

Secretin inhibits cholangiocarcinoma growth via dysregulation of the cAMP-dependent signaling mechanisms of secretin receptor

Paolo Onori¹, Candace Wise², Eugenio Gaudio³, Antonio Franchitto³, Heather Francis², Guido Carpino⁴, Vien Lee⁵, Ian Lam⁵, Timothy Miller⁶, David E. Dostal^{4,6} and Shannon S. Glaser^{6,7}

¹Experimental Medicine, University of L'Aquila, L'Aquila, Italy

²Division of Research and Education, Scott and White and Texas A&M Health Science Center, College of Medicine, MRB, Temple, TX 76504

³Department of Human Anatomy, University of Rome "La Sapienza," Rome, Italy

⁴Molecular Cardiology, Scott and White and Texas A&M Health Science Center, College of Medicine, MRB, Temple, TX 76504

⁵Department of Zoology, University of Hong Kong, Hong Kong, Hong Kong, China

⁶Department of Medicine, Scott and White and Texas A&M Health Science Center, College of Medicine, MRB, Temple, TX 76504

⁷Digestive Disease Research Center, Scott and White and Texas A&M Health Science Center, College of Medicine, MRB, Temple, TX 76504

Secretin plays a key role in the regulation of normal cholangiocyte physiology via secretin receptor (SCTR). SCTR expression is upregulated during extrahepatic cholestasis induced by bile duct ligation and closely associated with cholangiocyte proliferative responses. Although well studied in normal cholangiocytes, the role of secretin and the expression of SCTR in the regulation of cholangiocarcinoma proliferation are unknown. *In vitro*, secretin (10^{-7} M) displayed differential effects on normal cholangiocyte [H-69 and human intrahepatic biliary epithelial cell line (HIBEpiC)] and cholangiocarcinoma (Mz-ChA-1, HuH-28, TFK-1, SG231, CCLP1 and HuCC-T1) cell lines as such secretin is mitogenic for normal cholangiocytes and antiproliferative for cholangiocarcinoma. As expected in normal cholangiocytes (HIBEpiC), secretin increased intracellular cyclic adenosine monophosphate (cAMP) levels. However, the effect of secretin on intracellular cAMP levels was suppressed in Mz-ChA-1 cells. Secretin-stimulated intracellular cAMP levels in Mz-ChA-1 were restored by pretreatment with pertussis toxin, suggesting that the receptor coupled to G_{α_i} rather than G_{α_s} . SCTR expression was found to be downregulated in 4 of the 6 cholangiocarcinoma cell lines evaluated and in human cholangiocarcinoma biopsy samples. *In vivo*, secretin significantly inhibited the tumor size and more than doubled tumor latency, which was associated with a decrease in proliferating cell nuclear antigen and an increase in cleaved-caspase 3 expression levels. Our results demonstrate that secretin and/or the modulation of SCTR expression might have potential as a therapeutic tool in the treatment of cholangiocarcinoma.

Cholangiocytes are simple epithelia, which line the intrahepatic and extrahepatic ducts and are the target of chronic cholestatic liver diseases, that are characterized biliary inflam-

mation and by the dysregulation of the balance between cell growth and apoptosis.^{1,2} Cholangiocarcinoma results from the malignant transformation of cholangiocytes.³ The pathogenesis of cholangiocarcinoma is linked to chronic biliary inflammation, which occurs in cholestatic liver diseases such as primary sclerosing cholangitis (PSC).⁴ Cholangiocarcinoma is frequently diagnosed at late stages resulting in limited treatment options and a high rate of mortality.³ Cholangiocarcinoma responds poorly to both surgical resection and standard chemotherapy.³ With limited treatment strategies and reports of increasing incidence and prevalence of cholangiocarcinoma worldwide,⁵ it is critical to further our understanding of the factors controlling cholangiocyte growth and neoplastic transformation.

Secretin is a 27-amino acid peptide hormone secreted from the duodenal S endocrine cells in response to acidic contents leaving the stomach and is also expressed by several other cell types including the brain, reproductive system, kidney and lungs.⁶ Secretin receptors (SCTR) belong to the family of G-protein-coupled receptors,⁷ which signal through the activation of adenylyl cyclase and protein kinase A (PKA).⁷

Key words: cholangiocarcinoma, secretin, xenograft, secretin receptor

Abbreviations: cAMP: cyclic adenosine monophosphate; CFTR: cystic fibrosis transmembrane conductance regulator; CK-19: cytokeratin 19; DAPI: 4',6-diamidino-2-phenylindole; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; MTS: 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium: inner salt; PCNA: proliferating cell nuclear antigen; PKA: protein kinase A; SCTR: secretin receptor.

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Correspondence to: Shannon S. Glaser, Texas A&M Health Science Center, 702 SW H.K. Dodgen Loop, Temple, TX 76504, USA, Fax: +254-724-5944, E-mail: sglaser@tamu.edu

In the liver, SCTRs are expressed only on the basolateral membrane of rodent cholangiocytes.^{8,9} In the human liver, SCTR expression is limited to the biliary tract and cholangiocarcinoma.¹⁰ Secretin stimulates ductal bile secretion by a series of coordinated events. First, secretin binds to the basolateral SCTRs, which induces elevation of intracellular cyclic adenosine monophosphate (cAMP) leading to the activation of PKA.¹¹ Subsequently, PKA phosphorylates the cystic fibrosis transmembrane conductance regulator (CFTR) triggering the opening of this Cl⁻ channel leading to secretion of Cl⁻ at the apical membrane of cholangiocytes, which results in membrane depolarization.¹² The Cl⁻ efflux from CFTR creates a Cl⁻ gradient that favors activation of the apically located Cl⁻/HCO₃⁻ exchanger,¹³ which results in secretin-stimulated bicarbonate-enriched bile.^{14,15} Models of cholangiocyte hyperplasia are closely associated with increased secretin-stimulated choleresis, which is characterized by increased SCTR gene expression, elevated secretin-stimulated cAMP levels, enhanced Cl⁻/HCO₃⁻ exchanger activity and amplified secretin-stimulated bicarbonate secretion.^{1,14,16} However, any possible direct role of secretin in the regulation of normal, hyperplastic and neoplastic cholangiocyte proliferation has yet to be fully explored.

Based upon these previous reports and the importance of secretin and SCTR in the regulation of normal biliary physiology, it was deemed appropriate to evaluate the role of secretin and SCTR expression in the regulation of the proliferation of normal and malignant cholangiocytes. Consequently, the aim of this study was to determine the effect of secretin on cholangiocarcinoma proliferation *in vitro* and *in vivo* and to evaluate the expression of SCTR in cholangiocarcinoma and normal cholangiocyte cell lines and human biopsy samples.

Material and Methods

Materials

Reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise indicated. Human secretin was purchased from Bachem California (Torrance, CA). The rabbit anti-SCTR antibody was purchased from Sigma-Aldrich. The rabbit anti-active caspase-3 antibody and goat anti-rat SCTR antibody were purchased from Abcam (Cambridge, MA) and Santa Cruz Biotechnology (Santa Cruz, CA), respectively. The mouse anti-proliferating cell nuclear antigen (PCNA) and the goat anti-cytokeratin 19 (CK-19) and cytokeratin 7 (CK-7) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The cAMP enzyme immunoassay (EIA) kit was purchased from Cayman Chemical Company (Ann Arbor, MI). The IP-One ELISA was purchased from Cisbio US (Bedford, MA).

Cell culture

We used 6 human cholangiocarcinoma cell lines (Mz-ChA-1, HuH-28, TFK-1, SG231, CCLP1 and HuCC-T1) of different

origins. Mz-ChA-1 cells, from human gallbladder, were a gift from Dr. G. Fitz (University of Texas Southwestern Medical Center, Dallas, TX).¹⁷ HuH-28 cells, from human intrahepatic bile duct,¹⁸ and TFK-1 cells, from human extrahepatic bile duct,¹⁹ were acquired from the Cancer Cell Repository, Tohoku University, Japan. Mz-ChA-1, HuH-28 and TFK-1 cell lines were maintained at standard conditions as previously described.²⁰ The human immortalized, nonmalignant cholangiocyte cell line, H-69 (a gift from Dr. G.J. Gores, Mayo Clinic, Rochester, MN), was cultured as described.²¹ HuCC-T1, CCLP1 and SG231 cells, from intrahepatic bile ducts, were a kind gift from Dr. A.J. Demetris (University of Pittsburgh, Pittsburgh, PA) and were cultured as described.²²⁻²⁴ The normal human intrahepatic biliary epithelial cell line (HIBEpIC) was purchased from ScienCell Research Laboratories (Carlsbad, CA) and cultured as recommended by the vendor. All experiments were performed when cells reached 80% confluency and conducted in serum-free medium with serum deprivation for 24 hr before the experiment.

Expression of SCTR

Immunofluorescence. We detected SCTR expression in the Mz-ChA-1, HuH-28, TFK-1, SG231, CCLP1, HuCC-T1, H-69 and HIBEpIC cell lines by immunofluorescence. Cells were seeded into 6-well dishes containing a sterile coverslip on the bottom of each well. Cells were allowed to adhere overnight, washed once in cold phosphate-buffered saline (PBS), fixed to the coverslip with 4% paraformaldehyde (in 1× PBS) at room temperature for 5 min, permeabilized in PBS containing 0.2% Triton X-100 (PBST) and blocked in 4% bovine serum albumin (BSA) in PBST for 1 hr. The SCTR antibody was diluted (1:50) in 1% BSA in PBST, added to the coverslips and incubated overnight at 4°C. Cells were washed 3–10 min in PBST and a 1:50 dilution (in 1% BSA in PBST) of Cy3-conjugated secondary antibody (Jackson Immunochemicals, West Grove, PA) was added for 2 hr at room temperature. Cells were washed again and mounted into microscope slides with Prolong Antifade Gold containing 4',6-diamidino-2-phenylindole as a counterstain (Molecular Probes, Eugene, OR). Negative controls were performed with the omission of the respective primary antibodies. Sections were visualized using an Olympus IX-71 inverted confocal microscope.

Fluorescence-activated cell-sorting analysis. For profiling of SCTRs in cholangiocarcinoma cell lines, cells were dissociated in trypsin/ethylenediaminetetraacetic acid (EDTA), pelleted by centrifugation and resuspended at 0.5×10^6 cells/ml in 100- μ l binding buffer [PBS [pH 7.40], 1% fetal bovine serum (FBS), 2 mM EDTA and 0.2% saponin]. After addition of 1 μ g of goat antirat SCTR antibody (sc-26633, Santa Cruz) or vehicle (control), cells were incubated for 20 min at 22°C. Cells were then washed and incubated in 100- μ l binding buffer containing 2- μ g Alexa 488-conjugated chicken-anti-

goat secondary antibody (A-21467, Invitrogen Corp.). After washing with binding buffer, cells were resuspended in flow buffer (PBS [pH 7.40], 1% FBS, 2 mM EDTA) and scanned on a BD FACsCalibur flow cytometry, in which 10,000 events were recorded for each sample. Results were analyzed using CellQuest Pro software (BD Biosciences).

Real-time PCR. To evaluate the gene expression of SCTR in the selected cell lines, we used the RT² Real-time assay from SuperArray Bioscience Corporation (Frederick, MD). RNA was extracted with the RNeasy Mini Kit (Qiagen, Valencia, CA) and reverse transcribed using the Reaction ReadyTM First Strand cDNA synthesis kit (SuperArray, Frederick, MD). SYBR Green PCR master mix (SuperArray) was used in the experimental assay with RT² PCR rat primers designed specifically for SCTR and the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (SuperArray, Frederick, MD). Real-time RT-PCR was performed with an ABI Prism 7900HT System using a 2-step PCR cycling program at 95°C for 10 min followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. A $\Delta\Delta CT$ analysis was performed using HIBEpiC cells as the control.

Cholangiocarcinoma tissue array analysis. Immunoreactivity for SCTR and the cholangiocyte marker CK-19 were assessed in commercially available **Accumax tissue arrays** (Isu Abxis Co.) by immunohistochemistry, as described,²⁵ using specific antibodies. These tissue arrays contain 48 well-characterized cholangiocarcinoma biopsy samples from a variety of tumor-differentiation grades, as well as 4 control liver biopsy samples. At low magnification in cholangiocarcinoma tissue samples, the malignant cells form a mass composed by small glandular structures with oval and vesicular nuclei and with poor and irregular defined lumen structure. Usually, normal bile ducts (characterized by regular lumen structure) are separated from these malignant areas. In our analysis, we avoided areas that contained normal bile duct structures. Semiquantitative analysis was performed by 3 independent observers, in a blind fashion, using the following variables: (i) staining intensity was assessed on a scale from 1 to 4 (1, no staining; 4, intense staining) and (ii) the abundance of positively stained cells was given a score from 1 to 5 (1, no cells stained; 5, 100% stained). The staining index was then calculated by the staining intensity multiplied by the staining abundance that gave a range from 1 to 20.

Effect of secretin on malignant and normal cholangiocyte growth. Cholangiocyte growth was evaluated by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS)] proliferation assay (an index of changes in cell number²⁶; Promega Corporation, Madison, WI) in the Mz-ChA-1, HuH-28, TFK-1, SG231, CCLP1, HuCC-T1, H-69 and HIBEpiC cell lines. Immediately after trypsinization, cells were seeded into 96-well plates (5,000 cells per well) in a final

volume of 200 μ l of medium. After 24 hr of culture to allow reattachment, the cells were changed to serum-free medium and incubated for 24 hr. The cells were then treated with secretin (10^{-6} M to 10^{-12} M) for 48 hr. In additional experiments, Mz-ChA-1 and HIBEpiC cells were treated with secretin (10^{-7} M) for 24, 48 and 72 hr. At the end of each experiment, the MTS cell proliferation reagent (20 μ l) was added to each well, and the cells were incubated for 1–3 hr. Absorbance at 450 nm was measured using an automatic ELISA plate reader. In the case of Mz-ChA-1 cells, the secretin-induced reduction in proliferation was also assessed following pretreatment with pertussis toxin (PTX; 100 ng/ml). In all cases, data were expressed as the fold change of treated cells when compared with vehicle-treated controls.

The proliferative status of Mz-ChA-1 and HIBEpiC cells treated with vehicle and secretin was also determined by cell-cycle analysis by fluorescence activated cell sorting (FACS) with the Cell Cycle Phase Determination Kit (Accuri Cytometers, Ann Arbor, MI) according to the manufacturer's protocol.²⁷ Briefly, the cells were seeded in a 6-well plate at a density of 10^5 cells per well in 2 ml of culture medium. The cells were cultured overnight, the media were changed to serum free and the cells were incubated an additional 24 hr in a CO₂ incubator at 37°C. The cells were then stimulated with vehicle or secretin (10^{-7} M) for 48 hr. The cells were dissociated in trypsin/EDTA, pelleted by centrifugation and resuspended at $\sim 10^6$ cells/ml in $1 \times$ PBS containing 0.1% FBS. The cells were fixed in 70% ice-cold ethanol, followed by incubation in nuclei staining buffer [propidium iodide (PI)/RNase] for 30 min at 22°C according to the manufacturer's recommendations. Cell-cycle histograms were generated after analyzing the PI-stained cells by FACS (Accuri C6 Cytometers), in which 10^4 events were recorded for each sample. The samples were analyzed in the FL2 channel of the flow cytometer with a 488-nm excitation laser. The percentage of cells in G₀/G₁, S and G₂/M phases of the cell cycle was determined using software analysis CFlow Plus, Version 1.0.172.9 (Accuri Cytometers).

Evaluation of the intracellular mechanism by which secretin regulates normal and malignant cholangiocyte growth

Effect of secretin on cyclic AMP levels. SCTR has been well documented to stimulate physiological effects in both pancreatic and biliary epithelia cells via the receptor coupling with G α_s , resulting in the activation of adenylyl cyclase.^{6,9} To test if SCTR was functional in Mz-ChA-1 (cholangiocarcinoma) and HIBEpiC (normal cholangiocytes), we evaluated if secretin stimulation would trigger increased intracellular cAMP levels. After trypsinization, Mz-ChA-1 and HIBEpiC cell lines (1×10^6 cells per well) were plated in 6-well cell-culture plates and allowed to reattach overnight. The following day, the plates were stimulated with vehicle (0.2% BSA), secretin (10^{-7} M) and forskolin (an adenylyl cyclase activator, 10 μ M) for 10 min at room temperature. In selected

experiments, cells were pretreated with PTX (100 ng/ml) for 30 min at room temperature. Immediately following incubation, the media were aspirated from the wells and 1 ml of 0.1 M HCl (blocks endogenous phosphodiesterase activity) was added. The plate was incubated for 20 min at room temperature, and the cells were scraped off the plate surface with a cell scraper. The mixture was dissociated by pipetting up and down and then centrifuged at 1,000g for 10 min. The supernatant was retained, and cAMP levels were determined by a competitive EIA kit (Cayman).

Effect of secretin on D-myo-inositol-1,4,5-triphosphate (IP₃). Activation of intracellular IP₃/Ca²⁺ signaling has been shown to play a key role in the regulation of normal and cholestatic cholangiocyte proliferation via crosstalk between the IP₃/Ca²⁺- and cAMP-dependent signaling mechanisms.^{28–32} Depending upon the factor and the pathway-activated downstream of IP₃/Ca²⁺, activation of IP₃/Ca²⁺-dependent signaling can either augment or inhibit proliferation. For example, the hormone gastrin inhibits cholangiocyte proliferation in rats with bile duct ligation (BDL) via activation of IP₃/Ca²⁺-dependent activation of protein kinase C α (PKC α) resulting in the downregulation of cAMP levels.^{29,30,32} On the other hand, the bile acid taurocholate activates cholangiocyte proliferation and augments the effects of secretin on bicarbonate secretion via the activation of Ca²⁺-dependent signaling.^{31,32} To determine if the SCTR triggers the IP₃/Ca²⁺ signaling pathway as an alternative mechanism to cAMP, we determined the effects of secretin on intracellular IP₃ levels. After trypsinization, Mz-ChA-1 and HIBEpiC cell lines (5,000 cells per well) were plated in 6-well cell culture plates and allowed to reattach overnight. The following day, the plates were stimulated with vehicle (0.2% BSA) and secretin (10⁻⁷ M) for 10 min at room temperature. Following stimulation, the samples were processed according to the manufacturer's protocol for the IP-1 ELISA kit. The IP₃ lifetime within the cell is very short (less than 30 sec) before it is transformed into IP₂ and IP₁.³³ The kit is based upon the principle that when LiCl (lithium chloride) is added to the culture medium, the degradation of IP₁ into myo-inositol is inhibited and IP₁ can therefore accumulate in the cell.³³ Therefore, after receptor activation, IP₁ can be precisely quantified using the IP-1 assay.

Effect of secretin on cholangiocarcinoma xenograft growth in nude mice

Treatment schedule. Male 8-week-old BALB/c nude (*nu/nu*) mice were purchased from Taconic Farms (Germantown, NY). The mice were kept in a temperature-controlled environment (20–22°C) with a 12-h light–dark cycle with free access to drinking water and standard mouse chow. Study protocols were performed in compliance with the institutional guidelines. Mz-ChA-1 cells (3 × 10⁶) were suspended in 0.5 ml of extracellular matrix gel (Sigma-Aldrich) and injected subcutaneously in the left and right back flanks of the mice. Following 2 weeks

for tumor establishment, mice were divided into 2 groups: (i) the first group (*n* = 5) received 0.9% NaCl by subcutaneous injections near the tumor site (100 μ l); and (ii) the second group (*n* = 5) received secretin by subcutaneous injections near the tumor site (25 μ g/kg/BW every third day) for 57 days. The same operator did the injections and tumor measurements. Tumor size measured immediately before treatments with an electronic caliper. Tumor volume was determined as tumor volume (mm³) = 0.5 × [length (mm) × width (mm) × height (mm)]. Latency was determined as the time for the tumor to increase 150% of the initial volume. After 57 days, the mice were anaesthetized with sodium pentobarbital (50 mg/kg IP) and sacrificed according to institutional guidelines. Before sacrifice, serum was obtained, and aspartate aminotransferase (AST), alanine aminotransferase (ALT) and creatinine were measured. Tumor samples were collected and fixed in formalin, embedded in paraffin and processed for histopathology and immunohistochemistry. Liver blocks were obtained, fixed in formalin, embedded in paraffin, processed for histopathology and stained for H&E and Masson's trichrome for the detection of organ damage.

Morphologic analysis of tumor tissues. Tumor tissues were dissected from the mice. Neoplastic tissues were fixed in formalin, embedded in paraffin, processed for histopathology, stained for H&E for routine examination and Masson's trichrome for collagen visualization and examined by light microscopy. Tumor sections from each group (*n* = 4) were evaluated by immunohistochemistry for CK-7 (a cholangiocyte specific marker), PCNA (a marker of cellular proliferation³⁰) and cleaved caspase 3 (marker of apoptosis) as described.³⁴ Tumor sections were also stained for SCTR and vascular endothelial growth factor (VEGF)-A expression. Following staining, sections were counterstained with hematoxylin and examined with a microscope.

Statistical analysis

All data are expressed as mean \pm SEM. Differences between groups were analyzed by the Student unpaired *t*-test when 2 groups were analyzed and analysis of variance (ANOVA) when more than 2 groups were analyzed, followed by an appropriate post hoc test. A value of *p* < 0.05 was considered significant.

Results

Normal and malignant cholangiocytes express SCTR

The SCTR was detected in Mz-ChA-1, HuH-28, TFK-1, SG231, CCLP1, HuCC-T1, H-69 and HIBEpiC cell lines by immunofluorescence (Fig. 1a). We also evaluated SCTR expression in the cell lines by FACS analysis. SCTR expression was significantly decreased in the cholangiocarcinoma cell lines compared with the normal HIBEpiC with the exception of TFK-1 and HuCC-T1, which were significantly increased versus the normal HIBEpiC (Fig. 1b). However, the expression levels of the message for SCTR were significantly

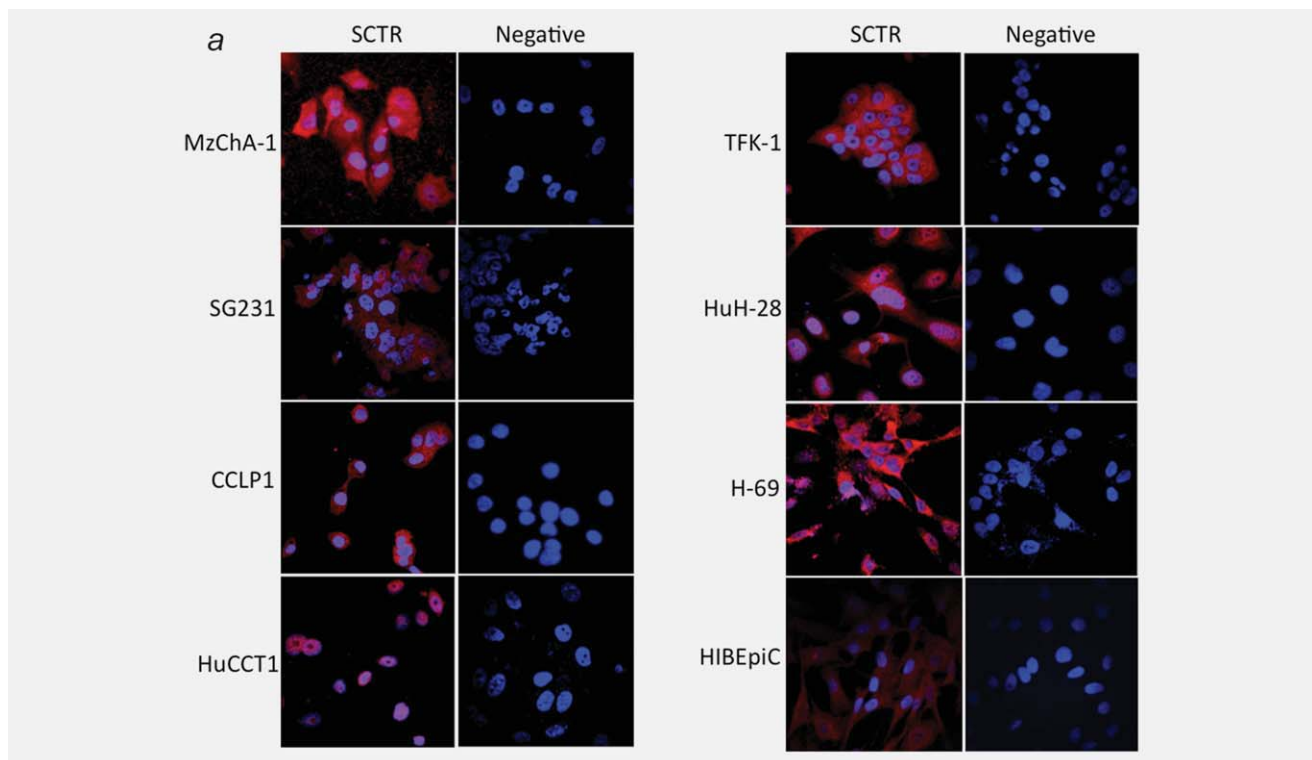


Figure 1. Expression of SCTR by normal and malignant cholangiocyte cell lines by immunofluorescence and real-time PCR. (a) All cholangiocarcinoma and normal cell lines were positive for SCTR by immunofluorescence, (b) FACS analysis and (c) by real-time PCR. (b) There was a significant reduction in SCTR expression in the cholangiocarcinoma cell lines compared to HIBEpIC with the exception of HuCC-T1 and TFK-1, which are significantly increased. $*p < 0.001$ versus HIBEpIC. (c) The expression of the message for SCTR was significantly decreased in the cholangiocarcinoma cell lines (with the exception of HuCC-T1 that was slightly but not significantly decreased) compared to the nonmalignant cell line HIBEpIC. Asterisk denotes significance ($p < 0.05$) compared with nonmalignant cell HIBEpIC.

decreased in all the cholangiocarcinoma cell lines (Mz-ChA-1, SG231, CCLP1, TFK-1 and HuH-28) compared to the nonmalignant cholangiocyte cell line HIBEpIC with the exception of HuCC-T1 in which SCTR expression of depressed but not statistically significant (Fig. 1c) compared to the primary cholangiocyte cell HIBEpIC. The expression level of the message of SCTR was elevated in the SV-40 transformed normal H-69 cell line, whereas the SCTR expression by FACS analysis was depressed compared to HIBEpIC. The discrepancies between the message and protein expression may merely be due to difference in message stability in the cholangiocarcinoma cell lines. However, all the cholangiocarcinoma cell lines express the message for SCTR.

Expression of SCTR in cholangiocarcinoma tissue array samples

Immunohistochemical analysis of human liver biopsy samples indicated that there was a significant suppression in SCTR immunoreactivity in cholangiocarcinoma samples compared to control, as assessed by 3 independent observers (Fig. 2; and data not shown). There were no differences in SCTR immunoreactivity in the cholangiocarcinoma biopsies as a function of tumor grade. Both nonmalignant bile ducts and cholangiocarcinoma samples stained positive for the cholangiocyte-specific

marker, CK-19 (Fig. 2). Representative grade 2/3 cholangiocarcinoma samples were presented in Figure 2.

Secretin inhibits cholangiocarcinoma growth *in vitro*

Secretin (10^{-6} M to 10^{-12} M) significantly, dose-dependently decreased the proliferation of the cholangiocarcinoma cell lines Mz-ChA-1, HuH-28, TFK-1, SG231, CCLP1 and HuCC-T1 (Fig. 3). To the contrary, secretin dose-dependently increased the proliferation of the nonmalignant cell lines, H69 and HIBEpIC (Fig. 3a). We also determined that secretin stimulated the time-dependent inhibition of proliferation in Mz-ChA-1 cells at 48 and 72 hr and the time-dependent stimulation of proliferation of HIBEpIC at 24, 48 and 72 hr (Fig. 3b), which was consistent with the dose-response curves. Consistent with the findings of MTS proliferation assay, secretin inhibited the proliferation of Mz-ChA-1 cells and stimulated the proliferation of HIBEpIC as determined by FACS analysis of the cell cycle using an Accuri C6 Flow Cytometer (Table 1).

Secretin fails to stimulate intracellular cAMP levels in Mz-ChA-1 cells

To evaluate potential downstream intracellular signaling mechanisms regulating secretin's effects, Mz-ChA-1 and

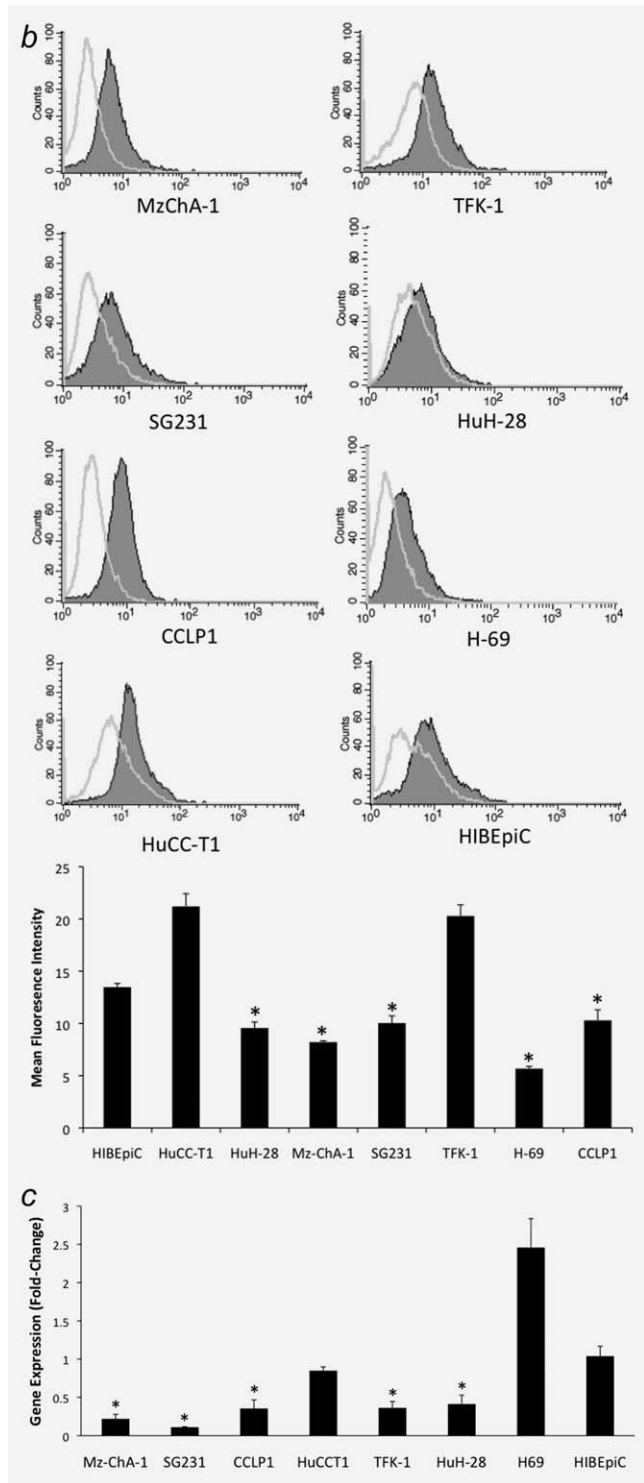


Figure 1. Continued

HIBepiC cells were stimulated with vehicle (0.5% BSA) and secretin (10^{-7} M) for 10 min. As expected, secretin stimulated a significant increase in intracellular cAMP levels in HIBepiC (Fig. 4). Also, as expected, the adenylyl cyclase activator, forskolin, stimulated a dramatic increase of intracellular cAMP levels in both Mz-ChA-1 and HIBepiC cells, which

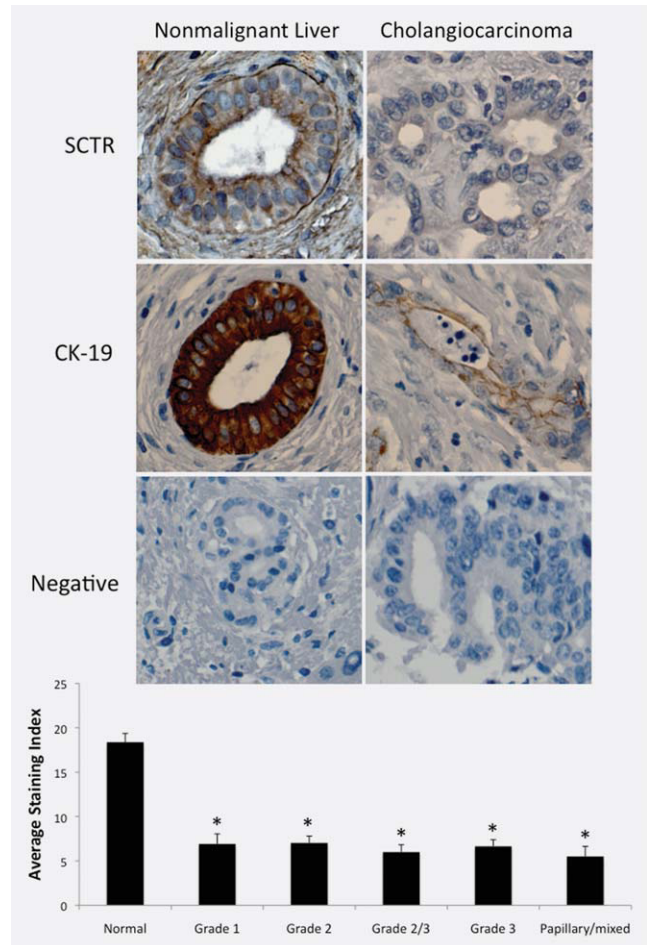


Figure 2. SCTR-expression levels assessment in biopsy samples from 48 cholangiocarcinoma patients and healthy controls by immunohistochemistry. [Top] Representative photomicrographs of the SCTR and CK-19 immunoreactivity in tumor grade 2/3. Negative staining was performed in the absence of the primary antibody for SCTR. Magnification, 40 \times . [Bottom] Staining intensity was assessed as described in Material and methods section. Data are represented as the average of all cholangiocarcinoma patients in a tumor grade category compared with control samples. SCTR immunoreactivity was significantly decreased in samples from patients with cholangiocarcinoma. Asterisk denotes significance ($p < 0.05$) compared with SCTR immunoreactivity in control biopsy samples.

was an indication of functional adenylyl cyclase. However, stimulation with secretin did not induce increased intracellular cAMP levels in Mz-ChA-1 cells (Fig. 4). There was a slight but nonsignificant depression in intracellular cAMP levels in Mz-ChA-1 cells stimulated with secretin. When Mz-ChA-1 cells were pretreated with PTX, the cells regained responsiveness to secretin as evidenced by an increase intracellular cAMP (Fig. 4), suggesting that the SCTR in the Mz-ChA-1 cells was coupled to $G\alpha_i$ rather than $G\alpha_s$. Pertussis toxin catalyzes the ADP-ribosylation of the α subunits of the

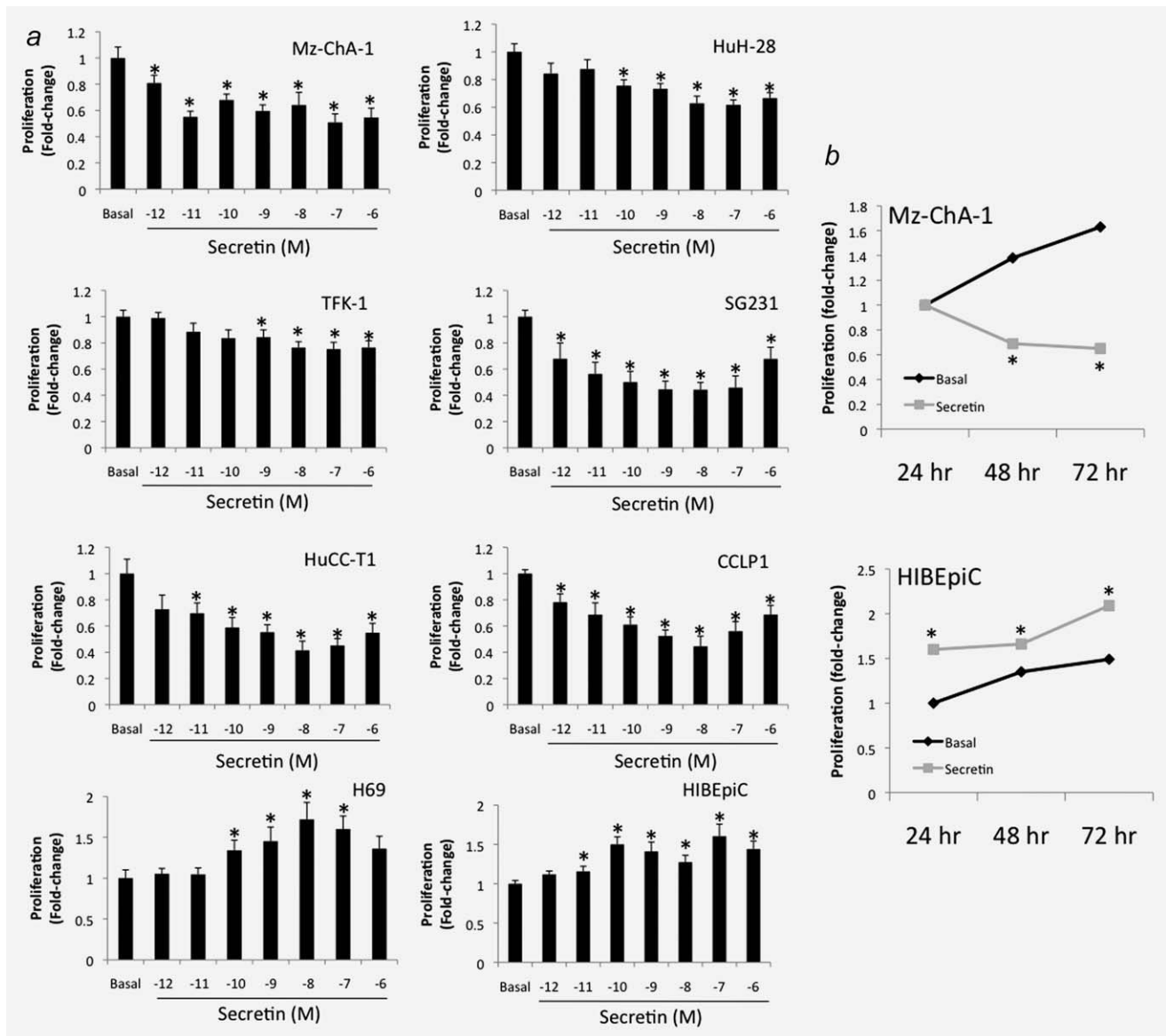


Figure 3. Effects of secretin on cholangiocarcinoma and normal cell line proliferation. (a) Cholangiocarcinoma and the normal cholangiocyte cell lines were treated with various concentrations of secretin (10^{-12} to 10^{-6} M) for 48 hr. Cell proliferation was assessed using an MTS cell-proliferation assay. Data are expressed as fold change in proliferation. Secretin significantly decreased the proliferation of all of the cholangiocarcinoma cell lines, whereas it increased the proliferation of the 2 normal cell lines, H69 and HIBEpiC. (b) Mz-ChA-1 and HIBEpiC cells were treated with secretin (10^{-7} M) for 24, 48 and 72 hr. Secretin induced a time-dependent inhibition of Mz-ChA-1 proliferation at 48 and 72 hr and stimulated the proliferation of HIBEpiC at 24, 48 and 72 hr. Data are the mean \pm SEM for $n = 6$ experiments. Asterisk denotes $p < 0.05$ compared with basal treatment within each cell line.

heterotrimeric guanine nucleotide regulatory protein $G\alpha_i$, thus blocking their coupling and activation.³⁵ Secretin had no effect on IP_3 levels as determined by the IP_3 ELISA in either the cholangiocarcinoma cell lines and the normal cell lines (data not shown).

Pretreatment with PTX restores secretin-induced proliferation in cholangiocarcinoma

Mz-ChA-1 cells were pretreated with PTX and subsequently stimulated with secretin for 48 hr. Pretreatment PTX restored

Table 1. Cell-cycle analysis of Mz-ChA-1 and HIBEpiC treated with vehicle (control) or secretin

Cell line	Treatment	G1 (%)	G2 (%)	S (%)
Mz-ChA-1	Vehicle	69.6	10.9	7.2
	Secretin	74.1	9.8	6.8
HIBEpiC	Vehicle	69.3	14.0	1.82
	Secretin	65.9	15.2	2.6

The percentage of cells in G_0/G_1 , S and G_2/M phases of the cell cycle were determined using software analysis CFlow Plus, Version 1.0.172.9 (Accuri Cytometers, Inc.).

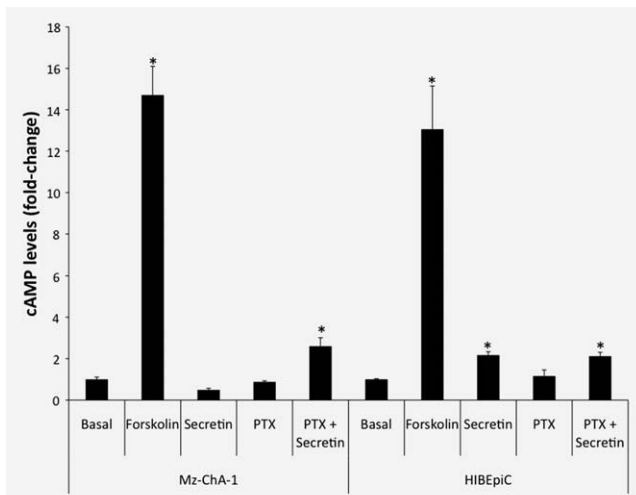


Figure 4. Effects of secretin on intracellular cAMP levels in Mz-ChA-1 cholangiocarcinoma and HIBEpIC normal human cell lines. Mz-ChA-1 and HIBEpIC cells were stimulated with vehicle (0.9% NaCl) and secretin (10^{-7} M) for 10 min in the presence and absence of pretreatment with PTX (100 ng/ml). Forskolin (10 μ M; an adenylyl cyclase activator) was used as a positive control to demonstrate the presence of active adenylyl cyclase. Secretin did not stimulate an increase in intracellular cAMP levels in Mz-ChA-1 cells, but the cells were responsive to forskolin indicated by a significant increase in cAMP levels. As expected, secretin stimulated a significant increase in intracellular cAMP levels in the normal HIBEpIC cell line. Secretin-induced cAMP levels were restored in Mz-ChA-1 cells following pretreatment with PTX. Data are the mean \pm SEM for $n = 5$ experiments. Asterisk denotes $p < 0.05$ compared with basal treatment within each cell line.

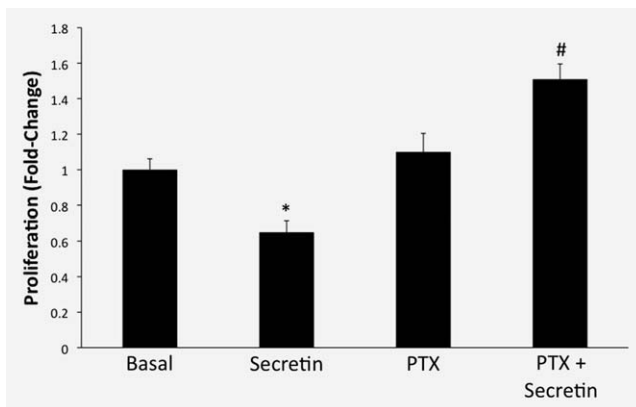


Figure 5. Effects of PTX pretreatment on secretin-induced inhibition of Mz-ChA-1 proliferation. Mz-ChA-1 cells were stimulated with vehicle (0.9% NaCl) or secretin (10^{-7} M) in the presence and absence of pretreatment with PTX (100 ng/ml), and proliferation was evaluated by MTS proliferation assay. Pretreatment with PTX prevented the decrease in Mz-ChA-1 proliferation induced by secretin. Data are the mean \pm SEM for $n = 6$ experiments. Asterisk denotes $p < 0.05$ compared with basal treatment. # denotes $p < 0.05$ compared to basal.

Table 2. Effect of SCT on cholangiocarcinoma xenograft growth and nude mice variables after 57 days of treatment

Variable	Vehicle (control)	Secretin
Body weight (g)	30.12 \pm 1.95	30.83 \pm 1.79 ^(NS)
Liver weight (g)	1.94 \pm 0.13	1.98 \pm 0.11 ^(NS)
Tumor latency (d)	11.57 \pm 2.56	24.04 \pm 1.59*
Tumor size (mm ³)	1,094.50 \pm 217.71	387.82 \pm 68.75**

Note: Data are a mean \pm SEM for $n = 5$ mice for vehicle and $n = 6$ mice for SCT treatments.

Abbreviations: NS, not significant; n.v., normal value.

* $p < 0.05$ versus the corresponding value of vehicle-treated mice. ** $p < 0.009$ versus the corresponding value of vehicle-treated mice.

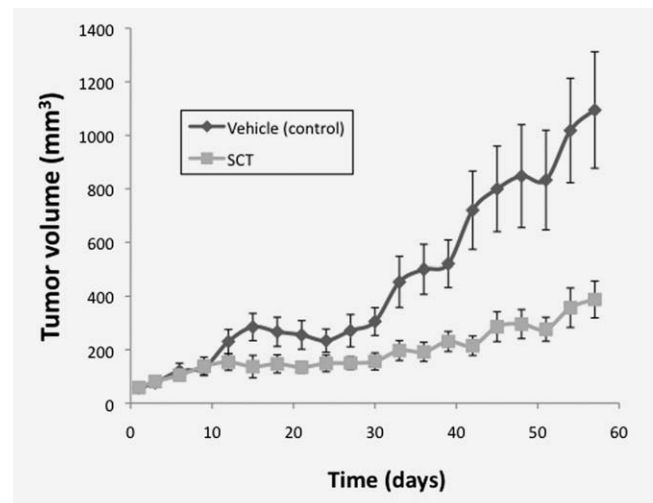


Figure 6. Secretin decreases tumor growth in an *in vivo* xenograft model of cholangiocarcinoma. Mz-ChA-1 cells were injected into the flank of athymic mice. After tumors were established, mice were treated with 25 μ g/kg/day secretin (into the implanted tumor) for 3 days/week for 57 d and tumor volume was assessed.

the secretin-induced proliferative response of Mz-ChA-1 cells (Fig. 5) to level similar to those observed for HIBEpIC (Fig. 3) as determined by MTS proliferation assay.

Secretin inhibits cholangiocarcinoma xenograft growth *in vivo*

At 57 days, a significant difference in tumor size was found in secretin-treated mice compared with mice injected with vehicle only (secretin 387.82 \pm 68.75 vs. control 1094.50 \pm 217.71 mm³; $p < 0.009$; Table 2 and Fig. 6). No significant difference in body weight was detected between the 2 groups (Table 2). There was a significant decrease in the tumor latency²⁶ in control mice versus mice treated with secretin (Table 2). Normal serum values of AST, ALT and creatinine were detected in the 2 groups (not shown). Histologic analysis of liver also indicated no significant organ damage caused by the chronic secretin treatment (data not shown).

Histologic analysis of the excised tumors revealed that all cells within tumors from secretin-treated and vehicle-treated

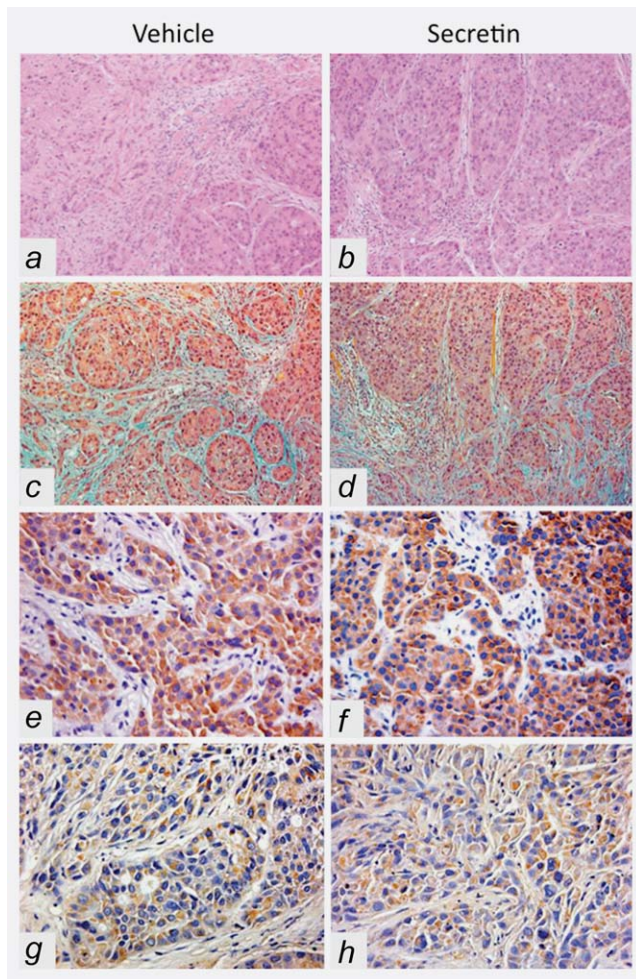


Figure 7. Histological and immunohistochemical staining for CK-7 and SCTR in xenograft tumors. Tumors were excised from mice treated with vehicle (0.9% NaCl) or secretin (25 µg/kg/BW every third day). (a, b) H&E staining was performed for routine staining and (c, d) Masson's trichrome staining for collagen visualization. There were no detectable changes in tissue necrosis or fibrosis. Tumors from both treatment groups stained positive for CK-7 (e, f) and SCTR (g, h). Representative photomicrographs of the histochemistry and immunoreactivity are shown. Magnification, 40×.

animals were CK-7 and SCTR-positive, indicating cholangiocyte phenotype (Fig. 7, Table 3). There were similar amounts of necrosis and fibrosis between the treatment groups (Fig. 7). By semiquantitative analysis, there was a decrease in the number of PCNA-positive cholangiocytes and an increase in the number of cells positive for cleaved caspase 3 (Table 3; Fig. 8). There was a significant reduction in PCNA-labeling index in the tumors from animals treated with secretin (38.61 ± 1.80) compared to those receiving vehicle (71.28 ± 2.10). There were no observable changes in the expression of VEGF-A between the treatment groups (Table 3; Fig. 8).

Table 3. Semi-quantitative analysis for CK-7, SCTR, PCNA, cleaved caspase 3 and VEGF-a expression by immunohistochemistry in excised tumors

Variable	Vehicle (control)	SCT
CK-7	+++	+++
SCTR	+++	++
PCNA	+++	++
Cleaved Caspase 3	–	+
VEGF-A	+++	+++

Analysis legend:
 (–) = 0–5%.
 (+/–) = 6–10%.
 (+) = 11–30%.
 (++) = 31–60%.
 (+++) = >61%.

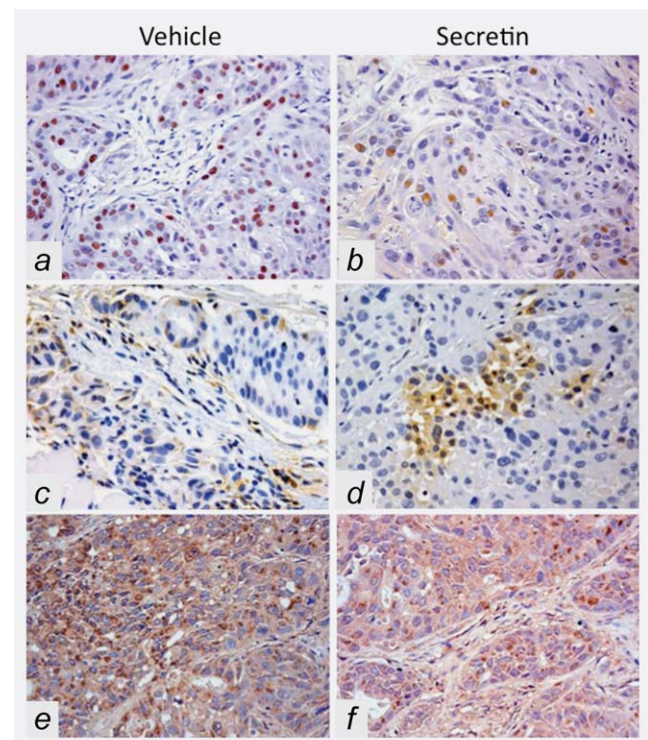


Figure 8. Immunohistochemical staining for PCNA, cleaved caspase 3 and VEGF-A in xenograft tumors. Tumors were excised from mice treated with vehicle (0.9% NaCl) or secretin (25 µg/kg/BW every third day). (a, b) PCNA protein expression was decreased in tumors treated with secretin; (c, d) cleaved-caspase 3 expression was increased in tumors treated with secretin; (e, f) Tumors from both treatment groups stained positive for VEGF-A with similar expression levels. Representative photomicrographs of the histochemistry and immunoreactivity are shown. Magnification, 40×.

Discussion

Secretin is a well-known, classical regulator of cholangiocyte biliary physiology that stimulates biliary bicarbonate secretion via the SCTR.^{9,14,36} Although much is known about the

action of this hormone on the physiological functions of cholangiocytes,³⁶ there is less information on the potential role that secretin may have in regulating cholangiocyte proliferative activities in normal and diseased states. The observation that there is high-SCTR expression in ductular reaction in liver cirrhosis¹⁰ suggests that secretin plays a crucial role in the pathogenesis of cholestatic liver diseases and may certainly have a role in cholangiocarcinogenesis. Because of the potential for secretin to play a key role in liver disease pathogenesis, it is clearly important to expand our knowledge of and determine if secretin plays a role in the regulation of cholangiocyte proliferation. In our study, we present the first novel evidence that secretin differentially regulates the proliferation of normal and malignant cholangiocytes. As such, secretin stimulates the proliferation of normal human cholangiocytes. To the contrary, secretin inhibits proliferation in cholangiocarcinoma cell lines in both *in vitro* and *in vivo* model systems.

Numerous studies have demonstrated that increased intracellular cAMP levels play a key role the regulation of biliary proliferation and in the maintenance of biliary mass during cholestasis and liver injury.^{1,34,37,38} In fact, direct activation of adenylyl cyclase by forskolin *in vitro* and *in vivo* stimulates the proliferation of rat cholangiocytes via the activation of the PKA/Src/MEK/ERK1/2 pathway, which is downstream of intracellular cAMP levels.³⁷ In addition, the expression of SCTR expression and cholangiocyte responsiveness to secretin is upregulated during extrahepatic cholestasis induced by BDL.⁹ During extrahepatic cholestasis, basal intracellular cAMP levels are elevated in cholangiocytes isolated from rats with BDL compared to those isolated from normal rats.⁹ Taken together, these data suggest that secretin and SCTR might play a key role in the regulation of cholangiocyte proliferative responses during cholestatic liver disease pathogenesis. In normal human cholangiocytes, we demonstrate that secretin stimulates a dose-dependent increase in cholangiocyte proliferation by MTS proliferation assay, which was confirmed by FACS analysis of the phase of the cell cycle. This observation is supported by our recent findings that, in mice lacking SCTR, there was a dramatic decrease in biliary proliferation induced by BDL.³⁹ Secretin also stimulated the proliferation of cholangiocytes in normal mouse cholangiocytes *in vitro* and *in vivo* when chronically administered to normal mice.³⁹ As expected, we show that secretin stimulated an increase in intracellular cAMP levels in normal human cholangiocytes, which is similar to findings in other human cell types and in cholangiocytes from animal models.^{6,9,14,39}

However, in the cholangiocarcinoma cell lines that were tested, we found that secretin stimulates a dose-dependent decrease in proliferation, which was also confirmed by FACS analysis of the cell cycle. This finding was in direct contrast to those in normal human cholangiocytes and suggested that deregulation of intracellular signaling downstream of SCTR might be liable for the differential effects observed. Indeed, we show in Mz-ChA-1 cholangiocarcinoma cells that secretin

fails to stimulate intracellular cAMP levels above basal values with a noted slight but not significant decrease. To confirm the presence of functional adenylyl cyclases, we stimulated Mz-ChA-1 cells with forskolin (an adenylyl cyclase activator). We found that forskolin stimulated a dramatic and significant increase in intracellular cAMP levels in Mz-ChA-1 cells, indicating a functional adenylyl cyclase system. We next proceeded to determine if perhaps the SCTR had undergone a switch in G-protein coupling from the adenylyl cyclase activating $G\alpha_s$ to that of the inhibitory $G\alpha_i$. To test this hypothesis, Mz-ChA-1 cells were pretreated with PTX, which catalyzes the ADP-ribosylation of the α subunits of the heterotrimeric guanine nucleotide regulatory protein $G\alpha_p$, thus blocking their coupling and activation.³⁵ In the Mz-ChA-1 cells, we found that pretreatment with PTX restored secretin-induced cAMP levels and proliferative responses to values similar to normal human cholangiocytes (i.e. HIBEpIC). Our findings indicate that SCTR aberrantly couples to $G\alpha_i$ in cholangiocarcinoma. Future studies will need to confirm the percentage of receptors aberrantly coupled and if this may be due to SCTR polymorphisms, which was beyond the scope of our current work.

Although the exact trigger for this switch in G-protein coupling is not known, previous studies have demonstrated that activation of elevated cAMP levels inhibits the proliferation of cholangiocarcinoma cells *in vitro*.⁴⁰ In this study, when cholangiocarcinoma cells were treated with the adrenergic agonist, UK14,304, there was a significant increase in intracellular cAMP levels and a subsequent decrease in cholangiocarcinoma proliferation.⁴⁰ Similarly, in the same study, treatment of Mz-ChA-1 cells with forskolin also significantly inhibited proliferation.⁴⁰ The inhibition of cholangiocarcinoma by increased intracellular cAMP was postulated to occur at the level of Raf-1 and B-Raf, whereby cAMP negatively regulates these 2 factors decreasing proliferation.⁴⁰ This negative regulatory mechanism bypasses Ras signaling, which has been shown to have unrestrained activation in many cancer cells, including 50% of the cases of cholangiocarcinoma, wherein mutated *Ras* genes produce abnormal proteins that remain locked in an active state, thereby inducing uncontrolled proliferative signals.⁴¹ Therefore, the switch in SCTR G-protein coupling from $G\alpha_s$ to that of the inhibitory $G\alpha_i$ may be an avoidance mechanism in cholangiocarcinoma to limit the production of the antiproliferative signaling molecule, cAMP.

We also found that there was a significant reduction in SCTR expression by FACS analysis in all but 2 of the cholangiocarcinoma cell lines compared to the nonmalignant, HIBEpIC. Immunohistochemical analysis of SCTR in human biopsies also revealed a significant suppression of SCTR immunoreactivity in cholangiocarcinoma samples compared to nonmalignant bile ducts. The downregulation of SCTR expression in cholangiocarcinoma could be another potential mechanism for the tumor cells to avoid the antiproliferative effects of secretin. The mechanism of the decrease in SCTR

expression is not known and could be due to promoter hypermethylation, which is a common event in the malignant transformation of cholangiocarcinoma.⁴² SCTR receptor expression status would be critical for the therapeutic potential of secretin in humans with cholangiocarcinoma and warrants further exploration.

Finally, we determined that secretin also inhibits the growth of cholangiocarcinoma in an *in vivo* xenograft mouse model. Secretin significantly inhibited the tumor size and more than doubled tumor latency compared to the vehicle control-treated animals. The inhibition of cholangiocarcinoma growth *in vivo* is consistent with our findings *in vitro*. In the secretin-treated tumors, there was a decrease in PCNA and an increase in cleaved caspase 3 expression levels. The tumors were positive for CK-7 and SCTR, markers of cholangiocyte phenotypes and there was no end organ damage present. Of interest, secretin did not alter the expression of VEGF-A, suggesting that its effects are predominantly on the regulation of cholangiocarcinoma proliferation and not on the process of angiogenesis as has been observed for γ -aminobutyric acid that inhibits cholangiocarcinoma growth *in vivo* and was associated with the downregulation of VEGF-A expression.⁴³

In conclusion, we present the novel finding that secretin decreases cholangiocarcinoma proliferation both *in vitro* and

in vivo, which is in contrast to normal human cholangiocytes. The effects of secretin on cholangiocarcinoma were mediated by differences in receptor coupling to $G\alpha_i$ downregulating the production of the antiproliferative cAMP. Modulation of cAMP signaling and most importantly SCTR might provide a unique opportunity for the development of novel therapeutic modalities for the treatment of cholangiocarcinoma. Future studies on the role of secretin and SCTR in the pathogenesis of the cholangiopathies (such as primary biliary cirrhosis and PSC, which often progress to cholangiocarcinoma) might also prove fruitful for our understanding of cholestatic disease pathogenesis.

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