Molecular Medicine in Practice

Tumor Suppressor MicroRNA-493 Decreases Cell Motility and Migration Ability in Human Bladder Cancer Cells by Downregulating RhoC and FZD4

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Abstract

The purpose of this study was to identify new tumor suppressor microRNAs (miRNA; miR) in bladder cancer, conduct functional analysis of their suppressive role, and identify their specific target genes. To explore tumor suppressor miRs in bladder cancer, miR microarray was conducted using SV-HUC-1, T24, J82, and TCCSUP cells. Expression of miR-493 in bladder cancer (T24, J82, and TCCSUP) cells was downregulated compared with normal SV-HUC-1 cells. Also, the expression of miR-493 was significantly lower in bladder cancer tissues than in their corresponding noncancerous tissues. Transfection of miR-493 into T24 or J82 cells decreased their cell growth and migration abilities. On the basis of this result, to identify potential miR-493 target genes, we used target scan algorithms to identify target oncogenes related to invasion and migration. miR-493 decreased 3'-untranslated region luciferase activity and protein expression of FZD4 and RhoC. miR-493 also decreased binding of RhoC and Rock-1. miR-493 is a new tumor suppressor miRNA in bladder cancer and inhibits cell motility through downregulation of RhoC and FZD4.

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Introduction

Bladder cancer is the ninth leading cause of death among men, accounting for 3% of total cancer (1). The most common histologic type of bladder cancer is urothelial carcinoma, which was formerly known as transitional cell carcinoma (2). Approximately 75% of patients are nonmuscle invasive urothelial carcinoma (pTa, pTis, pT1) and have a 5-year survival rate between 88% and 98% (3). The common treatment for these patients is endoscopic resection (2, 4). Patients with muscle invasive urothelial carcinoma are usually treated with radical cystectomy or chemoradiotherapy (2, 5). However, half of the patients with muscle-invasive urothelial carcinoma develop subsequent metastatic disease after the first aggressive treatment (2, 6). Previous studies have identified several potential molecular biomarkers for bladder cancer (7, 8). Namely, inactivation of tumor suppressor genes TP53 and Rb and Ras oncogene activation have been considered as important key players in bladder cancer carcinogenesis (7).

MicroRNAs (miRNA; miR) are well known as examples of noncoding RNAs (9) and human miRNAs now number 1,100 based on microRNA.org (http://www.microrna.org/microrna/home.do). miRNAs bind to the 3'-untranslated region (UTR) of target gene mRNA and repress translation or induce mRNA cleavage (10), thereby inhibiting translation from mRNA to protein. Aberrant expression of miRNAs occurs in bladder cancer. Decreased expression of tumor suppressor miRNAs result in increased expression of target oncogenes. In contrast, increased expression of oncogenic miRNAs leads to loss or decreased expression of target tumor suppressor genes. According to previous reports, a number of miRNA microarray studies have been conducted in samples of patients with bladder cancer, and the expression level of several miRNAs (miR-17-5p, miR-23a, miR-23b, miR-26b, miR-103-1, miR-185, miR-203, miR-205, miR-221, and miR-223) were upregulated in bladder cancer tissues (11). In contrast, some miRNAs (miR-30-3p, miR-125, miR-133a, miR-145, miR-195, and miR-199a) were downregulated in bladder cancer compared with normal bladder tissues (12). miRNA expression level in paired primary and metastatic bladder cancers was validated using real-time PCR. The data show that the expression levels of several miRNAs (miR-10b, miR-29a, miR-29b, miR-126, miR-142-5p, miR-146a, miR-146b-5p, miR-150, miR-155, and miR-342-3p) were upregulated in...
metastatic bladder cancer tissues and some miRNAs (miR-143, miR-145, and miR-320) were downregulated (13); miR-129 was found to be upregulated in progressive bladder tumor and significantly associated with short survival (14). miR-125b was downregulated in bladder tumor tissues compared with normal bladder tissues, and overexpression of miR-125b inhibited cell growth in bladder cancer cell lines (15). However, there have been few reports about the detailed functional analysis of these miRNAs in bladder cancer.

The aim of this study was to identify new tumor suppressor miRNAs that influence bladder cancer progression. Initially, we conducted miRNA microarray analysis to screen miRNAs related to bladder cancer using normal bladder cells (SV-HUC-1) and 3 bladder cancer cell lines (J82, T24, TCCSUP). We identified 10 miRNAs whose expression level in bladder cancer cell lines was significantly higher or significantly lower than normal bladder cell line, SV-HUC-1. Next, we checked the microarray results by real-time PCR. Among 10 miRNAs, the expression of miR-493, miR-141, and miR-1290 was lower in bladder cancer cell lines and these results were consistent with the microarray data. On the basis of microarray and real-time PCR results, we hypothesized that miR-493 may be a potential tumor suppressive miRNA in bladder cancer and found that miR-493 expression was significantly lower in bladder cancer tissues. Thus, we conducted functional assays using miR-493. Transfection of miR-493 into bladder cancer cells decreased cell growth, invasion, and migration. We also looked at potential target genes of miR-493 focusing on invasion and migration-related ones. We initially used a target scan algorithm (microRNA.org) to identify genes RhoC and FZD4 as targets of miR-493 and validated the results with a second target scan algorithm (TargetScan). We also conducted 3'-UTR luciferase assays and Western blot analysis to look at target gene protein expression in miR-493-transfected bladder cancer cells. Finally, we knocked down FZD4 and RhoC miRNAs using a short interfering RNA (siRNA) technique to examine and confirm the mechanism of miR-493 tumor suppressive function.

Materials and Methods

Cell lines and cell cultures
SV-HUC-1, T24, J82, and TCCSUP cells were purchased on February 2, 2010, from the American Type Culture Collection (ATCC). No authentication was done by the authors. T24, J82, and TCCSUP cells originating from transitional cell carcinoma were selected as model transitional cell carcinoma cells. SV-HUC-1 cells derived from normal uroepithelium were used as control cells. SV-HUC-1 cells were cultured in F-12K Medium (ATCC) with 10% FBS. T24 cells were cultured in McCoy’s 5A medium supplemented with 10% FBS. J82 cells were cultured in Minimum Essential Media (MEM) supplemented with 10% FBS. TCCSUP cells were cultured in MEM supplemented with 10% FBS, nonessential amino acids, and 1 mmol/L sodium pyruvate.

RNA extraction
The miRNA and total RNA were extracted from cell lines using a miRNeasy Mini Kit and an RNeasy Mini Kit (Qiagen). The miRNAs from clinical samples were extracted using laser capture microdissection techniques with a miRNeasy FFPE kit (Qiagen).

MicroRNA microarray
For miRNA microarray, total RNA was extracted from SV-HUC1, T24, J82, and TCCSUP cells using a miRNeasy Mini Kit. The miRNA microarray analysis was carried out and analyzed by Phalanx Bio Inc. We selected 10 miRNAs whose expression level in bladder cancer cell lines was significantly higher (top 5 miRNAs; miR-130b, miR-423-5p, miR-484, miR-503, miR-766) or significantly lower (top 5 miRNAs; miR-107, miR-141, miR-493, miR-933, miR-1290) than normal bladder SV-HUC-1 cells (Supplementary Table S1).

Tissue array samples
A human bladder cancer tissue array was purchased from US Biomax (catalog no.: BL801, US Biomax, Inc.) to detect miR-493 localization and confirm miR-493 expression levels in bladder cancer tissues by in situ hybridization. Tissue array patient information is shown in Supplementary Table S2.

Clinical samples
Twenty-three normal and 16 transitional cell carcinoma tissues in paraffin blocks were obtained from the Pathology Department of the Veterans Affairs Medical Center at San Francisco (San Francisco, CA). Informed consent was obtained from patients from the Veterans Affairs Medical Center at San Francisco.

Transfection
Pre-miR miRNA precursor (negative control/hsa-miR-493; Ambion), siRNA (control/RhoC/FZD4; Applied Biosystems, Invitrogen), Mock (without oligonucleotide), and cotransfection of Pre-miR miRNA precursor/pmirGLO Dual-Luciferase miRNA Target Expression Vector (Promega) were transiently transfected into cells by LipoFectamine 2000 (Invitrogen). Anti-miR miRNA inhibitors (negative control #1/has-miR-493; Applied Biosystems) were transiently transfected into cells by siPORT NeoFX Transfection Agent (Ambion).

Cell viability assay
Viability of T24 and J82 cells was measured by the MTS (CellTiter 96 Aqueous One Solution Cell Proliferation Assay; Promega) assay 4 days after transfection of Pre-miR miRNA precursor. Cell viability was determined by absorbance measurements at 490 nm using SpectraMax 190 (Molecular Devices). Data are presented as the mean value ± SD for triplicate experiments and compared with...
the level of miR-493/siRNA obtained in mock or control miRNA/siRNA transfected cells that is normalized to 100%.

Migration assay
Transwell membrane filter inserts (8.0 mm pore size; BD Biosciences) were set in 24-well plate. T24 and J82 cells were transfected with Pre-miR miRNA precursor or siRNA were harvested 72 hours after transfection and resuspended in serum-free MEM. Aliquots (5 × 10^4 cells/100 μL) of the prepared cell suspension were added into the upper chamber, and the lower chamber was filled with 1 mL of media containing 10% FBS. Cells were incubated for 4 hours at 37°C in a 5% CO2 tissue culture incubator. After 4 hours, no migrated cells were removed from Transwell membrane filter inserts using cotton-tipped swab, migrated cells were stained with Hema 3 STAIN SET (Fisher Scientific). Cells per 3 random fields of each membranes were counted with Nikon ECLIPSE TS100 (Nikon) at ×100 magnification. Data are presented as the mean value ± SD for triplicate experiments and compared with the level of miR-493/siRNA obtained in mock or control miRNA/siRNA transfected cells that is normalized to 100%.

Wound-healing assay
T24 and J82 cells were seeded to 6-well plates and transfected with Pre-miR miRNA precursor or control. At 24 hours after transfection, cells were transferred to 6-well plates to 12-well plates. After 48 hours, a wound was formed by scraping the cells with a 200 μL pipette tip and washed twice with medium. We observed cells at 0, 24, and 72 hours after scraping and photographed the cells with a microscope (Nikon). Data are presented as the mean value ± SD for experiments and compared with the level of miR-493/siRNA obtained in mock or control miRNA/siRNA transfected cells that is normalized to 100%.

Luciferase reporter assay
A pmirGLO Dual-Luciferase miRNA target expression vector was used for 3′-UTR luciferase assays (Promega). The target oncogenes of tumor suppressor miRNA-493 vector was used for 3′-UTR luciferase assays were made as described previously (16). For 3′-UTR luciferase assay, T24 cells were cotransfected with hsa-miR-493 and pmirGLO Dual-Luciferase miRNA target expression vectors with wild-type or mutant target sequence using Lipofectamine 2000. Luciferase assay was conducted using the Dual-Luciferase Reporter Assay System (Promega) at 48 hours after transfection. Data are presented as the mean value ± SD for triplicate experiments and compared with the level of wild-type sequence vectors obtained in mutant-type sequence vectors transfected cells that is normalized to 100%.

Quantitative real-time PCR
Extracted total RNA was reverse transcribed into single-stranded cDNA using a High Capacity cDNA Archive Kit (Applied Biosystems) and a TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems). Real-Time PCR was carried out using first-strand cDNA with TaqMan Fast Universal PCR Master Mix (Applied Biosystems). The assay numbers for the miRNA endogenous control (β-actin), target gene, miRNA endogenous control (RNU48), and target miRNAs were as follows: β-actin (Hs99999903_m1), RhoC (Hs00747110_s1), FZD4 (Hs00201853_m1), RNU48 (001006), miR-493 (002364), miR-107 (004433), miR-130b (004546), miR-141 (00463), miR-423-5p (002340), miR-484 (001182), miR-503 (001048), miR-766 (001986), miR-933 (002176), and miR-1290 (002863). Quantitative PCR was carried out with an Applied Biosystems Prism 7500 Fast Sequence Detection System (Applied Biosystems). Quantitative PCR parameters for cycling were as follows: 95°C for 20 seconds, 40 cycles of PCR at 95°C for 3 seconds, and 60°C for 30 seconds. All reactions were done in a 10-μL reaction volume in triplicate. The miRNA and miRNA expression level were determined using the 2^(-ΔΔC_t) method.

Western blot analysis
At 72 hours after transfection, cells were washed in ice-cold PBS and added to radioimmunoprecipitation assay lysis and extraction buffer (Fisher Scientific) containing Protease Inhibitor Cocktail 1 (Millipore). Dishes were incubated for 5 minutes on ice, and cells were collected with a cell lifter and rotated for 30 minutes at 4°C followed by centrifugation at 12,000 × g for 20 minutes at 4°C. Total protein was analyzed by Western blotting using primary antibodies, followed by antimouse and antirabbit IgG horseradish peroxidase–conjugated secondary antibodies (#7076, #7074; Cell Signaling Technology) and anti-rat IgG-B (sc-2041; Santa Cruz Biotechnology, Inc)/streptavidin–horseradish peroxidase (Invitrogen) and were visualized with LumigLO Reagent and peroxide reagent (Cell Signaling Technology). The primary antibodies used were anti-RhoC Antibody (#3430; Cell Signaling Technology), anti-RhoC Antibody (#7074; Cell Signaling Technology), anti-RhoC Antibody (#7076; Cell Signaling Technology).
anti-RhoA Antibody (#2117; Cell Signaling Technology), FZD4 Antibody (#LS-C6904; LifeSpan BioSciences, Inc.), and anti-β-actin (#3700; Cell Signaling Technology) antibodies. Western blot analysis was repeated independently 2 times.

**Immunoprecipitation**

At 72 hours after transfection, cells were washed in ice-cold PBS and added to cell lysis buffer (50 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1% Triton X-100) containing Protease Inhibitor Cocktail I (Millipore). Dishes were incubated for 5 minutes on ice, and cells were collected with a cell lifter and transferred to a 1.5-mL tube. Samples were sonicated on ice three times for 5 seconds followed by centrifugation at 14,000 × g for 10 minutes at 4°C. The supernatants were transferred to new 1.5-mL tubes. For cell lysate preclearing, protein G-agarose was added to sample tubes. Sample tubes were incubated at 4°C for 1 hour with gentle rocking followed by centrifugation at 14,000 × g for 10 minutes at 4°C. The supernatants were transferred to new 1.5-mL tubes. Anti-Rock-1 antibody (#ab45171; Abcam) or anti-control antibody (#2729; Cell Signaling Technology) was added to sample tubes, and these tubes were incubated with gentle rocking for overnight at 4°C. Protein G was added to sample tubes and these sample tubes were incubated with gentle rocking for 2 hours at 4°C followed by centrifugation at 14,000 × g for 30 seconds at 4°C. Samples were washed 5 times with cell lysis buffer. Sample buffer (2×) was added to sample tubes and vortexed followed by centrifugation at 14,000 × g for 1 minute. The supernatants were analyzed by Western blotting. Immunoprecipitation was repeated independently 2 times.

**In situ hybridization**

Tissue arrays were purchased from US Biomax, Inc. For in situ hybridization, IsHyb In Situ Hybridization (ISH) Kit was used according to the manufacturer’s instructions (BioChain). A tissue array slide was incubated overnight with a 1:500 dilution of miRCURY LNA Detection probe 5’-DIG labeled for miR-493 (EXIQON) and overnight at 4°C with a 1:200 dilution of AP-conjugated anti-digoxigenin antibody. BM Purple; AP substrate precipitating, (NBT/BCIP ready-to-use solution; Roche) was used as detector. Staining intensity of in situ hybridization was divided into 0, no staining; 1, positive staining; and 2, strong positive staining.

**siRNA knockdown of RhoC and FZD4 mRNA**

T24 and J82 cells were transfected with RhoC and FZD4 siRNA (RhoC Silencer Select Validated siRNA, siRNA ID s97 and FZD4 Silencer Select Validated siRNA, siRNA ID s15840; Applied Biosystems), RhoC and FZD4 siRNA (RHOC Stealth RNAi siRNA HSS100662 and FZD4 Stealth RNAi siRNA HSS188730; Invitrogen) or negative control siRNA using Lipofectamine 2000 according to the manufacturer’s instructions.

**Statistical analysis**

All statistical analyses were conducted using GraphPad prism 5 software (GraphPad Software). A value of *P* < 0.05 was considered as statistically significant.

**Results**

**Expression level of miR-493 in cell lines and primary tissues**

To identify tumor suppressor miRNAs in bladder cancer, we conducted an miR microarray using SV-HUC-1 as a normal bladder cell line and 3 bladder...
cancer cell lines, T24, J82, and TCCSUP, and selected 10 miRNAs for our study (Supplementary Table S1). Namely, the expression of 5 miRNAs was significantly higher (top 5) and that of 5 miRNAs were significantly lower (top 5) in bladder cancer cell lines than in normal bladder cells. To confirm the expression of these 10 miRNAs, we carried out real-time PCR and found that miR-107, miR-141 miR-493, and miR-1290 expression in bladder cancer cells was significantly lower than that in SV-HUC-1 ($P < 0.0001$; Figs. 1 and 2A). miR-933 expression was not detected in bladder cell lines using real-time PCR. The real-time PCR data showed similar results as the microarray data for miR-107, miR-141, miR-493, miR-503, and miR-1290, whereas some miRs showed opposite results (miR-130b, miR-423-5p, miR-484, and miR-766) as the expression was higher than in SV-HUC-1.

**Localization of miR-493 expression and decreased miRNA-493 expression in bladder cancer tissues**

To investigate miR-493 localization in human bladder tissues, we carried out *in situ* hybridization in bladder tissue array (normal and cancer tissues). As shown in Fig. 2B, miR-493 expression was significantly lower in bladder cancer tissues than in normal bladder tissues ($P = 0.0416$). Expression of miR-493 was observed in connective tissues, endothelial cells of blood vessels, and lamina muscularis mucosa in normal bladder tissues (data not shown). In urothelial cell carcinoma, miR-493 expression was lower than in layers of normal urothelium (Supplementary Fig. S1). We then compared miRNA-493 expression levels in bladder cancer tissues and normal bladder tissues by real-time PCR to validate the ISH results. Similar to the ISH results, the expression of miR-493 was significantly lower in bladder cancer tissues than in normal bladder tissues. The expression of miR-493 in the normal bladder cell line, SV-HUC-1, was used as reference (expression = 1). Consistent with the ISH results, the miR-493 expression was significantly lower in bladder cancer tissues ($P = 0.0080$) than in normal tissues (Fig. 2C). Regarding miR-107, miR-141, and miR-1290, we did not find any difference of expression between normal bladder tissues and bladder cancer tissues (Supplementary Fig. S2).

**Evaluation of the functional effects of miR-493 on T24 and J82 cells**

As the expression of miR-493 was significantly lower in bladder cancer cell lines and bladder cancer tissues than in normal bladder cells and tissues, we next focused on the functional effects of miR-493 on bladder cancer cells. miR-493 and miR control were transiently transfected into T24 and J82 cells. The expression level of miR-493 was significantly increased at 48 hours after transfection (Fig. 3A). Cell viability was decreased to 70% to 80% in miR-493-transfected cells compared with controls at 4 days after transfection ($P = 0.0286$; Fig. 3B). Cell motility was also significantly decreased in miR-493-transfected T24 and J82 cells ($P = 0.0068$ and $P < 0.0001$, respectively, Fig. 3C). Cell migration was also significantly decreased to about 30% in miR-493 transfected T24 and J82 cells ($P = 0.0007$ and $P < 0.0001$, respectively; Fig. 3D). Cell-cycle analysis...
based on flow cytometry was conducted with miR-493–transfected T24 cells. Significantly increased G1 cell-cycle arrest in the miR-493 transfectants was observed compared with controls \((P = 0.0002; \text{Fig. 3E})\). To see whether control miRNAs suppress cancer cells, mock (without oligonucleotide) and control miRNA were transfected into cancer cells, and real-time PCR, Western blot analysis, migration, and wound-healing assay were carried out. These results did not show significant data (Supplementary Fig. S3). To confirm whether miR-493 regulates cell motility, control or miR-493 inhibitors were transfected into SV-HUC-1 cells. miR-493 inhibitor slightly increased SV-HUC-1 cell motility (Supplementary Fig. S4).

**Identification of miR-493 target genes and its effect on their 3′-UTR-luciferase assays and protein expression**

On the basis of the observation that miR-493 affects cell motility and migration, we searched for target genes of miR-493 related to motility and migration using a target scan algorithm (microRNA.org; http://www.microrna.org/microrna/home.do) and identified RhoC and FZD4 as target oncogenes. As shown in Fig. 4A, FZD4 mRNA has one potential complimentary miR-493–binding site within its 3′-UTR. RhoC mRNA also has one potential complimentary miR-493–binding site within its 3′-UTR. We conducted RhoC and FZD4 3′-UTR luciferase assays and found that the relative luciferase activities with these sites were significantly decreased in miR-493–transfected T24 cells \((P = 0.0004\) and \(P = 0.0007\), respectively; Fig. 4B). With mutated plasmids, there was no significant difference in luciferase activity between controls and mutated miR-493 transfectants. To examine the inhibitory effect of miR-493 on protein expression levels, we conducted Western blot analysis 72 hours after miR-493 transfection into T24 and J82 cells. We observed that the protein levels of RhoC and FZD4 in miR-493–transfected T24 and J82 cells were significantly decreased compared with control cells;
of RhoC and FZD4 protein expression was also confirmed by Western blot analysis (Fig. 5A and B). To determine whether RhoC and FZD4 knockdown affects cell migration, migration assay was conducted using T24 and J82 cells. Cell migration was significantly decreased to 35% to 50% in si-RhoC and si-FZD4 transfectedants compared with control cells (P = 0.0015 and P = 0.0064, respectively; Fig. 5C and P = 0.0010 and P = 0.0007, respectively; Fig. 5D). To examine potential off-target effects of siRNA, other RhoC and FZD4 siRNAs from a different source were also used (Supplementary Fig. S5). As shown in Supplementary Fig. S5, the results were consistent with Fig. 5. These data suggest that the siRNAs used in this study did not induced the off-target effects in the siRNA experiments.

**Effect of miR-493 on the binding of RhoC to Rock-1 in bladder cancer cells**

To analyze whether overexpression of miR-493 affects the binding of RhoC to Rock-1, immunoprecipitation was carried out at 72 hours after transfection in T24 cells. We found that the binding of RhoC to Rock-1 in miR-493–transfected T24 cells was decreased compared with miR control transfectants (Fig. 6A). We also analyzed the binding of RhoC to Rock-1 after FZD4 siRNA transfection. At 72 hours after transfection, the binding was decreased in siFZD4-transfected cells (Fig. 6B). The schematic representation of the possible role of miR-493 in the FZD4/RhoC signaling pathway is shown in Fig. 6C.

**Discussion**

There have been several miRNA studies related to clinical bladder cancer and most have involved microarray screening (11–15). We used an miR microarray service to compare miRNA expression levels in a normal uroepithelium cell line (SV-HUC-1) and bladder cancer cell lines (T24, J82, and TCCSUP). On the basis of the miRNA microarray data, we initially chose 10 miRNAs whose expression was significantly higher (5 miRNAs) or lower (5 miRNAs) than the normal bladder cell line. Among the 10 miRNAs, the expression of miR-493 and miR-141 was lower in bladder cancer cell lines than in the normal cell line which is consistent with previous miRNA microarray results; however, there are no reports concerning the functional role of miR-493 in bladder cancer.

Thus, we focused on miR-493 as a potentially new candidate tumor suppressor miRNA in bladder cancer. In addition, we confirmed the miR-493 expression level in clinical bladder cancer tissues using ISH and real-time PCR. The expression of miR-493 was significantly lower in bladder cancer tissues than in normal bladder tissues. On the basis of these results, we hypothesized that miR-493 may play an important role as a tumor suppressor in bladder cancer. To test this hypothesis, we conducted functional analyses (MTS, migration, wound healing, and cell cycle) to look at miR-493 function using miR-493–transfected cells. As expected, miR-493 overexpression inhibited cell proliferation in bladder cancer cells (T24 and J82). Cell migration and motility were also dramatically reduced (Fig. 5A and B). The expression of miR-493 was significantly lower in bladder cancer tissues than in normal bladder tissues. On the basis of these results, we hypothesized that miR-493 may play an important role as a tumor suppressor in bladder cancer. To test this hypothesis, we conducted functional analyses (MTS, migration, wound healing, and cell cycle) to look at miR-493 function using miR-493–transfected cells. As expected, miR-493 overexpression inhibited cell proliferation in bladder cancer cells (T24 and J82). Cell migration and motility were also dramatically reduced.
decreased after miR-493 transfection. These results suggest that miR-493 may function as a tumor suppressor and play an important role in inhibition of cell growth and motility of bladder cancer cells.

As a next step, we also used microRNA.org to identify target oncogenes of miR-493 and identified potential candidate target oncogenes related to invasion and migration. TargetScan also showed RhoC and FZD4 as miR-493 target genes (Supplementary Fig. S6). We conducted 3′-UTR luciferase assay and observed that luciferase activity was decreased after cotransfection of miR-493 and a 3′-UTR vector containing the RhoC/FZD4 miR-493 target sequence. RhoC and FZD4 protein expression were also significantly downregulated in miR-493–transfected T24 and J82 cells indicating that RhoC and FZD4 are direct targets of miR-493.

It has been reported that RhoC protein is overexpressed in bladder tumor tissues compared with bladder nontumor tissues and higher RhoC protein expression in tumors was associated with clinicopathologic factors (grade and pT), with poorer disease-free survival and overall survival than low expression (17). Although there have been no reports about RhoC function in bladder cancer, in hepatocellular carcinoma cells, RhoC is essential for invasion and migration but not proliferation and apoptosis (18). This is consistent with our finding that cell migration in

Figure 5. Functional effects of RhoC and FZD4 siRNA knockdown on T24 and J82 cells. A, mRNA and protein expression level in si-control and si-RhoC–transfected bladder cancer cells (T24 and J82). WB, Western blot analysis. B, mRNA and protein expression level in si-control and si-FZD4–transfected bladder cancer cells (T24 and J82). At 48 hours after transfection, mRNA and protein were analyzed using real-time PCR and Western blot analysis. RhoC and FZD4 expression were normalized to β-actin. C and D, migration assay of si-RhoC and si-FZD4 transfectants. si-RhoC/si-FZD4 or si-control was transfected into T24 and J82 cells. At 72 hours after transfection, cells were added into the chamber. Cells were incubated for 4 hours at 37°C in a 5% CO2 tissue culture incubator; no migrated cells were removed from Transwell membrane filter inserts using cotton-tipped swab, and migrated cells were stained with Hema 3 STAIN SET. Representative photomicrographs are shown at ×100 magnification.
T24 and J82 RhoC knockdown was significantly decreased compared with control siRNA. However, cell growth in T24 and J82 RhoC knockdowns was not significantly different compared with control siRNA (data not shown). This cell growth inhibition result is consistent with RhoC function in hepatocellular carcinoma cells (18). It has been reported that RhoC is essential for invasion in some cancers (19–24), and RhoC expression is significantly correlated with poor prognosis (25–27). FZD4 knockdown decreased the binding of RhoC to Rock-1. We have also previously reported that miR-584 targets Rock-1 (16). As high Rock-1 expression in bladder tumors was associated with poor disease-free survival and overall survival than low expression (17), functional RhoC and Rock-1 in bladder cancer may be important to malignancy progression.

It has also been reported that miR-138 directly targets RhoC and Rock-2 in tongue squamous cell carcinoma and inhibits cell migration and invasion (28). Our microarray data show that miR-138 expression in TCCUP cells is significantly lower than in SV-HUC-1, but not in T24 and J82 cells (Supplementary Table S1).

Our study showed that miR-493 inhibited FZD4 and RhoC and this pathway is involved in Wnt-PCP pathway. Wnt signaling pathways include the following: (i) Wnt-β-catenin, (ii) Wnt-PCP, and (iii) Wnt-Ca²⁺, and these signaling pathways have been reported to be associated with cancer progression and poor prognosis (29). In bladder cancer, Ras oncogene activation plays a very important role in bladder cancer progression (7). Interestingly, Ahmad and colleagues have recently reported that Ras pathway activation cooperates with Wnt-β-catenin signaling to drive urothelial cell carcinoma (30). There are few reports about other Wnt signaling pathways (Wnt-PCP or Wnt-Ca²⁺) in bladder cancer. In conclusion, our study suggests that miR-493 may be a new tumor suppressive miRNA inhibiting cell invasion and migration by blocking FZD4 and RhoC in bladder cancer, implicating the Wnt-PCP pathway in bladder carcinogenesis.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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