H19 promotes cholestatic liver fibrosis by preventing ZEB1-mediated inhibition of EpCAM

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Supplementary Methods

Animal study

C57BL/6 mice were purchased from the Jackson Laboratory. *H19*$^{-/-}$ mice were kindly provided by Dr. Linheng Li **(1)**. Because H19 is a paternal imprinted gene, maternal *H19*-deleted mice were used for experiments and paternal *H19*-deleted mice were used as negative controls (neg. con.). Mice were fed a standard rodent chow diet (Harlan No. 2018) with free access to water and maintained in a 12 h light/dark (LD) cycle (light on 6 AM to 6 PM), temperature-controlled (23°C), and pathogen-free facility. *In vivo* experiments were performed on male mice at the age of 6 weeks unless stated otherwise (n=5 mice/group). For *in vivo* viral transduction **(2)**, mice were injected via tail vein with purified adeno-associated viral vector serotype 8 (AAV8) containing a liver-specific thyroxine-binding globulin (TBG) promoter driving H19 gene expression at $5x10^{10}$ virus particles per mouse. Sham and BDL surgeries were performed for one week post-AAV8-null or AAV8-H19 transduction for one month, using our established method **(3)**. For rescue experiment, mice with H19 overexpression for one month were injected via tail vein with mouse pShuttle-ZEB1 overexpression plasmid (GeneCopoeia, USA), EpCAM shRNA plasmid (Sigma-Aldrich, USA), or control plasmid at 50 μg per mouse using TurboFect *in vivo* Transfection Reagent (R0541; Thermo Scientific, Waltham, MA) as described previously **(4)**. All samples were collected under feeding conditions unless otherwise indicated. Protocols for animal use were approved by IACUC at the University of Connecticut.

Cell culture

Hepa1, HEK293T, HepG2, Hep3B, and Huh7 cells were purchased from ATCC and were made aliquots and stored in liquid nitrogen tank immediately after the first passage. The cholangiocyte-derived cell lines MSC, MLC, H69, HuCCT-1, CCLP-1, Mz-cha1 and SG231 were Dr. Meenakshisundaram Ananthanarayanan (Yale University). The cell lines were passaged for less than 6 months when used for experiments. Hepa1, HEK293T, HepG2, Hep3B, and Huh7 cells were maintained in DMEM (high glucose) medium containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. MSC cells and MSC cells were maintained in MEM medium containing 10% FBS and 1% penicillin-streptomycin. HuCCT-1 cells were maintained in RPMI-1640 medium with 10% FBS and 50 μg/ml gentamicin. SG-231 cells were maintained in alpha MEM medium with 10%FBS, 10 mM Hepes and 50μg/ml gentamicin. CCLP-1 cells were maintained in DMEM (high glucose) medium with 10% FBS and 50 μg/ml gentamicin. Mz-Cha1 cells were maintained in alpha-MEM medium with 10% FBS and Pen/Strep. All cell lines were maintained at 37°C in a humidified atmosphere of 5% CO2 in air.

Primary hepatocyte isolation

Primary hepatocytes were isolated by the two-step collagenase perfusion described previously(5). The cells were plated in William E medium supplemented with 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, and 2 mM glutamine and cultured in a humidified incubator at 37 °C, 5% CO2. Cells were plated for 24 h before the appropriate treatments.

Constructs

ZEB1 3'UTR luciferase construct was obtained from Addgene (6). ZEB1 CRISPR activator and CRISPR KO constructs were purchased from Santa Cruz. Epcam-Luc construct was kindly provided by Dr. Xinwei Wang (National Cancer Institute, NIH) (7). The mutant plasmid was constructed in our laboratory using Q5 Site-Directed Mutagenesis Kit (NEB, USA) (8).

Western blotting

The protein samples were extracted from liver tissue or cells as describe previously (9). The protein concentration was measured using the BCA method. Equal amounts of protein from different samples were separated in a 10% SDS-PAGE gel, then the samples were tranfered into polyvinylidene difluoride membranes (Millipore) from the gel. After incubating for 1 hour with 5% non-fat milk in TBST, The membranes were incubated overnight at 4℃ with anti-ZEB1 (1:1000, Sigma), anti- α-SMA (1:1000, Sigma), anti-EpCAM (1:1000, abcam), anti-p-Akt (1:1000, Cell-signaling), anti-Akt (1:1000, Cell-signaling), anti-p-ERK (1:1000, Cell-signaling), anti-ERK (1:1000, Cell-signaling), anti-SMAD2/3 (1:1000, Cell-signaling), anti- β -Actin (1:1000, Cell-signaling) or anti-α-Tubulin (1:1000, Santa Cruz) antibodies. Following the primary antibodies, the membranes were incubated in correspondence secondary antibodies at a 1:3000 dilution for 1 hour at room temperature. Immune complexes were detected using SuperSignal™ West Pico Chemiluminescent Substrate (Thermo Fisher). β-actin or α-Tubulin protein was evaluated as a loading control.

Real time PCR

RNA was isolated from the cells by TRIzol (Ambion) and cDNA synthesis was performed with High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher). And the quantitative PCR was performed using the Universal SYBR Green (Biorad). Relative quantification was calculated with normalization to HPRT1 (mouse) or GAPDH (human). Specific primers are included in the Supplementary Table S1.

Transient transfection and luciferase reporter assay

Cells were transfected with AAV8-H19 virus or negative control virus by reverse transfection and plated in 24-well plates. Twenty-four hours later, the cells were transfected with human ZEB1 CRISPR activator, human ZEB1 CRISPR KO constructs, human EpCAM promoter (EpCAM/luc) as indicated in the figure legends using transfection reagent X-tremeGENE HP (Roche) (4). Forty-eight hours after transfection, the cells were harvested, and the luciferase activity was measured using a dual-luciferase reporter assay system (Promega). pRL-TK was used as a control to normalize the luciferase activity.

Biotinylated RNA pull down assay

The biotinylated RNA pull down assay was performed as described previously (10). In briefly, Biotin-labelled RNA transcripts were synthesized by *in vitro* transcription with T7 RNA polymerase in the presence of Biotin-11-UTP (Thermo Fisher Scientific). PCR fragments were amplified with forward primers containing T7 RNA polymerase promoter sequences and reverse primers containing SP6 RNA polymerase promoter sequences. Purified PCR products were used as a DNA template for in vitro transcription. Whole cell lysates (300-500 μg per sample) were incubated with purified biotinylated RNA probes (~10 pmole) for 25 minutes at 30°C. RNA–protein complexes were further isolated by streptavidin sepharose high -performance beads (GE Healthcare, Marlborough, MA). The recruited proteins were detected by western blotting. Primers containing T7 or T3 promoter sequences used for synthesizing biotin-labeled H19 are listed in Supporting Tables S1.

Chromatin immunoprecipitation (ChIP) assay.

The ChIP assay was performed as described previously (11). Briefly, the cells were treated with 1% formaldehyde to cross-link the histones and genomic DNA. Cell lysates were prepared, and chromosomal DNA was sonicated to obtain average sizes between 200-1000 bp. The chromatin was incubated and precipitated with antibodies against ZEB1 or normal rabbit IgG as controls at 4°C overnight. The chromatin-antibody complexes were precipitated with Dynabeads-protein G. And then chromatin DNA fragments were purified with QIAGEN PCR purification kit (Qiagen, Valencia, CA, USA), and subjected to qPCR using primers listed in Table S1.

Analysis of serum bile acid (BA), ALT, AST, total and direct bilirubin

Serum total BA was determined by total BA assay kit (Cell Biolabs, #STA-631) according to the manufactures' protocols. Serum ALT and AST were detected by Infinity ALT/GPT kit (Thermo Fisher, TR71121) or Infinity AST/GOT (Thermo Fisher, TR70121) kit, respectively. Serum total and direct bilirubin were detected by total bilirubin kit (Pointe Scientific, B7576) and direct bilirubin kit (Pointe Scientific, B7538), respectively.

Bile acid pool size assay

The pool size was determined as the total bile acid content of the small intestine, gallbladder and liver, which were extracted in ethanol. Briefly, fresh mice tissues including gallbladder, liver, and entire small intestine were minced and extracted in 75% ethanol at 50 °C for 2 hr. The extract was then centrifuged, diluted with 75% ethanol, and further diluted with 25% phosphate-buffered saline before the enzymatic reaction. The total amount of BAs in the pool was determined using the total BA assay kit (Cell Biolabs, #STA-631). The pool size was expressed as micromoles of bile acid/100 g of body weight.

Fecal bile acid extraction

To determine fecal bile acid excretion, the feces from individually housed mouse over a 72 hr period were collected, dried and weighed. Then about 50mg feces were homogenize in 95% ethanol and extract at 60 °C for 4 hr. The extract was then centrifuged at 12000 rpm to pellet. Then the feces were reextracted with 80% ethanol, centrifuge and combine with supernatant above. The extract was diluted with 25% phosphate-buffered saline and subjected to bile acid measurement. The daily fecal output (grams/day/100 g of body weight) and fecal bile acid content (micromoles/g) were used to calculate the rate of bile acid excretion (micromoles/day/100 g of body weight).

Flow cytometry analysis

The assay was performed as described previously(12, 13). In brief, liver and spleens were harvested and mononuclear cells were purified by Ficoll-Paque. Red blood cells were removed by hypotonic ACK lysis buffer. The expression markers on T cells were determined by FACS analysis after surface or intracellular staining with anti-mouse specific antibodies conjugated with Alexa Fluor 488, Alexa Fluor 488 700, FITC, PE, allophycocyanin (APC), PerCP-cy5.5 or PE-cy7. These mouse antibodies included: anti-TCRγδ, anti-CD3, anti-CD4, anti-C8, anti-IFN-γ, anti-IL-4, anti-IL-17 and anti-Foxp3, which were purchased from Biolegend. For intracellular staining, T cells were stimulated with PMA (1 μg/ml) and ionomycin (2 μg/ml) (Sigma-Aldrich) for 5 hours in the presence of GolgiStop (BD Biosciences) before the intracellular staining with antibodies. All stained cells were analyzed on a LSR II cytometer (BD Bioscience) and data analyzed with FlowJo software (Tree Star).

Histological analysis of liver sections

Freshly harvested mouse livers were fixed in formalin for 48 hr and then embedded in paraffin. Paraffin sections at thickness of 4 μm were cut and subjected to xylene and ethanol rehydration prior to Hematoxylin and Eosin (H&E) and Masson Trichrome staining. Eight fields of each slide

were randomly taken under microscope and quantified by ImageJ software as described previously (14).

Statistical analysis

Data are shown as the mean ± standard error of the mean (SEM) and are representative of at least three independent experiments. Statistical analysis was carried out using Student's t test between two groups and one way ANOVA among multiple groups. $P<0.05$ was considered statistically significant.

Reference

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Table S1 The information of primary sclerosing cholangitis (PSC) and primary biliary cirrhosis (PBC) patients

Disease	age (year)	Gender	Individual ID
PSC	63	Female	UMN1690
PSC	51	Male	UMN1645
PSC	53	Female	UMN1635
PSC	33	Male	UMN1614
PSC	60	Male	UMN1600
PSC	43	Male	UMN1560
PSC	58	Male	UMN1537
PSC	61	Male	UMN1534
PSC	47	Male	UMN1520
PSC	58	Male	UMN1518
PSC	58	Male	UMN1489
PSC	53	Male	UMN1465
PSC	48	Male	UMN1383
PSC	59	Male	UMN1363
PSC	27	Female	UMN1358
PSC	58	Male	UMN1339
PSC	38	Male	UMN1315
PSC	32	Male	UMN1307
PSC	62	Male	UMN1270
PSC	38	Male	UMN1267
PSC	51	Female	UMN1240
PSC	50	Male	UMN1236
PSC	41	Male	UMN1222
PSC	22	Female	UMN1210
PSC	25	Male	UMn1199
PSC	55	Male	UMN1182
PSC	52	Male	UMN1179
PSC	34	Female	UMN1178
PSC	57	Female	UMN1171

PSC	32	Female	UMN1163
PSC	51	Male	UMN1155
PSC	41	Male	UMN1150
PBC	63	Female	UMN1682
PBC	52	Male	UMN1679
PBC	60	Female	UMN1595
PBC	65	Female	UMN1490
PBC	64	Female	UMN1448
PBC	67	Male	UMN1441
PBC	65	Male	UMN1388
PBC	35	Male	UMN1378
PBC	47	Female	UMN1374
PBC	63	Male	UMN1292
PBC	52	Female	UMN1269
PBC	46	Female	UMN1234
PBC	59	Female	UMN1218
PBC	46	Female	UMN1213
PBC	59	Female	UMN1183
PBC	60	Female	UMN1173
PBC	55	Female	UMN1064
PBC	62	Female	UMN1054
PBC	63	Female	UMN1038
PBC	58	Female	UMN1006
PBC	62	Male	UMN1003

Table S2 Primers used in the study

Name	Forward	Reverse
qPCR primers		
Human H19	TGGTGCACTTTACAACCACTG	ATGGTGTCTTTGATGTTGGGGC
Mouse H19	GAACAGAAGCATTCTAGGCTG	TTCTAAGTGAATTACGGTGGG
Human Zeb1	ACCCTTGAAAGTGATCCAGC	CATTCCATTTTCTGTCTTCCGC
Mouse Zeb1	TGATGAAAACGGAACACCAGA	GTTGTCCTCGTTCTTCTCATG
Human Epcam	CAATGCAGGGTCTAAAAGCTG	CACCCATCTCCTTTATCTCAGC
Mouse Epcam	TGGTGTCATTAGCAGTCATCG	TCAGTTCAGCACTCAGCAC
Mouse col1a1	GACGCCATCAAGGTCTACTGC	GGAAGGTCAGCTGGATAGCG
Mouse col1a2	AAGGATACAGTGGATTGCAGG	TCTACCATCTTTGCCAACGG
Mouse aSMA	AGAGTTACGAGTTGCCTGATG	ATGAAGGATGGCTGGAACAG
Mouse Ck7	TGAGATTGCGGAGATGAACC	CGATGCTGGACTCTAACTTGG
Mouse Ck19	CTCCCGAGATTACAACCACTAC	GTTCTGTCTCAAACTTGGTTCTG
Mouse cyp7a1	GGGATTGCTGTGGTAGTGAGC	GGTTATGGAATCAACCCGTTGT
Mouse cyp7b1	GGAGCCACGACCCTAGATG	GCCATGCCAAGATAAGGAAGC
Mouse Fxr	GCTTGATGTGCTACAAAAGCT	CGTGGTGATGGTTGAATGTCC
Mouse Fgfr4	TGTCAAATTCCGCTGTCCAG	ACCACACTTTCCATCACCAG
Mouse cyp8b1	CCTCTGGACAAGGGTTTTGTG	GCACCGTGAAGACATCCCC
Mouse Shp	CAGGTCGTCCGACTATTCTG	ACTTCACACAGTGCCCAGTG
Mouse Fos	TCCTTACGGACTCCCCAC	CTCCGTTTCTCTTCCTCTTCAG
Mouse Ccr2	TGCCATCATAAAGGAGCCA	AGCACATGTGGTGAATCCAA
ChIP primers		
Epcam (site 1)	GCCAGGTAAAAGCTCAAAGG	GCGGGAACTGGATAGAGGA
Epcam (site 2)	GATCCCTAACGCCGCCATG	GCCGCTGGTGCTCGTTGAT
Cloning construct primers		
Epcam promoter mutant	ATTAAATTGCacttTAAAAGCTCAAAGGTC TTTTTTATAG	TATACTCCTTTGAAATTAGAAAG
Primers for RNA pull down assay		
H19	TAATACGACTCACTATAGGG (T7)	GATTTAGGTGACACTATAG (SP6)

H19 fibrosis Fig. S1

Diagram of BDL models

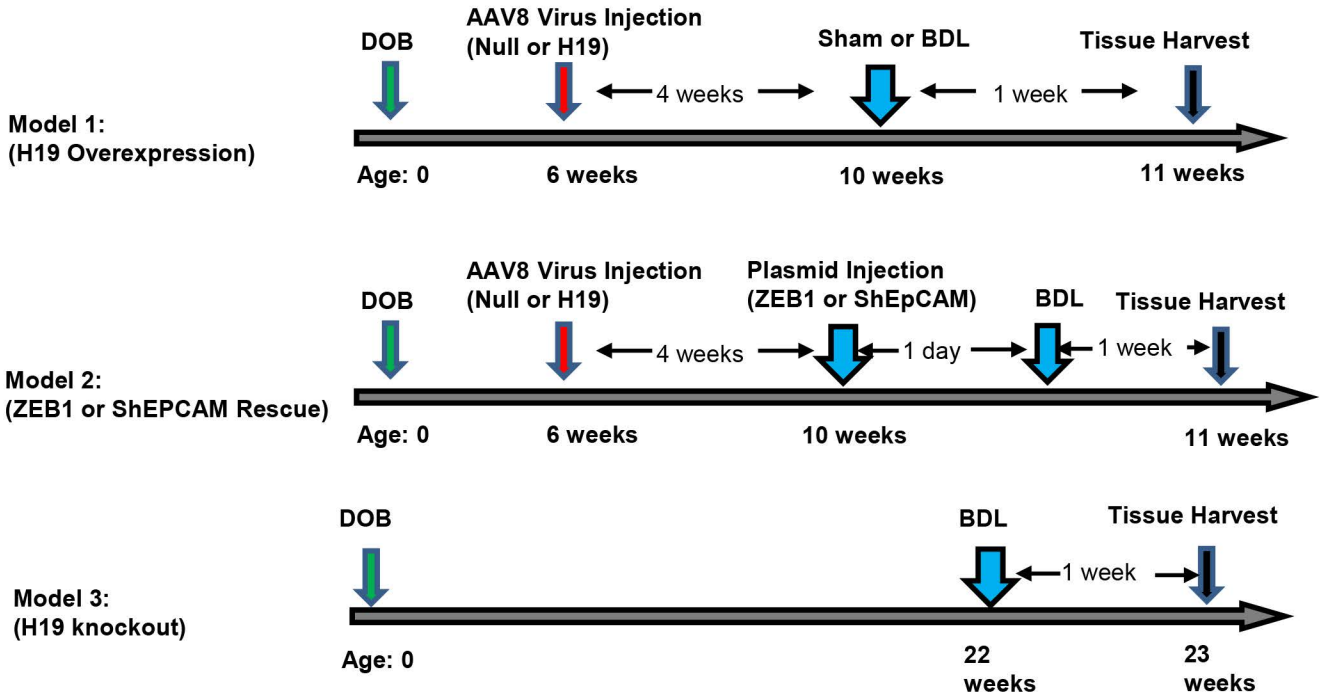

Fig. S1: **Diagram showing the mouse models used for this study with detailed timelines**.
Red below: The titers of viruses used for tail vein injection.

Virus particles:

AAV8-Null: $5x10^{10}$ viral particles/mouse
AAV8-H19: $5x10^{10}$ viral particles/mouse

H19 fibrosis Fig. S2

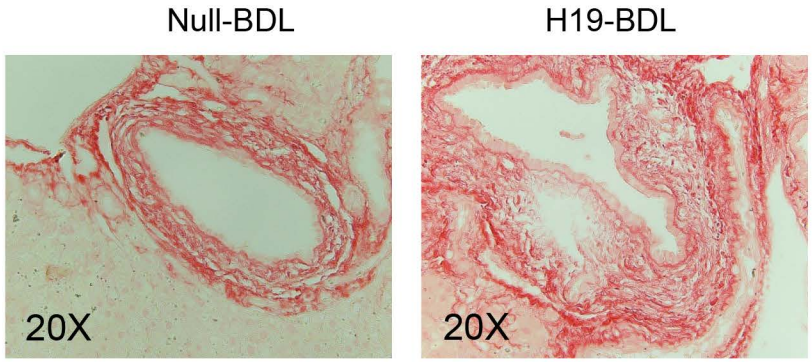

Fig. S2: **Sirius staining of liver sections from mice underwent BDL for one week after H19 overexpression.**

H19 fibrosis Fig. S3

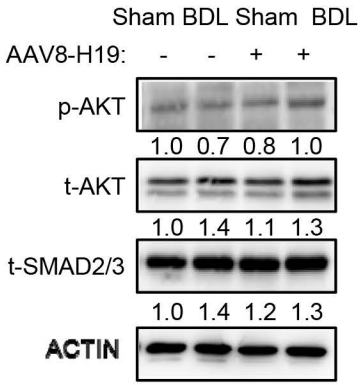

Fig. S3: **Western blot of protein expression in livers of Null and H19 mice under sham or BDL for 7 days.** Each band represented pooled sample (equal amounts of protein) from 5 individual mice.

H19 fibrosis Fig. S4

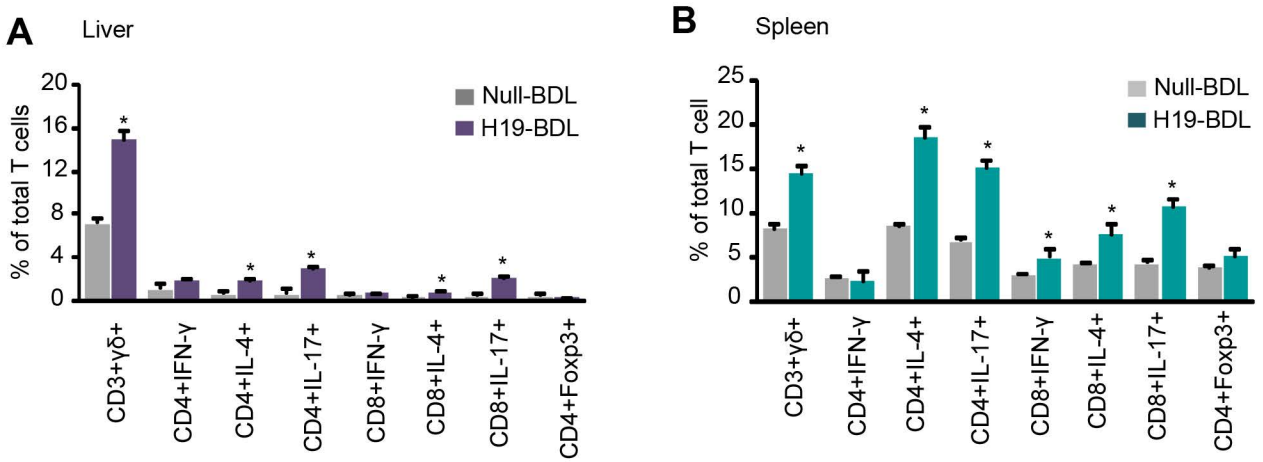

Fig. S4 Accumulation of T cell subsets in the H19-BDL mouse model. The proportion of different subsets of immune cell population in livers (A) and spleens (B) of Null and H19 mice under BDL were analyzed by FACS.

The same results were presented in main Fig. 2E-2F as fold change of H19 vs Null group.

H19 fibrosis Fig. S5

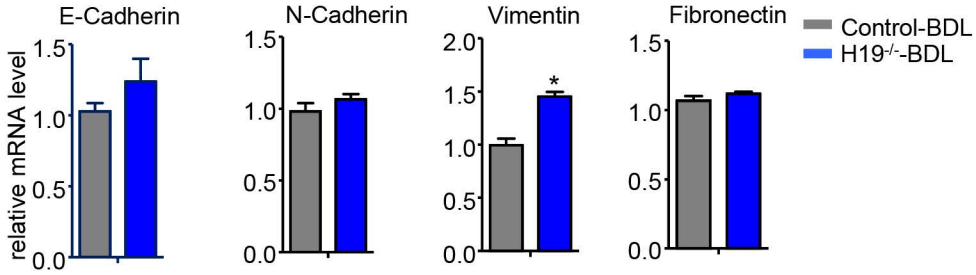

Fig. S5: qPCR of mRNA expression in livers from control and H19$^{-/-}$-BDL mice. Data were shown as mean ± SEM (n=5/group of triplicate assays). *P< 0.05 vs H19$^{-/-}$.

H19 fibrosis Fig. S6

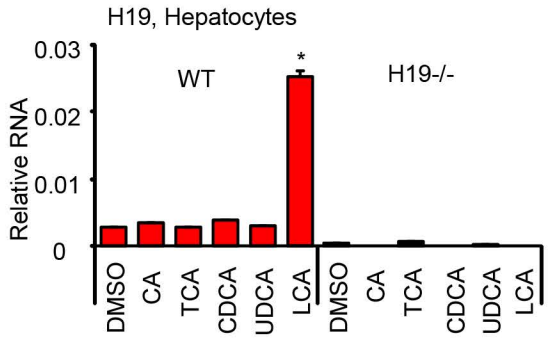

Fig. S6: **qPCR of H19RNA in primary hepatocytes.** Hepatocytes were isolated from WT and H19-/- mice, and treated with various BAs using the same experimental condition as in Fig. 6A. Cholic acid (CA, 100 μM), taurocholic acid (TCA, 100 μM), chenodeoxycholic acid (CDCA, 100 μM), ursodeoxycholic acid (UDCA, 100 μM) or lithocholic acid (LCA, 10 μM) for 24 hrs. Data were shown as mean ± SEM (triplicate assays). *P< 0.05 vs DMSO.

H19 fibrosis Fig. S7

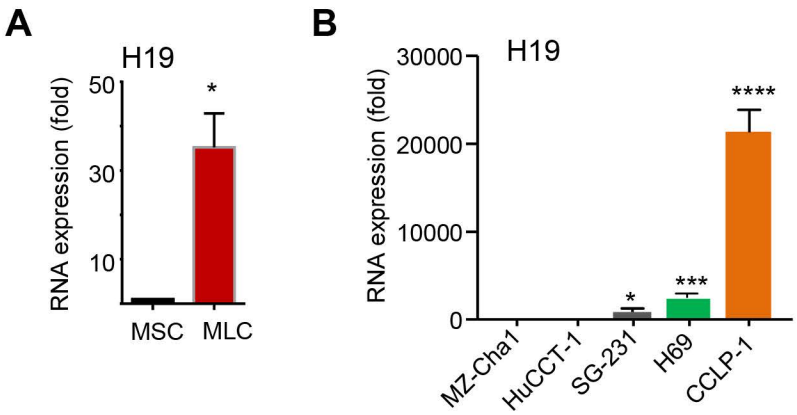

Fig. S7: **qPCR of H19RNA in cholangiocytes.** The basal levels of H19RNA expression in different cholangiocyte cell lines were detected by qPCR. Data were shown as mean ± SEM (triplicate assays). **P*< 0.05, *****P*< 0.001 vs MSC or MZ-cha1.