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*Original Article*

# MicroRNA-506 inhibits cell proliferation and invasion in prostate cancer by targeting HDAC4

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**Abstract:** Accumulating evidence shows the involvement of microRNAs (miRNAs) in carcinogenesis as either oncogenes or tumor suppressor genes. miR-506 has been implicated in several human cancers, but its roles and functional mechanisms in prostate cancer (PCa) have not yet been evaluated. The present study revealed that miR-506 was markedly down-regulated in PCa cells and tumor tissues. Ectopic expression of miR-506 in PCa cells suppressed cell proliferation and invasion, induced cell cycle arrest, and promoted cell apoptosis. Furthermore, miR-506 directly reduced the expression of HDAC4 by binding to its 3'-untranslated region. Knockdown of HDAC4 mimicked the effects of miR-506, whereas re-expression of HDAC4 restored the suppressive effect of miR-506. Taken together, our results indicate that miR-506 acts as a tumor suppressor in PCa and its suppressive effects are mediated by directly targeting HDAC4.

**Keywords:** miR-506, HDAC4, prostate cancer

## Introduction

Prostate cancer (PCa) is the most common malignant tumor in men and is now the second leading cause of cancer death in men in developed countries [1]. Every year, more than 900,000 new cases of PCa are diagnosed and more than 250,000 men will die due to PCa worldwide [2]. Despite advances in early detection and therapy, the overall survival rate has not significantly improved [3, 4]. Therefore, it is critical to improve understanding of the mechanisms underlying PCa development to identify novel targets for effective therapeutic strategies and improve patient survival.

MicroRNAs (miRNAs) are small, evolutionary conserved, non-coding single-stranded RNAs that regulate target gene expression by sequence-specific interactions with the 3'-untranslated regions (UTRs) of their target mRNAs [5-7]. miRNAs regulate many fundamental cellular activities, such as proliferation, differentiation, cell cycle and apoptosis, which are important in the development of cancer [8-10]. Recently, increasing evidence has indicated that deregulation of miRNAs is related to PCa initiation and development by directly exerting their functions as oncogenes or tumor suppressors [11-14]. For example, miR-18a is elevated in PCa and promotes tumorigenesis by suppressing STK4 *in vitro* and *in vivo* [15]. On the other hand, miR-224 and miR-218 are significantly down-regulated in PCa tissues and restoration of miR-224 or miR-218 significantly inhibits PCa cell migration and invasion [16, 17]. Extensive research has shown that miR-506 is down-regulated and acts as a tumor suppressor in many cancers [18-20]. However, the role of miR-506 in PCa has not been documented.

In this study, we demonstrate that miR-506 is significantly down-regulated in human PCa cell lines and tissues. Re-expression of miR-506 inhibited cell proliferation, migration and invasion, blocked cell cycle progression and promoted apoptosis by direct targeting of HDAC4. Thus, miR-506 may be a potential target for further studies on the therapeutics of PCa.

## Materials and methods

### *Tissue specimens*

Eight paired human PCa tissues and adjacent non-cancerous prostate tissues were obtained

from Wuxi No. 2 People's Hospital affiliated to Nanjing Medical University from 2011 to 2012. The tissues were immediately snap-frozen in liquid nitrogen until use. Written informed consent was obtained from each patient and the study was approved by the Institutional Research Ethics Committee of Wuxi No. 2 People's Hospital affiliated to Nanjing Medical University.

*Cell culture*

The normal prostate cell line (RWPE-1) and prostate carcinoma cell lines (LNCaP, DU145, PC-3) were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). RWPE-1 cells were maintained in keratinocyte serum-free medium (K-SFM, Invitrogen, Carlsbad, CA, USA). LNCaP, DU145 and PC-3 cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS). All cell lines were maintained in a 37°C humidified incubator with 5% $CO_2$.

*Vector construction and transfection*

The HDAC4 expression plasmid (pcDNA3.1-HDAC4) containing the coding sequence, but lacking the 3'-UTR, was constructed using PCR-generated fragments and pcDNA3.1 (+) vector. siRNA for HDAC4 (Ribobio, Guangzhou, China) was used to knockdown endogenous HDAC4 in this study. Cell transfection was performed using Lipofectamine2000 (Invitrogen) according to the manufacturer's protocol. Lentiviral vector expressing miR-506 (miR-506) and lentiviral vector expressing a scrambled RNA (vector) were purchased from GeneChem (Shanghai, China). Lentivirus infection was performed according to the manufacturer's instructions.

*RNA isolation and qRT-PCR*

Total RNA was extracted using TRIzol solution according to the manufacturer's protocol. Total RNA was synthesized into cDNA using the Prime Script RT reagent Kit (Takara, Dalian, China). Real-time PCR were carried out using the SYBR green Premix Ex Taq II (Takara), according to the manufacturer's protocols. β-actin was used as an endogenous control for mRNA expression and miR-506 expression was normalized to U6 small nucleolar RNA. Relative quantification analysis was calculated using the $2^{-\Delta\Delta Ct}$ method.

*Cell proliferation assay*

Cell proliferation was measured with the 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells ($2 \times 10^3$) were seeded into each well of a 96-well plate and cultured for 1, 2, 3 and 4 days. MTT (0.5 mg/ml in PBS) was added to each well. The cells were subsequently incubated for another 4 h at 37°C. The supernatants were carefully removed and 100 μl of dimethyl sulfoxide (DMSO) was added to each well and the absorbance was measured at 490 nm by a Microplate Reader.

*Cell cycle analysis and apoptosis assay*

For cell cycle analysis, harvested cells were fixed in ice-cold 70% ethanol. After washing, the cells were incubated at 37°C for 15 min and stained with propidium iodide (PI) for 20 min. DNA content was examined by flow cytometry on a FACScan (BD Biosciences, Bedford, MA, USA). The Annexin V/PI double staining method was used to examine cell apoptosis. Cells ($5 \times 10^5$ cells per well) were seeded in six-well plates. Following incubation for 48 h, the cells were trypsinized, harvested, resuspended in PBS buffer, double stained with Annexin V-FITC and PI and identified using a flow cytometer.

*Wound healing assay*

Cell migration was assessed by the scratch wound migration assay. Cells were seeded into 6-well plates and incubated in RPMI1640 medium for 24 h until the cell monolayer reach confluence. Wounds were made by scraping with a sterilized 200 μl pipette tip. The cells were washed 3 times with PBS to wash off unattached cells. The scratches were observed at 0 h and 48 h using an inverted microscope. The speed of wound closure was assessed as the rate of closure.

*Invasion assay*

Pre-coated Matrigel invasion chambers with a pore size of 8.0 μm (BD Biosciences) were used for the invasion assay, according to the manufacturer's protocol. Cells ($5 \times 10^4$ cells per well) re-suspended in 200 μl serum-free medium were placed in the upper chambers of the Matrigel-coated inserts. Invasion chambers were fitted into a 24-well plate containing 600

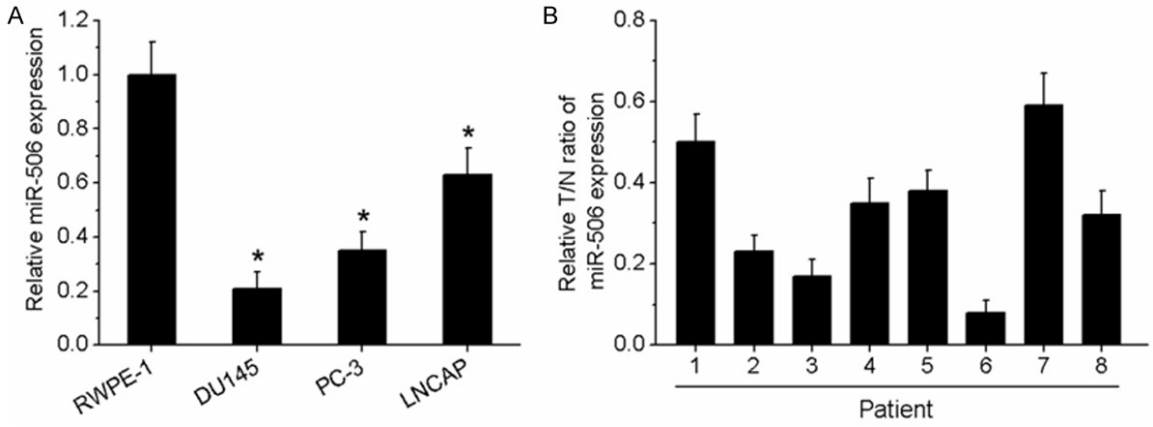


**Figure 1.** miR-506 expression is down-regulated in human PCa cell lines and tissues. A. The expression of miR-506 in three PCa cell lines and the normal human prostate cell line (RWPE-1) was examined by qRT-PCR and normalized to U6 expression. B. Relative expression of miR-506 in eight PCa tissues and adjacent non-cancerous prostate tissues.

μl culture medium with 10% FBS. The cells were incubated and allowed to migrate for 24 h at 37°C in a 5% $CO_2$ incubator. The cells that had invaded the lower surface were fixed in 100% ice-cold methanol for 10 min, stained with 0.5% crystal violet for 30 min, and wash with PBS. The relative number of cells that migrated through the pores was calculated from five-field digital images taken randomly under an inverted microscope.

*Luciferase reporter assay*

The 3'-UTR of HDAC4 and a mutant variant generated through site-directed mutagenesis were cloned into the pGL3-basic vector (Promega, Madison, WI, USA). For the luciferase activity assay, PC-3 and DU145 cells stably expressing miR-506 or vector were co-transfected with the pGL3 reporters and pRL-TK *Renilla* plasmid using Lipofectamine 2000. After 48 h of transfection, the cells were harvested, washed and lysed with lysis buffer. Luciferase activity was analyzed using the Dual-Glo luciferase assay kit (Promega). The firefly luciferase values were normalized to Renilla luciferase activity.

*Western blotting*

Western blotting was conducted according to previously described methods [21]. Total proteins were extracted in RIPA buffer [25 mM Tris·HCl (pH 7.6), 150 mM NaCl, 1% nonidet P-40, 1% sodium deoxycholate, 0.1% SDS, 1 × phosphoate inhibitor mixture and protease inhibitor mixture], separated on 12% SDS-PAGE (Invitrogen), and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA, USA). The membranes were then blocked in 5% non fat dried milk in Tris buffered saline with Tween-20 (TBST), incubated with the specific primary antibody, washed, and probed with secondary antibody conjugated to HRP (Pierce), according to the manufacturer's instructions and standard procedures. All results were visualized by autoradiography using an ECL kit (Amersham ECL Prime). Antibodies specific to HDAC4, GAPDH, and horseradish-peroxidase-coupled secondary antibodies were purchased from Santa Cruz Biotechnology.

*Tumor xenograft*

To produce a tumor xenograft assay, nude mice were divided into two groups each group consisting of 5 mice. Tumors were induced by subcutaneously injecting DU145-miR-506 cells or vector control cells ($1 \times 10^7$) into the mice (4-6 weeks old). Tumor growth was measured by a caliper when palpable. Tumor volume (V) was calculated according to the formula: $V = (L \times W^2)/2$, where L is the longest axis and W is the shortest axis. Mice were killed 25 days after inoculation. The tumors were then removed, weighed and snap-frozen in liquid nitrogen. All work was approved by the Animal Ethics Committee of Wuxi No. 2 People's Hospital affiliated to Nanjing Medical University.

*Statistical analysis*

All statistical analyses were carried out using SPSS 16.0 software. Experimental results from

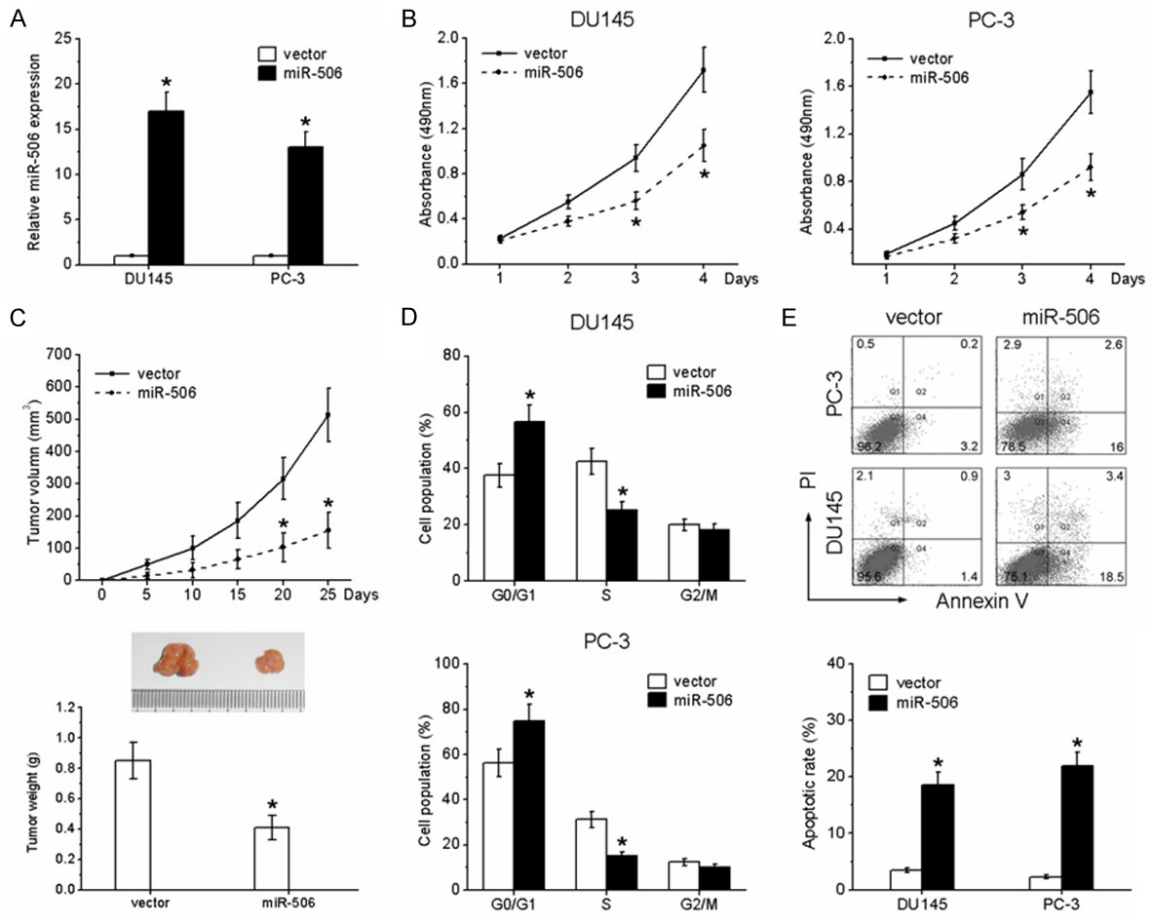


**Figure 2.** Ectopic expression of miR-506 inhibits proliferation, blocks cell cycle progression and promotes apoptosis of DU145 and PC-3 cells. (A) DU145 and PC-3 cells were infected with the miR-506 expression lentivirus (miR-506) or negative control (vector). Relative expression of miR-506 was detected by qRT-PCR. (B) Cell viability was determined by MTT assay. (C) DU145 cells stably expressing miR-506 or vector were injected subcutaneously into nude mice. Tumor volume was measured every 5 days. After 25 days, the mice were killed and tumors were weighed. Cell cycle (D) and apoptosis (E) were detected by flow cytometry analysis.

at least three separate experiments are expressed as mean ± standard deviation (SD). The two-tailed unpaired Student's *t*-test was used for simple comparison of two values. $P<0.05$ was considered statistically significant.

## Results

### *miR-506 expression is down-regulated in human prostate cancer cell lines and tissues*

We first determined miR-506 expression in three PCa cell lines (DU145, PC-3 and LNCaP) and the normal prostate cell line (RWPE-1) by qRT-PCR. The expression of miR-506 in PCa cell lines was significantly lower than that in RWPE-1 cells (**Figure 1A**). To further evaluate the expression and significance of miR-506 in human PCa, we determined the expression of miR-506 in eight human PCa tissues and adjacent non-cancerous prostate tissues. As shown in **Figure 1B**, compared with non-cancerous prostate tissues, the expression of miR-506 was markedly down-regulated in PCa tissues.

### *Ectopic expression of miR-506 inhibits cell proliferation both in vitro and in vivo*

To investigate the role of miR-506 in cell proliferation, we established PC-3- and DU145-miR-506 stable cells by infection with a miR-506 expression lentivirus. qRT-PCR analysis showed that miR-506 expression was significantly increased in these cells (**Figure 2A**). The MTT assay showed that ectopic expression of miR-506 significantly suppressed cell proliferation in both PCa cell lines, compared with vector control cells (**Figure 2B**). A tumor xenograft

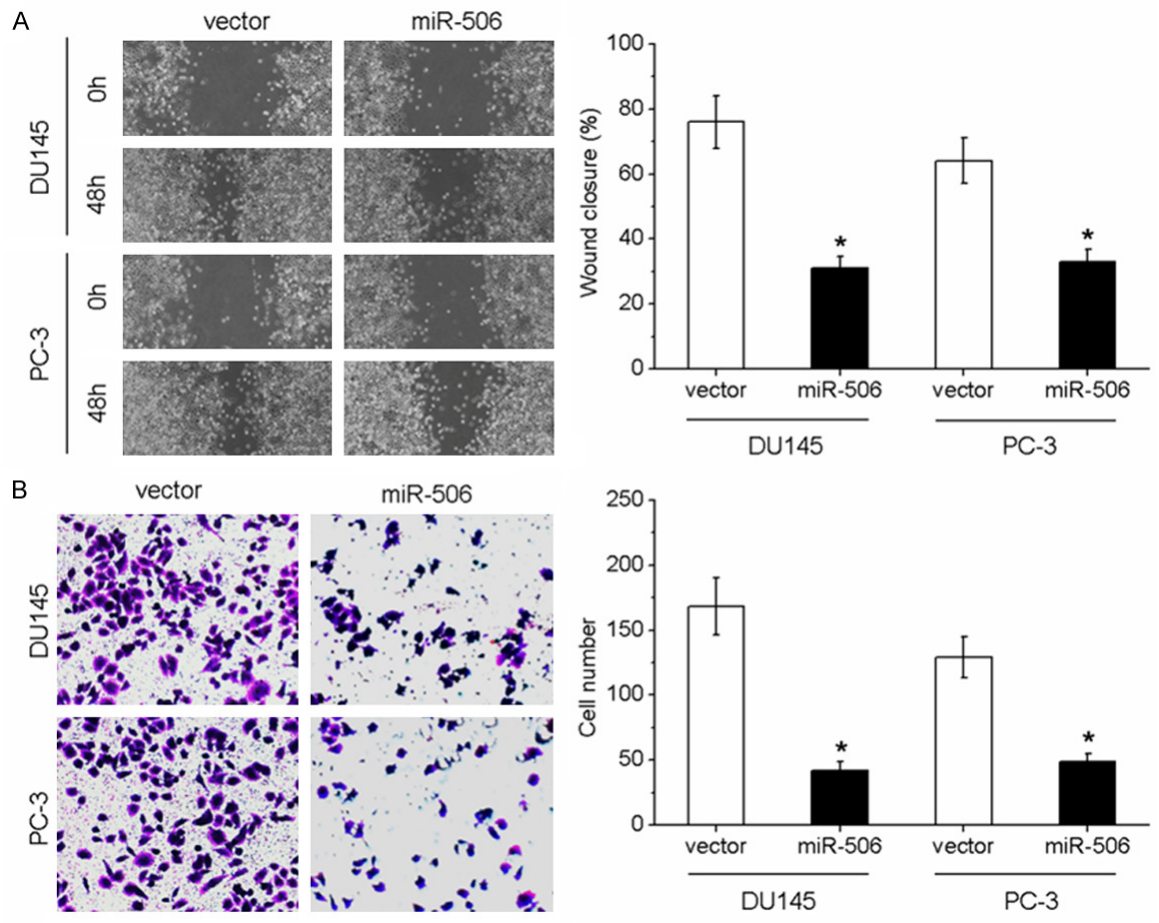


**Figure 3.** Ectopic expression of miR-506 suppresses cell migration and invasion. A. Migration of DU145 and PC-3 cells was determined by the wound healing assay. B. Invasion of DU145 cells was detected by the transwell invasion assay.

assay in a mouse model was performed to examine the *in vivo* function of miR-506, and revealed that the tumors derived from DU145-miR-506 cells were smaller and had lower weights than those formed by control cells (**Figure 2C**). To further investigate the possible mechanism of miR-506 function in human PCa cell growth, we adopted flow cytometry analysis to evaluate cell-cycle progression and apoptosis in both PCa cell lines. As shown in **Figure 2D**, G0/G1 phase cells were significantly increased and S phase cells decreased in miR-506-overexpressing cells, compared with vector cells. Furthermore, the apoptosis ratio was markedly higher in miR-506-overexpressing cells than that in vector cells (**Figure 2E**). Collectively, these results suggested that the growth-suppressive effect of miR-506 was partly due to a G0/G1 phase arrest and apoptosis.

*Ectopic expression of miR-506 suppresses cell migration and invasion*

We next investigated the potential role of miR-506 in PCa cell migration and invasion. The wound healing experiment indicated that the migration of DU145 and PC-3 cells was significantly suppressed when infected with the miR-506 expression lentivirus (**Figure 3A**). Similarly, the transwell assay clearly showed that overexpression of miR-506 resulted in a lower degree of invasion compared with that observed in control cells (**Figure 3B**). These results indicated that ectopic expression of miR-506 suppressed the migration and invasion of PCa cells.

*HDAC4 is a direct target of miR-506*

To investigate the molecular mechanism responsible for the tumor-suppressive abilities of

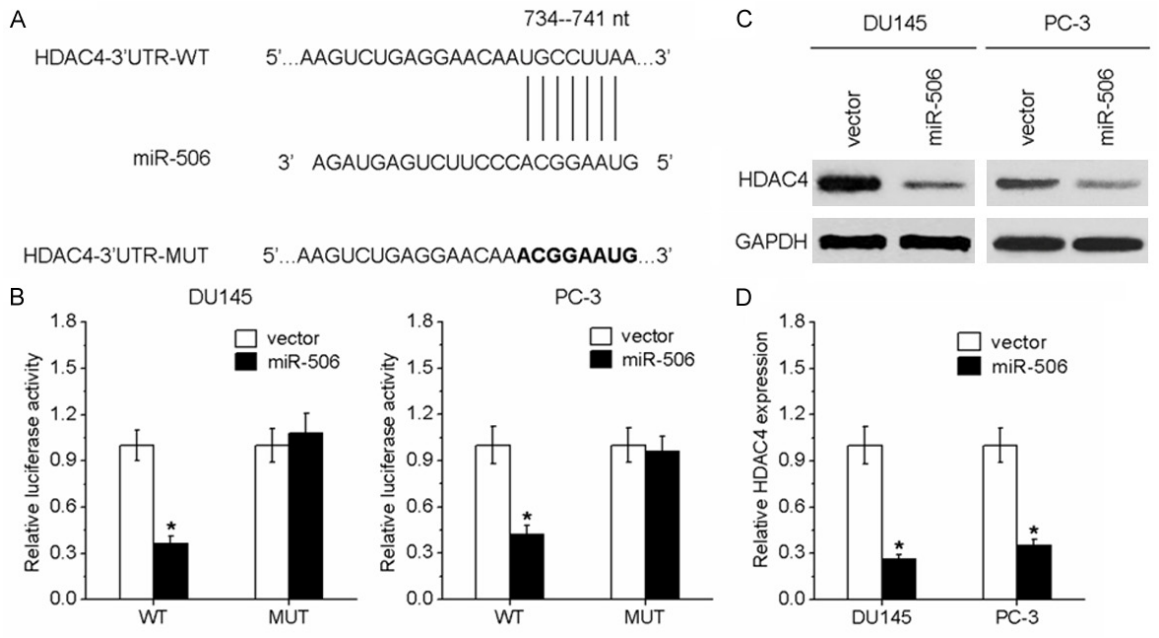


**Figure 4.** HDAC4 is a direct target of miR-506. (A) Schematic graph of the putative miR-506 target sequence in the wild-type HDAC4 3'-UTR (WT) and its mutant sequence containing altered nucleotides in the seeds sequence (MUT). (B) DU145 and PC-3 cells infected with the miR-506 or negative control lentivirus were transfected with wild-type or mutant reporter plasmids, and subjected to luciferase analysis. Western blotting (C) and qRT-PCR (D) analysis of the expression of HDAC4 in the indicated cells.

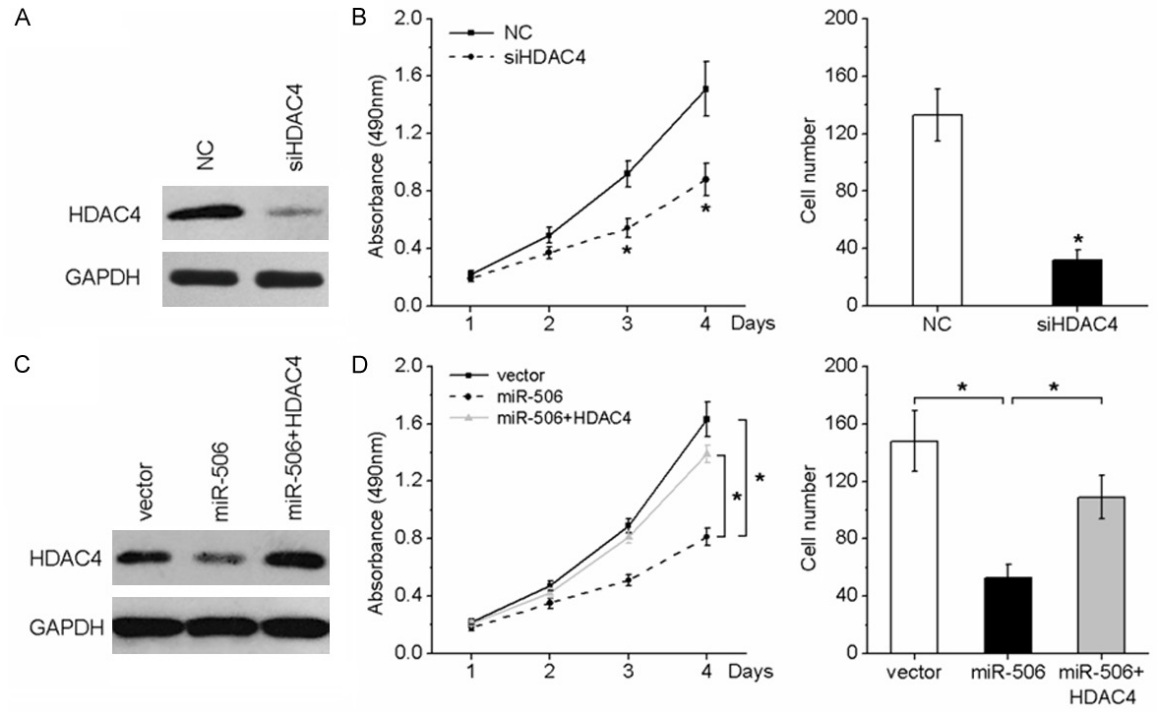


**Figure 5.** HDAC4 is involved in miR-506-induced suppression of PCa cell proliferation and invasion. (A) HDAC4 expression levels in DU145 cells transfected with HDAC4 siRNA (siHDAC4) or negative control siRNA (NC) were assessed by Western blot. (B) Cell viability and invasion assays of DU145 cells after transfection with siHDAC4 or negative control. DU145 cells stably expressing miR-506 or vector were transfected with pCDNA3.1-HDAC4 (HDAC4) or corresponding vector, and subjected to Western blot (C), MTT assay and transwell assay (D).

miR-506, we used two computational algorithms (TargetScan and miRanda) to identify its target genes, and HDAC4 was predicted to be a potential direct target of miR-506 (**Figure 4A**). To determine whether HDAC4 was regulated by miR-506, both wild-type and mutant HDAC4 3'-UTR fragments were cloned and inserted into a luciferase reporter system. They were transiently transfected into PC-3-miR-506 cells, DU145-miR-506 cells or their control cells, respectively. The results showed that miR-506 overexpression significantly reduced the luciferase activity of HDAC4-3'-UTR reporter, but had no effect on the mutant (**Figure 4B**). Moreover, we examined the effect of miR-506 on the expression of endogenous HDAC4 by western blot and qRT-PCR. As shown in **Figure 4C** and **4D**, HDAC4 protein and mRNA levels were lower in miR-506-overexpressing cells than in control cells. Taken together, these results suggested that HDAC4 is a direct target of miR-506 in human PCa cells.

*miR-506 suppresses cell proliferation and invasion by targeting HDAC4*

To determine whether the effects of miR-506 on cell proliferation and invasion were mediated by HDAC4, we inhibited endogenous HDAC4 expression via a HDAC4-specific siRNA (**Figure 5A**). Knockdown of HDAC4 significantly inhibited cell proliferation and invasion in DU145 cells (**Figure 5B**), which was similar to the inhibitory effects of miR-506. We also transfected DU145-miR-506 cells with HDAC4 plasmids lacking 3'-UTR. Re-expression of HDAC4 significantly abrogated the inhibitory effects of miR-506 on PCa cell proliferation and invasion (**Figure 5C** and **5D**). Together, these data suggested that HDAC4 is a functional downstream target of miR-506.

## Discussion

Aberrant expression of miRNAs is a frequent event in many types of human cancer [22-24]. Extensive research has shown that miR-506 is down-regulated and functions as a potential tumor suppressor in many cancers, including colorectal cancer (CRC), cervical cancer and hepatocellular carcinoma [18-20]. Ectopic expression of miR-506 significantly suppressed CRC cell proliferation and invasion both *in vitro* and *in vivo*, and increased sensitivity to chemotherapy [18]. Furthermore, miR-506 overexpression suppressed cell growth both *in vitro* and *in vivo*, and induced cell cycle arrest, apoptosis and chemosensitivity in cervical cancer [19]. Given these findings, we determined whether miR-506 expression is decreased in PCa and whether it has the ability to suppress PCa cell proliferation and invasion.

In the present study, we found that miR-506 was down-regulated in PCa cell lines compared with the normal epithelial prostate cell line. Low expression of miR-506 was also observed in eight human PCa tissues when compared to adjacent non-cancerous prostate tissues. We subsequently investigated the role of miR-506 deregulation in tumorigenesis and progression of PCa. The results showed that miR-506 overexpression inhibited cell proliferation, migration and invasion, induced cell cycle arrest and promoted apoptosis. Our data indicate that miR-506 functions as a tumor suppressor in PCa progression.

Using the TargetScan and miRanda databases, we identified several predicted target genes for miR-506, of which HDAC4 has been shown to play important roles in the regulation of chromatin remodeling by histone deacetylation, and is overexpressed in several types of cancer [25-27]. The up-regulation of HDACs is associated with dedifferentiation, enhanced proliferation and invasion, advanced disease stage, and poor prognosis [28-30]. For example, enhanced HDAC4 expression is more frequently observed in cases with papillary thyroid carcinoma compared to hyperplastic nodules. HDAC4 participates in the development and progression of thyroid malignancy and is also associated with malignant thyroid transformation in malignant thyroid lesions [29]. Knockdown of HDAC4 significantly inhibits hepatocellular carcinoma cell proliferation [31]. In this study, we confirmed that HDAC4 is a direct and functional target of miR-506 in PCa cells, and this conclusion is supported by the following evidence: (A) Complementary sequence of miR-506 was identified in the 3'-UTR of HDAC4 mRNA. (B) Overexpression of miR-506 decreased the luciferase reporter activity of wild-type 3'-UTR, but not mutant 3'-UTR of HDAC4. (C) miR-506 overexpression significantly repressed HDAC4 mRNA and protein levels in PCa cells. (D) More importantly, knockdown of HDAC4 significantly inhibited cell proliferation and invasion. The introduction of HDAC4 blocked the effects induced

by miR-506. Together, these results demonstrate that HDAC4 is a downstream mediator of miR-506 function in PCa.

In conclusion, our study demonstrated that miR-506 exerts tumor suppressor effects in PCa by down-regulating the expression of HDAC4. These data suggest that the miR-506/HDAC4 axis is a new and potentially effective target for the treatment of PCa.

## Disclosure of conflict of interest

None.

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