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Genome-wide Allelotyping of a New *in Vitro* Model System Reveals Early Events in Breast Cancer Progression[1]

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ABSTRACT

Toward the goal of identifying early genetic losses, which mediate the release of human breast epithelium from replicative suppression leading to cellular immortalization, we have used a newly developed *in vitro* model system. This system consists of epithelial cultures derived from noncancerous breast tissue, treated with the chemical carcinogen *N*-ethyl-*N*-nitrosourea, and continuously passaged to yield cell populations culminating in the immortal phenotype. Genome-wide allelotyping of early passage *N*-ethyl-*N*-nitrosourea-exposed cell populations revealed aberrations at >10% (18 of 169) loci examined. Allelic losses encompassing chromosomes 6q24-6q27, implicating immortalization-associated candidate genes, *hZAC* and *SEN6*, occurred in two independently derived cell lines before the Hayflick limit. Additional LOH sites were present in one cell line at 3p11–3p26, 11p15, and 20p12–13. Allelic losses reported in this cell line preceded detectable levels of telomerase activity and the occurrence of *p53*-related aberrations. Information gained from the search for early immortalization-associated genetic deletions in cultured cells was applied in a novel approach toward the analysis of morphologically normal terminal ductal lobular units microdissected from 20 cases of ductal carcinoma *in situ*. Notably, clonal allelic losses at chromosome 3p24 and 6q24 were an early occurrence in adjoining terminal ductal lobular units of a proportion of primary tumors, which displayed loss of heterozygosity (3 of 11 and 3 of 6, respectively). The biological insights provided by the new model system reported here strongly suggest that early allelic losses delineated in immortalized cultures and validated *in vivo* could serve as surrogate endpoints to assist in the identification and intervention of high-risk benign breast tissue, which sustains the potential for continuous proliferation.

INTRODUCTION

Cellular immortalization or infinite proliferative potential is a characteristic trait of cancer cells but occurs rarely in normal human cells. It is widely believed that the immortal phenotype is acquired through the selection of genetic and epigenetic alterations that activate proto-oncogenes and inactivate tumor suppressor genes during malignant progression. Although the full identity and precise sequence of cellular changes underlying immortalization is presently unknown, the widespread heterogeneity of neoplastic lesions of the human breast suggests that such changes may be acquired as stochastic events through multiple pathways (1). The application of surgical samples of pathological breast tissue for defining the sequence of key events in cellular immortalization is precluded by the inability to distinguish between defects conferring immortality and those related to other aspects of malignant transformation. Other limitations in defining temporal acquisition of the immortal phenotype include inaccessibility to precursor populations from the time of carcinogenic initiation through the course of malignant progression in the same patient. Thus, well-designed, biologically relevant cell culture models could facilitate a more pragmatic approach toward an in-depth understanding of the pathways to cellular immortalization.

Spontaneously immortalized breast cancer cell lines generally established from advanced malignant tumors have long played a critical role in functional experimentation. However, as they closely resemble tumor tissue in the above-mentioned limitations, these cell lines do not lend themselves well for defining causal changes underlying immortalization. Of more direct relevance to the acquisition of the immortal phenotype are spontaneously derived (2, 3) and carcinogen-induced cell lines (4), which have emerged from noncancerous breast epithelial cultures through continuous passaging. Experimental application of such cell lines and their derivatives have adequately established the usefulness of cell culture models for portraying well-defined genetic and phenotypic aspects characteristic of pathologically confirmed breast carcinoma (5–9).

In a reverse application, serially passaged cell populations could be effectively used for recapitulating the temporal sequence of immortalization-associated genetic changes in breast tissue from cancer patients. An important conceptual component of this strategy is the observation that specific genetic deletions commonly present in breast tumor cells are detectable in morphologically normal TDLUs[3] adjacent to carcinoma (10, 11). This finding implicates a proportion of TDLU in the cancerous breast as clonal precursors of malignant cells and proposes a new paradigm for identifying early aberrations related to malignant progression. However, direct searches of TDLU in this regard are severely hampered by qualitative and quantitative limitations of archived tissue. We have developed and used a new model system described here to assist in this search.

We report on the distinctive features of two novel human mammary epithelial cell lines immortalized with ENU and their application toward defining early genetic deletions, which may play a potentially causal role in cellular immortalization. First, by identifying specific molecular aberrations in continuously proliferating cells and subsequently validating their presence in pathological samples, we demonstrate that early passages of ENU-immortalized cell lines indeed have a counterpart in cancerous human breast tissue, which can be detected at the molecular level before the onset of proliferation-associated histological changes.

MATERIALS AND METHODS

ENU-Exposure and Cell Culture. Noncancerous breast tissue from a reduction mammoplasty procedure performed at the California Pacific Medical Center (San Francisco, CA) was obtained under Institutional Review Board-approved guidelines. For *in vitro* propagation of epithelial cells, organoids isolated from enzymatically dissociated tissue were plated in MM growth medium as described previously (12). The original primary culture initiated from organoids was designated as passage 1. Epithelial cultures at passage 2 were seeded at 5×10^4 cells/cm^2 into 10 independent 60-mm dishes for subsequent treatment and controls. Subconfluent cultures were treated twice within 7 days with 200 ng/ml ENU (Sigma). The cultures were not exposed to

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[3] The abbreviations used are: TDLU, terminal ductal lobular unit; ENU, *N*-ethyl-*N*-nitrosourea; TRF, terminal restriction fragment; RT-PCR, reverse transcription-PCR; DCIS, ductal carcinoma *in situ*; LOH, loss of heterozygosity; CK, cytokeratin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Table 1 *Chromosomal regions of allelic loss identified by genome-wide allelotyping analysis of self-selected early passage ENU-immortalized human breast epithelial cells*

		Presence of allelic loss				
Locus	Site	ENUt7-19′	ENUt7-28′	ENUt4A-31′	ENUt4A-44′	ENUt4J-31′
D3S2387	3p26.3	N[a]	Y	N	N	N
D3S4545	3p26.1	N	Y	N	N	N
D3S3038	3p22.3-23	N	Y	N	N	N
D3S2432	3p22.3	N	Y	N	N	N
D3S1766	3p21.1	N	Y	N	N	N
D3S4529	3p11.1	N	Y	N	N	N
F13A01	6p24.3-25.1	N	Y	N	Y	Y
D6S2439	6p22.1	N	Y	N	Y	Y
D6S1017	6p21.1	N	Y	N	Y	Y
D6S1053	6q11.2	N	Y	N	Y	N
GATA184A08[b]	6q24.2	Y	Y	N	Y	Y
D6S1277	6q26	N	Y	N	Y	Y
D6S1027[b]	6q27	Y	Y	N	Y	Y
D11S1984	11p15.5	N	Y	N	N	N
D11S1999	11p15.3	N	Y	N	N	N
D20S482	20p13	Y	Y	N	N	N
D20S604	20p12.1	N	Y	N	N	N
D20S481	20q13.12	N	Y	N	N	N

[a] N, no; Y, yes.
[b] These rows represent earliest allelic losses common to all ENU-exposed test populations.
′ indicates passage number.

any viral agents and were found to be free of *Mycoplasma* and human papilloma virus DNA sequences and SV40-large T antigen as determined by PCR assay and indirect immunostaining.

The epithelial origin of the cell lines was confirmed by indirect immunofluorescence of acetone-fixed monolayers seeded on microscope slides as previously described with mouse monoclonal antibodies to CKs 14 and 18 (13, 14).

Analysis of Immortalization-associated Allelic Loss. Initial PCR-based genotyping of ENU-treated populations was performed with 169 polymorphic markers comprising a low density Weber/RG set (details on markers are available on-line).[4] The mean heterozygosity index of the loci was 0.78. Twenty ng of DNA in a 10-μl PCR reaction mixture was amplified under the following conditions: denatured at 96°C for 30 cycles at 2 min each; denatured at 94°C for 45 s; annealed at 57°C for 45 s; extension at 72°C for 60 s; and final extension at 72°C for 7 min. Allelic size was determined with an ABI 377 DNA sequencer. Alterations in product size and/or allelic ratio were verified additionally in repeat PCR reactions with primers listed in Table 1. PCR products were resolved in 6–8% PAGE gels, visualized with Sybr-Green I (Molecular Probes) or with a silver stain kit (Pharmacia), scanned with Adobe PhotoShop, and quantitated by Image Quant. Allelic loss was defined as >50% reduction in the density of either allele compared with control DNA from untreated cells.

Determination of *p53* Status. Mutations in exons 4–9 of the *p53* gene were initially detected by PCR-single-strand conformational polymorphism analysis of cultures as described earlier (15) and confirmed by sequencing. For indirect immunostaining of paraffin-embedded tissue sections, anti-p53 mouse monoclonal antibody DO1 (Santa Cruz Biotechnology) at 1:100 dilution was used in conjunction with an ABC Immunoperoxidase kit (Vector Labs).

Telomerase Activity and Telomere Length Determination. Isolation of cell extract and the telomerase repeat amplification protocol was performed as per manufacturer recommendation (Trapeze Telomerase Detection Kit; Intergen). Briefly, the cell pellet was washed with PBS (Mg^{2+} and Ca^{2+} free), resuspended in 1× 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid lysis buffer at a concentration of 500 cells/μl, incubated on ice for 30 min, and centrifuged at 12,000 × *g* for 20 min at 4°C. PCR products were resolved by 10% PAGE and stained with Sybr Green-I (Molecular Probes). Heat-inactivated controls were included for each sample. A telomerase expressing positive control lysate was included in each test batch. Serial dilutions of lysates were tested to ensure that assays were in the linear range.

Genomic DNA was used to measure telomere lengths in an anti-digoxigenin alkaline phosphatase-mediated chemiluminescent assay based on Southern blotting and hybridization of TRFs with a digoxigenin-labeled probe specific for telomeric repeats (TeloTAGGG Assay; Roche Molecular Biochemicals). Control DNA samples representing known telomeric lengths of 3.9 kb (TeloLOW) and 10.2 kb (TeloHIGH) were run in parallel with the test samples. After exposure of the blot to X-ray film, an estimate of mean TRF length was obtained by visual comparison of the mean size of the smear in the test signal to the relative migration of molecular weight standards in 0.8% agarose gels.

RT-PCR Analysis. Total cellular RNA was isolated from cultured cells using RNeasy (Qiagen). Five hundred ng of total RNA were reverse transcribed with Superscript II Reverse Transcriptase (Life Technologies, Inc.) using random hexamers (Operon) in a reaction volume of 50 μl. PCR was performed using *GAPDH* as an internal control together with the test gene primers in a single 20-μl reaction volume. The upstream and downstream primer sequences were: *p16*, 5′-ATGGAGCCTTCGGCTGACTGG-3′ and 5′-GATCGGCCTCCGACCGTAAC-3′; *p21*, 5′-AGGATCCATGTCAGAACCGGCTGG-3′ and 5′-CAGGATCCTGTGGGCGGATTAGGGCT-3′; and *GAPDH*, 5′-TGATGACATCAAGAAGGTGGTGAA-3′ and 5′-TCCTTGGAGGCCATGTGGGCCAT-3′.

PCR conditions included 94°C for 4 min 30 s; 94°C for 30 s; 64°C for 30 s; 72°C for 45 s; and a final 5-min extension at 72°C (30 PCR cycles). Products were resolved in 2.0% agarose gels and stained with ethidium bromide (Sigma). A semiquantitative determination of the level of *p16* and *p21* expression was made by comparing the density of the 123-bp *p16* or 510-bp *p21* product with the 250-bp *GAPDH* product.

Growth in Soft Agar. Cell suspensions containing ENU-immortalized cells in 0.3% agarose (Sigma) in growth medium were plated on top of a 0.7% agarose gel layer in 24-well plates at 10^4 cells/well. Cells were fed weekly with 200 μl of growth medium. After 3–4 weeks, anchorage-independent colonies consisting of ≥30 cells were counted. Established breast cancer cell lines, BT-474 and MCF7, used as positive controls, were plated at 100 cells/well and colony counts made at 5–10 days after plating.

Tumorigenicity. Six-to-8-week old female CB-17 SCID-beige mice (Taconic) were used for evaluating tumorigenic potential of ENU-immortalized cultures. Suspensions of 10^7 cells/100 μl were mixed in equal volume with 1 mg of Matrigel (Becton Dickinson) and injected into the left scapular region. Mice were housed in microisolator cages of a Thoren unit and allowed food and water *ad libitum*. Animals were inspected for tumor growth once weekly up to 118 days. Control mice bearing MCF-7 or BT-474 xenografts were euthanized at 40–45 days after cell inoculation because of tumor ulceration or where tumor burden interfered with the mobility of the animal.

Tissue Microdissection and LOH Analysis. Archival tumor blocks were obtained under Institutional Review Board-approved guidelines from 20 cases of pathologically confirmed high-grade DCIS treated at the California Pacific Medical Center. Formalin-fixed sections stained with methyl green or H&E were used for manual microdissection of tumor and morphologically normal TDLU adjacent to carcinoma using scalpel blades. For normal TDLU adjacent to tumor, cells of the same lobular unit were pooled from five serial sections. Samples containing 500-5000 cells were incubated in lysis buffer [10 mM Tris-HCl, 1 mM EDTA, 1% Tween 20, and 400 μg/ml proteinase K] at 50

[4] Internet address: www.marshmed.org/genetics/sets/scrset6.txt.

cells/μl for 24–96 h at 50°C. DNA template equivalent to 50 cells/sample was amplified for LOH studies. Because of artifacts commonly encountered in allelic representation and reproducibility of allelic ratios in limited DNA samples isolated from fixed tissue, duplicate parallel PCR reactions were run on each sample. Data were recorded only when variation in allelic ratio between duplicates was <5%. Nonmalignant skin epithelium or lymphocytes derived from uninvolved nodes were used for determination of the constitutional genotype. LOH calls were noted as described above.

RESULTS

Growth and Phenotype of ENU-immortalized Human Breast Epithelial Cell Lines

A reduction mammoplasty specimen, designated G385E, used for culturing normal breast epithelial cells toward subsequent carcinogen exposure and derivation of continuously proliferating cell lines, was obtained from a 23-year-old donor with no known breast pathology. To immortalize human breast epithelial cells, we used the direct acting carcinogen ENU, an alkylating agent known to induce rodent mammary tumors (16). A single concentration of 200 ng/ml ENU, applied twice within an interval of 7 days, was tolerated by primary breast epithelial cultures without apparent cytotoxicity. Unlike culture conditions reportedly important in the derivation of spontaneously immortalized cell lines, such as low calcium concentration (3) or the use of a chemically defined growth medium (2), we have used the MM growth medium in our experiments. The advantage of using the MM medium, also used in the derivation of benzo(*a*)pyrene-transformed cells (4), was that it only supports short-term growth (15–25 population doublings) of normal breast epithelial cells (12). Consequently, untreated control cultures were completely nonproliferative by passage 5. Spontaneous immortalization was not observed in the cells of this individual. In 6 of 8 culture dishes independently exposed to ENU, designated ENUt 3–8, a relatively low rate of mitotically active cells continued to occur after growth cessation in untreated controls. Two independent cultures, ENUt4 and ENUt7, were passaged continually and used in additional studies described below. Both populations were initially maintained at relatively high density (1:2 splits). As shown in Fig. 1, the cultures were slow growing up to passage 20 and displayed population-doubling times of 13–16 days. In the next 20 passages, the doubling time was reduced almost by 60%. The ENUt4 and ENUt7 cell lines are currently at passages 97 and 103, respectively, with doubling times of ~72 h. Clonal growth potential on solid substrate was tested and confirmed at passage 26 for both lines.

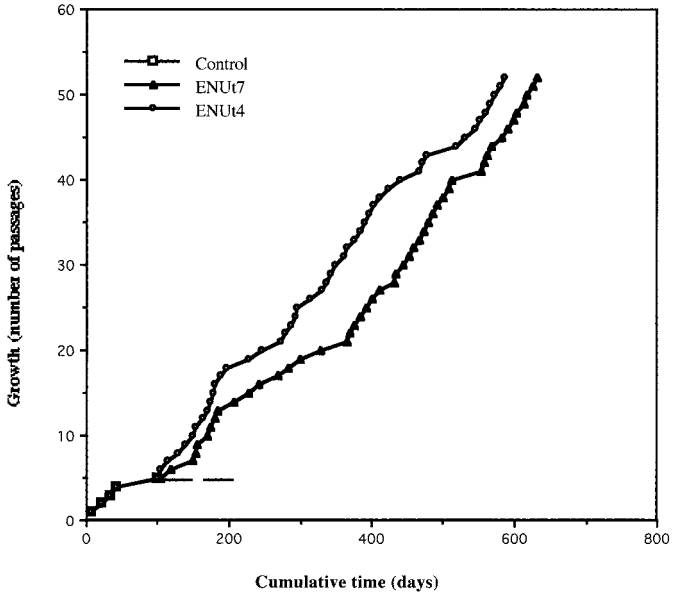

Fig. 1. Continuous growth of ENU-immortalized human breast epithelial lines, ENUt4 and ENU7. Untreated, control cultures displayed no growth after passage 5 (— — —).

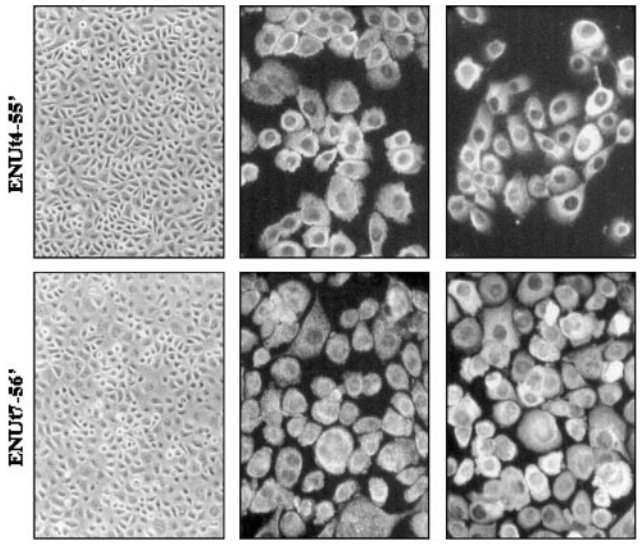

Fig. 2. Characteristic epithelial morphology of ENUt4 and ENUt7 cell lines (*left*: magnification ×40). Expression of CK 14 (*middle*: magnification ×400). Expression of CK 18 (*right*: magnification ×400). Passage number is indicated as ′.

DNA polymorphism-based verification of authenticity of the novel cell lines, determined by allelotyping, demonstrated >90% concurrence with the constitutional genotype of the donor (partial data are shown in figure 4). The characteristic epithelial phenotype of the cell lines is illustrated in Fig. 2. As previously described by direct methods of CK analysis of breast epithelial cultures propagated in MM medium (17), concurrent expression of basal and luminal specific CKs was also observed here by indirect immunolocalization. In both ENUt4 and ENUt7 cell lines, CK 14 and CK 18 were abundantly expressed (Fig. 2).

Molecular Analysis of ENUt4 and ENUt7 Cell Lines

To capture the early events in the dynamic multistep process of ENU-induced carcinogenic progression leading to infinite growth potential, cell aliquots of early passages of proliferating cultures were cryopreserved for future evaluation. Subsequent analyses of continuously passaged cells are described below for two subclones of the ENUt4 cell line, ENUt4A and ENUt4J, maintained separately from passage 26, and for uncloned mass cultures of the ENUt7 line.

Telomerase Activity and Telomere Restriction Fragment Length. To determine the role of altered telomerase regulation in the acquisition of immortality as previously reported (18), we assayed for detectable enzymatic activity. No telomerase activity was detectable in untreated control cultures of human breast epithelial cells. In the ENUt7 cell line, telomerase was first apparent at passage 30 and continued to be maintained with increasing passages of this cell line. In contrast, as shown in Fig. 3*a*, both subclones of the ENUt4 cell line sustained growth without telomerase induction for 73 population doublings (passage 73). In the case of the ENUt4 cell line, the induction of telomerase did not appear to be critical until passage 88, when activity was first detected (data not shown).

Mean TRFs of ENUt7 and both subclones ENUt4A and ENUt4J, measured at passages 60–101, were between 2.5 and 4.5 kb (Fig. 3*b*). In comparison to the untreated control epithelial cells (average TRF length, 7.5 kb) the telomeres of the ENU-immortalized cultures were considerably shortened as reported for other immortalized human

mammary epithelial cells, which have emerged gradually through the immortal conversion process (8).

Early Allelic Losses. To establish an initial baseline of genetic deletions associated with the potential for continuous growth in culture, allelotyping was first performed on DNA isolated from ENUt7 cells at passages 12 and 28 with 169 polymorphic markers providing a genome-wide scan with an average spacing of 24.2 cM. No abnormalities were detectable at passage 12. Cells at passage 28 displayed deviation from a 1:1 constitutional allelic ratio at 23 polymorphic sites. Subsequent LOH analysis confirmed allelic loss at 18 loci (Table 1). The next phase of LOH analysis at these loci was extended to ENU-treated populations, cryopreserved before passage 28 or thereafter.

As summarized in Table 1, the first evidence of allelic loss in the ENUt7 cell line was observed as early as passage 19 (~19 population doublings) at loci *GATA184A08* and *D6S1027* on chromosome 6q24 and 6q27, respectively, and at *D20S482* on chromosome 20p13. LOH at additional loci on chromosomes 3p, 6p, 6q, 11p, 20p, and 20q occurred within the next 9 passages. Notably, the acquisition of LOH at loci on 6p and 6q was common to both ENU-immortalized cell lines, although in the ENUt4 cultures, LOH was first observed several passages after its appearance in ENUt7 (Fig. 4). Initially, the LOH profiles of the two subclones, ENUt4A and ENUt4J, appeared somewhat dissimilar. At passage 31, whereas ENUt4J displayed several regions of loss on chromosome 6, in ENUt4A, only a single partial deletion at 3q13 was apparent. This population was replaced with cells that displayed all of the losses observed in ENUt4J by passage 44. However, the two ENUt4 subclones continued to be distinct from each other at locus *D6S1053*, where LOH was observed only in ENUt4A. On the basis of the similarities in the early LOH profiles, we conclude that sites of allelic loss common to the ENUt4 and ENUt7 cell lines may be indicative of inactivated candidate genes critical for suppressing the initiation of the pathway to ENU-induced cellular immortalization.

p53, p21, and p16 Alterations. Allelotyping of early passage ENU-immortalized cells did not reveal genetic deletions in the region encompassing the *p53* tumor suppressor gene at chromosome 17p. We asked whether a mutated *p53* gene might have played a role in the

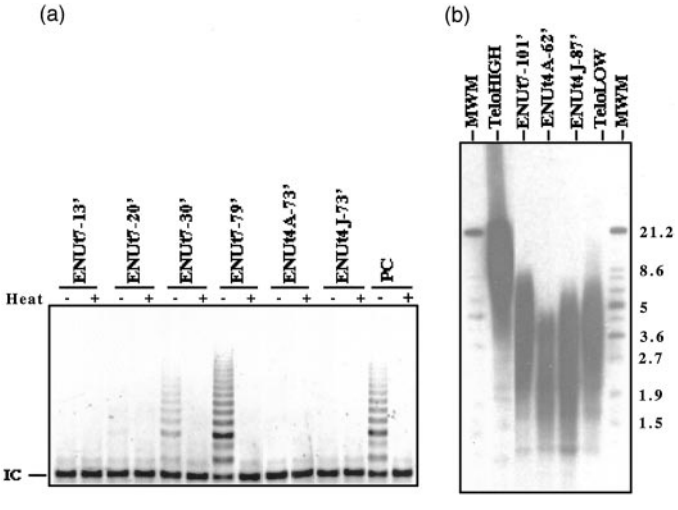

Fig. 3. *a*, telomerase activity in ENUt4 and ENUt7 cell lines. Extracts of 1000 cell equivalents were tested by the telomerase repeat amplification protocol assay; heat + indicates heat-treated controls. *IC* refers to an internal control to demonstrate the absence of PCR inhibitors in the cellular extract. *PC* (positive control) refers to telomerase activity in the breast cancer cell line T47D. *b*, TRF-based telomere length analysis in late passage ENUt cell populations. In all cases, telomeric chromosomal ends are considerably eroded but stabilized. TeloLOW and TeloHIGH are genomic DNA from control cell lines with a mean TRF length of 3.9 and 10.2 kb. MWM, molecular weight marker.

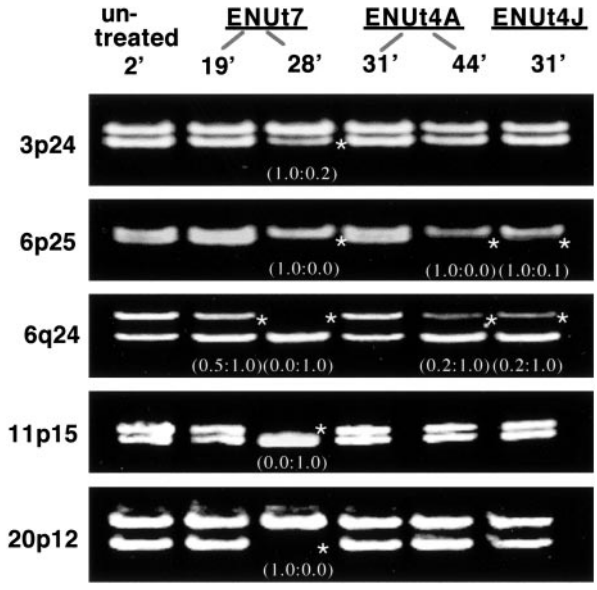

Fig. 4. PCR-based LOH analysis of DNA. Untreated control cells demonstrated a 1:1 allelic ratio at all loci tested. Earliest loss observed is partial LOH at chromosome 6q24 (locus *GATA 184A08*) in the ENUt7 cell line at passage 19. At passage 28, the culture shows complete LOH at this locus. Deletions at 3p24 (locus *D3S3038*), 6p25 (locus *F13A01*), 11p15 (locus *D11S1984*), and 20p12 (locus *D20S604*) were first observed at passage 28. In subclones ENU4A and ENUt4J, LOH was first detectable at passages 44 and 31 at 6p25 and 6q24, respectively. *Asterisks* denote allelic loss. Allelic ratio for each deleted locus is shown in *parentheses*.

acquisition of genome-wide LOH in these cell lines. In ENUt4A and ENUt4J cells, *p53* mutations have not been observed up to the most recent passage assayed (passage 63). In the ENUt7 cell line, *p53* mutations were observed only in a subset of cells at passage 28 when the entire culture homogeneously displayed complete allelic loss at chromosome 6q24-27. The occurrence of LOH at 6q24-27 thus appears to be *p53*-independent in these cells. At passage 31, the ENUt7 culture consisted of a mixed cell population with *p53* missense mutations within exon 7 at codon 239 and within exon 8 at codon 267. By passage 65, the cell line displayed mutations only within exon 7 (Fig. 5*a*).

Loss of expression of the *p53*-regulated *p21* gene, commonly encountered in breast cancer cell lines, was first detectable in ENUt7 cells at passage 74 (Fig. 5*b*). Similarly, for the *p16* gene, upstream of the *pRB* growth regulatory pathway, although relatively high transcript levels were detected up to passage 28, a visible reduction in gene expression was observed in the ENUt7 cell line after passage 31 (Fig. 5*c*). For both *p21* and *p16* genes, ENUt4 subclones continued to display high transcript levels at last evaluation (up to passage 74). Similar to the ENU-immortalized cell lines, heterogeneity in the loss of *p16* expression was also apparent in established breast cancer cell lines. For example, in MCF-7, T47D, and MDA 231 cells, *p16* transcripts were undetectable, whereas in SKBR3 and BT-474 cell lines, *p16* levels were relatively high (Fig. 5*c*).

Anchorage Independence and Tumorigenicity. With regard to the presence of classical transformation endpoints in ENU-immortalized cell lines, such as anchorage-independent growth and tumorigenicity in murine hosts, our analysis was limited to later passages. The cloning efficiency of ENUt4 (passage 66) and ENUt7 (passage 95) cells plated in 8–10 replicate wells in soft agar was 0.1–0.3%. Established breast cancer cell lines, MCF-7 and BT-474, displayed up to 100-fold greater cloning efficiencies (11 and 7%, respectively) under similar experimental conditions. Subsequent expansion and replating of ENUt4 and ENUt7 colonies picked from soft agar showed

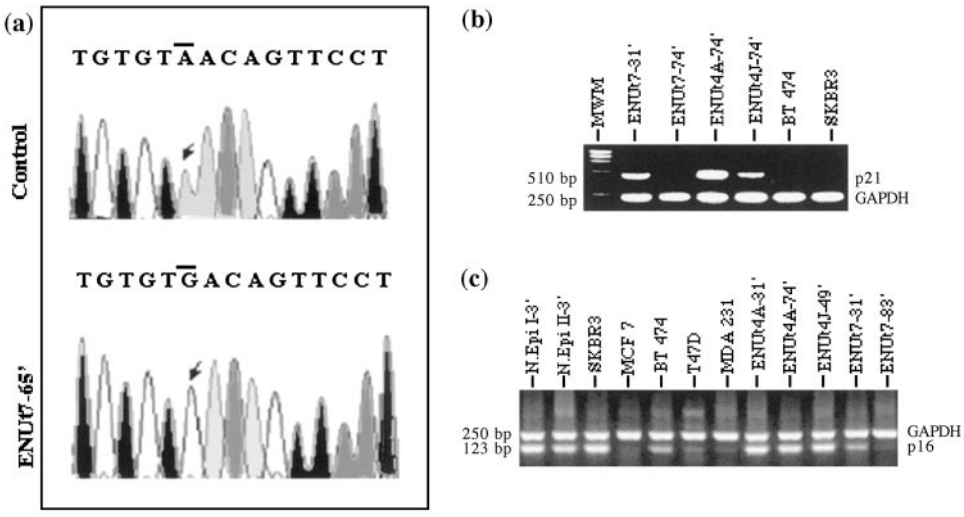

Fig. 5. Genetic alterations in ENU-immortalized human breast epithelial cells. *a*, *p53*: DNA sequence of late passage ENUt7 showing a homogeneous population of cells with a missense mutation from AAC to GAC at codon 239 of exon 7, resulting in the replacement of asparagine with aspartic acid. No mutations were detected in untreated control cells. *b*, detection of *p21* down-regulation in ENU-immortalized cells by semiquantitative RT-PCR analysis. Experimental conditions were optimized for coamplification of *p21* and *GAPDH* primers. Loss of *p21* expression observed in late passage ENUt7 but not in ENUt4A and 4J. A relatively high basal level of expression was maintained at earlier passages (data on early passage ENUt4A and 4J not shown). Breast cancer cell lines BT-474 and SKBR3 displayed reduction of *p21* expression similar to ENUt7–74′. MWM, molecular weight marker. *c*, RT-PCR analysis showing relative *p16* expression in normal breast epithelial cultures at passage 3 in two independent cases (*Lanes 1* and *2*), breast cancer cell lines (*Lanes 3–7*), and ENUt4 and ENUt7 populations (*Lanes 8–12*). Substantially lower *p16* expression is visible in three of five breast cancer cell lines. A gradual reduction in *p16* expression with increasing passage is seen only in the ENUt7 cell line.

relative increases in anchorage-independent cloning efficiency to 1 and 1.8%, respectively.

Inoculation of ENUt4 (passage 66) and ENUt7 (passage 95) cell lines into immune compromised mice ($n = 9$ for each cell line) did not yield measurable tumor growth up to 118 days of observation. Upon termination of the experiment, mice were euthanized, and the cell injection site was harvested and processed for histological analysis. No evidence of tumor formation was apparent at the microscopic level. In contrast, in independent experiments, palpable lesions within 10–15 days of inoculation and high tumor take were observed in the breast cancer cell lines MCF-7 ($n = 12$, 100% tumorigenicity) and BT-474 ($n = 24$, 92% tumorigenicity).

Contemporaneous Alterations in ENU-immortalized Cell Lines and Cancerous Breast Tissue

Allelic Losses. We asked whether allelic losses observed in early passage ENU-immortalized cells *in vitro* were contemporaneous with those *in vivo*. In other words, do such allelic losses also occur early during malignant progression? To address this, DNA was isolated from microdissected foci of preinvasive primary breast tumors classified as DCIS and compared with that of putative clonal progenitor cells, *i.e.*, morphologically normal adjacent TDLU in close proximity to the tumor (Fig. 6*A*). For evaluating the full spectrum of LOH-harboring loci observed in ENU-immortalized cells while DNA preparations from malignant sites were quantitatively adequate, the DNA yield of individual normal TDLU was a limiting factor. Therefore, LOH analysis of tissue-derived DNA was confined to two early candidate regions of deletion each on chromosome 3p and 6q. Nineteen of 20 DCIS cases examined were informative at one or more loci.

As summarized in Table 2, of the earliest deletions common to both ENU-immortalized cell lines, LOH at *GATA184A08* (6q24) was also observed in microdissected tumor cells of 6 of 11 informative cases. Notably, in 3 of 6 cases, LOH was displayed by morphologically normal adjacent TDLU as well (Fig. 6*B*). On the other hand, at locus *D6S1027* (6q27) whereas LOH occurred in the tumor cells of 4 of 8 informative cases, adjacent TDLU showed normal allelic ratios. At two loci encompassing 3p24 (*TRβ* and *D3S2423)*, 11 of 16 informative cases displayed allelic loss in the tumor cells. In 3 of 11 cases, LOH was also observed in morphologically normal TDLU (Fig. 6*C*). Deletions displayed by tumor/normal TDLU pairs were allele specific, providing strong supportive evidence for a clonal relationship between the two groups of microdissected cells. Of those DCIS cases

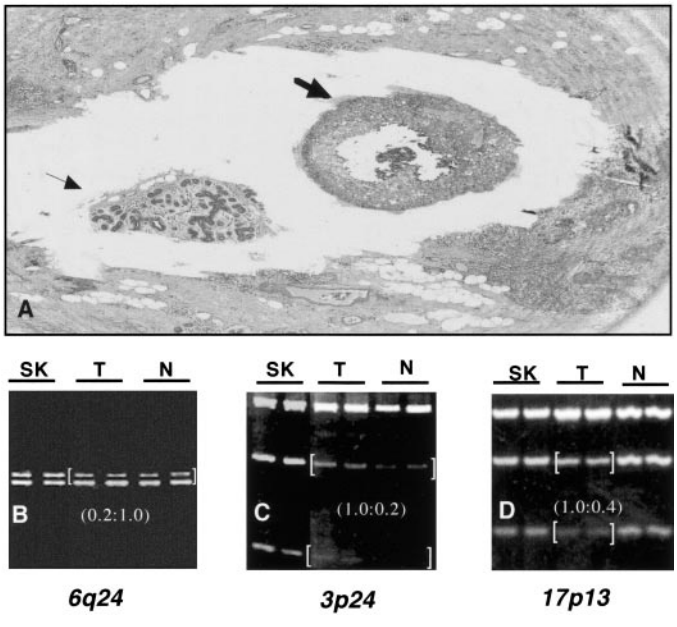

Fig. 6. Immortalization-associated LOH in microdissected populations from early stage breast tumor tissue. *A*, tissue surrounding the area of interest was scraped away to harvest highly enriched cell fractions from H&E-stained sections for LOH analysis. *Large arrow* indicates DCIS. *Small arrow* indicates a morphologically normal adjacent TDLU. For LOH analysis, each DNA sample was amplified in duplicate PCR runs and the products resolved within the same gel in parallel lanes to minimize experimental artifacts often observed with paraffin-embedded archival material. *Lanes SK* show 1:1 allelic ratio in uninvolved skin tissue of the patient. *Lanes T* denote microdissected tumor cells. *Lanes N* denote morphologically normal TDLU adjacent to carcinoma. The deleted allele is indicated within []. Altered allelic ratio is shown in parentheses. *B*, LOH displayed by tumor and normal TDLU at 6q24 (locus *GATA 184A08*). *C*, LOH displayed by tumor and normal TDLU at 3p24 (locus *TRβ*). *D*, LOH at 17p13 (locus *TP53.PCR8*) only in tumor DNA.

Table 2 *Pathological features and early genetic changes in breast tissue of patients diagnosed with DCIS*

Case	Patient age (yrs)	Tumor size (mm)	DCIS subtype	Comedo necrosis	Nuclear[a] *p53* in		LOH in	
					Tumor	TDLU	Tumor	TDLU
62	51	50	B[b]	Y[c]	N	N	Y (a)[d]	Y (a)
157	52	7	A	N	N	N	N	N
180	43	10	A	Y	N	N	Y (c)	N
351	52	20	A	Y	N	N	Y (a, c)	Y (a,c)
355	45	14	C	N	Y	N	Y (a, c)	Y (c)
499	47	10	B	Y	N	N	Y (c, d)	N
520	49	8	A	Y	N	N	N	N
524	42	na	A	N	N	N	N	N
583	76	12	A	N	N	N	Y (d)	N
625	56	12	A	Y	N	N	Y (b)	N
637	65	24	A	Y	N	N	Y (b)	N
752	70	45	C	Y	N	N	Y (b, c)	N
783	78	10	B	Y	N	N	Y (b)	N
788	71	19	A	Y	N	N	Y (a, b)	N
792	64	18	A	Y	Y	N	NI	
793	67	14	A	Y	Y	N	Y (c)	N
833	35	10	A	Y	N	N	Y (b, c, d)	Y (b,c)
852	75	19	A	Y	N	N	Y (b)	N
898	35	7	A	N	N	N	Y (a, b, d)	N
939	43	25	B	Y	N	N	N	N

[a] Presence of *p53* gene product was evaluated by immunohistochemistry.
[b] A, major category: solid; B, major category: micropapillary; C, major category: cribriform.
[c] Y, yes; N, no; na, not available; NI, noninformative.
[d] LOH sites indicated within (): 3p24: *TRβ* (a); *D3S2423* (b); 6q24: *GATA184AO8* (c); *D6S1027* (d).

where LOH was observed in morphologically normal TDLU in 2 patients, loss was either at 3p24 or at 6q24, whereas in another two cases, allelic loss was simultaneously displayed at both sites. No association was observed between the presence of LOH in TDLU and known clinical or pathologic features of the sample.

p53 Inactivation. Allelic loss encompassing the *p53* gene at 17p13 (loci *D17S1541* and *TP53.PCR5–8*) was common in the tumor cells and occurred in 12 of 18 informative cases. However, no deletions were found in normal adjacent TDLU (Fig. 6*D*). Similarly, immunopositive nuclei, indicative of a predominant class of *p53* mutations in cancer, were observed only in the malignant cells of 3 of 20 patients with these early stage tumors (Table 2). Nuclei of normal adjacent TDLU were negative for immunostaining in all cases. Thus, it appears that the occurrence of deletions or mutations, which inactivate the *p53* gene most likely are not an underlying factor in the accrual of allelic loss at chromosome 3p24 or 6q24 in morphologically normal TDLU adjacent to carcinoma.

DISCUSSION

Inactivation of growth-regulating genes involved in the immortalization of tumor cells occurs through multiple mechanisms, including loss-of-function mutation of one allele, presumably induced by a carcinogenic DNA insult, often accompanied by LOH. Genome-wide analyses of tumor tissue have demonstrated that allelic loss is common in breast cancer but occurrence sites vary widely between tumors (19–21). Sites of allelic loss shared by invasive tumor cells and accompanying preinvasive, or premalignant foci, such as DCIS and atypical hyperplasia, respectively, have suggested a clonal relationship between these microscopically distinct lesions (22) but have not revealed the sequence of acquisition of tumor-associated allelic losses conferring cellular immortality. Here, we have applied ENU-immortalized human mammary epithelial cells toward delineating the temporal relationship between early genomic deletions and the acquisition of known cellular alterations leading to infinite growth potential *in vitro* and in cancerous tissue.

Previous reports on the induction of immortalization in normal human mammary epithelial cells without viral oncogenes have demonstrated that functional telomerase and inactivation of the pRB/p16^{INK4} pathway are obligatory steps (9, 18). With regard to these and other known growth regulatory changes associated with immortalization, our data derived from the analysis of the ENUt7 cell line are in agreement. For example, at relatively early passages, the ENUt7 cell line displayed detectable telomerase activity, aberrations in *p53*, and in *p21* and *p16* gene expression reported in other immortal breast cell lines of normal or cancerous origin (23–25). Strikingly, in the ENUt4 line, also derived from the treatment of mammary epithelial cells from the same individual with the same chemical carcinogen, dramatic early changes in gene expression were not apparent. However, the possibility of nonfunctional *p21* and/or *p16* gene product have not been ruled out by our studies reported here with this cell line. Such variability specifically for this set of genes is indeed characteristic of immortalized cell lines and breast tumors (23–26). Similarly, for telomerase induction, the most commonly observed mechanism of telomere maintenance in human tumor epithelial cell lines, ENUt4 and ENUt7, displayed a temporal gap encompassing >50 population doublings.

A major goal in the derivation of ENU-immortalized lines was their rapid application toward gaining insights regarding genetic deletions, which occur early during breast tumorigenesis *in vivo*. The detection of a common genetic signature underlying the immortalization of ENUt4 and ENUt7 was largely facilitated and expedited by a genome-wide deletion analysis of early passage ENU-exposed cells. The chronological order of specific genetic losses showed consistent similarities in the two independently isolated cell lines, and most importantly at specific loci, it closely matched early sites of LOH in cancerous breast tissue. Although we have demonstrated the common genetic features of two lines from the same cell strain, repeated attempts for the derivation of additional immortalized cell lines from this or other reduction mammoplasty samples have not yet been made. Deletions detected before the Hayflick limit of cell replication (27), which were common to both ENUt4 and ENUt7, included LOH at loci on the long arm of chromosome 6, encompassing 6q24 to 6q27.

In breast cancer, chromosome 6q21-qter is one of the most frequently deleted regions (28). The candidate region, 6q24-25, harbors a potential gene known to inhibit tumor cell proliferation. *hZAC* (29), *LOT* (30), and *PLAGL1* (31) refer to the same candidate tumor suppressor gene isolated independently and localized to the 6q24-25 region. Down-regulation of *ZAC* is associated with spontaneous trans-

formation in rat ovarian epithelial cells (32). The gene encodes a zinc finger protein widely expressed in human tissues, including the mammary gland (33). *hZAC* is frequently inactivated in primary breast tumors, as well as in breast cancer cell lines and continually passaged cultures of normal human mammary epithelial cells (34). In *hZAC*-inactivated cancer cell lines, ectopic gene expression induces apoptosis and G_1 arrest (29). Additional support for the presence of one or more immortalization-related genes in the 6q21-qter region is also demonstrated by the induction of senescence upon microcell transfer of 6q26-27 into somatic cell hybrids (35). SV40-immortalized human fibroblasts derived from multiple tissues and individuals display a common region of loss at 6q26-27, potentially harboring a senescence-mediating locus, *SEN6* (36). *In vitro* induction of senescence by the *RNASE6PL* gene located at 6q27, a member of a highly conserved family of cytoplasmic RNAses, implicates it as a candidate gene in the SEN6 region (37).

In view of the previously known association of *ZAC* and *SEN6* with immortal transformation of mammalian cell cultures, the finding of LOH encompassing these chromosomal sites in ENU-immortalized cells is not entirely unexpected. To be noted, however, is our approach for delineating the temporal order of gene inactivation in the acquisition of immortalization *in vivo*. The observation of chromosomal deletions in the *ZAC* region within morphologically normal TDLU adjacent to carcinoma is the first demonstration to our knowledge of the loss of a potential tumor suppressor gene before the manifestation of histological changes associated with malignancy in sporadic breast cancer patients. Intriguingly, although 6q26-27 deletions have been observed in benign breast tumors (38), but unlike the region encompassing *hZAC*, we have not found loss of the *SEN6* region to be associated with morphologically normal TDLU within cancerous breast tissue. The absence of *p53* aberrations in normal TDLU adjacent to carcinoma is in agreement with other reports of the relatively late appearance of *p53* aberrations in sporadic breast tumors (39), supporting our view that allelic losses encountered before *p53* mutations *in vitro* and *in vivo* were most likely *p53* independent.

Whereas biallelic or complete inactivation is generally observed for genes targeted by frequent LOH in tumor cells, it is possible that for some genes, deletion of a single allele could have functional consequences preceding the histological changes characteristic of neoplasia. Similar to the causal role of haploinsufficiency reported for the tumor suppressor genes, *PTEN* and *p53*, in promoting tumorigenesis (40, 41), it is conceivable that LOH-based reduction in the dosage of *ZAC* and/or other genes at 6q24 in morphologically normal TDLU may suffice to impair the regulation of proliferative arrest of breast epithelium during normal cycles of growth and differentiation. Deletions at another chromosome site, 3p24, which were unique to the ENUt7 cell line, have been consistently observed in morphologically normal TDLU adjacent to carcinoma (Refs. 10, 11; data shown here). This LOH site harbors the *TRβ1* gene, a common target of allelic loss (11), as well as epigenetic inactivation in breast cancer cells (42). Similarly, LOH at 11p15.5 in ENUt7 cells, also reported in benzo(*a*)pyrene-immortalized human breast epithelial cells (43) may represent partial inactivation of the cyclin-dependent kinase inhibitor, $p57^{KIP2}$.

The consistent lack of early detectable alterations, other than LOH in the 6q24-27 region in ENUt4 cells, albeit surprising, suggests an initiating role for this deletion leading to the immortal phenotype of these cells. It can be speculated that to some degree mechanisms underlying the apparent differences in the two isogenic ENU-immortalized cell lines may reflect factors related to the genesis of genetically and histologically distinct multifocal and/or bilateral breast tumors in the same patient. Such differences could also be a determining predictive factor in downstream progression and cell behavior, particularly in terms of response to potential cancer therapeutic strategies. In conclusion, the ENU-immortalized cell lines described in this report have served reliably to recapitulate early immortalization-associated events *in vivo*. Despite vast differences in the microenvironmental milieu of normal TDLU within tumor tissue and that of ENU-immortalized cells in culture, the close parallel in early allelic losses observed between these entities suggests that cells harboring such alterations may be autonomous of certain types of microenvironmental growth regulatory signals. In this capacity, this new model system could contribute toward an improved understanding of the growth arresting circuitry, which goes awry in progressively aging epithelial cells, often resulting in the initiation and progression of malignancy.

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