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ORIGINAL ARTICLE

# Upregulation of miR-191 promotes cell growth and invasion via targeting TIMP3 in prostate cancer

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## Summary

***Purpose:*** *Prostate cancer (PCa) is the most frequently malignant neoplasm in men. MicroRNAs (miRs) have been identified to play important biological roles in a variety of tumors. Several studies showed that miR-191 was involved in the development of different cancers, but its role in prostate cancer remains unclear.*

***Methods:*** *Human PCa cell lines DU145, PC-3 and LNCAP, and benign prostate hyperplasia (BPH) and human prostate epithelial cell line RWPE-1 were used. The expression level of miR-191 in 48 paired prostate tumor and adjacent normal tissues was assessed along with the clinical patient features. Synthetic miR-191 mimics and inhibitors were used to overexpress or inhibit the miR-191 level. CCK8 and colony formation assay were used to evaluate the cell growth. The ability of cell invasion was studied by transwell assay. Dual-luciferase experiment was used to identify the target gene and western blot was performed to evaluate the protein level.*

***Results:*** *miR-191 was overexpressed in PCa tissue samples compared to the normal group as well in PCa-derived cell lines. Upregulation miR-191 in PC-3 cells significantly promoted while downregulation miR-191 in DU145 cells retarded the cell proliferation and invasion. Furthermore, TIMP3 were proved to be a direct target gene of miR-191 and knockdown of TIMP3 reversed the function of miR-191 downregulation.*

***Conclusion:*** *This study demonstrated that miR-191 promoted the cell growth and invasion ability in prostate cancer through downregulating TIMP3 and might be a potential target for the biotherapy for PCa.*

***Key words:*** *miR-191, prostate cancer, invasion, TIMP3*

## Introduction

PCa has taken the first place in incidence of all malignancies in men, and ranks second in mortality among all newly diagnosed cancer cases in developed countries [1]. Although the existing diagnostic and treatment program have greatly improved the survival rate of patients with PCa, the molecular biological mechanism related to the development and progression of PCa of this disease is still not very clear [2]. Several studies have demonstrated a series of oncogenes or tumor suppressor genes which influence the PCa process [3]. However, further studies are essential to detect the mechanism and provide targets for new therapies.

miRs are evolutionarily conserved, single-stranded non-coding RNAs which only ~22 nucleotides long [4]. They silence the expression of specific genes by pairing to the unique 3'-untranslated regions of target mRNAs on post-transcriptional level [5]. At present, several miRs have been proved to participate in the development and progression

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of PCa, such as miR-34a which inhibited PCa stem cells and metastasis by repressing CD44, miR-15a-miR-16-1 cluster that controlled multiple oncogenic activities of PCa by targeting several genes, and miR-424 that promoted PCa progression via impairing ubiquitination to activate STAT3 [6-8].

Among them, miR-191 was reported to be remarkable overexpressed in several cancers including intrahepatic cholangiocarcinoma, hepatocellular carcinoma, breast cancer, thyroid follicular carcinoma and colorectal cancer [9-13], while it was upregulated in PCa cancer-derived cell lines according to the Ottley et.al study [14]. However, the specific expression and mechanism of miR-191 in PCa still remains to be further explored.

The primary purpose of this study was to investigate the expression of miR-191 in prostate cancer and its role in the proliferation and invasion of PCa cells.

## Methods

*Clinical samples*

All 48 pairs of PCa tissue and adjacent normal tissue samples were collected from our hospital, while no patient had received chemotherapy or hormonotherapy before prostatectomy. Written informed consent was signed by all of recruited patients and the study was approved by the medical ethics committee of the affiliated hospital of Hebei University of Engineering (HS-03-2015-001). The tissue samples were kept in liquid nitrogen before the next RNA isolation. The clinical patient features were collected and assessed according to the standards of the American Joint Committee on Cancer (AJCC).

*Cell lines and cell culture*

Human PCa cell lines DU145, PC-3, LNCAP and benign prostate hyperplasia (BPH) and human prostate epithelial cell line RWPE-1 were purchased from the Cell Bank of Chinese Academy of Sciences. DU145 were cultured in Dulbecco's modified Eagle's medium (DMEM), PC-3 in F-12 and BPH in RPMI-1640 medium. RWPE-1 cells were cultured in K-SFM mixed with 0.05 mg/ml bovine pituitary extract (BPE) and 5 ng/ml epidermal growth factor (EGF) according to the manufacturer's instructions. All mediums were bought from Invitrogen (Carlsbad, CA, USA) and supplemented with 10% fetal bovine serum (FBS) (Gibco, USA) and the cells were maintained at 37°C with 5% $CO_2$.

*RNA isolation and qRT-PCR*

Total RNA was extracted using TRIzol reagent (Invitrogen, USA) and reversed using a miRNA Reverse Kit (Takara, Dalian, China). The miR-191 level was detected using SYBR Premix Ex Taq kits (Takara, Dalian, China) with ABI Prism 7900HT (ABI, USA) and U6 was used as internal control. Each experiment was performed in triplicate, and all the relative expression levels were measured by the $2^{-\Delta\Delta CT}$ method.

*Transfection of miR-191 mimics, inhibitors and siRNA-TIMP3*

The mimics and inhibitors for miR-191 and siRNA for TIMP3 were purchased from Genepharma (Shanghai, China). Cells were seeded into 6-well plates and cultured to a density of 50-70%, then incubated with miR-191 mimics, miR-191 inhibitors or siRNA-TIMP3 mixed with lipofectamine 2000 in serum-free medium for 48hrs according to the instructions. The transfection efficiency was confirmed by qRT-PCR.

*CCK8 assay*

CCK8 (Dojindo Laboratories, Kumamoto, Japan) assay was applied to detect the proliferation of cells. The cells seeded at a density of $1\times10^3$ cells with 100 µL medium were cultured for 24, 48, 72, 96 hrs after transfection. A volume of 10 µL CCK8 reagent was added into the wells and absorbance of 450 nm was measured.

*Colony formation assay*

A total of 300 cells made into a single cell suspension in 2mL medium were placed in 6-well plate and cultured for 15 days. Then, cells in the plate were fixed with ice methanol and stained with crystal violet. The number of colonies that contained more than 50 cells were calculated.

*Transwell invasion assay*

The Transwell inserts (Millipore, MA, USA) assay was used to measure the cell invasion ability. A total of $2\times10^5$ treated cells in 10% FBS medium were seeded into the top chamber of the insert which had plated Matrigel (BD, USA), and the lower chamber was immersed in FBS-free medium. After 24-h incubation, the membrane containing cells on its lower surface was fixed with pre-cooling methanol and stained with crystal violet. Then, the stained cells were imaged and calculated. Five random visions were taken using a microscope.

*Dual-luciferase assay*

The activity of luciferase was tested using the Dual-Luciferase reporter system (Promega, UK). The TIMP3 3'-UTR region containing the wild type or mutant miR-191 binding site was amplified and cloned into pGL3 luciferase vector (Promega, Madison, USA). Then, treated cells were co-transfected with the established vector and miR-191 mimics or scrambled using lipofectamine 2000. Then, the activity of luciferase was determined using luminometer (Promega) and measured as the fold-change to the basic pGL3 vector relatively.

*Protein extraction and western blot analysis*

To evaluate the relative TIMP3 protein expression level, cells were washed with pre-cooling PBS and then lysed by using RIPA (Beyotime, Jiangsu, China) to extract total protein. Then, the concentration of collected

protein was measured by using the BCA kit (Beyotime, China). After being chilled on ice, the protein was dispersed by 12% SDS-PAGE using Bio-Rad electrophoresis system (Bio-Rad, Hercules, CA, USA) and then shifted to PVDF membranes (Millipore, MA, USA). The membranes loaded with proteins were incubated in 5% skim milk dissolved in TBST buffer to block non-specific protein interactions at room temperature for 2 hrs. Then, the membranes were incubated at 4°C overnight with the primary antibody (1:1000) against TIMP3 (Abcam, USA). Afterwards, the membranes were incubated with horseradish peroxide- (HRP-) conjugated secondary antibody at room temperature for one hr after washing with TBST buffer for 10 min × 3 times. These bands were visualized using ECL Kit (Millipore, USA) after triple washing with TBST buffer following the instructions.

*Statistics*

All the study data were analyzed using SPSS 19.0 version and GraphPad prism 5.0 version software. The independent samples t-test and Fisher's exact test (SPSS, NY, USA) were used to access quantitative data and qualitative variables, respectively. Data was shown as mean ± SD and p value <0.05 was considered to show significant difference.

## Results

*MiR-191 expressed significantly higher in PCa tissue samples and cell lines*

To detect the expression level of miR-191 in prostate cancer, 48 pairs of tumor and adjacent normal tissue samples were analyzed using qRT-PCR, showing that miR-191 was significantly overexpressed in PCa samples compared to the normal group (Figure 1A). Also, the miR-191 level in 4 PCa-derived DU145, LNCAP, BPH PC-3 cell lines and normal prostate epithelial cell (RWPE-1) used as control were measured. miR-191 was upregulated in PCa cell lines, which confirmed the outcome in clinical samples (Figure 1B). Furthermore, we divided 48 patients into two groups according to the median level of miR-191 and analyzed the relationship between clinicopathological characteristics and the miR-191 expression. As clearly shown in Table 1, higher miR-191 level was associated with more metastatic capability, higher pre-operative PSA, Gleason score and advanced

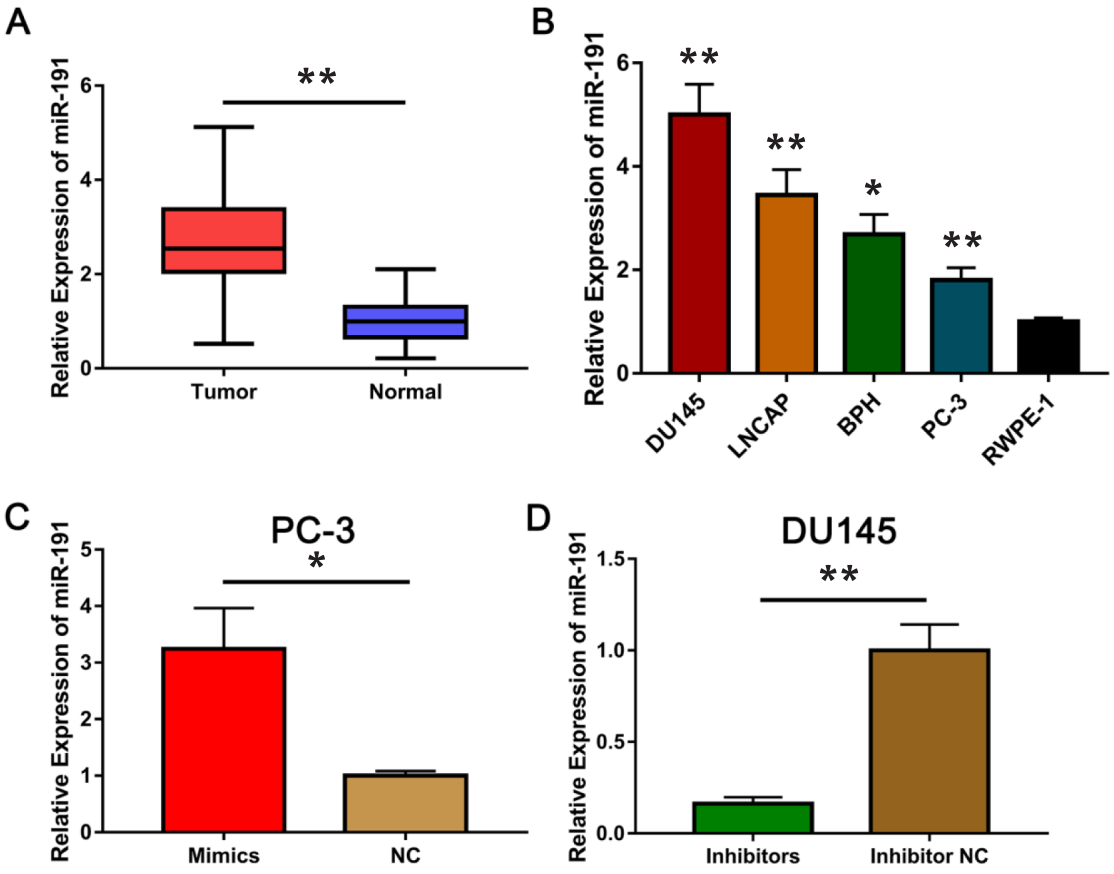


**Figure 1.** MiR-191 was upregulated in PCa tissues and cell lines. **A:** Analysis of the expression level of miR-191 in 48 pairs of PCa tumor and adjacent tissues. **B:** Analysis of miR-191 expression level in PCa cell lines (DU145, PC-3, LNCAP, BPH) and prostate epithelial cell line (RWPE-1). **C:** Expression of miR-191 in miR-191 mimics treated PC-3 cells. **D:** expression of miR-191 in miR-191 inhibitors treated DU145 cells. MiR-191 was detected by qRT-PCR and U6 was used as an internal control. Data are presented as the mean ± SD of three independent experiments. NC: normal control. *p<0.05, **p<0.01.

**Table 1.** Relationship between miR-191 level and the clinicopathological characteristics of 48 patients with prostate cancer

| | *miR-191 level** | | | |
|---|---|---|---|---|
| *Group* | *Total* | *High* | *Low* | p value |
| Age, years | | | | 0.245 |
| <70 | 21 | 8 | 13 | |
| >70 | 27 | 16 | 11 | |
| Metastasis | | | | 0.006 |
| Yes | 16 | 13 | 3 | |
| No | 32 | 11 | 21 | |
| Pre-operative PSA (mg/ml) | | | | 0.0061 |
| <4 | 9 | 1 | 8 | |
| 4-10 | 15 | 6 | 9 | |
| >10 | 24 | 17 | 7 | |
| T stage | | | | 0.043 |
| T1 | 26 | 9 | 17 | |
| T2/T3 | 22 | 15 | 7 | |
| Gleason score | | | | 0.0113 |
| <7 | 23 | 8 | 15 | |
| 7 | 7 | 2 | 5 | |
| >7 | 18 | 14 | 4 | |

*The median expression level of miR-191 was used as the cutoff value

T stage. These results indicated that miR-191 may behave as an oncogene in PCa.

To further verify the function mir-191 in PCa, we studied miR-191 overexpression in PC-3 cells and miR-191 knockdown DU145 cell line according to the expression level in cell lines using miR-191 mimics and inhibitors respectively. After transfection, PC-3 cells showed higher miR-191 expression while miR-191 level of DU145 cells decreased significantly comparing to each negative control (Figure 1C, 1D).

*MiR-191 affected the proliferation and invasion of PCa cells*

Using CCK8 and colony formation assay the proliferation and invasion ability of established PCa cells was analyzed. Upregulation miR-191 with mimics in PC-3 cells caused significantly increase in cell proliferation and colony formation (Figure 2A, 2B), however, downregulation miR-191 in DU145 cells led to a decrease of cell growth (Figure 2C, 2D). In addition, in order to evaluate the effect of miR-191 in cell invasion, we carried out transwell invasion assay and found a sharp enhancement after mir-191 overexpression but marked decline after miR-191 inhibition in the cell invasion ability (Figure 2E,2F). These loss- and gain- of function experiments indicated that upregulation of miR-191 could enhance the proliferation and invasion of PCa cells.

*TIMP3 was a direct target of miR-191*

To further explore the molecular mechanism involved in miR-191 regulation of PCa, we searched several database programs including Targetscan, miRwalk, and PicTar. After cross-analyzed information from these databases, TIMP3 was found to be a potential target of miR-191. Next, we tried to confirm this assumption via conducting dual-luciferase reporter vectors containing mutant or wild type (WT) miR-191 binding sites 3'-UTR (Figure 3A). The result of dual-luciferase assay revealed a significant decrease in the WT group but no difference in the mutant group (Figure 3B). In continuation, the protein expression of TIMP3 in miR-191 mimics or inhibitor-treated cells was measured by western blot analysis. Obviously, upregulation of miR-191 reduced the TIMP3 level in PC-3 cells (Figure 3C, 3E), while downregulation miR-191 in DU145 cells increased the TIMP3 expression (Figure 3D, 3F). These results suggested that TIMP3 was a direct target of miR-191.

*MiR-191 regulated cell growth and invasion via TIMP3*

Because downregulation miR-191 via inhibitors could decrease the cell proliferation and invasion capacity and might function via TIMP3, we further constructed siRNA of TIMP3 to reverse the effect of miR-191 inhibitors to verify the results.

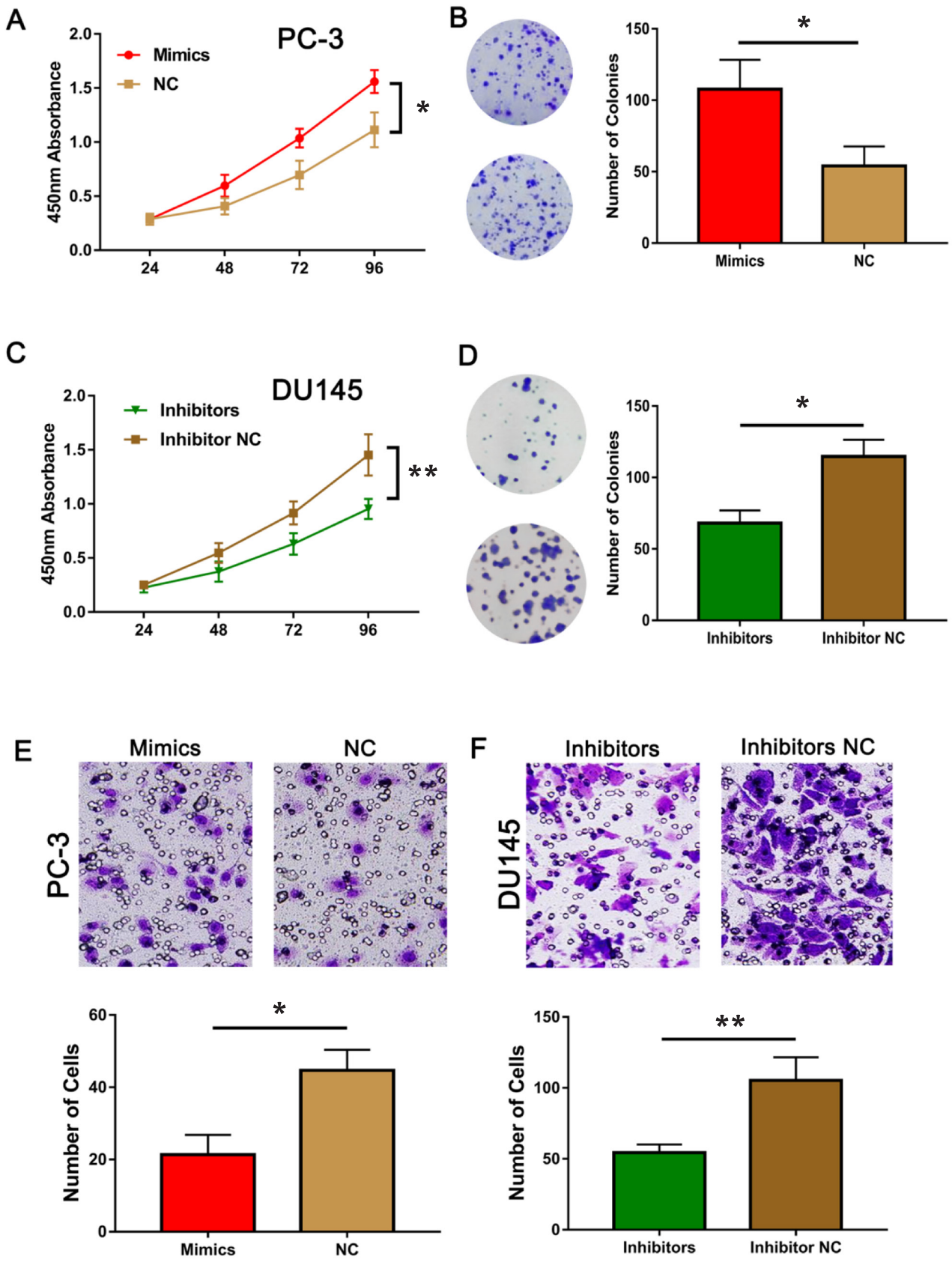


**Figure 2.** MiR-191 affected the proliferation and invasion of PCa cells. **A,C:** CCK8 assay was performed to determine proliferation of PC-3 **(A)** or DU145 **(C)** cells treated with miR-191 mimics or inhibitors compared to each negative control. **B,D:** Colony formation assay was performed to determine the growth of PC-3 **(B)** or DU145 **(D)** cells transfected with mimics or inhibitors, respectively. **E,F:** Transwell invasion assay was used to detect the invasion ability of miR-191 mimics treated PC-3 cells **(E)** or miR-191 inhibitors treated DU145 cells **(F)**. Data are presented as the mean ± SD of three independent experiments. NC: normal control. *p<0.05, **p<0.01.

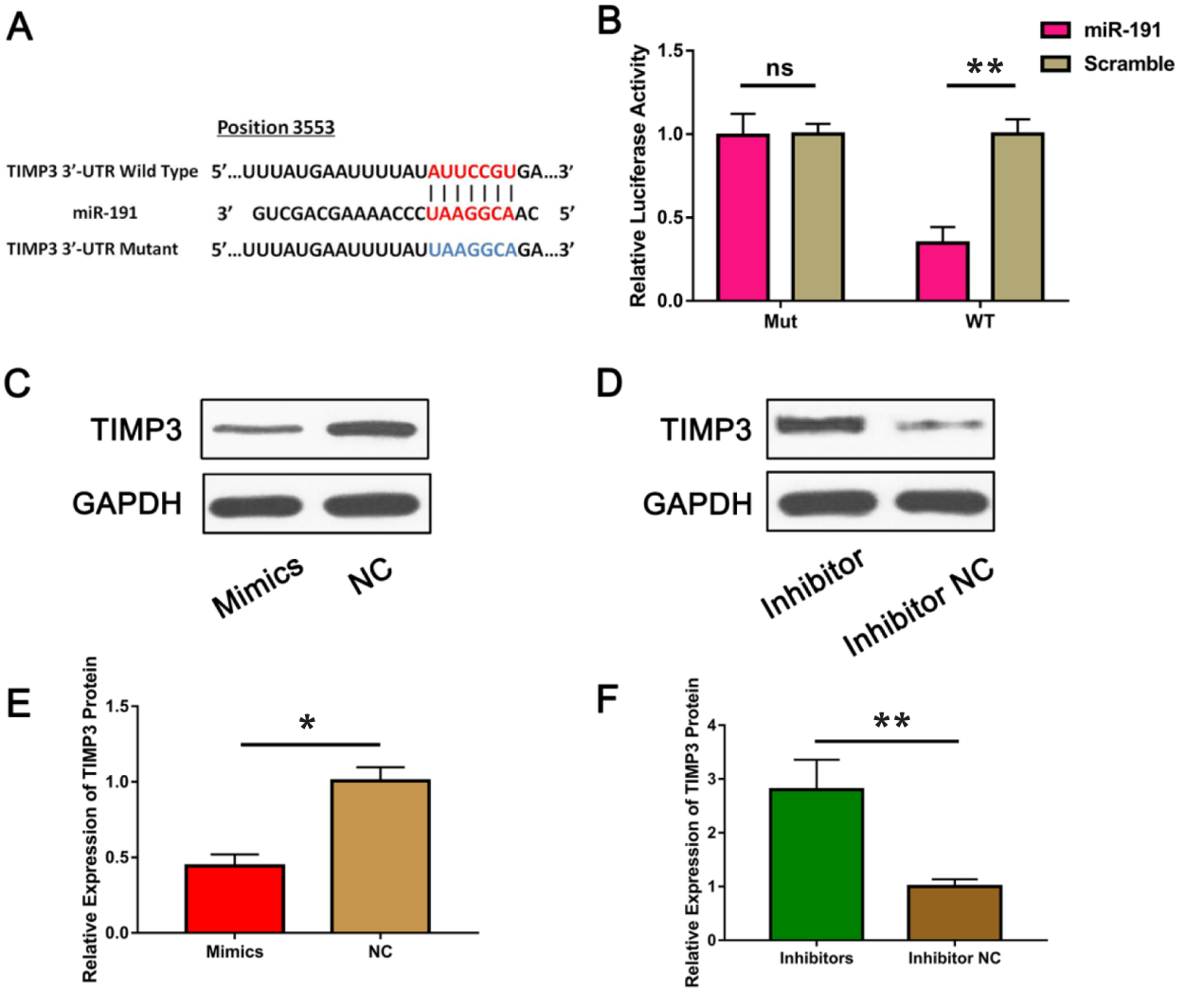


**Figure 3.** TIMP3 was a direct target of miR-191. **A:** The predicted binding sites of miR-191 in the 3'-UTR of TIMP3. **B:** Dual-luciferase reporter assay was used to determine the binding site. PC-3 cells treated by mimics or NC were transfected with pGL3 construct containing the wild type (WT) or mutant TIMP3 3'-UTR site. **C,D:** Levels of TIMP3 protein measured by western-blot in miR-191 overexpression PC-3 cells **(C)** and miR-191 knockdown DU145 cells **(D)**. **E,F:** The relative protein level of TIMP3. The protein levels were normalized to that of GAPDH. Data are presented as the mean ± SD of three independent experiments. NC: normal control; ns: non-sense. *p<0.05, **p<0.01.

As shown in Figure 4A, CCK8 assay showed that siRNA-TIMP3 transfection in miR-191 inhibitors-treated DU145 cells significantly rescued the cell proliferation ability. Furthermore, the decrease of invasion activity caused by miR-191 inhibitors was reversed by TIMP3 downregulation (Figure 4B, 4C). In addition, the TIMP3 protein expression was confirmed using western blot which showed TIMP3 decreased in siRNA-TIMP3 group (Figure 4D, 4E). These data demonstrated that miR-191 could promote PCa progression via downregulation of TIMP3.

## Discussion

The development and progression of PCa is a complex process of multi-step and multi-factor interaction [15]. PCa has different prognostic features and the therapeutic effects differ, causing difficulty for its treatment. Exploring the molecular mechanism related to the progress of PCa has become a hot topic in recent years, which can contribute to timely diagnosis, early prevention and treatment improvement of prostate PCa [16,17].

Non-coding RNAs, especially miRs have been proved to participate in many stages of cancer progression including tumor cell proliferation, invasion, metastasis, and drug resistance [18]. Recently, a considerable number of studies has revealed that abnormal expression of miRs is involved in tumor development and progression [19]. In PCa, miR-199a-3p suppressed PCa cell proliferation and invasion by targeting Smad1 and miR-543 promoted proliferation and epithelial-mesenchymal transition via RKIP; in addition, miR-181a could promote docetaxel resistance of PCa [20-24]. MiR-

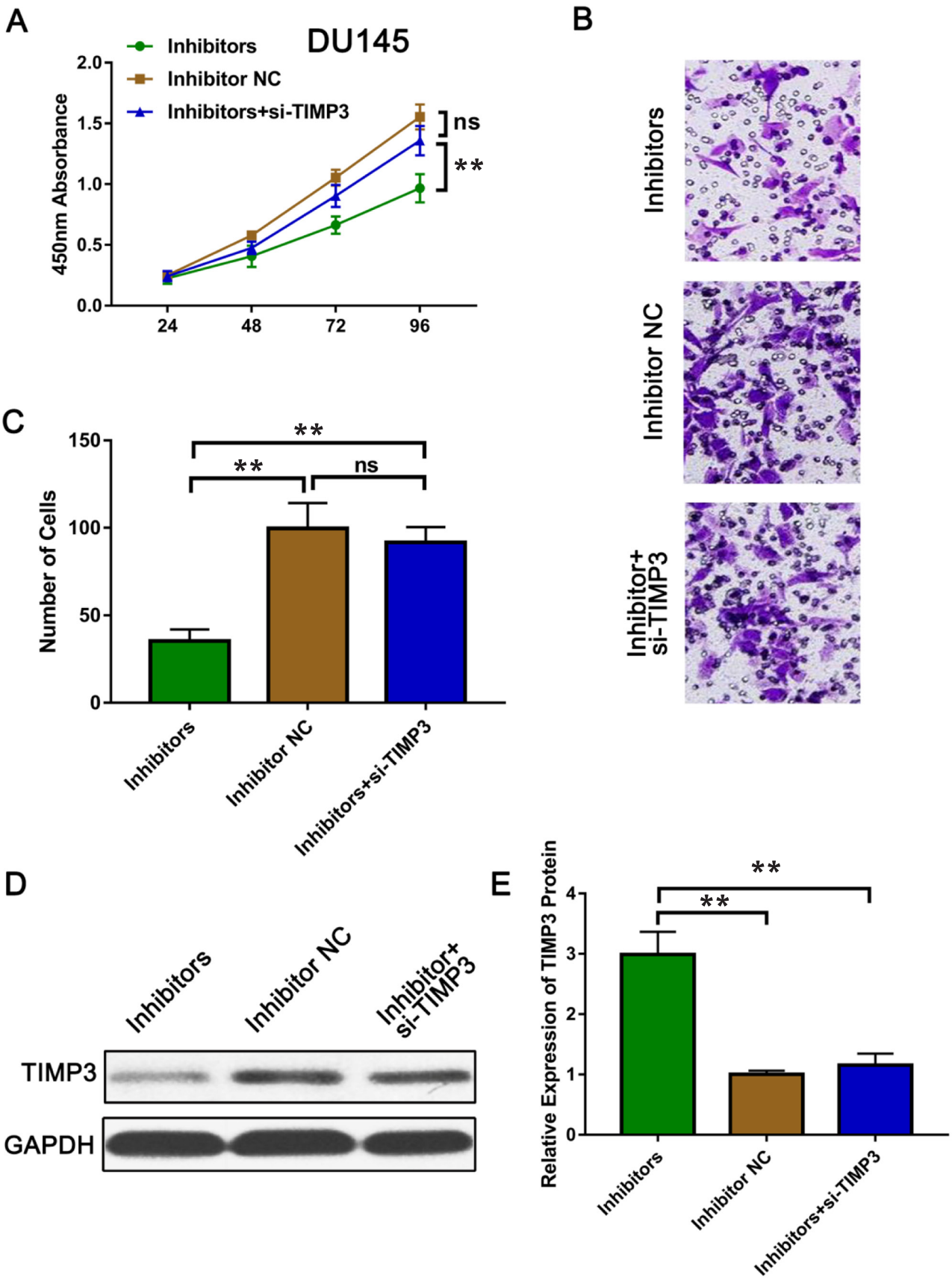


**Figure 4.** SiRNA-TIMP3 rescued the effects of miR-191 inhibitors in DU145 cells. **A:** Analysis of the cell proliferation ability by CCK8 assay in miR-191 inhibitors, inhibitor normal control (NC), or inhibitors+siRNA-TIMP3 treated DU145 cells; **B,C:** Cell invasion ability was measured by transwell assay; **D:** Western blot analyses of TIMP3 expression level. GAPDH was used as an internal control. **E:** Relative protein band densities of TIMP3. Data are presented as the mean ± SD of three independent experiments. ns: non-sense. **p<0.01.

191 has been identified as an oncogene in several tumors and is located in chromosome 3. It is reported that this miR promotes the cell proliferation and invasion of breast cancer via regulating TGF-β, while it accelerates tumorigenesis of human colorectal cancer through C/EBPβ [25,26]. But the role miR-191 played in PCa remains unclear. In our study, we first identified that the expression of miR-191 was significantly higher in tumor tissues than in adjacent normal tissues and higher miR-191 levels correlated to advanced tumor stage, higher pre-operative PSA level, Gleason score and increased metastatic potential. Furthermore, we studied miR-191 overexpression or knockdown in cell lines and employed several cell function experiments, which demonstrated that upregulation of miR-191 obviously promoted cell proliferation and invasion in PCa. As a conclusion, we considered miR-191 functions as a promotion factor in PCa.

MiRs could bind to the 3'-UTR region of target genes and influence their expression by changing cellular processes [27]. We further searched the potential target gene of miR-191 in some databases and found TIMP3 was a direct target of miR191. TIMP3 was an important inhibitor of matrix metalloproteinases (MMPs) which caused tumor growth and metastasis [28]. TIMP3 was downregulated in many malignant tumors such as thyroid cancer and melanoma and loss of TIMP3 caused PCa proliferation and metastasis [29-33]. To verify TIMP3 was a direct target of miR-191, dual-luciferase assay was used in PC-3 cells. Also, the protein level of TIMP3 was measured in miR-191 expression interference cells and showed a negative relationship between miR-191 and TIMP3 level. Also, siRNA-TIMP3 could reverse the effects of miR-191 inhibitors, which further confirmed that miR-191 promotes PCa cell proliferation and invasion via TIMP3.

Furthermore, one miR could target different genes and one gene could be regulated by different miRs [34]. We aimed at TIMP3 as the only target gene of miR-191, which could be a limitation of this study. Though *in vivo* assays might be needed for further study, our study demonstrated the function of miR-191 in PCa for the first time and elucidated TIMP3 as a target gene partially involved in the regulation mechanism of miR-191.

## Conclusion

This study demonstrated for the first time that miR-191 may function as an oncogene in PCa and promote cell proliferation and invasion via downregulating TIMP3. What's more, the results indicated that inhibition of miR-191 could reduce cell proliferation and invasion, which suggested miR-191 as a potential therapeutic target for PCa treatment.

## Conflict of interests

The authors declare no conflict of interests.

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