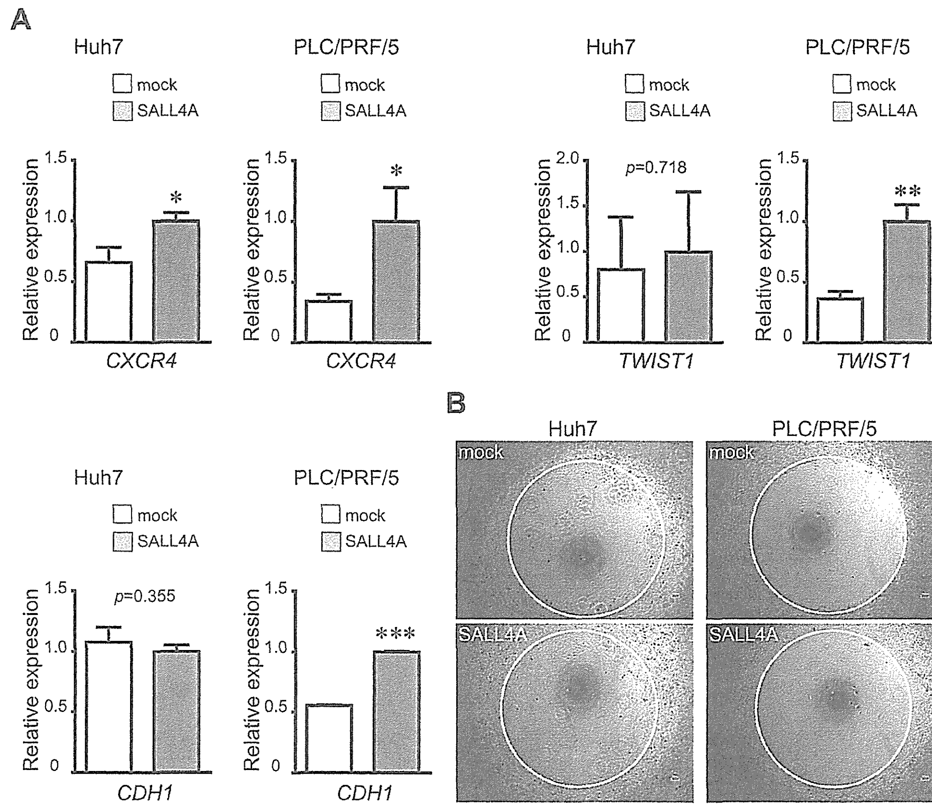

Figure 6. Oikawa et al.

Figure 6. Expression of EMT-related genes and migration assays in SALL4-over-expressing liver cancer cells. (A) Cells transduced by a over-expressing retroviral vector were cultured for 3 days. CXCR4, TWIST1, and CDH1 mRNA expression was detected using qRT-PCR. Data are expressed as mean ± SD (triplicate samples, ***$p<0.001$, **$p<0.01$, *$p<0.05$). (B) Migration assay in SALL4-over-expressing liver cancer cells.
157x144mm (300 x 300 DPI)

Acc

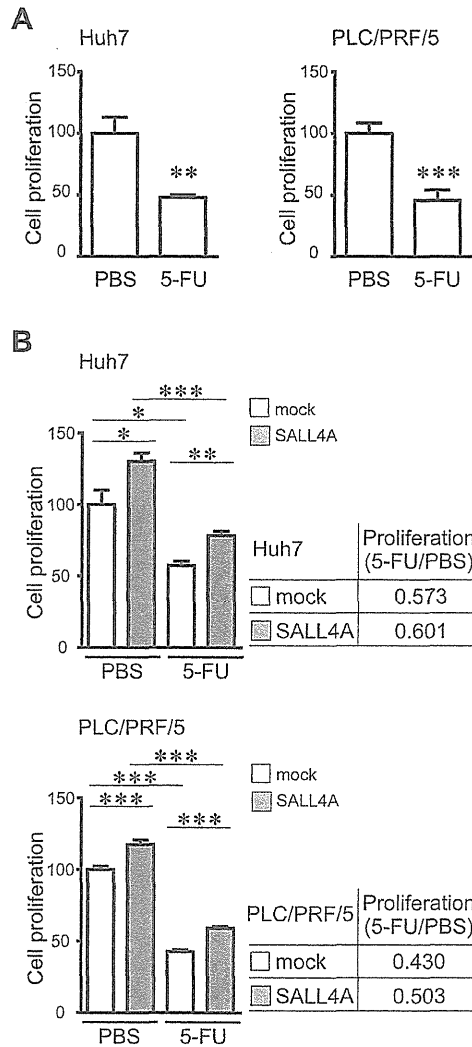

Figure 7. Chemo-resistance assays for SALL4-over-expressing liver cancer cells. Cells were transduced by a retroviral vector. Non-transduced (A) or transduced cells (B) were cultured in the presence or absence of 5-FU (2 μg/ml) for 7 days. The relative cell proliferation between PBS- and 5-FU-treated liver cancer cells is shown. Data are expressed as mean ± SD (triplicate samples, ***$p<0.001$, **$p<0.01$, *$p<0.05$).
77x185mm (300 x 300 DPI)

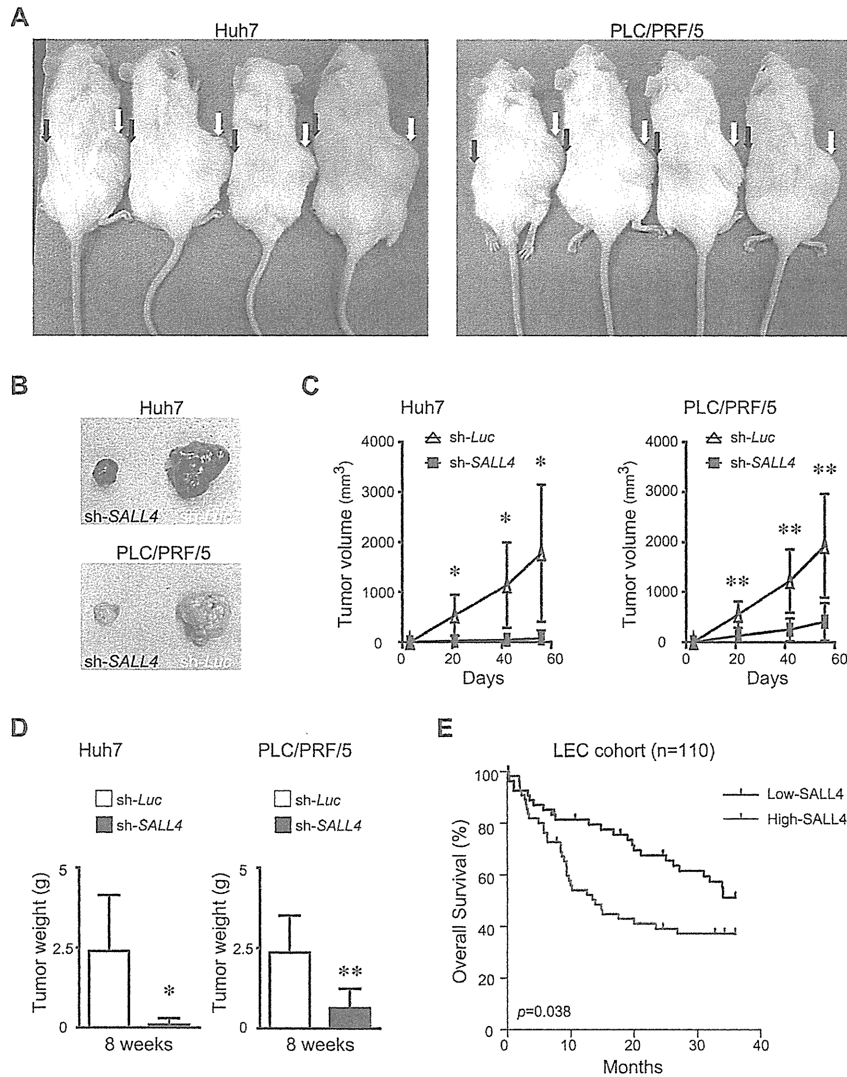

Figure 8. Oikawa et al.

Figure 8. Effect of SALL4 knockdown on xenograft tumor growth in vivo. (A) Control cells and SALL4-knockdown cells were implanted into recipient mice, respectively. White arrows show tumors derived from control cells and black arrows show tumors derived from SALL4-knockdown cells (Huh7 n=5, PLC/PRF/5 n=8). (B) Representative tumors derived from control versus SALL4-knockdown liver cancer cells at 8 weeks are shown. (C) The tumor growth curve over 8 weeks is shown. (D) The weight of the tumor at 8 weeks is shown. Data are expressed as mean ± SD (**$p<0.01$,*$p<0.05$). (E) Kaplan-Meier survival plot according to the relative level of SALL4 expression in HCC tumor samples, as determined by microarray analyses and with the use of the log-rank test. The median expression level was used to dichotomize low and high SALL4-expressing HCC tumors.
159x212mm (300 x 300 DPI)

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On-Line Supplement

SALL4, a Stem Cell Biomarker in Liver Cancers

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Supplementary Materials and Methods

Animals and Reagents

NOD/SCID mice (Sankyo Laboratory Co Ltd, Tsukuba, Japan) were used in this study. All mice were maintained in a specific pathogen-free animal facility at the Institute of Medical Science, University of Tokyo. All mice procedures were reviewed and approved by the animal care and use committee of the Institute of Medical Science, University of Tokyo. All animals were treated under the guidelines of the Institute of Medical Science, University of Tokyo. Fetal bovine serum was purchased from Tissue Culture Biologicals (Tulare, CA). DMEM, penicillin/streptomycin/ L-glutamine (100×), trypsin-EDTA solution (1×), protamine sulfate, 5-FU, bovine serum albumin, nicotinamide, insulin, transferrin, selenium, hydrocortisone, beta-mercaptoethanol, type IV collagenase and deoxyribonuclease were purchased from Sigma (St. Louis, MO). Roswell Park Memorial Institute (RPMI) 1640 and L-gultamine were purchased from Gibco (Grand Island, NY). Matrigel was purchased from Becton Dickinson (BD Biosciences, Bedford, MA). Horseradish peroxidase-conjugated anti-rabbit IgG antibody was purchased from GE Healthcare UK Ltd (Buckinghamshire, UK).

Sourcing of Human Livers and Liver Cancers

Fetal livers. Tissue samples were supplied by the accredited agency, Advanced Biological Resources and came from fetuses between 15-20 weeks gestation (**Supplementary Table 1A**) obtained by elective terminations of pregnancy.

Postnatal livers. Tissue samples derived from cadaveric neonatal, pediatric and adult donors (**Supplementary Table 1B**) were obtained through organ donation programs via United Network for Organ Sharing (UNOS), Organ Donation and Transplantation. Tissue samples were considered normal with no evidence of liver disease processes

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and obtained with informed consent from next kin for use of the livers for research purposes.

Non-cancerous liver and liver cancer specimens. Non-cancerous liver (**Supplementary Table 1C**) and tumor samples (**Supplementary Table 1D**) were obtained through the Cooperative Human Tissue Network (CHTN), the National Disease Research Interchange (NDRI), UNC and University of Minnesota. Tissue sample were obtained with informed consent for use of tumor for research purposes. This study was approved by the Institutional Review Board for Human Research Studies at the UNC at Chapel Hill.

Cell Isolation and Culture

For human HpSCs and hBTSCs, tissue processing to generate cell suspensions was conducted in RPMI 1640 supplemented with 0.1% bovine serum albumin, 1nM selenium and antibiotics. Enzymatic processing buffer contained 300U/ml type IV collagenase and 0.3 mg/ml deoxyribonuclease at 32°C with frequent agitation for 15-20 min. Enriched suspensions were pressed through a 75 gauge mesh and spun at 1200 RPM for 5 min before resuspension. Estimated cell viability by trypan blue exclusion was routinely higher than 95%. The cell suspension were seeded in plastic plate and in Kubota's Medium (KM), a serum-free, hormone defined medium (1) found effective for human hepatic stem/progenitors (2) and for biliary tree stem/progenitors (3, 4). It consists of any basal medium (here being RPMI 1640) with no copper, low calcium (0.3 mM), 10^{-9} M Selenium, 0.1% BSA, 4.5 mM Nicotinamide, 0.1 nM Zinc Sulfate heptahydrate , 10^{-8} M hydrocortisone, 5 µg/ml transferrin/Fe, 5 µg/ml insulin, 10 µg/ml high density lipoprotein, and a mixture of purified free fatty acids that are added bound

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to purified human serum albumin. The detailed protocol for the preparation of Kubota's Medium is given in a recent methods review (5).

Huh7 and PLC/PRF/5 were cultured in DMEM supplemented with 10% fetal bovine serum, 100 U /ml penicillin and 100 μg/ml streptomycin and incubated at 37°C in an atmosphere containing 5% CO_2.

Immunocytochemistry

Cells were fixed with 4% paraformaldehyde/PBS for 10 minutes. After 3 washing steps with PBS, cells were permeabilized using 0.5% TritonX-100 in PBS and blocked with 10% goat serum in PBS. Cells were then incubated overnight with a primary antibody with 10% goat serum in PBS at 4°C. Rabbit anti-SALL4 antibody, Rabbit anti-EpCAM antibody (Abcam, Cambridge, UK), mouse anti-EpCAM antibody (NeoMarkers, Fremont, CA), and mouse anti-NCAM antibody (BioLegend, San Diego, CA) were used as primary antibodies. See **Supplementary Table 3**.

As a negative control, cells were incubated with a mouse and rabbit IgG fraction with 10% goat serum/PBS. Cells were washed and incubated with an Alexa488- and Alexa 546-conjugated goat anti-mouse or rabbit IgG antibody (Invitrogen, Carlsbad, CA) for 1 h at room temperature. Nuclei were stained in a 10 μg/mL solution of 4',6-diamidino-2-phenylindole (DAPI).

Quantitative Reverse Transcription and Polymerase Chain Reaction

Total RNA was extracted from the cells using Trizol (Invitrogen). First-strand cDNA synthesized using the Primescript 1st strand cDNA synthesis kit (Takara, Otsu, Japan) was used as a template for PCR amplification. Quantitative analyses of mRNA levels were performed using Faststart Universal Probe Master (Roche Diagnostics, Mannheim, Germany) with ABI PRISM 7900HT Sequence Detection System (Applied Biosystems,

Foster City, CA). Primers were designed with the Universal Probe Library Assay Design Center (Roche Applied Science). Primer sequences are listed in **Supplementary Table 4**. The primers were annealed at 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C (15 s) and 60°C (1 min). Expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used generally as a standard.

Retroviruses and Lentiviruses

For use in the gain of function assays, retroviral vector pMXs-SALL4 (a gift of Prof. Yamanaka and Prof. Takahashi, University of Kyoto, Kyoto, Japan) was used (6). For use in loss of function assays, lentiviral vectors FG12 expressing shRNA sequences against human *SALL4* were constructed as reported and enhanced green fluorescent protein (EGFP) was co-expressed as an internal ribosomal entry site (IRES) sequence (7-9). Published SALL4 shRNA sequences were used for shRNA2 (10), with shRNA2 (5'-GATCCCCGATGCCTTGAAACAAGCCAAGCTACCTCAttcaagaga TGAGGTAGCTTGGCTTGTTTCAAGGCATCTTTTTA-3'/5'-AGCTTAAAAAGATGCCTT GAAACAAGCCAAGCTACCTCAtctcttgaaTGAGGTAGCTTGGCTTGTTTCAAGGCATC GGG-3').

Retorviruses and lentiviruses were generated as described. (8, 11) Retrovirus titers were determined by Retro-X q-RT-PCR titration kit (Clontech, Mountain View, CA). The lentiviral-infected cells were detected using FACS or by fluorescence microscopy. Lentiviral titers were determined by infection of NIH3T3 cells.

Western Blotting

Cultured liver cancer cell lines were lysed in RIPA lysis buffer containing 20 mM Tris-HCl (pH 7.5), 0.1% sodium dodecyl sulfate (SDS), 1% Triton, 1% sodium deoxycholate,

1mM EDTA, 5 μg/ml aprotinin, 1 mM NaF, and 2 mM Na_3VO_4. Extracted protein was mixed with SDS sample loading buffer (Bio-Rad Laboratories, Hercules, CA) containing β-mercaptoethanol, electrophoresed on a 7.5% or 10% SDS-polyacrylamide gel, and electrotransferred onto a Hybond-P membrane (GE Healthcare). Membranes were blocked using Tris-buffered saline (TBS) with Triton X-100 (20 mM Tris-HCl, 150 mM NaCl, 0.05% Triton X-100) containing 3% bovine serum albumin (BSA) and incubated with a primary antibody in TBS with Triton X-100. They then were washed in TBS with Triton X-100 and incubated with a horseradish peroxidase-conjugated secondary antibody. After washing, immunoreactive proteins were developed with ECL reagent, and blots were exposed to high-performance chemiluminescence film (GE Healthcare). Primary antibodies used were rabbit polyclonal anti-SALL4 (Abcam) and rabbit anti-actin (Sigma). See **Supplementary Table 3**.

Cell-Cycle Analysis using Flow Cytometer

Huh7 and PLC/PRF/5 cells were cultured on 6 well culture dishes ($1x10^5$ cells/well) and infected with mock and SALL4-over-expressing retroviruses. After 5 days of culture, cells were fixed with 70% EtOH (-20°C, overnight). Cells were washed with PBS and stained with 2 μg/ml propidium iodide and 0.2 mg/ml RNAase A in PBS. Cells were analyzed using FACS Calibur™ (BD Biosciences).

Migration Assay

Migration activity of liver cancer cells were analyzed using Oris Cell Migration Assay Kit (Platypus Tech, Madison, WI) according to manufacturer's protocol. Huh7 and PLC/PRF/5 cells were cultured on 6 well culture dishes ($1x10^5$ cells/well) and infected with mock and SALL4-over-expressing retroviruses. After 3 days of culture, cells were trypsinized and inoculated onto 96 wells culture plates ($1x10^6$ cells/well) with stoppers.

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After 24 h culture, stoppers were removed and cells were cultured for 24 more h. Cell migration was analyzed using Microscope TS100F (Nikon, Tokyo, Japan).

Statistics

We used the Microsoft Excel program to calculate SD and statistically significant differences between samples using Student's 2-tailed *t* test. Comparison of categorical variables was performed with the χ^2-test. P values of less than 0.05 were considered statistically significant.

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Supplementary Figure Legends

Supplementary Figure 1. Representative double immunostaining of EpCAM/CK19, SALL4/EpCAM and SALL4/CK19 expression in human normal livers. Fetal (16 weeks gestation) and neonatal (4 months) livers (A). (a, b) EpCAM and CK19 expression in fetal (a) and neonatal (b) livers. Sections were stained with anti-EpCAM and anti-CK19 antibody. Expression of EpCAM (***green***) or CK19 (***red***) was visualized with Alexa488- and Alexa546-conjugated antibodies. Nuclei (***blue***) were stained with DAPI. (c, d) SALL4 and EpCAM expression in fetal (c) and neonatal (d) livers. (e, f) SALL4 and CK19 expression in fetal (e) and neonatal (f) livers. Sections were stained with anti-SALL4 and anti-EpCAM or anti-CK19 antibody. Expression of SALL4 (***brown***) or EpCAM, CK19 (***red***) was visualized with 3,3'-diaminobenzidine and Warp Red. ***Arrows*** showed ductal plate cells, now recognized to be hHpSCs, and ***arrowheads*** indicated hHBs respectively. Magnification ×400. BD, bile duct; DP, ductal plate; PT, portal tract. (B) SALL4 expression in cultures of hBTSCs. The colony was stained with antibodies against EpCAM and SALL4. Expression of EpCAM (***green***) or SALL4 (***red***) was visualized with Alexa488- and Alexa546-conjugated antibodies. Nuclei (***blue***) were stained with DAPI. Magnification ×400.

Supplementary Figure 2. Representative immunostaining of SALL4 in non-cancerous liver and HCC specimens (A-D). (A) chronic hepatitis, (B) liver cirrhosis, (C) SALL4-negative HCC (T7, well-differentiated) and (D) SALL4-positive HCC (T48, well-differentiated). Sections were stained with anti-SALL4 antibody. Magnification ×200. BD; bile duct. (E, F) Representative double immunostaining of SALL4/EpCAM and SALL4/CK19 expression in CC specimens (T5, poorly-differentiated). Serial sections were stained with anti-SALL4, anti-EpCAM and anti-CK19 antibody. Magnification ×400.

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Supplementary Figure 3. Over-expression of SALL4 proteins in cultures derived from Huh7 and PLC/PRF/5 cells. Cells were infected with mock- or SALL4-expressing viruses and stained with anti-SALL4 antibody after fixation. SALL4 expression was detected immunocytochemically. Expression of SALL4 (***red***) was detected using Alexa546-conjugated antibody. Nuclei (***blue***) were stained with DAPI.

Supplementary Figure 4. Efficiencies of lentiviral transduction into Huh7 and PLC/PRF/5 cells. Transduction efficiency was estimated using flow cytometer (EGFP expression). The percentages of Huh7 cells infected with lentiviruses expressing-shRNA against *luciferase* or *SALL4* were respectively 97.0%, 97.9% and (upper panel). The percentages of PLC/PRF/5 cells infected with lentiviruses expressing-shRNA against *luciferase* or *SALL4* were respectively 94.2%, 97.6% and (lower panel).

Supplementary Figure 5. Morphological features of Huh7 and PLC/PRF/5 cells infected with control- and SALL4 knockdown-shRNA viruses. Cells were infected with lentiviruses expressing-shRNA against *luciferase* or *SALL4* and cultured for 7 days. Cells infected with lentiviruses were detected by expression of EGFP (***green***). Nuclei (***blue***) were stained with DAPI.

Supplementary Figure 6. Expression of hepatocytic differentiation (A) and stemness (B) genes in SALL4-over-expressing liver cancer cells. Cells were infected with a retroviral vector expressing SALL4 and cultured for 3 days. UGT2B7, HNF4α, TACSTD1, POU5F1, and CD90 mRNA expression was detected using qRT-PCR. Data are expressed as mean ± SD (triplicate samples, ***p<0.001, **p<0.01).

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Supplementary Figure 7. Expression of hepatocytic differentiation and EMT-related genes in SALL4-knockdown liver cancer cells. Cells were infected with a lentiviral vector expressing-shRNA against *luciferase* or *SALL4*. UGT2B7, HNF4α, TACSTD1, POU5F1, and CXCR4, TWIST1, CDH1 mRNA expression was detected using qRT-PCR. Data are expressed as mean ± SD (triplicate samples, *$p<0.05$).

Supplementary Figure 8. Representative immunostaining of SALL4 in HCC specimens (T37, moderately-differentiated) by using citrate buffer pH 6.0 (A) or EDTA buffer pH 8.0 (B) for antigen retrieval. Serial sections were stained with anti-SALL4 antibody. Magnification ×200.

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Supplementary Table 1A. Human Fetal Liver Tissue Samples

	Tissue sample	Gender	Gestational age
1	FL-521	M	16W
2	FL-634	F	19W
3	FL-645	F	19W
4	FL-649	N/A	20W
5	FL-655	M	15W
6	FL-657	F	18W

FL, human fetal liver

Supplementary Table 1B. Human Neonatal and Adult Liver Samples

	Tissue sample	Gender	Age	Cause of death
1	NL-2	N/A	N/A	N/A
2	NL-3	M	4M*	Chronic lung disease due to prematurity
3	AL-155	F	36Y	Cerebral hemorrhage
4	AL-156	M	68Y	Cerebral hemorrhage
5	AL-186	M	32Y	Head trauma

NL, neonatal liver; AL, adult liver

*born premature at 25 weeks and survived on neonatal intensive care unit for 4 months+30 days

Supplementary Table 1C. Non-cancerous Human Liver Samples

	Tissue sample	Gender	Age	Cause of disease
1	CH-1	M	57Y	Alcohol and HCV
2	CH-2	M	69Y	HCV
3	CH-3	F	50Y	Alcohol
4	LC-1	M	49Y	HCV
5	LC-2	M	77Y	Unknown

CH, chronic hepatitis; LC, liver cirrhosis; HCV, hepatitis C virus

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Supplementary Table 1D. Human Liver Cancer Samples

	Tumor sample	Gender	Age	Tumor Grading
1	T7-HCC	F	37Y	Well
2	T8-HCC	M	72Y	Moderately
3	T28-HCC	F	75Y	Well
4	T30-HCC	M	59Y	Poorly
5	T32-HCC	F	45Y	Poorly
6	T35-HCC	F	74Y	Well
7	T36-HCC	M	53Y	Well
8	T37-HCC	M	73Y	Moderately
9	T40-HCC	M	62Y	Moderately
10	T41-HCC	M	79Y	Well
11	T42-HCC	M	76Y	Moderately
12	T43-HCC	M	69Y	Well
13	T44-HCC	M	63Y	Poorly
14	T46-HCC	F	67Y	Moderately
15	T47-HCC	M	53Y	Moderately
16	T48-HCC	M	59Y	Well
17	T49-HCC	M	59Y	Poorly
18	T50-HCC	F	44Y	Moderately
19	T51-HCC	F	71Y	Moderately
20	T52-HCC	M	82Y	Well
21	T5-CC	F	51Y	Poorly
22	T13-CC	F	60Y	Moderately
23	T27-CC	M	53Y	Poorly
24	T34-CC	F	62Y	Moderately
25	T39-CC	F	78Y	Moderately
26	T45- HC-CC	M	48Y	Moderately
27	FL-HCC	M	27Y	Poorly

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T, tumor; CC, cholangiocarcinoma; HCC, hepatocellular carcinoma; HC-CC, combined hepatocellular and cholangiocarcinoma; FL-HCC, fibrolamellar hepatocellular carcinoma

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Supplementary Table 2. Clinico-pathologic Characteristics of Hepatocellular Carcinomas on the basis of SALL4 Immunostaining

	SALL4-positive HCC (n=17)	SALL4-negative HCC (n=3)	*p*-value
Mean age (years)			
≤60	6	2	0.306
>60	11	1	
Gender			
Male	12	1	0.212
Female	5	2	
Tumor size (cm)			
≤5	8	0	0.125
>5	9	3	
Liver cirrhosis [a]			
absent	5	2	0.375
present	8	1	
Tumor number			
1	13	2	0.718
2≤	4	1	
Vascular invasion [b]			
absent	9	2	0.938
present	5	1	
Tumor differentiation			
Well	7	1	0.804
Moderately/Poorly	10	2	
TNM stage [b]			
I, II	10	2	0.870
III, IV	4	1	

a A total of 4 patients for whom we did not have the available data.

b A total of 3 patients for whom we did not have the available data.

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Supplementary Table 3. Antibodies Used

Name	Host / isotype	Source	Catalog No.
SALL4	Mouse IgG1	Abcam	ab57577
SALL4	Rabbit IgG	Abcam	ab29112
EpCAM	Mouse IgG1	NeoMarkers	MS-181
EpCAM	Rabbit IgG	Abcam	ab32392
NCAM	Mouse IgG1	BioLegend	#318302
CK19	Mouse IgG2a	Abcam	ab7754
CK19	Rabbit IgG	Abcam	ab52907
Actin	Rabbit IgG	SIGMA	SAB4502631

EpCAM, epithelial cell adhesion molecules; NCAM, neural cell adhesion molecule; CK19, cytokeratin19

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Supplementary Table 4. Primers for qRT-PCR

Name	F/R	Primer Sequence	Probe Number	GenBank Accession
SALL4	F	CGCCCCGTGTGTCATGTAGTGAAC	#72	NM_020436.3
	R	TCCGAGAACAGCCGCACTGAGATGGAAG		
Cyclin D1	F	GAAGATCGTCGCCACCTG	#67	NM_053056.2
	R	GACCTCCTCCTCGCACTTCT		
Cyclin D2	F	GGACATCCAACCCTACATGC	#49	NM_001759.2
	R	CGCACTTCTGTTCCTCACAG		
CASP3	F	TTTTGGCAGTTTCAGTCTTCC	#18	AY_219866.1
	R	GATTCAAGTTTAAAACCGCTTGA		
ALB	F	AATGTTGCCAAGCTGCTGA	#27	NM_000477.5
	R	CTTCCCTTCATCCCGAAGTT		
TTR	F	ATGGCTTCTCATCGTCTGCT	#13	NM_000371.3
	R	GACCATCAGAGGACACTTGGA		
CK19	F	GCCACTACTACACGACCATCC	#71	NM_00276.4
	R	CAAACTTGGTTCGGAAGTCAT		
ABCG2	F	TGGCTTAGACTCAAGCACAGC	#56	NM_004827.2
	R	TCGTCCCTGCTTAGACATCC		
CXCR4	F	GCACTCACCTCTGTGAGCAG	#55	NM_001008540.1
	R	ATGTCCACCTCGCTTTCCT		
TWIST1	F	AAGGCATCACTATGGACTTTCTCT	#6	NM_000474.3
	R	GCCAGTTTGATCCCAGTATTTT		
CDH1	F	GCCGAGAGCTACACGTTCA	#80	NM_004360.3
	R	GACCGGTGCAATCTTCAAA		
UGT2B7	F	TGGATACCCCAGAATGACCT	#22	NM_001074.2
	R	GATCGGCAAACAATGGAATC		
HNF4α	F	ATTGACAACCTGTTGCAGGA	#3	NM_178849.1
	R	CGTTGGTTCCCATATGTTCC		

TACSTD1	F	CCATGTGCTGGTGTGTGAA	#3	NM_002354.2
	R	TGTGTTTTAGTTCAATGATGATCCA		
POU5F1	F	CTTCGCAAGCCCTCATTTC	#60	NM_002701.4
	R	GAGAAGGCGAAATCCGAAG		
CD90	F	CAGAACGTCACAGTGCTCAGA	#66	NM_006288.3
	R	GAGGAGGGAGAGGGAGAGC		
GAPDH	F	AGCCACATCGCTCAGACAC	#60	NM_002046.4
	R	GCCCAATACGACCAAATCC		

Primer list: CASP3, caspase-3; ALB, albumin; TTR, transthyretin; CK19, cytokeratin19; ABCG2, ATP-binding cassette-G2; CXCR4, C-X-C chemokine receptor type 4; CDH1, cadherin 1; UGT2B7, UDP-glucuronosyltransferase-2B7; HNF4α, hepatocyte nuclear factor 4; TACSTD1, tumor-associated calcium signal transducer 1; POU5F1, POU domain, class 5, transcription factor 1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase ; F, forward primer; R, reverse primer.

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