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Estrogen down regulates COMT transcription via promoter DNA methylation in human breast cancer cells

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ABSTRACT

Catechol-*O*-methyltransferase (COMT) acts as a 'gate-keeper' to prevent DNA damage during estrogen metabolism. Both experimental and epidemiological studies suggest the role of COMT in pathogenesis of human breast cancer (BCa). It was previously reported that inhibition of COMT enzyme activity in estradiol-treated human breast epithelial carcinoma-derived MCF-7 cells caused increased oxidative DNA damage and formation of mutagenic depurinating adducts. To improve our understanding of factors influencing estrogen metabolism in BCa, it requires a mechanistic study illustrating the regulation of this 'gate-keeper'. We investigated the epigenetic mechanisms underlying decreased *COMT* transcription in MCF-7 cells exposed to 17ß-estradiol (E2) and the phytoestrogen, genistein (GEN). CpG site-specific methylation at promoters for both soluble (S) and membrane-bound (MB) *COMT* transcripts were assessed. Both E2 and GEN induced CpG site-specific methylation within the distal promoter of *MB-COMT*. In addition, ChIP analysis showed that there was increased binding of DNMT3B, MBD2 and HDAC1 within this promoter. These epigenetic changes were associated with decreased *COMT* transcript levels. Interestingly, sulforaphane, an antioxidant commonly found in cruciferous vegetables, was able to reverse the estrogen-induced epigenetic changes and gene silencing of *COMT*. Our data provide a new insight in epigenetically targeting *COMT* transcription. Since reactive estrogen metabolites may contribute to breast cancer, our findings may help in developing prevention and/or intervention strategies for human BCa.

1. Introduction

Breast cancer (BCa) is the most commonly diagnosed cancer among women in the United States (www.cdc.gov/cancer/breast/statistics). Endogenous estradiol (E2) is suggested to be one of the major risk factors for BCa (Travis and Key, 2003). Catechol-*O*-methyltransferase (COMT) catalyzes the *O*-methylation of E2/estrone (E1) catechols which blocks their estrogenicity and further oxidation to reactive quinones that form mutagenic depurinating DNA adducts and also oxidative DNA damage through redox cycling (Lavigne et al., 2001; Yager, 2015; Cavalieri and Rogan, 2016). Inhibition of COMT enzyme activity has been shown to enhance oxidative DNA damage and adenine and guanine adduct levels in MCF-7 and MCF-10F cells treated with E2 and 4-OH E2 (Lavigne et al., 2001; Zahid et al., 2007). It suggest that a "gate keeper" role for *O*-methylation by COMT by preventing further E2/E1 catechol oxidative metabolism. Genetic variations of COMT were shown to be associated with BCa risk (reviewed by Yager, 2012), suggesting the potential role of COMT in development of BCa.

The *COMT* gene, located on chromosome 22, contains six exons and encodes membrane-bound (MB) and cytosolic (S) forms of the enzyme regulated by two promoters. Promoter 1 regulates expression of the 1.3 kb mRNA encoding S-COMT, a 24kd cytosolic protein and promoter 2 regulates expression of a 1.5 kb mRNA containing non-coding exons 1 and 2 and encoding MB-COMT, a 30kd membrane-bound protein (Yager, 2012). The additional N-terminal region in MB-COMT contains membrane anchoring hydrophobic amino acids (Zhu, 2002). MB-COMT is localized to the endoplasmic reticulum and variably on the plasma membrane and nuclear envelope in a variety of cell types (Tilgmann et al., 1992; Ulmanen et al., 1997) and in breast epithelial normal and tumor tissue (Weisz et al., 2000).

Abbreviation: BPA, bispenol A; BrdU, 5-bromo-2′-deoxyuridine; CEBP, CAAT/enhancer binding protein; ChIP, Chromatin immunoprecipitation; DMSO, dimethyl sulfoxide; DNMT, DNA methyltransferases; E2, estradiol; ER, estrogen receptor; EREs, estrogen response elements; GEN, genistein; HDAC, Histone deacetylases; MB-COMT, membrane-bound COMT; MBD, DNA Methylation Binding Domains; MSPCR, Methylation Specific qPCR; S-COMT, cytosolic COMT; SFN, sulforaphane

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COMT expression, protein level and activity are down regulated by E2. Using estrogen receptor (ER) positive MCF-7 cells, Xie et al. demonstrated an E2 dose dependent (1, 10 and 100 nM) decrease in *S-COMT* 1.3 kb mRNA that was maximum after 48 h (Xie et al., 1999). A similar decrease was not observed in ER negative HeLa cells. Their further analysis revealed that the E2-induced down regulation was related to the presence of two half palindromic estrogen response elements (EREs) in the promoter for S-COMT and two putative CAAT/enhancer binding protein (C/EBP) sites in the promoter for MB-COMT.

Although inhibition of COMT activity has been suggested to increase oxidative DNA damage and DNA adducts in MCF-7 cells and could be a risk factor for estrogen-associated cancers, mechanisms underlying regulation of COMT gene expression in human breast cancer cells were not fully understood. It has been suggested that DNA methylation at *COMT* promoters associate with the decrease in their transcript levels. The role of *COMT* gene methylation in disease pathogenesis has been investigated with mixed results in relation to various diseases (Norrholm et al., 2013; Teroganova et al., 2016 and van der Knaap et al., 2014). Especially, Swift-Scanlan and Smith et al. reported that in unspecified human breast cells lines, *COMT* gene expression was inversely correlated with CpG methylation within exon 3, which contains the translational start sites (ATG) for both *MB-* and *S-COMT* (Swift-Scanlan et al., 2014). On the other hand, *COMT* methylation was changed in respond to xenoestrogens. Nahar et al., 2014 demonstrated an association of increased site-specific methylation within the 5' *MB-COMT* regulatory region with higher levels of a xenoestrogen, bispenol A (BPA), in fetal human liver specimens. Genistein (GEN) is another xenoestrogen commonly found in large quantities in soybeans. It has been well documented that GEN induced changes in DNA methylation in tissues of reproductive organs (Tang et al., 2008; Rietjens et al., 2013; Guerrero-Bosagna and Skinner, 2014). GEN was shown to influence COMT activity in MCF-7 cells (Lehmann et al., 2008). However, in this case, the relationship between inhibition of COMT activity and *COMT* promoter methylation has not been studied.

In this present study, we aimed to determine the mechanisms of which E2 and GEN regulate *COMT* gene expression, via epigenetic modifications at the 5′ regulatory region of *COMT* in MCF-7 cells. Sulforaphane (SFN) is an inducer of phase II protective enzymes including glutathione-S-transferases and quinone reductase. Previously, it was reported that SFN (Nrf2 activator) may alter estrogen metabolism in breast MCF-10A cells and thus protect against estrogen-mediated DNA damage through alteration of E2 metabolizing enzymes such as *COMT, NQO1* and *GSTA1* (Yang et al., 2013). In this study, we investigated whether SFN would modulate the epigenetic regulation of *COMT* transcription. Data would provide new insights in regulating E2/E1 metabolism in human breast cancer cells, which may ultimately modulate breast cancer risk.

2. Materials and methods

2.1. Treatment with E2, GEN and SFN

Human Breast cancer cells, MCF-7 [ER positive], were obtained from the American Type Culture Collection (Manassas, VA, USA). MCF-7 cells were cultured as a monolayer in phenol-red–free Dulbecco's modified Eagle's medium (Mediatech Inc., Manassas, VA, USA) supplemented with 5% dextran-charcoal–stripped fetal bovine serum (Atlanta Biologicals, GA) and 0.01 mg/ml human recombinant insulin (ThermoFisher Scientific, Waltham, MA, USA). Dimethyl sulfoxide (DMSO), DL-sulforaphane (SFN), 17β-estradiol (E2), and genistein (GEN) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Cells were treated with E2 (1 nM), GEN (1 μM) or SFN (10 μM) and a combination of E2 or GEN and SFN for 48 h. Cells treated with DMSO acted as vehicle control (CTL). The maximum concentration of DMSO in the culture medium was 0.1% (*v*/v).

2.2. 5-bromo-2′-deoxyuridine (BrdU) cell proliferation assay

MCF7 cells were treated with vehicle control (DMSO), E2 (1 nM), GEN (1 μM) or SFN (10 μM) and a combination of E2 or GEN and SFN for 48 h. The BrdU Cell Proliferation Assay Kit (Cell Signaling Technology, Danvers, MA, USA) was used to detect the incorporation of 5-bromo-2′-deoxyuridine (BrdU) into DNA during cell proliferation using an anti-BrdU antibody. BrdU analog incorporation into DNA was measured.

2.3. Terminal deoxynucleotidyl transferase (TdT) dUTP nick end labeling (TUNEL) assay

MCF7 cells were treated with vehicle control (DMSO), E2 (1 nM), GEN (1 μM) or SFN (10 μM) and a combination of E2 or GEN and SFN for 48 h. The FragEL™ DNA Fragmentation Detection Kit (EMD Millipore, Burlington, MA, USA) was used to detect binding of TdT to exposed ends of DNA fragments generated in response to apoptosis and expressed as percent TdT labeling.

2.4. Quantification of mRNA levels by real-time PCR (qPCR)

Total RNA was reverse-transcribed into cDNA using iScript Reverse Transcription Supermix kit (BIO-RAD, Hercules, CA, USA). Using cDNA as a template, quantification of mRNA levels of the gene of interest was performed using TaqMan Universal PCR Master Mix (ThermoFisher Scientific, Waltham, MA, USA) and fluorogenic probes in TaqMan Gene Expression Assays specific for *COMT* (Hs00241349_m1) gene (both *MB-COMT*, NM000754.3; and *S-COMT*, NM_007310.2). mRNA levels of *COMT* in all samples were normalized to the levels of *RPL10A* (Hs03043870_g1) from the same sample and relative fold changes were calculated using the $2 - \Delta\Delta Ct$ method (Livak and Schmittgen, 2001).

2.5. Quantification of mRNA level of epigenetic modulators by qPCR

The mRNA levels of *DNMT1, DNMT3A, DNMT3B, MBD1–4, HDAC1–3,* and *HDAC6* were quantified by SYBR Green-based real-time PCR using SsoAdv SYBR Green PCR mix (BIO-RAD, Hercules, CA, USA). The sequences for primers are listed in Supplementary Table 1. The $2^{-\Delta\Delta Ct}$ method was used to calculate the relative expression level of the transcripts normalized to *RPL19*.

2.6. Bisulfite genomic sequencing of MB-COMT distal promoter

In silico analyses and detailed database searches (Li and Dahiya, 2002) were used to predict the CpG(s) in 5′ regulatory region of *MB-COMT*. 16 CpG sites were found in *MB-COMT* distal promoter (MB-COMT PR2). PCR primers were designed to amplify a CpG-rich region spanning from −621 to −1170 bp from the transcription start site, which contains 16 CpG sites within MB-COMT PR2. Primer sequences were BSPCR_MB-COMT-PR2-F: 5′- TTTTTTTGTTTGTTGTTTTTAT TAT-3′ for forward and BSPCR_MB-COMT-PR2-R: 5′- ATATAACCAAT TTCATTCCATACAC -3′ for reverse synthesis. In brief, 200 ng of genomic DNA was used for bisulfite treatment using the EZ DNA Methylation™ Kit (Zymo Research, Irvine, CA, USA). The bisulfite treated DNA was amplified with methylation specific primers using GoTaq Green Master Mix (Promega, Madison, WI, USA) and optimized PCR conditions (95 °C for 10 min, 35 cycles of 95 °C for 30 s, 56 °C for 1 min and 72 °C for 2 min, followed by an extension at 72 °C for 12 min). PCR products were purified and subcloned into pCR 2.1 vector (ThermoFisher Scientific, Waltham, MA, USA). 6–8 clones from each sample were sequenced (Macrogen, Rockville, MD, USA) to obtain direct measures of DNA methylation at each CpG site. Sequencing data was analyzed with the BiQ analyser (Bock et al., 2005) to examine the methylation status.

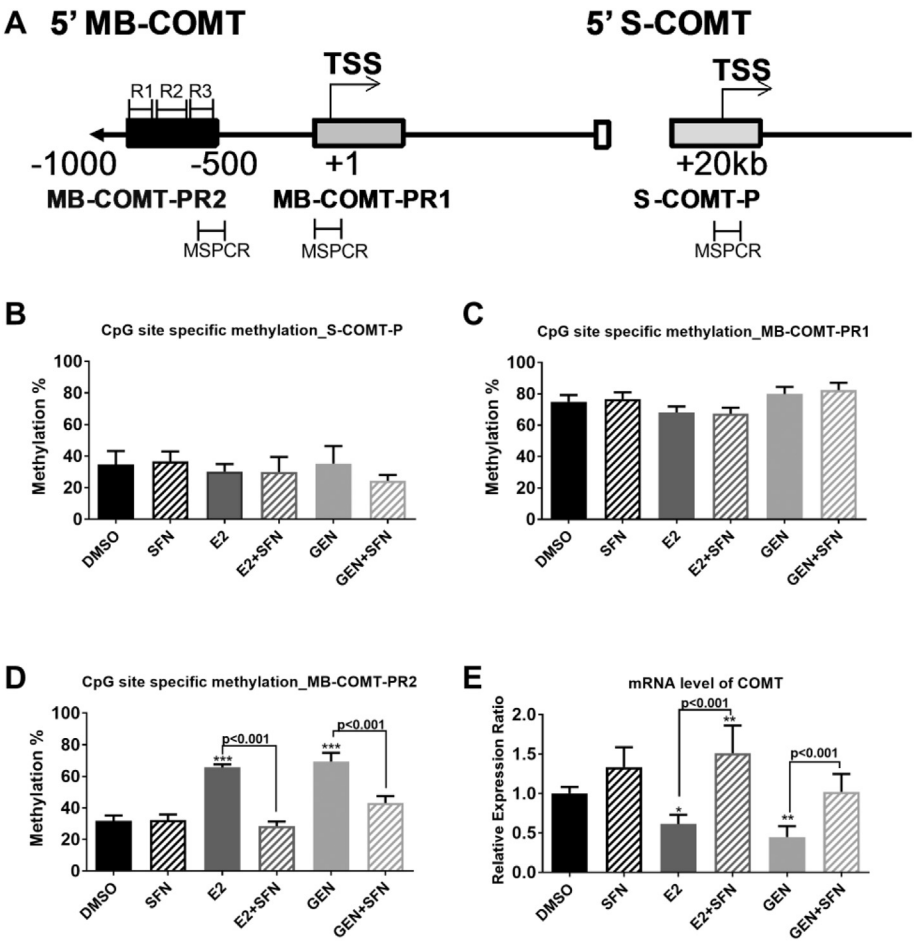

Fig. 1. Methylation levels of multiple promoters of *COMT* and mRNA level of *COMT* in MCF7 cells in response to E2, GEN and SFN. A. Schematic representing 5′ regulatory regions of the *COMT* promoters. MB-COMT-PR1: MB-COMT proximal promoter; MB-COMT-PR2: MB-COMT distal promoter; S-COMT-P: Promoter for the soluble form of COMT; TSS: Transcriptional Start Site. The PCR-amplified regions for methylation specific PCR (MSPCR) and chromatin immunoprecipitation assay (R1 to R3) are indicated by lines. B-D. Percentage of methylation at MB-COMT PR1, MB-COMT PR2 and S-COMT promoters was determined by MSPCR as described in methods. E. *COMT* mRNA levels were determined by qPCR. *P*-value (p) < 0.05 (*), < 0.01 (**) or < 0.001 (***) comparing mean to DMSO.

2.7. Methylation specific qPCR (MSPCR) of COMT promoters

Bisulfite treated DNA was amplified by qPCR using primers specific for methylated DNA within *S-COMT* promoter, S-COMT-P; *MB-COMT* proximal promoter, MB-COMT PR1; and *MB-COMT* distal promoter, MB-COMT-PR1. Sequences of MSPCR primers for MB-COMT-PR1 and S-COMT-P were reported (Sasaki et al., 2003). Forward primer for MSPCR_MB-COMT-PR2-F1: 5'-GGGTTAGTTATTATGTTTTTTATAGT CGT-3′; Reverse primer for MSPCR_ MB-COMT-PR2-R1: 5'-TATAACCA ATTTCATTCCATACACG-3′. Control DNAs (fully methylated and fully unmethylated) (Zymo Research, Irvine, CA, USA) were mixed in various concentrations and served as quantification standards when determining methylation degrees (%) of samples from qPCR.

2.8. Activity assays for DNA methylation and histone modification enzymes

Nuclear protein was isolated using the nuclear protein extraction kit (Epigentek, Farmingdale, NY, USA). Activity of DNMTs, MBD2, HDACs and HATs protein were measured using activity assay kits (Epigentek, Farmingdale, NY, USA).

2.9. Chromatin immunoprecipitation (ChIP) analysis

Chromatin immunoprecipitation was performed using the ChIP-IT Express kit (Active Motif, Carlsbad, CA, USA). Sheared chromatin was immunoprecipitated with antibodies against RNA Polymerase II, HAT/p300, DNMT1, DNMT3B, MBD2, HDAC1, HDAC6 (Epigentek, Farmingdale, NY, USA), H3K4Ac, H3K9Ac and H3K27Ac (Active Motif, Carlsbad, CA, USA). IgG was used as negative control. DNA released from reverse crosslink was purified prior to SYBR-green based qPCR; 1% of starting chromatin was used as input. Primers were designed to amplify 3 different regions of MB-COMT-PR2: ChIP-R1: 5'-CCCTCACA TCCGTGATTCTG-3′ for forward and 5'-TCAAAGCTTCTTGGCTGCAT-3′ for reverse synthesis; ChIP-R2: 5'-GTGACTGCCTCCCAGAACTC-3′ for forward and 5'-GAGGGCTGACTGTGTGTGTG-3′ for reverse synthesis; ChIP-R3: 5'-GCCCATTCACACACACAGTC-3′ for forward and 5'-GTTTC ATTCCATGCACGACA-3′ for reverse synthesis. PCR conditions were programmed as follows: 95 °C for 20 s, followed by 40 cycles 95 °C for 3 s, 60 °C for 30 s. The level of bound DNA sequences was calculated using percent input method (2ˆ-[Ct (ChIP) -Ct (Input)] * 100) by calculating the qPCR signal relative to input sample.

2.10. Statistics

Data from at least three independent experiments were collected. Technical triplicates were included in the qPCR, MSPCR and ChIP-qPCR assays. The results were expressed as the mean ± standard deviation. For the group comparisons, oneway ANOVA with corrected Tukey's multiple comparison tests was applied to determine if data between the treatment groups was statistically significant. All of the data were analyzed and plotted with Prism 6.0 (GraphPad, San Diego, CA, USA).

3. Results

3.1. Effects of estradiol and genistein on COMT expression and CpG-site specific DNA methylation within the MB-COMT distal promoter

We showed that E2 and GEN at hormonally active concentrations decreased *COMT* mRNA levels in MCF-7 cells in a dose-dependent manner (Supplementary Fig. 1A-B) which was demonstrated in other

groups (Xie et al., 1999; Lehmann et al., 2008). We chose E2 at 1 nM and GEN at 1 μM for a time-course study and found out decreased *COMT* expression was time-dependent and maximum by 48 h (Supplementary Fig. 1C-D). In addition, we did not observe any significant toxicity induced by E2 and GEN at these concentrations (Supplementary Fig. 2) and at this time point (48 h). Next, we sought to find out the mechanisms underlying regulation of *COMT* transcription in response to E2 and GEN. Suppression of *COMT* gene expression induced by E2 could be reversed by treatment of the cells with the DNA demethylating agent, 5-aza-2′-deoxycytidine (AZA), and histone deacetylases inhibitor, trichostatin A (TSA) (Supplementary Fig. 3A). Thus, we hypothesized that E2 and GEN modulated *COMT* gene expression via epigenetic modifications.

It has been suggested that DNA methylation at multiple promoters of *COMT* gene may selectively inactive COMT (Sasaki et al., 2003). We examined the methylation status of the promoters which regulate transcription of membrane- or cytosolic form of COMT in E2-treated MCF-7 cells (Fig. 1A). No changes were observed in the *MB-COMT* proximal promoter (MB-COMT PR1) and *S-COMT* promoter (S-COMT-P) (Fig. 1B-C). There is no study demonstrating changes in DNA methylation at the *MB-COMT* distal promoter (MB-COMT PR2) in E2- or GEN-treated MCF-7 cells. By *in silco* analysis, a total of 16 CpG sites were found in MB-COMT-PR2. We sequenced a 550 bp (−1170 to −621) region of MB-COMT PR2 (Supplementary Fig. 4). E2 significantly increased DNA methylation at CpG sites 6 ($p < .05$), 12, 14, 15 and 16 ($p < .01$). We next designed primers specific for methylated CpG sites 12 and 14–16 within MB-COMT PR2 and measured methylation changes by MSPCR. We showed that E2-induced methylation change at this CpG cluster could be completely reversed by AZA (Supplementary Fig. 3B), suggesting DNA methylation machinery induced by E2 occurs at this specific region. Both E2 and GEN significantly increased methylation at MB-COMT-PR2 (Fig. 1D). This increased methylation was associated with the decreased *COMT* gene expression (Fig. 1E). Taken together, these results indicate that down regulation of *COMT* expression by E2 and GEN was associated with increased CpG-site specific DNA methylation only at the *MB-COMT* distal promoter.

3.2. Effects of sulforaphane on COMT expression and CpG-site specific DNA methylation within the MB-COMT distal promoter

Previously, we reported that antioxidant SFN (Nrf2 activator) at 10 μM altered estrogen metabolism and thus protect against estrogen-mediated DNA damage in MCF-10A cells (Yang et al., 2013). In this study, we demonstrated that 10 μM SFN increased expression of Nrf2-targeted gene (NQO1) in MCF-7 cells (Supplementary Fig. 5) but did not exert any toxic effect on the cells (Supplementary Fig. 2A-B). We next determined the antioxidant effect of SFN on increased MB-COMT-PR2 methylation and *COMT* suppression caused by E2 and GEN. SFN alone did not change methylation level of MB-COMT-PR2 (Fig. 1D) as well as *COMT* mRNA level (Fig. 1E). Strikingly, we found that SFN reversed the effects of E2 and GEN on *MB-COMT* distal promoter methylation and modulated the promoter methylation to levels similar to that observed in control and SFN only treated cells (Fig. 1D). As shown in Fig. 1E, the reversal of E2- or GEN-induced *COMT* promoter methylation by SFN correlated to the increased *COMT* gene expression.

To assess more apical effects of SFN on E2- or GEN- treated MCF-7 cells, we examined cell proliferation and apoptosis in cells co-treated with SFN (Supplementary Fig. 2). E2 increased cell proliferation while SFN alone had no effect. Co-treatment with SFN reversed the increase in cell proliferation caused by E2. GEN alone did not modulate the cell proliferation while there was a decrease in level of BrdU incorporation when cells co-treated with SFN. On the other hand, cells underwent a small but significant increase in apoptosis upon co-treatment with E2 plus SFN. While SFN had large inhibitory effects on E2-induced proliferation, they were not accompanied by large increases in apoptosis. These results indicate that the changes modulated by SFN we are observing are not associated with its toxicity to the cells. Taken together, these results suggest that, at least in MCF-7 cells, SFN may protect from adverse effects (i.e. tumor cell growth) related to oxidative metabolism of E2 to its reactive quinone metabolites by blocking down regulation of *COMT* expression by E2.

3.3. Effects of estradiol, genistein and sulforaphane on activity and expression of DNA methylation and histone modification enzymes

We sought to characterize the epigenetic modifications of *MB-COMT* distal promoter in E2- or GEN-treated MCF-7 cells. We demonstrated that either DNMTs inhibitor (AZA) or HDACs inhibitor (TSA) modulated E2-induced *COMT* suppression and MB-COMT-PR2 methylation (Supplementary Fig. 3), suggesting *COMT* expression could be regulated by epigenetic modifications, perhaps through DNA methylation and histone deacetylation. Enzymes involved in DNA methylation (DNA methyltransferases, DNMTs; DNA methylation Binding Domains, MBDs), histone de- or acetylation (Histone acetyltransferases (HATs) such as enzyme p300, and histone deacetylases, HDACs) have a central role in epigenetic regulation of gene transcription in human breast cells (Mielnicki et al., 1999; Singal and Ginder, 1999; Zhang et al., 2005; Kar et al., 2014). In the present study, both E2 and GEN increased activity of MBD2, DNMTs and HDACs proteins while decreased that of HATs (Fig. 2A-D). SFN reversed the induction of DNMTs, MBD2 and HDACs activity by E2 or GEN but not the reduction of HATs activity. Therefore, we next identified which members of DNMTs, MBDs, HDACs and HATs showed alteration in gene expression in response to E2 or GEN in order to examine which epigenetic modification enzymes may involve in transcription of *COMT* (Table 1). Afterwards, we validated if any of these epigenetic modification enzymes were bound at the MB-COMT-PR2 in response to E2 and GEN (Fig. 3-6).

E2 increased expression of *DNMT1*, *DNMT3A, DNMT3B, MBD2, MBD4, HDAC1* and *HDAC6* expression levels (Table 1). There was a decrease in *HAT/p300* gene expression in response to E2. No changes were found in expression of other modification enzymes including *MBD1, MBD3*, *HDAC2* and *HDAC3* in E2-treated cells. GEN-treated cells showed increased expression in *DNMT1* and *HDAC6* but decreased expression in *HAT/p300*. There were no significant changes in *DNMT3A, DNMT3B, MBD4* and *HDAC1* expression in GEN-treated cells. SFN alone showed increased expression of *DNMT3A* and *MBD3* while reduced expression of *HDAC1* and *HDAC2*. Together with E2, SFN reduced the E2-induction of *DNMT1, DNMT3B, MBD2, HDAC1* and *HDAC6* gene expression but did not affect the E2-induced changes in *DNMT3A*, *MBD4* and *HAT/p300* gene expression. Similarly, SFN reversed the induction of *DNMT1* and *HDAC6* gene expression by GEN. These results suggest that both E2 and GEN induced alterations in gene expression of enzymes modifying DNA methylation and histone de- or acetylation. Some of these changes were modulated by SFN. We next sought to examine if these epigenetic changes contribute to transcription of *COMT*.

3.4. Estradiol, genistein and sulforaphane modulate binding of DNA methylation and histone modification enzymes at the MB-COMT distal promoter

As shown in Fig. 1D, DNA methylation occurred at a specific CpG cluster (R3, illustrated in Fig. 1A) of MB-COMT-PR2 in response to E2 and GEN exposure. We next investigated whether DNA methylation-related proteins (DNMT1, DNMT3B and MBD2) and histone modifying enzymes (HAT/p300, HDAC1 and HDAC6) along with RNA Pol II showed changes in their binding to R3 in response to E2 or GEN. E2 reduced binding of RNA Pol II while in contrast E2 increased recruitment of DNMT3B and MBD2 to this cluster (Fig. 3A, C-D). These changes were reversed by SFN exposure. Similar to the effects of E2, GEN exposure induced decreased RNA Pol II binding in contrast to the

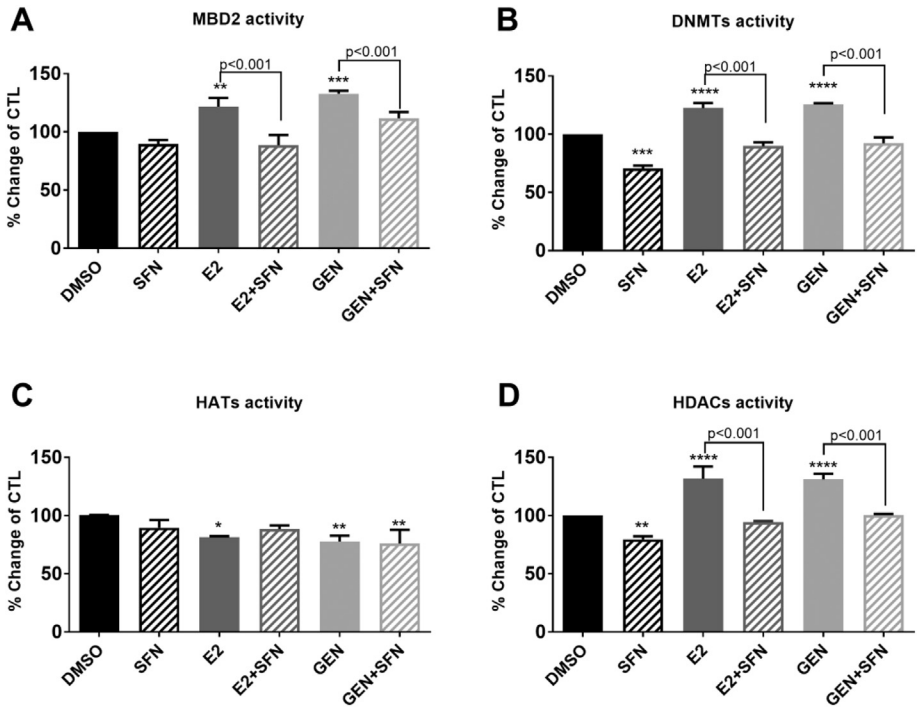

Fig. 2. E2 and GEN modulated activity of MBD2, DNMTs, HATs and HDACs. MCF7 cells were treated with vehicle control (DMSO), E2 (1 nM), GEN (1 μM) or SFN (10 μM) and a combination of E2 or GEN and SFN for 48 h. Isolated nuclear protein was subjected to protein activity assay for DNMTs, MBD2, HAT and HDACs as described in methods. P-value (p) < 0.05 (*), < 0.01 (**) or < 0.001 (***) comparing mean to DMSO.

increases in binding of DNMT3B and MBD2 to this region (Fig. 3A, C-D). When together with SFN, the changes in recruitment of RNA Pol II and MBD2 (but not DNMT3B) were completely abolished. There was no significant change in binding of DNMT1 in response to E2/GEN alone or E2/GEN plus SFN (Fig. 3B). Herein, these results suggest that DNMT3B and MBD2 may contribute to the decreased transcription of *COMT* in response to E2 or GEN, at least through CpG site-specific DNA methylation of this CpG cluster (R3).

In addition to DNA methylation, we demonstrated E2 and GEN modulated histone modifications of this CpG cluster (R3) (Fig. 4). E2 alone decreased HAT/p300 binding while increased HDAC1 binding to this cluster (Fig. 4A-B). E2-treated cells showed a decreased level of H3K4-, H3K9- and H3K27-acetylation (Fig. 4D-F) at this cluster. Similarly, GEN alone increased HDAC1 binding (Fig. 4B) but decreased levels of H3K4- and H3K9- acetylation (Fig. 4D-F). There was a trend for decreased HAT/p300 binding ($p = 0.055$) in GEN-treated cells (Fig. 4A). On the other hand, SFN alone decreased binding of HAT/p300 and HDAC1 at this cluster while it increased levels of H3K4-,

Table 1
Change in mRNA level of DNMTs, MBDs, HAT and HDACs in response to vehicle control (DMSO), E2 (1 nM), GEN (1 μM) or SFN (10 μM) and a combination of E2 or GEN and SFN.

Gene		DMSO	SFN	E2	E2 + SFN	GEN	GEN + SFN
DNMT1	Mean	1.00	0.94	2.51***	1.38^^	1.58*	0.50$^{\#}$
	SD	0.08	0.53	0.14	0.78	0.11	0.13
DNMT3A	Mean	1.00	1.39*	1.47**	1.58**	1.25	1.12
	SD	0.06	0.10	0.17	0.16	0.48	0.19
DNMT3B	Mean	1.01	0.76^^	2.26***	0.44^^^	1.56	0.35$^{\#}$
	SD	0.10	0.56	0.65	0.24	0.50	0.17
MBD1	Mean	1.00	1.06	0.92	1.31	0.85	1.11
	SD	0.02	0.03	0.31	0.15	0.29	0.04
MBD2	Mean	1.01	0.87	1.27**	0.82^^	1.02	0.53
	SD	0.14	0.27	0.06	0.16	0.29	0.17
MBD3	Mean	1.00	1.21*$^{\#}$	1.15	1.37^	0.67	0.66
	SD	0.04	0.06	0.01	0.12	0.14	0.02
MBD4	Mean	1.00	0.84^	1.23*	1.13	1.38	1.21
	SD	0.04	0.03	0.04	0.12	0.43	0.39
HAT/p300	Mean	1.00	0.82	0.68*	0.69*	0.53**	0.81
	SD	0.12	0.30	0.11	0.10	0.10	0.11
HDAC1	Mean	1.00	0.83**^^	1.22**	0.90^^^	1.29	0.38$^{\#\#\#}$
	SD	0.02	0.04	0.03	0.01	0.16	0.25
HDAC2	Mean	1.00	0.82**	0.94	0.89	0.92	1.28
	SD	0.05	0.04	0.05	0.05	0.36	0.60
HDAC3	Mean	1.00	0.75	1.15	2.94	0.98	1.01
	SD	0.06	0.02	0.04	0.25	0.08	0.29
HDAC6	Mean	1.00	0.83^^	1.65***	0.832^^^	1.72*	0.48$^{\#\#\#}$
	SD	0.02	0.15	0.08	0.19	0.65	0.15

Note: In all reported mean values, relative expression ratios (RER) were normalized to the average of the untreated controls (DMSO). P-value (p) < 0.05 (*), < 0.01 (**) or < 0.001 (***) comparing mean RER's to DMSO; p < .05 (^), < 0.01 (^^) or < 0.001 (^^^) comparing mean RER's to E2 and p < .05 ($^{\#}$), < 0.01 ($^{\#\#}$) or < 0.001 ($^{\#\#\#}$) comparing mean RER's to GEN.

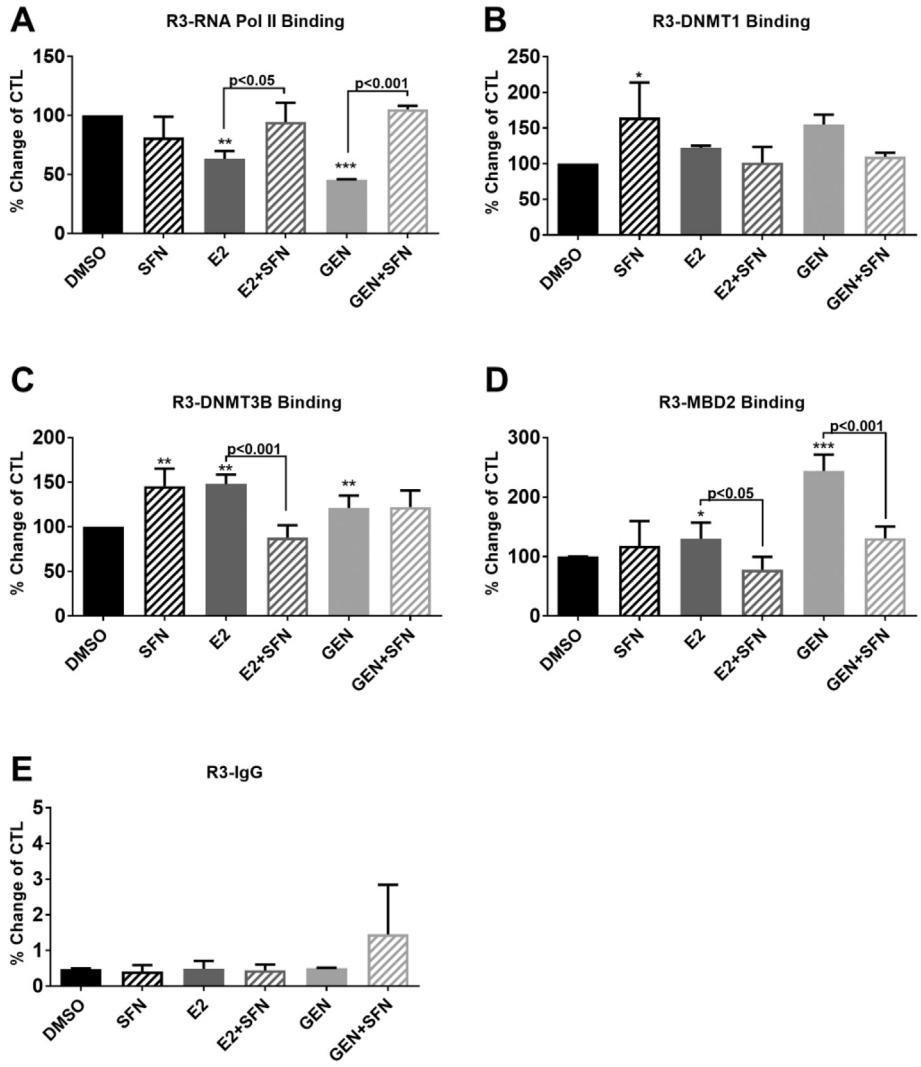

Fig. 3. Change in recruitment of RNA Polymerase II, DNA methylation-related proteins on the MB-COMT-PR2 promoter (Region 3, R3, enclosed CpG sites 12–16). % change in protein binding in treated samples as compared to vehicle control-treated samples is shown. IgG serves as the negative control. There was minimal binding of IgG at this region. P-value (p) < 0.05 (*), < 0.01 (**) or < 0.001 (***) comparing mean to DMSO.

H3K9- and H3K27-acetylation. Herein, we found SFN could reverse the E2- or GEN-induction of HDAC1 binding (Fig. 4B). In addition, SFN could modulate the E2-induced inhibitory effect of H3K4-, H3K9- and H3K27- acetylation (Fig. 4D-F). GEN-treated cells with SFN showed increased levels of H3K4- and H3K9- acetylation to the levels as control (Fig. 4D-E). SFN could not modulate the decrease in HAT/p300 binding induced by E2 or GEN (Fig. 4A). There was no change in HDAC6 binding in response to E2, GEN or SFN (Fig. 4C). Our data suggest that not only DNA methylation but also histone deacetylation at this cluster (R3) contributes to the E2- or GEN-induced *COMT* suppression. Not all epigenetic changes induced by E2 or GEN could be reversed by SFN, indicating SFN selectively modulated estrogen-induced epigenetic modifications of *COMT*.

When comparing changes in DNA methylation at every CpG site of MB-COMT-PR2, we showed differential methylation at CpG site 6 ($p < 0.05$) in E2-treated cells (Supplementary Fig. 4). However, in this region, we did not find E2 or GEN recruit the binding of DNA methylation-related proteins, DNMT1, DNMT3B and MBD2 at this region (Fig. 5B-D) although there was decreased binding of RNA Pol II (Fig. 5A). SFN alone induced a decrease in recruitment of RNA Pol II but increased binding of DNMT3B at this region. SFN did not modulate changes in binding of any of these proteins to this region when compared to that of E2-treated or GEN-treated cells. Surprisingly, we demonstrated that E2 or GEN induced histone modifications at this region (Fig. 6). E2 induced recruitment of HDAC1 (Fig. 6B) while decreased levels of H3K4-, H3K9- and H3K27 acetylation (Fig. 6D-F). GEN alone showed decreased binding of HAT/p300 (Fig. 6A) as well as levels of acetylation at H3K4- and H3K9- residues (Fig. 6D-E). On the other hand, SFN alone decreased HAT/p300 binding (Fig. 6A) while increased levels of H3K4- and H3K9- acetylation (Fig. 6D-E). When cells treated together with E2, SFN reversed the changes in HDAC1 binding (Fig. 6B) and H3K4- and H3K27- acetylation levels (Fig. 6D, F) induced by E2. On the other hand, SFN only modulated the inhibitory effect of GEN on H3K4 acetylation but not H3K9 acetylation (Fig. 6D). Herein, we demonstrated histone deacetylation (not DNA methylation) appeared to be the main epigenetic modifications occurred at R1, which might contribute to the E2- or GEN-induced *COMT* suppression. In contrast, we did not observe any changes in DNA methylation and histone acetylation as well as the binding of DNA methylation proteins and histone modifying enzymes at another CpG-rich region of MB-COMT-PR2 (R2, illustrated in Fig. 1A) where CpG sites 7–11 located (Supplementary Figs. 4, 6–7).

Our findings provided new evidence of how E2 and GEN epigenetically regulated *COMT* transcription. When comparing changes in DNA methylation and histone modifications (Table 2) at MB-COMT-PR2 in E2- or GEN- treated cells with untreated cells, we found the most

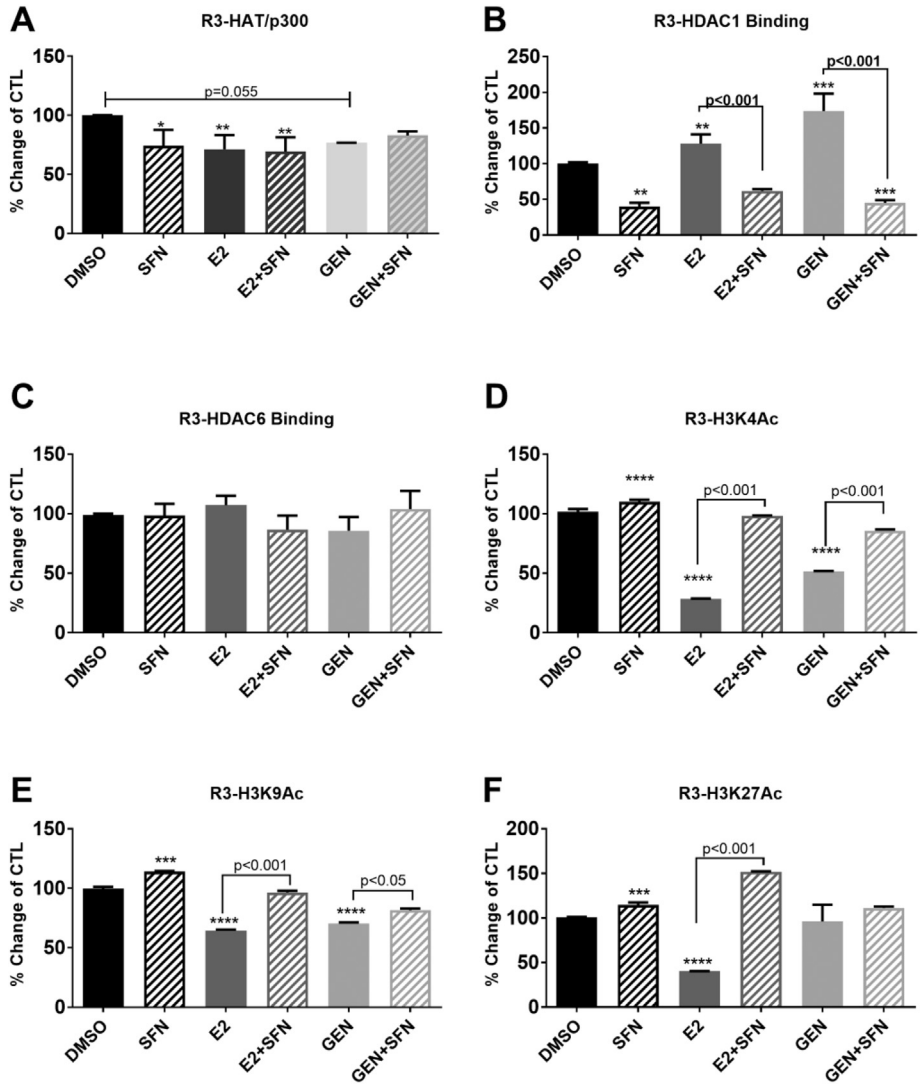

Fig. 4. Change in recruitment of histone modification proteins on the MB-COMT-PR2 promoter (Region 3, R3, enclosed CpG sites 12–16). % change in protein binding in treated samples as compared to vehicle control-treated samples is shown. IgG serves as the negative control. There was minimal binding of IgG at this region. P-value (p) < 0.05 (*), < 0.01 (**) or < 0.001 (***) comparing mean to DMSO.

changes in recruitment of epigenetic modulators existed at R1 and R3. Decreased *COMT* transcription could be explained by the CpG site-specific methylation at R3 but not R1 and R2, which were associated with the increased binding of DNMT3B and MBD2. Increased binding of DNMT3B and MBD2 to these regions might result in CpG site-specific DNA methylation. In addition to DNA methylation, we showed E2 and GEN modulated change in recruitment of histone modification enzymes (HAT/p300 and HDAC1) at both R1 and R3. Specifically, decrease levels of H3K4-, H3K9- and H3K27- acetylation associated to the E2 or GEN-induced *COMT* suppression. SFN was shown to selectively reverse the E2- or GEN-induced epigenetic modifications at MB-COMT-PR2.

4. Discussion

The goal of this study was to investigate the mechanisms underlying regulation of *COMT* transcription in MCF-7 cells given the fact that inhibition of COMT activity may result in the formation of DNA adducts and oxidative DNA damage which contributes to the initiation, promotion and progression of BCa (Yager et al., 2016). Estradiol or xenoestrogen has been shown to decrease *COMT* expression in breast epithelial cells (Lavigne et al., 2001; Zahid et al., 2007; Lehmann et al., 2008; Yue et al., 2013). In this study, we performed a mechanistic study at the *COMT* promoter and illustrated how E2 and GEN modulated the *COMT* transcription via epigenetic modifications at specific *COMT* promoter. E2 and GEN suppressed *COMT* gene expression in association with alterations in CpG site-specific DNA methylation and binding of DNA methylation-related proteins (5′ cytosine methylation) and histone modifying enzymes (N′ terminal deacetylation) at the *MB-COMT* distal promoter but not at other COMT promoters (*MB-COMT* and S-*COMT* promoters). It is supported by evidences that cancer cells may use multiple *COMT* promoters to selectively inactive COMT during development of cancer (Sasaki et al., 2003). On the other hand, Xie et al. reported that ER and C/EBP contributed to transcriptional activity of *S-COMT* promoter and *MB-COMT* promoter respectively (Xie et al., 1999). However, it is unclear if these transcription factors co-operate with enzymes modifying DNA methylation and histone acetylation at these promoters and results in selectively gene silencing of *COMT*. It requires future experiment to prove this hypothesis.

We found that E2 and GEN each has mostly similar effects on recruitment of the epigenetic modulators to the MB-COMT distal promoter (summarized in Table 2). These effects are associated with increased promoter methylation and gene suppression of *COMT*. It is noted that the array of epigenetic modulators recruited by E2 is not exactly the same as that by GEN. GEN was shown to modulate E2

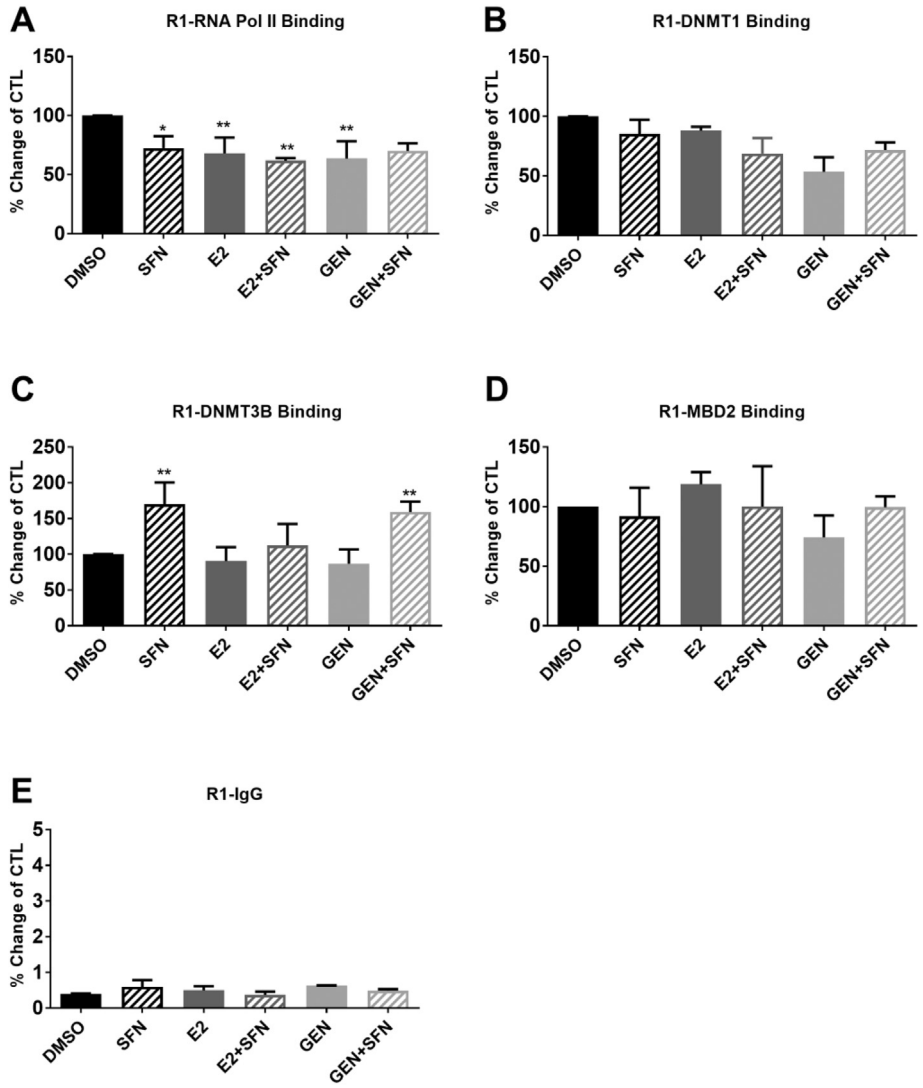

Fig. 5. Change in recruitment of DNA methylation-related proteins on the MB-COMT-PR2 promoter (Region 1, R1, enclosed CpG sites 1–6). % change in protein binding in treated samples as compared to vehicle control-treated samples is shown. IgG serves as the negative control. There was minimal binding of IgG at this region. P-value (p) < 0.05 (*) or < 0.01 (**) comparing mean to DMSO.

cellular bioavailability that may contribute to the regulations of E2/E1 metabolism (Hanet et al., 2008). It has been suggested that differential estrogenicity of GEN (either through ER-dependent or independent pathways) leads to distinct expression patterns of E2-responsive genes (Lin et al., 2008; Maggiolini et al., 2004). Thus, GEN may trigger alternative epigenetic or transcriptional machinery that contributes to *COMT* transcription although future study is required to support this claim.

We showed that the epigenetic effects of E2 and GEN on *COMT* transcription are region-specific. Both E2 and GEN induced DNA methylation at specific CpG cluster (CpG sites 12–16). Increased DNA methylation at this CpG cluster was associated with the decrease in *COMT* transcription. This CpG cluster encompassed transcription factor C/EBPα (illustrated in Supplementary Fig. 4). Methylation at this cluster may either interfere the binding of C/EBPα or disrupt the chromatin remodeling which results in gene suppression (Singal and Ginder, 1999; Zhang et al., 2005). Our finding is consistent with the report suggesting the possible role of the transcription factor, C/EBP, on suppression of E2-induced *COMT* transcription (Xie et al., 1999). Our data showing that E2 or GEN increased binding of DNMT3B and MBD2 further supports the presence of DNA methylation in this CpG cluster. Moreover, we showed E2 and GEN induced increased HDAC1 binding and decreased recruitment of HAT/p300 at this cluster. Furthermore, we demonstrated E2 and GEN decreased levels of H3K4-, H3K9- and H3K27-acetylation at this CpG cluster. It suggests that histone deacetylation might also contribute to the suppression of *COMT* transcription. In addition to this CpG-rich region (R3), another CpG cluster (R1) at the *MB-COMT* distal promoter was found to be responsive to histone modifications (H3K4/H3K9/H3K27-acetylation). Strikingly, we did not find any recruitment of DNA methylation-related proteins at this region. We acknowledged that we could not examine the effect of every epigenetic modification enzyme individually on *COMT* transcription as there are a large number of epigenetic modification enzymes. Perhaps, there are alternative epigenetic mechanisms contributed to *COMT* transcription but it requires further mechanistic study to prove this hypothesis. In this study, we showed that AZA and TSA completely reversed the E2-induced *COMT* suppression (Supplementary Fig. 3). Hence, we speculate that in MCF-7 cells, E2 and GEN epigenetically regulate *COMT* transcription, at least through promoter methylation and histone de- and acetylation along the *MB-COMT* distal promoter.

We demonstrated that phytoestrogen, GEN, inhibit *COMT* transcription epigenetically. GEN which is an isoflavone found in a soy-based diet is proposed to have the potential to prevent tumorgenesis and/or cancer progression through its selective estrogen receptor

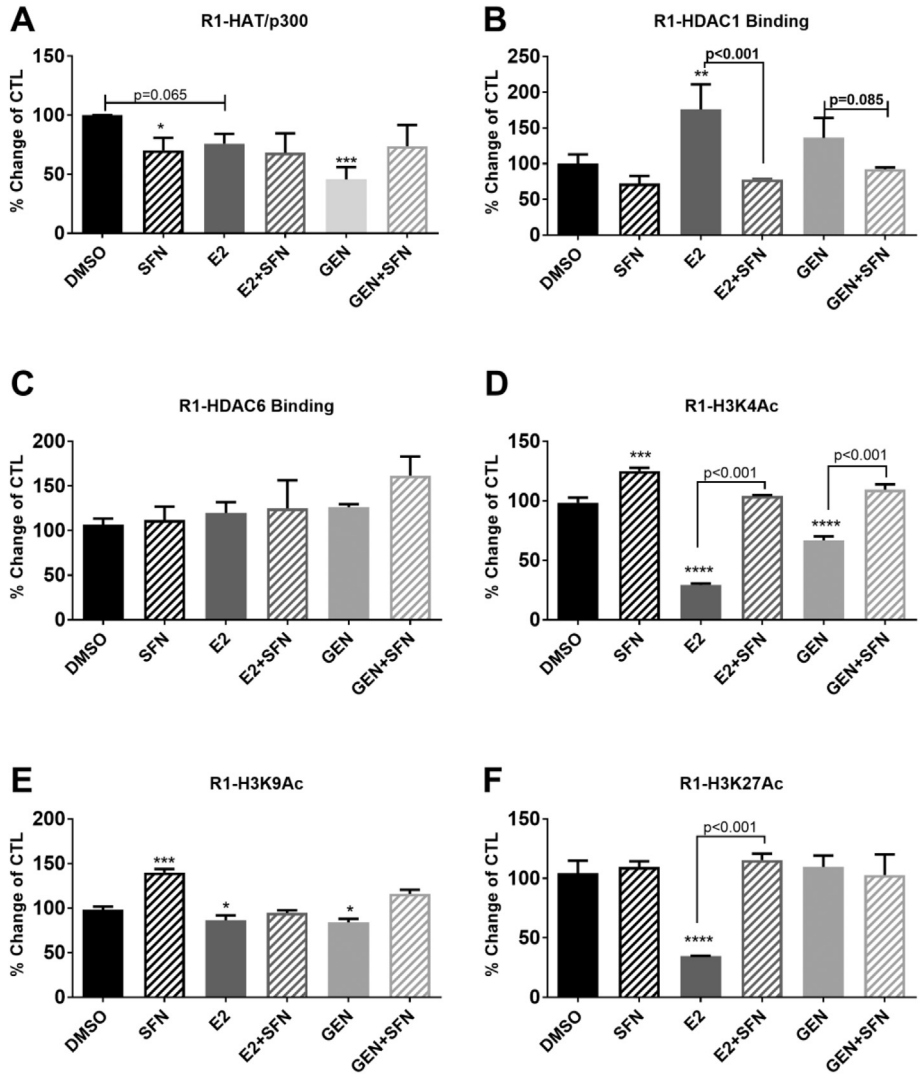

Fig. 6. Change in recruitment of histone modification proteins on the MB-COMT-PR2 promoter (Region 1, R1, enclosed CpG sites 1–6). % change in protein binding in treated samples as compared to vehicle control-treated samples is shown. IgG serves as the negative control. There was minimal binding of IgG at this region. P-value (p) < 0.05 (*), < 0.01 (**) or < 0.001 (***) comparing mean to DMSO.

modulators (SERM) activity. Nevertheless, whether use of GEN is beneficial for chemoprevention of BCa is controversial. There are both in vitro and in vivo studies that reported adverse effects of GEN on mammary tissues/cells via epigenetic mechanisms (Dolinoy et al., 2006; Tang et al., 2008; Greathouse et al., 2012). In the present study, we further suggest that there are epigenetic modifications (promoter methylation and histone de- and acetylation) underlying regulation of *COMT* expression in response to GEN. The questions of whether soy isoflavones, such as GEN, could epigenetically regulate transcription of genes involved in estrogen metabolism and tumorigenesis, deserve further studies both in vivo and in vitro.

Antioxidants such as SFN have been proposed to be chemopreventive agents to inhibit, delay or reverse the development of cancer (Bayat Mokhtari et al., 2018). SFN was shown in breast epithelial cell culture to suppress oxidative metabolism of E2/E1 and thus protect against estrogen-mediated DNA damage through alteration of E2 metabolizing

Table 2
Summary of epigenetic changes at the *MB-COMT* distal promoter (MB-COMT-PR2) in MCF-7 cells in response to E2 or GEN, and plus SFN.

	E2			GEN		
	DNA methylation	Increased Binding	Decreased Binding	DNA methylation	Increased Binding	Decreased Binding
R1 CpG site 1–6	Increase	HDAC1	H3K4Ac, H3K9Ac, H3K27Ac	Increase	ND	HAT/p300, H3K4Ac, H3K9Ac
Reversed by SFN	NO	YES	YES for H3K4Ac, H3K27Ac		ND	YES for H3K4Ac
R2 CpG site 7–11	No change	ND	ND	No change	ND	N/D
R3 CpG site 12–16	Increase	DNMT3B, MBD2, HDAC1	HAT/p300, H3K4Ac, H3K9Ac, H3K27Ac	Increase	DNMT3B, MBD2, HDAC1	HAT/p300 (p = .055), H3K4Ac, H3K9Ac,
Reversed by SFN	YES	YES for DNMT3B, MBD2, HDAC1	YES for H3K4Ac, H3K9Ac, H3K27Ac	YES	YES for MBD2, HDAC1	YES for H3K4Ac, H3K9Ac

Note. ND: Not detected significant changes in binding of epigenetic modulators.

enzymes such as *COMT, NQO1* and *GSTA1* (Yang et al., 2013). We showed that E2 or GEN suppressed *NQO1* and *GSTA1* (Supplementary Fig. 5) suggesting the existence of oxidative stress in response to estrogens. In contrast, SFN induced *NQO1* (Nrf2 responsive gene) in MCF-7 cells and reversed the E2- or GEN-induced *NQO1* suppression (Supplementary Fig. 5A). Yang et al. suggested that the effect of SFN on COMT activity may not be directly regulated by Nrf2 signaling because SFN did not affect the mRNA level of *COMT*. Similar to Yang et al., our results indicated that SFN alone did not significantly change *COMT* mRNA levels or CpG methylation of *COMT* promoters in MCF-7 cells. However, SFN reversed the epigenetic changes induced by E2 and GEN at the *MB-COMT* promoter resulting in the increased *COMT* expression. Our data suggest that, in addition to Nrf2-modulated antioxidant defense, SFN might modulate E2- or GEN-induced binding of epigenetic modulators (DNMT3B, MBD2 and HDAC1) to *COMT* promoter which may eventually result in modifications of COMT-mediated estrogen metabolism. It has been reported that SFN can modify the activities of DNMTs and HDACs in cancer cells (Tortorella et al., 2015). Thus, SFN may provide opportunities for cancer prevention by regulating the components of epigenetic machinery, especially DNMTs and MBDs (Kar et al., 2014) and epigenetic modifications of suppressor/oncogene transcription (Hsu et al., 2011). Our finding indicates that SFN may serve as epigenetic modifier to reverse the E2 or GEN-induced DNA methylation and histone acetylation at the *MB-COMT* promoter, may ultimately modulate the E2/E1 metabolism pathway in MCF-7 cells.

We suggest that epigenetic variation at *COMT* may play an equally important role as genetic variation in assessing the risk of human BCa. An abundance of epidemiology studies reported the association between genetic variations at *COMT* and BCa risk (Singh et al., 2005) but some produced conflicting results (Qin et al., 2012; Spurdle et al., 2017; Horvath, 2017; Movassagh et al., 2017). In addition, several studies suggesting environmental factors such as environmental chemicals with hormonal activity (Kocabaş et al., 2002; Cerne et al., 2011; Kallionpää et al., 2017), smoking (Saintot et al., 2003) and diets or consumption of nutritional supplements (Wang et al., 2011; Low et al., 2005) produce mixed results on the relation of genetic variations of *COMT* to human BCa risk. This raises possibilities for other mechanisms underlying the interaction between gene and environment. Swift-Scanlan et al. were the first to report site-specific DNA methylation changes at promoter regions for *S-COMT* and *MB-COMT* and their correlation to *COMT* gene expression in human BCa cells (Swift-Scanlan et al., 2014). In addition, they reported the association of environmental factors (e.g. socioeconomic status and alcohol use), with loci-specific DNA methylation and a functional SNP (Val^{158} Met of MB-COMT) in saliva-derived DNA using a cohort of 48 healthy participants. In the present study, we provide additional evidence that estrogens and phytoestrogens modulate *COMT* methylation in MCF-7 cells in a CpG site-specific manner. We found estrogens did not induce differential methylation at the CpG sites (located in *S-COMT* promoter and *MB-COMT* proximal promoter, Fig. 1) reported in Swit-Scanlan's study but at another CpG cluster flanking the TSS of *MB-COMT* distal promoter (MB-COMT-PR2, illustrated in Fig. 1A). Our data suggests that estrogens or antioxidants could alter *COMT* methylation at particular loci that may affect the expression of *COMT* transcripts. Future studies on epigenetic variations within the *COMT* gene may be beneficial for risk assessment for human BCa.

In the present study, we did not examine whether the CpG site-specific *COMT* methylation are associated with any genetic variants in response to E2, GEN or SFN. Some studies suggest that oxidative DNA damage causes genetic variation which may induce epigenetic changes that lead to gene silencing (Khobta et al., 2010; Leng et al., 2012). Future studies on both epigenetic and genetic variations within the *COMT* promoters, especially in large human cohort studies may help to reveal the mechanisms underlying gene regulation of *COMT* and improve the use of *COMT* epigenetic/genetic variants for the assessment of BCa risk. On the other hand, our study is limited to an ER positive BCa cell line, MCF-7. It has been shown that an ER-antagonist, ICI182780, could reverse the reduction of *COMT* expression in MCF-7 cells (Ho et al., 2013), indicating ER may mediate E2-induced *COMT* suppression. Future studies using several human BCa cells or clinical samples with known ER status might yield further insight on the influence of ERs on epigenetic regulation of *COMT*.

Taken together, we provide new evidence of targeting *COMT* transcription epigenetically in MCF-7 cells. We identified a new mechanism which consists of locus specific DNA methylation and higher chromatin configurations at MB-COMT distal promoter, contributes to E2- or GEN-induced *COMT* suppression. Furthermore, we indicated that SFN might serve as an epigenetic modifier to modulate the estrogens-induction of aberrant epigenetic changes of genes involved in estrogen metabolism. All in all, our findings may help in improving our understanding of factors involved in estrogen metabolism in human BCa.

Conflict of interest

All authors declare no conflict of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.taap.2019.01.016.

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