

ORIGINAL ARTICLE

MicroRNA-146b acts as a potential tumor suppressor in human prostate cancer

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Summary

Purpose: MicroRNAs (miRs) act as either tumor suppressors or oncogenes and are frequently deregulated in cancers. Although downregulation of miR-146b has been reported in various cancers, its role in prostate cancer is totally unknown.

Methods: The miR-146b expression in 18 human prostate cancer lines with case-matched adjacent normal tissues was measured by quantitative RT-PCR. Furthermore, the expression levels of miR-146b in normal prostate and prostate cancer cell lines were assessed. Cell proliferation, apoptosis, migration and invasion assays were performed in overexpressing or knockdown miR-146b cells.

Results: miR-146b expression was significantly reduced in all prostate tumor tissues. Furthermore, miR-146b was

significantly decreased in prostate cancer cells as compared to normal prostate cells. Loss-of-function and gain-of-function showed that miR-146b induced apoptosis and suppressed cell proliferation, migration and invasion of the prostate cancer cell lines.

Conclusion: Our results demonstrated that miR-146b expression is downregulated in prostate tumor tissues and is a potential tumor suppressor miR, suggesting that miR-146b might be a potential clinical marker and therapeutic target for prevention and treatment of prostate cancer.

Key words: apoptosis, invasion, metastasis, miR-146b, prostate cancer

Introduction

Prostate cancer is one of the most common malignancies in men worldwide [1] with most of the estimated 28,000 yearly deaths resulting from metastases [2]. Prostatic intraepithelial neoplasia is a precursor lesion of prostate cancer, which eventually progresses to adenocarcinoma and hormone-dependent or -independent diseases [3]. The invasiveness of prostate cancer and other cancers are potential causes of death and it is critical to know the genes involved in the progression from primary to metastatic disease [4]. Therefore, identifying genetic markers that can precisely predict the presence or absence of prostate cancer and its metastatic stages helps designing the therapeutic approach.

miRs are small noncoding RNAs that have been shown to regulate the lifespan of mRNAs by acting as negative regulators of gene expression through RNA interference pathways [5]. Since miRs play a pivotal role in the control of several biological processes, their dysregulated expression could result in the onset of diverse pathological conditions, including cancers [6]. In addition, the notion that miRs can interact with different mRNAs and that a single mRNA can be targeted by several miRs, highlights the complexity of miR-dependent regulation of gene expression. miR expression correlates with various cancers and these miR genes can act as tumor suppressors or oncogenes [7,8]. Moreover, a recent study found

that the absolute expression levels of many miRNAs were reduced significantly in tumors [9]. miR-146b is located on chromosome 10 at position q24.32 and it's an important tumor suppressor miR, which controls several cancer-related genes and processes [10]. Recent studies have shown the role of miR-146b in different cancer cell lines, including breast [11], glioblastoma [12], gallbladder [13] and lung [14]. miR-146b was shown to target multiple genes, including EGFR, MMP-16 and NF κ B and act as tumor suppressor [14,15]. However, the expression and function of miR-146b in human prostate cancer tissues and cell lines have not been examined.

In this study, we first checked the expression levels of miR-146b in tumor specimens and adjacent normal tissues of prostate cancer by quantitative RT-PCR. Further, we also examined the association of miR-146b expression in normal and prostate cancer cell lines and then analyzed its overexpression or knockdown effects on the ability of cell proliferation, apoptosis, migration and invasion of prostate cancer cells. The data demonstrated for the first time that miR-146b expression was downregulated in prostate tumor tissues and cells lines. Subsequent cellular functional studies demonstrated that miR-146b act as tumor suppressor by inhibiting cell proliferation, migration and invasion.

Methods

Human prostate normal and cancer cell lines

Human prostate normal (PNT2C2 and WPMY-1) and cancer (LNCaP, 22Rv1, PC-3 and DU145) cell lines were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). WPMY-1 cells were maintained in DMEM medium containing 10% fetal bovine serum (FBS), 100 units/ml penicillin and 100 μ g/ml streptomycin. All other cell lines were cultured in RPMI 1640 medium supplemented with 10% FBS, 100 units/ml penicillin and 100 μ g/ml streptomycin. All cells were incubated in a humidified atmosphere composed of 5% CO₂ at 37°C. All chemicals were purchased from Sigma-Aldrich (St Louis, MO), unless otherwise stated.

Tumor samples from patients

Human prostate tumor samples (N=18) along with adjacent normal tissues were immediately collected by biopsy or surgical resection from patients admitted to Huadong Hospital Affiliated to Sudan University, Jintan, Shanghai, China. The patients had not received preoperative chemotherapy or radiotherapy. This study was approved by the Huadong Hospital Affiliated to Su-

dan University Ethics Review Committees and written informed consent was obtained from all of the patients.

Quantitative RT-PCR (qRT-PCR) analysis

Total RNA was extracted from tumor tissues or cells using mirVana miRNA isolation kit (Ambion, USA). TaqMan microRNA probes (Invitrogen, Carlsbad, CA, USA) were used to measure mature miRNA-146b (assay ID number: 002361) and RNU6 (RNA, U6 small nuclear assay, ID number: 001093) expressions. RNU6 was used as endogenous control. The PCR reactions were carried in a 20 μ l mixture containing 150 ng of cDNA, 10 μ l of TaqMan 2X universal PCR master mix and 1 μ l of probes. The PCR reactions were run on the ABI Prism 7900 Fast Real-time PCR system for each gene and each sample in triplicate as follows: 95°C for 10 min, 45 cycles of a 15-s denaturing at 95°C, and 1 min annealing at 60°C. SDS 2.1 Software (ABI) was used to calculate miR-146b expression levels, normalized to endogenous control gene (RNU6), and relative to either normal tissue or mock controls.

Transfection of miR mimics and inhibitors

Synthetic miR-146b mimic (miR-146b mimic) and its scrambled control (control mimic) were purchased from GenePharma (Shanghai, China). The single-stranded modified RNA inhibitor antagomiR-146b (antago miR-146b) and its negative control (antago miR-NC) were obtained from GenePharma. Transfection of either mimic or inhibitors was carried out using Lipofectamine (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. In brief, about 3×10^5 cells/well were seeded in a six-well plate. One day after plating, the cells were transfected with 20 nM of either mimic or antagomiR with Lipofectamine (Life Technologies, Grand island, NY). The media were changed with fresh growth medium after 6-h of transfection. After 48 hrs of transfection, cells were collected for subsequent analysis.

Cell proliferation assay

WST-8 Cell Counting Kit-8 was used (CCK-8; Dojindo, Japan) to measure cell proliferation by flow cytometry as described previously [16]. Cells were transfected with either mimics or antagomiR or the negative control. After 48-h transfection, cells were seeded onto 96-well culture plates at a density of 600 cells/well. After 72 hrs of incubation, 10 μ l CCK-8 were added to each well of the 96-well assay plates, and the cells were cultured for 1 h at 37°C in 5% CO₂. The absorbance at 450 nm was measured using a DNM-9602 microplate reader

Apoptosis assay

Apoptotic cells was determined using FITC Annexin V kit (BD Biosciences, San Jose, CA) according to

the manufacturer's instruction as described previously [17]. Briefly, cells were trypsinized and pelleted by centrifugation at 1,000 rpm for 5 min. Then, the pellets were resuspended in binding buffer (500 μ l), added 5 μ l of Annexin V-FITC and 5 μ l of propidium iodide (50 μ g/ml) and incubated at room temperature for 5 min in the dark. Flow cytometry was performed in a FACS-can (BD Biosciences). For each data point, triple samples were analyzed and the experiment was reproduced three times.

Migration and invasion assays

Cell migration and invasion assays using the transwell system from BD Biosciences (San Jose, CA) were performed as per the manufacturer's instructions.

Briefly, cells were mixed with serum free media and added to each transwell insert, the medium in the lower chamber containing 10% FCS. The cells attached in the insert were removed using cotton swabs after incubation for 20 hrs at 37 °C. The migrated cells were fixed with 4% paraformaldehyde for 10 min and stained with 0.5% crystal violet. The amount of migrated cells was quantitatively assessed by reading the absorbance at 560 nm. Transwell cell invasion assays were performed similarly except that transwell inserts were pre-coated with growth factor-reduced Matrigel (BD Biosciences, San Jose, CA).

Statistics

SPSS 17.0 statistical software package was used

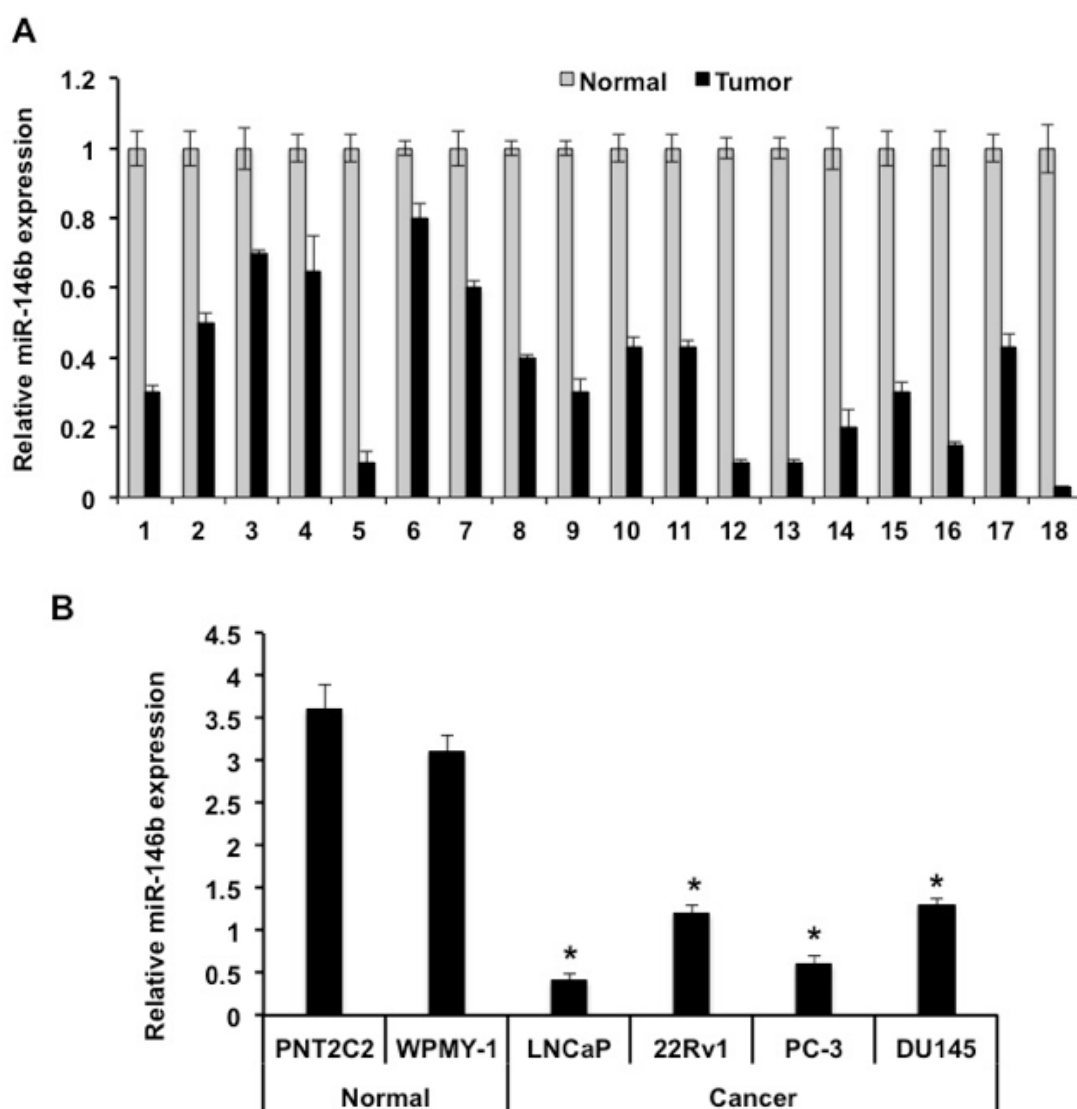


Figure 1. Downregulation of miR-146b expression in human prostate cancer tissues and cell lines. **A:** Total RNA was extracted from prostate tumor and adjacent normal tissues. **B:** shows prostate normal and cancer cell lines. miR-146 expression was determined by qRT-PCR, normalized to RNU6 and expressed as relative to corresponding normal tissue. The data are expressed as mean \pm standard deviation. Statistical analyses were performed with Student's t-test and * p <0.0001 denotes significant differences between prostate tumor tissues and their normal tissues in **A**. * p <0.0001 compared with prostate normal cell line PNT2C2 in **B**.

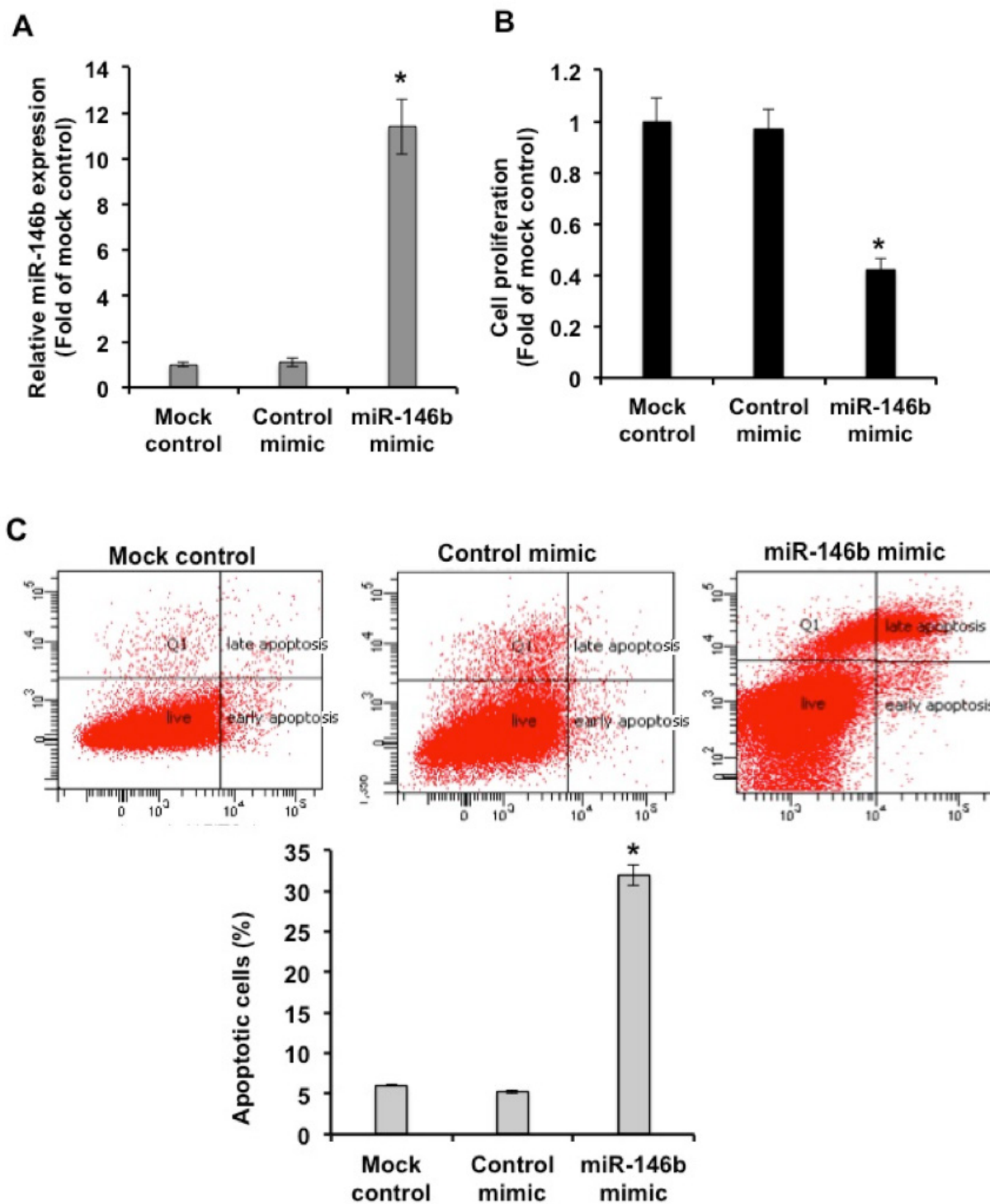


Figure 2. Ectopic expression of miR-146b inhibits cell proliferation and induces apoptosis in prostate cancer cells. **A:** Ectopic expression of miR-146b in PC3-cells. qRT-PCR analysis demonstrating the efficiency of miR-146b overexpression by transfection of 20 nM miR-146b mimic or scrambled (control mimic) or no transfection (mock control). **B:** PC-3 cells proliferation was measured by WST-8 assay kit after transfection with scrambled or miR-146b mimics. miR-146b mimic decreased PC-3 cell proliferation. **C:** PC-3 cells were harvested after 48 hrs of transfection with scrambled or miR-146b mimics and apoptosis was measured by FITC Annexin-V analysis. miR-146b mimic increased the percentage of apoptotic cells. Data represent three independent experiments and p values were determined by Student's t-test. * $p < 0.0001$ compared with mock control.

for analyses. Data were presented as mean \pm standard deviation (SD). One way ANOVA and Student's t-test

were used to statistically analyse significant differences. A p value < 0.05 was considered as statistically sig-

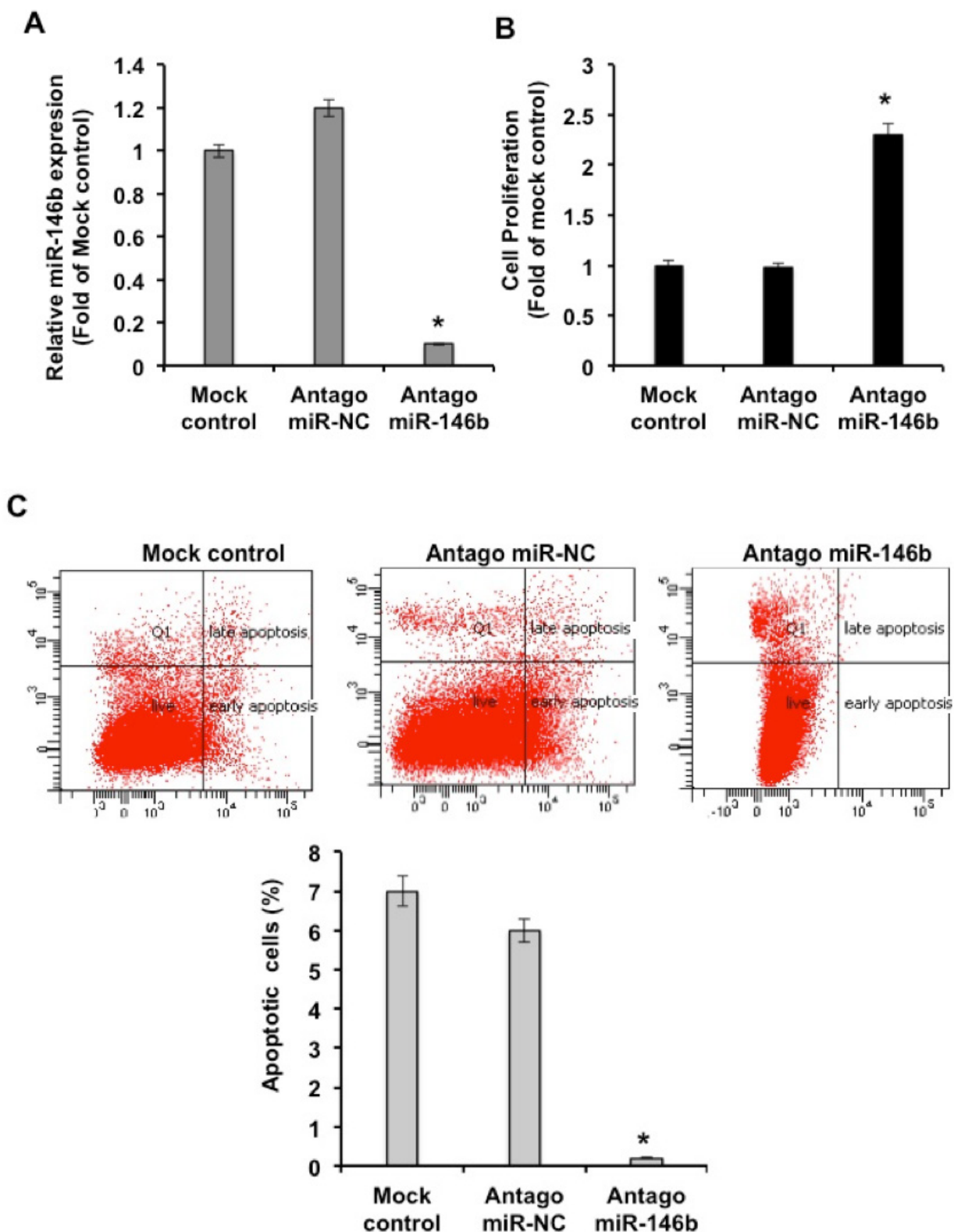


Figure 3. F. miR-146b knockdown increases cell proliferation and decreases apoptosis induction in prostate cancer cells. **A:** Knockdown of miR-146b in PC3-cells. qRT-PCR analysis demonstrating the efficiency of miR-146b inhibition by 20 nM of antagomiR-146b or antagomiR negative control (antagomiR-NC) or no transfection (mock control). **B:** PC-3 cells proliferation was measured by WST-8 assay kit after transfection with antagomiR-146b or antagomiR-NC or mock control. Knockdown of miR-146b increased the PC-3 cell proliferation. **C:** PC-3 cells were harvested after 48 hrs of transfection with antagomiR-146b or antagomiR-NC or mock control and apoptosis was measured by FITC Annexin-V analysis. Knockdown of miR-146b decreased the percentage of apoptotic PC-3 cells. Data are expressed as mean±standard deviation. Statistical analyses were performed with Student's t-test. *p<0.0001 compared with mock control.

nificant.

Results

miR-146b is downregulated in human prostate cancer tissues and cell lines

To assess the expression of miR-146b in human prostate tissues, we evaluated miR-146b expression in 18 matched pairs of prostate cancer and their adjacent normal tissues obtained by surgical resection or during autopsy using qRT-PCR. As shown in Figure 1A, the expression levels of miR-146b were considerably decreased ($p < 0.001$) in 4 samples (sample #3, 4, 6 and 7) as compared with matched normal tissues. The other 14 samples were significantly ($p < 0.0001$) downregulated in prostate cancer tissues relative to the corresponding normal tissues.

To test whether miR-146b is downregulated in prostate cancer cells, we examined its expression levels in normal and prostate cancer cell lines by qRT-PCR. As shown in Figure 1B the prostate cancer cell lines LNCaP, 22RV1, PC-3 and DU145 showed significantly reduced expression of miR-146b as compared to prostate normal cell lines PNT2C2 and WPMY-1 ($p < 0.0001$), suggesting that miR-146b expression might be functionally important in prostate cancer progression and metastasis. PC-3 cell line was chosen for further functional studies because it has high metastatic and invasive potential than other cells.

miR-146b inhibits prostate cancer cell proliferation and induces apoptosis in vitro

To further investigate the gain-of-function of miR-146b in prostate cancer, we performed over-expression experiments in PC-3 cells. As shown in Figure 2A, cells transfected with miR-146b mimics showed higher expression of miR-146b in relative to mock or scrambled control cells. Next, WST-8 cell proliferation assay showed a significant decrease ($p < 0.0001$) of the cell number in miR-146b transfected cells in comparison with control cells (Figure 2B). In contrast, miR-146b mimics showed significantly increased percent of apoptotic cells ($p < 0.0001$; Figure 2C), indicating that overexpression of miR-146b may lead to apoptosis.

We then analyzed the loss-of-function of miR-146b in PC-3 cells inhibiting miR-146b by antagomiR-146b. As shown in Figure 3A, cells transfected with antagomiR-146b showed lower expression of miR-146b relative to mock or negative control cells. The WST-8 cell proliferation assay showed

a significant increase of cell number in antagomiR-146b transfected cells as compared to control cells ($p < 0.0001$; Figure 3B). Conversely, miR-146b inhibitor decreased the percent of apoptotic cells (Figure 3C).

miR-146b inhibits migration and invasion of prostate cancer cells in vitro

To investigate the effect of miR-146b on the migratory capabilities of PC-3 cells in vitro, the transwell migration assays were conducted. As shown in Figure 4A, the migration rates were significantly lower in PC-3 cells transfected with miR-146b mimics relative to mock or scrambled control cells ($p < 0.0001$). However, the number of migrating cells were significantly increased ($p < 0.0001$) when cells transfected with antagomiR-146b were compared to mock or negative control cells (Figure 4B).

We then analyzed the effect of miR-146b on the invasive capacity of PC-3 cells in vitro. As shown in Figure 5A, significantly less PC-3 cells with miR-146b penetrated the matrigel-coated membrane when compared with mock or scrambled control cells ($p < 0.0001$). Conversely, miR-146b inhibitor significantly promoted the invasion of PC-3 cells (Figure 5B). Collectively, these results suggest that miR-146b exhibits a negative role on migration and invasion of prostate cancer.

Discussion

The global expression of miRs is deregulated in most cancer types. Recent findings revealed that miRs deregulation in human cancers occurs via multiple mechanisms, including transcriptional deregulation, epigenetic alterations, dysfunction of key proteins in the miRs biogenesis pathways, mutation and DNA copy number abnormalities. Studies have suggested that miR expression would be downregulated in human tumors relative to normal tissues, and other studies reported a tumor-specific mixed pattern of downregulation and upregulation of miR genes [18]. Several studies have reported that many miRs, including miR-1, 21, 143, 145, 154, 181, 200s, 203, and 205, play important roles in the prostate cancer metastasis by regulating different genes [19-25]. miR-146b is localized at q24.32 and downregulated in various cancers including breast [11], glioblastoma [12], gallbladder [13] and lung [14], but not in prostate cancer. Thus, the aim of this study was to investigate whether the expression of miR-146b in prostate cancer might lead to the discovery of

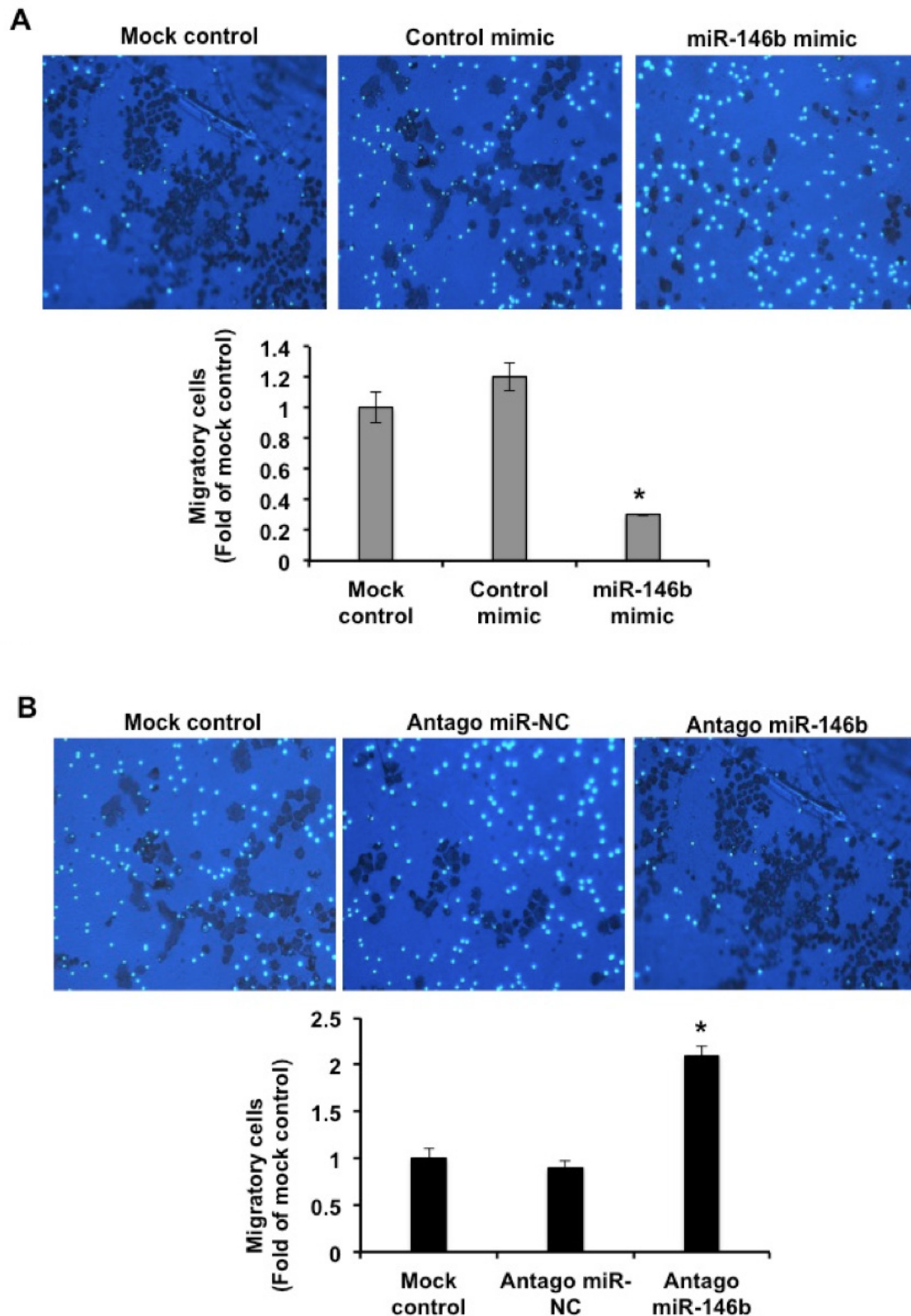


Figure 4. F.miR-146b inhibits migration ability of prostate cancer cells. PC-3 cells were transfected with either **A:** miR-146b mimic or **B:** antagomiR-146b or with corresponding controls. After 48 hrs of transfection, the migration ability of PC-3 cells was assessed as described in methods. Cells passing through the insert were stained and quantified using a spectrophotometer at 560nm and expressed as fold of control. Representative images are shown. miR-146b mimic decreased and antagomiR-146b increased the migration of PC-3 cells. For each data point, triple samples were analyzed. The data are expressed as mean±standard deviation and statistical analyses were performed with Student's t-test. *p<0.0001 compared with corresponding mock control.

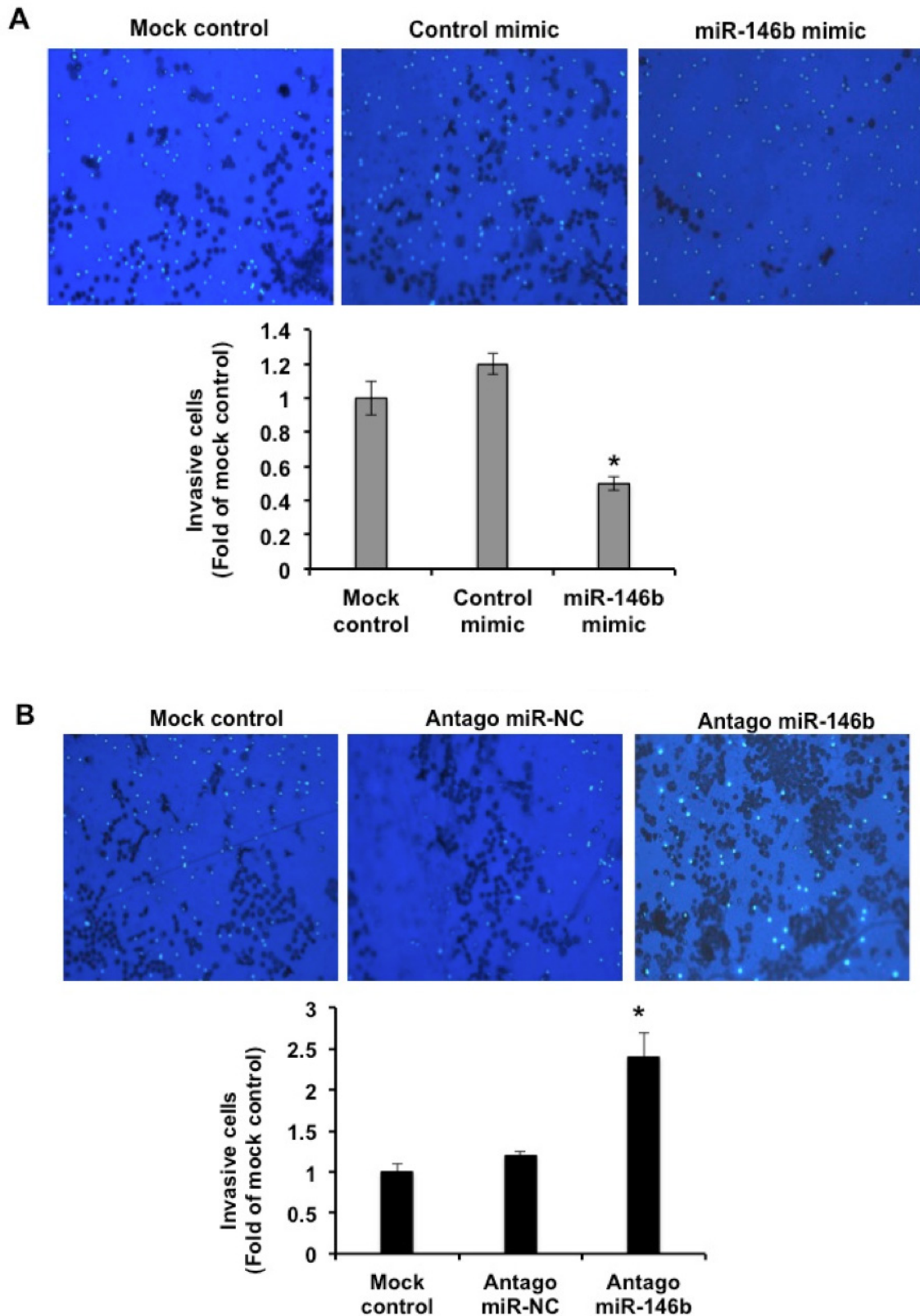


Figure 5. miR-146b inhibits invasiveness of prostate cancer cells. PC-3 cells were transfected with either **A:** miR-146b mimic or **B:** antagomiR-146b or with corresponding controls. After 48 hrs of transfection, the invasive ability of PC-3 cells was assessed as described in methods. Cells passing through the matrigel-coated insert were stained and quantified using a spectrophotometer at 560nm and expressed as fold of control. Representative images are shown. miR-146b mimic decreased and antagomiR-146b increased the invasion of PC-3 cells. For each data point, triple samples were analyzed. The data are expressed as mean±standard deviation and statistical analyses were performed with Student's t-test. * $p < 0.0001$ compared with corresponding mock control.

novel biomarkers for metastatic prostate cancer patients.

In the present study, qRT-PCR results showed that miR-146b is downregulated in prostate cancer tissues and cell lines relative to prostate normal cell lines and cancer-adjacent normal tissues. Subsequent loss-of-function and gain-of-function showed that miR-146b induced apoptosis and suppressed cell proliferation, migration and invasion in the prostate cancer cell lines. These results suggested that miR-146b has a tumor-suppressor role whose downregulation may contribute to the progression and metastasis of prostate cancer.

A previous study in glioblastoma has demonstrated that ectopic miR-146b suppressed the invasiveness and migration by targeting transcripts of the MMP16 gene [26]. Another study has demonstrated that miR-146b was shown to inhibit human breast cancer cells growth by targeting the EGFR gene [11]. Another similar study has also suggested a tumor suppressor role of miR-146b-5p by negatively regulating the nuclear factor-kB (NF-kB) pathway [15]. Consistent with previous reports, our data from qRT-PCR showed that miR-146b is downregulated in all prostate tumor tissues, suggesting that miR-146b may act as tumor suppressor in prostate tumorigenesis.

Consistent with a previous report by Xia et al. in 2009 [26], we found that miR-146b could

inhibit prostate cancer cells migration and invasion. The development of current metastatic models suggests that clonal expansion of cells with attained mutations can cause tumor progression that offers a growth advantage for cells harboring mutations and this facilitates metastasis of the primary tumor [27]. In addition, there could be a small proportion of cells among the primary tumor cells that have inherited alterations to facilitate metastasis and these are those cells that eventually contribute to metastasis [28,29]. The metastatic potential of these prostate cancer cells is due to their capability to suppress miR-146b expression by themselves. Taken together, miR-146b may function as a tumor metastatic suppressor by inhibiting cell proliferation and inducing apoptosis that could affect multiple many other cellular processes, such as carcinogenesis, invasion and metastasis.

In conclusion, miR-146b is frequently decreased in prostate cancer tissues and cell lines and is a potential tumor suppressor miR. Thus, our findings suggest that miR-146b might be a potential clinical marker and therapeutic target for prevention and possibly treatment of prostate cancer. However, further studies are needed to investigate the molecular mechanisms by which miR-146b suppresses metastatic prostate cancer.

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