

# Expression and roles of fatty acid synthase in hepatocellular carcinoma

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**Abstract.** Cell metabolism abnormalities are closely related to tumor occurrence and development. Fatty acid synthase (FASN) is the key molecule for catalyzing fatty acid synthesis. Increasing evidence indicates that FASN is highly expressed in a number of malignant tumors; it can promote the synthesis of endogenous fatty acids in tumor cells and then the synthesized fatty acids provide energy for the proliferation of tumor cells. However, there has been no systematic study focusing on FASN expression and function in hepatocellular carcinoma (HCC). The aim of the present study was to verify the high expression of FASN in HCC cells at the histological and cellular levels, and to construct FASN shRNA eukaryotic expression vector for interfering FASN expression in HCC cell line SK-Hep-1, in an effort to explore the role of FASN in the proliferation, apoptosis, invasion and migration of HCC cells. In the present study, we demonstrated that FASN was highly expressed in HCC tissues compared with tumor-adjacent tissue and normal liver cell line 7702 ( $P<0.05$ ). FASN expression in the high metastatic MHCC97H and SK-Hep-1 cell lines was increased compared with low metastatic HCC cell lines ( $P<0.05$ ). Then, we constructed a FASN shRNA eukaryotic expression vector; after HCC SK-Hep-1 cells were transfected, the cell proliferation, migration and invasion were inhibited, but FASN had no impact on the apoptosis of HCC cells. Collectively, these data indicate that FASN is possibly involved in the occurrence and metastasis of HCC. Thus, inhibition of FASN may be a promising approach for the treatment of HCC.

## Introduction

Fatty acid synthase (FASN) is a key enzyme for catalyzing endogenous fatty acid synthesis within the cells; the synthesized fatty acids are involved in the synthesis of cell constituent structure, such as cell biofilm. In the early 1990s, it was first discovered that FASN was highly expressed in breast cancer, and its expression level was closely related to tumor stage and prognosis. Evidence from subsequent studies has generally supported this finding and has highlighted the contribution of FASN in tumor occurrence and development in esophageal, lung and gastric cancer, malignant melanoma, ovarian, prostate and nasopharyngeal cancer (1-4). To date, there has been no systematic study focusing on FASN expression and function in hepatocellular carcinoma (HCC). The aim of the present study was to verify the high expression of FASN in HCC cells at the histological and cellular levels, and to construct FASN shRNA eukaryotic expression vector for interfering FASN expression in HCC cell line SK-Hep-1, in an effort to explore the role of FASN in the proliferation, apoptosis, invasion and migration of HCC cells.

## Materials and methods

**Cell line and culture.** Human normal liver cell line 7702 and HCC cell lines HepG2, SMMC7721, MHCC97L, MHCC97H and SK-Hep-1 were provided by the Stem Cell Bank, Chinese Academy of Sciences (Shanghai, China). All the cells were cultured with DMEM containing 10% fetal bovine serum in a 5% CO<sub>2</sub> incubator at 37°C. FASN mouse anti-human monoclonal antibody was purchased from Santa Cruz (USA). HRP-conjugated goat anti-mouse secondary antibody, BCA protein assay kit and SABC immunohistochemistry kit were purchased from Wuhan Boster Biological Engineering Co., Ltd. (Wuhan, Hubei, China). BLOCK-iT™ HiPerform™ Lentiviral Pol II miR RNAi Expression System with EmGFP and Lipofectamine 2000 (Invitrogen, USA) were used for the construction of vector. The apoptosis kit was purchased from Jingmei Biotech Co., Ltd. (Shenzhen, Guangdong, China).

**Harvesting HCC tissue specimens.** HCC tissue and tumor-adjacent tissue specimens were harvested from 20 patients with primary HCC undergoing resection of HCC in the First

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**Key words:** fatty acid synthase, hepatocellular carcinoma, proliferation, invasion, RNA interference

Affiliated Hospital of Xi'an Jiaotong University, China, in 2012. No patients received chemotherapy or radiotherapy prior to the surgery. HCC diagnosis was performed by imaging and histopathologic examination. There were 13 males and 7 females. All participating patients signed the informed consent and the study was approved by the Ethics Committee of the First Affiliated Hospital of Xi'an Jiaotong University, College of Medicine. The obtained tissue specimens were stored at  $-80^{\circ}\text{C}$ .

**Immunohistochemistry.** Paraffin sections were dewaxed in xylene, rehydrated in gradient alcohol and retrieved with citric acid at high temperature and high pressure. Then, the sections were immersed in 3% hydrogen peroxide for 10 min to eliminate endogenous peroxidase, and blocked with serum for 30 min. The sections were incubated with FASN antibody (1:250) at  $4^{\circ}\text{C}$  overnight, rinsed with phosphate-buffered saline (PBS) three times, and incubated with secondary antibody at  $28^{\circ}\text{C}$  for 30 min, and rinsed with PBS again. The sections were visualized with freshly prepared DAB and counterstained with hematoxylin for 1 min, followed by tap water washes, dehydration and mounting. The sections were observed with the microscope.

**Real-time quantitative PCR detection of FASN expression in different HCC cell lines.** FASN and GAPDH primers were synthesized with the SYBR-Green fluorescent method. The sequences of FASN upstream and downstream primers were: AAG GAC CTG TCT AGG TTT GAT GC and TGG CTT CAT AGG TGA CTT CCA. The sequences of GAPDH upstream and downstream primers were: TGT GGG CAT CAA TGG ATT TGG and ACA CCA TGT ATT CCG GGT CAA T. PCR conditions were:  $95^{\circ}\text{C}$  (30 sec); 35 cycles of  $94^{\circ}\text{C}$  (30 sec),  $58^{\circ}\text{C}$  (30 sec),  $72^{\circ}\text{C}$  (50 sec);  $58^{\circ}\text{C}$  (5 min),  $55^{\circ}\text{C}$  (30 sec) and  $95^{\circ}\text{C}$  (30 sec). Each sample was detected three times. The results of real-time quantitative PCR were analyzed with the  $\Delta\Delta\text{Ct}$  method according to the formula:  $\Delta\text{Ct} = \text{FASN gene Ct} - \text{GAPDH gene Ct}$ . The relative expression level of FASN was calculated using the  $2^{-\Delta\Delta\text{Ct}}$  method.

**Western blot analysis of FASN expression in different HCC cell lines.** After cells at logarithmic phase were cultured in fresh culture medium 12 h before the detection, total cell protein was extracted and quantitated with the BCA method. The proteins were boiled with 5X the sample buffer for 5 min, and added to each well at  $45\text{ }\mu\text{g}/\text{hole}$  for electrophoresis. Then, the proteins were transferred to a PVDF membrane and blocked with 5% skim milk for 1 h at room temperature, incubated with anti-FASN antibody (1:1,000) at  $4^{\circ}\text{C}$  overnight and secondary antibodies at room temperature for 1.5 h. The images were collected and analyzed with ECL system.

**Construction of FASN shRNA eukaryotic expression vector.** First, single-stranded sense and antisense oligo DNA was synthesized, and the sequences were: TGC TGT CAG GAA GAT AGC CGA GTT TTG GCC ACT GAC TGA CTC GGC ATG TAT CTT CCT GA and CCT GTC AGG AAG ATA CAT GCC GAG TCA GTC AGT GGC CAA AAC TCG GCA TGG CTA TCT TCC TGA C, respectively. After the single-stranded oligo DNA was annealed, the double-stranded shRNA

was inserted into the vector, which was constructed according to the instructions of BLOCK-iT™ HiPerform™ Lentiviral Pol II miR RNAi Expression System with EmGFP kit, and then the vector was transformed into competent bacteria.

**FASN shRNA transfection of SK-Hep-1 HCC cells.** SK-Hep-1 HCC cells were seeded onto the 6-well plates at the density of  $2 \times 10^5$  cells/hole in the incubator; when 80-90% confluent at the bottom, SK-Hep-1 cells were transfected with FASN shRNA. According to the instructions of the Lipofectamine 2000 kit, the constructed FASN interference vector and blank vector was diluted with serum-free culture medium in two centrifugation tubes (1.5 ml; tube 1 and 2), respectively, while Lipofectamine 2000 was diluted with 100  $\mu\text{l}$  of serum-free medium in another centrifugation tube (1.5 ml; tube 3). All tubes were placed at room temperature for 5 min and then 50  $\mu\text{l}$  of the diluted liposomes was collected from tube 3 and added into tubes 1 and 2, mixing gently and placing at room temperature for 25 min. The 6-well plates were supplemented with 100  $\mu\text{l}$  of the transfection solution, covering the cells at the bottom. The culture medium was replenished 5 h later and the transfection efficiency was detected 24 h later.

**Western blot analysis detection of interference effects of FASN shRNA.** At 48 h after transfection, total cell protein was extracted with the above method.

**3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2-tetrazolium bromide (MTT) detection of SK-Hep-1 cell proliferation.** The transfected cells were collected and counted at 48 h after transfection. The cells transfected with FASN interference vector and negative cells at 10,000/ml were seeded in 96-well plates. Each hole was supplemented with the previously prepared MTT working solution 20  $\mu\text{l}$  (5 mg/ml). The cells were mixed by pipetting and incubated for 4 h. The culture medium in each hole was discarded and was supplemented with 150  $\mu\text{l}$  of DMSO, shaking at room temperature for 10 min. The absorbance of each hole was measured using a microplate reader and averaged.

**Detection of SK-Hep-1 cell apoptosis.** At 48 h after transfection, the cells were washed three times with PBS, then collected and stained according to the instructions of the apoptosis kit (Jingmei Biotech). The apoptosis results were analyzed using flow cytometry.

**SK-Hep-1 cell migration and invasion.** Matrigel was thawed in a refrigerator at  $4^{\circ}\text{C}$  prior to use. Matrigel diluents were applied to coat the chamber, then dried. Each hole was supplemented with 50  $\mu\text{l}$  serum-free medium containing 10 g/l of bovine serum albumin, and cells were incubated at  $37^{\circ}\text{C}$  for 30 min. At 48 h after transfection, cells were collected and counted. Subsequently, 150  $\mu\text{l}$  of cell suspension at  $1.5 \times 10^5$  cells/ml was pipetted to the chamber, and the bottom of the 24-well plate was supplemented with 500  $\mu\text{l}$  bovine fetal serum-containing medium. The cells were cultured for an additional 48 h. After the culture was completed, the chamber was taken out and the lower layer of cells was fixed with 95% ethanol for 5 min, and stained with crystal violet solution at a concentration of 4 g/l. The cells were counted at five randomly selected visions.

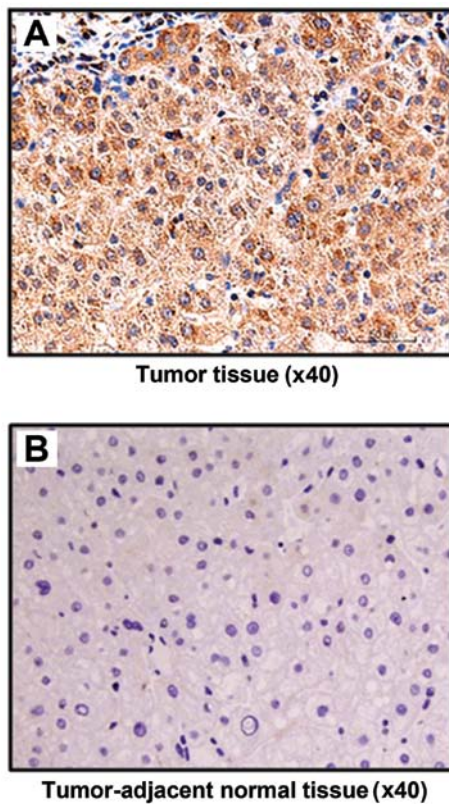


Figure 1. Expression of FASN in tumor and tumor-adjacent tissues of hepatocellular carcinoma. FASN, fatty acid synthase.

**Statistical analysis.** Experimental data were analyzed using SPSS 18.0 software and are expressed as the means  $\pm$  SD. The difference between groups was compared using one-way analysis of variance. A P-value of  $<0.05$  was considered to indicate a statistically significant result.

## Results

**FASN is highly expressed in HCC tissue.** Immunohistochemical staining results showed that FASN was positively expressed in all 20 HCC patients, accounting for the positive rate of 100% (20/20). However, the positive expression rate in tumor-adjacent tissue was only 10% (2/20); there were significant differences between the two groups ( $P<0.05$ ). The staining in HCC tissue was significantly more visible than that in tumor-adjacent tissue, and positive staining was placed in the cytoplasm (Fig. 1).

**FASN is highly expressed in HCC cell lines.** FASN mRNA and protein expression levels in normal liver cell line 7702 and HCC cell lines HepG2, SMMC7721, MHCC97L, MHCC97H and SK-Hep-1 were detected with real-time quantitative PCR and western blot analysis, respectively. The results showed that FASN mRNA and protein expression levels in HCC cell lines HepG2, SMMC7721, MHCC97L, MHCC97H and SK-Hep-1 were higher than those in normal liver cell line 7702 ( $P<0.05$ ; Figs. 2 and 3). In addition, highly metastatic liver cancer cell lines MHCC97H and SK-Hep-1 had a higher expression level than low metastatic liver cancer cell lines HepG2, SMMC7721 and MHCC97L ( $P<0.05$ ; Figs. 2 and 3).

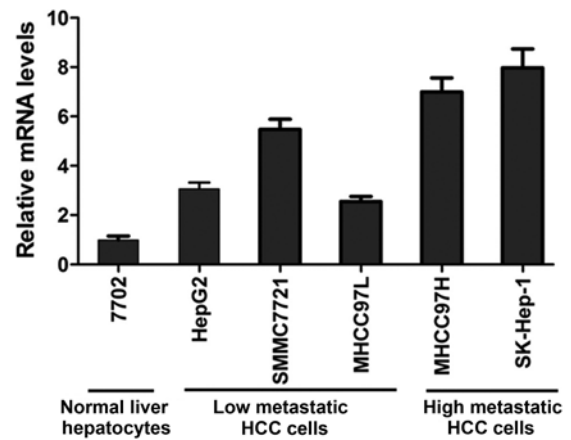


Figure 2. FASN mRNA expression levels in normal liver hepatocyte and hepatocellular carcinoma cell lines. FASN, fatty acid synthase.

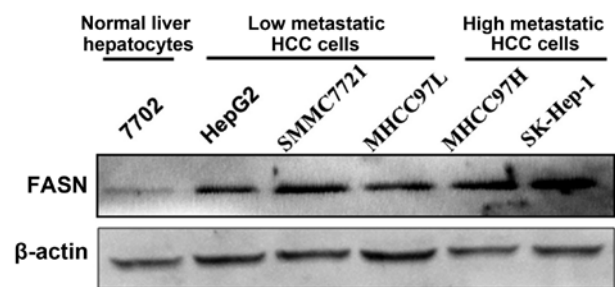


Figure 3. FASN protein expression levels in normal liver hepatocyte and hepatocellular carcinoma cell lines. FASN, fatty acid synthase.

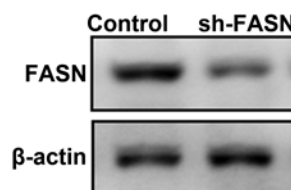


Figure 4. Analysis for interference efficiency of the FASN shRNA. FASN, fatty acid synthase.

**FASN shRNA interference of FASN expression.** To explore the function of FASN, we first constructed an interference vector of FASN expression, and identified its interference efficiency (Fig. 4).

As shown in Fig. 2, the FASN expression reached the peak in SK-Hep-1 cells, thus we transfected SK-Hep-1 cells with RNA interference vector to knock down the expression of FASN. The cell proliferation, apoptosis, migration and invasion were observed.

**Effects of sh-FASN on SK-Hep-1 cell proliferation and apoptosis.** After the FASN expression in SK-Hep-1 cells was knocked down, the cell proliferation and apoptosis were determined with MTT assay and flow cytometry. The results showed that the cell proliferation was significantly inhibited by the knockdown (Fig. 5), while the apoptosis did not change significantly (Fig. 6).

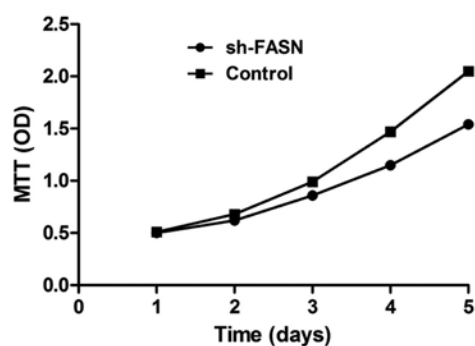


Figure 5. Effect of FASN knockdown on cell proliferation in hepatocellular carcinoma. FASN, fatty acid synthase.

*Effects of sh-FASN on SK-Hep-1 cell migration and invasion.* After FASN expression in SK-Hep-1 cells was knocked down, the cell migration and invasion were detected by Transwell chamber test. The results showed that the number of cells that migrated and invaded into the bottom of the membrane was significantly reduced after the knockdown (Figs. 7 and 8).

## Discussion

Increasing evidence indicates that cell metabolism abnormalities are closely related to tumor occurrence and development (5). Abnormal metabolism of tumor cells mainly refers to the abnormalities in the metabolism of glucose and lipid, the glycolytic activity and fatty acid synthesis activity of tumor cells are enhanced (6,7). FASN is the key molecule for catalyzing fatty acid synthesis, and human FASN gene is located at chromosome 17. FASN is highly expressed in a number of malignant tumors; it can promote the synthesis of endogenous fatty acids in tumor cells, then the synthesized fatty acids provide energy for the proliferation of tumor cells. Previous studies addressing prostate cancer revealed that FASN mRNA and protein expression levels in cancer tissue were significantly increased compared with normal prostate tissue around the lesions, and the expression levels gradually increased in normal epithelium, epithelial hyperplasia tissue, highly proliferative and prostate cancer tissues. These findings indicated the contribution of FASN in prostate cancer (8,9). FASN expression in renal carcinoma tissue was significantly higher than that in the tumor-adjacent normal tissue, indicating that FASN expression is involved in the lymph node metastasis, tumor classification and prognosis (10). A high FASN expression was also found in bladder cancer; the expression levels were significantly correlated with the tumor classification of bladder cancer, and high FASN expression can be regarded as an indicator of bladder cancer prognosis (11). Interference on FASN expression could inhibit the proliferation of endometrial cancer cells, while promoting apoptosis (12). Furthermore, FASN can promote the invasion and migration of osteosarcoma cells (13,14).

As FASN occurs in the progression of various tumors, investigations into FASN as a potential target for cancer therapy has been widely studied and has achieved considerable progress (15,16). Cerulenin is the first discovered FASN inhibitor, but its application in antitumor treatment has been limited due to unstable chemical property. C75 is a stable FASN inhibitor,

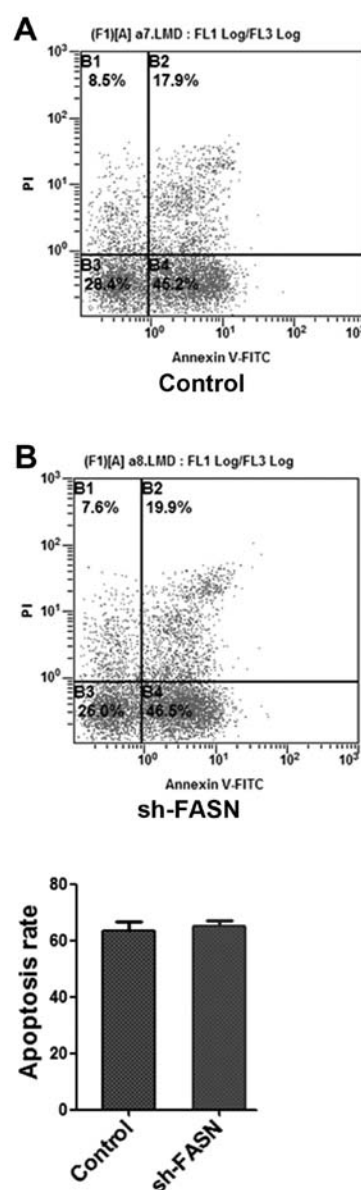


Figure 6. Effect of FASN knockdown on cell apoptosis in hepatocellular carcinoma. FASN, fatty acid synthase.

which can dose-dependently inhibit the expression of FASN in tumor cells, and was shown to exhibit antitumor activity in the *in vivo* experiments (17).

Previous studies demonstrated that fatty acid synthesis activity was significantly enhanced in HCC tissue. These metabolic changes can be explained by the high expression of several fatty acid synthetase in HCC cells. The fatty acid synthetase is mainly SREBP1, ACLY, ACC, FASN and SCD1, wherein FASN is the dominant one (18). However, these previous studies have some limitations, such as small size of HCC tissue samples, lack of detection of FASN expression levels in HCC cell lines, and lack of investigations into the biological significance of FASN high expression in HCC cells, that is the influence on malignant tumors. The present study demonstrated that FASN was highly expressed in HCC cell lines HepG2, SMMC7721, MHCC97L, MHCC97H and SK-Hep-1, compared with normal hepatocyte 7702. FASN

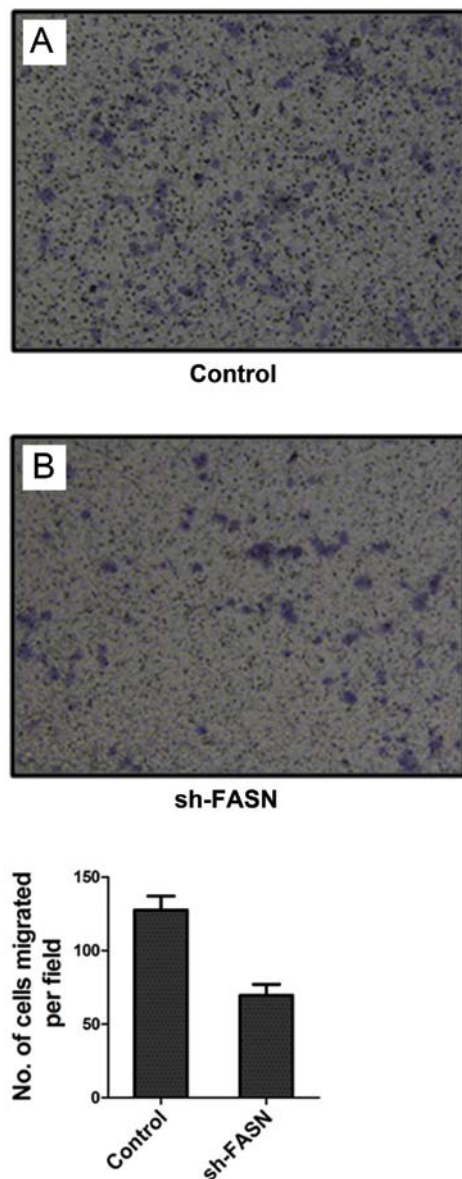


Figure 7. Effect of FASN knockdown on cell migration in hepatocellular carcinoma. FASN, fatty acid synthase.

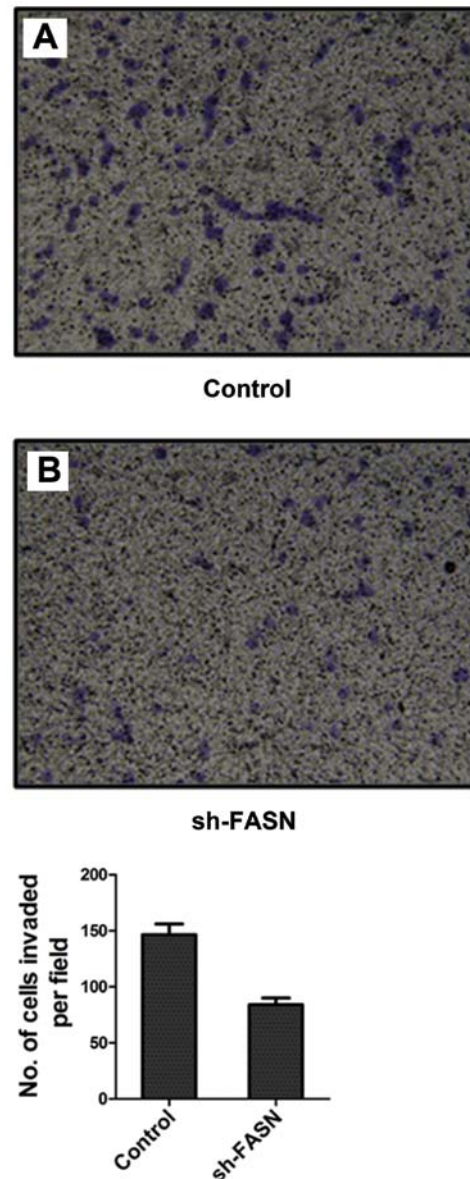


Figure 8. Effect of FASN knockdown on cell invasion in hepatocellular carcinoma. FASN, fatty acid synthase.

expression also showed significant differences between highly metastatic HCC cells (MHCC97H and SK-Hep-1) and low metastatic HCC cells (HepG2, SMMC7721 and MHCC97L). Our findings indicate that FASN expression may be involved in HCC metastasis. The high expression of FASN within cells may be regulated at the transcriptional level, rather than the post-transcriptional level, as detected by real-time quantitative PCR. In addition, immunohistochemical staining results also found a high expression of FASN in HCC tissue at the tissue level. Our findings demonstrated that FASN is highly expressed in both HCC cells and tissues. In the *in vitro* experiments, the eukaryotic expression vector targeting FASN interference was constructed and transfected into HCC cell line SK-Hep-1, to knock down FASN expression. Furthermore, cell proliferation, apoptosis, migration and invasion were observed. The results showed that the knock-down of FASN could inhibit cell proliferation, invasion and

migration, leaving cell apoptosis unaffected. It was previously reported that FASN promoted cell proliferation and inhibited apoptosis in endometrial cancer (12). Similarly, we supported their outcomes on the proliferation of HCC cells, although the apoptosis was not affected. The main reason for this discrepancy is that FASN may be involved in the development and progression of various types of tumor cells through different mechanisms. In addition, we confirmed that knockdown of FASN expression inhibited the invasion and migration of HCC cells, indicating the contribution of FASN to malignant tumor metastasis. Inhibiting FASN expression in HCC cells is a challenging issue and requires further investigation.

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