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Genetic and Epigenetic Changes in Mammary Epithelial Cells May Mimic Early Events in Carcinogenesis

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Studies of human mammary epithelial cells from healthy individuals are providing novel insights into how early epigenetic and genetic events affect genomic integrity and fuel carcinogenesis. Key epigenetic changes, such as the hypermethylation of the $p16^{INK4a}$ promoter sequences, create a previously unappreciated preclonal phase of tumorigenesis in which a subpopulation of mammary epithelial cells are positioned for progression to malignancy (Romanov *et al.*, 2001, *Nature*, **409:**633–637; Tlsty *et al.*, 2001, *J. Mammary Gland Biol. Neoplasia*, **6:**235–243). These key changes precede the clonal outgrowth of premalignant lesions and occur frequently in healthy, disease-free women. Understanding more about these early events should provide novel molecular candidates for prevention and therapy of breast cancer that target the process instead of the consequences of genomic instability. This review will highlight some of the key alterations that have been studied in human mammary epithelial cells in culture and relate them to events observed in vivo and discussed in accompanying reviews in this volume.

KEY WORDS: human mammary epithelial cells; breast carcinogenesis; genomic instability; $p16^{INK4a}$ hypermethylation; COX-2.

BREAST CANCER PROGRESSION

Breast cancer is a disease of the mammary tissue in which the epithelial cells and stromal cells collaborate to generate malignancy. *In vivo*, the disease is believed to progress through a continuum of stages described by pathologists (Fig. 1, bottom panel). In the earliest visible lesions, hyperplasias, the epithelial cells show architectural alterations but no cytologic atypia and the frequency of genetic changes is low. Luminal epithelial cells, typically form a single layer in the ductal structure, are seen to grow in a multilayered fashion in hyperplasias. In atypical ductal hyperplasia (ADH) the cells show cytologic atypia, however the number of genetic changes remains low. As the disease progresses to carcinoma *in situ* (CIS), cytologic atypia is more prominent, the mitotic index rises and the number of genetic changes increases dramatically. The defining difference between the premalignant CIS and malignant invasive disease is when the breast epithelial cells breach the basement membrane, invade the surrounding stroma and migrate to form micrometastases. Premalignant lesions in breast cancer share many characteristics with premalignant lesions in other tissues, and processes that contribute to progression may be similar in both. The long-sought goal of many studies has been to identify the molecular (causal) changes that underlie progression of normal cells to malignancy with hopes that such information will provide selective targets for effective treatment of the disease. In this review,

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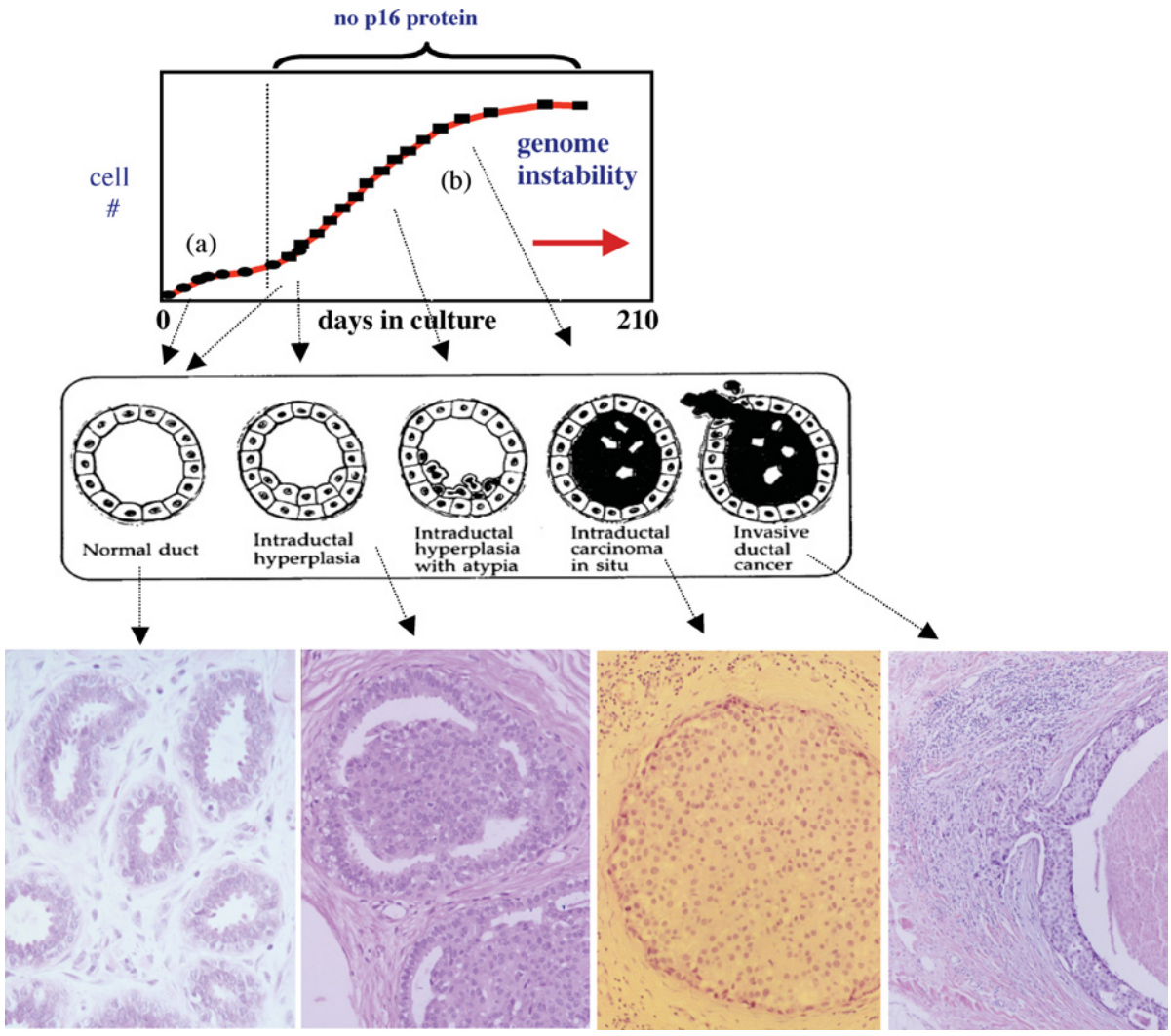

Fig. 1. Hypothetical relationship between epithelial cells *in vitro* and *in vivo*. The top panel illustrates the growth curve for HMEC isolated from reduction mammoplasties. The HMEC increase in number for ~20 population doublings when grown in culture and then enter a proliferation arrest, selection (a), whose termination is noted by the vertical dotted line. Clonal isolates become visible on the arrested cell lawn and continue proliferation for ~3–5 months in culture until there is no further increase in cell number, agonescence (b). These postselection HMEC (variants) have no detectable p16 protein and contain hypermethylated p16 promoter sequences. 10–20 population doublings prior to an obvious population growth plateau, the cells acquire chromosomal changes (genome instability). We postulate that the growth of variant HMEC *in vitro* may mimic the different premalignant stages of breast cancer (lower panels) as illustrated by the dotted arrows.

we describe the cellular and molecular evolution of human mammary epithelial cells in vitro, and illustrate striking similarities with the evolution of mammary cells as they progress from normal to premalignant to malignant *in vivo* (Fig. 1).

STUDYING CAUSAL EVENTS IN BREAST CANCER

An imposing barrier to the identification of causal alterations in early breast tumor cells has been the extensive heterogeneity in the number and type of genetic aberrations observed in vivo. Another major barrier is the inability to grow these cells in culture. The majority of mammary tumor cells available in culture are derived from highly-progressed metastatic lesions, thereby confounding attempts to understand the molecular events in premalignant cells. Other difficulties include the problems in obtaining premalignant cells (because of their small number) and the inability to reconstruct a continuum of progressive stages of disease over time from a

single sampling of tissue. An alternative approach to identifying causal events in breast cancer is to grow normal human mammary epithelial cells in culture and then define conditions that potentiate tumorigenicity. Hence the development of *in vitro* model systems becomes a focused effort in the field many years ago. To be useful, these models would need to be able to recapitulate the functional biology of *in vivo* transformation and allow the examination of cells throughout the process of initiation and progression. Heroic efforts over the past three decades have provided culture systems that allow for the isolation and propagation of human mammary epithelial cells (HMEC) (1–4). Understanding the contribution of specific genetic alterations to the transformation of these cells through the expression of viral oncoproteins and selected oncogenes is ongoing in many laboratories (5–8) and has increased our knowledge of oncogenesis.

Since over 90% of human cancers, including breast cancer, are of epithelial origin, we thought it important to compare fibroblasts and epithelial cells from the same tissue for differences in transformation-relevant signal transduction pathways. Since tight control of cell cycle progression and genomic stability are believed to be important barriers to the development of malignant lesions (9), we initiated a study of the control of genetic integrity in HMEC and human mammary fibroblasts (HMF) with the hopes of identifying early changes that permit the transformation of the epithelial cells. While we still do not understand why epithelial cells give rise to malignancy more often than fibroblasts, these analyses have led to some surprising observations that may provide a unique route to studying the early events in breast cancer. Recently, we demonstrated that, in contrast to human fibroblasts, HMEC do not exhibit a classical senescent arrest when grown *in vitro* (10). HMEC obtained from normal human tissues contain a subpopulation of "variant" cells that are resistant to the negative growth signals that initiate a proliferative arrest (selection) in the majority of the HMEC population after several passages in culture (11–13). These variant HMEC (vHMEC), which become visible while the majority of the population is arrested in selection, lack $p16^{INK4a}$ activity, a critical regulator of cell cycle checkpoint control, and proliferate for an extended period of time with eroding telomeric sequences. These cells subsequently exhibit telomeric dysfunction and generate the types of chromosomal abnormalities seen in the earliest lesions of breast cancer (10). Similar subpopulations are not observed in isogenic mammary fibroblasts (10). These differences between epithelial cells and fibroblasts may provide new insights into the mechanistic basis of neoplastic transformation. The existence of this subpopulation of variant HMEC, their ability to grow past proliferation barriers, and the accompanying acquisition of telomeric and centrosomal dysfunction (McDermott *et al.*, in preparation) may be pivotal events in the earliest steps of carcinogenesis allowing the acquisition of multiple, fundamental genetic changes necessary for oncogenic evolution.

CHARACTERIZATION OF HMF AND HMECS *IN VITRO*: IDENTIFICATION OF A SUBPOPULATION OF VARIANT EPITHELIAL CELLS

One of the first comparisons between fibroblasts and epithelial cells assessed their growth in culture and their entry into replicative senescence. Fibroblasts have provided the paradigm for cell senescence in culture. It is well known that human fibroblasts undergo a limited number of cell divisions prior to activating specific cell cycle checkpoints and entering into an irreversible arrest (variously termed the Hayflick limit (14), irreversible replicative senescence, and mortality stage 1 (M1)). Human mammary fibroblasts (HMF) from healthy individuals were grown as previously described (1) and characterized. Similar to previous studies in human skin fibroblasts (1,5,6,11,12), the HMF populations undergo a limited number of population doublings prior to entering a proliferative plateau (Fig. 1 and ref. 10). The cells enlarge in size, flatten in shape, vacuolate, and express senescence-associated β-galactosidase (SA-β-gal) (10). Low incorporation of bromodeoxyuridine (BrdU) and minimal expression of MCM2 protein indicate a low proliferative index. Additionally, Annexin-V staining indicates a low death index. Further characterization demonstrates that human foreskin fibroblasts and HMF both: (1) maintain genomic integrity (6,10); (2) maintain intact cell cycle checkpoint control (data not shown); (3) exhibit a 2N–4N DNA content ratio of $\geq$4 at the growth plateau (10); and (4) have a mean telomere restriction fragment (TRF) length that is similar at senescence (10). By the morphological, behavioral, and molecular criteria described above, HMF could be said to senesce in a manner similar to human skin fibroblasts (14). If senescence is the result

of a signal from shortened telomeres as has been previously postulated (15), one would predict that the expression of the catalytic subunit of telomerase, hTERT, would allow cells to bypass of "senescence" and continue proliferating. This prediction has been realized in the examination of human fibroblasts expressing hTERT (15,16); they exhibit an extended lifespan without acquiring properties of transformed cells (16).

At first glance, it appears that HMEC do not conform to this paradigm of senescence. In contrast to fibroblasts, HMEC obtained from normal human tissue demonstrate two growth phases (Fig. 1). After an initial phase of active growth (~15–20 population doublings) HMEC exhibit a growth plateau previously termed senescence, selection, or M0 (1,8,11). At this point in time the cell population is arrested in the G1 phase of the cell cycle. When the flasks containing arrested HMEC are cultured in serum-free media (MCDB 170), colonies of small, proliferative epithelial cells become visible. These cells (postselection or variant cells) are capable of undergoing an additional 20–50 population doublings before terminating in a population growth plateau that confusingly was also termed senescence or, alternatively, M1 (5,8,11). On the basis of these observations, it was previously postulated that senescence in HMEC involved two steps, with some cells transitioning past the initial plateau, proliferating, and ultimately entering "senescence" several months later (8). We now believe that the two growth phases represent the growth of two independent populations of mammary epithelial cells (described below) and that neither population enters replicative senescence as classically defined by experiments in human skin fibroblasts. In keeping with Stampfer's original designation, we also termed the plateau in which cells containing unmethylated p16 undergo a proliferative arrest, "selection." However, because of the recent appreciation for the origin of the (postselection) HMEC containing hypermethylated p16 promoter sequences, and to avoid a mechanistic implication of their behavior in vivo, we call these cells variants. We do not know if the environmental conditions of tissue culture, in any manner, reflect the conditions in vivo.

To analyze apparent cell-specific differences, we characterized the two in vitro population-growth plateaus in HMEC from healthy individuals grown as previously described (1) and compared them to the replicative senescence described in human skin fibroblasts. Similar to previous studies in human skin fibroblasts and HMEC (1,5,6,11,12), the epithelial cell populations undergo a limited number of population doublings prior to entering a proliferative plateau (Fig. 1 and ref. 10). Just as seen with the fibroblasts described above, the cells enlarge in size, flatten in shape, vacuolate, and express senescence-associated β-galactosidase (SA-β-gal) (10). Low incorporation of bromodeoxyuridine (BrdU) and minimal expression of MCM2 protein indicate a low proliferative index. Additionally, Annexin-V staining indicates a low death index. Further characterization demonstrated that preselection HMEC (1) maintain genomic integrity (6,10); (2) maintain intact cell cycle checkpoint control (data not shown); (3) exhibit a 2N to 4N DNA content ratio of $\geq$4 at the growth plateau (10); and (4) have a mean TRF length that is similar to human skin fibroblasts and HMF at replicative senescence (10). While the morphological, behavioral, and molecular criteria described above suggest that HMEC had entered replicative senescence in a manner similar to human skin fibroblasts, the expression of telomerase did not have a comparable outcome. Experiments (5,17) have demonstrated that expression of hTERT in these cells does not prevent their entry into the first growth plateau as described for fibroblasts. This demonstrates that the first growth plateau exhibited by HMEC grown in tissue culture does not correspond to the classical telomere-length-based replicative senescence. As described below, it is only in the epithelial cells lacking p16 expression that telomerase can "immortalize" a population (5,17). Intriguingly, recent experiments with fibroblasts also suggest that only fibroblast populations with low p16 activity can be "immortalized" by expression of telomerase (18). If this is so, it raises the question of why fibroblasts don't contain a subpopulation of cells that bypass the imposed arrest as seen in the epithelial population from the same individual.

Strikingly, HMEC and HMF appeared to differ in their ability to spontaneously overcome the observed proliferation barriers by several orders of magnitude. In skin fibroblasts, the terminal growth plateau, senescence, can last for years (>3 years, TDT unpublished data). Cells remain viable if fed routinely (10) and the frequency of spontaneous emergence is $<10^{-9}$ (data not shown; 10). Similarly, HMF fail to produce proliferating cells from senescence even after 5 months in continuous culture ($<6 \times 10^{-7}$, data not shown) (10). In contrast to fibroblasts and consistent with previous reports (1,11), epithelial populations maintained at the first plateau

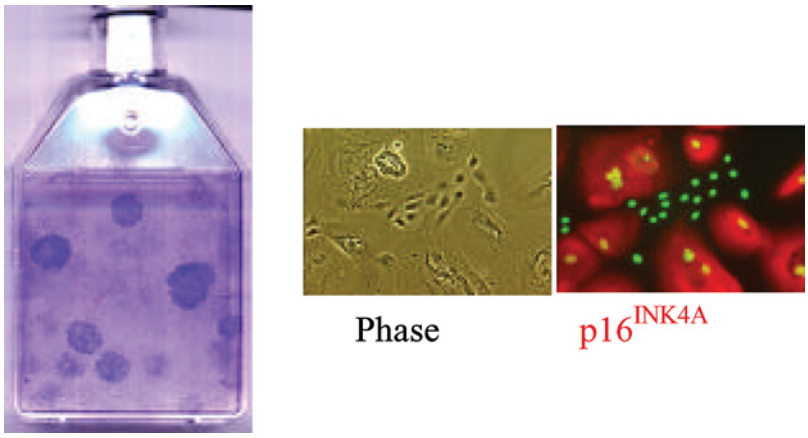

Fig. 2. Visualization of variant HMEC at selection. Preselection HMEC are plated in flasks prior to entering the proliferation barrier. Cells propagate, enter the first plateau, and change morphology, becoming large and flat. After ~2 weeks at the plateau, clonal expansions of small, proliferating cells are visible (phase, middle panel). ICC shows them to be devoid of $p16^{INK4a}$ (red, right panel). Green fluorescence identifies cell nuclei. The flask (left panel) was seeded with 10^5 cells and fed routinely until colonies were visible. The cells were stained with Wright's solution. The number of colonies allows measurement of the clonal events.

sporadically contain clusters of small, refractile cells ($\sim 10^{-4}$–10^{-5}) that continue to proliferate. Both the epithelial cells growing prior to the selection plateau (preselection) and the epithelial cells growing after the selection plateau (postselection HMEC or variant HMEC) exhibited typical heterogeneous expression of cytokeratins when examined by immunocytochemistry (ICC)(data not shown; ref. 4). For these and other reasons, the variant cells were believed to be the continued growth of the earlier population. As noted previously by several laboratories, HMECs emerging from the first population- growth plateau lack expression of the $p16^{INK4a}$ protein (11–13,19 and Fig. 2) due to the hypermethylation of the *p16*^{INK4a} promoter sequences. This observation provided a viable explanation for the continued growth of the variant cells in culture.

PROMOTER HYPERMETHYLATION OF THE *P16*^{INK4A} GENE AND CANCER

The lack of $p16^{INK4a}$ activity in the postselection variant HMEC is an intriguing finding (11–13) because it provides an epigenetic marker for the variant HMEC population. While the role of epigenetic *p16*^{INK4a} silencing in the growth of the variant HMEC cells has been relatively uncharacterized, the role of *p16*^{INK4a} silencing in the carcinogenic process has been extensively studied. The *p16*^{INK4a} gene product was initially isolated by two-hybrid screening for proteins associated with cyclin dependent kinase 4 (20), and was found to be a member of a family of proteins that bind and block the activity of cyclin D/cdk4 complexes and induce cell cycle arrest. Forced expression of $p16^{INK4a}$ protein induces a G1 arrest that is dependent on functional retinoblastoma protein (Rb) (21). Homozygous deletion of the chromosomal region containing *p16*^{INK4a} (and an additional family member *p15*^{INK4b}) is the most common genetic event in primary tumors (22). The dissection of the contribution of these two loci in the initiation and progression of different cancers has demonstrated that loss of *p16*^{INK4a} alone (with retention of *p19*^{Arf}) leads to tumor predisposition in mice (23). These animals have been shown to be highly susceptible to spontaneous and carcinogen-induced malignancy (24). The *p16*^{INK4a} gene can be inactivated by translocations, by mutations at many sites, and by hypermethylation (25). Point mutation in intron 2 (Asp 153) has been identified in tumors that leave cdk 4 binding intact while aborting inhibition of cdk activity (26). Other temperature sensitive mutations, Gly101-> Trp and Val126-> Asp, that abrogate binding to cdk4/6 and have been demonstrated to increase the fraction of G1 cells after transfection (27). The hypermethylation of *p16*^{INK4a} promoter sequences is also seen in over 20% of breast cancers.

The methylation of the *p16^{INK4a}* gene locus (and the concomitant silencing of p16^{INK4a} activity) is an effective way of modulating gene expression (25,28). In the mammalian genome, methylation can occur at CpG islands that are found in the proximal promoter regions of genes (28). The change in gene expression is heritable and is tightly linked to the formation of transcriptionally repressed chromatin structure. Cancers often exhibit changes in methylation in gene promoter sequences that are associated with loss of tumor suppressor function (29), providing an alternative to mutations that disrupt gene function. The importance of CpG island hypermethylation in cancer is obvious given the frequency of the process and the genes involved. The majority of tumor suppressor genes that cause genetic predisposition to cancer can be silenced by hypermethylation in non-familial cancers. The genes that can be methylated include repair genes (*MLH1, GST3*), cell cycle inhibitors (*p16^{INK4a}*, *p15*, p14^{ARF}), tumor suppressor genes (*VHL, BRCA1*), tissue remodeling enzymes and structures (*TIMP3, E-cadherin*), and receptors (estrogen receptor), to name a few (28). Methylation changes often precede the mutagenic events that drive tumor progression. The actual molecular mechanisms involved in methylation and silencing are unknown but under intense scrutiny. Undoubtedly, alterations in nuclear structure and chromatin organization are involved in generating the transcriptionally repressed region. While the role of methylation in gene expression changes is of obvious importance, methylation events can play additional roles in the clinical assessment of tumors, providing potential biomarkers to assess risk and predict disease progression.

CHARACTERIZATION OF VARIANT HMECS *IN VITRO:* ACQUISITION OF GENOMIC INSTABILITY

After they are observed, variant HMEC undergo exponential growth that usually extends for several months (Fig. 1, growth past the vertical dotted line), before entering a second population growth plateau (Fig. 1, agonescence). This plateau is critically different from the arrested state that terminates the proliferation of the HMEC population (P1). While previous studies have referred to this second plateau as "senescence" or "M1," these cells display attributes more similar to cells in crisis, than senescence. Variant HMEC at this stage are heterogeneous in size and morphology and demonstrate SA-β-gal staining (10). Furthermore, they continue to incorporate BrdU and retain high levels of MCM2 protein (>50% of nuclei strongly staining for MCM2). Upon FACS analysis, the 2N to 4N DNA ratio is approximately 1, similar to a population of cells in crisis (10). This high proliferative index is counterbalanced by an increase in cell death, such that the total number of cells remains constant. A significant fraction (~20%) of epithelial cells at the second plateau stain with Annexin-V, an indicator of cell death. In contrast, <1% of isogenic senescent HMF (or HMEC at the first plateau) are Annexin-V-positive. Thus, variant HMEC at the second plateau are unlike HMEC at the first plateau or fibroblast cells at senescence (10).

The cytogenetic analysis of variant HMEC at selected passages demonstrates that gross chromosomal abnormalities appear in virtually every metaphase spread as the cells approach the second growth plateau (10). In all cases, the abnormalities accumulate rapidly beginning 10–20 population doublings before the final passage of cells (Fig. 3) and coincide with slowing of the proliferation rates. In these cells, both the percent of abnormal metaphases and the number of abnormalities per metaphase increase. The abnormalities include multiple translocations, deletions, other rearrangements, telomeric associations, polyploidy, and aneuploidy. Substantial polyploidy (~25–35%) is detected by flow cytometric analysis at final passages of variant HMEC.

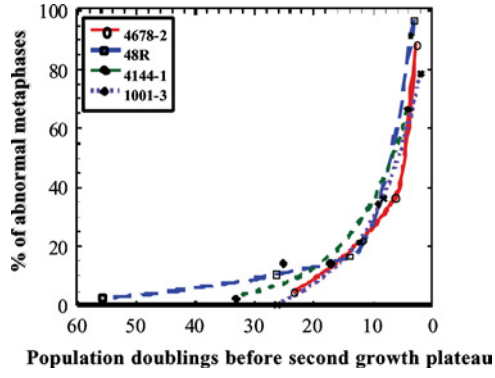

Fig. 3. Chromosomal instability in variant human mammary epithelial cells. The kinetics of accumulation of chromosomal abnormalities are diagrammed as a function of time. The percentage of metaphase are spreads with structural chromosomal abnormalities was plotted as a function of the number of population doublings before the cells entered the population growth plateau (agonescence), designated 0. Each line represents analysis of cells from different women. The women ranged in age from 16–50 years old. Karyotypes were performed at each point that comprises the given line.

Multi-polar mitoses are often observed. The accumulation of chromosomal abnormalities is independent of donor age (range = 16–50 y) and total proliferative potential of the epithelial populations (range = 30–60 PD). Characterization of these abnormalities has been described (10).

The timing and spectrum of chromosomal abnormalities, especially the numerous telomeric associations, suggest that late-passage variant HMEC are exhibiting telomeric dysfunction. Therefore various aspects of telomere metabolism were assessed in serial subcultures of HMEC and HMF. Both cell populations lack telomerase activity as measured by the TRAP assay (30) and exhibit a similar rate of telomere erosion (approximately 30 bp per population doubling; data not shown). Mean TRF length in isogenic HMEC at the first plateau and HMF at senescence is equivalent and similar to that in the earliest available passage of variant HMEC. Further proliferation of the variant HMEC is accompanied by continued shortening of their telomeres (10), down to a broad range of mean TRF lengths (mean ~3.5 kbp) at the second plateau.

Shortening of telomeres and their associated uncapping has previously been suggested to mediate chromosomal instability through the production of dicentric chromosomes (31). Resolution of dicentric chromosomes by chromosome breakage generates translocations, deletions, and duplications. Failure to resolve them can generate anaphase bridges, failed cytokinesis, and polyploid cells. These abnormalities are detected frequently in variant HMEC at the second plateau (10). Thus, the subpopulation of variant HMEC that emerge from the first proliferation barrier ultimately exhibit telomeric dysfunction. While variant HMEC at the second plateau exhibit many of the cellular characteristics of viral oncoprotein-induced crisis, spontaneous immortalization of variants (an important distinguishing hallmark of crisis) has yet to be detected. In addition, the p53 gene sequence is wild-type in these cells and still functional (10). Because of these (and other) differences (manuscript submitted), we have called these cells "agonescent" to distinguish them from cells in crisis. The Latin root "agon" defines a violent struggle that precedes death or a strong sudden display. The most prominent attributes of the late-passage variant HMEC are their dramatic accumulation of chromosomal rearrangements and the dynamic state of proliferation and death.

The variant HMEC described in this model system and the existence of an "agonescent" stage of proliferation provide a compelling argument for acquisition of massive random genomic instability that preceeds clonal outgrowth of tumor cells. Indeed, at the point in culture when virtually all of the variant HMEC cells exhibit chromosomal abnormalities via karyotypic analysis, analysis by comparative genomic hybridization (CGH) shows the population to be in a diploid non-rearranged state (manuscript in preparation). This is because CGH assesses clonal chromosomal changes that are present in a large fraction of the cell population and cannot detect random, non-clonal changes. It is intriguing to speculate that the relatively few chromosomal structural abnormalities observed in hyperplasias and atypical hyperplasias (32), and the transition to the dramatic increase of genomic instability detected in CIS in vivo using CGH analysis (33,34), is reflective of a pre-clonal phase of growth followed by clonal expansion in CIS. Therefore, this model system may have uncovered a previously unappreciated pivotal phase in tumorigenesis. In this pre-clonal phase, epithelial cells have the potential to acquire multiple, random chromosomal changes that provide fuel for clonal expansion.

GENE EXPRESSION PROFILING OF HMEC AND VARIANT HMEC IDENTIFY DIFFERENCES IN EXPRESSION

To further characterize the variant HMEC, we compared the expression profiles of isogenic sets of HMEC and variant HMEC. Total RNA from preselection HMEC was compared to RNA from both mid and late passages of variant HMEC using two color cDNA microarrays (chip content and methods available at http://dir.niehs.nih.gov/microarray). Following this analysis, we identified several genes that were differentially expressed between preselection cells and mid or late passage variant HMEC. One of these genes, prostaglandin-endoperoxide synthase 2 (COX-2) was significantly induced (average = 6.3 fold). We have verified this observation in multiple populations of HMEC using western analysis and immuno-cytochemistry and find that COX-2 RNA increases are accompanied by an increase in protein expression and enzyme activity (35). Subsequent studies demonstrate that this increase in COX-2 expression is causal for phenotypes often associated with malignant cells such as an increase in angiogenic potential invasion and proliferation and a decrease in apoptosis (35).

THE ORIGIN OF VARIANT HMEC: VARIANT MAMMARY EPITHELIAL CELLS ARE DETECTED IN VIVO

A compelling question raised by the above observations concerned the origin or generation of the variant HMEC in tissue culture. To address this question we undertook a Luria–Delbruck fluctuation analysis. The Luria–Delbruck fluctuation analysis is a combined experimental and statistical method that allows one to distinguish between variant cells arising by rare spontaneous mutations (adaptation model) and pre-existing variant cells appearing after an environmental selection (selection model) (36,37). We have previously used this analysis, which is based on variation seen in the emergence of colonies from parallel cultures, to analyze mutations in mammalian cells (37). The data from this study demonstrate that the variant HMEC are generated (or exist) prior to the plateau and do not arise through adaptation (38).

If the variant HMEC pre-exist in the population of HMEC prior to the selection plateau, they have either been generated by tissue culture manipulations and/or must be present in the human tissue from which the HMEC were originally obtained. To determine if cells with characteristics of variant HMEC (inactive $p16^{INK4a}$, over-expression of COX-2, and increased genomic instability) exist in vivo in healthy women, we took several approaches. Since the silencing (most often by methylation (11–13)) of the important cell cycle inhibitor *p16*^{INK4a} is a critical distinguishing characteristic of the variant HMEC, we analyzed morphologically normal tissue from reduction mammoplasties using solution-based methylation-specific PCR to determine if they contained detectable quantities of cells with *p16*^{INK4a} promoter methylation. Using techniques that have previously been developed to detect methylated sequences in paraffin embedded tissues (39,40), we measured the level of *p16*^{INK4a} promoter methylation in histological samples in collaboration with Drs. Steve Baylin and James Herman of Johns Hopkins University. Out of 15 samples, four demonstrated methylated *p16*^{INK4a} promoter sequences (38). Since the removal of tissue from the histological preparations includes both the epithelial cells of the mammary ducts and the stromal cells of the surrounding tissue, we additionally sought a method that would enable us to visualize the HMEC cells embedded in their natural tissue architecture. For this purpose we collaborated with Dr. Gerard Nuovo, who has developed an in situ method for detecting methylated sequences in histological sections from tumors (41). Ten samples of histological preparations of reduction mammoplasty tissue were examined for cells that contain methylated *p16*^{INK4a} promoter sequences. Three of the 10 samples demonstrated the unequivocal presence of cells (foci) that gave a positive signal, and were mapped using a novel method developed for the purpose of displaying multiple sets of data in the context of whole tissue (38). Close examination of the samples revealed that the cells producing a positive signal were luminal epithelial cells. Neither myoepithelial cells nor stromal cells produced positive signals in any of the samples analyzed. These data demonstrate that epithelial cells with hypermethylation of the p16 promoter sequences exist as foci in morphologically normal tissue of disease-free women (38).

While the in situ methylation specific PCR assay provided evidence that epithelial cells with a distinguishing characteristic of variant HMEC existed in morphologically normal tissue, the assay is too difficult and laborious to apply to large samples. For this purpose we sought to assess other characteristics of variant HMEC in vitro that may also be concomitantly expressed in the foci in vivo. One of the goals of the expression profile analysis was to identify such distinguishing characteristics of variant cells for the purpose of then examining their expression in vivo. To determine if the increased expression of COX-2 that is seen in a fraction of the cells containing p16 hypermethylation *in vitro* is also present *in vivo*, we analyzed serial histological sections of human mammary tissue for co-localization of these markers (35). Examination of the seven cases that were negative for cells with *p16*^{INK4a} hypermethylation did not exhibit intense staining of COX-2. In contrast, examination of the three cases that were positive for cells containing *p16*^{INK4a} hypermethylation exhibited areas of intense staining for COX-2 expression in adjacent serial slides. This intense staining colocalized with the areas of *p16*^{INK4a} promoter hypermethylation and extended to the adjacent areas. Maps were generated to demonstrate the localization of COX-2 in relation to *p16*^{INK4a} hypermethylation (35). Further analysis of these regions using an in situ hybridization technique has found them to also have shorter telomeres when compared to other areas of the same slide (Fordyce *et al*., in preparation).

The colocalization of intense COX-2 staining in cells with hypermethylated *p16*^{INK4a} sequences has important implications for the initiation and progression of malignancy in this tissue. COX-2 protein

is instrumental in prostaglandin synthesis, and increased expression in tumor cells is accompanied by several phenotypes that are critically relevant to cancer development (42). Overexpression of COX-2 leads to stimulation of mammary epithelial cell growth (43), increased biosynthesis of estrogens (44), and decreased immune surveillance (45). Additionally, expression of COX-2 leads to the production of mutagens (42), increased invasion, angiogenesis, and the inhibition of apoptosis (42,46,47). The observations described here suggest that the rare foci of cells containing hypermethylated $p16^{INK4a}$ promoters not only have the ability to accumulate genomic instability but also to induce critical oncogenic phenotypes such as angiogenesis and inhibition of apoptosis. *Thus, these cells represent a potent precursor population for oncogenic progression.*

POTENTIAL RELEVANCE OF VARIANT HMEC TO MALIGNANCY

The above data demonstrate that a sizable fraction of women (~30%) contain a subpopulation of human mammary ductal and lobular epithelial cells containing hypermethylated $p16^{INK4a}$ promoter sequences and overexpression of COX-2. As noted before, hemotoxylin and eosin (H and E) staining of adjacent serial sections demonstrated that the cells containing the coincident overexpression of COX-2 and methylated $p16^{INK4a}$ promoter sequences retained normal morphology as determined by pathologists. What is the relationship, if any, of these foci to the development of cancer? The first issue to address is that the fraction of women containing any frequency of foci exhibiting the "variant" characteristics is substantially higher than the fraction of women that are diagnosed with breast cancer. And, given that these determinations were done on a limited amount of tissue sampled from individual mammary glands, it may be that an even greater fraction of healthy women contain these foci and/or that the reservoir of these cells in healthy women is considerable. At the very least this could indicate that not all of these foci progress to cancer. Since less than 30% of the population develops breast cancer, it could reasonably be argued that subsequent events are necessary for progression. Of course, alternatively, it could be that these cells do not relate to carcinogenesis at all and may represent some stem cell population or dead end lineage.

Data exist for the progression of a fraction of less malignant lesions to more malignant ones. In this manner, fewer lesions at each stage would progress to the more advanced state. Using data generated from autopsy series, studies by Nielsen and colleagues (48) and Alpers and Wellings (49), among others, shed light on the prevalence of undetected premalignant breast disease. In the Nielsen study of double mastectomy specimens from 110 medicolegal autopsies, whose cause of death was unrelated to breast cancer, nearly one-third of patients harbored hyperplastic lesions (UDH, 32%), over one-quarter contained atypical ductal hyperplasia (ADH, 27%), almost one-fifth showed ductal carcinoma in situ (DCIS, 18%), and 2% had overt invasive breast cancer. Further, almost half of the women with ductal carcinoma in situ (DCIS) had bilateral (41%) and/or multifocal (45%) disease. Alpers and Wellings' study of 185 breast samples from random autopsies confirms this high prevalence of undetected premalignant breast lesions. Several other studies that sampled mastectomy tissue less frequently noted smaller numbers of premalignant lesions (50). Additionally, these numbers have been suggested to be high due to the difficulty in accurately diagnosing the various premalignant lesions. In the context of the observations made with the methylation of the $p16^{INK4a}$ promoter in healthy women *in vivo*, one would hypothesize that the methylation event is an early molecular event and that subsequent events would contribute to the multistep progression of this population of nascent tumor cells through the premalignant stages. If this is so, the characterization of the *in vitro* variant HMEC may provide molecular clues to the subsequent changes required for carcinogenesis.

If cells with "variant" characteristics do represent precursors to breast cancer, we would predict that some fraction of premalignant lesions would express the relevant characteristics. To test this hypotheis, we examined 65 cases of DCIS for the overexpression of COX-2 (51). We found that a large proportion of low- and high-grade DCIS overexpress COX-2 not only in the morphologically distinct DCIS lesion, but also in the adjacent surrounding morphologically normal epithelial cells. Studies are underway to assess the presence of cells with "variant" characteristics in even earlier premalignant lesions.

SIGNIFICANCE

These new observations in HMEC provide potential insights into tumorigenesis and identification of novel therapeutic targets. If cells that possess

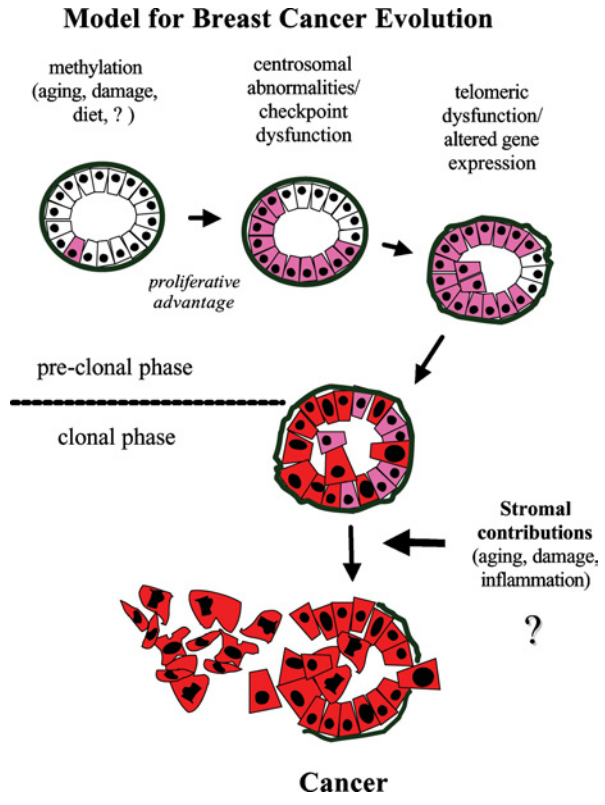

Fig. 4. Model for breast cancer evolution.

"variant" characteristics are related to the formation of breast cancer, they may provide potential markers for assessing susceptibility to neoplastic transformation in individuals, as well as, potential targets for prevention and therapy. Multiple markers clearly identify the different cellular states (10) and may allow the identification of these cells in different states *in vivo*. We *hypothesize* that the above-described properties of variant HMEC in vitro are critically relevant to their transformation processes *in vivo* and may provide insights into controlling progression to cancer (52). In the model presented in Fig. 4, cells that contain hypermethylated $p16^{INK4a}$ promoter sequences could continue to proliferate under conditions when the p16-expressing cells do not. Since continued proliferation of cells in the absence of p16 expression holds great potential for generating chromosomal abnormalities, this subpopulation of cells is free to accumulate mutations that may facilitate tumorigenesis. When the variant cells proliferate to the point of critically short telomeres, telomeric dysfunction fuels the generation of massive, random preclonal genomic instability to allow the emergence of clonal isolates that may progress to tumorigenicity. Selection pressures exerted by the microenvironment would be postulated to generate clonal isolates. The continuing telomeric dysfunction, coupled with the activation of pathways associated with overexpression of COX-2, provides a potent package of events to promote tumorigenesis.

This alternative perception of the tumorigenic process differs from other perceptions in that it recognizes an especially vulnerable stage of carcinogenesis that exists prior to the clonal outgrowth of tumorigenic cells. The majority of therapeutic targets at the present time address the consequences of genomic instability such as the targeting of Gleevec to the Philadelphia chromosome translocation. Analysis of this model system may provide targets to address the process of genomic instability rather than its consequences.

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