

Original Article

Targeting FAM64A: a promising strategy to halt liver cancer angiogenesis and enhance immunity

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Abstract: Liver cancer, a primary malignancy of liver cells, exhibits high morbidity and mortality. Family with sequence similarity 64, member A (FAM64A) acts as an oncogene and modulates tumor progression in multiple cancers. However, the specific role of FAM64A in liver cancer development remains poorly elucidated. Thus, this work uncovered the action of FAM64A on liver cancer pathogenesis and cancer immunity and reveals the underlying mechanism. In this study, the FAM64A expression in liver cancer and its association with the overall survival were investigated using bioinformatics analysis. Tumor tissues and adjacent normal tissues were obtained from 64 liver cancer cases. Cell viability, invasion, and angiogenesis were assessed by CCK-8 method, Transwell invasion assay, and tube formation assay, respectively. The secretion levels of IFN- γ , IL-2, IL-10 and TGF- β were tested utilizing the ELISA assay. A xenograft tumor was created to monitor tumor growth *in vivo*. Results revealed that FAM64A expression was elevated in liver cancer. Clinically, high FAM64A levels correlated with shorter overall survival. Silencing of FAM64A restricted the malignant phenotypes of liver cancer cells, including proliferation, invasion, angiogenesis and immune escape. Furthermore, FAM64A knockdown restricted the activation of the VEGFA/AKT signaling. Conversely, VEGFA overexpression offset the above influences of FAM64A on liver cancer cells. Moreover, silenced FAM64A suppressed tumor growth, decreased CD31 and PD-L1 levels, increased CD8 and IFN- γ level, and reduced activation of the VEGFA/AKT signaling. Taken together, FAM64A is up-regulated in liver cancer and closely correlated with poor prognosis. Silenced FAM64A restrained proliferation ability, invasion capacity, angiogenesis and immune escape of liver cancer cells through inactivating the VEGFA/AKT signaling. FAM64A may be a valuable candidate target for liver cancer therapy.

Keywords: Liver cancer, FAM64A, invasion, angiogenesis, immune escape, VEGFA/AKT pathway

Introduction

Liver cancer is a primary malignant tumor originating from hepatocytes, accounting for over 80% of global cancer patients [1, 2]. It consists of hepatocellular carcinoma (HCC) and intrahepatic cholangiocarcinoma (iCCA) [3]. Liver cancer is the sixth most common cancer, with approximately 906,000 newly diagnosed cases recorded in 2020 [4]. In 2022, liver cancer caused over 750,000 deaths globally, ranking it the third major cause of cancer-related mortality [3]. Current treatments for liver cancer mainly include surgical resection, targeted the-

rapy and liver transplantation [5]. Nevertheless, the prognosis for liver cancer remains unsatisfactory, especially in advanced stages with a one-year mortality rate exceeding 90% [6]. Accordingly, it is essential to elucidate the mechanisms underlying liver cancer pathogenesis and develop effective therapeutic strategies for liver cancer.

Immunotherapy is considered a promising cancer treatment strategy following surgery, radiation therapy and chemotherapy [7]. However, the complex nature of the tumor microenvironment exacerbates the difficulties related to

immunotherapy [8]. Tumor microenvironment is a highly structured ecosystem and contains cancer cells and mesenchymal cells, such as immune cells, cancer-associated fibroblasts, endothelial cells and pericytes [9]. The tumor microenvironment is crucial for tumor cells to progress from malignant growth to immune escape [9]. Immune escape refers to the phenomenon in which tumor cells evade immune recognition and attack through various mechanisms, enabling them to survive and proliferate [10]. Immune escape is predominantly mediated by downregulating major histocompatibility complex class I (MHC), up-regulating inhibitory ligands and secreting immunosuppressive cytokines, ultimately impairing effector T cell activity and cytotoxicity [11]. Immune escape is widely recognized as the primary barrier to anti-cancer therapy [12]. Thus, blocking immune escape may improve the efficacy of cancer immunotherapy.

Family with sequence similarity 64, member A (FAM64A) (also known as CATS, RCS1, or PIM-REG) is a clathrin assembly lymphoid myeloid leukemia gene (CALM)-interacting protein expressed in mitogen responses [13]. FAM64A expression is cell cycle-dependent and strongly associated with cell proliferation [14]. Emerging evidence has revealed that FAM64A acts as an oncogene and participated in cancer progression [15-17]. For example, Zhao *et al.* proved that FAM64A accelerated ovarian cancer proliferation and metastasis [15]. Interestingly, Zhang *et al.* found that elevated FAM64A expression was closely correlated with a poor prognosis in liver cancer and promoted the aggressive phenotypes of liver cancer cells [18]. Additionally, FAM64A has been shown to involve in immune regulation [16, 19]. Zhou *et al.* revealed that FAM64A affected the immune mechanism and IFN-signaling pathway in prostate cancer [16]. Consequently, FAM64A was presumed to regulate cancer immune escape. However, the precise role of FAM64A in liver cancer immunity remained poorly defined.

This work aimed to elucidate the role of FAM64A in the pathogenesis and immunity of liver cancer by investigating cell proliferation, invasion capacity, angiogenesis and immune escape *in vitro* and *in vivo* and reveal the underlying molecular mechanism.

Materials and methods

Bioinformatics analysis

The expression of FAM64A in liver cancer was explored using clinical data from the GEO database (GSE144269, GSE14520 and GSE54236) and TCGA database. The association of FAM64A expression with overall survival of liver cancer cases was investigated utilizing the online K-M plotter (<https://kmplot.com/analysis/>) and the GEO database (GSE54236 and GSE116174). The relationship between FAM64A and VEGFA expression was analyzed on the online database Gene Expression Profiling Interactive Analysis (GEPIA) using The Cancer Genome Atlas (TCGA) gene expression data of liver cancer cases.

Clinical samples

Tumor and adjacent normal tissues of sixty-four liver cancer cases that received tumor resection surgery at The First People's Hospital of Kunming were collected. Peripheral blood samples were collected from all cases 1 month prior to surgery. Cases that underwent chemotherapy or radiotherapy before surgery and had incomplete clinical data and follow-up records were excluded from this study. All enrolled participants signed informed consent. This research was approved from the Ethics Committee of The First People's Hospital of Kunming (No. YLS2025-005).

Cell culture

Transformed human liver epithelial-2 (THLE-2) cells, mouse liver cancer cells Hepa 1-6 and human liver cancer cells Huh7, Hep3B, HCCLM3, and SNU449 were offered by American Type Culture Collection (ATCC). THLE-2 cells were kept in BEGM medium containing 10% FBS. Hep3B cells were cultured in EMEM medium plus 10% FBS. Hepa 1-6, Huh7, SNU449, and HCCLM3 cells were grown in DMEM containing 10% FBS. All cells were maintained at the 37°C with 5% CO₂ atmosphere. In indicated assays, SNU449 cells were treated with 2.5 µg/mL of AKT inhibitor MK-2206 for 24 h.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNAs were obtained from liver cancer tissues and cells utilizing TRIzol reagent and sub-

jected to generate cDNA utilizing the First Strand cDNA Synthesis Kit (Yeasen, Shanghai, China). The qPCR assay was executed utilizing the qPCR SYBR Green Master Mix (Yeasen, Shanghai, China) following the manufacturer's instructions. The primers were: FAM64A: F, 5'-CCATTACGGCGATCAAGG-3'; R, 5'-CACAGGC-TCTAGGTCCT-3' [20]; LAG3: F, 5'-GCAGTGT-ACCTCAGAGCTGTC-3'; R, 5'-AAGCCAAAGGC-TCCAGTCACCA-3'; TIGIT: F, 5'-TGGTGGTCATCT-GCACAGCAGT-3'; R, 5'-TTTCTCCTGAGGTACCT-TCCAC-3'. VEGFA: F, 5'-TTGCCTTGCTGCTCTAC-CTCCA-3'; R, 5'-GATGGCAGTAGCTGCGCTGATA-3'. The PCR condition was: 95°C (5 min), 40 cycles of 95°C (10 s) and 60°C (30 s). The relative expression of FAM64A was normalized to *GAPDH* utilizing the $2^{-\Delta\Delta Ct}$.

Western blot

Liver cancer tissues and cells were lysed utilizing RIPA lysis (Yeasen, Shanghai, China), and protein concentration was determined using the BCA assay. Lysates were resolved in SDS-PAGE gel and transferred to a PVDF membrane. After blockade, the membranes were probed to anti-FAM64A (1:1000, H00054478-D01P, Thermo Fisher), PD-L1 (1:1000, ab205921, Abcam), VEGFA (1:1000, ab32152, Abcam), p-PI3K (1:1000, PA5-104853, Thermo Fisher), PI3K (1:1000, PA5-29220, Thermo Fisher), p-AKT (1:1000, PA5-104445, Thermo Fisher) and AKT (1:1000, RAB01302, Thermo Fisher) antibodies for 12 h at 4°C, followed by incubating with IgG H&L (HRP) (Abcam, Cambridge, MA, UK). The bands were checked utilizing the ECL Chemiluminescent Substrate Kit (Yeasen, Shanghai, China) and analyzed utilizing Image J (NIH, Bethesda, MD, USA).

Cell transfection

The shRNA against FAM64A (sh-FAM64A) and scramble shRNA (sh-NC) were acquired from GenePharma (Shanghai, China) and combined with the lentiviral vector pLKO.1. The 293T cells were co-introduced with the cloned pLKO.1, psPAX2 and pMD2G utilizing Lipofectamine 3000 (Invitrogen, MA, USA) and viral supernatants were collected after 48 h. Huh7 and Hep3B cells were then infected with the harvested virus to obtain stably transfected cells using 8 µg/mL polybrene.

To overexpress VEGFA in Huh7 cells, the full-length VEGFA plasmid (pcDNA3.1-VEGFA) was acquired from GenePharma (Shanghai, China) and transfected into Huh7 and Hep3B cells utilizing Lipofectamine 3000 following the supplier's instructions. To overexpress FAM64A in SNU449 cells, the full-length FAM64A plasmid (pcDNA3.1-FAM64A) was acquired from GenePharma (Shanghai, China) and transfected into SNU449 cells utilizing Lipofectamine 3000. To overexpress FOXM1 or STAT3 in Huh7 cells, the full-length FOXM1 plasmid (pcDNA3.1-FOXM1) or was full-length STAT3 plasmid (pcDNA3.1-STAT3) acquired from GenePharma (Shanghai, China) and transfected into Huh7 cells utilizing Lipofectamine 3000.

Cell counting kit 8 (CCK-8) assay

Huh7 and Hep3B cells were seeded in 96-well plates (1×10^4 cells/well) and maintained for 24 h. After that, CCK-8 (10 µL) (Yeasen, Shanghai, China) was added to cells for incubation. After 4 h, cell viability was measured by detecting the OD450 utilizing a microplate reader (Multiskan FC, Thermo Fisher Scientific, MA, USA).

Transwell invasion assay

Huh7, Hep3B or SNU449 cells (1×10^5) were plated in the top side of the Transwell which was pre-coated with Matrigel and maintained in culture medium devoid of serum. The lower chamber was immersed in medium containing 10% FBS. After 24 h, the invaded cells were dyed with crystal violet for 15 min. Different views were randomly selected and observed under an inverted microscope (ECLIPSE Ts2, Nikon, Tokyo, Japan).

Tube formation assay

A quantity of 1×10^5 Huh7, Hep3B, or SNU449 cells was introduced into the 6-well plates (1×10^5 cells/well) and maintained overnight. The medium was then replaced by fresh medium plus 2% FBS. After 48 h, cell supernatants were harvested and utilized as a conditioned medium for the tube formation assay after centrifugation at $400 \times g$ for 5 min at 4°C. HUVECs were plated in the 96-well plates (3×10^4 cells/well), which were pre-coated with Matrigel and cultured in conditioned medium for 24 h. The results were recorded utilizing a microscope

(ECLIPSE Ts2, Nikon, Tokyo, Japan) and quantified utilizing Image J.

Preparation of PBMCs and co-culture system

Peripheral blood mononuclear cells (PBMCs) were separated from peripheral blood utilizing the Ficoll density separation method. PBMCs were then activated by incubating with anti-CD3 antibody (2 µg/ml), anti-CD28 antibody (5 µg/ml), and IL-2 (100 U/ml). A co-culture system was prepared utilizing Transwell chambers. Activated PBMCs and Huh7 or Hep3B cells were respectively placed in the lower and upper transwell chambers with a ratio of 5:1 at 37°C incubator with 5% CO₂. After 48 h of co-culture, cells were washed with PBS and subjected to subsequent analysis.

Flow cytometry

The PBMCs were resuspended in PBS, prepared into the cell suspension (1×10⁷ cells/mL) and stained with 1 µl of anti-Tim-3-FITC antibody and anti-CD8-APC antibody (Thermo Fisher Scientific, Waltham, MA) in 100 µl of PBS for 30 min at 4°C without light. After centrifuging at 1000 rpm for 5 min, PBMCs were analyzed using a FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA).

For detecting MHC-I expression on Huh7 and Hep3B cells, Huh7 and Hep3B cells were stained with FITC-labeled HLA-ABC antibody (BioLegend, San Diego, CA, USA) for 30 min at 4°C without light. After centrifuging at 1000 rpm for 5 min, cells were analyzed using a FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA).

ELISA assay

PBMCs were co-cultured with Huh7 and Hep3B cells for 48 h. After that, the cell supernatant from PBMCs was harvested to assess the IFN-γ, IL-2, IL-10 and TGF-β secretion levels following the instructions of the corresponding ELISA kits (Proteintech, Wuhan, China).

Cell apoptosis

Cell apoptosis was assessed utilizing the Annexin V-FITC Apoptosis Detection Kit (Beyotime, Shanghai, China). Huh7 and Hep3B cells (1×10⁵) were harvested and resuspended us-

ing 195 µL binding buffer. After that, 5 µL Annexin V-FITC and 10 µL PI were added to cells for 15 min incubation in the dark. Cell apoptosis was analyzed by applying the FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA).

Protein degradation assay

After Huh7 cells were transfected with the sh-FAM64A using Lipofectamine 3000 for 40 h, the cells were treated with cycloheximide (CHX, 50 µg/ml) (Sigma, St Louis, MO, USA) for 2 h, 4 h, and 8 h, respectively [21]. After that, cells were subjected to western blot analysis to monitor the remaining levels of VEGFA. Quantifications were normalized to the untreated time point.

Co-immunoprecipitation (Co-IP) assay

Huh7 cells were lysed utilizing RIPA lysis (Yeasen, Shanghai, China). After centrifugation at 14000×g for 20 min at 4°C, the supernatants were treated with primary antibodies including FAM64A (H00054478-D01P, Thermo Fisher), FOXM1 (ab264210, Abcam), STAT3 (ab32500, Abcam) antibodies with gentle rocking overnight at 4°C. Subsequently, the immunocomplexes were incubated with the pre-washed protein A/G magnetic beads (EpiZyme) for 8 h with gentle rocking. After washing step, the antigen-antibody-bead complexes were resuspended with SDS sample buffer. The samples were heated to 100°C for 10 min and applied to western blot.

Chromatin immunoprecipitation (ChIP) assay

ChIP assay was performed using the EZ ChIP Kit (Millipore, Billerica, MA, USA) following the supplier's protocols. Huh7 cells were cross-linked with 1% formaldehyde for 10 min and quenched with 125 mM glycine for 5 min. After washing using PBS, Huh7 cells were treated by lysis buffer and sonicated for 10 min to shear chromatin to 200-500 bp fragments. The lysates were treated with anti-FOXM1 antibody or negative control anti-IgG antibody at 4°C overnight. Thereafter, Protein G Agarose was added to each IP and incubated for 2 h at 4°C with rotation. After washing step, the agarose was collected and reversed cross-links to free DNA with NaCl at 65°C overnight. The DNA was

purified using spin columns and determined utilizing qPCR assay.

Xenograft tumor experiment

Twelve C57BL/6 mice aged 6-8 weeks were offered by the Beijing Laboratory Animal Research Center (Beijing, China) and allocated to the sh-NC and sh-FAM64A groups. Hepa 1-6 cells were stably transfected with sh-NC and sh-FAM64A. To create xenograft tumors, C57BL/6 mice underwent a subcutaneous implantation of Hepa 1-6 cells (5×10^6) on the left flank. Tumor volume was gauged every 3 days from day 3 to 15 using the formula: $V = (\text{Length} \times \text{Width}^2)/2$. At the endpoint, animals were euthanized using CO₂ inhalation, followed by the excision of tumor tissues. The animal study was carried out in accordance with the national and international regulations and policies and authorized by the Ethics Committee of The First People's Hospital of Kunming (No. YLS2025-005).

Immunohistochemistry

Tumor tissues were processed by fixation with 4% PFA, followed by embedding in paraffin and sectioning into 5 µm slices. After deparaffinized using xylene, rehydrated in decreased concentrations of ethanol, heated for antigen retrieval and treated with 3% H₂O₂, the samples were probed to anti-FAM64A (1:200, MBS9144896, Biocampare), CD31 (1:200, ab281583, Abcam), PD-L1 (1:200, AB213480, Abcam), CD8 (1:200, ab316778) and IFN-γ (1:200, 29788-1-AP, Proteintech) antibodies overnight, followed by labelling with goat anti-rabbit IgG (Abcam, Cambridge, MA, UK) for 1 h and staining with 3, 3'-diaminobenzidine (DAB). The results were acquired utilizing a microscope (ECLIPSE Ts2, Nikon, Tokyo, Japan).

Statistical analysis

All data obtained from at least triplicates were presented as mean ± standard deviation (SD). Statistical analyses were performed utilizing GraphPad Prism 8.0.2 (GraphPad Software, Inc., USA). The paired *t*-test was used for comparative analysis of tumor tissues and paired normal tissues in liver cancer. Survival analyses were carried out utilizing Kaplan-Meier analysis with the log-rank test. The chi-square test was applied to assess the correlation

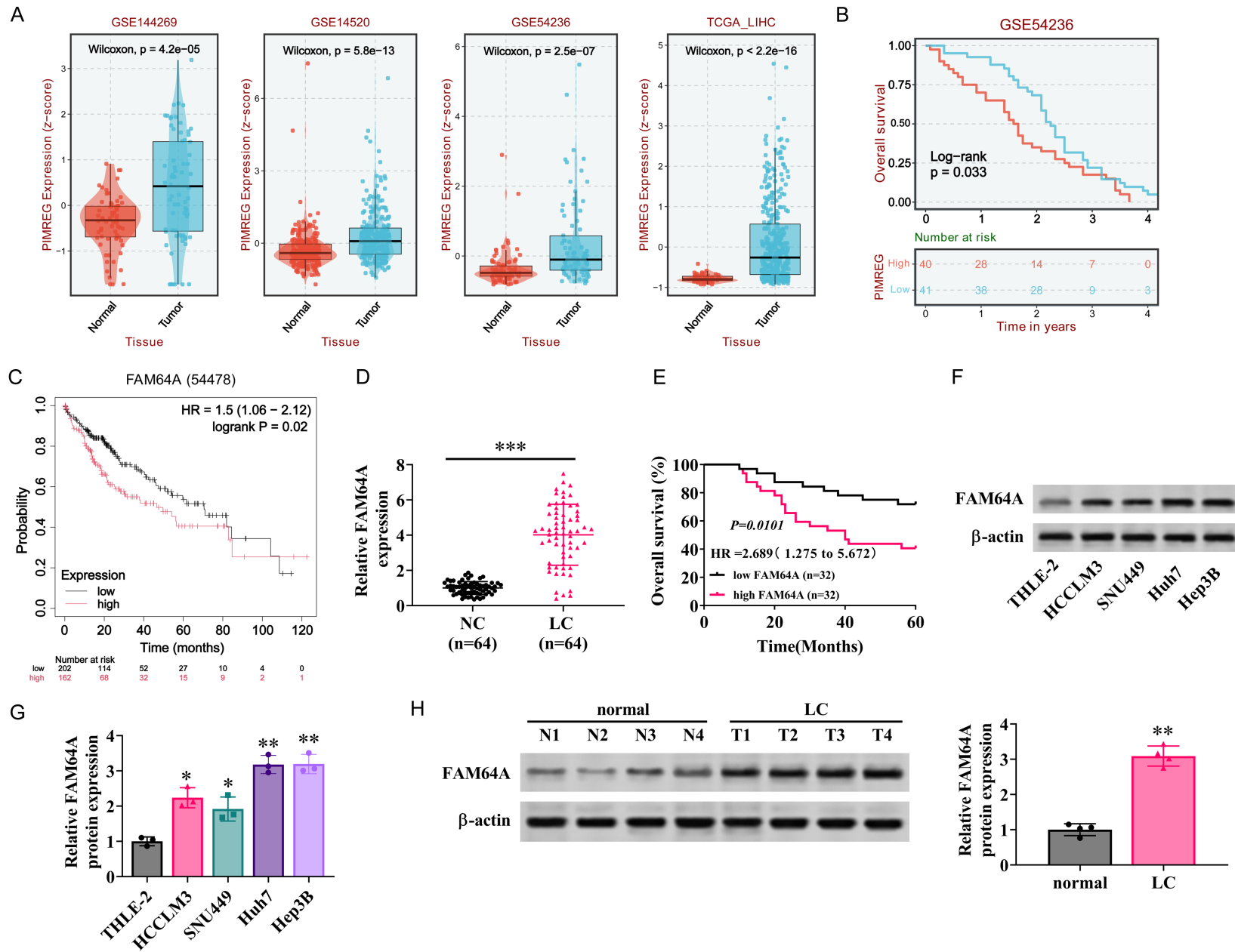
between FAM64A and the clinical pathological features of liver cancer patients. For other comparison procedures, the Student's *t*-test (non-paired) and One-way ANOVA with Tukey's *post hoc* test were utilized. Comparison of data at different time points was conducted utilizing repeated-measures ANOVA followed by Dunnett's *post hoc* test. Statistical significance was designated as $P < 0.05$.

Results

FAM64A is elevated in liver cancer and correlated to poor prognosis

To identify the function of FAM64A in liver cancer pathogenesis, the FAM64A expression in liver cancer was analyzed. Based on data from the GEO and TCGA databases, FAM64A was significantly up-regulated in liver cancer tissues compared with normal tissues ($P < 0.001$, **Figure 1A**). Liver cancer patients with high FAM64A expression exhibited poor overall survival ($P < 0.05$, **Figure 1B** and **1C**). Thereafter, these bioinformatics analysis results were validated in 64 liver cancer cases. Results showed that FAM64A mRNA and protein levels were significantly elevated in tumor tissues compared to paired normal tissues ($P < 0.01$, **Figure 1D** and **1H**). Besides, 64 liver cancer patients were allocated into the high FAM64A group ($n=32$) and low FAM64A group ($n=32$) utilizing the median value as a cutoff. The significance of FAM64A level and the clinical features of liver cancer were compared. Survival analysis demonstrated a significant prognostic correlation, with high FAM64A cases presenting shorter overall survival ($P=0.0101$, **Figure 1E**). Additionally, the FAM64A expression was correlated with lymph node involvement and TNM stage in liver cancer cases ($P < 0.05$), whereas no significant association was observed with age, gender, tumor size, or HBV infection (**Table 1**). Furthermore, the expression of FAM64A was also measured in liver cancer cells. Results showed that the expression of FAM64A was obviously enhanced in HCCLM3, SNU449, Huh7 and Hep3B cells compared to THLE-2 cells ($P < 0.05$, **Figure 1F** and **1G**). Notably, FAM64A presented higher expression in Huh7 and Hep3B cells compared to HCCLM3 and SNU449 cells (**Figure 1F** and **1G**). Accordingly, Huh7 and Hep3B cells were applied for subsequent assays. Therefore, FAM64A was

FAM64A regulates the liver cancer development



FAM64A regulates the liver cancer development

Figure 1. FAM64A is elevated in liver cancer and associated with poor prognosis. A. The expression of FAM64A in liver cancer was analyzed using the data from the GEO and TCGA databases. B. The correlation between FAM64A expression and overall survival of liver cancer cases was determined using the GEO data. C. K-M plotter survival analysis of liver cancer cases with low and high FAM64A expression was conducted. D. The expression of FAM64A in tumor tissues and adjacent normal tissue of 64 liver cancer cases was determined utilizing qRT-PCR. ***: $P<0.001$ versus NC group. E. The relationship between the FAM64A expression and the overall survival of liver cancer cases was determined utilizing Kaplan-Meier analysis. F, G. The protein level of FAM64A in transformed human liver epithelial-2 (THLE-2) cell line and human liver cancer cell lines Huh7, Hep3B, HCCLM3 and SNU449 were measured utilizing western blot. *: $P<0.05$, **: $P<0.01$ versus THLE-2 cells. H. The protein level of FAM64A in tumor tissues and adjacent normal tissue of 4 liver cancer cases was measured utilizing western blot. **: $P<0.01$ versus NC group.

Table 1. Correlation between FAM64A expression and the clinical pathological features of 64 liver cancer patients

Characteristic	All cases	FAM64A expression		P-value
		Low (n = 32)	High (n = 32)	
Age (years)				0.599
<55	42	20	22	
≥55	22	12	10	
Gender				0.614
Female	36	17	19	
Male	28	15	13	
Tumour size				0.211
<5 cm	31	18	13	
≥5 cm	33	14	19	
Lymph node involvement				0.012*
Absent	30	20	10	
Present	34	12	22	
TNM				0.042*
I+II	38	23	15	
III+IV	26	9	17	
HBV infection				0.316
Yes	34	19	15	
No	30	13	17	

A chi-square test was used for comparing groups between low and high FAM64A expression. * $P<0.05$.

enhanced in liver cancer and associated with poor prognosis.

Silenced FAM64A restricts the proliferation, invasion and angiogenesis of liver cancer cells

To assess the role of FAM64A in liver cancer pathogenesis, specific shRNAs targeting FAM64A were engineered. Three kinds of sh-FAM64A significantly suppressed FAM64A level in Huh7 and Hep3B cells ($P<0.05$, **Figure 2A**). Since the sh-FAM64A#1 exhibited the highest knockdown efficiency, it was selected for subsequent experiments. Functional assays revealed that knockdown of FAM64A reduced the viability and invasion capacity of Huh7 and Hep3B cells

($P<0.01$, **Figure 2B** and **2C**). Besides, HUVECs were cultured in the conditioned medium collected from Huh7 and Hep3B cells with FAM64A knockdown. Results proved that silencing of FAM64A impaired the tube formation ability of co-cultured HUVECs cells ($P<0.01$, **Figure 2D**). To further assess the action of FAM64A on liver cancer, the FAM64A overexpression plasmid was introduced into SNU449 cells. Results revealed that the FAM64A overexpression plasmid significantly elevated the FAM64A level ($P<0.01$, [Supplementary Figure 1A](#)). Overexpressed FAM64A promoted invasion ability of SNU449 cells and drove the tube formation ability of co-cultured HUVECs cells ($P<0.01$, [Supplementary Figure 1A-C](#)). Thus, silenced FAM64A restricted the proliferation, invasion, and angiogenesis of liver cancer

cells and overexpressed FAM64A promoted liver cancer cell malignant phenotypes.

Silenced FAM64A restricts immune escape in liver cancer cells

To substantiate the contribution of FAM64A to liver cancer progression, immune escape was assessed after co-culturing PBMCs with Huh7 and Hep3B cells that silenced FAM64A for 24 h. Results showed that silenced FAM64A enhanced the secretion levels of IFN- γ and IL-2 in co-cultured PBMCs ($P<0.01$, **Figure 3A**). Besides, FAM64A knockdown reduced IL-10 and TGF- β levels in co-cultured PBMCs ($P<0.05$, **Figure 3B**). Tim-3 was the early exhaustion-

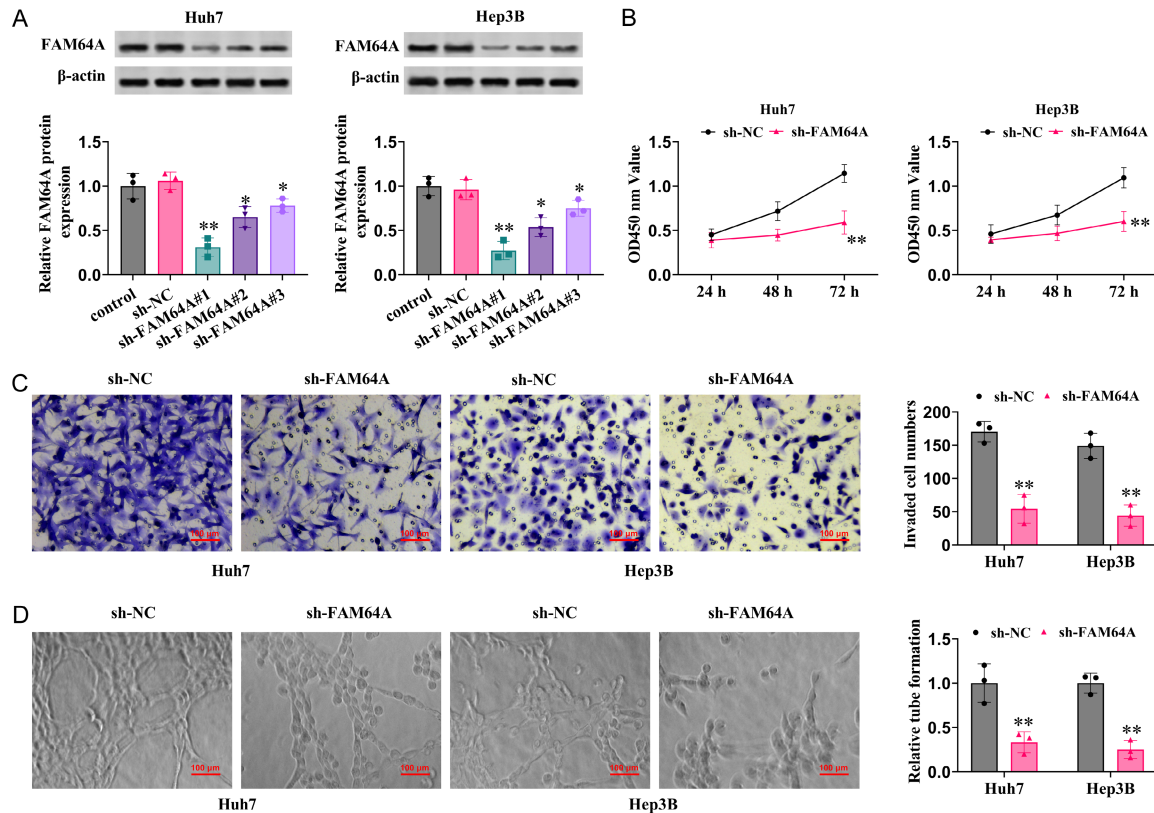


Figure 2. Silenced FAM64A restricts the proliferation, invasion and angiogenesis of liver cancer cells. Huh7 and Hep3B cells were transfected with sh-FAM64A. A. The protein level of FAM64A was measured utilizing western blot. B. Cell viability was determined by CCK-8 assay. C. Cell invasion ability was assessed using Transwell invasion assay. Scale bar: 100 μ m; magnification: 200 $\times$. D. The angiogenesis ability of HUVECs after cultured with conditioned medium collected from Huh7 and Hep3B cells was evaluated using the tube formation assay. Scale bar: 100 μ m; magnification: 200 $\times$. *: $P < 0.05$, **: $P < 0.01$ versus sh-NC group.

associated molecule. Thus, the proportion of Tim-3⁺CD8⁺ cells in co-cultured PBMCs was detected. Results found that silenced FAM64A reduced the proportion of Tim-3⁺CD8⁺ cells in co-cultured PBMCs ($P < 0.01$, **Figure 3C**). Furthermore, FAM64A knockdown significantly reduced the mRNA levels of early exhaustion-associated molecules LAG3 and TIGIT in purified CD8⁺ T cells ($P < 0.05$, **Figure 3D, 3E**). Silenced FAM64A also reduced the PD-L1 level of Huh7 and Hep3B cells ($P < 0.01$, **Figure 3F**). In contrast, overexpressed FAM64A elevated the PD-L1 level of SNU449 cells ($P < 0.01$, **Supplementary Figure 1D**). Meanwhile, silenced FAM64A enhanced MHC-I expression on the cell surface of Huh7 and Hep3B cells ($P < 0.01$, **Figure 3G**). Moreover, silenced FAM64A promoted the apoptosis of Huh7 and Hep3B cells after co-culture ($P < 0.01$, **Figure 3H**). Hence, silenced FAM64A restricted immune escape in liver cancer cells.

Silenced FAM64A restricts the activity of the VEGFA/AKT pathway in liver cancer

To ascertain the specific mechanism responsible for the regulatory roles of FAM64A in liver cancer pathogenesis, the pathway analysis was conducted. Analysis on GEPIA using expression data of liver cancer cases from the TCGA revealed a positive association between the expressions of FAM64A and VEGFA ($P = 0.0018$, **Figure 4A**). Besides, silenced FAM64A suppressed the mRNA expression of VEGFA (**Supplementary Figure 2A**). Silenced FAM64A suppressed VEGFA protein level and inhibited the phosphorylation levels of PI3K and AKT ($P < 0.01$, **Figure 4B and 4C**). Additionally, results showed no significant difference in VEGFA protein degradation rate after FAM64A knockdown compared with the control group (**Supplementary Figure 2B**). FAM64A is not a typical transcription factor, but it can regulate

FAM64A regulates the liver cancer development

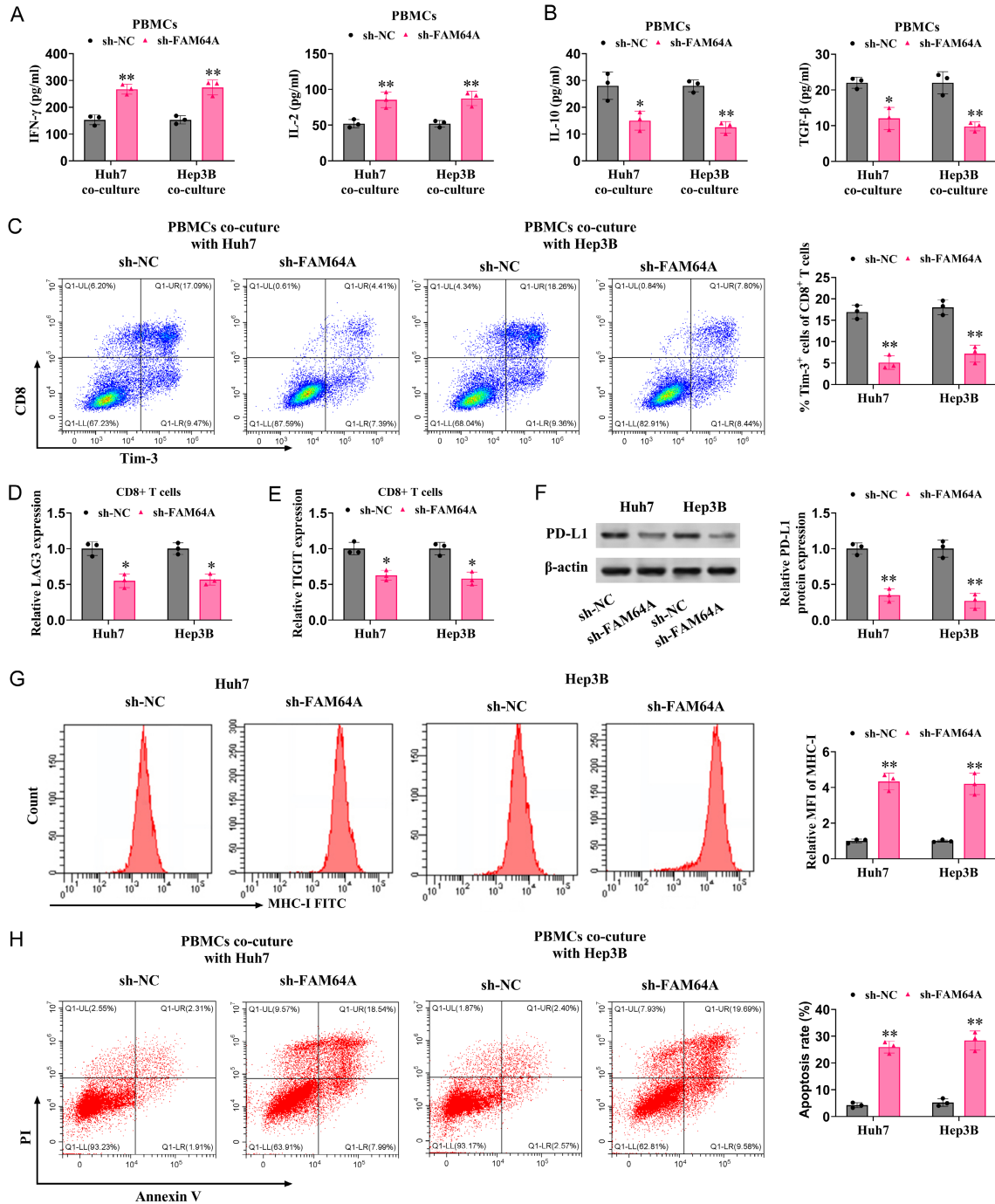


Figure 3. Silenced FAM64A restricts immune escape in liver cancer cells. PBMCs were co-cultured with Huh7 and Hep3B cells which were transfected with sh-FAM64A for 48 h. A. The levels of IFN- γ and IL-2 in PBMCs were measured using ELISA assay. B. The levels of IL-10 and TGF- β in PBMCs were detected using ELISA assay. C. The proportion of Tim-3⁺CD8⁺ cells in co-cultured PBMCs was assessed by flow cytometry. D. The LAG3 mRNA level in purified CD8⁺ T cells was detected utilizing qRT-PCR. E. The TIGIT mRNA level in purified CD8⁺ T cells was detected utilizing qRT-PCR. F. The PD-L1 protein level in Huh7 and Hep3B cells was measured utilizing western blot. G. The expression of MHC-I expression on the cell surface of Huh7 and Hep3B cells was measured utilizing flow cytometry. H. The apoptosis of Huh7 and Hep3B cells was tested using flow cytometry. *: $P < 0.05$, **: $P < 0.01$ versus sh-NC group.

gene transcription by interacting with transcription factors such as FOXM1 and STAT3.

To explore how FAM64A specifically regulates VEGFA expression via specific transcription

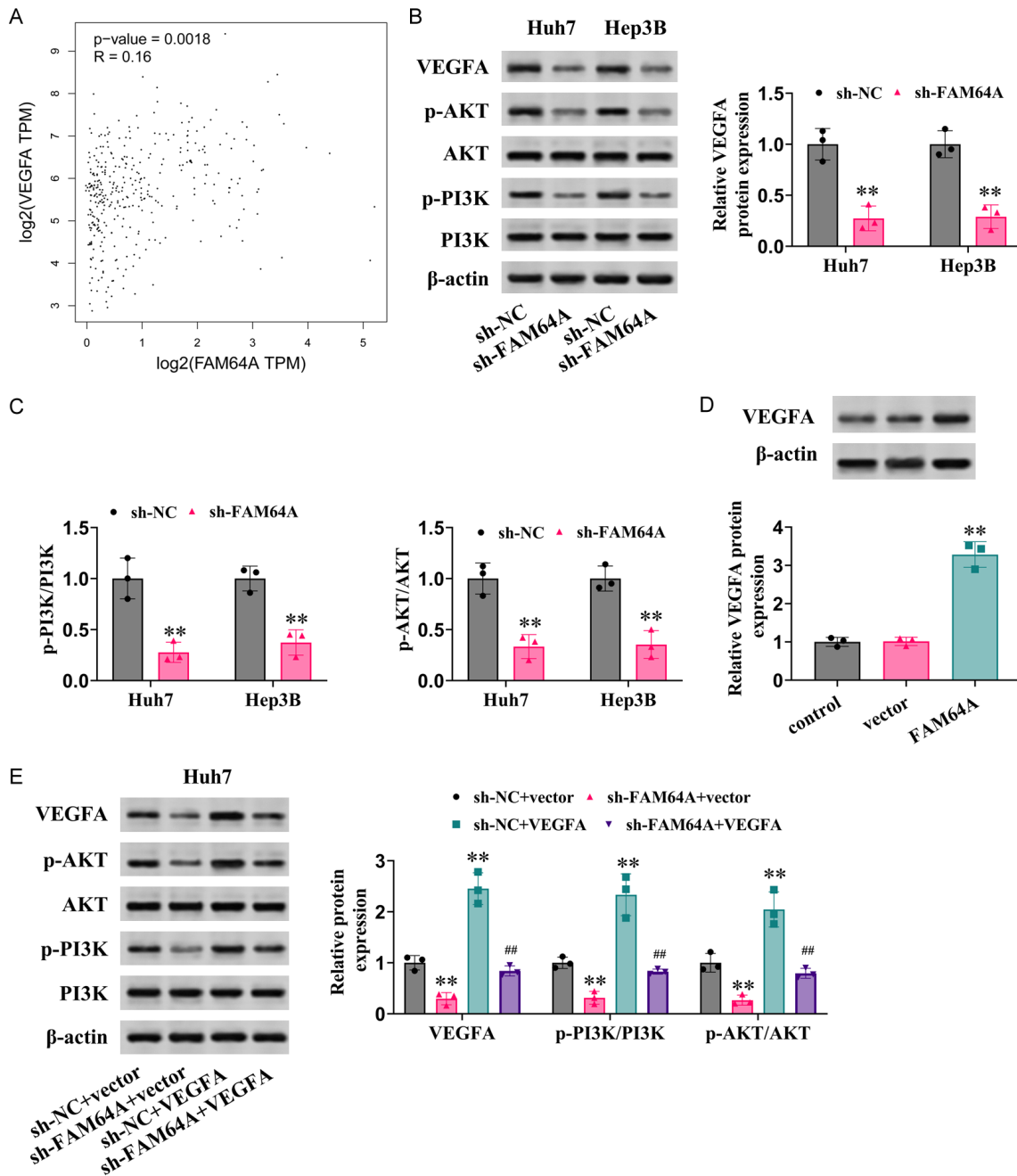


Figure 4. Silenced FAM64A restricts the activity of the VEGFA/AKT pathway in liver cancer. A. The correlation between FAM64A and VEGFA expression was analyzed on the GEPIA using the TCGA data of liver cancer cases. B, C. The protein levels of VEGFA, p-PI3K, PI3K, p-AKT and AKT in Huh7 cells transfected with sh-FAM64A were measured using western blot. D. The VEGFA level in Huh7 cells transfected with the VEGFA overexpression plasmid was measured using western blot. E. The protein levels of VEGFA, p-PI3K, PI3K, p-AKT and AKT in Huh7 cells co-transfected with sh-FAM64A and VEGFA overexpression plasmid were measured using western blot. **: $P < 0.01$ versus sh-NC group. #: $P < 0.01$ versus sh-FAM64A+vector group.

factors, Co-IP assay was conducted. Results showed that FOXM1 and STAT3 were identified as the components of the immunoprecipitated solution using FAM64A antibody

(Supplementary Figure 2C). Besides, FOXM1 and STAT3 antibodies also were able to pull down FAM64A (Supplementary Figure 2C). In addition, results showed that the inhibitory

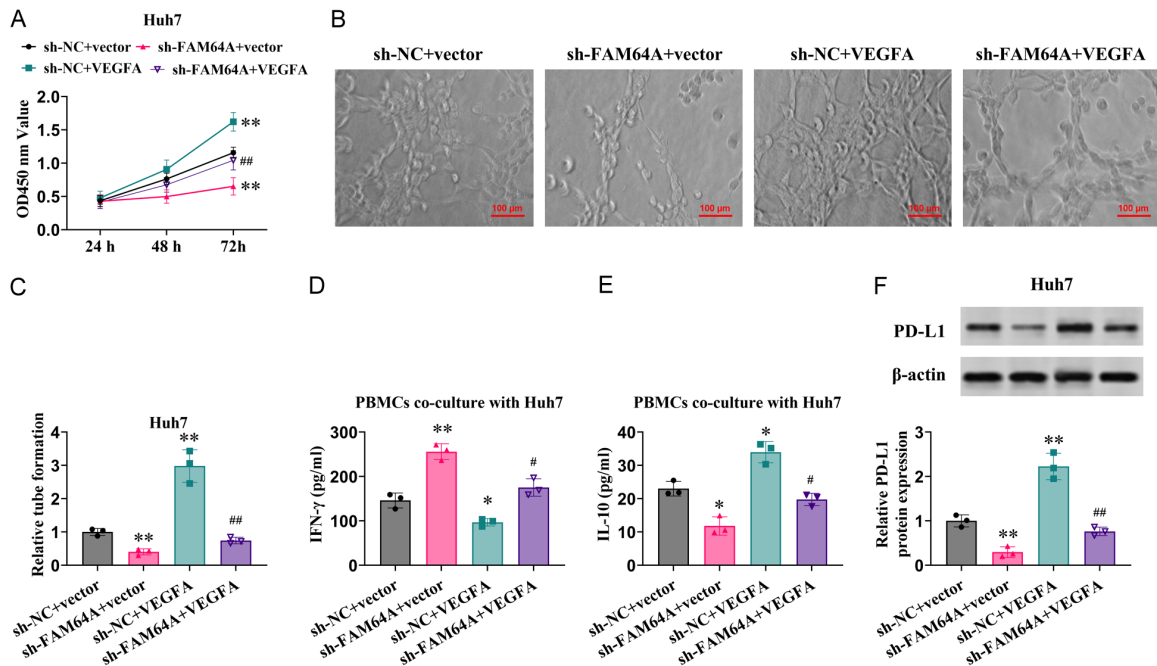


Figure 5. Silenced FAM64A restricts the angiogenesis and immune escape of liver cancer cells via inactivating the VEGFA/AKT pathway. Huh7 cells were co-transfected with sh-FAM64A and the VEGFA overexpression plasmid. (A) Cell viability was determined using CCK-8 assay. (B, C) Cell angiogenesis ability of HUVECs cells after cultured with conditioned medium from Huh7 cells was evaluated using the tube formation assay. Scale bar: 100 μ m; magnification: 200 $\times$. The levels of IFN- γ (D) and IL-10 (E) in PBMCs co-cultured with Huh7 cells for 24 h were measured using ELISA assay. (F) The PD-L1 protein level in Huh7 cells was measured utilizing western blot. *: $P < 0.05$, **: $P < 0.01$ versus sh-NC group. #: $P < 0.05$, ##: $P < 0.01$ versus sh-FAM64A+vector group.

effect of silenced FAM64A on VEGFA expression was abolished by FOXM1 instead of STAT3 ($P < 0.01$, [Supplementary Figure 2D](#)). Ch-IP assay revealed that VEGFA was immunoprecipitated by anti-FOXM1 antibody compared to the negative control group ([Supplementary Figure 2E](#)). However, silenced FAM64A decreased the enrichment level of VEGFA by anti-FOXM1 antibody ($P < 0.01$, [Supplementary Figure 2E](#)). These data demonstrated the interaction between FAM64A and FOXM1 or STAT3. Furthermore, the VEGFA overexpression plasmid was transfected into Huh7 cells to elevate the VEGFA level ($P < 0.01$, [Figure 4D](#)). Overexpressed VEGFA reversed the suppressive roles of silenced FAM64A on VEGFA level and the phosphorylation levels of PI3K and AKT ($P < 0.01$, [Figure 4E](#)). Collectively, silenced FAM64A inhibited the VEGFA/AKT signaling in liver cancer.

Silenced FAM64A restricts the angiogenesis and immune escape of liver cancer cells via inactivating the VEGFA/AKT pathway

To elucidate whether the VEGFA/AKT pathway mediates the regulation of FAM64A on liver

cancer pathogenesis, Huh7 cells were co-transfected with the sh-FAM64A and the VEGFA overexpression plasmid. Results revealed that overexpressed VEGFA enhanced Huh7 cell viability and tube formation ability of co-cultured HUVECs ($P < 0.01$, [Figure 5A-C](#)). Conversely, silenced FAM64A reduced Huh7 cell viability and the tube formation ability of co-cultured HUVECs, which was reversed by overexpressed VEGFA ($P < 0.01$, [Figure 5A-C](#)). Besides, overexpressed VEGFA reduced IFN- γ level but elevated IL-10 level in co-cultured PBMCs ($P < 0.05$, [Figure 5D and 5E](#)). However, silenced FAM64A elevated IFN- γ level and reduced IL-10 level, which was reversed by overexpressed VEGFA ($P < 0.05$, [Figure 5D and 5E](#)). Furthermore, overexpressed VEGFA elevated PD-L1 level in Huh7 cells ($P < 0.01$, [Figure 5F](#)). Silenced FAM64A inhibited PD-L1 level, but was abrogated by enhanced VEGFA ($P < 0.01$, [Figure 5F](#)). To further investigate the role of the VEGFA/AKT pathway in the regulation of FAM64A on liver cancer pathogenesis, SNU449 cells were co-transfected with the FAM64A overexpression plasmid and treated with the AKT inhibi-

FAM64A regulates the liver cancer development

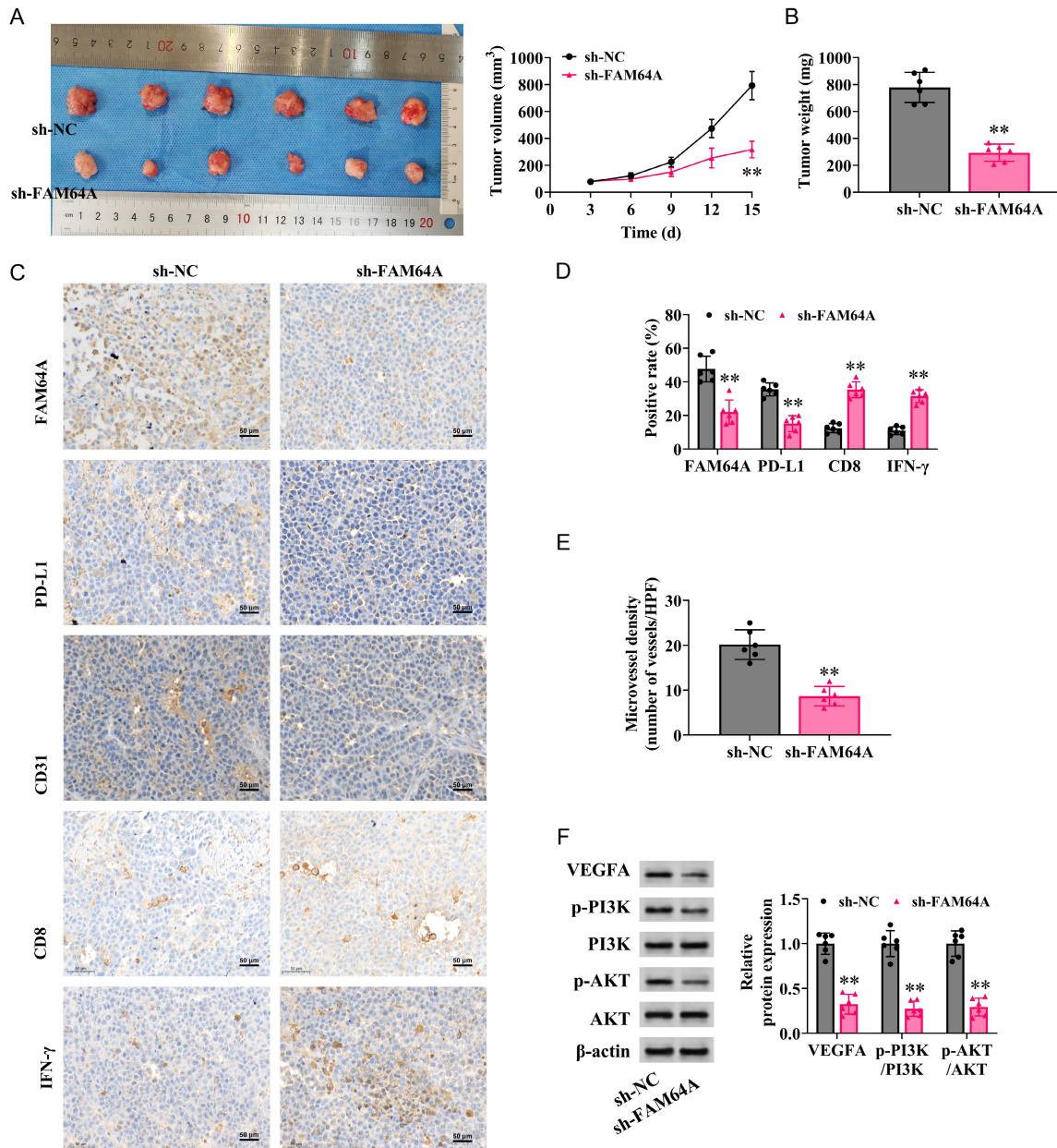


Figure 6. Silenced FAM64A restricts tumor growth and immune escape of liver cancer *in vivo*. Hepa 1-6 cells (5×10^6) stably transfected with sh-FAM64A were subcutaneously injected into the mice to construct xenograft tumor. A. Images of the xenograft tumors at 16 days were presented and the tumor volumes were recorded. B. Tumor weights were measured. C, D. The proportions of FAM64A, CD31, PD-L1, CD8, and IFN-γ positive cells in tumor tissues were measured utilizing Immunohistochemistry. Scale bar: 50 μm; magnification: 400×. E. Quantitative analysis of the microvessel density of subcutaneous tumors according to the staining status of CD31. HPF: High-power field. F. The levels of VEGFA, p-PI3K, PI3K, p-AKT and AKT in tumor tissues were measured utilizing western blot. **: $P < 0.01$ versus sh-NC group.

tor MK-2206. Results showed that FAM64A overexpression enhanced SNU449 cell invasion capacity and tube formation ability of co-cultured HUVEC, which was suppressed by MK-2206 ($P < 0.01$, Supplementary Figure 3A, 3B). Meanwhile, FAM64A overexpression in-

creased the PD-L1 level, while was abolished by MK-2206 ($P < 0.01$, Supplementary Figure 3C). Together, FAM64A knockdown restricted the angiogenesis and immune escape of liver cancer cells by inactivating the VEGFA/AKT pathway.

Silenced FAM64A restricts tumor growth and immune escape of liver cancer in vivo

To investigate the action of FAM64A on liver cancer pathogenesis *in vivo*, the xenograft tumors were established using Hepa 1-6 cells stably transfected with sh-FAM64A. Results proved that FAM64A knockdown obviously restrained tumor volume and tumor weight ($P < 0.01$, **Figure 6A, 6B**). Besides, knockdown of FAM64A decreased the proportions of FAM64A, CD31 and PD-L1 positive cells and increased CD8 and IFN- γ positive cells ($P < 0.01$, **Figure 6C, 6D**). Furthermore, the tumor microvessel density was quantified based on the staining status of CD31. Results revealed that FAM64A knockdown reduced tumor microvessel density ($P < 0.01$, **Figure 6E**). Moreover, knockdown of FAM64A reduced the VEGFA, p-PI3K and p-AKT levels, while it had no influence on the total PI3K and AKT levels in tumor tissues ($P < 0.01$, **Figure 6F**). These findings suggested that silenced FAM64A restricted tumor growth and immune escape of liver cancer *in vivo*.

Discussion

The suboptimal efficacy of current treatment strategies in liver cancer underscores the urgent need for novel therapeutic methods and targets. Herein, we explored the roles of FAM64A in liver cancer pathogenesis, especially in cancer immunity. The work revealed the aberrant high expression of FAM64A in liver cancer tissues and cell lines. Critically, *in vitro* and *in vivo* functional assays the findings demonstrated that FAM64A knockdown restricted the proliferation, angiogenesis, and immune escape of liver cancer by inactivating the VEGFA/AKT signaling.

To understand the action of FAM64A on the pathogenesis of liver cancer, the expression of FAM64A in liver cancer was measured. It was revealed that FAM64A was elevated in liver cancer, which was in sympathy with the previous reports [15, 17, 20]. Notably, high expression of FAM64A has also been reported in ovarian cancer, glioma and gastric cancer [15, 17, 20]. In addition, the results revealed that the FAM64A level was correlated with overall survival, lymph node involvement and TNM stage of liver cancer cases. Consistently, Fu *et al.* reported that elevated FAM64A was closely linked to the poor prognosis of glioma cases

[17]. Zhao *et al.* discovered that FAM64A overexpression was strongly related to the unfavorable prognosis of head and neck squamous cell carcinoma (HNSCC) [22]. Collectively, these findings confirmed that FAM64A was elevated in liver cancer and associated with poor prognosis.

Proliferative and invasive phenotypes are two fundamental hallmarks of malignant disease [23]. As two critical drivers for cancer, cell proliferation and invasion are responsible for tumor growth and metastasis [24]. FAM64A has been reported to be associated with cell proliferation [14]. Besides, increasing research revealed that FAM64A could modulate the invasion of cancer cells [20, 25]. In gastric cancer, FAM64A knockdown restrained proliferation and invasion through the EGFR/Akt/mTOR/NF- κ B signaling pathway [20]. In ovarian cancer, silencing FAM64A suppressed the proliferation and invasion via the Hippo pathway [25]. To explore the role of FAM64A in the pathogenesis of liver cancer, the proliferation and invasion ability of liver cancer cells were investigated after silenced FAM64A. It was found that silenced FAM64A restricted the proliferation ability and invasion capacity of liver cancer. Similarly, Zhang *et al.* demonstrated that FAM64A knockdown inhibited proliferation ability and invasion capacity of liver cancer cells [18].

Angiogenesis is a fundamental step in the transformation of tumors from benign to malignant states [26]. For solid tumors to expand beyond a few millimeters, the blood supply is essential [27]. Tumors can actually form this blood supply by releasing chemical signals that stimulate blood vessel formation [27]. Angiogenesis has been well described to modulate the progression of liver cancer [28, 29]. Zheng *et al.* demonstrated that a CD13-targeting peptide integrated protein repressed liver cancer development by blocking angiogenesis [28]. Li *et al.* proved that all-trans-retinoic acid inhibited hepatocellular carcinoma progression through restraining angiogenesis [29]. Here, the angiogenesis ability of liver cancer cells was investigated to explore the influence of FAM64A on liver cancer pathogenesis. The findings revealed that FAM64A knockdown restricted the angiogenesis ability. Up to now, the regulatory effect of FAM64A on angiogenesis has been reported for the first time.

Immune escape refers to the capability of tumor cells to evade immune recognition and offensive, allowing tumor cells to survive and proliferate [10]. It is widely regarded as the most significant barrier to effective anti-cancer therapy [12]. Therefore, targeting immune escape may be a potent strategy for cancer management and has the potential to enhance the efficacy of immunotherapy. Dong *et al.* revealed that bionic lipoprotein loaded with chloroquine blocked immune escape and improved anti-tumor immunotherapy [30]. Excitingly, FAM64A has been reported to participate in immune regulation [16, 19]. For instance, Zhou *et al.* discovered that FAM64A regulated the immune mechanism and the IFN-signaling pathway in prostate cancer [16]. Xu *et al.* revealed that FAM64A regulated T17 differentiation and inflammation-associated cancer development [19]. Consequently, we investigated whether FAM64A regulated immune escape in liver cancer. The findings revealed that FAM64A knockdown enhanced the secretion levels of IFN- γ and IL-2, inhibited the IL-10 and TGF- β levels, and reduced the proportion of Tim-3⁺CD8⁺ cells in co-cultured PBMCs. Furthermore, FAM64A knockdown reduced the mRNA expression of LAG3 and TIGIT in purified CD8⁺ T cells, suppressed the PD-L1 level, and promoted the apoptosis of Huh7 and Hep3B cells after co-culture. Within the tumor micro-environment, T cells, Tregs, Bregs, NK cells, DCs, MDSCs and macrophages are the primary immune cells [31]. Tumor-infiltrating T cells are critical effector cells in the immune system and can be allocated into CD4⁺ T cells (helper T cells) and CD8⁺ T cells (cytotoxic T cells), which can secrete anti-tumor cytokines such as interferon IFN- γ , IL-2, TNF- α and IL-17 [31]. Tregs and Bregs are immunosuppressive cell subsets that secrete TGF- β , IL-10 and IL-35 to inhibit the immune response of T cells [31]. During immune escape, the binding of PD-L1 and PD-1 limited the effector functions of T cells and induced T cell exhaustion [32]. LAG3 and TIGIT were early exhaustion-associated molecules of CD8⁺ T cells [33]. It is accepted that canonical T-cell exhaustion requires long-term persistent stimulation *in vivo* [34]. However, upregulation of Tim-3, LAG-3, and TIGIT within 24-48 h in tumor-T cell co-culture represents early, reversible inhibitory receptor upregulation induced by tumor-derived lactate and metabolic stress, not terminal exhaustion [35, 36]. Such short-

term assay is a well-accepted approach to measure acute T-cell dysfunction in tumor immunometabolism studies [37-39]. Tumor cells also can down-regulate or lose MHC I antigen presentation, and thereby become less stimulatory or even invisible to CD8⁺ T cells [40, 41]. A large number of cancers have been reported to lose expression of MHC I molecules to varying degrees, including liver cancer [40]. In this study, silenced FAM64A enhanced MHC-I expression on the cell surface of Huh7 and Hep3B cells. Collectively, the above findings proved that FAM64A knockdown restricted immune escape in liver cancer.

VEGFA is a vital signaling protein that plays a pivotal role in the process of angiogenesis [42]. The AKT signaling is the downstream target of VEGFA [43, 44]. Chen *et al.* reported that the up-regulation of lncRNA LIFR-AS1 facilitated pancreatic cancer progression through the miRNA-150-5p/VEGFA/AKT signaling [43]. Raji *et al.* showed that miR-383 enhanced cellular sensitivity to cisplatin by targeting VEGFA and its downstream AKT pathway [44]. Given that FAM64A could modulate the angiogenesis of liver cancer cells, we hypothesized that the VEGFA/AKT signaling might mediate the regulatory processes of FAM64A on the aggressive behaviors of liver cancer. As speculated, we found that silenced FAM64A suppressed VEGFA mRNA and protein level and inhibited the phosphorylation levels of AKT and PI3K. Besides, FAM64A knockdown did not exert a significant impact on the degradation rate of the VEGFA protein. The findings suggested that the regulatory effect of FAM64A on VEGFA expression predominantly occurs at the transcriptional or post-transcriptional level, rather than through influencing its protein degradation process. Meanwhile, we found the interaction between FAM64A and transcription factors FOXM1 or STAT3 in this study. Therefore, we inferred that FAM64A might affect the expression of VEGFA via interacting with FOXM1 or STAT3. Thereafter, we found that the inhibitory effect of silenced FAM64A on VEGFA expression was abolished by FOXM1 instead of STAT3. Additionally, VEGFA was immunoprecipitated by anti-FOXM1 antibody and silenced FAM64A decreased the enrichment level of VEGFA by anti-FOXM1 antibody. These data demonstrated that FAM64A could affect the expression of VEGFA via interacting with FOXM1. Furthermore, results dem-

onstrated that silencing FAM64A restricted the angiogenesis and immune escape of liver cancer cells by inactivating the VEGFA/AKT signaling pathway. Conversely, overexpressed FAM64A promoted invasion, angiogenesis and immune escape of liver cancer cells via activating the VEGFA/AKT pathway. To date, this study has, for the first time, comprehensively elucidated the impact of FAM64A on liver cancer pathogenesis and cancer immunity, along with its mechanism. Although our data highlight VEGFA/AKT pathway as the effective pathway mediating the regulatory effects of FAM64A on the malignant phenotypes of liver cancer, the potential involvement of other signaling cascades cannot be entirely ruled out. The lack of research on the crosstalk of signaling pathways is a limitation of this article, and further mechanistic investigations are essential.

To further validate the findings from the *in vitro* experiments, mice xenograft tumors were established to explore the influence of FAM64A on liver cancer pathogenesis *in vivo*. Consistent with the *in vitro* results, FAM64A knockdown restricted tumor growth, decreased the proportions of FAM64A, CD31 and PD-L1 positive cells, increased CD8 and IFN- γ positive cells and inhibited activation of the VEGFA/AKT signaling. Similarly, Zhao *et al.* also demonstrated that FAM64A knockdown restrained the tumor growth of ovarian cancer [15]. Collectively, silenced FAM64A restricted tumor growth and immune escape of liver cancer *in vivo*.

Conclusion

FAM64A is up-regulated in liver cancer and closely correlated with poor prognosis. FAM64A knockdown restrained the proliferation ability, invasion capacity, angiogenesis and immune escape of liver cancer cells by inactivating the VEGFA/AKT signaling. FAM64A may be a valuable candidate target for liver cancer therapy.

Acknowledgements

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Every participant signed the written informed consent.

Disclosure of conflict of interest

None.

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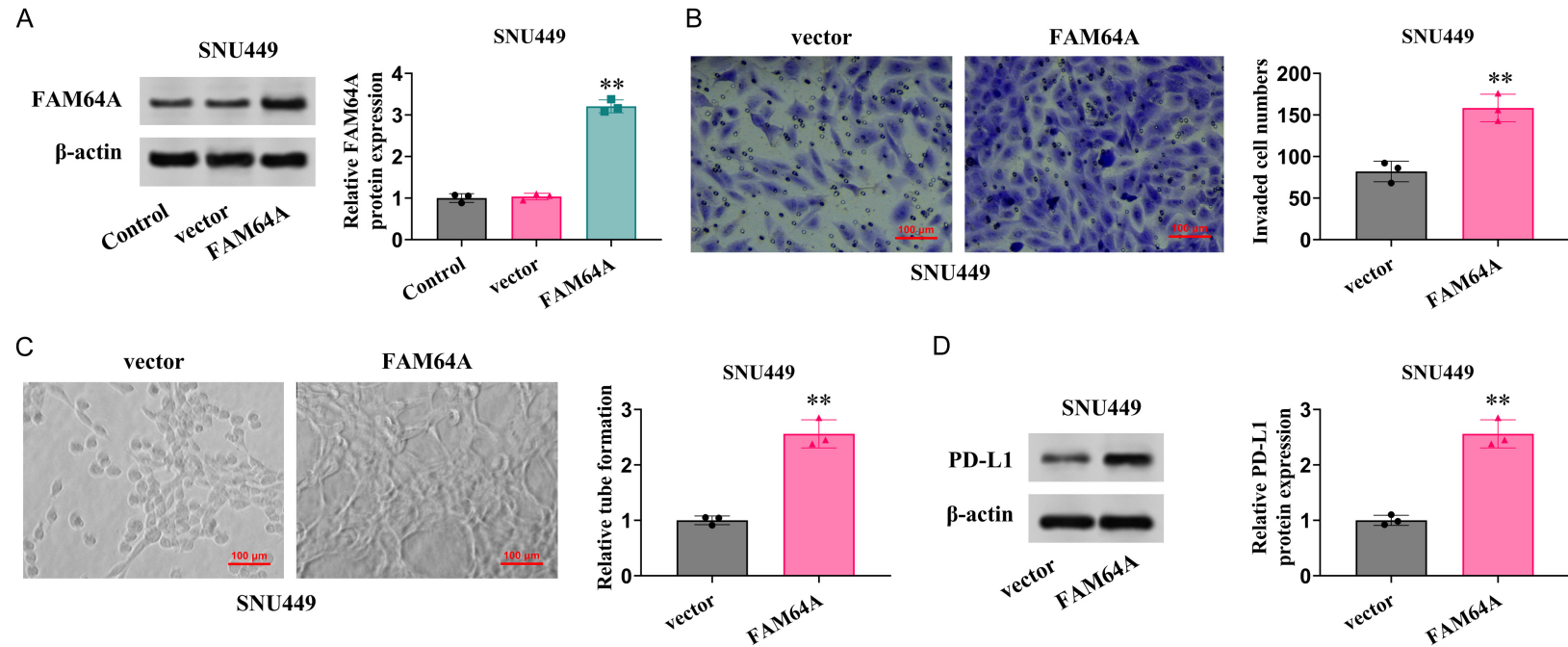
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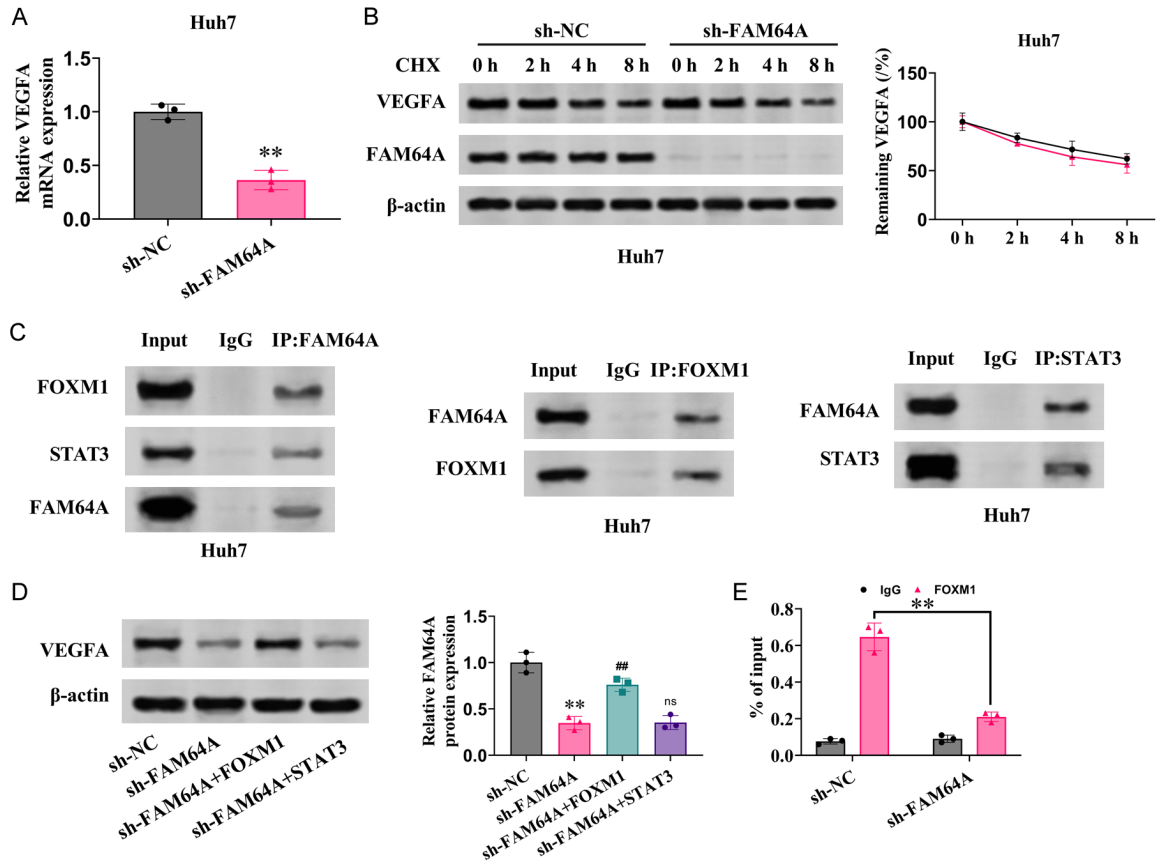
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FAM64A regulates the liver cancer development



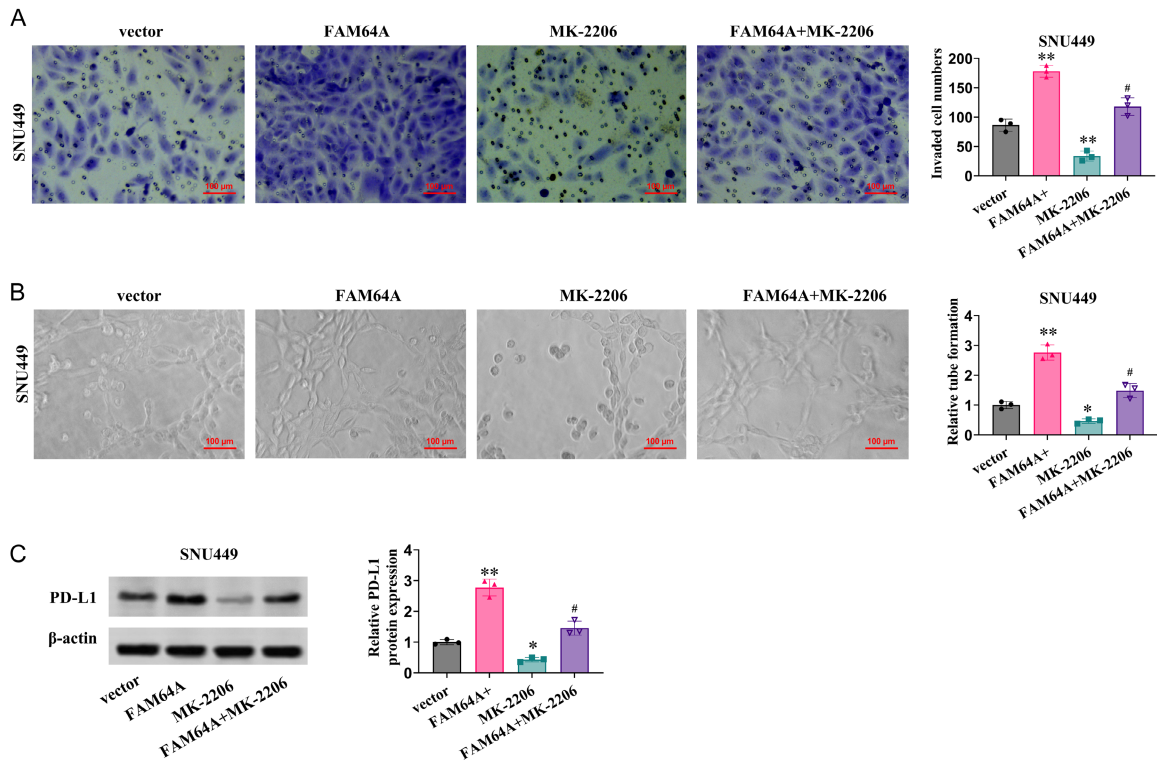
Supplementary Figure 1. Overexpressed FAM64A promoted the invasion, angiogenesis, and immune escape of liver cancer cells. SNU449 cells were transfected with the FAM64A overexpression plasmid. A. The protein level of FAM64A was measured utilizing western blot. B. Cell invasion ability was assessed using the Transwell invasion assay. Scale bar: 100 μ m; magnification: 200 $\times$. C. Cell angiogenesis ability of HUVECs cells after cultured with conditioned medium from SNU449 cells was evaluated using the tube formation assay. Scale bar: 100 μ m; magnification: 200 $\times$. D. The protein levels of PD-L1 were measured by western blot in SNU449 cells transfected with FAM64A overexpression plasmid. *: $P < 0.05$, **: $P < 0.01$ versus vector group.

FAM64A regulates the liver cancer development



Supplementary Figure 2. The effects of silenced FAM64A on VEGFA mRNA expression and protein degradation. A. The VEGFA mRNA expression in Huh7 cells was detected utilizing qRT-PCR after transfected with sh-FAM64A. B. The protein levels of VEGFA and FAM64A in Huh7 cells were measured utilizing western blot after transfected with sh-FAM64A and treated by CHX (50 μ g/mL) for 2 h, 4 h, and 8 h. C. Co-IP assays were performed to validate the interaction between FAM64A and FOXM1 or STAT3 in Huh7 cells. D. The level of VEGFA in Huh7 cells was measured using western blot after transfected with sh-FAM64A and overexpressed FOXM1 or STAT3. E. The direct binding of VEGFA and FOXM1 was determined by ChIP-qPCR assay after transfected with sh-FAM64A. **: $P < 0.01$ versus sh-NC group.

FAM64A regulates the liver cancer development



Supplementary Figure 3. Overexpressed FAM64A promoted invasion, angiogenesis and immune escape of liver cancer cells via activating the VEGFA/AKT pathway. SNU449 cells were co-transfected with the VEGFA overexpression plasmid and treated with the 2.5 μ g/mL of AKT inhibitor MK-2206. A. Cell invasion ability was assessed using Transwell invasion assay. Scale bar: 100 μ m; magnification: 200 $\times$. B. Cell angiogenesis ability of HUVECs cells after cultured with conditioned medium from SNU449 cells was evaluated using the tube formation assay. Scale bar: 100 μ m; magnification: 200 $\times$. C. The PD-L1 protein level in SNU449 cells was measured utilizing western blot. **: $P < 0.01$ versus vector group. #: $P < 0.05$ versus FAM64A group.