GASTROINTESTINAL, HEPATOBILIARY, AND PANCREATIC PATHOLOGY

The Neuropeptide Galanin Is Up-Regulated during Cholestasis and Contributes to Cholangiocyte Proliferation

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During the course of cholestatic liver diseases, mitotically dormant cholangiocytes proliferate and subsequently acquire a neuroendocrine phenotype. Galanin is a neuroendocrine factor responsible for regulation of physiological responses, such as feeding behavior and mood, and has been implicated in the development of fatty liver disease, although its role in biliary hyperplasia is unknown. Biliary hyperplasia was induced in rats via bile duct ligation (BDL) surgery, and galanin was increased in serum and liver homogenates from BDL rats. Treatment of sham and BDL rats with recombinant galanin increased cholangiocyte proliferation and intrahepatic biliary mass, liver damage, and inflammation, whereas blocking galanin expression with specific vivo-morpholino sequences inhibited hyperplastic cholangiocyte proliferation, liver damage, inflammation, and subsequent fibrosis. The proliferative effects of galanin were via activation of galanin receptor 1 expressed specifically on cholangiocytes and were associated with an activation of extracellular signal—regulated kinase 1/2, and ribosomal S6 kinase 1 signal transduction pathways and subsequent increase in cAMP responsive element binding protein DNA-binding activity and induction of Yes-associated protein expression. Strategies to inhibit extracellular signal—regulated kinase 1/2, ribosomal S6 kinase 1, or cAMP responsive element binding protein DNA-binding activity prevented the proliferative effects of galanin. Taken together, these data suggest that targeting galanin signaling may be effective for the maintenance of biliary mass during cholestatic liver diseases. *(Am J Pathol 2017, 187: 819—830; http://dx.doi.org/10.1016/j.ajpath.2016.12.015)*

The intrahepatic biliary tree is a network of interconnected ducts lined by cholangocytes,[1,2] which play a key role in determining the final composition of the bile reaching the duodenum.[1,3–6] Cholangiocytes are usually mitotically dormant but possess marked proliferative capacity[2,3] during experimental conditions, such as cholestasis induced by bile duct ligation (BDL)[3] and in human chronic cholestatic liver diseases.[2] In experimental models of cholestasis, subsequent to cholangiocyte proliferation are increased liver damage, expression of proinflammatory cytokines, and fibrosis.[7,8] A large number of endogenous signaling molecules have been shown to regulate cholangiocyte proliferation,[9–12] although the specific events triggering this process are not clear. Therefore, studies that dissect the mechanisms by which cholangiocytes proliferate are important and may reveal novel therapeutic targets for conditions of ductopenia.

Many different signal transduction pathways can regulate cholangiocyte proliferation. One common downstream target of these signaling pathways is the phosphorylation and activation of the extracellular signal—regulated kinase 1/2 (ERK1/2). Specifically, agents that activate ERK1/2 lead to cholangiocyte proliferation,[13,14] whereas agents that inhibit ERK1/2 phosphorylation are antiproliferative.[10,15] ERK1/2 has been shown to lead to the phosphorylation of

ribosomal S6 kinase 1 (RSK1) in several experimental models.[16] Indeed, we have previously shown that RSK1 is a downstream target of ERK1/2 in cholangiocarcinoma cells in response to IL-6 signaling,[17] although its role in hyperplastic cholangiocyte proliferation is unknown. Activated RSK1 is known to phosphorylate the cAMP responsive element binding protein (CREB),[18,19] a transcription factor that promotes cholangiocyte proliferation[20] and protects cholangiocytes from apoptosis.[21]

One potential downstream target of CREB transcriptional activity in the context of inducing cholangiocyte proliferation may be the Yes-associated protein (YAP). YAP expression is induced in a rodent model of cholestasis and in human cholestatic liver diseases and is thought to regulate the resulting hyperplastic cholangiocyte proliferation.[22] Furthermore, CREB has been shown to promote YAP transcriptional output by binding to the -608/439 sequence on the YAP (*YY1AP1*) promoter in liver cancer[23]; however, whether CREB activity can induce YAP expression to regulate hyperplastic cholangiocyte proliferation is unknown.

With the pathogenic switch from quiescence to proliferation, cholangiocytes undergo a wide array of phenotypical and morphological changes.[24] Besides proliferation, one of the major changes is the acquisition of a neuroendocrine phenotype, including an enhanced response to hormones and neuropeptides.[24] This neuroendocrine response allows cholangiocytes to synthesize and release neuroendocrine hormones and peptides thought to regulate their response to the initiating injury in an autocrine manner.[24]

Galanin is a 29—amino acid peptide that can be found in the small intestine and is widely distributed throughout the hypothalamus. It has been implicated in many diverse biological functions, including feeding, nociception, sleep cycle control, and regulation of mood. Galanin exerts its actions through one of three G-protein—coupled receptors: galanin receptor 1 (GalR1), galanin receptor 2 (GalR2), and galanin receptor 3 (GalR3). GalR1 and GalR2 are widely expressed in the brain, spinal cord, and throughout the periphery, including the liver.[25] GalR3 has been shown to be expressed only in discrete brain regions.[25] Activation of GalR1 activates mitogen-activated protein kinase ERK1/2—dependent pathways.[26] Conversely, GalR2 is thought to couple to $G_{q/11}$, leading to the activation of inositol trisphosphate/protein kinase C pathways.[26] The expression of galanin and its receptors and the effects of galanin signaling on cholangiocyte function during liver disease are not known.

Materials and Methods

Unless otherwise indicated, all chemicals were purchased from Sigma-Aldrich (St. Louis, MO) and were of the highest grade available. Vivo morpholino sequences were designed and purchased from Gene Tools Inc. (Philomath, OR) using the Rattus norvegicus galanin/galanin message-

associated peptide prepropeptide mRNA sequence from the National Center for Biotechnology Information database (NM_033237.1). The full-length recombinant galanin peptide Galanin 1-29 (Gal 1-29), the GalR1 agonist (M617), the GalR2 agonist (M1145), the ERK1/2 inhibitor (PD98059), and the RSK1 inhibitor (SL0101-1) were purchased from Tocris (Bristol, UK). Galanin and cytokeratin-19 (CK-19) antibodies were purchased from Abcam (Cambridge, MA). Proliferating cell nuclear antigen (PCNA) and GalR1 antibodies were purchased from Santa Cruz Biotechnology (Dallas, TX). Total CREB, phospho-specific and total ERK1/2, and total YAP primary antibodies were purchased from Cell Signaling Technology (Danvers, MA). The nucleofector kit for transient transfection of primary epithelial cells was purchased from Lonza (Walkersville, MD). The RSK1 activity enzyme immunoassay kit (detecting phospho-RSK1) was purchased from R&D Systems (Minneapolis, MN). The phospho-CREB DNA binding assay was purchased from Cayman Chemicals (Ann Arbor, MI). The total galanin enzyme immunoassay kit was purchased from Peninsula Laboratories (San Carlos, CA). Osmotic minipumps were obtained from Alzet Osmotic Pumps (Cupertino, CA).

Animal Treatment

Male Sprague-Dawley rats (150 g to 175 g) were purchased from Charles River (Wilmington, MA) and maintained in a temperature-controlled environment (20°C to 22°C) with a 12:12-hour light-dark cycle. Unless otherwise indicated, animals had free access to drinking water and standard rat chow. All animal experiments were approved by and were performed in accordance with the guidelines of the Baylor Scott & White Institutional Animal Care and Use Committee. Rats underwent BDL or sham surgery, as described previously,[27] and were treated with vehicle, Gal 1-29, or the GalR1 agonist M617 (both at 100 pmol/rat per day) via intraperitoneal i.p.-implanted minipumps for up to 4 weeks, commencing at the time of surgery. In parallel, rats were treated with galanin-specific vivo morpholino (5′-TAGTGCAGGTCCAAG-TGTCTCCGTC-3′, 4 μg/rat per day, i.v.) or a control sequence that has five bases altered (5′-TACTCCAGCTCC-AACTCTCTCCGTC-3′, 4 μg/rat per day, i.v.), for 3 days before sham or BDL surgery and throughout the experiment. On the days indicated after surgery, cholangiocytes and hepatocytes were isolated following the method described previously[4] and tissue and serum were collected for further analysis.

Galanin and GalR1 Expression

Serum levels of galanin were assessed after sham or BDL surgery by enzyme immunoassay following the manufacturer's instructions. Galanin and/or GalR1 expression was assessed in cholangiocytes and hepatocytes isolated from

sham or BDL tissue by real-time PCR[28] and in liver tissue by immunohistochemistry,[9,28] as previously described.

Cholangiocyte Proliferation, Serum Chemistry, Inflammation, and Fibrosis *in Vivo*

Cholangiocyte proliferation was assessed in liver sections by immunohistochemical staining for CK-19 to assess intrahepatic biliary mass and PCNA immunoreactivity as a marker of proliferative capacity using the method previously described.[29,30] After staining, sections were counterstained with hematoxylin and examined with a microscope (Olympus BX 40; Olympus Optical Co, Waltham, MA). More than 100 cholangiocytes were counted in a random, blinded manner (S.G.) in three different fields for each group of animals. Data were expressed as number of CK-19− and PCNA-positive cholangiocytes per portal tract.

Serum chemistry (aspartate aminotransferase, alanine aminotransferase, and total bilirubin) was assessed in all animal treatment groups using the Catalyst One chemistry analyzer (IDEXX Laboratories, Westbrook, ME). Proinflammatory cytokine expression (tumor necrosis factor-α and IL-1β) was assessed in total liver extracts by real-time PCR. The degree of fibrosis in liver sections was assessed by Sirius red staining (IHC World, Woodstock, MD) using the manufacturer's instructions. The degree of fibrosis was expressed as the average percentage of the total area. Furthermore, the expression of collagen 1A1, as a molecular marker of fibrosis, was assessed in total liver extracts by real-time PCR.

Cell Lines

A mouse cholangiocyte cell line (MCCL) was cultured as previously described.[9] Cells were plated onto 96-well plates at a density of 5000 cells per well and allowed to adhere overnight. Cells were then serum starved for 24 hours before treatment with various concentrations of Gal 1-29 (1 to 1000 nmol/L) M617 or M1145 (both at 1 to 50 nmol/L) in the absence or presence of inhibitors of ERK1/2 (PD98059; 20 nmol/L) or RSK1 (SL-0101-1; 20 nmol/L). Metabolism of MTS reagent was used as an indicator of cell proliferation, as described previously.[28] In parallel, to assess the role of CREB in the molecular effects of galanin, vectors containing wild-type CREB, and two mutated variants of CREB (CREB133 and kCREB) were transiently transfected into MCCL using the nucleofector kit for primary epithelial cells and electroporated using the Amaxa Nucleofector II Device (Lonza, Walkersville, MD) before plating. Overexpression of CREB was assessed by immunoblotting using a total CREB antibody with β-actin used as a loading control, as described,[28] and CREB activity was assessed using the phospho-CREB DNA binding assay (Cayman Chemicals) after stimulation for 6 hours with 100 nmol/L Gal 1-29 or 10 μmol/L forskolin as a positive control. Proliferation of MCCL cells after treatment with M617 or Gal 1-29 was assessed using MTS assay.

Cell Signaling Analyses

ERK1/2 activity in protein lysates from isolated cholangiocytes, total liver, and MCCL treatment groups described above was assessed by immunoblotting using phospho-specific and total ERK1/2 primary antibodies. All imaging was performed on an Odyssey 9120 Infrared Imaging System (LI-COR, Lincoln, NE), and band intensity quantifications were performed using ImageJ software version 1.49v (NIH, Bethesda, MD; http://imagej.nih.gov/ij). Data are expressed as fold change in fluorescent band intensity of the ratio between phospho-specific and total ERK1/2.

RSK1 activity was assessed in protein lysates from isolated cholangiocytes, total liver, and MCCL treatment groups described above using the total RSK1 activity enzyme immunoassay kit, following the manufacturer's instructions. Data are expressed as the average ± SEM amount of phospho-RSK per microgram of total protein.

CREB activity was assessed in nuclear extracts from isolated cholangiocytes, total liver, and MCCL treatment groups described above using the phospho-CREB DNA binding assay (Cayman Chemicals). Nuclear proteins were isolated following the methods described previously,[31] and 10 μg (isolated cholangiocytes) or 100 μg (total liver extracts) was used in the assay. Data are expressed as average ± SEM of the fold change in absorbance at 450 nm.

YAP expression was assessed in protein lysates from total livers and MCCL cells transiently transfected with wild-type and mutant CREB expression vectors by immunoblotting. All imaging was performed on an Odyssey 9120 Infrared Imaging System (LI-COR), and band intensity quantifications were performed using ImageJ software version 1.49v. Data are expressed as fold change in fluorescent band intensity of the ratio between YAP/β-actin.

Statistical Analysis

All statistical analyses were performed using Graphpad Prism software version 5.04 (Graphpad Software, La Jolla, CA). Results were expressed as means ± SEM. For data that passed normality tests, significance was established using the *t*-test when differences between two groups were analyzed, and analysis of variance when differences between three or more groups were compared followed by the appropriate post hoc test. If tests for normality failed, two groups were compared with a Mann-Whitney *U* test or a Kruskal-Wallis ranked analysis when more than two groups were analyzed. Differences were considered significant when the $P < 0.05$.

Results

Galanin Is Up-Regulated during Cholestasis and Contributes to Cholangiocyte Proliferation

In the serum 3 and 7 days after BDL surgery, we observed increased circulating galanin content associated with
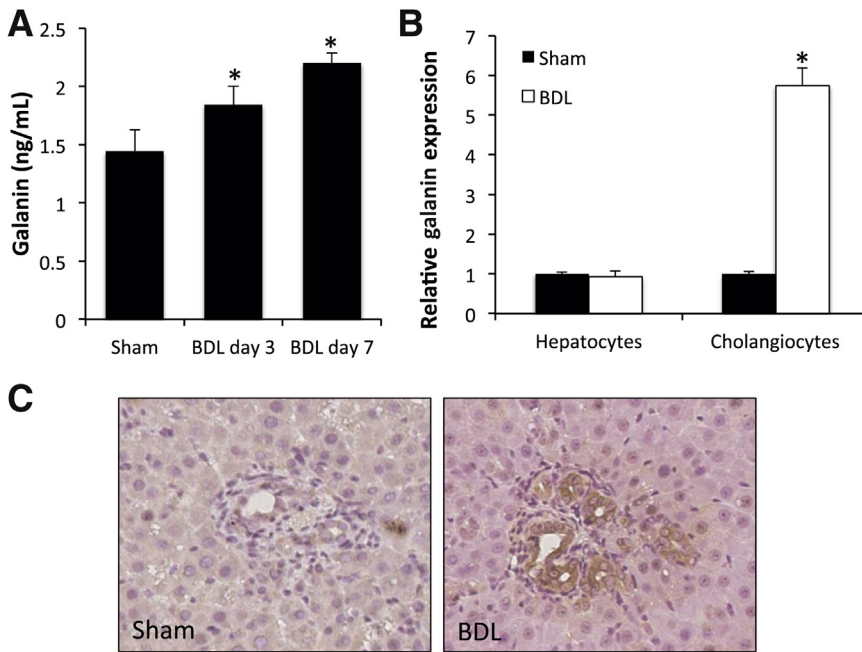
Figure 1 Galanin is up-regulated during bile duct ligation (BDL)—induced cholestasis. **A:** Concentrations of galanin in the serum of sham-operated, 3 days after BDL-operated, and 7 days after BDL-operated rats. **B:** Relative galanin mRNA expression in isolated hepatocytes and cholangiocytes from sham-operated and 7 days after BDL-operated rats. **C:** Galanin immunohistochemistry in liver sections from sham-operated and 7 days after BDL-operated rats. Data are expressed as means $\pm$ SEM (**A** and **B**). $n = 6$ (**A** and **B**). *$P < 0.05$ compared to sham-operated rats. Original magnification, $\times 20$ (**C**).

hyperplastic cholangiocyte proliferation (Figure 1A). This was paralleled by increased galanin expression in isolated cholangiocytes, but not hepatocytes, from BDL rats compared to sham (Figure 1B) and increased galanin immunoreactivity in cholangiocytes (Figure 1C), indicating that the increased circulating galanin observed during cholestasis may be derived from the liver.

To determine the role of galanin on cholangiocyte proliferation, rats were treated with recombinant Gal 1-29 for 7 days after sham or BDL surgery. There was increased intrahepatic bile duct mass and PCNA-positive cholangiocytes after BDL surgery when compared to sham treatment (Figure 2, A—C). Gal 1-29 treatment further increased intrahepatic bile duct mass, as assessed by CK-19 immunoreactivity, in both sham and BDL rats (Figure 2, A and B) when compared to saline-treated rats. In addition, there was a concomitant increase in PCNA-positive cholangiocytes after Gal 1-29 treatment in sham and BDL rats, compared to saline treatment (Figure 2, A and C). Interestingly, there were no differences in markers of liver damage (as assessed by serum aspartate aminotransferase, alanine aminotransferase, and total bilirubin), inflammation (assessed by expression of proinflammatory cytokines) (Table 1), and liver fibrosis (data not shown) between saline-treated and Gal 1-29 BDL rats. To determine whether the proliferative actions of Gal 1-29 were via direct actions on cholangiocytes, MCCL cells were treated with various concentrations of Gal 1-29. There was a dose-dependent increase in proliferative index, as assessed by MTS assay (Figure 2D).

Conversely, the effects of suppressing galanin expression were assessed after tail vein injection of galanin-specific vivo morpholino sequences before sham or BDL surgery. This

strategy reduced serum levels of galanin from 11.97 ± 0.415 ng/mL (after mismatched vivo morpholino treatment) to 3.877 ± 0.172 ng/mL (after galanin-specific vivo morpholino treatment). This treatment regimen also decreased galanin immunoreactivity in cholangiocytes after sham (data not shown) or BDL (Figure 3A). Although inhibiting galanin expression had no significant effect on intrahepatic bile duct mass in sham rats, this treatment decreased the intrahepatic bile duct mass in BDL rats compared to those treated with the mismatched vivo morpholino sequence (Figure 3, B and C). In addition, suppression of galanin expression reduced the liver damage and inflammation observed after BDL compared to BDL rats treated with the mismatched vivo morpholino sequence (Table 1). Furthermore, suppressing galanin expression reduced the degree of liver fibrosis observed after BDL, as demonstrated by Sirius red staining (Figure 3, D and E), and expression of collagen 1A1, a molecular marker of fibrosis (Figure 3F).

Galanin Exerts Its Effects through Activation of GalR1

To determine the mechanism by which galanin regulates hyperplastic cholangiocyte proliferation, MCCL cells were treated with specific agonists for GalR1 (M617) or GalR2 (M1145). Treatment with M617 (Figure 4A), but not M1145 (Figure 4B), increased MCCL cell proliferation, as demonstrated by MTS assay. In addition, the expression of GalR1 (but not GalR2) was increased in cholangiocytes isolated from BDL rats compared to sham by real-time PCR (Figure 4C) and by immunohistochemistry (Figure 4D). Furthermore, to determine the effects of GalR1 activation *in vivo*, sham and BDL rats were treated with M617 for 7 days. Activation of GalR1 increased
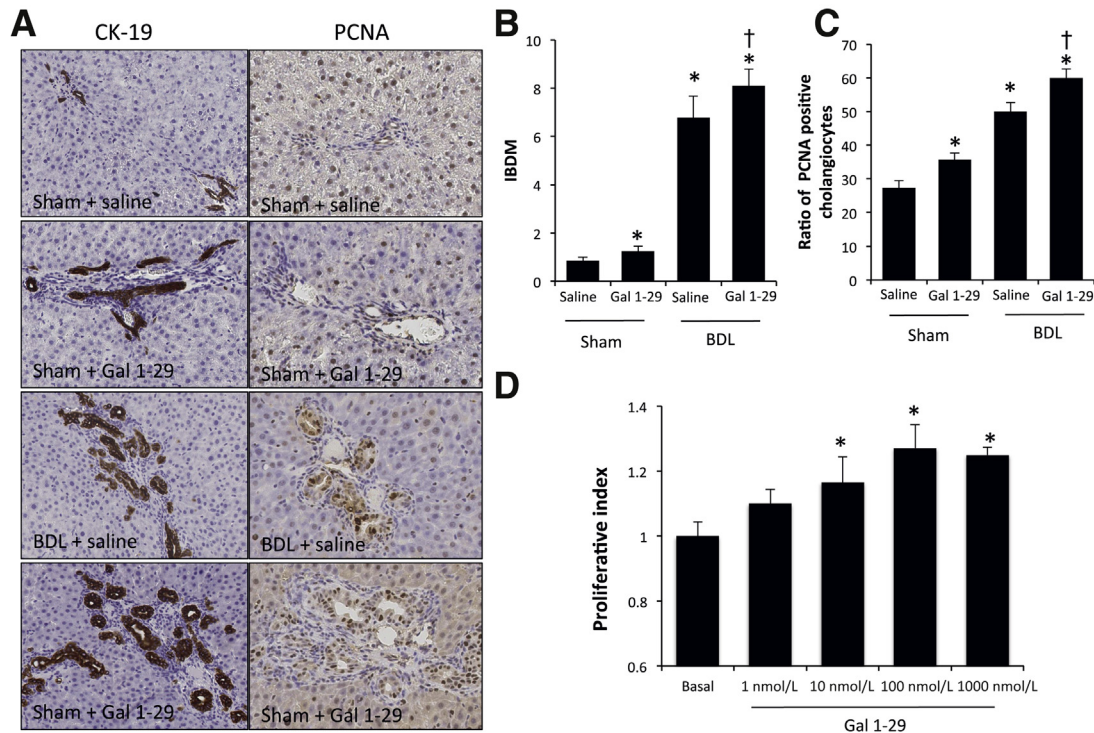
Figure 2 Galanin promotes cholangiocyte proliferation. **A:** CK-19 and proliferating cell nuclear antigen (PCNA) immunohistochemistry in liver sections from sham-operated and 7 days after bile duct ligation (BDL)—operated rats infused with saline or Gal 1-29. **B:** Quantification of intrahepatic bile duct mass (IBDM) based on percentage of CK-19—positive cells per field in liver sections from sham-operated and 7 days after BDL-operated rats infused with saline or Gal 1-29. **C:** Percentage of PCNA-positive cholangiocytes per field in liver sections from sham-operated and 7 days after BDL-operated rats infused with saline or Gal 1-29. **D:** Relative MTS metabolism, a measure of cell proliferation, in mouse cholangiocyte cell line (MCCL) cells treated with 1 to 1000 nmol/L of Gal 1-29. Data are expressed as means $\pm$ SEM (**B**—**D**). $n = 5$ (**B**—**D**). *$P < 0.05$ compared to sham-operated saline-infused rats or basal MCCL cells; $^{\dagger}P < 0.05$ compared to BDL-operated, saline-infused rats. Original magnification, $\times 20$ (**A**).

intrahepatic biliary mass after both sham and BDL, as demonstrated by increased CK-19 mRNA expression (Figure 5A) and immunoreactivity (Figure 5B). Interestingly, treatment of BDL animals with M617 did not significantly increase the degree of liver damage, inflammation (Table 1), or fibrosis observed from that in BDL alone (data not shown).

Activation of GalR1 by Galanin Leads to the Subsequent Activation of ERK1/2/RSK1/CREB/YAP-Dependent Pathways

GalR1 activation has previously been shown to lead to the activation of ERK1/2.[26] ERK1/2 activation has been demonstrated in cholangiocytes during BDL and can be

Table 1 Liver Damage and Inflammatory Markers during Cholestasis

Treatment	AST (U/L)	ALT (U/L)	Bilirubin (mg/dL)	IL-1β (fold change compared to sham)	TNF-α (fold change compared to sham)
Sham + saline	145.7 $\pm$ 32.7	55.2 $\pm$ 8.5	<0.1	1.0 $\pm$ 0.13	1.0 $\pm$ 0.12
Sham + Gal 1—29	136.4 $\pm$ 43.0	52.4 $\pm$ 2.8	<0.1	1.1 $\pm$ 0.02	1.0 $\pm$ 0.05
Sham + M617	194.0 $\pm$ 84.85	49.2 $\pm$ 14.3	<0.1	0.89 $\pm$ 0.02	0.92 $\pm$ 0.03
Sham + Gal mismatched VM	144.5 $\pm$ 10.61	61.6 $\pm$ 11.6	<0.1	1.0 $\pm$ 0.04	1.0 $\pm$ 0.10
Sham + Gal VM	167.1 $\pm$ 17.54	52.4 $\pm$ 2.1	<0.1	0.89 $\pm$ 0.02	0.97 $\pm$ 0.21
BDL + saline	758.4 $\pm$ 59.7*	331.5 $\pm$ 45.7*	12.7 $\pm$ 0.9*	2.7 $\pm$ 0.07*	2.5 $\pm$ 0.13*
BDL + Gal 1—29	694.5 $\pm$ 53.1*	395.3 $\pm$ 13.4*	11.6 $\pm$ 1.5*	2.9 $\pm$ 0.05*	2.4 $\pm$ 0.20*
BDL + M617	716.3 $\pm$ 39.2*	350.8 $\pm$ 29.6*	10.1 $\pm$ 0.7*	2.6 $\pm$ 0.02*	2.4 $\pm$ 0.34*
BDL + Gal mismatched VM	691.7 $\pm$ 61.5*	298.7 $\pm$ 16.7*	11.8 $\pm$ 1.1*	2.9 $\pm$ 0.06*	2.83 $\pm$ 0.31*
BDL + Gal VM	339.5 $\pm$ 30.4*†	179.3 $\pm$ 5.7*†	5.15 $\pm$ 0.6*†	1.4 $\pm$ 0.1*†	1.5 $\pm$ 0.11*†

*$P < 0.05$ versus corresponding sham sample.
$^{\dagger}P < 0.05$ versus BDL + saline.
ALT, alanine aminotransferase; AST, aspartate aminotransferase; BDL, bile duct ligation; Gal, galanin; TNF-α, tumor necrosis factor-α; VM, vivo morpholino.
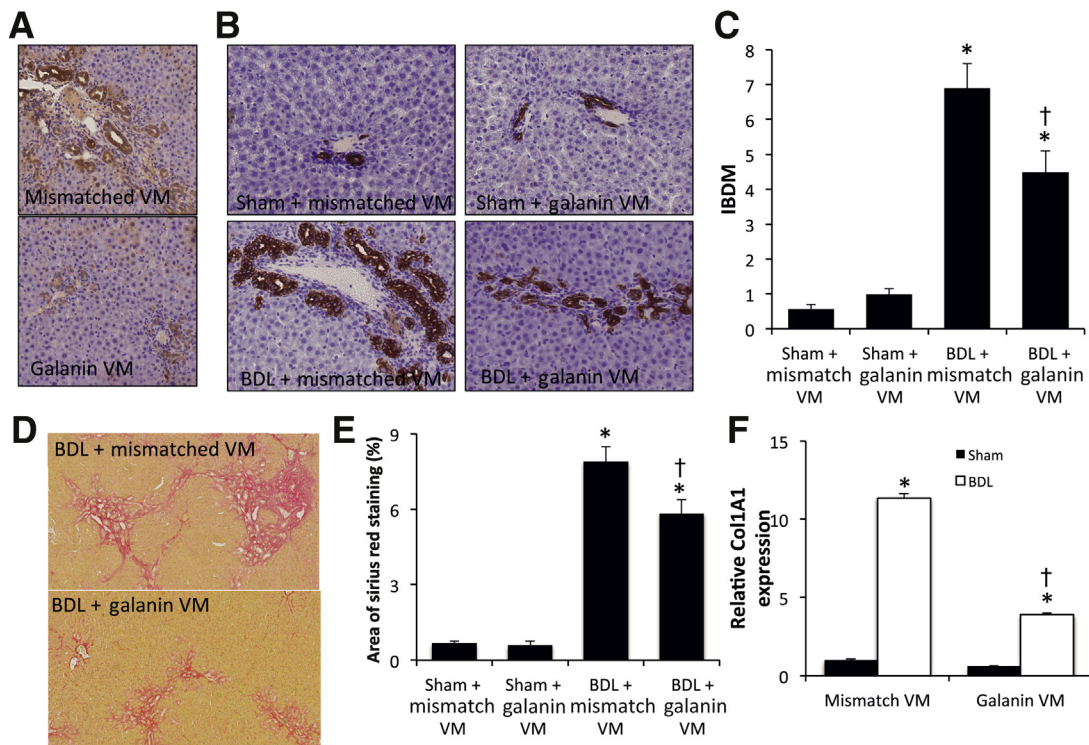
Figure 3   Galanin inhibition via vivo morpholino (VM) sequence injection reduces IBDM. **A:** Galanin immunohistochemistry in liver sections from 7 days after bile duct ligation (BDL)—operated rats injected with mismatched vivo morpholino or galanin vivo morpholino sequences. **B:** CK-19 immunohistochemistry in liver sections from 7 days after BDL-operated rats injected with mismatched vivo morpholino or galanin vivo morpholino sequences. **C:** Quantification of intrahepatic bile duct mass (IBDM) based on percentage of CK-19—positive cells per field in liver sections from 7 days after BDL-operated rats injected with mismatched vivo morpholino or galanin vivo morpholino sequences. **D:** Sirius red staining in liver sections from 2 weeks after BDL rats injected with mismatched vivo morpholino or galanin vivo morpholino sequences. **E:** Quantification of fibrosis based on the percentage of Sirius red staining—positive cells per field in liver sections from 7 days after BDL-operated rats injected with mismatched vivo morpholino or galanin vivo morpholino sequences. **F:** The expression of collagen 1A1 (Col1A1), as a marker of liver fibrosis, was assessed in total liver extracts from sham and BDL-operated rats treated with galanin vivo morpholino or mismatched control sequences. Data are expressed as means ± SEM (**C**, **E**, and **F**). $n = 4$ (three slides per rat; **C** and **E**); $n = 4$ (**F**). *$P < 0.05$ compared to sham-operated mismatch vivo morpholino—injected rats or basal mouse cholangiocyte cell line cells; †$P < 0.05$ compared to BDL-operated mismatch vivo morpholino—injected rats. Original magnification, ×20 (**A**, **B**, and **D**).

attributable to many factors[13,14]; we will therefore assess the effects of GalR1 activation only in sham animals to avoid confounding factors. Indeed, treatment of rats with both Gal 1-29 and M617 increased the phosphorylation of ERK1/2 in total liver extracts (Figure 6A). Conversely, treatment of rats with galanin vivo morpholino inhibited the BDL-induced activation of ERK1/2 (Figure 6B). Furthermore, treatment of MCCL cells *in vitro* with M617 increased ERK1/2 phosphorylation at 45 and 60 minutes after stimulation (Figure 6C). To determine the role of ERK1/2 activation on GalR1-induced cholangiocyte proliferation, MCCL cells were pretreated with an inhibitor of ERK1/2 activation, PD98059. Blocking ERK1/2 activation attenuated the proliferative effects of both M617 (Figure 6D) and Gal 1-29 (Figure 6E).

One of the reported downstream targets of ERK1/2 is RSK1.[16,17] Similar to ERK1/2, RSK1 activation was increased in cholangiocytes isolated from BDL rats compared to sham (Figure 7A). Furthermore, treatment of sham rats with both Gal 1-29 and M617 *in vivo* increased the phosphorylation of RSK1 in total liver extracts

(Figure 7B), whereas inhibition of galanin expression with vivo morpholinos in BDL rats decreased RSK1 phosphorylation (Supplemental Figure S1). Treatment of MCCL cells with M617 increased the phosphorylation of RSK1 45 and 60 minutes after treatment (Figure 7C), which could be attenuated by pretreatment with PD98059 (Figure 7D). In addition, the proliferative effects of both M617 and Gal 1-29 could be prevented by pretreatment with the RSK1 inhibitor SL-0101-1 (Figure 7, E and F).

The ERK1/2/RSK1 pathway has previously been shown to activate CREB,[18,19] a transcription factor associated with cholangiocyte proliferation,[20] although a definitive role for CREB activity in the proliferative process is unclear. Consistent with previous reports, CREB DNA-binding activity is increased in cholangiocytes isolated from rats after BDL compared to sham (Figure 8A). Furthermore, treatment of rats with both Gal 1-29 and M617 *in vivo* increased CREB DNA-binding activity in total liver extracts (Supplemental Figure S2A), whereas inhibition of galanin expression with vivo morpholinos in BDL rats decreased CREB DNA-binding activity (Supplemental Figure S2B).
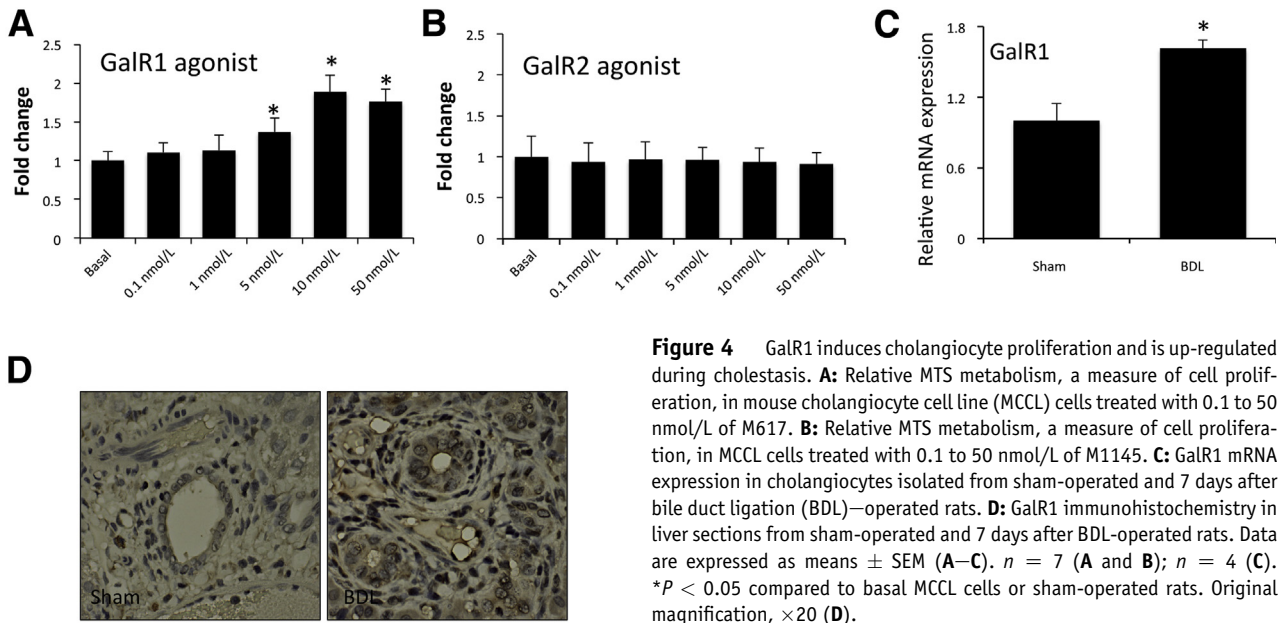
Figure 4 GalR1 induces cholangiocyte proliferation and is up-regulated during cholestasis. **A:** Relative MTS metabolism, a measure of cell proliferation, in mouse cholangiocyte cell line (MCCL) cells treated with 0.1 to 50 nmol/L of M617. **B:** Relative MTS metabolism, a measure of cell proliferation, in MCCL cells treated with 0.1 to 50 nmol/L of M1145. **C:** GalR1 mRNA expression in cholangiocytes isolated from sham-operated and 7 days after bile duct ligation (BDL)—operated rats. **D:** GalR1 immunohistochemistry in liver sections from sham-operated and 7 days after BDL-operated rats. Data are expressed as means $\pm$ SEM (**A**–**C**). $n = 7$ (**A** and **B**); $n = 4$ (**C**). *$P < 0.05$ compared to basal MCCL cells or sham-operated rats. Original magnification, $\times 20$ (**D**).

In vitro, treatment of MCCL cells with M617 directly increased CREB DNA-binding activity, which was attenuated by pretreatment with ERK1/2 and RSK1 inhibitors (Figure 8B). To assess the role of CREB DNA-binding activity in galanin-mediated proliferation, cholangiocytes were transfected with several CREB expression vectors. The wild-type CREB vector contains wild-type CREB that can be activated in response to factors known to increase CREB DNA-binding activity.[32] The vector containing mutant CREB133 expresses CREB with a serine to alanine mutation on amino acid residue 133 that blocks the phosphorylation and hence transcriptional activity of CREB[33]; the KCREB vector expresses CREB with a mutation in the DNA-binding domain, which also blocks its transcriptional activity.[32] Transient transfection of these vectors into MCCL cells increased the expression of total CREB compared to those transfected with the empty vector backbone (mock transfected) (Supplemental Figure S3A). Furthermore, stimulation with Gal 1-29 or forskolin (as a positive control) increased the CREB DNA-binding activity in mock-transfected cells as well as those transfected with

the wild-type CREB vector, but not the cells transfected with either mutant form of CREB (Supplemental Figure S3B). Treatment with M617 and Gal 1-29 increased proliferation of MCCL cells transfected with the empty vector and wild-type CREB vector, which was attenuated in cells transfected with the mutant CREB vectors (Figure 8C).

In an attempt to identify a possible downstream effector of CREB transcriptional activity, we assessed the effects of galanin activation on YAP expression. Because increased YAP expression occurs in cholangiocytes after BDL surgery and may be in response to many factors, we assessed the role of galanin-induced CREB activation on YAP expression only in the sham animals to avoid confounding factors. Indeed, treatment of rats with M617 or Gal 1-29 *in vivo* increased YAP expression in total liver extracts (Figure 9A). Furthermore, in MCCLs transiently transfected with a wild-type CREB expression vector, M617 and Gal 1-29 treatment increased YAP expression (Figure 9B). However, transient transfection of dominant negative mutant CREB expression vectors attenuated the stimulatory effects of M617 and Gal
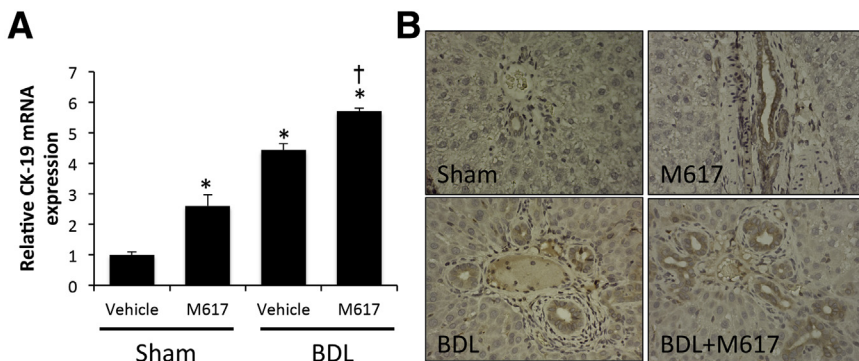

Figure 5 GalR1 agonism *in vivo* promotes cholangiocyte proliferation. **A:** CK-19 mRNA expression in total liver from sham-operated and 7 days after bile duct ligation (BDL)—operated rats infused with vehicle or M617. **B:** CK-19 immunohistochemistry in liver sections from sham-operated and 7 days after BDL-operated rats infused with vehicle or M617. Data are expressed as means $\pm$ SEM (**A**). $n = 5$ (**A**). *$P < 0.05$ compared to sham-operated saline-infused rats; †$P < 0.05$ compared to BDL-operated, saline-infused rats. Original magnification, $\times 20$ (**B**).
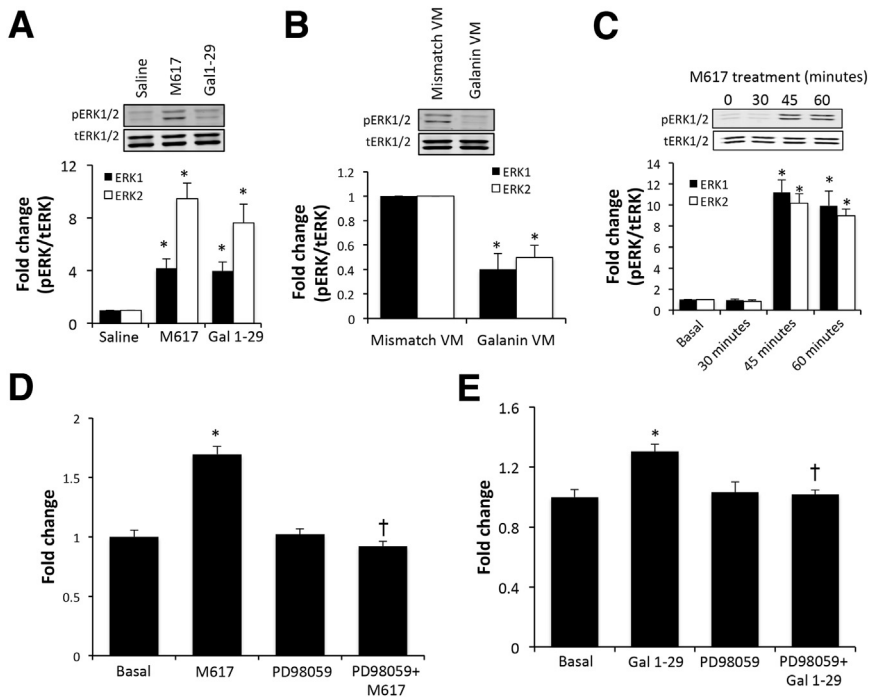
Figure 6 Galanin/GalR1 signaling promotes phosphorylation of ERK1/2, which drives cholangiocyte proliferation. **A:** Representative immunoblot and quantification for phospho-specific and total ERK1/2 (pERK1/2 and tERK1/2, respectively) in total liver extracts from sham-operated rats infused with saline, M617, or Gal 1-29. **B:** Representative immunoblot and quantification for pERK1/2 and tERK1/2 in total liver extracts from sham-operated rats injected with mismatch vivo morpholino (VM) or galanin vivo morpholino sequences. **C:** Representative immunoblot and quantification for pERK1/2 and tERK1/2 in mouse cholangiocyte cell line (MCCL) cells that were treated with 10 nmol/L M617 for up to 60 minutes. **D:** Relative MTS metabolism, a measure of cell proliferation, in MCCL cells treated with 10 nmol/L M617, 20 nmol/L PD98059, or both agents together. **E:** Relative MTS metabolism, a measure of cell proliferation, in MCCL cells treated with 100 nmol/L Gal 1-29, 20 nmol/L PD98059, or both agents together. Data are expressed as means $\pm$ SEM (**A–E**). $n = 4$ (**A–C**); $n = 7$ (**D** and **E**). *$P < 0.05$ compared to liver extracts from sham-operated saline infused, sham-operated mismatch vivo morpholino sequence—injected, or basal MCCL cells; †$P < 0.05$ compared to MCCL treated with M617.

1-29 on YAP expression (Figure 9B). Together, these data suggest that YAP, a known regulator of cholangiocyte proliferation,[22] may be a downstream target of the galanin-induced activation of the ERK1/2/RSK-1/CREB pathway that leads to enhanced cholangiocyte proliferation.

Discussion

The major findings of this study relate to the role of galanin in the regulation of hyperplastic cholangiocyte proliferation. Specifically, we demonstrate that galanin expression is increased in cholangiocytes in a rodent model of cholestasis, contributing to hyperplastic cholangiocyte proliferation via a mechanism involving the GalR1-dependent activation of ERK1/2/RSK1 and a subsequent increase in CREB transcriptional activity. Furthermore, strategies to inhibit galanin signaling attenuated hyperplastic cholangiocyte proliferation. Taken together, our data suggest that targeting galanin signaling may be effective for the maintenance of biliary mass during cholestatic liver diseases.

In animal models of cholestasis, as well as in human cholangiopathies, cholangiocytes proliferate or are damaged.[3,24] In the BDL rat model, widely used for evaluating the mechanisms of cholangiocyte hyperplasia,[3] there is an increase in ductal mass,[3,34,35] which is associated with increased liver damage, inflammation, and subsequent liver fibrosis.[7,8] In humans, cholangiocyte proliferation occurs in biliary obstruction, in chronic cholestatic liver diseases, and in many forms of liver injury.[24,35] Cholangiopathies share common pathological features, such as the damage of intrahepatic bile ducts and the proliferation of residual ducts (as a

mechanism of compensatory repair to maintain biliary homeostasis), but they progress toward ductopenia that represents the terminal stage of the disease.[24,35] Overt cholangiocyte proliferation, although considered compensatory, has been suggested to lead to neoplastic transformation of cholangiocytes, resulting in the formation of cholangiocarcinoma.[36] Therefore, different strategies (to stimulate or prevent proliferation) to maintain biliary mass homeostasis may be required, depending on the stage of the disease.

Associated with the pathogenic switch from quiescence to proliferation, cholangiocytes undergo a wide array of phenotypical and morphological changes.[24] Besides proliferation, one of the major changes is the acquisition of a neuroendocrine phenotype, including the expression of neuroendocrine markers (chromogranin A, glycolipid A2-B4, S-100 protein, and neural cell adhesion molecule) and acquisition of neuroendocrine granules.[24] This neuroendocrine response allows cholangiocytes to synthesize and release neuroendocrine hormones and peptides, which is thought to regulate their response to the initiating injury in an autocrine manner.[24] The work described herein is the first to suggest that among the neuroendocrine factors synthesized by cholangiocytes in response to biliary injury, the neuropeptide galanin may regulate the proliferative response of cholangiocytes. The data presented are exclusively in a rat model of cholestasis, and further studies assessing galanin expression in human cholestatic liver diseases are necessary.

There is a paucity of information regarding the effects of galanin on liver physiology and pathophysiology. Under physiological conditions, infusion of galanin into the hepatic
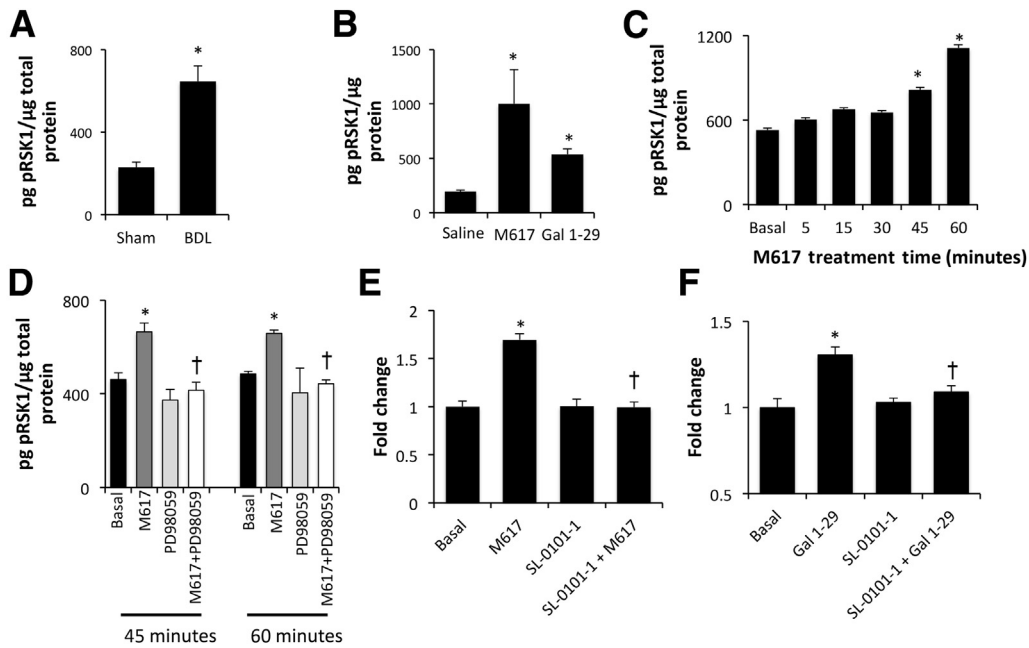
Figure 7 RSK1 phosphorylation promotes cholangiocyte proliferation and is induced by galanin/GalR1 signaling. **A:** Quantitative measurement of RSK1 phosphorylation in isolated cholangiocytes from sham-operated and 7 days after bile duct ligation (BDL)—operated rats. **B:** Quantitative measurement of RSK1 phosphorylation in total liver extracts from sham-operated rats infused with saline, M617, or Gal 1-29. **C:** Quantitative measurement of RSK1 phosphorylation in mouse cholangiocyte cell line (MCCL) cells treated with 10 nmol/L M617 for up to 60 minutes. **D:** Quantitative measurement of RSK1 phosphorylation in MCCL cells treated with 10 nmol/L M617, 20 nmol/L PD98059, or both agents for 45 or 60 minutes. **E:** Relative MTS metabolism, a measure of cell proliferation, in MCCL cells treated with 10 nmol/L M617, 20 nmol/L SL-0101-1, or both agents together. **F:** Relative MTS metabolism, a measure of cell proliferation, in MCCL cells treated with 100 nmol/L Gal 1-29, 20 nmol/L SL-0101-1, or both agents together. Data are expressed as means ± SEM. $n = 4$ (**A**–**D**); $n = 7$ (**E** and **F**). *$P < 0.05$ compared to isolated cholangiocytes from sham-operated rats, total liver extracts from sham-operated saline-infused rats, or basal MCCL cells; †$P < 0.05$ compared to MCCL treated with M617 or Gal 1-29. pRSK1, phosphorylated RSK1.

artery potentiates the effects of norepinephrine on hepatic glucose production.[37] Furthermore, in mice overexpressing galanin, there is an increase in body weight, serum cholesterol, serum triglycerides, and hepatic steatotosis contributing to the development of fatty liver disease independent of any orexigenic effect.[38] However, a role for galanin in the etiology of cholestatic liver diseases is unknown. Herein, we demonstrate that galanin, via activation of GalR1 expressed

on cholangiocytes, induces proliferation in both sham and BDL rats, whereas strategies to inhibit galanin signaling prevent hyperplastic biliary proliferation. Interestingly, the effects of galanin on cholangiocyte proliferation during BDL did not correlate to increased liver damage, inflammation, or fibrosis. We hypothesize that this is a caveat with the BDL model in that this model requires the continuous ligation of the common bile duct, inducing massive biliary
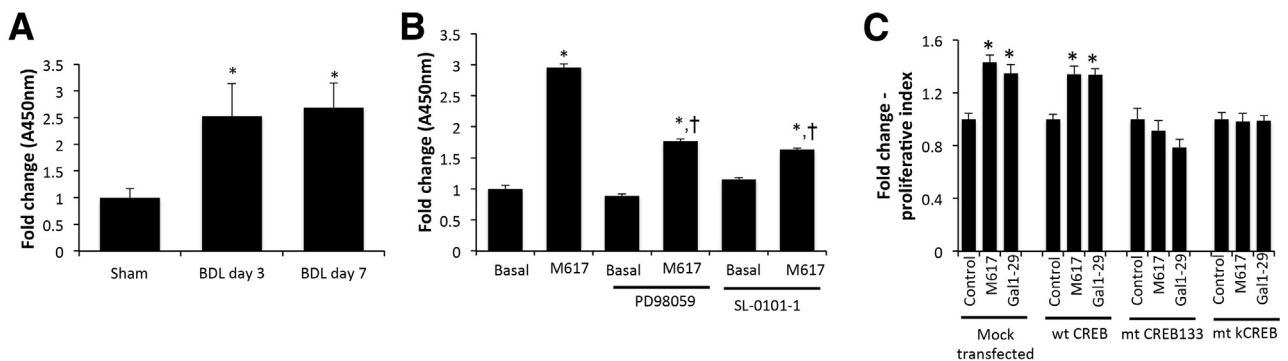

Figure 8 CREB DNA binding is induced by GalR1 signaling and promotes cholangiocyte proliferation. **A:** Relative CREB DNA-binding activity in isolated cholangiocytes from sham-operated, 3 days after bile duct ligation (BDL)—operated, and 7 days after BDL-operated rats. **B:** Relative CREB DNA-binding activity in mouse cholangiocyte cell line (MCCL) cells treated with 10 nmol/L M617, 20 nmol/L PD98059, 20 nmol/L SL-0101-01, or cotreatments of M617 with PD98059 and SL-0101-01. **C:** Relative MTS metabolism, a measure of cell proliferation, in MCCL cells that were mock transfected or had transient transfection of wild-type (wt) CREB or two mutated (mt) variants of CREB (CREB133 and kCREB) that were treated with 10 nmol/L M617 or 100 nmol/L Gal 1-29. Data are expressed as means ± SEM (**A**–**C**). $n = 4$ (**A** and **B**); $n = 7$ (**C**). *$P < 0.05$ compared to isolated cholangiocytes from sham-operated rats or basal MCCL cells; †$P < 0.05$ compared to MCCL treated with M617.
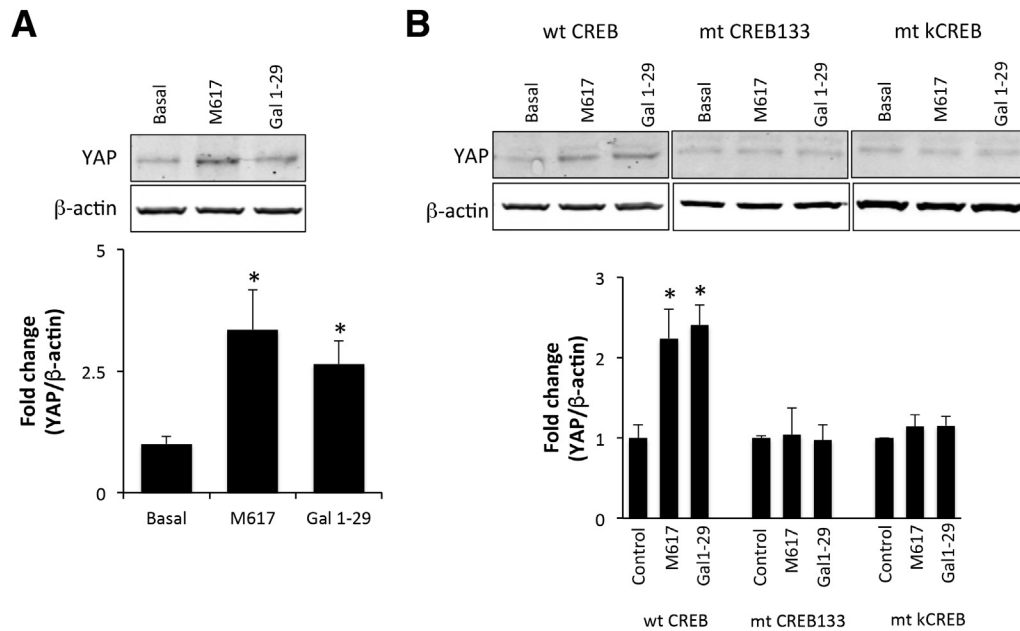
Figure 9 YAP expression is increased by GalR1 signaling in cholangiocytes. YAP protein expression was assessed in total liver extracts from rats infused with saline, M617, or Gal 1-29 (**A**) or in mouse cholangiocyte cell line (MCCL) cells transiently transfected with expression vectors containing wild-type (wt) CREB or two mutated (mt) variants of CREB (CREB133 and kCREB) before treatment with 10 nmol/L M617 or 100 nmol/L Gal 1-29 for 6 hours (**B**). YAP protein expression was assessed by immunoblotting. The protein expression of β-actin was used as the loading control. Data are expressed as fold change in fluorescent band intensity of the ratio between YAP/β-actin. Data are expressed as means ± SEM (**A** and **B**, **bottom panels**). $n = 4$ (**A** and **B**, **bottom panels**). *$P < 0.05$ compared to basal liver extracts from sham-operated rats or basal MCCL cells.

hyperplasia, liver damage, and inflammation, with no discernible ductopenia. Agents that may further stimulate cholangiocyte proliferation and liver fibrosis may be overwhelmed by the strong response observed after BDL alone. Furthermore, our data indicate that cholangiocytes from BDL rats already produce large amounts of galanin that may saturate the galanin-mediated signaling pathway; therefore, treatment of BDL rats with additional recombinant galanin may generate little effect. A more important observation is that blocking endogenous galanin expression inhibits cholangiocyte proliferation, liver damage, inflammation, and subsequent liver fibrosis. Together, these data suggest that galanin contributes to the pathology observed during cholestasis.

Downstream of GalR1 activation in cholangiocytes in the BDL model of extrahepatic biliary obstruction was the activation of ERK1/2 that modulated the proliferative effects of galanin signaling. This is consistent with previous reports indicating that GalR1 activation, in contrast to GalR2 and GalR3, leads to the activation of ERK1/2-mediated pathways.[26] In further support of our data, numerous studies demonstrate that agents that activate ERK1/2, regardless of whether it is via a calcium- or cAMP-dependent pathway, lead to cholangiocyte proliferation.[13,14] Conversely, agents that inhibit ERK1/2 phosphorylation are generally antiproliferative.[10,15] However, herein, we demonstrate that downstream of ERK1/2 activation is the activation of RSK1; inhibition of RSK1 attenuated the proliferative effects of galanin. The proliferative role of

RSK1 has not previously been demonstrated in cholangiocytes, but has been well established in many other cell types, in particular various types of cancer cells.[39—42] Indeed, RSK inhibitors are suggested as potential therapeutic agents for the treatment of certain cancers where the ERK1/2 pathway is dysregulated.[39,40,43] The data presented herein suggest that modulation of RSK1 activity may prove beneficial in the maintenance of biliary mass and warrants further investigation.

RSK1 is known to phosphorylate and regulate the activity of many targets, in particular the phosphorylation of CREB, which increases its transcriptional activity.[18,19] Herein, we demonstrate that the proliferative effects of galanin on cholangiocytes are via the activation of CREB, which can be attenuated by specific inhibitors of ERK1/2 and RSK1 activation. Furthermore, transfection of vectors containing dominant negative mutants of CREB, designed to inhibit endogenous CREB DNA-binding activity, prevented the proliferative response of cholangiocytes to galanin treatment. In support of these findings, increased CREB DNA-binding activity has been associated with agents that increase cholangiocyte proliferation,[20,44] although a definitive role for CREB activity in the proliferative response of cholangiocytes was not previously demonstrated in these publications. Given that many agents that regulate cholangiocyte proliferation activate an array of signal transduction pathways that seem to converge on CREB activity, the role of CREB in the etiology of cholestatic liver injury warrants further investigation. Indeed, one of the

potential downstream targets of CREB signaling may be the known regulator of cholangiocyte proliferation, YAP. CREB has previously been shown to increase YAP expression via the direct binding and transcriptional activation of the YAP promoter[23] in liver cancer cells. Given that YAP expression increases in cholangiocytes after BDL,[22] with no change in the p-YAP/total YAP ratio, it is conceivable that YAP may be a downstream target in the context of galanin-induced cholangiocyte proliferation. Indeed, the data presented herein suggest that galanin/GalR1-mediated signaling increases the expression of YAP and that CREB activation is required for this process to occur.

In conclusion, we have provided novel evidence for the increased expression of the neuropeptide galanin in cholangiocytes as part of the neuroendocrine response to biliary injury. The increase in galanin activates GalR1 and leads to the subsequent activation of the ERK1/2/RSK1/CREB/YAP signal transduction cascade via an autocrine mechanism. Further knowledge of the role of galanin signaling during cholestasis and the mechanism by which galanin exerts proliferative effects during cholestasis may lead to the development of innovative treatment paradigms for chronic cholestatic liver diseases.

Acknowledgment

Supplemental Data

Supplemental material for this article can be found at http://dx.doi.org/10.1016/j.ajpath.2016.12.015.

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