

AD_____

Award Number: DAMD17-00-1-0301

TITLE: The Roles of Histone Deacetylase and DNA Methylation in
Estrogen α Expression in Breast Cancer

PRINCIPAL INVESTIGATOR: Yi Huang, M.D., Ph.D.
Xiaowei Yang, M.D., Ph.D.

CONTRACTING ORGANIZATION: The Johns Hopkins University
School of Medicine
Baltimore, Maryland 21205-2196

REPORT DATE: June 2001

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

20020814 158

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)		2. REPORT DATE June 2001	3. REPORT TYPE AND DATES COVERED Annual Summary (1 Jun 00 - 31 May 01)	
4. TITLE AND SUBTITLE The Roles of Histone Deacetylase and DNA Methylation in Estrogen α Expression in Breast Cancer			5. FUNDING NUMBERS DAMD17-00-1-0301	
6. AUTHOR(S) Yi Huang, M.D., Ph.D. Xiaowei Yang, M.D., Ph.D.				
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) The Johns Hopkins University School of Medicine Baltimore, Maryland 21205-2196			8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012			10. SPONSORING / MONITORING AGENCY REPORT NUMBER	
11. SUPPLEMENTARY NOTES				
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited			12b. DISTRIBUTION CODE	
13. Abstract (<i>Maximum 200 Words</i>) (<i>abstract should contain no proprietary or confidential information</i>) The proposal tests the hypothesis that histone deacetylase activity contributes to the transcriptional repression of the methylated estrogen receptor α (ER) gene. It further postulates that inhibition of histone deacetylase (HDAC) and DNA methylation may act together to reactivate the ER gene. Studies to date show that the HDAC inhibitor trichostatin A, can reactivate ER expression in ER-negative breast cancer cell lines. Combined studies with HDAC inhibitors and demethylating agents are in progress.				
14. SUBJECT TERMS estrogen receptor α , histone deacetylase, trichostatin A, DNA methylation			15. NUMBER OF PAGES 32	
			16. PRICE CODE	
17. SECURITY CLASSIFICATION OF REPORT Unclassified Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited	

Table of Contents

Introduction.....	4
Body.....	4
Key Research Accomplishments.....	4
Reportable Outcomes.....	4
Conclusions.....	4
References.....	4
Appendices.....	4

INTRODUCTION

The proposal tests the hypothesis that histone deacetylase activity contributes to the transcriptional repression of the methylated estrogen receptor α (ER) gene in ER-negative human breast cancer cells. It was further postulated that inhibition of histone deacetylation and DNA methylation may act together to reactivate ER expression.

BODY

Technical Objective 1: To determine whether histone deacetylase activity is required for transcriptional silencing of ER gene.

Because the absence of ER gene expression has been associated with aberrant methylation of its CpG island in a significant fraction of breast cancers, the contribution of histone deacetylase activity to transcriptional inactivation of the methylated ER gene in a panel of ER-negative human breast cancer cells was tested. Treatment of these cells with trichostatin A, a specific histone deacetylase inhibitor, led to dose- and time-dependent expression of ER mRNA as detected by reverse transcription-PCR without alteration in ER CpG island methylation. Trichostatin A induced ER re-expression was associated with increased sensitivity to DNase I at the ER locus in MDA-MB-231 cells. These data implicate inactive chromatin mediated by histone deacetylation as a critical component of ER gene silencing in human breast cancer cells. These results were published in Yang et al, *Cancer Research* 60:6890-6894, 2000.

Technical Objective 2: To achieve maximal ER reactivation both in vitro and in vivo through the combination of demethylation and histone deacetylase inhibition.

The studies proposed in this objective are in progress.

KEY RESEARCH ACCOMPLISHMENTS

Demonstration that an inhibitor of histone deacetylase can reactivate ER expression in ER-negative human breast cancer cells.

REPORTABLE OUTCOMES

1. Yang X, Ferguson AT, Nass SJ, Phillips DL, Butash KA, Wang SM, Herman JG, and Davidson NE. Transcriptional activation of estrogen receptor α in human breast cancer cells by histone deacetylase inhibition. *Cancer Res* 60:6890-4, 2000.
2. Yan X, Yan L, and Davidson NE. DNA methylation in breast cancer. *Endocrine-related Cancer* 8:115-127, 2001.
3. Yan L, Yang X, and Davidson NE. Role of DNA methylation and histone acetylation in steroid receptor expression in breast cancer. *J Mammary Gland Biol Neoplasia* 6:183-192, 2001.

CONCLUSIONS

Administration of a histone deacetylase inhibitor, trichostatin A, can re-activate expression of ER in ER-negative breast cancer cell lines.

APPENDICES

Three manuscripts as listed above

Transcriptional Activation of Estrogen Receptor α in Human Breast Cancer Cells by Histone Deacetylase Inhibition¹

Xiaowei Yang, Anne T. Ferguson, Sharyl J. Nass, Dawn L. Phillips, Kim A. Butash, San Ming Wang, James G. Herman, and Nancy E. Davidson²

The Johns Hopkins Oncology Center, Johns Hopkins University, Baltimore, Maryland 21231 [X. Y., A. T. F., S. J. N., D. L. P., K. A. B., J. G. H., N. E. D.], and Section of Hematology/Oncology, University of Chicago Medical Center, Chicago, Illinois 60637 [S. M. W.]

Abstract

Recent findings have established a connection between DNA methylation and transcriptionally inactive chromatin characterized by deacetylated histones. Because the absence of estrogen receptor α ($ER\alpha$) gene expression has been associated with aberrant methylation of its CpG island in a significant fraction of breast cancers, we tested whether histone deacetylase activity contributes to the transcriptional inactivation of the methylated ER gene in a panel of ER-negative human breast cancer cells. Treatment of these cells with trichostatin A, a specific histone deacetylase inhibitor, led to dose- and time-dependent re-expression of ER mRNA as detected by reverse transcription-PCR without alteration in $ER\alpha$ CpG island methylation. Trichostatin A-induced ER re-expression was associated with increased sensitivity to DNase I at the ER locus in MDA-MB-231 cells. These data implicate inactive chromatin mediated by histone deacetylation as a critical component of ER gene silencing in human breast cancer cells. Therefore, histone deacetylation may be a potential target for therapeutic intervention in the treatment of a subset of ER-negative breast cancers.

Introduction

Interaction between 17β -estradiol and $ER\alpha$ ³ plays an important role in breast carcinogenesis and breast cancer treatment. That estrogen stimulates the growth of certain breast cancers is well established, and hormonal therapy via estrogen depletion or antiestrogen administration is widely used to block the action of estrogen in women with breast cancer. However, patients whose breast cancers lack ER seldom respond to endocrine therapy; therefore, a potential mechanism for hormone resistance is *de novo* or acquired loss of ER gene expression at the transcriptional level during disease progression (1, 2).

One possible mechanism for loss of ER in ER-negative breast cancers is cytosine methylation of the ER CpG island in the 5' regulatory region of the gene (3). Indeed the ER gene CpG island is extensively methylated in ER-negative breast cancer cells, and ~50% of unselected primary breast tumors but remains unmethylated in normal breast tissue and many ER-positive tumors and ER-positive cancer cell lines (4, 5). The functional importance of this finding is demonstrated by the fact that treatment of ER-negative human breast

cancer cells with the demethylating agent, 5-aza-dC, led to reactivation of expression of ER mRNA and functional ER protein (6). Recent studies indicate that silencing of a gene by methylation involves the generation of an inactive chromatin structure characterized by deacetylated histones. An abundant chromosomal methyl CpG-binding protein, MeCP2, was the first protein identified to link methylated DNA and a HDAC-containing transcriptionally repressive complex for gene silencing. Subsequently, several MBD proteins have been identified that, similar to MeCP2, couple methylated DNA to HDAC (7, 8). More recently, the well-known maintenance methyltransferase, Dnmt1, was found to interact physically with HDAC through its N terminus, thereby forming a transcriptionally inactive chromatin structure that represses transcription (9, 10). All of these findings demonstrate the important role of HDAC in transcriptional regulation. The HDACs deacetylate lysine groups of histones H3 and H4, allowing ionic interactions between positively charged lysines and negatively charged DNA, which result in a more compact nucleosome structure that limits transcription. The availability of specific HDAC inhibitors such as TSA (11) permits the study of the role of HDAC in silencing a variety of tissue-specific methylated genes (7, 12).

Here, we have tested whether loss of ER expression in some breast cancers is associated with transcriptional repression through HDAC activity on the methylated ER gene. Our data demonstrate that specific HDAC inhibition via TSA treatment can reactivate ER transcription in the presence of the methylated DNA. The activated gene transcription is associated with increased sensitivity of the ER promoter to DNase I treatment. These data suggest that inactive chromatin mediated by HDAC is critical to ER gene silencing.

Materials and Methods

Cell Lines, Reagents, and TSA Treatment. Human breast cancer cells (Hs578t, MCF-7/WT, MCF-7/AdR^R, T-47D, and MDA-MB-231) were grown in DMEM supplemented with 10% fetal bovine serum and 2 mM L-glutamine. TSA was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), dissolved in absolute ethanol at a stock concentration of 1 mg/ml (3.30 mM), and stored at -20°C . DNase I was purchased from Pharmacia Biotech (Pharmacia Biotech, NJ). Cells (MDA-MB-231, Hs578t, T-47D, or MCF-7/AdR^R) were seeded at a density of 6×10^5 cells/100-mm tissue culture dish (8×10^3 cells/cm²). After 24 h of incubation, the culture medium was changed to different concentrations of TSA- or vehicle (ethanol)-containing medium. Either total cellular RNA or genomic DNA was isolated after 0, 24, 48, or 72 h of TSA exposure.

RNA Isolation and RT-PCR Analysis of ER Expression. Total cellular RNA was isolated from cell lines with TRIzol reagent according to the recommendations of the supplier (Life Technologies, Inc., Rockville, MD). RNA (3 μg) was reversibly transcribed by Moloney murine leukemia virus reverse transcriptase (Life Technologies) using OligoDT₁₅ primer (Promega Corp., Madison, WI) in a final volume of 50 μl . Four % of synthesized cDNA (2 μl , derived from 150 ng of initial RNA) was used for PCR amplification of ER and the constitutively expressed housekeeping gene β -actin (13). Specific sense and antisense PCR primers used for the amplifications across the seventh

Received 7/10/00; accepted 10/31/00.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¹ This work was supported by Grants CA78352 (to N. E. D.) and 2-T32CA09110 (to X. Y. and S. J. N.) from the NIH, Contracts DAMD17-00-1-0301 (to X. Y.) and DAMD17-98-1-8116 (to S. J. N.) from the Department of Defense Breast Cancer Program, and Grant PF4231 (to A. T. F.) from the American Cancer Society.

² To whom requests for reprints should be addressed, at Johns Hopkins University School of Medicine, 1650 Orleans Street, CRB Room 409, Baltimore, MD 21231. E-mail: davidna@jhmi.edu.

³ The abbreviations used are: $ER\alpha$, estrogen receptor α ; 5-aza-dC, 5-aza-2'-deoxycytidine; MBD, methyl-CpG-binding domain; Dnmt, DNA methyltransferase; HDAC, histone deacetylase; MeCP2, methyl-CpG binding protein 2; MSP, methylation-specific PCR; TSA, trichostatin A; RT-PCR, reverse transcription-PCR.

intron of ER and the first intron of β -actin genes, yielding 470 and 400 bp of PCR products respectively, were described previously (6). PCR products were resolved by 2% agarose gel electrophoresis and visualized by ethidium bromide staining.

Quantitative Competitive PCR Analysis of ER Reactivation. A quantitative assay was performed to determine the level of ER mRNA in TSA- and vehicle-treated MDA-MB-231 cells as compared with the expression levels in the known ER-positive MCF-7 and T-47D cells using the method of Wang and Rowley (14). This assay involves coamplification of a wild-type target cDNA (wER) of unknown amount and a competitive template (cER) in known amounts. A truncated competitive template was generated with a sense primer containing a 22-bp deletion (primer 2) and the same antisense primer (primer 3) as the wild-type (Fig. 2A). After amplification, the competitive template was separated, gel purified, and quantified by Spectrophotometer DU 640 (Beckman, CA). Thus, target wild-type and known amounts of competitive templates can be coamplified with the pair of wild-type primers (primers 1 and 3) and differentiated by size. Because the most accurate results are obtained when wild-type and competitive templates are amplified at nearly equivalent concentrations, resulting in the signal ratio of wER:cER equivalent to 1, we first performed an initial titration in log and then in 2-fold dilutions to determine the approximate concentration of the wild-type ER cDNA in our experimental samples. RNAs under comparison were simultaneously reversibly transcribed to achieve equal efficiency for reverse transcription. The PCR reactions were carried out with 0.5 μ M of wild-type sense and antisense primers for 35 cycles. The wild-type and competitive PCR products were fractionated on 2.5% agarose gel, stained with ethidium bromide, and scanned by Densitometer (EagleSight Software of Eagle Eye II Imaging System; Stratagene, La Jolla, CA). The ratio between wild-type and control templates was determined and used to calculate the amount of target wild-type cDNA because the input of competitive template is known.

Genomic DNA Isolation and MSP Analysis of ER CpG Island. DNA was isolated by standard phenol-chloroform extraction. Isolated DNA was subjected to modification by sodium bisulfite to convert unmethylated cytosines but not methylated cytosines to uracil as described previously (15). Methylation status of the bisulfite-modified DNA at the ER locus was characterized by methylation-specific PCR using a method described previously (5).

DNase I Sensitivity Assay. This assay was performed according to the method of Keshet *et al.* (16) using cells from TSA- or vehicle-treated MDA-MB-231 and MCF 7 cells (1×10^7 cells/each) with the following modifications. The isolated DNA was digested with *EcoRI*, the recognition sites of which flank the ER promoter region to yield a 3.1-kb fragment that was separated by 1% agarose gel electrophoresis. DNAs blotted on nylon membrane were probed with a PCR-amplified, 561-bp DNA fragment corresponding to the ER CpG island (Fig. 4A). The sense and antisense oligonucleotides used to amplify the fragment are 5'-AGACCAGTACTTAAAGTTGGAG-GCC-3' and 5'-GGGAAACCCCCAGG-3'. The amplified DNA was cloned into pCR2.1-TOPO vector (Invitrogen, CA) according to the manufacturer's protocol. Colonies containing amplified DNA sequence, determined by Mini-Prep (Promega Wizard Mini-Prep kits), were grown and purified. The purified plasmids were sequenced via automated sequencing (Johns Hopkins Sequencing Core Facility). The specific ER CpG probe was prepared from sequence-confirmed plasmid and labeled with bio-16-dUTP (Boehringer Mannheim, IN) by PCR using the above-mentioned primer set. The 3.1-kb DNA band containing the ER promoter region was visualized by chemiluminescence using a streptavidin-conjugated, alkaline phosphatase-catalyzed substrate, CDP-star. The band signals that reflect the resistance to DNase I were quantified by densitometry (Stratagene), and the cumulative DNase I sensitivity was calculated as follows. The band density at each DNase I concentration was divided by the density of the control band and then multiplied by 100%. This value was subtracted from 100% to yield the percentage of DNase I sensitivity. The sum of the percentage of DNase I sensitivity at each dose was considered to be the cumulative DNase I sensitivity.

Results and Discussion

A growing body of data demonstrates the importance of histone acetylation and deacetylation and corresponding structural alteration of chromatin in gene transcriptional regulation (7, 8, 10). The ER-

negative cell line, MDA-MB-231, the ER CpG island of which is densely methylated, was used as a cell model to test whether HDAC activity contributes to repression of ER expression in ER-negative breast cancer cells. TSA, a specific and potent HDAC inhibitor, was used as a pharmacological tool. Exposure of MDA-MB-231 cells to increasing concentrations of TSA led to induction of ER mRNA synthesis in a dose-dependent manner (Fig. 1A). A detectable level of ER mRNA, as demonstrated by a RT-PCR product with predicted 470-bp size, was noted after treatment with 50 ng/ml (160 nM) TSA for 48 h, and ER transcript was clearly present after 100 ng/ml (330 nM) TSA for the same duration. A time course analysis showed that a weak signal could be seen after 24 h of 100 ng/ml TSA treatment, whereas ER transcript was readily observed after 48 or 72 h (Fig. 2B). Multiple experiments were done to achieve optimal conditions for TSA induction of ER mRNA in MDA-MB-231 cells. These showed that maximal ER reactivation was achieved with 100 ng/ml TSA for 48 h using an initial seeding density of 8×10^3 cells/cm². Higher inoculating cell densities reduced ER transcript signal (data not shown).

To ascertain whether HDAC activity could play a role in repression of ER expression more generally, the dose response and time course studies described above were extended to other ER-negative human breast cancer cells lines with methylated ER CpG islands. As shown in Fig. 1C, TSA treatment led to re-expression of ER mRNA in all three ER-negative cell lines tested. Optimal ER gene re-expression was observed after treatment of MDA-MB-231 cells with 100 ng/ml (330 nM), Hs578t cells with 400 ng/ml (1.32 μ M), and MCF-7/Adr^R with 25 ng/ml (82.5 nM) TSA for 48 h. Therefore, TSA treatment consistently induced ER re-expression in the panel of ER-negative cell lines, supporting a role for HDAC in ER gene silencing.

A quantitative competitive PCR assay was used to assess the magnitude of TSA-induced ER mRNA transcript in MDA-MB-231 cells. Fig. 2A shows the design of the primer sets used, and validation of the quantitative competitive PCR is shown in Fig. 2B. As shown in Fig. 2C, a 5-fold increase in ER transcript was obtained after TSA exposure in MDA-MB-231 cells (100 ng/ml for 48 h). This effect was specific for the methylated ER promoter because TSA treatment (50 or 100 ng/ml for 48 h) of ER-positive, unmethylated MCF-7 cells had

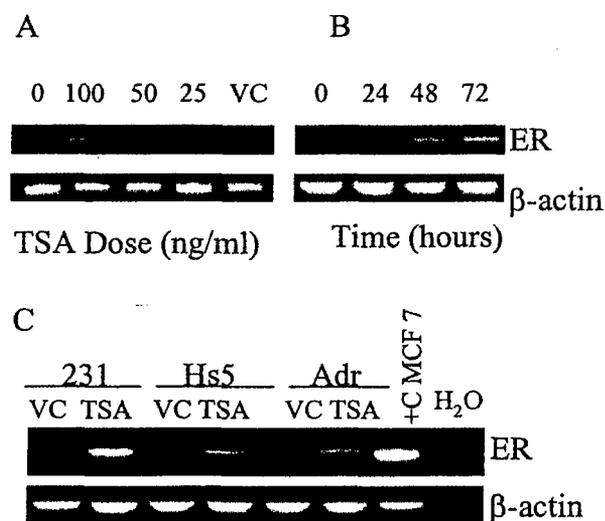
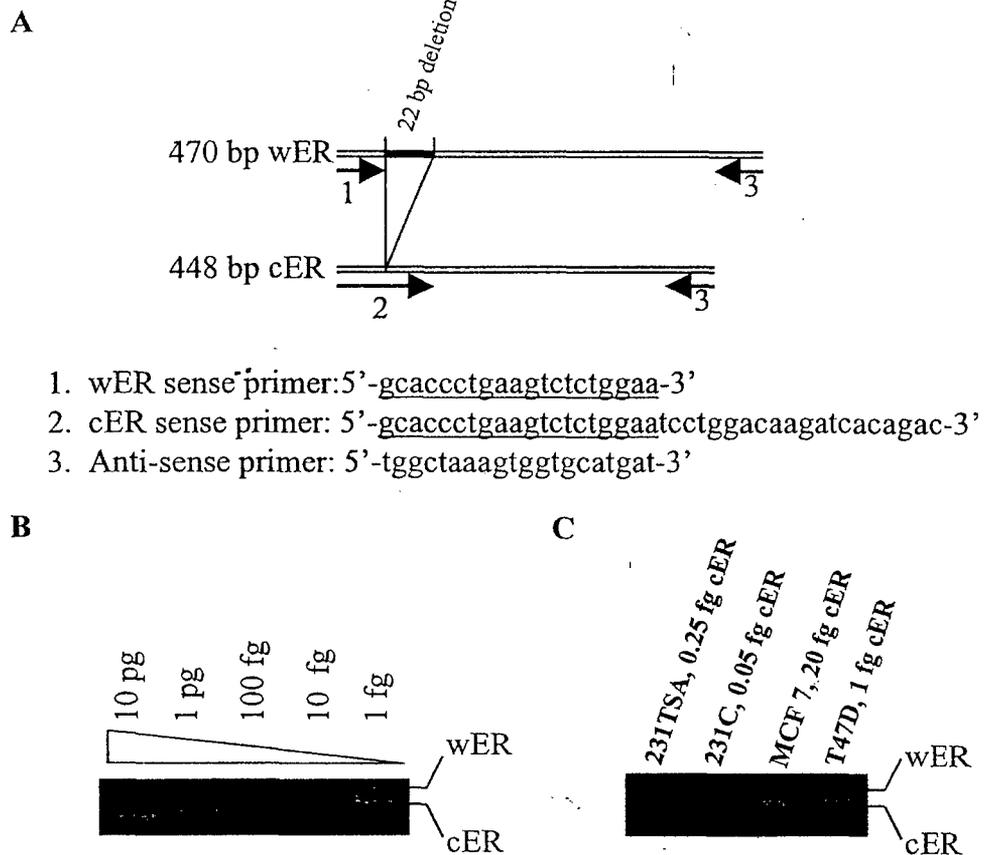


Fig. 1. Upper panels: A, dose response of TSA on ER transcription in MDA-MB-231 cells analyzed by RT-PCR. B, time course of TSA (100 ng/ml) on re-expression of ER mRNA for 48 h in MDA-MB-231 cells. C, RT-PCR analysis of ER mRNA re-expression by TSA for 48 h in a panel of known ER-negative breast cancer cell lines. The ER-positive prototype, MCF-7, was used as a RT-PCR-positive control. Lower panels: β -actin RT-PCR product provides a control for the amount of intact RNA used in the reactions. VC, vehicle-treated; 231, MDA-MB-231; Hs5, Hs578t; Adr, MCF-7/Adr.

Fig. 2. Quantitative competitive PCR analysis of ER expression in breast cancer cells. **A**, primer design. As shown above, the competitive sense primer 2 is a 40-bp oligonucleotide that harbors a 22-bp deletion in the middle of the primer. Its sequence at the 5' end is the same as the wild-type sense primer 1. Thus, primers 2 and 3 amplify cER, but wild-type primer pairs 1 and 3 amplify both wER and cER. **Arrow bars**, locations of sense and antisense primers. *wER*, wild-type ER; *cER*, competitive ER. **B**, validation of the competitive PCR. wER and cER from MCF-7 cells were amplified with the wild-type primers using different dilutions of competitive template DNA. The cER PCR product decreases and the wER PCR product increases when decreasing known amounts of cER and a constant but unknown amount of wER cDNA is used for PCR (see "Materials and Methods"). The amount of wER can be ascertained from the titration curve at the point where an equal amount of cER and wER is, in this example, between 100 and 10 fg. **C**, using this quantitative assay, TSA treatment (100 ng/ml for 48 h) of MDA-MB-231 cells induced a 5-fold increase in ER transcript. Equivalence of wER/cER signal was seen at 0.25 fg cER for TSA-treated cells and 0.05 fg cER for control cells (signal ratio of wER:cER = 1 in both TSA-treated and control cells; see "Materials and Methods"), thus demonstrating a 5-fold induction. Using the same calculations (signal ratio of wER:cER multiplied by amount of cER), the TSA-induced transcript reached about 1 and 10% of that found in ER-positive MCF-7 and T-47D cells, respectively. A representative example of four experiments that gave similar results is shown.



no effect on the level of ER mRNA expression using the same quantitative assay (data not shown). However, TSA treatment of MDA-MB-231 cells did not restore ER mRNA expression to the levels seen in cell lines with endogenous ER expression, as shown in Fig. 2C. Quantitative assay suggested that the level of ER transcript seen with TSA treatment of MDA-MB-231 cells represented about 1 and 10% of that seen in the ER-positive MCF-7 and T-47D cell lines, respectively. Several possibilities might account for this:

(a) It is possible that only partial reactivation is seen because only a fraction of cells responded to the treatment. Indeed, a similar pattern of partial reactivation was seen in MDA-MB-231 cells exposed to a demethylating agent, 5-aza-dC, in our previous study (6).

(b) It has been shown that a component of the repression mediated by MeCP2 transcriptional repression domain is partially HDAC independent; mSin3A could retain some ability to repress transcription, even in the absence of associated HDACs (7).

(c) Simultaneous inhibition of several components in the methylation-associated repressive complexes might be necessary to achieve maximal reactivation of the repressed genes (12).

The ability of TSA to reactivate ER expression raised the question of whether the ER CpG island remained methylated. Our previous studies with 5-aza-dC demonstrated that ER re-expression was associated with demethylation of the ER CpG island. However, a parallel study of the progesterone receptor in progesterone receptor-negative human breast cancer cells showed that the ligand-bound ER could overcome methylation-related repression of the progesterone receptor, even in the continuing presence of a methylated progesterone receptor CpG island (17). We therefore examined the methylation status of the ER CpG island in TSA-treated MDA-MB-231 cells using a sensitive MSP assay that allowed examination of methylation status across the ER CpG island (Fig. 3A). As shown in Fig. 3B, the ER CpG island was completely methylated across the entire CpG island in MDA-MB-231

cells treated with vehicle or TSA (100 ng/ml for 48 h). A single primer set, ER 5, was used to confirm this finding in the other TSA-treated, ER-negative human breast cancer cells, Hs578t and MCF-7/Adr^R (Fig. 3C). As expected, the unmethylated ER-positive, MCF-7 cells demonstrated an unmethylated pattern using all four primer sets that span the ER CpG island. In summary, TSA treatment of ER-negative breast cancer cells can lead to re-expression of ER mRNA without an apparent alteration in the methylation status of the ER CpG island.

Because acetylated histones are generally associated with transcriptionally active chromatin whereas deacetylated histones are often found in conjunction with an inactive chromatin state (18), we next studied whether HDAC inhibition could alter chromatin structure at the ER gene locus. Because nuclease susceptibility is one of the characteristics of active chromatin (19), we used a DNase I sensitivity assay to examine chromatin conformation of the ER gene in ER-negative MDA-MB-231 cells in the presence or absence of the HDAC inhibitor, TSA. Cells were treated with 100 ng/ml of TSA for 48 h, a treatment course shown previously to result in optimal re-expression of ER mRNA. Equal amounts of purified nuclei from control and TSA-treated MDA-MB-231 cells were exposed to increasing concentrations of DNase I as described in "Materials and Methods." Nuclei isolated from ER-positive MCF-7 cells served as a DNase I accessible control. As expected, the ER locus in MCF-7 cells was a highly DNase I sensitive region, whereas the ER locus in control MDA-MB-231 cells was relatively resistant to DNase I digestion (Fig. 4B). TSA treatment of MDA-MB-231 cells resulted in an ~2-fold increase in DNase I sensitivity (Fig. 4C), suggesting that inhibition of HDAC activity leads to a more open chromatin conformation, even in the presence of CpG island methylation.

Recently, the interaction between DNA methylation and histone deacetylation linked by methyl-binding proteins (MBDs), or the direct interaction of Dnmt1 with HDAC as well as other corepressors, has

Fig. 3. MSP analysis for the *ER* CpG island in TSA-treated ER-negative breast cancer cells. **A**, map of *ER* CpG island and location of ER MSP primers. **B**, MSP analysis of *ER* gene CpG island in TSA-treated MDA-MB-231 cells. Both control (231) and TSA-treated (231 TSA) MDA-MB-231 cells showed evidence of *ER* CpG methylation across primer sets examined. DNA from the ER-positive MCF-7 cell line was used as an unmethylated control. **C**, MSP analysis of *ER* gene CpG island by using primer set ER 5 in a panel of ER-negative breast cancer cells. Both vehicle- and TSA-treated cell pairs (MDA-MB-231 C and TSA; Hs578t C and TSA; and MCF-7/Adr^R C and TSA) showed methylated pattern, whereas ER-positive MCF-7 showed unmethylated pattern (MCF 7). Distilled water instead of bisulfite-treated genomic DNA was used as MSP-negative control (*H₂O*). m, methylated products; u, unmethylated products.

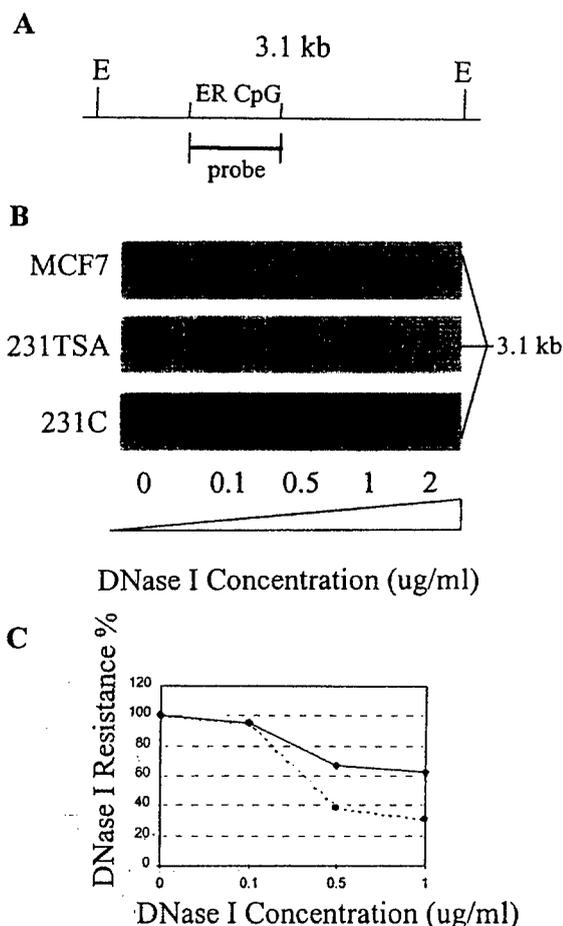
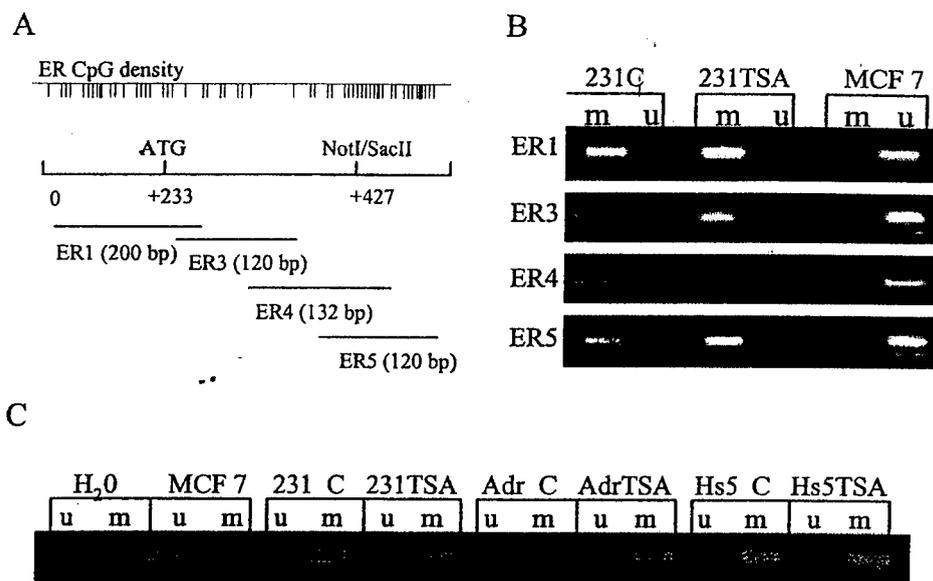


Fig. 4. Chromatin conformation at *ER* gene locus in breast cancer cells analyzed by DNase I sensitivity assay. **A**, map of the *ER* promoter region flanked by *EcoRI* (*E*), showing the location of the *ER* CpG island in the region and probe position. **B**, DNase I digestion profiles with the indicated concentrations of DNase I in TSA-treated (100 ng/ml for 48 h (231TSA) and vehicle-treated (231C) MDA-MB-231 cells are shown. A highly DNase I sensitive region was found in MCF-7 cells (MCF7). Presented is one of three experiments that showed similar results. **C**, quantitative analysis of DNase I sensitivity at 48 h after TSA treatment in MDA-MB-231 cells. The concentration of DNase I was plotted against the percentage of DNase I resistance. Densitometric analysis demonstrated a 1.8-fold increased sensitivity in TSA-treated (●, 231 TSA, cumulative DNase I sensitivity of 135) versus vehicle-treated (◆, 231C, cumulative DNase I sensitivity of 75) MDA-MB-231 cells (see "Materials and Methods").

been an area of active study. More recently, a nucleosome-stimulated ATPase Mi2, a part of chromatin remodeling machinery, was also shown to bind the methylated DNA through MBD3 and deacetylase in *Xenopus laevis* and mammalian cells, further illustrating the role of HDAC on gene transcription regulation (8). In addition to evaluating the role of HDAC in *in vitro* studies, it is of importance to study its role in silencing endogenous methylated genes. In some cases, HDAC inhibition alone seems to be sufficient to reactivate a methylated gene. For example, both sodium butyrate and TSA can restore transcription from methylated and silenced plant rRNA genes (20). Also, reactivation of transcription of the methylated *FMR1* gene was achieved by treatment with 4-phenylbutyrate, sodium butyrate or TSA (21). However, in other cases, both demethylation and HDAC inhibition appear to be necessary. For example, certain hypermethylated genes like *MLH1*, *TIMP3*, *CDKN2B*, and *CDKN2A* can be transcriptionally activated in colon cancer cells by TSA only after Dnmt1 inhibition by 5-aza-dC, suggesting an important role of Dnmt1 in transcription repression although recruiting HDAC is essential (12).

ER is a critical growth-regulatory gene in breast cancer, and its expression status is tightly linked to the prognosis and treatment outcome of breast cancer patients. Thus, it is important to understand its regulation. Our work suggests that histone deacetylation and DNA methylation may both play a role in ER transcription, and further studies will focus on the effects of TSA on ER protein expression. This is critical because it is possible that activation of the silenced ER by HDAC inhibition could open a new avenue for management of a subset of advanced breast cancer with hormonal resistance. Studies using primary breast cancers have shown that the antiestrogen, tamoxifen, confers a benefit to women whose breast cancer expresses ER by immunohistochemistry in as few as 1–10% of tumor cells. Thus, even partial re-expression of ER could be of clinical benefit (22).

References

- Hortobagyi, G. N. Treatment of breast cancer. *N. Engl. J. Med.*, 339: 974–984, 1998.
- Weigel, R. J., and de Coninck, E. C. Transcriptional control of estrogen receptor in estrogen receptor-negative breast carcinoma. *Cancer Res.*, 53: 3472–3474, 1993.
- Ottaviano, Y. L., Issa, J. P., Parl, F. F., Smith, H. S., Baylin, S. B., and Davidson, N. E. Methylation of the estrogen receptor gene CpG island marks loss of estrogen receptor expression in human breast cancer cells. *Cancer Res.*, 54: 2552–2555, 1994.
- Nass, S. J., Herman, J. G., Gabrielson, E., Iversen, P. W., Parl, F. F., Davidson, N. E., and Graff, J. R. Aberrant methylation of the estrogen receptor and E-cadherin 5' CpG

- islands increases with malignant progression in human breast cancer. *Cancer Res.*, *60*: 4346-4348, 2000.
5. Lapidus, R. G., Nass, S. J., Butash, K. A., Parl, F. F., Weitzman, S. A., Graff, J. G., Herman, J. G., and Davidson, N. E. Mapping of ER gene CpG island methylation-specific polymerase chain reaction. *Cancer Res.*, *58*: 2515-2519, 1998.
 6. Ferguson, A. T., Lapidus, R. G., Baylin, S. B., and Davidson, N. E. Demethylation of the estrogen receptor gene in estrogen receptor-negative breast cancer cells can reactivate estrogen receptor gene expression. *Cancer Res.*, *55*: 2279-2283, 1995.
 7. Nan, X., Ng, H. H., Johnson, C. A., Laherty, C. D., Turner, B. M., Eisenman, R. N., and Bird, A. Transcriptional repression by the methyl-CpG-binding protein MeCP2 involves a histone deacetylase complex. *Nature (Lond.)*, *393*: 386-389, 1998.
 8. Wade, P. A., Geggion, A., Jones, P. L., Ballestar, E., Aubry, F., and Wolffe, A. P. Mi-2 complex couples DNA methylation to chromatin remodeling and histone deacetylation. *Nat. Genet.*, *23*: 62-66, 1999.
 9. Rountree, M. R., Bachman, K. E., and Baylin, S. B. DNMT1 binds HDAC2 and a new co-repressor, DMAP1, to form a complex at replication foci. *Nat. Genet.*, *25*: 269-277, 2000.
 10. Robertson, K. D., Ait-Si-Ali, S., Yokochi, T., Wade, P. A., Jones, P. L., and Wolffe, A. P. DNMT1 forms a complex with Rb, E2F1 and HDAC1 and represses transcription from E2F-responsive promoters. *Nat. Genet.*, *25*: 338-342, 2000.
 11. Yoshida, M., Kijima, M., Akita, M., and Beppu, T. Potent and specific inhibition of mammalian histone deacetylase both *in vivo* and *in vitro* by trichostatin A. *J. Biol. Chem.*, *265*: 17174-17179, 1990.
 12. Cameron, E. E., Bachman, K. E., Myohanen, S., Herman, J. G., and Baylin, S. B. Synergy of demethylation and histone deacetylase inhibition in the re-expression of genes silenced in cancer. *Nat. Genet.*, *21*: 103-107, 1999.
 13. Yang, X., Uziely, B., Groshen, S., Lukas, J., Israel, V., Russell, C., Dunnington, G., Formenti, S., Muggia, F., and Press, M. F. *MDR1* gene expression in primary and advanced breast cancer. *Lab. Investig.*, *79*: 271-280, 1999.
 14. Wang, S. M., and Rowley, J. D. A strategy for genome-wide gene analysis: integrated procedure for gene identification. *Proc. Natl. Acad. Sci. USA*, *95*: 11909-11914, 1998.
 15. Herman, J. G., Graff, J. R., Myohanen, S., Nelkin, B. D., and Baylin, S. B. Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. *Proc. Natl. Acad. Sci. USA*, *93*: 9821-9826, 1996.
 16. Keshet, I., Lieman-Hurwitz, J., and Cedar, H. DNA methylation affects the formation of active chromatin. *Cell*, *44*: 535-543, 1986.
 17. Ferguson, A. T., Lapidus, R. G., and Davidson, N. E. Demethylation of the progesterone receptor CpG island is not required for progesterone receptor gene expression. *Oncogene*, *17*: 577-583, 1998.
 18. Luo, R. X., and Dean, D. C. Chromatin remodeling and transcriptional regulation. *J. Natl. Cancer Inst.*, *91*: 1288-1294, 1999.
 19. Eden, S., Hashimshony, T., Keshet, I., Cedar, H., and Thorne, A. W. DNA methylation models histone acetylation. *Nature (Lond.)*, *394*: 842, 1998.
 20. Chen, Z. J., and Pikaard, C. S. Epigenetic silencing of RNA polymerase I transcription: a role for DNA methylation and histone modification in nucleolar dominance. *Genes Dev.*, *11*: 2124-2136, 1997.
 21. Chiurazzi, P., Pomponi, M. G., Pietrobono, R., Bakker, C. E., Neri, G., and Oostra, B. A. Synergistic effect of histone hyperacetylation and DNA demethylation in the reactivation of the *FMR1* gene. *Hum. Mol. Genet.*, *8*: 2317-2323, 1999.
 22. Harvey, J. M., Clark, G. M., Osborne, C. K., and Allred, D. C. Estrogen receptor status by immunohistochemistry is superior to the ligand-binding assay for predicting response to adjuvant endocrine therapy in breast cancer. *J. Clin. Oncol.*, *17*: 1474-1481, 1999.

DNA methylation in breast cancer

X Yang, L Yan and N E Davidson

Breast Cancer Program, The Johns Hopkins Oncology Center, Johns Hopkins University School of Medicine, Baltimore, Maryland 21231, USA

(Requests for offprints should be addressed to N E Davidson, Department of Oncology, Johns Hopkins University School of Medicine, 1650 Orleans Street, CRB416, Baltimore, Maryland 21231, USA; Email: davidna@jhmi.edu)

Abstract

Like all cancers, breast cancer is considered to result in part from the accumulation of multiple genetic alterations leading to oncogene overexpression and tumor suppressor loss. More recently, the role of epigenetic change as a distinct and crucial mechanism to silence a variety of methylated tissue-specific and imprinted genes has emerged in many cancer types. This review will briefly discuss basic aspects of DNA methylation, recent advances in DNA methyltransferases, the role of altered chromatin organization and the concept of gene transcriptional regulation built on methylated CpGs. In particular, we discuss epigenetic regulation of certain critical tumor suppressor and growth regulatory genes implicated in breast cancer, and its relevance to breast cancer diagnosis, prognosis, progression and therapy.

Endocrine-Related Cancer (2001) 8 115–127

Introduction

Cytosine methylation and CpG islands in mammals

In vertebrate genomes, methylation of DNA occurs on cytosine residues of the CpG dinucleotides in DNA (Bird 1980). This epigenetic alteration in DNA is heritable but does not alter nucleotide sequence, in contrast to genetic changes (Feinberg 2001). Thus, unlike genetic changes, epigenetic modifications are potentially reversible (Baylin *et al.* 2001). About 3–6% of cytosines are methylated in mammals. Approximately 70–80% of CpG sites in the human genome are methylated (Vanyushin *et al.* 1970, Antequera & Bird 1993, Bird 1995). Cytosine residues in newly synthesized DNA are methylated by DNA-cytosine methyltransferase-1 (DNMT1) (Bestor 1988, Bestor & Verdine 1994). This enzyme transfers a methyl group from the methyl donor, S-adenosylmethionine, to nascent DNA using a hemimethylated DNA template in order to maintain DNA methylation patterns during cell division in mammals. CpG dinucleotides are not randomly distributed throughout the genome. Rather they are frequently clustered into CpG islands, regions that are rich in CpG sites. These islands extend about 0.5–3 kb, occur on average every 100 kb in the genome and are often found in the promoter area of genes (Cross & Bird 1995). Indeed, approximately half of all genes in the human (~45 000 genes) contain CpG islands (Antequera & Bird 1993). DNA methylation plays a role in

such diverse functions as gene imprinting (Forne *et al.* 1997, Reik & Walter 1998), X-chromosome inactivation (Heard & Avner 1994, Heard *et al.* 1997), normal development (Li *et al.* 1993, Weiss & Cedar 1997), repression of gene transcription (Keshet *et al.* 1985, Cedar 1988, Chan *et al.* 2000), and the suppression of parasitic DNA sequences (Yoder *et al.* 1997). It is believed to exercise its critical role in gene expression via several routes. First, methylation of a CpG island is associated with loss of transcription of the target gene. Secondly, 5-methylcytosine (5meC) residues are susceptible to deamination to thymine, resulting in a transition mutation (Schmutte & Jones 1998). Thirdly, it is possible that inappropriate hypomethylation could be linked to dysregulated gene induction (Muller *et al.* 2001).

DNMTs

To date, three members of the Dnmt gene family have been identified. Direct sequence analysis has revealed that the *Dnmt* gene family is highly conserved among eukaryotes, suggesting a central role of these proteins for development (Bestor 2000).

DNMT1 is the best known and studied member of the DNMT family. It is primarily a maintenance methylase, that is, it reproduces DNA methylation patterns from hemimethylated DNA during cell division (Bestor 1988). However, there is some evidence that DNMT1 may also have *de novo* methylase activity, at least in *in vitro* systems

(Laayoun & Smith 1995, Pradhan et al. 1997). *Dnmt1* gene knockout mice die in mid-gestation with reduced levels of DNA methylation (Li et al. 1992), disrupted imprinting and ectopic X-chromosome activation (Li et al. 1993), indicating that maintenance of DNA methylation is pivotal for development.

The human *DNMT1* gene is located at human chromosome 19p13.2 (Yen et al. 1992) and encodes a 200 kDa protein whose methyltransferase catalytic domain is located at the C-terminus of the protein. The large N-terminal portion of DNMT1 targets to replication foci through proliferating cell nuclear antigen (PCNA) (Chuang et al. 1997). Recent studies have identified new functions for this domain. First, its amino acid (653–730) sequence contains a CXXC motif that interacts directly with histone deacetylases (HDACs), which act to remove acetyl tails from histones in the nucleosome to generate a transcriptionally inactive chromatin structure (Fuks et al. 2000). Secondly, through its first 120 amino acids, it binds to a transcriptional co-repressor, DMAP1, that represses transcription independent of HDAC activity (Rountree et al. 2000). Lastly, amino acids 416–913 of the N-terminus of DNMT1 interact with the retinoblastoma protein, Rb (Robertson et al. 2000). Thus, the N-terminal portion of DNMT1 alone or in collaboration with other co-repressors and recruited HDACs significantly suppresses transcription *in vitro*.

A large body of data demonstrates that DNMT1 activity is elevated in neoplastic cells and this increased activity is associated with increased cell proliferation (El-Deiry et al. 1991), tumorigenesis (De Marzo et al. 1999) and tumor progression (Issa et al. 1993). For example, overexpression of DNMT1 can transform NIH-3T3 mouse fibroblast cells (Wu et al. 1993), and inhibition of this enzyme by antisense constructs can induce global DNA demethylation and revert the malignant phenotype (Ramchandani et al. 1997). In addition, *fos*-mediated transformation of normal fibroblasts is associated with increased DNMT1 expression and total methylation content in the genome (Bakin & Curran 1999). Finally, it is also reported that elevation of DNMT1 is an essential component of transformation induced by SV40 large T antigen via the Rb pathway (Slack et al. 1999).

However, increased DNMT1 expression is apparently not an obligatory feature of malignant cells (Eads et al. 1999). Somatic knockout of *DNMT1* expression in human colon cancer cells is not a lethal event. Further, total genomic methylated CpG content was reduced by only about 20% and certain gene-specific CpG island methylation patterns were maintained (Rhee et al. 2000). These findings, together with the observation that embryonic stem (ES) cells from *DNMT1* knockout mice are still capable of *de novo* methylation, suggest the possible existence of other DNMTs (Li et al. 1992, Lei et al. 1996).

One such DNMT, *Dnmt2*, was isolated by several groups (Okano et al. 1998). However, its catalytic domain lacks DNMT activity in the human and it is not discussed further.

Two isoforms of the DNMT3 enzyme family, *de novo* DNMTs 3a and 3b (*Dnmt3a* and *3b*) were recently isolated in the mouse (Okano et al. 1999). They methylate CpG dinucleotides of unmethylated and hemimethylated DNA *in vitro*. The two genes are expressed at high levels in ES cells and relatively low levels in adult somatic tissues. Human *DNMT3a* has been mapped to chromosome 2p23 whereas *DNMT3b* maps to chromosome 20q11.2 (Robertson et al. 1999, Xie et al. 1999).

Disruption of both *Dnmt3a* and *Dnmt3b* in mice by gene targeting blocks *de novo* methylation in ES cells and early embryos, but has no effect on maintenance of an imprinted methylation pattern (Okano et al. 1999). However, methylation capability is retained after inactivation of either *Dnmt3a* or *3b*, indicating some redundancy in the function of these two *de novo* methylases. *Dnmt3b* appears to be critical for the methylation of a particular compartment of the genome; loss of DNMT3b catalytic activity by gene mutation in the syndrome of immunodeficiency, centromeric instability and facial anomalies causes demethylation of only specific families of repeated sequences and CpG islands on the inactive X-chromosome (Hansen et al. 1999). Human DNMT3a is ubiquitously expressed but DNMT3b is expressed at low levels except in testis, thyroid and bone marrow. Overexpression of both *DNMT3b* and *DNMT3a* appears to characterize multiple types of human tumors (Xie et al. 1999). Four spliced forms of DNMT3b with altered enzymatic activity were expressed in a tissue-specific manner (Robertson et al. 1999). Future study will be needed to elucidate the possible roles of DNMT3 family members in tumorigenesis, *de novo* tissue-specific gene methylation and transcriptional regulation in somatic tissues.

Altered CpG island methylation, chromatin organization and transcriptional regulation

Much experimental evidence has documented the association of CpG island methylation and gene transcriptional inactivity but only recently have the underlying mechanisms of transcriptional silencing by methylation been partially clarified. One possible mechanism of transcriptional repression is the direct interference by methylation with the binding of sequence-specific transcription factors, such as AP-2, E2F and NFκB to DNA (Hermann & Doerfler 1991). A second possibility is that methylated CpG sequences recruit transcriptional co-repressors like mSin3A, DMAP1, TSG101 or Mi2, thereby contributing to transcriptional repression. Finally, chromatin structure is emerging as an important and more generalized mechanism to silence a variety of methylated tissue-specific and imprinted genes by HDAC family members. The deacetylation of lysine groups

of histones H3 and H4 allows ionic interactions between positively charged lysines and negatively charged DNA, resulting in a more compact nucleosome structure that limits gene activity. The discovery of the family of methyl-CpG-binding proteins provides a mechanistic linkage between DNA methylation and histone deacetylation as mediators of gene transcription. To date, six methyl-CpG-binding proteins including MeCP2, MBD1, MBD2a, MBD2b and MBD3 have been identified in vertebrates (Nan *et al.* 1998, Wade *et al.* 1998, Ng *et al.* 1999, 2000, Snape 2000). The common functional features for these proteins are that they bind to methyl-CpGs in DNA and frequently associate with members of the HDAC family, which currently includes eight distinct members (Ng & Bird 2000). That these processes might collaborate to regulate gene expression is demonstrated by a recent study, showing that multiple hypermethylated genes, such as *MLH1*, *TIMP-3*, *CDKN2B* and *CDKN2A*, could be robustly reactivated by a combination of DNMT1 and HDAC inhibition, suggesting that DNMT1 and HDAC are both essential in the silencing process in these colon cancer cells (Cameron *et al.* 1999). The above observation was confirmed by a very recent study that the known DNA methylation machinery protein, DNMT1 itself, is implicated in forming transcriptionally repressive complexes with HDAC as well as other co-repressors (Rountree *et al.* 2000).

DNA methylation patterns in normal tissues and cancer

Cell type and tissue-specific methylation patterns are established during early development, in part through the action of the *de novo* Dnmt3a and 3b (Okano *et al.* 1999). The sperm genome is extensively methylated while the oocyte genome is not. After fertilization, genes are demethylated and then remethylated before implantation. As the embryoblast differentiates, tissue-specific genes are demethylated in a tissue-specific fashion while housekeeping genes remain demethylated from fertilization through organogenesis (Bestor 1998).

CpG islands are generally unmethylated in normal adult tissues with the exception of transcriptionally silent genes on the inactive X-chromosome and imprinted genes like the H19 gene (Tremblay *et al.* 1995). Conversely, most neoplastic tissues demonstrate whole genomic hypomethylation and local promoter hypermethylation in certain critical tumor suppressor and growth regulatory genes (Baylin *et al.* 1998). The mechanism responsible for this type of pattern remains largely unclear. It is believed that the cell cycle checkpoint gene, *p21WAF1/CIP1*, may play a role in methylation regulation (Baylin *et al.* 1998). Since p21 competes with DNMT1 binding to PCNA, loss of p21 function may increase DNMT1 at replication sites (Chuang *et al.* 1997). In addition, mutation of another cell cycle gene, Rb, may play a role as

Rb mutation in its A/B pocket domain might disrupt the function of the transcriptionally repressive protein complex that involves Rb, DNMT1 and HDAC. Mistargeting of DNMT1 could then result (Robertson *et al.* 2000). Together, these observations are beginning to shed light on the paradox of global hypomethylation, increased CpG island hypermethylation, and increased DNMT1 activity in tumor cells.

DNA methylation and genetic instability

DNA methylation changes may ultimately lead to the genetic instability characteristic of cancer in several ways. First, 5mCs serve as sites of transition mutations by the hydrolytic deamination of 5mC to thymine. For example, such mutations frequently occur in the well-known p53 tumor suppressor gene (Magewu & Jones 1994). Similar point mutations characterize the mutations found in several other important genes like Rb, and c-H-ras-1 (Ghazi *et al.* 1990).

Secondly, epigenetic inactivation of certain critical genes in cancer by promoter methylation may predispose to genetic instability (Herman & Baylin 2000). For instance, methylation of *MLH1*, a gene involved in mismatch repair, precedes the MIN+ phenotype in sporadic colon, gastric and endometrial cancers (Esteller *et al.* 1999a). Further, there is a striking correlation between mismatch repair, genetic instability and methylation capacity in colon cancer cell models (Lengauer *et al.* 1997, 1998). In addition, promoter CpG island methylation and resulting inactivation of the detoxifying π -class glutathione S-transferase (GST) can lead to accumulation of oxygen radicals and subsequent DNA damage. The resulting adenine or guanine mutations are implicated in carcinogenesis in prostate, breast and other tissues (Lee *et al.* 1994, Henderson *et al.* 1998, Matsui *et al.* 2000). Further, a p53-inducible gene, 14-3-3 σ is methylated and inactivated in many breast cancers. Loss of its expression may also facilitate the accumulation of genetic damages (Ferguson *et al.* 2000).

Apart from regional hypermethylation of some critical tumor suppressor genes, genome-wide hypomethylation is an important feature in cancer. This also could contribute to genetic instability (Schmutte & Fishel 1999).

Methylation of critical tumor suppressor and growth regulatory genes in breast cancer

A large body of evidence has demonstrated that CpG island hypermethylation is implicated in loss of expression of a variety of critical genes in breast cancer. Some important genes inactivated by methylation in breast cancer are summarized in Table 1 and described below. They fall into several broad categories including cell cycle regulating, steroid receptor, tumor susceptibility, carcinogen

Table 1 Frequency of methylation of representative tumor suppression and growth regulatory genes in breast cancer.

Gene	Function	Incidence (%)	Reference
<i>p16^{INK4a}</i>	Cyclin-dependent kinase inhibitor	15	Herman et al. (1995)
<i>14-3-3σ</i>	G2 checkpoint	91	Ferguson et al. (2000)
<i>ERα</i>	Steroid receptor	50	Ottaviano et al. (1994)
<i>PR</i>	Steroid receptor	40	Lapidus et al. (1996)
<i>RARβ2</i>	Steroid receptor	25	Sirchia et al. (2000)
<i>BRCA1</i>	DNA damage repair	15	Dobrovic & Simpfendorfer (1997)
<i>GSTP1</i>	Carcinogen detoxification	30	Esteller et al. (1998)
<i>E-cadherin</i>	Epithelial cell-cell adhesion	50	Graff et al. (1995)
<i>TIMP-3</i>	Inhibition of MMPs	25	Bachman et al. (1999)

detoxification, cell adhesion and inhibitors of matrix metalloproteinases (MMPs) genes.

Methylation of cell cycle-related genes in breast cancer

p16/p16^{INK4A}/CDKN2A/MTS methylation and breast cancer

The *p16* gene is located on chromosome 9p21. It encodes a cyclin-dependent kinase inhibitor, *p16^{INK4A}*, that regulates the transition from G1- to S-phase via its effect on Rb phosphorylation (Liggett & Sidransky 1998). The transcription of the *p16^{INK4A}* gene can yield two distinct transcripts (α or β) that code for two functionally distinct proteins, *p16^{INK4A}* and *p19^{ARF}*. These two transcripts share identical second and third exons but have distinct first exons (Sharpless & DePinho 1999). Loss of *p16^{INK4A}* resulting from homozygous deletion, methylation of *p16* promoter or point mutation is a common feature of many cancers. Methylation of the 5' promoter and exon 1 regions is observed in both human breast cancer cell lines (Table 2) and 20–30% of primary breast cancers (Herman et al. 1995, Woodcock et al. 1999). The methylation phenotype is associated with loss of expression at both mRNA and protein levels although it does not correlate with some important clinical parameters in some relatively small cohort studies. For example, a study of 97 patients with breast cancer showed no association between *p16* methylation and overall or disease-free survival (Hui et al. 2000).

Finally, the stepwise inactivation of cyclin D-dependent kinase inhibitor *p16^{INK4A}* in human mammary epithelial cells

(HMEC) is associated with progressive methylation of the *p16* promoter CpG island. This allows HMECs to escape from M0 proliferation block, thereby identifying CpG methylation together with *p16* silencing as a possible contributor to breast tumorigenesis (Foster et al. 1998).

14-3-3 σ gene inactivation by methylation

The *14-3-3 σ* gene (also known as *HME1*), is localized at chromosome 1p35, and is a member of a gene family responsible for instituting the G₂ cell cycle checkpoint in response to DNA damage in human (Chan et al. 1999). Normally expression of σ is induced in response to DNA damage, and it causes cells to arrest in G₂. However, σ protein expression was downregulated in a significant fraction of primary bladder, colon and breast tumors (Celis et al. 1999). Studies of the molecular mechanisms responsible for the reduced expression have implicated hypermethylation of the CpG-rich exon 1 region of the gene, instead of genetic alterations such as loss of heterozygosity (LOH) and intragenic mutations in breast cancer (Ferguson et al. 2000). DNA from HMECs, immortal MCF-10A and HBL100 cells and two breast cancer cell lines, MCF-7 and MDA-MB-231, were unmethylated at the σ locus. In contrast, Hs578t and MDA-MB-435 cells were fully methylated as demonstrated by bisulfite genomic sequencing and methylation specific PCR (MSP) analyses. The use of 5-aza-2'-deoxycytidine (5-aza-dC) to treat the methylated non-expressing lines *in vitro* led to induction of transcription, further supporting the role of CpG island methylation in its repression. In addition, six DNA samples from microdissected normal mammary epithelial cells demonstrated an unmethylated pattern while 32 samples from microdissected breast carcinomas were methylated. Together, these cell line- and tissue-based studies support a role for methylation in the loss of *14-3-3 σ* expression in breast cancer.

Methylation of steroid receptor genes in breast cancer

The methylation of three members of the steroid hormone superfamily has been extensively studied in breast cancer models. These include estrogen receptor (ER) α , progesterone receptor (PR) and retinoic acid receptor- β (RAR β).

ER α methylation and hormone resistance

Steroid hormones, particularly estrogen, have long been linked to mammary carcinogenesis (Fishman et al. 1995). The role of estrogen and its catechol metabolite in breast cancer initiation and promotion is a continuing area of controversy (Yager 2000). That 17 β -estradiol stimulates the growth of certain breast cancers via functional ER is well recognized, and endocrine therapy is an established and important part of breast cancer management (Ruiz-Cabello et

Table 2 Methylation status of critical tumor suppressor and growth regulatory genes in human breast cancer cell lines.

Cell line	Gene							
	<i>p16</i>	<i>14-3-3σ</i>	<i>ER</i>	<i>PR</i>	<i>RAR-β2</i>	<i>BRCA1</i>	<i>GSTP1</i>	<i>E-cadherin</i>
HMECs	U/M	U	U	ND	U	U	ND	ND
HBL-100	U	U	ND	ND	ND	U	ND	ND
MCF-7	D	U	U	U	M	U	M	U
T47-D	M	ND	U	U	U	U	M	U
ZR75-1	U/M	ND	U	U	M	U	M	ND
MDA-MB-231	D	U	M	M	M	U	U	ND
MDA-MB-468	U	ND	M	M	M	U	ND	U
MDA-MB-435	ND	M	M	M	U	U	ND	M
Hs578t	U	M	M	M	U	U	U	ND
MCF7/Adr	ND	ND	M	ND	ND	U	ND	ND

U: unmethylated; M: methylated; U/M: partially methylated; D: biallelic deletion; ND: not done.

al. 1995, Davidson 2000). The presence of ER in breast tumors is a predictive marker for response to hormone therapy. However, up to one-third of breast carcinomas lack ER at the time of diagnosis and a proportion of cancers that are initially ER-positive lose ER during tumor progression (Hortobagyi 1998). Genetic alterations, such as homozygous deletion, LOH or ER gene mutation have not been reported to play a major role in loss of ER expression.

However, recent studies have shown that epigenetic alteration appears to play a role in inactivation of the gene (Ottaviano *et al.* 1994). The ER gene, located at chromosome 6q25.1, has a CpG island in its promoter and first exon regions. As demonstrated by Southern and methylation-specific PCR analyses, the ER CpG island is unmethylated in normal breast tissue and ER-positive tumor cell lines, such as MCF-7, T47-D and ZR75-1; it is methylated in ~50% of unselected primary breast cancers and most ER-negative cancer cell lines, e.g. MDA-MB-231, MDA-MB-435, MDA-MB-468, Hs578t and MCF-7/Adr (Table 2) (Lapidus *et al.* 1998). The ER CpG island methylation is associated with reduced or absent ER mRNA expression. The use of the methyltransferase inhibitors 5-aza-cytidine (5-aza-C) and 5-aza-dC, led to partial demethylation and restoration of ER mRNA expression, and synthesis of functional ER protein (Ferguson *et al.* 1995). Restored ER function was documented by eliciting estrogen response element-driven promoter activity from an exogenous plasmid as well as expression of the ER-responsive gene, PR.

In order to study *de novo* ER gene methylation *in vitro*, DNMT1 activity levels were measured in a panel of breast cancer cell lines. Expression of DNMT1 at both RNA and protein levels in ER-negative breast cancer cell lines is significantly elevated in ER-negative breast cancer cell lines compared with their ER-positive counterparts (Ottaviano *et al.* 1994). Detailed studies showed that DNMT1 expression was tightly correlated with S-phase fraction in ER-positive cells, while ER-negative cells expressed DNMT1 throughout the cell cycle, suggesting its expression is dysregulated in

ER-negative breast cancer cells (Nass *et al.* 1999). The data also showed that DNMT1 and p21 expression are inversely correlated in the breast cancer cell lines examined. Studies using DNMT1 antisense constructs showed that decreased expression of DNMT1 protein is linked to increased p21 protein expression. Since p21 competes with DNMT1 for targeting to PCNA, the increased p21 may lead to inhibition of DNA replication and methylation in these tumor cells (Chuang *et al.* 1997).

A related question is whether an inactive chromatin structure mediated by HDAC is involved in ER gene silencing. In fact, inhibition of HDAC by HDAC inhibitor (A, trichostatin TSA) induced ER transcript by 5-fold in a panel of well-characterized ER-negative MDA-MB 231, Hs578t and MCF-7/Adr cell lines. This transcriptional reactivation was associated with increased sensitivity to DNaseI at the ER locus without alteration of the methylated CpG sites, suggesting that open chromatin structure is associated with ER expression even in the presence of ER CpG island methylation (Yang *et al.* 2000). Our findings identified a role for both DNA methylation and histone acetylation in the regulation of ER gene transcription.

PR gene methylation

The PR gene, located at chromosome 11q13, also has a CpG island in its first exon (Lapidus *et al.* 1996). The PR gene encodes two isoforms, hPR_A (79 kDa) and hPR_B (109 kDa), which differ in both their N-terminal sequences and biological activities. The hPR_B transcript is preferentially induced by ER while the hPR_A is not. Since ligand-bound ER is a major transcriptional activator of hPR_B gene expression, the presence of PR is indicative of functional ER. PR gene methylation has been demonstrated by Southern analysis in ~40% of PR-negative breast tumors and several PR-negative breast cancer cell lines (Table 2). A possible functional role for PR CpG island methylation is suggested by the observation that treatment of PR-negative MDA-MB-231 cells with 5-aza-dC in the presence of estrogen led to partial demethylation of the PR CpG island

and re-expression of PR gene. Co-treatment with both 5-aza-dC and a pure anti-estrogen, ICI 182,780, prevented PR reactivation, suggesting that demethylation alone is not sufficient to reactivate PR expression. Rather it appears that ER-mediated chromatin remodeling is essential and sufficient to activate PR gene expression even in the presence of a methylated PR CpG island (Ferguson *et al.* 1998).

RAR β 2 gene methylation

RAR α , - β and - γ and retinoid X receptors- α , - β and - γ are also members of the nuclear receptor superfamily (Minucci & Pelicci 1999). All six of these receptors are ligand-activated transcription factors (Chambon 1996). The RAR β gene, located at chromosome 3p24, appears to play an important role in limiting the growth of certain tumor types, including breast, lung and others. RAR β 2 expression is often reduced or lost in breast cancer cells and they become resistant to induction by all-trans-retinoic acid (ATRA) (Swisshelm *et al.* 1994, Jing *et al.* 1996, Widschwendter *et al.* 1997). DNA methylation of the RAR β promoter is believed to be one of the factors linked to RAR β 2 downregulation in breast cancer (Widschwendter *et al.* 2000). RAR β promoter methylation has been demonstrated by Southern and methylation-specific PCR analyses in several RAR β 2-negative human breast cancer cell lines and about one-third of unselected primary breast cancer specimens (Sirchia *et al.* 2000). It is not observed in normal breast tissue or HMECs. There is no apparent correlation with ER status. As with other methylated genes, treatment of RAR β 2-negative cell lines with 5-aza-dC can partially re-induce RAR β 2 transcripts. Of note, the HDAC inhibitor TSA can also reactivate RAR β 2 expression in the presence of a methylated promoter, implicating inactive chromatin conformation as another possible regulatory process.

GSTP1 inactivation by methylation and its predisposition to genetic instability

Glutathione (GSH) and its corresponding cytosolic GSTs are involved in the detoxification pathway of xenobiotics and chemotherapeutic agents (Daniel 1993). They catalyze intracellular detoxification reactions by conjugating chemically reactive electrophiles to GSH, inactivating electrophilic carcinogens (Mannervik *et al.* 1985). The GSTs, encoded by several different genes at different loci, have been classified into α , μ , π and θ families. The π -class GST, encoded by the *GSTP1* gene, on chromosome 11, is of particular importance in breast cancer (Cairns *et al.* 1992, Gilbert *et al.* 1993). In cultured breast cancer cell lines an inverse relationship between *GSTP1* and *ER* gene expression has been reported, i.e. *GSTP1* was expressed in ER-negative but not in ER-positive lines (Table 2) although the underlying mechanism is unclear (Jhaveri & Morrow 1998). Treatment of the *GSTP1*-negative cell line MCF-7 with

5-aza-dC could induce mRNA expression and *de novo* synthesis of π -class protein. MSP-based studies of human tissues demonstrated that *GSTP1* promoter methylation is associated with gene inactivation in about 30% of primary breast carcinomas (Esteller *et al.* 1998). The detection of *GSTP1* methylation correlates with PR expression but there was no correlation with other clinical parameters such as the age at onset, histological type and grade, tumor size, nodal metastasis, DNA ploidy or ER status (Esteller *et al.* 1998). It is postulated that methylation-associated inactivation of *GSTP1* can result in adenine or guanine mutation by estrogen metabolites-DNA adduct formation and lead to genetic instability (Cavalieri *et al.* 1997).

BRCA1 methylation in sporadic breast cancer

The *BRCA1* gene, located at chromosome 17q21, is a well-known breast cancer susceptibility gene (Miki *et al.* 1994). Inhibition of *BRCA1* expression through antisense oligonucleotides increases the proliferation of normal and malignant mammary cells while overexpression of wild-type *BRCA1* suppresses MCF-7 breast cancer cell tumorigenesis in mice. Inherited mutations in the *BRCA1* gene account for one-half of inherited breast carcinomas (Friedman *et al.* 1994). However, in contrast to other tumor suppressor genes, somatic mutations in this gene have not been reported, despite the high degree of LOH at the *BRCA1* locus in sporadic breast and ovarian cancer (Merajver *et al.* 1995). Since *BRCA1* transcript and protein are either absent or reduced in sporadic breast cancer, DNA methylation has been proposed as an alternative mechanism to inactivate *BRCA1* (Dobrovic & Simpfendorfer 1997, Magdinier *et al.* 2000). By Southern analysis of the *BRCA1* promoter region, methylation was detected in 11% of sporadic breast cancer cases and was inversely correlated with expression of both ER and PR (Catteau *et al.* 1999). A study with 194 primary breast carcinomas demonstrated that the *BRCA1* promoter is methylated in 13% of unselected primary breast tumors (Esteller *et al.* 2000b). *BRCA1* methylation was especially associated with medullary and mucinous subtypes. As expected, *BRCA1* was unmethylated in all normal tissues examined as well as in 21 breast cancer cell lines (Table 2). The methylation was present in two breast cancer xenografts with concomitant loss of gene transcript. In this study one allele is lost by LOH and the other is inactivated by aberrant methylation, thereby resulting in biallelic inactivation and loss of functional *BRCA1* gene product. Finally, *BRCA1* methylation is only observed in breast and ovary cancers but not in tumors of colon or liver or leukemia, supporting a tissue-specific event for the process. Using chromatin immunoprecipitation and endonuclease chromatin accessibility assays, transcriptional repression of *BRCA1* by cytosine methylation is also mechanistically linked to histone deacetylation and inactive chromatin structure.

***E-cadherin* gene methylation and breast tumor progression**

The *E-cadherin* gene, located at chromosome 16q22.1, encodes a cell-surface adhesion protein that is important in maintaining homophilic cell–cell adhesion in epithelial tissues (Ilyas & Tomlinson 1997). Considerable evidence shows that loss of expression and function of E-cadherin protein contributes to increased proliferation, invasion and metastasis in breast cancer (Oka *et al.* 1992). Classical mutations and deletions clearly play a role in loss of the E-cadherin expression and function (Cleton-Jansen *et al.* 1994, Risinger *et al.* 1994). However, several studies demonstrate that epigenetic silencing of the *E-cadherin* gene by 5′CpG methylation occurs in some human breast cancer cell lines (Table 2) as well as about 50% of unselected primary breast cancers (Graff *et al.* 1995, Hiraguri *et al.* 1998). Its loss of expression is associated with tumor metastatic progression and decreased patient survival (Bringuier *et al.* 1993). Our recent work demonstrated that hypermethylation of the *E-cadherin* CpG island was evident in about 30% of ductal carcinomas *in situ* and increased significantly to nearly 60% of metastatic lesions (Nass *et al.* 2000), suggesting a role for this process in tumor progression.

Methylation and inactivation of *TIMP-3* gene

TIMP-3 belongs to a family of molecules that inhibit the proteolytic activity of the MMPs (Gomez *et al.* 1997, 1999). This protein can suppress primary tumor growth via its effects on tumor development, angiogenesis, invasion and metastasis (Uria *et al.* 1994). Methylation of its 5′CpG island has been associated with the loss of *TIMP-3* expression at both transcript and protein levels in several tumor types (Bachman *et al.* 1999). The *TIMP-3* promoter region is methylated in ~30% of human breast cancer cell lines as well as ~30% of primary breast tumors (Bachman *et al.* 1999). *TIMP-3* gene methylation has been associated with its loss of expression as its expression could be restored by 5-aza-dC treatment, again supporting a role for epigenetic mechanism in *TIMP-3* gene regulation.

Clinical implications of epigenetic regulation in breast cancer

Recognition of the important roles that DNA methylation and histone deacetylation play in gene expression in malignancy including breast cancer has led to consideration of how these findings can be exploited clinically. Two areas for exploration are the use of methylated markers for detection and prognosis, and the application of DNMT and HDAC inhibitors therapeutically to re-express silenced tumor suppressor and growth inhibitory genes.

Methylated markers for detection and prognosis

One of the tenets of epigenetic regulation is that CpG islands are generally unmethylated in normal adult tissues with rare exceptions. In contrast, as noted earlier, hypermethylation of various gene promoters is a common feature of malignant cells and these changes can occur early in the progression process. For example, about one-third of ductal carcinoma *in situ* lesions demonstrated methylation of the *E-cadherin* or *ER* promoter CpG islands (Nass *et al.* 2000). The availability of PCR-based strategies to assess methylation changes in minute quantities of biological materials raises the possibility that identification of methylated alleles might serve as a means of molecular risk assessment or detection (Herman *et al.* 1996). Indeed the ability to detect methylated genes in serum, urine, bronchoalveolar lavage fluid and lymph nodes derived from individuals with various types of malignancies has been described (Esteller *et al.* 1999b). In some cases methylated changes were also detected in samples banked well before the diagnosis of malignancy, raising the possibility that this type of analysis could facilitate risk assessment or early diagnosis strategies. One potential application in breast cancer would be the assessment of methylated markers using nipple duct lavage fluids or fine needle aspirates of the breast, as it could be hypothesized that their presence would predict a higher likelihood of subsequent breast cancer development. Other applications could include molecular staging of sentinel lymph nodes or bone marrow aspirates. Such studies will first require the development of a panel of methylated markers that would together identify virtually all breast cancers but that are unmethylated in normal tissues (Evron *et al.* 2001).

Finally, another possible clinical application for gene-specific methylation is prediction of prognosis or treatment outcome in certain cancers (Weinstein 2000). For example, the DNA-repair enzyme O⁶-methylguanine-DNA methyltransferase (MGMT) inhibits the ability of alkylating agents to kill tumor cells; loss of its expression by methylation might be expected to sensitize tumor cells to this class of agents. Indeed, methylation of MGMT is correlated with increased overall and disease-free survival and improved response to the alkylating agent carmustine in glioblastoma patients (Esteller *et al.* 2000a). Therefore, MGMT promoter methylation is a marker of good prognosis and predicts response to chemotherapy for glioblastoma patients.

DNMT and HDAC inhibitors for therapy of breast cancer

Although heritable, epigenetic changes are potentially reversible. Therefore, the prospect of intervening to reverse these changes as a possible means of reverting the malignant

phenotype is an attractive one. The availability of DNMT and HDAC inhibitors makes this a testable strategy.

DNMT inhibitors

The classic DNMT inhibitors, 5-aza-C and 5-aza-dC are cytosine analogs that are incorporated into replicating DNA (Jones & Taylor 1980, Jones 1985). The subsequent formation of covalent adducts between DNMT and 5-aza-C-substituted DNA irreversibly inactivates DNMT (Santi *et al.* 1984, Ferguson *et al.* 1997). One potential consequence then is the reactivation of previously methylated genes that have been transcriptionally silent. These analogs have been used clinically for treatment of patients with hemoglobinopathies, myelodysplasia and leukemia (Rivard *et al.* 1981, Ley *et al.* 1983). The clinical benefit observed has been associated with reactivation of previously silenced genes as, for example, the observation that 5-aza-treated sickle cell patients have improved hemoglobin and increased fetal hemoglobin (Nienhuis *et al.* 1985). Such strategies could potentially be applied to epithelial malignancies as well. For example, a gene microarray analysis of the effect of 5-aza-dC on HT29 colon cancer cells identified members of the interferon (IFN) response pathway as re-expressed genes (Karpf *et al.* 1999). Follow-up studies showed that 5-aza-dC treatment of these cells *in vitro* sensitized them to growth inhibition by exogenous IFN- α 2a. Thus unmasking of new therapeutic targets may be enhanced.

The elevated level of DNMT activity in cancer has also prompted development of more specific agents like antisense oligonucleotides to DNMT1. *In vitro* studies have shown that antisense treatment of human T24 bladder cancer cells and A549 non-small lung carcinoma cells can cause reduced DNMT1 levels, demethylation of the *p16^{INK4A}* gene promoter, re-expression of *p16^{INK4A}* protein, accumulation of the hypophosphorylated form of the Rb protein, increase in *p21^{WAF/CIP1}*, and cell growth arrest (Foumel *et al.* 1999). Thus, this specific targeting of DNMT1 can activate silent tumor suppressors.

HDAC inhibitors

Several structural classes of HDAC inhibitors have been identified. Phenylbutyrate, a short chain fatty acid that inhibits HDAC at millimolar concentrations and is approved for clinical use by the US Food and Drugs Administration (FDA), is an example of the first class (Carducci *et al.* 1996). The second class includes the hydroxamic acids, trichostatin A (Yoshida *et al.* 1990) and suberoylanalide hydroxamic acid (SAHA) (Richon *et al.* 1998). Nanomolar concentrations of TSA have been used widely in *in vitro* systems to inhibit HDAC activity and modify gene transcription. SAHA is also active in *in vitro* models at micromolar concentrations. It induces terminal cell differentiation with milk protein synthesis in MCF-7 cells (Richon *et al.* 1998). Further, its administration reduced the incidence of N-methyl-

nitrosourea-induced mammary carcinoma in rats without apparent toxicity (Desai *et al.* 1999). Phase I trials of SAHA are in progress (Marks *et al.* 2000). The third class of HDAC inhibitor, benzamide derivatives like MS-27-275, also showed marked activity against human tumor xenografts in mice (Saito *et al.* 1999). The fourth class includes cyclic tetrapeptides containing a 2-amino-8-oxo-9,10-epoxy-decanoyl (AOE) moiety such as trapoxin A (Kijima *et al.* 1993), while FR 901228 and apicidin are representative of the fifth class of cyclic peptides that lack an AOE moiety (Nakajima *et al.* 1998).

Combination strategies

Combined therapies may ultimately offer the best antineoplastic approach. If successful, such strategies could utilize agents that alone are ineffective but together result in the desired biological outcome. For example, sequential treatment of colon cancer cells with a demethylating agent followed by an HDAC inhibitor led to reactivation of multiple target genes whose expression was not affected by either agent alone (Cameron *et al.* 1999). Our own studies show that combined therapy of MDA-MB-231 cells with 5-aza followed by TSA results in re-expression of ER at lower doses than those needed with either single agent (Yang *et al.* 2001). Thus, it may be possible to use lower doses or shorter exposure durations to achieve the appropriate molecular effects, thereby increasing therapeutic effects, minimizing toxicity and improving ease of administration.

Alternatively, use of an 'epigenetic modifier' may enhance the response to another type of agent. The use of 5-aza and IFN in colon cancer cells was mentioned above. A human proof of principle for this general strategy is seen in the report of a patient with acute promyelocytic leukemia resistant to ATRA who developed a sustained remission after concomitant treatment with phenylbutyrate and ATRA (Warrell *et al.* 1998).

Conclusions

Taken together, substantial evidence demonstrates the importance of epigenetic mechanisms in the transcriptional regulation of critical tumor suppressor and growth regulatory genes in breast cancer. These genes include those that play crucial roles in DNA repair, cell cycle regulation, cell growth and cell-cell adhesion. These changes, along with the intrinsic ability of 5meC to function as a mutagen and the negative effects of dysregulated DNMT1 activity, can all contribute to breast cancer tumorigenesis and/or progression. A better understanding of epigenetic regulation of gene expression in a gene-specific and tissue-specific fashion will help efforts to modulate gene expression selectively with the ultimate goal of improved breast cancer prevention and therapy.

Acknowledgements

This work was supported by grants from the US National Institutes of Health (CA78352, P50CA88843 for N E D, and 2-T32CA09110 for X Y), and Department of Defense Breast Cancer Program (DAMD17-00-1-0310 for X Y) and (DAMD17-98-1-8116 for L Y).

References

- Antequera F & Bird A 1993 Number of CpG islands and genes in human and mouse. *PNAS* **90** 11995–11999.
- Bachman KE, Herman JG, Corn PG, Merlo A, Costello JF, Cavenee WK, Baylin SB & Graff JR 1999 Methylation-associated silencing of the tissue inhibitor of metalloproteinase-3 gene suggests a suppressor role in kidney, brain, and other human cancers. *Cancer Research* **59** 798–802.
- Bakin AV & Curran T 1999 Role of DNA 5-methylcytosine transferase in cell transformation by fos. *Science* **283** 387–390.
- Baylin SB, Herman JG, Graff JR, Vertino PM & Issa JP 1998 Alterations in DNA methylation: a fundamental aspect of neoplasia. *Advances in Cancer Research* **72** 141–196.
- Baylin SB, Esteller M, Rountree MR, Bachman KE, Schuebel K & Herman JG 2001 Aberrant patterns of DNA methylation, chromatin formation and gene expression in cancer. *Human Molecular Genetics* **10** 687–692.
- Bestor TH 1988 Cloning of a mammalian DNA methyltransferase. *Gene* **74** 9–12.
- Bestor TH 1998 Cytosine methylation and the unequal developmental potentials of the oocyte and sperm genomes. *American Journal of Human Genetics* **62** 1269–1273.
- Bestor TH 2000 The DNA methyltransferases of mammals. *Human Molecular Genetics* **9** 2395–2402.
- Bestor TH & Verdine GL 1994 DNA methyltransferases. *Current Opinion in Cell Biology* **6** 380–389.
- Bird AP 1980 DNA methylation and the frequency of CpG in animal DNA. *Nucleic Acids Research* **8** 1499–1504.
- Bird AP 1995 Gene number, noise reduction and biological complexity. *Trends in Genetics* **11** 94–100.
- Bringuier PP, Umbas R, Schaafsma HE, Karthaus HF, Debruyne FM & Schalken JA 1993 Decreased E-cadherin immunoreactivity correlates with poor survival in patients with bladder tumors. *Cancer Research* **53** 3241–3245.
- Cairns J, Wright C, Cattain AR, Hall AG, Cantwell BJ, Harris AL & Horne CH 1992 Immunohistochemical demonstration of glutathione S-transferases in primary human breast carcinomas. *Journal of Pathology* **166** 19–25.
- Cameron EE, Bachman KE, Myohanen S, Herman JG & Baylin SB 1999 Synergy of demethylation and histone deacetylase inhibition in the re-expression of genes silenced in cancer. *Nature Genetics* **21** 103–107.
- Carducci MA, Nelson JB, Chan-Tack KM, Ayyagari SR, Sweatt WH, Campbell PA, Nelson WG & Simons JW 1996 Phenylbutyrate induces apoptosis in human prostate cancer and is more potent than phenylacetate. *Clinical Cancer Research* **2** 379–387.
- Catteau A, Harris WH, Xu CF & Solomon E 1999 Methylation of the BRCA1 promoter region in sporadic breast and ovarian cancer: correlation with disease characteristics. *Oncogene* **18** 1957–1965.
- Cavaliere EL, Stack DE, Devanesan PD, Todorovic R, Dwivedy I, Higginbotham S, Johansson SL, Patil KD, Gross ML, Gooden JK, Ramanathan R, Cerny RL & Rogan EG 1997 Molecular origin of cancer: catechol estrogen-3,4-quinones as endogenous tumor initiators. *PNAS* **94** 10937–10942.
- Cedar H 1988 DNA methylation and gene activity. *Cell* **53** 3–4.
- Celis A, Rasmussen HH, Celis P, Basse B, Lauridsen JB, Ratz G, Hein B, Ostergaard M, Wolf H, Orntoft T & Celis JE 1999 Short-term culturing of low-grade superficial bladder transitional cell carcinomas leads to changes in the expression levels of several proteins involved in key cellular activities. *Electrophoresis* **20** 355–361.
- Chambon P 1996 A decade of molecular biology of retinoic acid receptors. *FASEB Journal* **10** 940–954.
- Chan MF, Liang G & Jones PA 2000 Relationship between transcription and DNA methylation. *Current Topics in Microbiology and Immunology* **249** 75–86.
- Chan TA, Hermeking H, Lengauer C, Kinzler KW & Vogelstein B 1999 14-3-3Sigma is required to prevent mitotic catastrophe after DNA damage. *Nature* **401** 616–620.
- Chuang LS, Ian HI, Koh TW, Ng HH, Xu G & Li BF 1997 Human DNA-(cytosine-5) methyltransferase-PCNA complex as a target for p21WAF1. *Science* **277** 1996–2000.
- Cleton-Jansen AM, Moerland EW, Kuipers-Dijkshoorn NJ, Callen DF, Sutherland GR, Hansen B, Devilee P & Cornelisse CJ 1994 At least two different regions are involved in allelic imbalance on chromosome arm 16q in breast cancer. *Genes, Chromosomes and Cancer* **9** 101–107.
- Cross SH & Bird AP 1995 CpG islands and genes. *Current Opinion in Genetics and Development* **5** 309–314.
- Daniel V 1993 Glutathione S-transferases: gene structure and regulation of expression. *Critical Reviews in Biochemistry and Molecular Biology* **28** 173–207.
- Davidson NE 2000 Combined endocrine therapy for breast cancer – new life for an old idea? *Journal of the National Cancer Institute* **92** 859–860.
- De Marzo AM, Marchi VL, Yang ES, Veeraswamy R, Lin X & Nelson WG 1999 Abnormal regulation of DNA methyltransferase expression during colorectal carcinogenesis. *Cancer Research* **59** 3855–3860.
- Desai D, El-Bayoumy K & Amin S 1999 Chemopreventive efficacy of suberanilohydroxamic acid (SAHA), a cytodifferentiating agent, against tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1 butanone (NNK)-induced lung tumorigenesis in female A/J mice. *Proceedings of the American Association for Cancer Research* **40** 365.
- Dobrovic A & Simpfendorfer D 1997 Methylation of the BRCA1 gene in sporadic breast cancer. *Cancer Research* **57** 3347–3350.
- Eads CA, Danenberg KD, Kawakami K, Saltz LB, Danenberg PV & Laird PW 1999 CpG island hypermethylation in human colorectal tumors is not associated with DNA methyltransferase overexpression. *Cancer Research* **59** 2302–2306.
- El-Deiry WS, Nelkin BD, Celano P, Yen RW, Falco JP, Hamilton SR & Baylin SB 1991 High expression of the DNA methyltransferase gene characterizes human neoplastic cells and progression stages of colon cancer. *PNAS* **88** 3470–3474.
- Esteller M, Corn PG, Urena JM, Gabrielson E, Baylin SB & Herman JG 1998 Inactivation of glutathione S-transferase P1 gene by promoter hypermethylation in human neoplasia. *Cancer Research* **58** 4515–4518.
- Esteller M, Catusas L, Matias-Guiu X, Mutter GL, Prat J, Baylin SB & Herman JG 1999a hMLH1 promoter hypermethylation is

- an early event in human endometrial tumorigenesis. *American Journal of Pathology* **155** 1767–1772.
- Esteller M, Sanchez-Cespedes M, Rosell R, Sidransky D, Baylin SB & Herman JG 1999b Detection of aberrant promoter hypermethylation of tumor suppressor genes in serum DNA from non-small cell lung cancer patients. *Cancer Research* **59** 67–70.
- Esteller M, Garcia-Foncillas J, Andion E, Goodman SN, Hidalgo OF, Vanaclocha V, Baylin SB & Herman JG 2000a Inactivation of the DNA-repair gene MGMT and the clinical response of gliomas to alkylating agents. *New England Journal of Medicine* **343** 1350–1354.
- Esteller M, Silva JM, Dominguez G, Bonilla F, Matias-Guiu X, Lerma E, Bussaglia E, Prat J, Harkes IC, Repasky EA, Gabrielson E, Schutte M, Baylin SB & Herman JG 2000b Promoter hypermethylation and BRCA1 inactivation in sporadic breast and ovarian tumors. *Journal of the National Cancer Institute* **92** 564–569.
- Evron E, Dooley W, Umbricht C, Rosenthal D, Sacchi N, Gabrielson E, Sorito AB, Hung DT, Ljung B-M, Davidson NE & Sukumar S 2001 Detection of breast cancer cells in ductal lavage fluid by methylation specific PCR. *The Lancet* **357** 1335–1336.
- Feinberg AP 2001 Cancer epigenetics takes center stage. *PNAS* **98** 392–394.
- Ferguson AT, Lapidus RG, Baylin SB & Davidson NE 1995 Demethylation of the estrogen receptor gene in estrogen receptor-negative breast cancer cells can reactivate estrogen receptor gene expression. *Cancer Research* **55** 2279–2283.
- Ferguson AT, Vertino PM, Spitzner JR, Baylin SB, Muller MT & Davidson NE 1997 Role of estrogen receptor gene demethylation and DNA methyltransferase. DNA adduct formation in 5-aza-2'deoxyctidine-induced cytotoxicity in human breast cancer cells. *Journal of Biological Chemistry* **272** 32260–32266.
- Ferguson AT, Lapidus RG & Davidson NE 1998 Demethylation of the progesterone receptor CpG island is not required for progesterone receptor gene expression. *Oncogene* **17** 577–583.
- Ferguson AT, Evron E, Umbricht CB, Pandita TK, Chan TA, Hermeking H, Marks JR, Lambers AR, Futreal PA, Stampfer MR & Sukumar S 2000 High frequency of hypermethylation at the 14–3–3 sigma locus leads to gene silencing in breast cancer. *PNAS* **97** 6049–6054.
- Fishman J, Osborne MP & Telang NT 1995 The role of estrogen in mammary carcinogenesis. *Annals of the New York Academy of Sciences* **768** 91–100.
- Forne T, Oswald J, Dean W, Saam JR, Bailleul B, Dandolo L, Tilghman SM, Walter J & Reik W 1997 Loss of the maternal H19 gene induces changes in Igf2 methylation in both cis and trans. *PNAS* **94** 10243–10248.
- Foster SA, Wong DJ, Barrett MT & Galloway DA 1998 Inactivation of p16 in human mammary epithelial cells by CpG island methylation. *Molecular and Cellular Biology* **18** 1793–1801.
- Fournel M, Sapieha P, Beaulieu N, Besterman JM & MacLeod AR 1999 Down-regulation of human DNA-(cytosine-5) methyltransferase induces cell cycle regulators p16(ink4A) and p21(WAF/Cip1) by distinct mechanisms. *Journal of Biological Chemistry* **274** 24250–24256.
- Friedman LS, Ostermeyer EA, Szabo CI, Dowd P, Lynch ED, Rowell SE & King MC 1994 Confirmation of BRCA1 by analysis of germline mutations linked to breast and ovarian cancer in ten families. *Nature Genetics* **8** 399–404.
- Fuks F, Burgers WA, Brehm A, Hughes-Davies L & Kouzarides T 2000 DNA methyltransferase Dnmt1 associates with histone deacetylase activity. *Nature Genetics* **24** 88–91.
- Ghazi H, Magewu AN, Gonzales F & Jones PA 1990 Changes in the allelic methylation patterns of c-H-ras-1, insulin and retinoblastoma genes in human development. *Development. Supplement* 115–123.
- Gilbert L, Elwood LJ, Merino M, Masood S, Barnes R, Steinberg SM, Lazarous DF, Pierce L, d'Angelo T, Moscow JA, Townsend AT & Cowan KH 1993 A pilot study of pi-class glutathione S-transferase expression in breast cancer: correlation with estrogen receptor expression and prognosis in node-negative breast cancer. *Journal of Clinical Oncology* **11** 49–58.
- Gomez DE, Alonso DF, Yoshiji H & Thorgeirsson UP 1997 Tissue inhibitors of metalloproteinases: structure, regulation and biological functions. *European Journal of Cell Biology* **74** 111–122.
- Gomez DE, De Lorenzo MS, Alonso DF & Andrade ZA 1999 Expression of metalloproteinases (MMP-1, MMP-2, and MMP-9) and their inhibitors (TIMP-1 and TIMP-2) in schistosomal portal fibrosis. *American Journal of Tropical Medicine and Hygiene* **61** 9–13.
- Graff JR, Herman JG, Lapidus RG, Chopra H, Xu R, Jarrard DF, Isaacs WB, Pitha PM, Davidson NE & Baylin SB 1995 E-cadherin expression is silenced by DNA hypermethylation in human breast and prostate carcinomas. *Cancer Research* **55** 5195–5199.
- Hansen RS, Wijmenga C, Luo P, Stanek AM, Canfield TK, Weemaes CM & Gartler SM 1999 The DNMT3B DNA methyltransferase gene is mutated in the ICF immunodeficiency syndrome. *PNAS* **96** 14412–14417.
- Heard E & Avner P 1994 Role play in X-inactivation. *Human Molecular Genetics* **3** 1481–1485.
- Heard E, Clerc P & Avner P 1997 X-chromosome inactivation in mammals. *Annual Review of Genetics* **31** 571–610.
- Henderson CJ, Smith AG, Ure J, Brown K, Bacon EJ & Wolf CR 1998 Increased skin tumorigenesis in mice lacking pi class glutathione S-transferases. *PNAS* **95** 5275–5280.
- Herman JG & Baylin SB 2000 Promoter-region hypermethylation and gene silencing in human cancer. *Current Topics in Microbiology and Immunology* **249** 35–54.
- Herman JG, Merlo A, Mao L, Lapidus RG, Issa JP, Davidson NE, Sidransky D & Baylin SB 1995 Inactivation of the CDKN2/p16/MTS1 gene is frequently associated with aberrant DNA methylation in all common human cancers. *Cancer Research* **55** 4525–4530.
- Herman JG, Graff JR, Myohanen S, Nelkin BD & Baylin SB 1996 Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. *PNAS* **93** 9821–9826.
- Hermann R & Doerfler W 1991 Interference with protein binding at AP2 sites by sequence-specific methylation in the late E2A promoter of adenovirus type 2 DNA. *FEBS Letters* **281** 191–195.
- Hiraguri S, Godfrey T, Nakamura H, Graff J, Collins C, Shayesteh L, Doggett N, Johnson K, Wheelock M, Herman J, Baylin S, Pinkel D & Gray J 1998 Mechanisms of inactivation of E-cadherin in breast cancer cell lines. *Cancer Research* **58** 1972–1977.
- Hortobagyi GN 1998 Treatment of breast cancer. *New England Journal of Medicine* **339** 974–984.
- Hui R, Macmillan RD, Kenny FS, Musgrove EA, Blamey RW, Nicholson RI, Robertson JF & Sutherland RL 2000 INK4a gene

- expression and methylation in primary breast cancer: overexpression of p16INK4a messenger RNA is a marker of poor prognosis. *Clinical Cancer Research* 6 2777–2787.
- Ilyas M & Tomlinson IP 1997 The interactions of APC, E-cadherin and beta-catenin in tumour development and progression. *Journal of Pathology* 182 128–137.
- Issa JP, Vertino PM, Wu J, Sazawal S, Celano P, Nelkin BD, Hamilton SR & Baylin SB 1993 Increased cytosine DNA-methyltransferase activity during colon cancer progression. *Journal of the National Cancer Institute* 85 1235–1240.
- Jhaveri MS & Morrow CS 1998 Methylation-mediated regulation of the glutathione S-transferase P1 gene in human breast cancer cells. *Gene* 210 1–7.
- Jing Y, Xu XC, Lotan R, Waxman S & Mira-y-Lopez R 1996 Human breast carcinoma slice cultures retain retinoic acid sensitivity. *Brazil Journal of Medical and Biological Research* 29 1105–1108.
- Jones PA 1985 Effects of 5-azacytidine and its 2'-deoxyderivative on cell differentiation and DNA methylation. *Pharmacology and Therapy* 28 17–27.
- Jones PA & Taylor SM 1980 Cellular differentiation, cytidine analogs and DNA methylation. *Cell* 20 85–93.
- Karpf AR, Peterson PW, Rawlins JT, Dalley BK, Yang Q, Albertsen H & Jones DA 1999 Inhibition of DNA methyltransferase stimulates the expression of signal transducer and activator of transcription 1, 2, and 3 genes in colon tumor cells. *PNAS* 96 14007–14012.
- Keshet I, Yisraeli J & Cedar H 1985 Effect of regional DNA methylation on gene expression. *PNAS* 82 2560–2564.
- Kijima M, Yoshida M, Sugita K, Horinouchi S & Beppu T 1993 Trapoxin, an antitumor cyclic tetrapeptide, is an irreversible inhibitor of mammalian histone deacetylase. *Journal of Biological Chemistry* 268 22429–22435.
- Laayoun A & Smith SS 1995 Methylation of slipped duplexes, snapbacks and cruciforms by human DNA(cytosine-5)-methyltransferase. *Nucleic Acids Research* 23 1584–1589.
- Lapidus RG, Ferguson AT, Ottaviano YL, Parl FF, Smith HS, Weitzman SA, Baylin SB, Issa JPJ & Davidson NE 1996 Methylation of estrogen and progesterone receptor gene 5' CpG islands correlates with lack of estrogen and progesterone receptor gene expression in breast tumors. *Clinical Cancer Research* 2 805–810.
- Lapidus RG, Nass SJ, Butash KA, Parl FF, Weitzman SA, Graff JG, Herman JG & Davidson NE 1998 Mapping of ER gene CpG island methylation-specific polymerase chain reaction. *Cancer Research* 58 2515–2519.
- Lee WH, Morton RA, Epstein JI, Brooks JD, Campbell PA, Bova GS, Hsieh WS, Isaacs WB & Nelson WG 1994 Cytidine methylation of regulatory sequences near the pi-class glutathione S-transferase gene accompanies human prostatic carcinogenesis. *PNAS* 91 11733–11737.
- Lei H, Oh SP, Okano M, Juttermann R, Goss KA, Jaenisch R & Li E 1996 *De novo* DNA cytosine methyltransferase activities in mouse embryonic stem cells. *Development* 122 3195–3205.
- Lengauer C, Kinzler KW & Vogelstein B 1997 Genetic instability in colorectal cancers. *Nature* 386 623–627.
- Lengauer C, Kinzler KW & Vogelstein B 1998 Genetic instabilities in human cancers. *Nature* 396 643–649.
- Ley TJ, DeSimone J, Noguchi CT, Turner PH, Schechter AN, Heller P & Nienhuis AW 1983 5-Azacytidine increases gamma-globin synthesis and reduces the proportion of dense cells in patients with sickle cell anemia. *Blood* 62 370–380.
- Li E, Bestor TH & Jaenisch R 1992 Targeted mutation of the DNA methyltransferase gene results in embryonic lethality. *Cell* 69 915–926.
- Li E, Beard C, Forster AC, Bestor TH & Jaenisch R 1993 DNA methylation, genomic imprinting, and mammalian development. *Cold Spring Harbor Symposia on Quantitative Biology* 58 297–305.
- Liggett WH Jr & Sidransky D 1998 Role of the p16 tumor suppressor gene in cancer. *Journal of Clinical Oncology* 16 1197–1206.
- Magdinier F, Billard LM, Wittmann G, Frappart L, Benchaib M, Lenoir GM, Guerin JF & Dante R 2000 Regional methylation of the 5' end CpG island of BRCA1 is associated with reduced gene expression in human somatic cells. *FASEB Journal* 14 1585–1594.
- Magewu AN & Jones PA 1994 Ubiquitous and tenacious methylation of the CpG site in codon 248 of the p53 gene may explain its frequent appearance as a mutational hot spot in human cancer. *Molecular and Cellular Biology* 14 4225–4232.
- Mannervik B, Alin P, Guthenberg C, Jansson H, Tahir MK, Warholm M & Jornvall H 1985 Identification of three classes of cytosolic glutathione transferase common to several mammalian species: correlation between structural data and enzymatic properties. *PNAS* 82 7202–7206.
- Marks PA, Richon VM & Rifkind RA 2000 Histone deacetylase inhibitors: inducers of differentiation or apoptosis of transformed cells. *Journal of the National Cancer Institute* 92 1210–1216.
- Matsui A, Ikeda T, Enomoto K, Hosoda K, Nakashima H, Omae K, Watanabe M, Hibi T & Kitajima M 2000 Increased formation of oxidative DNA damage, 8-hydroxy-2'-deoxyguanosine, in human breast cancer tissue and its relationship to GSTP1 and COMT genotypes. *Cancer Letters* 151 87–95.
- Merajver SD, Pham TM, Caduff RF, Chen M, Poy EL, Cooney KA, Weber BL, Collins FS, Johnston C & Frank TS 1995 Somatic mutations in the BRCA1 gene in sporadic ovarian tumours. *Nature Genetics* 9 439–443.
- Miki Y, Swensen J, Shattuck-Eidens D, Futreal PA, Harshman K, Tavtigian S, Liu Q, Cochran C, Bennett LM, Ding W, Bell R, Rosenthal J, Hussey C, Tran T, McClure M, Frye C, Hattier T, Phelps R, Haugen-Strano A, Katcher H, Yakumo K, Gholami Z, Shaffer D, Stone S, Bayer S, Wray C, Bogden R, Dayananth P, Ward J, Tonin P, Narod S, Bristow PK, Norris FH, Helvering L, Morrison P, Rostek P, Lai M, Barrett JC, Lewis C, Neuhausen S, Cannon-Albright L, Goldgar D, Wiseman R, Kamb A & Skolnick MH 1994 A strong candidate for the breast and ovarian cancer susceptibility gene BRCA1. *Science* 266 66–71.
- Minucci S & Pelicci PG 1999 Retinoid receptors in health and disease: co-regulators and the chromatin connection. *Seminars in Cell Development and Biology* 10 215–225.
- Muller K, Heller H & Doerfler W 2001 Foreign DNA integration: genome-wide perturbations of methylation and transcription in the recipient genomes. *Journal of Biological Chemistry* 276 14271–14278.
- Nakajima H, Kim YB, Terano H, Yoshida M & Horinouchi S 1998 FR901228, a potent antitumor antibiotic, is a novel histone deacetylase inhibitor. *Experimental Cell Research* 241 126–133.
- Nan X, Ng HH, Johnson CA, Laherty CD, Turner BM, Eisenman RN & Bird A 1998 Transcriptional repression by the methyl-CpG-binding protein MeCP2 involves a histone deacetylase complex. *Nature* 393 386–389.

- Nass SJ, Ferguson AT, El-Ashry D, Nelson WG & Davidson NE 1999 Expression of DNA methyl-transferase (DMT) and the cell cycle in human breast cancer cells. *Oncogene* **18** 7453–7461.
- Nass SJ, Herman JG, Gabrielson E, Iversen PW, Parl FF, Davidson NE & Graff JR 2000 Aberrant methylation of the estrogen receptor and E-cadherin 5' CpG islands increases with malignant progression in human breast cancer. *Cancer Research* **60** 4346–4348.
- Ng HH & Bird A 2000 Histone deacetylases: silencers for hire. *Trends in Biochemical Sciences* **25** 121–126.
- Ng HH, Zhang Y, Hendrich B, Johnson CA, Turner BM, Erdjument-Bromage H, Tempst P, Reinberg D & Bird A 1999 MBD2 is a transcriptional repressor belonging to the MeCP1 histone deacetylase complex. *Nature Genetics* **23** 58–61.
- Ng HH, Jeppesen P & Bird A 2000 Active repression of methylated genes by the chromosomal protein MBD1. *Molecular and Cellular Biology* **20** 1394–1406.
- Nienhuis AW, Ley TJ, Humphries RK, Young NS & Dover G 1985 Pharmacological manipulation of fetal hemoglobin synthesis in patients with severe beta-thalassemia. *Annals of the New York Academy of Sciences* **445** 198–211.
- Oka H, Shiozaki H, Kobayashi K, Tahara H, Kobayashi T, Takatsuka Y & Mori T 1992 Correlation between E-cadherin expression and metastasis in human breast cancer: preliminary report. *Nippon Geka Gakkai Zasshi* **93** 105.
- Okano M, Xie S & Li E 1998 Dnmt2 is not required for *de novo* and maintenance methylation of viral DNA in embryonic stem cells. *Nucleic Acids Research* **26** 2536–2540.
- Okano M, Bell DW, Haber DA & Li E 1999 DNA methyltransferases Dnmt3a and Dnmt3b are essential for *de novo* methylation and mammalian development. *Cell* **99** 247–257.
- Ottaviano YL, Issa JP, Parl FF, Smith HS, Baylin SB & Davidson NE 1994 Methylation of the estrogen receptor gene CpG island marks loss of estrogen receptor expression in human breast cancer cells. *Cancer Research* **54** 2552–2555.
- Pradhan S, Talbot D, Sha M, Benner J, Hornstra L, Li E, Jaenisch R & Roberts RJ 1997 Baculovirus-mediated expression and characterization of the full-length murine DNA methyltransferase. *Nucleic Acids Research* **25** 4666–4673.
- Ramchandani S, MacLeod AR, Pinar M, von Hofe E & Szyf M 1997 Inhibition of tumorigenesis by a cytosine-DNA, methyltransferase, antisense oligodeoxynucleotide. *PNAS* **94** 684–689.
- Reik W & Walter J 1998 Imprinting mechanisms in mammals. *Current Opinion in Genetics and Development* **8** 154–164.
- Rhee I, Jair KW, Yen RW, Lengauer C, Herman JG, Kinzler KW, Vogelstein B, Baylin SB & Schuebel KE 2000 CpG methylation is maintained in human cancer cells lacking DNMT1. *Nature* **404** 1003–1007.
- Richon VM, Emiliani S, Verdin E, Webb Y, Breslow R, Rifkin RA & Marks PA 1998 A class of hybrid polar inducers of transformed cell differentiation inhibits histone deacetylases. *PNAS* **95** 3003–3007.
- Risinger JI, Berchuck A, Kohler MF & Boyd J 1994 Mutations of the E-cadherin gene in human gynecologic cancers. *Nature Genetics* **7** 98–102.
- Rivard GE, Momparler RL, Demers J, Benoit P, Raymond R, Lin K & Momparler LF 1981 Phase I study on 5-aza-2'-deoxycytidine in children with acute leukemia. *Leukemia Research* **5** 453–462.
- Robertson KD, Uzvolgyi E, Liang G, Talmadge C, Sumegi J, Gonzales FA & Jones PA 1999 The human DNA methyltransferases (DNMTs) 1, 3a and 3b: coordinate mRNA expression in normal tissues and overexpression in tumors. *Nucleic Acids Research* **27** 2291–2298.
- Robertson KD, Ait-Si-Ali S, Yokochi T, Wade PA, Jones PL & Wolffe AP 2000 DNMT1 forms a complex with Rb, E2F1 and HDAC1 and represses transcription from E2F-responsive promoters. *Nature Genetics* **25** 338–342.
- Rountree MR, Bachman KE & Baylin SB 2000 DNMT1 binds HDAC2 and a new co-repressor, DMAP1, to form a complex at replication foci. *Nature Genetics* **25** 269–277.
- Ruiz-Cabello J, Berghmans K, Kaplan O, Lippman ME, Clarke R & Cohen JS 1995 Hormone dependence of breast cancer cells and the effects of tamoxifen and estrogen: 31P NMR studies. *Breast Cancer Research and Treatment* **33** 209–217.
- Saito A, Yamashita T, Mariko Y, Nosaka Y, Tsuchiya K, Ando T, Suzuki T, Tsuruo T & Nakanishi O 1999 A synthetic inhibitor of histone deacetylase, MS-27-275, with marked *in vivo* antitumor activity against human tumors. *PNAS* **96** 4592–4597.
- Santi DV, Norment A & Garrett CE 1984 Covalent bond formation between a DNA-cytosine methyltransferase and DNA containing 5-azacytosine. *PNAS* **81** 6993–6997.
- Schmutte C & Jones PA 1998 Involvement of DNA methylation in human carcinogenesis. *Biological Chemistry* **379** 377–388.
- Schmutte C & Fishel R 1999 Genomic instability: first step to carcinogenesis. *Anticancer Research* **19** 4665–4696.
- Sharpless NE & DePinho RA 1999 The INK4A/ARF locus and its two gene products. *Current Opinion in Genetics and Development* **9** 22–30.
- Sirchia SM, Ferguson AT, Sironi E, Subramanian S, Orlandi R, Sukumar S & Sacchi N 2000 Evidence of epigenetic changes affecting the chromatin state of the retinoic acid receptor beta2 promoter in breast cancer cells. *Oncogene* **19** 1556–1563.
- Slack A, Cervoni N, Pinar M & Szyf M 1999 DNA methyltransferase is a downstream effector of cellular transformation triggered by simian virus 40 large T antigen. *Journal of Biological Chemistry* **274** 10105–10112.
- Snape A 2000 MBDs mediate methylation, deacetylation and transcriptional repression. *Trends in Genetics* **16** 20.
- Swishelm K, Ryan K, Lee X, Ts'ou HC, Peacocke M & Sager R 1994 Down-regulation of retinoic acid receptor beta in mammary carcinoma cell lines and its up-regulation in senescing normal mammary epithelial cells. *Cell Growth and Differentiation* **5** 133–141.
- Tremblay KD, Saam JR, Ingram RS, Tilghman SM & Bartolomei MS 1995 A paternal-specific methylation imprint marks the alleles of the mouse H19 gene. *Nature Genetics* **9** 407–413.
- Uria JA, Ferrando AA, Velasco G, Freije JM & Lopez-Otin C 1994 Structure and expression in breast tumors of human TIMP-3, a new member of the metalloproteinase inhibitor family. *Cancer Research* **54** 2091–2094.
- Vanyushin BF, Tkacheva SG & Belozersky AN 1970 Rare bases in animal DNA. *Nature* **225** 948–949.
- Wade PA, Jones PL, Vermaak D & Wolffe AP 1998 A multiple subunit Mi-2 histone deacetylase from *Xenopus laevis* cofractionates with an associated Snf2 superfamily ATPase. *Current Biology* **8** 843–846.
- Warrell RP Jr, He LZ, Richon V, Calleja E & Pandolfi PP 1998 Therapeutic targeting of transcription in acute promyelocytic leukemia by use of an inhibitor of histone deacetylase. *Journal of the National Cancer Institute* **90** 1621–1625.

- Weinstein JN 2000 Pharmacogenomics – teaching old drugs new tricks. *The New England Journal of Medicine* **343** 1408–1409.
- Weiss A & Cedar H 1997 The role of DNA demethylation during development. *Genes and Cells* **2** 481–486.
- Widschwendter M, Berger J, Daxenbichler G, Muller-Holzner E, Widschwendter A, Mayr A, Marth C & Zeimet AG 1997 Loss of retinoic acid receptor beta expression in breast cancer and morphologically normal adjacent tissue but not in the normal breast tissue distant from the cancer. *Cancer Research* **57** 4158–4161.
- Widschwendter M, Berger J, Hermann M, Muller HM, Amberger A, Zeschneck M, Widschwendter A, Abendstein B, Zeimet AG, Daxenbichler G & Marth C 2000 Methylation and silencing of the retinoic acid receptor-beta2 gene in breast cancer. *Journal of the National Cancer Institute* **92** 826–832.
- Woodcock DM, Linsenmeyer ME, Doherty JP & Warren WD 1999 DNA methylation in the promoter region of the p16 (CDKN2/MTS-1/INK4A) gene in human breast tumours. *British Journal of Cancer* **79** 251–256.
- Wu J, Issa JP, Herman J, Bassett DE Jr, Nelkin BD & Baylin SB 1993 Expression of an exogenous eukaryotic DNA methyltransferase gene induces transformation of NIH 3T3 cells. *PNAS* **90** 8891–8895.
- Xie S, Wang Z, Okano M, Nogami M, Li Y, He WW, Okumura K & Li E 1999 Cloning, expression and chromosome locations of the human DNMT3 gene family. *Gene* **236** 87–95.
- Yager JD 2000 Endogenous estrogens as carcinogens through metabolic activation. *Journal of the National Cancer Institute Monographs* **27** 67–73.
- Yang X, Ferguson AT, Nass SJ, Phillips DL, Butash KA, Wang SM, Herman JG & Davidson NE 2000 Transcriptional activation of estrogen receptor alpha in human breast cancer cells by histone deacetylase inhibition. *Cancer Research* **60** 6890–6894.
- Yang X, Phillips DL, Ferguson AT, Herman JG & Davidson NE 2001 Synergistic reactivation of functional estrogen receptor by DNMT1 and HDAC inhibition in human breast cancer cells. *Proceedings of the American Association for Cancer Research* **42** 60.
- Yen RW, Vertino PM, Nelkin BD, Yu JJ, el-Deiry W, Cumaraswamy A, Lennon GG, Trask BJ, Celano P & Baylin SB 1992 Isolation and characterization of the cDNA encoding human DNA methyltransferase. *Nucleic Acids Research* **20** 2287–2291.
- Yoder JA, Walsh CP & Bestor TH 1997 Cytosine methylation and the ecology of intragenomic parasites. *Trends in Genetics* **13** 335–340.
- Yoshida M, Kijima M, Akita M & Beppu T 1990 Potent and specific inhibition of mammalian histone deacetylase both *in vivo* and *in vitro* by trichostatin A. *Journal of Biological Chemistry* **265** 17174–17179.

Role of DNA Methylation and Histone Acetylation in Steroid Receptor Expression in Breast Cancer^{1,4}

Lan Yan,² Xiaowei Yang,² and Nancy E. Davidson^{2,3}

DNA methylation is an epigenetic modification that is associated with transcriptional silencing of gene expression in mammalian cells. Hypermethylation of the promoter CpG islands contributes to the loss of gene function of several tumor related genes, including estrogen receptor α (ER) and progesterone receptor (PR). Gene expression patterns are also heavily influenced by changes in chromatin structure during transcription. Indeed both the predominant mammalian DNA methyltransferase (DNMT1), and the histone deacetylases (HDACs) play crucial roles in maintaining transcriptionally repressive chromatin by forming suppressive complexes at replication foci. These new findings suggest that epigenetic changes might play a crucial role in gene inactivation in breast cancer. Further, inhibition of DNA methylation and histone deacetylation might be a therapeutic strategy in breast cancer, especially for those cancers with ER and PR negative phenotypes.

KEY WORDS: Breast cancer; DNA methylation; histone acetylation; steroid receptor.

INTRODUCTION

DNA Methylation and Cancer-Related Gene Expression

Approximately 3–5% of the cytosine residues in the human genome are methylated (1). Seventy to eighty percent of these 5-methylcytosines are located in clusters of CpG dinucleotides, termed CpG islands, typically found in the 5' promoter region and first exon of certain genes (2). This methylation modification is

essential not only in mammalian development, but also in epigenetic regulation of gene expression, including genomic imprinting and X chromosome inactivation (3–5). Methylation of a CpG island is frequently associated with loss of expression of the target gene. Recent studies have provoked increasing interest in the role of DNA hypermethylation in tumorigenesis through its ability to alter the expression of tumor suppressor genes (6).

The DNA methylation reaction is catalyzed by a family of DNA methyltransferases (DNMTs) by use of the universal methyl donor, S-adenosylmethionine. Three distinct DNMTs have been identified in mammalian cells so far, namely DNMT1, DNMT2 and DNMT3. DNMT1 is constitutively expressed in mammalian cells and its function is to maintain the identical methylation pattern after DNA replication (7). That this function is critical is suggested by the finding that Dnmt1 gene knockout mice demonstrated global DNA demethylation and embryonic lethality (8, 9). On the other hand, somatic knockout of the DNMT1 gene in human colorectal carcinoma cells led to markedly decreased cellular DNMT activity, but only

¹ This work was supported by grants from the National Institutes of Health (CA78352, P50CA88843, and 2-T32CA09110), and the Department of Defense Breast Cancer Program (DAMD 17-00-1-0301 and DAMD 17-98-1-8116).

² Johns Hopkins Oncology Center, 1650 Orleans Street, Baltimore, Maryland 21231.

³ To whom correspondence should be addressed. e-mail: davidna@jhmi.edu

⁴ Abbreviations: estrogen receptor α (ER); progesterone receptor (PR); DNA methyltransferase (DNMT); histone deacetylase (HDAC); histone acetyltransferase (HAT); methylation-specific polymerase chain reaction (MSP); 5-azacytidine (5aza); 5-aza-2'-deoxycytidine (deoxyC); trichostatin (TSA); retinoic acid (RA); acute promyelocytic leukemia (APL).

a 20% decrease in overall genomic methylation was observed, mainly at juxtacentromeric satellites. Most of the gene-specific CpG islands analyzed remained fully methylated and silenced (10). These results suggest that DNMT1 might not be the only critical enzyme in maintaining human genome methylation status, and its exact role in mammalian development and gene transcription regulation needs to be further elucidated. DNMT2 is expressed at low levels in most adult tissues examined but recombinant mouse DNMT2 has no DNA methyltransferase activity (11-13). Thus the importance of DNMT2 is not clear. DNMT3 does have *de novo* methyltransferase activity and is highly expressed in embryonic stem cells. It has two isoforms, namely DNMT3A and DNMT3B. Unlike DNMT2, recombinant mouse DNMT3A and 3B can methylate cytosine residues in various native and synthetic DNA templates (14). DNMT3A expression is ubiquitous. It can be readily detected in most adult tissues, whereas DNMT3B expression is highly elevated in several tumor cell lines, including leukemia, melanoma, and colorectal cancer cell lines, to a level comparable to DNMT1 in these cell lines (15). How other DNMT family members, such as DNMT2, DNMT3A, DNMT3B, or other novel methylating proteins contribute to CpG methylation during normal development or tumorigenesis needs to be addressed in future study.

Methylation patterns in tumors are perplexing. In general, the level of 5-methylcytosine in tumor cells is lower than that in normal cells (16, 17). However, this global hypomethylation is observed in conjunction with regional hypermethylation at CpG islands, in turn associated with transcriptional inactivation of an increasing number of cancer-related genes. Thus far, a variety of genes, including tumor suppressor genes, DNA mismatch repair genes, cell cycle related genes, hormone receptors and tissue or cell adhesion molecules have been reported to be regulated by promoter CpG methylation. The inactivation of gene expression by aberrant CpG island hypermethylation is supported by studies using the demethylating agents, 5-azacytidine (5aza) and 5-aza-2'-deoxycytidine (deoxyC). Application of these agents to several cancer cell lines has been shown to demethylate CpG islands and reactivate expression of the previously silenced genes. In addition, recent studies suggest that hypermethylation of a CpG island not only can silence the gene it regulates, but also facilitate genetic alterations in tumor progression. P16^{INK4a} (18-21), MLH1 (22, 23), and GSTP1 (24, 25) are the best examples to support this "facilitation hypothesis."

Accumulating evidence suggests that hypermethylation of these three genes occurs in the early stages of tumorigenesis, predisposing cells to later genetic instability, which then contributes to tumor progression. Thus it appears that both epigenetic and genetic changes can contribute to the carcinogenic process.

Histone Acetylation, Chromatin Stability and Gene Expression

Chromatin structure and gene transcription are regulated partially by histone acetylation. During the S phase of the cell cycle, histone acetyltransferases (HAT) transfer an acetyl moiety to the ϵ -amino group of the amino acid, lysine, on histones, leading to neutralization of the positive charge and reduced affinity of histone for DNA. The ultimate consequence of this acetylation modification is the transformation of a tight-coiled inactive chromatin structure into a loose, transcriptionally active one (26). This process is reversed by histone deacetylation mediated by histone deacetylases (HDAC) during the G2 phase of the cell cycle. Deacetylated histones expose their positive charges to negatively charged DNA, leading to a condensed inactive chromatin structure. HDAC seems to play a role in gene silencing as well as in transcriptional activation (27, 28). In addition, HDACs are also involved in cell differentiation, cell-cycle arrest, apoptosis, chemosensitization, radiosensitization, antitumor effects and up-regulation of MHC class I (29). Three families of HDAC have been characterized so far, *S. cerevisiae* RPD3p (mammalian equivalents HDACs 1, 2 and 3), *S. cerevisiae* HDA1p (mammalian equivalents HDACs 4, 5 and 6), and *Zea mays* HD2 (29). The understanding of these deacetylases, especially the roles of HDAC1 and 2 in transcription regulation, is expanding.

Like DNA methylation, histone acetylation has recently been associated with tumorigenesis. For example, HAT CBP (a CREB-binding protein) is fused to the MLL gene in cases of acute leukemia or myelodysplasia secondary to therapy with drugs targeting DNA topoisomerase II (30, 31). This novel fusion protein leads to dysregulated histone acetylation that might contribute to the leukemogenic process (31). Histone acetylation may also play a role in breast cancer. The carboxy-terminal domains of proteins encoded by the breast cancer susceptibility genes, BRCA1 and BRCA2, can interact with Rb and Rb binding proteins as well as HDAC1 and HDAC2 (32). It has been proposed that the recruitment of HDACs

by BRCA proteins could cause gene deregulation in the progression of hereditary breast cancer (33, 34). For additional discussion of BRCA1 and BRCA2, see in this issue the article by Mielnicki *et al.* (35).

Molecular Mechanisms for the Epigenetic Regulation of Gene Expression

It appears that both DNA methylation and histone acetylation are involved in epigenetic regulation of gene expression in normal mammalian development as well as in tumorigenesis through their ability to modify chromatin structure during transcription. It also has been demonstrated that methylated inactive genes are associated with underacetylated histones whereas unmethylated active genes are linked to hyperacetylated histones (36, 37). These observations raise the following questions. How do DNA methylation and histone acetylation inhibit transcription? Do these processes interact with each other or act separately during this process? Are there any other factors involved?

Recent studies suggested that DNMT1 can form a repressive transcription complex at replication foci with HDAC2 and a newly identified protein, DMAP1 (DNMT1 associated protein), at the noncatalytic amino terminus of DNMT1. DMAP1 has intrinsic transcription repressive activity and interacts with DNMT1 throughout S phase, whereas HDAC2 joins the complex only during late S phase. These findings indicate that there is a connection between DNMT1 and HDAC, and histone deacetylation mediated by HDAC occurs after DNA replication (38). In another study, DNMT1 has been shown to form a transcription repressive complex with Rb, E2F1 and HDAC1 (39). Therefore, DNMT1, in addition to its function of maintaining CpG methylation, has direct inhibitory effects on transcription by formation of a repressive complex during DNA replication.

DNA METHYLATION AND HISTONE ACETYLATION IN REGULATION OF ER AND PR EXPRESSION IN BREAST CANCER

ER and PR Expression in Normal Mammary Gland and Breast Cancer

Estrogen and progesterone and their receptors play important roles in the development and function of the mammary gland as well as other female reproductive organs. At puberty, the hypothalamus and

pituitary gland stimulate the ovary to secrete estrogen that is responsible for the proliferation of the mammary epithelial cells and elongation and branching of mammary ducts. Progesterone is synthesized by the corpus luteum and placenta to promote the growth of mammary lobuloalveolar structures, especially during pregnancy in preparation for milk secretion after parturition (40). Although estrogen and progesterone receptors are expressed at very low basal levels in normal human mammary epithelial cells after a woman reaches sexual maturity (41, 42), they seem to play an important role in the initiation, progression and prognosis of breast cancer. They are also good predictors for endocrine therapy (43). Approximately two-thirds of breast cancers express ER transcript and synthesize ER protein at levels higher than in normal breast tissues and half of these ER-positive tumors express both ER and PR protein (ER+/PR+) (43). These tumors tend to be more differentiated and are more responsive to hormonal therapies. One-quarter of all breast cancers lack both ER and PR (ER-/PR-). In general, these tumors are associated with poorer differentiation, higher growth fraction, and worse clinical outcome than ER+/PR+ breast cancer (43). These cancers are estrogen-independent and rarely respond to hormonal therapies. Of note, up to one-third of initially ER+ tumors can evolve to an ER- status during tumor progression and become resistant to hormone therapy (44).

ER Methylation and DNMT Expression in Breast Cancer

The human ER cDNA and gene were cloned in 1986 (45-47). This 140 kb ER gene has eight exons and is located on chromosome 6q25.1. Since then, efforts have been devoted to the possible molecular genetic mechanisms for the loss of ER expression in breast cancers. Genetic changes, such as insertions, deletions, rearrangements, or point mutations of the ER gene were extensively sought in breast cancer cells since these in-frame or out-of-frame sequence alterations would lead to either absence of ER expression or expression of an unstable mutant that might be degraded shortly after protein translation by the ubiquitin protease system. Although several sequence mutations for the ER gene were identified and shown to be related to decreased ER expression and estradiol binding, they are uncommon and cannot explain the loss of ER expression in a significant fraction of human breast cancers (40). This finding suggests that mechanisms other than genetic changes

Table I. Frequency of ER Gene Methylation of Primary Breast Human Tissues by Use of Southern Blot Analysis^a

Genotype	ER gene methylation at the <i>NotI</i> site
ER+/ PR+ tumor	0/29 (0%)
ER+/ PR- tumor	0/24 (0%)
ER-/ PR- tumor	9/39 (25%)
ER-/ PR- metastases	2/2 (100%)
Normal breast	0/9 (0%)

^a Adapted from Ref (51).

might also contribute to the loss of ER expression in breast cancer.

One extensively studied epigenetic mechanism that might lead to loss of ER expression is CpG methylation. Interestingly, the ER gene has a CpG island in its promoter and first exon regions marked by a clustering of sites for methylation-sensitive restriction endonucleases (46, 48) (Fig. 1). In addition, absence of ER protein expression in human breast cancer specimens is frequently associated with loss of ER transcript (49). Those findings raise the possibility that absence of ER expression is associated with ER gene hypermethylation.

We have addressed this hypothesis using human breast cancer cell lines as a model system. Ottaviano *et al.* (50) reported that ER- human breast cancer cells displayed extensive methylation of the CpG island in the 5' promoter region of the estrogen

receptor gene, in association with silencing of ER gene expression. The 6.3 kb ER mRNA transcript was detected easily in three ER+ cell lines (MCF-7, T47D and ZR75-1), but none was detected in three ER- cell lines (Hs578t, MDA-MB-231 and MDA-MB-468). Southern blot analysis using methylation-sensitive restriction enzymes showed that the ER CpG island was methylated at a critical *NotI* site in multiple ER-negative breast cancer cell lines. As expected for an autosomal gene, the ER gene was unmethylated at the *NotI* site in the CpG island in all normal tissues studied including breast epithelium. In addition, the ER gene was unmethylated at multiple other restriction sites in its CpG island in all ER+ cell lines studied, but these sites were frequently methylated in ER-negative cell lines. Of note, the major DNA methyltransferase, DNMT1, was highly expressed in ER- cell lines at the mRNA level as well as the protein and enzymatic activity levels. Careful assessment of the relationship between ER expression and DNMT1 showed that DNMT1 protein expression was correlated with S-phase fraction in ER-positive cell lines, but not in ER-negative cell lines. Thus DNMT1 expression was elevated in ER-negative cell lines and was unlinked from cell cycle regulation (51).

A key question is whether these tissue culture findings have any relevance to human breast cancer. Using Southern blot analysis, Lapidus *et al.* (52) reported that the ER CpG island was methylated at the *NotI* restriction site in 9 of 39 (25%) of

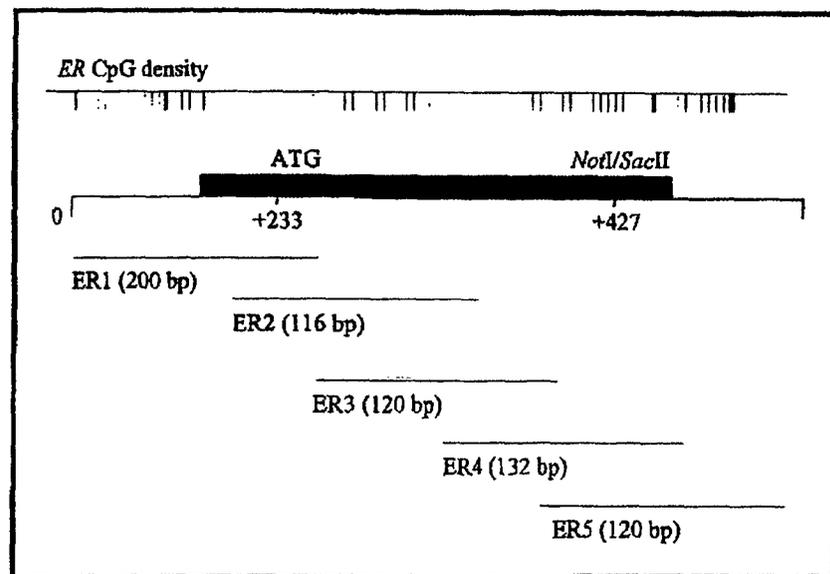


Fig. 1. Map of ER CpG island and locations of PCR products generated by use of specific ER MSP primers. Box represents exon 1 of ER gene.

primary ER- breast cancers but remained unmethylated in 53 ER+ breast cancers and 9 normal breast specimens (Table I). The possible explanations for the different frequency of *ER* methylation in cell lines and tissues include the presence of normal cells in primary cancers, heterogeneity of *ER* expression within breast cancers, methylation of other sites within the CpG island which were not examined, and the relative insensitivity of Southern blot analysis. These possibilities have been partially addressed through the development of a PCR-based assay termed methylation-specific PCR or MSP, a technique designed to analyze methylation of CpG dinucleotides across the entire *ER* CpG island (Fig. 1). By use of MSP, normal breast tissues and ER+ cell lines shown only an unmethylated product across all 5 primer sets used, whereas ER- cell lines shown methylated products across much of the island (52) (Table II). To confirm that *ER* CpG methylation could also be detected by MSP in primary human breast cancers, a panel of 33 primary breast cancers of known receptor status was studied. All 33 tumors showed at least some degree of methylation at one or more primer sites. By use of a semiquantitative scoring system for elevated CpG density, the percentage of tumors displaying substantial methylation can be calculated for each tumor set defined by receptor status. As shown in Table III, about one-third of ER+/PR+ tumors showed methylation, whereas the percentage increased to 100% in ER-/PR- tumors (53). Taken together, data derived from both our study and others (54) support a correlation between absence of *ER*

Table II. *ER* Gene Methylation of Human Breast Cancer Lines by Use of MSP^a

Source	ER1	ER2	ER3	ER4	ER5
Normal breast epithelial cells	-	-	-	-	-
ER+ cell lines					
MCF-7	-	-	-	-	-
T47D	-	-	-	-	-
ZR-75-1	-	-	-	-	-
ER- cell lines					
MDA-MB-231	+	+	+	+	+
Hs578t	+	+	±	+	+
MDA-MB-435	±	-	±	+	±
MDA-MB-468	+	±	±	±	±
MCF-7/Adr	+	+	±	±	±

^aThe results of normal breast epithelial cells are representative of five normal breast epithelial samples. "+": methylated; "-": unmethylated; "±": heterogeneous, both methylated and unmethylated PCR products. Adapted from Ref. (53).

Table III. *ER* Gene Methylation of Primary Human Primary Breast Cancers by Use of MSP

Receptor status	No. of tumors	No. of methylated tumors (percentage)
ER+/PR+	11	4 (36%)
ER+/PR-	11	8 (72%)
ER-/PR-	11	11 (100%)

^aAdapted from Ref. (53).

expression and aberrant CpG island methylation of *ER* gene. Whether methylation status actually acts solely or partially to silence *ER* transcription is a key question.

Demethylation of the *ER* Gene Results in Re-Expression of *ER*

If silencing of *ER* expression is a function of CpG island methylation of *ER* gene, it is possible that demethylation should result in the re-expression of *ER* in ER- breast cancer cells. In accord with this prediction, treatment of the ER- human breast cancer cell line, MDA-MB-231, with the demethylating agents, 5-aza and deoxyC, led to re-expression of *ER* (55). After treatment with either drug, the DNA from these cells became partially demethylated at several methylation-sensitive restriction enzyme sites, including *HhaI*, *NotI*, and *SacII*, within the *ER* promoter CpG island. Demethylation correlated with re-expression of the *ER* gene as detected by reverse transcriptase-PCR (RT-PCR) as demonstrated in Fig. 2 and Western Blot analysis. Functional activity of this *ER* protein was examined in two ways. First, its ability to activate expression of an endogenous estrogen-inducible gene, *PR*, was assessed. *PR* mRNA and protein were induced by estrogen treatment of deoxyC treated MDA-MB-231 or Hs578t cells. Second, the ability of the deoxyC-induced *ER* to transduce expression of a luciferase reporter gene linked to an estrogen response element (ERE) was assessed. MDA-MB-231 cells were stably transfected with an ERE-tk-luciferase construct with the expectation that deoxyC-induced *ER* expression would increase luciferase expression. As expected, increased luciferase activity (1.5–5.6 fold in 10 independent clones) over the background level of the untreated cells was observed (54). These results provide powerful evidence that DNA methylation of the *ER* CpG island plays a functional role in suppression of *ER* expression in ER- breast cancer cells.

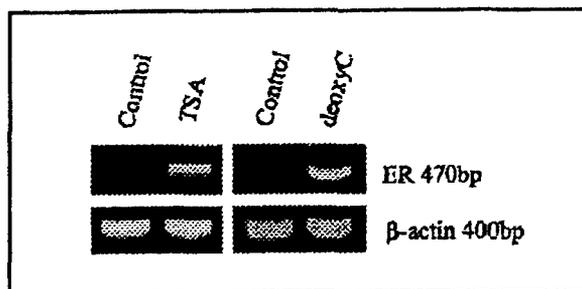


Fig. 2. RT-PCR analysis of ER mRNA re-expression by TSA (100 ng/ml \times 2 days) or deoxyC (2.5 μ M \times 4 days) treatment in ER $-$ MB-MDA-231 cells. β -actin RT-PCR product was used as a control for the amount of RNA used.

DNA Methylation and PR Gene Expression

As discussed in the Introduction, one quarter to one-third of breast cancers lack both ER and PR, whereas another one-third are ER $+$ but PR $-$. It also has been demonstrated that the expression of PR requires ER as a transcriptional activator (56). Therefore, the presence of PR in ER $+$ tumors may be a better predictor of hormone responsiveness than ER alone (43). Interestingly, the PR gene also has a typical CpG island in its first exon. The PR CpG island is 1 kb in size, has 70% GC content, and contains a wide array of methylation-sensitive restriction sites. Southern analyses targeted at three methylation-sensitive restriction sites in the PR CpG island showed that these sites are not methylated in normal breast tissue specimens but are hypermethylated in 40% of primary tumors that lack PR protein expression (52). Thus the question of why the PR hypermethylation could be responsible for a lack of PR expression was studied in MDA-MB-231 cells which lack both ER and PR (57). Treatment of these cells with the demethylating agent, deoxyC, led to partial demethylation of the ER and PR CpG islands and was associated with induction of PR protein expression as demonstrated by Western blot analysis. However, Ferguson *et al.* (57), using MDA-MB-231 cells stably transfected with an inducible expression vector for ER, found that the induction of PR gene expression by ligand-bound ER did not require demethylation of the PR CpG island. In addition, induction of PR transcription was inhibited by blocking the interaction of ER with SRC-1A, a coactivator of ER function. These results suggested that a transcription factor with the potential to remodel heterochromatin (ER) could activate PR gene expression without altering the methylation status of

the CpG island in the PR gene. These results raise the possibility that demethylation and histone acetylation are distinct but complementary mechanisms for destabilizing heterochromatin and activating gene transcription (58).

Histone Acetylation and ER Expression

As discussed earlier, histone acetylation and deacetylation are intimately involved in chromatin structure changes during transcription. In addition, new models of transcriptional suppressive complexes suggest that HDACs and DNMT1 are in direct contact. Recent studies indicate that silencing of a gene by methylation involves the generation of an inactive chromatin structure in which methyl CpG-binding protein (MeCP2) and the adapter protein, mSin3A, recruit histone deacetylase (59, 60). The deacetylation of lysine groups of histones H3 and H4 allows ionic interactions between positively charged lysines and negatively charged DNA, resulting in a more compact nucleosome structure that limits gene activation. The question emerges of whether epigenetic modification of histone acetylation alone could result in re-expression of genes that are inhibited by the transcriptional suppressive complex, or whether both histone acetylation and CpG demethylation are required for this event.

The answers to this question have been variable. For example, HDAC inhibitors like trichostatin (TSA) or phenylbutyrate alone restored retinoic acid receptor α (RAR α) expression in retinoic acid (RA) resistant acute promyelocytic leukemia (APL) cell lines as demonstrated by the differentiation of APL cells in the presence of RA (61–63). However, hypermethylated genes, such as MLH1, TIMP3, INK4B (p15) and INK4a (p16), could not be transcriptionally reactivated by TSA alone, but could be re-expressed in colon cancer cells with a combination of TSA and the demethylating agent, 5-aza (58).

To study the role of histone acetylation in ER expression, ER $-$ MDA-MB-231 cells were treated with HDAC inhibitor, TSA. A time- and dose-dependent reactivation of ER mRNA expression was observed (64). As shown in Fig. 2, TSA alone at 100 ng/ml for 2 days could reactivate ER expression as well as deoxyC in ER $-$ MDA-MB-231 breast cancer cell line. By use of quantitative competitive PCR assay, an increase of 5.0 fold of ER transcript expression was reported (64). MSP analysis of the ER CpG island showed no

change in its methylation after TSA treatment, suggesting that TSA's effects on ER re-expression were not associated with a change in methylation status. This finding was confirmed via a more rigorous assay, bisulfite genomic sequencing of the *ER* promoter region, a technique which permits the direct examination of the methylation status of each CpG dinucleotide within the promoter region. Multiple clones of control and TSA-treated cells were examined and did not show any change in the methylation status of the *ER* gene with TSA. A logical extension of these studies will be to examine the effect of combinations of demethylating agents and HDAC inhibitors on expression of *ER* as well as other methylated genes in breast cancer cells.

DNA Methylation and HDAC Inhibitors as Therapeutic Strategies in Breast Cancer

Abundant evidence indicates that epigenetic mechanisms play pivotal roles in the pathogenesis of cancer. Both DNA methylation and histone acetylation have been shown to be involved in the regulation of expression of cancer-related genes. The studies in breast cancer summarized earlier demonstrated that both demethylating agents and HDAC inhibitors could re-activate expression of a number of important growth regulatory genes in several breast cancer cell lines, thus raising the possibility of using these types of agents as therapeutic strategies for breast cancer.

Two demethylating agents, 5-aza and deoxyC, have been studied clinically, particularly in patients with hemoglobinopathies. For example, 5-aza has been successfully used to treat patients with β -thalassemia. It has been reported that loss of expression of the γ -globin gene is associated with methylation of its promoter (65). Infusion of 5-aza into β -thalassemia patients led to an increase of γ -globin expression and amelioration of anemia (66). Similarly, 5-aza can increase HbF production and reduce anemia in sickle cell disease (67). In addition, in an ongoing trial, the efficacy of treatment of nasopharyngeal tumors with 5-aza is being evaluated. The promoter of one of the Epstein Barr Virus-related proteins is heavily methylated in nasopharyngeal carcinoma. It has been proposed that demethylation of this promoter could lead to reactivation of an EBV latency protein that could then become the target for a secondary immune therapy (R. Ambinder, Johns Hopkins Oncology Center, personal communication). Another possible

approach is to use antisense oligonucleotides against DNA methyltransferase to inhibit DNA methylation. It has been shown that antisense oligonucleotides have *in vitro* and *in vivo* antitumor activity and a clinical trial of this approach is in progress (68, 69).

Several HDAC inhibitors have the potential to be used as anti-cancer agents (29). The most plausible and extensively studied are butyrate, TSA, and trapoxin (TPX) and their relatives. The IC_{50} of butyrate for HDAC is in the millimolar range, whereas TSA and TPX act at low nanomolar concentrations. One triglyceride analogue of the short-chain fatty acid butyrate, tributyrin, has been reported to induce transcription of p21, arrest of cells in G2/M and apoptosis in MCF-7 human breast cancer cell lines (70). Two butyrate derivatives, phenylbutyrate (PB) and phenylacetate (PA), have powerful growth inhibitory effects on several cell types including ovarian and prostate cancers (71). A clinical trial with sodium phenylacetate in patients with thalassemia showed an increase of fetal hemoglobin in some patients (72). Other HDAC inhibitors under study include FR901228, oxamflatin, depudecin, suberoylanilide hydroxamic acid (SAHA), m-carboxycinnamic acid bishydroxamide (CBHA) and apicidin.

A potential strategy is to combine demethylating agents and HDAC inhibitors. From a clinical point-of-view, the potential benefits of this combination could include lowering of drug concentration requirements, shorter periods of drug exposure, and lower toxicity, as well as the possibility of synergy. For example, synergistic effects were observed in a combination study with both a demethylating agent and a HDAC inhibitor in colon cancer cells. If cancer cells were treated with 100 nM 5-aza (a dose that had little effect on gene expression if used alone) for 24 hours followed by 300 nM TSA for 24 hours, (again a treatment that was ineffective alone), re-expression of several genes was obvious (58). A key question in development of this strategy will be the specificity and selectivity of gene re-expression patterns and how they might be exploited clinically.

Finally, it should be noted that demethylation and histone acetylation are only two mechanisms for epigenetic regulation of target genes like *ER*. Other transcriptional modulators might also contribute to the epigenetic regulation of gene expression. For example, it was reported that AP2 transcription factor can trans-activate the cloned human *ER α promoter in MDA-MB-231 cells (73).*

CONCLUSIONS

The evidence for epigenetic regulation of gene expression in tumorigenesis is accumulating. Two components, demethylation of C5-methylcytosines at the CpG island within the promoter region of target genes and histone acetylation of chromatin, have been studied extensively by use of demethylating agents and HDAC inhibitors. *ER* and *PR*, two critical genes in breast cancer development and treatment, have been reported to be densely methylated in ER- but not ER+ human breast cancer cell lines. Treatment of ER- human breast cancer cells with a demethylating agent or a HDAC inhibitor can lead to reactivation of ER expression in these cells. However, demethylation of *PR* CpG island is not required for PR expression. These data in combination with similar results observed with other critical genes in breast cancer and other types of cancer support the potential for compounds that can modulate epigenetic regulation in the treatment of human cancers.

REFERENCES

1. M. Ehrlich, M. A. Gama-Sosa, L. H. Huang, R. M. Midgett, K. C. Kuo, R. A. McCune, and C. Gehrke (1982). Amount and distribution of 5-methylcytosine in human DNA from different types of cells. *Nucleic Acids Res.* 10:2709-2721.
2. A. P. Bird (1986). CpG-rich islands and the function of DNA methylation. *Nature* 321:209-213.
3. S. U. Kass, D. Pruss, and A. P. Wolffe (1997). How does DNA methylation repress transcription? *Trends Genet.* 13:444-449.
4. M. S. Bartolomei and S. M. Tilghman (1997). Genomic imprinting in mammals. *Ann. Rev. Genet.* 31:493-525.
5. R. Jaenisch, C. Beard, J. Lee, Y. Marahrens, and B. Panning (1998). Mammalian X chromosome inactivation. *Novartis Foundation Symp.* 214:200-209.
6. S. B. Baylin and J. G. Herman (2000). DNA hypermethylation in tumorigenesis. *Trends Genet.* 16:168-174.
7. M. S. Tucker and T. H. Bestor (1997). Formation of methylation patterns in mammalian genome. *Mutation Res.* 386:119-130.
8. E. Li, T. H. Bestor, and R. Jaenisch (1992). Targeted mutation of the DNA methyltransferase gene results in embryonic lethality. *Cell* 69:915-926.
9. Y. Liu, E. J. Oakeley, L. Sun, and J. P. Jost (1998). Multiple domains are involved in the targeting of the mouse DNA methyltransferase in the DNA replication foci. *Nucleic Acids Res.* 26:1038-1045.
10. I. Rhee, K. W. Jair, R. W. C. Yen, C. Lengauer, J. G. Herman, K. W. Kinzler, B. Vogelstein, S. B. Baylin and K. E. Schuebel (2000). CpG methylation is maintained in human cancer cells lacking *DNMT1*. *Nature* 404:1003-1007.
11. J. A. Yoder and T. H. Bestor (1998). A candidate mammalian DNA methyltransferase related to pmt1p of fission yeast. *Hum. Mol. Genet.* 7:279-284.
12. M. Okano, S. Xie, and E. Li (1998). Dnmt2 is not required for *de novo* and maintenance methylation of viral DNA in embryonic stem cells. *Nucleic Acids Res.* 26:2536-2540.
13. V. Wyngaert, J. Sprengel, S. U. Kass, and W. H. Luyten (1998). Cloning and analysis of a novel human putative DNA methyltransferase. *FEBS Lett.* 426:283-289.
14. M. Okano, S. Xie, and E. Li (1998). Cloning and characterization of a family of novel mammalian DNA (cytosine-5) methyltransferases. *Nature Genet.* 19:219-220.
15. S. Xie, Z. Wang, M. Okano, M. Nogami, Y. Li, W. W. He, K. Okumura, and E. Li (1999). Cloning, expression and chromosome locations of the human DNMT3 gene family. *Gene* 236:87-95.
16. S. E. Goetz, B. Vogelstein, S. R. Hamilton, and A. P. Feinberg (1985). Hypomethylation of DNA from benign and malignant human colon neoplasms. *Science* 228:187-190.
17. L. Momparler and V. Bovenzi (2000). DNA methylation and cancer. *J. Cell. Physiol.* 183:145-154.
18. S. A. Belinsky, K. J. Nikula, W. A. Palmisano, R. Michels, G. Saccomanno, E. Gabrielson, S. B. Baylin, and J. G. Herman (1998). Aberrant methylation of p16^{INK4a} is an early event in lung cancer and a potential biomarker for early diagnosis. *Proc. Natl. Acad. Sci. U.S.A.* 95:11891-11896.
19. D. J. Wong, S. A. Foster, D. A. Galloway, and B. J. Reid (1999). Progressive region-specific *de novo* methylation of the p16 CpG island in primary human mammary epithelial cell strains during escape from M(0) growth arrest. *Mol. Cell. Biol.* 19:5642-5651.
20. G. J. Nuovo, T. W. Plaia, S. A. Belinsky, S. B. Baylin, and J. G. Herman (1999). In situ detection of the hypermethylation-induced inactivation of the p16 gene as an early event in oncogenesis. *Proc. Natl. Acad. Sci. U.S.A.* 96:12754-12759.
21. T. Kiyono, S. A. Foster, J. I. Koop, J. K. McDougall, D. A. Galloway, and A. J. Klingelutz (1998). Both Rb/p16^{INK4a} inactivation and telomerase activity are required to immortalize human epithelial cells. *Nature* 396:84-88.
22. J. G. Herman, A. Umar, K. Polyak, J. R. Graff, N. Ahuja, J. P. Issa, S. Markowitz, J. K. Wilson, S. R. Hamilton, K. W. Kinzler, M. F. Kane, R. D. Kolodner, B. Vogelstein, T. A. Kunkel, and S. B. Baylin (1998). Incidence and functional consequences of hMLH1 promoter hypermethylation in colorectal carcinoma. *Proc. Natl. Acad. Sci. U.S.A.* 95:6870-6875.
23. M. Esteller, L. Catusas, X. Matias-Guiux, G. L. Mutter, J. Prat, S. B. Baylin and J. G. Herman (1999). HMLH1 promoter hypermethylation is an early event in human endometrial tumorigenesis. *Amer. J. Pathol.* 155:1767-1772.
24. W. H. Lee, R. A. Morton, J. I. Epstein, J. D. Brooks, P. A. Campbell, G. S. Bova, W. S. Hsieh, W. B. Issacs, and W. G. Nelson (1994). Cytidine methylation of regulatory sequences near the pi-class glutathione S-transferase gene accompanies human prostatic carcinogenesis. *Proc. Natl. Acad. Sci. U.S.A.* 91:11733-11737.
25. M. Esteller, P. G. Corn, J. M. Urena, E. Gabrielson, S. B. Baylin, and J. G. Herman (1998). Inactivation of glutathione S-transferase P1 gene by promoter hypermethylation in human neoplasia. *Cancer Res.* 58:4515-4518.
26. M. Grunstein (1997). Histone acetylation in chromatin structure and transcription. *Nature* 389:349-352.
27. C. A. Mizzen and C. D. Allis (1998). Linking histone acetylation to transcriptional regulation. *Cell Mol. Life Sci.* 54:6-20.
28. C. A. Hassig, J. K. Tong, T. C. Fleischer, T. Owa, P. G. Grable, D. E. Ayer, and S. L. Schreiber (1998). A role for histone

- deacetylase activity in HDAC1-mediated transcriptional repression. *Proc. Natl. Acad. Sci. U.S.A.* 95:3519-3524.
29. U. H. Weidle and A. Grossmann (2000). Inhibition of histone deacetylases: A new strategy to target epigenetic modifications for anticancer treatment. *Anticancer Res.* 20:1471-1486.
 30. T. Taki, M. Sako, M. Tsuchida, and Y. Hayashi (1997). The t(11;16) (q23;p13) translocation in myelodysplastic syndrome fuses the MLL gene to the CBP gene. *Blood* 89:3945-3950.
 31. O. M. Sobulo, J. Borrow, R. Tomek, S. Reshmi, A. Harden, B. Schlegelberger, D. Housman, N. A. Doggett, J. D. Rowley, and N. J. Zeleznik-Le (1997). MLL is fused to CBP, a histone acetyltransferase, in therapy-related acute myeloid leukemia with a t(11;16) (q23;p13.3). *Proc. Natl. Acad. Sci. U.S.A.* 94:8732-8737.
 32. R. I. Yarden and L. C. Brody (1999). BRCA1 interacts with components of the histone deacetylase complex. *Proc. Natl. Acad. Sci. U.S.A.* 96:4983-4988.
 33. I. Irminger-Finger, B. D. Siegel, and W. C. Leung (1999). The functions of breast cancer susceptibility gene 1 (BRCA1) and its associated proteins. *Biol. Chem.* 380:117-128.
 34. H. Siddique, J. P. Zou, V. N. Rao, and E. S. P. Reddy (1998). The BRCA2 is a histone acetyltransferase. *Oncogene* 16:2283-2285.
 35. L. M. Mielnicki, H. L. Asch, and B. B. Asch (2001). Genes, Chromatin, and breast cancer: An epigenetic tale. *J. Mam. Gland Biol. Neoplasia*, 6(2).
 36. T. H. Bestor (1998). Methylation meets acetylation. *Nature* 393:311-312.
 37. S. Eden, T. Hashimshony, I. Keshet, H. Cedar, and A. W. Thorne (1998). DNA methylation models histone acetylation. *Nature* 394:842.
 38. M. R. Rountree, K. E. Bachman, and S. B. Baylin (2000). DNMT1 binds HDAC2 and a new co-repressor, DNMAP1, to form a complex at replication foci. *Nat. Genet.* 25:269-277.
 39. K. D. Robertson, S. Ait-Si-Ali, T. Yokochi, P. A. Wade, P. L. Jones, and A. P. Wolffe (2000). DNMT1 forms a complex with Rb, E2F1 and HDAC1 and represses transcription from E2F-responsive promoters. *Nat. Genet.* 25:338-342.
 40. R. G. Lapidus, S. J. Nass, and N. E. Davidson (1998). The loss of estrogen and progesterone receptor gene expression in human breast cancer. *J. Mam. Gland Biol. Neoplasia* 3:85-94.
 41. D. L. Ricketts, G. Turnbull, R. Ryall, N. S. B. Baskshi, N. S. B. Raswon, J-C. Gazet, C. Nolan, and R. C. Coombes (1991). Estrogen and progesterone receptor in the normal female breast. *Cancer Res.* 51:1817-1822.
 42. O. W. Peterson, P. E. Hoyer, and B. Van Deur (1987). Frequency and distribution of estrogen positive cells in normal, non-lactating human breast tissue. *Cancer Res.* 47:5748-5751.
 43. W. L. McGuire (1978). Hormone receptors: Their role in predicting prognosis and response to endocrine therapy. *Seminars Oncol.* 5:428-433.
 44. T. Kuukasjarvi, J. Kononen, K. Helin, K. Holli, and J. Isola (1996). Loss of estrogen receptor in recurrent breast cancer is associated with poor response to endocrine therapy. *J. Clin. Oncol.* 14:25284-25289.
 45. G. L. Greene, P. Hilna, M. Waterfield, A. Baker, Y. Hort, and J. Shine (1986). Sequence and expression of human estrogen receptor complementary DNA. *Science* 231:1150-1154.
 46. S. Green, P. Walter, V. Kumar, A. Krust, P-M. Bornet, P. Argos, and P. Chambon (1986). Human oestrogen receptor cDNA: Sequence, expression and homology to v-erb-A. *Nature* 320:134-139.
 47. M. Ponglikitmonkol, S. Green, and P. Chambon (1988). Genomic organization of the human oestrogen receptor gene. *EMBO J.* 7:3385-3388.
 48. R. Piva, R. Gambari, F. Zorzato, L. Kumar, and L. del Senno (1992). Analysis of upstream sequences of the human estrogen receptor gene. *Biochem. Biophys. Res. Commun.* 183:996-1002.
 49. R. J. Weigel and E. C. de Coninck (1993). Transcriptional control of estrogen receptor in estrogen receptor-negative breast carcinoma. *Cancer Res.* 53:3472-3474.
 50. Y. L. Ottaviano, J-P. Issa, F. F. Parl, H. S. Smith, S. B. Baylin, and N. E. Davidson (1994). Methylation of the estrogen receptor gene CpG island marks loss of estrogen receptor expression in human breast cancer cells. *Cancer Res.* 54:2552-2555.
 51. S. J. Nass, A. T. Ferguson, D. El-Ashry, W. G. Nelson, and N. E. Davidson (1999). Expression of DNA methyltransferase (DNMT) and the cell cycle in human breast cancer cells. *Oncogene* 18:7453-7461.
 52. R. G. Lapidus, A. T. Ferguson, Y. L. Ottaviano, F. F. Parl, S. B. Baylin, J-P. I. Issa, and N. E. Davidson (1996). Methylation of estrogen and progesterone receptor gene 5' CpG islands correlates with lack of estrogen and progesterone receptor gene expression in breast tumors. *Clin. Cancer Res.* 2:805-810.
 53. R. G. Lapidus, S. J. Nass, K. A. Butash, F. F. Parl, S. A. Weitzman, J. G. Graff, J. G. Herman, and N. E. Davidson (1998). Mapping of ER gene CpG island methylation by methylation-specific polymerase chain reaction. *Cancer Res.* 58:2515-2519.
 54. H. Iwase, Y. Omoto, H. Iwata, T. Toyama, Y. Hara, Y. Ando, Y. Ito, Y. Fujii, and S. Kobayashi (1999). DNA methylation analysis at distal and proximal promoter regions of the estrogen receptor gene in breast cancers. *Brit. J. Cancer* 80:1982-1986.
 55. A. T. Ferguson, R. G. Lapidus, S. B. Baylin, and N. E. Davidson (1995). Demethylation of the estrogen receptor gene in estrogen receptor-negative breast cancer cells can reactivate estrogen receptor gene expression. *Cancer Res.* 55:2279-2283.
 56. L. D. Read, C. E. Snider, J. S. Miller, G. L. Greene, and B. S. Katzenellenbogen. (1988). Ligand-modulated regulation of progesterone receptor messenger ribonucleic acid and protein in human breast cancer cell lines. *Mol. Endocrinol.* 2:263-271.
 57. A. T. Ferguson, R. G. Lapidus and N. E. Davidson (1998). Demethylation of the progesterone receptor CpG island is not required for progesterone receptor gene expression. *Oncogene* 17:577-583.
 58. E. E. Cameron, K. E. Bachman, S. Myohanen, J. G. Herman, and S. B. Baylin (1999). Synergy of demethylation and histone deacetylase inhibition in the re-expression of genes silenced in cancer. *Nat. Genet.* 21:103.
 59. X. Nan, H. H. Ng, C. A. Johnson, C. D. Laherty, B. M. Turner, T. N. Eisenman, and A. Bird (1998). Transcriptional repression by the methyl-CpG-binding protein MeCP2 involves a histone deacetylase complex. *Nature* 393:386-389.
 60. P. L. Jones, G. J. Veenstra, P. A. Wade, D. Vermaak, S. U. Kass, N. Landsberger, J. Strouboulis, and A. P. Wolffe (1998). Methylated DNA and MeCP2 recruit histone deacetylase to repress transcription. *Nat. Genet.* 19:187-189.
 61. R. J. Lin, L. Nagy, S. Inoue, W. Shao, W. H. Jr. Miller, and R. M. Evans (1998). Role of the histone deacetylase complex in acute promyelocytic leukaemia. *Nature* 391:811-814.
 62. F. Grignani, S. DeMatteis, C. Nervi, L. Tomassoni, V. Gelmetti, M. Ciocce, M. Fanelli, M. Ruthardt, F. F. Ferrara, I. Zamir, C. Seiser, F. Grignani, M. A. Lazar, S. Minucci, and P. G.

- Pellicci (1998). Fusion proteins of the retinoic acid receptor- α recruit histone deacetylase in promyelocytic leukaemia. *Nature* 391:815-818.
63. R. P. Warrell, Jr., L. Z. He, V. Richon, E. Calleja, and P. P. Pandolfi (1998). Therapeutic targeting of transcription in acute promyelocytic leukemia by use of an inhibitor of histone deacetylase. *J. Natl. Cancer Inst.* 90:1621-1625.
64. X. Yang, A. T. Ferguson, S. J. Nass, D. L. Phillips, K. A. Butash, S. M. Wang, J. G. Herman, and N. E. Davidson (2000). Transcriptional activation of estrogen receptor α in human breast cancer cells by histone deacetylase inhibition. *Cancer Res.* 60:6890-6894.
65. S. Charache, G. Dover, K. Smith, C. C. Talbot, Jr., M. Moyer, and S. Boyer (1983). Treatment of sickle cell anemia with 5-azacytidine results in increased fetal hemoglobin production and is associated with nonrandom hypomethylation around the γ - δ - β globin gene complex. *Proc. Natl. Acad. Sci. U.S.A.* 80:4842-4846.
66. T. J. Ley, I. DeSimone, N. P. Anagnou, G. H. Keller, R. K. Humphries, P. H. Turner, N. S. Young, P. Keller, and A. W. Nienhuis (1982). 5-azacytidine selectively increases γ -globin synthesis in a patient with β -thalassemia. *N. Engl. J. Med.* 307:1469-1475.
67. G. J. Dover, S. Charache, S. H. Boyer, G. Vogelsang, and M. Moyer (1985). 5-azacytidine increased HbF production and reduces anemia in sickle cell disease. Dose-response analysis of subcutaneous and oral dosing regimens. *Blood* 66:527-532.
68. A. R. MacLeod and M. Szyf (1995). Expression of antisense to DNA methyltransferase mRNA induces DNA demethylation and inhibits tumorigenesis. *J. Biol. Chem.* 270:8037-8043.
69. S. Ramchandani, A. R. MacLeod, M. Pinard, E. von Hofe, and M. Szyf (1997). Inhibition of tumorigenesis by a cytosine-DNA, methyltransferase, antisense oligonucleotide. *Proc. Natl. Acad. Sci. U.S.A.* 94:684-690.
70. B. G. Heerdt, M. A. Houston, G. M. Anthony, and L. H. Augenlicht (1999). Initiation of growth arrest and apoptosis of MCF-7 mammary carcinoma cells by tributyrin, a triglyceride analogue of the short-chain fatty acid butyrate, is associated with mitochondrial activity. *Cancer Res.* 59:1584-1591.
71. M. A. Carducci, J. B. Nelson, K. M. Chan-Tack, S. R. Ayyagari, W. H. Sweatt, P. A. Campbell, W. G. Nelson, and J. W. Simons (1996). Phenylbutyrate induces apoptosis in human prostate cancer and is more potent than phenylacetate. *Clin. Cancer Res.* 2:379-387.
72. A. F. Collins, H. A. Pearson, P. Giardina, K. T. McDonagh, S. W. Brusilow, and G. J. Dover (1995). Oral sodium phenylbutyrate therapy in homozygous β -thalassemia: A clinical trial. *Blood* 85:43-49.
73. L. A. McPherson and R. J. Weigel (1999). AP2 α and AP2 γ : A comparison of binding site specificity and trans-activation of the estrogen receptor promoter and single site promoter constructs. *Nucleic Acids Res.* 27:4040-4049.