

Hepsin Promotes Epithelial–Mesenchymal Transition and Cell Invasion Through the miR-222/PPP2R2A/AKT Axis in Prostate Cancer

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Ruiqian Li
Jun Li
Hong Yang
Yu Bai
Chen Hu
Hongyi Wu
Haiyang Jiang
Qilin Wang

Department of Urology, Third Affiliated
Hospital of Kunming Medical University,
Kunming City, Yunnan Province, People's
Republic of China

Purpose: To determine the role and underlying mechanism of hepsin in epithelial–mesenchymal transition (EMT) and cell invasion in prostate cancer.

Methods: The expression of hepsin in prostate cancer tissue samples and cell lines was measured by immunohistochemical staining and Western blotting. The EMT and cell invasion abilities of prostate cancer cells were detected by Western blot and transwell assays. RNA transfection was used to inhibit or overexpress related genes. The expression of miR-222 was detected by RT-qPCR. A dual-luciferase reporter gene assay was performed to determine the target of miR-222.

Results: Hepsin expression was upregulated in prostate cancer tissue samples and cell lines. Inhibition of hepsin attenuated EMT and cell invasion and downregulated the expression of miR-222. Decreased miR-222 expression enhanced the level of PPP2R2A, which in turn attenuated the AKT signaling. Activation of miR-222 or AKT could block the inhibitory effects on EMT and cell invasion induced by hepsin deficiency.

Conclusion: Hepsin promotes EMT and cell invasion through the miR-222/PPP2R2A/AKT axis in prostate cancer.

Keywords: prostate cancer, hepsin, miR-222, PPP2R2A, AKT signaling

Introduction

Prostate cancer (PC) is one of the most common malignancies with high mortality among men worldwide.^{1,2} Most prostate cancer patients initially respond to androgen ablation. Prostate cancer can metastasize to the liver, bone and lungs. PC metastasis is responsible for the poor prognosis of PC and the majority of deaths related to this disease. Metastasis is a very complicated process and closely related to tumor environment, cell adhesion and cell invasion.³ Moreover, epithelial–mesenchymal transition (EMT) is proposed to be an important mechanism regulating the initial steps in cancer metastasis. Thus, it is important to investigate new targets involved in PC EMT.

Most prostate cancer patients initially respond to androgen ablation. Hepsin is a type II transmembrane serine protease (TTSP) and studies indicated that androgen is related to TTSP family.⁴ Hepsin is frequently overexpressed in PC^{5,6} and correlated positively with aggressiveness.⁷ The functions of hepsin in malignant biological behaviors, including autophagy,⁸ bone metastasis⁹ and tumor growth,¹⁰ of PC have been well studied. Hepsin is also involved in the diagnosis and

Correspondence: Qilin Wang
Department of Urology, Third Affiliated
Hospital of Kunming Medical University,
Kunming City, Yunnan Province, People's
Republic of China
Tel +86 871-68179202
Email wql2079512108@163.com

prognosis of PC.¹¹ Moreover, several studies have demonstrated a role for hepsin in PC metastasis.^{12,13} However, the mechanism underlying EMT in PC is largely unknown. Our previous results suggested that hepsin was overexpressed in PC and promoted EMT and invasion in PC.

Hepsin is required for the activation of hepatocyte growth factor (HGF) and subsequently promotes the expression of a specific receptor of HGF, MET.¹⁴ A study showed that derepressed hepsin proteolytically augmented HGF/MET signaling.¹⁵ Accumulating evidence indicates that microRNAs (miRNAs) are involved in the regulation of the biological functions of cancers, such as autophagy,¹⁶ DNA damage,¹⁷ pyroptosis¹⁸ and metastasis.¹⁹ It has been reported that miR-222 can be regulated by MET.^{20,21} Thus, we hypothesized that miR-222 may be modulated by hepsin. Moreover, miR-222 has been implicated in the development of cancer through the modulation of phosphatase 2A subunit B (PPP2R2A).^{22,23} PPP2R2A is considered a tumor suppressor and participates in the proliferation, invasion and EMT of cancer cells.^{24–26} Additionally, PPP2R2A is reported to modulate aggressive behaviors of cancer cells by blocking the AKT signaling.²⁷ The AKT signaling is closely associated with EMT.^{28,29} In this study, we determined whether miR-222 and PPP2R2A are involved in hepsin-mediated PC EMT progression via the AKT signaling.

In the present study, we found that hepsin promotes EMT and cell invasion in PC cells. Then, we identified the regulatory mechanism involving hepsin in EMT through the miR-222/PPP2R2A/AKT pathway. Thus, our data suggest important roles for hepsin in PC EMT and invasion.

Materials and Methods

Cell Culture and Samples

Prostate epithelial cell line RWPE-1, and androgen-independent human prostate cancer PC3 and DU145 cell lines were obtained from the American Type Culture Collection (ATCC). PC3 cells were derived from metastatic bone and DU145 cells were derived from metastatic brain. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and a 1% penicillin/streptomycin solution. All the cell lines were reauthenticated by short tandem repeat analysis. For AKT-activating treatment, cells were treated with the 10 μ M AKT activator SC79 (SF2730, Beyotime, China).

This study has been approved by the Ethics Committee of Yunnan Provincial Oncology Hospital. The procedures related to human subjects were approved by Yunnan Provincial Oncology Hospital. All the patients provided informed consent for the use of their specimens before sampling. Twenty-six paired samples of prostate cancer tissue and benign tissue were obtained from the Oncology Hospital of Yunnan Province. Information of the CRC patients is summarized in [Supplementary Table S1](#).

RNA Transfection

A miR-222 expression plasmid and negative mimics were purchased from Shanghai Genepharma. PC3 and DU145 cells were transfected with the miR-222 mimic or NC mimic using Lipofectamine 2000 (Invitrogen, USA). PC3 and DU145 cells were transfected with siRNA targeting hepsin (5'-ggauccuuccaggccauaaatt-3' and 5'-uuuauaggccuggaagaucct-3') (GenePharma, China). A nontargeting siRNA (5'-uucuccgaacgugucagutt-3' and 5'-acgugacacguccgagaatt-3') (GenePharma, China) was used as a negative control (siNC). The transfection efficiency was determined by RT-qPCR and Western blotting.

Immunohistochemistry (IHC) Staining

Prostate cancer and adjacent tissue samples were fixed with 4% paraformaldehyde and dehydrated with ethanol. The samples were embedded in molten paraffin. Paraffin-embedded tissue sections were deparaffinized and heated. Then, the tissue sections were incubated with antibodies and analyzed by using a DAB kit (DA1010, Solarbio, China).

Cell Invasion Assay

The invasive abilities of PC3 and DU145 cells were measured with transwell invasion assays. The upper chambers were coated with FBS-free medium-diluted Matrigel matrix (35234, BD, Biosciences, China). Cells (1×10^5) were added to the upper chamber of the transwell plate and incubated at 37°C for 24 h. Then, the cells remaining in the upper chamber were removed with cotton swabs. The cells in the lower chamber were fixed and stained with 0.3% crystal violet. The quantification of invasive cells was performed with Image-Pro Plus 6.0.

Dual-Luciferase Assay

Bioinformatics analysis was performed by TargetScan. The 3'-UTR of PPP2R2A encompassing the target sequence for miR-222 was amplified and cloned into the

pGL4.49 luciferase reporter plasmid (pGL4.49-PPP2R2A-wt). A mutant 3'-UTR of PPP2R2A was inserted into the pGL4.49 luciferase reporter plasmid (pGL4.49-PPP2R2A-mut). PC3 and DU145 cells were cotransfected with pGL4.49-PPP2R2A-wt or pGL4.49-PPP2R2A-mut and miR-222 mimics or NC mimics. Luciferase activity was measured by using the Dual-Luciferase Reporter Assay Kit (Promega, USA) according to the manufacturer's instructions.

Western Blot Assay

Whole-cell lysates were prepared in RIPA lysis buffer (R0010, Solarbio, China) and quantified with the BCA Protein Assay Kit (PA115, TIANGEN BIOTECH, China). Cell lysates were separated by SDS-PAGE and then transferred to PVDF membranes (ISEQ00010, Millipore, USA). The membranes were blocked in 10% milk and incubated with primary and secondary antibodies. The following antibodies were used: anti-hepsin (1:2000, ab189246, Abcam, UK), anti-GAPDH (1:5000, ab9485, Abcam, UK), anti-PPP2R2A (1:1000, ab28370, Abcam, UK), anti-AKT (1:1000, ab18785, Abcam, UK), and anti-p-AKT (ser473) (1:1000, 9271, CST, USA), anti-N-cadherin (1:1000, ab18203, Abcam, UK), anti-MMP2 (1:1000, ab235167, Abcam, UK), anti-MMP9 (1:1000, ab38898, Abcam, UK). The intensity of Western blot bands was quantified by ImageJ.

Real-Time Quantitative PCR (RT-qPCR)

Total RNA was isolated with TRIzol reagent (Invitrogen, Thermo Fisher Scientific, USA). cDNA was synthesized with a PrimiScript RT reagent kit (RR047AA, Takara, Japan). RT-qPCR analysis was performed using the Bio-Rad CFX96 Real-Time PCR Detection System with SYBR Green PCR Master Mix (Bio-Rad). The primers used for RT-qPCR were as follows: miR-222 (5'-cgcagctacatctggctactg-3' and 5'-gtgcagggtccgaggt-3') and U6 (5'-cgcttcggcagcacatatac-3' and 5'-caggggccatgctaattctt-3').

Statistical Analysis

All experiments were independently performed with at least three biological repeats. All quantitative data are presented as the mean \pm SEM, and differences were analyzed by Student's *t* test or one-way ANOVA (* p <0.05, ** p <0.01, and *** p <0.001).

Results

Hepsin Expression is Upregulated in Prostate Cancer Samples and Cells and Positively Associated with EMT and Cell Invasion in Prostate Cancer Cells

To determine hepsin expression in prostate cancer samples, we performed IHC staining and Western blot of prostate cancer and benign tissue samples. IHC staining showed that prostate cancer tissue (T) has increased hepsin staining compared with benign tissue (N). (Figure 1A). Western blot results indicated that hepsin expression was higher in prostate cancer tissue (Figure 1B). We next investigated the expression levels in the prostate cancer cell lines PC3 and DU145 and prostate epithelial cell line RWPE-1. As shown in Figure 1C, the expression levels in PC3 cells and DU145 cells were increased. Collectively, our results indicated that hepsin might serve as an oncogene in prostate cancer.

To determine the role of hepsin in prostate cancer cell EMT, we established PC3 and DU145 cells with hepsin knockdown (si-hepsin) (Figure 1D). Western blot assays showed that the knockdown of hepsin decreased expression of EMT markers including N-cadherin, MMP2 and MMP9 (Figure 1E). Moreover, hepsin silencing attenuated prostate cancer cell invasion (Figure 1F). These results suggested that hepsin inhibition impaired EMT, accounting for the inhibition of cell invasion.

Hepsin Promotes EMT by Regulating miR-222 Expression

Studies have indicated that MET can be modulated by hepsin.^{15,30} Moreover, a study showed that miR-222 is a miRNA regulated by MET.³¹ miR-222 is involved in the development of prostate cancer.^{32,33} To determine whether miR-222 expression levels are altered in hepsin-deficient prostate cancer cells, we first analyzed miR-222 expression in hepsin-knockdown PC3 and DU145 cells by RT-qPCR. As shown in Figure 2A, hepsin knockdown decreased the expression of miR-222. We next transduced a miR-222 mimic into hepsin-knockdown PC3 and DU145 cells and found that the miR-222 mimic rescued N-cadherin, MMP2 and MMP9 expression in PC3 and DU145 cells (Figure 2A and B). Moreover, miR-222 mimic also reversed the hepsin-mediated inhibition of cell invasion (Figure 2C).

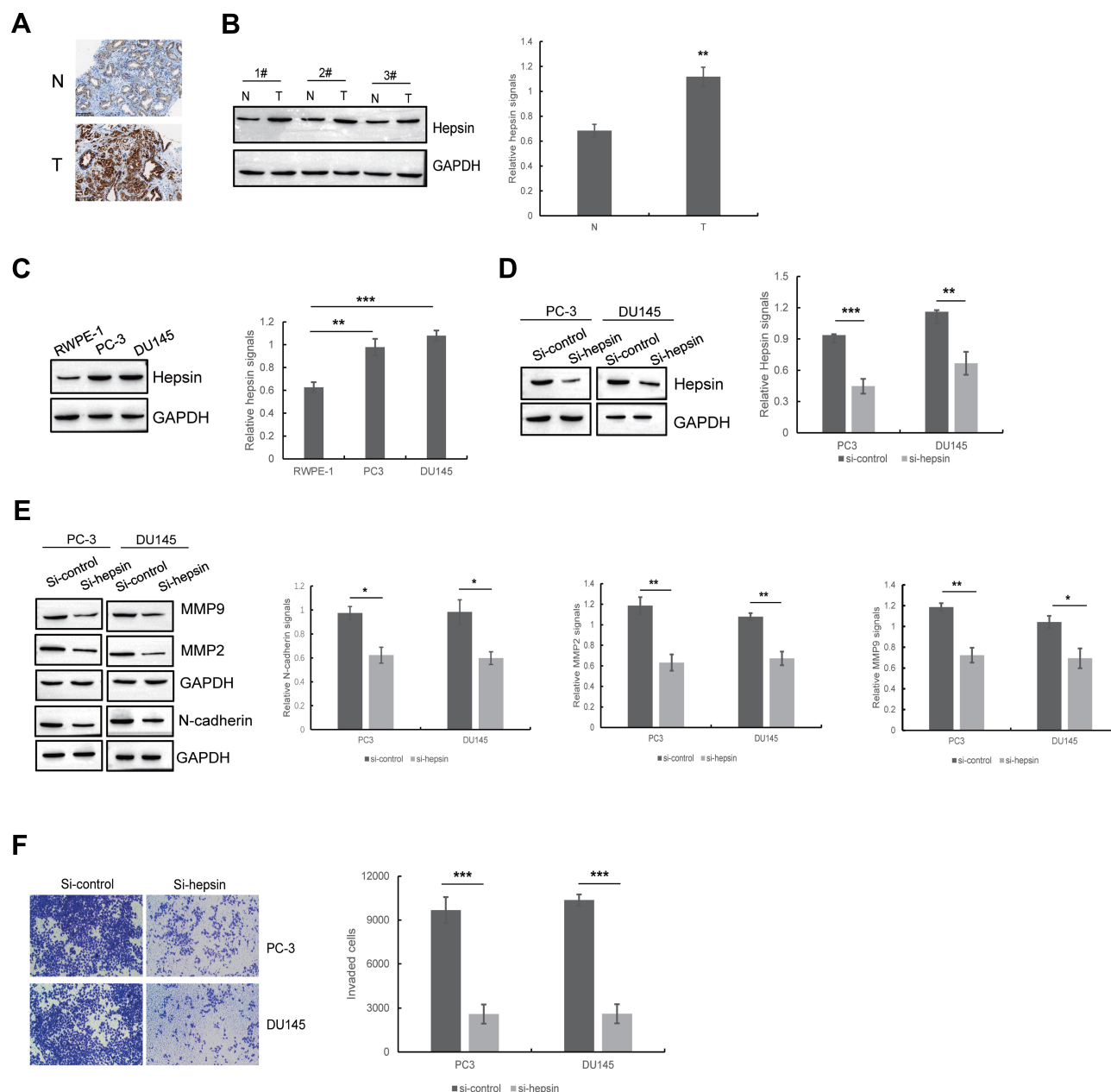


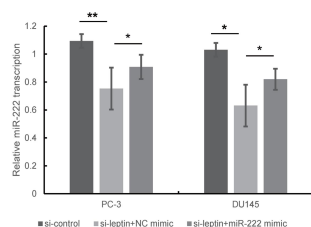
Figure 1 Hepsin is upregulated in prostate cancer samples and cells and positively associated with EMT and cell invasion in prostate cancer cells. **(A)** Representative immunohistochemistry staining of hepsin in human prostate cancer tissues and benign tissues. **(B)** Western blots of hepsin in prostate cancer tissues and benign tissues. A total of 26 paired tissue specimens were examined and only 3 paired tissue specimens were shown here. $**p < 0.01$. **(C)** The expression of hepsin in RWPE-1, PC3 and DU145 cell lines was detected by Western blotting. $**p < 0.01$, $***p < 0.001$. **(D)** Western blot showed the knockdown of hepsin by siRNA in PC3 and DU145 cells. $**p < 0.01$, $***p < 0.001$. **(E)** The effect of hepsin knockdown on EMT was determined by Western blotting. $*p < 0.05$, $**p < 0.01$. **(F)** The effect of hepsin knockdown on cell invasion was determined by transwell assays. $***p < 0.001$. Quantitative data are presented as the means \pm SEM ($n=3$), P value was determined by Student's t test or one-way ANOVA.

miR-222 Directly Targets the 3'-UTR of PPP2R2A

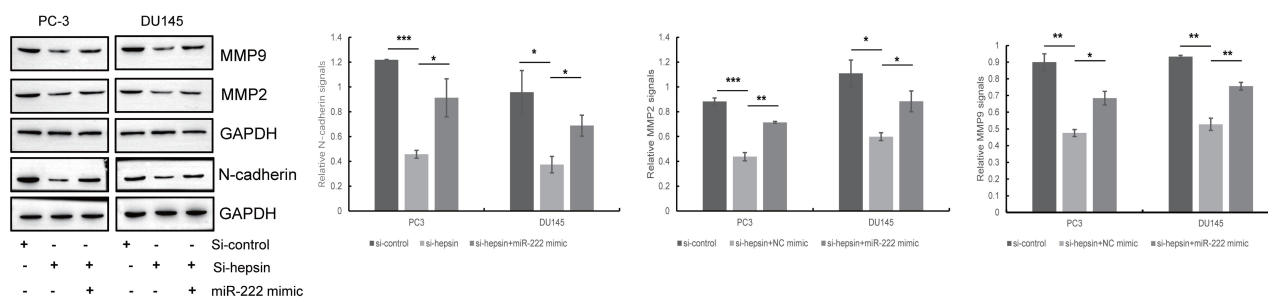
To investigate the target gene of miR-222, we made predictions by using TargetScan. PPP2R2A is a target gene of miR-222 that plays a significant role in the development of cancers, including breast cancer,³⁴ liver cancer³⁵ and prostate cancer.³⁶ To determine whether

miR-222 targets PPP2R2A, we transfected PC3 and DU145 cells with vectors containing the full-length wild-type or mutant 3'-UTR of PPP2R2A and conducted a dual-luciferase reporter gene assay. As shown in Figure 3A and B, miR-222 decreased the luciferase activity of pGL4.49-PPP2R2A-wt but had minimal effects on the luciferase activity of pGL4.49-PPP2R2A-mut. We

A



B



C

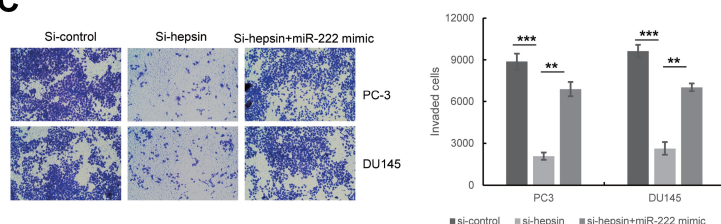


Figure 2 Hepsin promotes EMT through regulating miR-222 expression. **(A)** The effect of hepsin knockdown on miR-222 expression was detected by RT-qPCR. * $p < 0.05$, ** $p < 0.01$. **(B)** The effect of miR-222 on hepsin knockdown-induced inhibition of EMT was detected by Western blotting. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. **(C)** The effect of miR-222 on hepsin knockdown-induced inhibition of cell invasion was detected by transwell assay. * $p < 0.01$, *** $p < 0.001$. Quantitative data are presented as the means \pm SEM ($n=3$), P value was determined by Student's t test or one-way ANOVA.

next treated PC3 and DU145 cells with an NC mimic or a miR-222 mimic and then performed Western blot assays. Western blot analysis showed that miR-222 mimic treatment suppressed PPP2R2A expression (Figure 3C). All these results indicate that miR-222 directly targets PPP2R2A.

Hepsin Promotes EMT and Cell Invasion in Prostate Cancer Cells via the PPP2R2A/AKT Signaling Pathway

Several studies have indicated that PPP2R2A inhibition can enhance AKT signaling³⁷ and that AKT is responsible for the EMT process and cell invasion.^{38,39} Thus, we detected the AKT phosphorylation status in PC3 and DU145 cells upon hepsin knockdown. As shown in Figure 4A, the p-Akt (ser473)/Akt ratio was decreased in hepsin-knockdown PC3 and DU145 cells.

To check whether AKT signaling accounts for the EMT and cell invasion behaviors of prostate cancer cells, the AKT activator SC79⁴⁰ was used to treat hepsin-knockdown PC3 and DU145 cells (Figure 4B). SC79 administration blocked the anti-EMT effects of hepsin knockdown (Figure 4C). Moreover, SC79 administration also promoted cell invasion in hepsin-knockdown PC3 and DU145 cells (Figure 4D).

Discussion

In this study, we report that hepsin promotes cell invasion and EMT by modulating the miR-222/PPP2R2A/AKT pathway in prostate cancer. Inhibition of hepsin in prostate cancer cells represses miR-222, which then induces the expression of PPP2R2A. Activated PPP2R2A is able to suppress the AKT pathway. As a consequence, cell invasion and EMT are attenuated by hepsin knockdown. Therefore, our studies reveal the role and underlying

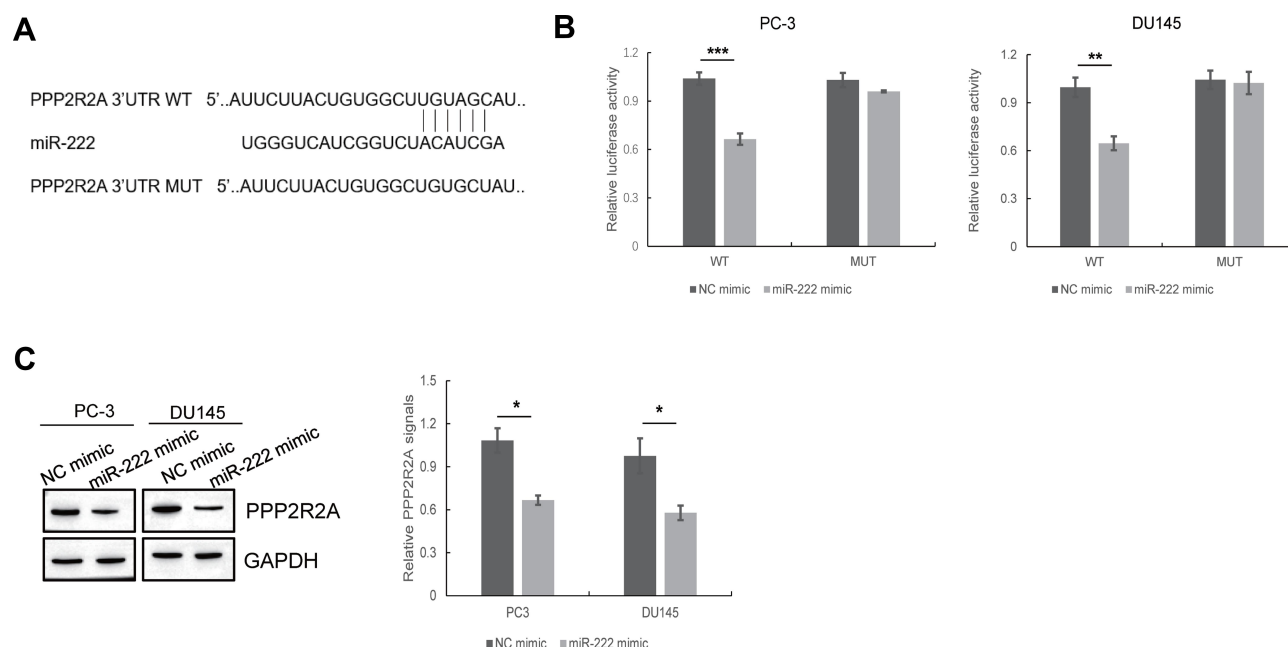


Figure 3 miR-222 directly targets the 3'UTR of PPP2R2A. **(A)** The predicted binding sites with miR-222 within the PPP2R2A 3'-UTR are shown according to Targetscan software. **(B)** The correlation between miR-222 and PPP2R2A was determined by dual-luciferase reporter gene assays. $^{**}p < 0.01$, $^{***}p < 0.001$. **(C)** The effect of miR-222 mimic on PPP2R2A expression was detected by Western blotting. $^{*}p < 0.05$. Quantitative data are presented as the means \pm SEM ($n=3$), P value was determined by Student's t test or one-way ANOVA.

mechanism of hepsin in cell invasion and EMT in prostate cancer.

Hepsin is a type II transmembrane serine protease that is critical for the development of prostate cancer. Zhang et al reported that hepsin modulates cell cycle progression in prostate cancer.⁴¹ Willbold et al demonstrated that the proteolytic activity of hepsin is related to ER stress and autophagy in prostate cancer cells.⁸ An inhibitor of hepsin was reported to block prostate cancer bone metastasis.⁹ Here, we show that hepsin is overexpressed in prostate cancer and that hepsin knockdown inhibits cell invasion and EMT in prostate cancer.

Previous studies have suggested that hepsin is a type II transmembrane serine protease that proteolytically activates HGF. Moreover, the expression of MET, a specific receptor of HGF, can also be upregulated by hepsin.^{14,42} Accumulating evidence has demonstrated that the miR-222 level is increased by MET.^{20,21} Based on these data, we hypothesize that miR-222 can be modulated by hepsin. The functional roles of miR-222 in cell invasion and EMT have been well studied. For example, miR-222 has been shown to promote EMT by targeting adiponectin receptor 1 in breast cancer.⁴³ Li et al demonstrated that miR-222 promotes invasion in

gastric cancer cells.⁴⁴ Here, we show that hepsin upregulates the expression of miR-222. In addition, miR-222 overexpression was shown to rescue the inhibitory effects on cell invasion and EMT mediated by hepsin knockdown.

Most miRNAs function by modulating the expression of their target genes. PPP2R2A is a target gene of miR-222 and is considered to be a tumor suppressor.^{24,45} Studies have indicated that PPP2R2A regulates cancer development via AKT pathways. Zhang et al reported that Pits2 promotes PPP2R2A-mediated AKT inhibition and represses pancreatic carcinogenesis. Zeng et al suggested that PPP2R2A is involved in bladder cancer chemoresistance via the AKT/mTOR axis.²² Our current data indicate that ectopic expression of miR-222 leads to the downregulation of PPP2R2A expression. The PPP2R2A-mediated AKT pathway is responsible for hepsin-induced cell invasion and EMT in prostate cancer.

Conclusion

Collectively, our results indicate that hepsin promotes cell invasion and EMT by increasing the expression of miR-222. As a consequence, upregulated miR-222 reduces PPP2R2A expression, which in turn activates the AKT

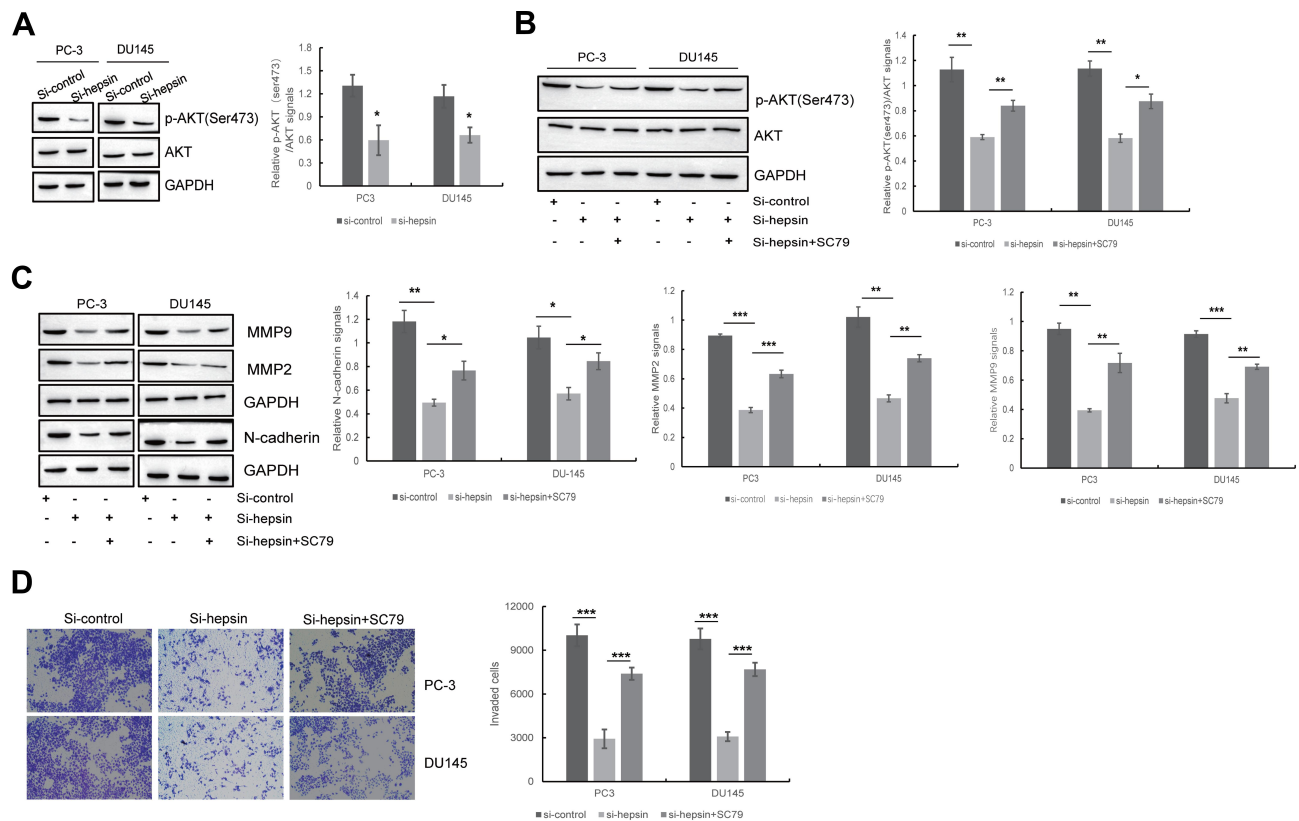


Figure 4 Hepsin promotes EMT and cell invasion of prostate cancer cells by PPP2R2A/AKT signaling pathway. **(A)** Western blot showed the effect of hepsin knockdown on AKT signaling pathway. * $p < 0.05$. **(B)** The expression of p-AKT (ser473) with AKT inhibitor treatment was detected by Western blot. * $p < 0.05$, ** $p < 0.01$. **(C)** The effect of AKT signaling pathway on hepsin knockdown-induced inhibition of EMT was detected by Western blotting. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. **(D)** The effect of miR-222 on hepsin knockdown-induced inhibition of cell invasion was detected by transwell assay. *** $p < 0.001$. Quantitative data are presented as the means \pm SEM ($n=3$), P value was determined by Student's t test or one-way ANOVA.

pathway. Our study is the first to uncover the mechanism involving hepsin in prostate cancer cell invasion and EMT.

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Disclosure

The authors declare that they have no competing interests.

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