

ORIGINAL ARTICLE

MicroRNA-140 inhibits proliferation and promotes apoptosis and cell cycle arrest of prostate cancer via degrading SOX4

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Summary

Purpose: To explore the regulatory roles of microRNA-140 and SOX4 in prostate cancer (PCa) tissues and paracancerous tissues, and their underlying mechanism.

Methods: MicroRNA-140 expressions in PCa tissues, paracancerous tissues and PCa cell lines were detected by quantitative real-time polymerase chain reaction (qRT-PCR). Proliferation, apoptosis and cell cycle of PCa cells after altering expressions of microRNA-140 and SOX4 were detected by MTT assay and flow cytometry, respectively. The regulatory effect of microRNA-140 on SOX4 was detected by Western blot and qRT-PCR. The binding condition of microRNA-140 on SOX4 was verified by luciferase reporter gene assay.

Results: MicroRNA-140 was downregulated in PCa tissues compared to paracancerous tissues. In particular, lower ex-

pression of microRNA-140 was found in PCa with Grade I+II compared to Grade III+IV. In vitro, microRNA-140 expression was negatively correlated with proliferative and invasive abilities, while positively correlated with apoptosis of PCa cells. MicroRNA-140 promoted cell cycle arrest in G0/G1 phase. SOX4 expression was inhibited by microRNA-140 overexpression in PCa cells.

Conclusions: Downregulated microRNA-140 promotes proliferation and cell cycle arrest, but inhibits apoptosis of PCa cells. MicroRNA-140 inhibits PCa development via degrading SOX4.

Key words: apoptosis, cell cycle arrest, MicroRNA-140, proliferation, prostate cancer, SOX4

Introduction

Prostate cancer (PCa) ranks second in tumor prevalence in the world. The rapidly increased incidence of PCa poses a huge burden on affected population and their families [1]. Therefore, it is urgent to find new targets for the prevention and treatment of this disease. MicroRNAs regulate a wide variety of vital processes. Dysfunction of microRNAs results in occurrence of various diseases, including different types of tumors [2]. Accumu-

lating researches have reported that microRNAs participate in tumor development [2]. Other studies have also demonstrated that microRNAs have certain impact on the transcription of downstream target genes [3]. The specific role of microRNAs in tumorigenesis has been well recognized, which provides an essential direction in improving the prediction and treatment efficacy. It is reported that microRNA-140 is greatly involved in the occur-

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rence and progression of PCa. The potential mechanism of microRNA-140 in regulating PCa, however, has not been completely elucidated [4].

SOX4 possesses a crucial role in embryonic development, maintenance of stem cells, and regulation of cell differentiation. SOX4 acts as a proto-oncogene in PCa. SOX4 expression is closely related to tumor stage, which also regulates invasion and metastasis of PCa via inducing epithelial-mesenchymal transition (EMT). Hence, SOX4 can serve as a prognostic factor for PCa [5-7]. Exploration on the relationship between microRNA-140 and SOX4 could provide a theoretical basis for investigating the pathogenesis of PCa, so as to improve the clinical outcomes of PCa patients.

Methods

Reagents and instruments

α -MEM, glutamine and penicillin were purchased from Gibco (Grand Island, NY, USA); Fetal bovine serum (FBS), 0.25% trypsin, LipofectamineTM2000, luciferase reporter gene assay kit, anti-SOX4 and β -actin were purchased from Sigma-Aldrich (St. Louis, MO, USA); MicroRNA-140 sequences, inhibitor and mimics were constructed from Ribo Bio (Guangzhou, China); PC-3, NHPE and DU-145 cell lines were purchased from Sciencell (Carlsbad, CA, USA); PCR reagents were purchased from Applied Biosystems (Foster City, CA, USA); bicinchoninic acid (BCA) kit was purchased from Best Bio (Shanghai, China); Inverted microscope was purchased from Nikon (Tokyo, Japan); Scanning electron microscope was purchased from Hitachi S-4800 (Tokyo, Japan); Flow cytometry was purchased from Bio-TEK Instruments, (Biotek Winooski, Vermont, USA). The sequences used in this experiment were as follows: Mi-

croRNA-140 mimic, 5'-UGGUAUCCCAUUUUGGUGA-3'; SOX4, 5'-AACGUGCCGUUUAAACCACU-3'.

Sample collection

Twenty-five pairs of PCa tissues and paracancerous tissues pathologically confirmed in our hospital from December 2017 to February 2018 were selected. Among them, 13 cases were Grade I+II and 12 were Grade III+IV. This study was approved by the ethics committee of the First Affiliated Hospital of Jinan University. Signed informed consents were obtained from all participants before study entry.

Cell culture

NHPE, PC-3 and DU-145 cells were cultured in RPMI 1640 (Roswell Park Memorial Institute-1640) containing 10% fetal bovine serum (FBS) and maintained in a 5% CO₂ incubator at 37°C. Cells were digested with 0.25% trypsin after cell confluence was up to 80-90%.

Cell transfection

PC-3 and DU-145 cells were seeded into the 96-well plates and 6-well plates at a density of 1×10^5 /mL. Cell transfection was performed according to the instructions of Lipofectamine 2000 manufacturer, when the confluence was up to 60-80%. Transfected cells were collected for the following experiments.

Quantitative real-time polymerase chain reaction (QRT-PCR)

Total RNA was extracted from the cells using the TRIzol kit (Invitrogen, Carlsbad, CA, USA), followed by measurement of RNA concentration using an ultraviolet spectrophotometer. The complementary deoxyribose nucleic acid (cDNA) was synthesized according to the instructions of the Primescript RT Reagent. PCR was performed based on the following reactions: 95°C for 15 s, 60°C for 30 s and 72°C for 45 s, for a total of 40

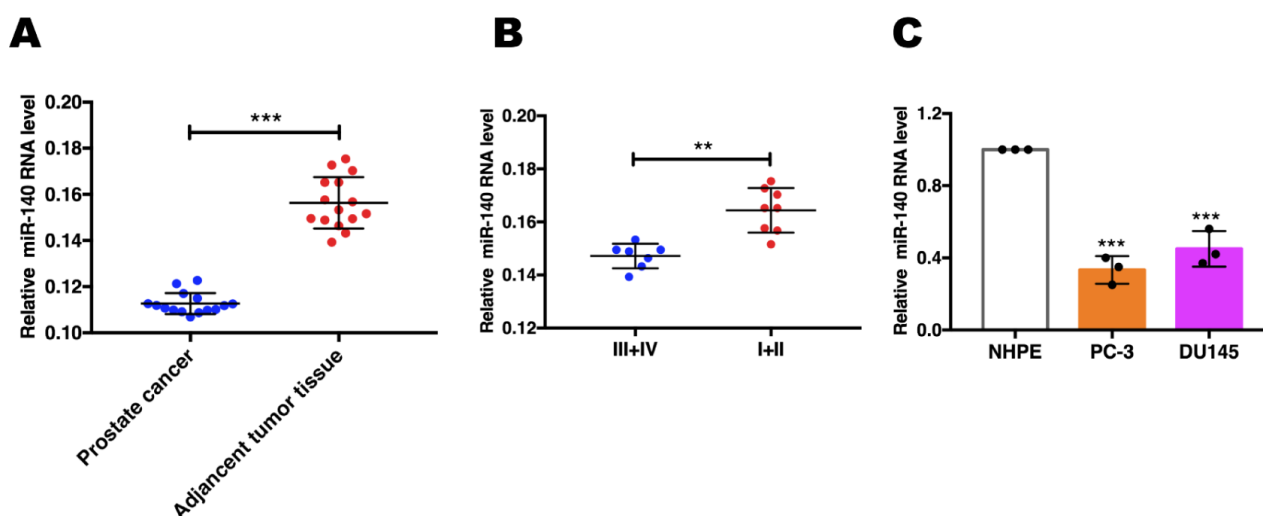


Figure 1. MicroRNA-140 was downregulated in PCa. **A:** MicroRNA-140 was downregulated in PCa tissues compared with paracancerous tissues. **B:** Lower expression of microRNA-140 was found in PCa with Grade I+II than in Grade III+IV. **C:** MicroRNA-140 was downregulated in PCa cell lines. ** $p < 0.01$, *** $p < 0.001$.

cycles. The relative expression level of the target gene was expressed by $2^{-\Delta\Delta Ct}$.

Western blot

The radioimmunoprecipitation assay (RIPA) protein lysate (Beyotime, Shanghai, China) was used to extract the total protein in each group of cells and tissues. The bicinchoninic acid (BCA) method was performed to quantify the protein concentration. Protein samples were electrophoresed on polyacrylamide gels and then transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA). After blocking with 5% skimmed milk, the membranes were incubated with primary antibody at 4°C overnight. The membranes were incubated with the secondary antibody after rinsing with the buffer solution (TBST). Chemiluminescence was used to expose the protein bands on the membrane.

MTT assay

After transfection for 0, 24, 48 and 72 hrs, PC-3 and DU-145 cells were collected. Twenty μ L of MTT solution was added to each well and incubated for 4 hrs. Subsequently, 150 μ L of dimethyl sulfoxide (DMSO) was added

in each well and the absorbance at 450 nm wavelength was measured using a microplate reader.

Cell cycle detection

1.0×10^5 /mL transfected cells were collected and centrifuged. After the supernatant was discarded, cells were resuspended in $1 \times$ binding buffer. 300-500 μ L of suspension was used to label the cell cycle indicators and detected using flow cytometry.

Cell apoptosis detection

Transfected PC-3 and DU-145 cells were collected and centrifuged. 1×10^5 /mL of cell suspension was washed with 0.1 mol/L phosphate buffered saline (PBS) and fixed with 700 mL/ethanol. Subsequently, cells were treated with 10 g/L Triton 100 for 10 min, 10 g/L RNase for 10 min and 2.5 g/L propionic iodide for 30 min, sequentially. Cell apoptosis was then detected using flow cytometry.

Luciferase reporter gene assay

PC-3 and DU-145 cells in logarithmic growth phase were seeded into 6-well plates at a density of 5×10^4 /mL.

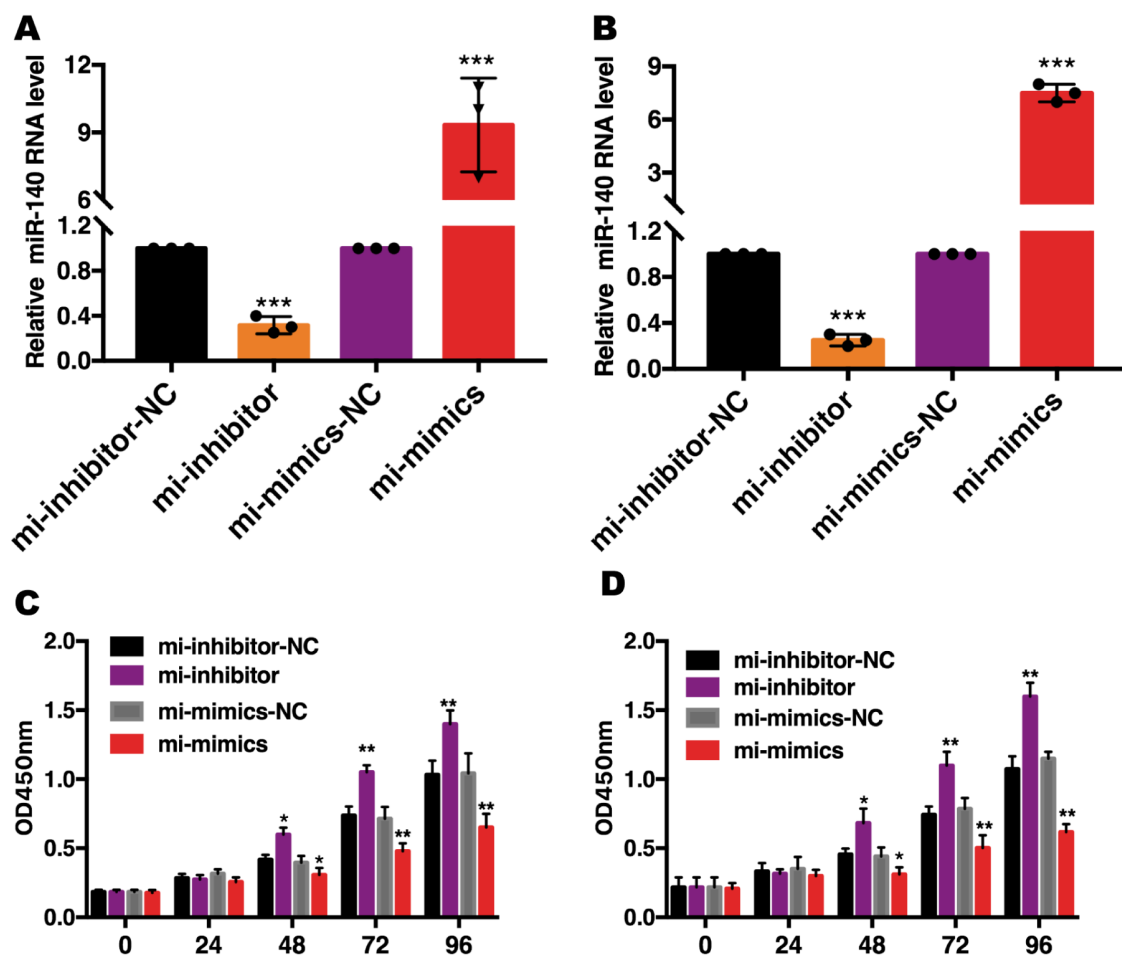


Figure 2. MicroRNA-140 inhibited the proliferation of PCa cells. **A,B:** The transfection efficiency of microRNA-140 mimics (**A**) and microRNA-140 inhibitor (**B**). **C:** Overexpression of microRNA-140 remarkably inhibited the proliferative ability of PCa cells. **D:** Knockdown of microRNA-140 remarkably promoted the proliferative ability of PCa cells. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Co-transfection of microRNA-140 negative control or microRNA-140 mimics and wild-type SOX4 (SOX4 WT) or mutant-type SOX4 (SOX4 MUT) was performed according to the instructions of Lipofectamine 2000. Luciferase activity was detected using luciferase reporter gene assay kit. Relative luciferase activity = Luciferase activity value of firefly / Luciferase activity value of Renilla.

Statistics

SPSS 20.0 statistical software package (IBM, Armonk, NY, USA) was used for data analyses. The *t*-test was used to analyze the difference between two groups. Comparison between groups was done using one-way ANOVA test followed by Student-Newman-Keuls (SNK) test. $p < 0.05$ was considered statistically significant.

Results

MicroRNA-140 was downregulated in PCa

MicroRNA-140 was downregulated in PCa tissues than that of paracancerous tissues (Figure 1A). In particular, lower expression of microRNA-140 was found in PCa with Grade I+II than that of Grade III+IV (Figure 1B). Meanwhile, microRNA-140 was

also downregulated in PCa cell lines compared to normal cell line, including PC-3 and DU145 cells (Figure 1C).

MicroRNA-140 inhibited the proliferation of PCa cells

We first constructed microRNA-140 mimics and microRNA-140 inhibitors. The transfection efficacy was verified by qRT-PCR (Figure 2A and 2B). Subsequently, cell proliferation was evaluated by MTT assay. Overexpression of microRNA-140 remarkably inhibited the proliferative ability of PCa cells (Figure 2C). MicroRNA-140 inhibitor transfection obtained the opposite results (Figure 2D), verifying the inhibitory effect of microRNA-140 on the proliferation of PCa cells.

MicroRNA-140 promoted apoptosis and cell cycle arrest of PCa cells

Flow cytometry results demonstrated that microRNA-140 overexpression remarkably promoted cell cycle arrest in G0/G1 phase (Figure 3A and 3B). Similarly, microRNA-140 overexpression also stimulated apoptosis of PCa cells (Figure 3C and 3D). We next detected relative indicators of

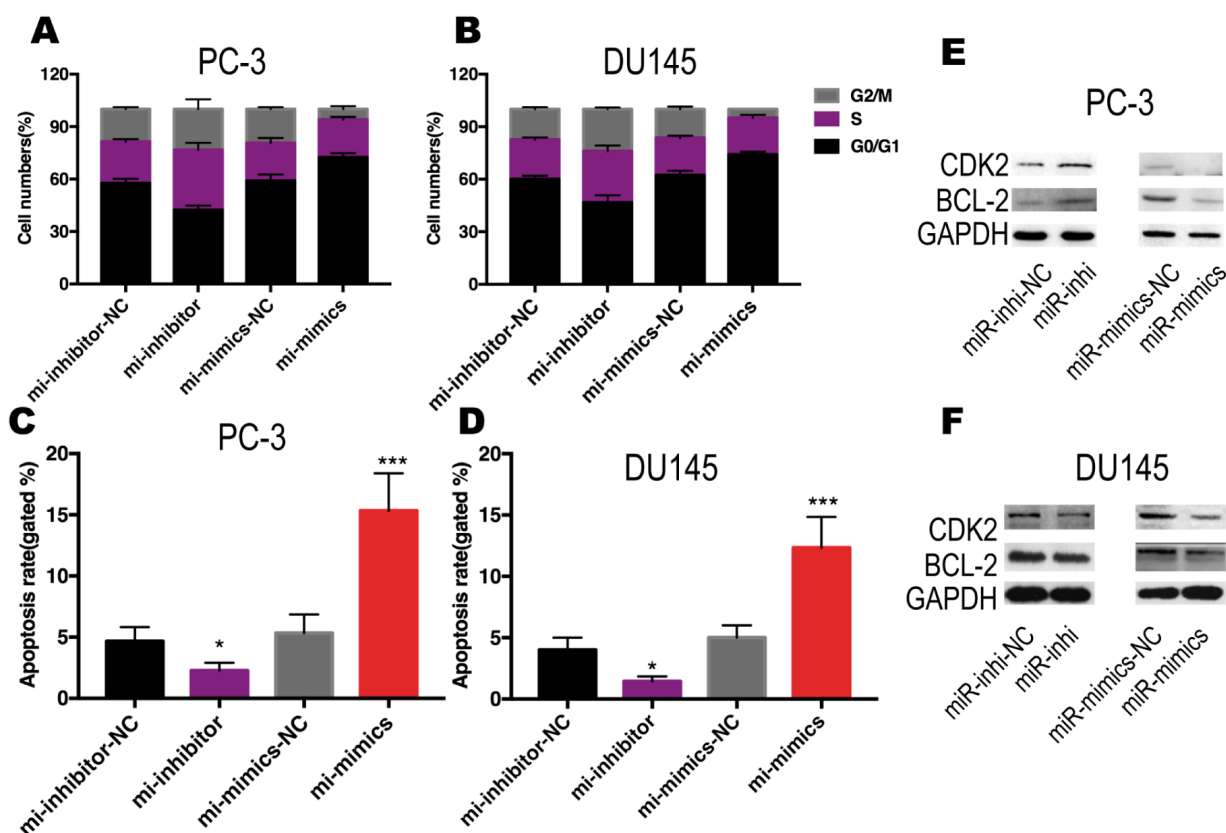


Figure 3. MicroRNA-140 promoted apoptosis and cell cycle arrest of PCa cells. **A:** MicroRNA-140 overexpression remarkably promoted cell cycle arrest in G0/G1 phase. **B:** MicroRNA-140 knockdown promoted cell cycle progression. **C:** MicroRNA-140 overexpression stimulated apoptosis of PCa cells. **D:** MicroRNA-140 knockdown inhibited apoptosis of PCa cells. **E,F:** Protein expressions of Bcl-2 (**E**) and CKD2 (**F**) were remarkably increased after microRNA-140 knock-down. * $p < 0.05$, *** $p < 0.001$.

apoptosis and cell cycle by Western blot. Protein expressions of Bcl-2 and CKD2 were remarkably increased after microRNA-140 knockdown (Figure 3E and 3F), suggesting the promotive role of microRNA-140 in apoptosis and cell cycle of PCa cells.

MicroRNA-140 degraded SOX4 expression

It is predicted that microRNA-140 could bind

to 3'-UTR of SOX4 by bioinformatics method (Figure 4A). Luciferase reporter gene assay results pointed out that microRNA-140 remarkably inhibited luciferase activity of SOX4 WT, indicating that microRNA-140 interferes with mRNA level of SOX4 (Figure 4B and 4C). Meanwhile, Western blot results also confirmed that microRNA-140 inhibited the protein expression of SOX4 (Figure 4D).

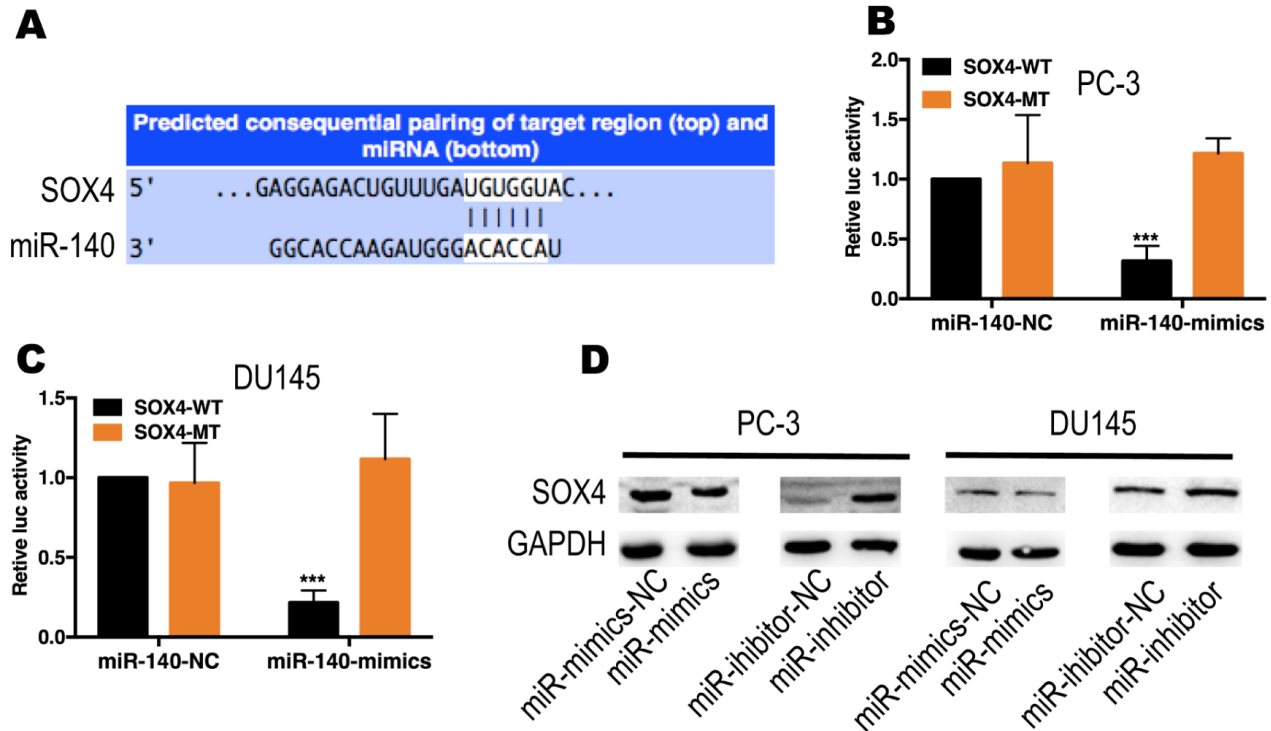


Figure 4. MicroRNA-140 degraded SOX4 expression. **A:** MicroRNA-140 could bind to 3'-UTR of SOX4. **B,C:** MicroRNA-140 remarkably inhibited luciferase activity of SOX4 WT. **D:** MicroRNA-140 inhibited protein expression of SOX4. *** $p < 0.001$.

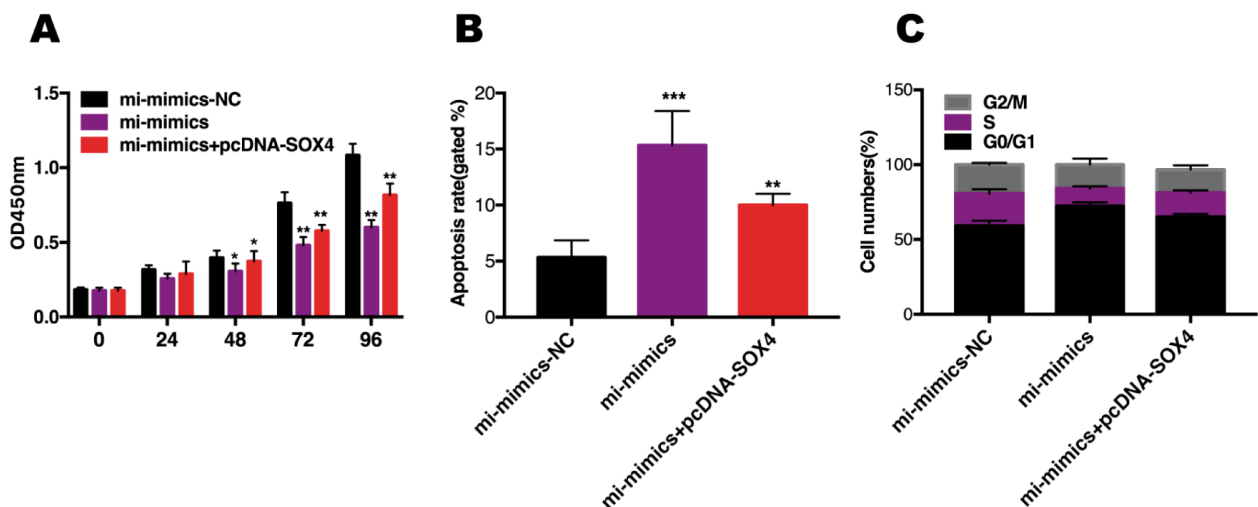


Figure 5. Cellular function of microRNA-140 in regulating PCa cells was partially reversed by SOX4 overexpression. Decreased proliferation (**A**), arrested cell cycle (**B**) and increased apoptosis (**C**) in PC-3 cells transfected with microRNA-140 mimics were partially reversed by SOX4 overexpression. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Cellular function of microRNA-140 in regulating PCa cells was partially reversed by SOX4 overexpression

Rescue experiments were performed to access the regulatory interaction between microRNA-140 and SOX4. Decreased proliferation, arrested cell cycle and increased apoptosis in PC-3 cells transfected with microRNA-140 mimics were partially reversed by SOX4 overexpression (Figure 5A-5C), further suggesting the regulatory role of microRNA-140 in SOX4.

Discussion

PCa is the most common malignancy in males in the developed countries. Globally, it is also the second leading cause of cancer-related death in men. Clinical studies have found that about 30% of PCa patients are already in advanced stage when first diagnosed. Currently, there are no effective prognostic indicators of PCa. It is urgent to explore novel prognostic factors and therapeutic targets for the individualized treatment of PCa patients. At present, key molecules in regulating tumorigenesis and progression have been served as biomarkers for prediction and treatment of tumors [8].

MicroRNAs are non-encoding single-stranded RNA molecules with 18-24 nucleotides in length [9-11]. They regulate target genes mainly through complete or incomplete complementary pair with 3'-UTR of these genes at post-transcriptional level [12]. MicroRNAs have been served as biomarkers in diagnosing various types of tumors [13,14]. Recent studies have found several differentially expressed microRNAs in PCa tissues and cells. Precise identification of certain microRNAs contributes to accurate diagnosis of PCa [15,16]. Studies have shown that microRNA-140 can inhibit the proliferation of oral squamous cell carcinoma through regulating integrin β 1. MicroRNA-140 can also inhibit invasion of breast cancer cells via regulating Slug expression [17]. SOX4 has been proved to exert a

crucial role in the maintenance and regulation of cell differentiation [18]. Dysregulated SOX4 would stimulate proliferation of tumor cells, thus exaggerating tumor deterioration [19,20].

In the present study, microRNA-140 was down-regulated in PCa tissues compared with paracancerous tissues. MicroRNA-140 expression was negatively correlated with proliferation and invasion, while positively correlated to apoptosis of PCa cells. Besides, microRNA-140 remarkably arrested cell cycle in G0/G1 phase. It is predicted that microRNA-140 could bind to SOX4 via TargetScan, miRWalk and miRDB. Hence, we speculate that microRNA-140 inhibits the proliferation of PCa cells via regulating SOX4. Our experiments revealed that overexpressed microRNA-140 remarkably decreases protein level of SOX4, whereas does not affect its mRNA level. It is suggested that microRNA-140 regulates proliferative ability of PCa cells via inhibiting SOX4 at post-transcriptional level. Additionally, rescue experiments demonstrated that the cellular functions of PCa cells affected by microRNA-140 could be partially reversed by SOX4 overexpression.

To sum up, microRNA-140 inhibited the proliferative ability of PCa cells via inhibiting mRNA translation of SOX4. Our study elucidated that microRNA-140 exerts a crucial role in preventing PCa development, which provides a novel direction in predicting and treating PCa.

Conclusion

Downregulated microRNA-140 promotes proliferation and cell cycle arrest, but inhibits apoptosis of PCa cells. MicroRNA-140 inhibits PCa development via degrading SOX4.

Conflict of interests

The authors declare no conflict of interests.

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