European Review for Medical and Pharmacological Sciences 2020; 24: 3775-3785

LncRNA FAS-AS1 inhibits the progression of non-small cell lung cancer through regulating miR-19a-5p

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Abstract. – OBJECTIVE: Non-small cell lung cancer (NSCLC) is one of the most common and deadly tumors in the world. LncRNA FAS-AS1 was abnormally expressed in various cancers, such as non-small cell lung cancer. However, the underlying mechanism of FAS-AS1 in NSCLC remains to be elucidated.

MATERIALS AND METHODS: The levels of FAS-AS1 and miR-19a-5p were measured using qRT-PCR in NSCLC cells. MTT and cell colony formation assays were performed to detect cell proliferative capacity. Transwell assay was carried out to measure cell migration and invasion. The relationship between FAS-AS1 and miR-19a-5p was confirmed using Luciferase reporter assay. Xenograft tumor experiment was conducted to detect the tumor growth in vivo.

RESULTS: FAS-AS1 was remarkably down-regulated in NSCLC cells. FAS-AS1 inhibited cell proliferation, migration, and invasion in NSCLC cells. Additionally, FAS-AS1 directly targeted miR-19a-5p and negatively regulated the expression of miR-19a-5p in NSCLC cells. Furthermore, FAS-AS1 overexpression restored the promotion of miR-19a-5p overexpression on proliferation, migration, and invasion of NSCLC cells. Additionally, suppression of FAS-AS1 abrogated the inhibitory effects of miR-19a-5p knockdown on the progression of NSCLC. FAS-AS1 suppressed the tumor growth *in vivo*.

CONCLUSIONS: FAS-AS1 suppressed cell proliferation, migration, and invasion by sponging miR-19a-5p in NSCLC, indicating that FAS-AS1 might be a potential biomarker and therapeutic target for NSCLC.

Key Words:
FAS-AS1, MiR-19a-5p, Non-small cell lung cancer, Progression.

Abbreviations

SCLC = small cell lung cancer; NSCLC = non-small cell lung cancer; lncRNAs = long non-coding RNAs; ncRNAs = non-coding RNAs; miRNAs = MicroRNAs.

Introduction

Lung cancer is the most common malignant tumor and the most important source of cancer mortality[1]. Lung cancer is classified into small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC)[2]. NSCLC accounts for 85% of lung tumors and is divided into adenocarcinoma, squamous cell carcinoma, and large cell carcinoma[3]. The 5-year survival rate for NSCLC patients is less than 16%[4]. Therefore, it is urgent to explore the molecular mechanisms of NSCLC to improve its unfavorable prognosis.

Long non-coding RNAs (lncRNAs) are a class of non-coding RNAs (ncRNAs) with a length of more than 200 nucleotides[5]. LncRNAs play an important role in the development and progression of various malignancies[6]. Recent studies[7] have elucidated that lncRNAs play an important role in the treatment of patients with NSCLC because lncRNAs play a vital role in diverse cellular processes. Fas-antisense 1 (FAS-AS1) regulated the alternative splicing of Fas in lymphomas and EZH2-mediated suppression of FAS-AS1 promoter could be released by DZNeP (3-Deazaneplanocin A) or overcome by ectopic expression of FAS-AS1, both of which increased levels of FAS-AS1 and correspondingly decreased sFas expression[8]. LncRNA FAS-AS1 was abnormally expressed in the development of non-small cell lung cancer[9], but the specific mechanism of FAS-AS1 is still unclear. Therefore, this study explored the related role and molecular mechanism of FAS-AS1 in the progression of non-small cell lung cancer through *in vitro* and *in vivo* experiments.

MicroRNAs (miRNAs) are highly conserved short non-coding RNAs consisting of 18-25 nucleotides[10]. MicroRNAs (miRNAs) have the function of post-transcriptional regulators involved in biological processes[11,12]. MiRNAs

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promoted or inhibited tumorigenesis and progression by regulating the expression of mRNAs[13]. High miR-19a expression was correlated with poor prognostic in patients with NSCLC[14]. MiR-19a-5p functioned as an oncogene and AC016405.3 inhibited glioblastoma multiforme cell proliferation and metastasis by up-regulating TET2 expression *via* sponging miR-19a-5p[15]. However, the role of miR-19a-5p in NSCLC remains unclear.

In this study, we demonstrated that FAS-AS1 expression was decreased and miR-19a-5p expression was increased in NSCLC cells. The functional analysis suggested that FAS-AS1 inhibited cell proliferation, migration, and invasion in NSCLC cells. In addition, the mechanism analyzed showed that FAS-AS1 directly targeted miR-19a-5p and negatively regulated the expression of miR-19a-5p in NSCLC cells. In conclusion, FAS-AS1 inhibited the progression of NSCLC by sponging miR-19a-5p, suggesting that FAS-AS1 might be a novel biomarker for NSCLC treatment.

Materials and Methods

Cell Culture

Human lung epithelial cell line (BEAS-2B) and the NSCLC cell lines (NCL-H1299, NCL-H460, and A549) were purchased from ATCC (American Type Culture Collection; Manassas, VA, USA). All cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA) at 37°C with 5% CO_2.

Cell Transfection

FAS-AS1 overexpression vector (FAS-AS1), pcDNA empty vector (NC), small interfering RNA (siRNA) against FAS-AS1 (si-FAS-AS1), the negative control siRNA (si-NC), miR-19a-5p mimic (miR-19a-5p), the negative control mimic (miR-NC), miR-19a-5p inhibitor (anti-miR-19a-5p), and the negative control inhibitor (anti-miR-NC) were purchased from RiboBio (Guangzhou, China). Short hairpin RNA against FAS-AS1 (sh-FAS-AS1) and the negative control (sh-NC) were purchased from GenePharma (Shanghai, China). Cell transfection was performed using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

Quantitative Real-Time PCR

Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. RNA reverse transcription was performed using High-Capacity cDNA Reverse Transcription Kits (Thermo Fisher Scientific, Waltham, MA, USA) or SYBR PrimeScript miRNA RT-PCR Kit (TaKaRa, Dalian, China). Quantitative real-time PCR (qPCR) was performed using SYBR Green Mixture (TaKaRa, Dalian, China). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and U6 were identified as internal controls. All experiments were repeated in triplicate. The primer sequences were as follows: FAS-AS1 (forward, 5'-GGTGTGACATTACTCCAGAGTTG-3'; reverse, 5'-AGGGCCAAGTCCAACTCCTT-3'), GAPDH (forward, 5'-GTCAACGGATTTGGTCTGTATT-3'; reverse, 5'-AGTCTTCTGGGTGGCAGTGAT-3-3'), U6 (forward, 5'-CTCGCTTCGGCAGCACA-3'; reverse, 5'-AACGCTTCACGAATTTGCGT-3'). The primers of miR-19a-5p were purchased from RiboBio (Guangzhou, China).

MTT Assay

Cell viability was evaluated using 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT; Sigma-Aldrich, St. Louis, MO, USA). Briefly, transfected cells were seeded in 96-well plates and MTT was added into each well after incubation for 0 h, 24 h, 48 h, and 72 h, respectively. Then, the cells were incubated for 4 h and dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO, USA) was added into each well. The absorbance was measured at 490 nm using Microplate Reader (Bio-Rad, Hercules, CA, USA).

Colony Formation Assay

The transfected cells were seeded into 6-well plates (200 cells/well) and cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS) for 12 days. The medium was replaced, and the state of the cells was observed every 3 days. The colonies were fixed with methanol for 15 min and stained with 0.5% crystal violet solution for 20 min. Colonies were photographed and counted

Transwell Assay

For migration assay, the transfected cells were resuspended in serum-free medium and seeded into the upper chamber of a 24-well transwell with 8 μm pore size (BD Biosciences, Franklin Lakes, NJ, USA). The lower chamber was added with com-

plete medium as chemoattractant. The cells were incubated at 37°C for 48 h, and the cells migrating to the lower surface were fixed with formaldehyde and stained with crystal violet. Finally, the cells were counted under a microscope. For invasion assay, the transwell was coated with Matrigel (BD Biosciences, Franklin Lakes, NJ, USA).

Luciferase Reporter Assay

The fragment of FAS-AS1 containing putative wild (FAS-AS1-wt) or mutant (FAS-AS1-mut) binding sites was cloned into pGL3 plasmids (Promega, Madison, WI, USA) to form reporter vectors. The corresponding vectors were cotransfected into NCL-H1299 and NCL-H460 cells with miR-19a-5p mimic (miR-19a-5p) or the control mimic (miR-NC) using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA). Luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) as described by the manufacturer. The activity of *Renilla* Luciferase was detected as endogenous reference.

Xenograft Tumor Experiment

BALB/c nude male mice (5-week-old) were maintained under pathogen-free conditions. NCL-H460 cells (2×10^7 cells/ml) transfected with NC, FAS-AS1, sh-NC or sh-FAS-AS1 were subcutaneously injected into nude mice. Tumor volume was calculated every 7 days. Five weeks after the inoculation, tumor weight was detected after mice were sacrificed. Tumor tissues were snap-frozen for RNA extraction. The xenograft analysis was approved by the Animal Ethics Committee of Zhenhai People's Hospital.

Statistical Analysis

All data were expressed as mean ± standard deviation (SD) at least three independent experiments. Student's *t*-test or one-way ANOVA followed by Tukey's post-hoc test was performed to analyze the differences using GraphPad Prism 7.0 software (GraphPad, San Diego, CA, USA). When p-value <0.05, the difference was considered statistically significant.

Results

FAS-AS1 Was Down-Regulated in NSCLC Cells and Inhibited Cell Proliferation

First, we detected the expression of FAS-AS1 in NSCLC cells using qRT-PCR and the results revealed that FAS-AS1 expression was significantly lower in NSCLC cells (NCL-H1299, NCL-H460, and A549) than that in human lung epithelial cells (BEAS-2B) (Figure 1A). Next, NCL-H1299 and NCL-H460 cells were transfected with NC, FAS-AS1, si-NC, and si-FAS-AS1, respectively. The results of qRT-PCR suggested that overexpression of FAS-AS1 significantly increased FAS-AS1 expression, while FAS-AS1 suppression strikingly decreased FAS-AS1 expression in NCL-H1299 and NCL-H460 cells (Figure 1B and 1C). MTT assay indicated that cell viability was remarkably reduced in FAS-AS1 group compared to NC group, and cell viability was markedly increased in si-FAS-AS1 group compared with si-NC group (Figure 1D and 1E). Furthermore, colony formation results showed that the cell proliferative capacity was significantly inhibited in FAS-AS1 group compared to NC group, whereas was notably induced in si-FAS-AS1 group compared with si-NC group (Figure 1F and 1G). All these data implied that FAS-AS1 might be a tumor suppressor in NSCLC and inhibited cell proliferation.

FAS-AS1 Inhibited Cell Migration and Invasion in NSCLC Cells

To investigate the effect of FAS-AS1 on migration and invasion of NSCLC cells, transwell assay was performed in NCL-H1299 and NCL-H460 cells transfected with NC, FAS-AS1, si-NC or si-FAS-AS1. The results demonstrated that overexpression of FAS-AS1 distinctly suppressed cell migration and invasion in NCL-H1299 and NCL-H460 cells (Figure 2A and 2C). Additionally, cell migration and invasion ability were significantly facilitated in NCL-H1299 and NCL-H460 cells transfected with si-FAS-AS1 compared to si-NC group (Figure 2B and 2D). From these data, it was elucidated that FAS-AS1 suppressed cell migration and invasion in NSCLC cells.

FAS-AS1 Directly Targeted MiR-19a-5p

To explore the mechanism of FAS-AS1, we predicted that miR-19a-5p had binding sites of FAS-AS1 using LncBase v2 (Figure 3A). Dual-Luciferase reporter assay was performed to validate whether FAS-AS1 targeted miR-19a-5p. The results suggested that mature miR-19a-5p strikingly inhibited the Luciferase activity of NCL-H1299 and NCL-H460 cells transfected with FAS-AS1-wt, while the Luciferase activity of cells transfected with FAS-AS1-mut was not

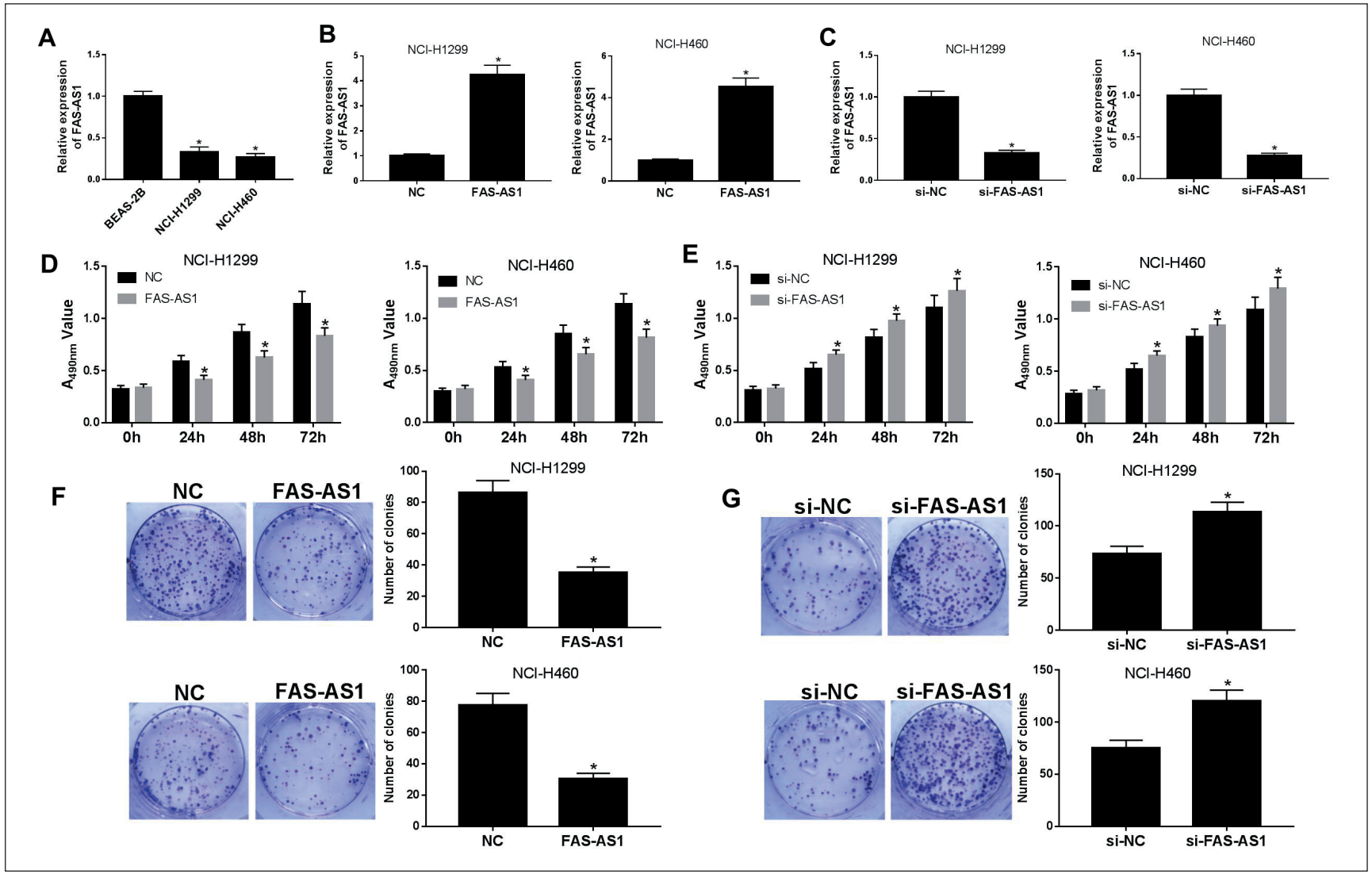

Figure 1. FAS-AS1 was down-regulated in NSCLC cells and inhibited cell proliferation. **A**, Relative expression level of FAS-AS1 was determined by qRT-qPCR in human lung epithelial cell line (BEAS-2B) and NSCLC cell lines (NCL-H1299, NCL-H460 and A549). **B-G**, NCL-H1299 and NCL-H460 cells were transfected with NC, FAS-AS1, si-NC and si-FAS-AS1, respectively. **B** and **C**, FAS-AS1 expression was detected by qRT-PCR. **D** and **E**, Cell viability was measured by MTT assay. **F** and **G**, Cell colony formation assay was performed to analyze cell proliferative capacity (magnification 300×). *$p < 0.05$.

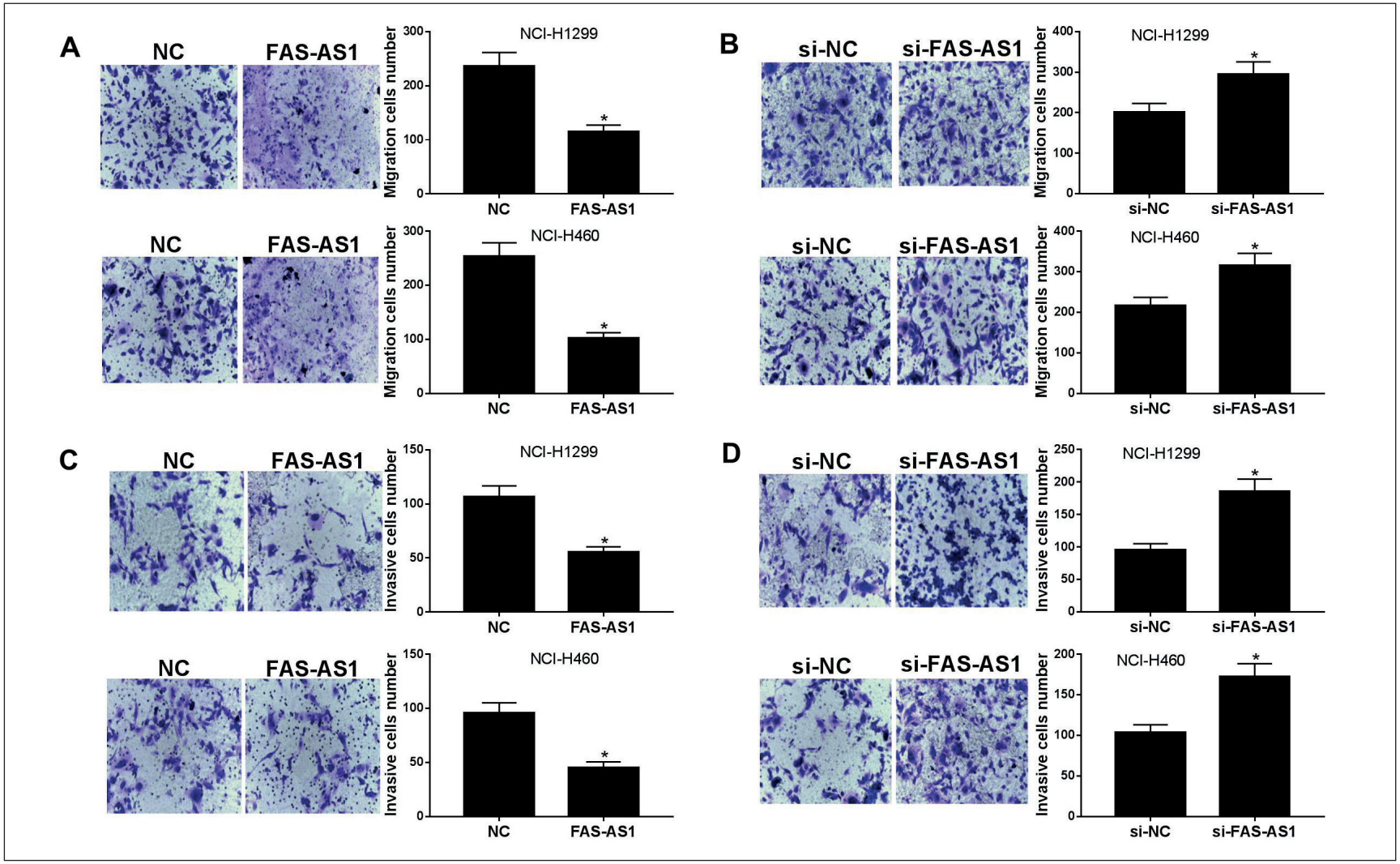

Figure 2. FAS-AS1 inhibited cell migration and invasion in NSCLC cells. **A** and **B**, Cell migration of NCL-H1299 and NCL-H460 cells transfected with NC, FAS-AS1, si-NC or si-FAS-AS1 was detected by transwell assay, (magnification 100X) **C** and **D**, Cell invasion of NCL-H1299 and NCL-H460 cells transfected with NC, FAS-AS1, si-NC or si-FAS-AS1 was determined by transwell assay (magnification 100X). *$p < 0.05$.

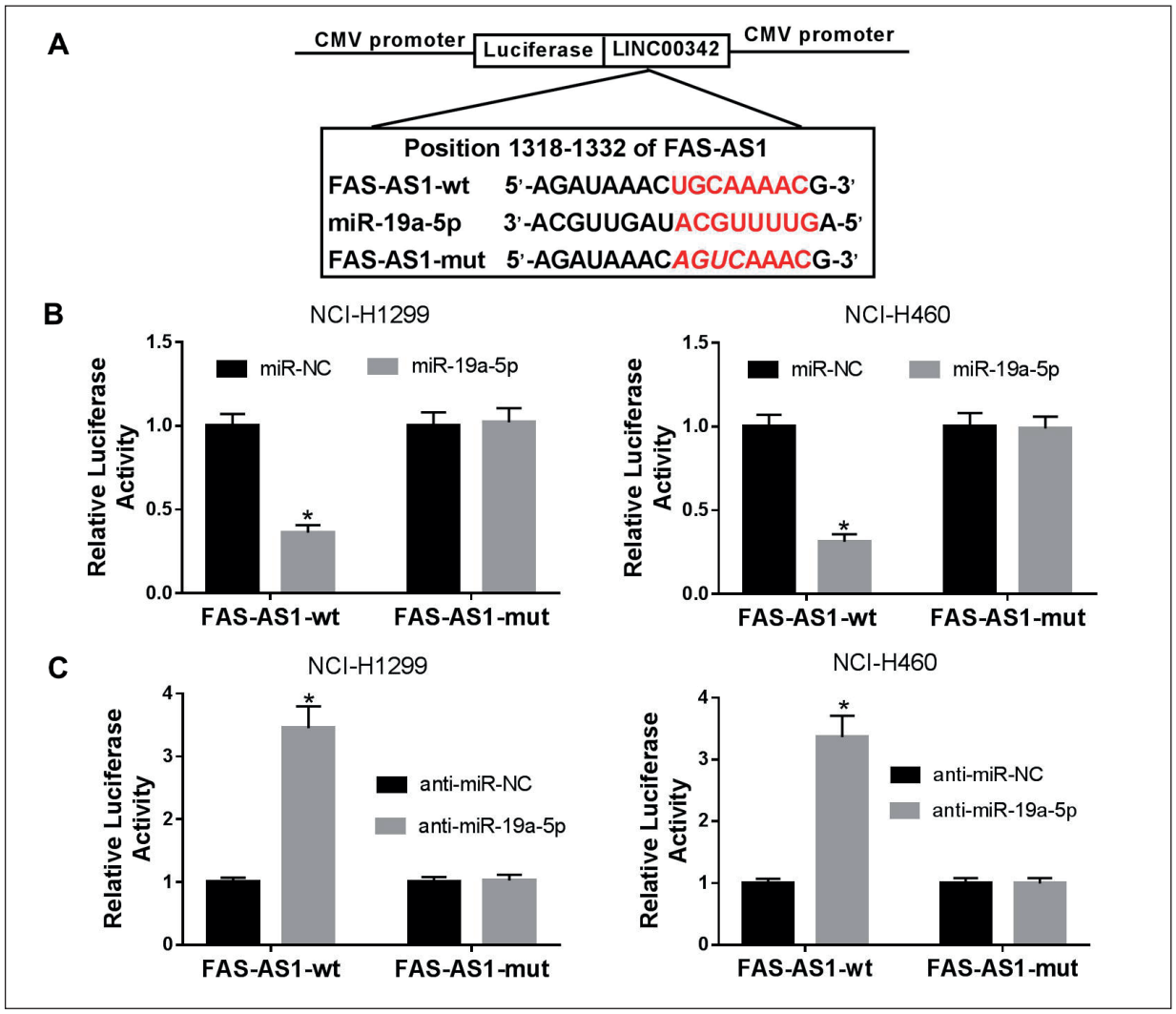

Figure 3. FAS-AS1 directly targeted miR-19a-5p. **A**, Putative binding sites of FAS-AS1 and miR-19a-5p. **B**, NCL-H1299 and NCL-H460 cells were cotransfected with FAS-AS1-wt or FAS-AS1-mut vectors and miR-19a-5p mimic or miR-NC, and Luciferase activity was examined at 48 h after transfection. **C**, NCL-H1299 and NCL-H460 cells were cotransfected with FAS-AS1-wt or FAS-AS1-mut vectors and anti-miR-19a-5p or anti-miR-NC, and Luciferase activity was determined at 48 h after transfection. $*p < 0.05$.

affected (Figure 3B). In addition, miR-19a-5p inhibitor strikingly increased the Luciferase activity of NCL-H1299 and NCL-H460 cells transfected with FAS-AS1-wt, whereas did not modulate the Luciferase activity when the binding sites were mutated (Figure 3C). These data demonstrated that FAS-AS1 directly targeted miR-19a-5p.

FAS-AS1 Regulated the Expression of MiR-19a-5p in NSCLC Cells

First, the expression of miR-19a-5p was detected in NSCLC cells using qRT-PCR and the results showed that miR-19a-5p was markedly upregulated in NCL-H1299 and NCL-H460 cells compared to human lung epithelial cells (BEAS-2B) (Figure 4A). Next, we measured miR-19a-5p expression in NCL-H1299 and NCL-H460 cells transfected with NC, FAS-AS1, si-NC or si-FAS-AS1. The results of qRT-PCR revealed that FAS-AS1 overexpression strikingly down-regulated miR-19a-5p expression (Figure 4B and 4C), while knockdown of FAS-AS1 remarkably upregulated miR-19a-5p expression in NCL-H1299 and NCL-H460 cells (Figure 4D and 4E). These results indicated that FAS-AS1 regulated the expression of miR-19a-5p in NSCLC cells.

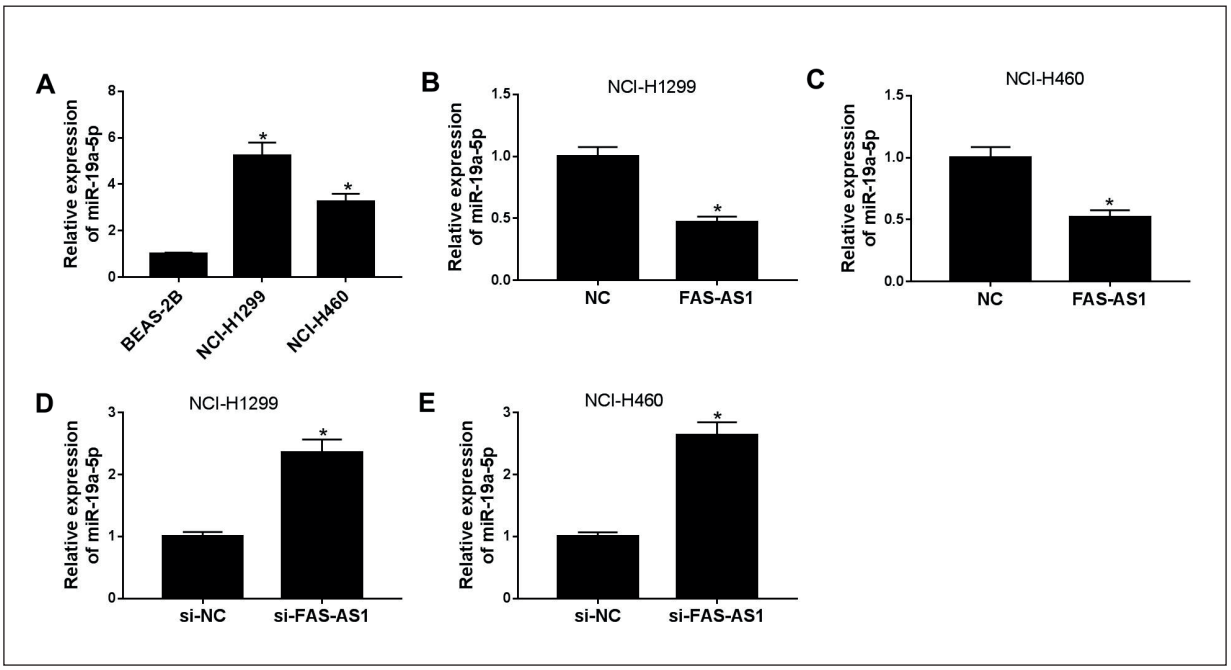

Figure 4. FAS-AS1 regulated the expression of miR-19a-5p in NSCLC cells. **A**, Relative expression of miR-19a-5p was measured by qRT-qPCR in human lung epithelial cell line (BEAS-2B) and NSCLC cell lines (NCL-H1299 and NCL-H460). **B** and **C**, Expression of miR-19a-5p in NCL-H1299 and NCL-H460 cells transfected with NC or FAS-AS1 was detected by qRT-PCR. **D** and **E**, Expression of miR-19a-5p in NCL-H1299 and NCL-H460 cells transfected with si-NC or si-FAS-AS1 was determined by qRT-PCR. *$p < 0.05$.

FAS-AS1 Restored the Effect of MiR-19a-5p on Proliferation, Migration, and Invasion of NSCLC Cells

To further investigate the relationship between FAS-AS1 and miR-19a-5p, NCL-H1299 and NCL-H460 cells were transfected with miR-NC, miR-19a-5p mimic (miR-19a-5p), miR-19a-5p+NC, miR-19a-5p+FAS-AS1, anti-miR-NC, anti-miR-19a-5p, anti-miR-19a-5p+si-NC, or anti-miR-19a-5p+si-FAS-AS1, respectively. The results showed that miR-19a-5p expression was significantly increased in miR-19a-5p group, while it was recovered to normal levels in miR-19a-5p+FAS-AS1 group (Figure 5A). The expression of miR-19a-5p was evidently decreased in anti-miR-19a-5p group, which was abrogated in anti-miR-19a-5p+si-FAS-AS1 group (Figure 5B). MTT assay and colony formation assay showed that cell proliferation was markedly promoted in miR-19a-5p group, whereas returned to normal levels in miR-19a-5p+FAS-AS1 group (Figure 5C and 5E). However, cell proliferation was drastically restrained in anti-miR-19a-5p group, whereas restored to normal levels in anti-miR-19a-5p+si-FAS-AS1 group (Figure 5D and 5F). Transwell assay revealed that cell migration and invasion were remarkably increased in miR-19a-5p group, while overexpression of FAS-AS1 and miR-19a-5p restored migration and invasion to normal levels (Figure 5G and 5I). In addition, cell migration and invasion were markedly increased in anti-miR-19a-5p+si-FAS-AS1 group compared to anti-miR-19a-5p group (Figure 5H and 5J). All these data indicated FAS-AS1 abrogated the promotion of miR-19a-5p on proliferation, migration, and invasion of NSCLC cells.

FAS-AS1 Blocked Tumorigenesis In Vivo

To confirm the effect of FAS-AS1 on tumor growth of NSCLC *in vivo*, the xenograft tumor model was constructed. NCL-H460 cells transfected with NC, FAS-AS1, sh-NC or sh-FAS-AS1 were inoculated into nude mice. The results showed that tumor volume in FAS-AS1 group was significantly lower than that in NC group, while tumor volume of sh-FAS-AS1 group was greatly higher than that of sh-NC group (Figure 6A). Furthermore, tumor weight was distinctly decreased in FAS-AS1 group compared with NC group, whereas was significantly increased in sh-FAS-AS1 group compared to sh-NC group (Figure 6B). In addition, we also detected the

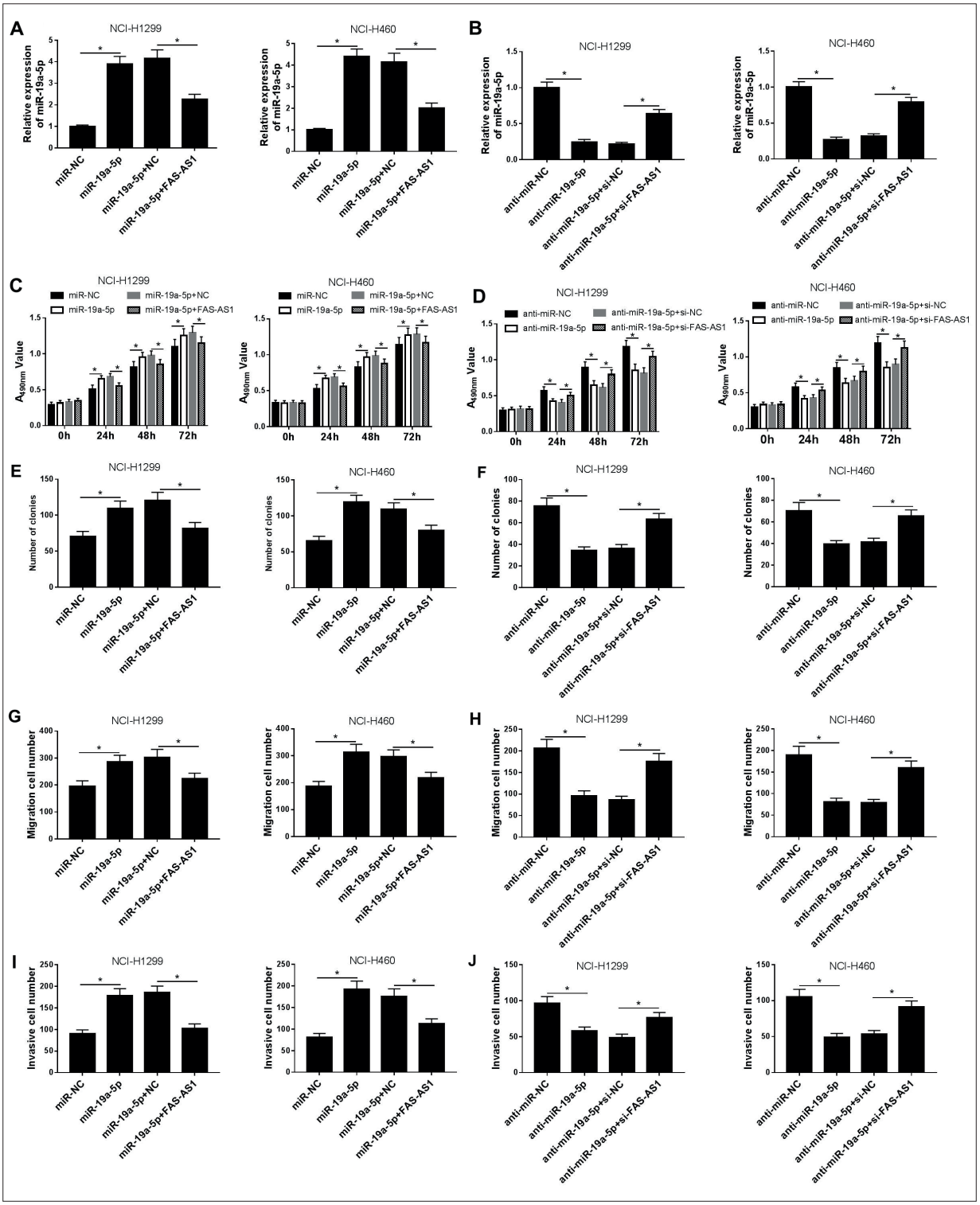

Figure 5. FAS-AS1 restored the effect of miR-19a-5p on proliferation, migration, and invasion of NSCLC cells. **A**, **C**, **E**, **G**, and **I**, NCL-H1299 and NCL-H460 cells were transfected with miR-NC, miR-19a-5p mimic (miR-19a-5p), miR-19a-5p+NC or miR-19a-5p+FAS-AS1. **B**, **D**, **F**, **H**, and **J**, NCL-H1299 and NCL-H460 cells were transfected with anti-miR-NC, anti-miR-19a-5p, anti-miR-19a-5p+si-NC or anti-miR-19a-5p+si-FAS-AS1. **A** and **B**, Expression of miR-19a-5p was examined by qRT-PCR. **C** and **D**, Cell viability was evaluated by MTT assay. **E** and **F**, Cell proliferative capacity was detected by colony formation assay. **G** and **H**, Transwell assay was used to detect cell migration ability. **I** and **J**, Transwell assay was performed to examine cell invasion ability. *$p < 0.05$.

expression levels of FAS-AS1 and miR-19a-5p in the tumor tissues by qRT-PCR assay after the mice were sacrificed. The results revealed that FAS-AS1 was significantly up-regulated in FAS-AS1 group, while was markedly down-regulated in sh-FAS-AS1 group (Figure 6C). Furthermore, miR-19a-5p was remarkedly down-regulated in FAS-AS1 group, whereas was significantly up-regulated in sh-FAS-AS1 group (Figure 6D). Thus, the xenograft assay indicated that FAS-AS1 suppressed the tumor growth *in vivo*.

Discussion

Non-small cell lung cancer (NSCLC) is one of the most common and deadly tumors worldwide[16]. Some studies[17,18] suggested that lncRNAs can function as potential tumor markers or therapeutic targets for NSCLC, because aberrant expression of lncRNAs plays important roles in tumorigenesis of various cancers by modulating many biological mechanisms. Abdolmaleki et al[19] demonstrated that FAS-AS1 expression was markedly lower in bladder cancer tissues than that in normal tissues, hinting that FAS-AS1 might act as a diagnostic marker in bladder cancer. In breast cancer, FAS-AS1 was remarkably down-regulated, and played a role in soluble Fas receptor (sFas) expression and breast cancer development[20]. A previous study[9] showed that lncRNA FAS-AS1 was down-regulated in non-small cell lung cancer tissues compared to the adjacent normal tissues. Consistent with this research, the expression level of FAS-AS1 was significantly lower in NSCLC cells than that in human lung epithelial cells. In the present study, we demonstrated that FAS-AS1 inhibited cell proliferation, migration, and invasion in NCL-H1299 and NCL-H460 cells.

LncRNAs serve as competitive endogenous RNAs to regulate miRNA expression[21,22]. We used LncBase v2 software to predict the targets of FAS-AS1 and selected miR-19a-5p for further investigations. MiR-19a-5p has been reported to play an important role in cell proliferation and metastasis in various cancers, including glioblastoma multiforme[15]. Many miRNAs play an important role in tumor progression. Cao et al[23] observed that miR-19a promoted gefitinib resistance and EMT in NSCLC cells by targeting c-Met. Wang and Chen[24] found that miR-19a was upregulated in NSCLC tissues and cells, and induced cell proliferation, migration, and invasion by targeting the suppressor of cytokine signaling 1 (SOCS1) and regulating STAT3 activation. Lin et al[25] suggested that high serum miR-19a expression was correlated with TNM stage and lymph node metastasis, indicating that high serum miR-19a expression was correlated with poor prognostic in patients with NSCLC. However, the underlying molecular mechanism of miR-19a-5p in NSCLC progression remains unclear. In the present study, we found that FAS-AS1 directly targeted miR-19a-5p and down-regulated the expression of miR-19a-5p. Therefore, we confirmed that FAS-AS1 suppressed cell proliferation, migration, and invasion in NSCLC cells by sponging miR-19a-5p.

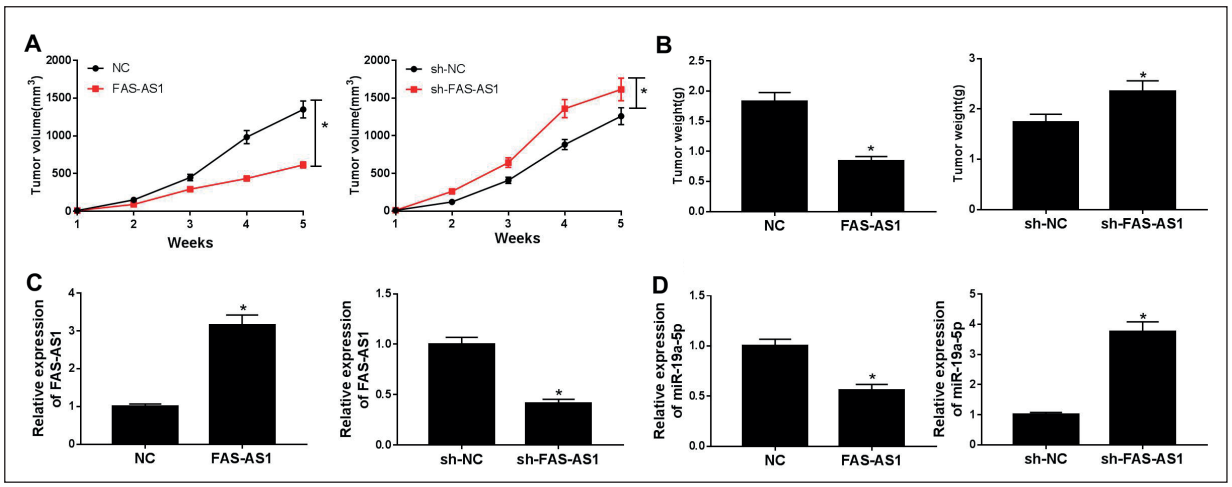

Figure 6. FAS-AS1 blocked tumorigenesis *in vivo*. **A-D**, NCL-H460 cells were transfected with NC, FAS-AS1, sh-NC or sh-FAS-AS1. **A**, Tumor volume was estimated every 7 days. **B**, Tumor weight was measured after mice were sacrificed. **C**, Expression of FAS-AS1 was examined by qRT-PCR. **D**, Expression of miR-19a-5p was measured by qRT-PCR. *$p < 0.05$.

Conclusions

All together, FAS-AS1 was down-regulated in NSCLC cells. FAS-AS1 overexpression suppressed proliferation, migration, and invasion of NSCLC cells. Conversely, FAS-AS1 knockdown induced proliferation, migration, and invasion of NSCLC cells. Overexpression of FAS-AS1 reversed the effects of miR-19a-5p overexpression on NSCLC progression. In addition, the suppression of FAS-AS1 abrogated the inhibitory effects of miR-19a-5p knockdown on the progression of NSCLC. To conclude, FAS-AS1 suppressed cell proliferation, migration, and invasion by sponging miR-19a-5p in NSCLC, which indicated that FAS-AS1 might be a novel therapeutic target for NSCLC.

Conflict of Interest

The Authors declare that they have no conflict of interests.

Ethics Approval and Consent to Participate

This investigation was approved by the Ethics Committee of The First Affiliated Hospital of Zhejiang University, Hangzhou. The methods used in this study were performed in accordance with relevant guidelines and regulations. Written consent was obtained from the participants or guardians of participants under 16 years old.

Availability of Data and Materials

All original data and materials are available from the corresponding author upon request.

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