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ORIGINAL ARTICLE

Prostate Cancer

Histone methyltransferase *SETDB1* is required for prostate cancer cell proliferation, migration and invasion

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SETDB1 has been established as an oncogene in a number of human carcinomas. The present study was to evaluate the expression of *SETDB1* in prostate cancer (PCa) tissues and cells and to preliminarily investigate the role of *SETDB1* in prostate tumorigenesis *in vitro*. Quantitative reverse transcription polymerase chain reaction (qRT-PCR) and immunohistochemistry (IHC) were used to detect the expression of *SETDB1* in PCa tissues, adjacent normal tissues, benign prostatic hyperplasia (BPH) tissues, PCa cell lines and normal prostate epithelial cells. The results suggested that *SETDB1* was upregulated in human PCa tissues compared with normal tissues at the mRNA and protein levels. The role of *SETDB1* in proliferation was analyzed with cell counting kit-8, colony-forming efficiency and flow cytometry assays. The results indicated that downregulation of *SETDB1* by siRNA inhibited PCa cell growth, and induced G0/G1 cell cycle arrest. The PCa cell migration and invasion decreased by silencing *SETDB1* which were assessed by using *in vitro* scratch and transwell invasion assay respectively. Our data suggested that *SETDB1* is overexpressed in human PCa. Silencing *SETDB1* inhibited PCa cell proliferation, migration and invasion.

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INTRODUCTION

Prostate cancer (PCa) is the most frequently diagnosed non-skin malignancy and the third leading cause of cancer-related deaths in men in the Western world.¹ Although most PCa are slow growing and responsive to the available hormone therapies, a significant number of cases become hormone refractory and metastatic. The majority of patients with metastatic PCa eventually develop castration-refractory PCa.^{2,3}

The precise cause of PCa progression has remained elusive, despite extensive research efforts and recent advances in our understanding of this disease. Comprehensive gene expression and genome analyses have suggested that a global pattern of gene expression and copy number alterations exist for PCa.^{4,5} In addition to genetic alterations, epigenetic aberrations also play key roles in the process of PCa development. The major epigenetic factors include DNA methylation, histone modification and noncoding RNAs.⁶ These epigenetic regulations are implicated in a variety of biological and pathogenic processes, such as aging, memory formation, embryological development and carcinogenesis.⁷

In eukaryotes, genomic DNA is packaged with histone proteins into chromatin, compacting DNA some 10 000-fold. Chromatin structure is regulated by a variety of posttranslational modifications, including DNA methylation, histone modifications and adenosine

triphosphate (ATP)-dependent chromatin remodeling. Histones can be modified by several posttranslational mechanisms, including the acetylation, methylation, phosphorylation, ubiquitination, sumoylation or ribosylation of distinct amino acids, resulting in either the activation or suppression of gene expression.^{8–12} Enzymes that tightly control the balance of covalent histone modifications include histone acetyltransferases, histone deacetylases, histone methyltransferases (HMTs) and demethylases.¹³

The gene *SETDB1* encodes an enzyme that methylates histone H3 on lysine 9 (H3K9).^{11,14} It is mapped to the human chromosome 1q21.^{15,16} *SETDB1* has been established as an oncogene in human lung cancer, melanoma and kidney tumors.^{16–18} Expression datasets for *SETDB1* in cervix squamous cell carcinoma and some other malignant tumors are available (<https://www.oncomine.org/>). To our knowledge, its role in PCa has not yet been elucidated, and there are no datasets showing its expression in PCa tissues or cell lines. Herein, we preliminarily explored *SETDB1* tumorigenesis in PCa.

MATERIALS AND METHODS

Cell culture

LNCAp-AD, PC3, DU145, LNCAp-AI, 22RV1 and RWPE-1 cell lines were obtained from our department cell line bank. LNCAp-AD, PC3 and

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DU145 were maintained in Roswell Park Memorial Institute (RPMI) medium 1640 (GIBCO, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS), 2 mmol⁻¹ L-glutamine and 25 mmol⁻¹ HEPES. LNCaP-AI, 22RV1 and C4-2 cells were maintained in phenol red-free RPMI medium 1640 (GIBCO) supplemented with 10% charcoal-stripped FBS, 300 mg l⁻¹ L-glutamine, 2000 mg l⁻¹ glucose and 2000 mg l⁻¹ NaHCO₃. All cell lines were maintained in a humidified incubator at 5% CO₂ and 37°C.

Quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR)

Total RNA was extracted from cell lines and frozen tissue specimens using TRIzol reagent (Invitrogen, Grand Island, NY, USA) and then reverse transcribed using PrimeScript RT reagent Kit (Takara, Otsu, Japan) according to the manufacturer's instructions. qRT-PCR was used to detect the expression of SETDB1. For qRT-PCR, 2 µl of cDNA was amplified in a 20 µl reaction containing SYBR Premix Ex Taq™ (Takara) using a two-step program. A melt-curve analysis was enabled at the end of the amplification. The relative expression of SETDB1 was normalized to beta-actin using the comparative Ct method. The primers are 5'-TCCCAGGATCTGCATAAAGG-3' and 5'-TCAGCAGGAGGGTGGTAATC-3' for SETDB1 and 5'-CGC GAG AAG ATG CCC AGA TC-3' and 5'-TCA CCG GAG TCC ATC ACG A-3' for β-actin. All experiments were performed in triplicate.

Tissue specimens and immunohistochemistry (IHC)

For the IHC studies, we used PCa (*n* = 108), cancer-adjacent normal tissue (*n* = 5) and benign prostatic hyperplasia (BPH) (*n* = 105). Clinical paraffin-embedded (FFPE) tissue specimens from PCa and BPH patients were obtained from the archives of Shanghai Changhai Hospital with informed consent and with the approval of the institutional ethics committee. A block with a viable tumor was chosen. For each specimen, a pathologist reviewed the hematoxylin and eosin stain slides. The tissue microarrays were constructed from 0.6 mm cores using a manual tissue arrayer from Leica Instruments (Leica, Mannheim, Germany). Tissue microarrays for IHC were placed on positively charged frosted glass slides. These slides were placed in a metal staining rack and incubated for 35 min at 70°C. Sections were dewaxed in xylene for 10 min, rehydrated in ethanol for 6 min and then rinsed twice in water for 6 min.

For antigen retrieval, the sections were immersed in 200 ml antigen retrieval solution containing three drops of HCl and heated in a microwave for 2–2.5 min. The tissue sections were then cooled at room temperature for 1 h before being washed with water for 5 min and in phosphate buffered saline (PBS) for another 5 min and then marked with a PAP pen. The sections were subsequently covered with the primary antibody (Abcam, San Diego, CA, USA) for SETDB1 overnight and then washed in PBS for 5 min. Then, the sections were covered with the secondary antibody (Abcam) and were incubated at room temperature for 15–30 min, followed by rinsing with PBS with a pH of 7.4–7.6 for 5 min. Tissue microarrays were then covered with undiluted streptavidin peroxidase solution and incubated at 45°C for 30 min, followed by rinsing with PBS for 5 min.

The slides were drained and blotted around the sections, and then undiluted substrate solution (3,3'-diaminobenzidine) containing 1 ml of DAB (diaminobenzidine) and one drop of chromogen was added to cover the sections. They were incubated for 3–5 min at room temperature followed by rinsing in PBS for 5 min. Finally, the sections were counterstained with hematoxylin for 30 s and then rinsed with tap water. After drying, the slides were mounted with DPX (distyrene,

plasticizer and xylene). The SETDB1 immunopositive cells were observed under a light microscope at 40× magnification. Malignant cells were considered immunopositive when they displayed a brownish nuclear stain. For scoring of the slides, three independent observers blindly examined the staining in the PCa, and a mean score was calculated. The positive reaction was scored according to the intensity of staining: 0, 1, 2, 3 and 4.

siRNA transfection

The 22RV1 cells were seeded at a density of 8 × 10⁴ cells per well into a six-well culture plate (Corning Costar, Cambridge, MA, USA) with RPMI 1640 medium containing 10% FBS or phenol-red free RPMI 1640 medium containing 10% Charcoal Stripped FBS. At the same time, cells were transfected with SETDB1-siRNA packaged by RNAiMAX transfection reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Forty-eight hours after transfection, the cells were harvested for further studies.

siRNA and siRNA knockdown experiments

The two siRNAs specific for SETDB1 named S5 and S9 and a nonspecific control siRNA were purchased from Invitrogen (Invitrogen, Shanghai, China). The specific siRNA sequences for SETDB1 used in this study were as follows:

S9 sense sequence: 5'-CGUGACUUCAUAGAGGAGU-3';
antisense sequence: 5'-ACUCCUCUAUGAAGUCACG-3';
S5 sense sequence: 5'-GAUCUAUCGAGGCUCUACA-3';
antisense sequence: 5'-UGUAGAGCCUCGAUAGAUC-3'.

An evaluation of the efficiency of the siRNA knockdown experiments was performed. We compared the SETDB1 expression in normal 22RV1 cells cultured for 72 h with S5 and S9 siRNA 72 h post-infected 22RV1 cells and siRNA control post-infected 22RV1 cells. The transfection method and gene expression assay method are the same as those described above.

Migration assay

Cells transfected with siRNA-SETDB1 or vector only were seeded into six-well plates, and cell growth was allowed to continue until confluence was reached. The cell monolayer was scratched with a 10 µl pipette tip, and dislodged cells were washed away with PBS. Cell incubation was continued under standard conditions, and the degree of cell migration into the scraped area was documented every 24 h.¹⁹

Invasion assay

Cell invasion assays were carried out using Boyden chambers containing Transwell (Corning Costar) membrane filter inserts with a pore size of 8 µm. For the invasion assay, the transwell membrane was coated with Matrigel (BD Biocoat, Bedford, MA, USA). Cells (3 × 10⁴) in 150 µl RPMI 1640 containing 0.2% bovine serum albumin were seeded on Boyden chambers (upper chamber). The lower chambers were filled with RPMI 1640 containing 10% FBS. After 36 h of migration at 37°C, cells were stained with crystal violet and counted under a microscope in five predetermined fields at 100× with ImageJ software (NIH software).

Cell proliferation assays

The proliferation of 22RV1 cells and siRNA-SETDB1-transfected 22RV1 cells was evaluated using a cell counting kit-8 (Dojindo, Kumamoto, Japan) assay according to the manufacturer's instructions. This assay is based on the cleavage of the tetrazolium salt WST-8 by mitochondrial dehydrogenase in viable cells. A total of 2 × 10³ cells per well were incubated with 100 µl of culture medium in 96-multiwell plates. Cells were cultured for 1, 2, 3, 4 or 5 days before the addition of 10 µl cell counting kit-8 (5 mg ml⁻¹) to the culture medium in each well.

After 1 h of incubation at 37 °C, the absorbance at 450 nm in each well was measured with a Thermomax microplate reader. Each experiment was triplicated, and the data represent the mean of all measurements.

Cell cycle analysis

The cell cycle distribution was analyzed by flow cytometry. 22RV1 cells and siRNA-SETDB1-transfected 22RV1 cells were cultured for 24 or 48 h, trypsinized, washed once with PBS and fixed in 70% ethanol for at least 1 h at -20 °C. Fixed cells were washed with PBS and incubated with propidium iodide staining solution (69 μmol l⁻¹ IP, 38 mmol l⁻¹ sodium citrate and 0.7 mg ml⁻¹ ribonuclease A, pH 7.4) for 30 min at 37 °C in the dark. The stained cells were analyzed with a FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). The CellQuest program was used for the acquisition and analysis of the samples.

Colony formation assay

22RV1 and LNCaP-AD cells were seeded at a density of 1 × 10⁴ cells per 100 mm dish and cultured in 0.35% soft agar in DMEM plus 10% FBS or phenol-red free RPMI 1640 medium (GIBCO) plus 10% charcoal-stripped FBS (GIBCO) at 37 °C for 48 h. Afterwards, these cells were transfected with siRNA-SETDB1 or control siRNA once every 3 days for a total of two times. Two weeks after the first transfection, the cells were stained with crystal violet and all the visible colonies were counted.

Statistical analysis

The data are presented as the mean ± standard deviation (s.d.). The Boyden chamber assay data were assessed by one-way ANOVA (analysis of variance). The other statistical analyses were assessed by *t*-test. SPSS (Statistical Package for Social Sciences) 11.0 software (SPSS Inc., Chicago, IL, USA) was used for all statistical analyses, with *P* < 0.05 considered statistically significant.

RESULTS

SETDB1 is overexpressed in human PCa tissues and cell lines

We performed qRT-PCR to investigate SETDB1 expression in PCa patients and BPH patients. SETDB1 was overexpressed in 19 out of 25 PCa tissues compared with adjacent normal tissues. On average, SETDB1 expression was 3.3-fold higher than in adjacent normal tissues (*P* < 0.001) (Figure 1a). Additionally, SETDB1 expression was significantly higher in PCa tissues than in BPH tissues, especially in androgen-independent PCa tissues (Figure 1b). Further studies on prostate cell lines showed that SETDB1 expression was also significantly higher in PCa cell lines than in normal prostate epithelial cell lines (RWPE-1). Respectively, LNCap-AD vs RWPE-1 was 1.45-fold higher, (*P* = 0.012), LNCap-AI vs RWPE-1 was 1.89-fold higher (*P* = 0.003), PC3 vs RWPE-1 was 3.39-fold higher (*P* < 0.001),

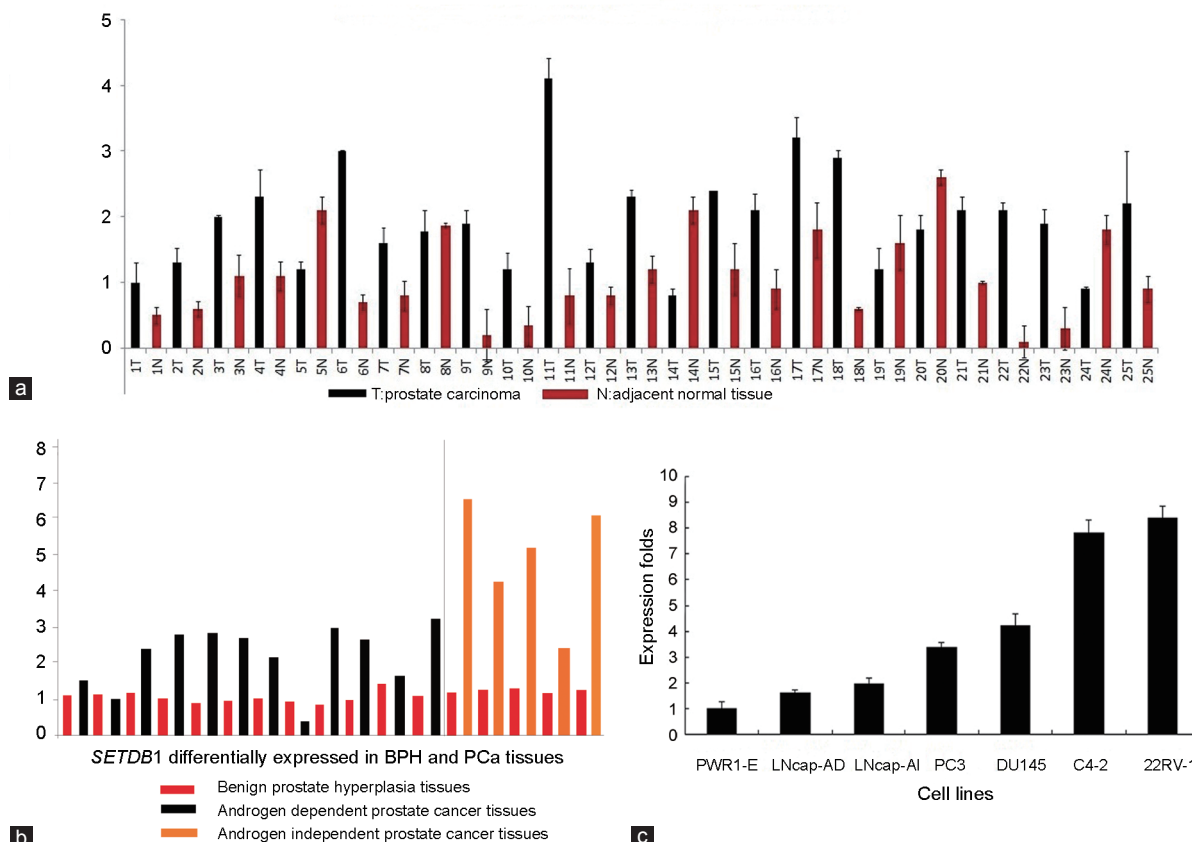


Figure 1: Quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR) examination of SETDB1 expression in prostate cancer (PCa) tissues, adjacent normal tissues and cell lines. (a) SETDB1 was overexpressed in PCa tissue compared with adjacent normal tissues (*n* = 19, *P* = 0.008). Expression of SETDB1 is higher in paired normal tissues than in PCa tissue (*n* = 6, *P* = 0.011). (b) SETDB1 expression was significantly higher in PCa tissues than in benign prostate hyperplasia (BPH) tissues. For androgen-dependent PCa (ADPC) (*n* = 12) vs BPH, the average fold increase is 2.34 (*P* = 0.006), whereas, for androgen-independent PCa (AIPC) (*n* = 5) vs BPH, the increase is 6.21-fold (*P* < 0.001). AIPC vs ADPC is 1.78-fold higher (*P* = 0.001). (c) SETDB1 expression was higher in PCa cell lines (22RV-1, LNCaP-AI, LNCaP-AD, C4-2, DU145 and PC3) than in normal prostate epithelial cell lines (RWPE-1).

DU145 vs RWPE-1 was 3.86-fold higher ($P < 0.001$) and C4-2 and 22RV1 were 7.89-fold higher (**Figure 1c**).

SETDB1 protein is overexpressed in human PCa tissues

To determine the extent of SETDB1 overexpression in human PCa, we measured SETDB1 protein levels using IHC in tissue microarrays containing specimen cores of PCa ($n = 108$), cancer-adjacent normal tissue ($n = 5$) and BPH ($n = 105$). Immunohistochemical staining for SETDB1 revealed cancer cells with concentrated staining in the nucleus, and PCa showed strong SETDB1 staining; whereas, weak staining and no staining were observed in BPH and cancer-adjacent tissue (score of BPH is 1.2 ± 0.5 and of PCa is 2.8 ± 0.7 , respectively, $P = 0.032$) (**Figure 2**).

Silencing of SETDB1 is associated with a decreased proliferation of PCa cells

To investigate the effects of SETDB1 suppression on the proliferation of human PCa cells, we previously described the knockdown efficiency of SETDB1-specific siRNA (**Figure 3a**). Subsequently, we downregulated the SETDB1 expression in PCa cells using siRNA. A total of 0, 24, 48, 72 and 96 h following transfection, PCa proliferation were measured by the MTT assay. As **Figure 3b** shows, cell proliferation was significantly reduced following siRNA-SETDB1 transfection compared with siRNA-control transfected cells and parental cells ($P = 0.049$). Furthermore, SETDB1 knockdown significantly reduced the clonogenicity of 22RV1 and LNCaP-AD cells (**Figure 3c**).

Silencing of SETDB1 is associated with decreased migration and invasiveness of PCa cells

An *in vitro* scratch assay was performed to evaluate the influence of SETDB1 on cellular migration. Cell numbers were normalized for better comparison. The short time required for the impedance to rise again after scratching the cell layer indicates that migration rather than proliferation is responsible for this effect. A microscopic analysis of the cells displayed a different rate of cells moving into the cell-free region. The siRNA-SETDB1 transfected cells displayed the lowest migratory ability compared with vector control cells (**Figure 4a**).

To evaluate the effects of SETDB1 on the invasiveness of tumor cells, PCa cells were transfected with siRNA-SETDB1 and standard *in vitro* chamber assays with a Matrigel model were performed. As shown in **Figure 4b**, the number of cells that digested Matrigel and penetrated through the transwell polycarbonate filter was significantly decreased by siRNA-SETDB1 transfection. Compared with the siRNA-control transfected cells and parental cells, siRNA-SETDB1-transfected cells decreased in invasiveness at 36 h post-transfection ($P = 0.002$).

Knockdown of SETDB1 resulted in G0/G1 phase cell cycle arrest

To investigate the effect of SETDB1 on cell cycle progression in 22RV1 cells, control-siRNA and SETDB1 siRNA-transfected 22RV1 cells were cultured for 24 and 48 h, and the percentages of cells in the G0/G1, S and G2/M phases were determined by a flow cytometric analysis. As shown in **Figure 5a and b**, siRNA-SETDB1 transfection increased the percentage of cells in G0/G1 from 55.9% to

66.9% ($P = 0.023$) and from 67.5% to 73.9% ($P = 0.041$) at 24 and 48 h, respectively, which was accompanied by a corresponding reduction in the percentage of cells in the S phase from 33.1% to 20.7% ($P = 0.019$)

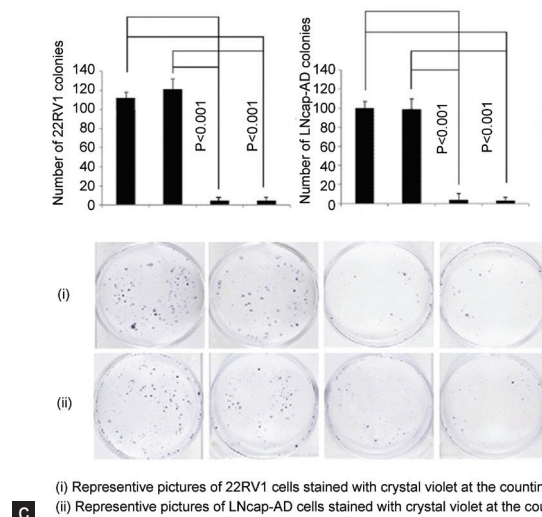
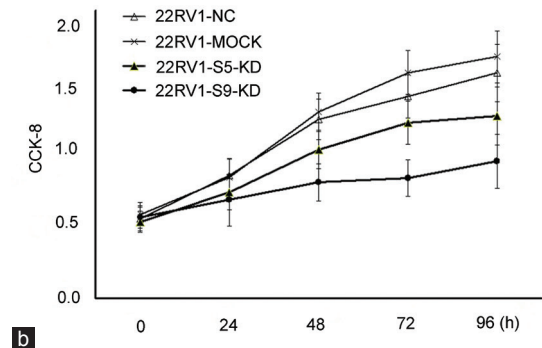
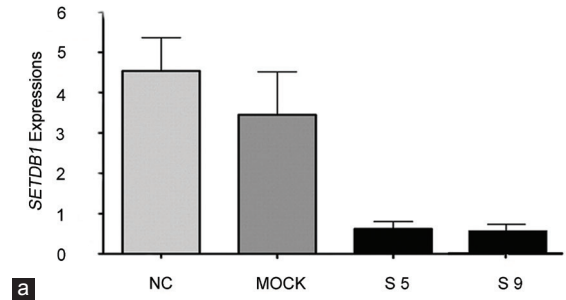


Figure 3: Knockdown of SETDB1 attenuated the growth and proliferation potential of PCa cells *in vitro*. (a) qRT-PCR analysis of mRNA levels in 22RV1 cells receiving siRNA or control siRNA, performed at 72 h post-transfection. All the values are the mean values (\pm standard error (s.e.)) of triplicate experiments. (b) Cell proliferation was significantly reduced following siRNA-SETDB1 transfection compared with siRNA-control transfected cells and parental cells. (c) Knockdown of SETDB1 resulted in a lower colony formation efficiency of PCa cells. The number of LNCap-AD and 22RV1 cell colonies was significantly reduced following siRNA-SETDB1 transfection compared with the control. The mean \pm standard deviation (s.d.) is shown.

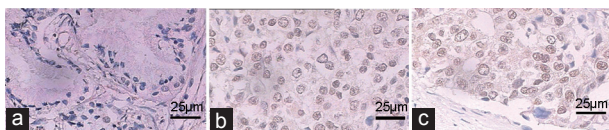


Figure 2: Representative images of the immunohistochemistry revealed differential expression of SETDB1 in BPH (a) and PCa (b and c). SETDB1 protein is overexpressed in PCa more so than in BPH tissues. PCa: prostate cancer; BPH: benign prostatic hyperplasia.

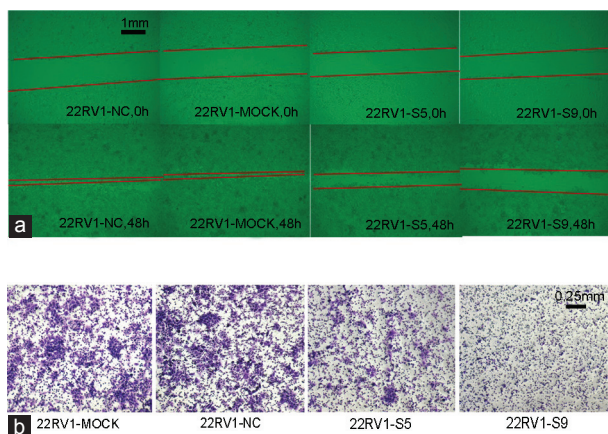


Figure 4: *SETDB1* silencing inhibits cellular migration in 22RV1 cells. (a) Representative 22RV1 cell images of the scratch closure *in vitro* scratch assay. siRNA-*SETDB1*-transfected cells displayed the lowest migratory ability compared with siRNA-control cells. (b) The number of cells that digested Matrigel and penetrated through the transwell polycarbonate filter was significantly decreased by siRNA-*SETDB1* transfection. Compared with the siRNA-control-transfected cells and parental cells, siRNA-*SETDB1*-transfected cells showed a 66.5% decrease in invasiveness at 36h post-transfection ($P < 0.001$).

and from 17.4% to 15.0% ($P = 0.045$) at 24 and 48 h, respectively. These data suggested that silencing *SETDB1* induces cell cycle arrest at the G0/G1 phase in 22RV1 cells.

DISCUSSION

Initial efforts in characterizing the tumorigenic process focused on genetic alteration.^{20,21} More recently, epigenetic changes have been proposed as an etiology.²² First identified as an H3K9-specific methyltransferase in 2002,²³ *SETDB1* modifies H3K943 and ING2 *in vitro*.²⁴ HMTs are fundamental players in the regulation of chromatin signaling. This is emphasized by several reports showing that HMT functional defects can lead to cancer,^{25,26} growth defects,²⁷ neurological disorders²⁸ and other human pathologies. Thus, HMTs have attracted interest in the field of cancer research due to their enzymatic activity, and thus potential therapeutic ability.²⁵

One well-studied example is *EZH2*, which is upregulated in several types of solid tumors and is associated with a poor prognosis.²⁹ Silencing *EZH2* has been shown to inhibit cell proliferation, cell invasion, tumor growth and metastasis.^{30,31} Inspired by these findings, we initially explored the implications for prostate tumorigenesis. In the current study, we provided *in vitro* evidence that *SETDB1* was overexpressed in PCa tissues compared with the adjacent normal prostate tissues. *SETDB1* is also upregulated in PCa cells compared with normal prostate epithelial cells. Furthermore, we showed that knockdown of *SETDB1* by siRNA could suppress cell growth, invasion and migration; decrease colony formation and induce cell cycle arrest. These findings preliminarily reveal the *SETDB1* tumorigenesis proficiency.

To demonstrate the gene expression changes that occur when *SETDB1* is overexpressed and there is cross-talk with other H3K9-specific methyltransferases, it is important to elucidate the *SETDB1* tumorigenesis in PCa, which requires more complex technological methods that we are currently unable to perform.

In conclusion, our study demonstrated for the first time that *SETDB1* is overexpressed in PCa tissues and in cell lines. Silencing *SETDB1* inhibited PCa cell growth, migration and invasion. We propose that *SETDB1* is a candidate oncogene of PCa.

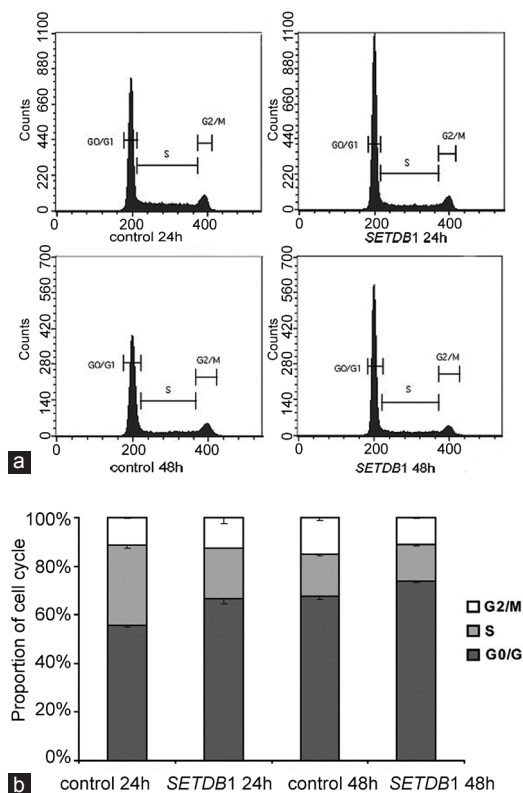


Figure 5: The knockdown of *SETDB1* resulted in G0/G1 phase cell cycle arrest. (a) 22RV1 cell analysis with flow cytometry (FACS). (b) The proportion of cells in the cell cycle FACS analysis of 22RV1 cells. Different cell cycle phases were quantified by propidium iodide staining, followed by FACS analysis for 22RV1 cells.

AUTHOR CONTRIBUTIONS

YS performed the gene expression analysis, participated in cellular function studies and data analysis and drafted the manuscript. MW performed all the clinical samples and participated in tissues selection and collection. RC, WDX, FBW, JL, JS participated in all gene expression analysis and cellular function studies. YWY, JTH performed the tissue specimens and immunohistochemistry analysis. CLX, JGH participated in the design, coordination of the study. YHS and SCR conceived the study, participated in the design, coordination of the study and drafting the manuscript.

COMPETING INTERESTS

The authors declare that they have no competing interests.

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