

Methylation-Mediated Repression of *GADD45α* in Prostate Cancer and Its Role as a Potential Therapeutic Target

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Abstract

Defects in apoptotic pathway contribute to uncontrolled proliferation of cancer cells and confer resistance to chemotherapy. Growth arrest and DNA damage inducible, alpha (*GADD45α*) is up-regulated on docetaxel treatment and may contribute to docetaxel-mediated cytotoxicity. We examined the mechanism of regulation of *GADD45α* in prostate cancer cells and the effect of its up-regulation on sensitivity to docetaxel chemotherapy. Expression of *GADD45α* in PC3 cells was higher than that in Du145 and LNCaP cells (17- and 12-fold, respectively; $P < 0.05$). Although the proximal promoter region was unmethylated in all three cell lines, methylation of a 4 CpG region upstream of the proximal promoter correlated inversely with gene expression levels. Methylation was reversed by treatment of Du145 and LNCaP cells with DNA methyltransferase inhibitors, leading to reactivation of *GADD45α* expression in these cells. The 5' 4 CpG region was also frequently methylated in prostate cancer tissues. Methylation of this region correlated inversely with gene expression in prostate cancer and benign prostate tissues. The methyl binding protein MeCP2 was associated with the methylated 4 CpGs in Du145 cells, and knockdown of MeCP2 in these cells (Du145 MeCP2⁻) led to a significantly increased expression of *GADD45α* (3-fold; $P = 0.035$) without affecting the methylation status of the gene. Enhanced sensitivity to docetaxel was observed by up-regulation of *GADD45α* in Du145 cells by recombinant expression of *GADD45α* or pretreatment with 5-azacytidine. Our results show that *GADD45α* is epigenetically repressed and is a potential target for treatment of prostate cancer. [Cancer Res 2009;69(4):1527–35]

Introduction

Apoptosis or programmed cell death is a crucial mechanism for maintenance of cell homeostasis. During carcinogenesis, there is increased cell proliferation and development of resistance to cytotoxic chemotherapeutic agents (1). The molecular mechanisms of this phenomenon are yet to be completely elucidated. It is believed that defects in apoptotic pathways play a key role in tumorigenesis. Genes involved in cell cycle control, apoptosis signaling, and DNA repair become methylated and epigenetically inactivated in most cancers (1–4).

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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Recently, there have been reports of methylation-mediated deregulation of proapoptotic genes, which could provide a mechanism through which tumor cells avoid apoptosis (5, 6). Growth arrest and DNA damage inducible, alpha (*GADD45α*) plays an important role in cellular response to DNA damage because it is involved in DNA repair, maintenance of genomic stability, cell cycle control, and apoptosis (7). *GADD45α* blocks G₂-M transition thereby causing cell cycle arrest in response to DNA-damaging agents such as UV radiation, ionizing radiation, and methylmethane sulfonate (8). The role of *GADD45α* in G₂-M arrest is shown by its ability to interact with Cdc2 kinase resulting in inhibition of Cdc2/cyclin B1 complex formation, which is required for G₂-M transition during cell cycle progression (9). Inhibition of growth by *GADD45α* occurs both in cell lines with functional p53 and those with a negative p53 status (7). *GADD45α* expression is critical for c-jun NH₂-terminal kinase activation and apoptosis in tumor cells (10–12). Recombinant *GADD45α* expression in tumor cell lines by transfection reduces cell proliferation (13). Treatment of cells with genotoxic agents up-regulates *GADD45α* expression resulting in induction of apoptosis (14).

Previous studies showed that *GADD45α* was down-regulated in prostate cancer compared with benign prostate tissues. Down-regulation of gene expression was not associated with hypermethylation of the proximal promoter region (15). In this study, we show that transcriptional repression of *GADD45α* in Du145 prostate cancer cells occurs by an atypical methylation of 4 CpGs located upstream of the proximal promoter region. Methylation of the 5' 4 CpG region is a frequent event in prostate cancer and correlates inversely with gene expression in prostate cancer and benign prostate tissues. Further, down-regulation of *GADD45α* in Du145 involves interaction of MeCP2 with *GADD45α* promoter. Induction of *GADD45α* by treatment of Du145 cells with DNA methyltransferase (DNMT) inhibitor increases sensitivity to docetaxel chemotherapy, thus identifying the role of *GADD45α* as a potential target gene in prostate cancer.

Materials and Methods

Cell culture. Du145, LNCaP, and PC3 prostate cancer cells were routinely cultured in RPMI 1640 (Mediatech) supplemented with 10% fetal bovine serum (Invitrogen), 2 mmol/L glutamine (Invitrogen), and 100 μg/mL penicillin-streptomycin (Invitrogen).

Tissue specimens. Tissue specimens used in this study were obtained from prostate cancer cases and benign prostatic hyperplasia (BPH) controls as previously described (15, 16).

Drug treatment. 5-Azacytidine was provided by Pharmion Corporation and 5-azadeoxycytidine was purchased from Sigma-Aldrich. Du145 cells were treated with varying concentrations of 5-azacytidine or 5-azadeoxycytidine for 72 h, after which RNA/DNA/protein were extracted. For determining the sensitivity to docetaxel, Du145 cells were seeded for 24 h,

after which they were treated with 5-azacytidine for 72 h followed by 5 nmol/L docetaxel for 72 h.

Plasmids and transfection. pSuppressor/MeCP2 siRNA, SureSilencing MBD2 shRNA, and pCMV/*GADD45α* plasmids were obtained from Imgenex, SuperArray Bioscience Corporation, and Origene Technologies, Inc., respectively. Transfection was carried out using Amaxa Nucleofection system (Amaxa, Inc.). To generate stable clones of Du145 and LNCaP MeCP2⁻ cells, transfected cells were selected with 350 μg/mL G418. Stable clones were then maintained in medium containing 350 μg/mL G418. Stable clones of Du145 and LNCaP MBD2⁻ cells were generated by transfection followed by selection in medium containing 5 and 2.5 μg/mL puromycin.

Quantitative reverse transcription-PCR. RNA extracted from untreated and drug treated cells using RNA Stat 60 (TelTest, Inc.) was reverse transcribed using Moloney murine leukemia virus reverse transcriptase (USB Corp.). Real-time PCR amplification was done in triplicates with cDNA using primers for *GADD45α*. The average C_t was then used to quantitate relative mRNA levels by the comparative C_t method. RNA from untreated cells was used as a calibrator. Both samples and calibrator were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The primer

sequences for *GADD45α* are 5'-CGCCTGTGAGTGAGTGC-3' (forward) and 5'-CTTATCCATCCTTTCGGTCTT-3' (reverse), and for GAPDH, 5'-GCTGAGTACGTCGTGGAGTC-3' (forward) and 5'-GGGGCAGAGATGATGACC-3' (reverse). The PCR reaction was carried out in a volume of 25 μL using iQ SYBR Green Supermix (Bio-Rad Laboratories) on MyiQ Single-Color Real-Time PCR Detection System (Bio-Rad Laboratories). Expression of *GADD45α* and of the reference gene *TBP* in tissues was done by real-time reverse transcription-PCR (RT-PCR) on an ABI 7900 instrument (Applied Biosystems) using assay systems supplied by the same company, as described (15).

Western blotting. Whole cell lysates were prepared using the M-PER Mammalian Protein Extraction Reagent (Pierce). Protein concentrations of the lysate were determined using bicinchoninic acid protein assay reagent (Pierce). For Western blot analysis, the proteins were resolved on a 4% to 20% SDS-PAGE gel and blotted onto a nitrocellulose membrane. The blot was then probed with rabbit anti-human *GADD45α* antibody (1:200 dilution; Santa Cruz Biotechnology), detected with antirabbit immunoglobulin-horseradish peroxidase (GE Healthcare Bio-Sciences Corp.), and visualized with SuperSignal West Femto Maximum Sensitivity Substrate kit (Pierce). To ensure equal loading in all the lanes, the blot was stripped

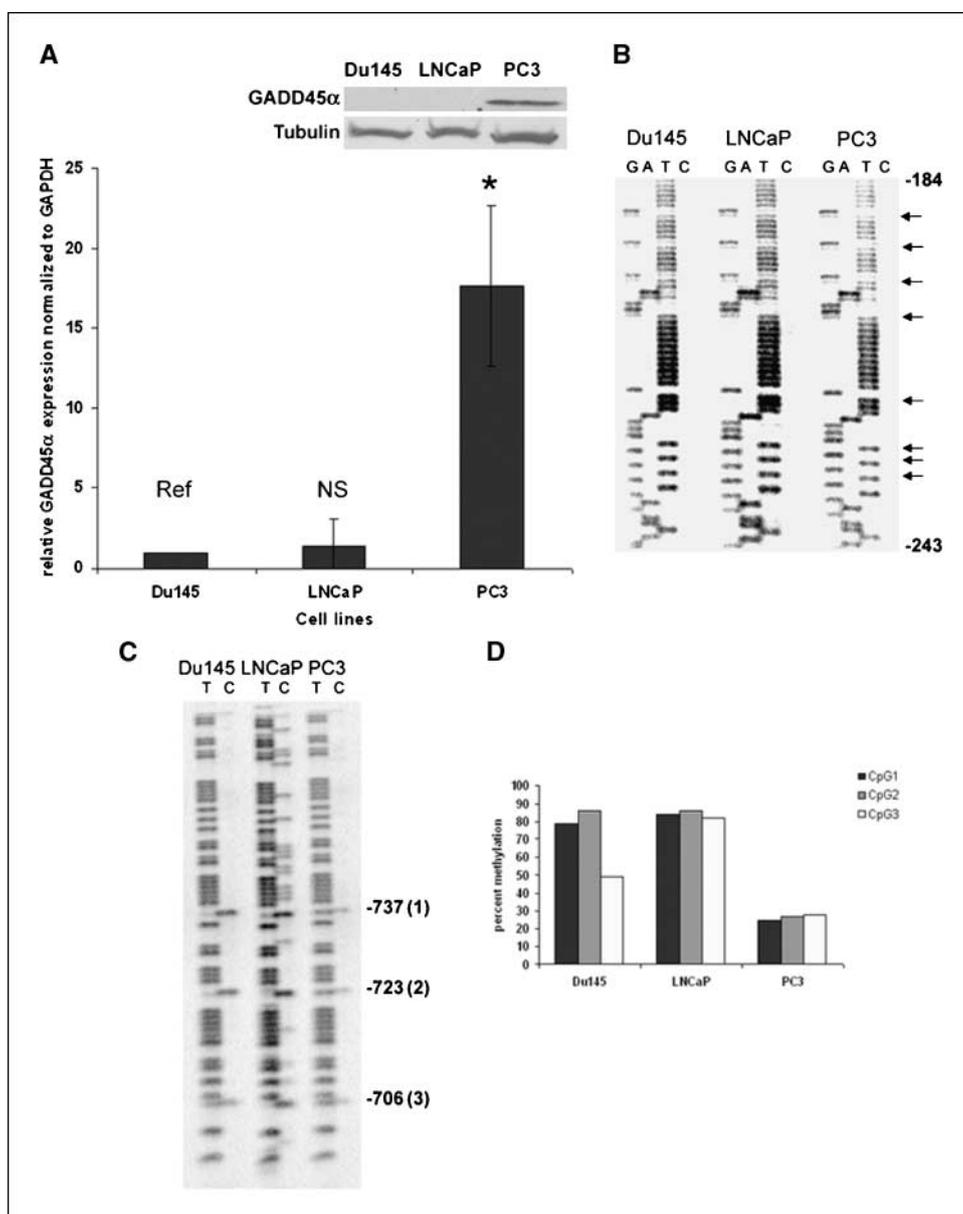
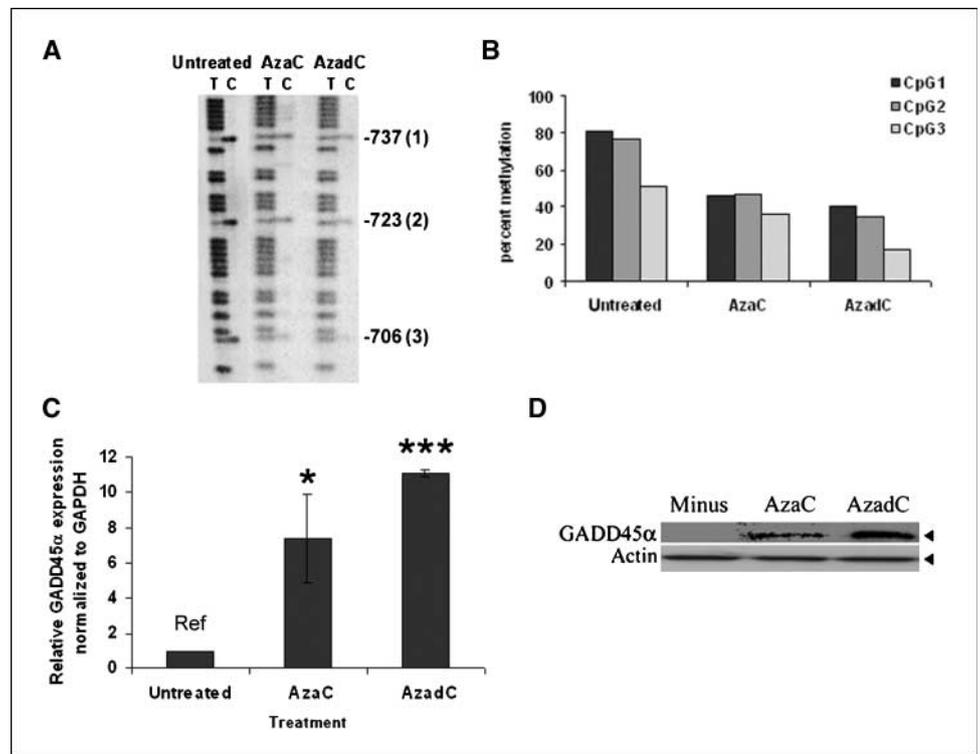


Figure 1. *GADD45α* expression and methylation in prostate cancer cells. **A**, RT-PCR analysis. cDNA synthesized from RNA was amplified by real-time PCR. Levels of *GADD45α* were normalized to that of GAPDH. **Inset**, Western blotting. Whole cell lysates were prepared from Du145, LNCaP, and PC3 cells and resolved on a 4% to 20% SDS polyacrylamide gel and blotted onto a nitrocellulose membrane. The blot was probed with anti-*GADD45α* antibody and detected by enhanced chemiluminescence (ECL). To ensure equal loading of the samples, the blot was stripped and reprobed with anti-tubulin antibody. **B**, bisulfite genomic sequencing analysis of the proximal promoter region. DNA extracted from cells was bisulfite treated and amplified using primers flanking the proximal promoter region. The PCR product was run on a 3% agarose gel and purified using Wizard SV gel and PCR clean-up system (Promega). The PCR product was sequenced using Thermosequencing Radiolabeled Terminator cycle sequencing kit (USB) and run on a 7 mol/L urea/5% polyacrylamide gel. The gel was dried and analyzed using Phosphorimager. **Arrows**, CpGs. CpGs in the *GADD45α* proximal promoter region are unmethylated in all three cell lines. **C**, bisulfite genomic sequencing analysis of the 5' 4 CpG region. The 4 CpGs at positions -737, -723, -706, and -690 (not shown) with respect to the transcription start site are methylated in Du145 and LNCaP, whereas they were unmethylated in PC3. **D**, methylation was quantitated using Quantity One software (Bio-Rad).

Figure 2. Reactivation of *GADD45 α* after treatment with DNMT inhibitors. **A**, bisulfite sequencing showing demethylation of CpGs in the *GADD45 α* 4 CpG region. Du145 cells were treated with 0.5 μ mol/L 5-azadeoxycytidine (*AzadC*) or 1 μ mol/L 5-azacytidine (*AzaC*) for 72 h. Control cells were left untreated. Bisulfite-treated DNA was amplified using primers flanking the 4 CpG region. The PCR product was run on a 3% agarose gel and purified using Wizard SV gel and PCR clean-up system (Promega). The PCR product was sequenced using Thermosequense Radiolabeled Terminator cycle sequencing kit (USB). **B**, methylation was quantitated using Quantity One software (Bio-Rad). **C**, quantitative RT-PCR. RNA extracted from the drug-treated cells was reverse transcribed and amplified by real-time PCR. Levels of *GADD45 α* mRNA were normalized to that of GAPDH. **D**, Western blot analysis. Whole cell lysates prepared from these cells were resolved on a 4% to 20% SDS polyacrylamide gel and blotted onto a nitrocellulose membrane. The blot was probed with anti-GADD45 α antibody and detected by ECL. To ensure equal loading of the samples, the blot was stripped and reprobed with anti-actin antibody.



and probed with antibody against housekeeping genes, anti-actin (Sigma-Aldrich) or anti-tubulin (Cell Signaling).

Immunofluorescence staining for MeCP2. Du145 wild-type (wt) and MeCP2⁻ cells were seeded in Lab-Tek chamber slides (Nalge Nunc International). Cells fixed in formaldehyde and permeabilized with 0.1% Triton X-100 were blocked in 3% bovine serum albumin prepared in PBS. Cells were then stained with rabbit anti-MeCP2 antibody followed by staining with secondary antibody conjugated with Texas red and visualized under a fluorescence microscope.

Bisulfite genomic sequencing and methylation analysis. DNA from cell lines, primary prostate cancer tissues, and benign prostate tissues were extracted using DNA Stat 60 (TelTest). Bisulfite conversion of the DNA was carried out as previously described (17). In brief, after bisulfite treatment, the DNA template was amplified with the following primers sets: 5'-AAAGTTTTTATTTTTAATGGTTT-3' (forward) and 5'-ATCAACC-TACTCCAACAATTTAAC-3' (reverse) for the proximal promoter region, and 5'-TTGGGTTGTTAGGGATTTTATATG-3' (forward) and 5'-AAAA-TCTTTCCACAAAAACAAA-3' (reverse) for the 5' 4 CpG region. Sequencing of the PCR-amplified product was done using the reverse primer. The [α -³³P]ddNTP terminator kit (USB) was used for sequencing. The sequencing gel was dried and exposed to a phosphorimager screen (Bio-Rad Laboratories). Methylation analysis was carried out by quantitating the intensity of C and T bands using Quantity One software (Bio-Rad Laboratories) and calculating the percentage of C/(C + T) bands, as previously described (18).

Bisulfite sequencing and methylation-sensitive single nucleotide primer extension analysis of tissue samples. DNA was extracted from prostate cancer and benign prostate tissues by the Qiagen blood and tissue DNA extraction kit with extended proteinase K treatment. A positive control for methylated DNA was generated by treating human leukocyte DNA from a healthy donor with SssI CpG methylase (New England Biolabs) according to the instruction of the supplier. Complete methylation was ensured by digestion with *HpaII*. Bisulfite treatment was carried out using the CpGenome DNA Modification Kit (Q-Biogene). The *GADD45 α* 5' 4 CpG region was amplified using the primers described above. For bisulfite sequencing, PCR products were cloned into the pCR4 vector using the TOPO TA cloning kit (Invitrogen). Successful cloning of the PCR product

was verified by restriction enzyme digestion, and multiple clones from each sample were sequenced at the central facility of the Center for Biological and Medical Research of the Heinrich Heine University by standard methods. Methylation-sensitive single nucleotide primer extension (MS-SNuPE) reactions were carried out using 1 μ L of the PCR product in a 25- μ L reaction mixture containing 1 \times PCR buffer (Sigma), 1 μ mol/L of SNuPE primer, 1 μ Ci of [³²P]dATP or [³²P]dGTP, and 1 unit of Taq polymerase (Sigma; refs. 19, 20). The primers used for MS-SNuPE analysis were as follows: CpG1, 5'-AACTAATCTAC-3'; CpG2, 5'-AACTAAAAATC-3'; CpG3, 5'-TTCCACCTACCC-3' (Supplementary Fig. S2).

Chromatin immunoprecipitation and real-time PCR. Chromatin immunoprecipitation assays were carried out with the kit from Upstate Biotechnology as described previously (21). Real-time PCR amplification of bound DNA was done in triplicate using 10 μ L of the immunoprecipitated DNA using primers amplifying the *GADD45 α* 4 CpG region.

Cell viability assay. Cell viability after drug treatment was analyzed using fluorescein di-*O*-acetate. Viable cells incorporate the nonfluorescent fluorescein di-*O*-acetate and rapidly hydrolyze it using acetyl esterase activity to fluorescein, which is retained within the cell. Nonviable cells no longer have esterase activity and will not be fluorescently stained. Fluorescence cell sorting was done in a Beckman Coulter XL flow cytometer (22).

Apoptosis assay. Apoptotic stress after drug treatment was measured by Annexin V/propidium iodide staining using APO-AF Annexin V-FITC apoptosis detection kit (Sigma-Aldrich). Fluorescence cell sorting was carried out as described above. Annexin V-FITC is detected as green fluorescence and propidium iodide staining is detected as red fluorescence.

Statistical analysis. Experiments were done in triplicates or quadruplicates. A two-sided *t* test or *z* test was used to compare two-group means and *P* \leq 0.05 was considered significant. Comparisons against a reference group are reported in the text as *P* values, and significance is displayed in figures as follows: NS, nonsignificant; *, 0.01 < *P* < 0.05; **, 0.001 < *P* < 0.01; and ***, *P* < 0.001.

Results

***GADD45 α* expression in prostate cancer cells correlates inversely to methylation of 5' 4 CpG region.** Transfection of

GADD45α has been shown to induce apoptosis in Du145 and PC3 prostate cancer cell lines (11); hence, we explored the role of *GADD45α* as a potential target for chemotherapy. We examined *GADD45α* expression in the three prostate cancer cell lines, LNCaP, Du145, and PC3, by real-time RT-PCR and Western blotting analyses. Compared with Du145, we found that LNCaP expressed similarly low levels ($P = 0.756$) whereas PC3 showed significantly elevated levels of *GADD45α* expression (17-fold, $P = 0.029$; Fig. 1A).

Because promoter methylation is commonly associated with silencing of gene expression, we examined the methylation status of the *GADD45α* promoter region in Du145, PC3, and LNCaP prostate cancer cells by bisulfite genomic sequencing. CpG islands in the proximal promoter region were unmethylated in all three cell lines (Fig. 1B). However, unlike known patterns of methylation of CpG islands, an atypical methylation pattern was observed. Similar to an earlier study in breast cancer cells (23), only 4 CpGs located ~700 bases upstream of the transcription start site were methylated in Du145 and LNCaP whereas they were unmethylated in PC3 (Fig. 1C). The average methylation of 3 CpGs was 71%, 84%, and 27% in Du145, LNCaP, and PC3, respectively (Fig. 1D). Levels of

expression of *GADD45α* in these cell lines correlated inversely with the methylation levels of CpGs in the 5' region.

Treatment with DNMT inhibitors reactivates *GADD45α* expression in Du145 cells. Several *in vitro* and *in vivo* studies have shown that treatment with inhibitors of DNMTs results in DNA demethylation and subsequent reactivation of otherwise silenced genes (24–26). To confirm the role of methylation of the 5' 4 CpG region in transcriptional repression of *GADD45α*, we analyzed the expression of *GADD45α* after treatment of Du145 cells with DNMT inhibitors, 5-azacytidine and 5-azadeoxycytidine. Levels of *GADD45α* mRNA and protein levels of *GADD45α* increased significantly after drug treatment (Fig. 2C and D), concomitant with demethylation of the 4 CpGs (Fig. 2A and B). When compared with untreated Du145 control, we observed a 7-fold increase in expression of *GADD45α* in cells treated with 5-azacytidine ($P = 0.049$) and an 11-fold increase with 5-azadeoxycytidine ($P = 0.000$). Similar results were observed in LNCaP cells when treated with DNMT inhibitors (data not shown). These results support the role of methylation in repressing *GADD45α* gene expression in Du145 and LNCaP cells.

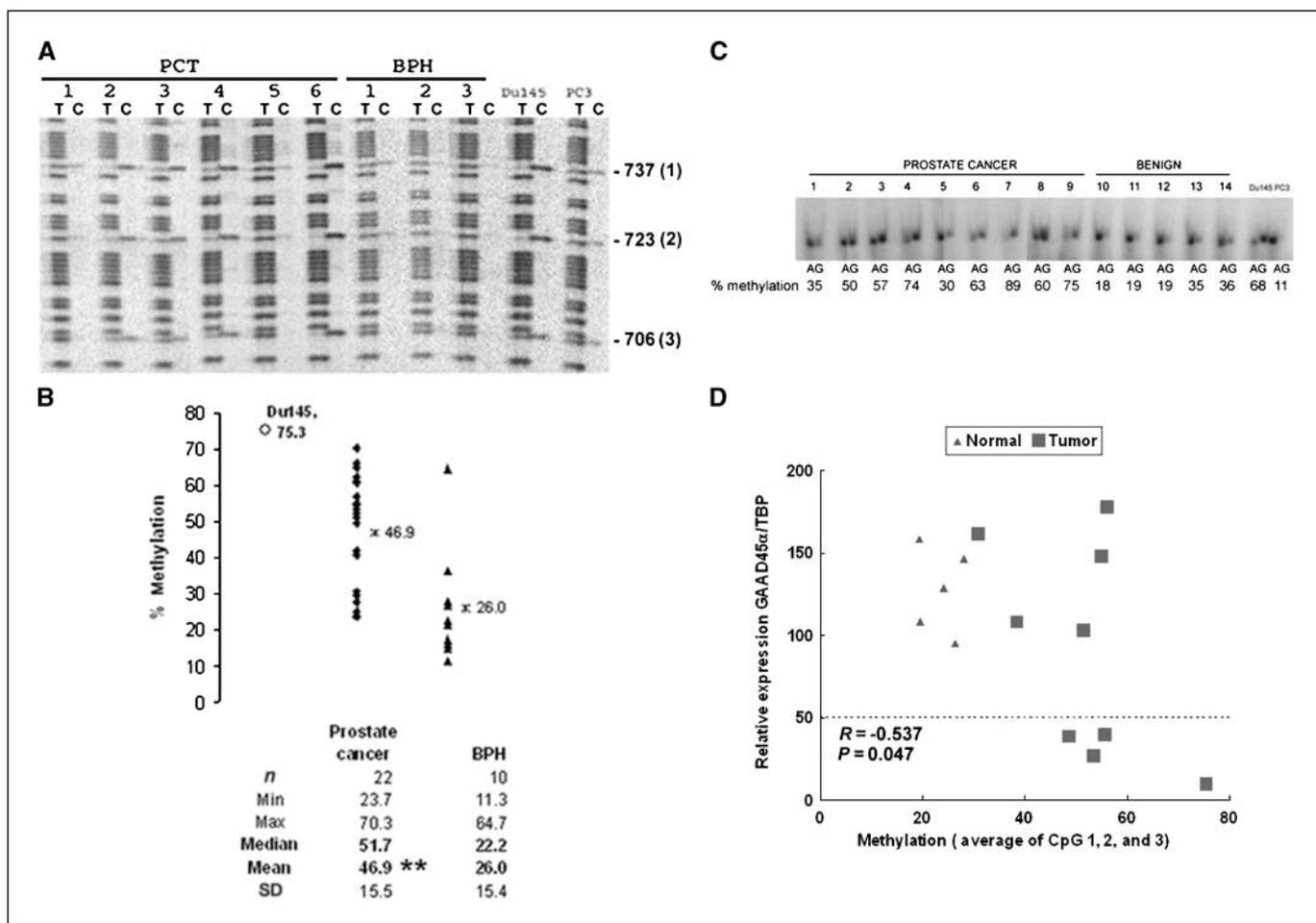


Figure 3. Quantitation of methylation of CpGs in the 4 CpG region of *GADD45α* promoter in prostate cancer tissues and BPH tissues. **A**, DNA extracted from tissues was bisulfite treated and amplified using primers flanking the 4 CpG region and sequenced as described before. **PCT**, prostate cancer tissues. **Arrows**, CpGs. Methylation of CpGs was quantitated using Quantity One (Bio-Rad). Percent methylation was calculated using the formula $C/C + T \times 100$. **B**, mean and SD of percent methylation in 22 prostate cancer patients and 10 BPH patients. The difference between the means is statistically significant, $P = 0.001$. **C**, representative MS-SNuPE analysis of methylation of *GADD45α* 4 CpG region (CpG site 2 shown) in prostate cancer and benign prostate tissues. **D**, correlation of expression of *GADD45α* with methylation of the 4 CpG region. Levels of *GADD45α* expression normalized to TBP levels were correlated with average methylation of CpG sites 1, 2, and 3 in the 5' 4 CpG region.

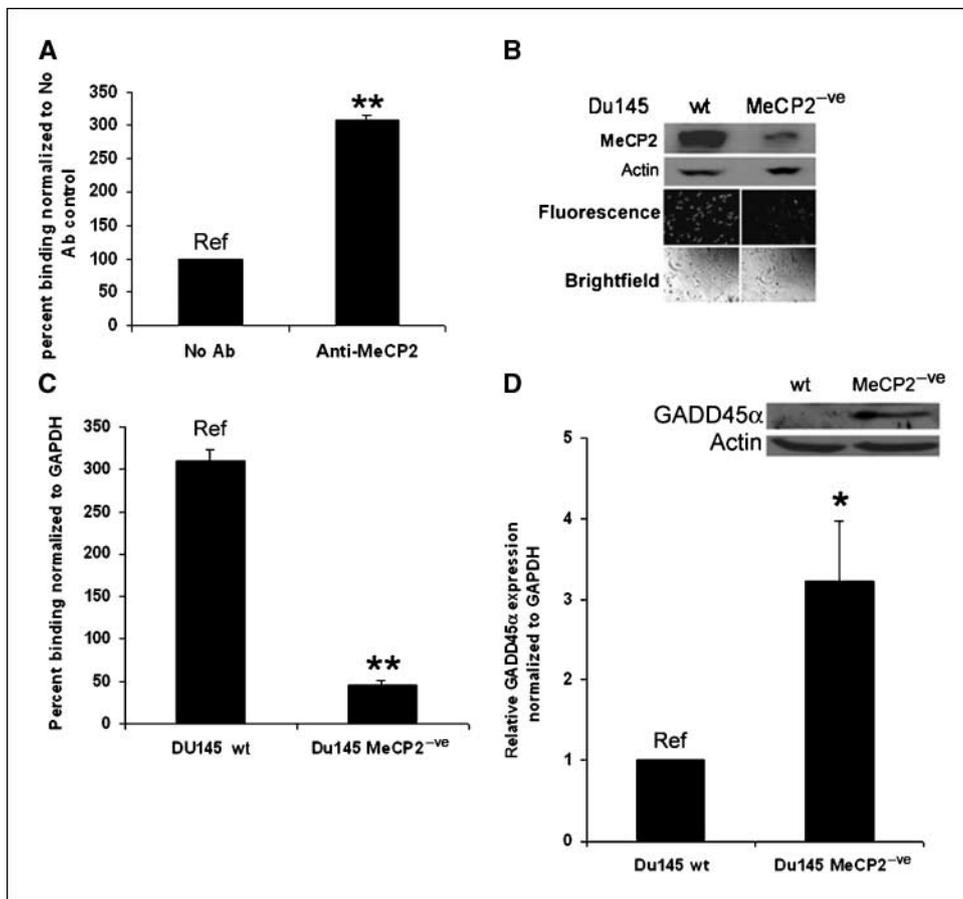


Figure 4. *A*, chromatin immunoprecipitation analysis of MeCP2 binding to *GADD45 α* 4 CpG region. Formaldehyde-fixed chromatin from Du145 cells was analyzed using chromatin immunoprecipitation kit (Upstate). DNA from bound fractions was amplified by real-time PCR. DNA binding to MeCP2 was calculated by normalizing to no-antibody (*No Ab*) control. *Ref*, reference group. *B*, reduced expression of MeCP2. For Western blotting analysis, whole cell lysates prepared from Du145 wt and MeCP2^{-ve} cells were resolved on a 4% to 20% SDS polyacrylamide gel and blotted onto a nitrocellulose membrane. The blot was probed with anti-MeCP2 antibody and detected by ECL. To ensure equal loading of the samples, the blot was stripped and reprobed with anti-actin antibody. Immunofluorescence staining (as described in Materials and Methods) showed reduced expression of MeCP2 in Du145 MeCP2^{-ve} cells compared with Du145 wt cells. *C*, chromatin immunoprecipitation analysis showing reduced binding of MeCP2 to *GADD45 α* 4 CpG region in Du145 MeCP2^{-ve} cells. Formaldehyde-fixed chromatin from Du145 wt and MeCP2^{-ve} cells was analyzed using chromatin immunoprecipitation kit (Upstate). DNA from bound fractions was amplified by real-time PCR. DNA binding to MeCP2 was calculated by normalizing to no-antibody control. *D*, increased *GADD45 α* expression in Du145 MeCP2^{-ve} cells. RNA extracted from cells was reverse transcribed and amplified by real-time PCR. Levels of *GADD45 α* mRNA were normalized to that of GAPDH. *Inset*, Western blotting. Whole cell lysates prepared from Du145 wt and MeCP2^{-ve} cells were resolved on a 4% to 20% SDS polyacrylamide gel and blotted onto a nitrocellulose membrane. The blot was probed with anti-*GADD45 α* antibody and detected by ECL. To ensure equal loading of the samples, the blot was stripped and reprobed with anti-actin antibody.

Methylation of *GADD45 α* 5' 4 CpG region is a frequent event in prostate cancer and correlates inversely with gene expression in primary prostate cancer and benign prostate tissues.

Previous results showed that *GADD45 α* was down-regulated in prostate cancer compared with benign prostate tissues (15). However, only proximal promoter methylation was evaluated and this region was found to be unmethylated in both cancer tissues and benign specimens. To determine if methylation of *GADD45 α* 5' 4 CpG region is present in prostate tumors, we compared the methylation of *GADD45 α* 4 CpG region in 22 prostate cancer tissues and 10 BPH tissues by bisulfite sequencing (Fig. 3A). We found a significant increase in methylation of this 4 CpG region in prostate cancer tissues compared with BPH (mean of 47% in prostate cancer tissues versus 26% in BPH, $P = 0.001$; Fig. 3B). Methylation of proximal promoter region was evaluated in randomly selected samples. These showed lack of methylation in proximal promoter despite methylation of the 4 CpG region (data not shown). Hence, atypical methylation of *GADD45 α* 4 CpG region is present in primary prostate cancer tissues and is not confined to prostate cancer cell lines.

To correlate *GADD45 α* expression with methylation of the 5' 4 CpG region, we examined methylation in five prostate cancer tissues with high *GADD45 α* expression, four prostate cancer tissues with low *GADD45 α* expression, and five benign prostate tissues from the previous study (15). All five benign tissues were found to have high *GADD45 α* expression. MS-SNuPE quantitation showed that methylation in each of 3 CpGs is significantly higher in tumor samples (average methylation of 3 CpGs, 52%) than in normal tissue samples (average methylation of 3 CpGs, 24%; $P < 0.0001$; Fig. 3C, data for CpG2 shown). Methylation of *GADD45 α* 5' 4 CpG region correlated inversely with gene expression in combined analysis of all tissue samples ($n = 14$, Pearson's correlation coefficient = -0.537 , $P = 0.047$; Fig. 3D). These results were also independently confirmed by bisulfite sequencing (see Supplementary Fig. S1).

Transcriptional repression of *GADD45 α* occurs via interaction of MeCP2 with its methylated promoter. Interaction of transcriptional repressor proteins with methylated DNA either directly by the methyl-binding domain (MBD) or indirectly by

interaction with other proteins contributes to transcriptional repression (27–29). To elucidate the mechanism of transcriptional silencing of *GADD45α* in prostate cancer cells, we analyzed the binding pattern of MeCP2 to the methylated 5' 4 CpG region upstream of *GADD45α* promoter in Du145 and LNCaP cells using chromatin immunoprecipitation assay. MeCP2 was found to interact with the methylated 4 CpG region in Du145 cells (mean percent binding: 310% normalized to no-antibody control, $P = 0.002$) in Du145 (Fig. 4A) but not in LNCaP cells (data not shown). In contrast, no binding of MBD2 to this region was observed in Du145 cells (data not shown).

It has been reported that knockdown of MeCP2 by siRNA transfection restores the expression of epigenetically silenced genes (30). To examine the role of MeCP2 in methylation-mediated transcriptional regulation of *GADD45α* in Du145 cells, we carried out transient knockdown of MeCP2 using a MeCP2 siRNA vector in Du145 cells and examined the changes in the expression pattern of *GADD45α*. *GADD45α* expression in Du145 MeCP2⁻ was increased by ~2-fold compared with wt Du145 cells, suggesting that MeCP2 interaction with the *GADD45α* 5' region has functional significance (data not shown). Consistent with chromatin immunoprecipitation analysis, transient transfection of MBD2 siRNA did not alter the gene expression levels in Du145 cells (data not shown). No enhancement of *GADD45α* expression was observed in LNCaP cells on stable knockdown of MeCP2 (data not shown). This was consistent with the chromatin immunoprecipitation analysis data that showed that MeCP2 does not bind to the *GADD45α* 4 CpG region in these cells (data not shown).

We further confirmed these findings by generating a stable Du145 MeCP2⁻ cell line in which MeCP2 was knocked down by transfection with pSuppressor/MeCP2 siRNA plasmid. Reduced expression of MeCP2 in Du145 MeCP2⁻ cells was observed by immunofluorescence staining with anti-MeCP2 antibody and also by Western blotting analysis, thus confirming the stable knockdown of MeCP2 in these cells (Fig. 4B). Consistent with these results, we found that the binding of MeCP2 to the *GADD45α* promoter is reduced in Du145 MeCP2⁻ cells (mean of 310% in Du145 wt versus 47% in MeCP2⁻, $P = 0.002$; Fig. 4C). Further, the levels of *GADD45α* increased by ~3-fold in Du145 MeCP2⁻ cells as compared with Du145 wt cells ($P = 0.035$; Fig. 4D) although methylation of the 4 CpGs remained unaltered (data not shown).

To elucidate the mechanism of *GADD45α* repression in LNCaP cells, we examined interaction of other MBDs using chromatin immunoprecipitation assay. We observed that interaction of MBD2 with *GADD45α* promoter and stable depletion of MBD2 by siRNA vector resulted in enhanced expression of *GADD45α* gene (data not shown).

Recombinant *GADD45α* expression results in increased sensitivity of Du145 cells to docetaxel. Up-regulation of *GADD45α* is a key event in docetaxel-induced apoptosis (10, 11, 31). Because *GADD45α* is down-regulated in Du145 cells, we hypothesized that expression of *GADD45α* would enhance sensitivity to docetaxel. Du145 cells were transiently transfected with pCMV/*GADD45α* or mock transfected with empty pCMV vector. Western blotting analysis confirmed the expression of *GADD45α* in these *GADD45α*-transfected cells (Fig. 5A). Increased apoptosis as measured by Annexin V/propidium iodide staining was observed in Du145 cells expressing *GADD45α* as compared with mock-transfected cells when treated with docetaxel for 72 hours (47% versus 31%, $P < 0.001$) as well as when left untreated (mean of 12% versus 4%, $P = 0.003$; Fig. 5B and C). Cells expressing

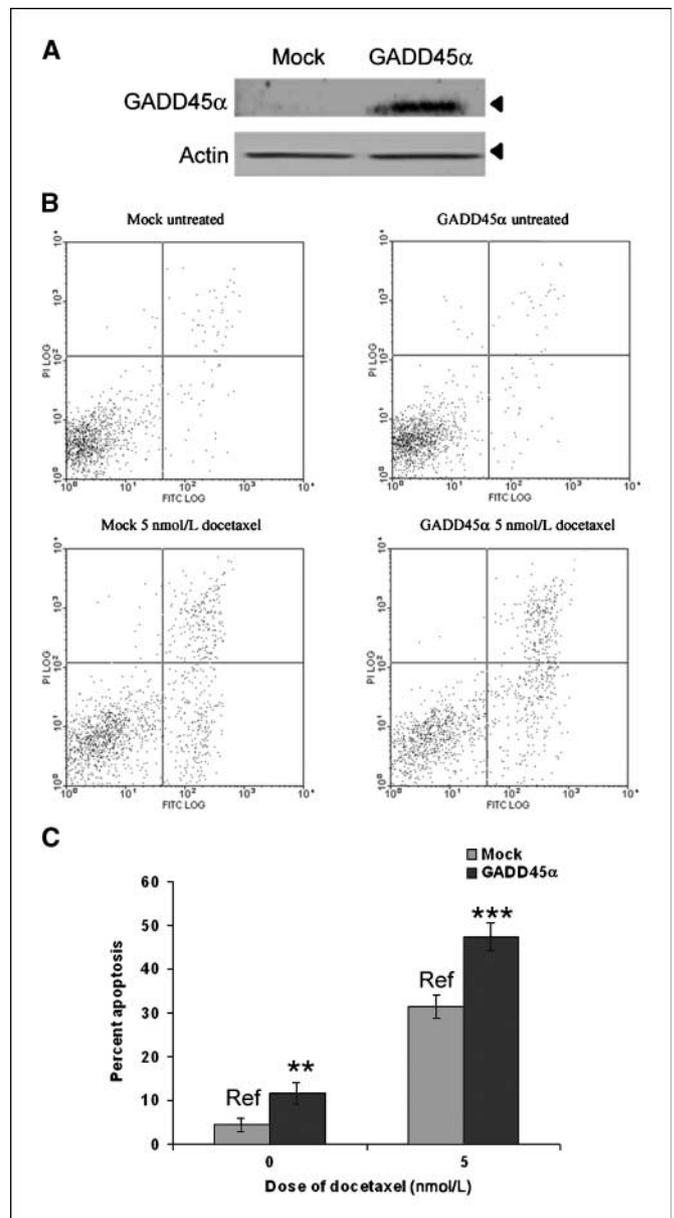


Figure 5. Effect of *GADD45α* expression on docetaxel sensitivity of Du145 cells. **A**, Western blot analysis of *GADD45α*- and mock-transfected cells. Whole cell lysates prepared from these cells were resolved on a 4% to 20% SDS polyacrylamide gel and blotted onto a nitrocellulose membrane. The blot was probed with anti-*GADD45α* antibody and detected by ECL. To ensure equal loading of the samples, the blot was stripped and reprobed with anti-actin antibody. **B** and **C**, Du145 cells were transfected with *GADD45α* expression vector or vector alone (*Mock*) using Amaxa nucleofection kit and seeded in 12-well plates for 24 h, after which they were treated with 5 nmol/L docetaxel for 72 h. Cytotoxicity was assayed by Annexin V/propidium iodide staining and analyzed by flow cytometry. Cells expressing *GADD45α* showed increased apoptosis when treated with docetaxel compared with untreated cells (47% versus 12%, $P < 0.001$). The mean difference in apoptosis between *GADD45α*-expressing and mock-transfected cells was greater in cells treated with docetaxel (mean difference, 16%) than in untreated cells (mean difference, 8%; $P < 0.001$).

GADD45α showed increased apoptosis when treated with docetaxel compared with untreated cells (47% versus 12%, $P < 0.001$). The mean difference in apoptosis between *GADD45α*-expressing versus mock-transfected cells was greater in cells treated with docetaxel (mean difference, 16%) than in untreated cells (mean difference, 7%; $P < 0.001$). This confirms the role of *GADD45α* in

augmenting docetaxel-mediated apoptosis in Du145 prostate cancer cells.

Up-regulation of GADD45 α by pretreatment with 5-azacytidine increases sensitivity of Du145 cells to docetaxel. Combination treatments can improve the antitumor activity of chemotherapeutic drugs (32). We hypothesized that induction of GADD45 α expression by 5-azacytidine pretreatment would increase sensitivity of Du145 cells to docetaxel chemotherapy. We performed time and dose response kinetics in Du145 cells to docetaxel and 5-azacytidine (Fig. 6A and B). Based on these results, we chose the subcytotoxic dose of 1 μ mol/L 5-azacytidine for pretreatment of the cells. As shown in Fig. 6C, we observed an increased sensitivity to docetaxel treatment. Annexin V/propidium iodide staining followed by flow cytometry showed that cells pretreated with 5-azacytidine showed higher apoptosis with docetaxel than previously untreated cells. The mean apoptotic fraction was 12% in 5-azacytidine-treated cells versus 4% in untreated cells ($P = 0.006$) and 25% in 5-azacytidine pretreated cells + docetaxel versus 16% in untreated cells + docetaxel ($P = 0.001$). Similar results were obtained with

fluorescein di-*O*-acetate cell viability assay (Fig. 6D). The additive effect of 5-azacytidine and docetaxel is likely a result of up-regulation of GADD45 α by 5-azacytidine treatment. We also examined the ability of 5'-azacytidine to enhance sensitivity to docetaxel treatment of LNCaP cells. The results were similar to those with Du145 cells (data not shown).

Discussion

This article describes epigenetic regulation of GADD45 α in prostate cancer cells and its potential role in docetaxel sensitivity. The key findings in this article are as follows. GADD45 α expression in prostate cancer cells correlates inversely with an atypical CpG methylation upstream of transcription start site. Methylation of GADD45 α occurs more frequently in prostate cancer tissues as compared with BPH tissues. Methylation of the 5' 4 CpG region correlates inversely with GADD45 α expression in prostate cancer and benign prostate tissues. Transcriptional repression of GADD45 α in Du145 cells involves interaction of MeCP2 with

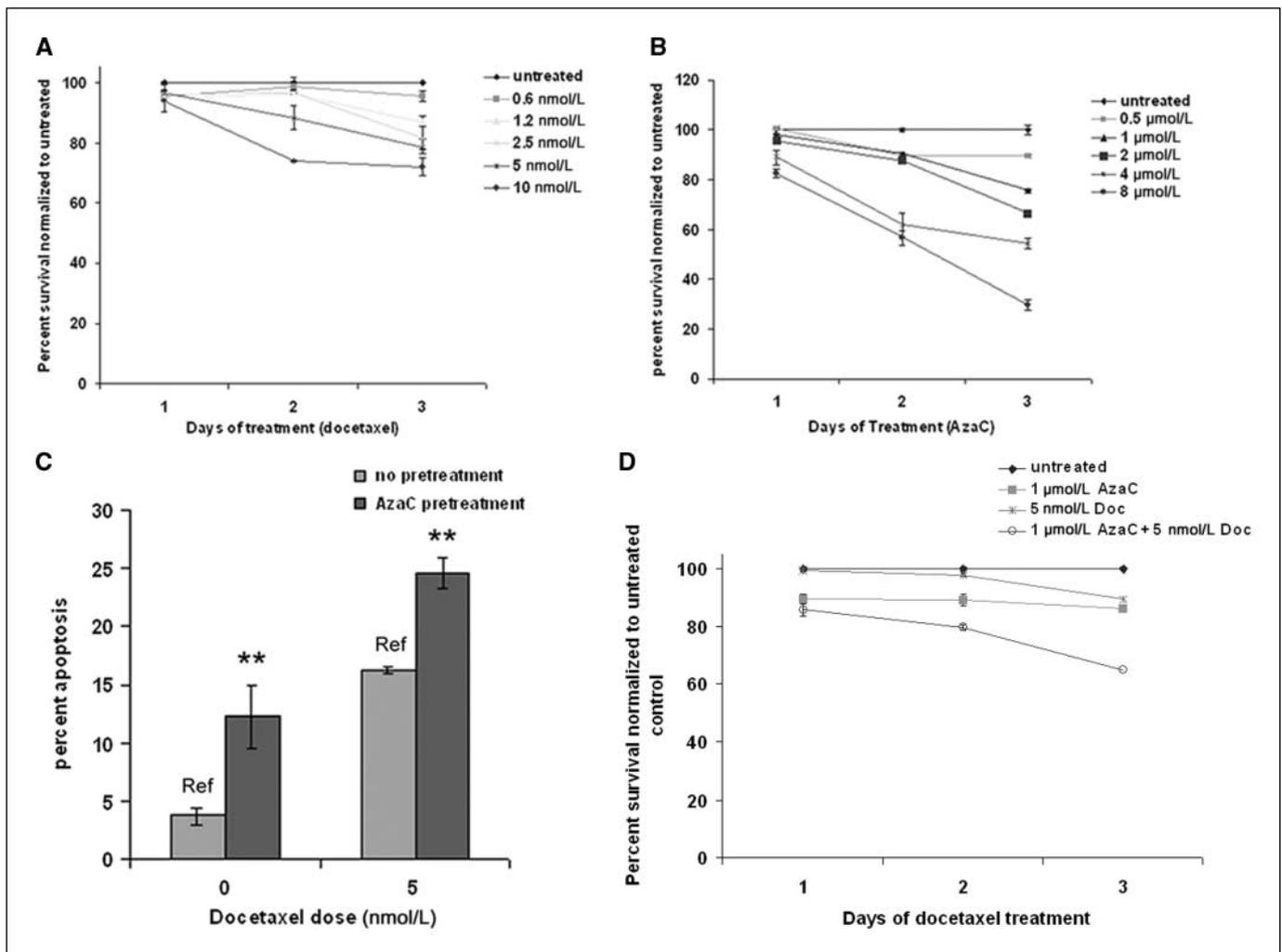


Figure 6. Dose and time response kinetics of Du145 cells to docetaxel (A) and 5-azacytidine (B). Cells seeded in 12-well plates were treated with different doses of drug and cell viability was assayed using fluorescein di-*O*-acetate (FDA) over 72 h. C and D, increased sensitivity of Du145 cells to docetaxel by pretreatment with 5-azacytidine. Cells seeded in 12-well plates were treated with 1 μ mol/L 5-azacytidine for 72 h, after which they were treated with 5 nmol/L docetaxel for 72 h. In parallel, control cells were left untreated for 72 h, after which they were treated with 5 nmol/L docetaxel for 72 h. Cytotoxicity was assayed by Annexin V/propidium iodide staining and analyzed by flow cytometry. Cells treated with 5-azacytidine and docetaxel showed increased apoptosis compared with cells treated with 5-azacytidine alone (61% versus 34%, $P = 0.005$; C). Cells seeded in 12-well plates were treated with 1 μ mol/L 5-azacytidine for 72 h, after which they were treated with 5 nmol/L docetaxel for 72 h. Cell viability was evaluated using fluorescein di-*O*-acetate followed by flow cytometry (D).

the 5' 4 CpG region of the gene. Induction of *GADD45α* expression by down-regulation of MeCP2 or by treatment with DNMT inhibitors leads to enhanced sensitivity of Du145 cells to docetaxel chemotherapy.

Although methylation-mediated inactivation of genes is attributed to methylation of dense CpG islands in the proximal promoter region (1–3, 6, 33, 34), a few examples to the contrary exist. Early evidence that sparse methylation could lead to transcriptional repression arose from the observation that *in vitro* methylation of human γ -globin and mouse α -globin sequences lacking CpG clusters resulted in loss of gene expression. Similar results were obtained when the human α -globin promoter with dense CpG clusters was methylated to a lower density (35). Atypical DNA methylation was described in the case of the *APAF1* gene, which is transcriptionally repressed in malignant melanoma. No methylation could be detected in its core promoter region. However, gene expression was restored by treatment with DNMT inhibitors, suggesting that methylation affects CpGs located outside of the promoter region (36). Conventional techniques to identify epigenetically regulated genes include MS-PCR and bisulfite sequencing of CpG islands located in the proximal promoter region of the gene. Often, these approaches do not address sparsely populated CpGs that are situated far upstream of the transcription start site. Another approach to identify epigenetically regulated genes is use of expression arrays to detect genes that are induced by 5-azacytidine. Using this strategy, *GADD45α* methylation outside of the proximal promoter region was detected in breast cancer (23). In the previous study, although *GADD45α* repression was observed in prostate cancer, evaluation of the proximal promoter region failed to detect methylated CpG dinucleotides (15). Consistent with these results, we did not detect methylation in the CpG rich proximal promoter region in the three prostate cancer cell lines analyzed. Instead, gene expression was regulated by methylation of 4 CpGs upstream of the proximal promoter region. This pattern of methylation is atypical because it occurs in a sparsely distributed CpG region situated far away from the actual promoter sequence. Moreover, these 4 CpGs are not contained within any recognized response element. We observed increased levels of methylation of *GADD45α* 4 CpG region in prostate cancer tissues compared with BPH tissues. Further, methylation levels of *GADD45α* 4 CpG region inversely correlated with gene expression in prostate tissues, suggesting the functional significance of methylation of the 4 CpG region in regulation of *GADD45α* expression. Therefore, methylation analysis of CpG dinucleotides away from the proximal promoter region may also be important for genes suspected to be repressed by methylation.

Epigenetic suppression of transcription involves proteins that bind specifically to methylated DNA, in particular MBD proteins. Transcriptional regulation by MBDs occurs by binding to methylated DNA, thus acting as a "locking" mechanism stabilizing gene repression caused by other components of chromatin modification systems (29). MeCP2, the archetypical MBD protein, has been shown to play a role in epigenetic transcriptional repression mechanism (37). MeCP2 binds to methylated CpGs of the *IκBα* promoter leading to the formation of a transcriptional repression complex that silences expression of the gene in myofibroblasts. Knockdown of MeCP2 led to enhanced *IκBα* promoter activity and restoration of mRNA and protein expression in these cells (30). In Du145 cells, *GADD45α* was found to be transcriptionally regulated via binding of MeCP2 to the methylated *GADD45α* 5' 4 CpG region. Transcriptional repression of the gene

was alleviated by knocking down MeCP2. Knockdown of MBD2, however, did not restore *GADD45α* expression in these cells. The specificity of binding of MeCP2 to sparsely methylated *GADD45α* sequence in Du145 could be explained by the fact that MeCP2 binds to single methylated CpG whereas MBD2/MeCP1 requires densely methylated CpG islands for binding (27). We found that the effect of MeCP2 knockdown on *GADD45α* expression was cell line specific because in LNCaP cells, there was no interaction of MeCP2 with *GADD45α* 5' 4 CpG region and, hence, no effect of MeCP2 knockdown on *GADD45α* expression. Conversely, in LNCaP cells, MBD2 interaction with *GADD45α* promoter was observed and depletion of MBD2 by siRNA vector resulted in enhanced expression of *GADD45α* gene. It has been postulated that a gene could be associated with different MBDs in different cells (29). Moreover, one of the models for gene regulation by MBDs proposes a random interaction of MBDs, thus accounting for the functional redundancy of these proteins (29). This explains why there are few instances of loss of a specific MBD protein resulting in over-expression of corresponding target genes.

Docetaxel is the only known chemotherapeutic agent shown to improve survival in patients with hormone-refractory prostate cancer as established by randomized controlled trials and therefore approved by the Food and Drug Administration for this use (38, 39). It has been known that docetaxel binds to microtubules inhibiting cancer cell proliferation (40). However, the precise molecular mechanisms for inhibiting cancer cell growth by docetaxel have not been fully elucidated. Resistance to chemotherapy, especially to docetaxel, has presented itself as a major obstacle in treatment of advanced prostate cancer. Currently, there is no standard of care defined for such patients. There is a continuous search for new treatment regimens to improve the efficacy of chemotherapeutic agents. Evidence from literature suggests that disruption of the apoptotic pathway seems to be a major mechanism of uncontrolled cell proliferation as well as resistance to chemotherapeutic agents (1). Gene expression profiling revealed that treatment of cells with docetaxel increased *GADD45α* expression, thus indicating *GADD45α* as a key element in docetaxel mediated apoptosis (14). Consistent with this observation, we found that sensitivity to docetaxel was enhanced in Du145 cells expressing *GADD45α* as compared with mock-transfected cells. Du145 cells treated with 5-azacytidine before docetaxel treatment showed increased sensitivity to docetaxel treatment likely due to increased *GADD45α* expression. Our results suggest that combination treatment with a DNMT inhibitor and docetaxel may improve the efficacy of docetaxel in treatment of metastatic prostate cancer.

Disclosure of Potential Conflicts of Interest

R. Singal: Commercial research grant and consultant/advisory board, Pharmion; honoraria from speakers bureau, Sanofi-Aventis. The other authors disclosed no potential conflicts of interest.

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Methylation-Mediated Repression of *GADD45* α in Prostate Cancer and Its Role as a Potential Therapeutic Target

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