

Knockout of Secretin Receptor Reduces Large Cholangiocyte Hyperplasia in Mice With Extrahepatic Cholestasis Induced by Bile Duct Ligation

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During bile duct ligation (BDL), the growth of large cholangiocytes is regulated by the cyclic adenosine monophosphate (cAMP)/extracellular signal-regulated kinase 1/2 (ERK1/2) pathway and is closely associated with increased secretin receptor (SR) expression. Although it has been suggested that SR modulates cholangiocyte growth, direct evidence for secretin-dependent proliferation is lacking. SR wild-type (WT) (SR^{+/+}) or SR knock-out (SR^{-/-}) mice underwent sham surgery or BDL for 3 or 7 days. We evaluated SR expression, cholangiocyte proliferation, and apoptosis in liver sections and proliferating cell nuclear antigen (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/day by way of 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, protein kinase A (PKA) activity, and ERK1/2 phosphorylation. SR expression was also stably knocked down by short hairpin RNA, and basal and secretin-stimulated cAMP 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 with 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 mitogen-activated protein kinase kinase inhibitors. Stable knock-down 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. (HEPATOLOGY 2010;52:204-214)

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, because secretin receptor (SR) is expressed in rodent and human liver by larger bile ducts.^{1,4-6} In large cholangiocytes, secretin

Abbreviations: BDL, bile duct ligation; BSA, bovine serum albumin; cAMP, cyclic adenosine monophosphate; CCl₄, carbon tetrachloride; ERK1/2, extracellular signal-regulated kinase; FACS, fluorescence-activated cell sorting; IBDM, intrahepatic bile duct mass; MEK, mitogen-activated protein kinase kinase; PCNA, proliferating cell nuclear antigen; PCR, polymerase chain reaction; PKA, protein kinase A; SEM, standard error of the mean; SR, secretin receptor; WT, wild-type.

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increases cyclic adenosine 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⁻/HCO₃⁻ anion exchanger 2¹⁰ and secretion of bicarbonate in bile.^{2,3}

Human cholangiocytes are the target cells in several cholangiopathies, including primary biliary cirrhosis and primary sclerosing cholangitis, 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 ligation (BDL) or acute carbon tetrachloride (CCl₄) administration.^{12,14} The proliferative response of the intrahepatic biliary epithelium to BDL is heterogeneous, because large (but not small) cholangiocytes proliferate through the activation of cAMP-dependent ERK1/2 signaling^{12,15} leading to enhanced ductal mass.^{5,12,14}

Because 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 (1) cholangiocyte hyperplasia (after BDL or 70% hepatectomy) is associated with enhanced SR expression and secretin-stimulated cAMP levels and bicarbonate secretion^{12,13,16-18} and (2) cholangiocyte damage (after CCl₄) decreases the functional expression of SR in large cholangiocytes.¹⁴ In pathological conditions—such as the CCl₄ 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 was obtained from Molecular Probes, Inc. (Eugene, OR). Porcine secretin was purchased from Peninsula Laboratories (Belmont, CA). The polyclonal SR antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was raised against a peptide mapping at the C terminus of SR 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 antibody was purchased from Caltag Laboratories Inc. (Burlingame, CA). Goat phosphorylated ERK1/2 and total ERK1/2 (44-42 kDa) polyclonal affinity purified antibodies were purchased from Santa Cruz Biotechnology. The RIA kits for the determination of intracellular cAMP levels in cholangiocytes were purchased from Perkin Elmer (Shelton, CT).

Animal Models. All animal experiments (Table 1) were performed in accordance with a protocol approved by the Scott & White and Texas A&M Health Science Center Institutional Animal Care and Use Committee and conformed to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (Publication No. 85-23, revised 1996). Our SR^{+/+} (wild-type [WT]) or SR knockout (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 g) of the N5 generation: (1) as normal treated with saline (0.9% NaCl) or secretin (2.5 nmol/kg/day, a dose similar to that used by us for another gastrointestinal hormone, gastrin, in rodents)¹⁸ by way of intraperitoneally implanted Alzet osmotic minipumps (Alzet, CA) for 7

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Table 1. Evaluation of Body Weight, Biliary Expression of SR, Lobular Necrosis, Percentage of PCNA- or TUNEL-Positive Large Cholangiocytes, and Large IBDM in Liver Sections

Groups	Body Weight (g)	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*	(-)	40.80 ± 2.29*	0.35 ± 0.02*	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*	(+)	60.62 ± 2.30	1.26 ± 0.06	ND
SR ^{-/-} BDL, 3 days	22.0 ± 0.5	ND	(++)	39.67 ± 2.16†	0.57 ± 0.06†	10.50 ± 1.08
WT BDL, 7 days	23.2 ± 0.7	41.33 ± 2.35*	(+)	47.67 ± 1.50	2.51 ± 0.12	ND
SR ^{-/-} BDL, 7 days	26.2 ± 0.6	ND	(++)	30.83 ± 2.07†	1.40 ± 0.11†	13.33 ± 0.88

Body weight values are derived from 10-20 animals per each group. Evaluations were performed in liver sections (4- to 5- μ m-thick). IBDM was measured as the area occupied by cytokeratin-19-positive bile duct/total area \times 100. Lobular necrosis was scored as follows: -, 0 foci; +/-, <2 foci; +, 2-4 foci; ++, >4 foci. Data are expressed as the mean \pm SEM.

Abbreviations: ND, not detected; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling.

* $P < 0.05$ versus the corresponding value of WT normal mice treated with NaCl for 1 week.

† $P < 0.05$ versus the corresponding value of WT mice with BDL for 3 and 7 days, respectively.

days; or (2) for sham operation or BDL (for 3 and 7 days).^{5,20,22} Because our previous studies²¹ showed 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 (after 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. Because 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 and that following BDL, large but not small cholangiocytes proliferate.⁵ Freshly isolated large cholangiocytes (\approx 99% by cytokeratin-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. Because immunohistochemistry shows that only large bile ducts from WT (but not knockout) animals express SR, we evaluated the expression of SR by way of immunofluorescence and real-time polymerase chain reaction (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 (Wetzlar, 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, messenger RNA and protein expression of SR were evaluated by way of real-time PCR²³ and western blot analysis, respectively.²⁰ For real-time PCR, RNA was extracted from cholangiocytes using 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 an SYBR Green PCR

master mix and specific primers designed against the mouse secretin receptor gene NM_001012322,²⁴ and glyceraldehyde 3-phosphate dehydrogenase, the house-keeping gene (SuperArray, Frederick, MD) in the real-time thermal cycler (ABI Prism 7900HT sequence detection system). A $\Delta\Delta C_t$ analysis was performed using normal large cholangiocytes as the control sample. Data are expressed as fold-change of relative messenger RNA levels \pm standard error of the mean (SEM) ($n = 6$).

Evaluation of Liver Histology, Cholangiocyte Apoptosis, and Proliferation. All liver sections were scored by two board-certified pathologists who were blinded to the identity of the samples. Lobular necrosis was evaluated in liver sections stained with hematoxylin-eosin.²⁵ Lobular necrosis was scored as follows: $-$, 0 foci; $+/-$, <2 foci; $+$, 2-4 foci; $++$, >4 foci.²⁵ Sections were examined in a coded fashion by BX-51 light microscopy (Olympus, Tokyo, Japan) equipped with a camera. We measured (1) the percentage of cholangiocyte apoptosis by semiquantitative terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling kit (Apoptag; Chemicon International, Inc.); (2) cholangiocyte proliferation by evaluation of the percentage of small and large cholangiocytes positive for PCNA⁵; and (3) intrahepatic bile duct mass (IBDM)⁵ of small ($<15 \mu\text{m}$)¹ and large ($>15 \mu\text{m}$)¹ bile ducts. IBDM was measured as the area occupied by cytokeratin-19-positive bile duct/total area $\times 100$. Proliferation was evaluated by immunoblots²⁰ 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 way of 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 knockout 7-day BDL mice, a 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 kits²⁰; 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 a phospho-imager.

In Vitro Effect of Secretin on the Proliferation, Protein Kinase A Activity, and ERK1/2 Phosphorylation of Large Cholangiocytes. Our small (negative control) and large cholangiocytes⁸ were treated at 37°C with 0.2% bovine serum albumin (BSA) (basal) or secre-

tin (100 nM) for 48 hours in the absence or presence of preincubation (1 hour) with H89 (protein kinase A [PKA] inhibitor, 30 μM) or PD98059 (mitogen-activated protein kinase kinase [MEK] inhibitor, 10 nM) before evaluating proliferation by CellTiter 96 Cell Proliferation Assay²⁰ (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 compared with vehicle-treated controls. In separate experiments, large cholangiocytes were treated with 0.2% BSA (basal) or secretin (100 nM) for 6 hours in the absence or presence of H89 (30 μM) or PD98059 (10 nM) before evaluating PCNA expression by way of immunoblotting,⁵ PKA activity,²⁰ and phosphorylation of ERK1/2 by way of immunoblotting.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 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 short hairpin RNA (SuperArray, 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 four 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 way of fluorescence-activated cell sorting (FACS) analysis and western blot analysis as described.²⁶

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 [data not shown] and 50% knockdown of protein expression by FACS)—were then treated with 0.2% BSA (basal) or secretin (100 nM for 5 minutes) before evaluation of cAMP levels by way of RIA^{4,7,9,18} or 0.2% BSA (basal) or secretin (100 nM) before measuring proliferation by way of 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 the mean \pm SEM. Differences between groups were analyzed using the Student unpaired t test when two

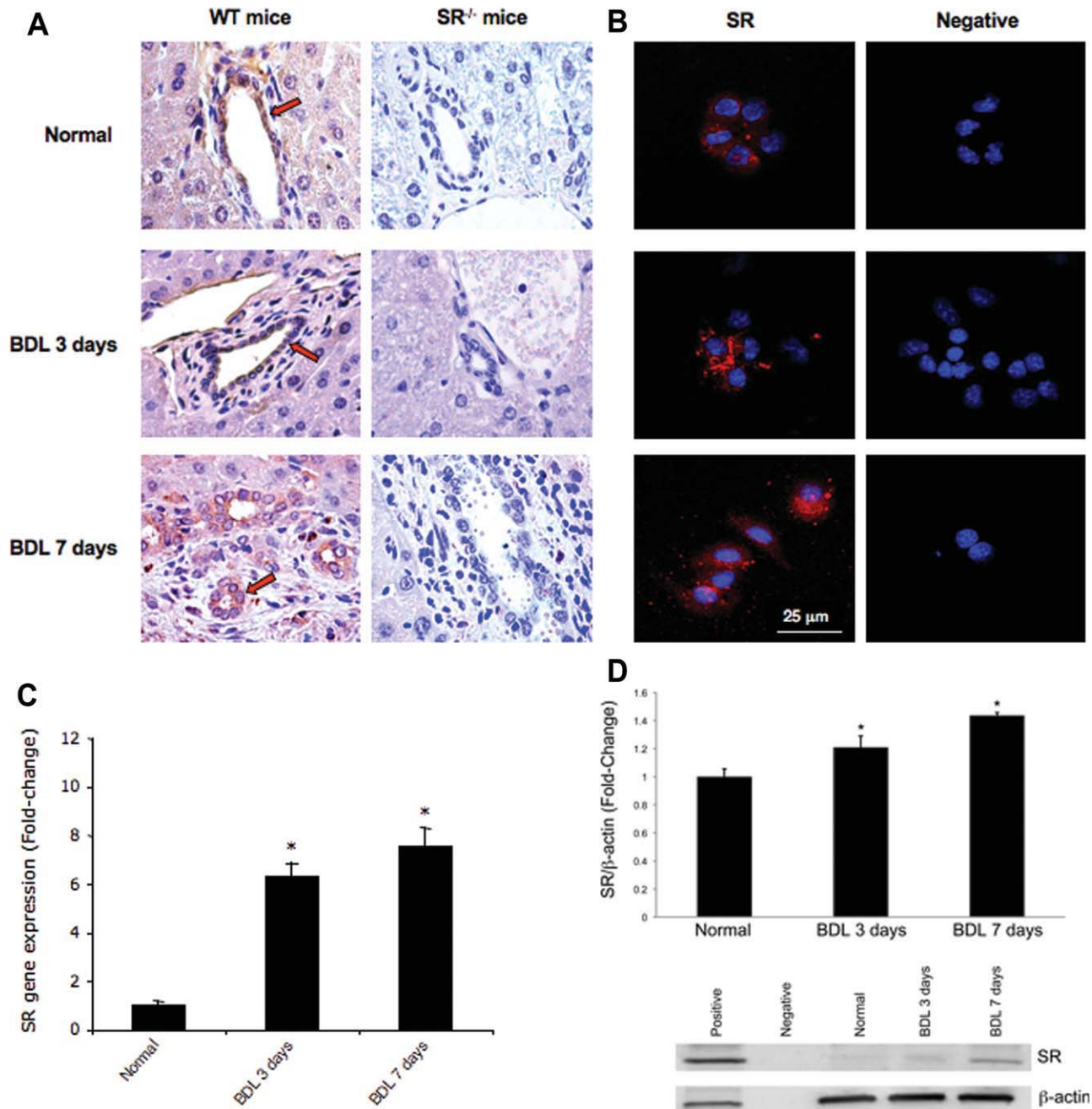


Fig. 1. Evaluation of SR expression by (A) immunohistochemistry in liver sections from WT and SR^{-/-} normal mice, and mice with BDL for 3 and 7 days, (B) immunofluorescence, (C) real-time PCR, and (D) immunoblots in freshly isolated large cholangiocytes from normal and 3- and 7-day BDL WT mice. (A) Large bile ducts from normal and BDL WT mice express SR (red arrows). Original magnification $\times 40$. (B) Specific immunoreactivity for SR in representative fields is shown in red; cell nuclei were stained with 4',6-diamidino-2-phenylindole (blue). Scale bar = 25 μ m. (C,D) Data are expressed as the mean \pm SEM of six experiments. * $P < 0.05$ versus normal.

groups were analyzed, and by way of analysis of variance 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 (Fig. 1A and Table 1). The expression of SR in large bile ducts was higher in: normal WT mice treated with secretin compared to saline-treated mice (Table 1) and WT BDL compared with normal WT mice (Table 1). There was no positive staining for SR in bile ducts from normal and BDL SR^{-/-} mice (Fig. 1A). The expression of SR was confirmed by way of

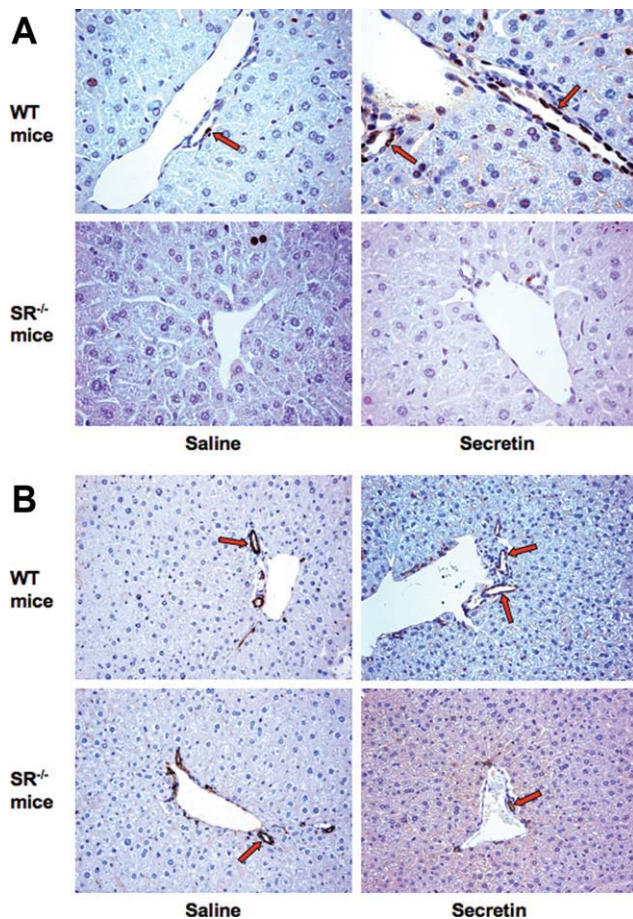


Fig. 2. Evaluation of the number of (A) large PCNA-positive cholangiocytes and (B) large IBDM in normal mice treated with saline or secretin for 1 week. In WT mice treated with secretin, there was an increase in the number of (A) large PCNA-positive cholangiocytes (red arrows) and (B) large IBDM (red arrows) compared with normal WT mice treated with saline. Original magnification $\times 40$ (A) and $\times 20$ (B).

immunofluorescence in large cholangiocytes purified from normal and BDL WT mice (Fig. 1B). Real-time PCR and immunoblot assay revealed that the expression of SR messenger RNA and protein was higher in large BDL cholangiocytes compared with normal large cholangiocytes (Fig. 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 mice compared with WT BDL mice. The chronic administration of secretin to normal WT mice increased the percentage of large PCNA-positive cholangiocytes and large IBDM compared with normal WT mice treated with saline (Fig. 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 (Fig. 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 with normal mice (Fig. 3A,B and Table 1). Similar to previous studies,¹⁶ large IBDM was enhanced in parallel with the increased duration of BDL (Fig. 3B and Table 1). Knockout of SR reduces large cholangiocyte proliferation and large IBDM induced by BDL^{5,20} compared with WT BDL mice (Fig. 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 with cholangiocytes from WT BDL mice (Fig. 4A). Basal cAMP levels of large cholangiocytes from $SR^{-/-}$ BDL mice were significantly lower than

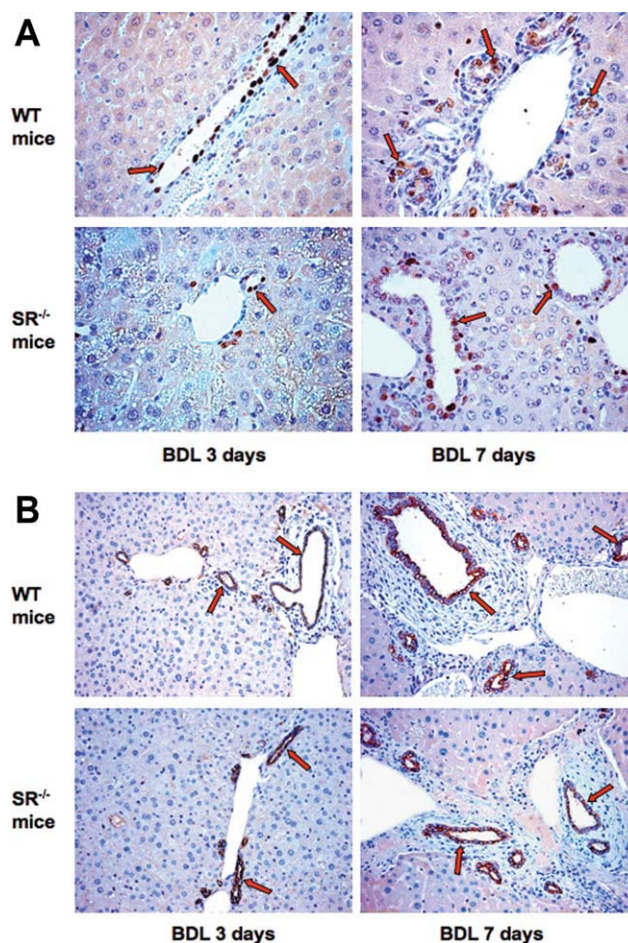


Fig. 3. Evaluation of the number of (A) large PCNA-positive cholangiocytes and (B) large IBDM in WT and $SR^{-/-}$ mice with BDL for 3 and 7 days. Original magnification $\times 40$ (A) and $\times 20$ (B).

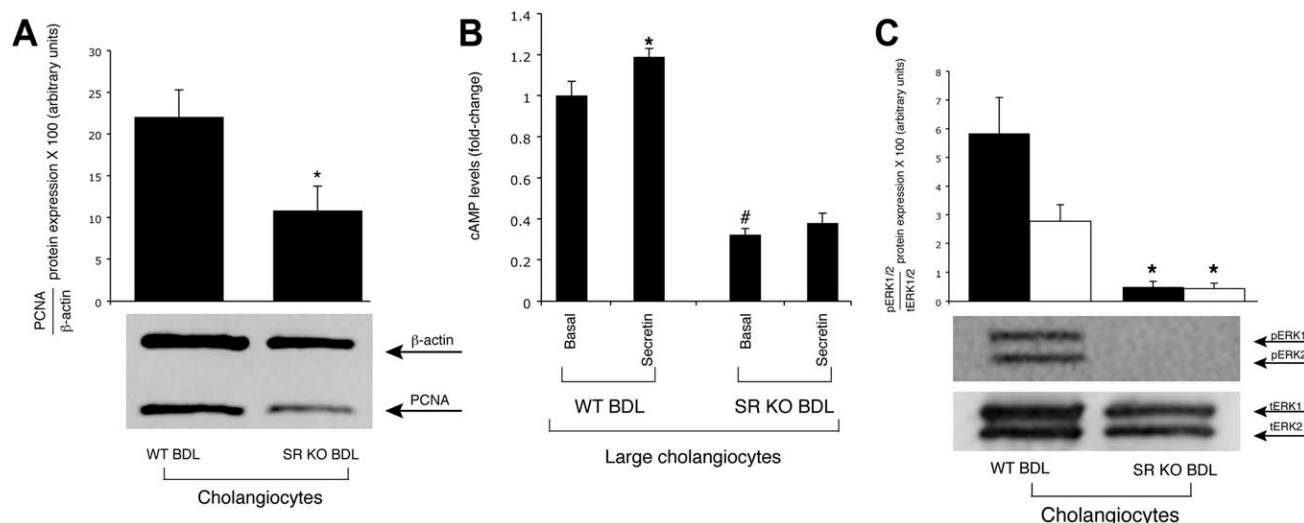


Fig. 4. Evaluation of (A) PCNA protein expression, (B) basal and secretin-stimulated cAMP levels, and (C) ERK1/2 phosphorylation in large cholangiocytes from WT and SR^{-/-} 7-day BDL mice. (A) Data are expressed as the mean \pm SEM of seven experiments. * P < 0.05 versus PCNA protein of large cholangiocytes from WT 7-day BDL mice. (B) Data are expressed as the mean \pm SEM of seven experiments. * P < 0.05 versus 0.05 versus basal cAMP levels of large cholangiocytes from WT 7-day BDL mice. (C) Data are expressed as the mean \pm SEM of seven experiments. * P < 0.05 versus 0.05 versus ERK1/2 phosphorylation of large cholangiocytes from WT 7-day BDL mice.

the corresponding levels of cholangiocytes from WT BDL mice (Fig. 4B). Secretin increased cAMP levels of large cholangiocytes from WT (but not SR^{-/-}) BDL mice (Fig. 4B). In large cholangiocytes from SR^{-/-} BDL mice, there was a decreased ERK1/2 phosphorylation compared with large cholangiocytes from WT BDL mice (Fig. 4C).

Secretin Stimulates *In Vitro* Large Cholangiocyte Proliferation. Large (but not small) cholangiocytes proliferate after the administration of secretin (Fig. 5A). Secretin-stimulation of large cholangiocyte proliferation was blocked by H89 and partially by the MEK inhibitor, PD98059 (Fig. 5A). Secretin increased PCNA expression of large cholangiocytes, an increase that was blocked by H89 and PD98059 (Fig. 5B). There was increased PKA activity (Fig. 5C) and ERK1/2 phosphorylation (Fig. 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 knockdown of SR protein expression by 50%, as demonstrated by FACS (Fig. 6B), was confirmed by way of western blot analysis (Fig. 6A). When we knocked down the gene for SR in large cholangiocytes, secretin did not increase cAMP levels (Fig. 6C) and proliferation (Fig. 6D, 48 hours of incubation) in these cells compared with 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 (Fig. 7) of SR-silenced large cholangiocytes compared with 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 (\approx 50%) during BDL compared with BDL WT 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 with BDL cholangiocytes. *In vitro*, secretin increased the proliferation of large cholangiocytes by activation of cAMP \rightarrow PKA \rightarrow ERK1/2 signaling. Silencing of the SR gene induces a decrease in the basal proliferative capacity of large cholangiocytes compared with 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 with normal large cholangiocytes. This finding was consistent with previous studies showing that: (1) in the rodent liver SR is only expressed by large cholangiocytes,^{1,4,5,9,12} (2) SR expression is up-regulated following BDL ligation in large cholangiocytes,^{14,17} and (3) the extent of secretin effects on cholangiocyte

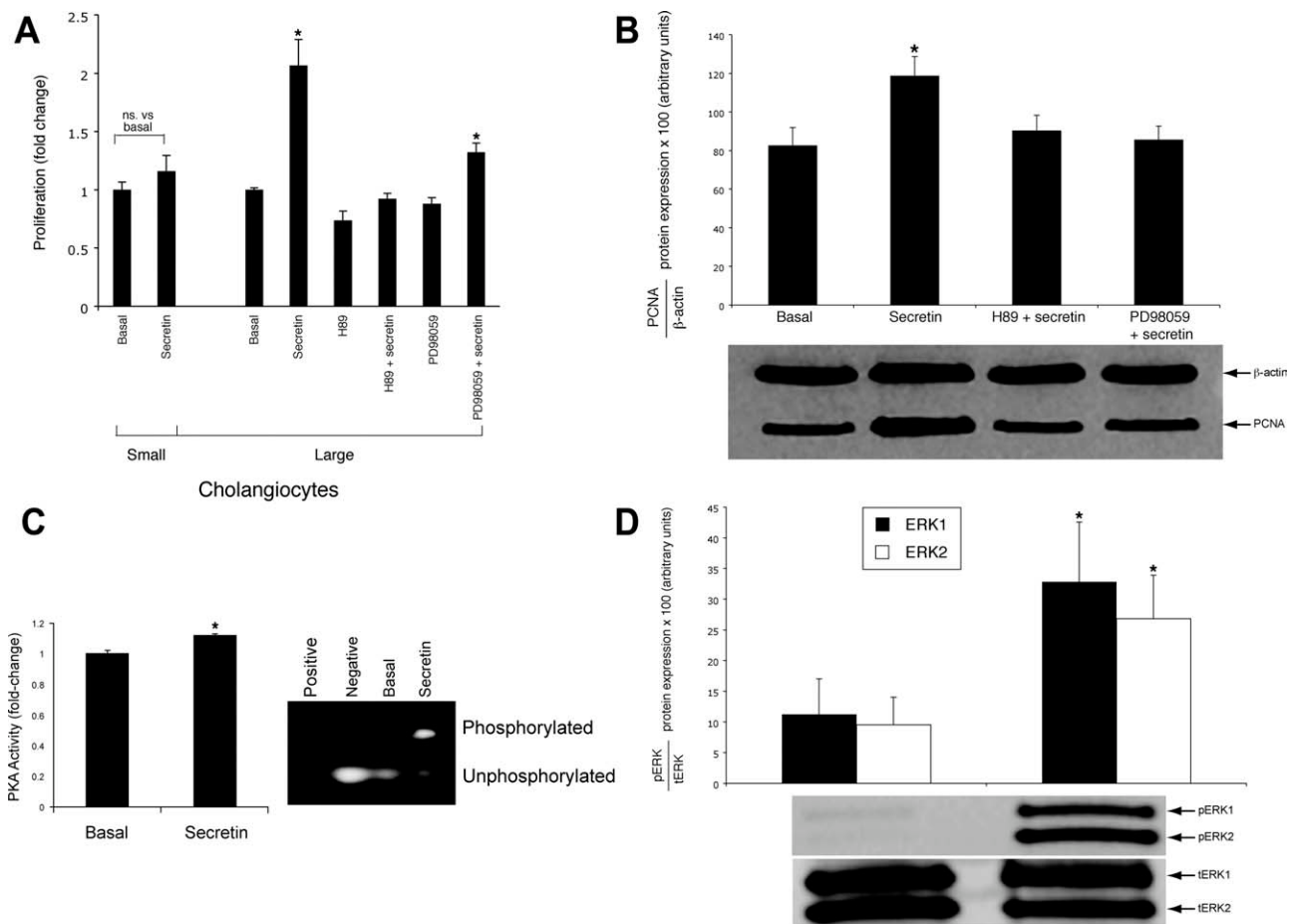


Fig. 5. (A) Effect of 0.2% BSA (basal) or secretin (100 nM) for 48 hours at 37°C on the proliferation of small and large cholangiocytes (MTS assay). Data are expressed as the mean \pm SEM of 14 experiments. * P < 0.05 versus its corresponding basal value. (B) Data are expressed as the mean \pm SEM of 14 experiments. * P < 0.05 versus its corresponding basal value. Secretin increased (C) PKA activity (n = 4) and (D) ERK1/2 phosphorylation (n = 7) in large cholangiocytes compared with large cholangiocytes treated with BSA. * P < 0.05 versus its corresponding basal value.

functions parallel with the duration of BDL.¹⁶ This finding parallels recent findings that mouse cholangiocytes share a similar heterogeneous profile as rat cholangiocytes⁵ and 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 up-regulated 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 neurohormonal factors.^{18,20,28} In a knock-out mouse model for α -calcitonin gene-related peptide, the lack of circulating α -calcitonin gene-related peptide

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 a key factor for the function of large cholangiocytes.^{1,4,7,9,13} Secretin stimulates bicarbonate secretion of large bile ducts through activation of cAMP-dependent CFTR \rightarrow Cl⁻/HCO₃⁻ anion exchanger 2.^{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

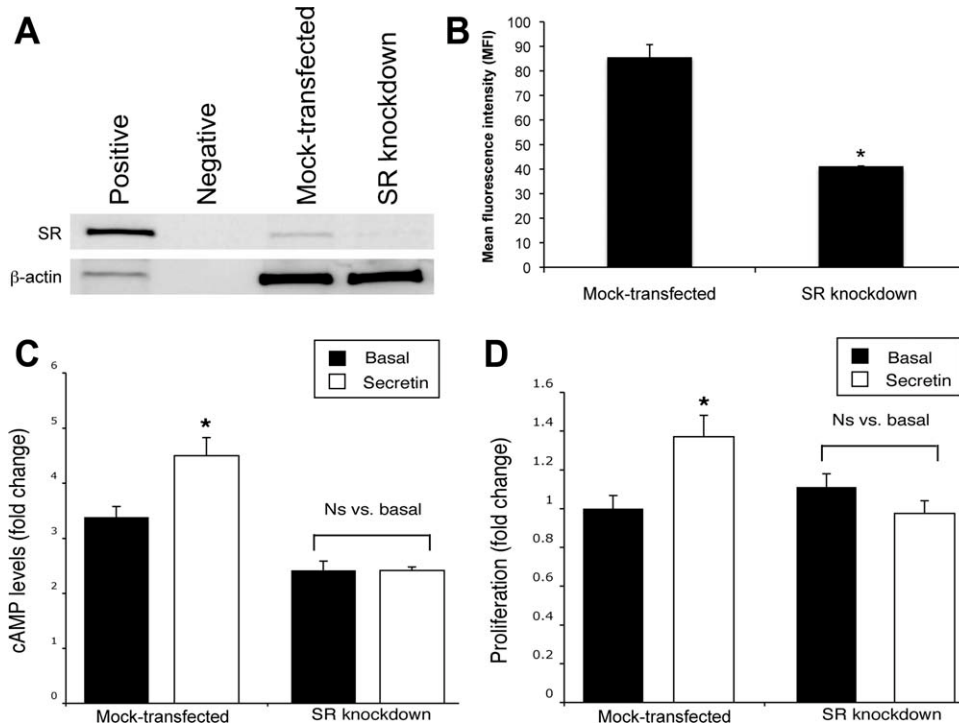


Fig. 6. Knockdown of secretin receptor protein expression in large cholangiocytes was evaluated by (A) western blotting and (B) FACS. Effect secretin receptor gene silencing on the effects of secretion on (C) cAMP levels, and (D) proliferation (by MTS assays) of large cholangiocytes. Data are expressed as the mean \pm SEM of six experiments. * $P < 0.05$ versus its corresponding value of mock-transfected large cholangiocytes.

forskolin administration prevents the impairment of cholangiocyte proliferation and enhancement of biliary apoptosis induced by vagotomy.³⁰ Furthermore, Banales et al. have shown³¹ that cAMP stimulates cholangiocyte proliferation through two downstream effectors (i.e., PKA and Epacs) in an animal model of

autosomal recessive polycystic kidney disease. Down-regulation 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

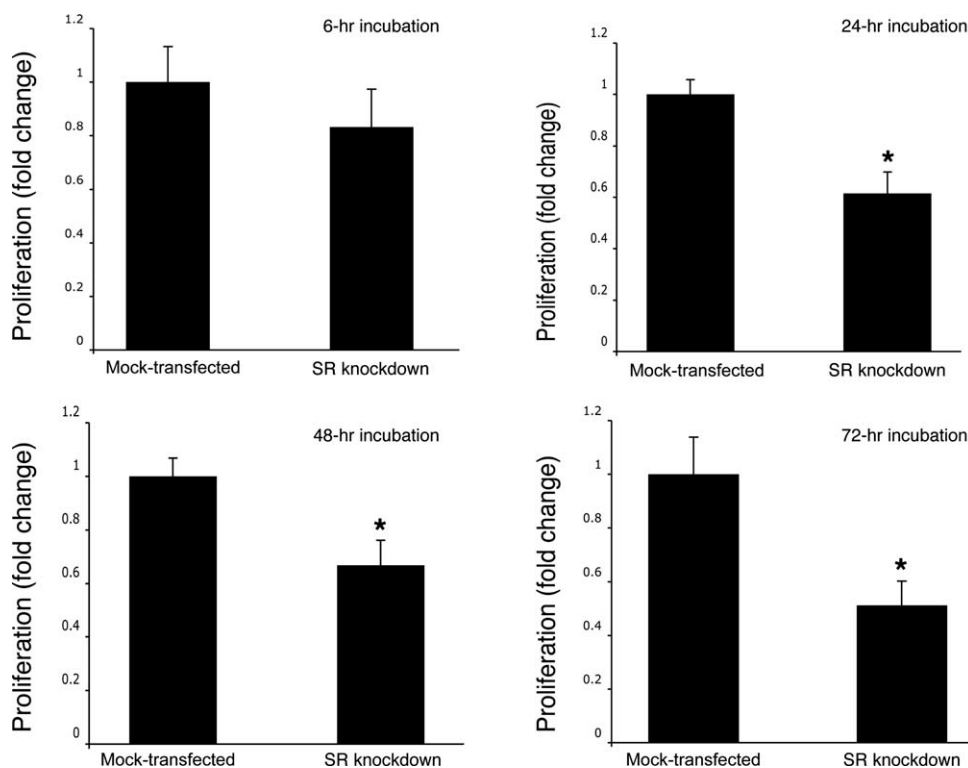


Fig. 7. Effect of secretin receptor gene silencing on the basal proliferative activity of large cholangiocytes following incubation for 6, 24, 48, and 72 hours with 0.2% BSA (MTS assay). Data are expressed as the mean \pm SEM of four experiments. * $P < 0.05$ versus its corresponding value of mock-transfected large cholangiocytes.

confirmed in BDL SR^{-/-} mice, which had reduced levels of phosphorylated ERK1/2 in isolated large cholangiocytes. As expected, large cholangiocytes 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 short hairpin RNA for SR. These SR stable knockdown cells displayed decreased basal and secretin-stimulated proliferative capacity compared with 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 with the mock-transfected controls are suggestive of 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 CCl₄ acute hepatotoxicity.¹⁴ We also have preliminary findings (unpublished data) that suggest that secretin has a protective role versus CCl₄-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 agonists³⁰ 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 proproliferative 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.

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