Accepted Manuscript

Secretin Stimulates Biliary Cell Proliferation by Regulating Expression of MicroRNA 125b and MicroRNA let7a in Mice

Shannon Glaser, Fanyin Meng, Yuyan Han, Paolo Onori, Billy K. Chow, Heather Francis, Julie Venter, Kelly McDaniel, Marco Marzioni, Pietro Invernizzi, Yoshiyuki Ueno, Jia-ming Lai, Li Huang, Holly Standeford, Domenico Alvaro, Eugenio Gaudio, Antonio Franchitto, Gianfranco Alpini

PII: S0016-5085(14)00241-8
DOI: 10.1053/j.gastro.2014.02.030
Reference: YGAST 58986

To appear in: *Gastroenterology*
Accepted Date: 11 February 2014

Please cite this article as: Glaser S, Meng F, Han Y, Onori P, Chow BK, Francis H, Venter J, McDaniel K, Marzioni M, Invernizzi P, Ueno Y, Lai J-m, Huang L, Standeford H, Alvaro D, Gaudio E, Franchitto A, Alpini G, Secretin Stimulates Biliary Cell Proliferation by Regulating Expression of MicroRNA 125b and MicroRNA let7a in Mice, *Gastroenterology* (2014), doi: 10.1053/j.gastro.2014.02.030.

Secretin Stimulates Biliary Cell Proliferation by Regulating Expression of MicroRNA 125b and MicroRNA let7a in Mice

Shannon Glaser,[1,2,4&]
Fanyin Meng,[1,2,3,4&]
Yuyan Han,[4]
Paolo Onori,[5]
Billy K Chow,[6]
Heather Francis,[1,2,3,4]
Julie Venter,[4]
Kelly McDaniel,[1]
Marco Marzioni,[7]
Pietro Invernizzi,[8]
Yoshiyuki Ueno,[9]
Jia-ming Lai,[10]
Li Huang,[10]
Holly Standeford,[1]
Domenico Alvaro,[11]
Eugenio Gaudio,[5]
Antonio Franchitto,[5,12#]
Gianfranco Alpini,[1,2,4,#]

From [1]Research, Central Texas Veterans Health Care System, [2]Scott & White Digestive Disease Research Center, Scott & White, [3]Academic Operations, Scott & White, [4]Department of Medicine, Division Gastroenterology, Texas A&M Health Science Center, College of Medicine, Temple, TX 76504, [5]Department of Anatomical, Histological, Forensic Medicine and Orthopedics Sciences, Sapienza, Rome, Italy, [6]School of Biological Sciences, The University of Hong Kong, Pokfulam Road, Hong Kong, China, Hong Kong, China, [7]Department of Medicine, Universita' Politecnica delle Marche, Ancona, Italy, [8]Liver Unit and Center for Autoimmune Liver Diseases, Humanitas Clinical and Research Center, Rozzano, Milan, Italy, [9]Division Gastroenterology, Yamagata University Faculty of Medicine, Yamagata, Japan, [10]Department of Hepatobiliary Surgery, the First Affiliated Hospital, Sun Yat-sen University, Guangzhou, China, [11]Department of Medico-Surgical Sciences and Biotechnologies, Polo Pontino, and [12]Eleonora Lorillard Spencer Cenci Foundation, Rome, Italy.

The authors have no conflict of interests to disclose.

This work was supported by the Dr. Nicholas C. Hightower Centennial Chair of Gastroenterology from Scott & White Hospital, a VA Research Career Scientist Award, a VA Merit award to Dr. Alpini, a VA CDA-2 Award to Dr. Francis, a VA Merit Award to Dr. Meng, by a Health and Labour Sciences Research Grants for the Research on Measures for Intractable Diseases from the Ministry of Health, Labor and Welfare of Japan, by Research Project funds from University of Rome "La Sapienza", MIUR grant

#2009X84L84_001 and FIRB Accordi di Programma 2010#RBAP10Z7FS to Prof. Gaudio, and a VA CDA-2 Award and NIH grant DK081442 to Dr. Glaser. [&]Drs. Glaser and Meng share the first authorship. [#]Drs. Franchitto and Alpini share the senior authorship.

Short title: Secretin modulation of cholangiocyte growth

Abbreviations: BDL = bile duct ligated; BSA = bovine serum albumin; cAMP = cyclic adenosine 3', 5'-monophosphate; CK-19 = cytokeratin-19; GAPDH = glyceraldehyde-3-phosphate dehydrogenase; IBDM = intrahepatic bile duct mass; NGF = nerve growth factor; PBC = primary biliary cirrhosis; PCNA = proliferating cell nuclear antigen; PSC = primary sclerosing cholangitis; Sct = secretin; *Sct*$^{-/-}$ = secretin KO mice; SR = secretin receptor; TUNEL = terminal deoxynucleotidyltransferase biotin-dUTP nick-end labeling; VEGF = vascular endothelial growth factor; WT = wild-type.

Address Correspondence to

Gianfranco Alpini, Ph. D.
VA Research Scientist Recipient
Professor, Medicine
Director, Scott & White Digestive Diseases Research Center
Dr. Nicholas C. Hightower Centennial Chair of Gastroenterology
Central Texas Veterans Health Care System
Texas A&M Health Science Center College of Medicine
Olin E. Teague Medical Center
1901 South 1st Street, Bldg. 205, 1R60
Temple, TX, 76504
Phone: 254-743-2625 and 254-743-1044
Fax: 743-0378 or 743-0555
E-mail: galpini@tamu.edu or galpini@medicine.tamu.edu

Shannon Glaser = Study concept and design; acquisition of data; analysis and interpretation of data; drafting of the manuscript; critical revision of the manuscript. No conflict of interest to disclose.

Fanyin Meng = Study concept and design; acquisition of data; analysis and interpretation of data for microRNA related experiments; drafting of the manuscript; critical revision of the manuscript. No conflict of interest to disclose.

Yuyan Han = Performed experiments on microRNA studies. She also wrote portions of the paper related to microRNAs. No conflict of interest to disclose.

Paolo Onori = Acquisition of IHC data; analysis and interpretation of data; critical revision of the manuscript. . No conflict of interest to disclose.

Billy K Chow = He provided KO mice and helped with the first draft of the manuscript. . No conflict of interest to disclose.

Heather Francis = Study concept and design; analysis and interpretation of data; drafting of the manuscript; critical revision of the manuscript. . No conflict of interest to disclose.

Julie Venter = All tissue culture studies. No conflict of interest to disclose.

Kelly McDaniel = Technical help on microRNA studies. No conflict of interest to disclose.

Marco Marzioni = Study concept and design; analysis and interpretation of data; drafting of the manuscript; critical revision of the manuscript. No conflict of interest to disclose.

Pietro Invernizzi = Study concept and design; analysis and interpretation of data; drafting of the manuscript; critical revision of the manuscript. No conflict of interest to disclose.

Yoshiyuki Ueno = Study concept and design; analysis and interpretation of data; drafting of the manuscript; critical revision of the manuscript. No conflict of interest to disclose.

Jia-ming Lai = Technical help on the microRNA studies and luciferase assay design. No conflict of interest to disclose.

Li Huang = Technical help on the microRNA studies and performed luciferase reporter assay. No conflict of interest to disclose.

Holly Standeford = Perform blots and real-time PCR. No conflict of interest to disclose.

Domenico Alvaro = Study concept and design; analysis and interpretation of data; drafting of the manuscript; critical revision of the manuscript. No conflict of interest to disclose.

Eugenio Gaudio = Study concept and design; analysis and interpretation of data; drafting of the manuscript; critical revision of the manuscript. No conflict of interest to disclose.

Antonio Franchitto = Study concept and design; acquisition of data; analysis and interpretation of data; drafting of the manuscript; critical revision of the manuscript. No conflict of interest to disclose.

Gianfranco Alpini = Study concept and design; acquisition of data; analysis and interpretation of data; drafting of the manuscript; critical revision of the manuscript. No conflict of interest to disclose.

Abstract:

Background & Aims: Proliferating cholangiocytes secrete and respond to neuroendocrine hormones including secretin. We investigated whether secretin secreted by S cells and cholangiocytes stimulates biliary proliferation in mice.

Methods: Cholestasis was induced in secretin knockout (*Sct*$^{-/-}$) and wild-type (control) mice by bile-duct ligation (BDL). At days 3 and 7 after BDL, control and *Sct*$^{-/-}$ mice received tail-vein injections of morpholinos against microRNA 125b or let7a. One week later, liver tissues and cholangiocytes were collected; immunohistochemical, immnoblot, luciferase reporter and real-time PCR assays were performed. Intrahepatic bile duct mass (IBDM) and proliferation were measured. Secretin secretion was measured in conditioned media from cholangiocytes and S cells, and in serum and bile.

Results: Secretin secretion was increased in supernatants from cholangiocytes and S cells and in serum and bile following BDL in control mice. BDL *Sct*$^{-/-}$ mice had lower IBDM, reduced proliferation, and reduced production of vascular endothelial growth factor A (VEGFA) and nerve growth factor (NGF) compared with BDL control. BDL and control mice given morpholinos against microRNA 125b or let7a had increased IBDM. Livers of mice given morpholinos against microRNA 125b had increased expression of VEGFA while those treated with morpholinos against microRNA let7a had increased expression of NGF. Secretin regulated VEGF and NGF expression that negatively correlated with microRNA 125b and let7a levels in liver tissue.

Conclusions: Following liver injury, secretin produced by cholangiocytes and S cells reduces microRNA 125b and let7a levels, resulting in upregulation of VEGF and NGF. Modulation of cholangiocyte expression of secretin could be a therapeutic approach for biliary diseases.

KEYWORDS: Biliary epithelium, cAMP, gastrointestinal hormones, heterogeneity

INTRODUCTION

Cholangiocytes are target cells in cholangiopathies including primary biliary cirrhosis (PBC) and primary sclerosing cholangitis (PSC), which are disorders associated with dysregulation of the balance between cholangiocyte proliferation/loss.[1, 2] The bile duct ligated (BDL) model mimics some features of human cholangiopathies.[3, 4] In rodents with BDL, large but not small cholangiocytes proliferate via activation of cAMP-dependent signaling leading to increased large intrahepatic bile duct mass (IBDM).[3, 5] The reaction of cholangiocytes to injury features a "neuroendocrine-like" trans-differentiation, which allows cholangiocytes to secrete a number of peptides and hormones that modulate cholangiocyte responses to injury by autocrine/paracrine mechanisms.[6-8]

The neuroendocrine hormone secretin is secreted by S cells that are localized primarily in the mucosa of the duodenum.[9] We have shown that secretin stimulates biliary growth by interaction with secretin receptors (SR, expressed only by large cholangiocytes)[10] and *in vivo* and *in vitro* knockout of SR reduces biliary proliferation by downregulating cAMP-dependent signaling.[11] No data exists regarding hepatic expression of secretin and role of secretin in the regulation of cholangiocyte growth/damage in biliary diseases.

MicroRNAs, which are post-transcriptional regulators that bind to complementary sequences on the 3'-UTR of target mRNA, alter gene translation and regulate hepatobiliary function.[12, 13] Following partial hepatectomy, microRNA 181b expression is upregulated in cholangiocytes,[14] whereas microRNA 125b is downregulated in hepatobiliary cancers.[13] In a model of cholestasis-associated cholangiocarcinoma, there was enhanced expression of microRNA let7a, which targets NF2/Merlin (critical regulator of cell proliferation/apoptosis).[15] The rationale for studying microRNA 125b and microRNA let7a is based on 3'-UTR sequence analysis and prediction algorithms, which reveal several microRNAs potentially targeting VEGF and NGF. MicroRNA 125b and microRNA let7a, two microRNA isoforms involved in hepatobiliary injury and cellular

proliferation,[13, 16] were identified as potential upstream microRNAs directly targeting VEGF/NGF from our most down-regulated miRNA list after BDL using combined analysis by TargetScan (http://targetscan.org/) and miRBase (http://microRNA.sanger.ac.uk/) databases[17], and through our most down-regulated microRNA list from microRNA microArray profiling data after BDL (show enhanced VEGF and NGF expression).

No information exists regarding mechanisms by which VEGF/NGF mediate secretin's trophic effects in cholangiocytes.[11, 18] We have shown that changes in biliary proliferation (by administration of VEGF to rats with hepatic artery ligation) were associated with changes in secretin-stimulated choleresis.[18] However, this study did not demonstrate a direct link between secretin and VEGF. Thus, we performed studies to evaluate if secretin stimulates biliary growth by autocrine/paracrine mechanisms through changes in microRNA 125b/microRNA let7a expression.

Materials and Methods

Materials

Reagents were purchased from Sigma Aldrich Co. (St. Louis, MO) unless otherwise stated. The normal human intrahepatic cholangiocyte line (HIBEpiC) was purchased from ScienCell Research Laboratories (Carlsbad, CA).[19] The antibodies used are listed in Suppl. File 1. MicroRNA precursors and anti-microRNA-specific inhibitors of microRNA 125b/microRNA let7a along with control microRNA precursors and inhibitors were purchased from Ambion (Austin, TX). pRL-TK microRNA let7a and pRL-TK controls were obtained from Addgene (Cambridge, MA) and Promega (Madison, WI), respectively. The cAMP EIA kit was purchased from Cayman Chemical (Ann Arbor, MI).

Animal Models

Animal procedures were performed according to protocols approved by Scott and White and Texas A&M HSC IACUC. Secretin (*Sct*) knockout (KO, $Sct^{-/-}$) mice were generated and characterized as described by us.[20] $Sct^{+/+}$ (wild-type, WT) and $Sct^{-/-}$ mice were maintained in a temperature-controlled environment with 12:12-hr light-dark cycles. We used male normal (or sham) and BDL (1 week) WT and $Sct^{-/-}$ mice (~25-30 g) of the N5 generation (Table 1). WT mice were purchased from Charles River Laboratories (Wilmington, MA). Since there were no differences in biliary growth between WT and $Sct^{-/-}$ mice and corresponding shams, we did not perform experiments on sham animals. Liver and body weight and liver to body weight ratio were measured.[4] Normal WT mice were treated with secretin (2.5 nmoles/kg BW/day) by osmotic minipumps for 1 week.[11] To reduce hepatic expression of microRNA 125b and microRNA let7a, normal or BDL WT mice (immediately after surgery)[4] were treated by two tail vein injections (one at day 3 and one at day 7) with Vivo-Morpholino sequences of microRNA 125b

(5'CATCACAAGTTAGGGTCTCAGGGAC3'), microRNA let7a (5'AACTATACAACCTACTACCTCATCC3'), or mismatched Morpholinos (5'CATCAgAAcTTAcGGTCTgAcGGAC3' for microRNA 125b) or (5'AAgTATAgAAgCTAgTAgCTCATCC3' for microRNA let7a), 30 mg/kg BW. We have previously shown the Vivo-Morpholino approach reduced biliary expression of arylalkylamine N-acetyltransferase (enzyme regulating melatonin secretion) in BDL rats.[21] One week later, liver tissue and cholangiocytes were collected. In RNA from isolated cholangiocytes, we measured the expression of microRNA 125b and microRNA let7a by real-time PCR. IBDM and semiquantitative expression of VEGFA and NGF was evaluated in liver sections.

Isolated Cholangiocytes, Hepatocytes and Biliary Cell Lines

Large cholangiocytes were isolated by counterflow elutriation followed by immunoaffinity separation.[10] Hepatocytes were isolated by standard collagenase perfusion. The *in vitro* experiments were performed in human HIBEpiC and large murine cholangiocyte lines.[22]

Evaluation of Secretin Expression in Liver and S Cells and Levels in Serum, Bile, and Supernatant from Cholangiocytes and S Cells

We evaluated the expression of secretin in liver sections (4 μm thick) by immunohistochemistry. Sections were imaged with Leica Microsystems DM 4500 B Light Microscopy (Weltzlar, Germany) with a Jenoptik Prog Res C10 Plus Videocam (Jena, Germany). Negative controls were included.

Since only large cholangiocytes express secretin (see results section) and proliferate following BDL,[23] we evaluated secretin expression (by real-time PCR and immunoblots, Suppl. File 1)[24] in large cholangiocytes and S cells and levels by EIA kits (Phoenix Pharmaceuticals, Inc., Burlingame, CA) in the medium of short-term (12 hr) cultures of

isolated cholangiocytes and S cells ($1x10^7$ cells/ml) from normal and BDL WT mice. We measured the levels of secretin secreted from basolateral and apical domains of cholangiocytes by plating the cell lines for 72 hr on collagen-coated filters of tissue culture inserts to produce a confluent monolayer.[25] To determine that secretin secreted from cholangiocytes is bioactive, we treated large cholangiocyte lines (following serum starvation for 24 hr) with cholangiocyte media from normal or BDL WT mice (in the absence/presence of pre-incubation with secretin antibody, 0.2 μg/200 μl for 30 min) before measuring cAMP (5 min stimulation)[5] levels by EIA and cell proliferation (48 hr stimulation) by MTS assays.[24]

S cell purity was evaluated by double immunofluorescence (Suppl. File 1) for chromogranin A/ secretin, and chromogranin A/SR[26]. We evaluated secretin levels in serum and bile from normal and BDL WT mice by EIA kits.

Evaluation of Liver Histomorphology, IBDM and Biliary Apoptosis

Liver histology was performed as described in Suppl. File 1. We determined IBDM of small (<15 μm diameter) and large (>15 μm diameter) bile ducts[27] and percentage of apoptotic cholangiocytes by terminal deoxynucleotidyltransferase biotin-dUTP nick-end labeling (TUNEL) kit (Apoptag; Chemicon International, Inc.) in liver sections.

Effect of Secretin or Knockout of the Secretin Gene on the Proliferation and Expression of VEGFA/C, NGF, MicroRNA 125b and MicroRNA let7a

Immunohistochemistry for VEGFA/C and NGF in liver sections was performed as described in Suppl. File 1. We evaluated the expression of PCNA, VEGFA/C, NGF, in isolated cholangiocytes and microRNA 125b and microRNA let7a in isolated cholangiocytes and hepatocytes by real-time PCR.[24] Large untransfected, vector- or

secretin-transfected cholangiocytes or HIBEpiC were treated with 0.2% BSA (basal) or secretin (10^{-7} M and/or 10^{-6} M) for 24 to 72 hr and 7 days before evaluating proliferation by MTS assay[24] and/or expression of PCNA, VEGFA/C, NGF, microRNA 125b and microRNA let7a by real-time PCR.[24] We knocked down secretin expression in large cholangiocytes (Suppl. File 1) before measuring proliferation by MTS assays (incubation for 24 to 72 hr and 7 days) and expression of VEGFA/C, NGF, microRNA 125b and microRNA let7a (incubation for 48 hr) by real-time PCR.[24]

To determine whether the expression of other microRNAs was affected by the knockout of secretin, we performed miScript miRNA PCR assay (SABioscience, Frederick, MD) in control and secretin shRNA large cholangiocytes and large cholangiocytes from normal and BDL WT and *Sct*$^{-/-}$ mice. Total RNAs (250 ng) were reverse-transcribed into cDNA with miScript II RT kit (SABioscience). For mature microRNA expression detection, the cDNA was mixed with QuantiTect SYBR Green PCR Master Mix and the mixture was added into a 96-well RT^2 microRNA PCR Assay (SABiosciences) that included primer pair for specific microRNA. The raw Ct was normalized to multiple housekeeping genes based on the established formula from the supplier.

Large cholangiocytes were transfected with microRNA 125b/microRNA let7a inhibitors or anti-microRNA control; or microRNA 125b/microRNA let7a precursors or scrambled controls to down-regulate or overexpress microRNA 125b/microRNA let7a, before measuring proliferation (by MTS assays) and expression of PCNA, VEGF-A and NGF by real-time PCR and immunofluorescence. Transfections, real-time PCR assays and immunofluorescence for the expression of target genes and luciferase reporter assays are described in Suppl. File 1.

Statistical Analysis

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 Expression in Liver and S Cells and Levels in Serum, Bile, and Supernatant from Cholangiocytes and S Cells

Normal large cholangiocytes (red arrows) express the protein for secretin; secretin expression increased in large BDL cholangiocytes (Figure 1A, Table 1) compared to normal control. No positive staining for secretin was observed in small cholangiocytes and hepatocytes from normal and BDL WT mice, and cholangiocytes from normal and BDL *Sct*$^{-/-}$ mice (Figure 1A, Table 1). The expression and levels of secretin were higher in large cholangiocytes and S cells from BDL compared to normal mice (Figure 1B-C, Table 2). Secretin levels were higher in bile and serum of BDL compared to normal mice (Table 2). Large cholangiocytes release secretin at both basolateral and apical domains (0.37±0.15 ng/1x10^6 cells (basolateral; n=31) and 0.074±0.01 ng/1x10^6 cells (apical; n=24)). Incubation of a large biliary cell line with medium from normal cholangiocytes increased cAMP levels and proliferation of these cells; the proliferative effects were amplified when large cholangiocytes were incubated with the biliary supernatant (containing more secretin) from BDL mice (Suppl. Figure 1A-B). Secretin-stimulation of cAMP levels and biliary proliferation were partly decreased by pre-incubation with a secretin-neutralizing antibody (Suppl. Figure 1A-B). The purity of S cells was demonstrated by positive staining for secretin/chromogranin A and chromogranin A/SR (Suppl. Figure 2A-B).

Evaluation of Liver Histomorphology, IBDM and Biliary Apoptosis

No differences in body weight were observed among the animal groups (Suppl. Table 2). There was increased liver to body weight ratio in BDL compared to normal mice and reduced liver to body weight ratio in *Sct*$^{-/-}$ BDL compared to BDL WT mice (Suppl. Table 2). No changes in inflammation, necrosis and steatosis were observed in *Sct*$^{-/-}$ compared to WT groups (not shown). There was no difference in IBDM of small and large cholangiocytes between normal WT and *Sct*$^{-/-}$ mice (Figure 1D, Table 1). There was increased large (green arrows) IBDM in BDL WT compared to normal mice (Figure 1D, Table 1). In *Sct*$^{-/-}$ BDL mice, there was reduced large IBDM (green arrows) compared to BDL WT mice (Figure 1D, Table 1) and enhanced biliary apoptosis (Table 1). In *Sct*$^{-/-}$ BDL mice there was increased IBDM of small cholangiocytes (red arrowheads) compared to BDL WT mice (Figure 1D, Table 1). In normal and BDL mice treated *in vivo* with microRNA 125b or microRNA let7a Vivo-Morpholinos, there was: (i) reduced biliary expression of microRNA 125b and microRNA let7a in cholangiocytes (Suppl. Figure 3A); (ii) increased large IBDM (Figure 1E); and (iii) enhanced expression of VEGFA (after treatment with microRNA 125b Vivo-Morpholinos) and NGF (after treatment with microRNA let7a Vivo-Morpholinos) in liver sections compared to control mice (Suppl. Figure 3B-C; Suppl. Table 1).

Effect of Secretin or Knockout of the Secretin Gene on Biliary Proliferation and Expression of VEGFA/C, NGF, MicroRNA 125b and MicroRNA let7a

In large ducts, expression of VEGFA/C and NGF increased following BDL and decreased in *Sct*$^{-/-}$ BDL compared to BDL WT mice (Figure 2A). In large cholangiocytes from *Sct*$^{-/-}$ BDL mice there was: (i) decreased expression for PCNA, VEGFA/C and NGF (Figure 2B); (ii) enhanced expression of microRNA 125b and microRNA let7a compared to BDL cholangiocytes (Figure 2C); and (iii) decreased expression of microRNA 125b

and microRNA let7a compared to normal cholangiocytes (Figure 2C). Opposite to cholangiocytes, in hepatocytes there was increased expression of microRNA 125b and microRNA let7a in normal WT mice treated with secretin and BDL mice compared to normal WT mice and reduced expression of microRNA 125b and microRNA let7a in BDL *Sct*$^{-/-}$ mice compared to BDL WT mice (Suppl. Figure 4). Since parenchymal cells do not express SR,[28] we propose that the opposite expression pattern of microRNA 125b and microRNA let7a in hepatocytes is not directly linked to secretin→SR axis, but may depend on the changes in the expression of specific transduction pathways (e.g., cAMP-dependent signaling) that are altered by BDL and lack of secretin,[11, 23] influencing hepatocyte functions by paracrine mechanisms. There is also a possibility that secretin can interact with other G protein coupled receptors of secretin family of receptors, which include receptors for include vasoactive intestinal peptide receptors and receptors for calcitonin and parathyroid hormone/parathyroid hormone-related peptides. Secretin and VIP can also interact at low affinity with the VIP and SR, respectively.[29] In rats, VIP binds to SR with similar affinity as the natural ligand, secretin, which may be a possible explanation for the responsiveness of hepatocytes to secretin or lack of secretin in our *in vivo* experiments.[30] Our data correlate with the previous finding that hepatocyte proliferation during BDL, which occurs at a much lower rate than cholangiocytes, is limited to the replenishment of damaged hepatocytes.[31] Further studies are warranted to elucidate mechanisms underlying the changes of microRNA 125b and microRNA let7a in hepatocytes in our model.

The expression of other microRNAs involved in hepatobiliary injury and proliferation was altered in secretin shRNA cholangiocytes, and large cholangiocytes from normal and BDL WT and *Sct*$^{-/-}$ mice compared to the corresponding controls (Suppl. Figure 5). Since microRNA 125b and microRNA let7a were found to specifically target VEGFA and NGF respectively, we focused on these two microRNAs.

Treatment of normal WT mice with secretin: (i) increased PCNA, VEGFA (but not VEGFC, not shown) and NGF expression (Suppl. Figure 6A); and (ii) decreased microRNA 125b and microRNA let7a expression in large cholangiocytes compared to saline-treated mice (Figure 2D). *In vitro*, secretin increased the expression of PCNA, VEGFA and NGF (Suppl. Figure 6B), and decreased expression of microRNA 125b and microRNA let7a in large cholangiocytes compared to basal (Figure 2E).

In vitro, secretin increased proliferation of non-transfected, control vector-transfected and secretin shRNA-transfected large cholangiocytes and HiBEpiC cells compared to basal (Figure 3A-C). We treated secretin shRNA-transfected cholangiocytes with secretin to demonstrate that replenishment of this hormone prevents the decrease in biliary proliferation (Figure 3B). In secretin shRNA-transfected cholangiocytes, there was: (i) decreased secretin expression and secretin secretion (Figure 4A) and reduced proliferation and expression of PCNA, VEGFA/C and NGF (Figure 4B-C); and (ii) increased expression of microRNA 125b and microRNA let7a compared to control cholangiocytes (Figure 4D-E). We further determined the effect of downregulation/overexpression of microRNA 125b and microRNA let7a (Suppl. Figure 7A-B) on biliary proliferation and the expression of PCNA, VEGFA and NGF (Figure 5A-D). The increase in biliary proliferation occurred in large cholangiocytes after transfection with microRNA 125b or microRNA let7a inhibitors compared to control (Figure 5A-C). The increase in biliary expression of VEGFA occurred in large cholangiocytes after transfection with microRNA 125b inhibitors, whereas enhanced biliary expression of NGF was observed when cholangiocytes were transfected with microRNA let7a inhibitors (Figure 5B, C). Following overexpression of microRNA 125b or microRNA let7a (Suppl. Figure 7B), there was reduced biliary proliferation (Figure 5B, D). Overexpression of microRNA 125b significantly reduced VEGFA expression, whereas reduced NGF expression was observed following overexpression of microRNA

let7a in cholangiocytes compared to control (Figure 5B, D).

Identification of the Targets for MicroRNA 125b and MicroRNA let7a

To verify that VEGF and NGF are targets of translational regulation by microRNA 125b and microRNA let7a in cholangiocytes, we performed studies using luciferase reporter constructs containing the microRNA 125b and microRNA let7a recognition sequence (Suppl. Figure 6C) from the 3′-UTR of VEGF and NGF inserted downstream of the luciferase gene. Transfection with microRNA 125b or microRNA let7a precursors decreased reporter activity in HiBEpiC cells. When these studies were repeated with reporter constructs containing random mutations in the recognition sequence, the effects of reporter deactivation by microRNA 125b and microRNA let7a precursors were abolished (Suppl. Figure 6C-D). These findings confirmed that VEGFA and NGF are biologically relevant targets of microRNA 125b and microRNA let7a, respectively.

Evaluation of the Interaction between Secretin and MicroRNA let7a in Cholangiocytes

To demonstrate the direct interaction between secretin and microRNA let7a, the pRL-Tk microRNA let7a constructs, which contains microRNA let7a binding site in the 3′-UTR of Renilla luciferase reporter, were co-transfected with shRNA for secretin in large mouse cholangiocytes. The inhibition of secretin leading to the restoration of microRNA let7a (which binds to the 3′-UTR of Renilla luciferase reporter) significantly decreased luciferase activity (Suppl. Figure 6D). Thus, confirming the direct inhibitory effects of secretin on the expression of microRNA let7a (Suppl. Figure 6D).

Discussion

This study demonstrated that secretin has autocrine and paracrine roles in the regulation of biliary growth during cholestasis. We demonstrated that secretin is expressed/secreted by large cholangiocytes and S cells at higher levels following BDL compared to normal WT mice. Higher levels of secretin were observed in serum and bile of BDL mice, which likely is due to enhanced secretion of this hormone by cholangiocytes into bile and serum, and S cells into serum.[9] Knockout of the *Sct* gene: (i) reduced BDL-induced increase in large IBDM and induced a concomitant increase in small IBDM; (ii) decreased expression of PCNA, VEGFA/C and NGF; and (iii) increased expression of microRNA 125b and microRNA let7a compared to BDL WT mice. Treatment of normal WT mice with secretin decreased biliary expression of microRNA 125b and microRNA let7a. Treatment of cholangiocyte lines with secretin increased the expression of PCNA, VEGFA/C and NGF along with reduced expression of microRNA 125b and microRNA let7a. In secretin-shRNA transfected cholangiocytes there was reduced expression of PCNA, VEGFA/C and NGF and increased expression of microRNA 125b and microRNA let7a. The silencing of microRNA 125b and microRNA let7a enhanced biliary proliferation and VEGFA and NGF expression, whereas overexpression of microRNA 125b and microRNA let7a decreased biliary proliferation, and VEGFA and NGF expression. The direct regulation of microRNA let7a by secretin as well as the target verification of microRNA 125b and microRNA let7a to VEGFA and NGF, respectively, was confirmed by luciferase assay. We conclude that S cells and cholangiocytes regulate biliary growth (by both autocrine/paracrine mechanisms) through the synthesis of secretin. Local manipulation of biliary secretin expression may be important for the management of biliary disorders.

Secretin mRNA is expressed in the CNS in the cerebellum, pituitary, brainstem and hypothalamus.[32] Limited data exists regarding the expression/synthesis of secretin in peripheral tissues besides S cells.[33] Secretin mRNA is detected in antral and corpus mucosae.[34] Secretin -positive cells are present in the lower part of the common bile duct in cholestatic patients.[35] Enhanced secretin serum levels have been demonstrated in dogs after ligation of pancreatic or bile ducts as well as in cirrhotic patients.[36, 37] The signaling associated with afferent pathways of parasympathetic innervation is up-regulated following BDL and abolished by vagotomy[38, 39], which may explain the enhanced synthesis of secretin from cholangiocytes and S cells.

Since SR is expressed in the basolateral domain of cholangiocytes, our findings raise questions about the role of secretin in bile and how it interacts with SR. Since secretin secreted into bile may not be eliminated in the feces, intestinal cells can reabsorb it by endocytosis thus reaching serum. Secretin might be an important factor for sustaining biliary proliferation during ductopenic diseases characterized by lack of secretin and SR expression and decreased bicarbonate secretion. Recent findings support the notion that biliary bicarbonate (stimulated by the secretin/SR axis) is a key protective mechanism for cholangiocytes in ductopenic states, in what has been defined as a “bicarbonate umbrella”. Studies have shown that such a protective layer of bicarbonate is defective in PBC and PSC.[40, 41] In fact, microRNA 506 is up-regulated in cholangiocytes from PBC, binds the 3’UTR binding assay of AE2 mRNA, prevents protein translation and decreases biliary secretion by reduced AE2 activity.[42, 43] Absence of choleretic response to secretin was observed in cholestatic and untreated PBC patients.[44]

The stimulatory effects of secretin on cell proliferation and VEGF and NGF expression were due to a direct interaction with SR, since secretin effects were reproducible in an *in vitro* culture system. Interestingly, an intense proliferative reaction was observed in *Sct*$^{-/-}$ BDL for small cholangiocytes *in vivo*, thus showing an opposite trend to what we found

for large cells in the BDL model.[11] Although the current study was not designed to evaluate such a phenomenon, we speculate that such an extensive ductular reaction is likely due to a compensatory mechanism since small cholangiocytes can proliferate and acquire large cholangiocyte phenotypes to repopulate damaged large ducts.[45, 46]

We performed experiments aiming to determine if the effects of secretin on biliary proliferation and VEGFA/C and NGF expression were mediated by direct interaction with specific microRNAs. We have shown that several mRNA and microRNAs (VEGF, PIGF and TIMP-3, mircroRNA 34a and microRNA 125b) are aberrantly expressed in diseased/injured human and mice liver compared to normal tissue.[14, 47] Specific microRNAs, such as microRNA let7a family members including microRNA let7a, regulate hepatobiliary cell proliferation and anti-inflammatory properties via repression of specific genes targeting downstream signaling pathways.[12] Through combined analysis of microRNA profiling (BDL vs. control mouse liver) and bioinformatics approaches, microRNA 125b and microRNA let7a were chosen as the potential mediators of secretin regulated VEGF and NGF signaling, respectively. Interestingly, the expression of VEGFC is independent of the secretin-microRNA 125b network, suggesting that changes in VEGFC expression are likely secondary to the increase/decrease in biliary proliferation mediated by secretin and other not yet identified factors. Negative regulation of microRNA let7a by secretin has also been confirmed through 3'-UTR region, whereas the detailed mechanism of transcriptional regulation of microRNA 125b by secretin requires further studies. Both microRNA 125b and microRNA let7a have been classified as critical modulators of cell proliferation in human liver and other organs, and numerous target genes were identified.[48] However, studies addressing the upstream regulators of microRNA 125b and microRNA let7a are limited, and the mechanisms remain unclear. Identification of specific secretin dependent microRNA 125b-VEGF/microRNA let7a as important regulators of cholangiocyte growth/proliferation *in*

vitro, as well as downstream signaling mechanisms underscores the essential role for secretin and related mRNAs/microRNAs in the mediation of hepatobiliary wound healing process.

Taking into account that secretin receptors are only expressed by large cholangiocytes in the liver,[10] up-regulated during biliary hyperplasia and down-regulated following cholangiocyte damage,[11, 23, 28] manipulation of the secretin/SR axis can be a key approach for managing the growth and/or damage of large, cAMP-dependent cholangiocytes. Further studies are needed to evaluate the precise contribution of S cells and cholangiocytes in secretin regulation of biliary homeostasis.

REFERENCES

1. Lleo A, Invernizzi P. Apotopes and innate immune system: Novel players in the primary biliary cirrhosis scenario. Dig Liver Dis 2013;45:630-6.
2. Lazaridis KN, Strazzabosco M, LaRusso NF. The cholangiopathies: disorders of biliary epithelia. Gastroenterology 2004;127:1565-77.
3. Glaser S, Gaudio E, Rao A, et al. Morphological and functional heterogeneity of the mouse intrahepatic biliary epithelium. Lab Invest 2009;89:456-69.
4. Alpini G, Lenzi R, Sarkozi L, et al. Biliary physiology in rats with bile ductular cell hyperplasia. Evidence for a secretory function of proliferated bile ductules. J Clin Invest 1988;81:569-78.
5. LeSage G, Glaser S, Marucci L, 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:G1289-301.
6. Gaudio E, Barbaro B, Alvaro D, et al. Vascular endothelial growth factor stimulates rat cholangiocyte proliferation via an autocrine mechanism. Gastroenterology 2006;130:1270-82.
7. Gigliozzi A, Alpini G, Baroni GS, et al. Nerve growth factor modulates the proliferative capacity of the intrahepatic biliary epithelium in experimental cholestasis. Gastroenterology 2004;127:1198-209.
8. Alvaro D, Mancino MG, Glaser S, et al. Proliferating cholangiocytes: a neuroendocrine compartment in the diseased liver. Gastroenterology 2007;132:415-31.
9. Polak JM, Coulling I, Bloom S, et al. Immunofluorescent localization of secretin and enteroglucagon in human intestinal mucosa. Scand J Gastroenterol 1971;6:739-44.

10. Alpini G, Roberts S, Kuntz SM, et al. Morphological, molecular, and functional heterogeneity of cholangiocytes from normal rat liver. Gastroenterology 1996;110:1636-43.

11. **Glaser S, Lam IP**, Franchitto A, et al. Knockout of secretin receptor reduces large cholangiocyte hyperplasia in mice with extrahepatic cholestasis induced by bile duct ligation. Hepatology 2010;52:204-14.

12. **Chen XM, Splinter PL**, O'Hara SP, et al. A cellular micro-RNA, let-7i, regulates Toll-like receptor 4 expression and contributes to cholangiocyte immune responses against Cryptosporidium parvum infection. J Biol Chem 2007;282:28929-38.

13. Meng F, Henson R, Lang M, et al. Involvement of human micro-RNA in growth and response to chemotherapy in human cholangiocarcinoma cell lines. Gastroenterology 2006;130:2113-29.

14. **Meng F, Francis H**, Glaser S, et al. Role of stem cell factor and granulocyte colony-stimulating factor in remodeling during liver regeneration. Hepatology 2012;55:209-21.

15. **Wang Y, Tang N**, Hui T, et al. Identification of endogenous reference genes for RT-qPCR analysis of plasma microRNAs levels in rats with acetaminophen-induced hepatotoxicity. J Appl Toxicol 2013;33:1330-1336.

16. Yang H, Li TW, Peng J, et al. A mouse model of cholestasis-associated cholangiocarcinoma and transcription factors involved in progression. Gastroenterology 2011;141:378-88, 388 e1-4.

17. John B, Enright AJ, Aravin A, et al. Human microRNA targets. PLoS Biol 2004;2:e363.

18. **Gaudio E, Barbaro B**, Alvaro D, et al. Administration of r-VEGF-A prevents hepatic artery ligation-induced bile duct damage in bile duct ligated rats. Am J Physiol Gastrointest Liver Physiol 2006;291:G307-17.

19. Onori P, Wise C, Gaudio E, et al. Secretin inhibits cholangiocarcinoma growth via dysregulation of the cAMP-dependent signaling mechanisms of secretin receptor. Inter J Cancer 2009;127:43-54.

20. **Lee VH, Lee LT**, Chu JY, et al. An indispensable role of secretin in mediating the osmoregulatory functions of angiotensin II. FASEB J 2010;24:5024-32.

21. Renzi A, DeMorrow S, Onori P, et al. Modulation of the biliary expression of arylalkylamine N-acetyltransferase alters the autocrine proliferative responses of cholangiocytes in rats. Hepatology 2013;57:1130-41.

22. Ueno Y, Alpini G, Yahagi K, et al. Evaluation of differential gene expression by microarray analysis in small and large cholangiocytes isolated from normal mice. Liver Int 2003;23:449-59.

23. Alpini G, Glaser S, Ueno Y, et al. Heterogeneity of the proliferative capacity of rat cholangiocytes after bile duct ligation. Am J Physiol Gastrointest Liver Physiol 1998;274:G767-75.

24. Francis H, Glaser S, DeMorrow S, et al. Small mouse cholangiocytes proliferate in response to H1 histamine receptor stimulation by activation of the IP_3/CaMK I/CREB pathway. Am J Physiol Cell Physiol 2008;295:C499-513.

25. Han Y, DeMorrow S, Invernizzi P, et al. Melatonin exerts by an autocrine loop antiproliferative effects in cholangiocarcinoma: its synthesis is reduced favoring cholangiocarcinoma growth. Am J Physiol Gastrointest Liver Physiol 2011;301:G623-33.

26. Chang CH, Chey WY, Erway B, et al. Modulation of secretin release by neuropeptides in secretin-producing cells. Am J Physiol Gastrointest Liver Physiol 1998;275:G192-202.

27. Alpini G, Glaser S, Robertson W, et al. Large but not small intrahepatic bile ducts are involved in secretin-regulated ductal bile secretion. Am J Physiol Gastrointest Liver Physiol 1997;272:G1064-74.

28. Alpini G, Ulrich CD, 2nd, Phillips JO, et al. Upregulation of secretin receptor gene expression in rat cholangiocytes after bile duct ligation. Am J Physiol Gastrointest Liver Physiol 1994;266:G922-8.

29. Gourlet P, Vilardaga JP, De Neef P, et al. The C-terminus ends of secretin and VIP interact with the N-terminal domains of their receptors. Peptides 1996;17:825-9.

30. Holtmann MH, Hadac EM, Ulrich CD, et al. Molecular basis and species specificity of high affinity binding of vasoactive intestinal polypeptide by the rat secretin receptor. J Pharmacol Exp Ther 1996;279:555-60.

31. Polimeno L, Azzarone A, Zeng QH, et al. Cell proliferation and oncogene expression after bile duct ligation in the rat: evidence of a specific growth effect on bile duct cells. Hepatology 1995;21:1070-8.

32. Samson WK, Lumpkin MD, McCann SM. Presence and possible site of action of secretin in the rat pituitary and hypothalamus. Life Sci 1984;34:155-63.

33. Mutoh H, Fung BP, Naya FJ, et al. The basic helix-loop-helix transcription factor BETA2/NeuroD is expressed in mammalian enteroendocrine cells and activates secretin gene expression. Proc Natl Acad Sci U S A 1997;94:3560-4.

34. Chey WY, Chang CH, Pan HJ, et al. Evidence on the presence of secretin cells in the gastric antral and oxyntic mucosa. Regul Pept 2003;111:183-90.

35. Gulubov MV, Hadjipetkov P, Sivrev D, et al. Endocrine cells in the human common bile duct in patients with obstructive jaundice. Hepatogastroenterology 2012;59:26-30.

36. Usami A, Mizukami Y, Onji M. Abnormal gastric motility in liver cirrhosis: roles of secretin. Dig Dis Sci 1998;43:2392-7.

37. Sasaki I, Naito H, Matsuno S, et al. Experimental studies on the influence of simultaneous ligation of the bile and pancreatic ducts upon gastric acid secretion and gut hormones. Tohoku J Exp Med 1986;150:91-9.

38. LeSage G, Alvaro D, Benedetti A, et al. Cholinergic system modulates growth, apoptosis, and secretion of cholangiocytes from bile duct-ligated rats. Gastroenterology 1999;117:191-9.

39. Chey WY, Chang TM. Neural control of the release and action of secretin. J Physiol Pharmacol 2003;54:105-12.

40. **Hohenester S, Wenniger LM**, Paulusma CC, et al. A biliary HCO_3^- umbrella constitutes a protective mechanism against bile acid-induced injury in human cholangiocytes. Hepatology 2012;55:173-83.

41. Beuers U, Hohenester S, de Buy Wenniger LJ, et al. The biliary HCO(3)(-) umbrella: a unifying hypothesis on pathogenetic and therapeutic aspects of fibrosing cholangiopathies. Hepatology 2010;52:1489-96.

42. Padgett KA, Lan RY, Leung PC, et al. Primary biliary cirrhosis is associated with altered hepatic microRNA expression. J Autoimmun 2009;32:246-53.

43. Banales JM, Saez E, Uriz M, et al. Up-regulation of microRNA 506 leads to decreased Cl^-/HCO_3^- anion exchanger 2 expression in biliary epithelium of patients with primary biliary cirrhosis. Hepatology 2012;56:687-97.

44. Prieto J, Garcia N, Marti-Climent JM, et al. Assessment of biliary bicarbonate secretion in humans by positron emission tomography. Gastroenterology 1999;117:167-72.

45. Mancinelli R, Franchitto A, Gaudio E, et al. After damage of large bile ducts by gamma-aminobutyric acid, small ducts replenish the biliary tree by amplification of calcium-dependent signaling and *de novo* acquisition of large cholangiocyte phenotypes. Am J Pathol 2010;176:1790-800.

46. Mancinelli R, Franchitto A, Glaser S, et al. GABA induces the differentiation of small into large cholangiocytes by activation of $Ca(^{2+})$/CaMK I-dependent adenylyl cyclase 8. Hepatology 2013;58:251-63.

47. Alpini G, Glaser S, Zhang JP, et al. Regulation of placenta growth factor by microRNA-125b in hepatocellular cancer. J Hepatol 2011;55:1339-45.

48. Cairo S, Wang Y, de Reynies A, et al. Stem cell-like micro-RNA signature driven by Myc in aggressive liver cancer. Proc Natl Acad Sci U S A 2010;107:20471-6.

Authors names in bold designate shared co-first authors.

LEGENDS

Figure 1 [**A**] Large normal cholangiocytes (red arrows) express secretin. The expression of secretin increased in large ducts (red arrows) from BDL WT mice (see Table 1). Orig. magn., x20. [**B-C**] The expression of secretin increased in large cholangiocytes and S cells from BDL compared to normal mice. Data are mean ± SEM of four real-time PCR reactions and immunoblots. ***p*<0.05 vs. normal WT mice. **[D]** In BDL WT mice, there was increased large IBDM compared to control mice (green arrows). Knockout of the *Sct* gene reduced large IBDM compared to BDL WT mice (green arrows). In *Sct*$^{-/-}$ BDL mice there was increased small IBDM compared to BDL WT mice (red arrowheads, see Table 1). Orig. magn., x20. **[E]** In mice treated *in vivo* with microRNA 125b or microRNA let7a Vivo-Morpholinos, there was increased IBDM compared to control mice. ***p*<0.05 vs. mice treated with mismatched Morpholinos.

Figure 2 [**A**] In large ducts, the expression of VEGF-A/C and NGF increased following BDL and decreased in SEC$^{-/-}$ BDL compared to controls. Orig. magn., x40. [**B**] In large cholangiocytes from SEC$^{-/-}$ BDL mice there was decreased expression for PCNA, VEGF-A/C and NGF compared to BDL cholangiocytes. Data are mean ± SEM of four experiments. ***p<0.05 vs. large BDL cholangiocytes. [**C**] The expression of microRNA 125b and microRNA let7a was lower in BDL compared to normal cholangiocytes. In cholangiocytes from SEC$^{-/-}$ BDL mice there was enhanced expression of microRNA 125b and microRNA let7a compared to BDL WT cholangiocytes. Data are mean ± SEM of four experiments. ***p<0.05 vs. normal cholangiocytes. #p<0.05 vs. large cholangiocytes from WT BDL mice. [**D-E**] Secretin (both *in vivo* and *in vitro*) decreased expression of microRNA 125b and microRNA let7a in cholangiocytes. Data are mean ± SEM of four experiments performed in purified cholangiocytes or cholangiocyte lines. [**D**]

*****p<0.05 vs. large cholangiocytes from saline-treated normal mice. [**E**] *****p<0.05 vs. the corresponding basal value.

Figure 3 Secretin increased the proliferation of [**A**] non-transfected, [**B**] vector-transfected and secretin shRNA large cholangiocytes and [**C**] HiBEpiC compared to the cell lines treated with BSA (basal). Data are mean ± SEM of six MTS assays.

Figure 4 [**A**] In large secretin-shRNA transfected cholangiocytes, there was decreased secretin expression and secretin secretion compared to control cholangiocytes (*p<0.05). Data are mean ± SEM of three real-time PCR reactions, three immunoblots and seven evaluations by ELISA kits. [**B-C**] In secretin-shRNA transfected cholangiocytes, there was reduced proliferation and expression of PCNA, VEGF-A/C and NGF and [**D-E**] increased expression of microRNA 125b and microRNA let7a in different transfection time points compared to control vector-transfected cholangiocytes. Data are mean ± SEM of four real-time PCR reactions. *****p<0.05 vs. the corresponding value of control vector-transfected cholangiocytes.

Figure 5 Effect of downregulation or overexpression of microRNA 125b and microRNA let7a (**Suppl. Figure 7A-B**) on biliary proliferation and expression of PCNA, VEGF-A and NGF. [**A, B, C**] The increase in proliferation occurred in cholangiocytes after incubation with anti-microRNA 125b or anti-microRNA let7a inhibitors. [**B, C**] Increased expression of VEGF-A occurred in large cholangiocytes after incubation with anti-microRNA 125b inhibitors, whereas enhanced NGF biliary expression was observed when cholangiocytes were treated with anti-microRNA let7a inhibitors compared to control. Following overexpression of microRNA 125b or microRNA let7a (**Suppl. Figure**

7B), there was reduced biliary proliferation [**B, D**]. Following overexpression of microRNA 125b, there was reduced VEGF-A expression, whereas reduced NGF expression was observed following overexpression of microRNA let7a in cholangiocytes [**B, D**). Data are mean ± SEM of four real-time PCR reactions. *****p<0.05 vs. controls.

Table 1 Measurement of the % of small and large bile ducts positive for secretin, the % of apoptotic small and large bile ducts, and the % IBDM of small and large cholangiocytes.

Treatment	Secretin (%)		IBDM (%)		Apoptosis	
	Small bile ducts	Large bile ducts	Small bile ducts	Large bile ducts	Small bile ducts	Large bile ducts
WT Normal	Not detected	28.8±2.0	0.04±0.005	0.18±0.03	<3%	<3%
Normal *Sct* $^{-/-}$	Not detected	ND	0.06±0.01	0.2±0.01	<3%	<3%
WT BDL	Not detected	48.0±2.8[a]	0.11±0.01	2.6±0.2[b]	7.4±0.6	15.1±1.5
BDL *Sct* $^{-/-}$	Not detected	Not detected	0.51±0.03[c]	1.8±0.16[d]	10.2±0.6	21.0±1.2[e]

Negative values correspond to <3%. [a]p<0.05 vs. % of secretin-positive large ducts from normal WT mice.

[b]p<0.05 vs. IBDM of large ducts from normal WT mice. [c]p<0.05 vs. IBDM of small ducts from normal BDL mice.

[d]p<0.05 vs. IBDM of large ducts from WT BDL mice.

[e]p<0.05 vs. % apoptosis of large ducts from BDL WT mice.

Table 2 Measurement of secretin levels in serum, bile, and supernatant from cholangiocytes and S cells isolated from normal and BDL WT mice.

Groups	Secretin levels in serum (ng/ml)	Secretin levels in bile (ng/ml)	Secretin levels in large cholangiocyte supernatant (ng/1x10^6 cells)	Secretin levels in S cell supernatant (ng/1x10^6 cells)
WT Normal Mice	0.56 ± 0.09 (n = 28)	9.57 ± 3.15 (n = 10)	1.44 ± 0.44 (n = 33)	0.25 ± 0.11 (n = 6)
WT BDL Mice	2.61 ± 0.78[a] (n = 24)	76.32 ± 28.18[a] (n = 6)	19.75 ± 5.48[a] (n = 12)	36.4 ± 10.2[a] (n = 9)

Data are mean ± SEM. [a]$p<0.05$ vs. the value of normal WT mice. BDL = bile duct ligation; WT = wild-type.

Figure 1

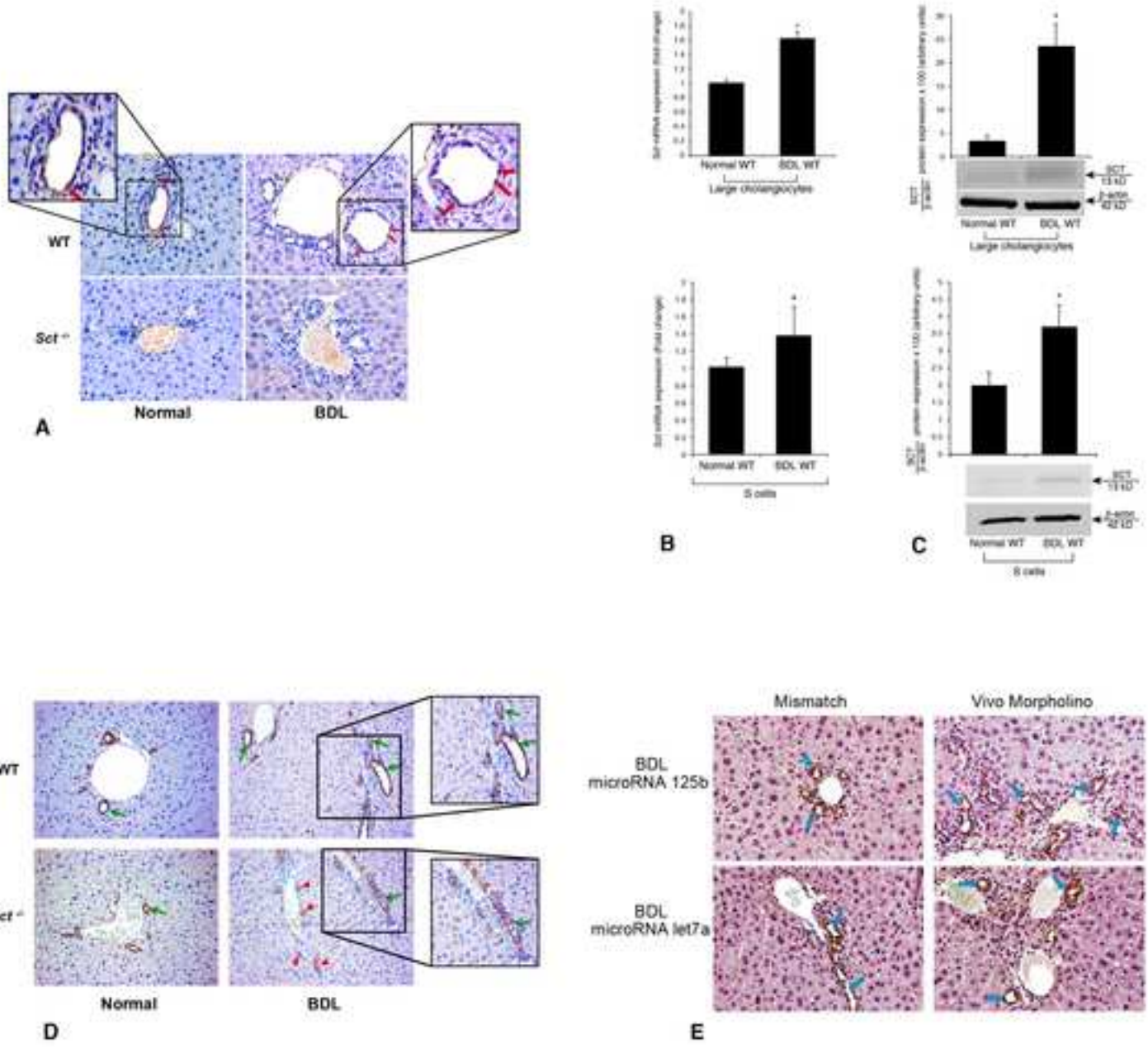

WT
Sct -/-
Normal
BDL
A
Normal WT
BDL WT
Large cholangiocytes
B
S cells
C
D
Mismatch
Vivo Morpholino
BDL
microRNA 125b
BDL
microRNA let7a
E

Figure 2

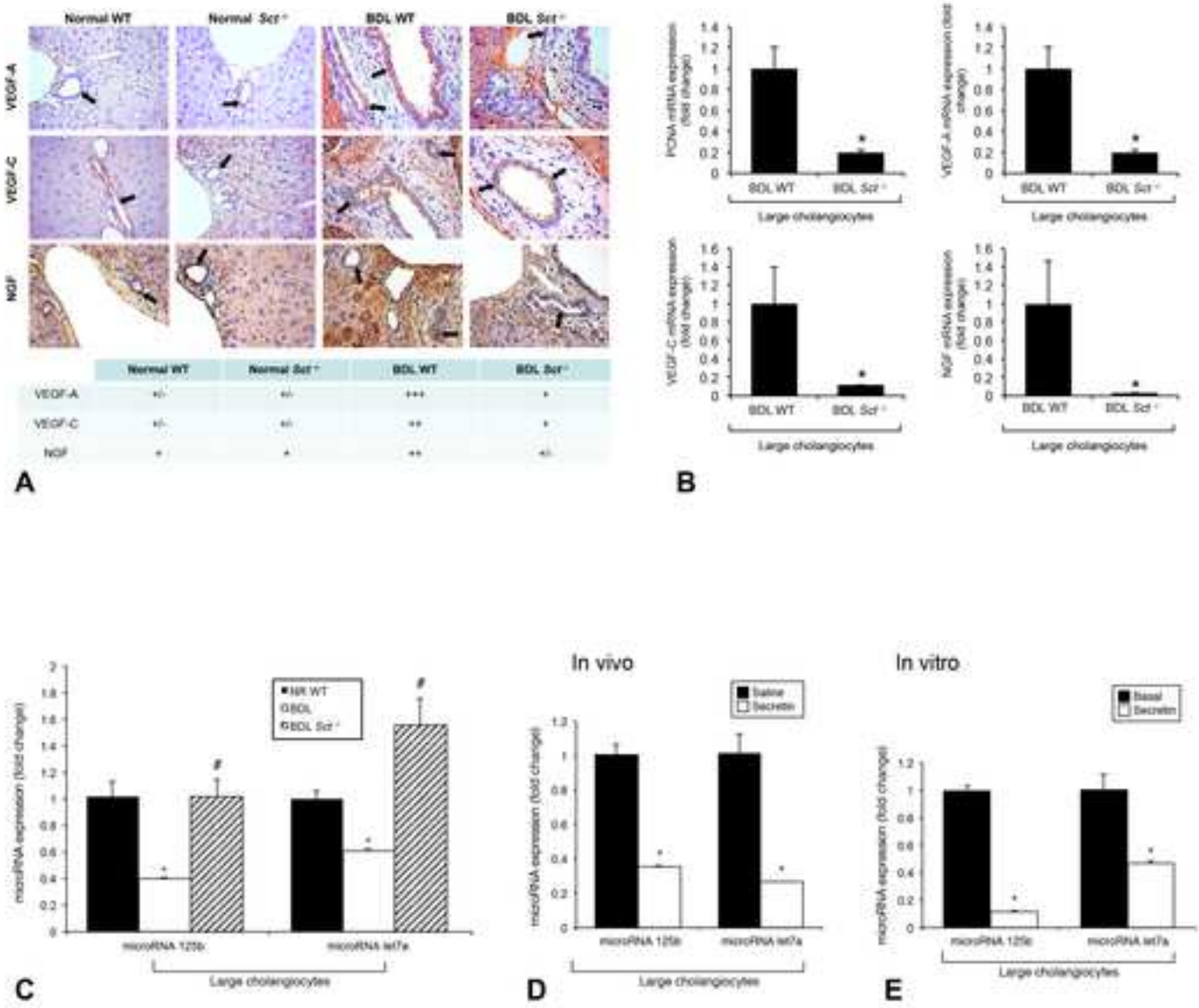
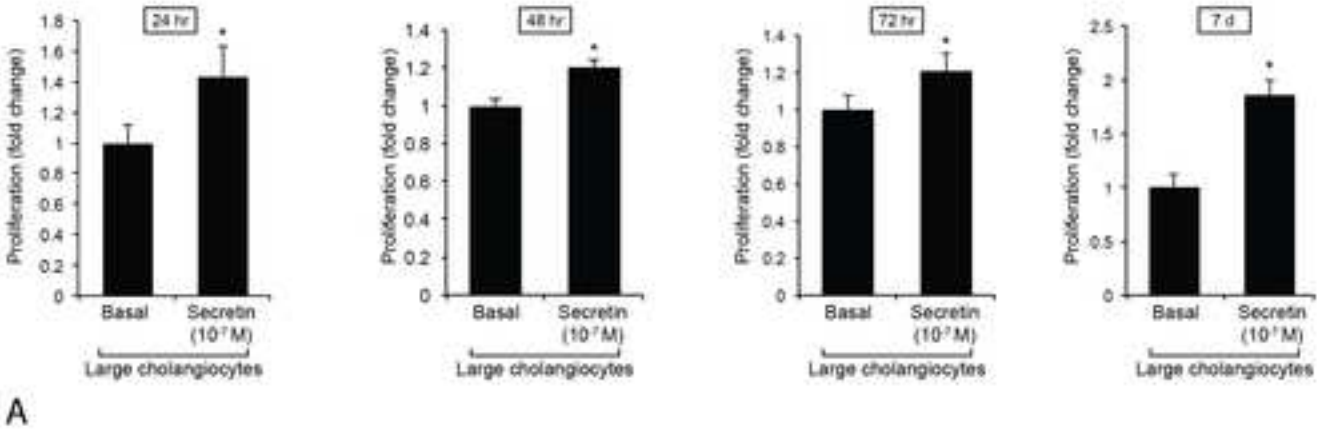
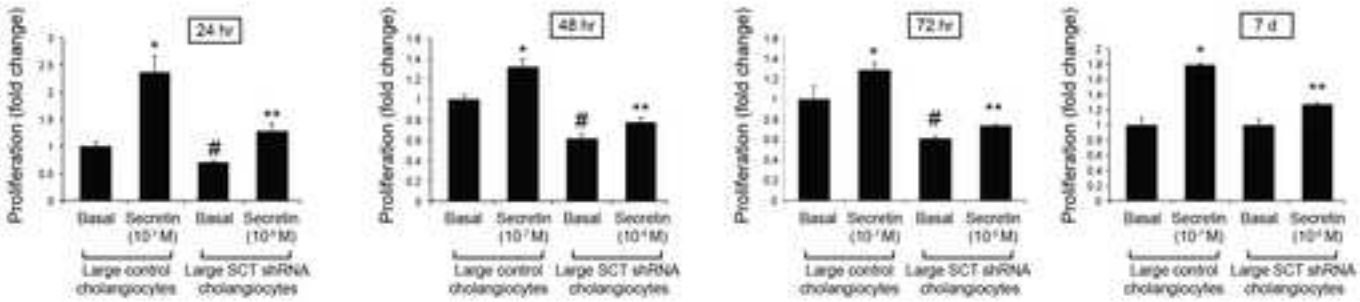

Normal WT
Normal Scr-/-
BDL WT
BDL Scr-/-
VEGF-A
VEGF-C
NGF
Normal WT
Normal Scr-/-
BDL WT
BDL Scr-/-
VEGF-A +/- +/- +++ +
VEGF-C +/- +/- ++ +
NGF + + ++ +/-
A
PCNA mRNA expression (fold change)
VEGF-A mRNA expression (fold change)
VEGF-C mRNA expression (fold change)
NGF mRNA expression (fold change)
BDL WT
BDL Scr-/-
Large cholangiocytes
B
microRNA expression (fold change)
NR WT
BDL
BDL Scr-/-
microRNA 125b
microRNA let7a
Large cholangiocytes
C
In vivo
Saline
Secretin
microRNA expression (fold change)
microRNA 125b
microRNA let7a
Large cholangiocytes
D
In vitro
Basal
Secretin
microRNA expression (fold change)
microRNA 125b
microRNA let7a
Large cholangiocytes
E

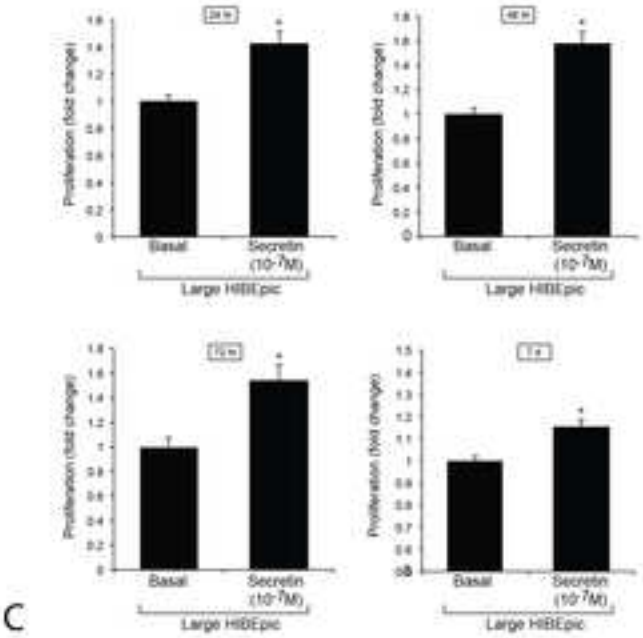
Figure 3


24 hr
48 hr
72 hr
7 d
Proliferation (fold change)
Basal
Secretin
(10⁻⁷M)
Large cholangiocytes

A


24 hr
48 hr
72 hr
7 d
Proliferation (fold change)
Basal
Secretin
(10⁻⁷M)
Large control cholangiocytes
Large SCT shRNA cholangiocytes

B


Proliferation (fold change)
Basal
Secretin
(10⁻⁷M)
Large HIBEpic

C

Figure 4

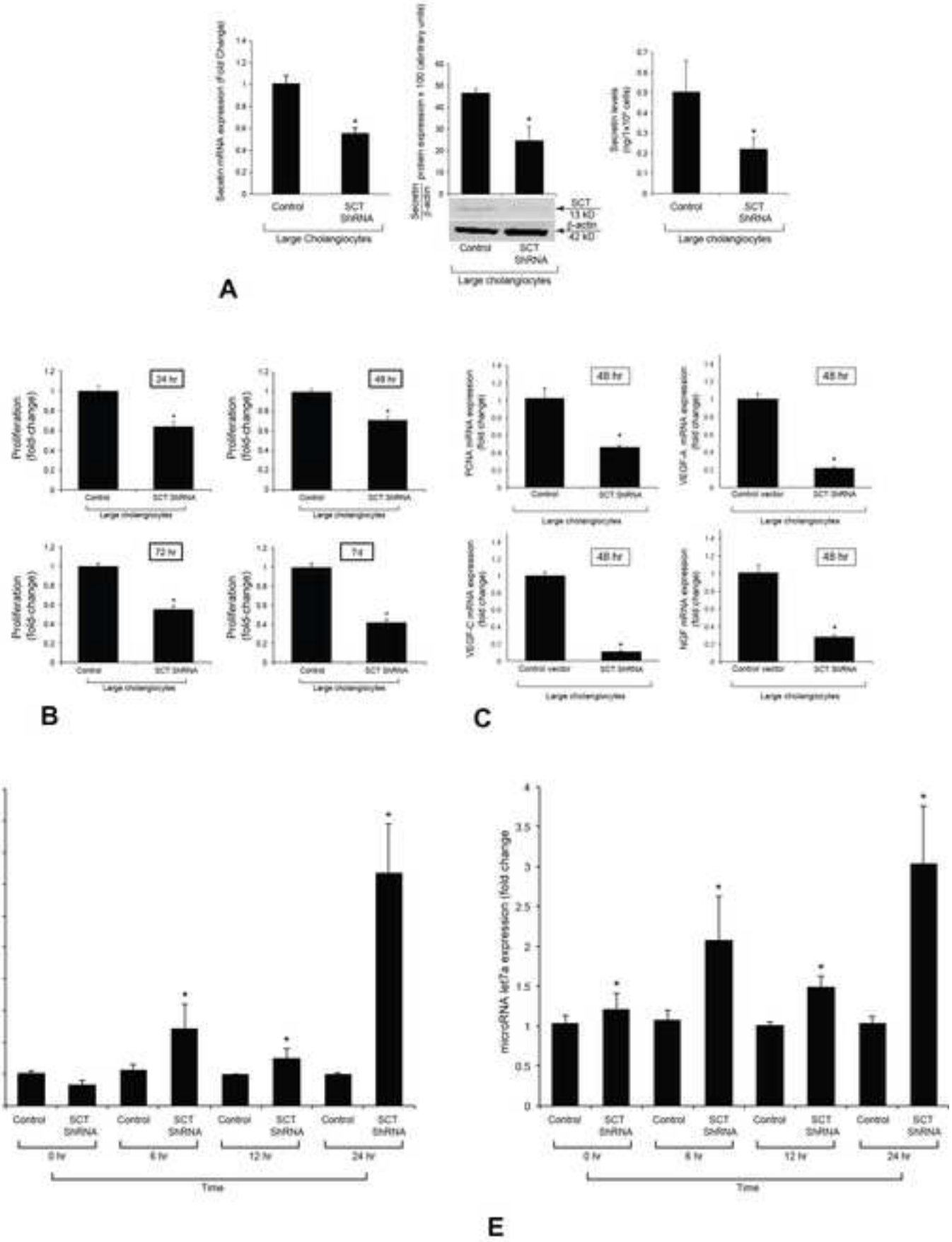
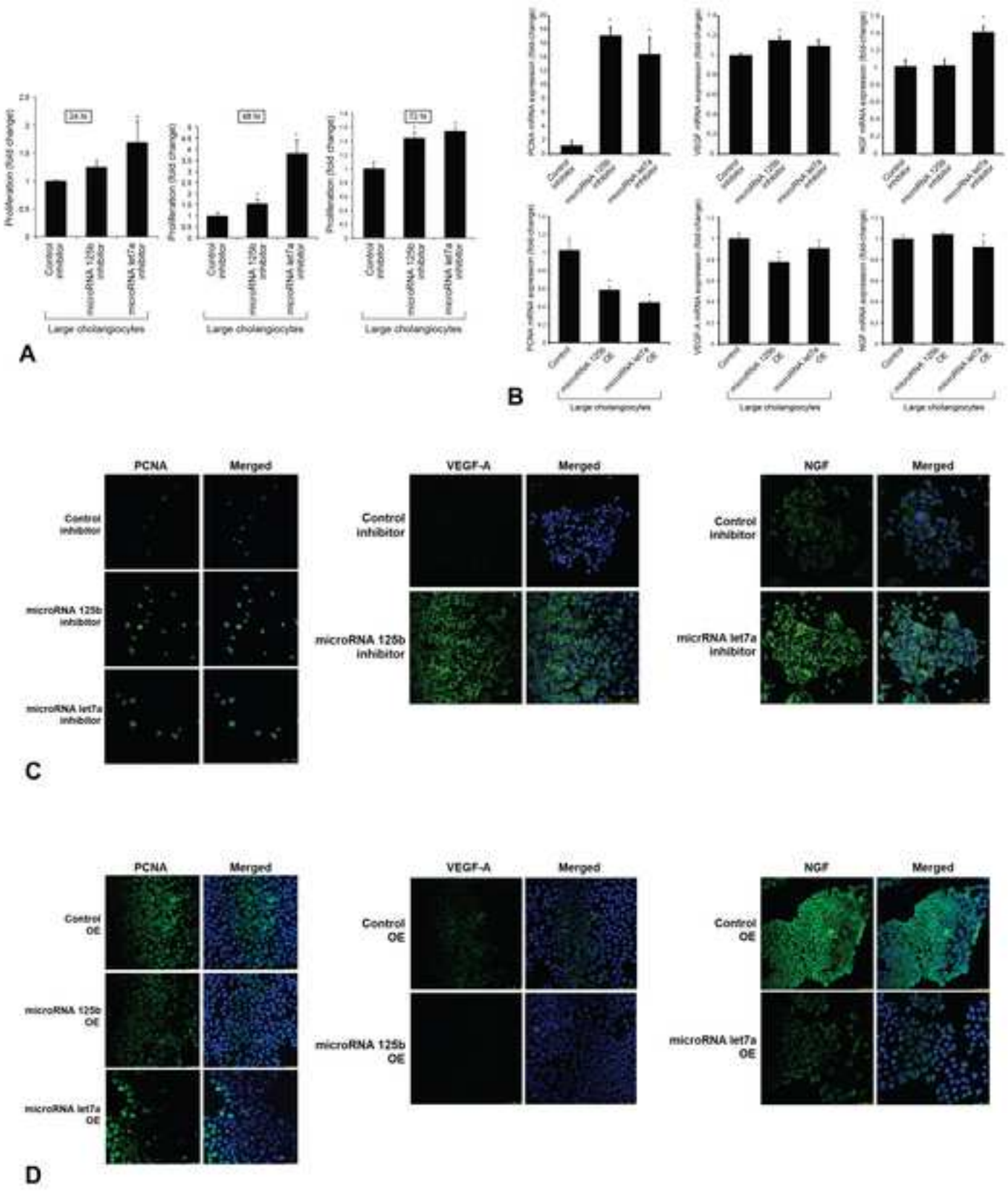

A
Control
SCT shRNA
Large Cholangiocytes
Large cholangiocytes
SCT 13 kD
β-actin 42 kD
B
C
D
E
microRNA 125b expression (fold change)
microRNA let7a expression (fold change)
0 hr
6 hr
12 hr
24 hr
Time

Figure 5


A
Control inhibitor
microRNA 125b inhibitor
microRNA let7a inhibitor
Large cholangiocytes
B
Control OE
microRNA 125b OE
microRNA let7a OE
Large cholangiocytes
C
PCNA
Merged
VEGF-A
NGF
Control inhibitor
microRNA 125b inhibitor
microRNA let7a inhibitor
D
Control OE
microRNA 125b OE
microRNA let7a OE

Supplemental Table 1 Measurement of the % IBDM of large cholangiocytes.

Parameter	**Normal WT + microRNA 125b Mismatch Morpholino**	**Normal WT + microRNA 125b Morpholino**	**BDL WT + microRNA 125b Mismatch Morpholino**	**BDL WT + microRNA 125b Morpholino**
IBDM (%)	0.21±0.007	0.25±0.005	2.2±0.06	2.7±0.02*
Parameter	**Normal WT + microRNA let7a Mismatch Morpholino**	**Normal WT + MicroRNA let7a Morpholino**	**BDL WT + microRNA let7a mismatch Morpholino**	**BDL WT + microRNA let7a Morpholino**
IBDM (%)	0.23±0.004	0.26±0.005	2.3±0.05	2.8±0.06*

Data are mean ± SEM. *p<0.05 vs. BDL mice treated with mismatch Morpholinos.

Supplemental Table 2 Measurement of body weight, liver weight and liver to body weight ratio in normal and BDL WT and BDL *Sct* $^{-/-}$) mice.

Parameter	Normal WT	Normal *Sct* $^{-/-}$	BDL WT	BDL *Sct* $^{-/-}$
Body weight (gm)	24.8±0.4 (n = 41)	30.7±0.8 (n = 16)	20.8±0.3 (n = 41)	25.35±0.9 (n = 16)
Liver weight (gm)	1.8±0.06 (n = 41)	2.5±0.1 (n = 16)	1.8±0.07 (n = 41)	1.96±0.08 (n = 16)
Liver to body weight ratio (%)	7.3±0.18 (n = 41)	8.1±0.4 (n = 16)	8.7±0.3[a] (n = 41)	7.78±0.3[b] (n = 16)

Data are mean ± SEM. [a]$p<0.05$ vs. liver to body weight ratio of normal WT mice. [b]$p<0.05$ vs. liver to body weight ratio of BDL mice.

Supplementary information:

Suppl. Figure 1 *In vitro e*ffect of the medium (in the absence/presence of secretin antibody) of cholangiocytes from normal and/or BDL WT mice on [**A**] cAMP levels and [**B**] proliferation of large cholangiocytes. Secretin-stimulation of cAMP levels and proliferation were partly decreased by pre-incubation with secretin antibody. *p<0.05 vs. basal. #p<0.05 vs. large cholangiocytes treated with medium from BDL cholangiocytes. Data are mean ± SEM of four experiments.

Suppl. Figure 2 Representative immunofluorescence images to colocalize: **[A]** Secretin (green staining) with chromogranin-A (red staining) and **[B]** secretin receptor (SR) (green staining) and chromogranin-A (red staining) in S cells from normal and BDL mice. In normal S cells, both secretin and SR (green staining) colocalize with chromogranin-A (red staining). In BDL S cells, there is co-staining of secretin and SR with chromogranin-A, but the expression of secretin and SR is higher compared to normal S cells. Nuclei are stained in blue with DAPI. Bar = 200 μm.

Suppl. Figure 3 [**A**] In normal and BDL mice treated *in vivo* with microRNA 125b or microRNA let7a Vivo-Morpholinos, there was reduced biliary expression of microRNA 125b and microRNA let7a in cholangiocytes. *p<0.05 vs. mismatch Morpholino treated mice. [**B**] In liver sections from BDL mice treated *in vivo* with microRNA 125b Vivo-Morpholinos there was enhanced VEGF-A expression. [**C**] In liver sections from BDL mice treated *in vivo* with microRNA let7a Vivo Morpholinos there was enhanced NGF expression. Orig. magn., x20.

Suppl. Figure 4 Evaluation of the expression of microRNA 125b and microRNA let7a in isolated hepatocytes. There was increased expression of microRNA 125b and microRNA let7a in normal WT mice treated with secretin and BDL mice compared to normal WT mice, and reduced expression of microRNA 125b and microRNA let7a in BDL $SEC^{-/-}$ mice compared to BDL WT mice. Data are mean ± SEM of four experiments. ***#** p<0.05 vs. normal WT mice. ******p<0.05 vs. BDL WT mice.

Suppl. Figure 5 Altered expression of microRNAs involved in hepatobiliary injury and proliferation in large SEC shRNA cholangiocytes, and large cholangiocytes from normal and BDL WT and $SEC^{-/-}$ mice compared to the corresponding controls. Real-time RT-PCR values represent means from triplicate measurements with multiple samples (*n* = 4–5).

Suppl. Figure 6 [**A**] Treatment of normal WT mice with secretin increased mRNA expression of PCNA, VEGF-A/C and NGF in cholangiocytes. Data are mean ± SEM of four real-time PCR reactions performed in cholangiocytes from four mice. *****p<0.05 vs. large cholangiocytes from saline-treated mice. [**B**] *In* vitro, secretin increased mRNA expression of PCNA, VEGF-A/C and NGF of large cholangiocytes. Data are mean ± SEM of four real-time PCR reactions. *****p<0.05 vs. the value of large cholangiocytes treated with BSA. [**C**] Targeting of VEGF and NGF 3′-UTR by microRNA 125b and microRNA let7a. Firefly luciferase activity was normalized to *Renilla luciferase* activity for each sample. **Left**: The sequences of the mutated target sites of VEGF and NGF with mutations to disrupt base pairing between the specific binding sites and microRNAs are also displayed. **Right**: The decreases in relative firefly luciferase with pMIR-WT-luc compared with the pMIR- MUT-luc constructs in microRNA 125b and microRNA let7a

overexpressed cells confirms that the VEGF and NGF complementary sequence in the 3′-UTR and the genes are the direct targets of modulation by microRNA 125b and microRNA let7a. The data represent the mean and standard errors from three independent transfections. *p< 0.05 relative to respective controls. [**D**] The pRL-Tk microRNA let7a constructs, which contains two microRNA let7a binding sites in the 3′-UTR of Renilla luciferase reporter, were co-transfected with shRNA for secretin in large cholangiocytes. The inhibition of secretin leading to the restoration of microRNA let7a (which binds to the 3′-UTR of Renilla luciferase reporter) caused a significant decrease in luciferase activity. This confirmed the direct inhibitory effects of secretin on the expression of microRNA let7a. Data are mean ± SEM of four repeats. *****p<0.05 vs. vector-transfected cholangiocytes.

Suppl. Figure 7 [**A-B**] microRNA expression of [**A**] silencing or [**B**] overexpression of microRNA 125b and microRNA let7a with anti-microRNA 125b inhibitors and anti-microRNA let7a inhibitors or microRNA 125b precursors and microRNA let7a precursors on large murine cholangiocytes. *****p<0.05 vs. controls.

Suppl. File 1

List of antibodies

The secretin polyclonal antibody (AB981) was purchased from Chemicon® International (Temecula, CA). The following antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA): 1) VEGF-A (JH121) mouse monoclonal antibody raised against full length VEGF of human origin; and 2) VEGF-C (H-190) rabbit polyclonal antibody raised against amino acids 230-419 of VEGF-C of human origin. The mouse anti-cytokeratin-19 (CK-19) antibody was purchased from Caltag

Laboratories Inc. (Burlingame, CA, USA). The rabbit polyclonal antibody to NGF (ab6199) was purchased from Abcam (Cambridge, MA).

Real-time PCR and Immunoblotting Analysis

For the quantitative evaluation of mRNA expression of the selected genes, total cellular RNA was extracted by the RNeasy Mini Kit (Qiagen Inc., Valencia, CA), and reverse transcribed using the Reaction Ready™ First Strand cDNA synthesis kit (SuperArray, Frederick, MD). For each reaction, we used 1.0 μg total RNA. These reactions were used as templates for the PCR assays using a SYBR Green PCR master mix and specific primers designed against the gene for mouse secretin (NM_011328), PCNA (NM_01145), VEGF-A (NM_031836), VEGF-C (NM_053653), NGF (NM_013609), glyceraldehyde-3-phosphate dehydrogenase (GAPDH, NM_008084, housekeeping gene, SABiosciences), in the real-time thermal cycler (ABI Prism 7900HT sequence detection system). For microRNA expression study, we utilized the TaqMan microRNA assay kit (Applied Biosystems, Foster City, CA) with the manufacture's protocol. Briefly, microRNA 125b, microRNA let7a and RNAU6 (housekeeping for microRNA 125b and microRNA let7a) were reverse transcribed using the TaqMan MicroRNA Reverse Transcription Kit, and the TaqMan microRNA real-time PCR amplification assays. For reverse transcription step, we used 10 ng of total RNA. The product from reverse transcription reaction was diluted by 1:15 for PCR amplification. A ΔΔCT (delta delta of the threshold cycle) analysis was performed using normal large cholangiocytes as controls. Data are expressed as fold-change of relative mRNA or microRNA levels ± SEM (n=6).

Immunoblots were performed in protein (10 μg) from whole cell lysate from purified cholangiocytes. Immunoblots were normalized by β-actin. Band intensity was determined by scanning video densitometry using the phospho-imager, Storm 860, (GE Healthcare, Piscataway, NJ) and the ImageQuant TL software version 2003.02 (GE Healthcare, Little Chalfont, Buckinghamshire, England).

Double Immunofluorescence

For double immunofluorescence, we used smears of S cells from normal and BDL mice. Staining was performed by fixing cells in acetone and following washes and incubation in 4% bovine serum albumin (BSA) in 1X Phosphate Buffered Saline Tween-20 (PBST), the cells were incubated with the selected primary antibodies: SR (Santa Cruz Biotechnology), secretin and chromogranin A (Santa Cruz Biotechnology). After one hour at room temperature, the cells were washed three times in PBS-T and then placed in the specific secondary antibodies (anti-goat Alexa Fluor 488 for SR, anti-human Alexa Fluor 488 for secretin and anti-rabbit Alexa Fulor 594 for chromogranin A, Invitrogen) in a dark room for 45 minutes. Finally, S cells were rinsed in buffer and mounted with Ultra-Cruz mounting medium (Santa Cruz Biotechnology). To demonstrate the specificity of the immunoreaction, negative controls were performed without the incubation with primary antibody. Images were taken by DM4500B light microscopy (Leica, Wetzlar, Germany).

Liver Histology

Paraffin-embedded liver sections (4 μm thick, 3 sections analyzed per group) were stained with hematoxylin and eosin (H&E) before evaluating necrosis, steatosis, and the degree of portal inflammation in a coded fashion. Histology of spleen, small and large

intestine, kidney, stomach and heart was determined in sections stained for hematoxylin and eosin. Sections were examined in a coded fashion with a BX-51 light microscope (Olympus, Tokyo, Japan) equipped with a camera.

Immunohistochemistry for VEGF-A/C and NGF in Liver Sections

In liver sections from WT and $SEC^{-/-}$ BDL mice, we evaluated the percentage of large bile ducts positive for VEGF-A/C and NGF. When 0%-5% of bile ducts were positive, we assigned a negative score; a +/- score was assigned when 6%-10% of ducts were positive; and a + score was assigned when 11%-30% of bile ducts were positive.

Knock-down of the Secretin Gene in Large Cholangiocytes

The cholangiocyte line with reduced *Sct* gene expression was generated using SureSilencing shRNA (SABiosciences, Valencia, CA) plasmid for mouse *Sct* containing a marker for neomycin resistance for the selection of stably transfected cells according to the instructions from the vendor. A total of 4 clones were assessed for the relative knockout of secretin by real-time PCR and a single clone with the greatest degree of knockout was selected for subsequent experiments. The extent of secretin knockout was also evaluated by immunoblots, and the amount of secretin released into the medium.

Transfections for Mature MicroRNAs

Large murine cholangiocytes were transfected with 5 μM microRNA 125b/microRNA let7a inhibitors or precursors or relative controls using Lipofectamine RNAiMAX transfection reagent (Life Technologies, Grand Island, NY) according to manufacturer's recommended protocol. Briefly, $1x10^5$ large murine cholangiocytes were plated on 6-well

plates. After 24 hours, 5µl microRNA 125b/microRNA let7a inhibitors or precursors with controls (5 nM) were diluted in 100 µl serum-free medium and mixed with 7 µl of each transfection reagent (Life Technologies) pre-diluted in 93 µl serum-free medium. After 20 minutes of incubation at room temperature, the complexes were added to the cells in a final volume of 1 ml of anti-biotic free medium.

Following transfection to knockout or overexpressing microRNA 125b and microRNA let7a, cells were harvested after 48 hr and small RNA was isolated with mirVana™ microRNA Isolation Kit according to the manufacture's instructions (Ambion). The expression of specific mature microRNAs was confirmed by real-time PCR analysis using TaqMan Human MicroRNA Assay kit (Applied Biosystems, Foster City, CA) with the TaqMan Universal PCR Master Mix reagent kit (Applied Biosystems) (details are shown above in real-time PCR section). Normalization was performed using RNA U6 as housekeeping RNA. To demonstrate that microRNA 125b and microRNA let7a regulates VEGF and NGF and biliary proliferation in large murine cholangiocytes, we measured by real-time PCR and immunofluorescence the expression of PCNA, VEGF-A and NGF after the confirmation of knocking down or overexpression of microRNA 125b and microRNA let7a.

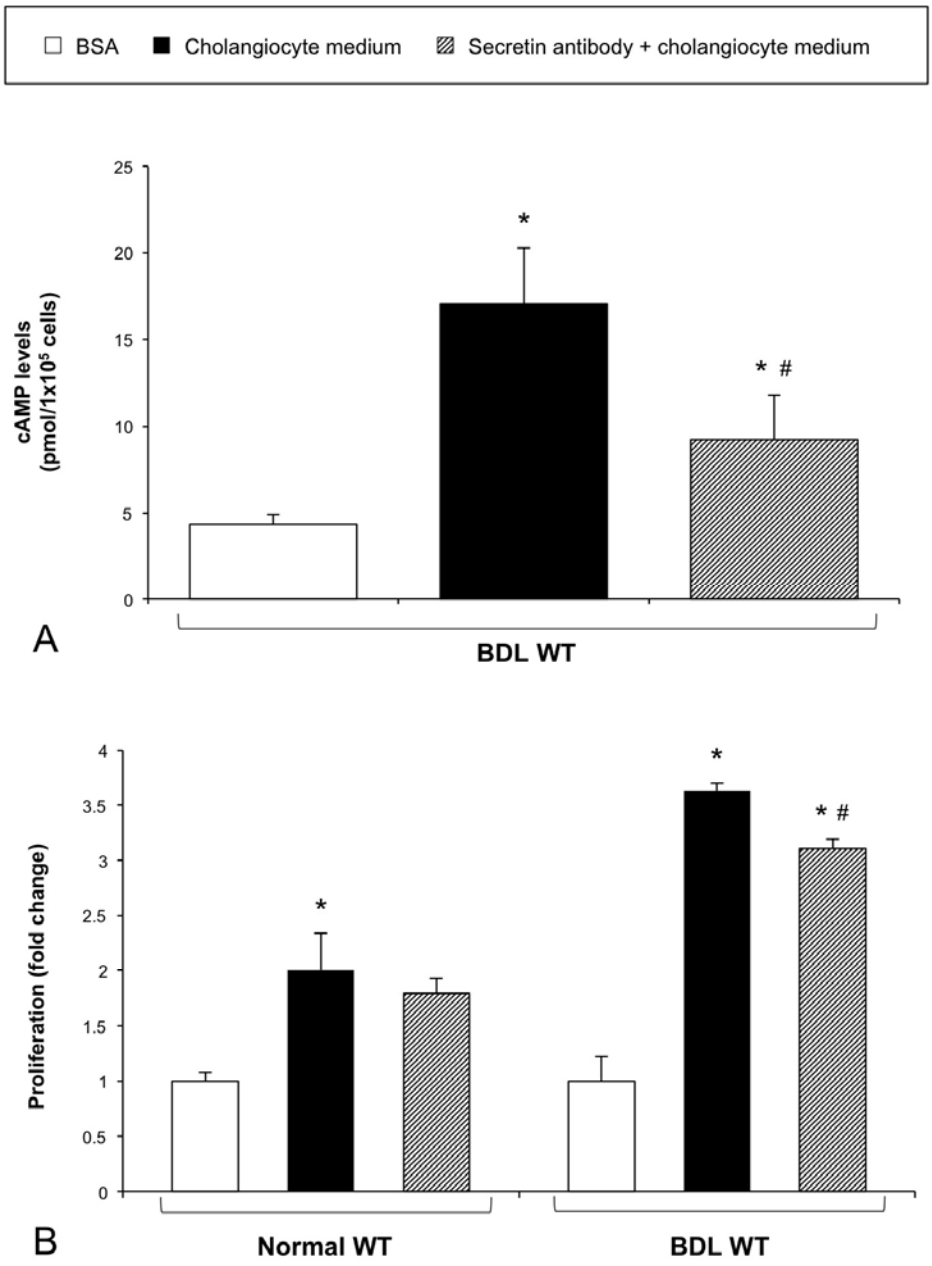
Immunofluorescence for VEGF, NGF and PCNA was performed after 72 hours as follows. Cells were cultured to confluency on 1.5% collagen-coated culture chambers (Chemicon International). Monolayers were washed twice and fixed in 100% ice-cold acetone for 10 minutes, then blocked in PBS containing 2% rabbit serum and 5% mouse serum for 1 hour at room temperature before incubation with primary antibodies, mouse anti-VEGF-A and anti-NGF (1:100), and rabbit anti–PCNA (1:100) overnight at 4℃ followed by secondary fluorescein isothiocyanate–conjugated anti-rabbit (1:200) for 1 hour at room temperature. After 3 washes, monolayers were mounted on glass slides with ProLong antifade mounting medium with DAPI (Molecular Probes). Images were

viewed and captured using Leica TCS SP5 confocal microscope system (Wetzlar, Germany).

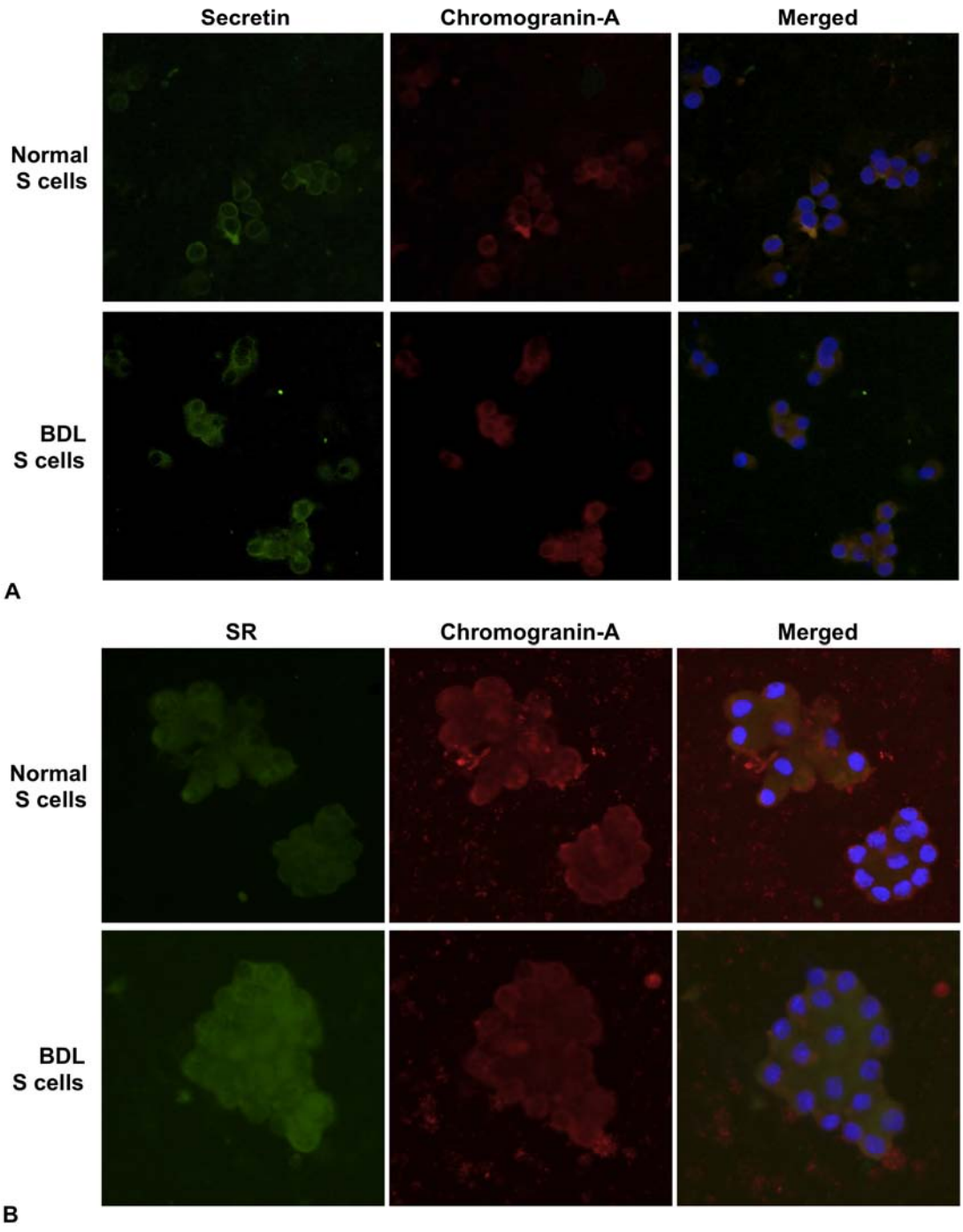
Luciferase Reporter Assay

HIBEpiC were co-transfected with 1 µg of firefly luciferase reporter plasmid and 100 nmol of microRNA precursor (pre-microRNA 125b and pre-miR-microRNA let7a respectively), or control pri-microRNA using DhamarFECT (Dharmacon, Thermofisher, Lafayette, CO) according to the manufacture's instructions. Next, cells were assayed using luciferase assay kits (Promega) after 72 hr of transfection. Each transfection experiment was performed in triplicate. Similarly, to demonstrate the regulation of secretin to microRNA let7a, we plated large murine cholangiocytes into 6-well plates. After 24 hours, the pRL-TK microRNA let7a which include two binding sites of microRNA let7a after the Renilla luciferase or the pRL-TK control were co-transfected with shRNA of secretin or shRNA control using DhamarFECT (Dharmacon, Thermofisher) according to the manufacture's instructions. After 48 hr, cells were lysed with passive lysis buffer (Promega). Aliquots of lysates were analyzed by dual luciferase reporter assay system. All signals from Renilla luciferase sensors were first normalized with that from firefly luciferase. The normalized values were re-normalized with the specific value of signals from the control plasmid.

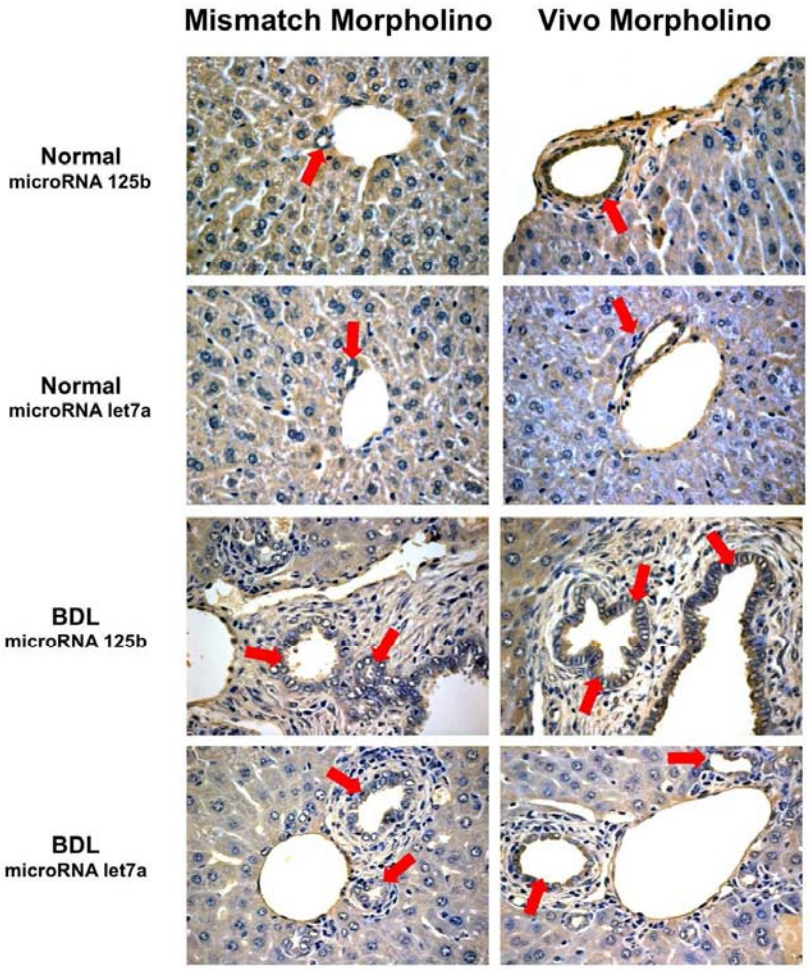
Supp. Figure 1



Suppl. Figure 2

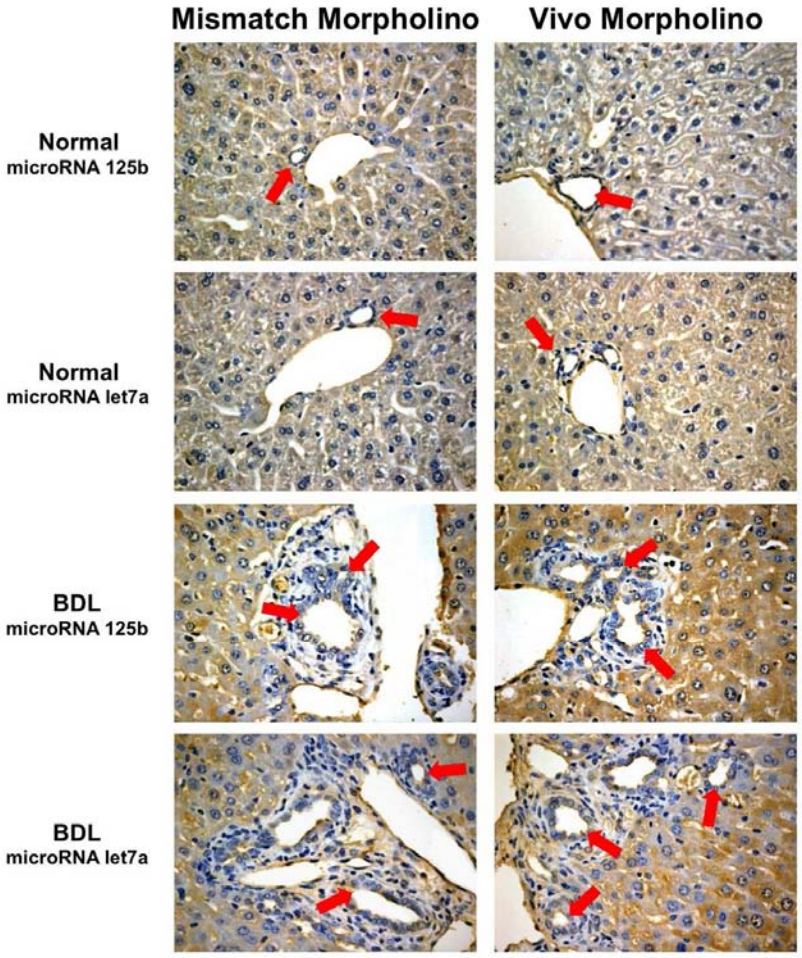


Suppl. Figure 3 B



VEGF-A			
Normal WT + microRNA 125b Mismatch Morpholino	Normal WT + microRNA 125b Vivo Morpholino	Normal WT + microRNA let7a Mismatch Morpholino	Normal WT + microRNA let7a Vivo Morpholino
-	+	-	+/-
BDL WT + microRNA 125b Mismatch Morpholino	BDL WT + microRNA 125b Vivo Morpholino	BDL WT + microRNA let7a Mismatch Morpholino	BDL WT + microRNA let7a Vivo Morpholino
+/-	+	+/-	+

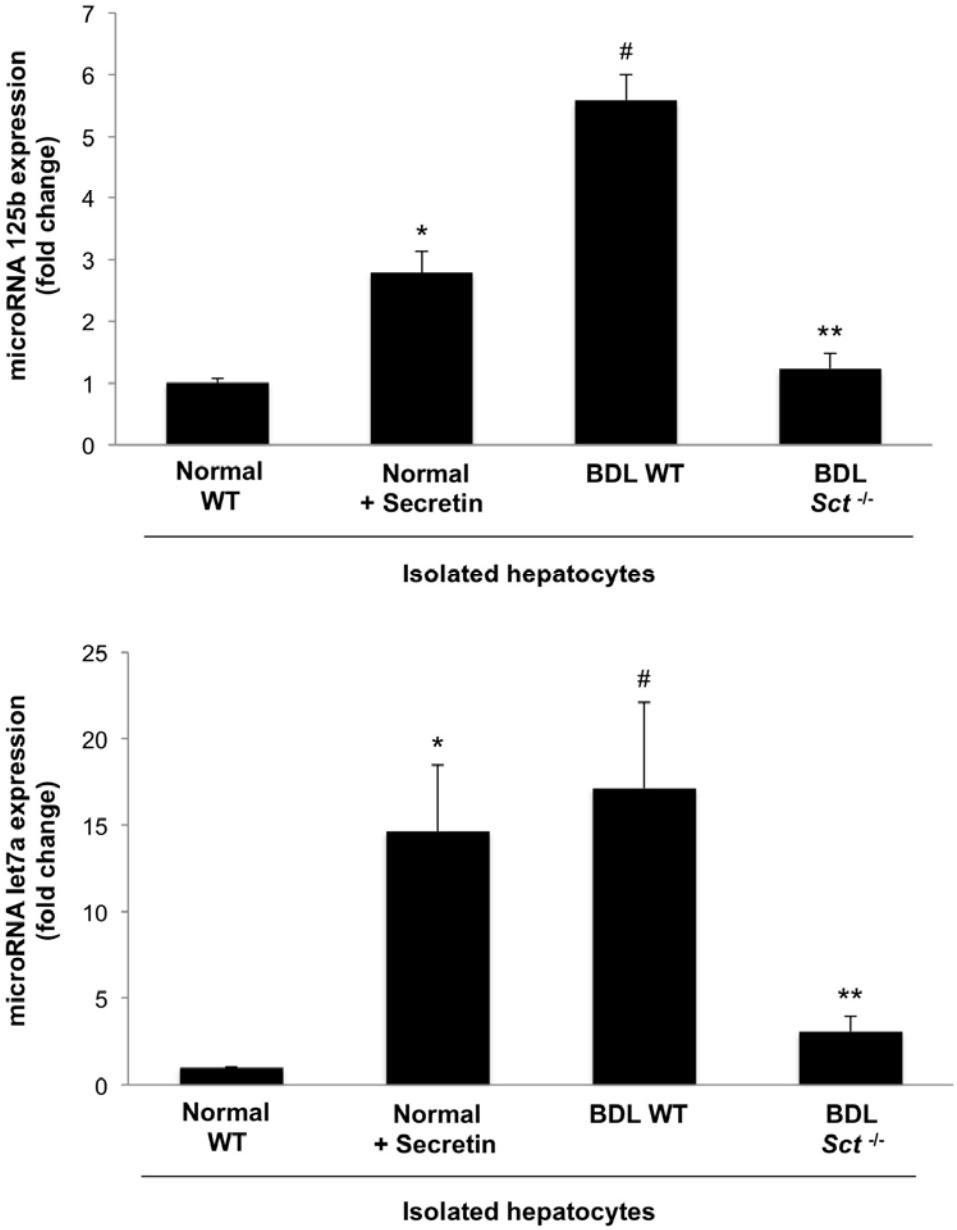
Suppl. Figure 3 C



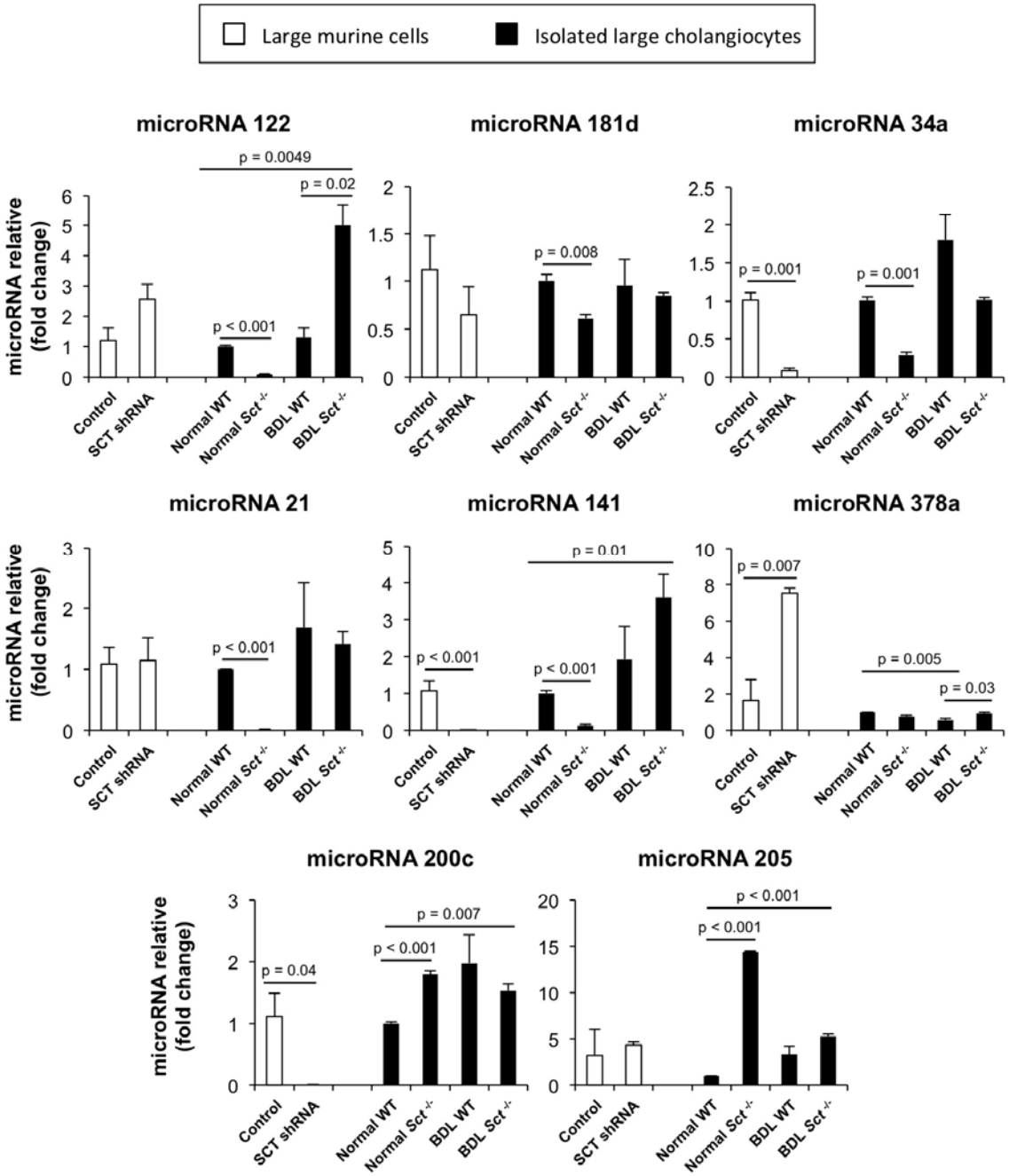
NGF			
Normal WT + microRNA 125b Mismatch Morpholino	Normal WT + microRNA 125b Vivo Morpholino	Normal WT + microRNA let7a Mismatch Morpholino	Normal WT + microRNA let7a Vivo Morpholino
-	+	-	+/-

BDL WT + microRNA 125b Mismatch Morpholino	BDL WT + microRNA 125b Vivo Morpholino	BDL WT + microRNA let7a Mismatch Morpholino	BDL WT + microRNA let7a Vivo Morpholino
+/-	+	+/-	+

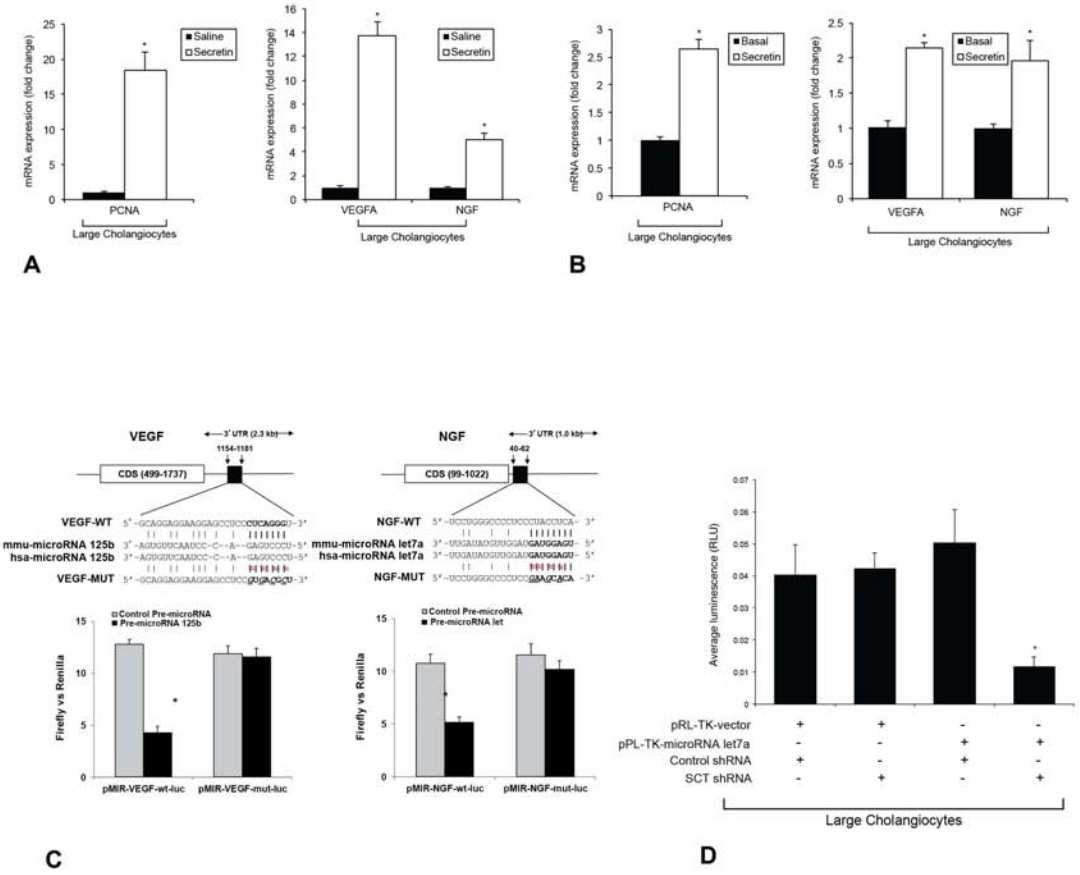
Suppl. Figure 4



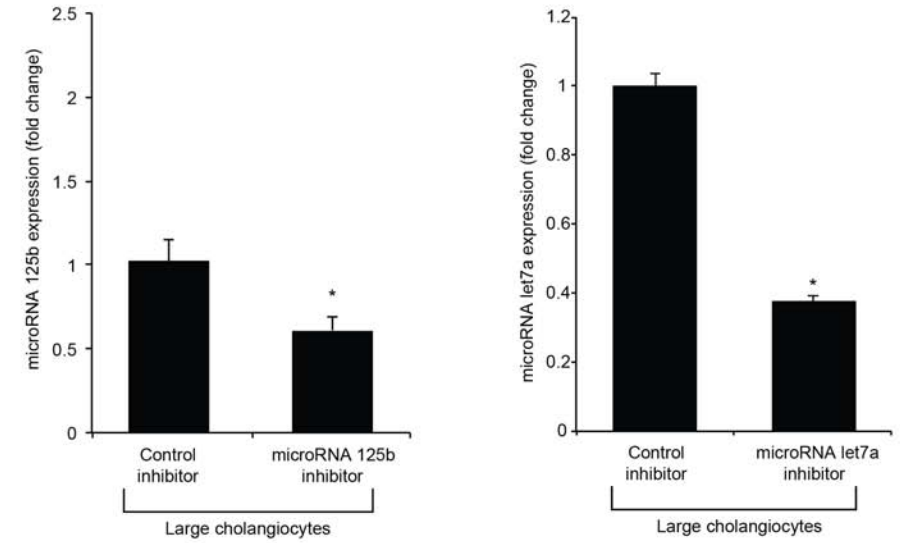
Suppl. Figure 5



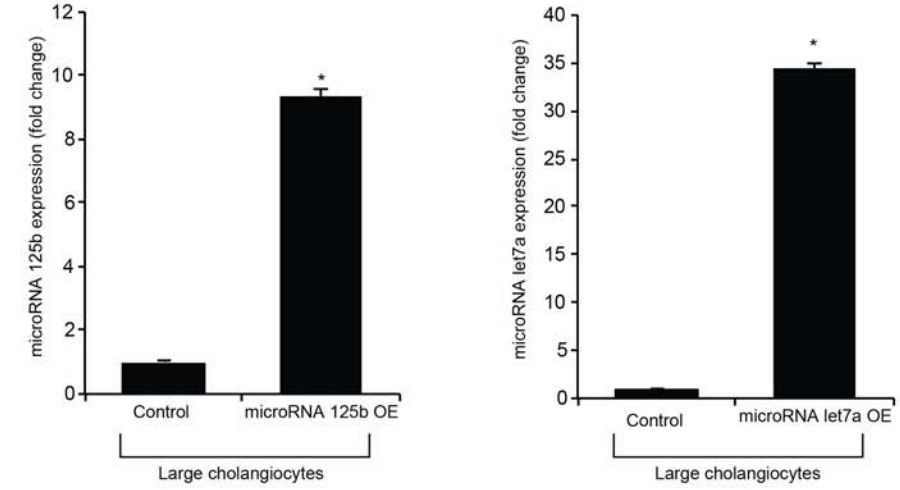
Suppl. Figure 6



Suppl. Figure 7



A



B