines that senescence should occur when the telomere length has decreased below a certain point (10). There are now two main lines of evidence in support of this hypothesis (11). The first is that progressive telomere shortening with cell division in vitro has now been documented in many studies. The reasons for this progressive shortening are not entirely clear, but may include the inability of the conventional DNA replication machinery to fill in the gap remaining after degradation of the terminal RNA primer and the activity of a putative 5’→3’ exonuclease (12) that shortens the C-rich strand thereby creating or increasing the length of a single-stranded G-rich telomeric tail (Figure 1). The second line of evidence is the observation that expression of telomerase, an enzyme that is able to maintain telomere length, allows some otherwise normal human cells to bypass senescence (13, 14).

It is possible that there may be clocks other than, or in addition to, telomere shortening (15), i.e. senescence may be triggered by multiple pathways (16). There is a rapidly growing list of treatments, many of which have no obvious relevance to telomere length, that cause a senescence-like state (often referred to as ‘premature senescence’) prior to the Hayflick limit being reached (15). Nevertheless, it is clear that immortalization and the activation of a telomere maintenance mechanism are closely associated: every immortalized human cell line examined to date has been found to have telomere maintenance activity (17). Furthermore, inhibition of telomere maintenance can result in a finite proliferative life span being reimposed on immortalized human cells (18, 19). In the majority of immortalized cell lines, and in ~85% of human cancers, telomere maintenance is dependent on the enzyme telomerase.

Abbreviations: ALT, alternative lengthening of telomeres; HPV, human papillomavirus; hTERT, human telomerase reverse transcriptase; hTR, human telomerase RNA; PD, population doublings; SF, surviving fraction; SV40, simian virus 40; TPA, terminal proliferation arrest.
Fig. 1. Shortening of telomeres during replication of DNA in somatic cells. (A) The C-rich 5’ strand of the telomere is shorter than the G-rich 3’ strand, so there is less C-rich template available to be replicated. (B) DNA synthesis requires annealing of RNA primers. (C) Degradation of the RNA primers leaves gaps. (D) Internal gaps are filled, but the terminal gap on the lagging strand is not. (E) A putative 5’→3’ exonuclease degrades an additional 130–210 nucleotides, further shortening the C-rich 5’ strands (12). There is thus less template that can be replicated in the next round of DNA synthesis.

Fig. 2. Extension of the G-rich telomeric strand by telomerase. The telomerase holoenzyme contains protein subunits including a catalytic subunit with reverse transcriptase activity and an RNA molecule that acts as the template for addition of TTAGGG repeats to telomeres.

Fig. 3. A possible mechanism for alternative (non-telomerase) lengthening of telomeres (ALT). Telomeres have been shown to form a loop structure, with the single-stranded G-rich tail invading a double-stranded region resulting in a displacement (D)-loop (33). It is possible that the G-rich tail can use the invaded region as a template for its elongation. Temporary resolution of this structure may permit partial filling-in of the C-strand.

and retrotransposon reverse transcriptases, and there is an additional motif, T, that is telomerase specific. There is evidence suggesting that a number of other proteins, including TEP1 (25,26), p23 and HSP90 (27), may form part of the telomerase complex.

Although telomerase activity has been detected in some normal human tissues, in most cases the level of activity does not appear to be sufficient to prevent telomere shortening (28). There is a very close temporal correlation between the immortalization event and the onset of telomerase activity (29). It has been found that transduction of normal cells with hTERT cDNA expression constructs induces telomerase activity (30), and this permits cells to bypass senescence (13,14).

Some mutant yeast cells lacking telomerase are able to survive by a mechanism that is dependent on the RAD52 gene which encodes a protein involved in DNA recombination (31). It is not known whether telomerase-negative immortalized mammalian cells all use the same telomere maintenance mechanism, but it is possible that some may use a mechanism similar to that in yeast in which homologous telomeric DNA acts as the copy template for extension of telomeres (32). It has recently been shown that telomeres may form a loop structure (33), so it is possible that any telomere in an ALT cell can use its own DNA sequence as the template (Figure 3). Normal cells and some telomerase-positive cells contain a repressor of the ALT mechanism (34). [Similarly, normal cells (18) and some ALT cells (35), contain telomerase repressors.]

Human tumors have not yet been surveyed extensively for ALT activity, but ALT has been found in a minority of the following types of tumors and/or tumor-derived cell lines: adrenocortical, breast, renal cell, and non-small cell lung carcinomas, melanomas and osteosarcomas (23,36; unpublished data). Some individual tumors use both ALT and telomerase (23). The recent finding that a specific form of nuclear body containing telomeric DNA, telomere-specific binding proteins and proteins involved in recombination is a morphologic marker of ALT that may be detected in paraffin-embedded tumor material (37) will facilitate clinical studies of ALT.
Fig. 4. Terminal proliferation arrest (TPA) states. Normal cells divide a limited number of times before permanently exiting the cell cycle and remaining in a viable non-proliferative state referred to as senescence (1). If p53 is inactivated in these cells, the cells may resume dividing a limited number of times, before they permanently exit the cell cycle (p53-minus TPA) (38,39). If p53 and the Rb/p16INK4a pathway are both disrupted, by example, by the presence of SV40 or HPV viral oncoproteins, the cells may bypass senescence but subsequently arrest in a state referred to as crisis (44). A rare cell (~1 in 10^5) may escape from crisis and become immortalized. Transduction of some normal cells with hTERT expression constructs may result in expression of telomerase and bypass of senescence.

Multiple barriers to immortalization

The terminal proliferation arrest (TPA) state referred to as senescence is not the only barrier to unlimited proliferation. Fibroblasts in which the normal function of p53 has been abrogated by transduction with a mutant p53 (38), by spontaneous loss of the single wild-type p53 gene in the case of Li–Fraumeni cells containing an inherited null-mutant p53 allele (39), or by expression of the human papillomavirus (HPV) E6 oncprotein that targets p53 for ubiquitin-mediated proteolysis (40), are able to proliferate for a limited number of population doublings (PD) beyond the point at which unaltered normal cells become senescent (Figure 4). Despite the absence of normal p53 function, these cells eventually enter a TPA state (referred to as ‘p53-minus TPA’ (41)).

Loss of p16^INK4a expression due to methylation of the gene’s CpG island in normal breast epithelial cells is associated with a finite extension of proliferative life span (42), and loss of pRb function due to expression of the HPV E7 oncprotein in fibroblasts also results in temporary escape from senescence (40). It seems very likely that pRb and p16^INK4a act in the same pathway for control of proliferative life span, because of the pro-apoptosis role of pRb, this may sequester p53 in the cytoplasm and prevent its normal effect on extension of proliferative life span (39,43).

Cells expressing viral oncoproteins that inactivate both p53 and pRb have an extended proliferative life span that terminates in a TPA state referred to as crisis (44) (Figure 4). The cell biology and gene expression patterns of cells in crisis are still very poorly defined. Approximately 1 in 10^5 cells escape from crisis and become immortalized (45). Thus, the TPA states may be regarded as a series of barriers to immortalization with crisis being the ultimate barrier. Senescence and crisis are sometimes referred to as M1 (for Mortality stage 1, although it should be noted that this is a viable proliferation arrest state) and M2 (Mortality stage 2), respectively (46), and in accordance with this nomenclature the intermediate TPA states have been referred to as Mint (40).

Cells that escape from senescence continue to undergo telomeric shortening until they reach the p53-minus TPA (39) or crisis (29) barriers. Cells that escape from crisis and become immortalized all activate a telomere maintenance mechanism. It thus seems likely that excessive telomere shortening has a key role in triggering crisis. In support of this concept, it is noteworthy that the excessive telomere shortening that occurs in late generation telomerase-negative mice results in a state resembling crisis (47). Forced expression of telomerase in pre-crisis cells by transduction with an hTERT expression construct resulted in bypass of crisis (48–51).

As mentioned above, forced expression of telomerase activity in otherwise normal fibroblasts and retinal pigment epithelial cells by expression of hTERT from a heterologous promoter permits bypass of senescence (13,14) and a large extension of proliferative life span, possibly immortalization (52–54). However, in a wide variety of cell culture models of immortalization (for example, transduction of DNA tumor virus oncogenes, chemical carcinogen treatment, irradiation and spontaneous immortalization of Li–Fraumeni syndrome cells), activation of a telomere maintenance mechanism is preceded by loss of p53 and Rb pathway function. It is possible that the consequences of losing the function of these tumor suppressor genes include facilitation of de-repression of hTERT expression; this would not be required when hTERT is expressed from a heterologous promoter. Interestingly, in two types of human epithelial cells grown under specific culture conditions, hTERT expression does not affect proliferative life span unless the Rb/p16^INK4a pathway is inactivated (55), so in these cells loss of the Rb/p16^INK4a pathway has a function in addition to creating a permissive environment for telomerase activation.

Other genes involved in senescence and immortalization

In addition to p53, p16^INK4a, Rb and the genes required for telomere maintenance, there may be other genes that are involved in senescence and immortalization (41). The chromosomal locations of a number of putative senescence genes have been identified; some of these do not have any known connection with p53, p16^INK4a, Rb or telomere maintenance, suggesting that senescence may be signaled by multiple pathways (16).

Not all cancers have mutations in p53 and it is possible that abnormalities of other genes that are upstream (or downstream) of p53, or that otherwise interact with p53, may have the same effect. In this regard, p14^ARF and MDM2 which control p53 availability in the nucleus (56,57), p33^ING1, which may be required for some of p53’s functions (58), and mortalin which may sequester p53 in the cytoplasm and prevent its normal function (59), are interesting candidates. Similarly, abnormal expression or function of genes that act in the same pathway as p16^INK4a and Rb, such as CDK4 or a D-cyclin, or proteins such as the basic helix–loop–helix transcription factor Id-1 that may interact with factors required for function of pRb (60) might substitute for abnormalities of these genes. Genes affecting both the p53 and Rb/p16^INK4a pathways, such as the Polycomb-group transcriptional repressor bmi-1 (61) that coordinately regulates p14^ARF and p16^INK4a, are also of particular interest.

As described above, there is evidence that normal cells contain repressors of the telomere maintenance mechanisms, telomerase and ALT. The chromosomal locations of these repressors are being mapped (18,62). The cloning of the upstream control region of the hTERT gene (63) should facilitate attempts to identify repressors of telomerase.
Is immortalization required for carcinogenesis?

Prior to the finding that most cancers have an activated telomere maintenance mechanism, the relationship between immortalization and cancer was somewhat controversial. An indirect line of evidence for a causal relationship is that the strains of HPV associated with malignancy are the same strains that can induce immortalization of human cells in culture (64–66). More direct evidence is provided by the observation that in many combined in vitro/vivo experimental systems immortalization is a prerequisite for the induction of tumors (51, 67–70; reviewed in ref. 71). For example, normal human fibroblasts and fibroblasts that were transduced with simian virus 40 (SV40) oncogenes but were not immortalized, were not transformed by an activated ras oncogene. In contrast, SV40-immortalized fibroblasts underwent malignant transformation (67) (Figure 5A). Interestingly, although such studies clearly showed that immortalization was a prerequisite for ras-induced tumorigenicity, evidence was subsequently obtained that at least one, currently unidentified, additional genetic change was also required (72, 73).

A clue to the molecular mechanisms underlying this relationship has been provided by studies of ras signaling in mouse fibroblasts (Figure 5B). Activated ras resulted in constitutive activation of the MEK/ MAPK mitogenic signaling pathway. In normal cells, this resulted in upregulation of p53 and p16INK4a and premature senescence (74).

As mentioned above, however, one argument against an important role of immortalization has been that many cancers do not appear to contain immortalized cells. Although there are some types of cancer, such as small cell carcinoma of the lung, where experienced cell culturists have had high success rate in obtaining permanent cell lines, there are other types, such as breast cancer, which are notoriously difficult in this respect. The debate has been whether this means that such cancers do not contain immortalized cells or whether suitable culture conditions have not yet been developed. Early support for the idea that cancers contain immortalized cells came from laboratory studies showing that normal tissues can be serially transplanted in animals only a finite number of times whereas cancers may be transplanted an apparently unlimited number of times (75). The more recent demonstration that most cancers contain telomerase activity (20), a marker of immortalization, suggests that the inability to obtain immortalized cell lines from many tumors is due to suboptimal cell culture systems.

A second argument against the proposition that immortalization is required for tumorigenesis is that the Hayflick limit appears to be consistent with the generation of large tumors. A fibroblast obtained from a middle-aged human is usually capable of a further 20–40 PD (2). Forty PD are capable of generating 2^40 cells which would be equivalent in total mass to ~1 kg of tumor. However, this simplistic calculation overlooks two key factors that greatly increase the number of PD required to form a tumor of significant size.

Table 1. Effect of cell death on number of cell divisions required for tumor formation

<table>
<thead>
<tr>
<th>Tumor size</th>
<th>No. of cells</th>
<th>Cell divisions required (SF=)</th>
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<td></td>
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<td>(1.0)</td>
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<tr>
<td>1 g</td>
<td>10^9</td>
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<tr>
<td>1 kg</td>
<td>10^{12}</td>
<td>40</td>
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<td></td>
<td></td>
<td>(0.75)</td>
</tr>
<tr>
<td>1 g</td>
<td>10^9</td>
<td>51</td>
</tr>
<tr>
<td>1 kg</td>
<td>10^{12}</td>
<td>68</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(0.60)</td>
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<tr>
<td>1 g</td>
<td>10^9</td>
<td>114</td>
</tr>
<tr>
<td>1 kg</td>
<td>10^{12}</td>
<td>152</td>
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*Fraction of cells that are able to replicate.

The calculation that 40 PD is capable of generating 2^40 cells assumes that every cell survives. It is clear, however, that this is not the case in real tumors for a variety of reasons including periods of inadequate blood supply leading to hypoxia and necrosis, especially at the center of the tumor. Genetic instability, as well as providing the engine for clonal evolution, also results in the production of many non-viable cells. Reliable estimates of the overall cell death rate within tumors are very difficult to obtain, but if the probability that any given cell is able to replicate is 75% [i.e. the surviving fraction (SF) is 0.75] then 67 PD would be required for the tumor size to reach 1 kg, and if the SF is 0.60 then 151 PD would be required (Table I).

Clonal evolution in oncogenesis

A second, and possibly more important consideration is that there is now very abundant evidence that tumorigenesis requires the accumulation of many genetic defects. This greatly increases the number of PD required to produce a tumor. After the first hit has occurred in a pre-existing population of cells within an essentially normal tissue, if each subsequent hit happens at an average rate of ~1 per 10^4 (= 2^13 cells), then 13 PD are required for the clonal expansion necessary per hit for SF = 1.0 (or ~23 PD for SF 0.75). (A mutation rate of 1 per 10^4 may occur in cells with a high level of genomic instability; if the rate is lower, the required number of PD will be greater.) Thus 13(x(n – 1)) PD are required to produce a cell with n hits and, simplifying a little by ignoring the contribution to the tumor mass of normal cells and cells with <n hits, a further 40 PD are required to produce a 1 kg tumor.
Senescence and immortalization

The in vitro replicative life span of cells from various species correlates with the maximum life span of members of those species (77). In vitro senescence is therefore studied as a model of organismal ageing (5,78,79). A compelling argument has been made for the proposition that the limited proliferative potential of somatic cells is the result of limited investment of cellular energy in the complex array of maintenance processes required to prevent an accumulation of defects in various cellular macromolecules, including, but certainly not limited to, DNA (80,81; reviewed in ref. 82). According to this view, the germline requires very high-fidelity maintenance processes, but the soma is disposable and consequently less energy needs to be expended upon repair and maintenance of its cells. Long-lived species clearly need to have somatic cell repair processes that are more precise than those of short-lived species, but no purpose is served by expending the energy required for maintaining cells such that they are capable of unlimited replication. Darwinian fitness is better served by investing this energy in reproduction instead. In this view the maximum replicative capacity of cells is determined by the genes that control the fidelity of the repair processes.

Whether or not the senescence growth arrest state is also predetermined in the sense that it is a programmed series of events executed by the cell, however, requires further study. An apparently major difficulty with the concept of senescence being a program executed by the cell is that programs must be produced by natural selection, and at first sight it seems unlikely that natural selection should act to produce a programmed cellular response that operates at the end of life, i.e. after organismal reproduction has ceased. This difficulty is resolved if the senescence arrest state also has a role in the period of life up to and including reproduction. Although the chance of a catastrophic accumulation of errors within any given somatic cell increases with increasing age of the organism, this may also occur in a stochastic manner in some cells early in life, as a result of environmental and/or endogenous events. There may be an advantage to the organism in being able to deal with this contingency by activating a permanent growth arrest state within such cells as an alternative to programmed cell death. At present it is only possible to speculate as to the nature of this advantage. In some situations it may be advantageous to retain some of a cell’s biochemical functions but to prevent it from replicating and propagating the genetic damage that it has accumulated. There may even be situations where it is advantageous to contain various damaged macromolecules within the membranes of a live but non-replicative cell. It may also be particularly useful to have a counting mechanism that keeps track of the number of times a cell has proliferated so that the senescence arrest state is triggered pre-emptively well before catastrophic accumulation of damaged macromolecules is likely to have occurred. Furthermore, the evidence presented above suggests that senescence and related forms of proliferation arrest are a major barrier to the development of cancer. If this is the case, then senescence may act to increase the chance of cancer-free pre-reproductive survival, which is of particular importance in a long-lived species such as Homo sapiens where reproduction occurs relatively late.

Fig. 6. The effect of clonal evolution on the number of population doublings (PD) required for tumorigenesis. For a hypothetical tumor that requires only two genetic 'hits', the first change occurs in a pre-existing population of genetically normal cells. If the second hit occurs in 1 per 10^4 (= 2^13) cells, then the required number of cells will be generated by 13 PD, assuming no cell death (i.e. SF = 1.0). A mutation rate of 1 per 10^8 may occur in cells with a high level of genomic instability; if the rate is lower, the required number of PD will be greater. A further 40 PD are required to produce a 1 kg tumor (Table I). The number of PD required for SF = 0.75 is shown on the right. In reality, the number of genetic changes required for tumorigenesis is much greater than two.

of n-hit cells (for SF = 1.0). Thus, the total PD required for a 1 kg tumor is 13×(n – 1) + 40 PD if the SF is 1.0, or 23×(n – 1) + 68 PD if the SF is 0.75. In Figure 6 this is shown schematically for n = 2 hits. Although the calculation is dependent on the mutation rate, the SF and the number of genetic hits required, for most common solid tumors where it is estimated that at least five (and maybe many more) critical genetic changes are essential (76), the number of PD required greatly exceeds the Hayflick limit. A corollary of this is that the situations where immortalization may not be essential for oncogenesis might be where there is a combination of some of the following factors: (i) a tumor arising early in life in cells where there is a large number of available PD; (ii) a high SF, for example in leukemias where there is no problem with blood supply; and (iii) where the number of genetic hits required is small, including genetically simple tumors and when tumorigenesis is initiated by infection with a virus that supplies the equivalent of multiple genetic hits.

Senescence and subsequent proliferation barriers as an anti-tumor mechanism

Although the above considerations suggest that senescence and the backup TPA states are an important barrier to tumorigenesis, the nature and function of senescence is still enigmatic. The various forms of permanent growth arrest may be subdivided according to whether they result in the death and subsequent destruction of the cell (e.g. apoptosis), or in live, metabolically active cells that are incapable of further replication (e.g. terminal differentiation or senescence). In contrast to senescence, the roles of apoptosis and terminal differentiation are much better understood.
Perspectives and prospects

The evidence indicates that senescence and the backup terminal proliferation arrest states represent a major barrier to tumorigenesis. Immortalization is not sufficient for tumorigenesis, but may be regarded as a necessary prerequisite for accumulating the total number of genetic changes required for malignancy. The genetic changes associated with immortalization, namely loss of p53 and RB/p16\(^{INK4a}\) function and activation of telomerase are among the most common known cancer-related changes, supporting the conclusion that immortalization is a key component of the cancer phenotype. In the case of p53, RB and p16\(^{INK4a}\), the reason that disrupted function of these proteins is so commonly selected for in cancer is likely to be that, among many other consequences, immortalization is thereby facilitated. In the case of telomerase activation, escape from proliferation barriers is most likely the main phenotype (together with prevention of excessive karyotypic instability arising from joining of unprotected chromosome ends) that is being selected for in tumors.

To put our current understanding of this subject into historical perspective, 20 years ago it was known that in contrast to tumor-derived cell lines, somatic cells have a finite proliferative life span in vitro (1,2), and that this could be abrogated by SV40 genes (44). The telomere hypothesis of senescence had been proposed (10), but was not widely known. Over the subsequent decade, a number of key studies showed that immortalization is required for tumorigenesis in a number of experimental systems (67,69,70). The SV40 large T antigen-interacting proteins, p53 and RB, were identified during this time, and ~10 years ago the first evidence that they were involved in immortalization was obtained (46). In the past decade, many genes interacting with, and related to p53 and RB have been described. There has been widespread interest in the potential role of telomerases in senescence, and the existence of telomere maintenance mechanisms in immortalized human cells was described. Some of the components of the telomerase holoenzyme have been identified.

As will be apparent from the preceding discussion, there are still many gaps in our understanding. Major gaps include the in vivo role of senescence, and other TPA states. The basic cell biology and gene expression patterns of cells in crisis are poorly understood. What triggers senescence and the genes and signaling pathways involved are only just beginning to be understood. Significant advances have been made in understanding the function of telomerase, but there is little definitive evidence as to the mechanism(s) involved in ALT. It will also be important to investigate the normal control mechanisms for telomerase and ALT activity.

Because the immortal phenotype appears to clearly distinguish cancer cells from normal somatic cells, an understanding of the cellular and molecular biology of immortalization may result in new tools for diagnosis of cancer, and also in novel forms of anti-cancer therapeutics. It is particularly encouraging to note that it is not necessary to correct all of the genetic defects that are required for immortalization in order to restore a limited life span: correcting only one of the defects may be enough (83).

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