Published in final edited form as:

Hepatology. 2018 August ; 68(2): 599–615. doi:10.1002/hep.29838.

Cholangiocyte-derived exosomal long noncoding RNA H19 promotes cholestatic liver injury in mouse and human

Xiaojaoyang Li¹, Runping Liu¹, Zhiming Huang², Emily C. Gurley^{1,3}, Xuan Wang¹, Juan Wang⁴, Hongliang He⁴, Hu Yang⁴, Guanhua Lai⁵, Luyong Zhang⁶, Jasmohan S Bajaj³, Melanie White³, William M Pandak³, Phillip B. Hylemon¹, and Huiping Zhou^{1,2,*}

¹Department of Microbiology and Immunology and McGuire Veterans Affairs Medical Center, Virginia Commonwealth University, Richmond, Virginia, USA

²Department of Gastroenterology, the First Affiliated Hospital, Wenzhou Medical University, Wenzhou, Zhejiang, China

³Division of Gastroenterology, Hepatology and Nutrition and McGuire Veterans Affairs Medical Center, Virginia Commonwealth University

⁴Department of Chemical and Life Science Engineering, Virginia Commonwealth University, Richmond, Virginia, United States

⁵Department of Pathology, Medical College of Virginia Campus, Virginia Commonwealth University, Richmond, Virginia, USA

⁶Guangdong Pharmaceutical University, Guangzhou, China

Abstract

Cholestatic liver injury is an important clinical problem with limited understanding of disease pathologies. Exosomes are small extracellular vesicles released by a variety of cells including cholangiocytes. Exosome-mediated cell-cell communication can modulate various cellular functions by transferring a variety of intracellular components to target cells. Our recent studies indicate that the long non-coding RNA H19 is mainly expressed in cholangiocytes and its aberrant expression is associated with significant down-regulation of small heterodimer partner (SHP) in hepatocytes and cholestatic liver injury in multidrug resistance 2 knockout (*Mdr2*^{-/-}) mice. However, how cholangiocyte-derived H19 suppresses SHP in hepatocytes remains unknown. Here, we report that cholangiocyte-derived exosomes mediate transfer of H19 into hepatocytes and promote cholestatic injury. The hepatic H19 level is correlated with the severity of cholestatic injury in both fibrotic mouse models, including *Mdr2*^{-/-} mice, a well-characterized model of primary sclerosing cholangitis (PSC), or carbon-tetrachloride (CCl₄)-induced cholestatic liver

Address correspondence to: Huiping Zhou, Ph.D., Department of Microbiology & Immunology, Virginia Commonwealth University, McGuire Veterans Affairs Medical Center, 1217 East Marshall Street, MSB#533, Richmond, VA, 23298-0678, USA, Tel: 804-828-6817; Fax: 804-828-0676, Huiping.zhou@vcuhealth.org.

Competing Financial interest: The authors declare no competing financial interest.

Author contributions.

XL and HZ conceived the original ideas, designed the study, analyzed the data and wrote the manuscript; XL, RL, ECG, XW, JW and HH carried out the experiments and data analysis. GL helped with histology analysis. JSB and MW were responsible for human sample collection; PBH, WMP, JSB, LZ and ZH helped with data analysis and reviewed the manuscript.

injury mouse models, and human PSC patients. Moreover, serum exosomal-H19 level is gradually upregulated during disease progression in *Mdr2*^{-/-} mice and cirrhotic patients. The H19-carrying exosomes from the primary cholangiocytes of wild type (WT) mice suppress SHP expression in hepatocytes, but not the exosomes from the cholangiocytes of *H19*^{-/-} mice. Furthermore, overexpression of H19 significantly suppressed SHP expression at both transcriptional and post-transcriptional levels. Importantly, transplant of H19-carrying serum exosomes of old fibrotic *Mdr2*^{-/-} mice significantly promoted liver fibrosis in young *Mdr2*^{-/-} mice.

Conclusion—Cholangiocyte-derived exosomal-H19 plays a critical role in cholestatic liver injury. Serum exosomal-H19 represents a novel non-invasive biomarker and potential therapeutic target for cholestatic diseases.

Keywords

bile acids; cholestasis; S1PR2; *Mdr2*^{-/-}; CCl4

Introduction

Primary sclerosing cholangitis (PSC) and primary biliary cholangitis (PBC) are important chronic cholestatic liver diseases. Despite extensive research, the pathogenesis of PSC and PBC remains unknown and liver transplantation remains as the only solution (1). Impaired bile flow and disrupted bile acid metabolism are major contributors to cholangiopathies (2). Our previous studies reported that taurocholate acid (TCA)-mediated activation of sphingosine 1-phosphate receptor 2 (S1PR2) not only promotes cholestatic injury in the bile duct ligation (BDL) mouse model, but also promotes the invasive growth of cholangiocarcinoma cells (3–5). In addition, estrogen is also a well-known risk factor for cholestasis (6). We recently reported that estrogen- and TCA-induced aberrant expression of long non-coding RNA (lncRNA) H19, an imprinted and maternally expressed transcript (7), was responsible for the suppression of small heterodimer partner (SHP) expression in hepatocytes and cholestatic liver injury in multidrug resistance 2 knockout (*Mdr2*^{-/-}) mice (6). However, hepatic H19 is mainly expressed in cholangiocytes, not in hepatocytes (6). There is an important gap in understanding of how cholangiocyte-derived H19 impacts hepatic cholestatic injury.

Emerging evidence indicates that exosomes, the small extracellular membrane-enclosed vesicles formed by the inward budding of endosomal membranes and released extracellularly *via* fusion with the plasma membrane, are important mediators of cell-to-cell communication (8, 9). Exosomes can be released by a variety of cells under normal and pathological conditions *in vitro* and *in vivo* (8, 10, 11). Recent studies have reported that exosome-mediated transfer of mRNAs, microRNAs, lncRNAs, proteins and lipids is implicated in various human diseases including liver diseases (8, 12–16). It also has been shown that liver epithelial cells, both cholangiocytes and hepatocytes, are exosome-releasing cells (17–20). In addition, exosomes exist in all major body fluids including blood, bile and urine (8). Biliary exosomes are involved in regulating intracellular signaling and cell proliferation in cholangiocytes (17).

Here we demonstrate that cholangiocyte-derived exosomes are rich in H19 under cholestatic conditions. Both estrogen and TCA increase the release of H19-carrying exosomes from cholangiocytes. Cholangiocytes are the source of H19 in hepatocytes during cholestatic disease progression. In addition, the H19 level in serum exosomes is correlated to the severity of cholestatic liver injury in mouse cholestatic injury models and human cirrhotic patients. We also show that H19 is a key molecule in cholangiocyte-derived exosomes that promotes cholestatic liver injury. These results suggest that H19-carrying exosomes not only can be used as novel non-invasive biomarkers, but also represent a potential therapeutic target for cholestatic liver diseases.

Materials and Methods

Animal Studies

FVB and C57/BL6 wild type (WT) mice (both male and female) were obtained from Jackson Laboratories (Bar Harbor, ME). *Mdr2*^{-/-} mice with FVB background were gifts from Dr. Gianfranco Alpini (Texas A&M HSC College of Medicine). *H19*^{-/-} mice (*H19* Exon1/+) with C57/BL6 background were provided by Dr. Karl Pfeifer at NIH. The *H19* Exon1/+ mouse carries a 1-kb deletion of *H19* exon1 that removes almost the entire exon1, including the region encoding miR-675, and reduces levels of even the partial *H19* transcript by > 100-fold globally (21). FVB WT and *Mdr2*^{-/-} mice at different ages (30 to 100-day old) were used. C57/BL6 WT and *H19*^{-/-} mice at 60- and 84-days old (both male and female) were used. All mice were housed in polysulfone microisolation cages maintained on ventilated rack and cage system with ¼-in corn cob bedding, in a 12 h light/12 h dark cycle with free access to standard chow and water *ad libitum*. Cotton nesting pads were used for housing enrichment. For the chronic carbon tetrachloride (CCl₄) study, 84-day old WT and *H19*^{-/-} mice were gavaged with CCl₄ (1 ml/kg) or sesame oil twice a week during light cycle as previously described (22). After 8 weeks, mice were sacrificed without fasting. For *in vivo* exosomal studies, exosomes were isolated from the serum of 100-day-old WT and *Mdr2*^{-/-} mice (both male and female) and used to treat 50-day-old *Mdr2*^{-/-} mice (both male and female) twice a week *via* tail vein injection. After 7 days, mice were sacrificed and blood and liver tissues were harvested for further analysis. All animal procedures were approved by the VCU Institutional Animal Care and Use Committee and are also performed in accordance with institutional guidelines for ethical animal studies.

Human liver samples

Frozen human liver tissues from PSC patients and normal controls were obtained from the Liver Tissue Cell Distribution System (Minneapolis, MN), which is funded by the National Institutes of Health (contract no. HSN276201200017C).

Human serum samples

Outpatients with compensated cirrhosis and age-matched healthy controls were prospectively recruited after written informed consent. Cirrhosis was diagnosed using fibroscan, liver biopsy or by endoscopic or radiological evidence of portal hypertension in patients with chronic liver disease. None of the patients were abusing alcohol or illicit drugs, had evidence of decompensation, or was on antibiotics or probiotics or hepatitis C

eradication therapy. Serum was collected and analyzed for exosomes. This protocol was approved by the Institutional Review Board of Virginia Commonwealth University.

Isolation of exosomes

Mouse large cholangiocytes (MLE) and mouse small cholangiocytes (MSE) were plated on 150-mm plates and treated with 17 β -Estradiol (E2) (100 nM), TCA (0.1 mM), glycocholic acid (GCA) (0.1 mM) and deoxycholic acid (DCA) (0.05 mM) for 48 h. Conditioned media were collected and cleared from cell debris by centrifugation at 2,000 g for 15 min followed by 16,000 g for 20 min at 4°C. Cell-derived exosomes in the conditioned media were isolated by ultracentrifugation at 100,000 g for 70 min at 4°C using WX Ultra 100 from Thermo Fisher Scientific (Waltham, MA). Pellets were resuspended in sterile PBS and stored at -80°C for further analysis. For isolation of exosomes from mouse or human serum, 0.5 ml of serum was diluted with PBS to 2 ml and centrifuged at 1,200 g for 15 min followed by 10,000 g for 30 min at 4°C. The supernatant was filtered with a 0.22 μ m filter to remove all particles bigger than 200 nm followed by ultra-centrifugation at 100,000 g for 70 min at 4°C. Pellets were resuspended in 50 μ l sterile PBS.

Dynamic light scattering (DLS) analysis

Isolated extracellular vesicles were resuspended with 50 μ l PBS and diluted with PBS to 1 ml volume in a cuvette. The size and size distributions of exosomes were analyzed by DLS analysis using a Malvern Zetasizer Nano ZS90 series instrument (Malvern, Worcestershire, UK). All samples were measured at 25°C using a 633 nm laser light set at a scattering angle of 90°, following an equilibration time of 120 s. The analysis was carried out using the Dispersion Technology Software v.5.10.

Transmission electron microscopy (TEM)

Concentrated exosomes were fixed in 2% paraformaldehyde in PBS at room temperature for 10 min. The fixed exosomes were placed on formvar-carbon-coated grids (TED PELLA, Inc., CA, USA). After being air-dried and washed with PBS, the grids were immersed in 1 % glutaraldehyde for 5 min, further contrasted and embedded in the mixture solution of 4 % uranyl acetate and 2 % methyl cellulose (9:1) on ice. Following removal of the extra contrasting solution, the grids were air dried and analyzed using Zeiss Libra 120 transmission electron microscopy (Carl Zeiss, Germany) at an accelerating voltage of 100 kV.

Statistics

Results were obtained from at least three independent experiments and were expressed as mean $\pm$ SD. Data were analyzed by two-tailed Student's t test or One-way ANOVA with Tukey's *post-hoc* test using GraphPad Prism 5 software (GraphPad, San Diego, CA). A *P* value of ≤ 0.05 was considered statistically significant. Error bars indicate SD.

All other methods are described in an online supplementary file.

Results

Estrogen and TCA increase the release of exosomal H19 from cholangiocytes

It is well-established that both conjugated bile acids (CBAs) and estrogen play etiopathogenic roles in the progression and development of cholestatic liver diseases or cholangiopathies (23–25). Our recent study showed that aberrant expression of hepatic H19 contributes to the gender disparity of cholestatic liver injury in *Mdr2*^{-/-} mice (6). H19 is mainly expressed in cholangiocytes, not hepatocytes. However, it is significantly upregulated in primary mouse hepatocytes from female *Mdr2*^{-/-} mice with severe cholestatic liver injury (6). It is unknown how H19 is upregulated in hepatocytes under cholestatic conditions. Exosomes are well-recognized as very important cell-cell communication vehicles by their transference of various intracellular regulatory molecules (13) and can be released by both cholangiocytes and hepatocytes (9, 26). However, it has not been determined how cholangiocyte-released exosomes play a role in estrogen- and TCA-induced cholestatic injury. We first examined the effect E2 and TCA had on exosome release in cultured MLE. As shown in Fig. 1A–C, TEM and DLS analysis indicate that the size of the MLE-released exosomes is between 50 to 150 nm in diameter. Interestingly, a combination of E2 and TCA not only increased the size of the exosomes, but also increased the number of exosomes released from MLEs. Among different bile acids, only TCA slightly increased the size of exosomes in MLE, but not in the MSE (Supplementary Fig. 1A–B and D–E). Western blot analysis further confirmed the expression of CD63 and lysosomal-associated membrane protein (LAMP) 1 and LAMP2, the common markers of exosomes (Fig. 1D). We have recently reported that both E2 and TCA induced H19 expression in MLEs (6). However, it is not clear if MLE-released exosomes also contain H19 and whether E2- and TCA-induced increase of H19 in MLEs correlates to H19 levels in exosomes. We isolated total exosomal RNA released from the MLEs treated with E2, TCA or both. As shown in Fig. 1E, MLE-released exosomes did contain H19 (confirmed by DNA sequencing, data not shown) and both E2 and TCA significantly and additively increased H19 levels in exosomes (Fig. 1F). Furthermore, TCA significantly increased H19 levels in MLE- but not MSE-derived exosomes (Supplementary Fig. 1C and 1F). Consistent with previous studies, TCA but not other bile acids (GCA and DCA) induced H19 upregulation both in MSE and MLE cells (Supplementary Fig. 2).

Effect of cholangiocyte-released exosomal H19 on SHP expression in hepatocytes

SHP plays an important role in maintaining hepatic cholesterol and bile acid homeostasis (27). The expression of SHP is rapidly induced by TCA in hepatocytes (28, 29). It has been reported that hepatic overexpression of B-cell lymphoma 2 (*Bcl-2*) induced H19 upregulation and rapid SHP protein degradation under cholestatic liver injury (30). Our recent studies also found that hepatic SHP protein levels are inversely related to H19 expression levels (6). Since SHP and H19 are mainly expressed in hepatocytes and cholangiocytes, respectively, cholangiocyte-derived exosomes may play a critical role in transferring H19 from cholangiocytes to hepatocytes. To determine the role of H19 in regulating hepatic SHP expression, we first examined whether E2 and TCA had a direct effect on H19 expression in hepatocytes. Similar to our previous findings, H19 expression levels in normal mouse primary hepatocytes (MPH) were very low and not induced by E2

and TCA (Fig. 2A, left panel). Interestingly, the purified exosomes from MLEs treated with TCA or E2 plus TCA significantly increased H19 and Bcl-2 expression in MPH (Fig. 2A, left panel and Supplementary Fig. 3A). However, treatment of MPH with exosomes from H19 knockdown MLEs did not increase H19 and Bcl-2 levels in MPH (Fig. 2A, right panel and Supplementary Fig. 3B). These results suggest that MLE-derived exosomes are the source of H19 for hepatocytes. As expected, E2/TCA-induced SHP upregulation was significantly inhibited in MPH by exosomes derived from E2/TCA-treated MLEs (Fig. 2B). To further delineate the crucial role of H19 in regulating cholestatic injury, we isolated mouse primary cholangiocytes (MPC) and MPH from WT and H19^{-/-} mice. The MPC cultured on Matrigel-coated plates reached confluence in five days (Supplementary Fig. 4A). The purity of the isolated MPC was checked by real-time RT-PCR with specific hepatic cell markers, cytokeratin 19 (CK-19) for MPC, cytochrome P450 1A2 (cyp1a2) for MPH, F4/80 for Kupffer cells (KCs), and desmin for hepatic stellate cells (HSC). As shown in Supplementary Fig. 4B, there was no cross-contamination in MPC with other hepatic cells. Similar to the findings in cultured MLEs, both E2 and TCA significantly induced H19 expression in MPC (Fig. 2C). The exosomes derived from WT MPC treated with E2 or TCA or both significantly increased H19 levels in WT MPH (Fig. 2D). Interestingly, the basal expression level of SHP was increased in MPH from H19^{-/-} mice compared to that in WT MPH (Fig. 2E). To further examine the role of cholangiocyte-derived exosomal H19 in regulating hepatic cholestatic injury, WT MPH were treated with MPC-derived exosomes from WT or H19^{-/-} mice. As shown in Fig. 2F (left panel), SHP mRNA was reduced in MPH treated with purified exosomes from the WT MPC treated with E2 and TCA, but not with the exosomes from H19^{-/-} MPC. In addition, exosomes from WT MPC treated with TCA or E2 plus TCA significantly increased Bcl-2 and sphingosine kinase 2 (SphK2) expression in WT MPH, but H19^{-/-} MPC-derived exosomes had no effect (Fig. 2F, right panel and Supplementary Fig. 4C). Our recent studies suggest that SphK2 is a key regulator of hepatic lipid metabolism (31). These results indicate that cholangiocyte-derived exosomal H19 plays a critical role in regulating key genes involved in hepatic bile acid and lipid metabolism.

Upregulation of serum exosomal H19 level is associated with the severity of cholestatic liver injury in fibrotic mouse models

The Mdr2^{-/-} mouse is a well-established animal model, which mimics the disease progression of human PSC. Mdr2^{-/-} mice spontaneously develop progressive biliary injury shortly after birth (32). It also has been reported that hepatic overexpression of H19 augmented BDL-induced liver fibrosis (33) and markedly suppressed the expression of SHP (6, 30). Our recent study demonstrates that H19 is mainly expressed in cholangiocytes, not hepatocytes (6). Previous studies showed that exosomes shuttled mRNA, lncRNA, microRNA, lipid and/or proteins between different cells or tissues and the molecular constituents of exosomes are promising biomarkers of various diseases (34, 35). In order to investigate whether cholangiocyte-derived exosomes are released into circulation, we isolated exosomes from the serum of 100-day old Mdr2^{-/-} mice and gender-/age-matched FVB WT mice. The isolated serum exosomes were confirmed by Western blot analysis for exosome markers, CD63, LAMP1 and LAMP2 (Fig. 3A). Our recent study reported that H19 expression was markedly induced in the liver of Mdr2^{-/-} mice, especially in female

Mdr2^{-/-} mice (6). We next examined whether H19 was present in serum exosomes by real-time RT-PCR. As shown in Fig. 3B, the H19 level was significantly increased in the serum exosomes from 100-day old *Mdr2*^{-/-} mice, especially from female *Mdr2*^{-/-} mice. Immunofluorescent staining further showed strong co-localization of CK-19 and CD63 in the liver of 100-day *Mdr2*^{-/-} mice, but not in age-matched WT mice (Fig. 3C). To further determine if serum exosomal H19 levels are correlated to the disease progression in *Mdr2*^{-/-} mice, we isolated serum exosomes from *Mdr2*^{-/-} mice at different ages. As shown in Fig. 3D, exosomal H19 levels gradually increased with the progression of cholestatic liver injury in *Mdr2*^{-/-} mice. At the age of 30 days, H19 was undetectable in the serum exosomes from both male and female *Mdr2*^{-/-} mice. At the age of 50, 75 or 100 days, serum exosomal H19 levels were much higher in female *Mdr2*^{-/-} mice compared to age-matched male *Mdr2*^{-/-} mice, which was also correlated to the severity of cholestatic liver injury (6). A previous study reported that hepatic H19 expression was increased in the CCl₄-induced liver fibrosis mouse model (30). Consistent with previous studies, both hematoxylin and eosin and Masson's trichrome staining indicated that 8-week of CCl₄ administration induced severe fibrotic liver injury in WT mice (Supplementary Fig. 5). Similarly, the hepatic H19 level was markedly upregulated in CCl₄-treated mice (Fig. 3E, left panel). In addition, serum exosomal H19 levels were also increased in CCl₄-treated mice compared to that in control groups, which is consistent with our findings in *Mdr2*^{-/-} fibrotic models (Fig. 3E, middle and right panel). We further confirmed that serum exosomes contained the cholangiocyte marker, CK-19, indicating that cholangiocytes were the source of serum exosomes (Figs. 3D, 3E and Supplementary Fig. 6). Similarly, immunofluorescence staining further showed strong co-localization of CK-19 and CD63 in the liver of CCl₄-administered mice, but not in control groups (Fig. 3F).

Effect of H19 overexpression on cholangiocytes and hepatocytes

In order to determine the direct effect of H19 on cholangiocytes and hepatocytes, we overexpressed H19 in MLE and MPH by transfection of a recombinant H19 expression plasmid. As shown in Figs. 4A and 4D, H19 was successfully overexpressed in MLE and MPH. Overexpression of H19 not only significantly upregulated the estrogen receptor (ER) α , SphK2, Bcl-2 and α -smooth muscle actin (α -SMA) in MLE cells (Fig. 4B–C and Supplementary Fig. 7), but also upregulated ER α , SphK2, Bcl-2 in MPH (Fig. 4E–F and Supplementary Fig. 8). Although overexpression of H19 had no effect on farnesoid X receptor (FXR) expression (both mRNA and protein), SHP mRNA and protein expression levels were significantly suppressed by H19 in MPH (Fig. 5A–C). Furthermore, overexpression of H19 significantly reduced the mRNA stability of SHP in MPH (Fig. 5D). In order to determine whether H19 had any effect on the transcription of SHP, we co-transfected 293 cells with PGL4.23-SHP-promoter and H19 overexpression plasmid. The SHP promoter activity was measured by luciferase assay. As shown in Fig. 5E (left panel), overexpression of H19 suppressed SHP promoter activity. Furthermore, the purified exosomes from human normal cholangiocyte H69 overexpressing H19 also inhibited SHP promoter activity (Fig. 5E, right panel). To further determine whether H19-mediated suppression of SHP transcription was inhibited by FXR activation, 293 cells were co-transfected with PGL4.23-SHP-promoter and pCMV-ICIS-hFXR plasmid together with transfection of H19 overexpression plasmid or treatment with exosomes isolated from H69

cells overexpressing H19 and then treated with FXR agonist, GW4064. As shown in Fig. 5F, both H19 overexpression (left panel) and exosomal H19 (right panel) inhibited SHP promoter activity under FXR activation. These results suggest that cholangiocyte-derived H19 suppresses SHP expression at both the transcriptional and post-transcriptional levels in hepatocytes.

Effect of transplant of serum exosomes from cholestatic Mdr2^{-/-} female mice on fibrotic liver injury *in vivo*

In order to further elucidate the role of exosomal H19 in promoting cholestatic liver injury *in vivo*, purified serum exosomes from 100 day-old female Mdr2^{-/-} mice (Mdr2^{-/-}-exosome) were used to treat 50 day-old Mdr2^{-/-} female mice *via* tail vein injection twice a week. The control group of mice were injected with the same amount of serum exosomes from WT mice (WT-exosome). After one week, mice were sacrificed. Quantitative image analysis showed that IRDye 800CW NHS ester (LI-COR, NE) labeled-serum exosomes were mainly delivered to the kidney and liver (Supplementary Fig. 9A). As shown in Fig. 6A, serum total bile acid (TBA) levels were increased in Mdr2^{-/-}-exosome-treated younger Mdr2^{-/-} mice, but alkaline phosphatase (ALP) (Fig. 6B), aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels were similar (Supplementary Fig. 9B–C). Hematoxylin and eosin (H&E) and Masson's Trichrome staining further indicated that Mdr2^{-/-}-exosome-treated younger Mdr2^{-/-} mice developed more severe cholestatic liver injury (Fig. 6C and Supplementary Fig. 9D), which was accompanied by upregulation of CK-19 and proliferating cell nuclear antigen (PCNA) expression (Fig. 6D and Supplementary Fig. 9E). Real-time RT-PCR analysis further showed that mRNA levels of c-Myc, α -SMA, PCNA, Ki67, ER α , S1PR2, cholesterol 7 α -hydroxylase (CYP7A1, rate-limiting enzyme for bile acid synthesis) and inflammatory mediators (TNF α , IL-1 β and IL-10) were significantly increased, but the mRNA levels of SHP, bile salt export pump (Bsep) and multidrug resistance-associated protein 2 (Mrp2) were markedly decreased in Mdr2^{-/-}-exosome-treated younger Mdr2^{-/-} mice (Fig. 6E and supplementary Fig. 10A). The mRNA level of organic solute transporter beta (Ostb) was decreased, but the mRNA level of Mrp3 was similar in Mdr2^{-/-}-exosome-treated younger Mdr2^{-/-} mice (Supplementary Fig. 10B–C). Interestingly, although the FXR protein level was not changed, SHP protein level was significantly decreased in Mdr2^{-/-}-exosome-treated younger Mdr2^{-/-} mice (Fig. 6F). In addition, the protein levels of c-Myc, S1PR2 and SphK2 were also significantly increased in Mdr2^{-/-}-exosome-treated younger Mdr2^{-/-} mice (Fig. 6F). The SHP levels in the liver of Mdr2^{-/-} mice treated with PBS or WT-exosomes remained the same (Supplementary Fig. 10D). These results suggest that cholangiocyte-derived exosomal H19 is a key player in promoting cholestatic liver injury.

Hepatic H19 is upregulated in human PSC patients

Given that the exosomes carrying H19 contribute to cholestatic liver injury, the H19 expression levels in serum exosomes could be a useful and critical marker of prognosis in cholestatic liver injury. To test this hypothesis, we first examined the expression of H19 in the liver of human PSC patients to determine its clinical relevance. As shown in Fig. 7A, H19 expression levels were markedly increased in the liver of human PSC patients. Consistent with our findings in Mdr2^{-/-} mice, CD63 expression was also upregulated in the

liver of human PSC patients (Fig. 7B). Our recent studies in *Mdr2*^{-/-} mice indicated that upregulation of hepatic H19 under cholestatic conditions is associated with activation of extracellular signal-regulated kinase 1/2 (ERK1/2), upregulation of c-Myc, S1PR2 and SphK2 and downregulation of FXR and SHP (6). Similarly, the phospho-ERK1/2 (p-ERK1/2), c-Myc, S1PR2 and SphK2 protein levels are significantly increased, but FXR and SHP protein levels are markedly down-regulated in human PSC livers (Fig. 7C).

To further examine the correlation of serum exosomal H19 levels with fibrotic liver injury, we isolated serum exosomes from human patients with compensated cirrhosis and age-/gender-matched normal controls. The isolation of exosomes was confirmed by real-time PCR for CD63 expression (Fig. 7F). Similar to the findings in *Mdr2*^{-/-} mice, the H19 levels were significantly increased in the serum exosomes from the cirrhotic patients (Fig. 7D). In addition, the CK-19 level was also increased, indicating cholangiocyte-released exosomes (Fig. 7E).

Discussion

Cholangiocytes are the primary target of cholestatic liver injury. Accumulation of bile acids in the liver activates the inflammatory response, which results in the destruction of intrahepatic bile ducts and eventually leads to cholangitis, fibrosis and potentially cirrhosis (2). It has been well-documented that in addition to bile acids, estrogen also contributes significantly in promoting cholangiocyte proliferation, a typical hallmark influencing disease progression of cholangiopathies (36, 37). Interestingly, our recent results showed that TCA/S1PR2- and estrogen-induced upregulation of H19 in cholangiocytes is correlated with the gender disparity of cholestatic liver injury in *Mdr2*^{-/-} mice (6). We also showed that aberrant expression of H19 contributes to dysregulation of hepatic bile acid metabolism *via* suppression of SHP expression in hepatocytes (6). However, it remains unclear how cholangiocyte-derived H19 regulates bile acid metabolism in hepatocytes. In the current study, we show that cholangiocyte-derived H19-carrying exosomes are responsible for TCA- and estrogen-mediated suppression of hepatic SHP expression and cholestatic liver injury.

Intercellular communication is critical for maintenance of physiological homeostasis for multicellular organisms. In addition to direct cell-to-cell contact, cells also communicate by sending and receiving regulatory signals. These signals can be from the environment or from other cells. As novel mediators of cell communication, exosomes have recently garnered much attention for their vital role not only in normal physiological homeostasis, but also in the pathogenesis of various human diseases, such as metabolic diseases, liver diseases and cancers (10, 38, 39). Exosomes can be released by many different types of cells, including dendritic cells, macrophages, lymphocytes, hepatocytes, cholangiocytes, mast cells and tumor cells and can transfer various signaling molecules, including protein, DNA, mRNA, microRNA, lncRNA and lipids, between neighboring and distant cells (13, 16, 40). Exosomes also have been identified in various bio-fluids, including serum, bile, urine, breast milk, lymph, saliva and malignant ascites, suggesting their potential impact on multiple organ systems (40, 41). Accumulating evidence indicates that liver-derived exosomes are closely associated with hepatic inflammation, stress response, apoptosis and fibrosis (38). Studies done by Masyuk, AI, *et al.* reported that biliary exosomes regulate microRNA

expression and intracellular signaling pathways and promote cholangiocyte proliferation through interaction with primary cilia (17). It has been reported that an increase in circulating exosomes is associated with disease states of obesity-related metabolic diseases including insulin resistance, diabetes, nonalcoholic fatty liver disease (NAFLD) and nonalcoholic steatohepatitis (NASH) (38). A recent study identified novel proteomic signatures in serum exosomes of cholangiocarcinoma, PSC and hepatocellular carcinoma patients (42). Several studies have reported that hepatocyte-derived exosomes are involved in drug-induced liver injury, alcoholic liver disease, lipid-induced hepatic injury and viral hepatitis C infection (16, 20, 43–45). However, the role of cholangiocyte-derived exosomes in cholangiopathies remains largely unknown.

In the present study, we report for the first time that the cholangiocyte-derived exosomes transfer lncRNA H19 into hepatocytes. Our recent studies identify that H19 is mainly expressed in cholangiocytes and aberrant expression of H19 is correlated to the suppression of hepatic SHP expression, activation of hepatic inflammation and severe cholestatic liver injury (6). H19 is an estrogen-regulated gene and is identified as a breast tumor oncogene (46). Aberrant expression of H19 was also observed in CCl₄-induced cirrhosis and BDL-induced fibrosis (30). We further showed that the expression level of H19 was not only increased in the liver, but also significantly increased in the serum exosomes in CCl₄-administered mice (Fig. 3). Similar to our previous findings that TCA and E2 induced H19 expression in MLE cells (6), TCA and E2 also increased H19 levels in large cholangiocyte- (Fig. 1E–F), but not in small cholangiocyte-derived exosomes (Supplementary Fig. 1). The *in vivo* transplant of serum-derived exosomes from old Mdr2^{-/-} mice with severe cholestatic liver injury into young Mdr2^{-/-} mice with benign liver injury significantly promoted fibrotic liver injury (Fig. 6), but had no effect on wild type mice (Data not shown). Serum exosomes often contain the markers of cell origin. As shown in Figs. 3D–E, serum exosomes from Mdr2^{-/-} mice and CCl₄-administered mice both contained significant amounts of CK-19, indicating a cholangiocyte origin. Similarly, serum exosomes from human cirrhotic patients not only contained higher H19, but also contained CK-19 (Fig. 7). Our data suggest that cholangiocyte-released H19-carrying exosomes also does not initiate cholestatic liver injury, but play a critical role in promoting cholestatic liver injury in Mdr2^{-/-} mice, CCl₄-treated mice and cirrhotic patients. As expected, H19 levels in serum exosomes of Mdr2^{-/-} mice were gradually increased during disease progression (Fig. 3D), suggesting that H19 in serum exosomes can be used as a diagnostic biomarker for assessing cholestatic injury.

Recently, it has been reported that hepatocyte-released exosomes promote hepatocyte proliferation *in vitro* and liver regeneration *in vivo* by mediating transfer of SphK2 into target cells and inducing upregulation of intracellular sphingosine-1-phosphate (S1P) (47). In the current study, we also found that overexpression of H19 induced SphK2 expression both in cholangiocytes and hepatocytes (Figs. 4B and 4E). Hepatic SphK2 expression levels are also increased in cholestatic Mdr2^{-/-} mice and human PSC patients (Figs. 6F and 7C). Furthermore, we show that TCA and E2 significantly increased S1P levels in cholangiocyte-derived exosomes (Supplementary Fig. 11). Although S1P itself did not induce H19 expression in hepatocytes, it enhanced exosome-induced H19 transfer (data not shown). It also has been reported that activation of S1P receptors is essential for cargo sorting into

multivesicular endosomes for exosomal release (48). However, the underlying mechanisms of exosome formation, targeting and function remain largely unknown.

We previously reported that E2 and TCA induced H19 expression *via* TCA/S1PR2- and estrogen/ER-mediated activation of the ERK1/2 signaling pathway in cholangiocytes (6). In the current study, we showed that E2 and TCA promoted exosomal release from cholangiocytes and enriched the content of H19 in exosomal cargo (Fig. 1). Similar to our findings in *Mdr2*^{-/-} mice (6), the hepatic ERK1/2 signaling pathway is highly activated in human PSC patients, which is accompanied by downregulation of FXR and SHP and upregulation of S1PR2 and SphK2 (Fig. 7C). Recent studies by Zhang Y, *et al.* reported that overexpression of Bcl-2 significantly induced hepatic H19 expression. Downregulation of H19 or overexpression of SHP rescued Bcl-2 overexpression-induced hepatic injury (30). In the current study, we further show that TCA-induced H19 expression in cholangiocytes plays a vital role in regulating hepatic SHP expression and cholestatic liver injury. The basal expression level of SHP is increased in H19^{-/-} mice (Fig. 2E). TCA-induced SHP expression is markedly suppressed by cholangiocyte-derived exosomal H19 (Fig. 2B).

In conclusion, together with our previous studies, we elucidated important mechanisms underlying cholestatic liver injury. As summarized in Fig. 8, under cholestatic conditions, TCA- and estrogen-induced activation of ERK1/2 signaling pathways *via* their cognate receptors upregulates H19 expression in cholangiocytes and exosomal release. H19-carrying exosomes can be delivered to hepatocytes and other target cells *via* circulation. In hepatocytes, exosomal-H19 suppresses SHP expression by inhibiting promoter activity and reducing mRNA stability. TCA-/estrogen-induced ERK1/2 activation in hepatocytes also can suppress SHP expression *via* activation of AMP-activated protein kinase (AMPK) (24). In addition to its regulatory role in bile acid and lipid metabolism, SHP has also been identified as an important regulator of innate immune response and an intrinsic negative regulator of the nucleotide-binding oligomerization domain-, leucine-rich repeat- and pyrin domain-containing 3 (NLRP3) inflammasome to prevent an excessive inflammatory response (49). Collectively, our findings highlight a novel mode of communication between cholangiocytes and hepatocytes and attribute a novel function for exosomes in the regulation of hepatic bile acid and lipid homeostasis. This study also sheds new light on the understanding of disease progression of cholangiopathies. In addition to direct interaction between cholangiocytes and hepatocytes and other soluble factors released by cholangiocytes, exosomes can transfer H19, lipids and other cytosolic components into hepatocytes and participate in the homeostasis of hepatic bile acids and lipids. These studies not only identified a novel serum biomarker for cholestatic liver diseases, but also opened the path for new strategies to treat cholangiopathies *via* manipulating the contents of cholangiocyte-derived exosomes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Grant Support: This work was supported by National Institutes of Health Grant R01 DK104893 (to HZ and PBH), R01DK-057543 (to PBH and HZ); VA Merit Award I01BX004033 (to HZ); I0CX001076 (to JB); National Natural

Science Foundation of China Grants 81070245 and 81270489 (to H. Z.); Wenzhou Medical University Research Fund (to HZ and ZH). Massey Cancer Center pilot grant (to HZ and PBH). Microscopy was performed at the VCU Microscopy Facility, supported in part by funding from NIH-NCI Cancer Center Grant P30 CA016059. NIH contract HSN276201200017C.

Abbreviations

PSC	primary sclerosing cholangitis
TCA	taurocholate
S1PR2	sphingosine 1-phosphate receptor 2
lncRNA	long non-coding RNA
SHP	small heterodimer partner
Mdr2^{-/-}	multidrug resistance 2 knockout
WT	wild type
CCl₄	carbon tetrachloride
MLE	mouse large cholangiocytes
MSE	mouse small cholangiocytes
E2	17 β -Estradiol
LAMP	lysosomal-associated membrane protein
Bcl-2	B-cell lymphoma 2
MPH	mouse primary hepatocytes
MPC	mouse primary cholangiocytes
CK-19	cytokeratin 19
SphK2	sphingosine kinase 2
ER	estrogen receptor
FXR	farnesoid X receptor
PCNA	proliferating cell nuclear antigen
ERK1/2	extracellular signal-regulated kinase 1/2
S1P	sphingosine-1-phosphate

References

1. Gidwaney NG, Pawa S, Das KM. Pathogenesis and clinical spectrum of primary sclerosing cholangitis. *World J Gastroenterol.* 2017; 23:2459–2469. [PubMed: 28465630]
2. Li Y, Tang R, Leung PSC, Gershwin ME, Ma X. Bile acids and intestinal microbiota in autoimmune cholestatic liver diseases. *Autoimmun Rev.* 2017; 16:885–896. [PubMed: 28698093]

3. Liu R, Li X, Qiang X, Luo L, Hylemon PB, Jiang Z, Zhang L, et al. Taurocholate Induces Cyclooxygenase-2 Expression via the Sphingosine 1-phosphate Receptor 2 in a Human Cholangiocarcinoma Cell Line. *J Biol Chem*. 2015; 290:30988–31002. [PubMed: 26518876]
4. Liu R, Zhao R, Zhou X, Liang X, Campbell DJ, Zhang X, Zhang L, et al. Conjugated bile acids promote cholangiocarcinoma cell invasive growth through activation of sphingosine 1-phosphate receptor 2. *Hepatology*. 2014; 60:908–918. [PubMed: 24700501]
5. Wang Y, Aoki H, Yang J, Peng K, Liu R, Li X, Qiang X, et al. The Role of Sphingosine 1-Phosphate Receptor 2 in Bile-Acid-Induced Cholangiocyte Proliferation and Cholestasis-Induced Liver Injury in Mice. *Hepatology*. 2017; 65:2005–2018. [PubMed: 28120434]
6. Li X, Liu R, Yang J, Sun L, Zhang L, Jiang Z, Puri P, et al. The role of long noncoding RNA H19 in gender disparity of cholestatic liver injury in multidrug resistance 2 gene knockout mice. *Hepatology*. 2017; 66:869–884. [PubMed: 28271527]
7. Gabory A, Ripoche MA, Le Digarcher A, Watrin F, Ziyat A, Forne T, Jammes H, et al. H19 acts as a trans regulator of the imprinted gene network controlling growth in mice. *Development*. 2009; 136:3413–3421. [PubMed: 19762426]
8. Hirsova P, Ibrahim SH, Verma VK, Morton LA, Shah VH, LaRusso NF, Gores GJ, et al. Extracellular vesicles in liver pathobiology: Small particles with big impact. *Hepatology*. 2016; 64:2219–2233. [PubMed: 27628960]
9. Martinez MC, Andriantsitohaina R. Extracellular Vesicles in Metabolic Syndrome. *Circ Res*. 2017; 120:1674–1686. [PubMed: 28495997]
10. Yokoi A, Yoshioka Y, Yamamoto Y, Ishikawa M, Ikeda SI, Kato T, Kiyono T, et al. Malignant extracellular vesicles carrying MMP1 mRNA facilitate peritoneal dissemination in ovarian cancer. *Nat Commun*. 2017; 8:14470. [PubMed: 28262727]
11. Yanez-Mo M, Siljander PR, Andreu Z, Zavec AB, Borrás FE, Buzas EI, Buzas K, et al. Biological properties of extracellular vesicles and their physiological functions. *J Extracell Vesicles*. 2015; 4:27066. [PubMed: 25979354]
12. Valadi H, Ekström K, Bossios A, Sjöstrand M, Lee JJ, Lotvall JO. Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. *Nat Cell Biol*. 2007; 9:654–659. [PubMed: 17486113]
13. Javeed N, Mukhopadhyay D. Exosomes and their role in the micro-/macro-environment: a comprehensive review. *J Biomed Res*. 2017; 31:386–394. [PubMed: 28290182]
14. Pan L, Liang W, Fu M, Huang ZH, Li X, Zhang W, Zhang P, et al. Exosomes-mediated transfer of long noncoding RNA ZFAS1 promotes gastric cancer progression. *J Cancer Res Clin Oncol*. 2017; 143:991–1004. [PubMed: 28285404]
15. Szabo G, Momen-Heravi F. Extracellular vesicles in liver disease and potential as biomarkers and therapeutic targets. *Nat Rev Gastroenterol Hepatol*. 2017; 14:455–466. [PubMed: 28634412]
16. Bala S, Petrasek J, Mundkur S, Catalano D, Levin I, Ward J, Alao H, et al. Circulating microRNAs in exosomes indicate hepatocyte injury and inflammation in alcoholic, drug-induced, and inflammatory liver diseases. *Hepatology*. 2012; 56:1946–1957. [PubMed: 22684891]
17. Masyuk AI, Huang BQ, Ward CJ, Gradilone SA, Banales JM, Masyuk TV, Radtke B, et al. Biliary exosomes influence cholangiocyte regulatory mechanisms and proliferation through interaction with primary cilia. *American Journal of Physiology-Gastrointestinal and Liver Physiology*. 2010; 299:G990–G999. [PubMed: 20634433]
18. Masyuk AI, Masyuk TV, LaRusso NF. Exosomes in the pathogenesis, diagnostics and therapeutics of liver diseases. *Journal of Hepatology*. 2013; 59:621–625. [PubMed: 23557871]
19. Worst TS, von Hardenberg J, Gross JC, Erben P, Schnolzer M, Hausser I, Bugert P, et al. Database-augmented Mass Spectrometry Analysis of Exosomes Identifies Claudin 3 as a Putative Prostate Cancer Biomarker. *Mol Cell Proteomics*. 2017; 16:998–1008. [PubMed: 28396511]
20. Hirsova P, Ibrahim SH, Krishnan A, Verma VK, Bronk SF, Werneburg NW, Charlton MR, et al. Lipid-Induced Signaling Causes Release of Inflammatory Extracellular Vesicles From Hepatocytes. *Gastroenterology*. 2016; 150:956–967. [PubMed: 26764184]
21. Kaffer CR, Grinberg A, Pfeifer K. Regulatory mechanisms at the mouse Igf2/H19 locus. *Mol Cell Biol*. 2001; 21:8189–8196. [PubMed: 11689707]

22. Kang DJ, Betrapally NS, Ghosh SA, Sartor RB, Hylemon PB, Gillevet PM, Sanyal AJ, et al. Gut microbiota drive the development of neuroinflammatory response in cirrhosis in mice. *Hepatology*. 2016; 64:1232–1248. [PubMed: 27339732]
23. Halilbasic E, Claudel T, Trauner M. Bile acid transporters and regulatory nuclear receptors in the liver and beyond. *J Hepatol*. 2013; 58:155–168. [PubMed: 22885388]
24. Li X, Liu R, Luo L, Yu L, Chen X, Sun L, Wang T, et al. Role of AMP-activated protein kinase alpha1 in 17alpha-ethinylestradiol-induced cholestasis in rats. *Arch Toxicol*. 2017; 91:481–494. [PubMed: 27090119]
25. Williamson C, Geenes V. Intrahepatic cholestasis of pregnancy. *Obstet Gynecol*. 2014; 124:120–133. [PubMed: 24901263]
26. Maas SL, Breakefield XO, Weaver AM. Extracellular Vesicles: Unique Intercellular Delivery Vehicles. *Trends Cell Biol*. 2017; 27:172–188. [PubMed: 27979573]
27. Lee YS, Chanda D, Sim J, Park YY, Choi HS. Structure and function of the atypical orphan nuclear receptor small heterodimer partner. *Int Rev Cytol*. 2007; 261:117–158. [PubMed: 17560281]
28. Studer E, Zhou X, Zhao R, Wang Y, Takabe K, Nagahashi M, Pandak WM, et al. Conjugated bile acids activate the sphingosine-1-phosphate receptor 2 in primary rodent hepatocytes. *Hepatology*. 2012; 55:267–276. [PubMed: 21932398]
29. Dent P, Fang Y, Gupta S, Studer E, Mitchell C, Spiegel S, Hylemon PB. Conjugated bile acids promote ERK1/2 and AKT activation via a pertussis toxin-sensitive mechanism in murine and human hepatocytes. *Hepatology*. 2005; 42:1291–1299. [PubMed: 16317705]
30. Zhang Y, Liu C, Barbier O, Smalling R, Tsuchiya H, Lee S, Delker D, et al. Bcl2 is a critical regulator of bile acid homeostasis by dictating Shp and lncRNA H19 function. *Sci Rep*. 2016; 6:20559. [PubMed: 26838806]
31. Nagahashi M, Takabe K, Liu R, Peng K, Wang X, Wang Y, Hait NC, et al. Conjugated bile acid-activated S1P receptor 2 is a key regulator of sphingosine kinase 2 and hepatic gene expression. *Hepatology*. 2015; 61:1216–1226. [PubMed: 25363242]
32. Popov Y, Patsenker E, Fickert P, Trauner M, Schuppan D. Mdr2 (Abcb4)^{-/-} mice spontaneously develop severe biliary fibrosis via massive dysregulation of pro- and antifibrogenic genes. *J Hepatol*. 2005; 43:1045–1054. [PubMed: 16223543]
33. Song Y, Liu C, Liu X, Trottier J, Beaudoin M, Zhang L, Pope C, et al. H19 promotes cholestatic liver fibrosis by preventing ZEB1-mediated inhibition of EpCAM. *Hepatology*. 2017; 66:1183–1196. [PubMed: 28407375]
34. Nedaenia R, Manian M, Jazayeri MH, Ranjbar M, Salehi R, Sharifi M, Mohaghegh F, et al. Circulating exosomes and exosomal microRNAs as biomarkers in gastrointestinal cancer. *Cancer Gene Ther*. 2017; 24:48–56. [PubMed: 27982021]
35. Balaj L, Lessard R, Dai L, Cho YJ, Pomeroy SL, Breakefield XO, Skog J. Tumour microvesicles contain retrotransposon elements and amplified oncogene sequences. *Nat Commun*. 2011; 2:180. [PubMed: 21285958]
36. Alvaro D, Mancino MG, Onori P, Franchitto A, Alpini G, Francis H, Glaser S, et al. Estrogens and the pathophysiology of the biliary tree. *World J Gastroenterol*. 2006; 12:3537–3545. [PubMed: 16773710]
37. Alvaro D, Gigliozzi A, Attili AF. Regulation and deregulation of cholangiocyte proliferation. *J Hepatol*. 2000; 33:333–340. [PubMed: 10952254]
38. Huang-Doran I, Zhang CY, Vidal-Puig A. Extracellular Vesicles: Novel Mediators of Cell Communication In Metabolic Disease. *Trends Endocrinol Metab*. 2017; 28:3–18. [PubMed: 27810172]
39. Blackwell RH, Foreman KE, Gupta GN. The Role of Cancer-Derived Exosomes in Tumorigenicity & Epithelial-to-Mesenchymal Transition. *Cancers (Basel)*. 2017; 9:105.
40. Kojima M, Gimenes-Junior JA, Langness S, Morishita K, Lavoie-Gagne O, Eliceiri B, Costantini TW, et al. Exosomes, not protein or lipids, in mesenteric lymph activate inflammation: Unlocking the mystery of post-shock multiple organ failure. *J Trauma Acute Care Surg*. 2017; 82:42–50. [PubMed: 27779585]

41. Keller S, Ridinger J, Rupp A-K, Janssen JWG, Altevogt P. Body fluid derived exosomes as a novel template for clinical diagnostics. *Journal of Translational Medicine*. 2011; 9:86. [PubMed: 21651777]
42. Arbelaiz A, Azkargorta M, Krawczyk M, Santos-Laso A, Lapitz A, Perugorria MJ, Erice O, et al. Serum extracellular vesicles contain protein biomarkers for primary sclerosing cholangitis and cholangiocarcinoma. *Hepatology*. 2017; 66:1125–1143. [PubMed: 28555885]
43. Cobb DA, Golden-Mason L, Rosen HR, Hahn YS. Hepatocyte-derived exosomes promote T follicular regulatory cell expansion during HCV infection. *Hepatology*. 2017; [Epub ahead of print]. doi: 10.1002/hep.29409
44. Holman NS, Mosedale M, Wolf KK, LeCluyse EL, Watkins PB. Subtoxic Alterations in Hepatocyte-Derived Exosomes: An Early Step in Drug-Induced Liver Injury? *Toxicol Sci*. 2016; 151:365–375. [PubMed: 26962055]
45. Momen-Heravi F, Bala S, Kodys K, Szabo G. Exosomes derived from alcohol-treated hepatocytes horizontally transfer liver specific miRNA-122 and sensitize monocytes to LPS. *Sci Rep*. 2015; 5:9991. [PubMed: 25973575]
46. Basak P, Chatterjee S, Weger S, Bruce MC, Murphy LC, Raouf A. Estrogen regulates luminal progenitor cell differentiation through H19 gene expression. *Endocr Relat Cancer*. 2015; 22:505–517. [PubMed: 25944846]
47. Nojima H, Freeman CM, Schuster RM, Japtok L, Kleuser B, Edwards MJ, Gulbins E, et al. Hepatocyte exosomes mediate liver repair and regeneration via sphingosine-1-phosphate. *J Hepatol*. 2016; 64:60–68. [PubMed: 26254847]
48. Kajimoto T, Okada T, Miya S, Zhang L, Nakamura S. Ongoing activation of sphingosine 1-phosphate receptors mediates maturation of exosomal multivesicular endosomes. *Nat Commun*. 2013; 4:2712. [PubMed: 24231649]
49. Yuk JM, Jin HS, Jo EK. Small Heterodimer Partner and Innate Immune Regulation. *Endocrinol Metab (Seoul)*. 2016; 31:17–24. [PubMed: 26754583]

Figure 1. Estrogen and TCA promote release of exosomes from cholangiocytes

MLE cells were plated with exosome-free MEM medium and then treated with vehicle control, E2 (100 nM), TCA (0.1 mM) or both for 48 h. (A) Representative TEM images of exosomes isolated from MLE medium. (B, C) The size distribution and number of isolated exosomes from MLE medium determined by DLS analysis. (D) Protein expression levels of CD63, LAMP1, LAMP2 and GRP78 were determined by Western blot analysis in isolated exosomes from MLE cell culture medium and total cell lysates. (E) Representative images of the DNA agarose gels of H19 and HPRT1. (F) The relative H19 mRNA levels in isolated

exosomes were determined by real-time RT-PCR and normalized using HPRT1 as an internal control. Statistical significance: * $P<0.05$, ** $P<0.01$, compared with vehicle control group, One-way ANOVA with Tukey's post-hoc tests (n=3).

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 2. Effect of cholangiocyte-released H19-carrying exosomes on hepatocytes (A, left panel) Relative H19 mRNA expression levels in MPH treated with E2 (100 nM) or TCA (0.1 mM) or both or exosomes (exos) isolated from MLE treated with E2 (100 nM) or TCA (0.1 mM) or both for 48 h. (A, right panel) Relative H19 mRNA expression levels in MPH treated with exos isolated from MLE cells infected with adenovirus of control shRNA (C-shRNA) or H19 shRNA and treated with E2 or TCA or both. (B) Relative SHP mRNA expression levels in MPH pre-treated with exos isolated from MLE cells treated with E2+TCA for 1 h then treated with vehicle control or E2+TCA for 3 h. (C) Relative H19

mRNA levels in WT mouse primary cholangiocytes (MPC) treated with E2 (100 nM) or TCA (0.1 mM) or both for 48 h. (D) Relative H19 mRNA levels in WT MPH treated with exos isolated from MPC treated with E2, TCA or both for 24 h. (E) Relative SHP mRNA and protein levels in MPH from WT and H19^{-/-} mice. (F) Relative SHP and Bcl-2 mRNA levels in WT MPH treated with exos isolated from WT and H19^{-/-} MPC treated with E2+TCA. All mRNA levels were determined by real-time RT-PCR and normalized using HPRT1. Statistical significance: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, compared with control group; ### $P < 0.001$, compared with E2+TCA group, One-way ANOVA with Tukey's post-hoc tests (n=3).

Figure 3. Characterization of H19-carrying exosomes from the serum of mice with fibrotic liver injuries

Serum exosomes were isolated from Mdr2^{-/-} mice both male (M) and female (F) at different ages as well as age-/sex-matched FVB WT mice as described in “Methods.” (A)

Representative immunoblot images of CD63, LAMP1, LAMP2 and GRP78 are shown. Total cell lysate of MLE was used as the negative control (NC). (B) Representative image of DNA agarose gel of H19 and relative levels of H19 in serum exosomes from 100-day-old WT and Mdr2^{-/-} mice. Statistical significance: \$\$\$*P*<0.001, compared with WT (M) mice; ****P*<0.001, compared with WT (F) mice; ##*P*<0.01, compared with Mdr2^{-/-} (M) mice. (C)

Relative H19 mRNA levels in serum-exosomes

Representative confocal images of immunofluorescent staining of hepatic CD63 and CK-19 of 100-day old WT and *Mdr2*^{-/-} mice. Scale bar = 50 μ m. (D) Representative DNA agarose gels of H19, CD63 and CK-19 and relative H19 mRNA levels in serum-derived exosomes from *Mdr2*^{-/-} mice of different ages (30–100 days). Statistical significance: * P <0.05, *** P <0.001, compared with male *Mdr2*^{-/-} mice, 2-tailed Student's t test (n=4). (E, left panel) The relative mRNA levels of hepatic H19 of CCl₄-treated mice. (E, middle and right panel) Relative H19 mRNA levels and representative DNA agarose gels of H19, CD63 and CK-19 in serum-derived exosomes from CCl₄- and vehicle-administered WT mice. (F) Representative confocal microscope images of immunofluorescent staining of hepatic CD63 and CK-19 of CCl₄- and vehicle-administered WT mice. Scale bar = 50 μ m. Statistical significance: ** P <0.01, *** P <0.001, compared with control groups, 2-tailed Student's t test (n=6).

Figure 4. Effect of H19 overexpression on mouse cholangiocytes and hepatocytes
 (A–C) MLE cells were transfected with control plasmid (CP) or H19 overexpression plasmid for 48 h. The mRNA levels of H19 were determined by real-time RT-PCR and normalized using HPRT1 as an internal control. (A) Relative mRNA levels of H19 and a representative DNA agarose gel image of real-time PCR products of H19 and HPRT1. (B) Relative protein expression levels of S1PR2 and SphK2 normalized using β-Actin as a loading control. (C) The mRNA levels of ERα, S1PR2 and SphK2 in MLE. (D–F) MPH cells were transfected with CP or H19 overexpression plasmid for 24 h. (D) Relative mRNA

levels of H19 and representative DNA agarose gel images of real-time PCR products of H19 and HPRT1. (E) Representative immunoblot images and relative protein expression levels of S1PR2 and SphK2 normalized using β -Actin as a loading control. (F) The mRNA levels of ER α , S1PR2 and SphK2 in MPH. Statistical significance: * $P<0.05$, ** $P<0.01$, *** $P<0.001$, compared with control group, One-way ANOVA with Tukey's *post-hoc* tests (n=3).

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 5. Effect of H19 on SHP expression in hepatocytes

(A–C) MPH cells were transfected with CP or H19 overexpression plasmid for 24 h. (A–B) The mRNA levels of FXR and SHP were determined by real-time RT-PCR and normalized using HPRT1 as an internal control. (C) Representative immunoblot images and relative protein expression levels of FXR and SHP normalized using β -Actin as a loading control. (D) MPH cells were transfected with CP or H19 overexpression plasmid for 24 h before addition of actinomycin D (5 μ g/ml) (time 0). Total cellular RNA was extracted at 0, 0.25, 0.5, 1 and 2 h after treatment with actinomycin D. Relative mRNA levels of SHP at different

time points were determined by real-time RT-PCR and compared to time 0. (E–F) 293 cells were transfected with pGL4.23-SHP-promotor, without (E) or with (F) co-transfection of pCMV-ICIS-hFXR plasmid for 24 h. Cells were then (E–F, left panel) transfected with H19 overexpression/control plasmid or (E–F, right panel) treated with exosomes (exos) isolated from H69 cells transfected with H19 overexpression/control plasmid for another 24 h. (F) Cells were then treated with 5 μ M GW4064 for another 8 h. Relative luciferase activity of SHP promotor was measured and normalized with protein content. Statistical significance: * P <0.05, ** P <0.01, *** P <0.001, compared with control groups, ## P <0.01, ### P <0.001, compared with control + GW4064 group, 2-tailed Student's t test (5D–E), One-way ANOVA with Tukey's *post-hoc* tests (5A–C, 5F) (n =3).

Figure 6. Effect of transplant of serum exosomes from cholestatic *Mdr2*^{-/-} female mice on fibrotic liver injury *in vivo*

Serum exosomes were isolated from 100-day-old WT (control group) or *Mdr2*^{-/-} mice (exosomes group) and used to treat 50-day old *Mdr2*^{-/-} mice twice a week *via* tail vein. Mice were sacrificed after one week. (A) TBA in the serum; (B) ALP level in serum; (C) Representative images of H&E and Masson's Trichrome staining; (D) Representative images of CK-19 and PCNA staining; (E) The relative mRNA levels of c-Myc, α-SMA, PCNA, Ki67, SHP, CYP7A1, Bsep, Mrp2, ERα and S1PR2 were determined by real-time RT-PCR and normalized using HPRT1 as an internal control; (F) Representative immunoblot

images and relative protein levels of c-Myc, FXR, SHP, S1PR2, SphK2 and normalized using β -Actin as a loading control. Scale bar= 100 μ m. Statistical significance: * P <0.05, ** P <0.01, *** P <0.01, compared with control group, 2-tailed Student's t test (n=4). Abbreviation: H&E, hematoxylin and eosin.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 7. The hepatic expression levels of H19 and related genes in human PSC patients and serum exosomes from cirrhotic patients

Total RNA was extracted from frozen liver tissues of normal control and PSC patients. Relative mRNA levels of H19 (A) and CD63 (B) were determined by real-time RT-PCR and normalized using HPRT1 as an internal control. (C) Representative immunoblot images and relative protein expression levels of c-Myc, p-ERK1/2, FXR, SHP, S1PR2 and SphK2 were normalized using t-ERK1/2 or β -Actin as a loading control. (D–F) Serum exosomes were isolated from normal controls and cirrhosis patients as described in “Methods.” Relative mRNA levels of H19, CK-19, and CD63 were determined by real-time RT-PCR and normalized using 18srRNA as an internal control. Representative DNA agarose gels of H19,

CK-19, CD63 and 18srRNA are shown. Statistical significance: * $P<0.05$, ** $P<0.01$, *** $P<0.001$, compared with normal control, 2-tailed Student's t test (PSC liver samples, $n=16$; cirrhotic patient's serum, $n=9$).

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 8. Schematic diagram of proposed mechanisms by which cholangiocyte-derived H19-carrying exosomes promote cholestatic liver injury

H19 is mainly expressed in cholangiocytes. In cholangiocytes, TCA/S1PR2- and E2-induced activation of ERK1/2 upregulates H19 expression level and promotes H19-carrying exosome release. Cholangiocyte-derived exosomes deliver H19 to hepatocytes directly or *via* circulation. Uptake of H19-carrying exosomes from cholangiocytes suppresses SHP expression by inhibiting promoter activity and destabilizing SHP mRNA in hepatocytes. In addition, TCA- and E2-induced activation of ERK1/2 in hepatocytes also activates AMPK, which further inhibits SHP transcription. Down-regulation of SHP expression results in increase of bile acid synthesis and eventually causes cholestatic liver injury.