Hepatic Suppression of Foxo1 and Foxo3 Causes Hypoglycemia and Hyperlipidemia in Mice

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Dysregulation of blood glucose and triglycerides are the major characteristics of type 2 diabetes mellitus. We sought to identify the mechanisms regulating blood glucose and lipid homeostasis. Cell-based studies established that the Foxo forkhead transcription factors Forkhead box O (Foxo)-1, Foxo3, and Foxo4 are inactivated by insulin via a phosphatidylinositol 3-kinase/Akt-dependent pathway, but the role of Foxo transcription factors in the liver in regulating nutrient metabolism is incompletely understood. In this study, we used the Cre/LoxP genetic approach to delete the Foxo1, Foxo3, and Foxo4 genes individually or a combination of two or all in the liver of lean or db/db mice and assessed the role of Foxo inactivation in regulating glucose and lipid homeostasis in vivo. In the lean mice or db/db mice, hepatic deletion of Foxo1, rather than Foxo3 or Foxo4, caused a modest reduction in blood glucose concentrations and barely affected lipid homeostasis. Combined deletion of Foxo1 and Foxo3 decreased blood glucose levels, elevated serum triglyceride and cholesterol concentrations, and increased hepatic lipid secretion and caused hepatosteatosis. Analysis of the liver transcripts established a prominent role of Foxo1 in regulating gene expression of gluconeogenic enzymes and Foxo3 in the expression of lipogenic enzymes. Our findings indicate that Foxo1 and Foxo3 inactivation serves as a potential mechanism by which insulin reduces hepatic glucose production and increases hepatic lipid synthesis and secretion in healthy and diabetic states. (Endocrinology 153: 631–646, 2012)
transcription factors, serves as a target of Akt in the insulin signaling cascade regulating gene expression of IGF-binding protein-1 (Igfbp1), glucose-6-phosphatase (G6pc), and phosphoenolpyruvate carboxykinase (Pck1) in hepatocytes (7–9). G6pc and Pck are the rate-limiting enzymes regulating gluconeogenesis and hepatic glucose production. Akt activation inhibits Foxo1 by phosphorylating serine/threonine residues at T24, S256, and S319 (7, 10), corresponding sites are also found in other Foxo members including Foxo3 and Foxo4 (11, 12). Phosphorylation of these residues enhances Foxo1 interaction with E3 ubiquitin ligase for Foxo1 degradation (7, 13–15). Importantly, because Foxo1 interacts with an insulin-responsive element (IRE) on the promoter region(s) of Igfbp1, G6pc, and Pck to promote hepatic gene expression, it is speculated that Foxo1 is a major mediator of insulin action in the control of a set of insulin-responsive genes and nutrient homeostasis (7). In fact, mice lacking the Foxo1 gene in the liver displayed reduced gluconeogenic gene expression, improved glucose tolerance, and reduced hepatic glucose production (16). Moreover, heploinsufficiency or hepatic Foxo1 deletion in mice lacking both the Irs1 and Irs2 genes reduces gluconeogenic gene expression and rescues diabetic phenotype of insulin resistance (17–19), suggesting that targeting hepatic insulin→Akt→Foxo1 signaling cascade provides a strategy to prevent the progression of diabetes mellitus.

Members of Foxo proteins, Foxo1, Foxo3, and Foxo4, contain conserved Akt phosphorylation sites; share a target consensus sequence similar to the IRE, and govern a variety of cellular processes including development, survival, and metabolism (20). Some functions of each member appear to be unique. For example, Foxo1-null mice are embryo lethal and Foxo3- and Foxo4-null mice are viable (21). Recently it has been shown that Foxo proteins synergistically promote hepatic glucose production (22), but their role in the control of lipid homeostasis is unclear.

In this study, we used genetic approaches to assess the role of hepatic Foxo1, Foxo3, and Foxo4 in regulating glucose and lipid homeostasis in mice and demonstrated that Foxo1 and Foxo3 differentially regulated glucose and lipid metabolism genes and that inactivation of both genes decreased hepatic glucose production and increased lipid synthesis and secretion in the lean and diabetic db/db mice.

Materials and Methods

Animals
Generation of mice with floxed (flanked by loxP) Foxo1, Foxo3, and Foxo4 has been described previously (23). To generate the liver-specific Foxo knockout mice, floxed Foxo1, Foxo3, or Foxo4 mice were crossed with Alb-Cre transgenic mice that express the Cre recombinase cDNA under the rat albumin promoter (3, 24). All mice were then backcrossed with C57BL/6J for four generations to achieve 93.75% C57BL/6J background with 6.25% 129/Sv background and fed with regular chow (Prolab isopro 5P76; Pittsburgh, PA). Diabetic db/db mice were resultant from db/+ breeding pair (C57BKS.Cg-m Lepr<sup>Δ/Δ</sup>), The Jackson laboratory, Bar harbor, ME), and db/db::Foxo knockout mice were generated by crossing db/+ mice with liver-specific Foxo1 knockout (F1KO), Foxo3 knockout (F3KO), or both Foxo1 and Foxo3 (F1/3KO) mice. All mice were housed in cages on a 12-h light, 12-h dark photcycle with free access to water and normal chow. If not specified elsewhere, the control and knockout mice were all male at the age of 8–12 wk. All animal experiments were performed according to procedures approved by the Texas A&M Health Science Center and Children’s Hospital Boston Institutional Animal Care and Use Committee.

Blood chemistry and metabolic analysis
Serum samples were collected from mice that were 18 h overnight fasting or random-fed ad libitum and analyzed for insulin (Crystal Chem Inc., Chicago, IL), free fatty acid, triglyceride, cholesterol, and ketone body (Wako, Richmond, CA) using commercial kits. Serum albumin levels were assessed using BCG reagent, and alkaline phosphatase activities were measured using QuantiChrom alkaline phosphatase assay kit-DALP-250 (Bioassay System, Hayward, CA). Blood glucose levels were measured using a portable glucometer (Bayer, Indianapolis, IN). For glucose tolerance tests, mice were fasted overnight and injected with 2 g/kg body weight D-glucose ip, as previously described (3). For insulin tolerance tests, random-fed mice were injected ip with 1 U/kg body weight of human insulin (Eli Lilly, Indianapolis, IN), and blood glucose levels were measured at the indicated time points. Bone mineral density and fat accumulation were determined using dual-energy x-ray absorptiometry with a Lunar PIXImus II mouse densitometer (GE LUNAR Corp., Madison, WI).

For very low-density lipoprotein (VLDL) triglyceride secretion analysis, mice were fasted for 5 h during daytime and injected 500 mg/kg body weight tyloxapol (Sigma, St. Louis, MO) via tail vein, as previously described (17). Blood samples were collected at 0, 30, 60, 90, and 120 min after injections and then analyzed for triglyceride concentrations.

Lipid determination and fatty acid analysis
The lipids from plasma were extracted in the presence of authentic internal standards by the method of Folch et al. (25). Two hundred microliters of plasma of classes within the extract were separated by preparative thin-layer chromatography, as previously described (26).

Liver glycogen and lipid assay
Liver glycogen, triglyceride, and cholesterol concentrations were analyzed as previously described (3, 17, 27).

Histological analysis
Liver tissue was fixed in 10% formaldehyde for hematoxylin and eosin (H&E) staining and periodic acid-Schiff (PAS) staining.
Frozen liver tissue was used for oil red O staining to evaluate hepatic lipid content.

**Mouse primary hepatocyte isolation and cell culture**

Primary mouse hepatocytes were isolated from 8- to 12-wk-old mice via collagenase (0.05% collagenase type I; Worthington, Lakewood, NJ) digestion and Percoll (Amersham Biosciences AB, Piscataway, NJ) gradient purification and cultured in collagen-coated plates. Freshly isolated hepatocytes were resuspended in DMEM/25 mM glucose supplemented with 10% fetal bovine serum and antibiotics for 4 h; after cell attachment, hepatocytes were cultured in serum-free medium DMEM/25 mM glucose overnight and then treated with or without 100 nM insulin for further analysis.

**Western blotting**

Serum were collected, resolved by SDS-PAGE, and transferred to nitrocellulose membrane for immunoblotting analysis using apolipoprotein (Apo) B-100 antibody (BioDesign International, Saco, ME). Protein lysates extracted from liver or primary hepatocytes were used to detect protein expression level of Foxo1, Foxo3, Foxo4, and β-actin. Antibodies against each were purchased from Cell Signaling Technology (Danvers, MA), and the immunoblotting protocol was previously described (28).

**Affymetrix GeneChip analysis**

Liver mRNA expression in control, F1KO, F3KO, and F1/3KO mice fasted for 18 h (n = 2/group) was determined on MG430 2.0 GeneChips (Affymetrix, Santa Clara, CA). A total of eight fasted mice were used. Total RNA was isolated from the liver tissues using Trizol (Invitrogen, Grand Islands, NY), and 15 μg of each RNA sample was used for labeling and hybridization which was performed at the Harvard Medical School Children’s Hospital Boston Genetic Core Facility. Affymetrix microarray Suite 5.0 was used to generate cell intensity files that were analyzed by robust multichip average analysis in planted in GeneSpring 7.2 (Agilent Technologies, Santa Clara, CA) and further analyzed by dChip software (http://biosun1.harvard.edu/complab/dchip).

**Mammalian cell culture and gene transfection of Foxo1 and Foxo3 in vitro**

To assess whether Foxo gene expression regulates hepatic gene expression in vitro, we cultured HepG2 cells from American Type Culture Collection (Manassas, VA) in 12-well plates in DMEM/5.5 mM glucose medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin for 4 h. After cell attachment, cells were transfected with plasmid DNA expressing green fluorescent protein (GFP), or human Foxo1, or human Foxo3 using TransIT-T293 transfection reagent (Minus, Madison, WI), as we previously described (7, 29). After 6 h of gene transfection, cells were cultured in in serum-free medium DMEM/5.5 mM glucose overnight and then treated with 30 mM high glucose for 24 h, a condition that promotes lipid synthesis in cells (30, 31). Then RNA was extracted from cells and measured by real-time PCR for relative expression of gene of interest.

**Real-time PCR**

One microgram of total RNA were reverse transcribed with random hexamer primers (Bio-Rad Laboratories, Hercules, CA). The relative mRNA abundance levels normalized to cyclophilin levels were determined with the threshold cycle method after amplification, using an iCycler IQ real-time PCR detection system with SYBER green (Bio-Rad Laboratories). Data are presented as means ± SEM. The procedure was essentially same as previously described (28). The PCR primer sequences used for mouse tissue or cell are the following: cyclophilin, 5′-ttgcttgcatcttg-3′ and 5′-tgccatcgcacgctct-3′; Foxo1, 5′-ag atgagtcgctgggc-3′ and 5′-gatggaactcctggtc-3′; Foxo3, 5′-ctctagccgcttcctgta-3′ and 5′-atgtagctcaggtatg-3′; Foxo4, 5′-ccacacactctgtgaggcc-3′ and 5′-ttccatacaagccact-3′; FoxA2, 5′-cgagcaccattacgccttc-3′ and 5′-ttggtagtctgacagtct-3′; Pck1, 5′-cc atgctgctatatctg-3′ and 5′-gacctctgctctctccatac-3′; G6pc, 5′-ca tcggaggctctgctc-3′ and 5′-ggcagatgattgataaactg-3′; Igfbp-1, 5′-cgctcagcacctatacag-3′ and 5′-ttcgatttctcgtc-3′; peroxisome proliferator activated receptor gamma coactivator 1α (Pgc1α), 5′-cctcagttacactcgagg-3′ and 5′-atgtagttcatctgctg-3′; Iris, 5′-cc cgctgctgccatatacg-3′ and 5′-gcacctggtgatgctcag-3′; Ir2s, 5′-actctccaggttctgct-3′ and 5′-gctctggagggcaggtat-3′; glucokinase (Gck), 5′-ctctgcctctctgcg-3′ and 5′-gctctgctctcctcactgcgg-3′; fatty acid synthase (Fasn), 5′-agcgctgctgctgtgctg-3′ and 5′-ggctctgtcatgtgtgtg-3′; 3-hydroxy-3-methylglutaryl-CoA reductase (Hmgcr), 5′-caacagctgctcagctg-3′ and 5′-tcctgctcgaagctg-3′. The PCR primer sequences used for human HepG2 cells are the following: H-Foxo1, 5′-ccaggtttctctctctg-3′ and 5′-ctctggagggcaggtatctg-3′; H-Foxo3, 5′-ccaggtttctctctctg-3′ and 5′-ctctggagggcaggtatctg-3′; H-Foxo4, 5′-ccaggtttctctctctg-3′ and 5′-ctctggagggcaggtatctg-3′; H-Pck1, 5′-ccaggtttctctctctg-3′ and 5′-ctctggagggcaggtatctg-3′; H-G6pc, 5′-ccaggtttctctctctg-3′ and 5′-ctctggagggcaggtatctg-3′; H-Igfbp-1, 5′-ccaggtttctctctctg-3′ and 5′-ctctggagggcaggtatctg-3′.

**Statistical analysis**

All results are presented as mean ± SEM determined by two-tailed Student’s t tests or one-way ANOVA. Paris comparisons of the means were made, and P < 0.05 was taken as a significant difference. The Bonferroni method was used to adjust the observed significance levels for the fact the multiple contrasts were being tested. Serial measurements made during glucose and insulin tolerance tests were summarized by determining the area under curve using Medcalc version 10.0 (http://www.medcalc.org), as previously described (3).

**Results**

**Gene expression of Foxo1 and Foxo3 in the liver of mice and generation of Foxo liver specific knockout mice**

We first examined the relative expression levels of each Foxo isoform in the liver of mice by real-time PCR in comparison with another forkhead gene, FoxA2 (known as hepatocyte nuclear factor-3β). The expression levels of Foxo1 and Foxo3 are almost equally abundantly expressed in adult liver, almost 2-fold higher than that of...
**FIG. 1.** Relative expression levels of Foxo genes in the liver of mice and mice lacking Foxo1, Foxo3, and Foxo4. A, The liver of random-fed C57/BL6 at the age of 10 wk was collected and RNA was analyzed by real-time PCR. Values are relative to FoxA2 gene RNA (mean ± SEM). *, P < 0.01 (n = 3). B, Genotyping of Cre, floxed Foxo alleles by PCR. Integration of Cre or insertion of 34 bp of loxp sites in Foxo1 and Foxo3 alleles caused an up-shift of the PCR product in 2% agarose gel and stained with ethidium bromide. The sizes of PCR products are indicated. L/L, Loxp homozygous; +/-, wild-type alleles; M, 100 bp DNA ladder. PCR primers 5'-gcttacagcagagatgttctcacatt, 5'-ccagagtctttgtatcaggcaaataa, and 5'-caagtccattaatccagcattga are for Foxo1; 5'-attcctttggaaatcaacaaaactg, 5'-tgctttgatactattccacaaaccc, and 5'-agatttatgccacactgcttctc are for Foxo3; 5'-gctttcttagtgagatgggaaa, 5'-attccttcctttcacactc, and 5'-ctctttggtgagtaaatgttttg for Foxo4; 5'-agatgttgcagcaatctc and 5'-ccagtgaaacagcattgctg are for Cre; NS, Nonspecific band. C–E, Protein content of Foxo1, Foxo3, and Foxo4 analyzed by specific immunoblotting of liver lysates extracted from 10-wk-old control (CNTR), F1KO, F3KO, or F4KO mice (n = 2 mice/group). F–H, Relative expression levels of Foxo1, Foxo3, and Foxo4 in the mutant mice. The liver of random-fed F1KO, F3KO, F4KO, F1/3KO, F1/4KO, F3/4KO, and F1/3/4KO mice at the age of 10–12 wk was collected and Foxo mRNA was analyzed by real-time PCR. Values are relative to the liver of control mice. *, P < 0.01 vs. control (n = 3/group).
Hepatic Foxo1 and Foxo3 inactivation synergistically alters lipid homeostasis

We next determined the effect of Foxo inactivation on lipid homeostasis in all of the mutant mice. F1KO, F3KO, F4KO, F1/4KO, and F3/4KO mice had no significant changes in the blood concentrations of triglyceride and total cholesterol; however, F1/3KO and F1/3/4KO mice had significantly elevated serum triglyceride and cholesterol concentrations by nearly 2-fold, compared with control or singular Foxo knockout mice (Fig. 3, A and B). We further performed experiments for quantitative analysis of the content of fatty acids in seven different fatty acid classes in the plasma sample of control and F1/3KO mice. The results indicated that the fatty acid concentrations in cholesterol ester, phosphatidylcholine, and phosphatidylethanolamine were significantly higher in F1/3KO mice compared with those of control mice (Fig. 3C). We also further measured the cholesterol contents in different lipoprotein complexes in the serum of mice. Total cholesterol content and high-density lipoprotein (HDL)- and very-low-density lipoprotein (VLDL)-/low-density lipoprotein (LDL)-associated cholesterol were all significantly increased in F1/3KO mice compared with control mice, although there was no significant change in F1KO and F3KO mice (Fig. 3D), suggesting there exists a synergistic effect on lipid metabolism following Foxo1 and Foxo3 inactivation.

Given the significant increase in VLDL/LDL cholesterol in F1/3KO mice (66 vs. 38 mg/dl in control, P < 0.01), we examined whether Foxo1 and Foxo3 inactivation altered lipid secretion. We measured lipid secretion rate by tail vein injection of tyloxapol. Tail vein injecting mice with tyloxapol coats lipoprotein particles and inhibits peripheral absorption (32). Upon tyloxapol injection, serum lipid concentrations were increased in all mice; however, the lipid concentration in F1/3KO mice was higher than that of control, F1KO, or F3KO mice at every time point measured after tyloxapol injection (Fig. 3E), suggesting that both Foxo1 and Foxo3 inactivation caused a higher rate of lipid secretion into blood circulation. The lipoprotein ApoB-100 level in serum was markedly increased in all mice after 2 h of tyloxapol injection; however, the ApoB-100 level was much higher in F1/3KO mice than that of control, F1KO, and F3KO mice (Fig. 3F). Together, these results demonstrate that hepatic Foxo1
and Foxo3 inactivation promotes lipid secretion and contributes to elevation of blood lipid concentrations.

We performed histological analysis of the liver from these mice. Although the morphology of hepatocytes in the mice of each group was unchanged by H&E staining, oil-red O stain indicated that F1/3KO livers displayed mild hepatosteatosis, and PAS staining for glycogen was not different (Fig. 4A). Further chemical measurements indicated that triglyceride content was increased by 30% in F1/3KO liver compared with control liver (74.3 ± 6.5 vs. 63.6 ± 5.6).
FIG. 3. Hepatic Foxo inactivation increases blood triglyceride and cholesterol levels and promotes lipid secretion. Concentrations of serum triglyceride (A) and total cholesterol (B) in 18-h fasted mice were measured. *, $P < 0.05$ (n = 6). C, The content of fatty acids in seven different lipid classes in serum from 18-h fasted control (CNTR) and F1/3KO mice were measured. Data were the average ± SEM from three different experiments in which samples from each group were from a pool of serum from five mice. CE, Cholesterol ester; FA, free fatty acids; TAG, triglycerides; LYP, lysophosphatidylcholine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; FC, free cholesterol. D, Serum total cholesterol, HDL-cholesterol, and LDL/VLDL-cholesterol levels were measured in 18-h fasted control, F1KO, F3KO, and F1/3KO mice using a commercial assay kit. Error bars represent SEM. *, $P < 0.05$ vs. control mice (n = 4). E, Liver triglyceride secretion was analyzed in 12-wk-old control, F1KO, F3KO, and F1/3KO mice that were fasted for 5 h and then injected with tyloxapol by tail vein. Serum triglyceride concentrations were measured at 30, 60, 90, and 120 min after tyloxapol injection. Error bars represent SEM. *, $P < 0.05$ vs. control (n = 3). F, Lipoprotein ApoB-100 levels in serum were analyzed by immunoblotting in 5-h fasted mice or mice after 2 h of tyloxapol injection (20 μg protein/each lane) from control, F1KO, F3KO, and F1/3KO mice. Data are representative from at least three different experiments. *, $P < 0.05$ vs. control (n = 3).
Hormone measurements indicated that serum insulin concentration was unchanged in the mutant mice in the fasting state, but significantly reduced in F1/3KO mice in the fed state compared with control mice (0.53 ± 0.04 vs. 0.75 ± 0.03 ng/ml, P < 0.05) (Fig. 4B).

**FIG. 4.** Foxo1 and Foxo3 inactivation induces mild hepatosteatosis. A, Liver morphology was analyzed by H&E stain in liver sections. Liver glycogen and lipid were analyzed by PAS reagent and oil red O stain in fed mice at the age of 10 wk, respectively, and representative sections are shown. (Magnification, ×200 for H&E and PAS, and ×400 for oil red O). B, Metabolic characteristics of the Foxo mutant mice. Data were presented as the average ± SEM. Mice at the age of 10 wk, after 18 h fasting in each group, and their littermate controls were analyzed, unless otherwise indicated. CNTR, Control; BMD, bone mineral density; BW, body weight; NEFA, nonesterified fatty acids; ALP, alkaline phosphatase. *, P < 0.05 vs. control mice (n = 6–8 mice/group).

91 ± 5.1 mg/g, P < 0.05) (Fig. 4B). To delineate the singular and synergistic contributions of hepatic Foxo1 and Foxo3 inactivation to the expression levels of genes key to metabolic regulation, we determined the Foxo1- and/or Foxo3-deficient liver transcriptome us-

**Hepatic Foxo1 and Foxo3 differentially regulate gluconeogenic and lipogenic gene expression**

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ing Affymetrix GeneChips. Analysis of RNA samples from the liver of F1KO, F3KO, and F1/3KO mice and corresponding control mice revealed that F1KO liver decreased expression of genes encoding G6pc and Pck1, rate-limiting enzymes for gluconeogenesis, whereas F3KO liver had no such effect (Fig. 5A). However, F3KO liver showed increased expression of genes of lipogenesis, including Fasn and Hmgcr, two key enzymes regulating the synthesis of triglyceride and cholesterol, although F3KO liver demonstrated no effect on the expression of Gck, and F1KO increased Gck gene expression. Real-time PCR analysis confirmed that F1KO significantly reduced the gene tran-
The transcriptional level of G6pc and Pck1 by 50% (log2-fold = −0.5 vs. control), and F3KO liver had little effect on expression of the two genes, but F1/3KO demonstrated almost completely diminished gene expression of G6pc and gluconeogenic transcription factor Pgc1α (log2-fold = −2.5 vs. control, P < 0.05) (Fig. 5B). By contrast, F3KO liver promoted expression of genes encoding Fasn and Hmgcr, revealing a stronger effect than that of F1KO liver. A synergistic effect of Fasn and Hmgcr stimulation in F1/3KO liver compared with singular F1KO and F3KO liver was also evident (Fig. 5B). Additionally, F1KO and F1/3KO liver reduced expression of other genes, including Irs2 and Igfbp1, whereas expression of Irs1 was unchanged (Fig. 5B). Together, these results indicate that Foxo1 inactivation impaired expression of genes regulating gluconeogenesis, and Foxo3 inactivation preferentially impaired the expression of genes regulating lipid synthesis, although the inactivation of both genes revealed a synergistic effect upon expression of genes controlling glucose and lipid production in the liver.

**Foxo1 and Foxo3 inactivation differently impairs the effect of insulin upon hepatic gene expression in vitro**

To examine whether Foxo deficiency impairs the effect of insulin on hepatic gene expression, we isolated and cultured primary hepatocytes from control, F1KO, F3KO, and F1/3KO mice and treated the cells with insulin. Western blot analysis revealed that 100 nm insulin stimulation for 30 min promoted Foxo1 ubiquitylation in control and F3KO hepatocytes and caused an upper shift in Foxo3 protein, an indicative of phosphorylation in control and F1KO hepatocytes (Fig. 6A). Insulin treatment for 6 h suppressed Pck1 and G6Pc expression by at least 30% in control and F3KO hepatocytes. In contrast, the inhibitory effect of insulin was completely abolished in F1KO and F1/3KO hepatocytes (Fig. 6B). In addition, the expression level of Pck1 or G6pc was slightly but significantly reduced in F1KO and F1/3KO cells compared with that of control hepatocytes. On the other hand, insulin stimulated expression of Fasn and Hmgcr by 1.5-fold in control cells, whereas F1KO, F3KO, and F1/3KO hepatocytes significantly increased expression of the two genes under both basal condition and insulin stimulation, and synergistic increases of Fasn and Hmgcr were evident in F1/3KO hepatocytes compared with F1KO or F3KO cells (Fig. 6C).

**Overexpression of Foxo1 and Foxo3 synergistically regulates hepatic gene expression**

To further confirm whether Foxo1 and Foxo3 synergistically affect expression of gluconeogenic and lipogenic gene expression in a gain-of-gene function system in hepatocytes, we cultured HepG2 cells, a human hepatoma cell line, and overexpressed Foxo1 by 600-fold or Foxo3 by 1500-fold compared with the endogenous Foxo1 or Foxo3 level in cells expressing control vector GFP (Fig. 7A). Although it is shown that Foxo3 stimulates Foxo1 gene transcription in fibroblasts (33), overexpression of Foxo3 had no such effect on the endogenous Foxo1 gene expression or vice versa in HepG2 cells (Fig. 7A). Without insulin stimulation, overexpression of Foxo1 or Foxo3 increased Pck1 and G6pc gene expression by at least 2-fold.

**FIG. 6. Effect of Foxo1 and Foxo3 inactivation upon insulin-regulated hepatic gene expression in mouse primary hepatocytes. A, Effect of insulin on Foxo1 and Foxo3 protein degradation. Mouse primary hepatocytes isolated from the liver of 12-wk-old control (CNTR), F1KO, F3KO, and F1/3KO mice were cultured and treated with 100 nm insulin for 0.5 h and then protein lysates analyzed by immunoblotting against antibody of Foxo1, Foxo3, or β-actin. B and C, Effects of insulin on Pck1 and G6pc (B) and Fasn, and Hmgcr (C) gene expression in primary hepatocytes deficient in Foxo genes. The primary hepatocytes from A were cultured and treated with 100 nm insulin for 6 h. Then RNA was extracted and levels of gene expression were analyzed by real-time PCR, and data are presented in comparison with control. *, P < 0.05 indicates the difference between basal level and insulin treatment; #, P < 0.05 indicates the difference between control and other groups without insulin treatment (n = 3 different experiments).**
and inhibited Fasn and Hmgcr gene expression by at least 50% compared with expression of GFP ($p < 0.05$). Overexpression of both Foxo1 and Foxo3 synergistically and significantly increased Pck1 and G6pc expression by more than 4-fold and inhibited Fasn and Hmgcr gene expression by more than 80% (Fig. 7, B and C).

**Effect of hepatic Foxo1 and Foxo3 inactivation upon glucose and lipid homeostasis in db/db mice**

To assess the role of hepatic Foxo inactivation in metabolic regulation in diabetes mellitus, we introduced the hepatic deletion of Foxo1, Foxo3, or both genes into the db/db mice and generated F1KO::db/db, F3KO::db/db, and F1/3KO::db/db mice. At 10 wk of age, blood glucose measurements indicated that F1KO::db/db mice had reduced fasting and fed blood glucose concentrations by 40%, whereas F3KO::db/db mice had a slight reduction compared with db/db mice; however, F1/3KO::db/db mice had reduced fasting and fed blood glucose concentrations by nearly 60% (Fig. 8A). Glucose tolerance tests revealed that F1KO, but not F3KO, improved glucose tolerance. F1/3KO::db/db mice showed a trend toward further improved glucose tolerance at the set point of time of glucose measurements but did not exhibit a significant difference in the blood glucose measurements compared with F1KO::db/db mice (Fig. 8B). In the insulin tolerance tests, F1/3KO::db/db mice slightly improved, even though insignificantly, the ability of insulin to lower blood glucose concentrations, similar to the F1KO::db/db mice (Fig. 8C).

The serum triglyceride concentration was significantly increased in F1/3KO::db/db mice compared with control db/db mice (Fig. 8D), whereas F1KO::db/db and F3KO::db/db mice did not display a significant difference. Total serum cholesterol, cholesterol in HDL- or LDL/VLDL-lipoportein complex was significantly higher in F1/3KO::db/db mice, compared with control and other sin-
gular mutant mice. The VLDL/LDL-cholesterol concentrations were increased by 36% in F1/3KO db/db mice compared with db/db mice (150 vs. 96 mg/dl in db/db mice, \( P < 0.01 \)) (Fig. 8E). The oil red O staining of liver

section analysis indicated that F1KO::db/db, F3KO::db/db, and F1/3KO::db/db mice displayed severe hepatoste-

We measured the gene expression level of G6pc and Pck1 in the liver of these mice. db/db liver demonstrated a markedly increased gene transcriptional level of G6pc, Pck1, and Gck, compared with nondiabetic db/+ liver (Fig. 8, G and H); however, the increase of G6pc and Pck1 in db/db liver was almost completely diminished in F1KO::db/db liver, whereas F3KO::db/db liver had few effects. By contrast, F3KO::db/db liver had a further increase in genes encoding Fasn and Hmgcr, although F1KO::db/db liver further increased Gck gene expression (Fig. 6H). The inhibitory effect of G6pc expression and stimulatory effect of Gck in F1KO::db/db liver compared with db/db liver was further enhanced upon both Foxo1 and Foxo3 deletion.

Discussion

In this study, we have generated Foxo1, Foxo3, and Foxo4 liver-specific knock-out mice and characterized the glucose and lipid metabolism in the lean and diabetic db/db mice lacking the hepatic Foxo genes, and demonstrated that Foxo genes differently regulate nutrient homeostasis. Foxo1, Foxo3, and Foxo4 are expressed in the liver of adult mice and all interact with the conserved DNA sequence such as the IRE: TGTTTTG or CAAAACA on the pro-

FIG. 8. Glucose and lipid homeostasis in db/db mice lacking hepatic Foxo1 and Foxo2 genes. A, Blood glucose concentrations were measured from 18-h fasting or random-fed db/db mice with different Foxo inactivation. Data are shown average ± SEM. *, \( P < 0.05 \) vs. db/db mice (n = 6). B, Glucose (B) and insulin tolerance tests (C) of 10-wk-old male db/db mice with hepatic Foxo inactivation. *, \( P < 0.05 \) vs. db/db mice. n.s, No significant difference (n = 6). D, Serum triglyceride concentrations of 18-h fasted db/db mice with hepatic Foxo deficiency. *, \( P < 0.05 \) vs. db/db mice (n = 4). E, Total cholesterol, HDL-cholesterol, and VLDL/LDL-cholesterol concentrations in serum from 18-h fasted mice. *, \( P < 0.05 \) vs. db/db control (n = 4). F, Liver sections of H&E and oil red O staining from db/+ and db/db mice with Foxo inactivation (magnification, \( \times 200 \)) are shown. Photos are representative from at least four sections per genotype. Relative expression levels of genes encoding Pck1 and G6pc (G) and lipogenic enzymes Gck, Fasn, and Hmgcr (H) were measured by real-time PCR in the liver of 18-h fasted mice. Expression level of each gene normalized by cyclophilin are shown at a log2 scale (mean ± sem), and expression level of control liver was set up as log21 (n = 3).
pression of genes encoding the lipogenic enzymes Fasn and Hmgcr. Inactivation of both Foxo1 and Foxo3 synergistically promoted expression of the lipogenic enzymes including Gck and enhanced the level of serum triglyceride, cholesterol, and lipid secretion and caused hepatosteatosis. Moreover, our data also show that Foxo1 inactivation in the liver of db/db mice diminished G6pc and Pck1 gene expression, reduced the blood glucose concentration, and Foxo3 inactivation elevated blood triglyceride and cholesterol concentrations in the context of Foxo1 inactivation.

Foxo proteins regulate liver metabolism by differentially controlling expression of the target genes, such as G6pc, Pck1, and Gck. There is at least one IRE motif present on the promoter region of Pck1 and at least three IRE motifs present on the promoter of G6pc (7, 34, 38). Although deficiency of Foxo1 rather than Foxo3 decreased expression of G6pc and Pck genes, deletion of both Foxo1 and Foxo3 has synergistic effects on lowering G6pc gene expression and stimulating Gck gene expression in F1/3KO liver, which may enhance the conversion of glucose to glucose-6-phosphate and lower hepatic glucose production, thereby reducing blood glucose concentration. Foxo3 binds the IRE and stimulates G6pc promoter activity in vitro (38), but Foxo1 is required for a full expression of G6pc promoter (7–9). Our data also support that Foxo3 contributes to the expression of G6pc in a Foxo1-dependent manner in vivo. Although Foxo4 is suggested to be involved in regulating glucose homeostasis because it is recently shown that F1/3/4KO mice displayed a further reduction in blood glucose concentration compared with F1/3KO mice (22), the unnoticeable role of Foxo4 in our studies may be from the difference of mouse genetic background most of which are C57/B6 background in our animals and the expression level of Foxo4 is also lower than that of Foxo1 or Foxo3 (Fig. 1A). Other forkhead transcription factor FoxA2 has been identified to bind the IRE (39). However, in vitro mutagenesis studies revealed that the effect of insulin on Pck1 and Igfbp1 gene transcription does not correlate with the binding of these factors, similar to Foxo3 (37). FoxA2 is suggested to mimic the action of Foxo1 by insulin (40), but recent data indicate that FoxA2 nuclear localization is not responsive to insulin (41) and liver-specific FoxA2 had little effect on glucose homeostasis (22). Other factors that can interact with Foxo1 include cAMP response element-binding protein/p300 (42), CCAAT/enhancer-binding protein-β (43), and Pgc1α (44). The formation of CCAAT/enhancer-binding protein-β-Foxo1-cAMP response element-binding protein/p300 complex may play a key role in promoting gluconeogenic enzymes and Igfbp1 gene expression via the IRE on the promoter regions (42). Alternatively, Foxo1 does not directly bind the promoter region of Gck in which a functional IRE has not been demonstrated, but Foxo1 acts as an inhibitor for expression of Gck gene by suppressing the effect of hepatocyte nuclear factor HNF-4α, a transcription factor that binds the Gck promoter and stimulates Gck gene expression (45). Upon insulin stimulation, Foxo1 is rapidly phosphorylated and ubiquitylated for degradation (13, 14, 28, 38, 46). How Foxo1 modification and degradation is involved in disrupting its transcriptional complex, terminating or triggering target gene transcription, and reducing hepatic glucose production or enhancing lipid metabolism genes needs further investigation.

Several mechanisms likely contribute to the lipid accumulation in the liver of F1/3KO mice. The increased substrate availability may play a key role in enhancing VLDL secretion in the F1/3KO mouse model in both lean and db/db background. With increased substrate availability, F1KO mice elevated VLDL secretion in association with streptozotocin-induced type 1 diabetes (47). Additionally, F1/3/4KO mice receiving a high-fat diet had a reduced level of nicotinamide adenine dinucleotide oxidation to nicotinamide adenine dinucleotide hydroxide ratio that inactivates sirtuin (Sirt)-1, which can promote hepatic lipid accumulation (48). Moreover, Sirt1 serves as a Foxo1 target gene (49) and Sirt1 activates AMP-activated protein kinase to lower lipid levels in HepG2 cells (31). However, inhibition of Foxo1 in both liver and adipose tissue disrupted hepatosteatosis of high-fat diet-fed mice (50), suggesting that Foxo1 in the adipose tissue also plays a key role in regulating hepatic lipid accumulation, in which during fasting state lipolysis from the adipose tissue provides substrates, such as fatty acids, for gluconeogenesis and maintenance of glucose homeostasis. Recently it has been shown that Foxo1 stimulates several key genes controlling the autophagic process (51), a catabolic pathway the inhibition of which promotes hepatic lipid accumulation (52). It is speculated that reduced autophagic process in the liver of F1/3KO liver may also suppress autophagosome formation, which contributes to hepatic lipid accumulation. Regardless, without hepatic Foxo1 and Foxo3, glucose flux favors the lipogenic pathway and enhances lipid secretion from the liver.

Inactivation of Foxo1 and Foxo3 in F1/3KO liver diminished expression of Irs2 and unaltered Irs1, and insulin resistance was not developed in the insulin tolerance tests, which is consistent with results in which we recently demonstrated that Irs1 plays a dominant role in the control of hepatic nutrient homeostasis (3). In the feeding state, hypoglycemia is accompanied by a reduced blood insulin concentration in F1/3KO mice; thus, insulin sensitivity as well as the glycogen content in the liver the level of which
was unchanged in F1/3KO mice is maintained, compared with control (Fig. 4B). It is also suggested that Irs2 plays a major role in the fasting or early postprandial period and that Irs1 plays a major role in the feeding or postprandial period for control of insulin action and glucose homeostasis (53). Diminished expression of Irs2 may also cause insulin resistance during the fasting or early postprandial period in fasting F1/3KO mice, in which hypoglycemia occurs with a normal blood insulin concentration (Fig. 4B). On the other hand, F1KO mice with a 50% decrease in hepatic Irs2 gene transcription in the fasting state reduced hepatic glucose production and required a 30% increase of glucose infusion rate to maintain euglycemia in hyperinsulinemic euglycemic clamp analysis, an indicative of enhanced insulin sensitivity (16). Consistent with this finding, the F1KO or F1/3KO mice in both lean and db/db background in our studies also displayed a trend toward improvement in whole-body insulin sensitivity (Figs. 2, E and F, and 8C). We showed that loss of hepatic Foxo1 and Foxo3 in vivo synergistically inhibited expression of G6pc and enhanced expression of Fasn and Hmgcr, whereas overexpression of Foxo1 and Foxo3 synergistically enhanced expression of G6pc and inhibited expression of Fasn and Hmgcr in vitro; however, the loss of Foxo1 or Foxo3 alone can be independent and not synergistic to alter gene expression levels of intracellular signaling components, secreted bioactive peptides, and hormones, including but not limited to the IGF-I, Igfbp1, and fibroblast growth factor-21 (Fig. 5A), which may also contribute to the regulation of insulin sensitivity in the target tissues or the whole body.

Hyperinsulinemia, hyperlipidemia, and hyperglycemia are three features of type 2 diabetes mellitus (54). Based on our studies, we expect that, during the early phase of type 2 diabetes hyperinsulinemia from pancreatic β-cell compensation promotes hepatic Akt activation and inhibits all FoxO, which contributes to reducing hepatic glucose production and increasing hepatic lipid synthesis and secretion and mild hepatostatosis. Lipids, such as palmitic acid, are known to serve as potent insulin desensitizers, and their accumulation in the liver can lead to insulin resistance (55, 56), attenuating Akt signaling and activating Foxo1 and Foxo3 that promote hepatic glucose production and contribute to hyperglycemia in concert with other elements. In the diabetic db/db mice, targeting Foxo1 and Foxo3 in the liver prevented the development of hyperglycemia, whereas hepatostatosis is not improved. In fact, increased triglyceride and VLDL is observed in insulin-resistant humans before the development of hyperglycemia (57). Although the molecular mechanisms of synergistic activation of gluconeogenesis and inhibition of lipogenesis by Foxo1 and Foxo3 within different cellular context and environment are unclear and await further investigation, a combinatory strategy targeting both the Foxo1 pathway to prevent hyperglycemia and suppressing the lipogenic pathway to lower lipid profiles would be a key in the control of type 2 diabetes mellitus in the future.

Acknowledgments

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High forkhead box protein 01 (Foxo1) activity in liver increases gluconeogenesis and hepatic glucose output; thus, Foxo1 is accused of contributing to the pathology of diabetes (for review, see Ref. 1). Until recently, other closely related members of the forkhead/winged-helix family of transcriptional regulators, Foxo3 and Foxo4, have received little attention because of negligible knockout phenotypes in mice (2, 3). In the current issue of *Endocrinology*, Zhang *et al.* (4) reveal new metabolic functions for these seemingly redundant family members through analysis of overlapping, liver-specific deletions of the three Foxo proteins. This elegant new study illustrates the increasing complexity of the transcriptional networks controlling metabolism and emphasizes the importance of considering these interactions when devising new therapeutic strategies.

The Fox transcriptional regulators (including Foxa, Foxo, Foxm, and Foxl proteins) have well-known roles in liver organogenesis (for review, see Ref. 5). However, in terms of liver metabolism, Foxo1 has gained the most notoriety due to its potent regulation of glucose homeostasis. Since the discovery of Foxo1 as a key regulator of gluconeogenic gene expression, there has been great interest in developing therapies to block hepatic Foxo1 activity in diabetic patients to reduce glycemia. Toward this end, Zhang *et al.* (4) show that a liver-specific knockout of Foxo1 can reduce blood glucose levels, and that concurrent reduction of Foxo3 further improves glycemia and whole-body insulin sensitivity. These particular data are not surprising, as another recent study reports similar findings (6). Although they differ in their conclusions concerning the role of Foxo4 in glycemia, these papers and others provide strong evidence that reducing Foxo activity in liver may indeed be useful to treat diabetes and metabolic syndrome. However, pertinent information contradicting this dogma is later revealed as Zhang *et al.* (4) further characterize the knockout metabolic phenotypes.

Zhang *et al.* observe that loss of both Foxo1 and Foxo3 also results in significant hypertriglyceridemia and hypercholesterolemia due to increased hepatic lipid secretion and mild steatosis. These new data not only reveal previously unrecognized metabolic functions for the mostly ignored Foxo3 but also demonstrate that Foxo-regulated metabolic networks may be far more complex than originally thought. By dissecting both global and target gene expression, Zhang *et al.* (4) suggest that Foxo1 and Foxo3 synergistically suppress key lipogenic pathways, in addition to enhancing genes controlling glucose metabolism. This has significant therapeutic relevance, as it implies that targeting Foxo proteins for the treatment of hyperglycemia may also have unforeseen negative consequences on cardiovascular health.

The metabolic outcome of reducing Foxo expression in diabetic models has been of great interest for a number of years (see Table 1 for summary). Thus, evidence of hyperlipemia in mice on a Foxo-null background can be found in other reports. An independent triple Foxo1/3/4 hepatic Foxo-null mouse line also displays hypertriglyceridemia and increased lipogenesis, but only after exposure to a high-fat diet (7). Unfortunately, this earlier study presents data only for the triple knockout and does not address the relative contributions of individual Foxo proteins. In contrast, Zhang *et al.* (4) systematically analyze the detailed phenotype of single- and double-knockout mice, shedding light on the role of each family member in liver metabolism. Interestingly, the new study does not include detailed phenotypic or expression data for the triple knockout, with the authors citing lack of an additive effect. These
recent reports provide strong evidence that hepatic Foxo family members regulate lipid metabolism. However, whether this is achieved in a coordinated or redundant fashion still remains unclear, as in vitro experiments exploring a synergistic relationship between Foxo1 and Foxo3 on liver gene expression are less convincing.

Of considerable note, hyperlipidemia caused by Foxo loss is most apparent on insulin-depleted or insulin-resistant backgrounds (Table 1). Insulin potently decreases the activity of all three Foxo proteins (for review see Ref. 8), and reciprocally, both Foxo1 and Foxo3 feed back to augment insulin activity (9, 10). Thus, direct effects on hepatic metabolism, and relatively modest direct effects on glucose tolerance and lipid metabolism, and relatively modest direct effects on glycemia. However, the impact of Foxo on hepatic lipid homeostasis remains controversial due to an abundance of conflicting data from both gain- and loss-of-function in vivo models (9, 14), leaving more questions than answers at this point. It is clear that we need more information about the activity of these factors at a molec-

### TABLE 1. Phenotypic comparison of energy homeostasis in hepatic Foxo-null mouse models

<table>
<thead>
<tr>
<th>Genotype (liver specific)</th>
<th>Background</th>
<th>Glycemia</th>
<th>Insulin</th>
<th>Serum lipids</th>
<th>Hepatic lipids</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Foxo1/−/−</td>
<td>WT</td>
<td>Decreased</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>4, 6, 11, 15</td>
</tr>
<tr>
<td>IRSR/−/−</td>
<td>Decreased</td>
<td>Resistant</td>
<td>Decreased</td>
<td>Decreased</td>
<td>Decreased</td>
<td>15</td>
</tr>
<tr>
<td>c-IRS1/2/−/−</td>
<td>Decreased</td>
<td>Resistant</td>
<td>Increased</td>
<td>—</td>
<td>—</td>
<td>12</td>
</tr>
<tr>
<td>WT + STZ</td>
<td>Decreased</td>
<td>Depleted</td>
<td>Increased</td>
<td>—</td>
<td>—</td>
<td>11</td>
</tr>
<tr>
<td>db/db</td>
<td>Decreased</td>
<td>Resistant</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>4</td>
</tr>
<tr>
<td>Foxo1/3/−/−</td>
<td>WT</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>4</td>
</tr>
<tr>
<td>db/db</td>
<td>—</td>
<td>Resistant</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>4</td>
</tr>
<tr>
<td>Foxo1/4/−/−</td>
<td>WT</td>
<td>Decreased</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>4</td>
</tr>
<tr>
<td>Foxo3/4/−/−</td>
<td>WT</td>
<td>Decreased</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>4</td>
</tr>
<tr>
<td>Foxo1/3/4/−/−</td>
<td>WT</td>
<td>Decreased</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>4</td>
</tr>
<tr>
<td>WT + HFD</td>
<td>NR</td>
<td>Resistant</td>
<td>Increased</td>
<td>Increased</td>
<td>Increased</td>
<td>7</td>
</tr>
</tbody>
</table>
| HFD, High-fat diet; STZ, streptozotocin; WT, wild type; —, no significant change; NR, not reported.

With age.

db/db Mouse insulin resistance due to obesity.

involved, but the mechanism by which these transcriptional regulators in liver so dramatically impact whole-body metabolism still remains a mystery.

The significant effects on glucose tolerance and lipid metabolism, and relatively modest direct effects on gluconeogenic and lipogenic gene expression, point a finger toward possible inter-organ cross talk. Clues from expression analysis in Foxo-null models suggest that dysregulation of hormones secreted from liver (hepatokines) may provide the answer. Zhang et al. (4) show that fibroblast growth factor 21 (FGF21) mRNA levels are significantly higher in Foxo3 knockout mice. Loss of Foxo1 also increases circulating FGF21 in mice when insulin is depleted (11) or signaling blocked (12). Interestingly, FGF21 stimulates lipolysis in peripheral tissues to increase circulating free fatty acids and significantly improves insulin sensitivity in mice (for review see Ref. 13), which may explain improved glucose tolerance in Foxo null-mice. This is but one possibility, but it is tempting to speculate that the dramatic metabolic effects of hepatic Foxo loss in vivo may actually be a consequence of indirect changes in adipose or muscle biology. Therefore, the question remains as to whether the observed metabolic effects are primarily due to changes in hepatic energy metabolism or the result of cross talk between liver and other organ systems.

There is little doubt that Foxo1 is a predominant regulator of glycemia. However, the impact of Foxo on hepatic lipid homeostasis remains controversial due to an abundance of conflicting data from both gain- and loss-of-function in vivo models (9, 14), leaving more questions than answers at this point. It is clear that we need more information about the activity of these factors at a molec-
ular level, including how they are regulated and their transcriptional targets, before we can understand the complex metabolic phenotypes of the knockout mice. Although the data presented by Zhang et al. (4) and others suggest a cooperative effort between the Foxo family members, there is little direct evidence indicating whether these proteins work in concert on the same targets or independently regulate different gene sets. Moreover, it is still unclear whether the effects on gene expression patterns are actually due to direct transcriptional regulation or indirect effects on other cellular pathways (i.e. insulin signaling). Future studies will benefit from global screening methods, such as chromatin immunoprecipitation sequencing technology and chromatin state maps, to reveal detailed gene networks regulated by this family.

Foxo activity and diabetes generally have a negative association. Zhang et al. (4) now highlight that Foxo proteins quietly keep triglyceride and cholesterol levels in check and may actually possess hidden metabolic benefits. This work opens up doors for new therapeutic strategies to target this family. However, warning flags from mouse models should emphasize caution when targeting the activity of this complex transcriptional network for treatment of metabolic disease.

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