

LncRNA HOXA11-AS promotes cell growth by sponging miR-24-3p to regulate JPT1 in prostate cancer

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Abstract. – **OBJECTIVE:** Long noncoding RNA (lncRNA) was found to play crucial roles in regulating cancer progression. HOXA11 antisense RNA (HOXA11-AS) was reported to serve an oncogenic lncRNA in cancers but its role in prostate cancer (PCa) remains to be explored.

MATERIALS AND METHODS: Expression levels of HOXA11-AS in PCa tissues and cells were analyzed with quantitative Real-Time PCR method. MTT assay, colony formation assay, transwell invasion assay, and flow cytometry assay were conducted to explore the biological roles of HOXA11-AS in PCa. Rescue experiments were conducted to investigate mechanisms of HOXA11-AS in regulating PCa progression.

RESULTS: We revealed that HOXA11-AS was up-regulated in PCa. Silencing of HOXA11-AS significantly inhibited PCa cell proliferation, colony formation, invasion, and promoted apoptosis in vitro. On the contrary, forcing of HOXA11-AS expression caused opposite effects on cancer cell behaviors. Furthermore, we showed that HOXA11-AS1 serves as a competing endogenous RNA (ceRNA) to regulate Jupiter microtubule associated homolog 1 (JPT1) *via* sponging microRNA-24-3p (miR-24-3p). Functionally, the overexpression of miR-24-3p or knockdown of JPT1 could partially reverse the effects of HOXA11-AS overexpression on PCa cell behaviors.

CONCLUSIONS: This newly identified HOXA11-AS/miR-24-3p/JPT1 axis may provide novel angle for the better control of PCa.

Key Words:

Prostate cancer, HOXA11-AS, MiR-24-3p, JPT1.

Tumor resection and radiotherapy are available treatment measures for early stage PCa patients³. Unfortunately, most patients were found at advanced stages due to the lack of early diagnosis markers, which results in a decrease of 5-year overall survival⁴. Previous researchers⁵ have identified numerous aberrantly expressed genes, including protein coding and non-coding genes in PCa progression.

The numbers of identified non-coding RNAs (ncRNAs) are continuously increasing in recent years due to the improvements of high-throughput sequencing technology⁶. ncRNAs can be generally classified into two groups based on length: short ncRNAs and long ncRNAs (lncRNAs)^{7,8}. lncRNAs were found participated in various cellular processes to affect PCa development⁹. Homeobox A11 antisense (HOXA11-AS), located at chromosome 7p15.2, was found aberrantly expressed in cancers and functioned as either oncogene or tumor suppressor gene. Bai et al¹⁰ reported that HOXA11-AS could stimulate non-small cell lung cancer *via* regulating microRNA-148a-3p (miR-148a-3p) and DNA methyltransferase 1 axis. On the contrary, Yin et al¹¹ found that HOXA11-AS could inhibit papillary thyroid cancer progression through miR-761/tripartite motif-containing 29 axis¹¹.

MiRNAs are small ncRNAs at the length of around 20 nucleotides¹². MiR-24-3p was an oncogenic miRNA in lung cancer and could promote cell migration and proliferation *via* regulating SRY-box 7¹³. MiR-24-3p was also found to suppress colon cancer progression *via* regulating tripartite motif-containing 11¹⁴.

In this study, we explored HOXA11-AS expression level in PCa cells and tissues. Further-

Introduction

Prostate cancer (PCa) ranks the second-most cause for cancer-related deaths worldwide^{1,2}.

more, *in vitro* and *in vivo* experiments were conducted to explore the functions and associated mechanisms of HOXA11-AS in PCa progression.

Materials and Methods

Study Subjects

This study protocol was approved by Ethic Committee of the Seventh Affiliated Hospital of Sun Yat-Sen University. 25 paired PCa tissues and adjacent normal tissues were obtained patients who underwent treatment at our hospital. Tumor stage was defined according to the eighth edition of the AJCC staging system. The patients who received anti-cancer treatment was excluded in this study. Written informed consent was obtained from all participates. Tissues were stored at -80°C for usage.

Cell Culture

PCa cell lines (PC-3, Du-145, and LNCaP) and normal prostate epithelial cell line RWPE-1 were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were incubated at Roswell Park Memorial Institute (RPMI-1640) medium in supplement with 10% fetal bovine serum (FBS, Invitrogen, Carlsbad, CA, USA), while the culture condition was maintained at 37°C with 5% of CO_2 .

Bioinformatic Analysis

For prediction HOXA11-AS targets, ENCORI (<http://starbase.sysu.edu.cn/>) was used and we found that miR-24-3p was a putative target. For prediction miR-24-3p targets, TargetScan (www.targetscan.org) was used and showed that Jupiter microtubule associated homolog 1 (JPT1) was a possible target.

Detection of Gene Expression at ENCORI

ENCORI website was used to explore gene expression level of HOXA11-AS, miR-24-3p, and JPT1 in PCa tissues and normal tissues. Moreover, the expression correlations of HOXA11-AS or JPT1 with miR-24-3p in PCa tissues were also analyzed at ENCORI.

Cell Transfection

Small interfering RNA against HOXA11-AS (si-HOXA11-AS) and control (si-con) were synthesized by GeneChem (Shanghai, China). pcD-

NA3.1 contains full sequence of HOXA11-AS (pHOXA11-AS) or JPT1 (pJPT1) was purchased at GenScript (Nanjing, China). MiR-24-3p mimic and control (mi-con) were also bought from GeneChem. Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) was used for cell transfection.

Quantitative Real Time-PCR (RT-qPCR)

miRNeasy Mini Kit (Qiagen, Dusseldorf, Germany) was used to isolate total RNA from tissues and cells. SuperMix (TransGen, Beijing, China) was used to synthesize first-strand cDNA after concentration quantification. RT-qPCR analysis was performed at ABI 7900 (Applied Biosystems, Foster City, CA, USA) using SYBR Green (TransGen, Beijing, China). Primers used were as follows: HOXA11-AS: F: 5'-CGGCTACAAGGAGATTTGG-3', R: 5'-AGGCTCAGGGATGGTAGTCC-3'; JPT1: F: 5'-ATAGCTCCCGAGTTTTGCG-3', R: 5'-TTG-GCCCAAGAAGCTTGA-3'; miR-24-3p: F: 5'-TGCGGTGGCTCAGTTCAGCAGGAAC-3', R: 5'-CAGTGCGTGTCTGCGGAGT-3'; glyceraldehyde-3-phosphate dehydrogenase (GAPDH): F: 5'-TCCCTGAGCTGAACGGGAAG-3', R: 5'-GGAGGAGTGGGTGTCGCTGT-3'; U6 small nuclear RNA (U6 snRNA): F: 5'-GGAACGATACAGAGAAGATTAGC-3', R: 5'-TGGAACGCTTACGAATTTGCG-3'. $2^{-\Delta\Delta\text{CT}}$ method was used to calculate relative gene level with GAPDH and U6 snRNA as internal controls.

Cell Proliferation Assay

1,000 cells were seeded in 96-well plate and incubated for 3 days. After the addition of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) solution (Beyotime, Haimen, Jiangsu, China) into the plate, the cells were further incubated for 4 h. DMSO was added to dissolve formazan, and then, detect absorbance at 490 nm using microplate reader.

Colony Formation Assay

800 cells were seeded into 6-well plate and incubated at the described condition for 14 days. Colonies were fixed by methanol and stained by crystal violet. Finally, colonies numbers were counted under microscope.

Transwell Invasion Assay

Matrigel coated 24-well transwell chamber (8 μm , Corning, NY, USA) was used to detect cell invasion ability. Cells with serum-free RPMI-1640

were plated into upper chamber. RPMI-1640 with 10% FBS was filled into lower chamber. After 48h incubation, the cells remained on the upper chamber were removed. Invaded cells were stained by crystal violet and counted from 5 independent fields.

Cell Apoptosis Assay

Flow cytometry was conducted to detect cell apoptosis rate using Annexin V- Fluorescein isothiocyanate (Annexin V-FITC) and Propidium iodide (PI) kit (Beyotime, Haimen, Jiangsu, China). Cells were collected, suspended in binding buffer, and incubated with Annexin V-FITC and PI for 15 min. Finally, cell apoptosis percentage was analyzed at FACSCalibur (BD Bioscience, Franklin Lakes, NJ, USA).

Dual-Luciferase Assay

Wild-type (WT) or mutant (MT) fragment of HOXA11-AS and JPT1 were cloned into Luciferase vector pMIR-REPORT (Promega, Madison, WI, USA) to obtain HOXA11-AS/JPT1-WT/MT. Cells were co-transfected with Luciferase constructs and miRNAs using Lipofectamine 2000 for 48 h. Dual-Luciferase assay was used to detect relative Luciferase activity with Renilla Luciferase as internal control.

RNA Pull-Down Assay

Biotin labeled miR-24-3p-WT or miR-24-3p-MT were transfected into PC-3 cells for 48 h. Then, cell lysates were incubated with streptavidin-labeled magnetic beads (Invitrogen, Waltham, MA, USA) in a RNase-free and yeast tRNA co-appearance atmosphere. Lysates were washed with salt buffer and extracted total RNA using TRIzol. RT-qPCR was used to detect relative gene expression levels.

RNA Immunoprecipitation (RIP) Assay

Cells were treated with RIP lysis buffer to obtain cell lysate. Then, Magna RI RNA-Binding Protein Immunoprecipitation Kit (Millipore, Billerica, MA, USA) was used for RIP assay. After that, magnetic beads were collected and incubated with anti-Argonaute 2 (anti-Ago2) or anti-Immunoglobulin G (anti-IgG) antibody. RNAs were isolated and subjected to RT-qPCR analysis.

Xenograft Tumors in Nude Mice

BALB/c nude mice were injected with short hairpin RNA against HOXA11-AS (sh-HOXA11-

AS) or control (sh-con) transfected PC-3 cells, tumor length, and width were recorded every 7 days for 4 times. Tumor weight in each group were measured after 4 weeks. The study was performed according to the guideline of China Council on Animal Care and Use. The study protocol was approved by Ethics Committee of the Seventh Affiliated Hospital of Sun Yat-Sen University.

Statistical Analysis

Data were analyzed using SPSS 21.0 software (IBM Corp., Armonk, NY, USA) and expressed as mean \pm SD. One-way analysis of variance and Tukey post-hoc test or Student's *t*-test were used to analyze differences in groups. Difference was considered as significance when $p < 0.05$.

Results

Increased Expression of HOXA11-AS in PCa

RT-qPCR analysis indicated HOXA11-AS level was higher in PCa tissues than that in normal tissues (Figure 1A). ENCORI website verified RT-qPCR results (Figure 1B). Then, we showed HOXA11-AS was significantly overexpressed in PCa cells compared with normal cells (Figure 1C). Since PC-3 cell has the highest HOXA11-AS level in PCa cells investigated, hence it was selected for functional analyses.

HOXA11-AS Facilitates PCa Cell Proliferation, Colony Formation and Impeded Cell Apoptosis

RT-qPCR showed HOXA11-AS expression level was increased by pHOXA1-AS and decreased by si-HOXA11-AS in PC-3 cells (Figure 2A). MTT assay showed HOXA11-AS overexpression significantly promoted cell proliferation compared with control groups (Figure 2B). Colony formation assay and transwell invasion assay indicated that colony formation and cell invasion ability in PC-3 cells were increased by pHOXA1-AS (Figure 2C and 2D). Flow cytometry assay showed cell apoptosis percentage was repressed after HOXA11-AS overexpression (Figure 2E). On the contrary, we showed that the knockdown of HOXA11-AS by si-HOXA11-AS has the opposite effects on PCa cell growth and invasion (Figure 2B-2E).

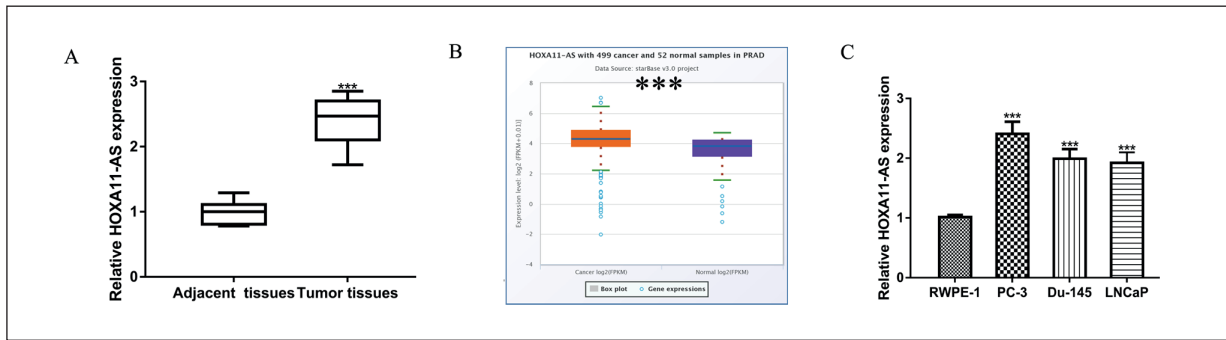


Figure 1. HOXA11-AS was overexpressed in PCa tissues and cells. **A**, HOXA11-AS was upregulated in PCa tissues based on RT-qPCR. **B**, HOXA11-AS was upregulated in PCa tissues based on ENCORI analysis. **C**, HOXA11-AS was upregulated in PCa cells based on RT-qPCR. HOXA11-AS: HOXA11 antisense RNA; PCa: prostate cancer; RT-qPCR: Quantitative real-time PCR.

HOXA11-AS Regulates MiR-24-3p in PCa

Through ENCORI analysis, we found that miR-24-3p was a putative target for HOXA11-

AS (Figure 3A). Dual-Luciferase assay showed co-introduction of HOXA11-AS-WT and miR-24-3p mimic reduced Luciferase activities in

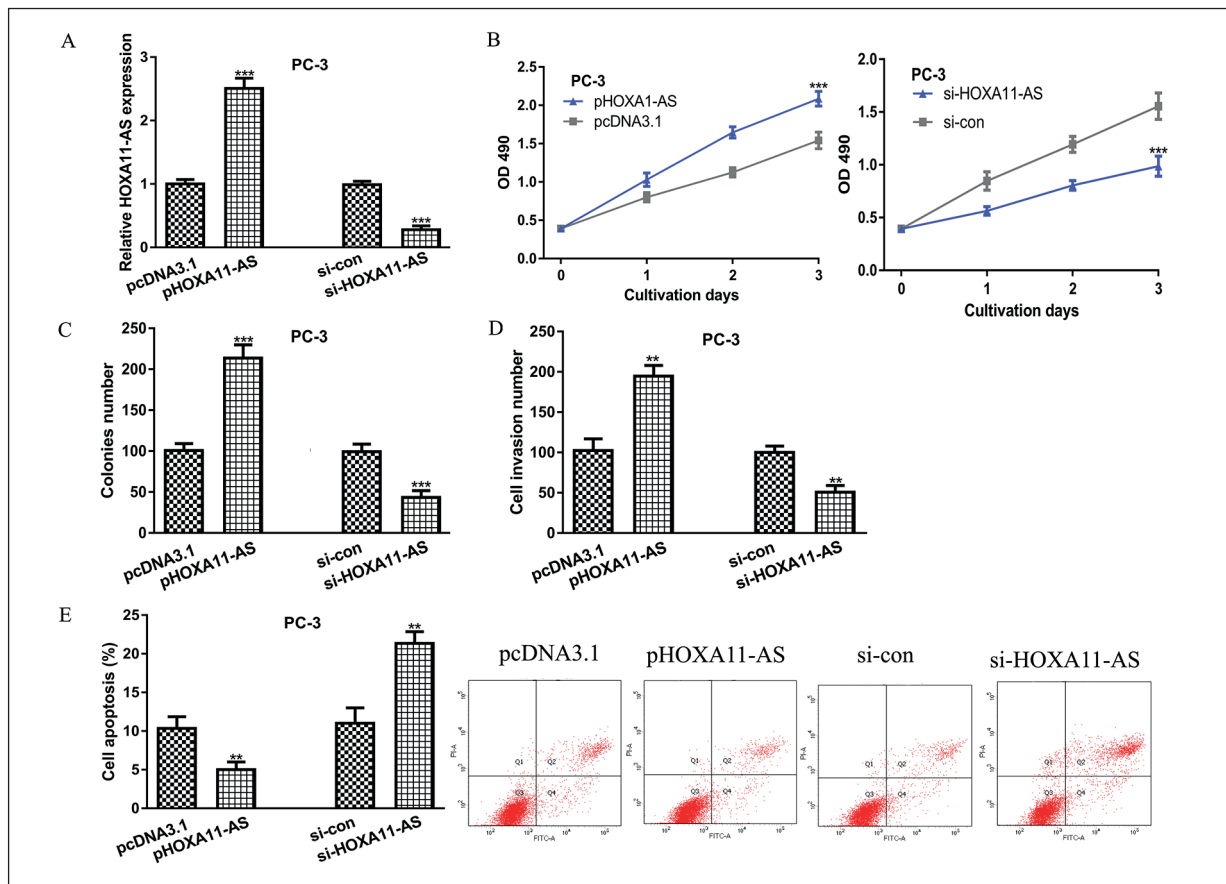


Figure 2. HOXA11-AS accelerates HCC cell proliferation, colony formation and invasion whereas impeded cell apoptosis. **A**, HOXA11-AS expression was elevated by pHOXA11-AS and reduced by si-HOXA11-AS2. HOXA11-AS overexpression promotes, whereas HOXA11-AS knockdown inhibits **(B)** cell proliferation, **(C)** colony formation, and **(D)** Cell invasion. **E**, HOXA11-AS overexpression inhibits, whereas HOXA11-AS knockdown promotes cell apoptosis. HOXA11-AS: HOXA11 antisense RNA; PCa: prostate cancer; RT-qPCR: Quantitative real-time PCR; si-HOXA11-AS: small interfering RNA against HOXA11-AS; si-con: negative control siRNA.

PC-3 cells (Figure 3B). RIP assay SHOWED HOXA11-AS and miR-24-3p was co-enriched in PC-3 cells (Figure 3C). According to RT-qPCR and ENCORI analyses results, we showed that miR-24-3p was decreased expression in both PCa tissues and cells (Figure 3D-3F). Moreover, we found that HOXA11-AS and miR-24-3p was negatively correlated in PCa tissues (Figure 3G).

HOXA11-AS Overexpression Stimulates PCa Cell Growth and Inhibited Cell Apoptosis Via Regulating MiR-24-3p

Then, we showed that miR-24-3p expression was increased by miR-24-3p mimic and decreased by pHOXA11-AS (Figure 4A). We found that miR-24-3p overexpression inhibits cell proliferation through MTT assay (Figure 4B). Colony numbers in miR-24a-3p mimic groups were found lower than in mi-con groups (Figure

4C). Transwell invasion assay showed that miR-24-3p overexpression could repress cell invasion (Figure 4D). Flow cytometry assay indicated that cell apoptosis rate of cells with miR-24-3p mimic transfection was significantly increased (Figure 4E). Importantly, we found that the roles of pHOXA11-AS on PCa cell behaviors could be partially abolished by miR-24-3p mimic (Figure 4B-4E).

MiR-24-3p Regulates JPT1 in PCa

TargetScan showed that JPT1 was a highly potential target of miR-24-3p (Figure 5A). Dual-Luciferase reporter assay validated that miR-24-3p could directly interact with 3'-UTR of JPT1 (Figure 5B). RIP assay showed the co-enrichment of HOXA11-AS, miR-24-3p and JPT1 (Figure 5C). RNA pull-down assay confirmed the interaction of miR-24-3p and JPT1 (Figure 5D). Results from RT-qPCR and ENCORI showed that JPT1 was

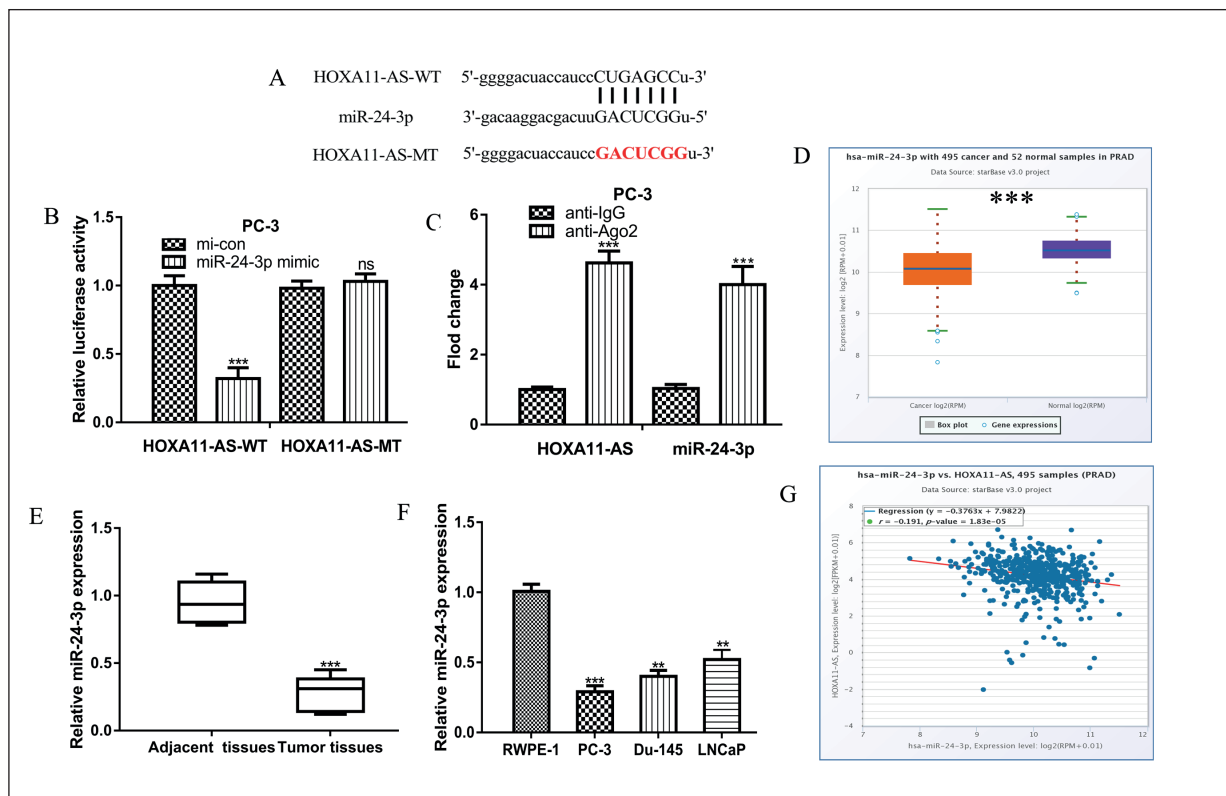


Figure 3. MiR-24-3p was a direct target of HOXA11-AS. **A**, We predicted binding modular between HOXA11-AS and miR-24-3p. **B**, Relationship between HOXA11-AS and miR-24-3p was validated by Luciferase activity assay. **C**, Co-enrichment of HOXA11-AS and miR-24-3p was validated by RIP assay. **D**, miR-24-3p was downregulated in PCa tissues based on ENCORI analysis. **E**, MiR-24-3p was downregulated in PCa tissues based on RT-qPCR. **F**, MiR-24-3p was downregulated in PCa cells based on RT-qPCR. **G**, Correlation of HOXA11-AS and miR-24-3p in PCa tissues. HOXA11-AS: HOXA11 antisense RNA; PCa: prostate cancer; RT-qPCR: Quantitative real-time PCR; miR-24-3p: microRNA-24-3p; mi-con: negative control miRNA; WT: wild type; MT: mutant; RIP: RNA immunoprecipitation.

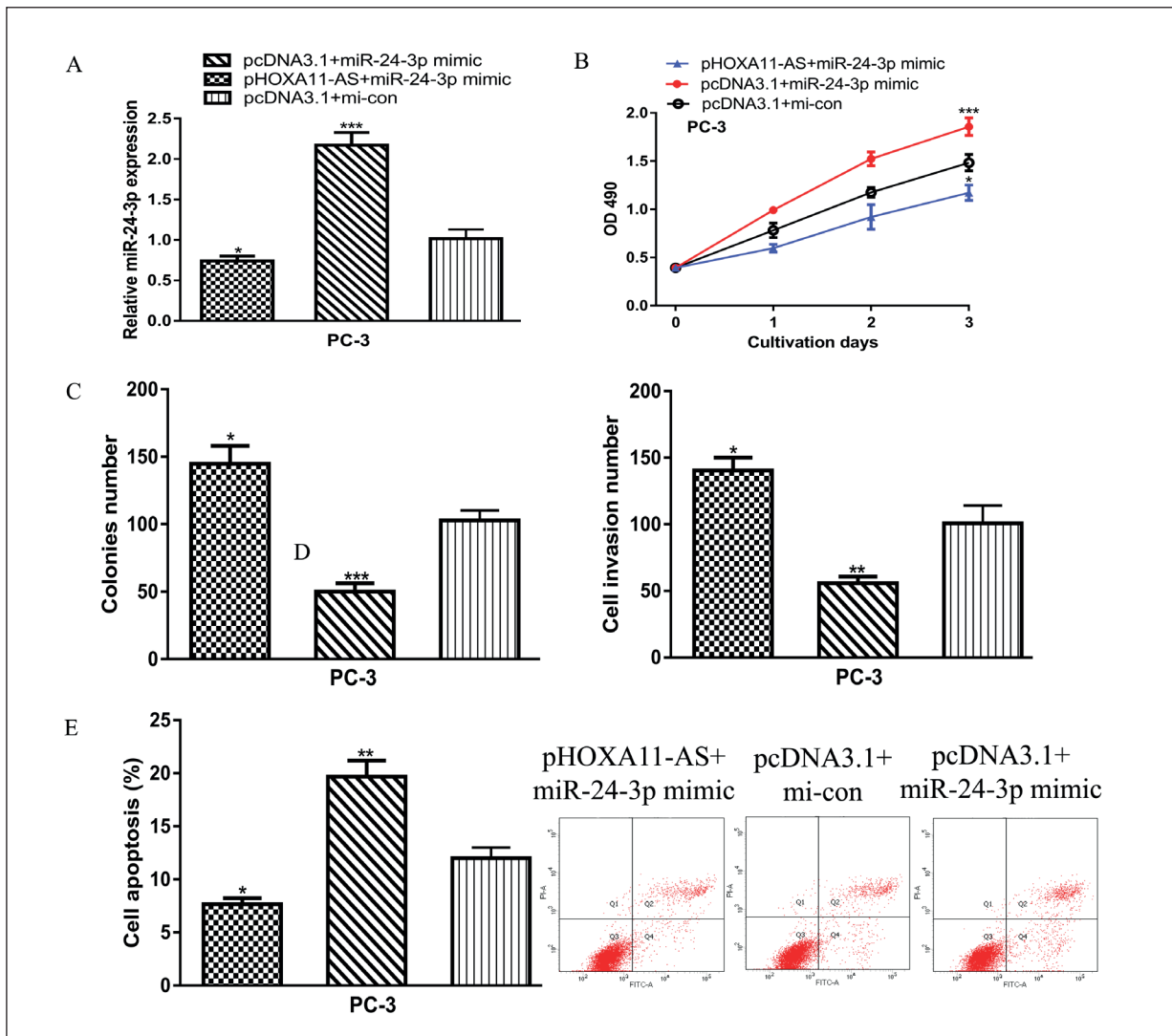


Figure 4. HOXA11-AS regulates PCa cell behaviors *via* miR-24-3p. **A**, MiR-24-3p expression, **(B)** cell proliferation, **(C)** colony formation, **(D)** cell invasion, and **(E)** cell apoptosis in PCa cells with pHOXA11-AS+miR-24-3p mimic, pcDNA3.1+miR-24-3p mimic, or pcDNA3.1+mi-con transfection. HOXA11-AS: HOXA11 antisense RNA; PCa: prostate cancer; RT-qPCR: Quantitative real-time PCR; miR-24-3p: microRNA-24-3p; mi-con: negative control miRNA.

highly expressed in PCa tissues and cells (Figure 5E-5G). Importantly, Pearson's correlation showed that miR-24-3p and JPT1 were negatively correlated in PCa tissues (Figure 5H). In the meantime, HOXA11-AS and JPT1 was positively correlated in PCa tissues (Figure 5I).

Roles of MiR-24-3p in PCa Were Exerted Via JPT1

We showed that JPT1 expression level was increased by pJPT1 and decreased by miR-24-3p mimic (Figure 6A). *In vitro* functional experiments showed that JPT1 overexpression promotes PCa cell growth and invasion (Figure 6B-6E). In

addition, we showed that JPT1 overexpression could attenuate that the roles of miR-24-3p mimic on PCa cell behaviors (Figure 6B-6E).

Knockdown of HOXA11-AS Suppresses PCa Tumorigenesis

To further validate the influences of HOXA11-AS on PCa tumorigenesis, animal experiments were conducted. Tumor volume and weight in the sh-con group were higher than sh-HOXA11-AS group (Figure 7A-7C). We then extracted RNA from tissues and showed that HOXA11-AS expression level was lower in sh-HOXA11-AS group than in sh-NC group (Figure 7D).

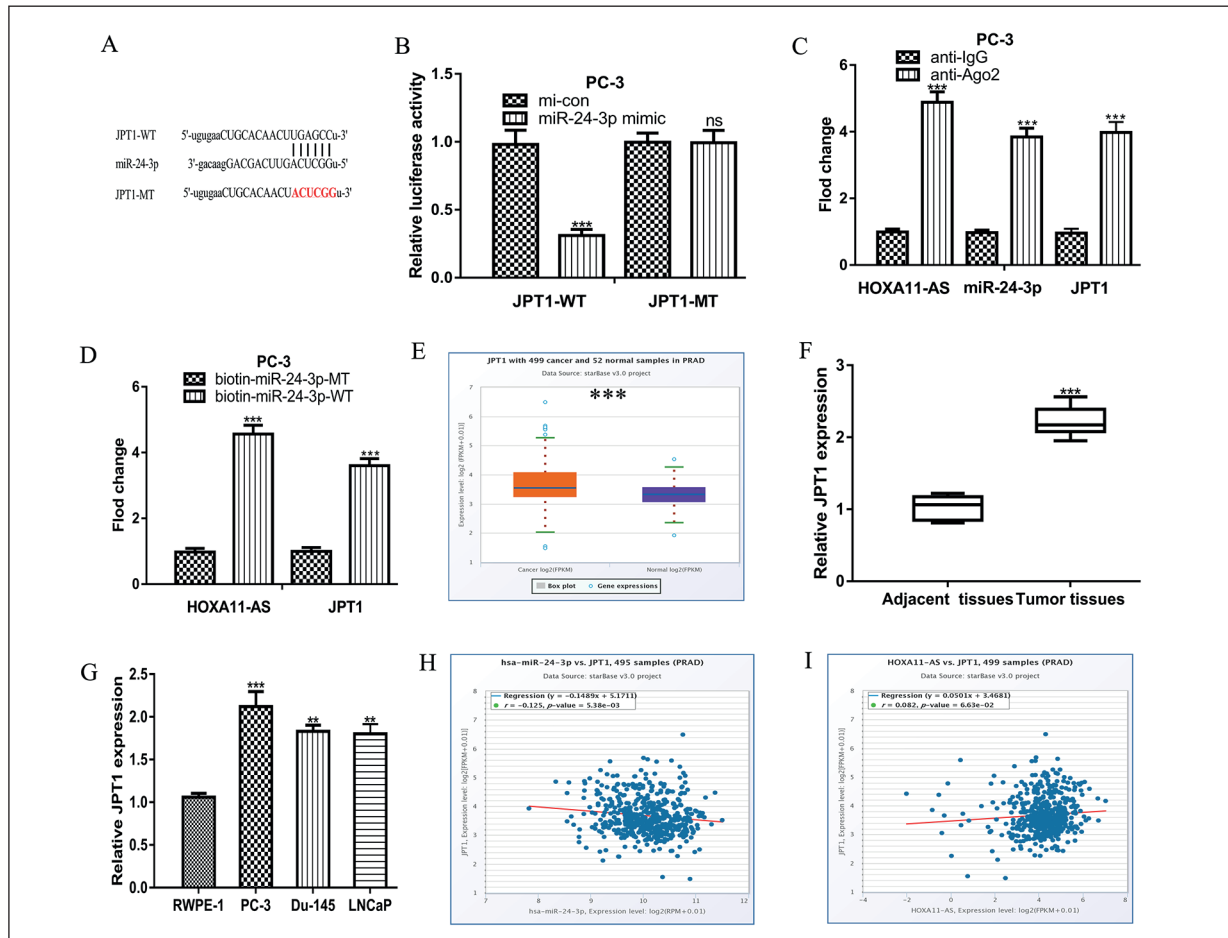


Figure 5. HOXA11-AS regulates JPT1 via miR-24-3p. **A**, We predicted binding modular between JPT1 and miR-24-3p. **B**, Relationship between JPT1 and miR-24-3p was validated by luciferase activity assay. **C**, Co-enrichment of HOXA11-AS, JPT1, and miR-24-3p was validated by RIP assay. **D**, RNA pull-down assay verified the connection of miR-24-3p and JPT1. **E**, JPT1 was upregulated in PCa tissues based on ENCORI analysis. **F**, JPT1 was upregulated in PCa tissues based on RT-qPCR. **G**, JPT1 was upregulated in PCa cells based on RT-qPCR. **H**, Correlation of JPT1 and miR-24-3p in PCa tissues. **I**, Correlation of HOXA11-AS and JPT1 in PCa tissues. HOXA11-AS: HOXA11 antisense RNA; PCa: prostate cancer; RT-qPCR: Quantitative real-time PCR; miR-24-3p: microRNA-24-3p; mi-con: negative control miRNA; WT: wild type; MT: mutant; RIP: RNA immunoprecipitation.

Discussion

LncRNAs including UCA1 and SNHG12 were found to regulate of PCa initiation and progression^{15,16}. LncRNA UCA1 was found significantly upregulated in PCa, and its knockdown could inhibit cell growth via targeting miR-143 and myosin VI axis¹⁶. Herein, HOXA11-AS levels were found remarkably increased in both PCa tissues and cells in comparison with normal counterparts. Functional assays showed that HOXA11-AS overexpression stimulates PCa cell proliferation, colony formation, invasion, and inhibits cell apoptosis *in vitro*, whereas HOXA11-AS downregulation has opposite effects. Moreover,

in vivo animal analyses showed that HOXA11-AS knockdown hindered tumorigenesis. Collectively, our results showed that HOXA11-AS plays an oncogenic role in PCa.

It has been shown that lncRNAs exerted their roles by sponging miRNA, which is also called competing endogenous RNA (ceRNA). For example, miR-148-3p and miR-761 were identified as downstream targets for HOXA11-AS^{10,11}. In non-small cell lung cancer, Bai et al¹⁰ showed that HOXA11-AS was able to stimulate cancer progression by interfering with miR-148-3p. In this study, we also tried to discover the miRNA molecular that have interaction with HOXA11-AS in PCa and found that miR-24-3p was a potential

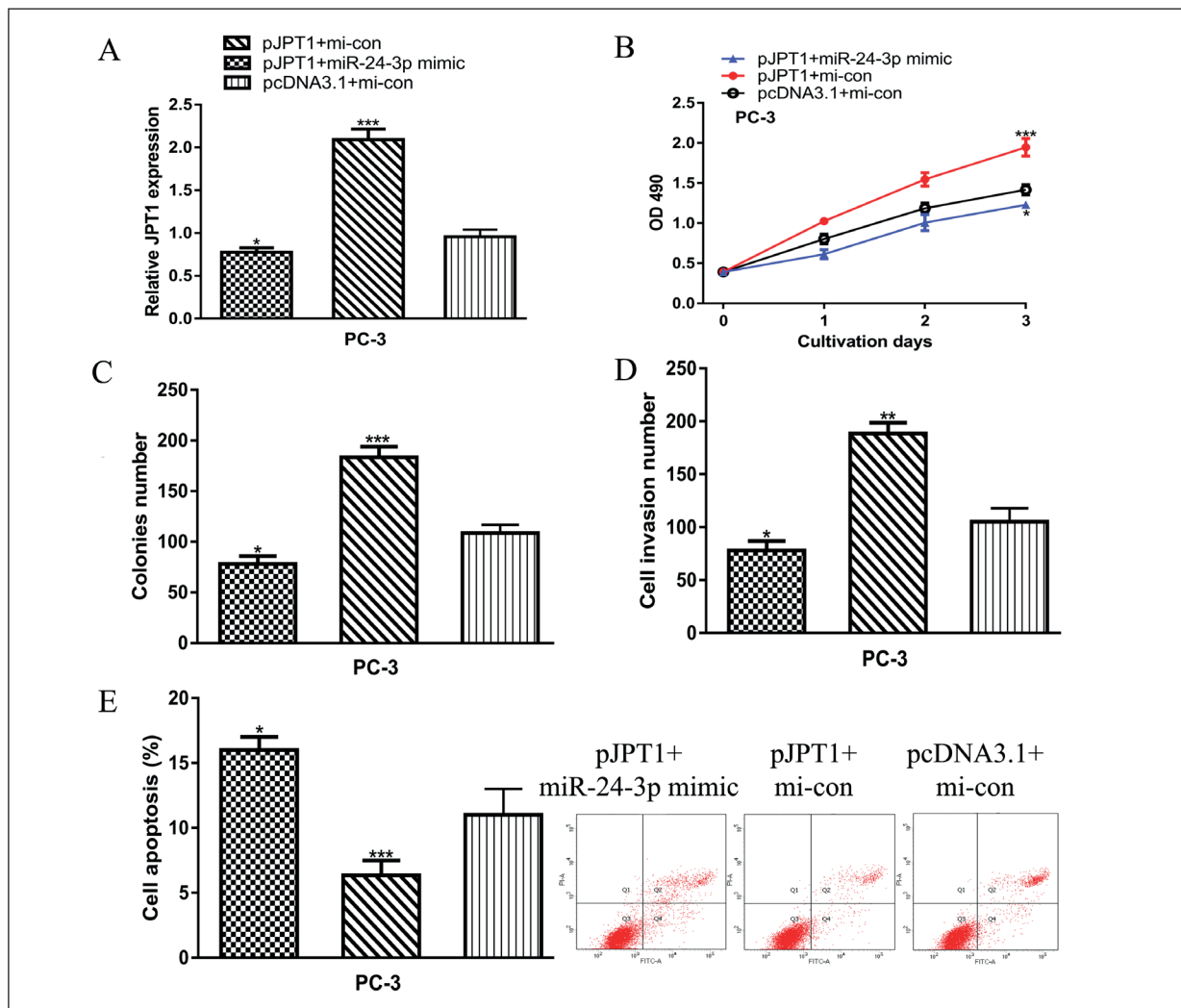


Figure 6. MiR-24-3p regulates PCa cell behaviors via regulating JPT1. **(A)**, JPT1 expression, **(B)** cell proliferation, **(C)** colony formation, **(D)** cell invasion, and **(E)** cell apoptosis in PCa cells with pJPT1+miR-24-3p mimic, pJPT1+mi-con, or pcDNA3.1+mi-con transfection. PCa: prostate cancer; RT-qPCR: Quantitative real-time PCR; miR-24-3p: microRNA-24-3p; mi-con: negative control miRNA.

target for HOXA11-AS. As reported in previous studies, miR-24-3p has dual roles in cancer progression^{13,14}. Here, functional experiments indicated that miR-24-3p overexpression could inhibit PCa cell growth, invasion, and promote apoptosis. Importantly, rescue experiments showed that miR-24-3p overexpression could attenuated the effects of HOXA11-AS on PCa cell behaviors. Hence, these results confirmed that HOXA11-AS functions as a ceRNA for miRNA.

JPT1, also known as HN1, was revealed to be used as biomarker to predict the response of endometrial cancer to metformin¹⁷. Moreover, Zhang et al¹⁸ reported that JPT1 could stimulate breast cancer carcinogenesis *via* enhancing MYC

activity. Here, we validated JPT1 was a target for miR-24-3p using Luciferase activity assay, RIP assay, and RNA pull-down assay. Functional assays revealed that JPT1 overexpression could promote PCa cell behaviors *in vitro*. Also, the roles of miR-24-3p on PCa cell events can be abolished by JPT1, indicating the direct regulatory relationship of miR-24-3p and JPT1.

Conclusions

Collectively, we showed that HOXA11-AS was elevated expression in PCa tumor tissues and cells compared with the normal counterparts. Al-

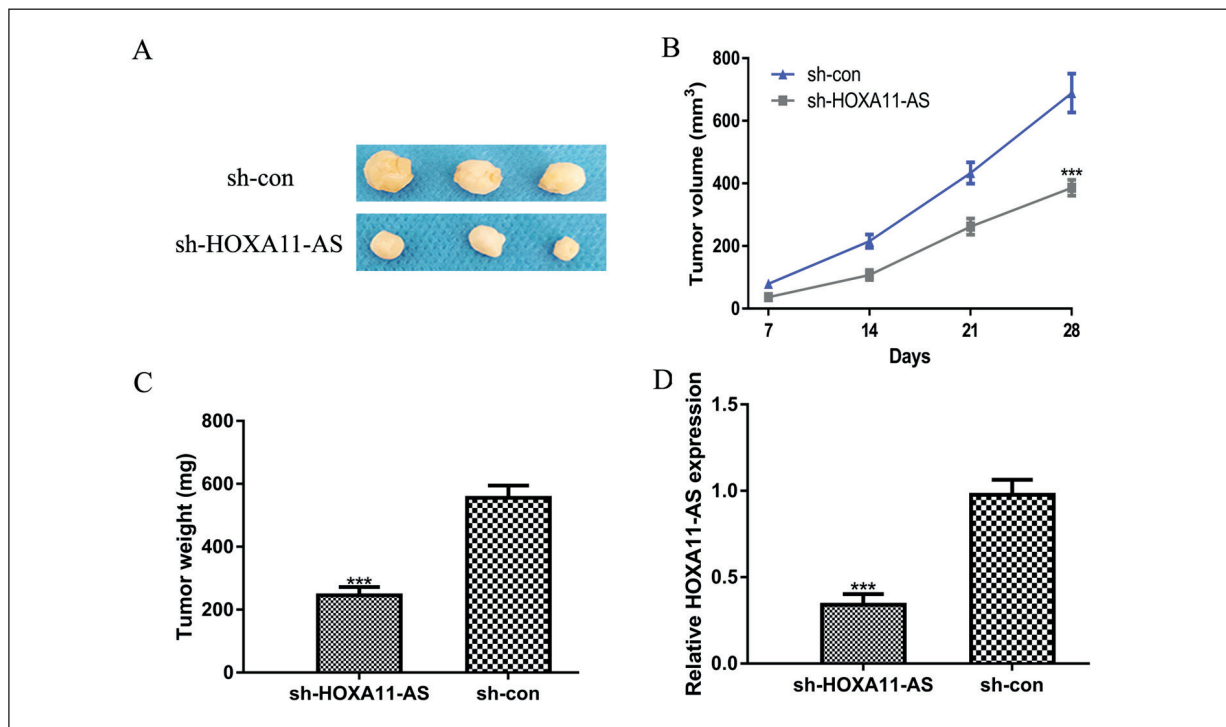


Figure 7. HOXA11-AS knockdown repressed PCa tumor growth. **A**, Images of excised tumor tissues. **B**, Tumor volume in sh-HOXA11-AS group was lower than that in sh-con group. **C**, Tumor weight in sh-HOXA11-AS group was lower than that in sh-con group. **D**, HOXA11-AS expression in sh-HOXA11-AS group was lower than that in sh-con group. HOXA11-AS: HOXA11 antisense RNA; PCa: prostate cancer.

so, we showed a novel mechanism for the roles of HOXA11-AS1 in cancer, as it can serve as ceRNA of miR-24-3p to upregulate JPT1 expression. Our results may help to understand the malignancy behaviors of PCa cells and discover novel targets for PCa treatment.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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