Histamine is formed by the conversion of L-histidine into histamine by histidine decarboxylase (HDC). We have previously shown that inhibition of HDC blocks cholangiocyte proliferation and silencing of HDC decreases vascular endothelial growth factor (VEGF) expression. We hypothesized that increased HDC expression during cholestatic liver injury is mediated by the down-regulation of the specific miRNA miR-125b, a post-transcriptional regulator. Mice were subjected to sham surgery or bile duct ligation (BDL), which induces large cholangiocyte proliferation, and subsequently treated with either saline or a-methyl-L-histidine (an HDC inhibitor) for 7 days. Liver blocks, serum, and large cholangiocytes were obtained, and intrahepatic bile duct mass, cholangiocyte proliferation (proliferating cellular nuclear antigen, VEGF-A/C, and intrahepatic bile duct mass, cholangiocyte proliferation (proliferating cellular nuclear antigen expression), and expression of both HDC and VEGF were measured. miRNA profiling was performed in isolated cholangiocytes. In vitro, miR-125b was overexpressed (or inhibited) or HDC was silenced before measuring HDC and VEGF-A/C expression and cholangiocyte proliferation. After BDL plus a-methyl-L-histidine, expression of intrahepatic bile duct mass, proliferating cellular nuclear antigen, VEGF-A/C, and HDC and levels of histamine all decreased compared with those of BDL alone. miR-125b was significantly down-regulated after BDL. In vitro, overexpression of miR-125b and knockdown of HDC both decreased HDC and VEGF expression and cholangiocyte proliferation. Manipulation of miR-125b—regulated HDC/VEGF expression may, thus, be a therapeutic approach for the treatment of aberrant cholangiocyte growth in biliary disorders. (Am J Pathol 2014, 184: 662–673; http://dx.doi.org/10.1016/j.ajpath.2013.11.008)
histamine receptor agonist increases small cholangiocyte proliferation.\textsuperscript{12} We have also shown that inhibition of HDC decreases cholangiocarcinoma growth \textit{in vitro} and \textit{in vivo}.\textsuperscript{10}

Cholangiocytes express VEGF-A and VEGF-C and secrete VEGF through an autocrine mechanism, thus providing the support needed to the proliferating biliary system after BDL.\textsuperscript{5,13} VEGF-A induces many cellular effects, including increasing cellular migration, mitogenesis, and angiogenesis and promoting cellular proliferation while inhibiting apoptosis.\textsuperscript{13,14} VEGF-C is an important mediator of cell growth and survival, as well as being a regulator of angiogenesis.\textsuperscript{15,16} HDC regulates both VEGF-A and VEGF-C expression.\textsuperscript{10,15} In HDC\textsuperscript{−/−} mice, angiogenesis is defective and histamine derived from nonmast cells regulates the angiogenesis of the inflammatory tissue.\textsuperscript{16}

miRNAs are post-transcriptional regulators that alter cellular translation. After 70\% partial hepatectomy, biliary miR-181b expression is significantly up-regulated compared with sham—operated on animals.\textsuperscript{17} Also, miR-125b is significantly down-regulated in hepatocellular cancer.\textsuperscript{18} Another study found that miR-125b inhibits hepatocellular cell migration and invasion,\textsuperscript{20} suggesting that this miRNA is involved in angiogenesis. On the basis of this background, we proposed to demonstrate that miR-125b regulates large biliary hyperplasia by modulating the HDC/VEGF axis.

### Materials and Methods

All reagents were obtained from Sigma-Aldrich, Co (St. Louis, MO), unless otherwise indicated. All primers and transfection plasmids were obtained from SA Biosciences (Frederick, MD), unless stated otherwise. Antibodies for immunohistochemistry (IHC) and Western blot analysis were obtained from Santa Cruz Biotechnology (Santa Cruz, CA), unless indicated otherwise. To determine histamine levels, we used an enzyme immunoassay kit from Cayman Chemical (Ann Arbor, MI).

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**Figure 1** Evaluation of HDC expression. **A:** HDC expression in bile ducts compared with normal mice and after treatment with the HDC inhibitor, α-methyl-DHis. Original magnification, ×20. **Inset:** Increased resolution of image. **B:** HDC gene expression in large cholangiocytes compared with BDL mice and after α-methyl-DHis treatment. Data are the means ± SEM of eight experiments from cumulative preparations of large cholangiocytes from at least eight animals from each treatment group. *P < 0.05 versus normal; †P < 0.05 versus BDL. **C:** After BDL, histamine secretion in serum compared with normal and after treatment with the HDC inhibitor compared with both normal and BDL. **D:** In isolated large cholangiocyte supernatant, histamine secretion after BDL compared with normal, and after BDL plus α-methyl-DHis treatment compared with normal and BDL. Data are the means ± SEM of 12 experiments from cholangiocyte supernatants collected from cumulative preparations of large cholangiocytes from at least six animals from each treatment group. *P < 0.05 versus normal; †P < 0.05 versus BDL.
Animal Models and Isolated Cholangiocytes

Mice were subjected to sham surgery (normal) or BDL and treated, immediately after surgery, by daily i.p. injections with vehicle (0.9% NaCl) or \( \alpha \)-methyl-\( \text{L} \)-histidine (\( \alpha \)-methyl-DHis; 150 \( \mu \)g/body weight; M8628; Sigma-Aldrich)\(^{10,21} \); after 3 and 7 days, liver blocks (paraffin and frozen, 4 to 5 \( \mu \)m thick), serum, organs, and isolated large cholangiocytes were collected. Large cholangiocytes were provided by Dr. Gianfranco Alpini (Scott and White, Central Texas Veterans Health Care System and Texas A&M Health Science Center, Temple).\(^4,22 \)

In Vitro Cell Lines

\textit{In vitro} studies were performed in SV-40—transformed large mouse cholangiocyte lines (LMCC)\(^{23} \) and human intrahepatic epithelial cells (HiBECs). HiBECs were purchased from Sciencell Research Laboratories (Carlsbad, CA).\(^{10} \) Cells were cultured according to previous studies.\(^{10,12,23} \)

Evaluation of HDC Expression and Histamine Secretion

To determine the expression of HDC in the liver, we performed IHC in liver sections (4 to 5 \( \mu \)m thick) and real-time and standard RT-PCR in isolated large cholangiocytes, hepatocytes, and LMCC. IHC was performed in paraffin-embedded liver sections. The antibody for HDC (sc-34458; Santa Cruz Biotechnology) was used at 1:50. Negative controls were performed in parallel using nonimmune serum as the primary antibody. Light microscopy (Leica Microsystems, Wetzlar, Germany) was performed to obtain and quantify images. Positive cells were counted in six nonoverlapping fields (\( \times \)20 magnification) for each slide, and the data expressed as percentage of positive cells. Light microscopy and IHC observation were performed in a blinded manner by two independent morphologists.

Real-time PCR was performed in total RNA extracted from isolated large cholangiocytes from all animal groups to determine gene expression of HDC. For all PCRs, mouse primers (SA Biosciences) were used and a \( \Delta \Delta \text{CT} \) analysis was performed. Total cellular RNA (1 \( \mu \)g) was used for PCRs, and cytokeratin-19 (a cholangiocyte-specific marker)\(^{14} \) or glyceraldehyde-3-phosphate dehydrogenase\(^{14} \) was used as the control. Standard RT-PCR was performed in LMCC to determine mRNA levels of HDC, as described.\(^{24} \)

Western blot analysis for HDC expression was performed on protein from whole cell lysates from purified large cholangiocytes, hepatocytes, and LMCC. Cells were trypsinized and lysed for immunoblots and SDS-PAGE, as described.\(^{10} \) Western blot analysis was performed with 10 to 20 \( \mu \)g of protein, and band intensity was determined by scanning video densitometry using the ImageQuant TL Software version 2003.02.

Figure 2 Evaluation of intrahepatic bile duct mass after \( \alpha \)-methyl-DHis treatment. Ductal mass after BDL compared with normal shown by IHC (A). Quantification of IHC (B). Original magnifications: \( \times \)20; \( \times \)40 (inset). *\( P < 0.05 \) versus normal; †\( P < 0.05 \) versus BDL.  

LMCC supernatants were measured by commercially available enzyme immunolink assays (Cayman Chemical).\(^{10} \)

Intrahepatic bile duct mass and cholangiocyte proliferation were determined in all animal groups by semi-quantitative IHC staining for cytokeratin-19 and proliferating cellular nuclear antigen (PCNA), respectively.\(^{11,25} \) In isolated large cholangiocytes from all animal groups and LMCC, protein lysates were collected and Western blot analysis was performed for PCNA to determine cholangiocyte proliferation. Western by scanning video densitometry using the ImageQuant TL Software version 2003.02.
**Determination of VEGF Expression**

VEGF cholangiocyte expression was evaluated by IHC in paraffin-embedded liver sections (4 μm thick), and real-time and standard RT-PCR in purified large cholangiocytes and LMCC, respectively. Sections were incubated overnight at 4°C with the following antibodies from Santa Cruz Biotechnology: VEGF-A (sc-152; 1:1000) and VEGF-C (sc-9047; 1:200). The immunoreactivity of VEGF-A/C in cholangiocytes was automatically quantified by ImageScope (Aperio Scanscope CS System, Vista, CA). Bile ducts were randomly selected by the operator, and an image analysis algorithm that automatically analyzes the selected areas was used to quantify the number of positive pixels in cholangiocytes. The algorithm was applied to the entire section of at least three different sections from each liver fragment. The percentage of positive pixels and the average intensity of positivity were obtained from the software, and quantification of IHC was obtained by multiplying the percentage of positive pixels by the average intensity of positivity.

Immunofluorescence and Western blot analysis were performed in LMCC to detect VEGF-A/C protein levels, as described. Cells were seeded on coverslips in a six-well plate (500,000 cells per well) and allowed to adhere overnight. Immunofluorescence was performed, as described, using a specific antibody for VEGF-A and VEGF-C (diluted 1:50 in 1% bovine serum albumin/PBS with Tween or nonimmune serum for negative controls). Images were obtained using an inverted Olympus IX-71 confocal microscope (Olympus, Tokyo, Japan). Quantification of immunofluorescent staining was performed manually and expressed as the percentage of VEGF-A/C-positive cholangiocytes per area (six areas were counted by a blinded operator). Primary antibodies for Western blot analysis were diluted at 1:50.

**Evaluation of miR-125b**

To determine whether HDC is a target of the predicted miR-125b, we extracted the mRNA sequence of HDC and scanned potential binding sites for miRNAs using both Targetscan (Whitehead Institute for Biomedical Research, Cambridge, MA) and PicTar 4-way software (Center for Comparative Functional Genomics, New York, New York) and performed miRNA profiling by microarray analysis.

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**Figure 3** Evaluation of PCNA expression after α-methyl-DHis treatment. A: The number of PCNA-positive cholangiocytes after BDL and after α-methyl-DHis treatment shown by IHC. Original magnifications: ×20; ×40 (inset). Quantification of IHC (B). PCNA protein expression after BDL and after treatment with α-methyl-DHIs compared with BDL mice (C). Data are the means ± SEM of at least four experiments from cumulative preparations of large cholangiocytes from at least eight animals from each treatment group. *P < 0.05 versus normal; †P < 0.05 versus BDL.
After identification of the target miR-125b, we measured miR-125b expression in isolated cholangiocytes from normal and BDL mice by microarray analysis performed by the Center for Targeted Therapy, Department of Experimental Therapeutics, The University of Texas MD Anderson Cancer Center (Houston) and verified by real-time PCR analysis, as previously described, using a specific TaqMan miRNA assay (Life Technologies, Carlsbad, CA).

Luciferase Reporter Assay

An intact putative miR-125b recognition sequence from the 3′—untranslated region (UTR) of HDC/VEGF (pMIR—HDC—wt—3′-UTR or pGL3—VEGF—3′-UTR) or with random mutations (pMIR—HDC—mut—3′-UTR) was cloned downstream of the firefly luciferase reporter gene. For the miR-125b—HDC target relationship, luciferase assays were performed at 72 hours after transfection with control and miR-125b precursor or inhibitor (50 nmol/L) in HiBEC cells using the Dual Luciferase Reporter Assay system (Promega, Madison, WI). To determine whether miR-125b directly targets VEGF, HiBEC cells were plated (2 × 10^6 cells per well) in 6-well plates. The pGL3—VEGF—3′-UTR firefly luciferase expression construct (1 μg) and a Renilla luciferase expression construct, pRL-TK (1 μg; Promega), were cotransfected with the miR-125b or control precursor or inhibitor. Luciferase assays were performed 48 hours after transfection using the dual Luciferase Reporter Assay System (Promega).

Genetic Modification of HDC and miR-125b in LMCC

To demonstrate that the effects of HDC and miR-125b are specific to cholangiocyte proliferation, we performed in vitro independent experiments to genetically knock down HDC or overexpress miR-125b in LMCC. By using an HDC shRNA plasmid, we generated a stably transfected HDC LMCC knockdown cell line (LMCC-HDC), along with a negative (LMCC-neg) cell line, as described. Overexpression of miR-125b (pre—miR-125b) was performed in standard media containing 10% fetal bovine serum and using pre—miR-125b (50 nmol/L) from Ambion Inc. (Austin, TX), along with appropriate controls (pre-miRNA), and miR-125b expression was measured by TaqMan real-time PCR assay (Applied Biosystem Inc., Grand Island, NY).

In LMCC-HDC or miR-125b overexpressed cells (and respective controls), HDC, VEGF-A, and VEGF-C gene and protein levels were determined. In LMCC-HDC and respective control cells, we also evaluated the expression of the mRNA for H1 to H4 histamine receptors by real-time PCR. Histamine secretion was measured in conditioned media from genetically modified cells using EIA, and cholangiocyte

![Figure 4](https://ajp.amjpathol.org)  **Figure 4**  *In vivo* expression of VEGF-A and VEGF-C in liver sections. VEGF-A and VEGF-C expression after BDL compared with normal, and after treatment with the HDC inhibitor, α-methyl-DHis, compared with BDL. Original magnifications: ×20; ×40 (Inset). Data are the means ± SEM from at least four animals per group. ∗P < 0.05 versus normal; †P < 0.05 versus BDL.
proliferation was determined by MTT assays\textsuperscript{12} and Western blot analyses\textsuperscript{12} for PCNA. Apical and basolateral membranes were collected from LMCCs, according to our previous studies.\textsuperscript{26,27} Specifically, cells were placed in transwell chambers and brought to confluence, and transepithelial resistance was measured before experimental analysis of histamine levels by EIA.

**Statistical Analysis**

Data are expressed as means ± SEM from at least three separate experiments performed in triplicate, unless noted otherwise. Differences between groups were analyzed by the Student’s unpaired t-test when two groups were analyzed, and by analysis of variance when more than two groups were analyzed, followed by an appropriate post hoc test. The null hypothesis was rejected at \( P < 0.05 \).

**Results**

**HDC Expression and Histamine Release in Vivo**

Prolonged administration (3 and 7 days) of \( \alpha \)-methyl-DHIs to normal mice induced no changes in intrahepatic bile duct production.
mass, liver weight, body weight, and liver/body weight ratio (data not shown). Therefore, we performed all studies in normal and BDL mice treated for 7 days with vehicle or \(\alpha\)-methyl-DHis. HDC was expressed mainly in large cholangiocytes (Figure 1A) and increased after BDL. Real-time PCR (Figure 1B) for HDC expression demonstrates that HDC gene expression is increased in large cholangiocytes after BDL and reduced in BDL + \(\alpha\)-methyl-DHis–treated mice. By immunoblotting, a slight expression of HDC was found in mouse hepatocytes, whereas a strong expression was found in isolated mouse cholangiocytes, thus strengthening the autocrine role of HDC in biliary proliferation (Supplemental Figure S1). Histamine levels increase in serum and cholangiocyte supernatant obtained from BDL mice when compared with normal mice, whereas treatment with \(\alpha\)-methyl-DHis significantly decreased histamine release in serum and cholangiocyte supernatant (Figure 1, C and D).

Evaluation of Intrahepatic Bile Duct Mass and Cholangiocyte Proliferation

After BDL, intrahepatic bile duct mass increases compared with normal mice; treatment with \(\alpha\)-methyl-DHis significantly decreases intrahepatic bile duct mass when compared with BDL alone (Figure 2). The number of PCNA-positive cholangiocytes increased after BDL and was significantly reduced when animals were treated with \(\alpha\)-methyl-DHis (Figure 3A). There was a significant decrease in PCNA protein expression in large cholangiocytes from \(\alpha\)-methyl-DHis–treated BDL mice compared with BDL mice (Figure 3B). We evaluated lobular damage, necrosis, and inflammation in the liver and other major organs (heart, lung, intestine, spleen, and stomach) in all groups and found that \(\alpha\)-methyl-DHIs has no pathological effects in our animal model, and no significant morphological changes were noted (data not shown). These data demonstrate that HDC is an important regulator of cholangiocyte proliferation.

**VEGF Expression in Vivo**

The expression of VEGF-A and VEGF-C significantly increased in cholangiocytes from mouse liver after BDL when compared with normal (Figure 4). Furthermore, after HDC inhibition, cholangiocyte VEGF-A/C expression decreased, suggesting that HDC regulates the expression of VEGF during biliary proliferation.

**In Vivo Evaluation of miR-125b and Its Target Proteins in BDL Mice**

The potential miRNAs predicted to target HDC were selected from the list of miRNAs in which expression levels were reduced in BDL mouse liver (<0.05-fold, \(P < 0.01\)). miR-125b is significantly decreased in BDL liver versus normal mice (Figure 5A) (ArrayExpress database; http://www.ebi.ac.uk/arrayexpress, accession number E-MTAB-2267). Real-time PCR analysis revealed that, in cholangiocytes isolated from BDL and normal mouse liver, miR-125b gene expression was significantly down-regulated after BDL compared with normal (Figure 5B). HDC is a predicted target of miR-125b, as shown by bioinformatic analysis (Figure 5C). To verify that HDC is a direct target of miR-125b, we used luciferase reporter constructs containing the miR-125b recognition sequence from the 3′-UTR of HDC inserted downstream of the luciferase gene (Figure 5C). Transfection with precursors or inhibitors of miR-125b significantly modulated reporter activity of HDC in LMCCs. miR-125b decreases HDC expression when compared with control pre-miRNA (top panel), HD1 to HD4 histamine receptor expression levels were measured after miR-125b overexpression compared with control pre-miRNA. Data are the means ± SEM of six experiments. *\(P < 0.05\) versus control transfected cells.
HDC in human cholangiocytes (Figure 5D). Studies were repeated with random mutations in the shared recognition sequence (Figure 5D), which resulted in abolition/enhancement of the HDC reporter activation by miR-125b overexpression or inhibition (Figure 5D). To determine that miR-125b also targets VEGF, we performed a comparison of bp between mature human miR-125b, human VEGF-A, mouse mmu-miR-125b, and mouse VEGF-A, and found a sequence conservation between the species (Supplemental Figure S2). Furthermore, by using luciferase assays, we found that both overexpression (miR-125b precursor) and inhibition (anti–miR-125b) of miR-125b induced a significant change of VEGF-A luciferase activity (Supplemental Figure S2), which demonstrated that VEGF-A is also a direct target of miR-125b in biliary epithelial cells.

In Vitro Evaluation of miR-125b Effects on HDC and VEGF Expression and Cholangiocyte Proliferation

The LMCCs express HDC and VEGF-A/C (Figure 6). Because miR-125b expression is greatly reduced after BDL (Figure 5B), we overexpressed miR-125b in LMCCs. Overexpression of miR-125b decreased HDC, H1 to H4 histamine receptors, and VEGF-A/C gene expression when compared with pre-miRNA control-transfected LMCCs (Figure 7). HDC and VEGF-A/C protein expression and histamine release were significantly reduced after miR-125b overexpression compared with miRNA control precursor (Figure 8, A–C). Cholangiocyte proliferation was reduced after miR-125b overexpression (Figure 8, D and E) compared with control precursor-transfected cells. To further confirm that miR-125b is an important regulator of HDC (and downstream targets), we silenced miR-125b (Supplemental Figure S3 and performed real-time PCR for HDC. We found that, after knockdown of miR-125b, there is an increase in HDC expression compared with control-transfected cells (Supplemental Figure S3). These data suggest that miR-125b is an important target and regulator of the HDC/VEGF axis.

Validation of HDC Effects in Vitro

To validate that the effects of miR-125b can be mimicked by silencing HDC, we generated a stably transfected HDC

![Figure 8](image-url)  
Effects of miR-125b in vitro. Effects of miR-125b overexpression in LMCCs on HDC, VEGF-A, and VEGF-C protein expression, histamine secretion, and cholangiocyte proliferation. HDC (A) and histamine secretion in conditioned medium from miR-125b overexpressed cells compared with control pre-miRNA transfected cells (B); C: Protein expression of VEGF-A/C after miR-125b overexpression compared with control pre-miRNA transfected cells. D and E: PCNA protein expression (D) and MTT assay (E) after miR-125b overexpression compared with control pre-miRNA transfected cells. Data are the means ± SEM of 12 experiments for secretion, the means ± SEM of 8 experiments for Western blot analysis, and the means ± SEM of 16 experiments for MTT assay (24-hour time point). *P < 0.05 versus control pre-miRNA—transfected cholangiocytes.
knockdown LMCC cell line (LMCC-HDC) lacking HDC and both shRNA control-transfected LMCC (LMCC-neg) and nontransfected LMCC (LMCC basal) cells as controls and measured the following: (i) HDC and VEGF-A/C expression, (ii) histamine secretion, and (iii) cholangiocyte proliferation. Loss of HDC (Figure 9, A and B) induced a decrease in these components. VEGF-A/C gene and protein expression was significantly decreased in LMCC-HDC (cells deficient in HDC expression) compared with control-transfected LMCC (LMCC-neg) (Figure 10) and nontransfected LMCC (data not shown) cells. LMCC secretes histamine at both the apical and basolateral membrane (Figure 9C), and histamine release (Figure 9D) and cholangiocyte proliferation (Figure 9, E and F) were diminished after knockdown of HDC when compared with controls. To determine whether histamine rescues the effects of genetic manipulation of HDC inhibition and further demonstrate an autocrine regulation by cholangiocytes, histamine was added back into the LMCC-HDC cells, secretion was measured by EIA, and the expression of H1 to H4 histamine receptors was evaluated by real-time PCR. Addition of histamine to LMCC-HDC increased histamine secretion (Figure 9D). Finally, we found that after knockdown of HDC (LMCC-HDC), the expression of all histamine receptors (H1 to H4) decreased compared with LMCC-neg (Figure 11), and when histamine was added to LMCC-HDC, H1 to H4 gene expression was significantly increased (Figure 11). These data demonstrate a role for HDC in regulating cholangiocyte proliferation and VEGF expression, potentially via one of the four histamine receptors.

Discussion

In the present study, we demonstrated the autocrine role of HDC on the modulation of cholangiocyte hyperplasia during extrahepatic bile duct obstruction, and that these effects are specific to cholangiocytes and regulated by miR-125b. We demonstrated that HDC expression is primarily expressed by cholangiocytes and increases after BDL that is coupled with a significant reduction in miR-125b expression. Traditionally,

![Figure 9](https://example.com/figure9.png)  
**Figure 9** Overall effects of HDC knockdown on biliary HDC expression, histamine secretion, and proliferation. By real-time PCR (A) and Western blot analysis (B), HDC gene and protein expression after stable knockdown transfection of HDC shRNA (LMCC-HDC) compared with control-transfected, LMCC-neg. Data are the means ± SEM of three experiments for real-time PCR and eight experiments for Western blot analysis. Histamine at the apical and basolateral membrane of LMCC (nontransfected, basal treatment) (C): histamine release in LMCC lacking HDC (LMCC-HDC): effects of addition of histamine (10 μmol/L) in LMCC-HDC histamine secretion (gray bar) compared with LMCC-neg and LMCC-HDC (D). By MTT assay (E) and PCNA protein expression (F), cholangiocyte proliferation after loss of HDC (LMCC-HDC) compared with cells with normal amounts of HDC (LMCC-neg). Data are the means ± SEM of 12 experiments. *P < 0.05 versus LMCC-neg, control-transfected cholangiocytes; †P < 0.05 versus LMCC-HDC.
HDC is expressed primarily by mast cells. However, in support of our findings, previous studies have shown that HDC expression and histamine synthesis are also found in gastric enterochromaffin-like cells. Histamine synthesis plays a role in neuroregulation, and HDC expression regulates day-night rhythms by the changes in the fluctuation of HDC expression in the hypothalamus. Numerous factors regulate cholangiocyte proliferation via an autocrine loop, including histamine, melatonin, and VEGF. We have shown that HDC is critical in regulating cholangiocyte hyperplasia, as shown by the loss of intrahepatic bile duct mass and cholangiocyte proliferation after inhibition of HDC. This concept, demonstrating that histamine is a trophic, growth-promoting factor. Our study shows that cholangiocytes contain the machinery to produce histamine and secrete increased levels of histamine after BDL, likely to sustain the increased ductal mass. Also, preliminary data from BDL rats show that histamine levels are significantly increased in bile (0.756 ± 0.1307 (normal) versus 47.85 ± 1.116 (BDL) nmol/L), which supports our autocrine hypothesis for histamine regulation of cholangiocyte proliferation. In genetically modified, HDC knockout mice, wound-healing time is slower when compared with mice...
with normal histamine levels.\textsuperscript{35,36} In our current and previously published studies, we have demonstrated that the HDC/histamine axis is a trophic pathway increasing cholangiocyte proliferation. In data presented herein and in preliminary data using HDC\textsuperscript{−/−} mice (data not shown), we found that inhibition of HDC (by pharmacological treatment or genetic manipulation) decreases cholangiocyte proliferation via decreased VEGF expression. In contrast, a recent study has shown that, in HDC\textsuperscript{−/−} mice, there is an increased rate of colon cancer and skin carcinogenesis.\textsuperscript{37} This study focused on the paracrine contribution of immature myeloid cell production of HDC and proposes that the balance of histamine synthesis is dysregulated in the tumor microenvironment of skin and colon cancer. Our study focuses on the autocrine release of HDC from the biliary epithelium, which is an unusual tissue. For example, gastrin (which has trophic effects in gastric, colonic, and pancreatic cancer cell lines) inhibits both biliary hyperplasia in BDL rats and cholangiocarcinoma growth of several human cell lines.\textsuperscript{38,39} We speculate that targeting HDC locally (by nanoparticles containing HDC inhibitors) may be an effective therapeutic approach for modulating HDC directly in the liver.

After BDL, the intrahepatic biliary epithelium experiences rapid cholangiocyte proliferation (that occurs before the growth of the peribiliary vascular plexus) that is characterized by increased proliferative activities, including the increased release of histamine and VEGF.\textsuperscript{5,40} In our study, systemic treatment of \textit{α}-methyl-D-\textit{His} decreased cholangiocyte VEGF-A/C expression after biliary injury, presumably to compensate for the decrease in biliary demand. To support our findings, VEGF and angiogenesis are decreased in HDC\textsuperscript{−/−} mice granulation tissue when compared with normal controls.\textsuperscript{16} When HDC\textsuperscript{−/−} mice are treated with an H2 histamine receptor agonist, wound healing is recovered and angiogenesis is restored.\textsuperscript{16}

Furthermore, our data demonstrate that, after loss of HDC (or in miR-125b−overexpressed cells), H1 to H4 histamine receptor expression decreases in a similar manner. Addition of histamine to cells with diminished HDC expression resulted in increased histamine secretion and increased receptor expression. We have shown, in our previous work, that there is a differential cholangiocyte response that results from specific receptor stimulation or inhibition, whereas in this study, it appears that knockdown of HDC affects all receptors, leading to a decrease in cholangiocyte proliferation. The exact role of the individual receptors will need to be further investigated.

Consistent with our previous findings in human cholangiocytes,\textsuperscript{10} our present study demonstrates that HDC regulates cholangiocyte VEGF-A/C expression; to expand this, we evaluated the effects of the specific miRNA miR-125b, on HDC. miR-125b expression is decreased in hepatocellular carcinoma, as well as cholangiocarcinoma tissues and cells, whereas overexpression of miR-125b decreases tumor growth.\textsuperscript{19} After BDL, miR-125b was significantly decreased, which may lead to up-regulation of HDC. After overexpression of miR-125b, the HDC/VEGF axis and cholangiocyte proliferation are all down-regulated. Further studies will focus on evaluating other targets (in addition to HDC) for miR-125b that may affect liver functions.

In summary, we have provided evidence that HDC plays a critical role in cholangiocyte proliferation. The HDC/VEGF axis that mediates cholangiocyte proliferation loses function when histamine is no longer being synthesized after the loss of HDC. Furthermore, we found that miR-125b regulates the HDC/VEGF axis and may be a potential target for future studies. The current study, coupled with our previous work regarding the role of histamine and VEGF on cholangiocyte proliferation, provides substantial evidence that histamine and HDC (regulated by miR-125b) are key mediators of cholangiocyte response during cholestatic liver injury (Figure 12 depicts an overall working model).

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**Supplemental Data**

Supplemental material for this article can be found at http://dx.doi.org/10.1016/j.ajpath.2013.11.008.

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