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RFWD3 acts as a tumor promotor in the development and progression of bladder cancer

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Summary. Background. Bladder cancer is one of the most commonly diagnosed malignancies of the urinary system with relatively poor prognosis and insufficient treatment strategies. RFWD3 is an E3 ligase whose function is rarely investigated in malignant tumors.

Methods. A tissue microarray was used for evaluating RFWD3 expression in clinical samples and its correlation with tumor characteristics and patients' prognosis. RFWD3 knockdown and overexpression cell models were constructed for conducting loss-of-function and gain-of-function assays. qPCR and western blotting were used for detecting mRNA and protein levels of RFWD3, respectively. MTT assay, colony formation assay, flow cytometry, wound-healing assay and transwell assay were carried out to demonstrate the change of cell phenotypes upon RFWD3 knockdown.

Results. RFWD3 expression was relatively higher in bladder cancer tissues than in normal tissues, which is correlated with higher N stage and poorer prognosis of patients. Knockdown of RFWD3 in bladder cancer cells significantly inhibited cell proliferation, colony formation, promote cell apoptosis and restrained cell migration. Overexpression of RFWD3 induced the opposite effects.

Conclusions. It was illustrated that RFWD3 possesses excellent tumor-promoting ability in bladder cancer. Accordingly, RFWD3 may be a promising therapeutic target in the targeted therapy of bladder cancer, which is worth further research.

Key words: Bladder cancer, RFWD3, Phenotypes, Tumor promoter

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Introduction

Bladder cancer is a common malignancy worldwide. According to histological classification, bladder cancer can be divided into bladder adenocarcinoma, squamous cell carcinoma and bladder urothelial carcinoma, of which bladder urothelial carcinoma accounts for more than 90% of all cases (Tran et al., 2021). Statistics released by GLOBOCAN in 2018 showed that the number of patients newly diagnosed with bladder cancer worldwide was about 550,000 in 2018, and 200,000 patients died, more than half of which appeared in Asia (Bray et al., 2018). Moreover, the incidence and mortality of bladder cancer have been increasing worldwide in recent years (Richters et al., 2020). Currently, treatment modalities for bladder cancer include surgical therapy, chemotherapy, targeted therapy, as well as immunotherapy. Among them, surgical treatment is still the most important and effective method, which greatly reduces the risk of metastasis and recurrence of bladder cancer with the help of adjuvant therapies (Crabb and Douglas, 2018; Lenis et al., 2020; Richters et al., 2020). In recent years, the advent of targeted therapy and immunotherapy has shed new light on cancer patients. Targeted drugs have been developed with the continuous efforts of researchers, such as the erdatini targeting fibroblast growth factor receptor (FGFR), bevacizumab and raltuzumab targeting the inhibition of tumor angiogenesis, and the immunotherapeutic drug BCG. Despite the major advances in the diagnosis and treatment of bladder cancer, the 5-year survival rate is less than 70% even after surgery, because the bladder is relatively prone to recurrence (Audenet et al., 2018; Hindy et al., 2019; Siracusano et al., 2020). Therefore, it is urgently necessary to further explore the mechanism of bladder cancer development to explore new molecular targets as well as treatments and maximize patient prognosis.

RING finger with WD repeat domain 3 (RFWD3) was originally screened and identified from the proteomics of ATM/ATR substrates. RFWD3 is composed of a ring finger domain, an SQ-rich region, a

coiled helical domain, and three WD40 domains (Dubois et al., 2017). When cells are subjected to ionizing radiation, DNA damage occurs leading to replication arrest, at which point some of the corresponding SQ sites of RFWD3 are phosphorylated. As a substrate for the checkpoint kinase ATM/ATR, RFWD3 responds to DNA damage by phosphorylation (Dubois et al., 2017). After DNA damage, RFWD3, which possesses *in vitro* E3 ubiquitination ligase activity, affects p53 stability by forming a complex with MDM2 and p53 through its E3 domain, implying that RFWD3 may have the nature of a tumor suppressor (Fu et al., 2010). However, in fact, RFWD3 was shown to be elevated in some types of malignant tumors by some recent reports, which has a certain promoting effect on tumor progression (Jia et al., 2020; Zhang et al., 2020a). Moreover, to the best of our knowledge, the regulation of bladder cancer by RFWD3 has not been reported.

In this study, for the purpose of evaluating the role of RFWD3 in the development and progression of bladder cancer, RFWD3 knockdown cell models were constructed for carrying out loss-of-function experiments. Accordingly, the effects of RFWD3 knockdown on cell proliferation, cell apoptosis, cell cycle and cell migration were assessed and indicated that the downregulation of RFWD3 in bladder cancer cells could inhibit the malignant phenotypes of cells and promote cell apoptosis, identifying RFWD3 as a tumor promoter and a promising therapeutic target of bladder cancer.

Materials and methods

Patient samples and immunohistochemical (IHC) staining

79 clinical tissue samples including 53 tumor tissues and 16 para-carcinoma normal tissues were collected and used for preparing a tissue microarray. Informed written consent was obtained from patients of all tissue samples, and ethical approval was obtained from Clinical Research Ethical Committee of the First Affiliated Hospital, Zhejiang University School of Medicine. The tissue samples were deparaffinized and rehydrated, and antigen was retrieved by citric acid buffer at 100°C for 10 min before staining. Primary antibody anti-RFWD3 (1:100, Cat. 19893-1-AP, proteintech) was used for incubating overnight at 4°C. After washing by PBS, secondary antibody was added, and then slices were dyed with DAB and hematoxylin for photographing. Staining percentage scores were ranked as 1 (1-25%), 2 (26-50%), 3 (51-75%) and 4 (76-100%), and staining intensity was graded 0 (weak color) to 3 (light yellow to strong brown). The final IHC scores were calculated by staining percentage and intensity.

Cell culture

Human bladder cancer cell lines EJ, RT4, J82 and T24 were purchased from ATCC. EJ and T24 cells were cultured in 90% RPMI-1640 with 10% fetal bovine serum (FBS), RT4 were cultured in McCoy's 5a Medium Modified growth medium containing 10% FBS. Four cell lines were grown in a cell incubator with 5% CO_2 at 37°C.

Infection of target cells by lentivirus

For overexpression, the RFWD3 construct was generated by subcloning human MPS-1 cDNA into vector (Shanghai Yibeirui Biomedical Science and Technoloty Co., Ltd, Shanghai, China). The short hairpin RNA for RFWD3 was constructed to explore the cellular function of RFWD3, and the RNA interference target sequences were as follow: 5'-GGGAGAAGACACTGT GGAGAA-3' for Human-RFWD3-1, 5'-TGAGAAC AAGAAGGAGGGTAT-3' for Human-RFWD3-2, and 5'-AAGGAAACAGGCCGAGTTAGA-3' for Human-RFWD3-3. Next, DNA oligo sequence was synthesized. BR-V108 virus vector was linearized with AgeI (NEB, #R3552L) and EcoRI (NEB, #R3101L) and linked with double-Stranded DNA oligo using Fermentas T4 DNA Ligase reaction system. The conjugated products were transformed into E. coli competent cells and cultured for clone formation; positive clones were identified by PCR. EndoFree Maxi Plasmid Kit was applied for plasmid collection and qualified plasmid was used for virus packaging. Qualified lentivirus with shRFWD3 was prepared for following experiments.

Infection of target cells by lentivirus

In this experiment, EJ and T24 cells (2×10^5 cells) for down-regulating or overexpression RFWD3 level were infected by lentivirus with corresponding lentiviruses (1×10^8 TU/mL× 40 mL) at a MOI of 20. For 72h culturing, the fluorescence of cells was observed by microscope for verification.

qRT-PCR

Total RNA from infected EJ and T24 cells and corresponding control groups were extracted separately, using Trizol reagent (Sigma, MO). Next, cDNA was obtained by reverse transcription using Promega M-MLV Kit (Promega Corporation, WI). SYBR Green Mastermixs Kit (Vazyme, Nanjing, China) was used for real-time qPCR with GAPDH as a housekeeping gene. The relative expression level of RNA was calculated by the $2^{-\triangle\triangle Ct}$ method. The primer sequences applied in qPCR: Upstream primer sequence for RFWD3: 5'-AGTTGGCGTAGGTGCATTCG-3', downstream primer sequence for RFWD3: 5'-AGCCATCACTGAAACCAG CA-3'; Upstream primer sequence for GAPDH: 5'-TGACTTCAACAGCGACACCCA-3', downstream primer sequence for GAPDH: 5'-CACCCTGTTGC TGTAGCCAAA-3'.

Western blotting assay

EJ and T24 cells were lysed for total proteins

extraction. Next, total proteins were quantified using the BCA Method. 20μg proteins were segregated by 12% SDS-PAGE and PVDF membrane, then PVDF membrane was blocked with 5% skimmed milk TBST solution. After blocking, anti-RFWD3 (biorbyt, #orb304552, 1:2000) and anti-GAPDH (Bioworld, #AP0063, 1:3000) primary antibodies were added and incubated with proteins overnight at 4°C. After the complex was washed with TBST solution 3 times for 10 min each, the Goat Anti-Rabbit (Beyotime, #A0208, 1:3000) secondary antibody was added. The protein signals were evaluated by enhanced chemiluminescence detection system.

MTT assay

The proliferation capacity of infected EJ and T24 cells was assessed by MTT assay. 1500 cells per well were seeded into 96-well plates and cultured for five days. 20 μL 5 mg/L MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide) solution was added in each well. After four hours' reaction, the culture medium was removed and 100 μL dimethyl sulfoxide (DMSO) was added to dissolve Formazan crystal. Finally, OD value at 490/570 nm was detected by enzyme reader.

Colony formation assay

The colony formation assay was performed to assess the long-term growth of EJ and T24 cells with RFWD3 knockdown or overexpression. Transfected cells (400 cells/well) were plated into 6-well plates and incubated for 14 days. The colonies were fixed with paraformaldehyde for 30 min and washed with PBS. Then Giemsa staining solution was added for 20 min of cell staining. Finally, the colonies were washed and photographed for counting colonies.

Cell apoptosis assay

Cell apoptosis analysis was carried out by Annexin V-APC/PI staining. Infected EJ and T24 cells were harvested at 1300 rpm for 5 min and washed with 4°C pre-cold D-Hanks (pH=7.2~7.4). Then cells were resuspended with 1×binding buffer and stained with Annexin V-APC for 15 min lightless. After further staining with PI, the cells were subjected to a FACSCalibur Flow Cytometry to evaluate the apoptosis level.

Wound-healing assay

Infected EJ and T24 cells were (5×10^4 cells/well) were seeded into 96-well plates. When cell confluence reached 90%, low-concentration fetal bovine serum (Cat. No. A11-102, Ausbian) was added. 96 wounding replicators (VP scientific) were used to make scratches on cell layer. Cell debris were washed, and culture continued in an incubator with 5% CO_2 at 37°C 8h. Photographs were captured by a fluorescence microscope at 0h, 4h and 8h. Finally, the migration area was analyzed to evaluate the migration rate.

Transwell assay

Transwell assay (3422 corning) was used for the migration assay. Infected EJ and T24 cells were resuspended in a low serum medium and were seeded on the upper chambers without serum. In the lower chamber 600 μL culture medium containing 30% FBS was added (with serum). After 24h, the non-metastatic cells in the upper chamber were gently removed by a cotton swab with PBS. Then the lower chamber was immersed in the staining solution with crystal violet for 5 min. After washing and drying, photos were taken under the microscope.

Statistical analysis

All experiments were in triplicate and data were analyzed by using GraphPad Prism 8 (San Diego, CA, USA). The differences were analyzed using Student's t-test between two groups. $P<0.05$ was considered to be significantly different.

Results

RFWD3 is upregulated in bladder cancer tissues

First of all, in order to reveal the expression pattern of RFWD3 in bladder cancer, a tissue microarray containing 53 tumor tissues and 16 para-carcinoma normal tissues was used for IHC staining. As shown in Fig. 1A and statistically analyzed in Table 1, it was demonstrated that RFWD3 expression is significantly upregulated in tumor tissues compared with that in normal tissues. Moreover, the correlation analysis taking tumor characteristics into account indicated the positive relationship between high RFWD3 expression and more advanced N stage (Fig. 1A, Table 2). Furthermore, the results of Kaplan-Meier survival analysis displayed the poorer prognosis of patients with relatively higher RFWD3 expression (Fig. 1B). All the above results provide elementary evidence about the tumor-promoting role of RFWD3 in bladder cancer.

Table 1. Expression patterns of RFWD3 in bladder cancer tissues and normal tissues revealed in immunohistochemistry analysis.

RFWD3 expression	Tumor tissue		Normal tissue	
	Cases	Percentage	Cases	Percentage
Low	28	52.8%	16	100%
High	25	47.2%	0	-

$P<0.001$.

Construction of RFWD3 knockdown cell models

For carrying out loss-of-function experiments to visualize the functions of RFWD3 in the development and progression of bladder cancer, we planned to evaluate the phenotypes of bladder cancer cells with or without RFWD3 knockdown. As the initial stage, the existence of RFWD3 in a variety of bladder cancer cell lines including RT4, T24, EJ and J82 was verified by qPCR (Fig. 2A). Subsequently, 3 shRNAs were designed using RFWD3 as the target, prepared and packaged into lentivirus for cell infection. As shown in Fig. 2B, the

Table 2. Relationship between RFWD3 expression and tumor characteristics in patients with bladder cancer.

Features	No. of patients	RFWD3 expression		P value
		low	high	
All patients	53	28	25	
Gender				0.295
Male	46	23	23	
Female	7	5	2	
Lymphatic metastasis (N)				0.014 (Spearman's ρ = 0.341)
N0	45	27	18	
N1	8	1	7	

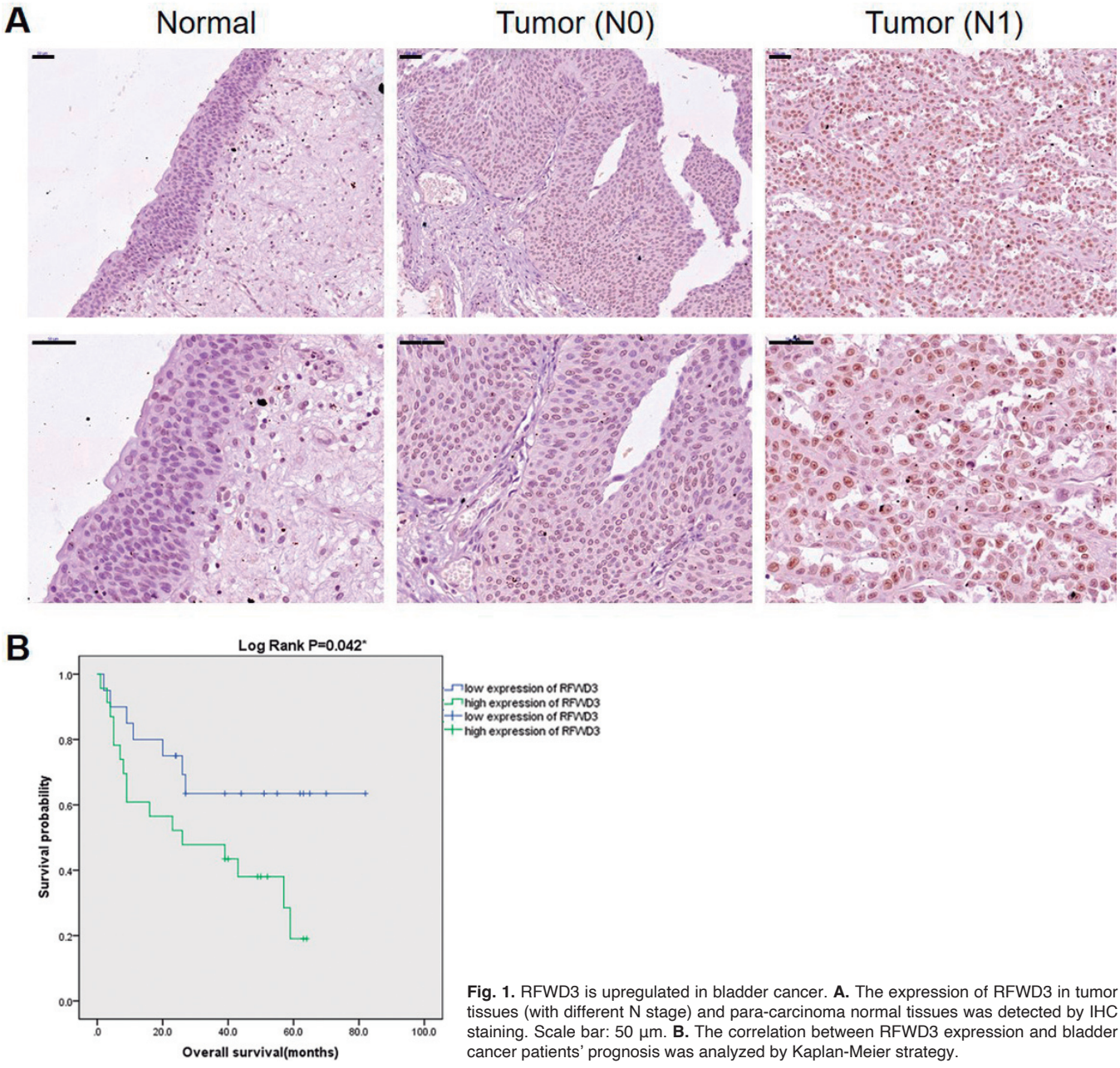

Fig. 1. RFWD3 is upregulated in bladder cancer. **A.** The expression of RFWD3 in tumor tissues (with different N stage) and para-carcinoma normal tissues was detected by IHC staining. Scale bar: 50 μm. **B.** The correlation between RFWD3 expression and bladder cancer patients' prognosis was analyzed by Kaplan-Meier strategy.

knockdown efficiencies of RFWD3 by the 3 shRNAs were assessed by qPCR and showed shRFWD3-3 as the most efficient one, which was used in the following experiments without further explanation. In the formal experiments, T24 and EJ cell lines were infected with shRFWD3 for constructing RFWD3 knockdown cell models. An infection efficiency of >80%, as detected by fluorescence imaging, was considered to be successful (Fig. 2C). The validity of the cell models was also guaranteed by both qPCR and western blotting. As shown in Fig. 2D, all the results showed the dramatical downregulation of RFWD3 in shRFWD3 groups, as well as the successful construction of cell models.

Knockdown of RFWD3 inhibits cell proliferation of bladder cancer cells

Rapid proliferation is an important feature of cancer cells. Therefore, the effects of RFWD3 knockdown on bladder cancer cell proliferation were first examined. As shown in Fig. 2E, bladder cancer cells with relatively low expression of RFWD3 possessed significantly lower

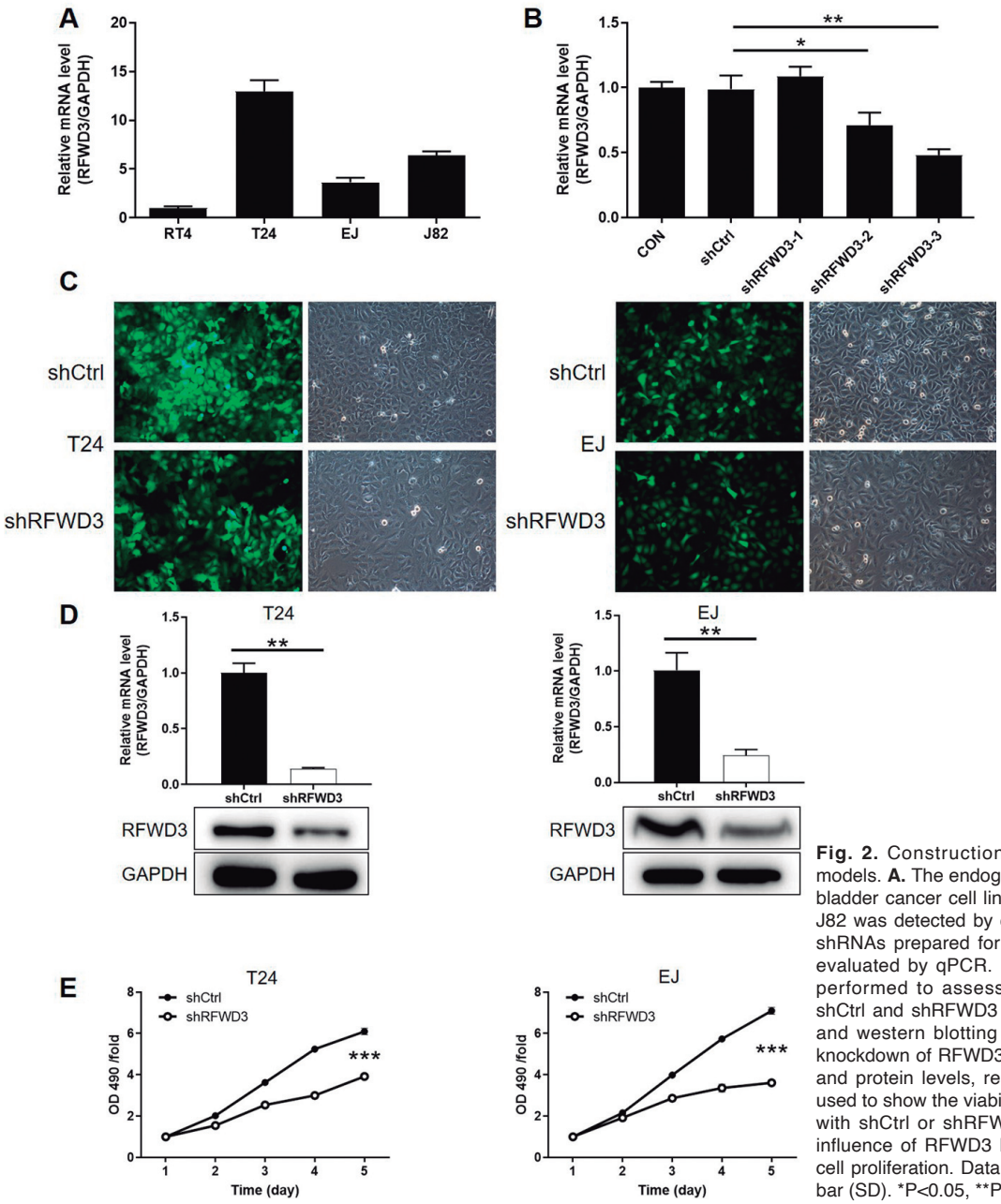

Fig. 2. Construction of RFWD3 knockdown cell models. **A.** The endogenous expression of RFWD3 in bladder cancer cell lines including RT4, T24, EJ and J82 was detected by qPCR. **B.** The efficiencies of 3 shRNAs prepared for knocking down RFWD3 were evaluated by qPCR. **C.** Fluorescence imaging was performed to assess the infection efficiencies of shCtrl and shRFWD3 in T24 and EJ cells. **D.** qPCR and western blotting were applied to examine the knockdown of RFWD3 in T24 and EJ cells on mRNA and protein levels, respectively. **E.** MTT assay was used to show the viability of T24 and EJ cells infected with shCtrl or shRFWD3 in 5 days, visualizing the influence of RFWD3 knockdown on bladder cancer cell proliferation. Data are shown as mean with error bar (SD). *P<0.05, **P<0.01, ***P<0.001.

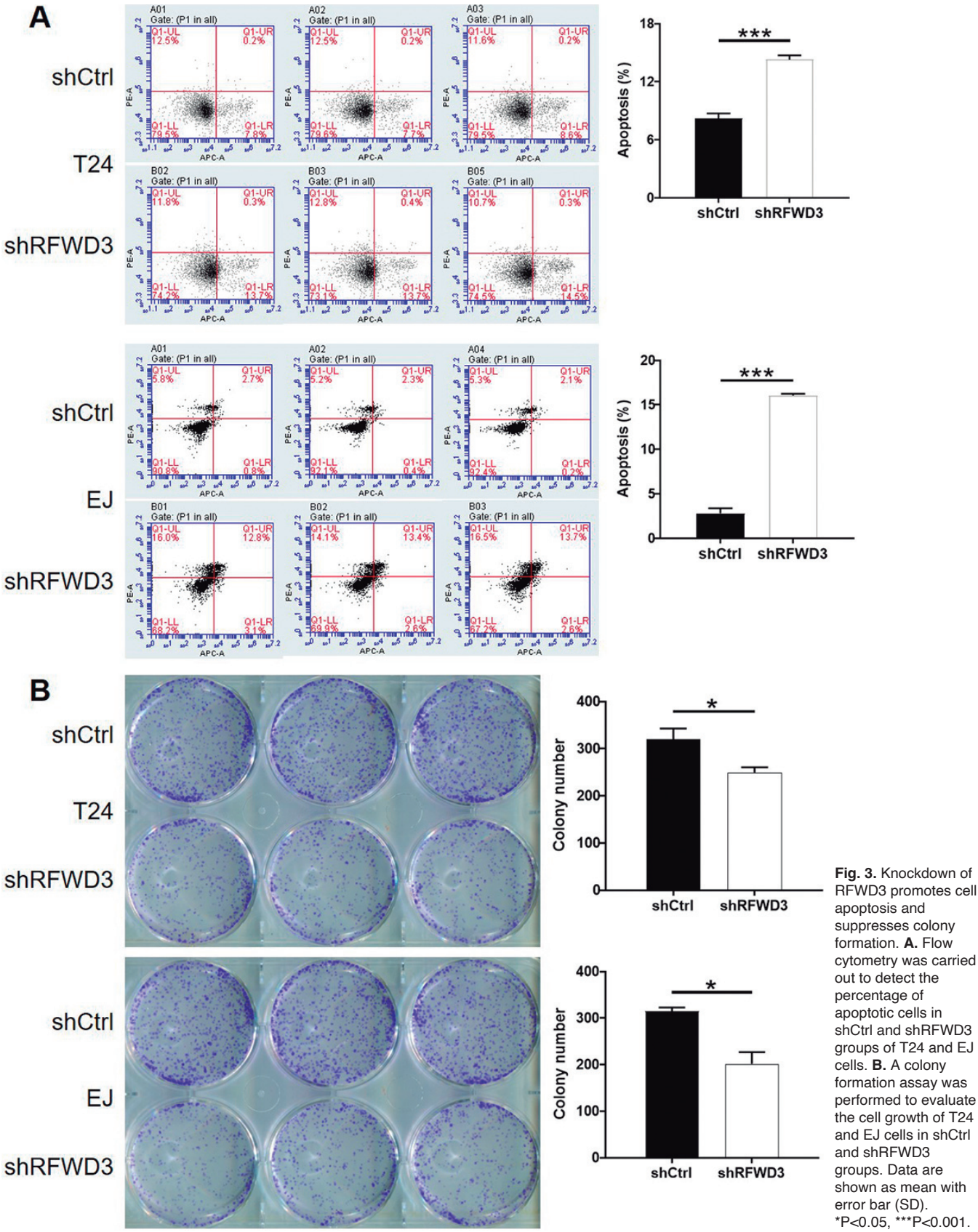

Fig. 3. Knockdown of RFWD3 promotes cell apoptosis and suppresses colony formation. **A.** Flow cytometry was carried out to detect the percentage of apoptotic cells in shCtrl and shRFWD3 groups of T24 and EJ cells. **B.** A colony formation assay was performed to evaluate the cell growth of T24 and EJ cells in shCtrl and shRFWD3 groups. Data are shown as mean with error bar (SD). *P<0.05, ***P<0.001.

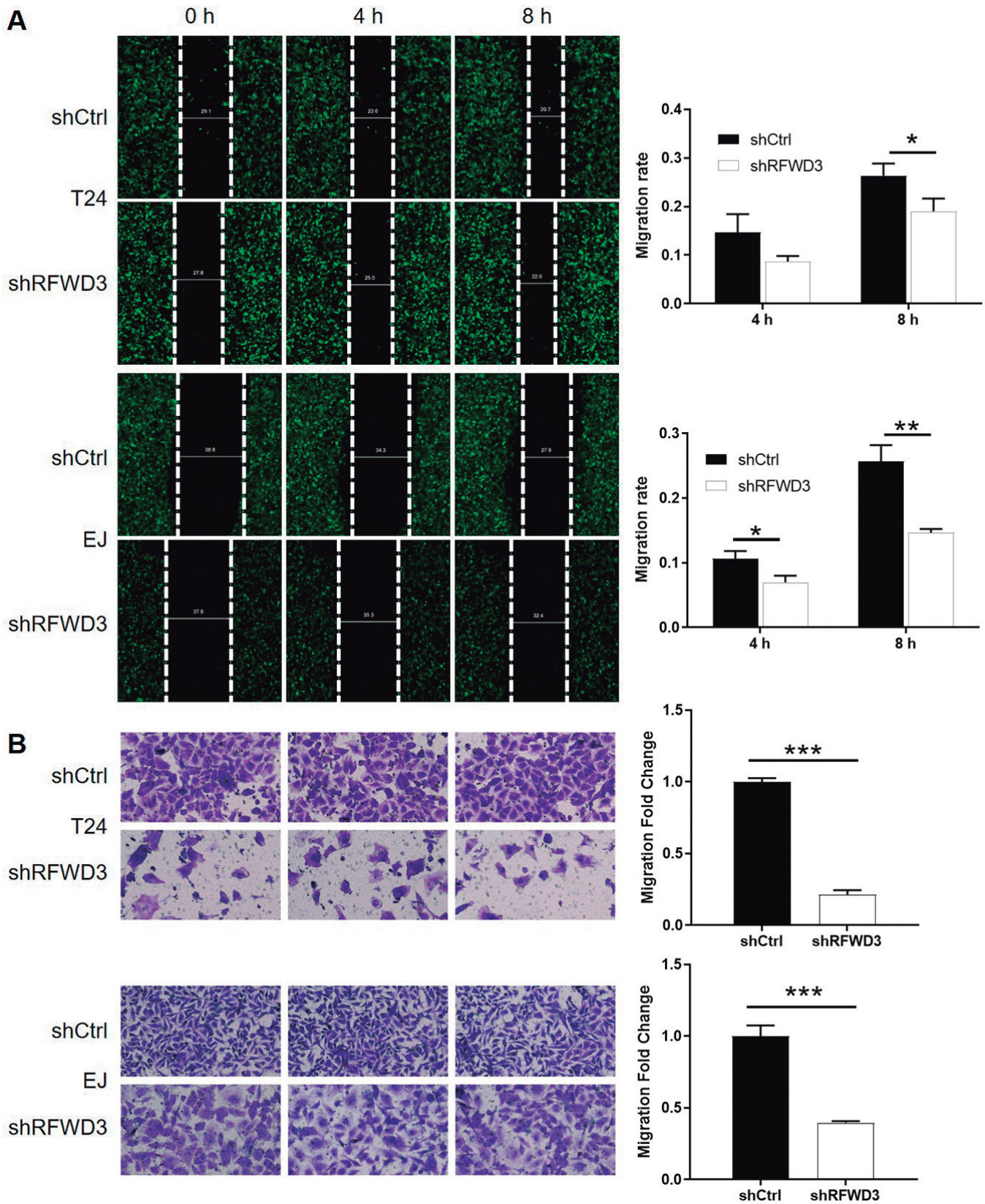

Fig. 4. Knockdown of RFWD3 inhibits cell migration of bladder cancer cells. Wound-healing assay **(A)** and Transwell assay **(B)** were both performed to test the effects of RFWD3 knockdown on bladder cancer cell migration. Data are shown as mean with error bar (SD). *P<0.05, ** P<0.01, ***P<0.001.

proliferative activity, suggesting the critical role of RFWD3 in the development of bladder cancer.

Knockdown of RFWD3 promotes cell apoptosis and suppresses colony formation

Subsequently, flow cytometry was performed to detect the cell apoptosis in T24 and EJ cells with or without RFWD3 knockdown. As shown in Fig. 3A, the percentage of apoptotic cells in shRFWD3 groups of T24 and EJ cells was significantly higher than in the shCtrl group, which explains the inhibition of cell proliferation by shRFWD3 to some extent. Moreover, the long-term growth rate of T24 and EJ cells with or without RFWD3 overexpression was evaluated by colony formation assay, indicating the significantly slower growth of cells with relatively lower expression of RFWD3 (Fig. 3B).

Knockdown of RFWD3 inhibits cell migration of bladder cancer cells

Tumor metastasis is another unpleasant characteristic of bladder cancer. So, we next tested the cell motility of T24 and EJ cells infected with shCtrl or shRFWD3. As shown in Fig. 4A, the results of wound-healing assay indicated the suppressed cell migration ability of bladder cancer cells with RFWD3 knockdown. Similar results were also obtained using transwell assay (Fig. 4B). Herein, our *in vitro* experiments showed that knockdown of RFWD3 could decrease the mobility of bladder cancer cells.

Overexpression of RFWD3 promotes development of bladder cancer in vitro

Although solid evidence has been provided by the loss-of-function assays to show the tumor-promoting feature of RFWD3, we further supplemented a series of gain-of-function assays for further verification. As shown in Fig. 5A,B, T24 and EJ cells with overexpressed RFWD3 were constructed and subjected to cell proliferation assay, indicative of the enhanced proliferation of cells with increased expression of RFWD3. Moreover, in contrast to the elevated cell apoptosis and suppressed colony formation and cell migration upon RFWD3 knockdown, RFWD3 overexpression decreased the percentage of apoptotic cells (Fig. 6A), while increasing colony formation (Fig. 6B) and cell migration abilities of bladder cancer cells (Fig. 7).

Discussion

Bladder cancer is one of the most commonly diagnosed malignancies of the urinary system with

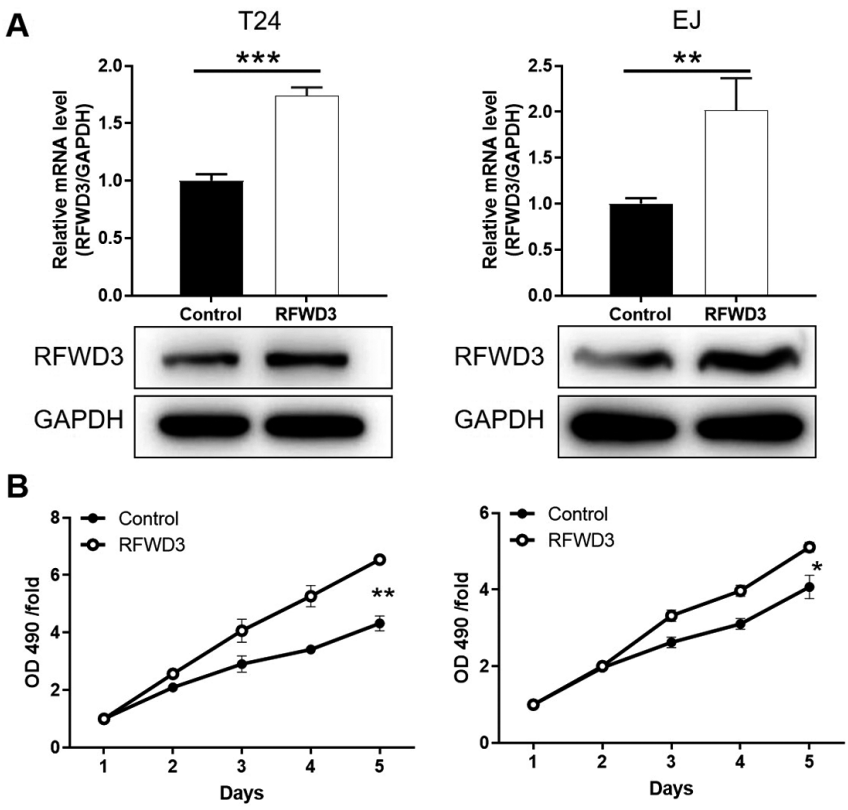

Fig. 5. Construction of RFWD3 overexpression cell models. **A.** qPCR and western blotting were applied to examine the overexpression efficiencies of RFWD3 in T24 and EJ cells on mRNA and protein levels, respectively. **B.** MTT assay was used to show the viability of T24 and EJ cells with or without RFWD3 overexpression in 5 days, visualizing the influence of RFWD3 overexpression on bladder cancer cell proliferation. Data are shown as mean with error bar (SD). *P<0.05, **P<0.01, ***P<0.001.

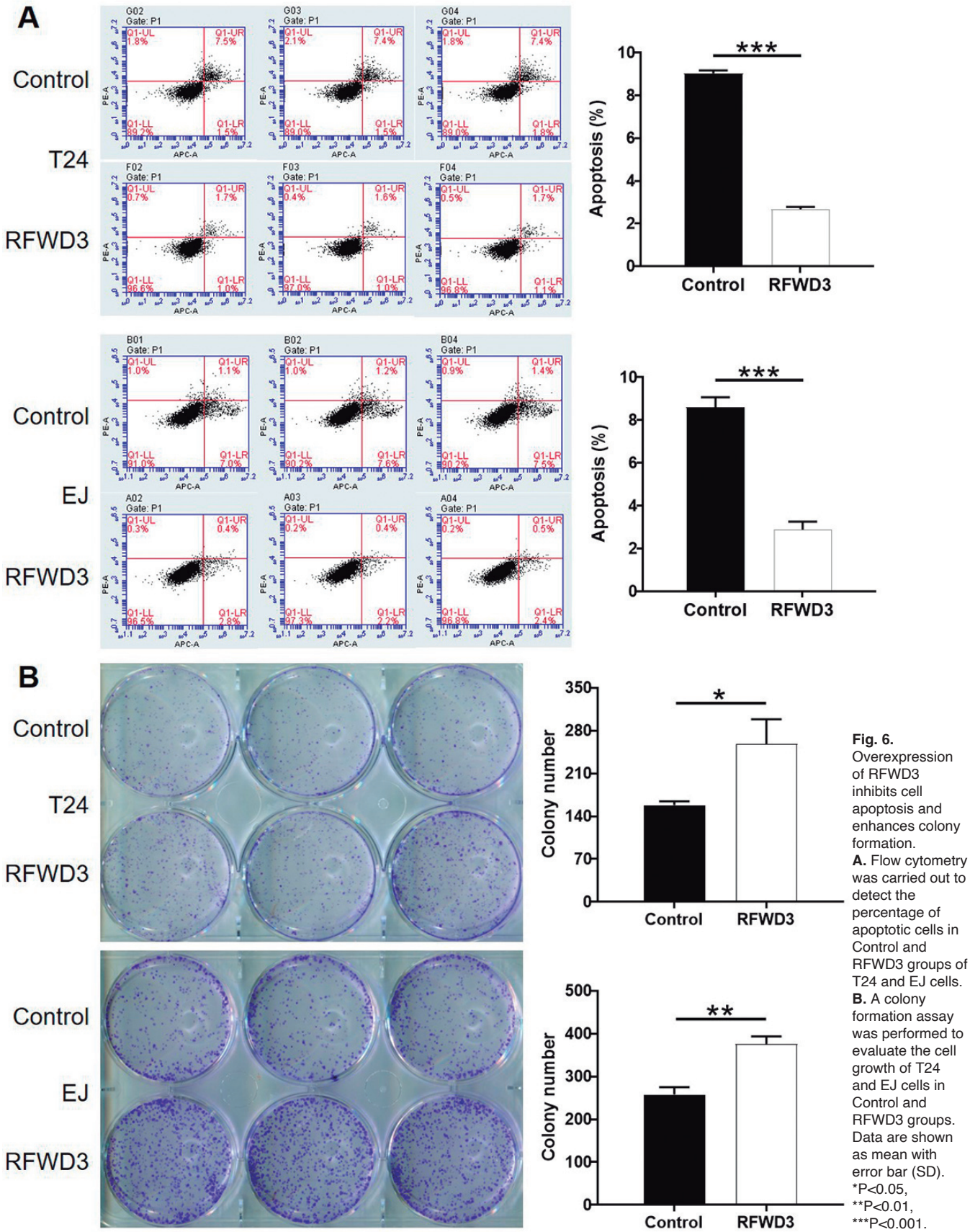

Fig. 6. Overexpression of RFWD3 inhibits cell apoptosis and enhances colony formation. **A.** Flow cytometry was carried out to detect the percentage of apoptotic cells in Control and RFWD3 groups of T24 and EJ cells. **B.** A colony formation assay was performed to evaluate the cell growth of T24 and EJ cells in Control and RFWD3 groups. Data are shown as mean with error bar (SD). *P<0.05, **P<0.01, ***P<0.001.

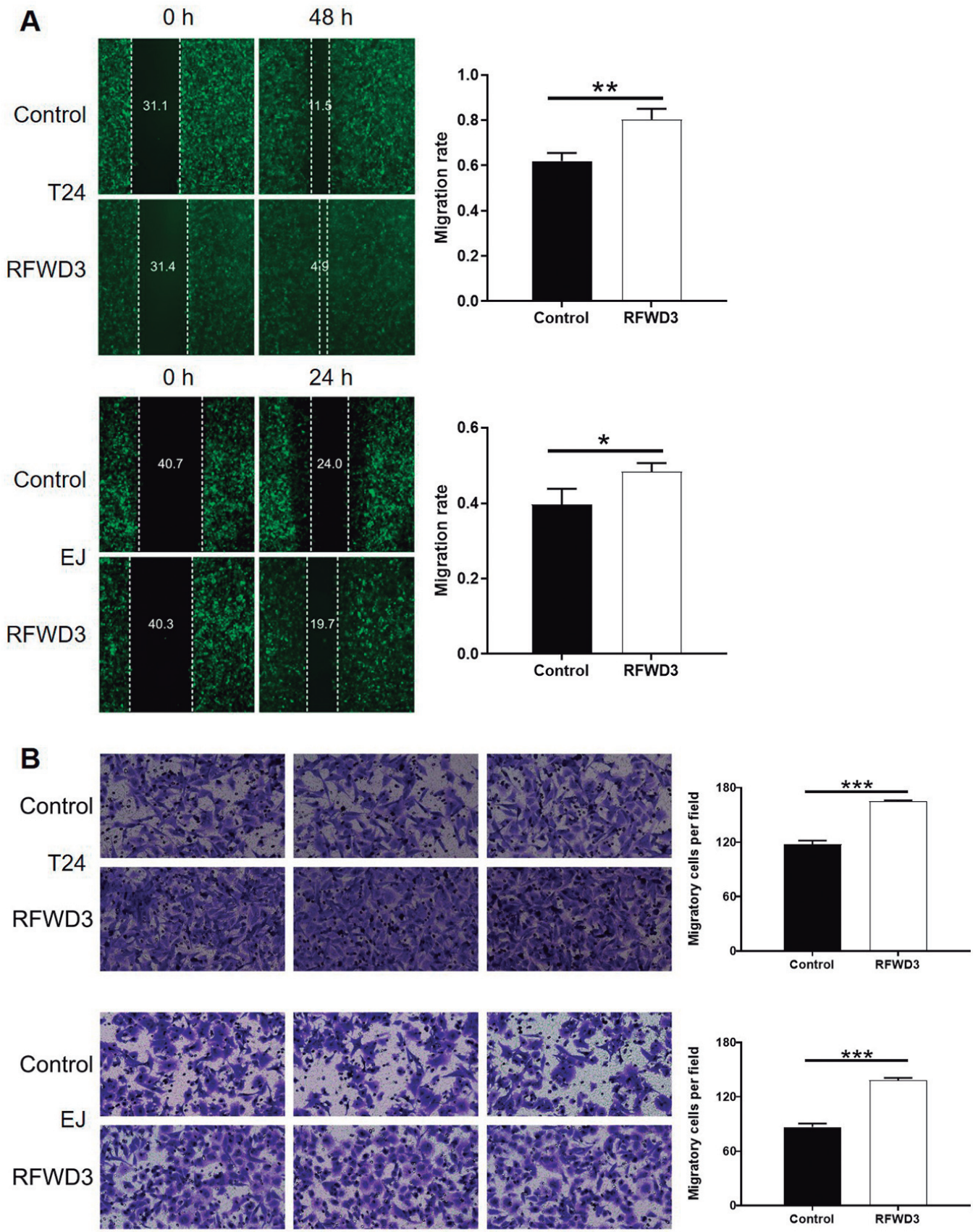

Fig. 7. Overexpression of RFWD3 promotes cell migration of bladder cancer cells. Wound-healing assay **(A)** and Transwell assay **(B)** were both performed to test the effects of RFWD3 overexpression on bladder cancer cell migration. Data are shown as mean with error bar (SD). *P<0.05, **P<0.01, ***P<0.001.

relatively poor prognosis and insufficient treatment strategies. In recent years, with the progress of genomics technology and molecular biology research technology, the discovery of more and more key molecules in tumor development has facilitated the emergence of various molecular targeted therapies (Alifrangis et al., 2019; Hindy et al., 2019). Despite this, the therapeutic efficiency of bladder cancer is still far from satisfying. Therefore, continuous intensive study of the molecular mechanisms of bladder cancer development is still of great significance and has been focused on by researchers. For example, a recent piece of work showed that transcriptional regulator YAP is overexpressed in bladder cancer and is a key activator of bladder cancer cell proliferation, which is mediated by the MAPK signaling pathway (Qiu et al., 2020). Zhang et al. demonstrated that a cell cycle regulator Cell division cycle 5-like (CDC5L) is highly expressed in bladder cancer tissues compared with normal tissues, which can trigger the acceleration of tumor development *in vitro* through promoting cell proliferation and colony formation (Zhang et al., 2020b). On the other hand, some non-coding RNAs such as circular RNA was also found to be able to regulate the progression of bladder cancer. For instance, Chen et al. recently reported that circ_0008532 can act as a tumor promoter of bladder cancer through promoting cell proliferation and cell migration/invasion and mechanistically regulating miR-155-5p/miR-330-5p/MTGR1 axis (Chen et al., 2020). Therefore, it could be concluded that the persistent exploration of novel key factors regulating development and progression of bladder cancer is of great clinical significance. Herein, our study revealed the role of RFWD3 in the development and progression of bladder cancer based on *in vitro* investigations.

At present, the functional research related to RFWD3 mainly focuses on its E3 ubiquitin ligase activity and the ubiquitination induced by RFWD3 (Gong and Chen, 2011; Lin et al., 2018; Duan et al., 2020; Hsu et al., 2020). According to a brand-new study, RFWD3 plays an important role in the ubiquitination of proteins in single-stranded DNA, the silence of which can lead to a profound defect in recruitment of key repair and signaling factors to damaged chromatin. Thus, RFWD3 is involved in the regulation of translesion DNA synthesis (Gong and Chen, 2011; Lin et al., 2018; Duan et al., 2020; Hsu et al., 2020). The study of Elia et al. showed that RFWD3 executes important functions in DNA damage repair by mediating ubiquitination of RPA and thus is responsible for homologous recombination repair at stalled forks (Elia et al., 2015). Otherwise, the functions of RFWD3 in stalled forks were also found to be related to its interaction with BRCA2 (Duan and Pathania, 2020). Recently, some researchers started to pay attention to the function of RFWD3 itself in the regulation of malignant tumors. Jia et al. discovered the overexpression of RFWD3 in gastric carcinoma and knocking it down might be an efficient strategy to slow down the development and progression of gastric cancer through negatively regulating malignant phenotypes of gastric cancer cells (Jia et al., 2020). Zhang et al. found the upregulation of RFWD3 in non-small cell lung cancer and its relationship with prognosis of patients (Zhang et al., 2020a). Recently, Xu et al. found that RFWD3 can enhance E2F1-mediated transcription of BIRC5/Survivin, thus promoting the progression of colorectal cancer (Xu et al., 2021). These results suggested that RFWD3 may be an important participator in the development and progression of malignant tumors. Therefore, this study was designed and carried out to fill the gap in the functional study of RFWD3 in bladder cancer.

Herein, out study aimed to explore the expression and function of RFWD3 in bladder cancer. First of all, the upregulation of RFWD3 in bladder cancer was established through IHC analysis of clinical tissue samples. The correlation between RFWD3 high expression and more advanced N stage and poorer prognosis was also observed. As the *in vitro* results indicated, RFWD3 was expressed in all tested bladder cancer cell lines. Knockdown of RFWD3 significantly inhibited cell proliferation, promoted cell apoptosis, induced cell cycle arrest in G2 phase and suppressed cell migration ability of bladder cancer cells. Moreover, RFWD3 overexpression induced the opposite effects. A current deficiency of this study, which is also the different from other works, lies in the temporary lack of exploration of downstream mechanisms, especially the correlation between the E3 ubiquitin ligase activity of RFWD3, and its bladder cancer regulatory ability has not been discussed. In fact, this work is only the beginning of our study on the function of RFWD3 in bladder cancer, and our subsequent study will deeply explore its intrinsic mechanism and elucidate the mechanism of action.

In summary, RFWD3 was identified as a key regulator in the development and progression of bladder cancer, which affected cell phenotypes *in vitro*. Therefore, RFWD3 may be considered as a potential therapeutic target in the treatment of bladder cancer.

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Author contributions. P. Jiang designed this program. Z. Xu and J. Sun operated the cell and animal experiments. X. Chen conducted the data collection and analysis. J. Sun and J. Tian produced the manuscript which was checked by S. Wu. All the authors have confirmed the submission of this manuscript.

Conflict of interest. The authors declare no conflict of interest.

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