FoxO1 regulates multiple metabolic pathways in the liver.

**Effects on gluconeogenic, glycolytic, and lipogenic gene expression**

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FoxO transcription factors are important targets of insulin and growth factor action, and they contribute to the regulation of cell growth, differentiation, and metabolism (1–3). FoxO proteins form a subgroup within the family of Forkhead box (or Fox) transcription factors (4). Early studies indicated that Forkhead proteins interact with insulin response sequences (IRs) in the promoter of the IGF-binding protein-1 (IGFBP-1) and the phosphoenolpyruvate carboxykinase (PEPCK) genes (5, 6) and that signaling through phosphatidylinositol 3’-kinase and protein kinase B (PKB) mediates IRS-dependent effects of insulin on gene expression (7). Genetic studies of *Caenorhabditis elegans* revealed that DAF-16, a FoxO transcription factor, is a major target of insulin-like signaling (8, 9). DAF-16 plays an important role in the adaptation to environmental stress, including nutrient restriction, and signaling through phosphatidylinositol 3’-kinase and PKB suppresses the function of DAF-16. Subsequent studies revealed that FoxO proteins contain highly conserved PKB phosphorylation sites (corresponding to Thr-24, Ser-256, and Ser-319 in human FoxO1) (10–12) and that phosphorylation at these sites suppresses transactivation and promotes nuclear exclusion of FoxO proteins through multiple mechanisms (13). FoxO proteins are targets for other signaling pathways (14–16), and other post-translational modifications also regulate the function of FoxO proteins, including deacetylation by sirtuins (NAD-dependent deacetylases) (17), and other post-translational modifications also regulate the function of FoxO proteins, including deacetylation by sirtuins (NAD-dependent deacetylases) (17), indicating that FoxO proteins are targets for both signaling and energy-sensing pathways (18).

FoxO transcription factors exert positive and negative effects on gene expression. In addition to binding directly to DNA target sites and stimulating transcription of target genes, FoxO proteins interact with and modulate the function of other transcription factors and co-activator proteins, including factors important for the regulation of gene expres-

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2 The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S4 and Tables S1, S2a, and S2b.

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4 The abbreviations used are: FoxO, Forkhead box-“Other”; ACC, acetyl-CoA carboxylase-alpha; AKT, atypical protein kinase C; ChREBP, carbohydrate response element binding protein; CREB, cyclic AMP response element-binding protein; CBP, CREB-binding protein; C/EBP, CCAAT enhancer binding protein; CoA, coenzyme A; GFP, green fluorescent protein; HBSS, Hank’s balanced salt solution; HDL, high density lipoprotein; IDL, intermediate density lipoprotein; IGFBP-1, insulin-like-growth factor binding protein-1; IRS, insulin response sequence; IRS-2, insulin receptor substrate-2; LDL, low density lipoprotein; LXR, liver X receptor; PBS, phosphate-buffered saline; PEPCK, phosphoenolpyruvate carboxykinase; PKB, protein kinase B; PPARγ, peroxisome proliferator-activated receptor-gamma; SREBP-1, sterol response element-binding protein-1; TGN, transgenic; TORC, transducer of regulated CREB; VLDL, very low density lipoprotein; WT, wild type; RT, reverse transcription.
FoxO1 Regulates Glycolysis and Lipogenesis

FoxO proteins play an important role in the adaptation to nutrient restriction in Caenorhabditis elegans and Drosophila (24, 25), and increasing evidence indicates that this function is conserved in mammals. In muscle, FoxO proteins promote the expression of atrogin-1, an F-box protein that targets proteins for ubiquitination and degradation by the proteasome, thereby providing amino acid substrate for gluconeogenesis (26). FoxO proteins also stimulate the expression of pyruvate dehydrogenase kinase-4 (27), which limits oxidative metabolism of glucose and conserves glucose for utilization in other tissues. FoxO proteins also stimulate the expression of lipoprotein lipase (28), and the translocation of CD36 to the plasma membrane where it can promote fatty acid uptake (29), and thereby enhances the metabolism of fatty acids. In the liver, FoxO1 interacts with insulin-responsive sequences in the promoters of IGFBP-1 (12, 30), the major short-term modulator of IGF bioavailability (31), and several enzymes involved in promoting hepatic glucose production, including PEPCK (32, 33) and glucose-6-phosphatase (34, 35). However, little is known regarding the effects of FoxO proteins on other metabolic pathways that are important in the adaptation to fasting and feeding in the liver.

To address this point, we studied transgenic mice targeting a constitutively active form of FoxO1 to the liver using the α1-antitrypsin promoter. Initial studies confirmed that the FoxO1 transgene is expressed in the liver in a tissue-specific manner and showed that FoxO1 transgenic mice are resistant to glucose lowering effects of insulin. Subsequent studies revealed that constitutively active FoxO1 also has prominent effects on post-prandial triglyceride levels and de novo lipogenesis, measured with 3H2O. Gene-array studies demonstrated that FoxO1 exerts effects on the expression of multiple genes involved in glucose utilization and lipogenesis in vivo, and studies using adenoviral vectors in isolated hepatocytes showed that these changes reflect a direct effect of FoxO1. Together, these findings indicate that FoxO proteins play an important role in regulating the expression of multiple metabolic pathways involved in the response to fasting and feeding in the liver.

EXPERIMENTAL PROCEDURES

Creation of Transgenic Mice—The creation of transgenic mice and all animal studies followed National institutes of Health guidelines and were approved by the institutional animal care committees at the University of Illinois at Chicago College of Medicine and the Jesse Brown Veterans Affairs Medical Center. The full-length cDNA coding for constitutively active human FoxO1, where Thr-24, Ser-256, and Ser-319 were replaced by alanines (36), was inserted downstream of the 650-bp rabbit β-globin intron and upstream of a fragment of the bovine growth hormone gene containing the polyadenylation signal (37). PCR primers were used to isolate from genomic DNA a 1200-bp DNA fragment containing the human α1-antitrypsin promoter and part of the noncoding first exon, and this promoter was placed upstream of the rabbit β-globin sequence, as shown in Fig. 1A. The function of this construct was confirmed in HepG2 cells in reporter gene assays by demonstrating that the expressed protein stimulates IGFBP-1 promoter activity in a sequence-specific fashion through known FoxO binding sites (7).

The linearized 4.2-kb targeting construct was released from the vector backbone with ClaI. The purified construct, devoid of plasmid sequences, was injected into the pronuclei of fertilized FVB/N mouse zygotes, and microinjected embryos were transferred into CD-1 foster mice in the University of Illinois at Chicago College of Medicine Transgenic Facility, according to the methods of Hogan et al. (38). The presence of the transgene in the genomic DNA of offspring was determined by PCR using sense and antisense primers that span the 3'-end of the β-globin intron and the 5'-end of the FoxO1 cDNA, as indicated in Fig. 1A, and results were confirmed by Southern blotting. Two founders were identified, including one that had very high copy number and was paraplegic due to spina bifida occulta and was not suitable for further study. The other founder demonstrated germ line transmission and was used for subsequent studies.

To examine the expression of the transgene in tissues from 2-month-old transgenic (TGN) and wild type (WT) mice, tissues were frozen in liquid nitrogen and then stored at −80 °C until extraction of total cellular RNA with TRIzol (Invitrogen) according to the manufacturer’s recommendations. In subsequent studies, RNA was isolated from liver and cells using the RNeasy kit (Qiagen) and treated with DNase I on the column according to the manufacturer’s protocol. RNA was quantified by absorbance at 260 nm and stored at −80 °C. cDNAs were synthesized with Superscript III Reverse Transcriptase (Invitrogen) according to the manufacturer’s instructions. Amplification of the transgenic FoxO1 mRNA was accomplished by PCR using primers that span the heterologous flanking sequence from the β-globin intron (sense) and the 5'-end of the FoxO1 cDNA (antisense), as above (Fig. 1A). PCR products were resolved by agarose gel electrophoresis and visualized with ethidium bromide.

Histochemistry—Livers were harvested from animals and fixed with 2% paraformaldehyde in phosphate-buffered saline (PBD) and then embedded in paraffin. Standard hematoxylin and eosin staining was performed on deparaffinized 7-μm-thick sections. For Oil Red O staining, livers were fixed with 2% paraformaldehyde in PBS, and then tissue was cryoprotected in 30% sucrose in PBS overnight at 4 °C. Tissue was then frozen under liquid nitrogen-cooled isopentane and stored at −80 °C until sectioning. The 7-μm-thick sections were prepared and stained, as previously described (39). Photographs were taken on an Olympus Provis microscope.

Animal Maintenance and Metabolic Studies—Mice were maintained on a 12/12 light/dark cycle and provided rodent chow (Diet SLA2, LabDiet, St. Louis, MO) in the animal care facility at the Jesse Brown Veterans Affairs Medical Center. Tail clippings were obtained at weaning (3 weeks after birth), and genotyping was performed by RT-PCR, as before. Mice were euthanatized by sedation with carbon dioxide and decapitation. Blood was collected from conscious mice in heparinized capillary tubes from clipped tails, or by cardiac puncture following sedation with CO2 at the time of euthanasia, unless otherwise specified. Livers were frozen immediately in liquid nitrogen and stored at −80 °C for subsequent studies.

Glucose and insulin tolerance testing was performed in 7- to 8-week-old mice following an 18-h overnight fast. Dextrose 2 g/kg or recombinant human insulin (0.5 unit/kg, Eli Lilly Co., Indianapolis, IN) was administered intraperitoneally, and glucose levels in tail blood were measured at baseline and 30, 60, 90, and 120 min later by glucometer (LifeScan, Milpitas, CA).

For studies related to lipogenic metabolism, mice were placed on a high carbohydrate (65% sucrose) diet that is low in polyunsaturated fatty
acids (TD.03303, Harlan Teklad, Madison, WI) for 4–5 days prior to studies, and housed in individual cages with non-digestible bedding (iso-PAD, Omni Bioresources, Cherry Hill, NJ). For fasting/refeeding studies, mice were fasted for 24 h (10 a.m. to 10 a.m.), and then allowed free access to high carbohydrate chow, and plasma samples obtained at appropriate time points.

**Biochemical Assays and FPLC Analysis of Plasma Lipids—** Plasma insulin levels were measured by enzyme-linked immunosorbent assay (Crystal Chem, Inc., Downers Grove, IL). In initial studies, plasma levels of triglycerides and cholesterol and amino leucine transferase activity were measured commercially (Ani Lytics, Gaithersburg, MD). In later studies, commercially available kits were used to measure triglycerides (Wako, Richmond, VA), cholesterol (Wako), and β-hydroxybutyrate (Wako), as per the manufacturer’s instructions. Hepatic triglyceride content was measured following two-step extraction with chloroform-methanol, after the method of Bligh and Dyer (40).

Plasma lipoprotein fractionation by FPLC was performed in the University of Cincinnati Mouse Metabolic Phenotype Center to characterize the distribution of triglyceride and cholesterol among the different lipoprotein classes. Plasma (200 µl) was chromatographed undiluted through two Superose 6 columns (Amersham Biosciences) linked in tandem and equilibrated with degassed buffer (50 mM EDTA, 150 mM NaCl, 1 mM EDTA, 7.7 mM NaN₃, pH 7.4), as previously reported (41). A flow rate of 0.6 ml/min was maintained by an Amersham Biosciences FPLC controller, and 0.5-ml fractions were collected into 1-ml screw-capped vials. Before the next sample was applied, the column was flushed with buffer until A₂₈₀ returned to a stable baseline. Because Superose 6 is a size-selective matrix, large particles elute first and small particles elute last. Eluted lipoprotein samples were stored at −80 °C until analysis. Cholesterol concentration in each 0.6-ml fraction was quantified by the Cholesterol/HP Kit (Roche Molecular Biochemicals, Indianapolis, IN) according to the manufacturer’s instructions. Triglyceride was determined using the Randox kit (Antrim, United Kingdom).

**Measurement of de Novo Lipogenesis in Liver and Fat Pads—** Lipogenesis was measured in vivo using ³H₂O after the method of Stansbie et al. (42). Eight-week-old male TGN and WT mice were studied at the end of a 24-h fast or 6 h after refeeding with lipogenic (high carbohydrate) chow. Here, ¹⁴C H₂O (17 µCi/g) (Amersham Biosciences, Piscataway, NJ) was injected intraperitoneally, and mice were sacrificed 1 h later. Retroroibital blood was obtained under isoflurane anesthesia, and mice were euthanized by cervical dislocation. Plasma was deproteinized with 20% (w/v) trichloroacetic acid, and diluted aliquots were taken for scintillation counting to estimate the specific activity of ³H in total body water. The liver and epididymal fat pad were weighed, and 0.8–1 g of liver or the entire fat pad was placed in 3 ml of 30% (w/v) potassium hydroxide for digestion at 70 °C for 20 min and then for an additional 2 h at 70 °C following the addition of 3 ml of 95% ethanol. Cooled samples were acidified by addition of 3 ml of 9 M H₂SO₄ and then extracted 3 times with 10 ml of petroleum ether. Ether extracts were washed three times with 10 ml of H₂O and brought to a final volume of 30 ml. 15-ml aliquots were dried under air in scintillation vials prior to addition of Ecoscint scintillation mixture (National Diagnostics, Atlanta, GA) and scintillation counting. Total extractable counts per minute (cpm) in liver or fat pad were calculated and adjusted for variations in specific activity based on plasma cpm.

**Western Blotting and Atypical Protein Kinase C Kinase Assay—** Protein extracts of liver tissue were prepared with T-Per (Pierce Chemical Co., Rockford, IL) in the presence of protease inhibitors (Protease Inhibitor Mixture, Cat. no. P8340, Sigma) and phosphatase inhibitors (Phosphatase Inhibitor Cocktails I and II, Cat. nos. P2850 and P5726, Sigma), and protein concentration was determined by the BCA Protein Assay kit (Pierce Biotechnology, Rockford, IL). Proteins were resolved by SDS-PAGE on 4–20% gradient gels (Bio-Rad) and electrotransferred onto nitrocellulose membranes (Amersham Biosciences). Membranes were blocked with 5% (w/v) nonfat dry milk, and then probed overnight at 4 °C with primary antibody prior to incubation with secondary antibody. Membranes were washed in Tris-buffered saline containing 0.1% (v/v) Tween 20 (Bio-Rad) and developed using the SuperSignal West Pico Chemiluminescent Substrate kit (Pierce Biotechnology).

Polyclonal anti-FLAG antibody was purchased from Sigma, and antibodies against total protein kinase B (PKB), serine 473-phosphorylated PKB, total glycerol synthase kinase-3 and serine 21-phosphorylated glycerol synthase kinase-3 were purchased from Cell Signaling (Beverly, MA). Antibodies against β-actin (C-11, sc-1615) and sterol response element binding protein-1 (SREBP-1) (2A4, sc-13551) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal sheep antibody against glucokinase was generously provided by Dr. M. Magnuson (Vanderbilt University) (43). Anti-rabbit-, -mouse-, -goat-, and -sheep secondary antibodies were purchased from Cell Signaling.

Liver aPKC kinase activity was measured as previously described (44). In brief, aPKCs were immunoprecipitated from tissue lysates with a rabbit polyclonal antiserum (Santa Cruz Biotechnology) that recognizes both PKC-ζ and PKC-λ, which are both expressed in liver. Immunoprecipitates were collected on Sepharose-AG beads (Santa Cruz) and incubated for 8 min at 30 °C in 100 µl of buffer containing 50 mM Tris/HCl (pH 7.5), 100 µM sodium vanadate, 100 µM sodium phosphate, 1 mM sodium fluoride, 100 µM phenylmethylsulfonyl fluoride, 4 µg of phosphatidylinerine (Sigma), 50 µM [γ-³²P]ATP (PerkinElmer Life Sciences), 5 mM magnesium chloride, and, as substrate, 40 µM serine analogue of the PKC-ε pseudosubstrate (BioSource, Camarillo, CA). After incubation, ³²P-labeled substrate was trapped on P-81 paper and counted.

**Gene Array Studies—** Transcriptional profiling of murine tissue was performed with the use of the Affymetrix Mouse Genome U74Av2 GeneChip array, which contains probe sets interrogating ~12,000 transcripts. Total cellular RNA was prepared from the livers of female wild-type and transgenic mice at the end of a 24-h fast or 6 h after refeeding (n = 3 for each group), and all subsequent manipulations, including labeling and hybridization, were performed independently for each sample. Labeling reactions and hybridizations were carried out according to the standard GeneChip® eukaryotic target labeling protocol (Affymetrix, Santa Clara, CA) in the University of Illinois at Chicago Core Genomics Laboratory. Briefly, 10 µg of total cellular RNA per sample was used to synthesize double-stranded cDNA, which then was transcribed in vitro in the presence of biotinylated dNTPs (Enzo Diagnostics, Farmingdale, NY). Biotinylated target cRNA was fragmented and brought up in hybridization mix. Successful labeling of all the samples (a minimum of 15 µg of in vitro transcription product per sample) was followed by test array hybridizations. Test hybridizations were performed with the use of “Test3” arrays (Affymetrix) to ensure quality of the biotinylated target.

All hybridizations were performed with arrays from the same manufacturing lot. Hybridizations were followed by binding to streptavidin-conjugated fluorescent marker. Detection of bound probe was achieved following laser excitation of the fluorescent marker and scanning of the resultant emission spectra using a scanning confocal laser microscope (Probe Array Scan, Agilent Technologies). Data acquisition was performed using Affymetrix GeneChip array 5.0 suite. Collected hybridization images were subjected to quality control to remove from analysis arrays, which failed to meet criteria both suggested by Affymetrix and developed internally in the laboratory. These quality requirements
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include: low Q-noise (1–10); low background (less than a 100); sample-dependent percent of probes, detected as present (20–50% for mammals); 3'/5' ratio of no more than 3; hybridization efficiency defined by intensities detected for the spike control probe sets (preferably higher than 2,000 fluorescent units); minimal deviation of scaling factors for the whole set of arrays to be analyzed.

Collected hybridization intensity signals were background corrected and normalized with the use of Affymetrix GeneChip array suite 4.0. The data then was analyzed to identify statistically significant differentially expressed transcripts by using the National Institute of Aging Array Analysis Tool (ngsun.grc.nia.nih.gov/ANOVA/). The following analysis settings were applied: variance model of 4, z value threshold to remove outliers equal to 8, error variance averaging window equal to 10, and false discovery rate equal to 0.05. Differentially expressed transcripts were annotated with the use of NetAffx Analysis Center (www.geneontology.org) according to the most current version of the Gene Ontology Data base (www.geneontology.org/).

**Results**

**Creation of FoxO1 Transgenic Mice**—To examine the role of FoxO proteins in the liver, we created transgenic mice targeting the expression of a constitutively active form of human FoxO1 (where the three predicted PKB phosphorylation sites (Thr-24, Ser-256, and Ser-319) have been replaced by alanine) to the liver under the control of the α1-antitrypsin promoter (Fig. 1A). RT-PCR studies demonstrated that the FoxO1 transgene is expressed in the liver of transgenic (TGN) but not wild-type (WT) mice and that the expression of the transgene is not detected in other tissues (including the pancreas, skeletal muscle, heart, epididymal fat, kidney, or brain) of transgenic mice (Fig. 1B), indicating that its expression is tissue-specific. As shown in Fig. 1C (left panel), expression of the endogenous mouse FoxO1 gene (solid bar) was not altered in TGN versus WT mice, and the human FoxO1 transgene (hatched bar) was expressed at a level equal to ~60% of that of the endogenous FoxO1 gene. The expression of insulin-like growth factor binding protein-1 (IGFBP-1), a known target of FoxO1, was increased ~10-fold in TGN versus WT mice (Fig. 1C, right panel). No evidence of injury in the liver of TGN was detected in histochemical studies (supplemental Fig. S1), and plasma levels of amino leucine transferase, a marker of hepatocyte injury, were not increased in TGN versus WT (data not shown). These results indicate that the constitutively active human FoxO1 transgene is expressed in the liver in a tissue-specific fashion and at near physiological levels and that it stimulates the expression of IGFBP-1, a known FoxO1 target in the liver, without evidence of hepatocellular injury.
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A. Transgene: vector

\[ \alpha_{\text{antipyr}} \text{FoxO1}_{\text{TSS-A}} \rightarrow h\text{FoxO1}_{\text{TSS-A}} \rightarrow b\text{GH}^3-\text{UTR} \]

\[ \beta\text{-globin} \]

B. Transgene: RT-PCR

| L | L | P | F | M | H | K | B |
|---|---|---|---|---|---|---|---|
| TGN | + | + | + | + | + | + | + |

C. Real-time PCR

| mRNA abundance (Relative copy #) | FoxO1 | IGFBP-1 |
|-------------------------------|--------|---------|
| WT                            |        |         |
| TGN                           |        |         |

**FIGURE 1.** FoxO1 transgenic mice. A, targeting vector. The cDNA coding for constitutively active human FoxO1 (where Thr-24, Ser-256, and Ser-319 have been replaced by alanines) is driven by the α1-antitrypsin promoter. The β-globin intron and the bovine growth hormone 3-untranslated region are located upstream and downstream from the FoxO1 CDS, respectively. The location of the sense (solid) and antisense (dashed arrow) primers used for screening genomic DNA, the detection of transgene expression in tissues by RT-PCR, and for quantitation of transgene expression by PCR is shown. B, tissue-specific expression of FoxO1 transgene. Total RNA was prepared from the liver (L) of a wild-type (-) and a FoxO1 transgenic (+) mouse and from the pancreas (P), epididymal fat (F), skeletal muscle (M), heart (H), kidney (K), and brain (B) of a transgenic mouse (+). RT-PCR was performed using primers specific for the FoxO1 transgene (above), and products were stained with ethidium bromide following agarose gel electrophoresis. In C, RT-PCR, left panel, expression of the endogenous mouse FoxO1 and human FoxO1 transgenes. The relative abundance of mRNA transcripts for the endogenous mouse FoxO1 gene (mFoxO1, solid bar) and the human FoxO1 transgene (hFoxO1, hatched bar) was determined by PCR using total RNA prepared from freely feeding female mice. PCR products produced with primers specific for mFoxO1 or the human FoxO1 transgene were quantified and used to generate standard curves to estimate copy number, as described under “Experimental Procedures.” Results were adjusted based on the relative abundance of mRNA transcripts for the ribosomal L32 protein and expressed relative to the level mouse FoxO1 transcripts detected in the liver of wild-type mice. Results are presented as the means ± S.E. of the mean. D, expression of IGFBP-1 in wild-type and transgenic mice. The relative abundance of IGFBP-1 mRNA transcripts in the livers of freely feeding wild-type (WT) and transgenic (TGN) female mice was quantified by real-time PCR. Results are expressed relative to the mean IGFBP-1 mRNA level in WT mice.

Fasting Glucose and Insulin Levels and Glucose and Insulin Tolerance Tests—As shown Fig. 2A, fasting blood glucose levels are modestly increased in male and female TGN mice compared with WT (first panel), while plasma insulin levels are increased ~2.5-fold in male and female TGN versus WT mice (second panel), indicating that the ability of circulating insulin to regulate fasting blood glucose levels is impaired in FoxO1 TGN mice, consistent with previous studies (47).

As shown in Fig. 2B, the ability to regulate blood glucose levels in response to an intraperitoneal glucose challenge is impaired in male and female TGN mice. The ability of insulin (0.5 unit/kg) to lower glucose levels also is impaired in male and female TGN versus WT mice (Fig. 2C), whereas higher doses of insulin (1 unit/kg) are effective in suppressing glucose levels in both WT and TGN mice (data not shown). These results indicate that the ability of insulin to suppress fasting blood glucose levels is impaired but not completely disrupted in FoxO1 transgenic mice.

Effects on Fasting and Post-prandial Lipid Levels—Insulin promotes lipogenesis and triglyceride production in the liver, and hypertriglyceridemia often accompanies insulin resistance. Nevertheless, we found that fasting plasma triglyceride levels are reduced in male FoxO1 transgenic mice compared with WT (first panel), insulin (second panel), triglycerides (third panel), and cholesterol (fourth panel) in 8-week-old male (M) and female (F) wild-type (WT, solid bars) and transgenic (TGN, open bars) mice at the end of 16 h are shown. * indicates p < 0.05, WT versus TGN. B, glucose tolerance test. Blood glucose levels were measured in 8-week-old male (left panel) and female (right panel) wild-type (solid line) and transgenic (dashed line) mice just before and 30, 60, 90, and 120 min after injection with 2 g/kg dextrose intraperitoneally. C, insulin tolerance test. Blood glucose levels were measured in 8-week-old male (left panel) and female (right panel) wild-type (solid line) and transgenic (dashed line) mice just before and 30, 60, 90, and 120 min after treatment with 0.5 unit/kg insulin.

To examine effects of the FoxO1 transgene on triglyceride metabolism further, we placed mice on a high carbohydrate (65% sucrose) diet...
that is low in polyunsaturated fatty acids to simulate de novo lipogenesis. Mice were placed on high carbohydrate chow for 4 days, then fasted for 24 h and refed to measure effects on fasting and post-prandial lipid levels. Initial studies were performed in male TGN and WT mice, where effects on lipid levels were expected to be most marked.

As shown in Fig. 3A, following a 24-h fast, plasma triglyceride levels are reduced in TGN (dashed line) versus WT (solid line) male mice, similar to results obtained on standard chow (Fig. 2A, third panel). After refeeding, plasma triglyceride levels rise progressively in WT mice (solid line), similar to previous reports (48). In contrast, the effect of refeeding on plasma triglycerides is markedly blunted in TGN (dashed line), despite similar food intake during refeeding (1.6 ± 0.2 and 1.5 ± 0.1 g consumed by TGN and WT, respectively).

To characterize effects on circulating lipids further, plasma was collected 6 h after refeeding in WT and TGN mice in a separate experiment, and triglyceride and cholesterol content in circulating lipoproteins was measured following fractionation by FPLC. As shown in the inset in the upper panel of Fig. 3B, total plasma triglycerides were markedly reduced in TGN versus WT mice 6 h after a high carbohydrate meal, as before. Fractionation by FPLC reveals that most triglycerides circulate in the form of very low density lipoproteins (VLDL) in WT mice (solid line), and VLDL triglyceride content is markedly reduced in TGN versus WT mice (p < 0.001). Triglyceride content also is reduced in intermediate/low density lipoproteins (IDL/LDL) in TGN versus WT mice (p < 0.01), and very little triglyceride is detected in the fractions eluting with high density lipoproteins (HDL).

Circulating levels of total cholesterol are modestly reduced in TGN versus WT male mice 6 h after refeeding (inset, lower panel of Fig. 3B). Cholesterol content in HDL and IDL/LDL lipoproteins is similar in TGN and WT mice, whereas VLDL cholesterol is reduced in TGN versus WT mice. This indicates that the reduction in total plasma cholesterol in TGN versus WT mice is due to differences in VLDL cholesterol content.

Hepatic Triglyceride Content, Fatty Acid Oxidation, and de Novo Lipogenesis — We performed additional studies to characterize the effects of refeeding in both male and female TGN and WT mice. As shown in Fig. 4A, post-prandial plasma triglyceride levels are markedly reduced in both male and female TGN mice compared with WT 6 h after refeeding, indicating that this effect is not limited to male mice.

We considered whether this result might be due to a defect in triglyceride secretion and the accumulation of triglycerides with the liver. However, as shown in Fig. 4B, intrahepatic triglyceride content is similar in TGN and WT mice 6 h after refeeding based on biochemical assay (Fig. 4B), and Oil Red staining also indicates that intrahepatic lipid content is similar in TGN and WT mice (supplemental Fig. S1). This indicates that the reduction in post-prandial plasma triglyceride levels in TGN mice is not due to impaired secretion and increased accumulation of intrahepatic triglyceride.

We also considered whether differences in post-prandial triglyceride levels might be due to an impaired ability to suppress fatty acid oxidation in TGN mice. As shown in Fig. 4C, plasma levels of β-hydroxybutyrate are suppressed to a similar extent in TGN and WT mice 6 h after refeeding, indicating that fatty acid oxidation is appropriately suppressed after refeeding in TGN mice.

To determine whether the ability to stimulate fatty acid synthesis in response to feeding is impaired in TGN versus WT mice, we measured de novo lipogenesis using 3H2O. Following intraperitoneal injection, 3H2O is distributed in the total body water space and 3H rapidly exchanges with hydrogen in NADPH, which provides reducing equivalents required for lipogenesis. As shown in the panel on the left in Fig.

![Figure 3](image_url)

**FIGURE 3.** Post-prandial triglyceride levels following a high carbohydrate meal. A, time course. Male wild-type (solid line) and transgenic (dashed line) mice were provided high carbohydrate chow (65% sucrose) for 4 days, then fasted for 24 h before refeeding. Triglyceride levels were measured in plasma samples obtained by tail bleeding at the end of a 24-h fast and 2, 4, 6, and 10 h after refeeding. B, FPLC analysis of triglyceride and cholesterol content in lipoproteins. Plasma samples were obtained from wild-type (solid lines) and transgenic (dashed line) male mice 6 h after refeeding and lipoproteins were separated by FPLC. Upper panel, triglyceride content was measured in plasma (inset) and in fractions collected during FPLC from TGN and WT mice. *, indicates p < 0.001, WT versus TGN. Lower panel, cholesterol content was measured in plasma (inset) and in fractions collected during FPLC. *, indicates p < 0.03, WT versus TGN.

4D, the incorporation of 3H into newly synthesized fatty acids is stimulated 30- to 40-fold in the liver of WT mice 6 h after refeeding, and this response to feeding is reduced by ~70% in TGN versus WT mice (p <
expression is either increased (TGN expression of those genes that are differentially expressed in TGN and TGN (versus WT mice) 6 h after refeeding (supplemental Fig. S2A). Similarly, aPKC activity increased after refeeding in both WT and TGN mice and tended to be increased in TGN versus WT mice 6 h after refeeding (supplemental Fig. S2C). Western blotting demonstrated that the phosphorylation of Ser-473, which is important for the activation of PKB, increases after feeding in WT mice, and Ser-473 phosphorylation is not reduced and tends to be increased in TGN versus WT mice (supplemental Fig. S2C). Similarly, the phosphorylation of Ser-21 in glycogen synthase kinase-3, a known target of PKB, is not reduced, and tends to be increased in TGN versus WT mice (supplemental Fig. S2C). Together, these results indicate that insulin secretion and the activation of signaling pathways thought to be important in promoting lipogenesis in the liver are not impaired in FoxO1 transgenic mice.

Effects on the Genomic Response to Feeding—We performed gene-array studies to better define effects of constitutively active FoxO1 on the response to fasting and feeding in the liver and verified results for selected genes by PCR. To emphasize the effect of refeeding, we performed these studies in female mice, where triglyceride levels are similar in TGN compared with control at the end of a 24-h fast but are markedly different 6 h after refeeding (Fig. 4A).

We first examined the genomic response to refeeding in WT and TGN separately. As shown in Fig. 5A, the expression of 495 genes was increased (fed/fasted > 1) and the expression of 368 genes was decreased (fed/fasted < 1) by refeeding in WT mice (solid bars). In contrast, the number of genes whose expression is increased (322) or decreased (230) with refeeding in TGN mice (open bars) was reduced by about one-third compared with WT. This indicates that the ability to stimulate and/or suppress the expression of some (but not all) nutritionally regulated genes is impaired in FoxO1 transgenic mice.

We next examined differences in gene expression in TGN versus WT mice at the end of a 24-h fast and 6 h after refeeding (Fig. 5B). At the end of a 24-h fast, when both the endogenous and transgenic FoxO proteins are active, we detected only 44 genes whose expression was either increased (TGN > WT, solid bar, n = 27) or decreased (TGN < WT, gray circle) in TGN versus WT mice 6 h after refeeding. The effect of refeeding on each gene in WT (x axis) and TGN (y axis) is plotted on a logarithmic scale, where an increase in gene expression (fed/fasted > 1) is shown as a positive value, and a reduction in gene expression (fed/fasted < 1) is shown as a negative value.

Because insulin plays an important role in stimulating lipogenesis, we considered whether insulin secretion and/or the activation of signaling pathways thought to mediate effects of insulin on lipogenesis, including atypical forms of protein kinase C (aPKCs) (49) and protein kinase B (PKB) (50), is(are) altered in TGN versus WT mice. Plasma levels of insulin levels are not reduced and tend to be increased in TGN versus WT mice 6 h after refeeding (supplemental Fig. S2A). Similarly, aPKC activity increased after refeeding in both WT and TGN mice and tended to be increased in TGN versus WT mice 6 h after refeeding (supplemental Fig. S2C). Western blotting demonstrated that the phosphorylation of Ser-473, which is important for the activation of PKB, increases after feeding in WT mice, and Ser-473 phosphorylation is not reduced and tends to be increased in TGN versus WT mice (supplemental Fig. S2C). Similarly, the phosphorylation of Ser-21 in glycogen synthase kinase-3, a known target of PKB, is not reduced, and tends to be increased in TGN versus WT mice (supplemental Fig. S2C). Together, these results indicate that insulin secretion and the activation of signaling pathways thought to be important in promoting lipogenesis in the liver are not impaired in FoxO1 transgenic mice.

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**A. Liver: TGN vs WT**

| Enzyme | Control | TGN |
|--------|---------|-----|
| BP-1   | 0.5     | 1.0 |
| G6Pase | 2.0     | 4.0 |
| PEPCK  | 3.0     | 6.0 |
| SREBP-1c | 0.2 | 0.1 |
| GK      | 0.1     | 0.05|
| TK      | 0.5     | 1.0 |
| PK      | 1.0     | 2.0 |
| ACC     | 2.0     | 4.0 |
| FAS     | 4.0     | 8.0 |

**B. Hepatocytes: FoxO1 vs GFP**

| Enzyme | Control | FoxO1 |
|--------|---------|-------|
| BP-1   | 0.5     | 1.0  |
| G6Pase | 2.0     | 4.0  |
| PEPCK  | 3.0     | 6.0  |
| SREBP-1c | 0.2 | 0.1 |
| GK      | 0.1     | 0.05|
| TK      | 0.5     | 1.0 |
| PK      | 1.0     | 2.0 |
| ACC     | 2.0     | 4.0 |
| FAS     | 4.0     | 8.0 |

Effects of FoxO1 on Gluconeogenic, Glycolytic, and Lipogenic Pathways—A complete list of the genes that are differentially expressed in TGN versus WT mice 6 h after refeeding is provided as supplemental information (supplemental Tables S2a and S2b). Approximately one-third of these genes are involved in glucose, lipid, protein, amino acid, or energy metabolism, indicating that FoxO proteins play a role in the regulation of multiple aspects of metabolism in the liver.

The genes that are directly involved in glucose, amino acid, or lipid metabolism in the liver, and whose expression was either increased or decreased in TGN versus WT mice are listed in Tables 1 and 2. Their role in glucose and lipid metabolism is presented schematically in supplemental Fig. S3. Results for a number of these genes were confirmed by real-time PCR, as indicated in Tables 1 and 2.

As shown in Table 1, the expression of several genes involved in promoting hepatic glucose production was increased in TGN versus WT mice, including PEPCK and glucose-6-phosphatase, consistent with previous studies (47). Interestingly the expression of aquaporin 9, which promotes hepatic uptake of glyceral (51), an important substrate for glyceral production, was also increased in TGN versus WT. The expression of multiple genes promoting the catabolism of proteins and amino acids, which also provides important substrate for gluconeogenesis, also was increased in TGN versus WT. The expression of multiple genes promoting the catabolism of proteins and amino acids, which also provides important substrate for gluconeogenesis, also was increased in TGN versus WT mice (Table 1).

This includes an F-box protein (F-box 21), which may target proteins for degradation by the proteasome, several genes coding for components of the proteasome, and genes coding for proteins involved in other pathways for protein turnover (cathepsin E, lysosomal membrane glycoprotein 2). These results suggest that FoxO proteins promote hepatic glucose production by multiple mechanisms; by stimulating the expression of genes directly involved in gluconeogenesis and by enhancing expression of genes involved in providing substrates for glucose production.

In contrast, the expression of multiple genes involved in promoting glyceral utilization by glycolysis, the pentose phosphate shunt, de novo lipogenesis, and sterol synthesis was reduced in TGN versus WT mice (Table 2). Gene-array studies indicate that the expression of glucokinase, which plays a critical role in promoting the formation of glucose 6-phosphate and lipogenesis in the liver, is markedly reduced in TGN versus WT. This result was confirmed by PCR (Table 2), and Western
blotting studies demonstrated that glucokinase protein levels are reduced in TGN versus WT (supplemental Fig. S4).

Metabolism of glucose 6-phosphate through the pentose phosphate shunt is critical for generating NADPH for lipogenesis and for the activation of the carbohydrate response element binding protein (ChREBP) (52). Gene-array studies show that expression of several genes in the shunt pathway (ribose-5-phosphate isomerase and transketolase) and several genes that also promote flux through the shunt (phosphoglucose

### TABLE 1

**Gene-array results: increased**
The genes whose expression is increased in transgenic (TGN) versus wild type (WT) mice after refeeding and directly related to glucose, protein or amino acid catabolism, or lipid trafficking or transport are listed. Gene-array studies were performed with Affymetrix U74Av2 chips using RNA obtained from the livers of TGN or WT female mice 6 h after refeeding with high carbohydrate chow to stimulate lipogenesis. The relative level of expression in transgenic versus wild-type mice (TGN/WT) is shown. Those genes where results were verified by quantitative PCR (qPCR) are identified (+).

| Accession | Gene description | TGN/WT | qPCR |
|-----------|------------------|--------|------|
| Gluconeogenesis |
| AA967194 | Aqua(glycero)porin 9 | 2.63 | + |
| M29944 | Carbonic anhydrase 2 | 1.7 |  |
| AF009605 | Phosphoenolpyruvate carboxykinase | 6.67 | + |
| U00445 | Glucose-6-phosphatase | 1.6 | + |
| Protein catabolism |
| AI853773 | F-box only protein 21 | 5.8 |  |
| U13393 | Proteasome subunit, β type 6 | 1.8 |  |
| U27933 | Proteasome subunit, β type 8 | 1.72 |  |
| AI009840 | Cathepsin E | 1.48 | |
| AI747533 | Lysosomal membrane glycoprotein 2 | 1.42 | + |
| Amino acid catabolism |
| D29987 | 4-Hydroxyphenylpyruvic acid dioxygenase | 2.72 |  |
| AI194855 | Tryptophan 2,3-dioxygenase | 1.71 |  |
| AI844995 | Glyoxylate/hydroxypropionate reductase | 1.65 |  |
| AA986718 | Alamine-glucosylate aminotransferase | 1.61 |  |
| L07645 | Histidine ammonia lyase | 1.5 |  |
| AI255553 | Tyrosine aminotransferase | 1.43 |  |
| Lipid trafficking/transport |
| CA26364 | Lipotein lipase | 2.55 | + |
| D78354 | Phospholipid scramblase 1 | 1.77 |  |
| AA655303 | Apolipoprotein M | 1.77 |  |
| AF005348 | Niemann Pick type C1 | 1.74 |  |
| AI848302 | Lipin 2 | 1.64 |  |
| M65034 | Fatty acid binding protein 2, intestinal | 1.58 | + |
| U37799 | Scavenger receptor class B, member 1 | 1.48 |  |
| AI785422 | Apolipoprotein A-V | 1.42 |  |

### TABLE 2

**Gene-array results: decreased**
The genes whose expression is decreased in TGN versus WT mice 6 h after refeeding and directly related to glucose and/or lipid metabolism are shown. The relative level of expression in transgenic versus wild-type mice (TGN/WT) in gene-array studies for each gene is shown, and those genes where the results of gene-array studies were verified by quantitative PCR (qPCR) are identified (+).

| Accession | Gene description | TGN/WT | qPCR |
|-----------|------------------|--------|------|
| Glycolysis |
| LI41631 | Glucokinase | 0.35 | + |
| M14220 | Glucose phosphate isomerase | 0.5 |  |
| AI655303 | Aldolase, B isoform | 0.62 | + |
| AI41389 | Enolase | 0.69 |  |
| D63764 | Pyruvate kinase, liver | 0.53 | + |
| Pentose phosphate shunt |
| L35034 | Ribose 5-phosphate isomerase | 0.55 | + |
| U05809 | Transketolase | 0.52 | + |
| Fatty acid/triglyceride synthesis |
| AW125336 | Pyruvate dehydrogenase (lipoamide) | 0.64 |  |
| AW125431 | Citrate synthase | 0.62 |  |
| A1B48354 | Citrate transporter, mitochondrial | 0.69 |  |
| AW121639 | ATP citrate lyase | 0.23 | + |
| J02652 | Malic enzyme, supernatant | 0.34 | + |
| X13135 | Fatty acid synthase | 0.47 | + |
| AW122533 | Fatty acid elongase (Elov6) | 0.32 |  |
| A1B80989 | Fatty acid elongase (Elov5) | 0.73 |  |
| M21285 | Stearoyl-coenzyme A desaturase 1 | 0.42 | + |
| A1B3021 | Fatty acyl-CoA synthetase 5 | 0.68 |  |
| AW290060 | Glycerol-3-phosphate dehydrogenase, cytoplasmic | 0.45 |  |
| D50430 | Glycerol-3-phosphate dehydrogenase, mitochondrial | 0.68 |  |
| U11680 | Glycerol-3-phosphate acyltransferase, mitochondrial | 0.25 | + |
| Sterol synthesis |
| A1B45689 | Sterol regulatory element binding factor 1 (SREBP-1) | 0.56 | + |
| M55797 | Acetyl-CoA acyltransferase, mitochondrial | 0.48 |  |
| AW045553 | Farnesyl diphasate synthetase | 0.51 |  |
| D42048 | Squalene epoxidase | 0.5 |  |
| AW049778 | Mevalonate (diphospho) decarboxylase | 0.58 |  |
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Gene profiling suggested that the expression of glucose-6-phosphate dehydrogenase might be modestly increased in TGN versus WT (supplemental Table S2a); however, PCR showed that the expression of glucose-6-phosphate dehydrogenase is reduced by ~70% in TGN versus WT, consistent with the other results indicating that genes involved in promoting flux through the shunt pathway are suppressed in TGN versus WT mice after feeding.

The expression of several other genes involved in glycolysis (enolase and pyruvate kinase) and in the utilization of pyruvate as a substrate for de novo lipogenesis (the lipoamide subunit of pyruvate dehydrogenase, citrate synthase, the mitochondrial citrate transporter, and ATP citrate lyase, which provides the sole source of cytoplasmic acetyl-CoA) was reduced in TGN versus WT mice (Table 2). The expression of acetyl-CoA carboxylase-α, which converts acetyl-CoA to malonyl-CoA, the substrate for fatty acid and steroid synthesis, was not detected in gene-array studies; however, PCR showed that the expression of acetyl-CoA carboxylase-α also was reduced by ~50% in TGN versus WT mice after refeeding (supplemental Fig. S3).

The expression of multiple genes involved in the synthesis, elongation, desaturation, and acylation of fatty acids and the formation of monoglycerides, the first step in triglyceride formation, also was suppressed in TGN versus WT mice, including fatty acid synthase, fatty acid elongases, stearoyl-CoA desaturase 1, fatty acyl-CoA synthase-5, glycerol-3-phosphate dehydrogenase, and glycerol-3-phosphate acyltransferase (Table 2). The expression of several genes in the sterol synthetic pathway was decreased (Table 2). The expression of hydroxy-methylglutaryl-CoA reductase, a rate-limiting step in sterol synthesis, tended to be decreased in transgenic mice in gene-array studies, but this effect did not achieve statistical significance. However, PCR confirmed that expression of hydroxy-methylglutaryl-CoA reductase also was decreased 30 and 50% in female and male TGN mice compared with WT.

The sterol response element-binding protein-1 (SREBP-1) plays a critical role in the regulation of lipogenesis and sterol synthesis in response to feeding, and gene profiling indicated that the expression of SREBP-1c was reduced in transgenic mice (Table 2). PCR studies showed that SREBP-1c was the major form of SREBP expressed in the liver (SREBP-1c > SREBP-1a > SREBP2), consistent with previous studies (53) and that the expression of SREBP-1c (but not SREBP-1a or -2) was reduced in TGN versus WT mice after refeeding (Table 2). Western blotting studies confirmed that hepatic SREBP-1 protein levels were reduced in TGN versus WT mice after refeeding (supplemental Fig. S4).

In contrast, we did not detect differences in the expression of several enzymes directly involved in the regulation of fatty acid oxidation (including carnitine palmitoyltransferase-1, acyl-CoA oxidase, or very long chain fatty acyl-CoA dehydrogenase) in TGN versus WT mice by gene array or PCR (data not shown). This indicates that the effects of the FoxO1 transgene on pathways involved in glucose utilization, lipogenesis, and sterol synthesis are selective.

Effects of FoxO1 in Isolated Hepatocytes—To determine whether these effects on gene expression are a direct effect of FoxO1 in the liver, we examined the effects of constitutively active FoxO1 on gene expression in primary mouse hepatocytes infected with adenoviral vectors expressing constitutively active FoxO1 plus green fluorescent protein (GFP) or GFP alone. Hepatocytes were treated with 100 nM insulin and 25 mM glucose for 24 h to provide a lipogenic milieu comparable to the fed state, and RNA was harvested for analysis of gene expression by real-time PCR. For comparison, the effects of the FoxO1 transgene on gene expression in the livers of TGN versus WT mice are shown in Fig. 6A. As shown in Fig. 6B, FoxO1 stimulated the expression of IGFBP-1, PEPCK, and glucose-6-phosphatase in isolated hepatocytes, consistent with previous reports. At the same time, FoxO1 suppressed the expression of SREBP-1c, glucokinase, transketolase, pyruvate kinase, acetyl-CoA carboxylase, and fatty acid synthase, supporting the concept that FoxO proteins contribute directly to the suppression of these genes in the liver.

Other Effects of FoxO1—Although not the focus of this study, it is important to note other results of our gene-array studies that provide insight into the role of FoxO proteins in the liver. Approximately one-third of the genes differentially expressed in TGN versus WT mice were involved in cell signaling, cell death or survival, or gene regulation (supplemental Tables S2a and S2b). This includes several genes that are important for insulin signaling, including insulin receptor substrate-2 (IRS-2) and the p85 regulatory subunit of phosphatidylinositol 3’-kinase (p85). PCR confirmed that the expression of IRS-2 was increased in TGN versus WT mice and in isolated hepatocytes expressing constitutively active FoxO1 (data not shown), consistent with previous studies demonstrating that FoxO proteins can stimulate IRS-2 expression (54). PCR also confirmed that the expression of p85 was reduced in TGN versus WT mice and by FoxO1 in isolated hepatocytes (data not shown). These changes in the expression of IRS-2 and p85 would be expected to increase the activation of phosphatidylinositol 3’-kinase and the activation of PKB (55–57) and may be part of a more general feedback loop by which FoxO proteins may limit their own function, as recently suggested (58). Gene profiling also suggested that the expression of the phosphatase and tensomodulin (PTEN) and TRB-3 genes, which also are important in modulating signaling by phosphatidylinositol 3’-kinase and PKB, also may be altered in transgenic mice, but these effects were not confirmed by PCR.

The expression of other genes involved in cell signaling also was altered in TGN versus WT mice (supplemental Tables S2a and S2b). PCR confirmed that the expression of IGF-binding protein-1 and the leptin and adiponectin receptors were increased in TGN versus WT mice, and in isolated hepatocytes expressing constitutively active FoxO1. Gene-array studies also indicated that the expression of the β3 adrenergic receptor and several intracellular proteins involved in mediating or modulating effects of cAMP are altered in TGN. The expression of several growth factors, cytokines and chemokines and/or their receptors, as well as membrane-associated or intracellular kinases, and factors involved in modulating signaling by G-protein receptors and calcium signaling pathways also were altered in TGN versus WT mice (supplemental Tables S2a and S2b). The expression of several proteins that enhance or inhibit the function of p300/CPB co-activator proteins also is altered in the liver of FoxO1 transgenic mice. Together, these findings indicate that FoxO proteins alter the expression of factors involved in the function of multiple regulatory pathways in the liver.

DISCUSSION

FoxO transcription factors are widely expressed (59) and are important targets of insulin action (2). FoxO1 is expressed in the liver, and previous studies have shown that it can stimulate the expression of IGF-binding protein-1 and several genes promoting hepatic glucose production, including PEPCK and glucose-6-phosphatase in liver cells and in vivo (47, 60). Using the a1-antitrypsin promoter, we created transgenic mice that express a constitutively active form of FoxO1 selectively in the liver and at near physiological levels and examined its effects on glucose and lipid metabolism and the genomic response to fasting and feeding. We found that fasting glucose levels are modestly increased despite hyperinsulinemia and that these mice have reduced glucose tolerance and are less sensitive to glucose lowering effects of insulin. Gene-
array and PCR studies show that the expression of PEPCK and glucose-6-phosphatase is increased in the liver of FoxO1 transgenic mice, and constitutively active FoxO1 stimulated the expression of these genes in isolated hepatocytes. These results support the concept that suppressing FoxO function is important for the ability of insulin to regulate fasting glucose levels and the expression of several genes directly involved in promoting hepatic glucose production.

The results of the present study indicate that FoxO proteins also promote hepatic glucose production through other mechanisms and exert other important effects on metabolism in the liver. In addition to altering the expression of PEPCK and glucose-6-phosphatase, we find that FoxO1 also can reduce the expression of glucokinase, the major enzyme responsible for the phosphorylation of glucose in the liver, and other genes involved in the metabolism of glucose through the glycolytic, pentose phosphate shunt, and lipogenic and sterol synthetic pathways. Presumably, reduced phosphorylation and metabolism of glucose would contribute to ability of the liver to secrete newly synthesized glucose in fasting, when insulin levels are low and endogenous FoxO proteins are active.

We also found that the expression of aquaporin 9 is increased in the liver of FoxO1 transgenic mice. Hepatic expression of aquaporin 9 is increased in fasting and insulin deficiency, and aquaporin 9 promotes hepatic uptake of glycerol, an important substrate for glucose production (51). Reporter gene studies have shown that insulin suppresses the expression of aquaporin 9 through an insulin response sequence (GAAAACAA) (51) that closely matches the consensus sequence for FoxO binding sites ((G/C)(T/A)AAA(C/T)AA), indicating that aquaporin 9 is likely to be a direct target for FoxO-mediated gene expression. Interestingly, the expression of aquaporin 7, which promotes glycerol export from adipocytes, also is increased in fasting and suppressed by insulin (51), and the aquaporin 7 promoter also contains putative FoxO binding sites (61). These observations suggest that FoxO proteins also may promote hepatic glucose production by enhancing glycerol transport in both adipose tissue (increased export) and the liver (increased import).

Amino acids also are an important substrate for gluconeogenesis, and increased protein turnover and amino acid availability are important for the adaptation to fasting. We find that the expression of a number of genes involved in promoting protein turnover and the catabolism of amino acids is increased in the liver of FoxO1 transgenic mice (Table 1). Previous studies have shown that the expression of the tyrosine aminotransferase gene is suppressed by insulin through a Forkhead response element (62), and the finding that its expression is increased in FoxO1 transgenic mice suggests that tyrosine aminotransferase may be directly regulated by FoxO proteins. Recent studies have shown that FoxO proteins exert important effects on protein turnover in skeletal muscle through the induction of atrogin-1, an F-box protein (F-box 32) that targets proteins for ubiquitination and proteasomal degradation (26, 63). Interestingly, the expression of another F-box protein (F-box 21) is increased ~6-fold in the liver of FoxO1 transgenic mice (Table 1), and we find that its expression is strongly regulated by fasting and refeeding in the liver of wild-type mice (data not shown). These observations suggest that FoxO proteins may be involved in regulating protein turnover in the liver, and that F-box proteins may play a role in this process, similar to muscle.

A major finding of this study is that the expression of multiple genes involved in promoting glucose utilization through glycolysis, the pentose phosphate shunt, de novo lipogenesis, and sterol synthesis is suppressed in transgenic mice expressing constitutively active FoxO1 in the liver. Based on the observation that fasting plasma triglyceride levels are modestly reduced in FoxO1 transgenic mice despite high insulin levels, we considered the possibility that constitutively active FoxO1 may impair the ability of insulin to stimulate lipid synthesis in the liver. To explore this possibility, we studied mice after a high carbohydrate meal, when lipogenesis is stimulated. These studies revealed that post-prandial triglyceride levels are markedly reduced, and metabolic studies with $^{3}$H$_2$O provided direct evidence that de novo lipogenesis is suppressed (by ~70%) in FoxO1 transgenic mice after refeeding mice. Gene-array studies indicated that this result reflects reduced expression of multiple genes involved in glycolytic and lipogenic metabolism. These findings were reproduced using adenoviral vectors to express FoxO1 in isolated hepatocytes, indicating that these results are not an artifact due to the site of insertion of the transgene, nor an indirect effect mediated due to changes in the hormonal milieu in vivo. These results appear to be specific, because the expression of many other genes is still appropriately regulated by fasting and refeeding in transgenic mice (Fig. 5A) including the expression of genes involved in fatty acid oxidation. To our knowledge, these studies provide the first evidence that FoxO proteins suppress glycolytic and lipogenic metabolism in the liver.

The fact that these effects were most apparent after refeeding, when the function of endogenous FoxO proteins (but not the transgenic constitutively active FoxO1 protein) is suppressed, demonstrates the importance of considering nutritional status when examining the role of FoxO proteins in vivo. In this context, it is interesting to note that the expression of constitutively active FoxO1 partially impaired, but did not fully disrupt the effects of refeeding on the expression of many the genes affected (Fig. 6C). This observation suggests that other factors also may play an important role in mediating the effects of food intake on gene expression in the liver. This is consistent with previous studies regarding the role of FoxO proteins in the regulation of IGFBP-1, PEPCK, and glucose-6-phosphatase, where both FoxO-dependent and -independent mechanisms mediate effects of insulin on gene expression (32, 34, 64), and with studies implicating other factors in the regulation of these genes by insulin, including SREBP-1c (65), C/EBP proteins (66), and TORC co-activator proteins (67). Given the critical role that many of the genes affected (e.g. SREBP-1c, glucokinase, and acetyl-CoA carboxylase) play in the regulation of carbohydrate and lipid metabolism, it is reasonable to expect that multiple and possibly redundant mechanisms might contribute to the integrated regulation of their expression.

Although the present study focused on the effects of FoxO1 on lipid synthesis, it is important to note that FoxO proteins also may exert effects on other aspects of lipid metabolism in the liver. We find that the expression of lipoprotein lipase is increased in the liver of FoxO1 transgenic mice, which can enhance the breakdown of triglycerides and metabolism of fatty acids. This is likely to reflect a direct effect of the transgene, because FoxO1 also has been reported to stimulate the expression of lipoprotein lipase in muscle cells (28). Interestingly, the expression of apolipoprotein AV, which can function together with lipoprotein lipase to promote lipolysis of circulating triglycerides, also is increased in TGN versus WT mice. FoxO1 also stimulates the translocation and activation of CD36, a fatty acid transport protein, in skeletal muscle (29). Although we did not detect an increase in CD36 expression, the expression of fatty acid binding protein-2 and several other genes involved in the regulation of lipid transport or trafficking is increased in the liver of transgenic mice (Table 1). Altomonte et al. (68) have reported that FoxO1 can stimulate the expression of apolipoprotein CIII in the liver, which can inhibit peripheral clearance of VLDL triglycerides. We did not detect an increase in apo-CIII expression in the liver of our transgenic mice or in isolated
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hepatocytes expressing constitutively active FoxO1. This difference may reflect differences in genetic background, the level, and/or distribution of FoxO1 expression, differences in the diets provided (high fat versus high carbohydrate chow), and nutritional status. Together, these results suggest that FoxO proteins may exert multiple and complex effects on lipid metabolism in the liver.

Additional studies are needed to examine the mechanism by which constitutively active FoxO1 suppresses the expression of genes involved glycolytic and lipogenic metabolism. Glucose flux through the pentose phosphate shunt and the production of xylulose 5-phosphate are important for the activation of the carbohydrate response element ChREBP (52, 69) and the induction of multiple genes involved in glucose metabolism and lipogenesis, including pyruvate kinase, ATP citrate lyase, acetyl-CoA carboxylase, fatty acid synthase, and others (70). We did not detect any effect of the FoxO1 transgene on the expression of ChREBP. However, reduced expression of glucokinase and other genes involved in the pentose phosphate shunt would be expected to limit the activation of ChREBP, and thereby limit the induction of lipogenic genes in response to feeding. ChREBP can function together with SREBP-1c to stimulate the expression of some lipogenic genes (52), suggesting that reduced expression of SREBP-1c may further limit glucose-regulated gene expression in FoxO1 transgenic mice.

Several factors may contribute to the reduction of glucokinase and SREBP-1c expression in FoxO1 transgenic mice. SREBP-1c is thought to play an important role in mediating effects of insulin on the expression of glucokinase (71, 72), indicating that reduced expression of SREBP-1c is likely to contribute to the reduction in glucokinase expression in FoxO1 transgenic mice. Other factors also are thought to contribute to the induction of glucokinase in response to insulin, including hypoxia-inducible factor-1α and hepatocyte nuclear factor-4 (73), and PPARγ also has been reported to stimulate glucokinase expression in liver-derived cells (74). FoxO1 can interact directly with and impair transactivation by hepatocyte nuclear factor-4 (21) and PPARγ (20), and FoxO1 proteins also can suppress the expression of hypoxia-inducible factor-1α (75), suggesting that FoxO proteins also could suppress the expression of glucokinase by impairing the function of these factors.

Similarly, there are several mechanisms by which FoxO1 may suppress the expression of SREBP-1c. Interestingly, glucose metabolism is thought to contribute to the regulation of SREBP-1c (72, 76), suggesting the possibility that reduced expression of glucokinase may contribute to the suppression SREBP-1c in FoxO1 transgenic mice. LXR, a nuclear receptor protein, is important in mediating the effect of sterols and insulin on the expression of SREBP-1c (77), and it is possible that FoxO1 may impinge the function of LXR, similar to other nuclear receptor proteins, including hepatocyte nuclear factor-4 and PPARγ. Other factors also contribute to the effect of insulin on the activity of the SREBP1c promoter (78), suggesting that FoxO1 may contribute the regulation of SREBP-1c through other mechanisms. In addition, our gene-array data indicate that FoxO1 can stimulate the expression of several proteins that can inhibit gene expression, including transducin enhancer of split 1 (groucho), and CREB-binding protein/p300 inhibitory protein (supplemental data), and increased expression of these proteins and/or other repressor proteins (79) also might contribute to reduced expression of SREBP-1c and glucokinase in FoxO1 transgenic mice. Additional studies to examine the mechanism(s) mediating the effect of FoxO1 and on the expression of SREBP-1c and glucokinase are needed.

As summarized in Fig. 7, the results of the present study suggest that

FoxO1 proteins may contribute to the regulation of multiple metabolic pathways important for the adaptation to fasting and feeding in the liver. Under fasting conditions, when insulin levels are low, endogenous FoxO proteins can function to promote gluconeogenesis and may enhance protein turnover and amino acid catabolism. At the same time, increased FoxO function may contribute to the suppression of glycolysis and metabolism through the pentose phosphate shunt and lipogenic and sterol synthetic pathways that is adaptive to fasting. In the fed state when insulin levels are high, the suppression of FoxO function is thought to be important for the ability of insulin to suppress the expression of genes involved in promoting hepatic glucose production. The results of this study indicate that reducing FoxO function also may be important for the ability to fully suppress protein/amino acid catabolism and promote glucose utilization and lipid synthesis in response to feeding. Together, these results suggest that FoxO proteins play an important role in the regulation of multiple metabolic pathways in the liver in response to fasting and feeding and contribute to the regulation of both anabolism and catabolism.

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