MicroRNA-320 was downregulated in non-small cell lung cancer and inhibited cell proliferation, migration and invasion by targeting fatty acid synthase

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Abstract. The expression and functions of microRNA (miR)-320 have been previously investigated in various types of cancer. However, to the best of our knowledge, no previous studies have investigated miR-320 in human lung cancer. The current study determined the expression, biological functions and molecular mechanisms of miR-320 in human lung cancer. The expression level of miR-320 in human non-small cell lung cancer (NSCLC) and normal adjacent tissue samples (NATs), NSCLC cell lines and non-tumorigenic bronchial epithelial cells was measured by reverse transcription-quantitative polymerase chain reaction. Following transfection with miR-320 mimics, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, cell migration and cell invasion assays, western blot analysis and luciferase assay were performed in human NSCLC cell lines. The results demonstrated that miR-320 was significantly downregulated in NSCLC tissue samples and cell lines compared with NATs and a control cell line, respectively. Statistical analysis demonstrated that expression of miR-320 was significantly associated with the TNM classification and metastasis. It was also observed that miR-320 inhibited cell growth, migration and invasion in NSCLC cells. Additionally, the present study provided evidence that miR-320 may directly target fatty acid synthase. These results suggest that miR-320 may serve as a therapeutic biomarker of NSCLC in the future.

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Introduction

Lung cancer is the leading cause of cancer-associated mortality worldwide (1). In the United States, it was estimated that there would be 221,200 new cases and 158,040 mortalities caused by lung cancer in 2015 (2). The occurrence and progression of lung cancer is complex and associated with various signaling pathways. Its incidence and mortality are increasing each year as a result of environmental pollution, particularly in China (3). Lung cancer can be divided into two clinically relevant subtypes: Small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC) (4). NSCLC, as the predominant form of lung cancer, accounts for 80-85% of all cases of lung cancer (5). Large cell carcinoma, adenocarcinoma, squamous carcinoma and adenosquamous carcinoma are types of NSCLC (6). Patients with NSCLC are often diagnosed in the late stages when it is locally advanced or has metastasized, rendering it one of the most lethal forms of cancer (7). Although there has been progress regarding early detection and treatment by radical surgical resection combined with chemotherapy and radiation therapy, the prognosis of patients with NSCLC remains poor and the 5-year survival rate is ≤15% (8,9). Thus, it is important to develop novel strategies and therapeutic targets for the treatment of NSCLC.

Increasing evidence has indicated that microRNAs (miRNAs) are deregulated in lung cancer (10-12). miRNAs are a type of endogenous non-protein-coding short RNA (19-22 nucleotides in length) that are widely expressed in eukaryotes (13). miRNAs are critical for the regulation of various diverse physiological and pathological processes, including proliferation, cell death, cell cycle, migration, invasion, development and differentiation. miRNAs function via complementary base pairing with target mRNAs in the 3' untranslated region (3' UTR), leading to cleavage or translation repression of the mRNA (14-16). Previous studies have demonstrated that more than half of miRNAs are located in cancer-associated genomic regions, suggesting that aberrant expression of miRNAs may be important during tumorigenesis and progression (17). miRNAs are stable molecules and may be useful for cancer diagnosis,

treatment and predicting prognosis (18,19). miRNAs have previously been demonstrated to function as oncogenes or tumor suppressors, and are aberrantly expressed in various types of human malignancy (20). Thus, investigating miRNAs may be critical to elucidate the prognostic value and therapeutic potential of miRNAs in lung cancer.

miRNA (miR)-320 has previously been reported to be frequently downregulated in multiple types of cancer. However, to the best of our knowledge, the expression, biological functions and molecular mechanisms of miR-320 in NSCLC have not been investigated. Thus, the aim of the present study was to elucidate the expression, biological functions and molecular mechanisms of miR-320 in NSCLC.

Materials and methods

Clinical specimens. The procedures were approved by the Ethic Committee on Human Experimentation of The First Affiliated Hospital of Dalian Medical University (Dalian, China) and written informed consent was also obtained from each patient. Samples of primary cancer tissue (n=81) and paired normal adjacent tissue (NAT) were obtained from patients who had undergone surgery at The First Affiliated Hospital of Dalian Medical University. None of the patients had been treated with radiotherapy or chemotherapy prior to surgery. All samples were rapidly placed in liquid nitrogen and stored at -80°C until use. The clinical data of NSCLC patients are presented in Table I.

Cell culture and transfection. NSCLC cell lines H23, H522 and non-tumorigenic bronchial epithelium BEAS-2B cells were obtained from American Type Culture Collection (Manassas, VA, USA). H23 and H522 cells were cultured in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 mg/ml streptomycin (Gibco; Thermo Fisher Scientific, Inc.) in a cell incubator at 37°C with an atmosphere of 5% CO_2. BEAS-2B cells were cultured in LHC-9 medium (Gibco; Thermo Fisher Scientific, Inc.) containing 10% FBS.

Mature miR-320 mimics (5'-AGCGGGAGAGUUGG-GUCGAAAA-3'), miRNA mimics negative control (NC; 5'-UUCUCCGAACGUGUCACGUTT-3'). and the luciferase reporter plasmid were obtained from Shanghai GenePharma Co., Ltd. (Shanghai, China). H23 and H522 were transfected with miR-320 mimics, NC or co-tranfected with luciferase reporter plasmid using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol.

RNA isolation and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from the tumor tissues, NATs and cells using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) following the manufacturer's protocol. Then, PrimeScript reverse transcription-PCR kit (Takara Biotechnology Co., Ltd., Dalian, China) was used to perform RT to obtain cDNA. For analysis of miR-320 expression levels, a SYBR PrimeScript miRNA RT-PCR kit (Takara Biotechnology Co., Ltd.) was used with the ABI 7500 Real-Time PCR detection system (Applied

Biosystems; Thermo Fisher Scientific, Inc.), with U6 serving as the internal control. Each sample was analyzed in triplicate. Primers were synthesized by Guangzhou RiboBio Co., Ltd. (Guangzhou, China). Data were analyzed using the $\Delta\Delta$Cq method (21)

Cell proliferation assay. The effect of miR-320 on NSCLC cell proliferation was analyzed using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Transfected cells (miR-320 or NC) were seeded in 96-well plates at a density of 3,000 cells/well in 100 μl medium. MTT assay was performed every 24 h for 120 h following transfection. Briefly, 20 μl MTT (5 mg/ml; Sigma-Aldrich, St. Louis, MO, USA) was added into each well and cells were incubated for 4 h at 37°C. Subsequently, the formazan was dissolved in 200 μl dimethyl sulfoxide. The absorbance at 490 nm was measured using a microplate reader (ELx800; Bio-Tek Instruments, Inc., Winooski, VT, USA). For each treatment group wells were assessed in triplicate.

Cell migration and invasion assay. The migration and invasion ability of NSCLC cells transfected with miR-320 mimics and NC was analyzed using Transwell chambers with an 8-μm pore polycarbonate membrane (EMD Millipore, Billerica, MA, USA). For cell migration assays, $2x10^4$ transfected cells in 100 μl serum-free RPMI-1640 medium were placed into the upper chambers. A volume of 500 μl RPMI-1640 medium supplemented with 20% FBS was added into the lower chambers. The cell invasion assays were performed according to the same procedure, although the Transwell chambers were pre-coated with Matrigel (BD Biosciences, San Jose, CA). Subsequent to incubation (12 h for migration assay and 24 h for invasion assay), cells that did not migrate or invade through the pores were carefully wiped away with cotton wool. Subsequently, the inserts were fixed with 100% methanol for 10 min, and stained with 0.5% crystal violet (Beyotime Institute of Biotechnology, Haimen, China) and imaged with an inverted microscope (IX71; Olympus Corporation, Tokyo, Japan) at x200 magnification.

Bioinformatics analysis. The target gene information of miR-320 was analyzed using Targetscan (www.targetscan.org/).

Western blotting. To determine the expression level of fatty acid synthase (FAS) at the protein level, western blot analysis was performed. Protein samples of the transfected cell lines were harvested using radioimmunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology) containing protease inhibitors and phosphatase inhibitors (Roche Diagnostics, Basel, Switzerland). Protein concentrations (30 μg) were measured by a bicinchoninic acid assay kit (Nanjing KeyGen Biotech Co., Ltd., Nanjing, China). Briefly, equal quantities of protein lysates were separated by 10% SDS-polyacrylamide gel electrophoresis for 20 min at 70 V and transferred to polyvinylidene fluoride membrane (EMD Millipore). After blocking with 5% non-fat milk Tris-buffered saline solution containing 0.1% Tween 20, and incubated with primary antibodies overnight at 4°C.

Primary antibodies in the current study included mouse anti-FAS monoclonal antibody (1:1,000 dilution; cat

Table I. A comparison of microRNA-320 expression in non-small cell lung cancer and clinicopathological features.

| Clinical features | Cases, n | miR-320 expression | | P-value |
		Low	High	
Gender				0.812
Male	38	30	8	
Female	43	33	10	
Age, years				0.544
<60	49	37	12	
≥60	32	26	6	
Smoking history, years				0.434
<10	34	25	9	
≥10	47	38	9	
Tumor differentiation, grade				0.591
I-II	45	34	11	
III-IV	36	29	7	
TNM classification, stage				0.026
I	22	13	9	
II	31	28	3	
III+IV	28	22	6	
Metastasis				0.011
No	33	21	12	
Yes	48	42	6	

The median expression level of miR-320 was used as a cutoff point to divide all patients into low and high expression groups.

no. ab106062; Abcam, Cambridge, MA, USA) and mouse anti-GAPDH monoclonal antibody (1:1,000 dilution; cat no. ab125247; Abcam). The membranes were then incubated with goat anti-mouse IgG horseradish peroxidase-conjugated secondary antibody (1:5,000 dilution; cat. no. ab6789; Abcam) was applied to the membranes for 1 h at room temperature. The protein blots were visualized with ECL reagents (Pierce; Thermo Fisher Scientific, Inc.), imaged using a FluorChem imaging system (ProteinSimple, San Jose, CA, USA) and normalized to GADPH. Protein bands were normalized to GAPDH and analyzed using the AlphaEase FC2 software (Alpha Innotech, San Leandro, CA, USA).

Luciferase assay. The luciferase assays were performed to determine whether FAS was a direct target of miR-320. NSCLC cells were plated in a 24-well plate at 40-50% confluency and were transfected with the luciferase reporter plasmid (synthesized by GenePharma Co., Ltd.), miR-320 mimics or NC in a 12-well plate using Lipofectamine 2000 according to the manufacturer's protocol. After 48 h, the activities of firefly and *Renilla* luciferases were measured with the Dual-Luciferase Reporter Assay system (Promega Corporation, Madison, WI, USA). The firefly luciferase activity was normalized to the *Renilla* luciferase activity. The wells were assessed in triplicate for each treatment group.

Statistical analysis. Data are presented as the mean ± standard deviation, and compared using SPSS software (version 11.0;

SPSS, Inc., Chicago, IL, USA). One-way analysis of variance followed by a least significant differences test was conducted to analyze the data. P<0.05 was considered to indicate a statistically significant difference.

Results

miR-320 expression in human NSCLC tissues and cell lines. To examine the function of miR-320 in NSCLC, miR-320 expression was analyzed by RT-qPCR. As demonstrated in Fig. 1A and B, the level of miR-320 was significantly downregulated in NSCLC tissue samples when compared with matched NATs (P=0.002). The expression level of miR-320 in NSCLC cell lines and non-tumorigenic bronchial epithelium BEAS-2B cells was also detected. As demonstrated in Fig. 1C, miR-320 expression level was also significantly decreased in H23 and H522 cells compared with BEAS-2B cells (P=0.001). These results indicate that miR-320 may be important during lung carcinogenesis.

Association between miR-320 expression level and clinicopathological features of patients with NSCLC. Statistical analysis was performed to determine whether the expression level of miR-320 was associated with the clinicopathological features of patients with NSCLC. The results demonstrated that the expression level of miR-320 was significantly associated with the TNM classification (P=0.028) and metastasis (P=0.016). However, no correlation was identified between the
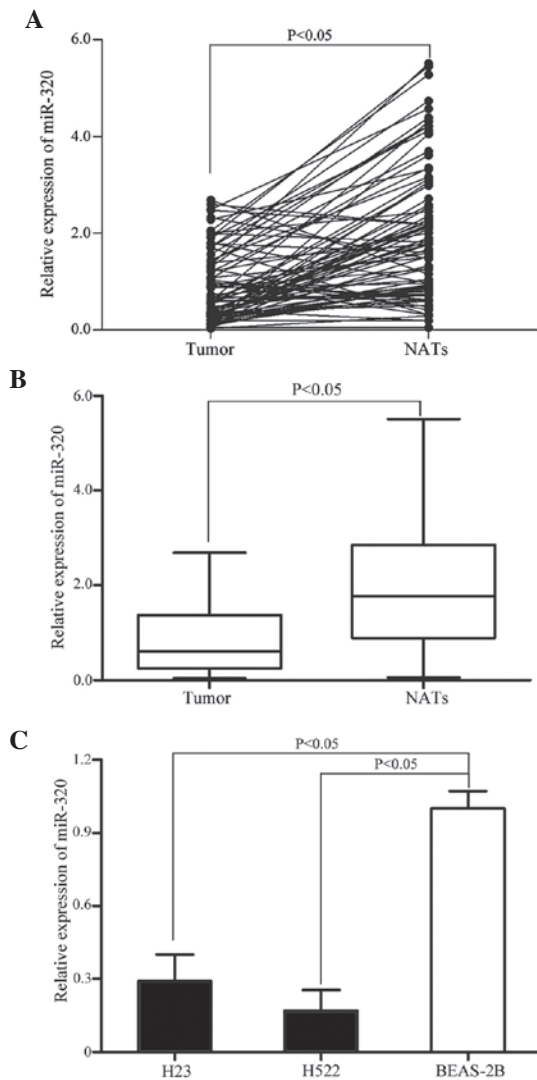
A

B

C

Figure 1. Expression of miR-320 in NSCLC tissues and cell lines. (A and B) miR-320 was significantly downregulated in NSCLC tissue samples compared with matched NATs. (C) miR-320 expression level was significantly decreased in H23 and H522 cells compared with non-tumorigenic bronchial epithelium BEAS-2B cells. NSCLC, non-small cell lung cancer; miR, microRNA; NATs, normal adjacent tissues.

miR-320 expression level and the other clinicopathological features (gender, age, smoking history and tumor differentiation).

miR-320 suppresses cell proliferation in H23 and H522 cells. The effect of miR-320 on NSCLC cell proliferation was measured by MTT assay. It was demonstrated that the absorbance in H23 (P=0.008) and H522 (P=0.019) cells transfected with miR-320 was significantly decreased when compared with the NC group (P<0.05; Fig. 2). This verified that miR-320 inhibits the proliferation of H23 and H522 cells.

miR-320 inhibits cell migration and invasion in H23 and H522 cells. The effect of miR-320 on NSCLC cell migration and invasion was measured using Transwell apparatus. As demonstrated in Fig. 3, overexpression of miR-320 significantly reduced the migration and invasion ability of H23 (P=0.021 for migration, 0.025 for invasion) and H522 (P=0.015 for

migration; P=0.020 for invasion) cells compared with the NC group (P<0.05). These results indicate that miR-320 may be involved in reducing the migration and invasion potential of NSCLC cells *in vitro*.

miR-320 directly targets FAS. FAS protein is important for cancer proliferation and metastasis, and was predicted to be a direct target gene of miR-320 (Fig. 4A); luciferase assays were performed to verify this. As demonstrated in Fig. 4B, miR-320 mimics significantly inhibited the luciferase activity of the wild type but not the mutant FAS 3' UTR constructs in H23 (P=0.034) and H522 cells (P=0.018).

Furthermore, western blot analysis was performed to determine the protein expression level of FAS in H23 and H522 cells following transfection with miR-320. The results of the current study demonstrate that the expression level of FAS was significantly inhibited in miR-320 mimic-transfected H23 (P=0.021) and H522 (P=0.028) cells compared with cells transfected with NC (P<0.05; Fig. 4C). These results demonstrate that FAS is a direct target gene of miR-320 *in vitro*.

Discussion

Increasing evidence indicates that the aberrant expression of miRNAs is a characteristic of multiple types of malignancy, including lung cancer (13,22). The expression of miR-320 has been observed to be downregulated in various types of human cancer, including breast cancer, oral squamous cell carcinoma (OSCC), colon cancer, acute myelogenous leukemia, osteosarcoma and glioma (23-28). However, to the best of our knowledge, no previous studies have investigated the expression of miR-320 in lung cancer. The present study demonstrated that miR-320 was significantly downregulated in NSCLC tissue samples and cell lines. Furthermore, the expression of miR-320 was significantly associated with the TNM classification and presence of metastasis. These results indicate that miR-320 may be important in NSCLC.

miR-320 has previously been demonstrated to be a tumor suppressor during tumorigenesis and progression in multiple types of cancer. In colon cancer, upregulation of miR-320 was demonstrated to inhibit cell growth, cell cycle, migration and invasion, whereas downregulation of miR-320 had the opposite effect on these biological processes (24). Additionally, it was demonstrated that overexpression of miR-320 enhanced the sensitivity of human colon cancer cells to fluorouracil and oxaliplatin by targeting forkhead box M1 *in vitro* (24). In OSCC, miR-320 was demonstrated to suppress tumor angiogenesis via downregulation of neuropilin 1 (26). Cheng *et al* (27) also reported that miR-320 inhibits osteosarcoma cell proliferation by targeting FAS. In human glioma, miR-320 decreased cell growth by directly targeting E2F transcription factor 1 (28). However, to the best of our knowledge, no previous studies have investigated the function of miR-320 in lung cancer. The present study demonstrated that miR-320 inhibits NSCLC cell proliferation, migration and invasion. The current study increased the knowledge of the functions of miR-320 in cancer. These findings indicate that miR-320 performs an essential function in these forms of cancer, and may have the potential to be developed as an anticancer therapeutic agent.
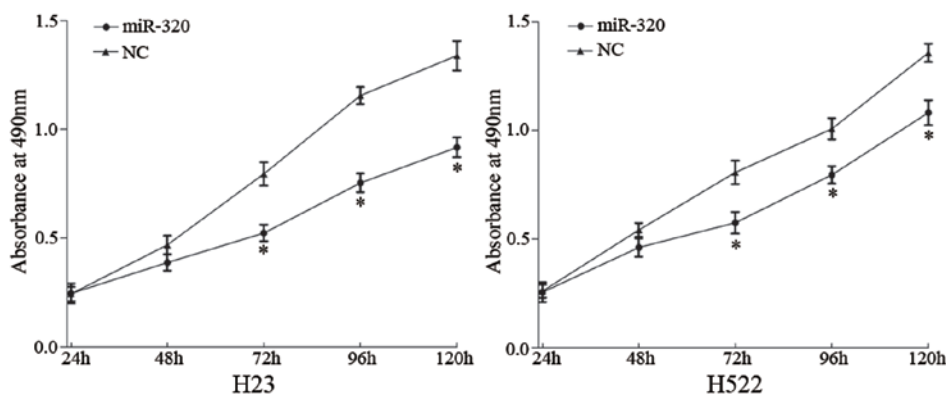
Figure 2. miR-320 inhibited cell proliferation in non-small cell lung cancer cells. The proliferation of H23 and H522 cells was determined by 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide assay after transfection with miR-320 or NC. *P<0.05, compared with NC. miR, microRNA; NC, negative control.
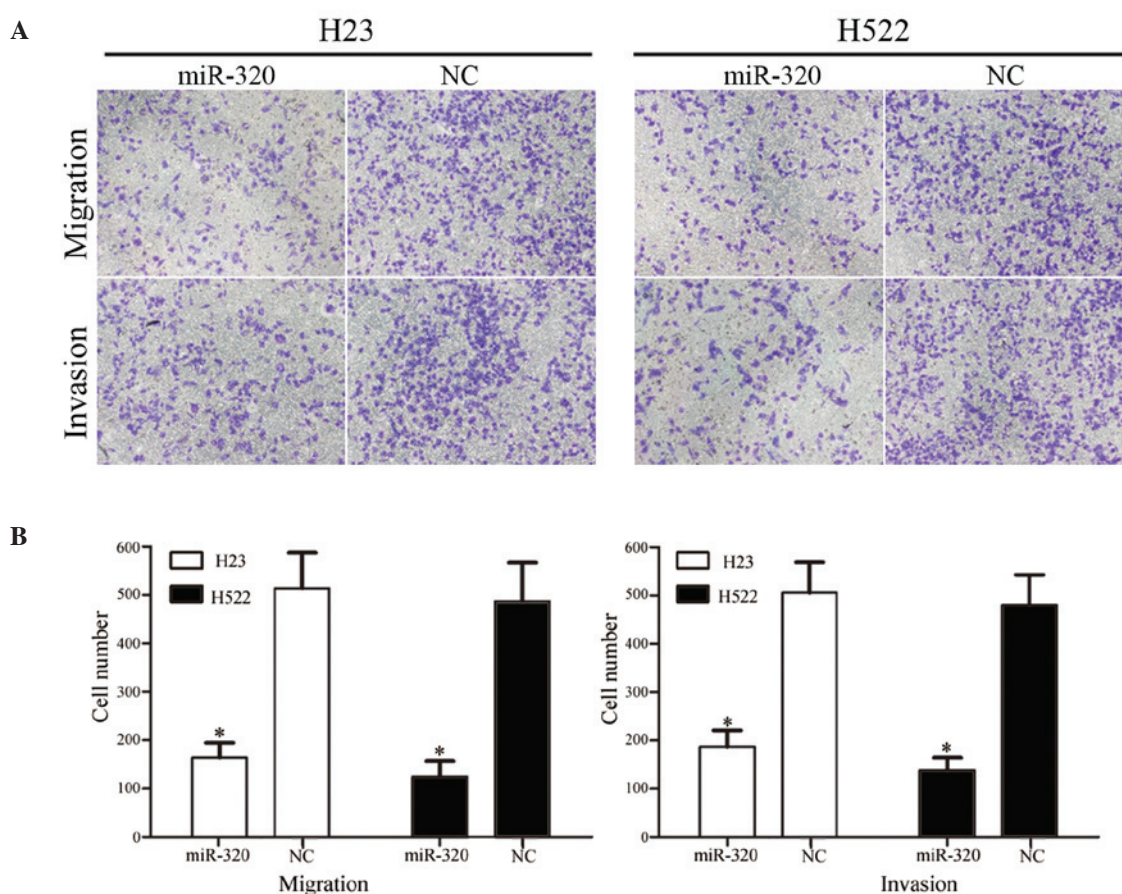

Figure 3. miR-320 inhibited cell migration and invasion in non-small cell lung cancer cells. The migration and invasion abilities of H23 and H522 cells transfected with miR-320 or NC were measured using Transwell chambers with an 8-μm pore polycarbonate membrane. (A) Microscopic images at x200 magnification, (B) quantification of migration and invasion. *P<0.05, compared with NC. Magnification, x200. miR, microRNA; NC, negative control.

Identification of miR-320 target genes is essential for understanding its mechanism in lung carcinogenesis and for the development of novel targeted therapies. The current study successfully demonstrated that FAS is a direct target gene of miR-320. Bioinformatics software (TargetScan) predicted that FAS was a direct target mRNA of miR-320 and luciferase assays demonstrated that miR-320 directly interacts with FAS 3' UTR. Furthermore, manipulation of miR-320 expression in NSCLC cell lines decreased the expression of FAS at the protein level. These findings suggest that miR-320 acts as a tumor suppressor in NSCLC by targeting FAS.

FAS, a multifunctional enzymatic complex, catalyzes the formation of palmitate from acetyl-coenzyme A and malonyl-coenzyme A (29). The endogenous FAS is expressed at very low levels in normal human tissues (30). However, increasing studies have demonstrated that the expression level of FAS is highly upregulated and involved in cancer proliferation and metastasis in various types of human tumor, including

A

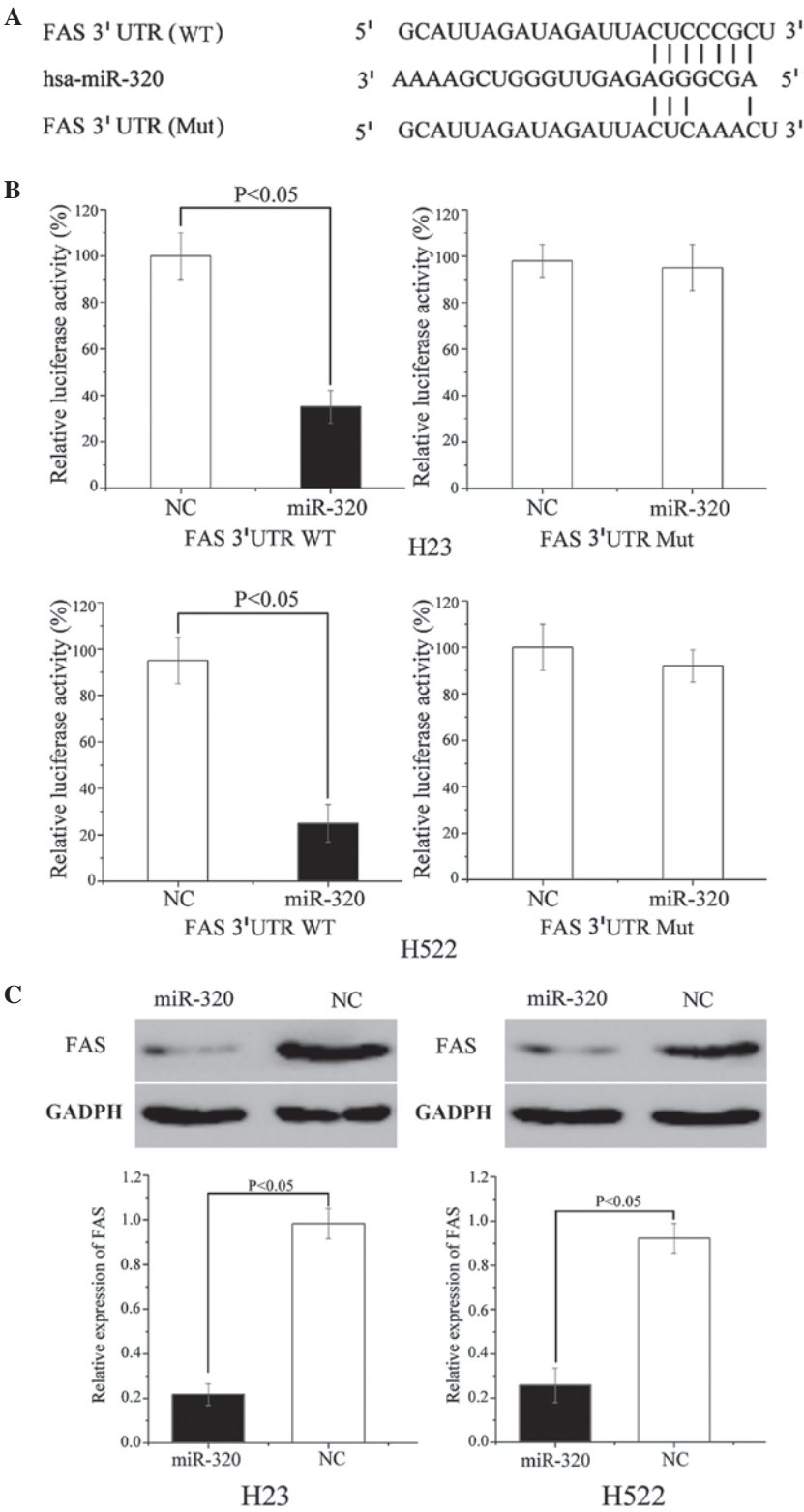|	
FAS 3' UTR (WT)	5' GCAUUAGAUAGAUUACUCCCGCU 3'
	|||||||
hsa-miR-320	3' AAAAGCUGGGUUGAGAGGGCGA 5'
	||| |
FAS 3' UTR (Mut)	5' GCAUUAGAUAGAUUACUCAAACU 3'

B

C

Figure 4. miR-320 directly targeted FAS. (A) TargetScan analysis demonstrated that FAS mRNA contained an miR-320 seven-nucleotide seed sequence of the FAS 3' UTR. (B) The luciferase activity was significantly decreased in H23 and H522 cells co-transfected with miR-320 mimic and FAS 3' UTR WT. (C) FAS was significantly downregulated at protein level in miR-320 mimic-transfected H23 and H522 cells compared with the NC. FAS, fatty acid synthase; UTR, untranslated region; miR, microRNA; WT, wild type; Mut, mutant; NC, negative control.

breast, colorectal, prostate, bladder, ovarian, esophageal, gastric and lung cancer, oral carcinoma, thyroid cancer and endometrial carcinoma, and also in mesothelioma, nephroblastoma, retinoblastoma, soft tissue sarcomas, melanoma and hepatocellular carcinoma (31-33). FAS was also previously reported to be correlated with various clinicopathological features of cancer. For example, overexpression of FAS in NSCLC was reported to be significantly associated with bone metastasis (34). These studies suggested that targeting FAS may present as a therapeutic approach.

FAS has previously been demonstrated to be regulated by multiple miRNAs in multiple types of cancer, including lung cancer. miR-601, miR-10b and miR-663 are involved in the biology of lung cancer via direct or indirect regulation of FAS (35-37). In osteosarcoma, miR-142-3p and miR-320 have been reported to act as tumor suppressors by regulation of FAS (27,38). Previous studies demonstrated that miR-424 and miR-195 regulate FAS to inhibit osteosarcoma cell migration and invasion (39,40). In colorectal and breast cancer, miR-196b and miR-21 modulate cell apoptosis by repressing FAS expression (41,42). In gastric cancer, altered miR-106a expression levels exert oncogenic effects in gastric carcinogenesis by targeting FAS (43). In prostate cancer, miR-185 and miR-342 have been demonstrated to decrease cell proliferation, migration and invasion by inhibiting FAS indirectly (44). In hepatocellular carcinoma, restoration of miR-449 suppresses cell proliferation by downregulating FAS (45). In the present study, overexpression of miR-320 in NSCLC cell lines demonstrated that miR-320 inhibits cell proliferation, migration and invasion via downregulation of FAS. Taken together, these results indicate that miR-320 may act as a tumor suppressor by inhibiting the oncogenic activity of FAS.

In conclusion, this was the first study, to the best of our knowledge, to demonstrate that miR-320 is downregulated in NSCLC, and is significantly associated with the TNM classification and metastasis. It was also demonstrated that miR-320 contributes to cell proliferation, migration and invasion by directly targeting FAS in NSCLC. The identification of FAS as a candidate target gene of miR-320 may provide an understanding of the potential carcinogenic mechanisms in NSCLC. These findings have therapeutic implications and may be exploited for developing treatment strategies for NSCLC.

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