Epigenetic Induction of Tissue Inhibitor of Matrix Metalloproteinase-3 by Green Tea Polyphenols in Breast Cancer Cells

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Aberrant epigenetic silencing of the tissue inhibitor of matrix metalloproteinase-3 (TIMP-3) gene that negatively regulates matrix metalloproteinases (MMPs) activity has been implicated in the pathogenesis and metastasis of breast cancer. In the present study, we demonstrate that green tea polyphenols (GTP) and its major constituent, epigallocatechin-3-gallate (EGCG) mediate epigenetic induction of TIMP-3 levels and play a key role in suppressing invasiveness and gelatinolytic activity of MMP-2 and MMP-9 in breast cancer cells. Treatment of MCF-7 and MDA-MB-231 breast cancer cells with 20 μM EGCG and 10 μg/mL GTP for 72 h significantly induces TIMP-3 mRNA and protein levels. Interestingly, investigations into the molecular mechanism revealed that TIMP-3 repression in breast cancer cells is mediated by epigenetic silencing mechanism(s) involving increased activity of the enhancer of zeste homolog 2 (EZH2) and class I histone deacetylases (HDACs), independent of promoter DNA hypermethylation. Treatment of breast cancer cells with GTP and EGCG significantly reduced EZH2 and class I HDAC protein levels. Furthermore, transcriptional activation of TIMP-3 was found to be associated with decreased EZH2 localization and H3K27 trimethylation enrichment at the TIMP-3 promoter with a concomitant increase in histone H3K9/18 acetylation. Our findings highlight TIMP-3 induction as a key epigenetic event modulated by GTPs in restoring the MMP:TIMP balance to delay breast cancer progression and invasion.

Key words: breast cancer; tissue inhibitors of matrix metalloproteinase-3; green tea polyphenols; invasion; metastasis; enhancer of zeste homolog 2; histone deacetylases

INTRODUCTION

Breast cancer is a heterogeneous malignancy, accounting for an estimated 226,870 new cases of invasive carcinoma and 39,510 cancer-related deaths among women in the United States in 2013 [1]. It is the second leading cause of women's cancer mortality, after lung cancer and majority of deaths occur due to tumor metastasis. The molecular pathway from tumor development to clinically evident metastasis is complex and consists of multiple sequential steps [2]. One of the early events in tumor invasion and metastasis is extracellular matrix (ECM) remodeling where matrix metalloproteinases (MMPs), a family of 25 individual metzincin endopeptidases in humans play an important role [3–6]. Degradation of multiple components of the ECM by MMPs generates various bioactive molecules called matrikines that contribute to tumor progression [7]. For example, a 5-kDa fragment generated upon osteopontin cleavage by MMP-9 promotes tumor cell invasion [8]. A substantial body of available data indicates the involvement of gelatinases/type IV collagenases (MMP-2 and MMP-9) secreted by tumor cells during cancer cell invasion and metastasis [9–11]. The gelatinases (MMP-2 and MMP-9) are produced in a latent form (pro-MMP) that require activation and are inhibited by tissue inhibitors of matrix metalloproteinases (TIMPs) [12]. TIMPs are natural inhibitors of MMPs and are involved in diverse biological processes

Abbreviations: 5-Aza-CdR, 5-aza-2′-deoxycytidine; DZNep, 3-deazaneplanocin A; ECM, extracellular matrix; EGCG, epigallocatechin-3-gallate; EZH2, enhancer of zeste homolog 2; GTP, green tea polyphenols; HDACs, histone deacetylases; MMP, matrix metalloproteinases; MSP, methylation-specific PCR; TIMP-3, tissue inhibitor of matrix metalloproteinase-3; H3K27me3, trimethylation of Histone H3 at Lysine 27; TSA, trichostatin A.

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including cell growth, tumor progression, apoptosis, invasion, metastasis, and angiogenesis [13]. Four different TIMP genes (TIMP-1, -2, -3, and -4) and proteins are described in humans and all four inhibit active form of most MMPs but with different affinities [14]. TIMPs inhibit MMPs by binding in a 1:1 stoichiometry through strong noncovalent interactions [15]. The balance between active MMPs and TIMPs determine tumor progression by regulating the net activity of MMPs [14]. Several studies suggest that TIMPs have tumor suppressive role and upregulation of TIMPs in cancer cells can inhibit invasion and metastasis [16–20]. Interestingly, in breast cancer low levels of tissue inhibitor of matrix metalloproteinase-3 (TIMP-3) protein expression has been shown to be associated with an aggressive tumor phenotype and poor disease-free survival [21]. TIMP-3 is a secreted protein which binds strongly to ECM through glycosaminoglycans, unlike other members of TIMP family [12,22]. Studies have demonstrated that TIMP-3 over-expression in tumor cells causes reduced primary growth and angiogenesis and induce apoptosis [23,24]. In breast cancer cells, TIMP-3 repression has been linked to promoter DNA hypermethylation, which has been reported in approximately 20–27% cases of primary breast tumors and invasive ductal carcinoma [25,26]. However, other epigenetic silencing mechanisms such as aberrant histone methylation and deacetylation patterns at the TIMP-3 gene locus as reported in certain other cancers [27], has not yet been characterized in breast cancer. Elucidation of epigenetic mechanism(s) of TIMP-3 silencing and its induction by small molecules could be an attractive target for prevention and/or therapy of breast cancer invasion and metastasis.

In recent years, numerous in vitro and in vivo experimental studies have demonstrated the anti-carcinogenic effects of green tea polyphenols (GTP) against a variety of solid tumors including breast cancer [28–30]. Epigallocatechin-3-gallate (EGCG), the major active polyphenol present in green tea, has shown to induce apoptosis, suppress proliferation, angiogenesis, and invasiveness in various breast cancer preclinical models [31,32]. For example, a significant reduction in DMBA-induced mammary tumor burden and invasiveness in female Sprague–Dawley rats has been demonstrated after consuming green tea as sole source in drinking fluid [33]. One of the important aspects of multifaceted role of EGCG in controlling tumor progression, and metastasis is the inhibition of MMPs. Previous studies have demonstrated that EGCG treatment of breast cancer cell lines MCF-7 and MDA-MB-231 inhibits invasion by suppressing the activity of gelatinases/type IV collagenases, viz. MMP-2 and MMP-9 [34,35]. More recent studies have demonstrated the role of GTPs as modifier of epigenetic mechanisms [36,37]. However, the effect of GTP or EGCG on TIMP-3 expression, in particular, has not yet been elucidated. The aim of the present study is to investigate whether GTP and/or EGCG could induce endogenously repressed TIMP-3 expression in breast cancer cells and decipher the molecular mechanism(s) involved in its re-expression. As in vitro model, we used two human breast carcinoma MCF-7 and MDA-MB-231 cell lines, which represent two different breast cancer phenotypes and vary in their invasive potential. In this study, we provide evidence that class I histone deacetylases (HDACs) and histone methyltransferase enhancer of zeste homolog 2 (EZH2) catalyzed histone modifications play key roles in TIMP-3 silencing, independent of promoter DNA hypermethylation in breast carcinoma MCF-7 and MDA-MB-231 cell lines. Our study demonstrates that GTP and EGCG treatment significantly induces TIMP-3 expression in breast cancer cells. A detailed study into the molecular mechanism revealed that GTP and EGCG treatment decreased EZH2 catalyzed trimethylation of Histone H3 at Lysine 27 (H3K27me3) with a corresponding increase in the deposition of transcriptionally active acetylated Histone H3 at Lysine 9/18 (H3K9/18 Ac) specifically at the TIMP-3 promoter.

MATERIALS AND METHODS

Antibodies and Reagents

Anti-TIMP-3 (SC-373839), anti-HDAC1 (SC-7872), anti-HDAC2 (SC-6296), anti-HDAC-3 (SC-11417), anti-HDAC-8 (SC-11405), anti-actin (SC-47778), goat anti-mouse IgG-HRP (SC-2005), bovine anti-goat IgG-HRP (SC-2350), and goat anti-rabbit IgG-HRP (SC-2004) antibodies were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX). Anti-H3K27me3 (mAbCam6002) was purchased from AbCam (Cambridge, MA); anti-acetyl H3 K9/18 (07–593) was purchased from Upstate (Lake Placid, NY); anti-Histone H3 (Clone A3S) and anti-EZH2 (07–689) were purchased from Millipore (Billerica, MA).

Cell Culture and Treatment

Breast cancer cell lines MCF-7 and MDA-MB-231 were obtained from Dr. Hung-Ying Kao's laboratory (Department of Biochemistry, School of Medicine, Case Western Reserve University). Cells were grown in DMEM containing high glucose supplemented with 10% fetal bovine serum, 50 U/mL penicillin, and 50 μg/mL streptomycin in 100-mm tissue culture plates at 37°C in a humidified atmosphere (5% CO_2). Cells were plated at 20% seeding density and allowed to settle for 24 h. Media was replaced and cells received the following treatment (optimum dose for each treatment was previously determined): 10 μM 5-aza-2′-deoxycytidine (5-Aza-CdR) (Sigma–Aldrich, St. Louis, MO), 40 μg/mL trichostatin A (TSA) (Sigma–Aldrich), 5 μM 3-deazaneplanocin A (DZNep; Cayman Chemical, Ann Arbor, MI), 10 μg/mL Polyphenon E (Mitsui, Norin, Japan) and 20 μM EGCG for indicated time period. Polyphenon E hereafter

referred as GTP and concentrations of 10 μg/mL Polyphenon E correspond to 14.0 μM of EGCG. The constituents present in Polyphenon E are described in our previous publication [37]. For combined treatment with TSA and DZNep, cells were cultured with TSA for 48 h followed by the addition of DZNep for 24 h. Every alternate day the media was replaced with the fresh media and GTP or EGCG was replaced with a fresh dose.

Transfection of Packaging Cells for Viral Production and Infection of Cells With Virus

Packaging cells 293T were plated in 100-mm plates with 20% cell density 1 d prior to transfection in DMEM media containing 10% heat inactivated FBS without penicillin–streptomycin. Cells were transfected with 6 μg of pLKO1 (control vector only), shEZH2-40075, shHDAC-1 4814, shHDAC-2 4819, shHDAC-3-4826, or shHDAC-8 4849 vector (Open Biosystems, Thermo Fisher, Pittsburgh, PA) along with second-generation packaging construct (pCMV-dR8.74) and pMD2G using lipid transfection Lipofectamine/Plus reagent (Invitrogen Corporation, Carlsbad, CA) as per manufacturer's protocol. Media was collected for two subsequent days and layered onto breast cancer cells MCF-7 and MDA-MB-231 to infect them with virus after adding 10 μL of 4 mg/mL polybrene per 10 mL and sterilize passing through 0.45 μM microfilters. Forty-eight hours after infection, virus-containing media was replaced with DMEM + 10% FBS. Twenty-four hours later cells were put under selection using 1 μg/mL puromycin for 2 wk. Selected cells were tested for gene knockdown using Western blot analysis of related protein in whole cell lysates.

RNA Extraction and Reverse Transcription—Semi-Quantitative PCR

Total RNA was extracted from cell lines after appropriate treatment at indicated time points using Trizol reagent. Quality and quantity of extracted RNA was determined using Nanodrop spectrophotometer. 2 μg of RNA from each sample was subjected to reverse transcription reaction using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). TIMP-3, MMP-2, MMP-9, and GAPDH expression levels were assessed semi-quantitatively using the primer sets as described previously [37]. The sequence details and optimized PCR conditions are provided in Table S1. Each reaction was carried out in a reaction volume of 20 μL containing 100 ng cDNA synthesized from 2 μg RNA, 10 μL 2× PCR master mix (Promega, Madison, WI) and forward and reverse primers (100 ng each in final reaction). PCR products were run in 2% ethidium bromide–agarose gel at 110 V in TBE buffer and visualized in an UV transilluminator. Images of ethidium bromide stained bands were captured using Kodak gel imaging system. For each gene, the band intensities were normalized against GAPDH. The normalized band intensities obtained for controls have been assigned the value of 1 or 100 and those obtained for various treatments were plotted as fold increase or percent with respect to control, respectively.

Bisulfite Modification and Methylation-Specific PCR

Genomic DNA from untreated, 5-Aza-CdR, GTP, and EGCG-treated cells was isolated using the protocol provided with DNA isolation kit (Quick-gDNA MiniPrep, Zymo Research, Irvine, CA). Bisulfite modification of genomic DNA (2 μg) was performed as per protocol provided with the EZ DNA Methylation-Gold Kit (Zymo) followed by additional desalting and purification using the DNA Clean and Concentrator-5 Kit (Zymo). DNA was suspended in 10 μL of water and stored at −20°C. PCR reaction was performed using methylation-specific PCR (MSP) primer sequences that specifically recognized unmethylated TIMP-3 sequence (−91 to 125 bp) were 5′-TTTTGTTTTGTTATTTTTTGTTTTTGGTTTT-3′ (upstream) and 5′-CCCCCAAAAACCCCACCTCA-3′ (downstream) and the methylated *TIMP-3* sequence were 5′-CGTTTCGTTATTTTTTGTTTTCGGTTTC-3′ (upstream) and 5′-CCGAAAACCCCGCCTCG-3′ (downstream) as previously described [38]. PCR products were resolved in 2% ethidium bromide–agarose gel along with low DNA mass ladder (Invitrogen) and visualized under UV light.

Western Blotting

Cells were lysed in the lysis buffer (Tris–HCl 50 mM, NaCl 150 mM, Triton X-100 1%, EGTA 1 mM, Sodium pyrophosphate 20 mM, pH 7.4 containing protease inhibitors cocktail [10 μL/mL], NaF [10 mM], DTT [1 mM], PMSF [0.1 mM], and sodium orthovanadate [1 mM]) on ice. To confirm equal loading, total protein concentration was determined using BCA method (Bio-Rad, Hercules, CA). Proteins were resolved using SDS–PAGE and then transferred to nitrocellulose membrane. Nonspecific binding sites on membrane were blocked using 5% nonfat skimmed milk and incubated with the primary antibody followed by the incubation with a secondary antibody. Proteins were detected using ECL Plus kit (GE-Amersham, Piscataway, NJ).

Chromatin Immunoprecipitation Assay

Untreated and treated cells were incubated with 1% formaldehyde for 15 min at room temperature for cross-linking. The reaction was then terminated using a 0.125-M final concentration of glycine. After cross-linking, chromatin was digested using monococcal nuclease enzyme and incubated with EZH2 antibody, H3K27me3 antibody, H3K9/18 acetylated antibody overnight at 4°C. After reversing the cross-linking by incubating the samples at 65°C overnight, DNA was purified using phenol–chloroform–isoamyl reagent

followed by ethanol precipitation. DNA was then dissolved in nuclease-free water. Immunoprecipitated DNAs, beads, or input controls were subjected to PCR amplification for 30 cycles of the following cycling conditions: stage 1, 95°C for 5 min (1 cycle); stage 2, 95°C for 30 s and 60°C for 30 s and 72°C for 1 min (30 cycles); and stage 3, 72°C for 7 min (1 cycle). Primers used for human TIMP-3 gene for region having YY1 binding site in the promoter region as reported [38]. PCR products were subjected to electrophoresis on a 2% ethidium bromide–agarose gel and visualized under UV light.

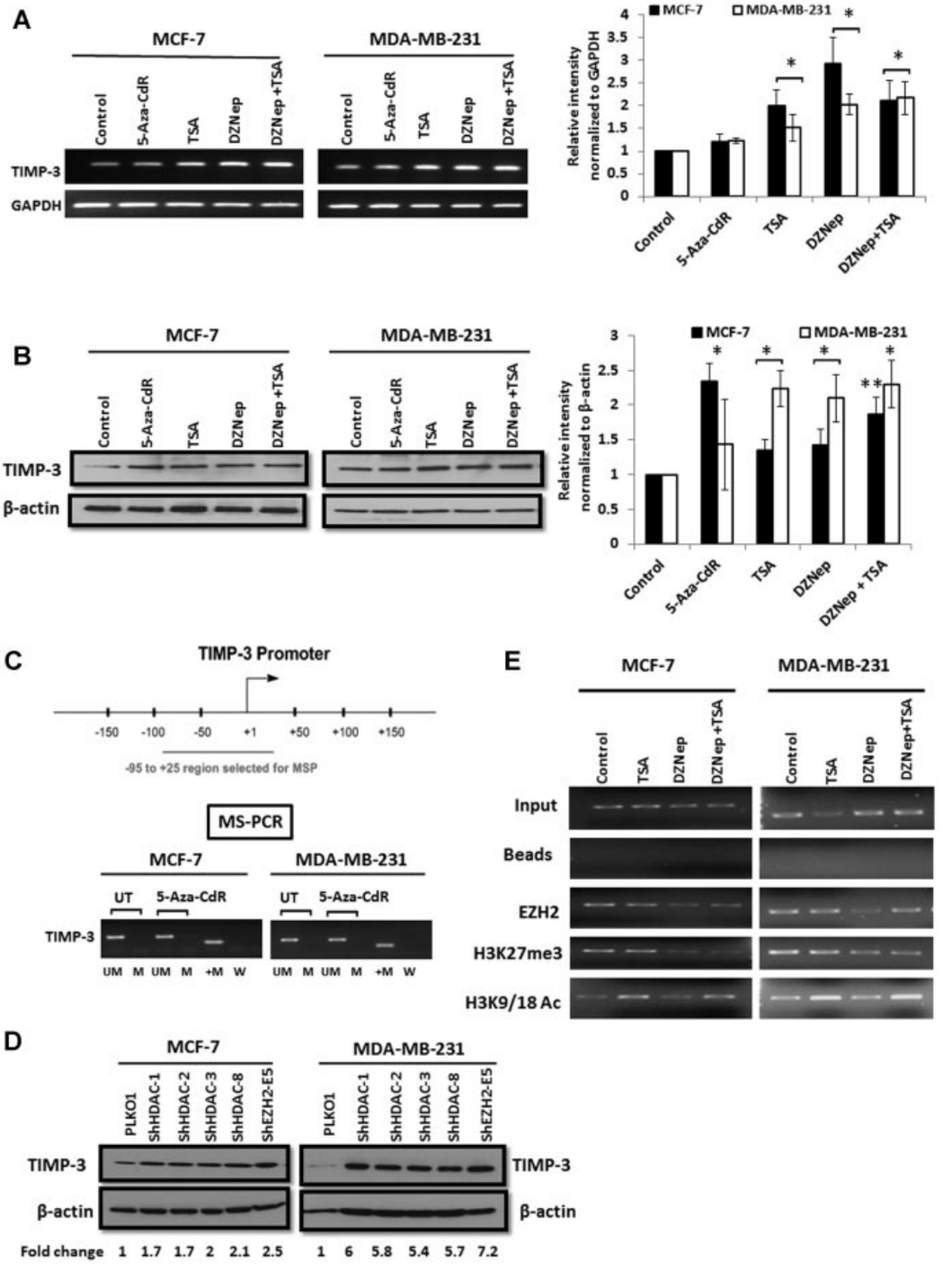

Figure 1.

Wound Healing Assay

Cells were seeded at 70–80% confluence into a 6-well culture dishes. After the cells grew to confluence, using a sterile pipette tip, at least three scratch wounds per plate was made. The cell monolayer was washed once with 1× PBS to remove any floating cells. Afterward, the culture medium was replenished with fresh dose of 10 μM 5-Aza-CdR, 40 μg/mL TSA, 5 μM DZNeP, 20 μM EGCG, and 10 μg/mL GTP. The cells were incubated at 37°C/5% CO_2 humidified incubator and photographed at indicated time points. Cell migration areas were calculated using Image J software.

Gelatin Zymography

MCF-7 and MDA-MB-231 cells (2×10^5) were seeded in 12-well plates containing DMEM (high glucose) supplemented with 10% FBS, 50 U/mL penicillin and 50 μg/mL streptomycin and incubated at 37°C in a humidified atmosphere (5% CO_2). After 24 h, cells were washed twice with PBS to remove serum-containing medium. In serum-free DMEM, cells received the following treatment: 10 μM 5-aza-2′-deoxycytidine (5-Aza-CdR), 40 μg/mL TSA, 5 μM DZNep, 10 μg/mL GTP, and 20 μM EGCG for 72 h time period. Culture supernatants were collected by centrifugation after each experiment and total protein concentration was estimated using BCA method. Culture supernatant volume corresponding to 20 μg total protein was then subjected to zymography on 7.5% SDS–PAGE gel co-polymerized with 0.1% gelatin. Gel was washed in 2.5% Triton X-100 for 30 min (three times) to remove SDS and was then incubated overnight in activation buffer (10 mM Tris–HCl pH 7, 5 mM $CaCl_2$, 1 μM $ZnCl_2$). After incubation, the gels were stained with 0.5% coomassie brilliant blue followed by destaining with methanol/glacial acetic acid/water (30:10:60) until clear bands were visible against blue background.

Statistical Analysis

The digital images were quantified using Image J software (NIH). Statistical analysis was performed using two-tailed Student's *t*-test. The level of significance designated statistically significant are as follows: $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$.

RESULTS

EZH2 and Class I HDACs Mediate Epigenetic Silencing of TIMP-3 in Human Breast Cancer Cells

Epigenetic silencing of TIMP-3 is a common event in breast cancer, however the epigenetic regulatory circuit causing TIMP-3 repression has not been completely elucidated. We used MCF-7 and MDA-MB-231 cells representing two different phenotypes of breast cancer condition, as an in vitro model, to dissect the epigenetic mechanisms causing transcriptional silencing of TIMP-3 gene. MCF-7 is estrogen responsive (ER+) and less-migratory whereas MDA-MB-231 is estrogen unresponsive (ER−) and highly invasive cells. We attempted to induce TIMP-3 expression with well characterized pharmacologic agents modulating epigenetic pathways related enzymes such as DNA methyltransferase (DNMT) inhibitor 5-Aza-CdR, HDAC inhibitor TSA and histone methylation inhibitor, DZNep. Treatment with 10 μM 5-Aza-CdR had no significant effect on TIMP-3 mRNA levels in both MCF-7 and MDA-MB-231 cells (Figure 1A). Unlike MCF-7 cells, in MDA-MB-231 cells, there was no significant effect of 5-Aza-CdR treatment on TIMP-3 protein levels (Figure 1B). For further

Figure 1. Histone methyltransferase EZH2 and class I HDACs-mediated epigenetic silencing of TIMP-3 in human breast cancer cells. (A) Effect of 5-Aza-CdR (10 μM), TSA (40 μg/mL), DZNep (5 μM), and combined DZNep (5 μM) + TSA (40 μg/mL), 72 h treatment on TIMP-3 mRNA levels as determined by semi-quantitative PCR in MCF-7 and MDA-MB-231 cells; GAPDH was used as an internal control. Image analysis (upper right panel) shows relative intensities of TIMP-3 bands normalized to GAPDH in MCF-7 and MDA-MB-231 cells. The normalized band intensities obtained for controls have been assigned the value of 1 (100%) and those obtained for various treatments were plotted as fold increase or percent with respect to control, respectively. Columns represent the mean TIMP-3 levels ± SD of three independent experiments. Two-tailed Student's *t*-test was used to compare TIMP-3 expression levels between individual treatment groups and control. $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$ versus control. (B) Western blot analysis of TIMP-3 expression and MDA-MB-231 after treatment with 5-Aza-CdR (10 μM), TSA (40 μg/mL), DZNep (5 μM), and combined DZNep (5 μM) + TSA (40 μg/mL) for 72 h treatment. Forty micrograms total protein was loaded in each lane and β-actin was used as loading control. Image analysis (lower right panel) shows relative intensities of TIMP-3 bands normalized to β-actin. TIMP-3 basal expression levels in control were taken as 1 (100%) and rest of the values for other treatment groups (as indicated) represent relative TIMP-3 expression with respect to control. Columns represent the mean TIMP-3 levels ± SD of three independent experiments. Two-tailed Student's *t*-test was used to compare TIMP-3 expression levels between individual treatment groups and control. $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$ versus control. (C) Analysis of CpG promoter methylation status of TIMP-3 in MCF-7 (panel 1) and MDA-MB-231 (panel 2) using methylation-specific PCR. MSP primer sequences were chosen to specifically recognize TIMP-3 promoter sequence in the region from −91 to 125 bp. The presence of a visible PCR product in lanes UM or M indicates the presence of unmethylated or methylated TIMP-3. Universal methylated human DNA standard (+M) and water (W) were used as positive and negative controls. The experiment was repeated three times. (D) Western blot analysis of TIMP-3 expression in MCF-7 (panel 1) and MDA-MB-231 cells (panel 2) after an infection with vector only (pLKO1), HDAC-1 (shHDAC1 4814), HDAC-2 (shHDAC-2 4819), HDAC-3 (shHDAC-3 4826), HDAC-8 (shHDAC-8 4849), EZH2 (shEZH2 E5) specific shRNA lentivirus. Forty micrograms total protein was loaded in each lane and β-actin was used as loading control. Fold change represents ratio of TIMP-3 level (after normalization to loading control) in specific shRNA treated cells versus only vector (pLKO1) treated cells. The normalized band intensities obtained for control (pLKO1) have been assigned the value of 1 and those obtained for various treatments were plotted as fold increase with respect to control, respectively (E) Chromatin Immunoprecipitation (ChIP) was performed to analyze EZH2 occupancy and local histone modifications (H3K27me3 and H3K9/18 Ac) induced by TSA (40 μg/mL), DZNep (5 μM) and combined DZNep (5 μM) + TSA (40 μg/mL), 72 h treatment at the TIMP-3 promoter in MCF-7 and MDA-MB-231. Details are provided in the Materials and Methods section.

investigation, we used MSP to analyze the methylation status of the TIMP-3 promoter with and without 5-Aza-CdR treatment. Our data show that TIMP-3 promoter was unmethylated in both MCF-7 and MDA-MB-231 as no methylation specific band could be detected in either of the cell lines used (Figure 1C). However unmethylated TIMP-3 associated bands were observed in both cell lines with or without 5-Aza-CdR treatment. Therefore, we conclude that TIMP-3 promoter methylation may not be the mechanism responsible for its repression in MCF-7 and MDA-MB-231 breast cancer cells.

Treatment with TSA or DZNep alone or in combination significantly induced TIMP-3 expression in both cell lines, suggesting the likely role of aberrant histone modification patterns in TIMP-3 repression (Figure 1A and B). To further validate our observations and confirm the role of aberrant histone deacetylation and methylation in TIMP-3 silencing in breast cancer cells, we knocked down class I HDACs (HDAC-1, -2, -3, and -8) and histone methyltransferase EZH2 in MCF-7 and MDA-MB-231 cells using specific shRNAs for each target (Figure S1). Compared to control cells (pLKO1), HDAC-2, HDAC-8, and EZH2 protein were markedly decreased in the corresponding knockdown cells. HDAC-1 and HDAC-3 were only modestly decreased and they may be considered as partial HDAC-1/-3 knockdown cells instead of complete HDAC-1/-3 knockout cells. The focus of our study was to investigate TIMP-3 levels in Class-I HDACs and EZH2 knockdown breast cancer cells. Interestingly, TIMP-3 protein levels were induced upon complete or partial class I HDACs or EZH2 knockdown in both MCF-7 and MDA-MB-231 cells (Figure 1D). Since HDACs work as a multi-protein transcriptional co-repressor complex, less abundance of any one of the members might disrupt the structural integrity and function of the enzyme complex. TIMP-3 protein level was also found to be 2.5- to 7.2-fold higher in breast cancer cell lines treated with EZH2 specific shRNA compared to only vector treated control cells (Figure 1D). Upregulation of TIMP-3 expression upon pharmacological inhibition (Figure 1A and B) or genetic knockdown of Class I HDACs and EZH2 (Figure 1D) strongly suggest that TIMP-3 repression in breast cancer is mediated by these histone modifying enzymes.

Furthermore, ChIP analysis revealed that there is decreased transcriptionally active H3K9/18 Ac enrichment and increased localization of EZH2 and corresponding repressive histone chromatin mark H3K27me3 at the control TIMP-3 promoter in both cell lines (Figure 1E). There was a marked increase in H3K9/18 Ac upon TSA treatment alone as well as in combination with DZNep in both MCF-7 and MDA-MB-231. Decrease in EZH2 and H3K27me3 enrichment at the TIMP-3 promoter after DZNep and combined DZNep + TSA treatment was found to be more prominent in MCF-7 as compared to MDA-MB-231 cells (Figure 1E). Combined DZNep and TSA treatment was found to be more effective in decreasing EZH2 localization and H3K27me3 while concomitantly increasing H3K9/18 Ac in both cell lines.

In conclusion, our data suggest that Class I HDACs and EZH2 play important roles in regulating TIMP-3 expression in MCF-7 and MDA-MB-231. Elevated levels of transcriptionally repressive H3K27me3 and decreased active H3K9/18 Ac at the TIMP-3 promoter may contribute its repression independent of promoter DNA methylation in these cells.

Transcriptional Induction of TIMP-3 by Green Tea Polyphenols in Human Breast Cancer Cells

Next, we addressed the potential of GTP and EGCG in inducing TIMP-3 expression in breast cancer cells. MCF-7 and MDA-MB-231 cells were treated with an optimum dose of 10 μg/mL GTP and 20 μM EGCG for 72 h. These doses have been previously used in our publications [37]. Cells treated with various other epigenetic modifying agents (as indicated) were used as controls for TIMP-3 induction. RT-PCR analysis exhibit that GTP and EGCG treatment significantly upregulated ($P < 0.05$) TIMP-3 expression in both cell lines (Figure 2A and B). In MCF-7 cells, there was approximately four- to fivefold and three- to fivefold induction in TIMP-3 protein levels after GTP and EGCG treatment for 3–7 d, respectively. Similarly in MDA-MB-231 cells, GTP and EGCG treated cells demonstrated a 1.3- to 2-fold and 1.8- to 2.5-fold induction in TIMP-3 protein, respectively (Figure 2C and D).

Green Tea Polyphenols Decrease Protein Levels of Class I HDACs and Histone Methyltransferase EZH2 in Human Breast Cancer Cells

Earlier we demonstrated that among several histone modifying enzymes, Class I HDACs and histone methyltransferase EZH2 mediate TIMP-3 silencing in breast cancer cells MCF-7 and MDA-MB-231 (Figure 1) and also that GTPs have the potential to induce TIMP-3 expression in these cells (Figure 2). So our next objective was to decipher the mechanism of TIMP-3 induction by EGCG and GTP. First we determined the protein levels of class I HDACs (HDAC-1, -2, -3, and -8) and histone methyltransferase EZH2 in MCF-7 and MDA-MB-231 cells treated with 10 μg/mL GTP and 20 μM EGCG which previously demonstrated elevated TIMP-3 levels (as shown in Figure 2) by Western blot analysis (Figure 3A and B). Cells treated with 10 μM 5-Aza, 40 μg/mL TSA, 5 μM DZNep, and 40 μg/mL TSA + 5 μM DZNep for 72 h served as controls. Compared to untreated MCF-7 cells, EGCG treatment caused a significant decrease in EZH2 levels from 50% and 36% at 3 and 7 d; whereas GTP treatment resulted in 69% and 65% decrease in EZH2 at 3 and 7 d. Similarly in MDA-MB-231 cells, EZH2 levels were significantly reduced to 54% and 64% by EGCG at 3 and 7 d whereas 83% and 48%

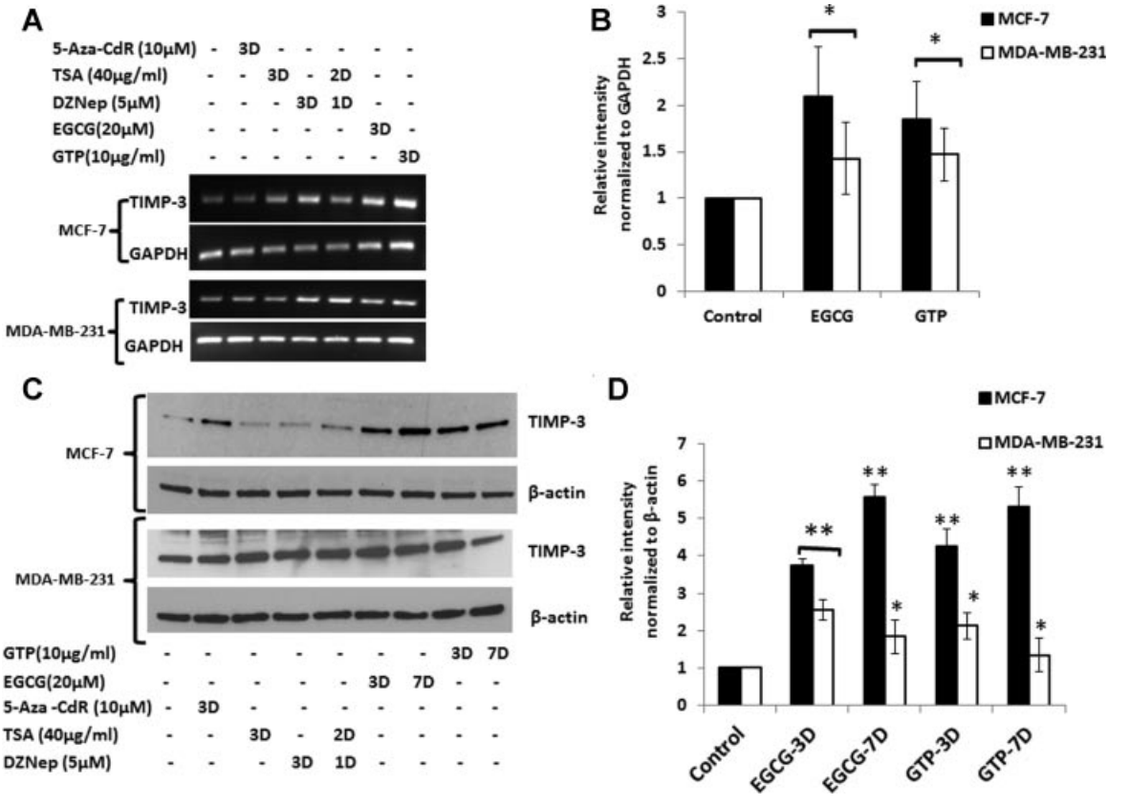

Figure 2. Green tea polyphenol-mediated induction of TIMP-3 in human breast cancer cells. MCF-7 and MDA-MB-231 cells were treated with 20 μM EGCG and 10 μg/mL GTP for 72 h. Other epigenetic modifying agent treatments (as indicated) were used as positive controls. (A) Change in TIMP-3 mRNA levels was detected by RT-PCR. GAPDH was used as internal control. (B) Image analysis shows relative intensities of TIMP-3 bands normalized to GAPDH in MCF-7 and MDA-MB-231. Columns represent the mean TIMP-3 levels ± SD of three independent experiments. Two-tailed Student's *t*-test was used to compare TIMP-3 expression levels between individual treatment groups and control. $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$ versus control. (C) Western blot analysis of TIMP-3 protein levels. Forty micrograms total protein sample was loaded in each lane and β-actin was used as loading control. (D) Image analysis shows relative intensities of TIMP-3 bands normalized to β-actin. Columns represent the mean TIMP-3 levels ± SD of three independent experiments. Two-tailed Student's *t*-test was used to compare TIMP-3 expression levels between individual treatment groups and control. $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$ versus control. Details are provided in the Materials and Methods section.

reduction in EZH2 was noted within 3 and 7 d of GTP treatment. In MCF-7, EGCG and GTP treatment for 3 d caused a significant decline in HDAC-2 protein levels while in MDA-MB-231, only prolonged exposure with EGCG for 7 d showed significant reduction of 68% in HDAC-2 levels. EGCG and GTP treatment caused no significant changes in HDAC-1 and HDAC-3 levels in MCF-7 cells unlike MDA-MB-231 where HDAC-1 and HDAC-3 levels were found to be significantly reduced with EGCG (3–7 d) and GTP (3 d) treated cells. In both cell lines, there was a marked decrease in HDAC-8 levels in EGCG and GTP treatment, compared to control groups. In summary, our results demonstrate that EGCG and GTP treatment caused a significant decrease in EZH2 and HDAC-8 protein levels in both breast cancer cell lines while the effect on other class I HDACs (HDAC-1, -2, and -3) varied depending on the cell phenotype and exposure time.

Green Tea Polyphenols Cause Decreased H3K27me3 and Increased Acetylated H3K9/18 Binding at TIMP-3 Promoter in Human Breast Cancer Cells

To address the direct association between GTP-mediated TIMP-3 induction (as shown in Figure 2A and B) and decrease in the levels of various histone modifying enzymes (as shown in Figure 3A and B) or their activity, we next studied the effect of GTP and EGCG treatment on histone modifications pattern specifically at the TIMP-3 promoter by ChIP assay using anti-EZH2, anti-H3K27me3, and H3K9/18 Ac antibodies. The immunoprecipitated DNA from MCF-7 and MDA-MB-231 cells was analyzed by PCR primers set [38], designed to amplify YY1 binding site in TIMP-3 promoter which plays a role in the recruitment of EZH2 containing polycomb repressive complex 2 (PRC2) complex. Our results show that GTP and EGCG treatment decreases EZH2 binding and

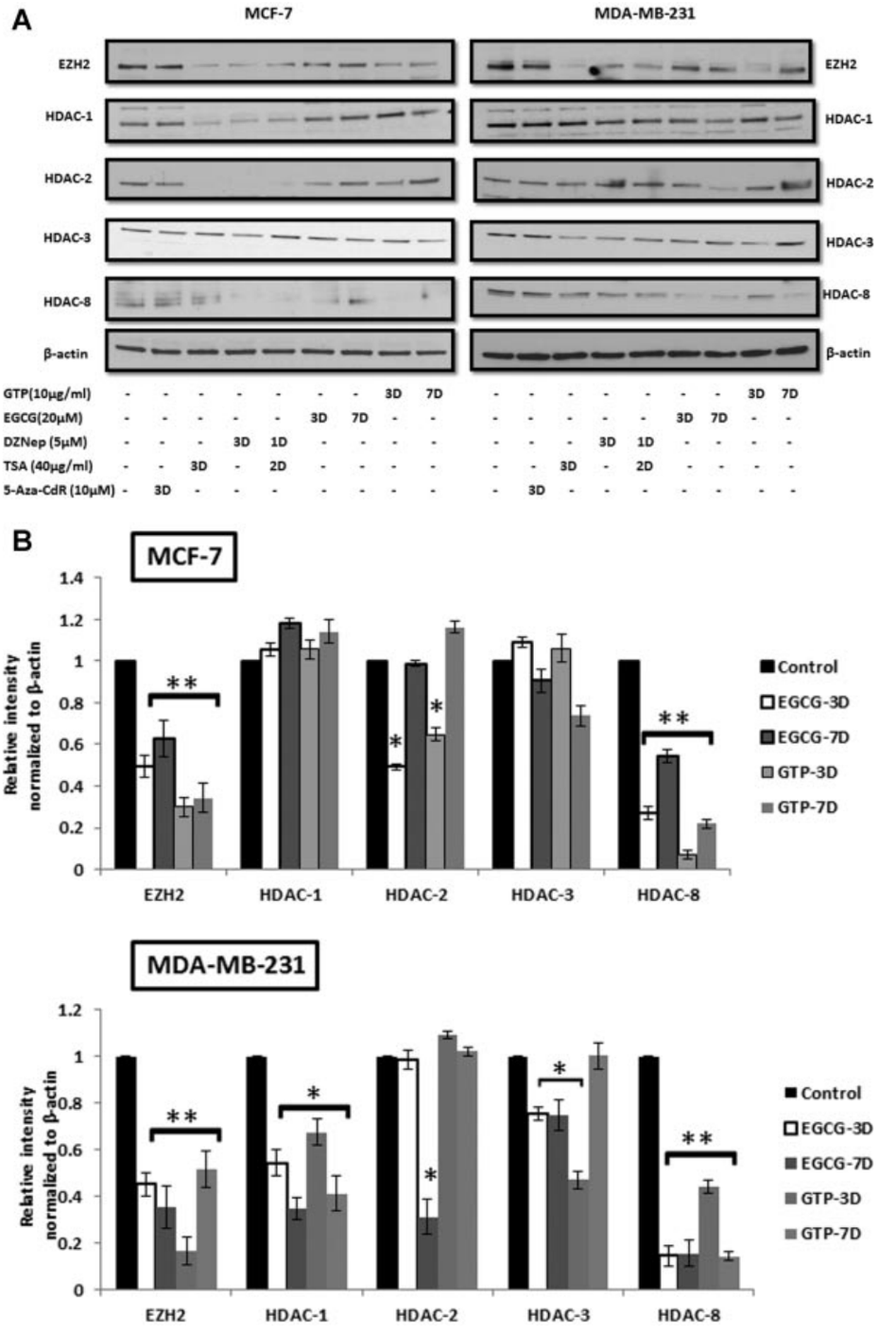

Figure 3. Green tea polyphenol-mediated decrease in class I HDACs and histone methyltransferase EZH2 protein levels in human breast cancer cells. (A) Western blot analysis of class I HDACs (HDAC-1, -2, -3, and -8) and EZH2 protein levels in MCF-7 (panel 1) and MDA-MB-231 (panel 2) cells treated with 5-Aza-CdR (10 μM), TSA (40 μg/mL), DZNep (5 μM), and combined DZNep (5 μM) + TSA (40 μg/mL), 20 μM EGCG, and 10 μg/mL GTP for 3 d (3D) or 7 d (7D) as indicated. Forty micrograms total protein sample was loaded in each lane and β-actin was used as loading control. (B) Image analysis represents relative intensities of class I HDACs and EZH2 protein bands normalized to β-actin in MCF-7 (upper panel) and MDA-MB-231 (lower panel). Columns represent mean ± SD of three independent assays. $P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$ versus control. Details are provided in the Materials and Methods section.

subsequently H3K27me3 enrichment at the TIMP-3 promoter. Furthermore, decrease in repressive H3K27me3 mark is followed by a corresponding increase in transcriptionally active Histone H3 acetylated at Lysine 9/18 enrichment at the TIMP-3 promoter (Figure 4A). These data suggest that GTP and EGCG treatment induce TIMP-3 expression in breast cancer cells by decreasing class I HDACs and EZH2 protein levels which corroborates with their less occupancy and decline in repressive chromatin H3K27me3 mark and with concomitant increase in H3K9/18 Ac levels at the TIMP-3 promoter.

Green Tea Polyphenols Cause Decrease in Global H3K27 Trimethylation With a Concomitant Increase in H3K9/18 Acetylation Levels in Human Breast Cancer Cells

Next we investigated whether decrease in class I HDACs and EZH2 protein levels affects global histone acetylation and methylation levels in breast cancer cells. As shown in Figure 4B, analysis of acid-extracted histones from MCF-7 and MDA-MB-231 cells treated with GTP and EGCG for 72 h show a marked global reduction in tri-methylated Histone H3 at Lysine-27 (H3K27me3) compared to untreated cells. In MCF-7, 49% and 60% decrease in H3K27me3 was observed whereas in MDA-MB-231 2.56% and 11.17% decrease in H3K27me3 was observed after EGCG and GTP treatment, respectively. There was concomitant increase in global Histone H3 acetylated at Lysine 9/18 (H3K9/18 Ac) in the above EGCG and GTP treated breast cancer cells. In MCF cells, EGCG and GTP treatment cause 1.5- to 2-fold increase in Histone H3 acetylation levels whereas in MDA-MB-231 the increase was more prominent with four- to sixfold induction. GTP and EGCG-mediated global reduction in histone methylation levels with a concomitant

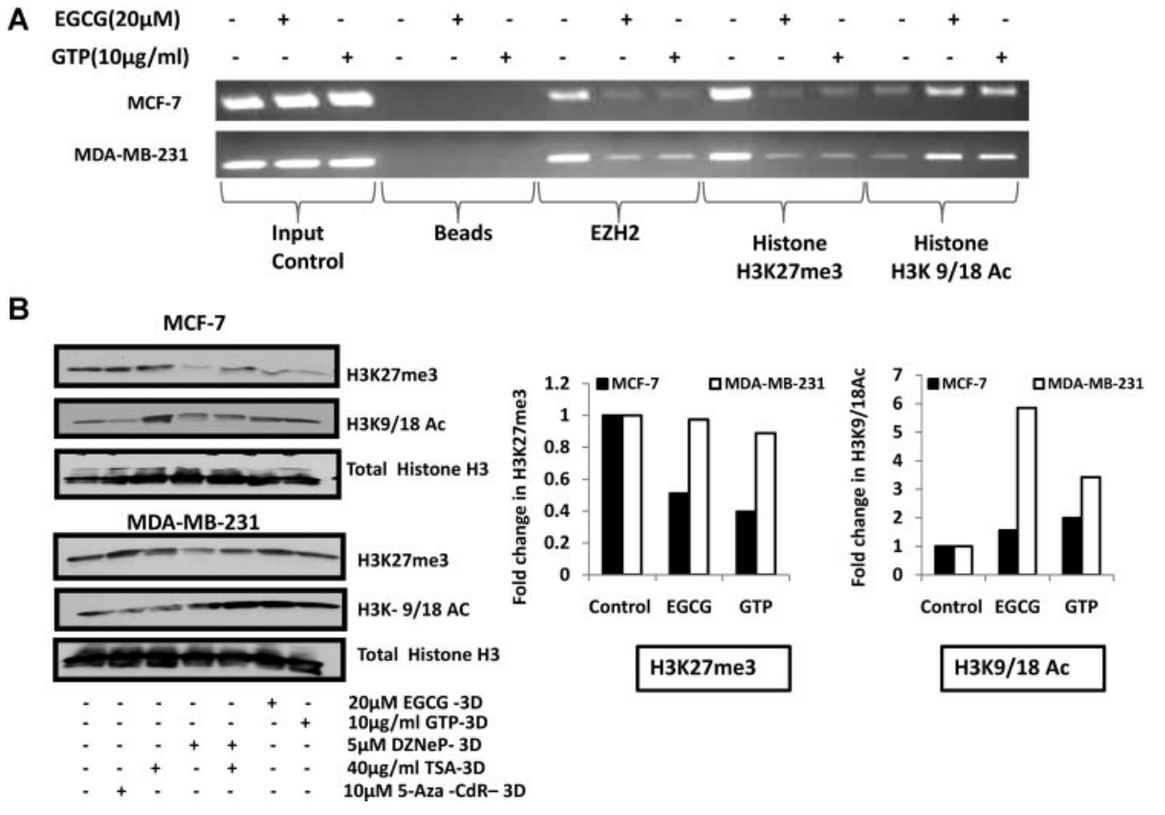

Figure 4. Green tea polyphenol-mediated decrease in H3K27 trimethylation with a concomitant increase in H3K9/18 acetylation levels in human breast cancer cells. (A) Chromatin immunoprecipitation assay was performed to analyze the local histone modifications induced by 20 μM EGCG and 10 μg/mL GTP treatment for 72 h at the TIMP-3 promoter. EGCG and GTP treatment caused decreased association of EZH2 and therefore less H3K27me3 enrichment at the TIMP-3 promoter with a concomitant increase in H3K9/18 Ac levels in MCF-7 (top panel) and MDA-MB-231 (bottom panel). The experiment was repeated at least twice. (B) Western blot analysis of acid-extracted total histones from MCF-7 (top panel) and MDA-MB-231 (bottom panel) cells treated with 5-Aza-CdR (10 μM), TSA (40 μg/mL), DZNep (5 μM), and combined DZNep (5 μM) + TSA (40 μg/mL), 20 μM EGCG, and 10 μg/mL GTP for 72 h. Twenty micrograms of total acid-extracted histone sample was loaded in each lane and total histone H3 served as internal control. Image analysis (right panel) shows ratio of relative intensities between histone H3 trimethylated at Lys 27 (H3K27me3) and histone H3 acetylated at Lys 9/18 (H3K9/18 Ac) bands normalized to total histone H3 in both cell lines. Details are provided in the Materials and Methods section.

increase in histone acetylation levels support transcriptional activation of TIMP-3 gene, which is regulated by both these epigenetic mechanisms.

Green Tea Polyphenols Suppress Invasiveness and Gelatinolytic Activity in Human Breast Cancer Cells

Given the significance of TIMP-3 levels in regulating tumor invasion and metastasis, we assessed the functional effect of GTP and EGCG-mediated TIMP-3 induction on breast cancer cell migration and gelatinolytic (MMP-2/-9) activity. Our results show that 20 μM EGCG and 10 μg/mL GTP treated MCF-7 cells resulted in 33% and 27% wound closure, respectively compared to untreated cells (95%) after 48 h treatment (Figure 5A and B). Similarly in MDA-MB-231 cells, which are highly invasive and migratory, 18% and 23% wound closure was observed in 20 μM EGCG and 10 μg/mL GTP posttreatment at 24 h after scratching. Combined treatment with 5 μM DZNep and 40 μg/mL TSA demonstrated comparable anti-migratory potential causing 50% wound closure in MCF-7 and MDA-MB-231 cells after 48 and 24 h treatment period. Treatment with 5-Aza-CdR, TSA, and DZNep alone, 70–95% wound area was occupied by cells 24–48 h after treatment.

TIMPs regulate MMPs activity by binding to them in 1:1 stoichiometry both in bound and secreted form and thus keep MMPs in an inactive state. Since MMP-2 and MMP-9 are prominent MMPs involved in ECM degradation and tumor cell migration that are regulated by TIMP-3, we determined the enzymatic activity of these two MMPs using gelatin zymography. As shown in Figure 6A and B, a significant decrease in gelatinolytic activity of 85% in MMP-9 and 53–60% in MMP-2 in MCF-7 cells was observed after EGCG and GTP treatment. In MDA-MB-231 cells, 20 μM EGCG treatment for 72 h caused 65–67% reduction in both MMP-2 and MMP-9 gelatinolytic activity. Treatment with 10 mg/mL GTP resulted in a more pronounced inhibition of gelatinolytic activity as compared to EGCG treatment, causing 95% decrease in MMP-9 and 80% decrease in MMP-2 in MDA-MB-231. In both cell lines, treatment with 5 μM DZNeP alone and in combination with 40 μg/mL TSA significantly reduced MMP-2/-9 activity. Our data suggest that EGCG and GTP treatment significantly inhibit migration and gelatinolytic activity as observed by combined treatment with DZNep and TSA in these breast cancer cells.

To confirm that TIMP-3 induction by GTPs play a major role in decreasing in MMP-2/MMP-9 activity, we studied the direct effect of EGCG and GTP on MMP-2 and MMP-9 expression levels in MCF-7 and MDA-MB-231 cells. MMP-2 mRNA levels were found to be negligible in both cell lines (Figure 6C). MMP-9 levels were relatively low in MCF-7 cells as compared to MDA-MB-231 cells. EGCG and GTP treatment had no significant effect on MMP-9 levels in either of the cell lines (Figure 6D). Although we did not observe any effect of EGCG/GTP on MMP-2/MMP-9 expression, further studies are needed to determine the exact quantitative contribution of TIMP-3 in MMP-2 and MMP-9 inhibition.

DISCUSSION

In recent years, GTPs have been demonstrated to elicit a multifunctional effect in impeding various molecular pathways involved in neoplastic transformation and metastasis in various cancer types including breast cancer [31–35]. This is the first study to demonstrate that the epigenetic induction of TIMP-3 may be one of the key mechanisms by which GTPs suppress MMP-2/-9 activity and tumor invasion in breast cancer cells. We also provided a novel mechanistic insight into the epigenetic regulation of TIMP-3 gene in breast cancer cells.

TIMP-3 is a ubiquitously expressed, ECM bound protein that acts as an endogenous inhibitor of MMPs and inhibit apoptosis, invasion and angiogenesis [12–14]. Reduced expression of TIMP-3 protein was associated with poorly differentiated and aggressive breast carcinoma of higher nuclear and histological grades [21]. Down-regulation of TIMP-3 expression was predominantly attributed to promoter DNA hypermethylation however only 20% breast tumor specimens display TIMP-3 methylation [25,26]. In the present study, we demonstrated that aberrant histone modification patterns at the TIMP-3 promoter contributes to its epigenetic silencing, independent of promoter DNA hypermethylation, at least, in breast cancer cell lines. We used two human breast adenocarcinoma cell lines: MCF-7 and MDA-MB-231 as our in vitro models as they represent two different phenotypes of breast cancer. Hypermethylation in TIMP-3 promoter was not detected in breast cancer cell lines used in our study and accordingly, there was no significant induction in TIMP-3 mRNA levels after treatment with demethylating agent 5-Aza-CdR. This observation is supported by another study which also reported that there was no TIMP-3 hypermethylation in mammary cancer cell lines [39]. Unlike MDA-MB-231 cells, there was a significant induction in TIMP-3 protein levels after 5-Aza-CdR treatment in MCF-7 cells. One possible explanation for this observation may be the direct effect of 5-Aza-CdR treatment on the modified histone marks such as H3K27me3 on the TIMP-3 promoter in MCF-7 cells. This observation is in line with a previous study which showed the effect of 5-Aza-CdR treatment on histone modification marks such as H3K27me3 in target genes which do not display promoter methylation [40]. We showed that treatment of breast cancer cells with TSA (HDAC inhibitor) and DZNep (histone methylation inhibitor) could reverse TIMP-3 repression. Furthermore, genetic knockdown studies confirmed the involvement of histone methyltransferase EZH2 and class I

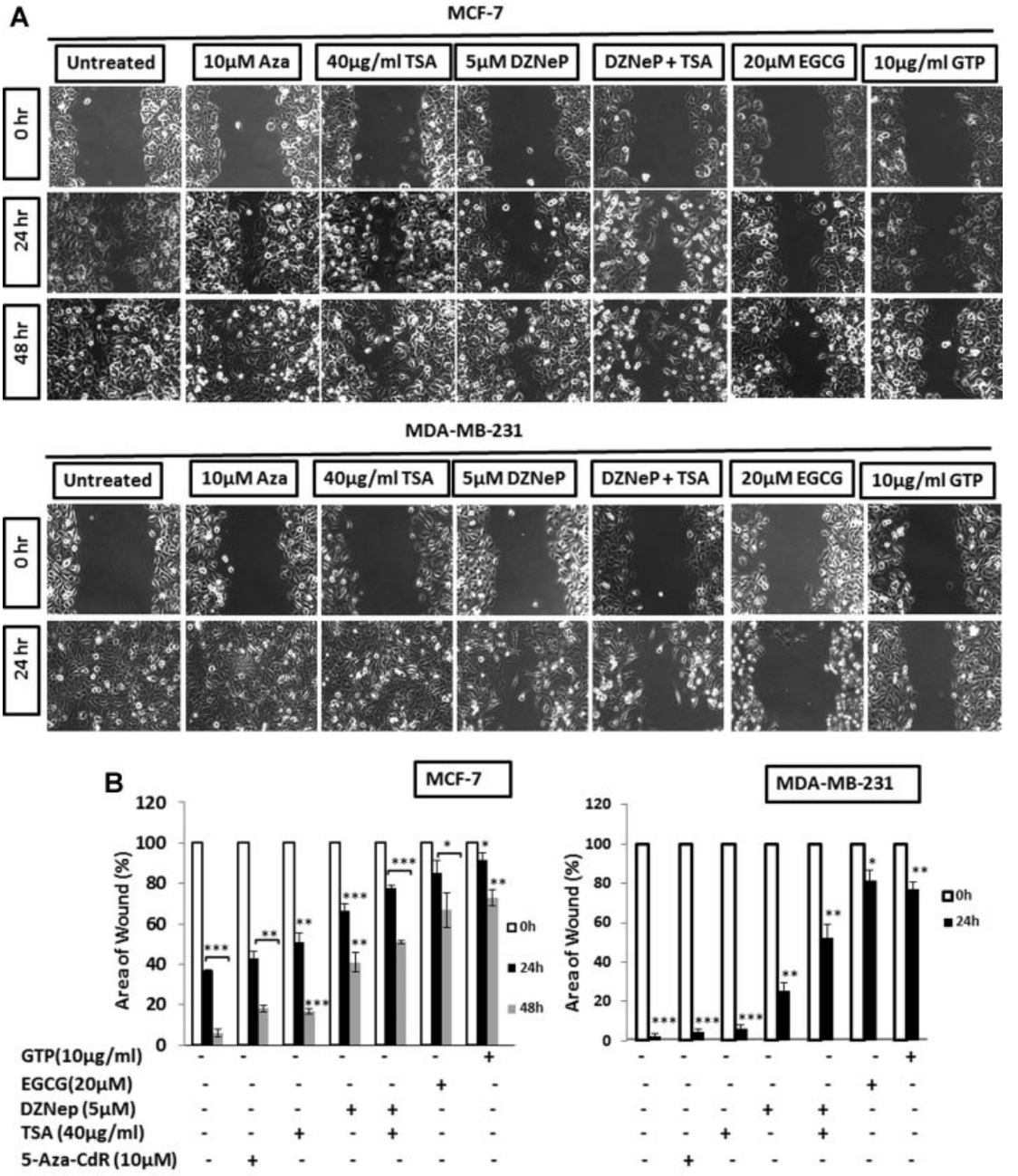

Figure 5. Green tea polyphenol treatment suppresses invasiveness in human breast cancer cells. (A) Wound healing assay of MCF-7 (top panel) and MDA-MB-231 (bottom panel) cells treated with 5-Aza-CdR (10 μM), TSA (40 μg/mL), DZNep (5 μM), and combined DZNep (5 μM) + TSA (40 μg/mL), 20 μM EGCG, and 10 μg/mL GTP. Images were taken before (0 h) and after wound closure (24 and 48 h). (B) The results were expressed as the percentage of the remaining area determined by normalizing the area of wound after 24 h or 48 h (as indicated) to the initial wound area at 0 h (set to 100%). Each bar represents the mean of five fields measured ± SD. Two-tailed Student's *t*-test was used to compare treatment groups and control. $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$ versus control. Details are provided in the Materials and Methods section.

HDACs in TIMP-3 repression. EZH2, the catalytic subunit of PRC2, tri-methylates Histone H3 at lysine-27 (H3K27me3), which serves as repressive mark in maintaining the silenced state of several genes during development and establishing embryonic stem cell identity ([41] and references therein). Recent findings in breast cancer implicate that EZH2 protein levels increase through successive

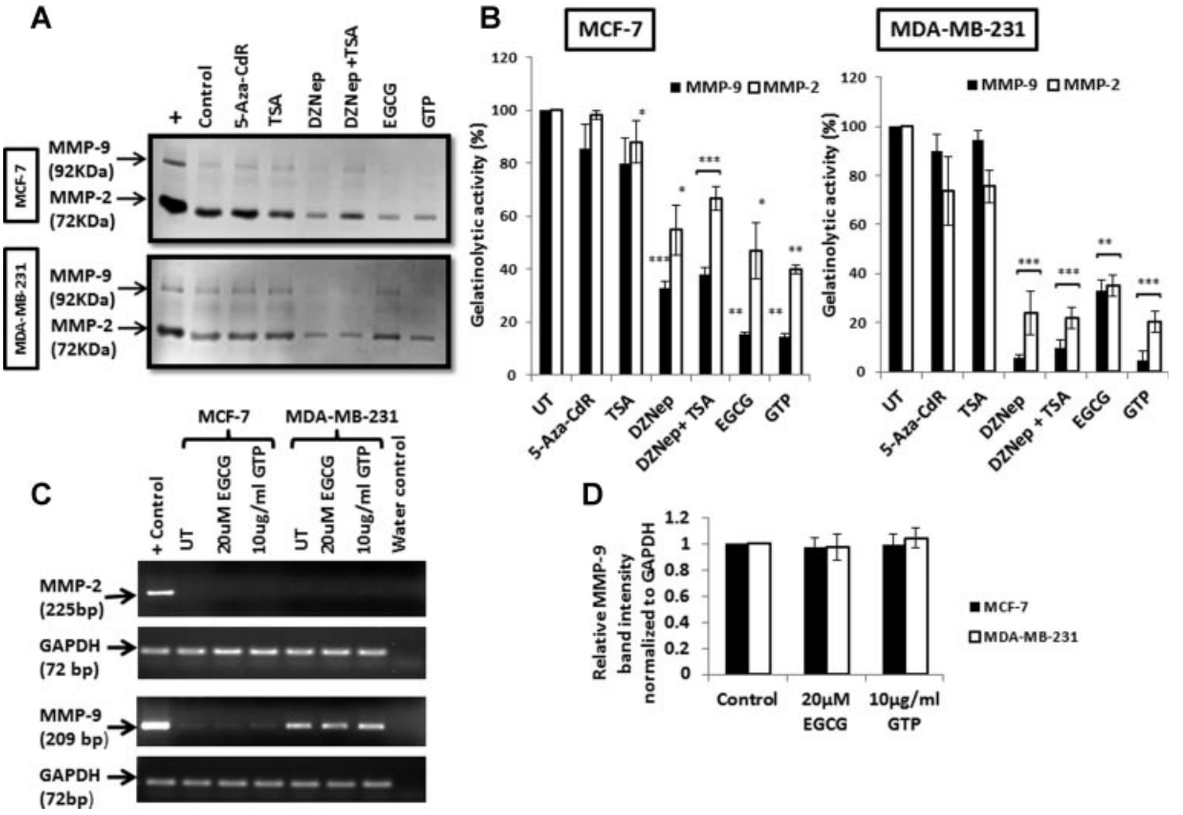

Figure 6. Green tea polyphenol treatment decrease gelatinolytic activity (Pro-MMP-2 and Pro-MMP-9) in human breast cancer cells. (A) Gelatin zymography assay for MMP-2 and MMP-9 activity in MCF-7 (top panel) and MDA-MB-231 (bottom panel) cells treated with 5-Aza-CdR (10 μM), TSA (40 μg/mL), DZNep (5 μM), and combined DZNep (5 μM) + TSA (40 μg/mL), 20 μM EGCG, and 10 μg/mL GTP for 72 h. Conditioned media (serum-free) was collected after 72 h treatment and culture supernatant volume equivalent to 40 μg total protein was used in the assay. HT1080 cell line conditioned medium was used as positive control and also served as molecular weight marker. (B) MMP-2 and MMP-9 band intensities (clearance zones) were quantified by densitometry using Image J software. Columns represent mean ± SD of three independent assays. $P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$ versus control. (C) RT-PCR analysis of MMP-2 (upper panel) and MMP-9 (lower panel) mRNA levels after treatment with 20 μM EGCG and 10 μg/mL GTP for 72 h in MCF-7 and MDA-MB-231 cells. GAPDH was used as an internal control. Each experiment was repeated at least three times (D) Image analysis of MMP-9 bands in MCF-7 and MDA-MB-231 cells by densitometry using Image J. Columns represent mean ± SD of three independent assays. Details are provided in the Materials and Methods section.

stages of neoplastic transformation [42,43], and enhanced EZH2 catalyzed H3K27me3 repressive mark results in the silencing of various critical tumor suppressor genes [44,45]. Similarly, abnormally high activity of HDACs has been linked to deacetylation and aberrant repression of several critical genes in breast cancer [46]. Our studies showed that decreased TIMP-3 expression in breast cancer cells MCF-7 and MDA-MB-231 is associated with elevated EZH2 and class I HDAC levels. ChIP studies demonstrated that decreased EZH2 and repressive H3K27me3 chromatin modification and a corresponding increase in H3K9/18 Ac by combined DZNep and TSA treatment de-repress TIMP-3 locus in both breast cancer cell lines. Recent studies in prostate cancer also demonstrated an inverse correlation between EZH2 and HDAC-1/2/3 levels to TIMP-3 expression [1].

To our knowledge, this is the first study that provides a detailed mechanistic insight into the epigenetic induction of TIMP-3 by GTP and EGCG in breast cancer. We showed that GTP and EGCG treatment significantly decreased EZH2 protein levels and hence its localization and catalytic activity, that is, H3K27me3 enrichment at the promoter region. Also, there was a significant decrease in HDAC-2 and HDAC-8 levels in MCF-7 and HDAC-1, HDAC-3 and HDAC-8 levels in MDA-MB-231 cells after treatment with GTP and EGCG, which might have altered the balance between the histone acetyltransferases (HATs) and HDACs in these breast cancer cells. This ultimately resulted in the deposition of increased transcriptionally active acetylated Histone H3 at Lysine9/18 (H3K9/18 Ac) at the TIMP-3 promoter. The underlying mechanism by which GTPs decrease EZH2 and Class I HDACs in breast cancer cells is not the focus of the present study and requires further investigation. However, the present findings are in line with our previous studies which suggest that

green tea catechins function as potent HDAC inhibitors and induce cell cycle arrest and apoptosis, by enhancing the proteasomal degradation of Class I HDACs (HDAC-1, -2, -3, and -8) in prostate cancer cells [37]. Also, a recent study reported that EGCG treatment decreased the protein levels of polycomb group members EZH2 and Bmi1 in epidermal squamous cell carcinoma cells [47,48]. It will be interesting to elucidate the effect of GTP/EGCG on histone acetyltransferases (HATs) which may change the dynamic relationship between HDACs/HATs in the tumor cells.

Focusing on the functional effect of EGCG/GTP-mediated TIMP-3 induction on MCF-7 and MDA-MB-231 cells, we demonstrated by gelatin zymography that there was significant decrease in MMP-2 and MMP-9 activity in conditioned media (serum-free) collected from EGCG and GTP treated cells for 72 h. Similar decrease in gelatinolytic activity was also observed in conditioned media collected from DZNep and TSA treated cells. EGCG has been shown to have multifunctional effect on MMP-2 and MMP-9 including their expression, activation, secretion, and the induction of some inhibitors such as RECK [34,35,50,51]. Based on previous studies it is arguable that the decrease in gelatinolytic activity might be due to direct effect of GTPs on MMP-2 and MMP-9 expression [34,50]. But our finding suggests that MMP-2 mRNA was negligible (not detected) in MCF-7 and MDA-MB-231 cells and is consistent with a previous study [52]. There was no further inhibitory effect of EGCG/GTP treatment on MMP-2 expression. MMP-9 expression levels also remained unaffected after EGCG/GTP treatment for 72 h in both cell lines. The reason for the divergent findings might have to do with either variability in sub-clones of cell lines used or different doses of EGCG and treatment period used during the experiments. Intriguingly, though MMP-2 mRNA levels were found to very low in the cell lines used in our study, the gelatinolytic activity associated with MMP-2 was detectable in zymography. The possible reason may be that gelatin zymography is a sensitive technique and can detect as low as 10 pg of MMP-2 [53]. Also conditioned media used in the experiment represents the sum total of all the secreted MMP-2 and MMP-9 (pro and active forms) in culture medium in a 72-h time period but RT-PCR analysis of expression levels reflect the mRNA levels at the end of 72 h. Here we show that GTP-mediated TIMP-3 may play a crucial role in shifting MMP: TIMP balance toward TIMPs and decrease the elevated gelatinolytic activity (MMP-2/-9) frequently observed in breast cancer cells. Further studies are needed to define the extent of contribution of TIMP-3 on MMP-2/-9 activity and the suppression of cell invasiveness.

Based on our results, we conclude that the in vitro and in vivo anti-invasive effects of EGCG/GTP reported in breast cancer cells may be due to, in part, through epigenetic induction of endogenous MMP inhibitor, TIMP-3. Also, our results provide the first detailed insight into the epigenetic regulatory mechanism causing TIMP-3 repression in breast cancer, highlighting the role of histone modifying enzymes EZH2 and HDACs. We demonstrated that GTP and EGCG-mediated decrease in EZH2 and class I HDACs levels caused a corresponding global decrease in repressive chromatin mark H3K27me3 and an increase in transcriptionally open chromatin, that is, H3K9/18 Ac, respectively, in MCF-7 and MDA-MB-231 cells. This coincides with decreased EZH2 localization and H3K27me3 enrichment at the TIMP-3 promoter with a concomitant increase in H3K9/18 Ac, after GTP and EGCG treatment. Epigenetic induction of TIMP-3 may be one of the key mechanisms by which green tea catechins shift the MMPs:TIMPs balance toward TIMPs and inhibit active MMPs in breast cancer.

We have used 10 μg/mL GTP and 20 μM EGCG which are physiologically attainable concentration and corresponds to drinking 2–3 cups of green tea by an adult individual [49]. Although case–control and epidemiological studies provide some clues that regular consumption of GTP and EGCG could decrease the risk of breast cancer invasion and/or progression, however, these studies do not provide further insight into the molecular mechanism, related to tumor inhibition. Based on our present findings, TIMP-3 can be used as a surrogate marker of GTP and EGCG response and warrants further investigation through well-designed clinical trial.

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