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MiR-492 exerts tumor-promoting function in prostate cancer through repressing SOCS2 expression

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Abstract. – OBJECTIVE: MiRNAs have been verified to play a role in the development and progression of prostate cancer (PCa). However, the role of miR-492 in PCa has not been mentioned. We aim to detect the expression of miR-492 in PCa and explore its underlying mechanism.

PATIENTS AND METHODS: The relative expression of miR-492 in PCa tissue samples to normal prostate tissues was detected using quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). The level of miR-492 in PCa-derived cell lines compared with the normal prostate cell line was also measured. Cell counting kit-8 (CCK-8) and colony formation assays were employed to investigate the cell proliferation ability. Transwell assay and wound-healing assays were utilized to explore the cell invasion and migration abilities. Luciferase assay and Western blot were utilized to explore the underlying mechanism of miR-492 in PCa cells.

RESULTS: MiR-492 expressed significantly higher in PCa tissues than that in the normal tissues. Its expression level was also over-expressed in PCa cells compared with that in the normal cells. The up-regulation of miR-492 promoted the growth, invasion, and migration of the cells, while down-regulation had the opposite effects. SOCS2 was identified as a potential target for miR-492 in PCa. Silencing of SOCS2 could neutralize the inhibitory function of miR-492 inhibitor in PCa cells.

CONCLUSIONS: This study demonstrated that miR-492 was over-expressed in PCa and exerted tumor-promoting function in PCa cells via repressing SOCS2 expression. This might provide a new sight for future accurate therapy for PCa.

Key Words:
Prostate cancer, MiR-492, Proliferation, Metastasis, SOCS2.

Introduction

The incidence of prostate cancer (PCa) is increasing year by year, and PCa is one of the most common malignant tumors in the male genitourinary system. Although PCa is relatively inferior in terms of malignancy, its occurrence and progression still seriously threaten the men's health[1,2]. Growth and metastasis are important biological characteristics of PCa, and some patients already have had distant metastasis at the time of diagnosis. However, the molecular mechanism of PCa growth and metastasis is still unclear[3]. Regulation of PCa proliferation, migration-related gene expression, inhibition of cell proliferation, and migration are hot spots in the PCa targeted therapy study.

MicroRNAs (miRNAs) are endogenous, non-coding RNAs found in somatic cells of eukaryotes that inhibit the expression of genes at the transcriptional level and act as oncogenes or tumor suppressor genes[4,5]. MiR-492 highly expressed in various tumors and played a role in promoting cancer. In hepatoblastoma, miR-492, which processes from the keratin 19 gene, regulates the cell metastasis *via* regulating the CD44[6,7]. Besides, in

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osteosarcoma, it suppresses the cell proliferation and metastasis targeting the PAK7[8]. High expression of miR-492 associates with poor prognosis of pancreatic cancer and leads to the increase of cell proliferation and cell circle of human breast cancer cells *via* down-regulating SOX7 protein level[9]. Furthermore, miR-492 was involved in the radiotherapy response in cervical squamous cell carcinomas and the Oxaliplatin resistance of colon cancer cells through CD147[10,11]. However, the function of miR-492 in PCa has not been mentioned before. Besides, the role of miR-492 in PCa is worthy of study.

The current work found that the expression of miR-492 in prostate cancer tissues and cell lines was significantly increased by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). MiR-492 mimics and inhibitor were transfected into prostate cancer cell line DU145 or PC-3. Besides, the effect of over-expression or under-expression of miR-492 on the proliferation and migration of prostate cancer cell line DU145 or PC-3 was examined. Moreover, downstream genes and proteins for miR-492 were explored. These findings might provide a new experimental basis for miRNA molecular targeted therapy of prostate cancer.

Patients and Methods

Prostate Cancer Tissues

The 76 prostate cancer tissues and 28 normal prostate tissues used in the study were obtained from the surgically resected specimens in the First Affiliated Hospital of Jinan University from 2013 to 2017. All patients with prostate cancer did not receive any radiotherapy, chemotherapy or hormonal therapy before the surgery. Specimens were preserved in liquid nitrogen after excision. All patients signed an informed consent form. This study was approved by the Ethics Committee of the First Affiliated Hospital of Jinan University.

Cell Lines and Culture

Roswell Park Memorial Institute-1640 (RPMI-1640) medium, keratinocyte serum-free medium (KSFM) medium, and fetal bovine serum (FBS) used for cell culture were purchased from HyClone (South Logan, UT, USA). The prostate cancer cell lines LNCap, 22RV1, DU145, PC3, and human normal prostate epithelial cells RWPE-1 were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA). Prostate cancer cells LNCap, 22RV1, DU145, and PC3 were cultured in RPMI-1640 medium containing 10% FBS. Besides, the human normal prostate epithelial cells RWPE-1 were cultured in KSFM medium containing 10% FBS. The cells were routinely cultured in an incubator at 37°C, with 5% CO_2, and saturated humidity.

Cell Transfection

MiR-492 mimics, inhibitor, negative control (NC) and inhibitor negative control (INC) were synthesized and constructed by Guangzhou Ribobio Co., Ltd (Guangzhou, China). The siRNA used for knocking down SOCS2 was constructed by GeneWiz (Suchou, China). The transfection procedure was performed by transfecting DU145 or PC3 cells in a logarithmic growth phase with reference to Lipofectamine™ 2000 instructions (Invitrogen, Carlsbad, CA, USA), and transiently transfected miR-492 mimics, NC, miR-492 inhibitor, INC, and siRNA-SOCS2, respectively. Transfection efficiency was detected by qRT-PCR.

QRT-PCR Detection of MiR-492 and SOCS2 mRNA Expression

Total RNA was extracted by TRIzol (Invitrogen, Carlsbad, CA, USA) method and reverse transcribed into cDNA using a reverse transcription kit (TaKaRa, Otsu, Shiga, Japan). The qRT-PCR assay was performed using a quantitative Real Time-PCR kit (Invitrogen, Carlsbad, CA, USA). The miR-492 assay used U6 as an internal reference and SOCS2 used glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as an internal reference. The qRT-PCR amplification conditions were: 95°C for 3 min; 95°C for 15 s, 58.8°C for 30 s, 72°C for 30 s, 35 cycles. The relative expression levels were analyzed by the $2^{-\Delta\Delta Ct}$ method. The primers used were as follows: SOCS2 Forward: 5'-ACTCTTGCCAAGTCTCGTCG-3', Reverse: 5'-ATCCTGGAGGACGGATGACA-3'; GAPDH Forward: 5'-ATGCCTCCTGCACCACCAACTGCTT-3', Reverse: 5'-TGGCAGTGATGGCATGGACTGTGGT-3'. All experiments were repeated independently three times or more.

Cell Counting Kit-8 (CCK-8) Method for Detecting Cell Proliferation Activity

The CCK-8 kit (Donjindo, Kumamoto, Japan) was used to detect cell proliferation. After 48 h of transfection, the experimental cells were digested and the cell density was adjusted. Besides, 4×10^3

cells per well were seeded in 96-well plates. After the incubation for 1, 2, 3, and 4 days, 10 μL/well of CCK-8 solution was added and incubated for 2 h. The absorbance (OD value) of each well at 490 nm was measured by a microplate reader, and four replicate wells per group.

Cloning Formation Experiment

After the established cells were treated into a single cell suspension, the cells were seeded in a 6-well plate at 500 cells per well. Then, they were cultured in a medium containing 10% FBS until the macroscopic colony formation was observed. After the methanol fixed, cells were stained with crystal violet. Colonies containing more than 50 cells were counted. The test was repeated 3 times.

Wound-Healing Assay

Cells were transfected and plated in a 6-well plate, next cultured to 100% confluence. After the cells were washed with phosphate-buffered saline (PBS), three scars were drawn in parallel at the bottom of the 6-well plate with a pipette tip (200 μL). Then, after the cells were washed with PBS, serum-free medium was added. The migration of the cells was observed after 48 h later.

Transwell Assay for Cell Invasion

The experiment used an 8 μm chamber purchased from Millipore (Billerica, MA, USA), and the upper layer of the chamber was added with Matrigel (BD Biosciences, Franklin Lakes, NJ, USA). After transfection for 48 h, cells were collected, and a single cell suspension was prepared with serum-free medium. A total of 4×10^4 cells was inoculated into each well in the upper chamber, while the whole medium was added to the lower chamber and was cultured for 36 h. The upper chamber was removed, and the cells that had not passed through the membrane were wiped off with a cotton swab. The cells were fixed in methanol for 10 min, stained with 0.1% crystal violet for 20 min, and observed under the microscope. The number of cells in each well was taken from four visions and averaged.

Dual Luciferase Reporter Gene

Luciferase reporter vectors (Promega, Madison, WI, USA) were co-transfected into DU-145 cells with miR-492 mimics or NC, respectively, according to the Lipofectamine™ 2000 instructions. The experimental groups were SOCS2-Wt+NC, SOCS2-Wt+miR-492, SOCS2-Mut+NC, and SOCS2-Mut+miR-492. The cells were collected after 48 h of incubation. The luciferase activity was measured using a microplate reader according to the instructions of the dual luciferase reporter assay kit (Promega, Madison, WI, USA). Relative luciferase activity was equal to Firefly luciferase activity value/Renilla luciferase activity value.

Western Blot Analysis of SOCS2 Protein and Related Protein Expression

After the transfection for 48 h, the cells were digested and extracted, and total protein was extracted by radioimmunoprecipitation assay (RIPA; Beyotime, Shanghai, China). A total of 50 μg of protein in each group was electrophoresed on sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and transferred to polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). 5% skim milk was used for blocking at room temperature for 2 h. The primary antibody of Anti-SOCS2 (1:1000) and GAPDH (1:2000) (Abcam, Cambridge, MA, USA) were incubated with the membrane at 4°C overnight. Next, the membrane was incubated with the secondary antibody at room temperature for 2 h. The membrane was exposed using enhanced chemiluminescence (ECL) kit (Millipore, Billerica, MA, USA).

Statistical Analysis

The data were statistically analyzed using Statistical Product and Service Solutions (SPSS) 20.0 statistical software (IBM, Armonk, NY, USA). The measurement data were expressed as mean ± standard deviation. The comparison of multiple sample means was analyzed by one-way ANOVA, followed by Post Hoc Test (Least Significant Difference). The two samples were compared by the *t*-test. $p<0.05$ was considered to be statistically significant.

Results

MiR-492 Was Over-Expressed in Prostate Cancer Tissues and Cell Lines

To evaluate the expression level of miR-492 in prostate cancer (PCa), we obtained 76PCa tissue samples and 28 normal prostate tissue samples. The expression of miR-492 was measured by qRT-PCR. Figure 1A showed that miR-492 expressed significantly higher in PCa tissues than in normal prostate tissues. Besides, the expression of miR-492 in four PCa cell lines including DU145,

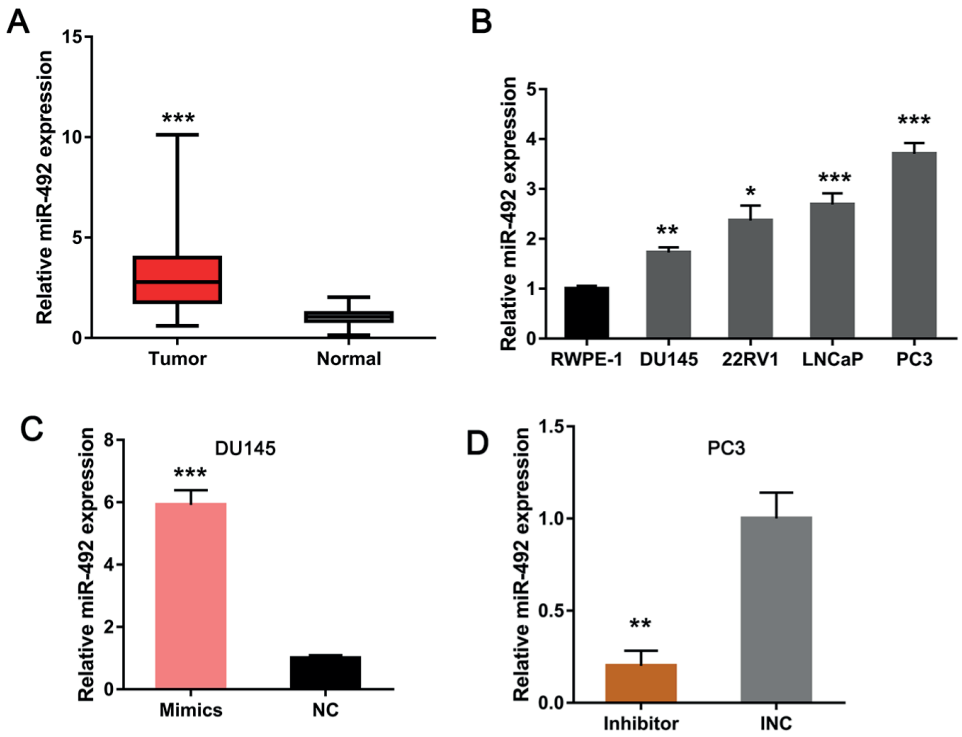

Figure 1. MiR-492 increased in PCa tissues and cell lines. ***A,*** Analysis of the expression level of miR-492 in 76 PCa tissue samples and 28 normal prostate tissue samples. ***B,*** Analysis of miR-492 expression level in PCa cell lines (DU145, LNCaP, 22RV1, PC3) and human normal prostate epithelial cell line RWPE-1. ***C,*** Expression of miR-492 in miR-492 mimics treated DU145 cells. ***D,*** Expression of miR-492 in miR-492 inhibitor-treated PC3 cells. *p<0.05, **p<0.01, ***p<0.001.

22RV1, LNCaP, and PC3 and in the normal prostate epithelial cell line RWPE-1 was also detected, respectively. The expression level of MiR-492 was markedly up-regulated in the PCa-derived cells (Figure 1B). These results indicated that miR-492 might act as a tumor-promoting gene in PCa.

Next, we chose DU145 cells and PC3 cells to change the expression of miR-492 for the subsequent experimental analysis. The miR-492 mimics enhanced the expression of miR-492 in DU145 cells compared to the NC group while the miR-492 inhibitor inhibited the expression of miR-492 in PC3 cells compared to the INC group (Figure 1C, 1D).

Abnormal Expression of MiR-492 Influenced Cell Proliferation of PCa Cells

To analyze the proliferation ability of cells, we recruited CCK8 and colony formation assays. CCK8 assay results indicated that over-expression of miR-492 markedly promoted the cell growth of DU145 at 3 and 4 days (Figure 2A). On the contrary, knockdown of miR-492 inhibited the proliferation of PC3 cells compared to the INC group (Figure 2B). At the same time, colony formation assay results showed that DU145 cells transfected with miR-492 mimics formed more colonies than the NC group. However, PC3 cells treated with miR-492 inhibitor developed fewer colonies than the INC group (Figure 2C, 2D). These results suggested that miR-492 could promote the proliferation of PCa cells.

Ectopic Expression of MiR-492 Affected Cell Invasion and Migration of PCa Cells

Next, we studied the effect of miR-492 on cell metastasis of PCa using transwell Matrigel assay and wound-healing assay. Clearly shown in Figure 3A, DU145 cells passed more cells through the membrane after the up-regulation of miR-492 but PC3 cells passed less after down-regulation of miR-492 (Figure 3A). Those results suggested that miR-492 promoted cell invasion of PCa. Then, wound-healing assay demonstrated that wound-healing rate of DU145 cells treated with miR-492 mimics was significantly higher than that in the NC group (Figure 3B). However, miR-492 inhibitor treatment slowed down the wound-healing rate of PC3 cells (Figure 3C). All

these results suggested that miR-492 promotes the invasion and migration of PCa.

SOCS2 was a Direct Target of MiR-492 in PCa

As have elucidated that miR-492 promoted cell proliferation and metastasis in PCa, we further aim to explore the underlying mechanism of miR-492 in PCa. By using bioinformatics analysis, we searched several databases (miRbase, PiTar, miRWalk) and found SOCS2 as a potential target for miR-492 in PCa. The binding site of SOCS2 3'-untranslated region (3'-UTR) with miR-492 was shown in Figure 4A. To verify our assumption, we conducted the dual-luciferase assay. We found that the luciferase activity of wild type 3'-UTR group (WT) was significantly decreased while the mutant (Mut) group increased compared to the relative control group (Figure 4B). By using qRT-PCR, we examined SOCS2 expression in PCa tissues and found a more significant decrease than that of the normal group (Figure 4C). The protein level of SOCS2 in established cells were measured using Western blotting. The protein level of SOCS2 was reduced in miR-492 mimics treated DU145 cells but increased in miR-492 inhibitor transfected PC3 cells compared to the control group, relatively (Figure 4D). All these indicated that SOCS2 serves as a direct target for miR-492 in PCa cells.

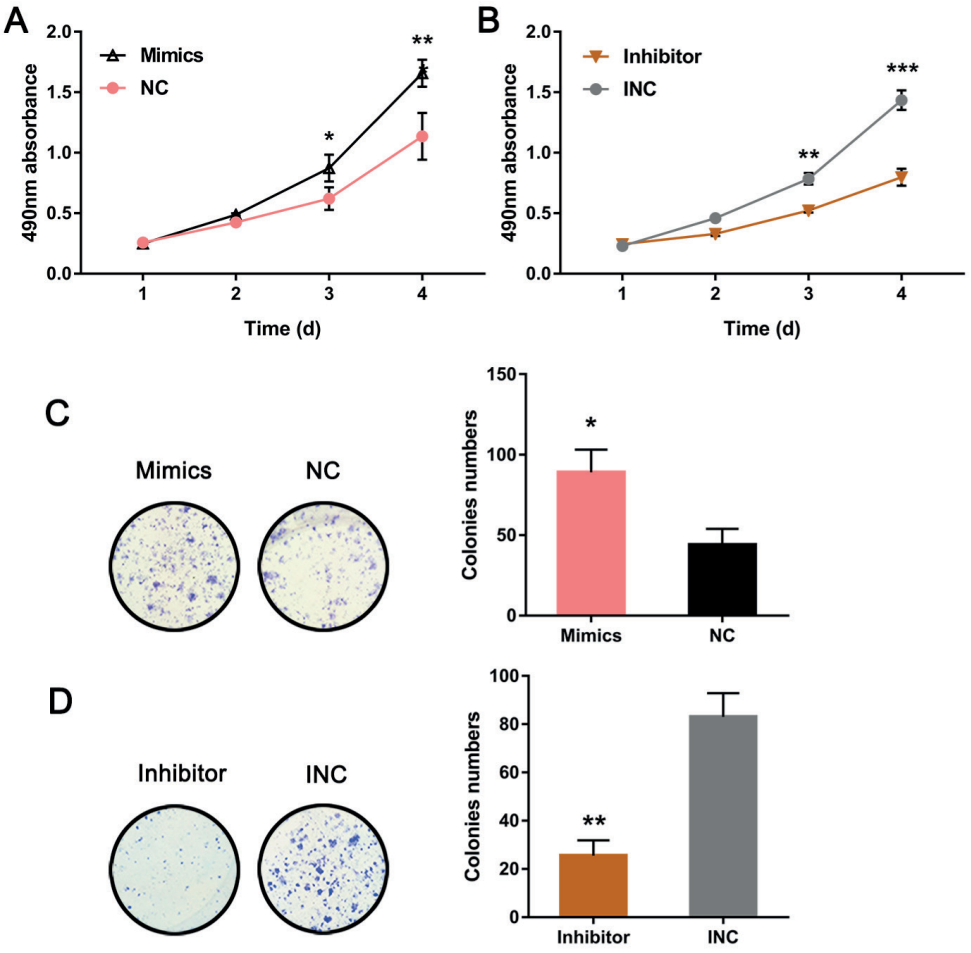

Figure 2. MiR-492 promoted the proliferation of PCa cells. ***A, B,*** CCK8 assay was performed to determine the proliferation of DU145 (***A***) or PC3 (***B***) cells treated with miR-492 mimics or inhibitors compared with each negative control. ***C, D,*** Colony formation analysis was performed to determine the cell growth of DU145 (***C***) or PC3 (***D***) cells transfected with mimics or inhibitor, respectively. *p<0.05, **p<0.01, ***p<0.001.

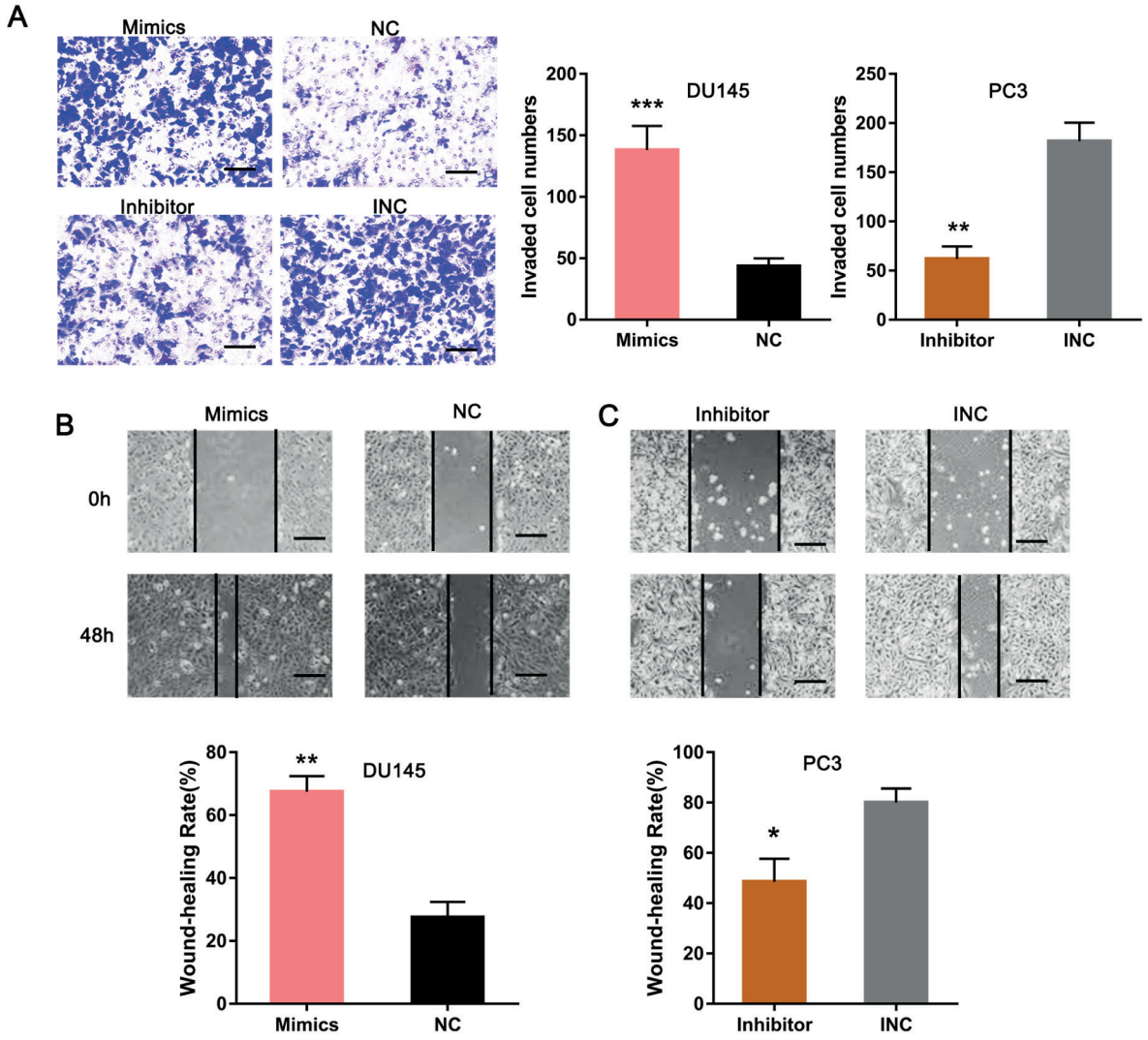

Figure 3. MiR-492 accelerated the invasion and migration of PCa cells. ***A,*** Transwell invasion assay was used to detect the invasion ability of miR-492 mimics treated DU145 cells or miR-492 inhibitor-treated PC3 cells. Magnification 400 × ***B,*** Wound-healing assay was used to detect the migration ability of miR-492 mimics treated DU145 cells or miR-492 inhibitors treated PC3 cells; magnification 40 ×, Data are presented as the mean ± SD of three independent experiments. *$p<0.05$, **$p<0.01$, ***$p<0.001$.

MiR-492 Promoted Cell Proliferation and Metastasis Via Repressing SOCS2 Expression

SOCS2 has been identified to be a target for miR-492 in PCa; we next designed the rescue experiments to re-confirm the results. By using siRNA for SOCS2, we knockdown the SOCS2 level in the miR-492 inhibitor-treated PC3 cells. Besides, the protein level of SOCS2 was detected using Western blotting. siRNA-SOCS2 markedly reduced SOCS2 protein level in PC3 cells which elevated by miR-492 knockdown (Figure 5A). Colony formation and CCK8 assays demonstrated that the cell proliferation decreased by miR-492 inhibitor was markedly restored by siRNA-SOCS2 (Figure 5B, 5C). Similarly, cell invasion ability reduced by miR-492 down-regulation was also partially reversed by SOCS2 inhibition (Figure 5D). Taken together, these results suggested that miR-492 serves as an onco-miR in PCa through regulating SOCS2 expression.

Discussion

In recent years, microRNAs (miRNAs) were found to have an important role in regulating tumor progression via degrading at the post-transcriptional level or interfering with the translation of target gene mRNA and then inhibiting the expression of target genes[12-14]. Studies have found that abnormal expression of miRNA is closely related to the progression of prostate cancer (PCa)[15]. For example, miR-34a directly repressing CD44 could inhibit PCa stem cells and its metastasis[6]. The invasion and migration of PCa cell could be promoted by miR-373 and miR-520c *in vivo* and *in vitro*[16]. In addition, up-regulation of miR-424 activates STAT3 to increase PCa progression *via* impairing ubiquitination[17]. MiR-379 together with miR-154* in the DLK1-DIO3 miRNA mega-clus-

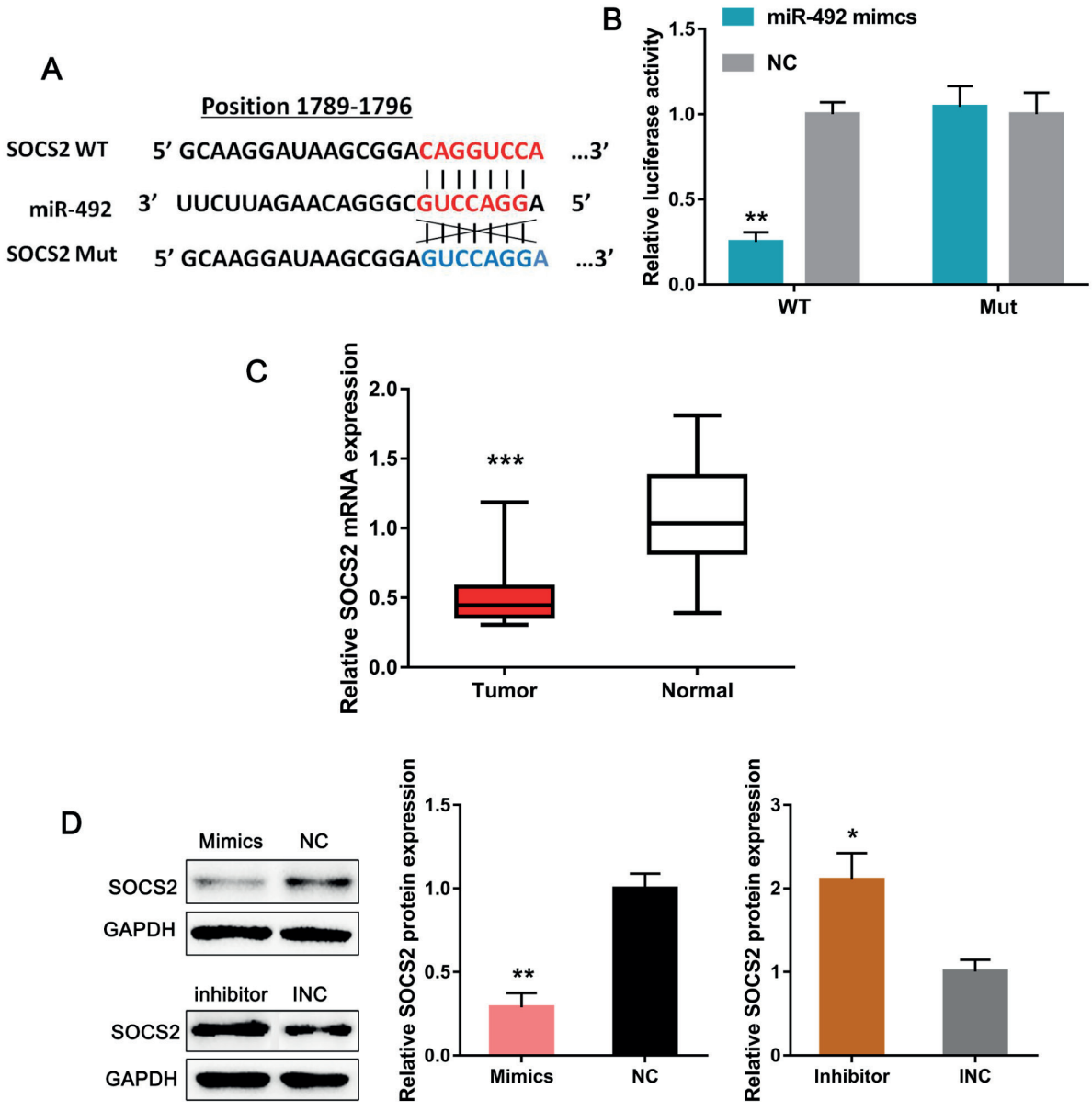

Figure 4. SOCS2 was a direct target of miR-492. ***A,*** The predicted binding sites of miR-492 in the 3'-UTR of SOCS2. ***B,*** Dual-luciferase reporter assay was used to determine the binding site. ***C,*** Expression of SOCS2 mRNA in PCa tissues compared with normal prostate tissues. ***D,*** Levels of SOCS2 and GAPDH protein measured by Western blotting in miR-492 over-expressed DU145 cells and miR-492 knockdown PC3 cells. The relative protein level of SOCS2 was compared with GAPHD. Data are presented as the mean ± SD of three independent experiments. *$p<0.05$, **$p<0.01$, ***$p<0.001$.

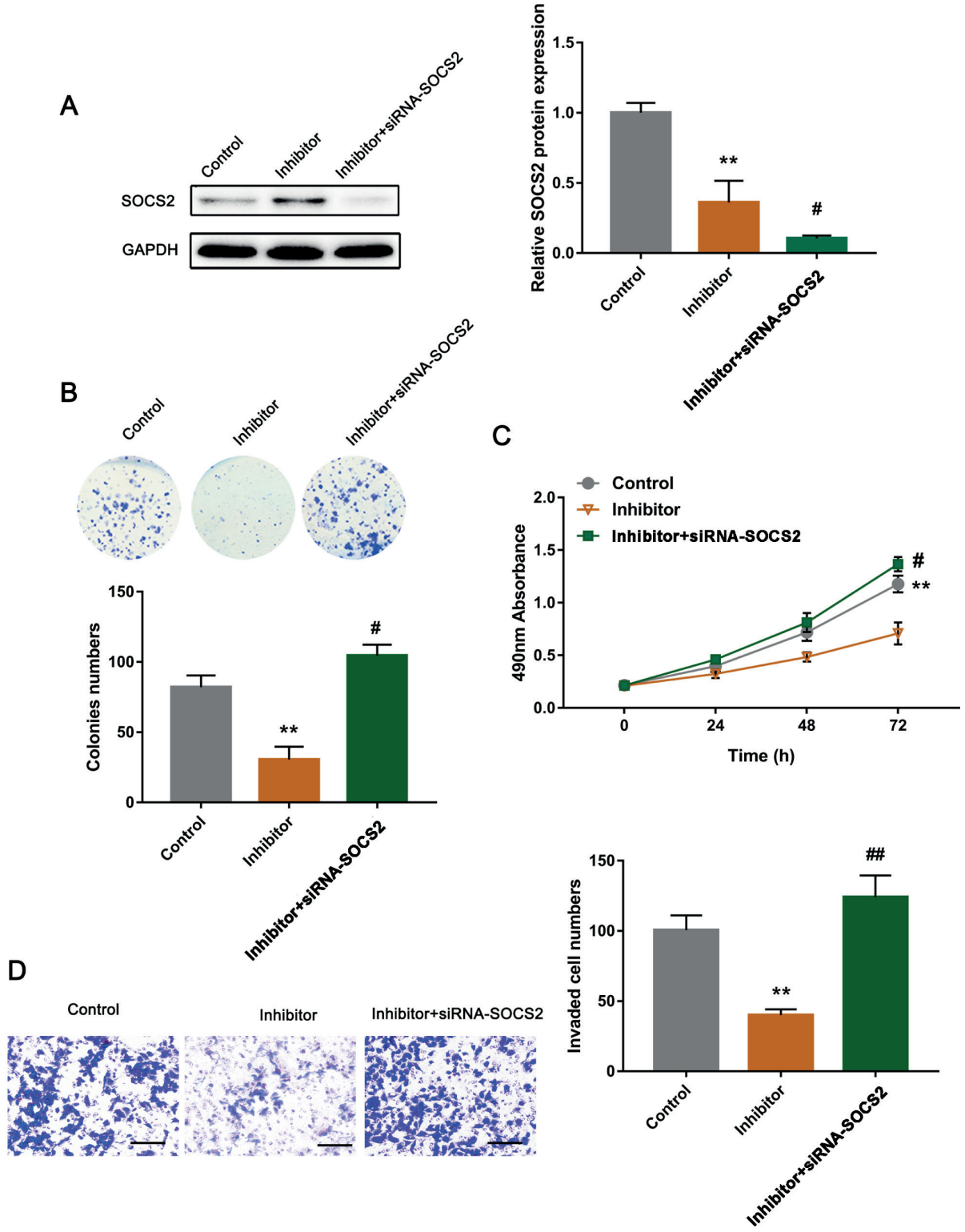

Figure 5. SOCS2 inhibition rescued the effects of miR-492 inhibitor in PC3 cells. ***A,*** Western blotting analyses of SOCS2. GAPDH was used as an internal control. ***B,*** Analysis of the cell proliferation ability by colony formation assay in control, inhibitor, or inhibitor + siRNA-SOCS2 treated PC3 cells; ***C,*** Analysis of the cell proliferation ability by CCK8 assay in control, inhibitor, or inhibitor + siRNA-SOCS2 treated PC3 cells; ***D,*** Cell invasion ability was measured by transwell assay; magnification 400 ×. Data are represented as the mean ± SD of three replicates. *p<0.05, **p<0.01, ***p<0.001.

ter influences the bone metastasis and epithelial-mesenchymal transition in PCa[18]. MiR-195 has tumor suppressive function through RPS6KB1 in PCa cells[19]. MiR-492 is highly expressed in a variety of tumors, and it regulates the growth and metastasis of tumors in a positive direction. The role and its specific mechanism of miR-492 in prostate cancer are still in the research stage.

This study found that the expression level of miR-492 in PCa tissues was higher than that in human normal prostate tissues, and the same trend was found in prostate cancer cell lines. It is suggested that the high expression of miR-492 may be involved in the progression of PCa. Overexpression of miR-492 prolonged the proliferation, invasion, and migration of prostate cancer cells, while prostate cancer cells with low expression of miR-492 showed opposite performance. These findings suggested that miR-492 is involved in promoting the proliferation and metastasis of prostate cancer cells.

Suppressor of cytokine signaling (SOCS2) is a newly discovered tumor suppressor gene belonging to the SOCS2 family. The SOCS family gene plays an important role in the development of glands and is also found to be associated with numerous tumors[20,21]. By negatively regulating the signal transduction of this pathway, SOCS could prevent the malignant transformation of cells and inhibit cell apoptosis, and its activation and expression have potential anti-tumor effects[22-24]. Promoting the expression of the SOCS2 gene may inhibit the proliferation and metastasis of PCa cells[25,26]. The bioinformatics technique and the dual luciferase reporter gene predicted and verified that SOCS2 serves as a potential target gene of miR-492. Overexpression of miR-492 significantly inhibited the expression of SOCS2, while knockdown of miR-492 significantly promoted the expression of SOCS2 gene. The knockdown of SOCS2 significantly restored the decrease of proliferation and metastasis ability of PCa cells caused by low expression of miR-492. These suggest that miR-492 may exert its cancer-promoting effect by inhibiting the expression of SOCS2 gene.

Conclusions

We showed that miR-492 is highly expressed in prostate cancer tissues and cell lines, and low expression of miR-492 could inhibit the proliferation and migration of PCa cells. The mechanism may be that the low expression of miR-492 leads to SOCS2 expression inhibited, ultimately inhibiting the proliferation and migration of PCa cells. Our research indicates that miR-492 could function as a cancer-promoting gene, which provides a new target for the biological treatment of PCa.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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