

## MicroRNA-940 Targets INPP4A or GSK3 $\beta$ and Activates the Wnt/ $\beta$ -Catenin Pathway to Regulate the Malignant Behavior of Bladder Cancer Cells

Rong Wang, Yunfeng Wu, Weihua Huang, and Weijun Chen

Department of Urology, The Jintan Hospital Affiliated with Jiangsu University, Changzhou, Jiangsu, P.R. China

In this report, we aimed to explore the role and regulatory mechanism of microRNA-940 (miR-940) in bladder cancer development. The expressions of miR-940 in bladder cancer tissues and cells were measured. miR-940 mimics, miR-940 inhibitor small interference RNA against INPP4A (si-INPP4A), and GSK3 $\beta$  (si-GSK3 $\beta$ ) and their corresponding controls were then transfected into cells. We investigated the effects of miR-940, INPP4A, or GSK3 $\beta$  on cell proliferation, migration, invasion, and apoptosis. Additionally, target prediction and luciferase reporter assays were performed to investigate the targets of miR-940. The regulatory relationship between miR-940 and the Wnt/ $\beta$ -catenin pathway was also investigated. miR-940 was upregulated in bladder cancer tissues and cells. Overexpression of miR-940 significantly increased bladder cancer cell proliferation, promoted migration and invasion, and inhibited cell apoptosis. INPP4A and GSK3 $\beta$  were the direct targets of miR-940, and knockdown of INPP4A or GSK3 $\beta$  significantly increased cancer cell proliferation, migration, and invasion and inhibited cell apoptosis. After miR-940 overexpression, the protein expression levels of c-Myc, cyclin D1, and  $\beta$ -catenin were significantly increased, and the expression levels of p27 and p- $\beta$ -catenin were markedly decreased. The opposite effects were obtained after suppression of miR-940. XAV939, a tankyrase 1 inhibitor that could inhibit Wnt/ $\beta$ -catenin signaling, significantly reversed the effects of miR-940 overexpression on cell migration and invasion. Our results indicate that overexpression of miR-940 may promote bladder cancer cell proliferation, migration, and invasion and inhibit cell apoptosis via targeting INPP4A or GSK3 $\beta$  and activating the Wnt/ $\beta$ -catenin pathway. Our findings imply the key roles of suppressing miRNA-940 in the therapy of bladder cancer.

**Key words:** Bladder cancer; miR-940; INPP4A; GSK3 $\beta$ ; Wnt/ $\beta$ -catenin pathway

### INTRODUCTION

Bladder cancer is a common urological malignancy worldwide and has a poor prognosis<sup>1,2</sup>. Despite a wide range of treatment modalities, the 5-year survival rate for patients with advanced bladder cancer is approximately 20%–40%<sup>3–5</sup>. A poor understanding of the molecular mechanisms underlying advanced bladder cancer may be responsible for the lack of effective therapies for this disease<sup>6</sup>. Thus, intensive study into the molecular mechanism is essential for improving diagnosis, prevention, and treatment of bladder cancer.

There is increasing evidence that microRNAs (miRNAs) play a pivotal role in the control of tumorigenesis via post-transcriptional regulation of gene expression<sup>7–10</sup>. In bladder cancer, deregulation of miRNAs is found to participate in cell proliferation, cell cycle arrest, cell apoptosis, drug resistance, and other functions<sup>11,12</sup>. As reported, miR-218 can inhibit cell proliferation, migration, and invasion in bladder cancer via targeting BMI-1<sup>13</sup>. miR-99a is down-regulated in bladder cancer tissues and possesses the

ability to suppress tumors in bladder cancer<sup>14</sup>. miR-214 is also found to exert a tumor-suppressive effect by targeting PDRG1 in bladder cancer<sup>15</sup>. Notably, miRNAs are considered to be potential markers for the detection, diagnosis, prognosis, or therapeutic intervention in bladder cancer<sup>15–17</sup>. Therefore, identification of key miRNAs associated with bladder cancer has great significance. Recently, miR-940 has been found to be involved in the pathologic processes of many diseases, including hepatocellular carcinoma<sup>18</sup>, prostate cancer<sup>19</sup>, and chronic heart failure<sup>20</sup>. Interestingly, miR-940 levels are found to be concordant with disease progression in bladder cancer<sup>21</sup>. However, the role and regulatory mechanism of miR-940 in the development of bladder cancer remain unknown.

In this study, we investigated the expression of miR-940 in bladder cancer tissues and cells. We then investigated the effects of overexpression and suppression of miR-940 on cell proliferation, migration, invasion, and apoptosis. Additionally, target prediction and luciferase reporter assays were performed to investigate the

targets of miR-940. The regulatory relationship between miR-940 and the Wnt/ $\beta$ -catenin pathway was also investigated. The objective of our study was to explore the role and regulatory mechanism of miR-940 in bladder cancer development.

## MATERIALS AND METHODS

### Patients

From September 2009 to August 2014, a total of 44 patients who were diagnosed with bladder cancer in our hospital were enrolled. The tumor tissues and their adjacent nontumor tissues (>2.0 cm distance from tumor edge) were taken out, snap frozen with liquid nitrogen, and stored at  $-80^{\circ}\text{C}$  for subsequent analysis. This study was approved by the ethics committee board of our hospital, and each patient signed an informed consent.

### Cell Culture

Human normal urothelial cell line SV-HUC-1 and human bladder cancer cell lines T24, UM-UC-3, and J82 were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA) and then incubated in high-glucose Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) in a humidified atmosphere with 5%  $\text{CO}_2$  at  $37^{\circ}\text{C}$ .

### Cell Transfection

Mature miR-940 mimic, miR-940 inhibitor, small interference RNA against INPP4A (si-INPP4A), and GSK3 $\beta$  (si-GSK3 $\beta$ ) and their corresponding controls were designed and synthesized by GenePharma (Shanghai, P.R. China) and then transiently transfected into cells which were seeded onto six-well plates ( $5 \times 10^5$  cells/per well) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) for 48 h following the manufacturer's instructions. Untreated cells were considered to be the control group.

### Real-Time PCR

Using TRIzol reagent (Invitrogen, New York, NY, USA), total RNA was extracted from tissues and cells. These were then reverse transcribed into cDNA using an

M-MLV Reverse Transcriptase kit (Invitrogen) following the instructions provided by manufacturer. Real-time PCR was performed to detect gene expression using a standard SYBR Green PCR kit (Toyobo, Osaka, Japan) on Rotor-Gene RG-3000A (Corbett Life Science, Sidney, Australia). Using U6 and  $\beta$ -actin as references for miRNAs and mRNAs, respectively, the relative quantitation of gene expression levels was determined using the comparative threshold (Ct) cycle ( $2^{-\Delta\Delta\text{Ct}}$ ) method. The primers used for target amplification are shown in Table 1.

### Cell Viability Assay

Cell viability was assessed by an MTT assay. Briefly, at various times following different transfections, T24 cells ( $2 \times 10^3$  cells/per well) were plated into each well of a 96-well plate. Approximately 20  $\mu\text{l}$  of MTT (5 mg/ml; Sigma-Aldrich, St. Louis, MO, USA) was then added to each well and incubated for 4 h at  $37^{\circ}\text{C}$ . After centrifugation, 150  $\mu\text{l}$  of dimethyl sulfoxide was added to each well to dissolve the formazan precipitates. Absorbance of each well at 490 nm was then measured using an MRX II absorbance reader (DYNEX Technologies, Chantilly, VA, USA).

### Colony-Forming Assay

After 48 h of transfection, the cells were harvested, resuspended in RPMI-1640 medium containing 10% FBS, plated into six-well plates (400 cells/well), and maintained under standard culture conditions for 14 days. The colonies were fixed with absolute methanol and then stained with crystal violet for estimation. Colonies with a diameter over 2 mm were counted under a microscope (IX83, Olympus).

### Detection of Cell Apoptosis by Flow Cytometry

After 48 h of transfection, the cells were harvested, washed with prechilled PBS, and then resuspended in a binding buffer with a concentration of  $1 \times 10^6$  cells/ml. Double staining with annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) was then performed using the Annexin-V-FITC Apoptosis Detection Kit (BD Biosciences, San Jose, CA, USA) according to

**Table 1.** Primers Used for Target Amplification

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')
miR-940	CACACATCCGTCTGGGGCTAGG	CTACAGAATGCCCGCCGCTGCT
INPP4A	GGAGCAGGTGATGCTTAGAAA	ATGATGAAGCCGCAGATGAG
GSK3 $\beta$	TGGCAGCAAGGTAACCACAG	CGGTTCTTAAATCGCTTGTCCTG
Bax	CTGAGCTGACCTTGGAGC	GACTCCAGCCACAAAGATG
Bcl-2	CTGGTGGACAACATCGCTCTG	GGTCTGCTGACCTCACTTGTG
Pro-caspase 3	TGTCATCTCGCTCTGGTACG	AAATGACCCCTTCATCACCA
Cleaved-caspase 3	GGTATTGAGACAGACAGTGG	CATGGGATCTGTTTCTTTGC
$\beta$ -Actin	GCACCACACCTTCTACAATG	TGCTTGCTGATCCACATCTG

the manufacturer's protocol, followed by flow cytometric analysis for cell apoptosis using the BD LSR II Flow Cytometer System (Becton-Dickinson, San Jose, CA, USA) with FACSDiva Software within 1 h.

#### Transwell Assay for Assessing Cell Migration and Invasion

Transwell chambers (8- $\mu$ m pore size; Costar, Switzerland) enveloped with serum-free or without 1 mg/ml BD Matrigel Matrix (BD Biosciences) were used to determine cell invasion and migration, respectively. The lower compartments of the Transwell chambers were prepared by the addition of RPMI-1640 medium with 10% FBS as a chemoattractant. After 48 h of transfection, T24 cells ( $8 \times 10^4$ ) were suspended in fresh medium without FBS, added to the inserts of Transwell chambers, and cultured for 24 h. After removing the cells on the upper surface using cotton buds, the cells on the lower surface of the Transwell chambers were fixed and stained with 0.1% crystal violet. Cells that migrated and invaded in five randomly selected visual fields of 200 $\times$  magnification of each insert were counted under a light microscope.

#### Target Prediction and Luciferase Reporter Assay

Using TargetScanHuman, INPP4A and GSK3 $\beta$  were predicted as the potential targets of miR-940, which was then verified by luciferase reporter assay. Briefly, the 3'-UTRs of INPP4A or GSK3 $\beta$  containing the miR-940 binding sites were cloned downstream of the luciferase reporter in the pmirGLO Dual-Luciferase miRNA Target Expression Vector (Promega, Madison, WI, USA). The cells were plated into 24-well plates and cotransfected with 100 ng of pmirGLO Dual-Luciferase miRNA Target Expression Vector and 20 nM miRNA mimic or mimic

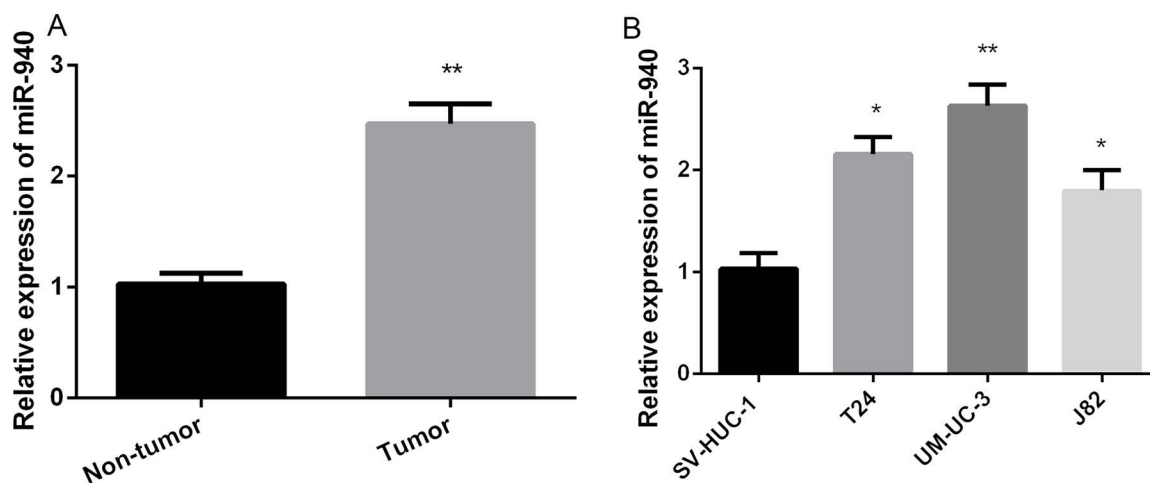
control. After 48 h of transfection, the cells were harvested for measuring of luciferase activity using the Dual-Glo Luciferase Assay Kit (Promega). *Renilla* luciferase activity was used as the internal control.

#### Western Blot

Cells were lysed with radioimmunoprecipitation assay (RIPA; Sangon Biotech, P.R. China) containing phenylmethanesulfonyl fluoride (PMSF; Sigma-Aldrich). After centrifugation at 12,000 bmp for 10 min, the supernatant was collected and the protein concentration was detected by the BCA protein assay kit (Pierce, Rockford, IL, USA). An equal amount of extracts was subjected to 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Darmstadt, Germany), and probed with primary antibodies to INPP4A, GSK3 $\beta$ , Bax, Bcl-2, pro-caspase 3, cleaved-caspase 3, c-Myc, cyclin D1, P27,  $\beta$ -catenin, p- $\beta$ -catenin, and GAPDH (1:1,000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4 $^{\circ}$ C. Followed by exposure to horseradish peroxidase-labeled appropriate secondary antibody (1:5,000 dilution; Santa Cruz Biotechnology) for 2 h, the blots were incubated with a chemiluminescent substrate kit (Pierce) and then detected using the enhanced chemiluminescence (ECL) method. GAPDH served as the internal control.

#### Statistical Analysis

All experiments were carried out in triplicate, and the data collected from multiple experiments were presented as the means  $\pm$  standard error of the mean (SEM). The differences between groups were measured using the Student's *t*-test and among groups were detected using one-way analysis of variance (ANOVA) followed



**Figure 1.** The relative expression of miR-940. (A) The relative expression of miR-940 in bladder cancer tissues and their adjacent nontumor tissues. (B) The relative expression of miR-940 in bladder cancer T24, UM-UC-3, and J82 cells and human normal urothelial SV-HUC-1 cells. \* $p < 0.05$ , \*\* $p < 0.01$ .

by least significant difference (LSD). All statistical analyses were conducted using SPSS 13.0 (SPSS, Chicago, IL, USA), and a value of  $p < 0.05$  was defined as statistically significant.

## RESULTS

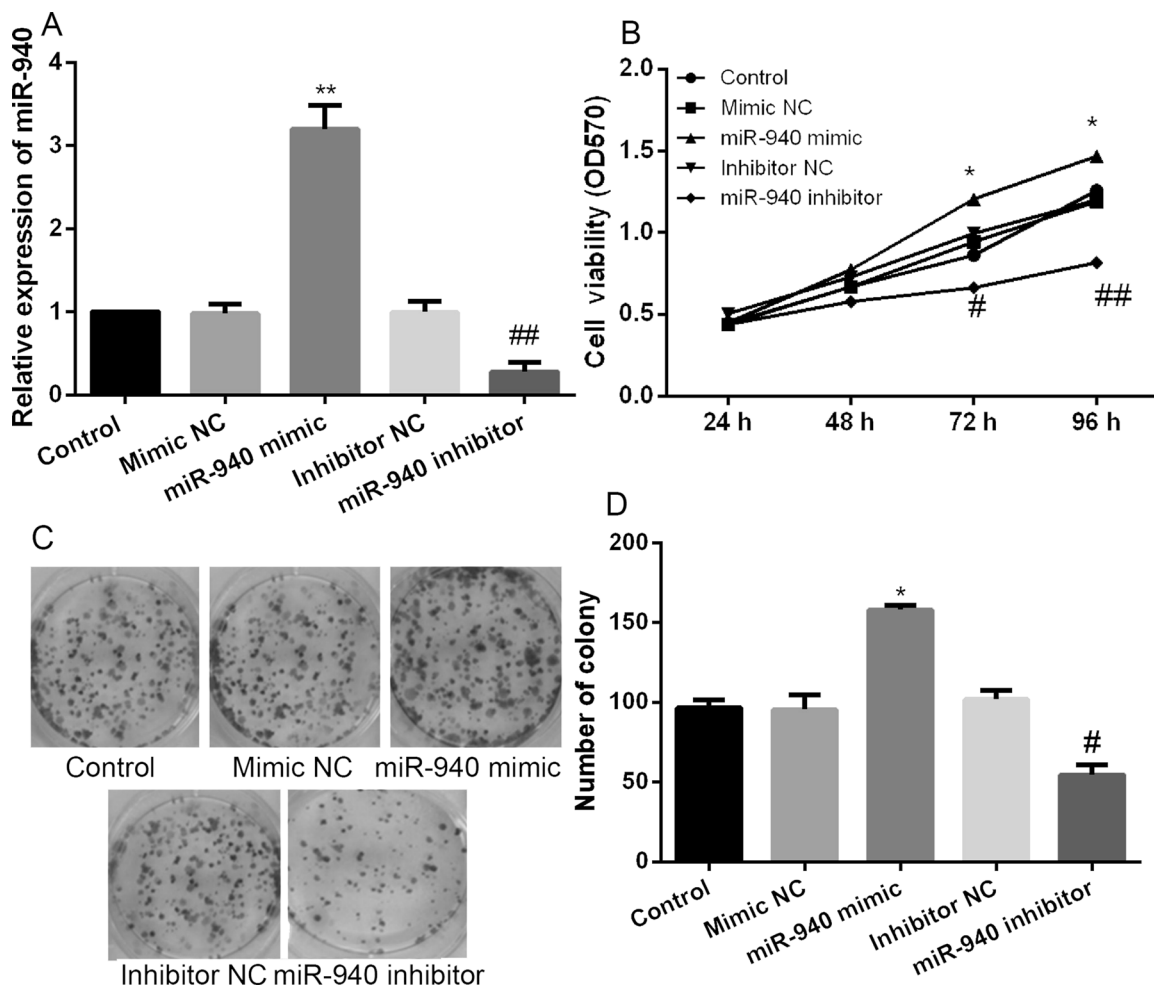
### *The Expression of miR-940 Was Upregulated in Bladder Cancer Tissues and Cells*

To investigate whether miR-940 played a key role in bladder cancer development, the expression of miR-940 in bladder cancer tissues and cells was detected. We found that miR-940 expression in bladder cancer tissues was significantly higher than in their adjacent nontumor tissues ( $p < 0.05$ ) (Fig. 1A). Moreover, the expression of miR-940 was upregulated in bladder cancer cells (T24, UM-UC-3, and J82) in comparison with human normal urothelial SV-HUC-1 cells ( $p < 0.05$ ) (Fig. 1B).

### *Overexpression of miR-940 Significantly Increased Bladder Cancer Cell Proliferation, Promoted Cell Migration and Invasion, and Inhibited Cell Apoptosis In Vitro*

To further investigate the key roles of miR-940 in bladder cancer development, bladder cancer T24 cells were transfected with miR-940 mimics and inhibitor. miR-940 expression in the miR-940 mimic group was significantly upregulated compared with that in the control or mimic NC groups ( $p < 0.05$ ) (Fig. 2A). The opposite expression changes of miR-940 were obtained in the miR-940 inhibitor group ( $p < 0.05$ ). These data indicated that miR-940 was successfully overexpressed and suppressed in bladder cancer T24 cells, which could be used for subsequent analysis.

We further detected the effect of miR-940 on bladder cancer cell proliferation. There was no significant

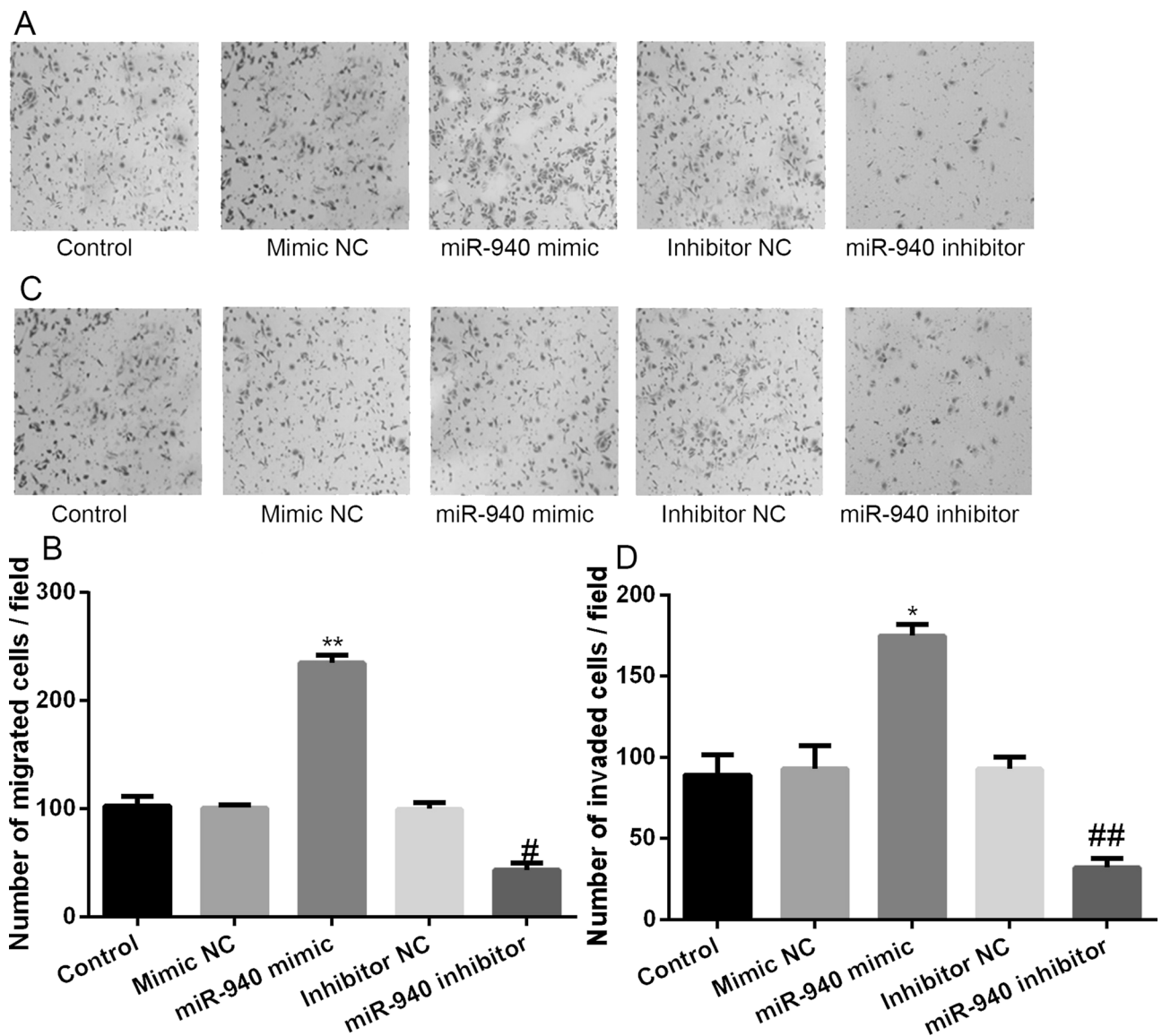


**Figure 2.** The effects of miR-940 on bladder cancer cell proliferation. (A) miR-940 expressions in different transfection groups. (B) The MTT assay showed the cell viability in the different transfection groups. (C, D) The colony-forming assay showed the number of colony in the different transfection groups. \* $p < 0.05$ , \*\* $p < 0.01$  compared to the mimic NC; # $p < 0.05$ , ## $p < 0.01$  compared to the Inhibitor NC.

difference in cell viability between the control and mimic NC groups, or between the control and inhibitor NC groups (Fig. 2B). However, in comparison with the control group, overexpression of miR-940 by transfection with the miR-940 mimic significantly increased cell viability, while suppression of miR-940 by transfection with the miR-940 inhibitor markedly decreased cell viability ( $p < 0.05$ ). In addition, consistent results were obtained from the colony-forming assays. Overexpression of miR-940 by transfection with the miR-940 mimic resulted in a significant increase in the number of colonies formed, while suppression of miR-940 by transfection with the miR-940 inhibitor had the opposite effect

( $p < 0.05$ ) (Fig. 2C and D). These data indicate that miR-940 significantly increased bladder cancer cell proliferation in vitro.

The effects of miR-940 on bladder cancer cell migration and invasion were also observed. Compared with the control or mimic NC groups, overexpression of miR-940 by transfection with the miR-940 mimic significantly promoted cell migration (Fig. 3A and B) and invasion (Fig. 3C and D), and the opposite effects were obtained after suppression of miR-940 by transfection with the miR-940 inhibitor ( $p < 0.05$ ) (Fig. 3A–D). These data indicate that miR-940 significantly promoted bladder cancer cell migration and invasion in vitro.



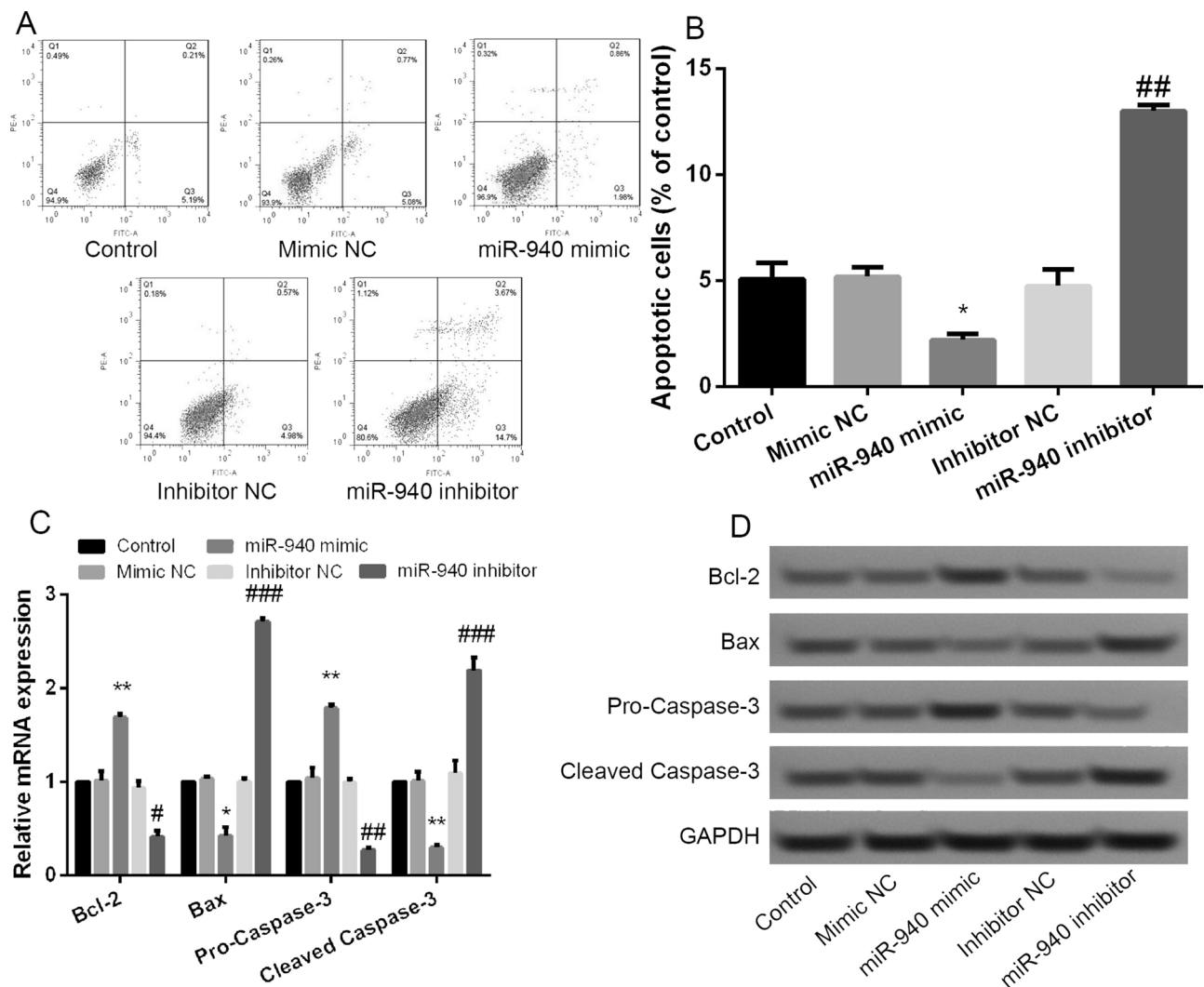
**Figure 3.** The effects of miR-940 on bladder cancer cell migration and invasion. (A, B) The Transwell assay showed the number of migrated cells in the different transfection groups. (C, D) The Transwell assay showed the number of invaded cells in the different transfection groups. \* $p < 0.05$ , \*\* $p < 0.01$  compared to the mimic NC; # $p < 0.05$ , ## $p < 0.01$  compared to the Inhibitor NC.



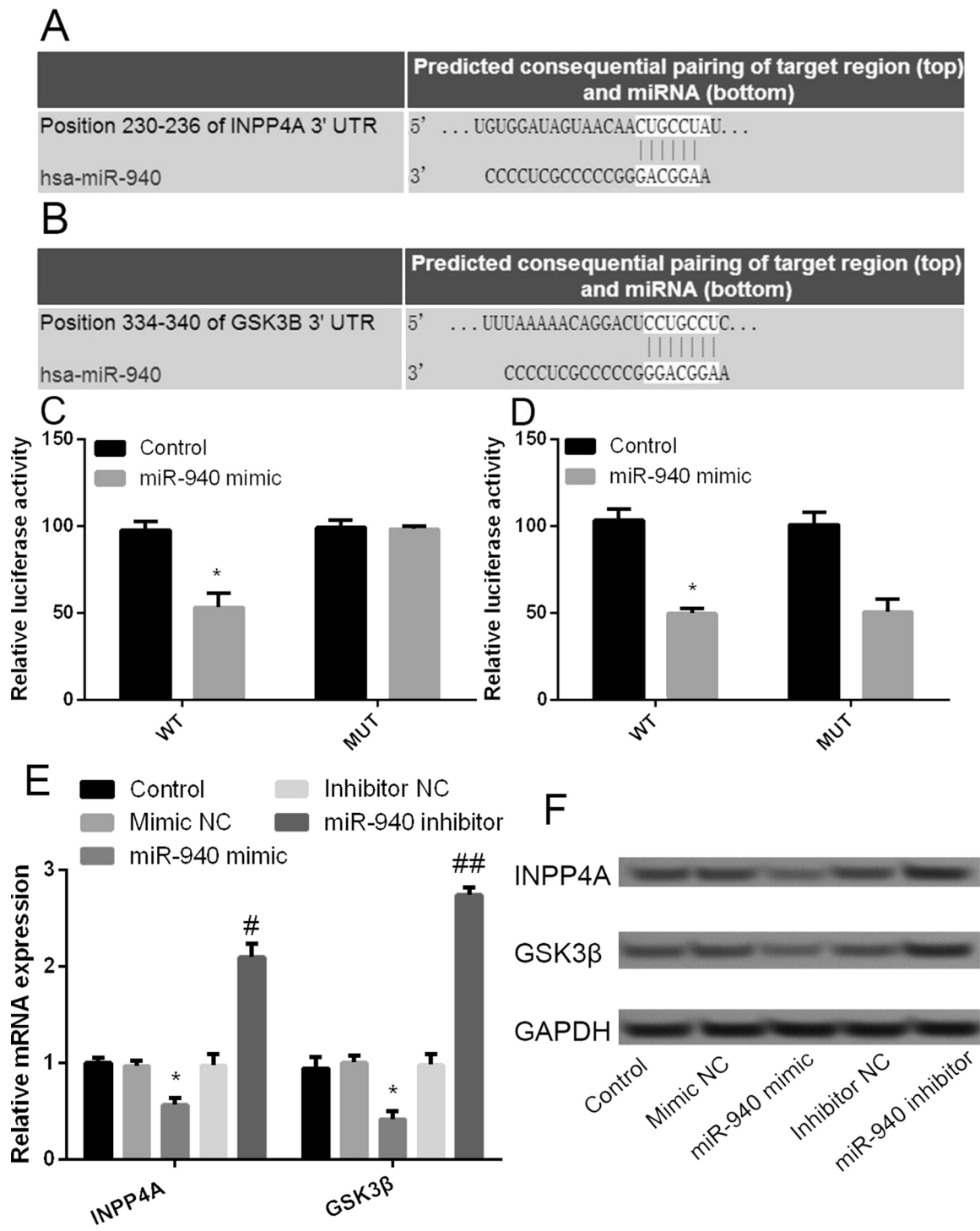
The effects of miR-940 on bladder cancer cell apoptosis were also measured. Overexpression of miR-940 by transfection with the miR-940 mimic significantly inhibited cell apoptosis compared with that in the control or mimic NC groups ( $p < 0.05$ ) (Fig. 4A and B). The expression levels of Bcl-2 and pro-caspase 3 in the miR-940 mimic group were significantly increased compared with those in the control or mimic NC groups, while the expression levels of Bax and cleaved caspase 3 were markedly decreased ( $p < 0.05$ ) (Fig. 4C and D). The opposite results were obtained after suppression of miR-940 by transfection with the miR-940 inhibitor (Fig. 4A–D). These data indicate that miR-940 significantly inhibited bladder cancer cell apoptosis in vitro.

#### *INPP4A and GSK3 $\beta$ Were the Direct Targets of miR-940*

According to the information from TargetScanHuman, INPP4A and GSK3 $\beta$  were predicted to be the potential targets of miR-940 (Fig. 5A and B). Dual-luciferase assays were conducted to verify the predicted results. We found that miR-940 could significantly inhibit the luciferase activity of the wild-type 3'-UTR of INPP4A and GSK3 $\beta$  mRNA, but not their mutant 3'-UTR ( $p < 0.05$ ) (Fig. 5C and D). Moreover, the expression levels of INPP4A and GSK3 $\beta$  in the miR-940 mimic group were significantly lower than those in the control or mimic NC groups, while the opposite expression changes of INPP4A and GSK3 $\beta$  were obtained in the miR-940 inhibitor group ( $p < 0.05$ )



**Figure 4.** The effects of miR-940 on bladder cancer cell apoptosis. (A, B) Flow cytometry showed the apoptotic cells in the different transfection groups. (C, D) The mRNA and protein expression levels of Bcl-2, Bax, pro-caspase 3, and cleaved caspase 3 in the different transfection groups. \* $p < 0.05$ , \*\* $p < 0.01$  compared to the mimic NC; # $p < 0.05$ , ### $p < 0.01$ , #### $p < 0.001$  compared to the Inhibitor NC.



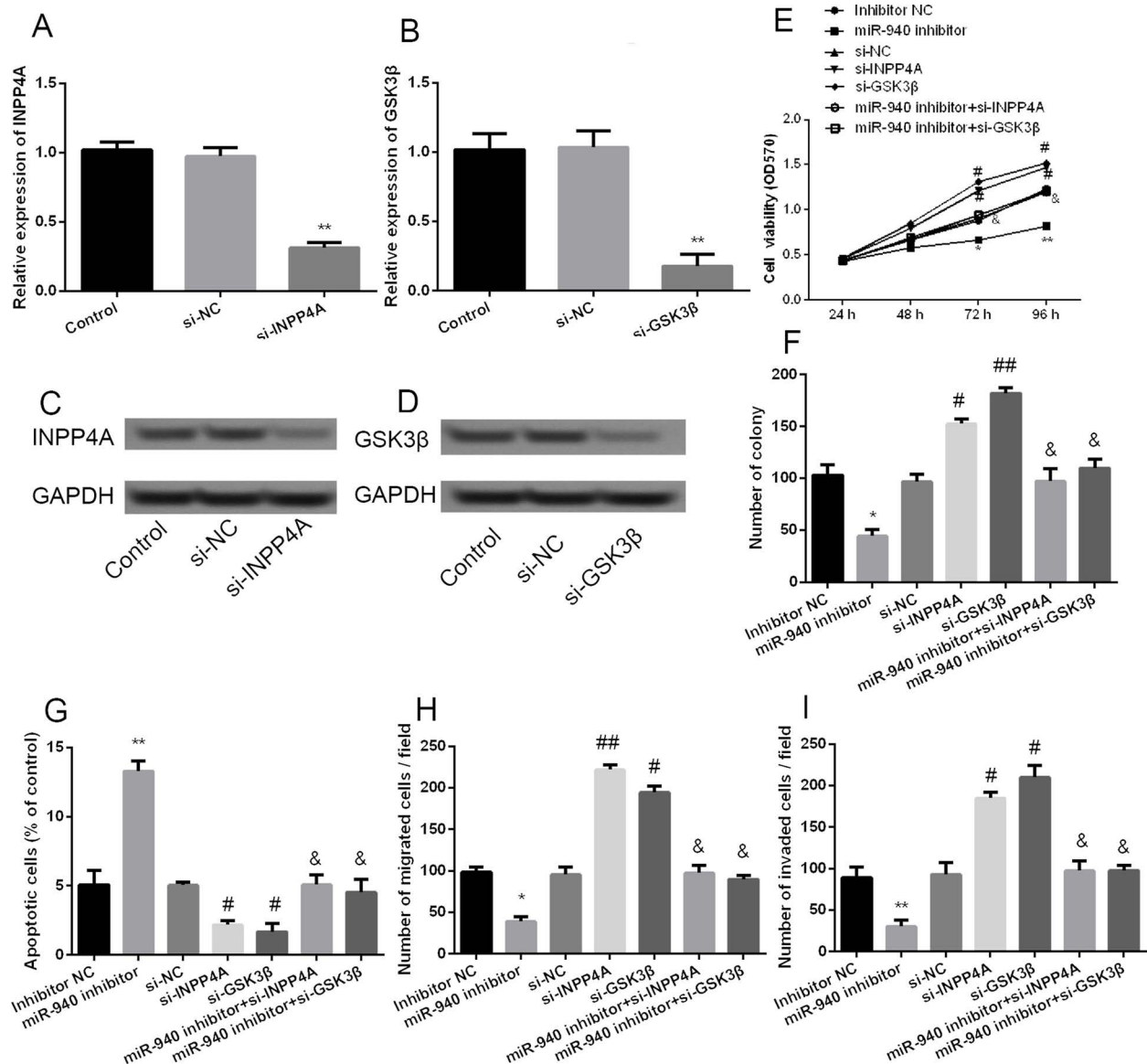
**Figure 5.** INPP4A and GSK3β were confirmed as the direct targets of miR-940. (A, B) The predicted information using TargetScanHuman. (C, D) The results of the dual luciferase assays. (E, F) The mRNA and protein expression levels of INPP4A and GSK3β in the different transfection groups. \* $p < 0.05$  compared to the mimic NC; # $p < 0.05$ , ## $p < 0.01$  compared to the Inhibitor NC.

(Fig. 5E and F). These data indicated that INPP4A and GSK3 $\beta$  were the direct targets of miR-940.

*Knockdown of INPP4A or GSK3 $\beta$  Significantly Increased Bladder Cancer Cell Proliferation, Promoted Cell Migration and Invasion, and Inhibited Cell Apoptosis In Vitro*

To further explore whether miR-940 played a crucial role in bladder cancer by targeting INPP4A or GSK3 $\beta$ ,

INPP4A and GSK3 $\beta$  were knocked down, respectively. In comparison with the si-NC group, the mRNA and protein expression levels of INPP4A and GSK3 $\beta$  in si-INPP4A and si-GSK3 $\beta$  were significantly decreased ( $p < 0.05$ ) (Fig. 6A–D), indicating that INPP4A and GSK3 $\beta$  were successfully knocked down. Our experiments further confirmed that knockdown of INPP4A or GSK3 $\beta$  after transfection with si-INPP4A or si-GSK3 $\beta$  resulted in a significant increase in cell proliferation



**Figure 6.** The effects of INPP4A and GSK3 $\beta$  on bladder cancer cell proliferation, migration, invasion, and apoptosis. (A, B) The mRNA and protein expression levels of INPP4A. (C, D) The mRNA and protein expression levels of GSK3 $\beta$ . (E) The MTT assay showed the cell viability in the different transfection groups. (F) The colony-forming assay showed the number of colonies in the different transfection groups. (G) The flow cytometry showed the apoptotic cells in the different transfection groups. (H) The Transwell assay showed the number of cells that migrated in the different transfection groups. (I) The Transwell assay showed the number of cells that invaded in the different transfection groups. \* $p < 0.05$ , \*\* $p < 0.01$  compared to the Inhibitor NC group; # $p < 0.05$ , ## $p < 0.01$  compared to the si-NC group; & $p < 0.05$  compared to the si-INPP4A or GSK3 $\beta$  group.



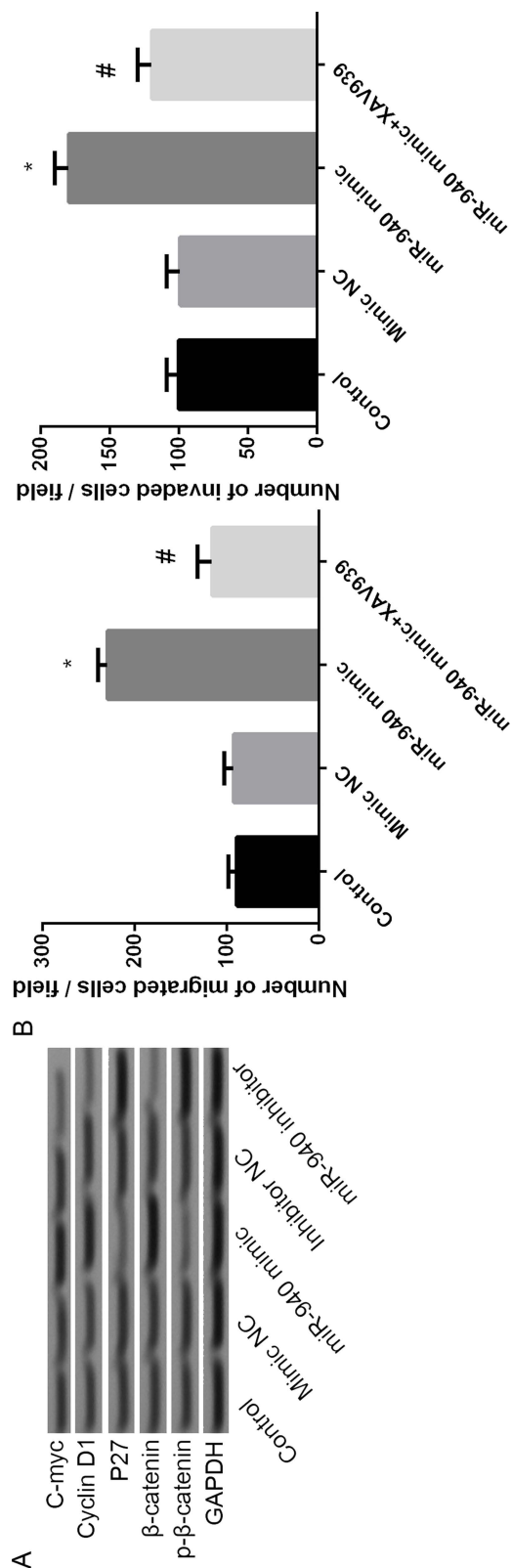
( $p < 0.05$ ) (Fig. 6E and F), marked inhibition of cell apoptosis ( $p < 0.05$ ) (Fig. 6G), and obvious enhancement of cell migration ( $p < 0.05$ ) (Fig. 6H) and invasion ( $p < 0.05$ ) (Fig. 6I) when compared with the corresponding treatment with si-NC, which were reversed after transfection with the miR-940 inhibitor and si-INPP4A or si-GSK3 $\beta$  at the same time. These data indicate that miR-940 may play a role in bladder cancer cell proliferation, migration, invasion, and apoptosis via targeting INPP4A or GSK3 $\beta$ .

#### miR-940 Activated the Wnt/ $\beta$ -Catenin Pathway

In a previous study, a mechanistic relationship between miR-940 and Wnt/ $\beta$ -catenin in the development of pancreatic carcinoma has been disclosed<sup>22</sup>. We therefore investigated the expression levels of Wnt/ $\beta$ -catenin pathway-related proteins to further explore the possible regulatory mechanism of miR-940 in the development of bladder cancer. The protein expression levels of c-Myc, cyclin D1, and  $\beta$ -catenin in the miR-940 mimic group were significantly increased compared with those in the control or mimic NC groups, while the expression levels of p27 and p- $\beta$ -catenin were markedly decreased ( $p < 0.05$ ) (Fig. 7A). The opposite expression changes in these proteins were obtained after suppression of miR-940 by transfection with the miR-940 inhibitor ( $p < 0.05$ ). In addition, XAV939, a tankyrase 1 inhibitor that could inhibit the activation of Wnt/ $\beta$ -catenin signaling, was used to treat cells to further verify whether miR-940 plays a key role in bladder cancer cell migration and invasion via regulating the Wnt/ $\beta$ -catenin pathway. The results showed that, compared with the miR-940 mimic group, the number of cells that migrated and invaded were significantly decreased in the miR-940 mimic + XAV939 group ( $p < 0.05$ ) (Fig. 7B), indicating that XAV939 significantly reversed the effects of miR-940 overexpression on bladder cancer cell migration and invasion. These data indicate that overexpression of miR-940 may play a role in bladder cancer development through activating the Wnt/ $\beta$ -catenin pathway.

## DISCUSSION

In this study, we found that miR-940 was upregulated in bladder cancer tissues and cells. INPP4A and GSK3 $\beta$  were confirmed as the direct targets of miR-940. Overexpression of miR-940 significantly increased bladder cancer cell proliferation, promoted migration and invasion, and inhibited cell apoptosis in vitro, and similar effects were obtained after knockdown of INPP4A or GSK3 $\beta$ . In addition, after miR-940 overexpression, the protein expression levels of c-Myc, cyclin D1, and  $\beta$ -catenin were significantly increased, and the expression levels of p27 and p- $\beta$ -catenin were markedly decreased. XAV939 significantly reversed the effects of miR-940 overexpression on bladder cancer cell migration and invasion. These data imply the key role of miR-940



**Figure 7.** Effects of miR-940 on cell signal-related protein expression and on cell metastasis. (A) The protein expression levels of the Wnt/ $\beta$ -catenin pathway-related proteins, including c-Myc, cyclin D1, p27, p- $\beta$ -catenin, and  $\beta$ -catenin. (B) The influences of the miR-940 inhibitor on cell migration and invasion. \* $p < 0.05$  compared to the Inhibitor NC group; # $p < 0.05$  compared to the miR-940 mimic group.

in regulating the malignant behaviors of bladder cancer cells and merit further consideration.

Intriguingly, one of the important findings in our study was that INPP4A and GSK3 $\beta$  were confirmed as the direct targets of miR-940. INPP4A was identified as a suppressor of glutamate excitotoxicity in the central nervous system and has been reported to protect neurons from excitotoxic cell death<sup>23</sup>. INPP4A is also demonstrated to control the activation of Akt and thereby regulate tumorigenesis<sup>24</sup>. Upregulation of miR-935 can also promote the development of pancreatic carcinoma via targeting INPP4A<sup>25</sup>. In addition, GSK3 is known to be a positive regulator of cancer cell proliferation in pancreatic cancer<sup>26</sup>, glioma<sup>27</sup>, and bladder cancer<sup>28</sup>. Upregulation of miR-92 can promote proliferation and invasion of bladder cancer cells via targeting GSK3 $\beta$  and activating Wnt/c-Myc/MMP7 signaling<sup>29</sup>. In our study, miR-940 could significantly inhibit the luciferase activity of the wild-type 3'-UTR of INPP4A and GSK3 $\beta$  mRNA. Knockdown of INPP4A or GSK3 $\beta$  significantly increased bladder cancer cell proliferation, promoted cell migration and invasion, and inhibited cell apoptosis, which were similar to the effects of miR-940 overexpression. Although the role of miR-940 in bladder cancer cells has not been fully investigated, our results prompt us to speculate that miR-940 may promote bladder cancer progression via targeting INPP4A or GSK3 $\beta$ .

Furthermore, as another aspect of the present analysis, our results showed that the protein expression levels of c-Myc, cyclin D1, and  $\beta$ -catenin were significantly increased, and the expression levels of p27 and p- $\beta$ -catenin were markedly decreased. XAV939 could also significantly reverse the effects of miR-940 overexpression on bladder cancer cell migration and invasion, indicating that miR-940 could activate the Wnt/ $\beta$ -catenin pathway to regulate bladder cancer cell migration and invasion. The genetic variant of the Wnt/ $\beta$ -catenin signaling pathway has been proven to be a key modulator and plays a role in bladder cancer etiology<sup>30</sup>.  $\beta$ -Catenin mutations are shown to be correlated with overexpression of c-Myc and cyclin D1 genes, which play a key role in the pathogenesis of bladder cancer<sup>31</sup>. In addition, accumulating evidence has reported that p27 is a well-known tumor suppressor, and miR-25 can promote cell proliferation in osteosarcoma through targeting p27<sup>32</sup>. The reduced p27 expression has been shown to be involved in the development of bladder cancer and is correlated with the poor prognosis of this disease<sup>33</sup>. Besides, a study of Wang et al. revealed that miR-92 could promote proliferation and invasion of bladder cancer cells via targeting GSK3 $\beta$  and activating Wnt/c-Myc/MMP7 signaling<sup>29</sup>. Although the relationship between miR-940 and the Wnt/ $\beta$ -catenin signaling pathway is largely unknown, our results imply

that miR-940 may promote bladder cancer development possibly via activation of the Wnt/ $\beta$ -catenin pathway.

In conclusion, our findings indicate that upregulation of miR-940 may inhibit bladder cancer cell apoptosis and promote bladder cancer cell proliferation, migration, and invasion targeting INPP4A or GSK3 $\beta$  and activating the Wnt/ $\beta$ -catenin signaling pathway. Our findings imply the key roles of suppressing miRNA-940 in the therapy of bladder cancer.

*ACKNOWLEDGMENTS: This study was supported by The Technology Projects of Jintan Science and Technology Bureau and The High-Level Personnel Training Program of Changzhou Municipal Health Planning Commission Grant No. TS2012053. The authors declare no conflicts of interest.*

## REFERENCES

1. Xue Y, Ma G, Zhang Z, Hua Q, Chu H, Tong N, Yuan L, Qin C, Yin C. A novel antisense long noncoding RNA regulates the expression of MDC1 in bladder cancer. *Oncotarget* 2015;6:484–93.
2. Cancer Research UK. Bladder cancer statistics. 2014.
3. Shelley MD, Wilt TJ, Barber J, Mason MD. A meta-analysis of randomised trials suggests a survival benefit for combined radiotherapy and radical cystectomy compared with radical radiotherapy for invasive bladder cancer: Are these data relevant to modern practice? *Clinical Oncol*. 2004;16:166–71.
4. Sudarshan S, Holman DH, Hyer ML, Voelkel-Johnson C, Dong JY, Norris JS. In vitro efficacy of Fas ligand gene therapy for the treatment of bladder cancer. *Cancer Gene Thera*. 2005;12:12–8.
5. Lu Q, Lu C, Zhou GP, Zhang W, Xiao H, Wang XR. MicroRNA-221 silencing predisposed human bladder cancer cells to undergo apoptosis induced by TRAIL. *Urol Oncol*. 2010;28:635–41.
6. Ismaili N, Heudel PE, Elkarak F, Kaikani W, Bajard A, Ismaili M, Errihani H, Droz JP, Flechon A. Outcome of recurrent and metastatic small cell carcinoma of the bladder. *BMC Urol*. 2009;9:1–8.
7. Gao WQ. Roles of microRNAs during prostatic tumorigenesis and tumor progression. *Oncogene* 2014;33:135–47.
8. Jiang C, Chen X, Alattar M, Wei J, Liu H. MicroRNAs in tumorigenesis, metastasis, diagnosis and prognosis of gastric cancer. *Cancer Gene Ther*. 2015;22:291–301.
9. Nanasinkam SP. MicroRNA regulation of tumorigenesis, cancer progression and interpatient heterogeneity: Towards clinical use. *Genome Biol*. 2014;15:1–9.
10. Zhan Q. Exploring the role of microRNAs in tumorigenesis. *Sci Bull. (Beijing)* 2014;59:2221–2.
11. Zhaoqi S, Qingxia W, Mmingxin Z, Junjun S. MicroRNAs in bladder cancer: Expression profiles, biological functions, regulation, and clinical implications. *Crit Rev Eukaryotic Gene Exp*. 2014;24:55–75.
12. Enokida H, Yoshino H, Matsushita R, Nakagawa M. The role of microRNAs in bladder cancer. *Investig Clin Urol*. 2016;57:S60.
13. Cheng Y, Yang X, Deng X, Zhang X, Li P, Tao J, Lu Q. MicroRNA-218 inhibits bladder cancer cell proliferation, migration, and invasion by targeting BMI-1. *Tumor Biol*. 2015;36:1–9.

14. Feng Y, Kang Y, He Y, Liu J, Liang B, Yang P, Yu Z. MicroRNA-99a acts as a tumor suppressor and is down-regulated in bladder cancer. *BMC Urol.* 2014;14:1–8.
15. Wang J, Zhang X, Wang L, Yang Y, Dong Z, Wang H, Du L, Wang C. MicroRNA-214 suppresses oncogenesis and exerts impact on prognosis by targeting PDRG1 in bladder cancer. *PLoS One* 2015;10:e0118086.
16. Pospisilova S, Pazourkova E, Horinek A, Brisuda A, Svobodova I, Soukup V, Hrbacek J, Capoun O, Hanus T, Mares J. MicroRNAs in urine supernatant as potential non-invasive markers for bladder cancer detection. *Neoplasma* 2016;63:799–808.
17. Ding M, Li Y, Wang H, Lv Y, Liang J, Wang J, Li C. Diagnostic value of urinary microRNAs as non-invasive biomarkers for bladder cancer: A meta-analysis. *Int J Clin Exp Med.* 2015;8:15432–40.
18. Bo Y, Liang Y, Wang D, Luo F. MiR-940 inhibits hepatocellular carcinoma growth and correlates with prognosis of hepatocellular carcinoma patients. *Cancer Sci.* 2015; 106:819–24.
19. Rajendiran S, Parwani AV, Hare RJ, Dasgupta S, Roby RK, Vishwanatha JK. MicroRNA-940 suppresses prostate cancer migration and invasion by regulating MIEN1. *Mol Cancer* 2014;13:1–15.
20. Xu T, Zhou Q, Che L, Das S, Wang L, Jiang J, Li G, Xu J, Yao J, Wang H. Circulating miR-21, miR-378, and miR-940 increase in response to an acute exhaustive exercise in chronic heart failure patients. *Oncotarget* 2016;7:12414–25.
21. Long JD, Sullivan TB, Humphrey J, Logvinenko T, Summerhayes KA, Kozinn S, Harty N, Summerhayes IC, Libertino JA, Holway AH. A non-invasive miRNA based assay to detect bladder cancer in cell-free urine. *Am J Translat Res.* 2014;7:2500–9.
22. Yang HW, Liu GH, Liu YQ, Zhao HC, Yang Z, Zhao CL, Zhang XF, Ye H. Over-expression of microRNA-940 promotes cell proliferation by targeting GSK3 $\beta$  and sFRP1 in human pancreatic carcinoma. *Biomed Pharmacother.* 2016;83:593–601.
23. Sasaki J, Kofuji S, Itoh R, Momiyama T, Takayama K, Murakami H, Chida S, Tsuya Y, Takasuga S, Eguchi S. The PtdIns(3,4)P(2) phosphatase INPP4A is a suppressor of excitotoxic neuronal death. *Nature* 2010;465:497–501.
24. Ivetac I, Gurung R, Hakim S, Horan KA, Sheffield DA, Binge LC, Majerus PW, Tiganis T, Mitchell CA. Regulation of PI(3)K/Akt signalling and cellular transformation by inositol polyphosphate 4-phosphatase-1. *EMBO Rep.* 2009; 10:487–93.
25. Wang C, Feng Z, Jiang K, Zuo X. Upregulation of microRNA-935 promotes the malignant behaviors of pancreatic carcinoma PANC-1 cells via targeting inositol polyphosphate 4-phosphatase type I gene (INPP4A). *Oncol Res.* 2017; 25:559–69.
26. Ougolkov AV, Fernandezzapico ME, Bilim VN, Smyrk TC, Chari ST, Billadeau DD. Aberrant nuclear accumulation of glycogen synthase kinase-3 $\beta$  in human pancreatic cancer: Association with kinase activity and tumor dedifferentiation. *Clin Cancer Res.* 2006;12:5074–81.
27. Kotliarova S, Pastorino S, Kovell LC, Kotliarov Y, Song H, Zhang W, Bailey R, Maric D, Zenklusen JC, Lee J. Glycogen synthase kinase-3 inhibition induces glioma cell death through c-MYC, nuclear factor-kappaB, and glucose regulation. *Cancer Res.* 2008;68:6643–51.
28. Naito S, Bilim V, Yuuki K, Ugolokov A, Motoyama T, Nagaoka A, Kato T, Tomita Y. Glycogen synthase kinase-3 $\beta$ : A prognostic marker and a potential therapeutic target in human bladder cancer. *Clin Cancer Res.* 2010;16: 5124–32.
29. Wang H, Ke C, Ma X, Zhao Q, Yang M, Zhang W, Wang J. MicroRNA-92 promotes invasion and chemoresistance by targeting GSK3 $\beta$  and activating Wnt signaling in bladder cancer cells. *Tumor Biol.* 2016;37(12):16295–304.
30. Pierzynski JA, Hildebrandt MA, Kamat AM, Lin J, Ye Y, Dinney CP, Wu X. Genetic variants within the Wnt/ $\beta$ -catenin signaling pathway as indicators of bladder cancer risk. *J Urol.* 2015;194:1771–6.
31. Shiina H, Igawa M, Shigeno K, Terashima M, Deguchi M, Yamanaka M, Ribeiro-Filho L, Kane CJ, Dahiya R.  $\beta$ -Catenin mutations correlate with over expression of C-myc and Cyclin D1 genes in bladder cancer. *J Urol.* 2002; 168:2220–6.
32. Cao J, Cui H, Li N. microRNA-25 promotes osteosarcoma cell proliferation by targeting the cell-cycle inhibitor p27. *Mol Med Rep.* 2014;10:855–9.
33. Xie Q, Han C, Lin F. Clinical significance of p27 expression in bladder cancer. *China J Modern Med.* 2002;18:005–9.