



Overexpression of F-box and WD repeat domain containing 7 prevents tumor growth of bladder cancer cells through regulating SREBP1a

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Background: This study aimed to explore the effect of F-box and WD repeat domain containing 7 (FBXW7) overexpression on bladder cancer, and to determine the regulatory effect of FBXW7 on sterol regulatory element-binding protein 1a (SREBP1a) in bladder cancer.

Methods: The function of F-box and FBXW7 in tumor growth of bladder cancer cells was investigated using *in vivo* and *in vitro* models. We constructed and transfected FBXW7 overexpression vectors into T24 and J82 bladder cancer cells. After transfections, the expression of FBXW7 at messenger RNA (mRNA) and protein levels in human bladder cancer cells was confirmed. To verify the effect of FBXW7 on tumor growth, cell proliferation and migration were detected in the tumor cells after overexpression of FBXW7. Additionally, an *in vivo* tumor model was produced by inoculating the tumor cells and the effect of FBXW7 was evaluated. Finally, the regulation mechanisms were determined.

Results: Our data showed that overexpression of FBXW7 in J28 and T24 cells significantly inhibited the migration and proliferation of J82 and T24 cells, arrested the cells at G0/G1 phase, and up-regulated phosphorylation of glycogen synthase kinase-3 β , while suppressing SREBP1a expression. *In vivo* data also showed that FBXW7 overexpression triggered apoptosis of tumor cells, prevented the pathological changes of tumor tissues, up-regulated p-GSK3 β expression, and suppressed SREBP1a expression. In addition, an interaction between FBXW7 and SREBP1a was confirmed by co-immunoprecipitation.

Conclusions: Together, our data indicate that overexpression of FBXW7 prevents tumor growth of bladder cancer cells, likely through suppressing SREBP1a expression.

Keywords: Bladder cancer; FBXW7; SREBP1a; p-GSK3

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Introduction

Bladder cancer is a common malignant tumor, especially in women (1), and possesses the highest incidence rate of urologic malignancy in China. It is the second most common urogenital tumors worldwide and has the ninth highest rate of cancer incidence (2,3). However, the

mortality rate of bladder cancer has only decreased by 5% in the past 15 years (4). The main reason is that the efficacy of chemotherapy for advanced bladder cancer is low, and an effective target treatment drug is still not available, as the progress and drug resistance mechanism of bladder cancer have remained unclear (5). Therefore, it is of great

significance to explore new molecular markers related to the occurrence and development of bladder cancer.

Hyperactivation of sterol regulatory element binding protein (SREBP) transcription factors directly upregulates lipogenic enzymes to trigger *de novo* lipogenesis in tumors (4). In mammals, 3 isoforms of SREBP protein have been identified: SREBP 1a, SREBP 1c, and SREBP2. Both SREBP1a and SREBP1c are produced by the *SREBP1* gene, while the *SREBP2* gene encodes SREBP2 (5). While SREBP1 mainly regulates the genes of fatty acid triglyceride and phosphate synthesis, SREBP2 preferentially activates cholesterol synthesis genes (5,6). Therefore, SREBP is essential to maintain the balance between protein and lipid biosynthesis and is involved in the development, migration, or poor prognosis of cancers (7,8). The imbalance of SREBP1 activates high-level *de novo* adipogenesis in cancer cells, which is one of the key factors driving the growth of malignant tumors (6). A new phosphorylation site ser-434 was identified in SREBPs, which regulates the degradation of SREBP1-dependent F-box and WD repeat domain containing 7 (FBXW7) (7). Interestingly, the expression of SREBP1 depends on glycogen synthase kinase-3 β (GSK-3 β) activity (9,10).

A member of the F-box protein family, FBXW7 is a substrate receptor of SKP1-CUL1-F-box protein (SCF) ubiquitin ligase, which plays an important role in regulating several important cellular processes, including cell growth, division, and differentiation (8,11). It is recognized as a typical tumor suppressor because it can promote ubiquitination and degradation of a variety of cancer proteins (including cyclin E, c-myc, c-jun, Notch1, and Mcl-1), thereby inhibiting the growth and survival of tumor cells (11). Function loss of FBXW7 is often reported in cancer (8,9). Therefore, it is very important to evaluate the effect of FBXW7 in cancer treatment. In this study, we aimed to explore the effect of FBXW7 overexpression on bladder cancer cells, and to determine the regulatory effect of FBXW7 on SREBP1a in bladder cancer. In doing so, we aimed to determine novel therapeutic targets for bladder cancer. We present the following article in accordance with the ARRIVE reporting checklist (available at <https://tau.amegroups.com/article/view/10.21037/tau-22-146/rc>).

Methods

Cell lines

Human bladder cancer cells J28 and T24 were purchased

from BeiNa Biotech and cultured in Dulbecco's Modified Eagle Medium (DMEM; Gibco, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT, USA) in an incubator with 5% CO₂ at 37 °C. The experiments were divided into 3 groups: a control group (without transfection), a control vector group (NC, non-functional version of FBXW7), and a FBXW7 overexpression group (FBXW7).

Cell transfection

The FBXW7 vector was constructed in pcDNA3.1 (+) plasmid. The J28 and T24 cells were transfected with a FBXW7 vector or control vector, respectively. When the cell confluence reached 70%, the transfection was carried out. The cell culture medium was replaced with serum-free medium, and combined with opti MEM (Thermo Fisher Scientific, Waltham, MA, USA), Lipofectamine 3000 (Thermo Fisher), and small interfering RNA [siRNA; 125 μ L/1 optical density (OD)]. At 4 h after transfection, the cells were refreshed with 1 mL of complete medium with 20% FBS, and the corresponding detection was carried out 48 h later.

Real-time fluorescent quantitative polymerase chain reaction

At 48 h after transfection, the cells were collected to extract total RNA using the TRIzol™ Plus RNA Purification Kit (CW0580S; CWBIO, Cambridge, MA, USA). The purity of RNA (OD260/OD280) was determined using a UV-Vis spectrophotometer (DeNovix, Wilmington, DE, USA). Complementary DNA (cDNA) was synthesized by a High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific). Thereafter, fluorescence quantitative polymerase chain reaction (qPCR) was carried out using the following reaction system: RNase free dH₂O 8.2 μ L, cDNA 1 μ L, upstream primer 0.4 μ L, downstream primer 0.4 μ L, and universal SYBR qPCR Master Mix 10 μ L. The reaction steps were as follows: pre-denaturation at 95 °C for 10 min; denaturation at 95 °C for 10 s; annealing at 58 °C for 30 s; and extension at 72 °C for 30 s, for a total of 40 cycles. The primer sequence was obtained from General Biosystems Co., Ltd (Anhui, China), and was as follows: *FBXW7* (F: 5'-AGCTGTCCAGCCCCCTTCTA-3'; R: 5'-GCACGCTTGTGATTCTCCTT-3'); *SREBP1a* (F: 5'-GCCTATTTGACCCACCCTAT-3'; R: 5'-TGGCACTGACTCTTCCTTGA-3'); β -*actin* (F:

5'-TGGCACCCAGCACAATGAA-3'; R: 5'-CTAAGTCA TAGTCCGCCTAGAAGCA-3'). The relative expression levels of *FBXW7* and *SREBP1a* were calculated by $2^{-\Delta\Delta CT}$ method and normalized to β -*actin*.

Western blotting

At 48 hours after transfection, the cells were collected to isolate the proteins. After 30 min pyrolysis on ice, the homogenates were centrifuged at 12,000 rpm for 15 min. The supernatant was collected, and protein concentration was determined by bicinchoninic acid (BCA) kit. Later, the protein was denatured, and 25 μ g protein was separated via 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) for 2 h. After that, the proteins were transferred to a nitrocellulose membrane. The rabbit anti-FBXW7 (1:1,000), rabbit anti-p-GSK3 β (1:400), and mouse anti-SREBP1a (1:500) was incubated overnight at 4 °C. The secondary antibody (1:100; catalogue nos. ab131368; Abcam, Cambridge, MA, USA) was added and co-incubated for 1–2 h at room temperature. Enhanced chemiluminescence (ECL) exposure liquid droplet (catalogue no. RPN2133; GE Healthcare Life Sciences, Chalfont, UK) was added on the membrane. Finally, the membrane was used for exposure utilizing a gel imaging system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Grey density was analyzed using Quantity One analysis software v1.4.6 (Bio-Rad Laboratories, Inc.).

Cell Counting Kit-8 (CCK-8)

CCK-8 assay was used to determine the cell proliferation as previously described (10). At 48 h after cell transfection, 10 μ L CCK-8 detection reagent was added to each well and incubated at 37 °C for 2 h; The OD value of each well at 450 nm wavelength was detected by microplate reader to calculate the cell survival rate.

Migration assay

When the cell confluence reached more than 90%, the migration assay was carried out. A 200 μ L pipette tip was used to scratch each well. The culture medium was discarded and washed with phosphate-buffered saline (PBS) 3 times. After changing to DMEM incomplete culture medium, the cells of each well were photographed. The cells were put back into the incubator, and the scratches of each well were photographed again 24 h later. The 24 h

data and 0 h data were determined, and the corresponding width was calculated.

Transwell assay

The J28 and T24 cells were seeded into the upper chamber with 1-day starvation of serum. The lower chamber contained DMEM with 10% FBS. The transwell system was placed in a CO₂ incubator at 37 °C for 24 h incubation. After that, the lower chamber was taken out and fixed with 4% paraformaldehyde (PFA) for 30 min at room temperature. The cells were stained with crystal violet (0.1%, C8470; Amresco, Solon, OH, USA) at room temperature for 5 min. At least 5 fields in each image were taken using light microscopy.

Flow cytometry

The J28 and T24 cells were seeded in 6-well plates. Approximately 3×10^6 cells in each group were collected after transfection. After washing with pre-cooled PBS, the cells were incubated with propidium iodide (PI) for 30 min. The cell cycles were detected using a flow cytometer [Becton, Dickinson, and Co. (BD) Biosciences, Franklin Lakes, NJ, USA] within 1 h.

In vivo tumor model

All animal experiments were approved by the Ethics Committee of the First Affiliated Hospital of Nanchang University (approval No. 20170312), in compliance with the institutional guidelines of First Affiliated Hospital of Nanchang University for the care and use of animals. A protocol was prepared before the study without registration. Male BALB/c-nu mice (4 weeks old) were purchased from Hunan SLAC Jingda Experimental Animal Co., Ltd. (Hunan, China; license No.: scxk (Xiang) 2019-0004) and housed under specific-pathogen-free conditions that were automatically maintained at a temperature of 23 ± 2 °C, relative humidity of 45–65%, and with a controlled 12 h light/dark cycle. A total of 19 mice implanted with the T24 cells were randomly divided into 3 groups: control group (n=7), FBXW7 NC group (n=7), and FBXW7 group (n=5), in which 1×10^6 normal T24 cells, FBXW7 NC T24 cells, and FBXW7 overexpressed T24 cells were inoculated into the axillary subcutaneously, respectively. At 30 days after tumor cell inoculation, the tumor tissues were collected for subsequent experiments.

Hematoxylin and eosin staining

The tumor specimens were fixed in 4% PFA in PBS (pH 7.4) at 4 °C overnight and then embedded in paraffin for tissue sectioning. The tissues were sectioned into 5 µm-thick sections. Subsequently, the slides were stained with hematoxylin (3%) and eosin (3%) for 5 min at room temperature. The images were captured under light microscopy.

TUNEL staining

Tumor tissues were fixed in 4% PFA in PBS (pH 7.4) at 4 °C overnight and then embedded in paraffin for tissue sectioning. The tissues were sectioned into 5 µm-thickness sections. Then, terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) solution was added to each section and incubated for 1.5 h at 45 °C. Thereafter, 4',6-diamidino-2-phenylindole (DAPI) (5 µg/mL) was added to stain the nuclei for 5 min at room temperature. The images were observed under a fluorescence microscope (magnification, ×200).

Immunohistochemistry

The tumor tissues were fixed in 4% PFA in PBS (pH 7.4) at 4 °C overnight and then embedded in paraffin for tissue sectioning. The tissues were sectioned into 5 µm-thick sections. The sections were blocked in 5% bovine serum albumin (BSA) for 2 h at room temperature and incubated with the primary antibodies overnight at 4 °C, namely, SREBP1α (1:200) and p-GSK3β (1:100). The sections were incubated with the secondary antibody and stained by 3,3'-diaminobenzidine (DAB) for 5–10 min.

Statistical analyses

All data were analyzed using the software SPSS 21.0 (IBM Corp., Armonk, NY, USA) and expressed as mean ± standard deviation (SD). The significant difference among groups was analyzed by one-way analysis of variance (ANOVA) and Bonferroni's test, with $P < 0.05$ as the significant difference.

Results

Effect of FBXW7 overexpression on migration and invasion of bladder cancer cells

The expression of FBXW7 in J82 and T24 cells were

detected by reverse transcription (RT)-qPCR and western blotting. Our results showed that the messenger RNA (mRNA; *Figure 1A*) and protein expression (*Figure 1B*) levels of FBXW7 in J82 and T24 cells were significantly increased in the FBXW7 vector group compared with those in control group and control vector group, which indicated that the FBXW7 vector was efficient to enhance FBXW7 expression.

Next, we determined the effects of FBXW7 overexpression on the biological activities of the tumor cells. The results showed that overexpression of FBXW7 significantly inhibited the migration (*Figure 1C*), as well as the invasion of J82 and T24 cells compared with control vector group (*Figure 1D*), quantitative data of invasion of J82 cells and T24 cells (*Figure 1E*).

Effect of FBXW7 overexpression on cell cycle distribution and proliferation of bladder cancer cells

The results showed that FBXW7 could significantly affect G0/G1, S, and G2/M phases of bladder cancer J82 and T24 cells (*Figure 2A*), and significantly inhibit the proliferation of J82 and T24 cells compared with the control and vector group (*Figure 2B*). Therefore, overexpression of FBXW7 could regulate the progression of bladder cancer by affecting the cell cycle distribution and inhibiting cell proliferation.

Effect of FBXW7 overexpression on expression of SREBP1a and p-GSK3β in bladder cancer cells

The expression of p-GSK3β and SREBP1a was detected. The results showed that the expression of SREBP1a in FBXW7 vector group was significantly lower than that in the control group (*Figure 2C, 2D*), while p-GSK3β was significantly higher in FBXW7 vector group than that in the control group (*Figure 2C, 2D*).

FBXW7 overexpression triggered apoptosis of the tumor cells

At 30 days after inoculation of different types of bladder cancer cells, the tumors were collected. Hematoxylin and eosin (HE) staining showed that tumor cells in the control and FCXW7 NC groups were diffusely distributed and pathological mitotic features were observable. In contrast, FCXW7 overexpression obviously altered the features of tumorigenesis (*Figure 3A*). Apoptosis increased in FBXW7 overexpression group compared with FCXW7 NC group (*Figure 3B, 3C*).

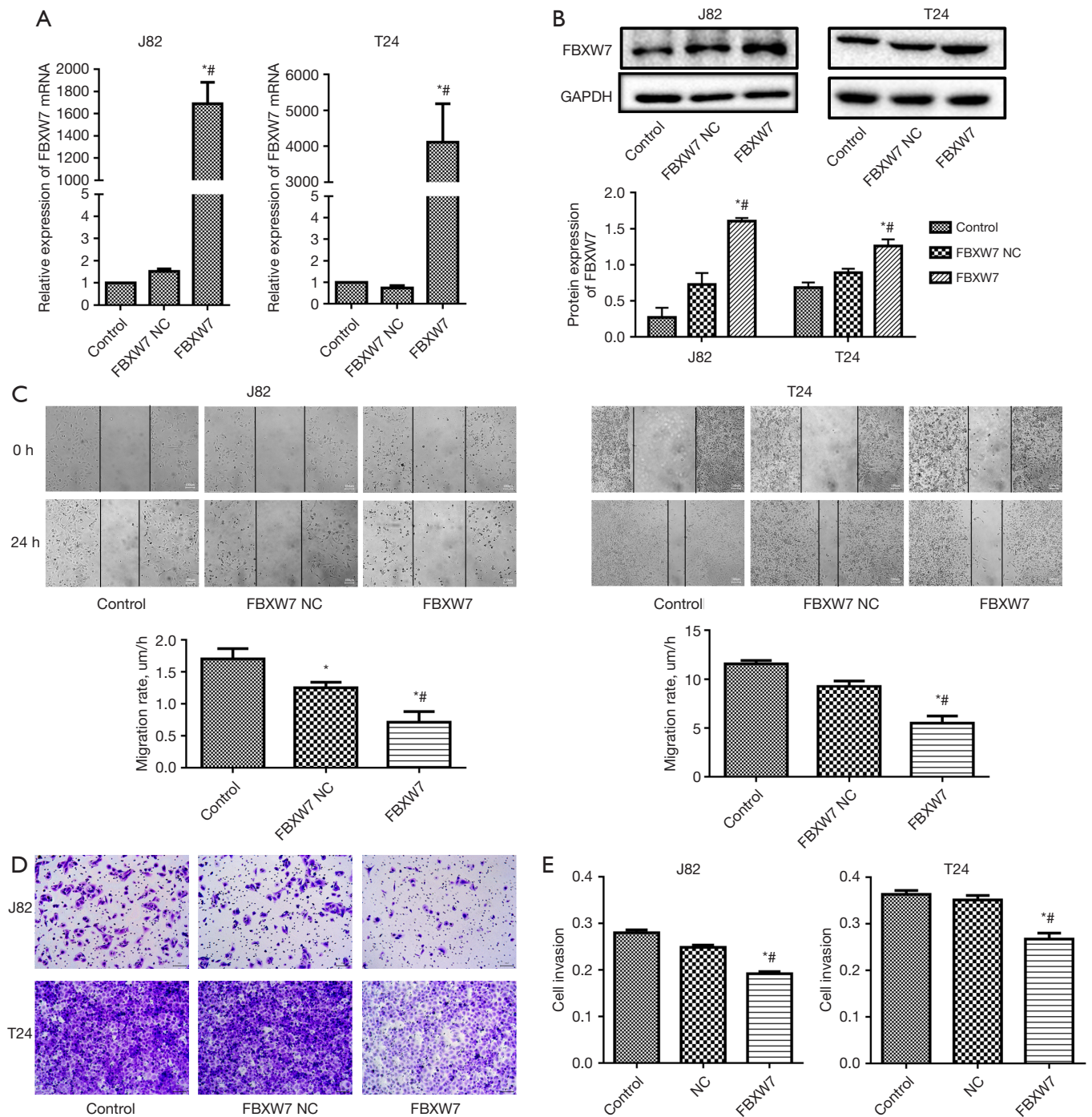


Figure 1 Effects of FBXW7 overexpression on migration of bladder cancer cells. (A) FBXW7 expression at mRNA level increased in the FBXW7 group in both J82 and T24 cells; (B) FBXW7 expression at protein level was also increased in FBXW7 group; (C) FBXW7 overexpression increased the migration rate in J82 cells and T24 cells. Left panel: Representative images and quantitative data of J82 cells; Right panel: Representative images and quantitative data of J82 cells. Magnification: 400 \times ; observational method: electron microscope. (D) representative images of cell invasion in J82 and T24 cells. Staining method: crystal violet staining; magnification: 400 \times ; (E) quantitative data of invasion of J82 cells and T24 cells. Compared with control, * $P < 0.05$; Compared with NC, # $P < 0.05$ ($n = 3$ in each group). mRNA, messenger RNA. FBXW7, Transfection of FBXW7 overexpression vector; NC, transfected with overexpressed empty vector.

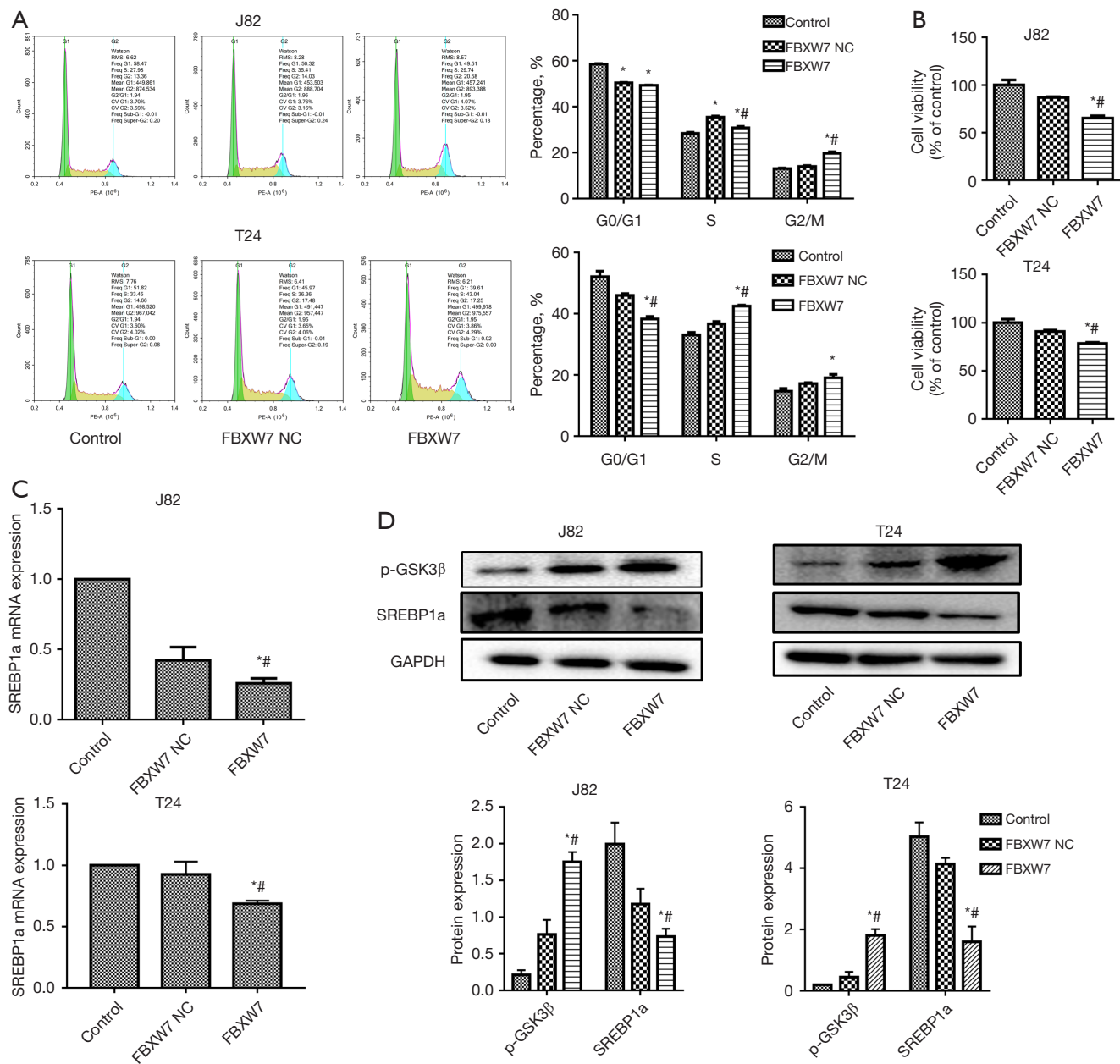


Figure 2 Effects of FBXW7 overexpression on cell cycling, proliferation, and expression of SREBP1a and p-GSK3 β in bladder cancer cells. Cell cycling and proliferation were detected after transfecting FBXW7. FBXW7 could affect G0/G1, S, and G2/M phases of bladder cancer J82 and T24 cells, and inhibit the proliferation of J82 and T24 cells compared with control and vector group. (A) Representative images of flow cytometry and quantitative data of cell cycle distribution; (B) cell proliferation of J82 and T24 cells detected by CCK-8 assay; (C) mRNA expression of SREBP1a in J82 cells and T24 cells; (D) Protein expression of SREBP1a and p-GSK3 β in J82 cells and T24 cells. Compared with control, * $P < 0.05$; Compared with NC, # $P < 0.05$ ($n = 3$ in each group). CCK-8, Cell Counting Kit-8; mRNA, messenger RNA. FBXW7, Transfection of FBXW7 overexpression vector; NC, transfected with overexpressed empty vector; SREBP1a, sterol regulatory element binding protein.

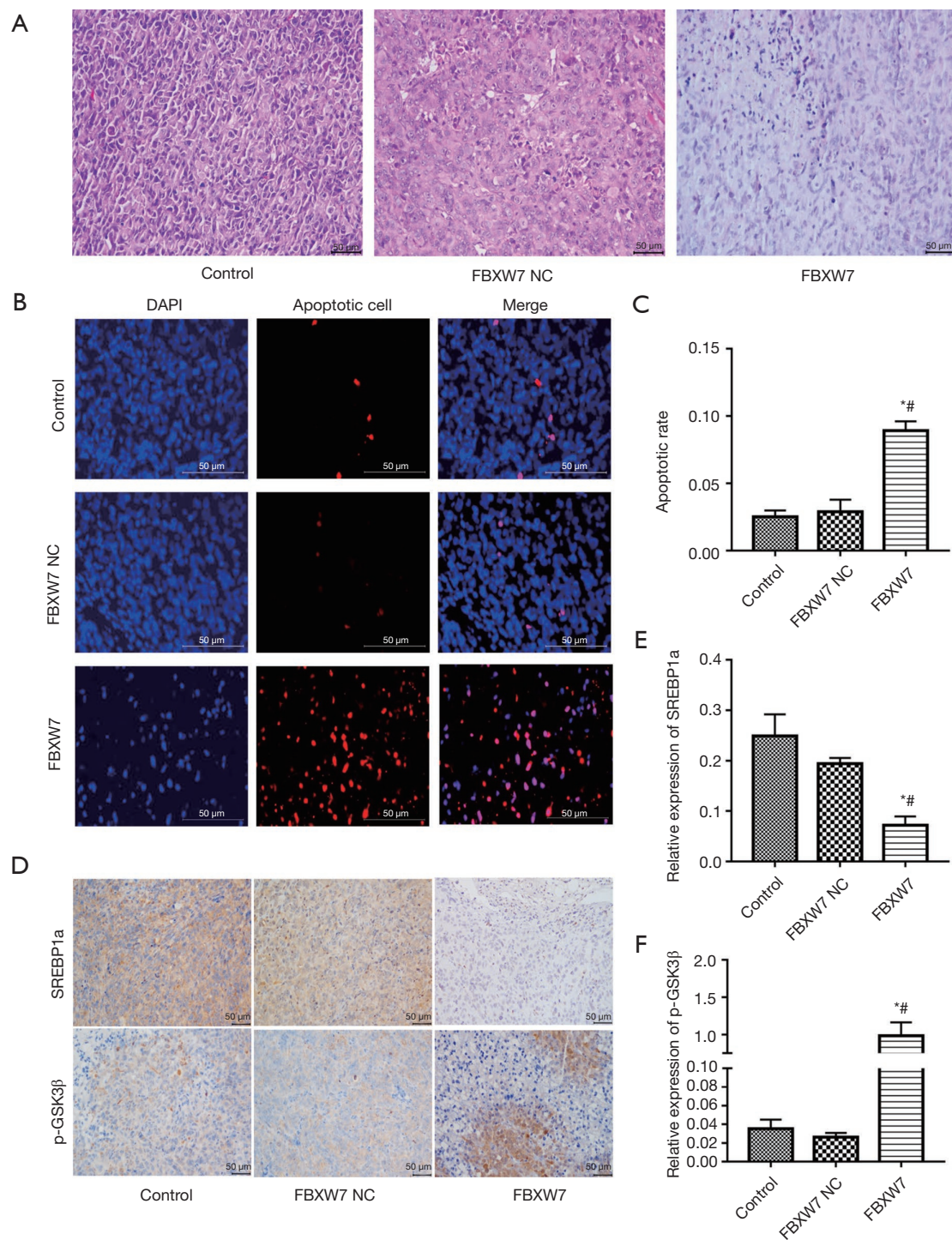


Figure 3 FBXW7 overexpression triggered morphological change and apoptosis of the tumor cells, and promoted p-GSK3β, but reduced SREBP1a expression. At 30 days after inoculation of different types of bladder cancer cells, the tumors were collected. (A) HE staining of the tumor tissue, magnification: 100×; (B) representative images of apoptosis, magnification: 400×, staining method: TUNEL staining; (C) quantification data; (D) representative images of IHC; scale bar: 50 μm; staining method: immunohistochemical staining; (E) quantification data of SREBP1a expression; (F) expression of p-GSK3β. Compared with control, *P<0.05; Compared with NC, #P<0.05 (n=3 in each group). HE, hematoxylin and eosin; IHC, immunohistochemistry. FBXW7, Transfection of FBXW7 overexpression vector; NC, transfected with overexpressed empty vector; SREBP1a, sterol regulatory element binding protein.

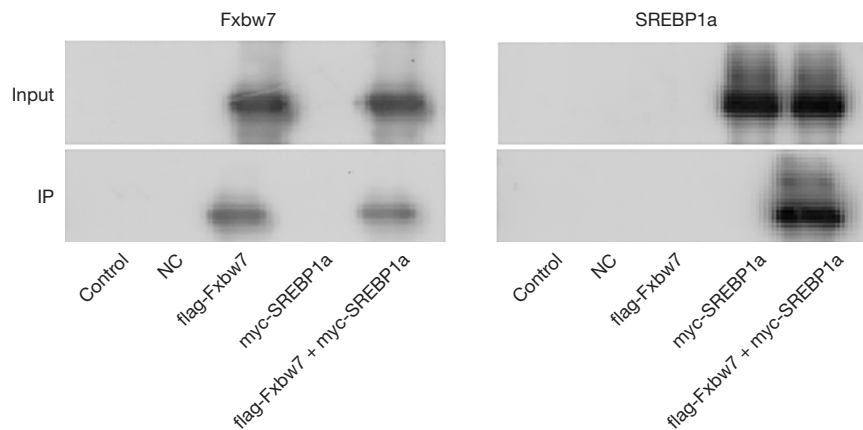


Figure 4 FBXW7 directly interacted with SREBP1a. Using FLAG-FBXW7 as bait protein, myc-SREBP1a protein could be detected in the product via co-immunoprecipitation experiment. IP, immunoprecipitation, NC, no-load carrier, FLAG-FBXW7, FLAG-tagged FBXW7 protein; myc-SREBP1a, myc-tagged SREBP1a protein.

FBXW7 overexpression promoted p-GSK3 β , but reduced SREBP1a expression in bladder cancer cells

Immunohistochemistry (IHC) was used to detect SREBP1a and p-GSK3 β in tumor tissues. As shown in *Figure 3D-3F*, SREBP1a and p-GSK3 β were expressed in nucleus as well as cytoplasm. Moreover, FBXW7 overexpression up-regulated p-GSK3 β , but reduced SREBP1a expression in bladder cancer cells.

FBXW7 directly interacted with SREBP1a

Using FLAG-FBXW7 as bait protein, myc-SREBP1a protein could be detected in the product via co-immunoprecipitation experiment (*Figure 4*), which suggested that FBXW7 interacted with SREBP1a.

Discussion

As bladder cancer is a severe type of malignant tumor with high metastasis, it is important to determine its pathogenesis to reduce the mortality of the disease (12,13). Tumor cell migration as well as invasion is crucial for tumorigenesis (14). Importantly, the invasion of cancer cells is related to cell migration (15,16). In this study, *in vitro* experiments were designed to explore the effect of FBXW7 on the migration and invasion of bladder cancer cells, and to disclose its mechanism. Our results demonstrated that FBXW7 overexpression could prevent migration and invasion of bladder cancer cells. Molecular mechanisms

showed that FBXW7 overexpression promoted p-GSK3 and suppressed SREBP1a expression. *In vivo* experiments further indicated that FBXW7 overexpression promoted tumor cell apoptosis, promoted p-GSK3, and suppressed SREBP1a expression. Moreover, a direct interaction was verified using co-immunoprecipitation. This study provides a new idea for the treatment of bladder cancer.

The *FBXW* gene encodes members of F-box protein family, which is the key component of skp1-cul1-f-box protein (SCF) ubiquitin ligase (17); FBXW7 targets a group of well-known oncoproteins, including c-myc, cyclin E, notch, c-jun and Mcl-1, for ubiquitination and degradation (18-20). Meanwhile, FBXW7 is involved in the regulation of many cellular processes, including cell proliferation, cell cycling, apoptosis, and differentiation (11,21,22). Previous studies have shown that FBXW7 is a tumor suppressor, because the decreased expression of FBXW7 promotes the occurrence and development of many types of cancer (23-25). Moreover, the expression level of FBXW7 is negatively correlated with the malignant progression of some tumors (25-27). It has been reported that the mutation of FBXW7 can promote the proliferation, invasion, and migration of cervical cancer cells (28), which is consistent with the results of this study. Overexpression of FBXW7 in bladder cancer cells J82 and T24 inhibits the migration, invasion, and proliferation of J82 and T24 cells, and affects the cell cycle.

As a member of SREBP transcription factor family, SREBP1a mainly regulates lipid homeostasis. It also activates high levels in *de novo* adipogenic tract cancer

cells, which is one of the keys to driving the growth of malignant tumors (29). Phosphorylation of thr-426 and Ser-430 in SREBP1a creates a docking site for ubiquitin ligase FBXW7, leading to degradation of transcription factors (7), while Prmt5-induced methylation prevents GSK3 β . The phosphorylation of SREBP1a at s430 results in its separation from FBXW7, and the degradation is avoided through ubiquitin proteasome pathway (29). In this study, overexpression of FBXW7 promoted p-GSK3 β expression, while suppressing the expression of SREBP1a. Interestingly, FBXW7 has different effects on SREBP1a in J82 and T24 bladder cancer cells, and FBXW7 has a stronger effect on SREBP1a in T24 bladder cancer cells.

This study demonstrated the exact function of FBXW7 on bladder cancer cell proliferation and migration. We selected 2 bladder cancer cells, J82 and T24. Additionally, *in vivo* data suggesting the function of FBXW7 in tumor cell migration were also demonstrated. In addition, a direct link between FBXW7 and SREBP1a was established.

In conclusion, our data indicated that FBXW7 inhibits bladder cancer cells, likely through activating GSK3 phosphorylation and suppressing SREBP1a expression. Zhu *et al.* confirmed that p65 overexpression can inhibit the transcription of PTEN, thus stabilizing the protein expression of FBXW7, thereby increasing the ubiquitination and degradation of RhoGDI α , and ultimately promoting the migration of HUMAN BC (30). This study proved that FBXW7 inhibits the expression of SREBP1a by promoting the activation of P-GSK3 β . This suggests that FBXW7 is regulated in different directions in cancer and has different effects on cancer. Therefore, this study enriched the mechanism of FBXW7's effect on cancer, and provided new ideas for FBXW7 as a target selection.

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Footnote

Reporting Checklist: The authors have completed the ARRIVE reporting checklist. Available at <https://tau.amegroups.com/article/view/10.21037/tau-22-146/rc>

Data Sharing Statement: Available at <https://tau.amegroups.com/article/view/10.21037/tau-22-146/dss>

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at <https://tau.amegroups.com/article/view/10.21037/tau-22-146/coif>). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All animal experiments were approved by the Ethics Committee of the First Affiliated Hospital of Nanchang University (approval No. 20170312), in compliance with the institutional guidelines of First Affiliated Hospital of Nanchang University for the care and use of animals.

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