SCF Promotes Cell-Cycle Progression to Late G_1, Whereas GM-CSF Is Necessary for S Entry. To further analyze the respective role of the combination of the cytokines in cell-cycle progression, we studied the expression levels of cyclin D1, Cdk2, and Cdk1 in small and large cholangiocyte subpopulations. Short-term cholangiocyte cultures, known to undergo one cell cycle under SCF stimulation, were used as controls.

In untreated and GM-CSF-stimulated cultures, as well as in untreated short-term cultures, cyclin D1 and Cdk1 were not detected (Fig. 3D), suggesting that cells were blocked in G_1 upstream from the mitogen-associated restriction point in mid-late G_1. In contrast, in GM-CSF+SCF-stimulated cultures and in SCF-treated short-term cultures, cyclin D1, Cdk2, and Cdk1 expression levels markedly increased, thus confirming the cell progression into the S and M phases. These results suggested that SCF alone promoted progression up to late G_1, and that GM-CSF stimulation was necessary for the G_1/S transition.

Contribution to Regenerative Potentials by Cytokines in Small Cells With Biliary Markers. Our previous studies have demonstrated that small cholangiocytes (originated from small bile duct branches and resistant to CCl_4-induced apoptosis)[2,20] *de novo* proliferate to compensate for the loss of large biliary mass.[2,4] It is possible that the combination of SCF and GM-CSF may alter the induction of remodeling potentials in cholangiocytes during liver regeneration. Alkaline phosphatase (AP) activity has been used as a biomarker for tissue repair and remodeling. We next compared StemTAG AP activity (Cell Biolabs, Inc., San Diego, CA) in mouse cholangiocytes after stimulation with SCF, GM-CSF, or a combination of the two. AP activity was scarcely detected 72 hours after stimulation with SCF or GM-CSF alone in small and large murine cells with biliary markers (Supporting Fig. 2A). In contrast, a more intense AP staining was observed when small cells with biliary markers were stimulated with the SCF+GM-CSF combination. mRNA levels of the SCF receptor were equally decreased after stimulation with either SCF alone or SCF combined with GM-CSF. In addition, GM-CSF receptor mRNA levels were similar between stimulation with SCF alone and SCF+GM-CSF (Supporting Fig. 2B). These results indicate that enhanced remodeling potential of small cells with biliary markers after stimulation with SCF+GM-CSF is the result of the activation of downstream signaling pathways, rather than increased levels of CSF receptors. Together, the data suggest that there are altered tissue-repair capabilities with SCF+GM-CSF in small mouse cells with biliary markers, but not in the presence of either SCF or GM-CSF alone.

Characterization of Tissue Remodeling Marker S100A4 in Cytokine-Treated Cholangiocytes. S100 calcium-binding protein A4 (S100A4), a member of the S100 family of proteins, is of particular interest as a marker of chronic tissue remodeling. It plays an important role in matrix remodeling by up-regulating the expression of matrix metalloproteinases (MMPs). We assessed the effect of cytokine combinations on S100A4 mRNA expression. Quantitative real-time PCR analysis revealed a 3.8 $\pm$ 0.2-fold increased expression of S100A4 after SCF+GM-CSF administration in small cells with biliary markers, compared to SCF alone ($P < 0.01$), suggesting a significant remodeling event after treatment (Fig. 4A, B). Interestingly, the same treatment combination also induced a moderate increase of S100A4 protein expression in human intrahepatic cholangiocytes (Fig. 4C, bottom panel). In contrast, no significant changes were observed in S100A4 expression in small and large cells with biliary markers by SCF or GM-CSF alone or other SCF combinations.

Involvement of SCF and GM-CSF in Transforming Growth Factor Beta–Dependent Signaling in Cholangiocytes. Transforming growth factor-beta (TGF-β) has been associated with intracellular matrix deposition and hepatic/biliary tissue repair and damage.[27] It is a cytokine that stimulates mesenchymal proliferation, inhibits epithelial growth, and is important in organogenesis. Because previous studies have suggested that TGF-β is produced in response to PH,[28] we next determined whether TGF-β would stimulate SCF and GM-CSF production and release. SMCC and LMCC *in vitro* were stimulated with 10 ng/mL of TGF-β or media alone and were harvested after 72 hours of incubation. Supernatants or supernatants plus cells were collected for SCF and GM-CSF measurement by ELISA assay. Supernatant levels of SCF/GM-CSF were used to estimate levels of soluble SCF/GM-CSF; for quantitation of soluble plus bound SCF/GM-CSF, supernatants plus cells were sonicated and SCF/GM-CSF levels in this solution were used as an estimate of soluble plus bound SCF/GM-CSF. Small mouse cholangiocytes produced significant amounts of both soluble and soluble plus bound SCF in response to TGF-β, when compared to controls, whereas large cholangiocytes only showed a slight increase at the time point studied (Fig. 5A-D).

Because TGF-β is a well-known S100A4 inducer and because the above experiments suggest that TGF-β

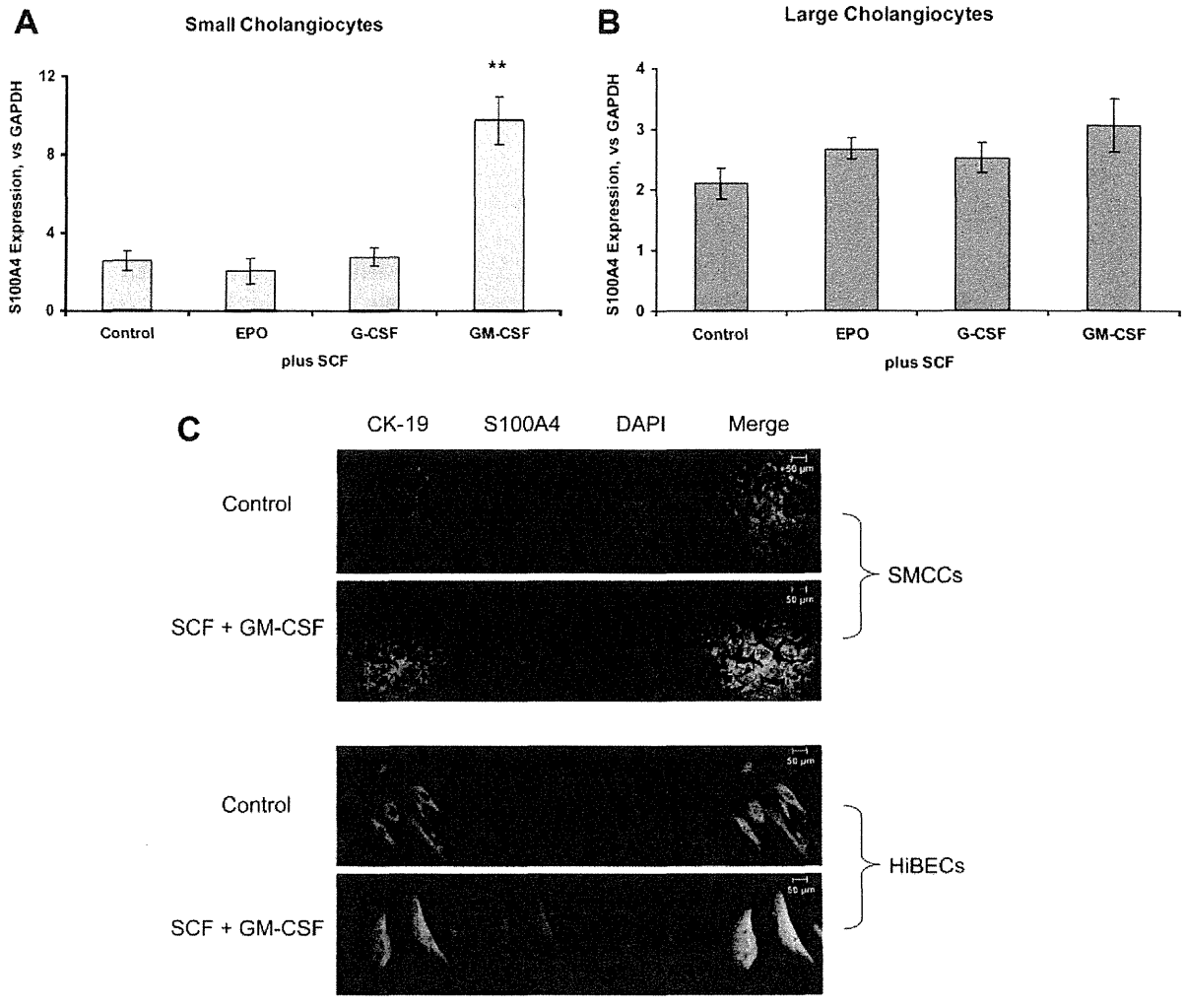

Fig. 4. SCF regulates mesenchymal marker S100A4 in combination with GM-CSF in small murine cholangiocytes. Total RNA was isolated from SMCCs (A) and LMCCs (B) treated with CSF+SCF, and quantitative real-time PCR for mesenchymal marker S100A4 was performed using a superarray quantitative PCR assay kit. Quantitative data representing the mean and SE from three experiments performed in triplicate are presented in the bar graph. The expression of S100A4 was normalized to that of the GAPDH gene control. SCF promotes synergistic mesenchymal transition or transformation in combination with GM-CSF only in small murine cells with biliary markers. Differences between any of the other groups were not significant. (C) Immunocytochemistry for S100A4 and CK-19 was performed in SMCCs and HiBECs. An increase in S100A4 expression is observed in both cell lines after SCF+GM-CSF treatment (10 ng/mL, 72 hours). $^{**}P < 0.01$, compared with expression in the SCF-only group.

can induce SCF/GM-CSF production and release, the next experiments were designed to evaluate whether SCF+GM-CSF and TGF-β-induced S100A4 expression in small cholangiocytes would occur via a related pathway. At a dose of 10 ng/mL, both TGF-β and SCF+GM-CSF induced significant cell remodeling after 72 hours of incubation, as measured by the incorporation of S100A4 mRNA expression (Fig. 5E). Next, the effects of SCF+GM-CSF blockade in the presence of TGF-β were measured. When small cholangiocytes were incubated with 10 ng/mL of TGF-β plus anti-SCF and GM-CSF antibodies (10 μg/L), a significant decrease in S100A4 mRNA expression was noted, suggesting that SCF+GM-CSF plays an important role in TGF-β-induced remodeling in this system. Meanwhile, when large cholangiocytes were incubated with the same combinations, only a slight decrease in S100A4 expression level was observed, which did not reach statistical significance (Fig. 5E).

SCF+GM-CSF Enhance miR-181 Targeting of Tissue Inhibitor of Metalloproteinases-3. Our recent data suggested that miRNA-181 is critical for hepatic cell remodeling and differentiation and target 3'-UTR of tissue inhibitor of metalloproteinases-3 (TIMP-3), leading to TIMP-3 mRNA degradation.[29] TIMP-3 has unique domains that interact with extracellular matrix

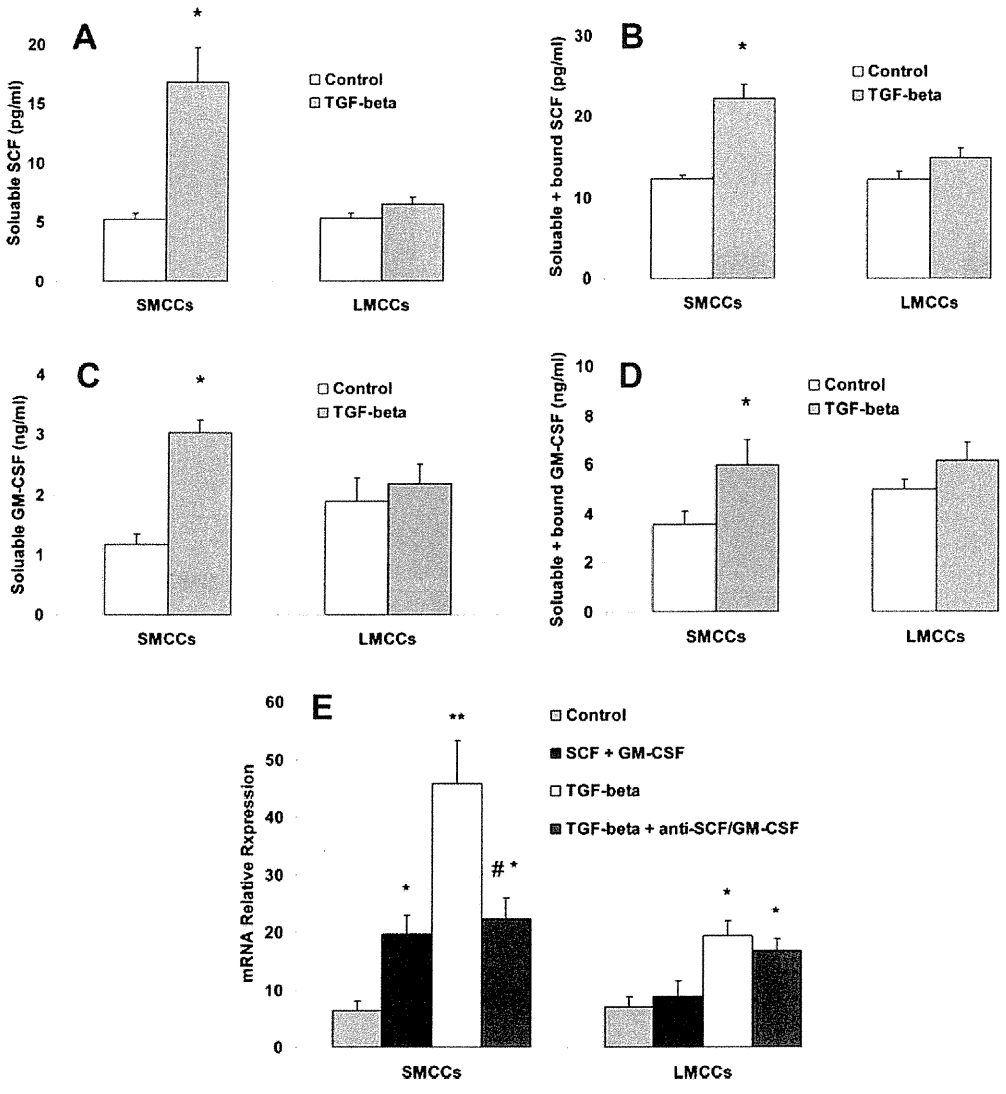

Fig. 5. TGF-β stimulates SCF and GM-CSF production and release in SMCCs. SMCC and LMCC mouse cells were stimulated with 10 ng/mL of TGF-ß or media alone. Supernatants and supernatants plus cells were harvested at 72 hours, and SCF and GM-CSF levels were measured by ELISA. Supernatant SCF and GM-CSF levels were used to determine levels of soluble SCF and GM-CSF (A and C). For quantitation of soluble plus bound SCF and GM-CSF (B and D), supernatants plus cells were sonicated and SCF and GM-CSF levels in this solution were measured as an estimate of soluble plus bound SCF. There were no significant differences noted in levels of soluble, compared with soluble plus bound, SCF in cells incubated in media alone. There were significant increases in soluble SCF and GM-CSF levels at the 72 hours time point from cells treated with TGF-ß in SMCCs ($*P < 0.05$ versus media alone). In addition, levels of soluble plus bound SCF and GM-CSF were significantly increased in SMCCs after TGF-ß treatment, compared with treatment with media alone ($*P < 0.05$ versus media alone). Furthermore, levels of soluble plus bound SCF and GM-CSF were significantly increased, compared with levels of soluble SCF alone, in SMCCs after TGF-ß treatment at 72 hours. Data are expressed as the mean ± SE. (E) Anti-SCF+GM-CSF partially blocked TGF-β-induced changes in small cells with biliary markers. SMCCs and LMCCs were incubated with 10 ng/mL of TGF-β with anti-SCF+GM-CSF antibodies (10 μg/L) or control antiserum for 72 hours, total RNA was isolated, and quantitative real-time PCR for mesenchymal marker S100A4 was performed using a superarray quantitative PCR assay kit. Quantitative data represent the mean and SE from three experiments. The expression of S100A4 was normalized to that of the GAPDH gene control. A significant decrease in S100A4 mRNA expression was noted in SMCCs treated with TGF-β with anti-SCF+GM-CSF antibodies, compared to control TGF-β group, suggesting that SCF+GM-CSF are involved in TGF-β-induced changes in this system. $*P < 0.05$; $**P < 0.01$, relative to control; $\#P < 0.05$, compared with expression in control TGF-β group.

(ECM) components and, unlike the other TIMPs, is mainly bound to tissue matrix.[30] Because miR-181 has been shown to be regulated by TGF-β, we performed experiments to determine whether SCF+GM-CSF would affect the miR-181 targeting of TIMP-3 in SMCCs. To this end, small cholangiocytes were transfected with pre-miR-181b or control pre-miRNA in combinations with the treatments of SCF+GM-CSF; cell lysates were obtained to determine the levels of TIMP-3 protein. Levels of TIMP-3 protein and

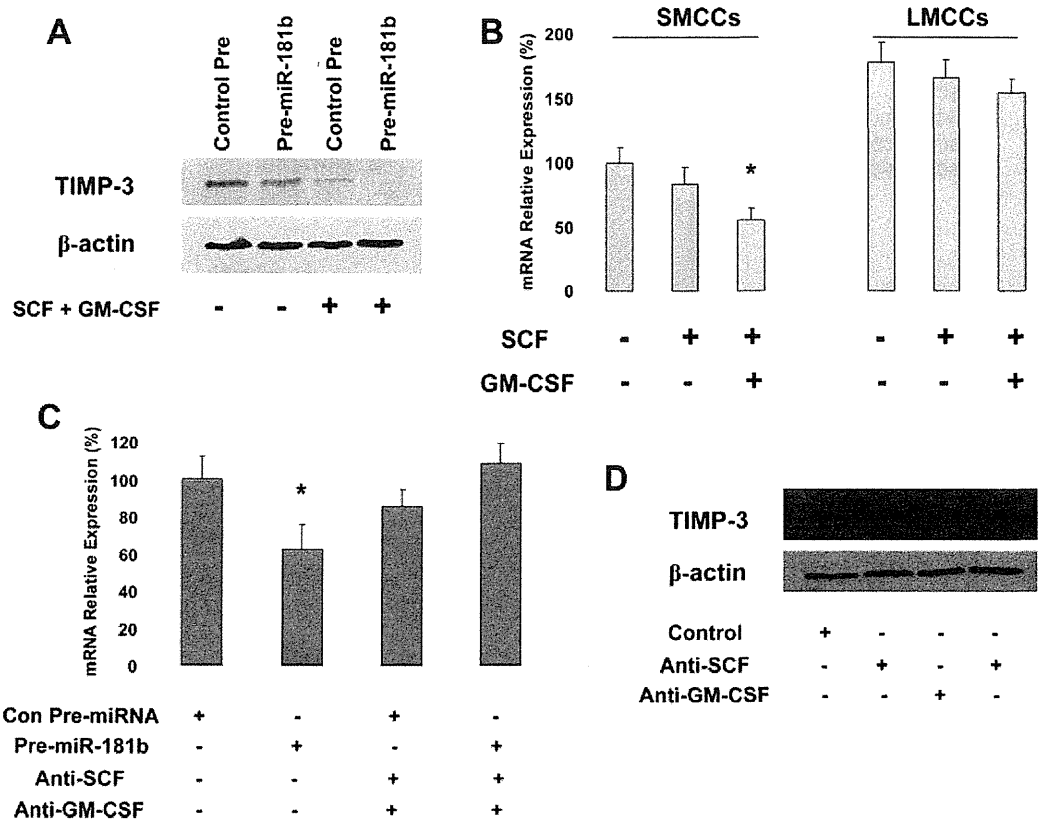

Fig. 6. SCF+GM-CSF facilitates miR-181b-induced TIMP-3 degradation. (A and C) SMCCs were transfected with control or miR-181b precursors with or without cotreatment with SCF+GM-CSF (10 ng/mL, 72 hours). (A) Western blotting with TIMP-3 antibody and β-actin was used as internal control, whereas (C) is real-time PCR analysis for TIMP-3 mRNA level, and GAPDH was used as internal control. (B) Total RNA from SMCCs and LMCCs treated with a different combination of SCF+GM-CSF indicated in the bottom were subjected to real-time PCR analyses for TIMP-3 mRNA level. GAPDH was used as internal control. mRNA expression values relative to control SMCCs group are shown (n = 6). (D) Western blotting using TIMP-3 antibodies in SMCCs treated with a different combination of SCF+GM-CSF for 72 hours, as indicated in the bottom. β-actin was used as the loading control. *$P < 0.05$, relative to the no treatment/control group.

mRNA were reduced in small cells with biliary markers and cotransfected with the miR-181b expression plasmid (pre-miR-181b); SCF+GM-CSF further reduced TIMP-3 protein (Fig. 6A). The level of TIMP-3 expression in small cells with biliary markers was lower than in LMCCs (Fig. 6B). Importantly, TIMP-3 was almost fully degraded in cells simultaneously treated with SCF+GM-CSF and transfected with miR-181b expression vectors; the ability of miR-181b targeting of TIMP-3 degradation was offset when SCF and GM-CSF were knocked down by specific antibodies (Fig. 6C). Levels of TIMP-3 protein in anti-SCF+GM-CSF-treated cells were also higher than in control small cells with biliary markers (Fig. 6C). Therefore, SCF+GM-CSF accelerates miR-181b-induced TIMP-3 protein degradation, whereas anti-SCF+GM-CSF counteracts this process. It is of note that TIMP-3 protein is almost absent when SCF+GM-CSF and miR-181b were treated/transfected simultaneously. These findings suggest that the level of TIMP-3 in SMCCs is regulated by SCF+GM-CSF and miR-181b.

Alteration of Matrix-Remodeling Enzymes: MMPs in Cholangiocytes After Treatments of SCF+GM-CSF. PH triggers the hepatocyte/proliferation of subpopulations sharing biliary epithelial markers/apoptosis and hepatic/biliary matrix remodeling, all of which are important events in the regenerating liver. MMPs are a family of zinc-containing neutral proteinases involved in matrix remodeling in both normal and pathophysiological processes. The onset of ECM production after PH correlates with a peak in the expression of TGF-β mRNA, which stimulates collagen synthesis. To confirm the functional relevance of SCF+GM-CSF-dependent modulation of regeneration, we assessed the expression of MMPs involved in cell remodeling. Treatment of small mouse cholangiocytes with SCF+GM-CSF significantly increased MMP-2

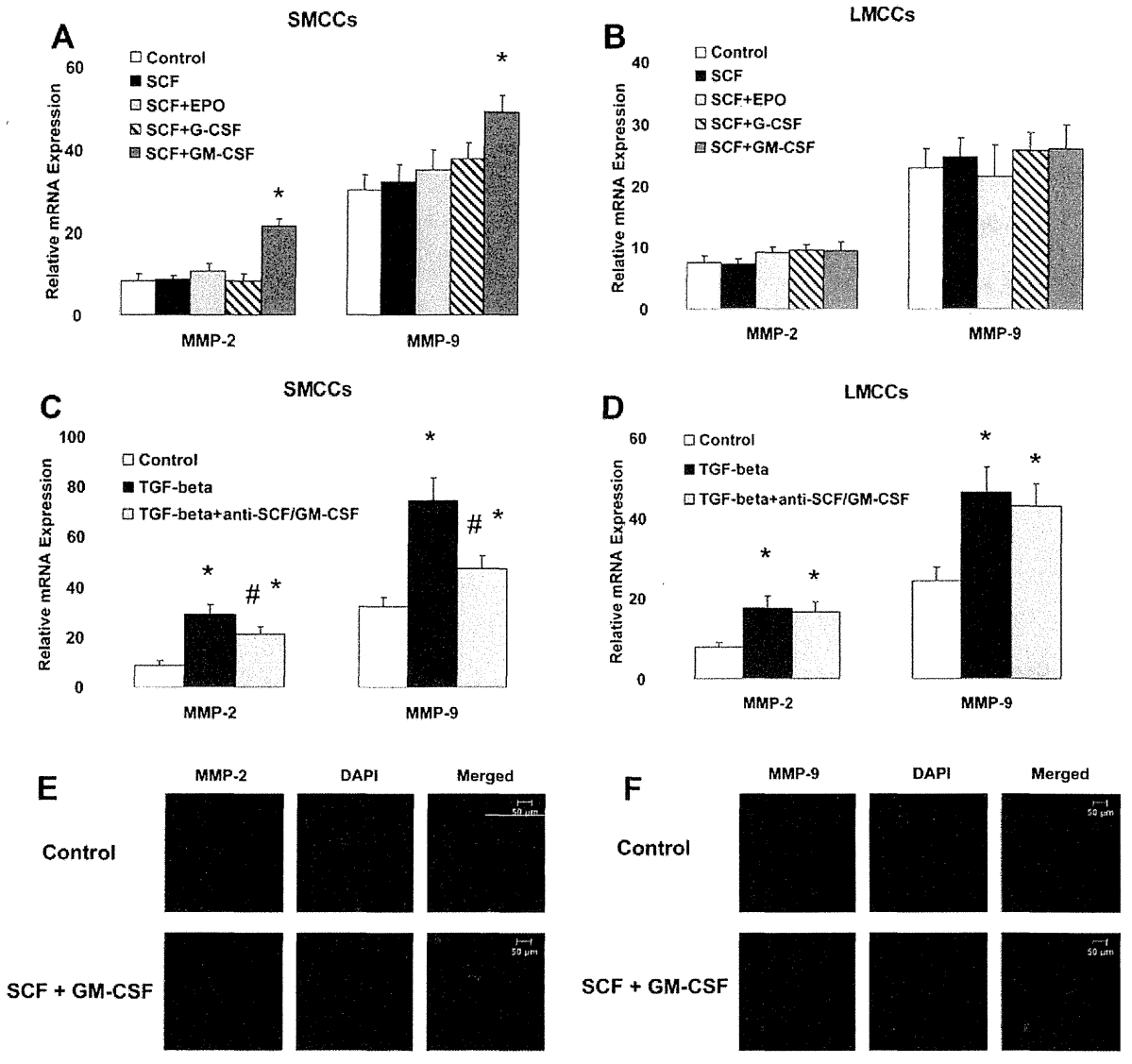

Fig. 7. SCF+GM-CSF modulate mRNA expression of MMPs. (A and B) LMCCs and SMCCs were treated with SCF and SCF plus EPO, G-CSF, GM-CSF, or controls. Quantitative real-time PCR was performed for MMP-2, MMP-9, and GAPDH mRNA expression. SCF+GM-CSF treatment increased MMP-9 mRNA expression in SMCCs. *$P < 0.05$, relative to SCF-only group. (C and D) SMCCs and LMCCs were incubated with 10 ng/mL of TGF-β with anti-SCF+GM-CSF antibodies (10 μg/L) or control antiserum for 72 hours, total RNA was isolated and quantitative real-time PCR for MMP-2 and MMP-9 was performed using a superarray quatitative PCR assay kit. Expression of MMPs was normalized to that of the GAPDH gene control. Inhibition of SCF+GM-CSF reduced both MMP-2 and MMP-9 mRNA expression in SMCCs, compared with controls. (E and F) Immunocytochemistry for MMP-2 (E) and MMP-9 (F) was performed in SMCCs. An increase in MMP-2 expression, along with the enhanced expression of MMP-9, was observed in SCF+GM-CSF-treated cholangiocytes. *$P < 0.05$; **$P < 0.01$, relative to control.

and MMP-9 mRNA expressions, when compared to SCF or GM-CSF alone. Compared with the controls, the expression of both MMP-2 and MMP-9 was increased in TGF-β-treated small cholangiocytes by 2.1- $\pm$ 0.4-fold and 3.6- $\pm$ 0.7-fold, respectively. Furthermore, the expression of both MMP-2 and MMP-9 were decreased after the addition of antibodies of SCF+GM-CSF in TGF-β treated small cells with biliary markers (Fig. 7). The up-regulations of MMP-2, MMP-9, as well as S100A4 were also observed in rat liver tissues as well as in IRCs 3 days after PH using the specific PCR Array kit from SABiosciences (#PAHS-033A; Frederick, MD) (Fig. 8A-C), along with the significant increase of miR-181b level and the reduced TIMP-3 expression by zymogen gel assay and real-time PCR analysis (Fig. 8D). Our findings provide evidence of a link between SCF+GM-CSF and the expression of mediators of cell remodeling in biliary cell subpopulations and lines. These data suggest that altered expression of SCF+GM-CSF after PH can

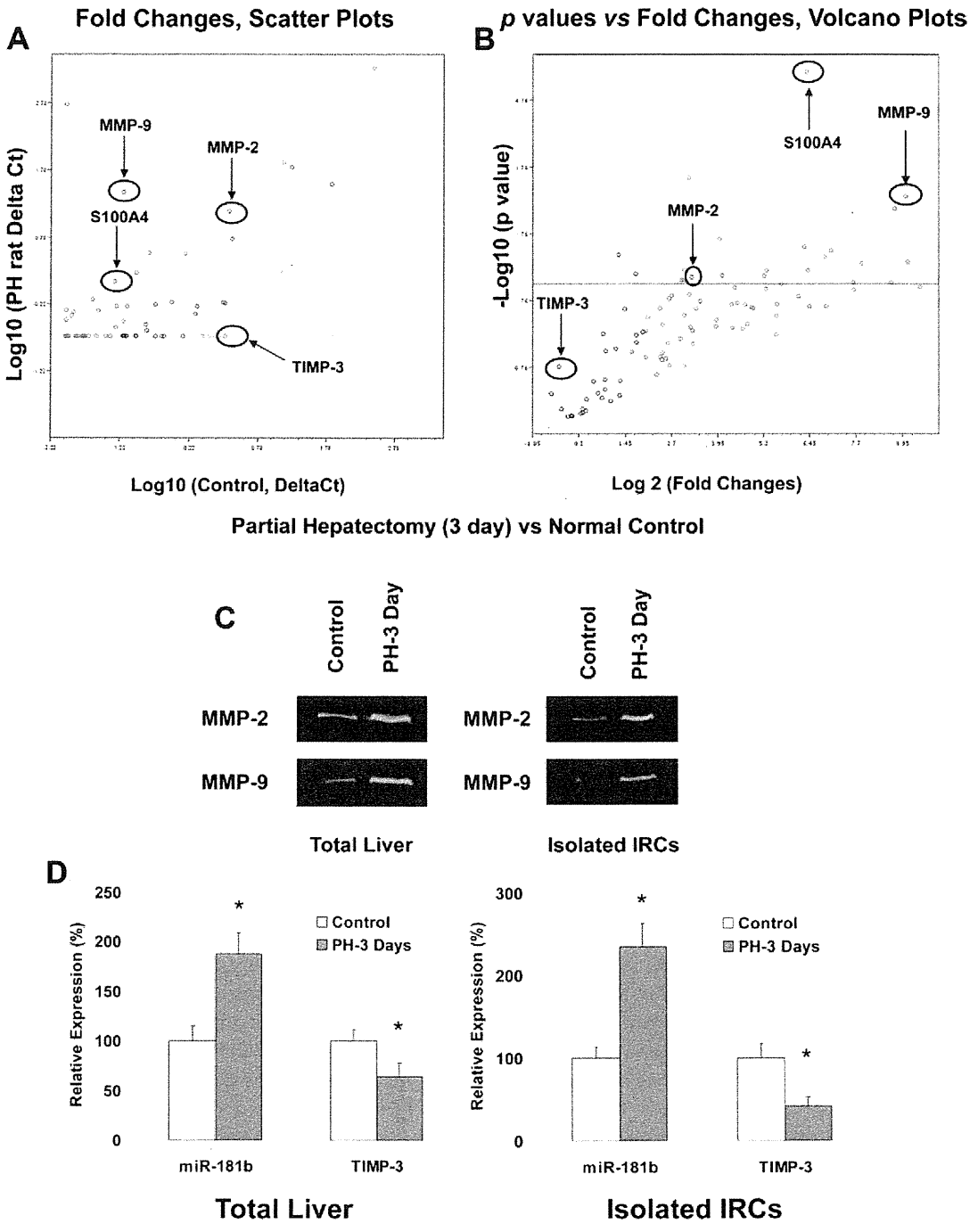

Fig. 8. Alterations of MMP-2, MMP-9, TIMP-3, miR-181b, and S100A4 in rat liver and isolated intrahepatic rat cholangiocytes after PH. Rats underwent 70% hepatectomy or sham control, and liver tissue was obtained for specific PCR array assay (PAHS-033A; SABiosciences, Frederick, MD) at 72 hours postoperatively. We choose this PCR array assay because this is the only 84-gene group PCR array, which includes S100A4 and other remodeling molecules. (A and B) Total RNAs from liver tissues/isolated IRCs of control and PH were characterized in technical triplicates, and the relative expression levels and *P* values for each gene in the related samples are plotted against each other in the scatter and volcano plots, depicting the relative expression levels (Log10) for selected genes in treated versus control panels (A) or in *P* values versus fold changes (B). Genes for which the difference in expression levels was greater than a factor of 3 and/or $P < 0.05$ are shown out of the cut-off lines on the graph. MMP-2, MMP-9 and S100A4 were among the top up-regulated genes in the PH group, when compared to sham control rats with $P < 0.05$. (C and D) Total RNAs from liver tissues/isolated IRCs of control and PH (3 days) were subjected to MMP zymogen gel assay (C) and real-time PCR assay (D), respectively. MMP-2, MMP-9, and miR-181b were significantly up-regulated, whereas TIMP-3 was reduced in total liver tissues as well as IRCs after PH (72 hours). $*P < 0.05$, relative to controls.

contribute to biliary remodeling by functional deregulation of activity of key signaling intermediates.

Discussion

Although a role for cholangiocytes in liver regeneration has been proposed, the molecular mechanisms by which cytokines modulate hepatobiliary remodeling and subsequently contribute to hepatic repair are unknown. We show that SCF+GM-CSF expressions are significantly increased after PH, compared to normal controls. Moreover, we demonstrate that the combination of SCF+GM-CSF stimulates specific cholangiocyte subpopulations in proliferation, remodeling, migration, and associated mesenchymal cell activation via TGF-β-dependent signaling and downstream effects involving the expression of miR-181b as well as matrix-remodeling enzymes TIMP-3, MMP-2, and MMP-9. The identification of SCF+GM-CSF as the important regulators of remodeling events *in vitro* emphasizes an essential synergistic role of these cytokines in mediating hepatic regeneration and repair and provides insight into the contribution of altered remodeling in recovering from severe liver injury.

Cholangiocytes and hepatocytes share embryologic origins. This common heritage contributes traits carried into adulthood. If specific cells exist within the adult liver with multipotential capacity, they should be able to differentiate into either hepatocytes or cholangiocytes in particular circumstances, such as when late-lineage-stage hepatocytes are lost or their regeneration is blocked. Plasticity between intrahepatic cholangiocytes and hepatocytes has been assumed,[17,31] suggesting that terminally differentiated cells of one lineage are able to directly differentiate into another lineage or undergo transdifferentiation.[32] However, the data are interpretable also as expansion and differentiation of a stem cell population.[4] The extent to which liver stem cells mediate liver regeneration has been hotly debated. One of the primary reasons for this controversy is the use of multiple definitions for the HSC.[33] Recent studies have demonstrated that meticulously isolated, rigorously characterized gallbladder epithelial cells cultured under defined *in vitro* conditions acquire hepatocyte-like properties, such as the ability to synthesize bile acids and take up low-density lipoprotein, without expression of oval cell or hematopoietic stem cell markers.[12] The recent discovery of biliary tree stem cell populations[34] again provides an alternative interpretation of the expansion and differentiation of stem cells, as opposed to transdifferentiation. Therefore, specific subpopulations of cells that express some of the known cholangiocyte markers can be hypothesized to contain a multipotent stem cell population when exposed to certain environmental conditions. Our studies have suggested that such cells could attain functional pluripotent characteristics after synergistic treatment of SCF+GM-CSFs, and subsequently, they could be used to repopulate damaged livers.

Inadequate liver regeneration is still an unsolved problem in major liver resection and living donor liver transplantation. The studies have implicated the usage of cytokines as the exogenous stimulators of liver regeneration in various animal models of liver resection and liver transplantation.[35] SCF and GM-CSF have been demonstrated to affect cellular differentiation and proliferation in various types of cells besides hepatobiliary epithelial cells. SCF enhances growth and differentiation when combined with other cytokines.[36] SCF and GM-CSF induce synergistic proliferation and differentiation in myeloid progenitor cells, including the megakaryoblastic cell line, MO7e.[37] Synergistic effects are of critical importance biologically, because hematopoietic stem cells and early progenitor cells require growth factors in combination for self-renewal and differentiation. Moreover, in the bone marrow microenvironment, the physiological actions of SCF occur in combination with other growth factors and ECM proteins. The combination of SCF+GM-CSF, promoting remodeling within the liver, supports the possibility of TGF-β-dependent mechanisms contributing to synergistic hepatic repair in response to SCF and GM-CSF.

Our current study indicates that there are relatively high constitutive SCF and GM-CSF levels within the liver during regeneration. SCF and GM-CSF are normally found as the transmembrane molecules under homeostatic conditions and are solubilized after inflammatory stimuli to induce the proper enzyme release to cleave it from the cell surface. This procedure may allow a substantial SCF and GM-CSF reservoir to be accumulated on the cell surface, ready for release upon the environmental condition changes. We hypothesize that solubilized SCF and GM-CSF released during liver regeneration could interact via their receptors with the surrounding specific subpopulations of cells with biliary marker populations, functioning as a remodeling agent within the damaged tissue. Although hepatobiliary homeostasis and regeneration likely involve multiple complex mechanisms and pathways, our current data strongly suggest that SCF and GM-CSF play significant synergistic roles in reestablishing the homeostasis of the biliary system.

The expression of downstream mediators of biliary remodeling could be modulated by SCF+GM-CSF.

Therapeutic strategies to increase SCF+GM-CSF may be potentially useful to rebuild the hepatobiliary system after liver injury. Knowledge of specific processes, such as biliary proliferation, migration, remodeling, and mesenchymal transition, that are regulated by CSFs, and the identification of critical targets for SCF and GM-CSF, provides novel insights into mechanisms in the development and remodeling of the intrahepatic biliary epithelium.

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Histamine stimulates the proliferation of small and large cholangiocytes by activation of both IP_3/Ca^{2+} and cAMP-dependent signaling mechanisms

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Although large cholangiocytes exert their functions by activation of cyclic adenosine 3′,5′-monophosphate (cAMP), Ca^{2+}-dependent signaling regulates the function of small cholangiocytes. Histamine interacts with four receptors, H1–H4HRs. H1HR acts by Gαq activating IP_3/Ca^{2+}, whereas H2HR activates $G\alpha_s$ stimulating cAMP. We hypothesize that histamine increases biliary growth by activating H1HR on small and H2HR on large cholangiocytes. The expression of H1–H4HRs was evaluated in liver sections, isolated and cultured (normal rat intrahepatic cholangiocyte culture (NRIC)) cholangiocytes. *In vivo*, normal rats were treated with histamine or H1–H4HR agonists for 1 week. We evaluated: (1) intrahepatic bile duct mass (IBDM); (2) the effects of histamine, H1HR or H2HR agonists on NRIC proliferation, IP_3 and cAMP levels and PKCα and protein kinase A (PKA) phosphorylation; and (3) PKCα silencing on H1HR-stimulated NRIC proliferation. Small and large cholangiocytes express H1–H4HRs. Histamine and the H1HR agonist increased small IBDM, whereas histamine and the H2HR agonist increased large IBDM. H1HR agonists stimulated IP_3 levels, as well as PKCα phosphorylation and NRIC proliferation, whereas H2HR agonists increased cAMP levels, as well as PKA phosphorylation and NRIC proliferation. The H1HR agonist did not increase proliferation in PKCα siRNA-transfected NRICs. The activation of differential signaling mechanisms targeting small and large cholangiocytes is important for repopulation of the biliary epithelium during pathologies affecting different-sized bile ducts.

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Cholangiocytes, which line intrahepatic and extrahepatic bile ducts,[1,2] modify canalicular bile while it is being delivered from the bile canaliculus to the small intestine.[3,4] Secretin stimulates secretion of ductal bile by interaction with secretin receptors (SRs)[1,3] that increases the synthesis of cyclic adenosine 3′,5′-monophosphate (cAMP), phosphorylation of protein kinase A (PKA), opening of cystic fibrosis transmembrane conductance regulator with subsequent activation of the Cl^-/HCO_3^- anion exchanger 2 (AE2), leading to enhanced bicarbonate secretion into bile.[1,3,5–7]

Cholangiocytes, which are constitutively mitotically dormant,[8,9] markedly proliferate or are damaged in human cholangiopathies[10] and in animal models of cholestasis, including bile duct ligation (BDL) and acute carbon tetrachloride (CCl_4) administration.[11] As cholangiocytes are the only cells expressing SR in the rodent liver,[12] changes in

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the expression of this receptor are key for functionally evaluating the degree of biliary growth/loss.[3,8,9,11,13,14] For example, treatment of normal rats with forskolin induces cholangiocyte hyperplasia and increases secretin-stimulated choleresis[13] that is similar to that seen in the BDL model.[3] In models of biliary damage (eg, after acute CCl_4 administration), there is loss of secretin-stimulated ductal secretion.[11] Models of biliary proliferation/loss are critically important to better understand the development of cholangiopathies, including primary biliary cirrhosis and primary sclerosing cholangitis. Two main signaling pathways are important for regulating cholangiocyte proliferation: the IP_3/Ca^{2+}- and the cAMP-dependent signaling pathways.[8,9,11,14–16] These two signaling pathways regulate both small (IP_3/Ca^{2+}) and large (cAMP) cholangiocyte growth/loss in rodent models of cholestasis.[8,9,11,14–16]

Histamine is derived from the conversion of histidine to histamine through the enzyme L-histidine decarboxylase,[17] and interacts with four G protein-coupled receptors: H1HR, H2HR, H3HR and H4HR.[18,19] Although endogenous histamine elicits a proliferative effect in different cell types,[20,21] the four histamine receptors (HRs) interact with independent G proteins to stimulate varying signaling pathways producing varying effects on cells.[22] The H1HR couples to $G\alpha_q$ mobilizing Ca^{2+}-dependent signaling in a number of cells including cholangiocytes,[15,22] whereas the H2HR mainly interacts with $G\alpha_s$ stimulating a cAMP-dependent pathway.[23] These two receptors stimulate the growth of numerous cell types, including cholangiocytes.[15,21] This is in contrast to H3 and H4HRs that have inhibitory effects on cell growth (including cholangiocytes)[16] by $G\alpha_i$ coupling that inhibits cAMP synthesis.[16] In our study, we tested the hypotheses that (1) histamine stimulates cholangiocyte proliferation of normal rats and (2) an H1HR agonist stimulates the proliferation of small cholangiocytes by activation of IP_3/Ca^{2+} signaling, whereas H2HR agonists increase the proliferation of large cholangiocytes by an intracellular mechanism requiring the activation of cAMP-dependent signaling.

MATERIALS AND METHODS

Materials

Reagents were purchased from Sigma Chemical (St Louis, MO), unless otherwise indicated. The HR agonists, histamine trifluoromethyl toluidide (HTMT dimaleate, H1HR agonist),[15] amthamine dihydrobromide (H2HR agonist)[24] and (R)-(α)-(-)-methylhistamine dihydrobromide (RAMH, a H3HR agonist)[16] were obtained from Tocris Bioscience (Ellisville, MO). The monoclonal mouse antibody against proliferating cellular nuclear antigen (PCNA, clone PC10, cat. no. M0879) was purchased from Dako (Kyoto, Japan). The substrate for γ-glutamyl transpeptidase (γ-GT), *N*-(γ-L-glutamyl)-4-methoxy-2-naphthylamide was purchased from Polysciences (Warrington, PA). Antibodies for HR subtypes were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and previously used in rat liver sections and in rat and mouse cholangiocytes.[15,16] In particular, H1HR (clone A-20, cat. no. sc-33970) is an affinity-purified goat polyclonal antibody raised against a peptide mapping within a cytoplasmic domain of H1HR of human origin. H2HR (clone H-70, cat. no. sc-50314) is a rabbit polyclonal antibody raised against amino acids 290–359 mapping within a C-terminal cytoplasmic domain of H2HR of human origin. H3HR (clone C-20, cat. no. sc-17921) is an affinity-purified goat polyclonal antibody raised against a peptide mapping near the C terminus of H3HR of human origin. H4HR (clone Q-20, cat. no. sc-33965) is an affinity-purified goat polyclonal antibody raised against a peptide mapping within a cytoplasmic domain of H4HR of human origin. The goat polyclonal affinity-purified phosphorylated-PKCα antibody (Ser 657, cat. no. sc-12356) was raised against a short amino-acid sequence containing phosphorylated Ser 657 of PKCα of human origin (Santa Cruz Biotechnology). PKCα (H-7, sc-8393) is a mouse monoclonal antibody specific for an epitope mapping between amino acids 645 and 672 at the C terminus of PKCα of human origin (Santa Cruz Biotechnology). The goat polyclonal affinity-purified pPKA IIα reg antibody (Ser 96, sc-12905-R) was raised against a short amino-acid sequence containing phosphorylated Ser 96 of p-PKA IIα reg of human origin (Santa Cruz Biotechnology). The PKAα cat affinity-purified rabbit polyclonal antibody (C-20, sc-903, Santa Cruz Biotechnology) against total PKA was raised against a peptide mapping at the C terminus of PKAα cat of human origin. The RNeasy mini kit to purify total RNA from cholangiocytes was purchased from Qiagen (Valencia, CA). The RIA kits for measurement of intracellular cAMP ([^{125}I] Biotrak Assay System, RPA509) and IP_3 (D-*myo*-inositol 1,4,5-trisphosphate (IP_3) [^{3}H] Biotrak Assay System, TRK1000) levels were purchased from GE Healthcare (Piscataway, NJ).

Experimental Models

Male Fisher rats (weighing 150–175 g) were purchased from Charles River (Wilmington, MA) and maintained in a temperature-controlled environment (20–22°C) with 12:12-h light–dark cycles. Animals were fed *ad libitum* standard chow and had free access to drinking water. Our studies were performed in normal rats treated by daily IP injections with vehicle (0.9% NaCl), histamine (0.5 mg/kg of BW),[25] HTMT dimaleate (H1HR agonist, 0.5 mg/kg of BW),[26] amthamine dihydrobromide (H2HR agonist, 0.5 mg/kg of BW),[27] RAMH (H3HR agonist, 10 mg/kg of BW)[16] or the H4HR agonist, clobenpropit (10 mg/kg of BW)[28] for 1 week. Before each experimental procedure, animals were injected with sodium pentobarbital (50 mg/kg weight, IP). Study protocols were performed in compliance with the institutional guidelines set forth by the Institutional Animal Care and Use Committee (Scott and White Texas A&M Health Science Center).

Purified Cholangiocytes and Normal Rat Intrahepatic Cholangiocyte Cultures

Virtually pure cholangiocytes were isolated by immunoaffinity separation[13,16,29] with a monoclonal antibody (a gift from Dr R Faris) against an unidentified antigen expressed by all intrahepatic rat cholangiocytes.[29] Cell count and viability (~97%) were measured by trypan blue exclusion. Purity (~99%) was assessed by histochemistry for γ-GT.[30] The *in vitro* experiments were performed in freshly isolated cholangiocytes and our polarized normal rat intrahepatic cholangiocyte culture (NRICs).[31] Cell number and frequency distribution of NRIC were measured using an automated cell counter (Cellometer Auto T4, Nexcelom Bioscience).[32]

Assessment of Histamine Receptor Expression

We evaluated the expression of HR subtypes by (1) immunohistochemistry[15,33] in normal liver sections; (2) immunofluorescence[15] in NRIC smears; and (3) RT-PCR[15] (1 μg total RNA) and immunoblots (10 μg protein) from freshly isolated normal cholangiocytes and NRICs. Immunohistochemistry in liver sections with the selected HR antibodies (1:100 dilution) was performed as described previously.[15,16] For all immunoreactions, negative controls (with pre-immune serum substituted for the primary antibody) were included. Light microscopy photographs of liver sections were taken by Leica Microsystems DM 4500 B Light Microscopy (Leica Microsystems, Weltzlar, Germany) using a Jenoptik Prog Res C10 Plus Videocam (Jena, Germany). Immunofluorescence for HR subtypes in NRICs was performed as described previously.[15] After staining, images were visualized using a confocal microscope (Olympus IX-71; Olympus, Tokyo, Japan). For all immunoreactions, appropriate negative controls were used.

Standard RT-PCR conditions were used for measurement of the mRNA expression for H1–H4R in pooled cholangiocytes (formed by small and large cholangiocytes)[31] and NRICs: 10 min at 95°C, 15 s at 95°C, 30 s at 55°C and 30 s at 72°C, 30 cycles at 4°C. Specific primers designed against the rat *H1HR* (NM_000861, expected fragment length 157 bp), *H2HR* (NM_022304, expected fragment length 181 bp), *H3HR* (NM_053506, expected fragment length 180 bp), *H4HR* (NM_131909, expected fragment length 181 bp) and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*, NM_002046, the housekeeping gene,[34] expected fragment length 175 bp) were purchased from (SABiosciences, Frederick, MD). We used RNA from the rat brain and water as positive and negative controls, respectively. The protein expression of H1–H4HR was measured by immunoblots in protein (10 μg) lysates from the brain (positive control) and isolated small and large normal cholangiocytes. Immunoblots were normalized by β-actin.[15,16]

In Vivo Studies Evaluation of the Intrahepatic Bile Duct Mass of Small, Large and Pooled Ducts

In the selected groups of animals, cholangiocyte growth was measured in liver sections (4 μm thick) by evaluation of the percentage of intrahepatic small, large and pooled bile ducts stained for cytokeratin-19 (CK-19, a specific marker of the biliary epithelium).[9,34] In each liver section, IBDM was measured as area occupied by CK-19-positive bile duct/total area × 100.[35] Sections were analyzed using a BX-51 light microscope (Olympus) with a video cam (Spot Insight; Diagnostic Instrument Inc., Sterling Heights, MI) and processed using an Image Analysis System (IAS: Delta Sistemi, Rome, Italy).

Cholangiocyte proliferation was also evaluated by immunoblots for PCNA[14] in protein (10 μg) lysates from the spleen (positive control) and isolated cholangiocytes from the selected experimental groups. Immunoblots were normalized by β-actin.[15,16] Band intensity was determined by scanning video densitometry using the phospho-imager, Storm 860 (GE Healthcare) and the ImageQuant TL software version 2003.02 (GE Healthcare, Little Chalfont, Buckinghamshire, England).

Effect of *In Vivo* Administration of Histamine on Basal- and Secretin-Stimulated Bile and Bicarbonate Secretion and cAMP levels

We measured the effect of chronic *in vivo* administration of histamine to normal rats on basal- and secretin-stimulated bile secretion (in bile fistula rats)[3,9] and cAMP levels (in purified cholangiocytes),[13,14,16] two functional parameters of cholangiocyte proliferation.[3,9,13,14] After anesthesia, rats were surgically prepared for bile collection as described previously.[3,9] When steady-state bile flow was reached (60–70 min from the intravenous infusion of Krebs–Ringer–Henseleit solution, KRH), rats were infused with secretin (100 nM) for 30 min, followed by intravenous infusion of KRH for 30 min. Bicarbonate concentration in bile was determined using an ABL 520 Blood Gas System (Radiometer Medical A/S, Copenhagen, Denmark).

After purification, cholangiocytes (1×10^5 cells) were incubated at 37°C for 1 h (to regenerate membrane proteins damaged by proteolytic enzymes during cell isolation)[5] and subsequently stimulated at room temperature for 5 min with 0.2% bovine serum albumin (BSA) or secretin (100 nM solution containing 0.2% BSA) before evaluation of cAMP levels by RIA.[13,14,16]

In Vitro Studies Evaluation of Cholangiocyte Proliferation and Signaling Mechanisms

Effects of histamine, H1HR or H2HR agonists (in the absence or presence of specific HR antagonists or inhibitors of IP_3/Ca^{2+} or cAMP signaling) on cholangiocyte proliferation and signaling mechanisms were evaluated in NRICs.[31] To evaluate dose and time dependency, NRICs were treated with 0.2% BSA (basal) or histamine (10, 50 and 100 μM) for 24 and 48 h before evaluating cell proliferation using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay (MTS)[15] (see above). In separate sets of experiments, NRICs were treated at 37°C for 24 h with: (1) 0.2% BSA; (2)

histamine (10 μM) in the absence or presence of pre-incubation with terfenadine (H1HR antagonist, 10 μM),[15] or cimetidine (H2HR antagonist, 10 μM)[36] or terfenadine + cimetidine (both at 10 μM + histamine, 10 μM); or (3) HTMT dimaleate (H1HR agonist, 10 μM),[15] amthamine dihydrobromide (H2HR agonist, 10 μM),[37] RAMH (H3HR agonist, 10 μM)[16] or clobenpropit (H4HR agonist, 10 μM)[37] before evaluation of proliferation by MTS assays.[15] To determine whether the stimulatory effects of histamine (the endogenous neurotransmitter) on NRIC proliferation are mediated by activation of H1 (mediated by Ca^{2+} signaling) and/or H2 (regulated by cAMP signaling) HRs, we stimulated NRICs at 37°C for 24 h with (1) 0.2% BSA; (2) histamine (10 μM); or (3) HTMT dimaleate (10 μM),[15] or amthamine dihydrobromide (10 μM)[37] in the absence or presence of pre-incubation with BAPTA/AM (5 μM),[15] Gö6976 (1 μM, Ca^{2+}-dependent PKCα inhibitor)[38] or Rp-cAMPs (100 μM, PKA inhibitor).[13]

Measurement of IP_3 and cAMP Levels and Phosphorylation of PKC and PKA

We performed experiments in NRICs aimed to demonstrate that HTMT dimaleate increases IP_3 levels and PKC phosphorylation, whereas amthamine dihydrobromide enhances cAMP levels and PKA phosphorylation. For evaluation of cAMP levels, NRICs were treated at room temperature for 5 min[5,13] with (1) 0.2% BSA or (2) HTMT dimaleate (10 μM)[15] or amthamine dihydrobromide (10 μM) before evaluation of cAMP levels by RIA.[5,13] For measurements of IP_3 levels, NRICs were treated at room temperature for 10 min with (1) 0.2% BSA or (2) HTMT dimaleate (10 μM)[15] or amthamine dihydrobromide (10 μM) before evaluation of IP_3 levels by RIA.[15]

Before evaluation of phosphorylation of PKC or PKA,[39] NRICs were treated at room temperature for 90 min with (1) 0.2% BSA or (2) HTMT dimaleate (10 μM)[15] or amthamine dihydrobromide (10 μM).[37]

Effect of PKCα Silencing on NRIC Proliferation

As (1) IP_3/Ca^{2+}-dependent PKCα has a key role in the regulation of cholangiocyte functions[14,39,40] and (2) HTMT dimaleate increases IP_3 levels and PKCα phosphorylation (see the 'Results' section), we evaluated the effect of PKCα silencing on NRIC proliferation after treatment of these cells with HTMT dimaleate. NRICs were plated into 6-well plates and allowed to adhere overnight. siRNA transfection (0.25–1 μg of PKCα siRNA was used) was performed according to the instructions provided by Santa Cruz Biotechnology. Diluted siRNA duplexes were added to the cells and allowed to incubate for 5 h at 37°C in a CO_2 incubator. For controls, a scrambled siRNA duplex (Santa Cruz Biotechnology) was added to corresponding wells. The extent of PKCα silencing was evaluated by measuring the protein expression of total PKCα in transfected *vs* control NRICs by immunoblots (see above).[15] PCNA protein expression was evaluated in NRIC cells treated with 0.2% BSA or HTMT dimaleate (10 μM) for 24 h in the absence or presence of PKCα siRNA (1.0 μg).

Statistical Analysis

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

RESULTS

Histamine Receptor Expression

By immunohistochemistry in liver sections, both small (yellow arrow) and large (red arrow) bile ducts express the four HR subtypes (H1–H4HRs) (Figure 1a). By immunofluorescence, NRICs (which are formed by small and large cholangiocytes as evaluated by frequency size distribution)[31] (Figure 1b) expressed the four HRs (Figure 1b). DAPI staining is seen in blue, whereas the red staining represents the specific HR. A merged image is also supplied along with negative staining (Figure 1b). By RT-PCR, purified pooled (containing small and large cells) cholangiocytes from normal rats and NRICs expressed *H1–H4HRs* (Figure 1c). In addition, by immunoblots, purified small and large normal cholangiocytes expressed H1–H4HRs (Figure 1c).

Evaluation of Intrahepatic Bile Duct Mass

Chronic administration of histamine and the H1HR agonist, HTMT dimaleate, increased small IBDM of normal rats compared with saline-treated rats (Figure 2a); the H2HR agonist, amthamine dihydrobromide, did not alter small IBDM (Figure 2a). Histamine and amthamine dihydrobromide (but not HTMT dimaleate) increased large IBDM of normal rats compared with control rats (Figure 2b). Histamine, the H1 and the H2HRs agonists increased overall IBDM (Figure 2a–c). No changes were observed when normal rats were treated with RAMH or clobenpropit (not shown). There was a significant increase in PCNA protein expression in freshly isolated pooled cholangiocytes from normal rats treated with histamine, H1HR or H2HR agonists compared with vehicle-treated rats (Figure 2d). These results rule out the potential role of the H3HR and H4HR agonists in activating cholangiocyte proliferation.

Effect of *In Vivo* Administration of Histamine on Basal- and Secretin-Stimulated Bile and Bicarbonate Secretion and cAMP Levels

Secretin increased intracellular cAMP levels in cholangiocytes from normal rats treated with saline (Figure 3). Chronic *in vivo* administration of histamine to normal rats increased both basal- and secretin-stimulated cAMP levels of cholangiocytes (a functional index of biliary growth)[3,11,14] compared with their corresponding values of purified cholangiocytes from saline-treated normal rats (Figure 3). As previously shown,[3] intravenous infusion of secretin did not

increase bile and bicarbonate secretion in normal rats (Table 1). Secretin increased bile and bicarbonate secretion in normal rats treated with histamine compared with normal rats treated with saline (Table 1), demonstrating that histamine stimulates biliary hyperplasia by activation of cAMP-dependent signaling.

In Vitro Studies Evaluation of Cholangiocyte Proliferation and Signaling Mechanisms

Dose- and time-response experiments demonstrated that histamine increases NRIC proliferation (by MTS assays) compared with BSA-treated NRICs (Figure 4). As the stimulatory effects of histamine on cholangiocyte proliferation were seen as early as after 24 h of incubation, all other stimulations were performed at 24 h at the histamine concentration of 10 μM, the smallest dose at which we observed a stimulatory effect on NRIC proliferation. Furthermore, we performed experiments aimed to demonstrate which HR subtypes regulate histamine-stimulated NRIC proliferation. Histamine, H1HR and H2HR (but not H3HR or H4HR) agonists increased NRIC proliferation (Figure 5a). Histamine stimulation of NRIC proliferation was blocked by terfenadine

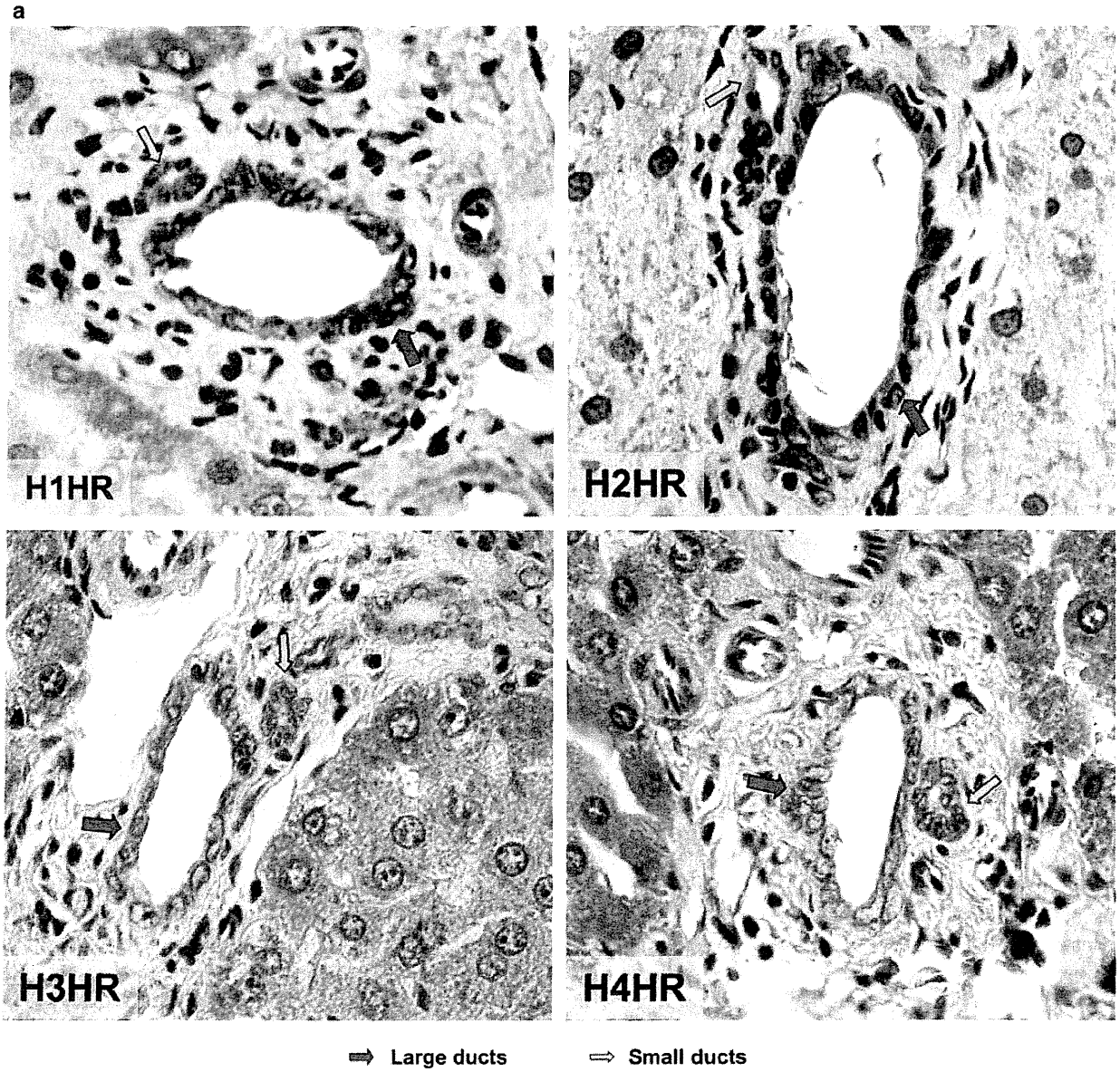


Figure 1 (**a**) By immunohistochemistry in liver sections, both small (yellow arrow) and large (red arrow) bile ducts express H1–H4HRs. Original magnification × 40. (**b**) Frequency size distribution shows that NRIC are formed by both small and large NRICs. NRIC by immunofluorescence (bar = 20 μm); DAPI staining is seen in blue, whereas the red staining represents the specific histamine receptor. A merged image is shown along with negative staining. (**c**) By RT-PCR, purified pooled cholangiocytes from normal rats and NRICs expressed *H1–H4HRs*. By immunoblots, purified small and large normal cholangiocytes expressed H1–H4HRs.

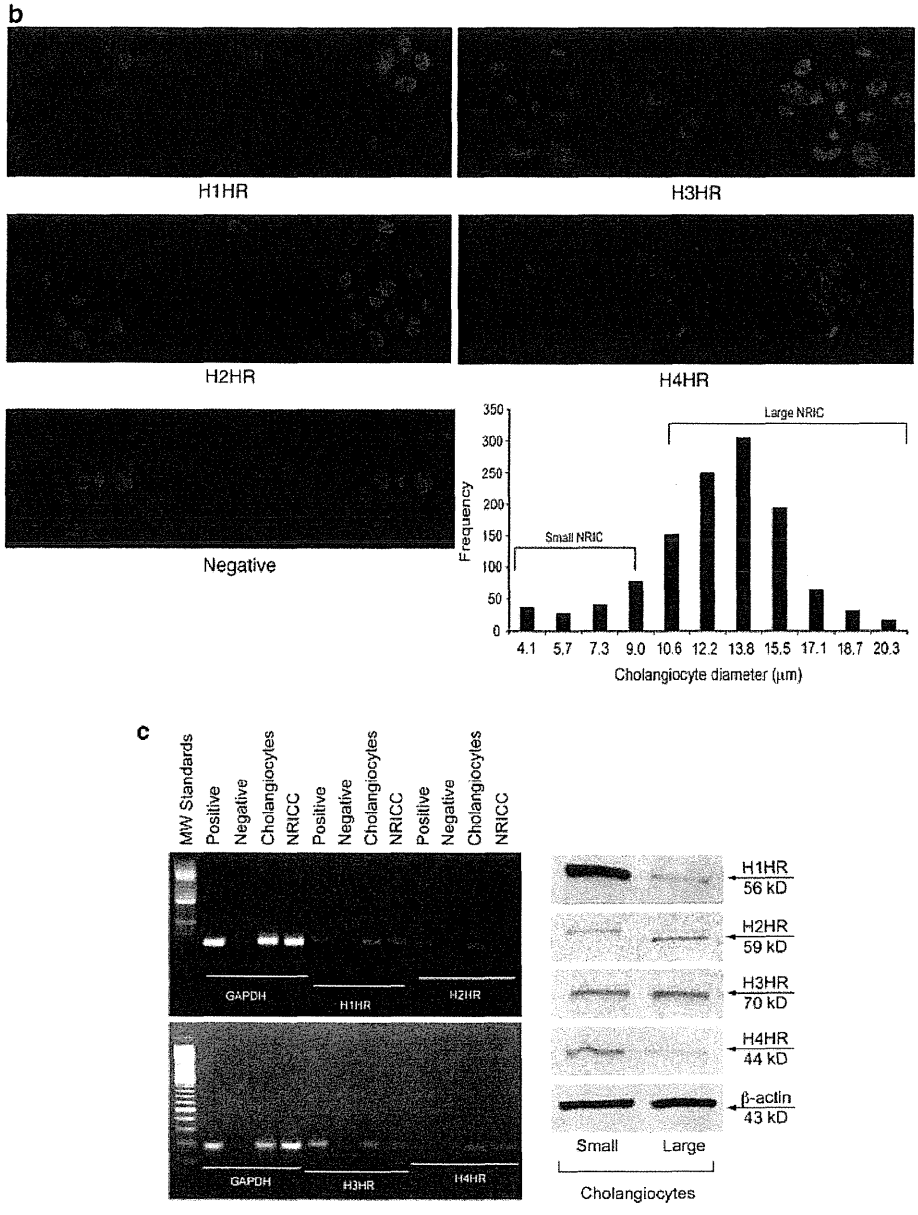


Figure 1 Continued.

(H1HR antagonist), cimetidine (H2HR antagonist) and at the same extent by terfenadine + cimetidine (Figure 5a), demonstrating that both H1HR (by activation of Ca^{2+} signaling; see Figure 6) and H2HR (by activation of cAMP signaling; see Figure 6) mediate histamine-induced increase in NRIC proliferation (Figure 5a). Furthermore, we demonstrated that (1) H1HR and H2HR (in addition to histamine) agonists stimulate NRIC proliferation (Figure 5b and c); (2) H1HR-induced NRIC proliferation was blocked by BAPTA/AM and Gö6976 but not by Rp-cAMPs (Figure 5b); and (3) H2HR-induced NRIC proliferation was blocked by Rp-cAMPs but not BAPTA/AM and Gö6976 (Figure 5c).

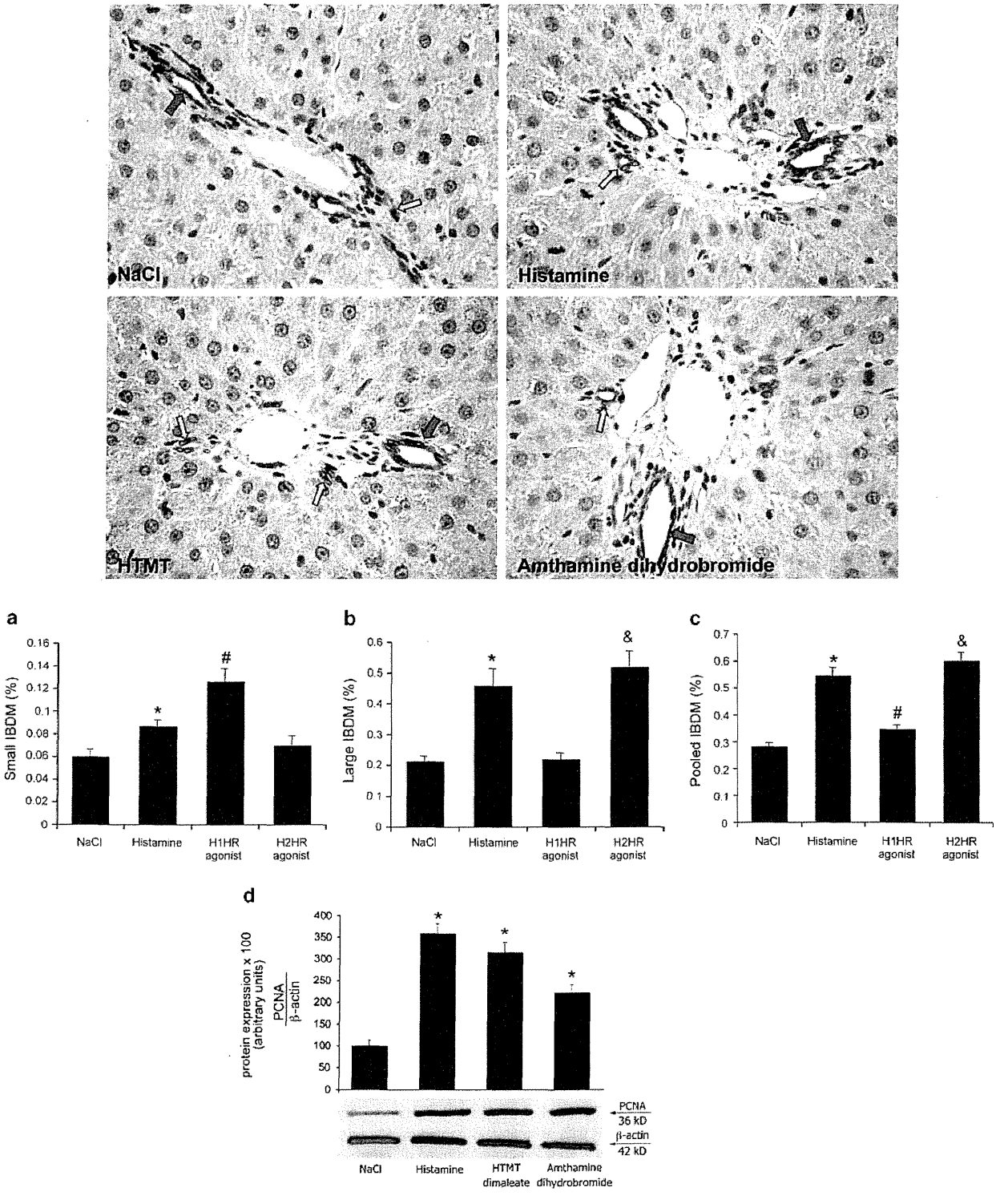
Measurement of IP_3 and cAMP Levels and PKC and PKA

We demonstrated that HTMT dimaleate (H1HR agonist) increases IP_3 levels (Figure 6a) and PKC phosphorylation (Figure 6c), whereas amthamine dihydrobromide (H2HR agonist) enhances cAMP levels (Figure 6b) and PKA phosphorylation (Figure 6d). HTMT dimaleate did not alter cAMP levels of NRIC, whereas amthamine dihydrobromide did not change IP_3 levels of NRIC (Figure 6a and b).

Effect of PKCα Silencing on NRIC Proliferation

With the finding that the H1HR agonist increased phosphorylation of PKCα, we sought to pinpoint the role of

Ca^{2+}-dependent PKCα (that has a key role in biliary growth)[14,39–44] on NRICs (after stimulation with HTMT dimaleate) using siRNA transfection. In PKCα-siRNA (1.0 μg)-transfected cells (90% knockdown efficiency, Figure 7a), HTMT dimaleate failed to increase NRIC proliferation (Figure 7b). As expected, in scrambled-transfected NRICs, HTMT dimaleate increased NRIC proliferation (Figure 7b).



DISCUSSION

We have demonstrated that intrahepatic bile ducts, freshly isolated cholangiocytes and NRICs express H1–H4HRs. We have shown that histamine increases normal cholangiocyte growth by interaction with H1HR and H2HR by activation of both IP_3/Ca^{2+}- and cAMP-dependent mechanisms, respectively. Furthermore, the H1HR agonist increases NRIC proliferation by activation of IP_3/Ca^{2+}/PKCα-dependent signaling likely targeting small Ca^{2+}-dependent NRIC. When PKCα was silenced, the H1HR agonist did not increase NRIC proliferation. In contrast, the H2HR (that signals through activation of $G\alpha_s$)[23] induces normal cholangiocyte growth by activation of a cAMP/PKA-dependent mechanism (independent of PKCα) by likely targeting large cAMP-dependent NRICs.

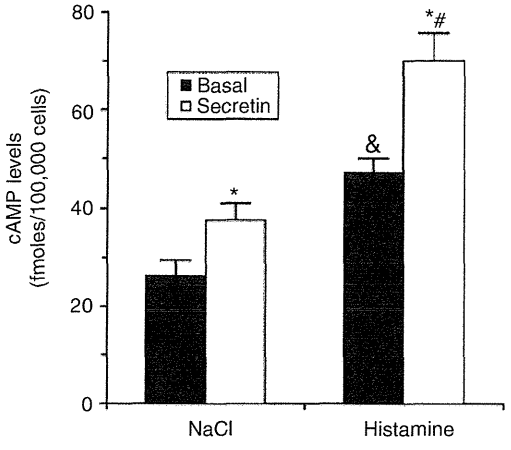


Figure 3 Evaluation of basal- and secretin-stimulated cAMP levels in purified cholangiocytes from normal rats treated with saline or histamine for 1 week. Histamine increased both basal- and secretin-stimulated cAMP of normal cholangiocytes compared with cholangiocytes from normal rats treated with saline. $^{\&}P<0.05$ *vs* basal cAMP levels of cholangiocytes from normal rats treated with saline. $^{*}P<0.05$ *vs* basal cAMP levels of cholangiocytes from saline-treated normal rats. $^{\#}P<0.05$ *vs* secretin-stimulated cAMP levels of cholangiocytes from saline-treated normal rats. Data are mean ± s.e.m. of six experiments.

The finding that normal rat cholangiocytes and NRICs express H1–H4HRs are supported by previous studies in normal mouse[15] and BDL rat cholangiocytes.[16] Moreover, we have previously demonstrated the expression of H3HR in hyperplastic cholangiocytes and cholangiocarcinoma cells, and defined the inhibitory role of H3HR in the growth of hyperplastic and neoplastic cholangiocytes.[16,45] HRs are also expressed in the liver by hepatocytes that are activated by endogenous histamine to release a wide array of effects.[46,47] For example, histamine activates IP_3 synthesis in guinea pig hepatocytes by activation of H1HR[47] and stimulates glycogenolysis in hepatocytes by interaction with H1HR by activating the Ca^{2+} messenger system.[46]

In animal models of cholangiocyte hyperplasia/loss, both IP_3/Ca^{2+}- and cAMP-dependent signaling pathways regulate the proliferative activities of small and large cholangiocytes, respectively.[3,8,9,16] For example, in the cholestatic BDL model, there is marked proliferation of large cholangiocytes by activation of cAMP signaling.[3,8,16] With regard to IP_3/Ca^{2+}-dependent signaling, stimulation of small cholangiocyte growth (eg, after feeding of the bile salt, taurocholate)

Table 1 Measurement of basal- and secretin-stimulated bile flow, bicarbonate concentration and secretion in normal rats treated with saline or histamine for 1 week

Treatment	Bile flow		Bicarbonate secretion	
	Basal (ml/min per kg BW)	Secretin (ml/min per kg BW)	Basal (mEquiv./min per kg BW)	Secretin (mEquiv./min per kg BW)
Normal rats+NaCl ($n=7$)	68.3 ± 6.2	73.6 ± 7.0	2.3 ± 0.2	2.5 ± 0.2
Normal rats+histamine ($n=7$)	74.5 ± 4.8	90.5 ± 5.2*	2.4 ± 0.2	3.1 ± 0.15*

BW, body weight.

When steady spontaneous bile flow was reached (60–70 min from the infusion of Krebs–Ringer–Henseleit (KRH), rats were infused for 30 min with secretin, followed by a final infusion of KRH for 30 min. Data are mean ± s.e.m. $^{*}P<0.05$ (by Student's unpaired *t*-test) *vs* the corresponding basal value of bile flow or bicarbonate secretion of normal rats treated with histamine for 1 week.

Figure 2 (**a–c**) Effect of chronic administration of saline, histamine, H1HR or H2HR agonists on small, large and pooled IBDM of normal rats. (**a**) Administration of histamine and the H1HR agonist, HTMT dimaleate increased small IBDM of normal rats compared with saline-treated rats; the H2HR agonist, amthamine dihydrobromide, did not alter small IBDM. (**b**) Histamine and amthamine dihydrobromide (but not HTMT dimaleate) increased large IBDM of normal rats compared with control rats. (**c**) The IBDM of pooled bile ducts was increased by histamine and both H1 and H2HR agonists. $^{*}P<0.05$ *vs* small, large and pooled IBDM of normal rats. $^{\#}P<0.05$ *vs* small and pooled IBDM of normal rats. $^{\&}P<0.05$ *vs* large and pooled IBDM of normal rats. Data are mean ± s.e.m. Ten randomly selected portal tracts were evaluated in three different slides. $^{*}P<0.05$ *vs* IBDM of normal rats treated with saline. (**d**) There was a significant increase in PCNA protein expression in freshly isolated pooled cholangiocytes from normal rats treated with histamine, H1HR or H2HR compared with vehicle-treated rats. $^{*}P<0.05$ *vs* PCNA of cholangiocytes from normal rats treated with saline. Data are mean ± s.e.m. of four blots from several different and cumulative preparations of cholangiocytes.

depends on the activation of the Ca^{2+}-dependent PKCα.[41] Moreover, activation of α1-adrenergic receptors stimulate the proliferation of small mouse cholangiocytes through Ca^{2+}-dependent activation of NFAT2 and Sp1.[48] In this study, we demonstrate that histamine stimulates small and large biliary growth by the differential activation of both Ca^{2+} and cAMP signaling. One possible explanation for the differential effects induced by histamine is likely due to the interaction with specific HRs expressed by different-sized cholangiocytes, ie, small and large. Indeed, we have shown that activation of H1HR increases the proliferation of small cholangiocytes by activation of IP_3/Ca^{2+}-CaMK I signaling.[15] In addition, activation of H3HR causes decreased large cholangiocyte hyperplasia by downregulation of cAMP-dependent signaling.[16] Based on this, we propose that histamine-induced increases in the proliferation of both small and large cholangiocytes are mediated by activation of IP_3/Ca^{2+} signaling in small cholangiocytes and NRICs (by likely interaction with H1HR) and cAMP transduction pathways in large cholangiocytes and NRICs (presumably mediated by H2HR). On the basis of this rationale and these previous findings, we also propose that the increase in secretin-stimulated choleresis (observed after *in vivo* administration of histamine to normal rats) likely depends on activation of H2HR in large cholangiocytes, the only biliary cell type in the liver that functions by a cAMP-dependent mechanism.[1,6,8,11]

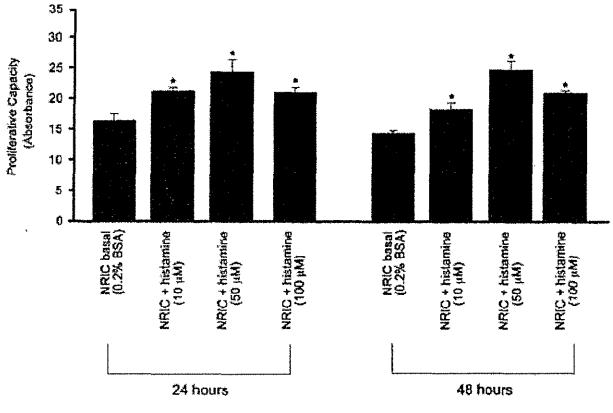


Figure 4 Dose- and time-response experiments demonstrated that histamine (at 10, 50 and 100 μM) increase NRIC proliferation (by MTS) compared with NRIC treated with BSA. *$P<0.05$ *vs* proliferation of NRIC treated *in vitro* with 0.2% BSA (basal). Data are mean ± s.e.m. of seven experiments.

We next performed *in vitro* studies in polarized NRICs (which contain both small and large cholangiocytes, Figure 1b) and demonstrated that histamine activation of NRIC proliferation is blocked by terfenadine (H1HR antagonist), cimetidine (H2HR antagonist) and at the same extent by simultaneous treatment with terfenadine + cimetidine. A shortcoming of this *in vitro* experiment is represented by the fact that both H1HR and H2HR antagonists block completely the stimulatory effects of histamine on NRIC proliferation, although one of the two receptors (H1HR or H2HH) are still active during treatment with these two inhibitors. We provide this explanation supported by our previous findings.[14,39,42,44,49,50] As we have previously demonstrated that cross-talk between Ca^{2+}- and cAMP-dependent signaling coordinately mediates biliary secretion and growth,[14,39,42,44,49,50] we propose that blockage of Ca^{2+} (by the H1HR antagonist) and cAMP (by the H2HR antagonist) signaling may reciprocally inhibit cAMP and Ca^{2+} signaling, respectively. This explanation is also supported by the fact that the inhibitory effects of terfenadine and cimetidine (when administered together) on NRIC proliferation were not additive.

We have also shown that (1) histamine stimulation of NRIC proliferation is dependent on the activation of both the IP_3/Ca^{2+} and the cAMP signaling pathways (blockage by both Ca^{2+} chelators and specific PKC and PKA inhibitors); (2) H1HR stimulation of small cholangiocyte growth

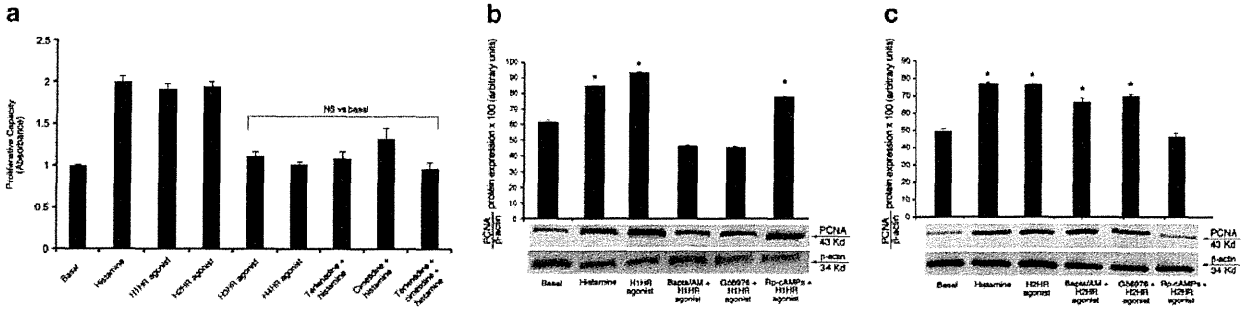


Figure 5 (**a**) Histamine, H1HR and H2HR (but not H3HR or H4HR) agonists increased NRIC proliferation. Histamine stimulation of NRIC proliferation was partly blocked by terfenadine and cimetidine. *$P<0.05$ *vs* proliferation of NRIC treated *in vitro* with 0.2% BSA (basal). Data are mean ± s.e.m. of seven experiments. H1HR agonist = HTMT dimaleate; H2HR agonist = amthamine dihydrobromide; H3HR agonist = RAMH; H4HR = clobenpropit. (**b** and **c**) By PCNA immunoblots, we demonstrated that: (**b** and **c**) H1HR and H2HR agonists (in addition to histamine) stimulate NRIC proliferation; (**b**) H1HR-induced NRIC proliferation is blocked by BAPTA/AM and Gö6976 but not Rp-cAMPs; and (**c**) H2HR-induced NRIC proliferation is blocked by Rp-cAMPs but not BAPTA/AM and Gö6976. *$P<0.05$ *vs* proliferation of NRIC treated *in vitro* with 0.2% BSA (basal). Data are mean ± s.e.m. of six experiments. H1HR agonist = HTMT dimaleate; H2HR agonist = amthamine dihydrobromide.

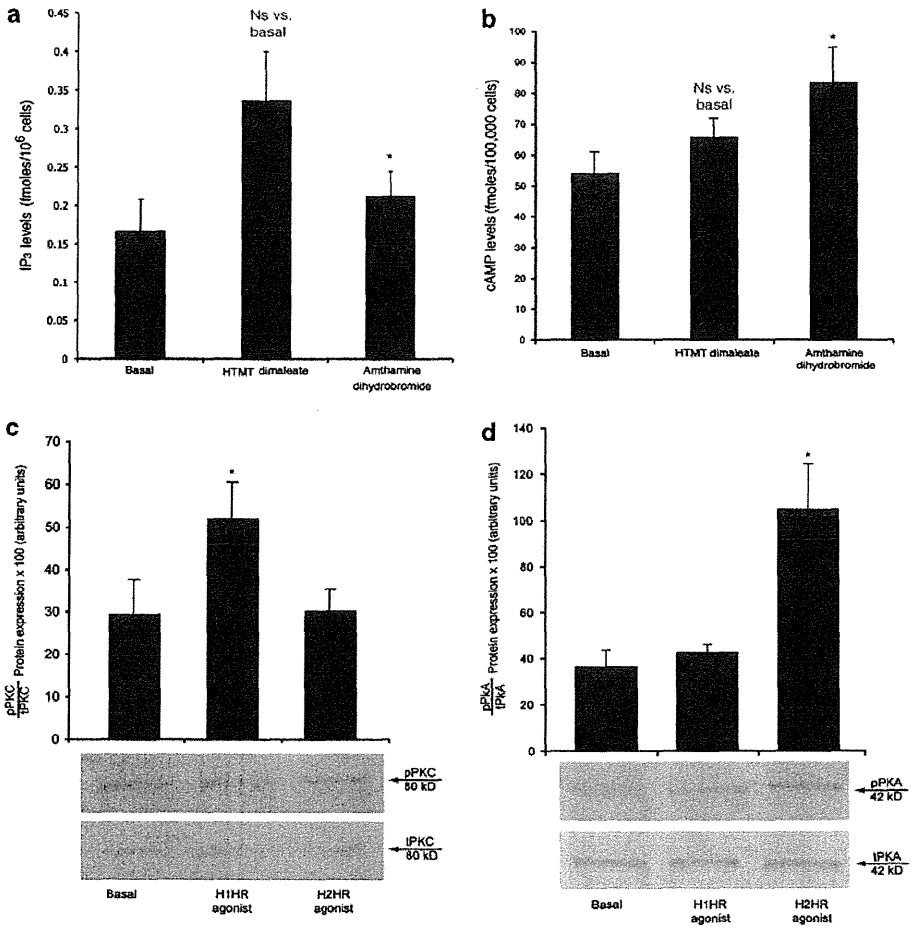


Figure 6 Effect of H1HR and H2HR agonists on (**a**) IP_3 levels and (**b**) cAMP levels, (**c**) PKC phosphorylation and (**d**) PKA phosphorylation in NRIC. We demonstrated that HTMT dimaleate (a H1HR agonist) increases IP_3 levels (**a**) and PKC (panel **c**) phosphorylation, whereas amthamine dihydrobromide (a H2HR agonist) enhances cAMP levels (**b**) and PKA phosphorylation (**d**). $^*P<0.05$ *vs* proliferation of NRIC treated *in vitro* with 0.2% BSA (basal). Data are mean ± s.e.m. of six experiments.

depends on the activation of $IP_3/Ca^{2+}/PKC\alpha$ signaling (proliferation blocked by Ca^{2+} chelators and PKC but not by PKA inhibitors); and (3) H2HR stimulation of large NRIC proliferation is mediated by cAMP-dependent PKA signaling (blockage by only PKA inhibitors). Further confirmation of differential signaling was found with evaluation of intracellular cAMP and IP_3 levels, as well as PKCα and PKA phosphorylation. H1HR stimulation activates IP_3 levels and PKCα phosphorylation, whereas H2HR increases cAMP levels and PKA phosphorylation. The importance of signaling through differential pathways is important because in cholangiopathies, there is proliferation/damage of only specific bile ducts of different sizes.[8,10,11,13,15,34] Cholangiopathies target both small and large cholangiocytes,[8,10,11,34] and, thus, it may be important for histamine to have the ability to modulate cholangiocyte proliferation and/or loss through the activation of HRs that will induce stimulatory (H1 and H2H) and inhibitory (H3 and H4HR) effects on biliary growth.

After finding that H1HR activates Ca^{2+}-dependent PKCα and knowing that PKCα has a critical role in cholangiocyte function,[14,39,41,42,50] we knocked down PKCα expression and evaluated the effect of HTMT dimaleate on NRIC proliferation. As no commercially kits are available for silencing PKA, we could not evaluate the role of PKA silencing on H2HR-induced large NRIC proliferation. Similar to what we have previously shown[45] in cholangiocarcinoma cells using the H3 agonist, RAMH, we found here that the reduction of PKCα in NRICs ablates the stimulatory effects of HTMT dimaleate. PKCα has a critical role in the function of numerous cells and the loss of PKCα signaling can have a negative or positive impact on the proliferation of a number of epithelial including cholangiocytes.[14,39–41,45,50] For example, PKCα has a stimulatory role in wound healing of human corneal epithelial cells after hepatocyte growth factor stimulation.[51] In cancer, PKCα has a stimulatory role enhancing the growth of certain cancers,[52] whereas the activation of PKCα

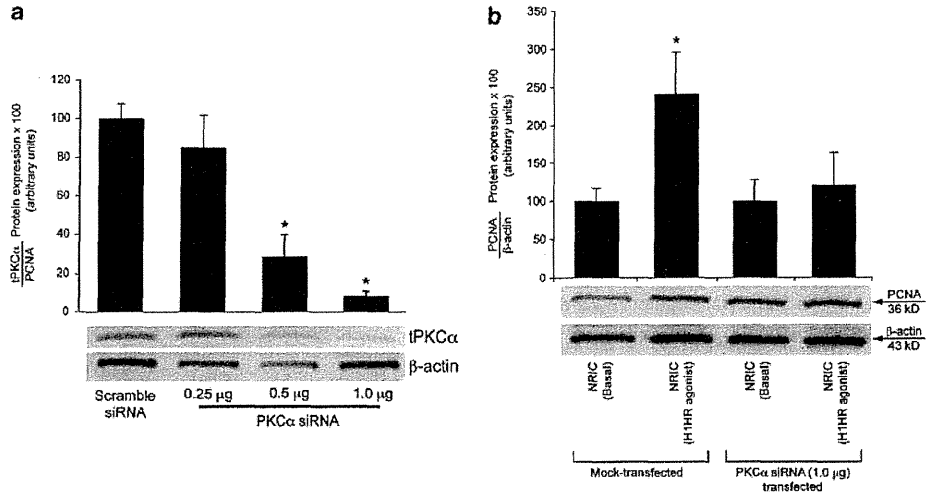


Figure 7 (**a**, **b**) Effect of (**a**) PKCα silencing on the (**b**) proliferation (by PCNA immunoblots) of NRIC after treatment with 0.2% BSA (basal) or HTMT dimaleate. In scrambled-transfected NRIC, HTMT dimaleate increased NRIC proliferation. In PKCα-siRNA (1.0 μg) transfected cells (90% silenced efficiency), HTMT dimaleate failed to increase NRIC proliferation. $^*P<0.05$ *vs* proliferation of NRIC treated *in vitro* with 0.2% BSA (basal). Data are mean ± s.e.m. of four experiments. H1HR agonist = HTMT dimaleate.

modulates the inhibition of cholangiocarcinoma growth.[42,45] Further studies are necessary to understand why activation of PKCα in biliary cells induces either inhibition or activation of hyperplastic and neoplastic biliary growth and secretin-stimulated ductal secretion (a functional index of biliary hyperplasia).[14,39,41–43,50] One potential explanation for the differential effects of PKCα on biliary growth may depend on the type of receptor (eg, M3 acetylcholine, insulin, gastrin or α1-adrenergic) or transporter (eg, Na^+-dependent apical bile acid transporter) that are upregulated or downregulated after vagotomy, the treatment with the selected gastrointestinal hormone or bile salt feeding.[14,39,41–43,50] A possible shortcoming of our study is that PKCα may be not the only factor as other Ca^{2+}-dependent (βI, βII and γ) and Ca^{2+}-independent (δ, ε, θ, η and ζ) PKC isoforms may be involved in H1HR stimulation of biliary growth.

Taken together, these findings implicate histamine and HRs as key factors in the regulation of normal cholangiocyte proliferation and function. Although endogenous histamine may activate numerous signaling mediators and pathways, it may be more important that stimulation of specific HRs is able to induce specific effects in target cells (possibly in small and large subpopulations of cholangiocytes) using different signaling pathways. In clinical situations in which certain subpopulations of cells are affected, the usage of specific agonists may help in the development of therapeutic treatments. For example, in a cholestatic state in which inflamed cholangiocytes are overproliferating, the usage of the H3HR agonist RAMH might aid in patient therapy to help decrease cholangiocyte proliferation. On the other hand, in a case of damaged or cholangiocyte loss, treatment with either an H1HR or H2HR agonist may increase proliferation when warranted and necessary for recovery.

As histamine is released by mast cells,[53] further studies are warranted to evaluate how mast cells may be contributing to cholangiocyte proliferation, a topic that is a topic of future work in our laboratory. In support of this novel topic, it has been demonstrated that during cholestasis, mast cell quantities increase.[53] Increased mast cell quantity would likely lead to increased histamine release into the microenvironment, thus modulating biliary functions. Furthermore, ongoing studies from our laboratory aim to demonstrate that bile acids (that have a key role in the regulation of biliary functions and stimulate histamine secretion from mast cells)[40,41,44,50,54] affect cholangiocyte growth by releasing histamine from proliferating mast cells.

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DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

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