

SPAG9 is involved in hepatocarcinoma cell migration and invasion via modulation of ELK I expression

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Background: Sperm-associated antigen 9 (SPAG9) is upregulated in several malignancies and its overexpression is positively correlated with cancer cell malignancies. However, the specific biological roles of *SPAG9* in hepatocellular carcinoma (HCC) are less understood.

Methods: We analyzed SPAG9 and ETS-like gene 1, tyrosine kinase (ELK1) expression in 50 paired HCC specimens and adjacent noncancerous liver specimens using immunohistochemistry. SPAG9 small interfering RNA (siRNA) was used to knockdown *SPAG9* expression in HCCLM3 and HuH7 cell lines. We used plasmids to upregulate *ELK1* expression and siRNA to downregulate *ELK1* expression in HuH7 cells. Quantitative real-time polymerase chain reaction and Western blot were used to evaluate the expression of *SPAG9* and *ELK1* at the mRNA and protein level, respectively. Wound healing, matrigel migration, and invasion analyses were performed to determine the effect of *SPAG9* and *ELK1* on HCC metastasis.

Results: SPAG9 and ELK1 were overexpressed in HCC tissue specimens and their expressions were higher in HCCLM3 and HuH7 cells compared to the low-metastatic HepG2 cells. Overexpression of SPAG9 was positively associated with tumor-node-metastasis staging ($P=0.032$), metastasis parameters ($P=0.018$) of HCC patients, and ELK1 expression ($r=0.422$, $P<0.001$) in HCC tissue specimens. In addition, knockdown of *SPAG9* in HCCLM3 and HuH7 cells using siRNA significantly suppressed cell migration and invasion. Furthermore, we observed inhibition of ELK1 expression and p38 signaling. However, *ELK1* overexpression reversed the inhibitory effects of *SPAG9* siRNA on HCC cell metastasis and ELK1 depletion inhibited HuH7 cell migration and invasion.

Conclusion: SPAG9 overexpression was positively correlated with HCC metastasis and SPAG9-induced migration and invasion were partially dependent on ELK1 expression in HCC cell lines. These results suggest that *SPAG9* may be a potential anti-metastasis target effective in HCC therapy.

Keywords: SPAG9, ELK1, hepatocellular carcinoma, metastasis

Introduction

Hepatocellular carcinoma (HCC) is the fifth most prevalent cancer and the third most common cause of cancer-related deaths worldwide.¹ There are several treatment options for HCC patients, including surgical resection, radiotherapy, chemoembolization, and liver transplantation. Despite the numerous treatments available, HCC prognosis remains poor because of the high incidence of recurrence and metastasis.² Therefore, more complete understanding of the mechanism involved in metastasis and the subsequent identification of novel therapeutic targets are necessary.

Cancer-testis (CT) antigens are a unique family of tumor antigens that have aberrant expression in various tumors and restricted expression in normal testis.³ CT antigens have been reported to play an important role in a number of malignant properties of cancer cells.^{4,5} Sperm-associated antigen 9 (SPAG9) protein, a new member of CT antigen family,



is highly expressed in 88% of breast cancer, 82% of cervical cancer, 74% of colorectal cancer, and 60% of astrocytoma.⁶⁻⁹ Its high expression is associated with lymph node metastasis in breast, prostate, and lung carcinomas, and *SPAG9* small interfering RNA (siRNA) treatment could reduce the cellular growth, migration, and invasion in these cancers.¹⁰⁻¹² Previous studies have shown that *SPAG9* is involved in c-Jun-NH2-kinase-signaling module and functions as a scaffolding protein for c-Jun-NH2-kinase binding and likely plays an important regulatory role in cell survival, proliferation, and tumor development.^{11,13,14} Recently, *SPAG9* was identified as a novel binding partner required for protein kinase C-related kinase 1 (PRK1)-mediated p38/ETS-like gene 1, tyrosine kinase (ELK1) activation in prostate cancer.¹⁵ Furthermore, p38 mitogen-activated protein kinase (MAPK) signaling activated transcription factors, including the ETS domain-containing protein ELK1,^{16,17} a modulator of tumor metastasis.¹⁸⁻²¹ However, the mechanism in which *SPAG9* promotes HCC metastasis by affecting cell migration and invasion remains unclear.

In this study, we showed that *SPAG9* silenced by siRNA suppressed HCC migration and invasion. In addition, our results suggest that these effects may have resulted from the modulation of *ELK1* and phosphorylated p38 expression.

Methods

Patients and specimens

Specimens of HCC tissues were obtained from 50 patients who underwent hepatic surgical resection without preoperative systemic chemotherapy at Shaoxing People's Hospital (Shaoxing, People's Republic of China). These specimens were collected from the biological specimen bank in Shaoxing People's Hospital after written informed consent was obtained from the patients. The Ethics Committee of Shaoxing People's Hospital approved this study. The main clinical and pathological information are as follows: 39 males and eleven females, aged 35–79 (50.5±9.3) years, 36 cases of hepatitis B surface antigen (HBsAg)-positive patients, 30 cases of liver cirrhosis, 31 cases with stages I–II, and 19 cases with stages III–IV. Clinical pathological information including histopathological diagnosis and tumor-node-metastasis (TNM) stage was extracted from medical records.

Immunohistochemistry staining

Immunostaining was performed using the avidin-biotin-peroxidase complex method (SP9001; Zhongshan Goldenbridge Biotechnology, Beijing, People's Republic of China). Antigen retrieval for *SPAG9* was conducted by heating the slides to 121°C for 90 seconds in a citrate buffer (pH 6.0) and, for *ELK1*, was conducted by heating the slides to 100°C

for 20 minutes in ethylene diamine tetracetic acid (pH 9.0) buffer. Next, endogenous hydrogen peroxidase was blocked by immersion in 3% hydrogen peroxide for 10 minutes. Goat serum was used to block nonspecific binding 37°C for 15 minutes. Then tissue sections were incubated with *SPAG9* antibody (1:1,000 dilution, ab12331; Abcam, Cambridge, UK) and *ELK1* antibody (1:50 dilution, ab32106; Abcam) at 4°C overnight. The next day, the sections were washed in phosphate-buffered saline and incubated with biotinylated anti-rabbit secondary antibody and horseradish peroxidase-labeled avidin chain enzyme working solution for 15 minutes at room temperature. After washing, the peroxidase reaction was developed with 3, 3'-diaminobenzidine tetrahydrochloride according to the manufacturer's instructions. Five randomly selected views were examined per slide and 100 cells were observed per view at 400× magnification. Expression score was determined by staining intensity and immunoreactive cell percentage. *SPAG9* cytoplasm staining and *ELK1* nuclear staining or cytoplasm staining without staining intensity were rated as 0, with weak staining in ≤25% as 1, with moderate or strong staining in 25%–50% as 2, strong staining in 50%–75% as 3, and strong staining in 75%–100% as 4. Tumor specimens were obtained by multiplying each score <4 into negative or low expression and ≥4 into positive or highly expressed.

Cell culture

Human HuH7 and HepG2 cell lines were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). The human HCC cell line HCCLM3 was provided by the Liver Cancer Institute, Fudan University (Shanghai, People's Republic of China). The cells were maintained in Dulbecco's Modified Eagle's Medium (SH30243.01B; Hyclone, Logan City, UT, USA) with 10% fetal bovine serum (10099-141-FBS; Life Technologies, Carlsbad, CA, USA), 100 U/mL penicillin, and 100 U/mL streptomycin (15140-122; Life Technologies) at 37°C in a humidified atmosphere containing 5% CO₂. Cells were grown on sterilized culture dishes and were passed every 3 days with 0.25% trypsin (Hyclone, SH30042.01, MD, USA).

Transfection and small interfering RNA treatment

SPAG9-specific siRNA targeting 5'-GCAATGACTCAGATGCATA-3', *ELK1*-specific siRNA targeting 5'-CACATCCCTTCTATCAGCGTGGATG-3', and control scrambled siRNA were chemically synthesized from RIBO-BIO (1451493202, S151113152330; Guangzhou, People's Republic of China). *ELK1* plasmid was purchased from Shanghai Genechem Co. (POSE144045095; Genechem,

Shanghai, People's Republic of China). Human HCC cells were seeded in six-well plates at a concentration of 3×10^5 per well and grown overnight, and then the cells were transfected with *SPAG9* or *ELK1* siRNA at a final concentration of 50 nM or 4 μ g of *ELK1* plasmids using lipofectamine 2000 reagent (11668-019; Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The *SPAG9*-specific siRNA sequence was 5'-GCAAUGACUCAGAUGCAUA-3' and 5'-UAUGCAUCAUCUGAGUCAUUGC-3'. The *ELK1*-specific siRNA sequence was 5'-CACAUCCCUU CUAUCAGCGUGGAUG-3' and 5'-CAUCCACGCUGA UAGAAGGGAUGUG-3'.

Wound-healing assay

Cells transfected with 50 nM *SPAG9* siRNA or control siRNA were seeded at a density of 1×10^6 per well into six-well plates. At 95% confluency, an artificial wound was created using 10 μ L pipette tip. After wounding, the medium was changed to fresh serum-free medium to remove cellular debris. Photomicrographs were taken at 0, 24, and 48 hours. The distances cells migrated into the wounded area were calculated by subtracting the distance 24 hours after wound healing from the initial distance.

Transwell migration and invasion assay

The transwell assay was performed as previously described.^{10,12} For migration assay, HuH7 and HCCLM3 cells transfected with *SPAG9* siRNA or control siRNA were plated at 1×10^5 cells/well in serum-free Dulbecco's Modified Eagle's Medium in the upper chamber of a 24-well Boyden chamber with a pore size of 8 μ m polycarbonate membrane filters (3422; Costar, Cambridge, UK). The invasion assay was performed in the same manner as the migration assay, except that the filter was precoated with 50 μ L matrigel (1:5 dilution, 356234; BD Bioscience, San Jose, CA, USA). Medium containing 10% fetal bovine serum in the lower chamber served as the chemoattractant. After incubation, the cells were fixed with methanol and stained with 0.05% Giemsa. Those on the upper surface of the filter were removed with a cotton swab. The filters were then rinsed in distilled water until no additional stain leached. The cells were then air dried for 20 minutes. The number of cells was counted within a field at 200 \times under a light microscope. For each membrane, a total of four fields were selected at random and the numbers were averaged.

Quantitative reverse transcription-polymerase chain reaction

Total RNA was extracted from HCC cells with TRIzol[®] (15596026; Thermo Fisher Scientific, Waltham, MA, USA)

and reverse transcribed using the first-strand cDNA synthesis kit (RT0212-03; Biomiga, San Diego, USA) according to the manufacturer's protocol. Quantitative reverse transcription-polymerase chain reaction (RT-PCR) was performed with the Light-cycler 480 PCR apparatus (Hoffman-La Roche Ltd, Basel, Switzerland) using SYBR Green PCR master mix (DRR014A; Takara Biotechnology, Dalian, People's Republic of China). PCR reactions were performed under the following conditions: 95°C for 30 seconds, 40 cycles of 95°C for 5 seconds, and 60°C for 30 seconds. The relative levels of gene expression were calculated by the $-\Delta\Delta C_t$ method using β -actin as a control and expressed as $2^{-\Delta\Delta C_t}$. PCR primers were used as follows: *SPAG9*: forward, 5'-TCCACTCATCTGTAGCCCAGT-3' and reverse, 5'-CCCATTGTCCATCAACTCCT-3'; *ELK1*: forward, 5'-AACTTTCAGGAGACCCGTCC-3' and reverse, 5'-CCATCCCAAGGGGTACTGTTA-3'; β -actin: forward, 5'-ACCCACACTGTGCCCATCTAC-3' and reverse, 5'-TCGGTGAGGATCTCATGAGGTA-3'.

Western blot analysis

Total proteins from cell lines were extracted in lysis buffer (P0013B; Beyotime, Jiangsu, People's Republic of China) with inhibitors of proteases and phosphatases (P1260; Solarbio, Beijing, People's Republic of China) and quantified using the Bradford method. Thirty five micrograms of protein were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (10%) (P1200; Solarbio). After transferring, the polyvinylidene fluoride membranes (IPVH00010; Millipore, Billerica, MA, USA) were incubated overnight at 4°C with the following antibodies: *SPAG9* (1:3,000, ab12331, Abcam), *ELK1* (1:500, ab32106, Abcam), p-p38 (1:1,000, ab4822, Abcam), and β -actin (1:5,000, ab198991, Abcam). After five washes with Tris-buffered saline with Tween, blots were probed with secondary antibodies conjugated with horseradish peroxidase (1:5,000, ab97051, Abcam) and were visualized using enhanced chemiluminescence (ECL)-plus (32132; Thermo Fisher Scientific). Finally, blots were exposed to a radiographic film.

Statistical analysis

All data were expressed as mean \pm standard deviation, and SPSS 17.0 software was used for statistical analyses. One-way analysis of variance and Student's *t*-test or Mann-Whitney test were used to analyze the significance between groups. The correlation between *SPAG9* expression and *ELK1* expression in HCC tissues was analyzed using Spearman rank test. A *P*-value less than 0.05 was considered statistically significant.

Results

Expression of SPAG9 is elevated in human HCC and associated with ELK

The expressions of SPAG9 and ELK1 were evaluated in 50 pairs of HCC and corresponding noncancerous liver specimens using immunohistochemical staining. As shown in Figure 1A, SPAG9 was localized mainly within the cytoplasmic compartment, while ELK1 was distributed throughout the whole cell, and was especially in the nucleus. Higher expression of SPAG9 and ELK1 was detected in 30/50 (60%) and 21/50 (42%) HCC tissues compared to the corresponding noncancerous tissues, respectively.

Next, we investigated the mRNA and protein expression of *SPAG9* and *ELK1* in HCC cell lines using Western blot and qRT-PCR. Our results indicated that *SPAG9* and *ELK1* mRNA and protein levels were higher in both HCCLM3 and HuH7 cells than HepG2 cells, a non-metastatic cell line (Figure 1B and C).

Immunohistochemical staining scores indicated that the increased expressions of SPAG9 and ELK1 observed in HCC were statistically significant compared to noncancerous tissues ($P < 0.001$, Figure 1D). Spearman rank analysis confirmed that ELK1 expression was positively associated with SPAG9 expression in HCC tissues (Figure 1E). As shown in Table 1,

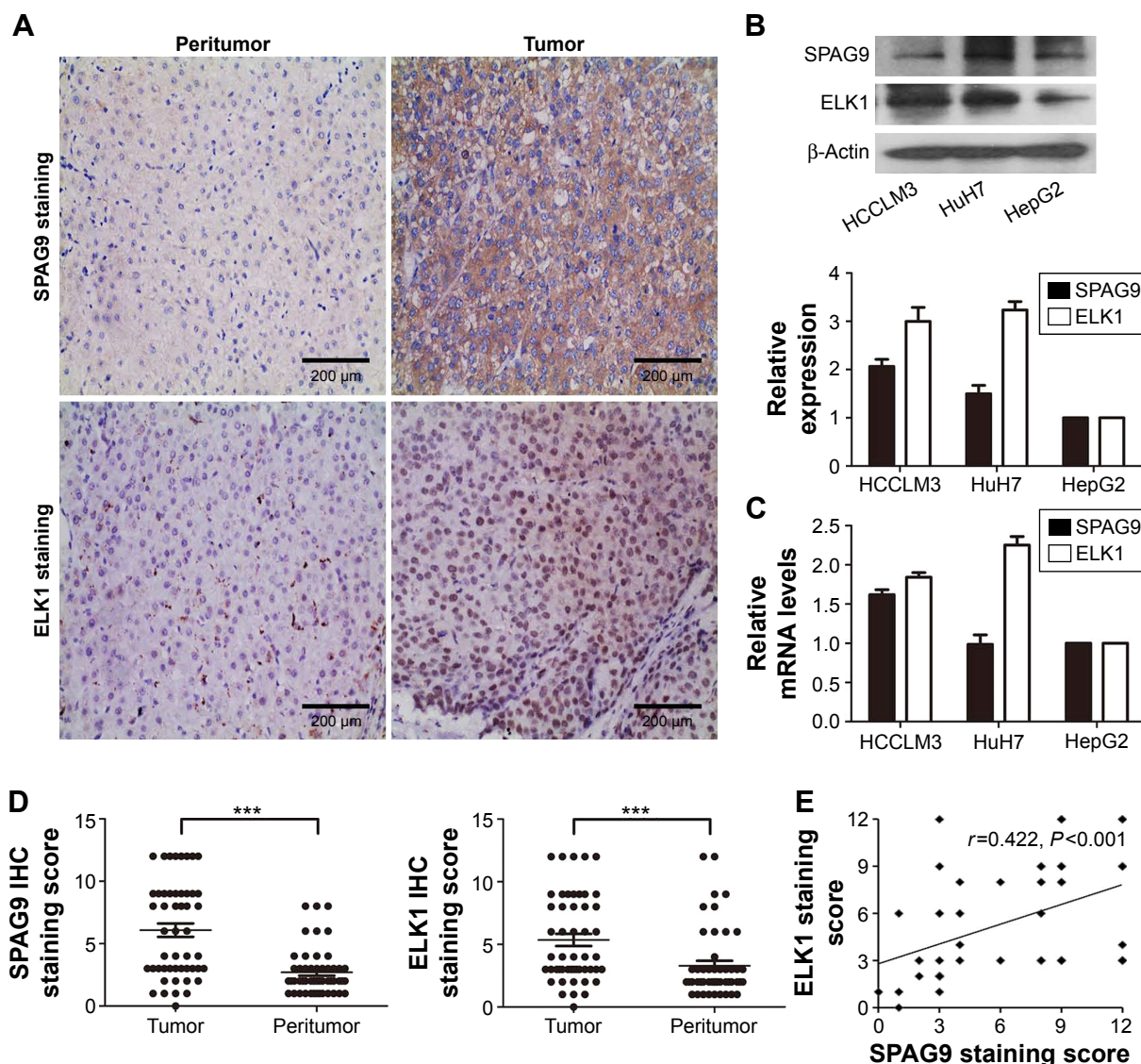


Figure 1 SPAG9 and ELK1 expression in HCC tissue specimens and cell lines.

Notes: (A) IHC staining of SPAG9 and ELK1 in hepatocarcinomas and adjacent noncancerous liver tissues. Weak SPAG9 and ELK1 staining can be observed in the adjacent noncancerous liver tissues (left panel) compared to the strong SPAG9 cytoplasmic staining and ELK1 nuclear staining in cancerous tissues (right panel) (magnification: 200×). (B) Protein and (C) mRNA levels of *SPAG9* and *ELK1* were examined in HCCLM3, HuH7, and HepG2 cell lines using Western blot and qRT-PCR analyses, respectively. (D) Mann-Whitney *U*-testing of IHC scores ($***P < 0.001$) (the bars show standard error), and (E) Spearman rank correlation analysis of SPAG9 and ELK1 expression in HCC tissues.

Abbreviations: SPAG9, sperm-associated antigen 9; IHC, immunohistochemistry; qRT-PCR, quantitative real-time polymerase chain reaction; ELK1, ETS-like gene 1, tyrosine kinase; HCC, hepatocellular carcinoma.

Table 1 The relationship between SPAG9 expression and the clinical features of HCC

Parameters	Total	SPAG9 expression		P-value
		Positive n (%)	Negative n (%)	
Age (year)				
≤50	20	13 (65.0)	7 (35.0)	0.769
>50	30	17 (56.7)	13 (43.3)	
Sex				
Male	39	24 (61.5)	15 (38.5)	0.736
Female	11	6 (54.5)	5 (45.5)	
HBsAg				
Presence	36	22 (61.1)	14 (38.9)	0.283
Absence	14	8 (57.1)	6 (42.9)	
Cirrhosis				
Presence	30	21 (70.0)	9 (30.0)	0.089
Absence	20	9 (45.0)	11 (55.0)	
TNM stage				
I+II	31	15 (48.4)	16 (51.6)	0.032*
III+IV	19	15 (78.9)	4 (21.1)	
Relapse or metastasis				
Presence	30	22 (73.3)	8 (26.7)	0.018*
Absence	20	8 (40.0)	12 (60.0)	
ELK1 status				
Positive	21	18 (85.7)	3 (14.3)	0.002*
Negative	29	12 (41.4)	17 (58.6)	

Note: * $P < 0.05$.

Abbreviations: SPAG9, sperm-associated antigen 9; HCC, hepatocellular carcinoma; HBsAg, hepatitis B surface antigen; TNM, tumor node metastasis; ELK1, ETS-like gene 1, tyrosine kinase.

SPAG9 overexpression was positively correlated with TNM staging, metastasis, and relapse parameters of HCC patients ($\chi^2=4.585$, $P=0.032$; $\chi^2=5.556$, $P=0.018$). These results suggest that SPAG9 expression is positively associated with ELK1 and highly correlated to HCC metastasis.

Knockdown of SPAG9 expression suppresses HCC cell migration and invasion

To further explore the role of SPAG9 in HCC metastasis, the migration and invasion activity of HCCLM3 and HuH7 cells was analyzed using a wound-healing and transwell assays, respectively. Interference of SPAG9 expression was achieved by transfecting cells with SPAG9-specific siRNA (Figure 2A and B). The results from the wound-healing assay showed that the migratory potential of SPAG9-silenced HCCLM3 and HuH7 cells was significantly reduced compared to scramble RNA-transfected cells (Figure 2C). The results from the transwell migration assay showed that SPAG9 siRNA-transfected cells had a significant reduction in migration compared to HCCLM3 and HuH7 control cells (116 ± 15 vs 80 ± 9 ; 326 ± 30 vs 141 ± 20 cells per fields, $P < 0.05$) (Figure 2D). Matrigel invasion assays also showed that

SPAG9 knockdown inhibited cell invasion in HCCLM3 and HuH7 cells compared to the controls (49 ± 9 vs 88 ± 12 ; 81 ± 10 vs 205 ± 15 , $P < 0.05$) (Figure 2D). These results demonstrate that SPAG9 is involved in the migration and invasion of HCCLM3 and HuH7 cells.

SPAG9 knockdown decreases ELK1 expression and inhibits p38 activation

Given that ELK1 expression was positively associated with SPAG9 expression in HCC tissues and cell lines, we investigated whether SPAG9 was able to modulate ELK1 expression. As depicted in Figure 3A and B, siRNA-mediated silencing of SPAG9 significantly decreased ELK1 expression at both the protein and mRNA level compared to cells transfected with scrambled siRNA. Furthermore, we examined the effect of SPAG9 silencing on p38 activation in HCC cells. We observed a strong reduction in phosphorylated p38 following SPAG9 knockdown (Figure 3C). These results suggest that SPAG9 may modulate ELK1 expression and p38 activation.

SPAG9-induced migration and invasion of HCC cells are dependent on ELK1 expression and ELK1 knockdown suppresses HCC cell migration and invasion

To further assess the functional relationship between ELK1 and SPAG9, we transfected SPAG9-silenced HuH7 cells with an ELK1 plasmid to upregulate ELK1 expression in these HuH7 cells. We observed that ELK1 overexpression attenuated the inhibitory effects of SPAG9 siRNA on cell migration and invasion ($P < 0.05$) (Figure 4A and 4B). Furthermore, the migration and invasion activity of HuH7 cells was inhibited by transfection with ELK1 siRNA ($P < 0.05$) (Figure 4C). As shown in Figure 4D, the protein level of ELK1 was significantly decreased 48 hours after siRNA transfection and the SPAG9 expression was not changed. These results suggest that SPAG9-induced migration and invasion of HCC cells are dependent on ELK1 expression, and ELK1 knockdown suppresses HCC cells migration and invasion.

Discussion

Given that metastasis plays an important role in recurrence and poor prognosis of HCC, it is necessary to identify novel therapeutic strategies for treatment of metastasis. Initially, it was reported that SPAG9 was involved in sperm-egg interactions.²² Recently, in vitro and in vivo studies have shown that siRNA-mediated knockdown of SPAG9 reduces tumor growth, as well as invasion and migration of various

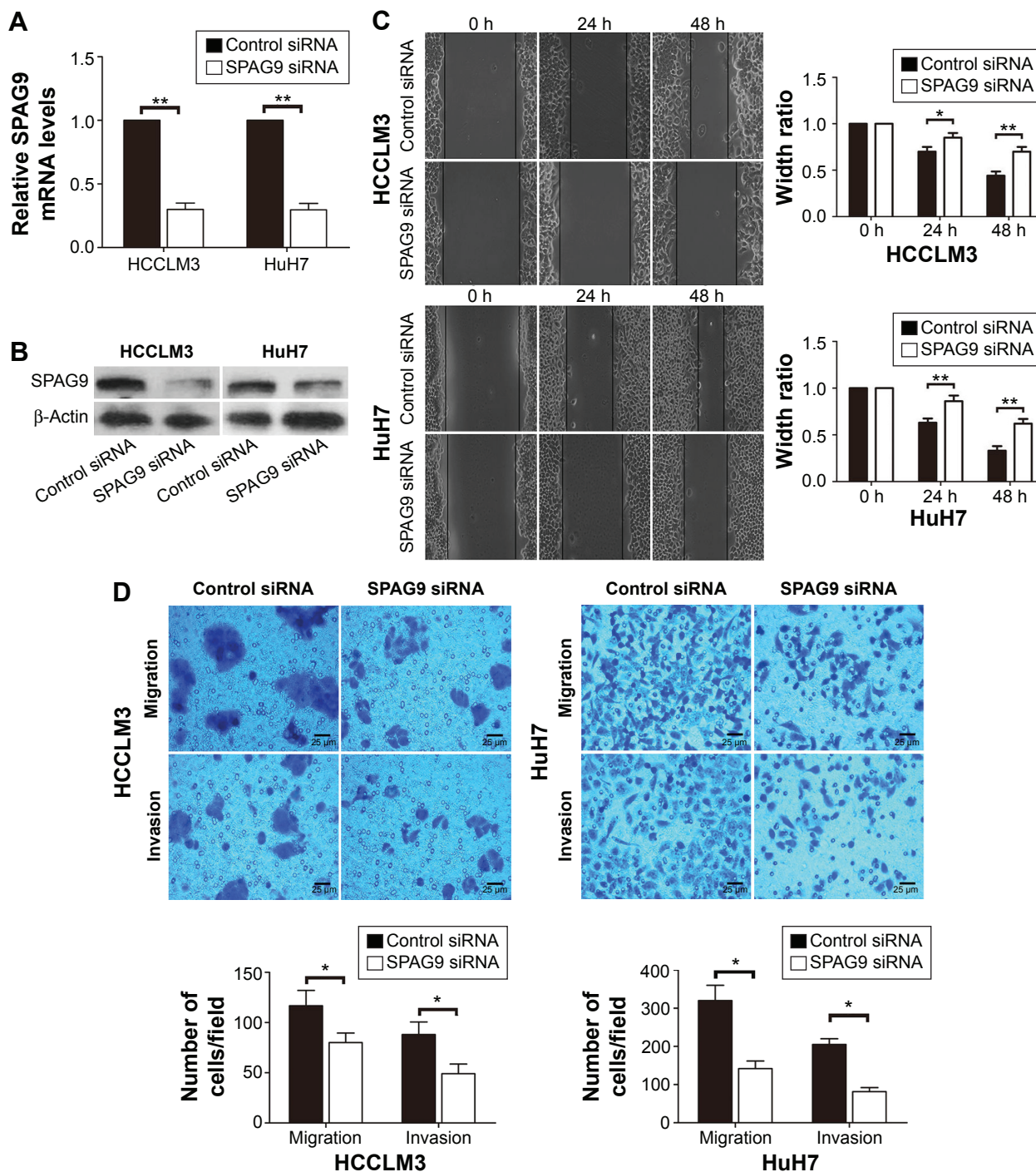
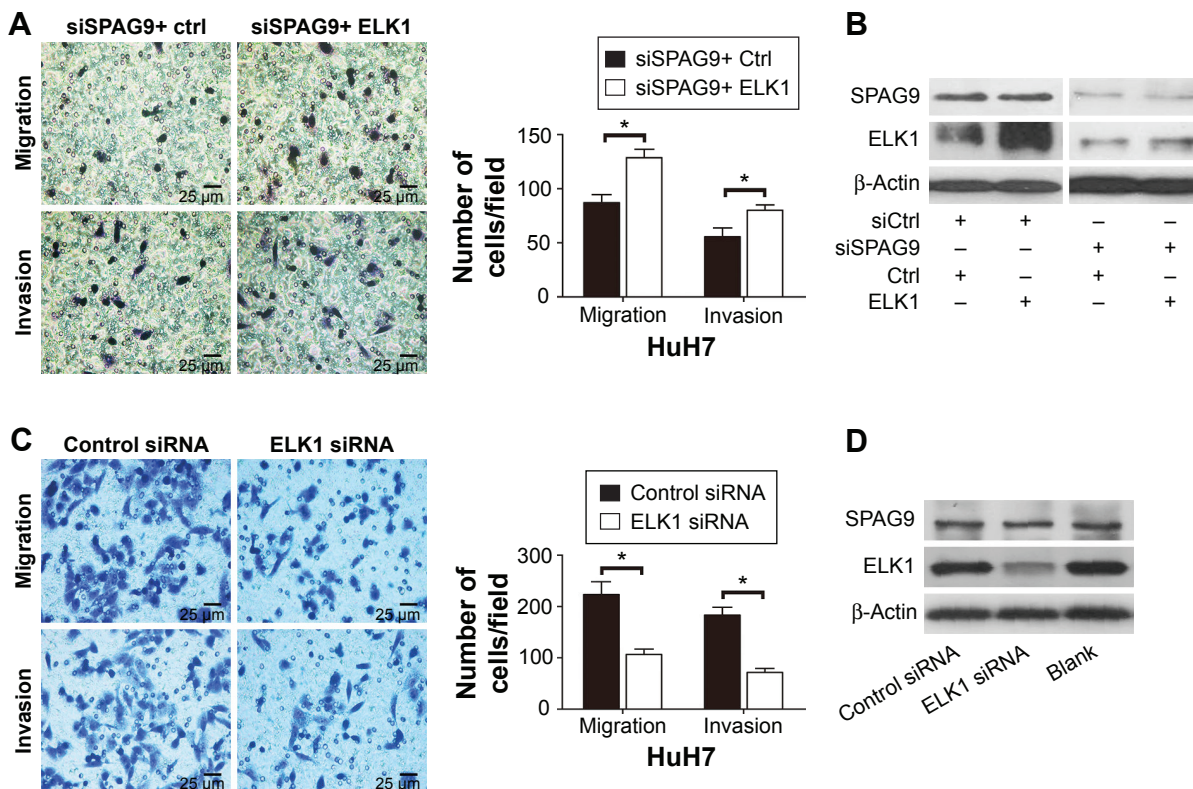
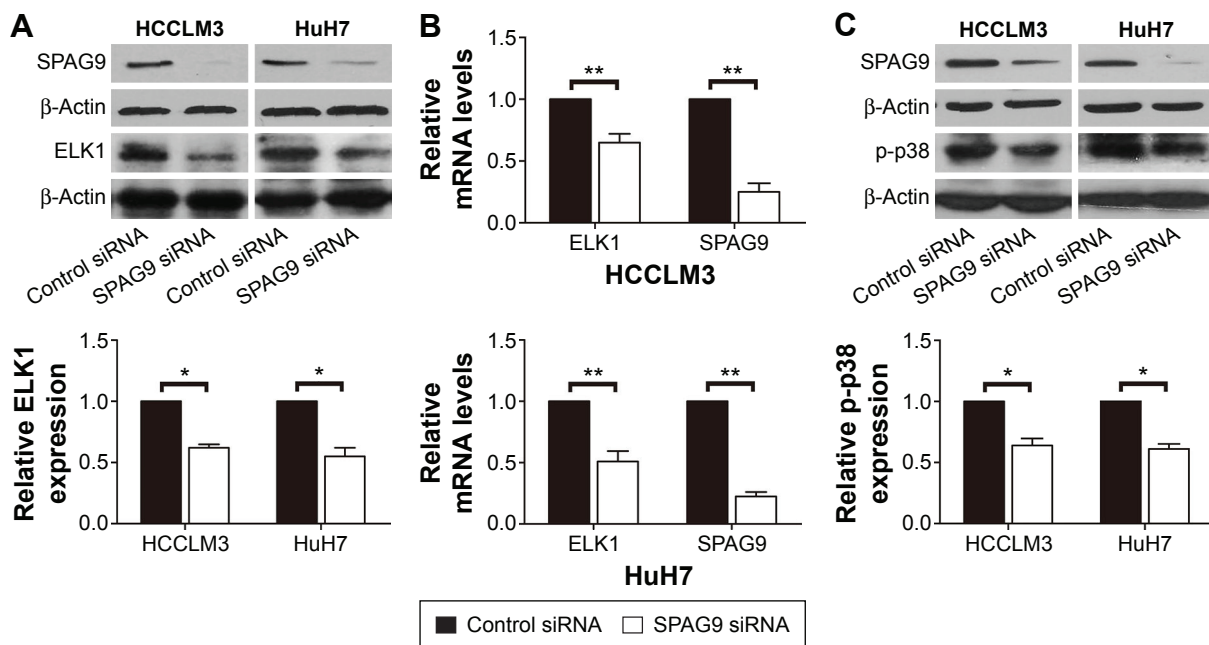


Figure 2 Reduction in SPAG9 expression suppresses cell migration and invasion of HCC cell lines. **Notes:** (A and B) SPAG9 siRNA-treated cells had decreased mRNA (A) and protein (B) levels compared to the control cells. (C) Results from the wound-healing assay showed that SPAG9 depletion decreased cell migration of HCCLM3 and HuH7 cells. (D) Results from the transwell migration and matrigel invasion assay showed that SPAG9 depletion decreased cell migration and invasion of HCCLM3 and HuH7 cells; * $P < 0.05$, ** $P < 0.01$. **Abbreviations:** SPAG9, sperm-associated antigen 9; siRNA, small interfering RNA; HCC, hepatocellular carcinoma; h, hour.

cancers.^{7,10,15,23} Therefore, SPAG9 appears to be a potential therapeutic target for HCC metastasis.

In this study, we found that SPAG9 was overexpressed in 60% (30/50) of human HCC tissues and was correlated with TNM staging. Moreover, SPAG9 expression was associated with relapse and metastasis in HCC patients (Table 1).

Interference of SPAG9 reduced migration and invasion of HCC cell lines (HCCLM3 and HuH7) indicating that SPAG9 is involved in the metastasis of HCC. Metastasis is a multistep process, including epithelial mesenchymal transition, mesenchymal epithelial transition, migration, and invasion.²⁴ Matrix metalloproteinase-9 (MMP9) was



reported to be downregulated after *SPAG9* knockdown, indicating that *SPAG9* may regulate invasion through MMP9 in astrocytomas and lung cancers.^{9,11} Furthermore, studies have reported that MMP2, MMP9, and vascular endothelial growth factor molecules participate in *SPAG9*-mediated migration and invasion of prostate cancer cells.¹² However, the molecular pathways involved in *SPAG9*-mediated metastasis in HCC remain unknown.

The ETS family proteins affect the expression of several oncogenes and tumor suppressor genes directly by regulating their promoters or indirectly through protein–protein interactions.²⁵ One such member, *ELK1*, has been reported to control breast cancer migration by regulating a set of target genes “unique” to *ELK1*.¹⁸ In addition, *ELK1* activation is involved in bombesin-induced prostate cancer cell growth.²⁶ Loss of *ELK1* expression inhibited the expression of protein kinase C alpha and decreased the cell migration and invasion ability of HCC.²⁰ In this study, we found *ELK1* was overexpressed in 42% (21/50) of HCC tissues specimens. In addition, *SPAG9* knockdown reduced *ELK1* expression, while *ELK1* overexpression reversed the inhibitory effects of *SPAG9* knockdown on cell migration and invasion, and *ELK1* depletion hindered the metastasis ability of HuH7 cells. This result indicated that *SPAG9*-enhanced migration and invasion may depend on *ELK1* expression. Experimental evidence demonstrated that the p38 MAPK pathway is involved in metastasis of tumor cells.²⁷ *SPAG9* acts as a scaffolding protein, which can activate p38 signaling.^{28,29} It promotes the invasion of gastric and prostate cancer through the upregulation of p38.^{15,30} In this study, we observed a reduction in p38 after *SPAG9* interference. Given that *ELK1* was one of the target genes of p38 signaling,^{17,31} we speculated that *SPAG9*-induced migration and invasion may be partly modulated by p38/*ELK1* signaling in HCC.

Conclusion

Our study showed that *SPAG9* and *ELK1* were upregulated in HCC tissue samples and that *SPAG9* overexpression was positively correlated with HCC metastasis. Furthermore, *SPAG9*-induced migration and invasion were dependent on *ELK1* expression, suggesting that *SPAG9* may be a potential anti-metastasis target for HCC therapy.

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Disclosure

The authors report no conflicts of interest in this work.

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