Hindawi
Andrologia
Volume 2023, Article ID 9821829, 10 pages
https://doi.org/10.1155/2023/9821829

Research Article

PRDM16, Negatively Regulated by miR-372-3p, Suppresses Cell Proliferation and Invasion in Prostate Cancer

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Received 3 March 2022; Accepted 26 June 2022; Published 18 March 2023

Academic Editor: Ralf Henkel

Prostate cancer (PCa) is one of the most prevalent malignant tumors. The alternation of microRNA (miRNA) expression is associated with prostate cancer progression, whereas its way to influence progression of prostate cancer remains elusive. The expression levels of PRDM16 mRNA and miR-372-3p in PCa cell lines were analyzed using qRT-PCR. The protein expression of PRDM16 in PCa cell lines was also analyzed using western blot. CCK-8, wound healing, and Transwell assays were applied to examine cell proliferation, migration, and invasion in prostate cancer cells, respectively. Dual-luciferase reporter assay was utilized to validate the interaction between miR-372-3p and PRDM16. In the present study, markedly decreased PRDM16 mRNA and protein expression levels were observed in prostate cancer cells. PRDM16 overexpression hampered cellular proliferation, migration, and invasion, while silencing PRDM16 had the opposite effect. Moreover, miR-372-3p could target the regulation expression of PRDM16. Rescue experiments demonstrated that upregulating miR-372-3p conspicuously restored the inhibitory effect of increased PRDM16 on cell proliferation, migration, and invasion in PCa. Overall, our study clarifies the biological role of miR-372-3p/PRDM16 axis in prostate cancer progression, which may be effective biomarkers for clinical treatment of prostate cancer.

1. Introduction

Prostate cancer is the most prevalent nonskin cancer, and it is a major cause of cancer-related deaths [1]. Prostate cancer is an epithelial malignancy in the prostate, and compression and metastasis appear as tumorigenesis, leading to ostealgia or pathological fractures [2]. Prostatectomy can greatly improve overall survival rate of patients with early prostate cancer [3], but relapse and metastasis still exist [4, 5]. The cure rate and 5-year survival rate of prostate cancer patients remain low [6]. Therefore, novel treatment options for preventing prostate cancer progression and prolonging patient's survival time in this area are of high unmet need.

The positive regulatory domain containing 16 (PRDM16) is featured by an N-terminal PR domain and multiplex Zn-finger repeats [7, 8]. It was confirmed that PRDM16 has intrinsic histone methyltransferase activity and catalyzes the H3K9me1 [9]. Li and Wu [10] investigated epigenetic mechanism of glioblastoma and revealed that both cancer susceptibility 2 and miR-101 result in overexpression of hypomethylation gene PRDM16. Moreover, PRDM16 also serves as a transcriptional modulator [11]. Zhang et al. [12] systematically revealed transcription factors pivotal for lung cancer oncogenesis, wherein PRDM16 is the potential tumor repressor. PRDM16 can also modulate multiple biologic processes, such as cancer cell proliferation and metastasis [13], epigenetic modification [14, 15], and potential biomarkers for cancer treatment [16]. PRDM16 is rarely studied relative to other PRDMs, and research on its function in prostate cancer is warranted.

Almost all normal cells are controlled by microRNAs (miRNAs) [17]. Noncoding RNAs are one of the main players in the regulation of multiple pathways and cellular processes [18]. miRNAs are short noncoding RNAs that are involved in many biological processes [19]. There are also differentially expressed miRNAs (DEmiRNAs) in prostate cancer; these DEmiRNAs modulate messenger RNA (mRNA) expression to affect incidence and progression of prostate cancer [20], such as miR-1182 [21], miR-515-5p [22], and miR-1301 [23]. miR-371-373 cluster has an essential function in stem cell pluripotency [24]. miR-371-373 cluster also affects tumor development and is aberrantly expressed in tumors [25–27]. A study [28] unearthed that miR-373 represses CD44 to hasten tumor invasion and metastasis. miR-372-3p targets FXYD6 to repress growth and metastasis of osteosarcoma cells [29]. Nonetheless, specific mechanism of miR-372-3p in regulating prostate cancer is elusive.

In this study, to comprehensively reveal the underlying mechanisms of PRDM16 in prostate cancer, we assayed its expression and effects on cell functions in prostate cancer. Additionally, bioinformatics methods and dual-luciferase reporter assays identified that PRDM16 was the target of miR-372-3p in prostate cancer. Upregulating miR-372-3p conspicuously restored the inhibitory effect of increased PRDM16 on cell proliferation, migration, and invasion in prostate cancer. The findings here may provide potential novel therapeutic targets and biomarkers for prostate cancer patients.

2. Materials and Methods

2.1. Bioinformatics Analysis. miRNA mature data (normal: 52 and tumor: 499) and mRNA expression profiles (normal: 52 and tumor: 499) of prostate cancer were acquired from The Cancer Genome Atlas (TCGA) database, along with the corresponding clinical data. "edgeR" package was utilized for differential expression analysis on miRNAs and mRNAs between the two groups ($|\log FC| > 1.5$, $padj < 0.05$) for acquisition of DEmiRNAs and DEmRNAs. The mRNA of interest was identified through a literature review. mirRDB, TargetScan, starBase databases were employed for predicting upstream regulatory genes of target mRNA. DEmiRNAs that had binding sites with target mRNA were obtained, and their correlation was analyzed to make a final determination of target miRNA.

2.2. Cell Lines and Cell Culture. Human normal prostate epithelial cell line RWPE-1 (ATCCCRL-11609) and prostate cancer cell lines PC-3 (ATCCCRL-1435), DU145 (ATCCHTB-81), LNCaP (ATCCCRL-1740), and VCaP (ATCCCRL-2876) were accessed from American Type Culture Collection. RPMI-1640 medium (Gibco, California, USA) plus 10% fetal bovine serum (FBS) was recommended for cell preparation in an environment at 37°C with 5% CO_2.

Negative control (NC) mimics and miR-372-3p mimics were synthesized by Shanghai GenePharma Co., Ltd. PRDM16 overexpression plasmid (oe-PRDM16) and empty plasmid (oe-NC) were purchased from the same company. Lipofectamine 2000 (Invitrogen, USA) was implemented to run the transfection. 48 h later, quantitative real-time polymerase chain reaction (qRT-PCR) or western blot was carried out to check transfection efficacy.

2.3. qRT-PCR. TRIzol reagent (Invitrogen, USA) was utilized for isolation of total RNA from cells. A spectrophotometer (BioTek) was utilized for quantitation. PrimeScript RT Reagent Kit (TaKaRa, Japan) was implemented to reverse transcribe 1 μg RNA into cDNA, which was quantified by qRT-PCR with SYBR Green Master Mix Kit (TaKaRa, Japan). miR-372-3p and PRDM16 were normalized to U6 and β-actin. Data analysis applied $2^{-\Delta\Delta Ct}$ method. Primer sequences are listed in Table 1.

2.4. Western Blot. Cells were collected from cell lysate (Cell Signaling Technology; Cat#: 9803). Protein samples were separated on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and were transferred onto polyvinylidene fluoride membrane (Roche, Switzerland). Membrane was sealed with skim milk for 1.5 h and was soaked in primary antibodies (PRDM16 (1 μg/ml, ab85874, Abcam, UK) and β-actin (1 : 1000, ab115777, Abcam, UK)) at 4°C overnight. Then, the membrane was soaked in horseradish peroxidase-labelled goat anti-rabbit IgG H&L (ab6721, Abcam, UK) for 2 h. Protein bands were evaluated on BeyoECL Plus (P0018, Beyotime, China). Target protein was evaluated on ImageJ version 1.38 (National Institutes of Health, USA), and it was normalized to β-actin level. β-Actin was considered an internal reference.

2.5. Cell Counting Kit-8 (CCK-8) Proliferation Assay. CCK-8 was utilized to assess proliferative capability of PCa cells after transfection. In short, after 4 h of cell incubation at a constant temperature of 37°C and 5% CO_2 in CCK-8 (Beyotime, China), viability of PC-3/DU145 cells was assayed at 24, 48, 72, 96, and 120 h. Absorbance was assessed at 450 nm with a microplate reader (Bio-Rad 680, USA).

2.6. Wound Healing Assay. Cells were cultivated in 6-well plates until 100% confluence. Next, a pipette tip was implemented to scratch a straight line in center of each well. After being rinsed two times with phosphate-buffered saline, cells were soaked with an FBS-free medium. At 0 h and 24 h, wound images of each well were captured. Image Pro Plus software (Media Cybernetics, USA) was employed to measure wound healing areas.

2.7. Transwell Invasion Assay. The 24-well Transwell chambers with Matrigel (Corning, NY, USA) were used for invasion detection. Briefly, 3×10^4 cells were inoculated into the upper insert with a serum-free medium. In the lower chamber, a medium containing 10% FBS was taken as a chemical attractant. Twenty-four hours later, cells on the membrane were wiped off. The remaining cells were soaked for 30 min in 95% ethanol and stained for another 30 min with 0.2% crystal violet. Invaded cells were counted and photographed under the inverted microscope.

Table 1: Primer sequences in qRT-PCR.

Target gene	Primer (5′-3′)
miR-372-3p	F: 5′-TAGCAGGATGGCCCTAGACC-3′
	R: 5′-TCCGTTGATATGGGCGTCAC-3′
U6	F: 5′-GCTTCGGCAGCACATATACTAAAAT-3′
	R: 5′-CGCTTCACGAATTTGCGTGTCAT-3′
PRDM16	F: 5′-AGGCTGCATCACAAAGATCTCC-3′
	R: 5′-CCTCACCTGGCTCAATGTCC-3′
β-Actin	F: 5′-CGTCGACAACGGCTCCGGCATG-3′
	R: 5′-GGGCCTCGTCACCCACATAGGAG-3′

2.8. Dual-Luciferase Reporter Gene Assay. Luciferase vectors expressing wild-type (WT) or mutant (MUT) 3′-untranslated region (3′-UTR) PRDM16 sequences were designed. PC-3 cells were cotransfected with luciferase vector and miR-372-3p mimics or NC mimics. Forty-eight hours later, a fluorescence reader (Promega Corporation, USA) was applied to compute luciferase signal.

2.9. Statistical Analysis. Statistical analysis was completed on SPSS 19.0 and GraphPad Prism 5.0. Data were shown as mean ± standard deviation. Student's t -test was for two-group comparison, while single factor analysis of variance was for multiple comparisons. $p < 0.05$ meant that the result reached statistical significance.

3. Results

3.1. PRDM16 Expression Decreases in Prostate Cancer. Differential analysis revealed 1,412 DEmRNAs in prostate cancer (Figure 1(a)). An existing study theorized the anticancer role of PRDM16 in cell proliferation and metastasis [30], but few investigations were carried out on the function of this gene in prostate cancer. Besides, through data from TCGA, PRDM16 was found to decrease in prostate cancer tissue (Figure 1(b)). qRT-PCR and western blot assays also assayed PRDM16 mRNA and protein expression in cell lines of prostate cancers. As illustrated in Figure 1(c), PRDM16 mRNA and protein levels were low in cancer cells relative to normal cells. All in all, PRDM16 was conspicuously lowly expressed in prostate cancer.

3.2. PRDM16 Upregulation Hampers Aggressive Phenotype of Prostate Cancer Cells. We investigated whether PRDM16 is a controller of prostate cancer cell behaviors. PC-3 and DU145 cells with relatively low PRDM16 expression were selected. First, PRDM16 was overexpressed in PC-3 and DU145 cells. PRDM16 expression in the oe-NC and oe-PRDM16 groups was compared through qRT-PCR and western blot. As presented in Figures 2(a) and 2(b), transfecting oe-PRDM16 notably increased PRDM16 expression in cells. CCK8 assay authenticated the suppressive effect of oe-PRDM16 on cell viability (Figure 2(c)). In wound healing assay, migration rate noticeably decreased with overexpressing PRDM16 (Figure 2(d)). Transwell confirmed that oe-PRDM16 hindered cell invasive capability (Figure 2(e)). These data validated that PRDM16 repressed phenotype progression of prostate cancer cells.

3.3. Increased miR-372-3p Downregulates PRDM16 in Prostate Cancer. Through bioinformatics analysis, upstream modulatory genes of PRDM16 were excavated. Following differential analysis, a total of 51 DEmiRNAs were acquired (36 upregulated DEmiRNAs and 15 downregulated DEmiRNAs) (Figure 3(a), Supplementary Figure 1). Next, mirRDB, TargetScan, and starBase databases were applied for predicting upstream modulatory genes of PRDM16. The results were taken intersection with upregulated DEmiRNAs to gain 2 DEmiRNAs that had binding sites with PRDM16 (Figure 3(b)). Moreover, it was previously authenticated that miR-372-3p facilitates malignant behaviors of cancer cells [29, 31, 32]. Finally, miR-372-3p as an upstream modulatory gene of PRDM16 was chosen for further study. Afterwards, expression analysis was conducted on miR-372-3p in downloaded data, and miR-372-3p level was conspicuously high in prostate cancer (Figure 3(c)). qRT-PCR assay also verified its marked high expression in prostate cancer cell lines (Figure 3(d)).

To validate binding of miR-372-3p and PRDM16, binding sites of PRDM16 3′UTR and miR-372-3p were predicted (Figure 3(e)). PC-3 cells were transfected with miR-372-3p mimics and NC mimics, separately. Dual-luciferase assay authenticated that miR-372-3p mimics remarkably reduced luciferase activity of cells expressing PRDM16 WT but had no influence on cells expressing PRDM16 MUT (Figure 3(f)). To further certify whether miR-372-3p targets PRDM16, PC-3 cells expressing miR-372-3p mimics were generated. qRT-PCR and western blot disclosed the decrease of PRDM16 levels by overexpressing miR-372-3p (Figures 3(g) and 3(h)). Hence, increased miR-372-3p targeted PRDM16 in prostate cancer.

3.4. miR-372-3p Facilitates Malignant Progression of Prostate Cancer Cells via PRDM16 Repression. As discussed above, miR-372-3p targeted PRDM16, and thus, we planned to overexpress miR-372-3p and PRDM16 in PC-3 and DU145 cells to testify influence of their modulatory mode on cell function.

In the first place, qRT-PCR and western blot were conducted to assay PRDM16 levels in various transfection groups. In comparison to the NC mimic+oe-PRDM16 group, PRDM16 mRNA and protein expressions dramatically downregulated with overexpressing miR-372-3p (Figures 4(a) and 4(b)). CCK8 verified that oe-PRDM16 restrained cell viability, while this impact was weakened by simultaneous miR-372-3p and PRDM16 overexpression (Figure 4(c)). As such, oe-PRDM16 hampered cell migration and invasion in comparison to the control group, but transfecting miR-372-3p mimics and oe-PRDM16 together markedly rescued this repressive effect (Figures 4(d) and 4(e)). Hence, we believed that miR-372-3p facilitated malignant progression of prostate cancer cells via PRDM16 repression.

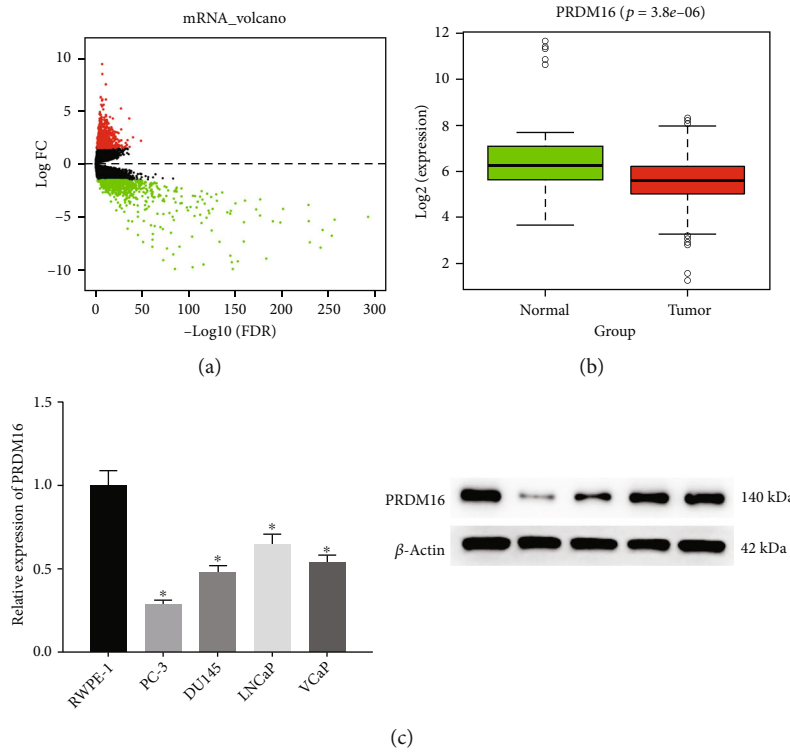

FIGURE 1: PRDM16 is lowly expressed in prostate cancer. (a) Volcano map of DEmRNAs in the normal and tumor groups in prostate cancer dataset. (b) Box plot of PRDM16 expression. (c) Compared with normal prostate cells (RWPE-1), PRDM16 mRNA and protein expressions decreased in prostate cancer cells (PC-3, DU145, and LNCaP); $^{*}p < 0.05$.

4. Discussion

Prostate cancer has a high fatality rate globally and is the most prevalent among male malignant tumors, which is increasingly the focus of basic and clinical researchers' attention [33]. In Asia, prostate cancer has become a serious medical, social, and economic issue, which is in complete accord with western countries [34]. Hence, there is increasing international concern about developing novel targets for prostate cancer and literature that recognizes the pivotal role of mRNAs as key modulators in incidence and progression of various malignancies [35].

PRDM16 is a methylation-associated gene. Hypomethylation of PRDM16 promoter is associated with dismal outcomes in patients with astrocytoma [36]. Through bioinformatics analysis, PRDM16 was found to be decreased in prostate cancer. PRDM16 is also a vital regulator of lipometabolism [37] and a potential diagnostic target in cancer [12]. As reported, PRDM16 exerts its effect through varying forms. For instance, a short-form PRDM16 is capable of fostering cell growth to be involved in progression of several malignancies [38]. Yoshida et al. [39] manifested that PRDM16 lacking a PR domain hampers cell growth mediated by TGFβ in adult T-cell leukemia cells. On the contrary, Takahata et al. [40] ascertained that total length PRDM16 interacts with SKI (TGFβ signaling suppressor), thereby preventing cell growth induced by TGFβ. Moreover, Fei et al. [41] disclosed that enforced expression of total length PRDM16 or PRDM16 lacking a PR domain can both repress transcription of MUC4 and restrain lung adenocarcinoma cell invasion and migration. Zhu et al. [42] also exhibited that PRDM16 is reduced in prostate cancer and affects cancer cell viability, which may represent a novel therapeutic target. As previously investigated, we found the decreased PRDM16 in prostate cancer and conducted cellular functional assays to reveal the repressive impact of PRDM16 overexpression on cell phenotype progression of prostate cancer. To sum up, PRDM16 exerts an important effect on tumor occurrence and progression, and different forms of PRDM16 function variously in multiple cancers. This conclusion provides a novel research direction for PRDM16.

Mature miRNAs are single-stranded RNA molecules about 22 nucleotides in length that regulate a wide range of biological functions from cell proliferation and death to cancer development and progression [43]. miRNAs can function as novel tumor oncogenes or tumor suppressor genes [44, 45]. miR-372-3p is a pivotal biomarker in liver cancer, and it cooperates with Rab11a to serve as controller in progression of liver cancer [46]. It is reported that the mature miRNA strand binds to the 3′UTR of its target genes [47]. We found that miR-372-3p and PRDM16 had binding sites, and experiments illustrated that miR-372-3p downregulates PRDM16. It theorized the abnormal expression of miR-372-3p in multiplex tumors as a tumor repressor or oncogene [31, 48, 49].

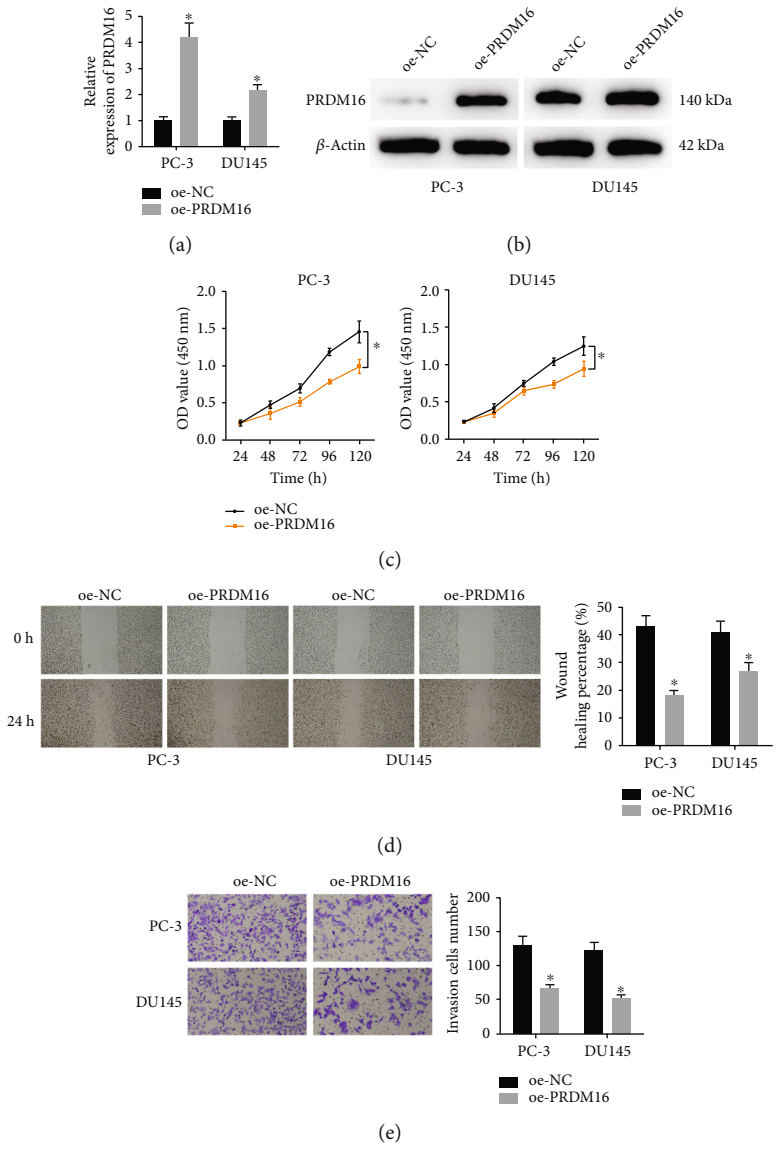

Figure 2: oe-PRDM16 restrains malignant progression of prostate cancer cells. (a) PRDM16 mRNA expression in the oe-NC and oe-PRDM16 groups of cells. (b) PRDM16 protein expression in each group of cells. (c) CCK8 assayed cell viability. (d) Wound healing assay assayed cell migratory ability (40x). (e) Transwell assayed cell invasive capability (100x); $^*p < 0.05$.

Yamashita et al. [50] unearthed that miR-372-3p upregulation is implicated in colorectal cancer patients' prognoses. miR-372 aberrant expression targets CDK2 Cyclin A1 to hinder cell growth of cervical cancer [51]. Besides, Cho et al. [52] unveiled that decreased miR-372-3p modulates LATS2 to restrain cell proliferation of gastric cancer. miR-372 may be an oncogenic miRNA and an independent biomarker of glioma [53]. In our research, rescue experiments denoted that miR-372-3p overexpression restored repressive effect of PRDM16 on malignant progression of prostate cancer, which suggested that it may act as an oncogene in prostate cancer. However, Kong et al.'s study reported that miR-372 may function as a tumor suppressor gene by regulating p65 in prostate cancer [54]. These findings confirmed the fact that miRNA plays both tumor-promoting and tumor-suppressing roles in the same cancer type, and the reason is that miRNAs in the same tumor are stimulated by external stimuli or have different levels of certain cytokines [55, 56]. Together, these findings unraveled that miR-372-3p hindered PRDM16 to facilitate cell aggressive phenotype of prostate cancer, thus becoming a controller of prostate cancer progression.

Our study confirmed the vital role of miR-372-3p/PRDM16 on prostate cancer progression. However, the upstream processes of miR-372-3p involving lncRNAs or circRNAs and the downstream processes of PRDM16 involving key signaling pathways have not been elucidated and need to be further investigated. In addition, the effect of the interaction between miR-372-3p and PRDM16 needs

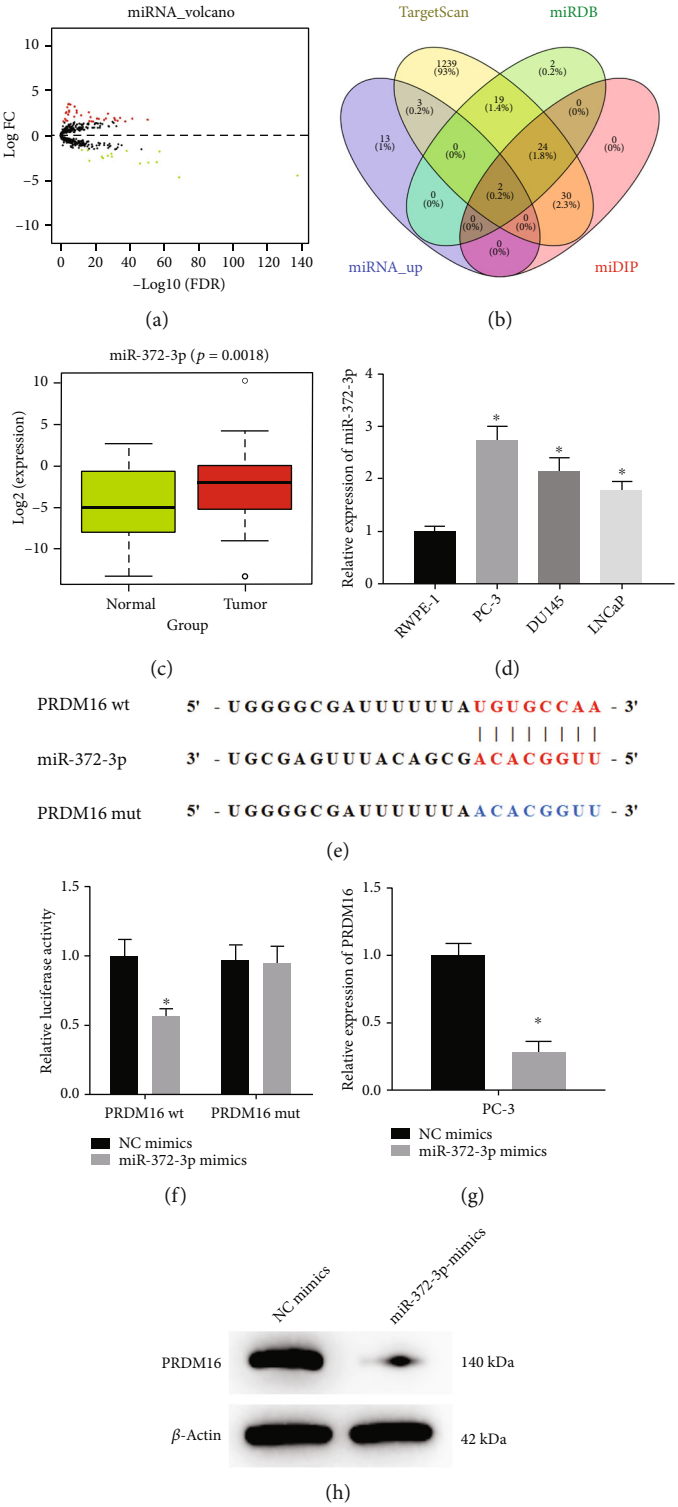

FIGURE 3: Increased miR-372-3p downregulates PRDM16 in prostate cancer. (a) Volcano map of DEmiRNAs in the normal and tumor groups in prostate cancer dataset. (b) The Venn diagram of upstream modulatory miRNAs of PRDM16 and upregulated DEmiRNAs. (c) Box plot of miR-372-3p expression. (d) In comparison to normal prostate cells (RWPE-1), miR-372-3p expression in PC-3, DU145, and LNCaP cell lines increased. (e) Binding sites of miR-372-3p and PRDM16. (f) Verification of binding of miR-372-3p and PRDM16 via dual-luciferase assay. (g, h) miR-372-3p overexpression downregulates PRDM16 mRNA and protein in PC-3 cells; $^{*}p < 0.05$.

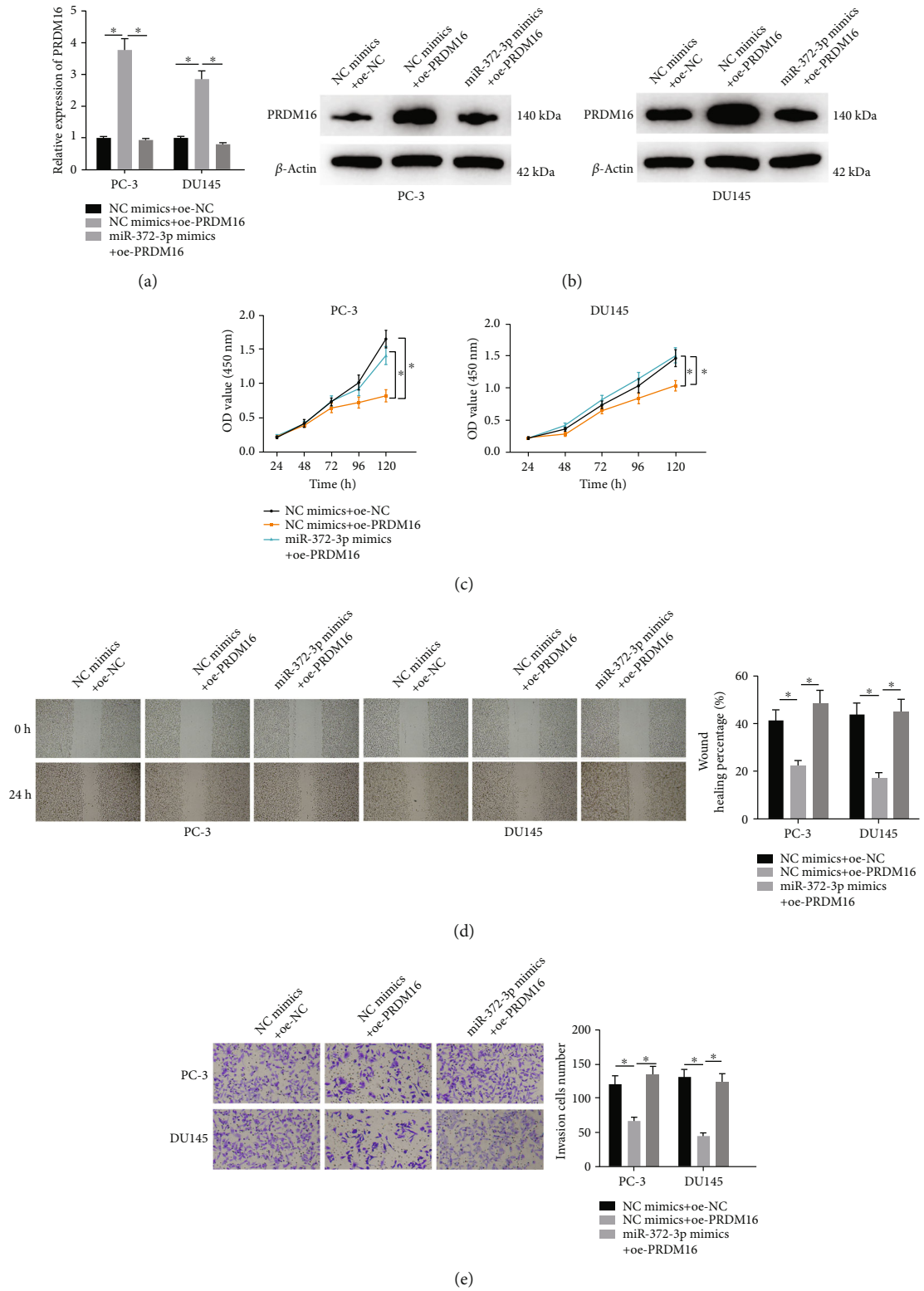

FIGURE 4: miR-372-3p facilitates malignant progression of prostate cancer cells via PRDM16 repression. (a) qRT-PCR assayed PRDM16 mRNA expression in the NC mimic+oe-NC, NC mimic+oe-PRDM16, and miR-372-3p mimic+oe-PRDM16 groups of PC-3 and DU145 cells. (b) Western blot assessed PRDM16 protein level. (c) CCK8 detected cell viability. (d) Wound healing assay assayed cell migratory capability (40x). (e) Invasion assay detected cell invasive capability (100x); $^{*}p < 0.05$.

to be confirmed *in vivo*. In conclusion, we demonstrated that miR-372-3p regulated prostate cancer progression *in vitro* by targeting PRDM16. *In vitro* experiments certificated that in prostate cancer, miR-372-3p enhanced cell aggressive phenotype through modulation of PRDM16.

Abbreviations

PCa: Prostate cancer
miRNAs: MicroRNAs
PRDM16: Positive regulatory domain containing 16
DEmiRNAs: Differentially expressed miRNAs
qRT-PCR: Quantitative real-time polymerase chain reaction
3′UTR: 3′-untranslated region
WT: Wild type
FBS: Fetal bovine serum
CCK-8: Cell Counting Kit-8
MUT: Mutant
TCGA: The Cancer Genome Atlas
mRNA: Messenger RNA
NC: Negative control.

Data Availability

All data supporting the conclusions of the study are included in this article. The materials and data in the current study are available from the authors upon reasonable request.

Consent

All authors agree to submit the manuscript for publication.

Conflicts of Interest

There are no potential conflicts of interest.

Authors' Contributions

GW and CQ completed the study design. BM carried out the literature search. CY obtained the data. PF contributed in writing the article. CF conducted the data analysis. SY drafted the manuscript. JH, LL, and AL revised the article and gave the final approval of the version to be submitted. All authors read and approved the final manuscript. Guangwei Yin and Chengquan Yan contributed equally to this work.

Supplementary Materials

Supplementary Figure 1: differentially expressed miRNAs between normal group and tumor group in prostate cancer dataset. *(Supplementary Materials)*

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