Research Article

Cancer Prevention Research

A Novel Prodrug of Epigallocatechin-3-gallate: Differential Epigenetic *hTERT* Repression in Human Breast Cancer Cells

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Abstract

Epigallocatechin-3-gallate (EGCG), a major component of green tea polyphenols (GTP), has been reported to downregulate telomerase activity in breast cancer cells thereby increasing cellular apoptosis and inhibiting cellular proliferation. However, the major concerns with GTPs are their bioavailability and stability under physiologic conditions. In the present study, we show that treatments with EGCG and a novel prodrug of EGCG (pro-EGCG or pEGCG) dose- and time-dependently inhibited the proliferation of human breast cancer MCF-7 and MDA-MB-231 cells but not normal control MCF10A cells. Furthermore, both EGCG and pro-EGCG inhibited the transcription of *hTERT* (human telomerase reverse transcriptase), the catalytic subunit of telomerase, through epigenetic mechanisms in estrogen receptor (ER)-positive MCF-7 and ER-negative MDA-MB-231 cells. The downregulation of *hTERT* expression was found to be because of *hTERT* promoter hypomethylation and histone deacetylations, mediated at least partially through inhibition of DNA methyltransferase and histone acetyltransferase activities, respectively. In addition, we also observed that EGCG and pEGCG can remodel chromatin structures of the *hTERT* promoter by decreasing the level of acetyl-H3, acetyl-H3K9, and acetyl-H4 to the *hTERT* promoter. EGCG and pEGCG induced chromatin alterations that facilitated the binding of many *hTERT* repressors such as MAD1 and E2F-1 to the *hTERT* regulatory region. Depletion of E2F-1 and MAD1 by using siRNA reversed the pEGCG downregulated *hTERT* expression and associated cellular apoptosis differently in ER-positive and ER-negative breast cancer cells. Collectively, our data provide new insights into breast cancer prevention through epigenetic modulation of telomerase by using pro-EGCG, a more stable form of EGCG, as a novel chemopreventive compound. *Cancer Prev Res; 4(8); 1243–54. ©2011 AACR.*

Introduction

Epigallocatechin-3-gallate (EGCG), a major component of green tea polyphenols (GTPs), has been shown to have anticancer, anti-inflammatory, and antitumor activity in various cancers including breast cancer (1–4). EGCG accounts for more than 50% of the total polyphenols, is the most active component of GTPs, and has been shown to induce apoptosis and cell cycle arrest in many cancer cells without affecting normal cells (1, 4–8). Therefore, it is likely that EGCG imparts its chemopreventive effects through many different mechanisms (8–10). One mechanism includes the inhibition of human telomerase reverse transcriptase (*hTERT*), the catalytic subunit of telomerase, an important enzyme required for maintenance of telomere length and tumorigenesis (2, 11, 12). Inhibition of telomerase has received wide attention in cancer prevention because of its high expression in cancer cells and very low expression in normal somatic cells (11–13). Furthermore, there has been a growing interest in epigenetic regulation by EGCG in chemoprevention because of its DNA methyltransferases (DNMTs) and histone acetyltransferases (HATs) inhibition activities (14–16). The DNMTs inhibition activity of EGCG has been shown to lead to global and local hypomethylation of a number of gene promoters (4, 10, 15).

DNA methylation of the promoter region of a gene has been shown to be an important factor in its ability to bind different transcription factors. *hTERT* is a promising target for cancer prevention and is regulated by several epigenetic alterations including histone acetylation and methylation at promoter sites (11–13, 17). Furthermore, the *hTERT* promoter region is paradoxically hypermethylated by specific DNMTs in cancer cells leading to its expression (18). The aberrant methylation pattern in the *hTERT* 5′-regulatory region prevents the binding of the methylation-sensitive transcriptional factors such as CTCF and E2F-1 to the *hTERT* promoter (18–20). Furthermore, MAD1 is also a

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transcription repressor of *hTERT* that binds to its 5′-CACGTG-3′ sequence (E-box), whereas c-MYC binds to the same promoter sites to activate *hTERT* expression (19, 21, 22). Binding of MAD1 recruits mSin3-HDACs complexes, which consequently results in decreased acetylation of histones H3 and H4 at the target gene promoters (22, 23). Therefore, EGCG mediates DNMTs and HATs inhibition which leads to DNA hypomethylation and histone deactylation, respectively, that are central pathways important for *hTERT*-targeted breast cancer prevention.

We and other investigators have shown that EGCG inhibits telomerase and induces cellular apoptosis in human breast cancer cells (4, 13, 20). However, the effects of EGCG on DNA methylation and histone acetylation in estrogen receptor (ER)-positive [ER (+)] and ER-negative [ER (−)] breast cancers have not yet been well studied. Because EGCG has DNMTs and HATs inhibitory activity, it is important to evaluate the impact of EGCG-induced chromatin modifications and its associated transcriptional factors binding on gene promoters important in cancer prevention such as *hTERT*. Therefore, the present study was undertaken to evaluate EGCG modulation of epigenetic regulation of *hTERT* expression and its promoter alterations associated with the induction of apoptosis and inhibition of cellular proliferations in both ER (+) and ER (−) human breast cancer cells. In addition, we also used a prodrug of EGCG (pro-EGCG or pEGCG and EGCG octaacetate) to enhance the bioavailability and stability of EGCG delivered into the cells (24, 25). Pro-EGCG was synthesized by modifying reactive hydroxyl groups with peracetate groups and found to be converted as well as accumulated into parental EGCG when cultured with human breast cancer cell (24). It was reported that pEGCG was better absorbed into the cells, converted into EGCG, and accumulated in greater quantity than natural EGCG (24). Furthermore, oral administration of pEGCG to CF-1 mice resulted in higher bioavailability compared with equimolar doses of EGCG (26). Our results indicate that pEGCG is more potent than EGCG but shares similar mechanisms as EGCG. Both EGCG and pEGCG inhibit *hTERT* expression by inducing DNA hypomethylation and promoter deacetylations mediated, at least partially, through inhibition of DNMTs and HATs, respectively. Furthermore, hypomethylation and deacetylation induced by EGCG further recruits *hTERT* transcriptional repressors such as E2F-1 and MAD1, thereby contributing to inhibition of *hTERT* expression and induction of cellular apoptosis in human breast cancer cells. Interestingly, the transcriptional binding of E2F-1 and MAD1 to regulate *hTERT* inhibition and induction of apoptosis occurred differently in ER (+) and ER (−) breast cancer cells. These novel findings are important in developing prevention and therapeutic strategies for ER (+) and ER (−) breast cancers.

Materials and Methods

Materials

EGCG (≥95% pure) was purchased from Sigma–Aldrich. Purified pro-EGCG (pEGCG; >98% pure) was prepared from EGCG as described previously (25). Structural and molecular differences between EGCG and pEGCG are described in Supplementary Fig S1. Both EGCG and pro-EGCG were prepared in dimethyl sulfoxide (DMSO) and stored at a stock concentration of 100 mmol/L at −20°C.

Cell culture and cell growth assay

The human breast cell lines were obtained from the American Type Culture Collection and no authentication was done by the authors. Breast cancer MCF-7 [ER (+)] and MDA-MB-231 [ER (−)] cells as well as normal control MCF10A cells were cultured as monolayer as described previously (27). MCF10A is a nontumorigenic human breast epithelial cell line and frequently used as a normal human breast control cell type (27–29). After seeding the cells for 24 hours, EGCG or pEGCG was added to the culture medium at the indicated concentrations and the maximum concentration of DMSO was 0.1% (v/v) in the medium. Cells treated only with DMSO served as a vehicle control. For cell growth assays, total viable cell numbers were calculated by using a hemocytometer and plotted against number of treatment days.

Assay for apoptosis by flow cytometry

Induction of apoptosis in human breast cancer cells caused by EGCG and pEGCG treatments were quantitatively determined by flow cytometry by using the Annexin V–conjugated Alexafluor 488 (Alexa488) Apoptosis Vybrant Assay Kit as described previously (30). Following treatment, cells were harvested by brief trypsinization, washed with PBS, and incubated with Alexa488 and propidium iodide for cellular staining in Annexin-binding buffer at room temperature for 10 minutes in the dark. The stained cells were analyzed by fluorescence- activated cell sorting (FACS) by using a FACS-Caliber instrument (BD Biosciences) equipped with Cell Quest 3.3 software.

Detection of apoptotic cells by Hoechst staining

Following treatment, cells were harvested and cytospinned on microscopic slides by using cytospin*4 centrifuge (Thermo Scientific, Fisher Scientific Products). Slides were washed with PBS and fixed in freshly prepared 0.1% ice-cold paraformaldehyde for 10 minutes. The cells were then washed with PBS and stained with Hoechst 33342 dye (50 μg/mL) for 1 minute in the dark. Hoechst-stained slides were randomly pictured under a fluorescence microscope and representative pictures are provided.

Quantification of *hTERT* expression by reverse transcriptase PCR and real-time PCR

Total RNA isolation and real-time quantification of *hTERT* expression were followed as described previously (27). Total RNA (2 μg) was reverse transcribed into cDNA by using the iScript cDNA Synthesis Kit (Bio-rad). The *hTERT* primers are as follows: sense 5′-CGGAAGAGTGTCTGGAGCAA-3′ and antisense 5′-GGATGAAGCGGAGTCTGGA-3′. The reaction conditions were 35 cycles at 94°C for 30 seconds, 52°C for 30 seconds, and 72°C for

25 seconds. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as an endogenous control. Real-time quantitative PCR (qPCR) was carried out as described earlier with following primers: sense 5′-AGGGGCAAGTCC-TACGTCCAGT-3′ and antisense 5′-CACCAACAAGAAAT-CATCCACC-3′ (27). The calculations for determining the relative level of gene expression were made by using the cycle threshold (C_t) method. The mean C_t values from duplicate measurements were used to calculate the expression of the target gene by using the formula: fold change in gene expression, $2^{-\Delta\Delta C_t} = 2^{-[\Delta C_t \text{ (treated samples)} - \Delta C_t \text{ (untreated control)}]}$, where $\Delta C_t = C_t\,(\text{hTERT}) - C_t\,(\text{GAPDH})$.

DNMTs, HDACs, and HATs activity assays

Cells were harvested at indicated time points, and nuclear extracts were prepared by using the nuclear extraction reagent (Pierce). The activities of DNMTs (Epigentek), HDACs (Active Motif), and HATs (Epigentek) were carried out by using the colorimetric kit according to the manufacturer's instruction as described previously (27). The enzymatic activities of DNMTs, HDACs, and HATs were detected by a microplate reader at 450 nm.

Bisulfite sequencing analysis

To assess the methylation status of the *hTERT* promoter, sodium bisulfite methylation sequencing was carried out by using the EpiTect-Bisulfite modification Kit following the manufacture's protocol (Qiagen). Approximately 2 μg of genomic DNA was used for bisulfite modification and then amplified by PCR by using Go Taq mix (Promega). Primers and PCR conditions were followed as described previously (31). PCR amplified DNA was purified by using the QIAquick PCR purification Kit (Qiagen) and sequenced by using the 3730 DNA Sequencer (Applied Biosystems). Percent methylation was calculated by using the following formula: (Number of methylated CpG × 100)/total number of CpG being assessed.

Chromatin immunoprecipitation analysis

Chromatin immunprecipitation (ChIP) analysis was conducted by using the EZ-ChIP Kit according to the manufacturer's instructions (Upstate Biotechnology) as described previously (27). The antibodies used in the ChIP assays were ChIP-validated acetyl-histone H3, acetyl-histone H3K9, acetyl-histone H4, dimethyl-histone H3K4, MAD1, c-MYC, and E2F-1 (Upstate Biotechnology). No antibody control was used to check ChIP efficiency. ChIP-purified DNA was quantified by using qPCR by using the Platinum SYBR Green detection system (Invitrogen) as described earlier (27). The primers for the *hTERT* promoter were as follows: forward, 5′-TCCCCTTCACGTCCGGCATT-3′; reverse, 5′-AGCGGAGAGAGGTCGAATCG-3′. For MAD1, c-MYC, and E2F-1, ChIP-purified DNA was amplified by using the following reverse transcriptase PCR (RT)-PCR primers: forward, 5′-CTCCGTCCTCCCCTTCAC-3′; reverse, 5′-CAGCGCTGCCTGAAACTC-3′, with a total of 30 cycles at 94°C for 15 seconds, 52°C for 30 seconds, and 72°C for 2 minutes. After amplification, PCR products were separated on 2% agarose gels and visualized by ethidium bromide staining by using Kodak 1D 3.6.1 image software and quantified. Quantitative data were analyzed by optical densitometry by using ImageJ Software version 1.36b (http://rsb.info.nih.gov/ij/).

Western blot analysis

For western blot analysis, protein extracts were prepared by using the radioimmunoprecipitation assay-lysis buffer (Upstate Biotechnology) following the manufacturer's protocol. For immunoblot analysis, 60 μg of protein was resolved on a 10% SDS-PAGE and transferred onto nitrocellulose membrane. After incubation in blocking buffer for 1 hour, the membranes were incubated with the primary antibodies specific for E2F-1 and MAD1 (Santa Cruz Biotechnology) and β-actin (Cell Signaling). The blot was then washed with TBS and 0.05% (v/v) Tween-20 and incubated with specific secondary antibody conjugated with horseradish peroxidase. Protein bands were then visualized by using the ECL detection system following the protocol of the manufacturer. The bands were analyzed by using Kodak 1D 3.6.1 image software.

siRNA knockdown of E2F-1 and MAD1

Approximately 2×10^5 cells were grown in 100 mm cell culture plates and allowed to incubate overnight. The E2F-1 and MAD1 siRNA (Santa Cruz Biotechnology) were prepared as 10 μmol/L stocks by using nuclease-free water. E2F-1 (6 nmol/L) and MAD1 (4 nmol/L) siRNA was delivered to the cells by using the Silencer siRNA Transfection Kit (Ambion/Applied Biosystems) according to the manufacturer's instructions. siCONTROL Non-Targeting siRNA (Santa Cruz Biotechnology) was used as a negative control. Cells were harvested and checked for E2F-1 and MAD1 knockdown after 6- and 9-day interval by using Western blot analysis. Pro-EGCG (20 μmol/L)-treated and nontreated cells were used to harvest RNA for PCR reactions by using total RNA extraction and real-time PCR procedures described in previous sections.

Apoptosis assay in siRNA knockdown cells

Breast cancer cells transfected with E2F-1, MAD1, and control siRNA as well as nontransfected cells were treated with 20 μmol/L pEGCG for 9 days. The cells were then lysed with nuclei lysis buffer and assayed for apoptosis by using the Cell Death Detection ELISA Kit (Roche) as described previously (27). Percent apoptosis was calculated by using the formula (100 × treatment cell absorbance/control cell absorbance) −100.

Statistical analysis

The statistical significance of differences between the values of treated samples and controls were determined with Kruskal–Wallis with Dunn's post test by using GraphPad Prism version 4.00 for Windows, GraphPad Software (www.graphpad.com). In each case, $P < 0.05$ was considered statistically significant.

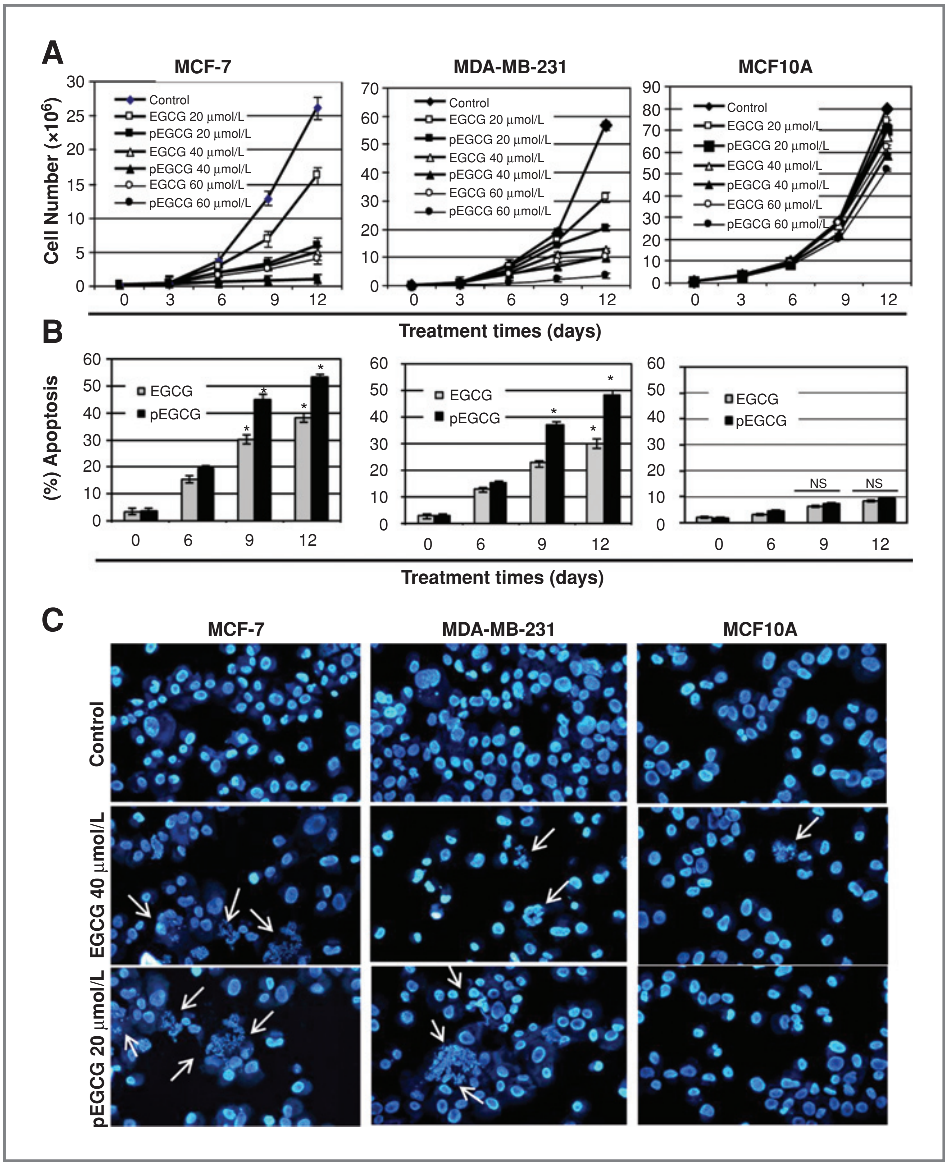

Figure 1. EGCG and pEGCG inhibit proliferation of breast cancer cells but have negligible effects on control MCF10A cells. A, breast cancer MCF-7 (left) and MDA-MB-231 (middle) cells as well as control MCF10A cells (right) were treated with EGCG and pEGCG (0, 20, 40, and 60 μmol/L) for 3, 6, 9, and 12 days. Growth curve kinetics was obtained by counting the total number of viable cells at the indicated time intervals by using trypan blue staining. Results were obtained from 3 independent experiments, mean ± SD. B, treatment with EGCG (40 μmol/L) and pEGCG (20 μmol/L) for 6, 9, and 12 days induced cellular apoptosis of human breast cancer MCF-7 (left) and MDA-MB-231 (middle) cells in a time-dependent manner. Control MCF10A cells (right) did not show a significant level of apoptosis at the same dosages and times of treatment. Percent apoptosis was assayed by using the Annexin V-Alexa Fluor 488 Apoptosis Vybrant Assay Kit. The experiment was repeated 2 times, and each point indicates the mean ± SD. Statistical significance, * $P < 0.05$. NS, not significant. C, treatment with EGCG (40 μmol/L) and pEGCG (20 μmol/L) for 9 days induced the cellular apoptosis in MCF-7 (left) and MDA-MB-231 (middle) cells but not in control MCF10A (right) cells. Apoptotic cells were detected by immunofluorescence staining under a fluorescence microscope and are shown as multinucleated cells (white arrows indicated). Representative photographs are shown from 3 repeated experiments.

Results

pEGCG is more potent than EGCG in inducing apoptosis and inhibiting cellular proliferation of human breast cancer cells

As shown in Figure 1, human breast cancer MCF-7 (left) and MDA-MB-231 (middle) cells as well as normal control human breast MCF10A (right) cells were treated with the indicated concentrations of EGCG and pEGCG for 3, 6, 9, and 12 days for cell growth assay. We observed a dose- and time-dependent cell growth inhibition with EGCG and pEGCG treatment both in MCF-7 and MDA-MB-231 cells (Fig. 1A). Doses of up to 60 μmol/L of EGCG and 40 μmol/L of pEGCG had negligible cell proliferation inhibition activity in control MCF10A cells whereas these same doses inhibited cellular proliferations for MCF-7 and MDA-MB-231 cells. In addition, significant levels of apoptosis were observed at 9 and 12 days with 20 μmol/L pEGCG treatments for both MCF-7 and MDA-MB-231 cells (Fig. 1B). However, EGCG required a higher dose (40 μmol/L) than pEGCG to induce a significant level of apoptosis at the same time intervals in both human breast cancer cells. Both EGCG (40 μmol/L) and pEGCG (20 μmol/L) were found to induce significant apoptosis in both MCF-7 and MDA-MB-231 cells, whereas the equivalent doses were found to have very negligible cellular apoptotic effects on normal MCF10A breast cells (Fig. 1B). Furthermore, Hoechast staining analysis clearly showed that treatment with EGCG (40 μmol/L) and pEGCG (20 μmol/L) induced more apoptotic cells in breast cancer cells, whereas negligible apoptotic cells were found in normal MCF10A cells (Fig. 1C). Collectively, these results

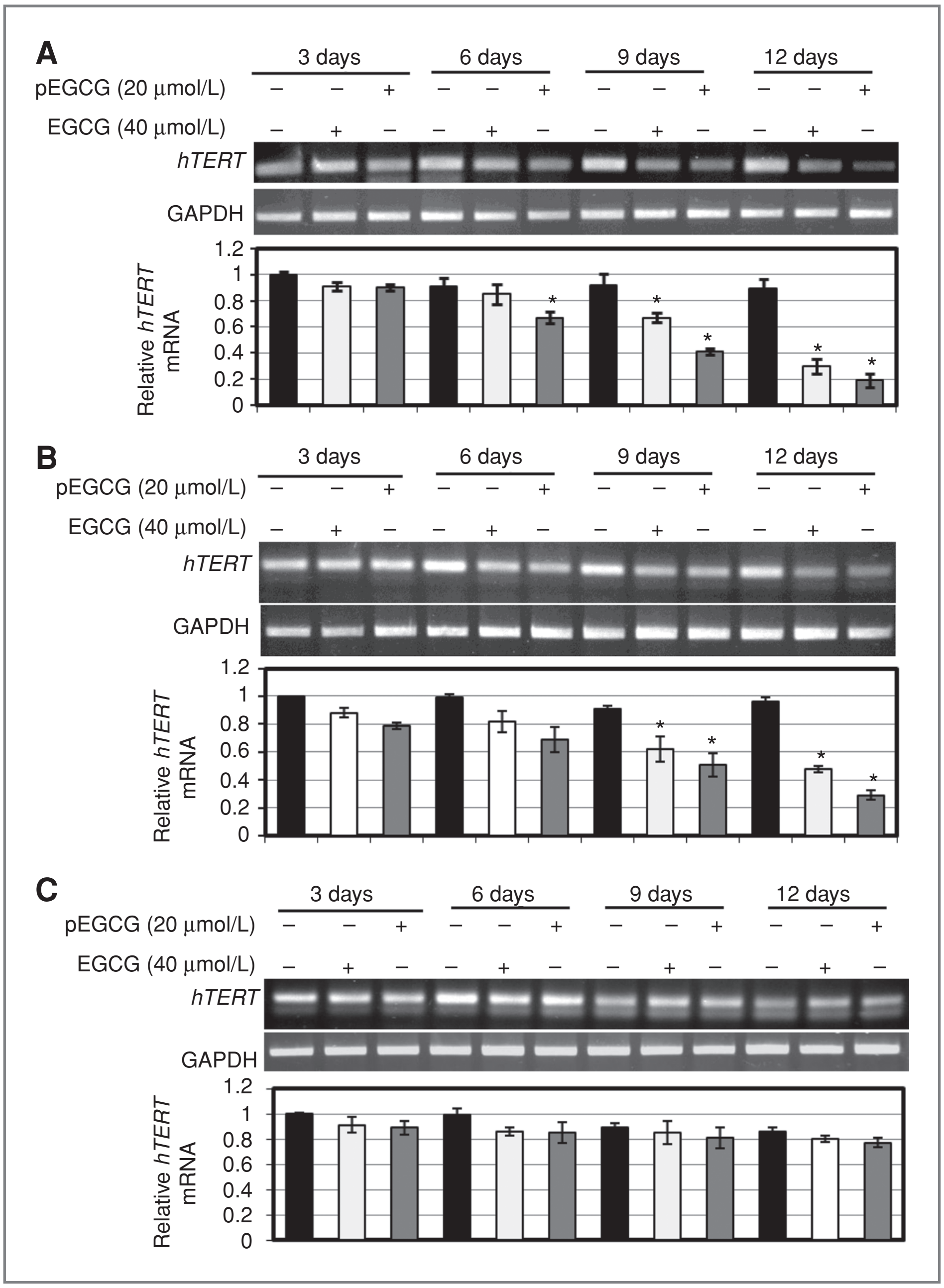

Figure 2. EGCG and pEGCG inhibits *hTERT* in human breast cancer cells. Both EGCG (40 μmol/L) and pEGCG (20 μmol/L) inhibit *hTERT* mRNA expression in MCF-7 (A) and MDA-MB-231 (B) human breast cancer cells, but negligible *hTERT* inhibition effects were found in control MCF10A (C) cells. The indicated breast cell types were treated with EGCG or pEGCG for 3, 6, 9, and 12 days. After treatment periods, relative mRNA levels of *hTERT* in each sample were assessed by using conventional gel-based PCR and quantified by using real-time PCR. Data are in triplicates from 3 independent experiments and were normalized to *GAPDH*. The values were plotted against respective controls as relative fold of induction ± SD. Significance against respective nontreated control, * $P < 0.05$.

indicate that 40 μmol/L of EGCG and 20 μmol/L of pEGCG, selectively inhibits breast cancer cells. Therefore, we chose 40 μmol/L EGCG and 20 μmol/L of pEGCG for further experiments. We used lower doses of EGCG and pEGCG than those previously reported 60 μmol/L IC_{50} values of EGCG and 50 μmol/L dosage of pEGCG (32, 24, 25), so that we could study the EGCG-induced epigenetic modifications on *hTERT* regulations without sudden cellular death. In addition, these lower doses should have higher translational potential in cancer chemoprevention as well as drug development and therapy.

EGCG and pEGCG inhibits *hTERT* expression in breast cancer cells

More than 90% of the cancer cells express higher levels of *hTERT*, the key catalytic subunit of telomerase, which serves as an important target for cancer chemoprevention (11–13, 33). Therefore, we investigated the effect of EGCG and pEGCG on *hTERT* expression in MCF-7 (Fig. 2A) and MDA-MB-231 (Fig. 2B) human breast cancer cells as well as normal breast MCF10A cells (Fig. 2C) by using conventional RT-PCR and real-time PCR. As shown in Figure 2, treatment with EGCG

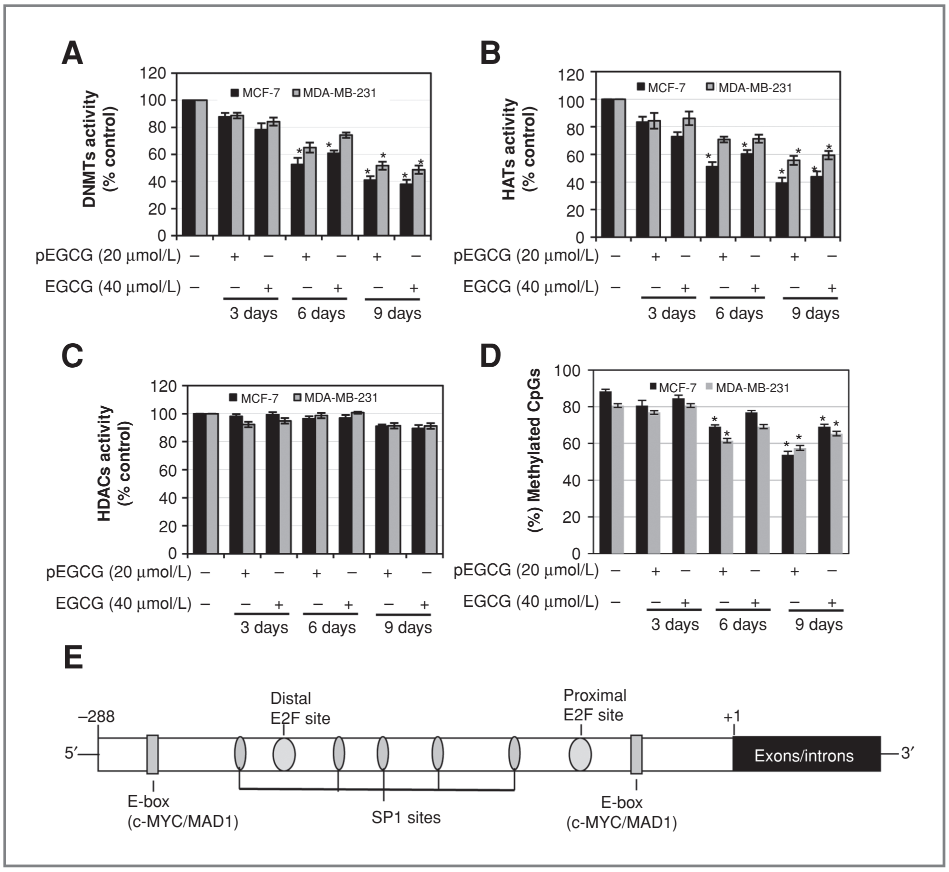

Figure 3. EGCG and pEGCG altered epigenetic-modulating enzymes activity and *hTERT* promoter methylation in breast cancer cells. A, EGCG (40 µmol/L) and pEGCG (20 µmol/L) inhibits DNMTs (A) and HATs (B) activity, but no significant effects on HDACs (C) activity were found in human breast cancer cells. MCF-7 and MDA-MB-231 cells were treated with the indicated concentration of EGCG and pEGCG for 3, 6, and 9 days. DNMTs, HATs, and HDACs activities were assayed and compared with mean percent control obtained from respective time intervals. Control values did not change considerably over the treatment times. Values are representative of 3 independent experiments and are represented as percent control ± SD; statistical significance, * $P < 0.05$. D, EGCG (40 µmol/L) and pEGCG (20 µmol/L) induced *hTERT* promoter DNA hypomethylation in human breast cancer cells as assayed by bisulfite sequencing. Breast cancer MCF-7 and MDA-MB-231 cells were treated with the indicated concentration of EGCG and pEGCG for 3, 6, and 9 days. Percent methylation was obtained by dividing the number of methylated CpGs by the total number of CpGs ($n = 26$) in the indicated *hTERT* promoter region assessed. Values are representative of 3 independent experiments and are represented as percent control ± SD; statistical significance, * $P < 0.05$. E, the *hTERT* transcription factors such as E2F-1, Sp1, and c-MYC/MAD1 (E-box-binding sites) are shown in the *hTERT* promoter.

(40 µmol/L) and pEGCG (20 µmol/L) time-dependently inhibited *hTERT* expression in both types of human breast cancer cells, although very negligible *hTERT* inhibitory activity was found in normal MCF10A cells. This is consistent with previous findings that inhibition of *hTERT* by chemopreventive compounds in cancer cells but not in normal cells is one of the important contributing factors in cancer chemoprevention (20, 27, 33). Furthermore, 20 µmol/L of pEGCG inhibited more *hTERT* expression than 40 µmol/L of EGCG at similar time points although both compounds inhibited significant levels of *hTERT* at 9 and 12 days of treatments. These results indicate that pEGCG is more potent than EGCG and both compounds act on *hTERT* leading to its downregulation specifically in breast cancer cells, which may play a critical role in inhibition of cancer cell proliferation and survival.

EGCG and pEGCG induced *hTERT* hypomethylation and altered epigenetic-modulating enzyme activities

Because *hTERT* is one of the most epigenetically regulated genes (11–13, 17), we assessed epigenetic-modulating enzymatic activity of the DNMTs (Fig. 3A), HATs (Fig. 3B), and HDACs (Fig. 3C) in MCF-7 and MDA-MB-231 breast cancer cells, by using EGCG or pEGCG treatments. Interestingly, both EGCG and pEGCG at the indicated concentrations significantly inhibited DNMTs and HATs activities at 6 and 9 days of treatment in human breast cancer cells. However, we did not find any significant alteration with HDACs activity in these breast cancer cells with EGCG- and pEGCG-treatments. EGCG inhibition of DNMTs activity might be because of the direct binding of EGCG to the active site of the DNMTs as reported previously (15). Furthermore, EGCG was also reported to have inhibitory activity of the HATs in HeLa nuclear extracts

(16). Because the *hTERT* promoter is hypermethylated in most cancer cells for its transcriptional activation, we assessed the methylation status of the *hTERT* promoter region from −288 to −31 covering 26 CpG dinucleotides and various overlapping transcription factor binding sites (Fig. 3E). We used bisulfite sequencing to detect the *hTERT* methylation patterns of EGCG- and pEGCG-treated human breast cancer cells. As shown in Figure 3D, control untreated MCF-7 and MDA-MB-231 breast cancer cells maintain a high level of methylation at promoter sites at 87.6% ± 3.24% and 79.4% ± 2.29%, respectively, whereas treatment with EGCG and pEGCG considerably reduced promoter methylation in a time-dependent manner. The EGCG- and pEGCG-mediated inhibition of DNMTs expression could be an important contributing factor in facilitating demethylation of *hTERT* promoter, which leads to transcriptional repression of *hTERT* expression (11–13).

EGCG and pEGCG induced chromatin modifications and binding of transcriptional repressors of the *hTERT* promoter

Previous studies have shown that *hTERT* expression is often modulated by epigenetic processes such as DNA methylations and histone acetylations (11–13, 17). We observed that EGCG and pEGCG time-dependently inhibited HATs activity without altering HDACs activity in human breast cancer cells (Fig. 3). Decreased HATs activity is often associated with histone hypoacetylation at the *hTERT* promoter, which is associated with transcriptional repression of *hTERT* expression (12, 22). Therefore, we sought to determine changes in histone modifications of the *hTERT* regulatory region by EGCG and pEGCG treatment in MCF-7 and MDA-MB-231 cells. EGCG and pEGCG treatments resulted in a time-dependent decrease in the acetylation of transcriptionally active chromatin markers; acetylated histone H3 (ac-H3), H3 at lysine 9 (ac-H3K9), and ac-H4 in both MCF-7 and MDA-MB-231 cells (Fig. 4A–C). We also found a decrease in the methylation status of active histone markers such as dimethyl-H3 lysine 4 in MCF-7 and MDA-MB-231 cells with EGCG and pEGCG treatments (Fig. 4D). These changes of histone acetylation and deacetylation allow transcriptional factors binding into the *hTERT* regulatory region by maintaining a repressive environment (16–18, 22). Active and inactive chromatin modulations can control the antagonistic binding of MAD1 and c-MYC to the two E-boxes of the *hTERT* promoter, which are the major repressors and activators, respectively, of *hTERT* (11, 12, 19). Indeed, we found that the MAD1 repressor of *hTERT* is increased in its binding in response to EGCG and pEGCG whereas the c-MYC activator is decreased in its binding to the *hTERT* promoter in MCF-7 and MDA-MB-231 (Fig. 5). Furthermore, EGCG- and pEGCG-induced promoter hypomethylation led to the binding of the methylation-sensitive *hTERT* repressor, *E2F-1*, to the *hTERT* promoter. Collectively, these results suggest that the EGCG- and pEGCG-induced chromatin modifications affect binding of key transcriptional repressors to the *hTERT* promoter and inhibition of DNMTs

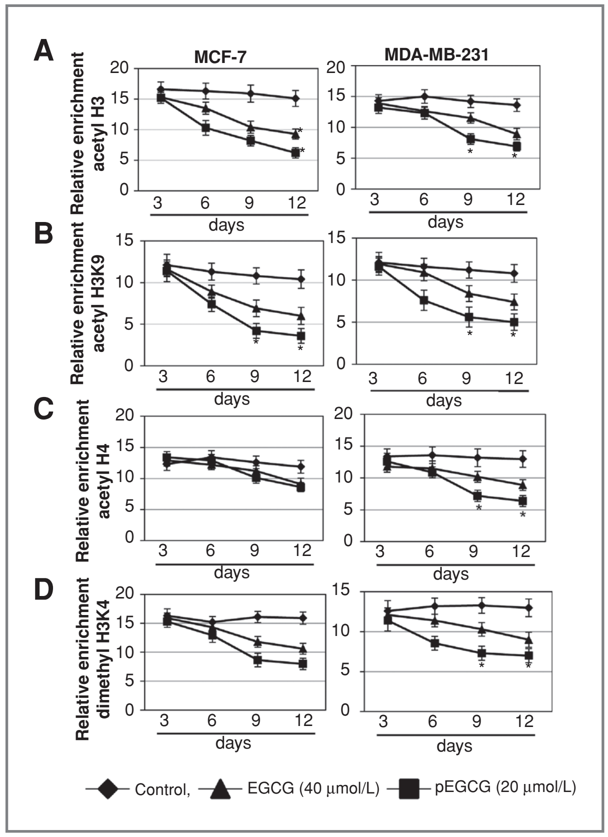

Figure 4. EGCG and pEGCG induced histone modification changes of the *hTERT* promoter in breast cancer cells. A, breast cancer MCF-7 (left) and MDA-MB-231 (right) cells were treated with EGCG (40 μmol/L) and pEGCG (20 μmol/L) for 3, 6, 9, and 12 days and analyzed by ChIP-qPCR assays by using chromatin markers including acetyl-H3 (A) acetyl-H3K9 (B), acetyl-H4 (C), and dimethyl-H3K4 (D) in the promoter region of *hTERT*. No antibody controls were assessed to verify the ChIP efficiency. qPCR primers and conditions were used as described in Materials and Methods. The *x*-axis represents the different treatment time in days, and the y-axis represents the relative enrichment of individual binding factors [the percentage of immunoprecipitates compared with the corresponding input samples (defined as 100)]. The experiment was repeated 3 times with triplicates in real-time PCR, and each point indicates the mean ± SD; statistical significance, * $P < 0.05$.

mediated CpG hypomethylation at the *hTERT* regulatory region contributed to *hTERT* downregulation in both MCF-7 and MDA-MB-231 breast cancer cells.

E2F-1 and MAD1 knockdown differentially regulates pEGCG-inhibited *hTERT* expression in ER (+) and ER (−) human breast cancer cells

We found that EGCG and pEGCG induced transcriptional repression of *hTERT* expression, at least partially, by altering the binding of repressor proteins such as MAD1 and E2F-1 to the *hTERT* promoter (Fig. 5). Therefore, we transiently transfected E2F-1 and MAD1 siRNA into the MCF-7 and MDA-MB-231 cells. Transfection of E2F-1 and MAD1 siRNA for 9 days considerably knocked down their expressions in both MCF-7 (Fig. 6A; left) and MDA-MB-231

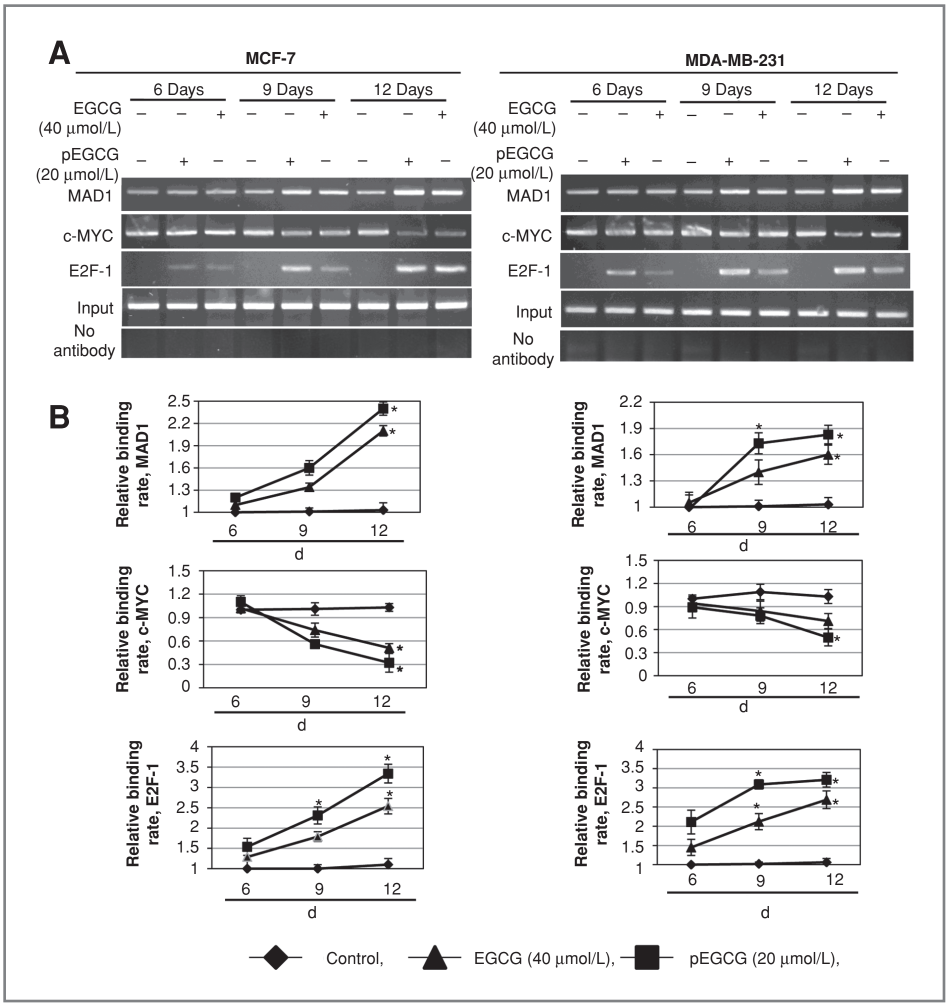

Figure 5. EGCG and pEGCG altered binding of transcriptional factors to the *hTERT* promoter in breast cancer cells. A, breast cancer MCF-7 (left) and MDA-MB-231 (right) cells were treated with EGCG (40 μmol/L) and pEGCG (20 μmol/L) for 6, 9, and 12 days, and ChIP assayed by using transcriptional factors such as c-MYC, MAD1, and E2F-1 in the promoter region of *hTERT*. No antibody controls were assessed to verify the ChIP efficiency. PCR primers and conditions were used as described in Materials and Methods. Photographs are representative of an experiment that was repeated in triplicates. B, ChIP data were calculated from the corresponding DNA fragments amplified by PCR by using Kodak 1D 3.6.1 image software; columns, mean; bars, SD; statistical significance, * $P < 0.05$. The relative binding ratio was calculated as the ratio between the net intensity of each bound sample divided by the input and the untreated control sample divided by the input (bound/input)/(control/input).

(Fig. 6B; right) cells without inducing any significant level of cellular toxicity (data not shown). Treatment with pEGCG (20 μmol/L) of ER (+) MCF-7 (Fig. 6C) and ER (−) MDA-MB-231 (Fig. 6D) breast cancer cells significantly inhibited *hTERT* expression and induced cellular apoptosis as observed in Figure 2A and B, respectively. Surprisingly, pEGCG inhibition of *hTERT* expression and its associated enhanced cellular apoptosis were significantly restored in E2F-1 knockdown MCF-7 cells but not in MDA-MB-231 cells. In contrast, pEGCG inhibition of *hTERT* expression was significantly restored by MAD1 knockdown-MDA-MB-231 cells but not in MCF-7 cells. Collectively, our data revealed for the first time that pEGCG inhibits *hTERT* expression and induced cellular apoptosis at least partially through the binding of E2F-1 and MAD1 to the *hTERT* promoter for ER (+) and ER (−) human breast cancer cells, respectively. Studies have indicated that ERα directly regulates E2F-1 expression, and knockdown of E2F-1 blocks estrogen regulation in E2F-1 target genes (34, 35). Therefore, the observed *hTERT* restoration by E2F-1 knockdown in MCF-7 cells but not in MDA-MB-231 cells is likely, at least in part, due to the ERα expression. However, in ER (−) breast cancer cells, pEGCG-induced binding of MAD1 plays an important role in *hTERT* regulation apparently because of the lack of ERα expression (Fig. 6D). Collectively, our results indicate that knockdown of *hTERT* repressors can reverse the inhibitory effect of pEGCG on *hTERT* expression and, perhaps most importantly, reverses the antiapoptotic effects of pEGCG. Therefore, these new findings suggest that the impedance of repressor binding to the *hTERT* promoter because of their epigenetic effects leads to cancer cell apoptotic properties by pEGCG.

Discussion

In accordance with previous findings, EGCG dose- and time-dependently inhibited the growth of both ER (+) and ER (−) human breast cancer cells (1, 4, 20, 24). pEGCG was found to be more potent than EGCG in inhibiting cellular proliferations and inducing cellular apoptosis in both ER (+) and ER (−) human breast cancer cells. Studies have clearly shown that pEGCG has increased stability compared with EGCG and is converted into parental EGCG in cultures with approximately 2.4-fold greater recovery in

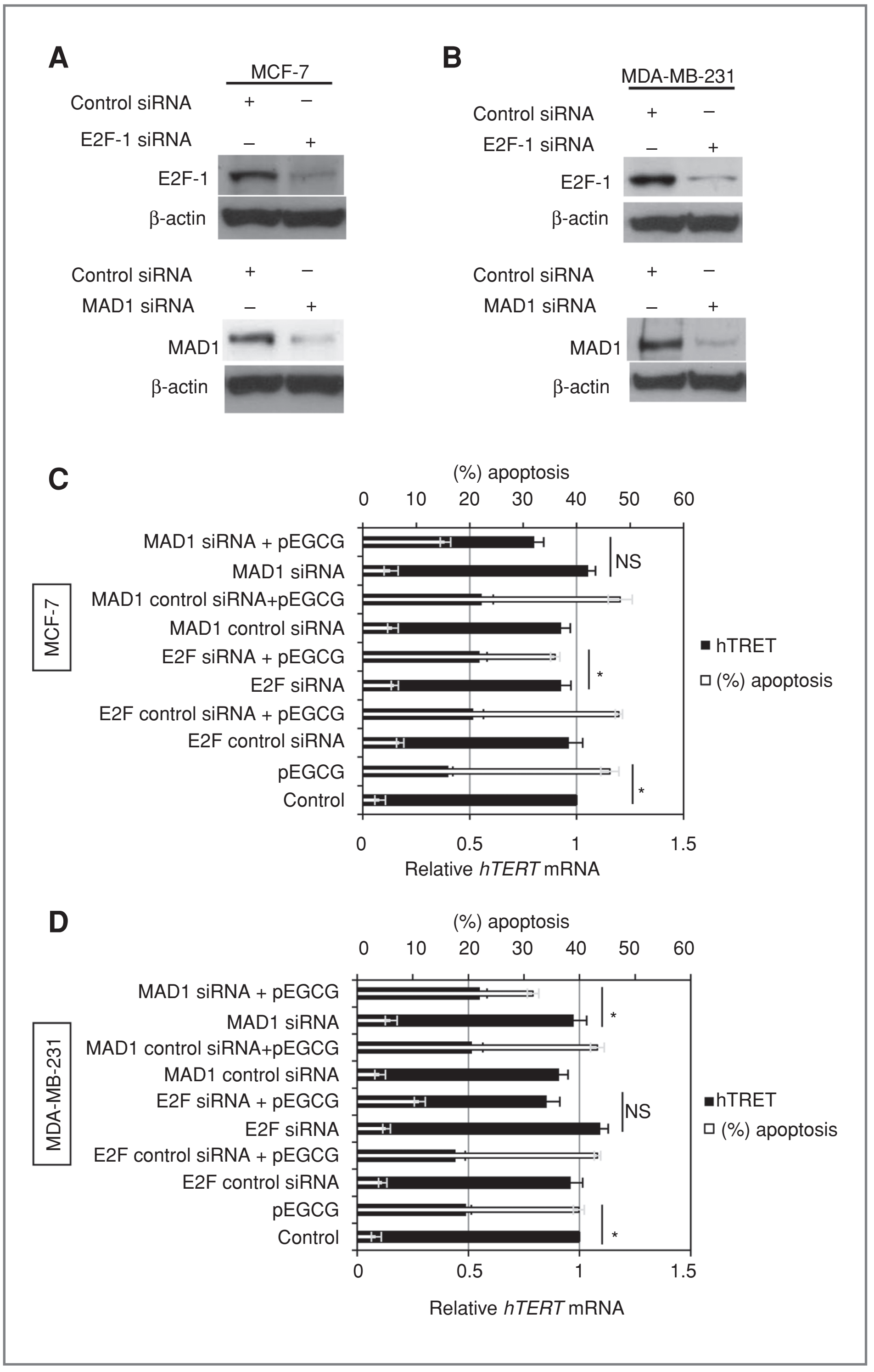

Figure 6. E2F-1 and MAD1 knockdown differentially regulate pEGCG-inhibited *hTERT* expression in ER (+) and ER (–) breast cancer cells. Breast cancer ER (+) MCF-7 (A) and ER (–) MDA-MB-231 (B) cells were subjected to treatments with 6 and 4 nmol/L of E2F-1 and MAD1 siRNA, respectively, or control siRNA fragments. Effects of siRNA interference with E2F-1 and MAD1 gene expression were assayed by Western blot analysis after 9 days by using specific antibodies (A). Data shown are representative of the 3 separate experiments. E2F-1 and MAD1 siRNA-transfected cells were treated with 20 μmol/L pEGCG for 9 days and analyzed for *hTERT* mRNA expression by RT-PCR as well as apoptosis by ELISA in MCF-7 (C) and MDA-MB-231 (D) breast cancer cells. Data are in triplicates from 2 independent experiments and were normalized to GAPDH for calculating relative *hTERT* mRNA. Statistical significance, * $P < 0.05$. NS, not significant.

cells than EGCG after 72-hour treatment (24, 26). Further pEGCG treatment increased the bioavailability of EGCG in breast, esophageal, and colon cancer cells (24, 26), which further substantiates our present findings.

We have shown that both EGCG and pEGCG time-dependently inhibited *hTERT* expression in both ER (+) and ER (−) human breast cancer cells but not in normal cells. This is in accordance with previous findings that EGCG inhibited telomerase through epigenetic modifications and induced cellular apoptosis in lung and ER (+) breast cancer cells (2, 4, 20). However, studies have been largely obscure on *hTERT* regulations in ER (−) human breast cancer cells. Interestingly, we found that EGCG and pEGCG inhibited *hTERT* expression similarly in both ER (+) and ER (−) breast cancer cells, although *hTERT* is a one of the targets for ligand-activated ER, and the presence of ER is a contributing factor, at least partially, for *hTERT* activation (36, 37). Therefore, it is likely that *hTERT* regulation in ER (+) and ER (−) breast cancer cells might act through different mechanisms. Furthermore, epigenetic regulation of *hTERT* is actively involved in cellular proliferation and apoptosis in various cancer cells. In most of the cancer cells the *hTERT* regulatory region is hypermethylated, which is associated with increased *hTERT* expression, whereas demethylation of this region inhibits *hTERT* transcription (18, 38). This phenomenon is opposite to the general model of gene activation, in which the presence of methylated cytosines in a promoter typically inhibits gene transcription (10, 39, 40).

Previously, we have shown that genistein and EGCG result in downregulation of the DNMTs, which is directly associated with repression of *hTERT* expression through *hTERT* promoter demethylation in breast cancer cells (20, 31). The EGCG-mediated DNMTs inhibition might be because of the possible direct interaction of EGCG with the DNMTs active site (15). Numerous studies have also reported that DNA methylation plays important roles in *hTERT* transcriptional regulation (10–12, 31). Together, our results suggest that EGCG-induced downregulation of DNMTs expression is not only involved in the demethylation processes of the *hTERT* control region in the process of anticarcinogenesis, but also enhances binding of methylation-sensitive transcription factors such as *E2F-1* to the *hTERT* regulatory region (23). Furthermore, our ChIP-analysis confirmed that EGCG- and pEGCG-induced demethylation at the CpG dinucleotides of the *hTERT* promoter resulted in an increased binding of E2F-1 to the *hTERT* proximal promoter which leads to repression of *hTERT* transcription.

In general, chromatin acetylation and deacetylation are catalyzed by HATs and HDACs, respectively, which play an important role in transcriptional regulations of *hTERT* expression (10, 16). EGCG was reported to have HATs inhibitory activity in HeLa nuclear extracts (16). Similarly, we found that EGCG treatment significantly inhibited HATs activities in human breast cancer cells; however, we did not find any significant alterations in HDACs activities. By contrast, sulforaphane (SFN), an isothiocyanate present in the cruciferous vegetables, also has *hTERT* inhibitory activity in human breast cancer cells, which specifically inhibits HDACs but not HATs activities (27). Unlike with the use of SFN, we found an EGCG-induced time-dependent decrease of transcriptional active chromatin markers such as ac-H3, ac-H3K9, and ac-H4 in human breast cancer cells. Chromatin remodeling resulting from reversible acetylation of histones has been suggested to be a critical component of transcriptional regulation of *hTERT* expression (22). Histone acetylation- and decetylation-modulated chromatin structure can be accessed with a number of transcription factors, including c-MYC and *MAD1*, which often regulates gene expression by recruiting HATs and HDACs, respectively (22). Our results also suggest that EGCG-induced MAD1 binding might recruit RBP2, a histone demethylase, to the *hTERT* promoter and reduced *hTERT* mRNA expression is accompanied by H3K4-demethylation (19). In addition, *hTERT* expression in normal and malignant human cells was found to have an inverse correlation with MAD1 expression (20, 41, 42). The MAD1-induced repression of *hTERT* transcription is mediated by the N-terminal SID of Mad1 that recruits HDACs to chromatin (41). Furthermore, there is a switch from MYC/MAX to MAD1/MAX binding and a decrease in histone acetylation at the *hTERT* promoter during HL60 differentiation (22). Taken together, it is apparent that DNMTs-induced promoter demethylation and chromatin remodeling alter binding of hTERT transcriptional repressors to the *hTERT* promoter is closely linked to the control of *hTERT* expression by EGCG and pEGCG in human breast cancer cells.

Our functional studies with E2F-1 and MAD1 siRNA revealed for the first time that pEGCG-inhibited *hTERT* expression and associated apoptosis is differently regulated in ER (+) MCF-7 and ER (−) MDA-MB-231 breast cancer cells. Previous studies have shown that EGCG can induce cellular apoptosis and can inhibit cellular proliferations in both ER (+) and ER (−) human breast cancer cells by similar mechanisms (1, 4). In addition, the present study also confirmed the fact that EGCG and pEGCG induced cellular apoptosis in both ER (+) and ER (−) human breast cancer cells but regulates *hTERT* through different transcriptional repressors. Our siRNA study revealed that *E2F-1* is an important transcriptional repressor required for ER (+) cancer cells. However, in ER (−) breast cancer cells, MAD1 seems to play an important role in *hTERT* repression and associated apoptosis. This might be partially due to the fact that estrogen stimulates ER-c-Myc protein interactions and lack of ER induces MAD1, c-MYC antagonist, for the target gene suppression (43).

In the present study, we not only used pEGCG to enhance the bioavailability and stability of EGCG but also explored the possible epigenetic mechanisms involved in *hTERT* repression. It is important to point out that *hTERT* gene control is unique, and the proposed mode of action is not the only way EGCG inhibits cancer cell growth. The optimal concentrations of EGCG and pEGCG used in this study are lower than the many other studies have used previously (1, 4, 15, 20). Furthermore, the concentrations we used selectively inhibited cellular proliferation and induced apoptosis in human breast cancer cells but not in control MCF10A cells as shown in Figure 1. Studies have shown that EGCG and pEGCG up to 50 mg/kg/d were well tolerated in experimental animals without noticeable toxicity (24, 44). Another important finding is that, for the first time, we showed that pEGCG treatment-induced chromatin changes resulted in a differentially regulated transcriptional repressor binding to the *hTERT* promoter in ER (+) and ER (−) human breast cancer cells. These findings have important implications for the application of EGCG in cancer prevention and drug development for ER (−) human breast tumors. However, further studies with spontaneous multistage breast tumor producing mouse models such as C3(1)/SV40 and Her2/neu are necessary to study the *in vivo* effect of EGCG and pEGCG during different stages of breast cancer progression. These *in vivo* mouse models will not only produce breast tumors which closely resemble the development, progression, and morphology of human breast tumors (45, 46) but also allow studying long-term bioavailability of EGCG, which is best suited for cancer chemoprevention models.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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