The apelin-apelin receptor axis triggers cholangiocyte proliferation and liver fibrosis during mouse models of cholestasis

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Short title: Apelin modulation of biliary damage.

Abbreviations: α -SMA: α -smooth muscle actin; ACTA2: actin alpha 2; ALT: alanine aminotransferase; AST: aspartate aminotransferase; ALK PHOS: alkaline phosphatase; BDL: bile duct ligation; CK-19: cytokeratin-19; CCl₄: carbon tetrachloride; CD68: cluster of differentiation 68; CDKN1A: cyclin dependent kinase inhibitor 1a; CDKN2A: cyclin dependent kinase inhibitor 2a; Ccl2: C-C motif chemokine ligand 2; COL1A1: collagen type I alpha 1; DAPI: 4',6-diamidino-2-phenylindole; DPI: diphenyleneiodonium chloride; ELISA: enzyme linked immunosorbent assay; ERK: extracellular signal-regulated kinase; Fn1: fibronectin1; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; GFAP: glial fibrillary acidic protein; HHStCs: human hepatic stellate cells; HIBEpCs: human intrahepatic biliary

epithelial cells; HSCs: hepatic stellate cells; hPSCL: human PSC cell lines; IBDM: intrahepatic bile duct mass; IL: interleukin; LCM: laser capture microdissection; Mdr2^{-/-}: multidrug resistance gene 2 knockout; NAC: N-Acetyl-L-cysteine; NADPH: nicotinamide adenine dinucleotide phosphate; PCNA: proliferating cell nuclear antigen; PBC: primary biliary cirrhosis; PDGFRB: platelet-derived growth factor receptor beta; PECAM-1: platelet endothelial cell adhesion molecule 1; PSC: primary sclerosing cholangitis; ROS: reactive oxygen species; SA- β -GAL: senescence-associated beta-galactosidase; TGF- β 1: transforming growth factor- β 1; TNF α : tumor necrosis factor α ; Thy-1: Thy-1 cell surface antigen; VEGFA: vascular endothelial growth factor A; VWF: von Willebrand factor; WT: wild-type.

Abstract

Background & Aims: Apelin is the endogenous ligand of its G-protein coupled receptor, APJ. Apelin serum levels are increased in human liver diseases. We evaluated whether apelin-APJ regulates ductular reaction and liver fibrosis during cholestasis.

Approach & Results: We measured the expression of apelin and APJ, and serum apelin levels in human primary sclerosing cholangitis (PSC) samples. Following bile duct ligation (BDL) or sham surgery, male wild-type mice were treated with ML221 (APJ antagonist) or saline for 1 wk. WT and apelin^{-/-} mice underwent BDL or sham for 1 week. Mdr2^{-/-} mice were treated with ML221 for 1 wk. Apelin levels were measured in serum and cholangiocyte supernatants, and cholangiocyte proliferation/senescence and liver inflammation, fibrosis and angiogenesis were measured in liver tissues. The regulatory mechanisms of apelin-APJ in: (i) biliary damage and liver fibrosis were examined in human biliary cells (HIBEpCs) treated with apelin; and (ii) HSC activation in apelin-treated human hepatic stellate cell lines (HHStCs). Apelin serum levels and biliary expression of apelin and APJ increased in PSC samples. Apelin levels were higher in serum and cholangiocyte supernatants from BDL and Mdr2^{-/-} mice. ML221 treatment or apelin^{-/-} reduced BDL- and Mdr2^{-/-}-induced cholangiocyte proliferation/senescence, liver inflammation, fibrosis and angiogenesis. *In vitro*, apelin induced HIBEpC proliferation, Nox4 expression, ROS generation and ERK phosphorylation. Pretreatment of HIBEpCs with ML221, DPI (Nox4 inhibitor), NAC (ROS inhibitor) or PD98059 (ERK inhibitor) reduced apelin-induced cholangiocyte proliferation. Activation of HHStCs was induced by apelin, but reduced by NAC.

Conclusions: Apelin-APJ axis induces cholangiocyte proliferation via Nox4/ROS/ERK dependent signaling and induces HSC activation via intracellular ROS. Modulation of the apelin-APJ axis may be important for managing cholangiopathies.

Keywords: Angiogenesis; biliary damage; cellular senescence; ductular reaction; reactive oxygen species.

Apelin (APLN) is the endogenous ligand of its G-protein coupled receptor, APJ (1). The *APLN* gene encodes a secreted 77-amino acid precursor called preproapelin, which is cleaved into a family of apelin fragments, including apelin 36, 17, 13 and 12. The most potent form is Pyr-apelin-13, which is detected in human plasma (2). Apelin and APJ are widely expressed in the central nervous system as well as peripheral organs including heart, brain, kidney, liver, and adipose tissue (3).

The apelin-APJ axis plays a key role in organ fibrosis, although the effect remains controversial. The apelin-APJ attenuates renal and myocardial fibrosis, while it has been shown to promote liver fibrosis (4, 5). Studies have shown that: (i) serum apelin levels are elevated in patients with chronic liver diseases; (ii) there is a significant correlation between apelin serum levels and Child-Turcotte-Pugh's and MELD score; and (iii) the expression of APJ is increased in the liver of cirrhotic patients (6). Furthermore, (i) apelin serum levels and apelin/APJ hepatic expression are increased in cirrhotic rats (7); and (ii) APJ blockade prevents the progression of liver fibrosis in CCl₄-treated rats (8).

In cholestatic liver diseases, such as primary biliary cholangitis (PBC) and primary sclerosing cholangitis (PSC), cholangiocytes (target of these diseases) are the key link between biliary injury and subepithelial fibrosis (9). In many cell types such as vascular smooth muscle cells, apelin activation of APJ regulates proliferation via extracellular signal-regulated kinase 1/2 (ERK1/2)-dependent signaling mechanisms (10). We have previously demonstrated that the ERK1/2 pathway plays a critical role in the regulation of biliary proliferation during cholestasis (11). Inhibition of apelin signaling with the

APJ antagonist, ML221, inhibits cholangiocarcinoma growth in a xenograft mouse model (12).

During the progression of liver fibrosis, quiescent hepatic stellate cells (HSCs) are activated to induce a transformation into a myofibroblast-like phenotype, leading to cell proliferation, expression of pro-fibrogenic mediators and production of extracellular matrix (ECM) components (13). Apelin is overexpressed in HSCs of cirrhotic rats and caused increased synthesis of collagen-I and platelet-derived growth factor receptor β (PDGFR β) in HSCs *in vitro* (14). Thus, we hypothesized that apelin-APJ triggers biliary damage via Nox4/ROS/ERK signaling pathway and apelin-APJ induces HSC activation through changes in reactive oxygen species (ROS) levels.

Materials and methods

Materials

Reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise indicated. The antibodies for cytokeratin-19 (CK-19) (ab52625), desmin (ab185033), CD68 (cluster of differentiation 68, ab955), Thy1 (Thy-1 cell surface antigen; ab181469) and GFAP (glial fibrillary acidic protein; ab7260) were purchased from Abcam (Cambridge, MA); F4/80 (70076s), p-ERK (4370) and ERK (4695) were purchased from Cell Signaling Technology (Beverly, MA); α -SMA (α -smooth muscle actin, c6198) were purchased from Sigma-Aldrich; apelin (BS-2425R) and apelin receptor (702069) were purchased from Thermo Fisher Scientific (Mountain View,

CA); CD31 (AF3628) were purchased from R&D Systems, Inc. (Minneapolis, MN). All selected primers were purchased from Qiagen (Germantown, MD) and specific information about the primers are listed in Supplementary Table 1. The iScript cDNA Synthesis Kit (170-8891) and iTaq Universal SYBR Green Supermix (172-5124) were purchased from Bio-Rad (Hercules, CA).

Animal models

All animal experiments were performed in accordance to protocols approved by the Baylor Scott & White Healthcare Institutional Animal Care and Use Committee and Texas A&M University Animal Care and Use Committee. C57BL/6 wild-type (WT) mice (control for apelin^{-/-} mice) were purchased from Charles River Laboratories (Wilmington, MA). Male Mdr2^{-/-} mice were originally purchased from Jackson Laboratories (Bar Harbor, ME); the colony is established in our facility. FVB/NJ mice (WT, control for Mdr2^{-/-} mice) were purchased from Jackson Laboratories. Mice were maintained in a temperature-controlled environment (20-22°C) with 12:12-hr light/dark cycles and fed with standard mouse chow along with free access to drinking water *ad libitum*. Male WT mice underwent bile duct ligation (BDL) or sham surgery and were treated with the apelin inhibitor, ML221 (15) (Tocris Bio-Techne, Minneapolis, MN, 150 µg/kg, 3×weekly via tail vein injections). The mice received the first ML221 injection at the time of the BDL surgery and were then treated for 1 wk. The apelin^{-/-} breeding pair was a gift from Dr. Hyung Chun (Yale University, New Haven, CT), which show normal physiological development (16). Male apelin^{-/-} mice (12 wk age)

underwent BDL or sham for 1 wk. Male $Mdr2^{-/-}$ mice (12 wk age) were treated with ML221 (150 $\mu\text{g/kg}$, 3 $\times$ weekly via tail vein injections) ($Mdr2^{-/-}$ + ML221) for 1 wk (12). For 12 days BDL (12 D BDL), WT mice underwent either BDL or sham, injections (ML221 150 $\mu\text{g/kg}$ via tail vein injections) were performed on day 7 and 9 post operatively (sacrifice at day 12). Animal groups are summarized in Supplementary Table 2. Following surgery and selected treatments, we collected serum, total liver samples, cholangiocytes, and cholangiocyte supernatant (after 6 hr incubation at 37°C) from the selected groups of animals. Other techniques are described in detail in Supplemental Information.

Human samples

The liver tissue samples (OCT-embedded blocks, late stage PSC patients $n=7$) and serum (healthy human $n=8$, late stage PSC patients $n=26$) were obtained under the Institutional Review Board approved protocol at Indiana University Purdue University Indianapolis. Healthy human liver OCT-embedded blocks ($n=4$) were purchased from Sekisui Xeno Tech (Kansas City, KS). Patient characteristics were listed in Supplementary Table 3.

Statistical analysis

All data are expressed as the mean $\pm$ SEM. Differences between groups were analyzed by unpaired Student's t test when two groups were analyzed and one-way

ANOVA when more than two groups were analyzed, followed by an appropriate *post hoc* test. The level of significance was set at $P < 0.05$.

Results

APJ mediated apelin expression is increased in cholangiocytes during cholestasis

To address the relevance of the apelin-APJ axis to human cholestatic liver diseases, we measured the immunoreactivity of apelin and APJ in liver sections from healthy controls and patients with late stage PSC. Liver sections of PSC patients showed increased biliary immunoreactivity of apelin and APJ compared with controls (Figure 1A-B). The mRNA expression of *APLN* (gene for apelin) and *APLNR* (gene for APJ) was increased in a human PSC cholangiocyte cell line (hPSCL, from an unidentified 46-year old male patient with stage 4 PSC without cholangiocarcinoma; a gift of Dr. Nicholas LaRusso, Mayo Clinic, Rochester, MN) compared with HIBEpiC (control for hPSCL) (17) (Figure 1C). Apelin serum levels were higher in PSC patients compared with healthy controls (Figure 1D). The levels of apelin in serum and cholangiocyte supernatant were increased in BDL mice compared with those of WT mice (Figure 1E). The expression of *Apln* and *Aplnr* was increased in cholangiocytes from BDL and *Mdr2*^{-/-} mice compared with control mice (Supplementary Figure 1A). By immunofluorescence, there was enhanced immunoreactivity for apelin and APJ (co-stained with CK-19) in cholangiocytes from BDL mice compared with WT mice (Supplementary Figure 1B-C). Apelin serum levels were increased in *Mdr2*^{-/-} mice compared with those of WT mice (Figure 1F).

The mRNA expression of *Apln* and *Aplnr* was decreased in cholangiocytes from BDL and *Mdr2*^{-/-} mice treated with ML221 compared with that of controls (Supplementary Figure 1A, 1D). The levels of apelin in serum and cholangiocyte supernatant (Figure 1E) and the expression of apelin and APJ (Supplementary Figure 1B-C) were decreased in BDL+ML221 compared with BDL mice. Apelin serum levels were decreased in *Mdr2*^{-/-} + ML221 compared with *Mdr2*^{-/-} mice (Figure 1F).

APJ blockade attenuates liver injury

BDL-induced liver injury was demonstrated by increased serum levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALK PHOS) and total bilirubin (Supplementary Table 4). However, AST, ALT, ALK PHOS and total bilirubin serum levels decreased in BDL mice treated with ML221 compared with BDL mice (Supplementary Table 4). By H&E staining, we observed moderate ductular reaction with bile stasis, stage 2 fibrosis with focal early bridging fibrosis, and rare hepatocyte necrosis in BDL mice, which was ameliorated in BDL+ML221 liver samples (Supplementary Figure 1E).

Apelin induces cholangiocyte proliferation during cholestasis via APJ

To determine if apelin regulates cholangiocyte proliferation, we performed BDL in apelin knockout (*apelin*^{-/-}) mice. The levels of apelin in serum and cholangiocyte supernatant were lower in *apelin*^{-/-} BDL compared with BDL mice (Supplementary Figure 1F). There was increased intrahepatic bile duct mass (IBDM) in BDL mice

compared with WT mice, values that were reduced in apelin^{-/-} BDL mice and BDL mice treated with ML221 (Figure 2A, C). Further, the mRNA expression of *Pcna* and *Ki67* was reduced in cholangiocytes from apelin^{-/-} BDL mice, as well as in BDL mice treated with ML221 compared with BDL mice (Figure 2B, D). There was increased IBDM in Mdr2^{-/-} mice (compared with WT mice), which was reduced in Mdr2^{-/-} mice treated with ML221 (Figure 2E). Furthermore, the mRNA expression of *Pcna* and *Ki67* decreased in cholangiocytes from Mdr2^{-/-} mice treated with ML221 compared with cholangiocytes from Mdr2^{-/-} mice (Figure 2F).

Apelin induces cholangiocyte profibrogenesis via APJ during cholestasis

The expression of the fibrosis markers *Colla1*, *Fnl* and *Tgfb1* was increased in cholangiocytes from BDL compared with WT mice but was decreased in BDL+ML221 mice and apelin^{-/-} BDL mice compared with BDL mice (Supplementary Figure 2A, B). To determine the direct effects of apelin on cholangiocyte profibrogenesis, we treated HIBEpiCs with apelin and measured the mRNA expression of fibrosis markers. The expression of fibrosis markers increased in apelin treated HIBEpiCs, which was reversed by pretreatment with ML221 compared with control values (Supplementary Figure 2C). To evaluate if TGF-β1 is involved in apelin induced cholangiocyte pro-fibrogenesis, HIBEpiCs were treated with apelin in the presence or absence of a TGF-β1 neutralizing antibody (Apelin-13+TGF-β1 Ab). The expression of the fibrosis markers *COL1A1*, *FNI* and *TGFB1* decreased in Apelin-13+TGF-β1 Ab treated HIBEpiCs compared with untreated HIBEpiCs (Supplementary Figure 2D).

Apelin knockout or APJ blockage attenuates biliary senescence in cholestatic liver

Biliary senescence is a key hallmark of cholangiopathies including PSC, which contributes to enhanced liver fibrosis (18). By senescence-associated β -galactosidase (SA- β -GAL) staining in liver sections, there was enhanced biliary senescence in liver samples from PSC patients compared with healthy controls (Figure 3A). Biliary senescence was increased in BDL liver sections (compared with WT mice), which was significantly decreased in BDL mice treated with ML221 as well as apelin^{-/-} BDL mice (Figure 3B). There was enhanced mRNA expression of the senescence markers *Cdkn2a*, *Cdkn1a* and *Ccl2* in cholangiocytes from BDL mice compared with WT mice, which was decreased in BDL+ML221 mice and apelin^{-/-} BDL mice (Supplementary Figure 3A). Furthermore, the expression of *Cdkn2a*, *Cdkn1a* and *Ccl2* was increased in cholangiocytes from Mdr2^{-/-} compared with WT mice, which was decreased in Mdr2^{-/-}+ML221 compared with Mdr2^{-/-} mice (Supplementary Figure 3B).

Apelin knockout or APJ blockade attenuates inflammation and angiogenesis in cholestatic liver

There was increased F4/80-positive areas in liver sections from BDL compared with WT mice (Figure 3C); F4/80-positive areas were reduced in liver sections from BDL+ML221 mice and apelin^{-/-} BDL mice compared with BDL mice (Figure 3C). Consistent with the histological findings, the upregulation of *Il1b*, *Il6*, *Il33* and *Tnfa* in BDL liver was reduced by apelin knockout or ML221 treatment (Supplementary Figure

3C). The expression of CD31 (a marker of endothelial cells) (19), was increased in liver samples from BDL compared with WT mice, but was decreased in BDL+ML221 mice and *apelin*^{-/-} BDL mouse liver compared with BDL mice (Figure 3D). Similarly, the mRNA expression of the angiogenesis markers (*Pecam1*, *Vegfa* and *Vwf*) was increased in BDL mice compared with WT mice, but decreased in BDL+ML221 mice and *apelin*^{-/-} BDL mouse liver compared with BDL mice (Supplementary Figure 3D).

Apelin induces liver fibrosis via APJ during cholestatic injury

Collagen deposition was increased in BDL mice compared with WT mice, but was significantly decreased in *apelin*^{-/-} BDL mice (Figure 4A, B). The mRNA expression of the fibrosis markers, *Acta2*, *Fn1*, *Tgfb1* and *Colla1*, was increased in total liver samples from BDL mice compared with WT mice, which was significantly reduced in *apelin*^{-/-} BDL mice compared with BDL WT mice (Supplementary Figure 4A). BDL-induced liver fibrosis was decreased in BDL mice treated with ML221 (Figure 4C, D). The mRNA expression of fibrosis markers was decreased in BDL+ML221 mouse liver compared with BDL mice (Supplementary Figure 4B). In addition, collagen deposition was increased in *Mdr2*^{-/-} compared with WT mice, but significantly decreased in *Mdr2*^{-/-} +ML221 compared with *Mdr2*^{-/-} mice (Figure 4E, F) Similarly, the mRNA expression of fibrosis markers was increased in *Mdr2*^{-/-} mouse liver compared with WT mice, which was significantly decreased in *Mdr2*^{-/-} +ML221 mice compared with *Mdr2*^{-/-} mice (Supplementary Figure 4C).

Apelin-APJ induces cholangiocyte proliferation via Nox4/ROS/ERK signaling pathway during cholestasis

Apelin regulates Nox4 expression levels and stimulates the formation of reactive oxygen species (ROS) that triggers vascular smooth muscle cell proliferation via ERK (20). We hypothesized that apelin-APJ induces cholangiocyte proliferation via Nox4/ROS/ERK signaling pathway in cholestasis. We demonstrated that the mRNA expression of *NOX4* was increased in hPSCL compared with HIBEpiCs (Figure 5A). Similarly, the mRNA expression of *Nox4* increased in cholangiocytes from BDL mice compared with WT mice, expression that was reduced in cholangiocytes from BDL+ML221 mice compared with BDL mice (Figure 5B). *In vitro*, apelin stimulation increased the mRNA expression of *NOX4* in HIBEpiCs, whereas ML221 pretreatment reversed apelin-induced *NOX4* expression (Figure 5C).

The levels of ROS in cholangiocyte cell lysate and cholangiocyte supernatant from BDL mice were increased compared with WT mice, but decreased in BDL+ML221 mice and apelin^{-/-} BDL mice compared with BDL mice (Figure 5D). ROS levels were higher in apelin-treated HIBEpiCs (compared with control HIBEpiCs), but decreased in HIBEpiCs pretreated with ML221, DPI, or NAC (Figure 5E-F).

There was increased ERK phosphorylation in cholangiocytes from BDL mice compared with WT mice, which was decreased in cholangiocytes from BDL+ML221 compared with BDL mice (Figure 6A). By immunofluorescence, we confirmed the increased activation of ERK with p-ERK nuclear localization in BDL mouse cholangiocytes, that decreased in BDL+ML221 mouse cholangiocytes compared with

BDL mouse cholangiocytes (Figure 6B). Furthermore, the phosphorylation of ERK was decreased in cholangiocytes from apelin^{-/-} BDL compared with BDL mice (Figure 6C). *In vitro*, the phosphorylation of ERK was increased in HIBEpiCs after apelin treatment, whereas pretreatment with ML221, DPI, NAC, or PD98059 decreased apelin-induced ERK phosphorylation (Figure 6D). Apelin treatment promoted HIBEpiC proliferation, but pretreatment of HIBEpiCs with ML221, DPI, NAC, or PD98059 attenuated apelin-induced biliary proliferation (Figure 6E). Similarly, the expression of *PCNA* and *KI67* was increased in apelin-treated HIBEpiCs (compared with untreated HIBEpiCs), which was reversed by pretreatment of ML221, DPI, NAC or PD98059 (Figure 6F).

Apelin-APJ induces HSC proliferation and activation via ROS during cholestasis

To unravel the mechanisms by which the apelin/APJ axis regulate liver fibrosis, we measured the expression of apelin and APJ in HSCs and portal fibroblasts in liver sections from healthy controls and patients with late stage PSC. HSC expression of apelin and APJ was increased in PSC liver compared with healthy controls (Supplementary Figure 5). There was no significant difference in the expression of apelin and APJ in portal fibroblasts from normal control liver and PSC liver (Supplementary Figure 6). Thus, we further evaluated the role of the apelin-APJ axis in HSC activation. By immunofluorescence, there was enhanced immunoreactivity for desmin and α -SMA in liver sections from BDL mice compared with WT mice (Figure 7A). However, the activation of HSC was decreased in BDL+ML221 mice and apelin^{-/-}

BDL mice compared with BDL mice (Figure 7A). Consistently, the mRNA expression of HSC proliferation (*PCNA* and *KI67*) and activation (*ACTA2* and *COL1A1*) markers was decreased in LCM-isolated HSCs from BDL+ML221 mice and *apelin*^{-/-} BDL mice compared with BDL mice (Supplementary Figure 7A, B). In addition, there was enhanced immunoreactivity for desmin and α -SMA in liver sections from *Mdr2*^{-/-} mice compared with WT mice, but significantly decreased in *Mdr2*^{-/-}+ML221 compared with *Mdr2*^{-/-} mice (Supplementary Figure 7C). When HHSteCs were incubated with cholangiocyte supernatant, the mRNA expression of *PCNA*, *KI67*, *ACTA2*, and *COL1A1* decreased in HHSteCs treated with supernatant from BDL+ML221 mice compared with HHSteCs treated with supernatant from BDL mice (Supplementary Figure 7D, E).

By immunofluorescence, we demonstrated that APJ was expressed in mouse HSCs and HHSteCs (Supplementary Figure 8A, B). To validate the paracrine effect of cholangiocyte apelin on HSC proliferation and activation, we treated HHSteCs with apelin *in vitro*. The expression of APJ was increased in HHSteCs treated with apelin (Supplementary Figure 8C). In apelin treated HHSteCs, the expression of the activation marker α -SMA was increased, whereas the expression of the quiescent marker GFAP was decreased (Supplementary Figure 8D). Studies have shown intracellular ROS generation triggers HSC proliferation and activation (21). In apelin treated HHSteCs, the intracellular ROS levels were higher than controls, whereas HHSteCs pretreated with ML221 or NAC reversed the generation of ROS (Figure 7B-C). Apelin treatment promoted HHSteC proliferation, but pretreatment of HHSteCs with ML221 or NAC

attenuated apelin-induced proliferation (Figure 7D). Consistently, the expression of *PCNA* and *KI67* increased in apelin treated HHStECs compared with control, which was reversed by pretreatment of ML221 or NAC (Figure 7E). The expression of activation markers of HSC was increased in apelin treated HHStECs compared with control, which was decreased by ML221 or NAC pretreatment compared with apelin treatment (Figure 7F). To evaluate if TGF- β 1 is involved in apelin induced HSC activation, HHStECs were treated with apelin in the presence or absence of the TGF- β 1 neutralizing antibody (Apelin-13+TGF- β 1 Ab). The expression of *COL1A1* and *ACTA2* was decreased in Apelin-13+TGF- β 1 Ab treated HHStECs compared with untreated HHStECs, but the fold change was not prominent (Supplementary Figure 8E).

Inhibition of APJ is therapeutic during cholestasis

Our data indicate that inhibition of APJ blockade may represent a valuable therapeutic approach for liver fibrosis. ML221 was injected into mice, followed by BDL for 7 days (Supplementary Figure 9A). A significant reduction in apelin serum levels was seen in mice treated with ML221 (Supplementary Figure 9B). By H&E staining, we demonstrated that ductular reaction with bile stasis, mild patchy active interface hepatitis, focal stage 2 fibrosis with focal early bridging fibrosis, and hepatocyte necrosis in BDL mice. These phenotypes were ameliorated in ML221 treated mice (Supplementary Figure 9C). In ML221 treated mice, BDL induced IBDM was reduced (Supplementary Figure 9D) along with attenuated BDL- induced liver fibrosis (Supplementary Figure 9E).

Discussion

Activation of the apelin-APJ axis induces a diverse array of biological effects, including angiogenesis (22) and fluid homeostasis (23). Differential effects of the apelin-APJ axis on fibrosis have been observed in other organs, which may be due to APJ acting as a dual receptor and revealing different distribution in various organs, or other undiscovered subtypes and functions (5). Apelin can also be degraded by the angiotensin-converting enzyme 2 (ACE2), a monocarboxypeptidase homologue to ACE (24), consistent with our finding of low levels of apelin in the serum of apelin^{-/-} mice, as opposed to no apelin. The current study evaluated whether the apelin-APJ axis plays a role in ductular reaction and liver fibrosis during cholestasis. We observed that the expression of the apelin/APJ axis increased in cholangiocytes of patients with PSC. This represents the first evidence linking apelin activation to cholestasis. Apelin levels in serum and cholangiocyte supernatant and the biliary expression of apelin and APJ were increased in both BDL and Mdr2^{-/-} mice. Interestingly, apelin serum levels were decreased in BDL and Mdr2^{-/-} mice treated with APJ antagonist ML221. Studies have indicated that APJ is also a mechanosensitive receptor (25). During cholestasis, accumulated bile elevates intrabiliary pressure, which induces mechanical stress on cholangiocytes that increases APJ expression and apelin synthesis. Our unpublished data showed that mechanical stress-stimulated (stretched) HIBepiCs with Flexcell

FX-6000 Tension System induced apelin synthesis. Treatment of BDL and Mdr2^{-/-} mice with ML221 or apelin knockout ameliorated biliary damage and liver fibrosis.

Cholangiocyte proliferation is a major hallmark of ductular reaction, which is associated with a paracrine activation of HSCs and increased phenotypes in cholangiopathies such as PSC and PBC (26). Proliferating cholangiocytes interact with other cells in the biliary microenvironment such as vascular endothelial cells, hepatocytes and hepatic cells, promoting liver inflammation and fibrosis (27). In our study, administration of ML221 reduced cholangiocyte proliferation in BDL and Mdr2^{-/-} mice and apelin knockout decreased cholangiocyte proliferation in BDL mice, which provide the evidence that apelin-APJ is key in modulating biliary homeostasis in cholestasis.

ERK1/2 is a key regulatory kinase regulating cell proliferation in different pathophysiological states including liver proliferation and fibrosis (28, 29). Consistent with our findings, apelin-APJ promotes the proliferation of several cell types including vascular smooth muscle cells through ERK activation (10). Our previous study demonstrated that ML221 treatment decreased cholangiocarcinoma growth both *in vitro* and *in vivo* by changes in ERK phosphorylation (12). In the current study, we observed that blockage of APJ or knockout apelin in BDL mice reduced the activation of ERK in cholangiocytes, which supports the concept of apelin-APJ regulation of ERK activation during cholestasis. *In vitro*, ML221 and PD98055 reduction of apelin-induced ERK phosphorylation and cholangiocyte proliferation further support

the concept that the apelin-APJ axis promotes biliary hyperplasia by activation of ERK signaling pathway.

Many different stimuli including growth factors, cytokines, ligands for heterotrimeric G protein-coupled receptors or transforming agents activate the ERK signaling pathway (30). There is evidence that ROS induce cell proliferation through ERK activation (31). Also, bile acids increase intracellular ROS levels, which trigger ERK phosphorylation and cholangiocytes proliferation (28). In support of these findings, our study has demonstrated increased ROS levels in cholestatic cholangiocytes, which was reduced by ML221 treatment and apelin knockout. Indeed, apelin increases ROS generation in endothelium (32) and induces ROS synthesis during vascular smooth muscle cell (VSMC) proliferation (33) through a ROS-ERK-dependent pathway (20). Our *in vitro* results showed that NAC and ML221 treatment reduced apelin induced cholangiocytes ROS generation and ERK activation. We propose that apelin-APJ promotes the proliferation of cholangiocytes by inducing intracellular ROS generation and activating the ERK signaling pathway.

When stimulated, many enzymes (e.g., cytochrome P450 mono-oxidase, and NADPH oxidase, Nox) involved in normal cell metabolism, trigger ROS accumulation in the body, leading to oxidative stress (34). In the current study, Nox4 gene expression was significantly increased in cholestatic cholangiocytes from BDL mice and PSC patients. Nox4, one of the major Nox isoforms expressed in the liver, regulates the progression of cholestatic liver injury and liver fibrosis (35). Also, Nox4 plays a crucial role in BDL-induced liver fibrosis (36) and PDGF-induced proliferation of hepatic

stellate cells (37). Supporting these studies, APJ blockage or apelin knockout reduced the expression of Nox4 in cholangiocytes from BDL mice as well as in biliary cell lines. A study has shown that in the endoplasmic reticulum stress autophagy, apelin can promote the expression of Nox4 in cardiomyocytes, and promote the production of ROS, leading to myocardial hypertrophy (38).

HSC activation is crucial for the deposition of the ECM during liver fibrosis. There is evidence that HSCs can be activated by cholangiocytes secreting inflammatory factors, chemokines, and neuropeptides, such as TGF- β , incretin, and calcitonin gene-related peptides through paracrine pathways (19, 39). The activation of APJ during hypoxia and inflammation induces a powerful proliferative effect in the human hepatic cell line, LX-2 (40). Furthermore, consistent with our data that APJ blockade reduces apelin-induced collagen-I and PDGFR β expression in HSC lines (14). However, the mechanisms of apelin induced HSCs activation are unclear. Thus, we aimed to determine the mechanisms by which biliary-secreted apelin promotes liver fibrosis by a paracrine mechanism. A previous study has shown that ethanol-increase ROS levels induces HSCs activation (41). Apelin has been shown to induce intracellular ROS synthesis in endothelial cells (32). In this study, *in vitro* pre-treatment of HHStecs with the ROS inhibitor NAC reduced apelin-13-induced HSC activation. These results indicate that apelin-APJ induced HSC proliferation and activation via intracellular ROS generation. ROS produced from other cells (e.g. hepatocytes) may impact HSC activation (42). In our study, we observed apelin-induced intracellular

ROS production in cholangiocytes suggesting that cholangiocytes-released ROS may impact HSC activation.

Cholangiocyte senescence is a hallmark of liver injury and has been found in the livers of patients with PSC and PBC as well as mouse models of PSC (43, 44). A study suggested the apelin-APJ axis as a novel promising therapeutic target for anti-aging (45). Supporting these findings, we demonstrated that ML221 treatment or apelin knockout reduce BDL and *Mdr2*^{-/-} induced biliary senescence and subsequently liver fibrosis. Inflammation is part of the liver wound healing response that, in chronic conditions, leads to the development of fibrosis and cirrhosis in cholangiopathies such as PBC and PSC (44, 46). There is evidence that pro-inflammatory factors can influence apelin expression and, likewise, apelin can influence pro-inflammatory factor expression (47). Blocking apelin-APJ can reduce expression of proinflammatory factors in the mesentery in a rat model of portal vein ligation (48). Apelin-APJ may be directly involved in the inflammatory response in cholestasis, and it may also reduce liver inflammation by reducing DR. Our data showed no significant difference in the expression of apelin and APJ in Kupffer cells from PSC compared with healthy controls (Supplementary Figure 10). The mechanisms of apelin-APJ in liver inflammation need to be further studied. Published literature indicates that angiogenesis may contribute to the progression of fibrosis during the wound healing process in chronic liver disease (49). Consistent with the finding that apelin-APJ regulates angiogenesis, we observed that ML221 treatment or apelin knockout reduce BDL-induce liver angiogenesis (50). For example, in CCl₄-induced cirrhosis,

blocking APJ reduces liver angiogenesis and decreases the progression of cirrhosis (8).

In conclusion, as presented in our working model, we demonstrated that modulation of the apelin-APJ axis plays an important role regulating cholangiocyte proliferation and liver fibrosis during cholestasis and may represent a novel therapeutic target for cholestatic liver diseases (Figure 8).

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Figure legends

Figure 1 APJ mediated apelin expression is increased in cholangiocytes during cholestasis. **A :** Immunofluorescence of apelin (co-stained with CK19) in liver sections from PSC patients (n=7), upper three panels: Orig. magn. $\times 40$, scale bar 50 μm ; lower panel: Orig. magn. $\times 40$ zoom5, scale bar 10 μm (red CK19, green apelin, blue DAPI). **B:** Immunofluorescence of APJ (co-stained) with CK19 in liver sections from PSC patients (n=7), upper three panels: Orig. magn. $\times 40$, scale bar 50 μm ; lower panel: Orig. magn. $\times 40$ zoom5, scale bar 10 μm (red CK19, green APJ, blue DAPI). **C:** The mRNA expression of *APLN* and *APLNR* in cholangiocytes from PSC patients (hPSCL) (mean $\pm$ SD, n = 3), $*P < 0.05$ vs. HIBEpiCs. **D:** Apelin level in PSC patients' serum (mean $\pm$ SD, n = 26), $*P < 0.05$ vs. Control; **E:** Apelin level in BDL mouse serum and cholangiocyte supernatant (mean $\pm$ SD, n = 3), $*P < 0.05$ vs. WT, $^{\#}P < 0.05$ vs. BDL. **F:** Apelin level in *Mdr2*^{-/-} mouse serum (mean $\pm$ SD, n = 3), $*P < 0.05$ vs. WT, $^{\#}P < 0.05$ vs. *Mdr2*^{-/-}.

Figure 2. Apelin induces cholangiocyte proliferation during cholestasis via APJ.

A : The immunohistochemical of CK19 in apelin^{-/-} BDL mouse liver sections (mean $\pm$ SD, n = 3), Orig., magn., 20 $\times$; scale bars represent 100 μm , $*P < 0.05$ vs. WT, $^{\#}P < 0.05$ vs. BDL. **B:** The mRNA expression of *Pcna* and *Ki67* in cholangiocytes from apelin^{-/-} BDL mice (mean $\pm$ SD, n = 3), $*P < 0.05$ vs. WT, $^{\#}P < 0.05$ vs. BDL. **C :** Immunohistochemistry for CK19 in BDL+ML221 mouse liver sections (mean $\pm$ SD, n = 3), Orig., magn., 20 $\times$; scale bars represent 100 μm , $*P < 0.05$ vs. WT, $^{\#}P < 0.05$ vs.

BDL. **D:** The mRNA expression of *Pcna* and *Ki67* in cholangiocytes from BDL+ML221 mice (mean $\pm$ SD, n = 3), * P <0.05 vs. WT, # P <0.05 vs. BDL. **E :** Immunohistochemistry for CK19 in *Mdr2*^{-/-} mouse liver sections (mean $\pm$ SD, n = 3), Orig., magn., 20 $\times$; scale bars represent 100 μ m, * P <0.05 vs. WT, # P <0.05 vs. *Mdr2*^{-/-}. **F:** The mRNA expression of *Pcna* and *Ki67* in cholangiocytes from *Mdr2*^{-/-} mice (mean $\pm$ SD, n = 3), * P <0.05 vs. WT, # P <0.05 vs. *Mdr2*^{-/-}.

Figure 3 Apelin knockout or APJ blockade attenuates biliary senescence, inflammation and angiogenesis in cholestatic liver. **A:** SA- β -GAL staining in liver sections from PSC patients (n = 7), Orig., magn., 20 $\times$; scale bars represent 100 μ m. **B:** SA- β -GAL staining in mouse liver sections (mean $\pm$ SD, n = 3), Orig., magn., 20 $\times$; scale bars represent 100 μ m, * P <0.05 vs. WT, # P <0.05 vs. BDL. **C:** The immunohistochemical of F4/80 in mouse liver sections (mean $\pm$ SD, n = 3), Orig., magn., 20 $\times$; scale bars represent 100 μ m, * P <0.05 vs. WT, # P <0.05 vs. BDL. **D:** Immunofluorescence for CD31 (co-stained with CK19) in mouse frozen liver sections (n=3), Orig., magn., 40 $\times$; scale bars represent 50 μ m (purple CK19, green CD31, blue DAPI).

Figure 4 Apelin induces liver fibrosis via APJ during cholestatic. **A:** Sirius red staining in *apelin*^{-/-} BDL mouse liver sections (mean $\pm$ SD, n = 3), Orig., magn., 20 $\times$; scale bars represent 100 μ m, * P <0.05 vs. WT, # P <0.05 vs. BDL. **B:** Hydroxyproline levels in *apelin*^{-/-} BDL mouse liver (mean $\pm$ SD, n = 3), * P <0.05 vs. WT, # P <0.05 vs.

BDL. **C:** Sirius red staining in BDL +ML221 mouse liver sections (mean $\pm$ SD, n = 3), Orig., magn., 20 $\times$; scale bars represent 100 μ m, * P <0.05 vs. WT, # P <0.05 vs. BDL. **D:** Hydroxyproline levels in BDL +ML221 mouse liver (mean $\pm$ SD, n = 3), * P <0.05 vs. WT, # P <0.05 vs. BDL. **E:** Sirius red staining in Mdr2^{-/-} mouse liver sections (mean $\pm$ SD, n = 3), Orig., magn., 20 $\times$; scale bars represent 100 μ m, * P <0.05 vs. WT, # P <0.05 vs. Mdr2^{-/-}. **F:** Hydroxyproline levels in Mdr2^{-/-} mouse liver (mean $\pm$ SD, n = 3), * P <0.05 vs. WT, # P <0.05 vs. Mdr2^{-/-}.

Figure 5 Apelin-APJ induces cholangiocyte proliferation via Nox4/ROS/ERK signaling pathway during cholestasis. **A:** The mRNA expression of *NOX4* in cholangiocytes from PSC patients (hPSCL) (mean $\pm$ SD, n = 3), * P <0.05 vs. HIBEpiCs. **B:** The mRNA expression of *Nox4* in mouse cholangiocytes (mean $\pm$ SD, n = 3), * P <0.05 vs. WT, # P <0.05 vs. BDL. **C :** The mRNA expression of *NOX4* in apelin treated HIBEpiCs (mean $\pm$ SD, n = 3), * P <0.05 vs. Control, # P <0.05 vs. apelin-13. **D:** ROS level in mouse cholangiocyte supernatant and cholangiocyte cell lysate (mean $\pm$ SD, n = 3), * P <0.05 vs. WT, # P <0.05 vs. BDL. **E:** Fluorescence Microplate Assay for intracellular ROS regeneration in apelin treated HIBEpiCs (mean $\pm$ SD, n = 3), * P <0.05 vs. Control, # P <0.05 vs. apelin-13. **F:** Confocal Microscopy Assay for intracellular ROS regeneration in HIBEpiCs (green ROS), Orig., magn., 20 $\times$; scale bars represent 100 μ m.

Figure 6 Apelin-APJ induces cholangiocyte proliferation via Nox4/ROS/ERK signaling pathway during cholestasis. **A:** The expression of p-ERK in mouse cholangiocytes (mean $\pm$ SD, n = 3), * P <0.05 vs. WT, # P <0.05 vs. BDL. **B:** Immunofluorescence for p-ERK (co-stained with CK19) in frozen liver sections (n=3), upper three panels: Orig. magn. $\times 40$, scale bar 50 μ m; lower panel: Orig. magn. $\times 40$ zoom5, scale bar 10 μ m (red CK19, green p-ERK, blue DAPI). **C:** The expression of p-ERK in cholangiocytes from apelin^{-/-} BDL mice (mean $\pm$ SD, n = 3), * P <0.05 vs. WT, # P <0.05 vs. BDL. **D:** Expression of p-ERK in apelin treated HIBEpiCs (mean $\pm$ SD, n = 3), * P <0.05 vs. Control, # P <0.05 vs. apelin-13. **E:** The measurement of proliferation in apelin treated HIBEpiCs by MTS (mean $\pm$ SD, n = 3), * P <0.05 vs. Control, # P <0.05 vs. apelin-13. **F:** The mRNA expression of *PCNA* and *KI67* in apelin treated HIBEpiCs (mean $\pm$ SD, n = 3), * P <0.05 vs. Control, # P <0.05 vs. apelin-13.

Figure 7 Apelin induces hepatic stellate cell proliferation and activation via APJ during cholestasis. **A:** Immunofluorescence for α -SMA (co-stained with desmin) in BDL mouse frozen liver sections (n=3), Orig., magn., 20 $\times$; scale bars represent 50 μ m (red α -SMA, green desmin, blue DAPI). **B:** Fluorescence Microplate Assay for intracellular ROS regeneration in apelin treated HHSteCs (mean $\pm$ SD, n = 3), * P <0.05 vs. Control, # P <0.05 vs. apelin-13. **C:** Confocal Microscopy Assay for intracellular ROS regeneration in apelin treated HHSteCs (green ROS), Orig., magn., 20 $\times$; scale bars represent 100 μ m. **D:** The measurement of proliferation in apelin treated HHSteCs by MTS (mean $\pm$ SD, n = 3), * P <0.05 vs. Control, # P <0.05 vs. apelin-13. **G:**

The mRNA expression of *PCNA* and *KI67* in apelin treated HHSteCs (mean $\pm$ SD, n = 3) * P <0.05 vs. Control, # P <0.05 vs. apelin-13. **H:** The mRNA expression of *ACTA2* and *COL1A1* in apelin treated HHSteCs (mean $\pm$ SD, n = 3) * P <0.05 vs. Control, # P <0.05 vs. apelin-13.

Figure 8. Working model of apelin-APJ regulation of cholangiocyte proliferation and liver fibrosis in cholestasis. APJ mediates apelin expression and secretion increased in cholangiocytes during cholestasis. On one hand, cholangiocytes secrete apelin which binds to APJ on cholangiocytes and promotes cholangiocytes proliferations via Nox4/ROS/ERK pathway by autocrine. On the other hand, cholangiocyte-derived apelin binds to APJ on HSC and induces intracellular ROS generation and HSC activation by paracrine. In addition, apelin-APJ is also involved in biliary senescence and expression of a profibrogenic cholangiocyte phenotype. Taken together, apelin-APJ promotes liver fibrosis during cholestasis.

Supplementary Figure 1. APJ mediated apelin expression is increased in cholangiocytes during cholestasis. A: The mRNA expression of *Apln* and *Aplnr* in BDL mouse isolated cholangiocytes (mean $\pm$ SD, n = 3), * P <0.05 vs. WT, # P <0.05 vs. BDL; **B:** Immunofluorescence of apelin (co-stained with CK19) in mouse frozen liver sections (n=3), upper three panels: Orig., magn., 20 $\times$; scale bars represent 100 μ m; lower panel: Orig. magn. $\times$ 20 zoom 5, scale bar 20 μ m (red CK19, green apelin, blue DAPI); **C:** Immunofluorescence of APJ co-staining with CK19 in mouse frozen liver

sections (n=3), upper three panels: Orig., magn., 20×; scale bars represent 100 μ m; lower panel: Orig. magn. $\times 20$ zoom5, scale bar 20 μ m (red CK19, green APJ, blue DAPI); **D:** The mRNA expression of *Apln* and *Aplnr* in *Mdr2*^{-/-} mouse isolated cholangiocytes (mean $\pm$ SD, n = 3), **P*<0.05 vs. WT, #*P*<0.05 vs. *Mdr2*^{-/-}; **E:** Liver histology was evaluated in liver sections from BDL and BDL+ML221 mice with H&E staining (n=3; Orig., magn., 10×; scale bars represent 200 μ m); **F:** Apelin level in apelin^{-/-} mouse serum and cholangiocyte supernatant (mean $\pm$ SD, n = 3), **P*<0.05 vs. WT, #*P*<0.05 vs. BDL.

Supplementary Figure 2. Apelin induces cholangiocyte profibrogenesis via APJ during cholestasis

A: The mRNA expression of fibrosis markers *Colla1*, *Fn1* and *Tgfb1* in cholangiocytes from BDL and apelin^{-/-} BDL mouse (mean $\pm$ SD, n = 3), **P*<0.05 vs. WT, #*P*<0.05 vs. BDL. **B:** The mRNA expression of fibrosis markers *Colla1*, *Fn1* and *Tgfb1* in cholangiocytes from BDL+ML221 mouse (mean $\pm$ SD, n = 3), **P*<0.05 vs. WT, #*P*<0.05 vs. BDL. **C:** The mRNA expression of fibrosis markers *COL1A1*, *FN1* and *TGFB1* in apelin-13 treated HIBEpiCs (mean $\pm$ SD, n = 3), **P*<0.05 vs. Control, #*P*<0.05 vs. apelin-13. **D:** The mRNA expression of fibrosis markers *COL1A1*, *FN1* and *TGFB1* in apelin-13 and TGF- β 1–neutralizing antibody treated HIBEpiCs (mean $\pm$ SD, n = 3), **P*<0.05 vs. Control, #*P*<0.05 vs. apelin-13.

Supplementary Figure 3. Evaluation of senescent, inflammatory and angiogenesis

markers in different *in vivo* models. **A:** The mRNA expression of *Cdkn2a*, *Cdkn1a* and *Ccl2* in BDL mouse liver (mean $\pm$ SD, n = 3), **P*<0.05 vs. WT, #*P*<0.05 vs. BDL. **B:** The mRNA expression of *Cdkn2a*, *Cdkn1a* and *Ccl2* in *Mdr2*^{-/-} mouse liver (mean $\pm$ SD, n = 3), **P*<0.05 vs. WT, #*P*<0.05 vs. *Mdr2*^{-/-}. **C:** The mRNA expression of *Il1b*, *Il6*, *Il33* and *Tnfa* in mouse liver (mean $\pm$ SD, n = 3), **P*<0.05 vs. WT, #*P*<0.05 vs. BDL. **D:** The mRNA expression of *Pecam1*, *Vegfa* and *Vwf* in mouse liver (mean $\pm$ SD, n = 3), **P*<0.05 vs. WT, #*P*<0.05 vs. BDL.

Supplementary Figure 4. Apelin induces liver fibrosis via APJ during cholestatic

injury. **A:** The mRNA expression of fibrosis markers *Acta2*, *Colla1*, *Fnl* and *Tgfb1* in apelin^{-/-} BDL mouse liver (mean $\pm$ SD, n = 3), **P*<0.05 vs. WT, #*P*<0.05 vs. BDL; **B:** The mRNA expression of fibrosis markers *Acta2*, *Colla1*, *Fnl* and *Tgfb1* in BDL +ML221 mouse liver (mean $\pm$ SD, n = 3), **P*<0.05 vs. WT, #*P*<0.05 vs. BDL; **C:** The mRNA expression of fibrosis markers *Acta2*, *Colla1*, *Fnl* and *Tgfb1* in *Mdr2*^{-/-} mouse liver (mean $\pm$ SD, n = 3), **P*<0.05 vs. WT, #*P*<0.05 vs. *Mdr2*^{-/-}.

Supplementary Figure 5. Apelin and APJ expression was increased in PSC

hepatic stellate cells. **A:** Immunofluorescence of apelin (co-stained with desmin) in liver sections from PSC patients (n=7), Orig. magn. $\times 20$, scale bar 100 μ m (red desmin, green apelin, blue DAPI). **B:** Immunofluorescence of APJ (co-stained) with desmin in liver sections from PSC patients (n=7), Orig. magn. $\times 20$, scale bar 100 μ m (red desmin,

green APJ, blue DAPI). **C:** The mRNA expression of *APLN* and *APLNR* in HSCs from PSC patients (mean $\pm$ SD, n = 3), * P <0.05 vs. Control.

Supplementary Figure 6. No significant different expression of Apelin and APJ in portal fibroblasts between PSC and normal control. **A:** Immunofluorescence of apelin (co-stained with Thy1) in liver sections from PSC patients (n=7), Orig. magn. $\times 20$, scale bar 100 μ m (red Thy1, green apelin, blue DAPI). **B:** Immunofluorescence of APJ (co-stained) with Thy1 in liver sections from PSC patients (n=7), Orig. magn. $\times 20$, scale bar 100 μ m (red Thy1, green APJ, blue DAPI). **C:** The mRNA expression of *APLN* and *APLNR* in LCM-isolated portal fibroblasts from PSC patients (mean $\pm$ SD, n = 3), * P <0.05 vs. Control.

Supplementary Figure 7. Apelin induces hepatic stellate cell activation via APJ during cholestatic injury. **A:** The mRNA expression of *Pcna* and *Ki67* in LCM-isolated mouse HSCs (mean $\pm$ SD, n = 3), * P <0.05 vs. WT, # P <0.05 vs. BDL; **B:** The mRNA expression of *Acta2* and *Col1a1* in LCM-isolated mouse HSCs (mean $\pm$ SD, n = 3), * P <0.05 vs. WT, # P <0.05 vs. BDL; **C:** Immunofluorescence for α -SMA (co-stained with desmin) in *Mdr2*^{-/-}+ML221 mouse frozen liver sections (n=3), Orig., magn., $20\times$; scale bars represent 100 μ m (red α -SMA, green desmin, blue DAPI); **D:** The mRNA expression of *PCNA* and *KI67* in HHStECs treated with mouse cholangiocyte supernatant (mean $\pm$ SD, n = 3), * P <0.05 vs. Control, # P <0.05 vs. BDL cho-sup; **E:** The mRNA expression of *ACTA2* and *COL1A1* in HHStECs treated with

mouse cholangiocyte supernatant (mean $\pm$ SD, n = 3), * P <0.05 vs. Control, # P <0.05 vs. BDL cho-sup.

Supplementary Figure 8. APJ is expressed in mouse HSCs and HHSteCs. A:

Immunofluorescence of APJ co-staining with desmin in mouse frozen liver sections (n=3), Orig., magn., 40 $\times$; zoom2, scale bars represent 25 μ m (green desmin; red APJ, blue DAPI). **B:** Immunofluorescence of APJ in HHSteCs (n=3), Orig., magn., 40 $\times$; scale bars represent 50 μ m (green APJ, blue DAPI). **C:** Immunofluorescence of APJ in apelin treated HHSteCs (n=3), Orig., magn., 20 $\times$, zoom 4; scale bars represent 25 μ m (green APJ, blue DAPI). **D:** Immunofluorescence of α -SMA and GFAP in apelin treated HHSteCs (n=3), Orig., magn., 20 $\times$, zoom5; scale bars represent 20 μ m (green α -SMA, red GFAP, blue DAPI). **E:** The mRNA expression of fibrosis markers *ACTA2* and *COL1A1* in apelin-13 and TGF- β 1–neutralizing antibody treated HHSteCs (mean $\pm$ SD, n = 3), * P <0.05 vs. Control, # P <0.05 vs. apelin-13.

Supplementary Figure 9. APJ blockade can be therapeutic during cholestasis. A:

Schematic overview of experimental design for 12 days BDL model. **B:** Apelin level in 12 days BDL mouse serum (mean $\pm$ SD, n = 3), * P <0.05 vs. WT, # P <0.05 vs. 12 D BDL. **C:** Liver histology was evaluated in liver sections from 12 D BDL and 12 D BDL+5 D ML221 mice with H&E staining (n=3; Orig., magn., 10 $\times$; scale bars represent 200 μ m). **D:** The immunohistochemical of CK19 in 12 D BDL and 12 D BDL+5 D ML221 mouse liver sections (mean $\pm$ SD, n = 3), Orig., magn., 20 $\times$; scale

bars represent 100 μm , $*P<0.05$ vs. WT, $^{\#}P<0.05$ vs. 12 D BDL. **E:** Sirius red staining in in 12 D BDL and 12 D BDL+5 D ML221 mouse liver sections (mean $\pm$ SD, $n = 3$), Orig., magn., 20 $\times$; scale bars represent 100 μm , $*P<0.05$ vs. WT, $^{\#}P<0.05$ vs. 12 D BDL.

Supplementary Figure 10. No significant different expression of apelin and APJ in Kupffer cells from PSC and normal control. A: Immunofluorescence of apelin (co-stained with CD68) in liver sections from PSC patients ($n=7$), Orig. magn. $\times 20$, scale bar 100 μm (red CD68, green apelin, blue DAPI). **B:** Immunofluorescence of APJ (co-stained) with CD68 in liver sections from PSC patients ($n=7$), Orig. magn. $\times 20$, scale bar 100 μm (red CD68, green APJ, blue DAPI). **C:** The mRNA expression of *APLN* and *APLNR* in LCM-isolated Kupffer cell from PSC patients (mean $\pm$ SD, $n = 3$), $*P<0.05$ vs. Control.

Figure 1

Figure 2

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Figure 3

Figure 4

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Figure 5

Figure 6

Figure 7

Figure 8

The apelin-apelin receptor axis triggers cholangiocyte proliferation and liver fibrosis during mouse models of cholestasis

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Short title: Apelin modulation of biliary damage.

Abbreviations: α -SMA: α -smooth muscle actin; ACTA2: actin alpha 2; ALT: alanine aminotransferase; AST: aspartate aminotransferase; ALK PHOS: alkaline phosphatase; BDL: bile duct ligation; CK-19: cytokeratin-19; CCl₄: carbon tetrachloride; CD68: Cluster of differentiation 68; CDKN1A: cyclin dependent kinase inhibitor 1a; CDKN2A: cyclin dependent kinase inhibitor 2a; Ccl2: C-C motif chemokine ligand 2; COL1A1: collagen type I alpha 1; DAPI: 4',6-diamidino-2-phenylindole; DPI: diphenyleneiodonium chloride; ELISA: enzyme linked immunosorbent assay; ERK: extracellular signal-regulated kinase; Fn1: fibronectin1; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; GFAP: Glial fibrillary acidic protein; HHStECs: human hepatic stellate cells; HIBEpICs: human intrahepatic biliary epithelial cells; HSCs: hepatic stellate cells; hPSC: human PSC cell lines; IBDM: intrahepatic bile duct mass; IL: interleukin; LCM: laser capture microdissection; Mdr2^{-/-}: multidrug resistance gene 2 knockout; NAC: N-Acetyl-L-cysteine; NADPH: nicotinamide adenine dinucleotide phosphate; PCNA: proliferating cell nuclear antigen; PBC: primary biliary cirrhosis; PDGFRB: platelet-derived growth factor receptor beta; PECAM-1: platelet endothelial cell adhesion molecule 1; PSC: primary sclerosing cholangitis; ROS: reactive oxygen species; SA- β -GAL: senescence-associated beta-galactosidase; TGF- β 1: transforming growth factor- β 1; TNF α : tumor necrosis factor α ; Thy-1: Thy-1 cell surface antigen; VEGFA: vascular endothelial growth factor A; VWF: von Willebrand factor; WT: wild-type.

Supplemental Information

Materials and methods

Materials

Reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise indicated. The antibodies for cytokeratin-19 (CK-19) (ab52625), desmin (ab185033), CD68 (cluster of differentiation 68, ab955), Thy1 (Thy-1 cell surface antigen; ab181469) and GFAP (Glial fibrillary acidic protein; ab7260) were purchased from Abcam (Cambridge, MA); F4/80 (70076s), p-ERK (4370) and ERK (4695) were purchased from Cell Signaling Technology (Beverly, MA); α -SMA (α -smooth muscle actin, c6198) were purchased from Sigma-Aldrich; apelin (BS-2425R) and apelin receptor (702069) were purchased from Thermo Fisher Scientific (Mountain View, CA); CD31 (AF3628) were purchased from R&D Systems, Inc. (Minneapolis, MN). All selected primers were purchased from Qiagen (Germantown, MD) and specific information about the primers are listed in Supplementary Table 1. The iScript cDNA Synthesis Kit (170-8891) and iTaq Universal SYBR Green Supermix (172-5124) were purchased from Bio-Rad (Hercules, CA).

Animal models

All animal experiments were performed in accordance to protocols approved by the Baylor Scott & White Healthcare Institutional Animal Care and Use Committee and Texas A&M University Animal Care and Use Committee. C57BL/6 wild-type (WT) mice (control for apelin^{-/-} mice) were purchased from Charles River Laboratories (Wilmington, MA). Male Mdr2^{-/-} mice were originally purchased from Jackson Laboratories (Bar Harbor, ME); the colony is established in our facility. FVB/NJ mice (WT, control for Mdr2^{-/-} mice) were purchased from Jackson

Laboratories. Mice were maintained in a temperature-controlled environment (20-22°C) with 12:12-hr light/dark cycles and fed with standard mouse chow along with free access to drinking water *ad libitum*. Male WT mice underwent bile duct ligation (BDL) or sham surgery and were treated with the apelin inhibitor, ML221 (1) (Tocris Bio-Techne, Minneapolis, MN, 150 µg/kg, 3×weekly via tail vein injections). The mice received the first ML221 injection at the time of the BDL surgery and were then treated for 1 wk. The apelin^{-/-} breeding pair was a gift from Dr. Hyung Chun (Yale University, New Haven, CT), which show normal physiological development (2). Male apelin^{-/-} mice (12 wk age) underwent BDL or sham for 1 wk. Male Mdr2^{-/-} mice (12 wk age) were treated with ML221 (150 µg/kg, 3×weekly via tail vein injections) (Mdr2^{-/-} + ML221) for 1 wk (3). For 12 days BDL (12 D BDL), WT mice underwent either BDL or sham, injections (ML221 150 µg/kg via tail vein injections) were performed on day 7 and 9 post operatively (sacrifice at day 12). Animal groups are summarized in Supplementary Table 2. Following surgery and selected treatments, we collected serum, total liver samples, cholangiocytes, and cholangiocyte supernatant (after 6 hr incubation at 37°C) from the selected groups of animals. Other techniques are described in detail in Supplemental Information.

Human samples

The liver tissue samples (OCT-embedded blocks, late stage PSC patients n=7) and serum (healthy human n=8, late stage PSC patients n=26) were obtained under the Institutional Review Board approved protocol at Indiana University Purdue University Indianapolis. Healthy human liver OCT-embedded blocks (n=4) were purchased from Sekisui Xeno Tech (Kansas City, KS). Patient characteristics were listed in Supplementary Table 3.

Isolated mouse cholangiocytes, hepatic stellate cells (HSCs), portal fibroblasts and Kupffer cells

Mouse cholangiocytes were obtained by immunoaffinity separation using a mouse IgG_{2a} monoclonal antibody (a gift from Dr. R. Faris, Brown University, Providence, RI), against an unidentified antigen expressed by all rodent cholangiocytes (4). Mouse HSCs, human HSCs, human portal fibroblasts and human Kupffer cells were isolated by laser capture microdissection (LCM) (4). Following immunofluorescent staining, desmin/Thy1/CD68-positive cells were dissected from the slides by the LCM system Leica LMD7000 (Buffalo Grove, IL). RNA from LCM cells were extracted with the Arcturus PicoPure RNA isolation kit (Thermo Fisher Scientific, Mountain View, CA).

Immunofluorescent staining

Frozen liver sections (6 μ m) were placed on glass slides and fixed in 4% paraformaldehyde and permeabilized by 0.1% Triton X-100 at room temperature. Afterwards, sections or cells were blocked with 5% bovine serum albumin and incubated with the selected primary antibodies overnight at 4°C: (i) apelin/APJ co-stained with CK-19, CD68, Thy1 or desmin for the expression of apelin and APJ in cholangiocytes; (ii) p-ERK co-stained with CK-19 for the phosphorylation of ERK in proliferating cholangiocytes; (iii) desmin co-stained with α -SMA for the proliferation of HSC, and co-stained with APJ for the expression of APJ in HSCs; (iv) APJ for the immunoreactivity of APJ in human hepatic stellate cell lines (HHStECs, Sciencell, Carlsbad, CA); (v) GFAP and α -SMA for the activation of HHStECs; and (vi) CD31 co-stained with CK-19 for the evaluation of angiogenesis. Next, the samples were incubated with appropriate Cy3/Cy2/Cy5-conjugated secondary antibodies (1:100, Jackson ImmunoResearch Laboratories) for 1 hr at room

temperature. Stained sections were observed under an Olympus Fluoview 300 confocal microscope (Olympus, Center Valley, PA) with 4',6-diamidino-2-phenylindole (DAPI) (Thermo Fisher Scientific) as a nuclear counterstain.

Immunohistochemical staining

We performed immunohistochemical analyses in rodent and human liver sections by standard techniques (4). Briefly, following deparaffinization and antigen retrieval, paraffin-embedded liver sections (5 μ m) were incubated with the selected primary and secondary antibodies. Following staining, slides were mounted with a cover slip. Then, liver sections were examined by Image Pro-Analyzer software (Olympus, Tokyo, Japan). Changes in intrahepatic bile duct mass (IBDM, stained with CK-19) (4, 5) and Kupffer cell number (by staining for F4/80) (4) in liver sections were semi-quantitatively measured by Visiopharm software (10 different fields analyzed from three different samples from five different animals).

Assessment of liver histology, serum chemistry, biliary senescence and liver fibrosis

Liver histology was evaluated by standard H&E staining in paraffin-embedded liver sections (4-6 μ m). Following H&E staining, images were captured by Olympus Image Pro-Analyzer software 7.0 (Olympus, Tokyo, Japan) and evaluated by a board-certified pathologist. Serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALK PHOS), and total bilirubin were measured by a Dimension RxL Max Integrated Chemistry system (Dade Behring Inc., Deerfield IL) at the Department of Chemistry, Baylor Scott & White Healthcare, Temple TX. Biliary senescence was evaluated in frozen liver sections (10 μ m) by staining for senescence-associated- β -galactosidase (SA- β -gal) using the commercially available

cellular senescence assay kit (Millipore Sigma, Billerica, MA) (4, 5). Collagen deposition in liver sections (5 μ m) was assessed by Sirius Red staining (10 different fields were analyzed from three different samples from three different animals) (4, 5). Liver fibrosis was also measured by hydroxyproline levels in total liver samples using the hydroxyproline Assay Kit (4).

Determination of apelin and reactive oxygen species (ROS) levels

Commercially available Enzyme Linked Immunosorbent Assay (ELISA) kits for measuring apelin levels in human and mouse serum, cell culture medium, cell lysate and cholangiocyte supernatant were purchased from Sigma-Aldrich (6). The commercially available kits for measuring ROS levels in liver samples and cultured cells were purchased from Abcam (Cambridge, MA) and Cell Biolabs, Inc. (San Diego, CA). ELISA and ROS kits were performed according to the manufacturer's instructions.

Immunoblots

Immunoblots were performed by standard techniques (5). Protein bands were visualized by Fujifilm Image Reader LAS-4000 (Fujifilm, Japan) chemiluminescence densitometric analysis was performed using Image J. β -actin was used as an internal control. For the phosphorylation of ERK, data was presented as p-ERK/ERK.

Total RNA Isolation and Real-time PCR Assays

RNA was isolated from liver tissues and cell lysates using mirVanaTM miRNA Isolation Kit (Ambion, Mountain View, CA) according to the manufacturer's protocol. Quantitation of RNA was done using Nanodrop 2000 (Thermo Fisher Scientific, Mountain View, CA). cDNA synthesis

was carried out using the Affinity Script Multi Temperature cDNA Synthesis Kit (Bio-Rad, Hercules, CA) or TaqMan™ MicroRNA Reverse Transcription Kit (Thermo Fisher Scientific). Real-time PCR was performed using RT² SYBR Green (Bio-Rad,) or TaqMan™ Universal PCR Master Mix (Thermo Fisher Scientific) for a ViiA 7 Real-Time PCR System according to the manufacturer's protocol (Applied Biosystems). Glyceraldehyde-3-phosphate dehydrogenase was used as housekeeping; data were analyzed using the PCR array data analysis template (Applied Biosystems). All samples were tested in triplicate, and average values were used for quantification.

In vitro studies

The *in vitro* studies were performed in human primary cholangiocyte cell line (HIBEpiCs; Sciencell, Carlsbad, CA) and HHSteCs. All cells were grown in 5% CO₂ in a humidified incubator maintained at 37°C. When the cells were treated with ML221 (10 μM) (3), DPI (10 μM, Nox4 inhibitor) (7), NAC (5 mM, ROS inhibitor) (8), PD98059 (10 μM, ERK inhibitor) (9) or TGF-β1–neutralizing antibody (10 μg/ml, R&D Systems, Inc. Minneapolis, MN) (10) these compounds were added to the medium 1 hr prior to apelin (10 μM) treatment.

Cell proliferation assay: Cell proliferation was measured using the MTS CellTiter 96 Aqueous One Solution Reagent assays according to the manufacturer's instructions (Promega Corporation, Madison, WI). Briefly, cells were seeded in 96-well culture plates and treated accordingly in different groups for 24 hr (apelin, apelin+ML221, apelin+DPI, apelin+NAC, apelin+PD98059). After treatments, MTS was added to each well for 1 hr. The absorbance was measured at 490 nm. Cell proliferation was evaluated directly based on optical density.

Detection of ROS: Free radicals and other reactive species were detected using the Cellular ROS/Superoxide Detection Assay Kits (Abcam) according to the manufacturer's instructions.

Fluorescence Microplate Assay: Cells were seeded in 96-well plates. After 24 hr, the culture medium was removed. The cells were then incubated with the ROS/Superoxide Detection Mix and treatments (apelin, apelin+ML221, apelin+DPI, apelin+NAC) for 60 min at 37°C. After incubation, intracellular ROS levels were determined using a fluorescence microplate reader.

Fluorescence/Confocal Microscopy Assay: Cells were seeded onto 35 mm Thermo Scientific Nunc Glass Bottom Dishes (Thermo Fisher Scientific, Mountain View, CA). After 24 hr, the culture medium was removed. The cells were then incubated with the ROS/Superoxide Detection Mix and treatments (apelin, apelin+ML221, apelin+DPI, apelin+NAC) for 60 min at 37°C. After incubation, Fluorescence images were captured using Leica AF 6000 Modular Systems.

Statistical analysis

All data are expressed as the mean $\pm$ SEM. Differences between groups were analyzed by unpaired Student's *t* test when two groups were analyzed and one-way ANOVA when more than two groups were analyzed, followed by an appropriate *post hoc* test. The level of significance was set at $P < 0.05$.

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Supplementary Figure 1

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Supplementary Figure 2

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Supplementary Figure 3

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Supplementary Figure 4

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Supplementary Figure 5

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Supplementary Figure 6

Supplementary Figure 7

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Supplementary Figure 8

Supplementary Figure 9

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Supplementary Table 1 List of real-time PCR primers used

Gene	Species	Detected transcript	Source
<i>Acta2</i>	Mouse	NM_007392	QIAGEN
<i>Apln</i>	Mouse	NM_013912	QIAGEN
<i>Aplnr</i>	Mouse	NM_011784	QIAGEN
<i>Col1a1</i>	Mouse	NM_007742	QIAGEN
<i>Ccl2</i>	Mouse	NM_011333	QIAGEN
<i>Cdkn2a</i>	Mouse	NM_010233	QIAGEN
<i>Cdkn1a</i>	Mouse	NM_008084	QIAGEN
<i>Fn-1</i>	Mouse	NM_008361	QIAGEN
<i>Gapdh</i>	Mouse	NM_031168	QIAGEN
<i>Il1b</i>	Mouse	NM_133775	QIAGEN
<i>Il6</i>	Mouse	NM_001081117	QIAGEN
<i>Il33</i>	Mouse	NM_015760	QIAGEN
<i>Ki67</i>	Mouse	NM_001040654	QIAGEN
<i>Nox4</i>	Mouse	NM_001111099	QIAGEN
<i>Pcna</i>	Mouse	NM_011045	QIAGEN
<i>Pecam1</i>	Mouse	NM_008816	QIAGEN
<i>Tgfb1</i>	Mouse	NM_011577	QIAGEN
<i>Tnfa</i>	Mouse	NM_013693	QIAGEN
<i>Vegfa</i>	Mouse	NM_009505	QIAGEN
<i>Vwf</i>	Mouse	NM_011708	QIAGEN
<i>ACTA2</i>	Human	NM_001141945	QIAGEN
<i>APLN</i>	Human	NM_017413	QIAGEN
<i>APLNR</i>	Human	NM_005161	QIAGEN
<i>COL1A1</i>	Human	NM_000088	QIAGEN
<i>FN1</i>	Human	NM_002026	QIAGEN
<i>GAPDH</i>	Human	NM_001256799	QIAGEN
<i>KI67</i>	Human	NM_001145966	QIAGEN
<i>NOX4</i>	Human	NM_016931	QIAGEN
<i>PCNA</i>	Human	NM_002592	QIAGEN
<i>TGFB1</i>	Human	NM_000660	QIAGEN
<i>VEGFA</i>	Human	NM_001025366	QIAGEN

Supplementary Table 2 List of animal groups

Models	Groups	Mice	Treatment	Number	Age	Weight
WT BDL	WT	C57	Sham+Saline	6	12 weeks	25-30gm
	WT+ML221	C57	Sham+ML221	9	12 weeks	25-30gm
	BDL	C57	BDL+Saline	6	12 weeks	25-30gm
	BDL+ML221	C57	BDL+ML221	9	12 weeks	25-30gm
Apelin^{-/-} BDL	WT	C57	Sham	3	12 weeks	25-30gm
	Apelin ^{-/-}	Apelin ^{-/-}	Sham	6	12 weeks	25-30gm
	BDL	C57	BDL	3	12 weeks	25-30gm
	Apelin ^{-/-} BDL	Apelin ^{-/-}	BDL	6	12 weeks	25-30gm
Mdr2^{-/-}	WT	FVB	Saline	6	12 weeks	25-30gm
	WT+ML221	FVB	ML221	6	12 weeks	25-30gm
	Mdr2 ^{-/-}	Mdr2 ^{-/-}	Saline	6	12 weeks	25-30gm
	Mdr2 ^{-/-} +ML221	Mdr2 ^{-/-}	ML221	6	12 weeks	25-30gm
12 D BDL	WT	C57	Sham+Saline	3	12 weeks	25-30gm
	WT+5 D ML221	C57	Sham+ML221	3	12 weeks	25-30gm
	12 D BDL	C57	12 days BDL+5 days Saline	3	12 weeks	25-30gm
	12 D BDL +5 D ML221	C57	12 days BDL+5 days ML221	3	12 weeks	25-30gm

Supplementary Table 3: Characteristics of human control and PSC samples

Diagnosis	Age	Gender	Race	Origin	Sample Type
Healthy	52	F	W	Sekisui Xeno Tech (Kansas City, KS)	Frozen liver
Healthy	46	M	W	Sekisui Xeno Tech (Kansas City, KS)	Frozen liver
Healthy	17	F	W	Sekisui Xeno Tech (Kansas City, KS)	Frozen liver
Healthy	38	M	W	Sekisui Xeno Tech (Kansas City, KS)	Frozen liver
PSC	60	M	W	Indiana University School of Medicine	Frozen liver
PSC	N/A	N/A	N/A	Indiana University School of Medicine	Frozen liver
PSC	57	M	W	Indiana University School of Medicine	Frozen liver
PSC	33	M	W	Indiana University School of Medicine	Frozen liver
PSC	47	F	W	Indiana University School of Medicine	Frozen liver
PSC	42	M	W	Indiana University School of Medicine	Frozen liver
PSC	63	M	W	Indiana University School of Medicine	Frozen liver
Healthy	68	M	N/A	Indiana University School of Medicine	Serum
Healthy	68	M	N/A	Indiana University School of Medicine	Serum
Healthy	68	M	N/A	Indiana University School of Medicine	Serum
Healthy	51	M	N/A	Indiana University School of Medicine	Serum
Healthy	51	M	N/A	Indiana University School of Medicine	Serum
Healthy	67	M	N/A	Indiana University School of Medicine	Serum
Healthy	51	M	N/A	Indiana University School of Medicine	Serum
Healthy	30	M	N/A	Indiana University School of Medicine	Serum
Healthy	45	N/A	N/A	Indiana University School of Medicine	Serum
PSC	14	F	W	Indiana University School of Medicine	Serum
PSC	58	M	W	Indiana University School of Medicine	Serum
PSC	63	M	W	Indiana University School of Medicine	Serum
PSC	45	M	W	Indiana University School of Medicine	Serum
PSC	58	F	W	Indiana University School of Medicine	Serum
PSC	44	M	W	Indiana University School of Medicine	Serum
PSC	66	M	W	Indiana University School of Medicine	Serum
PSC	42	M	B	Indiana University School of Medicine	Serum
PSC	43	M	W	Indiana University School of Medicine	Serum
PSC	27	F	W	Indiana University School of Medicine	Serum
PSC	58	M	W	Indiana University School of Medicine	Serum
PSC	61	M	W	Indiana University School of Medicine	Serum
PSC	55	M	W	Indiana University School of Medicine	Serum
PSC	68	M	W	Indiana University School of Medicine	Serum
PSC	38	M	W	Indiana University School of Medicine	Serum
PSC	49	F	W	Indiana University School of Medicine	Serum
PSC	28	M	W	Indiana University School of Medicine	Serum
PSC	62	M	W	Indiana University School of Medicine	Serum
PSC	57	M	W	Indiana University School of Medicine	Serum
PSC	51	F	W	Indiana University School of Medicine	Serum
PSC	43	F	B	Indiana University School of Medicine	Serum
PSC	57	M	W	Indiana University School of Medicine	Serum

PSC	63	M	W	Indiana University School of Medicine	Serum
PSC	33	M	W	Indiana University School of Medicine	Serum
PSC	32	M	W	Indiana University School of Medicine	Serum
PSC	38	M	W	Indiana University School of Medicine	Serum

PSC: Primary Sclerosing Cholangitis

**Supplementary Table 4 Evaluation of serum levels of ALT, AST, ALK PHOS
and Total Bilirubin and liver to body weight ratio**

Treatment		WT	WT+ML221	BDL	BDL+ML221
Liver weight (gm)		1.33±0.08 n=9	1.34±0.07 n=9	1.51±0.10 n=9	1.25±0.07 n=9
Body weight (gm)		26.8±1.20 n=9	26.63±1.00 n=9	20.45±0.87 n=9	19.50±0.86 n=9
LW/BW (%)		4.95±0.12 n=9	5.00±0.12 n=14	7.33±0.26* n=12	6.43±0.13 [#] n=9
Serum Chemistry	ALT (units/L)	141.6±15.9 n=3	215.0±12.6 n=3	941.7±61.3* n=3	556.7±36.6 [#] n=3
	AST (units/L)	243.3±72.6 n=3	326.7±20.9 n=3	1136.7±94.9* n=3	683.3±33.8 [#] n=3
	ALK PHOS (units/L)	<50 n=3	<50 n=3	340.0±38.2* n=3	215.0±20.0 [#] n=3
	total Bilirubin (mg/dL)	<0.5 n=3	<0.5 n=3	17.3±0.17* n=3	10.5±0.29 [#] n=3

ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; ALK PHOS: alkaline phosphatase.

*** $P < 0.05$ vs. WT, [#] $P < 0.05$ vs. BDL.**