MicroRNA-106a suppresses proliferation, migration, and invasion of bladder cancer cells by modulating MAPK signaling, cell cycle regulators, and Ets-1-mediated MMP-2 expression

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Abstract. Despite the clinical significance of tumorigenesis, little is known about the cellular signaling networks of microRNAs (miRs). Here we report a new finding that mir-106a regulates the proliferation, migration, and invasion of bladder cancer cells. Basal expression levels of mir-106a were significantly lower in bladder cancer cells than in normal urothelial cells. Overexpression of mir-106a suppressed the proliferation of bladder cancer cell line EJ. Transient transfection of mir-106a into EJ cells led to downregulation of ERK phosphorylation and upregulation of p38 and JNK phosphorylation over their levels in the control. Flow cytometry analysis revealed that mir-106a-transfected cells accumulated in the G1-phase of the cell cycle, and cyclin D1 and CDK6 were significantly downregulated. This G1-phase cell cycle arrest was due in part to the upregulation of $p21^{CIP1/WAF1}$. In addition, mir-106a overexpression blocked the wound-healing migration and invasion of EJ cells. Furthermore, mir-106a transfection resulted in decreased expression of MMP-2 and diminished binding activity of transcription factor Ets-1 in EJ cells. Collectively, we report the novel mir-106a-mediated molecular signaling networks that regulate the proliferation, migration, and invasion of bladder cancer cells, suggesting that mir-106a may be a therapeutic target for treating advanced bladder tumors.

Introduction

Since microRNAs (miRs) were first discovered in the early 1990s, their functional roles in regulating cellular physiology of both normal and cancerous cells have been highlighted by many studies (1). In cancer cells, miR dysregulation was first identified in a study on chronic lymphocytic leukemia (CLL), in which mir-15 and mir-16 were frequently deleted at the chromosome locus 13q14.3 (2). This deletion induced upregulation of BCL-2, which led to anti-apoptotic advantages to the cells. It has been accepted that miRs can serve as either oncogenes or tumor suppressor genes. During transformation of normal cells, oncogenic miRs are often overexpressed, while tumor-suppressing miRs are downregulated (3-5). The expression profile of miRs in each tumor type is unique, and many researchers have therefore attempted to evaluate their clinical significance as diagnostic tools (6-8). For example, Puerta-Gil and colleagues reported that miRs in urine, such as mir-143, mir-222, and mir-452, might be useful diagnostic markers for bladder cancer (9). Wang *et al* also demonstrated that mir-141 is associated with diagnosis of bladder cancer using 114 bladder tumors and matched normal bladder tissues (10). Recently, clinical trials using miRs as therapeutics have gained popularity; 138 clinical trials related to miRs are ongoing or have been completed to date (https://clinicaltrials.gov/). Although bladder cancer is one of the most fatal diseases in Western countries, sufficient clinical studies on bladder cancer have not yet been performed. This might be due in part to the heterogeneity of the disease and the absence of signature mutations (11). Therefore, in bladder cancers, identifying effective molecular targets is critical. Iyer and coworkers attempted to verify genetic alterations in bladder cancers using 97 advanced-stage bladder tumors. In their integrative analysis, driver mutations in the RTK-RAS-RAF axis, the PI3K/AKT/mTOR axis, and in regulators of G1/S cell cycle phases were identified (12). In accordance, our previous study verified that mir-20b regulates the growth and migration of bladder cancer cells through cell cycle accumulation in G1/S phase and matrix metalloproteinase (MMP) regulation (13).

In this study, we report the novel finding that mir-106a regulates the proliferation and migration of bladder cancer cells via modulating MAPKs (ERK p38, and JNK), $p21^{CIP1/WAF1}$/cyclin D1/CDK 6, and Ets-1/MMP-2.

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Materials and methods

Materials. Antibodies for ERK, phospho-ERK, p38, phospho-p38, JNK, and phospho-JNK were purchased from Cell Signaling Technology (Danvers, MA, USA). Polyclonal antibodies against cyclin D1, cyclin E, CDK2, CDK6, p53, $p21^{CIP1/WAF1}$, $p27^{KIP1}$, GAPDH, Ets-1, Sp-1, ATF-2, and CREB were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The anti-MMP-2 antibody was obtained from Chemicon International (Billerica, MA, USA). mir-106a (5'-AAAAGUGCUUACAG UGCAGGUAG-3') and mir-106a inhibitor were obtained from Genolution (Seoul, Korea).

Cell cultures. Human bladder carcinoma cell lines (EJ, 5637, and T24) were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, l-glutamine, and antibiotics (Biological Industries, Beit Haemek, Israel) at 37˚C in a 5% CO_2 humidified incubator. Normal human urothelial cells (HUCs) were purchased from ScienCell Research Laboratories (Carlsbad, CA, USA). The cells were grown in the medium specific for HUCs with supplements according to the manufacturer's protocol.

Quantitative real-time RT-PCR (qRT-PCR). MicroRNA expression was measured using a Rotor-Gene 6000. Real-time PCR assays were performed using a miScript PCR Starter kit (Qiagen Korea, Seoul, Korea) as previously described (14). RT-PCR conditions were as follows: 1X initial enzyme activation for 15 min at 95˚C, then 50 cycles of denaturation for 15 sec (94˚C), annealing for 30 sec (55˚C) and extension for 30 sec (70˚C). The melt curve was performed from 70-99˚C at a heating rate of 1˚C/5 sec. Spectral data were analyzed using Rotor-Gene Real-Time Analysis Software 6.0 Build 14. All experiments were performed in triplicate. Expression of miRNAs was normalized to U6 RNA.

Bioinformatics analysis. Screening of putative targets of mir-106a was performed by utilizing the miRanda algorithm (http://www.microrna.org/microrna/home.do) and the NCBI mRNA database (NCBI mRNA DB: http://www.ncbi.nlm.nih.gov/).

Cell proliferation. Cell proliferation was studied using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay as previously described (15). Cellular morphology images were obtained using phase-contrast microscopy.

Transfection. Cells were transfected with mir-106a and its inhibitor using Lipofectamine 2000 transfection reagent (Invitrogen Corp., Carlsbad, CA, USA) according to the manufacturer's protocol. After transfection for 48 h, cells were subjected to further experiments including MTT, immunoblotting, invasion, wound-healing migration, zymography, and electrophoretic mobility shift assays (EMSA).

Flow cytometry cell cycle analysis. Cells were harvested and fixed in 70% ethanol. After washing the cells with 1X ice-cold PBS, they were treated with RNase (1 mg/ml) followed by propidium iodide (50 mg/ml). Cell cycle phase distribution was analyzed using a Becton-Dickinson FACStar flow cytometer equipped with Becton-Dickinson Cell Fit software.

Immunoblot analysis. Preparation of cell lysates and measurement of protein concentrations were performed as described previously (13). Lysates were then electrophoresed on 10% SDS polyacrylamide gels (SDS-PAGE) under denaturing conditions and transferred to nitrocellulose membranes (Hybond, Amersham Corp.). Membranes were blocked with 5% (w/v) non-fat dry milk in TBS [10 mM Tris-HCl (pH 8.0), 150 mM NaCl] followed by incubation with primary antibodies at 4˚C overnight. Then, blots were incubated with secondary antibodies for 90 min. Detection was performed using a Chemiluminescence reagent kit (Amersham Corp.). Experiments were repeated at least thrice.

Immunoprecipitation and immune complex kinase assays. Cell lysates were collected from cell pellets using ice-cold lysis buffer. Briefly, cell lysates were centrifuged at 10,000 x g for 5 min. The supernatants were precipitated by protein-A sepharose beads pre-coated with indicated antibodies at 4˚C for 2 h. Then the beads were washed 4 times with 1 ml lysis buffer and twice with a kinase buffer as previously described (13). Finally, pellets were resuspended in 25 μl of the kinase buffer containing 1 μg of glutathione S-transferase (GST)-pRb C-terminal (pRb amino acids 769-921) fusion protein (Santa Cruz Biotechnology), 20 μM ATP, and 5 μCi of [γ^{32}P]-ATP (4,500 μCi/mmol; ICN). Subsequently, resuspended pellets were incubated for 20 min at 30˚C with occasional mixing. The kinase reactions were terminated by the addition of 25 μl of 2X Laemmli sample buffer and were heated at 100˚C for 5 min. Samples were resolved on 10% SDS-polyacrylamide gels, and then the gels were dried. Radioactive bands were detected on a film.

Wound-healing migration assay. Cells ($3x10^5$) were seeded per well in 6-well plates. Monolayers were scratched with a 2-mm-wide pipette tip. After washing three times with 1X PBS, plates were incubated at 37˚C in serum-free medium. Migration of cells into the scratched area was visualized under an inverted microscope at x40 magnification.

Invasion assay. Invasion assays were performed using an invasion assay kit (Cell Biolabs, USA) according to the manufacturer's instructions. Cells ($2.5x10^4$) were resuspended in serum-free medium and plated in the upper chamber of the apparatus. Medium with 10% FBS was added to the lower chamber as a chemoattractant. After incubation for 24 h, cells in the lower chamber were fixed, stained, and photographed. The invasion potential of the cells was estimated using a commercial cell invasion assay kit (Chemicon International).

Gelatin zymography. Conditioned cell culture media was electrophoresed in a polyacrylamide gel containing 1 mg/ml gelatin. The gel was then washed with 2.5% Triton X-100 for 2 h at room temperature, followed by overnight incubation at 37˚C with a buffer containing 10 mM $CaCl_2$, 150 mM NaCl and 50 mM Tris-HCl (pH 7.5). The gel was photographed on a light box after staining with 0.2% Coomassie blue. Areas of

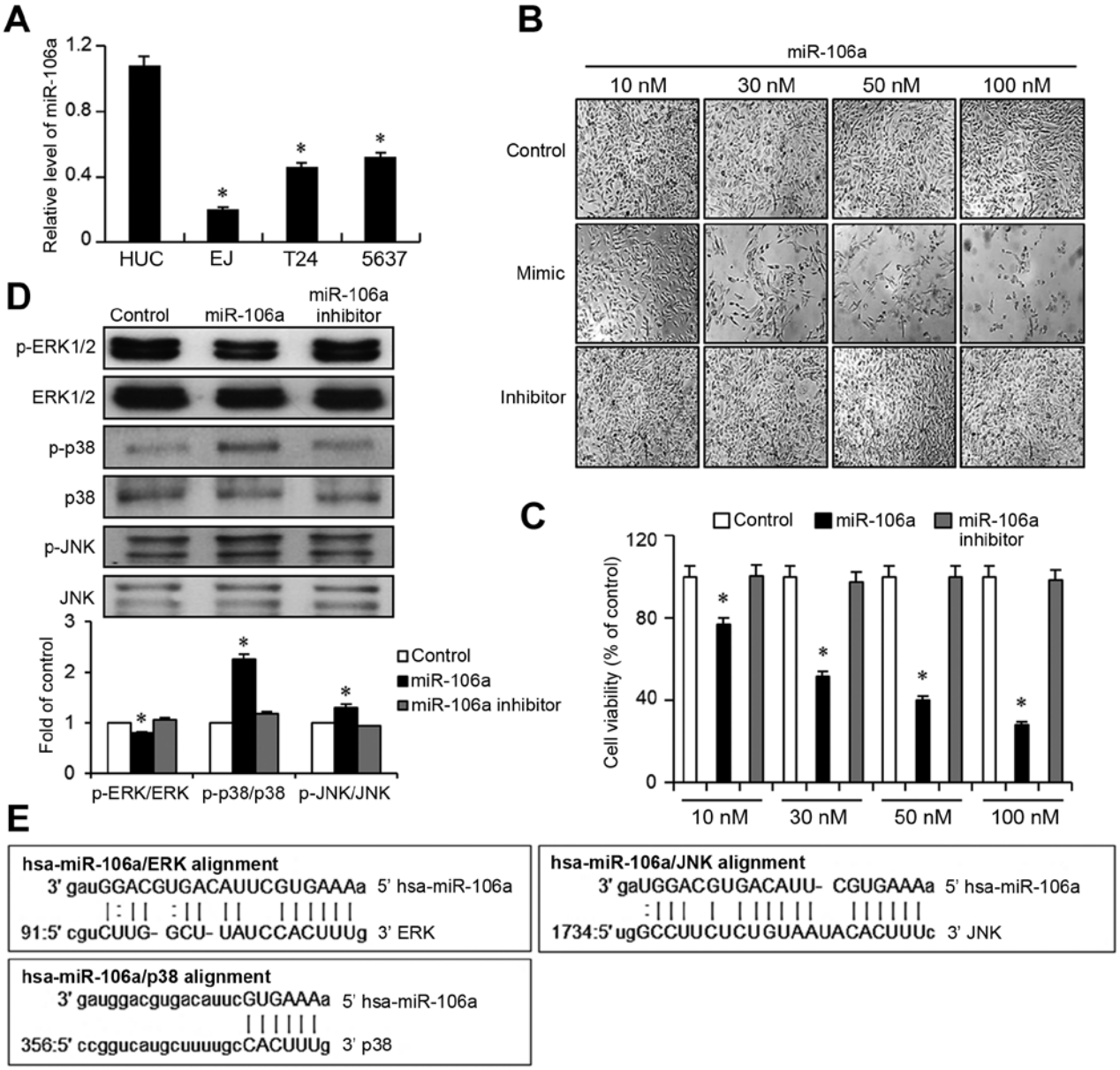

Figure 1. Basal expression of mir-106a is reduced in bladder cancer cells and mir-106a transfection blocks the proliferation of bladder cancer EJ cells via the MAPK pathway. (A) The expression of mir-106a was investigated in 3 bladder cancer cell lines (EJ, T24, and 5637) and normal human urothelial cells (HUC) using quantitative real-time PCR analysis. mir-106a expression was normalized to U6 expression. Results are expressed as the means ± SE from triplicate experiments. *Significantly different from control at P<0.01. (B) Cellular morphologies of EJ cells transfected with the indicated doses (nM) of mir-106a, mir-106a inhibitor, or Lipofectamine alone (control) were captured by phase contrast images 48 h after transfection. (C) Proliferation rate of EJ cells transfected with mir-106a as measured by MTT assay 48 h after transfection. Results are expressed as the means ± SE from triplicate experiments. *Significantly different from control at P<0.01. (D) Modulation of MAPKs by the transfection of mir-106a in EJ cells. After 48 h of transfection, activation of each MAPK (phospho-ERK1/2, phospho-p38, and phospho-JNK) was assessed by immunoblotting. (E) Sequence alignments of mir-106a to the 3'-UTR of ERK, p38, and JNK were obtained from the miRanda bioinformatics DB and NCBI mRNA DB. Results are expressed as the means ± SE from triplicate experiments. *Significantly different from control at P<0.01.

gelatinase activity were verified as a clear band in a dark blue field.

Preparation of nuclear extracts and EMSA. Nuclear proteins were prepared as described previously (16). Briefly, cells were washed, scraped, and resuspended in a buffer containing 10 mM HEPES (pH 7.9), 10 mM KCl, 1 mM DTT, 0.5 mM PMSF, 0.1 mM EDTA, and 0.1 mM EGTA. Then the cells were lysed, homogenized, and centrifuged. The nuclear pellets were extracted with an ice-cold high salt buffer containing 0.4 M NaCl, 20 mM HEPES (pH 7.9), 1 mM EDTA, 1 mM EGTA, 1 mM DTT, and 1 mM PMSF. After centrifugation, supernatants were collected. Protein concentration was measured using the Bradford reagent method (Bio-Rad). Electrophoretic mobility shift assay (EMSA) was accomplished as previously described (16). Briefly, the oligonucleotides spanning the MMP-2 *cis* element of interest were end-labeled with ^{32}P-ATP by T4 polynucleotide kinase (Promega, Madison, WI, USA). The nuclear extracts were incubated with a radiolabeled oligonucleotide probe (10,000 cpm) for 20 min at 4˚C in a

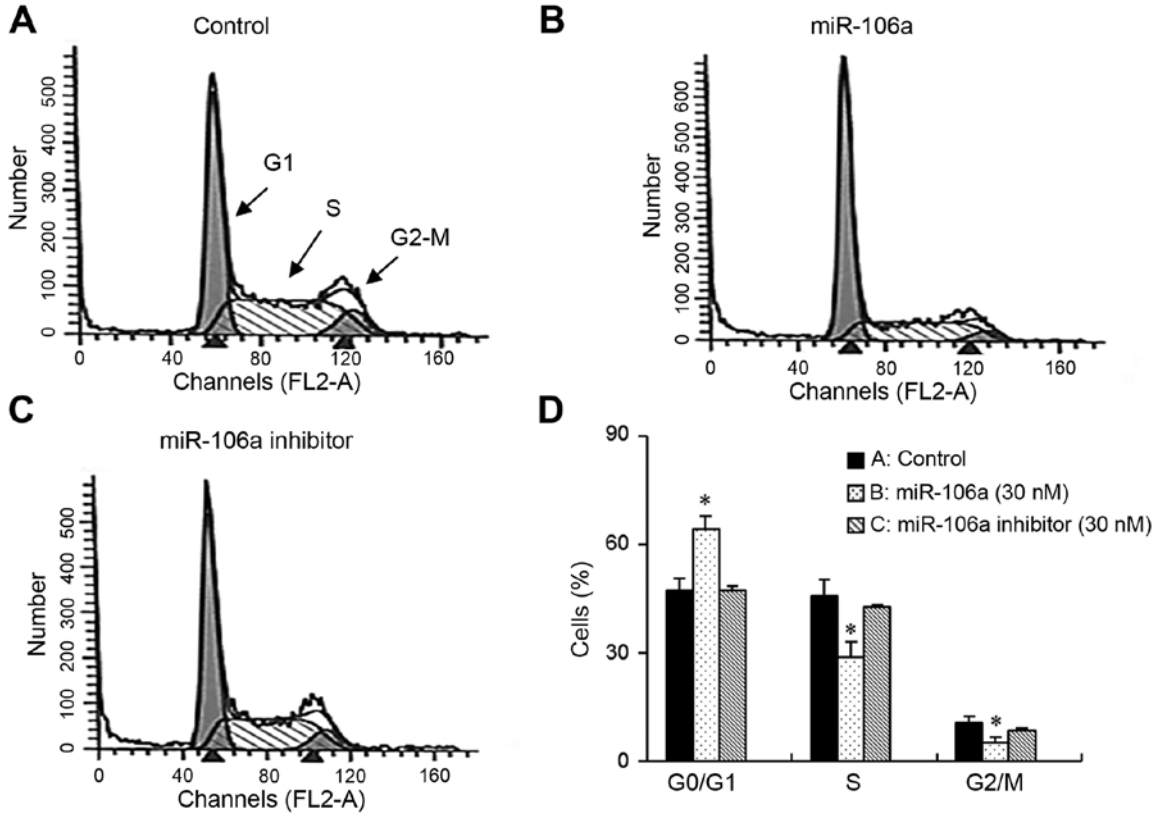

Figure 2. Transfection of a mir-106a mimic induces G1-phase cell cycle arrest in EJ cells. (A-C) After 48 h of transfection, flow cytometry analysis was performed to determine the effect of mir-106a on cell cycle phase distribution. (D) The percentage of cell distribution in each cell cycle phase is represented as the means ± SE from triplicate experiments. *Significantly different from control at P<0.01.

binding buffer containing 25 mM HEPES (pH 7.9), 50 mM NaCl, 0.5 mM EDTA, 0.5 mM DTT, 2.5% glycerol, and 2 μg of poly dI/dC. The DNA-protein complexes were separated on 6% SDS-PAGE gels at 4˚C with TBE running buffer (89 mM Tris, 89 mM boric acid, and 1 mM EDTA). The gel was washed, dried, and then exposed to X-ray film for 10 h. The sequences for the oligonucleotides were as follows: Ets-1, GATCTCGA GCCGGAAGTTCGA; Sp-1, GCCCATTCCTTCCGCCCCC AGATGAAGCAG; ATF-2, AGAGATTGCCTGA CGTCAGA GAGCTAG; CREB, AGAGATTGCCTGACGTCA GAGAGC TAG; and C/EBP, TGCAGATTGCGCAATCTGCA. For the competition of Ets-1, Sp-1, ATF-2, and CREB, each specific polyclonal antibody was supplied to the binding reaction prior to the incubation of radiolabeled probe.

Statistical analysis. Where appropriate, data are represented as the means ± SE. Data were evaluated by factorial ANOVA and a Fisher's least significant difference test where appropriate. Statistical significance was considered at P<0.05.

Results

Expression of mir-106a is downregulated in bladder cancer cells. In order to investigate the role of mir-106a in bladder cancers, we first measured the basal expression levels of mir-106a in bladder cancer cell lines EJ, T-24, and 5637, and compared their miR expression with normal human urothelial cells (HUC). Quantitative real-time PCR results showed that all bladder cancer lines tested exhibited significantly lower levels of mir-106a than HUC cells (Fig. 1A). Because mir-106a expression was minimal in EJ cells, ~20% of the expression seen in HUCs, EJ cells were subjected to further experiments. We next assessed the effect of mir-106a on the proliferation of EJ bladder cancer cells by transfecting a mir-106a mimic, a mir-106a inhibitor, or Lipofectamine 2000 only (control). As seen in Fig. 1B, transfection of mir-106a significantly inhibited the proliferation of EJ cells in a dose-dependent manner. Control or inhibitor treatment elicited no changes in cell growth. In accordance, mir-106a-transfected EJ cells showed a dose-dependent decrease in cell viability (Fig. 1C). Based on the cell growth study, subsequent experiments were performed using 30 nM (around the IC_{50}) of mir-106a. We then hypothesized that transfection of miR106a into EJ cancer cells may modulate molecular effectors associated with proliferation. To narrow the candidates, we utilized the microRNA bioinformatics database miRanda (http://www.microrna.org/microrna.home.do). Nucleotide sequence alignment suggested that mitogen-activated protein kinases (MAPKs) including ERK, p38, and JNK might be targets of mir-106a (Fig. 1E). To validate this screening result, we performed immunoblot assays to determine whether the MAPKs were modulated by the transfection of mir-106a. Interestingly, introduction of mir-106a into EJ cancer cells led to a downregulation of ERK phosphorylation, while p38 phosphorylation was increased >2-fold and JNK phosphorylation was increased moderately (Fig. 1D).

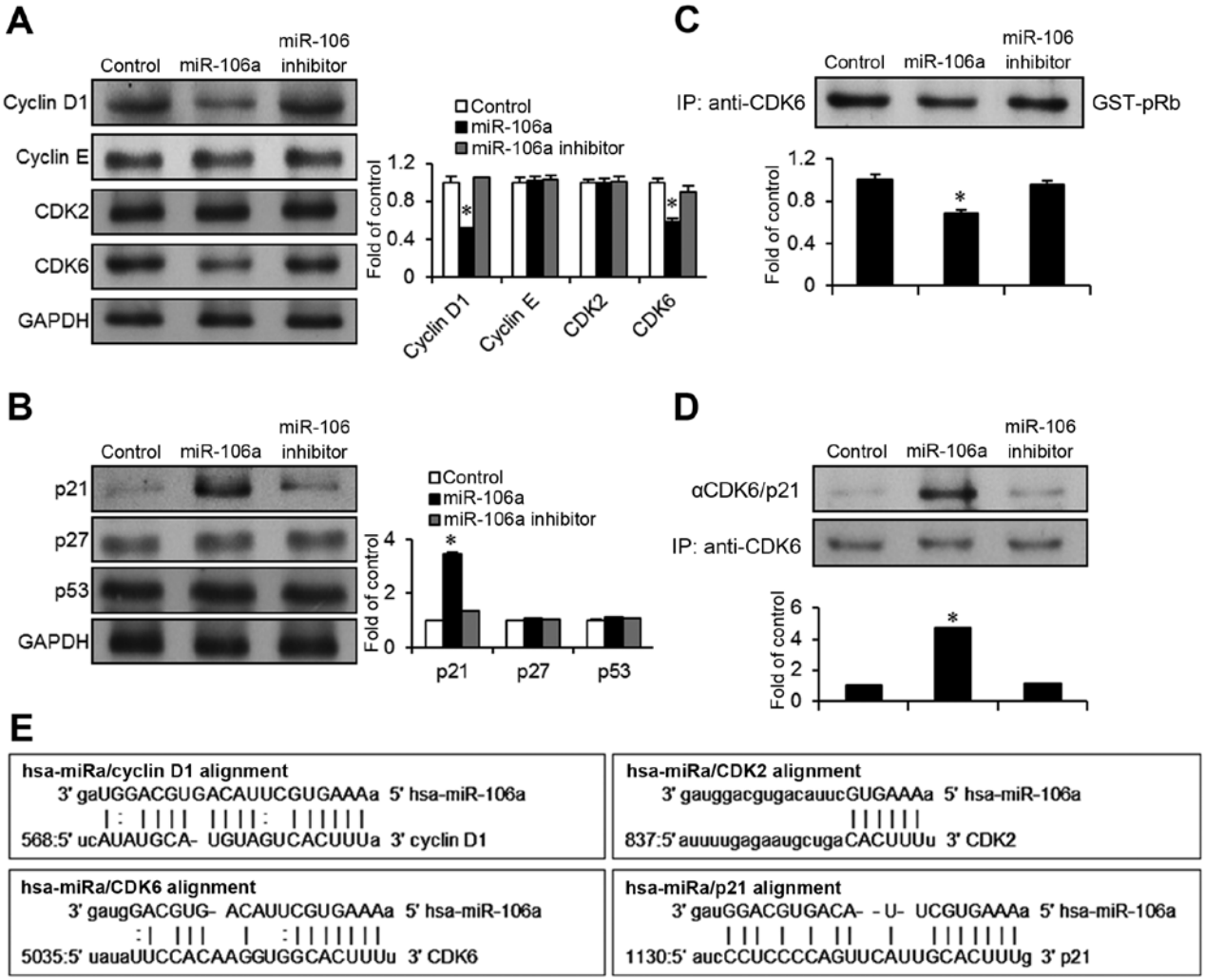

Figure 3. mir-106a inhibits the expression of cyclin D1 and CDK6, leading to the induction of $p21^{CIP1/WAF1}$ in EJ cells. (A and B) Immunoblot assays were performed after transfection of mir-106a, mir-106a inhibitor, or Lipofectamine alone (control). GAPDH was used as a loading control. (C) After transfection, immunoprecipitation (IP) was performed using an anti-CDK6 antibody. Kinase activity was assessed by adding GST-pRb as the substrate. (D) Pull-down assays were performed after immunoprecipitation of total cell lysates using a CDK6 antibody followed by immunoblotting using either a $p21^{CIP1/WAF1}$ or CDK6 antibody. (E) Sequence alignment of the mir-106a base-paring site in the 3'-UTR of cyclin D1, CDK6, CDK2, and $p21^{CIP1/WAF1}$. All results are expressed as the means ± SE from triplicate experiments. *Significantly different at $P<0.01$.

mir-106a leads to G1-phase cell cycle arrest in bladder cancer cells. We speculated that the growth inhibition of bladder cancer cells induced by mir-106a might be due to cell cycle arrest. Therefore, we investigated cell cycle phase distribution in mir-106a-transfected cells using flow cytometry. As demonstrated in the DNA histograms, mir-106a resulted in an accumulation in the G1 phase of the cell cycle. However, control cells and cells treated with the mir-106a inhibitor showed no changes in cell cycle distribution (Fig. 2A-C). In accordance with the accumulation of cells in G1, the number of cells in S-phase was reduced (Fig. 2D).

mir-106a targets cyclin D1/CDK6 leading to upregulation of $p21^{CIP1/WAF1}$ in bladder cancer cells. In order to identify molecular targets of mir-106a, we used a microRNA database (miRanda) to find potential cell cycle regulators associated with the inhibition of G1-S cell cycle progression. We identified cyclin D1, CDK2, CDK6, and $p21^{CIP1/WAF1}$ as candidates based on the miRanda bioinformatics algorithm (Fig. 3E). Immunoblot assays were performed to investigate possible changes in the expression of those candidates after transfection with mir-106a. Interestingly, mir-106a transfection induced a downregulation in both cyclin D1 and CDK6 in EJ cancer cells (Fig. 3A). However, cyclin E and CDK2 levels were unchanged. In addition, among the cell cycle inhibitors examined, $p21^{CIP1/WAF1}$ expression was significantly increased >3-fold over expression in the control (Fig. 3B). However, $p27^{KIP1}$ and p53 were not altered by mir-106a transfection.

Since cyclin D1/CDK6 forms a complex that phosphorylates the Rb protein to aid cell cycle progression, we investigated the kinase activity of CDK6. Cell lysates from the mir-106a transfectants were immunoprecipitated with a CDK6 antibody, and then GST-pRb was added as the substrate with a kinase buffer containing radiolabeled ^{32}P-ATP. The kinase activity of CDK6 was reduced >30% in mir-106a transfectants compared to the control or transfection with the mir-106a inhibitor (Fig. 3C). This suggests that mir-106a clearly targets cyclin D1/CDK6-associated growth signals in bladder cancer

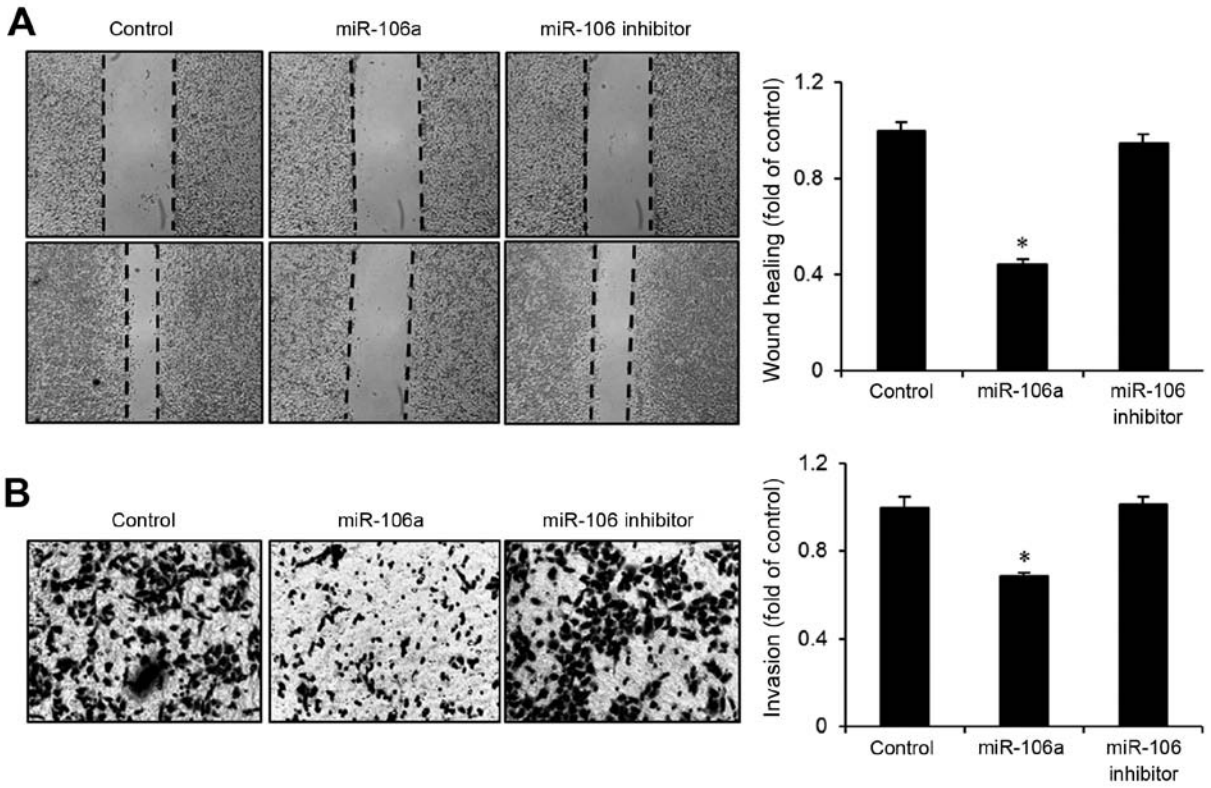

Figure 4. Transfection of mir-106a represses migration and invasion of EJ cells. (A) After transfection of mir-106a, scratch wound healing assays were performed. Wound closure ratios are shown. (B) Boyden chamber assays were employed to validate the invasion of EJ cells. Results are expressed as the means ± SE from triplicate experiments. *Significantly different at P<0.01.

cells. Upregulation of $p21^{CIP1/WAF1}$ expression by mir-106a was again validated in immunoprecipitation experiments with a CDK6 antibody followed by immunoblotting with an antibody against $p21^{CIP1/WAF1}$ or CDK6 (control). Complex formation of $p21^{CIP1/WAF1}$ with CDK6 was increased >4-fold in the mir-106a-transfected cells compared to the control (Fig. 3D). Taken together, these results demonstrate that $p21^{CIP1/WAF1}$ is a critical effector in mir-106a-mediated G1-phase cell cycle arrest and growth inhibition.

mir-106a suppresses migration and invasion of bladder cancer cells. When normal bladder cells are transformed, they frequently acquire the ability to invade other tissues. We investigated whether the transfection of mir-106a suppresses the migration and invasion of bladder cancer cells. In wound-healing assays, mir-106a transfectants exhibited slower growth into the wounded area than control cells or cells transfected with the mir-106a inhibitor (Fig. 4A). Wound-closure rate was ~60% slower after mir-106a transfection. Similarly, Boyden chamber assays showed that invasiveness of EJ bladder cancer cells was reduced >30% after mir-106a transfection (Fig. 4B). These results suggest that mir-106a possesses a tumor suppressive function in the migration and invasion of bladder cancer cells.

mir-106a downregulates MMP-2 expression via activation of Ets-1. Transformation of bladder epithelia often includes the upregulation of proteases such as MMPs to create spaces for the cancer cells to migrate. Using the miRanda sequence alignment algorithm for mir-106, we found several putative targets including MMP-2, Ets-1, Sp-1, ATF-2, CREB, and C/EBP (Fig. 5C). In order to verify whether MMP-2 is modulated by mir-106a transfection, we performed gelatin zymography assays. In conditioned culture medium obtained from mir-106a transfectants, gelatinase activity of MMP-2 was significantly lower (>60%) than in the control. Correspondingly, in immunoblot assays, MMP-2 protein expression was significantly lower than that in the control or the mir-106 inhibitor (Fig. 5A). We next investigated which MMP-2-mediated transcription factors are modulated by mir-106a. Because Ets-1, Sp-1, ATF-2, CREB, and C/EBP were the candidates identified from the miRanda DB search, we assessed alterations in binding activities of these factors in mir-106a transfectants using EMSA assays. Among transcription factors tested, only Ets-1 exhibited a reduced activity in the binding motif (Fig. 5B). Activities of Sp-1, ATF-2, CREB, and C/EBP were unchanged by the introduction of mir-106a. These results suggest that mir-106a abrogates the expression of MMP-2 modulated by the transcription factor Ets-1, resulting in the inhibition of migration and invasion in bladder cancer EJ cells.

Discussion

Bladder cancer is one of the most notorious malignancies in Western countries. The incidence is estimated to be 1 in 26 for males, and ~74,000 new cases were diagnosed in 2015 in the US (17). In this study, we report the novel finding that mir-106a regulates the proliferation, migration, and invasion

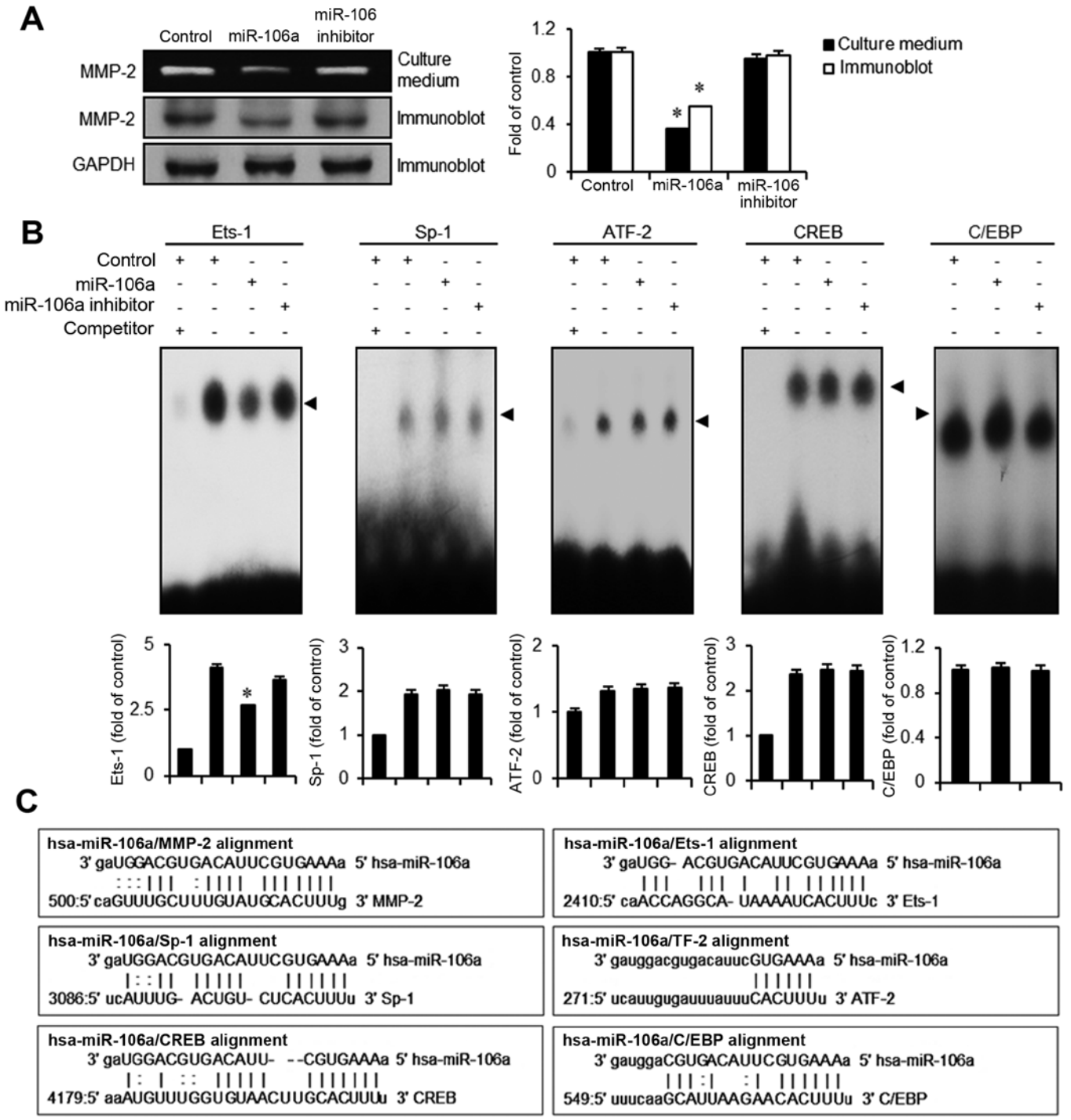

Figure 5. Overexpression of mir-106a leads to inhibition of MMP-2 by hindering Ets-1 binding activity in EJ cells. (A) Activity of MMP-2 was measured using gelatin zymography for the mir-106a-transfected EJ cells. (B) Assessment of alteration in transcription factor binding motif activation in mir-106a-transfected EJ cells using EMSA. For the competition (competitor) assay, each polyclonal antibody (1 μg) against Ets-1, Sp-1, ATF-2, and CREB was added. (C) Sequences of target sites for mir-106a in the 3'-UTR of MMP-2 and transcription factors. Results are expressed as the means ± SE from triplicate experiments. *Significantly different at P<0.01.

of bladder cancer cells through modulating effectors such as early signaling molecules (ERK, p38MAPK, and JNK), cell cycle regulators (cyclin D1, CDK6, and $p21^{CIP1/WAF1}$), and extracellular protein degradation enzymes (MMP-2 and Ets-1).

Initially, we examined the basal expression of mir-106a in three bladder carcinoma cell lines. The basal expression level of mir-106a was significantly lower in cancer cell lines in comparison to control. The basal expression of mir-106a in cancers is controversial and seems to be dependent on tumor type (18-21). For example, Volinia and colleagues performed miRnome analysis to verify whether the expression of microRNA correlates with malignancies, and a total of 540 clinical specimens including lung, breast, stomach, prostate, colon, and pancreatic cancers were investigated. mir-106a was determined to be upregulated in colon, pancreas, and prostate cancers. However, in breast cancers, mir-106a

expression was downregulated with respect to its expression in corresponding normal tissues (22). Controversially, Diaz and coworkers reported that mir-106a expression was significantly downregulated in tumor specimens from 110 colorectal cancer patients, and is correlated with poor survival (P<0.04) (21). In agreement with some previous results, our results showed lower expression of mir-106a in bladder cancer cells compared to normal urothelial cells. Cell viability assays showed a dose-dependent decrease of EJ cancer cell proliferation after transfection with mir-106a. Interestingly, ERK phosphorylation was significantly reduced by mir-106a transfection, whereas phosphorylation of p38 and JNK was upregulated. Hyperactivation of MAPKs, particularly ERK, or genetic mutations in the RAS-RAF-MEK-ERK signaling cascade have been reported as critical events in tumorigenesis of various tissue types. For example, in melanoma, BRAF mutation at position 600 (V600E) has been reported as a mutation hot spot or signature mutation (23-25). Even melanomas with wild-type BRAF frequently show increased phosphorylation of ERK through autocrine stimulation with growth factors such as fibroblast growth factor (FGF) and insulin-like growth factor (IGF) (26,27). This suggests that cancer cells may be addicted to growth stimuli from ERK activation. Therefore, it is noteworthy that mir-106a inhibits ERK signaling in bladder cancers. In addition to the inhibition of phospho-ERK signaling, activation of stress-activated protein kinases p38 and JNK (28) was observed in mir-106a transfectants. These results suggest that mir-106a exerts an antiproliferative effect on bladder cancer cells through modulating the MAPK signaling pathway.

Cell cycle analysis showed that mir-106a-transfected bladder cancer cells accumulated in G1-phase, suggesting that cyclins might be associated with mir-106a-mediated cell cycle arrest. In bladder cancers, cyclin D1 and E were reported to be key regulators associated with disease initiation and progression (29,30). We performed an immunoblot assay and a kinase complex assay to verify which cyclin participated in the mir-106a signaling pathways. Interestingly, only cyclin D1 was shown to be modulated by mir-106a. This result was supported by the observation that the activity of its corresponding kinase CDK6 was equally diminished by transfection with mir-106a. As a matter of fact, cumulative results have demonstrated that a 6-7 base seed sequence was sufficient for miRNA regulation (31,32). Therefore, we employed the 6 base sequence of CDK2 to know whether mir-106a regulate the CDK2 level in bladder cancer cells. In this study our results indicated that 6 base seed sequence of CDK2 did not influence the miRNA regulation. This result suggested that CDK2 was not affected by the introduction of mir-106a in bladder cancer cells. We next investigated whether CDK inhibitors were correspondingly altered by mir-106a transfection. Among CDK inhibitors, $p21^{CIP1/WAF1}$ was significantly upregulated in mir-106a transfectants. This was further verified by immunoprecipitation assays using a CDK6 antibody followed by immunoblotting with a $p21^{CIP1/WAF1}$ antibody. A recent study demonstrated that CDK6 might be an effective target of mir-29c for suppressing bladder cancer progression (33). In agreement with those results, our data showed that mir-106a inhibited cyclin D1 and CDK6 expression in bladder cancer cells. This study demonstrated that mir-106a inhibits proliferation of bladder cancer EJ cells by targeting the $p21^{CIP1/WAF1}$/cyclin D1/CDK6 cell cycle cascade.

Previous studies have shown that MMP-2 upregulation in bladder cancer is a key step in migration and invasion (6,13,16). MicroRNAs, including mir-430 and mir-20b, are known to be capable of regulating migration and invasion by coordinating MMP-2 expression (13,34). In this study, mir-106a overexpression blocked the migration and invasion of bladder cancer cells. The inhibitory effect was in part due to the inhibition of MMP-2 activity as verified by gelatin zymography assays and immunoblotting. MMP-2 expression in bladder cancers is tightly regulated by transcription factors including p53, AP-1, Ets-1, C/EBP, CREB, PEA3, Sp1, ATF2, and AP-2 (35). In this study, Ets-1 was identified as an essential transcription factor responsible for the mir-106a-mediated MMP-2 inhibition in bladder cancer cells. Our results show that mir-106a may suppress migration and invasion by downregulating Ets-1 and MMP-2 expression in bladder cancer cells.

In conclusion, we verified that the expression level of mir-106a is abnormally downregulated in bladder cancer cells. Introduction of mir-106a impaired the proliferation of bladder cancer EJ cells through modulating MAPK (ERK/p38/JNK) signaling and cell cycle regulators ($p21^{CIP1/WAF1}$/cyclin D1/CDK6). In addition, mir-106a overexpression impeded MMP-2 expression by inactivating the Ets-1 binding motif, which led to a reduction in the migration and invasion of bladder cancer cells. Therefore, our data suggest that mir-106a might serve as a potential target for treating bladder malignancies. Furthermore, our results indicate that bioinformatics might be a useful tool for predicting the targets of miRs involved in the regulation of tumor cells.

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