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Methylation of *p16*^{INK4a} Promoters Occurs *in Vivo* in Histologically Normal Human Mammary Epithelia[1]

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ABSTRACT

Cultures of human mammary epithelial cells (HMECs) contain a subpopulation of variant cells with the capacity to propagate beyond an *in vitro* proliferation barrier. These variant HMECs, which contain hypermethylated and silenced *p16*^{INK4a} (*p16*) promoters, eventually accumulate multiple chromosomal changes, many of which are similar to those detected in premalignant and malignant lesions of breast cancer. To determine the origin of these variant HMECs in culture, we used Luria-Delbrück fluctuation analysis and found that variant HMECs exist within the population before the proliferation barrier, thereby raising the possibility that variant HMECs exist *in vivo* before cultivation. To test this hypothesis, we examined mammary tissue from normal women for evidence of *p16* promoter hypermethylation. Here we show that epithelial cells with methylation of *p16* promoter sequences occur in focal patches of histologically normal mammary tissue of a substantial fraction of healthy, cancer-free women.

INTRODUCTION

HMEC[4] populations do not exhibit classical replicative senescence (1, 2). After 10–15 PDs in culture, an overwhelming majority of HMECs encounter a proliferation barrier and activate a cell cycle arrest that is phenotypically similar to fibroblast senescence (normal karyotype, low proliferation, and death indexes; Ref. 1). A subpopulation of HMECs are capable of proliferating beyond this arrest. To avoid possible mechanistic implications, we will, for the purposes of this article, refer to this cell population as variant HMECs, or vHMECs. At the proliferation arrest, vHMECs appear as colonial outgrowths of small cells among the background of large vacuolated nonproliferating cells (1, 3). Importantly, vHMECs exhibit several properties that distinguish them from the initial population of explanted cells, including promoter hypermethylation-mediated silencing of *p16* gene expression (4–7). The origin of the vHMEC subpopulation is currently unknown.

Subsequent to the proliferation arrest, vHMECs proliferate an additional 30–50 generations beyond the time that the bulk population activates the proliferative arrest. These cells eventually reach a second population growth plateau that we previously termed agonescence (2) and that is phenotypically different from human mammary fibroblast senescence and the HMEC first plateau. Agonescent vHMEC populations have both high proliferation and high death indexes, although they exhibit no net increase in cell number (1). Furthermore, nearly 100% of vHMECs approaching agonescence exhibit chromosomal defects, including aneuploidy, telomeric associations, and various other classes of structural abnormalities (1). Such chromosomal instability is reminiscent of the abundant and heterogeneous chromosomal changes observed in premalignant and malignant breast cancer lesions (8, 9).

To gain insight into the origin of vHMECs, we asked whether this cell subpopulation exists before the first population growth plateau. We found that this vHMEC subpopulation exists before this proliferation barrier. Seeking to extend our *in vitro* observations to the tissue from which HMECs are cultured, we examined histologically normal mammary tissue for *p16* promoter hypermethylation, a defining characteristic of vHMECs. We report here that a significant fraction of normal women contain mammary epithelial cells with *p16* hypermethylation.

MATERIALS AND METHODS

Cells and Cell Culture. Isolation of HMECs has been described previously (3). HMECs were cultured in modified MCDB 170 (MEGM, BioWhittaker, Walkersville, Maryland), supplemented with isoproterenol (10^{-5} M; Sigma) and transferrin (5 μg/ml, Sigma). We studied HMECs from reduction mammoplasty specimens from four different individuals: 184, 48, 240 (kindly provided by Martha Stampfer, Lawrence Berkeley National Laboratories, Berkeley, California), and RM9 (organoids derived in the laboratory of T.D.T.). Routine cell culture was essentially as described (1), except that cells were seeded at 6.7×10^3 cells/cm^2. PDs were calculated using the equation, PD = log(*A*/*B*)/log2, where *A* is the number of cells collected and *B* is the number of cells plated initially.

Fluctuation Analysis. Fluctuation analysis experiments (Fig. 1, *A* and *B*, *SET 2*) were conducted by (*a*) imposing a population bottleneck on early-passage HMEC populations; (*b*) allowing *in vitro* expansion of the initial populations, subcultivating the cell populations as needed to prevent confluence; until (*c*) the cultures ceased increasing in cell number (1). In parallel with SET 2 of samples 184 and 48, mass cultures were propagated (Fig. 1, *A* and *B*, *SET 1*) under standard culture conditions. Although *in vitro* propagation of cells, by its very nature, favors cells with a higher proliferation rate, we postulate here that selection for vHMECs occurs predominantly at the first growth plateau. We make this postulation because the proliferation rates and colony-forming efficiencies of early-passage HMEC populations and early-passage vHMEC populations are equivalent (data not shown). When the cell populations ceased expansion, colonies were fixed and stained by standard protocols. Colonies were scored positively if they met the following criteria: colony diameter $\geq$6 mm; staining significantly darker than background; and microscopic confirmation that >90% of the cells in the colony were uniformly small. To validate these colony-scoring criteria, we performed immunocytochemical staining for p16 on representative colonies. Only those colonies that met these criteria were p16-negative (data not shown).

The small subpopulations of founder cells (population bottlenecks) in HMEC 48, 240, and RM9 were derived 8, 10, and 12 days, respectively, after the initial seeding of organoids onto the primary tissue culture flask. HMEC 184 populations were derived from second-passage frozen cell populations.

Binomial distributions were calculated according to the formula (using one Poisson assumption, because $x \ll n$:

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[4] The abbreviations used are: HMEC, human mammary epithelial cell; vHMEC, variant HMEC; *p16*, *p16*^{INK4a} (also known as *CDKN2A* and *MTS-1*); MSP, methylation-specific PCR; ISH, *in situ* hybridization; CIS, carcinoma *in situ*; PD, population doubling.

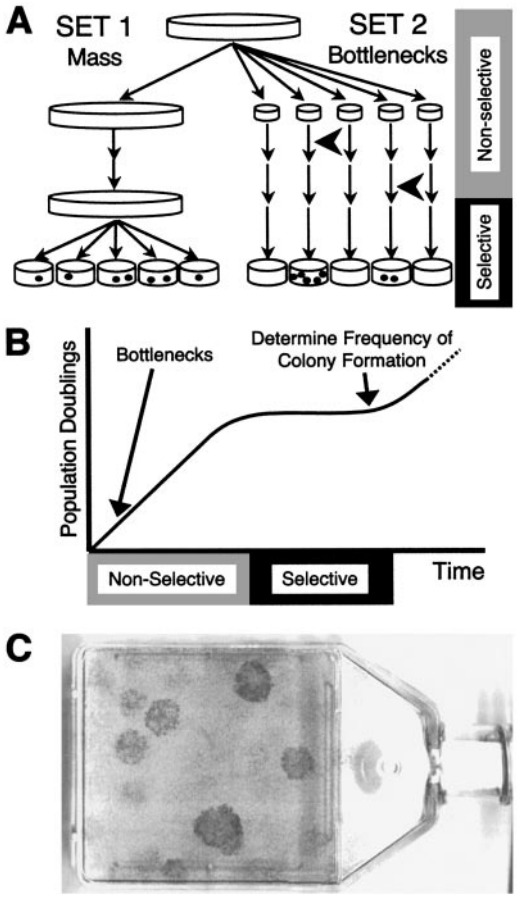

Fig. 1. Schematic diagrams of fluctuation analyses and representative colonies. *A*, general schematic diagram. SET 1 samples the variance of the mass population; the distribution should be binomial, and the variance should approximate the mean ($V = m$). SET 2 measures the distribution of colony formation in bottleneck populations that have been segregated and propagated before application of the selective pressure. In this example, variants have arisen at times before selection (*arrowheads*), making the variance greater than the mean ($V > m$). *Black dots*, colonies. *B*, fluctuation analysis experimental design as defined in this study. *C*, colonies of vHMECs in a 25-cm^2 flask stained for scoring.

$$p(x) = f^x(1-f)^{n-x}\,(n^x/x!)$$

where x is the number of events (colonies), n is the total number of cells plated, and f is the observed frequency. This equation allowed correction for differential cell proliferation from subpopulation to subpopulation. The expected distributions of colony numbers were determined by multiplying the probability distribution by the total number of replicates.

Immunohistochemical Analysis. p16 immunostaining was performed on 5-μm sections of paraffin-embedded tissue using the p16^{INK4a} Ab-4 antibody (clone 16P04; NeoMarkers, Inc., Fremont, CA). Briefly, deparaffinized slides were blocked with 3% hydrogen peroxide, followed by heat-mediated antigen retrieval by microwaving in 10 mM citrate buffer (pH 6.0). Slides were incubated for 1 h at room temperature with a 1:200 dilution of the antibody in PBS and 1% BSA. Antibody staining was visualized using biotinylated-antimouse antibodies (Vector Laboratories, Burlingame, CA) and ABC-HRP Elite (Vector Laboratories, Burlingame, CA), followed by diaminobenzidine reaction. Sections were counterstained with light hematoxylin and then dehydrated and coverslipped with mounting medium.

MSP. The *p16* CpG island methylation status was assessed by using a modification of the protocol described previously (10). Briefly, DNA was extracted according to standard protocols, denatured by NaOH, modified by sodium bisulfite, purified using Wizard DNA purification resin (Promega), treated again with NaOH, precipitated with ethanol, and resuspended in water. A nested approach was used, first amplifying the bisulfite-modified DNA with the flanking primers 5′-AGA AAG AGG AGG GGT TGG TTG G-3′ (upper primer) and 5′-ACR CCC RCA CCT CCT CTA CC-3′ (lower primer), "R" being a mixture of A and G. After this step, 4 μl of each 1:1000-diluted flanking PCR reaction was used as a template for MSP, using the primers previously described (10). Ten μl of each PCR reaction were loaded onto nondenaturing 6% polyacrylamide gels, stained with ethidium bromide, and visualized under UV illumination.

MSP-ISH. Formalin-fixed, paraffin-embedded tissues (5-μm sections) were used to determine the incidence and cellular distribution of *p16* hypermethylation in clinical samples. MSP-ISH was performed as described previously (11). After pepsin digestion of specimens, the DNA was bisulfite modified (CpG Wiz p16 methylation assay; Intergen Discovery Products, Gaithersburg, MD). After a manual hot start (94°C, 3 min), 35 cycles were conducted (55°C, 1.5 min; 94°C, 1 min). PCR used the methylation-specific primer set described previously (10). After PCR, ISH was performed using a methylated allele-specific internally digoxigenin-labeled probe (1 μg/ml), diluted with Hybrisol VII (Ventana Medical Systems). The amplicon and probe

Table 1 *Fluctuation analysis reveals that vHMECs exist before the proliferative arrest*

	Cell population					
	184		48		240	RM9
Category	SET 1	SET 2	SET 1	SET 2	SET 2	SET 2
Replicates (n)	26	19	16	24	15	23
Bottleneck population size		1.0×10^4		1.0×10^4	1.3×10^4	1.0×10^3
PD[a] (mean ± SD)		2.7 ± 0.4		5.1 ± 0.2	7.0 ± 0.5	7.9 ± 1.1
No. cells plated (per replicate) for colony formation	1.0×10^4	$\sim 6.5 \times 10^4$	2.0×10^5	$\sim 3.4 \times 10^5$	$\sim 1.7 \times 10^6$	$\sim 2.4 \times 10^5$
No. replicates with X colonies						
$X = 0$	14	14	11	20	9	20
$X = 1$	10	1	5	1	2	0
$X = 2$	1	1	0	1	1	0
$X = 3$	1	0	0	0	0	0
$X = 4$	0	0	0	1	1	0
$X \geq 5$	0	3[b]	0	1[b]	2[b]	3[b]
No. colonies per replicate						
Range	0–3	0–15	0–1	0–17	0–10	0–51
Mean (m)	0.58	1.63	0.31	1.0	1.7	3.5
Variance (V)[c]	0.57	15	0.23	12	9.2	125
V/m	0.99	9.1	0.73	12	5.5	36
χ^2	0.4	97.7	0.3	33.6	55.7	103
P[d]	0.98	<0.001	0.99	<0.001	<0.001	<0.001
Frequency (f)[e]	5.8×10^{-5}	2.3×10^{-5}	1.6×10^{-6}	2.9×10^{-6}	9.7×10^{-7}	1.1×10^{-5}

[a] PDs from bottleneck until growth plateau.
[b] Actual SET 2 colony numbers (≥5): for specimen 184, colony numbers were 5, 8, 15; for specimen 48, colony numbers were 17; for specimen 240, colony numbers were 7, 10; for specimen RM9, colony numbers were 12, 17, 51.
[c] $V = SD^2$.
[d] Ps were calculated using the χ^2 test (Microsoft Excel), with degrees of freedom (df) as follows: 184, 4; 48, 3; 240, 3; RM9, 4.
[e] Mean frequency for a whole SET.

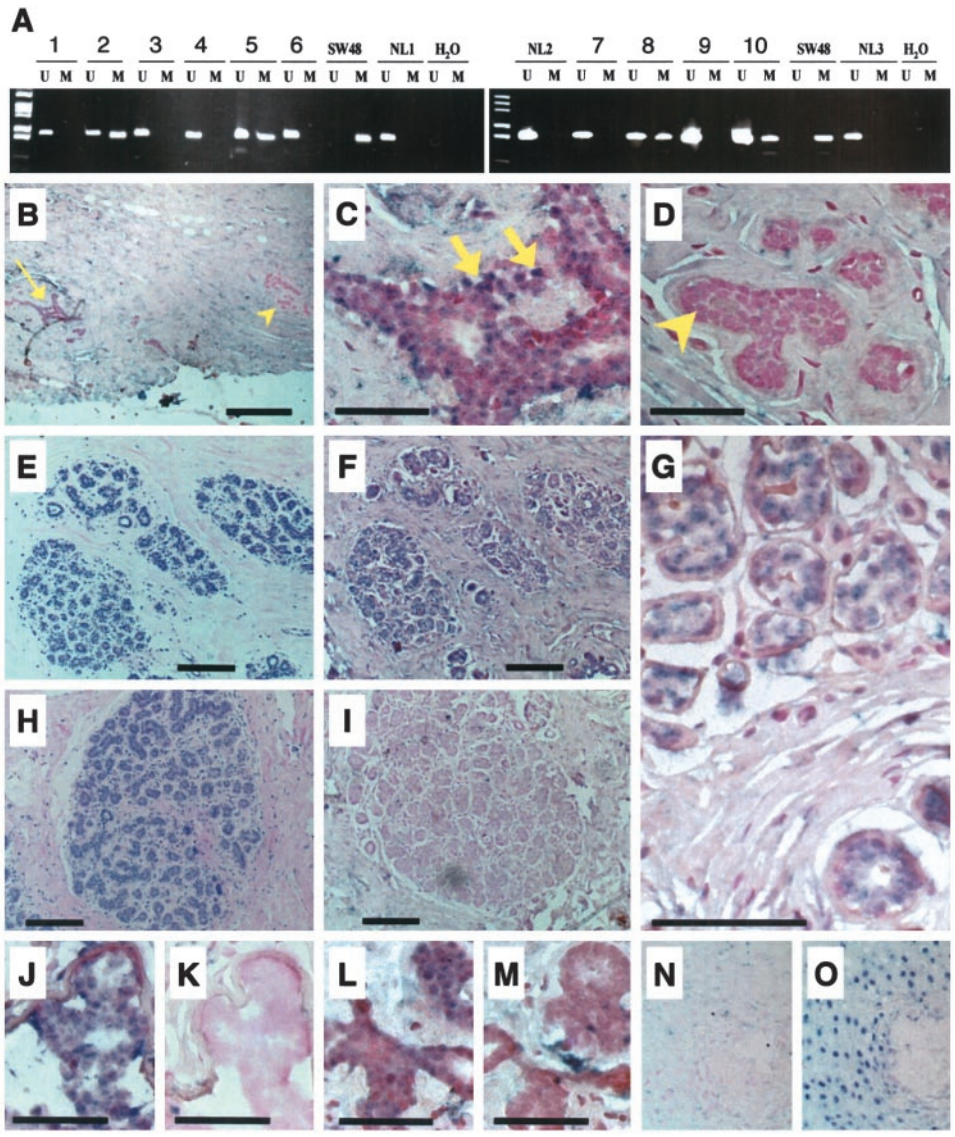

Fig. 2. Methylation of *p16* in reduction mammoplasty samples. *A*, detection by MSP. The presence of a visible PCR product in those lanes marked "*U*" indicates the presence of unmethylated alleles; the presence of product in those lanes marked "*M*" indicates the presence of methylated alleles. The cancer cell line SW48 was used as positive control for methylation, normal lymphocytes (*NL1*, *NL2*, and *NL3*) were used as negative controls for methylation, and water (H_2O) was used as negative PCR control. Sample, patient numbers: *1*, 1514; *2*, 7643; *3*, 10824; *4*, 12993; *5*, 8285; *6*, 9468; *7*, 9624; *8*, 9698; *9*, 11755; *10*, 10434. pBR322/Msp digest is shown *at left* as molecular weight markers. *B–L*, detection of *p16* promoter hypermethylation in histologically normal mammary epithelia *in situ* by MSP-ISH. *B*, field of view with adjacent positively (*arrow*) and negatively (*arrowhead*) stained regions of epithelial cells (patient 9698, MSP-ISH). *Blue signal*, hybridization to the MSP product by ISH; *pink signal*, nuclear counterstain. *C*, *D*, higher magnification of the areas indicated in *B* by the *arrow* and *arrowhead*, respectively. *E*, *F*, representative lobular epithelial cells with *p16* hypermethylation (patient 10811; *E*, H&E; *F*, MSP-ISH). *G*, region showing positive epithelial cell staining and negative adjacent stromal fibroblast staining (patient 10811, MSP-ISH). *H*, *I*, representative unmethylated lobular epithelial region (patient 10434; *H*, H&E; *I*, MSP-ISH). *J–O*, representative examples of MSP-ISH control experiments. Serial sections adjacent to samples positive for *p16* hypermethylation were processed in parallel, with or without the PCR amplification step. This tests the dependency of "positive" ISH results on prior PCR amplification. Shown are representative results for samples from individuals 10811 (*J*, *K*) and 9698 (*L*, *M*). *J*, *L*, representative images of complete MSP-ISH reactions demonstrating *p16* promoter hypermethylation. *K*, *M*, representative images of control reactions in which the PCR step was omitted. *N*, *O*, MSP-ISH of negative and positive control samples. In parallel with each MSP-ISH experiment, we processed two samples whose *p16* methylation status was known (11). *N*, negative control: benign reactive squamous cervical tissue. *O*, positive control: cervical CIS. *Standard bars*, 460 μm (*B*), 150 μm (*E*, *F*, *H*, *I*), and 45 μm (*C*, *D*, *G*, *J–M*).

were codenatured (95°C, 5 min), hybridized (37°C, 2 h), washed (1× SSC +2% BSA, 52°C, 10 min), incubated with antidigoxigenin alkaline phosphatase conjugate (1:200; Roche Molecular Biochemicals), and then exposed to the chromogen, nitroblue tetrazolium, and 5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP; Enzo Diagnostics) at 37°C. The final counterstain, nuclear Fast Red, stains the negative cells pink in contrast to the blue signal. To confirm normal histology, adjacent serial sections were stained with H&E, as per standard histological procedures. To confirm staining specificity, adjacent serial sections were treated as above, but omitting the PCR amplification step.

RESULTS

vHMECs Exist Before the First Population Growth Plateau. The chromosomal abnormalities that accumulate in agonescent vHMEC populations are similar to those chromosomal changes present in the earliest lesions of breast cancer. An understanding of the origin of the vHMEC subpopulation of cells is, therefore, critically important. Specifically, do vHMECs arise as a result of the proliferation arrest of the first population growth plateau, *i.e.*, via induction during a stress or adaptation response ("adaptation"); or do they preexist within the population before the bulk population activates the arrest, and at that time, become selected because of a growth advantage ("selection")? We used Luria-Delbrück fluctuation analysis to distinguish between these two models.

Luria-Delbrück fluctuation analysis is a combined experimental and analytical approach that can be used to determine the origin of variant cells that are resistant to a selective pressure (12, 13). Cells are grown under two sets of conditions, SET 1 and SET 2 (Fig. 1*A*), and then are analyzed for their ability to generate variants. In SET 1 growth conditions, the cells are aliquoted from a mass culture immediately before the selection pressure, thereby measuring the frequency of resistant cells. These aliquots represent a random sampling of cells from the mass population at that time, and the number of variants per aliquot should display a binomial distribution (where, under the condition of a low frequency event, the variance, *V*, approximates the mean, *m*).

In SET 2 growth conditions, cells are aliquoted into many replicates of small subpopulations of founder cells (population bottlenecks) and are allowed to proliferate for several generations before the selection pressure. If the variant cells are generated by conditions found at the arrest (adaptation), then previous propagation history should be irrelevant and each subpopulation would have an equal probability of generating variants. The adaptation model predicts that the number of colonies per subpopulation in SET 2 will exhibit a binomial distribu-

tion ($V \approx m$). In contrast, under the selection model, if the variant cells preexist before the selective pressure, then the variability from subpopulation to subpopulation in SET 2 will be higher than that predicted by the binomial distribution ($V > m$).

To apply fluctuation analysis to the question of the origin of vHMECs, we defined the selective pressure as the self-imposed proliferative arrest of the first population plateau, thereby operationally making the first period of exponential proliferation the nonselective period (Fig. 1*B*). We then measured the frequency and variance of the vHMECs that grew beyond the proliferative arrest. HMECs were cultured according to SET 2 and SET 1 conditions as follows: replicate subpopulations of small numbers of HMECs ($1.0 \times 10^3 - 1.3 \times 10^4$; Table 1; SET 2) were cultured separately, while the parental population was cultured in parallel *en mass*, (SET 1). Cell populations were allowed to proliferate exponentially (subcultivated as necessary), until the cell number ceased to increase and the cells became large and vacuolated (*i.e.,* they activated the proliferative arrest; data not shown). Cultures were then fed regularly until colonies of p16-negative vHMECs (data not shown) were clearly distinguishable from the background (14–21 days). At this time, the plates were fixed, stained, and scored for the frequency of cells that could grow beyond the proliferation barrier (Fig. 1*C*).

Fluctuation analyses performed on cell populations derived from four different individuals (HMEC 184, 48, 240, and RM9) indicated that, whereas the distribution of colonies from the SET 1 populations exhibited the expected binomial distribution ($V \approx m$; Table 1), the distribution of colonies among the SET 2 subpopulations did not ($V > m$; Table 1; $P < 0.001$ by χ^2 test for each experiment). Similar observations were made in nine independent experiments, four of which are summarized fully here (Table 1). These data are consistent with the model that vHMECs are not generated at the time of the proliferative arrest but, rather, exist within the population before the arrest and are selected at the arrest. For this reason, we will henceforth in this paper use the terms "preselection" and "postselection" to describe HMECs *in vitro*, in keeping with their original description (3).

Histologically Normal Human Mammary Tissue Contains Epithelial Cells with Hypermethylated *p16* Promoters. The magnitude of the colony formation frequencies observed in the bottleneck populations of the fluctuation analysis suggested that variant cells not only existed in the population before the proliferative arrest but also were present very early within the culture, and perhaps even *in vivo*. To test the hypothesis that these variant cells exist *in vivo*, we assessed mammary tissue from reduction mammoplasty patients (a patient population with no overt increased risk for breast cancer; Ref. 14) for a defining characteristic of postselection HMECs, namely *p16* promoter hypermethylation (4–7). We used the sensitive MSP assay (10) to ascertain *p16* promoter methylation status in DNA isolated from histologically normal mammary tissue sections. Strikingly, we detected methylated *p16* promoter sequences in DNA isolated from 7 of 15 women (47%; Fig. 2*A*; Table 2). All of the samples that contained methylated-specific PCR product also contained unmethylated-specific PCR product, indicating a mixture of methylated and unmethylated alleles (Fig. 2*A* and data not shown).

Because the MSP analysis was performed on DNA obtained from an entire histological section, it did not provide information about the cell type that generated the positive PCR signal. To address this issue, we performed MSP-ISH (11) on a partially overlapping set of samples also analyzed by MSP. We detected *p16* promoter methylation in 29% (4 of 14) of the MSP-ISH samples analyzed (*e.g.,* Fig. 2, *B, C, F, G, J,* and *L*; Table 2). Seventy-one % (10 of 14) of reduction mammoplasty specimens analyzed by MSP-ISH contained undetectable levels of methylation (see Fig. 2*I*; Table 2). Adjacent serial sections were stained with H&E to allow cytological evaluation of the regions of positive staining. Microscopic analysis revealed that methylated *p16* alleles were present in histologically normal mammary epithelial cells in both lobular (Fig. 2, *E–G*) and ductal (data not shown) regions. Neither myoepithelial cells nor stromal cells (including fibroblasts) contained detectable methylated alleles (Fig. 2*G*). Although several of the breast reduction samples were positive by both the MSP and MSP-ISH analyses, five samples demonstrated discordance between the two methods of analysis (Table 2). Possible explanations for this discordance include differential sensitivities of the two techniques, the apparent focal nature of the hypermethylated cells, and/or sampling differences.

Table 2 p16 *promoter methylation status in normal breast specimens*

Patient identification	Age (yr)	MSP[a]	MSP-ISH[b]
9698	27	+	+
5308	56	+	+
10811	26	+	+
7643	31	+	−
8285	18	+	−[c]
10434	32	+	−
4508	69	+	−
9624	16	−	+
11755	22	−	−
1514	45	−	ND[d]
10824	46	−	ND
12993	21	−	ND
9468	40	−	ND
8275	33	−	ND
5105	45	−	ND
10966	26	ND	−
12075	30	ND	−
12610	31	ND	−
11139	32	ND	−
11018	25	ND	−[e]
Median age (yr)	31		
Methylated		31	26.5
Unmethylated		36.5	30.5
Samples methylated (%)		7/15 (47)	4/14 (29)

[a] MSP scoring: −, unmethylated product but not methylated product detected; +, methylated product detected.
[b] MSP-ISH scoring: −, no positively stained nuclei present in entire section; +, positive nuclei detected.
[c] Few epithelial cells present in section.
[d] ND, not done.

We then analyzed the distribution pattern and approximate frequency of cells positively staining for *p16* promoter methylation within the tissue and exhibited the data using a tissue map (Fig. 3). This method of presenting the data allows a display of spatial information and heterogeneity. In general, the samples fell into three major categories: (*a*) as already mentioned, the majority of samples (10 of 14) contained an undetectable number of cells per histological section with methylated *p16* promoter sequences ($\ll$1% positive epithelial cells per section; *e.g.,* Figs. 2, *H* and *I*, and 3, *A–G*, and data not shown). By calculating the total area occupied by epithelial cells per section and the mean number of epithelial cell nuclei per unit area, we estimate that the average histological section contains ~30,000 epithelial cell nuclei. Thus, in 10 of the 14 samples, the frequency of detection is less than 1/30,000, or 3.3×10^{-5}. It is currently unknown whether repeated sampling from different sites of the same breast will reveal similar or different frequencies; (*b*) two samples contained rare foci or an intermittent scattering of cells with methylated *p16* promoter sequences (samples 9624 and 5308; Fig. 3*H* and data not shown; note frequent juxtaposition of methylated clusters and unmethylated clusters); and (*c*) finally, two samples contained a considerable number of cells per section (~10–50% positive epithelial cells per section, although the frequency varied greatly from region to region; samples 9698 and 10811; Fig. 3, *I* and *J*). Large adjoining

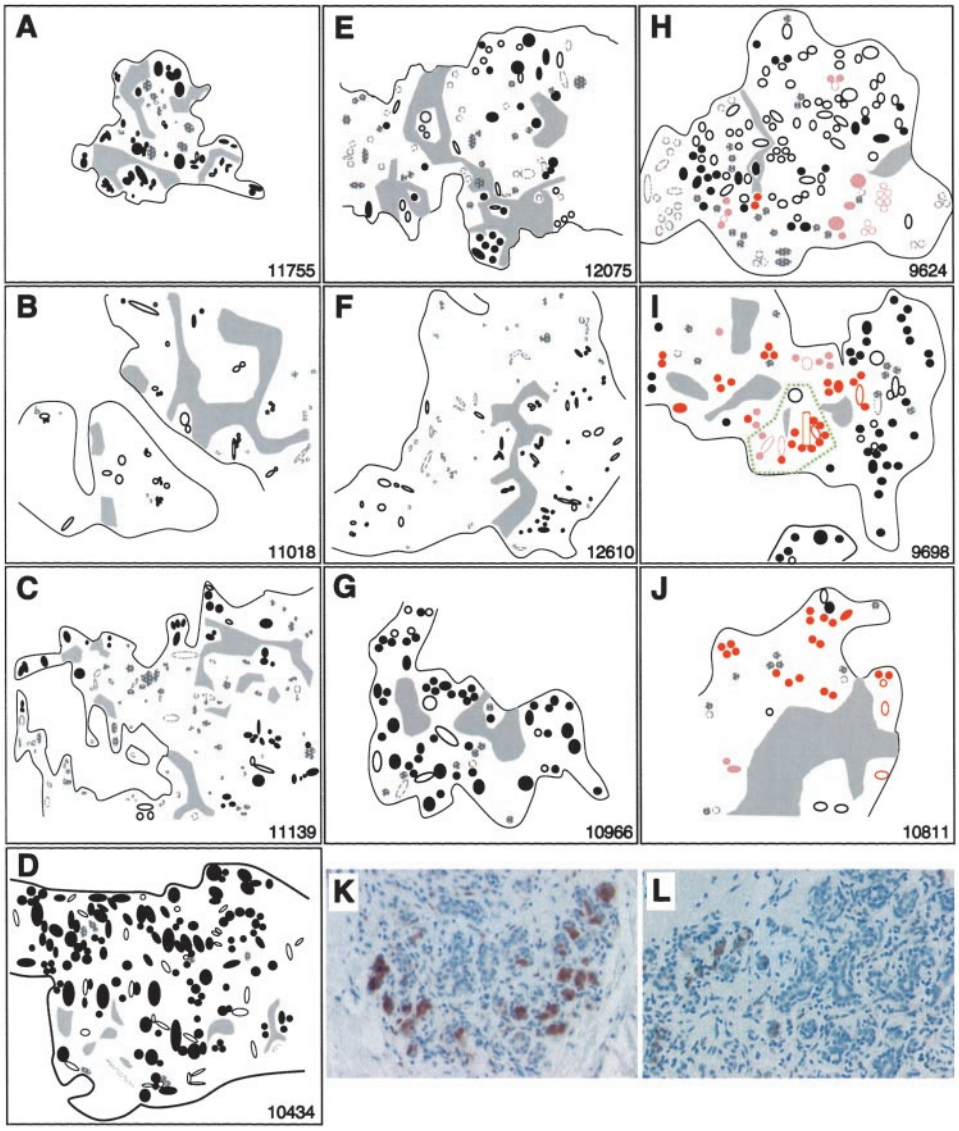

Fig. 3. Distribution of epithelial cells with methylated *p16* promoters in histologically normal mammary tissue. *A–J*, we generated a gridded map of epithelial cell clusters by examining the H&E section. We classified epithelial clusters as ductal (*open ovals*) or lobular (*filled ovals*) and then assessed, in the adjacent serial section, the methylation status of each cluster (MSP-ISH). Methylation status of lobular and ductal epithelial cell clusters was scored as negative (*black*, <1% cells methylated), low frequency of positive (*pink*, 1–50% cells methylated), or high frequency of positive (*red*, >50% cells methylated). Epithelial cell clusters that could not be assessed because of processing problems are *stippled gray*. Regions of adipose tissue are represented in *light gray*. *A–G*, examples of tissue without detectable *p16* methylation. *H–J*, examples of tissue containing cells with hypermethylated *p16* promoter sequences. *A–J*, lower right of panels, patient identification numbers. *K*, patient *10966*, and *L*, patient *10811*, representative immunohistochemical detection of p16 protein expression.

regions of positivity (e.g., as outlined by the *green dashed line* in Fig. 3*I*) may indicate clonal origin of variant cells or a field effect.

Promoter hypermethylation is frequently associated with the silencing of gene expression (15–17). Consistent with this association, we found that regions of *p16* hypermethylation in reduction mammoplasty samples corresponded to regions of low-to-undetectable p16 protein expression (*e.g.*, Fig. 3*L*, sample 10811; and data not shown). Specimens with undetectable hypermethylation varied in their levels of p16 protein expression; some contained low-to-undetectable expression levels (data not shown), whereas others expressed p16 abundantly in focal patches (*e.g.*, Fig. 3*K*, sample 10966). The basis for the induction of p16 expression in mammary tissue is currently under investigation.

To confirm the specificity of the MSP-ISH results, an adjacent serial section from each reduction mammoplasty was processed in parallel, but omitting the PCR step. Omission of PCR resulted in the loss of nuclear hybridization (Fig. 2, *K* and *M*), thereby attesting to the specificity of the post-PCR methodology. Furthermore, with each set of MSP-ISH reactions, we processed a cervical CIS sample, shown previously by various methods to contain extensive *p16* promoter hypermethylation, as well as a benign cervical sample previously shown to contain unmethylated promoter sequences (11). As expected, after MSP-ISH, the cervical CIS sample showed abundant nuclear hybridization (Fig. 2*O*) and the signal was undetectable in the benign cervical tissue (Fig. 2*N*). In further control experiments, the CIS sample stained negatively when (*a*) PCR was omitted, (*b*) primers were omitted from the PCR, or (*c*) the sodium bisulfite modification reaction was omitted (data not shown).

DISCUSSION

Dysregulation of the p16/cyclin D1/pRb pathway is common to many different cancer types (18, 19). Sporadic breast carcinomas frequently exhibit heterozygous or homozygous deletion of the *INK4a* locus (20), and/or silencing of the *p16* gene by promoter hypermethylation (21). We show here that hypermethylation of the *p16* promoter can occur in morphologically normal mammary epithelial cells from a sizeable fraction of women with no overt increased risk for breast cancer. The clinical relevance of this event in normal women is currently under investigation. It is possible that, in the breast, *p16* hypermethylation serves a "normal," as-yet-undetermined biological function unrelated to carcinogenesis. However, because of the extensive clinical and experimental evidence implicating the *p16* gene as a tumor suppressor, we favor the interpretation that the observed *p16*

hypermethylation is a common and early event in sporadic breast cancer. In keeping with the current multistep model of initiation and progression in breast cancer, we posit that additional epigenetic and/or genetic lesions beyond *p16* silencing would be necessary to manifest the malignant phenotype.

If the *p16* promoter-hypermethylated variant epithelial cells indeed represent precursors to breast cancer, our observations suggest that premalignant breast lesions are more frequent than generally appreciated. Studies by Nielsen *et al.* (22) and Alpers and Wellings (23) have shown a surprising degree of undetected premalignant and malignant lesions. In the Nielsen study of double mastectomy specimens from 110 medicolegal autopsies, in which the cause of death was unrelated to breast cancer, nearly one-third of the women harbored hyperplastic lesions (32%), more than one-quarter contained atypical ductal hyperplasia (27%), almost one-fifth showed ductal CIS (18%), and 2% had overt invasive breast cancer. Furthermore, almost one-half of the women with ductal CIS had bilateral (41%) and/or multifocal (45%) disease (22). Alpers and Wellings' study of 185 breast samples from random autopsies confirmed this high prevalence of undetected premalignant breast lesions (23). Other studies have reported lower frequencies of premalignant lesions (24, 25), but sampling methods and clinical definitions varied among these studies (26). These data indicate that the initiation of premalignant lesions, identified by morphological alterations within the tissue, is by no means a rare event.

Our observation of *p16*-methylated variant cells in histologically normal tissue may be identifying premalignant lesions before the morphological changes reported above. Several recent studies have shed light on the genomic status of histologically normal breast tissue. Deng *et al.* (27) showed that a common genomic alteration in primary invasive breast cancers (loss of 3p) often occurred in adjacent morphologically normal ductal tissue. Using a broader range of markers, Larsen *et al.* (28) showed that 22% of microdissected histologically normal breast samples showed microsatellite instability and/or loss of heterozygosity. Kandel *et al.* (29), furthermore, showed that p53 mutations, including missense mutations previously detected in breast cancer, could be detected in normal and benign breast tissue. These observations, along with the epigenetic alteration reported here, support the hypotheses that early premalignant breast lesions are more frequent, and harbor more genetic and epigenetic alterations, than previously suspected. We anticipate that further study of vHMECs *in vitro* and *in vivo* will continue to provide insights into early changes in breast cancer.

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