Supplementary Materials and Methods

Materials. U0126 and ICI182,780 were purchased from Sigma (St. Louis, MO). JTE-013 was purchased from Cayman Chemicals (Ann Arbor, MI). Antibody against p-AMPK (Thr-172, 2531) was purchased from Thermo Fisher Scientific (Waltham, MA). Antibody against Bcl-2 (ab692) was from Abcam (Cambridge, MA). Antibody against sphk2 (s3573) was purchased from Sigma (St. Louis, MO). Antibody against p-ERK (E-4, sc-7383), ERK1 (C-16, sc-93), ERK2 (C-14, sc-154), FXR (sc-13063), SHP (sc-30169), TGR5 (sc-48688), s1pr2 (EDG5, sc-25491), c-Myc (sc-70465) and PCNA (sc-56) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Taurocholate (TCA), 17β-Estradiol (E2) and other chemicals were purchased from Sigma (St. Louis, MO). Estradiol ELISA kit was purchased from Cayman (Ann Arbor, MI). All cell culture supplement components were obtained from Gibco (Waltham, MA). The Bio-Rad protein assay reagent, Precision Plus Protein Kaleidoscope Standards, and iQ^{TM} SYBR Green Supermix were obtained from Bio-Rad (Hercules, CA). IRDye secondary antibody was from LI-COR (Lincoln, NE).

Animal Studies. FVB wild-type mice (both male and female) were purchased from Jackson Laboratories (Bar Harbor, ME). Mdr2 knockout mice (Mdr2$^{-/-}$) were gifts from Dr. Gianfranco Alpini (Texas A&M HSC College of Medicine). Mice were housed under 12h light/12 dark cycle and fed standard chow and tap water *at libitum.* Mice at different ages (60, 100 and 180 days) were used in this study. At the end of experiment, the mice were weighted and sacrificed. Blood and bile were collected to measure liver enzyme activities, bile acid and hydroxyproline concentrations. The livers were harvested and either fixed and processed for histological analysis or frozen in liquid nitrogen and stored at -80°C for further analysis. For *in vivo* knockdown of H19 experiment, female Mdr2$^{-/-}$mice (60 days) were injected with purified adenovirus of H19 shRNA (2 x10^{10} virus particles per mouse) via tail vein. After 7 days, mice were harvested as described above. All animal study protocols were approved by the

Institutional Animal Care and Use Committee of Virginia Commonwealth University. In addition, all experiments were performed in accordance with institutional guidelines and regulations.

Measurement of the liver functional enzyme activities, hydroxyproline and total bile acid levels. Serum levels of alkaline phosphatase (ALP), aspartate aminotransferase (AST), alanine aminotransferase (ALT) and hydroxyproline were measured using commercially available assay kits from Sigma (St. Louis, MO) according to the manufacturers' procedures. Total bile acid (TBA) was measured by use of an assay kit from Crystal Chem (Downers Drive, IL). Liver samples were homogenized in RIPA buffer and total protein lysates were used for analysis. Results were normalized with total protein amount. Absorptions were determined by the Victor3 Multilabel Plate Counter from PerkinElmer (Waltham, MA).

Measurement of estradiol levels in serum. Serum levels of estradiol were measured by use of commercially available ELISA kits from Cayman (Ann Arbor, MI) according to the manufacturer's procedures. Absorbance at 450 nm was determined by the Victor3 Multilabel Plate Counter from PerkinElmer (Waltham, MA).

Isolation and culture of primary mouse hepatocytes, Kupffer cells and cholangiocytes. Primary mouse hepatocytes were isolated using a two-step collagenase digestion method as previously described (23). Cells were plated in collagen-coated plates at a density of 70% confluence using William's E medium containing penicillin, dexamethasone (0.1 μM) and thyroxine (0.1 μM). After cells were attached (3-4 h), the medium was replaced to remove dead cells. For cholangiocytes isolation, after the two-step collagenase perfusion, puncture and shaking with HBSS buffer, biliary tree was isolated using two-step digestion with collagenase XI-hyaluronidase and bovine pancreas trypsin. Cholangiocytes were further collected using 70μM cell strainer (BD Biosciences) and purified by immunoaffinity separation. Primary mouse kupffer cells were isolated using Percoll gradient centrifugation method as previously described (24).

Cell culture and treatment. Normal mouse large cholangiocytes (MLE) used in this study were originally obtained from Dr. Gianfranco Alpini (Texas A&M HSC College of Medicine) and were

cultured with minimum essential medium (MEM) medium containing 10% fetal bovine serum (FBS), penicillin G (100 U/mL), streptomycin (100 µg/mL). Human embryonic kidney cells (HEK293 cells; American Type Culture Collection, Manassas, VA) used for amplification of adenovirus were cultured with Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS, penicillin G (100 U/mL), streptomycin (100 µg/mL). Cells were kept at 37°C with 5% CO_2 in a humidified cell culture incubator. MLE cells were treated with TCA (0.1 mM), GCA (0.1 mM) or E2 (100 nM) with or without pretreatment of JTE-013 (10 µM) for 48 h. For the H19 knockdown study, MLE cells were incubated with adenovirus of H19 shRNA or control adenovirus for 6h and refurnished with fresh medium. After 24h, cells were treated with TCA (0.1 mM) or E2 (100 nM) for 48h.

Western blot analysis. Total cell lysates were prepared from mouse liver tissue or MLE cells with RIPA buffer (25). The protein concentrations were determined using Bio-Rad protein assay kit as previously described (26). 100 µg of protein were resolved on a 10% SDS-PAGE gel and transferred to Nitrocellulose membranes. After blocking with 5% BSA in TBS buffer for 1 h at room temperature, membranes were incubated with specific primary antibodies for overnight at 4°C. Immunoreactive bands were detected using IRDye secondary antibodies. Images were recorded using LI-COR Odyssey imaging system.

RNA isolation and quantitative RT-PCR. Total RNA was isolated from mouse liver tissues or cultured MLE cells using TRIzol Reagent (QIAGEN, Valencia, CA, USA). 2 µg of RNA were isolated, quantified and reverse transcribed using High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA). The mRNA levels of targeted genes were determined by real-time PCR using iQ™ SYBR Green Supermix and normalized using HPRT1 as an internal control as described previously. Primer sequences were shown in online table1.

Adenovirus shRNA for knock down H19. Adenoviruses with the validated sequence for knock down mouse H19 (GCATGACAGACAGAACATT) was a gift provided by Dr. Li Wang (The Institute for Systems Genomics in University of Connecticut). GFP Adenoviruses was from our

own lab. The concentrated adenovirus was amplified and produced by HEK293 cells. Briefly, after being transfected with adenovirus of H19 siRNA for 24h, HEK293 cells were collected and lysed with five consecutive freeze-thaw cycles. The supernatants were then ultracentrifuged with gradient cesium chloride ($CsCl_2$) for 21h using Sorvall WX Ultra 100 (Thermo Fisher Scientific, Waltham, MA). Viruses were removed from the centrifuge tubes with a syringe and dialyzed for overnight. Titers were measured using adenovirus titration kits from Clontech (Takara, Japan), according to manufacturer's instruction.

Histopathology, Masson's Trichrome staining and immunohistochemistry. After being scarified, livers were collected and fixed in 4% formaldehyde and embedded in paraffin. 4.5 μm sections were prepared and stained with hematoxylin and eosin (H&E) following the previous protocol (27). Liver fibrosis was determined by Masson Trichrome staining according to manufacturer's instruction (28). For immunohistochemical studies, paraffin-embedded liver sections were deparaffinized, hydrated and incubated with antibodies following the manufacturer's procedures for VECTASTAIN (vector laboratories, Burlingame, CA). Antibody against cytokeratin polypeptide 19 (CK-19) (dilution, 1:100) was purchased from DSHB (Iowa, IA). Antibody against PCNA (dilution, 1:50) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The images of staining sections were obtained by Zeiss Axio Scope.A1 microscope (Carl Zeiss, Germany), and analyzed by ZEN software (Carl Zeiss, Germany). The numbers of PCNA or CK19 positive cells were quantified by counting the number of cells from three different fields in each section.

Statistical analysis. The results were presented as mean ± SE and were from at least three independent experiments. One-way ANOVA and post-test was used to analyze the differences between multiple groups by GraphPad Prism 5 (GraphPad, San Diego, CA). P values of ≤ 0.05 were considered statistically significant.

Supplementary figure legends

Supplementary Figure 1. Gender disparity of liver injury in Mdr2$^{-/-}$ mice. 100-day-old Mdr2$^{-/-}$ mice and age-matched FVB wild type mice were sacrificed. (A) Liver levels of ALP and TBA. (B) Serum AST, ALT levels. (C) Ratios of liver to body weight and spleen to body weight. The mRNA levels of PCNA (D) and proinflammatory chemokines (F) were determined by real-time RT-PCR and normalized using HPRT1. (E) The numbers of PCNA or CK19 positive cells. Statistical significance: *P<0.05, **P<0.01, compared with male (M) wild type (WT) mice; $^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001, compared with female (F) WT mice; $^{\$\$}$P<0.01, $^{\$\$\$}$P<0.001, compared with (M) Mdr2$^{-/-}$ mice (n=6).

Supplementary Figure 2. Age disparity of liver injury in Mdr2$^{-/-}$ mice. Both male (M) and female (F) Mdr2$^{-/-}$ mice at 60-day and 100-day-old were sacrificed. (A) Serum AST and ALT levels. (B) Ratio of liver to body weight. (C) Ratio of spleen to body weight. (D) The mRNA levels of proinflammatory chemokines were determined by real-time RT-PCR and normalized using HPRT1. (E) The numbers of PCNA or CK19 positive cells. Statistical significance: *P<0.05, **P<0.01, compared with 60-day-old (M) Mdr2$^{-/-}$ mice; $^{\#}$P<0.05, $^{\#\#}$P<0.01, compared with 60-day-old (F) Mdr2$^{-/-}$ mice; $^{\$}$P<0.05, $^{\$\$}$P<0.01, $^{\$\$\$}$P<0.001, compared with 100-day-old (M) Mdr2$^{-/-}$mice (n=6).

Supplementary Figure 3. The expression of Bcl-2, FXR and SHP in wild type and Mdr2$^{-/-}$ mice. 100-day-old WT and Mdr2$^{-/-}$ mice were sacrificed. The mRNA levels of Bcl-2 (A-C), FXR (E) and SHP (F) were determined by real-time RT-PCR and normalized using HPRT1. (B-D) Protein levels of Bcl-2 were determined by western blotting analysis and normalized with β-actin. Representative images were shown. Statistical significance: *P<0.05, compared with (M) WT mice; $^{\#}$P<0.05, $^{\#\#}$P<0.01, compared with (F) WT mice; $^{\$}$P<0.05, $^{\$\$\$}$P<0.001, compared with (F) Mdr2$^{-/-}$mice (n=6).

Supplementary Figure 4. The expression of H19, SHP, Ki67, PCNA, Collagen 1 and Bcl-2 in different age of Mdr2$^{-/-}$mice. 60-day-old, 100-day-old and 180-day-old Mdr2$^{-/-}$ mice were sacrificed. The mRNA levels of (A) H19, (B) SHP, (C) Ki67, (D) PCNA, (E) Collagen 1 and (F) Bcl-2 were determined by real-time RT-PCR and normalized using HPRT1. Statistical significance: $^{\$\$\$}$P<0.001, compared with 60-day-old (M) Mdr2$^{-/-}$mice; $^{\#}$P<0.05, $^{\#}$P<0.01, $^{\#\#\#}$P<0.001, compared with 60-day-old (F) Mdr2$^{-/-}$mice (n=6).

Supplementary Figure 5. The expression of TGR5 and ERα and serum levels of estrogen in Mdr2$^{-/-}$mice. 100-day-old WT and Mdr2$^{-/-}$ mice were sacrificed. The mRNA levels of TGR5 (A) and ERα (D) were determined by real-time RT-PCR and normalized using HPRT1. (B) Protein level of TGR5 was determined by western blotting analysis and normalized using β-actin. (C) Serum levels of estrogen in 60-day, 100-day, 180-day-old WT and Mdr2$^{-/-}$ mice. Primary mouse hepatocytes, cholangiocytes and kupffer cells were isolated as described in Method. (E) The mRNA levels of ERα in differential primary cells were detected by real-time RT-PCR, and normalized using HPRT1 as an internal control. Statistical significance: *P<0.05, compared with (M) WT mice; $^{\#}$P<0.05, compared with (F) WT mice; $^{\$}$P<0.05, compared with (M) Mdr2$^{-/-}$ mice (n=6). For serum levels of estrogen, **P<0.01, compared with 60-day-old (M) Mdr2$^{-/-}$ mice; $^{\#}$P<0.05, compared with 60-day-old (F) Mdr2$^{-/-}$ mice; $^{\Psi}$P<0.05, compared with 100-day-old (M) WT mice; $^{\$\$\$}$P<0.001, compared with 100-day-old (M) Mdr2$^{-/-}$ mice; $^{\varphi\varphi}$P<0.01, compared with 180-day-old (M) Mdr2$^{-/-}$ mice (n=4).

Supplementary Figure 6. The effects of H19 knockdown on serum ALT and AST in female Mdr2$^{-/-}$ mice. 60-day-old (F) Mdr2$^{-/-}$ mice were injected with control or purified H19 adenovirus shRNA (2 x10^{10} virus particles per mouse) as described in Methods. (A) AST, ALT levels in serum (n=6).

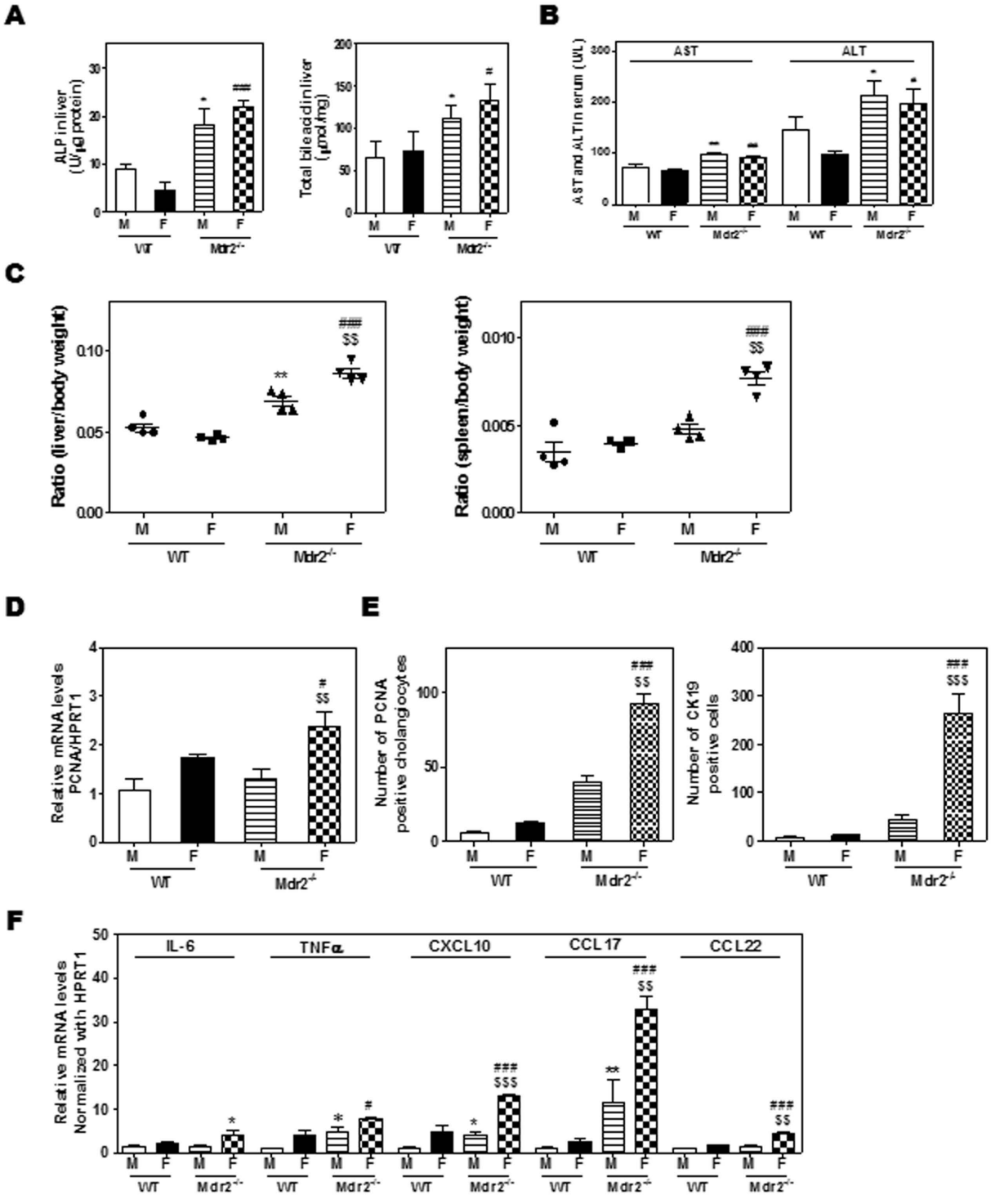
Supplementary Figure 7. The effects of H19 knockdown on relative gene expression in female Mdr2$^{-/-}$ mice. 60-day-old (F) Mdr2$^{-/-}$ mice were injected with control or purified H19 adenovirus shRNA as described in Methods. The mRNA levels of CYP7A1 (A), FXR (B), bile

acid receptors (E) and cholesterol synthesis genes (F) were determined by real-time RT-PCR and normalized using HPRT1. (C) Representative images of Western blot analysis and the relative protein levels of PCNA, c-Myc, S1PR2, Bcl-2 and SHP normalized with β-actin as an internal control. (D) The numbers of PCNA or CK19 positive cells. Statistical significance: *P<0.05, **P<0.01, ***P<0.001, compared with control virus group (n=6).

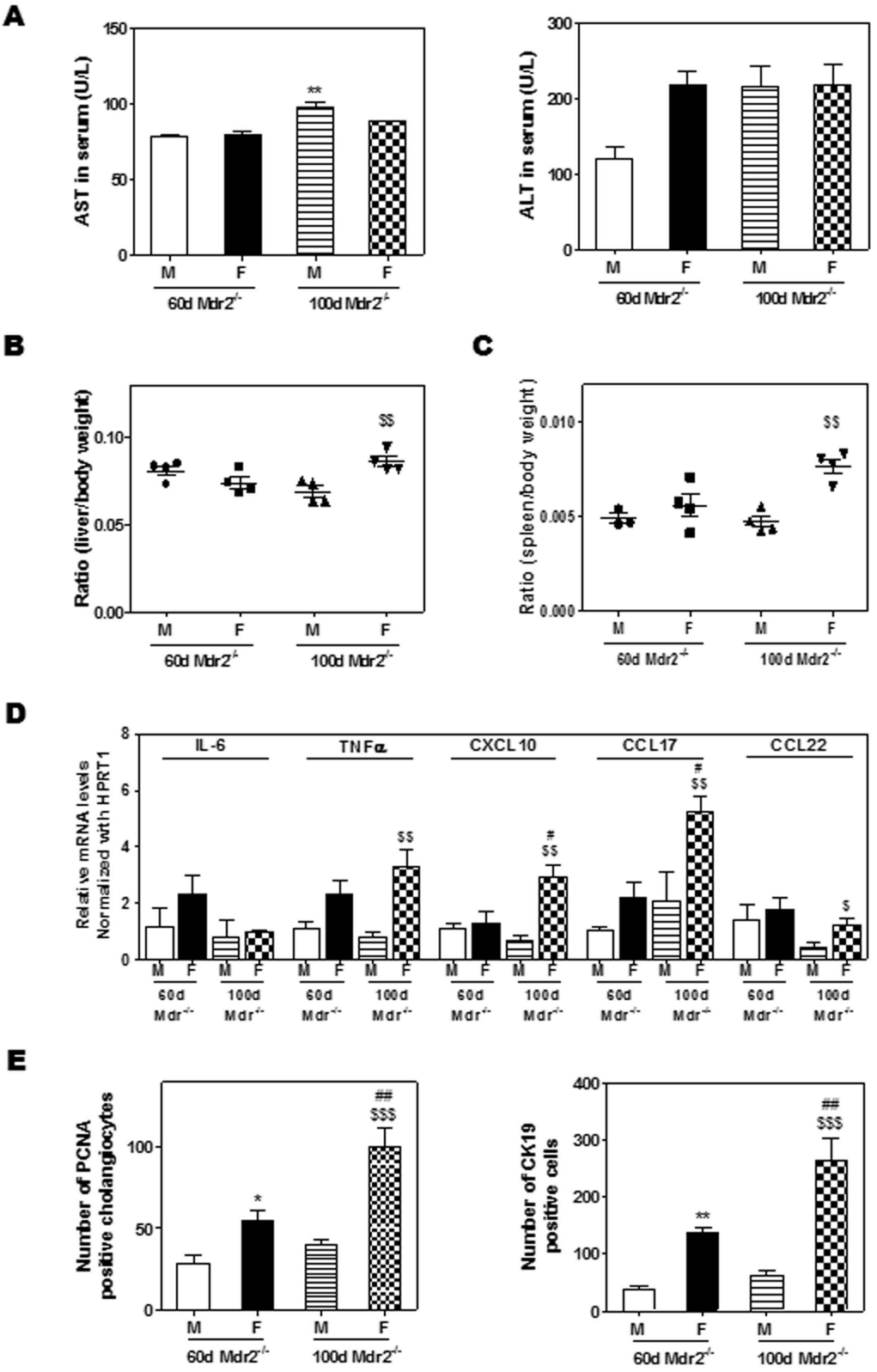
Supplementary Figure 8. The expression of H19 and relative genes in human normal and PSC patient liver. Total RNA was isolated from human normal liver tissues and PSC patient liver tissues. The expression levels of (A) H19, (C) c-Myc, Ki67 and Collagen 1, (D) FXR, SHP and S1PR2 were determined by real-time RT-PCR and normalized using HPRT1 as an internal control. (B) Representative image of DNA agarose gel of H19 PCR products. Statistical significance: *P<0.05, **P<0.01, compared with normal (M) human; $^{\#}$P<0.05, $^{\#}$P<0.01, compared with normal (F) human; $^{\$\$}$P<0.01, compared with PSC (M) (n=8).

Supplementary Figure 9. Sphk2 is critical for cholestatic liver injury in Mdr2$^{-/-}$ mice. 100-day-old WT, 60-day-old and 100-day-old Mdr2$^{-/-}$ mice were sacrificed. 60-day-old (F) Mdr2$^{-/-}$ mice were injected with control or purified H19 adenovirus shRNA. (A-C) The mRNA levels of Sphk2 were determined by real-time RT-PCR and normalized using HPRT1. (A-C) Protein levels of Sphk2 were determined by Western blotting analysis and normalized with β-actin. Representative images were shown. Statistical significance: *P<0.05, compared with control virus group; $^{\#\#}$P<0.01, compared with (F) WT mice; $^{\$}$P<0.05, $^{\$\$}$P<0.01, compared with (M) Mdr2$^{-/-}$ mice (n=6).

Supplementary Figure 1



Supplementary Figure 2



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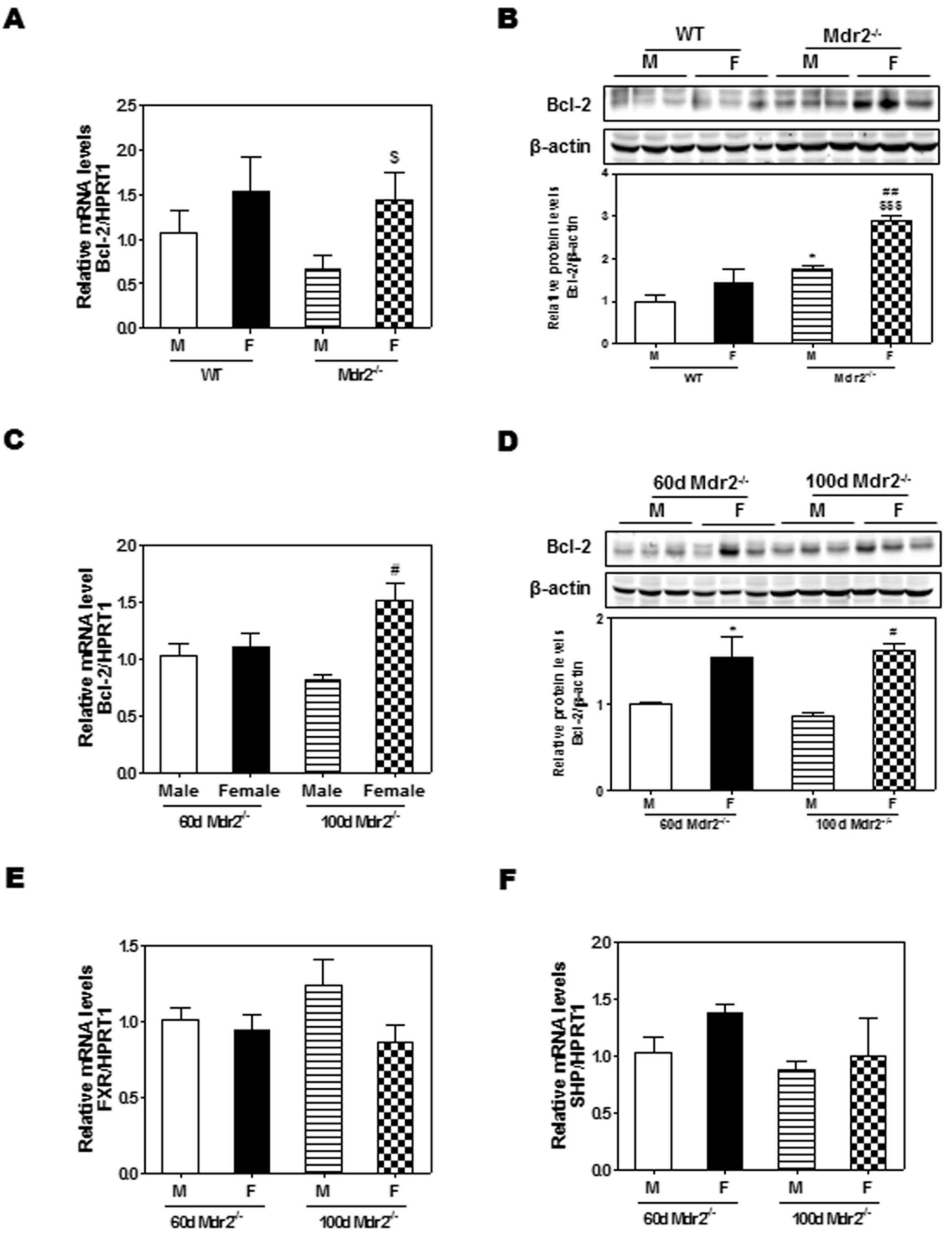

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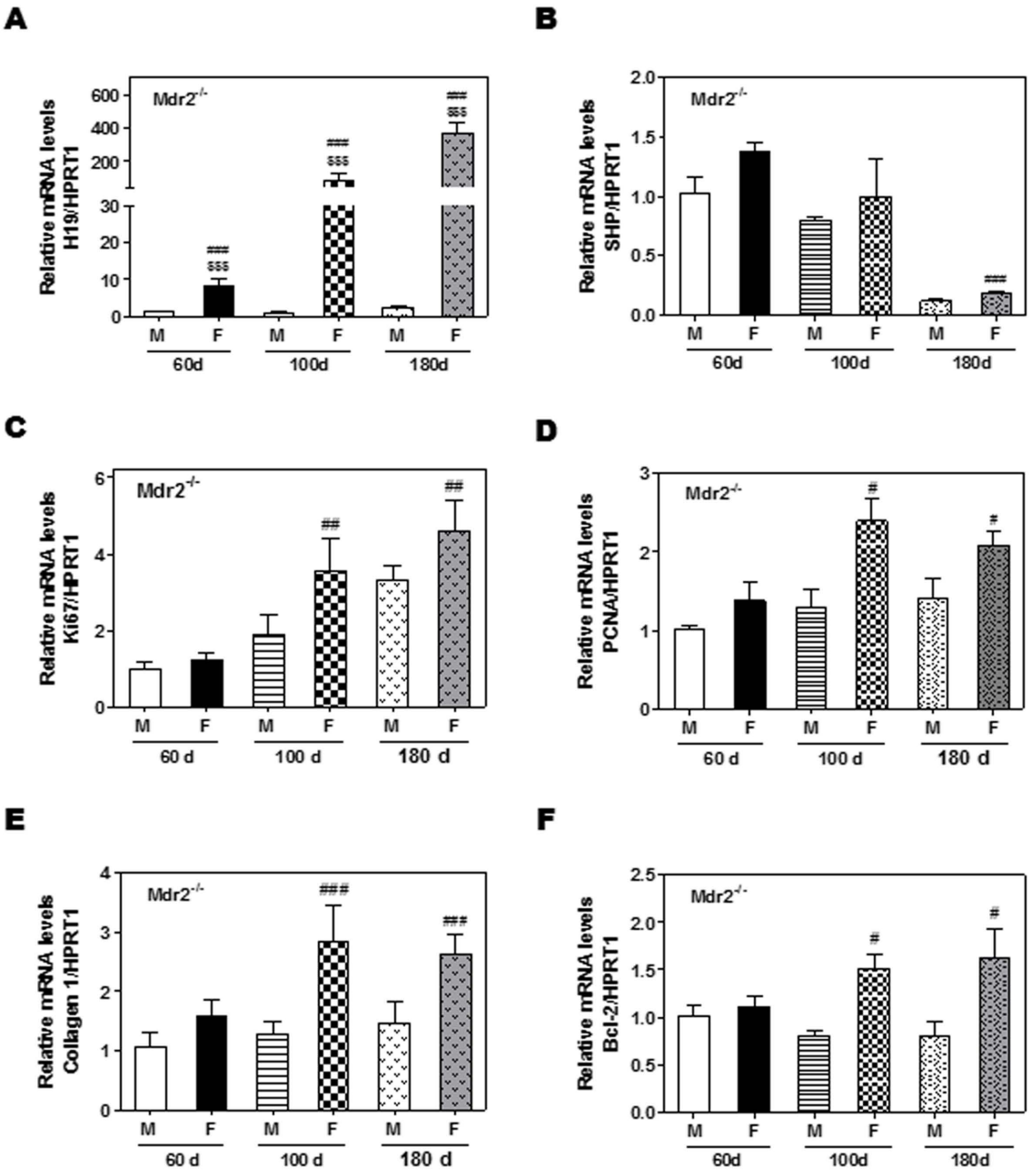

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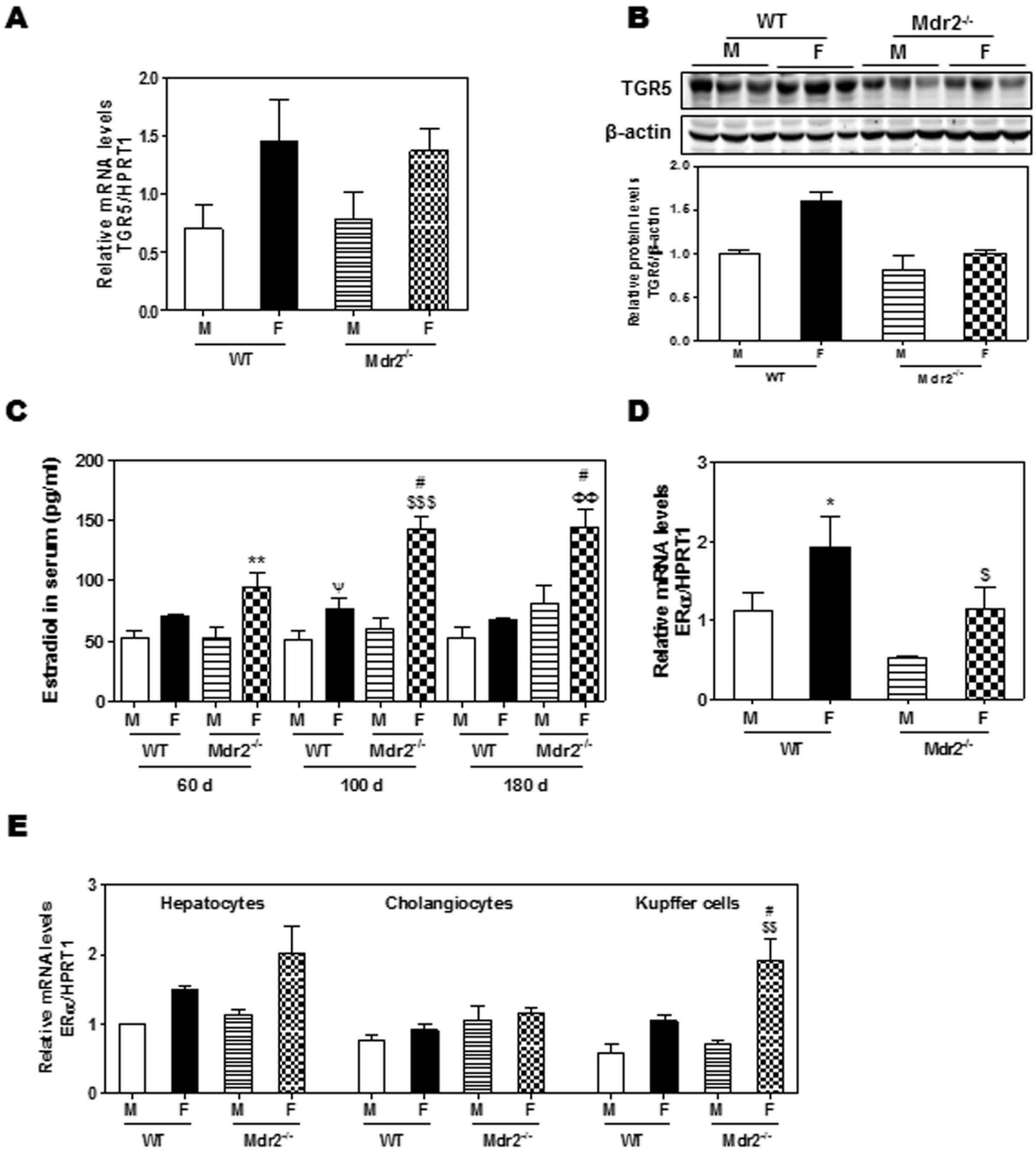

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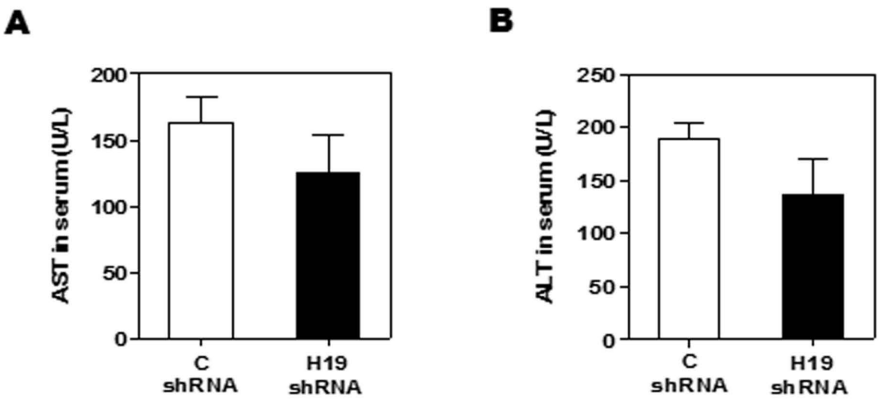

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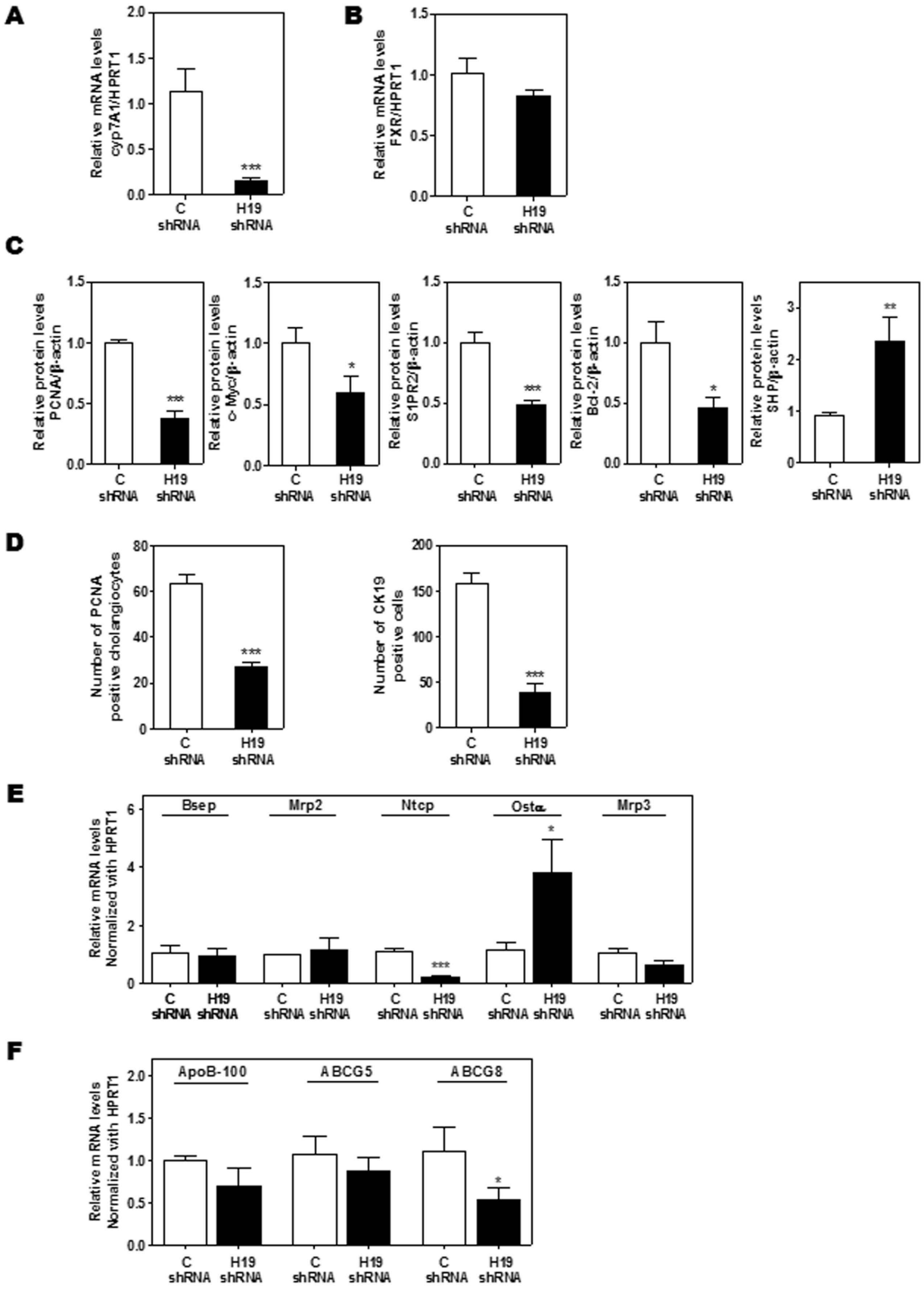
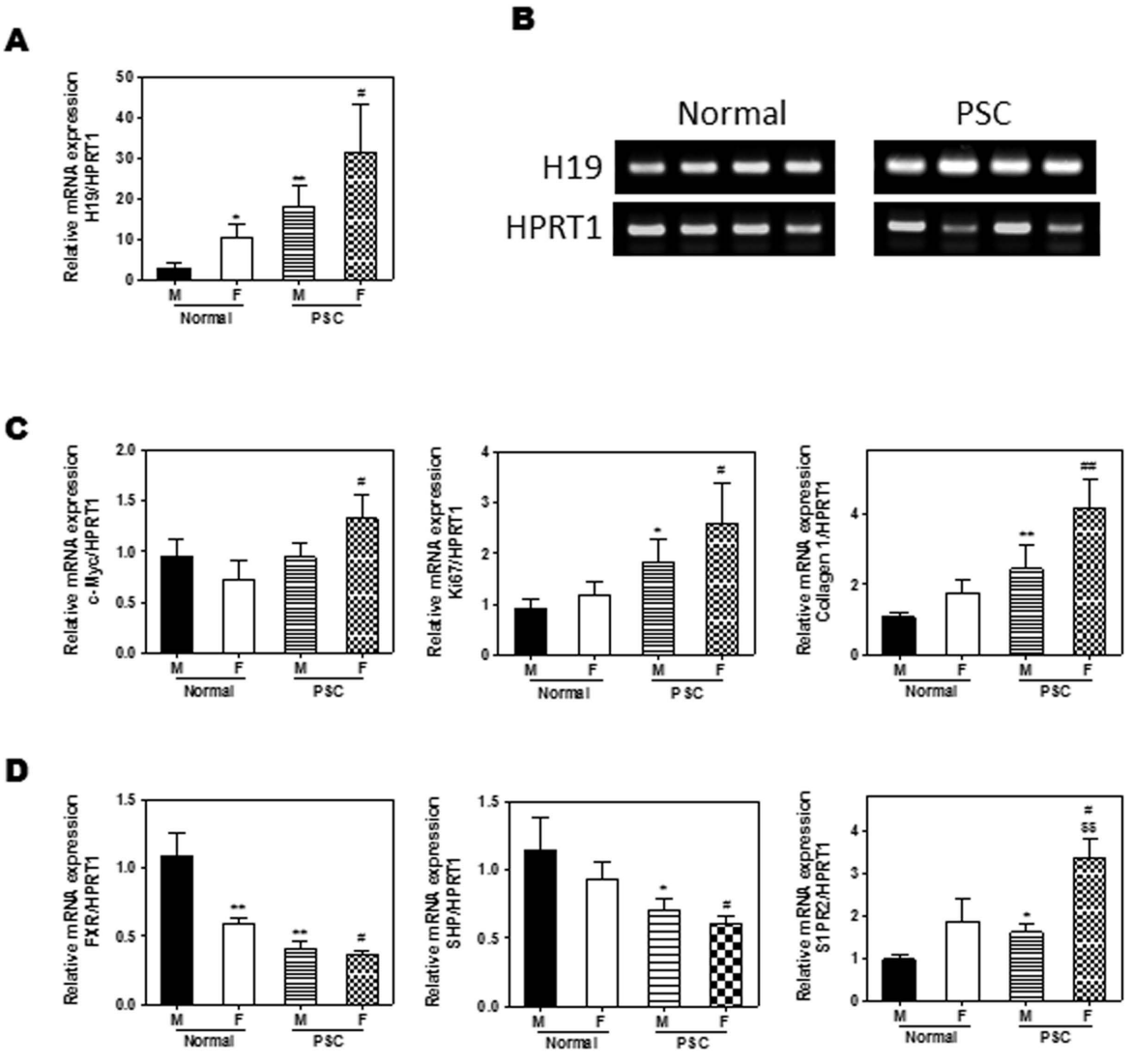


Supplementary Figure 8



Supplementary Figure 9