Published in final edited form as:
Hepatology. 2010 July ; 52(1): 204–214. doi:10.1002/hep.23657.

Knockout of secretin receptor reduces large cholangiocyte hyperplasia in mice with extrahepatic cholestasis induced by bile duct ligation

Shannon Glaser[2,4,#], **Ian P Lam**[6,#], **Antonio Franchitto**[5], **Eugenio Gaudio**[5], **Paolo Onori**[7], **Billy K Chow**[6], **Candace Wise**[2,3,4], **Shelley Kopriva**[2,4], **Julie Venter**[2,4], **Mellanie White**[2,4], **Yoshiyuki Ueno**[9], **David Dostal**[4], **Guido Carpino**[8], **Romina Mancinelli**[4,5], **Valorie Chiasson**[4], **Sharon DeMorrow**[2,4], **Heather Francis**[2,3,4], and **Gianfranco Alpini**[1,2,4]

[1] Research, Central Texas Veterans Health Care System, Texas A&M Health Science Center, College of Medicine, Temple, TX 76504

[2] Scott & White Digestive Disease Research Center, Scott & White, Texas A&M Health Science Center, College of Medicine, Temple, TX 76504

[3] Division of Research and Education, Scott & White, Texas A&M Health Science Center, College of Medicine, Temple, TX 76504

[4] Department of Medicine, Division Gastroenterology, Texas A&M Health Science Center, College of Medicine, Temple, TX 76504

[5] Dept. Human Anatomy, University of Rome “La Sapienza”, Rome, Italy

[6] School of Biological Sciences, The University of Hong Kong, Pokfulam Road, Hong Kong, China

[7] Experimental Medicine, University of L’Aquila, L’Aquila, Italy

[8] Dept Health Science, University of Rome “Foro Italico”, Italy

[9] Div. Gastroenterol, Tohoku University Graduate School of Medicine, Aobaku, Sendai, Japan

Abstract

During bile duct ligation (BDL), the growth of large cholangiocytes is regulated by the cAMP/ERK1/2 pathway and is closely associated with increased secretin receptor (SR) expression. Although it has been suggested that the SR modulates cholangiocyte growth, direct evidence for secretin-dependent proliferation is lacking. $SR^{+/+}$ (wild-type, WT) or SR knockout ($SR^{-/-}$) mice underwent sham surgery or BDL for 3 or 7 days. We evaluated: (i) SR expression, cholangiocyte proliferation, and apoptosis in liver sections; and (ii) PCNA protein expression and ERK1/2 phosphorylation in purified large cholangiocytes from WT and $SR^{-/-}$ BDL mice. Normal WT mice were treated with secretin (2.5 nmoles/Kg BW/day by osmotic minipumps for 1 week) and biliary mass was evaluated. Small and large cholangiocytes were used to evaluate the *in vitro* effect of secretin (100 nM) on proliferation, PKA activity and ERK1/2 phosphorylation. SR expression was also stably knocked down by shRNA, and basal and secretin-stimulated cAMP

Address correspondence to: Gianfranco Alpini, Ph. D., VA Research Scientist Career Award Recipient, Professor, Medicine, Dr. Nicholas C. Hightower Centennial Chair of Gastroenterology, Director of the Scott & White Digestive Diseases Research Center, Research Central Texas Veterans Health Care System, Scott & White and Texas A & M Health Science Center College of Medicine, Medical Research Building, 702 SW H.K. Dodgen Loop, Temple, TX, 76504, Phone: 254-742-7044 or 254-742-7046, Fax: 254-724-9278 or 254-724-5944, galpini@tamu.edu or galpini@medicine.tamhsc.edu. Shannon S. Glaser, Ph.D., Assistant Professor, Department of Medicine, Texas A&M Health Science Center, Scott & White Hospital, Medical Research Building, 702 SW H.K. Dodgen Loop, Temple, TX, 76504, Phone: (254) 742-7058, Fax: (254) 724-5944, sglaser@medicine.tamhsc.edu.
[#]Dr. Glaser and Lam share the same authorship.

levels (a functional index of biliary growth) and proliferation were determined. SR was expressed by large cholangiocytes. Knockout of SR significantly decreased large cholangiocyte growth induced by BDL, which was associated with enhanced apoptosis. PCNA expression and ERK1/2 phosphorylation were decreased in large cholangiocytes from $SR^{-/-}$ BDL compared to WT BDL mice. *In vivo* administration of secretin to normal WT mice increased ductal mass. *In vitro*, secretin increased proliferation, PKA activity and ERK1/2 phosphorylation of large cholangiocytes that was blocked by PKA and MEK inhibitors. Stable knockdown of SR expression reduced basal cholangiocyte proliferation. SR is an important trophic regulator sustaining biliary growth.

Conclusion—The current study provides strong support for the potential use of secretin as a therapy for ductopenic liver diseases.

Keywords

Biliary epithelium; cAMP; gastrointestinal hormones; heterogeneity; MAPKinase

---

Cholangiocytes line the intrahepatic biliary system, which modifies the bile of canalicular origin into its final composition before reaching the small intestine (1,2). Several gastrointestinal peptides/hormones including bombesin, gastrin and secretin regulate cholangiocyte secretory activity (1–3). Among these factors, secretin plays a key role in the biliary secretion of water and bicarbonate since its receptor (SR) is expressed in rodent and human liver by larger bile ducts (1,4–6). In large cholangiocytes, secretin increases cyclic adenosine 3′, 5′-monophosphate (cAMP) levels (1,4,5,7,8) and induces the opening of the Cl^- channel (cystic fibrosis transmembrane conductance regulator, CFTR) (9) leading to the activation of the Cl^-/HCO_3^- anion exchanger 2 (AE2) (10) and secretion of bicarbonate in bile (2,3).

Human cholangiocytes are the target cells in several cholangiopathies including primary biliary cirrhosis (PBC) and primary sclerosing cholangitis (PSC), diseases associated with dysregulation of the balance between cholangiocyte proliferation/apoptosis (11). Rodent cholangiocytes, which are normally mitotically quiescent (12,13), markedly proliferate in animal models of cholestasis including extrahepatic bile duct obstruction (BDL) or acute carbon tetrachloride (CCl_4) administration (12,14). The proliferative response of the intrahepatic biliary epithelium to BDL is heterogeneous since large (but not small) cholangiocytes proliferate by activation of cAMP-dependent ERK1/2 signaling (12,15) leading to enhanced ductal mass (5,12,14).

Since SR is only expressed by large cholangiocytes in the liver (1,4,5,9,12,14), changes in the functional expression of this receptor have been suggested as a pathophysiological tool for evaluating changes in the degree of cholangiocyte growth/loss (5,12,14). Indeed, we have shown that: (i) cholangiocyte hyperplasia (e.g., after BDL or 70% hepatectomy) is associated with enhanced SR expression and secretin-stimulated cAMP levels and bicarbonate secretion (12,13,16–18); and (ii) cholangiocyte damage (e.g., after CCl_4) decreases the functional expression of SR in large cholangiocytes (14). In pathological conditions, such as the CCl_4 model, which is characterized by lack or damage of the hormonally responsive large cholangiocytes, small cholangiocytes proliferate and express SR *de novo* (14).

The hormonal actions of secretin through SR have been studied in the pancreas, stomach and biliary epithelium (19). Although it has been suggested that SR modulates cholangiocyte growth (2,12–14), the direct link between SR expression and its possible role in the regulation of biliary proliferation has not been elucidated. The aim of our study was to

determine the role that SR plays in sustaining large cholangiocyte growth during cholestasis induced by BDL.

Materials and Methods

Materials

Reagents were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise stated. The nuclear dye 4,6-diamidino-2-phenylindole (DAPI) was obtained from Molecular Probes, Inc., Eugene, OR. Porcine secretin was purchased from Peninsula Laboratories (Belmont, CA). The polyclonal secretin receptor antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA) was raised against a peptide mapping at the C-terminus of secretin receptor of human origin and cross-reacts with mouse (20). The antibody against proliferating cell nuclear antigen (PCNA) was purchased from Santa Cruz Biotechnology. The mouse anti-cytokeratin-19 (CK-19) antibody was purchased from Caltag Laboratories Inc. (Burlingame, CA, USA). The goat p-ERK1/2 and total ERK1/2 (44-42 kDa) polyclonal affinity purified antibodies were purchased from Santa Cruz Biotechnology Inc. The RIA kits for the determination of intracellular cAMP levels in cholangiocytes were purchased from Perkin Elmer, Shelton, CT.

Animal Models

All animal experiments (see Table 1) were performed in accordance with a protocol approved by the Scott and White and Texas A&M Health Science Center Institutional Animal Care and Use Committee and conform to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Our $SR^{+/+}$ (wild-type, WT) or secretin receptor knockout (KO, $SR^{-/-}$) (21) mice were maintained in a temperature-controlled environment (20–22°C) with a 12:12-hour light-dark cycle. We used adult male WT and $SR^{-/-}$ mice (approximately 25–30 gm in weight) of the N5 generation: (i) as normal treated with saline (0.9% NaCl) or secretin (2.5 nmol/Kg body weight per day, a dose similar to that used by us for another gastrointestinal hormone, gastrin, in rodents) (18) by intraperitoneally implanted Alzet® osmotic minipumps (Alzet, CA) for 7 days; or (ii) for sham-operation or BDL (for 3 and 7 days) (5,20,22). Since our previous studies (21) described that $SR^{-/-}$ mice have a renal defect in water reabsorption and associated polyuria and polydipsia, experiments were performed to determine whether the response of $SR^{-/-}$ mice to BDL was due to the lack of SR, rather than severe dehydration. Thus, we evaluated changes in body weight and mortality rate in the experimental groups of Table 1. In addition, both WT and $SR^{-/-}$mice (post-BDL or administration of secretin) received oral hydration therapy, consisting of up to 1 ml of normal saline subcutaneously up to twice daily along with water in gel form on the ground and food supplements. Since there were no differences in cholangiocyte proliferation between normal WT and normal $SR^{-/-}$ mice and their corresponding sham mice, we did not show the results from the sham animals.

Immortalized and Freshly Isolated Cholangiocytes

The *in vitro* studies were performed in freshly isolated or immortalized (5,8) large cholangiocytes. The rationale for performing these studies only in large cholangiocytes is based on the fact that secretin stimulated *in vivo* the proliferation of only large bile ducts (results section); and following BDL, large but not small cholangiocytes proliferate (5). Freshly isolated large cholangiocytes (~99% by CK-19 immunohistochemistry) (5,20) were purified by centrifugal elutriation (4,9,14) followed by immunoaffinity separation by a monoclonal antibody, rat IgG_{2a} (provided by Dr. R. Faris, Brown University, Providence, RI), against an antigen expressed by all mouse cholangiocytes (5). Our large mouse

cholangiocyte lines, which display morphological, phenotypic and functional features similar to that of freshly isolated large cholangiocytes were cultured as described (5,8,9).

Evaluation of Secretin Receptor Expression

We evaluated the expression of SR by immunohistochemistry in paraffin-embedded liver sections from the experimental groups of Table 1. Since immunohistochemistry shows that only large bile ducts from WT (but not $SR^{-/-}$) animals express SR (results section), we evaluated the expression of SR by immunofluorescence and real-time PCR in freshly isolated large cholangiocytes from normal and 3- and 7-day BDL WT mice. Semiquantitative immunohistochemical analysis of SR expression in sections was performed as described (5). Light microscopy photographs of liver sections were taken by Leica Microsystems DM 4500 B Light Microscopy (Weltzlar, Germany) with a Jenoptik Prog Res C10 Plus Videocam (Jena, Germany). Immunofluorescence for SR was also performed in large cholangiocytes from normal and 3- and 7-day BDL WT mice (5,20). Images were visualized using an Olympus IX-71 confocal microscope. For all immunoreactions, negative controls (with normal serum from the same species substituted for the primary antibody) were included.

In freshly isolated large cholangiocytes from normal and BDL WT mice, mRNA and protein expression of SR were evaluated by real-time PCR (23) and Western blot analysis, respectively (20). For real-time PCR, RNA was extracted from cholangiocytes by the RNeasy Mini Kit (Qiagen Inc, Valencia, CA), and reverse transcribed using the Reaction Ready™ First Strand cDNA synthesis kit (SuperArray, Frederick, MD). These reactions were used as templates for the PCR assays using a SYBR Green PCR master mix and specific primers designed against the mouse secretin receptor gene NM_001012322 (24), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), the housekeeping gene (SuperArray, Frederick, MD) in the real-time thermal cycler (ABI Prism 7900HT sequence detection system). A ΔΔCT (delta delta of the threshold cycle) analysis was performed using normal large cholangiocytes as the control sample. Data are expressed as fold-change of relative mRNA levels ± SEM (n=6).

Evaluation of Liver Histology, Cholangiocyte Apoptosis and Proliferation

All liver sections were scored by two board-certified pathologists, blinded to the identity of the samples. Lobular necrosis was evaluated in liver sections stained for hematoxylin and eosin (25). Lobular necrosis was scored as follows: − = 0 foci; +/− = <2 foci; + = 2–4 foci; and ++ = >4 foci (25). Sections were examined in a coded fashion by BX-51 light microscopy (Olympus, Tokyo, Japan) equipped with a camera. We measured in liver sections: (i) the percentage of cholangiocyte apoptosis by semiquantitative terminal deoxynucleotidyltransferase biotin-dUTP nick-end labeling (TUNEL) kit (Apoptag; Chemicon International, Inc.); (ii) cholangiocyte proliferation by evaluation of the percentage of small and large cholangiocytes positive for PCNA (5), and (iii) intrahepatic bile duct mass (IBDM) (5) of small (<15 μm diameter) (1) and large (>15 μm diameter) (1) bile ducts. The IBDM was measured as area occupied by CK-19 positive-bile duct/total area × 100. Proliferation was evaluated by immunoblots (20) for PCNA in protein (10 μg) from lysate from spleen (positive control) and large cholangiocytes from WT and $SR^{-/-}$ BDL mice. Blots were normalized by β-actin (5). The intensity of the bands was determined by scanning video densitometry using the Storm 860 and the ImageQuant TL software version 2003.02 (GE Healthcare, Little Chalfont, Buckinghamshire, England).

Measurement of cAMP Levels and Phosphorylation of ERK1/2

These experiments were performed in large cholangiocytes from WT and $SR^{-/-}$ 7-day BDL mice, a time period where a marked ductal hyperplasia is observed (2,12). We evaluated

basal and secretin-stimulated cAMP levels (a functional parameter of cholangiocyte growth) (13,18) by commercially available RIA kits (20); and phosphorylation of ERK1/2 by immunoblots in protein (10 μg) from cholangiocyte lysate. The intensities of the bands were determined by scanning video densitometry using the phospho-imager.

In Vitro Effect of Secretin on the Proliferation, PKA Activity and ERK1/2 Phosphorylation of Large Cholangiocytes

Our small (negative control) and large cholangiocytes (8) were treated at 37°C with: (i) 0.2% BSA (basal) or secretin (100 nM) for 48 hours in the absence or presence of pre-incubation (1 hour) with H89 (PKA inhibitor, 30 μM) or PD98059 (MEK inhibitor, 10 nM) before evaluating proliferation by CellTiter 96 Cell Proliferation Assay (20) (Promega Corp., Madison, WI). Absorbance was measured at 490 nm on a microplate spectrophotometer (Molecular Devices, Sunnyvale, CA). Data were expressed as the fold-change of treated cells as compared to vehicle-treated controls. In separate experiments, large cholangiocytes were treated with (i) 0.2% BSA (basal) or secretin (100 nM) for 6 hours in the absence/presence of H89 (30 μM) or PD98059 (10 nM) before evaluating PCNA expression by immunoblots (5), activity of PKA (20) and phosphorylation of ERK1/2 by immunoblots (5). The intensity of the bands was determined as described above.

Stable Transfected Knockdown of Secretin Receptor in Large Cholangiocytes

To provide conclusive evidence that SR is a key pro-proliferative regulator sustaining large cholangiocyte growth, we stably knocked down the expression of this receptor in large cholangiocyte lines (8). The mouse cell line lacking SR was established using SureSilencing shRNA (Super-Array, Frederick, MD) plasmid for mouse SR containing a marker for neomycin resistance for the selection of stably transfected cells, according to the instructions provided by the vendor as described (23). A total of 4 clones were assessed for the relative knockdown of the SR gene using real time PCR and a single clone with the greatest degree of knockdown was selected for subsequent experiments. In selected and mock-transfected clones, the degree of SR knockdown was also evaluated by FACS analysis and Western blot analysis, as we have previously described (26).

Then, the two cell lines, mock-transfected clone (transfected with control vector) and the SR knockdown clone (80% knockdown efficiency of the message by real-time PCR (not shown) and 50% knockdown of protein expression by FACS (see Figure 6B) were treated with: (i) 0.2% BSA (basal) or secretin (100 nM for 5 minutes) before evaluation of cAMP levels by RIA (4, 7, 9, 18); or (ii) 0.2% BSA (basal) or secretin (100 nM) before measuring proliferation by MTS assay (48-hour incubation). The mock-transfected and SR knockdown clones in large cholangiocytes were incubated in culture medium before evaluating basal proliferative activity by MTS proliferation assay (after incubation for 6, 24, 48 and 72 hours).

Statistical Analysis

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

Results

Evaluation of Secretin Receptor Expression

In liver sections, we demonstrated that large but not small bile ducts from normal and BDL WT mice express SR (Figure 1A and Table 1). The expression of SR in large bile ducts was higher in: (i) normal WT mice treated with secretin compared to saline-treated mice (Table

1); and (ii) WT BDL compared to normal WT mice (Table 1). There was no positive staining for SR in bile ducts from normal and BDL $SR^{-/-}$ mice (Figure 1A). The expression of SR was confirmed by immunofluorescence in large cholangiocytes purified from normal and BDL WT mice (Figure 1B). By real-time PCR and immunoblots, the expression of SR mRNA and protein was higher in large BDL cholangiocytes compared to normal large cholangiocytes (Figure 1C–D).

Evaluation of Liver Weight, Lobular Necrosis, Cholangiocyte Apoptosis and Proliferation

No significant differences in body weight and mortality rates were observed among the experimental groups of Table 1. No difference in lobular necrosis was observed in normal WT and $SR^{-/-}$ mice, whereas the typical necrosis present in the BDL model showed only a smaller increase (not significant) in $SR^{-/-}$ BDL compared to WT BDL mice. The chronic administration of secretin to normal WT mice increased the percentage of large PCNA-positive cholangiocytes and large IBDM compared to normal WT mice treated with saline (Figures 2A–B and Table 1); secretin did not increase the proliferation of small ducts that do not express SR (not shown) (5). In normal $SR^{-/-}$ mice, secretin did not induce changes in cholangiocyte proliferation or apoptosis (Figures 2A–B and Table 1). Following BDL, there was an increase in the percentage of PCNA expressing cholangiocytes and IBDM in large bile ducts compared to normal mice (Figures 3A–B and Table 1). Similar to previous studies (16), large IBDM was enhanced in parallel with the increased duration of BDL (Figure 3B and Table 1). Knockout of SR reduces large cholangiocyte proliferation and large IBDM induced by BDL (5,20) compared to WT BDL mice (Figures 3A–B and Table 1).

Evaluation of Proliferation, cAMP Levels, and Phosphorylation of ERK1/2 in Isolated Large Cholangiocytes

In large cholangiocytes from 7-day $SR^{-/-}$ BDL mice, there was decreased PCNA expression compared to cholangiocytes from WT BDL mice (Figure 4A). Basal cAMP levels of large cholangiocytes from $SR^{-/-}$ BDL mice were significantly lower than the corresponding levels of cholangiocytes from WT BDL mice (Figure 4B). Secretin increased cAMP levels of large cholangiocytes from WT (but not $SR^{-/-}$) BDL mice (Figure 4B). In large cholangiocytes from $SR^{-/-}$ BDL mice, there was a decreased ERK1/2 phosphorylation compared to large cholangiocytes from WT BDL mice (Figure 4C).

Secretin Stimulates *in Vitro* Large Cholangiocyte Proliferation

Large (but not small) cholangiocytes proliferate after the administration of secretin (Figure 5A). Secretin-stimulation of large cholangiocyte proliferation was blocked by H89 and partially by the MEK inhibitor, PD98059 (Figure 5A). Secretin increased PCNA expression of large cholangiocytes, an increase that was blocked by H89 and PD98059 (Figure 5B). There was increased PKA activity (Figure 5C) and ERK1/2 phosphorylation (Figure 5D) in large cholangiocytes treated with secretin compared to BSA-treated cells.

Silencing of the Secretin Receptor Gene Decreases the Proliferative Capacity of Large Cholangiocytes

The knock down of SR protein expression by 50%, as demonstrated by FACS (Figure 6B), was confirmed by Western blot analysis (Figure 6A). When we knocked-down the gene for SR in large cholangiocytes, secretin did not increase cAMP levels (Figure 6C) and proliferation (Figure 6D, 48 hours of incubation) in these cells compared to the increase shown in large mock-transfected cholangiocytes. In support of the hypothesis that SR is a key trophic regulator in the regulation of biliary growth, there was a decrease in the basal proliferative capacity (Figure 7) of SR-silenced large cholangiocytes compared to large mock-transfected cholangiocytes.

Discussion

In our study, we show that SR is an important trophic regulator sustaining large cholangiocyte proliferation during extrahepatic cholestasis. In the $SR^{-/-}$ mouse model, we show that proliferation of large cholangiocytes (12,14) is reduced (~50%) during BDL compared to BDL wild-type mice, concomitant with elevation of biliary apoptosis. The reduction of cholangiocyte hyperplasia was associated with a decrease in both basal and secretin-stimulated cAMP levels and phosphorylation of ERK1/2 in large cholangiocytes compared to BDL cholangiocytes. *In vitro*, secretin increased the proliferation of large cholangiocytes by activation of cAMP⇒PKA⇒ERK1/2 signaling. Silencing of the SR gene induces a decrease in the basal proliferative capacity of large cholangiocytes compared to large mock-transfected cholangiocytes.

In our evaluation of SR expression, we found a time-dependent increase in the expression of SR in large cholangiocytes during BDL compared to normal large cholangiocytes. This finding was consistent with previous studies showing that: (i) in the rodent liver SR is only expressed by large cholangiocytes (1,4,5,9,12); (ii) SR expression is upregulated following BDL ligation in large cholangiocytes (14,17); and (iii) the extent of secretin effects on cholangiocyte functions parallel with the duration of BDL (16). This finding parallels recent findings that: (i) mouse cholangiocytes share a similar heterogeneous profile as rat cholangiocytes (5); and (ii) freshly isolated and immortalized large mouse cholangiocytes are the only cell types to express the SR (5,8,14). In human, SR expression is present in the biliary tract in normal bile ducts and ductules and the majority of cholangiocarcinomas, but is not present in hepatocytes or hepatocellular carcinoma (26,27). Consistent with animal models of cholestasis, SR expression was upregulated in ductular reactions in liver cirrhosis (27).

In our *in vivo* model, the level of the reduction of cholangiocyte proliferation is consistent with the paradigm that cholangiocyte proliferation is regulated in autocrine and paracrine mechanisms by a number of stimulatory neuro-hormonal factors (18,20,28). In a knockout mouse model for alpha-calcitonin gene related peptide (α-CGRP), the lack of circulating α-CGRP also reduces biliary proliferation during BDL to a similar degree as the lack of SR (20), which indicates that the regulation of biliary proliferation during extrahepatic cholestasis is multifactorial and a complex regulatory system (18,20,28).

The trophic effects of secretin were dependent upon the activation of the cAMP/PKA/ERK1/2 signaling. The second messenger system, cAMP, is key factor for the function of large cholangiocytes (1,4,7,9,13). Secretin stimulates bicarbonate secretion of large bile ducts by activation of cAMP-dependent CFTR⇒Cl^-/HCO_3^- anion exchanger 2 (AE2) (1,4,7,9,13). Also, the activation of the cAMP/PKA/ERK1/2 pathway modulates cholangiocyte proliferation (12,15,18,29). In fact, the direct stimulation of adenylyl cyclase activity by the chronic administration of forskolin stimulates normal cholangiocyte proliferation both *in vivo* and *in vitro*, which is associated with activation of the PKA/Src/MEK/ERK1/2 pathway (29). Maintenance of cAMP levels by forskolin administration prevents the impairment of cholangiocyte proliferation and enhancement of biliary apoptosis induced by vagotomy (30). Furthermore, Banales et al. have shown (31) that cAMP stimulates cholangiocyte proliferation via two downstream effectors (i.e., PKA and Epacs) in an animal model of autosomal recessive polycystic kidney disease. Downregulation of cAMP levels and cAMP-dependent signaling reduces biliary growth and increases cholangiocyte damage by apoptosis (12,14,20,30). The involvement of the cAMP-dependent ERK1/2 pathway in secretin-dependent biliary proliferation during cholestasis was confirmed in BDL $SR^{-/-}$ mice, which had reduced levels of phosphorylated ERK1/2 in isolated large cholangiocytes. As expected, large cholangiocyte isolated from $SR^{-/-}$ did not

respond to secretin, which was evidenced by lack of accumulation of intracellular cAMP levels.

Finally, we demonstrated that SR expression is critical for basal cholangiocyte proliferation in large mouse cholangiocytes that have stable knockdown of SR by transfection with shRNA for SR. These SR stable knockdown cells displayed decreased basal and secretin-stimulated proliferative capacity as compared to control-transfected cholangiocytes. As expected, these stable knockdown SR cells lacked secretin stimulated intracellular cAMP levels. Decreased basal proliferative rates that we observed in the cells with stable knockdown of SR compared to the mock-transfected controls are suggestive on the regulation of the basal proliferative rates by secretin perhaps in an autocrine mechanism. Consistent with our current study, we have previously shown that secretin stimulates the proliferation of two normal human cholangiocyte cell lines, H-69 and HiBEpiC (26). Collectively, the findings of our study revealed that secretin is a trophic factor for cholangiocytes that differentially regulated the growth of large cholangiocytes by acting on the specifically expressed SR under normal and pathological conditions.

De novo SR expression in small cholangiocytes is often found in models of liver damage that alter the SR-dependent functional capacity of large cholangiocytes such as CCl_4 acute hepatoxicity (14). We also have preliminary findings (Alpini, unpublished findings) that suggest that secretin has a protective role vs. CCl_4-induced damage of large cholangiocytes (14). These findings are consistent with the lack of secretin-dependent signaling resulting in an increase in the basal apoptotic activity in cells lacking SR that we observed in the SR knockdown cells. In addition, our other studies in which large cholangiocyte damage was prevented by administration of bile acids (such as, taurocholate) (32) and cAMP agonists (30) suggest that secretin, a cAMP agonist, would have a role as a protective factor during large bile duct damage. Further studies are necessary to confirm this role, but are suggestive that secretin or other cAMP agonists could prevent biliary loss in ductopenia pathologies such as drug induced vanishing bile duct syndrome or graft versus host disease.

The discovery of a novel pro-proliferative function of secretin in cholangiocytes along with the demonstration that *in vitro* and *in vivo* molecular manipulations of the SR gene ablated the proliferative and apoptotic responses of large cholangiocytes, may shed light on the development of new therapeutic approach for the management of cholestatic liver diseases. Overexpression of SR or secretin administration might open new avenues for the treatment of ductopenic liver diseases.

Acknowledgments

This work was supported partly by the Dr. Nicholas C. Hightower Centennial Chair of Gastroenterology from Scott & White, the VA Research Scholar Award, a VA Merit Award and the NIH grant DK58411 to Dr. Alpini, a NIH grant DK081442 to Dr. Glaser, (DK078532) to Dr. DeMorrow and by University funds to Dr. Onori and PRIN 2007 and Federate Athenaeum funds from University of Rome “La Sapienza” to Prof. Gaudio.

References

1. Alpini G, Glaser S, Robertson W, Rodgers RE, Phinizy JL, Lasater J, LeSage G. Large but not small intrahepatic bile ducts are involved in secretin-regulated ductal bile secretion. Am J Physiol Gastrointest Liver Physiol 1997;272:G1064–1074.
2. Alpini G, Lenzi R, Sarkozi L, Tavoloni N. Biliary physiology in rats with bile ductular cell hyperplasia. Evidence for a secretory function of proliferated bile ductules. J Clin Invest 1988;81:569–578. [PubMed: 2448343]
3. Kanno N, LeSage G, Glaser S, Alpini G. Regulation of cholangiocyte bicarbonate secretion. Am J Physiol Gastrointest Liver Physiol 2001;281:G612–625. [PubMed: 11518673]

4. Alpini G, Roberts S, Kuntz SM, Ueno Y, Gubba S, Podila PV, LeSage G, et al. Morphological, molecular, and functional heterogeneity of cholangiocytes from normal rat liver. Gastroenterology 1996;110:1636–1643. [PubMed: 8613073]
5. Glaser S, Gaudio E, Rao A, Pierce LM, Onori P, Franchitto A, Francis HL, et al. Morphological and functional heterogeneity of the mouse intrahepatic biliary epithelium. Lab Invest 2009;89:456–469. [PubMed: 19204666]
6. Martinez-Anso E, Castillo JE, Diez J, Medina JF, Prieto J. Immunohistochemical detection of chloride/bicarbonate anion exchangers in human liver. Hepatology 1994;19:1400–1406. [PubMed: 8188169]
7. Kato A, Gores GJ, LaRusso NF. Secretin stimulates exocytosis in isolated bile duct epithelial cells by a cyclic AMP-mediated mechanism. J Biol Chem 1992;267:15523–15529. [PubMed: 1322400]
8. Ueno Y, Alpini G, Yahagi K, Kanno N, Moritoki Y, Fukushima K, Glaser S, et al. Evaluation of differential gene expression by microarray analysis in small and large cholangiocytes isolated from normal mice. Liver Int 2003;23:449–459. [PubMed: 14986819]
9. Alpini G, Ulrich C, Roberts S, Phillips JO, Ueno Y, Podila PV, Colegio O, et al. Molecular and functional heterogeneity of cholangiocytes from rat liver after bile duct ligation. Am J Physiol Gastrointest Liver Physiol 1997;272:G289–297.
10. Banales JM, Arenas F, Rodriguez-Ortigosa CM, Saez E, Uriarte I, Doctor RB, Prieto J, et al. Bicarbonate-rich choleresis induced by secretin in normal rat is taurocholate-dependent and involves AE2 anion exchanger. Hepatology 2006;43:266–275. [PubMed: 16440368]
11. Lazaridis KN, Strazzabosco M, LaRusso NF. The cholangiopathies: disorders of biliary epithelia. Gastroenterology 2004;127:1565–1577. [PubMed: 15521023]
12. Alpini G, Glaser S, Ueno Y, Pham L, Podila PV, Caligiuri A, LeSage G, et al. Heterogeneity of the proliferative capacity of rat cholangiocytes after bile duct ligation. Am J Physiol Gastrointest Liver Physiol 1998;274:G767–775.
13. LeSage G, Glaser S, Gubba S, Robertson WE, Phinizy JL, Lasater J, Rodgers RE, et al. Regrowth of the rat biliary tree after 70% partial hepatectomy is coupled to increased secretin-induced ductal secretion. Gastroenterology 1996;111:1633–1644. [PubMed: 8942744]
14. LeSage G, Glaser S, Marucci L, Benedetti A, Phinizy JL, Rodgers R, Caligiuri A, et al. Acute carbon tetrachloride feeding induces damage of large but not small cholangiocytes from BDL rat liver. Am J Physiol Gastrointest Liver Physiol 1999;276:G1289–1301.
15. Alvaro D, Onori P, Metalli VD, Svegliati-Baroni G, Folli F, Franchitto A, Alpini G, et al. Intracellular pathways mediating estrogen-induced cholangiocyte proliferation in the rat. Hepatology 2002;36:297–304. [PubMed: 12143037]
16. Alpini G, Lenzi R, Zhai WR, Slott PA, Liu MH, Sarkozi L, Tavoloni N. Bile secretory function of intrahepatic biliary epithelium in the rat. Am J Physiol Gastrointest Liver Physiol 1989;257:G124–133.
17. Alpini G, Ulrich CD 2nd, Phillips JO, Pham LD, Miller LJ, LaRusso NF. Upregulation of secretin receptor gene expression in rat cholangiocytes after bile duct ligation. Am J Physiol Gastrointest Liver Physiol 1994;266:G922–928.
18. Glaser S, Benedetti A, Marucci L, Alvaro D, Baiocchi L, Kanno N, Caligiuri A, et al. Gastrin inhibits cholangiocyte growth in bile duct-ligated rats by interaction with cholecystokinin-B/Gastrin receptors via D-myo-inositol 1,4,5-triphosphate-, $Ca(^{2+})$-, and protein kinase C alpha-dependent mechanisms. Hepatology 2000;32:17–25. [PubMed: 10869284]
19. Lam IP, Siu FK, Chu JY, Chow BK. Multiple actions of secretin in the human body. Int Rev Cytol 2008;265:159–190. [PubMed: 18275888]
20. Glaser S, Ueno Y, DeMorrow S, Chiasson VL, Katki KA, Venter J, Francis HL, et al. Knockout of alpha-calcitonin gene-related peptide reduces cholangiocyte proliferation in bile duct ligated mice. Lab Invest 2007;87:914–926. [PubMed: 17618297]
21. Chu JY, Chung SC, Lam AK, Tam S, Chung SK, Chow BK. Phenotypes developed in secretin receptor-null mice indicated a role for secretin in regulating renal water reabsorption. Mol Cell Biol 2007;27:2499–2511. [PubMed: 17283064]
22. Miyoshi H, Rust C, Roberts PJ, Burgart LJ, Gores GJ. Hepatocyte apoptosis after bile duct ligation in the mouse involves Fas. Gastroenterology 1999;117:669–677. [PubMed: 10464144]

23. DeMorrow S, Francis H, Gaudio E, Ueno Y, Venter J, Onori P, Franchitto A, et al. Anandamide inhibits cholangiocyte hyperplastic proliferation via activation of thioredoxin 1/redox factor 1 and AP-1 activation. Am J Physiol Gastrointest Liver Physiol 2008;294:G506–519. [PubMed: 18096608]
24. Strausberg RL, Feingold EA, Grouse LH, Derge JG, Klausner RD, Collins FS, Wagner L, et al. Generation and initial analysis of more than 15,000 full-length human and mouse cDNA sequences. Proc Natl Acad Sci U S A 2002;99:16899–16903. [PubMed: 12477932]
25. Baumgardner JN, Shankar K, Hennings L, Badger TM, Ronis MJ. A new model for nonalcoholic steatohepatitis in the rat utilizing total enteral nutrition to overfeed a high-polyunsaturated fat diet. Am J Physiol Gastrointest Liver Physiol 2008;294:G27–38. [PubMed: 17947452]
26. Onori P, Wise C, Gaudio E, Franchitto A, Francis H, Carpino G, Lee V, et al. Secretin inhibits cholangiocarcinoma growth via dysregulation of the cAMP-dependent signaling mechanisms of secretin receptor. Inter J Cancer. 2009 In press.
27. Korner M, Hayes GM, Rehmann R, Zimmermann A, Scholz A, Wiedenmann B, Miller LJ, et al. Secretin receptors in the human liver: expression in biliary tract and cholangiocarcinoma, but not in hepatocytes or hepatocellular carcinoma. J Hepatol 2006;45:825–835. [PubMed: 16935383]
28. Alvaro D, Mancino MG, Glaser S, Gaudio E, Marzioni M, Francis H, Alpini G. Proliferating cholangiocytes: a neuroendocrine compartment in the diseased liver. Gastroenterology 2007;132:415–431. [PubMed: 17241889]
29. Francis H, Glaser S, Ueno Y, LeSage G, Marucci L, Benedetti A, Taffetani S, et al. cAMP stimulates the secretory and proliferative capacity of the rat intrahepatic biliary epithelium through changes in the PKA/Src/MEK/ERK1/2 pathway. J Hepatol 2004;41:528–537. [PubMed: 15464232]
30. LeSage G, Alvaro D, Benedetti A, Glaser S, Marucci L, Baiocchi L, Eisel W, et al. Cholinergic system modulates growth, apoptosis, and secretion of cholangiocytes from bile duct-ligated rats. Gastroenterology 1999;117:191–199. [PubMed: 10381927]
31. Banales JM, Masyuk TV, Gradilone SA, Masyuk AI, Medina JF, LaRusso NF. The cAMP effectors Epac and protein kinase a (PKA) are involved in the hepatic cystogenesis of an animal model of autosomal recessive polycystic kidney disease (ARPKD). Hepatology 2009;49:160–174. [PubMed: 19065671]
32. Marzioni M, LeSage GD, Glaser S, Patel T, Marienfeld C, Ueno Y, Francis H, et al. Taurocholate prevents the loss of intrahepatic bile ducts due to vagotomy in bile duct-ligated rats. Am J Physiol Gastrointest Liver Physiol 2003;284:G837–852. [PubMed: 12684215]

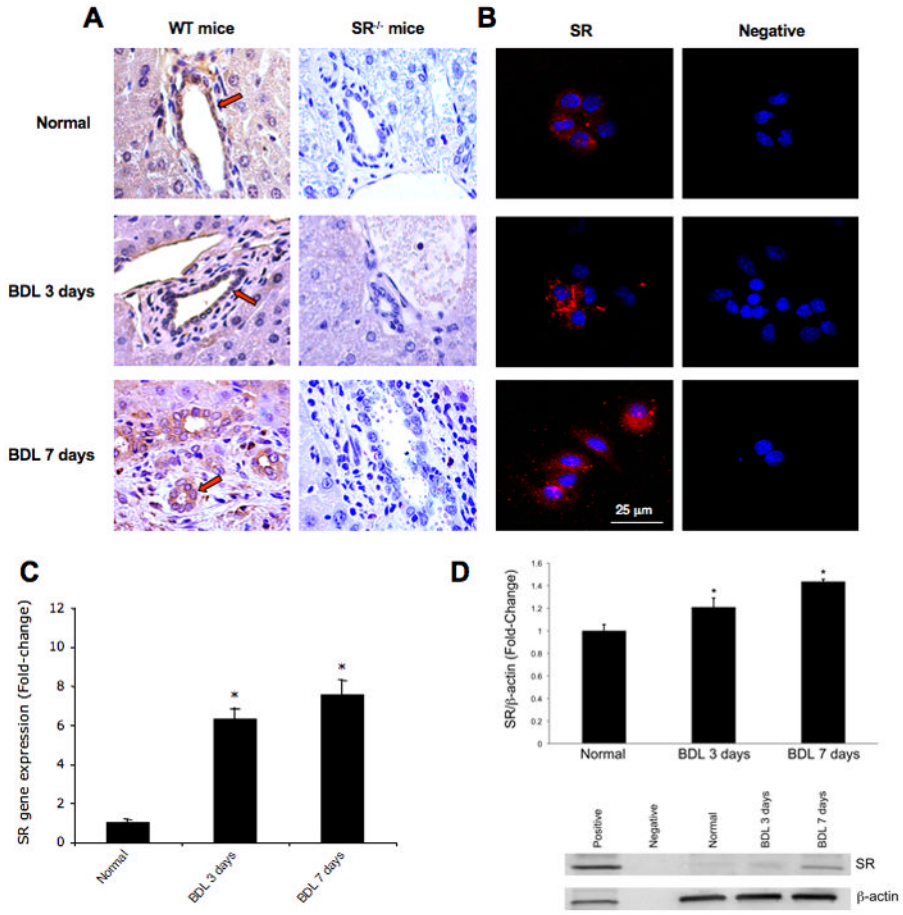

Figure 1.

Evaluation of SR expression by: [**A**] immunohistochemistry in liver sections from WT and $SR^{-/-}$ normal mice, and mice with BDL for 3 and 7 days, [**B**] immunofluorescence, [**C**] real-time PCR or [**D**] immunoblots in freshly isolated large cholangiocytes from normal and 3- and 7-day BDL WT mice. [**A**] Large (red arrows) bile ducts from normal and BDL WT mice express SR. Orig. magn., × 40. [**B**] Specific immunoreactivity for SR in representative fields is shown in red; cell nuclei were stained with DAPI (blue). Bar size = 50 μm. [**C, D**] Data are mean ± SEM of 6 experiments. *****p<0.05 vs. normal.

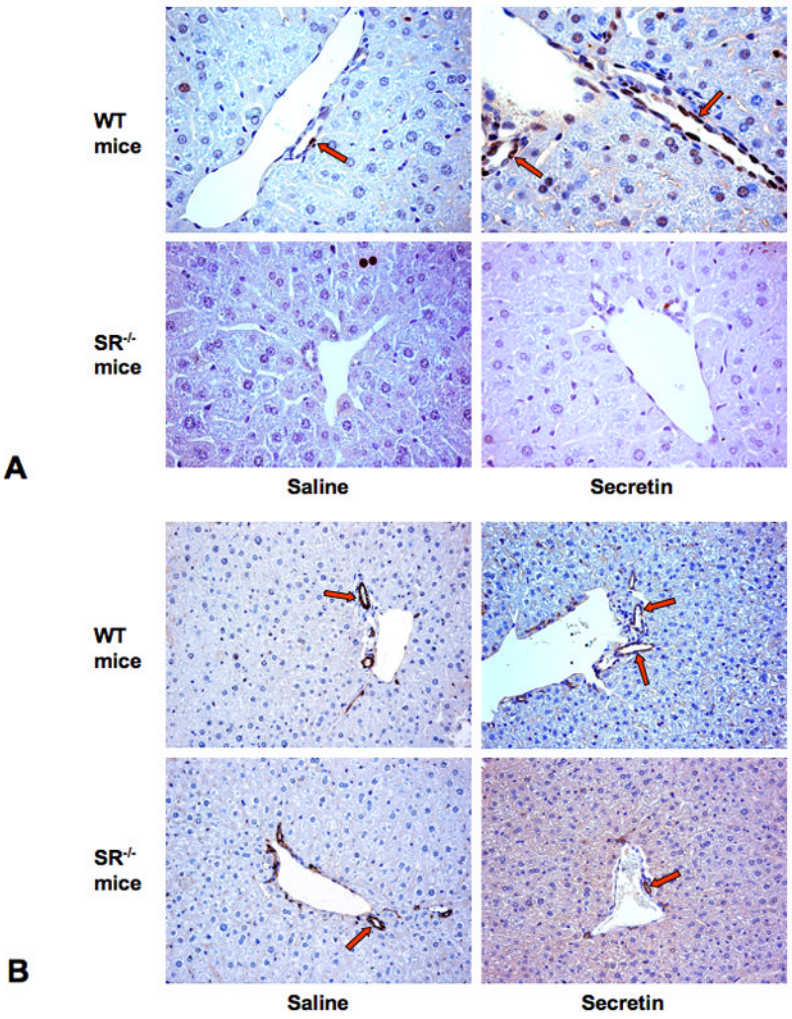

Figure 2.
Evaluation of the number of large [**A**] PCNA-positive cholangiocytes and large [**B**] IBDM in normal mice treated with saline or secretin for 1 week. In WT mice treated with secretin there was an increase in the number of large (**A**, red arrows) PCNA-positive cholangiocytes and large IBDM (**B**, red arrows) compared to normal WT mice treated with saline. Orig. magn., ×40 [**A**] and ×20 [**B**].

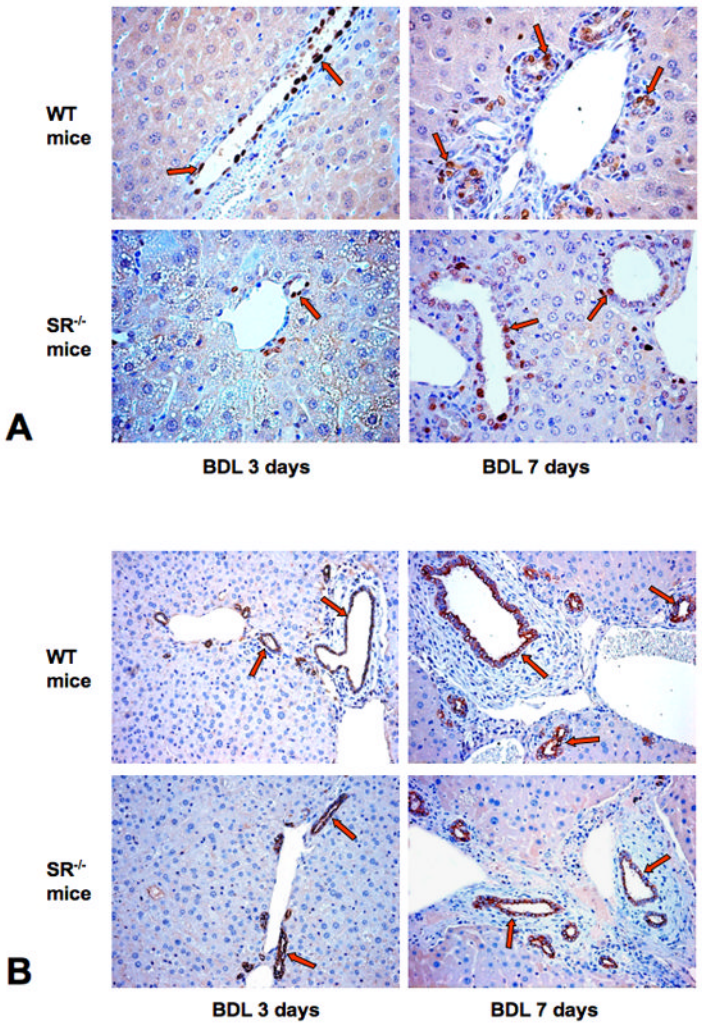

Figure 3.
Evaluation of the number of large [**A**] PCNA-positive cholangiocytes and large [**B**] IBDM in WT and $SR^{-/-}$ mice with BDL for 3 and 7 days. Orig. magn., ×40 [**A**] and ×20 [**B**].

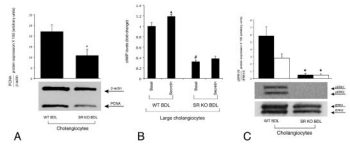

Figure 4.

Evaluation of [**A**] PCNA protein expression, [**B**] basal and secretin-stimulated cAMP levels, and [**C**] ERK1/2 phosphorylation in large cholangiocytes from WT and $SR^{-/-}$ 7-day BDL mice. [**A**] Data are mean ± SEM of 7 experiments. *****p<0.05 vs. PCNA protein of large cholangiocytes from WT 7-day BDL mice. [**B**] Data are mean ± SEM of 7 experiments. *****p<0.05 vs. basal cAMP levels of large cholangiocytes from WT 7-day BDL mice. [**C**] Data are mean ± SEM of 7 experiments. *****p<0.05 vs. ERK1/2 phosphorylation of large cholangiocytes from WT 7-day BDL mice.

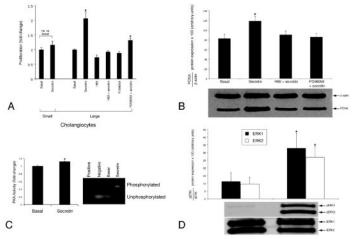

Figure 5.

[**A**] Effect of 0.2% BSA (basal) or secretin (100 nM) for 48 hours at 37°C on the proliferation (by MTS assays) of small and large cholangiocytes. Data are mean ± SEM of 14 experiments. *****p<0.05 vs. its corresponding basal value. [**B**] Data are mean ± SEM of 14 experiments. *****p<0.05 vs. its corresponding basal value. Secretin increased PKA activity [**C**, n = 4] and ERK1/2 phosphorylation [**D**, n =7] in large cholangiocytes compared to large cholangiocytes treated with BSA. *****p<0.05 vs. its corresponding basal value.

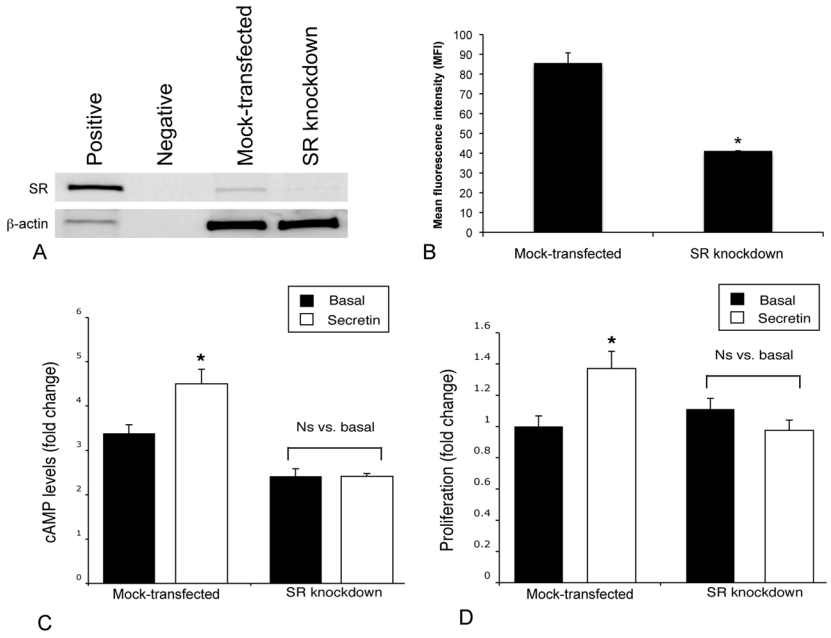

Figure 6.

Knockdown of secretin receptor protein expression in large cholangiocytes was evaluated by [**A**] Western blotting and [**B**] FACS. Effect secretin receptor gene silencing on the effects of secretion on [**C**] cAMP levels, and [**D**] proliferation (by MTS assays) of large cholangiocytes. Data are mean ± SEM of 6 experiments. *****$p<0.05$ vs. its corresponding value of mock-transfected large cholangiocytes. cAMP = cyclic adenosine 3′, 5′-monophosphate.

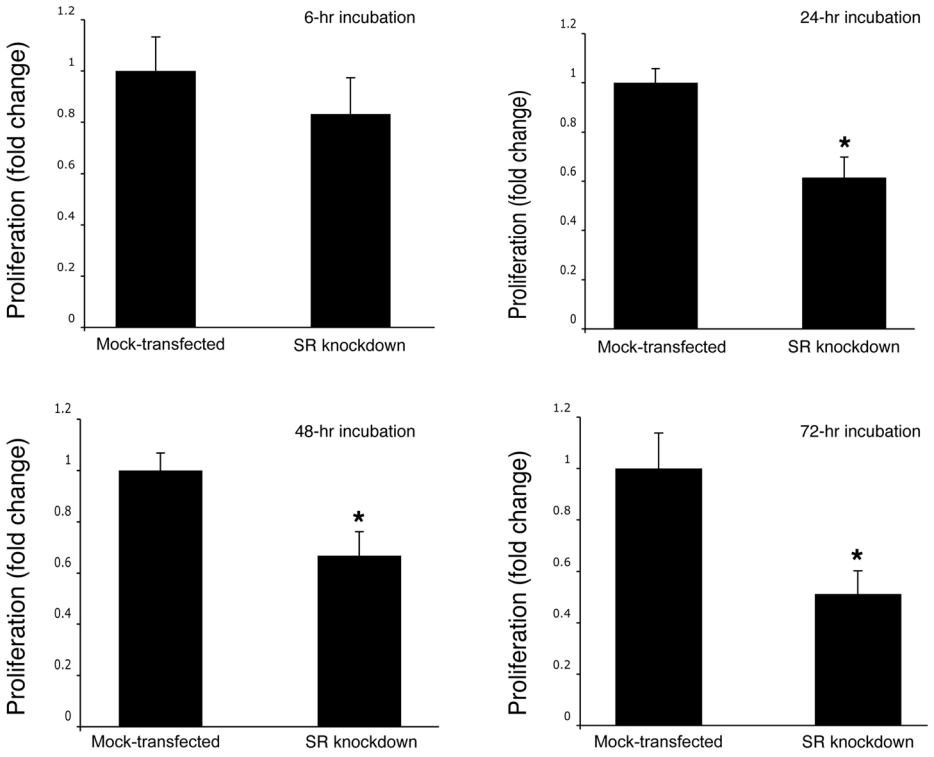

Figure 7.

Effect of secretin receptor gene silencing on the basal proliferative activity (by MTS assays, following incubation for 6, 24, 48 and 72 hours with 0.2% BSA) of large cholangiocytes. Data are mean ± SEM of 4 experiments. *****$p<0.05$ vs. its corresponding value of mock-transfected large cholangiocytes.

Table 1

Evaluation of: (i) body weight; (ii) the biliary expression of secretin receptor; (iii) lobular necrosis; (iv) the percentage of PCNA- or TUNEL-positive large cholangiocytes; and (v) large IBDM in liver sections.

Groups	Body weight (gm)	Percentage of large cholangiocytes positive for SR	Lobular necrosis	Percentage of large cholangiocytes positive for PCNA	Large IBDM	Percentage of large cholangiocytes positive by TUNEL
WT normal + NaCl 1 week	27.8 ± 0.8	19.83 ± 1.96	(−)	6.20 ± 0.97	0.17 ± 0.03	ND
WT normal + secretin 1 week	25.6 ± 0.5	30.60 ± 2.04[a]	(−)	40.80 ± 2.29[a]	0.35 ± 0.02[a]	ND
Normal $SR^{-/-}$ + NaCl 1 week	28.6 ± 0.7	ND	(−)	4.20 ± 0.66	0.18 ± 0.02	ND
Normal $SR^{-/-}$ + secretin 1 week	29.0 ± 1.8	ND	(−)	5.33 ± 1.08	0.18 ± 0.03	ND
WT BDL 3 days	23.2 ± 0.7	39.0 ± 2.07[a]	(+)	60.62 ± 2.30	1.26 ± 0.06	ND
$SR^{-/-}$ BDL 3 days	22.0 ± 0.5	ND	(++)	39.67 ± 2.16[b]	0.57 ± 0.06[b]	10.50 ± 1.08
WT BDL 7 days	23.2 ± 0.7	41.33 ± 2.35[a]	(+)	47.67 ± 1.50	2.51 ± 0.12	ND
$SR^{-/-}$ BDL 7 days	26.2 ± 0.6	ND	(++)	30.83 ± 2.07[b]	1.40 ± 0.11[b]	13.33 ± 0.88

Body weight values derive from 10–20 animals per each group. These evaluations were performed in liver sections (4–5 μm thick). IBDM = intrahepatic bile duct mass. IBDM was measured as area occupied by CK-19 positive-bile duct/total area x 100. BDL = bile duct ligation; ND = not detected; SR = secretin receptor; WT = wild-type. Lobular necrosis was scored as follows: − = 0 foci; +/− = <2 foci; + = 2–4 foci; and ++ = >4 foci.

[a] $p<0.05$ vs. the corresponding value of WT normal mice treated with NaCl for 1 week.

[b] $p<0.05$ vs. the corresponding value of WT mice with BDL for 3 and 7 days, respectively. Data are mean ± SEM.