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Original Article

POU2F1 promotes growth and metastasis of hepatocellular carcinoma through the FAT1 signaling pathway

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Abstract: Increasing evidence suggests that POU domain class 2 transcription factor 1 (POU2F1) participates in carcinogenesis and cancer progression via promotion of cell proliferation and metastasis; however, the functional role of POU2F1 in hepatocellular carcinoma (HCC) is largely unknown. In this study, we determined that POU2F1 was significantly up-regulated in HCC tumor tissue and cell lines. We demonstrated that POU2F1 over-expression promoted HCC cell proliferation, colony formation, migration, and invasion, while silencing of POU2F1 inhibited these malignant phenotypes. In vivo experiments indicated that knockdown of POU2F1 inhibited HCC cell metastasis and xenograft growth, whereas ectopic expression of POU2F1 promoted these cellular functions. Microarray analysis suggests that FAT atypical cadherin 1 (FAT1) can function downstream of POU2F1. Functionally, we demonstrated that POU2F1 knockdown induced growth suppression and metastasis inhibition of HCC cells and inactivated the FAT1 pathway, indicating that POU2F1 is a potential novel therapeutic target in HCC.

Keywords: POU2F1, hepatocellular carcinoma, metastasis, FAT1

Introduction

Hepatocellular carcinoma (HCC) is one of the most prevalent cancers, and the third most common cause of cancer-related death worldwide [1]. The treatment of HCC is complex. Despite the development of precision medicine in oncology, hepatic resection and transplantation are still the most effective therapeutic methods for HCC; however, the tumor recurrence rate remains very high in patients with HCC, and the overall rate survival is poor and requires improvement [2]. The poor prognosis associated with this type of cancer can mainly be attributed to intrahepatic and extrahepatic metastases. Exploration of the molecular mechanisms underlying tumor migration and invasion has potential to contribute greatly to understanding of tumor progression and metastasis. Therefore, the further research to elucidate the molecular mechanism of HCC progression and identify new therapeutic targets is required [3].

POU domain class 2 transcription factor 1 (POU2F1), also referred to as octamer binding transcription factor-1 (OCT-1), is encoded by a gene locus on chromosome 1q24. It is a ubiquitous transcription factor that regulates the expression of target genes associated with cell cycle regulation [4]. POU2F1 is involved in cell differentiation through regulation of housekeeping genes such as H2B and snRNAs, and also participates in immunity and inflammation via modulation of tissue-specific target gene expression. Additionally, POU2F1 participates in cellular responses to DNA damage [5]. It is reported as over-expressed in osteosarcoma tumors and was identified as an independent prognostic factor in gastric carcinoma. POU2F1 is highly expressed in head and neck squamous cell carcinoma cell lines, and in patient tumor samples compared to tissue-matched controls [6]. Knockdown of POU2F1 caused significant reduction in the proliferation and invasion of head and neck squamous cell carcinoma cells [7]. Hence, accumulating evidence suggests

that POU2F1 is involved in carcinogenesis and cancer progression. POU2F1 has also been identified as a factor that can promote liver cancer cell proliferation and inhibit apoptosis through CAPN6; however, the functional role of POU2F1 in HCC metastasis remains unknown [8].

In this study, we evaluated the expression of POU2F1 in HCC tissues compared with adjacent non-cancerous normal specimens, and analyzed its relationship with patient outcomes. We further evaluated the role of POU2F1 in HCC cell growth and metastasis in vitro and in vivo. Finally, we investigated pathways downstream of POU2F1 and their role in HCC and clinical outcomes. Our findings suggest that POU2F1 has a pivotal role in HCC progression and could serve as a potential therapeutic target.

Methods

Cell culture

Human HCC cells (SNU-423, SNU-387, SNU-449, HUH-6, HUH-7, HepG2, HUH-7) and LO2 cells were purchased from the Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China). These cells were cultured in DMEM or RPMI 1640 medium with 10% fetal bovine serum.

Tissue specimens

Study protocols were provided by the Alenabio biotechnologies co., LTD (Xian, Shanxi, China), and written informed consent was obtained from patients based on the Declaration of Helsinki. A total of 74 fresh primary HCC cancer and paired adjacent normal tissue specimens were provided by Alenabio biotechnologies co., LTD (Xian, Shanxi, China). Tumor staging was determined according to the American Joint Committee on Cancer criteria. Informed consent was obtained from each patient before study. None of these patients underwent preoperative chemotherapy and/or radiation therapy.

Real-time PCR

Total RNA was extracted from HCC cancer tissues and the matched adjacent normal tissues using Trizol reagent (Invitrogen, Carlsbad, CA, USA). 1 μg of total RNA from each sample were reverse transcribed into cDNA using PrimeScript™ RT Master Mix Kit (Takara, Dalian, China). Real-time PCR was performed using SYBR Premix Ex Taq II Kit (Takara, Dalian, China) according to the instructions of the manufacturer [12]. The specific primers used were as follows: POU2F1 sense, 5'-ATGAACAATCCGTCAGAAACCAG-3' and antisense 5'-GATGGAGATGTCCAAGGAAAGC-3'; GAPDH sense, 5'-GGAAGGTGAAGGTCGGAGTCA-3' and antisense 5'-GTCATGATGGCAACAATATCCACT-3'.

Immunohistochemistry

The paraffin-embedded sections were deparaffinized in xylene, rehydrated in descending percentages of ethanol and heated in citrate buffer (pH 6.0) for antigen retrieval. After washing steps, slides were blocked with 3.0% hydrogen peroxide and 10% goat serum and incubated with a primary antibody at 4°C overnight. The following antibodies were used: rabbit anti-POU2F1 antibody (1:500; Abcam, MA, USA) and rabbit anti-Ki67 (1:400; Cell signaling technology, MA, USA). After the sequential incubation with biotinylated secondary antibody, streptavidin-horseradish peroxidase complex and diaminobenzidine (DAB), the slides were counterstained with hematoxylin, dehydrated, and mounted. Finally, sections were observed and imaged under light microscope [13].

Establishment of stable expression and knockdown cell lines

A POU2F1 or FAT1 expression construct was generated by sub-cloning PCR-amplied full-length human POU2F1 cDNA or FAT1 cDNA into the pMSCV retrovirus plasmid. Human POU2F1-targeting short hairpin RNA (shRNA) oligonucleotides sequences were cloned into pSuper-retro-puro to generate pSuper-retro-POU2F1-RNAi(s). Human FAT1-targeting short hairpin RNA (shRNA) oligonucleotides sequences were cloned into pSuper-retro-puro to generate pSuper-retro-FAT1-RNAi(s). Lipofectamine RNAi MAX and Lipofectamine 2000 reagent (Invitrogen) were used for transient knockdown by shRNA or transient overexpression, respectively. All experiments were performed according to the manufacturer's instructions.

MTT proliferation assay

Cell proliferation was measured by MTT assay. Briefly, lentivirus infected cells were plated in

96-well plates at a density of 3,000 cells per well. At various time points (24, 48, 72 and 96 h), 10 μl of MTT solution was added to each well and incubated at 37°C for 4 h, and then the absorbance was measured at 490 nm. The experiment was performed independently in triplicate [14].

Colony formation assay

Two hundred HCC cells were seeded into 6 cm plates and incubated for 14 days. Cells were then fixed with 4% paraformaldehyde and dyed with 1% crystal violet. Colonies were counted and photographed. The experiment was performed independently in triplicate [15].

Apoptosis assay

Forty-eight hours after transfection, the cells were digested with ethylenediamine tetraacetic acid (EDTA)-free trypsin, collected in flow cytometry tubes, and centrifuged at 1,000 rpm for 5 min. The supernatants were aspirated and the cells washed three times with cold PBS. The cells were then centrifuged once more and the supernatants discarded. We added 150 μL of binding buffer (Annexin-V-FITC Apoptosis Detection Kit, Sigma-Aldrich) and 5 μL of Annexin-V-FITC to each tube. The tubes were then mixed and incubated for 15 min at room temperature in the dark. Following the incubation, 100 μl of binding buffer and 5 μl of PI were added to each tube. Apoptotic cells were detected and analyzed by flow cytometry.

Wound healing assay

The indicated cells were seeded in 60 mm dishes to produce a confluent monolayer. After starvation in serum-free cultured medium for 12 hours, a wound was made by scratching the cell monolayer with a 200 μl pipette tip. The cell monolayer was washed with PBS to remove cell debris and incubated with fresh complete medium. The area of wound scratch was photographed at 0 h and 24 h, respectively. The wound healing effect was determined by measuring the percentage of the remaining cell-free area compared with the area of the initial wound [16].

Cell invasion assay

Invasion of cells was measured in Matrigel (BD) -coated Transwell inserts (6.5 mm, Costar, USA) containing polycarbonate filters with 8-μm pores as detailed previously. The inserts were coated with 50 μl of 1 mg/ml Matrigel matrix according to the manufacturer's recommendations. 200 μl 2 × 10^5 cells were plated in the upper chamber, whereas 600 μl of medium with 10% fatal bovine serum were added to lower well. After 24 h incubation, cells that migrated to the lower surface of the membrane were fixed and stained with 1% crystal violet. For each membrane, five random fields were counted [17].

Western blot analysis

Briefly, whole-cell lysates were prepared and protein concentration was estimated using a BCA Protein Assay kit (Pierce, USA). The immune-blots were transferred onto PVDF membranes and membranes were incubated with monoclonal antibody against POU2F1, FAT1 and GAPDH overnight at 4°C [18]. Then PVDF membranes were incubation with peroxidase conjugated goat anti-rabbit (Abcam). The blots were visualized using enhanced chemiluminescence detection kit (Thermo).

Animal studies

Animal studies were approved by the Institutional Animal Care and Use Committee of Suqian First Hospital. Cells were transfected with POU2F1 or POU2F1-shRNA and their negative control vector. 100 μl cells (1 × 10^6) were subcutaneously inoculated into 5-week-old BALB/c nude mice. On day 21 after implantation, tumors were harvested and weighed. The tumor volume was calculated (volume = 0.5 × length × width^2). IHC analysis was performed as previously described to determine the expression of POU2F1 and Ki67. For metastasis assays, shPOU2F1 B16F10 or POU2F1 overexpressing cells were injected into the lateral tail vein (1 × 10^6 cells) of 6-7 week-old C57/BL/6 mice. Lungs were fixed in formalin and were photographed using a Leica microscope (Leica, Wetzlar, Germany).

Statistical analysis

The data were presented as mean ± SD. Differences in the results of two groups were evaluated using either two-tailed Student's t test or one-way ANOVA followed by *post hoc* Dunnett's test. The differences with $P < 0.05$ were considered statistically significant.

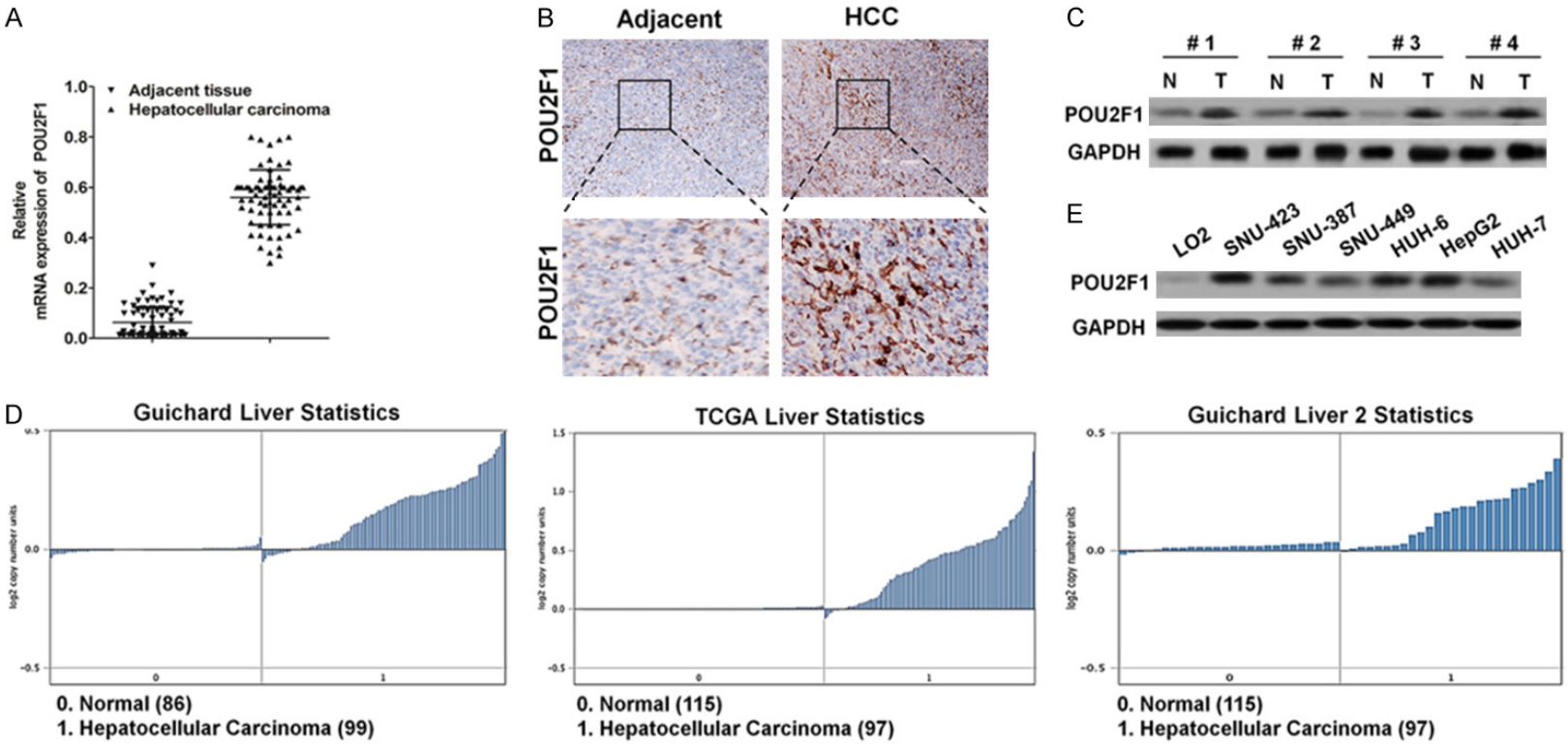


Figure 1. Overexpression of POU2F1 in HCC patient tissues and cell lines. A. Relative expression of POU2F1 mRNA in 74 HCC cancer and paired adjacent normal tissue samples determined by qPCR. PCR values were normalized to levels of GAPDH expression. B. Expression of POU2F1 protein in HCC and adjacent tissues determined by IHC analysis. Scale bars: upper panel, 100 μm; lower panel, 50 μm. C. Expression of POU2F1 protein in HCC cancer (T) and adjacent normal (N) tissues, determined by western blot assay. GAPDH was used as loading control. D. Box plots showing increased levels of POU2F1 in HCC (right), compared with normal adjacent tissues (left) in three microarray data sets. **P < 0.01, compared with normal tissues. E. Immunoblotting analysis of the levels of POU2F1 in various HCC cancer cell lines. GAPDH was used as loading control.

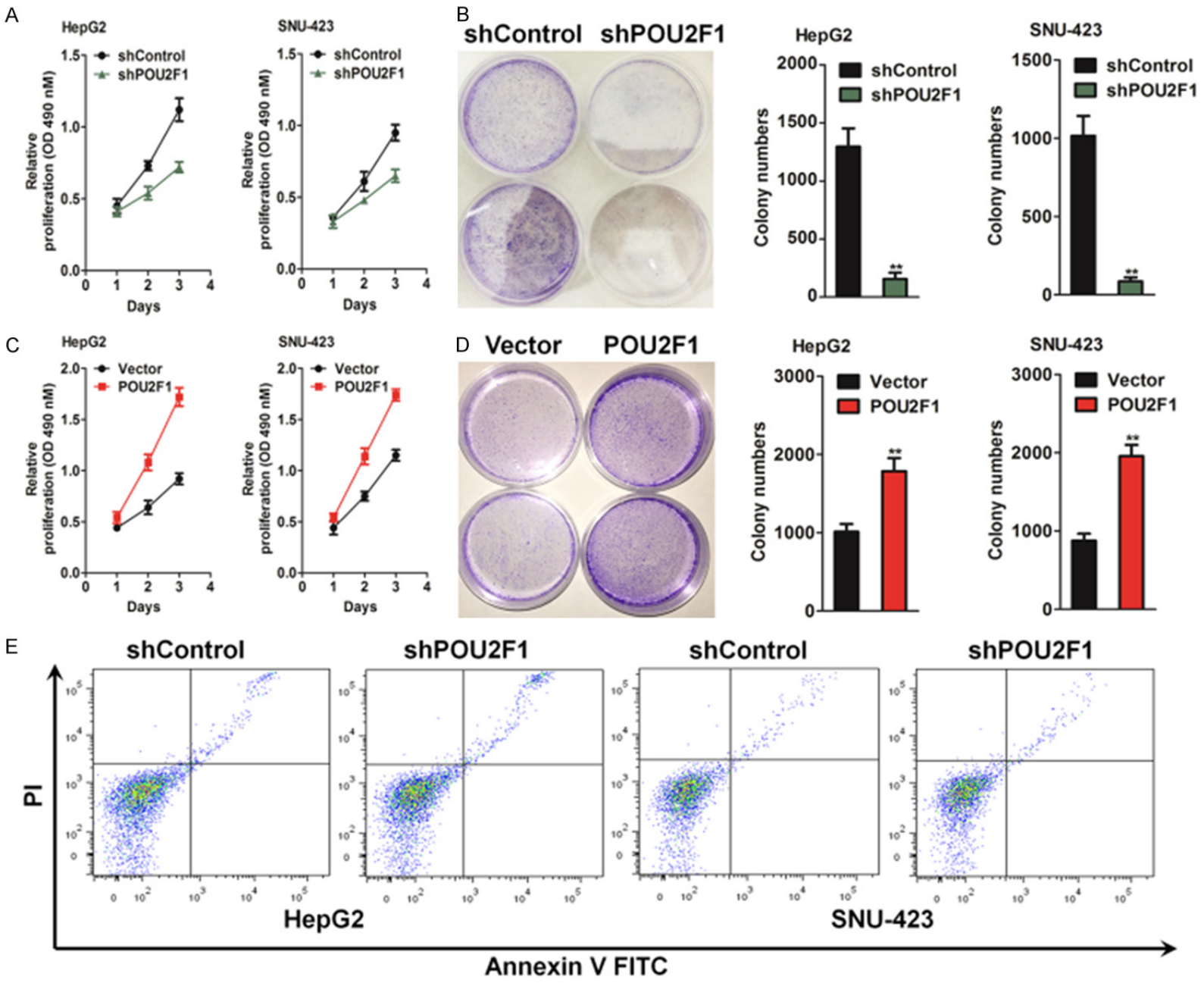

A
HepG2
shControl
shPOU2F1
Relative proliferation (OD 490 nM)
Days
SNU-423
B
shControl
shPOU2F1
HepG2
SNU-423
Colony numbers
C
Vector
POU2F1
D
E
PI
Annexin V FITC

Figure 2. POU2F1 accelerates liver cancer cell growth in vitro and in vivo. A. MTT analysis of HepG2 and SNU-423 cells transfected with POU2F1 shRNA. 3×10^4 cells were seeded in 96-well plates and cultured for 24, 48, 72, and 96 h. Data are presented as means ± standard deviation (SD). B. Colony formation analysis of HepG2 and SNU-423 cells. Cells (n = 200) were seeded in 6 cm plates and cultured for 14 days. **P < 0.01 compared to control cells. C. MTT analysis of POU2F1 over-expressing HepG2 and SNU-423 cells. 3×10^4 cells were seeded in 96-well plates and cultured for the indicated amount of time. Data are presented as means ± SD. D. Colony formation analysis of POU2F1 over-expressing HepG2 and SNU-423 cells. **P < 0.01 compared to cells transfected with control vector. E. Apoptosis levels determined using Annexin V/PI staining by flow cytometry.

Results

Overexpression of POU2F1 in HCC tissue samples and cell lines

First, we investigated the expression levels of POU2F1 in liver cancer tissue samples. POU2F1 mRNA levels were significantly up-regulated in HCC compared with non-tumor tissues, as revealed by qPCR (n = 74) (**Figure 1A**). This result was supported by the findings of immunohistochemistry (IHC) analysis (**Figure 1B**), demonstrating increased POU2F1 protein expression in HCC tissues. Quantification of POU2F1 protein expression by western blotting generated data consistent with the IHC findings, with POU2F1 expression in HCC tissues significantly higher than that in adjacent non-tumor tissue (**Figure 1C**). To further investigate whether POU2F1 is involved in HCC progression, we examined its expression pattern using data from the publicly accessible Oncomine microarray database [9]. POU2F1 expression was markedly elevated in HCC compared with matched normal tissues in three independent clinical data sets (**Figure 1D**). The prognostic value of POU2F1 in HCC was analyzed using survival analysis and SurvExpress, an online biomarker validation tool and database for cancer gene expression data. Kaplan-Meier analysis of overall HCC data revealed a positive correlation between overexpression of POU2F1 and lower patient survival rates (Supplementary Figure 1). The association between cancer progression and reduced POU2F1 expression was also confirmed in a panel of HCC cell lines (**Figure 1E**). POU2F1 was expressed at relatively high levels in HCC cell lines (SNU-423, SNU-387, SNU-449, HepG2, HUH-6, and HUH-7), but was markedly lower in an untransformed liver cell line (LO2), partially due to a decrease in POU2F1 mRNA levels, as determined by qPCR (Supplementary Figure 2).

POU2F1 promotes HCC cell growth in vitro and in vivo

To investigate the biological role of POU2F1 in hepatocellular carcinoma cells, HepG2 and SNU-423 cell lines with up- and down-regulation of POU2F1 were established. As shown in Supplementary Figure 3A, POU2F1-shRNA silenced the expression of POU2F1 in both cell lines. Consequently, cell proliferation was remarkably suppressed in liver cancer cells with POU2F1 down-regulated, compared with that of cells transfected with control-shRNA, as determined by both MTT (**Figure 2A**) and colony formation assays (**Figure 2B**). In contrast, overexpression of POU2F1 dramatically accelerated cell growth (Supplementary Figure 3B and **Figure 2C**) and colony formation (**Figure 2D**) in vitro. We then examined the effect of POU2F1 silencing/overexpression on apoptosis of HepG2 and SNU-423 cells. Overexpression/silencing of POU2F1 had no effect on apoptosis of HepG2 or SNU-423 cells in vitro, as determined by Annexin V/PI assays (**Figure 2E**). To investigate the influence of POU2F1 up- or down-regulation on liver cancer growth in vivo, we established a subcutaneous xenograft model in nude mice, using HepG2 and SNU-423 cells. As shown in **Figure 3A**, the mean tumor volume in mice inoculated with cells with POU2F1 down-regulated (POU2F1-shRNA group) was significantly smaller than that in the control-shRNA group. Conversely, up-regulation of POU2F1 led to significantly increased growth of the xenografts (**Figure 3B**). IHC staining of Ki67 revealed that knock-down of POU2F1 inhibited liver cancer growth in vivo, while ectopic expression of POU2F1 exerted the opposite effect (**Figure 3C** and **3D**). Collectively, these data indicate that POU2F1 has an important role in liver cancer cell proliferation in vitro and growth in vivo.

POU2F1 promotes liver cancer cell metastasis in vitro and in vivo

Next, we investigated the effects of POU2F1 on liver cancer cell metastasis in vitro. Compared to controls, silencing of POU2F1 led to impaired HepG2 and SNU-423 cell migration in vitro, as determined by wound closure analysis (**Figure 4A**) and Transwell invasion assay (**Figure 4B**). In

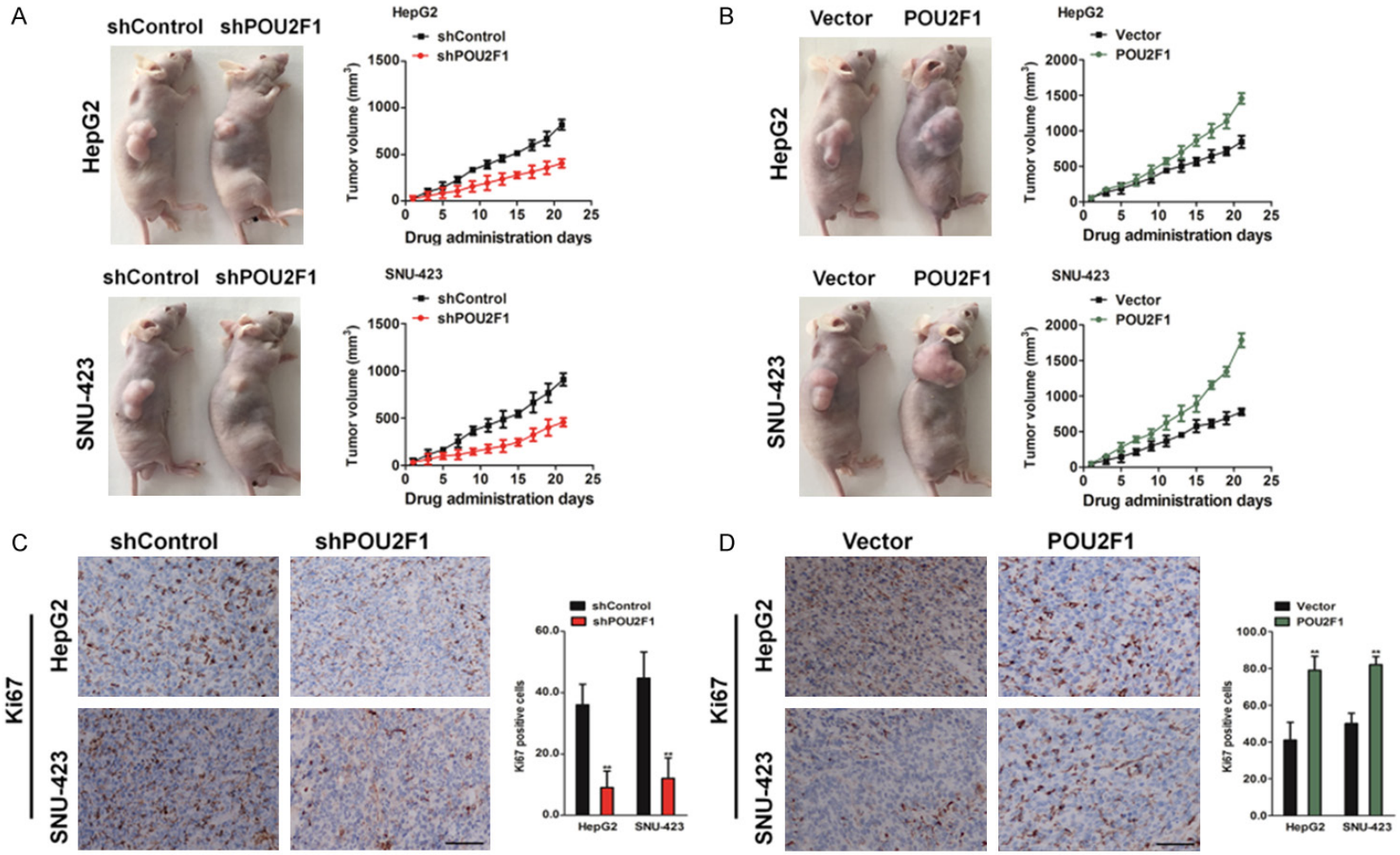
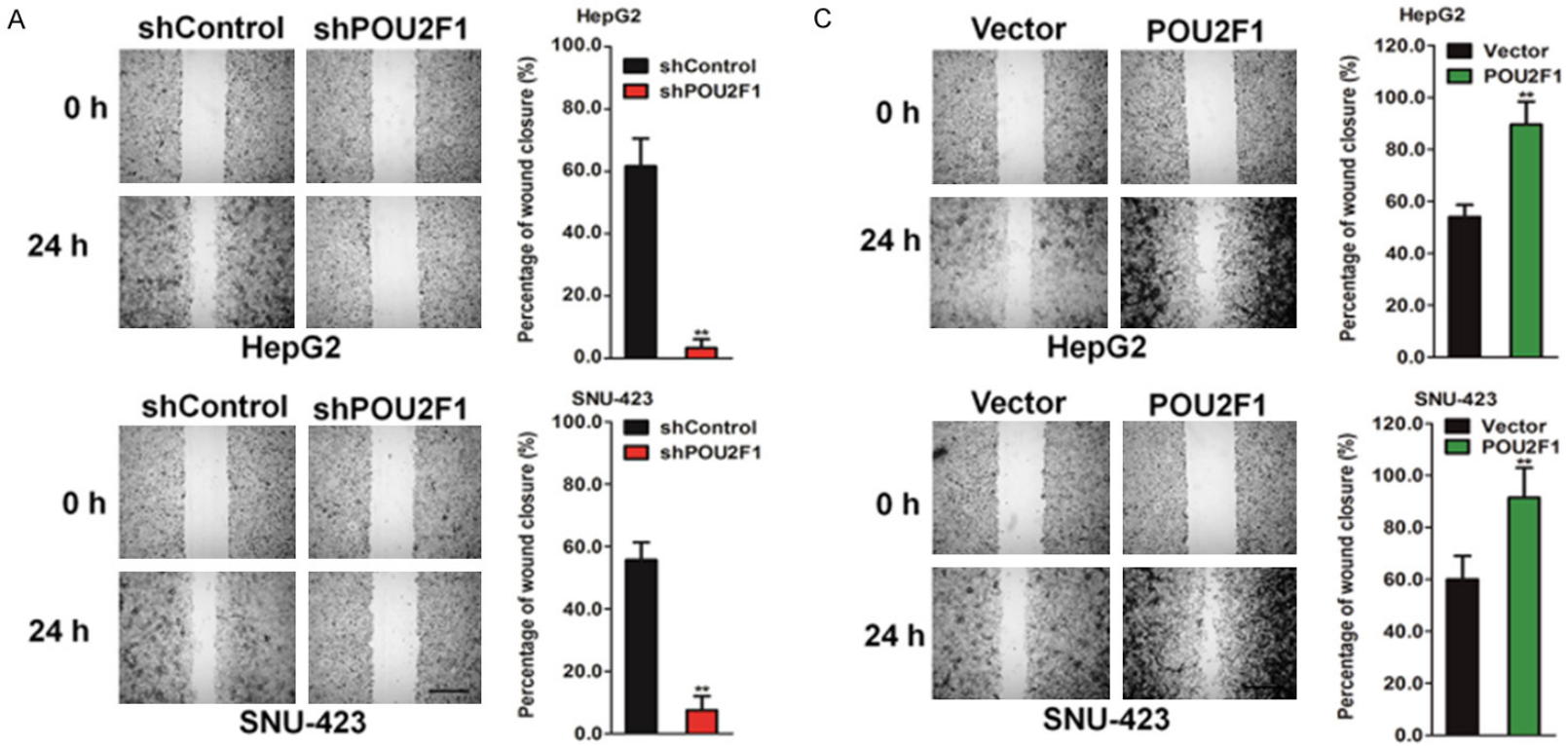


Figure 3. Effect of POU2F1 knockdown or over-expression on liver cancer cell growth in vivo. A. POU2F1 knockdown HepG2 or SNU-423 cells (3×10^6; 100 μl) were implanted subcutaneously into Balb/c-nude mice to form xenografts. After 21 days, the xenografts were harvested, the mean tumor volume in different groups determined, and samples processed for immunohistochemistry. B. Images of xenografts in POU2F1 over-expressing HepG2 or SNU-423 groups at the end of the experiment. Tumors were isolated 21 days later. C. Immunohistochemistry analysis of POU2F1 and Ki67 in xenografts from mice grafted with POU2F1 silenced liver cancer cells. **P < 0.01 compared with mice inoculated with control cells. Scale bar, 100 μm. D. Immunohistochemistry analysis of POU2F1 and Ki67 in xenografts from mice inoculated with POU2F1 over-expressing liver cancer cells. **P < 0.01 compared with mice inoculated with control cells. Scale bar, 100 μm.


A
shControl
shPOU2F1
0 h
24 h
HepG2
SNU-423
Percentage of wound closure (%)
100.0
80.0
60.0
40.0
20.0
0.0
shControl
shPOU2F1
**
C
Vector
POU2F1
120.0

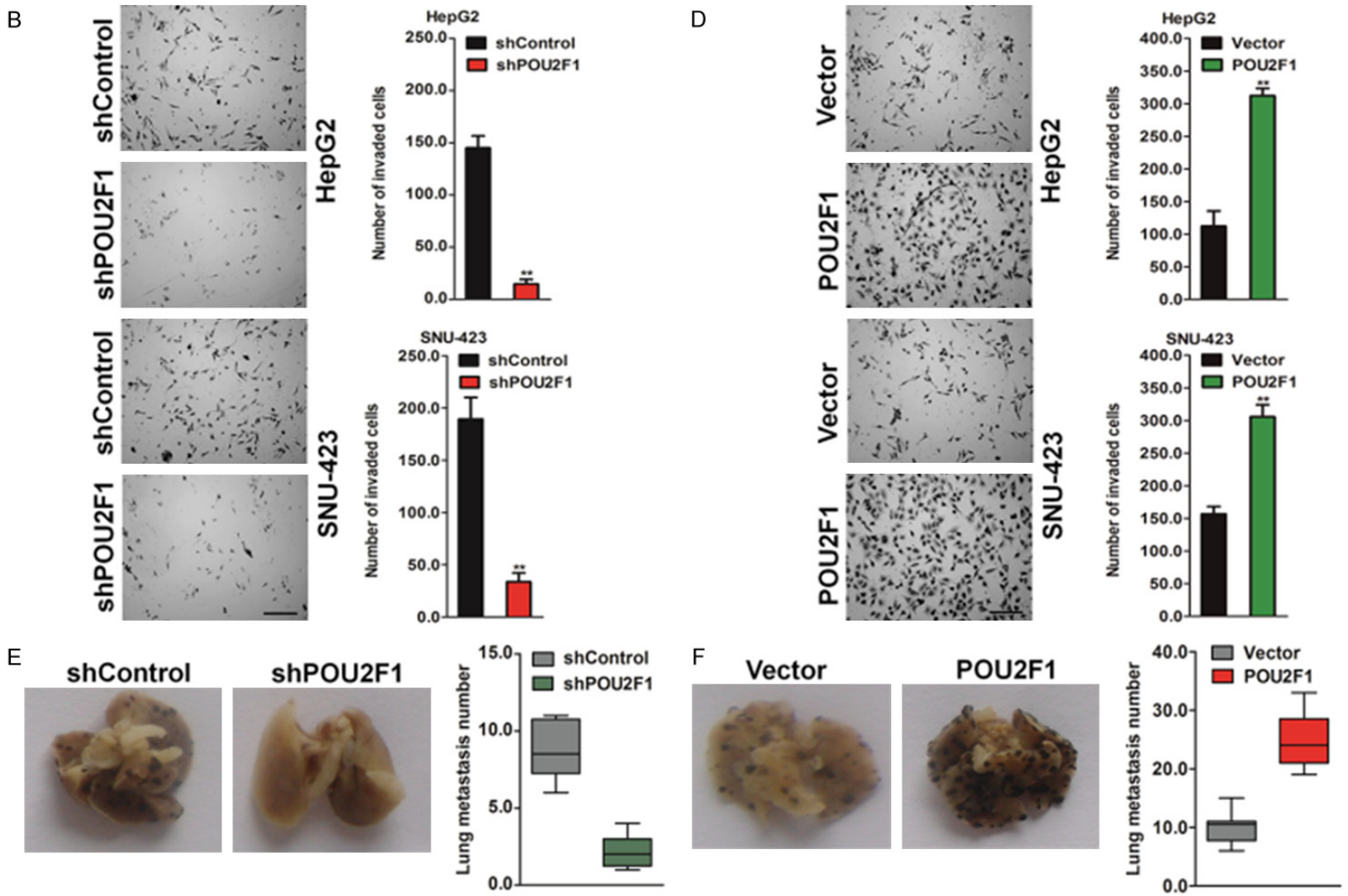


Figure 4. Effect of POU2F1 on liver cancer cell metastasis. A. Representative wound healing images of HepG2 and SNU423 cells at 0 and 24 h. Black lines indicate the cell boundaries. Wound healing rates were quantified in HepG2 and SNU423 cells. Data are expressed as means ± SD of wound closure rates relative to the control group. **P < 0.01 compared to mice inoculated with control cells. Scale bar, 200 μm. B. Representative images of invasion of POU2F1 silenced and control cells. Cell invasion was quantified in HepG2 and SNU423 cells. **P < 0.01 compared with mice inoculated with control cells. Scale bar, 100 μm. C. Representative images of wound healing of POU2F1 over-expressing HepG2 and SNU423 cells. Quantification of wound closure data are presented as a bar chart (right). Data are

expressed as means ± SD. **P < 0.01 compared with mice inoculated with control cells. Scale bar, 200 μm. D. The number of invasion cells were quantified in POU2F1 over-expressing HepG2 and SNU423 groups. Cells on the lower surface of the membrane were quantified in five randomly selected fields. **P < 0.01 compared with mice inoculated with control cells. Scale bar, 100 μm. E. Lung metastases were counted in mice inoculated with B16F10 cells treated with POU2F1 shRNA or control cells and numbers of pulmonary metastatic nodules were subjected to statistical analysis (box plot, right). F. Lung metastases were counted in mice inoculated with B16F10 cells over-expressing POU2F1 or control cells and numbers of lung metastatic nodules were subjected to statistical analysis (box plot, right).

contrast, the same assays demonstrated that overexpression of POU2F1 significantly promoted HepG2 and SNU-423 cell migration and invasion in vitro (**Figure 4C** and **4D**). To examine the role of POU2F1 in cancer cell metastasis in vivo, an experimental metastasis assay was performed. Control and POU2F1 knock-down melanoma B16F10 cells (data not shown) were injected into the lateral tail vein of C57BL/6 mice. Two weeks post inoculation, animals were sacrificed and all the major organs checked for the generation of tumor metastasis. Metastases were primarily observed in the lungs, as previously reported. We found that injection of B16F10 control cells resulted in the formation of numerous lung colonies, whereas these cells with POU2F1 silenced exhibited significantly suppressed pulmonary metastasis, generating only one third of the number of lung colonies. In addition, B16F10 control cells produced nodules that occupied a higher percentage of the total lung area, while metastatic nodules generated from shPOU2F1 cells were observed as discrete black foci (**Figure 4E**); POU2F1 overexpression led to the opposite outcome. These results imply that POU2F1 silencing did indeed perturb cancer cell metastasis, not only in vitro but also in vivo.

POU2F1 stimulates liver cancer cell progression via the FAT1 signaling pathway

Since POU2F1 has a key role in liver cancer carcinogenesis, we performed a microarray assay to analyze the expression of a panel of genes with different roles in cellular migration and invasion processes by silencing of POU2F1 in HepG2 and SNU-423 cells (**Figure 5A**). Among those genes analyzed, FAT1 was down-regulated in both types of cells and was of particular interest. The results of qPCR and western blotting assays confirmed that the expression of FAT1 was greatly reduced in HepG2 and SNU-423 cells after silencing of POU2F1 (**Figure 5B**). To determine the clinical relevance of alteration in FAT1 expression levels, we first analyzed FAT1 protein expression from clinical specimens based on the human protein atlas (www.proteinatlas.org). FAT1 had strong positive expression in HCC, and weak expression in normal liver (**Figure 5C**). To explore the clinical significance of FAT1, we analyzed the expression of FAT1 in liver cancer using Oncomine (http://www.oncomine.org). The expression of FAT1 in 35 hepatocellular carcinoma tissues was significantly higher than that in normal liver tissues (**Figure 5C**) using Wurmbach Liver statistics [10]. The prognostic value of FAT1 in HCC was analyzed using survival analysis with SurvExpress, an online biomarker validation tool and database for cancer gene expression data. Kaplan-Meier analysis of overall HCC data demonstrated a positive correlation between overexpression of FAT1 and lower overall survival rates (**Figure 5D**). To assess whether FAT1 regulates liver cancer cell metastasis independently, HepG2 and SNU-423 cells were transfected with FAT1-targeting shRNA or a control (shCtrl). Down-regulation of FAT1 expression by the specific shRNA was confirmed by western blot analysis (Supplementary Figure 4). Wound healing assays demonstrated that treatment of cells with FAT1 shRNA significantly decreased their migration in vitro (**Figure 5E**). Using Matrigel invasion assays, FAT1 knock down in HepG2 and SNU-423 cells was demonstrated to significantly suppress cell invasion (**Figure 5F**).

FAT1 has a functional role in POU2F1 regulated HCC metastasis

To determine whether FAT1 is directly involved in POU2F1 function in HCC cells, FAT1 was knocked down using FAT1 specific shRNA (shFAT1) in HepG2 POU2F1 over-expressing cells. The cells were then subjected to MTT and colony formation assays. Reduction of FAT1 levels in HepG2 cells over-expressing POU2F1 led to decreased tumor cell proliferation (**Figure 6A**) and colony formation (**Figure 6B**). Wound healing and Transwell Matrigel invasion assays we-

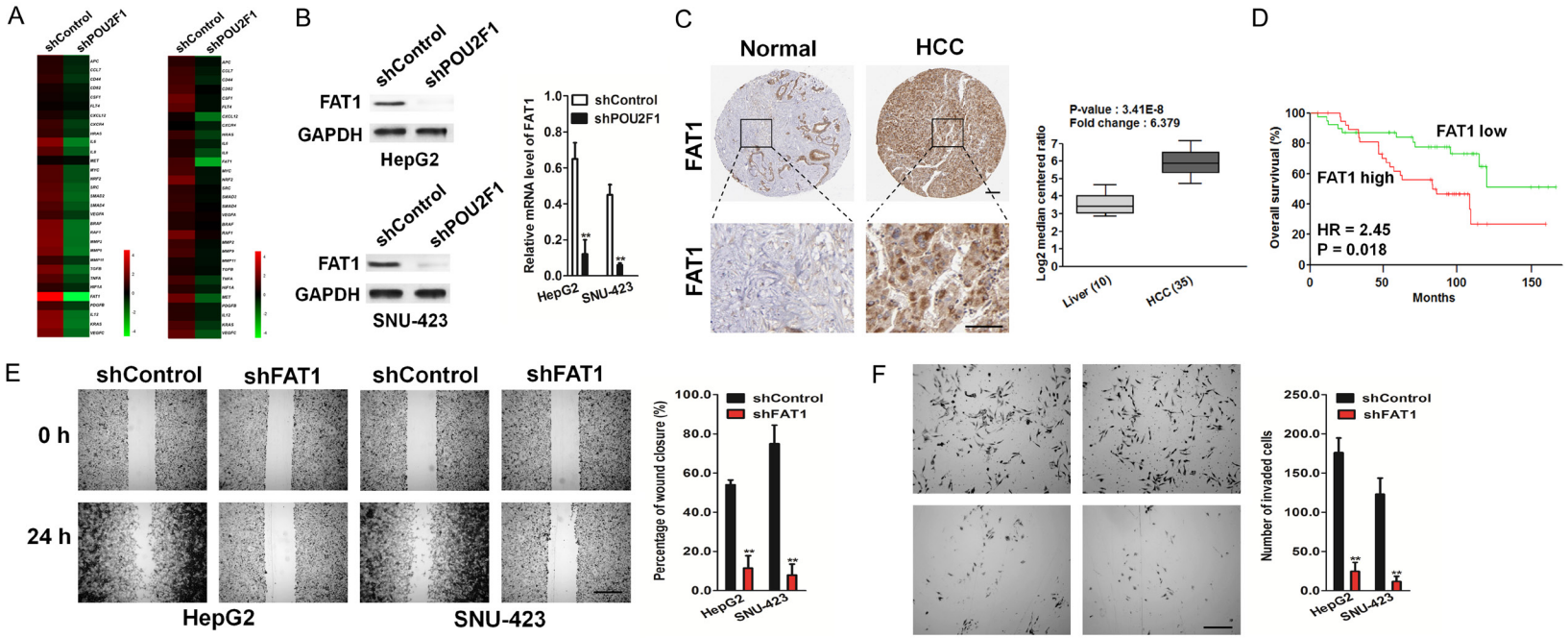
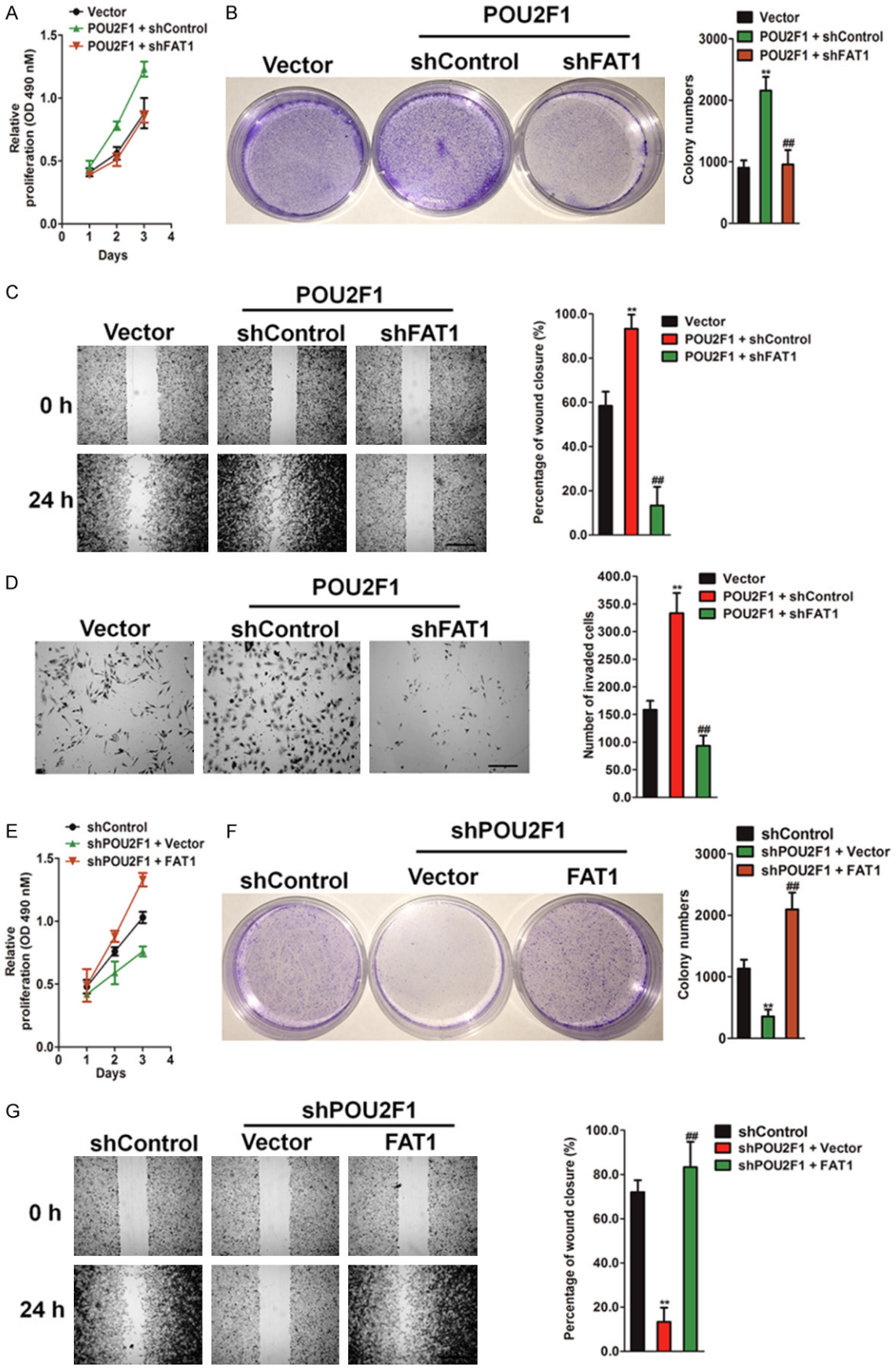


Figure 5. Effects of POU2F1 knockdown on the expression of genes involved in metastatic processes in HCC cells. A. PCR array screening of 31 genes involved in the migration and invasion processes. HepG2 and SNU-423 cells were transfected with shControl or shPOU2F1. After RNA extraction and reverse transcription, the resulting cDNA was applied to the array and amplified. Data are reported as means ± SD of three independent experiments. B. Western blot analysis of total cell lysates from control cells and cells transfected with FAT1 shRNA (left panel). qPCR analysis of FAT1 mRNA levels in indicated cells (right panel). PCR values were normalized to the expression levels of GAPDH. Data are presented as means ± SD from three independent measurements. C. Left panel: FAT1 expression in normal liver tissue and HCC specimens. Images were taken from the Human Protein Atlas (http://www.proteinatlas.org) online database. Scale bar (upper panel), 100 μm. Scale bar (lower panel), 50 μm. Right panel: Box plots generated using gene expression data in Oncomine comparing expression of the FAT1 gene in normal (left plot) and HCC (right plot) tissues. D. Kaplan-Meier plots showing overall survival in HCC. Red, patients with high expression of FAT1; green, patients with low expression of FAT1. P = 0.018. E. Cells were subjected to a wound healing assay. Representative images of wound closure are shown. Scale bar, 200 μm. Results are expressed as means ± SD. F. Cells were subjected to matrigel invasion assays. Representative images of invaded cells are shown. Scale bar, 100 μm. Five fields were counted per experiment. Results are expressed as means ± SD. **P < 0.01 compared with control cells.


A
Vector
POU2F1 + shControl
POU2F1 + shFAT1
Relative proliferation (OD 490 nM)
Days
B
POU2F1
Vector
shControl
shFAT1
Colony numbers
C
Percentage of wound closure (%)
0 h
24 h
D
Number of invaded cells
E
shControl
shPOU2F1 + Vector
shPOU2F1 + FAT1
F
shPOU2F1
shControl
Vector
FAT1
G

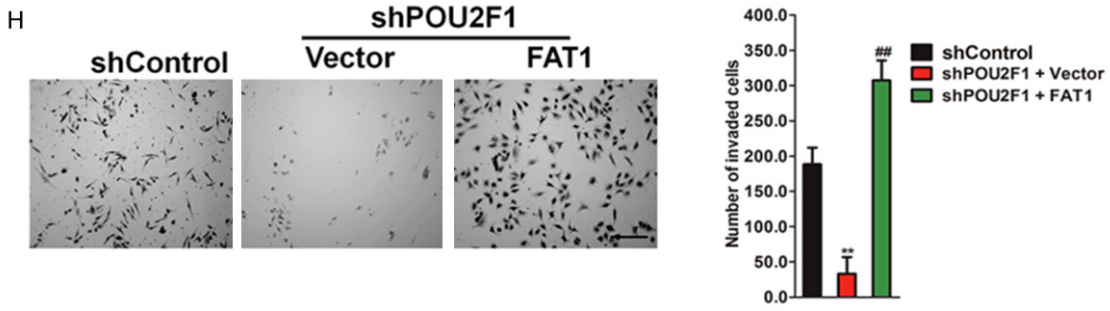


Figure 6. POU2F1 facilities the growth and metastasis of HCC cells via the FAT1 pathway. A. Cells over-expressing POU2F1 were transfected with either shControl or shFAT1 and subjected to MTT proliferation assays. B. Colony formation assays were performed to determine the growth of cells co-transfected with plasmids expressing POU2F1 and shFAT1. Columns in the bar chart (right) represent data collected from three independent experiments. C. HepG2 cells over-expressing POU2F1 were transfected with either shControl or shFAT1. Wound healing assays were performed to assess the migration of indicated cells. Scale bar, 200 μm. D. Representative images of invaded cells are shown. Scale bar, 100 μm. Cells were counted in each of five fields for each experiment, and experiments were performed in triplicate. Results are expressed as means ± SD. E. HepG2 cells treated with shPOU2F1 were transfected with either control vector or a plasmid expressing FAT1. The indicated cells were subjected to MTT growth assays. F. The indicated cells were subjected to colony formation analysis in vitro. Representative images of colonies are shown. G. Co-transfected cells were subjected to wound healing assays. Experiments were performed in triplicate. Results are expressed as means ± SD. Scale bar, 200 μm. H. Transwell assays were conducted in the presence of shPOU2F1, to evaluate cell invasiveness after transfection. Representative images were acquired after staining with crystal violet. Scale bar, 100 μm. Columns in bar chart (right) represent data collected from three independent experiments and are expressed as means ± SD. $^{**}P < 0.01$ compared to shControl cells, $^{\#\#}P < 0.01$ compared to cells co-transfected with shPOU2F1 and vector.

re also performed, and it was demonstrated that reduction of FAT1 reduced migration of POU2F1 over-expressing HCC cells (**Figure 6C**) and their invasiveness (**Figure 6D**) compared with control-transfected HepG2 cells. To confirm the role of FAT1 in POU2F1-mediated cell growth and metastasis, FAT1 was introduced into POU2F1-silenced HepG2 cells. As expected, introduction of FAT1 largely restored the impaired proliferation (**Figure 6E**) and colony formation (**Figure 6F**) in POU2F1-silenced cells. Furthermore, in HepG2 cells treated with POU2F1 shRNA, wound healing and transwell invasion assays were performed in the presence of ectopic FAT1 expression. As shown in **Figure 6G**, **6H**, the POU2F1 shRNA-mediated reductions in cell migration and invasion in vitro were completely restored by FAT1 over-expression. These data indicate that FAT1 signaling is involved in POU2F1 promoted HCC cell metastasis.

Discussion

POU2F1 is a member of the POU homeodomain family, and is expressed ubiquitously [11]. Analyses of information in public databases revealed that POU2F1 showed higher expression levels in kidney, ovary, and esophageal cancer, compared with normal controls, whereas expression of POU2F1 was reduced in some brain tumors, bladder cancer, and liposarcoma. A study that focused specifically on intestinal-type gastric cancer showed that 74% of 42 gastric carcinoma samples displayed increased POU2F1 protein levels. According to our results, POU2F1 protein was over-expressed in HCC tissues compared with adjacent non-cancerous liver tissue specimens. These observations indicate that it may be possible to use expression levels of POU2F1 as a diagnostic tool to distinguish HCC tissues from non-malignant liver tissues. Furthermore, over-expression of POU2F1 in HCC correlated significantly with poor prognoses of patients with HCC.

POU2F1 has recently been suggested to play a crucial role in the tumorigenesis of several types of human cancer, and can act, not only as an oncogene, but also as a tumor suppressor gene in different cancers. Therefore, a logical hypothesis is that POU2F1 may act as an important oncogene contributing to the tumorigenesis and progression of HCC. To confirm this hypothesis, a series of in vitro and in vivo experiments, including proliferation, colony forma-

tion, migration, and invasion assays, was employed to investigate the role of POU2F1 in HCC cell growth, migration, and invasion. The results demonstrate that POU2F1 knockdown inhibits malignant phenotypes, such as proliferation, colony formation, migration, and invasion in vitro. In contrast, the ectopic over-expression of POU2F1 in HCC cells substantially promoted their aggressive phenotypes. These data support our hypothesis that POU2F1 may function as an oncogene in HCC, expanding on its previously reported role as a tumor suppressor in other cancers.

The pathways through which POU2F1 may be regulated and how POU2F1 stimulates HCC cell metastasis remain unclear and should be the subject of future investigations. A recent study showed that POU2F1 regulates expression of the metastasis genes Twist1, Snai1, and Snai2, during hypoxia-dependent loss of PER2 in breast cancer. The transcription factors encoded by these genes, including Twist1, Snai1, Snai2, and ZEB1, are implicated in E-cadherin transcriptional suppression, as well as Vimentin activation, leading to cell migration and invasion. However, the molecular mechanisms underlying POU2F1-induced metastasis in HCC remain unknown. In this study, we examined the possible pathways through which POU2F1 participates in HCC cell metastasis. We detected a significant association between the expression levels of FAT1 and those of POU2F1 in HCC cells. The cadherin gene, FAT1, located on chromosome 4q34-35 within a region frequently deleted in human cancers, encodes a large protein with extracellular cadherin repeats [12]. In solid tumors, aberrant expression of FAT1 is associated with disease progression. FAT1 regulates cell growth and migration through specific protein-protein interactions via its cytoplasmic tail, and deregulated FAT1 expression is associated with different human diseases, including cancer [13]. FAT1 suppresses cancer cell growth by binding β-catenin and antagonizing its nuclear localization, while silencing of FAT1 results in up-regulation of Wnt/β-catenin target genes, such as c-myc and cyclin D1, promoting tumor growth [14]. Our results demonstrate that FAT1 is also a key effector of POU2F1 in the regulation of malignant phenotypes in HCC. FAT1 is overexpressed in a variety of cancers and/or cancer cell lines, including lymphoma, and testicular, colorectal, and breast cancers. Indeed, our analysis of TCGA gene expression data demonstrated that FAT1 is overexpressed at least 6-fold in HCC, compared with normal liver tissues. Importantly, we provide evidence that FAT1 regulates HCC cell growth and invasion in vitro and that it is functionally important in POU2F1 mediated cell growth and metastasis.

In conclusion, up-regulation of POU2F1 correlates with poor prognoses in patients with HCC. A novel role for POU2F1 in HCC tumorigenesis and progression was elucidated in this study, which demonstrated that up-regulation of POU-2F1 stimulates HCC cell growth, colony formation, migration, and invasion via FAT1 signaling. Additional studies are clearly warranted to elucidate the mechanism by which POU2F1 regulates this pathway, and to validate the utility of POU2F1 as a therapeutic target in HCC.

Disclosure of conflict of interest

None.

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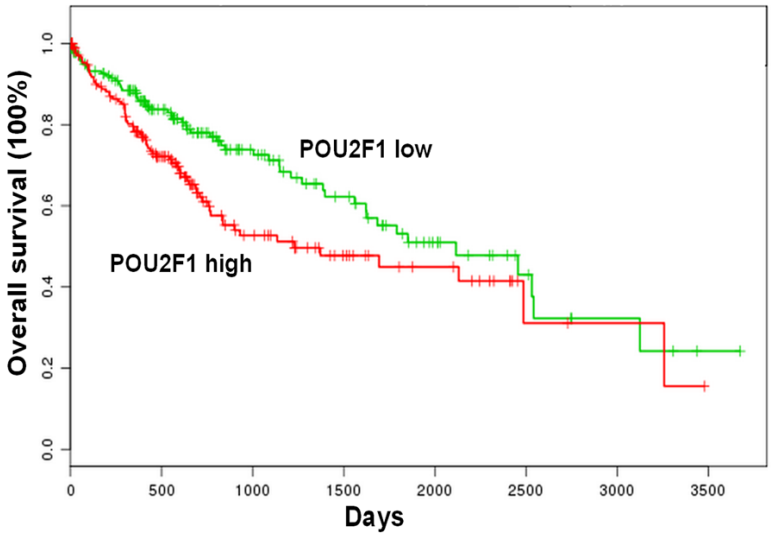
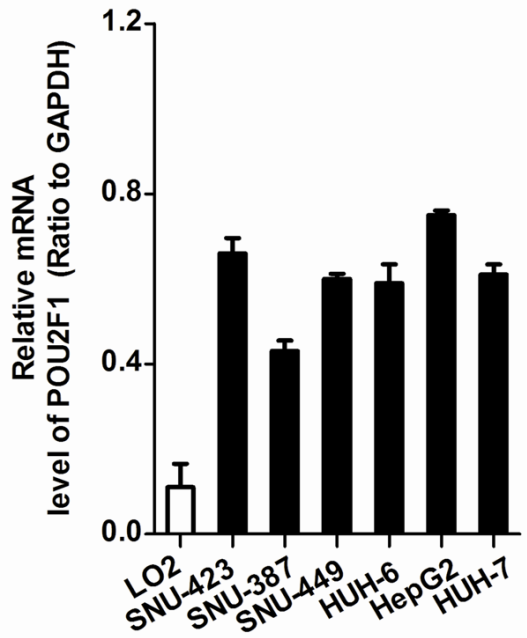
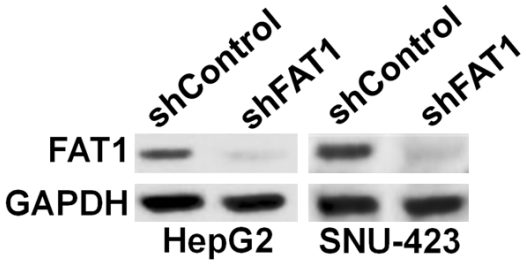


Supplementary Figure 1. Kaplan-Meier plots showing overall survival in HCC. In red: patients with high expression of POU2F1 and in black, patients with low expressions of POU2F1. Hazard Ratio = 1.54, P = 0.001.



Supplementary Figure 2. qPCR analysis of POU2F1 mRNA levels in LO2 and various HCC cell lines. PCR values were normalized to the levels of GAPDH. Data are presented as the mean ± SD from three independent measurements.

Supplementary Figure 3. Western blot analysis of total cell lysates obtained from control cells and cells transfected with POU2F1shRNA or POU2F1 over-expressing vector.



Supplementary Figure 4. Western blot analysis of total cell lysates obtained from control cells and cells transfected with FAT1 shRNA.