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Sensitivity of human prostate cancer cells to chemotherapeutic drugs depends on EndoG expression regulated by promoter methylation

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Abstract

Analysis of promoter sequences of all known human cytotoxic endonucleases showed that endonuclease G (EndoG) is the only endonuclease that contains a CpG island, a segment of DNA with high G+C content and a site for methylation, in the promoter region. A comparison of three human prostate cancer cell lines showed that EndoG is highly expressed in 22Rv1 and LNCaP cells. In PC3 cells, EndoG was not expressed and the *EndoG* CpG island was hypermethylated. The expression of EndoG correlated positively with sensitivity to cisplatin and etoposide, and the silencing of EndoG by siRNA decreased the sensitivity of the cells to the chemotherapeutic agents in the two EndoG-expressing cell lines. 5-aza-2′-deoxycytidine caused hypomethylation of the *EndoG* promoter in PC3 cells, induced EndoG mRNA and protein expression, and made the cells sensitive to both cisplatin and etoposide. The acetylation of histones by trichostatin A, the histone deacetylase inhibitor, induced EndoG expression in 22Rv1 cells, while it had no such effect in PC3 cells. These data are the first indication that EndoG may be regulated by methylation of its gene promoter, and partially by histone acetylation, and that EndoG is essential for prostate cancer cell death in the used models.

Keywords

Endonuclease G; DNA methylation; Prostate cancer; Cell death; Cisplatin

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1. Introduction

Prostate cancer is the most commonly diagnosed non-cutaneous malignancy. It is the second leading cause of cancer deaths among men in the United States and overseas [1–3]. Prostate cancer, as well as cancer in general, is recognized as both a genetic and epigenetic disease, because both genetic and epigenetic changes play crucial roles in the transformation of cells to malignancy and in the progression of cancer [4]. Epigenetic changes are believed to be the most common alteration at the DNA level in prostate cancer [5,6].

Two types of DNA epigenetic changes occur in prostate cancer: regional DNA hypermethylation and regional/global DNA hypomethylation. Hypermethylation of the promoter region that contains CpG island occurs in a large number of genes and is associated with gene silencing in the vast majority of prostate cancer cases [1,3,4]. Studies have shown that hypermethylation of this region holds promise as a tumor biomarker for early diagnosis and risk assessment of prostate cancer. Furthermore, the prevalence of epigenetic changes in prostate cancer and the potential reversibility of DNA methylation alterations by DNA methylation inhibitors suggest that these changes are a viable target for cancer chemotherapy and chemoprevention strategies [7–9].

One of the key properties of cancer cells that leads to chemotherapy failure is the cells' ability to avoid cell death by deregulation of apoptosis [10,11]. The two best characterized and most broadly used chemotherapeutic drugs that inhibit DNA methylation are 5-aza-2′-deoxycytidine (AzaC), clinically referred to as decitabine [12,13], and another recently developed inhibitor of DNA methylation, zebularine [14]. One of the main goals of the application of these drugs is the restoration of apoptosis by normalizing DNA methylation [8]. Evidence has accumulated that a number of genes involved in cell death pathways are often silenced by DNA hypermethylation, contributing to inactivation of the apoptosis that is commonly observed in prostate cancer cells [15–18]. However the potential role of DNA methylation in modulating cell death endonucleases in cancer cells has not been studied.

Cytotoxic endonucleases, also called "cell death endonucleases," are the recently recognized group of enzymes responsible for premortem and postmortem DNA fragmentation associated with cell death by apoptosis or necrosis [19,20]. Major representatives of this group of enzymes include: deoxyribonuclease I (DNase I) [21], deoxyribonuclease II (DNase II) [22], EndoG [23], caspase-activated DNase (CAD) [24], and DNase gamma [25]. Cell death endonucleases were found in all studied cells and tissues, including the prostate [26,27]. These enzymes differ in certain catalytic characteristics and DNA sequence specificity, and yet produce a similar type of DNA damage, including single- and double-strand DNA breaks. Endonuclease-generated breaks have been shown to strongly interfere with DNA synthesis in both normal and cancer cells [28]. While often considered downstream effectors of apoptotic cascades, endonucleases can cause DNA fragmentation and imminent, irreversible cell death when acting alone after overexpression or introduction into the cell [21,22,24]. Some cell death endonucleases seem to be dispensable in normal apoptosis [29–31]. However, recent studies from our group and others demonstrated that inactivation of endonucleases causes protection of normal and cancer cells against a variety

of injuries *in vitro* and *in vivo* [32–35], suggesting that the endonucleases are essential for injury-related cell death. One of the endonucleases, EndoG, seems to be particularly important in cancer cells because it regulates their sensitivity to chemotherapeutic agents [32]. EndoG has been recently recognized as a key endonuclease in the caspase-independent apoptosis [36,37] and necrosis [38,39].

The molecular mechanism of EndoG regulation is complex and largely unknown. As described below, the sequence analysis of human *EndoG* gene has shown the presence of a CpG island in the promoter region, suggesting that this gene is regulated by DNA methylation. In view of these considerations, we undertook the present study to investigate the role of DNA methylation in the regulation of EndoG in human prostate cancer cells.

2. Materials and methods

2.1. Prostate cancer cells

Human prostate cancer cell lines, including well-differentiated, 22Rv1 (ATCC # CRL-2505) and LNCaP cells (ATCC # CRL-1740), and poorly differentiated PC-3 cells (ATCC #CRL-1435) were obtained from the American Type Culture Collection (ATCC, Manassas, VA). The cells were maintained in accordance with ATCC recommendations. All cells were seeded at density 0.5×10^6 cells per 100 mm plate, and the media was changed every other day for 6 days. Cisplatin (Bedford laboratories, Bedford, OH) or etoposide (Sigma-Aldrich) was added to serum-free media for 24 h. To evaluate cytotoxicity, the lactate dehydrogenase (LDH) release assay kit (Promega, Madison, WI) was used. Toxicity was expressed as the percentage of LDH released to the medium to that of the total LDH. Cell viability was measured using clonogenic assay as described by us previously [65].

2.2. EndoG siRNA silencing

EndoG silencing was performed according to an algorithm similar to one previously described for breast cancer cells [32]. Briefly, 22Rv1 cells were seeded in Permanox 8-well chambers and grown to 70–80% confluence. To knockdown EndoG mRNA, cells were transfected with designed DY547-siRNA duplexes labeled by 5′UTR of its sense strand (sense siRNA 5′-DY547-AUGCCUGGAACAACCUGGAdTdT-3′ antisense siRNA 3′-UCCAGGUUGUUCCAGGCAUdTdT-5′) or Control Non-Targeting siRNA #1 (Dharmacon, Lafayette, CO). Control siRNA was labeled in the same way as the targeted siRNA. The cells were transfected with complexes containing 50 μM siRNA mixed with TransIT-TKO transfection reagent (Mirus, Houston, TX) according to manufacturer recommendations in serum-free medium for 48 h. Then cells were washed and treated with 80 uM cisplatin for an additional 24 h, washed again and fixed with 4% paraformaldehyde, containing 0.012% saponine (w/v).

2.3. Immunohistochemistry and image analysis

The fixed cells were rehydrated and probed with polyclonal anti-EndoG antibody (Millipore, Billerica, MA) at 1:200 dilution at +4°C overnight. Secondary anti-rabbit-AlexaFluor 594 conjugate (Invitrogen, Carlsbad, CA) at 1:400 dilution was applied for 1 h at room temperature. After subsequent washing the cells were mounted under coverslips using the

Prolong® Antifade kit with DAPI (Invitrogen). The cells were visualized using an Olympus IX-81 microscope (Olympus America Inc., Center Valley, PA); images and acquisitions were made with a digital camera HAMAMATSU ORCA-ER (Hamamatsu Photonics K.K., Hamamatsu City, Japan) and software Slidebook 4.1 (SciTech Pty Ltd., Australia). For quantification three experimental points were taken (10 fields of view in each). Cells were masked, and integral optical density (IOD) of each channel was measured using the automated option of the mentioned software. The data were presented as averages of IOD_x/IOD_{DAPI} for each channel.

2.3. RNA extraction and real-time RT-PCR

The total RNA was extracted using RNeasy Mini kit from Qiagen (Valencia, CA) according to manufacturer recommendations. The quality of RNA was determined using 1.2% formaldehyde-agarose gel. Reverse transcription reaction was performed using the GeneAmp Gold RNA PCR core kit (Applied Biosystems, Foster City, CA) and Oligo $d(T)_{16}$. In general, 1 μg of total RNA was reverse-transcribed in a 50-μl reaction followed by real-time RT-PCR in a 25-μl reaction using SmartCycler (Cepheid, Sunnyvale, CA). Reaction mix was prepared using Platinum SYBR Green qPCR Supermix-UDG (Invitrogen, Carlsbad, CA) according to manufacturer recommendations and primers: 5′-CTACCTGAGCAACGTCGCG-3′ and 5′-TCCAGGTTGTTCCAGGCATT-3′. 18s ribosomal subunit RNA was amplified in parallel reaction using primers 5′-TTCGAACGTCTGCCCTATCAA-3′ and 5′-ATGGTAGGCACGGCGACTA-3′. Two-temperature cycles with annealing/extension temperature at 62°C for EndoG and 64°C for 18s were used. The melting curve analyses were performed at the end of the reaction (after the 45^{th} cycle) between 60°C and 95°C to assess the quality of the final PCR products. The C(t) values were calculated by using the basal fluorescence at 15 units. cDNA samples were diluted for real-time 1:5 and 1:200 for EndoG and 18s, respectively. Three replicate reactions were performed for each sample, and the average C(t) was calculated. The standard curve of the reaction effectiveness was performed using the serially diluted (5 points) mixture of all experimental cDNA samples for EndoG and 18s separately. Calculation of the relative RNA concentration was performed using Cepheid SmartCycle software (Version 2.0d). Data are presented as ratio of EndoG/18s mRNA.

2.5. Cell extracts

Cells ($2–4 \times 10^6$) were rinsed twice with phosphate buffered saline (PBS). Cells were suspended in buffer containing 50 mM Tris-HCl pH 7.9, 0.25 M sucrose, and the Complete Mini Proteinase Inhibitor Cocktail (Roche Diagnostics, Mannheim, Germany) (1 tablet/10 ml). Cells were then homogenized using a minihomogenizer (Fisher Scientific, Houston, TX). The samples were sonicated in the Virsonic 475 (Virtis, Gardiner, NY) (5 × 20 sec) to decrease viscosity. DNA was removed by centrifugation at 195,000 × g for 2 h. The extracts were dialyzed against storage buffer (55% Glycerol, 10 mM Tris-HCl pH 7.6, 0.5 mM dithiothreitol) and kept at −20°C for up to 2 weeks without loss of endonuclease activity. The protein concentration was measured using the Bradford method [66].

2.6. Plasmid incision assay (PIA)

This assay was used for the measurement of endonuclease activity as previously described [67]. The activity was measured in 20 μl samples containing 1 μg plasmid pBR322 DNA (New England Biolabs, Beverly, MA), 10 mM Tris-HCl, pH 7.7, 25 μg/ml bovine serum albumin V fraction, 0.5 mM dithiothreitol, 5 mM $MnCl_2$, and 2 μl of cell extract in serial dilutions 1:5. After 1 h incubation at 37°C, the reaction was stopped by the addition of 5 μl 1% SDS and 100 mM EDTA. Then digested DNA was subjected to 1% agarose gel electrophoresis at 7 V/cm for 1 h at room temperature. The gel was stained in 0.5 μg/ml ethidium bromide solution for 20 min and photographed under UV light. The EagleEye scanning densitometer (Stratagene, La Jolla, CA) was utilized to quantify the relative amount of endonuclease-treated plasmid DNA present in a covalently closed circular DNA (form 1), open circular DNA (form 2), or linear DNA (form 3). One DNase/endonuclease unit was the amount of the enzyme required to convert 1 μg DNA form 1 to DNA forms 2 and 3.

2.7. Methylation-sensitive McrBC-PCR assay

Methylation status of EndoG promoter was determined by McrBC-PCR assay as described previously [68,69]. Genomic DNA (1 μg) was digested with 20 units of McrBC endonuclease (New England Biolabs, Beverly, MA) for 12 h at 37°C. Undigested DNA served as the control. Following McrBC treatment, PCR was used to amplify the promoter sequence. The primers were designed to amplify CpG islands located within the 5′ region of the EndoG gene: sense 5′GCCGCGAGTCGTACGTGCTGTGCTA, reverse 5′GGGGCGCGACGTTGCTCAGGTAGA. Each PCR reaction contained 0.25 μg of undigested or McrBC-digested DNA and 50 pmol of each primer in 25 μl of 1X GC-RICH PCR System (Roche Diagnostic, Indianapolis, IN). The cycling conditions consisted of an initial denaturation at 95°C for 10 min, followed by 30 cycles of denaturation at 95°C for 30 s, primer annealing at 66°C for 60 s, and extension at 72°C for 60 s. The semi-quantitative aspect of the procedure was verified by a linear increase in PCR product recovery with increasing cycle number and DNA template concentration. The PCR products were separated on 2% agarose gels, stained with ethidium bromide, and photographed. The band intensity was measured by ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

2.8. Western blotting

Protein was separated in 11.5% gel according to the Laemmli procedure [70]. The total protein extract from cells (10–100 μg) was dissolved in 50 mM Tris-HCl, pH 6.8, 1% SDS, 2 mM EDTA, 1% 2-mercaptoethanol and 7.5% glycerol, and denatured by heating at 100°C for 10 min. Electrophoresis was performed at 100 V for 2 h. Proteins were transferred onto the nitrocellulose membrane in Novex transferring buffer (Invitrogen) at 40 V for 3 h, and stained with Ponseau S (Sigma) to control equal protein load as described elsewhere [71]. After soaking in the blocking solution overnight at 4°C, the membrane was incubated with polyclonal anti-EndoG antibody (Millipore) diluted 1:1000 and washed in Tris-buffered saline (TBS). Primary antibodies were detected with anti-rabbit IgG-horseradish peroxidase (HRP) using SuperSignal chemiluminiscent kit (Pierce Biotechnology, Rockford, IL).

2.9. Statistical analysis

The results were expressed as mean ± standard error of mean (SEM). Statistical analysis was performed using SPSS software (SPSS Inc., Chicago, IL USA). To evaluate the significance of differences between two the groups of experiments, the analysis of variance (ANOVA) and Student's t test were used. Additionally to evaluate the significance of several time points in comparison to one control point, the Bonferroni adjustment of the t-test was used. A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. CpG islands in promoter regions of the genes of cell death endonucleases

The mammalian genome depends on patterns of methylated cytosines for normal function, but until recently the structural organization of the methylation landscape of the human genome was unclear [40]. It has been reported that the human genome consists of short (<4 kb) unmethylated domains enriched in promoters, CpG islands, and first exons, embedded in a matrix of long methylated domains [40]. A large number of studies have shown that methylation of promoter CpG islands plays an important role in gene silencing [41,42]. The broadly accepted definition of a CpG island as a 200-bp fragment of DNA with G + C content greater than 50% and observed CpG/expected CpG ratio higher than 0.6 failed to exclude many sequences (such as *Alu* repeats and unknown sequences) that are not associated with regulatory regions of genes [43]. Recent studies indicate that the usage of a modified algorithm to search for CpG islands using a more stringent definition (G + C content higher than 55% and a length greater than 500 bp with observed CpG/expected CpG ratio 0.65) resulted in the exclusion of the majority of *Alu* repetitive and unknown sequences associated with the 5′ region of genes [43]. In view of these considerations, we applied this algorithm to the analysis of endonuclease genes, which could be regulated by DNA methylation. All known human cell death endonucleases and their sequence variants were analyzed using the CpG Island Searcher program (available at http://www.cpgislands.com [44]): DNase 1, DNase 1L1 variants 1, 2, 3 and 4; DNase 1L2, DNase 1L3 (DNase gamma), DNase 2α, DNase 2β variants 1 and 2, L-DNase II (LEI), caspase-activated DNase (CAD) and EndoG. This analysis showed that EndoG is the only gene that satisfied the criteria of containing a long CpG island in the promoter and exon 1 of the gene (Fig. 1).

3.2. Methylation of EndoG promoter in human prostate cancer cells

The methylation status of the EndoG promoter/exon 1 in prostate cancer cells was determined by using the methylation-sensitive McrBC-PCR method. McrBC is an endonuclease, that does not act on unmethylated DNA, but cleaves DNA containing 5-methylcytosine in one or both strands and thus nullifies PCR amplification [45]. This experiment showed that in the three prostate cancer cell lines, EndoG promoter methylation was lowest in 22Rv1 cells and highest in PC3 cells (Fig. 2).

3.3. EndoG expression

We next determined whether and how EndoG expression correlates with the methylation of the *EndoG* gene promoter. The expression of EndoG determined by real-time RT-PCR

showed that the level of EndoG inversely correlates with the level of the methylation in the *EndoG* promoter (Fig. 3A). Plasmid incision assay in the presence of Mn2+ ion was used to measure activity of EndoG, the only known Mn-dependent endonuclease, as described by us previously [46]. As expected, EndoG activity was higher in 22Rv1 and LNCaP cells than in PC3 cells (Fig. 3B,C). The Western blotting data described below also confirmed these observations.

3.4. Sensitivity of prostate cancer cells to cisplatin and etoposide

To determine whether the level of EndoG expression affects the sensitivity of prostate cancer cells to chemotherapeutic drugs, we exposed the three cell lines to two anticancer agents, cisplatin (0–100 μM) and etoposide (0–300 μM), which are known to induce cell death *in vitro* [16,47]. As expected, the two cell lines that expressed EndoG, 22Rv1 and LNCaP, were highly sensitive to both chemotherapeutic agents (Fig. 4). EndoG-deficient PC3 cells, in contrast, were insensitive to these drugs in the range of concentrations used.

3.5. Cisplatin-induced death of prostate cancer cells can be prevented by EndoG silencing

Although EndoG is known to participate in cell death, we needed to determine whether the role of EndoG was the same in prostate cancer cells subjected to injury by cytotoxic agents as has been described in other cells. To determine a causal relationship, we silenced EndoG in 22Rv1 cells by applying siRNA. To show that siRNA was delivered to the cells, we used DY547-labeled siRNA. After DY547-siRNA transfection, 22Rv1 cells were exposed to 80 μM cisplatin, a concentration that had induced significant cell death in the experiments described above. Next, we conducted TUNEL assay to measure DNA fragmentation, and observed that it was decreased, showing that silencing of EndoG leads to significant decrease of EndoG expression and protects cells from DNA fragmentation (Fig. 5A, B). As expected, EndoG silencing resulted in the increased viability of cisplatin-treated 22Rv1 cells as measured using clonogenic assay (Fig 5C). Our results suggest that EndoG is responsible for cisplatin-induced death in prostate cancer cells.

Inhibition of DNA methylation induces EndoG and increases sensitivity of PC3 cells to cisplatin and etoposide—To determine whether EndoG expression is regulated by DNA methylation, we treated PC3 cells with 5′-aza-2-deoxycytidine (decitabine), a DNA methylation inhibitor. Using McrBC-PCR method, we determined that the treatment of PC3 cells with decitabine inhibited methylation of the CpG island in the *EndoG* gene (Fig. 6A,B). The same concentration of decitabine also increased EndoG expression as determined by real-time RT-PCR and Western blotting (Fig. 6C,D). These data suggest that EndoG expression is regulated by DNA methylation. Importantly, the induction of EndoG by demethylation caused a significant increase in sensitivity to cisplatin and etoposide (Fig. 7).

3.6. Role of histone acetylation in regulation of EndoG expression in prostate cancer cells

Histone modification, in particular histone acetylation, is another epigenetic mechanism that is important in regulation of genes in prostate cancer [7,8,48]. To determine whether and how histone acetylation regulates EndoG expression, two prostate cancer cell lines were treated with the histone deacetylase inhibitor trichostatin A (TSA), and EndoG protein

expression was studied using Western blotting. The exposure of the cells to TSA induced high levels of EndoG expression in EndoG-positive 22Rv1 cells, whereas in EndoG-deficient PC3 cells, EndoG was not induced (Fig. 8A). As expected, EndoG induction by TSA caused increased sensitivity to cisplatin (Fig. 8B). These data demonstrate that chromatin acetylation is important for EndoG expression. Taken together with the above experiments, these data also indicate that DNA methylation plays a primary role in EndoG regulation as compared to histone acetylation. In other words, the CpG island of the *EndoG* gene has to be hypomethylated in order to allow regulation of EndoG expression by histone acetylation.

4. Discussion

Existing studies of endonucleases in either normal prostate tissue or prostate cancer cells are limited [49–55]. The degradation of genomic DNA into nucleosome-sized fragments was shown to be an early event in castration-induced androgen withdrawal that involves the death of the androgen-dependent epithelial cells, following an increase in endonuclease activity [49–51]. Anticancer drugs were shown to induce death of cancer cells through endonuclease-mediated DNA fragmentation [52,53], and inhibition of endonuclease activity had a protective effect [52]. Although endonuclease-mediated DNA fragmentation is commonly used as a marker of apoptosis in prostate cancer [54,55], there are virtually no studies performed on the mechanisms of DNA fragmentation by endonucleases or the regulation of these enzymes in prostate cancer.

In general, there is no difference between apoptosis and necrosis in terms of endonuclease-mediated DNA fragmentation. In both processes, the fragmentation of DNA is produced by the same group of cytotoxic endonucleases. Our recent studies showed that if the impact is weak or the used model is strictly apoptotic, for example, in etoposide-induced cell injury [32], EndoG and other endonucleases work as apoptotic enzymes. Strong *in vitro* injuries of cells or *in vivo* injuries usually force endonucleases to act as executioners of necrosis [33,35]. In the present study, as before [32,33,46], we use LDH release as a universal method to assess cell death.

DNA methylation of cell death-related genes was shown to be involved in pathogenesis and to correlate with the prognosis of prostate cancer [56]. Demethylation is known to regulate DNA damage-and death-related genes. For example, Pulukuri & Rao [57] demonstrated that demethylation of p53 and p21Waf1/Cip1 pathway in LNCaP cells led to the inhibition of cell proliferation as well as the induction of apoptosis. Our data are similar to those in a recent report by Das et al. [48] wherein it was observed that the loss of TMS1/ASC gene expression associated with complete methylation of the promoter region in LNCaP cells was restored by AzaC, while TSA had no effect. The role of DNA methylation in regulation of cytotoxic endonucleases has not been studied.

To study the potential regulation of EndoG by methylation, we first compared the promoter and exon 1 regions of all known human endonucleases involved in cell death. This comparison showed that EndoG promoter/exon 1 contains the longest CpG island of all cell death endonuclease sequences, and thus it is likely to be methylated. We found that two

human prostate cancer cell lines, 22Rv1 and LNCaP, in which EndoG is highly expressed, contain unmethylated EndoG CpG islands, while in PC3 cells, EndoG is silenced and the EndoG CpG island is methylated. By using specific siRNA, EndoG was shown to be responsible for cisplatin or etoposide-induced cell death in 22RV1 and LNCaP cells. Unlike the two other prostate cancer cell lines, the EndoG-deficient PC3 cells were insensitive to cisplatin or etoposide-induced cell death. Inhibition of methylation by AzaC increased EndoG expression in PC3 cells and made them sensitive to the cytotoxic chemotherapeutic drugs.

The induction of EndoG by promoter hypomethylation may explain the synergy between AzaC and cisplatin during treatment of prostate cancer DU145 as observed by others [47]. The observation that EndoG is important in inducing the death of prostate cancer cells may explain the resistance of prostate cancer to cisplatin and camptothecin reported in some studies [58,59]. Our data provide further evidence that EndoG is the endonuclease that is important in cell death induced by cell injury.

It is interesting that PC3 is not only chemoresistant but is also an invasive cell line, while the two other prostate cancer cell lines are not invasive. This finding is in agreement with our previous study in which we determined that endonuclease activity in the differentiated non-invasive human breast cancer cell lines was higher than that in the poorly differentiated invasive cells [32]. In these cells, the expression of EndoG was strongly correlated with the degree of estrogen receptor expression and showed an inverse correlation with vimentin and matrix metalloproteinase-13. The EndoG-positive, well-differentiated, non-invasive cells were more sensitive to etoposide- or camptothecin-induced cell death than the EndoG-negative, poorly differentiated, invasive cells. These findings suggest that the presence of EndoG in non-invasive cancer cells determines their sensitivity to cytotoxic agents. Downregulation of endonucleases was also observed in diethylnitrosamine (DEN)-induced hepatomas in rats, compared to normal liver tissue [60]. The decrease was proportional to the degree of dedifferentiation, and endonucleases activity was lowest in poorly differentiated tumors. Immortalization of rat fibroblasts with the S1A segment of SA7 adenovirus also led to a significant decrease of endonuclease activity [61]. However, the particular endonuclease that was decreased in tumor cells was not identified in these studies.

Future studies may be necessary to determine the role of other epigenetic mechanisms in regulation of EndoG and their role in chemoresistance to prostate cancer and cancers of other organs. Chemotherapy is currently one of the frequently used therapeutic strategies for prostate cancer [62–64], and measurement of EndoG may be a potentially useful approach to evaluate chemosensitivity of cancer cells to determine optimal conditions for chemotherapy. Manipulating with sensitivity to cytotoxic agents to alter cancer progression has been suggested as a therapeutic approach for prostate cancer in several studies [10,11]. If *in vivo* studies confirm our observation that EndoG is a potential key mediator of prostate cancer cell death regulated by the methylation of *EndoG* gene promoter, future epigenetic therapeutics will need to be targeted to EndoG.

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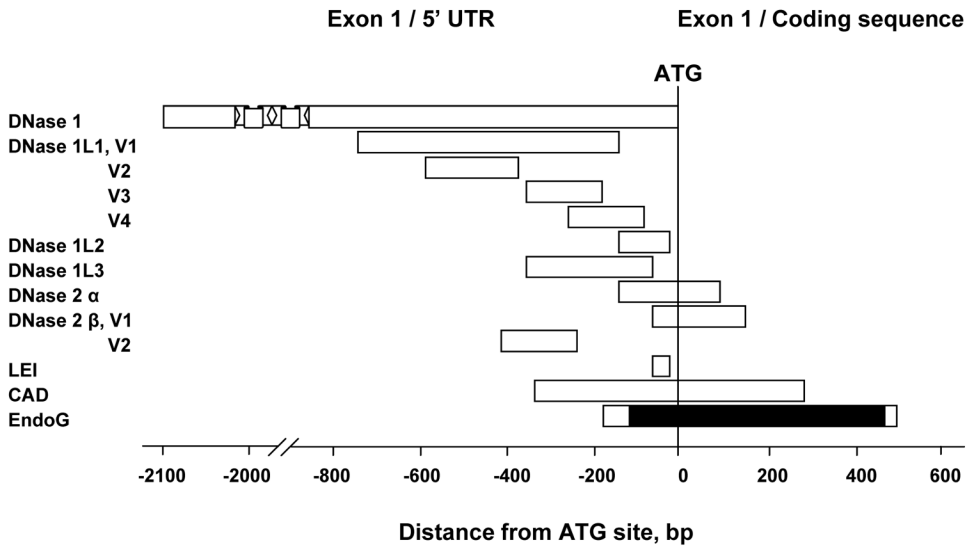

Fig. 1.

Identification of CpG island only in promoter and exon 1 regions of EndoG gene among all known human cell death endonucleases including sequence variants. The criteria for CpG island identification were: length > 500bp, GC > 55%, observed/expected ratio > 0.65). DNase 1 (GenBank accession # NM 005223.3), DNase 1L1 variants 1 (NM 006730.2), 2 (NM 001009932.1), 3 (NM 001009933.1) and 4 (NM 001009934.1), DNase 1L2 (NM 001374.2), DNase 1L3 (DNase γ) (NM 004944.2), DNase 2α (NM 001375.2), DNase 2β variants 1 (NM 021233.2) and 2 (NM 058248.1), L-DNase II (LEI) (NM 030666.2), caspase-activated DNase (CAD) (NM 004402.2) and EndoG (NM 004435.2). CpG island in EndoG promoter/exon 1 is shown in solid black color.

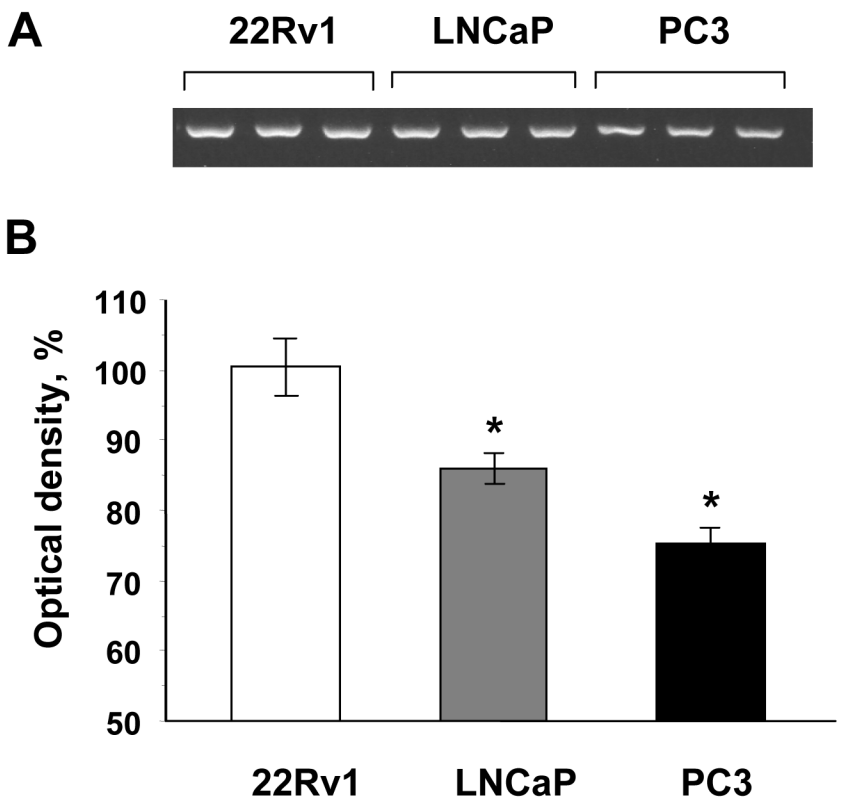

Fig. 2.

Methylation of EndoG promoter/exon1 region in human prostate cancer cell lines. (A) EndoG promoter/exon1 DNA methylation measured by the McrBC-PCR screening method. (B) Quantification by densitometry. Optical density of 22Rv1 cells was considered 100%. n = 3 per group, * $p < 0.05$ compared to 22Rv1 cells.

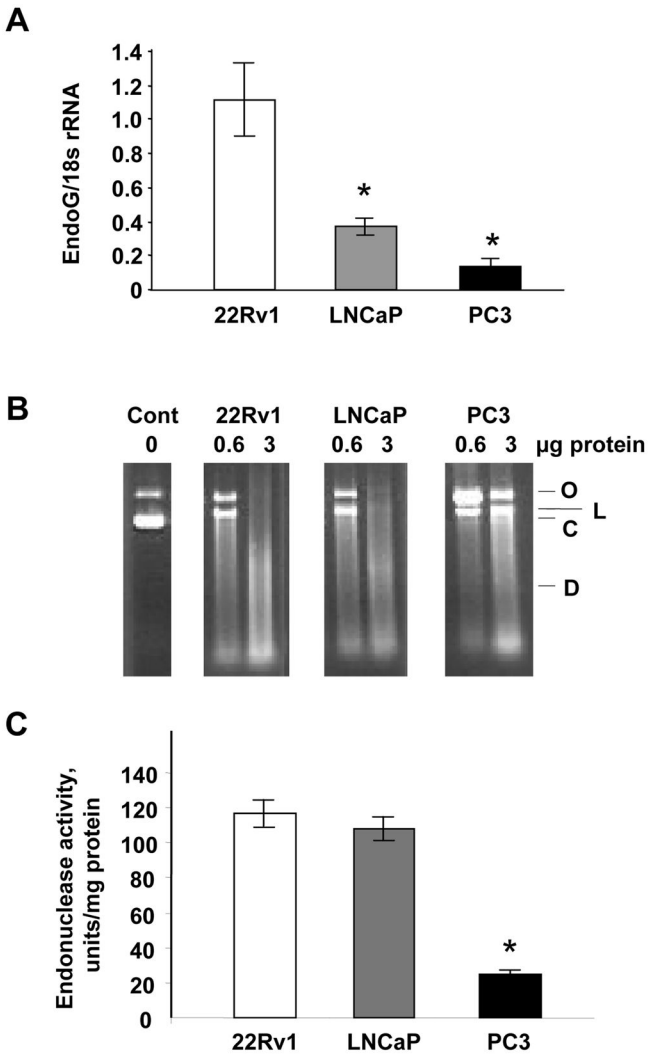

Fig. 3.

EndoG expression in human prostate cancer cell lines. (A) Expression of EndoG measured by using real-time RT-PCR. n = 4 per group, *p <0.001 compared to 22Rv1 and <0.05 compared to LNCaP cells. (B) Mn-dependent endonuclease (EndoG) activity in total protein extracts from the cells as measured using plasmid incision assay. O, open circular DNA; L, linear DNA; C, covalently closed supercoiled DNA, D, digested (fragmented) DNA. Each cell extract was tested using two concentrations of protein, 0.6 or 3 µg per sample. (C) Quantification of the plasmid incision assay. n = 4 per group, * p<0.001 compared to 22Rv1 or LNCaP cells.

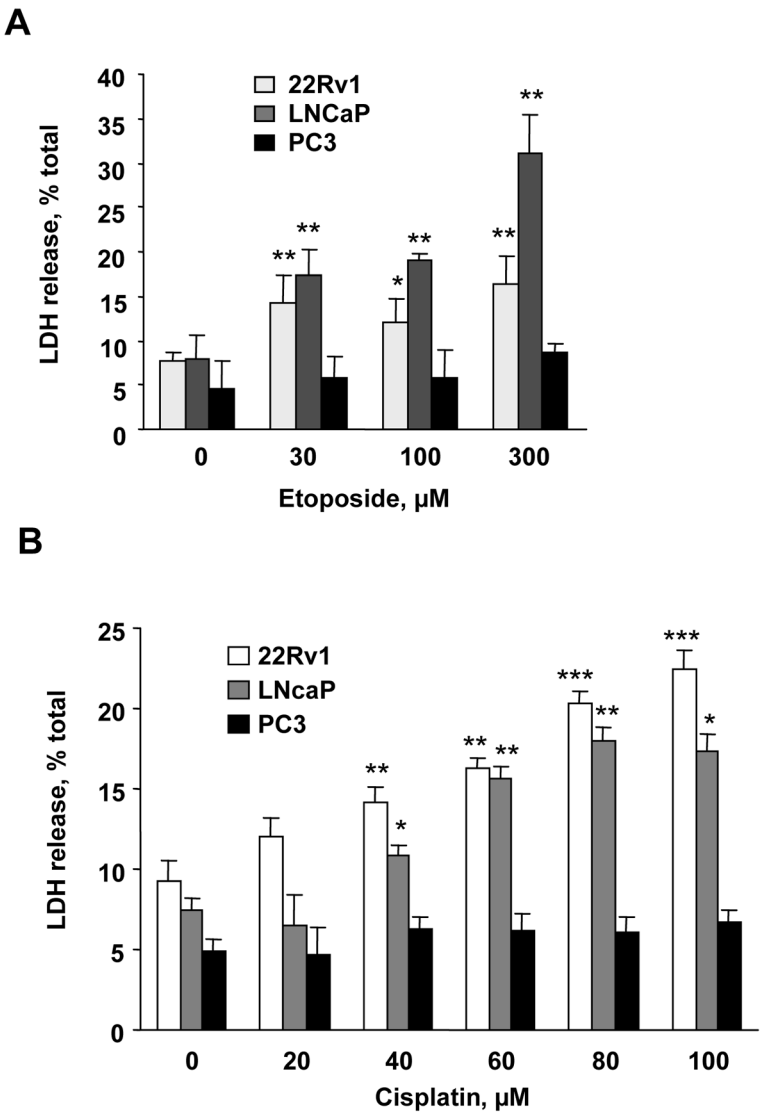

Fig. 4.

Sensitivity of prostate cancer cells to cisplatin and etoposide. (A) 22Rv1, LNCaP and PC3 cells were treated with cisplatin (0–100 μM) or (B) etoposide (0–300 μM) for 24 h. Cell death was measured using LDH release assay. n = 4 per group, *p <0.05, **p <0.01, ***p < 0.001 compared to zero point for the same cell line.

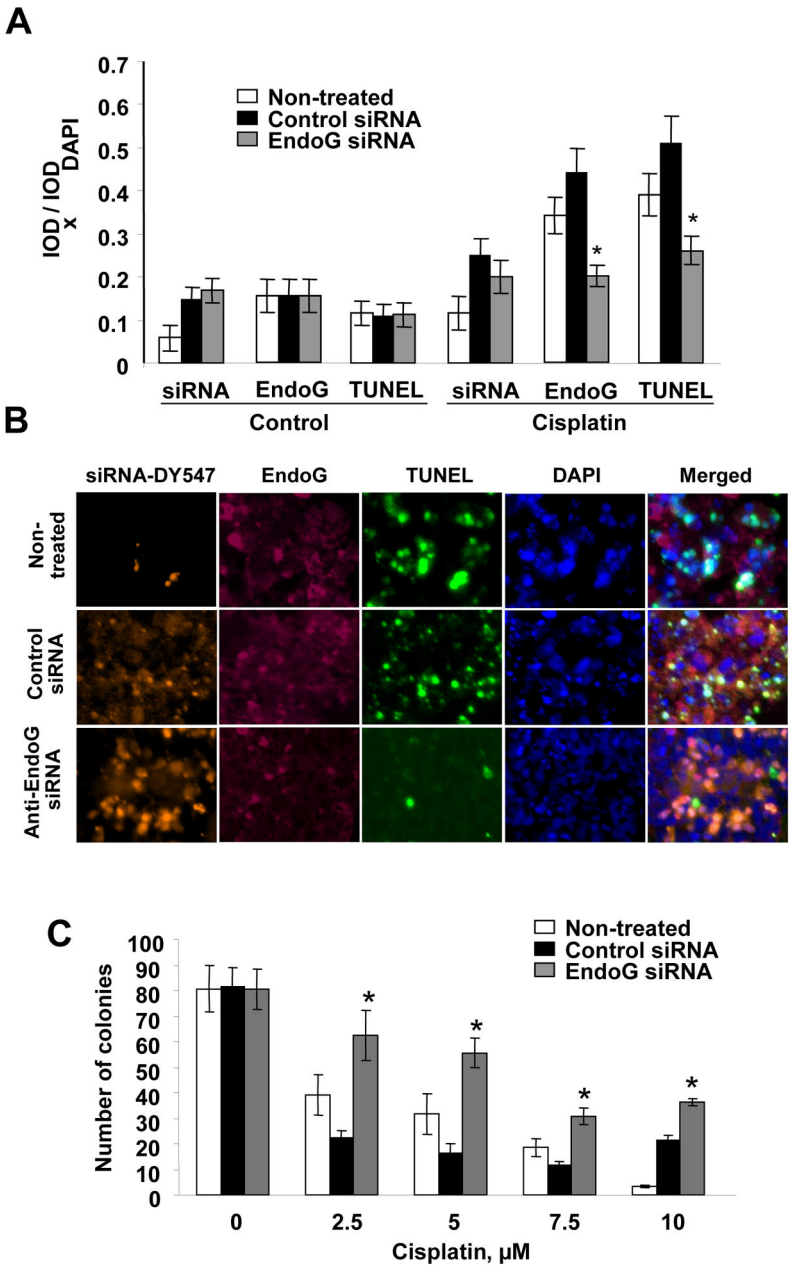

Fig 5.

Cisplatin-induced death of prostate cancer cells can be prevented by silencing EndoG. (A) EndoG-positive 22Rv1 cells treated with DY547-labeled anti-EndoG siRNA had EndoG expression and DNA fragmentation (TUNEL) significantly decreased compared to the cells treated with transfection reagent only (“non-treated”) or with non-specific control siRNA, and were protected from cell death induced by cisplatin (80 μM) exposure for 24 h. n=4 per group, *p<0.05 compared to non-treated or cells treated with control siRNA. (B) Representative images of the cells after treatment with cisplatin. Bar, 10 μm. (C) Silencing of EndoG in 22Rv1 cells by anti-EndoG siRNA caused increased viability of the cells during

exposure with cisplatin (2.5–10 μM) (n=4 per group, *p<0.01 to non-treated or cells treated with control siRNA).

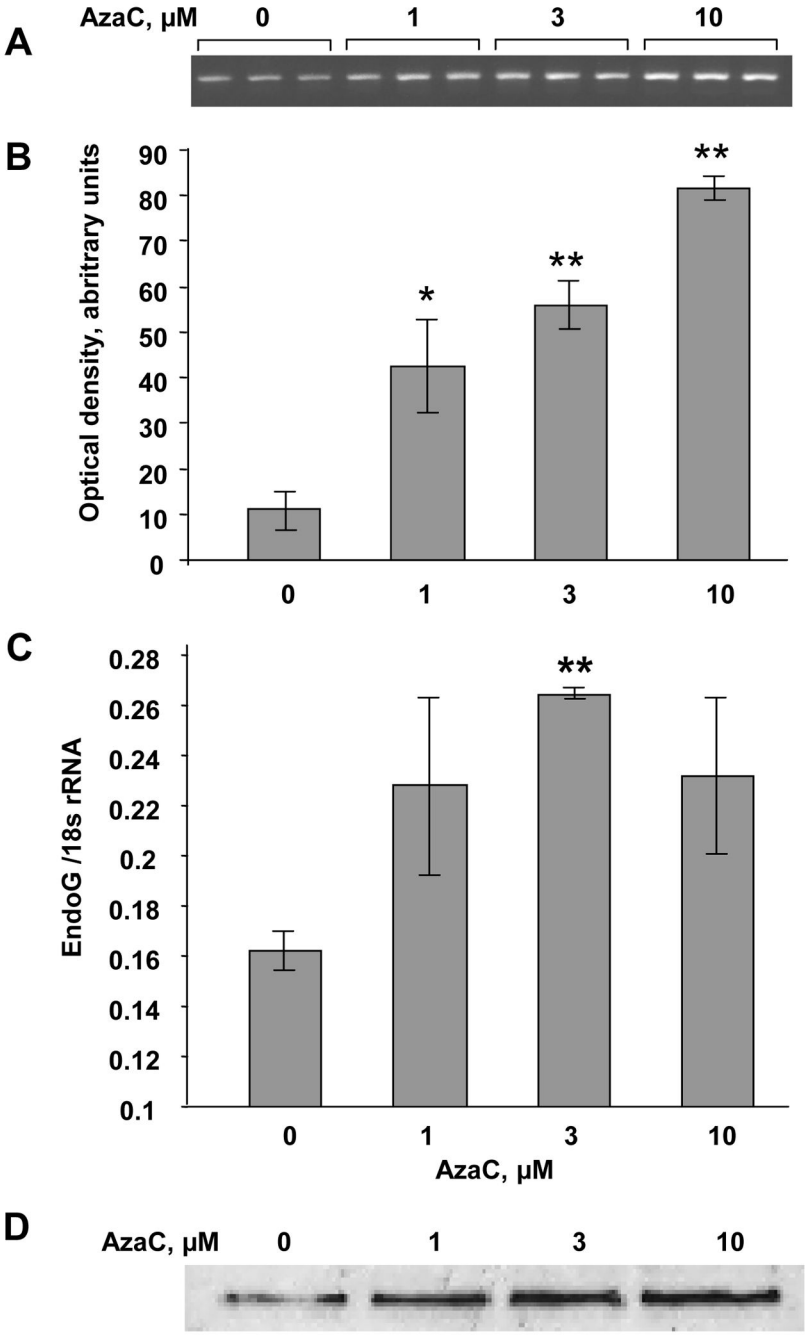

Fig. 6.

Inhibition of DNA methylation induces EndoG expression in PC3 cells. The cells were exposed with AzaC (0–10 μM) for 24 h. (A, B) EndoG promoter methylation was measured using the McrBC-PCR screening method. n = 3 per group, n = 3 per group, * p <0.05, ** p <0.01 compared to zero point. (C) EndoG expression assessed by real-time RT-PCR. n = 4 per group, * p <0.01, (D) EndoG protein expression determined by Western blotting. The membrane was stained with Ponseau S to control equal protein load (75 μg/well).

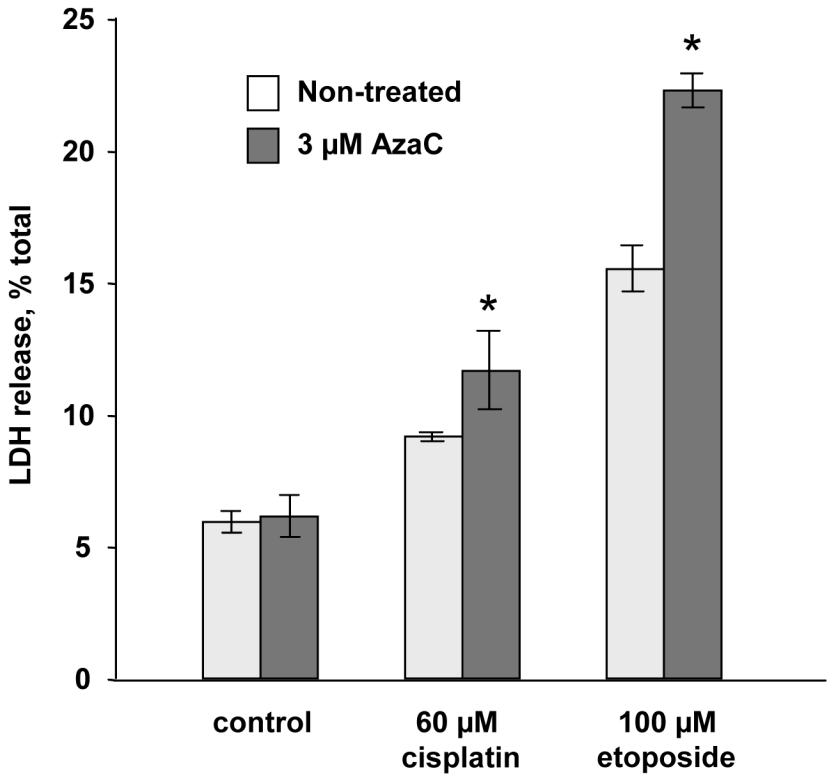

Fig. 7.

Inhibition of DNA methylation increases cisplatin and etoposide sensitivity of PC3 cells. The cells were exposed to AzaC (3 μM) for 24 h. EndoG mRNA induction was confirmed by real time RT-PCR (in Figure 6C above). The cells were then exposed to cisplatin (60 μM, 24 h) or etoposide (100 μM, 24 h). Cell death was measured by the LDH release. n=4 per group, *p<0.05 compared to corresponding non-treated cells.

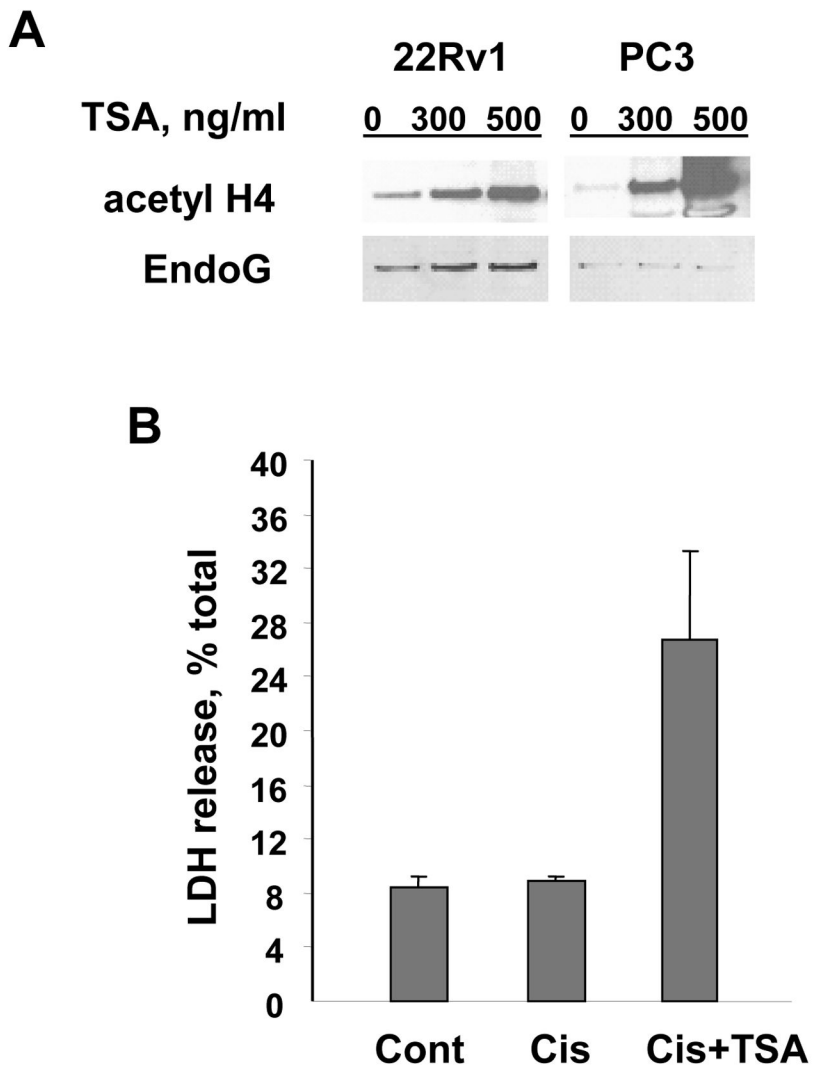

Fig. 8.

Histone acetylation induced by TSA causes EndoG induction and increased sensitivity to cisplatin. (A) Histone acetylation induced by TSA caused EndoG induction only in EndoG-expressing 22Rv1 cells but not in EndoG-negative PC3 cells as determined by Western blotting. The membranes were stained with Ponseau S to control equal protein load (30 μg/well). Histone H4 acetylation was assessed using polyclonal acetyl-histone H4 antibody (Upstate, Lake Placid, NY). (B) Induction of EndoG by TSA (100ng/ml) in 22Rv1 cells was associated with the increased sensitivity to cisplatin (Cis, 25 μM).