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Original Paper

miR-186-5p Functions as a Tumor Suppressor in Human Osteosarcoma by Targeting FOXK1

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Key Words
miR-186-5p • Osteosarcoma • FOXK1 • Suppressor

Abstract
Background/Aims: Aberrantly expressed miRNAs play a vital role in the development of some cancers, such as human osteosarcoma (OS). However, the detailed molecular mechanisms underlying miR-186-5p-involved osteosarcoma are unclear. ***Methods:*** qRT-PCR and western blot analysis were employed to measure the expressions of miR-186-5p and forkhead box k1 (FOXK1). CCK-8 assay evaluated the effect of miR-186-5p and FOXK1 on cell proliferation. Transwell assay confirmed cell migration and invasion. Eventually, the dual-luciferase reporter assay validated 3′-untranslated region (3′-UTR) of FOXK1 as a direct target of miR-186-5p. ***Results:*** Down-regulation of miR-186-5p was identified in OS tissues and cell lines, and negatively correlated with distant metastasis, Enneking stage and poor 5-year prognosis as well as the expression of forkhead box k1 (FOXK1) protein. Further assays demonstrated that miR-186-5p overexpression had inhibitory effects on in-vitro cell proliferation, cell cycle, and in-vivo tumor growth. miR-186-5p overexpression also inhibited the epithelial-to-mesenchymal transition (EMT), migration and invasion of OS cells. Importantly, miR-186-5p directly targeted FOXK1 3′-UTR and negatively regulated its expression. Silencing of FOXK1 expression enhanced the inhibitory effects of miR-186-5p on OS cell proliferation, migration and invasion. ***Conclusion:*** These findings highlighted miR-186-5p as a tumor suppressor in the regulation of progression and metastatic potential of OS, and may benefit the development of therapies targeting miR-186-5p in patients with OS.

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Introduction

Osteosarcoma is a commonly malignant bone cancer with approximately 3, 000, 000 new cases every year [1]. Concurrent chemo-radiotherapy should be a treatment option for advanced patients with OS [2]. Although the patients with OS can accept the resection surgery, less than 20% of patients suffered from recurrence because OS cells often metastasize to other organs [3]. Thus, it is crucial to develop a novel therapeutic strategy to treat patients with OS more effectively.

MicroRNAs (miRNAs) are small non-coding RNAs with 19-23 nucleotides in length. MiRNAs regulate the expression of target genes by binding to 3′-UTR of target genes at a post-transcriptional level. miR-186-5p has been identified to be involved in the initiation and development of tumors [4-7]. Li J et al. reported that miR-186-5p upregulation inhibited proliferation, metastasis and EMT of colorectal cancer cell by targeting ZEB1 [7]. Besides, FOXK1, a member of the FOX transcription factor family, has been documented to play a crucial role in cell proliferation, metastasis, and metabolism [8]. Recently, increasing evidence indicated FOXK1 was dysregulated in varies of human cancers, including colorectal cancer [9], esophageal cancer [10] and hepatocellular carcinoma [11]. However, the expression and role of miR-186-5p and FOXK1 in OS have never been investigated.

In the present study, we aimed to investigate the expression and significance of miR-186-5p in OS tissues and cells, and discovered the inhibitory effects of miR-186-5p on proliferation, migration and invasion of OS cells via targeting FOXK1 expression using in-vitro and in-vivo assays.

Materials and Methods

Tissue samples

A total of 60 primary OS tissues and matched adjacent non-tumorous tissues were collected from patients with OS in Shandong Provincial Third Hospital, Shandong Cancer Hospital (Jinan, China), and Wuhan Third Hospital, Tongren Hospital of Wuhan University (Wuhan China). Written informed consent was obtained from all patients. All the tissue samples were obtained after surgery resection and immediately snap-frozen in liquid nitrogen for subsequent experiments. None of the OS patients had received radiation therapy or chemotherapy prior to surgery. The present study was approved by the Ethics Committee of Shandong Provincial Third Hospital (Jinan, China).

Cell culture

Human OS cell lines (U2OS, MG-63 and Saos-2) and the normal human osteoblast cell line hFOB1.19 were purchased from the American Type Culture Collection. Human embryonic kidney-293T (HEK293T) cells were obtained from Shandong University. All cell lines were cultured in the Dulbecco's Modified Eagle's Medium (DMEM, Thermo Fisher Scientifc, Inc.) supplemented with 10 % fetal bovine serum (FBS, Gibco, Grand Island, NY.) and maintained in a humidified atmosphere containing 5 % CO_2 at 37 °C.

Reverse transcription-quantitative polymerase chain reaction (qRT-PCR)

Total RNA from cells was extracted using TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.) according to manufacturer's protocol. First-strand cDNA synthesis was performed using the TaqMan MicroRNA Reverse Transcription kit and miR-186-5p RT primers (Applied Biosystems; Thermo Fisher Scientific, Inc.). The temperature protocol of first-strand cDNA synthesis was: 16°C for 30 min, 42°C for 30 min and 85°C for 5 min. qRT-PCR was performed using a miR-186-5p TaqMan MicroRNA Assay and TaqMan Universal Master Mix II on an ABI PRISM 7500 (Applied Biosystems; Thermo Fisher Scientific, Inc.). Thermocycler conditions used as follow: 95°C for 5 min, followed by 40 cycles at 95°C for 30 sec, 55°C for 40 sec and 72°C for 30 sec. The expression of miR-186-5p was normalized to that of U6. Data analysis was performed using the $2-\Delta\Delta CT$ method. For RT-PCR, the amplified PCR products were resolved by electrophoresis on 2% agarose gels and were visualized by ethidium bromide staining.

Western blot analysis

The proteins were extracted by lysing the cells in sample loading buffer. The composition of the loading buffer was 1.5% SDS, 10% glycerol, 5 mM β-mercaptoethanol, bromphenol blue and 75 mM Tris (pH 7.0). Whole cell lysates were separated by SDS-PAGE (12% gel) and the proteins were transferred onto a polyvinylidene fluoride membrane. Subsequently, the membranes were incubated with anti-FOXK1 (1: 200; ab18196; Abcam, Cambridge, MA, USA) and anti-GAPDH antibodies (1: 200; Abcam, Cambridge, MA, USA) at 4°C overnight. Subsequent to incubation, the membranes were incubated with a goat anti-mouse horseradish peroxidase-conjugated immunoglobulin G secondary antibody (cat. no. sc-2005; dilution, 1:5, 000; Santa Cruz Biotechnology, Inc.) at room temperature for 2 h. Finally, the immunoreactive bands were visualized using an enhanced chemiluminescence western blotting kit (Pierce; Thermo Fisher Scientific, Inc.).

Cell transfection

miR-186-5p mimics, negative control miRNAs (miR-NC), and luciferase reporter plasmid were synthesized by Shanghai GenePharma Co., Ltd. (Shanghai, China). FOXK1 siRNA (si-FOXK1) and negative control siRNAs (si-control) were obtained from Ambion (Thermo Fisher Scientific, Inc.). Cells in the exponential phase of growth were seeded in a 6-well plate and cultured in DMEM without antibiotics. Cell transfection was performed using Lipofectamine™ 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) when the cell density reached 50–60%, according to the manufacturer's protocol.

Cell proliferation assay

Cells transfected with miR-186-5p mimics or miR-NC were seeded in 96-well plates at 2×10^3/well, respectively, and cultured for 24, 48, 72, and 96 h. Ten microliters CCK-8 (Dojindo, Kumamoto, Japan) was added to the cells for 3-4 h, and their viability was measured at 450 nm using SpectraMax M5 Microplate Reader, according to the manufacturer's instructions.

Cell migration and invasion assays

Briefly, 1×10^4 cells were placed on the top of transwells with 8.0-μm pore polycarbonate membrane inserts (Corning, New York, NY) for the migration assay. For the invasion assay, the inserts were coated with a thin layer of Matrigel basement membrane matrix (BD Biosciences San Diego, CA). Serum (10%) was used as the chemoattractant. After 24 h, the cells on the lower surface of the inserts were fixed with methanol for 15 min, stained with 1% crystal violet solution for 15 min, and counted using a light microscope.

Cell apoptosis analyses

Cell apoptosis analyses were performed using a Phycoerythrin-Annexin V Apoptosis Detection kit I (BD Pharmingen; BD Biosciences, San Jose, CA, USA). For cell apoptosis analysis, 1×10^5 cells were seeded in 6-well plates. At 78 h after transfection, cells were harvested and labeled with Annexin V for 15 min. Subsequently, 50 μg/ml of propidium iodide was added for 1 h at 37°C to each sample prior to flow cytometry using the BD LSR II (BD Biosciences).

Plasmid construction and luciferase reporter assay

TargetScan (http://www.targetscan.org/) was used to predict potential target genes of miR-186-5p and identified FOXK1 as a potential target. Firstly, we cloned miR-186-5p mimics into pmiR-RB-REPORT™ vector, and inserted the wild type (Wt) target sequences on FOXK1 into pcDNA3.1 plasmid vector (pcDNA3.1-FOXK1-3'-UTR-Wt). Secondly, the mutation (Mut) fragment was inserted into pcDNA3.1 plasmid vector (pcDNA3.1-FOXK1-3'-UTR-Mut). Luciferase reporter activity was measured after co-transfection with miR-186-5p mimics or negative control and pcDNA3.1-FOXK1-Wild type (Wt) or pcDNA3.1-FOXK1-Mutant (Mut) into cells. The experiment kit used was the Dual-Luciferase® Reporter Assay System (Promega, Madison, WI, USA). Renilla luciferase was used as normalization.

Animal experiments

A total of 30 female BALB/c nu/nu mice (6-8 weeks old and weight 18–20 g; Jinan, China) were housed together in specific pathogen-free conditions with a temperature of 25°C, 55–65% relative humidity, and a 12-h light-dark cycle with standard chow and water ad libitum. 2×10^6 cells infected with lentiviral vectors expressing the miR-186-5p or negative control were inoculated subcutaneously into the flanks of nude mice (five mice per group). The mice were closely monitored for tumor growth, and tumor size was measured every 4 days. Tumor volume was calculated using the following formula: Tumor volume=0.5 × width^2 × length. After 4 weeks, the mice were sacrificed by cervical dislocation and tumor weights were measured. All animal procedures were performed in accordance with protocols approved by the Institute Research Ethics Committee of Shandong Provincial Third Hospital.

Statistical analysis

Data were analyzed using SPSS version 19.0 software (SPSS, Inc., Chicago, IL, USA). Data are expressed as the mean ± standard deviation (SD). Comparisons were performed using an unpaired Student's t-test or Mann-Whitney U test for two groups or with a one-way analysis of variance, followed by the least significant difference post hoc test, for three groups. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

miR-186-5p is down-regulated in OS tissues and cell lines

To figure out the role of miR-186-5p in the development of OS, the expression level of miR-186-5p was evaluated in OS tissues (n=60) and adjacent normal tissues (n=60). The qRT-PCR analysis indicated that the miR-186-5p level was significantly decreased in OS cancer tissues compared with adjacent normal tissues ($P < 0.001$; Fig. 1A-B). Subsequently, the expression of miR-186-5p in human OS cell lines was assessed. Consistent with OS cancer tissues, we found that miR-186-5p expression was downregulated in human OS cell lines (U2OS, MG-63 and Saos-2) compared with the normal human osteoblast cell line hFOB1.19 ($P < 0.001$; Fig. 1A-B). Thus, these results suggested that the reduced expression of miR-186-5p may be involved in the malignant progression of OS.

FOXK1 is up-regulated in OS tissues and cells

To evaluate whether miR-186-5p was negatively correlated with FOXK1 expression, the expression of FOXK1 mRNA and protein was subsequently tested in OS tissues and cells. As shown in Fig. 1A and 1B, FOXK1 mRNA and protein expressions were significantly up-regulated in OS tissues as compared with adjacent non-cancerous tissues ($P<0.01$; Fig. 1B). Similar to cancer tissues, FOXK1 mRNA and protein were up-regulated in human OS cell lines (U2OS, MG-63 and Saos-2) compared with the normal human osteoblast cell line hFOB1.19 ($P < 0.001$; Fig. 1A, 1B). Thus, our results indicated that up-regulated expression of FOXK1 may also be involved in the progression of OS.

Associations of miR-186-5p and FOXK1 levels with clinicopathological factors and prognosis

In order to investigate whether miR-186-5p and FOXK1 levels were correlated with clinicopathological factors in patients with OS, statistical analysis was carried out. The results demonstrated that the low expression level of miR-186-5p was negatively associated with distant metastasis (Yes v.s. No; P=0.003) and Enneking stage (I-IIA v.s. IIB-III; P=0.001) in patients with OS, whereas no significant associations were observed between miR-186-5p expression and gender (male v.s. female; P=0.439), age (<18 v.s. ≥18; P=0.390), anatomical location (tibia/femur v.s. Other; P=0.188) or tumor size (<8 cm v.s. ≥8 cm; P=0.075). In addition, FOXK1 mRNA and protein expressions were also associated with distant metastasis (P=0.001, P=0.003, respectively) and Enneking stage ($P < 0.001$, $P < 0.001$, respectively) in patients with OS. To further confirm the role of miR-186-5p and FOXK1 in 5-year overall

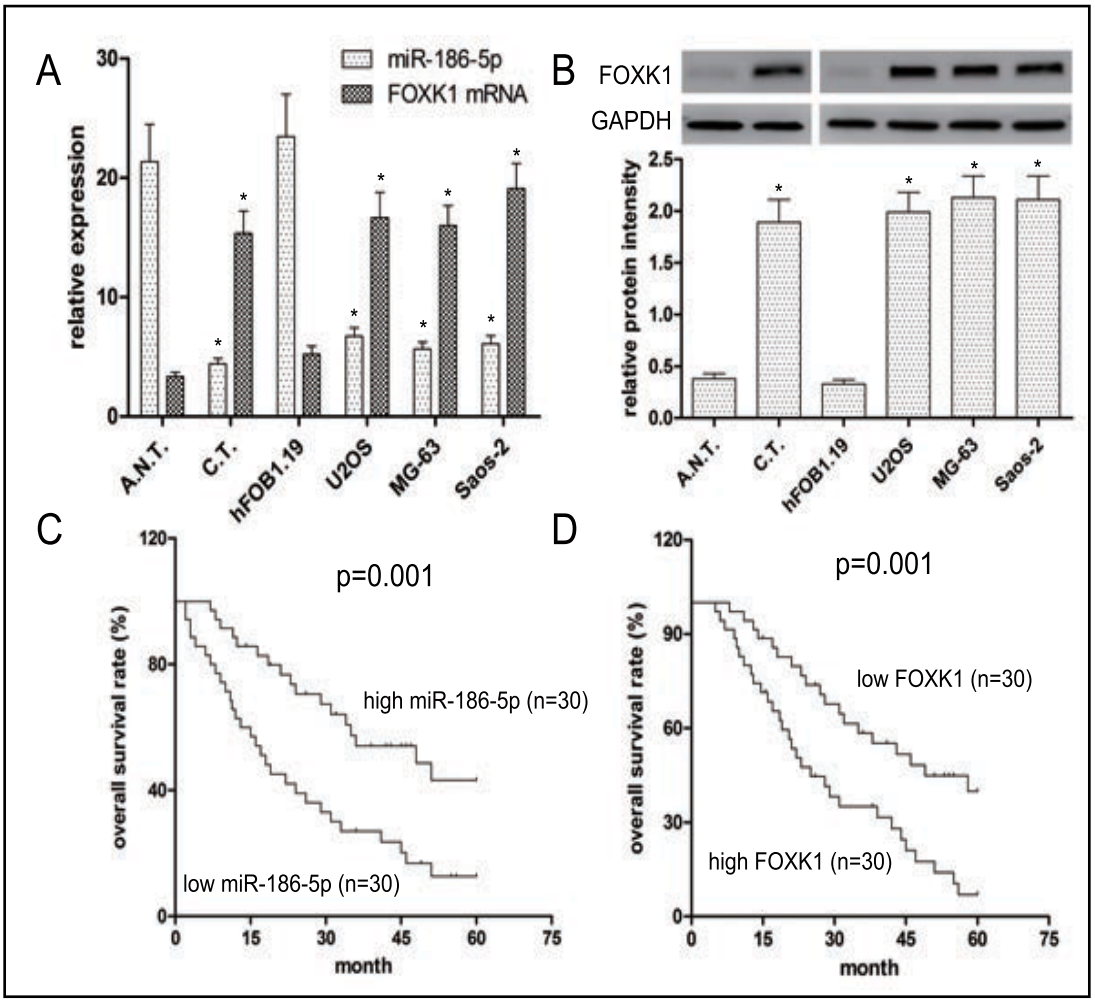

Fig. 1. Expressions of miR-186-5p and FOXK1 in OS tissues and cell lines, and their association with prognosis. (A) qRT-PCR was performed to measure the expression of miR-186-5p and FOXK1 mRNA in OS cells, cancer tissues (C.T.) and adjacent normal tissues (A.N.T.). The bands were measured by densitometry with Quantity One quantitation analysis software package. U6 and GAPDH were used for loading normalization. (B) Western blot was performed to measure the relative expression of FOXK1 protein in human OS tissues and cell lines (U2OS, MG-63 and Saos-2) compared with normal tissues and human osteoblast cell line hFOB1.19. (C,D) The Kaplan Meier plotter was employed to analyze the prognosis of patients with OS. Data are presented as mean ± SD of at least three independent experiments *P<0.001 vs. control.

survival of patients, we conducted Kaplan-Meier analysis. Patients with high miR-186-5p or low FOXK1 protein expression had a better prognosis than those with low miR-186-5p or high FOXK1 protein expression (both P=0.001; Fig. 1C-D). The Cox proportional regression analyses revealed that miR-186-5p and FOXK1 protein expression were the independent prognostic factor for OS patients (P < 0.001).

miR-186-5p inhibits proliferation and tumor growth of OS cells in vitro and in vivo

To explore the function of miR-186-5p in OS cells, the stable infection of miR-186-5p mimics were performed in U2OS, MG-63 and Saos-2 cell lines. The qRT-PCR assay revealed that the expression of miR-186-5p was successfully up-regulated by the stable infection of miR-186-5p mimics (P < 0.001; Fig. 2A). CCK-8 assay indicated that up-regulation of miR-186-5p inhibited the proliferation of U2OS, MG-63 and Saos-2 cells (P < 0.001; Fig. 2B). We then determined the effect of miR-186-5p on the tumorigenic potential *in vivo*. U2OS, MG-63

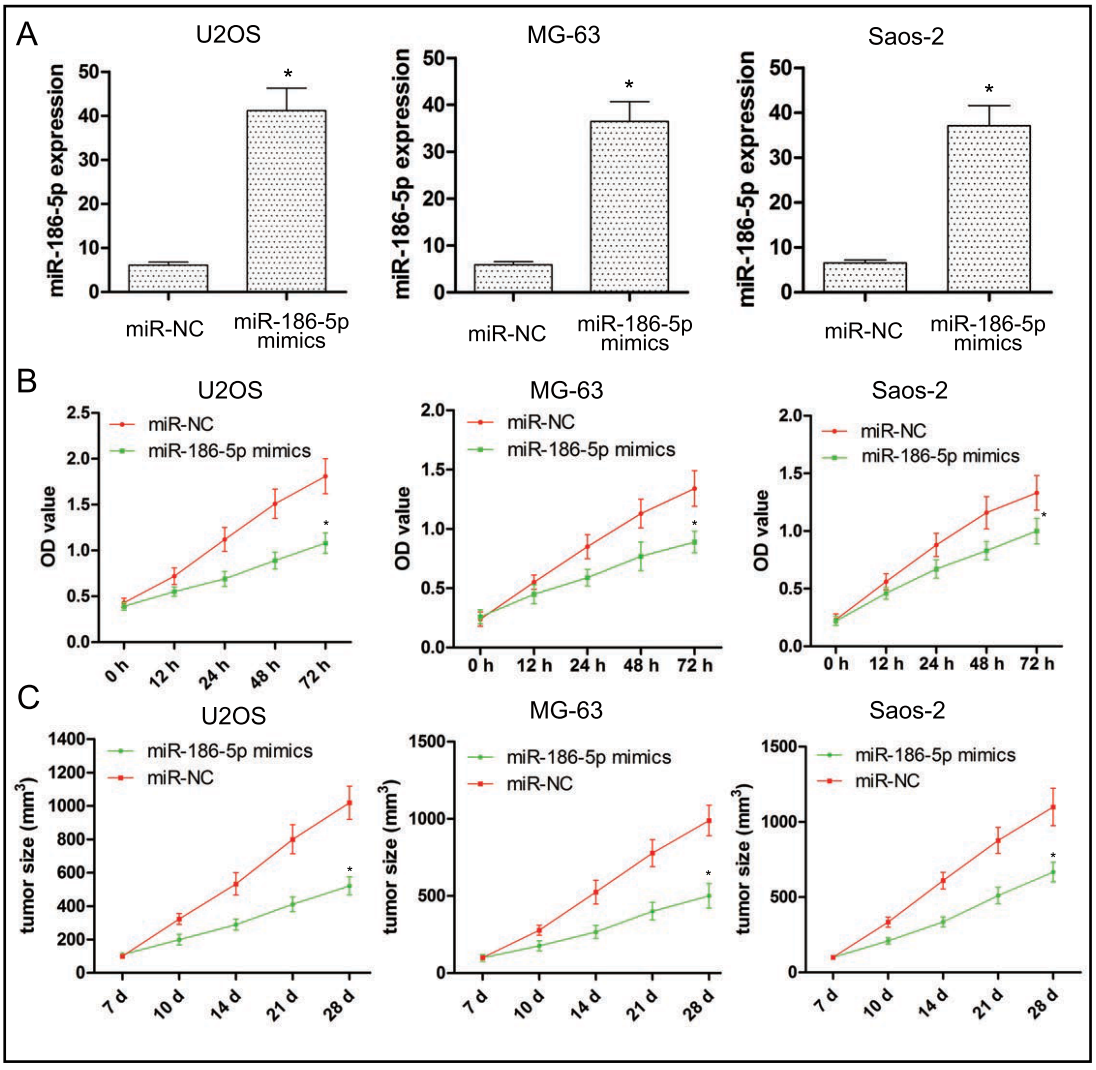

Fig. 2. miR-186-5p suppresses cell proliferation. (A) U2OS, MG-63 and Saos-2 cells were transiently transfected with miR-186-5p mimics or miR-NC, respectively. (B) CCK-8 assay was performed to detect the cell proliferation ability. (C) U2OS, MG-63 and Saos-2 cells expressing miR-186-5p or miR-NC were subcutaneously injected into the flanks of nude mice. The mice were closely monitored for tumor growth, and tumor size was measured at the indicated times. Photographs of tumors isolated from mice by surgical excision at the final experiment day. Values presented as mean ± SD. *P<0.001, v.s. control.

and Saos-2 cells expressing miR-186-5p or miR-NC were subcutaneously injected into the flanks of nude mice. The results revealed that the mice with miR-186-5p-overexpressing cells had significantly smaller tumor volumes than those with the control cells ($P < 0.001$; Fig. 2C).

To explore whether cell proliferation was affected due to cell cycle arrest and apoptosis, the cell cycle status was analyzed by flow cytometry. We found treatment with miR-186-5p mimics resulted in a shift in the cell cycle distribution in U2OS, MG-63 and Saos-2 cells. Specifically, miR-186-5p mimics increased the percentage of cells in the G1/G0 phase of the cell cycle as compared with miR-NC (Fig. 3A). Further, using flow cytometry, we confirmed that miR-186-5p mimics induced apoptosis in U2OS, MG-63 and Saos-2 cells compared with miR-NC (Fig. 3B). These findings revealed that miR-186-5p played a proliferation-suppressive role in OS cells.

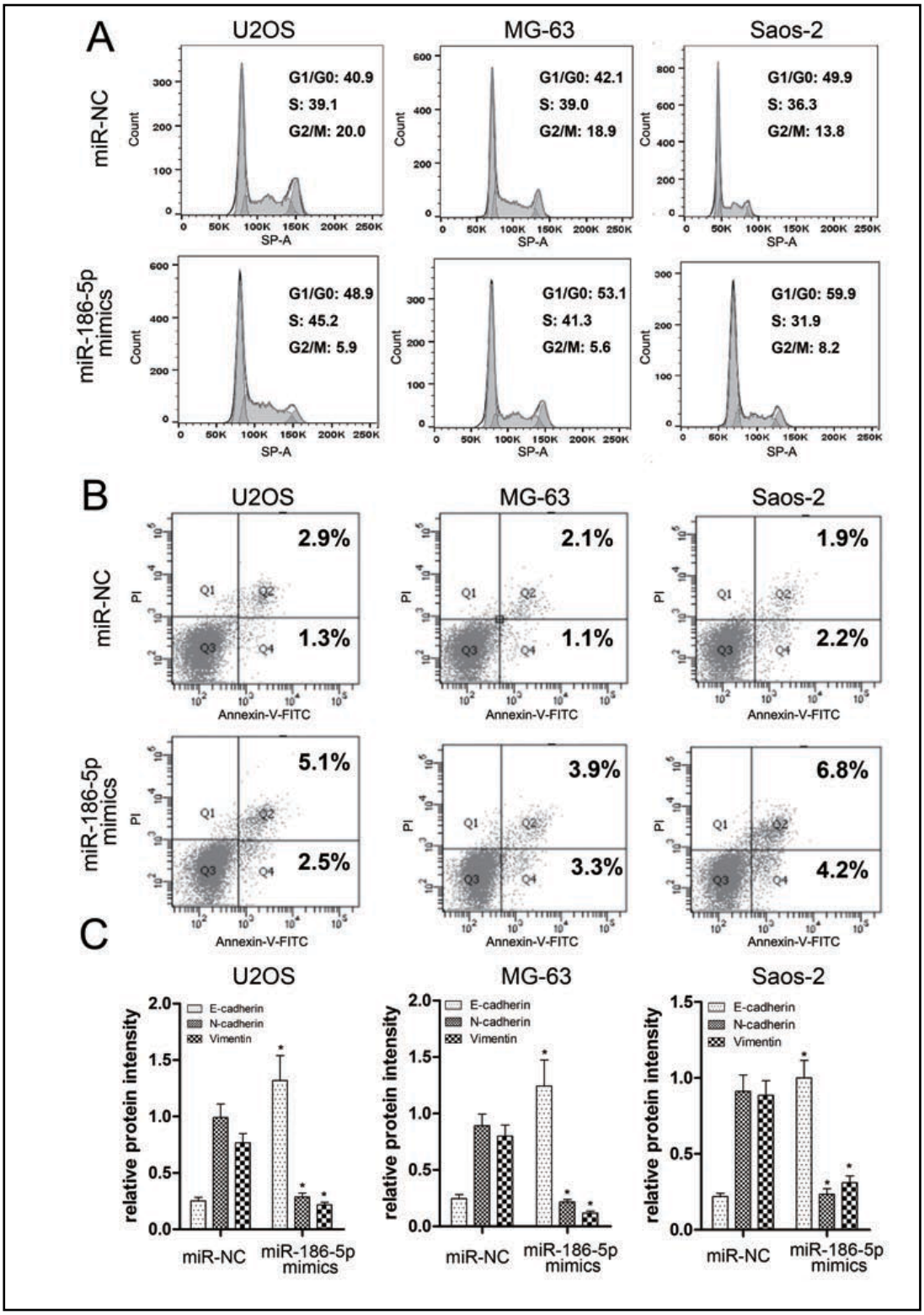

Fig. 3. miR-186-5p suppresses cell cycle and EMT, and induces apoptosis. (A) Cell cycle of OS cells was analyzed by flow cytometry. (B) Annexin/PI assay was used to detect apoptosis of OS cells. (C) Western blot was used to analyze EMT markers E-cadherin, N-cadherin, and Vimentin in OS cells transfected with miR-186-5p mimics or miR-NC. GAPDH served as an internal control. Values presented as mean ± SD. *P<0.001, v.s. control.

miR-186-5p suppresses EMT, cell migration and invasion in vitro

Here, we investigated the potential involvement of miR-186-5p in the regulation of OS metastasis. Firstly, we evaluated the expression of EMT biomarkers in U2OS, MG-63 and Saos-2 cells by western blotting. The level of epithelial marker E-cadherin increased, while the level of N-cadherin and mesenchymal marker Vimentin decreased after overexpression of miR-186-5p ($P < 0.001$; Fig. 3C), suggesting that miR-186-5p inhibited the EMT of OS cells. Functional transwell assay revealed that U2OS, MG-63 and Saos-2 cells expressing miR-186-5p all had a less capacity of cell migration and invasion than their controls ($P < 0.001$; Fig. 4A, B). Therefore, miR-186-5p played a suppressive role in regulation of EMT, migration, and invasion in OS cells.

FOXK1 is a novel target of miR-186-5p

TargetScan (http://www.targetscan.org/) was used to predict potential target genes of miR-186-5p and identified FOXK1 as a potential target with highly matched base sequences or high scores (Fig. 4C). We next used the dual-luciferase reporter assay to reveal the relationship of miR-186-5p and FOXK1. The fragments containing the miR-186-5p binding wild sequence or mutated sequence in the 3′-UTRs of FOXK1 were cloned into the pmiR-RB-REPORT™ vector luciferase reporter. These reporter constructs were co-transfected with miR-186-5p mimics or miR-NC into HEK293T cells, and the luciferase activities were subsequently measured. The data showed that the luciferase activity of FOXK1 was reduced significantly after co-transfection of wild-type luciferase reporter constructs and miR-186-5p mimics, whereas there was little change in the activity following co-transfection of mutant luciferase reporter constructs and miR-186-5p mimics in HEK293T cells ($P < 0.001$; Fig. 5A-B).

In order to investigate the potential regulatory role of miR-186-5p on FOXK1 protein, the association between miR-186-5p and FOXK1 was further illustrated using Spearman's correlation analysis. The analysis indicated an inverse correlation between miR-186-5p and FOXK1 protein expression in OS tissues ($R^2=0.7135$; $P < 0.001$; Fig. 5C). Consistently, western blotting showed that miR-186-5p mimics significantly downregulated the expression of FOXK1 proteins, which was confirmed by co-transfection of miR-186-5p mimics and FOXK1 cDNA containing Wt and Mut 3′-UTR in HEK293T cells ($P < 0.001$). These results suggested that miR-186-5p negatively regulated the expression of FOXK1 at a post-transcriptional level via directly targeting its 3′-UTR.

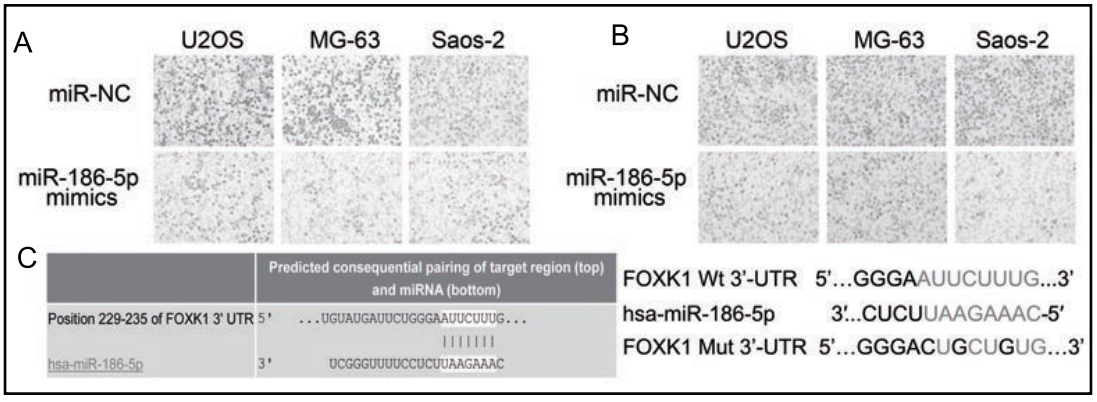

Fig. 4. miR-186-5p inhibits the migration and invasion of OS cells in vitro, and targets a predicted target FOXK1 3′-UTR. (A,B) Transwell assay was performed to determine the migration and invasion ability of U2OS, MG-63 and Saos-2 cells. Representative images showed migrative and invasive cells in the lower chamber stained with crystal violet. (C) TargetScan (http://www.targetscan.org/) was used to predict potential target genes of miR-186-5p and identified FOXK1 as a potential target. A schematic showed reporter constructs of wild-type FOXK1 3′-UTR (upper panel) and 3′-UTR with mutated miR-186-5p-binding sites (lower panel). Values presented as mean ± SD. *P<0.001, v.s. control.

FOXK1 is critical for the biological functions of miR-186-5p in OS

We further investigated whether FOXK1 could mediate the biological functions of miR-186-5p in OS. Specific FOXK1 siRNAs significantly reduced the FOXK1 expression in three OS cells with or without miR-186-5p mimics ($P < 0.001$). Knockdown of FOXK1 significantly enhanced inhibitory effects of miR-186-5p overexpression on the proliferation, migration and migration in U2OS, MG-63 and Saos-2 cells ($P < 0.001$; Fig. 6). These data suggested that FOXK1 was indeed involved in the biological functions of miR-186-5p in OS.

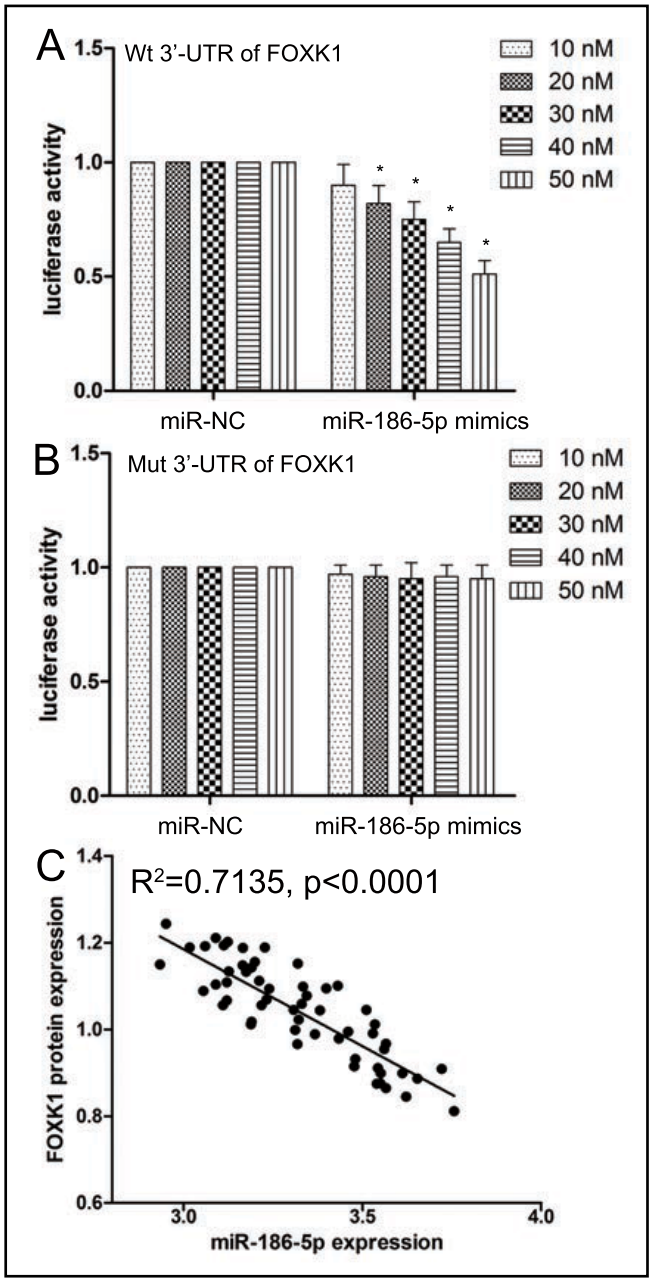

Fig. 5. miR-186-5p targets FOXK1 3'-UTR directly. (A,B) Relative luciferase activity was detected by fluorescent intensity. Wt: wild-type FOXK1 3′-UTR transfection; Mut: mutated-type FOXK1 3′-UTR transfection. (C) Spearman's rank correlation analysis of the expression levels between miR-186-5p and FOXK1 protein in OS tissues. Values presented as mean ± SD. *P<0.001, v.s. control.

Discussion

To date, several studies have emphasized the importance of miR-186-5p that could serve as a diagnostic and therapeutic biomarker in cancer patients [4-7]. In addition, FOXK1 was also identified to play pivotal roles in cancer cell proliferation, cell growth, and metabolism [8]. For example, Yang Q et al. suggested knockdown of FOXK1 eliminated cell cycle-dependent oscillations and decreased cell proliferation rate and the development of the malignant phenotype in human osteosarcoma U2OS cells [12]. More reports also indicated the role of FOXK1 in regulating tumorigenesis and development [13, 14]. However, the relationship and interaction of miR-186-5p and FOXK1 were not investigated in OS.

Here, we demonstrated that down-regulation of miR-186-5p was identified in OS tissues and cell lines, and negatively correlated with distant metastasis, Enneking stage and poor 5-year prognosis as well as the expression of forkhead box k1 (FOXK1) protein using qRT-PCR and western blot analysis. Then, we assumed that miR-186-5p exerted an important effect on the development of OS. During OS progression, advanced cancer cells frequently exhibit a conspicuous

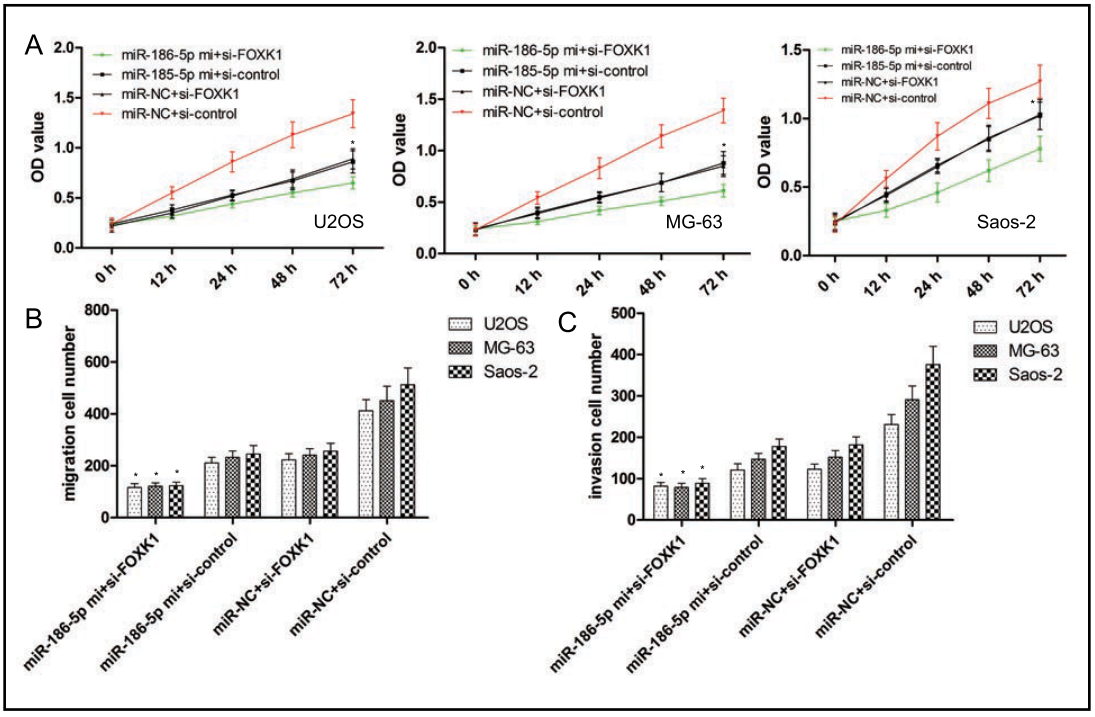

Fig. 6. FOXK1 is critical for the biological functions of miR-186-5p in OS cells. (A) Expression of FOXK1 in U2OS, MG-63 and Saos-2 cells after co-transfection of miR-186-5p mimics with si-control or si-FOXK1. And then CCK-8 assay was performed to detect the cell proliferation ability. (B,C) Transwell assay was performed to determine the migration and invasion ability. Values presented as mean ± SD. *P<0.001, v.s. control.

downregulation of epithelial markers and a loss of intercellular junctions, resulting in a loss of epithelial polarity and reduced intercellular adhesion. Further, we indicated that overexpression of miR-186-5p also attenuated OS cell proliferation, cell cycle, EMT and induced apoptosis. Recent studies also showed FOXK1 promoted migration, metastasis, and dissemination to facilitate the development and progression of tumors [15, 16]. To my point of view, these findings suggested that miR-186-5p may regulate the development of OS probably by targeting FOXK1.

Previous studies have revealed that miR-186 potentially regulated the invasion and metastasis of bladder cancer via VEGF-C [17]. miR-186 is also down-regulated in NSCLC tissues and miR-186 reduces invasion ability of lung cancer cells by directly targeting cdc42 and modulating EMT process [18]. In the present study, our findings indicated that FOXK1 was a direct target gene, and silencing of FOXK1 synergized the effects of miR-186-5p on the proliferation, migration, and invasiveness of OS cells. According to these findings, miR-186-5p is proposed to be one of tumor-suppressors, and may serve as a therapeutic target in the treatment of patients with OS.

Conclusion

In conclusion, this is the first study to demonstrate that miR-186-5p is downregulated in OS, and decreases cell proliferation, migration and invasion by directly targeting FOXK1 in OS. Thus, the expression and function of miR-186-5p and its target gene FOXK1 in OS may aid in understanding the molecular mechanisms of tumorigenesis, as well as providing a therapeutic target in patients with OS.

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Disclosure Statement

The authors have no conflicts of interest to declare.

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