

RESEARCH PAPER

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DNA methylome changes by estradiol benzoate and bisphenol A links early-life environmental exposures to prostate cancer risk

Ana Cheong^{a,b,*}, Xiang Zhang^{a,b,*}, Yuk-Yin Cheung^{a,**}, Wan-ye Tang^{a,b,#}, Jing Chen^{a,##}, Shu-Hua Ye^c, Mario Medvedovic^{a,b,d}, Yuet-Kin Leung^{a,b,d}, Gail S. Prins^{c,e}, and Shuk-Mei Ho^{a,b,d,f}

^aDepartment of Environmental Health, University of Cincinnati College of Medicine, Cincinnati, OH, USA; ^bCenter for Environmental Genetics, University of Cincinnati College of Medicine, Cincinnati, OH, USA; ^cDepartment of Urology, College of Medicine, University of Illinois at Chicago, Chicago, IL, USA; ^dCincinnati Cancer Center, Cincinnati, OH, USA; ^eUniversity of Illinois Cancer Center, Chicago, IL, USA; ^fCincinnati Veteran Affairs Hospital Medical Center, Cincinnati, OH, USA

ABSTRACT

Developmental exposure to endocrine-disrupting chemicals (EDCs), 17 β -estradiol-3-benzoate (EB) and bisphenol A (BPA), increases susceptibility to prostate cancer (PCa) in rodent models. Here, we used the methylated-CpG island recovery assay (MIRA)-assisted genomic tiling and CpG island arrays to identify treatment-associated methylome changes in the postnatal day (PND)90 dorsal prostate tissues of Sprague-Dawley rats neonatally (PND1, 3, and 5) treated with 25 μ g/pup or 2,500 μ g EB/kg body weight (BW) or 0.1 μ g BPA/pup or 10 μ g BPA/kg BW. We identified 111 EB-associated and 86 BPA-associated genes, with 20 in common, that have significant differentially methylated regions. Pathway analysis revealed cancer as the top common disease pathway. Bisulfite sequencing validated the differential methylation patterns observed by array analysis in 15 identified candidate genes. The methylation status of 7 (*Pitx3*, *Wnt10b*, *Paqr4*, *Sox2*, *Chst14*, *Tpd52*, *Creb3l4*) of these 15 genes exhibited an inverse correlation with gene expression in tissue samples. Cell-based assays, using 5-aza-cytidine-treated normal (NbE-1) and cancerous (AIT) rat prostate cells, added evidence of DNA methylation-mediated gene expression of 6 genes (exception: *Paqr4*). Functional connectivity of these genes was linked to embryonic stem cell pluripotency. Furthermore, clustering analyses using the dataset from The Cancer Genome Atlas revealed that expression of this set of 7 genes was associated with recurrence-free survival of PCa patients. In conclusion, our study reveals that gene-specific promoter methylation changes, resulting from early-life EDC exposure in the rat, may serve as predictive epigenetic biomarkers of PCa recurrence, and raises the possibility that such exposure may impact human disease.



Abbreviations: AA, African-American; Acrbp, Acrosin binding protein 21; AIT, Rat prostate cancer cell line; Akt, Protein kinase B; 5-aza, 5-aza-cytidine; BPA, Bisphenol A; Btd3, BTB domain containing 3; BW, Body weight; Ccdc67, Coiled-coil domain containing protein 67; Chad, Chondoadherin; Chst14, Carbohydrate sulfotransferase 14; Creb3l4, Cyclic AMP responsive element binding protein 3-like 4; Ctrl, Control; DMR, Differentially methylated region; Dna-se2b, Deoxyribonuclease II β ; Dnmt3a, DNA methyltransferase 3a; Dnmt3b, DNA methyltransferase 3b; E₂, 17 β -estradiol; EB, 17 β -estradiol-3-benzoate; EDC, Endocrine-disrupting chemical; ER, Estrogen receptor; ERK1/2, Extracellular-signal-regulated kinase 1/2; ERR γ , Estrogen related receptor gamma; ESR1/ER α , Estrogen receptor α ; ESR2/ER β , Estrogen receptor β ; GPER1/GPR30, G protein-coupled estrogen receptor 1; Hmgn5, High mobility group nucleosome binding domain 5; Habb2, Hyaluronan binding protein 2; Hpcal1, Hippocalcin-like 1; IPA, Ingenuity Pathway Analysis; Krt83, Keratin 83; Mbd2, Methyl-CpG binding domain protein 2; Mbd4, Methyl-CpG binding domain protein 4; MIRA, Methylated-CpG island recovery assay; NbE-1, Rat prostate epithelial cell line; Nfia, Nuclear factor I/A; Osbp16, Oxysterol binding protein-like 6; Paqr4, Progesterin and adipoQ receptor family member 4; PCa, Prostate cancer; Pde4d, Phosphodiesterase type IV variant; Phpt1, Phosphohistidine phosphatase 1; Pitx3, Paired-like homeodomain 3; PIN, Prostatic intraepithelial neoplasia; PND, Postnatal day; Prkar1a, Protein kinase cAMP-dependent type 1 regulatory subunit α ; Rbpjl, Recombination signal binding protein for immunoglobulin kappa J region-like; Rnf186, Ring finger protein 186; Rpl19, Ribosomal protein L19; SD, Sprague Dawley; SEM, Standard error of mean; Sox2, Sex determining region Y box 2; Tacstd2, Tumor-associated calcium signal transducer 2; Tbx4, T-box 4; TCGA, The Cancer Genome Atlas; TGF β , Transforming growth factor β ; Tmem27, Transmembrane protein 27; TNBC, Triple negative breast cancer; Tpd52, Tumor protein D52; Tymp, Thymidine phosphorylase; Wnt10b, Wingless-type MMTV integration site family, member 10B


ARTICLE HISTORY

Received 25 April 2016
Revised 25 June 2016
Accepted 27 June 2016

KEYWORDS

Developmental origin of health and disease (DOHaD); early-life reprogramming; endocrine-disrupting chemicals (EDCs); epigenetics; Ingenuity[®] Pathway Analysis (IPA[®]); methylated-CpG island recovery assay (MIRA); NimbleGen rat DNA methylation promoter array; Sprague Dawley rats; stem cell pluripotency; The Cancer Genome Atlas (TCGA)

CONTACT Shuk-Mei Ho, PhD  hosm@ucmail.uc.edu  Room 128, Department of Environmental Health, Kettering, Complex, 160 Panzeca Way University of Cincinnati Medical Center Cincinnati, OH, 45267-0056, USA.

 Supplemental data for this article can be accessed on the [publisher's website](#).

*These authors contributed equally to this work.

**Current address: Life Giving Foundation Education Center, Cheung Sha Wan, Kowloon, Hong Kong

#Current address: Department of Environmental Health Sciences, John Hopkins Bloomberg School of Public Health, Baltimore, MD, USA

##Current address: Division of Biomedical Informatics, Cincinnati Children's Hospital Medical Center, Cincinnati, OH, USA

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Introduction

Exposure to environmental agents is a risk factor for multiple diseases. Evidence is mounting in support of the causal link between exposure to xenoestrogens and human diseases,^{1–3} including cancer.^{4–7} Bisphenol A (BPA) is a ubiquitous environmental xenoestrogen widely used during the production of polycarbonate plastics, epoxy resins, carbonless receipt paper, and hundreds of other manufactured products.¹ The fact that urinary BPA is detectable in >95% of the US population⁸ indicates that BPA readily leaches into the environment, contaminates our food and water, and enters our bodies. Chronic exposure to BPA in rodents⁹ and in humans¹⁰ is associated with prostate cancer (PCa) and its prelesions. Moreover, BPA is detectable in umbilical cord blood^{11–14} and sera of newborns,¹⁵ which elevates the concern of early-life BPA exposure and development of later-life disease. As an endocrine disrupting chemical (EDC), BPA binds to estrogen receptors (ERs) ESR1, ESR2, and GPER1,¹⁶ which are expressed in the rodent and human prostate epithelial cells.^{17,18} While affinity for nuclear ERs is low compared to estradiol-17 β , BPA has equivalent activation capacities for membrane ERs^{16,19} and rapid actions of low-dose BPA have been documented in prostate cells.¹⁹ Finally, BPA can also signal through non-ER pathways.^{20,21}

Like BPA, 17 β -estradiol-3-benzoate (EB) is a ubiquitous environmental agent. It is an estradiol analog commonly used in livestock for inducing weight gain and synchronizing estrus cycles in heifers and cattle.²² Since EB binds ERs with high affinity,²³ unintended exposures may trigger estrogenic responses that compromise health. In rodents, neonatal EB exposure (30–125 ng/day in mouse and 125–500 ng/day in rats) has been shown to induce the production of morphologically abnormal sperm during adulthood.²⁴ Similarly, rats neonatally exposed to 25 μ g EB developed prostates with a greater risk of malignant changes with aging, including severe prostatic intraepithelial neoplasia (PIN), a precancerous condition.^{25–27} Since production of morphologically abnormal sperm²⁴ and induction of increased susceptibility to hormonal carcinogenesis²⁵ are similarly observed following neonatal BPA exposure, these findings strongly implicate early-life as a susceptible window for environmental xenoestrogens to predispose later-life disease risk and EB can serve as a positive estrogen control in studies of weaker estrogenic agents such as BPA. While emerging evidence indicates that epigenetic modifications are involved,^{25,28–33} the molecular underpinnings of this process remain to be fully clarified.

To initially interrogate whether neonatal exposure to EB and BPA reprogrammed the prostate epigenome, we exposed rats to an environmentally relevant dose of BPA [10 μ g/kg body weight (BW)] or EB (2,500 μ g/kg BW) on postnatal days 1, 3, and 5. We observed that this transient developmental exposure increased the dorsal and lateral prostate lobe susceptibility to adult-onset E₂-induced carcinogenesis, markedly augmenting the incidence and severity of lesions as compared to oil-treated controls.^{25,32,34} Using methylation-sensitive restriction fingerprinting to identify altered DNA methylation marks, we found that neonatal EB and BPA exposures were associated with the dysregulation of *phosphodiesterase type IV variant 4 (Pde4d4)*,²⁵ *hippocalcin-like 1 (Hpcal1)*; also known as *visinin-like protein-3*, *Vilip-3*, and *high mobility group nucleosome binding domain 5*

(*Hmgm5*; formerly known as *nucleosome binding protein 1*, *Nsbp1*)³² through aberrant promoter methylation detected on postnatal day (PND)10, 90, and 200. Together, these findings provided the first evidence that early-life environmental exposure to EDC reprogrammed the prostate epigenome and identified a developmental basis of PCa risk with aging. Moreover, the expression of *DNA methyltransferase3a* and *3b (Dnmt3a* and *Dnmt3b)* and *methyl-CpG binding domain protein2* and *4 (Mbd2* and *Mbd4)* was upregulated in PND10 and 90 prostate tissues,³² providing a mechanistic basis for reprogramming of DNA methylation marks upon early-life EDC exposure. Thus, we propose that differential methylation of genes that persist in the PND90 prostate primes the tissue for heightened sensitivity to a secondary exposure to rising estradiol later in life, as occurs in aging males.³⁵ This is particularly relevant since elevated estrogens have been associated with increased PCa risk in men⁶ and are sufficient to transform the human prostate epithelium.¹⁷

While these findings had a major impact on the field of EDC research and developmental basis of carcinogenesis,³⁶ a genome-wide search for additional DNA methylation targets of neonatal exposure to xenoestrogens is warranted for an unbiased discovery of other epigenetic marks that may underlie increased PCa risk with aging. To accomplish this goal, we herein used the methylated-CpG island recovery assay (MIRA)-assisted genomic tiling and CpG island array analysis and identified distinct and common EB-/BPA-associated genes in PND90 prostate tissues from rats with neonatal exposure to these xenoestrogens. Among 25 epigenetically regulated candidate genes, the promoter methylation status of 7 genes (*Pitx3*, *Wnt10b*, *Paqr4*, *Sox2*, *Chst14*, *Tpd52*, *Creb3l4*) was inversely correlated to gene expression. These validated genes have functional connectivity associated with stem cell pluripotency. Of clinical relevance, expression of these genes was found to be associated with recurrence-free survival of 497 patients in The Cancer Genome Atlas (TCGA) PCa cohort, suggesting that they may have utilities for predicting disease progression and patient stratification based on disease aggressiveness.

Results

Methylation array revealed differential promoter methylation of genes associated with neonatal EB or BPA exposure in the PND90 prostate

We performed genome-wide methylation analysis, using MIRA-assisted genomic tiling and CpG island array, in dorsal prostate tissues from PND90 rats neonatally exposed to EB or BPA (Fig. 1). Using a 750 bp sliding window approach and a selection criteria of $P < 10^{-5}$ and mBar >0.6 or <−0.25 (Fig. 1), we identified a total of 177 differentially methylated regions (DMRs), which were randomly distributed among chromosomes (Supplemental Figure S1). Since this array was designed to target CpG sites only at the gene promoter region, the genes described hereafter refer to those identified with DMRs at the 5'-promoter region. Of the 177 genes with identified DMRs, 111 genes were EB-associated, 86 genes were BPA-associated, and 20 genes were common between the 2 groups (Fig. 1; Supplemental Table S1).

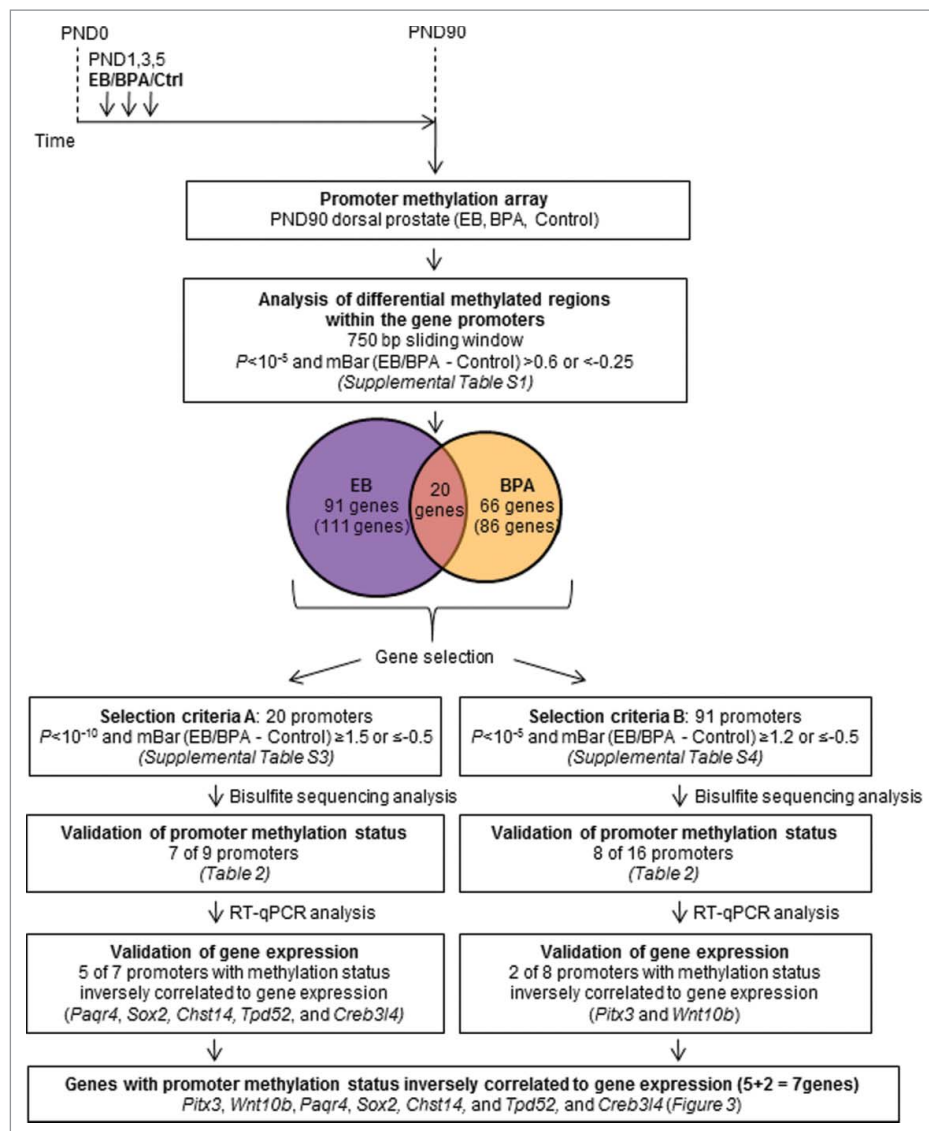


Figure 1. Schematic diagram of the experimental and analytical procedures. Sprague-Dawley (SD) rats were neonatally [postnatal day (PND) 1, 3, 5] treated with 17β-estradiol-3-benzoate (EB) at 25 μg/pup or 2,500 μg/kg body weight (BW), bisphenol A (BPA) at 0.1 μg/pup or 10 μg/kg BW, or corn oil as a control (Ctrl). DNA extracted from PND90 dorsal prostate tissue was subjected to promoter methylation array analysis, and genes with differentially methylated regions and inverse expression correlation were identified and validated.

Biological significance of differentially methylated regions associated with neonatal EB and BPA exposure in PND90 prostate

To identify biological processes related to the identified exposure-associated DMRs, we performed functional network analysis using Ingenuity Pathway Analysis (IPA). As shown in Table 1, the top 3 networks related to the EB-associated genes were identified as: 1) “tissue morphology, embryonic development, organ development;” 2) “cell-to-cell signaling and interaction, cell-mediated immune response, cellular growth and proliferation;” and 3) “post-translational modification, cellular assembly and organization, cellular function and maintenance.” Upon merging the molecular interactions of these top 3 networks, the EB-associated genes were found to converge at protein kinase B (AKT) and extracellular-signal-regulated kinase (ERK)1/2. While the EB-associated genes had implications in the development and function of several physiological systems, including

embryonic, tissue, organ, and reproductive (Table 1), the top related disease was “cancer,” which included PCA.

Similarly, the top 3 networks related to the 86 BPA-associated genes were identified as: 1) “cell-to-cell signaling and interaction, cell-mediated immune response, cellular growth and proliferation;” 2) “nucleic acid metabolism, small molecule biochemistry, molecular transport;” and 3) “cellular assembly and organization, cellular function and maintenance, cellular compromise” (Table 1). The molecular interactions of these networks converged at transforming growth factor β (TGFβ), and the 86 genes had implications in digestive and cardiovascular system development and function, organ and tissue morphology, and organismal development (Table 1). Like the EB-associated genes, BPA-associated genes were also highly associated with “cancer,” including PCA.

Interestingly, the top 3 networks related to the 20 associated genes common of both EB- and BPA-exposure were: 1) “cancer, organismal injury and abnormalities, renal and urological disease;” 2) “cell-to-cell signaling and interaction, nervous

Table 1. Top networks and bio-functions of the genes associated with neonatal 17 β -estradiol-3-benzoate (EB) or bisphenol A (BPA) exposure in postnatal day (PND)90 prostate.

EB-associated genes Number of genes = 111	BPA-associated genes Number of genes = 86
Top networks	Top networks
1. Tissue morphology, Embryonic development, Organ development	1. Cell-to-cell signaling and interaction, Cell-mediated immune response, Cellular growth and proliferation
2. Cell-to-cell signaling and interaction, Cell-mediated immune response, Cellular growth and proliferation	2. Nucleic acid metabolism, Small molecule biochemistry, Molecular transport
3. Post-translational modification, Cellular assembly and organization, Cellular function and maintenance	3. Cellular assembly and organization, Cellular function and maintenance, Cellular compromise
4. Molecular transport, Nucleic acid metabolism, Small molecule biochemistry	4. Cell cycle, Cell-to-cell signaling and interaction, Cellular growth and proliferation
5. Lipid metabolism, small molecule biochemistry, Endocrine system development and function	5. Lipid metabolism, Molecular transport, Small molecule biochemistry
Top bio-functions	Top bio-functions
Diseases and disorders	Diseases and disorders
1. Cancer	1. Antimicrobial response
2. Reproductive system disease	2. Cancer
3. Metabolic disease	3. Connective tissue disorders
4. Auditory disease	4. Developmental disorder
5. Cardiovascular disease	5. Gastrointestinal disease
Physiological system development and function	Physiological system development and function
1. Embryonic development	1. Digestive system development and function
2. Organ development	2. Organ morphology
3. Organismal development	3. Cardiovascular system development and function
4. Reproductive system development and function	4. Organismal development
5. Tissue development	5. Tissue morphology

Data were analyzed using Qiagen's Ingenuity Pathway Analysis (IPA; Qiagen, www.qiagen.com/ingenuity).

system development and function, cardiovascular disease;" and 3) "cancer, connective tissue disorders, dermatological diseases and conditions" (Supplemental Table S2C). While these 3 networks converged at tumor protein 53 (TP53), overall, the 20 genes were significantly related to digestive, hepatic, and nervous system development and function, and embryonic and organismal development (Supplemental Table S2). They also had implications in "connective tissue disorders," "developmental disorder," "hereditary disease," "inflammatory disease," and "metabolic disease" (Supplemental Table S2).

Selection of top candidate genes for methylation validation by bisulfite sequencing

To tighten the selection of differentially methylated candidate genes, we initially sorted the 177 identified genes based on $P < 10^{-10}$ and $mBar (EB/BPA - Control) \geq 1.5$ or ≤ -0.5 (Selection criteria A, Fig. 1). Under this stringent selection criteria, 20 candidate genes were identified (Supplemental Table S3), of which the top 9 most differentially methylated genes, based on differences in $mBar$ values (Fig. 2), across

exposure groups (*Btbd3*, *Chst14*, *Creb3l4*, *Paqr4*, *Phpt1*, *Rbpjl*, *Sox2*, *Tacstd2*, and *Tpd52*) were selected for validation. As shown in Figure 2, primers for bisulfite sequencing validation for each gene were designed to amplify the region with the greatest methylation changes between the EB/BPA-treated groups and the control group. In most cases, the interrogated regions were closely aligned to the predicted CpG island based on MethPrimer program.³⁷

Promoter methylation status was validated by performing bisulfite sequencing analysis on the same PND90 prostate tissue samples used in the methylation array (Table 2; Supplemental Table S3). In accordance with the methylation patterns observed in the array, the DMRs of 7 out of 9 genes were confirmed. The DMRs of *Chst14*, *Paqr4*, *Phpt1*, *Rbpjl*, and *Sox2* were hypomethylated, while those of *Creb3l4* and *Tpd52* were hypermethylated in the EB/BPA-exposure groups when compared to controls (Table 2).

To include more genes in the analysis, we also sorted the 177 exposure-associated genes using wider selection parameters of $P < 10^{-5}$ and $mBar (EB/BPA - Control) \geq 1.2$ or ≤ -0.5 (Selection criteria B, Fig. 1). Using selection criteria B, 91 candidate genes were identified (Supplemental Table S4). Bisulfite sequencing analysis was performed on the top 16 most differentially methylated genes across exposure groups (*Acrbp*, *Ccdc67*, *Chad*, *Dnase2b*, *Habp2*, *Krt83*, *Nfia*, *Osbp16*, *Pitx3*, *Prkar1a*, *Rnf186*, *Tbx4*, *Tmem27*, *Tymp*, *Uox*, and *Wnt10b*) (Table 2; Supplemental Table S4). Promoter methylation validation of the 16 genes, using PND90 dorsal prostate tissues, confirmed promoter hypomethylation of *Pitx3* and *Wnt10b*, and promoter hypermethylation of *Acrbp*, *Chad*, *Osbp16*, *Rnf186*, *Tmem27*, and *Tymp* (Table 2).

Overall, using 2 selection criteria, there were 25 top differentially methylated candidate genes identified, of which the promoter methylation status of 15 were validated (*Chst14*, *Creb3l4*, *Paqr4*, *Phpt1*, *Rbpjl*, *Sox2*, and *Tpd52* from selection criteria A; and *Acrbp*, *Chad*, *Osbp16*, *Pitx3*, *Rnf186*, *Tmem27*, *Tymp*, and *Wnt10b* from selection criteria B) (Table 2).

Validation of promoter methylation status and gene expression correlation

To elucidate the biological relevance of the DMRs within these gene promoters, we next performed gene expression analyses on the PND90 prostate tissues. As shown in Figure 3, expression of *Pitx3*, *Wnt10b*, *Paqr4*, *Sox2*, and *Chst14* was significantly upregulated in the EB-exposure group when compared to the control. Of these genes, only *Sox2* was significantly upregulated in the BPA-exposure vs. control group. In contrast, expression of *Tpd52* was significantly downregulated in both exposure groups, and *Creb3l4* showed a trend of downregulation in exposure groups, when compared to the control group (Fig. 3). Since gene expression of *Acrbp*, *Chad*, *Osbp16*, *Rnf186*, *Tmem27*, and *Tymp* in the prostate tissues of the exposure groups did not associate with their validated promoter methylation status, they were not studied further (Table 2; data not shown).

To investigate the collective biological relevance of the 7 candidate genes (*Pitx3*, *Wnt10b*, *Paqr4*, *Sox2*, *Chst14*, *Tpd52*, and *Creb3l4*), we performed functional network analysis using IPA. We found that the candidate genes were associated with 2 networks: 1) "embryonic development, organismal

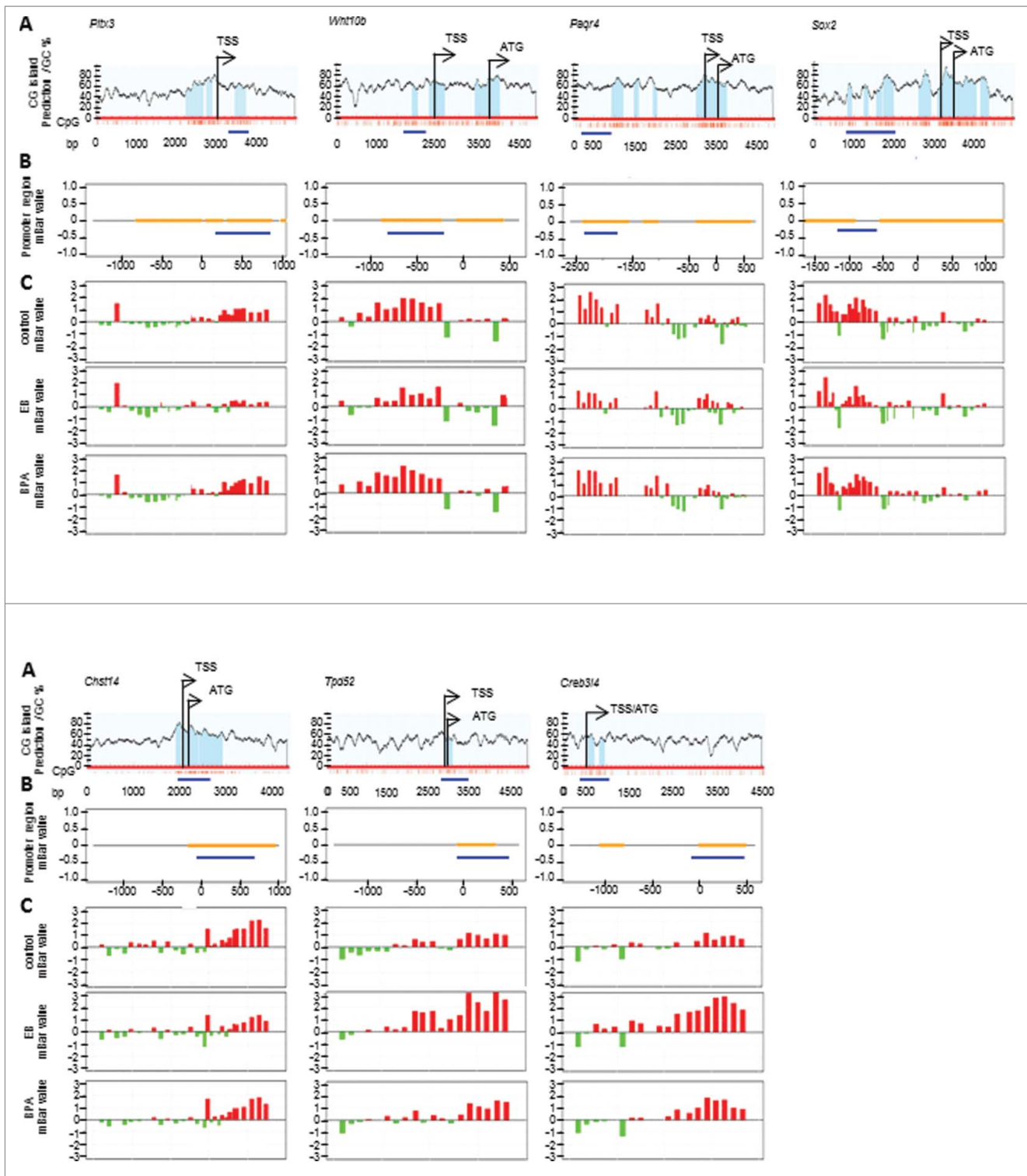


Figure 2. Representative results from genome-wide methylation study. A) Predicted CpG islands (light blue shaded areas) in the promoter region of differentially methylated genes (*Pitx3*, *Wnt10b*, *Paqr4*, *Sox2*, *Chst14*, *Tpd52*, and *Creb3l4*) identified in this study. TSS stands for transcriptional start site whereas ATG stands for translational start codon. Individual CpG sites are represented by red vertical lines. Dark blue horizontal line marks the region selected for BS-sequencing; B) Position of the BS-sequenced region (blue line) relative to the NimbleGen probes covered regions (dirty yellow lines) of each gene; C) Significant methylation level of each gene in vehicle-(control), EB- and BPA-treated groups, which were measured by NimbleGen array probes (mBar values). The height of the mBar represents the probe intensity; red and green bars represent positive and negative methylation value, respectively, relative to their respective input control.

development, gene expression” and 2) “cell cycle, embryonic development, gene expression” (Supplemental Table S5), which converged at *Sox2* (Fig. 4). While these 7 candidate genes had implications in embryonic, organ, and organismal development, as well as auditory, vestibular, and nervous system

development and function, “cancer” was determined to be the top-related disease associated with the differentially methylated genes with inverse expression correlation (Supplemental Table S5). Complementary reported bio-functions are summarized in Table 3.

Table 2. Validation of promoter methylation status and gene expression in postnatal day (PND)90 prostate.

Gene	Selection criteria [#]		Validated in EB and/BPA		Gene name
	A	B	Methylation ^a	Expression ^b	
Acrbp		✓	Hyper		Acrosine binding protein
Btbd3	✓				BTB domain containing protein 3
Ccdc67		✓			Coiled-coil domain containing protein 67
Chad		✓	Hyper		Chondroadherin
Chst14	✓		Hypo	↑	Carbohydrate sulfotransferase 14
Creb3l4	✓		Hyper	↓	cAMP responsive element binding protein 3-like 4
Dnase2b		✓			Deoxyribonuclease II beta
Habp2		✓			Hyaluronan binding protein 2/FSAP
Krt83		✓			Keratin 83
Nfia		✓			Nuclear factor I/A
Osbpl6		✓	Hyper		Oxysterol binding protein-like 6
Paqr4	✓		Hypo	↑	Progesterin and adipoQ receptor family member 4
Phpt1	✓		Hypo		Phosphohistidine phosphatase 1
Pitx3		✓	Hypo	↑	Paired-like homeodomain 3
Prkar1a		✓			Protein kinase cAMP-dependent type 1 regulatory subunit alpha
Rbpjl	✓		Hypo		Recombination signal binding protein for immunoglobulin kappa J region-like
Rnf186		✓	Hyper		Ring finger protein 186
Sox2	✓		Hypo	↑	Sex determining region Y box 2
Tacstd2	✓				Tumor-associated calcium signal transducer 2
Tbx4		✓			T-box 4
Tmem27		✓	Hyper		Transmembrane protein 27
Tpd52	✓		Hyper	↓	Tumor protein D52
Tymp		✓	Hyper		Thymidine phosphorylase
Uox		✓			Urate oxidase
Wnt10b		✓	Hypo	↑	Wingless-type MMTV integration site family, member 10B

[#]Selection criteria used for identifying genes with differential promoter DMR methylation in PND90 prostate of rats neonatally exposed to 17 β -estradiol-3-benzoate (EB) or bisphenol A (BPA) vs. control group

Selection criteria A: $P < 10^{-10}$ and mBar (EB/BPA - Control) ≥ 1.5 or ≤ -0.5 ; Selection criteria B: $P < 10^{-5}$ and mBar (EB/BPA - Control) ≥ 1.2 or ≤ -0.5 .

^aPromoter methylation validated by bisulfite sequencing: Hyper=hypermethylated; Hypo=hypomethylated

^bGene expression validated by qPCR analysis: \uparrow = up-regulated and \downarrow = downregulated

Highlighted in light grey, genes with promoter methylation status validated

Highlighted in dark grey, genes with promoter methylation status and gene expression validated to be inversely correlated.

Effect of 5-aza-cytidine on the expression of differential methylation candidate genes in rat prostate cells

To determine whether the expression of the 7 identified candidate genes (*Pitx3*, *Wnt10b*, *Paqr4*, *Sox2*, *Chst14*, *Tpd52*, and *Creb3l4*) were directly regulated by DNA methylation, we compared gene expression in a rat prostate epithelial cell line NbE-1³⁸ and the rat PCa cell line AIT³⁹, either with or without a 4-day treatment of the DNA methylation inhibitor, 5-aza-cytidine (5-aza) at doses of 0.5 μ M and 1 μ M. When compared to the untreated control, 5-aza treatment significantly upregulated *Pitx3*, *Wnt10b*, *Chst14*, and *Creb3l4* in NbE-1 cells, and *Pitx3*, *Wnt10b*, *Sox2*, *Chst14*, *Tpd52*, and *Creb3l4* in AIT cells (Fig. 5). Although the same treatment increased the expression of *Sox2* and *Tpd52* in NbE-1 cells, the change was not significant. Overall, the 5-aza treatment had no effect on the expression of *Paqr4* in both NbE-1 and AIT cells.

Association of the identified candidate genes with the recurrence of PCa

To gain clinical significance, RNAseq data from 497 PCa specimens and their associated clinical data (Biotab) were retrieved from the TCGA database. Extensive data transformation and biostatistical analyses revealed that the expression of these 7 genes in human PCa samples correlated with recurrence-free

survival, i.e., the cohort of patients was segregated into those with longer (Group 1) and shorter (Group 2) duration to cancer re-appearance after initial treatment. For the initial 5-year survival after diagnosis, Group 2 patients had a shorter time to recurrence (poorer recurrence-free survival) when compared with Group 1 (Fig. 6A, Hazard ratio = 1.786, log rank test $P = 0.0411$), suggesting Group 2 had significantly more aggressive disease. Consistent with this finding, we found that Group 2 had significantly more cases with higher tumor Gleason score (≥ 7) when compared with Group 1 (Odds ratio = 0.3423; $P < 0.0001$) (Fig. 6B). It is well established that men with higher grade tumors have higher risk of dying from PCa.⁴⁰

Discussion

In the present study, we conducted a comprehensive genome-wide search for DNA methylation targets of developmental exposure to EB and BPA, with a view to identifying candidate epigenetic biomarkers for predicting PCa risk. We reported the genome-wide promoter methylation changes in PND90 dorsal prostate tissues related to neonatal exposure to EB or BPA. Overall, we identified 111 EB-associated and 86 BPA-associated DMRs, with 20 in common between the 2 groups. Using two selection criteria, 25 candidate genes were selected, of which 15 were validated to have treatment-associated promoter methylation changes using

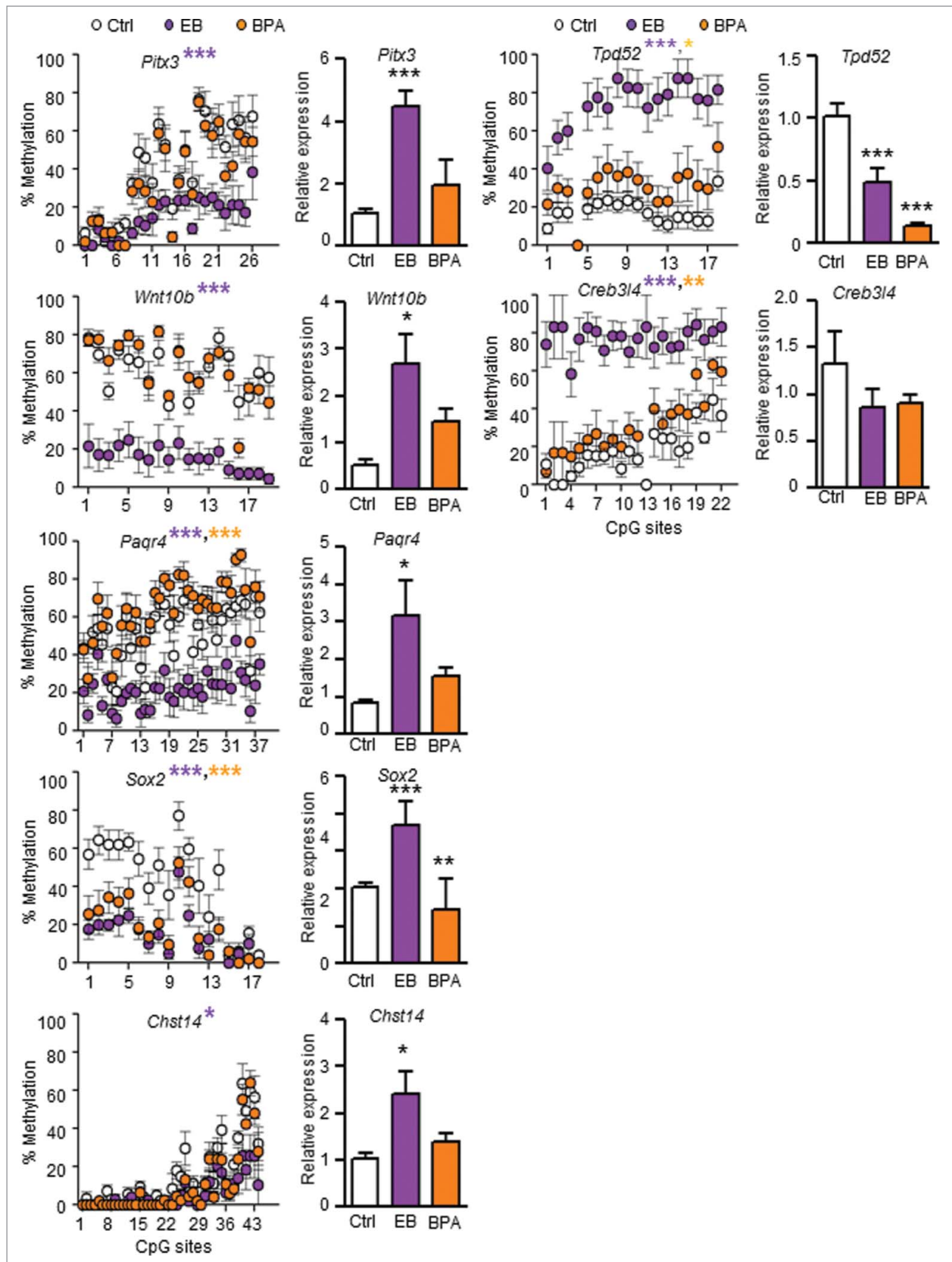


Figure 3. Effect of neonatal exposure to EB or BPA on promoter methylation and gene expression in PND90 dorsal prostate. Promoter methylation status (left panel: Scatter Plot) and expression (right panel: Bar Graph) of candidate genes, in PND90 prostate tissues from SD rats treated with either corn oil (Ctrl; white), EB (green), or BPA (red), were analyzed using bisulfite sequencing and qPCR, respectively. Each circle in the scatter plot represents mean \pm SEM of methylation percentage (averaged from 6 individual samples/animals) at a single CpG site in the gene promoter region. The % methylation of each CpG site in each sample was determined from bisulfite sequencing data derived from 8–12 clones. Gene expression data were expressed as mean \pm SEM from 3 individual samples. Statistical significance was determined by one-way ANOVA and Tukey test when compared to Ctrl. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

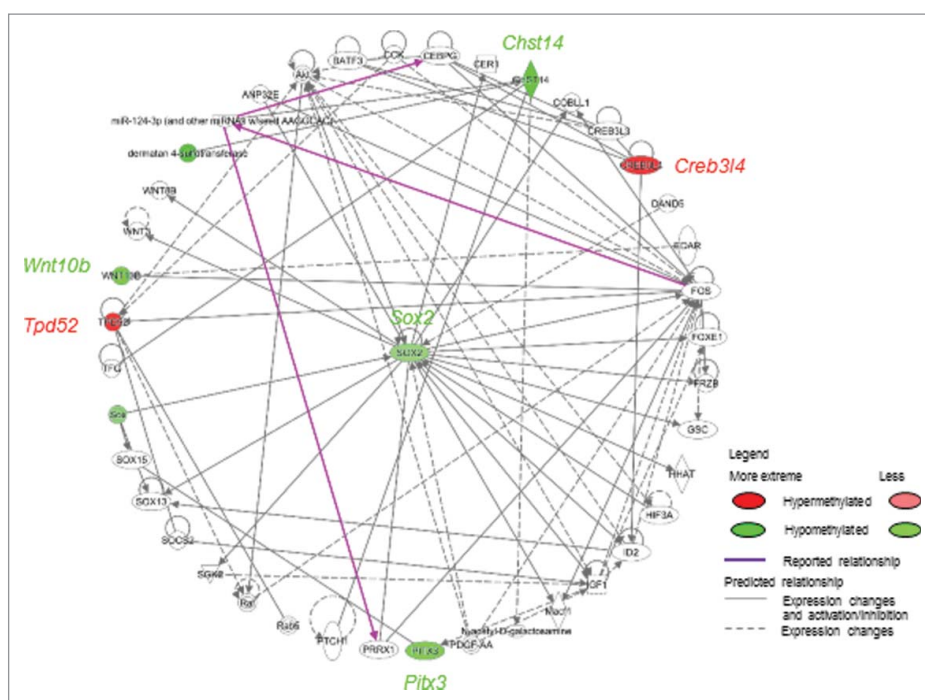


Figure 4. Signaling pathways associated with differentially methylated candidate genes were predicted by Ingenuity Pathway AnalysisTM. Genes with promoter hypermethylation are shown in red and genes with promoter hypomethylation are shown in green, with color intensity signifying the magnitude of differential methylation. Gray arrows indicate predicted association pathways; purple arrows indicate reported directional pathways.

bisulfite sequencing analysis, and 7 identified to exhibit an inverse correlation between promoter methylation status and gene expression. EB treatment upregulated the expression of *Pitx3*, *Wnt10b*, *Paqr4*, *Sox2*, and *Chst14* through promoter hypomethylation, and downregulated *Tpd52*, and *Creb3l4* through promoter hypermethylation. Similarly, BPA treatment upregulated *Sox2* and *Chst14* through promoter hypomethylation, and downregulated *Tpd52* through promoter hypermethylation, but had no significant effects on the other genes. Of the 7 candidate genes identified in the rat, *WNT10b*, *SOX2*, *TPD52*, and *CREB3L4* were upregulated in PCa (Table 3), and *PAQR4*, *WNT10b*, *PITX3*, and *TPD52* were differentially methylated between PCa and adjacent normal prostate tissues.⁶⁹ Clustering analysis, based on the expression of these 7 genes in PCa tissues of 497 PCa patients from the TCGA data sets, further revealed an association with shorter recurrence-free survival. Taken together, DNA methylation-mediated dysregulation of this unique signature of 7 genes established a causal link between early-life EDC exposure and PCa risk, and raises the possibility that such exposure may impact the human disease.

Neonatal development is a susceptible window for the DNA methylation reprogramming effects of EDCs. Since the prostate is estrogen responsive, it is highly vulnerable to insult by estrogenic agents during development.^{27,34,41} Using a neonatal EB/BPA exposure rat model, we previously reported that treatment-associated dysregulation of *Pde4d4*, *Hmgn5*, and *Hpcal1* through promoter methylation changes, persisted in the prostate throughout life.^{25,32} While we observed an increase in the incidence and severity of PIN in PND200 rats upon E₂-induced carcinogenesis,^{25,32} the current study aimed to interrogate the underlying mechanism for the early-life EDC exposure-associated increase in PCa risk, by performing

genome-wide promoter methylation analysis on PND90 prostate tissue. The 111 EB- and 86 BPA-associated genes with identified DMRs were mostly related to cell-to-cell signaling and interaction, cell-mediated immune response, and cellular growth and proliferation, with cancer as the major disease outcome. Specifically, most of these treatment-associated genes were related to 2 oncogenic rapid signaling molecules AKT and ERK1/2. AKT is upregulated in prostate tumors when compared to benign prostatic tissues^{42,43} and is associated with a stage-dependent increase in tumor cell proliferation.⁴⁴ Its activation has also been shown to suppress androgen/androgen-receptor induced apoptosis in PC3 cells.⁴⁵ Unlike AKT, expression of ERK1/2 is similar between PCa and normal tissues, yet its activation is associated with increased cell proliferation in PCa tissues.⁴⁶ Moreover, the concomitant activation of Akt and Erk1/2 promotes PCa cell growth and tumorigenicity in a rodent PCa model.⁴⁷ Taken together, these reports suggest a possible role for the identified treatment-associated genes in the malignant transformation of rat prostate epithelial cells.

Interestingly, functional connectivity of the 7 candidate genes was linked to embryonic stem cell pluripotency, further evidenced by convergence at *Sox2*. SOX2 is a stem cell marker upregulated in PCa.⁴⁸⁻⁵⁰ Overexpression of SOX2 in human DU145 PCa cells promotes tumorigenesis,⁴⁸ increases cell proliferation and migration, and reduces apoptosis.^{51,52} Of the other identified genes, *TPD52*, an oncogene, has been reported to be upregulated in high-grade PIN^{33,53} and PCa.⁵²⁻⁵⁴ *CREB3L4*^{55,56} and *WNT10B*⁵⁷ have also been found to be upregulated in prostate tumors. On the contrary, the expression of *PITX3* and *CHST14* has yet to be studied in PCa tissues. While only 4 of the 7 genes have been functionally characterized in PCa cells and tissues, all genes, except *PAQR4*, are related to

Table 3. Reported bio-function(s) and PCa associations of the seven identified candidate genes with DMRs inversely correlated with gene expression as a result of neonatal 17 β -estradiol-3-benzoate/bisphenol A (EB/BPA) exposure.

Gene	Promoter methylation		Gene expression		Reported bio-function(s)	Gene expression in PCa	Other pathological diseases	Reported stem/progenitor cell bio-function
	EB ^a	BPA ^b	EB ^a	BPA ^b				
Pitx3	*		*		Knockdown of Pitx3 promoted the loss of lens epithelial cells by inducing early activation of cell cycle inhibitors ⁷¹	Not reported	Downregulated in Chinese patients with Parkinson's disease ⁷²	Expression of Pitx3 initiated differentiation of mid-brain derived neural stem cells ⁷³
Wnt10b	*		*		Upregulating Wnt10b expression in endometrial cells Ishikawa increased cell proliferation and reduced apoptosis ⁷⁴ Overexpression of Wnt10b in dermal papilla cells increased cell proliferation ⁷⁵ Overexpression of Wnt10b induced S-phase cell progression in mouse mammary epithelial and human BCa cells ⁵⁰ Overexpression of WNT10B in TNBC increased cell proliferation by upregulating oncogene high mobility group A hook-2. ⁷⁶	Upregulated in prostate tumors when compared to normal prostate tissues ⁵⁷ Upregulated in prostate tumors in a stage-dependent manner ⁷⁷	Upregulation is associated with poor survival of patients with osteosarcoma ⁵ Upregulated in endometrial cancer tissues when compared to hyperplastic or normal tissue samples ⁷⁴	Treatment of human embryonic stem cell colony culture induces the formation of prostate organoids ⁷⁸ Wnt10b conditioned medium promoted proliferation, morphological changes, and chemotaxis of immortalized and primary mouse spermatogonial stem cells ⁷⁹
Paqr4	*		*		Not reported	Not reported	Pancreatic cancer ⁸⁰	Not reported
Sox2	*	*	*	*	Overexpression of Sox2 in DU145 increased cell migration and proliferation, reduced apoptosis, ^{51,52} and promoted tumorigenesis ⁴⁸ Conditional Sox2 deletion in pre-existing skin papilloma and skin squamous-cell carcinoma (SCC) reduced tumor growth and cancer cell propagation in serial transplantation ⁸¹ Overexpression of Sox2 in chick embryos inhibited neural differentiation ⁸²	Upregulated in PCa ⁸³ Upregulated in PCa with higher Gleason score ^{48,49}	Downregulated in colon cancer ⁸⁴ and gastric cancer ⁷ Upregulated in squamous cell lung cancer ⁸⁵ , SCC ⁸¹ and nasopharyngeal carcinoma ⁸⁶ , and frequently expressed in basal-like breast cancer tumors when compared to luminal and HER2+ tumors ⁸⁷	Overexpression of Sox2 protein in mouse embryonic stem (ES) cells trigger their differentiation into cells that express markers for various differentiated cell types ⁸⁸ Overexpression of Sox2 suppresses neural differentiation through downregulating GFAP expression ⁴ . Sox2 expression maintains osteoblast self-renewal ⁸⁹ Deletion of Sox2 in neural stem cells (NSC) reduced the size and formation of neurospheres ⁹⁰ Inhibiting Sox2 signaling decreased progenitor marker expression and promoted neuronal differentiation ⁸²
Chst14	*		*		Loss of Chst14 in neuronal stem cells reduce cell proliferation ⁹¹	Not reported	Downregulated in colorectal cancer ⁸⁴	Loss of Chst14 decreased neurogenesis and proliferation of neural stem cells ⁹¹
Tpd52	*		*	*	Overexpression of TPD52 increased LNCaP cell proliferation and migration ⁵² Mice inoculated with TPD52 overexpressing fibroblasts have been shown to develop lung metastasis ⁹²	Upregulated in high-grade PIN ^{53,93} and PCa ⁵²⁻⁵⁴ Higher expression in Gleason score 4 than 3 prostate tumors, ⁹³ in localized PCa with Gleason score 4+4=8 than 3+3=6 localized PCa ⁹⁴ , and in hormone naive and refractory metastatic PCa ⁹⁵	Upregulated in ovarian cancer ⁹⁶ Upregulated in testicular germ cell tumors ⁹⁷	Higher expression in the earlier passages of differentiating human dental pulp stem cells ⁹⁸
Creb3l4	*				Overexpression of Creb3l4 in LNCaP cells upregulated genes related to PCa ⁹⁹ .	Upregulated in PCa when compared to adjacent normal tissues. ^{55,56}	Not reported	Loss of Creb3l4 increased apoptosis of meiotic/post-meiotic germ cells in the mouse seminiferous tubules ¹⁰⁰

^aTreatment with 2,500 μ g EB/kg BW/day on PND1, 3, and 5.

^bTreatment with 10 μ g BPA/kg BW/day on PND1, 3, and 5.

*Indicates a statistically significant change ($P < 0.05$) in promoter DMR methylation (validated by bisulfite sequencing) or gene expression (validated by qPCR) between treatment and control groups

Light grey, promoter hypomethylation or decreased gene expression; dark grey, promoter hypermethylation or increased gene expression

stem cell proliferation, cancer cell proliferation migration, and/or tumorigenesis (reported bio-functions are summarized in Table 3).

We have previously shown that prostate stem/progenitor cells isolated from the human adult prostate are more proliferative and able to retain their "stemness" properties when

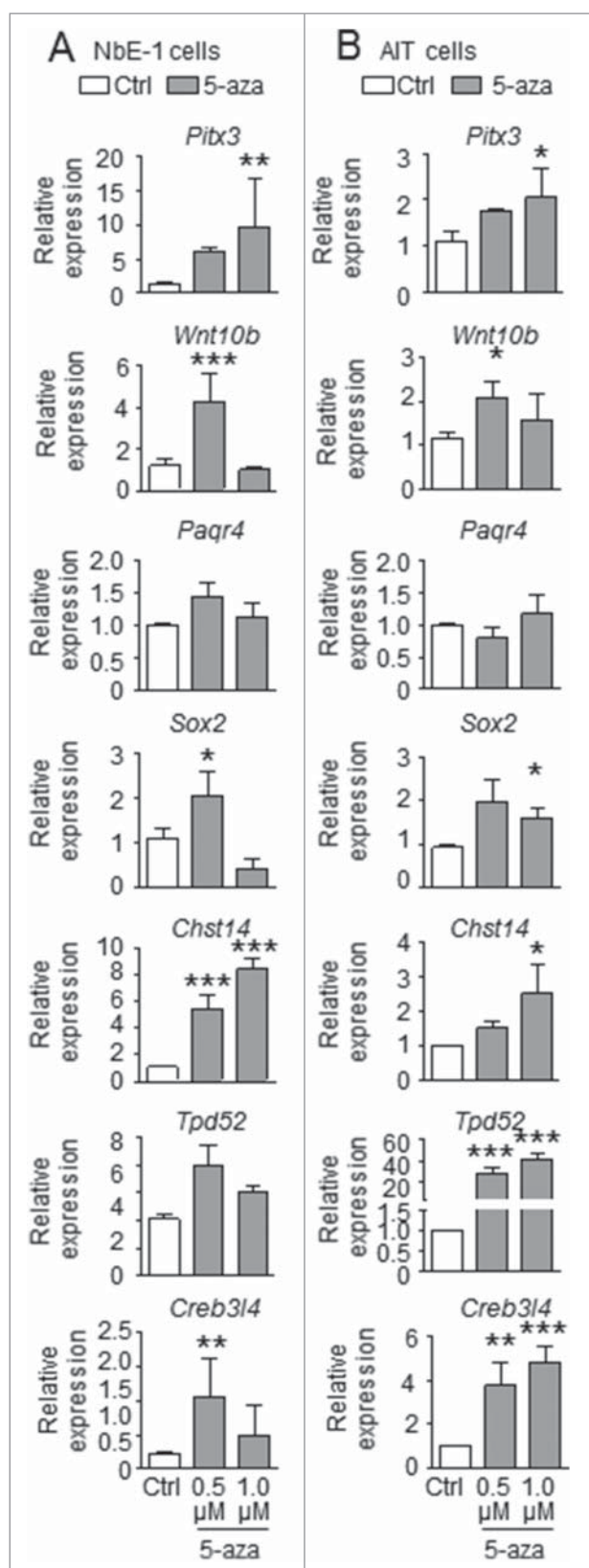


Figure 5. Effect of 5-aza-cytidine treatment on gene expression in NbE-1 and AIT cells. Gene expression was analyzed by qPCR in rat (A) normal prostate epithelial NbE-1 cells and (B) prostate cancer AIT cells treated with DMSO (Ctrl), or 0.5 μ M or 1 μ M 5-aza-2-deoxycytidine (5-aza), a DNA methylation inhibitor, for 8 d. Data (mean \pm SEM) is normalized to corresponding *Rpl19* levels, and is expressed as fold change vs. Ctrl. * $P < 0.05$, and ** $P < 0.001$ by one-way ANOVA and Tukey test when compared to Ctrl.

treated with E_2 or BPA.^{17,19} Suppression of a class of non-coding RNAs, the small nucleolar RNA C/D box,³⁰ associated with distinct histone modifications^{19,30} has been postulated as a mechanism causing a slow-down in biosynthesis and stem cell differentiation, thus extending the proliferation phase of these cells. In concordance, our current study showed that neonatal exposure to xenoestrogens led to the dysregulation of a panel of stem cell function-related genes through DNA methylation in the adult prostate, lending further credence to this hypothesis. In this regard, neonatal exposure to xenoestrogens may allow for an extended proliferation phase and increase the number of stem/progenitor cells in the prostate. As such, these cells are known targets for carcinogen-induced malignant transformation in adult-life, leading to increased cancer susceptibility.⁵⁸

In addition to their reported involvement in PCa, of clinical relevance, we report here that the expression of the novel 7-gene set is associated with shorter recurrence-free survival of PCa patients in the TCGA cohort. These patients were also mostly diagnosed with prostate tumors of high Gleason score (≥ 7). Of interest, we also found that 4 out of these 7 genes were differentially methylated between PCa tissues and their adjacent normal tissues in the cohort reported by Kim et al.⁵⁹ Thus, this gene signature may have diagnostic/prognostic value by serving as biomarkers for predicting disease initiation and progression.

Although neonatal exposure to either EB or BPA increased PCa risk in the rodent model, their DNA methylation mediated effects on the prostate are different. There were more EB-associated than BPA-associated genes identified from our promoter methylation array. Genes with methylation changes validated were also mostly EB-associated. Moreover, the EB-associated genes linked to AKT and ERK1/2 as the major signaling molecules, whereas the BPA-associated genes linked to TGF β , an oncogenic molecule upregulated in PCa.⁶⁰ This indicates that they activate different signaling pathways to increase PCa risk. Furthermore, EB-treatment dysregulated *Pitx3*, *Wnt10b*, *Paqr4*, and *Creb3l4* in PND90 prostate tissues through promoter methylation changes, but BPA exposure had no significant effects. This suggests that EB had a stronger effect on the prostate than BPA, which could in part be related to a markedly higher dose of EB utilized (2500 μ g/kg BW) when compared to the lower environmentally relevant dose of BPA (10 μ g/kg BW). Alternatively, it has been reported that the action of BPA can be independent of the classical estrogen receptors (ER α and ER β) and mediated via estrogen-related receptor γ (ERR γ) and G protein-coupled receptor 30 (GPER1), to name a few.^{61,62} However, this does not explain why the expression of *Acrbp*, *Chad*, *Osbp16*, *Phpt1*, and *Tmem27* did not correlate to their confirmed promoter methylation status, nor why the expression of *Paqr4* was unaffected by 5-aza treatment in NbE-1 and AIT cells. In human prostatespheres, E_2 /BPA-mediated small nucleolar RNA box C/D suppression was shown to be associated with the occupancy of histone marks the trimethylated histone 3 at lysine 9 and lysine 27, and the loss of trimethylated histone 3 at lysine 4, rather than DNA methylation.³⁰ Thus, EB and BPA may dynamically alter the PND90 prostate transcriptome through other epigenetic mechanisms not yet explored.

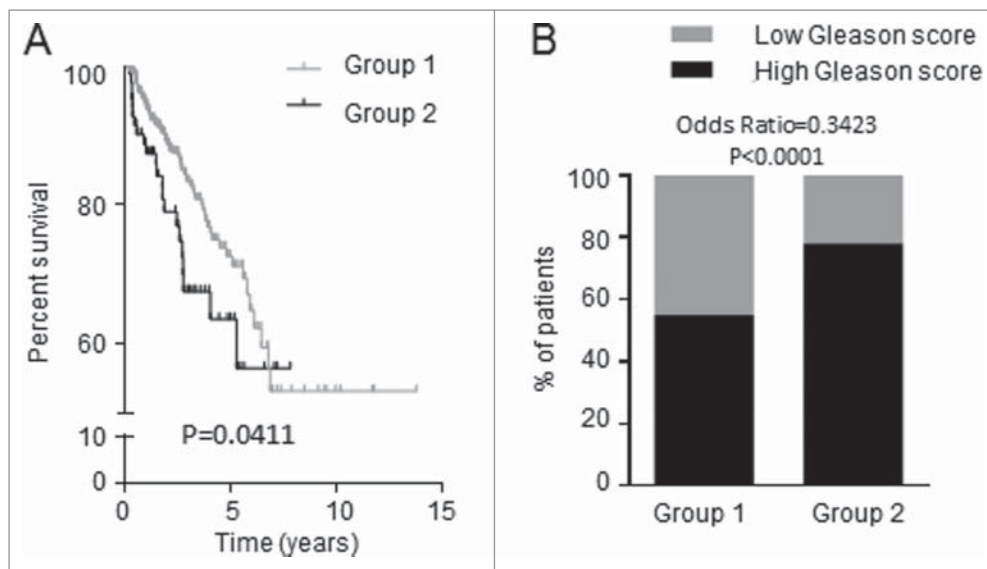


Figure 6. Expression of the 7 candidate genes was associated with shorter recurrence-free survival of PCa patients. TCGA data consisting of 497 PCa patients were dichotomized into Groups 1 and 2 by K-means clustering analysis based on the 7 candidate genes. (A) Group 1 patients have longer time to recurrence than Group 2 patients; the two groups differ in recurrence-free survival. (B) Proportion of PCa patients having tumors with high (≥ 7) and low (< 7) Gleason score.

In summary, we have identified a set of 7 genes (*Pitx3*, *Wnt10b*, *Paqr4*, *Sox2*, *Chst14*, and *Tpd52*, and *Creb3l4*) with differential methylation at the promoter region, in PND90 dorsal prostate tissue of rats neonatally exposed to EDCs. The methylation pattern of these 7 genes was inversely correlated to gene expression. Cell-based studies using 5-aza-cytidine-treated normal (NbE-1) and cancerous (AIT) prostate epithelial cell lines further confirmed that gene expression was regulated by DNA methylation, in 6 of the 7 genes. To provide clinical relevance, 4 of the 7 genes (*PITX3*, *WNT10B*, *PAQR4*, and *TPD52*) have been reported to be differentially methylated between PCa and normal adjacent tissues. Moreover, expression of the 7 genes is associated with the recurrence-free survival of PCa patients. Taken together, we conclude that DNA methylation changes in our novel 7-gene signature may be an epigenetic signature associated with increased cancer susceptibility in the adult gland due to early-life exposure.

Materials and methods

Animal housing and treatments

All animal treatments in this study were in accordance with the accepted standards of humane animal care and approved by the Animal Use Committee at the University of Illinois. Pregnant Sprague-Dawley (SD) rats used on postnatal day (PND) 90 were from Zivic-Miller Laboratories (Pittsburgh, PA) and shipped on gestational day 12. Animal husbandry and conditions were controlled to avoid inadvertent BPA and phytoestrogen exposures, as reported previously.^{21,63}

A schematic diagram of the experimental and analytical procedures is outlined in Figure 1. Pregnant dams were monitored and the day of birth was designated as PND0. Anogenital distance was used to segregate male and female pups. Male pups were randomly assigned to one of 3 treatment groups, $n = 30/\text{group}$: (a) controls given tocopherol-stripped corn oil vehicle alone (Ctrl), (b) 25 μg EB/pup or

2,500 μg EB/kg BW (Sigma-Aldrich Chemical Co., E8515), or (c) 0.1 μg BPA/pup or 10 μg BPA/kg BW (Sigma-Aldrich Chemical Co., 239658). To avoid litter effects, pups from each litter were equally divided among treatment groups and toe clipped for permanent identification. Pups were subcutaneously injected in the nape of the neck with the respective steroids on PND1, 3 and 5, weaned on PND21, and siblings were housed until PND90. The dose and route of EB and BPA was based on our published study^{25,64} and utilized to maintain continuity of results.²⁷ We had previously characterized the BPA pharmacokinetics in PND3 neonates following a single subcutaneous injection of 10 $\mu\text{g}/\text{kg}$ BW of the endocrine disruptor.³⁴ The mean sera unconjugated BPA level reached a C_{max} of 1.77 ng/ml in 0.5 h but rapidly declined to 0.7 ng/ml and 0.54 ng/ml at 1 h and 2 h, respectively, post-injection. These findings suggest a rather rapid rate of biodegradation of BPA in PND3 neonates, making it unlikely that BPA will reach higher levels upon multiple injections (PND1, 3, 5). However, since we did not have actual measurements of circulating BPA in the neonates during this period, this conclusion may simply be a conjecture. Yet, it should be noted that these levels and those reported before in rodent neonates^{34,65} are well within the range reported for human developmental exposures.^{12,15,34}

Genomic DNA extraction from dorsal prostate

Genomic DNA was extracted from the rat dorsal prostate using DNeasy Blood & Tissue kit (Qiagen, Valencia, CA) and in the presence of RNase A (Qiagen 19101) according to the manufacturer's protocol. All reagents were supplied in the kit. In brief, the tissue was lysed in Buffer ATL with proteinase K at 56°C until the tissue was completely lysed. The lysate was incubated with 100 mg/ml RNase A and mixed thoroughly with equal portions of Buffer AL and 100% ethanol. The mixture was loaded on to a DNeasy Mini spin column, and washed with Buffer AW1 and Buffer AW2 consecutively by

centrifugation. Genomic DNA was eluted with DEPC water diluted Buffer AE.

Methylated CpG island Recovery Assay (MIRA)-assisted Methylation array analysis

A total of 5, 4, and 5 PND90 prostate genomic DNA samples from EB, BPA, and Ctrl group, respectively, were used to perform the MIRA-assisted genome-wide methylation analysis. In brief, 4.5 μg genomic DNA was sonicated by Bioruptor (Diagenode, Belgium) to obtain 300–500 bp fragments. The methylated DNA fraction was enriched using MethylCollector Ultra kit (ActiveMotif, 55005) via MIRA-assisted methylated DNA enrichment with proper enrichment controls. It should be noted that the MIRA-, also known as MBD2-, assisted procedure enriches CpG-dense sequences that are likely in CpG islands.⁶⁶ The methylation-enriched DNA and its corresponding Input were then amplified using GenomePlex WGA kit (Sigma-Aldrich, WGA2), labeled with Cy3/Cy5 dyes, and co-hybridized in Rat ChIP 385K Promoter 2 array set (Roche NimbleGen, Madison, WI) according to the manufacturer's protocol. The arrays have $\sim 780,000$ probes with probe sizes ranging from 50–70 mers in length and median probe spacing of 105 bp, covering $\sim 16,000$ regions primarily in promoter regions of $\sim 23,000$ transcripts from known genes. These regions principally contain CpG-dense DNA sequences. The probes are typically placed at 4,500 bp upstream and 1,125 bp downstream from the transcriptional start site (TSS). The estimated coverage approximates 97Mb and 3.7% coverage of the rat genome.⁶⁷ Thus, the design of the experiments was to focus on CpG rich regions within or near known or predicted gene promoters. This design has the advantage of increasing the hit rate and reducing noise, but does have the deficit of biasing toward gene promoters and having low coverage of the overall rat genome and DMRs not assisted with gene promoters.

Methylation array data analysis

Data analysis was performed using R and Bioconductor packages.⁶⁸ The log-ratios of Cy5 to Cy3 (M values) were first normalized using GC-loess normalization. The average M value was then calculated for each probe across all samples in the same group. The significantly enriched regions were identified using sliding window analysis for each group separately. In this analysis, a window of 750 bps around each probe was considered at a time and RandomSet statistic⁶⁹ was calculated on the average M values of all probes in the window to estimate the enrichment of the probes in the window when compared to the background. Since there is no enrichment of immunoprecipitated DNA among the probes in the window, the RandomSet statistic, which is defined as the average of the average M values in the window, is approximately distributed as the normal random variable. The *P*-value of each window was obtained based on the RandomSet statistic and adjusted by the false discovery rate. Windows that were significantly enriched ($P < 10^{-5}$) in any of the groups (EB, BPA, and Ctrl) were mapped to genes and were further filtered, where differentially methylated genes were selected based on the difference

of mBar values of treatment group (EB/BPA) when compared to Ctrl and at different cutoff levels of *P*-value (Fig. 1). Using this approach, a methylation bar plot was generated for each promoter region in the 3 groups (Fig. 2). In a given promoter region, the difference in height of the bars (mBar) between treated and Ctrl groups represents the difference in probe intensity, i.e., the difference in promoter methylation status. When compared with the control, a positive and negative mBar difference in the treated group indicates promoter hyper- and hypo-methylation, respectively. Primers were then designed based on these differentially methylated regions for validation using bisulfite sequencing analysis.

Bisulfite PCR sequencing analysis

Based on the methylation array bar plot, significant differential methylated regions flanking the gene transcription start sites were selected for methylation validation using bisulfite sequencing. Primers used for bisulfite sequencing were designed with MethPrimer.³⁷ (Supplemental Table S6) Genomic DNA was bisulfite modified using an EZ DNA Methylation kit (Zymo Research, D5001). In brief, 500 ng genomic DNA diluted with M-Dilution buffer was incubated at 37°C for 15 min, mixed with CT reagent and incubated for 16 hours at 50°C. The reaction was then mixed with Binding buffer, loaded to a spin column, washed, incubated with desulphonation buffer at room temperature for 20 min, and eluted with 40 μl elution buffer. Bisulfite PCR was performed using 2 μl bisulfite modified DNA and Platinum Taq DNA Polymerase (Invitrogen, 10966026) in a 25 μl reaction according to the manufacturer's protocol. Following 40 cycles of PCR amplification with the annealing temperature at 55°C, the amplicons were gel-purified and TA-cloned into pGEMT-easy vector (Promega, A1360). Plasmids from a single *E. coli* colony were amplified using TempliPhi DNA amplification kits (GE Healthcare, 25640010) and sequenced (Macrogen USA, Rockville, MD). The methylation status of each CpG site was analyzed using BiQ Analyzer.⁷⁰

To validate the promoter methylation status of genes identified from the PND90 prostate microarray analysis, the same amount of DNA from each animal of the same treatment group was mixed for bisulfite sequencing with ~ 12 clones per group. To further compare methylation pattern of promoter DMR of the 7 selected gene candidates, bisulfite sequencing analysis on 6 samples each from individual animal was performed with ~ 8 –12 clones selected per sample.

Treatment of NbE-1 and AIT cells

Rat prostatic epithelial cell lines, the immortalized normal prostatic epithelial cell line NbE-1³⁸ and the tumorigenic cell line AIT³⁹ were maintained in DMEM/F12 medium (Invitrogen, 11330057) supplemented with 5% fetal bovine serum (HyClone, 35-010-CV), 1X insulin-transferrin-selenium (Invitrogen, 41400045), 1 mM sodium pyruvate (Invitrogen, 11360070), and 100 mM minimum nonessential amino acids (Invitrogen, 11140076). The cells were seeded and treated with 0.5 μM or 1.0 μM 5-aza-2-deoxycytidine (5-aza-dC; Tocris,

2624), a DNA methylation inhibitor, every 2 d for 8 d as previously described.^{25,32} Treatment with 0.1% dimethylsulfoxide (DMSO; Sigma D4540) was used as control (Ctrl) in each experimental set. Cell lysates were stored in TRIzol reagent for RNA extraction.

RNA extraction and real-time RT-qPCR

Dorsal prostate tissue was homogenized in TRIzol reagent (Invitrogen, 15596) using Precellys 24 homogenizer (Bertin Technologies, France) and extracted according to the manufacturer's protocol. One microgram total RNA was reversed transcribed using SuperScript III Reverse Transcriptase (Invitrogen, 18080085) in a 20 μ l reaction. Primers for real-time RT-qPCR were designed using Primer-BLAST program (Supplemental Table S7). qPCR analysis was performed in a 7500 Fast Real-Time System (ABI, Foster City, CA) in triplicate in a 20 μ l reaction. Target gene expression was normalized against the individual endogenous *Rpl19*³⁴ and relative gene expression was calculated using the $2^{-\Delta\Delta C_t}$ method.³⁰ Similarly, total RNA from treated cells was extracted using TRIzol reagent (Invitrogen, 15596-018) according to the manufacturer's protocol and reversed transcribed for qPCR analyses as previously described.³⁰

Functional connectivity analysis

Ingenuity Pathway Analysis (IPA; Qiagen, Redwood City) was performed as previously described⁹ for analyzing, integrating, and interpreting the data generated from the methylation array.

Association with the recurrence-free survival of PCa patients

RNaseq data (RNaseqV2, level 3) from 497 PCa subjects as well as their associated clinical data (Biotab) were downloaded from the TCGA database on Feb 1st 2016. Expression levels of the 7 genes were pulled from normalized RNaseq gene results. The data were variance stabilizing transformed before dichotomizing into 2 groups by K-means clustering analysis. Recurrence-free survival was determined based on "days to new tumor event after initial treatment." Survival analysis with log rank test as well as hazard ratio were calculated using Graph-Pad Prism software (La Jolla, CA).

Statistical analysis

Data are expressed as mean \pm SEM. There were at least 3 individual experimental sets for gene expression analysis. Bisulfite sequencing (BS) analysis of the 7 selected gene candidates was performed on 6 individual samples, with \sim 8–12 clones per group. One-way ANOVA and Tukey test were used for comparison among groups. $P < 0.05$ was considered as statistically significant.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

Acknowledgments

The results published here are in whole or in part based upon data generated by the TCGA Research Network: <http://cancergenome.nih.gov/>. We thank The Genomics, Epigenomics, and Sequencing Core for the array service. We also thank Lynn Birch, Wen Yang Hu, Saikumar Karyala, Miral Patel, Hong Xiao and for their technical assistance and Jennifer Veevers for her excellent editing of the manuscript.

Funding

This study was supported in part by grants from the National Institutes of Environmental Health Sciences: R01CA015776 (SMH), R01ES015584 (GSP, SMH), RC2ES018758 (GSP, SMH, MM), RC2ES018789 (SMH, MM), U01ES019480 (SMH, MM), U01ES020988 (SMH, MM), CA172220 (GSP), and P30ES006096 (SMH, MM), the United States Department of Veterans Affairs I01BX000675 (SMH), and the Department of Defense Prostate Cancer Research Program W81XWH-06-1-0373 (WT) and W81XWH-15-1-0496 (AC).

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