miR-197 is downregulated in cervical carcinogenesis and suppresses cell proliferation and invasion through targeting forkhead box M1

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Received May 6, 2016; Accepted June 15, 2017

DOI: 10.3892/ol.2018.8565

Abstract. Cervical cancer is the second most common type of cancer in females worldwide. It has been demonstrated that microRNAs (miRs) serve important roles in the occurrence and development of various types of cancer, including cervical cancer. The results of the present study revealed that miR-197 was downregulated in cervical cancer tissues and cell lines. Restoration of miR-197 expression significantly inhibited cell viability and invasion of cervical cancer. Additionally, forkhead box M1 (FOXM1) was identified as a direct target gene of miR-197. Bioinformatic analysis revealed that FOXM1 was a potential target gene of miR-197. Luciferase reporter assay, reverse transcription-quantitative polymerase chain reaction and western blot analysis demonstrated that miR-197 decreased FOXM1 expression through direct binding to its 3'-untranslated region. Furthermore, the effects of FOXM1 underexpression were comparable with the effects induced by miR-197 overexpression in cervical cancer cells, suggesting that FOXM1 acted as a downstream effector in miR-197-mediated proliferation and invasion of cervical cancer cells. The results of the present study suggested that miR-197 inhibited growth and metastasis of cervical cancer by directly targeting FOXM1.

Introduction

Cervical cancer is the second most common type of cancer in females worldwide, with an estimated 530,000 novel cases and 275,000 mortalities annually (1). The occurrence and development of cervical cancer involves a sequential progression from normal cervical epithelium to preneoplastic cervical intraepithelial neoplasia and subsequently to invasive cervical cancer (2). Studies have indicated that numerous risk factors contribute to the initiation and development of cervical cancer including early sexual intercourse, increased number of sexual partners and infection with high risk types of human papillomavirus (3,4). Surgery with adjuvant chemotherapy is the current therapeutic intervention for patients with cervical cancer. Despite marked progress in the diagnosis, surgical methods and application of comprehensive therapy, prognosis remains unsatisfactory (5). The 5-year overall survival rate for patients with locally advanced cervical cancer is between 30 and 50% compared with between 5 and 15% for patients with metastatic disease (6). Therefore, it is imperative to fully understand the underlying molecular mechanism of cervical cancer and provide novel diagnostic and prognostic markers, and identify novel therapeutic targets for treatment of this disease.

MicroRNAs (miRs) are endogenously expressed RNAs of between 20 and 23 nucleotides in length, which comprise a large family of non-coding and single-strand RNAs (7). These small molecules have been demonstrated to regulate gene expression at the post-transcriptional level through binding to the 3'-untranslated regions (3'-UTRs) of their target genes in a base-pairing manner, resulting in translational inhibition or mRNA degradation (8,9). The association between miRs and cancer has become a popular area of research. Comparison between human cancer tissues and their matched normal tissues have revealed distinct miR expression profiles (10). The abnormal expression of miRs in various types of cancer is associated with a broad range of physiological and pathological processes including proliferation, apoptosis, cell cycle, migration, invasion and metastasis (11,12). In human cancer, miRs may act as tumor suppressors or oncogenes which primarily depends on the roles of their target genes (13). Highly expressed miRs function as oncogenes by blocking tumor suppressor gene function, whereas downregulated miRs function as tumor suppressor genes through negative regulation of oncogenes (14,15). Therefore, elucidating the expression pattern, functional roles and underlying molecular

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Key words: microRNA-197, cervical cancer, forkhead box M1, proliferation, invasion

mechanism of miRs may be particularly useful in improving cancer treatments.

In the present study, the expression level of miR-197 was determined in cervical cancer, and the functional roles of miR-197 were investigated in cervical cancer cells and the direct target genes of miR-197 were identified in cervical cancer. The results of the present study determined the differential expression of miR-197 in cervical cancer and demonstrated that miR-197 suppressed cell proliferation and invasion in cervical cancer though directly targeting forkhead box M1 (FOXM1).

Materials and methods

Ethical statement and human tissue samples. The present study was approved by the Ethics Committee of Xiangyang Central Hospital (Xiangyang, China). Written informed consent was obtained from all patients prior to collection of tissue samples. A total of 46 pairs of human cervical cancer and adjacent normal cervical tissues were obtained from patients with cervical cancer who underwent cervical surgical resection without radiotherapy and/or chemotherapy treatment at Xiangyang Central Hospital. The tissue samples were frozen in liquid nitrogen immediately following surgery and stored at -80˚C until use.

Cell lines, culture conditions and cell transfection. The normal human cervical epithelial cell line (Ect1/E6E7), cervical cancer cell lines (HeLa, C33A, CaSki and SiHa) and HEK293 T cell line were purchased from Chinese Center for Type Culture Collection (Wuhan, China). All cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% (FBS) and 1% antibiotic/antimycotic (all from Thermo Fisher Scientific, Waltham, MA, USA) at 37˚C in a humidified atmosphere containing 5% CO_2. Chemically synthesized miR-197 mimics, negative control (NC), FOXM1 small interfering RNA (siRNA) and NC siRNA were obtained from Guangzhou RiboBio Co., Ltd. (Guangzhou, China). The miR-197 mimic sequence was 5'-UUCACCACCUUCUCCA CCCCAGC-3' and the NC sequence was 5'-UUCUCCGAA CGUGUCACGUTT-3'. The FOXM1 siRNA sequence was 5'-GGACCACUUUCCCUACUUUTT-3' and the NC siRNA sequence was 5'-UUCUCCGAACGUGUCACGUTT-3'. Cells were transfected with miR-197 mimics, NC, FOXM1 siRNA or NC siRNA using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA from tissues and cells were isolated using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. To determine the levels of miR-197, RT was performed using the miScript Reverse Transcription kit (Qiagen China Co., Ltd., Shanghai, China), followed by qPCR using SYBR Premix Ex Taq II (Takara Biotechnology Co., Ltd., Dalian, China). The qPCR was performed with cycling conditions as follows: 5 min at 95˚C, followed by 40 cycles of 95˚C for 30 sec and 65˚C for 45 sec. To monitor FOXM1 expression, cDNA was synthesized from total RNA using Moloney murine leukemia virus reverse transcriptase (Invitrogen; Thermo Fisher Scientific. Inc.). RT-qPCR was performed using a SYBR-Green Master Mix kit (Roche Applied Science, Shanghai, China). The thermocycling conditions for qPCR were as follows: 95˚C for 10 min, followed by 40 cycles of 95˚C for 15 sec and 60˚C for 1 min. The primers were designed as follows: miR-197, 5'-ATT ACTTTGCCCATATTCATTTTGA-3' (forward) and 5'-ATT CTAGAGGCCGAGGCGGCCGACATGT-3' (reverse); U6, 5'-GCTTCGGCAGCACATATACTAAAAT-3' (forward) and 5'-CGCTTCACGAATTTGCGTGTCAT-3' (reverse); FOXM1, 5'-GAAGAACTCCATCCGCCACA-3' (forward) and 5'-GCC TTAAACACCTGGTCCAATGTC-3' (reverse); and β-actin, 5'-TGGCATTGTTACCAACTGGGTC-3' (forward) and 5'-TCACGGTTGGCCTTAGGGTTC-3' (reverse). Relative expression of miR-197 and FOXM1 mRNA was normalized to U6 and β-actin, respectively. Relative expression was calculated using the $2^{-\Delta\Delta Cq}$ method (16).

MTT assay. Cells were seeded separately in 96-well plates at a density of 3,000 cells/well 24 h after transfection. The MTT (5 mg/ml; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) assay was performed 0, 24, 48, 72 and 96 h after incubation. In brief, 10 μl MTT solution was added to each well and the plates were then incubated for an additional 4 h at 37˚C. Subsequently, the culture medium was removed, 200 μl dimethyl sulfoxide (Sigma-Aldrich; Merck KGaA) was added to each well and the plates were incubated at 37˚C for 30 min. Finally, the optical density at 490 nm was measured using a Versamax microplate reader (Molecular Devices, LCC, Sunnyvale, CA, USA).

In vitro cell invasion assay. A 24-well Boyden chamber with an 8-μm pore-size polycarbonate membrane (Corning Life Sciences, Cambridge, MA, USA) was used to evaluate the invasion ability of cervical cancer cells. The membranes were coated with Matrigel (BD Biosciences, San Jose, CA, USA) according to the manufacturer's protocol. Cells ($5x10^4$) were collected and reseeded into the upper chamber in 200 μl FBS-free culture medium (Gibco; Thermo Fisher Scientific, Inc.) 48 h after transfection. In the lower chamber, 500 μl DMEM (Gibco; Thermo Fisher Scientific, Inc.) containing 20% FBS was added as a chemoattractant. Following incubation for 48 h, cells remaining on the top of the membranes were carefully removed. Then cells migrating across the membranes were fixed, stained with 0.1% crystal violet (Beyotime Institute of Biotechnology, Haimen, China) at room temperature for 20 min and counted under a light microscope in 5 fields.

Target predication and luciferase reporter assay. To explore the potential target genes of miR-197, bioinformatic analysis was performed using miRanda (www.microrna.org/microrna) and TargetScan (www.targetscan.org).

To explore the direct interaction between miR-197 and FOXM1, a luciferase reporter assay was performed. HEK293 T cells were seeded in 24-well plates at a density of between 40 and 50% confluence and transfected with miR-197 mimics or NC, along with pGL3-FOXM1-3'UTR wild-type (Wt) or pGL3-FOXM1-3'UTR mutant (Mut). pGL3-FOXM1-3'UTR Wt and pGL3-FOXM1-3'UTR Mut luciferase reporter vectors, synthesized by Shanghai GenePharma Co., Ltd.

(Shanghai, China). The luciferase activity was measured using Dual-Luciferase Reporter assays (Promega Corporation, Madison, WI, USA) 48 h post-transfection and the transfection efficiency was normalized to the paired *Renilla* luciferase activity. Results were obtained from three independent experiments.

Western blotting. Proteins were isolated from transfected cells using radioimmunoprecipitation lysis buffer (Beyotime Institute of Biotechnology) in the presence of protease inhibitor cocktail (0.1 mg/ml phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate and 1 mg/ml aprotinin; Pierce; Thermo Fisher Scientific, Inc.). Following centrifugation at 10,000 x g for 15 min, supernatants were transferred to new tubes for quantification. The concentration of total protein was determined with bicinchoninic acid protein assay (Aidlab Biotechnologies Co., Ltd., Beijing, China). Equal amounts of protein (20 μg) were separated using SDS-PAGE (10% gel), transferred onto polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA) and blocked with 5% non-fat milk in Tris-buffered saline-Tween-20 (TBST) buffer for 0.5 h at room temperature. The membranes were then probed overnight at 4˚C with mouse anti-human monoclonal FOXM1 (1:1,000 dilution, sc-166709; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and GADPH (1:1,000 dilution, sc-59540; Santa Cruz Biotechnology, Inc.) antibodies. Following washing three times with TBST, the membranes were incubated with a goat anti-mouse horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, Inc.) for 2 h at room temperature. Finally, an enhanced chemiluminescent reagent (EMD Millipore) was added to develop the signal bands. The intensity of signal bands was analyzed with GeneTools (version 3.03; SynGene, Frederick, MD, USA).

Statistical analysis. All values are presented as the mean ± standard deviation. Differences between groups were assessed using SPSS software (version 13.0; SPSS, Inc., Chicago, IL, USA). Data were analyzed with Student's t-test or one-way analysis of variance. Students-Newman-Keuls was performed to compare between two groups in multiple groups study. P<0.05 was considered to indicate a statistically significant difference.

Results

miR-197 downregulation in cervical cancer tissues and cell lines. The expression level of miR-197 was measured in 46 pairs of cervical cancer and adjacent normal cervical tissues using RT-qPCR. It was identified that miR-197 was significantly downregulated in cervical cancer tissues compared with adjacent normal cervical tissues (P<0.05; Fig. 1A). miR-197 expression in human cervical cancer cell lines (HeLa, C33A, CaSki and SiHa) and a normal human cervical epithelial cell line (Ect1/E6E7) was also detected. As presented in Fig. 1B, the expression levels of miR-197 were significantly decreased in the four cervical cancer cell lines compared with Ect1/E6E7 cells (P<0.05).

Effects of miR-197 on viability and invasion of cervical cancer cells. To investigate the roles of miR-197 in cervical cancer, HeLa and SiHa cells expressing a relatively low level

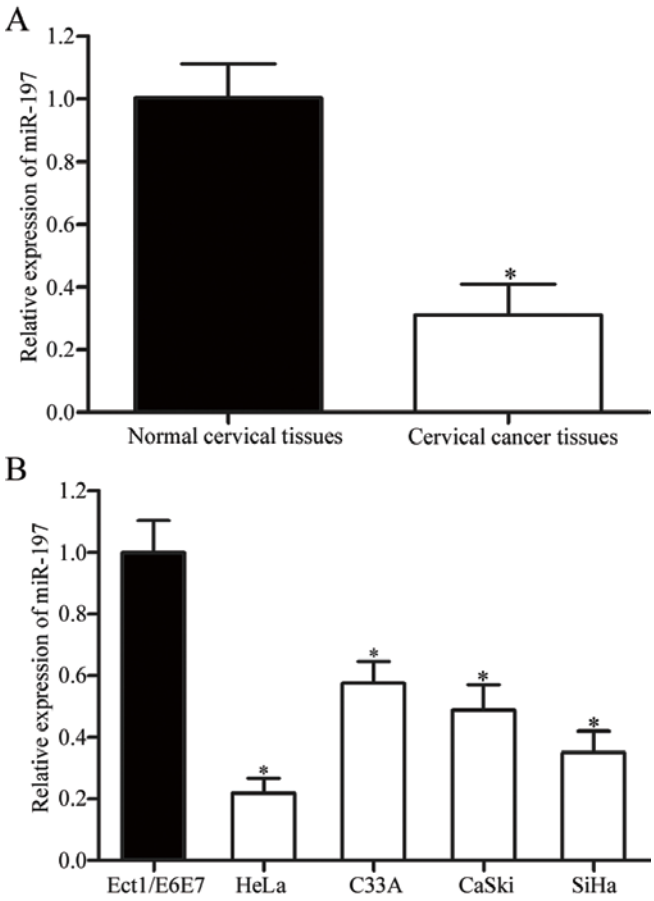

Figure 1. miR-197 expression is decreased in cervical cancer tissues and cell lines. (A) The expression level of miR-197 in cervical cancer tissues was decreased compared with adjacent normal cervical tissues. (B) miR-197 expression was significantly downregulated in four cervical cancer cell lines compared with a normal human cervical epithelial cell line. *P<0.05 vs. control group. miR, microRNA.

of miR-197 were transfected with miR-197 mimics or NC. Following transfection, the expression level of miR-197 was significantly increased in HeLa and SiHa cells transfected with miR-197 mimics (P<0.05; Fig. 2A).

MTT assays were performed to investigate the effect of miR-197 on the viability of cervical cancer cells. Overexpression of miR-197 was identified to significantly suppress the viability of HeLa and SiHa cells (P<0.05; Fig. 2B). To investigate the role of miR-197 on the invasion of the cervical cancer cells, *in vitro* cell invasion assays were performed. As shown in Fig. 2C, restoration of miR-197 expression inhibited the invasive ability of HeLa and SiHa cells compared with cells transfected with NC (P<0.05). The results of the present study demonstrated that miR-197 acted as a tumor suppressor in cervical cancer.

miR-197 directly targets the 3'-UTR of FOXM1 mRNA to inhibit its expression. To investigate the molecular mechanism underlying the suppressive roles of miR-197 in cervical cancer, bioinformatic analysis was performed to explore potential target genes. Among these putative targets of miR-197, FOXM1 was selected for further investigation (Fig. 3A). Luciferase reporter assays revealed that upregulation of miR-197 significantly decreased the firefly luciferase activity

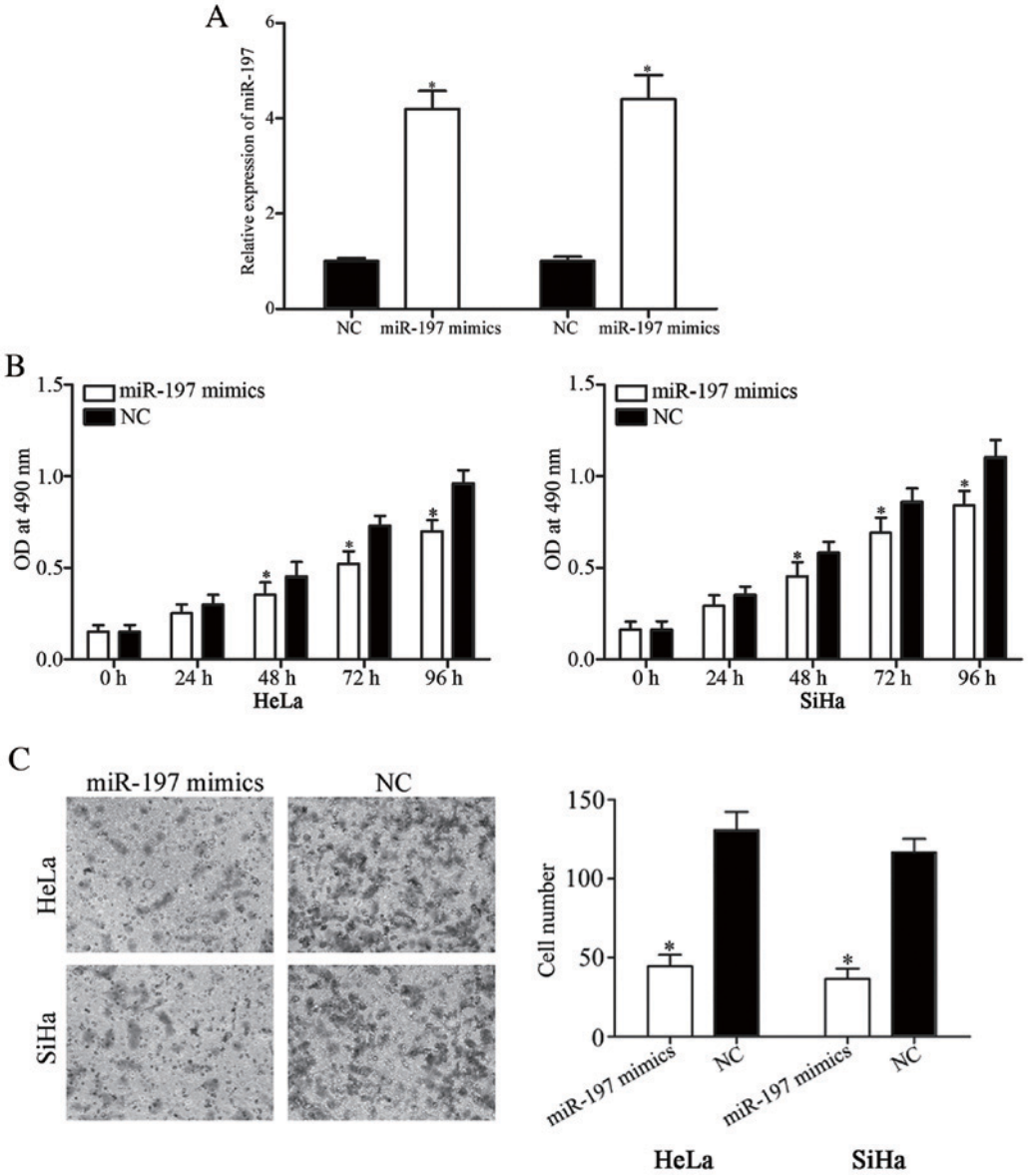

Figure 2. miR-197 suppresses the proliferation and invasion of cervical cancer cells. (A) The expression of miR-197 in HeLa and SiHa cells transfected with miR-197 mimics or NC was detected using the reverse transcription-quantitative polymerase chain reaction. (B) The MTT assay was performed to evaluate cell viability in HeLa and SiHa cells treated with miR-197 mimics or NC. (C) The invasive capacity of HeLa and SiHa cells transfected with miR-197 mimics or NC was assessed using an *in vitro* cell invasion assay. *P<0.05 compared with control group. OD, optical density; NC, negative control; miR, microRNA.

of pGL3-FOXM1-3'UTR Wt (P<0.05; Fig. 3B), whereas no such inhibitory effect was identified when miR-197 was transfected with pGL3-FOXM1-3'UTR Mut (P>0.05).

The effects of miR-197 on FOXM1 expression were also evaluated. As presented in Fig. 3C and D, miR-197 overexpression suppressed FOXM1 expression in HeLa and SiHa cells at the mRNA and protein level (P<0.05). These results indicated that FOXM1 was a direct target gene of miR-197.

Effects of FOXM1 silencing on viability and invasion of cervical cancer cells. To investigate the functional roles of FOXM1 in cervical cancer, loss-of-function studies using FOXM1 siRNA were performed. RT-qPCR was performed to evaluate its transfection efficiency. The results revealed that FOXM1 siRNA significantly suppressed FOXM1 expression in HeLa and SiHa cells when compared with cells transfected with NC siRNA (P<0.05; Fig. 4A).

The MTT assays revealed that cell viability was inhibited in the HeLa and SiHa cells transfected with FOXM1 siRNA compared with cells transfected with NC siRNA (P<0.05; Fig. 4B). Furthermore, the number of invading cells was also significantly decreased in FOXM1 siRNA transfectants compared with NC siRNA transfectants in HeLa and SiHa cells. These results suggested that the roles of FOXM1 underexpression were similar with the functions induced by miR-197 overexpression in cervical cancer cells, suggesting that FOXM1 acted as a downstream effector in the miR-197-mediated viability and invasion of cervical cancer cells.

Discussion

It has been demonstrated that miRs serve a critical role in the carcinogenesis and progression of human cancers (17,18).

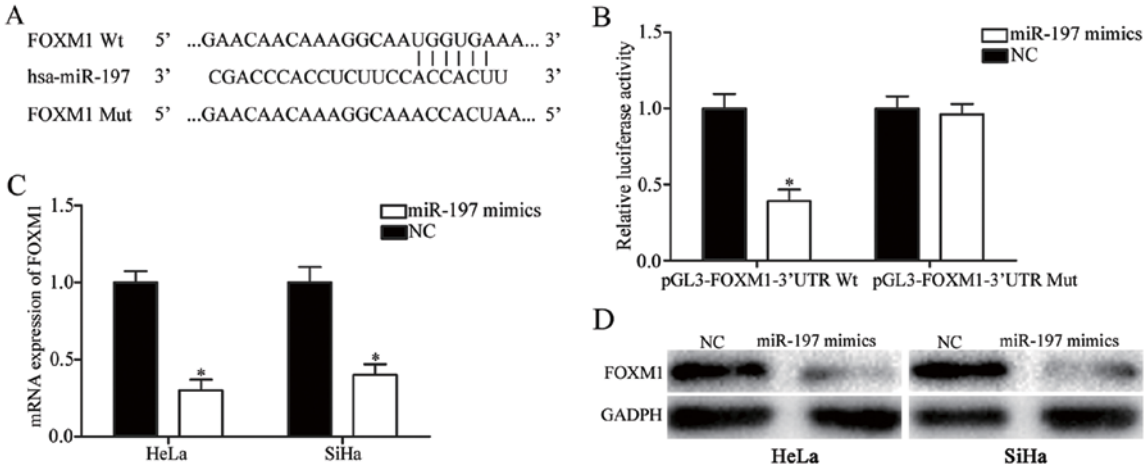

Figure 3. Direct regulation of FOXM1 by miR-197 through binding to its 3'-UTR. (A) The miR-197-binding sites in the 3'-UTR of FOXM1 Wt and Mut sequences. (B) HEK293 T cells were co-transfected with pGL3-FOXM1-3'UTR Wt or pGL3-FOXM1-3'UTR Mut, together with miR-197 mimics or NC. Luciferase activity was measured at 48 h after transfection. (C) FOXM1 mRNA expression in HeLa and SiHa cells transfected with miR-197 mimics or NC was measured using the reverse transcription-quantitative polymerase chain reaction. (D) Protein expression of FOXM1 was measured using western blot analysis in HeLa and SiHa cells transfected with miR-197 mimics or NC. *P<0.05 vs. control group. Wt, wild-type; Mut, mutant; NC, negative control; UTR, untranslated region; miR, microRNA; FOXM1, forkhead box M1; hsa, *Homo sapiens*.

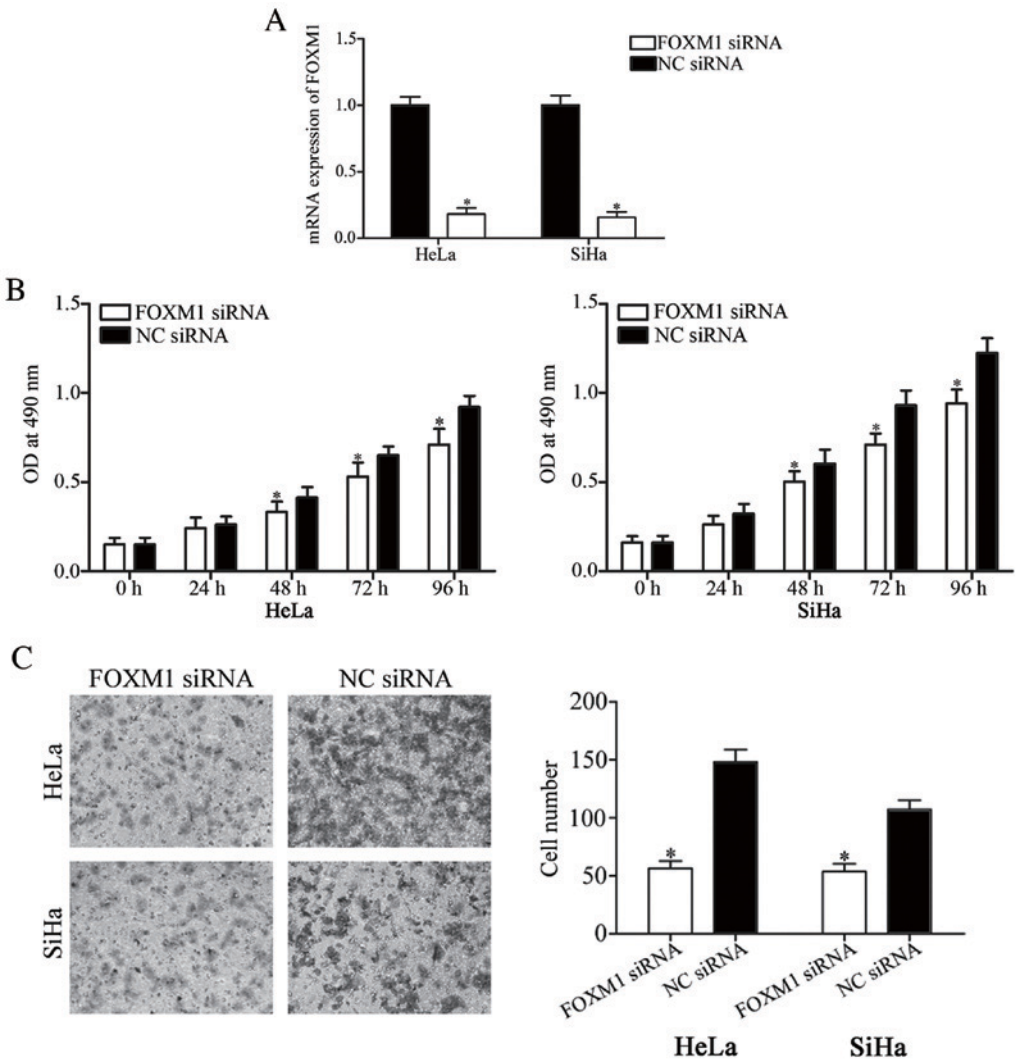

Figure 4. FOXM1 siRNA inhibits proliferation and invasion of cervical cancer cells. (A) The reverse transcription-quantitative polymerase chain reaction was used to measure FOXM1 expression in HeLa and SiHa cells treated with FOXM1 siRNA or NC siRNA. (B) The MTT assay was performed to evaluate cell viability in HeLa and SiHa cells transfected with FOXM1 siRNA or NC siRNA. (C) The invasive capacity of HeLa and SiHa cells transfected with FOXM1 siRNA or NC siRNA was assessed using an *in vitro* cell invasion assay. *P<0.05 vs. control group. FOXM1, forkhead box M1; siRNA, small interfering RNA; NC, negative control; OD, optical density.

Therefore, understanding the roles of miRs may be important for providing new insights into the involved molecule mechanism in cancer initiation and development and defining novel markers for cancer prognosis, diagnosis and treatment (19). Previously, miR-197 has been reported to be downregulated in glioblastoma (20), uterine leiomyoma (21), multiple myeloma (22) and esophageal cancer (23), and upregulated in non-small cell lung cancer (24), taxol-resistant ovarian cancer (25) and hepatocellular carcinoma (26).

The results of the present study revealed that miR-197 was downregulated in cervical cancer tissues and cell lines compared with adjacent normal cervical tissues and normal human cervix epithelial cell line, respectively. These results demonstrated that miR-197 may act as a tissue-specific miR.

It has also been reported that miR-197 is associated with the clinicopathological features in patients with cancer. For example, in non-small cell lung cancer, miR-197 was associated with increased tumor size and squamous cell carcinoma histological type. Furthermore, miR-197 expression was identified as a novel independent predictor of unfavorable prognosis for patients with non-small cell lung cancer (24). In esophageal cancer, results of Kaplan-Meier estimator analysis suggested the expression levels of miR-197 was markedly associated with survival time. Furthermore, Cox's multi-factor analysis model revealed that miR-197 expression was associated with prognosis, tumor length and expression, and survival time (23). These results indicated that miR-197 may be a biomarker of, and be involved in, the progression of human cancer.

miR-197 has been reported to be involved in physiological and pathological processes in numerous types of cancer. For example, restoration of miR-197 decreased cell proliferation through negative regulation of Grb-associated-binding protein 2 (20). In uterine leiomyoma, upregulation of miR-197 inhibited cellular proliferation and promoted cell cycle arrest in G0/G1 phase *in vitro* (21). Wu *et al* (27) also demonstrated that miR-197 may inhibit uterine leiomyoma cell proliferation and migration, and induce apoptosis *in vitro*. miR-197 overexpression may enhance taxol resistance in ovarian cancer cells while also increasing cell proliferation and invasion (25). Yang *et al* (22) revealed that miR-197 overexpression suppressed cell viability, colony formation and migration, and induced apoptosis in multiple myeloma cells. In hepatocellular carcinoma, miR-197 underexpression repressed cell migration and invasion *in vitro* and *in vivo* (26). The results of the present study demonstrated that miR-197 expression decreased cell proliferation and invasion of cervical cancer cells. These results suggested that miR-197 acted as a tumor suppressor in cervical cancer and that low expression level of miR-197 in cervical cancer may contribute to abnormal proliferation and invasion of cervical cancer cells, and promote tumor growth and metastasis.

The various effects of miR-197 in distinct tissues may be a result of the specific targets repressed in each tissue. In the present study, FOXM1 was identified as a novel target gene of miR-197. miR-197 target genes in cervical cancer were analyzed using the TargetScan and miRanda databases. Bioinformatic analysis results indicated that FOXM1 may be a direct miR-197 target gene. FOXM1 was selected for further study for the following reasons: i) FOXM1 is a member of the forkhead superfamily of transcription factors and has been identified to be upregulated in a number of types of human cancer including lung, breast, liver, pancreatic and cervical cancer, as well as in glioblastoma (28-30); and ii) functional studies have revealed FOXM1 to be involved in numerous biological processes including cell proliferation, cell cycle progression, cell differentiation, DNA damage repair, tissue homeostasis, angiogenesis and apoptosis (31).

Luciferase reporter assays were performed in order to investigate the hypothesis. The present study revealed that miR-197 decreased the firefly luciferase activity of a Wt FOXM1 3'-UTR luciferase reporter vector, but did not affect the luciferase activity of a mut FOXM1 3'-UTR luciferase reporter vector. Furthermore, restoration of miR-197 expression suppressed FOXM1 expression at the mRNA and protein levels in cervical cancer cells. Finally, the roles of FOXM1 underexpression were similar to the functions induced by miR-197 overexpression in cervical cancer cells, suggesting that FOXM1 acted as a downstream effector in miR-197-mediated proliferation and invasion of cervical cancer cells. The results of the present study demonstrated that miR-197 directly decreased FOXM1 expression by binding to the 3'-UTR of the FOXM1 gene.

In conclusion, the results of the present study indicated that miR-197 was downregulated in cervical cancer. In addition, miR-197 overexpression may suppress cell viability and invasion of cervical cancer. Furthermore, FOXM1 was identified as a novel direct target of miR-197. These results suggest the therapeutic potential of miR-197 in the treatment of cervical cancer.

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