Oncology and Translational Medicine
DOI 10.1007/s10330-022-0601-1

June 2023, Vol. 9, No. 3, P121–P132

ORIGINAL ARTICLE

MiR-183-5p promotes the progression of non-small cell lung cancer through targeted regulation of FOXO1

Yan Deng, Zhengguang He, Xiaobin Luo, Rong Qiu (✉), Yong Zhao, Wen Luo

Department of Respiratory and Critical Care Medicine, Suining Central Hospital, Suining 629000, China

Abstract

Objective To investigate miR-183-5p targeting to forkhead box protein O1 (FOXO1) and its corresponding effect on the proliferation, migration, invasion, and epithelial-mesenchymal transition (EMT) of non-small cell lung cancer (NSCLC) cells.

Methods NSCLC tissues and adjacent normal tissues from 60 patients with NSCLC adenocarcinoma were obtained via pathological biopsy or intraoperative resection. Several cell lines were cultured *in vitro*, including the human normal lung epithelial cell line BEAS-2B and human NSCLC cell lines A549, SPCA-1, PC-9, and 95-D. miR-183-5p and FOXO1 mRNA expression in tissues and cells were detected by qRT-PCR; the corresponding correlations in NSCLC tissues were analyzed using the Pearson test, and the relationship between miR-183-5p expression and clinicopathological parameters was analyzed. The miR-183-5p-mediated regulation of FOXO1 was verified by bioinformatics prediction alongside double luciferase, RNA-binding protein immunoprecipitation (RIP) assay, and pull-down experiments. A549 cells were divided into control, anti-miR-NC, anti-miR-183-5p, miR-NC, miR-183-5p, miR-183-5p+pcDNA3.1, and miR-183-5p+pcDNA3.1-FOXO1 groups. Cell proliferation, invasion, migration, apoptosis, and cell cycle distribution were detected using an MTT assay, clone formation assay, Transwell assay, scratch test, and flow cytometry, respectively. The expression of EMT-related proteins in the cells was analyzed by western blotting. The effect of miR-185-3p silencing on the development of transplanted tumors was detected by analyzing tumor formation in nude mice.

Results miR-183-5p expression was significantly higher in NSCLC tissues and cells than in adjacent normal tissues, whereas FOXO1 mRNA expression was significantly down-regulated. There was a significant negative correlation between miR-183-5p and FOXO1 mRNA in NSCLC tissues ($P < 0.05$). Additionally, the expression of miR-183-5p was significantly correlated with tumor size, tumor differentiation, and tumor-node-metastasis stage in patients with NSCLC ($P < 0.05$). miR-183-5p targeted and inhibited FOXO1 expression. Compared to the anti-miR-NC group, the cell proliferation, scratch healing rate, N-cadherin and vimentin protein expression, and the proportion of S phase cells were significantly lower in the anti-miR-183-5p group, whereas the protein expression of E-cadherin and α-catenin and the proportion of G0/G1 phase cells were significantly higher; additionally, the frequency of colony formation and invasion were significantly lower in the anti-miR-183-5p group ($P < 0.05$). Compared to the miR-NC group, the cell proliferation, scratch healing rate, N-cadherin and vimentin protein expression, and the proportion of S phase cells in the miR-183-5p group were significantly higher, whereas the E-cadherin and α-catenin protein expression and the proportion of G0/G1 phase cells were significantly lower; furthermore, the frequency of colony formation and invasion were significantly higher in the miR-183-5p group ($P < 0.05$). Compared with the miR-183-5p+pcDNA3.1 group, the OD value, scratch healing rate, N-cadherin and vimentin protein expression, and the proportion of S phase cells were significantly lower in the miR-183-5p+pcDNA3.1-FOXO1 group, whereas E-cadherin and α-catenin protein expression and the proportion of G0/G1 phase cells were significantly higher; additionally, the frequency of colony formation and invasion was significantly lower in the miR-183-5p+pcDNA3.1-FOXO1 group ($P < 0.05$). Overall, silencing miR-185-3p inhibited the growth of transplanted tumors and promoted FOXO1 expression.

Conclusion Overexpression of miR-183-5p can inhibit apoptosis and promote the proliferation, migration, invasion, and EMT, of NSCLC cells by down-regulating FOXO1 expression.

Key words: non-small cell lung cancer; miR-183-5p; forkhead box protein O1; malignant biological behavior; targeted regulation

Received: 12 October 2022
Revised: 23 May 2023
Accepted: 15 June 2023

✉ Correspondence to: Rong Qiu. Email: 443182163@qq.com
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Lung cancer is a malignant tumor with the highest morbidity and mortality of all cancers in China [1]. According to histological characteristics, lung cancer is divided into small cell lung cancer and non-small cell lung cancer (NSCLC), with NSCLC accounting for approximately 80% of total lung cancer diagnoses [2]. Approximately 90% lung cancer patient deaths are attributed to distal metastasis [3]; therefore, it is of particular importance to elucidate the mechanism of lung cancer cell metastasis and invasion. MicroRNA (miRNA) is a type of non-coding RNA that plays a key role in post-transcriptional gene regulation and target protein degradation. In recent years, miRNAs have been determined to be closely associated with the biological function of many kinds of tumor cells, including NSCLC cells [4, 5]. miR-144-3p expression is down-regulated in NSCLC tissues and cells; in contrast, its overexpression promotes apoptosis and inhibits the proliferation and migration of NSCLC cells [6]. Additionally, miR-183-5p is closely related to the occurrence and development of various diseases; for example, miR-183-5p is highly expressed in acute myeloid leukemia cells. The up-regulation and silencing of miR-183-5p expression are related to cell proliferation, differentiation, and development of acute myeloid leukemia; therefore, miR-183-5p is a novel prognostic biomarker and therapeutic target [7]. Wang *et al.* [8] established that miR-183-5p acts as a tumor-promoting factor in NSCLC, and down-regulation of its expression can inhibit cell proliferation and promote apoptosis, thereby inhibiting NSCLC progression. However, at present, few studies have evaluated the relationship between miR-183-5p and NSCLC progression, and its mechanism is yet to be elucidated. Forkhead box protein O1 (FOXO1) belongs to the FOXO family; it is abnormally expressed in colon and gastric cancer and is closely associated with the prognosis of patients [9, 10]. Additionally, some studies have confirmed that FOXO1 is associated with the proliferation, migration, invasion, and anti-radiation of NSCLC cells; therefore, this protein may be a potential therapeutic target for the enhancement of NSCLC radiosensitivity [11]. Through bioinformatics prediction, a targeted relationship between miR-183-5p and FOXO1 has been found. Down-regulation of miR-183-5p can promote the expression of FOXO1 and inhibit the proliferation, invasion, and angiogenesis of colorectal cancer cells; therefore, miR-183-5p can inhibit the progression of colorectal cancer [12]. However, the effects of miR-183-5p and FOXO1 on the biological functions of NSCLC cells have not yet been reported. Therefore, this study was conducted to explore the effects of miR-183-5p on the proliferation, migration, invasion, and epithelial-mesenchymal transition (EMT) of NSCLC cells through the targeted regulation of FOXO1 to provide further molecular targets for the treatment of NSCLC.

Materials and methods

Samples and participants

Lung cancer tissue (taken from > 5 cm of tumor foci) and corresponding paracancerous tissue samples were collected from 60 patients with lung cancer adenocarcinoma that were treated in our hospital from September 2020 to September 2022 and then preserved in liquid nitrogen. The clinical data, such as sex, age, and tumor diameter, were collected. No patients were treated with radiotherapy or chemotherapy. All the samples were collected following informed consent from the patients and their families. The research scheme was approved by the Ethics Committee of our hospital (051237L).

Reagents and instruments

Normal human lung epithelial cell line BEAS-2B and human NSCLC cell lines A549, SPCA-1, PC-9, and 95merD were purchased from Shanghai Cell Resource Center (Chinese Academy of Sciences, China). Reagents used include the following: Dulbecco's modified eagle medium (DMEM; Gibco Company, USA); fetal bovine serum (Sijiqing, China); Lipofectamine 2000 kit, Opti-MEM medium, TRIzol reagent, reverse transcriptase kit, trypsin (Thermo Fisher Scientific Company, USA); MTT solution (Sigma-Aldrich Co., Ltd., USA); Matrigel (Millipore Co., Ltd., USA); BCA Kit (Shanghai enzyme Union Biotechnology Co., Ltd., China); horseradish peroxidase labeled rabbit anti-IgG secondary antibody (LI-COR Biosciences Co., Ltd., USA); ECL developer (Shanghai Biyuntian Biotechnology Co., Ltd., China). The pcDNA3.1 vector and FOXO1 overexpression plasmid were purchased from Addgene (Addgene, USA); N-cadherin, vimentin, E-cadherin, and α-catenin primary antibodies were purchased from Abcam (Abcam Co., Ltd., UK). The equipment is listed as follows: NanoDrop 2000 ultramicro spectrophotometer (Thermo Fisher Scientific Co., Ltd., USA), BD FACSAria flow cytometer (BD Biosciences, USA), and IX71 inverted microscope (Olympus, Japan).

Cell culture, grouping, and transfection

The human NSCLC and normal lung epithelial cell lines were cultured in DMEM containing 10% fetal bovine serum and 100 U/mL penicillin and streptomycin at 37 °C and 5% CO_2. When adherent growth of the cells was observed, and confluence reached 85%, 25% trypsin was added for cell digestion. The digested cells were placed in the DMEM culture medium to continue culturing and passage; then, the expression of miR-183-5p and FOXO1 mRNA in each cell line was detected using qRT-PCR.

A549 cells were divided into 7 groups: control (untreated), anti-miR-NC (anti-miR-183-5p negative control), anti-miR-183-5p (anti-miR-183-5p

Table 1 PCR primer sequences

Genes	Upstream primer (5'-3')	Downstream primer (5'-3')
miR-183-5p	ATCTCACCAAACGACCGACT	TCCTGGCATCATTGGAGGAG
FOXO1	GGATGTGCATTCTATGGTGTACC	TTTCGGGATTGCTTATCTCAGAC
U6	GCGCGTCGTGAAGCGTTC	GTGCAGGGTCCGAGG

transfection), miR-NC (miR-183-5p negative control), miR-183-5p (miR-183-5p transfection), MiR-183-5p+pcDNA3.1 (transfected with miR-183-5p and pcDNA3.1-FOXO1 negative control pcDNA3.1), and miR-183-5p+pcDNA3.1-FOXO1 (transfected with miR-183-5p and pcDNA3.1-FOXO1). Transfection was conducted using the Lipofectamine 2000 kit, containing 500 μL Opti-MEM medium (100 nM mimic/inhibitor or its negative control), and 2 μL Lipofectamine 2000; the corresponding reagents were added to a 24-well plate inoculated with the A549 cells. After 48 h of culture, the cells were collected for follow-up experiments.

Target gene prediction and dual luciferase reporter assay

The related target genes of miR-183-5p were predicted using the DIANA-mirPath v.3 tools (http://diana.imis.athena-innovation.gr); consequently, the *FOXO1* gene was determined to be a potential target gene of miR-183-5p. The 3′ untranslated region (UTR) of FOXO1 wild type (FOXO1 3′UTR-WT) and FOXO1 mutant 3′UTR (FOXO1 3′UTR-MUT) luciferase expression vectors were constructed and co-transfected into A549 cells with miR-NC and miR-183-5p, using the Lipofectamine 2000 kit. Luciferase activity was detected according to the manufacturer's instructions, and the experiment was repeated 3 times.

RNA-binding protein immunoprecipitation (RIP) assay

A Magna RIP RNA binding protein immunoprecipitation kit (Merck Millipore, USA) was used for RIP analysis. The specific procedure was as follows: the cultured A549 cells were collected and resuscitated with RIP lysis buffer; the cells were then incubated with magnetic beads conjugated with anti-AGO2 antibody or IgG antibody overnight at 4 °C; the following day, after washing 3 times, the magnetic beads were incubated with protease K. Finally, the total RNA was extracted, and cDNA was synthesized; the relative expression of miR-183-5p and FOXO1 mRNA were determined using qRT-PCR.

RNA pull-down

Approximately 2×10^7 A549 cells were cultured in two 15 cm petri dishes. Then, the biotin-labeled miRNA probe (miR-183-5p-Bio group) and negative control probe (Bio-NC group) were transfected. After 48 hours of culture, the cells were washed twice with precooled phosphate-buffered saline (PBS). An miRNA pull-down kit (Guangzhou Boxin Biotechnology Co., Ltd., China) was used for miRNA pull-down test. According to the manufacturer's instructions, magnetic bead sealing, cell lysis, hybridization incubation, elution, and RNA precipitation were conducted in turn. After completing the pull-down experiment, enrichment was detected by qRT-PCR.

qRT-PCR of miR-183-5p and FOXO1 mRNA in tissues and cells

Total RNA in tissues and cells was extracted using TRIzol reagent; this RNA was then reverse transcribed into cDNA according to manufacturer's instructions. For post-transcriptional amplification, 1 μL of cDNA was used; U6 was used as the internal reference. qRT-PCR reaction conditions were as follows: an initial pre-denaturation at 95 °C for 30 s, followed by 40 cycles of 95 °C for 10 s and 60 °C for 30 s. The relative expression levels of miR-183-5p and FOXO1 mRNA were calculated using the $2^{-\Delta\Delta CT}$ formula. PCR primer sequences are shown in Table 1.

MTT assay

The transfected A549 cells were inoculated into a 96-well plate at a density of 5×10^3 cells per well and incubated with 180 μL of DMEM for 0, 24, 48, and 72 h. Additionally, 20 μL of MTT solution and 150 μL of dimethyl sulfoxide were added to each well after 10 min. The optical density (OD) of each well was measured at 490 nm using an enzyme labeling instrument (Thermo Fisher Scientific, USA).

Clone formation experiment

The transfected A549 cells were inoculated in a 6-well plate at a density of 1×10^3 cells per well and cultured for 2 weeks. When the clone colony was visible to the naked eye, the culture was terminated, fixed with formaldehyde, stained with Giemsa, washed, and dried. The colony formation of cells was observed and counted under light microscope. This experiment was repeated 3 times.

Transwell chamber experiment

A Transwell system (aperture 8 mm; Corning Costar, USA) with Matrigel (Millipore) was used to measure cell migration ability. The transfected A549 cells were

suspended in a serum-free RPMI 1640 medium and inoculated into the upper chamber, which contained RPMI 1640 medium supplemented with 20% fetal bovine serum. After 24 h of culture, methanol was used to fix the samples; cells were then stained with 0.1% crystal violet. An IX71 inverted microscope (Olympus) was used to image the samples to observe the samples and count the number of invading cells.

Scratch test

The transfected A549 cells were inoculated into a 6-well plate. After reaching 80% confluence, the tip of the pipette was used to form scratches in the middle of each hole. PBS was used to wash the cells; serum-free medium was then added. Using a microscope, cells were observed and the scratch width was determined; cells were then cultured for 24 h, followed by further observation and determination of the scratch width. Consequently, the scratch healing rate could be calculated.

Flow cytometry

The transfected A549 cells were collected and fixed overnight at -20 °C. The cells were collected by centrifugation, washed with PBS, then resuscitated with 450 μL PBS and 50 μL of 0.5 mg/mL propidium iodide (PI) in a water bath at 37 °C for 30 min; cells were then centrifuged and resuscitated with PBS. The cell cycle distribution was measured and analyzed using a BD FACSAria flow cytometer.

The transfected A549 cells were collected and washed with PBS. The cells were suspended with precooled 1 × binding buffer (500 mL). Then, 5 μL of Annexin-V-FITC and 2.5 μL of PI were added to the samples. Apoptosis was detected using a BD FACSAriaTM flow cytometer.

Tumorigenesis experiment in nude mice

A549 cells (2×10^6) stably transfected with anti-miR-NC or anti-miR-183-5p were subcutaneously injected into 6-week-old female BALB/c nude mice, which were purchased from Beijing Weitong Lihua Experimental Animal Technology Co., Ltd (China). Animal health was monitored every day, and tumor size was measured every week. After 4 weeks, the mice were euthanized, the tumor tissue was removed, the tumor volume was measured, and the relative expression levels of miR-183-5p and FOXO1 mRNA in the tumor tissue were detected by qRT-PCR.

Western blotting

Total protein was extracted from the transfected A549 cells and corresponding total protein concentration was determined. The total protein was separated using electrophoresis and transferred into 5% skimmed milk. The cells were blocked with 5% skimmed milk for 1 h, and then incubated overnight at 4 °C with N-cadherin (1:2000), vimentin (1:2000), E-cadherin (1:7000), or α-catenin (1:5000). After washing the polyvinylidene difluoride membrane, rabbit anti-IgG secondary antibody labeled with horseradish peroxidase (1:10000) was added and incubated at Billerica for 2 h. ECL chromogenic agent was added to avoid light exposure. The gray level of the strip was analyzed using a Li-Cor Odyssey infrared imaging system (version 3.0 software, LI-COR Biosciences); β-actin was used as internal reference.

Statistical analysis

The data were analyzed using SPSS software (version 22, SPSS Inc., USA). The counting and measurement data were expressed in the form of rate (%) and mean ± standard deviation; the differences between two groups were compared using a χ^2 test and t-test for counting and measurement data, respectively. $P < 0.05$ was defined as statistically significant.

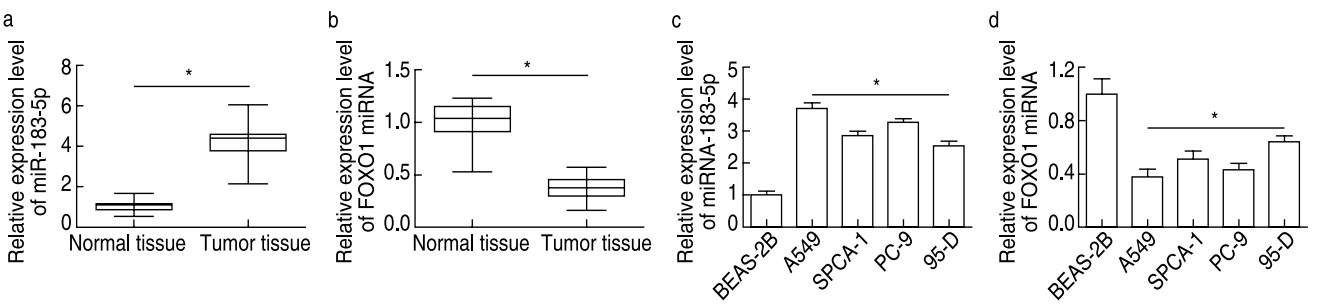

Fig. 1 The expression of miR-183-5p and FOXO1 in NSCLC tissues and cells. (a) miR-183-5p expression in NSCLC and normal tissues detected by qRT-PCR; (b) FOXO1 mRNA expression in NSCLC and normal tissues detected by qRT-PCR; (c) miR-183-5p is higher in NSCLC cell lines and lower in a normal human lung epithelial cell line (BEAS-2B); (d) FOXO1 mRNA expression is lower in NSCLC cell lines and higher in a normal human lung epithelial cell line (BEAS-2B). *$P < 0.05$

Results

Expression of miR-183-5p and FOXO1 in NSCLC tissues and cells

The qRT-PCR results (Fig. 1) demonstrated that the expression level of miR-183-5p in NSCLC tissues was significantly higher than that in normal tissues, whereas the expression level of FOXO1 mRNA was significantly lower than that in normal tissues ($P < 0.05$). Further, compared with the normal human lung epithelial cell line BEAS-2B, the miR-183-5p expression in the NSCLC cell lines A549, SPCA-1, PC-9, and 95-D was significantly higher, whereas the FOXO1 mRNA expression was significantly lower ($P < 0.05$).

Relationship between miR-183-5p expression and clinicopathological parameters in patients with NSCLC

According to the median relative expression of miR-183-5p in NSCLC tissues, 60 NSCLC patients were divided into the miR-183-5p low expression ($n = 30$) and miR-183-5p high expression ($n = 30$) groups. The relationship between miR-183-5p expression and clinicopathological parameters of NSCLC patients was analyzed. As shown in Table 2, miR-183-5p expression was significantly correlated with tumor size, tumor differentiation, and tumor-node-metastasis (TNM) stage in NSCLC patients ($P < 0.05$). Nonetheless, there was no significant correlation between miR-183-5p expression and age, sex, smoking, histological type, or lymph node metastasis ($P > 0.05$).

Table 2 Relationship between miR-183-5p expression and clinicopathological parameters in patients with NSCLC [*n* (%)]

Pathological features	Case	miR-183-5p		χ^2	*P*
		High expression	Low expression		
Age (years)				2.411	0.121
≤ 49	32	13 (40.63)	19 (59.38)		
> 49	28	17 (60.71)	11 (39.29)		
Gender				2.443	0.118
Male	34	14 (41.18)	20 (58.82)		
Female	26	16 (61.54)	10 (38.46)		
Smoking history				2.400	0.121
No	30	12 (40.00)	18 (60.00)		
Yes	30	18 (60.00)	12 (40.00)		
Tumor size (cm)				16.484	< 0.001
≤ 3	21	18 (85.71)	3 (14.29)		
> 3	39	12 (30.77)	27 (69.23)		
Tumor differentiation				11.380	0.001
High-middle grade	33	10 (30.30)	23 (69.70)		
Low grade	27	20 (74.07)	7 (25.93)		
Lymph node metastasis				0.287	0.592
No	22	12 (54.55)	10 (45.45)		
Yes	38	18 (47.37)	20 (52.63)		
TNM stage				10.000	0.002
I-II	24	6 (25.00)	18 (75.00)		
III	36	24 (66.67)	12 (33.33)		

Targeted inhibition of FOXO1 expression by miR-183-5p

The DIANA TOOLS prediction results indicated that there are potential binding sites for miR-183-5p in the FOXO1 3′UTR (Fig. 2a). The double luciferase assay (Fig. 2b) demonstrated that the luciferase activity of wild type FOXO1 in the miR-183-5p group was significantly lower than that in the miR-NC group ($P < 0.05$), indicating that miR-183-5p conferred targeted down-regulation of FOXO1. Additionally, the Pearson test results (Fig. 2c) showed that there was a significant negative correlation between miR-183-5p and FOXO1 mRNA expression in NSCLC ($P < 0.05$). Further, RIP results demonstrated that both miR-183-5p and FOXO1 could bind to AGO2 protein, indicating that miR-183-5p may bind to FOXO1 protein, thereby reducing FOXO1 expression (Fig. 2d). Following RNA pull-down analysis (Fig. 2e), clear bands were observed in the miR-183-5p-Bio pull-down group, but no clear bands were observed in the Bio-NC pull-down group; this indicated that FOXO1 could bind to and be pulled down by the miR-183-5p biotin probe in this assay. Additionally, the corresponding miR-4319 enrichment in the miR-183-5p-Bio group was significantly higher than that in the Bio-NC group ($P < 0.05$).

miR-183-5p overexpression regulates FOXO1 to promote proliferation, migration, invasion, and EMT of NSCLC cells

The results from the MTT assay (Fig. 3a), clone formation (Fig. 3b), Transwell assay (Fig. 3c), scratch test (Fig. 3d), and western blot analysis (Fig. 4) demonstrated that there was no significant difference in OD value, frequency of colony formation, frequency of invasion, scratch healing rate, and N-cadherin, vimentin, E-cadherin and α-catenin protein expression among the control, anti-miR-NC, and miR-NC groups ($P < 0.05$). Compared to the anti-miR-NC group, the OD value, scratch healing rate, N-cadherin and vimentin protein expression, and frequency of colony formation and invasion were significantly lower, whereas the E-cadherin and α-catenin protein expression were significantly higher in the anti-miR-183-5p group ($P < 0.05$). Additionally, the OD value, scratch healing rate, N-cadherin and vimentin protein expression, and frequency of colony formation and invasion were significantly higher, and E-cadherin and α-catenin protein expression were significantly lower in the miR-183-5p group than in the miR-NC group ($P < 0.05$). Further, compared to the miR-183-5p+pcDNA3.1

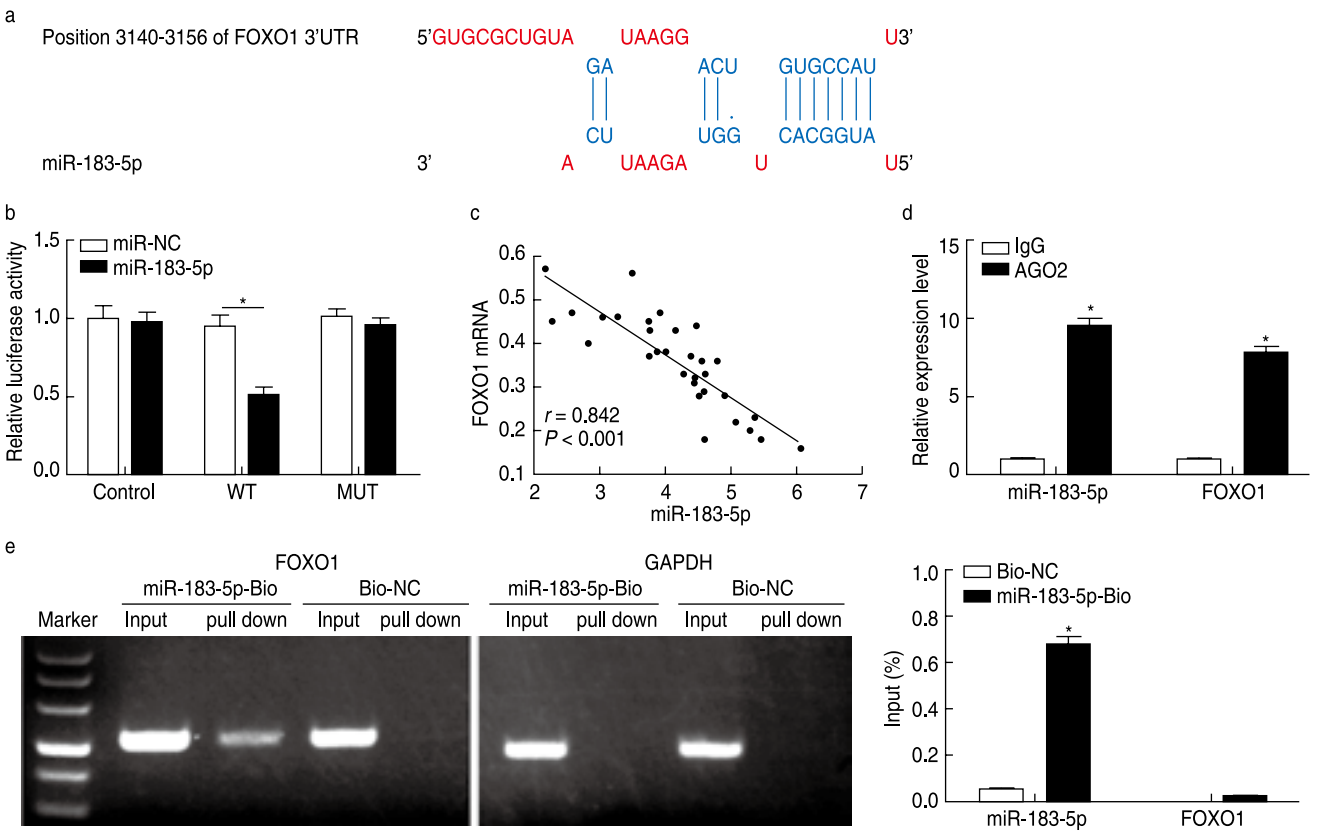

Fig. 2 miR-183-5p targets and inhibits FOXO1 expression. (a) DIANA TOOLS predicts the potential binding sites of miR-183-5p to FOXO1; (b) Verification of the targeting relationship between miR-183-5p and FOXO1 (double luciferase test); (c) Pearson test to analyze the correlation between miR-183-5p and FOXO1 mRNA expression in NSCLC tissues; (d) RIP assay results; (e) Pull-down assay results. *$P < 0.05$

group, the OD value, scratch healing rate, N-cadherin and vimentin protein expression, and frequency of colony formation and invasion were significantly lower, whereas E-cadherin and α-catenin protein expression were significantly higher in the miR-183-5p+pcDNA3.1-FOXO1 group ($P < 0.05$).

miR-183-5p overexpression targets the effects of FOXO1 on the cell cycle and apoptosis of NSCLC cells

Flow cytometry was used to analyze the effects of miR-183-5p overexpression on the cell cycle and apoptosis of NSCLC cells by targeted regulation of FOXO1. The PI single staining results indicated that (Fig. 5a) there was no significant difference in the proportion of cells in G0/G1 phase and S phase among the control, anti-miR-NC, and miR-NC groups ($P > 0.05$). Compared to the anti-miR-NC group, the proportion of G0/G1 phase cells in the anti-miR-183-5p group was significantly higher, whereas the proportion of S phase cells was significantly lower. Additionally, the proportion of G0/G1 phase cells was lower and S phase cells was higher in the miR-183-5p group than in the miR-NC group. Further, compared to the miR-183-5p+pcDNA3.1 group, the proportion of G0/G1 phase cells in the miR-183-5p+pcDNA3.1-FOXO1 group was significantly higher, whereas the proportion of S phase cells was significantly lower. However, there was no significant difference in the proportion of cells in the G2/M phase among the different groups ($P > 0.05$). The Annexin-V-FITC and PI double staining results showed that there was no significant difference in the apoptosis rate among the control, anti-miR-NC, and miR-NC groups ($P > 0.05$; Fig. 5b). Nonetheless, compared to the anti-miR-NC group, the apoptosis rate of the anti-miR-183-5p group was significantly higher. Compared to the miR-NC group, the apoptosis rate of the miR-183-5p group was significantly lower. Additionally, the apoptosis rate of the miR-183-5p+pcDNA3.1-FOXO1 group was significantly higher than that of the miR-183-5p+pcDNA3.1 group ($P < 0.05$).

Silencing miR-183-5p expression inhibits tumor growth *in vivo*

A549 cells stably infected with anti-miR-NC and anti-miR-183-5p were subcutaneously injected into nude mice; the corresponding tumor growth four weeks later

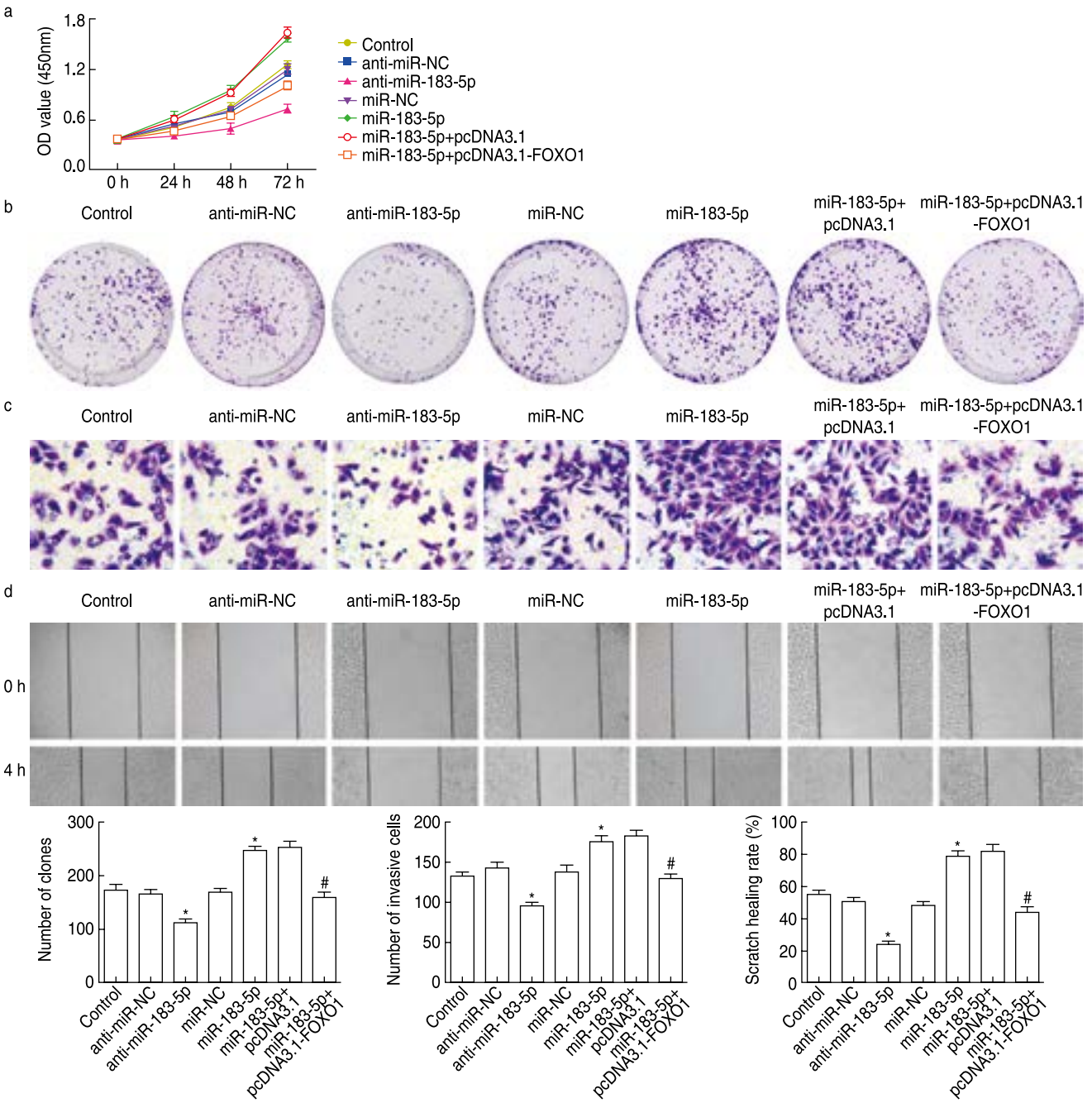

Fig. 3 Overexpression of miR-183-5p regulates FOXO1, thereby promoting the proliferation, migration, invasion, and EMT of NSCLC cells. (a) MTT assay for cell viability; (b) Clone formation test for cell proliferation; (c) Transwell chamber test for cell invasion; (d) Scratch test for cell migration. $^{*}P < 0.05$, compared with the control, anti-miR-NC, and miR-NC groups; $^{\#}P < 0.05$, compared with the miR-183-5p+pcDNA3.1 group

is shown in Fig. 6a. Compared to the anti-miR-NC group, the volume (Fig. 6b) and mass (Fig. 6c) of the transplanted tumor in the anti-miR-183-5p group were significantly lower; additionally, the relative miR-183-5p expression in the transplanted tumor (Fig. 6d) was significantly lower and the relative FOXO1 mRNA expression (Fig. 6e) was significantly higher in the anti-miR-183-5p group than that in the anti-miR-NC group.

Mechanism of miR-183-5p-targeted inhibition of FOXO1 in the proliferation, migration, invasion, and EMT of NSCLC cells

By detecting the expression of miR-183-5p and FOXO1, we found that both were significantly up-

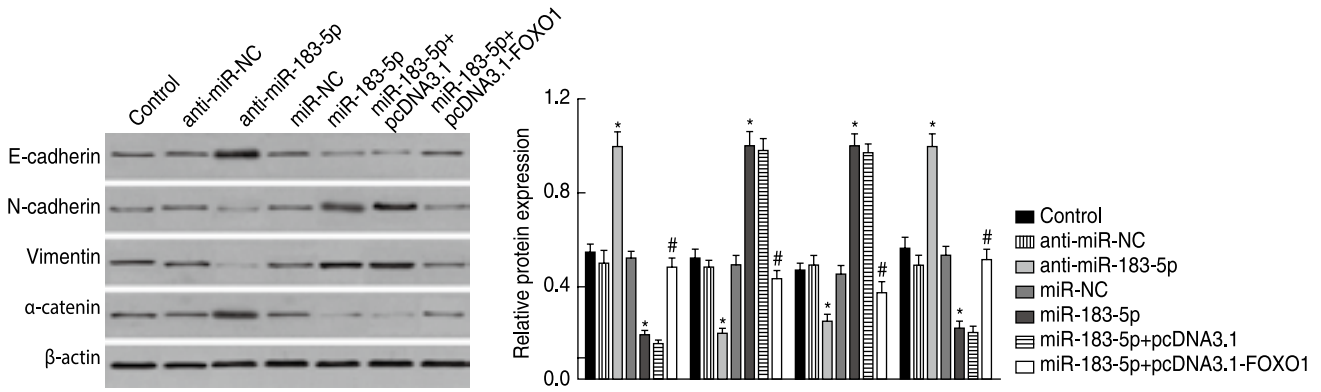

Fig. 4 Western blot analysis for the expression of EMT-related proteins in the cells of each group. **P* < 0.05, compared with the control, anti-miR-NC, and miR-NC groups; #*P* < 0.05, compared with the miR-183-5p+pcDNA3.1 group

regulated in NSCLC tissues and cells. Additionally, miR-183-5p could specifically inhibit the expression of FOXO1. To further observe the effects of miR-183-5p and FOXO1 on the malignant biological behavior of NSCLC cells, we detected the proliferation, invasion, migration, EMT-related protein expression, cell cycle, and apoptosis of NSCLC cells. Overexpression of miR-183-5p was observed to promote cell proliferation, invasion, migration, EMT, and cell cycle progression, whilst inhibiting apoptosis and FOXO1 expression. Nonetheless, up-regulation of FOXO1 expression can ameliorate these malignant effects. Therefore, it was inferred that miR-183-5p overexpression promotes the malignant biological behavior of NSCLC cells by targeting the inhibition of FOXO1 expression (Fig. 7).

Discussion

In recent years, the role of miRNA in cancer progression has been continuously explored. During tumor occurrence and development, miRNA can regulate the biological functions of tumor cells by regulating the expression of tumor suppressor genes or proto-oncogenes. Additionally, several miRNAs play an important role in the occurrence and development of NSCLC [13, 14]. Zhao *et al.* [15] confirmed that miR-641 inhibits NSCLC tumor development; therefore, up-regulation of miR-641 expression inhibits the proliferation, migration, and invasion of NSCLC cells, thus reducing the cisplatin resistance of NSCLC cells. Further, Luo *et al.* [16] established that miR-195-5p expression is down-regulated in NSCLC, and miR-195-5p overexpression can inhibit the proliferation of NSCLC cells, arrest the cell cycle in G0/G1 phase, and promote apoptosis; ultimately, this provides a potential target for the treatment of NSCLC. Alternatively, the miR-183-5p expression is significantly up-regulated in lung cancer tissue; therefore, miR-183-5p has been established as the differentially expressed of miRNA in lung cancer tissue, indicating that this miRNA may play an important role in the occurrence and development of lung cancer [17]. The prognosis of patients with NSCLC largely depends on their corresponding clinical stage and tumor differentiation [18]. In the current study, miR-183-5p was determined to be highly expressed in NSCLC tissues and cells; further, its corresponding expression levels were determined to be related to tumor size, TNM stage, and tumor differentiation in NSCLC patients. Overexpression of miR-183-5p was determined to promote NSCLC cell proliferation, invasion, migration, EMT, and cell cycle progression, whilst also inhibiting apoptosis, which is consistent with the corresponding results obtained by Wang *et al.* [8]; this indicates that miR-183-5p plays a role as a cancer-promoting factor in NSCLC. However, at present, few studies have been conducted on the effects of miR-183-5p on NSCLC; therefore, the specific mechanism of action of this miRNA is yet to be fully elucidated.

Forkhead box proteins are a type of transcription factor that primarily exist in invertebrates and mammals. FOXO is one of the largest subfamilies of the forkhead box family. At present, four FOXO subtypes (FOXO1, FOXO3a, FOXO4, and FOXO6) have been identified in mammals, all of which have homologous genes in nematodes [19]. Specifically, FOXO1 has been widely studied as a hypertranscription factor with complex activity, which can regulate the expression of genes involved in apoptosis, the cell cycle, metabolism, stress responses, and differentiation [20]. The abnormal expression of FOXO1 is also involved in the occurrence and development of various cancers. Ma *et al.* [21] demonstrated that FOXO1 expression is significantly down-regulated in ovarian cancer tissues and cells, and its low expression is significantly correlated with paclitaxel resistance, poor prognosis, short disease-free survival

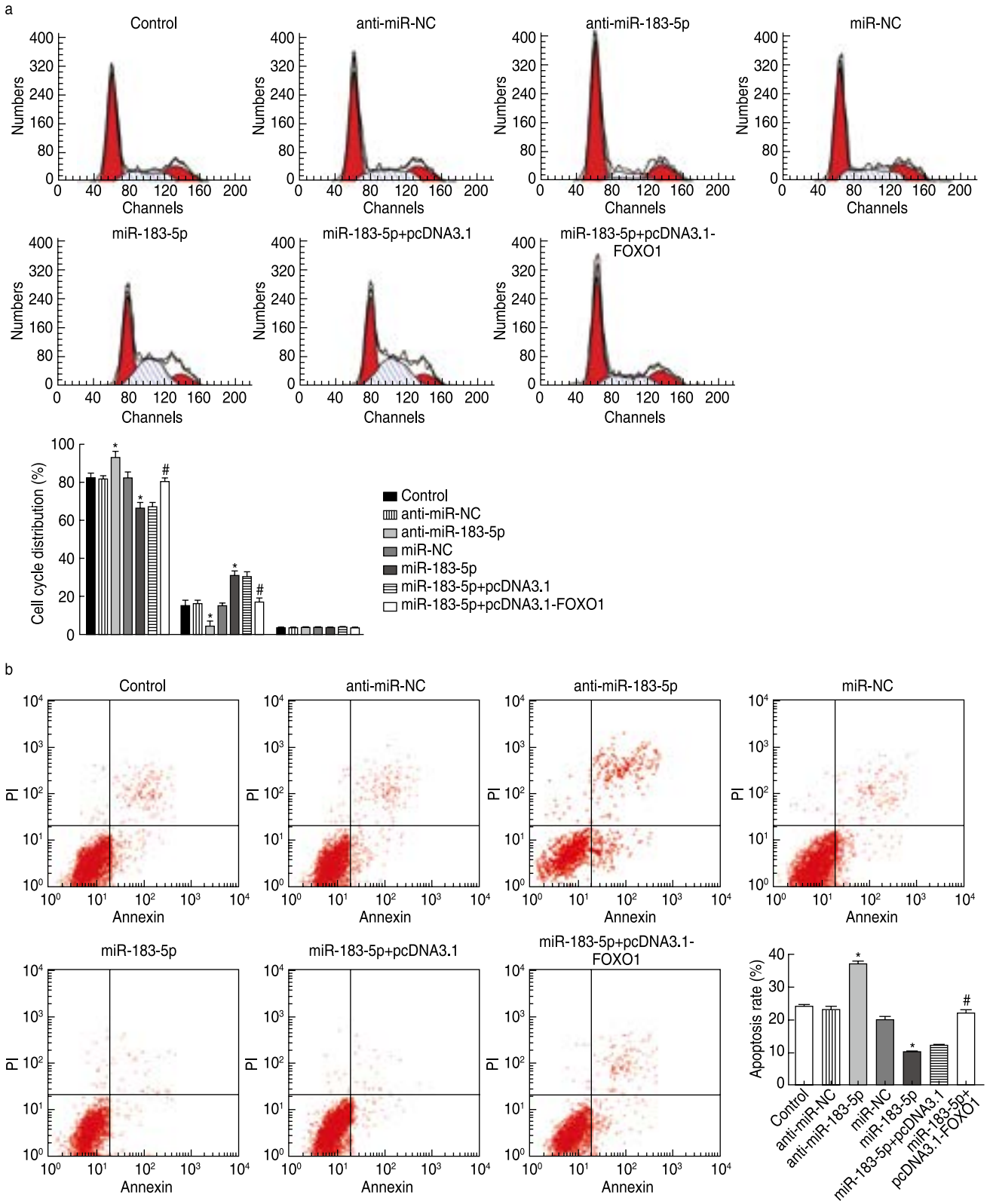

Fig. 5 miR-183-5p overexpression targets the effect of FOXO1 on the cell cycle and apoptosis of NSCLC cells. (a) PI single staining to compare the cell cycle distribution of each group; (b) Annexin-V-FITC and PI double staining to compare the apoptosis rate of cells in each group. **P* < 0.05, compared with the control, anti-miR-NC, and miR-NC groups; #*P* < 0.05, Compared with the miR-183-5p+pcDNA3.1 group

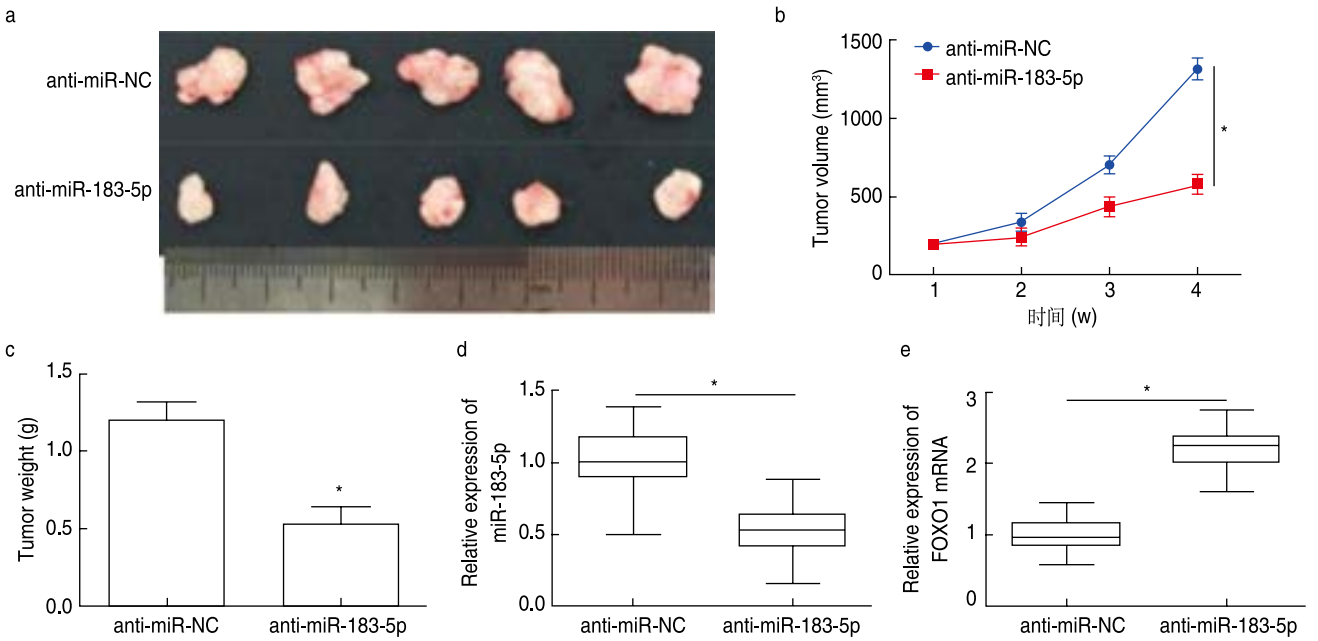

Fig. 6 Silencing miR-183-5p expression inhibits tumor growth in vivo. (a) Images of tumors 4 weeks after inoculation with anti-miR-NC or anti-miR-183-5p; (b) Comparison of the transplanted tumor volumes; (c) Comparison of the transplanted tumor weights; (d) Comparison of the relative miR-183-5p expression in transplanted tumors; (e) Comparison of the relative FOXO1 mRNA expression in transplanted tumors. *P < 0.05, compared with the anti-miR-NC group

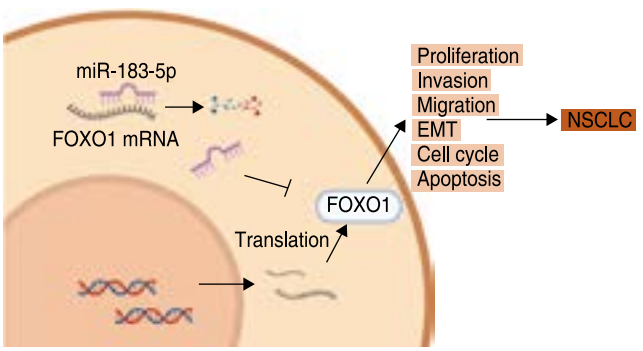

Fig. 7 Mechanism of miR-183-5p-targeted inhibition of FOXO1, resulting in an increase in the proliferation, migration, invasion, and EMT of NSCLC cells

and overall survival in patients with ovarian cancer. Therefore, FOXO1 can be used as a novel therapeutic target and prognostic marker for ovarian cancer. Further, Gheghiani *et al.* [22] have indicated that FOXO1 is a differentially expressed gene in prostate cancer, with its expression being significantly down-regulated in prostate cancer. This low FOXO1 expression is closely associated with the occurrence of prostate cancer and poor prognosis. However, few studies have evaluated the role of FOXO1 in the development of NSCLC; therefore, the function of FOXO1 in NSCLC remains unclear. In the current study, FOXO1 expression is down-regulated in NSCLC tissues and cells; in contrast, FOXO1 up-regulation was observed to inhibit the proliferation, invasion, migration, EMT, and other malignant biological behaviors of NSCLC cells. A variety of miRNAs can participate in the occurrence and development of cancer through targeting the regulation of FOXO1 expression. Bioinformatic prediction indicated that there were complementary binding sites between the 3'UTR of FOXO1 and miR-183-5p. Additionally, Han *et al.* [23] confirmed that down-regulation of miR-183-5p can promote the expression of FOXO1, thereby inhibiting the proliferation, migration, invasion, and glycolysis of thyroid cancer cells, and ultimately inhibiting the progression of thyroid cancer. However, the effects and mechanisms of miR-183-5p and FOXO1 on the biological processes of NSCLC cells are yet to be reported.

The double luciferase, RIP, and RNA pull-down experiments in this study also confirmed that miR-183-5p could specifically inhibit the expression of FOXO1, and that there was a significant negative correlation between miR-183-5p and FOXO1 mRNA expression in NSCLC; this indicated that FOXO1 may be regulated by miR-183-5p. In the present study, we interfered with miR-183-5p and FOXO1 expression in NSCLC cells. Overall, after co-transfection of miR-183-5p and FOXO1, the miR-183-5p-mediated up-regulation of proliferation, invasion, and migration of NSCLC cells was eliminated; this indicated that FOXO1 could reverse the effect of miR-183-5p on NSCLC cells. EMT is a key step in the early stages of cancer metastasis and primarily manifests as the transformation of epithelial cells to stromal

cells. In this study, overexpression of miR-183-5p was determined to promote the expression of N-cadherin and vimentin and inhibit the expression of E-cadherin and α-catenin. Nonetheless, this could be reversed by FOXO1 overexpression; therefore, it was established that miR-183-5p could affect EMT in NSCLC cells by regulating the expression of FOXO1. In addition, this study found that miR-183-5p can affect the cell cycle and apoptosis of NSCLC cells by regulating FOXO1 expression. *In vivo* experiments in this study also confirmed that down-regulating miR-183-5p expression can inhibit the growth of transplanted tumors and promote the FOXO1 expression.

In conclusion, the overexpression of miR-183-5p can promote the proliferation, migration, invasion, EMT, and cell cycle process of NSCLC cells, whilst also inhibiting apoptosis. Additionally, this mechanism was determined to occur via the miR-183-5p-mediated down-regulation of FOXO1 expression. This study provides a novel experimental basis for the progression of NSCLC and indicates that miR-183-5p may be a potential target for the corresponding clinical treatment of NSCLC. Nonetheless, some limitations remain regarding the understanding of miR-183-5p in NSCLC. First, the effects of silent FOXO1 expression on the proliferation, invasion, migration, and other malignant biological behaviors of NSCLC cells have yet to be explored; second, the specific mode of cell death initiated by this process has not been further evaluated. Therefore, these limitations need to be addressed in future research.

Acknowledgments
Not applicable.

Funding
Not applicable.

Conflicts of interest
The authors indicated no potential conflicts of interest.

Author contributions
All authors contributed to data acquisition, data interpretation, and reviewed and approved the final version of this manuscript.

Data availability statement
Not applicable.

Ethical approval
This study was approved by the Ethics Committee of Suining Central Hospital (051237L).

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DOI 10.1007/s10330-022-0601-1
Cite this article as: Deng Y, He ZG, Luo XB, *et al*. MiR-183-5p promotes the progression of non-small cell lung cancer through targeted regulation of FOXO1. Oncol Transl Med. 2023;9(3):121–132.