

MicroRNA-425-5p promotes the development of prostate cancer via targeting forkhead box J3

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Abstract. – **OBJECTIVE:** MicroRNAs (miRNAs) have critical roles in the progression of prostate cancer (PCa) and have the potential to be used as prognosis biomarkers. In this study, we aimed to investigate the role of miR-425-5p in the progression of PCa.

PATIENTS AND METHODS: miR-425-5p expression in PCa tumor tissues and cell lines was measured by Quantitative Real-time PCR (RT-qPCR). Effects of miR-425-5p expression on PCa cell proliferation, colony formation, cell migration, and cell invasion were measured.

RESULTS: We found miR-425-5p expression was elevated in both PCa tissues and cell lines. Importantly, we found overexpression of miR-425-5p promoted proliferation, colony formation, migration and invasion of PCa cell lines *in vitro*. Forkhead box J3 (FOXJ3) was validated as a downstream target of miR-425-5p. Finally, we found the stimulation effects of miR-425-5p on PCa cell behaviors were fulfilled through directly regulating the expression of FOXJ3, which validated FOXJ3 as a functional target of miR-425-5p in PCa.

CONCLUSIONS: Taken together, our results demonstrated miR-425-5p may contribute the malignancy progression of PCa in a mechanism involving FOXJ3, implicating miR-425-5p may be developed as therapeutic target for PCa in the future.

Key Words:

miR-425-5p, FOXJ3, Prostate cancer, Proliferation, Migration, Invasion.

Introduction

Prostate cancer (PCa) was estimated to be the third most commonly diagnosed cancer type worldwide in 2018¹. Meanwhile, the incidence and mortality of PCa will be doubled with annual increase rate of approximately 5% by 2030 as predicted². The usage of prostate specific antigen (PSA) has greatly helped us to distinguish PCa³. However, PSA has showed a serious drawback, lack of specificity that often resulted in overdi-

agnosis⁴. Hence, we need to find novel strategy to diagnose PCa. microRNAs (miRs) belong to a large number of non-coding RNAs that have critical roles in the initiation and progression of human cancers^{5,6}. miRNAs can function as either tumor suppressor or oncogene in human cancers depending on the context⁷. It has also been demonstrated⁷ that a miRNA may have dual functions in different cancers. miRNAs regulate gene expression by binding with the 3'-untranslated regions (UTRs) of target genes and resulted in direct cleavage of the message RNAs (mRNAs) or translation inhibition⁸. miR-425-5p, located on chromosome 3, was reported to abnormally expressed in various human cancers and implicated miR-425-5p deeply participated in the development of these cancer types⁹⁻¹². For example, miR-425-5p was found significantly up-regulated in gastric cancer tumor tissues and cell lines investigated⁹. Also, they found gastric cancer cell migration and invasion ability was attenuated when miR-425-5p expression was silenced, whereas the overexpression of miR-425-5p will cause the opposite effects⁹. A research¹⁰ conducted in hepatocellular carcinoma (HCC) revealed that miR-425-5p overexpression promoted HCC cell invasion and metastasis but not proliferation and apoptosis *in vitro*. Moreover, the overexpression of miR-425-5p was correlated with poor long-term survival and disease-free survival¹⁰. Besides that, miR-425-5p was also revealed to be a potential prognosis biomarker for cervical cancer and a therapeutic target for metastasis colorectal cancer^{11,12}. However, to the best of our knowledge, the expression status and role of miR-425-5p in PCa remain unclear.

In this study, we examined miR-425-5p expression in PCa tumor tissues and cell lines. We further investigated the effects of miR-425-5p expression on PCa cell behaviors *in vitro*. The exacted underlying mechanism of miR-425-5p in PCa was also examined.

Materials and Methods

Patients and Human Tissues

Nineteen pairs of PCa tumor tissues and adjacent normal tissues were obtained from the Second Hospital of Fujian Medical University. All these tissues were immediately snap-frozen in liquid nitrogen for further usage. The study protocol was approved by the Ethics Committee of our Hospital. All the patients provided the written informed consent.

Cell Lines and Cell Transfection

PC3 and DU145 PCa cell lines, and normal prostate epithelial cells (RWPE-1), were purchased from Cell Bank of Chinese Academy of Sciences (Shanghai, China). PCa cell lines were incubated in F-12K medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA). RWPE-1 cells were cultured in Dulbecco's modified Eagle's medium (Gibco; Thermo Fisher Scientific, Waltham, MA, USA). The entire incubation medium was supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Waltham, MA, USA). These cells were maintained in a humidified atmosphere at 37°C containing 5 % CO₂. miR-425-5p mimic, miR-425-5p inhibitor, and negative control miRNA (NC-miR) were purchased from GenePharma (Shanghai, China). Small-interfering RNA targeting FOXJ3 (si-FOXJ3) and negative control siRNA (NC-siR) were also synthesized by GenePharma (Shanghai, China). Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) was used for transfection following the manufacturer's instructions.

Quantitative Real-Time PCR (RT-qPCR)

Total RNA extraction was conducted using mir-Vana miRNA Isolation kit (Ambion; Thermo Fisher Scientific, Waltham, MA, USA). Complementary DNA (cDNA) was obtained from RNA reverse-transcribe using PrimeScript RT Reagent Kit (TaKaRa, Dalian, China). Quantitative Real-time PCR (RT-qPCR) was conducted at Applied Biosystems 7900 Real-time PCR system (Applied Biosystems, Foster City, CA, USA) using SYBR Premix Ex Taq kit (TaKaRa, Otsu, Shiga, Japan) with the following procedures: 95°C for 20 s (1 cycle), 95°C for 3 s, and 60°C for 30 s (40 cycles). Relative expression levels were calculated with U6 snRNA as internal control. Primers used were as follows: miR-425-5p: forward: 5'-TGCGGAATGACACGATCACTCCCG-3', reverse:

5'-CCAGTGCAGGGTCCGAGGT-3'; U6 snRNA: forward: 5'-CTCGCTTCGGCAGCACACA-3', reverse: 5'-AACGCTTCACGAATTTGCGT-3'.

Western Blot

Total protein was extracted using Radio Immunoprecipitation Assay (RIPA) lysis buffer (Beyotime, Shanghai, China) and separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto polyvinylidene difluoride (PVDF) membrane (Beyotime, Shanghai, China). Membranes were blocked with 5 % fat-free milk and incubated with primary antibody against FOXJ3 (ab183112, Abcam, Cambridge, MA, USA) and glyceraldehyde 3-phosphate dehydrogenase (ab181602, Abcam, Cambridge, MA, USA) at 4°C for overnight. Following, the horseradish peroxidase (HRP) conjugated secondary antibody (ab6721, Abcam, Cambridge, MA, USA) was used to incubate with the membrane at room temperature for 2 h. Finally, BeyoECL Star (Beyotime, Shanghai, China) was used to visualize the band signals. Relative expression level was calculated with GAPDH as internal control.

Cell Counting Kit-8 (CCK-8) Assay

Cells were incubated at 96-well plate at the density of 5 x 10³ cells/well and incubated for 24 h. Then, 10 µl cell Counting Kit-8 (CCK-8, Beyotime, Shanghai, China) reagent was added to each well at 0, 24, 48, and 72 h and further incubated for 2 h. Optical density at 450 nm was measured using a microplate reader (Bio-Rad, Inc., Hercules, CA, USA).

Colony Formation Assay

Cells were seeded in 6-well plate at the density of 1 x 10³ cells/well and cultured for 2 weeks. Next, the colonies developed were fixed with methanol and stained with crystal violet. The colonies number above 50 was counted under a microscope and the average number was calculated from 5 independent fields.

Cell Migration Assay

Cells were seeded in 6-well plates and grown to approximately 100 % confluence. Then, a pipette tip was used to create a scratch in the cell surface and cultured in serum-free F-12K medium. At 0 and 48 h, the wound was observed under microscope and measured by Image J 1.42 software (National Institutes of Health, Bethesda, MD, USA).

Cell Invasion Assay

3×10^4 cells were plated to the upper chamber containing serum-free F-12K medium. The lower chamber was filled with F-12K medium containing 10% FBS. The chambers were pre-coated with 80 μ l Matrigel (BD Bioscience, Franklin Lakes, NJ, USA). After incubation for 48 h, the non-invasive cells were wrapped with cotton swab while the invasive cells were fixed with 0.1% paraformaldehyde and stained with 4% crystal violet. The invasive numbers were counted under a microscope.

Luciferase Activity Assay

The wild-type (wt) 3'-UTR of FOXJ3 was cloned from genome and cloned into the pMir-report vector (Promega, Madison, WI, USA) and designated as wt FOXJ3. The mutant (mut) type was built using the site-direct mutagenesis kit (TaKaRa, Otsu, Shiga, Japan) and named as mut FOXJ3. Cells were seeded into a 24-well plate and co-transfected with miR-425-5p mimic or NC-miR and wt or mut FOXJ3. Luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) after 48 h of transfection with Renilla-luciferase as internal control.

Statistical Analysis

Results collected from at least three independent experiments were analyzed with SPSS 22 software (IBM, Armonk, NY, USA) and presented as mean \pm standard deviation (SD). Differences in groups were analyzed with Student's *t*-test or one-way ANOVA and Tukey post-hoc test. *p*-values less than 0.05 were regarded as significant.

Results

miR-425-5p Expression was Upregulated in PCa tissues and Cell Lines

miR-425-5p expression was examined by RT-qPCR and normalized to U6 snRNA. The results showed that miR-425-5p expression was significantly upregulated in PCa tissues compared with the adjacent normal tissues (Figure 1A). Moreover, the expression of miR-425-5p in PCa cell lines (PC3 and DU145) and normal prostate epithelial cells (RWPE-1) was also examined. It was found that miR-425-5p expression was remarkably higher in PCa cell line than in RWPE-1 cell line (Figure 1B).

Cell Transfection Efficiency

The synthetic miRNAs transfection efficiency was also investigated by RT-qPCR. The results in Figure 1C indicated that the transfection of miR-425-5p mimic significantly increased the expression of miR-425-5p, while the transfection of miR-425-5p inhibitor decreased the expression of miR-425-5p.

FOXJ3 was a Direct Target of miR-425-5p

TargetScan algorithm was used to predict the targets of miR-425-5p. We found the 3'-UTR of FOXJ3 contains a binding site for miR-425-5p (Figure 2A). The luciferase reporter activity assay showed that miR-425-5p mimic decreased the luciferase activity of cells transfected with wt FOXJ3 but not mut FOXJ3 (Figure 2B). Moreover, we showed that miR-425-5p mimic transfection significantly inhibited the protein expression of FOXJ3, while the miR-425-5p

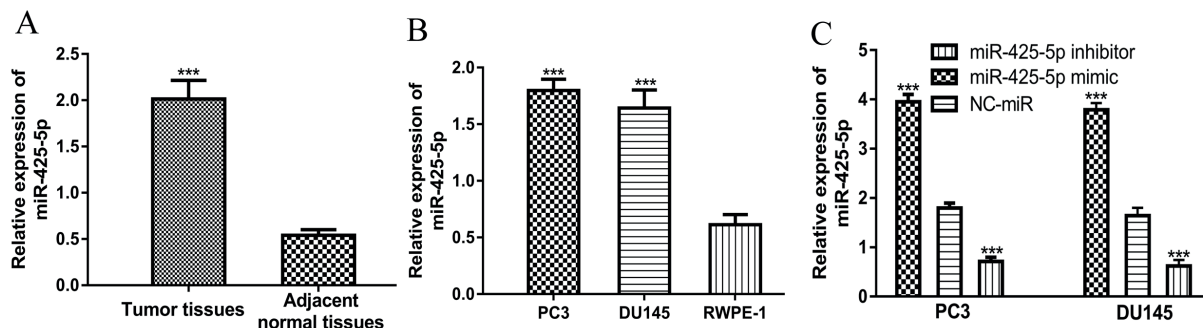


Figure 1. Overexpression of miR-425-5p in PCa tissues and cell lines. (A) miR-425-5p expression in PCa tissues and normal adjacent tissues. (B) miR-425-5p expression in PCa cell lines (PC3 and DU145) and normal prostate epithelial cells (RWPE-1). (C) miR-425-5p expression in PCa cell lines (PC3 and DU145) with synthetic miRNAs transfection. (***) $p < 0.001$ miR-425-5p: microRNA-425-5p; NC-miR: negative control miRNA; PCa: prostate cancer.

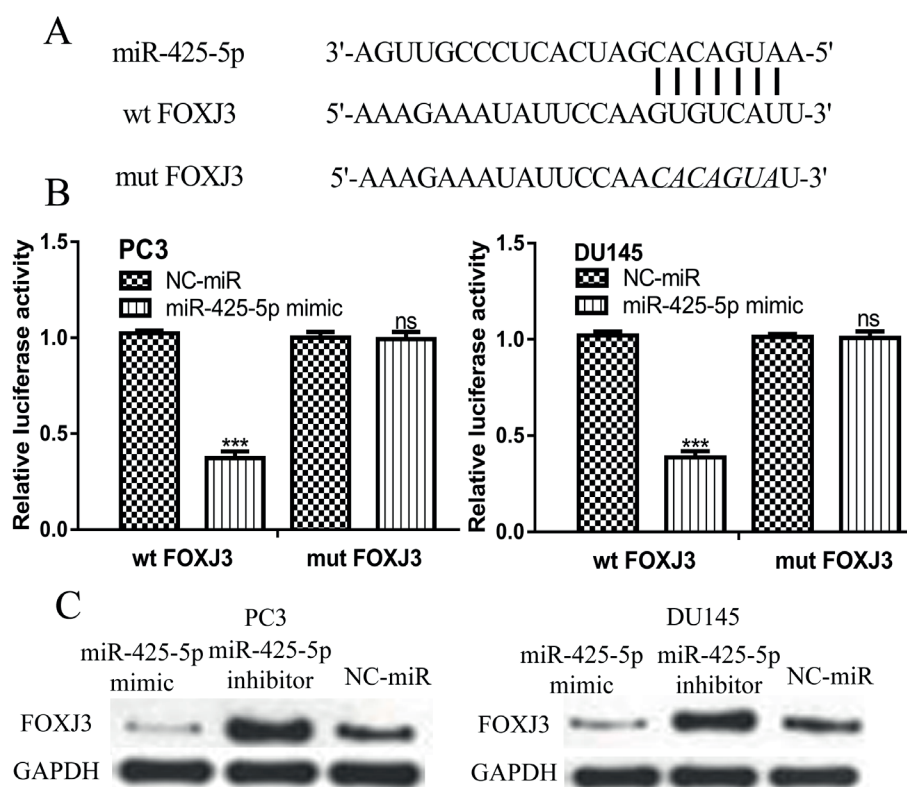


Figure 2. FOXJ3 was a direct target of miR-425-5p in PCa. (A) Putative binding sequence between miR-425-5p and FOXJ3. (B) Luciferase activity in PCa cell lines (PC3 and DU145) with miR-425-5p mimic or NC-miR and wt or mut FOXJ3. (C) FOXJ3 expression in PCa cell lines (PC3 and DU145) with synthetic miR-425-5p transfection. (ns not significant, *** $p < 0.001$) miR-425-5p: microRNA-425-5p; NC-miR: negative control miRNA; PCa: prostate cancer; FOXJ3: Forkhead box J3; wt: wild type; mut: mutant.

inhibitor transfection caused the opposite effects (Figure 2C). Collectively, these results demonstrated that FOXJ3 was a direct target of miR-425-5p in PCa.

Overexpression of miR-425-5p Promotes Cell Proliferation, Migration and Invasion

To reveal the biological role of miR-425-5p in PCa, the effects of miR-425-5p expression on PCa cell proliferation, migration and invasion were investigated. As shown in Figure 3A, cell proliferation rate of PC3 and DU145 cells was suppressed by miR-425-5p inhibitor but enhanced by miR-425-5p mimic. To further characterize the effects of miR-425-5p on cell proliferation, colony formation assay was conducted. The number of colonies formed in miR-425-5p mimic transfected group was significantly higher than those in NC-miR group (Figure 3B). The wound-healing assay showed that cell migration ability was enhanced by

miR-425-5p mimic but inhibited by miR-425-5p inhibitor (Figure 3C). Transwell invasion assay showed the overexpression of miR-425-5p promoted the invasion of PC3 and DU145 cells (Figure 3D).

Silence the Expression of FOXJ3 Attenuated the Effects of miR-425-5p

To further confirm FOXJ3 as a direct target of miR-425-5p, we co-transfected si-FOXJ3 and miR-425-5p inhibitor into the investigated cells. FOXJ3 expression was significantly reduced by si-FOXJ3 but this effect can be attenuated by miR-425-5p inhibitor (Figure 4A). The *in vitro* mechanism analysis results revealed that the inhibition effects of miR-425-5p inhibitor on PCa cell behaviors including cell proliferation, colony formation, cell migration and cell invasion, could be reversed by si-FOXJ3 (Figure 4B-E). These results validated FOXJ3 as a functional target of miR-425-5p.

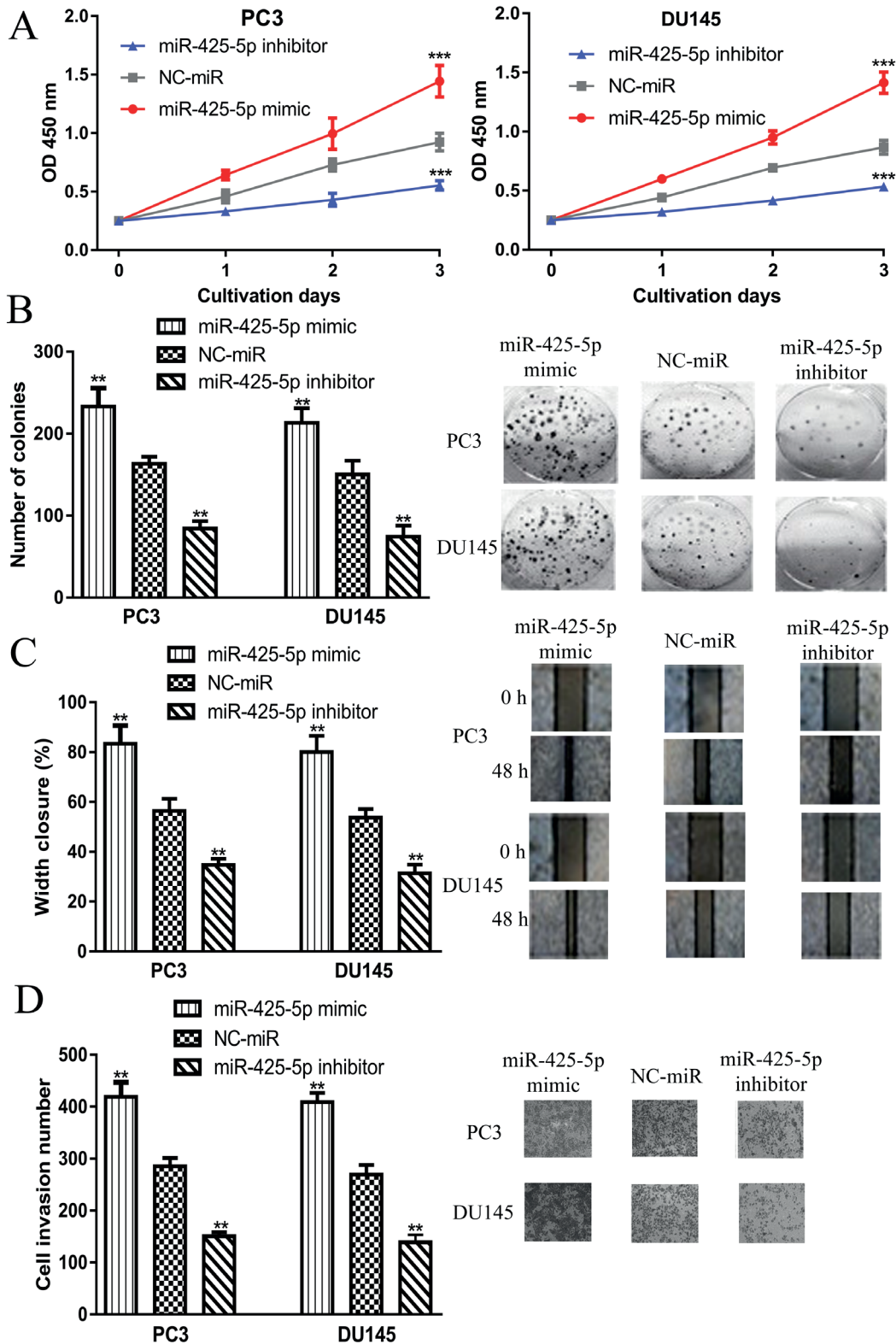


Figure 3. Overexpression of miR-425-5p promote PCa cell proliferation, colony formation, cell migration and cell invasion. (A) Cell proliferation, (B) Colony formation, (C) Cell migration and (D) Cell invasion in PCa cell lines (PC3 and DU145) with synthetic miRNAs transfected. (** $p < 0.01$, *** $p < 0.001$) miR-425-5p: microRNA-425-5p; NC-miR: negative control miRNA; PCa: prostate cancer.

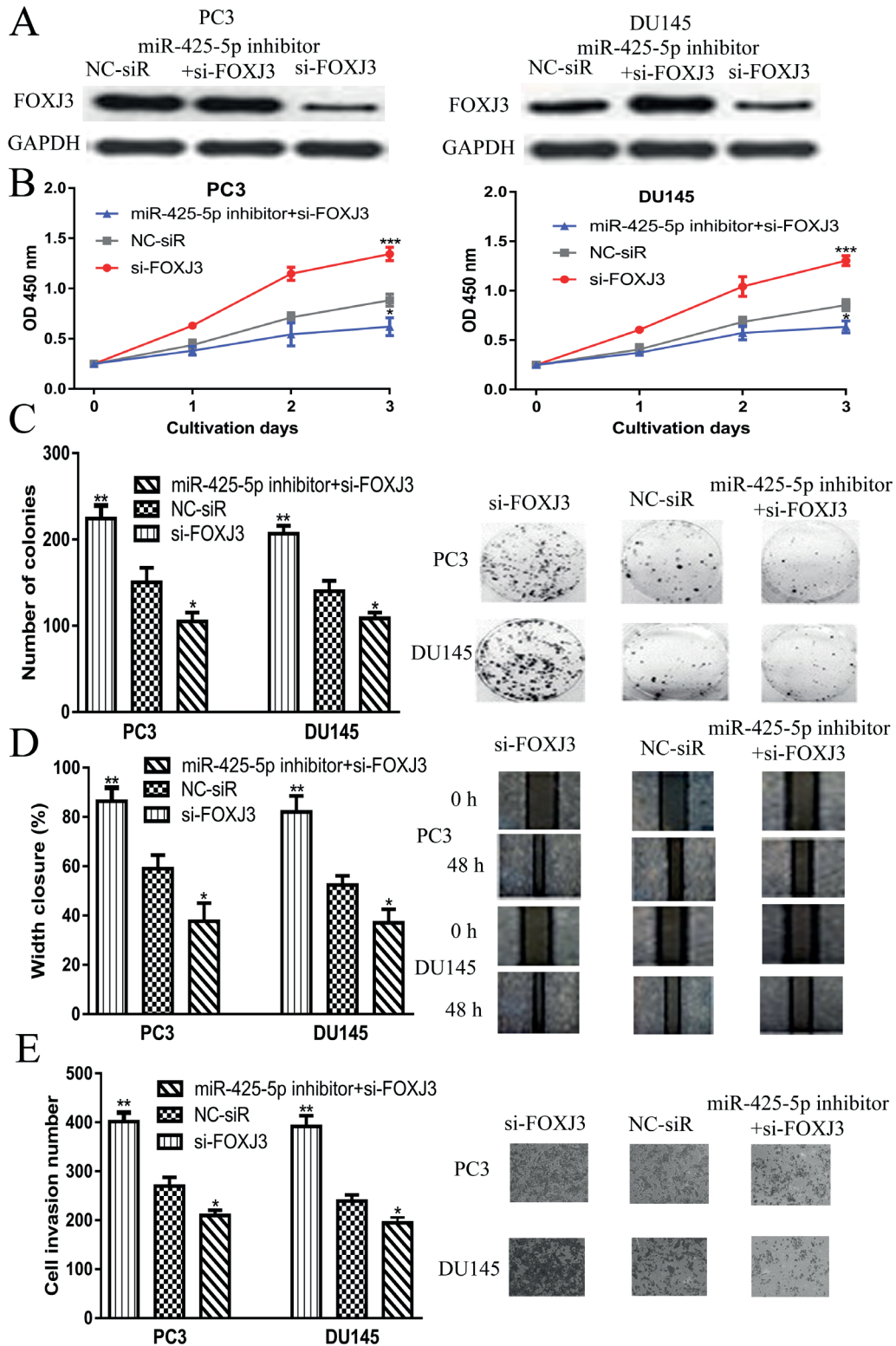


Figure 4. Silence FOXJ3 expression attenuated the effects of miR-425-5p inhibitor on PCa cell behaviors. (A) FOXJ3 expression, (B) Cell proliferation, (C) Colony formation, (D) Cell migration and (E) Cell invasion in PCa cell lines (PC3 and DU145) with si-FOXJ3 or miR-425-5p inhibitor expression. (* $p < 0.05$, *** $p < 0.001$) miR-425-5p: microRNA-425-5p; FOXJ3: Forkhead box J3; si-FOXJ3: small interfering RNA targeting FOXJ3; NC-siR: negative control siRNA; PCa: prostate cancer.

Discussion

Extensive studies^{5,13-15} have been conducted to understand the progression of human cancers including PCa. Moreover, new treatment methods have been developed to better control the progression of tumors¹⁶⁻¹⁸. miRNAs were reported closely correlated with the development PCa and showed the potential to be used as therapeutic targets for PCa¹⁹⁻²². miR-139 was reported to inhibit the proliferation of PCa cells by interfering with cell cycle through targeting Notch1¹⁹. miR15a/16 was found to suppress transforming growth factor- β (TGF- β) signaling pathways to regulate PCa cell progression and metastasis²⁰. miR-425-5p was demonstrated to be an oncogene in several cancer types but its role in PCa was not reported yet⁹⁻¹². In this study, we showed that the expression of miR-425-5p in PCa tissues was significantly higher than that in the adjacent normal tissues, which showed the hyperexpression status of miR-425-5p in PCa. Moreover, the overexpression of miR-425-5p in PCa cell lines was also observed when compared with normal cell line. Furthermore, we examined the biological role of miR-425-5p in PCa cell lines and showed that the overexpression of miR-425-5p promotes PCa cell proliferation, colony formation, cell migration and cell invasion *in vitro*, while the downregulation of miR-425-5p has the opposite effects. To determine the molecular mechanisms of miR-425-5p in regulating PCa cell behaviors, we explored the potential targets of miR-425-5p in PCa. With the online algorithm TargetScan, we found FOXJ3 contains a prediction-binding site for miR-425-5p. FOXJ3, a key transcription factor of mitochondrial biogenesis, was identified to be downregulated in colorectal cancer and lung cancer^{23,24}. Also, it was determined that FOXJ3 expression could be regulated by miRNAs including miR-517 and miR-494²³⁻²⁵. In this present study, we showed FOXJ3 was also a target of miR-425-5p by luciferase activity reporter assay and western blot assay. We found the transfection of miR-425-5p mimic decreased FOXJ3 expression in the PCa cell lines investigated. Further functional experiments showed that silencing the expression FOXJ3 attenuated the inhibitory effects of miR-425-5p inhibitor on PCa cell behaviors.

Conclusions

We showed that miR-425-5p was overexpressed in PCa and its overexpression promotes PCa cell

proliferation, colony formation, cell migration, and cell invasion. Mechanistically, we showed that miR-425-5p participates in regulating PCa cell behaviors through targeting FOXJ3. Taken together, our results may provide a possible therapeutic target for PCa but still need further validation in clinical.

Conflict of Interest

The Authors declare that they have no conflict of interest.

Acknowledgement

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