SMYD3 as an Oncogenic Driver in Prostate Cancer by Stimulation of Androgen Receptor Transcription

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Background
Androgen receptor (AR) is critical for prostate tumorigenesis and is frequently overexpressed during prostate cancer (PC) progression. However, few studies have addressed the epigenetic regulation of AR expression.

Methods
We analyzed SMYD3 expression in human PC with Western blot and immunohistochemistry. SMYD3 expression was knocked down using short hairpin RNA (shRNA) or small interfering RNA (siRNA). Cell proliferation, colony formation, and apoptosis analyses and xenograft transplantation were performed to evaluate the impact of SMYD3 depletion on PC cells. AR expression and promoter activity were determined using real-time quantitative polymerase chain reaction, western blot, and luciferase reporter assay. AR promoter association with Sp1, SMYD3, and histone modifications was assessed by chromatin immunoprecipitation. Differences in AR mRNA abundance and promoter activity were analyzed using Wilcoxon signed-rank tests, SMYD3 expression was analyzed using with Mann–Whitney U tests for unpaired samples, and tumor weight was analyzed with Student t test. All statistical tests were two-sided.

Results
The upregulation of SMYD3 protein expression was observed in seven of eight prostate tumor specimens, compared with matched normal tissues. Immunohistochemical analysis showed a strong SMYD3 staining in the nuclei of PC tissues in eight of 25 (32%) cases and in the cytoplasm in 23 out of 25 (92%) cases, whereas benign prostate tissue exhibited weak immunostaining. Depletion of SMYD3 by siRNA or shRNA inhibited PC cell proliferation (72 hours relative to 24 hours: control shRNA vs SMYD3 shRNA 1: mean fold change = 2.76 vs 1.68; difference = 1.08; 95% confidence interval = 0.78 to 1.38, P < .001), colony formation, cell migration, invasion, and xenograft tumor formation. Two functional SMYD3-binding motifs were identified in the AR promoter region.

Conclusions
SMYD3 promotes prostate tumorigenesis and mediates epigenetic upregulation of AR expression.


Androgen–androgen receptor (AR) is the central signaling pathway in normal growth of the prostate gland and prostate carcinogenesis. When activated by the binding of androgen ligands, AR becomes phosphorylated and translocates into the nucleus. Within the nucleus, AR recognizes and occupies its binding motifs in the genome and activates the transcription of its target genes, which are essential for prostate development and prostate cancer (PC) progression (1).

The dysregulated AR expression in PC and its underlying mechanisms have been of considerable research interest because these represent the most therapeutically relevant targets in this disease. Several lines of evidence have demonstrated that the progression from androgen-dependent prostate cancer (ADPC) to castrate-resistant prostate cancer (CRPC) is associated with the upregulation of AR, whereas AR inhibition represses PC growth (2). Moreover, AR mRNA levels are frequently increased in CRPC cases, of which only 10% to 20% have AR gene amplification, suggesting that AR transcription is altered during PC progression (2).

Recent studies have shed light on the importance of epigenetic events in the carcinogenesis and progression of PC, including the facilitation of AR signaling by histone-modifying enzymes. However, the epigenetic regulation of AR expression in PC has remained poorly understood (3). SET and MYND domain-containing protein 3 (SMYD3) is a histone methyltransferase that contains a SET domain and has histone H3-K4 di-/tri- and H4-K5-methyltransferase activities (4). It recognizes and occupies the DNA binding motif(s) 5′-CCCTCC-3′ in the promoter regions of its downstream target genes and di-/trimethylates H3-K4, thereby leading to transcriptional activation (5). Expression of SMYD3 is undetectable or very weak in many types of normal human tissue, whereas overexpression of SMYD3 has been linked with the development and progression of colorectal, hepatocellular, and breast cancers (6). The role of SMYD3 in the development and progression of PC, however, has not yet been studied.
Methods

Cell Lines and Tissue Specimens
LNCaP, PC3, and DU145 cells were obtained from American Type Culture Collection (Manassas, VA). The tissue donation program was approved by the Ethical Committee of Shandong University Qilu Hospital. Benign and PC tissue specimens were obtained by biopsy or surgical procedures from 33 patients at the First Hospital of Peking University, Shandong University Qilu Hospital, and Qingdao Municipal Hospital. All patients had histologically confirmed PC, and the presence of tumor tissue within the specimens was checked. Clinical staging was determined according to Tumor node metastasis (TNM) classification of International Union against Cancer (Supplementary Table 1, available online).

Plasmids and Transfection of Cells
PGL3-basic vector containing AR 2-kb promoter (AR-promoter) and 6-kb Prostate-Specific Antigen (PSA) promoter (PSA-promoter) were obtained courtesy of Professor Huiqing Yuan (Shandong University) (7,8). PC cells were transfected with plasmid using LipofectAMINE2000 (Invitrogen, Carlsbad, CA). Mutagenesis was performed with QuickChange II Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA).

Cell Proliferation and Xenograft Tumor Growth
Colony formation and cell proliferation assay were performed as described (9,10). The mouse experiments and animal care procedures were approved by the Ethics Committee of Shandong University Qilu Hospital. Six-week-old male BALB/c nude mice were obtained from Shanghai Laboratory Animal Center (Shanghai, China). LNCaP cells (10^5) stably expressing SMYD3 or control short hairpin RNA (shRNA) were suspended in 150 µL of phosphate-buffered saline and inoculated subcutaneously in the inguinal areas (n = 8 per group). After 7 and 10 weeks, the mice were killed by carbon dioxide asphyxiation, and the tumors were collected for analysis.

Chromatin Immunoprecipitation (ChIP)
ChIP assay was performed as previously described (11). Additional details are in the Supplementary Methods (available online).

Wounding Assay and Transwell Assay
Cultured cells at 75% confluence were subjected to wounding as previously described (12). Transwell assay was performed using Transwell chamber migration assay (8-µm pore size; BD Biosciences, San Jose, CA) (13). For invasion assays, transwell chambers were first covered with matrigel (BD Biosciences), and an experimental procedure similar to the migration assays was completed. Additional details are available in the Supplementary Methods (available online).

Real-time Quantitative Polymerase Chain Reaction, Immunohistochemistry (IHC), and Immunoblotting
We extracted total cellular RNA using RNeasy mini-kits (Qiagen, Hilden, North Rhine-Westphalia, Germany). Levels of target mRNA were calculated based on the computed tomography (CT) values and normalization of β2-M expression. IHC was performed on 4-mm sections of paraffin-embedded specimens using SMYD3 antibody (Abcam, Cambridge, UK). For Western blot analysis, total cellular proteins were extracted with Sodium dodecyl sulfate (SDS) lysis buffer, and 30 µg of the protein were resolved sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride (PVDF) membrane. The membranes were probed with the SMYD3 antibody, AR antibody (Santa Cruz Biotechnology, Santa Cruz, CA) or BCL-xL antibody (Cell Signaling Technology, Danvers, MA). Additional details are available in the Supplementary Methods (available online).

CCK-8 Assay
Cell proliferation was assessed by the cell counting kit 8 (CCK-8) assay according to the manufacturer’s Instruction (Dojindo Molecular Technologies, Rockville, MD). LNCaP cells in a 96-well plate were incubated with CCK-8 solutions for 1 hour at 37°C. Absorbance of each well was quantified at 450 nm by an enzyme-linked immunosorbent assay microplate reader.

Small interfering RNA (siRNA) Treatment and shRNA Transfection
Chemically modified Stealth siRNA were purchased from Invitrogen (see Supplementary Methods, available online). SMYD3 shRNA vectors (GV102, SMYD3-shRNA 1, 2, and Control shRNA) were purchased from GeneChem (Shanghai, China). Transfection of the shRNA-SMYD3 plasmid into the LNCaP cells was performed using LipofectAMINE2000. Stable shRNA expressing colonies were selected using G418 (Sigma-Aldrich, St. Louis, MO).

Luciferase Reporter Assay
Cells cultured in 24-well plates were transfected with AR or PSA-promoter reporter construct or mutant variants and pcDNA vector or siRNA. A Renilla luciferase–containing plasmid, which is driven by thymidine kinase promoter, was always included in transfection to control transfection efficiency. Luciferase activity was determined by using a dual luciferase reporter assay system following the manufacturer’s instructions (Promega, Madison, WI).

Statistical Analysis
Differences in AR mRNA abundance and promoter activity between paired samples were analyzed using Wilcoxon signed-rank tests. The SMYD3 expression in benign prostate and PC tissues and ChIP assays were analyzed using Mann–Whitney U tests for unpaired samples. Student t test was used for analyses of tumor weight. All statistical tests were two-sided and computed using SigmaStat 3.1 software (Systat Software, Chicago, IL). P values of less than .05 were considered statistically significant when comparing two related or two independent samples. We used a Bonferroni correction to adjust for multiple tests and considered only values less than .05/n (n: the number of hypotheses in a test ) to be statistically significant.

Results
Enhanced Expression of SMYD3 in Primary PC Cells
We first determined SMYD3 expression in primary PC using immunoblotting and observed an enhanced SMYD3 expression in seven of eight freshly frozen prostate tumor specimens, in comparison with their matched noncancerous tissue (Figure 1A). We further analyzed SMYD3 expression level in primary tumors from 25
The proliferation of LNCaP cells, either transfected with SMYD3 siRNA or stably expressing SMYD3 shRNA, was monitored with cell counting or CCK-8 assay. SMYD3 depletion resulted in a remarkable decrease in cell proliferation (Figure 2, A and B) (cell growth at 72 hours relative to 24 hours: control shRNA vs SMYD3 shRNA 1: mean fold change = 2.76 vs 1.68, difference = 1.08, 95% confidence interval [CI] = 0.78 to 1.38, \( P < .001 \); cell growth at 72 hours relative to 24 h: control shRNA vs SMYD3 shRNA 2: mean fold change = 2.76 vs 1.81, difference = 0.95, 95% CI = 0.75 to 1.15, \( P < .001 \)) by blocking cells in the S phase (Figure 2, C and D). Moreover, LNCaP cells stably expressing SMYD3 shRNA (LNCaP-SMYD3-shRNA) had a markedly lower number of colonies compared with control shRNA transfected cells (LNCaP-Con-shRNA) (Figure 2E).

We then examined apoptosis using anti-annexin-V and propidium iodide staining. The fluorescence activated cell sorting (FACS) analysis showed that control siRNA transfection was toxic and led to 13% apoptosis (95% CI = 10.1% to 15.9%) of LNCaP cells and that spontaneous apoptosis (7% ± 1.6%) occurred in LNCaP-Con-shRNA cells. SMYD3 depletion using siRNA and shRNA induced 44% (95 CI = 38.7% to 49.3%) and 27% (95 CI = 22.1% to 31.9%) of LNCaP cells to undergo apoptosis, respectively (Figure 2, F and G). We further observed a remarkable downregulation of the pro-survival factor B–cell lymphoma–extra large (Bcl-xL) in the LNCaP-SMYD3-shRNA cells (Figure 2H).

To determine whether the above results obtained from in vitro observations could be recapitulated in an in vivo setting, we performed the experiments with the xenograft model of PC in nude mice using LNCaP-SMYD3-shRNA or LNCaP-Con-shRNA.
Figure 2. SET and MYND domain-containing protein 3 (SMYD3) and tumorigenicity of LNCaP cells. A) Cell counting kit 8 assay was performed to measure the growth inhibition rate of LNCaP cells at 24, 48, and 72 hours after small interfering RNA (siRNA) treatment. Error bars correspond to 95% confidence intervals of three independent transfections performed in triplicate. *P < .001 using two-sided t test. B) LNCaP cells stably expressing one of two different SMYD3 short hairpin RNAs (shRNAs) (1 or 2) or control shRNA were seeded into cell culture plates. Cell growth was then measured over 3 days by counting the number of cells (*n = 4*). Error bars correspond to 95% confidence intervals of three independent experiments performed in triplicate. *P < .001 using two-sided t test. C and D) Representative examples of propidium iodide staining of LNCaP cells. Fluorescence activated cell sorting (FACS) analysis was performed on LNCaP cells stably expressing SMYD3/control shRNA or 48 hours after transfection with SMYD3/control siRNA, respectively. Three independent experiments were performed (left). The percentage of cells in each transfected population in each cycle phase was calculated (right). E) Representative images of clonogenic assays of LNCaP cells stably expressing one of two different SMYD3 shRNA vectors (1 or 2) or control shRNA. Two hundred cells per well (in 6-well plates) were incubated for 14 days, and the number of foci were counted. Three independent experiments were performed in triplicates. F and G) Representative images of FACS analysis. LNCaP cells transfected with SMYD3/control siRNA for 48 hours or the LNCaP cells stably expressing shRNA were analyzed using propidium iodide/annexin V double staining. Three independent experiments were performed. H) Western blot analysis of Bcl-xL on LNCaP cells transfected with SMYD3/control siRNA or stably expressing SMYD3/control shRNAs. β-actin level was measured as a loading control. I) Transwell migration assays on stable LNCaP cells. The cells that migrated to the lower compartments were counted in 12 representative fields for each well after 24, 48, and 72 hours of incubation; error bars correspond to 95% confidence intervals. Two-sided t tests were used to calculate the P values based on three experiments in triplicates. J) Transwell invasion assays on stable LNCaP cells, as described in the Methods. The cells were counted in 12 representative fields for each well after 24, 48, and 72 hours of incubation; error bars correspond to 95% confidence intervals. Two-sided t tests were used to calculate the P values based on three experiments in triplicates. K) LNCaP cells expressing SMYD3 shRNA or control shRNA were wounded and then left for 48 hours. Contrast-phase images are representative of three different experiments, each performed in triplicate.
cells. Nude mice were inoculated subcutaneously at the inguinal area at $10 \times 10^6$ per injection site and killed for evaluation 7 and 10 weeks after xenotransplantation. Markedly smaller tumors were observed in mice receiving LNCaP-SMYD3-shRNA cells (Figure 3, A–H). IHC on xenograft tissues using antibodies against proliferating cell nuclear antigen and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) demonstrated that SMYD3 inhibition resulted in decreased proliferating cell nuclear antigen staining (compromised proliferation) but increased TUNEL signals (apoptosis induction) in LNCaP cells (Figure 3, I and J).

**Effect of SMYD3 Expression on Migration and Invasion of PC Cells**

Epigenetic changes of cancer cells may be linked to cell movement (14). Because overexpression of SMYD3 induces matrix metalloproteinase 9 (MMP-9) expression in transformed leukocytes and fibrosarcoma cells and MMP-9 is involved in the regulation of AR-mediated cell mobile phenotype (15,16), we asked whether SMYD3 could play a role in the migration and invasion of LNCaP cells. The impact of SMYD3 inhibition on serum-induced movement of LNCaP cells was determined using transwell cell migration and invasion assays. We found that LNCaP-SMYD3-shRNA cells exhibited reduced ability to pass through the pores (Figure 2, I and J) (cell migration at 72 hours: control shRNA vs SMYD3 shRNA $1 = 181$ vs $63$, difference $= 118$, 95% CI $= 99$ to $137$, $P < .001$; cell invasion at 72 hours: control shRNA vs SMYD3 shRNA $1 = 61$ vs $32$, difference $= 29$, 95% CI $= 20$ to $38$, $P = .003$). LNCaP-SMYD3-shRNA or LNCaP-Con-shRNA cells were wounded and allowed to migrate for 48 hours. As shown in Figure 2K, the wounded area was remarkably reduced in LNCaP-Con-shRNA cells compared with LNCaP-SMYD3-shRNA cells.
Figure 4. SET and MYND domain-containing protein 3 (SMYD3) and androgen receptor (AR) transcription and expression in human prostate cancer (PC) cells. A and B) Real-time polymerase chain reaction assay and Western blot analysis of AR transcription and expression in LNCaP, PC3, and DU145 cells transfected with SMYD3 small interfering RNAs (siRNAs), control siRNA, PC-vector, or PC-SMYD3 vector respectively. The real-time reverse-transcription polymerase chain reaction data were normalized to the mRNA level of beta-2 microglobulin. The efficiency of SMYD3 siRNA knocking down was evaluated using Western blot analysis, and β-actin level was measured as a loading control. Error bars correspond to 95% confidence intervals. Wilcoxon signed-rank tests for the paired samples were used to calculate the two-sided P values based on six independent transfections. C) SMYD3 depletion is associated with decreased AR activity. SMYD3 siRNA or control siRNA was contransfected with wild-type (WT) Prostate-Specific Antigen (PSA)-reporter plasmid into LNCaP cells. Five
Effect of SMYD3 Expression on AR Transcription and Expression in Human PC Cells

We then sought to probe the molecular mechanism underlying SMYD3-mediated effects on PC cells. From a preliminary gene expression microarray analysis, we found that AR expression was repressed in SMYD3-depleted LNCaP cells. As shown in Figure 4, A and B, SMYD3 inhibition by siRNA led to statistically significantly reduced AR mRNA and protein abundance 48 hours after transfection. Because proliferation and migration of PC cells were proposed to be linked with AR-mediated transcription (12), we examined the possible association between SMYD3 expression and AR target gene transactivation. SMYD3 knockdown led to a greater than twofold reduction of PSA-promoter activity (Figure 4C). We next ectopically expressed SMYD3 in androgen-independent PC cell lines PC3 and DU145 with low but detectable AR expression (17) and found that transfection of the SMYD3 expression vector (pcDNA-SMYD3) induced AR mRNA and protein expression in both cell lines (Figure 4, A and B).

We then examined whether SMYD3 up regulates AR expression at the transcriptional level. We found that ectopic expression of SMYD3 led to approximately 80% and 140% increase of AR promoter activity in PC3 and DU145 cells, respectively, in a dose-dependent manner (Figure 4E) (relative luciferase activity in pcDNA SMYD3–transfected PC3 cells: 1.6 μg vs 0 μg = 1.88 vs 1.00, difference = 0.88, 95% CI = 0.59 to 1.77, P = .03; relative luciferase activity in pcDNA SMYD3–transfected DU145 cells: 1.6 μg vs 0 μg = 2.45 vs 1.00, difference = 1.48, 95% CI = 1.29 to 1.67, P = .03). In contrast, SMYD3 knockdown repressed AR promoter activity by 40% in LNCaP cells (Figure 4F) (Relative luciferase activity in SMYD3 siRNA transfected cells: 0 pmol vs 15 pmol = 1.00 vs 0.57, difference = 0.43, 95% CI = 0.23 to 0.63, P = .028).

SMYD3 binds to a putative motif 5′-CCCTCC-3′ in the promoters of its target genes (5). Interestingly, we identified five putative SMYD3 binding elements within 0.8 kb upstream from the transcription start site of AR gene (Figure 4D). We thus introduced site-directed mutation into each potential SMYD3 binding motif (MT1–MT5) and identified MT2 and MT5 as the functional motifs for the transcriptional activity of the AR gene in LNCaP cells. Correspondingly, mutation on MT2 or MT5 abolished SMYD3-mediated AR promoter transactivation in PC3 and DU145 cells. In contrast, disruption of the other motifs did not affect or only slightly affected AR promoter activity, indicating that MT1, MT3, and MT4 were nonfunctional/noncritical for SMYD3 in PC cells (Figure 4, G and H) (relative luciferase activity in LNCaP cells: WT vs MT2 = 1.00 vs 0.31, difference = 0.69, 95% CI = 0.59 to 0.79, P = .008; WT vs MT5 = 1.00 vs 0.39, difference = 0.61, 95% CI = 0.52 to 0.70, P = .008; relative luciferase activity in PC3 cells: WT vs MT2 = 1.79 vs 0.74, difference = 1.05, 95% CI = 0.88 to 1.22, P = .006; WT vs MT5 = 1.79 vs 0.69, difference = 1.10, 95% CI = 0.89 to 1.31, P = .006; relative luciferase activity in DU145 cells: WT vs MT2 = 2.42 vs 0.70, difference = 1.72, 95% CI = 1.46 to 1.98, P = .008; WT vs MT5 = 2.42 vs 0.73, difference = 1.69, 95% CI = 1.38 to 2.00, P = .006).

Correlation Between SMYD3 and the AR Promoter

Given the findings above, we sought to determine whether SMYD3 physically binds to the AR promoter in PC cells. ChIP assay was performed with primers encompassing the SMYD3 binding motifs in the AR promoter using SMYD3 antibody (Figure 5A). As shown in Figure 5B, the AR promoter was indeed occupied by SMYD3 protein, and this association was abolished when SMYD3 expression was knocked down in LNCaP cells. Correspondingly, ectopically expressed SMYD3 led to its accumulation at the AR promoter region in low AR-expressed PC3 cells (Figure 5C).

Effect of SMYD3 Expression on Chromatin Remodeling and Sp1 Accumulation in the AR Promoter Region

Because SMYD3 induces target gene transcription by di-/trimethylating H3-K4 in the promoter region, we proceeded to determine whether SMYD3 contributes to AR expression by altering histone modification and subsequent transcription factor occupation. SMYD3 expression in LNCaP cells was knocked down using siRNA, and thereafter the status of H3-K4 mono-/di-/trimethylation at the AR promoter was examined using ChIP assay. SMYD3 depletion decreased H3-K4 di- and trimethylation, but not mono-methylation at the AR promoter region (Figure 5B), indicating that SMYD3 is required for di-/trimethylation of H3-K4 at the AR promoter.

H3-K4 di-/trimethylation provides docking sites for certain protein complexes possessing histone acetyltransferase activity, which in turn leads to increased accessibility for transcriptional activator(s) on promoters (18). Moreover, the proximal promoter independent transfections were performed. Bar graphs are relative luciferase activity. Luciferase activity assay was performed 48 hours after transfection. Variation in transfection efficiency was normalized by thymidine kinase–driven Renilla luciferase activity. Error bars correspond to 95% confidence intervals. A Wilcoxon signed-rank test for the paired samples was used to calculate the two-sided P values. D) Sequence of the AR promoter region. Five putative SMYD3 binding sites are underlined. The sequence that was mutated in the transcriptional activity analysis of cis-acting elements (MT1–MT5) is indicated by dots, and substitutions are given above. +1 indicates the first nucleotide upstream of the transcription start site (TSS); the arrow indicates the first nucleotide of the first exon. E) Various amounts of SMYD3 expression vectors pcDNA-SMYD3 were cotransfected with WT pGL3-AR-promoter vector into PC3 or DU145 cells. Luciferase activity assay was performed 48 hours after transfection. Six independent transfections were performed. Error bars correspond to 95% confidence intervals. Wilcoxon signed-rank tests for the paired samples were used to calculate the two-sided P values. F) Increasing doses of SMYD3 siRNA were cotransfected with WT pGL3-AR-promoter vector into LNCaP cells. Luciferase activity assay was performed 48 hours after transfection. Six independent transfections were performed. Error bars correspond to 95% confidence intervals. A Wilcoxon signed-rank test for the paired samples was used to calculate the two-sided P value. G) WT or SMYD3 motif mutant (MT1–MT5) AR promoter activity in LNCaP cells. Three independent experiments were performed in triplicates. Bar graphs represent the means, and error bars correspond to 95% confidence intervals. Wilcoxon signed-rank tests were used to calculate the two-sided P values. A Bonferroni correction was used to adjust for a multiple comparison and P less than .05/2 was considered to be statistically significant. H) pcDNA-SMYD3 was cotransfected with WT pGL3-AR-promoter vector or mutant reporter plasmid (MT1–MT5) into PC3 or DU145 cells. Luciferase activity assay was performed 48 hours after transfection. Three independent experiments were performed in duplicate. Error bars correspond to 95% confidence intervals.
of AR gene harbors a GC-box, where transcription factor Sp1 plays a key role in regulating AR transcription (19,20). We thus examined whether abolished SMYD3 expression could affect the H3 acetylation and the occupancy of Sp1 in the AR promoter region. As expected, SMYD3 depletion led to decreased acetylation of histone H3 and Sp1 binding at the AR promoter in LNCaP cells (Figure 5B). Concordantly, the increased Sp1 occupancy on the AR promoter in PC3 cells transfected pcDNA-SMYD3 or backbone vector. Omission of antibodies (No Ab) was included in the whole experimental procedure, together with the polymerase chain reaction amplification of unrelated GAPDH gene, as appropriate controls. Data shown are from four independent transfections. Mean values of ChIP signals are normalized to 1% input. Input control was from nonimmunoprecipitated total chromatin DNA. Error bars correspond to 95% confidence intervals. Mann–Whitney U tests were used to calculate the two-sided P values.

Figure 5. SET and MYND domain-containing protein 3 (SMYD3) and androgen receptor (AR) expression, chromatin remodeling, and Sp1 occupancy at the AR promoter. A) Schematic presentation of the AR promoter and polymerase chain reaction primer locations (relative to transcription start site) for chromatin immunoprecipitation assay. GC-box and functional SMYD3 motifs are indicated. B) Quantitative chromatin immunoprecipitation (ChIP) assay for H3-K4 tri-/di-/mono-methylation, H3 acetylation, and Sp1 and SMYD3 occupancy at the AR promoter in LNCaP cells treated with the SMYD3 small interfering RNA (siRNA) or control siRNA. C) Quantitative ChIP assay for H3-K4 tri-/di-/monomethylation, H3 acetylation, and Sp1 and SMYD3 occupancy at the AR promoter in PC3 cells transfected pcDNA-SMYD3 or backbone vector.
Discussion

In this study, we showed that the expression of SMYD3 was upregulated in human PC tissue and SMYD3 had protumorigenic effects on PC. We further explored the underlying mechanisms and identified the AR gene as a direct target of SMYD3.

AR signaling pathway is critical for PC formation/progression and enhanced AR expression is important for the development of PC (2). Several studies have shown that epigenetic aberrations play important roles in PC growth and metastasis (21,22). However, few studies have addressed the epigenetic regulation of AR expression in PC cells. In this study, we revealed a unique role of histone methyltransferase SMYD3 in the PC pathogenesis by demonstrating the SMYD3 activation of AR transcription. To our knowledge, this is the first study showing that histone H3-K4 methylation/demethylation is involved in the regulation of AR expression and that SMYD3 is aberrantly upregulated in PC.

Histone-modifying enzymes are typically recruited to various gene promoters by their interaction with specific transcription factors. However, SMYD3 is unique because it has a DNA binding capacity and directly interacts with target promoters by recognizing a canonical responsive motif 5′-CCCTCC-3′. As presented here, the AR promoter harbors two functional SMYD3 binding elements through which SMYD3 associates with it and methylates local histone H3-K4. It has been proposed that methylated H3-K4 can contribute to transcriptional regulation by recruiting transcription complexes that contain histone acetyltransferase activity, leading to an accessible promoter for transcription factors. The transcription factor Sp1 activates AR transcription by direct binding to the GC-box in the AR promoter (20). By controlling the access of Sp1 to and histone H3 acetylation at the AR promoter, SMYD3-mediated H3-K4 di/trimethylation may function as a licensing element for AR expression.

SMYD3 directly interacts with the ligand-binding domain of the estrogen receptor and is recruited to the proximal promoter regions of estrogen receptor target genes upon gene induction (23). We performed immunoprecipitation on PC3 cells cotransfected with FLAG-tagged SMYD3 and untagged AR-expressing vectors but failed to observe their physical association (data not shown). In addition, potential trans-acting elements of AR transcription include NF-kappaB, LEF1, RB1/E2F, and Twist1 (10,24–26). It is currently unclear whether SMYD3 cooperates or interacts with any of the above transcription factors to control AR transcription. This issue is under investigation in our laboratory.

The relationship between SMYD3 and AR transcription prompted us to vigorously address the functional role of SMYD3 in PC pathogenesis. Our findings suggest that SMYD3 is important for PC proliferation/survival and tumorigenicity and its function is consistent with the observed role of AR in PC pathogenesis. Thus, the SMYD3-AR axis might be an important driving-force for PC. However, because SMYD3 has been shown to exert protumorigenic effects through various mechanisms, it remains to be defined to what extent AR contributes to the oncogenic activities mediated by SMYD3 in PC. The direct target genes of SMYD3 identified so far include AR, MMP-9, Bcl-xL, Wingless-Type MMTV Integration Site Family, Member 10B1(WNT10B), E2F transcription factor 1 (E2F1), NK2 Homeobox 8 (Nkx2.8), and telomerase reverse transcriptase (bTERT), and they all play important roles in cancer development and/or progression (27). Interestingly, MMP-9 is also regulated by AR signaling and is closely associated with the invasiveness of PC cells (15). Taken together, SMYD3 and AR may contribute to PC pathogenesis in three ways: First, SMYD3 directly activates AR transcription, thereby regulating AR downstream oncogenic effectors. For instance, SMYD3 depletion led to apoptosis of LNCaP cells coupled with the downregulation of Bcl-xL expression. Bcl-xL is a well-characterized AR target gene through which AR maintains PC cell survival (28,29), and conceivably, its diminished expression might contribute to apoptosis induction mediated by SMYD3 knockdown. Second, SMYD3 and AR cooperatively regulate their common targets such as MMP-9. Third, SMYD3 exerts a protumorigenic effect by regulating its targets (eg, hTERT) independently of the AR signaling.

Previous studies have shown that the SMYD3 inhibition led to G1 arrest of cell cycle in non-PC cancer cell lines (30,31). Intriguingly, we found that the S phase arrest occurred in SMYD3-depleted LNCaP cells, but there were no notable changes in G1. It is currently unclear whether this scenario is unique to LNCaP or PC cells or what the underlying mechanisms might be. Further studies are required to answer these questions.

As a histone methyltransferase, SMYD3 is localized in nuclear compartments of cells to regulate H3-K4 methylation, chromatin configuration, and gene transcription. However, we noticed the cytoplasmic distribution of SMYD3 in primary PC cells and LNCaP cells. It is currently unclear what causes the aberrant cytoplasmic distribution of SMYD3 in PC cells and whether cytoplasmic SMYD3 has any novel functional activities.

A major obstacle for treating PC is developing resistance to androgen depletion therapy. Restoration of AR activity through different mechanisms is the critical event in the transition from ADPC to CRPC stage. The most common alteration in the AR pathway during the transition is the overexpression of AR itself (25). Given the important function of SMYD3 in PC cells, it will be interesting to elucidate its role in the transition from ADPC to CRPC. Our preliminary data showed that SMYD3 expression was indeed inhibited by synthetic androgen R1881 in PC cells. It is imperative to evaluate the impact of androgen depletion therapy on SMYD3 expression in cellular and animal models and to determine the expression of SMYD3 in CRPC tissues.

Our study had some limitations. Although we found very weak expression of SMYD3 in noncancerous prostate tissues and overexpression in PC specimens, the numbers of noncancerous patients and PC patients were small, and the observation should be interpreted with caution. Furthermore it remains to be defined why most PC tumors exhibited cytoplasmic overexpression of SMYD3 and whether SMYD3 can be used to predict patient outcomes. In short, recruitment of more PC patients is needed to assess clinical significance of SMYD3 in PC.

In summary, by demonstrating the SMYD3-mediated oncogenic activity and AR expression in PC, we provide a potential avenue for controlling AR-mediated PC progression by inhibiting SMYD3 expression or activity.
References

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Notes
C. Liu and C. Wang contributed equally to this study.

The authors were fully responsible for the design of the study, analysis and interpretation of results, the decision to submit the manuscript, and the writing of the manuscript.

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