Tumor Suppressor p16^{INK4A} Regulates Polycomb-mediated DNA Hypermethylation in Human Mammary Epithelial Cells

Altering DNA in DNA methylation are important in cancer, but the acquisition of these alterations is poorly understood. Using an unbiased global screen for CpG island methylation events, we have identified a non-random pattern of DNA hypermethylation acquired in p16-repressed cells. Interestingly, this pattern included loci located upstream of a number of homeobox genes. Upon removal of p16^{INK4A} activity in primary human mammary epithelial cells, polycomb repressors, EZH2 and SUZ12, are up-regulated and recruited to HOX9, a locus expressed during normal breast development and epigenetically silenced in breast cancer. We demonstrate that at this targeted locus, the up-regulation of polycomb repressors is accompanied by the recruitment of DNA methyltransferases and the hypermethylation of DNA, an endpoint, which we show to be dependent on SUZ12 expression. These results demonstrate a causal role of p16^{INK4A} disruption in modulating DNA hypermethylation, and identify a dynamic and active process whereby epigenetic modulation of gene expression is activated as an early event in breast tumor progression.

Aberrant epigenetic changes have been documented in cancer, which act as alternatives to mutation or deletion to disrupt tumor suppressor gene function (1–3). However, the control of this aberrant epigenetic remodeling is unknown. Both changes in the levels of chromatin remodeling proteins, including polycomb group (PcG)^2 proteins, and genome-wide loss and localized gains in DNA methylation have been reported in many tumors (1–3). PcG proteins form polycomb repressor complexes (PRCs) to control cell fate determination, stem cell renewal, cell growth, and cell division. The PRC2 complex contains at least four different subunits, EED, EZH2, SUZ12, and AEBP2 (4), many of which are downstream transcriptional targets of E2F in multiple cell types (5, 6). The PRC2-associated PcG proteins, EZH2 and SUZ12, are overexpressed in a wide spectrum of human tumors (7–9) and increased EZH2 in cancer cells generates a variant PRC that may alter the targeting of gene silencing (10). Changes in DNA methylation patterns have been identified in cancer and result in the silencing of important tumor suppressor genes involved in cell cycle regulation, differentiation, apoptosis, DNA repair, and metastasis (1–3). Recent studies suggest that DNA hypermethylation is the consequence of gene silencing (11–15).

The product of the {INK4A} locus, p16, encodes a cyclin-dependent kinase (CDK) inhibitor that functions as a negative regulator of cyclin/CDK complexes. It binds preferentially to CDK4/6 and prevents their association with D-type cyclins, thus inhibiting pRB phosphorylation and progression through the cell cycle (16, 17). Cells with loss of p16^{INK4A} activity exhibit increased E2F activity. In humans, p16^{INK4A} has a role in the maintenance of normal cellular properties, preventing centrosome dysfunction and genomic instability (18). In human tumors, inactivation of p16^{INK4A} is as an early event (16, 17) and loss of p16/Rb activity occurs through deletion, mutation or hypermethylation of p16^{INK4A} or other members of the pRb pathway in the majority of tumors (17).

HOX genes encode DNA binding transcription factors that are spatially and temporally regulated during embryonic development and are critical to the regulation of gene expression, morphogenesis, and differentiation (19). In breast cancer, there are numerous examples of the aberrant repression of HOX genes (20, 21). In contrast to the transforming properties of HOX9 in lymphocytes, it has been reported that HOX9 expression is necessary for lactational differentiation (22) and is absent in breast cancer (23). However, the role of HOX9 in the mammary gland is unclear.

Rare foci of morphologically normal epithelial cells with hypermethylation of p16^{INK4A} promoter sequences and loss of p16^{INK4A} activity have been observed in vivo in disease-free tissue from breast (24) and other organs (25, 26) and are hypothesized to be precursors to cancer (27). In the breast, these rare p16^{INK4A}-silenced cells (variant human mammary epithelial cells or vHMEC) can be isolated from breast tissue samples and propagated in culture where they have been shown to exhibit selected pre-neoplastic phenotypes and biomarkers (18, 24, 28–33). Expression of these biomarkers are observed in the majority of premalignant lesions (29, 34), contributing to...
the evidence that these p16INK4A-silenced cells are early manifestations of malignancy and provide an excellent model system to study early events in cancer formation.

In the present study, we show that loss of tumor suppressor p16INK4A activity leads to the up-regulation of polycomb proteins, EZH2 and SUZ12 and DNA hypermethylation. We have identified a non-random pattern of DNA hypermethylation using an unbiased global screen for CpG island methylation events in primary human mammary epithelial cells with loss of p16INK4A activity. We demonstrate that polycomb proteins play a causal role in targeting DNA hypermethylation to the HOXA9 locus. In primary breast tumors, we find HOXA9 is epigenetically silenced. These studies suggest that epigenetic changes, controlled by loss of p16INK4A activity, are some of the earliest events in breast tumor progression.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Plasmids**—Isolation and culture of HMEC in modified MCDB 170 (MEGM, Cambrex) has been described (35). HMF were grown in RPMI1640 supplemented with 20% fetal bovine serum. We studied HMEC/vHMEC/HMF from reduction mammaryplasty specimens from ten individuals: 48 and 240 (kindly provided by Martha Stamper), RM9, RM13, RM15, RM16, RM18, RM20, RM21, and RM163 (cells derived in our laboratory). vHMEC clones were generated using standard ring cloning procedures. Population doublings (PD) 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. Cell cycle profiles were analyzed by FACS.

pBABE-myc-EZH2 has been described (36). The SUZ12 ORF was excised from pCMV-SUZ12 (a kind gift from Dr. Peggy Farnham) and cloned into pBABE-Hygro. The shRNA specific for p16INK4A encoded inverted repeats of 27 bp corresponding to nt 381–407 of the human CDKN2A p16INK4A cDNA (GenBank™ accession No. NM000077), separated by an 8-nt spacer (37). The shRNA specific for GFP was purchased from Open Biosystems. Constructs were packaged in Phoenix A cells for viral propagation. Retroviral infection of HMEC/vHMEC was carried out using standard methods. Puromycin selection (4 μg/ml) or hygromycin selection (20 μg/ml) was applied 24 h after retroviral infection and continued for 5 d after which cells were harvested. Transient transfection of HMEC with siSUZ12 (SMART pool M006957) or siControl (D001206) (Dharmacon) was carried out using Lipofectamine (Invitrogen). In siRNA/shRNA combination treatment, retroviral infection was followed 12 h later by siSUZ12 or siControl transfection. Another 24 h later, puromycin selection was applied (4 μg/ml) for 5 d after which the cells were harvested. In order to induce demethylation, we incubated vHMEC with 5-aza-2’-deoxycytidine (1 μM) and drug-containing media was replaced every 24 h for 3 days.

**Bisulfite Treatment, PCR Analysis, and Western Blotting**—We isolated genomic DNA (Promega) from cells and performed bisulfite treatment basically as described (38). We digested 2 μg of genomic DNA with EcoRV and then denatured it in 0.3 M NaOH for 20 min at 37 °C. We used an 8-h incubation at 55 °C followed by removal of free bisulfite using a Wizard Clean Up kit (Promega). PCR was performed using primers to amplify region gb/AC004080: 43040–42674 (MR1) or 40432–40134 (HOX promoter). Nested PCR products were cloned into pCR2.1 (Invitrogen) followed by nucleotide sequencing using the Big-Dye Termination method (ABI). Methylation-specific PCR primers to the HOXA9 regulatory region were designed by the MethPrimer program (39) and identified methylated or unmethylated alleles (A9MS: 5′-TCGCGGTAGATGGAGG-GTGGAGA-3′, A9MA: 5′-AAAAATAAAAAACGAAAAAC-AAACGAA-3′, A9US: 5′-TGGGTTTAGATGGAGGT-TGGGA-3′, A9UA: 5′-AAAAATAAAAAACGAAAAAC-AAACGAA-3′).

Total RNA was isolated from cells and cDNA synthesized using standard methods. Quantitative real-time PCR (TaqMan) was performed on cDNA (40) using the standard curve method with primer/probe sets for HOXA9 (ABI: Hs00365956_m1), p16INK4A (ABI: Hs00233365_m1) SUZ12 (ABI: Hs01093658_m1), EZH2 (ABI: Hs00544830_m1), EED (ABI: Hs00243609_m1), PCNA (ABI: Hs00427214_g1), and for GUSB (ABI: Hs99999908_m1). The expression of GUSB (external control) was used to normalize for variances in input cDNA.

For Western blot analysis, 30 μg of protein from total cell extracts or nuclear or cytoplasmic extracts were fractionated in gradient (4–20%) polyacrylamide gels (FMC) and transferred to Hybond-P (Amersham Biosciences) membrane. Lysates were exposed to rabbit polyclonal anti-p16INK4A (Upstate), anti-SUZ12 (Abcam), anti-HOXA9, anti-EZH2, anti-EED or anti-Myc tag (Upstate), followed by horseradish peroxidase-conjugated goat-anti-rabbit antibody (Calbiochem). Actin, tubulin (Sigma), or Lamin B (Santa Cruz Biotechnology) were used as loading controls.

**RLGS**—We carried out RLGS as described (41). Briefly, non-specific, sheared ends of genomic DNA (2–5 μg) were blocked in a 10-μl reaction by the addition of nucleotide analogues (αS,dGTP, αS-dCTP, dATP, ddTTP) with DNA polymerase 1 (2 units; 37 °C, 20 min) followed by enzyme inactivation (65 °C, 30 min). The buffer was then adjusted and the DNA was digested (37 °C, 2 h) with 20 units NotI (Promega). We used Sequenase (version 2.0, USB) to fill in the NotI ends with [α-32P]dCTP (New England Nuclear) and [α-32P]dCTP (Amersham Biosciences) for 30 min at 37 °C. The labeled DNA was digested (37 °C, 1 h) with EcoRV (20 units; Promega) and a proportion was electrophoresed through a 60-cm long, 0.8% agarose tube gel (first dimension separation). Theagarose gel was then equilibrated in HinfI digestion buffer and the DNA digested in the gel with HinfI (700 units; Promega) at 37 °C for 2 h. The agarose gel was then placed horizontally (rotated 90° relative to the first electrophoresis) across the top of a non-denaturing 5% polyacrylamide gel, the two gels connected with molten agarose and the DNA was electrophoresed in the second dimension. The gels were dried and exposed to x-ray film in the presence of intensifying screens (Quanta III, DuPont) for 2–10 days.

The central portion of the RLGS profile (grid sections 2A through 5E) exhibits the best fragment resolution and was used for methylation analysis. Of greater than 1000 fragments in this region, 762 which were non-overlapping with other fragments, single copy intensity and present on HMECs from three different individuals were included in the analysis. Of these 762 fragments, 42 showed complete or near complete absence in at least
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one vHMEC population. To test for the presence of heterogeneity in methylation frequencies among islands, we compared methylation frequencies of the islands to what would be expected under null hypotheses of no preferential methylation sites. To avoid making parametric assumptions, a permutation approach was employed. To test for equal methylation frequency among the islands, we emulated the null hypothesis by fixing the marginal number of methylation events for each clone and permuting methylation events, thus randomly assigning methylation events occurring in a given clone to the islands. For each iteration, the proportion of resulting methylation frequency for each island was retained. The procedure was repeated 10,000 times. Unadjusted \( p \) values were assigned by calculating the proportion of permutations in which the resulting methylation frequency for a given island exceeded the observed frequency. Under null hypothesis, the \( p \) values should follow a Uniform (0, 1) distribution. We used a one-sample Kolmogorov-Smirnov test to assess the degree of plausibility for the null hypothesis of no differential methylation and obtained a \( p \) value of 0. After Bonferroni adjustment for 762 tests, 16 islands were significantly hypermethylated (\( p \) value = 0 with upper boundary of 0.0762 because of the limit of 10,000 permutations). Alternatively, by considering maximum frequency observed in each permutation and comparing the observed frequencies to the distribution of the maximum, we obtained a \( \max T \) adjusted \( p \) value for each island (42). Sixteen islands were assigned \( \max T \) adjusted \( p \) value less than 0.01. This insures the probability of less than 1% of having at least one false positive. All calculations were performed in a freely available \( R \) statistical environment (43). Bioinformatics programs were used to find the position of RLGS fragments in the genome.

Luciferase Reporter Assays—1 \( \times 10^7 \) vHMEC were seeded in 24-well plates. After 24 h of treatment with 5-aza-2'-deoxycytidine (1 \( \mu M \)), 1 \( \mu g \) of the promoter reporter (pGL3, EhpB4, EhpB4nt) was transfected into vHMEC using a cationic lipid (Invitrogen). Lysates were harvested 48 h later and dual luciferase analysis performed (Promega). Equal amounts of CMV-driven constructs were transfected in each experiment, and cotransfection of a Renilla luciferase reporter (20 ng) was used to allow standardization for transfection efficiency. Drug-containing media was replaced every 24 h. All experiments were performed in triplicate.

Chromatin Immunoprecipitation—For chromatin immunoprecipitations, DNA-protein cross-linking was performed as described (44) with slight modifications. Cells were incubated with 0.5 mM DSG (Pierce) at room temperature for 30 min, then formaldehyde was added to a final concentration of 1%, and cells were incubated at room temperature for 10 min. DNA shearing was performed in nuclei lysis buffer (1% SDS, 10 mM formic acid was added to a final concentration of 1%, and 0.1M NaHCO3. Samples were incubated with protein A/G-agarose and bound complexes were eluted with 1% SDS, 0.1 M NaHCO3. Samples were digested with Proteinase K. Following purification of DNA fragments (QiaQuick PCR purification kit, Qiagen), promoter sequences were amplified using PCR. The following primer set was used: MR2 nt 42764–42556 of AC004080.

**RESULTS**

Loss of p16\(^{INK4A}\) Activity Up-regulates Selected PcG Proteins—Because cells with loss of p16\(^{INK4A}\) activity exhibit increased E2F activity, a known regulator of PcG expression, we wished to use microarray analysis to determine if factors involved in epigenetic silencing show altered expression in vHMEC compared with HMEC. Two members of the polycomb group family of proteins, SUZ12 and EZH2, were among the genes that were up-regulated when vHMEC were analyzed by microarray. To verify the gene expression profiling data, we assessed the mRNA levels of several PRC2 genes (SUZ12, EZH2, and EED) in HMEC and vHMEC populations using quantitative real-time PCR. Both SUZ12 and EZH2 mRNA is increased in vHMEC compared with HMEC, 4-fold and 9-fold, respectively (Fig. 1A). These striking differences in PRC gene expression are independent of proliferative differences between HMEC and vHMEC, because both cell populations double with similar kinetics (30 h and 26 h, respectively) and exhibit a similar S phase fraction under the conditions of these analyses (15 and 20%, respectively; data not shown). Additionally the increase in expression of EZH2 and SUZ12 is also observed after normalization of expression to PCNA levels (supplemental Fig. S1A). The levels of a third component of the PRC2 complex, EED, shown to be an E2F target gene in some cell types (5), remain unchanged. We verified that the up-regulation of SUZ12 and EZH2 mRNAs was indeed accompanied by an increase in protein expression via Western blot analysis (Fig. 1, A and B) and documented that EZH2 localized to the nuclear compartment, while SUZ12 was localized to both the nuclear and cytoplasmic compartment in vHMEC (supplemental Fig. S1B). SUZ12 and EZH2 protein expression is low in HMEC but is increased significantly in vHMEC, whereas the levels of EED remain unchanged (Fig. 1B). HMEC obtained from nine different individuals all display a low level of EZH2 when compared with their isogenic vHMEC population. The basal levels of EZH2 expression in HMEC vary between individuals and extremes of expression are shown in Fig. 1, B and C.

To determine if there is a causal relationship between loss of p16\(^{INK4A}\) activity and increased expression of PcG genes in these primary human cells, we assessed lysates from HMEC after infection with retrovirus containing a small hairpin RNA (shRNA) to either GFP or p16\(^{INK4A}\) by Western blot analysis.
Whereas HMEC infected with retrovirus containing a shRNA to GFP (shGFP) have low levels of SUZ12 and EZH2, HMEC infected with retrovirus containing a shRNA to p16INK4A (shp16) show high levels of EZH2 and SUZ12 expression upon decreased p16INK4A levels (Fig. 1C), comparable to the levels of EZH2 and SUZ12 seen in vHMEC (Fig. 1, B and C). To determine whether p16INK4A expression is sufficient to repress PcG EZH2 and SUZ12 seen in vHMEC (Fig. 1, control or wild-type p16INK4A by Western blot analysis. We infected vHMEC after infection with retrovirus containing empty vector control or wild-type p16INK4A by Western blot analysis. We find vHMEC infected with retrovirus containing wild-type p16INK4A have decreased expression of both SUZ12 and EZH2 when compared with vHMEC infected with retrovirus containing empty vector (Fig. 1C). These determinations were made at a time point when vHMEC expressing wild-type p16INK4A were still engaged in the cell cycle (4 days), a time prior to the eventual proliferative arrest that they enter at day 7. Other CDK inhibitors at the CDKN2 locus, p14ARF and p15, are expressed in both HMEC and vHMEC (33) and data not shown.

We also assessed the expression of PcG proteins in human mammary fibroblasts (HMF). There is no expression of SUZ12 and EZH2 in HMF (Fig. 1C, parent). Furthermore, expression of PcG proteins cannot be induced in HMF infected with retrovirus containing shp16 (Fig. 1C), demonstrating that loss of p16INK4A activity is sufficient to drive overexpression of SUZ12 and EZH2 in mammary epithelial cells but not human mammary fibroblasts. Taken together, our results demonstrate that, in HMEC, expression of both SUZ12 and EZH2 can be directly influenced by loss of p16INK4A activity in a manner that is independent of proliferation status.

**Increased Expression of Selected PcG Proteins Is Accompanied by Targeted DNA Hypermethylation**—To determine whether p16INK4A-mediated up-regulation of PcG proteins is associated with DNA hypermethylation, we compared the methylation profiles of populations of primary human mammary cells using RLGS, a technique that allows the unbiased screening of genome-wide CpG island methylation events (45). We compared in vivo uncultured breast tissue and organoids, as well as in vitro cultured primary cells (HMEC and HMF) to vHMEC that exhibited overexpressed PcG proteins. The uncultured breast tissue, obtained by biopsy, and the paired organoid sample, partially processed to remove most stromal components and enriched in fibroblasts and epithelial cells, retain the epithelial cells in their physiological environment and allow us to detect potential culture effects. We propagated the in vitro cultures (HMEC, vHMEC, and HMF) from the isogenic organoids and analyzed them at points in the growth curves indicated by arrows in Fig. 2A. RLGS profiles for each sample were generated by digesting genomic DNA with NotI, followed by end-labeling, gel electrophoresis, further in-gel digestion and second-dimension gel electrophoresis. Comparison of RLGS profiles between vHMEC and the other samples identified twenty-four loci that were methylated only in vHMEC and not in HMEC, HMF, organoids, or breast tissue (Fig. 2B and supplemental Fig. S2). The differential pattern of CpG island methylation in vHMEC was non-random by statistical analysis (p < 0.01) (Fig. 2B), reminiscent of that observed in human tumors (45). Many of these loci were upstream of known genes, some encoding homeobox transcription factors (Fig. S2).

Of particular interest in this pattern was a locus, 3D33, which is critical in cell fate decisions and is silenced in breast cancer. This candidate locus (Fig. 2C; a NotI-EcoRV fragment; gb/AC004080: 44621–43088) was found by BLAST analysis to be located ~4-kb upstream of the HOXA9 transcriptional start site.
site, in a regulatory region that controls gene expression of the adult isoform of HOXA9 (4, 46). A comparison of RLGS data at the 3D33 locus (HOXA9) documents hypermethylation in vHMEC when compared with uncultured tissue samples taken by biopsy, organoids prepared from the same biopsy and finally, cultured HMEC and HMF propagated from the organoid sample (Fig. 2D, RM16). We analyzed mass populations of vHMEC as well as individual subclones. Early isolates of individual

**Figure 2D**

(A) Population doubling (PD) of vHMEC (red) compared to HMEC (black) over 150 days of culture.

(B) Methylation frequency at Loci 1 to 762 for RM16 tissue, organoid, fibroblast, HMEC, vHMEC.

(C) Section 3D of the tissue showing p16 expression.

(D) Summary of p16 and HOXA9 expression across different cell types.

(E) Schematic of gene expression and methylation at the 3D33 locus.
vHMEC subclones displayed heterogeneity in their hypermethylation status at the HOXA9 regulatory locus (Fig. 2D, RM240). For example, clones 240c1 and 240c5 exhibited hypermethylation at early passage (Fig. 2, D and E), while another, 240c3, acquired hypermethylation at a later passage (data not shown). Methylation-specific PCR analysis at the 3D33 region (using primers directed within the hypermethylated region) also documented DNA hypermethylation in vHMEC (Fig. 2B). In these cells the process of DNA hypermethylation must be selectively targeted because several other loci that are often found to be hypermethylated (e.g. BRCA1, E-CAD, IGFBP3) are not hypermethylated to any appreciable extent under these experimental conditions (data not shown). In this report we focus the remaining analyses on the epigenetic regulation of the HOXA9 regulatory region identified by RLGS.

To validate that the 3D33 locus is hypermethylated in vHMEC compared with HMEC, a genomic region was amplified by PCR using bisulfite-treated DNA templates derived from these samples. The region encompasses regulatory sequences upstream of the transcription start site of HOXA9 (MR1 (35CpGs): gb/AC004080: 43040–42674). Ten individual alleles were sequenced for each sample and the patterns were analyzed for CpG methylation (supplemental Fig. S3). Differential hypermethylation between vHMEC and their isogenic HMEC at the 3D33 locus occurred in each individual tested, particularly within the region containing a site that regulates transcriptional silencing (PRC2) of HOXA9 (Fig. 2E and Ref. 4). We observed some heterogeneity in the levels of DNA methylation among HMEC from nine different individuals analyzed by this method, with each HMEC sample displaying some basal DNA methylation (compare Figs. 2E and 4, A and B). The basal methylation detected in HMEC samples, that was also observed in the absence of cell culture (in organoid, Fig. 2E), was in contrast to the robust hypermethylation detected in the mass populations of vHMEC. Additionally, bisulfite sequencing also confirmed the DNA hypermethylation in two early passage vHMEC subclones (48c1 and 240c5) that had been observed by RLGS.

DNA Hypermethylation of the HOXA9 Regulatory Region Controls Transcriptional Expression—Whereas regulatory sequences upstream of the transcription start site of HOXA9 were hypermethylated in vHMEC, the methylation pattern of the region located at the HOXA9 proximal promoter did not change significantly between HMEC and vHMEC (supplemental Fig. S4). Most often, but not always (47), transcriptional changes correlate with hypermethylation at promoter sequences (48). To determine whether the DNA hypermethylation within the regulatory region (3D33) is associated with decreased expression of HOXA9, we performed quantitative real-time PCR (Taqman) analysis of HOXA9 expression levels. The HOXA9 gene has two alternative transcripts: one with a fetal expression pattern and the other predominantly expressed in adult tissues (46). Whereas the fetal transcript is not expressed in HMEC or vHMEC (data not shown), the adult transcript is modulated in these cells showing a 3-fold decrease of expression in vHMEC mass populations (48 and 240) compared with isogenic HMEC populations (Fig. 3A). We next assessed HMEC after infection with retrovirus containing empty vector or shp16. HMEC infected with retrovirus containing shp16 show a 3-fold decrease of expression of HOXA9 compared with control HMEC (Fig. 3A). This further suggests that loss of p16INK4A activity silences HOXA9.

Early passage subclones, 48c1 and 240c5, also exhibited a decrease in HOXA9 expression that correlated with their DNA hypermethylation status. These two subclones were used for further analysis of epigenetic regulation. To determine whether DNA methylation is necessary for silencing this locus, we exposed the subclones to the global demethylating agent 5-aza-dC for 72 h. HOXA9 transcript levels increase significantly upon drug treatment (Fig. 3B) as compared with sham treatment controls. As expected, the transcript levels of p16INK4A also increase (Fig. 3B). Given the unusual relationship of a methylated locus (p16INK4A) potentially controlling the methylation of a second locus (HOXA9), we needed a direct demonstration that 5-aza-dC treatment was modulating HOXA9 methylation and not working indirectly through the demethylation of p16INK4A. We found that vHMEC infected with retrovirus containing shp16 and subsequently treated with 5-aza-dC also demonstrated HOXA9 expression upon 5-aza-dC treatment. Hence, this demonstrates that the 5-aza-dC effect on HOXA9 is direct and is observed even when p16INK4A is still silenced (Fig. 3B and data not shown). The transcription of some genes can be further increased by combination treatments using both a DNA demethylating agent and the HDAC inhibitor, trichostatin A (49). In vHMEC, combination treatment with trichostatin A gives no additional increase in transcript levels of HOXA9 or p16INK4A (data not shown).

EphB4 is a downstream target of HOXA9 transcriptional activation (50) that is expressed in mammary epithelial cells (51). To assess the functionality of re-expressed HOXA9 protein, we utilized EphB4-Luc promoter constructs as a read-out. The vHMEC clone 48c1 transfected with CMV-driven EphB4-Luc promoter construct demonstrates a mean of ~12-fold activation following 5-aza-dC treatment (background <4-fold).
This activity is abrogated upon mutation of the HOXA9 binding site in EphB4-Luc (EphB4mt) or when 5-aza-dC is not added. Taken together, these results demonstrate that HOXA9 transcriptional repression in vHMEC is maintained by DNA methylation, and that functional protein can be re-expressed by treatment with a DNA-demethylating agent.

Targeted DNA Hypermethylation Is the Consequence of Transcriptional Silencing—This model system can be used to assess the causal and sequential relationship between p16\textsuperscript{INK4A} inactivation and DNA hypermethylation, as well as, the potential role of PcG proteins. To determine whether loss of p16\textsuperscript{INK4A} expression in HMEC is sufficient to acquire DNA hypermethylation at the HOXA9 regulatory region, we used...
bisulfite sequence analysis to assess HMEC after infection with retrovirus containing either shGFP or shp16. Whereas HMEC infected with retrovirus containing shGFP have levels of DNA methylation of HOXA9 similar to those in uninfected controls, HMEC infected with retrovirus containing shp16 show the acquisition of HOXA9 DNA hypermethylation (Fig. 4A).

To determine if the PcG proteins play a causal role in DNA hypermethylation, we examined DNA hypermethylation at the HOXA9 regulatory region in the presence or absence of small interfering RNAs to SUZ12 (siSUZ12). Strikingly, the acquisition of DNA methylation at HOXA9 was abrogated when siSUZ12 was added in addition to shp16. Under these conditions, as expected, HOXA9 expression was maintained (Fig. 4A). Thus, the inactivation of p16INK4A and ensuing increase of PcG proteins in HMEC facilitates DNA hypermethylation. In contrast, the inactivation of p16INK4A coupled with the specific absence of PcG protein SUZ12 in HMEC fails to facilitate DNA hypermethylation and gene silencing at this locus demonstrating that SUZ12 up-regulation is necessary for these processes. When HMF are infected with retrovirus containing a shp16, there is no increase in methylation at the HOXA9 locus (Fig. 4A). Because HOXA9 controls lactational differentiation and tubulogenesis, phenotypes not expressed in mammary fibroblasts, we would not expect these loci to be hypermethylated in fibroblasts.

To determine whether overexpression of PcG proteins is sufficient to acquire DNA hypermethylation at HOXA9, we infected HMEC with retrovirus containing an empty vector, an EZH2 cDNA or a combination of EZH2 cDNA and SUZ12 cDNA. When we infected HMEC with retrovirus containing a single PcG expression vector, EZH2 cDNA, we observed no DNA hypermethylation at the HOXA9 locus (Fig. 4B). However, upon infecting HMEC with retrovirus containing a combination of both EZH2 and SUZ12 cDNA, we observed the acquisition of DNA hypermethylation at the HOXA9 locus. HMEC overexpressing EZH2 alone or both EZH2 and SUZ12 together, expressed these proteins at levels observed in vHMEC (Fig. 4B). Taken together, these data demonstrate that loss of p16INK4A and up-regulation of specific PcG proteins play causal roles in the acquisition of DNA hypermethylation at the HOXA9 regulatory region in mammary epithelial cells. Specifically, this DNA hypermethylation is downstream of PcG-mediated transcriptional repression. Overexpression of EZH2 by itself is not sufficient to acquire DNA hypermethylation while overexpression of SUZ12 and EZH2 is both necessary and sufficient to activate DNA hypermethylation.

**SUZ12 and DNMTs Are Enriched at the HOXA9 Regulatory Region**—To determine whether silencing factors and DNA methyltransferases are located specifically at the HOXA9 regulatory region, we performed chromatin immunoprecipitation (ChIP) on mammary epithelial cells (Fig. 5) using primers for the HOXA9 regulatory region (Fig. 4A, MR2). We observed selective enrichment for DNA methyltransferases in vHMEC and HMEC infected with retrovirus containing shp16. Whereas the mRNA levels of DNMT1, an E2F target gene, are elevated in vHMEC, the mRNA levels of DNMT3a and DNMT3b are not elevated in vHMEC (data not shown). DNMT1 together with DNMT3a and DNMT3b were enriched at the HOXA9 regulatory region in vHMEC and HMEC infected with retrovirus containing shp16 (Fig. 5), strongly suggesting that DNA methyltransferases participate in the DNA hypermethylation at HOXA9 and act to lock-in the repressed transcriptional state. SUZ12 and 3mH3-K27 selective enrichment is observed in vHMEC and HMEC infected with retrovirus containing shp16, suggesting that the PRC2 complex binds to the same region as DNMT1, DNMT3a and 3b (Fig. 5). This further implicates PRC2 in targeting the DNA hypermethylation to HOXA9. As a control for a successful ChIP procedure, histone H3 enrichment is observed in both HMEC and vHMEC, whereas no enrichment is observed in mock (IgG) immunoprecipitated samples.

**Epigenetic Silencing of HOXA9 in Primary Breast Tumors**—To determine whether HOXA9 is epigenetically silenced in breast tumors, we assessed 46 cases of primary breast cancer and 9 normal breast tissue samples by immunohistochemical staining using anti-HOXA9 antibody. We observed robust HOXA9 staining in normal breast epithelial cells in 9 out of 9 normal tissue samples (Fig. 6A). In contrast, expression of HOXA9 is absent or abrogated in 43 out of 46 breast tumor samples (Fig. 6A). To determine whether the absence of HOXA9 expression in primary breast tumors correlates with DNA hypermethylation of the HOXA9 regulatory region, we assessed the DNA methylation status of a second set of 50 cases of primary breast tumors. Using methylation-specific PCR (MSP) primers to amplify bisulfite-treated genomic DNA, we observed that 22 out of 50 (44%) of the primary breast tumors analyzed showed evidence of DNA hypermethylation, whereas none (0/3) of the normal breast samples analyzed by this method showed DNA hypermethylation (Fig. 6B). This is likely to be under-representative of the number of tumors harboring HOXA9 DNA methylation, since primary tumors are heterogeneous in their tissue composition (diluting a tumor cell contribution) and our sampling of DNA methylation used one primer set. We then validated this DNA hypermethylation by using bisulfite sequencing of the HOXA9 regulatory region. The presence of DNA hypermethylation in tumors which showed methylation (T2) as assessed by MSP, and the minimal DNA methylation in unmethylated samples from normal breast (N2), or tumors which showed no DNA hypermethylation (T4), was validated upon sequence analysis in each of three cases (Fig. 6B). In many of these cases, HOXA9 DNA hypermethylation and silencing was found to be co-incident with SUZ12 overexpression and p16INK4A DNA hypermethylation (data not shown). Taken together, these results demonstrate that HOXA9 is expressed in normal breast tissue but undergoes epigenetic silencing due to DNA hypermethylation in a large proportion of primary breast tumors.

**DISCUSSION**

The p16INK4A locus has been extensively studied as a recipient of DNA hypermethylation but not as an upstream regulator of DNA hypermethylation activity. Classically, p16INK4A has been thought to participate in cell cycle control allowing cells to...
A

22%  
HMEC shGFP

65%  
HMEC shp16

50%  
HMEC shp16 siControl

12%  
HMEC shp16 siSUZ12

11%  
HMF vector

9%   
HMF shp16

B

MR1

16%  
HMEC pB-Hygro/ pB-Puro

51%  
HMEC pB-H-SUZ12/ pBP-EZH2

18%  
HMEC pBP-EZH2

HMEC

Vector EZH2

myc-EZH2

β-actin

HMEC

Vector SUZ12

SUZ12

β-actin
We present striking findings that, in breast epithelial cells, loss arrest proliferation in response to environmental stress signals.

SUZ12 and DNMTs are enriched at the HOXA9 regulatory region. Chromatin was extracted from HMEC, vHMEC, and HMEC infected with retrovirus containing shp16 (RM13), DSG, and formaldehyde cross-linked, and immunoprecipitated using antibodies against SUZ12 (Abcam), DNMT3a (Novus), DNMT3b (Abcam), DNMT1 (Lmmgenex), 3mH3-K27 (Upstate), histone H3 (Abcam), or rabbit IgG (mock). PCR was performed using ChIP template or no template (blank) with primers specific for (Upstate), histone H3 (Abcam), or rabbit IgG (mock). PCR was performed using primers specific for 3mH3-K27 linked, and immunoprecipitated using antibodies against SUZ12 (Abcam), with retrovirus containing shp16 (RM13), DSG, and formaldehyde cross-linking. Chromatin was extracted from HMEC, vHMEC, and HMEC infected with retrovirus containing shp16 together with transfection of siRNA targeting SUZ12 or empty vector. These data demonstrate that loss of p16INK4A and targeted downstream DNA hypermethylation is strictly dependent on the prior up-regulated expression of PcG proteins in primary mammary epithelial cells.

We find that the p16INK4A-dependent hypermethylation of the HOXA9 locus occurs within an upstream regulatory region 4 kb from the transcription start site. Other regulatory regions, upstream of promoter sequences, have been reported that regulate the expression of neighboring genes (52). However, most reports of DNA hypermethylation in cancer have investigated promoters or CpG islands within genes. Our use of RLGS, an unbiased screen of CpG islands genome-wide, highlights the point that regulatory regions are also modulated by this mechanism and should be included in global screening strategies. We expect that loss of p16INK4A drives DNA hypermethylation at a defined set of promoters and regulatory regions and are currently investigating other candidates.

These studies emphasize that cell context plays a pivotal role in determining the function of HOX genes in the differentiation networks of mammalian cells. HOX9 functions as a transcription factor acting as both an activator and repressor of a variety of genes in cell-specific patterns suggesting that the transcriptional effects of HOX9 are largely dependent on the cell context. Enforced HOX9 expression is leukemogenic in mice, and HOX9 is frequently activated in human acute myeloid leukemia (AML). Many HOX9 target genes are expressed in CD34+ stem cells or are members of gene families involved in proliferation or myeloid differentiation. These data suggest that HOX9 may mediate important biologic effects in normal and leukemic hematopoiesis (53). In striking contrast, the HOXA9 region is often silenced, either through deletion or hypermeth-
FIGURE 6. Epigenetic silencing of HOXA9 in breast cancer. A, immunohistochemical staining of normal breast epithelia and primary breast tumors with anti-HOXA9 antibody. Representative examples are shown from 46 breast tumor specimens and nine normal breast specimens. B, representative examples are shown of MSP analysis from 50 breast tumor (T) and 3 normal breast (N) DNA samples, using primer sets that specifically amplify either methylated (m) or unmethylated (u) DNA. Control templates from human genomic lymphocyte DNA either treated with SssI methylase (+) or untreated (−) are shown. Bisulfite-treated DNA was sequenced at the HOXA9 regulatory region from three samples in each of three cases. Methylated CpGs (closed circles) and unmethylated CpGs (open circles) are shown for representative examples of 10 alleles for each sample: DNA from normal tissue (N2), tumor DNA showing HOXA9 methylation (T2) and tumor DNA showing unmethylated HOXA9 (T4).
ylation, in several tumor types (this report and Ref. 54) suggesting that in these tissues HOXA9 plays a suppressive, rather than oncogenic, role in tumorigenesis. In these tissues hypermethylation of HOXA9 is associated with cancer (this report and Ref. 54) and decreased survival (54). The function of HOXA9 itself in mammary cells provides an excellent example of a novel mechanism by which p16INK4A inactivation may contribute to malignant transformation in breast cancer. In mammary epithelial cells, HOXA9 acts as a positive regulator of terminal differentiation and its expression is important for cell fate determination (22). We hypothesize that the up-regulation of PcG proteins and the targeted down-regulation of expression of HOXA9 by DNA hypermethylation inhibits terminal differentiation through aberrant silencing of a homeobox gene and promotes the maintenance of a more progenitor-like phenotype. These studies predict that HOXA9 functions as a tumor suppressor gene in mammary epithelial cells.

Mouse models hint at a role for p16INK4A pathways in controlling the progenitor-differentiation switch in mammary tissue. In transgenic MMTV-cyclin D1 mice, overexpression of cyclin D1 prevents normal mammary terminal differentiation by abating the p16INK4A pulse that usually decreases cyclin D1 levels and represses E2F target genes (55). In mammals, BMI-1-mediated repression of p16INK4A activity has been shown to be necessary for generating pluripotent hematopoietic and neural stem cells (56, 57). BMI-1-null mice fail to suppress p16INK4A expression resulting in the absence of stem cells, thus firmly establishing an important, but largely undefined role for p16INK4A in cell fate processes (58). In Drosophila, the up-regulation of E(z), the homolog of EZH2, is critical in maintaining epigenetic patterns of pluripotent stem cells. Furthermore, in undifferentiated myoblasts, increased EZH2 expression inhibits differentiation, dictating the decision between progenitor and differentiated states (36). Importantly, the vHMEC used in our RLGS analysis exhibit overexpression of BMI-1 (data not shown) and p16INK4A inactivation, providing us with a physiologically relevant system for precisely defining the role of p16INK4A in the epigenetic program of primary epithelial cells.

Morphologically normal foci of epithelial cells exhibiting p16INK4A inactivation have been found in several tissues (24–26) and may be precursors to cancer. Our previous work demonstrates that cells lacking p16INK4A activity exhibit phenotypes associated with malignancy (18, 33). The acquisition of genomic instability and the activation of stress pathways such as COX-2 provide these cells with the mutagenic potential to survive adverse environments as well as the ability to migrate, evade apoptosis and immune surveillance, and summon sustaining vasculature (28). Examination of archived tissue from women with DCIS (ductal carcinoma in situ) reveals epithelial cells that over-express markers of premalignant stress activation pathways and mirror the distinctive expression patterns of these markers observed in vitro (34). These epithelial cells are found within the premalignant lesion as well as in the field of morphologically normal tissue that surrounds the lesion. Here, we show p16-dependent epigenetic silencing of HOXA9 and extend this to primary breast tumors in vivo, implying an important role for HOXA9 in breast carcinogenesis. The present work suggests that cells lacking p16INK4A activity also induce critical activities that allow cells to evade differentiative processes that would be expected to terminate proliferation. All of these properties are critical to malignancy. PcG proteins and/or targeted DNA hypermethylation events may be useful biomarkers to detect the earliest events in breast cancer.

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