Chromatin Remodeling in Mammary Gland Differentiation and Breast Tumorigenesis

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DNA methylation and histone modifications have essential roles in remodeling chromatin structure of genes necessary for multi-lineage differentiation of mammary stem/progenitor cells. The role of this well-defined epigenetic programming is to heritably maintain transcriptional plasticity of these loci over multiple cell divisions in the differentiated progeny. Epigenetic events can be deregulated in progenitor cells chronically exposed to xenoestrogen or inflammatory microenvironment. In addition, epigenetically mediated silencing of genes associated with tumor suppression can take place, resulting in clonal proliferation of undifferentiated or semidifferentiated cells. Alternatively, microRNAs that negatively regulate the expression of their protein-coding targets may become epigenetically repressed, leading to oncogenic expression of these genes. Here we further discuss interactions between DNA methylation and histone modifications that have significant contributions to the differentiation of mammary stem/progenitor cells and to tumor initiation and progression.

In the breast, a subpopulation of cells located in the basal layer of terminal end ducts have been implicated as stem cells (Dontu et al. 2003). A study using serial transplantation of this cell population in mice unequivocally showed their stem/progenitor functions (Stingl et al. 2006). These slowly dividing cells have the capacity of self-renewal and, in response to hormonal stimuli, give rise to transient populations that may undergo terminal differentiation into different epithelial lineages (Péchoux et al. 1999; Gudjonsson et al. 2002; Dontu et al. 2004, 2005; Villadsen et al. 2007). Differential

transcriptional programs are used to maintain lineage specification and homeostasis of differentiated cells. Estrogen receptor α (ER α)-negative progenitors usually differentiate into myoepithelial cells, which form the basal layer of mammary ducts whereas other progenitors give rise to luminal epithelial cells, some of which appear to be ER α -positive (Dontu et al. 2004, 2005).

The maintenance of stem/progenitor cells and their differentiation fate follows a well-defined epigenetic program. Without altering nucleotide sequences or DNA copy-number,

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the event heritably transmits transcriptional information from progenitors to their differentiated progeny. This epigenetic program is coordinately regulated by DNA methylation, histone modifications, and noncoding RNAs (Jaenisch and Bird 2003; Jones and Baylin 2007; Feinberg 2008; Suzuki and Bird 2008; Cedar and Bergman 2009; Davalos and Esteller 2009; Guil and Esteller 2009). Increasing evidence has indicated that spatiotemporal interactions among different epigenetic components play essential roles in modulating transcriptional activation or deactivation. In a permissive environment, the chromatin structure of a target locus exists in an open conformation that allows transcription factors and coregulators to bind to their cognate sequences and initiate transcription (Cairns 2009). In a nonpermissive environment, these loci are inaccessible to these transcription factors, resulting in transcriptional silencing. DNA methylation and histone modifications are known to regulate this transcriptional plasticity, which is necessary for normal development and maintenance of mammary epithelial lineages (Dontu et al. 2005; Bloushtain-Qimron et al. 2009). Deregulation of these epigenetic programs may result in clonal proliferation of transformed progenitor cells, also called cancer-initiating cells. Subtypes of these aberrant cells may develop into diverse groups of epithelial tumors, including luminal A and B (ER α -positive), basal (ER α -negative), ERBB2-overexpressing, and normal-like phenotypes (Sorlie et al. 2001).

In this article, we discuss the importance of DNA methylation and histone modifications in modulating chromatin structure for gene transcription. The emerging role of noncoding RNA in cell differentiation and neoplasm is discussed in a preceding article. Potential uses of DNA methyltransferases and histone deacetylase inhibitors in epigenetic therapies are extensively reviewed elsewhere (Balch et al. 2005; Lo and Sukumar 2008; Batty et al. 2009). Here we focus on describing the contributions made by DNA methylation and histone modifications to mammary epithelial differentiation. We also highlight the significance of epigenetic deregulation in breast tumorigenesis.

DNA METHYLATION

Methylation Variations in Normal Tissues and Mammary Epithelial Subtypes

The human genome contains 28 million CpG dinucleotides (Table 1). Approximately 10% (2 million) of these dinucleotides are primarily distributed in GC-rich repetitive sequences (e.g., ribosomal DNA and α satellites) and single-copy CpG islands (Antequera and Bird 1993, 1999). These CpG islands (1 to 2-kb) are usually associated with protein-coding genes or noncoding RNAs, with the majority located in the promoter regions (70%) and less within intergenic regions (10%) (Jaenisch and Bird 2003; Jones and Baylin 2007). These GC-rich sequences are targets for enzymatic modifications by DNA methyltransferases (DNMTs), which add a methyl group to the fifth position of cytosine of a CpG dinucleotide and convert it into methylcytosine. De novo DNA methylation is usually carried out by DNMT3A and DNMT3B whereas maintenance of methylation is mediated by DNMT1 (Bestor 2000). Recent evidence suggests that DNMT3A and DNMT3B also cooperate with DNMT1 for the maintenance function, which serves to transmit methylated DNA patterns from parental to daughter cells (Jones and Liang 2009).

Table 1. Information of CpG dinucleotides in the human genome^a

Total number of CpG dinucleotides	28,340,937
CpG dinucleotides within CpG islands	2,107,414
CpG dinucleotides outside of CpG islands	26,233,523
Total number of CpG islands	27,934
CpG islands within ± 1 kb TSS	14,909
Gene-associated CpG island outside of ± 1 kb TSS	7,093
CpG islands not associated with known genes	5,932
Total bp covered by promoter CpG islands	13,992,078
Total bp covered by gene-body CpG islands	3,700,617
Total bp covered by intergenic CpG islands	3,663,451

^aBased on the UCSC Human Genome Assembly hg18.

Repetitive sequences, such as *Alu*, *LINE*, and α satellites, are usually methylated in the normal genome (Jaenisch and Bird 2003; Jones and Baylin 2007). This acquisition of DNA methylation may act to repress the transcription or retrotransposition of repetitive sequences. However, CpG islands near the promoters of genes, with the exception of imprinted and inactive X-chromosome loci, are thought to remain unmethylated in the genome (Jaenisch and Bird 2003; Jones and Baylin 2007). This prevailing notion that CpG islands are normally “protected” from DNA methylation has been challenged by detailed studies on candidate loci or global screening of the genome (see examples in Table 2 for different technologies used for methylation analysis). Together, the findings indicate that normal methylation could occur in 5%–15% of human CpG islands not associated with imprinting or X-inactivation (Weber et al. 2005; Shen et al. 2007; Weber et al. 2007). In this regard, tissue-specific DNA

methylated regions are present in the genome and have been proposed to repress transcripts during cell differentiation and organogenesis (Futscher et al. 2002; Ohgane et al. 2005; Brena et al. 2006b). This differential methylation may contribute to phenotypic variations of individuals, most dramatically shown in studies of monozygotic twins (Fraga et al. 2005). DNA methylation also changes subtly in normal aging cells, with a progressive loss of methylation content in repetitive sequences, but a progressive increase of density in selected CpG islands (Issa et al. 1994; Issa 2003).

Cell type-specific methylation patterns are similarly found in the mammary gland. Polyak and colleagues (Bloushtain-Qimron et al. 2008, 2009) used the methylation-specific digital karyotyping (MSDK) method to determine DNA methylation profiles in subtypes of mammary epithelial cells. Based on the characteristics of cell surface markers, four different types—myoepithelial (CD10+), luminal epithelial

Table 2. Summary of candidate gene and genome-wide techniques for DNA methylation analysis^a

Categories	Techniques
Candidate gene, cloning, and gel-based approaches	Bisulfite sequencing; MSP (methylation-specific PCR); COBRA (combined bisulfite restriction analysis); MethyLight ; Pyrosequencing ; MassARRAY ; MS-AP PCR (methylation-sensitive arbitrarily primed PCR) MSRF (methylation-sensitive restriction fingerprinting); MS-RDA (methylation-sensitive representational analysis); and RLGS (restriction landscape genome scanning)
Microarray-based approaches	DMH (differential methylation hybridization); MTA (methylation tissue array); MSO (methylation-specific oligonucleotide); HELP (<i>HpaII</i> tiny fragment enrichment by ligation-mediated PCR); AIMS (amplification of intermethylated sites); MSNP (methylation single nucleotide polymorphism chip-based method); MMASS (microarray-based methylation assessment of single sample); PMAD (promoter-associated methylated DNA amplification); MSDK (methylation-specific digital karyotyping) MIAMI (microarray-based integrated analysis of methylation); MCAM (methylated CpG island amplification and microarray); MeDIP-chip (methylated DNA immunoprecipitation on microarray); MIRA-chip (methylated-CpG island recovery assay on microarray); McrBC ; MethylScope ; Pharmacologic unmasking analysis ; and Infinium BeadArray
Next generation sequencing	RRBS (Reduced representation bisulfite sequencing); BS-seq ; MethylC-seq ; Padlock probes coupled with whole-genome bisulfite sequencing; MRE-seq ; HELP-seq ; MeDIP-seq ; and MIRA-seq

^aDescriptions of these techniques are provided in review articles (Balch et al. 2008; Brena et al. 2006b; Chang et al. 2008; Zuo et al. 2009).

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(CD24+ and MUC1+), and progenitor (CD44+) were isolated for methylation profiling (Bloushtain-Qimron et al. 2008). CD44+ cells display stem/progenitor characteristics and, on signaling activation, undergo lineage-specific differentiation. Genes important for pluripotency and self-renewal were hypomethylated and highly expressed in CD44+ cells compared with the other three differentiated cells (Bloushtain-Qimron et al. 2008). Examples are *SUZ12* gene targets in which promoter methylation was found in CD24+ cells, but not in CD44+ cells. *SUZ12*, a member of polycomb-repressor complex 2, is known to regulate the transcription of genes essential for maintaining the pluripotency and self-renewal of stem/progenitor cells (Lee et al. 2006; Pasini et al. 2007). Acquired DNA methylation may result in permanent silencing of *SUZ12* targets

during mammary epithelial differentiation (see Fig. 1). Epigenetic control of gene silencing, marked by promoter methylation, therefore is an essential step of mammary epithelial differentiation.

DNA Methylation Alterations in Transformed Breast Epithelial Cells

It has been hypothesized that mammary stem/progenitor cells are primary targets for cellular transformation. Compared to terminally differentiated cells that have a high turnover rate, these cells have a long life span and are slowly dividing within the stem/progenitor compartment (Dontu et al. 2003; Cheng et al. 2008). These primitive cells are therefore susceptible to molecular injuries because of persistent exposures to different environmental or

Figure 1. Model of mammary gland differentiation and differential accumulation of DNA methylation in normal and transformed epithelial cells. A hierarchy of stem/progenitor cells, analogous to that in the hematopoietic system, may exist in mammary gland. Stem cells undergo self-renewal and differentiation within their compartment. The primitive progenitor cells give rise to myoepithelial and luminal progenitors, which then differentiate into different epithelial subtypes (Note: other cell types, such as alveolar epithelial cells, are omitted in this model). Acquired DNA methylation in promoter CpG islands occurs in genes required for differentiation of progenitor cells. The resulting epigenetic repression (marked in “green” and “yellow” lollipops for myoepithelial and luminal epithelial cells, respectively) is also essential for maintaining the homeostasis of mature epithelial cells. Deregulation of differentiation signaling may disrupt this epigenetic programming (e.g., aberrant acquisition of promoter methylation marked in “red” lollipops), leading to neoplastic transformation and clonal proliferation.

inflammatory stimulants (Fenton 2006; Yager and Davidson 2006; Cheng et al. 2008). Environmental chemicals, such as xenoestrogens, can mimic estrogenic actions that aberrantly influence epithelial differentiation in mammary glands (Cheng et al. 2008; Hsu et al. 2009). The action is mediated in part through nuclear hormone receptors (e.g., ER α) that activate or deactivate the transcription of target genes (Cheng et al. 2008; Hsu et al. 2009). Alternatively, xenoestrogens may stimulate phosphorylation of membrane-bound proteins, which subsequently activate different kinase signal transduction pathways that are required for transcriptional regulation (Jensen and Jordan 2003).

Persistent exposure to these environmental chemicals therefore increases breast cancer risk. Animal and epidemiologic studies suggest an imprinting phenomenon in which early exposure to xenoestrogens may promote a carcinogenic process observed later in adult life (Fenton 2006; Maffini et al. 2006). Specifically, long-term exposure to low-dose xenoestrogens may alter epigenetic reprogramming of stem/progenitor cells during epithelial differentiation (Ho et al. 2006; Cheng et al. 2008). This molecular alteration may deregulate transcriptional programs important for epithelial differentiation. Repressive chromatin is subsequently established and sets the stage for heritable silencing of target genes (Cheng et al. 2008; Hsu et al. 2009). De novo DNA methylation occurs and gradually spreads from pre-existing methylated CpG sites to neighboring areas in the genome (Cheng et al. 2008; Hsu et al. 2009). These methylated sites may serve as seeds to propagate the repressive information to the core regions of targeted CpG islands. A ChIP-chip analysis in breast cancer cells using antibodies against methyl-CpG binding domain proteins, which bind like “magnets” to methylated cytosines, identified a large number of hypermethylated CpG islands (Ballestar et al. 2003). More recently, using the methylation-dependent immunoprecipitation on microarray (MeDIP-chip, see Table 2) approach, our study found that $\sim 0.5\%$ of human CpG islands become hypermethylated in epithelial cells derived from estrogen-exposed mammary

progenitors compared with the nonestrogen control cells in an in vitro model (Cheng et al. 2008). Ontological analysis revealed significant methylation enrichment in CpG islands of polycomb protein-regulated targets, which are known to regulate pluripotency and self-renewal of stem/progenitor cells (Bracken et al. 2006).

This methylation alteration may precede morphologic transformation of normal breast epithelia and is an early event of tumorigenesis. As a result, a large field defect of premalignant cells is generated in an affected breast area (Fig. 2). Additional accumulation of genetic and epigenetic damages is needed in the affected area to generate invasive carcinomas. Methylation alterations have frequently been found in histologically normal breast tissues adjacent to primary tumor sites (Yan et al. 2003, 2006). Overall methylation levels of two candidate loci, *RASSF1A* and *RUNX3*, are significantly higher in these adjacent tissues compared to control samples obtained from individuals undergoing reduction mammoplasties (Yan et al. 2006; Cheng et al. 2008). These methylation levels, however, are lower than those of primary tumor sites, and there is a gradient pattern

Figure 2. Model of epigenetic field cancerization occurring in the human breast. Depicted here is a large affected area of methylation gradient in the upper-left side of the breast. Chronic exposures to xenoestrogens may alter epigenetic programs of normal differentiation. Methylation accumulation of promoter CpG islands takes place in silenced genes associated with neoplastic development. A tumor site may serve as the epicenter (depicted in red) from which the highest number of methylated CpG islands and the highest density of methylation within an island are observed in the breast.

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in which higher degrees of methylation are seen in normal tissues closest to the tumor site compared to those which are 2–4 cm away (Yan et al. 2006; Cheng et al. 2008).

DNA Hypermethylation in Epithelial Breast Tumors

Increased methylation of promoter CpG islands and increased numbers of methylated islands are associated with tumor progression (Nephew and Huang 2003; Novak et al. 2009). From a geographic perspective, a tumor site can serve as the “epicenter center” from which a maximum accumulation of promoter methylation can take place in the human breast (see Fig. 2) (Yan et al. 2006; Cheng et al. 2008). These methylation accumulations are known to contribute to different stages of neoplastic development, including initiation, invasion, metastasis, and endocrine resistance. As of today, candidate gene and global screening approaches have now identified >150 loci frequently hypermethylated in breast cancer, including genes encoding for cell cycle regulation (e.g., *p16^{INK4a}*, *p15*, and *p14^{ARF}*), DNA repair (e.g., *MLH1* and *GST3*), tumor suppression (e.g., *BRCA1* and *VHL*), tissue remodeling (e.g., *TIMP3* and *E-cadherin*), and hormone receptor (e.g., *ESR1* and *ESR2*) (Tables 3 and 4). In addition to protein-coding genes, *microRNAs* with tumor and metastasis suppressor functions also undergo CpG island methylation-associated silencing in transformed cells (Saito et al. 2006; Lujambio et al. 2007, 2008; Hsu et al. 2009).

Methylation-mediated silencing of these loci may contribute, in part, to the development of malignant phenotypes. For example, hypermethylation of CpG islands in the *p16^{INK4a}*, or *CDKN2A*, gene is known to be associated with cell cycle deregulation in breast cancer cells (Herman et al. 1995). This gene normally encodes a cyclin-dependent kinase which is responsible for the maintenance of the retinoblastoma protein in an active state for transcriptional regulation of downstream targets (Kamb et al. 1994). Lack of the *p16^{INK4a}* expression promotes aberrant proliferation of primary mammary epithelial cells that normally undergo

senescence in vitro (Huschtscha et al. 1998; Hinshelwood and Clark 2008; Hinshelwood et al. 2009). This epigenetic silencing may acquire additional genetic and epigenetic changes in cancer cells (Romanov et al. 2001; Tlsty et al. 2001). Specifically, the *p16^{INK4a}* silencing results in the up-regulation of polycomb repressors, EZH2 and SUZ12, which are known to recruit DNA methyltransferases to target genes leading to de novo methylation of their promoters (Reynolds et al. 2006).

Another gene that undergoes methylation-mediated gene silencing is *BRCA1* and this event disrupts cellular functions which are required for DNA repair, protein ubiquitination, and chromatin remodeling (Narod and Foulkes 2004). Inactivation of one *BRCA1* allele, i.e., the first hit of Knudsen’s hypothesis, is attributed to genetic mutations or loss of heterozygosity in breast neoplasm (Meric-Bernstam 2007). Hypermethylation of *BRCA1* may be a second hit during breast tumorigenesis (Birgisdottir et al. 2006) and in invasive carcinomas it is frequently associated with shorter patient survival (Xu et al. 2009).

In addition to single-copy genes, hypermethylation occurs in 18S and 28S ribosomal genes in breast cancer (Yan et al. 2000). Approximately 400 copies of ribosomal loci, arranged in “head-to-tail” arrays, are located on the short arms of human acrocentric chromosomes. The transcription domain of these loci displays sequence characteristics of a CpG island. Increased methylation in these repetitive GC-rich sequences may result in down-regulation of ribosomal gene expression in breast cancer cells (Yan et al. 2000; Chan et al. 2005). Because this aberrant event is preferentially found in undifferentiated and ER α -negative tumors, hypermethylation of ribosomal DNA can be an additional prognostic biomarker for breast cancer (Yan et al. 2000).

DNA Hypomethylation in Epithelial Tumors

Global hypomethylation is frequently observed in cancer cells because of a deficiency in the production of S-adenosylmethionine (SAM) (Ehrlich 2002, 2006). SAM is the principal methyl

Table 3. Summary of genes commonly hypermethylated or hypomethylated in breast cancer^a

Functions	Genes
Angiogenesis	<i>CXCR4, HIF1A, IL2, IL10, NOS3, and VEGF</i>
Apoptosis	<i>APAF1, BAK1, BAX, BIM, BME, BCL2, BCL2L1, BCL2L2, CASP8, DR4, DR5, FADD, MCL1, SFRP1, PYCARD, TRAIL, and XIAP</i>
Binding protein	<i>CEBPD, FABP3 (MDGI), GATA3, GNAL, HRAS, IGFBP3, IGFBP7, LDLRAP1, LRP2, PRKDCB, and SOCS1</i>
Cell cycle regulation	<i>CCNA1, CCNA2, CCND1, CCND2, CDKN1A, CDKN1B, CDKN1C, CDKN2A, CTPS, DBC1, DNAJC15, GADD45, EPB41L3, GREM1, IGF2, RAD9A, SFN, RNR1, SCGB3A1, and TYMS</i>
Proteases, transcriptases, and enzymes	<i>KLK6, KLK10, hTERT, ABL1, AK5, AKT1, DAPK, FLT3, RAF1, TEK, PTGS2, CYP1B1, HS3ST2, HSD17B4, GSTP1, NAT1, SAT2, SULT1A1, SYK, TDH, and WRN</i>
DNA repair/detoxify carcinogen	<i>MGMT, MLH1, PRKDC, RPA2, TYMS, XRCC5, and XRCC6</i>
Enhancers and transcription factors	<i>F0XA2, H0XA5, H0XD11, IRF7, ID4, MY0D1, PAX5, PAX6, P0U3F1, RUNX3, SIM1, SIM2, TWIST1, and WT1</i>
Hormone receptors and kinases	<i>AR, CALCA, EPHA3, EPO, EGFR, ERBB2, ESR1, ESR2, GALR2, LTB4R, PGR, RARB, STK11, TGFB1, TGFB2, TNFRSF12, and UPA</i>
Immune responses	<i>CsADMI (TSLC1), CD40, CD44, CD80, CD86, and ICAM1</i>
Invasion/metastasis suppressors	<i>COL9A1, MMP2, MMP9, MT1A, PLS3, RECK, ROBO1, S100A4, SLIT2, THBS1, TIMP1, TIMP2, TIMP3, TJP2, TPM1, and VCAN</i>
Transporters, glycoproteins, and membrane-associated proteins	<i>ABCB1, AMN, CDCP1, CDH1, CDH3, CDH13, CFTR, DSC3, EDNRB, GJB2, GPC3, LAMA3, LAMB3, LAMC2, MUC2, NEFL, PCDH10, RARRES1, RBP1, TMEFF2, TSPAN2, SLC5A5, and SLC6A20</i>
Tumor suppressors	<i>APC, BRCA1, CAV1, CST6, DAB2, DCC, DLC1, FHIT, GSN, H19, HIC1, LATS1/LATS2, PLAGL1, PTEN, RASSF1A, RBL1, RBL2, PRDM2, SNCG, SERPINB5, TP53, TSC1, TSC2, TUSC3, VHL, and WIF1</i>
Tumor antigens	<i>ABO, MAGEA1, and MME</i>

^aSee also www.pubmeth.org

donor for DNA methylation reactions in normal cells. As a result of this deficiency in cancer cells, hypomethylation of repeat elements (e.g., *Alu*, *LINE*, and α satellites) occurs, contributing to reactivation of transposable elements, and the promotion of chromosomal translocation,

deletion, and duplication, as well as genomic instability (Ehrlich 2002, 2006). This aberrant epigenetic event has been known to correlate with advanced clinical stage, tumor size, and histological grade in breast cancer (Soares et al. 1999). Several lines of investigation also

Table 4. Analysis of histone modifications and nucleosome positioning using candidate gene and global approaches^a

Category	Examples
Histone modifications	ChIP-qPCR or -cloning (chromatin immunoprecipitation followed by quantitative PCR of candidate genes or cloning); ChIP-PET (ChIP-paired-end-tagging); ChAP (ChIP coupled to arbitrarily primed PCR); SACO (serial analysis of chromatin occupancy); ChIP-seq (ChIP coupled to massively parallel sequencing); and Mass spectrometry
Nucleosome positioning	Nuclease protection assay ; DNase-chip or -seq (mapping of DNase I hypersensitive sites on microarray or with massively parallel sequencing); and Micrococcal nuclease array

^aDescriptions of these techniques are provided in review articles (Lo and Sukumar 2008; Balch et al. 2008; Wu et al. 2006).

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indicate hypomethylation of CpG islands may contribute to loss of imprinting and reactivation of testis-antigen related genes (Cho et al. 2003; De Smet et al. 2004; Kaneda and Feinberg 2005). Promoter hypomethylation may be linked to reactivation of proto-oncogenes (e.g., *synuclein* γ , *ID4*, and *annexin A4*) that are associated with tumor metastasis and endocrine resistance (Gupta et al. 2003; Pakneshan et al. 2004; Fan et al. 2006). In some cases, hypo- and hyper-methylation of the same CpG island co-exist in advanced tumors. This heterogeneity has been found in the *E-cadherin* promoter, suggesting that, in breast cancer cells, DNA methylation is a dynamic and plastic event which accompanies progression to a metastatic state (Graff et al. 2000).

Hypomethylation of promoter CpG islands may require a functional DNA demethylase yet to be characterized in cancer cells. In this case, a demethylation event is supposed to occur in an inactive, methylated promoter, leading to transcriptional reactivation of an oncogene. However, experimental proof for genuine promoter hypomethylation is frequently inconclusive and difficult to obtain because the outgrowth of a subpopulation of cancer cells may confound this epigenetic observation. For example, the oncogene of interest may have never been silent in a minor population of cancer-initiating cells while the majority of other cells display promoter hypermethylation of the gene. The increased expression of this oncogene may simply result from rapid expansion of these few cells that eventually take over the whole population during tumor progression. If this scenario indeed occurs, it cannot be a bona fide demethylating event for oncogene activation. Alternatively, active demethylation may be mediated by the 5-methyl-CpG binding domain protein 4 (MBD4) (Zhu 2009). Though MBD4 functions as a T-G mismatch glycosylase, it has recently been found to induce active demethylation of methylated CpG sites (Kim et al. 2009; Zhu 2009). MBD4 mutant with a deletion in the glycosylase catalytic domain abrogates this demethylating ability (Kim et al. 2009). Clearly, elucidating the functions of MBD4 in promoter hypomethylation during

mammary gland differentiation and breast tumorigenesis is an important area in need of further investigation.

HISTONE MODIFICATIONS

Combinatorial Variations Associated with Gene Transcription

Histone modification is another epigenetic mechanism important for chromatin remodeling during stem/progenitor differentiation. The basic subunit of chromatin is the nucleosome, a complex consisting of 146 bp of genomic DNA wrapped around a core histone octamer composed of one histone H3-H4 tetramer and two histone H2A-H2B dimers (Kouzarides 2007). The histone amino-terminal “tails” extending from a nucleosome octamer are frequently subject to posttranslational modifications, including acetylation, phosphorylation, ubiquitination, and methylation. Among these various modifications, histone methylation and acetylation are shown to be relatively stable and therefore considered potential marks for carrying epigenetic information through cell divisions (Barski et al. 2007; Wang et al. 2008). The sum total of these covalent alterations is referred to as the histone code that can be “written” by histone modifying enzymes (e.g., acetyltransferases, deacetylases, methyltransferases, or demethylases) and read by various binding proteins that act to further modify chromatin structure (Wang et al. 2009). Among these proteins, EZH2, a known component of polycomb repressor complex, can cooperatively work with histone deacetylases and methyltransferases to modify histone tails for initiating transcriptional repression. EZH2 also works with DNA methyltransferases for subsequent maintenance of gene silencing (Sparmann and van Lohuizen 2006). Table 4 lists techniques for analysis of histone modifications and chromatin structure of active and inactive genes.

Both packaging of DNA into chromatin and nucleosome positioning influence gene transcription by either enhancing or inhibiting the accessibility of general and site-specific transcription factors to target loci. In general,

acetylation of histone lysine residues is associated with open chromatin structure and active transcription whereas the methylation of these residues can be associated with either an active or repressive chromatin structural conformation (Fig. 3) (see examples in Table 5). For example, active transcription of a promoter can be marked by acetylation at specific lysine residues of histone H3 (e.g., lysine 5, 8, and 12), histone H4 lysine 16, and dimethylation of histone H3 lysine 4. In contrast, when genes are silenced, these active marks are replaced by repressive marks, including

mono-, di-, and tri-methylation of H3K9, H3K27, and H4K20. Combinatorial epigenetic alterations likely mark differential degrees of gene silencing, starting from a transient to a more rigid state of repression.

When binding target sequences in the chromatin, transcription factors may act as docking molecules for recruitment of other DNA- and histone-modifying proteins to target genes for transcriptional regulation (Jenuwein and Allis 2001). For example, Wang and coworkers (Ko et al. 2008) showed that YY1 physically interacts with SUZ12 and acts as a mediator in the recruitment of polycomb group proteins

Figure 3. Models of open or closed chromatin configurations associated with gene transcription status. Binding of regulatory factors and their co-factors to a target locus results in the formation of either an activator or repressor complex. This complex may act as docking molecules for recruitment of acetyltransferases, deacetylases, methyltransferases, or demethylases that post-translationally modify the amino-terminal tail of histone. Modified histone marks are associated with specific transcription status of a target gene. Active transcription is usually associated with histone acetylation of H3K9 or methylation of H3K4 residues, whereas gene repression is linked to methylation of H3K9, H3K27 and H4K20 residues.

Table 5. Representative examples of histone tail methylation and its associated genomic functions^a

Histone methylation mark	Function	Genomic location
H3K4me1	Active	TSS, enhancer
H3K4me2	Active	TSS
H3K4me3	Bivalent	TSS, enhancer
H3K9me1	Active	TSS
H3K9me2	Repressive	10-kb of TSS
H3K9me3	Repressive	10-kb of TSS
H3K27me1	Active	Downstream of TSS
H3K27me2	Repressive	—
H3K27me3	Bivalent	Promoter, gene-body
H3K36me1	Active	TSS
H3K36me3	Active (?)	Downstream of TSS
H3K79me1	No preference	—
H3K79me2	No preference	—
H3K79me3	Repressive	Promoter, gene-body
H3R2me1	Modestly active	—
H3R2me2	Modestly active	—
H4K20me1	Active	Downstream of TSS
H4K20me3	No preference	—
H2A+H4R3me2	No preference	—
H2BK5me1	Active	Downstream of TSS

^aSee a publication by Zha and colleagues (Barski et al. 2007). K: lysine; R: arginine; TSS: transcription start site.

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and DNA methyltransferases that promote transcriptional repression. Several studies have found that open chromatin regions are usually correlated with gene-dense regions and that closed chromatin regions exist in both euchromatic G-bands and heterochromatic C-bands (Gilbert et al. 2004; Weil et al. 2004; Crawford et al. 2006). These studies also show active genes can exist within closed chromatin domains.

Histone Modifications in Normal Epithelial Differentiation and Breast Tumorigenesis

The fact that somatic cells possess an identical genome, yet display diverse phenotypes, can partly be attributed to combinatorial variations of histone modifications that are functionally associated with gene transcription (Balch et al. 2007). Recent advances in technologies have allowed for the examination of global histone modifications within a cell, and this accomplishment has provided insight into the organization of the genome (Balch et al. 2008). In human tumors, modifications of histone H4 generally result in a loss of monoacetylated K16 and trimethylated K20 forms (Fraga et al. 2005). These histone changes are associated with DNA hypomethylation in various tumor types, including breast cancer (Fraga et al. 2005; Tryndyak et al. 2006). More recently, Zhao and colleagues (Barski et al. 2007) have comprehensively examined 20 histone marks, RNA polymerase II, and other binding proteins across the human T-cell genome. This study found that combinatorial histone variations contribute to the maintenance of transcriptional plasticity and to the fate of specific cell lineages during differentiation. In a separate study, Bernstein and co-workers (Mikkelsen et al. 2007) constructed global chromatin maps for mouse embryonic stem cells (ESCs) and their differentiated cells. A large number of genes important for differentiation were found to possess a “bivalent” feature, consisting of post-translational histone modifications commonly found in transcriptionally active (H3K4me2) and a repressive (H3K27me3) chromatin. Coexistence of these histone marks is likely to maintain progenitor cells in a “ready

state” for lineage-specific gene activation or repression. For example, the *Sox2* gene possesses these bivalent modifications in ESCs, but only has the H3K27me3 modification in differentiated cells, indicating that this locus is poised for repression during terminal differentiation (Mikkelsen et al. 2007).

At present, chromatin-state maps have not been systematically generated for breast progenitor cells and their differentiated progeny. It is likely that bivalent genes are present to maintain pluripotency of the progenitor genome. During epithelial differentiation, these bivalent loci may acquire activating or repressive chromatin marks that specify specific cell lineages (Balch et al. 2007). Terminally differentiated cells may therefore display unique chromatin signatures distinctly associated with their developmental fates. In an initial study, Polyak and coworkers (Bloushtain-Qimron et al. 2008) have further shown that bivalent loci identified in ESCs might also be present in CD44+ breast progenitor cells, as genes highly expressed in CD44+ compared to CD24+ cells were enriched in Suz12 targets in ESCs. As discussed earlier, CpG islands of these genes were found to be hypomethylated in CD44+ cells compared to those of differentiated CD24+ cells. Although the aforementioned bivalent marks play an important role in maintaining transcriptional plasticity in CD44+ cells, it remains to be determined whether other histone modifications also contribute to this ready state for activation and deactivation of genes during mammary epithelial differentiation.

As indicated earlier, propagation of neoplastic lesions can be initiated by CD44+ progenitor cells, which have been exposed to aberrant microenvironments. In this regard, deregulation of TGF- β (Hinshelwood et al. 2007) and AKT1 (Lin et al. 2008) signaling has been implicated in abnormal differentiation of mammary epithelial cells. In vitro studies have found that cultured mammary epithelial cells routinely enter a phase of growth arrest, but a subpopulation of progenitor cells may continue to proliferate into preneoplastic clones (Hinshelwood and Clark 2008). It has been hypothesized also that aberrant progenitor cells

retain bivalent features of differentiation-control genes, but many of these genes encoding tumor suppressor functions may undergo epigenetic silencing during neoplastic development. In addition to H3K27me₃, two additional repressive marks, H3K9me₂ and H3K9me₃, may be present in these genes, leading to permanent silencing of tumor-suppressor genes and subsequent propagation of transformed phenotypes (Bloushtain-Qimron et al. 2008; Cheng et al. 2008; Hsu et al. 2009). Transition from bivalent to fully repressive histone modifications therefore contributes to the development of breast cancer. A systematic cataloging of different histone marks for normal progenitors and cancer-initiating cells is an important task for future research of mammary gland development and tumorigenesis.

Connecting Histone Modifications and DNA Methylation for Gene Silencing

The interplay between histone modifications and DNA methylation has a critical role in nucleosome remodeling and subsequent regulation of gene transcription (Ballestar and Esteller 2005; Esteller 2006). The aforementioned polycomb target genes, known to be associated with stem/progenitor differentiation, provide the best example of how these two epigenetic components cooperate to initiate and maintain gene silencing (Cheng et al. 2008). These genes are generally associated with promoter CpG islands and are usually protected from de novo DNA methylation in stem/progenitor cells (Meissner et al. 2008). During epithelial differentiation, EZH2, as part of the polycomb repressor complex 2, catalyzes trimethylation of H3K27 on target nucleosomes (Sparmann and van Lohuizen 2006). This modification may serve as a dock site for the recruitment of a second repressor complex, PRC1, which additionally modifies target loci into compact chromatin configuration. These polycomb complexes may be constitutively present and can readily reattach to target sequences following DNA replication of daughter cells. Further recruitment of DNA methyltransferases and methyl-CpG binding protein may occur in

promoter CpG islands for de novo DNA methylation (Leu et al. 2004; Hinshelwood et al. 2009). This epigenetic repression occurs in a subset of PRC2-mediated target genes essential for tumor suppression (Cheng et al. 2008) and can be heritably maintained over multiple divisions in the differentiated progeny.

This epigenetic reprogramming can be disrupted in mammary progenitor cells as a result of chronic exposure to xenoestrogens or inflammatory microenvironment. Deregulated signaling related to epithelial differentiation may aberrantly up-regulate the expression of EZH2 and other polycomb repressor proteins, such as SUZ12, BMI1, G9a, and SUV39H (Dimri et al. 2002; Schultz et al. 2002; Kleer et al. 2003; Lehnertz et al. 2003). As a result, polycomb-mediated silencing of genes associated with tumor suppressor functions takes place, resulting in clonal proliferation of undifferentiated or semi-differentiated cells. Further accumulation of DNA methylation likely causes the permanent silencing of these genes in proliferating cells. In addition to tumor-suppressor genes, epigenetic silencing may lead to up-regulation of oncogenes. In this regard, microRNAs that negatively regulate the expression of their target loci may become transcriptionally silent, leading to aberrant de-repression of these oncogenes in cancer cells (Huang et al. 2009).

The cooperation between histone methylation and DNA methylation is highly dynamic in different cancer cell types. In some cancer types, epigenetically silent loci are highly enriched for trimethylation of H3K27 (H3K27me₃), but show low levels of DNA methylation (Kondo et al. 2008). In other cases, this trimethylation mark can be lost while DNA methylation is accumulated in silent loci (Gal-Yam et al. 2008). It is possible that these two repressive marks become more independent of each other in terms of maintenance silencing during cancer development, because genetic knockdown of EZH2 does not change profiles of de novo DNA methylation in a transfection study (McGarvey et al. 2007). Therefore, H3K27me₃ can be replaced by DNA methylation for long-term repression. This reprogramming may be more widespread in cancer

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cells that show high degrees of genomic instability.

Concluding Remarks

Epigenetic disruption is a characteristic of human cancer. The reduction of the total amounts of DNA methylation compared with their normal counterparts has been the first epigenetic alteration described in human tumors. Global DNA hypomethylation contributes to the origin of cancer cells by generation of chromosomal instability, reactivation of transposable elements, and loss of imprinting. Most importantly, CpG methylation can be acquired in promoter regions of tumor-suppressor genes, such as *BRCA1* and *p16^{INK4a}*, leading to the inactivation of these cancer-protecting proteins. Furthermore, methylation mediated-silencing was observed in microRNA loci with tumor-suppressive functions. Human tumors also show a distorted histone code. Breast tumors undergo massive and adaptive changes in their natural history, i.e., these cancer cells can metastasize to distant sites where they create new blood and lymph vessels to feed on and eliminate metabolites. They can also phenotypically alter their response to treatment with drugs, hormones, or radiation. In this regard, these cancer cells may have a limited ability to undergo fast genetic changes to adapt to hostile microenvironments. However, the expansion of a subset of breast cancer cells can occur through rapidly occurring epigenetic changes. For example, the cell adherence *E-cadherin* gene becomes methylated and silenced in breast cancer cells once they have metastasized to other organs or tissues. One of the essential differences between genetic and epigenetic alterations in cancer cells is that, unlike the former, DNA methylation and histone modifications are reversible under the right circumstances. Thus, epigenetic changes can be one of weak points in self-defense mechanisms of cancer cells, because those hypermethylated tumor-suppressor genes in their long “sleep” can be awakened and reactivated with the right drug regimen and exert their normal growth-inhibitory functions. Two families of

epigenetic drugs-DNA demethylating agents and histone deacetylase inhibitors hold great promise for future cancer treatments.

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