Mechanisms of epigenetic silencing of the Rassf1a gene during estrogen-induced breast carcinogenesis in ACI rats

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Breast cancer, the most common malignancy in women, emerges through a multistep process, encompassing the progressive sequential evolution of morphologically distinct stages from a normal cell to hyperplasia (with and without atypia), carcinoma in situ, invasive carcinoma and metastasis. The success of treatment of breast cancer could be greatly improved by the detection at early stages of cancer. In the present study, we investigated the underlying molecular mechanisms involved in breast carcinogenesis in Augustus and Copenhagen-Irish female rats, a cross between the ACI strains, induced by continuous exposure to 17β-estradiol. The results of our study demonstrate that early stages of estrogen-induced breast carcinogenesis are characterized by altered global DNA methylation, aberrant expression of proteins responsible for the proper maintenance of DNA methylase pattern and epigenetic silencing of the critical Rassf1a (Ras association domain family 1, isoform A) tumor suppressor gene. Interestingly, transcriptional repression of the Rassf1a gene in mammary glands during early stages of breast carcinogenesis was associated with an increase in trimethylation of histones H3 lysine 9 and H3 lysine 27 and de novo CpG island methylation and at the Rassf1a promoter and first exon. In conclusion, we demonstrate that epigenetic alterations precede formation of preneoplastic lesions indicating the significance of epigenetic events in induction of oncogenic pathways in early stages of carcinogenesis.

Introduction

Breast cancer is the most common malignancy in women. Despite the statistically significant decline in breast cancer incidence during 2002–2007, breast cancer continues to be the second leading cause of cancer death among women (1–3). The incidence of invasive breast cancer, the most serious form of breast cancer, in the USA was estimated to increase to 192 370 new cases in 2009 compared with 182 460 in 2008 (3,4). The success of the treatment of breast cancer relies on the ability to detect the disease early, which, in turn, greatly depends on better understanding the underlying molecular mechanisms involved in breast cancer initiation and progression. Classic molecular cancer biology focuses on the role of direct genetic changes in the etiology of cancer (5–7). However, it is now well recognized that alterations in epigenetic mechanisms, e.g. aberrant DNA methylation and histone modifications, also play a fundamental role in carcinogenesis by silencing tumor suppressor genes in all major human cancers (8,9), including breast cancer (10,11). Currently, more than one hundred individual genes have been identified that are frequently hypermethylated in breast cancer alone (11). However, the main question as to whether or not detection of these hypermethylated genes can be used as early diagnostic and therapeutic targets for breast cancer management and prevention, remains unresolved. This is mainly due to a lack of knowledge concerning how specific epigenetic changes may be related mechanistically to neoplastic transformation and uncertainty regarding the temporal sequence of epigenetic alterations occurring between the transition of a normal cell through intermediate tumorigenic stages to a tumor cell (12,13). Investigating these molecular mechanisms in humans is often impractical and, in most cases, unethical (14). In contrast, relevant animal models of mammary gland carcinogenesis provide an opportunity for the study of breast cancer initiation and progression. Considering data obtained in recent epidemiological studies showing a causative role of estrogen for human breast cancer development, especially in premenopausal women (15), and the fact that estrogen-induced mammary gland tumorigenesis in Augustus and Copenhagen-Irish (ACI) female rats is remarkably similar to human ductal breast cancer (16,17), the present study was conducted (i) to identify critical tumor suppressor genes that are epigenetically silenced at early preneoplastic stages of breast cancer development and (ii) to define the underlying mechanisms associated with transcriptional silencing of these genes. We demonstrate that the appearance of the earliest preneoplastic morphological changes, such as atypical alveolar and ductal hyperplasia in mammary glands, during estrogen-induced breast tumorigenesis in female ACI rats, is accompanied by an alteration of global DNA methylation, dysregulation in the expression of proteins required for the proper maintenance of DNA methylation pattern, hypermethylation of the Rassf1a gene, a substantial increase in trimethylation of histone H3 lysine 9 (H3K9me3) and trimethylation of histone H3 lysine 27 (H3K27me3) at the Rassf1a promoter and loss of Rassf1a protein. These results demonstrate clearly that epigenetic dysregulation is one of the underlying events in the mechanism of breast carcinogenesis.

Materials and methods

Animals, treatment and tissue preparation

Intact female ACI rats were purchased from Harlan Sprague–Dawley (Indianapolis, IN). The animals were housed two per cage in a temperature-controlled (24°C) room, with a 12 h light–dark cycle and given ad libitum access to water and NIH-31 pellet diet. At 8 weeks of age, the rats were allocated randomly into two groups of 20 rats each. One group received no treatment (control group). The other group received a single pellet, containing 25 mg of 90 day release 17β-estradiol (E2; Innovative Research of America, Sarasota, FL) that was implanted subcutaneously in the shoulder region, which results in the development of mammary gland adenomas and adenocarcinomas after 24 weeks after estrogen initiation. Five rats per group were humanely euthanized using an overdose of CO2 after 6 and 12 weeks of treatment. All animal experimental procedures were carried out in accordance with animal study protocols approved by the National Center for Toxicological Research Animal Care and Use Committee. The paired caudal inguinal mammary glands (and fat pad) were excised from the overlying skin. One gland was frozen immediately in liquid nitrogen and stored at −80°C for subsequent analyses. The contralateral gland and fat pad were carefully spread onto a 5 x 8 cm glass slide and excess fat and other tissue were trimmed. The gland was then placed flat in a cassette in toto. This provided a histological specimen with frontal (coronal) plane orientation, in which the gland profile is comparable with that of a mammary whole mount. This orientation allows visualization of the arborizing pattern of the duct system and associated alveoli more clearly and completely than is possible using a transverse section of the gland. The specimens were then fixed in 10% neutral buffered formalin for 48 h, processed, embedded in paraffin, sectioned at 4 μ and mounted on glass slides. The sections were stained with hematoxylin and eosin for histopathological examination.

Determination of global DNA methylation status by quantification of 5-methyl-2′-deoxycytidine level

The levels of 5-methyl-2′-deoxycytidine in mammary gland DNA were determined by high-performance liquid chromatography combined with...
electrospray tandem mass spectrometry (HPLC-ES-MS/MS) as detailed in Song et al. (18).

Determination of gene-specific methylation by methylation-specific polymerase chain reaction
The methylation status of the promoter CpG islands of the Rassf1a, p16INK4A, Socs1, Cx26 and Cdh1 genes was determined by methylation-specific polymerase chain reaction (PCR) analysis (19,20). Supplementary Figure 1, available at Carcinogenesis online, shows a diagram of promoter CpG islands of the Rassf1a, p16INK4A, Socs1, Cx26 and Cdh1 genes. The primer sets used for methylation-specific polymerase chain reaction analysis were described previously (19,20). Negative control PCR amplifications were performed by using both sets of modified primers with untreated DNA. The absence of PCR products confirms that unmodified DNA could not be amplified with the modified sets of primers.

Determination of the Rassf1a methylation status by bisulfite-sequencing analysis
Genomic DNA was modified by standard bisulfite treatment and amplified by PCR. The primers were designed to amplify a 532 bp fragment of the CpG island located in the promoter and first exon of the Rassf1a gene (GenBank Access. #s AB212726 and NM_001037555). Amplified PCR products were cloned into pCR2.1-TOPO vector (Invitrogen, Carlsbad, CA) and 10 clones from each sample (n = 4 animals per group) were sequenced using an ABI 3730 sequencer (Applied Biosystems, Foster City, CA).

Western immunoblot analysis of protein expression
Protein levels of DNA methyltransferase (DNMT) 1, DNMT3A, DNMT3B, methyl-CpG-binding protein MeCP2, Rassf1a, Cdkn2a, Socs1, Cx26, Cdh1 and β-actin were determined by western immunoblot analysis as described previously (21,22).

Chromatin immunoprecipitation assay for the Rassf1a-, p16INK4A-, Socs1-, Cx26- and Cdh1-associated histone lysine methylation
Formaldehyde cross-linking and chromatin immunoprecipitation assays were performed, as described previously (22), using a Chromatin Immunoprecipitation Assay Kit (Millipore, Billerica, MA). Briefly, 100 mg of mammary gland tissue were minced in liquid nitrogen and suspended in 0.2 ml phosphate buffered saline (10 mM Na2HPO4, 2 mM KH2PO4, 137 mM NaCl and 2.7 mM KCl, pH 7.4). Proteins were cross-linked to DNA by addition of formaldehyde to the tissue suspension to a final concentration of 1% and incubation for 10 min at room temperature. The cross-linking reaction was quenched and chromatin was sheared to an average length of 0.2–1.0 kb by sonication. Chromatin was immunoprecipitated with primary anti-H3K9me3 and anti-H3K27me3 antibodies (Millipore). In addition, 1/20 of the solution was collected before adding antibody to determine the amount of input DNA.

Fig. 1. Histomorphological changes in mammary gland of female ACI rats exposed to 17β-estradiol for 6 and 12 weeks. (A) Mammary gland from untreated (control) ACI female rats (original magnification ×100); (B) Typical alveolar hyperplasia after 6 weeks of continuous E2 treatment (original magnification ×100); (C) Typical hyperplasia showing alveolar lobule (AL) and a normal appearing intralobular duct (D) after 6 weeks of continuous E2 treatment (original magnification ×400); (D) Typical hyperplasia after 12 weeks of continuous E2 treatment (original magnification ×100); (E) Mammary gland hyperplasia with atypical (arrows) and typical (arrowheads) hyperplastic alveoli after 12 weeks of continuous E2 treatment (original magnification ×100) and (F) Mammary gland hyperplasia with atypical hyperplastic ducts (arrows) lined with variably sized epithelial cells (arrowheads). Duct lumen (L) contains intensely eosinophilic secretion; compare with normal appearing duct in panel C. Original magnification ×400.

Epigenetic silencing of the Rassf1a gene
As a control, the procedure was performed without the addition of the antibody (mock precipitation). Purified DNA from immunoprecipitates and from input DNA was analyzed by quantitative real-time PCR on an Applied Biosystems 7500 Real-Time PCR System using Power SYBR® Green PCR Master Mix (Applied Biosystems). The results were normalized to the amount of input DNA.

Statistical analysis
Results are presented as mean ± SD. Statistical analyses were conducted using one-way analysis of variance, with pairwise comparisons being conducted by Student–Newman–Keuls test.

Results

Early morphological changes during E2-induced breast carcinogenesis
Exposure of ACI female rats to a constant elevated level of E2 produced significant histological changes in the mammary glands. Within the first 6 weeks of exposure, the morphological changes were characterized predominantly by an increased number and size of normal appearing alveolar lobules and ducts per unit area of tissue (Figure 1A and B). This lobular hyperplasia manifested some variability in alveolar and cellular size due to the secretory activity and the number of cytoplasmic lipid vacuoles. The secretory epithelium of alveoli was well differentiated and the intralobular ducts and ductules were lined by a single layer of cuboidal epithelium with a low cytoplasmic to nuclear ratio (Figure 1C).

By 12 weeks, the number and size of foci of alveoli per unit area of glandular tissue (Figure 1D) increased compared with 6 week exposure (Figure 1B). Furthermore, between 6 and 12 weeks, foci of both atypical alveoli and ducts had developed (Figure 1E and F, respectively). The atypical hyperplastic alveolar foci were distinctly different in appearance than typical hyperplastic alveoli (Figure 1E). The alveolar epithelial cells and their nuclei were often larger than those in secretory cells in adjacent normal appearing alveoli. The cytoplasm was more basophilic and nuclear membranes and nuclei were prominent. In many instances, the alveolar lumens were slightly enlarged and irregularly shaped compared with typical alveoli. Atypical small- and intermediate-sized ducts were either clustered together forming separate distinct foci or were nestled between or admixed with normal appearing alveolar glands. The epithelial cells lining these atypical ducts (Figure 1F) were often less uniform in size and larger than those lining normal appearing ducts (Figure 1C). The lumens of atypical ducts were commonly filled with a deeply eosinophilic secretion.

Level of 5-methylcytosine during E2-induced breast carcinogenesis
Table I shows the changes in the 5-methyl-2'-deoxycytidine content in mammary gland DNA during early stages of breast carcinogenesis as determined by HPLC-ES-MS/MS analysis. In the mammary glands of control ACI rats, the extent of DNA methylation did not change over the 12 week period (Table I). In the mammary glands of rats exposed to E2, DNA became significantly demethylated after 6 weeks of exposure. However, after 12 weeks of E2-exposure, the extent of DNA methylation significantly increased as compared withagematched control rats (Table I).

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<tr>
<th>Time of treatment</th>
<th>Percent 5-methylcytosine (± SD)</th>
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<tr>
<td></td>
<td>Control</td>
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<tr>
<td>6 weeks</td>
<td>3.10 ± 0.16</td>
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<tr>
<td>12 weeks</td>
<td>2.97 ± 0.09</td>
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*Significantly different from age-matched controls, (n = 5, mean ± SD).

Expression of DNMT1, DNMT3A, DNMT3B and methyl-CpG-binding protein MeCP2 during E2-induced breast carcinogenesis
Since the accurate maintenance of DNA methylation patterns depends on the function and cooperation of several factors, and especially on

Fig. 2. Western blot analysis of DNMT1, DNMT3A, DNMT3B and MeCP2 proteins in mammary glands of control rats and rats continuously exposed to E2 for 6 and 12 weeks. Mammary gland tissue lysates were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and subjected to western immunoblotting using specific antibodies against DNMT1, DNMT3A, DNMT3B and MeCP2. Equal sample loading was confirmed by immunostaining against β-actin. These results were reproduced in two independent experiments. (A) Representative western immunoblot images. (B) Quantitative analysis of DNMT1, DNMT3A, DNMT3B and MeCP2 protein levels. Data are presented as relative to age-matched control rats. Control values at each time point were considered 100%. The histograms in each of the panels are the mean ± SD. *Significantly different from the control at the same time point.
the function of DNMTs, we measured levels of several critical proteins involved in the maintenance of faithful DNA methylation during breast carcinogenesis. Figure 2 shows changes in the protein levels of the maintenance of DNMT1, \textit{de novo} DNMT3A and DNMT3B and MeCP2 proteins in the mammary glands of ACI rats exposed to \textit{E}2 and in age-matched control rats. Exposure of ACI rats to \textit{E}2 resulted in an early increase in the protein levels of \textit{de novo} DNMT3A and DNMT3B and MeCP2 in mammary glands, especially after 12 weeks of exposure. At that time levels of DNMT3A, DNMT3B and MeCP2 in the breast tissue of rats treated with \textit{E}2 were 1.24, 1.75 and 1.65 times, respectively, greater than the control values.

Epigenetic changes in \textit{Rassf1a}, \textit{p16INK4A}, \textit{Socs1}, \textit{Cx26} and \textit{Cdh1} tumor suppressor genes during \textit{E}2-induced breast carcinogenesis

The increased expression of DNMT3A, DNMT3B and MeCP2 in mammary glands may cause methylation of normally unmethylated promoter CpG islands. This prompted us to investigate the status of promoter methylation of several critical tumor suppressor genes, including \textit{Rassf1a}, \textit{p16INK4A}, \textit{Socs1}, \textit{Cx26} and \textit{Cdh1}, that are frequently hypermethylated in human breast cancer (10). Figure 3A shows the early appearance of promoter hypermethylation of the \textit{Rassf1a} gene in mammary glands of \textit{E}2-exposed rats as detected by methylation-specific polymerase chain reaction. In contrast, we did not detect methylation changes in the promoter regions of \textit{p16INK4A}, \textit{Socs1}, \textit{Cx26} and \textit{Cdh1} genes (data not shown).

The greater intensity of methylated bands after 12 weeks of \textit{E}2-exposure than after 6 weeks indicates a progressive time-dependent hypermethylation of the \textit{Rassf1a} promoter (Figure 3A). This was further substantiated and confirmed by the quantitative bisulfite sequencing analysis of \textit{Rassf1a} promoter methylation (Figure 3B).

Another major mechanism of tumor suppressor gene silencing in cancer is trimethylation of histones (H3K9me3 and H3K27me3) (23–25). Analysis of histone lysine trimethylation in the promoter regions of \textit{Rassf1a}, \textit{p16INK4A}, \textit{Socs1}, \textit{Cx26} and \textit{Cdh1} genes demonstrated an enrichment of histone H3K9me3 and H3K27me3 at the gene promoters (Table II). The most significant increase in the levels of H3K9me3 and H3K27me3 was found at the promoter region of the \textit{Rassf1a} and \textit{Socs1} genes.

In order to determine whether or not these epigenetic alterations are associated with inhibition of gene expression, we analyzed the levels of \textit{Rassf1a}, \textit{Cdkn2a}, \textit{Socs1}, \textit{Cx26} and \textit{Cdh1} proteins in the mammary glands of control rats and rats exposed to \textit{E}2. Figure 4 shows a complete loss of \textit{Rassf1a} protein and substantial decrease of \textit{Socs1} protein in mammary glands of rats exposed to \textit{E}2 for 6 and 12 weeks. In contrast, the levels of \textit{Cdkn2a}, \textit{Cx26} and \textit{Cdh1} did not change significantly.

**Fig. 3.** \textit{Rassf1a} promoter methylation in mammary glands of control rats and rats continuously exposed to \textit{E}2 for 6 and 12 weeks. (A) MSP analysis of the \textit{Rassf1a} first exon methylation. Bisulfite-modified DNA was PCR amplified with two sets of primers specific to unmethylated (U) and methylated (M) cytosine residues in the promoter region of \textit{Rassf1a} gene. The PCR consisted of initial denaturation at 95°C for 7 min followed by 37 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 60 s and extension at 72°C for 60 s. Presence of methylated PCR products after amplification with methylation-specific primers indicates appearance of \textit{de novo} methylation of the first exon of \textit{Rassf1a} gene in mammary glands of \textit{E}2-exposed rats. (B) Bisulfite sequencing analysis of \textit{Rassf1a} promoter methylation. Bisulfite-modified DNA from control (\( n = 4 \)) and \textit{E}2-treated rats (\( n = 4 \)) was PCR amplified and PCR products were cloned into pCR2.1-TOPO vector (Invitrogen). Ten independent clones from each DNA sample were sequenced. Each row represents an individual clone and each column represents an individual CpG site. Open and closed circles represent unmethylated and methylated CpG sites, respectively. Open circles indicate unmethylated in four of four samples; half filled circles indicate methylated in two of four samples; three quarter-filled circles indicate methylated in three of four samples and closed circles indicate methylated in four of four samples.
In this report, we demonstrate that deregulation of cellular epigenetic processes plays an important role in the mechanism of $E_2$-induced breast carcinogenesis in ACI rats. This was evidenced by altered mammary gland tissue of rats treated with $E_2$. More importantly, these epigenetic changes occurred after only 6 weeks of $E_2$ treatment and preceded the appearance of atypical alveolar and ductal hyperplasia, well-accepted early putative preneoplastic morphological lesions (26–28), which were detected only after 12 weeks of exposure. The progressive dynamic nature of epigenetic alterations suggests their crucial role in breast carcinogenesis.

The results of our study demonstrate that long-term exposure of rats to $E_2$ results in significant initial loss of global DNA methylation driven by profound $E_2$-induced cell proliferation in breast tissue (Figure 1). This loss of DNA methylation was accompanied by an upregulation of the de novo DNMT3A and methyl-CpG-binding protein MeCP2. It is well established that accurate maintenance of DNA methylation status is critical for normal cell functioning and that the accurate maintenance of the DNA methylation pattern depends on cooperation between individual DNMTs, especially under conditions when normal DNA methylation levels are compromised (29). Therefore, the increased expression of DNMT3A, DNMT3B and MeCP2 in mammary glands of $E_2$-exposed rats may be a cellular compensatory reaction aimed at restoring the altered DNA methylation pattern. However, the increased expression of DNMTs, especially DNMT3B, may initiate aberrant gene-specific de novo methylation events and result in gene silencing (30,31).

In this report, we demonstrate that one of the earliest changes occurring in mammary glands during estrogen-induced breast carcinogenesis in ACI rats is a loss of Rassf1a protein expression. The results of numerous studies have implicated Rassf1a downregulation in the loss of cell cycle control, resistance to apoptosis and enhanced genetic instability (32–35). Any of these events may contribute to tumorigenesis. Thus, downregulation of the Rassf1a gene may be a crucial event in breast carcinogenesis. This suggestion is supported by recent reports on frequent inactivation of RASSF1A in human tumorigenesis (32).

Previously, RASSF1A transcriitional silencing in cancer cells has been linked to gene deletions, point mutations and inappropriate promoter hypermethylation (32,35,36). Hypermethylation of the RASSF1A promoter region has been frequently found in breast cancer (37–39) and even in epithelial hyperplasia at preneoplastic stages of breast carcinogenesis (40). Likewise, in our study, we also detected aberrant, progressive Rassf1a CpG island methylation, especially in the first exon of the Rassf1a gene, at early stages of breast carcinogenesis. The results of the elegant study conducted by Yan et al. (38) have convincingly demonstrated that RASSF1A promoter hypermethylation rather than first exon methylation is responsible for the silencing of the RASSF1A gene, suggesting the involvement of mechanisms other than CpG methylation in the loss of Rassf1a protein during early stages of $E_2$-induced mammary gland carcinogenesis (Figure 4). Similarly, a recent study conducted by Hinshelwood et al. (41) has shown that silencing the p16$^{INK4A}$ gene in primary human mammary epithelial cells during neoplastic cell transformation occurred prior to de novo promoter CpG island methylation.

The results of recent studies have established the significance of aberrant histone modification, especially increased trimethylation of histones H3K9 and H3K27, as an additional major mechanism of transcriptional gene silencing (43,44). Furthermore, it has been shown that methylation of histone H3K9 and histone H3K27 is a primary signal that is sufficient for initiating gene silencing in vivo (43,44). In this respect, our results demonstrating a substantial increase in Rassf1a- and Socs1-associated H3K9 and H3K27 trimethylation and gene repression provide support for this mechanism. Similarly, in our previous study, we have shown that inhibition of Socs1 gene expression was driven primarily by H3K9me (22). However, the results of our study and previous reports of other investigators (38), demonstrate that only a combination of CpG island hypermethylation and histone lysine methylation resulted in complete transcriptional silencing of Rassf1a gene.

In conclusion, the results of our study demonstrate the fundamental role of epigenetic alterations in estrogen-induced mammary gland carcinogenesis in ACI rats. Importantly, we provide evidence that epigenetic alterations precede the formation of preneoplastic lesions indicating the significance of epigenetic events in induction of oncogenic pathways in early stages of carcinogenesis.

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References


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