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Original Article

miR-24-3p stimulates migration, invasion and proliferation of prostate cancer cells by targeting suppressor of cytokine signaling 6

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Abstract: Prostate cancer is among the most widespread malignancies affecting men in the world. Its aggressive evolution has been associated with altered expression of suppressor of cytokine signaling 6 (SOCS6) but very little is known about the mechanism by which this alteration occurs. The purpose of this study was to explore the role of SOCS6 in prostate cancer cells and the involvement of its regulating microRNA (miR), miR-24-3p. Prostate cancer cell lines were used to determine the transcription level of miR-24-3p and SOCS6 by quantitative reverse-transcriptase-polymerase chain reaction (qRT-PCR) and Western blot. Cell proliferation and cell migration assays were done to determine the effect of miR-24-3p mimics and inhibitors on cell proliferation, invasion and migration. Luciferase reporter assay with SOCS6 3'-UTR was performed to confirm the control of SOCS6 expression by the miR. The results showed that miR-24-3p was up-regulated in prostate cancer cells whereas SOCS6 protein was downregulated. Overexpression of miR-24-3p in prostate cancer cells promoted cell proliferation, inhibited apoptosis, and increased cell migration and invasion. Luciferase reporter assays showed that SOCS6 is a direct target of its negative regulator miR-24-3p and overexpression of SOCS6 reverses the effects of miR-24-3p on the metastatic phenotype of prostate cancer cells. These results show case miR-24-3p up-regulation in prostate cancer and a mechanism for inhibition of SOCS6 expression. Thus, the miR-24-3p/SOCS6 pathway could be a relevant avenue for prostate cancer treatment.

Keywords: Prostate cancer, SOCS6, miR-24-3p

Introduction

Prostate cancer is one of the most common cancers diagnosed in men. It counts for 15% of all diagnosed cancers in men and is the fifth leading cause of death from cancer in 2012 [1]. It is a slowly growing cancer without any particular symptoms in the earlier stage andis mostly diagnosed in men above 50 years old [2]. Despite its relatively slow growth, prostate cancer cells can migrate and invade other organs such as bones where metastases occur frequently [3, 4]. Molecular mechanisms involved in metastasis are complex and not completely understood [5]. It is widely accepted that the cell microenvironment plays a critical role in the induction and silencing of cellular signaling pathways that govern cell biology. Among the microenvironment elements involved in this process are cytokines that have been associated to the biology of many cancer cells [6, 7]. Cytokine-induced inhibitors of the downstream elements of their receptors, such as suppressor of cytokine signaling 6 (SOCS6), could play an important role in the biology of cancer cells [8].

In previous studies, SOCS6 was shown to be significantly downregulated in colorectal and prostate cancers [9, 10]. The mechanism by which SOCS6 is down-regulated in cancer cells is not known but several studies point to epigenetic regulation of its expression by microRNAs (miRs) [11]. Moreover, the expression profile of miRs is extremely altered in prostate cancer [12]. In effect, epigenetic factors involving miRs are involved in cell transformation and cancer maintenance making them interesting markers for early cancer diagnosis [13]. miRs are also responsible for some treatments

making them particularly good biomarkers for decision making regarding the appropriate treatment strategy [14]. miRs are also applicable as specific cancer therapeutics [15] and thus knowing more about the mechanism of action of miRs is an advantage in defining new strategies for cancer diagnosis and treatment [16].

The function and mode of action of miR-24-3p in a diversity of cancers enclosing glioma, hepatocellular carcinoma, lung cancer, breast cancer, bladder cancer and colorectal cancer hasbeen conveyed in previous studies [17-25]. However, despite some studies reporting miR-24-3p as a tumor suppressor while others demonstrated its role as an oncogene, the potential role of miR-24-3p in prostate cancer is not entirely understood and whether and how the miR-24-3p/SOCS6 axis is involved needs to be clarified.

Based on the hypothesis that miR-24-3p deregulation in prostate cancer cells may affectdisease progression, the present study was designed to explore the potential relationship between miR-24-3p and SOCS6 and their effect on prostate cancer cellbiology.

Material and methods

Cell culture

Prostate cancer cell lines LNCaPFGC, PC3 and DU 145 were used in this study and compared to human normal prostate epithelial cell line RWPE1. RWPE-1 cells were cultured in keratinocyte serum-free medium (K-SFM, Invitrogen, Carlsbad, USA) supplemented with 50 mg/ml bovine pituitary extract, 100 U/ml penicillin and 100 mg/l streptomycin. Other cells were cultured in RPMI 1640 medium (Gibco, USA) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin antibiotics (Gibco, USA). All cultures were incubated at 37°C in 5% CO_2, 95% air atmosphere.

RNA extraction and quantitative real-time RT-PCR

Total RNA was extracted from harvested cells (5 × 10^4 cells) using TRIzol reagent (Invitrogen). The TaqMan miR reverse transcription kit (Applied Biosystems, Foster City, CA) was used on the extracted RNA to generate miR complementary DNAs that were further amplified and quantified by normalizing to U6 snRNA. SOCS6 mRNA expression level was determined by reverse transcription using the SuperScript III first-strand synthesis system supermix (Invitrogen) and a SYBR Green PCR kit (TaKaRa, Dalian, China). The level of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was used as a reference for quantification. The ΔΔCT method was used for quantifications.

Cell transfection

PC-3 Cells in a logarithmic growth phase were transfected with miR-24-3p mimic, miR-24-3p inhibitor (antisense oligonucleotide), or miR-control. Transfection was performed for each cell line with 50 nM of oligonucleotides using riboFECT™ CP transfection reagent (Ribo-bio, China) according to the user manual. Transfected cells were cultured and forwarded for further experiments.

Cell viability test

Cell lines and transfected cells were seeded in 96-well plates at 10^4 cells per well. Cell proliferation was measured using Cell Counting Kit-8 (CCK-8) (Dojindo Laboratories, Tokyo, Japan) in accordance with the manufacturer's recommendations. Absorbance was measured at 450 nm using a multilableplate reader. Each experiment was performed with three replicates.

Cell migration and invasion

Transwell assay with FBS in the lower chamber was used to determine the migration rate of studied cell lines and transfected cells. Cells were suspended in serum free medium and loaded to the upper chamber (10^5 cells/well). The transwell membrane (Millipore, Billerica, MA, USA) with 8 μm pores size was coated with Matrigel (BD Biosciences, San Jose, USA)for tracking cell invasion. Fresh medium was loaded in the lower chamber with 10% FBS as the chemoattractant. After 24 h of incubation at 37°C, the invaded cells were fixed with 100% methanol, stained with 1% crystal violet for 20 minutes, and counted under a microscope.

In silico analysis

For prediction of miR-24-3p targets, the online bioinformatics tool Targetscan (www.targetscan.org) was used.

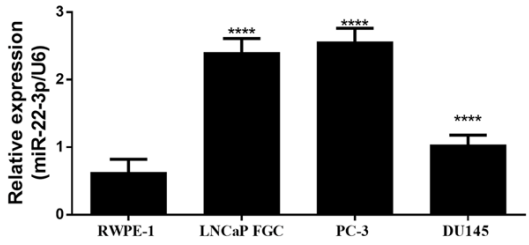

Figure 1. miR-24-3p is up-regulated in prostate cancer cells. Expression level of miR-24-3p in prostate cancer cell lines and RWPE1 cells was measured by qRT-PCR. ****P < 0.0001 when compared to normal cells.

Luciferase reporter assays

Cell lines were seeded in 24 well plates and transfected the following day with expression plasmids constructs harboring the luciferase gene with the SOCS6 3'-UTR or a mutated SOCS6 3'-UTR. Transfection was performed using Lipofectamine 2000 (Invitrogen). Two days after transfection, cells were lysed and analyzed by luciferase reporter assay using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI) according to the manufacturer's recommendations.

Western blot

The expression levels of proteins were measured by Western blot on all cell lines and those transfected with miR-24-3p mimics or inhibitors. Cells were harvested 48 h after transfection, washed twice with cold phosphate-buffered saline (PBS) and lysed in 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Nonidet P-40, 10 mM KCl, 1 mM EDTA, 20 mM NaF, 0.25% Na deoxycholate, 5 mM dithiothreitol (DTT). Protease inhibitors (Roche, USA) and phosphatase inhibitors (ThermoFisher Scientific) were added to the mix and total cellular lysates (20 µg for each sample) were subjected to 10% SDS-polyacrylamide gel electrophoresis. At the end of electrophoresis, proteins in the gel were transferred to nitrocellulose membrane and blocked with 5% (w/v) skimmed milk for one hour at room temperature. Membranes were then incubated overnight at 4°C with monoclonal primary antibodiesagainst SOCS6, caspase 3, and cleavedcaspase 3 (Abcam, UK). After incubation with the primary antibody, membranes were washed three times with TBS-T and then incubated one hour at room temperature with the horseradish peroxidase-conjugated goat anti-mouse secondary antibody, (Santa Cruz Biotechnology, USA). Membranes were washed three times with TBS-T and protein bands were stained using the BM chemiluminescence Blotting Substrate (POD) (Roche, USA) according to the manufacturer's instructions. Image J software (NCBI) was used for densitometry analysis.

Statistical analysis

Data were expressed as mean ± standard deviation (SD) of three independent experiments performed in triplicate. Statistical analyses were performed using one-way or two-way analysis of variance (ANOVA). Values of $P < 0.05$ were considered statistically significant compared to the respective control.

Results

miR24-3p is up-regulated in prostate cancer cells

Prostate cancer cell lines and prostate normal epithelial cells were subjected to whole transcriptome extraction followed by reverse transcription qPCR to quantify the expression level of miR-24-3p. These experiments revealed a significant increase in the amount of miR-24-3p expressed in all cancer cell lines in comparison to prostate epithelial RWPE1 cell lines (**Figure 1**). As shown in **Figure 1**, miR-24-3p level was the most upregulated in PC-3 cell line. This cell line was therefore selected for further experiments.

miR24-3p overexpression promotes the proliferation, invasion and migration of prostate cancer cells

miR-24-3p mimic,the inhibitor, or theirrespective controls were transfected in PC-3 cells after checking the expression profile of miR-24-3p in prostate cancer cells by qRT-PCR. The efficiency of miR-24-3p expression after transfection is reported in **Figure 2A**. To evaluate the effect of miR-24-3p on the phenotype of PC-3 cells, the proliferation, apoptosis rate and cell migration and invasion were measured in these cells transfected with miR-24-3p mimic or inhibitor and compared with control cells. Results of these assays are shown in

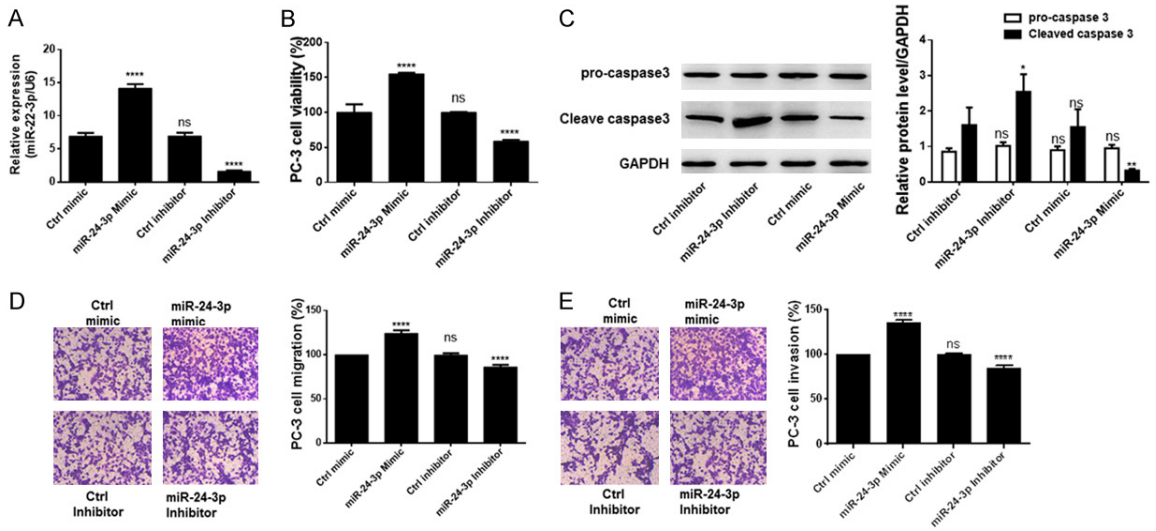

Figure 2. Overexpression of miR-24-3p in prostate cancer cells induces aggressive cancer cell behavior. A. miR-24-3p expression profile in cells transfected with miR-24-3p mimics, the inhibitors, or control. B. miR-24-3p overexpression induced the viability of prostate cancer cells. C. Western blot analysis of procaspase 3 and cleaved-caspase 3 indicated that miR-24-3p inhibited cell apoptosis. D. Cell migration rate in transfected cells. E. Rate of cell invasion after transfection. All experiments were performed in triplicate. *P < 0.05; **P < 0.01; ****P < 0.0001 and ns means non-significant when compared to the Ctrl mimic group (Ctrl inhibitor group in the case of western blot analysis).

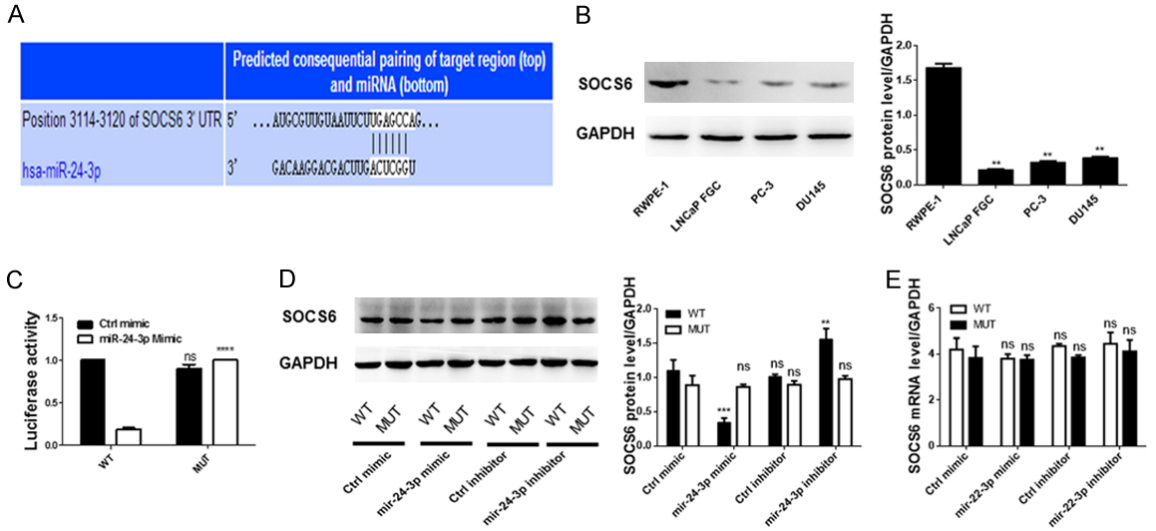

Figure 3. SOCS6 is a direct target of its inhibitor miR-24-3p. A.Targetscan predicted SOCS6 3'-UTR as seed sequence for miR-24-3p. B. Expression level of SOCS6 protein in prostate cancer cell lines and RWPE1 cells. C. Luciferase reporter assay indicated miR-24-3p as a negative regulator of its direct target SOCS6. D. Western blot analysis of SOCS6 protein level in PC-3 cells co-transfected with miR-24-3p mimics, the inhibitors, or control and the mutated or wild type 3'-UTR vector. E. SOCS6 mRNA level in PC-3 cells transfected with miR-24-3p mimics, inhibitors or control. All experiments were performed in triplicate. **P < 0.01; **P < 0.01; ****P < 0.0001 and ns means non-significant when compared to the normal control cells, Ctrl mimic group or wild type (WT) group.

Figure 2B-E. There was a high increase in proliferation of PC-3 cells over-expressing miR-24-3p in comparison to controls (**Figure 2B**). Furthermore, cells over-expressing the miR showed a significant reduction in cell apoptosis as evidenced by the level of cleaved-caspase 3 (**Figure 2C**). Moreover, miR-24-3p overexpression induced an increase in cell invasion

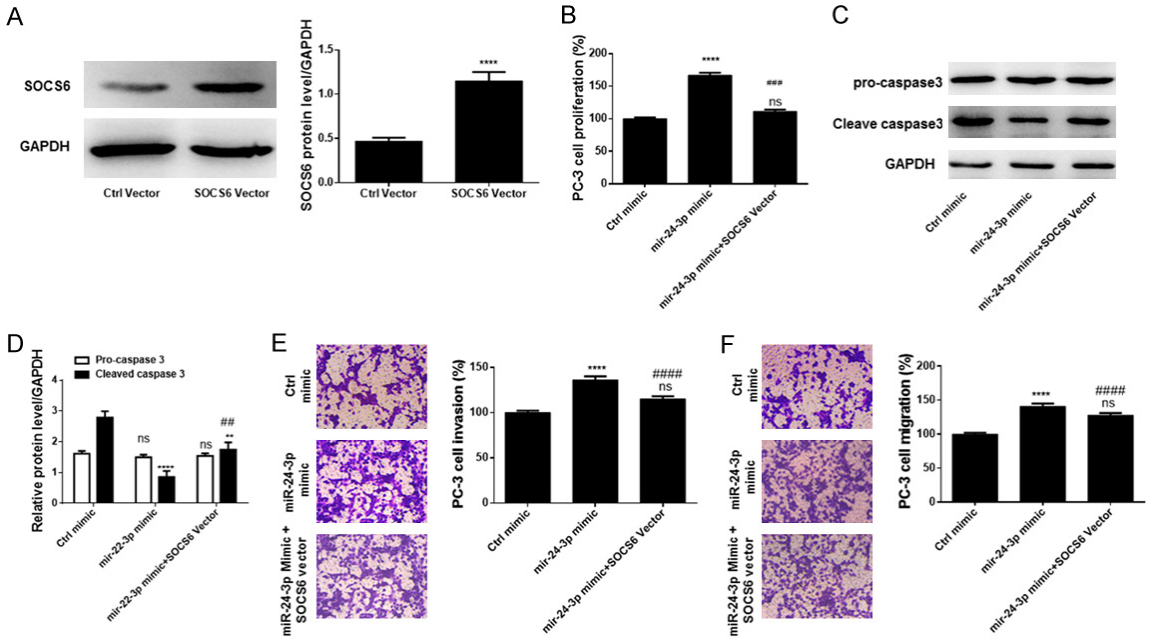

Figure 4. Ectopic expression of SOCS6 inverted the action of miR-24-3p. A. Ectopic SOCS6 expression efficiency. B. Effect of SOCS6 overexpression on miR-24-3p-induced cell proliferation. C. Western blot analysis of the effect of SOCS6 overexpression on expression of caspase 3 and cleaved caspase 3. D. Densitometry analysis of bands obtained from the Western blot analysis. E. Effect of SOCS6 overexpression on cell migration. F. Effect of SOCS6 overexpression on cell migration. All experiments were performed in triplicate. **P < 0.01; ****P < 0.0001 and ns means non-significant when compared with Ctrl vector or Ctrl mimic. ##P < 0.01; ###P < 0.001 and ####P < 0.0001 when compared with miR-24-3p mimic group.

and migration (**Figure 2D**, **2E**). In contrast, transfection with miR-24-3p inhibitors in PC-3 cells significantly inhibited cell proliferation, migration, and invasion but increased the rate of apoptosis as measured by the level of cleaved-caspase 3. Altogether, these observations imply that miR-24-3p promotes the metastatic phenotype of prostate cancer cells.

miR-24-3p is a post-transcriptional negative regulator of SOCS6

Targetscan analysis showed *in silico* that miR-24-3p targets among others the SOCS6 mRNA in the 3'-UTR region (position 3114-3120) as presented in **Figure 3A**. Western blot analysis of SOCS6 in prostate cancer cell lines and prostate epithelial cells showed a significantly decreased level of protein in cancer cell lines in comparison to RWPE1 cells (**Figure 3B**). The luciferase reporter gene expression assay was performed with the luciferase gene under the control of a SOCS6 3'-UTR fragment (mutated or wild type). In the case of wild-type SOCS6 3'UTR, miR-24-3p drastically decreased the expression level of luciferase whereas the miR had no significant effect on the expression of luciferase linked to mutated SOCS6 3'-UTR (**Figure 3C**). PC-3 cells transfected with miR-24-3p mimics or inhibitors and vectors harboring SOCS6 gene with either mutated or wild type 3'-UTR were also subjected to Western blotting and SOCS6 mRNA quantification by qRT-PCR. The results showed that expression of miR-24-3p or its inhibitor did not affect the level of SOCS6 mRNA while overexpressing the miR significantly decreased the protein level (**Figure 3D**, **3E**). These results indicate that miR-24-3p is a post-transcriptional negative regulator of SOCS6.

Overexpression of SOCS6 reverses the effect of miR-24-3p

When PC-3 cells were transfected with a SOCS6 expression vector, the protein was abundantly expressed in the cells despite transfection with the miR-24-3p mimics (**Figure 4A**). These cells were used as models to investigate the effect of SOCS6 overexpression on miR-24-3p action. Overexpression of SOCS6 significantly inverted the effect of miR-24-3p

on PC-3 cells as shown in **Figure 4B**, **4F**. Ectopic overexpression of SOCS6 attenuated the miR-24-3p-induced cell proliferation, migration, and invasion, but increased the rate of apoptosis in cells expressing miR-24-3p. Overall, these results indicate that miR-24-3p accelerates migration, invasion, and proliferation of prostate cancer cells by directly and negatively regulating SOCS6.

Discussion

SOCS6 is one of the understudied members of the cytokine-induced STAT inhibitors family that has been shown to be induced by cell proliferative cytokines and growth factors [26, 27]. As SOCS6 action is to inhibit these cytokines and downstream proliferation signaling of cellular growth factors, it makes sense to consider that SOCS6 exerts an anti-proliferative activity as reportedin several previous studies [28-30]. SOCS6 could exert the same anti-proliferative activity in prostate cells in a comparable manner explaining the fact that this protein is highly produced in normal cells but be present in low amounts in prostate cancer cells as found in this *in vitro* study. Prostate cancer could rise when the protein level of SOCS6 decreases, thus abrogating the control action of this protein on the proliferative signal induced by different cytokines. This observation has already been made clinically and *in vitro* by other authors [31-33]. One of the unanswered questions in this possible mechanism of cell transformation is the mechanism by which SOCS6 is downregulated in cancer cells. Giving an answer based on experimental data to this question was the central objective in the present study.

A comparison between prostate normal cells and prostate cancer cell lines was performed regarding the expression of miR-24-3p. The results show that the level of miR-24-3p is significantly high in cancer cell lines. Meanwhile, Western blot results showthat thelevel of SOCS6 protein is decreased in cancer cell lines as compared to prostate epithelial cells. Bioinformatics show that miR-24-3p anneals to SOCS6 mRNA in the 3-'UTR whereby miR-24-3p directly targets SOCS6 mRNA and down-regulates its translation. The reporter gene expression assay confirmed that SOCS6 mRNA is a direct target of the miR. To our knowledge, this is the first time miR-24-3p has been experimentally shown to downregulate SOCS6 in prostate cancer cell lines. In fact, previous studies demonstrated that SOCS6 expression is under the control of other miRssuch as miR-21, miR-142-3p, miR-183, or miR-494-3p in diverse cancer types [34-36]. miR-24-3p can thus control the expression of SOCS6. The present study could not exclude the action of other miRs but the results in the luciferase reporter gene assay not only confirm that SOCS6 3'-UTR is determinant in the expression control but also highlight the importance of miR-24-3p in down-regulating SOCS6 in prostate cancer cell lines.

According to the results of the present study, SOCS6/miR-24-3p regulatory axis plays an irrevocable role in prostate cancer biology. In effect, miR-24-3p overexpression was sufficient to induce cell proliferation and increase cell migration and invasion. Moreover, ectopic expression of SOCS6 in cancer cells attenuated the proliferation and cell invasion induced by the miR. miR-24-3p could therefore be responsible for prostate cancer induction, its maintenance and also for the spreading of cancer cells to other organs where metastases occur. This explains why SOCS6 downregulation is associated with aggressive cancer such as colorectal cancer [9]. Both SOCS6 and miR-24-3p appear to be good markers for prostate cancer prognosis and offer a potentially high effective pathway target for prostate cancer treatment.

Overall, the present study identifies SOCS6 and its regulatory miR, miR-24-3p as determinant factors in prostate cancer biology. They are central to prostate cancer cell proliferation and metastases. This axis (miR-24-3p/SOCS6) is a promising therapeutic target for prostate cancer that needs to be more deeply investigated in future studies.

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Disclosure of conflict of interest

None.

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