Title

Loss of Chromosomal Integrity in Human Mammary Epithelial Cells Subsequent to Escape from Senescence

Journal

Journal of Mammary Gland Biology and Neoplasia, 6(2)

ISSN

1083-3021

Authors

Tlsty, Thea D
Romanov, Serguei R
Kozakiewicz, B Krystyna
et al.

Publication Date

2001-04-01

DOI

10.1023/a:1011369026168

Peer reviewed

Journal of Mammary Gland Biology and Neoplasia, Vol. 6, No. 2, 2001

Loss of Chromosomal Integrity in Human Mammary Epithelial Cells Subsequent to Escape from Senescence

Thea D. Tlsty,[1,3] Serguei R. Romanov,[2] B. Krystyna Kozakiewicz,[1] Charles R. Holst,[1] Larisa M. Haupt,[1] and Yongping G. Crawford[1]

The genomic changes that foster cancer can be either genetic or epigenetic in nature. Early studies focused on genetic changes and how mutational events contribute to changes in gene expression. These point mutations, deletions and amplifications are known to activate oncogenes and inactivate tumor suppressor genes. More recently, multiple epigenetic changes that can have a profound effect on carcinogenesis have been identified. These epigenetic events, such as the methylation of promoter sequences in genes, are under active investigation. In this review we will describe a methylation event that occurs during the propagation of human mammary epithelial cells (HMEC)[4] in culture and detail the accompanying genetic alterations that have been observed.

KEY WORDS: Genomic instability; methylation; senescence; carcinogenesis.

INTRODUCTION

DNA methylation is the best-studied epigenetic event associated with cancer (1). Methylation of cytosine residues in CpG sequences has been shown to silence promoter activity of specific genes and is a frequent event in cancer progression (2). The mechanism by which this methylation is targeted is mostly unknown at the present time but insights into the process are beginning to emerge (3). The creation and ablation of methylation patterns is a frequent event in carcinogenesis and is believed to be instrumental in tumor progression (4–6). Several genes important in cancer initiation and progression (retinoblastoma gene, E-cadherin, insulin-like growth factor-2 gene, von Hippel-Lindau gene, the hMLH1 gene and $p16^{INK4A}$) show altered methylation patterns during cancer progression (2). In this review we focus on the cyclin dependent kinase inhibitor $p16^{INK4A}$ which has been reported to be frequently methylated in a variety of tumors. This gene resides on the tip of human chromosome 9p within a complex locus that controls two other tumor suppressor genes $p19^{ARF}$ and p 15. In addition to the reported methylation of the $p16^{INK4A}$ gene in cancers, methylation of this gene has also been reported in tissue culture studies.

Irreversible senescence and tight control of genomic stability are believed to be important barriers to the development of malignant lesions. Our current understanding of these processes derives largely from studies in fibroblasts. In a recent study of normal human mammary epithelial cells *in vitro* a high frequency spontaneous epigenetic event was identified that resulted in cells in which both these critical cellular properties are lost (7). The methylation of the $p16^{INK4A}$ promoter and the silencing of the gene accompany the emergence of mammary epithelial cells

[1] Department of Pathology, University of California at San Francisco, San Francisco, California 94143-0506.

[2] Thurston Arthritis Research Center, and Center for Inflammatory Disorders, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599-7280.

[3] To whom correspondence should be addressed at 513 Parnassus Ave, Box 0506, Department of Pathology, University of California at San Francisco, San Francisco, California 94143-0506. e-mail: ttlsty@itsa.ucsf.edu

[4] Abbreviations: human mammary epithelial cells (HMEC); human mammary fibroblasts (HMF); the catalytic subunit of telomerase (hTERT); senesence associated-β-galactosidase (SA-β-gal); human papilloma virus (HPV).

1083-3021/01/0400-0235$19.50/0

from senescence (7–10). In addition to growth beyond senescence, these mammary epithelial cells have an altered response to some types of genomic damage and accumulate genomic abnormalities with a startling frequency (7).

Fibroblasts and Senescence

Our current understanding of senescence and other barriers (e.g., checkpoint control) to unrestricted proliferation in human cells derives mostly from studies in fibroblasts. In tissue culture, human skin fibroblasts proliferate for a limited number of population doublings and then enter a growth plateau, termed senescence or Hayflick limit, an insurmountable barrier to further population expansion (11). For years, the only way that fibroblasts could escape senescence was by expression of viral oncoproteins such as SV40 TAg. More recent experiments in which the expression of the catalytic subunit of telomerase (hTERT) was manipulated have demonstrated that fibroblasts can grow in culture for an extended period of time beyond which they would normally enter senescence (12). Expression of the viral oncoproteins results not only in extended proliferation but also entry into a second growth plateau called crisis, a second bottleneck from which immortal cell populations can arise. Cellular characteristics at crisis differ greatly from those at senescence, with cell division, cell death and genomic instability being notably prominent in crisis and negligible in senescence. Senescence is believed to be a critical barrier to the development of cancer and, as such, it was important to extend these early studies on fibroblasts to other cell types that are the biologically relevant counterparts of the transformed cells found in tumors.

Interestingly, mammary epithelial cells do not conform to the paradigm of senescence first described in fibroblasts. In the mid-1980s Stampfer and colleagues (13) documented the growth profiles of human mammary epithelial cells. Normal human mammary epithelial cells were shown to have an extremely short life span *in vitro* (averaging 20 population doublings) before they ceased proliferation and entered a growth plateau. When cultured in serum-free media, these primary mammary epithelial cells were observed to undergo a process termed self-selection, resulting in a subpopulation of cells which have an extended but still finite life span (13, 14). These post-selection cells are capable of undergoing an additional 20–70 population doublings. Based on these observations, it was previously postulated that senescence in mammary epithelial cells involves two steps. The initial growth arrest (termed M0) describes the growth arrest of the pre-selection cells and a second (termed M1) describes senescence in aged post-selection cells (15). As discussed later, these terms are not in keeping with the biochemical data. These data indicate that the first growth plateau, selection, has many of the hallmarks of senescence except that it is not an insurmountable barrier to further growth. The second growth plateau, previously called senescence, has many (but not all) hallmarks of the viral oncoprotein-induced crisis seen in fibroblast cells. In the next section the biochemical data in fibroblasts will be described and compared with similar findings in human mammary epithelial cells.

Human Mammary Fibroblasts Proliferate and Senesce as Described Previously for Other Human Fibroblasts

In a recent study (7), isogenic human mammary fibroblasts (HMF) and human mammary epithelial cells (HMEC) from the same gland were isolated from healthy tissues and grown as previously described (13, 14, 16). The growth kinetics of mammary fibroblasts were similar to those previously described in human skin fibroblasts (17, 18). The cells underwent a limited number of population doublings prior to entering a phase with no increase in cell number. This plateau in population growth, variously termed the Hayflick limit (11), irreversible replicative senescence (19), and mortality stage 1 (M1) (20, 21), exhibits several distinctive features. The cells enlarge in size, flatten in shape, become vacuolated, and express senescence-associated β-galactosidase (22) (SA-β-gal). Few cells incorporate bromodeoxyuridine (BrdU, a thymidine analog), indicating a low proliferative index. Additionally, few fibroblasts at this plateau stain with annexin-V, indicative of a low death fraction. In skin fibroblasts, this growth plateau lasts from months to years; the cells remain viable if fed on a routine schedule (23, 24). No spontaneous emergence of proliferating cells from senescent populations of normal human mammary fibroblasts has ever been detected (25, 26).

The mammary fibroblasts were similar to previously characterized human foreskin fibroblasts in several additional ways. In both cell types the genomic integrity was maintained, with a normal diploid karyotype being retained throughout growth in culture (7). In both types of fibroblasts, cell cycle checkpoint

control was intact as assayed by flow cytometry after exposure to negative growth stimuli such as γ-radiation and Colcemid (16, 27). Additionally, cells that arrested at senescence did so with the majority of cells in G_0/G_1, exhibiting a 2N to 4N DNA content ratio of ~4. Finally, in both types of fibroblasts the telomeres were eroded to a mean minimal length (~6 kbp) as they entered senescence. By the morphological, behavioral, and biochemical criteria described earlier, human mammary fibroblasts senesce in a manner similar to human skin fibroblasts.

Comparison of Proliferation and Senescence in Human Mammary Epithelial Cells and Mammary Fibroblasts

In contrast, *in vitro*, mammary epithelial cells demonstrated two distinctive stages of growth. When placed in culture, mammary epithelial cells underwent an initial ~10–20 population doublings prior to entering a growth plateau (P1) in which there was no increase in cell number for several weeks. During this transient plateau, the majority of the cells in culture were large, flat and expressed SA-β-gal. This first phase of epithelial cell growth and the resultant first growth plateau, P1, exhibited all the afore-mentioned characteristics of mammary fibroblasts as they expand in culture and enter senescence (7). In both types of cells (human mammary fibroblasts and epithelial cells) a diploid karyotype and intact cell cycle checkpoint control were maintained. The mean telomere length eroded, halting at ~6.0 kbp when the cell number no longer increased. Furthermore, few epithelial cells at this first growth plateau incorporated BrdU or stained positively for annexin-V. The majority of cells accumulated in G_0/G_1, as previously reported (15), exhibiting a 2N to 4N DNA content ratio of ~9. An interesting difference between the epithelial cells and fibroblasts, even when obtained from the same gland, was the number of population doublings they each undergo before entering the first growth plateau. The basis for this difference is unknown. Based on the similarities with fibroblast populations, one can conclude that the mammary epithelial cells completed the initial exponential growth phase by entering a senescence-like state, referred to as selection.

Human Mammary Epithelial Cells Spontaneously Overcome Senescence at High Frequency

Both mammary and skin fibroblasts failed to produce proliferating cells from their first plateau, however mammary epithelial cells can be observed to produce colonies at a high frequency (Fig. 1). When cultured in serum-free media (but not serum-containing media), primary mammary epithelial cells undergo a process that was years ago termed self-selection (8, 13, 14). When examined by immunocytochemistry, preselection mammary epithelial cells express both basal (5/6/14) and luminal (8/17/18) cytokeratins (28). To measure the frequency of emergence of cells from the first plateau, the epithelial cells are grown in serum-containing media until P1 and then placed in serum-free media. As the cultures are maintained, distinct populations of cells sporadically and spontaneously emerge, forming clusters of small, refractile cells (Fig. 1). The cells that emerge from P1 maintain epithelial-like morphology and continue to exhibit heterogeneity in markers characteristic of both basal and luminal mammary epithelial cells. The clones of proliferating epithelial cells emerge from the background of large flat cells at a frequency of 1×10^{-4} to 1×10^{-5} as estimated by colony formation (7). After a second period of exponential growth, these epithelial cells enter a second growth plateau, P2 (Fig. 2), that we have termed agonescence (see later).

Several groups have shown that cells that emerge from the P1 growth plateau lose expression of the $p16^{INK4A}$ protein, a cyclin-dependent kinase inhibitor (CKI), predominantly by methylation of CpG islands in the $p16^{INK4A}$ promoter (8–10). This methylation event occurs without genetic manipulation simply in response to environmental change. The culture conditions described earlier allow reproducible measurement of these methylation events as mammary epithelial cells emerge from P1.

The basis for the methylation of p16 during this process is presently unknown. Since the frequency at which the methylation event occurs is similar to the frequency at which point mutations often occur, the possibility exists that the methylation of p16 is the result of mutation in a gene that regulates the methylation of this locus. Hypothetically this mutation could result in any number of changes including hyperactivity of a methyltransferase or alteration of chromatin structure at this locus. The existing data indicate that the methylation is not due to a gross chromosomal alteration because careful analysis of the HMECs as they emerge from P1 indicate that the chromosome complement is normal. Further investigation is needed to clarify the nature of the events that result in the methylation of p16 as the cells emerge from P1.

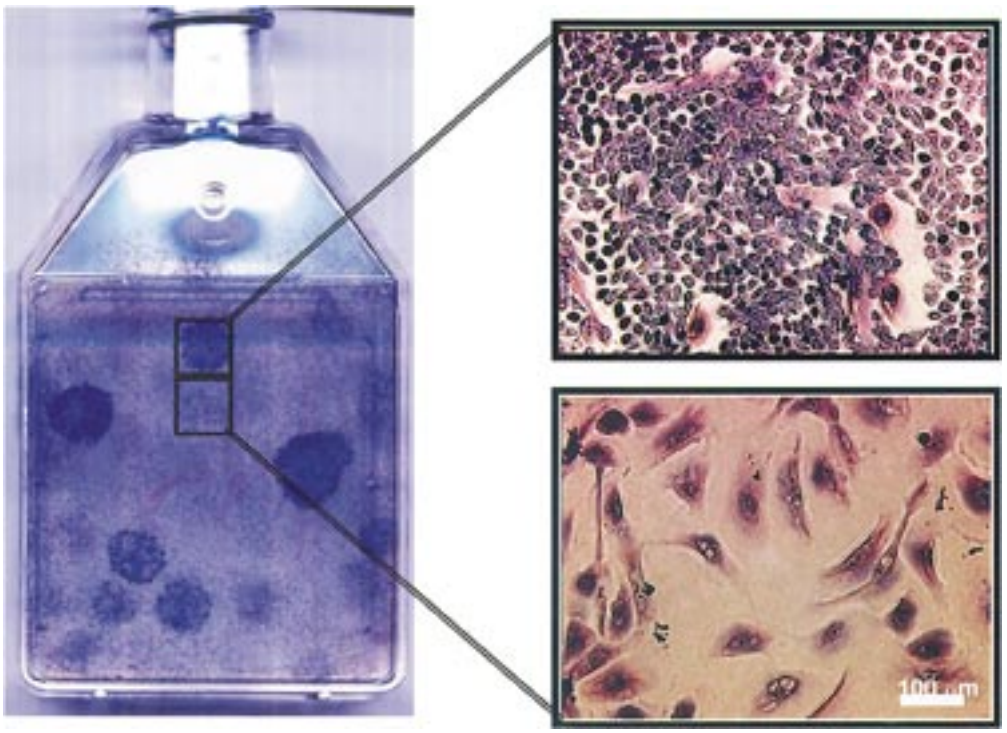

Fig. 1. Selection of mammary epithelial cells in MEGM medium. Emergence of proliferating cells from the first growth plateau. Populations of epithelial cells were serially subcultured in MM media until they reached the first growth plateau. The arrested cells were then trypsinized and plated. The following day the medium was changed to MEGM. Cell cultures produced colonies of small rapidly growing cells.

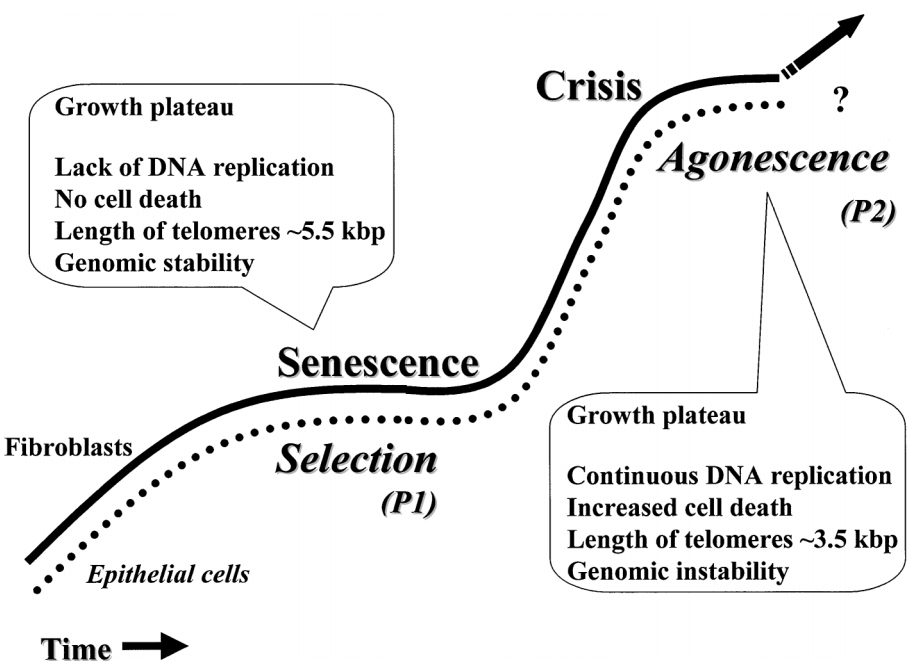

Fig. 2. Schematic diagram comparing growth plateaus of human foreskin fibroblasts and human mammary epithelial cells. Fibroblasts bypass senescence if made to express viral oncoproteins such as SV40 T antigen.

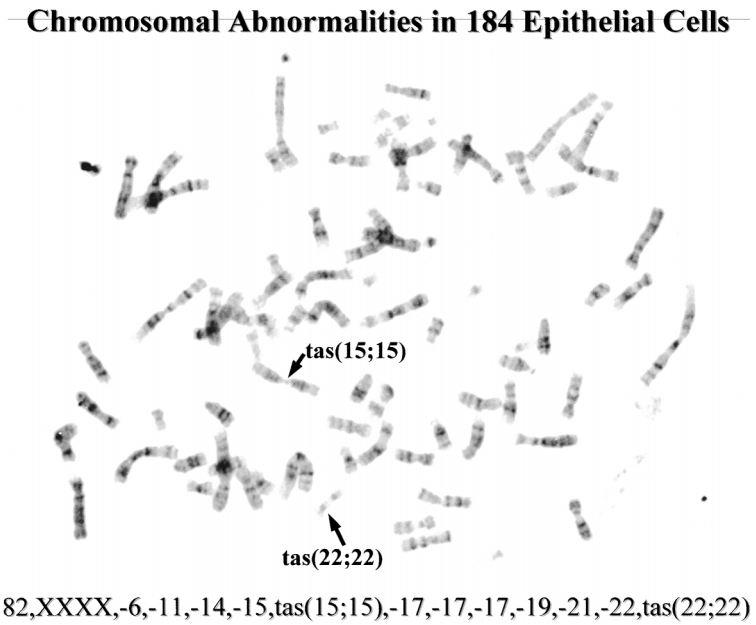

Fig. 3. Genomic instability in human mammary epithelial cells upon growth past the senescent barrier. The karyotypic integrity of mammary epithelial cells is lost as they approach the second growth plateau *in vitro*. Please note the telomeric associations (tas) that connect chromosome 15 to an additional copy of 15 and chromosome 22 to an additional copy of 22 (arrows). This cell carries four copies of the X chromosome as well as lacks a tetraploid complement of chromosomes 6,11,14,15,17,19,21, and 22.

Human Mammary Epithelial Cells that Emerge from Senescence Accumulate Genetic Changes

Cytogenetic analysis of post-selection mammary epithelial cells demonstrated that following the emergence from selection, the cells retain a normal chromosome complement for many population doublings (i.e., several months in culture). However, gross chromosomal abnormalities appeared in virtually every metaphase spread as the cells approached P2 (7) (Fig. 3). The abnormalities included a plethora of translocations, deletions, other rearrangements, telomeric associations and, less frequently, polyploidy or aneuploidy. In all cases, the abnormalities accumulated rapidly in a discreet window beginning 10–20 population doublings before the final cell passage. At five population doublings before P2, 66–100% of metaphases exhibited structural abnormalities, almost half exhibited telomeric associations and many contained a polyploid complement of chromosomes. Interestingly, the onset of genomic instability coincided with the entry of the cells into P2. To examine the proliferative capacity of the cells containing chromosomal abnormalities, a post-selection population of mammary epithelial cells containing chromosomal abnormalities was continuously pulsed with BrdU for 100 hours. The vast majority of these cells incorporated BrdU, indicating the continued proliferation of cells containing these alterations. In addition, the accumulation of chromosomal abnormalities was independent of donor age. The donors in this study ranged from 16–50 years of age.

The timing and spectrum of chromosomal abnormalities, especially the numerous telomeric associations, suggested that post-selection mammary epithelial cells were defective in responding to endogenous damage signals such as shortened, and hence uncapped, telomeres. No telomerase activity was detected in either the mammary fibroblast or epithelial cell populations (29). The dynamics of telomere erosion were similar in both cell types. Interestingly, as noted before, the mean terminal restriction fragment (TRF) lengths in isogenic mammary epithelial cells at P1 and mammary fibroblasts at senescence were similar. As the post-selection epithelial cells proliferated in culture, further shortening of their telomeres occurred until a critical length was reached at P2. Erosion of telomeres to a similar critical length has been

reported in fibroblasts induced to extend proliferation by expression of viral oncoproteins such as SV40 TAg and high-risk Human Papilloma Virus (HPV) E6/E7 (30). Shortening of telomeres and their associated uncapping has previously been suggested to mediate chromosomal instability (31–33) and hence might, in part, explain the abundant telomeric association and chromosome fusion and breakage events observed in this cytogenetic analysis.

The failure of the mammary epithelial cells to halt growth when they contained aberrant chromosomes and/or short telomeres could reflect a defect in a checkpoint control mechanism. We had previously examined the response of post-selection mammary epithelial cells to γ-radiation at different passages in culture (16). Whereas fibroblasts respond to γ-radiation by activating the G_1 and G_2 checkpoints, typically reducing the S-phase fraction greater than five-fold, isogenic post-selection epithelial cells exhibited a substantially less pronounced G_1 response, reducing the S-phase fraction only about two-fold. This assessment of checkpoint integrity in post-selection mammary epithelial cells demonstrated that, although some checkpoint pathways remain intact in post-selection mammary epithelial cells [i.e., those that respond to UV radiation (16)], others were less effective (i.e., the G_1 checkpoint that responds to γ-radiation).

Ploidy changes in several populations of mammary epithelial cells were analyzed more fully. Since the drop in mitotic index as cells approached P2 made it difficult to obtain large numbers of metaphase spreads for cytogenetic analysis, ploidy was analyzed using flow cytometry. The post-selection epithelial populations, at the final passage, accumulated a substantial polyploid subpopulation, i.e., cells with >4N DNA content (~25–30%). In contrast, few mammary fibroblasts at latest passage and few epithelial cells at P1 had a DNA content of >4N (<3%).

Mammary Epithelial Cells Enter a Crisis-Like State in the Absence of Any Viral Oncoprotein Expression

Following the post-selection exponential growth phase (lasting several months), the mammary epithelial cells entered a second growth plateau, P2, where the cells were heterogeneous in size and morphology, exhibited extensive vacuolation as well as SA-β-gal staining. Unlike the mammary epithelial cells at P1, mammary epithelial cells at P2 continued to incorporate BrdU. The ratio of cells with a 2N to 4N DNA content in the P2 epithelial populations was approximately 1, typical of a population of cells in crisis. In contrast, fibroblasts in senescence, or epithelial cells at P1, arrest with a 2N DNA content and demonstrate a 2N to 4N DNA content ratio of $\geq$4 (7, 19). Hence, although there is a negligible increase in cell number in these cultures, the cells continue to synthesize DNA. Further, time-lapse video-microscopy documented mitotic events in the P2 population even four months after reaching plateau.

Because dividing cells were detected at P2, increased cell death could be responsible for the lack of a net increase in cell numbers over time. Mammary cells were analyzed for standard markers of cell death including annexin staining, propidium iodide permeability, and DNA fragmentation (34). Microscopic analysis revealed a significant fraction of annexin-V-positive cells in epithelial populations at P2 but very few in the isogenic mammary fibroblasts at senescence and epithelial populations at P1. All annexin-V-positive epithelial cells also counterstained with propidium iodide, suggesting necrosis, not apoptosis, as a primary cause of cell death in the epithelial P2 populations. DNA fragmentation characteristic of apoptosis was not detectable.

The characteristics displayed by post-selection epithelial cells during proliferation and P2 were similar to those described previously in human fibroblasts expressing viral oncoproteins (21, 27, 35, 36). The expression of SV40 TAg or HPV16 E6/E7 in human foreskin fibroblasts causes relaxation of checkpoint control, a concomitant extension of proliferative capacity, the continued shortening of telomeres and, in the terminal passages of cell culture, the acquisition of genomic instability (27). Early studies with SV40 TAg described cells exhibiting these characteristics as being in crisis (35–38) and later it was speculated that crisis was a second barrier to immortalization (mortality stage 2, or M2) (18, 20, 21, 26, 39, 40). Previous studies (41) measured mutant drug resistant cells emerging from M2 populations at a frequency of 10^{-2}. The expression of HPV E6/E7 in mammary epithelial cells also causes these cells to enter a crisis from which immortalized cells emerge (42–44).

An overview of these data indicates that mammary fibroblasts at senescence behave similarly to P1 epithelial cells, which have previously been termed M1 (15, 42) or senescence, but differ dramatically from epithelial cells at P2. The characteristics of mammary epithelial cells at P2 show several similarities as well as important differences from the characteristics

of fibroblast cells undergoing viral oncoprotein-induced crisis. Senescence, crisis, P1, and P2 in both cell types shared a lack of overall increase in cell number and SA-β-gal staining. The lack of correlation of SA-β-gal staining with cell senescence had previously been noted in fibroblast studies (45). In contrast, the P2 of mammary epithelial cells had a strong similarity to crisis in viral oncoprotein-expressing fibroblasts with respect to BrdU incorporation, cell death assays, and genomic instability. An important distinction between crisis and the mammary epithelial cells in P2 is the frequency of immortalization, which seems to be higher in viral oncoprotein-expressing cells in crisis.

The current terminology describing the various growth plateaus of human mammary epithelial cells has been confusing since each of the plateaus (P1 and P2) has been termed senescence by various investigators (8–10, 15, 46). Senescence was first defined from a population perspective and later described by a set of cellular characteristics. Although the first growth plateau (P1) of mammary epithelial cells exhibits many cellular characteristics of senescence, it is not an insurmountable barrier to further growth. P1 thus does not fit the definition of senescence. Similar to senescence, crisis has previously been defined based on the characteristics of the population and individual cells. The major difference between mammary epithelial cells at P2 and human fibroblasts in oncoprotein-induced crisis is frequency of cellular immortalization. To date, P2 has been an insurmountable barrier to immortalization while crisis has not been. In this respect especially, the P2 of mammary epithelial cells does not fit the characteristics of crisis.

Implications for Studies of Normal Human Epithelial Cells

Examination of the cellular characteristics at P1 and P2 in mammary epithelial cells reveals that neither of the plateaus conforms to the current definitions of senescence or crisis. It seems logical that the first plateau be called by its original descriptive name, selection. However, it would be useful to suggest a new name for P2 since it is neither senescence nor viral oncoprotein-induced crisis. The most prominent attributes of the mammary epithelial cells at P2 are their dramatic accumulation of chromosomal rearrangements and the dynamic state of proliferation and death. The Latin root "agon" is defined as a violent struggle that precedes death or a strong sudden display. An agonescent cell would be one that is engaged in the violent struggle that precedes death. The presence of agonescent cells may herald an increased susceptibility to malignant transformation as well as viral oncoprotein-induced immortality (42).

The importance of the proposed nomenclature goes beyond mere terminology because it pertains to the actual condition of the cells. Despite the infrequent emergence of cells from the selection process [$\sim 10^{-4}$–10^{-5} (7, 10)] and despite the loss of p16 gene product, post-selection mammary epithelial cells are commonly regarded as normal (8, 9, 42, 47). However, the data presented in this review refute this view. The epithelial cells that emerge from the first growth plateau are prone to chromosomal abnormalities and are compromised in several cell cycle checkpoint controls. These cells cannot be considered "normal" in several critical pathways. Therefore, caution should be exercised when using these cells to study cellular phenomena associated with human neoplastic transformation.

These observations in mammary epithelial cells have several important ramifications. Critically, they identify a high frequency spontaneous epigenetic event that occurs in mammary epithelial cells and not in the corresponding fibroblasts. This epigenetic event is associated with the emergence of epithelial cells from selection (escape from senescence) and the entrance into an extended proliferative state that is vulnerable to mutagenic changes (Fig. 4). Only by

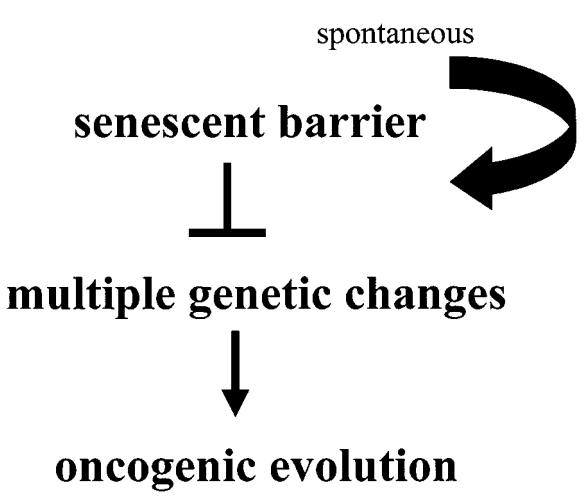

Fig. 4. Human mammary epithelial cells can spontaneously overcome the senescent barrier to further proliferative growth. This event allows the subsequent accumulation of chromosomal aberrations, which are known to fuel oncogenic evolution.

the expression of viral oncoproteins, such as SV40 TAg or HPV E6/E7 do fibroblasts enter this biologic window of growth and genetic vulnerability. This data therefore indicate that mammary epithelial cells and fibroblasts differ in their ability to traverse these barriers. Further studies of these cell type-specific differences may finally illuminate the mechanism for frequent neoplastic transformation in mammary epithelial cells as opposed to the negligible frequency of transformation in fibroblasts from the same gland (48).

Our *in vitro* observations would predict that methylation of p16 *in vivo* may be important in the neoplastic transformation of epithelial cells. Recent studies examining breast, prostate, renal and colon cancer cell lines has demonstrated that *de novo* methylation of the 5′ CpG island is a frequent mode of inactivation of $p16^{INK4A}$. Additionally, this gene has been found to be one of the most frequently altered genes in human tumors (49). The methylation of $p16^{INK4A}$ silences the gene and is believed to allow the altered cells to enter a growth period that is missing proper cell cycle controls. More recently studies utilizing PCR-SSCP indicate that deletions and transcriptional silencing by methylation may represent the main mechanism of $p16^{CDKN2}$ inactivation in breast carcinomas (50, 51). Since the chromosomal abnormalities discussed in this review are similar to the ones seen in the earliest lesions of breast cancer, it will be interesting to study the methylation of this gene throughout tumor progression *in vivo*.

ACKNOWLEDGMENTS

This work was supported by grants from the NIH and NASA to TDT.

REFERENCES

1. S. B. Baylin and J. G. Herman (2000). DNA hypermethylation in tumorigenesis: Epigenetics joins genetics. *Trends in Genetics* **16:**168–174.
2. B. Tycko (2000). Epigenetic gene silencing in cancer. *J. Clin. Invest.* **105:**401–407.
3. J. G. Herman and S. B. Baylin (2000). Promoter-region hypermethylation and gene silencing in human cancer, *Curr. Top Microbiol. Immunol.* **249:**35–54.
4. T. Sugimura and T. Ushijima (2000). Genetic and epigenetic alterations in carcinogenesis, *Mutation Res.* **462**, 235–246.
5. K. D. Robertson and P. A. Jones (2000). DNA methylation: Past, present and future directions, *Carcinogenesis.* **21:**461–467.
6. R. L. Momparler and V. Bovenzi (2000). DNA methylation and cancer, *J. Cell Physiol.* **183:**145–154.
7. S. R. Romanov, B. K. Kozakiewicz, C. R. Holst, M. R. Stampfer, L. Haupt, and T. D. Tlsty (2001). Normal human mammary epithelial cells spontaneously escape senescence and acquire genomic changes, *Nature* **409:**633–637.
8. L. I. Huschtscha, J. R. Noble, E. L. M. Neumann, P. Barry, J. R. Melki, S. J. Clark, and R. R. Reddel (1998). Loss of $p16^{INK4A}$ Expression by methylation is associated with lifespan extension of human mammary epithelial cells, *Cancer Res.* **58:**3508–3512.
9. S. A. Foster, D. J. Wong, M. T. Barrett, and D. A. Galloway (1998). Inactivation of p16 in human mammary epithelial cells by CpG island methylation, *Mol. Cell. Biol.* **18:**1793–1801.
10. A. J. Brenner, M. R. Stampfer, and C. M. Aldaz (1998). Increased p16 expression with first senescence arrest in human mammary epithelial cells and extended growth capacity with p16 inactivation, *Oncogene* **17:**199–205.
11. L. Hayflick (1965). The limited *in vitro* lifetime of human diploid cell strains, *Exp. Cell Res.* **37:**614–636.
12. A. G. Bodnar, M. Ouellette, M. Frolkis, S. E. Holt, C.-P. Chiu, G. B. Morin, C. B. Harley, J. W. Shay, S. Lichtsteiner, and W. E. Wright (1998). Extension of life-span by introduction of telomerase into normal human cells, *Science* **279:**349–352.
13. S. L. Hammond, R. G. Ham, and M. R. Stampfer (1984). Serum-free growth of human mammary epithelial cells: Rapid clonal growth in defined medium and extended passage with pituitary extract, *Proc. Natl. Acad. Sci. U.S.A.* **81:**5435–5439.
14. M. R. Stampfer (1985). Isolation and growth of human mammary epithelial cells, *Tissue Cult. Meth.* **9:**107–116.
15. S. A. Foster and D. A. Galloway (1996). Human papillomavirus type 16 E7 alleviates a proliferation block in early passage human mammary epithelial cells. *Oncogene* **12:**1773–1779.
16. K. M. Meyer, S. M. Hess, T. D. Tlsty, and S. A. Leadon (1999). Human mammary epithelial cells exhibit a differential p53-mediated response following exposure to ionizing or UV light, *Oncogene* **18:**5792–57805.
17. J. Campisi (1997). The biology of replicative senescence, *Eur. J. Cancer.* **33:**703–709.
18. D. Wyndford-Thomas (1999). Cellular senescence and cancer, *J. Pathol.* **187:**100–111.
19. S. Goldstein (1990). Replicative senescence: The human fibroblast comes of age. *Science* **249:**1129–1133.
20. J. W. Shay, W. E. Wright, and H. Werbin (1991). Defining the molecular mechanisms of human cell immortalization, *Biochim. Biophys. Acta* **1072:**1–7.
21. W. E. Wright, O. M. Pereira-Smith, and J. W. Shay (1989). Reversible cellular senescence: Implications for immortalization of normal human diploid fibroblasts, *Mol. Cell. Biol.* **9:**3088–3092.
22. G. P. Dimri, X. Lee, G. Basile, M. Acosta, G. Scott, C. Roskelley, E. E. Medrano, M. Linskens, I. Rubelj, O. Pereira-Smith, M. Peacocke, and J. Campisi (1995). A biomarker that identifies senescent human cells in culture and in aging skin *in vivo*, *Proc. Natl. Acad. Sci. U.S.A.* **92:**9363–9367.
23. J. Campisi, G. Dimri, and E. Hara (1996). Control of replicative senescence. In E. L. Schneider and L. W. Rowe (eds.), *Handbook of the Biology of Aging*, Academic Press, San Diego, California, pp. 121–146 (1996).
24. R. J. Pignolo, M. O. Rotenberg, and V. J. Cristofalo (1994). Alterations in contact and density-dependent arrest state in senescent WI-38 cells. *In Vitro Cellular and Developmental Biology. Animal.* **30A:**471–476.

25. J. J. McCormick and V. M. Maher (1988). Towards an understanding of the malignant transformation of diploid human fibroblasts, *Mutation Res.* **199:**273–291.
26. J. W. Shay and W. E. Wright (1989). Quantitation of the frequency of immortalization of normal human diploid fibroblasts by SV40 large T-antigen, *Exp. Cell Res.* **184:**109–118.
27. A. E. White, E. M. Livanos, and T. D. Tlsty (1994). Differential disruption of genomic integrity and cell cycle regulation in normal human fibroblasts by the HPV oncoproteins, *Genes Dev.* **8:**666–677.
28. J. Taylor-Papadimitriou, M. Stampfer, J. Bartek, A. Lewis, M. Boshell, E. B. Lane, and I. M. Leigh (1989). Keratin expression in human mammary epithelial cells cultured from normal and malignant tissue: Relation to *in vivo* phenotypes and influence of medium, *J. Cell Sci.* **94:**403–413.
29. M. R. Stampfer, A. Bodnar, J. Garbe, M. Wong, A. Pan, B. Villeponteau, and P. Yaswen (1997). Gradual phenotypic conversion associated with immortalization of cultured human mammary epithelial cells, *Mol. Biol. Cell* **8:**2391–2405.
30. J. Zhu, H. Wang, J. M. Bishop, and E. H. Blackburn (1999). Telomerase extends the lifespan of virus-transformed human cells without net telomere lengthening [see comments], *Proc. Natl. Acad. Sci. U.S.A.* **96:**3723–3728.
31. C. M. Counter, A. A. Avilion, C. E. Le Feuvre, N. G. Stewart, C. W. Greider, C. B. Harley, and S. Bacchetti (1992). Telomere shortening associated with chromosome instability is arrested in immortal cells which express telomerase activity, *EMBO J.* **11:**1921–1929.
32. L. Filatov, V. Golubovskaya, J. C. Hurt, L. L. Byrd, J. M. Philips, and W. K. Kaufmann (1998). Chromosomal instability is correlated with telomere erosion and inactivation of G2 checkpoint function in human fibroblasts expressing human papillomavirus type 16 E6 oncoprotein, *Oncogene* **16:**1825–1838.
33. J. D. Griffith, L. Comeau, S. Rosenfield, R. M. Stansel, A. Bianchi, H. Moss, and T. de Lange (1999). Mammalian telomeres end in a large duplex loop, *Cell* **97:**503–514.
34. W. Wei and J. Sedivy (1999). Differentiation between senescence (M1) and crisis (M2) in human fibroblast cultures. *Exp. Cell Res.* **253:**519–522.
35. G. H. Stein (1985). SV40-transformed human fibroblasts: Evidence for cellular aging in pre-crisis cells, *J. Cell. Physiol.* **125:**36–44.
36. R. G. Oshima, O. L. Pellett, J. A. Robb, and J. A. Schneider (1997). Transformation of human cystinotic fibroblasts by SV40: Characteristics of transformed cells with limited and unlimited growth potential, *J. Cell. Physiol.* **93:**129–136.
37. M. L. Steinberg and V. Defendi (1983). Transformation and immortalization of human keratinocytes by SV40, *J. Invest. Dermatol.* **81:**131s–136s.
38. N. Stewart and S. Bacchetti (1991). Expression of SV40 large T antigen, but not small t antigen, is required for the induction of chromosomal aberrations in transformed human cells, *Virology* **180:**49–57.
39. J. W. Shay, O. M. Pereira-Smith, and W. E. Wright (1991). A role for both RB and p53 in the regulation of human cellular senescence, *Exp. Cell Res.* **196:**33–39.
40. J. A. Bond, M. F. Haughton, J. M. Rowson, P. J. Smith, V. Gire, D. Wyndford-Thomas, and F. S. Wyllie (1999). Control of replicative life span in human cells: Barriers to clonal expansion intermediate between M1 senescence and M2 crisis, *Mol. Cell. Biol.* **19:**3103–3114.
41. D. I. Schaefer, E. M. Livanos, A. E. White, and T. D. Tlsty (1993). Multiple mechanisms of N-(phosphonoacetyl)-L-aspartate drug resistance in SV40-infected precrisis human fibroblasts, *Cancer Res.* **53:**4946–4951.
42. J. W. Shay, W. E. Wright, D. Brasiskyte, and B. A. Van Der Haegen (1993). E6 of human papillomavirus type16 can overcome the M1 stage of immortalization in human mammary epithelial cells but not in human fibroblasts, *Oncogene* **8:**1407–1413.
43. V. Band, D. Zajchowski, V. Kulesa, and R. Sager (1990). Human papilloma virus DNAs immortalize normal human mammary epithelial cells and reduce their growth factor requirements, *Proc. Natl. Acad. Sci. U.S.A.* **87:**463–467.
44. C. L. Halbert, G. W. Demers, and D. A. Galloway (1991). The E7 gene of human papillomavirus type16 is sufficient for immortalization of human epithelial cells, *J. Virol.* **65:**473–478.
45. W. Wei and J. M. Sedivy (1999). Differentiation between senescence (M1) and crisis (M2) in human fibroblasts cultures, *Exp. Cell Res.* **253:**519–522.
46. T. Kiyono, A. Hiraiwa, S. Ishii, T. Takahashi, and M. Ishibashi (1994). Inhibition of p53-mediated transactivation by E6 of type 1, but not type 5, 8, or 47, human papillomavirus of cutaneous origin, *J. Virol.* **68:**4656–4661.
47. T. Kiyono, S. A. Foster, J. I. Koop, J. K. McDougall, D. A. Galloway, and A. J. Klingelhutz (1998). Both Rb/p16^{INK4A} inactivation and telomerase activity are required to immortalize human epithelial cells, *Nature* **396:**84–88.
48. J. W. Berg and R. V. Hutter (1995). *Cancer* **75:**257–269.
49. J. G. Herman, A. Merlo, L. Mao, R. G., Lapidus, J. P. Issa, N. E. Davidson, D. Sidransky, and S. B. Baylin (1995). Inactivation of the CDKN2/p16/MTS1 gene is frequently associated with aberrant DNA methylation in all common human cancers, *Cancer Res.* **55:**4525–4530.
50. V. G. Gorgoulis, E. N. Koutroumbi, A. Kotsinas, P. Zacharatos, C. Markopoulos, L. Giannikos, V. Kyriakou, Z. Voulgaris, I. Gogs, and C. Kittas, (1998). Alterations of p16-pRb pathway and chromosoome locus 9p21-22 in sporadic invasive breast carcinomas, *Mol. Med.* **4:**807–822.
51. D. M. Woodcock, M. E. Linsenmeyer, J. P. Doherty, and W. D. Warren (1999). DNA methylation in the promoter region of the p16 (CDKN/MTS-1/INK4A) gene in human breast tumours, *Brit. J. Cancer* **79:**251–256.