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The secretin/secretin receptor axis modulates liver fibrosis through changes in TGF-β1 biliary secretion

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Abstract

The secretin/secretin receptor (SR) axis is upregulated by proliferating cholangiocytes during cholestasis. Secretin stimulates biliary proliferation by downregulation of let-7a and subsequent upregulation of the growth-promoting factor NGF. It is not know if the secretin/SR axis plays a role in subepithelial fibrosis observed during cholestasis. Our aim was to determine the role of secretin/SR axis in the activation of biliary fibrosis in animal models and human primary sclerosing cholangitis (PSC). Studies were performed in Wild-type (WT) mice with bile duct ligation (BDL), BDL $SR^{-/-}$ mice or $Mdr2^{-/-}$ mouse models of cholestatic liver injury. In selected

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studies, the SR antagonist (Sec 5–27) was used to block the secretin/SR axis. Biliary proliferation and fibrosis were evaluated as well as the secretion of secretin (by cholangiocytes and S cells), the expression of markers of fibrosis, TGF-β1, TGF-β1R, let-7a and downstream expression of NGF. Correlative studies were performed in human control and PSC liver tissue biopsies, serum and bile. SR antagonist reduced biliary proliferation and hepatic fibrosis in BDL WT and Mdr2$^{-/-}$ mice. There was decreased expression of let-7a in BDL and Mdr2$^{-/-}$ cholangiocytes that was associated with increased NGF expression. Inhibition of let-7a accelerated liver fibrosis due to cholestasis. There was increased expression of TGF-β1, TGF-β1R. Significantly higher expression of secretin, SR and TGF-β1 was observed in PSC patient liver samples compared to healthy controls. In addition, there was higher expression of fibrosis genes and remarkably decreased expression of let-7a and increased expression of NGF compared to the control.

Conclusion—The secretin/SR axis plays a key role in regulating the biliary contribution to cholestasis-induced hepatic fibrosis.

Keywords

Biliary Fibrosis; Cholangiocytes; microRNA; Nerve Growth Factor; Cholestasis

Cholangiocytes line the intrahepatic biliary system and participate in several different cellular processes including the modification of ductal bile before reaching the duodenum (1). Cholangiocytes are the target cells in several human cholangiopathies including primary biliary cirrhosis and primary sclerosing cholangitis (PSC), which are diseases characterized by extensive fibrosis (2). Cholangiocytes are normally mitotically dormant and proliferate in response to liver injury during extrahepatic bile duct obstruction (BDL) and in the Mdr2$^{-/-}$ mouse model of PSC (3). Proliferating cholangiocytes display neuroendocrine characteristics and secrete and respond to several neuropeptides and gastrointestinal hormones that modulate cholangiocyte responses to injury via autocrine/paracrine mechanisms (4).

Secretin is secreted by duodenal S cells, brain and cholangiocytes (4–6). Secretin exerts its effects through secretin receptor (SR), which is expressed in the liver by large cholangiocytes (7–9). Enhanced biliary proliferation during cholestasis is associated with increased SR expression on cholangiocytes and increased cAMP dependent secretin-stimulated ductal secretion (4, 10, 11).

Administration of secretin stimulates cholangiocyte proliferation via activation of SR through a cAMP/PKA/ERK1/2 dependent mechanism (4, 10). Knockout of SR reduces cholangiocyte hyperplasia in cholestatic mice indicating that secretin is an important trophic regulator sustaining biliary growth (10). Recently, we explored the role of secretin in the regulation of biliary proliferation in a secretin knockout (Sct$^{-/-}$) model during extrahepatic cholestasis (4). In this study, we found that following liver injury, secretin produced by cholangiocytes and S cells reduces the expression level of miRNA let-7a resulting in upregulation of the target gene NGF that is a proliferative factor for cholangiocytes (12).

During the progression of liver diseases, cholangiocytes, through the products released during their neuroendocrine activation, have been suggested as a link between biliary injury

and activation of hepatic fibrogenesis (13). No information exists on the role that the secretin/SR axis plays in the activation of hepatic fibrogenesis during cholestasis. Thus, we evaluate the role of the secretin/SR axis in hepatic fibrogenesis in the BDL and Mdr2 knockout animal models of PSC by modulating the axis with the SR antagonist (Sec 5–27) (14).

Materials and Methods

Materials

Reagents were purchased from Sigma-Aldrich Co. (St. Louis, MO) unless otherwise indicated. The RNeasy Mini Kit for RNA purification and mouse PCR primers were purchased from Qiagen (Valencia, CA); details are provided in Suppl. File 1. The primary antibodies for cytokeratin-19 (CK-19) and CD31 (marker of endothelial cells) (15) were purchased from Abcam (Cambridge, MA). The antibodies against TGF-β1 and phospho-SMAD2/3 were purchased from Cell Signaling Technology (Danvers, MA). The TGF-β1 receptor antibody was purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX). The ELISA Kits to measure TGF-β1 levels were purchased from Affymetrix Inc. (Santa Clara, CA). The EIA kits (EK-067-04) to measure secretin levels in mice were purchased from Phoenix Pharmaceuticals, Inc., Burlingame, CA. The EIA kits (EK6-067-03) to measure secretin levels in human serum and bile were purchased from Phoenix Pharmaceuticals, Inc.

Animal Models

All animal experiments were performed according to protocols approved by the Baylor Scott & White IACUC Committee. C57/BL6 wild-type (WT) mice (25–30 gm) were purchased from Charles River (Wilmington, MA). The $SR^{-/-}$ mouse colony (16) is established in our facility. Male FVB/NJ WT and $Mdr2^{-/-}$ (17) mice (25–30 g) were purchased from Jackson Laboratories (Sacramento, CA). Animals were maintained in a temperature-controlled environment (20–22°C) with 12:12-h light/dark cycles and fed *ad libitum* standard chow with free access to drinking water. The studies were performed in: (i) normal and 1 wk BDL WT and $SR^{-/-}$ mice; and (ii) normal or BDL WT mice that immediately after surgery were treated with saline, secretin (2.5 nmoles/kg BW/day) (4) or the SR antagonist, Sec 5–27 (10 μg/kg BW/day, Thermo Scientific, Waltham, MA) by IP implanted osmotic minipumps for 1 wk. Sec 5–27 has been previously used in another *in vivo* study (14). Since the effects of secretin on biliary hyperplasia are mediated by downregulation of miRNA let-7a-dependent NGF (4), we used normal and BDL WT mice that immediately after surgery were treated (to reduce the hepatic expression of miRNA let-7a) by two tail vein injections (one day 3; one day 7, 30 mg/kg BW) with Vivo-Morpholino sequences of miRNA let-7a (5'-AACTATACAACCTACTACCTCATCC-3'), or mismatched Morpholinos (5'-AAgTATAgAAgCTAgTAgCTCATCC-3' for miRNA let-7a). We have used this dose and route of administration of Vivo-Morpholino sequences of miRNA let-7a in BDL WT mice (4). $Mdr2^{-/-}$ mice were treated with saline or Sec 5–27 (10 μg/kg BW/day) by minipumps for 1 wk before collecting serum, liver blocks and cholangiocytes. Before each procedure, animals were treated with euthasol (200–250 mg/kg BW). In all groups, we measured liver and body weight and liver to body weight ratio (index of liver cell growth) (1).

Isolated cholangiocytes and intrahepatic murine cholangiocyte lines (IMCL)

Cholangiocytes were obtained by immunoaffinity separation (4). The *in vitro* studies were performed in vector or Sct stable-transfected IMCL (4). The IMCL control (stable transfected with the empty vector) or IMCL lacking Sct was established using SureSilencing short hairpin RNA (Super-Array, Frederick, MD) plasmid for mouse Sct containing a marker for neomycin resistance for the selection of stably transfected cells. The human hepatic stellate cells (HHSteC) were purchased from Sciencell (Carlsbad, CA).

Measurement of Serum Chemistry, Intrahepatic Bile Duct Mass (IBDM), Expression of Sct/SR Axis and NGF and Levels of Secretin and TGF-β1 in Serum and Cholangiocyte Supernatant

The serum levels of glutamate pyruvate transaminases (SGPT), glutamic oxaloacetic transaminase (SGOT), alkaline phosphatase (ALP) and total bilirubin were measured by a Dimension RxL Max Integrated Chemistry system (Dade Behring Inc., Deerfield IL), Chemistry Department, Baylor Scott & White. We determined IBDM in liver sections (4–5 μm thick, 10 fields analyzed from 3 samples from 3 different animals) as the area occupied by CK-19 positive-bile ducts/total area × 100. Sections were examined by the Olympus Image Pro-Analyzer software (Olympus, Tokyo, Japan). The expression of Sct, SR and NGF in cholangiocytes was evaluated by *q*PCR (4). We evaluated the secretion of secretin in short-term (6 hr) cultures of S cells from normal WT and Mdr2$^{-/-}$ mice (4). The levels of secretin and TGF-β1 in serum and/or cholangiocyte supernatants from mice and/or patients were measured by commercially available kits.

Measurement of Liver Fibrosis, Expression of TGF-β1, TGF-β1R, FN-1 and miRNA let-7a and SMAD2/3 Phosphorylation

Liver fibrosis was evaluated by: (i) Sirius Red staining in liver sections (4–5 μm thick, 10 fields analyzed from 3 samples from 3 different animals); and (ii) measurement of hydroxyproline levels in total liver samples using the Hydroxyproline Assay Kit (MAK008, Sigma-Aldrich Co.). Slides were scanned by a digital scanner (Leica Microsystems, SCN400, Buffalo Grove, IL) and quantified using Image-Pro Premier 9.1 (Media Cybernetics, MD). For hydroxyproline measurements, we used 3 liver samples from 3 different mice.

We evaluated by immunohistochemistry the expression of TGF-β1 and TGF-β1R in liver sections (4–5 μm) from WT and Mdr2$^{-/-}$ mice. Semiquantitative analysis was performed using our published grading system (18): 0–5%=negative; 6–10%=+/−; 11–30%=+; 31–60%=++; >61%=+++.

By *q*PCR, we measured the mRNA expression of TGF-β1 and TGF-β1R in endothelial cells and cholangiocytes (isolated by laser capture microdissection, LCM) from WT and Mdr2$^{-/-}$ mice. Frozen liver sections (n=3, 10 μm thick) were placed on PEN-membrane slides that were fixed in 100% methanol for 3 minutes, rinsed with 1× PBS and incubated with a primary CD31 (marker of endothelial cells) (15) or CK-19 antibody overnight at 4°C. Following washes, sections were incubated with a fluorescent secondary antibody overnight at 4°C. Next, CD-31 or CK-19 positive cells were cut out from the slides by the LCM

system Leica LMD7 (Leica Microsystems, Buffalo Grove, IL) and collect into a tube. The RNA was extracted with the Arcturus PicoPure RNA isolation kit (Mountain View, CA), and the expression of TGF-β1 and TGF-β1R was evaluated.

To evaluate the activation of TGF-β1 signaling by secretin we measured in cholangiocytes the phosphorylation of SMAD2/3 (downstream signaling specific to TGF-β1 signaling in liver fibrosis) (19) by immunoblots. Protein expression was quantified by the LI-COR Odyssey Infrared Imaging System (LI-COR Bioscience, Lincoln, NE).

We measured the expression of TGF-β1, TGF-β1R and FN-1 in RNA (1 μg) from total liver. In RNA from total liver and/or cholangiocytes from WT and Mdr2$^{-/-}$ mice, we measured the expression of miRNA let-7a by *q*PCR. In normal and BDL WT mice treated with miRNA let-7a Vivo-Morpholino or mismatched Morpholinos, we measured liver fibrosis by Sirius red staining in liver sections. *In vitro*, ICML were treated with let-7a precursor antisense inhibitors or controls for 24 hr before evaluating the mRNA expression of COLA1 *q*PCR.

Measurement of Expression of Secretin, SR, TGF-β1, TGF-β1R and Fibrotic Genes and Levels of Secretin and TGF-β1 in Normal and PSC Samples

Liver sections (4–5 μm thick, 2 sections evaluated for each protein measured) from three control and three PSC patients (stage 4) were obtained from Dr. Invernizzi (Humanitas Research Hospital, Rozzano, Italy) under a protocol approved by the Ethics Committee by the Humanitas Research Hospital; the protocol was reviewed by the Veterans' Administration IRB and R&D Committee. The protocol was approved by the Texas A&M HSC Institutional Review Board. The expression of secretin, SR, TGF-β1 and TGF-β1R in human liver sections was evaluated by immunohistochemistry. Sections were analyzed in a coded fashion using an Olympus BX-51 light microscope (Tokyo, Japan) with a Videocam (Spot Insight; Diagnostic Instrument, Inc., Sterling Heights, MI).

Total RNA was extracted from paraffin embedded sections from samples obtained from three control and three PSC patients using the kit RNeasy® FFPE kit (Qiagen, Valencia, CA). From these samples, the mRNA expression (from cDNA samples) of selected genes was evaluated by *q*PCR using human primers purchased from Qiagen; details on primers are provided in Suppl. File 1. The levels of secretin in bile (n=9 control; n=5 PSC patients, early and advanced stage) and serum (n=67 control; n=21 PSC patients, early and advanced stage and TGF-β1 (in serum; n=5 for control, and n=7 for PSC samples, early and advanced stage) from control and PSC patients were evaluated by EIA kits.

In Vitro Effect of Secretin on Biliary TGF-β1 and let-7a expression and TGF-β1 Secretion

***In Vitro* Effect of Cholangiocyte Supernatant on the Activation of HHSteC—**Our ICML (that express the SR) (10) were treated with 0.2% BSA (basal) or secretin (100 nM) for 24 hr before measuring the expression of: (i) TGF-β1 mRNA and let-7a miRNA by *q*PCR; (ii) SMAD2,3 phosphorylation by immunofluorescence; and (iii) the levels of biliary TGF-β1 by ELISA kits. Control IMCL or IMCL cells lacking Sct were incubated for 24 hr before evaluating the expression of TGF-β1 mRNA and let-7a miRNA by *q*PCR, and the levels of biliary TGF-β1 by ELISA kits.

To demonstrate that secretin fibrotic effects are not due to a direct interaction with stellate cells (that do not express SR, Alpini, unpublished observations, 2016), we treated these cells with secretin (100 nM) for 24 hr before evaluating the expression of fibrotic genes by *q*PCR. HHSteC were incubated for 12 hr with the supernatant (after 6 hr incubation) of cholangiocytes from normal and BDL mice (containing different amount of TGF-β1) in the absence/presence of LY2109761 (TGF-β1 receptor antagonist) (20) (Cayman Chemical Company, Ann Arbor, MI) before evaluating the mRNA expression of PCNA, FN-1, α-SMA and SMAD2,3.4 in HHSteC.

Statistical Analysis

Data are expressed as mean±SEM. Differences between groups were analyzed by Student's unpaired t-test when two groups were analyzed and ANOVA when more than two groups were analyzed, followed by an appropriate post hoc test.

RESULTS

Measurement of Serum Chemistry, IBDM, Expression of Sct/SR Axis and NGF and Levels of Secretin and TGF-β1 in Serum and Cholangiocyte Supernatant

Liver to body weight ratio increased in normal mice treated with secretin and in BDL WT compared to normal WT mice (Suppl. Table 1). Liver to body weight ratio decreased in BDL $SR^{-/-}$ mice and in BDL WT mice treated Sec 5–27 compared to BDL WT mice (Suppl. Table 1). Liver to body weight ratio increased in $Mdr2^{-/-}$ compared to WT mice but it was similar between normal WT and $Mdr2^{-/-}$ mice treated with Sec 5–27 (Suppl. Table 2A).

Serum levels of transaminases, ALP and bilirubin were higher in BDL WT and in $Mdr2^{-/-}$ mice compared to WT mice (Suppl. Tables 2A–B). Biochemical indexes of liver injury were improved in: (i) $SR^{-/-}$ BDL compared to BDL WT mice; and (ii) $Mdr2^{-/-}$ mice treated with Sec 5–27 compared to $Mdr2^{-/-}$ mice (Suppl. Table 2A–B). Serum levels of transaminases (but not bilirubin and ALP) were reduced in BDL WT mice treated Sec 5–27 compared to BDL WT mice (Suppl. Table 2B). In BDL WT and $Mdr2^{-/-}$ mice there was enhanced IBDM compared to normal mice (Figure 1A–B). The administration of Sec 5–27 to BDL WT or $Mdr2^{-/-}$ mice decreased IBDM compared to WT mice (Figure 1A–B).

The biliary expression of secretin, SR, and TGF-β1 was higher in cholangiocytes from $Mdr2^{-/-}$ mice, but was similar to that of normal WT mice in $Mdr2^{-/-}$ mice treated with Sec 5–27 (Figure 2A). Secretin serum levels increased in normal WT mice treated with secretin and in BDL WT compared to normal WT mice (Figure 2B). There was a decrease (although not significant) in secretin serum levels in $Mdr2^{-/-}$ compared to normal WT mice (Suppl. Table 2A). Secretin levels were higher in cholangiocyte supernatant (collected from 3 cumulative preparations from 4 mice, n=12) from BDL WT mice (107.5±31.3 vs. 1.81±0.8 ng/ml, normal WT mice, $p<0.05$; n=6) and $Mdr2^{-/-}$ mice (10.40±5.6 vs. 30.28±6.2 ng/ml, normal WT mice, $p<0.05$; n=23), but decreased in S cells (from 6 individual mice) from 12 wk $Mdr2^{-/-}$ mice compared to normal WT mice (258.1±18.4 vs. 66.7±13.4 ng/ml, normal WT mice, $p<0.05$; n=6 evaluations). TGF-β1 levels increased in serum and cholangiocyte supernatant from BDL WT and $Mdr2^{-/-}$ mice compared to WT animals (Figure 2C–D).

Measurement of Liver Fibrosis, Expression of TGF-β1, TGF-β1R, FN-1 and let-7a

Secretin increased the percentage of collagen in normal and BDL WT mice (Figure 3A); no difference was observed between normal WT, $SR^{-/-}$ normal mice and normal WT mice treated with Sec 5–27 (Figure 3A). BDL WT mice display higher collagen deposition compared to normal WT mice (Figure 3A). In BDL $SR^{-/-}$ mice and BDL WT mice or $Mdr2^{-/-}$ treated with Sec 5–27 there was reduced collagen deposition compared to BDL WT or $Mdr2^{-/-}$ mice (Figure 3A–B). Similar changes in liver fibrosis were observed by measurement of hydroxyproline levels in liver samples (Figure 3A–B).

We demonstrated increased immunoreactivity for TGF-β1 and TGF-β1R in $Mdr2^{-/-}$ compared to normal WT mice (Figure 3C). There was enhanced mRNA expression of TGF-β1 and TGF-β1R in LCM-isolated cholangiocytes (but not endothelial cells) from $Mdr2^{-/-}$ compared to normal WT mice (Figure 3D).

Administration of secretin increased the mRNA expression of TGF-β1 and FN-1 in total liver compared to normal WT mice (Figure 4A). In BDL WT and $Mdr2^{-/-}$ mice, there was increased expression of TGF-β1 and FN-1 in total liver compared to normal WT mice, increase that was blocked by Sec 5–27 or knockdown of SR (Figure 4B–C). Secretin increased the phosphorylation of SMAD2,3 in cholangiocytes from normal WT mice (Figure 4D). In cholangiocytes from $Mdr2^{-/-}$ mice there was increased phosphorylation of SMAD2,3 compared to normal WT mice that was reduced by Sec 5–27 (Figure 4D). *In vitro*, secretin increased SMAD2.3 phosphorylation in IMCL (Suppl. Figure 1). There was decreased expression of miRNA let-7a and enhanced expression of NGF in cholangiocytes from $Mdr2^{-/-}$ compared to normal WT mice, changes that were prevented by Sec 5–27 (Figure 4E). There was enhanced: (i) collagen deposition in normal and BDL WT mice treated with miRNA let-7a Vivo-Morpholino compared to control mice (Figure 5A); and (ii) mRNA expression of COLA1 in IMCL treated with the let-7a inhibitor compared to basal (Figure 5B).

Expression of Sct, SR and fibrotic genes in Normal and PSC Human Samples

In PSC liver sections there was increased expression of Sct, SR, TGF-β1 and TGF-β1R compared to normal samples (Figure 6A). We demonstrated: (i) increased mRNA expression of PCNA, Sct, SR, TGF-β1, TGF-β1R, FN-1, COLA1, α-SMA and NGF; (ii) enhanced levels of secretin in bile (although not significant) and increased TGF-β1 serum levels (Figure 6B), and reduced secretin serum levels (patient data information in Suppl. Table 3); and (iii) decreased expression of miRNA let-7a in PSC samples compared to controls (Figure 6B).

In Vitro Effect of Secretin on Biliary TGF-β1 Expression and Levels

In Vitro Paracrine Effect of TGF-β1 Released by Cholangiocytes on the Activation of Stellate Cells—

In vitro, secretin did not increase the expression of fibrotic genes in stellate cells (not shown). In IMCL lacking Sct there was reduced expression of TGF-β1 levels and TGF-β1 and enhanced expression of let-7a compared to vector-transfected IMCL (Figure 2E–G). In IMCL treated with secretin there was: (i) increased TGF-β1 levels and TGF-β1 mRNA expression and reduced let-7a expression (Figure 2E–G).

When HHSteC were treated with cholangiocyte supernatant from BDL mice there was increased expression of PCNA, FN-1, α-SMA and SMAD2,3, 4 in HHSteC, which was reduced by LY2109761 (Figure 7). No effects were observed with normal cholangiocyte supernatant (Figure 7).

DISCUSSION

We demonstrated that: (i) secretin stimulates hepatic fibrogenesis in normal and BDL mice; and (ii) liver fibrosis is reduced in BDL $SR^{-/-}$ mice and BDL and $Mdr2^{-/-}$ mice treated with Sec 5–27. Secretin-induced increase in hepatic fibrosis was associated with increased: (i) expression of TGF-β1/TGF-β1R axis and SMAD2/3 phosphorylation; (ii) levels of secretin in serum and cholangiocyte supernatants; and (iii) TGFβ1 levels in cholangiocyte supernatants. To elucidate the mechanisms by which the secretin/SR axis regulates liver fibrosis, we evaluated the expression of let-7a-dependent NGF that regulates biliary hyperplasia during cholestasis (4). There was decreased let-7a expression in $Mdr2^{-/-}$ mice followed by increased expression of NGF that was reduced by Sec 5–27. Administration of let-7a Vivo-Morpholinos increased fibrogenesis in BDL mice. We demonstrated increased expression of the Sct/SR axis and enhanced levels of secretin in bile (although not significant) and reduced secretin serum levels from late stage PSC samples. The changes in Sct/SR axis correlated with increased expression of fibrogenic genes, TGF-β1 serum levels and decreased let-7a expression and enhanced NGF expression in PSC samples.

In support of the use of Vivo-Morpholino to modulate liver fibrosis, studies have demonstrated that Morpholino oligomers are powerful antisense tools to block translation or interfere with RNA processing (21). We have previously used Vivo-Morpholinos to reduce the hepatic expression of arylalkylamine N-acetyltransferase (22) and miRNA let-7a and miR-125b (4) in rats and mice.

In addition to cholangiocytes (4), the secretin/SR axis promotes cell proliferation in several cell lines including epithelial cells from cysts of patients with autosomal dominant polycystic kidney disease (23). Administration of Sec 5–27 significantly inhibited biliary proliferation in BDL and $Mdr2^{-/-}$ mice, demonstrating that the Sct/SR axis is key in regulating biliary homeostasis by modulating the balance between cholangiocyte proliferation/loss. Supporting this concept, studies have shown that Sec 5–27 reduces secretin-induced proliferation and secretin-stimulated intracellular signaling in other systems (24, 25).

The activation of biliary proliferation is thought to play a role in the initiation and progression of liver fibrosis through autocrine (by secreting pro-fibrogenic factors including PDGF-B chain) (26) and paracrine mechanisms by activating stellate cells (27). To further explore the role of the secretin/SR in the promotion of liver fibrogenesis, we evaluated the role of let-7a, which is downregulated during cholestasis and modulates of NGF expression (4), which was downregulated in cholestatic cholangiocytes with a corresponding increase in liver fibrosis. These findings support the concept that cholangiocyte proliferation is regulated by autocrine/paracrine mechanisms by neurohormonal factors including the secretin/SR axis and downstream, NGF. The importance of let-7a in biliary proliferation and

liver fibrosis is supported by other studies. Down-regulation of microRNA let-7a contributed to the overexpression of type I collagen in systemic and localized scleroderma (28). Overexpression of let-7 in cancer cell lines reduces cell mitosis, demonstrating that let-7 functions as a tumor suppressor in lung cells (29). Further studies are necessary to pinpoint the role of other pro-fibrogenic factors that may be modulated by let-7a, in addition to NGF, during hepatic fibrogenesis. NGF may be linked to secretin/SR axis modulated hepatic fibrosis since NGF transcriptionally upregulates TGFβ1 expression and secretion in renal tubular cells (30).

Secretin increased hepatic fibrosis and the expression of TGFβ1 and selected fibrogenic markers. There was increased TGF-β1R expression in bile ducts. Interestingly, TGF-β1R is a predicted target gene by let-7a and the target relationship between TGF-β1R and let7 family members has been confirmed during renal fibrosis (31). These data suggest that secretin-induced synthesis of TGF-β1 may elicit an autocrine TGF-β-1/TGF-β1R in cholangiocytes, which may contribute to biliary response to injury. Given that TGF-β1 is a known activator of stellate cells, our data suggest that the induction of TGF-β1 by secretin in cholangiocytes may stimulate liver fibrogenesis that parallels cholangiocyte proliferation (32, 33). The role of cholangiocytes in TGF-β1 stimulation of liver fibrosis is supported by our finding showing that were no changes in the expression of TGF-β1 and TGF-β1R in endothelial cells from Mdr2$^{-/-}$ compared to WT mice. Further studies are necessary to pinpoint how secretin induces the release of TGF-β1 from cholangiocytes either through a direct interaction with the TGF-β1/TGF-β1R axis or as direct consequence of enhanced biliary proliferation by secretin. The stimulatory effects of secretin on liver fibrosis may also be indirectly mediated by the activation of macrophages by the NGF released from cholangiocytes by secretin (4). This view is supported by previous studies showing that NGF activates murine macrophages during inflammation (34).

Lastly, we performed studies in human PSC samples. Similar to Mdr2$^{-/-}$ mice, there was up-regulation of the secretin/SR axis in PSC liver tissues. The expression of secretin and SR was limited to the biliary epithelium, which was similar to another study in human liver tissues (9). Similar to the Mdr2$^{-/-}$ model, there was increased expression of fibrotic genes that is consistent with the periductal concentric fibrosis that is a characteristic finding in PSC (35). There was downregulation of let-7a and up-regulation of NGF in PSC samples relative to normal controls suggesting that NGF may play a role in the autocrine pathways driving the activation of biliary proliferation and subsequent fibrogenesis in PSC. In contrast to a previous study (36), there was elevation of serum TGF-β levels in patients with late stage PSC, which may be due to a difference in the disease staging.

Our findings demonstrated that biliary secretin levels are enhanced during cholangiocyte proliferation in Mdr2$^{-/-}$ and BDL mice and PSC samples. In contrast, in the same conditions, secretin serum levels are decreased in human PSC samples and Mdr2$^{-/-}$ mice as a consequence of impaired release from gut S cells. We speculate that: (i) the liver increased the production of biliary secretin (4) and expression of SR possible in response to lower circulating secretin levels; and (ii) the reduced secretin serum levels in patients with PSC may be due to intestinal damage resulting in decreased secretion of secretin. In fact, PSC has been associated with ulcerative colitis and Crohn's disease (37, 38). Another possible

explanation is that the decreased bile secretion in cholestatic mice and PSC may affect fatty acid and bile acid concentration (major determinant of secretin release) (39) in the gut lumen with consequent impaired secretin release from S cells (40).

In conclusion, the secretin/SR axis and let-7a signaling pathway regulates biliary proliferation and hepatic fibrosis (by modulating the TGF-β1/TGF-β1R axis in cholangiocytes and stellate cells) during cholestasis (Suppl. Figure 2). It is critical to maintain a balance of secretin synthesis (from cholangiocytes and S cells) (4) and the expression of the secretin/secretin receptor axis to regulate the homeostasis of the biliary epithelium. While increased secretin synthesis is beneficial to sustain biliary proliferation during ductopenia, inhibition of secretin synthesis is important to reduce biliary hyperplasia and liver fibrosis during aberrant cholangiocyte proliferation. Targeting the secretin/SR axis or the let-7a signaling pathway may provide a therapeutic approach for the treatment of human cholangiopathies including PSC.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ALP	alkaline phosphatase
α-SMA	α-smooth muscle actin
BDL	bile duct ligated
BSA	bovine serum albumin
cAMP	cyclic adenosine 3', 5’-monophosphate
CFTR	cystic fibrosis transmembrane conductance regulator
CK-19	cytokeratin-19
COLA1	collagen, type I, alpha 1
FN-1	fibronectin-1
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
IBDM	intrahepatic bile duct mass

NGF	nerve growth factor
PCNA	proliferating cell nuclear antigen
PSC	primary sclerosing cholangitis
***q*PCR**	real-time PCR
PDGF	platelet-derived growth factor
Sct	secretin
SGOT	glutamic oxaloacetic transaminase
SGPT	glutamate pyruvate transaminases
SR	secretin receptor
TGF-β1	transforming growth factor-β1
TGF-β1R	transforming growth factor-β1 receptor
WT	wild-type

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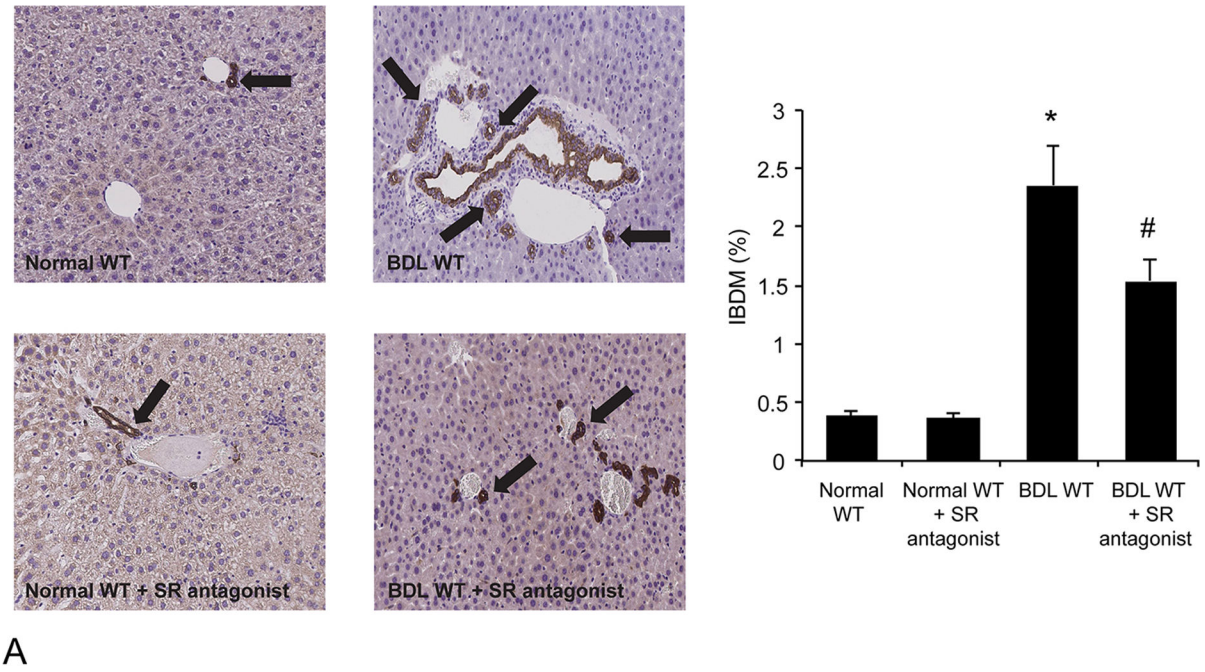

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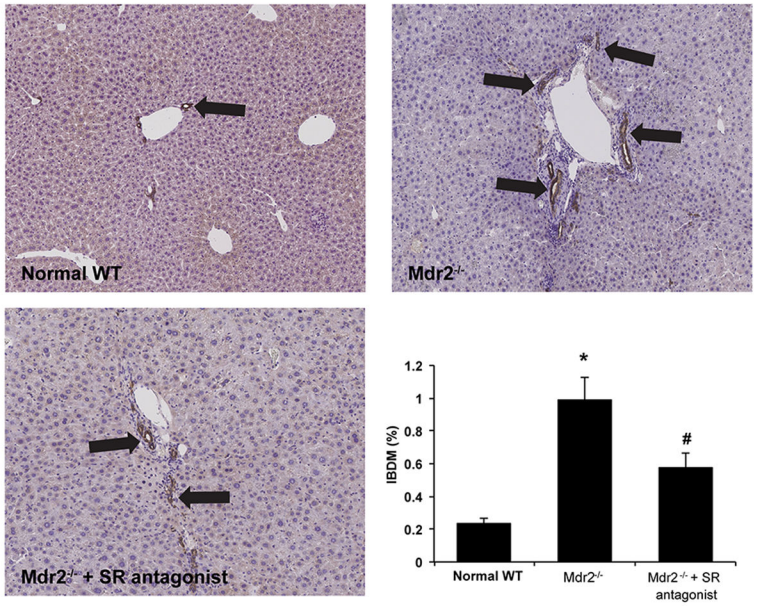


B

Figure 1.

[**A–B**] In BDL WT and Mdr2$^{-/-}$ mice there was increased IBDM compared to normal WT mice. The administration of Sec 5–27 to BDL WT or Mdr2$^{-/-}$ mice decreased IBDM compared to control mice. Ten fields were analyzed from liver samples from 3 different animals. *****p<0.05 vs. normal mice. $^{\#}$p<0.05 vs. BDL WT or Mdr2$^{-/-}$ mice.

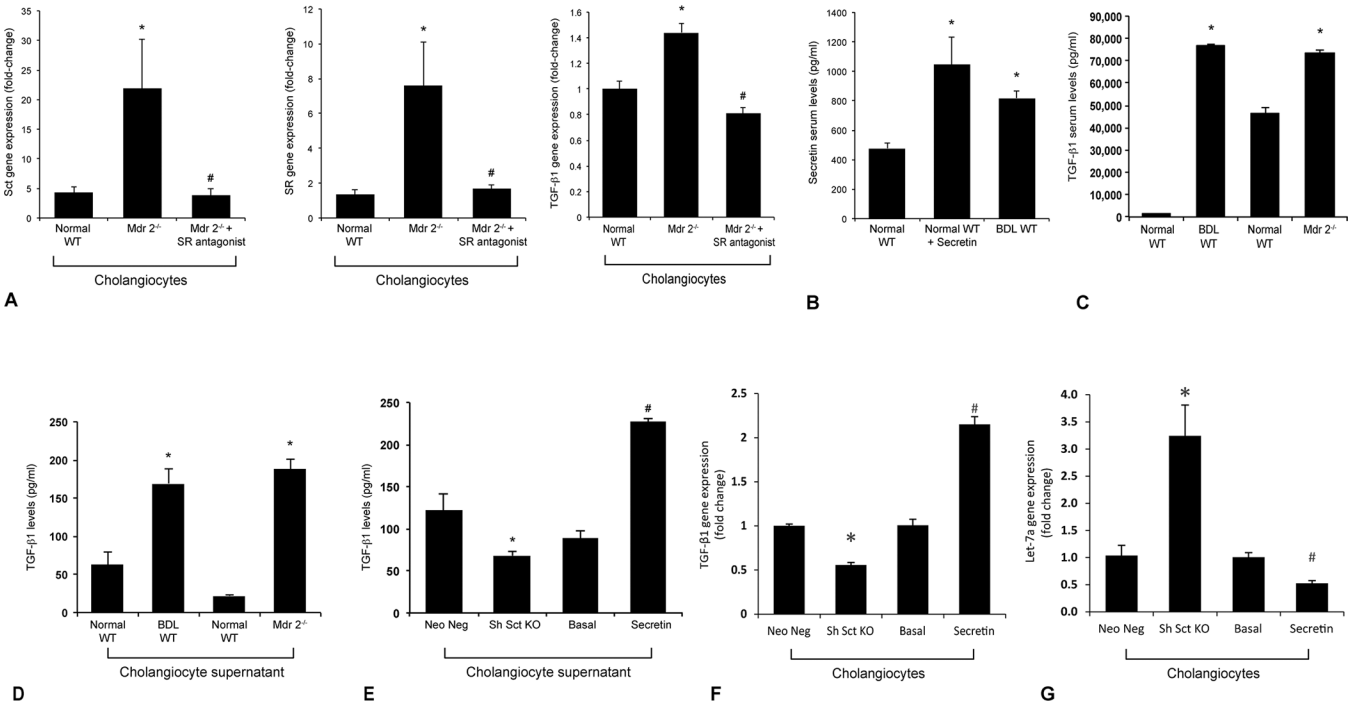
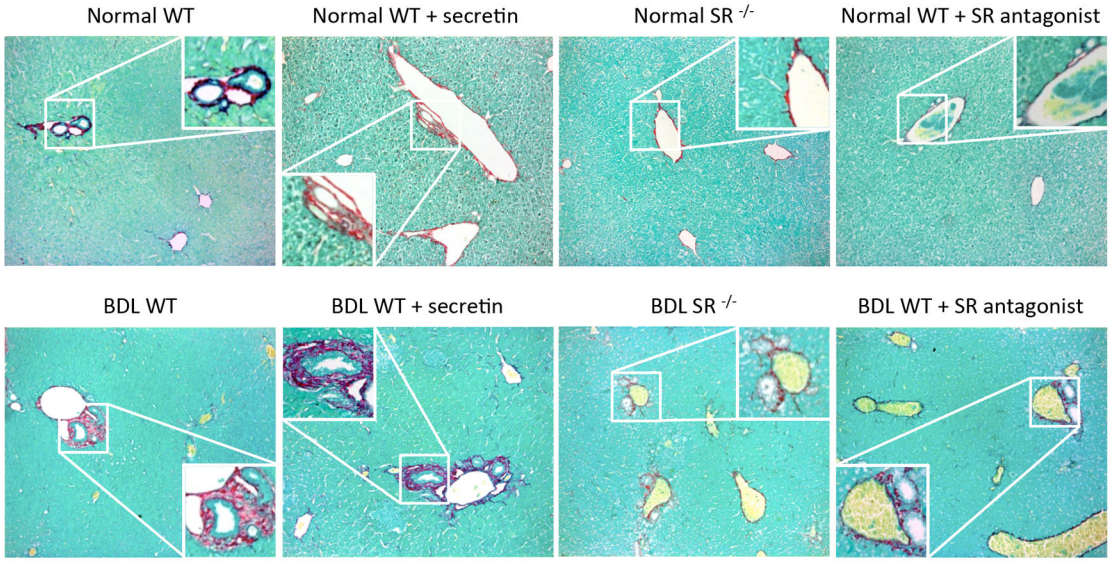


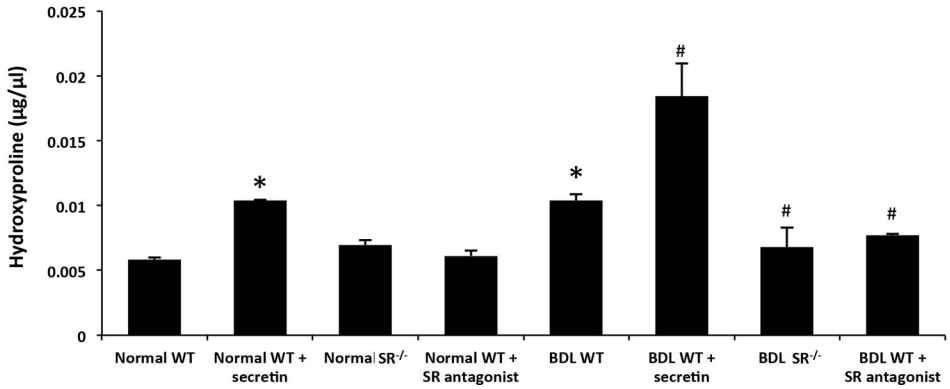
Figure 2.

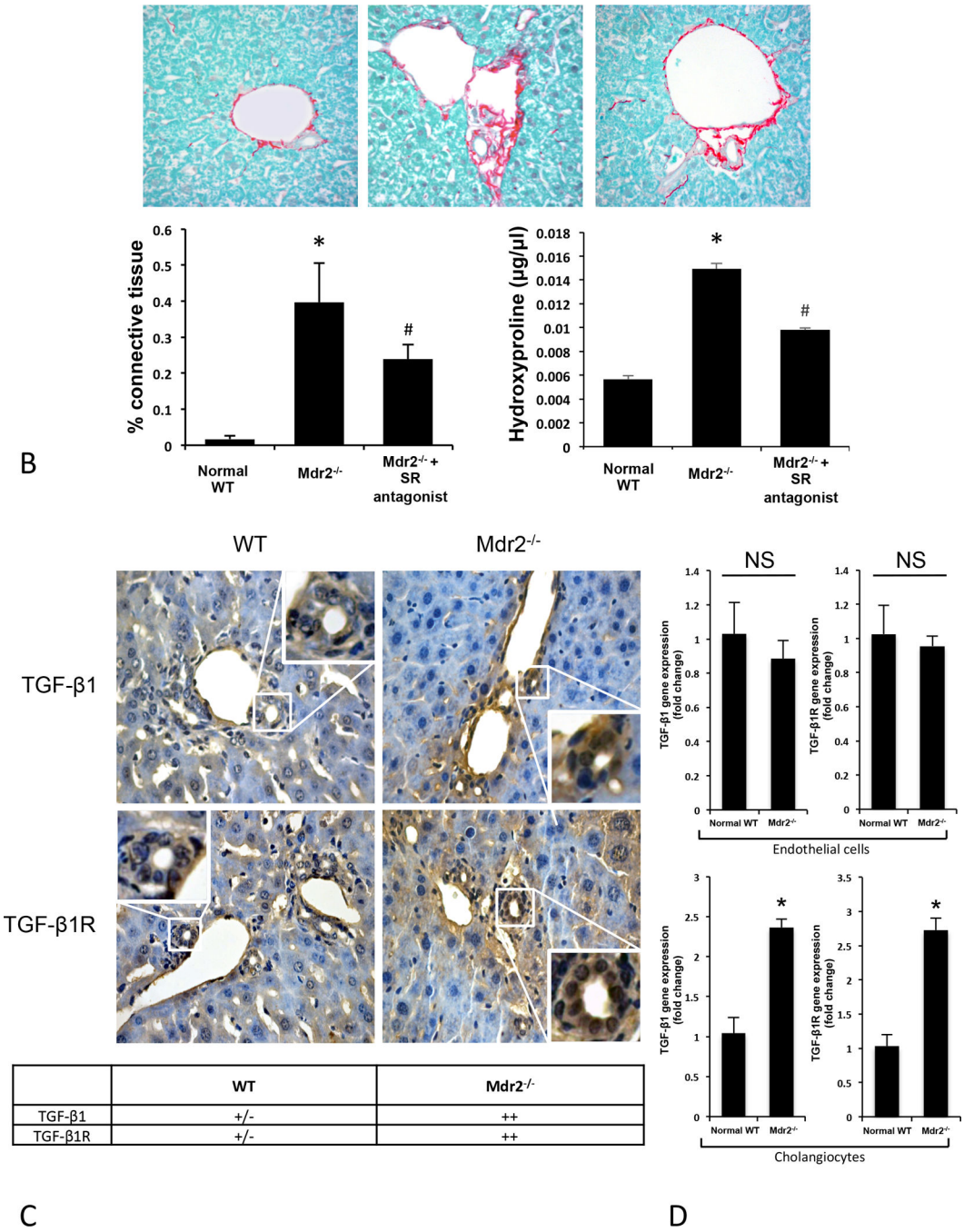
[**A**] The expression of Sct, SR, and TGF-β1 was higher in cholangiocytes from Mdr2$^{-/-}$ mice but similar to that of WT mice in Mdr2$^{-/-}$ mice treated with Sec 5–27. Data are mean ±SEM of 6 evaluations from 3 cumulative preparations of cholangiocytes from 4 mice, n=12. [**B**] Secretin serum levels increased in normal mice treated with secretin and BDL WT mice compared to normal mice. Data are mean±SEM of n=6 from six individual mice. *****p<0.05 vs. normal WT mice. $^{\#}$p<0.05 vs. Mdr2$^{-/-}$ mice. [**C–D**] TGF-β1 levels increased in serum and cholangiocyte supernatant of BDL WT and Mdr2$^{-/-}$ mice compared to WT mice. Data are mean±SEM of 6 evaluations from supernatant from 3 cumulative preparations of cholangiocytes from 4 mice, n=12. *****p<0.05 vs. normal WT mice. [**E–G**] In IMCL lacking Sct there was reduced expression of TGF-β1 levels in IMCL supernatant and TGF-β1 mRNA expression and enhanced expression of let-7a miRNA in IMCL compared to vector-transfected IMCL. Data are mean±SEM of 3 evaluations from 3 preparations of IMCL. *p<0.05 vs. empty vector transfected IMCL (Neo neg). In IMCL treated with secretin there was increased TGF-β1 levels and increased TGF-β1 mRNA expression, but decreased expression of let-7a miRNA compared to basal. Data are mean±SEM of n=3 from 3 preparations of IMCL. $^{\#}$p<0.05 vs. basal.

A



	Normal WT	Normal WT + secretin	Normal SR$^{-/-}$	Normal WT + SR antagonist
% connective tissue	0.24 ± 0.02	0.361 ± 0.05*	0.14 ± 0.01	0.22 ± 0.01
	BDL WT	**BDL WT + secretin**	**BDL SR$^{-/-}$**	**BDL WT + SR antagonist**
% connective tissue	4.06 ± 0.06*	4.91 ± 0.22$^{\#}$	2.07 ± 0.09$^{\#}$	2.55 ± 0.20$^{\#}$





	WT	Mdr2$^{-/-}$
TGF-β1	+/-	++
TGF-β1R	+/-	++

Figure 3.

[**A**] BDL WT mice display higher fibrotic tissue compared to normal mice. Secretin increased the percentage of connective tissue in normal and BDL WT mice. In BDL SR$^{-/-}$ mice and BDL WT mice treated with Sec 5–27 there was reduced collagen deposition compared to BDL WT mice. Ten fields were analyzed from 3 samples from 3 different animals. Orig. magn., ×10. The hydroxyproline content match the data on liver fibrosis by Sirius red staining; we used 3 different liver samples from 3 different animals. *****p<0.05 vs. normal mice. $^{\#}$p<0.05 vs. BDL mice. [**B**] In Mdr2$^{-/-}$ treated with Sec 5–27 there was

reduced collagen deposition compared to Mdr2$^{-/-}$ mice. *p<0.05 vs. normal WT mice. $^{\#}$p<0.05 vs. Mdr2$^{-/-}$ mice. The hydroxyproline content match the data on liver fibrosis by Sirius red staining. Orig. magn., ×40. [**C**] We demonstrated increased immunostaining for TGF-β1 and TGF-β1R in Mdr2$^{-/-}$ compared to normal mice. Orig. magn., ×40. [**D**] There was enhanced expression of TGF-β1 and TGF-β1R in LCM-isolated cholangiocytes (but not endothelial cells) from Mdr2$^{-/-}$ compared to normal WT mice. *****p<0.05 vs. normal mice.

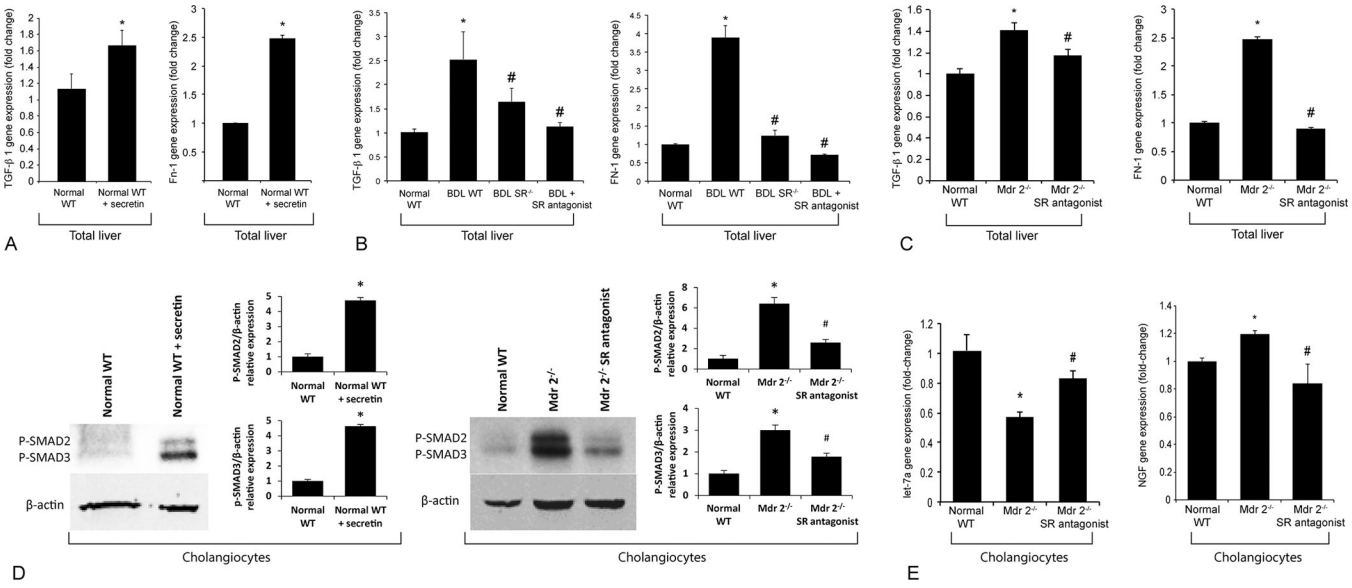


Figure 4.

[**A**] Secretin increased the expression of TGF-β1 and FN-1 in normal WT mice. [**B–C**] In BDL WT and Mdr2$^{-/-}$ mice, there was increased expression of TGF-β1 and FN-1 compared to normal mice that was blocked by Sec 5–27 or knockout of SR. [**D**] Secretin increased the phosphorylation of SMAD2,3 in cholangiocytes from normal WT mice. In cholangiocytes from Mdr2$^{-/-}$ mice there was increased phosphorylation of SMAD2,3 compared to normal mice that was reduced by Sec 5–27. [**E**] There was decreased expression of miRNA let-7a and enhanced expression of NGF in cholangiocytes from Mdr2$^{-/-}$ compared to normal mice, changes that were prevented by Sec 5–27. PCR data (mean±SEM of 6 evaluations) in liver samples from 6 separate animals. PCR data (mean±SEM of 3 evaluations) in cholangiocytes from 3 cumulative cells preparations from 4 mice, n=12. Immunoblots for SMAD2/3 in cholangiocytes from 3 separate animals; mean±SEM n=3. *p<0.05 vs. normal mice. #p<0.05 vs. BDL WT or Mdr2$^{-/-}$ mice.

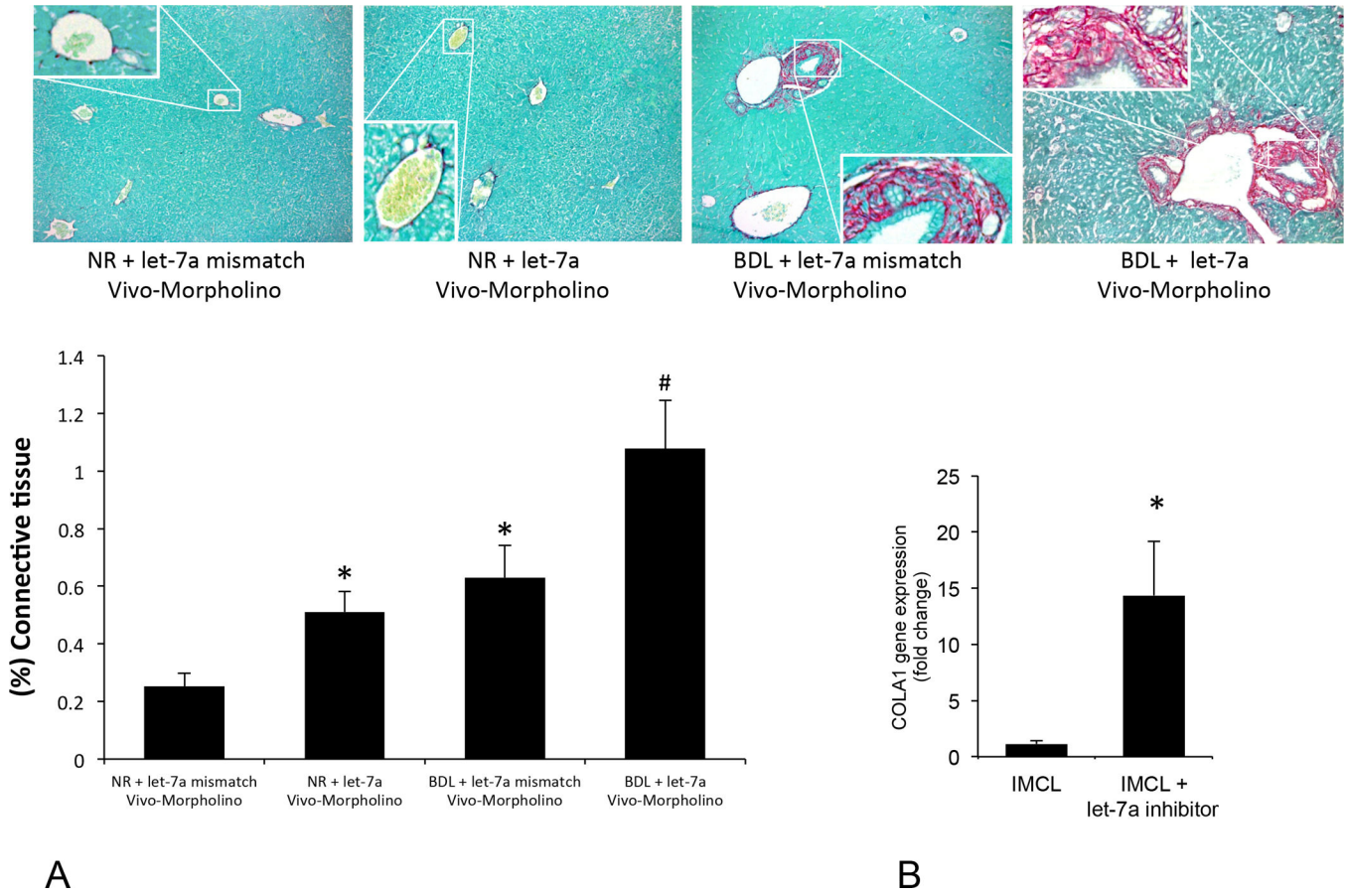
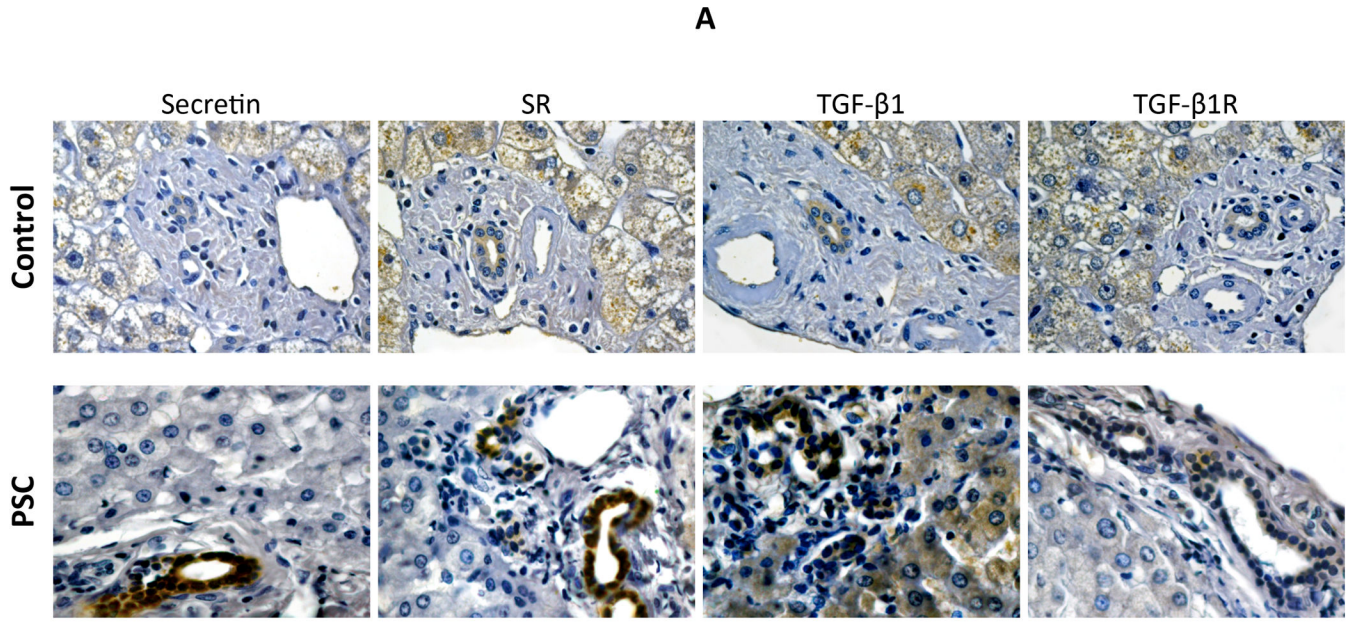


Figure 5.

[**A**] There was enhanced collagen deposition in normal and BDL WT mice treated with miRNA let-7a Vivo-Morpholino compared to control mice. Ten fields were analyzed from 3 samples from 3 different animals. Orig. magn., ×10. *****p<0.05 vs. control mice. #p<0.05 vs. mismatch-treated BDL WT mice. NR WT= normal wild-type. [**B**] There was increased mRNA expression of COLA1 in cholangiocyte lines treated with let-7a inhibitor compared to basal. Data are mean±SEM of 3 evaluations from 3 preparations of IMCL. *****p<0.05 vs. basal values.



	Secretin	SR	TGF-β1	TGF-β1R
Control	+/-	+	+	+/-
PSC	+++	+++	+++	++

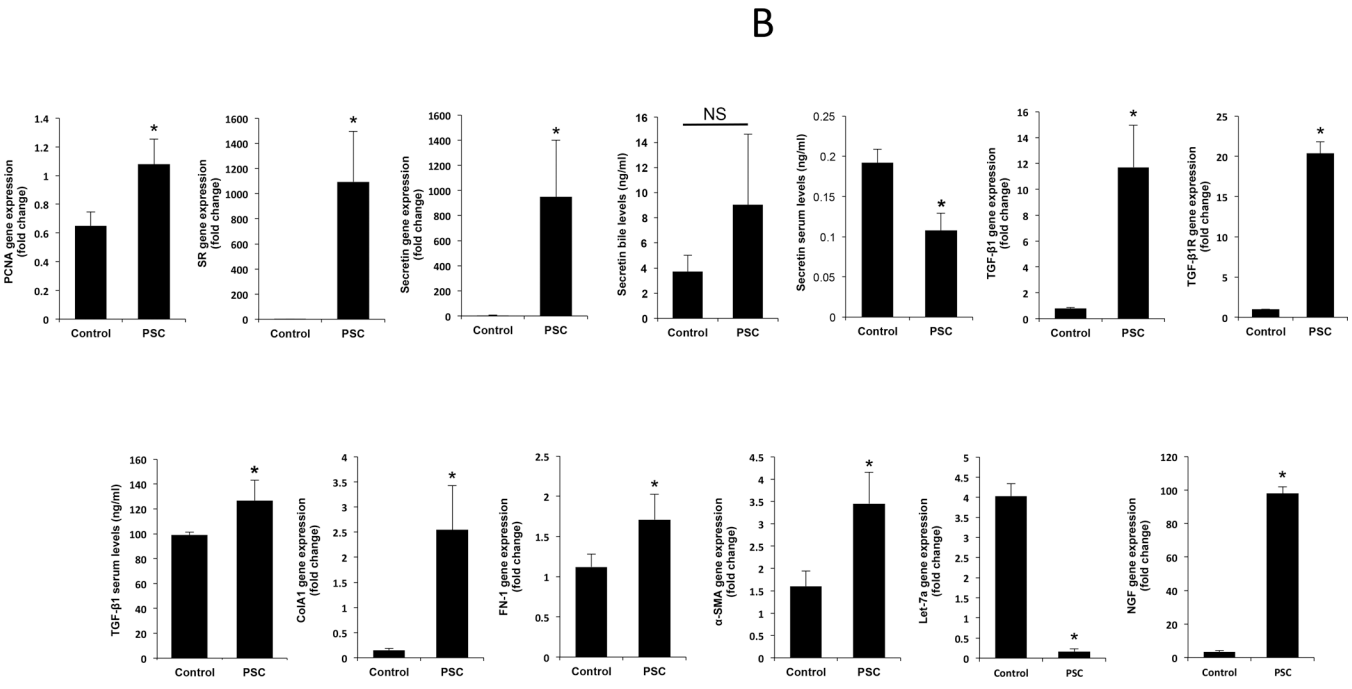


Figure 6.

[**A**] In PSC liver sections there was increased immunoreactivity for Sct, SR, TGF-β1 and TGF-β1R compared to normal samples. Semiquantitative analysis was performed in 5 fields in one section obtained from one normal and one PSC patient. Orig. magn., ×40. [**B**] There was: (i) increased mRNA expression of PCNA, Sct, SR, TGF-β1, TGF-β1R, FN-1, COLA1, α-SMA and NGF; enhanced levels of secretin in bile (n=9 control; n=5 PSC patients, early and advanced stage) and TGF-β1 (n=5 control; n=7 PSC samples, early and advanced stage) in serum and reduced secretin levels in serum (n=67 control; n=21 PSC patients, early and advanced stage); and (iii) decreased expression of miRNA let-7a in PSC samples compared to control samples. Data are mean±SEM of 3 PCR reactions from 3 different samples from 3 normal and 3 PSC patients. *****$p<0.05$ vs. control human samples.

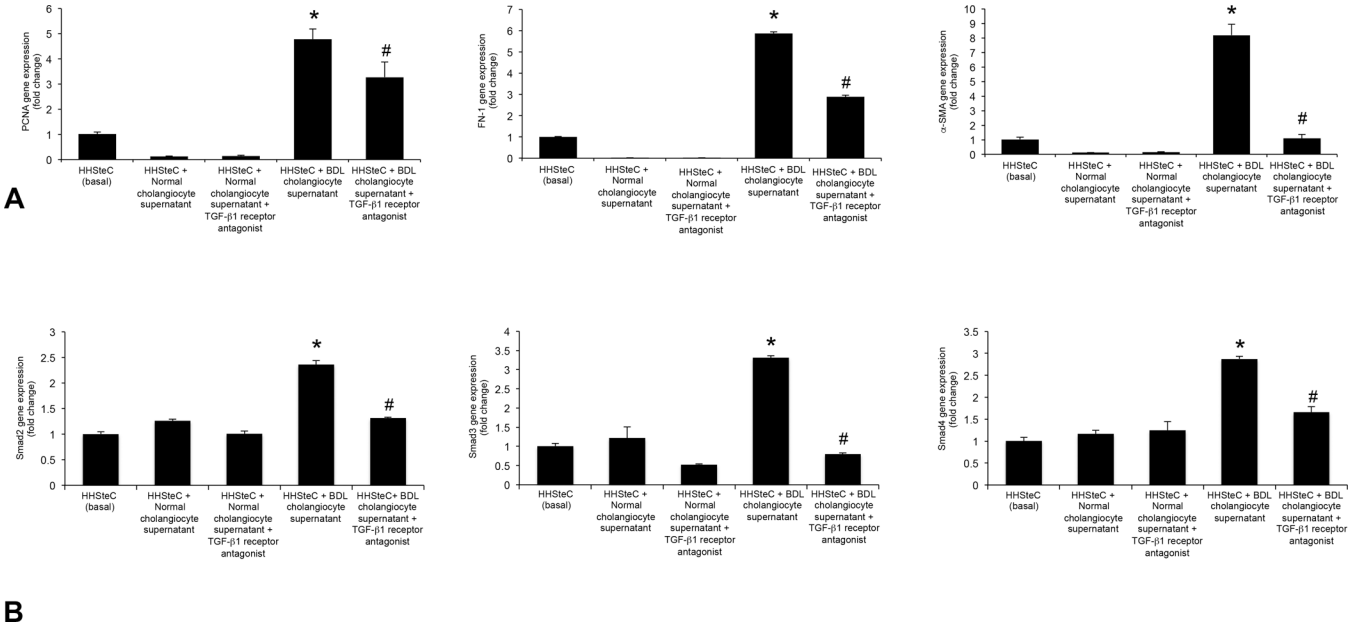


Figure 7.

There was increased expression of PCNA, FN-1, α-SMA and SMAD2,3,4 when HHSteC were treated with BDL cholangiocyte supernatant, increase that was reduced by LY2109761. Data are mean±SEM of n=3 from 3 preparations of IMCL. *$p<0.05$ vs. basal. $^{\#}p<0.05$ vs. HHStec treated with BDL cholangiocyte supernatant.