# MicroRNA-16-5p regulates cell survival, cell cycle and apoptosis by targeting *AKT3* in prostate cancer cells

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**Abstract.** Prostate cancer (PCa) is a malignancy with the highest morbidity rate in 105 countries worldwide and was a major cause of cancer-associated death in men in 2018. Accumulating evidence suggests that microRNAs (miRNAs/miRs) have important functions in the carcinogenesis of PCa, and may provide novel treatment targets. Previous studies have indicated that miR-16-5p is associated with PCa. However, the relevance and importance of miR-16-5p in PCa carcinogenesis are still not completely understood. In the current study, we aimed to investigate the role and mechanism of miR-16-5p in PCa carcinogenesis. The results showed that miR-16-5p was markedly downregulated in PCa cells, and MTS assay, colony formation, flow cytometric analyses demonstrated that miR-16-5p inhibited PCa cell survival, regulated cell cycle distribution and induced apoptosis. Moreover, luciferase reporter assay and western blot analysis showed that miR-16-5p directly targets *AKT3* (AKT serine/threonine kinase 3), which is associated with PCa carcinogenesis, and the effects of the downregulation of AKT3 were similar to the effects of upregulation of miR-16-5p in PC-3 cells. In conclusion, our data clarify that miR-16-5p has anticancer functions in PCa cells, and our findings provide experimental evidence to highlight the potential value of miR-targeting treatment strategies for PCa.

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## Introduction

Prostate cancer (PCa) is a malignancy with the highest morbidity in 105 countries worldwide and was a major cause of cancer-associated death in men in 2018 (1). In Europe, PCa is the most common solid neoplasm, with an incidence rate of 214 cases per 1000 men, outnumbering lung and colorectal cancer. PCa affects elderly men more often and therefore is a more serious health concern in developed countries (2,3). In the US, PCa is the most prevalent cancer in males. In 2020, the estimated number of new cases was approximately 191,930, and the number of PCa-associated deaths was approximately 33,330 (4). Due to improved early diagnosis and therapeutics, the mortality rate of patients with PCa has been reduced in the US. However, the morbidity and mortality rates of PCa have been steadily rising in Asia (1,4,5). At present, the main treatment options for patients with PCa include radical prostatectomy, hormone therapy, radiotherapy, chemotherapy or combination therapy (6). Various new targets and treatment methods are also under study, Antognelli *et al* found that glyoxalase 1 had a significant role in prostate cancer progression, and provided an additional candidate for risk assessment in prostate cancer patients and an independent prognostic factor for survival (7,8). Nevertheless, the majority of patients with PCa will relapse and gradually develop castration-resistant prostate cancer (CRPC), which accounts for the majority of PCa mortality (9). In addition, prostate-specific antigen (PSA) is currently the most important biochemical marker for the diagnosis of PCa. Because of the limited specificity of PSA, clinically irrelevant tumors and benign abnormalities are also detected that potentially lead to over-treatment (10-13). Therefore, exploring the underlying mechanisms of PCa occurrence, and identifying novel therapeutic and early diagnosis targets are necessary to improve therapeutic outcomes.

MicroRNAs (miRNAs/miRs) are endogenous single-stranded RNA molecules that are 21-26 nucleotides in length. These highly conserved small non-coding RNAs act as posttranscriptional modulators by binding to the 3'-untranslated regions (UTR) of specific mRNAs (14). Compelling evidence suggests the role of miRNAs in cancer biology having potential as

diagnostic, prognostic and predictive biomarkers (15). Previous studies suggest that miRNA dysregulation mediates the occurrence and development of tumors, including PCa (16,17). For example, Liu *et al* (18) found miR-34a inhibited PCa progenitor cells and metastasis through targeting CD44. Guelfi *et al* supported the notion of a relatively high diagnostic value of the *let-7* family for PCa detection. This research confirmed the potential use of miRNAs as non-invasive biomarkers in the diagnosis of PCa, potentially reducing the invasiveness as actual clinical strategy (19). Another report showed that the downregulation of p63 and its target miR-205 increased the migration of PCa cells (20). The progressive confirmation of the key role of miRNAs in cancer, their stability in biological fluids and their resistance to various storage conditions make miRNAs excellent candidates for the development of minimally invasive biomarkers for cancer diagnosis and prognosis (21).

miR-16-5p is dysregulated in several types of cancer, and is located on chromosome 13q14, which is generally deleted in B cell chronic lymphocytic leukemia (22,23). Notably, the 13q allele deletion has also been reported in PCa, and the frequency of this deletion is associated with tumor progression. The frequency in the early stage is ~30%, rises to 70% in advanced stages and is ~90% in the metastatic stage (24). Therefore, these data suggest that miR-16-5p may have important functions in PCa carcinogenesis. However, the relevance and significance of miR-16-5p in PCa carcinogenesis are still not completely understood.

The PI3K/AKT/mTOR pathway influences cell viability, apoptosis, malignant transformation and metastasis (25,26). In PCa, the PI3K/AKT/mTOR pathway is always abnormally activated. A previous study showed that aberrant PI3K/AKT/mTOR signaling contributes to 42% of elementary and 100% of advanced PCa cases (27). Various miRNAs have also been demonstrated to mediate PCa carcinogenesis through the PI3K/AKT/mTOR pathway. Zhang and Wu illustrated that miR-511-3p can inhibit the expression of its target gene *AKT3* (AKT serine/threonine kinase 3), and functions as a tumor inhibitor and prognostic factor in PCa (28). In addition, *AKT3* is also a latent target for miR-16-5p as predicted using bioinformatics methods (29). However, it has not been fully clarified whether miR-16-5p influences PCa carcinogenesis through the PI3K/AKT/mTOR signaling pathway.

The present results showed that miR-16-5p is prominently downregulated in PCa cells, and miR-16-5p was able to inhibit PCa cell survival, regulate cell cycle distribution and induce apoptosis. Moreover, it was identified that miR-16-5p directly targets *AKT3*, which is closely associated with PCa carcinogenesis, therefore miR-16-5p may exert these functions by modulating the PI3K/AKT/mTOR pathway. Additionally, the effects of the downregulation of AKT3 were similar to those noted following upregulation of miR-16-5p in PC-3 cells. In conclusion, these data clarified that miR-16-5p has anticancer functions in PCa cells, which provides experimental evidence to highlight the potential value of miRNA-targeting treatment strategies for PCa.

## Materials and methods

*Cell lines and cell culture*. Human PCa cell lines PC-3, LNCaP, DU145 and normal prostate epithelial RWPE-1 cells were kindly provided by the Stem Cell Bank, Chinese Academy of Sciences (Shanghai, China). Cells were cultured in the recommended medium containing 10% fetal bovine serum (FBS) (Biological Industries) in an incubator at 37˚C constant temperature with 5% $CO_2$.

*Cell viability analysis*. Cell viability analysis was investigated using methyl tetrazolium salt (MTS) assay. Cells were plated in 96-well plates with 5,000 cells per well and incubated overnight. Then the cells were transfected and incubated for 24, 48 and 72 h to detect viability using the MTS Cell Proliferation Assay (Promega Corp.). At the indicated times, 20 $\mu$l MTS was added to each well and incubated for 90 min in a 37˚C constant temperature incubator. Then the absorbance was detected with a multifunction microplate reader (Tecan Infinite M200, Swiss) at 490 nm.

*Clonogenic analysis*. Cells were harvested at 24 h after transfection and plated into 60-mm dishes with 1,000 cells per dish. After incubation at 37˚C for 14 days, the cells were washed three times with PBS and fixed with 4% paraformaldehyde for 15 min, and then the colonies were stained with 0.5% crystal violet dye for 30 min. Only colonies with ≥50 cells were counted as surviving colonies under a microscope (DE20; Carl Zeis). The colony formation rate was calculated with the following equation: Colony formation rate=(number of clones)/(number of seeded cells) x100%.

*Cell cycle detection*. Cells were cultured for 24 h after transfection, then collected and fixed with pre-chilled 75% ethanol in phosphate-buffered saline (PBS) for 24 h at -20˚C. Next, cells were centrifuged at 100 x g for 5 min and washed three times with PBS. Then cells were resuspended in 50 $\mu$g/ml propidium iodide (BD Biosciences) and 100 $\mu$g/ml RNase (Takara Bio, Inc.) for 30 min in the dark, and analyzed with Amnis® FlowSight flow cytometer (Luminex Corp.). The samples were collected with a minimum of 20,000 cells, and the results were analyzed with ModFit software (v3.2, Verity Software House Inc.).

*Cell apoptosis detection*. AnnexinV/PI Cell Apoptosis kit (BD Biosciences) was used to measure apoptosis. Forty eight hours after transfection, cells (1x$10^6$/ml) were harvested and washed three times with PBS. Then, the residual liquid was removed and cells were resuspended in 75-150 $\mu$l binding buffer. Next, 5 $\mu$l FITC-Annexin V and 5 $\mu$l PI was added to the binding buffer, and incubation was carried out for 10-15 min, protected against exposure to light. After incubation, the samples were detected with Amnis® FlowSight flow cytometer (Luminex Corp.). The apoptotic cell population was calculated using IDEAS Application (v6.0; Luminex Corp.). Both $PI^-$/Annexin $V^+$ and $PI^+$/Annexin $V^+$ cells were considered as apoptotic cells.

*Cell transfection*. MicrON miR-16-5p-mimics or micrON mimics negative control (mimic-NC) and siRNA against AKT3 (si-AKT3) or negative control (NC) were purchased from Ribobio. One day before transfection, cells (1x$10^6$/ml) were cultured in a 60-mm dish at approximately 50-60% confluence, and then transfection was carried out with Lipofectamine 3000 (Thermo Fisher Scientific, Inc.).

Table I. Sequence of the primers for RT-qPCR.

| Gene | Sequence |
|---|---|
| miR-16-5p | 5'-TAGCAGCACGTAAATATTGGCG-3' |
| RNU6 | F 5'-CTCGCTTCGGCAGCACA-3' |
| | R 5'-AACGCTTCACGAATTTG-3' |
| AKT3 | F 5'-CTGAGGACCGCACACGTTTCTA-3' |
| | R 5'-TGGCCATCTTTGTCCAGCATTA-3' |
| GAPDH | F 5'-TCGCTCTCTGCTCCTCCTGTTC-3' |
| | R 5'-CGCCCAATACGACCAAATCC-3' |

F, forward; R, reverse.

Following 6 h of incubation, the old medium was discarded and fresh medium supplemented with 10% FBS was added.

*Quantitative RT-PCR analysis*. Total RNA was obtained using TRIzol reagent (Thermo Fisher Scientific, Inc.) according to the previous report (23). The quantitative RT-PCR of miR-16-5p was mainly divided into two steps. First, the total RNA was transcribed into cDNA using the Mir-X™ miRNA First Strand Synthesis kit (Takara). Then amplification reaction was proceeded using SYBR-Green Advantage qPCR Premix (Takara) in the QuantStudio 5 Real-Time PCR-System (Thermo Fisher Scientific, Inc.) with specific primers (Table I). The quantitative RT-PCR of mRNA was also divided into two steps. First, the total RNA was transcribed into cDNAs by QuantiTect Reverse Transcription kit (Qiagen); and then amplified in the same way as miR-16-5p with individual primers (Table I). The PCR thermocycling conditions were as follows: Initial denaturation at 95˚C for 30 sec; 40 cycles of denaturation at 95˚C for 5 sec, annealing at 60˚C for 34 sec. The results were analyzed using the comparative threshold cycle ($2^{-\Delta\Delta Cq}$) method (30), and the relative expression levels were normalized by the expression levels of RNU6 or GAPDH.

*Dual-luciferase reporter assay*. Wild-type (wt) or mutant-type (mut) *AKT3* 3'-UTR was chemically synthesized (BGI-Tech, China) and cloned into psiCHECK2 luciferase vector. Cells ($1x10^5$) were seeded in 48-well plates and cotransfected with the constructed luciferase vector plus corresponding miRNA. After 48 h transfection, firefly and *Renilla* luciferase activities were measured by Dual Luciferase Reporter Assay kit (Promega Corp.).

*Western blot analysis*. Twenty-four hours after transfection, cells were harvested and total protein was obtained by ice-cold RIPA cell lysis buffer (Beyotime Institute of Biotechnology) with fresh protease and phosphatase inhibitor (Thermo Fisher Scientific, Inc.). After incubation for 30 min on ice, the supernatant was collected through centrifuging for 15 min at 15,294 x g at 4˚C. Then the concentration of protein was determined by the BCA Assay kit (Boster Biological Technology). According to the standard procedures, protein lysate (30 $\mu$g) was loaded on 8-15% SDS-PAGE and transferred onto a pre-activated PVDF membrane (Merck Millipore). Then the membranes were blocked with 5% skim milk for 1-1.5 h. Next, antibodies against cyclin D1 (1:1,000 dilution; cat. no. 2922; Cell Signaling Technology, Inc.), AKT (1:1,000 dilution; cat. no. 4691; Cell Signaling Technology, Inc.), phosphorylated (p-)AKT (Ser473) (1:2,000 dilution; cat. no. 4060; Cell Signaling Technology, Inc.), Rb (1:1,000 dilution; cat. no. 9313; Cell Signaling Technology, Inc.), p-Rb (Ser807/811) (1:500 dilution; cat. no. 8516; Cell Signaling Technology, Inc.) and AKT3 (1:1,000 dilution; product code ab152157; Abcam), GAPDH (1:10,000 dilution; product code ab181602; Abcam), p-mTOR (Ser2448) (1:1,000 dilution; product code ab109268; Abcam), mTOR (1:300 dilution; cat. no. bsm-50414M; Bioss, Inc.) were used as primary antibodies and incubated overnight at 4˚C. On the second day, the membranes were washed three times with TBST, and HRP-linked anti-mouse (1:10,000 dilution; cat. no. 7076; Cell Signaling Technology, Inc.) or anti-rabbit (1:10,000 dilution; cat. no. 7074; Cell Signaling Technology, Inc.) IgG secondary antibodies were added and incubation was carried out for 1 h at room temperature. The results were normalized by GAPDH levels. The blots were detected using an enhanced chemiluminescence kit (Thermo Fisher Scientific, Inc.) and imaged using chemiluminescence gel imaging system (AI680; GE Healthcare, Inc.).

*Bioinformatics analysis*. The interaction between miR-16-5p and *AKT3* was predicted by MiRanda, TargetScan 7.1, picTar and RNA 22. Starbase v3.0 project (http://starbase.sysu.edu.cn/) was used for the analysis of miR-16-5p and AKT3 expression levels in PCa samples. The expression data of genes in cancers were downloaded from TCGA project via Genomic Data Commons Data Portal.

*Statistical analysis*. The data from at least three independent experiments are expressed as the means ± standard deviations (SD). Statistical analysis was performed using the two-tailed Student's t-test or ANOVA with Tukey's post hoc test. $P<0.05$ was considered to indicate a statistically significant difference.

## Results

*miR-16-5p is downregulated in PCa cell lines and suppresses cell survival*. Abnormal expression of miR-16-5p has been found in several cancer types (31-33), therefore the present study investigated the expression level of miR-16-5p using RT-qPCR in three human PCa cell lines (LNCaP, PC-3 and DU145) and one normal prostate epithelial cell line (RWPE-1). The results showed that miR-16-5p was markedly downregulated in the three PCa cell lines when compared to the RWPE-1 cells (Fig. 1A), indicating that miR-16-5p may function in the carcinogenesis of PCa. To further investigate this, the role of miR-16-5p in cell survival was evaluated. A custom synthesized miR-16-5p mimic was transfected into PCa cells to upregulate miR-16-5p levels. This resulted in an 8-fold increase in the miR-16-5p expression levels (Fig. 1B). MTS analysis was performed to evaluate cell proliferation. Compared with the untransfected (mock) group, overexpression of miR-16-5p significantly suppressed the proliferation of PC-3 cells (Fig. 1C). Meanwhile, overexpression of miR-16-5p

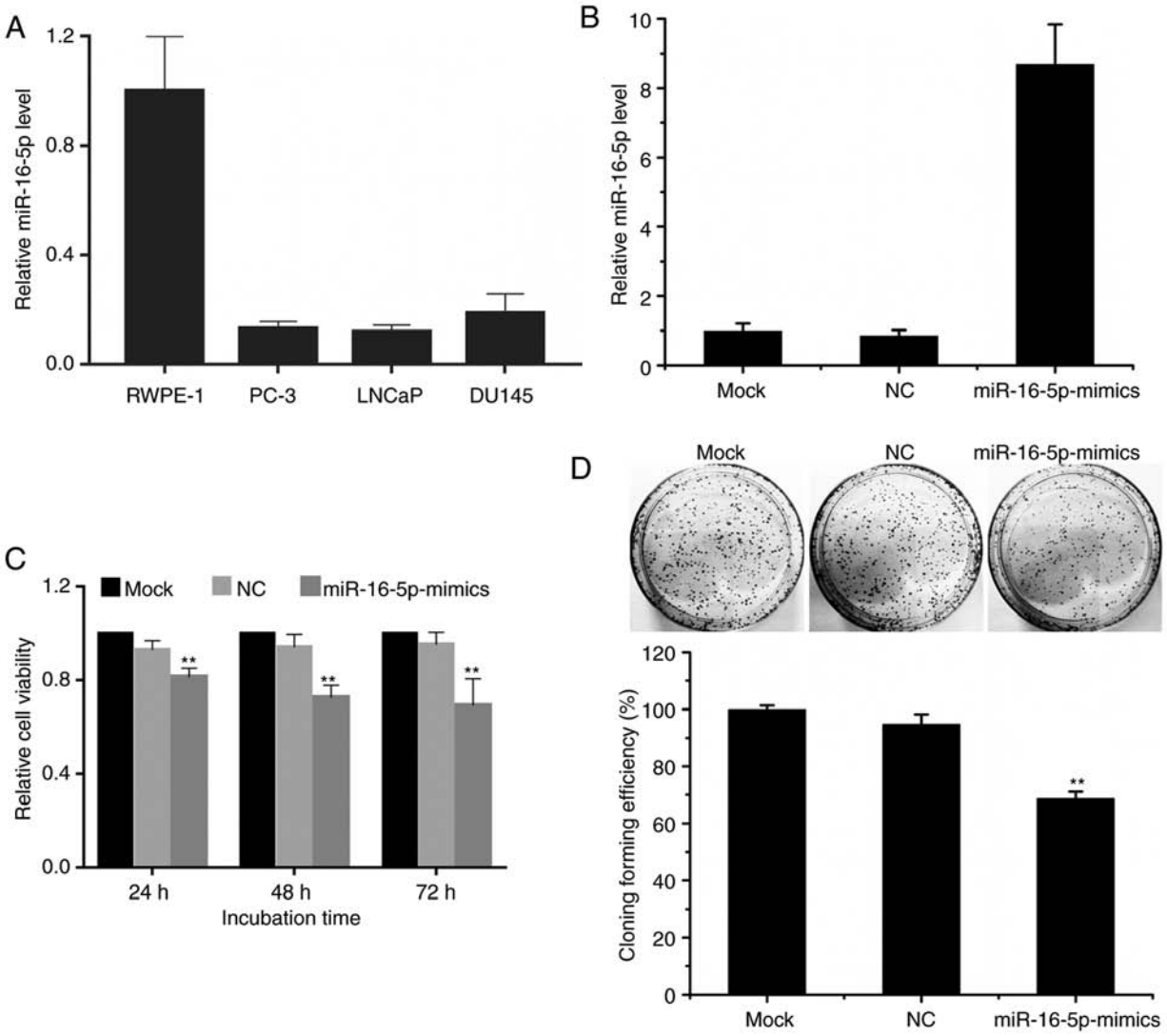


Figure 1. miRNA-16-5p is downregulated in PCa cells and suppresses cell survival. (A) Relative expression of miR-16-5p in three human PCa cell lines (LNCaP, PC-3 and DU145) and one normal prostate epithelial cell line (RWPE-1) was analyzed using reverse transcription-quantitative PCR. (B) miR-16-5p-mimics and negative control mimic (NC) were transfected into PC-3 cells and the relative expression of miR-16-5p was determined at 24 h after transfection. (C) Proliferation of PC-3 cells was detected at the indicated time points (24, 48 and 72 h) after transfection using the methyl tetrazolium salt assay. (D) Colony formation of PC-3 cells was detected 14 days after transfection. Values are presented as mean ± standard deviation (n≥3). $^{**}$P<0.01 vs. mock. miRNA/miR, microRNA; PCa, prostate cancer; NC, negative control mimic.

also significantly inhibited the colony formation of PC-3 cells (Fig. 1D). These data demonstrated that miR-16-5p was notably downregulated in PCa cells and suppressed cell survival.

*miR-16-5p induces apoptosis in PCa cells*. In order to analyze the effect of miR-16-5p on cell apoptosis, miR-16-5p-mimics and the mimic-negative control (mimic-NC) were transfected into PC-3 cells, and cell apoptosis was detected using the Annexin V/PI double staining method. Compared with the untransfected (mock) group, miR-16-5p mimics significantly increased the expression levels of miR-16-5p in the PC-3 cells (Fig. 2A), and compared with the untransfected (mock) group, overexpression of miR-16-5p caused a significant increase in the percentage of apoptotic cells. The rate of apoptosis was 38.7% in miR-16-5p upregulated cells compared with 2.73 and 6.13% in the mock and mimic-NC groups, respectively (Fig. 2B and C), indicating that overexpression of miR-16-5p induced apoptosis.

*miR-16-5p regulates cell cycle distribution*. As previous studies have suggested that cell cycle distribution is associated with tumorigenesis (34,35), the effect of miR-16-5p on cell cycle distribution was analyzed using flow cytometry. Forced overexpression resulted in a 10-fold increase in miR-16-5p expression levels in PC-3 cells (Fig. 3A). Compared with the mock group, a significantly decreased number of cells in the S phase were observed in the miR-16-5p overexpression group (Fig. 3B and C). Meanwhile, the ratio of $G_0/G_1$ phase cells was significantly increased in miR-16-5p-overexpression cells, leading to $G_0/G_1$ phase arrest. Therefore, these data suggest that miR-16-5p can induce $G_0/G_1$ arrest and reduce the ratio of S phase cells, resulting in suppressed cell proliferation.

*AKT3 is a direct target of miR-16-5p*. miRs act as regulators of target gene expression, thus four online miRNA target-prediction databases (MiRanda, TargetScan 7.1, picTar and RNA22) (29,36-38) were used to identify the probable

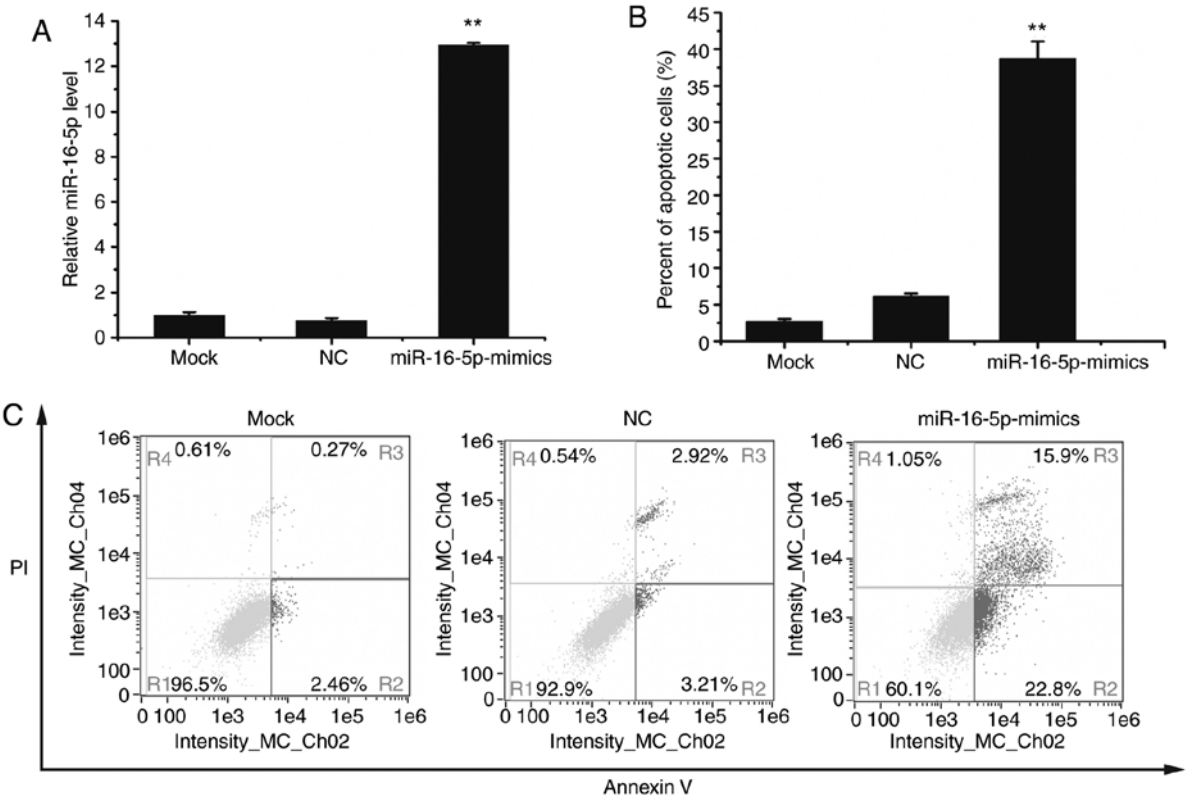


Figure 2. miRNA-16-5p induces apoptosis in PCa cells. miR-16-5p-mimics and negative control mimic (NC) were transfected into PC-3 cells. (A) Relative expression of miR-16-5p was determined 24 h after transfection by RT-qPCR. (B and C) The apoptotic rate of PC-3 cells was determined 48 h after transfection using Annexin V/PI staining analysis. Values are presented as the mean ± standard deviation (n≥3). $^{**}$P<0.01 vs. mock. miRNA/miR, microRNA; PCa, prostate cancer; NC, negative control mimic.

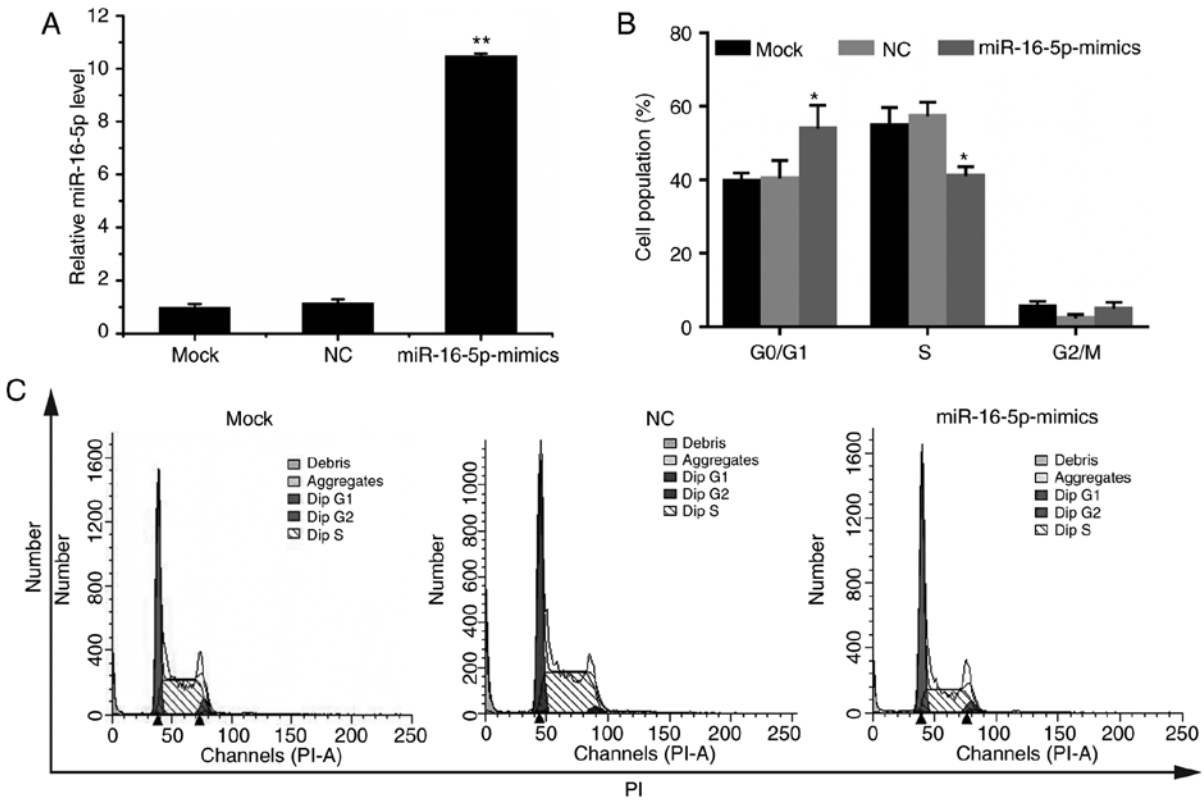


Figure 3. miRNA-16-5p regulates cell cycle progression in PCa cells. miR-16-5p-mimics and the negative control mimic (NC) were transfected into PC-3 cells. (A) Relative expression of miR-16-5p was determined 24 h after transfection. (B and C) Cell cycle distribution was analyzed 24 h after transfection using flow cytometry. Values are presented as the mean ± standard deviation (n≥3). $^{*}$P<0.05, $^{**}$P<0.01 vs. mock. miRNA/miR, microRNA, PCa, prostate cancer; NC, negative control mimic.

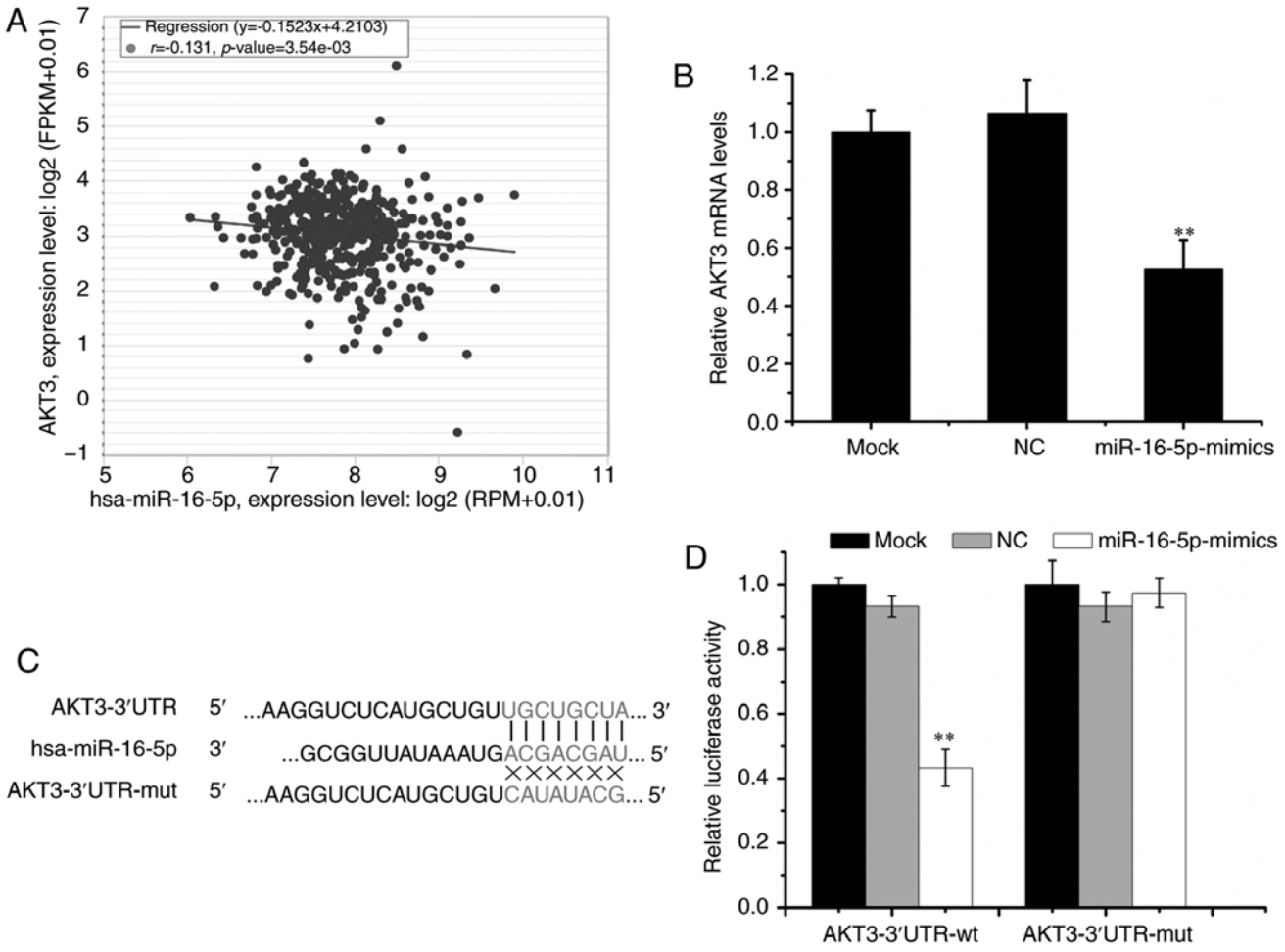


Figure 4. *AKT3* is a direct target of miRNA-16-5p in PCa cells. (A) Correlation investigation between AKT3 and miR-16-5p from 495 PCa samples (Date source: starBase v3.0 project). (B) miR-16-5p-mimics and negative control mimic (NC) were transfected into PC-3 cells and the relative expression of *AKT3* mRNA was determined 24 h after transfection using reverse transcription-quantitative PCR. (C) Schematic diagram of miRNA-16-5p binding site in the *AKT3*-3'UTR-wt or the site-directed mutant *AKT3*-3'UTR-mut. (D) Effects of miR-16-5p-mimics and NC on luciferase activities in cells co-transfected with *AKT3*-3'UTR-wt or *AKT3*-3'UTR-mut vectors. Values are presented as mean ± standard deviation (n≥3). $^{**}$P<0.01 vs. mock. miRNA, microRNA; PCa, prostate cancer; NC, negative control mimic; wt, wild-type; mut, mutant; 3'UTR, 3' untranslated region. *AKT3*, AKT serine/threonine kinase 3.

targets of miR-16-5p. Several carcinogenesis-associated proteins were predicted as targets of miR-16-5p, and *AKT3*, a key downstream molecule of the PI3K/AKT/mTOR signaling pathway, was predicated as a miR-16-5p target in all databases. Additionally, the correlation investigation between AKT3 and miR-16-5p from 495 PCa samples (39) indicated that there was a negative correlation between AKT3 and miR-16-5p (r=-0.131, P=3.54e-03, Fig. 4A) (Date source: starBase v3.0 project) (40). To investigate the association between miR-16-5p and *AKT3*, miR-16-5p overexpression was induced and the mRNA levels of *AKT3* were analyzed using RT-qPCR. The results showed that miR-16-5p inhibited the transcription of *AKT3* (Fig. 4B). Furthermore, the 3'UTR of *AKT3* mRNA contained a conserved region that was complementary with the seed sequence of miR-16-5p (Fig. 4C). Therefore, the psiCHECK2-*AKT3*-3'UTR-wt and psiCHECK2-*AKT3*-3'UTR-mut vectors were constructed to identify whether *AKT3* mRNA is a direct target of miR-16-5p. The results showed that upregulation of miR-16-5p lessened the luciferase activity of wild-type vector cells compared with the mutant vector cells (Fig. 4D). It was then clarified that ectopic expression of miR-16-5p also significantly decreased the protein levels of AKT3 compared with the untransfected (mock) group (Fig. 5A and B). *AKT3* plays an important role in the PI3K/AKT/mTOR signaling pathway, and the PI3K/AKT/mTOR pathway is always aberrantly activated in PCa (25,41-43). Our western blot analysis showed that miR-16-5p regulated the expression of AKT3, AKT, p-AKT, mTOR, p-mTOR, Rb, p-Rb and CCND1 (Fig. 5A), indicating that the mechanism by which miR-16-5p modulates PCa development may involve the PI3K/AKT/mTOR signaling pathway. Overall, these data demonstrated that *AKT3* was negatively associated with miR-16-5p and was a direct target of miR-16-5p.

*Knockdown of AKT3 inhibits cell survival, regulates cell cycle distribution and induces apoptosis*. As *AKT3* was shown to be a direct target of miR-16-5p and upregulation of miR-16-5p influences PCa carcinogenesis, it was ascertained whether the regulatory action of miR-16-5p is mediated by directly targeting *AKT3*. To validate this, AKT3 was knocked down using a specific siRNA in PCa cells, and then cell survival, cell cycle distribution and cell apoptosis were evaluated after transfection with si-AKT3. Western blotting confirmed that

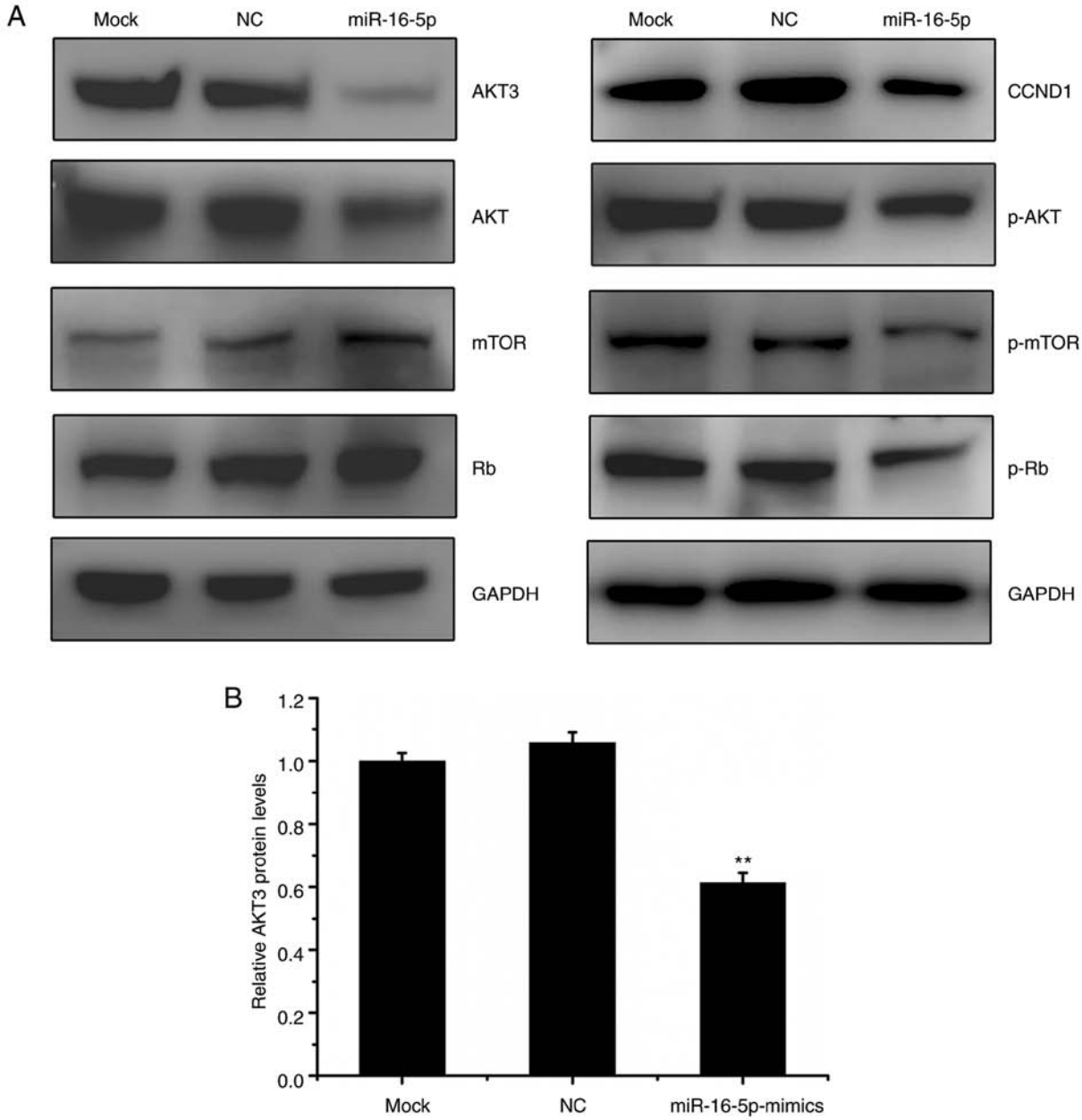


Figure 5. miRNA-16-5p modulates the PI3K/AKT/mTOR pathway in PCa cells. miR-16-5p-mimics and negative control mimic (NC) were transfected into PC-3 cells. (A and B) Relative expression of AKT3 protein and key molecules of the PI3K/AKT/mTOR signaling pathway were determined 48 h after transfection using western blotting. Values are presented as mean ± standard deviation (n≥3). $^{**}$P<0.01 vs. control. miRNA/miR, microRNA; PCa, prostate cancer; NC, negative control mimic; p-, phosphorylated; PI3K, phosphoinositide 3-kinase; mTOR, mammalian target of rapamycin; AKT3, AKT serine/threonine kinase 3.

the expression of AKT3 was reduced after transfection with three different siRNAs (S1, S2 and S3) when compared to the mock group, and si-AKT-3 exhibited the strongest inhibition effect on AKT3 levels. Thus, siRNA3 was selected to knock down AKT3 expression in PC-3 cells and is subsequently referred to as si-AKT3 (Fig. 6A). Then cell proliferation was detected using MTS analysis after transfection with si-AKT3 and it was demonstrated that knockdown of AKT3 significantly suppressed the proliferation of PC-3 cells compared with the mock group (Fig. 6B). Meanwhile, compared with the mock group, knockdown of AKT3 also significantly inhibited colony formation in PC-3 cells (Fig. 6C and D). Next, it was determined whether knockdown of AKT3 influenced cell cycle progression and apoptosis. Cell cycle distribution was detected as aforementioned, and the results showed that knockdown of AKT3 led to a significantly decreased number of cells in the S phase and a significantly increased number of cells in the $G_0/G_1$ phase compared with the mock group, coinciding with the suppression of cell survival (Fig. 7A and B). For cell apoptosis, downregulation of AKT3 by siRNA induced a significantly higher percentage of apoptotic cells compared with the mock cells (Fig. 7C and D). Taken together, these data suggest that downregulation of AKT3 through siRNA had similar effects to forced overexpression of miR-16-5p in regards to cell viability, cell cycle progression and apoptosis. Therefore, it was speculated that miR-16-5p suppresses tumor growth, regulates cell cycle progression and induces apoptosis by targeting *AKT3*.

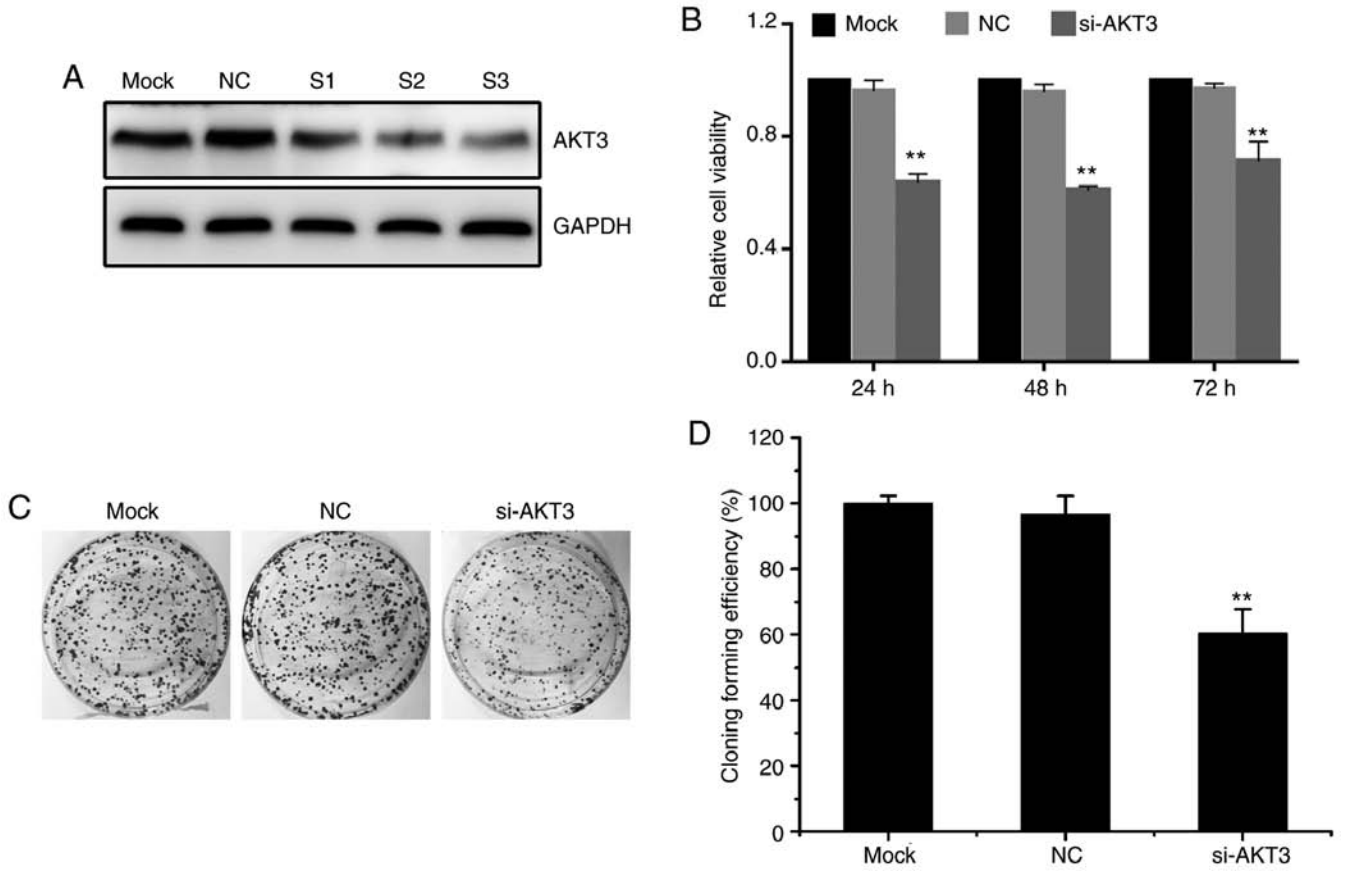


Figure 6. Knockdown of AKT3 inhibits cell survival in PCa cells. si-AKT3 (S1, S2 and S3) and NC were transfected into PC-3 cells. (A) Expression levels of AKT3 protein were determined 24 h after transfection using western blotting. (B) Cell viability of PC-3 cells was detected at the indicated time points after transfection using the methyl tetrazolium salt assay. (C and D) Colony formation of PC-3 cells was detected 14 days after transfection. Values are presented as mean ± standard deviation (n≥3). $^{**}$P<0.01 vs. mock. PCa, prostate cancer; NC, negative control; si, small interfering; AKT3, AKT serine/threonine kinase 3.

## Discussion

Accumulating evidence has shown that abnormal expression of miRNAs is common in several cancer types, indicating that miRNA dysregulation is associated with tumor development and progression (44). Mearini *et al* showed miR-146a-5p, miR-141-3p and miR19a-3p were differentially expressed in urine sediments from patients with bladder cancer, compared with subjects with bladder inflammation and age-matched healthy controls (45). Previous research has reported that miRNAs regulate the occurrence and progression of tumors by negatively modulating tumor-associated genes. Therefore, miRNAs are also viewed as potential diagnostic biomarkers and molecular treatment targets (15,44,46). The applications of these new generation therapies are being investigated in pre-clinical trials, including some miRNA-based therapies. For example, treatment with MRX34, a miR-34 analog packed with lipid nanoparticles, has been approved for human clinical trails in aggressive non-small cell lung cancer (NSCLC) (47). In addition, one phase I clinical trail is investigating the treatment of patients with scleroderma using miR-29 mimics, while another is investigating the LNA-based anti-miR-155 strategy in patients with cutaneous T cell lymphoma (44). Thus, miRNA-based therapeutics may be a therapeutic option for patients with cancer in the future, and exploring the function and mechanisms of specific cancer-associated miRNAs may help inform these treatments.

miR-16-5p resides on chromosome 13q14, which is generally deleted in B cell chronic lymphocytic leukemia (22). miR-16-5p is also dysregulated in several cancer types, such as gastric cancer, breast cancer and lung cancer (31,48,49). In prostate cancer (PCa), the 13q allele deletion has also been reported, and the frequency of this is associated with tumor progression. The frequency in the early stage is ~30%, rises to 70% in advanced stage and is ~90% in metastatic stage (50). Bonci *et al* found that miR-16 deletion or dysfunction of miR-16 targets is associated with poor prognosis in PCa (51). These data indicate that miR-16-5p may have pivotal roles in PCa oncogenesis. The present study demonstrated that miR-16-5p is significantly downregulated in PCa cells and suppresses PCa cell survival, regulates cell cycle distribution and induces apoptosis. The underlying mechanisms of these functions may involve direct targeting of *AKT3* and modulation of the PI3K/AKT/mTOR signaling pathway. Recently, a phase I clinical trail using miR-16-5p mimics was approved for patients with NSCLC or malignant pleural mesothelioma (44). It is anticipated that the success this clinical trail may highlight the value of miR-16-5p targeting as a promising therapeutic strategy for patients with PCa.

The PI3K/AKT/mTOR pathway is always aberrantly activated in PCa and is closely associated with cell viability, metastasis, invasion and resistance to therapy (52). Aberrations of the PI3K/AKT/mTOR signaling pathway have been identified in 40% of patients with early-stage PCa and 70-100%

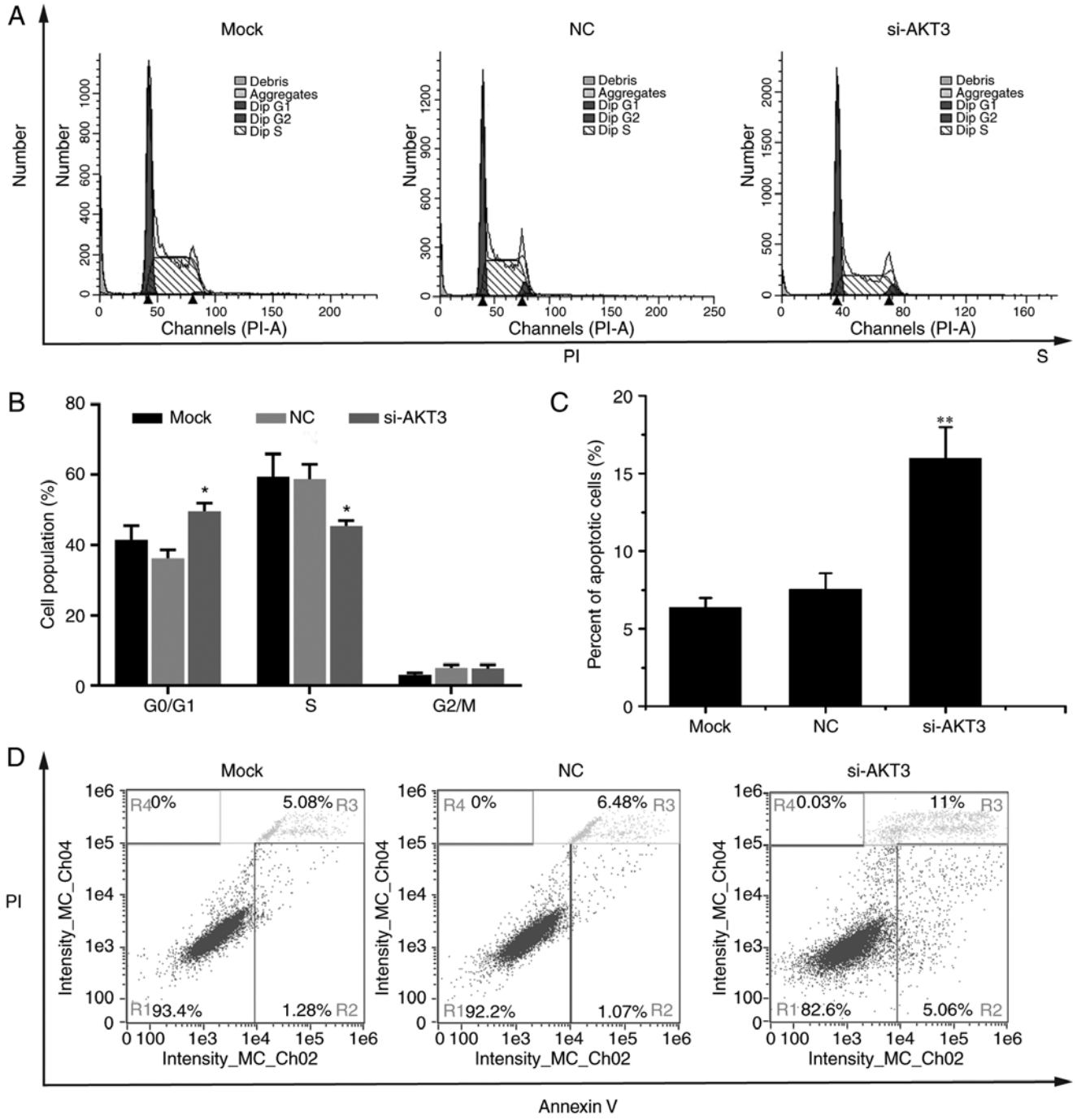


Figure 7. Knockdown of AKT3 regulates cell cycle distribution and induces apoptosis in PCa cells. The si-AKT3 and NC were transfected into PC-3 cells. (A and B) Cell cycle distribution was analyzed 24 h after transfection using flow cytometry. (C and D) The apoptotic rate of PC-3 cells was determined 48 h after transfection using Annexin V/PI staining analysis. Values are presented as mean ± standard deviation (n≥3). $^{*}$P<0.05, $^{**}$P<0.01 vs. mock. PCa, prostate cancer; si, small interfering; NC, negative control; AKT3, AKT serine/threonine kinase 3.

of advanced cases. One of the main reasons of this unusual activation is the deletion of *PTEN* (53). Deletion or mutation of *PTEN* is always assocaited with metastasis, progression to castration-resistant prostate cancer (CRPC) and poor prognosis in PCa (54). Serine/threonine kinase AKT is located on a central signaling node in the PI3K/AKT/mTOR signaling pathway, and three isoforms of AKT have been identified: AKT-1, AKT-2 and AKT-3. A previous study demonstrated that the expression levels of AKT3 in PCa are much higher compared with those in normal prostate cells (42). Thus, it was speculated that *AKT3* has an important function in PCa. Notably, *AKT3* was predicated as a miR-16-5p target in online target-prediction databases, and the correlation investigation between AKT3 and miR-16-5p from 495 samples indicated that there was a negative correlation between AKT3 and miR-16-5p. Therefore, miR-16-5p may function as an anticancer miRNA in PCa through directly targeting *AKT3*. The present results support this hypothesis, demonstrating that miR-16-5p regulates cell survival, cell cycle and apoptosis by targeting *AKT3* in PCa cells. In the PI3K/AKT/mTOR pathway, AKT is activated by phosphorylation. Activated AKT then phosphorylates a number of substrates that regulate diverse cellular functions, such as cell survival,

differentiation, apoptosis, glucose transport and migration (55). mTOR, another substrate of AKT, also has essential functions in tumorigenesis (41,56,57). In the present study, both knockdown of AKT3 and overexpression of miR-16-5p repressed PCa cell viability, regulated cell cycle distribution and induced apoptosis. Additionally, western blotting showed that miR-16-5p regulated the expression of AKT3, AKT, p-AKT, mTOR and p-mTOR, the key components of the PI3K/AKT/mTOR signaling pathway. As mentioned above, PI3K/AKT/mTOR is closely related to the progression of prostate cancer by modulating cell viability, cell cycle, metastasis and invasion. We also observed that the expression of cell cycle-related proteins Rb, p-Rb and CCND1 was regulated by miR-16-5p, indicating that the mechanism by which miR-16-5p modulates PCa development may involve the PI3K/AKT/mTOR signaling pathway.

In conclusion, the present study demonstrated that miR-16-5p was markedly downregulated in PCa cells and suppressed cell survival, regulated cell cycle distribution and induced apoptosis in PCa cells. Additionally, *AKT3* was negatively associated with miR-16-5p and was found to be a direct target of miR-16-5p. Knockdown of AKT3 had similar effects as the forced expression of miR-16-5p in regards to cell viability, cell cycle progression and apoptosis. Hence, it was speculated that miR-16-5p functions as an anticancer miRNA by direct targeting *AKT3*, and the mechanisms underlying this may involve the PI3K/AKT/mTOR signaling pathway. The present study may provide experimental support to highlight the value of miRNA-targeting treatment strategies for PCa.

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**Availability of data and materials**

All data generated or analyzed during this study are included within this article.

**Authors' contributions**

HZ, FW and CD designed the research. FW, AM, WW, LL and LG conducted the experiments. YX, JY, YC and JS analyzed the data. FW wrote the manuscript. All authors carefully reviewed the manuscript. All authors read and approved the final version of this manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

**Ethics approval and consent to participate**

Not applicable.

**Patient consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

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