



Original Article



Krüppel-like Factor 13 Promotes HCC Progression by Transcriptional Regulation of HMGCS1-mediated Cholesterol Synthesis

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Abstract

Background and Aims: Krüppel-like factor (KLF) has a role in the occurrence, development and metabolism of cancer. We aimed to explore the role and potential molecular mechanism of KLF13 in the growth and migration of liver cancer cells. **Methods:** The expression of KLF13 in hepatocellular carcinoma (HCC) tissues was higher than that in normal tissues according to analysis of The Cancer Genome Atlas (TCGA) database. Lentiviral plasmids were used for overexpression and plasmid knockdown of KLF13. Real-time quantitative polymerase chain reaction (qPCR) and western blotting were used to detect mRNA and protein expression in HCC tissues and cells. Cell counting kit-8 (CCK-8), colony formation, cell migration and invasion, and flow cytometry assays were used to assess the *in vitro* function of KLF13 in HCC cells. The effect of KLF13 on xenograft tumor growth *in vivo* was evaluated. The cholesterol content of HCC cells was determined by an indicator kit. A dual-luciferase reporter assay and chromatin immunoprecipitation sequencing (ChIP-seq) revealed the binding relationship between KLF13 and HMGCS1. **Results:** The expression of KLF13 was upregulated in HCC tissues and TCGA database. KLF13 knockdown inhibited the proliferation, migration and invasion of HepG2 and Huh7 cells and increased the apoptosis of Huh7 cells. The opposite effects were observed with the overexpression of KLF13 in SK-Hep1 and MHCC-97H cells. The overexpression of KLF13 promoted the growth of HCC in nude mice and KLF13 transcription promoted the expression of HMGCS1 and the biosynthesis of cholesterol. KLF13 knockdown inhibited cholesterol biosynthesis mediated by HMGCS1 and inhibited

the growth and metastasis of HCC cells. **Conclusions:** KLF13 acted as a tumor promoter in HCC by positively regulating HMGCS1-mediated cholesterol biosynthesis.

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Introduction

Hepatocellular carcinoma (HCC) is the sixth most common malignant tumor and the third leading cause of cancer-related death worldwide.¹ Approximately half of the new HCC cases in the world are diagnosed in China.² Although significant progress has been made in diagnosis and treatment, including neoadjuvant chemotherapy and targeted therapy, the long-term survival of patients with liver cancer is still not satisfactory because of the high rate of intrahepatic and distal metastasis.³ According to epidemiological statistics, primary HCC cases in China account for more than half of the cases worldwide each year, and the proportion of HCC cases in China caused by hepatitis B virus is as high as 92%.⁴

The Krüppel-like factor (KLF) family comprises transcription factors containing conserved zinc ester domains that regulate the transcriptional activity of genes by binding to the DNA sequence of target genes.⁵ In humans, the KLF family consists of 17 members, KLF1–17. They are divided into three groups by their transcriptional activity. KLF3, KLF8, and KLF12, act as repressors to inhibit target gene transcription, KLF1, KLF2, KLF4, KLF5, KLF6, and KLF7, activate target gene transcription, and KLF9, KLF10, KLF11, KLF12, KLF13 and KLF16, also function as repressors, but the actions of these KLFs are different from those in the first group. KLFs have different roles in tumors.^{6–8} Based on two studies, KLF13 may have different functions in different tumors. In prostate cancer, the expression of KLF13 was low, and the overexpression of KLF13 not only inhibited the activity of AKT but also significantly inhibited the growth of prostate cancer tumors.⁹ However, in oral cancer cells, the expression of KLF13 was significantly increased, and downregulation of intracellular KLF13 inhibited the proliferation of cancer cells.¹⁰

Keywords: Hepatocellular carcinoma; KLF13; HMGCS1; Cholesterol.

Abbreviations: CCK-8, cell counting Kit-8; ChIP, chromatin immunoprecipitation; DMEM, Dulbecco's modified Eagle's medium; EMT, epithelial-mesenchymal transition; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HCC, hepatocellular carcinoma; HE, hematoxylin-eosin; HMGCS, HMG-CoA synthase; IHC, immunohistochemical; KLF13, Krüppel-like factor; PCNA, proliferating cell nuclear antigen; PVDF, polyvinylidene fluoride; qPCR, quantitative polymerase chain reaction; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TBST, Tris-buffered saline with Tween-20; TCGA, The Cancer Genome Atlas.

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In addition, FBW7-mediated KLF13 degradation was shown to be involved in the life cycle and immune function of HPV.¹¹ However, the role of KLF13 in HCC is not clear.

HMG-CoA synthase (HMGCS) has two subtypes, cytoplasmic HMGCS1 and mitochondrial HMGCS2. HMG-CoA produced by intracellular HMGCS1 is converted to methylene hydroxylic acid under the action of HMG-CoA reductase, which initiates the isoprene pathway that has cholesterol as the final product.¹² HMGCS1 has a carcinogenic role in the gastrointestinal tract. Dipyridamole enhances the killing effect of trametinib on colon cancer cells by knocking down HMGCS1.¹³ The methylene hydroxylic acid pathway enzyme HMGCS1 promotes the progression of gastric cancer.¹⁴ However, the role of HMGCS1 in HCC is unclear.

In this study, we evaluated the role of KLF13 in HCC. We found that KLF13 expression was upregulated in HCC tissues versus normal tissues. Functional experiments showed that KLF13 promoted cell cycle progression, ethynyl-deoxyuridine (EdU) incorporation, cell proliferation, migration, and invasiveness. It was also shown that KLF13 promoted HMGCS1-mediated cholesterol synthesis. In addition, silencing HMGCS1 attenuated the proliferation and migration of HCC cells with a high expression of KLF13. Our study shows that KLF13 promoted HCC tumor progression by modulating cholesterol metabolism.

Methods

Patients and specimens

Human HCC tissues and paired adjacent noncancerous liver tissues included in this study were obtained from patients at the Affiliated Hospital of Guizhou Medical University between 2019 and 2020 and used for real-time quantitative polymerase chain reaction (qPCR, $n=28$), western blotting ($n=8$), and immunohistochemistry ($n=20$) assays. Paired noncancerous tissues were isolated from a site at least 3 cm away from the tumor border and pathological diagnosis was performed. All patients underwent tissue collection and further analytical tests before surgery. The samples were stored at -80°C for further study.

Cell lines and cell culture

LO2 normal human hepatocytes and Huh7, LM3, HepG2, MHCC-97H, and SK-Hep1 HCC cell lines were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Cells were cultured in high glucose Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (Biological Industries) and 1% Corning penicillin/streptomycin solution. The cell cultures were incubated in a humidified chamber at 37°C with a concentration of 5% CO_2 .

Lentivirus transduction and small interfering RNA (siRNA) transfection

To construct a stable overexpression cell line, a lentivirus was used as a vector to carry the KLF13 sequence, and an empty vector was used as a negative control. The constructs were transfected into SK-Hep1 and MHCC-97H cells. A recombinant plasmid containing an siRNA targeting the KLF13 gene was constructed to knock down of KLF13 in Huh7 and HepG2 cells. To stably knockdown HMGCS1 in HCC cells, small-hairpin RNA was designed and inserted into a vector (GeneChem, Shanghai, China). We used Lipofectamine 3000 Reagent (Invitrogen) to transfect shRNA into HCC cells

according to the manufacturer's operating procedures. For the inhibition of HMGCS1 gene expression, 10 nM siRNA was transfected into HCC cells using Lipofectamine 3000 Reagent (Invitrogen). siRNA was synthesized and purchased from RiboBio (Guangzhou, China). The shRNA sequences were KLF13-RNAi (7394-1) CGGGAAATCTTCGCACCTCAA, KLF13-RNAi (7396-1) CTAGCGGACCTCAACCAGCAA.

qPCR

Total RNA was extracted from tumor tissues, paracancerous tissues and HCC cells by TRIzol reagent (Invitrogen), and 1 μg total RNA was reverse transcribed using PrimeScript RT Reagent Kits (Vazyme, Nanjing, China) according to the manufacturer's instructions. qPCR was performed with SYBR Premix Ex Tag (Takara) on a 7500 Real-time PCR system. The qPCR conditions were 95°C for 30 s, followed by 40 cycles of 95°C for 5 s and 60°C for 34 s. Relative gene expression was calculated by the $2^{(-\Delta\Delta\text{Ct})}$ method. GAPDH was used as the internal reference and normalized to the control sample. The primer sequences were KLF13 forward: 5'-CTCACACAGGTGAGAGGC-3'; reverse: 5'-GTGCTTGGTCAGGTGGTC-3'; GAPDH, forward: 5'-GATCATCAGCAATGCCTC-3'; reverse: 5'-GTCCTTCACGATACCAA-3'. HMGCS1, forward: 5'-TATGATTGCATTGGCGGC-3'; reverse: 5'-CCCGGTGTAGCATCTTGTG-3'.

Western blotting

Total protein was extracted from tissue and cells using RIPA buffer with phenylmethanesulfonyl fluoride protease inhibitor. Protein concentration was determined with bicinchoninic acid (BCA) assay kits (Thermo Fisher). The proteins were separated by 10% or 12% Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and then transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA, USA). The membranes were blocked with 5% skim milk, incubated with primary antibodies overnight at 4°C and then with the designated secondary antibody at room temperature for 1 h after washing with once with tris-buffered saline and Polysorbate 20 (TBST). The signals were detected with an enhanced chemiluminescence (ECL)-Plus kit (Amersham Biosciences). Primary antibodies against the following proteins were used: KLF13 (1:1,000; Proteintech, 18352-1-AP), GAPDH (1:7,000; Proteintech, 10494-1-AP), HMGCS1 (1:1,000; Proteintech, 17643-1-AP), E-cadherin (1:5,000; Proteintech, 20874-1-AP), N-cadherin (1:6,000; Proteintech, 22018-1-AP), vimentin (1:5,000; Abcam, ab92547), CDK2 (1:2,000; Proteintech, 10122-1-AP), CDK4 (1:2,500; Proteintech, 11026-1-AP), cyclinD1 (1:1,500; Proteintech, 26939-1-AP), cleaved-caspase-3 (1:500; Abcam, ab32042), and KLF13-flag (1:50; Cell Signaling).

Immunohistochemistry

The expression of KLF13, proliferating cell nuclear antigen (PCNA) and Ki67 in tissues was assayed by immunohistochemical (IHC) staining. HCC and tumor xenograft tissues were fixed in paraformaldehyde, paraffin-embedded, and sectioned at 4 μm for the IHC assay. Briefly, after deparaffinization, hydration, antigen retrieval, addition of peroxidase labeled streptavidin, and blocking with goat serum, the tissue sections were incubated with primary antibody in a 4°C refrigerator overnight, followed by incubation with secondary antibody at room temperature for 20 m. The antigen was visualized after incubation with the chromogen diaminobenzidine at room temperature for 5 m. The antigens were observed by microscopy to determine the degree

of staining. Hematoxylin counter-staining and hydrochloric acid-alcohol differentiation were performed using primary antibodies against KLF13 (1:100), PCNA (1:200; Protein-tech), and Ki67 (1:100; Cell Signaling Technology).

Cell proliferation assay

Cell proliferation was measured with a cell counting kit (CCK)-8 assay. HCC cells were plated at a density of 3×10^3 into 96-well plates with 100 μ L culture medium in each well and incubated for the appropriate times. Subsequently, 10 μ L CCK-8 reagent was added to each well and incubated at 37°C for 1.5 h. The absorbance (optical density, OD) was determined at 450 nm.

Colony formation assay

Cells were seeded in 6-well culture plates at 1,000 cells/well. After incubation for 14 days in high glucose DMEM, the cells were washed three times with phosphate buffered saline (PBS) solution, fixed with 4% paraformaldehyde and stained with hematoxylin. The colonies were counted by light microscopy.

Migration and invasion assays

For the wound healing migration assays, approximately 6×10^5 HCC cells were seeded in six-well plates. After the HCC cells had attached, a wound was made by scraping a 200 μ L plastic pipette tip along the bottom of the plate. The cells were washed with PBS three times, the detached cells were removed, and serum-free medium was added. Wound healing was recorded for 48 h. Cell migration and invasion assays were performed using 24-well Transwell filter chambers and Matrigel. Cells (4×10^4) in 200 μ L serum-free culture medium were seeded into the top chamber, and 700 μ L medium supplemented with 10% FBS was added to the bottom chamber, followed by incubation at 37°C in an incubator containing 5% CO₂ for 48 h (migration assay) or 72 h (invasion assay). Cells on the lower surface of the membrane were stained with 0.1% crystal violet, photographed, and counted in four random fields per group using a light microscope.

EdU staining

BeyoClick EdU cell proliferation kits were used in conjunction with Alexa Fluor 594 for EdU staining, following the manufacturer's instructions. Briefly, 2×10^5 HCC cells were inoculated into six-well plates with cover slides. After 15 h, 10 μ M EdU chromogenic agent was added to each well and incubated at 37°C for 1.5–2 h. The cells were washed with PBS, fixed with 4% paraformaldehyde, permeabilized with 0.3% TritonX-100 and stained with Click Additive Solution. The cell nuclei were stained with Hoechst 33342 for 10 min. The EdU-positive cells were counted by fluorescence microscopy.

Cell cycle and cell apoptosis assays

A total of 1×10^4 cells were collected, washed three times with PBS and stained with 7 μ L Annexin V and 10 μ L 7-AAD at room temperature in the dark for 5 m. The percentage of apoptotic cells was determined with a FACScan flow cytometer and with FlowJo software. The distribution of the

cell cycle was determined by flow cytometry after propidium iodide (PI) staining. Briefly, cells were inoculated into 6-well plates with three replicates for each condition. When the cells reached 90% confluency, they were fixed overnight in 75% ethanol, and PI staining was assayed with a C6 flow cytometer.

Cholesterol assay

Cholesterol content was assayed with a kit (APPLYGEN, Beijing, China) following the manufacturer's protocol. HCC cells were seeded into six-well plates. Then 100 μ L of the lysis fluid was added to 1×10^6 cells, mixed by shaking and then allowed to stand for 10 m. The cholesterol content was corrected by the protein concentration per mg and the optimal absorbance (OD) was measured at 550 nm.

Dual-luciferase reporter assays

To determine the effect of KLF13 on the HMGCS1 promoter, pcDNA-KLF13 or pcDNA-vector (NC) was cotransfected into HepG2 and Huh7 cells with pGL3-based constructs containing an HMGCS1 normal (NC) vector, WT or MT promoter sequence, and Renilla luciferase reporter plasmids. HCC cells were inoculated in six-well plates 24 h before transfection. Afterwards, 2.5 μ g of KLF13 vector, pcDNA-KLF13 or pcDNA-vector, with the addition of 2.5 μ g of HMGCS1 vector or WT or MT promoter, were cotransfected into cells using Lipofectamine 3000 (Invitrogen). Firefly and Renilla signals were measured with a luciferase reporter assay kit (Genomeditech, Shanghai, China) after transfection for 48 h. The Renilla luciferase vector served as the internal control to determine the transfection efficiency.

ChIP-qPCR assay and ChIP-sequencing

To determine whether KLF13 binds to the promoter of the HMGCS1 gene, ChIP assays were performed using a SimpleChIP enzymatic chromatin IP kit (Cell Signaling Technology) following the manufacturer's protocols. Immunoprecipitated DNA was amplified by promoter-specific primers. The qPCR primer sequences were forward: 5'-TTT-GTCCCCGCTCTTCTC-3', reverse: 5'-CGATGACTCGCTAG-GATTTTCC-3'. The PCR products were assayed on 2% agarose gels. ChIP-sequencing was performed by Aksomics (Shanghai, China). DNA samples (10 ng) were prepared for Illumina sequencing as follows: (1) DNA samples were blunt-ended; (2) A dA base was added to the 3' end of each strand; (3) Illumina genomic adapters were ligated to the DNA fragments; (4) PCR amplification was performed to enrich the ligated fragments; (5) size selection of ~200–1,500 bp enriched product was done with AMPure XP beads. The completed libraries were quantified with an Agilent 2100 Bioanalyzer. The libraries were denatured with 0.1 M NaOH to generate single-stranded DNA molecules, captured on Illumina flow cell, amplified *in situ*. The libraries were then sequenced on the Illumina NovaSeq 6000 following the NovaSeq 6000 S4 Reagent Kit (300 cycles) protocol. After the sequencing platform generated the sequencing images, image analysis and base calling were performed with Off-Line Basecaller software (OLB V1.8). After passing a Solexa Chastity quality filter, the clean reads were aligned to the human genome (UCSC HG19) with BOWTIE software (V2.2.7). Aligned reads were used for peak calling of the ChIP regions using MACS V1.4.2. The peaks in samples were annotated by the nearest gene using the newest UCSC RefSeq database.

Xenograft tumorigenesis assay

SK-Hep1-KLF13 and SK-Hep1-vector cells were prepared (5×10^6 cells in 200 μ L PBS) and inoculated subcutaneously into the right axilla of a group of five immunodeficient 4-week-old female nude mice. Tumor volume was calculated as $=(\text{length} \times \text{width}^2)/2 \text{ mm}^3$. The mice were sacrificed at 28 days after implantation. Tumor were weighed immediately after sacrifice. This procedure was conducted at the Guizhou Medical University, which is the location of the Animal Care Welfare committee that approved the study.

Statistical analysis

GraphPad Prism 8 and SPSS software ver. 20.0 (IBM Corp., Armonk, NY, USA) were used for the statistical analysis. Two-tailed Student's t-tests was used to compare between-group differences. One-way analysis of variance was used for multiple-group comparisons. *P*-values <0.05 , <0.01 , and <0.001 indicated statistical significance.

Results

KLF13 is overexpressed in HCC tissues

To explore the clinical significance of KLF13 in HCC, we used TCGA database and found upregulation of KLF13 mRNA levels in HCC (Fig. 1A). To verify that result, we assessed the expression of KLF13 in 28 pairs of HCC tissues and nontumorous adjacent liver tissues by qPCR. The results showed that the level of KLF13 mRNA in 71.4% of the tumor tissues was higher than that in the corresponding nontumor tissues (Fig. 1B). Western blot and IHC further confirmed that the expression of KLF13 in HCC tissues was upregulated compared with adjacent normal tissues (Fig. 1C, D). According to the Barcelona staging standard, both KLF13 protein and mRNA expression were significantly higher in advanced HCC tissues than in early HCC tissues (Fig. 1E, F). In addition, the expression level of KLF13 in HCC cell lines was assessed. The expression of KLF13 in HCC cells and LO2 normal liver cells was compared in western blots and qPCR assays (Fig. 1G, H). Taken together, our findings indicate that KLF13 was upregulated in HCC and may have been correlated with disease progression.

KLF13 overexpression promoted the proliferation and migration of HCC cells

To explore the biological function of KLF13 in HCC cells, we established stable models of KLF13 overexpression in SK-Hep1 and MHCC-97H cells with lentiviruses based on the expression level of KLF13 in HCC cell lines. Western blotting and qPCR results showed that KLF13 was effectively overexpressed in SK-Hep1 and MHCC-97H cells (Fig. 2A, B). CCK-8 and colony formation assays showed that the overexpression of KLF13 promoted the proliferation of SK-Hep1 and MHCC-97H cells (Fig. 2C, D). Migration and invasion assays showed that the overexpression of KLF13 promoted the mobility and invasiveness of HCC cells (Fig. 2E, F). We also assessed the epithelial-mesenchymal transition (EMT) markers E-cadherin, N-cadherin and vimentin. Western blot results showed that KLF13 overexpression inhibited the expression of E-cadherin and increased the expression of N-cadherin and vimentin in SK-Hep1 and MHCC-97H cells (Fig. 2G, H).

KLF13 knockdown suppressed the proliferation and migration of HCC cells

We created a stable model of KLF13 knockdown in HepG2 and Huh7 cells with plasmids. Western blot and qPCR results showed that KLF13 was effectively knocked down in HepG2 and Huh7 cells (Fig. 3A, B). Similarly, the knockdown of KLF13 inhibited the proliferation of HCC cells by CCK-8 assay (Fig. 3C). The results showed that KLF13 knockdown inhibited the migration and invasion of HCC cells (Fig. 3D, E). KLF13 knockdown also affected the expression of EMT-related proteins, as shown by the increase in E-cadherin and the inhibition of vimentin and N-cadherin expression (Fig. 3F). Taken together, our findings indicate that KLF13 was a cancer-promoting factor in HCC.

KLF13 overexpression inhibited the apoptosis of HCC cells

We analyzed the role of KLF13 in apoptosis. The apoptosis of vector-transfected and KLF13-overexpressing SK-Hep1 was assayed by 7-AAD and annexin V staining. The results demonstrated that the overexpression of KLF13 led to a decrease in the apoptosis rate, and the knockdown of KLF13 led to an increase in apoptotic cells (Fig. 4A). The cell cycle distribution also demonstrated that overexpression of KLF13 decreased the number of cells in the G1/G0 peak and increased the number of cells in the S peak; knockdown of KLF13 produced the opposite result (Fig. 4B). EdU is incorporated into newly synthesized DNA chain during DNA replication. We performed an EdU assay to further analyze DNA synthesis. EdU assays showed that proliferation was significantly increased in KLF13-overexpressing SK-Hep1 and MHCC-97H cells compared with control cells (Fig. 4C). In contrast, proliferation was significantly decreased in HepG2 and Huh7 cells (Fig. 4D). We analyzed the expression of the cell cycle protein markers cyclinD1, CDK2, and CDK4, and the apoptosis proteins cleaved-caspase3 and Bcl2 by western blotting. The results indicated that the protein expression of the G1 phase regulatory proteins cyclinD1, CDK2 and CDK4 was significantly higher, while the apoptosis protein cleaved caspase-3 was significantly lower after KLF13 overexpression (Fig. 4E). KLF13 knockdown had the opposite effects (Fig. 4F).

KLF13 overexpression enhanced the tumorigenicity of HCC cells in vivo

To explore the oncogenic role of KLF13 in HCC cell growth in vivo, we developed a subcutaneous xenograft model. HCC cells mixed with PBS were injected into 4-week-old BALB/c female nude mice. The effect on tumor growth was assessed by measuring the tumor size after injection. The tumors formed by KLF13-overexpressing SK-Hep1 cells were significantly larger than those formed by the vector control cells (Fig. 5A–C). Moreover, we also tested the proliferation marker expression of Ki67 and PCNA at the tissue level. IHC results showed that the expression of Ki67 and PCNA in the control group was lower than that in the KLF13 overexpression group (Fig. 5D). The results confirm that KLF13 promoted HCC cell-induced tumor growth.

KLF13 promotes cholesterol biosynthesis by promoting HMGCS1 expression through transcription

KLFs, including KLF13, have an important role in hepatic

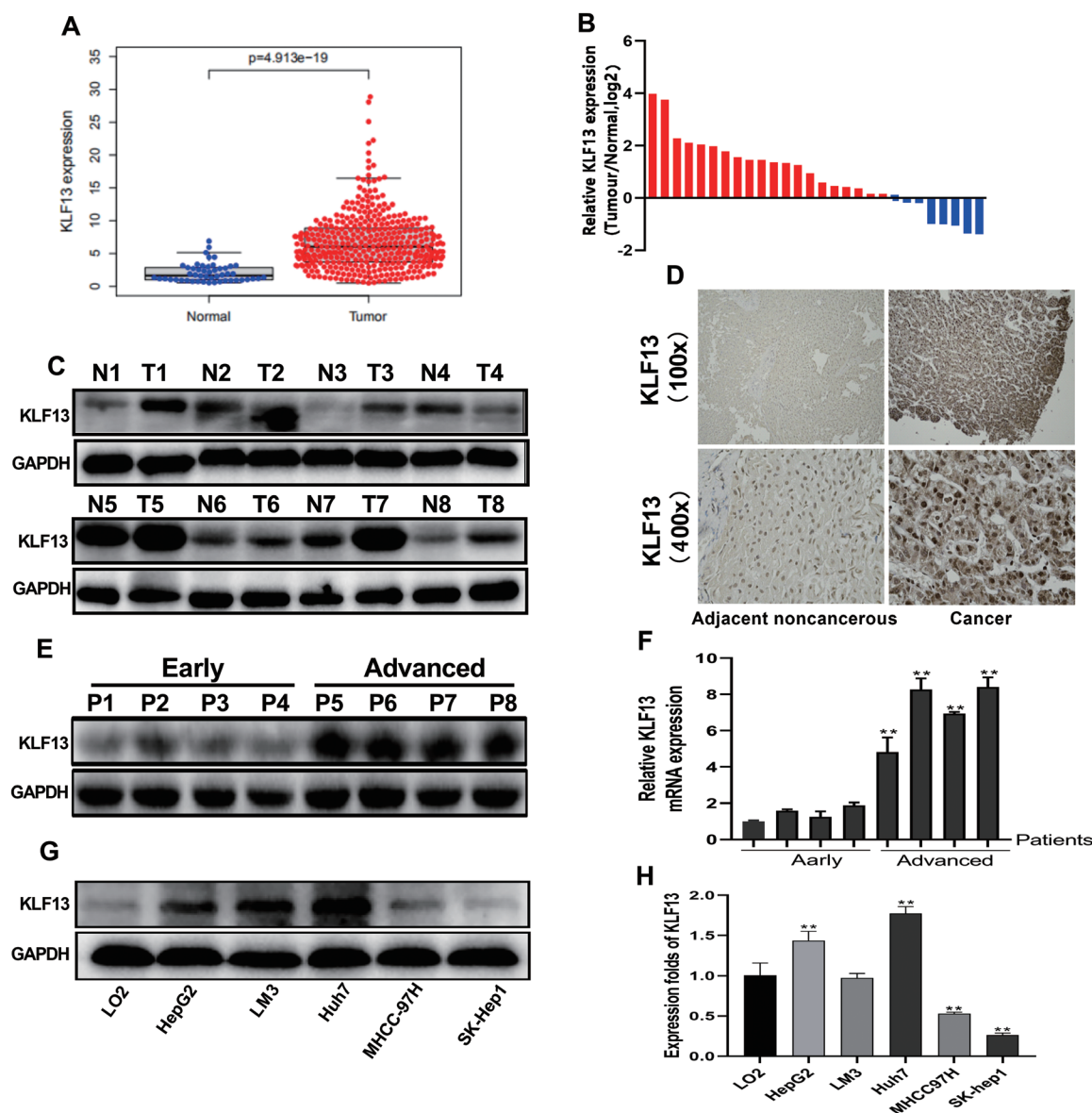


Fig. 1. KLF13 is upregulated in HCC tissues. (A) Krüppel-like factor 13 (KLF13) mRNA expression in liver cancer tissue ($n=371$) and normal tissue ($n=50$) in hepatocellular carcinoma (HCC) patients included in the cancer genome atlas (TCGA) database (<http://ualcan.path.uab.edu/>). (B) Quantitative polymerase chain reaction (qPCR) assay of KLF13 mRNA expression in HCC and paracarcinoma tissue. (C) Western blot assays of KLF13 protein expression in paracarcinoma ($n=8$) and HCC tissue ($n=8$). (D) KLF13 expression was assayed by immunohistochemical staining in HCC and paracarcinoma tissues ($\times 100$ visual field, $\times 400$). (E, F) Western blot and qPCR assays of KLF13 expression in four advanced HCC tissues and four early HCC tissues. (G, H) Western blot and qPCR assays of expression KLF13 in normal hepatocytes and HCC cell lines. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

lipid metabolism.¹⁵ The biosynthesis of cholesterol is beneficial to the proliferation of cancer cells. Thus, we analyzed whether KLF13 regulates cholesterol biosynthesis in HCC cells. First, cholesterol content was assessed in HCC cells. The results showed that KLF13 overexpression increased the cholesterol level in both SK-Hep1 and MHCC-97H cells (Fig. 6A). Similarly, KLF13 knockdown suppressed cholesterol biosynthesis in HepG2 and Huh7 cells (Fig. 6B). HMGCS1 is an important regulator of cholesterol biosynthesis. We examined whether KLF13 regulates the expression of HMGCS1. Western blot and RT-qPCR results showed that KLF13 overexpression upregulated HMGCS1 at the mRNA and protein levels in SK-Hep1 and MHCC-97H cells (Fig. 6C). In contrast, KLF13 knockdown led to downregulation

of HMGCS1 expression in HepG2 and Huh7 cells (Fig. 6D). We purified the KLF13-binding DNA in HepG2 cells by using KLF13 specific antibody and subjected to ChIP-seq analyses, the peak distribution of gene elements of HepG2-ip showed that only 6.54% peaks were located in gene promoter regions (Fig. 6E). ChIP-seq found that HMGCS1 was in the data (Fig. 6F), suggesting that HMGCS1 was a possible target gene of KLF13. To investigate whether KLF13 directly regulated HMGCS1, bioinformatic analysis of the HMGCS1 promoter was performed to predict the putative binding site of KLF13 in the HMGCS1 promoter, and then dual-luciferase reporter and ChIP-qPCR assays to verify that KLF13 transcriptionally activated the HMGCS1 promoter (Fig. 6G, H).

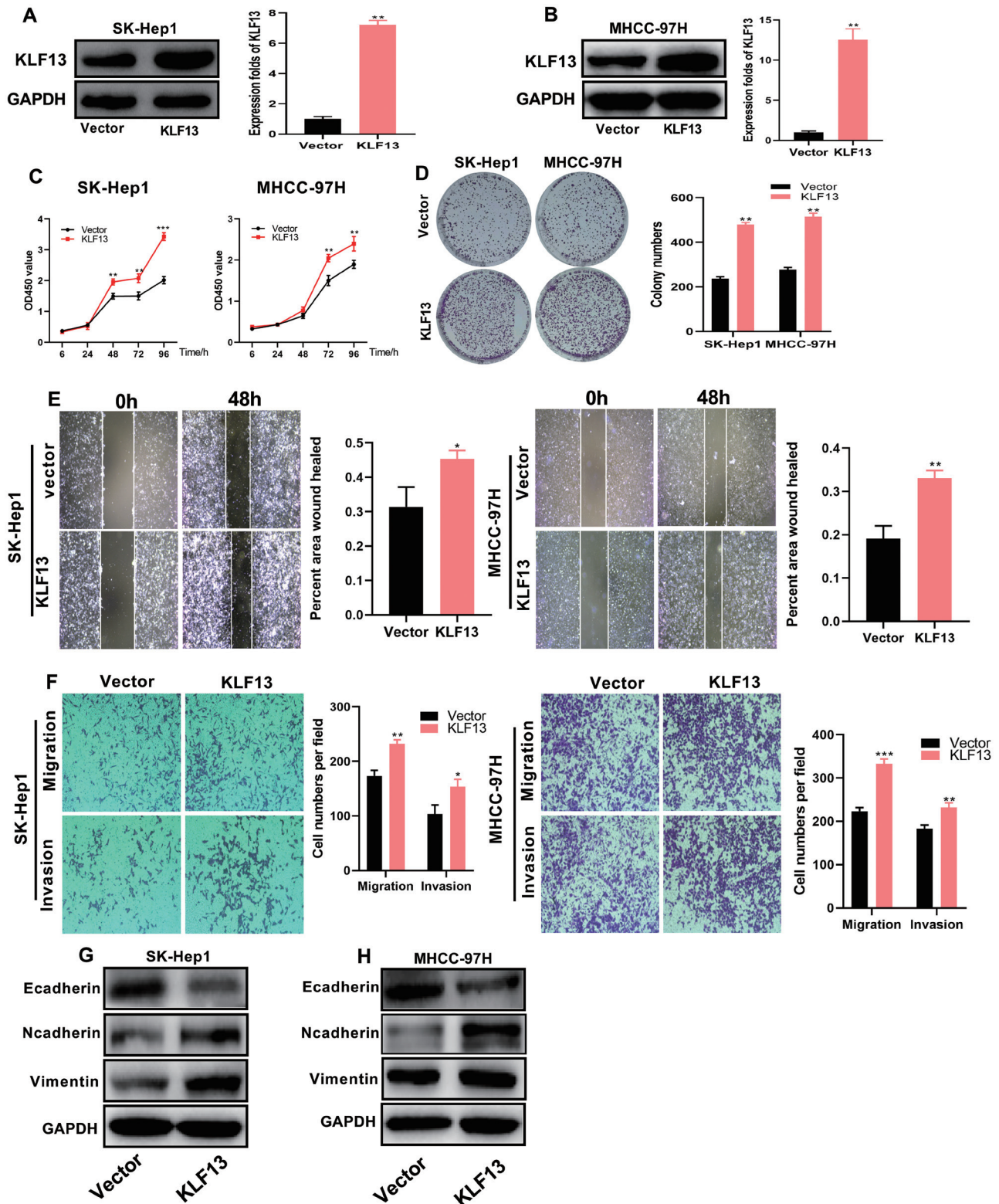


Fig. 2. KLF13 promotes the proliferation and migration of HCC cells. (A, B) Western blot and qPCR assays of Krüppel-like factor 13 (KLF13) gene and protein expression after KLF13 lentivirus or vector lentivirus transfection into SK-Hep1 and MHCC-97H cells. (C, D) Cell Counting Kit-8 (CCK8) analysis of cell proliferation, and colony formation (left, representative images; right, quantitative results). (E) Migration of hepatocellular carcinoma (HCC) cells with KLF13 overexpression compared with control vector were assayed by wound healing. (F) Effects of HCC cells overexpressing KLF13 compared with control vector on migration and invasion. (G, H) Expression of the epithelial-mesenchymal transition (EMT) markers E-cadherin, N-cadherin, and vimentin assayed by western blotting. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

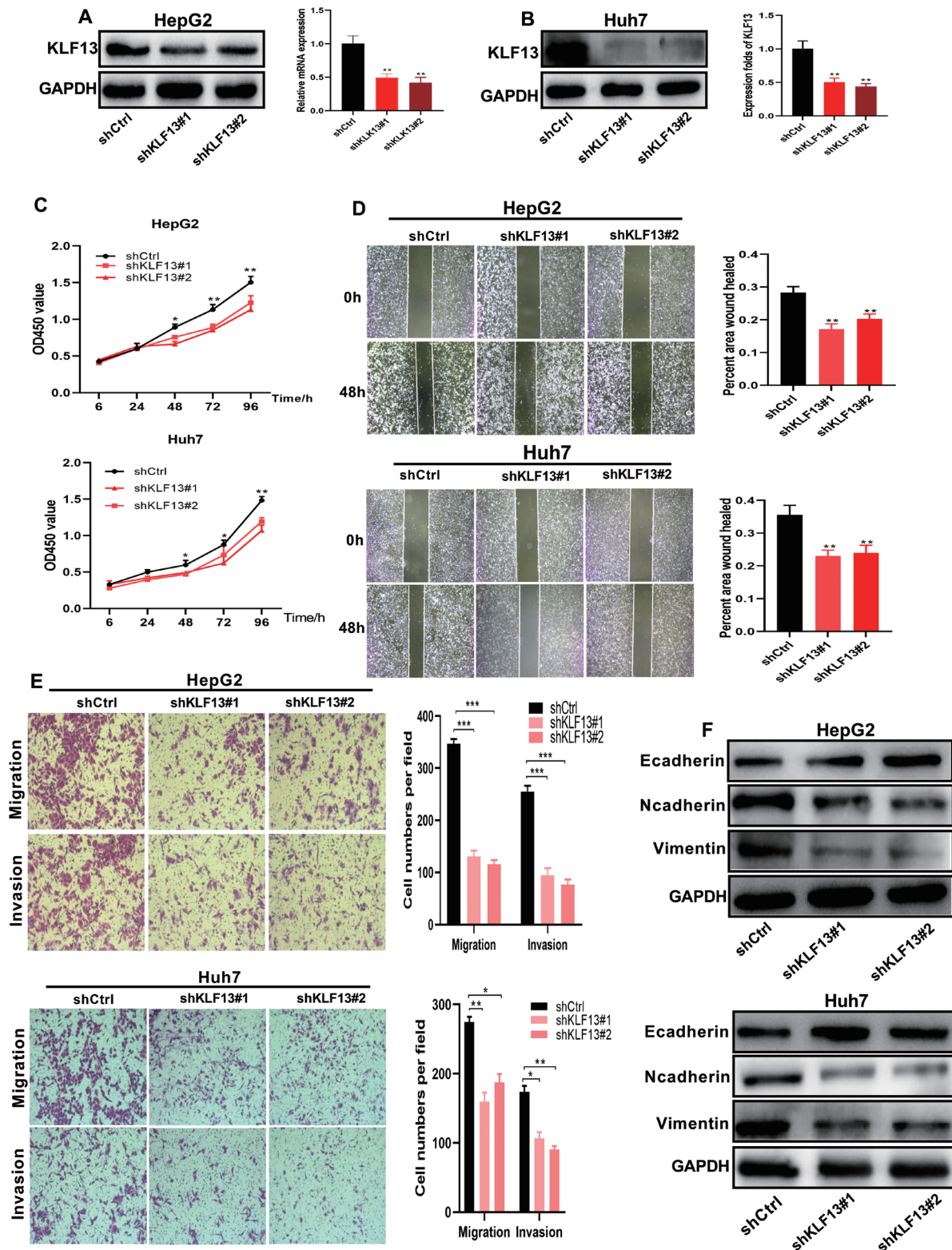


Fig. 3. shRNA-KLF13 suppresses the proliferation and migration of HCC cells. (A, B) Western blot and qPCR assays of Krüppel-like factor 13 (KLF13) gene and protein expression after shRNA-KLF13 plasmid or shRNA-Ctrl plasmid transfection into HepG2 and Huh7 cells. (C) Cell Counting Kit-8 (CCK8) assays indicate that shRNA-KLF13 inhibited the proliferation of hepatocellular carcinoma (HCC) cells. (D) Representative images of wound healing in HCC cell cultures. Cell migration was quantified as the percentage decrease of the wound area. (E) Effects of KLF13 knockdown on migration and invasion of HCC cells *in vitro*. (F) Expression of the epithelial-mesenchymal transition (EMT) markers E-cadherin, N-cadherin, and Vimentin examined by Western blot. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

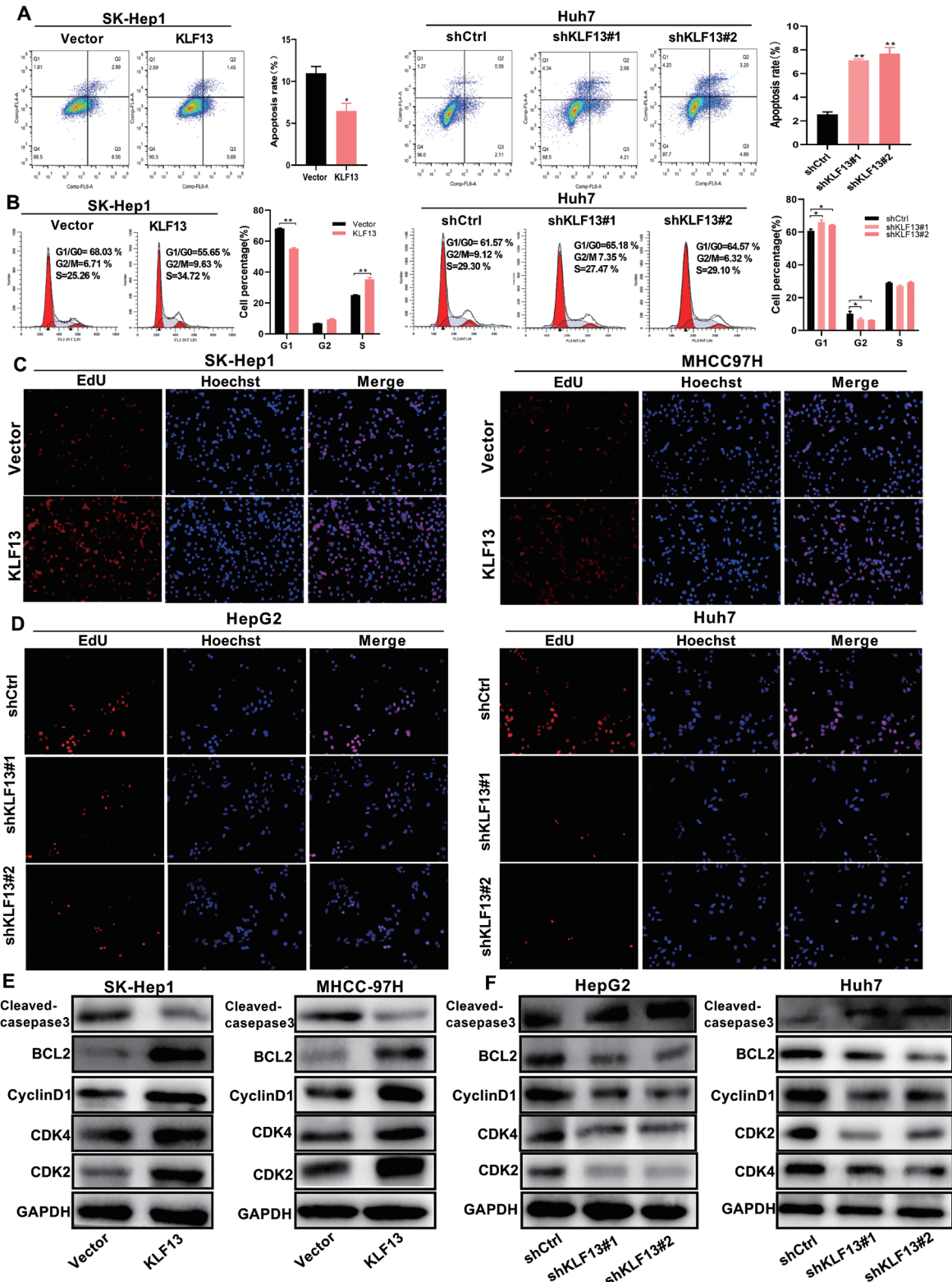


Fig. 4. KLF13 inhibits apoptosis and promote cell cycle progression in HCC cells. (A) Results of Annexin V and 7-AAD staining in HCC cells by flow cytometry. (B) Cell cycle distribution was determined flow cytometry (left, representative images; right, quantitative results). (C, D) Representative images of ethynyl-deoxyuridine (EdU) assay in HCC cells. (E, F) Expression of the cell cycle-related proteins cyclinD1, cyclinE, CDK2, CDK4, and the apoptosis proteins cleaved-caspase3 and Bcl2 determined by western blotting. Data are means \pm standard deviation of three independent assays and were compared using Student's t-test (Sk-hep-1 and MHCC-97H cells), or one-way analysis of variance followed by a Dunnett *t*-test (HepG2 and Huh7 cells). * p <0.05, ** p <0.01, *** p <0.001.

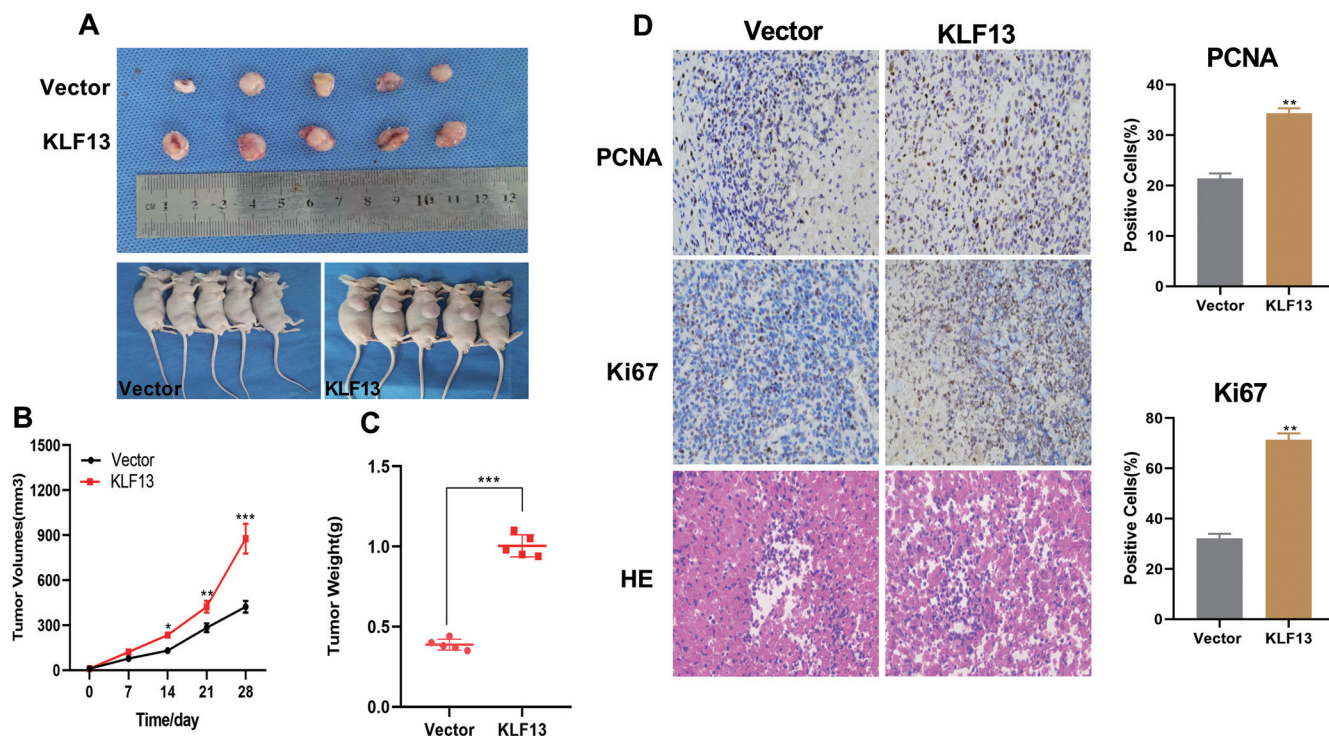


Fig. 5. KLF13 promotes cell growth of HCC *in vivo*. (A) Nodules in the Krüppel-like factor 13 (KLF13) and control vector groups ($n=5$). (B) One-week measurement of increase in nodule size in each group. (C) Average tumor weight increase in the KLF13 group compared with the control vector group. (D) Hematoxylin and eosin staining of xenograft tumors of nude mice and expression of PCNA and Ki-67 assayed by immunochemical staining. Quantitative results of PCNA and Ki67 are on the right. * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

Knockout of HMGCS1 inhibited the proliferation and migration of HCC cells overexpressing KLF13

KLF13 may promote the proliferation and migration of HCC cells through HMGCS1-mediated cholesterol synthesis. The gene should be knocked out in SK-Hep1 and MHCC-97H cells overexpressing KLF13 and vector. To test that hypothesis, HMGCS1 was knocked down in HCC cells overexpressing KLF13. Western blot assays showed that HMGCS1 expression was significantly reduced (Fig. 7A, B). Downregulation of HMGCS1 reduced cholesterol abundance in KLF13-overexpressing and vector SK-Hep1 and MHCC-97H cells (Fig. 7C). Importantly, CCK-8 and EdU analysis showed that silencing HMGCS1 inhibited the proliferation of KLF13-overexpressing and vector SK-Hep1 and MHCC-97H cells (Fig. 7D, E). In addition, migration and invasion assays demonstrated that silencing HMGCS1 inhibited the migration of HCC cells overexpressing KLF13 and vector (Fig. 7F).

Discussion

KLFs are a family of transcriptional regulators that bind to DNA and are involved in a variety of cellular processes, such as cell proliferation, differentiation, migration, inflammation, and pluripotency.¹⁶ Structurally, all members of the KLF family have three zinc finger DNA-binding domains at the C-terminus, while their N-terminal regions are highly diversified.¹⁷ Highly proliferative cancer cells need to produce membranes rapidly, and an increase in cholesterol synthesis contributes to that process.¹⁸ The increase in cholesterol is positively correlated with the occurrence of

HCC associated with steatohepatitis.¹⁹ Cholesterol metabolism substantially contributes to cancer progression, including cell proliferation, migration and invasion.²⁰⁻²² Therefore, cholesterol is required for the growth and survival of cancer cells, and inhibiting the biosynthesis of intracellular cholesterol has promise as an anticancer strategy. HMGCS1 is a key enzyme in cholesterol synthesis. In this study, we found that KLF13 regulated cholesterol biosynthesis in HCC cells by regulating HMGCS1. KLF transcription factors have different roles in different tumors, showing different transcriptional activities because they can interact with different activators or inhibitors.²³ Therefore, KLFs have different physiological and pathological functions. Recently, it was found that KLFs have an important role in the development of cancer, including HCC. Overexpression of miR-7 suppresses the progression of HCC by targeting suppressed KLF-4.²⁴ MiR-9 promotes the migration and invasion of HCC through KLF17.²⁵ The transcription factor KLF9 inhibits the growth of HCC cells by positively regulating the expression of p53 and provides a strategy for HCC clinical treatment.²⁶ A protective effect of the KLF6 transcription factor was observed on the apoptosis of HCC cells.²⁷ KLF8 promotes proliferation and metastasis in HCC through the Wnt/ β -catenin signaling pathway.^{28,29} A relationship has been observed between the downregulation of KLF17 expression, tumor metastasis and poor prognosis in HCC patients.³⁰ DNMT1-mediated KLF13 epigenetic silencing plays a role in regulating glioma cell proliferation and invasion, and the up-regulation of KLF13 may be found to be a new treatment.³¹ However, the exact function of KLF13 in HCC remains to be explored. Here, we found that KLF13 was a tumor promoter in HCC. The expression of KLF13 was increased in HCC tissue. Overexpression of KLF13 promoted the proliferation, migration, cell cycle progression, and DNA synthesis of

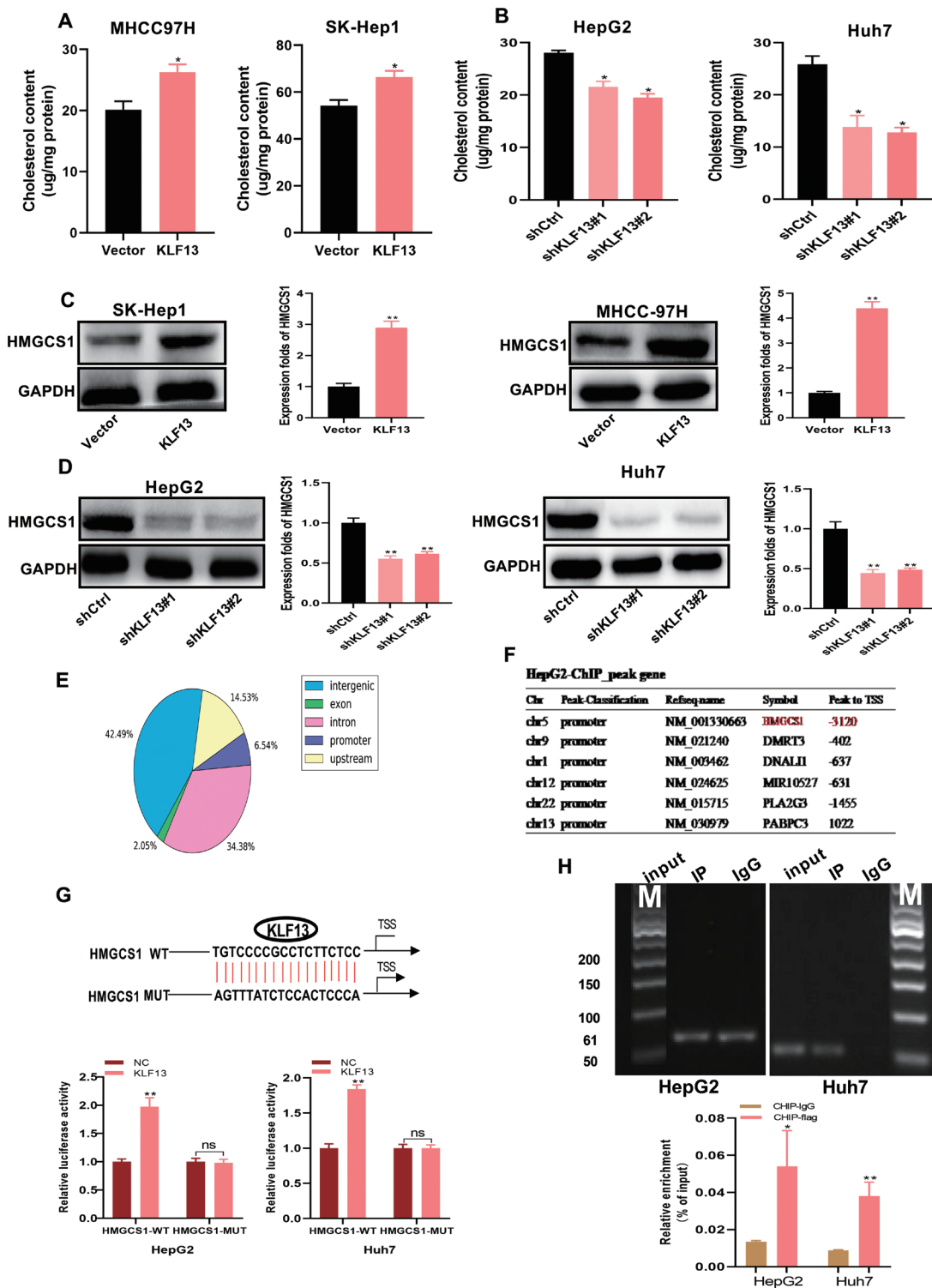


Fig. 6. KLF13 increases cholesterol biosynthesis and transcriptionally promotes HMGCS1. (A) Cholesterol content was assayed in control vector and SK-Hep1 and MHCC-97H cells overexpressing Krüppel-like factor 13 (KLF13). (B) Cholesterol content was assayed in shCtrl, shKLF13#1 and shKLF13#2 HCC cells. (C) Western blot and qPCR assays of HMG-CoA synthase 1 (HMGCS1) expression in control vector and in HCC cells overexpressing KLF13. (D) Western blot and qPCR assays of HMGCS1 in shCtrl and shKLF13#1, and shKLF13#2 HCC cells. (E) Peak distribution of the gene elements classified by location (UCSC annotation data) and showing intergenic regions, exons, introns, promoters, and upstream regions. (F) A list of a part of the results in HepG2-IP_vs_HepG2-input. (G) A dual-luciferase reporter assay demonstrated the luciferase activity of wild-type and mutated HMGCS1 promoters in HCC cells transfected with the KLF13 or KLF13 normal control plasmids. (H) ChIP-qPCR assays of KLF13 overexpression in HepG2 and Huh7 to determine KLF13 binding to HMGCS1 promoter. The enrichment fragments pulled down by anti-KLF13 antibody were analyzed by agarose gel electrophoresis. * $p < 0.05$; ** $p < 0.01$.

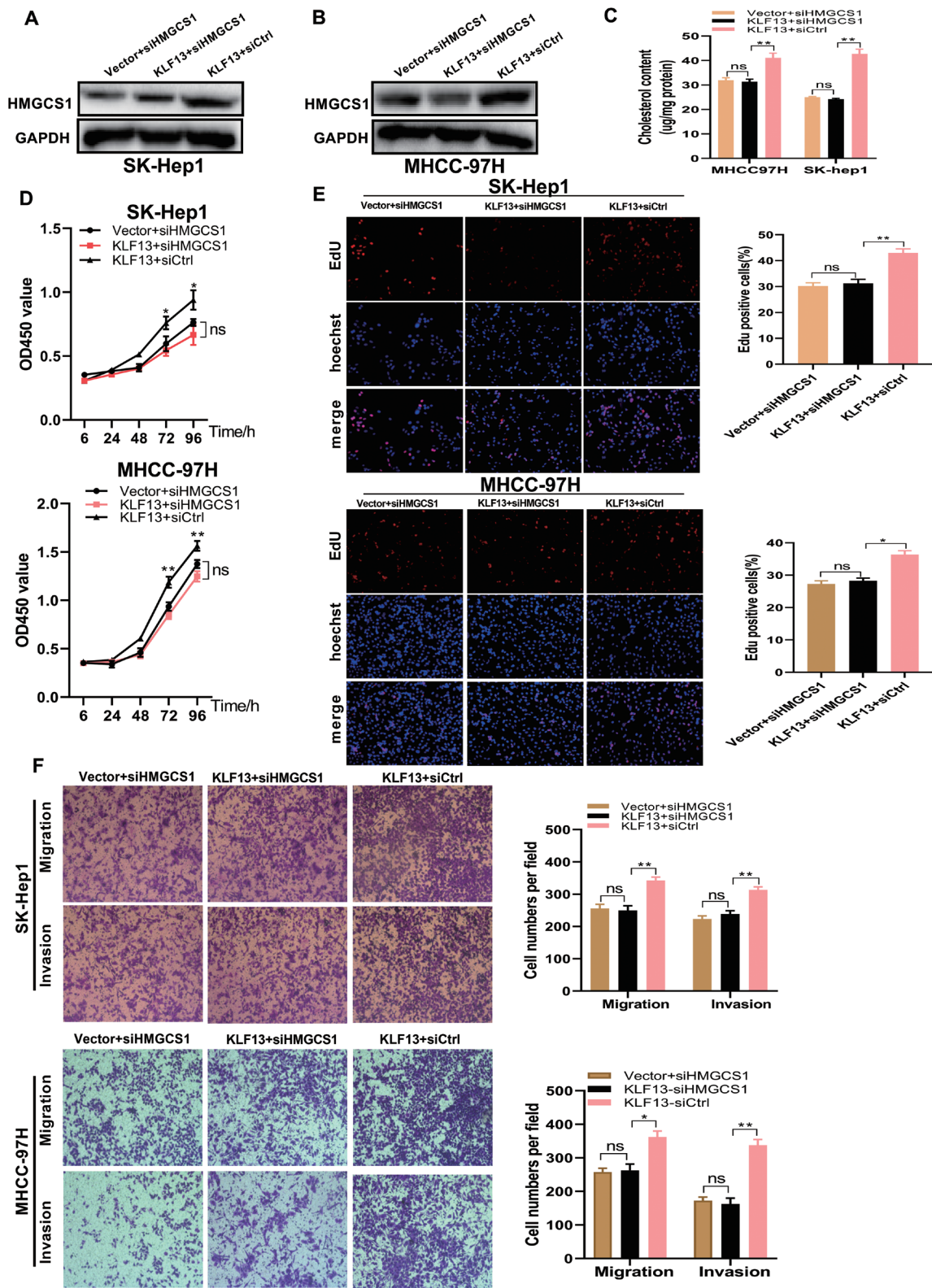


Fig. 7. HMGCS1 suppresses the proliferation and migration of HCC cells. (A, B) Western blot assays of gene expression in hepatocellular carcinoma (HCC) cells overexpressing Krüppel-like factor 13 (KLF13) after transfection with vector, siCtrl, or siHMGCS1. (C) Cholesterol content was assayed in HCC cells overexpressing KLF13 and transfected by control vector siCtrl, or siHMGCS1. (D, E) CCK8 and EdU assay showed that the downregulation of HMGCS1 suppressed the proliferation of HCC cells overexpressing of KLF13 HCC cells. (F) Effects of overexpression KLF13 HCC cells and vector transfected siCtrl or siHMGCS1 on migration and invasion. * $p < 0.05$; ** $p < 0.01$.

HCC cells. Our study showed that the expression of KLF13 was positively correlated with the occurrence and development of HCC. HMGCS1 is an enzyme that promotes the synthesis of cholesterol and fatty acid synthesis.³² Analysis of TCGA datasets showed that the *HMGCS1* gene is amplified in various cancers, such as stomach, breast, liver and lung.³³ The STAT3-miR-223-TGFBR3/HMGCS1 axis modulates the progression of cervical carcinoma.³⁴ However, given the diversity in the expression of HMGCS1 its activity in HCC needs further study. We found that overexpression of KLF13 upregulated the expression of HMGCS1 in HCC cells. A luciferase reporter assay showed that KLF13 positively regulated the transcriptional activity of HMGCS1. ChIP-qPCR assays revealed that KLF13 directly bound to the HMGCS1 promoter. We also observed that knockdown of HMGCS1 inhibited cholesterol synthesis, proliferation, and migration in HCC cells overexpressing KLF13. The results suggest that HMGCS1 was involved in the tumor-promoting effect of KLF13 in HCC. Therefore, we studied the biological function of KLF13 in controlling tumor development and HCC cell progression and clarified the potential mechanism of HMGCS1-mediated HCC progression. However, further studies are needed to determine how KLF13 regulates HMGCS1 and to assess the promoting effect of cholesterol on liver cancer. The results will help us to understand the treatment options for patients with HCC with high expression of KLF13.

Taken together, the study data demonstrate that KLF13 was upregulated in HCC tissue. The overexpression of KLF13 promoted HCC cell proliferation, migration and tumorigenesis. KLF13 had a tumor-promoting role in HCC by positively regulating HMGCS1-mediated cholesterol biosynthesis. Mechanistically, KLF13 transcriptionally upregulated HMGCS1 and enhanced cholesterol synthesis in HCC. In addition, the biological effect of overexpression KLF13 in HCC cells was reversed by silencing HMGCS1. Therefore, we believe that KLF13 is a valuable, potential therapeutic target for HCC.

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Conflict of interest

The authors have no conflict of interests related to this publication.

Author contributions

Study concept and design (CCC), acquisition of data (CCC), analysis and interpretation of data (CCC, XMX), drafting of the manuscript (CCC), critical revision of the manuscript for important intellectual content (XKZ, SZ), and administrative, technical, or material support, study supervision (HYL).

Ethical statement

The study was approved by the Ethics Committee of The Affiliated Hospital of Guizhou Medical University. All subjects gave written informed consent and the study was performed following the ethical guidelines of the Declaration of Helsinki. The animal study was reviewed and approved by the Research Ethics Committee of The Affiliated Hospital of Guizhou Medical University. This study was carried out in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health.

Data sharing statement

All data are available upon request.

References

- Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin* 2018;68(6):394–424. doi:10.3322/caac.21492, PMID:20207593.
- Torre LA, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J, Jemal A. Global cancer statistics, 2012. *CA Cancer J Clin* 2015;65(2):87–108. doi:10.3322/caac.21262, PMID:25651787.
- Maluccio M, Covey A. Recent progress in understanding, diagnosing, and treating hepatocellular carcinoma. *CA Cancer J Clin* 2012;62(6):394–399. doi:10.3322/caac.21161, PMID:23070690.
- Zhou M, Wang H, Zeng X, Yin P, Zhu J, Chen W, *et al*. Mortality, morbidity, and risk factors in China and its provinces, 1990–2017: a systematic analysis for the global burden of disease study 2017. *Lancet* 2019;394(10204):1145–1158. doi:10.1016/S0140-6736(19)30427-1, PMID:31248666.
- Kaczynski J, Cook T, Urrutia R. Sp1- and Krüppel-like transcription factors. *Genome Biol* 2003;4(2):206. doi:10.1186/gb-2003-4-2-206, PMID:12620113.
- Zhu B, Liu Q, Han Q, Zeng B, Chen J, Xiao Q. Downregulation of Krüppel-like factor 1 inhibits the metastasis and invasion of cervical cancer cells. *Mol Med Rep* 2018;18(4):3932–3940. doi:10.3892/mmr.2018.9401, PMID:30132534.
- Wang R, Xu J, Zhu W, Qiu T, Li J, Zhang M, *et al*. MiR-326/Sp1/KLF3: A novel regulatory axis in lung cancer progression. *Cell Prolif* 2019;52(2):e12551. doi:10.1111/cpr.12551, PMID:30485570.
- Zhao L, Zhang Y, Liu J, Yin W, Jin D, Wang D, *et al*. miR-185 Inhibits the Proliferation and Invasion of Non-Small Cell Lung Cancer by Targeting KLF7. *Oncol Res* 2019;27(9):1015–1023. doi:10.3727/096504018X15247341491655, PMID:29716672.
- Wang Q, Peng R, Wang B, Wang J, Yu W, Liu Y, *et al*. Transcription factor KLF13 inhibits AKT activation and suppresses the growth of prostate carcinoma cells. *Cancer Biomark* 2018;22(3):533–541. doi:10.3233/CBM-181196, PMID:29843216.
- Henson BJ, Gollin SM. Overexpression of KLF13 and FGFR3 in oral cancer cells. *Cytogenet Genome Res* 2010;128(4):192–198. doi:10.1159/000308303, PMID:20539070.
- Kim DS, Zhang W, Millman SE, Hwang BJ, Kwon SJ, Clayberger C, *et al*. Fbw7-mediated degradation of KLF13 prevents RANTES expression in resting human but not murine T lymphocytes. *Blood* 2012;120(8):1658–1667. PMID:22797700.
- Hegardt FG. Mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase: a control enzyme in ketogenesis. *Biochem J* 1999;338:569–582. PMID:10051425.
- Zhou S, Xu H, Tang Q, Xia H, Bi F. Dipyridamole Enhances the Cytotoxicities of Trametinib against Colon Cancer Cells through Combined Targeting of HMGCS1 and MEK Pathway. *Mol Cancer Ther* 2020;19(1):135–146. doi:10.1158/1535-7163.MCT-19-0413, PMID:31554653.
- Wang IH, Huang TT, Chen JL, Chu LW, Ping YH, Hsu KW, *et al*. Mevalonate Pathway Enzyme HMGCS1 Contributes to Gastric Cancer Progression. *Cancers (Basel)* 2020;12(5):1088. doi:10.3390/cancers12051088, PMID:32349352.
- Hsieh PN, Fan L, Sweet DR, Jain MK. The Krüppel-Like Factors and Control of Energy Homeostasis. *Endocr Rev* 2019;40(1):137–152. doi:10.1210/er.2018-00151, PMID:30307551.
- Tetreault MP, Yang Y, Katz JP. Krüppel-like factors in cancer. *Nat Rev Cancer* 2013;13(10):701–713. doi:10.1038/nrc3582, PMID:24060862.
- Lu XJ, Shi Y, Chen JL, Ma S. Krüppel-like factors in hepatocellular carcinoma. *Tumor Biol* 2015;36(2):533–541. doi:10.1007/s13277-015-3127-6, PMID:25652467.
- Mullen PJ, Yu R, Archer MC, Penn LZ. The interplay between cell signalling and the mevalonate pathway in cancer. *Nat Rev Cancer* 2016;16(11):718–731. doi:10.1038/nrc.2016.76, PMID:27562463.
- Liang JQ, Teoh N, Xu L, Pok S, Li X, Chu ESH, *et al*. Dietary cholesterol promotes steatohepatitis related hepatocellular carcinoma through dysreg-

- ulated metabolism and calcium signaling. *Nat Commun* 2018;9(1):4490. doi:10.1038/s41467-018-06931-6, PMID:30367044.
- [20] Ding X, Zhang W, Li S, Yang H. The role of cholesterol metabolism in cancer. *Am J Cancer Res* 2019;9(2):219–227. PMID:30906624.
- [21] Wang Y, Liu C, Hu L. Cholesterol regulates cell proliferation and apoptosis of colorectal cancer by modulating miR-33a-PIM3 pathway. *Biochem Biophys Res Commun* 2019;511(3):685–692. doi:10.1016/j.bbrc.2019.02.123, PMID:30827510.
- [22] Liu Z, Liu X, Liu S, Cao Q. Cholesterol promotes the migration and invasion of renal carcinoma cells by regulating the KLF5/miR-27a/FBXW7 pathway. *Biochem Biophys Res Commun* 2018;502(1):69–75. doi:10.1016/j.bbrc.2018.05.122, PMID:29782853.
- [23] Bouwman P, Philipsen S. Regulation of the activity of Sp1-related transcription factors. *Mol Cell Endocrinol* 2002;195(1-2):27–38. doi:10.1016/s0303-7207(02)00221-6, PMID:12354670.
- [24] Wu W, Liu S, Liang Y, Zhou Z, Liu X. MiR-7 inhibits progression of hepatocarcinoma by targeting KLF-4 and promises a novel diagnostic biomarker. *Cancer Cell Int* 2017;17:31. doi:10.1186/s12935-017-0386-x, PMID:28239300.
- [25] Sun Z, Han Q, Zhou N, Wang S, Lu S, Bai C, *et al*. MicroRNA-9 enhances migration and invasion through KLF17 in hepatocellular carcinoma. *Mol Oncol* 2013;7(5):884–894. doi:10.1016/j.molonc.2013.04.007, PMID:23684102.
- [26] Sun J, Wang B, Liu Y, Zhang L, Ma A, Yang Z, *et al*. Transcription factor KLF9 suppresses the growth of hepatocellular carcinoma cells in vivo and positively regulates p53 expression. *Cancer Lett* 2014;355(1):25–33. doi:10.1016/j.canlet.2014.09.022, PMID:25242357.
- [27] Sirach E, Bureau C, Péron JM, Pradayrol L, Vinel JP, Buscail L, *et al*. KLF6 transcription factor protects hepatocellular carcinoma-derived cells from apoptosis. *Cell Death Differ* 2007;14(6):1202–1210. doi:10.1038/sj.cdd.4402114, PMID:17347668.
- [28] Shen YN, He HG, Shi Y, Cao J, Yuan JY, Wang ZC, *et al*. Krüppel-like factor 8 promotes cancer stem cell-like traits in hepatocellular carcinoma through Wnt/ β -catenin signaling. *Mol Carcinog* 2017;56(2):751–760. doi:10.1002/mc.22532, PMID:27478926.
- [29] Yang T, Cai SY, Zhang J, Lu JH, Lin C, Zhai J, *et al*. Krüppel-like factor 8 is a new Wnt/ β -catenin signaling target gene and regulator in hepatocellular carcinoma. *PLoS One* 2012;7(6):e39668. doi:10.1371/journal.pone.0039668, PMID:22761862.
- [30] Liu FY, Deng YL, Li Y, Zeng D, Zhou ZZ, Tian DA, *et al*. Down-regulated KLF17 expression is associated with tumor invasion and poor prognosis in hepatocellular carcinoma. *Med Oncol* 2013;30(1):425. doi:10.1007/s12032-012-0425-3, PMID:23325444.
- [31] Wu R, Yun Q, Zhang J, Bao J. Downregulation of KLF13 through DNMT1-mediated hypermethylation promotes glioma cell proliferation and invasion. *Onco Targets Ther* 2019;12:1509–1520. doi:10.2147/OTT.S188270, PMID:30863117.
- [32] Pandya AA, Mullen PJ, Goard CA, Ericson E, Sharma P, Kalkat M, *et al*. Genome-wide RNAi analysis reveals that simultaneous inhibition of specific mevalonate pathway genes potentiates tumor cell death. *Oncotarget* 2015;6(29):26909–26921. doi:10.18632/oncotarget.4817, PMID:26353928.
- [33] Ye J, DeBose-Boyd RA. Regulation of cholesterol and fatty acid synthesis. *Cold Spring Harb Perspect Biol* 2011;3(7):a004754. doi:10.1101/cshperspect.a004754, PMID:21504873.
- [34] Zhang J, Jiang M, Qian L, Lin X, Song W, Gao Y, *et al*. The STAT3-miR-223-TGFBR3/HMGCS1 axis modulates the progression of cervical carcinoma. *Mol Oncol* 2020;14(9):2313–2331. doi:10.1002/1878-0261.12737, PMID:32491253.