YMTHE, Volume *30*

Supplemental Information

An SCD1-dependent mechanoresponsive pathway promotes HCC invasion and metastasis through lipid metabolic reprogramming

Hua-Hua Liu, Yang Xu, Cao-Jie Li, Shu-Jung Hsu, Xia-Hui Lin, Rui Zhang, Jie Chen, Jun Chen, Dong-Mei Gao, Jie-Feng Cui, Xin-Rong Yang, Zheng-Gang Ren, and Rong-Xin Chen

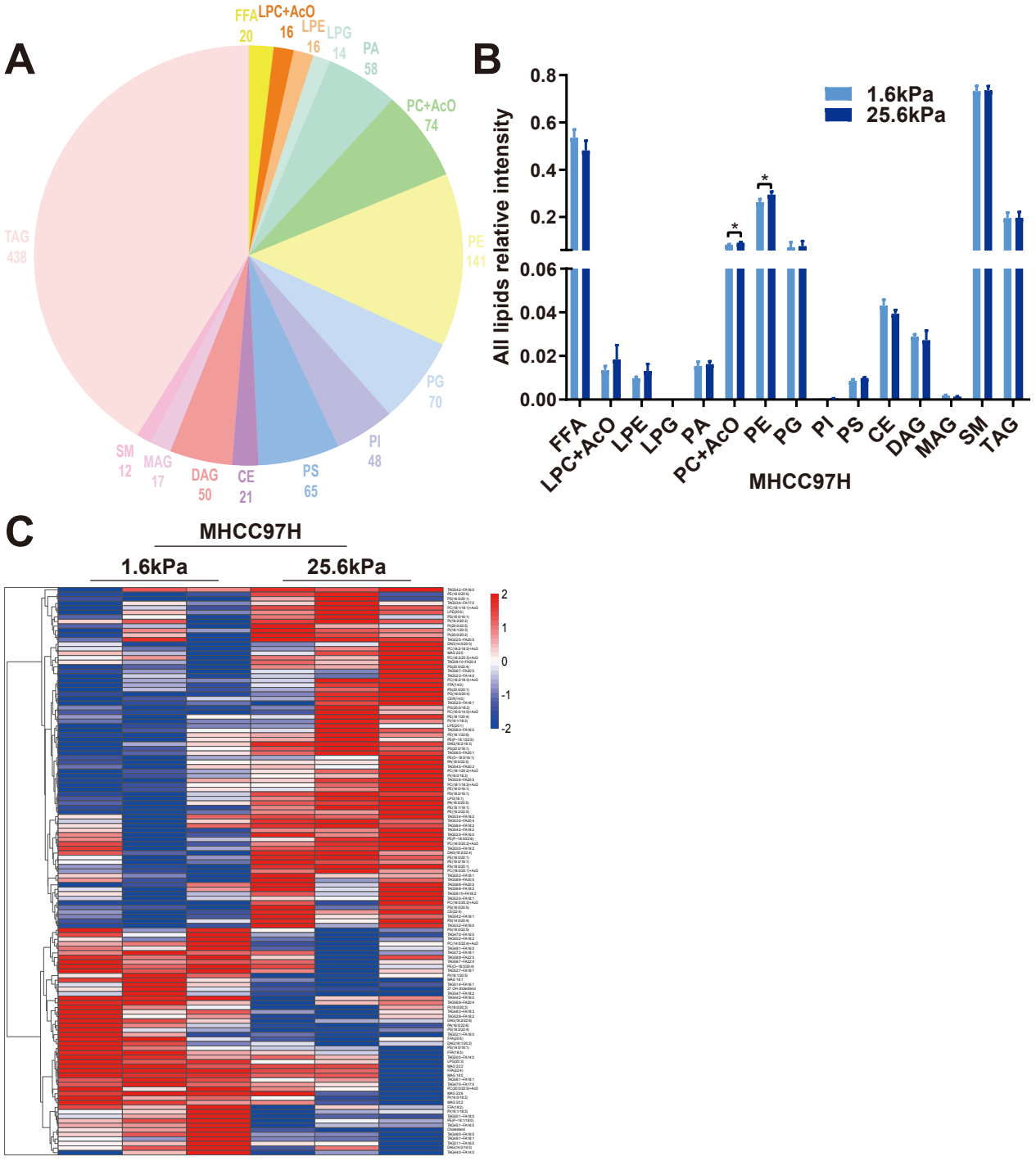

Figure S1. Lipid profiles in HCC cells are altered by matrix stiffness.

(A) Lipid classes were identified in MHCC97H cells cultured on supports at 1.6 kPa or 25.6 kPa in 3D Matrigel overlay culture. (B) Relative intensity of major lipid species in MHCC97H cells on 1.6 kPa or 25.6 kPa support. (C) Lipidomic changes occurring in HCC cells on stiff (25.6 kPa) vs. soft (1.6 kPa) supports were clustered using hierarchical clustering analysis and is shown as a heatmap. Pseudo-colors indicate an increase (red) or a decrease (blue), respectively.

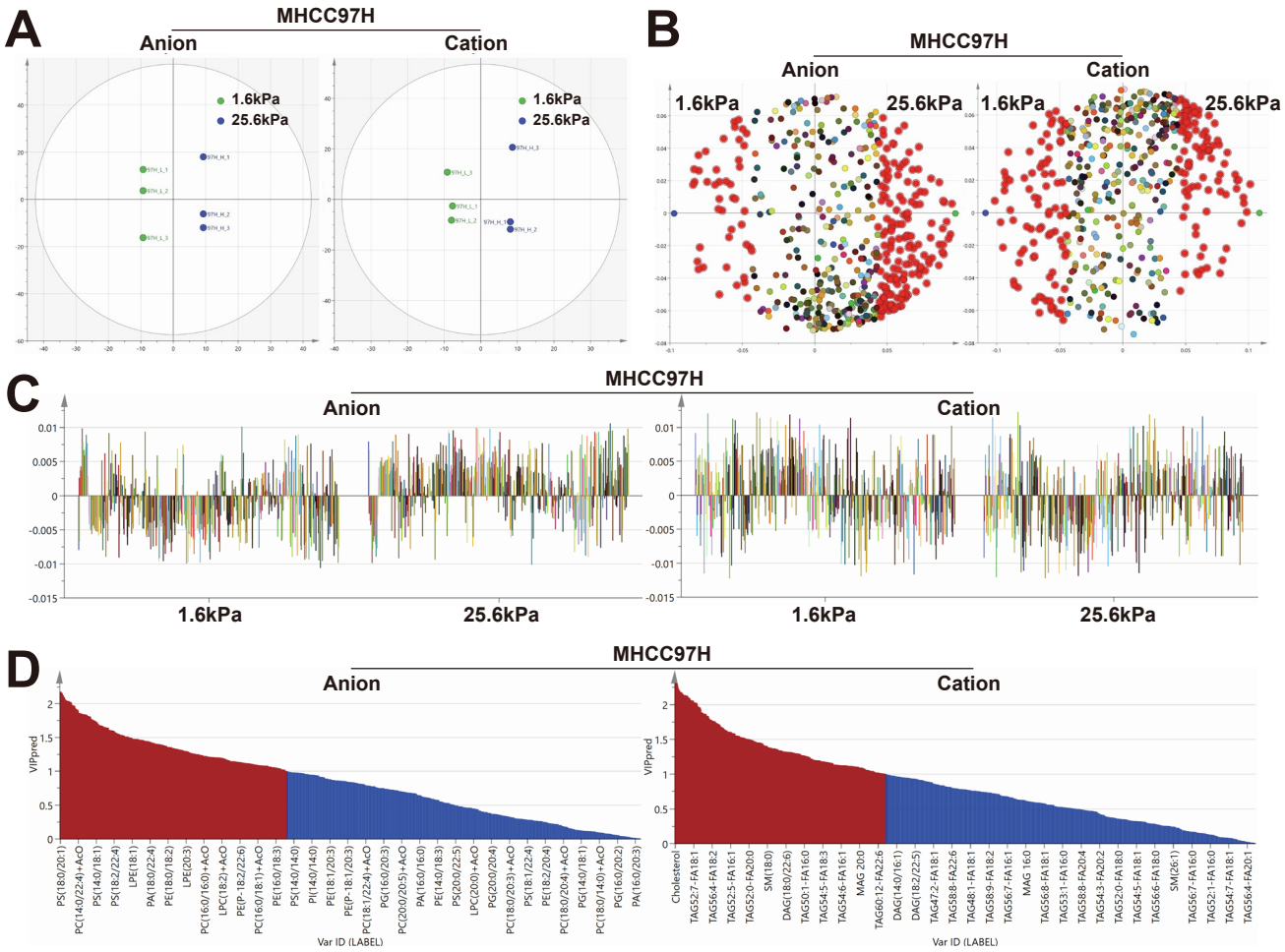

Figure S2. Identification of lipids in HCC cells regulated by matrix stiffness.

(A) Principal component analysis plot for exploratory data of anions and cations in MHCC97H cells on 1.6 kPa or 25.6 kPa support. (B) Lipid molecules in HCC cells on stiff (25.6 kPa) vs. soft (1.6 kPa) support. (C) Distribution of lipids in HCC cells on stiff (25.6 kPa) vs. soft (1.6 kPa) support. (D) Major lipid molecules in HCC cells on stiff (25.6 kPa) vs. soft (1.6 kPa) support were identified by SIMCA software.

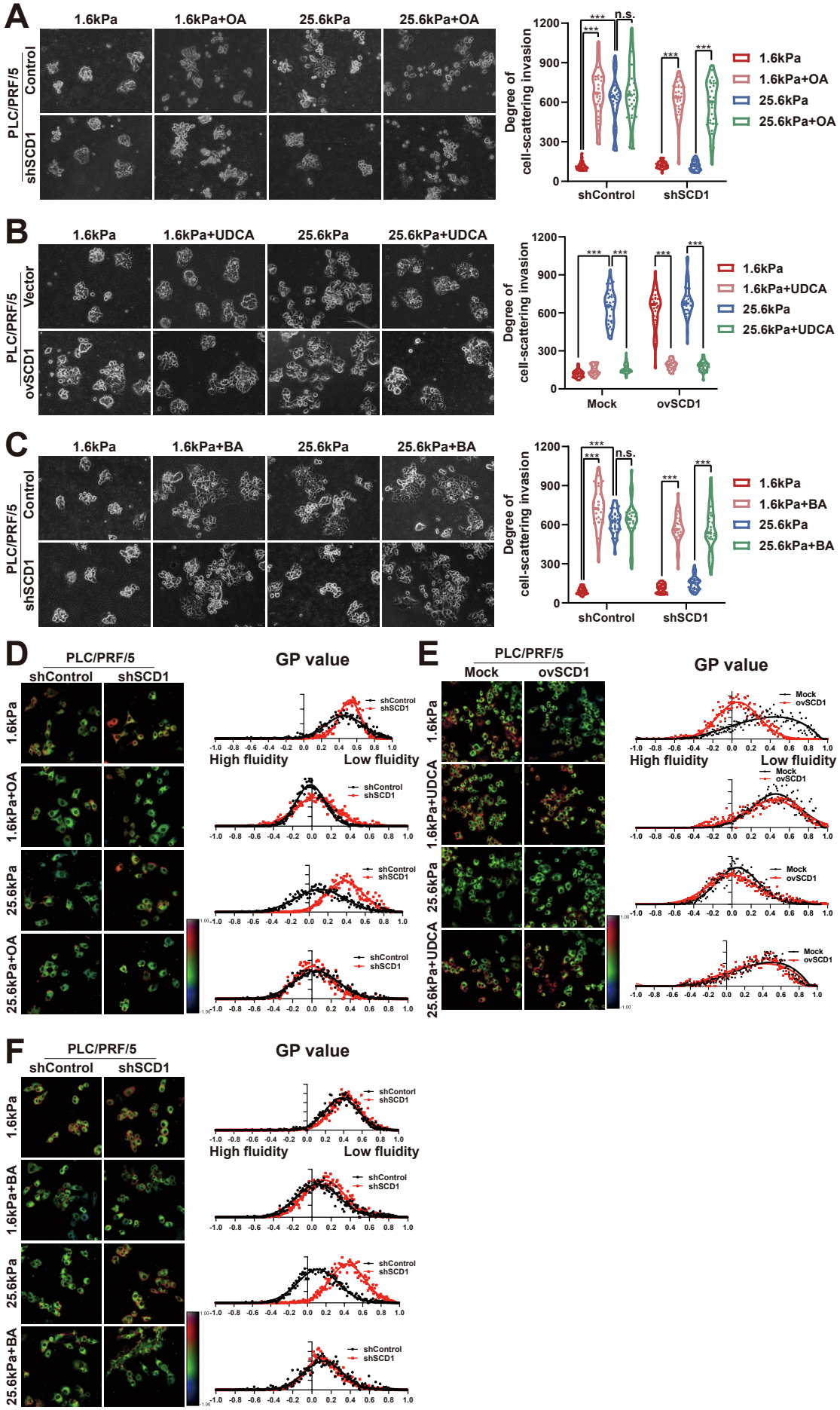
A
1.6kPa
1.6kPa+OA
25.6kPa
25.6kPa+OA
PLC/PRF/5
Control
shSCD1
Degree of cell-scattering invasion
1200
900
600
300
0
***
n.s.
shControl
shSCD1
1.6kPa
1.6kPa+OA
25.6kPa
25.6kPa+OA
B
1.6kPa
1.6kPa+UDCA
25.6kPa
25.6kPa+UDCA
PLC/PRF/5
Vector
ovSCD1
Mock
ovSCD1
1.6kPa
1.6kPa+UDCA
25.6kPa
25.6kPa+UDCA
C
1.6kPa
1.6kPa+BA
25.6kPa
25.6kPa+BA
PLC/PRF/5
Control
shSCD1
shControl
shSCD1
1.6kPa
1.6kPa+BA
25.6kPa
25.6kPa+BA
D
PLC/PRF/5
shControl
shSCD1
GP value
High fluidity
Low fluidity
1.6kPa
1.6kPa+OA
25.6kPa
25.6kPa+OA
E
PLC/PRF/5
Mock
ovSCD1
GP value
High fluidity
Low fluidity
1.6kPa
1.6kPa+UDCA
25.6kPa
25.6kPa+UDCA
F
PLC/PRF/5
shControl
shSCD1
GP value
High fluidity
Low fluidity
1.6kPa
1.6kPa+BA
25.6kPa
25.6kPa+BA

Figure S3. Membrane fluidity mediates matrix stiffness-induced cellular invasion in HCC cells.

(A) PLC/PRF/5 cells expressing control (shControl) or SCD1 shRNA (shSCD1) were cultured on polyacrylamide hydrogels with the indicated rigidities (1.6 kPa and 25.6 kPa) in the presence of OA in 3D Matrigel overlay culture. (B) HCC cells were transfected with Mock or SCD1 expressing plasmid (ovSCD1) and cultured on polyacrylamide hydrogels with the indicated rigidities (1.6 kPa and 25.6 kPa) in 3D Matrigel overlay culture and then treated with membrane-stabilizing agent UDCA. (C) PLC/PRF/5 cells expressing control (shControl) or SCD1 shRNA (shSCD1) were cultured on polyacrylamide hydrogels with the indicated rigidities (1.6 kPa and 25.6 kPa) in 3D Matrigel overlay culture and treated with membrane-agonist compound BA. The in vitro invasion of HCC cells is quantified by the degree of cell scattering using ImageJ software and GraphPad Prism 8. (D) Hue-saturation-brightness images of PLC/PRF/5 cells expressing shControl or shSCD1 in the presence of OA. (E) Hue-saturation-brightness images of PLC/PRF/5 cells transfected with Mock or ovSCD1 treated with/without UDCA. (F) Hue-saturation-brightness images of PLC/PRF/5 cells expressing shControl or shSCD1 treated with/without compound BA. ***, $P < 0.001$.

Table S1. Clinicopathological characteristics of 110 patients with HCC.

Variable		Number of patients (%)
Age(years)	≥50	51(46.4)
	< 50	59(53.6)
Gender	Female	6(5.5)
	Male	104(94.5)
ALT(U/L)	≥40	42(38.2)
	< 40	68(61.8)
Child Pugh	B	1(0.9)
	A	109(99.1)
HBsAg	Yes	101(91.8)
	No	9(8.2)
AFP (ng/ml)	≥400	47(42.7)
	< 400	63(57.3)
Liver Cirrhosis	Yes	84(76.4)
	No	26(23.6)
Encapsulation	Complete	67(60.9)
	Incomplete	43(39.1)
Differentiation	Low	36(32.7)
	High	74(67.3)
Tumor size(cm)	≥5	35(31.8)
	< 5	75(68.2)
Tumor number	Multiple	11(10)
	Single	99(90)
Vascular Invasion	Yes	42(38.2)
	No	68(61.8)
MVI	Yes	42(38.2)
	No	68(61.8)
PVTT	Yes	16(14.5)
	No	94(85.5)
BCLC stage	B+C	19(17.3)
	0+A	91(82.7)
Collagen (%)	≥11.98	55(50)
	< 11.98	55(50)
SCD1	High	58(52.7)
	Low	52(47.3)

Table S2. Univariate and multivariate analysis of factors associated with the RFS in patients with HCC.

	Category	Univariate analysis		Multivariate analysis	
		HR (95% CI)	*P*	HR (95% CI)	*P*
Age(years)	≥50/ < 50	1.212(0.767-1.913)	0.410		
Gender	Male/Female	1.710(0.688-4.250)	0.248		
ALT(U/L)	≥40/ < 40	1.278(0.802-2.036)	0.303		
Child Pugh	B/A	1.102E-07(0-inf)	0.996		
HBsAg	Yes/No	1.292 (0.560-2.979)	0.547		
AFP (ng/ml)	≥400/ < 400	1.472(0.928-2.335)	0.101		
Liver Cirrhosis	Yes/No	1.170(0.672-2.038)	0.579		
Encapsulation	Complete /Incomplete	0.563(0.355-0.895)	0.015	1.017 (0.564-1.836)	0.955
Differentiation	Low/High	1.317(0.812-2.134)	0.264		
Tumor size(cm)	≥5/ < 5	2.369(1.471-3.815)	< 0.001	2.116 (1.259-3.560)	0.005
Tumor number	Single/Multiple	1.005(0.461-2.191)	0.991		
Vascular Invasion	Yes/No	1.871(1.178-2.974)	0.008	0.715(0.090-5.669)	0.751
MVI	Yes/No	1.792(1.129-2.845)	0.013	1.842 (0.236-14.387)	0.560
PVTT	Yes/No	2.469(1.338-4.553)	0.004	1.816 (0.353-9.347)	0.475
BCLC stage	B+C/0+A	2.023 (1.138-3.597)	0.016	0.596(0.138-2.575)	0.488
Collagen (%)	≥11.98/ < 11.98	2.550(1.580-4.116)	< 0.001	0.837(0.482-1.454)	0.527
SCD1	High/Low	2.591(1.588-4.229)	< 0.001	1.583(0.926-2.706)	0.093
Collagen+SCD1	High/Low	8.554(3.879-18.865)	< 0.001	8.947(3.531-22.670)	< 0.001

$P < 0.05$ was regarded as statistically significant and *P* value was calculated by Cox proportional hazards regression.

Table S3. Univariate and multivariate analysis of factors associated with the OS in patients with HCC.

	Category	Univariate analysis		Multivariate analysis	
		HR (95% CI)	*P*	HR (95% CI)	*P*
Age(years)	≥50/ < 50	1.736(0.998-3.017)	0.051	1.904(1.022-3.547)	0.042
Gender	Male/Female	1.873(0.674-5.205)	0.229		
ALT(U/L)	≥40/ < 40	1.254(0.719-2.186)	0.425		
Child-Pugh	B/A	6.754(0.892-51.133)	0.064	17.668(0.789-395.492)	0.070
HBsAg	Yes /No	1.025 (0.370-2.843)	0.962		
AFP (ng/ml)	≥400/ < 400	2.172(1.257-3.754)	0.006	1.631(0.910-2.925)	0.101
Liver cirrhosis	Yes /No	1.270(0.637-2.533)	0.497		
Encapsulation	Complete /Incomplete	0.541(0.314-0.932)	0.027	1.040(0.502-2.153)	0.916
Differentiation	Low/High	1.515(0.866-2.651)	0.145		
Tumor size(cm)	≥5/ < 5	2.214(1.277-3.836)	0.005	2.324(1.274- 4.239)	0.006
Tumor number	Single/Multiple	0.878(0.349-2.207)	0.781		
Vascular invasion	Yes /No	2.160(1.251-3.730)	0.006	6.561(0.820-52.486)	0.076
MVI	Yes /No	1.900(1.098-3.271)	0.022	0.276(0.037-2.075)	0.211
PVTT	Yes /No	1.863(0.934-3.717)	0.078	1.452(0.156-13.504)	0.743
BCLC stage	B+C/ 0+A	1.936 (1.014-3.695)	0.045	0.728(0.094-5.628)	0.761
Collagen (%)	≥11.98/ < 11.98	2.295(1.294-4.070)	0.005	0.492(0.135-1.786)	0.281
SCD1	High/Low	2.562(1.419-4.626)	0.002	1.463(0.709-3.017)	0.303
Collagen+SCD1	High/Low	2.847(1.592-5.092)	< 0.001	5.278(1.396-19.949)	0.014

$P < 0.05$ was regarded as statistically significant and *P* value was calculated by Cox proportional hazards regression.

Supplemental Methods

Cell culture

Human HCC cells MHCC97H (established by Liver Cancer Institute, Zhongshan Hospital of Fudan University, Shanghai, China), PLC/PRF/5, Hep3B and Huh7 (purchased from the cell bank of Chinese Academy of Sciences, Shanghai, China) were grown in Dulbecco's modified Eagle media (Gibco,) supplemented with 10% fetal bovine serum (FBS; Biosun, China) and 1% penicillin-streptomycin (Gibco, USA) at 37°C in a 5% CO_2 incubator. The authenticity of HCC cell lines was validated by DNA Short Tandem Repeat (STR) analysis.

Antibodies and reagents

Primary antibodies, including anti-SCD1 (Santa Cruz, 1:200 for immunostaining, 1,000 for Western blot), anti-α-Tubulin (Beyotime Biotechnology, China; 1:1,000), anti-N-cadherin (CST,1:1,000 for Western blot), anti-E-cadherin (CST, 1:1,000 for Western blot), anti-human laminin (Abcam, 1:200), anti-β1-integrin clone 6S6 (Millipore Sigma, USA; 50 μg/mL), control IgG1 (CST, USA) and anti-Ubiquitin(P4D1) (CST, USA) were used. The secondary antibodies included anti-mouse conjugated with Alexa Fluor 488 (Life Technologies) and anti-mouse conjugated horseradish peroxidase (HRP) antibodies (Beyotime Biotechnology). Focal adhesion kinase (FAK) inhibitor (PF562271, Selleck, China) was solubilized in dimethylsulfoxide (DMSO) and utilized at a concentration of 0.5 μM.

Generation of stable gene knockdown or overexpression cell lines

Stable gene knockdown or overexpression cell lines were generated using lentiviral plasmid vectors (Genechem, China). Briefly, the lentiviruses carrying SCD1 shRNA or control shRNA, carrying SCD1 cDNA or control vector were infected into HCC cells MHCC97H and PLC/PRF/5 with the multiplicity of infection (MOI) of 20 and 10, respectively. SCD1 shRNA1, 5'-CGTCCTTATGACAAGAACATT-3'; SCD1 shRNA2, 5'-CTACGGCTCTTTCTGATCATT-3'; SCD1 shRNA3, 5'-CCCACCTACAAGGATAAGGAA-3'; Control shRNA, 5'-TTCTCCGAACGTGTCACGT-3'. Infected cells were then selected for stable cell subclones with 3-6 μg/mL puromycin. SCD1 protein levels were analyzed by Western blot.

Polyacrylamide hydrogels with variable stiffness

A collagen-coated polyacrylamide hydrogel system with incremental stiffness ranging from 1.6 kilo-Pascal (kPa) to 25.6 kPa was constructed between glass coverslips by mixing acrylamide and bis-acrylamide (Usen, China) at variable ratios, TEMED, and ammonium persulfate (APS, Sigma, USA)

according to the formula described by Dou et al.[1] A 1 mm-thick flat hydrogel was formed and cut into appropriate sizes after polymerization. After rinsing with phosphate-buffered saline (PBS) and sterilizing by microwave heating, the gels were treated with dopamine hydrochloride solution (1 mg/mL in 50 mM HEPES, pH 8.5, Sigma) for 20 min, followed by incubation with collagen I solution (0.06 mg/mL in PBS, pH 7.4) (BD Biosciences, USA) for 60 min and pre-incubation in FBS-free culture medium at 4 °C overnight.

Three-dimensional (3D) cell culture

The 3D cell culture was carried out using the method described previously by Wei et al.[2] Briefly, HCC cells were seeded on the polyacrylamide hydrogels with different stiffness in six-well plates and cultured in 2% Matrigel DMEM supplemented with 10% FBS and 1% penicillin and streptomycin. The media were replaced every 4 days. The formed cell spheres at 10-day post culture were photographed using a contrast-phase Olympus BX51 inverted microscope (200× magnification) under a bright field. The cell spheres protruding into the Matrigel were scored as round, compact morphology or spread, cell-dissociated and invasive phenotype. The degree of cell scattering was defined as a coefficient of variation (percentage) of scattered distribution of cells in each cell sphere, quantified by ImageJ software in 50 cell spheres from five random fields per well, indicating cell invasion.

Reverse transcription and quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted from cells using TRIzol reagent (Invitrogen, USA). The cDNA was synthesized from 1 μg RNA using a cDNA Synthesis Super Mix Kit (Yeasen, China) and used as template for a real-time PCR amplification with gene specific primers and FastStart Universal SYBR Green Master (Rox) Kit (Roche, USA) on an Applied Biosystems 7500 PCR system. The expression value of the target gene was calculated using the 2-ΔΔCt method normalized to that of β-actin and expressed as fold-change. The following primers were used for SCD1 amplification: sense primer, 5′-GCCCCTCTACTTGGAAGACGA-3′ and antisense primer, 5'-AAGTGATCCCATACAGGGCTC-3'. The β-actin was amplified using sense primer, 5′-CATGTACGTTGCTATCCAGGC-3' and antisense primer, 5'-CTCCTTAATGTCACGCACGAT-3'.

Western blot

Western blot analysis was performed as described previously.[3,4] Briefly, the protein was extracted from total cells lysates, separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to polyvinylidene difluoride (PVDF) membrane (Merck Millipore, USA). The

membrane was blocked with 5% fat-free milk and probed with primary antibodies overnight at 4 °C, followed by incubation with horseradish peroxidase (HRP)-conjugated secondary antibody. The reaction was developed by an enhanced chemiluminescence assay. For IP, cell lysates incubated with IP antibodies against SCD1 (1:100, Abcam, USA) rotationally at 4 ℃ overnight. On the second day, Protein A Magnetic Beads (73778, CST) were added for pulling down SCD1 at room temperature for 20 min.

Lipidomic profile analysis

Total lipids were extracted using a modified method by Bligh and Dyer.[5] Briefly, 1×10^6 cells were sonicated for 30 s in 900 μL ddH_2O. Then, the suspension was mixed with 1 mL chloroform (HPLC grade) (Billerica, MA, USA) and 2 mL methanol (Billerica, MA, USA) for 1 min, followed by the addition of 10 μL internal standard cocktails (Alabaster, AL, USA) and 1 mL chloroform for 1 min. Subsequently, 1 mL ddH_2O was added to the mixture before centrifugation at 2000 ×*g*, 4 °C for 15 min. After stratification, the lower layer of chloroform phase was withdrawn into a new glass tube (Kimble, USA), evaporated under nitrogen gas, and reconstituted with 200 μL of dichloromethane: methanol (1:2) containing 10 mM ammonium acetate (Billerica). Of this, 180 uL was transferred into the sample bottle. Finally, lipid samples were subjected to LC-MS analysis as described previously (5). Each experiment was repeated three times.

Lipid compositions were identified by mass spectrometry and compared to internal standards for quantification as reported previously.[6] Under multi-reaction monitoring (MRM) mode, the normal-phase silica liquid chromatography-coupled (NPLC) triple quadrupole mass spectrometer (QTRAP® 6500, SCIEX, USA) was used to extract the lipid for the positive and negative electrospray ionization mode, and Q-Trap was used to scan the precursor/product ion to obtain the chemical structures of the lipids. The peak area of each pair obtained by mass spectrometry was processed and quantified using MultiQuant™ software (AB, SCIEX), and lipidomic data were processed with SIMCA software (v 14.1). The relative signal intensities were calculated by normalization to cell numbers and total signal intensities. The peak changes between samples were compared by manual quantification. Multivariate statistical analysis and cluster analysis were carried out using MetaboAnalyst 4.0 (http://www.metaboanalyst.ca).

Immunofluorescence

Briefly, cells were washed with PBS, fixed with 4% paraformaldehyde (Beyotime Biotechnology), permeabilized with 0.5% TritonX-100 (Beyotime Biotechnology), and blocked with 5% bovine serum albumin at room temperature for 30 min. Then, the samples were incubated with primary antibodies

overnight at 4 ℃, followed by incubation with secondary antibody for 60 min at room temperature and counterstained for nucleus with 4',6-diamidino-2-phenylindole (DAPI, Thermo Fisher, USA). The images were acquired using an Olympus BX51 microscope (200× magnification), and the signals were quantified using ImageJ analysis software (National Institutes of Health, Bethesda, MD, USA).

SCD1 degradation assay

SCD1 protein stability in HCC cells on polyacrylamide gels with different stiffness was analyzed by Western blot in the presence of protein synthesis inhibitor cycloheximide (Selleck, China) at a concentration of 20 μg/mL or cycloheximide with proteasome inhibitor MG-132 (MCE, China) at 10 μM. At each time point, cells were harvested and lysed with RIPA buffer. Equivalent amounts of total protein were analyzed by Western blot.

Immunohistochemistry

The paraffin-embedded tumor tissue sections were deparaffinized with xylene and dehydrated with alcohol. After microwave antigen retrieval and blocking with 5% goat serum, sections were incubated with primary antibodies overnight at 4 ℃ and secondary antibodies at room temperature for 30 min, followed by development with 3,3'-diaminobenzidine (DAB) and counterstaining with hematoxylin. The images were captured under Olympus BX51 microscope (×100 magnification) and analyzed using Image-Pro Plus 6.0 (Media Cybernetics, USA).

Analysis of The Cancer Genome Atlas (TCGA) data

The publicly available data were downloaded from the TCGA Research Network. For the survival analysis, we examined the expression of the SCD1and COL1A1 in TCGA-HCC cohort (368 tumoral samples). The median of all samples was used as the threshold and the tumor samples were splitted into high- and low-expression groups. Kaplan-Meier survival curves were generated and group comparisons were assessed by a log-rank test.

Supplemental References

1. Dou, C., Liu, Z., Tu, K., Zhang, H., Chen, C., Yaqoob, U., et al. (2018). P300 Acetyltransferase Mediates Stiffness-Induced Activation of Hepatic Stellate Cells into Tumor-Promoting Myofibroblasts. Gastroenterology 154, 2209-2221.e2214.

2. Wei, S.C., Fattet, L., Tsai, J.H., Guo, Y., Pai, V.H., Majeski, H.E., et al. (2015). Matrix stiffness drives epithelial-mesenchymal transition and tumour metastasis through a TWIST1-G3BP2 mechanotransduction pathway. Nat Cell Biol 17, 678-688.

3. Zhang, R., Ma, M., Lin, X.H., Liu, H.H., Chen, J., Chen, J., et al. (2018). Extracellular matrix collagen I promotes the tumor progression of residual hepatocellular carcinoma after heat treatment. BMC Cancer 18, 901.

4. Sun, J., Zhou, C., Zhao, Y., Zhang, X., Chen, W., Zhou, Q., et al. (2021). Quiescin sulfhydryl oxidase 1 promotes sorafenib-induced ferroptosis in hepatocellular carcinoma by driving EGFR endosomal trafficking and inhibiting NRF2 activation. Redox Biol 41, 101942.

5. Bligh, E.G., and Dyer, W.J. (1959). A rapid method of total lipid extraction and purification. Can J Biochem Physiol 37, 911-917.

6. Gao, D., Zhang, L., Song, D., Lv, J., Wang, L., Zhou, S., et al. (2019). Values of integration between lipidomics and clinical phenomes in patients with acute lung infection, pulmonary embolism, or acute exacerbation of chronic pulmonary diseases: a preliminary study. J Transl Med 17, 162.