Original Article
Downregulation of FoxM1 inhibits cell growth and migration and invasion in bladder cancer cells

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Abstract: The FoxM1 (Forkhead Box M1) transcription factor plays a key role in regulation of cell growth, cell cycle, and transformation. Higher expression of FoxM1 has been observed in various types of human cancers including bladder cancer. However, the exact function of FoxM1 in bladder cancer has not been elucidated. To investigate the cellular and molecular function of FoxM1 in bladder cancer, we measured the consequences of downregulation and upregulation of FoxM1 in bladder cancer cells using MTT assay, wound healing assay, and invasion assay. We found that downregulation of FoxM1 inhibited cell growth, but induced apoptosis in bladder cancer cells. Moreover, we found that inhibition of FoxM1 retarded cell migration and invasion. In line with this, upregulation of FoxM1 led to cell growth promotion and inhibited cell apoptosis in bladder cancer cells. Consistently, upregulation of FoxM1 led to increased cell migration and invasion. Our Western blotting results identified that downregulation of FoxM1 increased p27 level and inhibited VEGF, while overexpression of FoxM1 reduced p27 level and increased VEGF. Our findings suggest that FoxM1 could be a useful target for the treatment of bladder cancer.

Keywords: FoxM1, bladder cancer, cell growth, apoptosis, invasion

Introduction
Bladder cancer is one of the common malignancies, which has about 400,000 new patients diagnosed yearly worldwide [1]. Tobacco smoking is one of main risk factors [2]. Non-muscle-invasive bladder cancer (NMIBC) is the main type of bladder cancer, which often has recurrence after transurethral resection [3]. Thus, some patients develop to muscle-invasive bladder cancer after multiple recurrences [4-6]. Therefore, it is required to determine the molecular insight onto bladder tumorigenesis and explore new therapeutic strategies to reduce recurrences in bladder cancer.

FoxM1 (Forkhead Box M1) signaling plays a critical role in governing cell proliferation and apoptosis [7, 8]. FoxM1 (also named as HFH-11, MPP2, Win, and Trident) is a member of the Fox transcription factor family [9, 10]. It has been reported that FoxM1 is a key cell cycle regulator through regulation of some cell cycle genes such as Cdc25A (cell division cycle 25A), Cdc25B, cyclin B, cyclin D1, p21<sup>cip1</sup> and p27<sup>kip1</sup> [11, 12]. It has been known that dysfunction of FoxM1 is associated with tumorigenesis. Upregulation of FoxM1 has been observed in a variety of human cancers including lung cancer, prostate cancer, hepatocellular carcinoma, breast cancer, and pancreatic cancer [13-16]. These reports suggest that FoxM1 could be an oncoprotein in tumorigenesis [17]. However, the role of FoxM1 in bladder cancer has not been elucidated. Therefore, we sought to determine the function of FoxM1 in bladder cancer.

In the current study, we explored the consequence of down-regulation of FoxM1 by its siRNA on bladder cancer cell growth, apoptosis, and invasion. We also examined the effect of FoxM1 overexpression on the process of cell growth and invasion of bladder cancer cells. We found that inhibition of FoxM1 suppressed cell growth and induced apoptosis in bladder cancer cells. Moreover, down-regulation of FoxM1 inhibited cell migration and invasion of bladder cancer cells. Notably, overexpression of FoxM1...
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enhanced cell growth and invasion, but inhibited apoptosis in bladder cancer cells. Strikingly, down-regulation of FoxM1 increased p27 level and decreased VEGF (vascular endothelial growth factor) expression, whereas over-expression of FoxM1 reduced p27 level and increased VEGF expression in bladder cancer cells. Our findings demonstrated that inactivation of FoxM1 could represent a novel strategy for anti-cancer therapies for bladder cancer.

Materials and methods

Cell culture, reagents and antibodies

The human bladder cancer RT4 cells were incubated in a 5% CO₂ humidified atmosphere at 37°C in DMEM (Dulbecco’s modified Eagle’s medium) medium (Gibco Company, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin, and 100 µg/ml streptomycin. Anti-FoxM1 and anti-p27 antibodies were obtained from Cell Signaling Technology. Anti-tubulin antibody was bought from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Lipofectamine 2000 was purchased from Invitrogen (Carlsbad, CA). MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] was obtained from Sigma (St. Louis, MO).

Transfection

Bladder cancer cells were seeded in six-well plates and transfected with control siRNA or FoxM1 siRNAs (GenePharma, Shanghai, China) using Lipofectamine 2000 according to manufacturer’s protocol. RT4 cells were also transfected with FoxM1 cDNA or pcDNA3.0 as control using Lipofectamine 2000 [18].

MTT assay

The transfected cells (5 × 10⁵) were seeded in a 96-well culture plate. After 48 h and 72 h, cells were then incubated with MTT reagent (0.5 mg/ml) for 2 h at 37°C. Cell growth assay was conducted by determining the absorbance at 560 nm using a Benchmark Microplate Reader (Bio-Rad, Hercules, CA, USA). All values were normalized to those of the controls [19].

Apoptosis assay

The transfected cells were cultured in six-well plate for 48 h. Then, the cells were trypsinized, collected, and washed with PBS. Cells were collected by centrifugation and the pellets were resuspended in 500 µl binding buffer with 5 µl propidium iodide (PI) and 5 µl FITC-conjugated anti-Annexin V antibody. Apoptosis was analyzed by a FACScalibur flow cytometer [20].

Real-time reverse transcription-PCR analysis

Total RNA from the transfected cells was isolated with Trizol. One microgram of total RNA from each sample was reversed-transcribed into cDNA by TaqMan reverse transcription reagents kit (Applied Biosystems, Foster City, CA). RT reaction was conducted at 25°C for 10 min, followed by 48°C for 30 min and 95°C for 5 min. The primers used in the PCR reaction are FoxM1 forward primer (5’-AAC CGC TAC TTG ACA TTG G-3’) and reverse primer (5’-GCA GTG GCT TCA TCT TCC -3’); β-actin forward primer

Figure 1. Down-regulation of FoxM1 by its siRNA in bladder cancer cells. A. Real-time RT-PCR analysis was used to determine the efficacy of FoxM1 siRNA in RT4 bladder cancer cells. *P < 0.01 vs Control siRNA. B. Top panel: Western blot analysis was used to measure the FoxM1 expression in RT4 bladder cancer cells transfected with different FoxM1 siRNAs. Bottom panel: Quantitative results for Top panel. *P < 0.01, vs Control siRNA.
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(5’-CCA CAC TGT GCC CAT CTA CG-3’) and reverse primer (5’-AGG ATC TTC ATG AGG TAG TCA GTC AG-3’).

Western blotting analysis

Cells were lysed in lysis buffer [50 mmol/L Tris (pH 7.5), 100 mmol/L NaCl, 1 mmol/L EDTA, 0.5% NP40, 0.5% Triton X-100, 2.5 mmol/L sodium orthovanadate, 10 µL/mL protease inhibitor cocktail, and 1 mmol/L PMSF]. The protein concentrations were measured by Bio-Rad assay system. Equal amount of proteins were fractionated by SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) and then transferred to nitrocellulose membranes. The membranes were immunoblotted by primary antibodies. The anti-FoxM1 (1:2000), anti-p27 (1:1000), anti-VEGF (1:2000), and anti-tubulin (1:4000) antibodies were used. The expression of tubulin was used as internal control.

Wound healing assay

Cells were seeded in 6-well plates and grown to almost confluency. Then, monolayers of cells were scratched with 200 µL small yellow pipette tips and washed twice with PBS. The scratched area was photographed with a microscope at 0 h and 20 h, respectively [21].

Transwell invasion assay

Cell invasion was assessed using BD BioCoat Matrigel invasion chambers. Briefly, transfected cells were seeded in DMEM without serum in the upper chamber of the system. The bottom chamber was added with complete medium. After 20 hours of incubation, the non-invading cells were removed. The cells that had invaded through Matrigel matrix membrane were stained with Wright’s-Giemsa or 4 µg/ml Calcein AM in hanks buffered saline at 37°C for one hour. The labeled invasive cells were photographed under a microscope.

Figure 2. Down-regulation of FoxM1 inhibited cell proliferation and induced apoptosis. A. MTT assay was used to measure cell proliferation in RT4 bladder cancer cells after FoxM1 siRNA transfection. The transfected cells (5 × 10^5) were seeded in a 96-well plate. After 48 h and 72 h, cells were incubated with MTT reagent (0.5 mg/ml) for 2 h at 37 °C. Cell growth was determined by measuring absorbance at 560 nm. All values were normalized to those of the controls. *P < 0.05 vs Control siRNA. B. Flow cytometry was used to measure cell apoptosis in RT4 bladder cancer cells after FoxM1 siRNA transfection. The transfected cells were cultured in the 6-well plate for 48 h. Then, the cells were collected by centrifugation and resuspended in binding buffer with 5 µl propidium iodide and 5 µl FITC-conjugated anti-Annexin V antibody. Apoptosis was analyzed by a FACScalibur flow cytometer.
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Statistical analysis

The data were presented as mean ± SD. Student’s t-test was performed to evaluate statistical significance. The value of \( P < 0.05 \) was considered as significance.

Results

Downregulation of FoxM1 by its siRNA inhibited cell growth

In order to ascertain the function of FoxM1 in the progression of bladder cancer, we conducted a series of experiments to achieve our goal. The bladder cancer cells were transfected with FoxM1 siRNA to down-regulate the expression of FoxM1. The efficacy of FoxM1 for knockdown by siRNA was validated by real-time RT-PCR and Western blotting in bladder cancer cells. Our RT-PCR results showed that FoxM1 mRNA was significantly inhibited in FoxM1 siRNA transfected cells, compared with control siRNA transfected cells (Figure 1A). We also observed that FoxM1 protein expression was barely detectable in FoxM1 siRNA transfected cells (Figure 1B and Supplementary Figure 1). MTT was per-
formed to measure cell viability in FoxM1 siRNA transfected cells. Our MTT data showed that downregulation of FoxM1 expression led to cell growth inhibition in bladder cancer cells (Figure 2A).

**Downregulation of FoxM1 induced apoptosis in bladder cancer cells**

To further investigate whether the growth inhibitory effect of FoxM1 knockdown is due to apoptosis, we conducted apoptosis assay by Annexin V-FITC/PI method in RT4 cells after FoxM1 siRNA transfection. We found that downregulation of FoxM1 increased the percentage of apoptotic cells in RT4 cells transfected with FoxM1 siRNA. Specifically, cell apoptosis was increased from 10.47% in control siRNA treatment group to 23.62% in FoxM1 siRNA treatment group in RT4 cells (Figure 2B). This finding indicated that depletion of FoxM1 triggered apoptosis, which might contribute to cell growth inhibition in bladder cancer cells.

**Downregulation of FoxM1 decreased VEGF expression and increased p27 level**

To further explore the mechanism of FoxM1-mediated tumor progression, we investigated whether FoxM1 siRNA treatment could regulate VEGF and p27 in bladder cancer cells. Our Western blotting data showed that p27 level was dramatically increased in the FoxM1 siRNA transfected cells (Figure 4). We also found that VEGF expression was remarkably decreased in cells after FoxM1 transfection in bladder cancer cells (Figure 4A and Supplementary Figure 1). Our findings indicated that downregulation of FoxM1 exerts its anti-tumor function partly via inhibition of VEGF and upregulation of p27 in bladder cancer cells.

**Over-expression of FoxM1 enhanced cell growth and inhibited apoptosis**

To further validate the function of FoxM1 in bladder cancer cells, RT4 cells were transfecteded human FoxM1 vector or empty vector alone.
We found that over-expression of FoxM1 by its cDNA transfection promoted bladder cancer cell growth (Figure 5A). Moreover, over-expression of FoxM1 inhibited cell apoptosis in bladder cancer cells (Figure 5B). Taken together, FoxM1 was critically involved cell growth in bladder cancer cells.

**Over-expression of FoxM1 promoted cell migration and invasion**

To further determine the role of FoxM1 in cell migration and invasion, we used Transwell chamber assay to measure the cell invasion in RT4 cells after FoxM1 cDNA transfection. We found that FoxM1 cDNA transfected cells showed significant promotion of cell invasion compared to empty vector-transfected control cells (Figure 6A and 6B). Wound healing assay was performed to measure the migratory activity in bladder cancer cells after overexpression of FoxM1. We found that FoxM1 cDNA transfected RT4 cells showed a remarkably increase in cell migration (Figure 6C). Altogether, overexpression of FoxM1 enhanced cell motility in bladder cancer cells (Figure 7 and Supplementary Figure 1).

**Discussion**

FoxM1 plays a pivotal role in regulation of cell proliferation, differentiation, and apoptosis. Abnormal expression of FoxM1 has been reported in various human malignancies including bladder cancer [22-24]. For example, FoxM1
was found to be overexpressed in bladder tumor samples as compared with normal bladder tissues [22]. Similar study showed that overexpressed FoxM1 is observed in bladder cancer clinical specimens [24]. Another study also supported that FoxM1 expression was up-regulated in the majority of the bladder cancer tissue specimens at both mRNA and protein levels [23]. Moreover, FoxM1 expression was significantly correlated with TNM (tumor, node, and metastasis) stage and histological grade and metastasis, suggesting that FoxM1 up-regulation was associated with poor prognosis in bladder cancer [23]. Interestingly, recent study identified that FoxM1 was differentially expressed between muscle-invasive bladder cancer subtypes [25]. Although FoxM1 was associated the poor prognosis in bladder cancer, the mechanism of FoxM1-mediated tumor progress has not been fully elucidated. In the present study, we found that FoxM1 promoted cell growth and migration and invasion partly through downregulation of p27 and upregulation of VEGF in bladder cancer cells.
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Emerging evidence has suggested that FoxM1 functions as an oncoprotein in multiple human cancers [26, 27]. We determined the biological function of FoxM1 in bladder cancer cells via downregulation or overexpression of FoxM1. Consistent with one study [23], we found that down-regulation of FoxM1 inhibited cell proliferation, migration, and invasion in bladder cancer cells. Keeping with one report [24], downregulation of FoxM1 induced cell apoptosis in bladder cancer. Mechanistically, depletion of FoxM1 inhibited cell growth and triggered apoptosis partly via upregulation of p27 in bladder cancer cells. VEGF is an important molecule that involved in tumor cell invasion and metastasis [28, 29]. It has been reported that FoxM1 regulated VEGF expression in various cell types [30-32]. In this study, we found that FoxM1 down-regulation led to a significant reduction of VEGF expression in bladder cancer cells. Consistently, over-expression of FoxM1 resulted in a remarkably increased in the expression of VEGF in bladder cancer cells. Our findings indicated that downregulation of FoxM1 could potentiate the anti-motility activity in part through inhibition of VEGF in bladder cancer cells.

Since FoxM1 plays an oncogenic role in human cancers, down-regulation of FoxM1 could be a useful for the treatment of cancer. Multiple natural compounds have been discovered to inhibit the expression of FoxM1. For instance, plum-bagin induced growth inhibition of human glioma cells via downregulation of the expression and activity of FoxM1 [33]. Genistein inhibited cell growth accompanied by induction of apoptosis through attenuation of FoxM1 in pancreatic cancer cells [34]. Diarylheptanoids suppressed cell growth via modulation of FoxM1 in pancreatic cancer cells [35]. Sorafenib inhibited cell proliferation and invasion via suppressing FoxM1 in human hepatocellular carcinoma cells [36]. Therefore, inhibition of FoxM1 by these natural agents could be effective therapeutic approach for human cancer treatment.

Disclosure of conflict of interest

None.

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Supplementary Figure 1. The original images for Western blotting bands in Figures 1B, 4A and 7A.