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ORIGINAL ARTICLE

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UNC5D, suppressed by promoter hypermethylation, inhibits cell metastasis by activating death-associated protein kinase 1 in prostate cancer

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Prostate cancer (PCa) death primarily occurs due to metastasis of the cells, but little is known about the underlying molecular mechanisms. This study aimed to evaluate the expression of *UNC5D*, a newly identified tumor suppressor gene, analyze its epigenetic alterations, and elucidate its functional relevance to PCa metastasis. Meta-analysis of publicly available microarray datasets revealed that *UNC5D* expression was frequently downregulated in PCa tissues and inversely associated with PCa metastasis. These results were verified in clinical specimens by real-time PCR and immunohistochemistry assays. Through methylation analysis, the downregulated expression of *UNC5D* in PCa tissues and cell lines was found to be attributable to the hypermethylation of the promoter. A negative correlation was observed between methylation and *UNC5D* mRNA expression in PCa samples. The ectopic expression of *UNC5D* in PCa cells effectively reduced their ability to migrate and invade both in vitro and in vivo, and siRNA-mediated knockdown of *UNC5D* yielded consistent results. *UNC5D* can recruit and activate death-associated protein kinase 1, which remained to be essential for its metastatic suppressor function. In conclusion, these results suggested that *UNC5D* as a novel putative metastatic suppressor gene that is commonly down-regulated by hypermethylation in PCa.

KEYWORDS
expression, metastasis, methylation, prostate cancer, *UNC5D*

Abbreviations: DAPK, death-associated protein kinase 1; GEO, Gene Expression Omnibus; IHC, immunohistochemistry; IP, immunoprecipitation; PCa, prostate cancer.

1 | INTRODUCTION

Prostate cancer is one of the most commonly diagnosed malignancies among men and is the fourth most common cancer among men worldwide.[1] Mortality occurs due to metastasis of PCa that is present either at the time of diagnosis or after failure of primary treatment. Metastatic disease indicates poor prognosis in men with PCa, and correlates with significant mortality.[2] Most of the metastatic PCa cases eventually develop resistance to primary androgen deprivation therapy, a condition known as metastatic castration-resistant prostate cancer.[2] However, little is known about the molecular mechanisms involved in the metastasis of PCa cells, which ultimately limits the development of effective therapies that aimed to prevent the spread of PCa cells. Therefore, identification of novel genes that are functionally involved in PCa development and progression or new markers that have the capability to predict or classify metastatic disease in the early stage could help to investigate potential diagnostic and therapeutic targets.

In recent years, rapid development has been observed in next-generation sequencing technology, which indicates that genetic and epigenetic changes are the primary drivers of PCa.[3] For human PCa, epigenetic alterations are not only involved in the initial tumorigenesis process, but also in the continuous regulation of subsequent progression of the malignancy.[4,5] An in-depth understanding regarding the epigenetic changes in PCa provides new opportunities for the discovery of biomarkers for screening, diagnosis, and risk stratification of PCa.[6] Methylation of the promoter DNA leads to the silencing of the genes involved, and plays crucial roles in tumorigenesis. In addition, abnormal DNA methylation occurs even earlier than genetic events, such as loss of heterozygosity and microsatellite instability,[7,8] and much earlier than the changes of protein biomarkers. This in turn produced DNA methylation abnormality as a biomarker that is more sensitive and valuable than genetic aberrations and protein biomarkers.

Members of the *UNC5* family, including four homologues (*UNC5A-D*), were originally identified as netrin receptors and are considered to participate in the regulation of cell migration and morphogenesis during development.[9] The expression of *UNC5* homologues was downregulated in many human malignancies due to genetic and epigenetic alterations.[10] *UNC5* receptors have been reported to function as "dependence receptors" owing to their dependence on the availability of netrin-1 for cell survival.[10,11] Although *UNC5H* receptors share high homology with each other, their functions are not exactly the same.[10,12,13] *UNC5D/H4* is the most recently identified member of *UNC5* family.[14] Like other members of this family, *UNC5D* has also been characterized as a tumor suppressor gene in several cancers, such as neuroblastoma,[15,16] renal cell carcinoma,[17] and bladder cancer.[18] *UNC5D* has been observed with high expression in the human prostate tissue, but underexpressed in the castration-resistant stage of prostate cancer.[17,19] However, little is known about the role and underlying mechanism of *UNC5D* in prostate cancer pathogenesis and progression.

In the present study, we evaluated the expression status of *UNC5D* in normal prostate and primary and metastatic PCa, and clarified whether the downregulated expression of *UNC5D* was mainly attributable to the methylation alterations on the CpG island in the promoter. Both in vitro and in vivo functional assays were applied to characterize the inhibitory effects of *UNC5D* on the metastasis of PCa cells. Molecular mechanisms for the suppressive function of *UNC5D* were also explored in this study.

2 | MATERIALS AND METHODS

2.1 | Prostate cancer clinical specimens

Prostate cancer and corresponding noncancerous tissues were obtained from 82 PCa patients, including 60 patients with primary tumor and 22 patients with metastatic tumor, who underwent surgery at Tianjin Medical University Cancer Institute and Hospital (Tianjin, China). All PCa patients gave written informed consent on the use of clinical specimens for medical research. All procedures undertaken in studies involving human participants were in accordance with the 1964 Helsinki Declaration ethical standards and approved by the Research Ethics Committee of Tianjin Medical University Cancer Institute and Hospital.

2.2 | Cell lines, Abs, and drug treatments

All PCa cell lines used in this study were obtained from the Cell Bank of the Chinese Academy of Medical Sciences (Beijing, China), and maintained in RPMI-1640 supplemented with 10% FBS (Gibco-BRL, Gaithersburg, MD, USA) and 1% penicillin/streptomycin. Antibodies specific to UNC5D, β-actin, and DAPK1 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Anti-phospho-DAPK1 Ab (Ser-308) was purchased from Cell Signaling Technology (Beverly, MA, USA). For demethylation assays, cell lines were treated with 10 mmol/L 5-aza-2-deoxycytidine (Sigma-Aldrich) for 3 days with exchange of reagents and medium every 24 hours. For experiments using the DAPK1 inhibitor ((4Z)-4-(3-Pyridylmethylene)-2-styryl-oxazol-5-one; Sigma-Aldrich), cells were plated in 24-well plates, pretreated for 60 minutes with 25 μmol/L DAPK inhibitor, and then maintained with DAPK1 inhibitor at the concentration of 1 μmol/L (in media with 0.1% DMSO). Media alone with 0.1% DMSO was used as vehicle control.

2.3 | Methylation analysis of *UNC5D*

Genomic DNA (500 ng) was bisulfite converted following EZ DNA-Methylation Gold kit instructions (Zymo Research, Irvine, CA, USA). Methylation-specific PCR and bisulfate genomic sequencing analysis were carried out as described previously.[20] Methylation negative or positive was determined by the presence or absence of the electrophoresis band. Polymerase chain reaction for MethyLight assays and the calculation for the percentage of methylated reference was

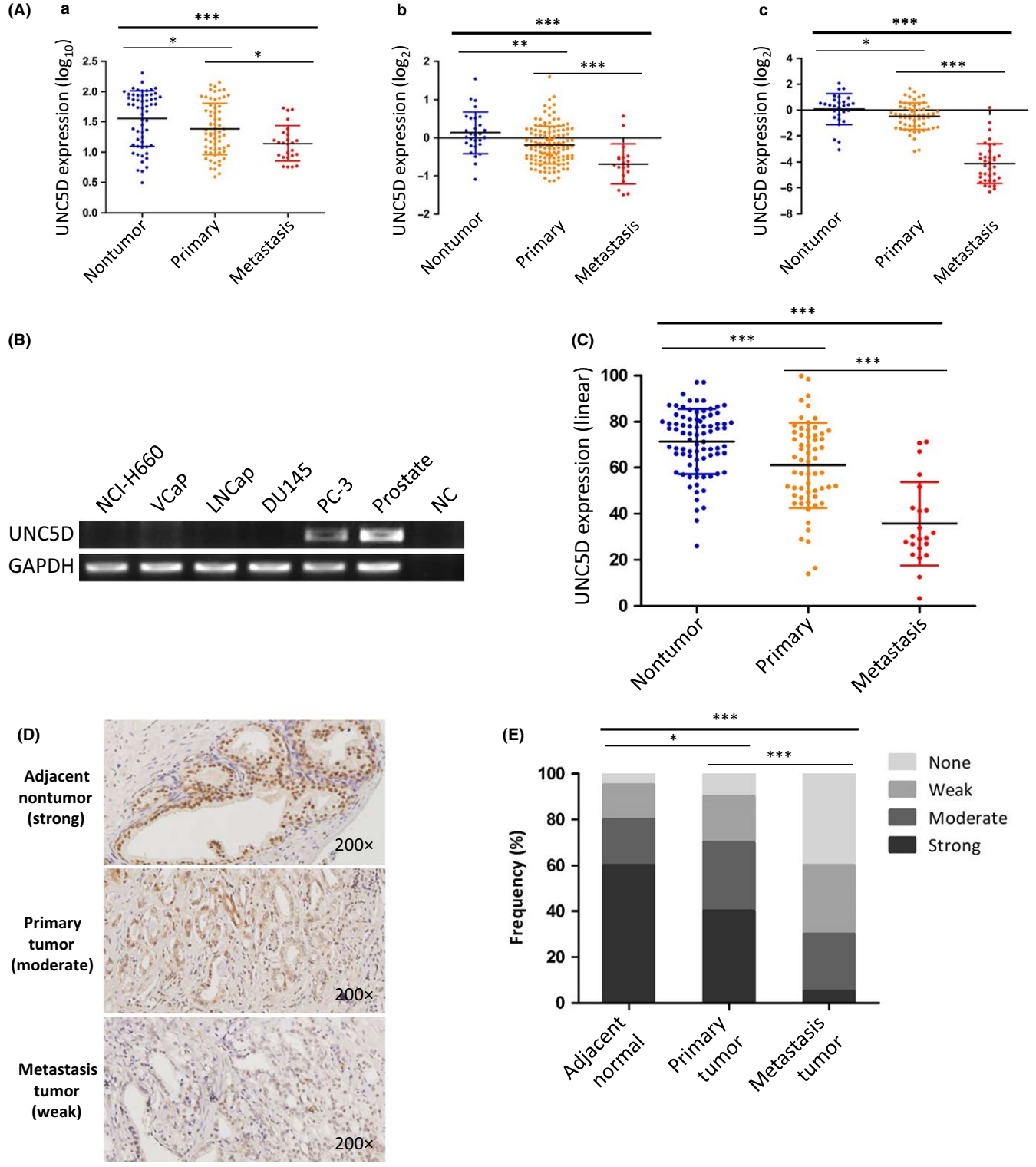


FIGURE 1 Expression of *UNC5D* is downregulated in primary and metastatic prostate cancer (PCa). A, meta-analysis of *UNC5D* expression in prostate nontumor tissues and primary and metastatic PCa tissues through the publicly available microarray datasets, Gene Expression Omnibus (GEO) and Oncomine. a, GEO datasets; $\log_{10}$ scale at *Y*-axis; b and c, Oncomine datasets; $\log_2$ scale at *Y*-axis. Mean and SD values are shown. B, Expression of *UNC5D* mRNA in 5 PCa cell lines, nontumor prostate tissue as the positive control, and water as the negative control. C, Scatterplots of the relative expression of *UNC5D* detected by quantitative RT-PCR in prostate nontumor tissues and primary and metastatic PCa tissues. Linear scale at *Y*-axis, mean and SD values are shown. D, Representative immunohistochemistry staining of *UNC5D* expression in adjacent nontumor, primary tumor, and metastatic tumor, respectively (magnification, 200×). E, Comparison of the relative protein levels of UNC5D in prostate nontumor tissues, primary and metastatic PCa tissues as measured by immunohistochemistry; Fisher's exact test was used. **P* < .05; ***P* < .01; ****P* < .001

undertaken as described previously.[21] Sequence information for the primers and probes used is listed in Table S1.

2.4 | Immunohistochemistry and scoring

Tissue sections were incubated with anti-UNC5D Ab (Sigma-Aldrich) at 1:200 dilution overnight at 4°C. The sections were then washed and subsequently incubated with a universal secondary Ab for 1 hour at room temperature. The scoring method that combined intensity and percentage of positivity was previously described,[22] and extent and intensity measures for each core were combined as weak (score 1), moderate (score 2), and strong (score 3).

2.5 | Transfection, infection, and western blot analysis

Adenoviruses expressing *UNC5D*, *UNC5D-ΔDD* (UNC5D without the death domain[17]), or MOCK were packaged by the Vector Gene Technology Company (Beijing, China). Prostate cancer cell lines were infected with 20 MOI of the adenoviral vector. JetPRIME transfection reagent (Polyplus-transfection, Illkirch, France) was used for all transfections in this study. All western blots were detected by electrochemiluminescence (GE Healthcare Life Sciences, Uppsala, Sweden). Beta-actin (Sigma-Aldrich) was used as the internal control.

2.6 | Knockdown of *UNC5D*

Two *UNC5D*-specific Stealth siRNA, targeting the noncoding region of *UNC5D* and a negative control siRNA, were purchased from Invitrogen (Carlsbad, CA, USA). Transfection was carried out using the jetPRIME transfection reagent (Polyplus-transfection) according to the manufacturer's instructions. After transfection for 72 hours, the cells were harvested for further analysis. Sequence information for the siRNA used is listed in Table S1.

2.7 | Immunoprecipitation

The IP was carried out as described previously.[23] Cells were lysed in lysis buffer for cell IP (Thermo Fisher Scientific, Waltham, MA, USA) for 30 minutes on ice. After sufficient centrifugation, the supernatant was incubated with anti-DAPK1, anti-UNC5D, or anti-IgG Affinity Gel (Sigma-Aldrich) at 4°C overnight. The beads were washed 3 times with 25 mmol/L Tris-HCl (pH 7.4), 100 mmol/L NaCl, and 1% (w/v) Triton X-100. Approximately 5% of the whole lysate (Input) was used as a positive control. The precipitates were analyzed by SDS-PAGE and immunoblotting.

2.8 | Wound healing assay

Cell motility was determined by measuring the movement of cells to close an artificial wound. Cells were seeded in 24-well plates at 80% confluence. Cells were wounded with a 200-μL pipette tip, washed with PBS, and incubated with medium containing 1% FBS. The distance traveled by cells was monitored by phase-contrast microscopy (Olympus, Tokyo, Japan) at indicated time points.

2.9 | Cell migration and invasion assay

For cell migration assay, PCa cells were seeded into Transwell inserts (8 μmol/L pore; BD Biosciences, San Jose, CA, USA). The bottom chamber contained medium with 10% FBS. After the cells were incubated for 20 hours, the noninvading cells remaining on the upper surface of the membrane were removed by scraping and cells attaching to the bottom of the membranes were fixed in 10% formalin for 30 minutes and stained with 0.05% crystal violet. For the cell invasion assay, cells were seeded in the Matrigel-coated Transwell inserts (BD Biosciences) and the invasion time was extended to 36 hours.

2.10 | In vivo metastasis assay

For lung metastasis assays, the androgen-independent DU145 cells were infected with adenoviruses expressing *UNC5D* or MOCK. Twenty-four hours after infection, the cells were trypsinized and washed with PBS. A total of one million cells in 100 μL were injected into the tail vein of nude mice. At 6 weeks after injection, the animals were killed and lungs were harvested and fixed in 10% formaldehyde/PBS before being assessed for evidence of metastases by H&E staining. In in vivo experiments, the female Nu/Nu nude mice at age of 4 weeks were maintained under specific pathogen-free conditions. All animal protocols were in accordance with guidelines for animal care and were approved by the Animal Ethics Committee of Tianjin Medical University.

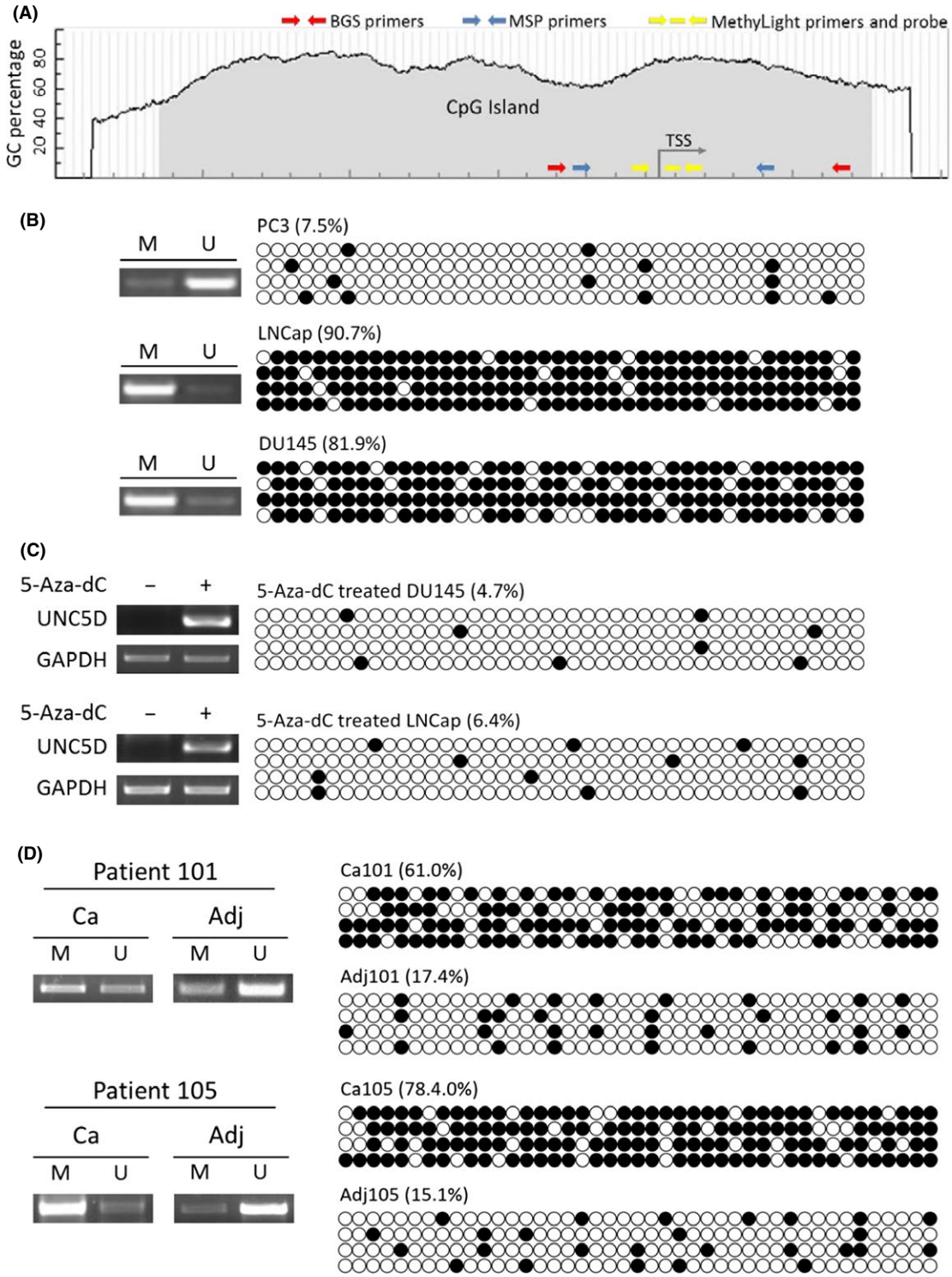
2.11 | Statistical analysis

Quantitative data were expressed as data plots or mean ± SD. Mann-Whitney *U* test was applied to compare the variables of the 2 specimen groups. For multiple comparison, one-way ANOVA analysis followed by Tukey's multiple comparison test. Differences between the mean values of 2 experiment groups were analyzed by Student's *t* test. To compare the relative protein levels of UNC5D prostate non-tumor tissues and primary and metastatic PCa tissues, and to analyze the association between promoter methylation and the expression of *UNC5D*, Fisher's exact test was used. The Pearson correlation coefficients method was used to evaluate the association between the percentage of methylated reference and the expression of *UNC5D*. All statistical tests were undertaken using SPSS (version 13.0; SPSS, Chicago, IL, USA). Value of $P < .05$ was taken as statistical significance.

3 | RESULTS

3.1 | Downregulated expression of *UNC5D* in primary and metastatic PCa

First, a meta-analysis of *UNC5D* expression was carried out through the publicly available microarray datasets GEO (https://www.ncbi.



(E)

Primary tumor (n = 60)	Methylation (–)	Methylation (+)	*P*
UNC5D high expression	21	10	.0009
UNC5D low expression	10	19	

FIGURE 2 Promoter methylation contributes to the suppressed expression of *UNC5D* in cell lines and primary prostate cancer (PCa). A, Schematic diagram of the CpG island of *UNC5D* and detection primers. B, Methylation status of *UNC5D* promoter in PCa cell lines. Left, results of methylation-specific PCR (MSP) analysis; right, results of bisulfate genomic sequencing (BGS) analysis. Circles, CpG sites analyzed; row of circles, an individual promoter allele that was cloned, randomly selected, and sequenced; filled circle, methylated CpG sites; open circle, unmethylated CpG site. C, Results of *UNC5D* expression (right) and BGS analysis (left) in DU145 and LNCap cells after demethylation (5-aza-2-deoxycytidine [5-Aza-dC]) treatment. D, *UNC5D* promoter methylation was evaluated by MSP and BGS analysis in primary PCa and adjacent nontumor tissues. Results from 2 representative pairs are shown. E, Association between the expression of *UNC5D* and the aberrant methylation of the *UNC5D* promoter ($P < .01$, Fisher's exact test)

nlm.nih.gov/geo/) and Oncomine (https://www.oncomine.org/resource/main.html). Datasets meeting the following criteria were included: (a) the datasets were about human prostate cancer; (b) nontumor gland, primary tumor, and metastatic tumor were included; (c) the mRNA expression of *UNC5D* was measured in these databases; and (d) the number of cases exceeds 100. Finally, a total of 3 independent human prostate microarray databases (one GEO database, GSE6919; two Oncomine databases, Grasso and Taylor Prostate) were enrolled in this analysis, including 115 nontumor glands adjacent to tumor or not, 254 primary PCa, and 79 metastatic PCa samples. Compared with nontumor tissue, the relative level of *UNC5D* was significantly lower in the primary tissue in all the three datasets (Figure 1A). The results showed a more notable decline in the expression of *UNC5D* in metastatic PCa compared to the primary tumor (Figure 1A).

Then we examined the expression of *UNC5D* in 5 prostate cancer cell lines. Complete lack of *UNC5D* expression was observed in NCI-H660, VCaP, LNCap, and DU145 cells. Only PC-3 was detected with relatively weak *UNC5D* expression compared with the normal prostate tissue (Figure 1B). Real-time PCR (Figure 1C) and IHC staining (Figure 1D) assays yielded consistent results with the meta-analysis. The IHC staining was further scored by taking into account the staining intensity and percentage of cells showing positive staining. The specimens were divided into 3 groups (nontumor, primary tumor, and metastatic tumor), and the percentages with strong, medium, and weak expression levels were calculated separately. As shown in Figure 1E, the UNC5D protein was significantly downregulated in primary PCa tissues compared to noncancerous tissues, and least expression was observed in the metastatic tumors.

3.2 | Promoter hypermethylation of *UNC5D* in cell lines and primary PCa

The mechanism for the downregulated expression of *UNC5D* in PCa was explored. Methylation detection primers against the *UNC5D* promoter were designed to analyze the methylation status of *UNC5D* in PCa cell lines and tissues (Figure 2A). Methylation-specific PCR and bisulfate genomic sequencing analysis revealed that the *UNC5D* promoter was highly methylated in DU145 and LNCap, not expressing *UNC5D*, but much less methylated in PC-3, expressing *UNC5D* (Figure 2B). To further determine the silence effects of methylation, DU145 and LNCap were treated with DNA methyltransferase inhibitor 5-aza-2-deoxycytidine. Restoration of *UNC5D* expression was observed in those cells, accompanied by significantly declined methylation levels (Figure 2C). Methylation analysis was further extended to primary tumors. The results showed that the primary PCa tissues have higher levels of methylation than the paired noncancerous tissues (Figure 2D). The mean mRNA expression level of *UNC5D* was selected to classify patients with primary PCa into the *UNC5D* high-expression group (n = 31) and *UNC5D*

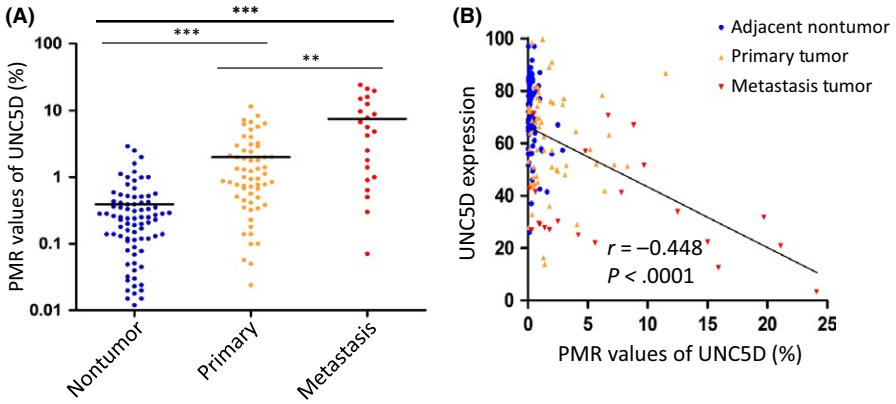
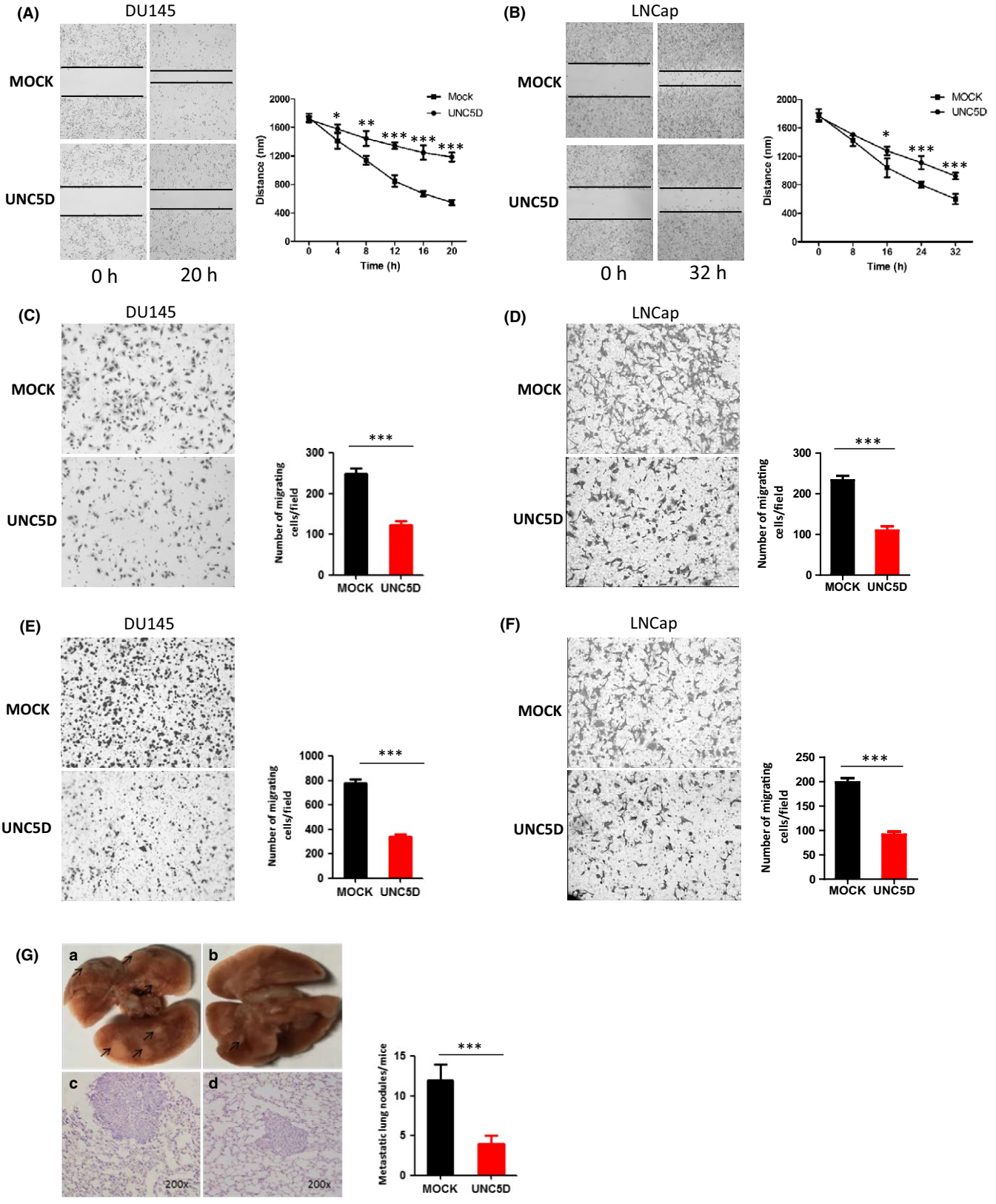


FIGURE 3 Methylation level of *UNC5D* is positively correlated with the metastasis of prostate cancer (PCa). A, Scatterplots of the methylation levels of *UNC5D* percentage of methylated reference (PMR) detected by MethyLight assay in prostate nontumor tissue and primary and metastatic PCa tissues. Log_{10} scale at *Y*-axis, mean values are shown. B, Correlation between the expression of *UNC5D* and the methylation levels of *UNC5D* promoter ($r = -0.448$, $P < .0001$, Pearson's correlation coefficients method). The nontumor tissues and primary and metastatic PCa tissues are displayed separately as indicated. $^{**}P < .01$; $^{***}P < .001$



low-expression group (n = 29). Ten of 31 in the *UNC5D* high-expression group, and 19 of 29 in the *UNC5D* low-expression group were positively methylated. *UNC5D* expression was significantly associated with promoter hypermethylation (Figure 2E; $P = .0009$, Fisher's exact test).

3.3 | Positive correlation between methylation level of *UNC5D* and the metastasis of PCa

MethyLight assay was used to analyze the methylation in an accurate and quantitative manner. This detection system containing 9

FIGURE 4 Restoring *UNC5D* expression suppresses the migration and invasion of prostate cancer (PCa) cell lines in vitro and in vivo. DU145 and LNCap cells were infected with *UNC5D*-expressing or MOCK adenoviruses, and the migration and invasion abilities were assessed. A,B, Results of wound healing assay. Left, representative images showing cell migration in wound healing assay; right, wound closure over time as measured by the distance between the front edges. Transwell migration (C,D) and Matrigel invasion (E,F) assays were undertaken to compare cell motilities between the two groups. The number of migrated or invaded cells was calculated and the results are expressed as mean ± SD of 3 independent experiments. G, In vivo lung metastasis assay was carried out to evaluate the effect of *UNC5D* on tumor metastasis. Representative images of lungs from nude mice injected with DU145-MOCK (a) or DU145-UNC5D (b) are shown. Tumors on the surface of the lung are indicated by arrows. Representative images of H&E staining of lung sections with tumors on the surface (c) and internal (d) of the lung are shown (magnification, 200×). The numbers of all metastatic nodules are summarized (right). Mean and SD values are shown. $^{*}P < .05$; $^{**}P < .01$; $^{***}P < .001$

CpG sites (3 in the forward primer, 2 in the reverse primer, and 4 in the TaqMan probe; Table S1), and the oligonucleotides with all these sites methylated will be annealed and amplified. All the metastatic tumors, primary tumors, and paired noncancerous tissues were detected. As shown in Figure 3A, the methylation levels in the promoter of *UNC5D* were the highest in metastatic tumors, followed by primary tumors, and the lowest in adjacent nontumor tissues. In addition, a significant inverse correlation between *UNC5D* expression and promoter methylation was observed for PCa samples (Figure 3B, $r = -0.448$, $P < .0001$). Taken together, these data indicated that the promoter methylation was the major cause of *UNC5D* downregulation in PCa.

3.4 | Restoration of *UNC5D* expression suppresses the migration and invasion of PCa cell lines

Frequent downregulation of *UNC5D* in PCa tissues, especially metastatic tumor tissues, suggests a potential role for *UNC5D* in the tumorigenesis and metastasis of PCa. To test this possibility, expression of *UNC5D* was restored in DU145 and LNCap cells, and the capabilities of tumor cells in migration and invasion were monitored. In vitro wound healing assay revealed that the motility of DU145 (Figure 4A) and LNCap (Figure 4B) cells has been significantly weakened. In vitro Transwell migration and Matrigel invasion assays

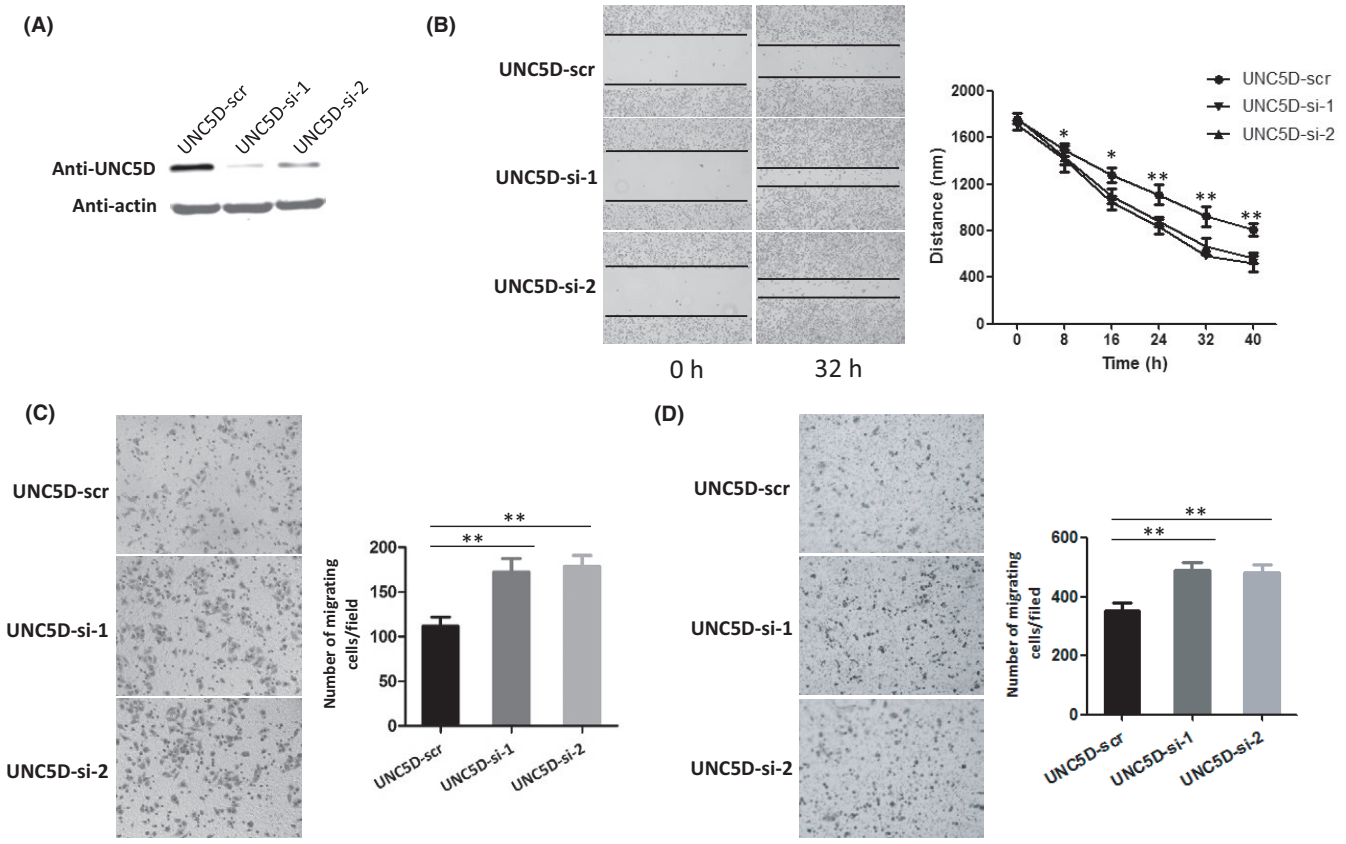
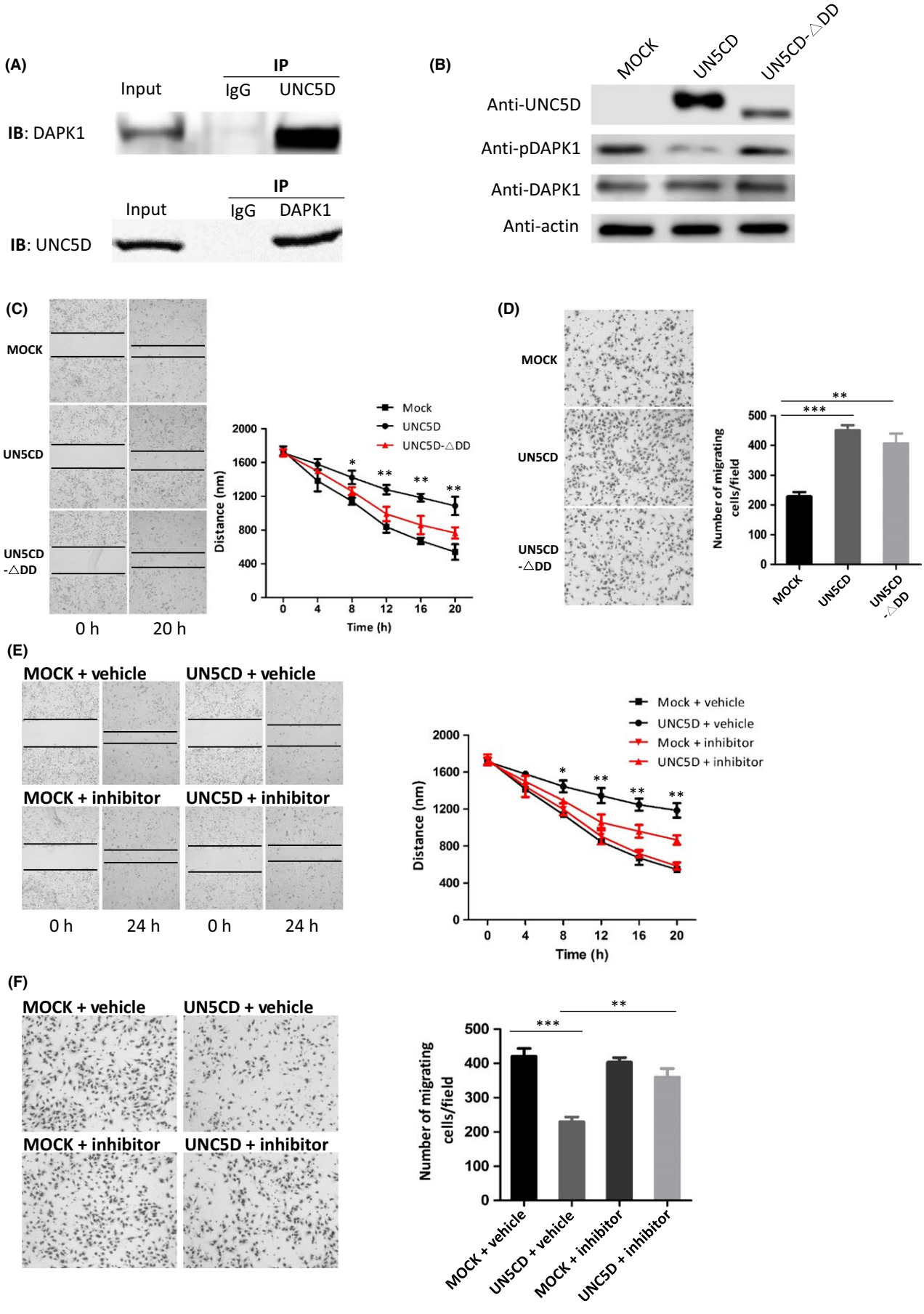


FIGURE 5 Silencing of *UNC5D* enhances the metastatic ability of tumor cells. A, Knockdown efficiency of *UNC5D* by siRNA was evaluated by western blot 48 hours after transfection. *UNC5D*-scr, negative control siRNA; *UNC5D*-si-1/2, independent siRNAs targeting *UNC5D*. Wound healing assay (B), Transwell migration (C) and Matrigel invasion (D) assays were undertaken to compare cell motilities of these cells. Results are expressed as mean ± SD of 3 independent experiments. $^{*}P < .05$; $^{**}P < .01$; $^{***}P < .001$


(A)
IP
Input
IgG
UNC5D
IB: DAPK1
IP
Input
IgG
DAPK1
IB: UNC5D
(B)
MOCK
UN5CD
UN5CD-△DD
Anti-UNC5D
Anti-pDAPK1
Anti-DAPK1
Anti-actin
(C)
MOCK
UN5CD
UN5CD -△DD
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UN5CD
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Number of migrating cells/field
(E)
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UN5CD + vehicle
MOCK + inhibitor
UN5CD + inhibitor
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FIGURE 6 *DAPK1* is essential for the metastasis suppressor function of *UNC5D*. A, Interaction between *UNC5D* and *DAPK1*. DU145 cells were infected with *UNC5D*-expressing adenoviruses. Cell lysates were immunoprecipitated (IP) with anti-UNC5D and IgG control (upper) or DAPK1 and IgG control (lower). Western blot analysis was undertaken to detect *DAPK1* and *UNC5D*, respectively. B, DU145 cells were infected with MOCK, *UNC5D*-ΔDD- or *UNC5D*-expressing adenoviruses. Western blot analysis was used to detect the expression of *UNC5D* and *UNC5D-ΔDD*, as well as the activation of *DAPK1*. The DAPK1 protein and actin were used as internal controls. C,D, The essential role of the death domain for the function of *UNC5D*. DU145 cells were infected with MOCK, *UNC5D*-ΔDD- or *UNC5D*-expressing adenoviruses. Wound healing (C) and Transwell migration (D) assays were used to compare cell motilities of these cells. E,F, DU145 cells were infected with MOCK or *UNC5D*-expressing adenoviruses, then the inhibitor of DAPK1 or the vehicle control (media with 0.1% DMSO) were added in the wound healing assay (E) and Transwell migration assay (F) to compare cell motilities. Results are expressed as mean ± SD of 3 independent experiments. $^{*}P < .05$; $^{**}P < .01$; $^{***}P < .001$

found that enforced expression of *UNC5D* significantly inhibited the migratory and invasive abilities of DU145 (Figure 4C,E) and LNCap (Figure 4D,F) cells compared with control cells. To test the suppressive effects of *UNC5D* on metastasis in vivo, DU145-MOCK and DU145-UNC5D cells were injected into the tail vein of nude mice (10 per group). Lung metastatic nodules were counted at 6 weeks after injection. The number of metastatic nodules formed on the surface and inside the lung were significantly less in mice injected with DU145-UNC5D cells compared to those injected with DU145-MOCK cells (Figure 4G).

3.5 | Silencing of *UNC5D* enhances the metastatic ability of PCa cells

To further confirm the inhibitory effects of *UNC5D* on PCa cell metastasis, *UNC5D* expression was knocked down by siRNA in PC-3 cells, showing endogenous expression of *UNC5D* to a certain extent. Knockdown effects were confirmed by western blot analysis after 48 hours of transfection of siRNA (Figure 5A). Wound healing assay showed that knockdown of *UNC5D* significantly increased the cell motility in PC-3 cells compared with the control cells transfected with scrambled-siRNA (Figure 5B). The effect of *UNC5D* knockdown in PC-3 cells was also confirmed by in vitro Transwell migration and Matrigel invasion assays (Figure 5C,D). This experiment further confirmed the metastatic-suppressive effect of *UNC5D* in PCa.

3.6 | *DAPK1* is essential for the metastasis suppressor function of *UNC5D*

UNC5D shares high homology with other members that were discovered previously, in both sequence and structure.[10] *UNC5A*, *UNC5C*, and especially *UNC5B* are capable of binding with the serine/threonine kinase, DAPK1,[24] which is a key intracellular kinase with both apoptosis-inducing[25,26] and motility-inhibitory functions.[27,28] The apoptosis-inducing biological effect of *UNC5B* is achieved by recruiting and activating DAPK1.[24,26] This subsequently led us to speculate whether the antimetastatic effect of *UNC5D* in PCa is mediated by DAPK1.

The expression of *DAPK1* in PCa tissues and cell lines was analyzed. Three publicly available microarray datasets (2 from GEO, both from GSE6919; 1 from Oncomine, Taylor Prostate) were selected to undertake the meta-analysis of *DAPK1* expression. Although usually reported as a tumor suppressor gene, expression of *DAPK1* was actually upregulated in the PCa tissues compared with the nontumor prostate tissue (Figure S1A-C). Moreover, the expression level of *DAPK1* was even higher in metastatic PCa than in primary tumors, according to the two datasets (Figure S1B,C). These results suggested that *DAPK1* could play a regulatory role in the tumorigenesis and metastasis of PCa. The expression of *DAPK1* in PCa cell lines was also evaluated by western blot analysis. DU145 and VCaP cells showed relatively strong expression of *UNC5D*, whereas LNCap, PC-3, and HCI-H660 cells showed lower *UNC5D* expression (Figure S1D).

We then investigated whether *UNC5D* could recruit and activate *DAPK1*. The co-IP assays were carried out in DU145 cells infected with adenoviruses expressing *UNC5D*. As shown in Figure 6A, *UNC5D* and *DAPK1* were IP with each other. Decreased levels of phosphorylation at Ser-308 showed the active state of *DAPK1*.[29,30] Western blot analysis indicated that *DAPK1* was obviously activated in DU145-UNC5D cells more than DU145-MOCK cells (Figure 6B). As reported, the death domains of *UNC5* family members remained crucial for their biological function in tumor cells.[10] Therefore, DU145 cells were also infected with adenoviruses expressing *UNC5D-ΔDD*. As shown in Figure 6B, *UNC5D-ΔDD* was observed with significantly diminished capability of activating *DAPK1*. To determine the indispensable role of the death domain for *UNC5D*, in vitro wound healing and cell Transwell migration assays were carried out. The antimetastatic effect of *UNC5D-ΔDD* was significantly diminished in both wound healing or Transwell migration assays (Figure 6C,D). These data support that the death domain of *UNC5D* was essential for the metastatic-suppressive function of *UNC5D*, possibly due to its indispensable role in activating *DAPK1*. Finally, *DAPK1* inhibitor was used to further confirm the pivotal role of the activation of *DAPK1* in the functioning of *UNC5D*. In vitro wound healing and cell t=Transwell migration assays (Figure 6E,F) showed that the antimetastatic effects of *UNC5D* were significantly reduced by *DAPK1* inhibitor. Taken together, these data illustrated that *DAPK1* could interact with *UNC5D* and might mediate its inhibitory function.

4 | DISCUSSION

Epigenetic changes are common features of PCa, and play key roles in the initiation and progression of cancer.[5] As for *UNC5D*, hypermethylation alterations at the promoter region are one of the main causes for its repression in a variety of tumors,[15-18] and are the same

in PCa cells according to our study. However, there are seldom reports on the methylation status of *UNC5D* in metastatic tumors. Our data indicated that the methylation changes exist not only in primary PCa, but also in metastatic tumors, and in a more reinforced manner, accounting for further downregulation of *UNC5D* in the metastatic tumors. Large-scale cancer genome sequencing studies in the genome of invasive PCa showed catastrophic hypermethylation alterations, involving hundreds to thousands of CpG islands, and the majority of these were highly methylated across the metastasis process within the individuals.[31] Thus, cancer metastasis could be driven by cumulative expression disorders, where hypermethylation occurs in genes that are key components in the metastatic pathways. In this study, we tried to determine whether *UNC5D* might act as one such key component.

Recently, it has been reported that *UNC5* family receptors act as dependent receptors for netrin-1, and these receptors induce apoptosis when not engaged with their ligand.[10] However, it is not always the case. Except from apoptosis induction, the *UNC5* homolog family also plays other important roles that are independent of netrin-1. *UNC5B* could interact with FLRT3 and Rnd1 to modulate cell adhesion in *Xenopus* embryos,[12] and the UNC5D/FLRT2 complex regulates the radial migration of cortical cells.[13] These findings indicated that whether the dependent receptors induce apoptosis depends on various factors both intracellularly and extracellularly.[32] For tumor cells, the factors that influenced apoptosis are considered to be more complicated. It is well known that many malignant cells gain resistance to apoptosis to evade chemotherapy.[33,34] Typical examples include renal cell carcinoma cells[35] and advanced PCa cells.[36] Both *UNC5C* and *UNC5D* have no apoptosis-inducing effects on renal carcinoma cells,[17,37] whereas *UNC5D* exerted tumor suppressive effects mainly by cell cycle arrest in renal carcinoma cells.[17] In this study, we showed that *UNC5D* has the capability to suppress the metastatic ability of PCa cells. Moreover, the suppressive function of *UNC5D* was dependent on *DAPK1* in the downstream. *DAPK1* is a key intracellular kinase that has both apoptosis-inducing and motility-inhibitory functions.[25-28] In apoptosis-sensitive cells, *DAPK1* participates in a wide range of apoptotic signals.[27,28] In tumor cells that are resistant to *DAPK*-induced apoptosis, *DAPK* has a motility-inhibitory effect and functions as a determining factor in tumor cell invasion.[27,28,38] *DAPK1* has been reported as a tumor suppressor gene, with promoter hypermethylation and downregulated expression in various tumors.[39-41] However, interestingly, our work revealed that *DAPK1* was significantly upregulated in PCa. This explains, to some extent, that the PCa cells were classified as cells resistant to *DAPK*-induced apoptosis, making *DAPK1* a cell motion regulator. As a death-associated protein kinase, *DAPK1* could be activated by the death domain of *UNC5* homologues.[10] *UNC5D-ΔDD*, *UNC5D* without death domain, did not activate *DAPK1* and the metastatic inhibitory effects of *UNC5D* in PCa cells were diminished significantly. Therefore, activation of *DAPK1* remains to be essential for the function of *UNC5D* in PCa. Although the underlying mechanisms for the upregulated expression of *DAPK1* are still unknown, considering its indispensable role for the metastatic inhibitory effects of *UNC5D*, high expression of *DAPK1* induces *UNC5D* as a potential target for PCa therapy.

Collectively, we have identified *UNC5D* as a novel candidate metastasis suppressor that is silenced by promoter hypermethylation in PCa. We present clinical evidence that *UNC5D* expression is negatively associated with its methylation level, as well as PCa metastasis. In addition, we validated the metastasis suppressor function of *UNC5D* in PCa cells, which is dependent on the downstream activation of *DAPK1*. Our findings proposed that *UNC5D* could be a potential diagnostic biomarker and therapeutic target for metastatic PCa.

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CONFLICTS OF INTEREST

Authors declare no conflicts of interest for this article.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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