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Original Article

microRNA-506 regulates proliferation, migration and invasion in hepatocellular carcinoma by targeting F-spondin 1 (SPON1)

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Abstract: Our previous study indicates microRNA-506 (miR-506) is downregulated in hepatocellular carcinoma (HCC). In the current study, we investigate the effects of miR-506 on proliferation, migration and invasion in HCC. We report that enforced expression of miR-506 inhibits proliferation, migration and invasion in vitro, and suppresses tumor growth in vivo. Conversely, suppression of miR-506 exhibits promoting effects on proliferation, migration and invasion in vitro, and on tumor growth in vivo. In addition, miR-506 binds to the 3'UTR of F-spondin 1(SPON1), and enforced expression of miR-506 decreases accumulation of SPON1. Moreover, enforced expression of SPON1 and suppression of SPON1 alleviates effects of miR-506 mimics and inhibitors on proliferation, migration and invasion in vitro, respectively. In conclusion, microRNA-506 regulates proliferation, migration and invasion in HCC by targeting SPON1.

Keywords: microRNA-506, proliferation, migration and invasion, hepatocellular carcinoma, SPON1

Introduction

Hepatocellular carcinoma (HCC) is one of the most lethal malignancies in worldwide [1-3]. The major therapy for HCC patients is surgery combines chemotherapy. However, the patient outcome is not very optimistic due to complicated reasons; including drug resistance, high recurrence rate and patients not qualify for surgery. Figuring out the mechanisms of carcinogenesis, migration and invasion in HCC will help improving efficiency of HCC therapy. MicroRNAs (miRNAs) is the regulator of carcinogenesis, migration and invasion. microRNA 506 (miR-506) has been reported as suppressive miRNA and plays a role in colon cancer [4], breast cancer [5], gastric cancer [6], oral cell carcinoma [7], cervical cancer [8], ovarian cancer [9], clear cell renal cell carcinoma [10] and nasopharyngeal carcinoma[11].

F-spondin 1(SPON1) is an extracellular matrix protein and expresses in a high levels in the floor plate [12]. Recombinant SPON1 promotes the attachment of spinal cord and sensory neuron cells and the outgrowth of neurites in vitro. SPON1 may contribute to the growth and guidance of axons in both the spinal cord and the PNS [12]. SPON1 regulates cementoblastic differentiation of human periodontal ligament cells [13], differentiation of nerve precursor [14], and differentiation of clastic precursors [15]. Recently, SPON1 was reported as a novel ovarian cancer marker

In the current study, we report miR-506 inhibits proliferation, migration and invasion in HCC by down-regulating SPON1. Our data provides supplementation in mechanisms of carcinogenesis, migration and invasion in HCC and helps establishing novel strategy for HCC therapy.

Materials and methods

Cell culture

Human hepatocelullar carcinoma cell line huh7 and hepatoma cell line hepG2 were obtained from Shanghai Cell Institute Country Cell Bank, (Shanghai, China). huh7 and hepG2 cells were

Table 1. Primers for qRT-PCR

ID	Sequence
SPON1-F	TCTTAGACTGCTGTGCCTGC
SPON1-R	AACTTGTTTGACGCCTTCGC
GAPDH-F	AGAAGGCTGGGGCTCATTTG
GAPDH-R	AGGGGCCATCCACAGTCTTC

grown in 1640 medium with 10% fetal bovine serum (FBS) (GIBCo/BRL, MD), supplemented with 100 U/ml penicillin G and 100 μg/ml streptomycin (Sigma-Aldrich Corp., St. Louis, MO). Cells were maintained at 37°C in a humidified 5% CO_2 incubator.

MicroRNA and siRNA transfection

Negative control of microRNA (mimics NC), mimics of miR-506, negative controls of microRNA inhibitor (inhibitor NC), inhibitors of miR-506 and siRNAs targeted for SPON1 were purchased from GenePharma (China). For miRNA and siRNA transfection, 5×10^4 HCC cells plated in triplicate in 6-well plates. 100 nM mimics or inhibitors of miR-506 or 100 nM siRNAs of SPON1 were transfected into HCC cells using lipofectamine 2000 (invitrogen) according to manufacturer's protocol. The efficiency of microRNA and siRNAs transfection were confirmed by quantitative real-time reverse transcription PCR (qRT-PCR).

RNA extraction and quantitative qRT-PCR

Total RNA was extracted using TRIZOL reagent (Ambion) according to manufacturers' protocols. cDNA used to examined SPON1 were synthesized by using PrimeScriptTM RT reagent kit (TaKaRa) according to manufacturers' protocols. cDNA used to examined miR-506 were synthesized by using miRcute miRNA cDNA first strand synthesis kit (TIANGEN, Beijing) according to manufacturers' protocols. Expression of SPON1 was examined using SYBR® Premix Ex TaqTM II (TaKaRa) and GAPDH was served as internal reference. Expression of miR-506 was examined using miRcute miRNA qPCR detection kit (TIANGEN, Beijing) and U6 was served as internal reference. All experiments were performed in duplicate and repeated twice. Results are represented as fold induction using the 2-ΔΔCt method. Primers of miR-506 and U6 were purchased from GenePharma (China). Primers used to examine expression of SPON1 were listed in **Table 1**.

Proliferation assays

3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) assays were performed according to the manufacturer's protocol. Briefly, 5000 cells/well were plated in triplicate in 96-well plates. The MTT reagent was prepared at 5 mg/ml in Phosphate Buffered Saline (PBS). This MTT stock solution was then added to each well at a 1:10 dilution. Cells were incubated for 4 h, and the resulting crystals were dissolved in 100 μl Dimethyl sulfoxide (DMSO). The absorbance at 490 nm was measured using a multi-well plate reader.

Transwell assays for migration and invasion

The transwell assay for migration was done by using chambers with polycarbonate filters (pore size, 8 μm) (Becton Dickinson Labware). The transwell assay for migration was done by using chambers with matrixgel and polycarbonate filters (pore size, 8 μm) (Becton Dickinson Labware). Briefly, 24 hours after miRNA transfection, HCC cells were harvested and 5×10^4 transfected cells in 200 μL of 0.1% serum medium were placed in the upper chamber. The lower chamber was filled with 10% fetal bovine serum medium (600 μL). After 24 h incubation and removal of the cells on the upper chamber of the filter with a cotton swab, the cells on the underside were fixed with 4% paraformaldehyde, stained with 0.1% crystal violet in 20% ethanol, and counted in five randomly selected fields under phase contrast microscope. The migrated or invasive cells were monitored by photographing at 400× magnification with LEICA Microscope. The assays were performed in triplicate.

Tumorigenicity assays in nude mice

Six-week-old female athymic nude mice were subcutaneously injected in the right armpit region with 1×10^7 cells in 0.1 mL of PBS. Two groups of mice (n = 6/group) were tested. Group 1 (NC) was injected with huh7 cells transfected with NC; and group 2 (miR-506) was injected with huh7 cells transfected with mimics of miR-506. The tumor sizes were measured every 7 days with calipers. The tumor volume was calculated with the formula: (L × W2)/2, where

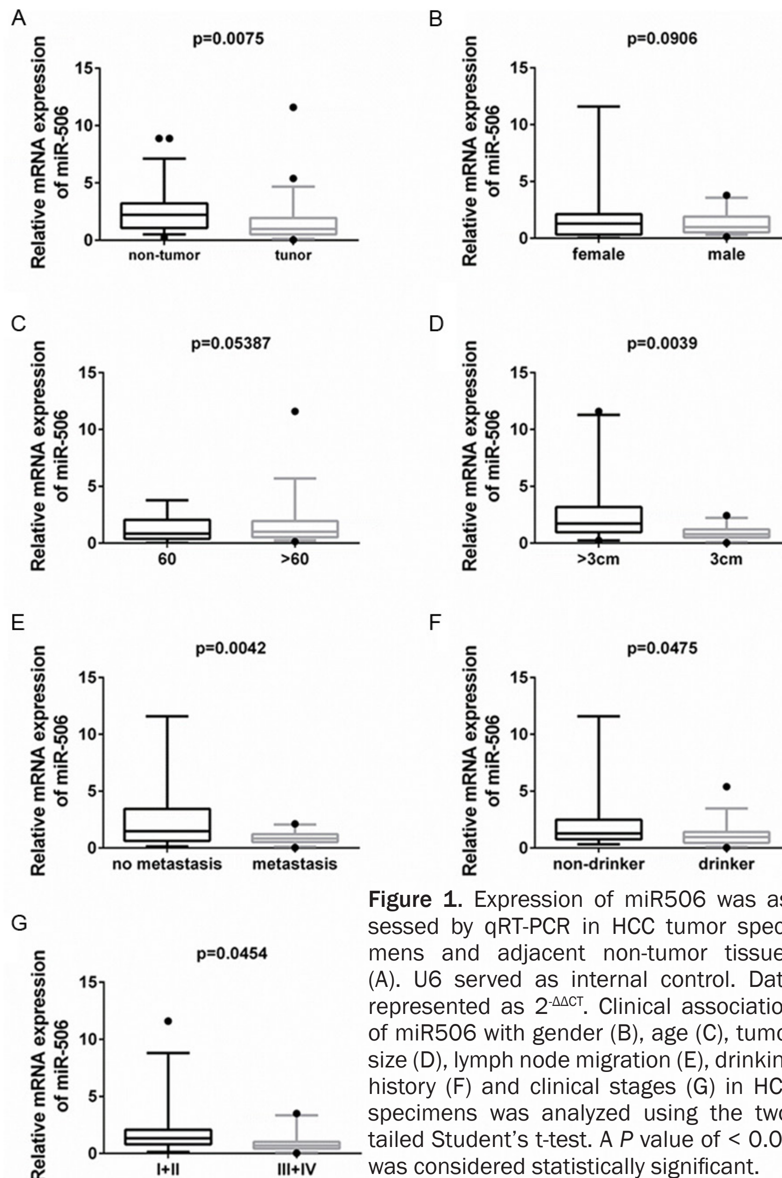


Figure 1. Expression of miR506 was assessed by qRT-PCR in HCC tumor specimens and adjacent non-tumor tissues (A). U6 served as internal control. Data represented as $2^{-\Delta\Delta CT}$. Clinical association of miR506 with gender (B), age (C), tumor size (D), lymph node migration (E), drinking history (F) and clinical stages (G) in HCC specimens was analyzed using the two-tailed Student's t-test. A *P* value of < 0.05 was considered statistically significant.

L is the length and W is the width of the tumor. After the mice were killed at five weeks, the weights of the tumors were measured. All experimental procedures involving animals were in accordance with the Guide for the Care and Use of Laboratory Animals (NIH publication no. 80-23, revised 1996) and were performed according to the institutional ethical guidelines for animal experiments.

Immunoblot

Immunoblot was performed according to standard Western blot procedures as previously described. Briefly, proteins were separated by 10% SDS-PAGE and then transferred to nitrocellulose membrane (Bio-Rad). After blocking in 5% nonfat milk, the membranes were incubated with the following primary antibodies: rabbit anti-SPON1 monoclonal antibody (mAb; 1:300; Abcam) and mouse anti-GAPDH mAb (1:1,000; Abcam). The proteins were visualized with enhanced chemiluminescence reagents (Pierce).

Luciferase reporter assays

Huh7 cells were seeded in 96-well plates at 6,000 cells per well the day before transfection. A mixture of 100 ng luciferase reporter constructs (psiCHECK-SPON1-WT and psiCHECK-SPON1-mutant) and 200 ng of NC or miR-506 mimics was transfected into huh7 cells with Lipofectamine 2000. Forty-eight hours later, Firefly and Renilla luciferase activities were measured with a Dual-Luciferase Reporter System (Promega) according to the manufacturer's protocol.

Statistical analysis

Data were shown as mean ± SD unless otherwise noted. The Student's t test was used to analyze difference between two groups. A *P* value of < 0.05 was considered statistically significant.

Results

miR-506 is down-regulated in HCC

Our previous study using microchip showed miR-506 is down-regulated in HCC tissues (data not shown). To further verify this finding, we examined expression of miR-506 in 56 HCC tissues and their corresponding non-cancerous tissues by quantitative real time RT-PCR. We

Table 2. The association of miR-506 with clinicopathological features of 50 patients with HCC

Characteristic	No. of patients		$2^{-\Delta\Delta Ct}$	P
	No	%	Mean ± SD	
Age, years				
> 60	38	76%	1.585 ± 0.3231	0.5387
≤ 60	12	24%	1.211 ± 0.3147	
Sex				
Male	35	70%	1.211 ± 0.1458	0.0906
Female	15	30%	2.158 ± 0.7759	
Size				
> 3 cm	20	40%	2.377 ± 0.5722	0.0039
≤ 3 cm	30	60%	0.9072 ± 0.1114	
Histological grade				
I, II	28	56%	1.948 ± 0.4226	0.0454
III, IV	22	44%	0.9193 ± 0.1684	
Lymph node status				
No Migration	16	32%	2.444 ± 0.7146	0.0042
Migration	34	68%	0.9283 ± 0.09725	
Drinking history				
Drinker	32	64%	1.116 ± 0.1801	0.0475
Nondrinker	18	36%	2.169 ± 0.6163	

found expression of miR-506 in HCC tissues were significantly lower than their non-cancerous counterparts (**Figure 1A**). We also analyzed correlation between expression of miR-506 and clinpathological parameters (**Table 2**). Our data showed expression of miR-506 correlate with tumor size (**Figure 1D**), migration (**Figure 1E**), drinking history (**Figure 1F**) and clinical stage (**Figure 1G**), but not with sex (**Figure 1B**) and age (**Figure 1C**).

miR-506 inhibits cell proliferation, migration and invasion in vitro

We investigated effects of miR-506 on cell proliferation, migration and invasion in huh7 and hepG2 cells. Enforced expression of miR-506 and suppression of miR-506 was achieved by transfecting mimics and inhibitors of miR-506 into huh7 and hepG2 cells, respectively. NC was served as control for transfection. The efficiency of transfection was verified by quantitative real time qRT-PCR. Our data showed enforced expression of miR-506 inhibits cell proliferation (**Figure 2B**), migration (**Figure 2C**) and invasion (**Figure 2D**) of huh7 and hepG2 cells. Conversely, suppression of miR-506 promotes cell proliferation (**Figure 2B**), migration (**Figure 2C**) and invasion (**Figure 2D**) of huh7 and hepG2 cells.

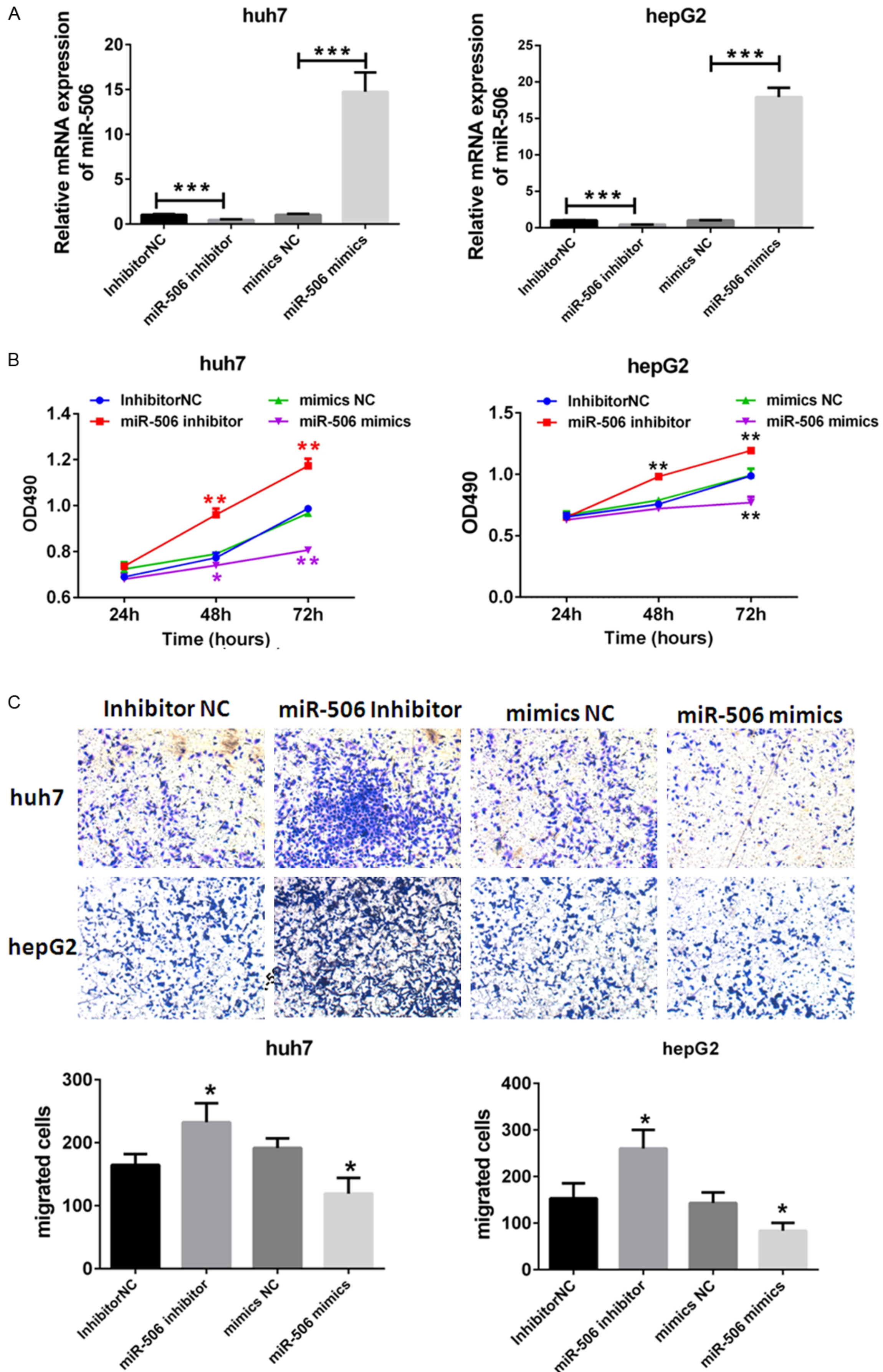
miR-506 inhibits tumor growth in vivo

In order to verify our finding in vitro, we investigated effect of miR-506 on tumor growth in nude mice. Huh7 cells transfected with mimics or inhibitor of miR-506 were injected subcutaneously into nude mice. NC was served as control. The efficiency of transfection was verified by quantitative real time RT-PCR. We found huh7 cells transfected with mimics of miR-506 formed significantly smaller tumors in nude mice compared with controls. Conversely, huh7 cells transfected with inhibitor of miR-506 formed significantly bigger tumors in nude mice compared with controls (**Figure 3**). These data implicated miR-506 regulates tumor growth in vivo.

miR-506 binds to 3'UTR of SPON1 and decreases expression of SPON1

In order to explore the mechanism underlying inhibitory effects of miR-506 on proliferation, migration and invasion, we identified target of miR-506 using online program. SPON1 is the candidate with higher scores and miR-506 binding site in their 3'UTR (**Figure 4A**). In order to investigate whether SPON1 is potential targets of miR-506 or not, we created luciferase reporter constructs containing wild type or mutated 3'UTR of SPON1, and examined whether miR-506 binds to 3'UTR of SPON1. Our data showed enforced expression of miR-506 decreased relative luciferase activities in huh7 cells transfected with psiCHECK-SPON1-WT, but not in huh7 cells transfected with psiCHECK-SPON1-mutant (**Figure 4B**). These results indicate miR-506 binds to 3'UTR of SPON1.

Next, we examined expression of SPON1 in huh7 cells transfected with mimics or inhibitor of miR-506, and in tumors isolated from nude mice which injected with huh7 cells transfected with mimics or inhibitor of miR-506. Our data showed enforced expression of decreased accumulation of SPON1 in huh7 cells (**Figure 4C**) and tumors isolated from nude mice (**Figure 4D**), which indicates miR-506 regulates expression of SPON1.


A
huh7
Relative mRNA expression of miR-506
***
InhibitorNC
miR-506 inhibitor
mimics NC
miR-506 mimics
hepG2
B
OD490
Time (hours)
24h
48h
72h
C
Inhibitor NC
miR-506 Inhibitor
mimics NC
miR-506 mimics
migrated cells

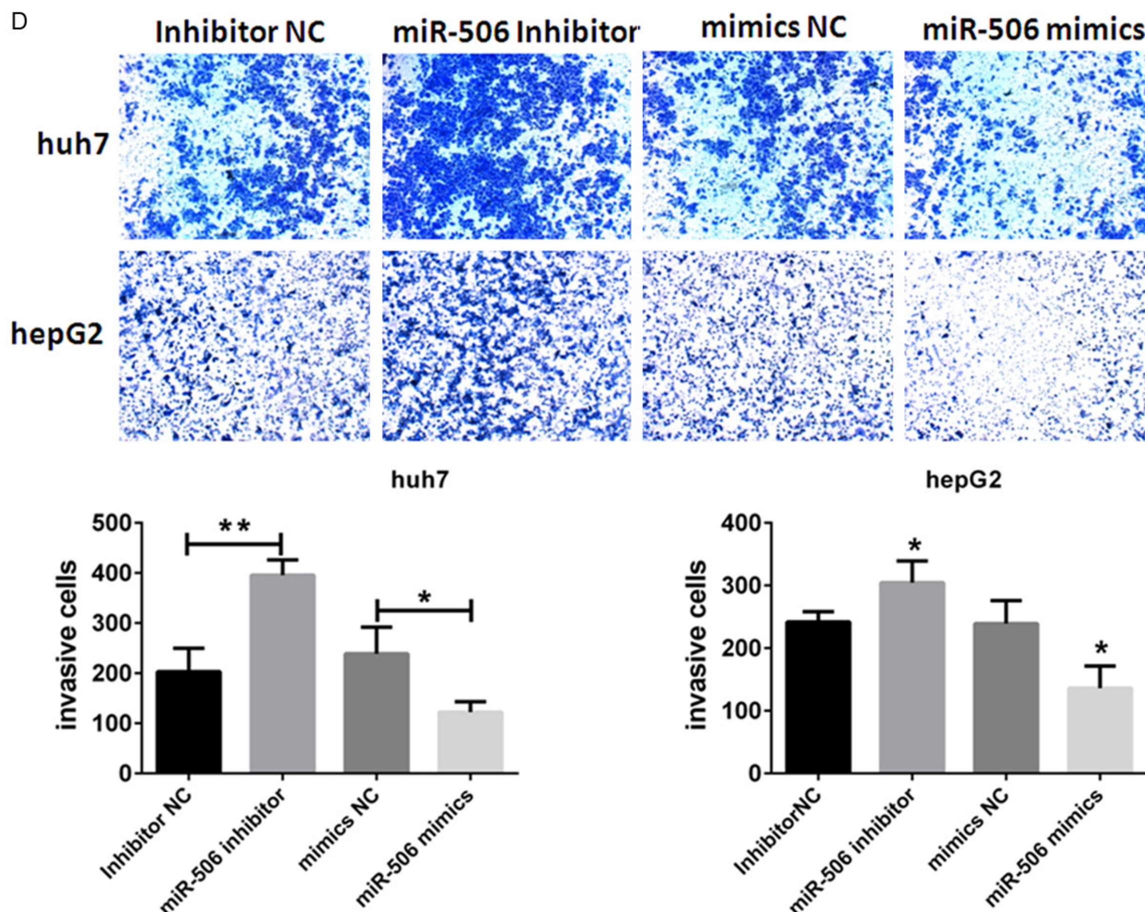


Figure 2. miR506 inhibits proliferation, migration and invasion in huh7 cells. A. Expression of miR-506 in huh7 and hepG2 cells. U6 served as internal control. Data represent as means ± SD of three independent experiments. ***P < 0.001 vs NC. B. MTT assay of huh7 and hepG2 cells. Cells were transfected with mimic or inhibitor of miR506 and MTT assay was performed as previously described. Data represents means ± SD of at least three independent experiments. *P < 0.05 vs NC. **P < 0.01 vs NC. C. Transwell assay for migration in huh7 and hepG2 cells. magnification, 40×. Data represents means ± SD of at least three independent experiments. *P < 0.05 vs NC. D. Transwell assay for invasion in huh7 and hepG2 cells. magnification, 40×. Data represents means ± SD of at least three independent experiments. *P < 0.05 vs NC. **P < 0.01 vs. NC.

Enforced expression of SPON1 alleviates effects of miR-506 on proliferation, migration and invasion

We investigated whether enforced expression of SPON1 affects the role of miR-506 in huh7 cells. pcDNA-SPON1 were co-transfected with miR-506 and proliferation, migration and invasion of huh7 cells were evaluated. pcDNA 3.1 empty vector was served as control. Enforced expression of SPON1 was confirmed by qRT-PCR (**Figure 5A**). Our data showed huh7 cells transfected with pcDNA-SPON1 and miR-506 grew much faster (**Figure 5B**) and presented more stronger migration (**Figure 5C**) and invasion (**Figure 5D**) ability than those transfected with empty vector and miR-506. These results indicate enforced expression of SPON1 alleviates effects of miR-506 on proliferation, migration and invasion.

Silencing SPON1 alleviates effects of miR-506 inhibitor on proliferation, migration and invasion

In order to further verify our finding that SPON1 mediates the role of miR-506 in HCC, we investigated if silencing SPON1 affects the effects of miR-506 inhibitor on proliferation, migration and invasion. Silencing SPON1 in huh7 cells using siRNA targeted SPON1 was confirmed by qRT-PCR (**Figure 6A**). Our data showed huh7 cells, in which expression of SPON1 and miR-506 were both suppressed, grew much slower (**Figure 6B**) and presented more weaker migration (**Figure 6C**) and invasion (**Figure 6D**) ability

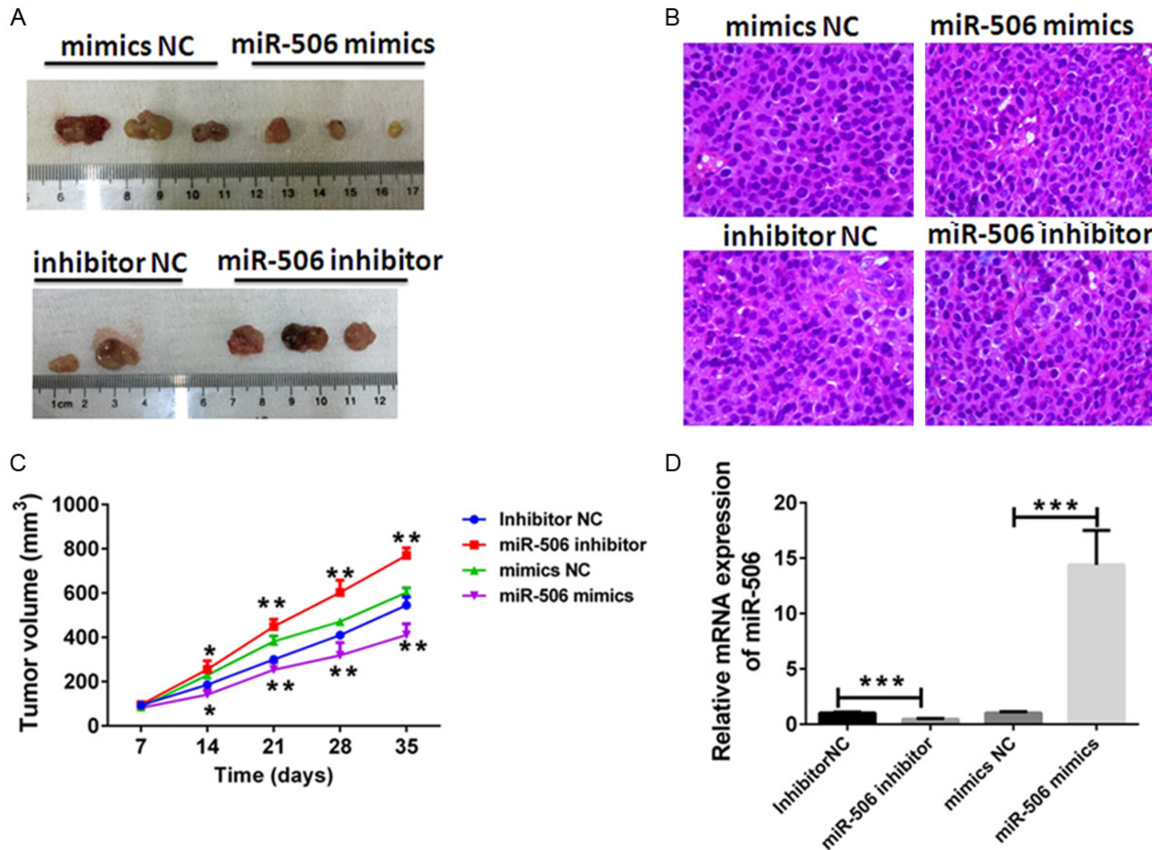


Figure 3. miR-506 inhibits tumor growth in nude mice. A. Representative pictures of xenograft tumors isolated from nude mice injected subcutaneously with huh7 cells which were transfected with mimic or inhibitor of miR506. B. HE staining of tumor tissues. magnification, 200×. C. Growth curve of tumors. *P < 0.05. **P < 0.01. D. Expression of miR-506 in tumor tissues. U6 served as internal control. Data represent as means ± SD of three independent experiments. ***P < 0.001.

than those cells that only expression of miR-506 was inhibited. These results indicates silencing SPON1 alleviates effects of miR-506 inhibitor on proliferation, migration and invasion, and supports our hypothesis that miR-506 regulates proliferation, migration and invasion of HCC cells through regulating SPON1.

Discussion

High mortality, high recurrence rate, and low cure rate make HCC one of the most lethal cancers in the world, especially in Asia [1-3, 16-18]. Dysregulation of microRNA is one of the most important mechanisms which contributes to HCC. Wen *et. al*. reported that miR-506 was downregulated in approximately 80% of the cervical cancer samples examined [8]. Yang *et. al*. reported that miR-506 was downregulated in clear cell renal cell carcinoma [10]. Our previous study using microchip identified miR-506 is downregulated in HCC (data not shown). In the current study, we reported expression of miR-506 in 50 HCC tissues were significantly lower than their non-cancerous counterparts (**Figure 1A**) by using qRT-PCR, which is consistent with previous report [19]. Moreover, we showed expression of miR-506 correlate with tumor size (**Figure 1D**), migration (**Figure 1E**), drinking history (**Figure 1F**) and clinical stage (**Figure 1G**), but not with sex (**Figure 1B**) and age (**Figure 1C**). These data indicates expression of miR-506 as a potential diagnostic marker or target for developing therapeutic strategies to treat HCC.

The function of miR-506 was mainly reported in cancer research. miR-506 was first reported in colon cancer and plays an important role in chemotherapeutic drug resistance [4]. miR-506 regulates epithelial mesenchymal transition in breast cancer [5] and gastric cancer [20]. Multiple research groups showed miR-506 inhibits proliferation, migration and inva-

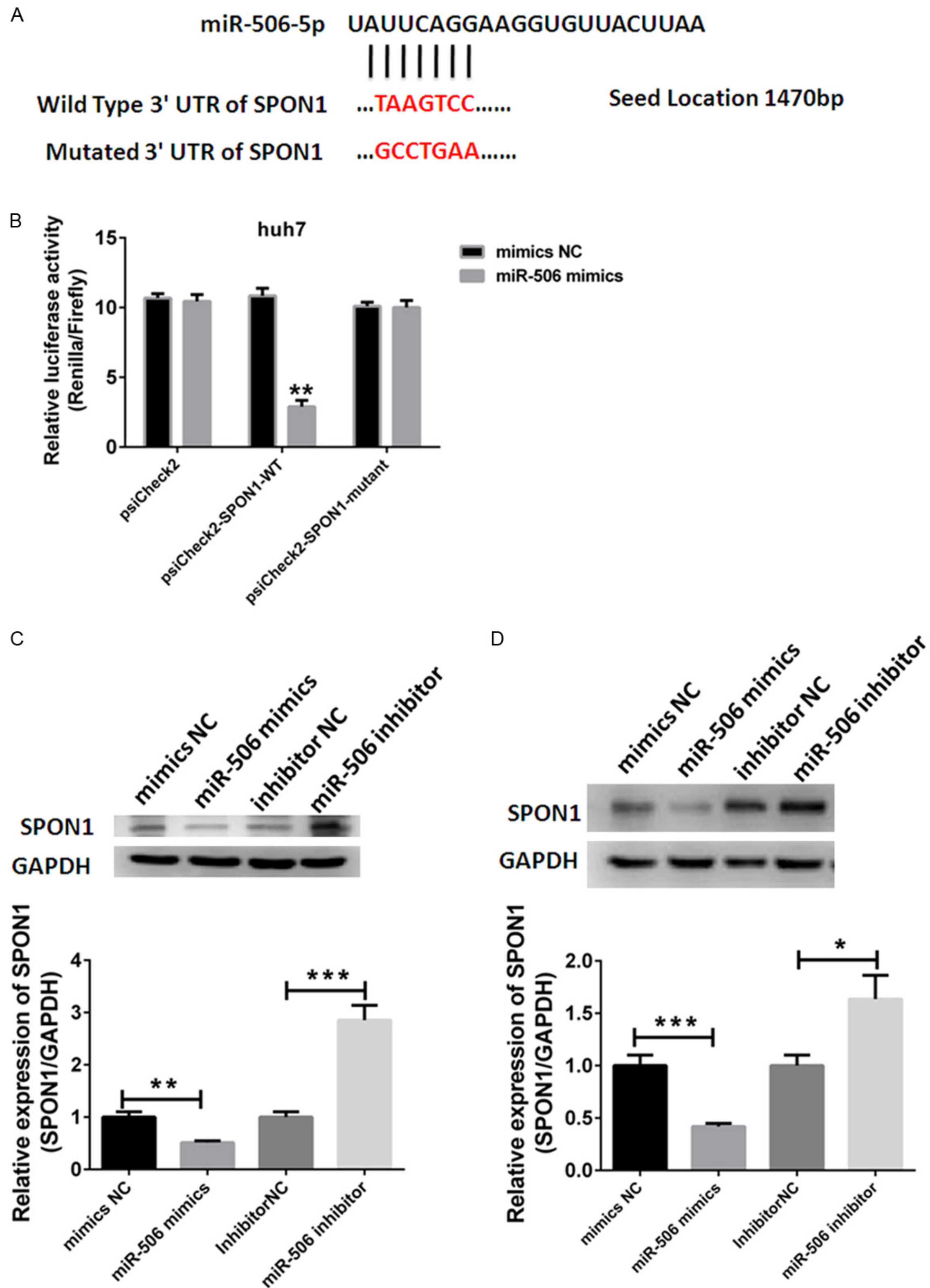


Figure 4. miR-506 negatively regulates SPON1 by binding to the SPON1 3'UTR. A. The putative miR-506 binding site in 3'UTR of SPON1 is indicated with red characters. B. Luciferase reporter assay of huh7 cells transfected with luciferase reporter construct containing wild type (psiCheck2-SPON1-WT) or mutated 3'UTR of SPON1 (psiCheck2-SPON1-mutant). The luciferase activity was normalized to Renilla luciferase activity. Data represents means ± SD of at least three independent experiments. **$P < 0.01$. C. Protein expression of SPON1 in huh7 cells. D. Protein expression of SPON1 in tumor tissues isolated from nude mice. *$P < 0.05$. **$P < 0.01$. ***$P < 0.001$.

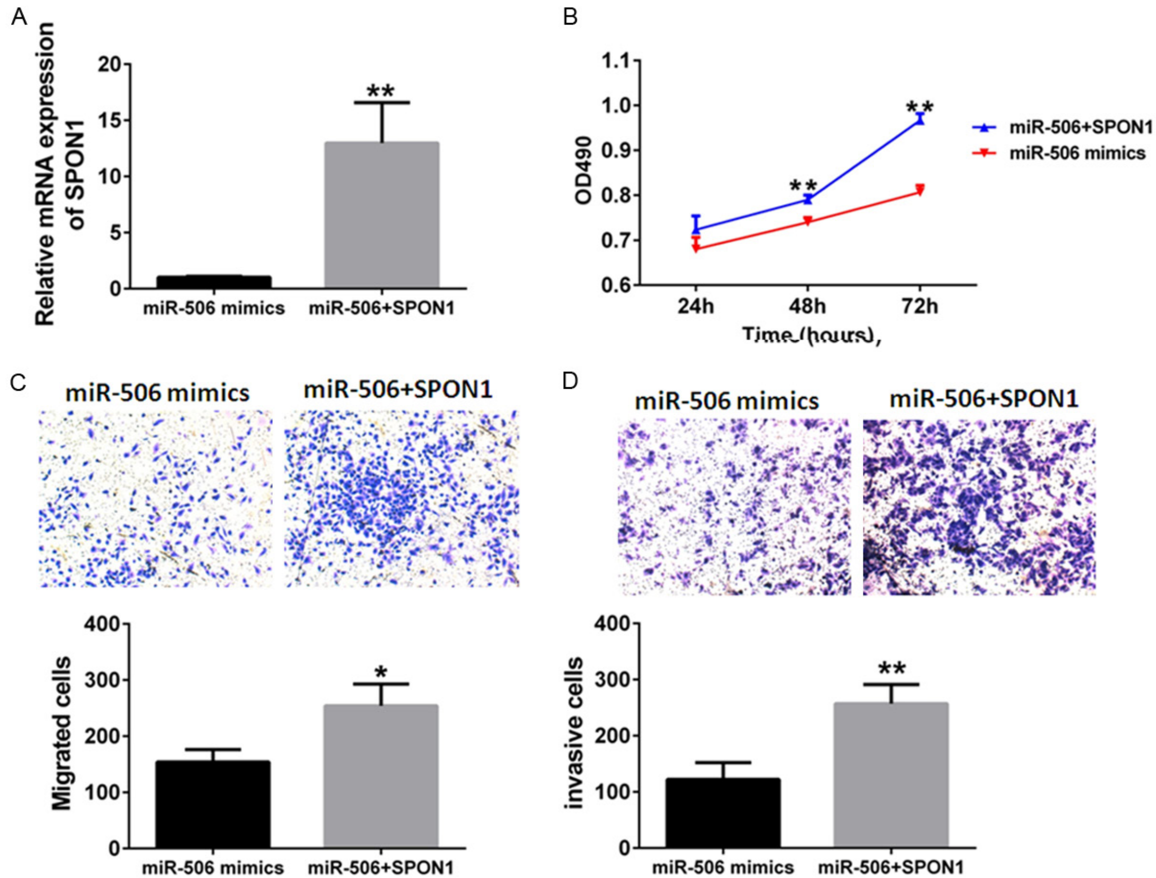


Figure 5. Enforced expression of SPON1 alleviates effects of miR-506 mimics on proliferation, migration and invasion. A. Expression of SPON1 in huh7 cells. U6 served as internal control. Data represent as means ± SD of three independent experiments. **P < 0.01 vs miR-506 mimics. B. MTT assay of huh7 cells. Data represents means ± SD of at least three independent experiments. *P < 0.05 vs miR-506 mimics. C. Transwell assay for migration in huh7 cells. original magnification, 40×. Data represents means ± SD of at least three independent experiments. **P < 0.01 vs miR-506 mimics. D. Transwell assay for invasion in huh7 cells. original magnification, 40×. Data represents means ± SD of at least three independent experiments. **P < 0.01 vs miR-506 mimics.

sion in various type of cancer [6-11, 19]. Our study showed miR-506 inhibits proliferation, migration and invasion in huh7 cells (**Figure 2**) and suppresses tumor growth in nude mice (**Figure 3**), which indicates miR-506 may act as a tumor suppressor in hepatocellular carcinoma, and reveals the therapeutic value of miR-506 in cancer therapy.

miRNAs play an important role in initiation and progression of cancer by targeting key regulator. The dominant mechanism is that miRNA binds to 3'UTR of target gene and leads to consequential degradation of mRNA, which results in decreased accumulation of target gene in cancer cells. miR-506 has been demonstrated to downregulate multiple genes through targeting their 3'UTR, including PPAR alpha [4], Cl(-)/HCO3- anion exchanger 2 (AE2) [21], CDK4 [9], CDK6 [9], ABCC4 [22], YAP [6, 19], CD151 [23], GATA6 [7], Gli3 [8], FLOT1 [10] and FOXQ1 [11]. In this study, our data showed miR-506 binds to 3'UTR of SPON1 (**Figure 4B**), an extracellular matrix protein, which involves in cell adhesion. Enforced expression of miR-506 caused decreased accumulation of SPON1 in HCC cells (**Figure 4C**) and tumors isolated from nude mice injected with HCC cells (**Figure 4D**). Moreover, enforced expression of SPON1 and silencing SPON1 alleviates effects of miR-506 mimics and inhibitor on proliferation, migration and invasion in vitro, respectively (**Figures 5** and **6**). These data indicates SPON1 is a direct target of miR-506.

In conclusion, our data demonstrated that miR-506 inhibits proliferation, migration and invasion in vitro and suppresses tumor growth in

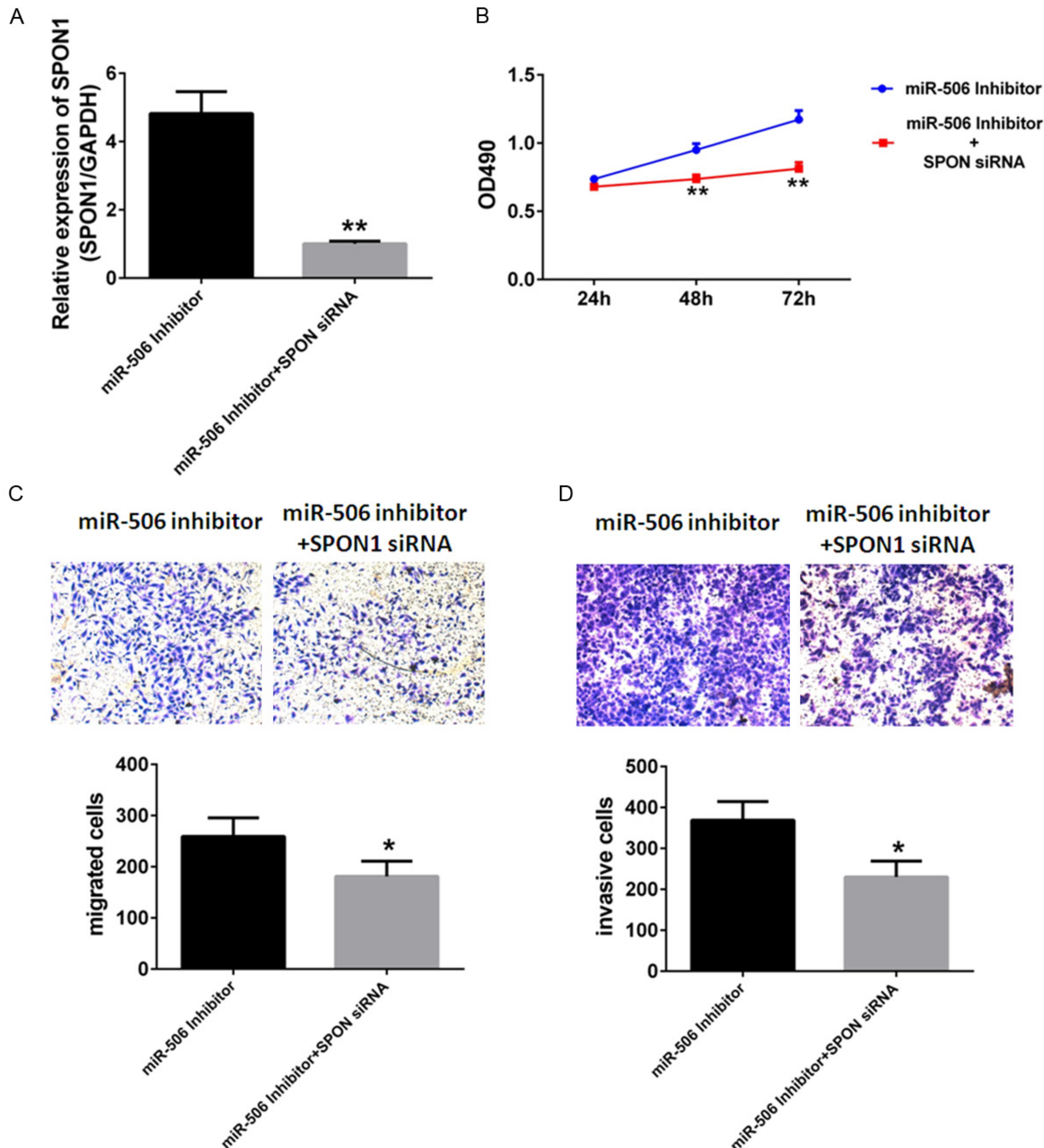


Figure 6. Silencing SPON1 alleviates effects of miR-506 inhibitors on proliferation, migration and invasion. A. Expression of SPON1 in huh7 cells. U6 served as internal control. Data represent as means ± SD of three independent experiments. **P < 0.01 vs miR-506 inhibitors. B. MTT assay of huh7 cells. Data represents means ± SD of at least three independent experiments. **P < 0.01 vs miR-506 inhibitors. C. Transwell assay for migration in huh7 cells. original magnification, 40×. Data represents means ± SD of at least three independent experiments. *P < 0.05 vs miR-506 inhibitors. D. Transwell assay for invasion in huh7 cells. original magnification, 40×. Data represents means ± SD of at least three independent experiments. *P < 0.05 vs miR-506 inhibitors.

vivo, and that SPON1 is a direct target of miR-506.

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Disclosure of conflict of interest

None.

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