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miR-361-5p suppresses lung cancer cell lines progression by targeting FOXM1

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Lung cancer is the most common type of cancer and the leading cause of death in worldwide. MicroRNAs are known to be key players in a variety of biological processes, including tumorigenesis. In present study, we investigated the effect of miR-361-5p on lung cancer progression. We found that miR-361-5p was down-regulated in lung cancer. Overexpression of miR-361-5p suppressed lung cancer proliferation and invasion. Mechanistically, FOXM1 was identified as a direct target of miR-361-5p. Furthermore, miR-361-5p inhibits EMT-like phenotype through down-regulation of FOXM1 expression in lung cancer cells. In conclusion, our results indicated that miR-361-5p acts as a tumor suppressor in lung cancer.

Key words: miR-361-5p, FOXM1, EMT, lung cancer

Lung cancer is the most common type of cancer and the leading cause of death in worldwide. It is the most frequently diagnosed cancer in males and the second in the females [1]. Most lung cancer patients at an early stage have no symptoms, nearly two-thirds are at an advanced stage (stage III or IV) when they are seeking medical advices [2]. Despite improvements in lung cancer diagnosis and treatment, the prognosis of lung cancer remains very poor with the 5-year survival rate at a dismal less than 5% for patients who had locally advanced or metastatic disease [2]. Therefore, a better understanding of the mechanisms of lung cancer is urgently needed.

MicroRNAs (miRNAs) are a class of small (19-24 nucleotides) non-coding functional RNAs, highly conserved and widespread in the whole body. MicroRNA mediates post-transcriptional regulation of gene expression by targeting 3'untranslated regions (3'UTR) of mRNAs, now known to be key players in a variety of biological processes, including development, differentiation, growth, metabolism and tumorigenesis [3-6]. Increasing evidences confirmed that microRNA plays a crucial role in lung cancer tumorigenesis [7-10]. Emerging evidences have indicated that miR-361-5p was associated with various types of cancers. For instance, loss of miR-361-5p has been associated with increasing the malignancy grade in human cutaneous squamous cell carcinoma [11], prostate cancer [12], colorectal and gastric cancer [13, 14] and hepatocellular carcinoma [15]. In contrast, miR-361-5p has been implied as an oncogene in cervical cancer [16]. Besides, miR-361-5p was reported to related to Late-onset hypogonadism [17] and acute myocardial infarction [18]. But the role of miR- 361-5p in the lung cancer is still limited.

In the present study, we found that the expression of miR-361-5p was decreased in lung cancer tissues. Moreover, overexpression of miR-361-5p inhibited cell proliferation and invasion, and promotes cell apoptosis in lung cancer cell lines. Furthermore, FOXM1 was identified as a new direct target of miR-361-5p. FOXM1 mediated miR-361-5p regulated EMT-like phenotype in lung cancer.

Materials and methods

Cell culture. Human lung cancer cell lines A549, NCI-H1299, NCI-H460, HCC827 and embryonic kidney cell line HEK-293T were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). A549 and HCC827 cells were cultured in DMEM medium (Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco). NCI-H1299 and

NCI-H460 were cultured in RPMI-1640 medium (Gibco) supplemented with 10% FBS. HEK-293T was cultured in DMEM medium with high glucose (Gibco) supplemented with 10% FBS. All cells were cultured with 1% streptomycin/penicillin (Gibco) and incubated at 37°C in a 5% CO_2 atmosphere. Normal human bronchial epithelial cells (NHBE) were maintained in a culture medium according to the protocol provided by Clonetics™. All cells were expanded immediately and multiple aliquots were cryopreserved. Cells were then used within 6 months of resuscitation.

Tissue specimens. The study was approved by the Institutional Review Board of Tianjin Medical University Cancer Institute and Hospital and informed consent was obtained from all patients. Specimens from 20 patients with primary lung cancers and the paired adjacent histologic normal tissues (> 2 cm distance from the margin of the resection) were immediately frozen in liquid nitrogen and stored at –80°C.

Plasmids, miRNAs and transfection. miRNA-361-5p mimic, mimic control, inhibitor and inhibitor control were chemically synthesized by RiBoBio (Guangzhou, China). A 3'UTR fragment containing the predicted miR-361-5p targeting site of FOXM1 was cloned into pGL3-control plasmid. The miR-361-5p mutated binding site was also constructed. All constructs were verified by DNA sequencing. miRNAs were transfected with Fugene6 (Promega) and plasmids were transfected with ViaFect (Promega) according to the manufacture's protocol.

RNA extraction and quantitative real-time PCR (RT-qPCR). For mRNA detection, total RNAs of cells were extracted with RNAiso Plus (TakaRa) according to the manufacture's protocol. Reverse transcription was performed with PrimeScript RT reagent kit (TakaRa) according to the manufacture's protocol. RT-qPCR was performed using SYBR Premix Ex Taq II (TaKaRa) on a Bio-Rad iQ5 Optical System (Bio-Rad). β-actin was used as a reference gene.

For miRNA detection, reverse transcription was performed with TaqMan MicroRNA Reverse Transcription kit and RT-qPCR was performed with TaqMan miR-361-5p and U6 RNA (used as a normalizer) assays (Life Technologies) according to the manufacture's protocol. Relative expression was quantified using the $2^{-\Delta Ct}$ method. The sequences of primers were list on Table 1.

Proliferation assays. MTT and colony formation were used to evaluate the ability of cell proliferation. For MTT assay, 48 h after transfection, 5,000 cells were seeded in 96-wells plates per well. After incubation for indicated time, the cells were incubated with 20 μl MTT (5 mg/mL; Sigma-Aldrich) at 37°C for 4 h. Then, the medium was removed and Formazan was dissolved in 150 μl of dimethyl sulfoxide (DMSO; Sigma-Aldrich). The absorbance was detected at 570 nm using a micro-plate auto-reader (Bio-Rad, Richmond).

For colony formation assay, 48 h after transfection, 500 cells were seeded in 6-wells plates per well and cultured as previously described for about 10 days until visible colonies. The colonies were fixed with 4% Paraformaldehyde (Solarbio) for 30-60 minutes and then stained with 0.2% crystal violet (Solarbio) for 30-60 minutes at room temperature. The colonies were counted and compared with control cells.

Transwell assay. The invasion abilities of lung cancer cells *in vitro* were evaluated by Matrigel coated Transwell inserts (BD Biosciences). 5×10^4 cells in 200 μl serum-free medium were added to upper chamber of transwell and 600 μl 20% FBS contained medium was added in lower chamber. After 24 hours, the migrant cells were fixed with 4% paraformaldehyde (Solarbio) for 30 minutes and stained with Giemsa (Solarbio) for 30-60 minutes. All operations were done at room temperature. The cells were observed under a microscope and the number of migrated cells was counted in five predetermined fields.

Cell apoptosis assay. The cell apoptosis assay was performed with Annexin V Staining Kit (BD Biosciences). Cells were washed and resuspended in the binding buffer. Then incubated in the Annexin V and PI and analyzed using BD FACSCanto II.

Luciferase reporter assay. Cells were seeded in 24-wells plates, cultured overnight, and co-transfected with corresponding plasmids and miRNAs. 48 h after transfection, luciferase activity was detected with a dual luciferase assay kit (Promega) according to the manufacturer's protocol.

Table 1. Sequences of Primers used for RT-qPCR

Gene	**Up** 5' → 3'	**Low** 5' → 3'
FOXM1	TGGGGAGGAAATGCCACACTTAG	TAGGACTTCTTGGGTCTTGGGGTG
SLUG	TTCGGACCCACACATTACCT	TTGGAGCAGTTTTTGCACTG
SNAIL	GTCCGTCTGCCGCACCTGAG	ACACGGCGGTCCCTACAGC
TWIST	ACGAGCTGGACTCCAAGATG	CACGCCCTGTTTCTTTGAAT
ZEB1	AGGCAGATGAAGCGAGATGT	TCTGGTCCTCTTCAGGTGCT
FOXM1	ATACGTGGATTGAGGACCACT	TCCAATGTCAAGTAGCGGTTG
ACTB	AGGCCAACCGCGAGAAGATGACC	GAAGTCCAGGGCGACGTAGCAC
miR-361-5p	ATAAAGTGCTGACAGTGCAGATAGTG	TCAAGTACCCACAGTGCGGT
U6	CTCGCTTCGGCAGCACA	AACGCTTCACG AATTTGCGT
miR-361-5p mimics	ACGCCUGGAGAUUCUGAUAAUU	

Western blot. Total protein was extracted by lysing the cells with RIPA buffer and protease inhibitor cocktail (Roche). After denatured, proteins were run in the 10% SDS/PAGE gel and transferred to a PVDF membrane (Millipore). Membrane was blocked in 10% skim milk for 1 hour at room temperature and incubated in primary antibodies overnight at 4°C. Then membrane was incubated with the corresponding secondary antibody for 1 hour at room temperature. Protein bands were visualized by the ECL system (Millipore).

Xenograft. 2×10^6 miR-361-5p-overexpressed A549 and control cells suspended in 100 μl of phosphate-buffered saline were injected subcutaneously in the groin of BALB/c nude mice. Tumour volume (V) was monitored every 5 days by measuring the tumour length (L) and width (W) with a vernier caliper and calculated using the formula $V = 0.5 \times L \times W^2$. The animals were sacrificed, and the tumor tissues were resected.

Statistical analysis. Each experiment was performed in triplicate. Data from experiments was expressed as mean ± SD. All statistical analyses were performed using SPSS18.0 software system for Windows (SPSS Inc.). Differences between groups were compared using student *t* test. *P* value less than 0.05 were considered significant.

Results

miR-361-5p is down-regulated in lung cancer. To investigate the role of miR-361-5p in lung cancer development and progression, we first examined the expression of miR-361-5p in lung cancer cell lines and bronchial epithelial cells by RT-qPCR. We observed that miR-361-5p was expressed at higher levels in NHBE cell line compared with that of lung cancer cell lines (Figure 1A). Moreover, we found that miR-361-5p was down-regulated in lung cancer tissues compared with the paired adjacent non-malignant tissues (Figure 1B). These results suggested that miR-361-5p is down-regulated in lung cancers.

miR-361-5p is a tumor suppressor in lung cancer. To study the biological function of miR-361-5p, we overexpressed or depleted the expression of miR-361-5p in A549 and H460 cells, by transfection of miR-361-5p mimic or inhibitor, respectively (Figure 2A and Figure 3A). We evaluated the effect of miR-361-5p on cell proliferation by MTT and colony formation assays. Colony formation assay showed that number of colonies was lower in miR-361-5p-overexpressed A549 (Figure 2B), whereas it was higher in miR-361-5p-depleted H460 (Figure 3B) compared with that of control cells. In addition, MTT

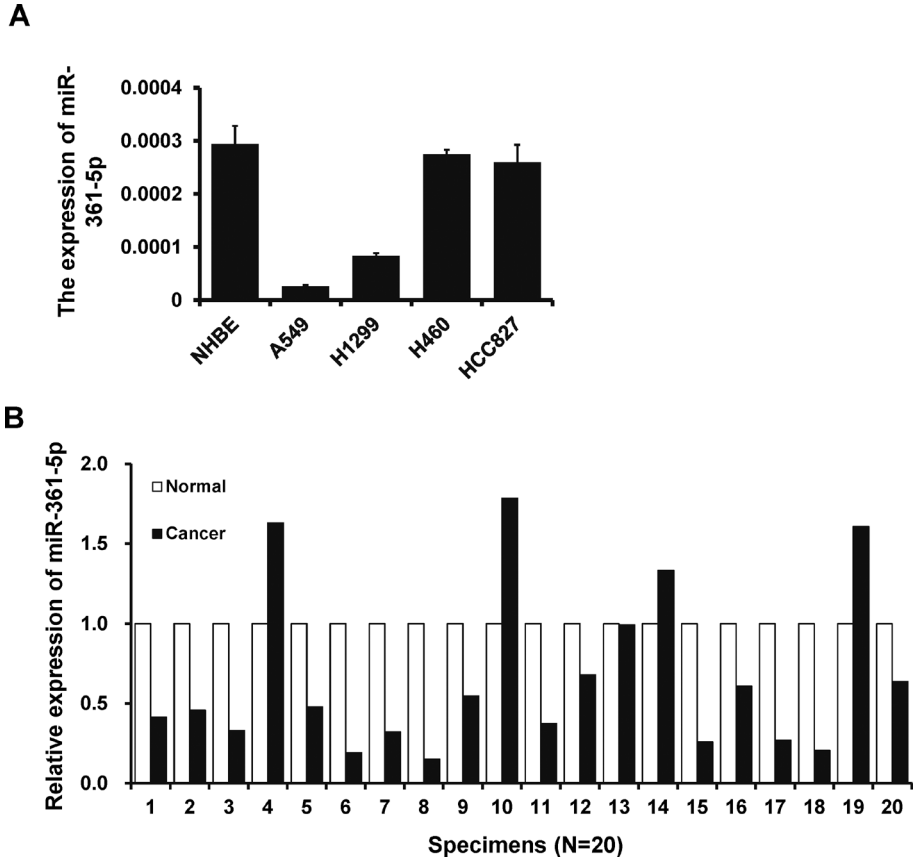

Figure 1. miR-361-5p is down-regulated in lung cancer. A RT-qPCR analysis of miR-361-5p expression in human lung cancer cell lines and NHBE cell line. B The relative expression of miR-361-5p in lung cancer and the paired adjacent non-malignant tissues.

assay showed that overexpression of miR-361-5p inhibited cell viability in A549 cells (Figure 2C), whereas depletion of miR-361-5p promotes cell viability in H460 cells (Figure 3C). Next, we performed Transwell assay to evaluate the effect of miR-361-5p on cell invasion. The results showed that overexpression of miR-361-5p reduced cell invasion in A549 cells (Figure 2D), while its inhibition enhanced cell invasion in H460 (Figure 3D) compared to that of control cells. Furthermore, the number of apoptotic cells was significant higher in miR-361-5p-overexpressed A549 compared with control cells (Figure 2E). The *in vivo* analysis indicated that overexpression of miR-361-5p inhibits tumor growth in A549 cells (Figure 2F and 2G). These results indicated that miR-361-5p inhibits the lung cancer cell proliferation and invasion, and promoted cell

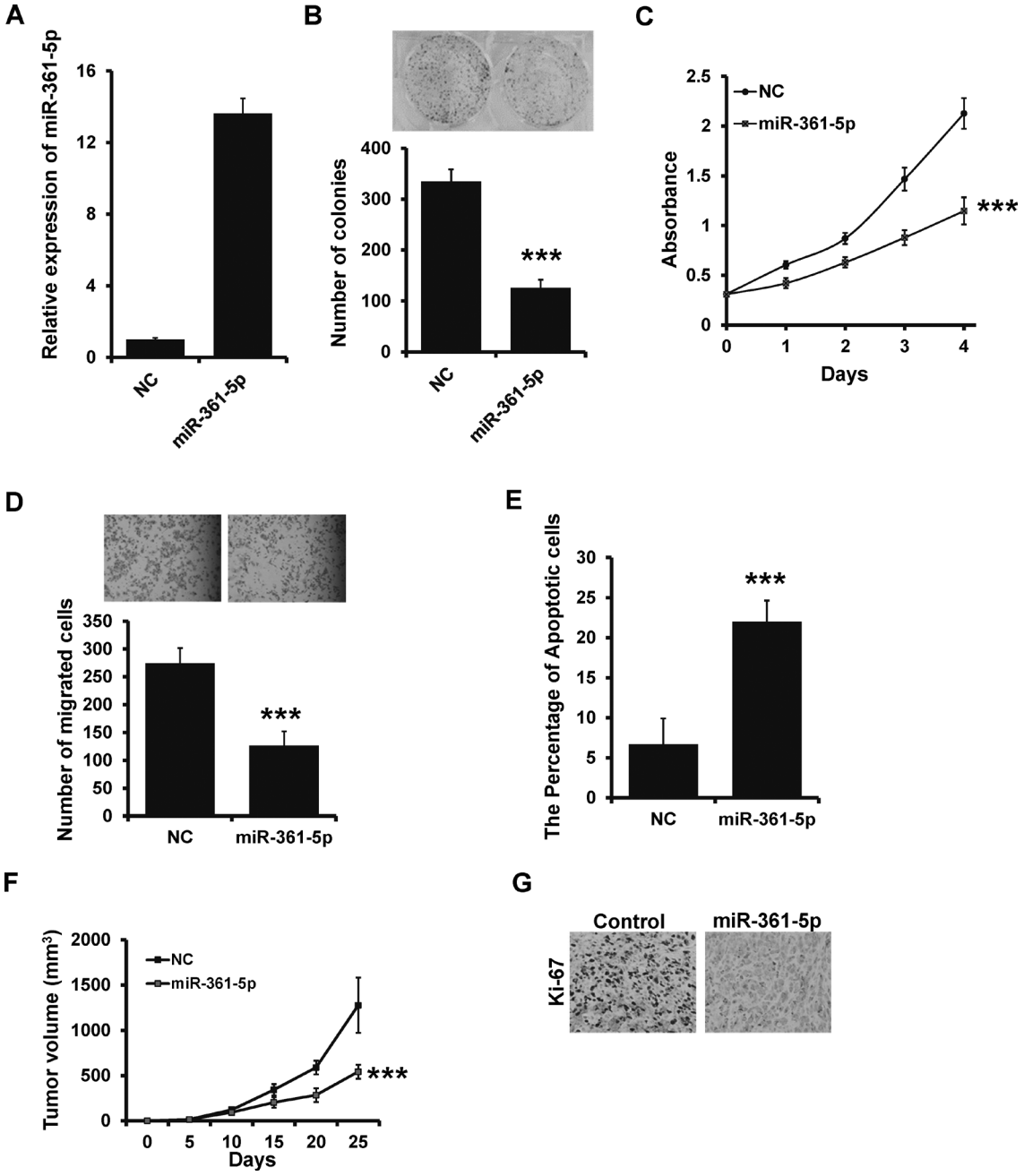

Figure 2. Overexpression of miR-361-5p suppresses cell proliferation and invasion in A549 cells. A RT-qPCR analysis of miR-361-5p expression in A549 cells transfected with miR-361-5p-mimic or mimic-control. B and C MTT (B) and colony formation assays of cells as in (A). D Transwell analysis of cells as in (A). E The percentage of apoptotic cells in cells as in (A) by FACS. F Tumor growth curves presented by tumor volume indicate that miR-361-5p inhibits A549 xenograft growth. G Ki-67 staining of xenograft sections. *** P <0.001.

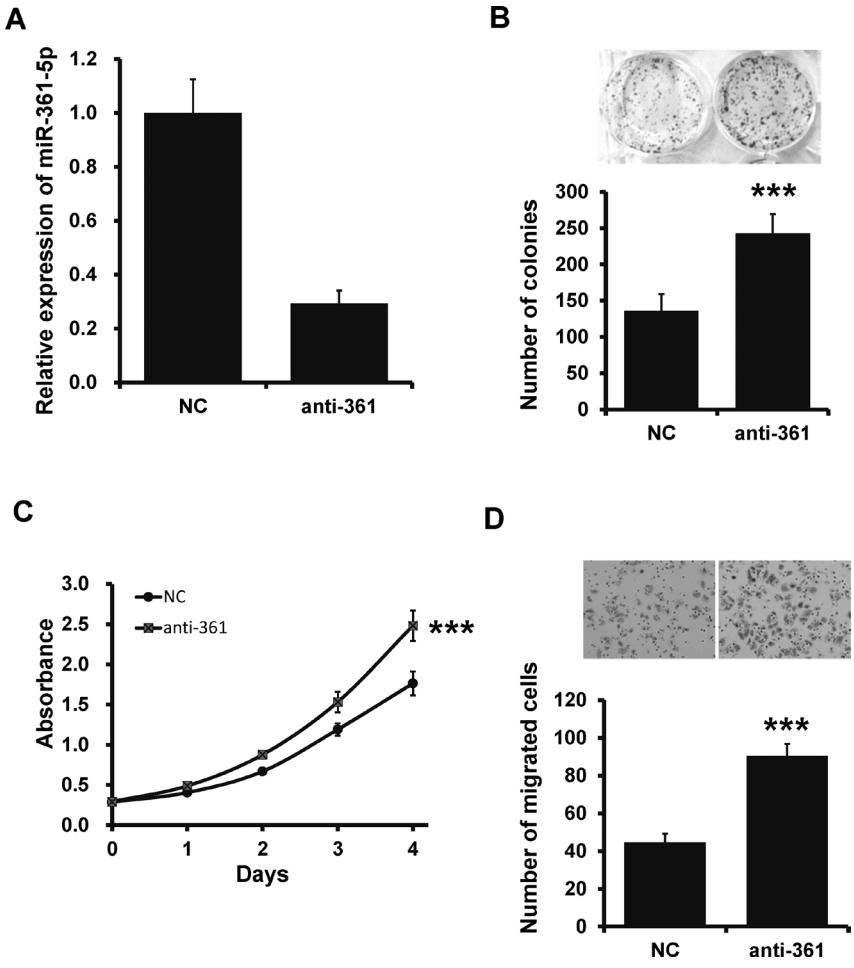

Figure 3. Knockdown of miR-361-5p promotes cell proliferation and invasion in H460 cells. A RT-qPCR analysis of miR-361-5p expression in H460 cells transfected with miR-361-5p-inhibitor or inhibitor-control. B and C MTT (B) and colony formation assays of cells as in (A). D Transwell analysis of cells as in (A). *** P <0.001.

apoptosis, suggesting that miR-361-5p is a tumor suppressor in lung cancer.

FOXM1 is a direct target of miR-361-5p. miRNA target analysis tools TargetScan 7.1 and miRNA.org were used to explore potential target of miR-361-5p. FOXM1 was predicted to be a potential target of miR-361-5p (Figure 4A). To confirm targeting of FOXM1 by miR-361-5p, we performed luciferase activity assay. The 3'UTR of FOXM1 was cloned to the downstream of LUC coding gene of pGL3-control plasmid (FOXM1-3'UTR-Wt). The site-directed mutant of the seed region was also constructed (FOXM1-3'UTR-Mu). The FOXM1-3'UTR-Wt or FOXM1-3'UTR-Mu was co-transfected with miR-361-5p mimic or the control into HEK-293 cells. As shown in Figure 4B, overexpression of miR-361-5p decreased the luciferase activity of FOXM1 3'UTR. In addition, site-directed mutagenesis of the seed region abolished the inhibitory effect of miR-361-5p (Figure 4C). We further examined the FOXM1 expression in miR-361-5p-overexpressed A549 and miR-361-5p-depleted H460 cells by RT-qPCR and Western Blot. As shown in Figure 4D and 4E, overexpression of miR-361-5p decreased the expression level of FOXM1 mRNA (Figure 4D; left) and protein (Figure 4E) in A549 cells, while the expression was increased in miR-361-5p-depleted H460 cells (Figure 4D, right and Figure 4E). Together, these results indicated that FOXM1 is a target gene of miR-361-5p.

miR-361-5p inhibits EMT-like phenotype in lung cancer cells. FOXM1 is a critical factor in EMT. Thus, we detected the effect of miR-361-5p on the expression of EMT-related transcription factors. We observed that the expression of SLUG, SNAIL, TWIST and ZEB1 was significantly down-regulated in miR-361-5p-overexpressed A549 cells by RT-qPCR (Figure 5A). We observed that miR-361-5p-overexpressed cells displayed a cobblestone-like morphology (Figure 5B). Furthermore, overexpression of miR-361-5p decreased the expression of mesenchymal phenotypic marker of Vimentin, while increased the expression of epithelial phenotypic marker of E-cadherin by western blot (Figure 5C). To further

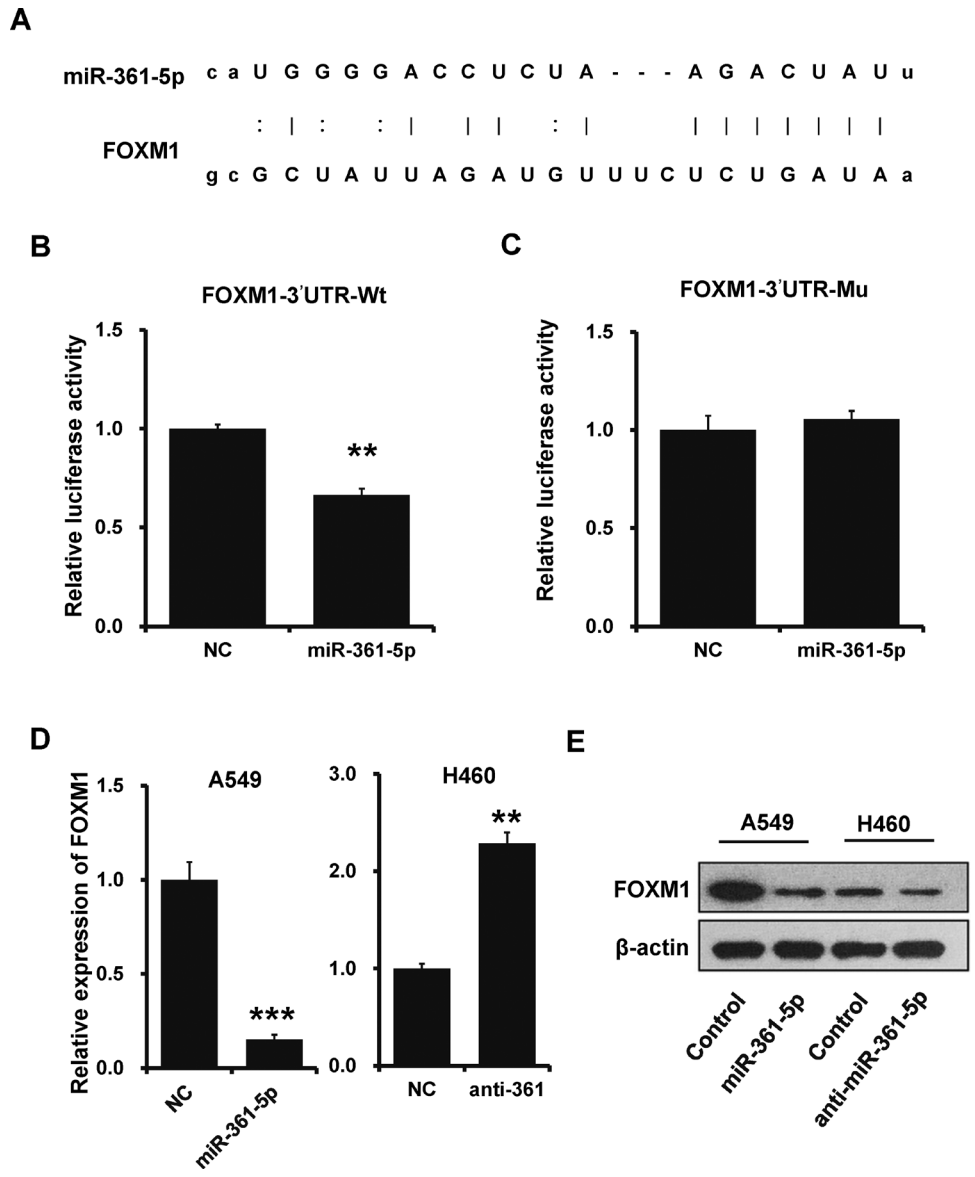

Figure 4. FOXM1 is a direct target of miR-361-5p. A Predicted binding sites of miR-361-5p with FOXM1 3'UTR. B and C Luciferase reporter system analysis was performed to validate miR-361-5p targeting FOXM1. A 3'UTR fragment containing the predicted miR-361-5p targeting site of FOXM1 was fused downstream of the LUC gene in pGL3-control plasmid (FOXM1-3'UTR-Wt). A miR-361-5p mutated binding site was also constructed (FOXM1-3'UTR-Mu). D and E The expression levels of FOXM1 in lung cancer cells by RT-qPCR (D) and western blot (E). ** P<0.01, *** P<0.001

study the role of miR-361-5p in EMT process, we treated A549 with 10 ng/ml TGFβ1. Overexpression of miR-361-5p reversed the expression of Vimentin and E-cadherin induced by TGFβ1 treatment (Figure 5D). Together, these results indicated that miR-361-5p inhibits EMT phenotype in lung cancer cells.

miR-361-5p inhibits lung cancer cell proliferation and invasion in FOXM1-dependent manner. To further confirm miR-361-5p inhibits lung cancer cell proliferation and invasion through down-regulating FOXM1, we rescued FOXM1 expression in miR-361-5p-overexpressed A549 cells. MTT, colony formation and transwell assays indicated that overexpression of FOXM1 eliminated the inhibition caused by miR-361-5p (Fig. 6A-C). In addition, overexpression of FOXM1 rescued the expression of Vimentin and E-cadherin (Fig. 6D). The expression of miR-361-5p also negatively related to the expression of FOXM1 in normal breast tissues (Fig. 6E) and breast cancer tissues (Fig. 6F). Together, these results indicated that miR-361-5p suppresses lung cancer progression through down-regulation of FOXM1.

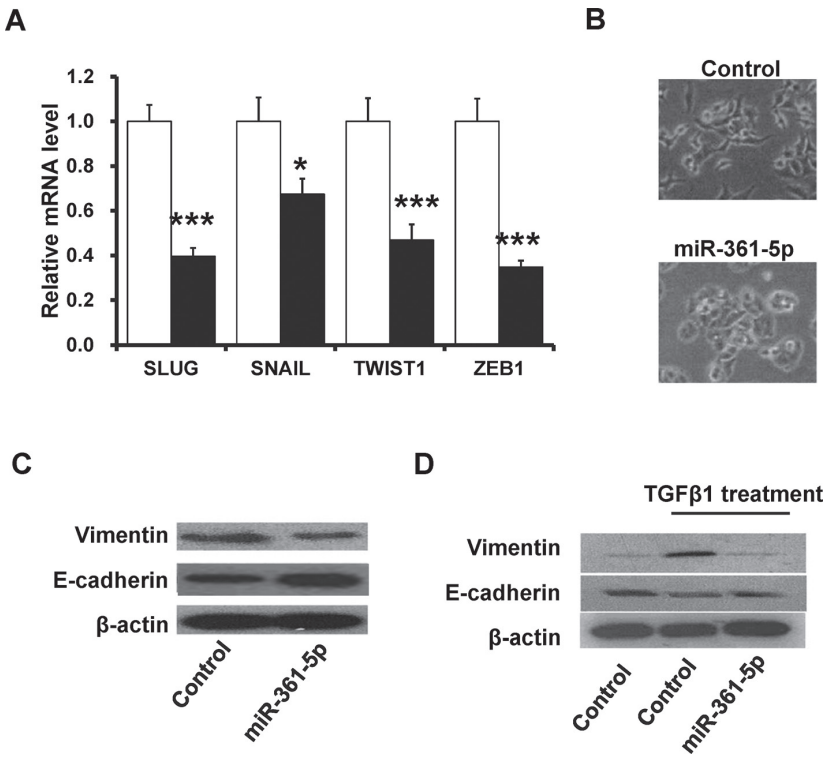

Figure 5. miR-361-5p inhibits EMT-like phenotype in lung cancer cells. A RT-qPCR analysis of EMT markers in A549 cells transfected with miR-361-5p-mimic. B Cellular morphology of the miR-361-5p-overexpressed A549 and control cells. C Western Blot analysis of the expression level of Vimentin and E-cadherin in A549 cells transfected with miR-361-5p-mimic. D Western blot analysis of the expression level of Vimentin and E-cadherin after treatment with TGFβ1 in A549 cells transfected with miR-361-5p-mimic. * P<0.05, *** P <0.001.

Discussion

Emerging evidences have indicated that miRNAs are closely involved in the regulation of various processes during tumorigenesis and progression, including tumor growth, cell invasion, and tumor metastasis. Previous studies have shown that miR-361-5p was dysregulated and played an important role in several types of human cancers. For example, miR-361-5p has been implied as an anti-oncogene in human cutaneous squamous cell carcinoma, prostate cancer, colorectal and gastric cancer and hepatocellular carcinoma. miR-361-5p might affect cancer development or progression by modulating vascular endothelial growth factor A (VEGFA) in human cutaneous squamous cell carcinoma [11]. And it acted as a tumor suppressor in prostate cancer by targeting signal transducer and activator of transcription-6) [12]. Moreover, miR-361-5p inhibited colorectal and gastric cancer growth and metastasis by targeting staphylococcal nuclease domain containing-1 (SND-1), forming of SND1-pre-miR-361-5p complex [13] and suppressed cell proliferation and invasion in hepatocellular carcinoma by directly targeting Chemokine receptor 6 (CXCR6) [15]. However, Wu et al. reported that miR-361-5p was upregulated in transferred cervical cancer cells and acted as an oncogene to enhance cell proliferation and promote cell invasion through mediation of epithelial-to-mesenchymal transition (EMT) [16]. Furthermore, miR-361-5p was reported to be associated with Late-onset hypogonadism (LOH). Chen et al. found that miR-361-5p level was positively associated with serum testosterone concentrations. miR-361-5p are differentially expressed between LOH and healthy controls [17]. Consistent with their studies, our results indicated that miR-361-5p is down-regulated in lung cancer and acts as a tumor suppressor.

FOXM1 is a member of the FOX superfamily of transcription factors. FOXM1 has been reported to be associated with cell proliferation [19], cell cycle control [20], DNA damage and repair [21], tumor development and progression [22], and chemotherapy resistance [23]. FOXM1 is a typical proliferation-associated transcription factor that regulates genes in the cycle control and transactivates the human c-myc P1 and P2 promoters directly via their TATA-boxes. In addition, FOXM1 is a master tumor metastasis-associated regulator that induces an epithelial–mesenchymal–like transition [24]. Increased expression of FOXM1 is associated with poor prognosis and serves as an independent predictor of poor survival in many cancers [22]. In the present study, we

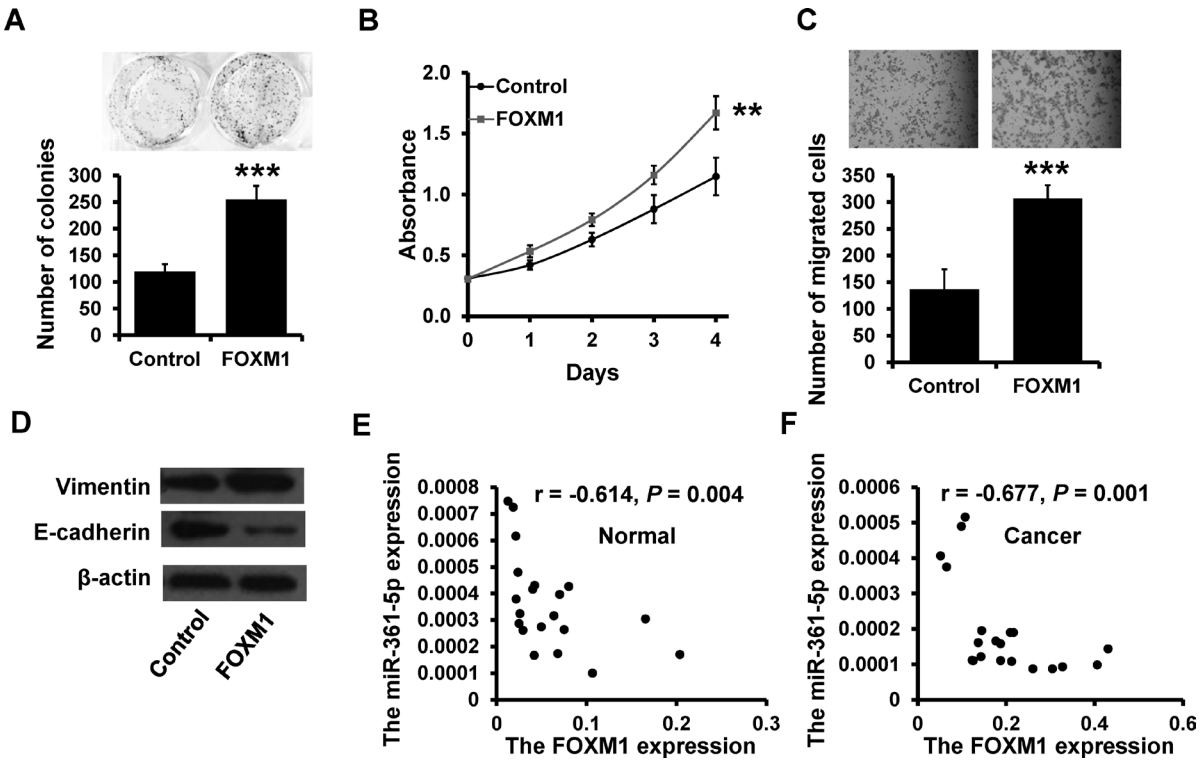

Figure 6. miR-361-5p suppresses lung cancer progression in a FOXM1-depedent manner. A and B Colony formation (A) and MTT (B) analysis of cell proliferation in miR-361-5p-overexpressed A549 cells transfected with FOXM1 or vector. C Transwell analysis of cells as in (A). D Western Blot analysis of the expression level of Vimentin and E-cadherin in cells as in (A). E and F The expression of miR-361-5p and FOXM1 in normal breast tissues (E) and breast cancer tissues (F). *** P <0.001. ** P <0.01.

revealed that FOXM1 is a direct target of miR-361-5p. miR-361-5p inhibits lung cancer progression by down-regulation of FOXM1.

The epithelial to mesenchymal transition (EMT), where in epithelial cells lose epithelial phenotypic markers and gain mesenchymal phenotypic markers, is a potential mechanism by which tumor cells gain metastatic features [25]. FOXM1 has been previously identified a master EMT regulator in cancer progression [26-30]. Our results showed that overexpression of miR-361-5p decreased the expression of EMT-related transcription factors and suppressed the TGF-β-induced EMT, suggesting that miR-361-5p inhibits the lung cancer progression through suppression the EMT-like phenotype by down-regulation of FOXM1

In conclusion, our results indicated that miR-361-5p inhibits lung cancer cell proliferation and invasion by directly regulating FOXM1 expression. miR-361-5p might be a novel agent for lung cancer therapy. However, further studies are still needed to explore the relationship between miR-361-5p expression and clinical characteristic and prognosis of patients with lung cancer.

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