Ghrelin reverses ductular reaction and hepatic fibrosis in a rodent model of cholestasis

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The orexigenic peptide ghrelin (Ghr) stimulates hunger signals in the hypothalamus via growth hormone secretagogue receptor (GHS-R1a). Gastric Ghr is synthetized as a preprohormone which is proteolytically cleaved, and acylated by a membrane-bound acyl transferase (MBOAT). Circulating Ghr is reduced in cholestatic injuries, however Ghr’s role in cholestasis is poorly understood. We investigated Ghr’s effects on biliary hyperplasia and hepatic fibrosis in Mdr2-knockout (Mdr2KO) mice, a recognized model of cholestasis. Serum, stomach and liver were collected from Mdr2KO and FVBN control mice treated with Ghr, des-octanoyl-ghrelin (DG) or vehicle. Mdr2KO mice had lower expression of Ghr and MBOAT in the stomach, and lower levels of circulating Ghr compared to WT-controls. Treatment of Mdr2KO mice with Ghr improved plasma transaminases, reduced biliary and fibrosis markers. In the liver, GHS-R1a mRNA was expressed predominantly in cholangiocytes. Ghr but not DG, decreased cell proliferation via AMPK activation in cholangiocytes in vitro. AMPK inhibitors prevented Ghr-induced FOXO1 nuclear translocation and negative regulation of cell proliferation. Ghr treatment reduced ductular reaction and hepatic fibrosis in Mdr2KO mice, regulating cholangiocyte proliferation via GHS-R1a, a G-protein coupled receptor which causes increased intracellular Ca2+ and activation of AMPK and FOXO1, maintaining a low rate of cholangiocyte proliferation.

Ghrelin (Ghr) is a gastric orexigenic peptide with multiple roles in physiology, including food intake and appetite regulation by gut-brain signaling pathways, as well as metabolism functions related to energy homeostasis1–4. Ghr is synthetized predominantly in the stomach and gastrointestinal tract as a preprohormone which is proteolytically processed to a 28-amino acid peptide, and then acylated at serine 3 residue by a membrane-bound acyl transferase (MBOAT)5. Both des-acylated and octanoyl-Ghr are secreted into the systemic circulation during fasting, but only octanoyl-Ghr binds to and activates growth hormone secretagogue receptors type 1a (GHS-R1a), located predominantly in the hypothalamic neurons that regulate hunger and metabolism6–9. GHS-R1a is a G-protein-coupled receptor expressed in many tissues including the hypothalamus, pituitary gland, adrenals, thyroid, pancreas, and liver6. The expression of GHS-R1a mRNA increases up to eightfold in the hypothalamus during fasting, enhancing Ghr-mediated stimulation of neuropeptide Y (NPY) and agouti-related peptide (AgRP) neurons, with roles in inducing appetite9,10.

In the serum of healthy human subjects, two forms of the peptide have been identified, i.e. the acylated Ghr with a half-life of approximately 10 min, and the unacylated or des-ghrelin (DG), which is more stable, having a half-life of 35 min11. Systemic Ghr and DG have been reported to be in a ratio of 1/4 to 1/9 respectively in humans, and 1/13 in the mouse11,12. Acylation of DG to Ghr has been demonstrated to occur primarily in the stomach by MBOAT also known as Ghr O-acyl transferase or GOAT13,14. However, the process of Ghr deacylation is still poorly understood, though it has been suggested that it could take place in the liver since Ghr was found in significant amounts in hepatic tissue11,15. Other studies suggest that Ghr is deacylated by serum...
butyrylcholinesterase, and both Ghr and DG undergo renal clearance following the typical clearance pathway for small peptides.

Interestingly, Ghr was found to be dysregulated in patients with liver injuries. Fasting Ghr levels were decreased in adults with cirrhosis, while DG was not changed or increased as compared to healthy volunteers. In Child C cirrhosis patients though, Ghr levels were unchanged or elevated, independent of the etiology of liver disease. In patients with primary biliary sclerosis (PBC), serum Ghr levels were decreased compared to the control groups. A study on the influence of polymorphism in Ghr gene (GHRL) on hepatic fibrosis in patients with chronic hepatitis C, found that certain mutations in GHRL were associated with more severe liver fibrosis compared to wild type GHRL. Only a few publications describe the effects of exogenously administered Ghr on the liver in animal models of hepatic injuries, suggesting that Ghr may have antioxidant and anti-inflammatory outcomes protecting against liver fibrosis due to biliary obstruction.

In the present study, we used the Mdr2-knockout (Mdr2KO) mouse model of cholestasis to assess the effects of Ghr on biliary hyperplasia and hepatic fibrosis. In Mdr2KO mice, the ablation of ABCC4 gene encoding for the multidrug resistance protein 2 (Mdr2), a membrane protein with flippase activity and a role in the hepatocanalicular transport of phospholipids, results in bile retention in the liver and cholestasis-induced hepatic fibrosis. Mdr2 is the rodent homologue of human Mdr3 protein, an ATP-binding cassette transporter with an important role in normal bile secretion. Several clinical studies demonstrated that genetic mutations in ABCC4 gene encoding for Mdr3 were associated with symptomatic intrahepatic cholelithiasis, familial hepatic cholestasis and cirrhosis. Therefore, a large number of mechanistic studies of potential therapeutic drugs for treating symptoms of cholestasis-related injuries of the liver have been performed on Mdr2KO mice. In the study described herein, we assessed Ghr levels in serum, stomach and liver of cholestatic versus normal mice, as well as how exogenous administration of Ghr and DG influence the expression of fibrogenic genes in vivo. Using cholangiocytes in vitro, we also dissected the molecular mechanism underlining Ghr-mediated regulation of cholangiocyte proliferation.

Results

The mice used in our experiments were genotyped, and it was confirmed that FVBN mice were Mdr2+/+, while Mdr2KO mice were Mdr2−/− (Suppl Fig. 1).

Time-course of serum Ghr in Mdr2KO mice as compared to FVBN control mice. Ghr peptide level was measured in serum of male and female Mdr2KO and FVBN mice from two weeks to six months old (Fig. 1). In FVBN controls, Ghr concentration increased gradually with age until four months, when it reached a plateau. In Mdr2KO mice, there was a significant increase in circulating Ghr in mice from two weeks up to one month, but no further enhancement was detected in later age groups. Interestingly, at each timepoint tested, the level of serum Ghr was lower in Mdr2KO mice as compared to FVBN mice.

Expression of Ghr and MBOAT in the stomach of Mdr2KO and FVBN control mice. We assessed Ghr expression in the stomach of Mdr2KO and FVBN mice, since Ghr is mainly produced in this part of the gastrointestinal system. Ghr mRNA level was significantly lower in cholestatic mice than in normal controls (Fig. 2A). At the translational level, Ghr peptide was also decreased in Mdr2KO mice as compared to FVBN controls (Fig. 2B,C). Similarly, the enzyme which activates Ghr by acylation, MBOAT, was found to be less abundant in Mdr2KO mice over FVBN controls (Fig. 2D–F). There were no significant gender-related differences in Ghr and MBOAT gastric expression.

Cellular distribution of GHS-R1a and Ghr in the liver of Mdr2KO and FVBN control mice. To investigate the expression of GHS-R1a and Ghr in isolated cholangiocytes, hepatocytes and hepatic stellate cells (HSC) from livers of Mdr2KO mice vs FVBN controls, we used laser capture microdissection (LCM). For optimal precision, cholangiocytes, hepatocytes and HSC were labeled for two different markers, i.e. cytokeratin (CK) 19 and CK7 for cholangiocytes, CK8 and albumin (Alb) for hepatocytes, desmin and alpha smooth muscle

Figure 1. Time-course of serum Ghr in Mdr2KO mice vs FVBN controls. Male and female mice from two weeks to six months old were tested for circulating Ghr by EIA. Number of animals, 4; number of serum sample replicates in ELISA test, 3; p < 0.05. *, Mdr2KO vs FVBN mice. #, all mice older than two weeks vs two week old, male FVBN mice.
Figure 2. Gastric Ghr and MBOAT expression in Mdr2KO vs FVBN mice. Stomach tissue from 2 month old male and female Mdr2KO and FVBN mice was tested for RNA and protein expression of Ghr and MBOAT, as described in "Methods" section. (A) Ghr mRNA expression was assessed by RT-qPCR. (B) Quantification of Ghr by analysis of IHC images as shown in (C). (D) MBOAT mRNA as assessed by RT-qPCR. (E) Quantification of MBOAT by image analysis of IHC images. (D) representative images of MBOAT IHC. Number of animals, 4. Number of sample replicates for RT-qPCR, 3. For IHC: 10 images at × 20 magnification for each scanned IHC liver section were taken and analyzed using Image J software, as described in the Methods section. p < 0.05, *, Mdr2KO vs FVBN mice. #, Female vs male.
Ghr treatment of Mdr2KO mice attenuates serum biomarkers of liver disease. The effects of exogenous Ghr and its des-acylated form, DG, on the serum liver enzymes, when administered to cholestatic vs control mice, were assessed. While DG had no effect on alanine aminotransferase (ALT) and aspartate aminotransferase (AST), Ghr induced a significant reduction in serum levels of these liver enzymes in Mdr2KO mice (Fig. 4A,B). Serum bilirubin and albumin were slightly changed in Mdr2KO mice compared to FVB/N control mice treated with vehicle, and the treatment with DG or Ghr corrected these changes (Suppl. Table 1). The inflammatory chemokine CCL2 (C–C chemokine 2), also known as monocyte chemotactic/chemoattractant protein 1 (MCP1) was found to be more than eightfold–increased in serum of male and female Mdr2KO mice compared to FVB/N controls (Fig. 4C). Administration of DG did not change the high plasma level of CCL2, however treatment with Ghr significantly reduced CCL2 in Mdr2KO mice by twofold (Fig. 4C). Serum tumor growth factor beta (TGFβ), the product of the most active profibrogenic gene in hepatic cholestasis, was also tested and found to be increased 3 to 4-fold in both male and female Mdr2KO mice compared to FVB/N control counterparts (Fig. 4D). Treating the Mdr2KO mice with DG had no effect on TGFβ level, while Ghr reduced approximately by half the plasma TGFβ concentration (Fig. 4D). In summary, Ghr, but not DG, was effective in reducing the serum levels of ALT, AST, CCL2 and TGFβ which were remarkably increased in Mdr2KO mice as compared to FVB/N controls.

Ghr decreases intrahepatic bile duct mass (IBDM) and cholangiocyte proliferation in Mdr2KO mice. Because no gender-related differences were found in serum levels of major biomarkers of liver dysfunction in Mdr2KO mice when treated with vehicle, DG or Ghr, we used only male mice for the subsequent experiments.

The effects of DG and Ghr on the expression of CK19 in the liver of cholestatic vs control mice was assessed at mRNA and protein level (Fig. 5A–C). Ghr but not DG, caused a significant reduction in the excessively large IBDM of Mdr2KO mice. Proliferating cell nuclear antigen (PCNA) expression was assessed in cholangiocytes of Mdr2KO mice (Fig. 5D–F). Both the mRNA and protein of PCNA were drastically decreased in Mdr2KO mice after treatment with Ghr, but not with DG.

Hematoxylin and eosin (H&E) staining of liver sections from Mdr2KO mice and FVB/N controls was used to assess the status of hepatocytes, as well as the size and frequency of biliary mass (Fig. 5G). It can be observed that there were no effects of DG or Ghr on the liver histology in FVB/N controls, as expected (top images in Fig. 5G). However, vehicle treated Mdr2KO mice exhibited an increased number of bile ducts, surrounded by thick layers of small cells including string-like shaped HSC. The treatment of Mdr2KO mice with DG had only a small effect on the enlarged biliary mass, however Ghr was very efficient in reducing the size of IBDM. No significant damage was noted in regard to the shape of hepatocytes in Mdr2KO mice, neither steatosis or other histological changes were noted.

These data suggest that exogenously administered Ghr reduces proliferation of cholangiocytes and decreases the size of HSC layers around bile ducts in cholestatic mice.

Ghr treatment alleviates liver fibrosis in Mdr2KO mice. Genes known to be upregulated in cholestasis-induced hepatic fibrosis, including desmin and αSMA markers of HSC, as well as structural proteins of extracellular matrix (ECM) such as collagen types I and III, and integrins, were tested in Mdr2KO mice and FVB/N controls treated with vehicle, DG or Ghr (Fig. 6). All the tested genes were downregulated in Ghr-treated Mdr2KO mice while being insignificantly affected by DG. Thus, the expression of both desmin and αSMA was strongly increased in Mdr2KO mice compared to FVB/N controls (Fig. 6A–F). The treatment with DG did not change the excessive amount of HSC markers detected in Mdr2KO mice, while Ghr induced a significant decrease of them. Collagen types I and III were assessed using Sirius-Red staining of liver sections, and were found to be produced in excess in Mdr2KO mice treated with vehicle or DG but were reduced in Mdr2KO mice treated with Ghr (Fig. 6G–I). To confirm these results, we also assayed the liver hydroxyproline concentrations (Suppl. Fig. 3), and determined that hydroxyproline was excessively expressed in the livers of Mdr2KO mice, and it was not affected by DG, however it was significantly decreased following Ghr treatment (Suppl. Fig. 3). The β6 component of integrins was demonstrated to be more than sevenfold increased at mRNA level in the livers of Mdr2KO mice compared to FVB/N controls, and it was strongly diminished in Mdr2KO mice treated with Ghr, while DG had only small alleviating effect (Fig. 6J). Moreover, IHC assessment of integrin αvβ6 indicated...
Figure 3. Expression of GHS-R1a and Ghr in liver cells of Mdr2KO vs FVBN mice. (A, B) Liver frozen sections from Mdr2KO and FVBN mice, were immunolabeled for CK19 (cholangiocytes), CK7 (hepatocytes) and desmin (HSC). The labeled cells were dissected by laser capture microdissection (LCM) and used for RNA isolation and quantitative determination of GHS-R1a and Ghr mRNAs (fold change relative to GAPDH gene control). Number of animals for each type of treatment, 4. Number of sample replicates for RT-qPCR assay, 3. p < 0.05. *, Mdr2KO vs FVBN mice. C, D At protein level, GHS-R1a and Ghr were detected in same cell types by confocal microscopy colocalization with CK19, CK8 and desmin. (C) Representative images of GHS-R1a (red) colocalization with cholangiocytes, hepatocytes and HSC (green). (D) Representative images of Ghr (red) with cholangiocytes, hepatocytes and HSC (green). The pixels with both green and red fluorescence signals are shown in yellow (also pointed out by arrows). Cell nuclei were stained with DAPI (blue).
that the massive increase in this integrin expression within the thick layers of HSC around enlarged bile ducts in Mdr2KO mice was counteracted by treatment with Ghr, while DG had no effect (Fig. 6K–L).

The expression of additional genes with roles in liver fibrogenesis was tested at mRNA level in Mdr2KO mice treated with vehicle, DG or Ghr, and compared to FVBN controls subjected to similar treatments (Fig. 7). Thus, fibronectin (FN1), integrin component αv, matrix metalloproteinase-2 (MMP2), and tissue inhibitor of MMP-1 (TIMP1) were abnormally increased in Mdr2KO mice treated with vehicle. Ghr but not DG, was effective in reducing mRNA expression of these genes (Fig. 7A–D). Profibrogenic genes such as TGFβ, PDGFα (platelets derived growth factor alpha) and connective tissue growth factor (CTGF) which are major indicators of liver fibrosis, were assayed using qPCR and shown to be significantly increased in Mdr2KO mice compared to FVBN controls (Fig. 7E–G). Interestingly, DG, the less active form of Ghr, had a trend to decrease the expression of these genes, and it had a significant effect on CTGF. Ghr was more effective than DG, drastically lowering the expression of all tested growth factors. Finally, several proinflammatory genes including CCL2, interleukin (IL)-1β, IL-6 were assessed in livers of Mdr2KO mice vs FVBN controls (Fig. 7H–J). CCL2 was the most increased (more than 25-fold), followed by IL-6 (fourfold) and IL-1β (twofold) in Mdr2KO mice compared to FVBN controls. DG did not decrease CCL2 mRNA, but had a significant effect on IL-1β and IL-6, while Ghr reduced the expression of all tested cytokines (Fig. 7H–J).

In summary, major indicators of hepatic fibrosis in Mdr2KO mice including biomarkers of proliferating and activated HSC, ECM structural proteins and modulators, profibrogenic and proinflammatory genes were strongly reduced in mice treated with Ghr. The expressions of fewer genes, namely profibrogenic growth factors and cytokines were also diminished by DG.

**Ghr reduces apoptosis and necrosis in Mdr2KO mouse livers.** To investigate whether DG and Ghr influence apoptosis and necrosis in the Mdr2KO model of cholestasis-induced liver fibrosis, we measured products of these processes using a fluorescence microscopy procedure. Thus, we used a kit for specific staining of phosphatidylserine (PS), a hallmark of apoptosis, and of nuclei of necrosis-damaged cells, and demonstrated that both DG and Ghr significantly decreased apoptosis and necrosis in the liver of Mdr2KO mice (Fig. 8). By image analysis we determined that Ghr was more effective than DG in diminishing apoptosis and necrosis markers.
Figure 5.  Ghr reduces the IBDM in Mdr2KO mice. Liver samples from male Mdr2KO and FVBN mice treated with vehicle, DG or Ghr, were tested for expression of CK19 mRNA, by RT-qPCR (A) and CK protein by IHC (B, C).  (B) Representative images of CK19 (marker of cholangiocytes) IHC.  (C) Quantification of CK19 expression by image analysis using ImageJ software. PCNA, a marker of cell proliferation was also tested at RNA (D) and protein (E, F) level using RT-qPCR and IHC, respectively.  (E) Representative images of PCNA IHC.  (F) Quantification of PCNA-expressing cholangiocytes by analysis of IHC images.  p < 0.05.  * DG or Ghr vs vehicle.  #, Mdr2KO vs FVBN mice. Number of animals in each treatment group, 4. Number of sample replicates in RT-qPCR assay, 3. Number of images from each animal in a treatment group, in IHC assay, 10.  G, Representative images of H&E staining of liver tissue from male and female FVBN (control) mice and Mdr2KO (cholestatic) mice treated with vehicle, DG or Ghr.
Figure 6. Ghrelin reduces the expression of fibrosis markers in livers of Mdr2KO mice. Liver samples from male FVBN and Mdr2KO mice treated with vehicle, DG or Ghr, were used. Desmin mRNA (A) and protein (B, C), αSMA mRNA (D) and protein (E, F), markers of HSC proliferation and activation were detected and quantified by RT-qPCR, IHC and image analysis as described under Methods. (G) Collagen 1A1 (Col1A1) mRNA was assayed by RT-qPCR. (H, I) Collagens I and III were assessed by Sirius Red staining of paraffin sections of liver from FVBN and Mdr2KO mice. (J) Integrin αvβ6 mRNA expression was determined by RT-qPCR. (K) Representative images of integrin αvβ6 IHC in sections of liver. (L) Quantification of αvβ6 protein expression in the liver using analysis of IHC images. Number of animals in each treatment group, 4. Number of sample replicates in RT-qPCR assays, 3. Number of images for each IHC sample, 10. p <0.05. *, DG or Ghr vs vehicle. #, Mdr2KO vs FVBN mice.
Figure 7. Ghrelin attenuates expression markers of hepatic fibrosis and inflammation in Mdr2KO mice. The expression of ECM components FN1 (A), Integrin αv (B), MMP2 (C) and TIMP1 (D) at RNA level was performed by RT-qPCR in male Mdr2KO and FVBN mice that had been treated with vehicle, DG or Ghr. The expression of profibrogenic genes including TGFβ (E), PDGFα (F), CTGF (G), as well as proinflammatory cytokines CCL2 (H), IL-1β (I) and IL-6 (J), was also tested by RT-qPCR in the same groups. Number of animals per treatment group, 4. Number of sample replicates in RT-qPCR assay, 3. p < 0.05, *, DG or Ghr vs vehicle. #, Mdr2KO vs FVBN mice.
Ghr attenuates cholangiocyte proliferation via AMP-activated protein kinase (AMPK) and forkhead box protein O1 (FOXO1) activation pathway. Activation of AMPK in response to Ghr was tested by measuring phospho-AMPK (p-AMPK) in mouse cholangiocytes in vitro (Fig. 9A). The cells were treated with Ghr in the absence or presence of Ca²⁺ chelator BAPTA or AMPK inhibitor dorso morphine (DM), which are known to block AMPK activation. Phospho-AMPK was quantified at various timepoints up to 2 h using an ELISA kit. Ghrelin induced a significant increase in p-AMPK as early as 15 min after treatment and the effect of Ghr lasted up to several hours. The inhibitors of AMPK phosphorylation and activation, BAPTA and DM, counteracted the effect of Ghr up to two hours.

The AMPK-mediated activation of FOXO1 initiated by Ghr signaling in cholangiocytes was also tested (Fig. 9B). FOXO1 activation in cholangiocytes treated with Ghr only or Ghr plus BAPTA or Ghr plus DM was tested, demonstrating that Ghr induced a two-fold increase in binding of activated FOXO1 to specific DNA response elements. This activation of FOXO1 was not detected in the presence of BAPTA and DM (Fig. 8B), suggesting that FOXO1 activation is dependent on AMPK activation.

It is known that FOXO1 is located inside the nuclei when activated, binding promoters of target genes with roles in cell proliferation control. We assessed the nuclear translocation induced by Ghr in cholangiocytes...

Figure 8. Treatment of Mdr2KO mice with Ghr and DG reduces apoptosis and necrosis markers in the liver. Frozen sections of livers from male Mdr2KO (A) and FVBN (B) mice treated with vehicle, DG or Ghr, were processed for fluorescence labeling of apoptosis (green) and necrosis (red) markers, while the cell nuclei were stained in blue using DAPI, as described under “Methods”. Panels (A) and (B) present representative images of each treatment group. (C, D) Graphs of quantitative determinations of apoptosis and necrosis, respectively, by image analysis using Image J software. Number of animals in each treatment, 4. Number of images per each sample, 10. p < 0.05, *, DG or Ghr vs vehicle. #, Mdr2KO vs FVBN mice.
(Fig. 9C). In cells treated with vehicle, FOXO1 was mostly cytoplasmic, detected around nuclei, while after 15 min–2 h of Ghr treatment, FOXO1 was detected inside nuclei in many cells. The inhibitors of AMPK activation, BAPTA and DM, prevented Ghr-induced accumulation of FOXO1 inside nuclei, and even caused upregulation of FOXO1 in the cytoplasm. Some proliferating cells were detected one hour or longer after treatments with BAPTA or DM in addition to Ghr.

To further test the possibility that Ghr affects the proliferation rate of cholangiocytes via AMPK-FOXO1 signaling pathway, we assessed cell proliferation after treatment with Ghr alone or in addition to BAPTA or DM (Fig. 9D). Ghr suppressed cell proliferation while BAPTA and DM prevented this effect (Fig. 9D). To confirm these results, we also measured the incorporation of IDU into nuclei of mouse cholangiocytes treated with vehicle, Ghr, Ghr plus BAPTA, and Ghr plus DM (Fig. 9E,F). The results suggested that Ghr reduced cell proliferation, however BAPTA and DM attenuated this effect.

Taken together, these data demonstrate that Ghr inhibits cholangiocyte proliferation via a mechanism involving Ca²⁺ and AMPK-mediated nuclear translocation of FOXO1.

Silencing of GHS-R1a with siRNA in mouse cholangiocytes reverses the effects of Ghr. In order to assess the role of GHS-R1a in signaling FOXO1 nuclear translocation and suppression of cell proliferation rate, we knocked down GHS-R1a mRNA, and confirmed the reduction of GHS-R1a expression (Fig. 10A). Mouse cholangiocytes transfected with GHS-R1a-specific siRNA, were then treated with vehicle or Ghr for 15 to 60 min, and AMPK phosphorylation was measured (Fig. 10B). Similarly, cells transfected with negative control siRNA were used as controls. P-AMPK induced by Ghr, was reduced in GHS-R-siRNA transfected cells compared to cells transfected with negative control siRNA, suggesting that Ghr's effect is dependent of GHS-R1a. FOXO1 transactivation measurements indicated that GHS-R1a knockdown impaired Ghr-induced FOXO1 transactivation (Fig. 10C). These data were confirmed by the immunofluorescence colocalization of FOXO1 with nuclei in cholangiocytes transfected with GHS-R1a-siRNA vs negative control siRNA (Fig. 10D). Moreover, the assessment of Ghr-induced reduction of cell proliferation rate was performed by two procedures: one measuring the percent of live cells (Fig. 10E), and the other by measuring the incorporation of IDU into newly synthesized DNA during cell replications (Fig. 10F). Both procedures indicated that knockdown of GHS-R1a reduced the ability of Ghr to stimulate cholangiocyte proliferation.

Discussion

Since its discovery, Ghr has been largely investigated as an endogenous gastric orexigenic peptide with high affinity for GHS-R1a, or the control of glucose metabolism and body weight, however, Ghr's role in the cholestatic liver is poorly understood. In the present study using Mdr2KO mice as a model of biliary cholestasis-induced hepatic fibrosis, we investigated endogenous Ghr levels in chronic cholestasis, as well as the effects of exogenously administered Ghr and its less active form DG on biliary and hepatic pathology. Serum levels of total Ghr in Mdr2KO mice were lower than in FVBN control mice, demonstrating that hepatobiliary dysfunction resulted in strong downregulation of GHR gene expression in the stomach, the main source of systemic DG and Ghr. These results indicate that during cholestasis, the gastric biosynthesis and activation of DG to fully active Ghr, are drastically impaired, further aggravating the liver injury.

It has been previously reported that Ghr can promote cell survival and inhibit apoptosis, having therapeutic effects on myocardial, intestinal and pancreatic injuries. In our study we demonstrate that Ghr attenuates DG to fully active Ghr, are drastically impaired, further aggravating the liver injury. Since its discovery, Ghr has been largely investigated as an endogenous gastric orexigenic peptide with high affinity for GHS-R1a, or the control of glucose metabolism and body weight. Ghr's role in the cholestatic liver is poorly understood. In the present study using Mdr2KO mice as a model of biliary cholestasis-induced hepatic fibrosis, we investigated endogenous Ghr levels in chronic cholestasis, as well as the effects of exogenously administered Ghr and its less active form DG on biliary and hepatic pathology. Serum levels of total Ghr in Mdr2KO mice were lower than in FVBN control mice, demonstrating that hepatobiliary dysfunction resulted in strong downregulation of GHR gene expression in the stomach, the main source of systemic DG and Ghr. These results indicate that during cholestasis, the gastric biosynthesis and activation of DG to fully active Ghr, are drastically impaired, further aggravating the liver injury.

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The possibility of Ghr being a hepatoprotective factor was investigated in only a few previous studies. Iseri et al. (2008) demonstrated that exogenous Ghr had anti-inflammatory and antioxidant effects in BDL rats, and proposed a potential therapeutic value of Ghr in protecting against hepatic fibrosis and oxidative injury due to biliary obstruction. Later on, Moreno et al. (2010) studied the effect of recombinant Ghr on acute, carbon tetrachloride-induced liver injury, demonstrating that markers of hepatic fibrosis were increased in GHR null mice as compared to wild type mice. A more recent study proposed a role of Ghr in protecting against liver injury via nitric oxide release. In the present study, we investigated the expression of Ghr and its receptor GHS-R1a in the liver of Mdr2KO mice compared to FVBN control mice, and found that both of these genes were downregulated in the cholestatic mice. The cellular distribution of Ghr and its receptor GHS-R1a in various types of cells in the liver was also studied, suggesting that Ghr and its receptor were expressed more in cholangiocytes and hepatocytes than in HSC, in FVBN mice, and both Ghr and GHS-R1a were downregulated in these cells of Mdr2KO mice. These results indicate that in addition to Ghr being decreased in the liver of cholestatic mice, GHS-R1a was also downregulated in these mice, impairing Ghr function in the liver. Treatment of cholestatic mice with Ghr increased the expression of GHS-R1a, suggesting a positive feedback loop of GHS-R1a regulation by Ghr. When Ghr was exogenously administered to cholestatic mice, there was a remarkable improvement of the IBDM and fibrosis markers.

The influence of DG and Ghr on proinflammatory and profibrogenic genes in Mdr2KO mice vs FVBN control mice was also addressed in our study. Ghr significantly reduced the liver levels of cytokines such as CCL2, IL-1β and IL-6, as well as profibrogenic genes including TGFβ, PDGFA, CTGF. Interestingly, DG was also effective...
A. Graph showing p-AMPK (OD/mg protein) levels over time (15 min, 30 min, 1 h, 2 h) for different treatments: Vehicle, Ghr, Ghr+BAPTA, Ghr+DM. Bars marked with * indicate significant changes compared to Vehicle, and bars marked with # indicate significant changes compared to Ghr.

B. Graph showing FOXO1 Activity with Fold change for Vehicle, Ghr, Ghr+BAPTA, Ghr+DM. Bars marked with * indicate significant changes compared to Vehicle, and bars marked with # indicate significant changes compared to Ghr.

C. Fluorescence images of FOXO1, DAPI, and Merge for Vehicle, Ghr, Ghr+BAPTA, Ghr+DM treatments. Scale bars indicate measurement size.

D. Graph showing Cell Proliferation by MTS for Vehicle, Ghr, Ghr+BAPTA, Ghr+DM. Bars marked with * indicate significant changes compared to Vehicle.

E. Graph showing percent IDU-labeled cells (%) for Vehicle, Ghr, Ghr+BAPTA, Ghr+DM. Bars marked with * indicate significant changes compared to Vehicle.

F. Fluorescence images showing IDU-labeled cells for Vehicle, Ghr, Ghr+BAPTA, Ghr+DM treatments. Scale bars indicate measurement size.
in attenuating the expression of the tested profibrogenic genes, especially CTGF. However, a significant effect in terms of expression of ECM proteins such as FN1, Col 1A1, integrin αvβ6, was observed only for Ghr in its acylated form.

We focused on a possible effect of Ghr on cholangiocytes in particular, based on the observation that the IBDM was significantly reduced in cholestatic mice when treated with Ghr. Using mouse cholangiocytes in culture, we demonstrated that Ghr-activated GHS-R1 receptor initiated a signaling pathway conducive to FOXO1 activation and negative regulation of cell proliferation. Thus, our results indicate that Ghr treatment of cholangiocytes caused activation of AMPK via a Ca2+-dependent signaling, since BAPTA, a Ca2+ chelator, prevented this process. Moreover, Ghr induced FOXO1 nuclear translocation, FOXO1 activation, and a significant decrease in proliferation of cholangiocytes. Both BAPTA and an inhibitor of AMPK activity, dorso morphine, had a negative effect on Ghr-induced nuclear localization and activation of FOXO1. Several studies on FOXO1 roles in the regulation of cell cycle progression have found that FOXO1 has a growth-inhibitory effect through apoptosis or cell cycle arrest. Thus, FOXO1 is a member of FOXO transcription factors that induce cell cycle arrest at G1 phase by modulation of CDK (cycle-dependent kinase) inhibitors p27KIP1 and p21 WAF, as well as cyclins D1 and D3. The subcellular localization of FOXO1 can be influenced by post-translational modifications including acetylation by sirtuins, or serine/threonine phosphorylation by nuclear or cytoplasmic protein kinases. FOXO1 was demonstrated to be involved in progranulin (PGRN)-induced proliferation of cholangiocytes, where PGRN decreased sirtuin 1 expression and increased acetylation of FOXO1 resulting in the cytoplasmic accumulation of FOXO1. In cholangiocarcinoma cells, IL-6-dependent activation of PGRN increased cell proliferation by a mechanism involving protein kinase B (Akt) phosphorylation followed by nuclear exclusion of FOXO1. In this study, we demonstrate that unlike PGRN, Ghr induces the activation of FOXO1 and its translocation from cytoplasm into nuclei, decreasing the cell proliferation of cholangiocytes in vitro. We also showed that GHS-R1a knockdown with siRNA, impaired Ghr signaling in cholangiocytes, suggesting that Ghr effects on AMPK/FOXO1-mediated control of cholangiocyte proliferation is mediated by this receptor.

In regard to Ghr-induced activation of AMPK via GHS-R1a, it is known that GHS-R1a, once activated, acts as a G-protein coupled receptor upon plasma membrane phospholipases which then produce inositol triphosphate (IP3), a secondary messenger to endoplasmic reticulum causing the release of Ca2+ into the cytoplasm. The increase in Ca2+ stimulates calmodulin-dependent protein kinase kinase (CaMKK) to phosphorylate and activate AMPK. AMPK is a cellular energy sensor and its beneficial role in ameliorating hepatic fibrosis has been extensively reviewed. However, the role of AMPK as related to Ghr and its receptor in cholangiocytes is newly described in this work. Previously published data show that AMPK becomes activated in response to various stimuli including low levels of ATP, increased metabolic and oxidative stress, and promotes expression of genes related to energy production and resistance to cellular stress, in a FOXO-dependent manner. In cholestasis, cholangiocytes undergo a process of activation also known as ductular reaction, in which the cell proliferation rate is abnormally increased, and FOXO1, a member of FOXO family of transcription factors with roles in cell fate regulation, is expressed in cholangiocytes. Our data suggest that Ghr acts via GHS-R1a and modulates the nuclear vs cytoplasmic distribution of FOXO1, causing a reduction in cholangiocyte proliferation.

In conclusion, Ghr levels in serum, liver and stomach of Mdr2KO mice were significantly lower compared to FVBN control mice. Exogenously administered Ghr reduced serum liver enzyme levels, biliary hyperplasia and hepatic fibrosis in the Mdr2KO mouse model of hepatic cholestasis. In vitro experiments using mouse cholangiocytes demonstrated that Ghr induced activation of AMPK and FOXO1 and reduced cell proliferation. All these results suggest that Ghr and its receptor GHS-R1a have relevant roles in modulating bile duct proliferation and liver fibrogenesis in the context of cholestasis.

Methods
Chemicals, kits, antibodies, tissue culture media. All chemicals were purchased from MilliporeSigma (Burlington, MA) unless otherwise stated, and were of the highest grade available. Ghr1-36, BAPTA (Ca2+ chelator, inhibitor of AMPK phosphorylation) and DM (inhibitor of AMPK activity) were purchased from Tocris Bioscience (Minneapolis, MN). Hydroxyproline assay kit was purchased from Millipore Sigma (St. Louis,
A. GHS-R1a mRNA

B. P-AMPK

C. FOXO1 Activity

D. Immunofluorescence images

E. Cell proliferation by MTS

F. IDU-labeled cells (%)
Assessment of mRNA expression. Assessment of gene expression at the mRNA level in liver tissue or cultured cells was performed by real time quantitative PCR (RT-qPCR) for Ghr, GHS-R1a, CK19, PCNA, desmin, αSMA, ColIα1, MMP2, TIMP1, integrin β6, integrin αv, FN1, TGFβ, PDGFα, CTGF, CCL2, IL-1β, IL-6. Fold changes in gene expression were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Total RNA was isolated by using RNeasy kit, followed by cDNA synthesis with iScript kit from Bio-Rad Life Sciences (Hercules, CA), and RT-qPCR using iTaq Universal SYBR-Green Supermix from the same company. RT2 qPCR Primer Assays were purchased from Qiagen (Frederik, MD). A list of all primers used, is presented in supplemental Table 1. AriaMx Real-Time PCR system thermal cycler from Agilent Technologies (Santa Clara, CA) was used for running RT-qPCR. The data was analyzed as previously described.

Assessment of biliary hyperplasia and liver fibrosis in Mdr2KO and FVBN mice. The IBDM was assessed by immunohistochemistry (IHC) for CK19 to estimate biliary hyperplasia. The hepatic fibrosis markers desmin, αSMA and integrin αvβ6 were assayed by IHC of liver tissue from mice treated with vehicle, DG or Ghr. Liver tissue sections (4 µm thick) were immunolabeled with primary antibodies specific to proteins of interest, and then processed for staining with VectaStain kits (Burlingame, CA). Solutions of 2–5 µg/mL primary antibodies were used. The IHC slides were scanned with a Leica SCN400 scanner at 20 × magnification, followed by screenshots at 10 × magnification, and image analysis with ImageJ software version 1.41, downloaded from the NIH website (https://imagej.nih.gov). For all samples and controls, the percent areas of stained pixels were calculated and compared for significant differences. Liver samples were also assayed by using Sirius Red specific staining of collagens I and III which are increased in hepatic fibrosis, with the kit from IHC World (Ellicott City, MD).
Assessment of serum Ghr, liver enzymes, cytokines and growth factors. Ghrelin was assayed in serum of Mdr2KO and FVBN mice, using an ELISA kit purchased from Phoenix Pharmaceuticals, Inc (Burlingame, CA), according to the manufacturer’s instructions. Serum markers for liver function, ALT and AST, from mice treated with vehicle, Ghr or DG were assessed using the IDEXX Catalyst One analyzer from IDEXX Laboratories (Westbrook, ME). Proinflammatory cytokine CCL2 and profibrogenic growth factor TGFβ1 were assessed using ELISA kits from ThermoFisher (Waltham, MA).

Assessment of AMPK and FOXO1 activation in mouse cholangiocytes. Cholangiocytes were treated with vehicle. Ghr, Ghr and BAPTA or Ghr and DM for 15 min to 2 h and then processed for ELISA for p-AMPK with the PathScan Phospho-AMPK (Thr172) ELISA kit from Cell Signaling Technology Inc. (Danvers, MA), and TransAM FKHR kit from Active Motif (Carlsbad, CA). Prior to the ELISA assay for FKHR or FOXO1 activity, the cell nuclei were isolated using a Nuclear Extract kit from Active Motif (Carlsbad, CA).

Cell proliferation of cholangiocytes treated with Ghr in the absence or presence of BAPTA or DM was assessed using the MTS kit from Abcam (Cambridge, MA), according to manufacturer’s instructions. Incorporation of IDU (5-Iodo-2′-deoxyuridine from Millipore Sigma, Saint Louis, MO) into DNA in proliferative cholangiocytes was also performed using BrdU/IdU-specific antibody from BD Biosciences (San Jose, CA). Briefly, the cells were incubated with culture medium containing 25 μM IDU for 2 h at 37 °C under 5% CO2, then washed with PBS, fixed with 4% paraformaldehyde, permeabilized with 0.5% Triton in PBS (PBS-Triton), followed by treatments with 1 N and 2 N HCl for 10 min each on ice, methanol/acetic acid (3:1 vol/vol) for 10 min at room temperature and washed with PBS-Triton. The cells were further immunolabeled with BrdU/IDU antibody (5 μg/mL) at 4 °C overnight followed by secondary antibody-conjugated with fluorescent dye for 1 h at room temperature. The proliferative cells were counted on confocal images, and the results were expressed in % cells that had IDU incorporated in their nuclei.

Assessment of Ghr and GHS-R1α expression in the liver by LCM. Frozen sections of liver (8 μm thick) from two month old male and female FVBN and Mdr2KO mice were processed for IF by blocking of non-specific binding with 4% bovine serum albumin (BSA) in phosphate buffer saline (PBS) supplemented with 0.5% Tween 20 (PBST), followed by two hour incubation with 2–5 μg/mL primary antibody in PBST/BSA at 4 °C, and subsequent labeling with Alexa Fluor 488-conjugated secondary antibody incubation for 1 h at room temperature. The liver sections were labeled with antibodies specific to markers of cholangiocytes, hepatocytes and HSC. Subsequently, a Leica LMD7000 microdissection system (Temple Health & Bioscience District, Temple TX) was used to isolate the specific liver cells. The RNA isolation from batches of 500–1000 cells, was achieved using Arcturus PicoPure Frozen RNA Isolation kit from Thermo Fisher Scientific (Waltham, MA). The expression of Ghr, GHS-R1α and β-actin was then assessed by the same procedure described for RT-qPCR.

Assessment of Ghr distribution in different types of liver cells by confocal microscopy. The presence of Ghr in cholangiocytes, HSC and hepatocytes, was detected by double fluorescent labeling of frozen liver sections with a mix of antibodies specific to Ghr and one of the three cell markers, i.e. CK19, CK8 and desmin. The overlay of red fluorescence-labeled Ghr with the green fluorescence-labeled cells was observed by using a confocal laser scanning system from Leica Microsystems Inc. (Buffalo Grove, IL).

Assessment of apoptosis and necrosis in liver from FVBN and Mdr2KO mice. We used an Apoptosis/Necrosis detection kit from Abcam (catalog number ab176749) and followed the manufacturer’s instructions with a few adjustments for frozen sections. Thus, frozen sections of livers from Mdr2KO and FVB/N mice were washed briefly with PBS, fixed with 4% paraformaldehyde for 5 min at room temperature and incubated with 1 × Apopxin Green Indicator to stain phosphatidylserine which is exposed by apoptotic cells. The liver sections were then washed briefly with PBS followed by incubation with 1 × 7-AAD (7-aminactinomycin D), a membrane impermeable dye which labels nuclei of damaged cells. Cells labeled by 7-AAD appear in red fluorescence in confocal microscopy. In the end the sections were mounted in Prolong Gold antifade mountant with DAPI from InVitrogen (Carlsbad, CA).

Knockdown of GHS-R1α mRNA in mouse cholangiocytes. Mouse cholangiocytes were plated in six-well plates (for ELISA assays) or in chamber slides (for IF assays) at 30–40% confluency and transfected the same day using Lipofectamine 2000 according to manufacturer’s instructions. The GHS-R1α and negative control siRNAs were purchased from Ambion through Thermofisher (Waltham, MA). Validation for GHS-R1α knockdown was performed in cholangiocytes 48 h after the siRNA transfection, using RT2 qPCR Primer Assays for GHS-R1α purchased from Qiagen (Frederik, MD). Also, at 48 h after the transfection, the cells were treated with vehicle or Ghr for 30 min, and then processed for ELISA or IF and confocal microscopy. Cell proliferation of transfected cells was also assessed using the MTS kit from Abcam. In this experiment the cells were plated in 96-well plate with transfection reagents, incubated for 48 h, followed by treatments with vehicle or Ghr in the presence of MTS reagent for 4 h.

Statistics. Quantifications by RT-qPCR, ELISA and image analysis were analyzed by calculating the average and standard error of the mean (SEM) of at least three replicates for each group of tested animals. The number of animals (n) used for each treatment or control group was 4–5, as specified in the Results section for each experiment. The statistical difference was calculated between two groups by using the Student’s T-test, and was
considered significant for p values less than 0.05. When multiple groups of animals were compared, a two-way ANOVA was used followed by an appropriate post-hoc test using GraphPad Prism software (San Diego, CA).

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References
1. Al Massadi, O., Lopez, M., Ferro, J., Dieguez, C. & Nogueiras, R. What is the real relevance of endogenous ghrelin? Peptides 70, 1–6 (2015).
2. Lim, C. T., Kola, B. & Korbonits, M. The ghrelin/GOAT/GHS-R system and energy metabolism. Rev. Endocr. Metab. Disord. 12, 173–186 (2011).
3. St-Pierre, D. H., Wang, L. & Tache, Y. Ghrelin: A novel player in the gut-brain regulation of growth hormone and energy balance. News Physiol. Sci. 18, 242–246 (2003).
4. Takahashi, H. et al. Ghrelin enhances glucose-induced insulin secretion in scheduled meal-fed sheep. J. Endocrinol. 189, 67–75 (2006).
5. Meier, U. & Gressner, A. M. Endocrine regulation of energy metabolism: Review of pathobiocmical and clinical chemical aspects.
6. Muller, T. D. et al. Ghrelin. Mol. Metab. 4, 437–460 (2015).
7. Callahan, H. S. et al. Postprandial suppression of plasma ghrelin level is proportional to ingested caloric load but does not predict intermeal interval in humans. J. Clin. Endocrinol. Metab. 89, 1319–1324 (2004).
8. Cummings, D. E., Frayo, R. S., Marmonier, C., Aubert, R. & Chapelot, D. Plasma ghrelin levels and hunger scores in humans initiating meals voluntarily without time- and food-related cues. Am. J. Physiol. Endocrinol. Metab. 287, E297-304 (2004).
9. Howard, A. D. et al. A receptor in pituitary and hypothalamus that functions in growth hormone release. Science 273, 974–977 (1996).
10. Liu, B., Garcia, E. A. & Korbonits, M. Genetic studies on the ghrelin, growth hormone secretagogue receptor (GHSR) and ghrelin O-acetyl transferase (GOAT) genes. Peptides 32, 2191–2207 (2011).
11. Tong, J. et al. The pharmacokinetics of acyl, des-acyl, and total ghrelin in healthy human subjects. Eur. J. Endocrinol. 168, 821–828 (2013).
12. Ariyasu, H. et al. Transgenic mice overexpressing des-acyl ghrelin show small phenotype. Endocrinology 146, 355–364 (2005).
13. Yang, J., Brown, M. S., Liang, G., Grishin, N. V. & Goldstein, I. L. Identification of the acyltransferase that octanoylates ghrelin, an appetite-stimulating peptide hormone. Cell 132, 387–396 (2008).
14. Gutierrez, J. A. et al. Ghrelin octanoylation mediated by an orphan lipid transferase. Proc. Natl. Acad. Sci. U S A 105, 6320–6325 (2008).
15. Banks, W. A., Burney, B. O. & Robinson, S. M. Effects of triglycerides, obesity, and starvation on ghrelin transport across the blood-brain barrier. Peptides 29, 2061–2065 (2008).
16. De Vriese, C. et al. Ghrelin degradation by serum and tissue homogenates: Identification of the cleavage sites. Endocrinology 145, 4979–5005 (2004).
17. Yoshimoto, A. et al. Plasma ghrelin and desacyl ghrelin concentrations in renal failure. J. Am. Soc. Nephrol. 13, 2748–2752 (2002).
18. Dornelles, C. T. et al. Ghrelin, leptin and insulin in cirrhotic children and adolescents: Relationship with cirrhosis severity and nutritional status. Regul. Pept. 180, 26–32 (2013).
19. Tacke, F. et al. Ghrelin in chronic liver disease. J. Hepatol. 38, 447–454 (2003).
20. Marchesi, G. et al. Plasma ghrelin concentrations, food intake, and anorexia in liver failure. J. Clin. Endocrinol. Metab. 89, 2136–2141 (2004).
21. Takahashi, H., Kato, A., Onodera, K. & Suzuki, K. Fasting plasma ghrelin levels reflect malnutrition state in patients with liver cirrhosis. Hepatol. Res. 34, 117–123 (2006).
22. Breidert, M., Zimmermann, T. F., Schneider, R., Ehninger, G. & Brabant, G. Ghrelin/leptin imbalance in patients with primary biliary cirrhosis. Exp. Clin. Endocrinol. Diabetes 112, 123–126 (2004).
23. Moreno, M. et al. Ghrelin attenuates hepatocellular injury and liver fibrogenesis in rodents and influences fibrosis progression in humans. Hepatology 51, 974–983 (2010).
24. Isrei, S. O. et al. Ghrelin alleviates biliary obstruction-induced chronic hepatic injury in rats. Regul. Pept. 146, 73–79 (2008).
25. Smit, J. J. et al. Homozygous disruption of the murine mdr2 P-glycoprotein gene leads to a complete absence of phospholipid from bile and to liver disease. Cell 75, 451–462 (1993).
26. Lamert, F. et al. Spontaneous cholecysto- and hepatolithiasis in Mdr2-/- mice: a model for low phospholipid-associated cholelithiasis. Hepatology 39, 117–128 (2004).
27. Katzenellenbogen, M. et al. Molecular mechanisms of liver carcinogenesis in the mdr2-knockout mice. Mol. Cancer Res. 5, 1159–1170 (2007).
28. Trauner, M., Fickert, P. & Wagner, M. MDR3 (ABC14) defects: A paradigm for the genetics of adult cholestatic syndromes. Semin. Liver Dis. 27, 77–98 (2007).
29. Rosmorduc, O., Hermelin, B. & Poupon, R. MDR3 gene defect in adults with symptomatic intrahepatic and gallbladder cholesterol cholelithiasis. Gastroenterology 120, 1459–1467 (2001).
30. Iacuquenin, E. et al. The wide spectrum of multidrug resistance 3 deficiency: From neonatal cholestasis to cirrhosis of adulthood. Gastroenterology 120, 1448–1458 (2001).
31. Petrescu, A.D., et al. Glucocorticoids cause gender-dependent reversal of hepatic fibrosis in the MDR2-knockout mouse model. Int. J. Mol. Sci. 18 (2017).
32. Petrescu, A. D. et al. Coordinated targeting of galanin receptors on cholangiocytes and hepatic stellate cells ameliorates liver fibrosis in multidrug resistance protein 2 knockout mice. Am. J. Pathol. 190, 586–601 (2020).
33. Meng, F. et al. Ursodeoxycholate inhibits mast cell activation and reverses biliary injury and fibrosis in Mdr2(-/-) mice and human primary sclerosing cholangitis. Lab Invest. 98, 1465–1477 (2018).
34. Kennedy, L. et al. Blocking H1/H2 histamine receptors inhibits damage/fibrosis in Mdr2(-/-) mice and human cholangiocarcinoma tumorigenesis. Hepatology 68, 1042–1056 (2018).
35. Jones, H. et al. Inhibition of mast cell-secreted histamine decreases biliary proliferation and fibrosis in primary sclerosing cholangitis Mdr2(-/-) mice. Hepatology 64, 1202–1216 (2016).
36. Schmidt, M. et al. Cell cycle inhibition by FoxO forkhead transcription factors involves downregulation of cyclin D. Mol. Cell Biol. 22, 7842–7852 (2002).
37. Frescas, D., Valenti, L. & Accili, D. Nuclear trapping of the forkhead transcription factor FoxO1 via Sirt-dependent deacetylation promotes expression of glucoconogenic genes. J. Biol. Chem. 280, 20589–20595 (2005).
38. Serrif, M., Goldstone, A. P. & Korbonits, M. Ghrelin in obesity and endocrine diseases. Mol. Cell Endocrinol. 340, 15–25 (2011).
39. Sun, Y. et al. Ghrelin suppresses Purkinje neuron P-type Ca(2+)-channels via growth hormone secretagogue type 1a receptor, the betagamma subunits of Go-protein, and protein kinase a pathway. Cell Signal. 26, 2530–2538 (2014).
40. Andrews, Z. B. The extra-hypothalamic actions of ghrelin on neuronal function. Trends Neurosci. 34, 31–40 (2011).
41. Castaneda, T. R., Tong, J., Datta, R., Cuiller, M. & Tschop, M. H. Ghrelin in the regulation of body weight and metabolism. Front. Neuroendocrinol. 31, 44–60 (2010).
42. Gonzalez-Rey, E., Chorny, A. & Delgado, M. Therapeutic action of ghrelin in a mouse model of colitis. Gastroenterology 130, 1707–1720 (2006).
43. Granata, R. et al. Acylated and unacylated ghrelin promote proliferation and inhibit apoptosis of pancreatic beta-cells and human islets: involvement of 3’5’-cyclic adenosine monophosphate/protein kinase A, extracellular signal-regulated kinase 1/2, and phosphatidylinositol 3-Kinase/Akt signaling. Endocrinology 148, 512–529 (2007).
44. Li, L. et al. Cardioprotective effects of ghrelin and des-octanoyl ghrelin on myocardial injury induced by isoproterenol in rats. Acta Pharmacol. Sin. 27, 527–535 (2006).
45. Ceronowicz, P., et al. Essential role of growth hormone and IGF-1 in therapeutic effect of ghrelin in the course of acetic acid-induced colitis. Int. J. Mol. Sci. 18 (2017).
46. Kabil, N. N., Seddiek, H. A., Yassin, N. A. & Gamal-Eldin, M. M. Effect of ghrelin on chronic liver injury and fibrogenesis in male rats: Possible role of nitric oxide. Peptides 52, 90–97 (2014).
47. Dijkers, P. F., Medema, R. H., Lammers, J. W., Koenderman, L. & Cofer, P. J. Expression of the pro-apoptotic Bcl-2 family member Bim is regulated by the forkhead transcription factor FKHR-L1. Curr. Biol. 10, 1201–1204 (2000).
48. Xing, Y. Q. et al. The regulation of FOXO1 and its role in disease progression. Life Sci. 193, 124–131 (2018).
49. Kops, G. J. et al. Control of cell cycle exit and entry by protein kinase B-regulated forkhead transcription factors. Mol. Cell Biol. 22, 2025–2036 (2002).
50. Dijkers, P. E. et al. Forkhead transcription factor FKHR-L1 modulates cytokine-dependent transcriptional regulation of p27(KIP1). Mol. Cell. Biol. 20, 9138–9148 (2000).
51. Medema, R. H., Kops, G. J., Bos, J. L. & Burgering, B. M. AFX-like Forkhead transcription factors mediate cell-cycle regulation by Ras and PKB through p27kip1. Nature 404, 782–787 (2000).
52. Frampton, G. et al. The novel growth factor, progranulin, stimulates mouse cholangiocyte proliferation via sirtuin-1-mediated inactivation of FOXO1. Am. J. Physiol. Gastrointest. Liver Physiol. 303, G1202–1211 (2012).
53. Demorrow, S. Programulin: A novel regulator of gastrointestinal cancer progression. Transl. Gastrointest. Cancer 2, 145–151 (2013).
54. Frampton, G. et al. Interleukin-6-driven progranulin expression increases cholangiocarcinoma growth by an Akt-dependent mechanism. Gut 61, 268–277 (2012).
55. Carling, D. AMPK signalling in health and disease. Curr. Opin. Cell Biol. 45, 31–37 (2017).
56. Hardie, D. G., Ross, F. A. & Hawley, S. A. AMPK: A nutrient and energy sensor that maintains energy homeostasis. Nat. Rev. Mol. Cell Biol. 13, 251–262 (2012).
57. Hurley, R. L. et al. The Ca2+/calmodulin-dependent protein kinase isoforms are AMP-activated protein kinase kinases. J. Biol. Chem. 280, 29060–29066 (2005).
58. Liang, Z. et al. AMPK: A novel target for treating hepatic fibrosis. Oncotarget 8, 62780–62792 (2017).
59. Greer, E. L., Banko, M. R. & Brunet, A. AMP-activated protein kinase and FoxO transcription factors in dietary restriction-induced longevity. Ann. N. Y. Acad. Sci. 1170, 688–692 (2009).
60. Yun, H. et al. AMP-activated protein kinase mediates the antioxidant effects of resveratrol through regulation of the transcription factor FoxO1. FEBS J. 281, 4421–4438 (2014).
61. McMillin, M., Frampton, G., Grant, S. & DeMorrow, S. The neuropeptide galanin is up-regulated during cholestasis and contributes to cholangiocyte proliferation. Am. J. Pathol. 187, 819–830 (2017).
62. Quinn, M. et al. Suppression of the HPA axis during extrahepatic biliary obstruction induces cholangiocyte proliferation in the rat. Am. J. Physiol. Gastrointest. Liver Physiol. 302, G182–193 (2012).
63. Hall, C. et al. Regulators of cholangiocyte proliferation. Gene Expr. 17, 155–171 (2017).
64. Maugham, M. L. et al. No effect of unacylated ghrelin administration on subcutaneous PC3 xenograft growth or metabolic parameters in a Rag1-/- mouse model of metabolic dysfunction. PLoS ONE 13, e0198495 (2018).
65. Livak, K. J. & Schmittgen, T. D. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta CT(T)) method. Methods 25, 402–408 (2001).

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Author contributions
S.D. formulated the study, designed the project, wrote the manuscript. A.P. performed assays and data analysis, organized figures, wrote the manuscript. S.G. and E.W. performed animal experiments, tissue collection, assays, data analysis. G.F., A.N., E.R., M.M. performed assays and data analysis. All authors critically edited and approved the final manuscript.

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Competing interests
The authors declare no competing interests.

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