

# MicroRNA-18b-5p Inhibits the Malignant Progression of Prostate Cancer Through Downregulating TRAF5

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**Objective:** Extensive efforts have been made for translating the mechanisms of microRNAs (miRNAs) in prostate cancer (PCa). However, the specific role of miR-18b-5p in PCa is still in obscurity. Herein, miR-18b-5p/TRAF5 axis-oriented exploration in PCa has been launched.

**Methods:** miR-18b-5p and TRAF5 expression in PCa tissues and cells was detected by RT-qPCR. *In-vitro* experiments were conducted to investigate the biological functions of miR-18b-5p and TRAF5 in PCa cells. The underlying mechanism of miR-18b-5p was revealed by luciferase reporter assay, miRNA Pull down, RT-qPCR, and rescue assay.

**Results:** Lower miR-18b-5p and higher TRAF5 expression were observed in PCa tissues and cell lines. miR-18b-5p overexpression or TRAF5 downregulation impaired proliferation, diminished migratory and invasive properties, as well as advanced apoptosis in PCa cells. miR-18b-5p could regulate TRAF5 expression by directly binding to its 3'-untranslated region. Overexpression of TRAF5 abolished the suppressive effects of restored miR-18b-5p on PCa cell progression.

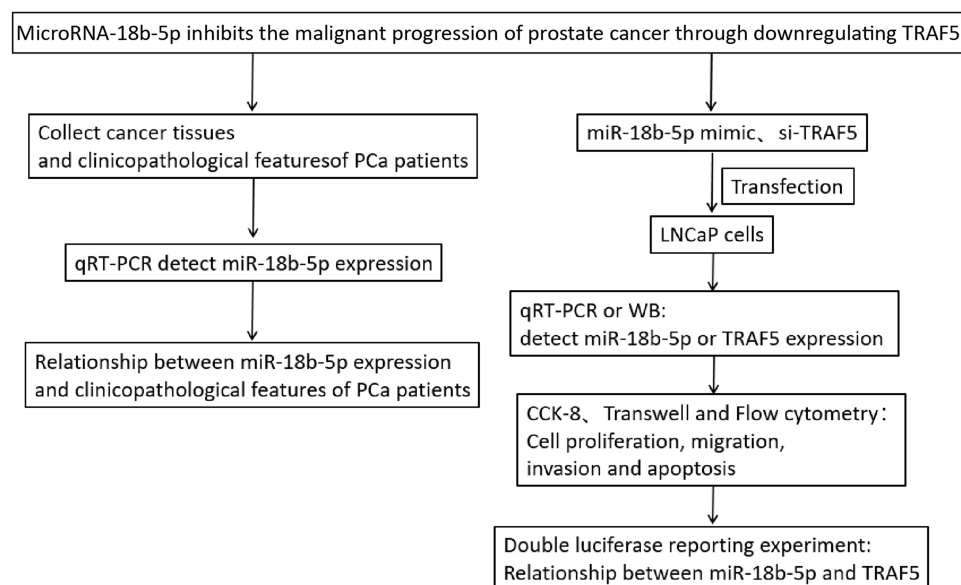
**Conclusion:** This study elucidates that upregulated miR-18b-5p impedes PCa cell progression via downregulating TRAF5, which may provide a novel therapeutic basis for PCa.

**Keywords:** prostate cancer, microRNA-18b-5p, TRAF5, proliferation, invasion, migration, apoptosis

## Introduction

Prostate cancer (PCa) ranks one of the most encountered malignancy in male.<sup>1</sup> Pathologically, several risk factors are devoted to the initiation of PCa, including aging, family history and ethnicity.<sup>2</sup> Radical prostatectomy (RP) is an eligible therapeutic choice in the light of local PCa.<sup>3</sup> Statistically, though received RP, the average life expectancy for patients with PCa is assumed to 22 years.<sup>4</sup> In 2021, approximately 248,530 cases of prostate cancer are expected to be diagnosed in the USA, with an estimated 34,130 deaths.<sup>5</sup> Unexpectedly, in addition to having a reduced life expectancy, PCa patients are also prone to clinical and biochemical recurrence.<sup>6</sup> Besides, PCa is distinctly heterogeneous, which can be subdivided into multiple intermediate clinical states, and each of which could benefit from different therapeutic modalities.<sup>7</sup> A biopsy-confirmed Gleason score is crucial in determining the treatment approach for PCa, whether surgical (radical prostatectomy) or non-surgical (active surveillance, watchful waiting, radiation therapy, and hormone therapy).<sup>8</sup> Bladder EpiCheck is one of several urinary tests studied to identify bladder tumors,<sup>9</sup> and several blood or serum-based biomarkers are used for diagnosing PCa, including those approved by the US FDA (such as PSA, fPSA, and Prostate Health Index) and biomarkers approved under the Clinical Laboratory Improvement Amendments (CLIA), such as the 4Kscore.<sup>5</sup> Focal treatment is a viable and minimally invasive option for treating localized PCa, as it carries a lower

## Graphical Abstract



risk of adverse outcomes compared to a radical approach.<sup>10</sup> Given that, the requirement for novel curative choices for PCa is in urgency.

Many different genetic alterations and complicated epigenetic interactions are the basis of the occurrence and progression of PCa.<sup>11</sup> microRNAs (miRNAs) are short, non-coding, conserved, oligonucleotides that are regulatory in nature and are frequently dysregulated in diverse cancers including PCa.<sup>12,13</sup> Some articles have demonstrated that circulating miRNAs have enormous potential as noninvasive diagnostic and prognostic markers for PCa.<sup>14,15</sup> Advancements in molecular biology and experimental techniques for analyzing miRNAs have enabled more detailed studies of the pathological mechanisms underlying septic acute kidney injury.<sup>16</sup> Meanwhile, therapeutic genome editing using CRISPR/Cas-miRNA targeting is advancing from early-stage research to preclinical development.<sup>17</sup> Many miRNAs, including miR-18b-5p, has been revealed to discriminate PCa patients from those with benign prostatic hyperplasia (BPH).<sup>18</sup> In the meantime, transfection with has-miR-18b-5p inhibitor has been revealed to attenuate the impact of tumor-associated fibroblasts for promoting proliferation and migration of PCa cells.<sup>19</sup> Besides, several promising candidate biomarkers, including miR-18b-5p, have been identified to improve treatment strategies in PCa.<sup>20</sup> It has been revealed that miR-18b-5p is downregulated in follicular fluid and ovarian granulosa cells of polycystic ovary syndrome (PCOS) women.<sup>21</sup> Xue et al have found that transfection of miR-18b-5p mimic inhibits the growth and aggressive phenotypes of ovarian cancer cells.<sup>22</sup> These findings demonstrate that miR-18b-5p could lead to advancements in diagnostics, therapeutics, or prognostics. Nevertheless, the concrete mechanism of miR-18b-5p in PCa remains to be elucidated. miRNAs function by binding to the 3' untranslated regions (3'UTRs) of mRNAs, allowing the detection of miRNA-mRNA interactions in vitro using a dual-luciferase assay system with co-transfection of miRNA and a luciferase reporter containing the target mRNA fragment into mammalian cells.<sup>23,24</sup> In this work, TRAF5 was demonstrated to be a target gene of miR-18b-5p via online databases DIANA-microT, miRDB, and ENCORI. Tumor necrosis factor receptor-associated factors (TRAFs) are play a crucial role in mediating cellular receptor signaling transduction to downstream pathways and play multifaceted roles in modulating cell survival and carcinogenesis.<sup>25</sup> As previously reported, TRAF1/5/6 control the pathogenesis of chronic inflammatory diseases, and particularly, TRAF5 deficiency enhances lipid uptake and foam cell formation in macrophages.<sup>26</sup> Peng et al have supported that miR-218-5p impedes the invasion and migration capabilities of PCa cells via simultaneously targeting TRAF1/2/5.<sup>27</sup> Additionally,

Huang et al have stated that miR-141-3p inactivates NF- $\kappa$ B signaling through targeting TRAF5 and TRAF6, thereby restraining bone metastasis of PCa.<sup>28</sup> These studies confirmed the specific contribution of TRAF5 to PCa progression. On the whole, the multilateral interplay among miR-18b-5p and TRAF5 in PCa has not been fully explored. Therein, this study was performed to explore the mechanism by which miR-18b-5p targets and suppresses TRAF5 expression to affect the biological functions of PCa cells.

## Materials and Methods

### Ethics Statement

The clinical data used were approved by the ethics committee of The People Hospital of Jilin Province (approval number: 2015019). All participants supplied written informed consent. This study complied with the Helsinki Declaration.

### Specimens

Specimens were collected from patients who received surgical resection in The People Hospital of Jilin Province, including 59 cases of PCa and 20 cases with BPH. The inclusion criteria for PCa patients were: 1) No prior treatment before surgery; 2) Pathologically confirmed prostate cancer; Exclusion criteria were: exclusion of recurrent tumors, metastatic tumors, patients with other malignancies, and those with severe underlying diseases. The inclusion criteria for BPH patients were: 1) Diagnosis of BPH according to the diagnostic criteria;<sup>29</sup> 2) Requirement for surgical resection treatment; Exclusion criteria included: patients with hypertension, diabetes, or other underlying diseases, patients with prostate cancer or other malignancies, and those with incomplete clinical data. The obtained tissue samples were immediately stored in liquid nitrogen. The relatively complete clinicopathological data were compiled, and each patient was listed according to their age, gender, preoperative prostate-specific antigen (PSA) level, Gleason grade, presence or absence of distant metastases, and clinical stage.

### Bioinformatics Analysis

Using online analysis tools ENCORI (<https://rnasysu.com/encori/>, last update: 10/18/2024),<sup>30</sup> miRDB 6.0 (<https://mirdb.org>) (last update: 6/2019),<sup>31</sup> and DIANA microT\_CDS v5.0 ([http://dianalab.e-ce.uth.gr/html/dianauniverse/index.php?r=microT\\_CDS](http://dianalab.e-ce.uth.gr/html/dianauniverse/index.php?r=microT_CDS), last update: 1/27/2012),<sup>32</sup> the input parameter “has-miR-18b-5p” was used to obtain downstream regulatory genes of miR-18b-5p, and a Venn diagram of target genes was drawn to identify the intersecting genes, among which TRAF5 was included. The downstream regulatory genes of miR-18b-5p from each database are listed in [Supplementary materials](#).

### Cell Culture and Transfection

Human PCa cell lines LnCaP, Vcap, DU145, 22Rv1 and human normal prostate epithelial cells RWPE1 were supplied by American Type Culture Collection (VA, USA). All the cells were incubated in a 37°C incubator containing 5% CO<sub>2</sub>. DU145, 22RV1, VCaP, and LnCaP cells were cultured using RPMI-1640 medium (Gibco, Carlsbad, CA, USA), and RWPE-1 cells were grown in Keratinocyte serum-free medium (Gibco) supplemented with epidermal growth factor and bovine pituitary extract (KGM). All media contain 10% FBS (Gibco). The miR-18b-5p mimic, small interfering RNA (siRNA) specific for TRAF5 and TRAF5 expressing vector, and the corresponding negative controls were obtained from GenePharma (Shanghai, China). Cell transfection was conducted with reference to Lipofectamine 2000 reagent (11668–019, Invitrogen Inc., Carlsbad, CA, USA) following the manufacturer’s protocol. Cell functional experiments were performed at 48 h after the transfection.<sup>33</sup>

### RT-qPCR

Total RNA extraction from 100 mg tissues and cells (50–80 × 10<sup>5</sup>/per sample) was implemented via Trizol kit (Invitrogen). miRNA detection was performed according to Mir-X miR First-Strand Synthesis kit (Takara, Dalian, China) and SYBR Premix Ex Taq II (Takara), while mRNA was performed with PrimeScript RT reagent Kit (Takara) and SYBR Premix Ex Taq II (Takara), and 1 µg of total RNA was used for reverse transcription. CFX96 real-time PCR detection system (Bio-Rad Laboratories, Hercules, CA, United States) was utilized for RT-qPCR. Data evaluation was

conducted by the  $2^{-\Delta\Delta Ct}$  method with U6 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as internal controls.<sup>34</sup> The primer sequences for genes are listed in Table 1.

### Western Blot

Proteins were extracted using RIPA lysis buffer. Protein concentration was measured using the BCA protein assay kit (Beyotime, Shanghai, China). Thirty micrograms of protein from each sample were loaded and electrophoresed using polyacrylamide gel electrophoresis. Proteins were transferred to PVDF membranes (Millipore, MA, USA) by wet transfer. After blocking the membranes with 3% bovine serum albumin (BSA) at room temperature for 60 min, the membranes were incubated overnight at 4°C with the primary antibody, rabbit anti-TRAF5 (ab303522; 1:1000, Abcam, Cambridge, MA, UK). The membranes were then incubated with HRP-conjugated goat anti-rabbit IgG antibody (ab97051, 1:2000, Abcam) at room temperature for 2 h. Detection was performed using a digital chemiluminescence system (C-DiGit<sup>®</sup> Blot Scanner, Li-Cor, NE, USA), and analysis was carried out using Image J (USA). GAPDH (ab9485, 1:2500, Abcam) was used as the internal reference. The relative expression of the target protein was determined by the ratio of the gray value of the target band to the gray value of the internal reference band. Each experiment was repeated three times.

### CCK-8 Assay

The transfected cells were seeded in a 96-well culture plate at a density of  $1 \times 10^3$  cells per well. At three time points (24, 48, and 72 h), 10  $\mu$ L of CCK8 solution (Dojindo, Kumamoto, Japan) was added to each well, and the plates were incubated at 37°C for 2 h. The absorbance at 490 nm (OD value) was measured using a spectrophotometer (BioTek Instruments, Winooski, VT, USA). The experiment was repeated three times, and a growth curve was plotted.<sup>35</sup>

### Transwell Assay

Cell migration and invasion were assessed using the Transwell<sup>®</sup> assay (with or without Matrigel<sup>®</sup>). Cell migration assay: Groups of transfected cells (200  $\mu$ L,  $5 \times 10^4$  cells/mL) were inoculated in the upper chamber of the transfected wells. After 24 h of incubation, cells migrating to the lower chamber were stained with Giemsa. Cell invasion assay: Matrigel<sup>®</sup>-precoated Transwell<sup>®</sup> chamber (Corning) was used, and groups of transfected cells (200  $\mu$ L,  $5 \times 10^4$  cells/mL) were inoculated in the upper chamber of transwell coated with matrigel. After 24 h of incubation, cells retained in the lower half were stained with Giemsa. Images were obtained using a DMI4000B microscope (Leica, Wetzlar, Germany) and 5 fields of view were randomly selected for cell counting. The relative cell motility activity was calculated using the following formula: relative cell motility activity = number of migrated or invaded cells from different treatment groups  $\div$  number of migrated or invaded cells from the control group. Three replicate wells were set up for each group, and each experiment was repeated three times.<sup>36,37</sup>

**Table 1** Primer Sequences for Genes

Gene	Primer Sequence (5'-3')
miR-18b-5p	Forward: 5'-CCGAGtaagggtcatctagtgcagttag-3' Reverse: 5'-CTCAACTGGTGTCTGTGA-3'
TRAF5	Forward: 5'-CTGTGCTGTAAACGGATAAACGG-3' Reverse: 5'-TAGCTGCTGGATTTTACTTTCTTTC-3'
U6	Forward: 5'-GCTTCGGCAGCACATATACTAAAAT-3' Reverse: 5'-CGCTTCACGAATTTGCGTGTTCAT-3'
GAPDH	Forward: 5'-GGAGCGAGATCCCTCCAAAT-3' Reverse: 5'-GGCTGTTGTCATACTTCTCATGG-3'

**Abbreviations:** miR-18b-5p, microRNA-18b-5p; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

## Flow Cytometry

Cell apoptosis was estimated annexin V-fluorescein isothiocyanate (FITC)/PI double staining. In short, the harvested cells were rinsed and then resuspended in 200  $\mu$ L of binding buffer. Subsequently, the cell suspension was reacted for 15 min with the 10  $\mu$ L of annexin V-FITC (ab14085, Abcam, Cambridge, UK) and 5  $\mu$ L of PI at room temperature void of light. The FITC and PI signals were tested using a flow cytometer at an excitation wavelength of 488 nm.<sup>38</sup>

## Dual Luciferase Reporter Gene Assay

TRAF5-3'UTR fragments containing the miR-18b-5p binding site were cloned into pGL3 plasmids to create luciferase reporter vectors. Additionally, the TRAF5-3'UTR-MUT fragments, which had mutated miR-18b-5p binding sites, were inserted into the pGL3 vector. Using liposome-based transfection, the recombinant plasmids (pGL3-TRAF5-3'UTR, pGL3-TRAF5-3'UTR-MUT) and the Renilla reference plasmid were co-transfected with either miR-18b-5p mimic or NC-mimic into HEK293T cells. Forty-eight hours after transfection, luciferase activity was measured using a luciferase reporter assay system (Promega, Madison, WI, USA) and a luciferase assay kit (K801-200, BioVision Technologies, San Francisco, CA, USA). The relative luciferase activity was calculated by normalizing the firefly luciferase activity to the Renilla luciferase activity, with Renilla luciferase serving as the internal control.<sup>38</sup>

## Biotinylated miRNA Pulldown Assay

Biotin-labeled miR-18b-5p and miR-NC probes (Genepharma, 100 nM) were transfected into LNCaP cells. The experiment was conducted following the miRNA pulldown kit (BersinBio, Guangzhou, China) guidelines. In short, cells transfected for 48 h were harvested and lysed. Next, the lysate was mixed with streptavidin magnetic beads and incubated overnight at 4°C with rotation. Lastly, the eluted RNA was purified and utilized for RT-qPCR analysis to ascertain the relative enrichment levels of TRAF5.

## Statistical Analysis

All data were analyzed by SPSS 26.0 statistical software (SPSS, Chicago, IL, USA) and GraphPad Prism 9 (GraphPad Software, San Diego, CA, USA). Measurement data were depicted as mean  $\pm$  standard deviation. The normality of the data was tested using the Shapiro–Wilk test. Comparisons between two groups of measurement data obeying normal distribution were performed by the unpaired independent samples *t*-test. One-way analysis of variance (ANOVA) was indicative of comparisons among multiple groups, after which Tukey's multiple comparisons test for pairwise comparisons. Categorical data expressed as percentage were analyzed by chi-squared test.  $P < 0.05$  was regarded with statistical significance.

## Results

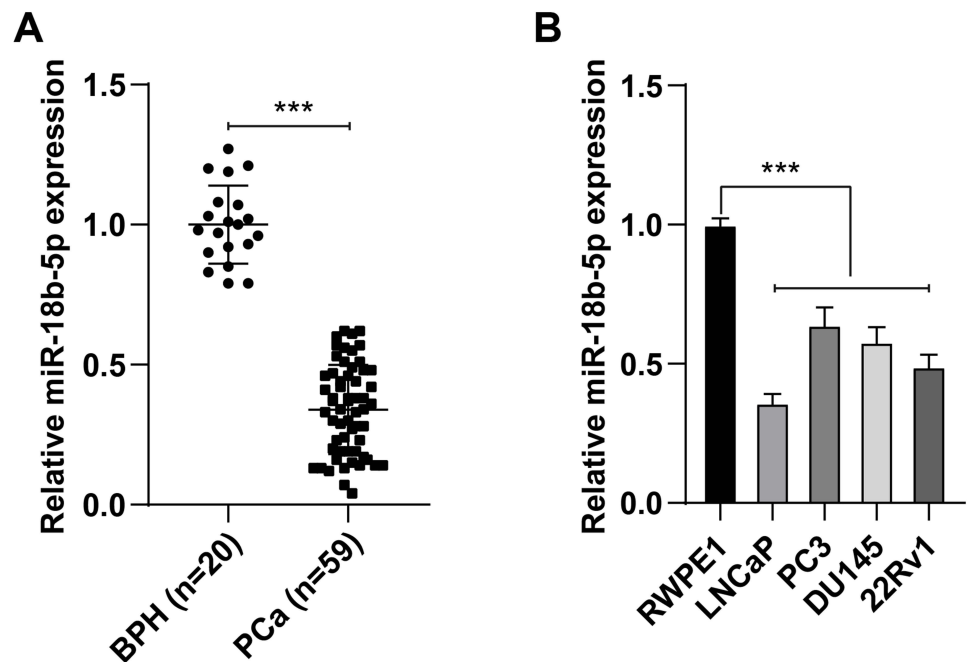
### miR-18b-5p is Lowly Expressed in PCa Tissues and Cells

As previously reported, miR-18b-5p show high sensitivity and specificity for predicting PCa, respectively,<sup>18</sup> but their specific mechanisms are unknown. In this work, to further unravel miR-18b-5p expression in PCa, RT-qPCR was implemented to test miR-18b-5p expression in 59 cases of PCa and 20 cases of BPH tissues, and the results disclosed that miR-18b-5p expression was significantly downregulated in PCa tissues compared with BPH tissues (Figure 1A,  $P < 0.05$ ).

miR-18b-5p expression in PCa cell lines (LNCaP, Vcap, DU145 and 22Rv1) and human normal prostate epithelial cells (RWPE1) was tested by RT-qPCR, which signified that lower expression levels of miR-18b-5p were observed in LNCaP, Vcap, DU145 and 22Rv1 cells compared with RWPE1 cells (Figure 1B,  $P < 0.05$ ), with the lowest miR-18b-5p expression in LNCaP cells, and therefore, the LNCaP cell line was selected for subsequent cell experiments.

### miR-18b-5p Expression Correlates with Gleason Score and Distant Metastasis in PCa Patients

Based on the median expression of miR-18b-5p (median value = 0.34), PCa patients were assigned into the miR-18b-5p low ( $n = 30$ ,  $\leq 0.34$ ) and high expression ( $n = 29$ ,  $> 0.34$ ) groups. It was outlined that miR-18b-5p low expression was



**Figure 1** miR-18b-5p is lowly expressed in PCa tissues and cells. **(A)** Expression of miR-18b-5p in 59 cases of prostate cancer and 20 cases of benign prostatic hyperplasia tissues was tested by RT-qPCR. **(B)** Expression of miR-18b-5p in RWPE1, LNCaP, Vcap, DU145 and 22Rv1 cells was examined by RT-qPCR. All experiments were repeated three times. Comparisons between two groups were performed using the unpaired independent samples t-test, and comparisons among multiple groups were conducted using one-way ANOVA followed by post hoc analysis with Tukey's test. \*\*\*  $P < 0.001$ .

significantly associated with higher Gleason score, advanced pT stage and distant metastasis, while had no relation with age and preoperative PSA (Table 2).

### Restored miR-18b-5p Restrains Cell Malignant Phenotypes in PCa Cells

Previous results have shown that miR-18b-5p is downregulated in PCa. To investigate the effect of miR-18b-5p on the malignant biological behaviors of PCa cells, miR-18b-5p mimic and mimic NC were transfected into LNCaP cells. After overexpression treatment of miR-18b-5p, miR-18b-5p expression was evaluated in LNCaP cells using RT-qPCR, and the findings suggested that miR-18b-5p expression was significantly elevated in LNCaP cells in response to miR-18b-5p mimic treatment (Figure 2A). CCK-8, Transwell, and flow cytometry assay were implemented to assess PCa cell activities, with the results highlighting that (Figure 2B–2E) impaired proliferation, diminished migratory and invasive

**Table 2** Association Between Clinicopathological Characteristics of PCa Patients and miR-18b-5p Expression

Clinicopathological Data	Case (n)	miR-18b-5p Expression		P
		Low Expression (n = 30)	High Expression (n = 29)	
Age (years)				
< 65	18	8	10	0.514
≥ 65	41	22	19	
Gleason grade				
< 7	13	2	11	0.011
7	17	9	8	
≥ 7	29	19	10	

(Continued)



**Table 2** (Continued).

Clinicopathological Data	Case (n)	miR-18b-5p Expression		P
		Low Expression (n = 30)	High Expression (n = 29)	
Tumor stage				
≤T2b	39	15	24	0.008
>T3a	20	15	5	
Preoperative PSA (ng/mL)				
≤ 10	12	5	7	0.476
> 10	47	25	22	
Distant metastasis				
Absence	41	15	26	0.001
Presence	18	15	3	

**Note:** The data in this table are enumeration data, using chi-square test.

**Abbreviation:** PSA, prostate specific antigen.

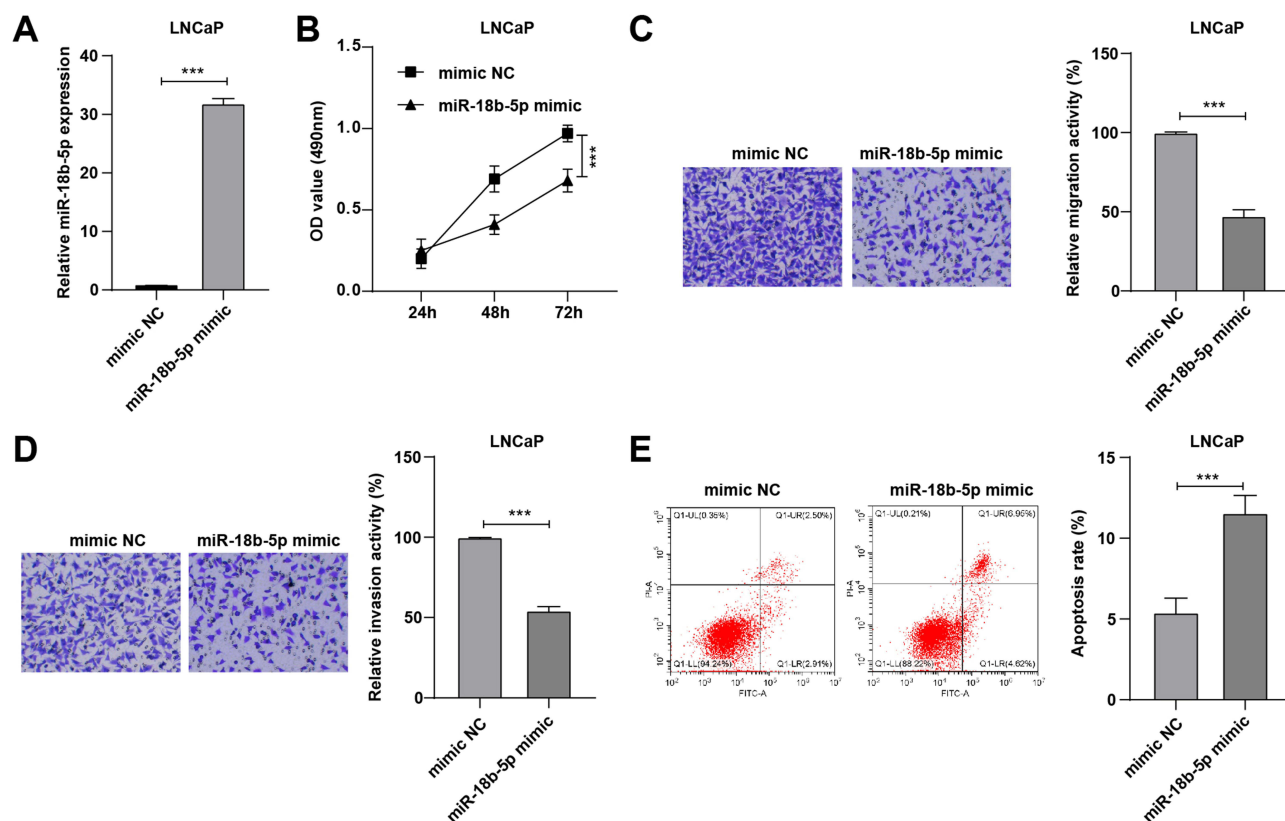
properties, as well as advanced apoptosis, were induced by overexpressed miR-18b-5p in LNCaP cells. It is suggested that overexpression of miR-18b-5p results in suppression of malignant behaviors of PCa cells.

### miR-18b-5p Targets TRAF5

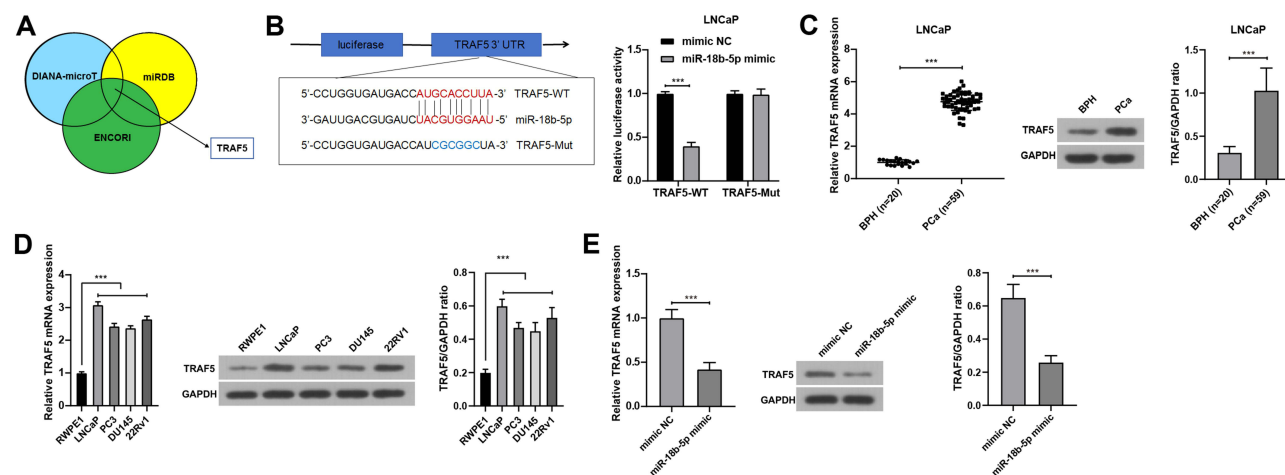
For miR-18b-5p target regulation of genes in PCa is not clear. Therefore, the online databases DIANA-microT, miRDB, and ENCORI were utilized to predict the mRNA targets regulated by miR-18b-5p. By taking the intersection of the results, TRAF5 was identified as one of the potential mRNA targets (Figure 3A). In combination with previous literature, it was found that TRAF5 may be involved in PCa development.<sup>27,28</sup> Following the outcomes of the dual luciferase reporter gene assay, it was exhibited that miR-18b-5p mimic significantly reduced the wild-type TRAF5 luciferase activity in LNCaP cells while having no significant effect on the mutant-type TRAF5 luciferase activity (Figure 3B), indicating that miR-18b-5p could specifically bind to TRAF5, and TRAF5 was a target gene of miR-18b-5p. Meanwhile, TRAF5 mRNA and protein expression levels were tested by RT-qPCR and Western blot in PCa tissues and PCa cell lines, and the results revealed that TRAF5 mRNA and protein expression levels were highly expressed in both PCa tissues and PCa cells (Figure 3C and 3D). Moreover, the results of RT-qPCR and Western blot experiments revealed that TRAF5 mRNA and protein expression levels were decreased after overexpression of miR-18b-5p (Figure 3E). The above results imply that miR-18b-5p targets to inhibit TRAF5 expression.

### Depleted TRAF5 Impedes Cell Malignant Phenotypes in PCa Cells

To further validate the impact of TRAF5 on the biological functions of PCa cells, after interference treatment of TRAF5, RT-qPCR and Western blot assays were adopted to evaluate the transfection efficiency. Compared to the si-NC group, the TRAF5 mRNA and protein expression levels were reduced in the si-TRAF5-1, si-TRAF5-2, and si-TRAF5-3 groups, with the lowest expression observed in the si-TRAF5-1 group, which was selected for subsequent experiments (Figure 4A). CCK-8, Transwell, and flow cytometry assay were implemented to assess PCa cell activities, with the results demonstrating that (Figure 4B–4E) impaired proliferation, diminished migratory and invasive properties, as well as advanced apoptosis, were induced by silenced TRAF5 in LNCaP cells. It is summarized that downregulation of TRAF5 leads to suppression of malignant behaviors of PCa cells.

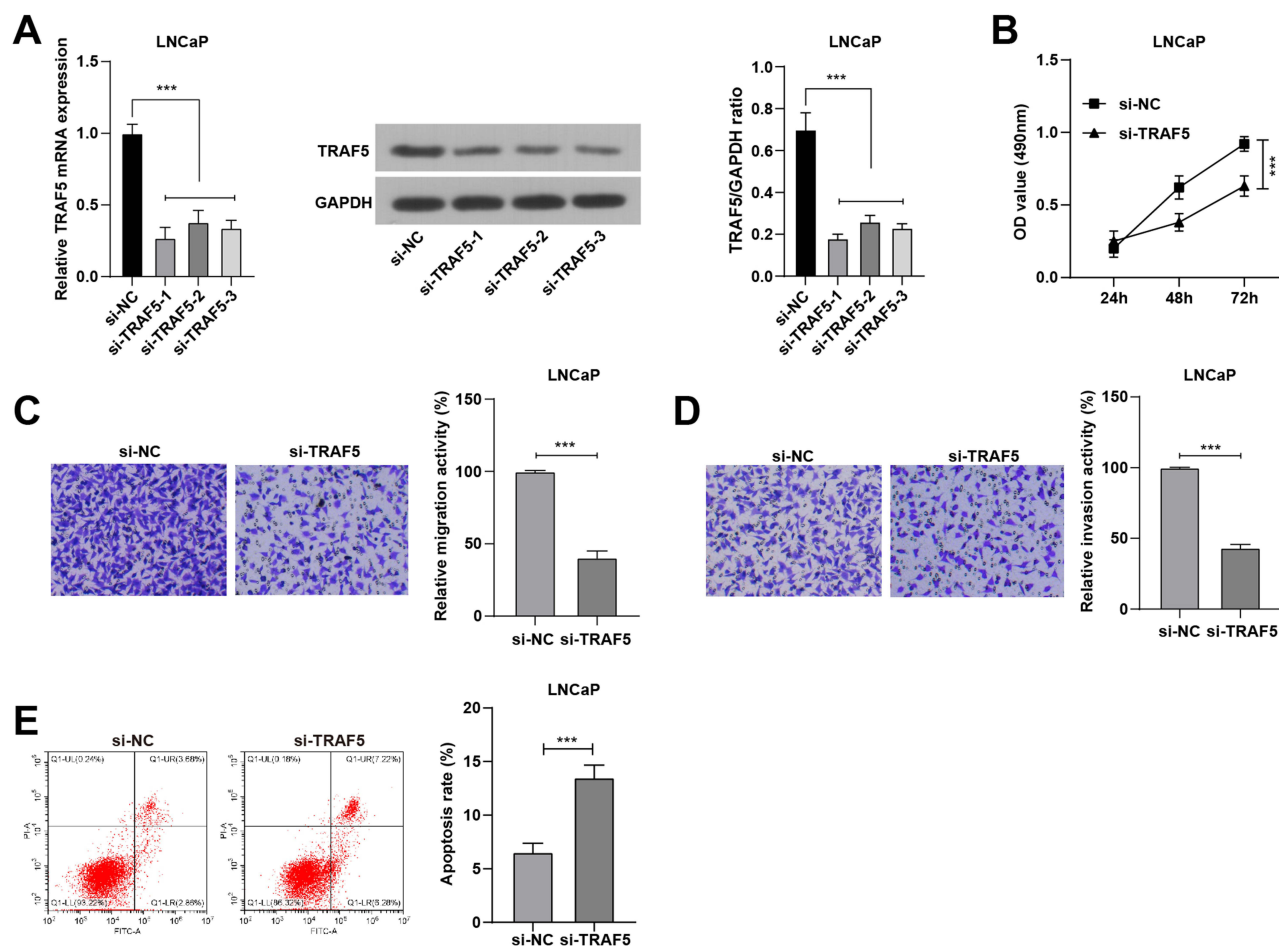


**Figure 2** Restored miR-18b-5p restrains cell proliferation, migration, invasion, and advances apoptosis in PCa cells. **(A)** Detection of miR-18b-5p overexpression transfection efficiency in LNCaP cells by RT-qPCR. **(B)** Cell proliferation of LNCaP cells was evaluated by CCK-8 assay. **(C and D)** Cell migratory and invasion potentials of LNCaP cells were estimated by Transwell assay. **(E)** Apoptosis rate of LNCaP cells assayed by flow cytometric annexin V-FITC/PI double staining, FITC, fluorescein isothiocyanate. All experiments were repeated three times. Comparisons between two groups were performed using the unpaired independent samples t-test. \*\*\*  $P < 0.001$ .



**Figure 3** MiR-18b-5p targets TRAF5. **(A)** DIANA-microT, miRDB and ENCORI databases predict miR-18b-5p targeting downstream mRNAs. **(B)** Luciferase reporter assay was performed to determine the effect of miR-18b-5p mimic on the luciferase activity of LNCaP cells transfected with TRAF5 wild type and TRAF5 mutant type. **(C and D)** TRAF5 expression was tested by RT-qPCR in 59 cases of prostate cancer and 20 cases of benign prostatic hyperplasia tissues, as well as RWPE1, LNCaP, Vcap, DU145 and 22Rv1 cells. **(E)** Detection of TRAF5 expression after overexpression of miR-18b-5p in LNCaP cells by RT-qPCR. All experiments were repeated three times. Comparisons between two groups were performed using the unpaired independent samples t-test, and comparisons among multiple groups were conducted using one-way ANOVA followed by post hoc analysis with Tukey's test. \*\*\*  $P < 0.001$ .





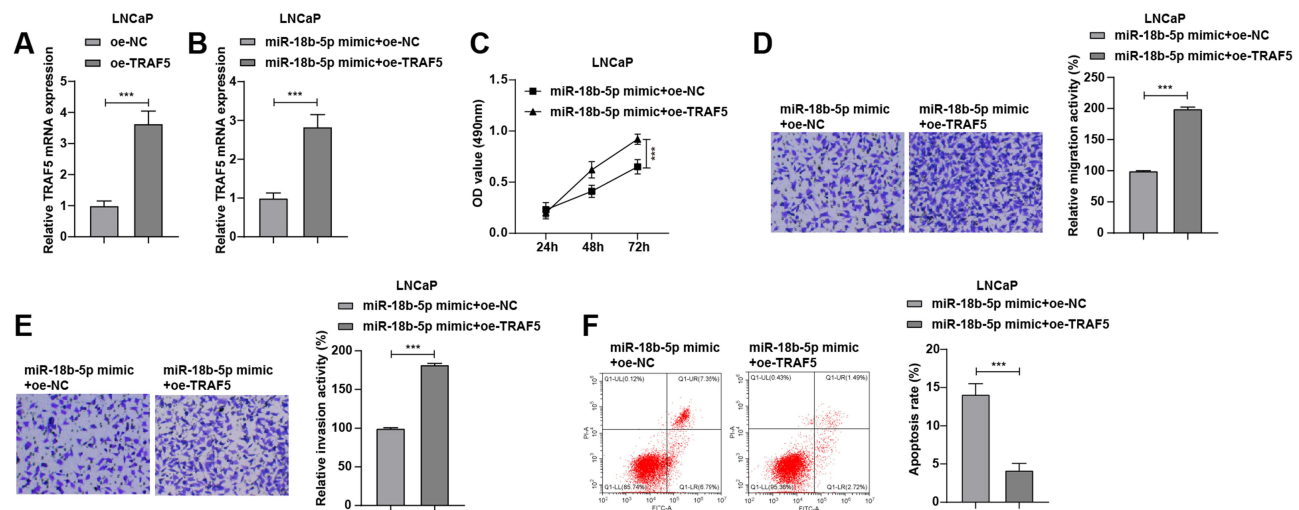
**Figure 4** Depleted TRAF5 impedes cell proliferation, migration, invasion, and advances apoptosis in PCa cells. **(A)** Detection of TRAF5 interference transfection efficiency in LNCaP cells by RT-qPCR. **(B)** Cell proliferation of LNCaP cells was evaluated by CCK-8 assay. **(C and D)** Cell migratory and invasion potentials of LNCaP cells were estimated by Transwell assay. **(E)** Apoptosis rate of LNCaP cells assayed by flow cytometric annexin V-FITC/PI double staining, FITC, fluorescein isothiocyanate. All experiments were repeated three times. Comparisons between two groups were performed using the unpaired independent samples *t*-test, and comparisons among multiple groups were conducted using one-way ANOVA followed by post hoc analysis with Tukey's test. \*\*\* *P* < 0.001.

## Overexpression of TRAF5 Abolishes the Suppressive Effects of Restored miR-18b-5p on PCa Cell Progression

To further probe whether miR-18b-5p impacted the biological function of PCa cells through TRAF5, LNCaP cells were divided into the miR-18b-5p mimic + oe-NC group and miR-18b-5p mimic + oe-TRAF5 group. A TRAF5 overexpression vector and its corresponding NC were constructed and transfected alone or co-transfected with miR-18b-5p mimic into LNCaP cells. RT-qPCR results showed that, compared to the oe-NC group, the TRAF5 mRNA expression level was significantly increased in the oe-TRAF5 group (Figure 5A). Moreover, compared to the miR-18b-5p mimic + oe-NC group, the miR-18b-5p mimic + oe-TRAF5 group also showed a significant increase in TRAF5 mRNA expression levels (Figure 5B). It was observed that TRAF5 overexpression attenuated the effects of miR-18b-5p overexpression in LNCaP cells (Figure 5C–5F).

## Discussion

PCa is a common tumor in men varying from indolent to aggressive presentations.<sup>39</sup> In despite of extensive studies focusing on miRNAs, the definite position of miR-18b-5p in PCa remains in obscurity. With this in mind, this study focused on miR-18b-5p, which was found to be downregulated in PCa. We hypothesized that it might play a crucial role in PCa and could be a potent therapeutic target for PCa if its mechanism in this disease is better understood.



**Figure 5** Overexpression of TRAF5 abolishes the suppressive effects of restored miR-18b-5p on PCa cell progression. **(A)** RT-qPCR detection of TRAF5 overexpression transfection efficiency in LNCaP cells. **(B)** RT-qPCR detection of TRAF5 mRNA expression levels in LNCaP cells after co-transfection of miR-18b-5p and TRAF5. **(C)** Cell proliferation of LNCaP cells was evaluated by CCK-8 assay. **(D and E)** Cell migratory and invasion potentials of LNCaP cells were estimated by Transwell assay. **(F)** Apoptosis rate of LNCaP cells assayed by flow cytometric annexin V-FITC/PI double staining. FITC, fluorescein isothiocyanate. All experiments were repeated three times. Comparisons between two groups were performed using the unpaired independent samples t-test. \*\*\* $P < 0.001$ .

Currently, early-stage disease can be detected by PCa screening protocols, and they lack sufficient sensitivity and specificity to contribute to unnecessary biopsies and overtreatment. Besides, there are currently no tools to forecast tumor progression, risk reclassification, as well as treatment response.<sup>40</sup> With the increasing demand for accurate biomarkers, miRNAs are promising markers for PCa screening, monitoring, prognosis, and response to treatment.<sup>41</sup> As previously reported, miR-253p and miR-18b-5p show high sensitivity and specificity for predicting PCa, respectively,<sup>18</sup> but their specific mechanisms are unknown. In this work, to further unravel miR-18b-5p expression in PCa, we tested miR-18b-5p expression in PCa tissues and cells, and the results disclosed that miR-18b-5p expression was significantly down-regulated in PCa tissues and cells. Subsequently, we also found that miR-18b-5p expression was significantly associated with Gleason score and distant metastasis in PCa patients, and impaired proliferation, diminished migratory and invasive properties, as well as advanced apoptosis, were induced by overexpressed miR-18b-5p in LNCaP cells. Similar to our findings, some articles have also demonstrated the roles of miR-18b-5p in other diseases. For example, Dai et al have supported that low expression of miR-18b-5p is found in pulpitis, and miR-18b-5p protects against lipopolysaccharide-induced cell injury via targeting HIF3A.<sup>42</sup> Another publication had unveiled that miR-18b-5p serves as a tumor repressor in breast cancer, and breast cancer cell proliferative, colony-forming, migratory, and invasive potentials are attenuated on transfecting miR-18b-5p mimic.<sup>43</sup> Nevertheless, the concrete capability of miR-18b-5p in PCa warrants following verification.

miR-18b-5p had been demonstrated to serve as a tumor suppressor in ovarian cancer and decrease downstream target mRNAs via binding with their 3'UTRs.<sup>22</sup> Our study showed that miR-18b-5p had a target binding site with TRAF5. In combination with previous literature, it was found that TRAF5 may be involved in PCa development.<sup>27</sup> Following the outcomes of the dual luciferase reporter gene assay, it was exhibited that miR-18b-5p could specifically bind to TRAF5, and TRAF5 was a target gene of miR-18b-5p. Meanwhile, TRAF5 expression was tested by RT-qPCR in PCa tissues and cell lines, and the results revealed that TRAF5 was highly expressed in both PCa tissues and cells. Meanwhile, down-regulated TRAF5 impaired proliferation, diminished migratory and invasive properties, as well as advanced apoptosis. Overexpression of TRAF5 abolished the suppressive effects of restored miR-18b-5p on PCa cell progression. Accordingly, Peng et al have supported that miR-218-5p impedes the invasion and migration capabilities of PCa cells via simultaneously targeting TRAF1/2/5.<sup>27</sup> Huang et al have found that miR-141-3p blocks the activation of the NF- $\kappa$ B signaling through directly targeting TRAF5/6, thereby weakening invasion, migration potency of PCa cells.<sup>28</sup> However,

little study has been conducted to indicate the interaction between miR-18b-5p and TRAF5, which needs in-depth exploration in the future.

As the global population continues to age in the coming decades, PCa in elderly men will pose a significant disease burden.<sup>44</sup> Currently, there is no optimal approach for managing PCa. In this study, we investigated the biological functions of miR-18b-5p and TRAF5 in PCa cells, and found that miR-18b-5p could downregulate TRAF5 to inhibit the malignant progression of PCa. From a clinical standpoint, early warning can be provided before facing the challenging issue of limited treatment options and poor prognosis for patients with PCa.

However, our study has limitations. We used only one cell line and one siRNA to verify the in vitro function, and a larger scale of researches with more cell lines and more siRNAs should be programmed for thorough comprehension of the miR-18b-5p/TRAF5 axis in PCa. Meanwhile, although TRAF5 is shown as a target, exploring the downstream signaling pathways affected by TRAF5 in PCa could add depth to the mechanistic understanding. Besides, further validation of the miR-18b-5p/TRAF5 axis in in vivo models or larger patient cohorts would bolster its translational relevance.

## Conclusion

To sum up, the specific mechanism of miR-18b-5p elevation or TRAF5 reduction encumbering PCa cell progression has been elucidated in this study, which imposes more possibilities on PCa-oriented therapy. The results of this experiment show that miR-18b-5p could downregulate TRAF5 to inhibit the malignant progression of PCa, which provides a new perspective on the mechanism of PCa formation. Additionally, this work underscores that miR-18b-5p might be a potential therapeutic target for PCa. Our findings demonstrate that miR-18b-5p could lead to advancements in diagnostics, therapeutics, or prognostics in PCa.

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## Disclosure

The authors declare that they have no conflicts of interest in this work.

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