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14. ABSTRACT he purpose of this project is to investigate whether epigenetic mechanisms may contribute to reduced expression of the tumor suppressor gene BRCA-1 in sporadic breast cancers. The scope is to test the role of xenobiotics and food compounds that bind the aromatic hydrocarbon receptor (AhR). AhR-ligands include the dioxin-like and tumor promoter 2,3,7,8 tetrachlorobenzo-p-dioxin (TCDD). The activated AhR regulates transcription through binding to xenobiotic response elements (XRE=GCGTG) and interactions with transcription cofactors. Major findings: Gestational exposure to TCDD increased levels of methylation of a CpG region comprising an XRE harbored in the BRCA-1 promoter. This enrichment was paralleled by increased recruitment of DNMT1, decreased BRCA-1 mRNA and protein expression, reduced mammary gland structures associated with differentiation, and mRNA increases of cell cycle regulatory protein cyclin D1 and cdk4. Conversely, cotreatment with the dietary compound resveratrol (Res) had protective effects on TCDD-induced BRCA-1 methylation, BRCA-1 expression, and changes in mammary gland development. Significance. These data provide new insight into the impact of gestational exposure on epigenetic events induced by the AhR on the BRCA-1 gene.					
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Introduction

Subject: Epigenetic alterations such as histone and DNA modifications have been proposed to play a key role in the onset of a variety of tumors by inducing stable, heritable changes in BRCA-1 gene expression [1-13]

Purpose of experiments covered by this reporting period:

- 1) **Objective 1:** Test whether activation of the AhR leads to specific histone and DNA modifications that cause silencing of the BRCA-1 gene in mammary tissue, and the preventative effects of the dietary AhR antagonist resveratrol.
Timed pregnant rat females were assigned to four groups: Group 1, gavaged with 1 µg TCDD/kg body weight on day 15 post-conception; Control Group 2, gavaged with an equivalent volume of sesame oil (~0.2 ml/animal); Group 3, prophylactic group receiving RES from 7 days post breeding and then continued until the end of gestation; and Group 4 receiving RES at 7 days before gavage with TCDD and then continued until the end of gestation. Measurements include changes in expression of BRCA-1, BRCA-1 promoter methylation and chromatin remodeling including histone modifications and chromatin accessibility.
- 2) **Objective 2:** Test if tumors induced by an AhR-ligand and mammary carcinogen causes epigenetic silencing of BRCA-1. Six-weeks old female offspring were administered a single dose of DMBA (10mg/rat administered by gavage in sesame oil). Measurements included tumor data, BRCA-1 promoter methylation, histone modifications, and chromatin remodeling.

Body

Objective 1. Test whether activation of the AhR leads to specific histone and DNA modifications that cause silencing of the BRCA-1 gene in mammary tissue, and the preventative effects of the dietary AhR antagonist resveratrol.

1.1. TCDD increase levels of methylation in the rat BRCA-1 promoter. To test the hypothesis that in utero activation of the AhR may reduce BRCA1 expression through silencing of the BRCA1 gene, levels of promoter methylation were compared in mammary glands from offspring exposed to TCDD and control animals. In preliminary experiments, the number of cycles was determined that allowed linear amplification of the selected BRCA-1 fragment using unmethylated (U) and methylated (M)-specific primers flanking a CpG island containing an XRE consensus sequence (**Figure 1 A and B**). Analysis of 46 day-old mammary tissue documented that TCDD increased by ~75% the ratio between methylated and unmethylated BRCA-1 promoter alleles compared to control and Res (**Figure 1C and D**). Consistent with results presented in the 2010 Progress Report and published in 2011 (Papoutsis et al., J. Nutr. Biochem, Dec 22, please see paper in appendix) documenting the antagonistic effects of Res against activation of the AhR, TCDD-induced methylation was reduced by ~23% in mammary tissue obtained from animals cotreated with TCDD plus Res.

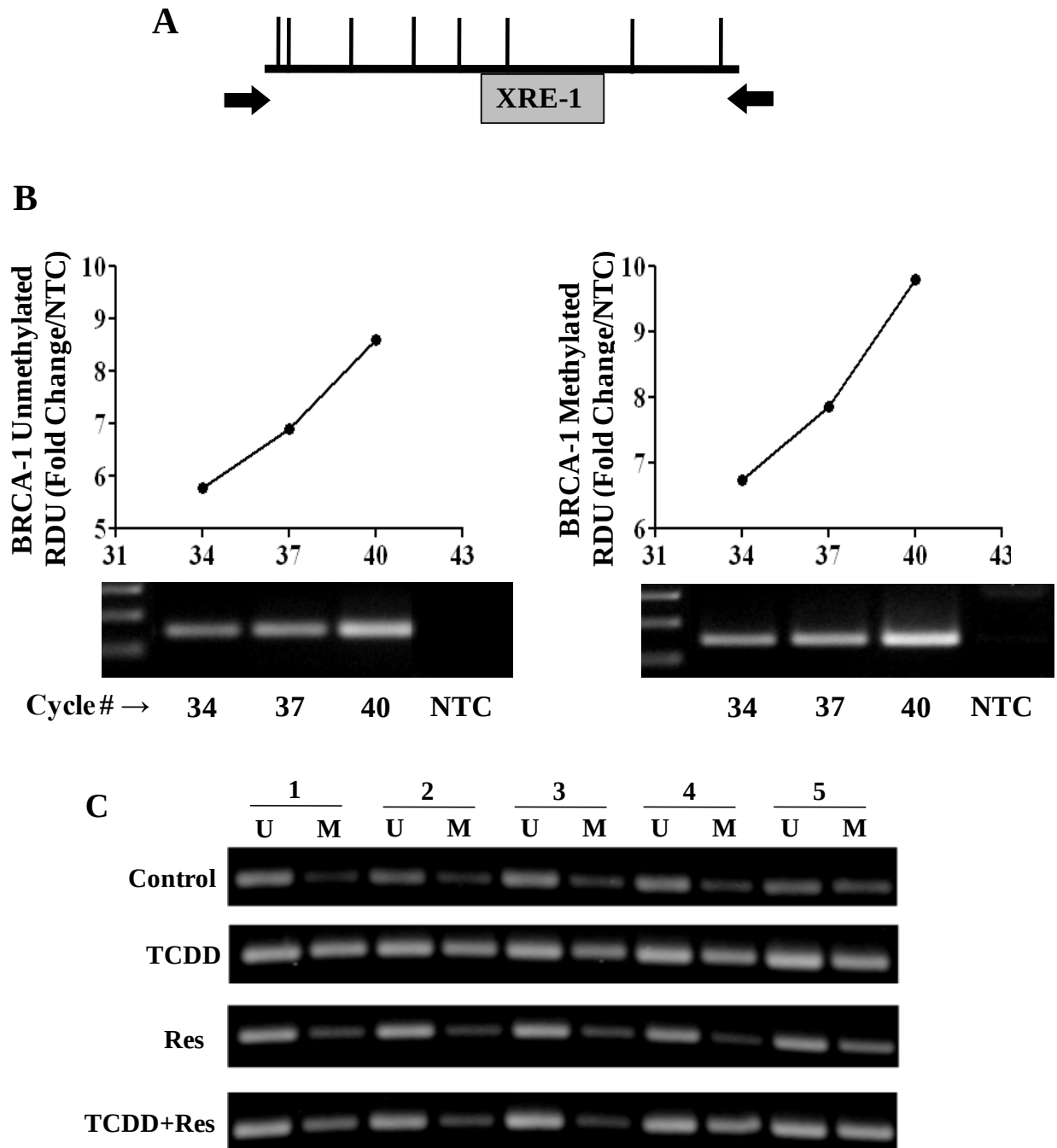


Figure 1. TCDD induces methylation of BRCA-1 XRE. (A) Schematic representation of the BRCA-1 CpG island. Vertical bars indicate the location of CpG sites around XRE. Arrows comprise the BRCA-1 promoter region amplified by MSP. (B) Documents cycle titrations used to identify the appropriate cycle number to accurately represent amplification in the linear range. (C) Genomic DNA was isolated from mammary tissue from 46-day old rats and bisulfite-modified. DNA was analyzed for BRCA-1 promoter methylation using MSP. U=unmethylated and M=methylated promoter sequences. PCR products were separated on a 2% agarose gel and visualized by ethidium bromide staining. The size of the BRCA-1 amplicon is 142 bp.

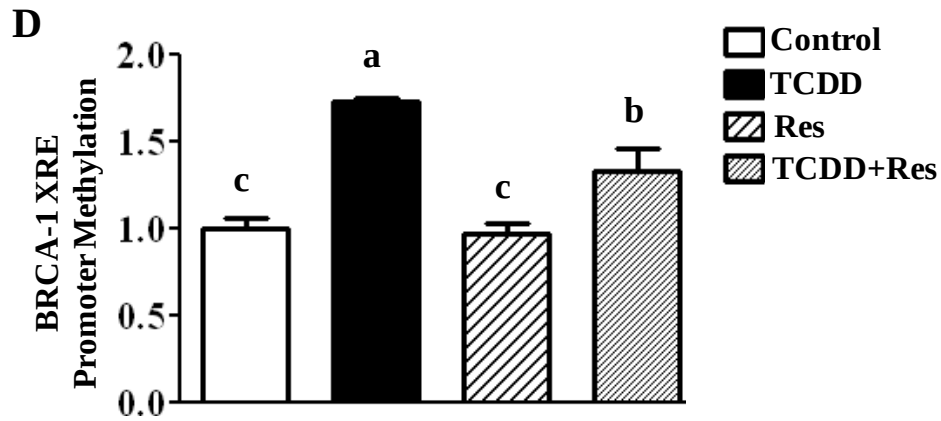


Figure 1 cont'd. TCDD induces methylation of BRCA-1 XRE. (D) Quantitation of BRCA-1 promoter methylation. Bars are means $\pm$ SEM, (CV < 5%). Means without a common letter differ, $P < 0.05$.

1.2. TCDD increases BRCA-1 promoter occupancy of DNMT1. Chromatin immunoprecipitation (ChIP) assay experiments indicated that *in utero* exposure to TCDD increased by 5-fold the occupancy of the DNMT1 on the BRCA1 promoter compared to control (**Figure 2**). Maternal dietary supplementation with Res reduced by ~60% DNMT1 occupancy on the BRCA-1 promoter, compared with TCDD exposed tissues. The increased recruitment of DNMT1 was suggestive of a model of transcriptional silencing that is conserved trans-generationally through maintenance of a hypermethylated BRCA-1. These findings also support the notion that *in utero* exposure to TCDD may contribute to breast cancer in adult offspring through persistent epigenetic silencing of BRCA-1 involving DNMT [14-16]

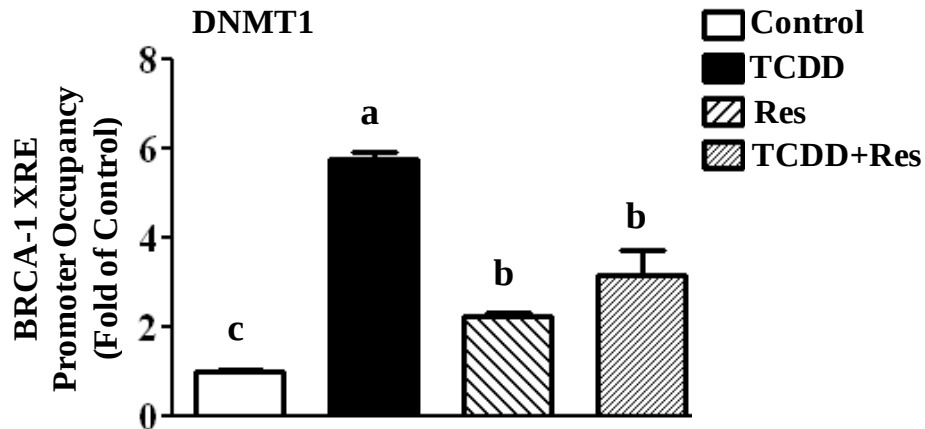


Figure 2. TCDD induces the recruitment of DNMT1 at the BRCA-1 promoter. ChIP analysis of DNMT1 from representative mammary tissue collected at 46 d. Bars are means $\pm$ SEM, (CV < 5%). Means without a common letter differ, $P < 0.05$.

1.3. Resveratrol prevents TCDD-dependent reduction of BRCA-1 mRNA and protein expression.

BRCA-1 mRNA levels were analyzed by RT-PCR using GAPDH as an internal control. Mammary tissue of 46 day-old offspring treated *in utero* with TCDD had a 50% reduction in BRCA-1 mRNA levels compared to control (**Figure 3A**). Conversely, the cotreatment with Res antagonized this effect whereas Res alone increased mRNA levels by ~0.5-fold compared to control. These results corroborated the proposed hypothesis and previous results suggesting antagonists of the AhR may prevent against AhR-dependent repression of BRCA-1 expression [17].

To investigate the correlation between methylation status of the BRCA-1 promoter and protein expression levels, lysate preparations of mammary tissue were analyzed by Western blot analysis. Gestational exposure to TCDD reduced BRCA-1 expression by ~35% compared to control. Conversely, Res alone increased BRCA-1 protein by ~0.75-fold compared to control. (**Figure 3B and 3C**). Taken together, these results are consistent with studies reporting that hypermethylation of BRCA-1 is linked to reduced BRCA-1 mRNA and protein expression. In addition, BRCA-1 repression in offspring mammary tissue was associated to AhR activation, since TCDD induced by ~2.0 fold CYP1A1 mRNA level, compared to control and Res treated groups (**Figure 3D**).

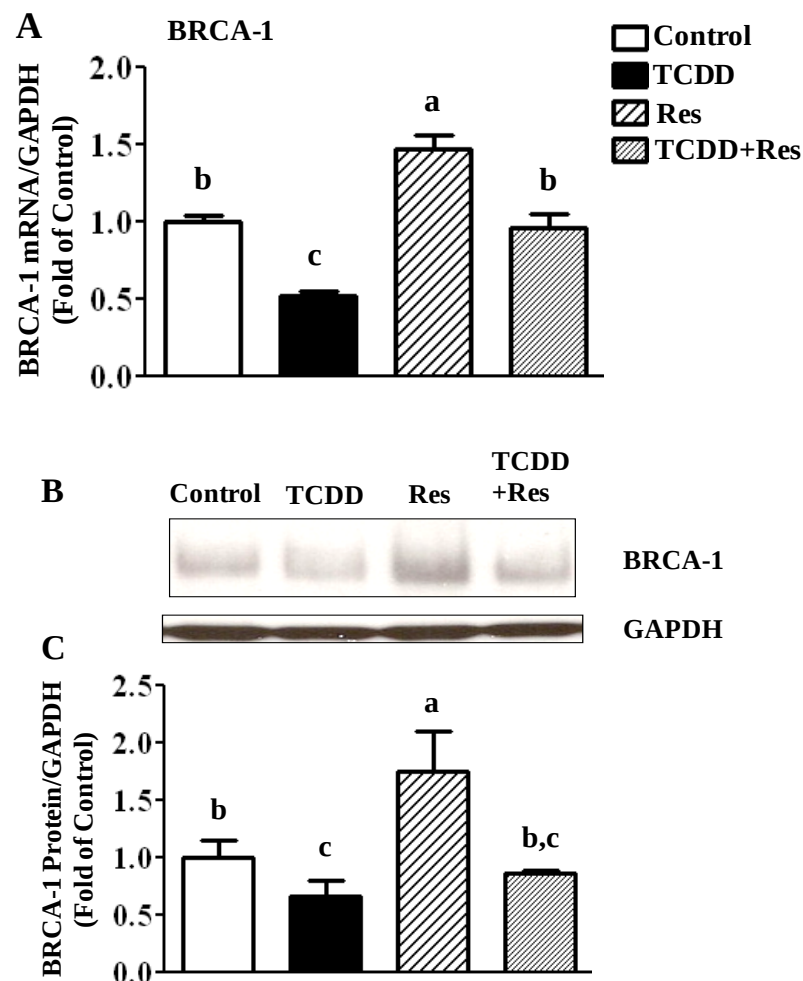


Figure 3. TCDD reduces BRCA-1 mRNA and protein expression levels. Representative results of mammary gland tissue collected at 46 d. (A) BRCA-1 mRNA levels were measured by real-time reverse transcription and normalized with GAPDH. (B) Representative results of mammary gland tissue lysates collected from 46 day-old samples. Bands are immunocomplexes for BRCA-1 and GAPDH. (C) Bars are means $\pm$ SEM, (CV < 5%). Means without a common letter differ, $P < 0.05$.

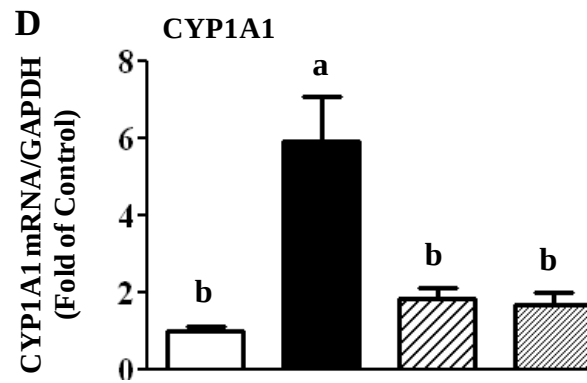


Figure 3 cont'd. TCDD reduces BRCA-1 mRNA and protein expression levels. Representative results of mammary gland tissue collected at 46 d. (D) CYP1A1 mRNA measured by real-time reverse transcription and normalized with GAPDH. Bars are means $\pm$ SEM, (CV < 5%). Means without a common letter differ, $P < 0.05$.

1.4. TCDD increases cyclin D1 and cdk4 mRNA transcripts. Cyclin D1 and cdk4 are cell cycle regulatory proteins needed for progression into S-phase and increased expression levels are associated with proliferating cells. Amplification and overexpression of cyclin D1 are frequently observed in a variety of tumors, including those of the breast, and may contribute to tumorigenesis. RT-PCR analysis confirmed that in mammary tissue of female offspring, both cyclin D1 and cdk4 mRNA transcripts were up-regulated by ~3.0 fold following in-utero exposure to TCDD (**Figure 4A and 4B**). These results were in agreement with data from the morphological analysis presented in the 2010 Annual Progress Report illustrating that TCDD increased terminal end bud structures.

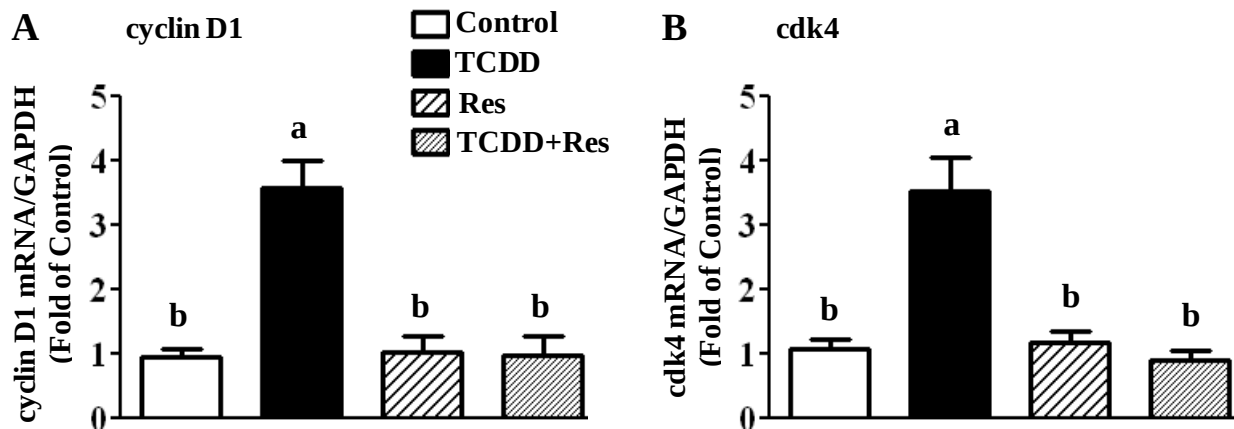


Figure 4. TCDD increases cell cycle regulatory proteins. Mammary gland tissues were collected at 46 d of age and levels of mRNA measured by real-time PCR for (A) cyclin D and (B) CDK4 and normalized with GAPDH. Bars are means $\pm$ SEM, (CV < 5%). Means without a common letter differ, $P < 0.05$.

Objective 2.

2.1. BRCA-1 expression is repressed in DMBA-induced mammary tumors. Based on results of Objective 1, we examine the status of BRCA-1 in DMBA-initiated mammary tumors. Lysates were prepared from non-tumor mammary tissue and mammary tumor tissue from the same animals. Analysis by Western blot highlighted that BRCA-1 protein was expressed at various levels in non-tumor mammary gland tissue of animals treated with DMBA (D), but was significantly reduced in mammary tumors. **Figure 5** shows representative Westerns from DMBA-treated non-tumor (D) and DMBA-treated tumor (T) samples.



Figure 5. BRCA-1 expression is repressed in DMBA-induced mammary tumors. Female Sprague Dawley rats were gavaged with 10 mg DMBA at 50 days postpartum. Mammary gland tissue lysates were prepared from (D) = DMBA-treated non-tumor mammary tissue, (T) = mammary tumor tissue, (C) = control mammary tissue. Bands are immunocomplexes for BRCA-1 and GAPDH.

2.2. AhR mRNA transcripts levels are overexpressed in DMBA-induced mammary tumors. To gather evidence if the observed reduced expression of BRCA-1 protein from mammary tumors was associated with increased AhR activity, levels of AhR-regulated transcripts were evaluated. RT-PCR analysis illustrated that AhR mRNA levels were much higher in all mammary tumors as compared to non-tumor mammary tissue originating from the same animals (**Figure 6**). These data are in good agreement with those of previous reports indicating association between activation of AhR and induction of mammary tumors [18,19].

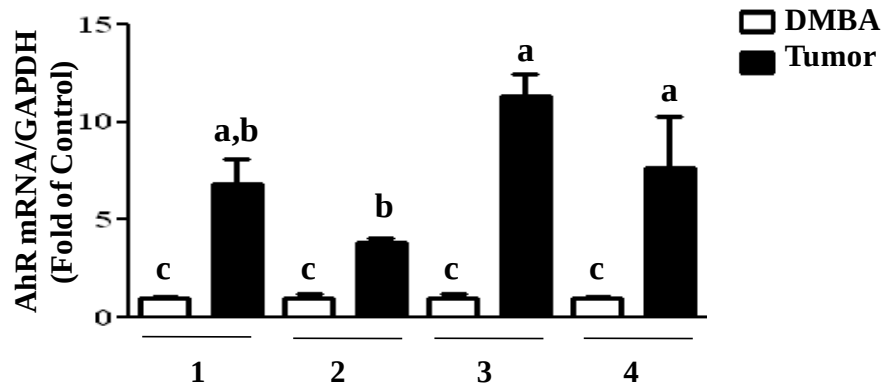


Figure 6. DMBA increases AhR mRNA in mammary tumors. Female Sprague Dawley rats were gavaged with DMBA at 50 days postpartum. Results are from non-tumor mammary tissue (white bars) and tumor tissue (black bars) and represent mRNA levels of AhR normalized to GAPDH. Bars are means $\pm$ SEM, (CV < 5%). Means without a common letter differ, $P < 0.05$.

2.3 DMBA increases levels of methylation of BRCA-1 promoter harboring XRE. To investigate whether the repression BRCA-1 expression in DMBA tumors was associated with hypermethylation of the BRCA-1 promoter, semi-quantitative MSP was used to amplify a CpG island flanking an XRE in the rat BRCA-1 promoter containing 8 CpG sites (**Figure 7A**). When compared to non-DMBA treated control mammary tissue, methylation levels were increased in DMBA treated rats by ~ 2.0 -fold (**Figure 7B**). Taken together, these data support the hypothesis that activation of the AhR by an AhR-ligand and mammary carcinogen leads to epigenetic repression of the BRCA-1 gene through promoter hypermethylation.

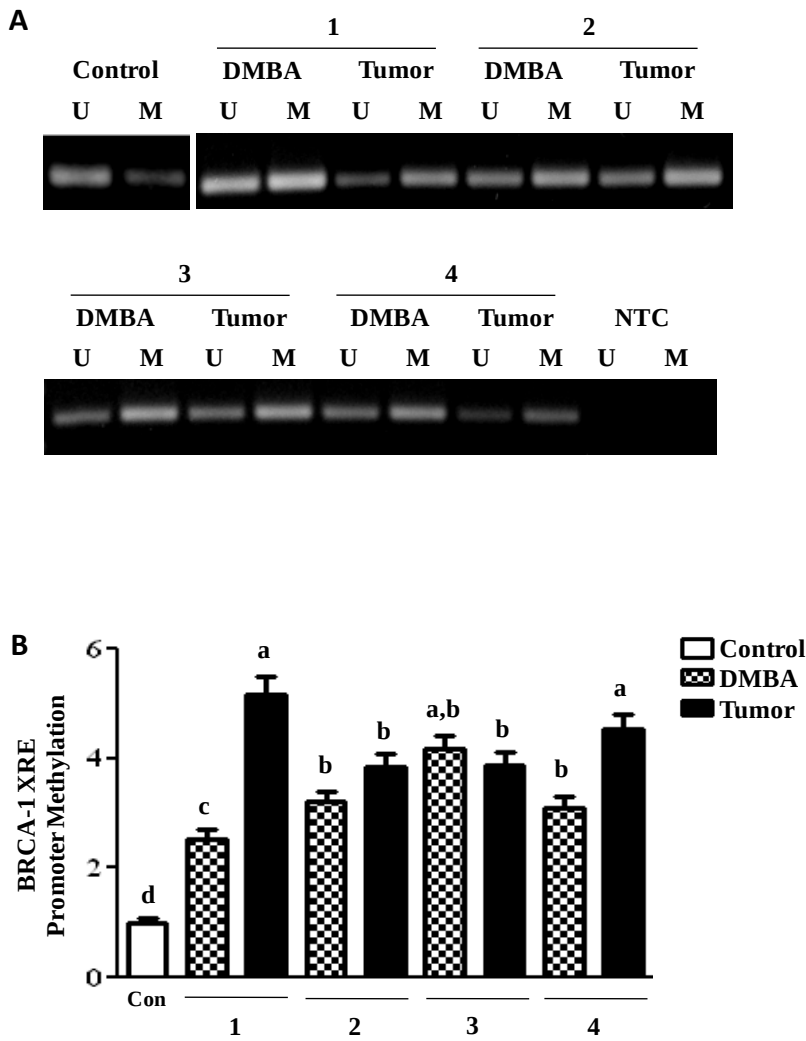


Figure 7. DMBA-induced tumors express higher levels of BRCA-1methylation. (A) Female Sprague Dawley rats (n=4) were gavaged with 10 mg DMBA at 50 days postpartum. Genomic DNA was isolated from non-tumor mammary tissue and tumor tissue and bisulfite-modified. DNA was analyzed for BRCA-1 promoter methylation using MSP. U=unmethylated and M=methylated promoter sequences. PCR products were separated on a 2% agarose gel and visualized by ethidium bromide staining. The size of the BRCA-1 amplicon is 142 bp. (B) Bars are means $\pm$ SEM, (CV < 5%). Means without a common letter differ, $P < 0.05$.

Key Research Accomplishments

1. Obtained evidence in-utero activation of the AhR with the agonist TCDD increase levels of BRCA-1 promoter methylation in mammary tissue of rat female offspring. This is paralleled by increased association of the DNA methyltransferase-1 (DNMT-1) with the BRCA-1 promoter. Conversely, the dietary AhR antagonist may be a useful prototype for prevention [20].
2. In-utero activation of the AhR leads to reduced BRCA-1 expression, and increased expression of markers of proliferation (cyclin D, cdk4) and AhR activation (CYP1A1) in mammary tissue of female offspring.
3. Induction of mammary tumors with the AhR-ligand and mammary carcinogen DMBA leads to repression of BRCA-1 expression, which is paralleled by BRCA-1 promoter methylation.
4. Reduced expression of BRCA-1 in DMBA-induced mammary tumors is paralleled by increased expression of AhR

Reportable Outcomes

1. Manuscripts:

Andreas J. Papoutsis, Jamie L. Borg, Ornella I. Selmin, and Donato F. Romagnolo. BRCA-1 Promoter Hypermethylation and Silencing Induced by the Aromatic Hydrocarbon Receptor-ligand TCDD are Prevented by Resveratrol in MCF-7 Cells. *J. Nutr. Biochem.* Dec 22, 2011

Andreas J. Papoutsis, Jamie L. Borg, Ornella I. Selmin, and Donato F. Romagnolo. In-utero activation of the AhR and epigenetic silencing of BRCA-1 in mammary tissue of rat offspring (In submission, *Cancer Research*).

Donato F Romagnolo and Ornella I. Selmin. Flavonoids in Cancer Prevention: Review of the Evidence. *J Nutr Gerontology*. Accepted for pub. (In Press)

2. Presentations:

Donato F. Romagnolo. Diet-nuclear receptor interactions and epigenetic diseases. Symposium: Nutritional Regulation of Epigenetic Changes. 2012 Annual Meeting of the FASEB/ASN, San Diego, CA. April 25, 2012.

Andreas J. Papoutsis. Ornella I. Selmin, Donato F. Romagnolo. Epigenetic regulation of the breast cancer type-1 gene by the activated AhR and preventative effects of resveratrol. 2012 Annual Meeting of the FASEB/ASN, San Diego, CA. April 23, 2012.

Donato F. Romagnolo, Andreas J. Papoutsis. Ornella I. Selmin. BRCA-1 Promoter Hypermethylation and Silencing Induced by Ligands of the Aromatic Hydrocarbon Receptor and Preventative Effects of Resveratrol. 2011 Meetings of the American Institute for Cancer Research, November 2011. Washington, D.C.

Donato F. Romagnolo. Epigenetics of Breast Cancer. Town Hall Meeting. Arizona Cancer Center, December 14, 2011.

3. Degrees supported by this award:

Dissertation project of Dr. Andreas J. Papoutsis, in Nutritional Sciences and Cancer Biology, The University of Arizona, Tucson, AZ.

4. Funding applied based on work supported by this award:

5. Employment:

Ornella Selmin, Research Associate Scientist.

Jamie Borg: Research Technician

Conclusions

Results included in this Progress Report indicate gestational activation of the AhR increases levels of methylation of a CpG region comprising an XRE harbored in the rat BRCA-1 promoter. This enrichment is paralleled by increased recruitment of DNMT1, decreased BRCA-1 mRNA and protein expression, reduced mammary gland structures associated with differentiation, and mRNA increases of cell cycle regulatory protein cyclin D1 and cdk4. Conversely, cotreatment with the dietary compound resveratrol exerts protective effects on AhR-induced BRCA-1 methylation, BRCA-1 expression, and on mammary gland development.

Development of mammary tumors following exposure to the AhR ligand and carcinogen DMBA is paralleled by hypermethylation of BRCA-1 promoter and reduced BRCA-1 expression.

These data provide new insight into the impact of gestational exposure on epigenetic events induced by the activated AhR on the BRCA-1 gene and provide the basis for testing of this hypothesis in human tumors.

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BRCA-1 promoter hypermethylation and silencing induced by the aromatic hydrocarbon receptor-ligand TCDD are prevented by resveratrol in MCF-7 Cells[☆]

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Abstract

Epigenetic mechanisms may contribute to reduced expression of the tumor suppressor gene BRCA-1 in sporadic breast cancers. Through environmental exposure and diet, humans are exposed to xenobiotics and food compounds that bind the aromatic hydrocarbon receptor (AhR). AhR-ligands include the dioxin-like and tumor promoter 2,3,7,8 tetrachlorodibenzo-*p*-dioxin (TCDD). The activated AhR regulates transcription through binding to xenobiotic response elements (XREs=CGGTG) and interactions with transcription cofactors. Previously, we reported on the presence of several XREs in the proximal BRCA-1 promoter and that the expression of endogenous AhR was required for silencing of BRCA-1 expression by TCDD. Here, we document that in estrogen receptor- α -positive and BRCA-1 wild-type MCF-7 breast cancer cells, the treatment with TCDD attenuated 17 β -estradiol-dependent stimulation of BRCA-1 protein and induced hypermethylation of a CpG island spanning the BRCA-1 transcriptional start site of exon-1a. Additionally, we found that TCDD enhanced the association of the AhR; DNA methyl transferase (DNMT)1, DNMT3a and DNMT3b; methyl binding protein (MBD)2; and trimethylated H3K9 (H3K9me3) with the BRCA-1 promoter. Conversely, the phytoalexin resveratrol, selected as a prototype dietary AhR antagonist, antagonized at physiologically relevant doses (1 μ mol/L) the TCDD-induced repression of BRCA-1 protein, BRCA-1 promoter methylation and the recruitment of the AhR, MBD2, H3K9me3 and DNMTs (1, 3a and 3b). Taken together, these observations provide mechanistic evidence for AhR agonists in the establishment of BRCA-1 promoter hypermethylation and the basis for the development of prevention strategies based on AhR antagonists.

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1. Introduction

The aberrant hypermethylation of tumor suppressor genes has been recognized as a predisposing event in breast carcinogenesis [1]. For example, BRCA-1 promoter hypermethylation has been linked to loss or silencing of BRCA-1 expression in sporadic breast tumors [2–7] and the development of high-grade breast carcinomas [8–10]. Higher incidence (30%–90%) of BRCA-1 hypermethylation has been reported in infiltrating tumors [2,10–12], suggesting that epigenetic repression of BRCA-1 may accompany the transition to more invasive phenotypes. Moreover, BRCA-1 promoter methylation was found to be positively associated with increased mortality among women with

breast cancer [13]. Although these studies provide strong correlative evidence between hypermethylation of the BRCA-1 gene and increased risk of sporadic breast cancer, the mechanisms that contribute to epigenetic silencing of BRCA-1 remain largely unknown.

Several factors contribute to establishment of epigenetic states and include DNA methyltransferases (DNMTs), which are responsible for methylation of cytosines in CpG dinucleotides [14]. DNMTs are classified as maintenance (DNMT1) or *de novo* DNMTs (DNMT3a and DNMT3b) [15]. Remodeling events that reinforce the action of DNMTs and spreading of epigenetic marks to larger chromatin domains are posttranslational modifications of histones by histone deacetylases (HDACs) and methyltransferases, and recruitment to hypermethylated DNA of methyl binding proteins (MBDs) and heterochromatin proteins (HPs) [16].

Mapping of promoter elements provides important clues about mechanisms of transcriptional activation or repression by transcription factors and nuclear receptors. The BRCA-1 gene comprises two transcription start sites on exon-1 and exon-1b [17]. Previously, we reported that an activator protein-1 (AP-1)/estrogen receptor- α (ER α) site located upstream of the BRCA-1 exon-1b mediated the responsiveness of the BRCA-1 gene to 17 β -estradiol (E2) [18]. Also, in previous studies, we identified multiple putative xenobiotic response elements (XREs=CGGTG) for the aromatic hydrocarbon receptor (AhR) in exon-1a (XRE-1=1598–1602), the intervening sequence (XRE-2=1756–1760) and exon-1b (XRE=2069–2073) [19]. The

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presence of multiple XREs in the BRCA-1 promoter suggests a potential role for the AhR in regulation of BRCA-1 transcription. Ligands of the AhR include the compound 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) found in industrial pollution and cooked foods. The activation of the AhR by TCDD was reported to induce transcriptional repression of the tumor suppressor genes p16 and p53 through promoter hypermethylation [20].

Recently, we reported that TCDD repressed E2-dependent activation of BRCA-1 transcription. This inhibition was paralleled by increased recruitment of HDAC-1 and AhR, and reduced association of p300, SRC-1 and acetylated H4 at the BRCA-1 promoter [19]. Conversely, siRNA for the AhR or cotreatment with the AhR antagonist resveratrol restored BRCA-1 protein expression [21]. Whereas these studies provided a proof-of-principle for the involvement of the AhR in repression of BRCA-1 expression, they did not link the reduction in BRCA-1 expression with establishment of BRCA-1 promoter hypermethylation and utilized concentrations of resveratrol (5–20 $\mu\text{mol/L}$) considered largely above the C_{max} (2.4 $\mu\text{mol/L}$) attained in pharmacokinetic studies [22]. Here, we report that increased occupancy of the AhR at the BRCA-1 promoter is coupled to hypermethylation of a CpG island spanning the transcriptional start site of exon-1a. Conversely, histone modifications and recruitment of chromatin modifying enzymes associated with BRCA-1 transcriptional repression were reversed by resveratrol at physiologically relevant concentrations (1 $\mu\text{mol/L}$) [23]. These data provide a mechanistic framework to predict epigenetic silencing of BRCA-1 by AhR agonists and the basis for the development of preventative strategies based on dietary AhR antagonists including resveratrol.

2. Materials and methods

2.1. Cell culture and reagents

MCF-7 breast cancer cells were obtained from the American Type Culture Collection and maintained as described previously [24]. TCDD was supplied by the National Cancer Institute, Division of Cancer Biology, Chemical and Physical Carcinogenesis Branch, and distributed by Midwest Research Institute under contract (64 CFR 72090, 64 CFR 28205). 17 β -Estradiol and resveratrol were purchased from Sigma. Treatments were carried out in phenol red-free medium containing 5% charcoal-stripped fetal bovine serum.

2.2. Western blot analysis

Western blot analysis was performed as previously described [21]. Equal amounts of proteins were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and subsequent immunoblotting was carried out with antibodies against BRCA-1 and GAPDH (Cell Signaling). The immunocomplexes were detected using enhanced chemiluminescence (GE Healthcare/Amersham).

2.3. BRCA-1 promoter methylation-specific polymerase chain reaction (PCR)

MCF-7 cells were harvested, and genomic DNA was isolated using DNeasy Blood & Tissue Kit (Qiagen). DNA (1 μg) was subjected to bisulfite modification using CpGenome DNA Modification Kit (Millipore). The modified DNA (50 ng) was analyzed using CpG WIZ Fast DNA BRCA-1 and CpG WIZ ER α Amplification Kits (Millipore). The PCR amplification products were separated on 2% agarose gels and visualized using ethidium bromide staining. PCR products were gel-isolated and ligated overnight into a pGEM-T Easy vector. The ligation product was used to transform One Shot Top-10 chemically competent *Escherichia coli* (Invitrogen) cells. Recombinant clones were grown on LB/ampicillin agar plates, and plasmid DNA was purified using QIAprep Spin Miniprep Kit (Qiagen). At least 8 clones were sequenced for each treatment to identify methylated CpG sites in the BRCA-1 promoter.

2.4. Chromatin immunoprecipitation (ChIP) assay and quantitative real-time PCR

ChIP assays were performed using the EZ ChIP kit (Millipore) as previously described [18]. Chromatin was immunoprecipitated with antibodies against AhR (Santa Cruz Biotechnologies), MBD2 (Millipore), trimethylated H3K9 (H3K9me3), DNMT1, DNMT3a and DNMT3b (Cell Signaling Solutions). Anti-mouse immunoglobulin G (IgG) was used as a control to check for specificity of antibodies. DNA fragments were amplified by quantitative real-time PCR using the SYBR Green PCR Reagents kit (Biorad). Briefly, PCR reactions were done at a final volume of 25 μL consisting of the following: 12.5 μL of 23 SybrGreen buffer; 1 μL each of XRE-2: forward

(5'-AGCTCGCTGAGACTTCTGGAC-3') and reverse (5'-GTCAGCTTCGGAAATCCACTCTC-3'), exon-1a: forward (5'-CTCCCATCTCTGATTGACCTTGAT-3') and reverse (5'-CAGGAAGTCTCAGCGAGCTCAC-3') and exon-1b: forward (5'-CTGACAGATGGG-TATTCTTTGACG-3') and reverse (5'-GCATATTCAGTCTCTATCAGAG-3'); 8.5 μL nuclease free water and 2 μL DNA purified from the ChIP assay. The standard curve was generated using as a template a plasmid containing the BRCA-1 promoter. Bound DNA was normalized to input DNA.

2.5. DNA protein-binding (pull-down) assay

The binding of AhR to CYP1A1 oligonucleotides was performed as previously described [21]. Briefly, nuclear extracts were prepared using the NE-PER Nuclear and Cytoplasmic Extraction reagents (Pierce Biotechnology), quantitated using the BCA Protein Assay kit (Pierce Biotechnology) and incubated with biotin-labeled, double-stranded DNA oligonucleotides and streptavidin agarose beads. Following dissociation from DNA, nuclear proteins were separated by SDS-PAGE and analyzed by Western blot analysis with an antibody against the AhR (Santa Cruz Biotechnology). Sequences of biotin-labeled oligonucleotides (Sigma) were CYP1A1 XRE: forward (5'-CGGCTCTGTGTCACGCACTCCGAGTCA-3') and reverse (5'-TGAGCTCGGAGTTCGT-GAGAGAGCCC-3').

2.6. Statistical analysis

Densitometry after Western blotting was performed using Kodak ID Image Analysis Software. Statistical analysis was performed using GraphPad Prism 5.0 software. Data from factorial experiments were analyzed by two-way analysis of variance. Post hoc multiple comparisons among all means were conducted using Tukey's test after main effects and interactions were found to be significant at $P \leq 0.05$. All immunoblottings were repeated at least three times.

3. Results

3.1. Resveratrol prevents TCDD-dependent reduction of BRCA-1 expression

Previous studies by our group [19,25] and other investigators [26] documented that BRCA-1 protein expression was induced by E2 in ER α -positive human breast and ovarian cancer cells. Conversely, AhR agonists were shown to disrupt E2-dependent activation of BRCA-1 promoter activity [19,21]. To examine the dose-response effects of TCDD on E2-regulated BRCA-1 protein, we pretreated MCF-7 cells with various concentrations of TCDD followed by cotreatment with E2. The dioxin-like compound TCDD was utilized because, compared to other AhR agonists (i.e., benzo[a]pyrene), it is not metabolized and interpretation of results is not confounded by effects due to secondary metabolites. Western blot analysis of cell lysates indicated a U-shaped relationship with increased BRCA-1 expression at lower (<5 nmol/L) and higher (>10 nmol/L) levels of TCDD treatment (Fig. 1). Based on these results, we selected the dose of 10 nmol/L TCDD for subsequent experiments. This concentration has been used previously to investigate the mechanisms of TCDD-induced initiation in breast epithelial cells and the preventative effects of resveratrol [27]. We found that the pretreatment for 12 h with resveratrol followed by the cotreatment with resveratrol plus E2 for 24 h dose-dependently (10>1>0.5 $\mu\text{mol/L}$) prevented the repressive effects of TCDD on BRCA-1 protein (Fig. 2A). The inhibition of BRCA-1 expression by TCDD (~40%) was completely reversed by the cotreatment with 1 $\mu\text{mol/L}$ resveratrol. The latter dose is lower than the maximal levels (2.4 $\mu\text{mol/L}$) of resveratrol reported in pharmacokinetic studies with human subjects [22]. In keeping with earlier reports documenting estrogen-like effects of resveratrol at higher concentrations [28] (Fig. 2A), a ~1.0-fold induction of BRCA-1 protein was observed in the presence of 10 $\mu\text{mol/L}$ resveratrol plus E2 compared to treatment with E2 plus TCDD. Additionally, 1 $\mu\text{mol/L}$ resveratrol treatment alone resulted in a ~1.0-fold induction of BRCA-1 protein compared to baseline expression (Fig. 2C and 2D). To date, most of the *in vitro* experiments that investigated the effects of resveratrol in breast cancer cells have utilized doses exceeding ~10–20 $\mu\text{mol/L}$ [29]. Results of dose-dependent experiments depicted in Fig. 1 suggest that the stoichiometric ratios among AhR ligands and relative binding

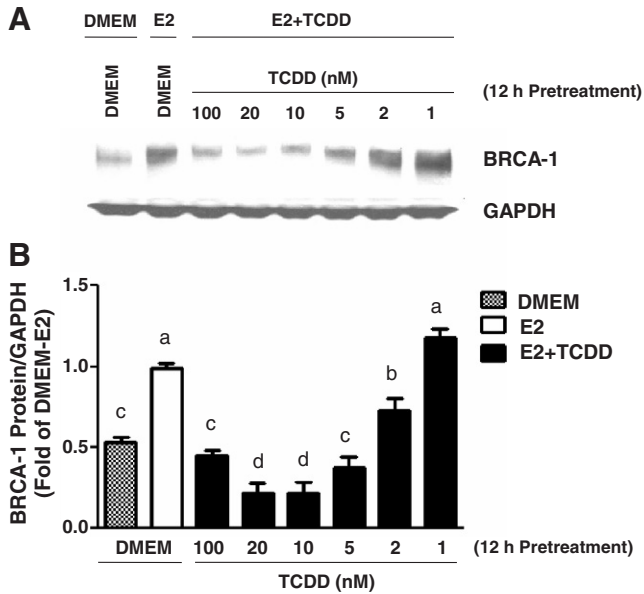


Fig. 1. TCDD reduces E2-dependent activation of BRCA-1 protein expression. (A) MCF-7 cells were precultured in DMEM or DMEM with various doses of TCDD for 12 h. Then, cells were cultured for an additional 24 h in the presence of DMEM, E2 (10 nmol/L) or E2 plus the corresponding TCDD concentration used in the pretreatment. Bands are immunocomplexes for BRCA-1 and GAPDH. In (B), bars are means $\pm$ S.E.M.; $n=3$ (Coefficient of Variation [CV] <5%). Means without a common letter differ; $P<.05$.

affinity for the AhR (Res<TCDD) likely impact the E2-dependent regulation of BRCA-1 expression.

3.2. TCDD-induced hypermethylation of a BRCA-1 CpG island flanking exon-1a is prevented by resveratrol

To investigate whether the repression of E2-induced BRCA-1 expression by TCDD was associated with hypermethylation of the BRCA-1 promoter, we cultured MCF-7 cells in the presence of various amounts of TCDD and resveratrol for 6 days. This time point has been used in previous studies to detect induction of CpG methylation by TCDD in the p16 promoter [20]. DNA samples obtained from MCF-7 cells were subjected to PCR analysis using methylated (M)- and unmethylated (U)-specific primers spanning the BRCA-1 transcription start site of exon-1a (Fig. 3A). This BRCA-1 promoter region harbors a CpG island reported to be methylated in sporadic breast tumors [11]. In the presence of E2, a band was amplified with U primers, whereas the DNA amplification product of M primers was undetectable (Fig. 3B, lane 2). The latter result was in agreement with the previously reported absence in MCF-7 cells of 5-methylcytosine in the BRCA-1 promoter region flanking exon-1a [30]. However, a PCR fragment was amplified with M-specific primers from DNA samples treated with 100 nmol/L TCDD (lane 4), but not in MCF-7 cells cotreated with TCDD plus 20 μ mol/L resveratrol (Fig. 3B, lane 6). Amplification with U- and M-specific primers from control (Con) DNA corroborated the validity of the experimental conditions for methylation analysis and PCR amplification.

Based on these results, we tested the effects of lower levels of TCDD (10 nmol/L) and resveratrol (1.0 and 10 μ mol/L). The results depicted in Fig. 3C suggested that the treatment with 10 nmol/L TCDD induced hypermethylation of the BRCA-1 promoter (lane 6), whereas resveratrol exerted preventative effects (lanes 8 and 10). The hypermethylation of the BRCA-1 promoter at 6 days correlated with reduced BRCA-1 protein expression in TCDD-treated cells, whereas the treatment with resveratrol reversed this repressive effect (Fig. 3D). Compared to treatment with E2 alone or E2 plus resveratrol, the

addition of TCDD to the culture medium did not induce significant changes in the amplification of methylated fragments for the ER α promoter (Fig. 3E, lane 4). These results were in agreement with those of other studies reporting the presence of unmethylated ER α promoter in MCF-7 cells [6] and pointed to promoter-specific mechanisms of regulation by the AhR.

To obtain direct evidence that the treatment with TCDD induced methylation of the CpG island flanking exon-1a, we sequenced multiple clones originated from U and M PCR products. Eight out of eight clones obtained from genomic DNA of MCF-7 cells treated with E2 plus two concentrations (10 and 100 nmol/L) of TCDD showed cytosine methylation of all 10 CpG sites including the CpG cytosine within the XRE-1 (Table 1). Also, a cytosine at two CpNGp

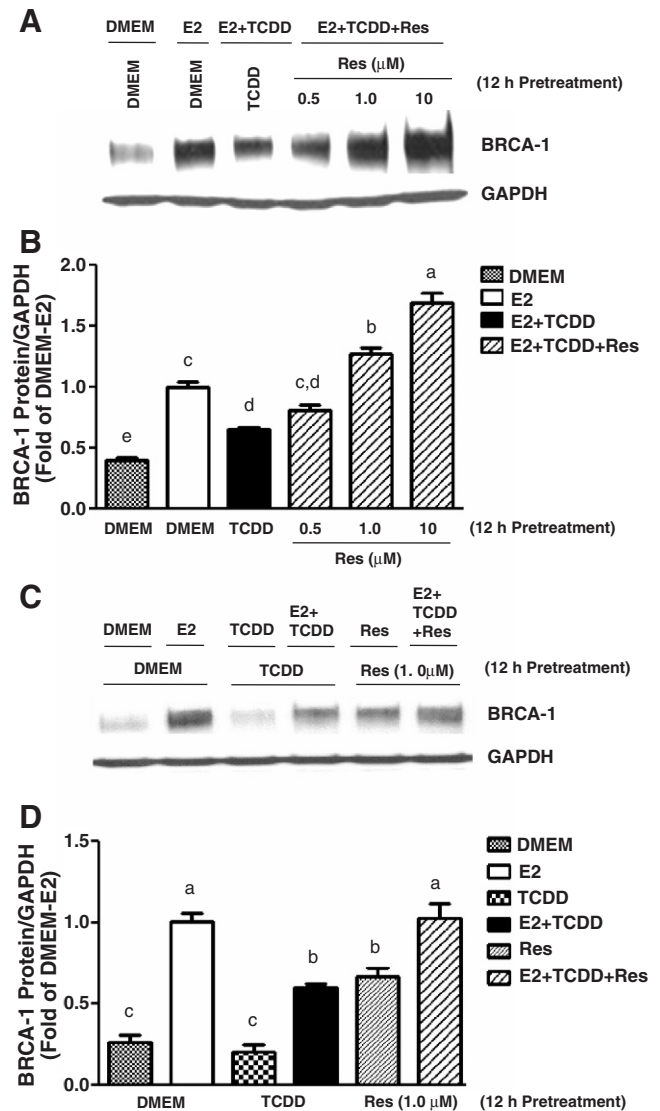


Fig. 2. Resveratrol antagonizes TCDD-dependent repression of BRCA-1 protein expression. (A) Breast cancer MCF-7 cells were precultured in DMEM, DMEM with 10 nmol/L TCDD or DMEM with 0.5, 1 or 10 μ mol/L resveratrol (Res) for 12 h. Then, cells were cultured for an additional 24 h in the presence of DMEM, E2 (10 nmol/L), E2 plus 10 nmol/L TCDD or E2 plus 10 nmol/L TCDD plus the corresponding Res concentration used in the pretreatment. In (C), MCF-7 cells were precultured for 12 h in DMEM, DMEM with 10 nmol/L TCDD or DMEM with 1 μ mol/L Res. Cells were then cultured for an additional 24 h in the presence of DMEM, E2 (10 nmol/L), 10 nmol/L TCDD, E2 plus 10 nmol/L TCDD, 1 μ mol/L Res or E2 plus 10 nmol/L TCDD plus 1 μ mol/L Res. Bands are immunocomplexes for BRCA-1 and GAPDH. In (B and D), bars are means $\pm$ S.E.M.; $n=3$ (CV<5%). Means without a common letter differ; $P<.05$.

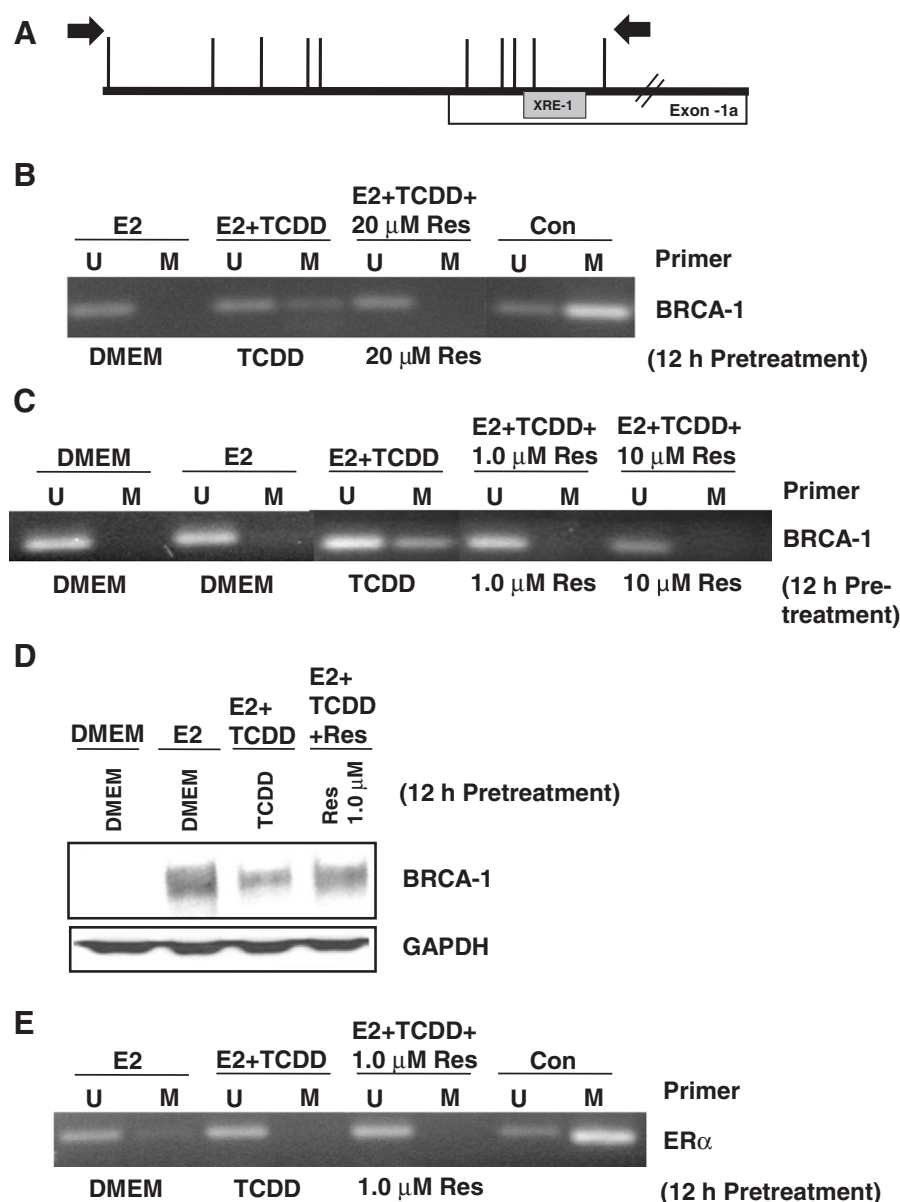


Fig. 3. Resveratrol prevents TCDD-induced methylation of BRCA-1 exon-1a. (A) Schematic representation of the BRCA-1 CpG island. Vertical bars indicate the location of CpG sites around exon-1a. Arrows comprise the BRCA-1 promoter region amplified by PCR. (B) MCF-7 cells were precultured in DMEM, DMEM with 100 nmol/L TCDD or DMEM with 20 μmol/L resveratrol (Res) for 12 h. Then, cells were cultured for an additional 144 h in the presence of E2 (10 nmol/L), E2 plus 100 nmol/L TCDD or E2 plus 100 nmol/L TCDD plus 20 μmol/L Res. In (C) and (E), cells were treated as described in (B) except with doses of 10 nmol/L TCDD and 1 or 10 μmol/L Res. Genomic DNA was isolated, and bisulfite-modified. DNA was analyzed for BRCA-1 (B and C) or ERα (E) promoter methylation using methylated specific PCR. U=unmethylated and M=methylated promoter sequences. Genomic DNA supplied by manufacturer was used as positive Con for PCR. In (D), cells were pretreated with DMEM, 10 nmol/L TCDD or 1 μmol/L Res for 12 h followed by cotreatment for 144 h in the presence of DMEM, E2 (10 nmol/L), E2 plus 10 nmol/L TCDD or E2 plus 10 nmol/L TCDD plus 1 μmol/L Res. Bands are immunocomplexes for BRCA-1 and GAPDH.

trinucleotides was found to be consistently methylated following treatment with TCDD (10 and 100 nmol/L). In contrast, none of the cytosines at CpG and CpNpG nucleotides were methylated in clones amplified using U-specific primers from DNA samples originated in

the presence of Dulbecco's modified Eagle's medium (DMEM), E2 or E2 plus TCDD and resveratrol.

3.3. Resveratrol antagonizes TCDD-induced recruitment of the AhR and chromatin remodeling factors at the BRCA-1 promoter

In control ChIP experiments, we observed that TCDD induced the recruitment of the AhR to the BRCA-1 promoter segment flanking XRE-2 (Fig. 4B, lane 2), whereas the intensity of the PCR amplification product was reduced following cotreatment with resveratrol (Fig. 4B, lane 3 and Fig. 4C). However, no AhR enrichment was observed following incubation of chromatin with IgG (Fig. 4B, lanes 3–6) irrespective of treatment, and no differences

Sequences of PCR products generated after amplification of bisulfite-treated DNA	
Primers	PCR sequence (5' – 3')
Unmethylated	TGGTAGTTTTTTGGTTTTGTGGTAATGGAAAAGTGTGGGAATT ATAGATAAATAAATTGTGATTGTGTGGTGTGAGTTTGTGA
Methylated	CGGTAGTTTTTTGGTTTTCGTGGTAACGGAAAAGCGCGGAATT ACAGATAAATAAATCGCGATTGCGCGGCTGAGTTCGTTGA

Bolded are CpG methylated cytosines. Underlined are non-CpG methylated cytosines.

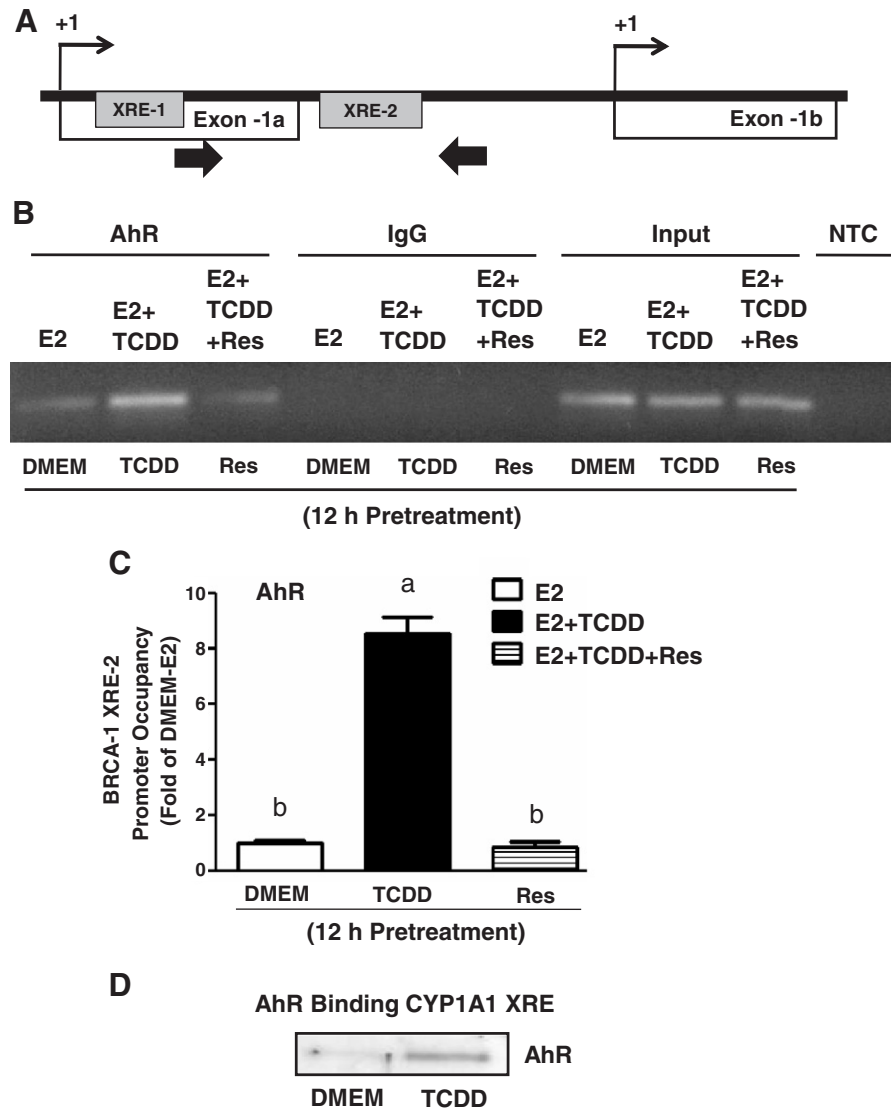


Fig. 4. Resveratrol antagonizes TCDD-dependent recruitment of the AhR to the BRCA-1 promoter spanning XRE-2. (A) Schematic representation of the BRCA-1 promoter. Arrows represent the position of oligonucleotides used to examine by ChIP assay the recruitment of AhR to the BRCA-1 promoter region flanking the XRE-2. (B and C) Pretreatment of MCF-7 cells with DMEM, 10 nmol/L TCDD or 1 μ mol/L Res for 12 h was followed by cotreatment for 144 h with E2 (10 nmol/L), E2 plus 10 nmol/L TCDD or E2 plus 10 nmol/L TCDD plus 1 μ mol/L Res. (B) Reverse transcriptase PCR products were separated on a 2% agarose gel and visualized by ethidium bromide staining. Inputs are control bands amplified from chromatin before immunoprecipitation. The size of the amplicon is 180 bp. (C) Bars are means $\pm$ S.E.M.; $n=3$. Means without a common letter differ; $P < .05$. (D) DNA protein binding assay was performed by incubating nuclear extracts harvested from MCF-7 cells cultured in the DMEM or TCDD for 12 h and with CYP1A1 oligonucleotides. Bands represent AhR immunocomplexes visualized by Western blotting after immunoprecipitation of DNA–nuclear protein complexes with an AhR antibody.

in input chromatin were recorded (Fig. 4B, lanes 7–9). Moreover, we performed DNA pull-down assay for the AhR using CYP1A1 oligonucleotides containing a consensus XRE (Fig. 4D). These data validated the experimental conditions for AhR activation and recruitment to XRE and ChIP experiments.

Next, we examined how TCDD influenced the recruitment of chromatin remodeling factors to the BRCA-1 promoter segment comprising the start site of exon-1a and XRE-1 (Fig. 5A). We found that TCDD stimulated the recruitment of the AhR (~ 8.0 -fold) (Fig. 5B), MBD2 (0.8-fold) (Fig. 5C) and H3K9me3 (~ 1.0 -fold) (Fig. 5D) to this region. Also, TCDD induced the recruitment of DNMT1 (1.6-fold) (Fig. 5E), DNMT3a (1.8-fold) (Fig. 5F) and DNMT3b (0.9-fold) (Fig. 5G) to the BRCA-1 promoter. In contrast, the addition of 1 μ mol/L resveratrol in the pretreatment and cotreatment media reduced the association of these factors to levels lower than those measured in DMEM-E2 chromatin samples.

Because *cis*-epigenetic states such as DNA methylation may impact adjacent chromatin domains [16], we examined the influence of TCDD treatment on recruitment of DNMTs to the BRCA-1 promoter segment comprising the start site of exon-1b (Fig. 6A). Results of ChIP analyses indicated that TCDD enhanced (average ~ 1.0 -fold) the recruitment of DNMT1, DNMT3a and DNMT3b (Fig. 6B–D). Moreover, TCDD stimulated (~ 1.5 -fold) the association of MBD2 and H3K9me3 (Fig. 6E–F). Trimethylated H3K9 is a suppressor chromatin marker coupled to DNA methylation by DNMT1 [31].

Taken together, these findings suggested that recruitment of AhR to the BRCA-1 promoter segment flanking exon-1a and exon-1b was coupled to reduced BRCA-1 expression and enhanced BRCA-1 promoter hypermethylation. The increased association of *de novo* (DNMT3a and DNMT3b) and maintenance (DNMT1) DNA methylation activities correlated with increased association of MBD2 and H3K9me3. Conversely, resveratrol antagonized the

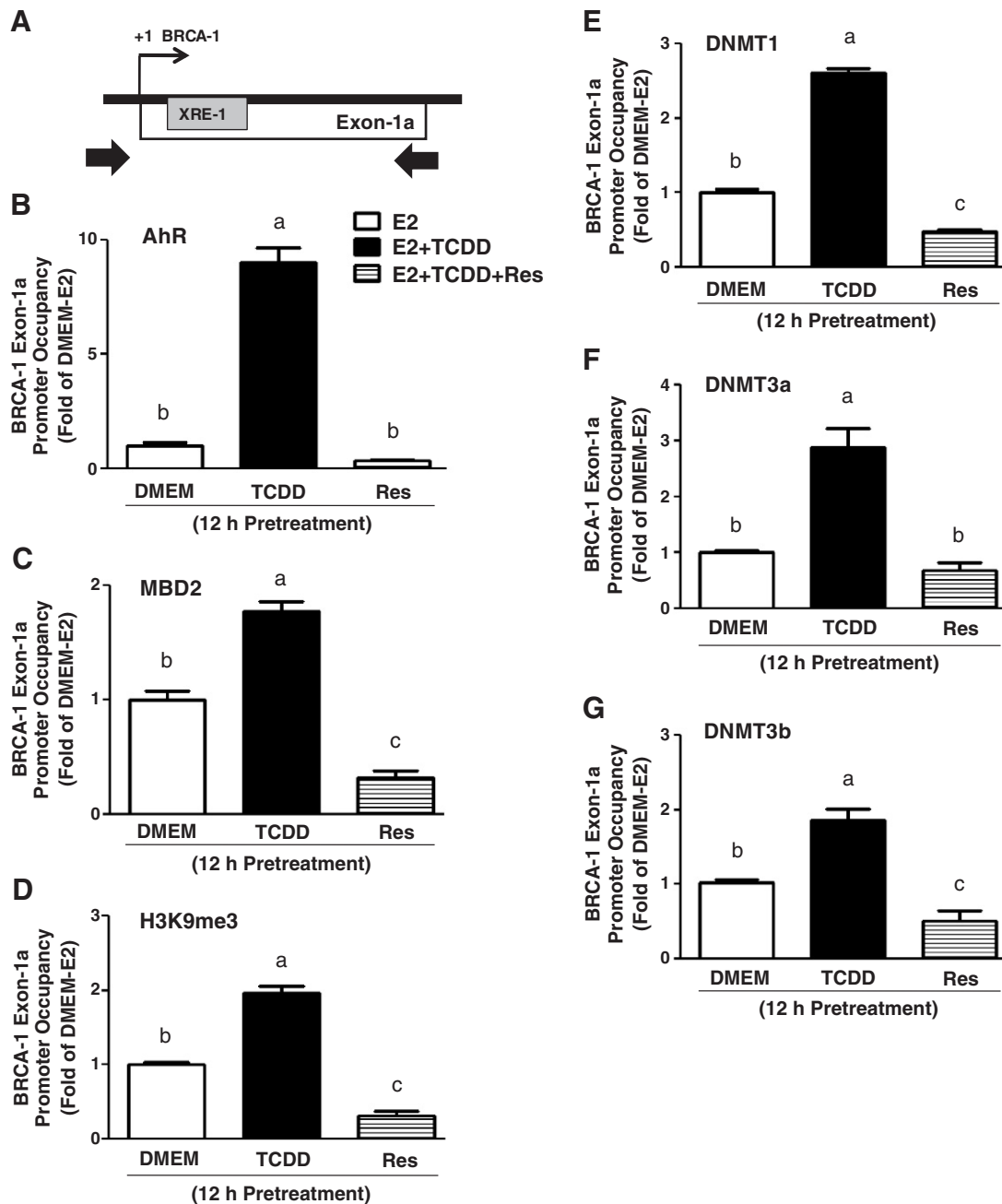


Fig. 5. Resveratrol antagonizes TCDD-dependent recruitment of the (B) AhR, (C) MBD2, (D) H3K9me3, (E) DNMT1, (F) DNMT3a and (G) DNMT3b to the BRCA-1 promoter spanning exon-1a (A). Arrows represent the position of oligonucleotides used to examine by ChIP assay the recruitment of factors to the exon-1a region of the BRCA-1 promoter. Pretreatment of MCF-7 cells with DMEM, 10 nmol/L TCDD or 1 μ mol/L Res for 12 h was followed by cotreatment for 144 h with E2 (10 nmol/L), E2 plus 10 nmol/L TCDD or E2 plus 10 nmol/L TCDD plus 1 μ mol/L Res. Bars are means $\pm$ S.E.M.; $n=3$. Means without a common letter differ; $P < .05$.

TCDD-induced establishment of these epigenetic marks and reduction of BRCA-1 expression.

4. Discussion

Epigenetic abnormalities play a seminal role in the genesis of cancer [14]. The loss of tumor suppressor functions including those controlled by BRCA-1 due to nongenetic influences may substantially increase the risk of developing sporadic breast cancer [32]. Frequent hypermethylation of BRCA-1 has been observed in subsets of sporadic breast tumors [11,12]. Hypermethylation at CpG islands neighboring the transcription initiation sites of the BRCA-1 gene may contribute to

organization of a compact heterochromatin and silencing of BRCA-1 expression. Because DNA methylation is potentially reversible, identifying the mechanisms that induce BRCA-1 promoter hypermethylation may offer new avenues for the development of prevention strategies based on drugs [33] and dietary agents [34].

A plausible role for the AhR in epigenetic regulation of tumor suppressor genes has been provided by the observation that TCDD repressed transcriptional activity and induced promoter hypermethylation of p16 [20]. The latter effect was dependent on expression of the AhR, whose activation has been associated with increased susceptibility to mammary carcinogenesis [35], altered mammary gland development [36,37] and disruption of endocrine functions

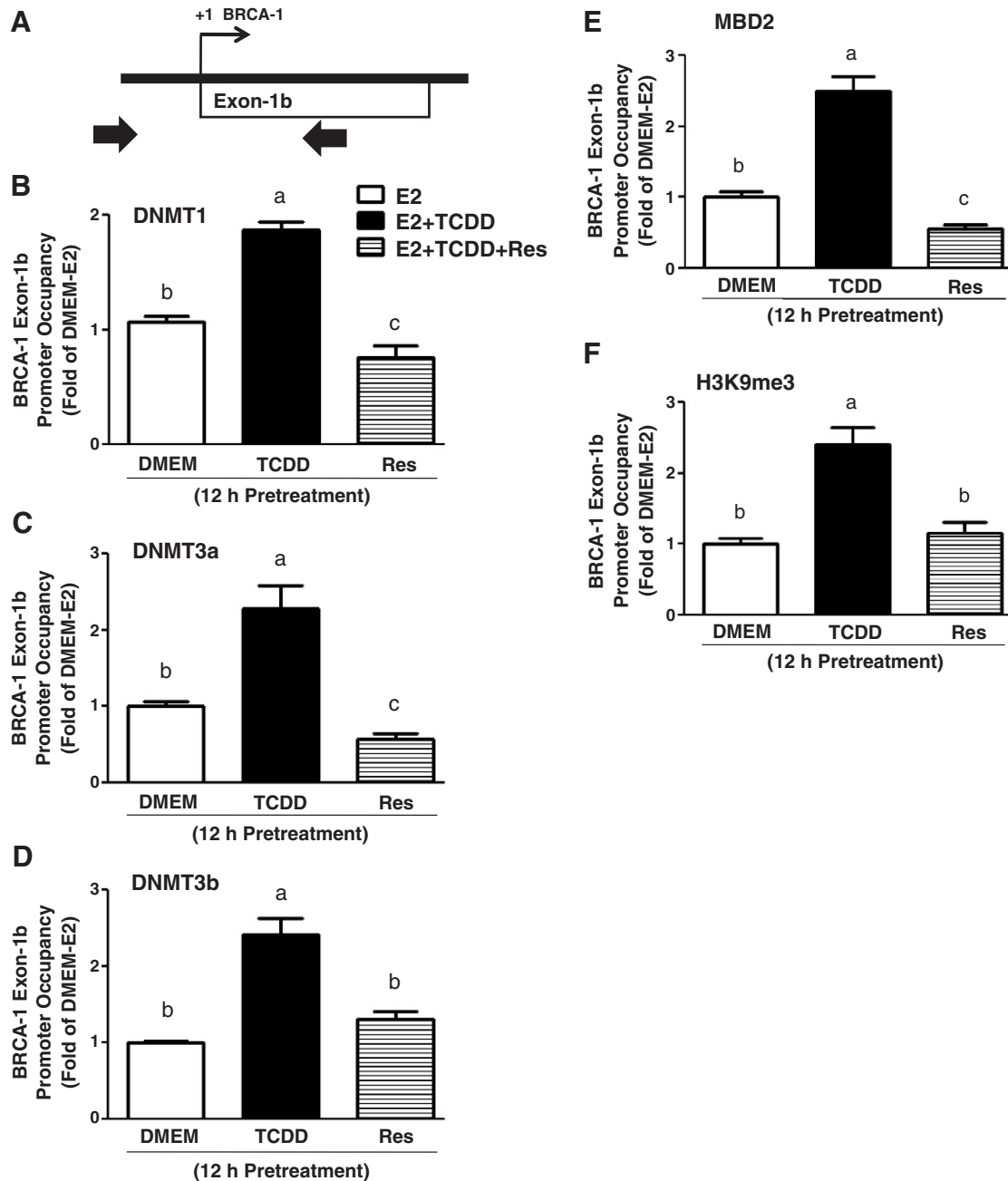


Fig. 6. Resveratrol prevents the TCDD-dependent recruitment of (B) DNMT1, (C) DNMT3a, (D) DNMT3b, (E) MBD2 and (F) H3K9me3 to the BRCA-1 promoter spanning exon-1b (A). Arrows represent the position of oligonucleotides used to examine by ChIP assay the recruitment of factors to the exon-1b region of the BRCA-1 promoter. Pretreatment of MCF-7 cells with DMEM, 10 nmol/L TCDD or 1 μ mol/L Res for 12 h was followed by cotreatment for 144 h with E2 (10 nmol/L), E2 plus 10 nmol/L TCDD or E2 plus 10 nmol/L TCDD plus 1 μ mol/L Res. Bars are means $\pm$ S.E.M.; $n=3$. Means without a common letter differ; $P < .05$.

[38,39]. The accumulation of TCDD has been reported in breast milk [40,41]. Therefore, based on the information that the BRCA-1 gene harbors multiple XRE, we investigated the epigenetic regulation of BRCA-1 under conditions of AhR activation. For these studies, we used the MCF-7 cell line because, in these cells, the BRCA-1 gene is unmethylated around exon-1a [30], BRCA-1 expression is inducible by E2 [18], and the AhR pathway is operative [19].

We documented that, under conditions of TCDD exposure previously shown to induce transformation of mammary epithelial cells [27] and repress E2-induced BRCA-1 expression [19,21], we detected hypermethylation of a CpG island spanning the BRCA-1 transcriptional start site of exon-1a. We also found that TCDD induced

methylation of the CpG within the XRE-1 binding domain, which is located immediately downstream from the start site of exon-1a. Moreover, we observed that a cytosine at two CpNpG trinucleotides was consistently methylated in clones that originated after treatment of MCF-7 cells with TCDD. Methylation of non-CpG cytosines has also been reported for the maspin gene [42].

It is unclear whether the methylation of the BRCA-1 CpG island flanking exon-1a detected in TCDD-treated cells represents a molecular signature specific to AhR activation or a general pattern of BRCA-1 transcriptional repression. However, the CpG island of exon-1a comprises 10 CpG sites found to be hypermethylated in sporadic breast tumors [11] and with histological characteristics

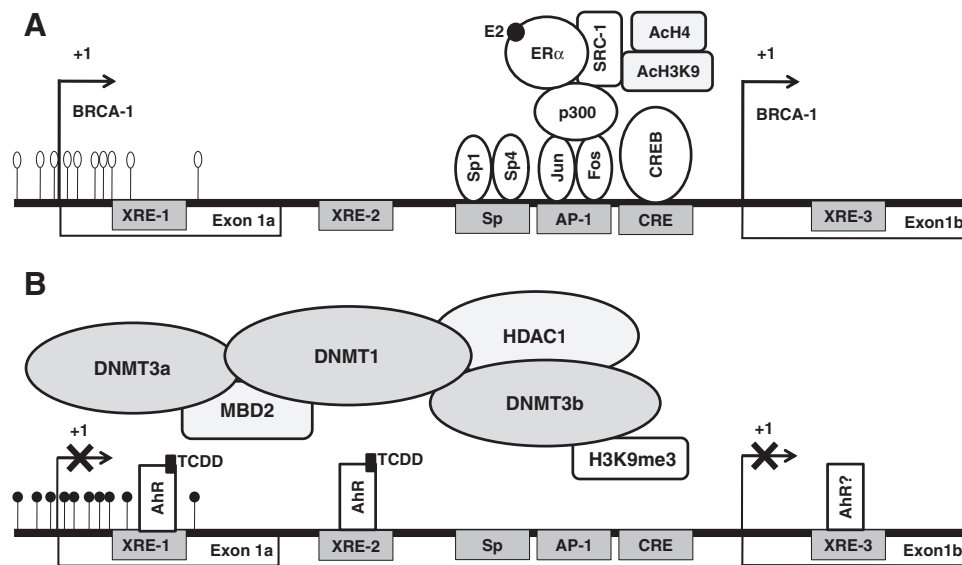


Fig. 7. Proposed model for transcriptional regulation of BRCA-1 promoter. (A) The activation of BRCA-1 by E2 is associated with unmethylated BRCA-1 promoter (open circles), the assembly of a p300/ER α /SRC-1 heterocomplex at an AP-1 site [18], the association of AcH4 and AcH3K9 [21], and the recruitment of Sp1 and Sp4 to the Sp-binding region with constitutive presence of CRE-binding protein (CREB) at the cAMP response site [59]. (B) The activation of the AhR (i.e., TCDD) and its recruitment to XRE lead to hypermethylation (black circles) of the BRCA-1 promoter. This is accompanied by the recruitment of MBD2, H3K9me3, HDAC and DNMTs, which methylate CpG sites and lead to epigenetic silencing. The epigenetic repression of BRCA-1 may be prevented by AhR antagonist (i.e., resveratrol).

frequently seen in breast tumors of BRCA-1 mutation carriers [43,44]. A recent study [12] that included only ductal and/or lobular infiltrating tumors selected from patients over 55 years of age reported that 86% (24/28) of the BRCA-1 low-expressing tumors carried hypermethylated BRCA-1 at this CpG region. Also, it is unknown whether reduced BRCA-1 expression in sporadic breast tumors correlate with higher levels or activity of AhR. Interestingly, studies in sporadic breast tumors reported deregulation of AhR target genes [45], and increased AhR activity was reported in premalignant and malignant mammary tissue [46,47]. These cumulative observations support the concept that constitutive AhR activation may herald mammary tumor formation [48].

The increased recruitment of DNMTs and MBD2 to the BRCA-1 promoter regions flanking the BRCA-1 exon-1a and -1b start sites was clearly suggestive of stimulation by TCDD of maintenance (DNMT1) and *de novo* (DNMT3a and DNMT3b) DNMT activities. Significant overexpression of DNMT3b has been reported in sporadic breast tumors that lack or have low BRCA-1 expression [12]. MBD2 represents the methyl-CpG-binding readout activity of the MeCp1 complex, which through an HDAC-dependent mechanism represses transcription [49]. MBD2 binding has been reported in methylated promoters of various tumor suppressor genes including p16 [50] and 14-3-3 σ [51]. Also, we observed that TCDD stimulated the recruitment of H3K9me3. The latter is a hallmark of transcriptionally repressive heterochromatin [52] and contributes to DNMT1-mediated maintenance of DNA methylation [31]. Methylated H3K9 attracts HP-1, which in turn binds DNMT1, thereby enhancing methylation of CpGs [15]. The results presented here are in keeping with a model in which the recruitment of the AhR to XRE elements neighboring the BRCA-1 start sites of exon-1a and exon-1b may favor spreading of heterochromatin domains and transcriptional silencing [16] (Fig. 7).

Although a strong correlation between ER promoter and BRCA-1 promoter methylation has been documented for a subset of primary breast cancers [6], we did not detect significant ER α promoter hypermethylation or loss of ER α protein expression [21] in TCDD-treated MCF-7 cells. The ability of TCDD to establish BRCA-1, but not ER α , promoter hypermethylation may be due to differences in chromatin organization or CpG promoter content [52].

Conversely, the treatment with the phytoalexin resveratrol antagonized the TCDD-dependent repression of BRCA-1 protein expression; induction of BRCA-1 promoter methylation; recruitment of AhR, DNMTs and MBD-2; and enrichment of H3K9me3 at the BRCA-1 promoter. These preventative effects of resveratrol were attributed to its antagonism towards the AhR [24,53,54], and occurred at concentrations (1 μ mol/L) lower than the C_{max} achieved in pharmacokinetic studies [22,55] and proposed to be attainable in humans through intake of 1 g/d or less [23]. Whether the preventative effects of resveratrol observed in this study were due to the parent compound or its metabolites [56] remains unknown and should be the subject of future investigations.

In summary, the data presented here support the hypothesis that transcriptional repression of BRCA-1 by the AhR involves promoter hypermethylation of a critical CpG regulatory region in the BRCA-1 gene and it is accompanied by the recruitment of chromatin remodeling factors. In contrast, we document for the first time that resveratrol, selected as a prototype AhR antagonist, may prevent hypermethylation of a BRCA-1 CpG island reported to be hypermethylated in sporadic breast tumors. The fact that many food constituents possess ligand properties towards the AhR [57,58] may offer new avenues for the development of prevention strategies for the prevention of BRCA-1 silencing in sporadic breast tumors.

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