Depletion of hepatic forkhead box O1 does not affect cholelithiasis in male and female mice

Xiaoyun Feng1,2*, Cuiling Zhu1,3*, Sojin Lee1, Jingyang Gao1,3, Ping Zhu1,4, Jun Yamauchi1, Chenglin Pan1,5, Sucha Singh6,7, Shen Qu3, Rita Miller1, Satdarshan P. Monga6,7, Yongde Peng2 and H. Henry Dong1,7#

1 Division of Endocrinology and Diabetes, Department of Pediatrics, UPMC Children’s Hospital of Pittsburgh, University of Pittsburgh School of Medicine, Pittsburgh, PA 15224, USA
2 Department of Endocrinology & Metabolism, Shanghai General Hospital, Shanghai Jiaotong University, Shanghai 200080, P.R. China
3 Department of Endocrinology & Metabolism, Shanghai 10th People's Hospital, Tongji University School of Medicine, Shanghai 200072, P.R. China
4 Department of Endocrinology and Metabolism, Guangzhou Red Cross Hospital, Medical College of Jinan University, Guangzhou 510220, P.R. China
5 Department of Pediatrics, Shanghai 10th People's Hospital, Tongji University School of Medicine, Shanghai 200072, P.R. China
6 Division of Experimental Pathology, Department of Pathology, University of Pittsburgh School of Medicine, Pittsburgh, PA 15224, USA
7 Pittsburgh Liver Research Center, Department of Pathology, University of Pittsburgh School of Medicine, Pittsburgh, PA 15224, USA

Running title: FoxO1 in gallstone disease

* Authors who contributed equally to this study.
# To whom correspondence should be addressed: H. Henry Dong, UPMC Children’s Hospital of Pittsburgh, Rangos Research Center, 4401 Penn Avenue, Pittsburgh, PA 15224, USA. Tel: +1 (412)692-6324, Fax: +1 (412)692-5809, Email: dongh@pitt.edu

Keywords: cholelithiasis, gallstone, liver, farnesoid X receptor (Fxr), forkhead box O1 (FoxO1), bile acid metabolism, mice, obesity, insulin resistance, metabolic disorder

Abstract: Cholelithiasis is one of the most prevalent gastroenterological diseases and is characterized by the formation of gallstones in the gallbladder. Both clinical and preclinical data indicate that obesity, along with comorbidity insulin resistance, is a predisposing factor for cholelithiasis. Forkhead box O1 (FoxO1) is a key transcription factor that integrates insulin signaling with hepatic metabolism and becomes deregulated in the insulin resistant liver, contributing to dyslipidemia in obesity. To gain mechanistic insights into how insulin resistance is linked to cholelithiasis, here we determined FoxO1’s role in bile acid homeostasis and its contribution to cholelithiasis. We hypothesized that hepatic FoxO1 deregulation links insulin resistance to impaired bile acid metabolism and cholelithiasis. To address this hypothesis, we used the FoxO1LoxP/LoxP-Albumin-Cre system to generate liver-specific FoxO1-knockout mice. FoxO1-knockout mice and age- and sex-matched wild-type littermates were fed a lithogenic diet, and bile acid metabolism and gallstone formation were assessed in these animals. We showed that FoxO1 affected bile acid homeostasis by regulating hepatic expression of key enzymes in bile acid synthesis and in biliary cholesterol and phospholipid secretion. Furthermore, FoxO1 inhibited hepatic expression of the bile acid receptor farnesoid X receptor (Fxr) and thereby counteracted hepatic Fxr signaling. Nonetheless, hepatic FoxO1 depletion neither affected the onset of gallstone disease nor impacted the disease progression, as FoxO1-knockout and control mice of both sexes had similar gallstone weights and incidence rates. These results argue against the notion that FoxO1 is a link between insulin resistance and cholelithiasis.

Introduction: Cholelithiasis, manifested by the presence of gallstones in the gallbladder, is one of the most prevalent gastroenterological diseases. Currently there are about 15% of adults with gallstones in industrial countries (1,2). The prevalence of gallstone disease appears to increase with age (2,3). Although most gallstones remain asymptomatic, symptoms can ensue in about 25% of cases with complications ranging from biliary...
Bile acids play a critical role in the pathogenesis of cholelithiasis (15,16). Synthesized from cholesterol primarily in the liver, bile acids are conjugated with taurine or glycine and are secreted into the bile. Conjugated bile acids, known as bile salts, are stored in the gallbladder in fasting states. After meals, bile acids are released from the gallbladder into the intestine, where bile acids function as physiochemical detergents to facilitate the absorption of dietary lipids and fatsoluble vitamins (15,16). In the post-absorption phase, bile acids are absorbed from the intestine and are circulated back to the liver, where bile acids inhibit their own biosynthesis in a feedback regulatory loop. About 95% of bile acids are re-absorbed in the intestine and transported back to the liver. Such enterohepatic circulation of bile acids is instrumental for preventing bile acid overproduction, as abnormally higher bile acid concentrations in the liver can damage cell membranes, impair liver function, and cause cholestasis and cirrhosis (15,16). Key to the feedback regulation of bile acid biosynthesis is the farnesoid X receptor (Fxr), a member of the nuclear receptor superfamily (17-19). Expressed abundantly in the liver and to lesser extent the intestine, Fxr functions as a bile acid receptor (15,17). Upon its binding by bile acids, Fxr is translocated from the cytoplasm into the nucleus, where Fxr becomes active in suppressing hepatic expression of cholesterol 7 alpha-hydroxylase (Cyp7A1), the rate-limiting enzyme in the oxidation of cholesterol for bile acid biosynthesis (20). Such Fxr-dependent feedback loop is pivotal for maintaining normal bile acid homeostasis in the body. Indeed, Fxr-deficient mice are associated with impaired bile acid metabolism and increased susceptibility to developing gallstone disease (17,21). Conversely, pharmacological activation of Fxr activity by its agonists protects against the formation of gallstones in the gallbladders in animal models and at-risk human subjects (21-23). Furthermore, human subjects harboring genetic mutations in the FXR gene are associated with heightened risk of developing cholelithiasis (24,25).

In this context, a preclinical study by Biddinger et al. (44) reports that hepatic insulin resistance directly promotes the formation of gallstones in mice, when fed a lithogenic diet (LD) – a dietary regimen that is shown to stimulate gallstone formation in a number of animal models (45,46). They show that mice with conditional insulin receptor (Ir) depletion in the liver develop gallstone disease, as result of genetically inherited insulin resistance in Ir-knockout mice (44). Nonetheless, the underlying mechanism connecting Ir deficiency to gallstone disease remains undefined. It is known that the Forkhead box O1 (FoxO1) transcription factor acts downstream of the Ir signaling pathway to mediate the inhibitory action of insulin on target gene expression (47-49). FoxO1 activity is subject to insulin inhibition. In the absence of insulin, FoxO1 is active in the nucleus, in which FoxO1 binds to target promoters and stimulates target gene expression (47-49). In response to insulin, FoxO1 undergoes Akt-dependent phosphorylation and translocation from the nucleus to the cytoplasm (50). This effect accounts for insulin-dependent inactivation of FoxO1 activity. As a result of insulin resistance, hepatic FoxO1 activity becomes
abnormally higher, due to the inability of FoxO1 to undergo Akt-dependent phosphorylation and translocation in obesity and type 2 diabetes (50). Likewise, genetic Ir depletion in the liver is expected to intercept insulin inhibition of FoxO1 activity, contributing to constitutive FoxO1 activity in insulin resistant liver of Ir-knockout mice, although this point was not addressed in the study (44). Together these data implicate FoxO1 at the interface of insulin resistance and gallstone disease, inspiring us to hypothesize that genetic FoxO1 depletion would protect against the development of cholelithiasis in mice. To test this hypothesis, we generated mice with conditional FoxO1 depletion in the liver, using the FoxO1<sup>LoxP/LoxP</sup> Albumin-Cre system. Liver-conditional FoxO1-knockout mice vs. age/sex-matched wild-type littermates were fed on LD, followed by the determination of the effect of hepatic FoxO1 depletion on bile acid metabolism and gallstone disease. To corroborate these studies, we developed a high fat-containing lithogenic diet (HFLD) with 60% fat content, as the low fat content (15% fat) in LD is inadequate to induce obesity (45,46). We replicated our studies by feeding liver-conditional FoxO1-knockout mice vs. age/sex-matched wild-type littermates the newly customized HFLD to further test our hypothesis that FoxO1 deregulation, resulting from overnutrition and insulin resistance, is culpable for the pathogenesis of cholelithiasis in obesity.

Results:
Effect of FoxO1 on hepatic Fxr expression:
Fxr is a key regulator of bile acid homeostasis. Fxr-deficient mice are associated with increased susceptibility to developing cholesterol gallstone disease (21). Fxr is abundantly expressed in the liver and its hepatic expression is down-regulated in response to insulin-deficiency or insulin resistance (51). To understand the underlying mechanism, we determined hepatic regulation of Fxr expression by FoxO1, whose hepatic activity is reciprocally upregulated in insulin deficient and insulin resistant states (47,48,50). We transduced human hepatoma HepG2 cells with Adv-FoxO1 or Adv-Empty vector, as described (52), followed by the determination of hepatic Fxr expression. Adenovirus-mediated FoxO1 production resulted in a significant reduction of hepatic Fxr expression at both mRNA and protein levels (Fig. 1A-D). These results raise the hypothesis that FoxO1 might target the Fxr gene for trans-repression. Implicit in this hypothesis is the revelation of a consensus FoxO1 binding site [T(G/ A)TTT(T/G)(G/T)] in the proximal region of the Fxr promoter in both rodents and humans (Fig. 1E), indicative of an evolutionarily conserved mechanism by which FoxO1 regulates Fxr expression in the liver. To determine whether FoxO1 inhibits Fxr expression via its cognate binding to the Fxr promoter, we subjected FoxO1-expressing HepG2 cells to chromatin immunoprecipitation (ChIP) assay, using anti-FoxO1 or control IgG. Immunoprecipitated DNA was analyzed by PCR assay, using specific primers flanking the FoxO1 consensus site in the Fxr promoter. FoxO1 was shown to bind to the Fxr promoter, as evidenced by the detection of the Fxr promoter DNA in the protein-DNA complexes that were immunoprecipitated by anti-FoxO1 antibody, but not by control IgG (Fig. 1F).

FoxO1 is known to modulate target gene expression via a trans-repression mechanism (53-56). To determine whether FoxO1 binding to the Fxr promoter translated into its inhibitory effect on Fxr promoter activity, we cloned the human Fxr promoter into the pGL3-basic plasmid encoding the firefly luciferase reporter gene, as described (52). We then transfected the Fxr promoter-directed luciferase reporter system in combination with control plasmid pGL4.75 expressing Renilla luciferase into HepG2 cells, followed by the determination of Fxr promoter activity in a dual luciferase assay. We showed that Fxr promoter activity was markedly enhanced in the presence of Fxr agonist, and this effect was reduced by 2-fold to the baseline in response to adenovirus-mediated FoxO1 production in HepG2 cells (Fig. 1G). These results suggest that FoxO1 counteracts Fxr by inhibiting hepatic Fxr production via a trans-repression mechanism.

Characterization of L-FoxO1-KO mice on regular chow.
Based on the findings that FoxO1 inhibits Fxr, a key bile acid receptor whose activation protects against gallstone disease (21), we hypothesize that increased FoxO1 activity, resulting from insulin resistance, might be a risk factor for gallstone disease. Consistent with this hypothesis are the observations that FoxO1 is markedly upregulated in insulin resistant liver of mice with dietary obesity or type 2 diabetes, as well as in the liver of human subjects with metabolic disease (50,57). To address this hypothesis, we depleted FoxO1 conditionally in the liver of mice, using the Albumin-Cre system that is well characterized to ablate the LoxP-floxed gene of interest conditionally in the liver (58).
We crossed FoxO1\textsuperscript{LoxP/LoxP} and Albumin-Cre mice. The resulting progenies, FoxO1\textsuperscript{LoxP/LoxP;::Albumin-Cre\textsuperscript{v/v}} and FoxO1\textsuperscript{LoxP/LoxP;::Albumin-Cre\textsuperscript{v/v}} mice were subjected to validation of hepatic FoxO1 depletion, using both real-time qRT-PCR and anti-FoxO1 immunoblot assays. FoxO1\textsuperscript{LoxP/LoxP;::Albumin-Cre\textsuperscript{v/v}} (designated L-FoxO1-KO), as opposed to FoxO1\textsuperscript{LoxP/LoxP;::Albumin-Cre\textsuperscript{v/v}} (WT control), had close to 85% reduction in hepatic FoxO1 expression at both mRNA and protein levels (Fig. 2A-C). The degree of reduction in hepatic FoxO1 production corresponds to near complete FoxO1 depletion in hepatocytes, given the fact that there are about 10-20% of other cell types including endothelial cells, macrophages and stellate cells in the liver (59). As control, we determined hepatic expression of other members of the FoxO subfamily. No significant differences were detectable in hepatic FoxO3, FoxO4 and FoxO6 mRNA levels were unchanged between L-FoxO1-KO and WT mice (Supplemental Fig. 1A-C). These results preclude the possibility that hepatic FoxO1 depletion would beget compensatory increases in hepatic expression of other FoxO members in L-FoxO1-KO mice.

L-FoxO1-KO and WT littermates had similar weight gain on regular chow (Fig. 2D). However, L-FoxO1-KO mice, as opposed to WT littermates, had lower fasting blood glucose levels and plasma insulin levels (Fig. 2, E and F), consistent with previous observations that hepatic FoxO1 depletion impairs hepatic gluconeogenesis, contributing to the reduction of fasting glycemia (60,61). As a result, L-FoxO1KO mice had significantly lower blood glucose levels during glucose tolerance test (Fig. 2, G and H). In contrast, no significant differences were detected in plasma TG and cholesterol levels (Fig. 2, I and J).

Glucose and lipid metabolism in L-FoxO1-KO mice on LD:
To address whether FoxO1 is a contributing factor for the pathogenesis of cholelithiasis, we determined hepatic FoxO1 expression in WT mice on LD vs. regular chow. We detected a marked induction of hepatic FoxO1 expression at both mRNA and protein levels in WT mice following a 12-week LD feeding (Fig. 3, A-C). These data lent a pivotal support for our hypothesis that hepatic FoxO1 depletion would protect against gallstone disease in mice. To test this hypothesis, we fed L-FoxO1-KO and WT littermates (8 weeks old, male, n=7-9/group) the LD for 12 weeks. Both groups had similar weight gain (Fig. 3D), accompanied by similar plasma levels of triglycerides, cholesterol, phospholipids and bile acids (Supplemental Table 2). Likewise, L-FoxO1-KO and WT littermates had similar blood glucose levels under fed conditions (Fig. 3E). L-FoxO1-KO and WT littermates also had similar postprandial blood glucose profiles in response to glucose challenge (Fig. 3F). However, L-FoxO1-KO mice, when fasted for 16 h, displayed significantly lower fasting blood glucose levels (Fig. 3E), in keeping with previous findings that hepatic FoxO1 deficiency reduces the capacity of liver to undergo gluconeogenesis in response to prolonged fasting (61). L-FoxO1-KO mice also had lower plasma insulin levels, in accordance with their lower fasting blood glucose levels (Fig. 3G).

Hepatic triglyceride, cholesterol and bile acid content in L-FoxO1-KO mice on LD:
To determine the impact of FoxO1 depletion on hepatic lipid metabolism, we euthanized L-FoxO1-KO and WT littermates after 12 weeks of LD feeding. Liver tissues were procured for histological examination after staining with Oil Red O and H&E (Fig. 4, A-D). LD feeding resulted in steatosis. Nonetheless, L-FoxO1-KO and WT littermates had similar degrees of fat infiltration into the liver. To corroborate these findings, we quantified hepatic lipid content, demonstrating that L-FoxO1-KO and WT littermates had similar hepatic triglyceride levels (Fig. 4E), accompanied by similar hepatic cholesterol content in the liver (Fig. 4F). Likewise, hepatic bile acid levels remained unchanged in L-FoxO1-KO vs. WT mice (Fig. 4G).

Impact of hepatic FoxO1 depletion on hepatic bile acid metabolism:
To determine the effect of FoxO1 on bile acid homeostasis, we employed real-time qRT-PCR assay to profile hepatic expression of Fxr and its downstream effector genes, whose functions are cardinal for bile acid homeostasis, including bile acid biosynthesis, biliary cholesterol and phospholipid secretion and bile salt excretion (Supplemental Fig. 2). Hepatic Fxr mRNA levels were significantly upregulated in L-FoxO1-KO liver (Fig. 5A). We validated this finding with anti-FoxO1 immunoblot assay, demonstrating that hepatic FoxO1 depletion resulted in 2.5-fold increases in Fxr protein levels in the liver of L-FoxO1-KO vs. WT mice (Fig. 5B). These results reinforce the idea that FoxO1 counteracts Fxr activity by inhibiting Fxr production in the liver. As result of hepatic FoxO1 depletion, Fxr expression became de-repressed,
contributing to increased Fxr expression at both mRNA and protein levels in the liver of L-FoxO1-KO mice.

We then examined the effect of hepatic FoxO1 depletion on hepatic expression of cholesterol 7α-hydroxylase (Cyp7A1), sterol 12-alpha-hydroxylase (Cyp8B1), sterol 27α-hydroxylase (Cyp27A1), and oxysterol 7α-hydroxylase (Cyp7B1), four key enzymes in bile acid biosynthesis. Expressed abundantly in the liver, Cyp7A1, followed by Cyp8B1, catalyzes the rate-limiting step in the classic bile acid synthesis pathway (16), whereas Cyp27A1 and Cyp7A1 function to catalyze the alternative pathway of bile acid synthesis. Both classic and alternative pathways contribute about equally to bile acid synthesis in the liver of rodents, although the classic pathway is predominant for bile acid synthesis in the liver of humans (16). We showed that hepatic expression of Cyp7A1, Cyp27A1 and Cyp7B1, but not Cyp8B1, were down-regulated in the liver of L-FoxO1-KO mice (Fig. 5, C-F). These effects correlated inversely with hepatic up-regulation of Fxr, a nuclear receptor that is characterized as a negative regulator of bile acid synthesis (Fig. 5A and 5B).

To assess the effect of FoxO1 depletion on biliary cholesterol secretion, we determined hepatic expression of ATP-binding cassette G sub-family members 5 and 8 (Abcg5 and Abcg8). Expressed predominantly in the liver, Abcg5 and Abcg8 genes encode two cholesterol transporters that act as obligate heterodimers to facilitate biliary cholesterol secretion into bile (62). Here we showed that hepatic expression of both Abcg5 and Abcg8 genes was reduced to a similar extent in the liver of L-FoxO1-KO mice, although the amplitude of reduction in hepatic Abcg5 and Abcg8 mRNA levels did not reach a significant level, when compared to that in WT littermates (Fig. 5G and 5H).

In addition, we determined the impact of FoxO1 deficiency on hepatic expression of ATP-binding cassette subfamily B, member 4 (Abcb4) and member 11 (Abcb11), two key functions for maintaining normal bile acid homeostasis in the liver. We detected a significant reduction of Abcb4 mRNA expression in FoxO1-deficient liver (Fig. 5I). In contrast, hepatic Abcb11 mRNA expression remained unchanged in L-FoxO1-KO vs. WT mice (Fig. 5J).

**Effect of FoxO1 depletion on gallstone disease in mice on LD:**

To determine the impact of FoxO1 depletion on gallstone formation, we procured individual gallbladders from euthanized mice in both male and female sexes after 12 weeks of LD feeding, followed by examination of gallstones in incised gallbladders in a dissecting microscope. We then extracted all pieces of gallstones from individual gallbladders of mice with positive gallstone formation. We detected a similar incidence rate (~50%) of gallstone disease in L-FoxO1-KO and WT mice, irrespective of sex (Fig. 6 A-E). Furthermore, no significant differences were detectable in gallstone weight, indicative of a similar rate of gallstone disease progression in L-FoxO1-KO and WT groups (Fig. 6F). Likewise, no differences in gallbladder weight or liver weight were seen in male FoxO1-KO vs. WT mice (Fig. 6G and 6H). These effects were reproducible in female L-FoxO1-KO and WT mice (Supplemental Fig. 3).

**Characterization of L-FoxO1-KO mice on HFLD:**

We noted that the conventional LD contains about 15% fat, a dietary regimen that is insufficient to induce obesity in mice. To overcome this limitation, we customized a HFLD for feeding L-FoxO1-KO and age/sex-matched WT mice. As expected, a 12-week HFLD feeding resulted in increased adiposity in mice. Nonetheless, male L-FoxO1-KO and WT mice had similar fat mass and lean mass, correlating with similar weight gain and equivalent food intake (Fig. 7 A-D). L-FoxO1-KO and WT groups had similar fasting blood glucose and plasma insulin levels (Supplemental Table 3), accompanied by postprandial glucose disposal post intraperitoneal glucose injection (Fig. 7E). Likewise, both L-FoxO1-KO and WT mice developed similar degrees of insulin resistance, as evidenced by the HOMA-IR index (Supplemental Table 3). We recapitulated these findings by performing insulin tolerance test, during which L-FoxO1-KO and WT mice had similar blood glucose profiles (Fig 7F). We reproduced these results in female L-FoxO1-KO vs. WT mice (Supplemental Fig. 4 and Supplemental Table 4).

To determine the impact of HFLD feeding on lipid metabolism, we determined plasma lipid profiles in L-FoxO1-KO and WT mice. We found that HFLD feeding, as opposed to feeding on LD, resulted in marked increases in plasma phospholipid and bile acid levels, accompanied by corresponding decreases in plasma cholesterol levels in mice (Supplemental Table 3). Nevertheless, male L-FoxO1-KO and WT mice had
similar plasma levels of TG, cholesterol, phospholipid, and bile acids on HFLD (Supplemental Table 3). In contrast, female L-FoxO1-KO mice, as opposed to age-matched female WT littermates, had significantly higher plasma TG and cholesterol levels without alterations in plasma bile acid levels (Supplemental Table 4). To preclude the possibility that this lack of alterations in plasmid lipid profiles was due to differential lipid excretion, we determined lipid levels in feces collected from individual mice in fed states during a 24-h period, showing that fecal TG, cholesterol and bile acid concentrations were unchanged between L-FoxO1-KO and WT groups in both sexes (Supplemental Tables 3 and 4).

To determine the impact of HFLD on hepatic lipid metabolism, we quantified hepatic fat content, revealing that HFLD feeding resulted in severe steatosis in both WT and L-FoxO1-KO groups. We confirmed these results by histological examination of liver sections stained with H&E and Oil red O (Fig. 8, A-D). Nonetheless, no significant differences in hepatic TG, cholesterol and bile acid levels were seen between L-Foxo1-KO and WT mice in both males (Fig. 8 E-G) and females (Supplemental Fig. 5).

Is FoxO1 a candidate molecule for linking insulin resistance to gallstone disease?
To address the hypothesis that FoxO1 integrates insulin resistance to gallstone disease, we euthanized mice after 12 weeks of HFLD feeding, followed by the examination of gallstone formation in the gallbladder in a dissecting microscope. We found that L-FoxO1-KO and WT mice had a similar incidence rate (~50%) of gallstone disease (Fig. 9 A-E). Furthermore, we extracted all pieces of gallstones from individual mice with positive gallstone formation, demonstrating that L-FoxO1-KO and WT groups had a similar gallstone weight, relative to gallbladder weight and liver weight (Fig. 9 F-H). These results indicate that hepatic FoxO1 depletion did not protect against the development of cholelithiasis in HFLD-fed mice. This effect ensued in both male and female L-FoxO1-KO mice.

Discussion:
Cholelithiasis is characterized by the formation of cholesterol stones in the gallbladder. While the etiology of cholelithiasis is incompletely understood, its close association with obesity and type 2 diabetes implicates insulin resistance as a predisposing factor for gallstone disease (26-33). Nonetheless, given the controversial data in the literature, it remains a long-standing question as to how insulin resistance contributes to the pathogenesis of gallstone disease. Our goal in this study is to address the hypothesis that FoxO1 integrates insulin resistance to gallstone disease and hepatic FoxO1 depletion would protect against the formation of gallbladder stones in mice with insulin resistance and obesity. This hypothesis dwelled on three lines of evidence in the literature. First, hepatic FoxO1 activity becomes abnormally higher, due to an impaired ability of insulin to inhibit FoxO1 activity in insulin resistant liver, contributing to the deregulation in glucose and lipid metabolism in obesity and type 2 diabetes (47,48,50). Second, FoxO1 deregulation is associated with abnormal bile acid metabolism in mice (63). Third, insulin resistance, resulting from genetic Ir depletion in the liver, is associated with increased susceptibility to developing gallstone disease in mice (44). To address this hypothesis, we determined the effect of FoxO1 on gallstone disease in L-FoxO1-KO mice and sex/weight-matched WT littermates following 12 weeks of LD feeding. We detected a similar incidence rate and similar mean weight of gallstones in mice between L-FoxO1-KO and WT groups. We recapitulated these findings in both male and female mice. These results suggest that hepatic FoxO1 depletion neither affected the onset of gallstone disease nor impacted the disease progression, regardless of sex.

We noted that LD contains 15% fat. Although potent for inducing gallstone disease, LD did not induce obesity and insulin resistance in mice. We then overcame this limitation by feeding mice a newly developed HFLD with 60% fat. As expected, a 12-week HFLD feeding resulted in moderate obesity with a concomitant induction of insulin resistance in L-FoxO1-KO and WT groups. Furthermore, HFLD feeding resulted in a significant induction of hepatic fat infiltration, characteristic of non-alcoholic fat disease. However, HFLD feeding did not exacerbate the incidence rate of gallstone disease. L-FoxO1-KO and WT littermates had a similar incidence rate of gallstone formation (~50%) and a similar mean weight of gallbladder stones. These results were reproducible in male and female mice. Taken together, our data argue against the notion that FoxO1 is culpable for linking insulin resistance to the pathogenesis of cholelithiasis.

These results, which defied our original hypothesis, were totally unexpected. An intuitive explanation is that hepatic FoxO1 depletion begot a compensatory
upregulation of FoxO3, FoxO4 or FoxO6, three other FoxO members with functional redundancy in the liver (64). This would marginalize the effect of hepatic FoxO1 depletion on the pathogenesis of gallstone disease. However, this possibility was ruled out, as we did not detect significant increases in hepatic expression of other FoxO isoforms in L-FoxO1-KO mice. Instead, we showed that FoxO1 exerted a direct effect on hepatic bile acid metabolism, as hepatic FoxO1 depletion resulted in a significant reduction in hepatic expression of Cyp7A1, the rate-limiting enzyme in the classic bile acid biosynthesis pathway. In keeping with our findings, three independent groups reported that FoxO1 is a positive regulator of the Cyp7A1 gene in rodents (65-67). FoxO1 binds to its consensus site within the Cyp7A1 promoter and stimulates Cyp7A1 expression in the liver (65,67). This effect is counteracted by insulin, a mechanism that accounts in part for insulin-dependent inhibition of bile acid biosynthesis in the liver (68). Likewise, insulin also exerts an inhibitory effect on hepatic expression of Cyp27A1 and Cyp7B1, two key enzymes that catalyze bile acid biosynthesis in the alternative pathway (16). We detected a significant reduction in hepatic expression of Cyp27A1 and Cyp7B1 in the liver of L-FoxO1-KO vs. WT mice. These results underscore the physiological importance of FoxO1 in regulating bile acid biosynthesis in the liver.

Aside from its direct impact on bile acid biosynthesis, FoxO1 appears to play an important role in regulating biliary cholesterol secretion. We showed that FoxO1 loss-of-function attenuated hepatic expression of Abcg5 and Abcg8, two cholesterol transporters that function in heterodimers to facilitate biliary cholesterol secretion was attenuated in the liver of L-FoxO1-KO mice. These results are consistent with the previous findings that FoxO1 targets the Abcg5 and Abcg8 genes for transactivation (44). Consistent with their functions in obligate heterodimers, Abcg5 and Abcg8 are coordinately regulated at the transcriptional level, as they share a common bi-directional promoter (374 bp), from which Abcg5 and Abcg8 genes are transcribed in opposing directions. Clinical studies indicate that human subjects harboring a common ABCG8-Asp19His allele are associated with increased susceptibility to developing gallstone disease (69,70). Preclinical studies show that elevated Abcg5 and Abcg8 expression, resulting from insulin resistance, increases biliary cholesterol secretion, translating to a significant risk of cholesterol gallstone formation (44). Indeed, FoxO1 activity becomes unchecked in insulin resistant states (47,48,50). Our data along with previous observations provide further physiological underpinning for the idea that FoxO1 is liable for coupling insulin resistance to biliary cholesterol hypersecretion in the pathogenesis of cholesterol gallstone disease.

Another significant finding derived from our studies is the revelation of the mechanism by which FoxO1 counteracts Fxr, a bile acid receptor whose function is instrumental for maintaining normal bile acid homeostasis in the body (15,17). We showed that FoxO1 binds to its consensus site within the Fxr promoter and inhibits Fxr expression in hepatocytes. We reproduced these data in vitro and in vivo. As a result of hepatic FoxO1 depletion, Fxr is markedly upregulated in the liver of L-FoxO1-KO mice. These data suggest that FoxO1 counterbalances the effect of Fxr function on bile acid metabolism by inhibiting Fxr production in the liver. While hepatic Fxr expression becomes markedly down-regulated in insulin-deficient and insulin resistant livers, contributing in part to the alterations in bile acid metabolism in diabetes, the underlying mechanism still remains elusive (51). Our data provide important mechanistic insights into the mechanism by which hepatic Fxr expression is down-regulated in diabetes. In response to insulin deficiency or insulin resistance, FoxO1 activity becomes abnormally higher (47,48,50), contributing to the suppression of hepatic Fxr production and impaired bile acid metabolism in diabetes (51).

Moreover, we found that hepatic FoxO1 depletion resulted in a significant reduction in hepatic expression of Abcb4, also known as the multidrug resistance gene (MDR3) in humans (71). Abcb4 is expressed mainly in the liver and acts as a phospholipid transporter for transporting phosphatidylcholine into the bile (71). Genetic ABCB4 variants are associated with intrahepatic cholestasis and cholesterol gallstone disease in humans (72,73). Our data indicate that Abcb4 is down-regulated in FoxO1-deficient liver, suggesting that the Abcb4 gene is a FoxO1 target. Consistent with this interpretation is that hepatic Abcb4 expression is up-regulated, coinciding with the induction of hepatic FoxO1 activity in dietary obese mice (74,75). Further investigation is needed to assess the contribution of FoxO1 signaling via Abcb4 to bile acid metabolism and risk of gallstone disease in obesity and type 2 diabetes.
In conclusion, our studies revealed that FoxO1 plays an independent role in maintaining normal bile acid homeostasis by regulating hepatic expression of key genes involved in bile acid synthesis, biliary cholesterol secretion, and phospholipid secretion. Furthermore, FoxO1 acts to attenuate bile acid signaling by counteracting Fxr activity in the liver. Although abnormally higher FoxO1 activity, resulting from insulin resistance, is associated with impaired bile acid metabolism and risk of cholelithiasis, hepatic FoxO1 deficiency did not confer a protective effect on the development of gallstone disease in mice on LD and HFLD diets, respectively. We acknowledge a limitation in the scope of our studies, as we only employed FoxO1-deficient models. Our data could not preclude the possibility that hepatic FoxO1 overproduction would predispose to developing gallstone disease in mice. Therefore, further studies are warranted to understand the molecular basis that mechanistically links insulin resistance to gallstone disease.

**Experimental Procedures:**

**Animal studies:** FoxO1<sup>loxP/loxP</sup> mice encoding two LoxP sites flanking the 2nd exon of FoxO1 were described (76). We re-derived the FoxO1<sup>loxP/loxP</sup> allele in the C57BL/6 mice, followed by crossing with C57BL/6 mice for >5 generations. To deplete FoxO1 in the liver, we crossed FoxO1<sup>loxP/loxP</sup> mice with C57BL/6-Albumin-Cre mice (Jackson Laboratory, MN). The resulting progenies were genotyped by PCR assay for the FoxO1<sup>loxP/loxP</sup> and albumin-Cre alleles, as described (64). Mice were fed standard rodent chow and water ad libitum in sterile cages with a 12-h light/dark cycle in a pathogen-free barrier facility of Children’s Hospital of Pittsburgh of UPMC. To induce gallstone disease, mice were fed on LD containing 15.8% fat, 1.25% cholesterol and 0.5% sodium cholate (Teklad Diet TD88051, Envigo). To induce both insulin resistance and gallstone disease, we developed HFLD containing 60% fat, 1.25% cholesterol and 0.5% sodium cholate (Teklad Diet, Customized TD190158). For blood chemistry, mice were fasted for 16 h and tail vein blood was sampled. Blood glucose levels were measured, using Glucometer (Contour Next BG Monitoring System). Plasma insulin levels were determined, using ultrasensitive mouse insulin enzyme-linked immunosorbent assay (ALPCO, Windham, NH). Fat mass and lean mass of mice were determined using the EchoMRI-100 system (Echo Medical Systems, Houston, TX). The homeostasis model for insulin resistance (HOMA-IR) was determined by multiplying fasting blood glucose (mmol/L) and fasting plasma insulin (µIU/mL) levels, divided by 22.5. Plasma levels of triglyceride and cholesterol were determined, using Thermo Infinity triglyceride and cholesterol reagents (ThermoFisher Scientific, Middletown, VA). Plasma phospholipid levels were measured, using the phospholipid assay kit (Sigma-Aldrich). Plasma bile acid levels were determined, using the bile acid assay kit (Cell Biolabs, Inc.). Mice were euthanized to collect liver and gallbladder tissues for analysis. All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of University of Pittsburgh.

**Luciferase reporter assay:** We used the dual luciferase reporter system (Promega, Madison, WI) for determining promoter activity. To clone the human Fxr promoter (1.8 kb), we subjected human genomic DNA isolated from HepG2 cells to PCR assay using the primers (5'--CATCTCTGCTGGAGAGATG-3' and 5'--AGAGTTCCAGTGTACGTGC -3') that flank the human Fxr promoter (-1,721/+133 nt). The Fxr promoter DNA was cloned into the pGL3-Basic plasmid (Promega) encoding the luciferase reporter gene. To determine Fxr promoter activity, HepG2 cells were transduced in 12-well plates with 50 pfu/cell of Adv-FoxO1 or Adv-Empty vector, followed by transfection with 2-µg of pFxr-Luc encoding the Fxr promoter-directed luciferase reporter system. pGL4.75 expressing Renilla luciferase (Promega) was included as control for normalizing transfection efficiency. Plasmid pGL3-basic encoding promoter-less luciferase was used as control for determining the baseline luciferase activity. After 24-h incubation in the absence or presence of Fxr agonist GW4064 (final concentration, 1 µM, Sigma-Aldrich), cells were starved in serum-free medium for 6 h and were subjected to the dual luciferase activity assay for determining Fxr promoter activity.

**ChIP assay:** Chromatin immunoprecipitation (ChIP) assay was performed as described (77). Due to extremely low FoxO1 expression in HepG2 cells, we used HepG2 cells pre-transduced with Adv-FoxO1 vector (50 pfu/cell) in the ChIP assay for determining FoxO1 interaction with the human Fxr promoter, using anti-FoxO1 antibody or anti-β-galactosidase IgG as control. The immunoprecipitates were subjected to PCR analysis using the primers (5'-ATCTAACCAGGCTGTGCT-3' and 5'-ACTGAACAGAGCTGGGAG-3') flanking the consensus FoxO1 binding site (-481/-250 nt) within the
human Fxr promoter. As input control, aliquots of cell lysates (5 µl) prior to immunoprecipitation were analyzed in the same PCR assay. As off-target control, the immunoprecipitates were subjected to PCR assay, using the primers (5'-GCTCAAGTGAACACTGCTTCT-3’ and 5’-TCATCTGTGGTAGGTAAATGGGA-3’) flanking the human Fxr cDNA region (-139/+53 nt).

**RNA isolation and real-time qRT-PCR:** Total RNA was prepared from the liver or islets using the TRIzol Reagent (Invitrogen, Calabas, CA). Real-time quantitative RT-PCR was used for quantifying mRNA concentrations using the Roche LightCycler-RNA amplification kit (Roche Diagnostics, Indianapolis, IN), as described (52). All primers (Supplemental Table 1) were obtained commercially from Integrated DNA Technologies (Coralville, IA).

**Western blot assay:** Mice were euthanized for tissue procurement. Aliquots of liver tissues (10 mg) were homogenized in 350-µl M-PER supplemented with 3.5-µl Halt Protease Inhibitor Cocktail (Pierce). After centrifugation in microtubes at 13,000 rpm for 10 min, aliquots of protein lysates (20 µg) were subjected to immunoblot analysis, using rabbit anti-Fxr (Abcam Cat#A1978) and mouse anti-actin (Sigma-Aldrich, Cat#A155124) antibodies, respectively. Polyclonal rabbit anti-FoxO1 antibody was described elsewhere (75).

**Liver histology:** Liver tissues were embedded in the Histoprep tissue-embedding media. Frozen sections (6 µm in thickness) were stained with Oil Red O, followed by counter-staining with hematoxylin. Paraffin-embedded sections (6 µm in thickness) were stained with hematoxylin and eosin.

**Gallstone determination:** Mice were euthanized after 6-h fasting. Gallbladder was procured from individual mice. Gallbladder was incised under a dissecting microscope and the gallbladder content containing gallstones were collected into individual microtubes. The gallstone sample was dried and gallstone weight was determined.

**Hepatic triglyceride content:** Liver tissues (10 mg) were homogenized in 200-µL HPLC-grade acetone. After incubation with agitation at room temperature for 16 h, aliquots (10 µL) of acetone-extracted lipid suspension were used for determining triglyceride concentration, using the Infinity triglyceride reagent (ThermoFisher Scientific). Hepatic triglyceride content was defined as mg of triglyceride per gram of total liver proteins.

**Hepatic cholesterol content:** Liver tissues (10 mg) were homogenized in 200 µL of Chloroform: Isopropanol (7:11 in volume) at 4°C. After centrifugation in microtubes at 10,000 rpm for 5 min, aliquots (20 µL) of hexane-isopropanol-extracted cholesterol suspension were used for determining cholesterol concentration, using the Infinity cholesterol reagent (ThermoFisher Scientific). Hepatic cholesterol content was defined as mg of cholesterol per gram of total liver proteins.

**Hepatic bile acid content:** Liver tissues (10 mg) were homogenized in 300 µL of PBS buffer, followed by determination of bile acid concentration, using the bile acid assay kit (Cell Biolabs Inc.). Hepatic bile acid content was defined as mg of bile acids per gram of total liver proteins.

**Fecal triglyceride content:** Feces were collected from individual mice during a 24-h period under fed conditions. Aliquots of feces (50 mg) were homogenized in 200-µL HPLC-grade acetone, followed by 30-min incubation with agitation at room temperature. Fecal TG content, defined as mg of TG per gram of feces, was determined in acetone-extract lipid suspension, using the Infinity triglyceride reagent (ThermoFisher Scientific).

**Fecal cholesterol content:** Aliquots of feces (50 mg) were homogenized in 200 µL of Chloroform: Isopropanol (7:11 in volume) at 4°C. After centrifugation in microtubes at 10,000 rpm for 5 min, aliquots (20 µL) of hexane-isopropanol-extracted fecal suspension were used for determining fecal cholesterol content, defined as mg of cholesterol per gram of feces.

**Fecal bile acid content:** Aliquots of feces (50 mg) were homogenized in 200 µL of PBS buffer. After centrifugation in microtubes at 10,000 rpm for 5 min, aliquots (20 µL) of supernatants were used for determining bile acid content, using the bile acid assay kit (Cell Biolabs Inc.).

**Statistics:** Statistics of data were analyzed by unpaired Student’s t-test, using the Graphpad Prism 7.0. Data are expressed as means ± SD. P values <0.05 were
considered statistically significant.

Data Availability: We state that all data are in the manuscript.

Acknowledgments: We thank Dr. Ronald DePinho for providing the FoxO1loxP/loxP mice. This work is supported in part by UPMC Children's Hospital of Pittsburgh and National Health Institute grant 1R01DK120310-01A1. Cuiling Zhu is a visiting scholar who is sponsored by scholarship #201806260074 from China Scholarship Council.

Conflict of Interest: The authors declare that they have no conflicts of interest with the contents of this article.

References:
1. Shaffer, E. A. (2006) Gallstone disease: Epidemiology of gallbladder stone disease. Best Pract Res Clin Gastroenterol 20, 981-996
2. Lammert, F., and Sauerbruch, T. (2005) Mechanisms of disease: the genetic epidemiology of gallbladder stones. Nat Clin Pract Gastroenterol Hepatol 2, 423-433
3. Everhart, J. E., Khare, M., Hill, M., and Maurer, K. R. (1999) Prevalence and ethnic differences in gallbladder disease in the United States. Gastroenterology 117, 632-639
4. Marschall, H. U., and Einarsson, C. (2007) Gallstone disease. J Intern Med 261, 981-996
5. Wittenburg, H. (2010) Hereditary liver disease: gallstones. Best Pract Res Clin Gastroenterol 24, 747-756
6. Zheng, Y., Xu, M., Heianza, Y., Ma, W., Wang, T., Sun, D., Albert, C. M., Hu, F. B., Rexrode, K. M., Manson, J. E., and Qi, L. (2018) Gallstone disease and increased risk of mortality: Two large prospective studies in US men and women. J Gastroenterol Hepatol 33, 1925-1931
7. Sandblom, G., Videhult, P., Crona Guterstam, Y., Svenner, A., and Sadr-Azodi, O. (2015) Mortality after a cholecystectomy: a population-based study. HPB (Oxford) 17, 239-243
8. Sandler, R. S., Everhart, J. E., Donowitz, M., Adams, E., Cronin, K., Goodman, C., Gemmen, E., Shah, S., Avdic, A., and Rubin, R. (2002) The burden of selected digestive diseases in the United States. Gastroenterology 122, 1500-1511
9. Lammert, F., and Miquel, J. F. (2008) Gallstone disease: from genes to evidence-based therapy. J Hepatol 48 Suppl 1, S124-135
10. Rebholz, C., Krawczyk, M., and Lammert, F. (2018) Genetics of gallstone disease. Eur J Clin Invest 48, e12935
11. Maurer, K. J., Carey, M. C., and Fox, J. G. (2009) Roles of infection, inflammation, and the immune system in cholesterol gallstone formation. Gastroenterology 136, 425-440
12. Wang, H. H., Portincasa, P., Mendez-Sanchez, N., Uribe, M., and Wang, D. Q. (2008) Effect of ezetimibe on the prevention and dissolution of gallstone gallstones. Gastroenterology 134, 2101-2110
13. Venneman, N. G., Besselink, M. G., Keulemans, Y. C., Vanberge-Henegouwen, G. P., Boermeester, M. A., Broeders, I. A., Go, P. M., and van Erpecum, K. J. (2006) Ursodeoxycholic acid exerts no beneficial effect in patients with symptomatic gallstones awaiting cholecystectomy. Hepatology 43, 1276-1283
14. Ferkingstad, E., Oddsson, A., Gretarsdottir, S., Benonisdottir, S., Thorleifsson, G., Deaton, A. M., Jonsson, S., Stefansson, O. A., Norddahl, G. L., Zink, F., Arnadottir, G. A., Gunnarsson, B., Halldorsson, G. H., Helgadottir, A., Jansson, B. O., Kristjansson, R. P., Sveinbjornsson, G., Sverrisson, D. A., Masson, G., Olafsson, I., Eyjolfsinsson, G. I., Sigurdardottir, O., Holm, H., Jonsdottir, I., Olafsson, S., Steingrimsdottir, T., Rafnar, T., Bjornsson, E. S., Thorsteinsdottir, U., Gudbjartsson, D. F., Sulem, P., and Stefansson, K. (2018) Genome-wide association meta-analysis yields 20 loci associated with gallstone disease. Nat Commun 9, 5101
15. Chiang, J. Y. (2009) Bile acids: regulation of synthesis. J Lipid Res 50, 1955-1966
16. Chiang, J. Y. L., and Ferrell, J. M. (2018) Bile Acid Metabolism in Liver Pathobiology. Gene Expr 18, 71-87
17. Claudel, T., Zollner, G., Wagner, M., and Trauner, M. (2011) Role of nuclear receptors for bile acid metabolism, bile secretion, cholestasis, and gallstone disease. Biochim Biophys Acta 1812, 867-878
18. Teodoro, J. S., Rolo, A. P., and Palmeira, C. M. (2011) Hepatic FXR: key regulator of whole-body energy metabolism. Trends Endocrinol Metab 22, 458-466
19. Li, T., and Chiang, J. Y. (2013) Nuclear receptors in bile acid metabolism. Drug Metab Rev 45, 145-155
20. Chiang, J. Y., Kimmel, R., Weinberger, C., and Stroup, D. (2000) Farnesoid X receptor responds to bile acids and represses cholesterol 7alpha-hydroxylation gene (CYP7A1) transcription. *J Biol Chem* **275**, 10918-10924

21. Moschetta, A., Bookout, A. L., and Mangelsdorf, D. J. (2004) Prevention of cholesterol gallstone disease by FXR agonists in a mouse model. *Nat Med* **10**, 1352-1358

22. Magouliotis, D. E., Tasiopoulou, V. S., Svakos, A. A., Svokos, K. A., Chatedaki, C., Sioka, E., and Zacharoulis, D. (2017) Ursodeoxycholic Acid in the Prevention of Gallstone Formation After Bariatric Surgery: an Updated Systematic Review and Meta-analysis. *Obes Surg* **27**, 3021-3030

23. Stokes, C. S., Glud, L. L., Casper, M., and Lammert, F. (2014) Ursodeoxycholic acid and diets higher in fat prevent gallbladder stones during weight loss: a meta-analysis of randomized controlled trials. *Clin Gastroenterol Hepatol* **12**, 1090-1100 e1092; quiz e1061

24. Hirobe-Jahn, S., Harsh, S., Renner, O., Richter, D., Muller, O., and Stange, E. F. (2015) Association of FXR gene variants with cholelithiasis. *Clin Res Hepatol Gastroenterol* **39**, 68-79

25. Kovacs, P., Kress, R., Rocha, J., Kurtz, U., Miquel, J. F., Mendez-Sanchez, N., Uribe, M., Bock, H. H., Schirin-Sokhan, R., Stumvoll, M., Mossner, J., Lammert, F., and Wittenburg, H. (2008) Variation of the gene encoding the nuclear bile salt receptor FXR and gallstone susceptibility in mice and humans. *J Hepatol* **48**, 116-124

26. Katsika, D., Tuublady, C., Einarsson, C., Lichtenstein, P., and Marschall, H. U. (2007) Body mass index, alcohol, tobacco and symptomatic gallstone disease: a Swedish twin study. *J Intern Med* **262**, 581-587

27. Al-Jiffry, B. O., Shaffer, E. A., Saccone, G. T., Downey, P., Kow, L., and Toulou, J. (2003) Changes in gallbladder motility and gallstone formation following laparoscopic gastric banding for morbid obesity. *Can J Gastroenterol* **17**, 169-174

28. Shiffman, M. L., Sugerman, H. J., Kelly, J. H., Brewer, W. H., and Moore, E. W. (1993) Gallstones in patients with morbid obesity. Relationship to body weight, weight loss and gallbladder bile cholesterol solubility. *Int J Obes Relat Metab Disord* **17**, 153-158

29. Bonfrate, L., Wang, D. Q., Garruti, G., and Portincasa, P. (2014) Obesity and the risk and prognosis of gallstone disease and pancreatitis. *Best Pract Res Clin Gastroenterol* **28**, 623-635

30. Lv, J., Yu, C., Guo, Y., Bian, Z., Yang, L., Chen, Y., Li, S., Huang, Y., Fu, Y., He, P., Tang, A., Chen, J., Chen, Z., Qi, L., and Li, L. (2017) Gallstone Disease and the Risk of Type 2 Diabetes. *Sci Rep* **7**, 15853

31. Tung, T. H., Ho, H. M., Shih, H. C., Chou, P., Liu, J. H., Chen, V. T., Chan, D. C., and Liu, C. M. (2006) A population-based follow-up study on gallstone disease among type 2 diabetics in Kinmen, Taiwan. *World J Gastroenterol* **12**, 4536-4540

32. Acosta, A., and Camilleri, M. (2014) Gastrointestinal morbidity in obesity. *Ann N Y Acad Sci* **1311**, 42-56

33. Wang, F., Wang, J., Li, Y., Yuan, J., Yao, P., Wei, S., Guo, H., Zhang, X., Yang, H., Wu, T., and He, M. (2018) Gallstone Disease and Type 2 Diabetes Risk: A Mendelian Randomization Study. *Hepatology*

34. Li, V. K., Pulido, N., Martinez-Suartez, P., Fajnwaks, P., Jin, H. Y., Szomstein, S., and Rosenthal, R. J. (2009) Symptomatic gallstones after sleeve gastrectomy. *Surg Endosc* **23**, 2488-2492

35. Sarr, M. G. (2006) Patients developed symptomatic gallstones between 3 and 21 months after Roux-en-Y gastric bypass (RYGB), neither prophylactic cholecystectomy nor treatment with ursodeoxycholic acid is necessary after open RYGB. *Surg Obes Relat Dis* **2**, 233; author reply 233

36. Cazoo, E., Gestic, M. A., Utrini, M. P., Machado, R. R., Jimenez, L. S., da Silva, A. P., Baracat, J., Callejas-Neto, F., Pareja, J. C., and Chaim, E. A. (2016) Influence of Insulin Resistance Status on the Development of Gallstones Following Roux-En-Y Gastric Bypass: a Prospective Cohort Study. *Obes Surg* **26**, 769-775

37. Johanna, S. (2016) Gallstones and Bariatric Surgery: To Treat or Not to Treat? *World J Surg* **40**, 2911-2912

38. Morais, M., Faria, G., Preto, J., and Costa-Maia, J. (2016) Gallstones and Bariatric Surgery: To Treat or Not to Treat? *World J Surg* **40**, 2904-2910

39. Li, V. K., Pulido, N., Fajnwaks, P., Szomstein, S., Rosenthal, R., and Martinez-Duartez, P. (2009) Predictors of gallstone formation after bariatric surgery: a multivariate analysis of risk factors comparing gastric bypass, gastric banding, and sleeve gastrectomy. *Surg Endosc* **23**, 1640-1644

40. Johansson, K., Sundstrom, J., Marcus, C., Hemmingsson, E., and Neovius, M. (2014) Risk of symptomatic gallstones and cholecystectomy after a very-low-calorie diet or low-calorie diet in a commercial weight loss program: 1-year matched cohort study. *Int J Obes (Lond)* **38**, 279-284
41. Erlinger, S. (2000) Gallstones in obesity and weight loss. *Eur J Gastroenterol Hepatol* **12**, 1347-1352
42. Bouchard, G., Johnson, D., Carver, T., Paigen, B., and Carey, M. C. (2002) Cholesterol gallstone formation in overweight mice establishes that obesity per se is not linked directly to cholelithiasis risk. *J Lipid Res* **43**, 1105-1113
43. Hyogo, H., Roy, S., and Cohen, D. E. (2003) Restoration of gallstone susceptibility by leptin in C57BL/6J ob/ob mice. *J Lipid Res* **44**, 1232-1240
44. Biddinger, S. B., Haas, J. T., Yu, B. B., Bezy, O., Jing, E., Zhang, W., Unterman, T. G., Carey, M. C., and Kahn, C. R. (2008) Hepatic insulin resistance directly promotes formation of cholesterol gallstones. *Nat Med* **14**, 778-782
45. Holzbach, R. T. (1984) Animal models of cholesterol gallstone disease. *Hepatology* **4**, 191S-198S
46. Wang, T. Y., Portincasa, P., Liu, M., Tso, P., and Wang, D. Q. (2018) Mouse models of gallstone disease. *Curr Opin Gastroenterol* **34**, 59-70
47. Accili, D., and Arden, K. C. (2004) FoxOs at the crossroads of cellular metabolism, differentiation, and transformation. *Cell* **117**, 421-426
48. Barthel, A., Schmoll, D., and Unterman, T. G. (2005) FoxO proteins in insulin action and metabolism. *Trends Endocrinol Metab* **16**, 183-189
49. Sparks, D. J., and Dong, H. H. (2009) FoxO1 and hepatic lipid metabolism. *Current Opinion in Lipidology* **20**, 217-226
50. Lee, S., and Dong, H. H. (2017) FoxO integration of insulin signaling with glucose and lipid metabolism. *J Endocrinol* **233**, R67-R79
51. Duran-Sandoval, D., Mautino, G., Martin, G., Percevault, F., Barbier, O., Fruchart, J. C., Kuipers, F., and Staels, B. (2004) Glucose regulates the expression of the farnesoid X receptor in liver. *Diabetes* **53**, 890-898
52. Kamagate, A., Qu, S., Perdomo, G., Su, D., Kim, D. H., Slusher, S., Meseck, M., and Dong, H. H. (2008) FoxO1 mediates insulin-dependent regulation of hepatic VLDL production in mice. *J Clin Invest* **1113**, 2347-2364
53. Bowell, P., Otto, T. C., Adi, S., and Lane, M. D. (2003) Convergence of peroxisome proliferator-activated receptor gamma and FoxO1 signaling pathways. *J Biol Chem* **278**, 45485-45491
54. Kibbe, C., Chen, J., Xu, G., Jing, G., and Shalev, A. (2013) FOXO1 competes with carbohydrate response element-binding protein (ChREBP) and inhibits thioredoxin-interacting protein (TXNIP) transcription in pancreatic beta cells. *J Biol Chem* **288**, 23194-23202
55. Armoni, M., Harel, C., Karni, S., Chen, H., Bar-Yoseph, F., Ver, M. R., Quon, M. J., and Karniel, E. (2006) FOXO1 represses peroxisome proliferator-activated receptor-gamma1 and -gamma2 gene promoters in primary adipocytes. A novel paradigm to increase insulin sensitivity. *J Biol Chem* **281**, 18981-18989
56. Langlet, F., Haesler, R. A., Linden, D., Ericson, E., Norris, T., Johansson, A., Cook, J. R., Aizawa, K., Wang, L., Buettnner, C., and Accili, D. (2017) Selective Inhibition of FOXO1 Activator/Repressor Balance Modulates Hepatic Glucose Handling. *Cell* **171**, 824-835 e818
57. Valenti, L., Rametta, R., Dongiovanni, P., Maggioni, M., Ludovica Fracanzani, A., Zappa, M., Lattuada, E., Roviaro, G., and Fargion, S. (2008) Increased Expression and Activity of the Transcription Factor Foxo1 in Nonalcoholic Steatohepatitis. *Diabetes* **57**, 1355-1362
58. Postic, C., Shiota, M., Niswender, K. D., Jetton, T. L., Chen, Y., Moates, J. M., Shelton, K. D., Lindner, J., Cherrington, A. D., and Magnuson, M. A. (1999) Dual roles for glucokinase in glucose homeostasis as determined by liver and pancreatic beta cell-specific gene knock-outs using Cre recombinase. *J Biol Chem* **274**, 305-315
59. Krenkel, O., and Tacke, F. (2017) Liver macrophages in tissue homeostasis and disease. *Nat Rev Immunol* **17**, 306-321
60. Altomonte, J., Richter, A., Harbaran, S., Suriawinata, j., Nakae, J., Thung, S. N., Meseck, M., Accili, D., and Dong, H. (2003) Inhibition of Foxo1 Function Is Associated with Improved Fasting Glycemia in Diabetic Mice. *Am. J. Physiol.* **285**, E718-E728
61. Matsumoto, M., Pocai, A., Rossetti, L., Depinho, R. A., and Accili, D. (2007) Impaired regulation of hepatic glucose production in mice lacking the forkhead transcription factor foxo1 in liver. *Cell Metab* **6**, 208-216
62. Yu, L., Li-Hawkins, J., Hammer, R. E., Berge, K. E., Horton, J. D., Cohen, J. C., and Hobbs, H. H. (2002) Overexpression of ABCG5 and ABCG8 promotes biliary cholesterol secretion and reduces fractional absorption of dietary cholesterol. *J Clin Invest* **110**, 671-680
63. Haeusler, R. A., Pratt-Hyatt, M., Welch, C. L., Klaassen, C. D., and Accili, D. (2012) Impaired generation of 12-hydroxyxlated bile acids links hepatic insulin signaling with dyslipidemia. *Cell Metab* **15**, 65-74

64. Haeusler, R. A., Kaestner, K. H., and Accili, D. (2010) FoxOs function synergistically to promote glucose production. *J Biol Chem* **285**, 35245-35248

65. Li, T., Kong, X., Owsley, E., Ellis, E., Strom, S., and Chiang, J. Y. (2006) Insulin regulation of cholesterol 7alpha-hydroxylase expression in human hepatocytes: roles of forkhead box O1 and sterol regulatory element-binding protein 1c. *J Biol Chem* **281**, 28745-28754

66. Zhang, W., Patil, S., Chauhan, B., Guo, S., Powell, D. R., Le, J., Klotsas, A., Matika, R., Xiao, X., Franks, R., Heidenreich, K. A., Sajan, M. P., Farese, R. V., Stolz, D. B., Tso, P., Koo, S. H., Montminy, M., and Unterman, T. G. (2006) FoxO1 Regulates Multiple Metabolic Pathways in the Liver: Effects on gluconeogenic, glycolytic, and lipogenic gene expression. *J Biol Chem* **281**, 10105-10117

67. Park, W. H., and Pak, Y. K. (2011) Insulin-dependent suppression of cholesterol 7alpha-hydroxylase is a possible link between glucose and cholesterol metabolisms. *Exp Mol Med* **43**, 571-579

68. Twisk, J., Hoekman, M. F., Lehmam, E. M., Poger, M., and Princhen, H. M. (1995) Insulin suppresses bile acid synthesis in cultured rat hepatocytes by down-regulation of cholesterol 7 alpha-hydroxylase and sterol 27-hydroxylase gene transcription. *Hepatology* **21**, 501-510

69. Katsika, D., Magnusson, P., Krawczyk, M., Grunhage, F., Lichtenstein, P., Einarsson, C., Lammert, F., and Marschall, H. U. (2010) Gallstone disease in Swedish twins: risk is associated with ABCG8 D19H genotype. *J Intern Med* **268**, 279-285

70. von Kampen, O., Buch, S., Nothnagel, M., Azocar, L., Molina, H., Brosch, M., Erhart, W., von Schonfels, W., Egberts, J., Seeger, M., Arlt, A., Balschun, T., Franke, A., Lerch, M. M., Mauerle, J., Kratzer, W., Boehm, B. O., Huse, K., Schnewind, B., Tiemann, K., Jiang, Z. Y., Han, T. Q., Mittal, B., Srivastava, A., Fenger, M., Jorgensen, T., Schirin-Sokhan, R., Tonjes, A., Wittenburg, H., Stumvoll, M., Kalthoff, H., Lammert, F., Tepel, J., Buschel, K., Becker, T., Schreiber, S., Platzer, M., Volzke, H., Krawczak, M., Miquel, J. F., Schafmayer, C., and Hampe, J. (2013) Genetic and functional identification of the likely causative variant for cholesterol gallstone disease at the ABCG5/8 lithogenic locus. *Hepatology* **57**, 2407-2417

71. Zhao, Y., Ishigami, M., Nagao, K., Hanada, K., Kono, N., Arai, H., Matsuo, M., Koka, N., and Ueda, K. (2015) ABCB4 exports phosphatidylcholine in a sphingomyelin-dependent manner. *J Lipid Res* **56**, 644-652

72. Rosmorduc, O., Hermelin, B., and Poupon, R. (2001) MDR3 gene defect in adults with symptomatic intrahepatic cholestasis. *Gastroenterology* **120**, 1459-1467

73. Weber, S. N., Bopp, C., Krawczyk, M., and Lammert, F. (2019) Genetics of gallstone disease revisited: update inventory of human lithogenic genes. *Curr Opin Gastroenterol* **35**, 82-87

74. Ghoneim, R. H., Ngo Sock, E. T., Lavoie, J. M., and Piquette-Miller, M. (2015) Effect of a high-fat diet on the hepatic expression of nuclear receptors and their target genes: relevance to drug disposition. *Br J Nutr* **113**, 507-516

75. Qu, S., Almoutte, J., Perdomo, G., He, J., Fan, Y., Kamagate, A., Meseck, M., and Dong, H. H. (2006) Aberrant Forkhead box O1 function is associated with impaired hepatic metabolism. *Endocrinology* **147**, 5641-5652

76. Paik, J. H., Kollipara, R., Chu, G., Ji, H., Xiao, Y., Ding, Z., Miao, L., Tothova, Z., Horner, J. W., Carrasco, D. R., Jiang, S., Gilliland, D. G., Chin, L., Wong, W. H., Castrillon, D. H., and DePinho, R. A. (2007) FoxOs are lineage-restricted redundant tumor suppressors and regulate endothelial cell homeostasis. *Cell* **128**, 309-323

77. Kim, D. H., Perdomo, G., Zhang, T., Slusher, S., Lee, S., Phillips, B. E., Fan, Y., Giannoukakis, N., Gramignoli, R., Strom, S., Ringquist, S., and Dong, H. H. (2011) FoxO6 Integrates Insulin Signaling With Gluconeogenesis in the Liver. *Diabetes* **60**, 2763-2774

**Footnotes:** FoxO1, Forkhead box O1; Fxr, Farnesoid X receptor; Cyp7A1, Cholesterol 7 alpha-hydroxylase; Cyp8B1, Sterol 12-alpha-hydroxylase; Cyp27A1, Sterol 27 alpha-hydroxylase; Cyp7B1, Oxysterol 7 alpha-hydroxylase; Abcg5, ATP-binding cassette G sub-family member 5; Abcg8, ATP-binding cassette G sub-family member 8; Abcb4, ATP-binding cassette subfamily B, member 4; Abcb11, ATP-binding cassette subfamily B, member 11; TG, Triglyceride; ChIP, Chromatin immunoprecipitation; LD, Lithogenic diet; HFLD, High fat-containing lithogenic diet

**Figure legends:**
HepG2 cells were transduced with Adv-FoxO1 and Adv-Empty vectors, followed by the determination of hepatic expression of Fxr at mRNA and protein levels, using real-time qRT-PCR and immunoblot assays, respectively. (A) Fxr mRNA levels. (B) Fxr protein levels. (C) FoxO1 protein levels. (D) Immunoblots. Data in panels B and C were quantitative analysis of Fxr and FoxO1 protein bands relative to that of β-actin protein in panel D. (E) The schematic depiction of the human Fxr promoter. The nucleotides corresponding to the FoxO1 DNA binding motif are underlined. (F) FoxO1 binds to the human Fxr promoter. FoxO1-expressing HepG2 cells were subjected to ChIP assay, using anti-FoxO1 or anti-β-galactosidase IgG as control. Immunoprecipitates were analyzed by PCR assay to detect the presence of the human Fxr promoter. (G) FoxO1 inhibits Fxr promoter activity. HepG2 cells were transduced with Adv-FoxO1 or Adv-Empty vector, followed by transfection with pFxr-Luc encoding the Fxr promoter-directed luciferase reporter system. pGL4.75 expressing Renilla luciferase was included as control for normalizing transfection efficiency. After 24-h incubation in the absence or presence of Fxr agonist GW4064, cells were subjected to dual luciferase activity assay for determining Fxr promoter activity. Data were obtained from 3 independent experiments. *P<0.05 and **P<0.001 vs. control.

Fig. 2. Characterization of L-FoxO1-KO mice on regular chow. L-FoxO1-KO and WT littermates (male, 12 weeks old, n=6) fed regular chow were euthanized after 16-h fasting. Liver tissues were procured and subjected to real-time qRT-PCR and immunoblot analysis, using anti-FoxO1 and anti-actin antibodies. (A) Hepatic FoxO1 mRNA levels. (B) Hepatic FoxO1 protein levels. (C) Anti-FoxO1 and anti-actin immunoblots. (D) Body weight. (E) Fasting blood glucose. (F) Fasting plasma insulin levels. Fasting blood glucose and plasma insulin levels were determined in mice after 16-h fasting. (G) Glucose tolerance. Mice were fasted for 16, followed by intraperitoneal injection of glucose (2 g/kg). (H) Area under curve of blood glucose profiles during glucose tolerance. (I) Plasma TG levels. (J) Plasma cholesterol levels. *P<0.05 vs. WT control.

Fig. 3. Glucose metabolism in L-FoxO1-KO mice on LD. Two groups of WT mice (male, 8 weeks old, n=7/group) were fed a regular chow (RD) and lithogenic diet (LD) for 12 weeks. Mice were euthanized after 16-h fasting, and liver tissues were subjected to real-time qRT-PCR and immunoblot analysis for determining hepatic FoxO1 expression. (A) Hepatic FoxO1 mRNA levels. (B) Hepatic FoxO1 protein levels. (C) Anti-FoxO1 and anti-actin immunoblots. In addition, L-FoxO1-KO and WT littermates (male, 8 weeks old, n=7-9/group) were fed on LD for 12 weeks, followed by the determination of glucose metabolism. (D) Body weight. (E) Blood glucose levels under fed and fasting conditions. Fasting blood glucose levels were determined after 16-h fasting. (F) Glucose tolerance. Mice were fasted for 16, followed by intraperitoneal injection of glucose (2 g/kg). (G) Plasma insulin levels. Plasma insulin levels were determined after 16-h fasting. Similar results were obtained from female L-FoxO1-KO and WT littermates on LD. *P<0.05 and **P<0.001 vs. WT control.

Fig. 4. Effect of FoxO1 depletion on hepatic lipid metabolism on LD. Liver tissues procured from euthanized mice in WT control (A and C) and L-FoxO1-KO (B and D) groups on LD were subjected to H&E (A and B) and oil red O staining (C and D), respectively. In addition, aliquots of liver tissues (10 mg) were used for the determination of hepatic triglyceride (E), cholesterol (F) and bile acid contents (G). N=7-9 per group. Bar, 100 μm.

Fig. 5. Effect of FoxO1 depletion on hepatic gene expression. Total RNA from liver tissues of euthanized mice in L-FoxO1 and WT groups were subjected to real-time qRT-PCR analysis for determining the expression of mRNAs corresponding to key enzymes in bile acid receptor signaling, bile acid synthesis, biliary cholesterol and phospholipid secretion. *P<0.05 vs. WT control. N=7-9 per group.

Fig. 6. Effect of hepatic FoxO1 depletion on gallstone formation on LD. Gallbladder was procured from euthanized mice in WT control (A and C) and L-FoxO1-KO group (B and D) in males (A and B) and females (C and D) for the determination of gallstone weight relative to gallbladder weight. (E) Gallstone incidence rate. (F) Gallstone weight. (G) Gallbladder weight. (H) Liver weight. As we did not detect significant differences in gallstone incidence rates between male and female mice in WT or L-FoxO1-KO group, we calculated the overall gallstone incidence rate as the number of gallstone-positive mice out of total number of mice in both sexes in WT (5 out of 11 mice) vs. L-FoxO1 (8 out of 16 mice) groups fed on LD. Gallbladders were marked by arrow. Bar, 1 cm.
**Fig. 7. Glucose metabolism in L-FoxO1-KO mice on HFLD.** L-FoxO1-KO and WT littermates (male, 5 weeks old, n=7/group) were fed on HFLD for 12 weeks, followed by the determination of glucose metabolism. (A) Growth Curve. (B) Fat mass. (C) Lean mass. (D) Food intake. (E) Glucose tolerance test. Glucose tolerance. Mice were fasted for 16 h, followed by intraperitoneal injection of glucose (2 g/kg). (F) Insulin tolerance test. Mice were injected intraperitoneally with insulin (0.75 IU/kg), followed by the determination of blood glucose levels.

**Fig. 8. Effect of FoxO1 depletion on hepatic lipid metabolism on HFLD.** Liver tissues from euthanized WT (A and C) and L-FoxO1-KO (B and D) male mice fed on HFLD were subjected to histological examination after H&E (A and B) and Oil red O (C and D) staining, respectively. Furthermore, aliquots of liver tissues (10 mg) were used for the determination of hepatic triglyceride (E), cholesterol (F) and bile acid contents (G). N=7 per group. Bar, 100 µm.

**Fig. 9. Effect of hepatic FoxO1 depletion on gallstone disease on HFLD.** Gallbladder was procured from euthanized mice in WT (A) and L-FoxO1-KO (B) groups for the determination of gallstone weight relative to gallbladder weight. Gallstones were procured from the gallbladders of WT (C) and L-FoxO1-KO (D) mice and were visualized in a light microscope. (E) Gallstone incidence rate. (F) Gallstone weight. (G) Gallbladder weight. (H) Liver weight. No significant differences in gallstone incidence rates between male and female mice in WT or L-FoxO1-KO group. As a result, the gallstone incidence rate was determined as the number of gallstone-positive mice out of total number of mice in both sexes in WT (7 out of 14 mice) vs. L-FoxO1 (8 out of 17 mice) groups fed on HFLD. Gallbladders were marked by arrow. Bar=1 cm in Panels A and B. Bar=100 µm in Panels C and D.
Figure 6

(A) WT Male
(B) L-FoxO1-KO Male
(C) WT Female
(D) L-FoxO1-KO Female

(E) Gallstone incidence rate (%)
(F) Gallstone weight (μg/mg gallbladder)

Gallstone incidence rate: 5 out of 11 for WT, 8 out of 16 for L-FoxO1-KO.

(G) Gallbladder weight (mg/g liver)
(H) Liver weight (mg/g body weight)
Figure 7

A. Body weight (g) over age (weeks)

B. Fat mass (% body weight)

C. Lean mass (% body weight)

D. Food intake (Kcal/g body weight)

E. Blood glucose (mg/dL) over time (min)

F. Blood glucose (mg/dL) over time (min)
Figure 8

(A) WT H&E staining
(B) L-FoxO1-KO H&E staining
(C) WT Oil red O staining
(D) L-FoxO1-KO Oil red O staining

(E) Hepatic TG
(F) Hepatic cholesterol
(G) Hepatic bile acids

WT vs. L-FoxO1-KO comparison graphs.
Figure 9

(A) WT

(B) L-FoxO1-KO

(C) WT

(D) L-FoxO1-KO

(E) Gallstone incidence rate (%)

7 out of 14

8 out of 17

(F) Gallstone weight (µg/mg gallbladder)

WT

L-FoxO1-KO

(G) Gallbladder weight (mg/g liver)

WT

L-FoxO1-KO

(H) Liver weight (mg/g body weight)

WT

L-FoxO1-KO
Depletion of hepatic forkhead box O1 does not affect cholelithiasis in male and female mice
Xiaoyun Feng, Cuiling Zhu, Sojin Lee, Jingyang Gao, Ping Zhu, Jun Yamauchi, Chenglin Pan, Sucha Singh, Shen Qu, Rita Miller, Satdarshan P. Monga, Yongde Peng and H. Henry Dong

J. Biol. Chem. published online April 9, 2020

Access the most updated version of this article at doi: 10.1074/jbc.RA119.012272

Alerts:
  • When this article is cited
  • When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts