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# Methylation of a Single Intronic CpG Mediates Expression Silencing of the *PMP24* Gene in Prostate Cancer

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## Abstract

**BACKGROUND—**We previously demonstrated that a putative anti-tumor gene, *peroxisomal membrane protein 4, 24 kDa (PMP24 or PXMP4)*, is silenced via DNA methylation of a CpG island in its 5′ flanking region (5′-CGI) in prostate cancer (PCa) cells.

**METHODS—**To identify demethylation hypersensitive site(s) in *PMP24* 5′-CGI, PC-3 cells with methylated 5′-CGI were treated with a low-dose of 5-aza-2′-deoxycytidine (5-aza-dC) just sufficient to reactivate gene expression, referred as the *limited demethylation approach*. Gel shift assays and promoter analyses were performed to demonstrate the role of the hypersensitive site in *PMP24* gene regulation. Transfection of a methylated oligonucleotide corresponding to the hypersensitive site was conducted to determine the effect of site-specific methylation on the gene expression. Bisulfite sequencing analysis was performed to reveal the methylation status of *PMP24* promoter in cultured cells and microdissected samples. *In situ* hybridization was applied to determine expression positivity of *PMP24* mRNA.

**RESULTS—**A 5-aza-dC hypersensitive site encompasses two CpG dinucleotides in intron 1 was identified. Methylation of the first, but not the second, CpG dinucleotide of this site disrupted DNA-protein interactions and suppressed the gene expression. Using archival specimens, we found the first CpG dinucleotide of the hypersensitive site is hypermethylated with a loss of *PMP24* mRNA expression in microdissected PCa cells when compared to normal prostatic epithelial cells.

**CONCLUSIONS—**These findings support a critical role for a single intronic CpG dinucleotide in *PMP24* gene regulation through DNA methylation. The data suggest that methylation-mediated silencing of *PMP24* is a molecular event associated with prostate carcinogenesis.

### Keywords

*PXMP4*; intronic regulatory sequence; limited demethylation approach; methylation-demethylation hypersensitive site; 5-aza-2′-deoxycytidine

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## INTRODUCTION

DNA methylation is widely involved in tumorigenesis, and changes in both its level and pattern have been reported in virtually all types of cancers, including prostate cancer (PCa) [1–3]. Aberrant hypermethylation of promoter CpG islands (CGIs) in cancer is of particular interest because of its close association with gene silencing, especially when the silencing occurs to tumor suppressor genes such as *BRCA1* [4], *MLH1* [5], and *p16* [6]. Tumor suppressor genes often can be reactivated by reducing the extent of methylation in their regulatory CGIs through treatment with epigenetic modifiers such as 5-aza-2′-deoxycytidine (5-aza-dC), offering an attractive option for cancer treatment [7,8]. In this regard, 5-aza-dC and other DNA methyltransferase inhibitors have been tested in multiple clinical trials for the treatment of patients with hematologic malignancies [9]. However, the efficacy of these drugs in activating tumor suppressor genes is dependent on both the cell type and the target gene [10,11], and their anti-cancer activity may involve both DNA methylation-dependent and -independent pathways [12]. Therefore, the complex relationship between aberrant promoter CGI methylation and gene silencing requires further exploration, with an anticipatory outcome of improving the clinical potential of demethylating agents as cancer therapies.

CGIs located in the 5′ regulatory region of the genes usually encompass the promoter, the first exon, and occasionally the first intron [13]. Hypermethylation of 5′-CGIs is thought to repress gene transcription by interfering with transcription initiation. However, other studies have shown that exons and introns further downstream can contribute to gene regulation via DNA methylation [14,15]. Studies have linked DNA methylation-regulatory proteins (eg, DNA methyltransferases and methyl CpG binding-domain proteins) and histone modification proteins (eg, histone deacetylases and acetylases) to higher-order chromatin remodeling events (eg, nucleosome destabilization) as a mechanism of epigenetic regulation of gene expression [2,16]. These proteins, working in concert, facilitate the assembly of a repressive chromatin that is inaccessible to gene-specific transcription factors (TFs) and/or the general transcriptional machinery involved in the initiation of transcription. DNA methylation also directly affects gene transcription by steric interference of TF binding to *cis*-elements in the promoter region [17]. We previously demonstrated the correlation between the methylation of three CpG clusters (methylation hotspots) in the *estrogen receptor* β (*ERβ)* promoter-CGI and the silencing of the gene in clinical PCa specimens [18]. More recently, we found that the activator protein 2 (AP-2) interacts with the most 5′ methylation hotspot (16-mer) and elicits the transcription of *ER*β [19], hence establishing this methylation hotspot as a regulatory *cis*-element. These findings illustrate how methylation of the *cis*-acting element of a key TF of a gene conveys epigenetic suppression.

In the prostate, androgen signaling plays a critical role in cancer development [20–22]. In a previous study, using methylation-sensitive restriction fingerprinting [23], we identified the *peroxisomal membrane protein 4, 24 kDa (PMP24* or *PXMP4)* [24], as a gene that undergoes DNA hypermethylation-mediated transcriptional silencing during the transition of LNCaP, a PCa cell line, from androgen dependence to androgen independence [25]. PMP24 is a 24 kDa peroxisomal intrinsic membrane protein [24] with unknown function although recent research has established an indispensable role of peroxisomes in catalyzing a number of essential metabolic functions including fatty acid beta-oxidation, ether phospholipid biosythesis, fatty acid alpha-oxidation and glyoxylate detoxification [26]. We found that *PMP24* has a 5′-CGI with 43 CpG dinucleotides encompassing the proximal promoter, exon 1, and part of intron 1 [25]. The gene is actively transcribed in LNCaP and immortalized human normal epithelial cells (NPrEC) [27] and its 5′-CGI is unmethylated in these cells. In the androgen-independent subline, LNCaP$^{CS}$, generated by maintaining LNCaP in medium

with charcoal-stripped (CS) serum for over 30 passages, *PMP24* is silenced and exhibits a densely methylated 5′-CGI. *PMP24* is also silenced in PC-3, an androgen-independent PCa cell line. Treatment of $LNCaP^{CS}$ and PC-3 with 5-aza-dC readily re-activates expression of *PMP24* and induces demethylation of its 5′-CGI. Ectopic expression of *PMP24* in $LNCaP^{CS}$ and PC-3 induces a significant reduction in cell growth and colony-formation potential on soft agar, suggesting a tumor-suppressing role for *PMP24* [25].

In this study, we aimed to identify specific CpGs in the 5′-CGI of *PMP24*, whose methylation status is essential for gene regulation. A *limited demethylation approach* with fine-tuned, low concentrations of 5-aza-dC was used to identify hypersensitive CpG dinucleotides. In parallel, methylated sense oligonucleotides targeting specific CpGs was used to achieve *in cellulo* sequence-specific methylation [18]. Deletion- and site-directed mutants were then used to validate the transcriptional activities of the putative regulatory CpGs. Laser capture microdissection (LCM) was used to acquire pure populations of normal and malignant prostatic epithelial cells from clinical specimens for subsequent assessment of their *PMP24* 5′-CGI methylation status *in vivo*. The expression of *PMP24* transcript in PCa foci and benign glands were evaluated by *in situ* hybridization. These studies conjointly identified a single CpG dinucleotide in the intronic region of the 5′-CGI whose methylation status is critical to the transcription of *PMP24 in vitro* and *in vivo*. Our findings implicate an involvement of methylation-mediated silencing of *PMP24* in prostate carcinogenesis.

## METHODS AND METHODS

### Prostate Samples and Laser Capture Microdissection

Formalin-fixed, paraffin-embedded sections were obtained from archival collections of radical prostatectomy specimens [18]. Seven cases of specimens were used to acquire seven LCM samples of benign prostate epithelial cells. Fourteen cases of PCa tissues were used for LCM to obtain 16 samples of PCa cells. For *in situ* hybridization, sections from another seven cases of prostate specimens which contained both benign glands and PCas were used. The use of these samples was reviewed and approved by the respective institutional review boards at the University of Cincinnati and University of Massachusetts Medical School.

### Cell Culture and 5-aza-dC Treatment

Prostate cancer cell lines were purchased from the American Type Culture Collection (Manassas, VA) and maintained according to the supplier's suggestions. An immortalized human normal prostate epithelial cell line (NPrEC) was generated in our laboratory and maintained as previously described [27]. For the demethylation assay, PC-3 cells were seeded at a density of $1 \times 10^5$ cells per T25 flask at day 0, one day before treatment with 5-aza-dC (Sigma, St. Louis, MO). The cells were treated for a total of 5 days with medium containing fresh 5-aza-dC changed every two days. The cells were collected at day 6 for bisulfite sequencing and real-time RT-PCR analyses.

### Bisulfite Sequencing Analyses and Real-time RT-PCR

The LCM bisulfite-sequencing analysis was targeted on a 372 bp region within the CGI encompassing 37 CpG dinucleotides. The primers P24-bisF1/P24-bisR1 and P24-bisF2/P24-bisR2 (Table I) were used in the first round and nested PCR, respectively. The amplified region was from −151 to +221 (Figure 1A), with the translation start site designated as +1. The real-time RT-PCR primers for *PMP24* transcript were PMP24f and PMP24r (Table I). *GAPDH* and *18S* RNA was used as the controls [19].

### Gel Shift Assay

A 30-bp putative *cis*-element encompassing the hypersensitive site was used for the gel shift assay (Table I). Fluorescence dye IRDye 800-labeled probe (Li-Cor, Lincoln, NB), together with the unlabeled (cold) wild-type or mutated oligonucleotides (Table I), were used in the assay to determine the binding specificity. Methylated probe was generated by *in vitro* methylation of oligonucleotides with *M. SssI* CpG methyltransferase (NEB, Beverly, MA) and purified with the QIAquick nucleotide removal kit (Qiagen, Valencia, CA). Nuclear proteins were extracted by Nuclear Extract Kit from Active Motif (Carlsbad, CA). DNA-protein binding reactions were set up with 5 μg of NPrEC nuclear extract in 1× binding buffer (20 mM HEPES, pH 7.9, 100 mM KC1, 2.5 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM DTT) containing 5% glycerol and 0.1 mg/ml poly d(I-C) with or without unlabeled competitive oligonucleotides. After 10 min of incubation at 22°C, 0.05 pmol of IRDye-labeled probe was added to each reaction and incubated for another 20 min before separation on 6% DNA Retardation Gels (Invitrogen, Carlsbad, CA) and detected by the Odyssey Infrared Imaging System (Li-Cor).

### Construction of Promoter-Luciferase Plasmids

Luciferase reporter vector pGL3 basic (Promega, Madison, WI) was used in the promoter-reporter analysis. As the 5′ UTR and a part of the open reading frame of *PMP24* gene was included, the vector was modified with an insertion of a 0.6-kb internal ribosomal entry site (*IRES*) [28] immediately upstream of the luciferase gene and named pGL3/IRES. The *IRES* element eliminates the potential interference from the open reading frame of *PMP24* on the translation of the luciferase reporter gene. The promoter region from −415 to +330 of the *PMP24* gene was inserted upstream of *IRES* in pGL3/IRES vector and designated as pGL3/IRES/(−415/+330). Additional constructs with nested 3′-end deletions of the promoter including pGL3/IRES/(−415/+194) and pGL3/IRES/(−415/+93) were generated using the Delete-A-Base kit (Promega). Plasmids with mutations of the two intronic CpG dinucleotides in the promoter-hypersensitive region *cis*-element (Figure 1 and Table I) were generated from pGL3/IRES/(−415/+330) using the GeneTailer Site-Directed Mutagenesis System (Invitrogen) and referred to as pGL3/IRES/(−415/+330)/mutCG1 and pGL3/IRES/(−415/+330)/mutCG2, respectively. The sequence of the mutated region is the same as the corresponding mutated probe sequence used in the gel shift assay (Table I). The promoter sequence was confirmed by sequencing (Macrogen Inc, Korea).

### Promoter Luciferase Assay

Transient transfection of the promoter-reporter plasmid into LNCaP cells was performed with Lipofectamine and Plus Reagents (Invitrogen) according to the manufacturer's instructions. In brief, $6 \times 10^4$ cells were plated in each well of a 24-well plate for 48 h before transfection with 0.2 μg of promoter-luciferase plasmid and 40 ng of *CMV* promoter-driven *LacZ* gene plasmid which expresses β-galactosidase as the internal control. The cells were then incubated with complete medium for 24 h before luciferase assay (Bright-Glo Luciferase Assay Kit, Promega). Luciferase and β-galactosidase assays were performed as previously described [29]. Relative luciferase activity of each sample was normalized by its β-galactosidase activity.

### Delivery of Methylated Oligonucleotides to NPrEC Cells

A 16-bp phosphorothioated oligonucleotide (Proligo, Boulder, CO) with 5′-methylcytosines (mC) in CpG dinucleotide, named metODN (Table I), was used for targeting the 5-aza-dC-hypersentive site (Figure 4). Unmethylated phosphorothioated oligonucleotide was used as a control. NPrEC cells that express *PMP24* with an unmethylated promoter background were seeded at $3 \times 10^5$ cells per well in a 6-well plate for 24 h before transfection. Two rounds of

transfection of the oligonucleotides at 2 μM were carried out using Lipofectamine and Plus reagents. The experiment was repeated once to generate four samples for each regimen. Transfected cells were harvested after 96 h for real-time RT-PCR and bisulfite sequencing analyses.

### SiRNA-mediated Knockdown and Ectopic Expression in NPrEC Cells

SiRNA knockdown of *AP-2α*, *-2γ* and *Sp1* were performed as previously described [19,29]. TransIT TKO kit (Mirus, Madison, WI) was used according to the manufacturer's recommendation for siRNA transfection to NPrEC cells. The ectopic expression of *AP-2* isoforms in NPrEC cells was achieved by transfection of expression plasmids [19] into these cells using the human PrEC Nucleofector kit purchased from Lonza (Valais, Switzerland).

### *In Situ* Hybridization (ISH) of PMP24 Transcript and Evaluation of Expression

ISH was performed on sections of formalin-fixed paraffin-embedded clinical specimens containing benign glands and cancer foci. The criteria for the probe design were based on the previously described approach [30,31] and a scrambled probe served as a negative control (Exiqon, Denmark). The sequences of the probes are listed in Table I. The oligo DNA template for the cRNA probe was generated as reported [29]. The DIG-labeled cRNA probes were synthesized according to the protocol (Roche Applied Science, Indianapolis, IN). The ISH for *PMP24* transcript was performed based on a published method [32] with the following modifications: acetylation with 0.1 M triethanolamine and 0.25% acetic anhydride was performed for 10 min prior to prehybridization. Hybridization was carried out at 55°C for 16 hr. The signals were developed with BCIP/NBT substrate (Millipore, Berillica, MA), and amplified by the TSA DNP (AP) System (Perkin Elmer, Waltham, MA). The sections were counterstained with methyl green for nuclear staining.

In each sample, 3–5 representative foci of benign glands and PCa were selected to evaluate *PMP24* transcript expression (positive or negative), and were represented as the percentage of cells expressing the transcript. Evaluation was conducted by two individuals (XZ and MTL) under light microscopy and the percentage of positivity in each focus was averaged.

### Bioinformatics and Statistical Analyses

Promoter CpG island was analyzed by Methprimer [33]. Putative TF binding sites in the *PMP24* 5′-CGI was predicted with the MatInspector (www.genomatix.de) based on the TRANSFAC database.

In the promoter-reporter assays, a one-way analysis of variance (ANOVA), followed by Tukey's HSD *post hoc* test (SigmaPlot version 9.01; Systat Software Inc., Chicago, IL), was used for the comparisons of the promoter activity between the mutated or truncated promoter constructs with the wild-type promoter of pGL3/IRES(−415/+330). In the real-time RT-PCR, a two-tailed, unpaired *t*-test was performed between two groups. The statistical significance of the differences of methylation percents between microdissected benign (n=7) and cancer foci (n=16) was evaluated. Logistic regression was performed to compare methylation percents for each CpG dinucleotide. In order that inferences may be applied to any set of samples and not just the samples that were observed, a random sample effect was modeled. Probabilities (*p*-values) were calculated testing the difference between mean methylation percents in the two groups for each CpG dinucleotide. The analysis was preformed using the GLIMMIX procedure in the Statistical Analysis System (SAS, version 9.2; SAS Institute Inc., Cary, NC). Unless otherwise stated, $p<0.05$ was considered as statistically significant and error bars represented standard deviation.

# RESULTS

## Identification of a Single Intronic CpG Cluster Exhibiting Hypersensitivity to 5-aza-dC-demethylationand Its Correlation with Gene Re-activation

The *PMP24* 5′-CGI encompasses the promoter, first exon, and a part of intron 1 (Figure 1A). *PMP24* expression, quantified by real-time RT-PCR, was highest in NPrEC, followed by LNCaP and DU145 (Figure 1B). LNCaP$^{CS}$ and PC-3 cells are devoid of expression. The degree of methylation in the *PMP24* 5′-CGI was inversely related to gene expression [25]. To identify the CpG dinucleotide whose methylation status is most important for gene regulation, we first used the PC-3 cell as a model. This cell line lacks *PMP24* expression, and the 5′-CGI of the gene is heavily methylated. By exposing PC-3 cells to various concentrations of 5-aza-dC, we found the minimal concentration of the drug (0.1 μM) that reactivated expression of the gene (Figure 1C). The gene expression level in response to 5-aza-dC treatment exhibited a concentration-dependent manner. We reasoned that, if only a few CpG dinucleotides were demethylated at this low concentration of 5-aza-dC and the gene expression was reactivated, these dinucleotides must play a pivotal role in gene regulation. Because of the lack of standard terminology to describe our approach, we arbitrarily referred to it as a "*limited demethylation approach*" and to these sites as "*hypersensitive sites*" (to demethylating agents). Using this approach, we found only one CpG cluster comprising two CpGs (CpG 29 and 30) that exhibited hypersensitivity to 5-aza-dC demethylation, along with reactivation of gene expression (a 5-aza-dC hypersensitive site, demethylated at 0.1 μM, Figure 1D). Higher concentrations of 5-aza-dC (5 μM), however, extended demethylation to other CpGs flanking this site and to multiple regions of the CGI with less site-specificity. These results, taken together, suggested that the methylation status of these two CpG dinucleotides in the *PMP24* 5′-CGI may play a pivotal role in the regulation of gene expression.

## The 5-aza-dC-Hypersensitive Site Binds Nuclear Protein(s) in a Sequence- and Methylation Status-specific Manner

We then performed a gel-shift assay to determine if one or both of the CpGs (CpG 29 and 30, Figure 1A) in the 5-aza-dC-hypersensitive site was essential for protein interaction. Nuclear extracts were prepared from NPrEC cells because they have high levels of *PMP24* expression and thus should contain all essential proteins supporting transcription. A 30-mer oligonucleotide corresponding to the hypersensitive site was synthesized and labeled as the probe (Table I). As shown in Figure 2, following incubation of the labeled probe with NPrEC nuclear extracts, a sequence-specific DNA-protein complex (indicated by the solid arrow; lane 2) was formed; this complex could be completely blocked by co-incubation with an excess of unlabeled wild-type oligonucleotide (WT, lane 3). We then investigated the effect of cytosine methylation on this DNA-protein interaction. When the oligonucleotide was methylated, it completely lost its ability to compete with the labeled probe, indicating that methylation interfered with DNA-protein interaction (MetWT, lane 4). Mutation at both CpGs (MutCG1-2, lane 5) or only at the first CpG site (MutCG1, lane 6) in the oligonucleotide resulted in the loss of their ability to compete with the labeled probe. In contrast, a mutation at the second CpG dinucleotide of the oligonucleotide alone did not abolish its ability to block the DNA-protein complex formation (MutCG2, lane 7), suggesting that the first, but not the second, CpG dinucleotide was crucial for DNA-protein-interaction. Collectively, these data indicated that the 5-aza-dC-hypersensitive site interacts with nuclear protein(s) in a sequence-specific manner and that the interaction is sensitive to cytosine methylation, with the first intronic CpG dinucleotide being crucial to the interaction.

### The First CpG in the Hypersensitive Site Is Crucial for PMP24 Promoter Activity

To evaluate the individual contribution of the first and the second CpGs in the 5-aza-dC-hypersensitive site of the *PMP24* 5′-CGI on promoter activity, we generated site-specific mutants and deletion mutants from a minimal proximal promoter (−415/+330 wt). Mutation of CpG1 (mutCG1) significantly decreased the promoter activity by 82% ($p<0.001$) in LNCaP cells as compared with that of the wild-type promoter in the pGL3/IRES/(−415/+330) plasmid (Figure 3). In comparison, mutation of CpG2 (mutCG2) showed activity comparable to that of the wild-type promoter ($p>0.05$). Nested deletion at the 3′ flanking region did not significantly affect the promoter activity (pGL3/IRES/(−415/+194), $p>0.05$) until the entire 5-aza-dC-hypersensitive region was removed (pGL3/IRES/(−415/+93), 69% decrease, $p<0.001$). These results demonstrated that the promoter activity depends critically on the first CpG located at the 5-aza-dC-hypersensitive site (corresponding to CpG 29 in the CGI depicted in Figure 1A).

### Site-specific Methylation of the Hypersensitive Region Downregulated *PMP24* Expression

To directly demonstrate that methylation of the hypersensitive site can impede *PMP24* expression, we used methylated oligonucleotides (MetODN, Table I). MetODNs are 18–25-bp oligonucleotides designed to be corresponding to the targeted sequence at which one wants to induce *in cellulo* methylation [34,35]; each has at least three methylated CpG dinucleotides. It is believed that, following transfection, the MetODNs will form hemi-methylated DNA intermediates with the targeted sequence during cell replication. Since hemi-methylated DNAs are preferred substrates for DNA methyltransferase I, site-specific methylation could be attained after multiple rounds of cell proliferation. MetODNs targeting the 5-aza-dC-hypersensitive site and its unmethylated counterpart (CtrlODN) were transfected into NPrEC cells, which have a completely unmethylated *PMP24* 5′-CGI [25]. The expression level of *PMP24* decreased by 44% ($p<0.001$) in the MetODN-treated cells, but that of the CtrlODN-transfected cells showed no change as compared with the untransfected cells ($p>0.05$, Figure 4A). In concordance with the *PMP24* expression level, bisulfite sequencing analysis of the MetODN-transfected cells confirmed selective methylation of in the hypersensitive site, with methylation occurring predominately at the first CpG dinucleotide of the hypersensitive site (Figure 4B). In contrast, transfection of the unmethylated CtrlODN did not elicit any methylation and had no effect on gene expression.

### *In Silico* Predicted Transcription Factors Failed to Regulate *PMP24* Expression in NPrEC

We spent much effort to identify the TFs that bind to the 5-aza-dC hypersensitive site and regulate *PMP24* gene expression. We used both MatInspector from Genomatix and TRANSFAC to search for putative transcription factor-binding sites in the hypersensitive region. *In silico* analyses predicted putative AP-2- and Sp1-binding sites at this region. We therefore used NPrEC cells as a model system for targeted gene knockdown experiments since *PMP24* 5′-CGI is completely unmethylated and the gene is expressed in this cell model. Using real-time RT-PCR, we first established that NPrEC express significant levels of *AP-2α* and *AP-2γ* but little or no *AP-2β*, *-2δ* and *-2ε* transcripts. It also expresses high levels of *Sp1* mRNA. We then conducted two-round siRNA-mediated knockdown experiments [19] to down-regulate *AP-2α*, *AP-2γ*, or *Sp1* expression in NPrEC. Although the knockdown experiments effectively repressed the siRNA-targeted genes (>80%), they failed to change the level of *PMP24* expression (data not shown). We also overexpressed *AP-2α*, *AP-2γ* and their dominant negative mutant in NPrEC, but these efforts failed to change *PMP24* transcript levels in the transfected cells (data not shown). These data lead us to conclude that the 5-aza-dC hypersensitive site does not harbor an AP-2- or a Sp1 *cis*-element; rather an unknown TF may interact with it to regulate *PMP24* expression.

### Differential Methylation of a Single CpG Dinucleotide in Clinical Prostate Specimens Correlates with *PMP24* Transcript Expression

Using cell model experiments, we indentified a single CpG dinucleotide in the intronic region of *PMP24* 5′-CGI (CpG 29 in Figure 1A) playing a crucial in gene regulation via DNA methylation. To better understand the clinical relevance of our *in vitro* data, we compared the degree of methylation at each CpG dinucleotide of the *PMP24* 5′-CGI in microdissected samples of prostate epithelial cells from morphologically normal tissue versus that in microdissected PCa cells. As shown in Figure 5A, a single CpG dinucleotide, CpG29 of the CGI, was found to be significantly hypermethylated ($p$=0.03) in PCa cells when compared to normal prostate epithelial cells; statistical significance was not observed for any other CpGs within the CGI (error bars not shown). ISH staining showed *PMP24* transcript expression in all benign glands foci but marked reduction in expression in many, but not all, PCa foci (Figure 5B). Quantitatively, the levels of transcript expression, represented as percent of positively stained cells (% of positivity), were significantly higher in foci of benign glands than those with PCa (Figure 5C, $p$<0.01). Thus, an inverse association was observed between methylation of CpG29 in *PMP24* 5′-CGI and the level of gene expression in clinical samples.

## DISCUSSION

Our previous study showed that *PMP24* in PCa cells is silenced by a mechanism associated with the hypermethylation of its promoter CGI and the silenced gene can be reactivated by treatment with 5-aza-dC [25]. In this study, by carefully varying the concentration of 5-aza-dC until it was just enough to reactivate *PMP24* expression in PC-3 cells, we successfully identified a single CpG dinucleotide, within the *PMP24* 5′-CGI, whose methylation status plays a crucial role in gene regulation. Several features of this CpG dinucleotide are intriguing: 1) it is harbored within the first intron; 2) its methylation status critically controls *PMP24* expression; 3) it is one of the many CpG dinucleotides in a relatively large CGI; 4) nuclear proteins bind to it in a sequence-specific manner but it does not match to the *cis*-regulatory elements predicted by *in silico* analysis; and 5) it exhibits hypersensitivity to both demethylation by 5-aza-dC and MetODN-mediated methylation *in cellulo*. Most interestingly, however, we found that this CpG exactly matches a single CpG (CG29) in the *PMP24* 5′-CGI whose degree of methylation was found to be significant different between normal prostate epithelial cells and PCa cells obtained by LCM of clinical samples. Importantly, ISH data support a regulatory role of this CpG in regulating gene expression via DNA methylation under the *in vivo* setting. In short, we have identified a single intronic CpG, within a relatively large CGI, that is highly susceptible to methylation-demethylation and thereby can play a dynamic role in the regulating *PMP24* expression. Methylation of this CpG appears to be a molecular event associated with *PMP24* silencing during prostate carcinogenesis.

Traditionally, the majority of studies on gene silencing by DNA methylation are focusing on CGIs in the promoter region. It is believed that hypermethylation of a promoter CGI increases the binding of methyl-CpG-binding proteins, along with the recruitment of other chromatin modifiers, and leads to chromatin remodeling and transcriptional repression. Up till recently, not much is known about the relevance of non-promoter CGI(s) or, in the case of our study, a single CpG, in gene regulation via cytosine methylation. Nevertheless, Jones and associates [36,37] offered an attractive model to explain the role of non-promoter CGI in epigenetic gene regulation. They demonstrated that non-promoter CGIs are more susceptible to *de novo* methylation than promoter islands and may serve as foci for the seeding of *de novo* methylation which can then spread into adjacent islands that contain enhancer elements for transcriptional regulation. Whether the reverse scenario, i.e., demethylation, is true remains unknown. Specifically, pertaining to our results, it will be

important to determine, in future studies, whether the demethylation of a single CpG (CG29) in the *PMP24* CGI can initiate "spreading" of demethylation to adjacent sites, leading to the expansion of chromatin relaxation and increased gene reactivation. In support of this possibility, in this study, we did observe the spreading of demethylation from CG29 to adjacent CGs when PC-3 cells were exposed to higher levels of 5-aza-dC (Figure 1D) along with progressive increases in gene expression (Figure 1C).

Recent reports have broadened our understanding of the importance of intronic CGIs in epigenetic gene regulation. For example, hypermethylation of an intron 1 CGI was shown to silence the *EGR2* gene and the CGI was found to harbor *cis*-elements similar to enhancers commonly found in promoter regions [38]. Similarly, an intron 4 CGI that exhibited all the characteristics of a *bona fide cis*-element including direct interaction with the 5′ proximal promoter was shown to regulate the expression of *IL-10* [39]. In another scenario, transcriptional silencing of the *MCJ* gene was found to associate with methylation of a CGI in its intron 1 region and reduction of histone acetylation within the CGI as well as in the promoter region of the gene [40]. This finding suggests that methylation of an intronic CGI can alter the chromatin structure of the entire upstream region, leading to the repression of gene expression. These studies provide emerging evidence that intronic CGIs are equally essential in gene regulation as their counterparts in promoter regions.

In the literature there is only a handful of single CpG identified to be essential for gene regulation via methylation. The scarcity of single CpG dinucleotide that plays the most critical role in gene regulation is due to the way 5-aza-dC experiments normally conducted. Most investigations were aimed at achieving maximal gene reactivation and therefore adopted a prolonged treatment protocol using relatively high concentrations of the demethylating agent [41]. Using this traditional approach, it is difficult to ascertain if any of the CpG dinucleotides exhibit differential susceptibility towards methylation-demethylation or to locate the critical regulatory sequences within a CGI. The fact that we have identified CG29 within the *PMP24* 5′-CGI as the key regulatory site is due to the careful choice of the time and the dose of 5-aza-dC for demethylation treatment. The lowest concentration of 5-aza-dC was used to identify the CpG(s) most sensitive to demethylation along with gene reactivation. Intriguingly, when we used the methylation sense ODN to induce site-specific methylation CG29 is also the most susceptible CpG for methylation with concordant gene silencing of *PMP24*. Therefore, we can conclude that the limited demethylation approach has proven useful in identifying hypersensitive methylation-demethylatoin CpG(s) essential for gene regulation. These sites may serve as dynamic switches to activate or inactivate of a gene through cytosine methylation.

It is noteworthy to mention that this site may harbor a yet-to-be identified *cis*-element whose methylation status determines gene expression. Unfortunately, forced expression and siRNA-mediated knockdown experiments failed to identify this site as an AP-2- or a Sp1-*cis*-element. Regarding to the function of *PMP24* in peroxisomes, essential cell organelles involved in lipid metabolism and detoxification [42], however, not much is known about the function of *PMP24* except our earlier observations demonstrating that it has an anti-tumor action on PCa cells [25]. Future studies are required to elucidate the TF-hypersensitive site interaction and biological function of this gene.

Finally, using LCM samples from clinical prostate specimens combined with bisulfite sequencing analysis and ISH, we established an inverse correlation between methylation of CG29 in the *PMP24* 5′-CGI and expression of the gene transcript in clinical specimens. In essence, normal prostate epithelial cells express *PMP24* and have a hypomethylated CG29 in the gene's 5′-CGI whereas PCa cells exhibit significant reduction in *PMP24* expression and have a hypermethylated CG29 site. These data strongly suggest that CG29 is essential to

*PMP24* gene regulation via cytosine methylation *in vivo* and the DNA methylation-mediated silencing of the gene is a molecular event associated with prostate carcinogenesis. The fact that CG29 identified in clinical samples matches the 5-aza-dC hypersensitive site identified by limited demethylation in cell model systems inspires confidence that this site serves as a versatile switch for *PMP24* regulation in cell model systems and in tissue.

## CONCLUSION

By using the limited demethylation approach, we identified a single CpG dinucleotide in the *PMP24* 5′-CGI to be essential for gene regulation via methylation. The importance of this site in mediating gene silencing through cytosine methylation was also illustrated using LCM-sampling of benign glands and PCa cells from clinical specimens along with ISH staining. Collectively, these data provide a strong disease relevance to this unique intronic CpG dinucleotide that plays a pivotal role in *PMP24* regulation.

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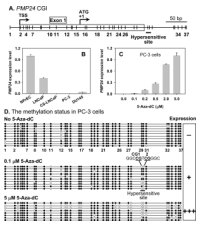

**Fig. 1. Treatment of 5-aza-dC by *limited demethylation approach* preferentially demethylated specific CpG dinucleotide in *PMP24* promoter CGI**

**A**: The organization of *PMP24* promoter CGI. The major 5-aza-dC-hypersensitive site at intronic CpG 1 and 2 that corresponding to CG29 and CG30 is marked with a line. **B**: Relative *PMP24* expression levels, as determined by real-time RT-PCR, among the normal and malignant prostate cell lines, which correlated with the extent of promoter methylation [25]. **C**: Treatment with 5-aza-dC reactivated *PMP24* gene expression in a dose-dependent manner, with 0.1 μM the lowest concentration causing gene reactivation (n=4). **D**: Using the limited demethylation approach, we found one major region in *PMP24* CGI that is hypersensitive to 5-aza-dC demethylation after PC-3 cells were treated briefly with a very low dose of 5-aza-dC. Figure 1A and 1D represent the same region with the same scale.

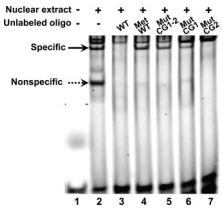


**Fig. 2. The first CpG site in the 5-aza-dC-hypersensitive region is essential for DNA-protein interaction**

A 30-bp labeled oligonucleotide (WT) corresponding to the hypersensitive site was incubated with a nuclear extract from NPrEC cells. A gel shift assay demonstrated the formation of a sequence-specific DNA-protein complex, as indicated by the solid arrow. The presence of 100-fold molar excess of unlabeled WT probe (lane 3) or mutCG2 (mutation at the second CpG dinucleotide, lane 7) diminished the shifted band (DNA-protein complex), while the same excess amount of MetWT (methylated WT sequence, lane 4), mut1-2CGs (mutations at both CpG1 and 2, lane 5), and mutCG1 (mutation at the first CpG dinucleotide, lane 6) showed no competition with the labeled probe. Dotted arrow indicates nonspecific DNA-protein interaction as any excessive unlabeled oligos can compete for the protein binding regardless of their sequences and methylation status (lane 3 to 7). Using nuclear extract from different passage numbers of NPrEC cells, three independent experiments were done and one representative figure is shown.

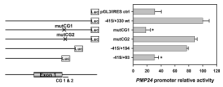

**Fig. 3. *PMP24* promoter analysis**

Promoter luciferase reporter pGL3/IRES/(−415/+330)mutCG1 and pGL3/IRES/(−415/+330)/mutCG2 that have mutations at the first or second CpG dinucleotide respectively, were generated from the WT promoter reporter pGL3/IRES/(−415/+330). Nested 3′ deletion of the promoter generated two additional plasmids of pGL3/IRES/(−415/+194) and pGL3/IRES/(−415/+93) with or without the hypersensitive site (+128), respectively. Deletion of the hypersensitive site (intronic CG1 &2) or the first CpG dinucleotide but not deletion of the second significantly decreased the promoter activity (**p*<0.001) as compared with the activity of the WT promoter (n=3). The position of the translation start site was designated as +1. The *IRES* element immediately upstream of the luciferase gene is not shown.

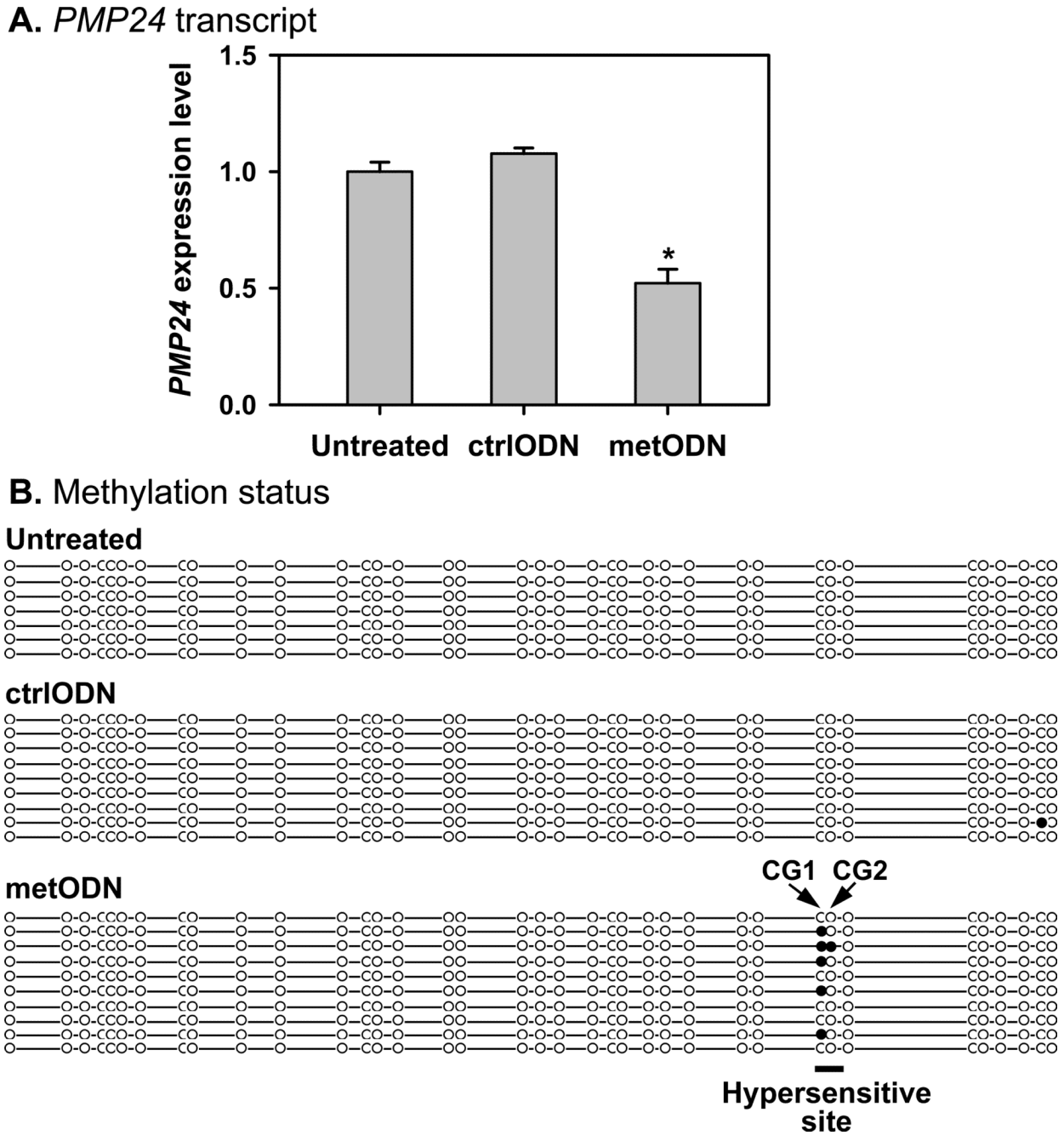


**Fig. 4. Methylation of the first CpG in the 5-aza-dC-hypersensitive site repressed *PMP24* gene expression in NPrEC cells**

**A**: Compared with non-methylated oligonucleotide (ctrlODN)-transfected control, real-time RT-PCR analysis showed significant downregulation of *PMP24* transcript in the cells transfected with a methylated oligonucleotide (metODN) targeting the 5-aza-dC-hypersensitive site (*, n=3, $p<0.001$). Transfection with ctrlODN did not significantly change *PMP24* expression as compared with the untreated cells. **B**: The metODN induced ~50% methylation at the first CpG dinucleotide but almost no methylation at the second CpG dinucleotide of the hypersensitive site.

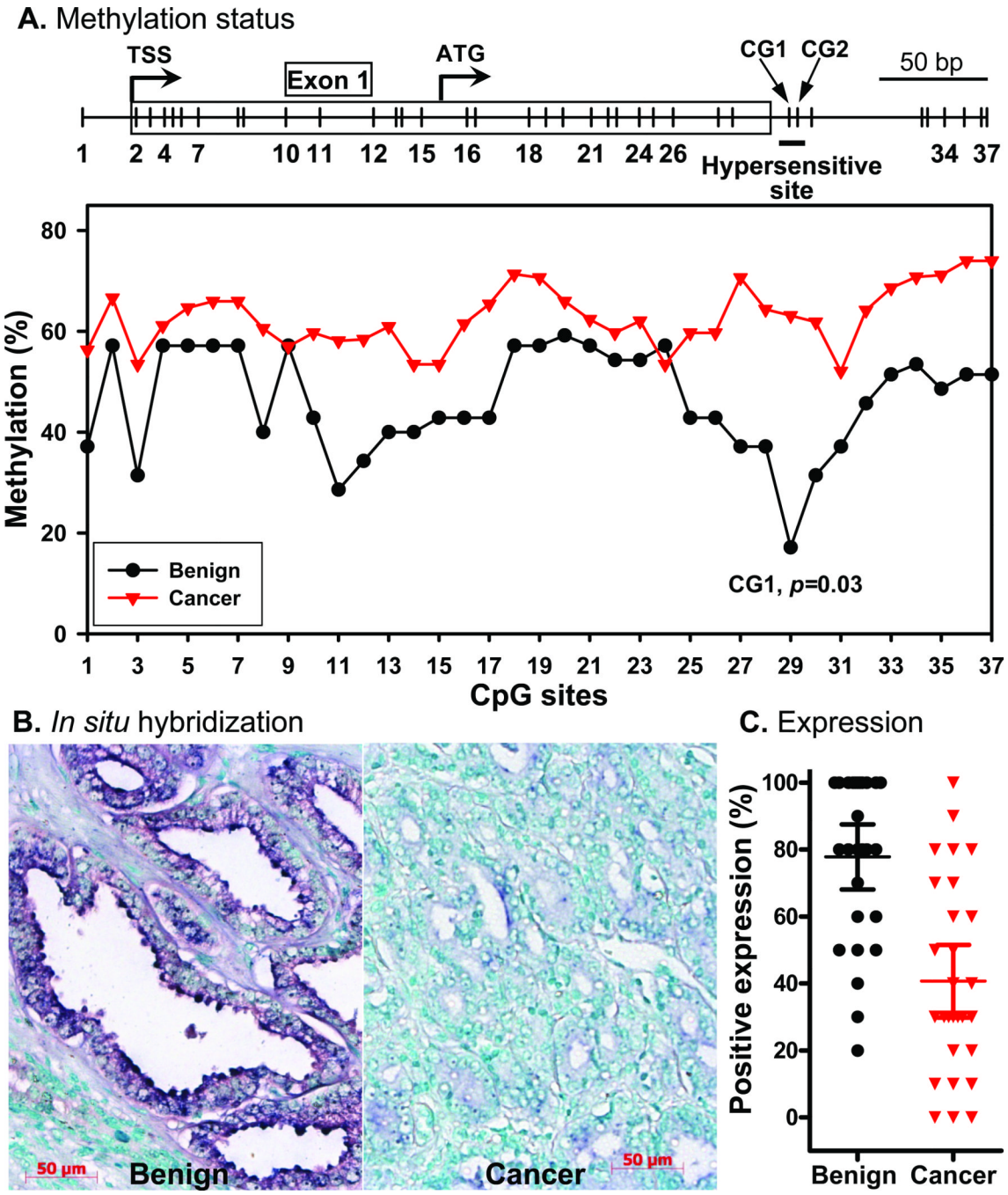


**Fig. 5. Methylation status of *PMP24* CGI in prostate LCM samples**

**A**: Bisulfite sequencing result from the benign (n=7) and cancer foci (n=16) of LCM samples. Overall, the CGI is hypermethylated in the cancer group with the average methylation percentage of 63%, whereas in the benign group the average methylation percentage is 46%. One hypomethylated site, CG29 (methylation percentage 17%), was identified in the benign but not in the cancer group (63%). This site exactly matches the 5-aza-dC hypersensitive region shown in Figure 1. CG29 but not other sites exhibited significant difference of the methylation status ($p$=0.03) between the two groups. **B**: Representative ISH staining results of benign prostatic glands and PCa foci. **C**: Comparison of the *PMP24* mRNA expression positivity between benign glands and PCa foci (3–5 foci/

case) from seven cases of specimens analyzed by ISH ($p<0.01$). Error bar: 95% confident interval.

**Table I**

Oligonucleotide sequences used in the study.

| Assay | Name | Primer sequences (5′➔3′) |
|---|---|---|
| Bisulfite sequencing | P24-bisF1 | GAG TGT TAT TGT TTT ATG AGA TAA GTA |
| | P24-bisR1 | AAC TCC CAT TAC ACA AAT AAT AAC |
| | P24-bisF2 | TGA TTG GTT TTT TAT GTA TGA GT |
| | P24-bisR2 | AAA ACT ACT CCA AAA CAA ACA A |
| Real-time RT-PCR | PMP24f | AGG GCT CTG CTC GTA GTC GTC |
| | PMP24r | GCC ATT CCG GAA GAG AAA GGT |
| Gel shift | WT | CTG TGT GAG TGG GGC CGT CGG GCC GGG CTG |
| | MutCG1 | CTG TGT GAG TGG GAT TAT CGG GCC GGG CTG |
| | MutCG2 | CTG TGT GAG TGG GGC CGT TAA ACC GGG CTG |
| | MutCG1-2 | CTG TGT GAG TGG GGT TAT TAA GCC GGG CTG |
| *In cellulo* methylation | metODN | G*G*G* mCGT mCGG GCmC GG*G* C*T |
| *In situ* hybridization | T7 | CAG TAA TAC GAC TCA CTA TA |
| | P24probe | TGG TAG CGG CGC TTG CGC AGC AGT GCG TTG ACG ACT ACG AGC AGA CCC TAT AGT GAG TCG TAT TAC TG |
| | Scramble probe | GTG TAA CAC GTC TAT ACG CCC A |

Note: Primers for the real-time RT-PCR are located at exon 1 and 2, with the PCR size 153 bp. In the gel-shift assay, CpG dinucleotides in the WT probe and mutated nucleotides at the first and second CpG dinucleotides (mutCG1 and mutCG2, respectively) are underlined. mC indicates methylated C.

* indicates nucleotide phosphorothioated bonds. For *in situ* hybridization, the underlined is the consensus promoter sequence of T7 RNA polymerase.