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Supplementary Information for

A hepatocyte differentiation model reveals two subtypes of liver cancer with different oncofetal properties and therapeutic targets

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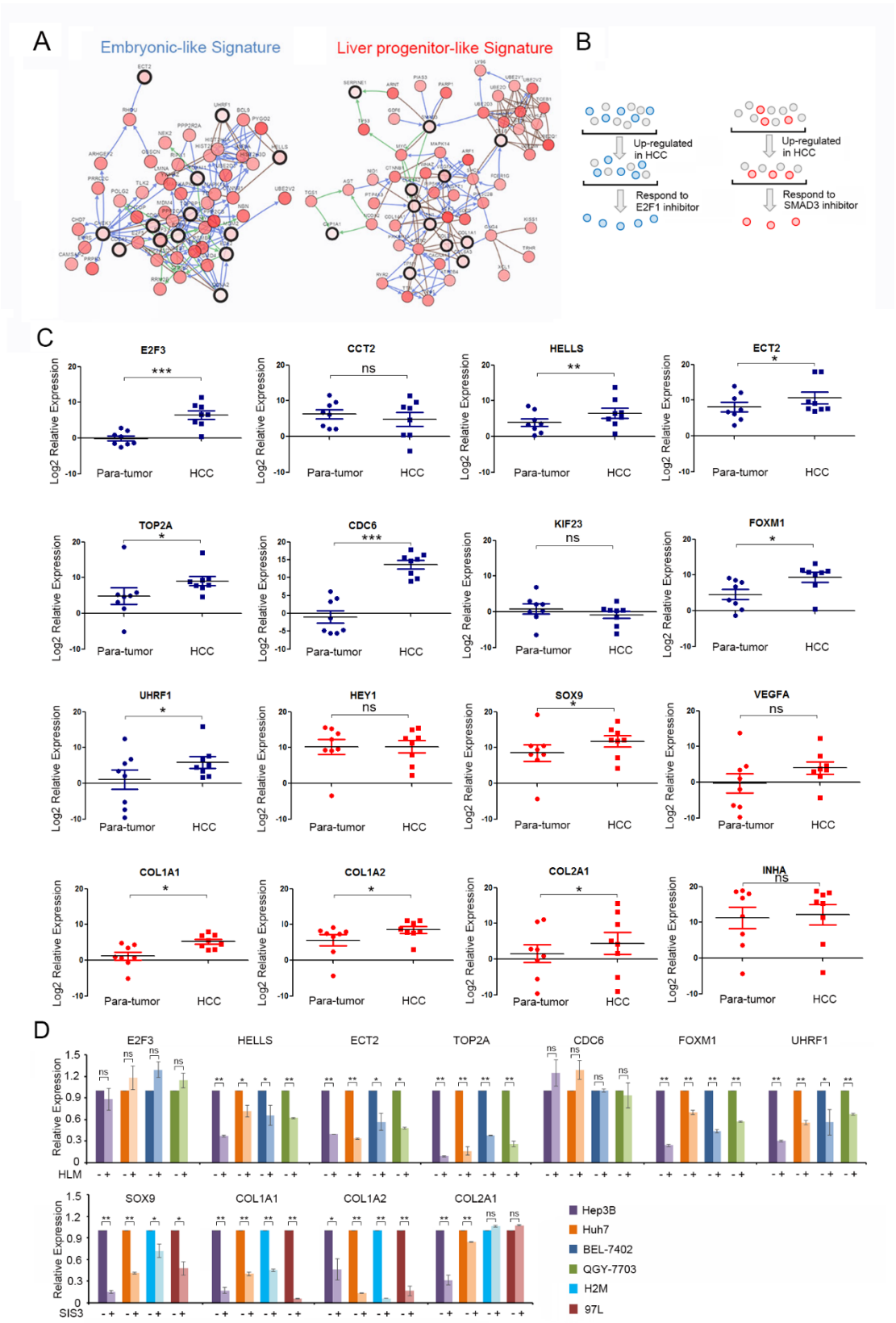
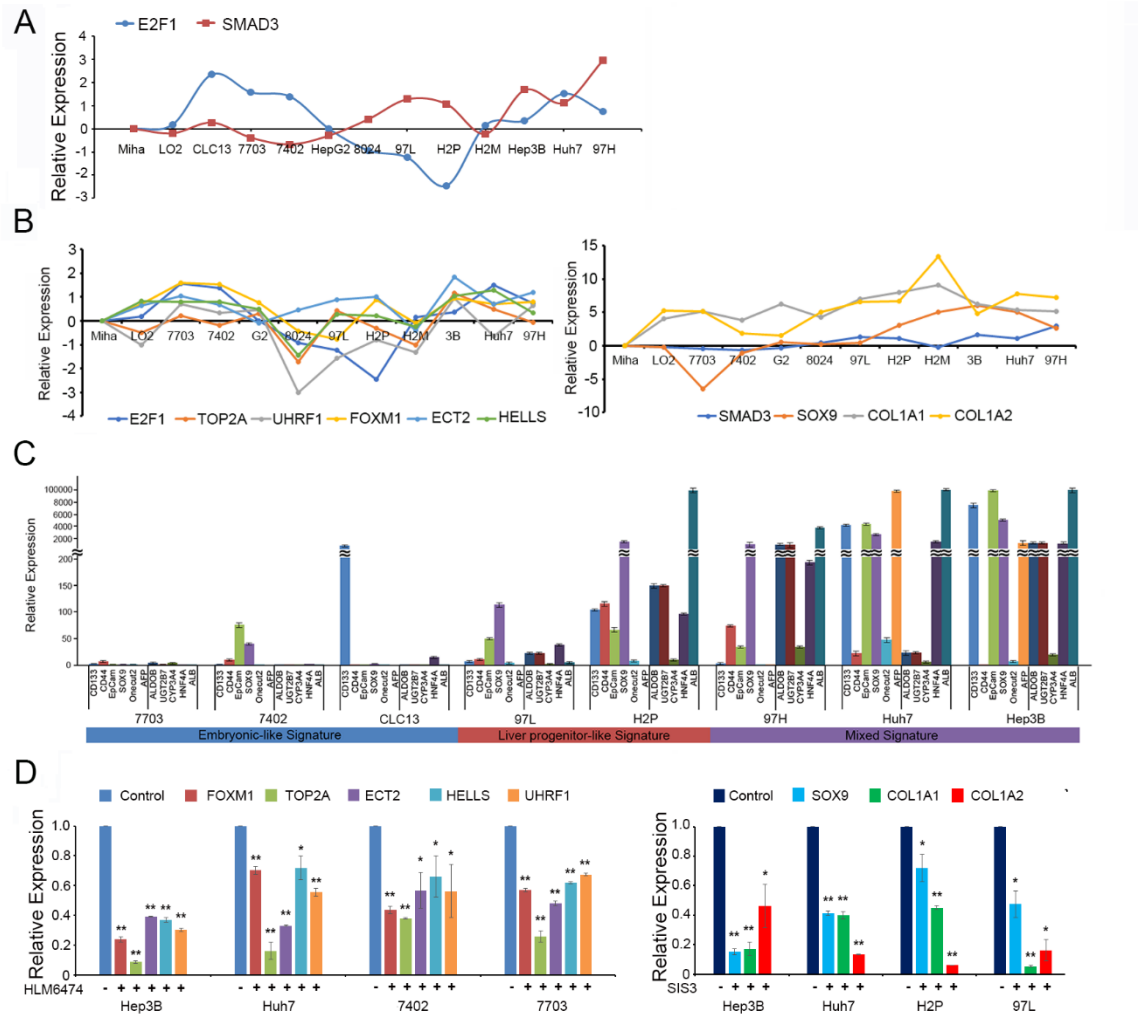


Liu et al. Appendix Fig. S1

Fig. S1. Network analysis of core module genes with Embryonic-like signature and Liver progenitor-like signatures, and relative expression of candidate oncofetal genes in paired HCC clinical samples and cell lines.

(A) The selected core module genes from the Embryonic-like subgroup or Liver progenitor-like subgroup were subjected for network analysis. The regulatory or interacting proteins were connected with arrow lines. The color represents the extent of upregulation in tumor tissues. (B) The top-ranked predicted targets which both up-regulated in HCC clinical samples and respond to small molecular inhibitors in HCC cell lines were selected as the core module genes of the two subtypes of liver cancer respectively. (C)The relative expression of ES-like signature genes and LP-like signature genes were screened in 8 paired HCC samples. Paired sample t test was used for statistical analysis. *, $P<0.05$, ** $P<0.01$, ***, $P<0.001$. (D) E2F1 inhibitor HLM6474 and SMAD3 inhibitor SIS3 were used to treat HCC cells with ES-like signature, LP-like signature or mixed signature. The relative expressions of candidate core module genes were detected by qPCR.



Liu et al. Appendix Fig. S2

Fig. S2. Relative expression of signature biomarkers and their responses to specific inhibitors.

(A) The expression of E2F1 and SMAD3 were detected by quantitative real time PCR (qPCR) in series of HCC cell lines as well as immortalized liver cell lines. (B) The relative expression of core module genes was detected by qPCR in series of HCC cell lines. (C) The relative expression of liver progenitor cell markers (CD133, CD44, EpCam, Sox9, Onecut2, AFP) and hepatocyte differentiation markers (ALDOB, UGT2B7, CYP3A4, HNF4A, ALB) were detected by quantitative real-time PCR in HCC cell lines with ES-like signature (7703, 7402), LP-like signature (97L, H2P) and Mixed signature (97H, Huh7, Hep3B). (D) HCC cell lines were treated with E2F1 specific inhibitor HLM6474 (50μM) and SMAD3 specific inhibitor SIS3 (20μM) respectively. The relative expression of the core module genes was detected by qPCR. *, $P<0.05$, **, $P<0.01$ by independent t test.

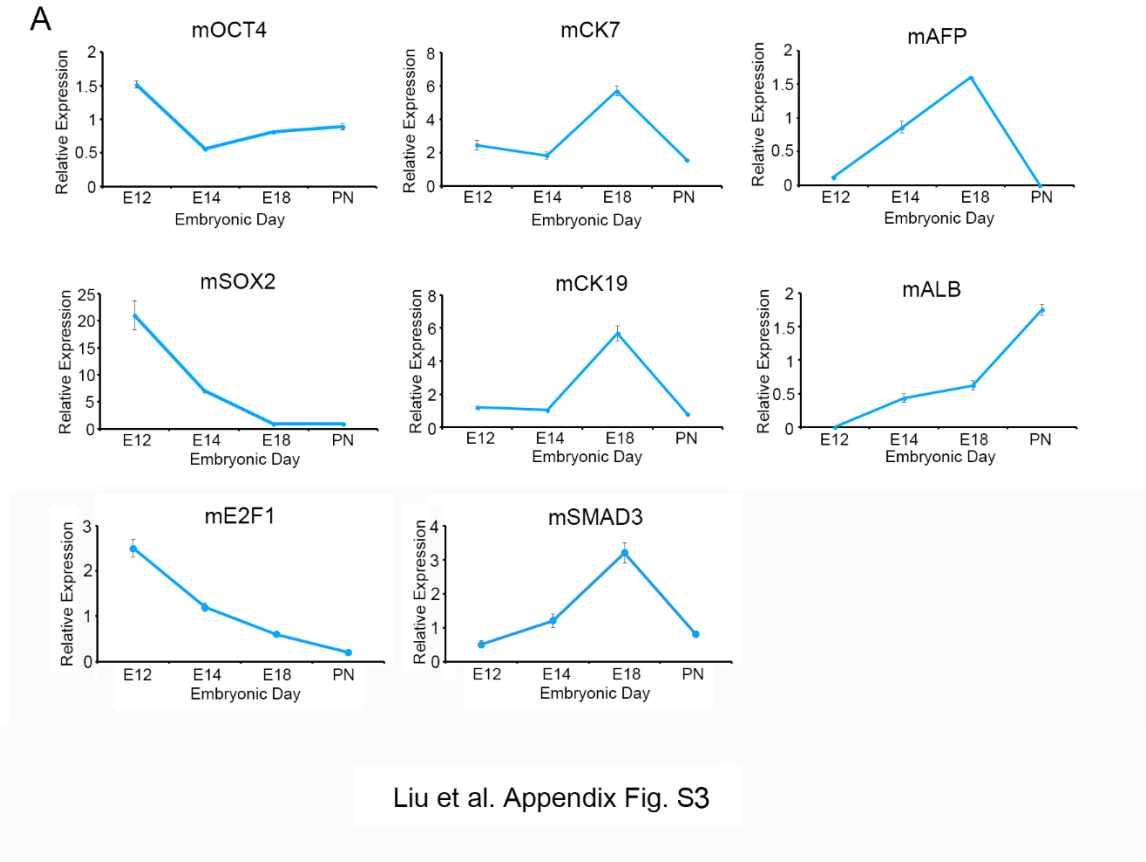
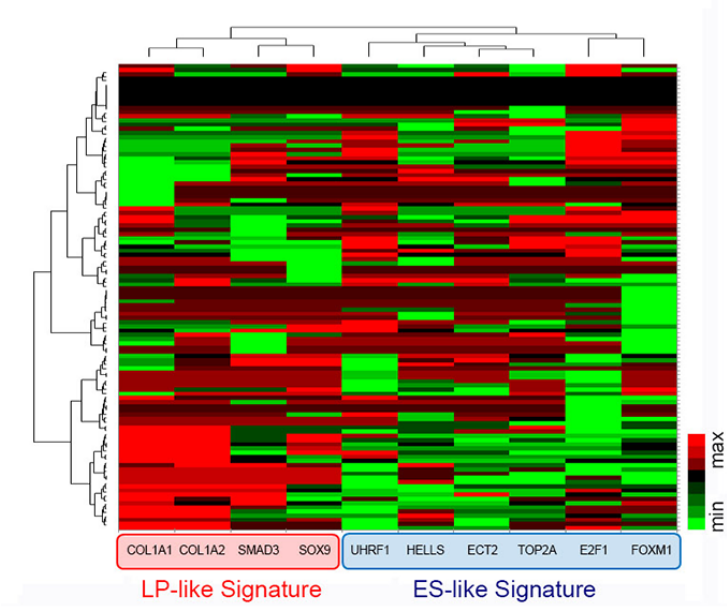


Fig. S3. Relative expression of signature biomarkers during mice fetal liver development. (A)Mice fetal liver at different developmental stages (E12, E14, E18, Perinatal) were collected, and the total RNA were extracted. The relative expressions of representative biomarkers including mOCT4, mSOX2, mCK7, mCK19, mAFP, mALB, mE2F1, and mSMAD3 were detected by qPCR.



Liu et al. Appendix, Fig. S4

Fig. S4 Hierarchical clustering analysis was used to detect the selected core module genes in an independent cohort of clinical HCC patients.

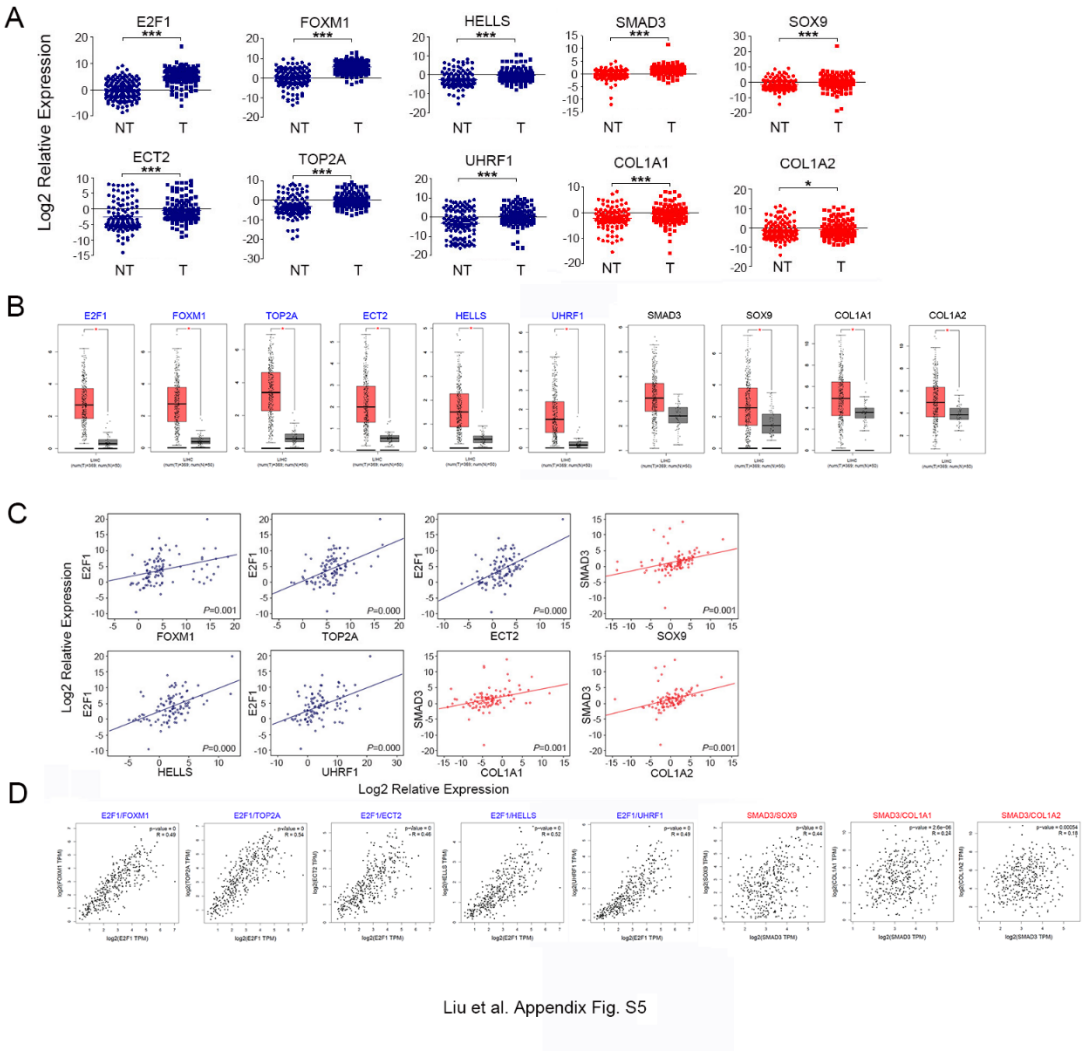
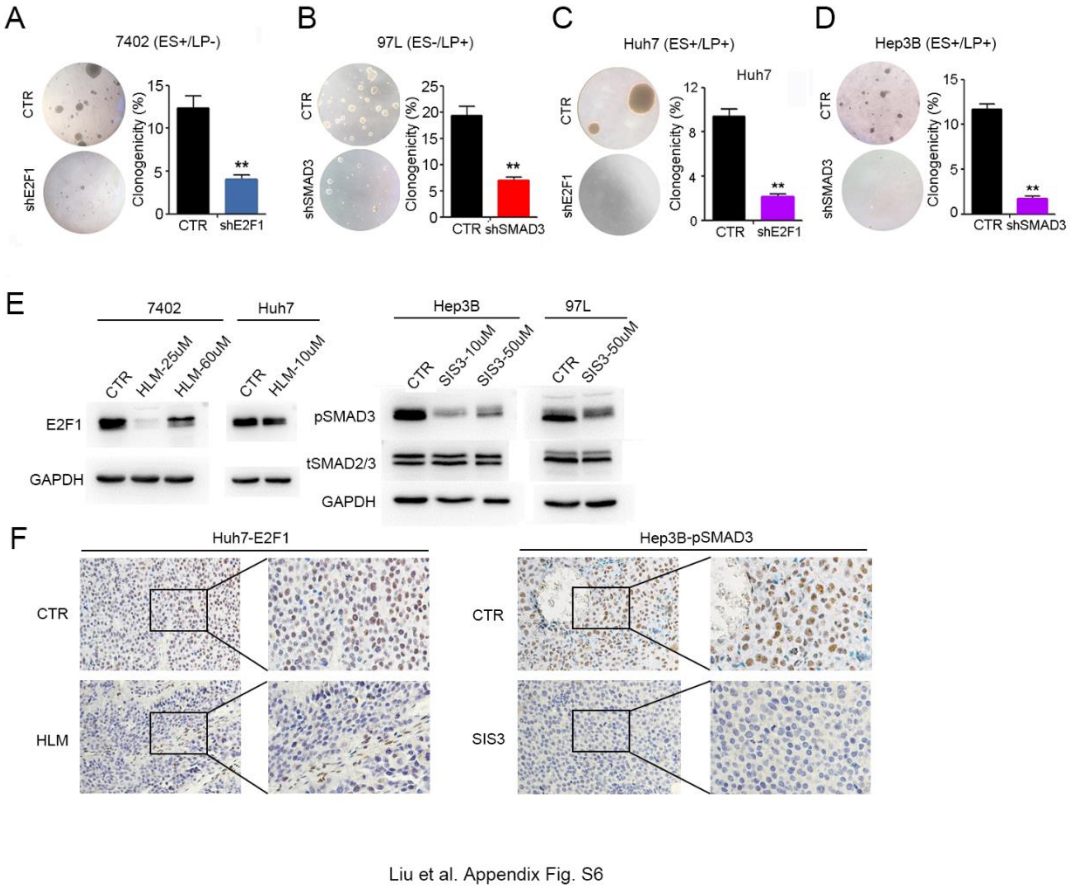


Fig. S5 Relative expression of core module oncofetal genes and their correlations in paired HCC clinical samples.

(A) The relative expression of ES-like signature genes and LP-like signature genes were examined by qPCR in HCC patients from HKU_HCC cohort (N=99). Paired sample t test, ***, P<0.001. (B) The relative expression of ES-like signature genes and LP-like signature genes were examined in the TCGA_HCC cohort using GEPIA web server (N=369). Independent t test, *, P<0.05. (C) The correlation of ES-like oncofetal driver E2F1 with core module genes (FOXM1, TOP2A, ECT2, HELLS, UHRF1) and the correlation of LP-like oncofetal driver SMAD3 with core module genes (SOX9, COL1A1, COL1A2) were examined by qPCR in HCC patients from HKU_HCC cohort (N=99). Pearson's $\chi 2$ test. (D) The correlation of ES-like oncofetal driver E2F1 with core module genes (FOXM1, TOP2A, ECT2, HELLS, UHRF1) and the correlation of LP-like oncofetal driver SMAD3 with core module genes (SOX9, COL1A1, COL1A2) were examined in the TCGA_HCC cohort using GEPIA web server (N=369). Pearson's $\chi 2$ test.



Liu et al. Appendix Fig. S6

Fig. S6 Expression of oncofetal drivers and functional roles in HCC.

(A-D) Cells were seeded in 0.4% bactoagar on the bottom layer of solidified 0.6% bactoagar in six-well plates at a density of 3,000 cells/well. The clonogenicity was counted 3 weeks later. (E) The expression of E2F1 was examined by western blot in HCC cell lines treated with control or HLM. The expression of pSMAD3 and total SMAD3 was examined by western blot in HCC cell lines treated with control or SIS3. (F) The staining of E2F1 was examined by IHC in xenograft tumors treated with control or HLM in Huh7 cells. The staining of pSMAD3 was examined by IHC in xenograft tumors treated with control or SIS3 in Hep3B cells.

Table S1. Activation scores of the upstream regulators and the predicted downstream targets in two subtypes of liver cancer

Upstream Regulator	Fold Change in Tumor	Activation Z score	P-value of overlap
ES-like			
E2F1	15.387	5.977	3.40E-22
FOXM1	8.134	3.750	8.38E-03
E2F3	4.403	2.157	3.54E-02
MYCN	2.648	5.082	1.80E-07
LP-like			
SMAD3	2.675	3.948	3.87E-02
Gene Name	**Fold Change in Tumor**		**Prediction**
TOP2A	18.106		Activated
E2F1	15.387		Activated
E2F3	14.025		Activated
UHRF1	13.165		Activated
CDC6	12.232		Activated
HELLS	9.538		Activated
ECT2	8.865		Activated
CDC45	8.414		Activated
FOXM1	8.134		Activated
Gene Name	**Fold Change in Tumor**		**Prediction**
COL2A1	314.265		Activated
SOX9	6.886		Activated
HEY1	4.047		Activated
VEGFA	3.391		Activated
COL1A1	2.815		Activated
SMAD3	2.675		Activated
INHA	2.409		Activated

Table S2. Clinical pathological correlation of liver cancer subtypes in HKU-HCC Patients.

Clinical pathological features	Total	Liver cancer subtypes				P*
		ES-/LP-	ES+/LP-	ES-/LP+	ES+/LP+	
Gender						
Male	84	31	18	16	20	0.451
Female	15	3	3	2	6	
Age						
< 60	84	26	19	14	25	0.129
≥ 60	15	8	2	4	1	
Preoperative AFP						
< 300 ng/ml	54	20	11	11	12	0.162
≥300 ng/ml	45	14	10	7	14	
Recurrence or Metastasis						
No	52	22	10	7	13	0.386
Yes	44	12	11	10	11	
HBsAg						
Negative	20	7	2	4	7	0.585
Positive	71	23	17	12	19	
HCVAb						
Negative	89	29	19	15	26	0.491
Positive	2	1	0	1	0	
Cirrhosis						
No	30	13	8	5	4	0.124
Mild	34	7	8	9	10	
Moderate	20	5	4	2	9	
Severe	8	5	0	1	2	
Tumor Size						
< 5 cm	27	7	3	4	13	**0.042**
≥5 cm	66	23	17	13	13	
Tumor Number						
1	72	23	14	15	20	0.743
2	11	4	2	1	4	
3	10	3	4	1	2	
Vascular Invasion						
No	87	30	19	17	21	**0.016**
Yes	6	0	1	0	5	
Capsule						
Complete	41	16	6	7	12	0.554
Incomplete	33	7	9	8	9	
No	19	7	5	2	5	
TNM Stage						
Ⅰ	50	14	11	12	13	0.274
Ⅱ	2	0	0	0	2	
ⅢA	12	5	4	1	2	
ⅢB	2	0	1	1	0	
ⅢC	1	0	0	1	0	
Ⅳ	0	0	0	0	0	

Differentiation						
Well	16	5	2	7	2	
Moderate	70	25	14	8	23	**0.005**
Poor	4	0	3	0	1	

*: Two–sided $\chi 2$ test;

Table S3. Distribution of liver cancer subtypes in previously reported classification.

Subtypes	ES+/LP-	ES+/LP+	ES-/LP+	ES-/LP-	Num	*P* value*
Cluster A/B[1]						
Cluster A	63 (17.0%)	81 (21.8%)	21 (5.7%)	11 (3.0%)	176	**0.000**
Cluster B	26 (7.0%)	12 (3.2%)	49 (13.2%)	108 (29.1%)	195	
HB/HC[2]						
HB	19 (5.1%)	55 (14.8%)	49 (13.2%)	20 (5.4%)	143	**0.000**
HC	70 (18.9%)	38 (10.2%)	21 (5.7%)	99 (26.7%)	228	
EpCam[3]						
EpCam-	65 (17.5%)	40 (10.8%)	52 (14.0%)	104 (28.0%)	261	**0.000**
EpCam+	24 (6.5%)	53 (14.3%)	18 (4.9%)	15 (4.0%)	110	
S1-S3[4]						
S1	15 (4.0%)	58 (15.6%)	33 (8.9%)	6 (1.6%)	112	**0.000**
S2	38 (10.2%)	31 (8.4%)	12 (3.2%)	21 (5.7%)	102	
S3	36 (9.7%)	4 (1.1%)	25 (6.7%)	92 (24.8%)	157	
G1-G6[5]						
G1	5 (1.3%)	26 (7.0%)	10 (2.7%)	6 (1.6%)	47	**0.000**
G2	13 (3.5%)	13 (3.5%)	6 (1.6%)	13 (3.5%)	45	
G3	36 (9.7%)	40 (10.8%)	2 (0.5%)	8 (2.2%)	86	
G4	26 (7.0%)	8 (2.2%)	19 (5.1%)	45 (12.1%)	98	
G5-6	9 (2.4%)	6 (1.6%)	33 (8.9%)	47 (12.7%)	95	

*****, Two–sided $\chi 2$ test;

[1], The top rated 11 survival genes with hazard ratio greater than 2.5 (TOPBP1, GTPBP4, PTMA, YWHAB, SUMO2, PDCD5, NLRP2, SLBP, BUB3, DLGAP5, HDAC2) were selected to classify Cluster A and Cluster B subgroups of HCC patients, according to Lee et al. Hepatology 2004. Each gene with relative expression greater than median value was given 1 score. Patients with average score of the 11 signature genes greater than 0.5 was classified as Cluster A subtype, and less than 0.5 was classified as Cluster B subtype.

[2], The top 6 representative hepatoblast signature genes (KRT7, KRT19, VIM, FOS, FOSL2, JUNB) were selected to classify HB and HC subgroups of HCC patients, according to Lee et al. Nat Med 2006. Each gene with relative expression greater than median value was given 1 score. Patients with average score of the 6 signature genes greater than 0.5 was classified as HB subtype, and less than 0.5 was classified as HC subtype.

[3], The relative expression of EpCam and AFP was selected to classify EpCam+ and EpCam- subgroups of HCC patients, according to Yamashita et al. Gastroenterology 2009. Gene with relative expression greater than median value was considered positive. Only EpCam and AFP double positive patients were classified as EpCam+ subtype, and the rest were classified as EpCam- subtype.

[4], The top rated 10 genes representing S1-S3 subgroups of HCC patients (S1: IQGAP1, S100A11, RAB31, CD37, POSTN, ARHGDIB, ALOX5AP, LAPTM5, CSPG2, ARPC2; S2: COL2A1, GPC3, AFP, AHCY, TARBP1, ARID3A, FGFR3, SMARCC1, RPS5, EIF4A2; S3: GLYAT, SERPINC1, APOC4, MTHFD1, GPT, HPD, SERPING1, DPAGT1, PCK1, HGD) were selected according to Hoshida et al. Cancer Res 2009. Each gene with relative expression greater than median value was given 1 score. Patient were assigned to different subgroups according to their relative scores.

[5], The top rated 10 genes representing G1-G6 subgroups of HCC patients (G1:IGF2, PEG3, SOX9, SPINT2, AFP, MYH4, MGC11242, SPINT1, ZNF83, ST14; G2:NAV3, ENPP2, AF1Q,

EPHA1, MEIS2, DEGS, RRAS2, CD58, INHBB, MOSPD1; G3: G6PD, HN1, PFN2, NDRG1, PHB, MAD2L1, NRAS, ENO1, NME1, HCAP-G; G5-6:PAP, SPARCL1, GLUL, LAMA3, TBX3, REG1A, C1QTNF3, CTNNA2, LEF1, RHOBTB1) were selected according to Boyault et al. Hepatology 2007. Each gene with relative expression greater than median value was given 1 score. Patient were assigned to different subgroups according to their relative scores.

Table S4. Sequences of primers used in qPCR.

Primer	Sequence (5'-3')
qRT-E2F3-F	AAAGCCCCTCCAGAAACAAGA
qRT-E2F3-R	CCTTGGGTACTTGCCAAATGT
qRT-CCT2-F	GAACGCCTAGCTCTTGTCACA
qRT-CCT2-R	GCACCACGCAAAACAATGGTA
qRT-18S-F	AACCCGTTGAACCCCATT
qRT-18S-R	CCATCCAATCGGTAGTAGCG
qRT-HELLS-F	AGAAGGCATGGAATGGCTTAGG
qRT-HELLS-R	GCCACAGACAAGAAAAGGTCC
qRT-ECT2-F	ATACCCCTAACAGCAATCGCA
qRT-ECT2-R	GAAATGGTGACACGTCTGTCT
qRT-TOP2A-F	TGGCTGTGGTATTGTAGAAAGC
qRT-TOP2A-R	TTGGCATCATCGAGTTTGGGA
qRT-CDC6-F	ACCTATGCAACACTCCCCATT
qRT-CDC6-R	TGGCTAGTTCTCTTTTGCTAGGA
qRT-KIF23-F	TACCCATTTGAATCGTGAGTCCA
qRT-KIF23-R	CTCTGGTCCGGTTAGTTCTTTC
qRT-FOXM1-F	ATACGTGGATTGAGGACCACT
qRT-FOXM1-R	TCCAATGTCAAGTAGCGGTTG
qRT-UHRF1-F	AGGTGGTCATGCTCAACTACA
qRT-UHRF1-R	CACGTTGGCGTAGAGTTCCC
qRT-HEY1-F	GAAGTTGCGCGTTATCTGAGC
qRT-HEY1-R	ATGCGAAACCAGTCGAACTCG
qRT-SOX9-F	AGCGAACGCACATCAAGAC
qRT-SOX9-R	CTGTAGGCGATCTGTTGGGG
qRT-VEGFA-F	AGGGCAGAATCATCACGAAGT
qRT-VEGFA-R	AGGGTCTCGATTGGATGGCA
qRT-COL1A1-F	ATCAACCGGAGGAATTTCCGT
qRT-COL1A1-R	CACCAGGACGACCAGGTTTTC
qRT-COL1A2-F	GGCCCTCAAGGTTTCCAAGG
qRT-COL1A2-R	CACCCTGTGGTCCAACAACTC
qRT-COL2A1-F	CCAGATGACCTTCCTACGCC
qRT-COL2A1-R	TTCAGGGCAGTGTACGTGAAC

qRT-INHA-F	TTCCACTACTGTCATGGTGGT
qRT-INHA-R	AGTGCTGCGTGAGAAGGTTG
qRT-E2F1-F	CATCCCAGGAGGTCACTTCTG
qRT-E2F1-R	GACAACAGCGGTTCTTGCTC
qRT-SMAD3-F	GCGTGCGGCTCTACTACATC
qRT-SMAD3-R	GCACATTCGGGTCAACTGGTA
qRT-MCM10-F	AAGCCTTCTCTGGTCTGCG
qRT-MCM10-R	CTGTGGCGTAACCTTCTTCAA
qRT-CCNA2-F	GGATGGTAGTTTTGAGTCACCAC
qRT-CCNA2-R	CACGAGGATAGCTCTCATACTGT
qRT-CDC25A-F	TTCCTCTTTTTACACCCCAGTCA
qRT-CDC25A-R	TCGGTTGTCAAGGTTTGTAGTTC
qRT-ORC1-F	ACCGAGATTCACATCCAGATTGG
qRT-ORC1-R	CGAGCACGTTTCTTAGGAGGA
qRT-CCNB1-F	AACTTTCGCCTGAGCCTATTTT
qRT-CCNB1-R	TTGGTCTGACTGCTTGCTCTT
qRT-POLD1-F	CAGTGCCAAGGTGGTGTATGG
qRT-POLD1-R	CTTGCTGATAAGCAGGTATGGG
qRT-MCM8-F	TTTACAGCGATAGCTCTCCTTTG
qRT-MCM8-R	AGGTGCATCTCTTAGTTCAGTTG
qRT-CDC45-F	GTGGGCCATCGTTGGACTAAC
qRT-CDC45-R	TCAAAGGAGATCCGTGTGCAG
qRT-cMYC-F	GGCTCCTGGCAAAAGGTCA
qRT-cMYC-R	CTGCGTAGTTGTGCTGATGT
qRT-OCT4-F	CAAAGCAGAAACCCTCGTGC
qRT-OCT4-R	TCTCACTCGGTTCTCGATACTG
qRT-SOX2-F	TGGACAGTTACGCGCACAT
qRT-SOX2-R	CGAGTAGGACATGCTGTAGGT
qRT-NANOG-F	TTTGTGGGCCTGAAGAAAACT
qRT-NANOG-R	AGGGCTGTCCTGAATAAGCAG
qRT-GLI1-F	AACGCTATACAGATCCTAGCTCG
qRT-GLI1-R	GTGCCGTTTGGTCACATGG
qRT-GLI2-F	CATGGAGCACTACCTCCGTTC
qRT-GLI2-R	CGAGGGTCATCTGGTGGTAAT

qRT-GLI3-F	ACTTCCGCCTTATCTAGTAGCC
qRT-GLI3-R	CCACGGGTTGCTGAGATCAT
qRT-ZIC2-F	CACCTCCGATAAGCCCTATCT
qRT-ZIC2-R	GGCGTGGACGACTCATAGC
qRT-NOTCH1-F	GAGGCGTGGCAGACTATGC
qRT-NOTCH1-R	CTTGTACTCCGTCAGCGTGA
qRT-NOTCH2-F	CAACCGCAATGGAGGCTATG
qRT-NOTCH2-R	GCGAAGGCACAATCATCAATGTT
qRT-NOTCH3-F	CGTGGCTACACTGGACCTC
qRT-NOTCH3-R	AGATACAGGTGAACTGGCCTAT
qRT-RBPJ-F	CTGACTCAGACAAGCGAAAGC
qRT-RBPJ-R	AGGAACACACCAATGTCATCAC
qRT-HEY1-F	GCCCGCCCTTGTCAGTATC
qRT-HEY1-R	ATGCGAAACCAGTCGAACTCG
qRT-HEY2-F	GCCCGCCCTTGTCAGTATC
qRT-HEY2-R	CCAGGGTCGGTAAGGTTTATTG
qRT-HEYL-F	GGCTGCTTACGTGGCTGTT
qRT-HEYL-R	GACCCAGGAGTGGTAGAGCAT
qRT-TGFB1-F	CAATTCCTGGCGATACCTCAG
qRT-TGFB1-R	GCACAACTCCGGTGACATCAA
qRT-TGFB2-F	CCATCCCGCCCACTTTCTAC
qRT-TGFB2-R	AGCTCAATCCGTTGTTCAGGC
qRT-GDF6-F	CCTATCACTGCGAGGGTGTAT
qRT-GDF6-R	GATGGGAGTCAATTTGGTGGG
qRT-BMP4-F	TAGCAAGAGTGCCGTCATTCC
qRT-BMP4-R	GCGCTCAGGATACTCAAGACC
qRT-SMAD6-F	GCTACCAACTCCCTCATCACT
qRT-SMAD6-R	CGTACACCGCATAGAGGCG
qRT-TGFBR1-F	CACAGAGTGGGAACAAAAAGGT
qRT-TGFBR1-R	CCAATGGAACATCGTCGAGCA
qRT-AXIN2-F	AGCCAAAGCGATCTACAAAAGG
qRT-AXIN2-R	AAGTCAAAAACATCTGGTAGGCA
qRT-LEF1-F	AGAACACCCCGATGACGGA
qRT-LEF1-R	GGCATCATTATGTACCCGGAAT

qRT-TCF7L1-F	TCGTCCCTGGTCAACGAGT
qRT-TCF7L1-R	ACTTCGGCGAAATAGTCCCG
qRT-TCF7L2-F	AGAAACGAATCAAAACAGCTCCT
qRT-TCF7L2-R	CGGGATTTGTCTCGGAAACTT
qRT-PPARD-F	GCCTCTATCGTCAACAAGGAC
qRT-PPARD-R	GCAATGAATAGGGCCAGGTC
qRT-FOSL1-F	CAGGCGGAGACTGACAAACTG
qRT-FOSL1-R	TCCTTCCGGGATTTTGCAGAT
qRT-FZD3-F	AATATGGACGTGTCACACTTCC
qRT-FZD3-R	GGATATGGCTCATCACAATCTGG
qRT-FZD6-F	GCGATAGCACAGCCTGCAATA
qRT-FZD6-R	AATGGTAAGAATCACCCACCAC
qRT-COL4A4-F	AGGCTCAACTGGTCTAAGAGG
qRT-COL4A4-R	GCAGGGTCACCTTTGTTTCC
qRT-COL8A1-F	AAAGGGGAAATTGGGCCTATG
qRT-COL8A1-R	CTGGTTGCCCTGGTAACCC
qRT-ITGB1-F	CAAGAGAGCTGAAGACTATCCCA
qRT-ITGB1-R	TGAAGTCCGAAGTAATCCTCCT
qRT-ITGB6-F	CTCAACACAATAAAGGAGCTGGG
qRT-ITGB6-R	AAAGGGGATACAGGTTTTTCCAC
qRT-LAMA3-F	TGCTAACAGTATCCGGGATTCT
qRT-LAMA3-R	TCTTGGTTCAAGCCATTTGCC
qRT-LAMB3-F	CCAAAGGTGCGACTGCAATG
qRT-LAMB3-R	AGTTCTTGCCTTCGGTGTGG
qRT-AFP-F	CTTTGGGCTGCTCGCTATGA
qRT-AFP-R	GCATGTTGATTTAACAAGCTGCT
qRT-CK7-F	TCCGCGAGGTCACCATTAAC
qRT-CK7-R	GCTCTGTCAACTCCGTCTCAT
qRT-CK19-F	AACGGCGAGCTAGAGGTGA
qRT-CK19-F	GGATGGTCGTGTAGTAGTGGC
qRT-EPCAM-F	AATCGTCAATGCCAGTGTACTT
qRT-EPCAM-R	TCTCATCGCAGTCAGGATCATAA
qRT-mOCT4-F	GGCTTCAGACTTCGCCTCC
qRT-mOCT4-R	AACCTGAGGTCCACAGTATGC
qRT-mSOX2-F	GCGGAGTGGAAACTTTTGTCC

qRT-mSOX2-R	CGGGAAGCGTGTACTTATCCTT
qRT-mKRT7-F	AGGAGATCAACCGACGCAC
qRT-mKRT7-R	GTCTCGTGAAGGGTCTTGAGG
qRT-mKRT19-F	GGGGGTTCAGTACGCATTGG
qRT-mKRT19-R	GAGGACGAGGTCACGAAGC
qRT-mAFP-F	CTTCCCTCATCCTCCTGCTAC
qRT-mAFP-R	ACAAACTGGGTAAAGGTGATGG
qRT-mALB-F	TGCTTTTTCCAGGGGTGTGTT
qRT-mALB-R	TTACTTCCTGCACTAATTTGGCA
qRT-mE2F1-F	CTCGACTCCTCGCAGATCG
qRT-mE2F1-R	GATCCAGCCTCCGTTTCACC
qRT-mSMAD3-F	CACGCAGAACGTGAACACC
qRT-mSMAD3-R	GGCAGTAGATAACGTGAGGGA
qRT-m18S-F	AGGGGAGAGCGGGTAAGAGA
qRT-m18S-R	GGACAGGACTAGGCGGAACA