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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=GCGTG) 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 17β-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 μmol/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.

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 (ERα) site located upstream of the BRCA-1 exon-1b mediated the responsiveness of the BRCA-1 gene to 17β-estradiol (E2) [18]. Also, in previous studies, we identified multiple putative xenobiotic response elements (XREs=GCGTG) 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 μmol/L) considered largely above the Cmax (2.4 μmol/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 μmol/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). 17β-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 ERα 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′-AGCTCGCTGAGACTTCCTGGAC-3′) and reverse (5′-GTCAGCTTCGGAAATCCACTCTC-3′), exon-1a: forward (5′-CTCCCATCCTCTGATTGTACCTTGAT-3″) and reverse (5′-CAGGAAGTCTCAGCGAGCTCAC-3′) and exon-1b: forward (5′CTGACAGATGGG-TATTCTTTGACG-3′) and reverse (5′-GCATATTCCAGTTCCTATCACGAG-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′-CGGCTCTTGTCACGCAACTCCGAGCTCA-3′) and reverse (5′TGAGCTCGGAGTTGCGT-GAGAAGAGCCG-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 $P \leq .05$. 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 ERα-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 μmol/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 μmol/L resveratrol. The latter dose is lower than the maximal levels (2.4 μmol/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 μmol/L resveratrol plus E2 compared to treatment with E2 plus TCDD. Additionally, 1 μmol/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 μmol/L [29]. Results of dose-dependent experiments depicted in Fig. 1 suggest that the stoichiometric ratios among AhR ligands and relative binding

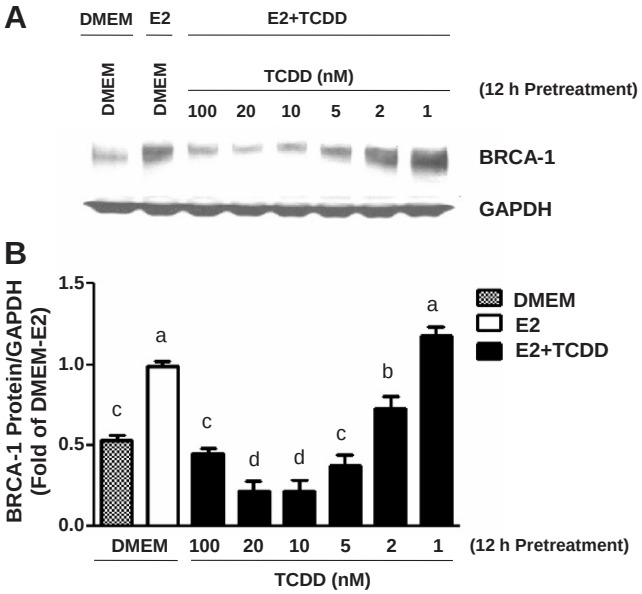

Fig. 1. TCDD reduces E2-dependent activation of BRCA-1 protein expression. (A) MCF-7 cells were precultured in DMEM or DMEM with various doses of TCDD for 12 h. Then, cells were cultured for an additional 24 h in the presence of DMEM, E2 (10 nmol/L) or E2 plus the corresponding TCDD concentration used in the pretreatment. Bands are immunocomplexes for BRCA-1 and GAPDH. In (B), bars are means ± 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 μmol/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 μmol/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 ERα promoter (Fig. 3E, lane 4). These results were in agreement with those of other studies reporting the presence of unmethylated ERα 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

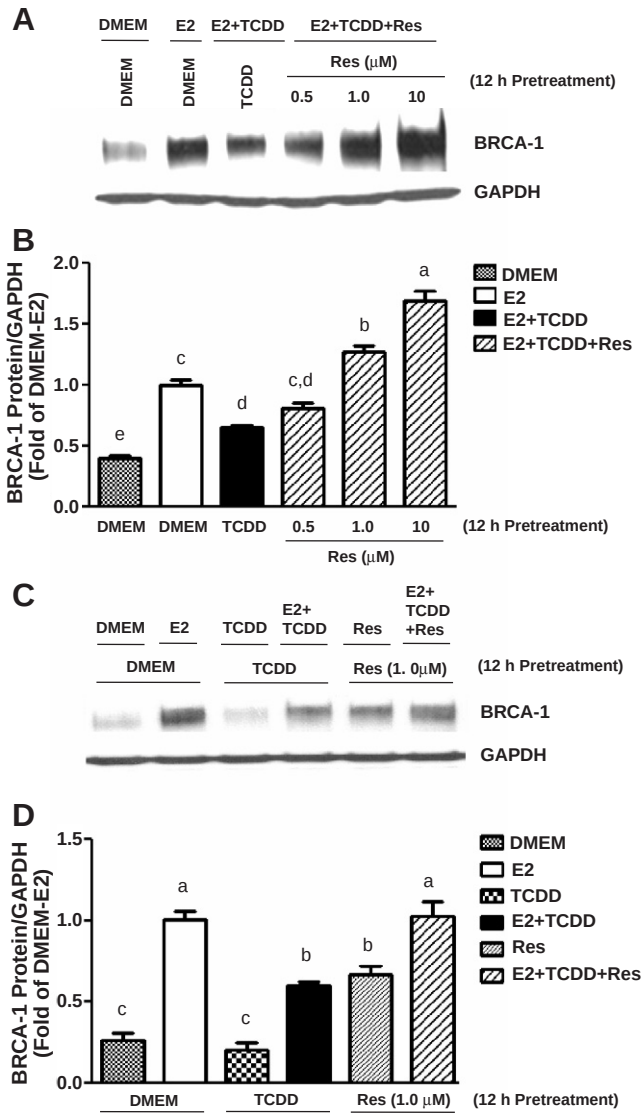

Fig. 2. Resveratrol antagonizes TCDD-dependent repression of BRCA-1 protein expression. (A) Breast cancer MCF-7 cells were precultured in DMEM, DMEM with 10 nmol/L TCDD or DMEM with 0.5, 1 or 10 μmol/L resveratrol (Res) for 12 h. Then, cells were cultured for an additional 24 h in the presence of DMEM,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

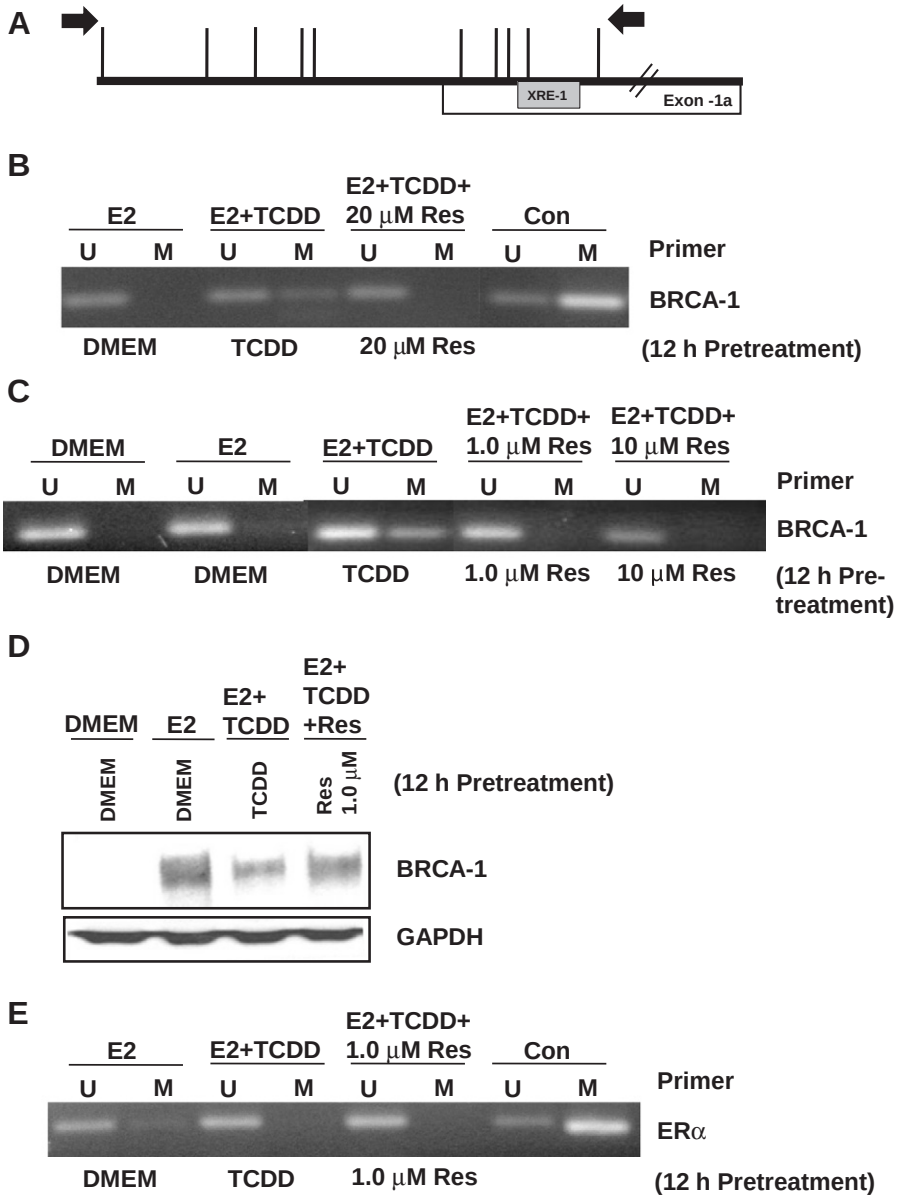

Fig. 3. Resveratrol prevents TCDD-induced methylation of BRCA-1 exon-1a. (A) Schematic representation of the BRCA-1 CpG island. Vertical bars indicate the location of CpG sites around exon-1a. Arrows comprise the BRCA-1 promoter region amplified by PCR. (B) MCF-7 cells were precultured in DMEM, DMEM 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.

Table 1
Sequences of PCR products generated after amplification of bisulfite-treated DNA

Primers	PCR sequence (5' – 3')
Unmethylated	TGGTAGTTTTTTGGTTTTTGTGGTAATGGAAAAGTGTGGGAATT ATAGATAAATTAAAATTGTGATTGTGTGGTGTGAGTTTGTTGA
Methylated	**CG**GTAGTTTTTTGGTTTT**CG**TGGTAA**CG**GAAAAG**CGCG**GGAATT AC̲AGATAAATTAAAAC̲TG**CG**ATTG**CGCG**G**CG**TGAGTT**CG**TTGA

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

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

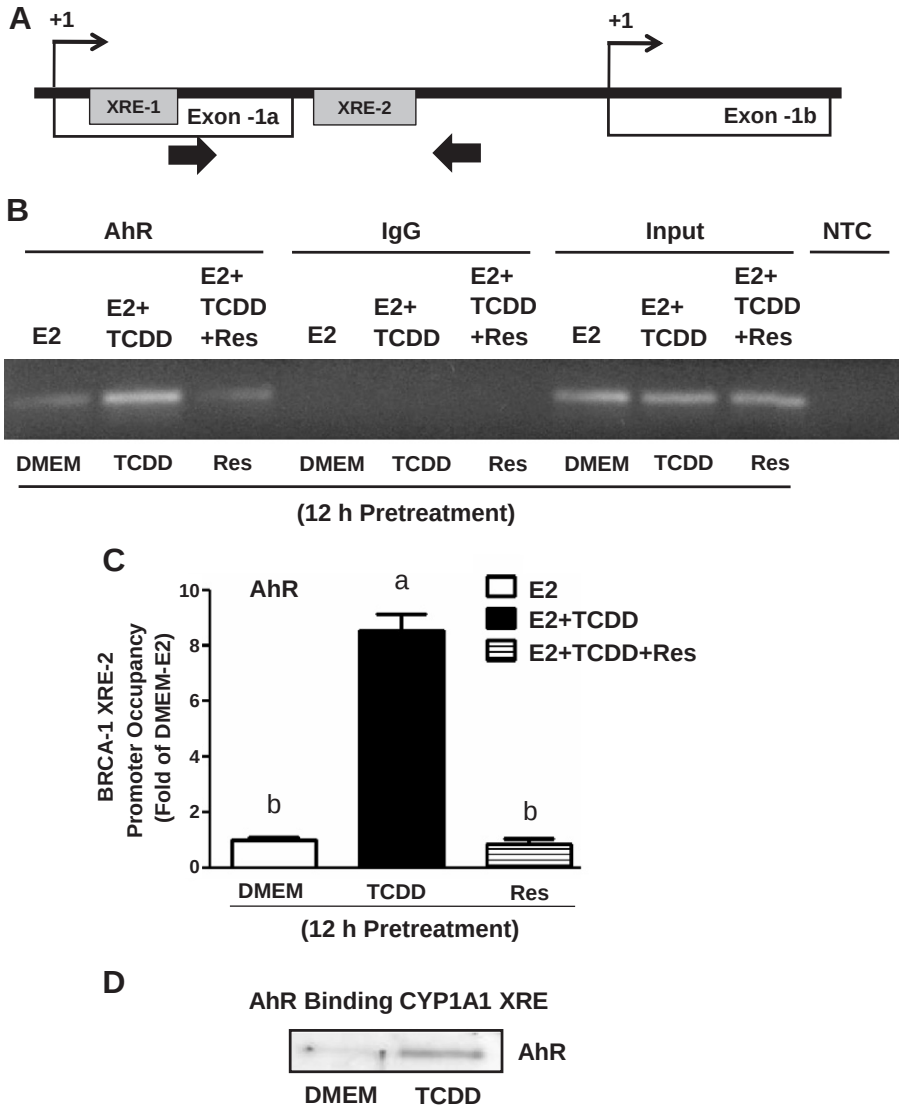

Fig. 4. Resveratrol antagonizes TCDD-dependent recruitment of the AhR to the BRCA-1 promoter spanning XRE-2. (A) Schematic representation of the BRCA-1 promoter. Arrows represent the position of oligonucleotides used to examine by ChIP assay the recruitment of AhR to the BRCA-1 promoter region flanking the XRE-2. (B and C) Pretreatment of MCF-7 cells with DMEM, 10 nmol/L TCDD or 1 μmol/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 μmol/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 ± 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 μmol/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

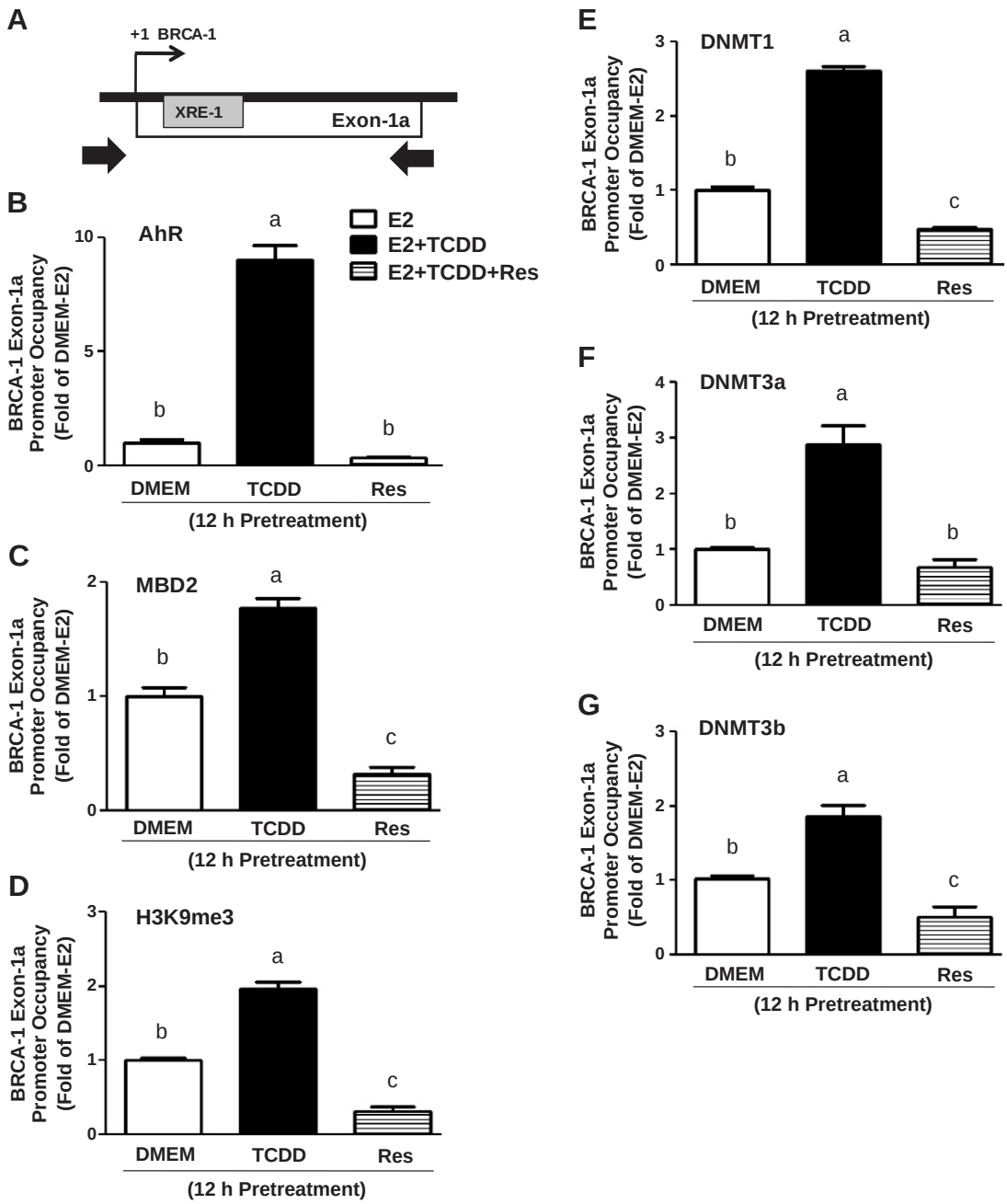

Fig. 5. Resveratrol antagonizes TCDD-dependent recruitment of the (B) AhR, (C) MDB2, (D) H3K9me3, (E) DNMT1, (F) DNMT3a and (E) DNMT3b to the BRCA-1 promoter spanning exon-1a (A). Arrows represent the position of oligonucleotides used to examine by ChIP assay the recruitment of factors to the exon-1a region of the BRCA-1 promoter. Pretreatment of MCF-7 cells with DMEM, 10 nmol/L TCDD or 1 μmol/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 μmol/L Res. Bars are means ± 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

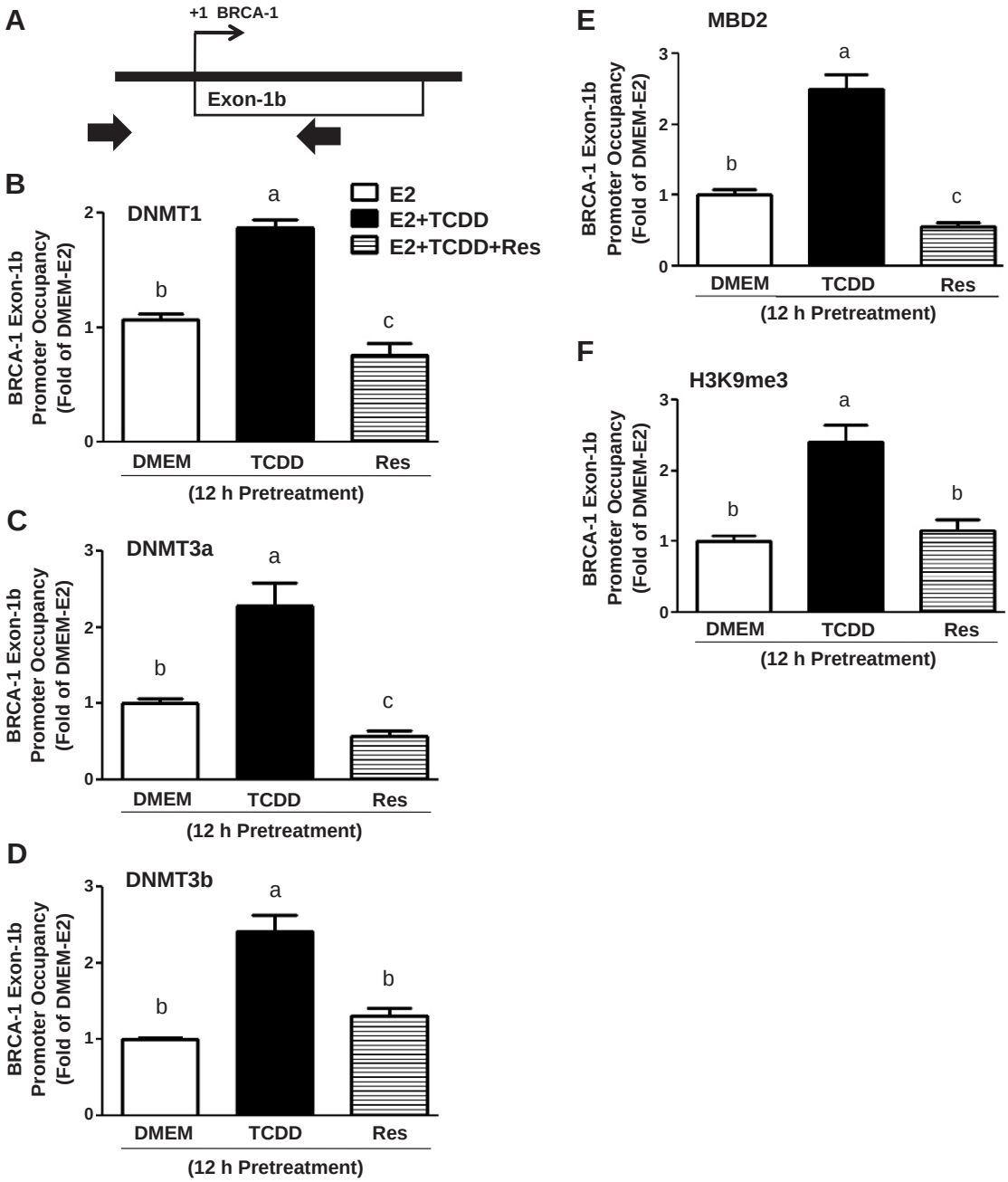

Fig. 6. Resveratrol prevents the TCDD-dependent recruitment of (B) DNMT1, (C) DNMT3a, (D) DNMT3b, (E) MBD2 and (F) H3K9me3 to the BRCA-1 promoter spanning exon-1b (A). Arrows represent the position of oligonucleotides used to examine by ChIP assay the recruitment of factors to the exon-1b region of the BRCA-1 promoter. Pretreatment of MCF-7 cells with DMEM, 10 nmol/L TCDD or 1 μmol/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 μmol/L Res. Bars are means ± 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

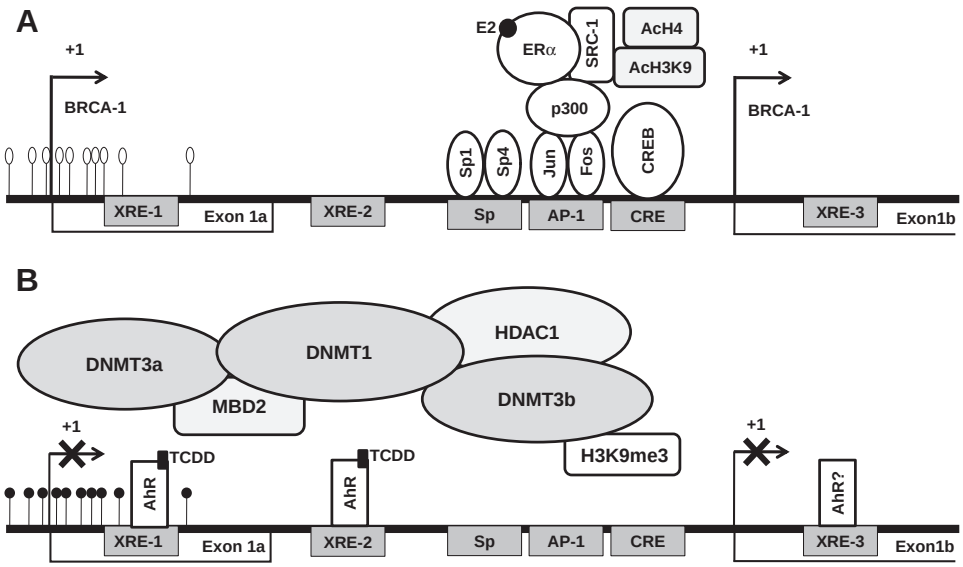

Fig. 7. Proposed model for transcriptional regulation of BRCA-1 promoter. (A) The activation of BRCA-1 by E2 is associated with unmethylated BRCA-1 promoter (open circles), the assembly of a p300/ERα/SRC-1 heterocomplex at an AP-1 site [18], the association of AcH4 and AcH3K9 [21], and the recruitment 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 MeCpP1 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 14-3-3σ [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 ERα promoter hypermethylation or loss of ERα protein expression [21] in TCDD-treated MCF-7 cells. The ability of TCDD to establish BRCA-1, but not ERα, 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 μmol/L) lower than the Cmax 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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