



The Role of Hepatic FoxO1 in Insulin Resistance

Citation

Ling, Alisha Viva. 2015. The Role of Hepatic FoxO1 in Insulin Resistance. Doctoral dissertation, Harvard University, Graduate School of Arts & Sciences.

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The Role of Hepatic FoxO1 in Insulin Resistance

A dissertation presented

by

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to

The Division of Medical Sciences

in partial fulfillment of the requirements
for the degree of
Doctor of Philosophy
in the subject of

Biological and Biomedical Sciences

Harvard University

Cambridge, Massachusetts

May 2015

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Abstract

Metabolic syndrome is a major health concern in the US, affecting a third of all adults and amplifying the risk of cardiovascular disease and diabetes. The central pathophysiological root of metabolic syndrome is widely considered to be insulin resistance, though the mechanisms linking insulin resistance to this clinical constellation of obesity, dyslipidemia, hypertension and hepatic steatosis are poorly understood. In insulin resistance, insulin suppression of the forkhead box protein O1 (FOXO1) transcription factor is lost, and FoxO1 remains inappropriately over-active. FoxO1 has an established role activating gluconeogenesis, however, its regulation of lipid metabolism, especially of cholesterol metabolism, has remained largely unstudied. Here, we investigate the role of hepatic FoxO1 in mediating the dysregulation of lipid metabolism.

Using a mouse model liver-specific knockout of the insulin receptor and FoxO1, we show that loss of hepatic FoxO1 restores normal gene expression of gluconeogenic and cholesterologenic, but not lipogenic genes. We identify *Cyp8b1* as a target of exquisitely sensitive control by FoxO1. *Cyp8b1* expression is increased in a mouse model of metabolic syndrome and decreased with acute knockdown of FoxO1, and also increased in humans with metabolic syndrome. Via *Cyp8b1*, FoxO1 controls cholic acid synthesis, which in turn increases dietary cholesterol absorption, intrahepatic cholesterol, and secretion of FGF15 and GLP1 from the small intestine. To confirm this extrahepatic role of hepatic FoxO1 as a regulator of cholesterol metabolism, we re-introduced CYP8B1 in the absence of FoxO1.

To further investigate the well-documented association between insulin resistance and cardiovascular disease, we used non-biased profiling methods to identify the enzyme flavin-containing monooxygenase 3 (*Fmo3*) to be a target of insulin. FMO3 produces trimethylamine N-oxide (TMAO), which has recently been suggested to promote atherosclerosis in mice and humans.

We show that *FM03* is suppressed by insulin *in vitro*, increased in obese/insulin resistant mice, and increased in obese/insulin resistant humans. Knockdown of FM03 in insulin-resistant mice suppressed FoxO1, and entirely prevented the development of hyperglycemia, hyperlipidemia, and atherosclerosis. Overall, this dissertation examines the role of hepatic FoxO1 as a potent mediator of the metabolic dysfunction in insulin resistance and metabolic syndrome, and the development of cardiovascular disease.

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List of Abbreviations

ASO	antisense oligonucleotides
CA	cholic acid
CDCA	chenodeoxycholic acid
CVD	cardiovascular disease
<i>Fdps</i>	farnesyl diphosphate synthase
FPLC	Protein Liquid Chromatography
<i>Fmo3</i>	Flavin-containing monooxygenase 3
FoxO1	Forkhead box protein O1
FXR	Farnesoid X receptor
HDL	High-density lipoprotein
<i>Hmgcr</i>	3-hydroxy-3-methylglutaryl-CoA reductase
<i>InsR</i>	Insulin receptor
LDKO	Liver Double Knockout (FoxO1 ^{lox/lox} InsR ^{lox/lox} :alb-Cre ⁺)
LDL	Low-density lipoprotein
<i>Ldlr</i>	LDL receptor
LFKO	Liver-specific FoxO1 Knockout (FoxO1 ^{lox/lox} InsR ^{wt/wt} :alb-Cre ⁺)
LIRKO	Liver Insulin Receptor Knockout (FoxO1 ^{wt/wt} InsR ^{lox/lox} :alb-Cre ⁺)
Lox	FoxO1 ^{wt/wt} InsR ^{lox/lox}
<i>Pck1</i>	Phosphoenolpyruvate carboxykinase
qPCR	Quantitative PCR
<i>sqs</i>	squalene synthase
STZ	streptozotocin
<i>Tbp</i>	TATA-box-binding protein
TMA	Trimethylamine
TMAO	Trimethylamine N-oxide
UDCA	ursodeoxycholic acid
VLDL	Very low-density lipoprotein

Acknowledgments

“There are in fact two things—science and opinion. The former begets knowledge, the latter ignorance.”

— Hippocrates

My thanks go to my dissertation advisor, Sudha Biddinger, and the members of the Biddinger lab: past and present: Ji Miao and Dong-Ju Shin, Xiao Wei Sun, Mary Haas, Mary Gearing; Amy Levenson, Praveen Mantheena, Abhiruchi Mehta and Jordan Grant.

For their encouragement and wisdom, I wish to especially thank the members of the Dissertation Advisory Committee: Gary Ruvkun; Morris White; and Zoltan Arany; and the members of the Dissertation Examination Committee: Susan Fried; Steve Shoelson; and Alexander Soukas.

I am deeply indebted to my wonderful advisors and mentors: David Cardozo, Richard Mitchell, Matthew Frosch, David Frank, and Allan Goldstein.

I thank the HST, MDPHD and BBS programs, and in particular Patty Cunningham, Amy Cohen, and Kate Hodgins, who have been steadfast in their support.

Finally, I wish to acknowledge my family: my brother, Will; my late grandparents, Gung Gung and Paw Paw; and my mother, without whom this would not have been possible. Happy Mother’s Day 2015!

Chapter 1

Introduction

1.1 Metabolic Syndrome & Insulin Resistance

Metabolic syndrome is a major health concern in the US, now affecting a third of all adults. (Ervin, 2009) Formerly known as syndrome X, the dysmetabolic syndrome, and the insulin resistance syndrome, metabolic syndrome amplifies the risk of diabetes and of cardiovascular disease. (Cornier et al., 2008) Already the leading cause of death in the US overall, cardiovascular disease is an even greater concern for people with diabetes as it is the cause of death for 65% of this population. (Grundy et al., 1999) The ever growing epidemic of obesity and metabolic syndrome only highlights our need for a better understanding of the specific mechanisms that link insulin resistance, metabolic syndrome, and diabetes to the development of cardiovascular disease.

Metabolic syndrome is a constellation of clinical findings, including central obesity, glucose intolerance, dyslipidemia (high plasma triglycerides and low plasma HDL), and hypertension, and amplifies the risk of diabetes and cardiovascular disease. (DeFronzo and Ferrannini, 1991; Lindsay and Howard, 2004; Reaven, 1988) Insulin resistance is widely considered to be at the pathophysiological root of metabolic syndrome. (Biddinger and Kahn, 2006)

An important mouse model used in the study of insulin resistance is the Liver Insulin Receptor Knockout (LIRKO) mouse. First described in 2000 (Michael et al., 2000), the LIRKO mouse

exhibits hyperglycemia, a pro-atherogenic profile of lipoproteins, and is unusually susceptible to atherosclerosis (Biddinger et al., 2008b). While useful in the study of insulin signaling, the LIRKO mouse differs from metabolic syndrome in that it has total hepatic insulin resistance, rather than selective insulin pathway resistance. In metabolic syndrome and in diabetes, insulin fails to suppress gluconeogenesis yet continues to activate lipogenesis. By contrast, the LIRKO mouse fails to suppress gluconeogenesis and also fails to activate lipogenesis and therefore does not exhibit hypertriglyceridemia, unlike metabolic syndrome.(Brown and Goldstein, 2008)

Table 1.1 Clinical criteria for the metabolic syndrome.

According to the National Cholesterol Education Program (NCEP) Adult Treatment Panel (ATP III), clinical identification of metabolic syndrome requires 3 of the following criteria.

Risk Factor	Defining Level
Abdominal obesity	Waist circumference
Men	>102 cm (>40 in)
Women	>88 cm (>35 in)
Triglycerides	≥150 mg/dL
HDL cholesterol	
Men	<40 mg/dL
Women	<50 mg/dL
Blood pressure	≥130/≥85 mmHg
Fasting glucose	≥110 mg/dL

1.2 FoxO1

The forkhead box protein O1 transcription factor (FoxO1) is a potent regulator of metabolism, growth, and stress response. In metabolism, FoxO1 regulates glucose production, bile acid metabolism, lipoprotein metabolism and fatty acid metabolism. FoxO1’s role in hyperglycemia and glucose intolerance is well-established. However, its role in the regulation of lipid metabolism remains poorly understood. A thorough investigation of FoxO1’s role in regulating lipid metabolism will grant us a better understanding of the mechanisms linking insulin resistance to the clinical sequelae of metabolic syndrome and to cardiovascular disease and to develop new therapeutic targets to prevent the development of cardiovascular disease in patients with diabetes or metabolic syndrome.

The dominant regulator of FoxO1 activity is inhibition by insulin via the insulin receptor substrate (IRS 1/2), phosphatidylinositol 3-kinase (PI3K), AKT phosphorylation cascade. When phosphorylated, FoxO1 is excluded from the nucleus and subsequently degraded. In insulin resistance, this insulin suppression of FoxO1 is absent leaving FoxO1 disinhibited and an important driver of metabolic dysregulation.

As a signal of the fasting state, FoxO1 is a critical activator of hepatic glucose production and induces transcription of G6pc and Pck1. In the absence of insulin signaling in the liver, FoxO1 tonically drives the expression of G6pc and Pck1. Multiple mouse models of defective insulin signaling have examined the role of FoxO1 in regulating hepatic glucose production. One study used liver-specific knockout of Irs1 and Irs2 as a model and found that concurrent liver-specific knockdown or knockout of FoxO1 rescued hyperglycemia, insulin resistance, and growth retardation. (Dong et al., 2008) Another study looked at liver-specific knockout of Akt1 and Akt2 and found that hepatic deletion of FoxO1 in these mice normalized hyperglycemia, glucose intolerance, and hyperinsulinemia, despite the continued defect in insulin signaling. (Lu et al., 2012) One study knocked out hepatic FoxO1 from a mouse model deficient in insulin receptor in all tissues that develops lethal neonatal diabetic ketoacidosis and steatosis. Hepatic knockout of FoxO1 was sufficient to rescue this phenotype and extend survival, though these mice eventually develop severe diabetes by 3-4 months of age. (Matsumoto et al., 2007) Yet another group is currently studying the effect of liver-specific knockout of FoxO1 in the LIRKO mouse (which they have named "LIRFKO") on glucose homeostasis. They have found that their LIRFKO mice have normalized glucose tolerance tests and ameliorated insulin tolerance tests. Despite the complete absence of hepatic insulin signaling, insulin was fully able to suppress hepatic glucose output in LIRFKO mice, unlike in LIRKO mice. (O-Sullivan et al.) While not yet published, this work was presented at the 16th International Congress of Endocrinology and the Endocrine Society's 96th Annual Meeting and Expo.

There have also been numerous studies examining hepatic FoxO1 regulation of Srebp-1c, the key transcription factor that activates lipogenesis, with conflicting results (see Table 1.2). Of particular relevance to this work is a recent report by Haeusler et al proposing that ablation of hepatic FoxO1 increases plasma and hepatic triglyceride levels by lowering levels of Cyp8b1 and its product, cholic acid, and thus decreasing FXR activation. (Haeusler et al., 2012)

Table 1.2 Conflicting studies examining FoxO1 regulation of Srebp-1c and lipogenesis

Gain of function studies	Manipulation	<i>Srebp-1c</i> mRNA	SREBP-1C protein	<i>Fasn</i> mRNA	Plasma triglycerides	Additional Comments
(Deng et al., 2012)	FoxO1-CA, in vivo	↓ (fasting & refed)	↓	↓		
(Qiang et al., 2012)	constitutively deacetylated FoxO1				↓	
(Zhang et al., 2006a)	transgenic FoxO1-CA	↓ (in vivo & in vitro)			↓ (males only)	
(Qu et al., 2006)	adenoviral GOF, in vivo	↔	↔	↑		
(Qu et al., 2006)	hepatic FoxO1-CA (S253A), in vivo	↑	↑			
(Matsumoto et al., 2006)	hepatic FoxO1-CA			↑	↓	hepatic steatosis
(Altomonte et al., 2004)	FoxO1 cDNA, in vitro & in vivo				↑	↑ plasma ApoCIII
(Kamagate et al., 2008)	transgenic FoxO1-CA	↑	↑	↑	↑	↑ plasma ApoB

(FoxO1-CA = constitutively active FoxO1)

Table 1.2 (Continued)

Loss of function studies	Manipulation	<i>Srebp-1c</i> mRNA	<i>Fasn</i> mRNA	Plasma triglycerides	Additional Comments
(Haeusler et al., 2012)	hepatic FoxO1-KO, in vivo	↔	↑	↑ (on LDLR-/- background)	
(Deng et al., 2012)	hepatic FoxO1-KO, in vivo	↑			
(Qu et al., 2006)	(dominant negative adenovirus)				↑ hepatic triglycerides
(Matsumoto et al., 2007)	hepatic FoxO1-KO			↔	
(Kamagate et al., 2008)	FoxO1 RNAi	↔	↓	↔	↓ plasma ApoB
(Haeusler et al., 2010)	STZ +/- hepatic FoxO1-KO	↑ (fasted)	↑ (fasted)		↑VLDL secretion, ↑plasma FFAs
(Zhang et al., 2012)	hepatic KO Foxo1			↔	
	hepatic KO Foxo3			↔	
	hepatic KO Foxo1/3			↑	
	hepatic KO Foxo1/3/4			↑	

1.3 Bile Acids

Bile acid synthesis and regulation of Cyp8b1

Bile acids are synthesized in the liver by a collection of enzymes in the cytochrome P450 family. (Russell, 2003) Although some of the genes involved in bile acid synthesis are expressed in other tissues, bile acid synthesis occurs exclusively in the liver. (Chiang, 1998; Russell, 1992; Russell and Setchell, 1992)

Primary bile acids are synthesized in the liver via the classical pathway (75% of total primary bile acids) and the alternative pathway (25% of total primary bile acids). (Russell, 2003) The classical pathway requires the activity of CYP7A1 and CYP8B1 and generates both cholic acid (CA) and chenodeoxycholic acid (CDCA), whereas the alternative pathway involves CYP27A1 and CYP7B1 and exclusively generates CDCA and its derivatives. (Li-Hawkins et al., 2002; Russell, 2003)

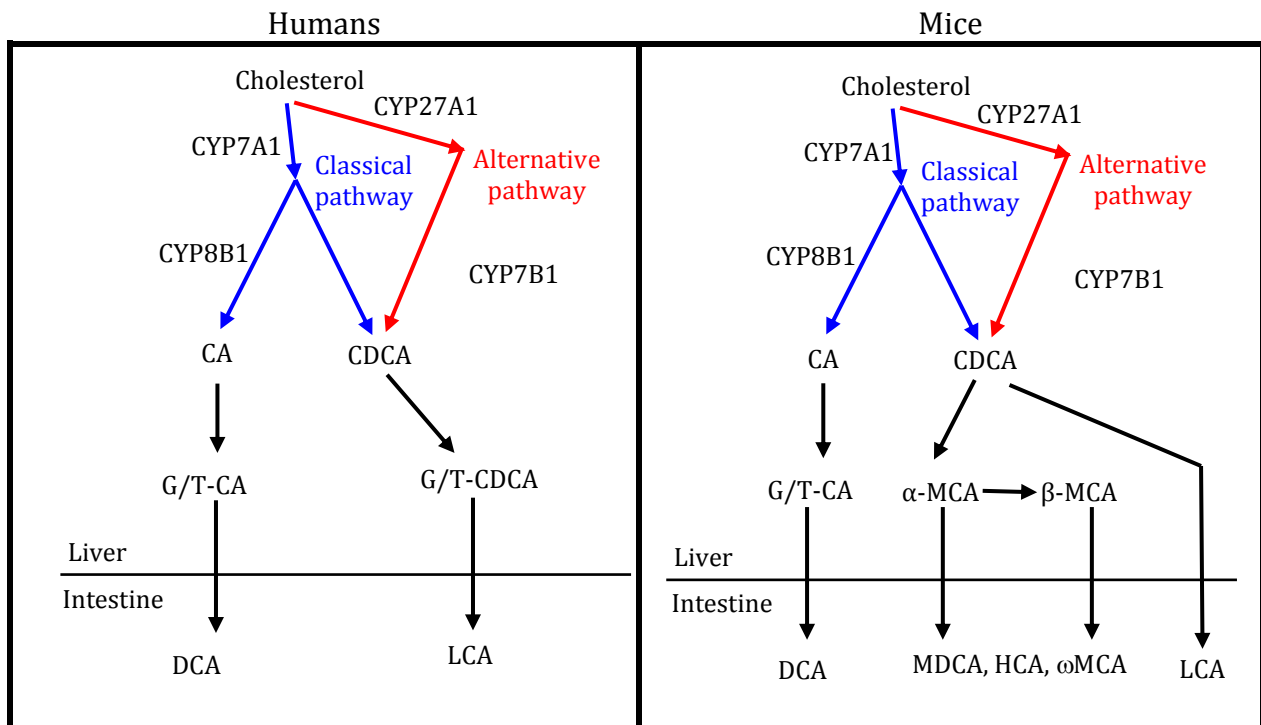


Figure 1.1 Simplified schematic of bile acid synthesis

CYP7A1, also named cholesterol 7 α -hydroxylase, catalyzes the rate-limiting step of the classical pathway and is thought to regulate the total amount of bile acid synthesis and the bile acid pool size. (Chiang, 2009a; Fiorucci et al., 2009) CYP8B1, also named sterol 12 α -hydroxylase, catalyzes the synthesis of CA, and thus regulates the balance of the bile acid pool between CA and CDCA and their respective derivatives. (Bjorkhem et al., 1983)

Both Cyp8b1 and Cyp7a1 are subject to a negative feedback loop that was described well over 50 years ago. (Beher et al., 1961) The products of Cyp8b1 and Cyp7a1 activity, bile acids, activate FXR, a bile acid nuclear receptor. FXR activation increases expression of SHP, which directly suppresses transcription of Cyp8b1 and Cyp7a1. A second negative feedback loop regulating Cyp8b1 and Cyp7a1 expression acts via TGR5, a G-protein coupled receptor in the ileum that is also activated by bile acids. When activated, TGR5 increases expression and secretion of FGF15 peptides, which bind hepatic cell surface receptor FGFR4 to suppress transcription of Cyp8b1 and Cyp7a1, independently of FXR and SHP.

Transcription of Cyp8b1 is also inhibited by cholesterol and by insulin (Chiang, 2003; Ishida et al., 2000; Vlahcevic et al., 2000) and stimulated by cholestyramine, a bile acid sequestrant which prevents the reabsorption of bile acids in the gastrointestinal tract. (Vlahcevic et al., 2000) Hepatocyte nuclear factor 4 α (HNF4 α) also activates Cyp7a1 and Cyp8b1 transcription. (Chiang, 2009b) A recent study has shown suppression of Cyp8b1 in the absence of FoxO1, with a deficiency in CA and downstream products of CA. (Haeusler et al., 2012)

Primary bile acids are those bile acids synthesized in the liver from cholesterol. In humans, CA and CDCA are the two predominant primary bile acids. In mice, CDCA is efficiently converted to β -muricholic acid (β MCA) and the two major predominant species are CA and β MCA. (Russell, 2003; Stanimirov et al., 2012; Thomas et al., 2008b) Secondary bile acids are generated by the

dehydroxylation of primary bile acids by gut bacteria. This dehydroxylation converts CA into deoxycholic acid (DCA) and CDCA into lithocholic acid (LCA). (Fiorucci et al., 2009)

Functions of bile acids

The main functions of bile acids are traditionally considered to be facilitating the dietary absorption of lipids, cholesterol, and fat-soluble vitamins and providing a pathway of cholesterol catabolism. In addition to being digestive surfactants, bile acids also function as powerful endocrine signals, which can be modulated by both the total amount of bile acids in the blood and by which bile acid species are more prevalent. (Hylemon et al., 2009)

The two most well-described bile acid receptors are farnesoid X receptor (FXR) and TGR5 (also known as GP-BAR1 or M-BAR). (Duboc et al., 2014) FXR is a nuclear receptor expressed in the liver, small intestine, adrenal gland, and kidney. Hepatic FXR signaling decreases gluconeogenesis, triglyceride synthesis and secretion, and is a pathway of negative feedback decreasing bile acid synthesis. FXR triggers the transcription of the small heterodimer partner (SHP), which suppresses transcription of key enzymes of bile acid synthesis and SREBP-1c, a master regulator of lipogenesis. (Kerr et al., 2002; Teodoro et al., 2011)

TGR5 is a G-protein coupled receptor, expressed in a wide array of tissues. (Keitel et al., 2007) In brown adipose tissue, TGR5 activation stimulates type 2 iodothyronine deiodinase, which activates thyroid hormone intracellularly and increases energy expenditure. (Pols et al., 2011) This increased energy expenditure appears to be mediated by the uncoupling proteins (UCP1, UCP3) that generate heat and dissipate energy by dissipating the mitochondrial proton gradient. In the intestinal L-cells, increased TGR5 stimulation leads to secretion of glucagon-like peptide-1 (GLP-1), an incretin that signals satiety in the hypothalamus and increases glucose-induced insulin secretion from the pancreas. (Katsuma et al., 2005; Reimann et al., 2008) In vivo studies with TGR5

stimulation by pharmacological and genetic techniques lead to improved glucose tolerance and liver and pancreas function in obese mice (Thomas et al., 2008a; Thomas et al., 2009), with a significant body weight reduction in mice fed a high fat diet. (Watanabe et al., 2006)

Other roles of bile acids include a role in lipid homeostasis via FXR and SHP mediated inhibition of SREBP-1c (Watanabe et al., 2004), glucose homeostasis via FXR repression of gluconeogenic genes and induction of glycogen synthesis (Nguyen and Bouscarel, 2008; Zhang et al., 2006c), and increased energy expenditure via TGR5 intracellular activation of thyroid hormone in brown adipose tissue (Watanabe et al., 2006). Bile acids are also reported to have immunomodulatory function via TGR5 in CD14-positive monocytes, alveolar macrophages and Kupffer cells (Calmus et al., 1992; Ichikawa et al., 2012), neuronal modulation via CDCA antagonism of the NMDA and GABA receptors (Schubring et al., 2012), and promote liver regeneration via FXR and TGR5 (Ding et al., 2015; Fan et al., 2015). Since enterohepatic circulation sends bile acids out to the gut lumen and back via subsequent reabsorption in the terminal ileum, the bile acids are exposed to the gut microbiome and deconjugated and hydrolyzed by the gut bacteria. In this way, it has been postulated that bile acids could function as a method of communication with and surveillance of the gut microbiome. (Groh et al., 1993; Nicholson et al., 2012; Ridlon et al., 2006)

Specific bile acid species fulfill these functions of bile acids with variable efficiency. The size, shape, and hydrophobicity of bile acids impact their ability to bind receptors, to emulsify fats, and to be transported in and out of cells. Thus, the balance between CA and CDCA exerts a strong influence over bile acid signaling because hydrophobic bile acids (eg. CDCA) are generally more potent activators of FXR than hydrophilic species (eg. muricholic acids). (Chiang, 2009a; Fiorucci et al., 2009; Makishima et al., 1999). Secondary bile acids, such as LCA and DCA, are more potent ligands of TGR5 than primary bile acids, CA and CDCA. (Chen et al., 2011) Cholic acid is much more efficient in stimulating dietary cholesterol absorption than CDCA and MCAs. (Reynier et al., 1981)

These differences in bile acid activity mean that difference in bile acid species commonly found in humans vs. mice is an important caveat to our model system. In humans, CDCA is a potent FXR agonist and one of the dominant bile acid species. In mice, CDCA is efficiently converted to muricholic acids (MCAs), which are weak FXR agonists.

1.4 Flavin-containing monooxygenase 3 and Trimethylamine N-oxide

TMAO Predicts Cardiovascular Risk

Recently, trimethylamine N-oxide (TMAO) has been identified as a predictor of cardiovascular disease. (Wang et al., 2011) This study found dose-dependent associations between fasting serum TMAO and presence of cardiovascular disease, peripheral artery disease, coronary artery disease, and history of myocardial infarction. Fasting plasma TMAO has since been reported to predict the risk of major adverse cardiovascular events in a 3 year study of 4007 patients. (Tang et al., 2013)

TMAO is a metabolite of dietary choline, an essential nutrient found in foods high in phosphatidylcholine, a.k.a. lecithin, such as red meat and eggs. (Koeth et al., 2013) Dietary choline is converted by gut bacteria into trimethylamine (TMA), which is efficiently absorbed from the gut and then oxidized in the liver by flavin-containing monooxygenase 3 (FMO3) into TMAO. In a 3-year prospective case-control study, fasted serum TMAO predicted the risk of major adverse cardiovascular events (death, myocardial infarction, and stroke). (Tang et al., 2013)

Flavin-containing Monooxygenase 3 (FMO3)

FMO3 efficiently oxidizes TMA to TMAO, and is the principal hepatic FMO isoform responsible for this conversion. Thus upregulation of hepatic FMO3 has been reported to increase serum TMAO and knockdown of hepatic *Fmo3* decreases serum TMAO.

FMOs metabolize numerous foreign chemicals, including drugs, pesticides, and dietary metabolites. In humans, defective FMO3 is unable to convert TMA to TMAO and leads to trimethylaminuria, also called fish-odor syndrome due to the secretion of odorous TMA in urine, in sweat, and on the breath. (Hernandez et al., 2003; Mitchell and Smith, 2001) Of the 5 isoforms of the FMO family, *Fmo3* is the most highly expressed in the liver and is the primary member responsible for the conversion of TMA to TMAO. Thus, serum TMAO has been reported to increase with upregulation of hepatic FMO3 and to decrease with knockdown of hepatic *Fmo3*. (Bennett et al., 2013) Another study of *Fmo3* knockdown in Western diet fed LDLR knockout mice found that a 90% decrease in FMO3 protein levels corresponded to a 50% decrease in plasma TMAO and decreased atherosclerotic lesion size at the aortic root. (Shih et al., 2015)

1.5 Mice as a Model Organism

The mouse, *Mus musculus*, is the most commonly used model organism in medical research. There are many powerful advantages to selecting mice as a model organism, including the ability to make targeted gene knockouts and their striking anatomic, physiologic and genetic similarity to humans. Since the use of mice as model organisms is so prevalent, there are a plethora of well-established and characterized mouse lines available. In pragmatic terms, mice are also a convenient choice because of their comparatively short generation time, relative ease with regards to breeding, and low cost maintenance compared to larger mammals.

Despite the powerful genetic tools available in mice, there are several caveats to bear in mind when using them to study bile acid metabolism and lipoprotein metabolism. The dominant bile acid species in humans are CA and CDCA, whereas the dominant bile acid species in mice are CA and MCAs. CDCA is a potent FXR agonist, whereas MCAs have much weaker affinity for FXR. (Russell, 2009)

With regards to the study of dyslipidemia and the development of atherosclerosis, the native lipoprotein profile of mice is very different from that of humans. Circulating cholesterol is carried in the plasma mainly in high density lipoprotein (HDL) particles in mice versus in low density lipoprotein (LDL) particles in humans. (Fernandez et al., 1999; Kapourchali et al., 2014; Xiangdong et al., 2011) This difference is likely because mice lack the cholesteryl ester transfer protein, (Barter et al., 2003) which transfers cholesteryl esters from HDL to apoB-containing proteins such as LDL and very low density lipoprotein (VLDL). Additionally, absorption of dietary cholesterol is also much lower in mice than in humans. (Carter et al., 1997)

1.6 Overview of the Dissertation

In this dissertation, I examine the role of FoxO1 in driving the metabolic dysfunction of insulin resistance that promotes the development of cardiovascular disease. In Chapter 2, I use mouse models of liver-specific knockout mouse models to show that ablation of FoxO1 from LIRKO mice results in complete normalization of glucose tolerance and hepatic glucose production and normalized expression of genes involved in cholesterol synthesis. I demonstrate that FoxO1 regulates the expression of cholesterogenic genes differently by cell autonomous and systemic pathways. I also demonstrate that FoxO1 tightly controls the expression of Cyp8b1 and synthesis of cholic acid, thus impacting dietary cholesterol absorption and intrahepatic cholesterol. In Chapter 3, I describe the regulation of Fmo3 by insulin and show that it is increased in mouse and human models of diabetes and obesity. I demonstrate that knockdown of Fmo3 suppresses FoxO1 via Srebp2 and that, in LIRKO mice, prevents the development of hypercholesterolemia and atherosclerosis. In the final chapter, I discuss my conclusions and discuss future directions regarding the role of hepatic FoxO1 as a driver of metabolic syndrome and cardiovascular disease.

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Chapter 2

Cyp8b1 Mediates Systemic Effects of Insulin and Hepatic FoxO1 on Cholesterol Metabolism

2.1 Abstract

The role of FoxO1 as a driver of hyperglycemia in insulin resistance is well-established, but its role in mediating lipid metabolism remains poorly understood. Here we use mouse models of liver-specific knockout of the insulin receptor and of FoxO1 to investigate the extent to which FoxO1 mediates lipid abnormalities that occur with hepatic insulin resistance. Ablation of hepatic FoxO1 in LIRKO mice restored gene expression of cholesterol synthesis genes. Further investigation revealed that FoxO1 differentially regulates the expression of cholesterologenic genes in *in vitro* cell autonomous versus *in vivo* non-cell autonomous settings. Here, we show that FoxO1 exerts extremely sensitive control over the expression of Cyp8b1 and through Cyp8b1 is able to control cholic acid synthesis, cholesterol absorption and intrahepatic cholesterol. In the absence of FoxO1, Cyp8b1, proportion of cholic acid, and cholesterol absorption were all decreased. Finally, we demonstrated that FoxO1 suppression of cholesterol synthesis genes *in vivo* is mediated through this pathway by re-introducing Cyp8b1 via adenovirus. Importantly, Cyp8b1 was also found to be increased in mouse and human models of metabolic syndrome. Taken together, these data indicate that hepatic FoxO1 regulates systemic cholesterol metabolism via Cyp8b1 and dietary cholesterol absorption.

2.2 Introduction

Insulin resistance is central to metabolic syndrome and to diabetes. Clinical features of metabolic syndrome include insulin resistance with associated hyperinsulinemia, impaired glucose tolerance, impaired insulin-mediated glucose uptake, dyslipidemia (hypertriglyceridemia and low plasma HDL), and hypertension. (Melmed and Williams, 2011).

There is ample evidence that FoxO1 is suppressed by insulin and a key regulator of hepatic glucose production, and a driver of hyperglycemia in insulin resistance. Multiple mouse models of insulin resistance – hepatic, whole body or otherwise – have shown improvement if not outright complete rescue of hyperglycemia, glucose intolerance and overall glucose homeostasis upon ablation of hepatic FoxO1, making FoxO1 an attractive therapeutic target. (Dong et al., 2008; Lu et al., 2012; Matsumoto et al., 2007; O-Sullivan et al.) While the role of hepatic FoxO1 in disturbing glucose homeostasis in insulin resistance is well-established, FoxO1's role in mediating lipid metabolism remains unclear.

Cholesterol, triglyceride and bile acid metabolism are also profoundly disturbed in metabolic syndrome. Free fatty acid release from adipose tissue is increased, leading to increased triglyceride synthesis in the liver and increased and secretion of triglyceride-rich very low density lipoproteins (VLDL). (Eckel et al., 2005) Bile acids are also reported to be altered in people with insulin resistance. One study found that DCA is increased in people with diabetes, whereas CDCA and CA are decreased. (Cariou et al., 2011) Another group found that human insulin resistance is associated with increased plasma levels of 12 α -hydroxylated bile acids (predominantly composed of CA and DCA). (Haeusler et al., 2013) Cholesterol absorption from the gut has been reported to be decreased 45% in obese subjects with diabetes compared to non-diabetic obese subjects. (Simonen et al., 2002)

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FoxO1 regulation of lipogenesis is unclear (see Table 1.2), though it is known to target microsomal triglyceride transfer protein (MTTP, which recruits triglycerides to developing VLDL particles) (Kamagate et al., 2008), and apolipoprotein C-III (Apo-CIII, which is secreted to the blood and inhibits lipoprotein lipase). (Altomonte et al., 2004) Together, these two genes act to increase plasma levels of triglycerides. FoxO1 has also been reported to regulate bile acids by inducing transcription of Cyp7a1 (Shin and Osborne, 2009) and Cyp8b1 (Haeusler et al., 2012).

Here, we investigate the role of hepatic FoxO1 in regulating lipid metabolism and in mediating lipid dysregulation in the setting of insulin resistance. Using liver-specific knockout mouse models of the insulin receptor and of FoxO1, we confirm that loss of FoxO1 rescues glucose intolerance in LIRKO mice and restores glucose production to normal levels. We demonstrate that hepatic FoxO1 suppresses cholesterol synthesis gene expression *in vivo* and induces it *in vitro*. Finally, we identify Cyp8b1 as a highly-regulated target of FoxO1, through which FoxO1 controls cholic acid synthesis, cholesterol absorption, and intrahepatic cholesterol. Overall, our studies show that insulin regulates cholesterol metabolism via cell autonomous and systemic non-cell autonomous mechanisms.

2.3 Methods

Animals

Generation of mice with floxed (flanked by *loxP*) insulin receptor (Michael et al., 2000) and FoxO1 (Matsumoto et al., 2007) have been described previously. To generate liver-specific knockout mice, floxed mice were intercrossed with *Alb-Cre* transgenic mice that express the Cre recombinase cDNA under the rat albumin promoter. All mice were maintained on a C57BL/6 background. Except where otherwise specified, we used male mice in all experiments, fed standard chow ad libitum, and sacrificed in the non-fasted state at 2pm.

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Adenovirus injection

Adenoviruses carrying either Cyp8b1 or GFP (ViraQuest) were administered to 2-3 month old mice via tail vein injection at a dose of 3×10^9 PFU/mouse. Plasma was collected 2 days after injection after a four-hour fast, and the mice were sacrificed 1 week after injection.

Blood chemistry and metabolic analysis

Plasma analytes.

Plasma samples collected from mice either after 4 hours of fasting, or at the time of sacrifice in the non-fed state. Plasma samples were analyzed using the following assays: insulin, ELISA (Crystal Chem); c-peptide, ELISA (Alpco); leptin, ELISA (Crystal Chem); total cholesterol or triglycerides, colorimetric assay (Thermo Scientific).

Triglyceride secretion.

Mice were fasted for 4 hours and then administered 500mg/Kg body weight Triton WR1339 via retro-orbital injection. Blood samples were collected at 0, 90, and 180 minutes after injection, and then analyzed for triglyceride concentrations using the Thermo Scientific Infinity Triglycerides Reagent.

Glucose tolerance tests.

Mice were fasted for 14 hours overnight and then administered 1g glucose per kg body weight by intraperitoneal injection. Blood glucose levels were monitored via tail nick at 0, 15, 30, 60, 90 and 120 minutes after glucose injection.

Fractional cholesterol absorption

Fractional cholesterol absorption measurements were performed as previously described. (Temel et al., 2005) 13-week old male mice were gavaged 0.025 μ Ci of [4- 14 C]cholesterol (Perkin Elmer) and 0.75 μ Ci of [22,23- 3 H]sitosterol (American Radiolabeled Chemicals) dissolved in 50 μ L of

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soybean oil. Each mouse was then individually housed in a cage with a wire bottom, with free access to regular chow and water. After 72 hours, the feces were collected and desiccated in a vacuum oven overnight at 80°C, then homogenized using a mortar and pestle. Duplicate aliquots of the feces were saponified in 2mL 95% ethanol and 200µL 50% KOH, while heated to 65°C for 1 hour with periodic vortexing. Neutral lipids were extracted by adding hexane and deionized water, vortexing, and centrifuging at 2000g for 10 minutes. The upper hexane phase was transferred to a scintillation vial and dried overnight at room temperature. Ultima Gold Cocktail (Perkin Elmer) was added to the vials and the [¹⁴C]cholesterol and [³H]sitosterol counts were measured using a Liquid Scintillation Analyzer Tri-Carb 2900TR (Perkin Elmer). Percentage cholesterol absorption was calculated using the following equation:

$$\frac{(^{14}\text{C}/^3\text{H dose ratio} - ^{14}\text{C}/^3\text{H feces ratio})}{^{14}\text{C}/^3\text{H dose ratio}} \times 100\%$$

HPLC bile acid analysis

Conjugated bile acids were analyzed by high-performance liquid chromatography. An octadecylsilane column (RP C-18, Beckman Instruments, Fullerton, CA) was used with isocratic elution at 0.75 mL/min. The eluting solution was composed of a mixture of methanol and 0.01 M KH₂PO₄ (67%, v/v), adjusted to an apparent pH of 5.3 with H₃PO₄. Bile acids were quantified by measuring their absorbance at 204 nm. Bile acid amidates (taurine and glycine) have similar extinction coefficients. Bile acids were tentatively identified by matching their relative retention times with those of known standards.

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Hepatic lipid measurements

Livers were homogenized in 50 mM NaCl and lipid was extracted with chloroform and methanol (2:1). Lipid extract was then analyzed by a colorimetric assay per manufacturers' instructions (Thermo Scientific).

Cell culture

HEK293 and FAO cells were grown in RPMI medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin and maintained at 37°C in a humidified atmosphere with 5% CO₂.

Primary hepatocyte isolation

Primary mouse hepatocytes were isolated from 2-3 month old mice as previously described.(LeCluyse, 2001) After isolation, cells were suspended in William's E medium (Life Technologies) containing penicillin-streptomycin, 100nM glutamine, and 10% fetal bovine serum in wells coated with rat tail collagen I (BD Biosciences) for 3-6 hours. Cells were then washed twice with PBS and incubated overnight in M199 medium supplemented with 100nM dexamethasone, 100nM triiodothyronine, 1nM insulin, and penicillin-streptomycin. The next day, cells were washed twice with PBS and incubated for 6 hours in M199 medium supplemented with 100nM dexamethasone, 100nM triiodothyronine and penicillin-streptomycin, with or without the addition of 100nM insulin or 1μM GW4064 (Tocris). Cells were then harvested and RNA was prepared for qPCR analysis.

Adenoviruses

Control adenovirus and adenovirus expressing constitutively-active human FoxO1 were obtained from Terry Unterman and amplified in HEK293A cells. (Zhang et al., 2006a) As previously described, the recombinant adenoviruses expressed GFP and human FoxO1 where Thr-24, Ser-256, and Ser-319 were replaced by the nonphosphorylatable alanine.

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Adenovirus over-expressing mouse Cyp8b1 on a CMV promoter was purchased from Vector Biolabs and then amplified by ViraQuest. Control adenovirus containing eGFP on a CMV promoter was purchased from ViraQuest.

Gene expression analysis / Real-time PCR

RNA was isolate from frozen livers using the RNEasy kit (Qiagen). RNA from primary hepatocytes was isolated with Trizol (Life technologies). cDNA was synthesized using a reverse transcription kit (Applied Biosystems), then diluted ten-fold for real-time PCR analysis with SYBR green reagents (Thermo Scientific). Primer sequences can be found in Table 2.1.

Table 2.1 Sequences of qPCR primers.

Species	Gene	Forward Primer	Reverse Primer
Mouse	<i>18s</i>	GTAACCCGTTGAACCCCAT	CCATCCAATCGGTAGTAGCG
	<i>Acc</i>	TGACAGACTGATCGCAGAGAAAG	TGGAGAGCCCCACACACA
	<i>ApoC3</i>	ACATGGAACAAGCCTCCAAG	TGGTTGGTCCTCAGGGTTAG
	<i>Cyp27a1</i>	GGAGGATTGCAGAAGTGGAG	TGCGGGACACAGTCTTTACTT
	<i>Cyp51</i>	AGCTGTACGCAGACCTGGAT	ACGCCCGTCCTTGTATGTAG
	<i>Cyp7a1</i>	GTCCGGATATCAAGGATGCA	AGCAACTAAACAACCTGCCAGTACTA
	<i>Cyp7b1</i>	GAGGTTCTGAGGCTGTGCTC	TCCCTCCTTTGAAAAACGTG
	<i>Cyp8b1</i>	GGACAGCCTATCCTTGGTGA	GGCCCCAGTAGGGAGTAGAC
	<i>Fasn</i>	GCTGCGGAAACTTCAGGAAAT	AGAGACGTGTCACTCCTGGACTT
	<i>Fdps</i>	CCGACCTTTCCCGTCACA	ATGGAGATGGGCGAGTTCTTC
	<i>FoxO1</i>	AGGAACTGAGGAGCAGTCCA	ATGATTCCCAATGGCACAGT
	<i>G6pc</i>	TCTGTCCCGGATCTACCTTG	GTAGAATCCAAGCGCGAAAC
	<i>Gk</i>	AAGCTGCACCCGAGCTTC	CCAGCATGCAAGCCTTCTT
	<i>Hmgcr</i>	AGCCGAAGCAGCACATGAT	CTTGTGGAATGCCTTGTGATTG
	<i>Igfbp1</i>	CTGCCAAACTGCAACAAGAA	GACCCAGGGATTTCTTTCC
	<i>Mtp</i>	TACCCGTTCTTGGTCTGCAT	CTGTTGATGAGGGGGAGT
	<i>Pck1</i>	ATCCCAACTCGAGATTCTGC	CATGGCTGCTCCTACAAACA
	<i>Pgc1a</i>	ACAGCTTTCTGGGTGGATTG	TCTGTGAGAACCGCTAGCAA
	<i>Scd1</i>	CATCATTCTCATGGTCCTGCT	CCCAGTCGTACACGTCATTTT
	<i>Sqs</i>	CCAACTCAATGGGTCTGTTCT	TGGCTTAGCAAAGTCTTGCAACT
	<i>Srebp1c</i>	GGCCCGGAAGTCACTGT	GGAGCCATGGATTGCACATT
	<i>Srebp2</i>	CTCCTGGGTGAGACCTACCA	ATGCTTGGTGTCTGAGTGG
	<i>Tbp</i>	ACCCTTCACCAATGACTCCTATG	TGACTGCAGCAAATCGCTTGG

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Species	Gene	Forward Primer	Reverse Primer
RAT	<i>36b4</i>	TTCCCACTGGCTGAAAAGGT	CGCAGCCGCAAATGC
	<i>Fdps</i>	CAGCTGCATTTGTTGTCCTG	TTGATGGGGAGAAGGAACAC
	<i>G6pc</i>	CTACCTTGCGGCTCACTTTC	ATCCAAGTGCGAAACCAAAC
	<i>Hmgcr</i>	GATGCTCAAGCTGCCTTGTT	TTGTGTGTGGCACTGTGATG

Table 2.1 (Continued)

Liver lysate extraction and western blotting

Tissue samples were lysed in RIPA buffer. 40ug of lysate were loaded onto SDS-PAGE gels and transferred onto a PVDF membrane (Thermo Scientific). Blots were blocked in SuperBlock buffer (Thermo Scientific), incubated overnight with primary antibody and detected with secondary antibody conjugated with HRP. Antibody complexes were visualized by enhanced chemiluminescence using X-ray film.

Statistical analyses

Human studies

For clinical parameters, p-value was determined by Mann-Whitney test for continuous variables and by χ^2 test for categorical variables. For gene expression studies, the expression values were not normally distributed. Therefore, a log transformation was performed after which the mean and SEM were calculated, and a t-test was performed. Data are presented as the back-transformed values of the mean and SEM.

Mouse Studies

Sample sizes were based on standard lab protocols and previous studies, rather than power calculations, as the effect sizes were not known a priori. Animals were randomized to control and experimental groups. For Figures 2-4 and 8 showing Ctrl, LFKO, LIRKO, and LDKO in vivo data, statistically significant effects of hepatic insulin receptor knockout and hepatic FoxO1 knockout were identified using a two-way ANOVA model with interaction. In each ANOVA model, the

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significance of the interaction term was assessed first, and, if the interaction was not statistically significant ($p > 0.05$), the significance of the main effects was assessed. Similarly, two-way ANOVA models with interaction were used to test statistically significant effects of genotype and ASO knockdown treatment in Figure 7a. The differences between groups in Figure 9 were assessed by a two-tailed unequal variance Student's t-test. Bars and error bars correspond to the mean \pm SEM. Representative results of two to four independent experiments are shown.

Cell Culture Studies

Gene expression studies were performed with triplicate wells. Average or representative results of two to five independent experiments are shown.

2.4 Results

Knockout of hepatic FoxO1 in LIRKO mouse has profound impact glucose and cholesterol disturbances, but not triglyceride metabolism

To investigate the extent to which FoxO1 mediates the profound metabolic dysregulation in LIRKO mice, we generated mice with liver-specific knockout of the insulin receptor (LIRKO), FoxO1 (LFKO), or both (LDKO), using a Cre-loxP strategy. To achieve hepatocyte-specific knockout, the floxed mice were crossed with albumin-Cre mice. All mice were maintained on a C57/Bl6 background. InsR and FoxO1 knockout were confirmed by protein immunoblot of liver lysates (see Figure 2.1)

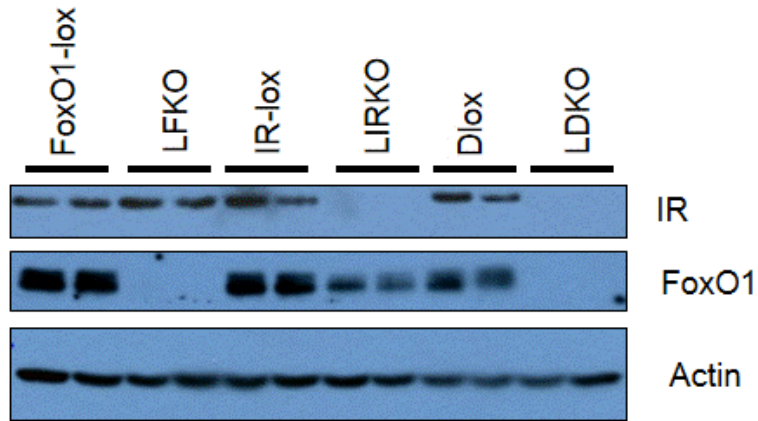


Figure 2.1 Validation of liver knockout by Western blot

When subjected to intraperitoneal glucose tolerance testing, 6-8-week-old male LIRKO mice exhibited pronounced glucose intolerance compared to controls. By contrast, this phenotype was completely rescued in the LDKO mice, which were indistinguishable from control mice (see Figure 2.2a). The defect in glucose tolerance in LIRKO mice is thought to be due to failure of the insulin-insensitive livers to shut off hepatic glucose production. (Michael et al., 2000) When we measured hepatic gene expression, the expression of Pck1 and G6pc, whose gene products catalyze the rate-limiting steps of gluconeogenesis, and Pgc1 α , a transcriptional coactivator that also promotes gluconeogenesis, were all significantly increased more than 2-fold in LIRKO mice and rescued in LDKO mice (see Figure 2.2b). Igfbp1 is uninvolved in hepatic gluconeogenesis but is a transcriptional target of FoxO1 and thus a useful marker. Igfbp1 expression was increased more than 20-fold in LIRKO mice and rescued in LDKO mice.

Consistent with previous reports, we measured profound hyperinsulinemia in LIRKO mice. Plasma insulin after a 4 hour fast was more than 5-fold higher in LIRKO mice than Ctrl mice and c-peptide, which can be used to estimate insulin secretion, was increased more than 2-fold in LIRKO mice than in Ctrl mice. Despite the complete absence of insulin clearance by the liver, insulin secretion and plasma insulin levels were normal in LDKO mice (see Figure 2.2c-d).

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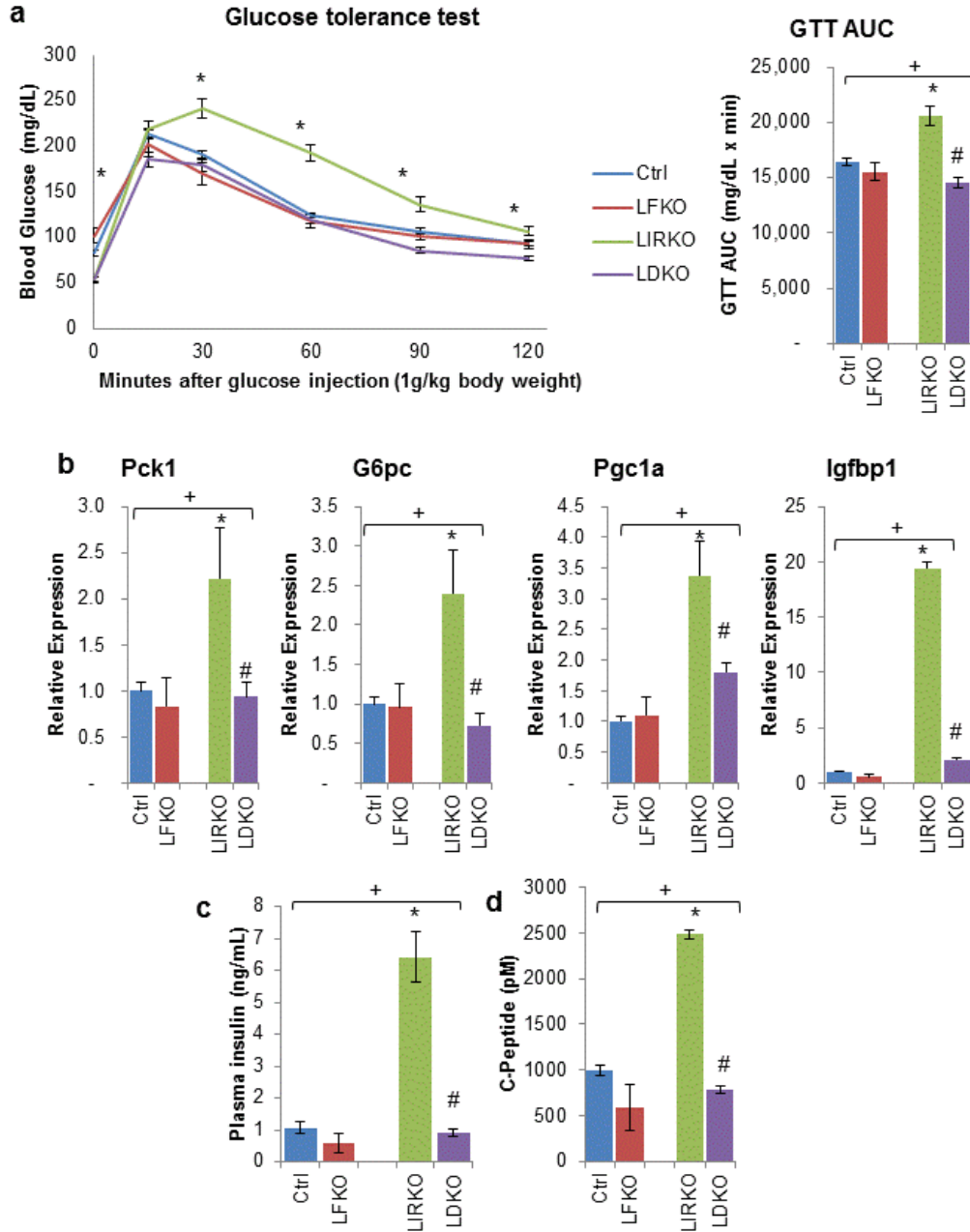


Figure 2.2 Glucose intolerance, hepatic glucose production and hyperinsulinemia in the LIRKO mouse is rescued by knockout of hepatic FoxO1

(a) Glucose tolerance test (right panel is area under the curve) performed in 6-8 week old male mice (n≥8) (b) Hepatic gene expression of gluconeogenic genes, Pck1, G6pc, and Pgc1a and Igfbp1, a target of FoxO1, in 8-10 week old male mice sacrificed in the non-fasted state. All results normalized to Tbp. (n>5, *p<0.05) (c) Plasma insulin levels and (d) plasma c-peptide level in 7-9 week old male mice after a 4 hour fast. Statistically significant effects of hepatic insulin receptor knockout and hepatic FoxO1 knockout were identified using a two-way ANOVA model with interaction. Statistical effects. *p < 0.05 for hepatic insulin receptor knockout effect; #p < 0.05 for hepatic FoxO1 knockout effect; +p < 0.05 for their interaction.

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Consistent with previous reports (Biddinger et al., 2008; Michael et al., 2000), the LIRKO mice in our studies exhibited decreased plasma triglycerides from 49 ± 3 mg/dL in Ctrl mice to 19 ± 1 mg/dL in LIRKO mice, as well as decreased triglyceride secretion (see Figure 2.3a-b). Triglyceride secretion was assayed by measuring plasma triglycerides 0, 90, and 180 minutes after injection of tyloxapol, an inhibitor of lipoprotein lipase. Knockout of hepatic FoxO1 alone had no impact on either plasma triglycerides or triglyceride secretion, but LDKO mice had partial rescue of triglyceride secretion and normalization of plasma triglycerides compared to LIRKO mice.

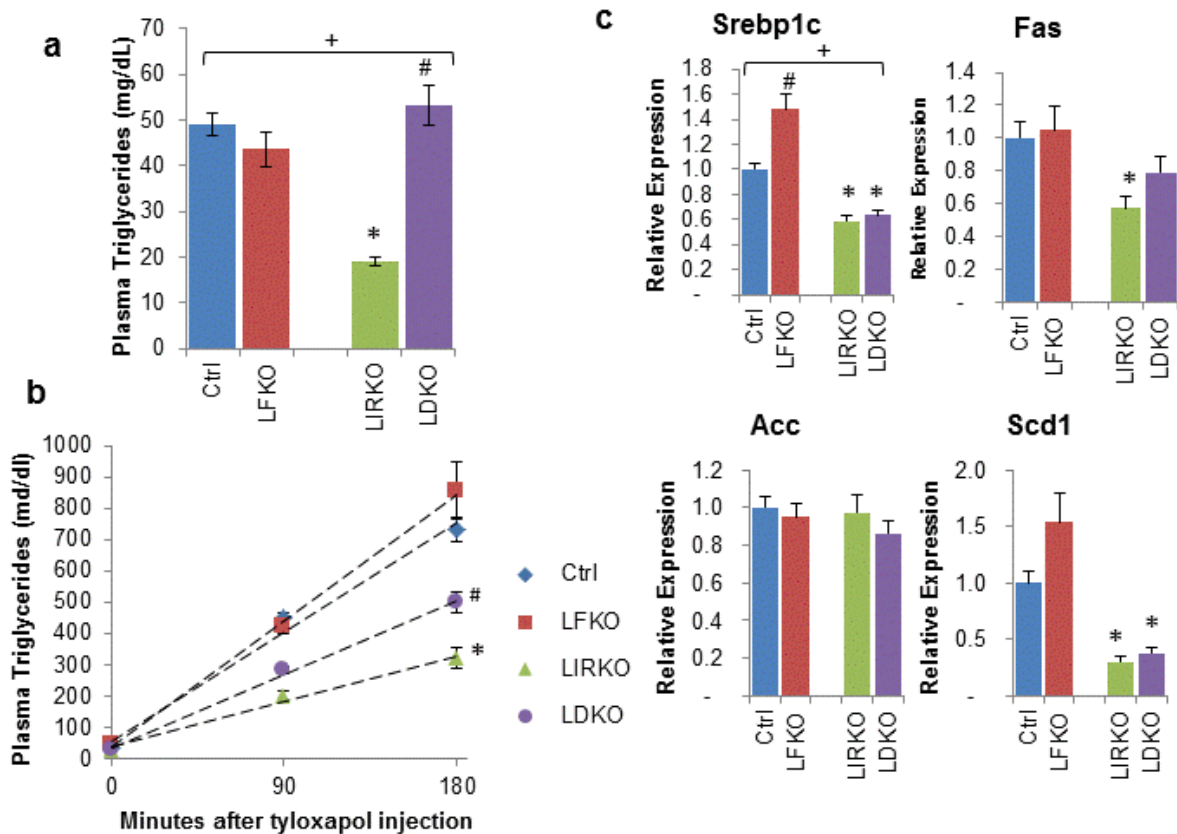


Figure 2.3 Hepatic ablation of FoxO1 does not rescue lipogenic gene expression in the LIRKO liver

(a) Plasma triglycerides measured in 6-8 week old male mice after a 4 hour fast. (b) Triglyceride secretion measured in Ctrl, LFKO, LIRKO, and LDKO mice. Mice were injected with tyloxapol, a lipoprotein lipase inhibitor, and then plasma samples collected at 0, 90, and 180 minutes. * $p < 0.05$ between Ctrl and LIRKO mice, # $p < 0.05$ between LIRKO and LDKO mice. (c) Hepatic gene expression of Srebp1c and its lipogenic targets measured in 8-10 week old male mice sacrificed in the non-fasted state. Results are normalized to Tbp. * $p < 0.05$ for hepatic insulin receptor knockout effect; # $p < 0.05$ for hepatic FoxO1 knockout effect; + $p < 0.05$ for their interaction.

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In LFKO mice, liver expression of lipogenic genes was inconsistently elevated (see Figure 2.3c). This is similar to the findings of other groups which have reported no change in Srebp1c and its targets in the setting of liver FoxO1 knockout, but have reported increases in lipogenic gene expression when liver FoxO1 knockout is in the setting of western type diet or whole body Ldlr knockout. (Haeusler et al., 2012)

When measuring hepatic gene expression of Srebp2 and genes of cholesterol synthesis, we found expression to be suppressed in the livers LIRKO mice by up to 80%, and restored to normal levels in LDKO mice (see Figure 2.4b). Indeed, while LFKO measurements were not statistically significant, expression of cholesterologenic genes consistently trended upward compared to controls. Taken together, these data imply that the effect of hepatic FoxO1 activity on cholesterol synthesis in vivo was suppressive.

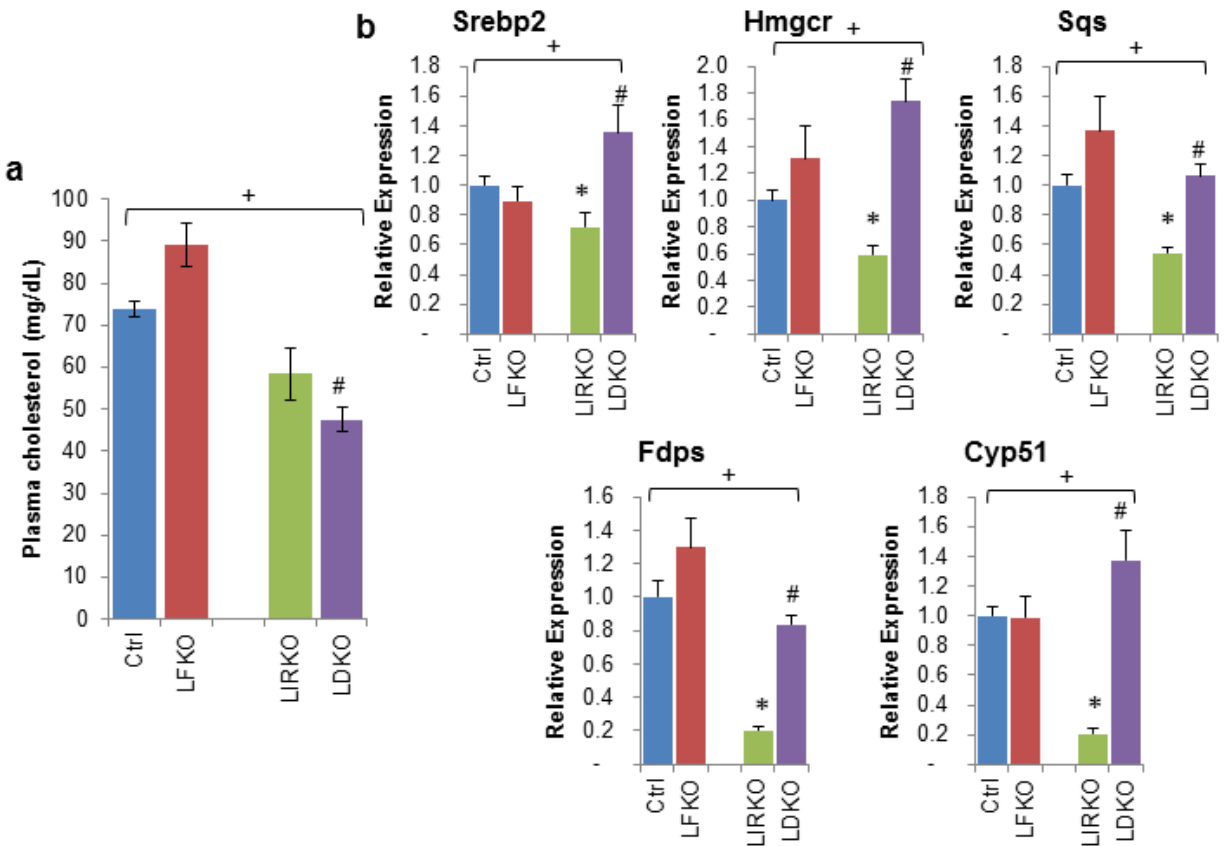


Figure 2.4 Hepatic FoxO1 suppresses expression of cholesterol synthesis genes in vivo

(a) Plasma cholesterol measured in 6-8 week old male mice after a 4 hour fast. (b) Hepatic gene expression of Srebp2 and its cholesterologenic targets measured in 8-10 week old male mice sacrificed in the non-fasted state. Results are normalized to Tbp. *p < 0.05 for hepatic insulin receptor knockout effect; #p < 0.05 for hepatic FoxO1 knockout effect; +p < 0.05 for their interaction.

FoxO1 upregulates expression of cholesterol synthesis genes in vitro

To probe whether the effect of FoxO1 in suppressing expression of cholesterol synthesis genes in the liver was cell autonomous, we investigated hepatocytes in vitro with decreased and increased FoxO1 levels. Primary mouse hepatocytes were harvested from 2-3 month old female FoxO1-lox and LFKO mice and gene expression measured by qPCR. Expression of Pck1, G6pc and Pgc1 α , known targets of FoxO1, was suppressed in the FoxO1 knockout hepatocytes. Expression of cholesterol synthesis genes was decreased in hepatocytes lacking FoxO1 (see Figure 2.5a).

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FAO hepatoma cells were transfected with adenovirus carrying either LacZ, or a constitutively-active FoxO1. Expression of Pck1, G6pc, and Igfbp1, known targets of FoxO1 were appropriately increased with over-expression of constitutively active FoxO1. Expression of Hmgcr and Fdps were also increased with FoxO1 overexpression (see Figure 2.5b). Taken together, these results indicate that FoxO1 up-regulates expression of cholesterol synthesis gene in vitro. FoxO1 therefore regulates the expression of cholesterol synthesis genes in both a cell autonomous and an extrahepatic pathway.

FoxO1 exerts exquisitely sensitive control over Cyp8b1 expression

Similar to previous reports, we found that Cyp8b1 expression is decreased in the livers of mice that lack FoxO1. (Haeusler et al., 2012) To investigate the strength of FoxO1's regulation of Cyp8b1 expression, we used in vitro models with FoxO1 knockout and FoxO1 over-expression. FAO cells with FoxO1 over-expression showed strong up-regulation of Cyp8b1 (see Figure 2.6a). By contrast, primary mouse hepatocytes lacking FoxO1 (FoxO1-KO) showed 90% decreases in Cyp8b1 expression (see Figure 2.6b-c). Treatment with GW4064, a pharmacological FXR agonist, suppressed Cyp8b1 as expected in control (FoxO1-lox) hepatocytes, but was unable to do so in FoxO1 knockout hepatocytes. Thus, the expression of Cyp8b1 appears to be tied to the degree of FoxO1 activity.

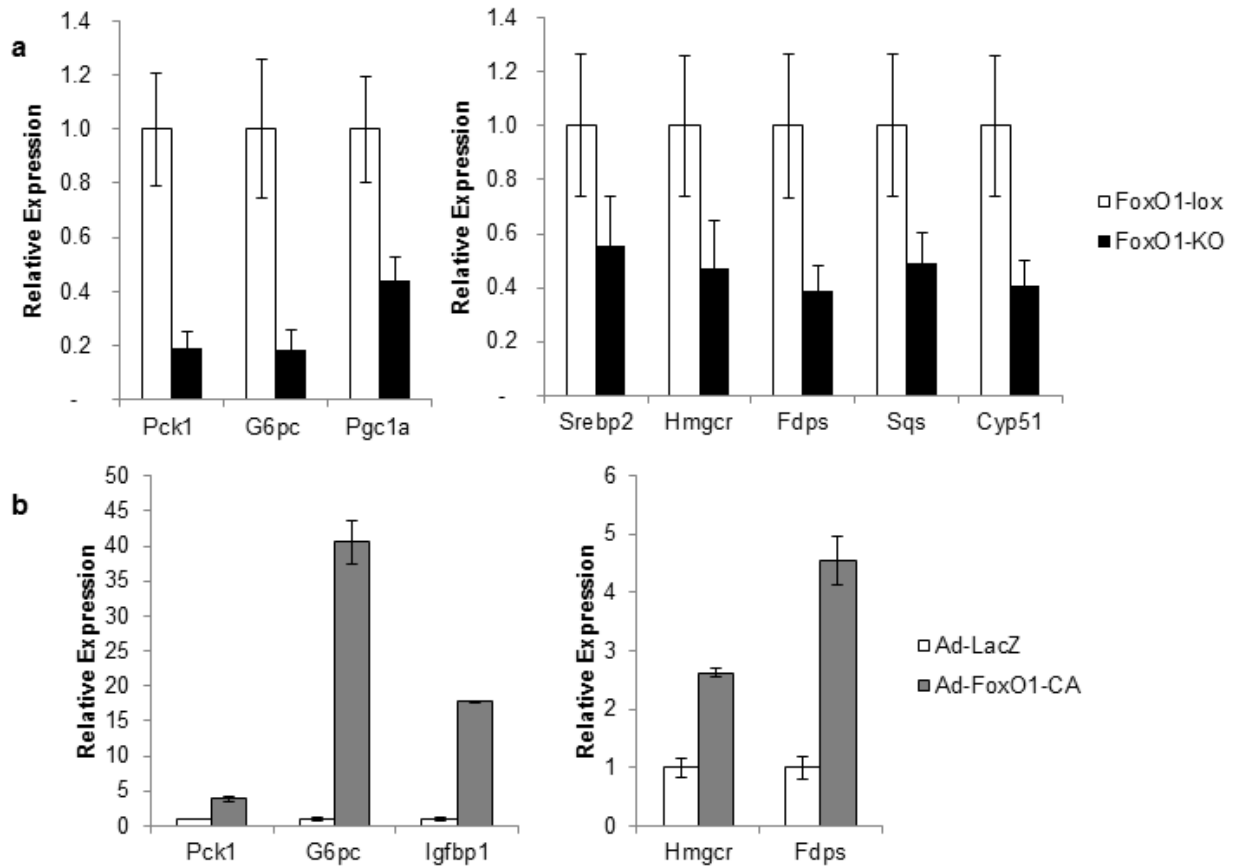


Figure 2.5 Hepatic FoxO1 increases expression of cholesterol synthesis genes in hepatocyte cell culture

(a) Gene expression measurements in mouse primary hepatocytes. Cholesterol synthesis genes are suppressed in the absence of FoxO1. Results are normalized to 18s, and represent data from three replicate experiments. (b) Gene expression measurements in rat FAO hepatoma cells. Cholesterol synthesis genes are induced by the over-expression of a constitutively active FoxO1. Results are normalized to 36b4.

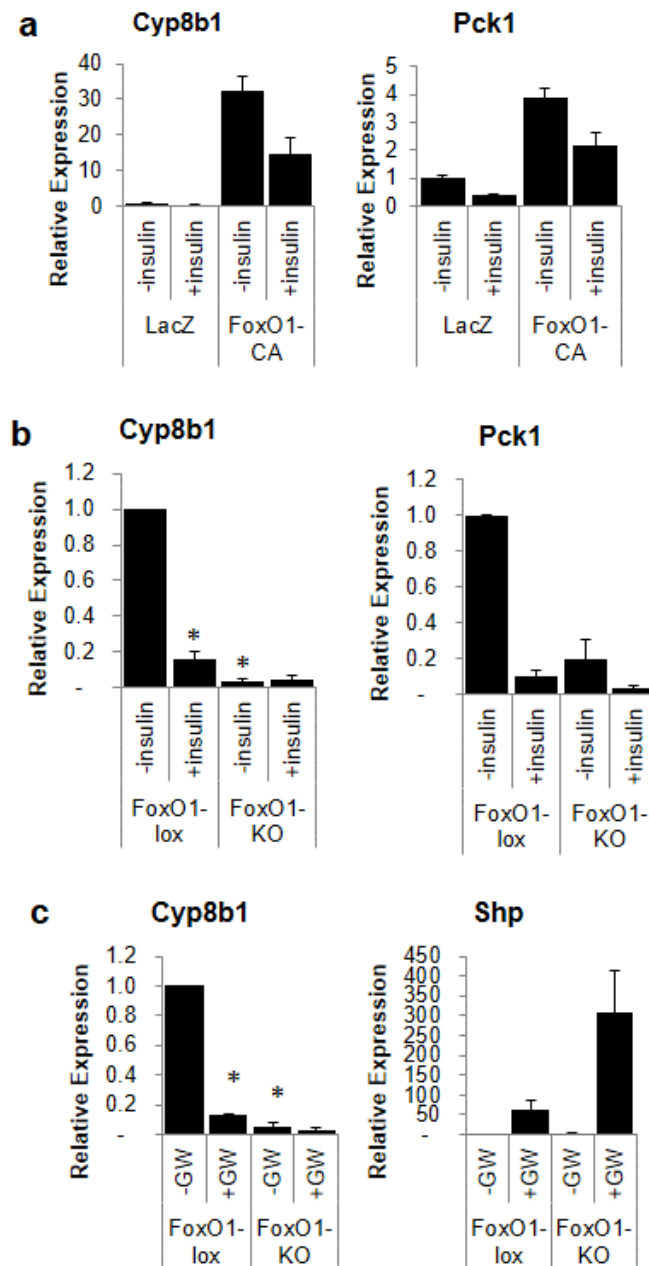


Figure 2.6 Hepatic FoxO1 is both necessary and sufficient for the expression of Cyp8b1

(a) Gene expression measurements in rat FAO hepatoma cells. Cyp8b1 is upregulated by the over-expression of a constitutively active FoxO1. Results are normalized to 36b4. (b-c) Gene expression measurements in mouse primary hepatocytes. Results are normalized to 18s, and represent data from three replicate experiments. (b) Cyp8b1 and Pck1 mRNA in control (FoxO1-lox) and knockout (FoxO1-KO) hepatocytes treated for 6 hours with 0nM or 100nM of insulin. (c) Cyp8b1 and Shp mRNA in control (FoxO1-lox) and knockout (FoxO1-KO) hepatocytes treated for 6 hours with 0 μ M or 1 μ M of GW4064.

Cyp8b1 is increased in models of metabolic syndrome

Hepatic gene expression was measured in control B6 and in *ob/ob* mice treated with control or with FoxO1 antisense oligonucleotide for acute FoxO1 knockdown. Cyp8b1 was increased by 50% in *ob/ob* mice compared to control mice. In *ob/ob* mice, a 50% knockdown of FoxO1 was sufficient to cause a 40% decrease in hepatic Cyp8b1 mRNA (see Figure 2.7a). These data show that regulation of Cyp8b1 by FoxO1 occurs in a mouse model of metabolic syndrome, and does not require complete ablation of FoxO1.

Liver biopsies were taken from obese patients (see Figure 2.7b) and Cyp8b1 expression measured. Patients with metabolic syndrome were found to have 40% higher expression of Cyp8b1 (see Figure 2.7c). This data is an important confirmation that the regulation of Cyp8b1 by FoxO1 is maintained and relevant in humans.

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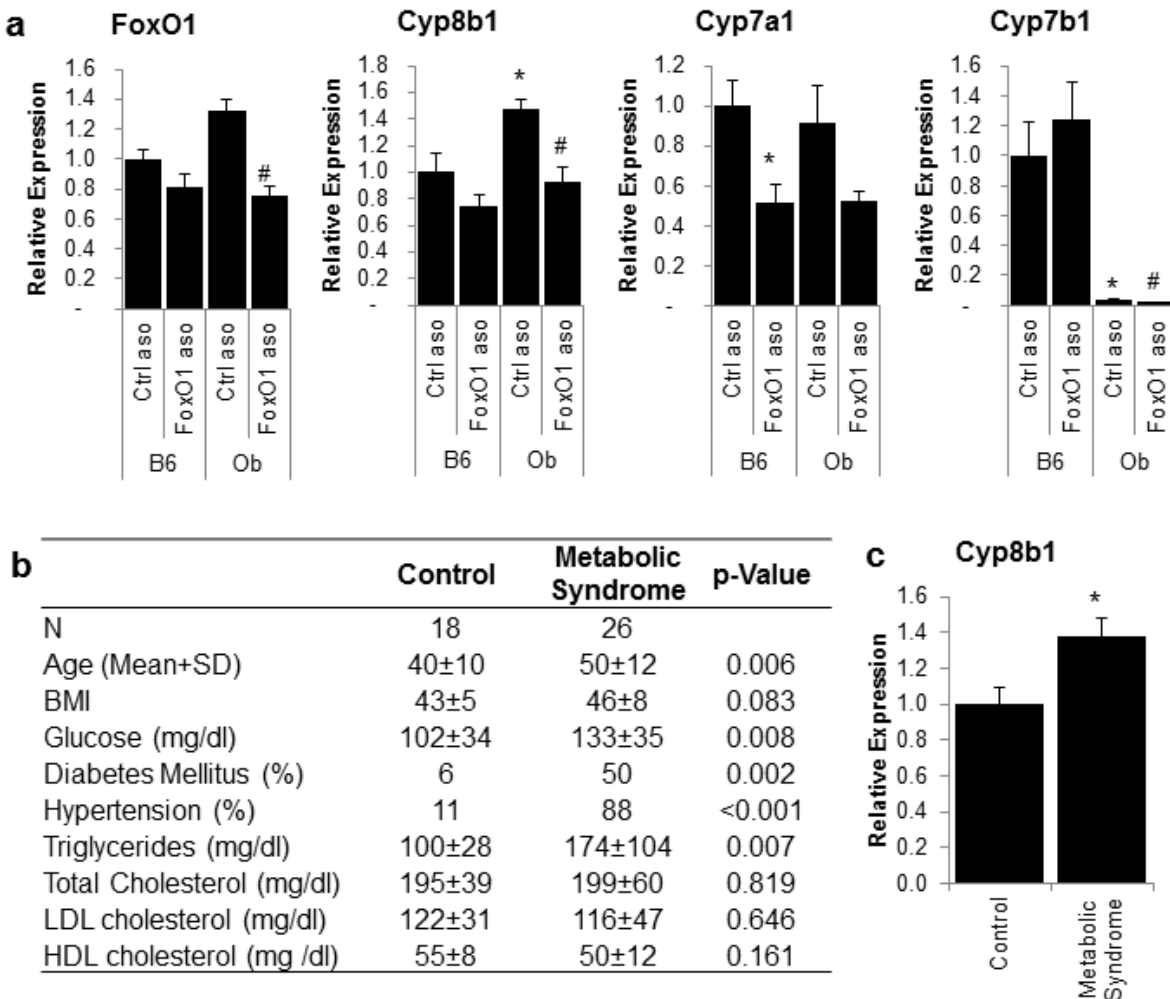


Figure 2.7 Cyp8b1 expression is increased in models of metabolic syndrome

(a) Hepatic gene expression in Control B6 and *ob/ob* mice treated with 5 weeks of ASO-mediated FoxO1 knockdown ($n=5$, $*p<0.05$ versus B6 Ctrl aso, $\#p<0.05$ versus Ob Ctrl aso). Statistically significant effects of genotype and ASO treatment were identified using a two-way ANOVA model with interaction. $*p < 0.05$ for genotype effect; $\#p < 0.05$ for ASO treatment effect; $+p < 0.05$ for their interaction. (b-c) Liver biopsies were taken from female obese patients undergoing gastric bypass surgery. (b) Biochemical and physiological data from these patients is shown. (c) FMO3 expression was measured by real-time PCR.

Via Cyp8b1, hepatic FoxO1 controls cholic acid synthesis, cholesterol absorption, and intrahepatic cholesterol

We examined the effects of FoxO1 regulation of Cyp8b1 expression. Hepatic gene expression measurements of Cyp8b1 revealed it to be decreased in LFKO and LDKO mice, as

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expected (see Figure 2.8a). HPLC analysis of the bile acids in bile collected from the gallbladder revealed increased proportion of cholic acid in LIRKO mice compared to controls and decreased proportion of cholic acid in both LFKO and LDKO mice (see Figure 2.8b). A canonical function of bile acids is the absorption of cholesterol and cholic acid is much more efficient in stimulating dietary cholesterol absorption than CDCA. (Reynier et al., 1981) We therefore proceeded to measure cholesterol absorption and found that cholesterol absorption was increased in LIRKO mice and decreased in both LFKO and LDKO mice compared to controls (see Figure 2.8c). These results exactly parallel the cholic acid measurements. After absorption, cholesterol is packaged with triglycerides and apolipoprotein B-48 into chylomicrons, the bulk of which are delivered to the liver. Hepatic cholesterol measurements were increased in LIRKO mice, with no change in LFKO and LDKO mice (see Figure 2.8d). The liver can sense increases in hepatic cholesterol and responds by decreasing hepatic cholesterol synthesis (Lu et al., 2001), which was observed in the LIRKO mice (see Figure 2.4b). Taken together, these data show that hepatic FoxO1, by virtue of its regulation of Cyp8b1 expression, governs cholic acid synthesis and subsequently cholesterol absorption. Thus we demonstrate a pathway by which hepatic FoxO1 regulates systemic cholesterol metabolism.

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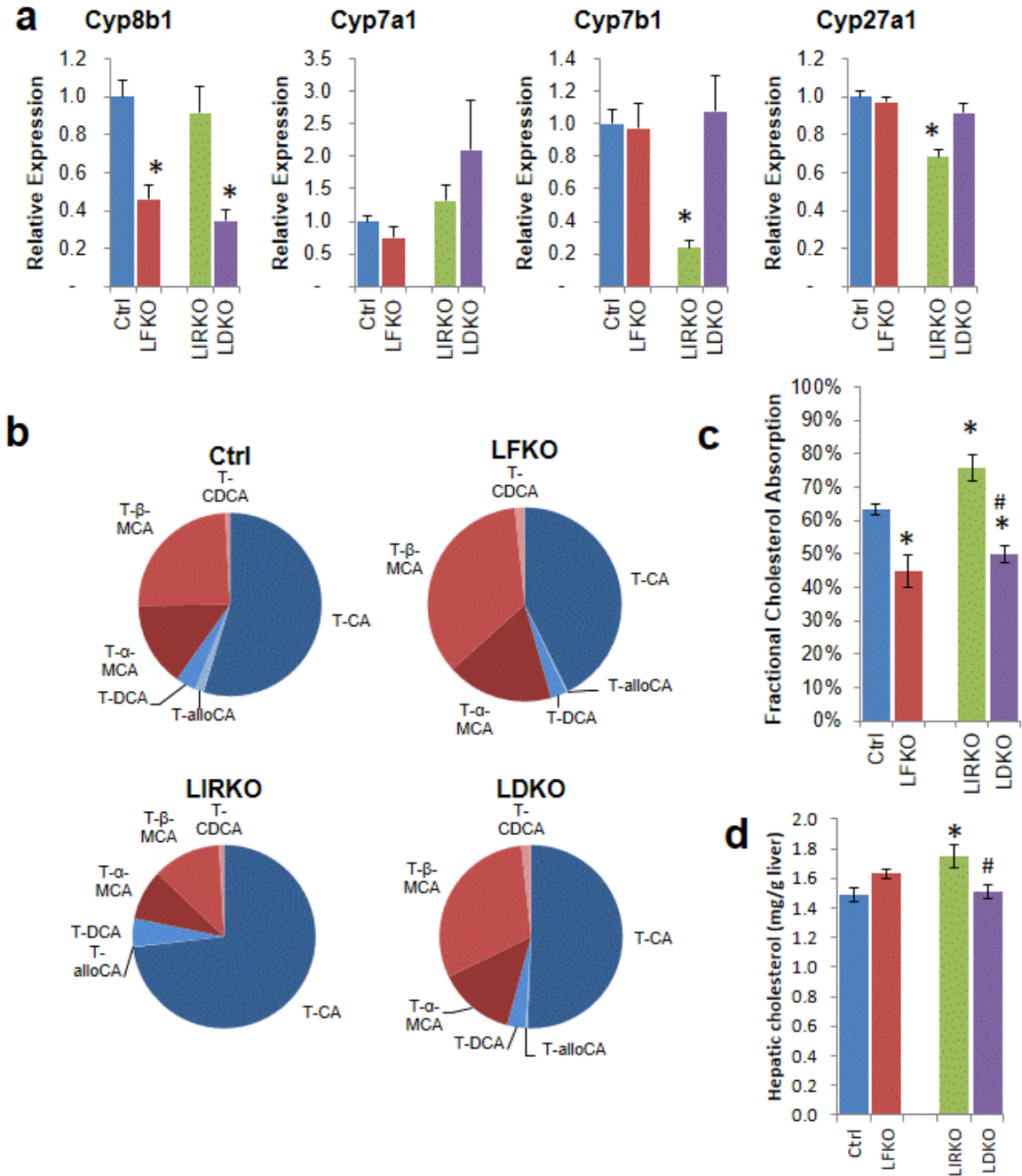


Figure 2.8 Hepatic FoxO1 controls cholic acid synthesis, cholesterol absorption, and intrahepatic cholesterol

(a) Hepatic gene expression of key bile acid synthesis enzymes in Control, LFKO, LIRKO and LDKO mice measured in 8-10 week old male mice sacrificed in the non-fasted state. Data are normalized to Tbp. (n>8, p<0.05 versus floxed control) (b) Distribution of bile acid species analyzed by HPLC. (c) Fractional cholesterol absorption (n=6, *p<0.05 versus Ctrl, #p<0.05 versus LIRKO) (d) Hepatic cholesterol measurement in 8-10 week old mice (n>5, *p<0.05 versus Ctrl, #p<0.05 versus LIRKO). *p < 0.05 for hepatic insulin receptor knockout effect; #p < 0.05 for hepatic FoxO1 knockout effect; +p < 0.05 for their interaction.

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Over-expression of Cyp8b1 in LDKO mice is sufficient to suppress expression of cholesterol synthesis genes and induce FGF15

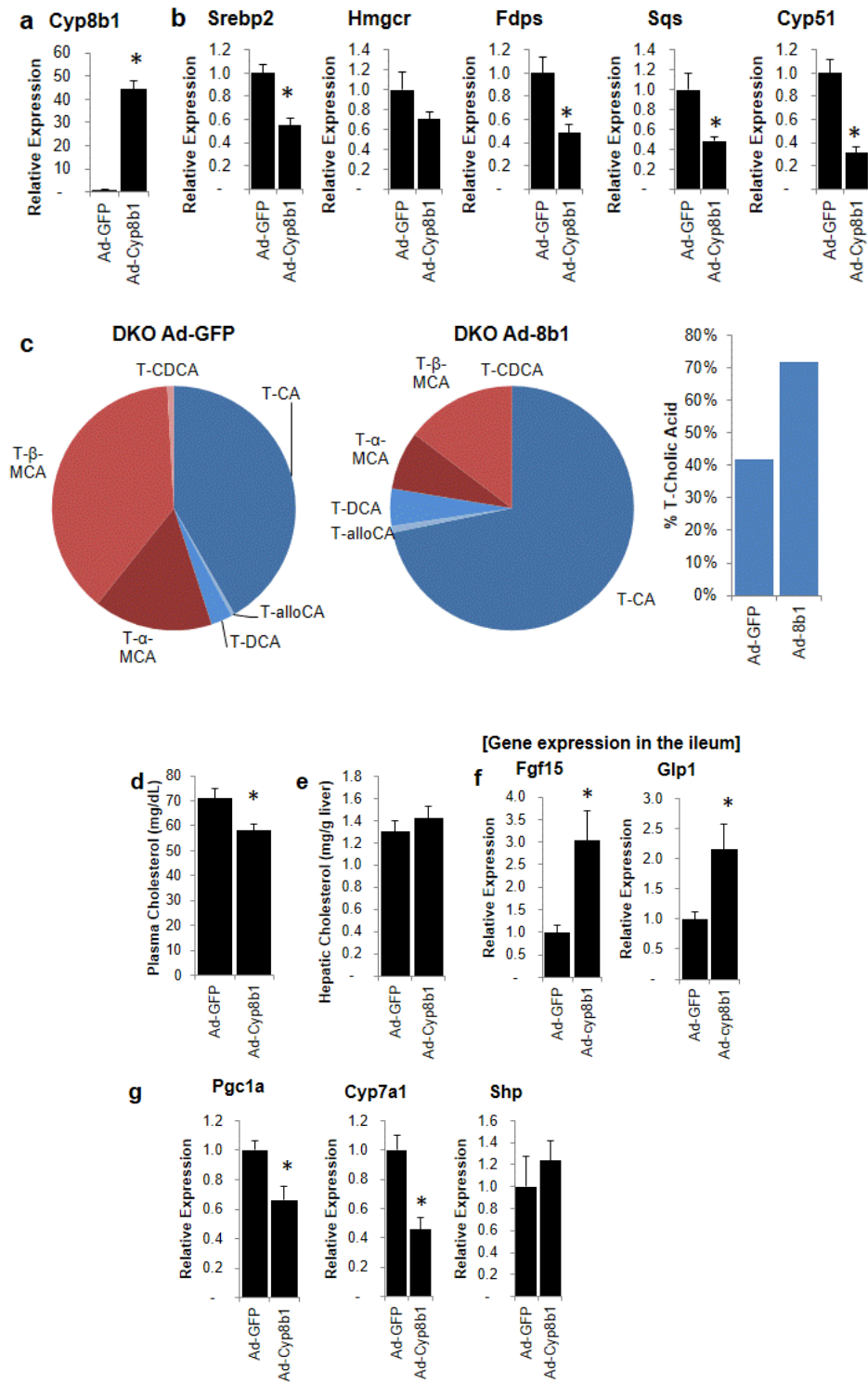
To investigate the degree to which FoxO1 regulation of cholesterol metabolism was mediated by Cyp8b1, we over-expressed Cyp8b1 in LDKO to test whether this would reverse the beneficial effects of hepatic FoxO1 ablation in LIRKO mice. Hepatic gene expression of Cyp8b1 was measured to confirm robust over-expression (see Figure 2.9a). Hepatic gene expression of cholesterologenic genes (Srebp2, Fdps, Sqs, and Cyp51) were consistently and significantly decreased with over-expression of Cyp8b1. Hmgcr expression was also decreased, though not significantly (see Figure 2.9b). Bile acids analysis showed an increase in the proportion of cholic acid from 40% of total bile acids to 70% (see Figure 2.9c). Hepatic cholesterol levels trended upwards with Cyp8b1 over-expression, but were not significantly increased (see Figure 2.9e). Gene expression in the ileum showed that the increased cholic acid levels robustly up-regulated Fgf15 and Glp1 (see Figure 2.9f). Pgc1 α and Cyp7a1 are both targets of negative regulation by Fgf15 and liver expression of both were significantly suppressed (see Figure 2.9g). Hepatic expression of Shp was unchanged (see Figure 2.9g). This indicates that the suppression of Cyp7a1 was due to Fgf15 signaling through FGFR4 and Pgc1 α , and not FXR and SHP.

Figure 2.9 Over-expression of Cyp8b1 in LDKO mice is sufficient to suppress cholesterol synthesis and induce FGF15

(a-b) Hepatic gene expression of in 2-3 month old male LDKO mice with one week of adenoviral delivery of GFP or Cyp8b1. Data are normalized to Tbp. (n≥5, p<0.05) (a) Hepatic gene expression of Cyp8b1 to validate over-expression. (b) Hepatic gene expression of cholesterol synthesis genes. (c) Distribution of bile acid species analyzed by HPLC. Percent CA versus total indicated. (d) Plasma cholesterol measured after 4 hour fast. (e) Hepatic cholesterol measurement. (f) Gene expression in the ileum of Fgf15 and Glp1, and hepatic gene expression of Pgc1a.

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Figure 2.9 (Continued)



2.5 Discussion

In this study, we examined the role of hepatic FoxO1 in mediating lipid dysregulation in insulin resistance. We generated mouse models of liver-specific knockout of the insulin receptor, FoxO1 or both. Similar to other mouse models of selective hepatic insulin resistance, ablation of hepatic FoxO1 in LIRKO mice was sufficient to normalize glucose tolerance, indicating a role for FoxO1 in insulin regulated glucose homeostasis, likely via both direct and indirect pathways. Loss of hepatic FoxO1 tended to induce lipogenic genes, consistent with similar mouse models, (Dong et al., 2008; Haeusler et al., 2012) but failed to do so in the absence of insulin signaling. Knockout of FoxO1 resulted in increased expression of cholesterol synthesis genes *in vivo*, particularly in the absence of insulin signaling, but the absence of FoxO1 decreased expression of cholesterol synthesis genes *in vitro*. The discrepancy in these results implies that insulin action through non-FoxO1 pathways is required for the induction of lipogenic gene expression. Thus, FoxO1 knockout in LIRKO mice restores glucose and cholesterol but not triglyceride metabolism.

Further investigation of the regulation of cholesterol synthesis gene expression revealed that FoxO1 regulates cholesterol synthesis through both cell autonomous and systemic effects. *In vivo*, expression of cholesterol synthesis was suppressed by hepatic FoxO1 and increased with FoxO1 knockout. By contrast, *in vitro*, FoxO1 was necessary and sufficient to drive cholesterologenic gene expression. Review of previous FoxO1 ChIP-Seq studies in hepatocytes did not reveal FoxO1 binding sites near cholesterol synthesis genes. (Shin et al., 2012)

We identified Cyp8b1 expression to be exquisitely sensitive to the action of FoxO1. We used gain of function and loss of function approaches and found FoxO1 to be both necessary and sufficient for Cyp8b1 expression. Compared to the canonical target genes of FoxO1, Cyp8b1 mRNA levels were much more responsive to FoxO1 knockout or over-expression.

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Our work demonstrates that Cyp8b1 mediates many effects of FoxO1. First, the Cyp8b1 gene product catalyzes the rate-limiting step in cholic acid synthesis. Increases in cholic acid promote cholesterol absorption from the intestine. To demonstrate that Cyp8b1 mediates the extra-hepatic effects of FoxO1, we over-expressed Cyp8b1 in LDKO mice. As predicted, the proportion of cholic acid in bile was increased and cholesterol synthesis gene expression was suppressed. Interestingly, we found that Cyp8b1 over-expression was sufficient to increase Fgf15 and Glp1 expression in the small intestine. Glp1 is an incretin that stimulates insulin secretion and inhibits glucagon release. (Baggio and Drucker, 2007) In mice, Fgf15 is secreted from the intestine, and binds the hepatic cell surface receptor FGFR4, where it then exerts negative feedback on Cyp7a1 and Cyp8b1 expression in a non-FXR non-SHP dependent manner, and via CREB acts to suppress Pgc1 α and gluconeogenesis. (Russell, 2009) The decreases in both Pgc1 α and Cyp7a1 without any changes in Shp expression indicate that these changes were not a result of hepatic FXR/SHP signaling but of the FGF15/FGFR4 axis.

Having found Cyp8b1 expression to be increased in the livers of humans with metabolic syndrome, we would therefore predict these people to have increased cholic acid and/or its derivative, deoxycholic acid, as well as increased expression of plasma FGF19, the human ortholog of FGF15. While this has not been extensively studied, one group measuring bile acids in the plasma of type 2 diabetes mellitus patients found decreased CDCA. Total bile acid content and CA also tended to decrease, but were not significant. (Brufau et al., 2010) While CA levels were also decreased, it appears that CA made up proportionally greater fraction of total bile acids in the diabetic patients.

In addition to decreasing cholic acid, we would expect inhibiting or knocking down Cyp8b1 in humans to increase the proportion of CDCA within the pool of bile acids. Through its potent activation of FXR, CDCA treatment has been shown to decrease plasma lipids in

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hypertriglyceridemic patients. (Bateson et al., 1978) Congruently, administration of bile acid sequestrants has more recently been reported to induce lipogenesis in an FXR-dependent manner. (Herrema et al., 2010)

Our work demonstrates a role for hepatic FoxO1 in regulating systemic cholesterol metabolism through Cyp8b1 and cholic acid signaling. This further highlights FoxO1 as a potential therapeutic target for inhibition or knockdown in the clinical setting. Additional, since Cyp8b1 is increased in humans with metabolic syndrome, these results suggest that Cyp8b1 inhibition or knockdown would also have a therapeutic benefit by decreasing cholesterol absorption.

2.6 Attribution of Experiments in Chapter 2

Contributors to this work include Sudha B. Biddinger, Mary E. Gearing, Jordan Grant, Mary E. Haas, Lee Hagey, Abhiruchi Mehta, Ji Miao, Dong-Ju Shin, David Vicent, and the Morbid Obesity Study Group. A.V.L. and S.B.B. designed experiments and reviewed results. J.M, D.S, M.E.H, M.E.G, A.M, and J.G assisted in mouse experiments. L.H. performed HPLC analysis of bile samples. D.V and the Morbid Obesity Study Group acquired and analyzed human liver biopsy specimens. A.V.L. performed all other experiments and measurements.

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Chapter 3

Flavin monooxygenase 3 as a potential player in diabetes-associated atherosclerosis

3.1 Abstract

Despite the well-documented association between insulin resistance and cardiovascular disease, the key targets of insulin relevant to the development of cardiovascular disease are not known. Here, using non-biased profiling methods, we identify the enzyme flavin monooxygenase 3 (*Fmo3*), to be a target of insulin. FMO3 produces trimethylamine N-oxide (TMAO), which has recently been suggested to promote atherosclerosis in mice and humans. We show that *FMO3* is suppressed by insulin in vitro, increased in obese/insulin resistant male mice, and increased in obese/insulin resistant humans. Knockdown of FMO3 in insulin resistant mice suppresses FoxO1, a central node for metabolic control, and entirely prevents the development of hyperglycemia, hyperlipidemia and atherosclerosis. Taken together, these data indicate that FMO3 is required for FoxO1 expression and the development of metabolic dysfunction.

3.2 Introduction

Obesity, metabolic syndrome and Type 2 diabetes are related disorders which have reached epidemic proportions in our society, with almost one in three adults meeting criteria for metabolic syndrome and 5-10% of the population with Type 2 diabetes. One of the major forms of morbidity and mortality associated with these disorders is cardiovascular disease (CVD). Diabetes increases

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the risk of cardiovascular disease by two- to four-fold and most individuals with diabetes ultimately die of cardiovascular disease. (Grundy et al., 1999; Isomaa et al., 2001) Importantly, the risk of CVD in diabetic patients remains high even after optimal treatment with statin drugs. (Cholesterol Treatment Trialists et al., 2010; Ginsberg, 2013) This indicates the urgent need for developing better treatments to prevent CVD in diabetic patients.

Our ability to develop new therapies has been hampered by our lack of understanding of the specific mechanisms by which diabetes promotes CVD. (Kahn et al., 2005) On the one hand, it is clear that defects in insulin action play a central role in the pathophysiology of diabetes. (Biddinger and Kahn, 2006) However, insulin is a pleiotropic anabolic hormone, with many targets. (Biddinger and Kahn, 2006) The goal of these studies was to identify novel targets of insulin action relevant to the development of diabetes-associated CVD.

3.3 Methods

Animal, diets and treatments

Animals were housed in a twelve-hour light/dark cycle (7 a.m. to 7 p.m). Unless otherwise indicated, mice were given standard chow and water ad libitum, and sacrificed in the non-fasted state, at 2 p.m. The type and length of the diet other than chow were indicated in the main text and figure legends. The Western diet (TD88137) and Paigen diets (TD09237) were obtained from Harlan Teklad. All procedures were approved by the Institutional Animal Care and Research Advisory Committee at Boston Children's Hospital.

LIRKO (Cre+/-, IR lox/lox) mice (Michael et al., 2000) and their littermate flox controls (Cre-/-, IR lox/lox) were maintained on a mixed genetic background including 129/sv, C57BL/6, FVB and DBA. Male ob/ob mice and their lean, wildtype C57BL/6J controls were purchased from Jackson Laboratories.

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For antisense oligonucleotides (ASO) experiments, four to six week old mice were administered chemically modified ASO (50 mg kg⁻¹ body weight) by intraperitoneal injection weekly for seven weeks, or sixteen weeks in the case of the atherosclerosis studies. Mice were sacrificed one day after the final dose. Control (ISIS-141923, 5'-CCTTCCCTGAAGGTTCCCTCC-3'), FM03-specific (ISIS-555847, 5'-TGGAAGCATT GCCTTTAAA-3') and insulin receptor-specific (ISIS 401145 5'-GTGTTTCATCATA GGTCCGTT-3') chimeric 20-mer phosphorothioate oligonucleotides containing 2'-O-methoxyethyl groups at positions 1 to 5 and 16 to 20 were diluted in normal saline before injection. Glucose tolerance and insulin tolerance tests were performed after five weeks of ASO administration, unless otherwise indicated. For streptozotocin (STZ) treatment, eight to nine week old male C57BL/6J mice were injected with STZ (180 mg kg⁻¹ body weight) or vehicle (0.1 M citric acid, pH = 4.2), and sacrificed seven days later. For the lovastatin/ezetimibe treatments, eight to ten week old male mice were given free access to powdered chow with or without supplementation of 0.1% lovastatin and 0.025% ezetimibe (both w/w), and sacrificed seven days later.

Glucose tolerance test (GTT), insulin tolerance test (ITT) and pyruvate tolerance test (PTT)

Mice were fasted (~16 hours for GTT and PTT and 4 hours for ITT) and then were intraperitoneally injected with 1 g/kg body weight glucose (GTT), 1 U/kg body weight insulin (ITT) or 2 g/kg body weight of sodium pyruvate (PTT). (Guo et al., 2009; Haas et al., 2012)

Phenotypic and biochemical characterization

Blood glucose was measured with a glucometer using whole blood from tail bleeds. Plasma total cholesterol (Thermo Scientific) and total triglycerides (Thermo Scientific) were measured using colorimetric assays per manufacturers' instruction. To measure hepatic lipids, livers were homogenized in 50 mM NaCl and lipid was extracted with chloroform and methanol (2:1) followed by a colorimetric assay per manufacturers' instruction. (Miao et al., 2014)

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Plasma metabolites were extracted with methanol followed by LC/MS analysis. TMAO $m/z = 76$ peaks were monitored and areas under the curve were calculated. (Roberts et al., 2012) Fast Protein Liquid Chromatography (FPLC) Analysis (Brown et al., 2008; Lee et al., 2004) was performed by the Lipid, Lipoprotein and Atherosclerosis Analysis Core Laboratory at Wake Forest University.

Microarray and metabolomics analysis

Total RNA (RNeasy, Qiagen) was isolated from the livers of non-fasted male mice (RNA from two to three mice were pooled for each chip) and hybridized to Affymetrix MG-U74A-v2 chips per manufacturers' instructions. Raw data was processed in R (www.r-project.org) using the open-source Bioconductor packages, *affy* (Gautier et al., 2004) and *limma*. (Wettenhall and Smyth, 2004) Samples were background corrected and normalized using robust multichip averaging (RMA). (Gautier et al., 2004) Adjusted p-values were calculated with the *limma* package by applying the Benjamini-Hochberg correction. Alternatively, metabolomics was performed (Rhee et al., 2011; Roberts et al., 2012) on individual livers harvested from male Flox and LIRKO mice; p-value was adjusted for multiple comparisons using a Bonferroni correction. Heat maps were generated in Excel for significantly altered genes or metabolites (adjusted p-value < 0.05) and were colored by \log_2 fold changes.

Gene expression analysis

Gene expression was measured using real-time PCR. Total RNA was isolated by Trizol (Life technologies) or RNeasy kit (Qiagen). cDNA was synthesized by a reverse transcription kit (Applied Biosystems). The resultant cDNA was diluted five- to ten-fold and used for real-time PCR analysis with SYBR green reagents (Thermo Scientific) in Applied Biosystems 7900 HT or 7000 instruments. Results were normalized to the house keeping genes 18S (*in vivo* studies) or 36B4 (*in vitro* studies).

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In some cases, gene expression data, after normalization to 18S, were expressed as a heat map made using GenePattern. (Reich et al., 2006) Primers sequences are listed in **Table 3.1**.

Table 3.1 Sequences of qPCR primers.

Species	Gene	Forward Primer	Reverse Primer
Mouse	<i>Fmo3</i>	GGAAC TTGCACTTTGCCTTC	TAGGAGATTGGGCTTTGCAC
	<i>Fmo1</i>	AAACAAGCATAGCGGGTTTG	ATCCGGTTTTGCGTTGATAG
	<i>Fmo2</i>	AGCTGTGGTCTTCGAGGATG	GGCAAGCTACACAAGCCTTT
	<i>Fmo4</i>	CGGAGCAGCTCATTA AAAAGG	CTGAGTGAGCTCGTCCATGT
	<i>Fmo5</i>	TGCCCTCACA AAGTGA AATG	GCTGGCTGTCCACATACCTT
	<i>FoxO1</i>	TCCCATCATGACAGAGCAGGATGA	CAGACTGGGCAGCGTAGACGC
	<i>Pck1</i>	ATCCCAACTCGAGATTCTGC	CATGGCTGCTCCTACAAACA
	<i>G6pc</i>	TGGCTTTTTCTTTCCTCGAA	TCGGAGACTGGTTCAACCTC
	<i>Igfbp1</i>	ATTAGCTGCAGCCCAACAGA	GACACACCAGCAGAGTCCAG
	<i>Srebp-2</i>	GCGTTCTGGAGACCATGGA	ACAAAGTTGCTCTGAAAACAAATCA
	<i>Ldlr</i>	GAGGAGCAGCCACATGGTAT	GCTCGTCTCTGTGGTCTTC
	<i>Hmgcr</i>	CTTGTGGAATGCC TTGTGATTG	AGCCGAAGCAGCACATGAT
	<i>Fdps</i>	ATGGAGATGGGGCAGTTCTTC	CCGACCTTTCCCGTCACA
	<i>Fdft1</i>	CCAACTCAATGGGTCTGTTCTT	TGGCTTAGCAAAGTCTTCCA ACT
	<i>Cyp51</i>	AGCTGTACGCAGACCTGGAT	ACGCCCGTCTTGTATGTAG
	<i>Srebp-1c</i>	GGCCCGGAAGTCACTGT	GGAGCCATGGATTGCACATT
	<i>Fasn</i>	GCTGCGGAAACTTCAGGAAAT	AGAGACGTGTCACTCCTGGACTT
	<i>Cyp27a1</i>	GGAGGATTGCAGAACTGGAG	TGCGGGACACAGTCTTTACTT
	<i>Cyp7a1</i>	GTCCGGATATTCAAGGATGCA	AGCAAATAAACAACCTGCCAGTACTA
	<i>Cyp8b1</i>	GGAACAGCCTATCCTTGGTGA	GGCCCCAGTAGGGAGTAGAC
	<i>Abca1</i>	CATCCTCTCCAGAGCAAAA	CCACATCCACA ACTGTCTGG
	<i>Abcg5</i>	TGCCATTCTTTAAAAATCC	GATGAACTGGACCCCTTGG
	<i>Abcg8</i>	GGGGCTGATGCAGATTCA	GTAGCTGATGCCGATGACAA
	<i>Bsep</i>	AAGGACAGCCACACCAACTC	CCAGAACATGACAAACGGAA
	<i>Oatp1</i>	TAATCGGGCCAATCTTC	ACTCCATAATGCCCTTGG
	<i>Ntcp</i>	TCCGTCGTAGATTCTTTGC	AGGGGGACATGAACCTCAG
	<i>Mrp2</i>	TCTGTGAGTGCAAGAGACAGGT	TCCAGGACCAAGAGATTTGC
	<i>Lxr</i>	ACTCAGTTACAACCGGAAGA	GAGCAA ACTCAGCATCATTGAG
	<i>Fxr</i>	CACGGTTGTAAATACAGACTAGATAG	TTGATTTAATTAGGCCAAAAGG
	<i>Shp</i>	CTGCCTGGAGTCTTTCTGGA	GGTACCAGGGCTCCAAGACT
	<i>36b4</i>	AGATTCCGGATATGCTGTTGGC	TCGGGTCTTAGACCAGTGTTT
	<i>18s</i>	GTAACCCGTTGAACCCATT	CCATCCAATCGGTAGTAGCG
	<i>Tat</i>	CACGGCTAGACACAGCTCAA	CTATCCAGTCGGGAGGAGGT

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Table 3.1 (Continued)

Species	Gene	Forward Primer	Reverse Primer
Rat	<i>38b4</i>	TTCCCACTGGCTGAAAAGGT	CGCAGCCGCAAATGC
	<i>Fmo3</i>	GTGTTTCCAGACTTCCC	ATACCACCAGTCAGAAAT
	<i>Fmo1</i>	GGTCCTGAAAGGTGCGACTA	CCAAATCCGCTATGCTTGTT
	<i>Fmo2</i>	CCTGGTGCCTCAGAACAAAT	CTTTGATGGCTCCGTACAGC
	<i>Fmo4</i>	CAGTGGAATAGCAGCACAGG	TTCAAAGGGTAGCCACCAAC
	<i>Fmo5</i>	TGAAGGTGAAAGGCAATGTG	TGTAGCCTGTGGCAAAGATG
	<i>Pck1</i>	ATGAAGTTTGATGCCCAAGG	ACCCCCATCACTTGTCTCAG
	<i>Srebp-1c</i>	GCGCTACCGTTCCTCTATCA	ACATCTGTGCCTCCTCCACT
	<i>Tat</i>	AGCTTCTCAAGTCCAATGC	TTCATCTCAAGTCCAATGC

Alternatively, for quantification miR-182 (mmu-miR-182), Taqman assays (Applied Biosystems) were performed on RNA samples prepared using Trizol and the expression was normalized to housekeeper U6 snRNA. The value of the control group was set to 1.

Construction of plasmids

Full length of mouse *Fmo3* cDNA was cloned into pcDNA3 vector by introducing KpnI and NotI restriction enzyme sites using PCR. Control shRNA against LacZ (GTTACGGCGACAAGTGC), shRNA against mouse FMO3 (#1: GCATTTACCAATC GGTCTTCA; #2 GCTGGGCAGCACAAGTAATAA; #3GCTTCCACAGCAGGGACTATA) and shRNA against SREBP-2 (GGACAACACACAATATCATTG) were constructed using the Block-it U6 system (Life Technologies).

Adenovirus

Control adenovirus and adenovirus expressing human SREBP-2 were purchased (Eton Biosciences) and amplified in 293A cells (Life Technologies). Adenovirus expressing the precursor form of mmu-miR-182 was generated using the AdEasy™ XL Adenoviral Vector System. The DNA sequence from 157-bp upstream through 224-bp downstream of the mouse pri-miR-182 sequence was amplified by PCR with primers containing NheI and XhoI restriction sites on either end. This

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fragment was cloned into the pGEM-T vector (Promega), sequenced, transferred into the pShuttle vector and subsequently cloned into the pAd-Easy-1 vector (Agilent).

Cell culture

Cells were maintained at 37°C in a 5% CO₂ mammalian cell culture incubator. H2.35 mouse hepatoma cells (ATCC) and 293A cells were maintained in DMEM media (25 mM glucose, Life Technologies) containing penicillin-streptomycin and 10% fetal bovine serum. Cells were tested every four months to ensure no mycoplasma contamination. Transient transfection was performed using lipofectamine 2000 (Life Technologies) when cells reach 70% confluency according to a protocol suggested by the manufacturer. Cells were then harvested for protein or RNA extraction 48 hours post transfection.

Primary hepatocyte studies

Primary rat hepatocytes were isolated from eight week old male Sprague-Dawley rats (Harlan). (Chen et al., 2004) After isolation, cells were suspended in William's E medium (Life Technologies) containing penicillin-streptomycin, 100 nM glutamine (medium A) and 10% fetal bovine serum and 1 X 10⁶ rat hepatocytes were placed on rat tail collagen I (BD Biosciences) coated 6-well plates. Four hours later, cells were washed twice with PBS and incubated overnight in medium A supplemented with 100 nM dexamethasone, 100 nM triiodothyronine and 1 nM insulin (fasting medium). Cells were then washed twice with PBS and incubated for six hours in either medium A containing 100 nM dexamethasone and 100 nM triiodothyronine with or without 100 nM insulin supplementation, or medium A containing 100 nM dexamethasone with or without 100 nM glucagon supplementation.

Similarly, male mouse hepatocytes were isolated, plated at a density of 0.5 X 10⁶ cells per well. Cells were incubated overnight in medium A without dexamethasone, washed, and then incubated for six hours with either vehicle (DMSO) or 100 nM dexamethasone.

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For adenoviral infection, rat hepatocytes were infected with 10 to 50 MOI adenovirus 4 hours after cells were placed on plates. 24 hours later, cells media were replaced with fresh media. Cells then were collected 48 hours post viral infection.

Western blotting

Nuclear and cytoplasmic extracts were prepared by using a commercial kit (Thermo Scientific) per the manufacturer's instruction. Alternatively, whole cell lysates were prepared from liver (Miao et al., 2014), primary mouse hepatocytes (Haas et al., 2012) and H2.35 cells. (Haas et al., 2012) 10 to 50 µg of lysates were loaded onto SDS-PAGE gels and transferred onto a PVDF membrane (Thermo Scientific). After blocking in SuperBlock buffer (Thermo Scientific), blots were incubated overnight with primary antibody (1:1000 to 2000 dilution). Secondary antibody conjugated with horseradish peroxidase (Thermo Scientific) and chemiluminescent ECL reagents (Thermo Scientific) were used to develop blots. ImageJ (NIH) was used to quantify densitometry of the bands on film. LDLR antibody was a kind gift from Dr. Alan Attie. (Dirlam et al., 1996)

Atherosclerosis

Animals were perfused with saline. The abdominal aortas were dissected free of adventitial fat, fixed with formalin and stained with Oil-Red-O. Aortas were then open longitudinally and pinned onto surfaces of black wax. Images of stained aortas were taken with a digital camera and lesion areas were quantified using computer-aided software (Image-Pro).

Human studies

Human studies were approved by the Ethics Committee of the Hospital Clínico San Carlos, and all subjects gave informed consent. Liver biopsies were obtained from morbidly obese patients undergoing bariatric surgery or control patients undergoing surgery for gastroesophageal reflux disease (4 patients), achalasia (1 patient), and cholelithiasis (2

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patients), during the years 2004-2009 at the Hospital Clínico San Carlos. The bariatric surgery patients and controls were post-hoc age and sex matched. Clinical and biochemical data taken at the time of surgery were obtained from the chart. Clinical and biochemical data were available for all patients undergoing bariatric surgery. For the controls, glucose and total cholesterol levels were available in 6 patients, triglycerides in 5 patients, and HDL and LDL in three patients. For gene expression, RNA was isolated (TRI Reagent, Sigma-Aldrich), cDNA was synthesized (High-Capacity cDNA Reverse Transcription Kit, Applied Biosystems), and FMO3 gene expression was measured (TaqMan human FMO3 Gene Expression Assay Hs00199368_m1 and human 18s rRNA as reference gene, Applied Biosystems). Expression was calculated via the $\Delta\Delta C_t$ method.

Statistical analysis

Human Studies

For clinical parameters, p-value was determined by Mann-Whitney test for continuous variables and by χ^2 test for categorical variables. For gene expression studies, the expression values were not normally distributed. Therefore, a log transformation was performed after which the mean and SEM were calculated, and a t-test was performed. Data are presented as the back-transformed values of the mean and SEM.

Mouse Studies

Sample sizes were based on standard lab protocols and previous studies, rather than power calculations, as the effect sizes were not known a priori. Animals were randomized to control and experimental groups. Atherosclerosis was measured in a blinded fashion. Animals whose body weights were two or more standard deviations lower than the average of their groups were excluded (one animal in the Flox control ASO group in (Figure 3.3)). Differences between groups were assessed by a two-tailed unequal variance Student's t-test. Bars and error bars correspond to

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the mean and SEM, respectively. Representative results of two to four independent experiments are shown.

In vitro Studies

Gene expression studies were performed with triplicate wells and immunoblotting experiments were performed using duplicate wells. Average or representative results of two to five independent experiments are shown.

3.4 Results

To search for novel targets of insulin without the bias of an *a priori* hypothesis, we performed metabolic and transcriptional profiling on the livers of chow-fed male Liver Insulin Receptor Knockout (LIRKO) mice and their littermate Flox controls. The livers of LIRKO mice are unable to respond to insulin, allowing us to identify the targets of insulin action on the liver *in vivo*. (Michael et al., 2000) LIRKO mice show hyperglycemia, hyperinsulinemia, and changes in lipid metabolism that are similar to individuals with mutations in the insulin receptor. (Biddinger et al., 2008a; Haas et al., 2012; Semple et al., 2009) Moreover, they are markedly susceptible to atherosclerosis. (Biddinger et al., 2008b)

Of the 175 metabolites profiled in the livers of LIRKO mice—including organic acids, bile acids, purines, pyrimidines, and other compounds—the metabolite that showed the greatest fold change in LIRKO versus Flox livers was TMAO (Figure 3.1a). In both humans and mice, TMAO concentrations correlate positively with CVD risk, and dietary supplementation with either TMAO or compounds that can be metabolized to TMAO increases atherosclerosis in mice. (Koeth et al., 2013) In parallel, microarray profiling revealed that the second most highly upregulated transcript in LIRKO livers was *Fmo3*, the gene encoding the enzyme that produces TMAO. (Bennett et al., 2013) (Figure 3.1b) This was surprising, given that *Fmo3* had previously been shown to be

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regulated primarily by sex hormones and bile acids. (Bennett et al., 2013) Taken together, these striking findings indicate that one of the most profound effects of insulin on the male mouse liver *in vivo* is to suppress *Fmo3* and TMAO.

Further studies in the livers of male LIRKO mice using real time PCR analysis confirmed that *Fmo3* was increased more than 1000-fold in the livers of LIRKO mice relative to their littermate Flox controls. (Figure 3.1c) In parallel, FMO3 protein was expressed robustly in LIRKO livers, but undetectable in Flox livers (Figure 3.1d)

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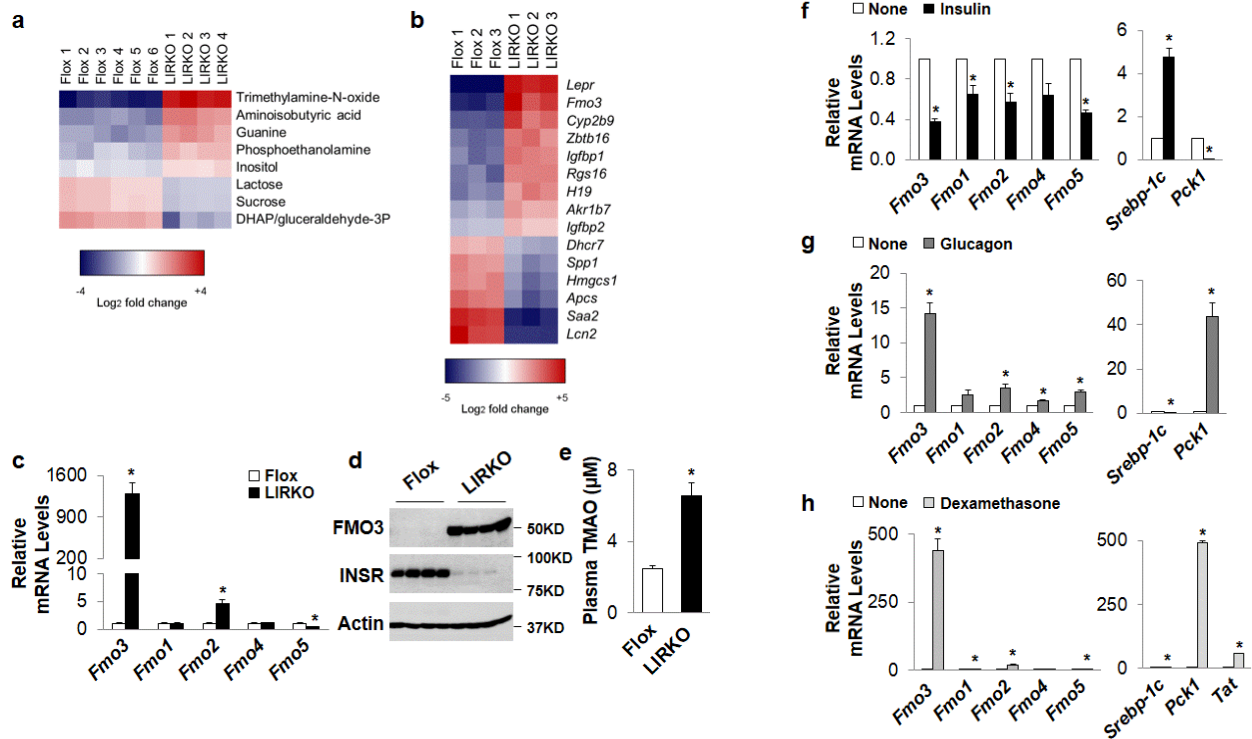


Figure 3.1 FMO3 is suppressed by insulin.

(a to e) The livers of two month old non-fasted male Flox and LIRKO mice were subjected to metabolic profiling (a) and microarray analysis (b); all metabolites and genes with an adjusted p-value less than 0.05 are shown. Alternatively, hepatic gene expression (c) was measured using real-time PCR and protein levels (d) were measured by western blotting whole cell lysates. Plasma TMAO levels (e) were measured using LC/MS. Data represent the mean and SEM; n = 5 - 11; * p < 0.05 (Student's t-test). (f to h) Gene expression was measured in primary hepatocytes from male rats (f, g) or mice (h) after six hours of stimulation with insulin (f), glucagon (g), or dexamethasone (h). Average or representative results of two to four independent experiments are shown; * p < 0.05 (Student's t-test); n = 3 replicates per condition.

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Finally, TMAO levels in the plasma were elevated approximately 2.5-fold (Figure 3.1e). Other *Fmo* genes were more modestly changed, with a three-fold increase in *Fmo2* and a 50% decrease in *Fmo5* (Figure 3.1c).

To determine whether insulin could directly suppress *Fmo3*, we treated primary rat hepatocytes with insulin (Figure 3.1f). Within six hours of treatment, insulin suppressed the gluconeogenic enzyme phosphoenolpyruvate carboxykinase (*Pck1*) by more than 90%, and induced sterol regulatory element binding protein-1c (*Srebp-1c*) more than four-fold, as expected.

Diabetes is associated not only with defects in insulin action, but also multiple other changes in the hormonal milieu. In particular, there is increased action of glucagon and glucocorticoids, hormones which antagonize insulin action. (Bujalska et al., 1997; Di Dalmazi et al., 2012; Li and Zhuo, 2013; Torrecilla et al., 2012). We found that glucagon increased *Fmo3* fourteen-fold, and dexamethasone (a synthetic glucocorticoid) increased *Fmo3* more than 400-fold (Figure 3.1g, h). Similarly, *Pck1* was induced 40-fold by glucagon, and more than 400-fold by dexamethasone (Figure 3.1g, h).

The other genes in the *Fmo* family showed similar, but more modest, responses to insulin, glucagon and dexamethasone. Insulin decreased expression of *Fmo1*, *Fmo2*, and *Fmo5* by 35-50%, whereas glucagon increased them two- to three-fold (Figure 3.1f, g). Dexamethasone increased *Fmo1* two-fold, *Fmo2* twenty-fold, and *Fmo5* six-fold (Figure 3.1h).

To determine whether FMO3 might play a role in the development of the diabetic phenotype, we used second generation antisense oligonucleotides (ASO) to knockdown FMO3 in LIRKO mice. We studied three groups: Flox mice treated with a control ASO, LIRKO mice treated with a control ASO, and LIRKO mice treated with an ASO against *Fmo3*. Each mouse was injected

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with 50 mg kg⁻¹ body weight of ASO by intraperitoneal injection each week. This ASO was previously shown to reduce hepatic *Fmo3* expression by 90% and plasma TMAO by almost 50%. (Bennett et al., 2013)

The FMO3 ASO decreased *Fmo3* mRNA by approximately 75% in LIRKO mice and markedly reduced FMO3 protein (Figure 3.2a, b); it also reduced *Fmo2* mRNA by 50% but it did not significantly change the other *Fmo* genes (data not shown). The FMO3 ASO did not alter body weight, nor did it produce hepatic or renal toxicity (data not shown). As expected, FMO3 ASO treatment normalized plasma TMAO levels in LIRKO mice (Figure 3.2c).

Figure 3.2 Knockdown of FMO3 suppresses FoxO1 via SREBP-2 and miR-182

(a to k) Four to six week old male Flox and LIRKO mice were treated with control (Con) or FMO3 ASO for seven weeks and sacrificed in the non-fasted state. Hepatic gene expression **(a, f, h, i, j, k)** was measured by real-time PCR, and in **(h)** expressed as a heat map, with each column representing data from a single mouse. The data are row-normalized with red and blue representing high and low expression, respectively. Protein levels were measured by western blotting whole cell lysates **(b, g)** or nuclear fractions **(i)**. **(c)** TMAO was measured in plasma collected at the time of sacrifice using LC/MS. Glucose **(d)** and insulin **(e)** tolerance testing were performed after five weeks of ASO treatment. Data represent the mean and SEM; $n = 5 - 7$; * $p < 0.05$ (Student's t-test) LIRKO versus Flox mice treated with the control ASO; # $p < 0.05$ (Student's t-test) control versus FMO3 ASO treatment of LIRKO mice. **(l to o)** Primary mouse hepatocytes were infected with control adenovirus or adenovirus expressing SREBP-2 **(l to n)** or miR-182 **(o)**. Alternatively, shRNA expression plasmids were transfected into H2.35 hepatoma cells **(p, q)**. Gene expression was measured by real-time PCR **(m, n, q)**. Data represent mean and SEM; * $p < 0.05$ (Student's t-test); $n = 3$ wells per condition; results are representative of three independent experiments. **(l, o, p)** Whole cell lysates were subjected to western blotting **(l, o, p)** and quantification **(o, p right panels)**. **(o, p)** Data represent the mean and SEM of three independent experiments; * $p < 0.05$ (Student's t-test). Representative images are shown in **l** and the left panels of **o, p**. **(r to u)** Eight to ten week old male LIRKO mice were fed a chow diet with or without supplementation of lovastatin and ezetimibe (L+E) for one week and euthanized in the non-fasted state. Hepatic gene expression **(r, s, u)** was measured by real-time PCR. Hepatic FoxO1 protein **(t)** was measured by western blotting whole cell lysates. Data represent the mean and SEM; $n = 5 - 7$; # $p < 0.05$ (Student's t-test).

Figure 3.2 (Continued)

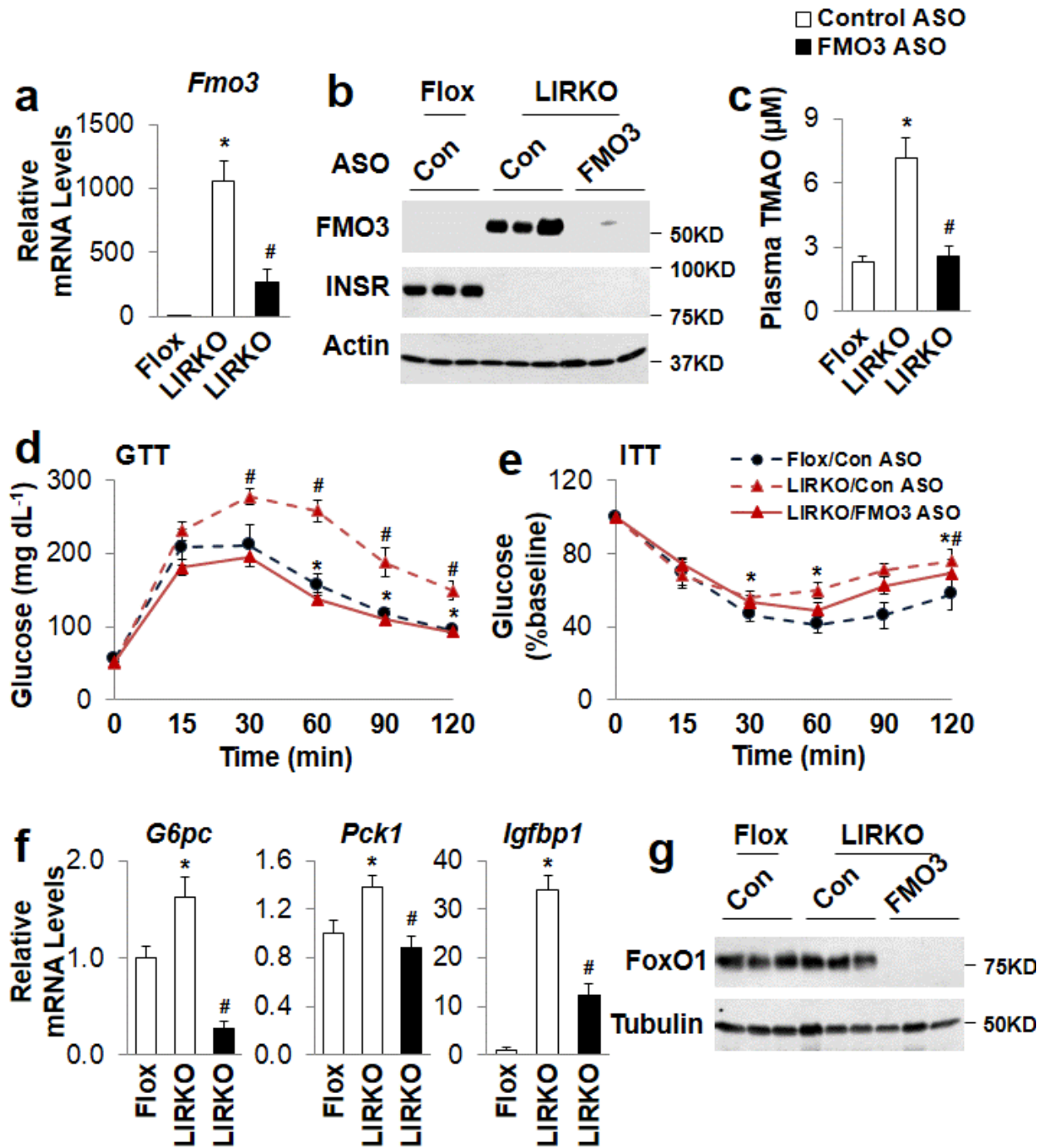


Figure 3.2 (Continued)

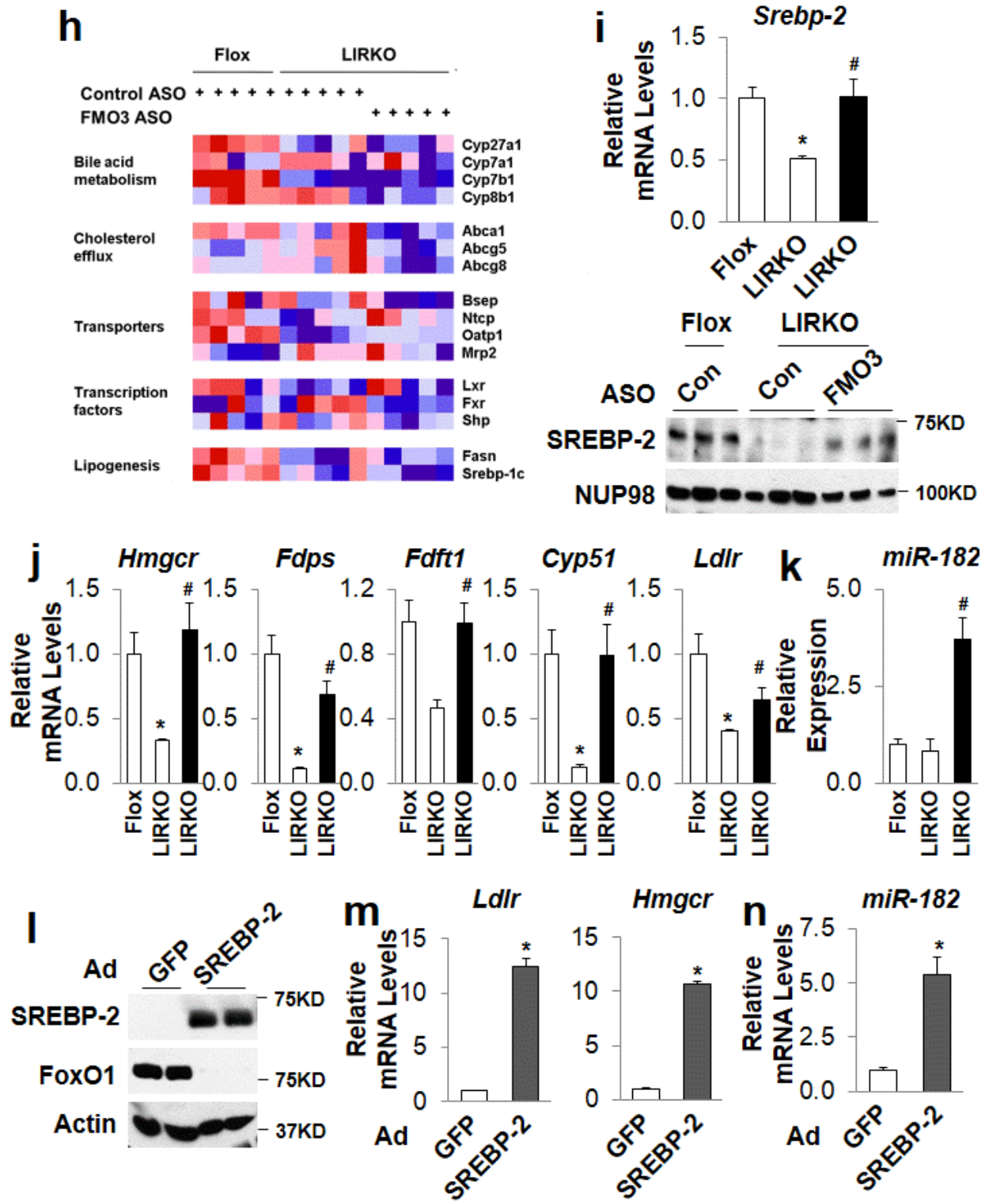
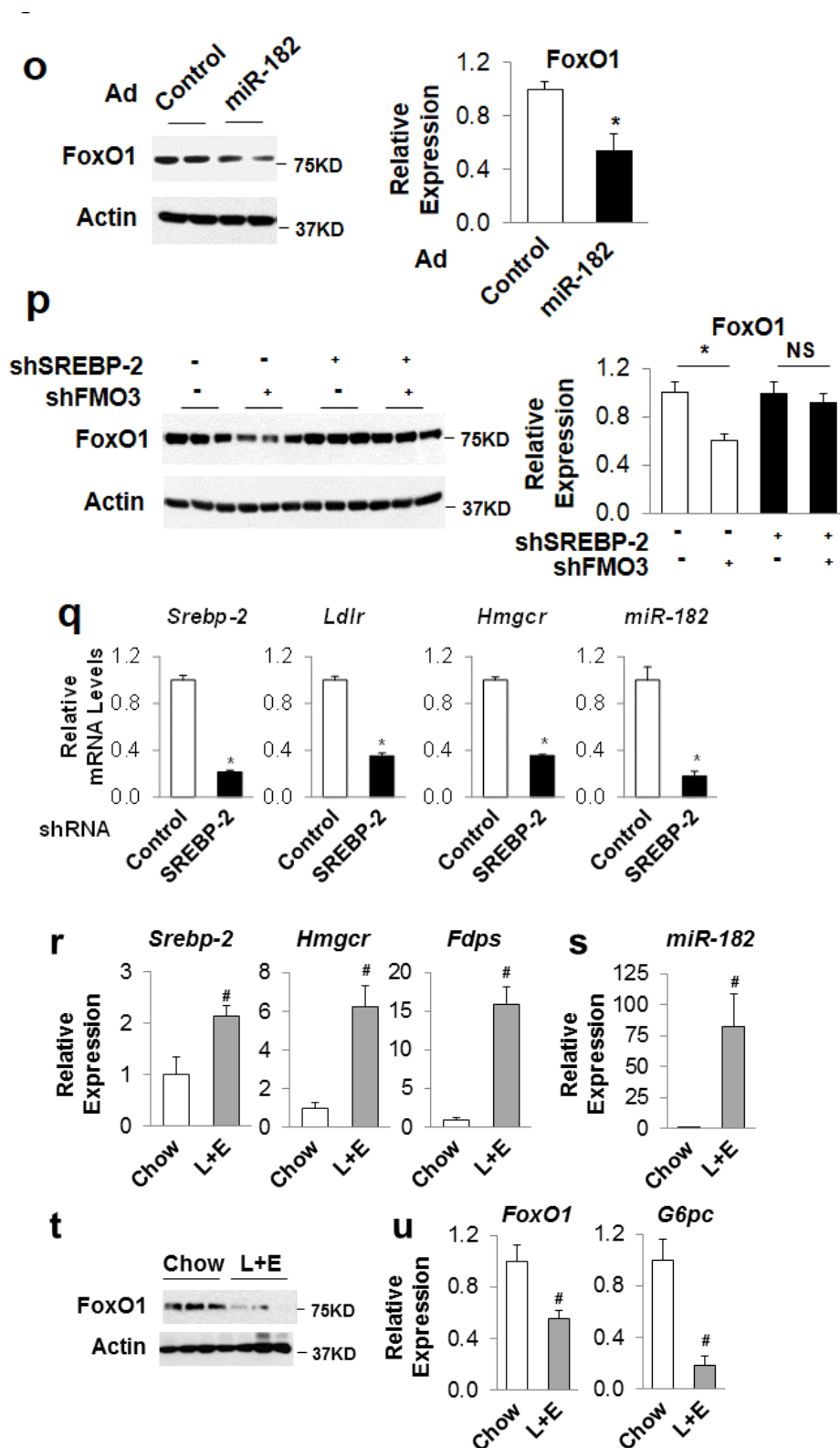


Figure 3.2 (Continued)



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Surprisingly, knockdown of FMO3 completely normalized glucose tolerance and improved insulin tolerance in LIRKO mice (Figure 3.2d, e). In parallel, FMO3 ASO significantly reduced expression of the gluconeogenic enzymes, glucose 6-phosphatase (*G6pc*) and *Pck1*, and insulin-like growth factor binding protein 1 (*Igfbp1*) (Figure 3.2f).

G6pc, *Pck1*, and *Igfbp1* are all targets of the transcription factor forkhead box O1 (FoxO1). FoxO1 drives gluconeogenic gene expression and is inhibited by insulin. (Haas and Biddinger, 2009; Matsumoto et al., 2007) We found that FMO3 ASO treatment markedly reduced FoxO1 protein levels (Figure 3.2g). The effects of FMO3 knockdown on FoxO1 were unlikely to be due to off-target effects of the ASO, as a second ASO targeting FMO3 also reduced FoxO1 protein in LIRKO livers and three additional shRNA constructs targeting FMO3 reduced FoxO1 protein levels in parallel with their ability to knockdown FMO3 *in vitro* (data not shown).

FMO3 is known primarily for its role in xenobiotic metabolism and more recently, cholesterol metabolism, via its product TMAO. (Warrier et al., 2015) Though the specific molecular targets remain unclear, mice with dietary induced increases in TMAO have been shown to have altered cholesterol absorption, reverse cholesterol transport and bile acid metabolism. (Bennett et al., 2013; Wang et al., 2011) We therefore examined the effects of FMO3 ASO treatment on a panel of genes involved (Figure 3.2h). The bile acid synthetic enzyme *Cyp8b1*, which determines the bile salt profile and is known to be driven by FoxO1, was decreased by knockdown of FMO3. However, we did not observe consistent, significant effects of the FMO3 ASO on any of the following: the other bile acid enzymes (*Cyp7a1*, *Cyp7b1*, or *Cyp27a1*); the cholesterol and bile transporters (*Abca1*, *Abcg5*, *Abcg8*, *Ntcp*, *Mrp2*, *Oatp1* or *Bsep*); the transcriptional regulators, *Lxr*, *Fxr*, and *Shp*; or the lipogenic enzyme *Fasn* and its regulator *Srebp-1c*.

On the other hand, we observed profound and consistent changes in the cholesterol synthetic enzymes and their regulator, Sterol Regulatory Element Binding Protein- 2 (SREBP-2).

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(Horton, 2002) SREBP-2 is induced by the depletion of cholesterol in the endoplasmic reticulum. (Radhakrishnan et al., 2008) We have previously shown SREBP-2 to be decreased in LIRKO livers. (Miao et al., 2014) Interestingly, knockdown of FMO3 largely normalized expression of *Srebp-2* mRNA and the amounts of nuclear SREBP-2 protein (Figure 3.2i). The SREBP-2 targets, 3-hydroxy-3-methylglutaryl-CoA reductase (*Hmgcr*), farnesyl diphosphate synthase (*Fdps*), squalene synthase (*Fdft1*), *Cyp51* and the LDL receptor (*Ldlr*) were also normalized in LIRKO mice by treatment with FMO3 ASO (Figure 3.2j).

One potential link between FoxO1 and SREBP-2 is miR-182. This microRNA has been shown to directly target the 3' UTR of FoxO1 and thereby suppress FoxO1 expression. (Guttilla and White, 2009; Kim et al., 2012; Stittrich et al., 2010) Importantly, miR-182 is encoded by a miRNA locus that is activated directly by SREBP-2. (Jeon et al., 2013) We therefore measured miR-182 expression in the livers of LIRKO mice treated with FMO3 ASO. Consistent with the induction of SREBP-2 in these livers (Figure 3.2i), miR-182 was increased four-fold (Figure 3.2k).

To directly test the roles of SREBP-2 and miR-182 in the regulation of FoxO1, we overexpressed SREBP-2 via an adenovirus in primary mouse hepatocytes (Figure 3.2l). Overexpression of SREBP-2 was sufficient to induce *Ldlr*, *Hmgcr* and miR-182 (Figure 3.2m, n). In parallel, FoxO1 protein was suppressed (Figure 3.2l). Similarly, overexpression of miR-182 also suppressed FoxO1 protein (Figure 3.2o).

We also knocked down SREBP-2 in mouse hepatocyte derived H2.35 cells using an shRNA construct. This construct reduced expression of *Srebp-2* and its targets, *Hmgcr*, *Ldlr* and miR-182 (Figure 3.2q). Moreover, knockdown of SREBP-2 abolished the effects of FMO3 knockdown on FoxO1 protein levels, indicating that SREBP-2 is required for the effects of FMO3 knockdown on FoxO1 (Figure 3.2p; right panel shows quantification).

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Taken together, these data suggest that knockdown of FMO3, perhaps by lowering endoplasmic reticulum cholesterol, induces SREBP-2 and miR-182, and thereby suppresses FoxO1. Consistent with this, knockdown of FMO3 reduced total hepatic cholesterol in the livers of LIRKO mice.

To determine whether a reduction in hepatic cholesterol was sufficient to suppress FoxO1, we treated LIRKO mice with lovastatin and ezetimibe for one week. (Bennett et al., 2008) This combination of drugs reduces hepatic cholesterol by inhibiting cholesterol synthesis and absorption. (Horton et al., 2002) As expected, lovastatin/ezetimibe treatment strongly induced *Srebp-2* and its targets in LIRKO livers (Miao et al., 2014) (Figure 3.2q). It also induced miR-182 (Figure 3.2r) and suppressed *FoxO1* mRNA, FoxO1 protein, and *G6pc* mRNA (Figure 3.2s, t).

We also asked whether knockdown of FMO3 could prevent atherosclerosis in LIRKO mice. Though LIRKO mice are not hypercholesterolemic relative to Flox mice on a chow diet, they do develop hypercholesterolemia and atherosclerosis in response to the stress of the Paigen diet (15% fat, 1% cholesterol, 0.5% cholic acid). (Biddinger et al., 2008b) We therefore challenged the mice with the Paigen diet for four months. *Fmo3* mRNA was only 30-fold increased in LIRKO versus Flox livers on the Paigen diet, perhaps because of the presence of cholic acid, which induces *Fmo3* expression in normal mice. (Bennett et al., 2013) Nonetheless, in LIRKO mice, treatment with ASO against FMO3 reduced *Fmo3* mRNA by approximately 50%; more importantly, FMO3 protein and TMAO levels were normalized by FMO3 ASO treatment (Figure 3.3a-c).

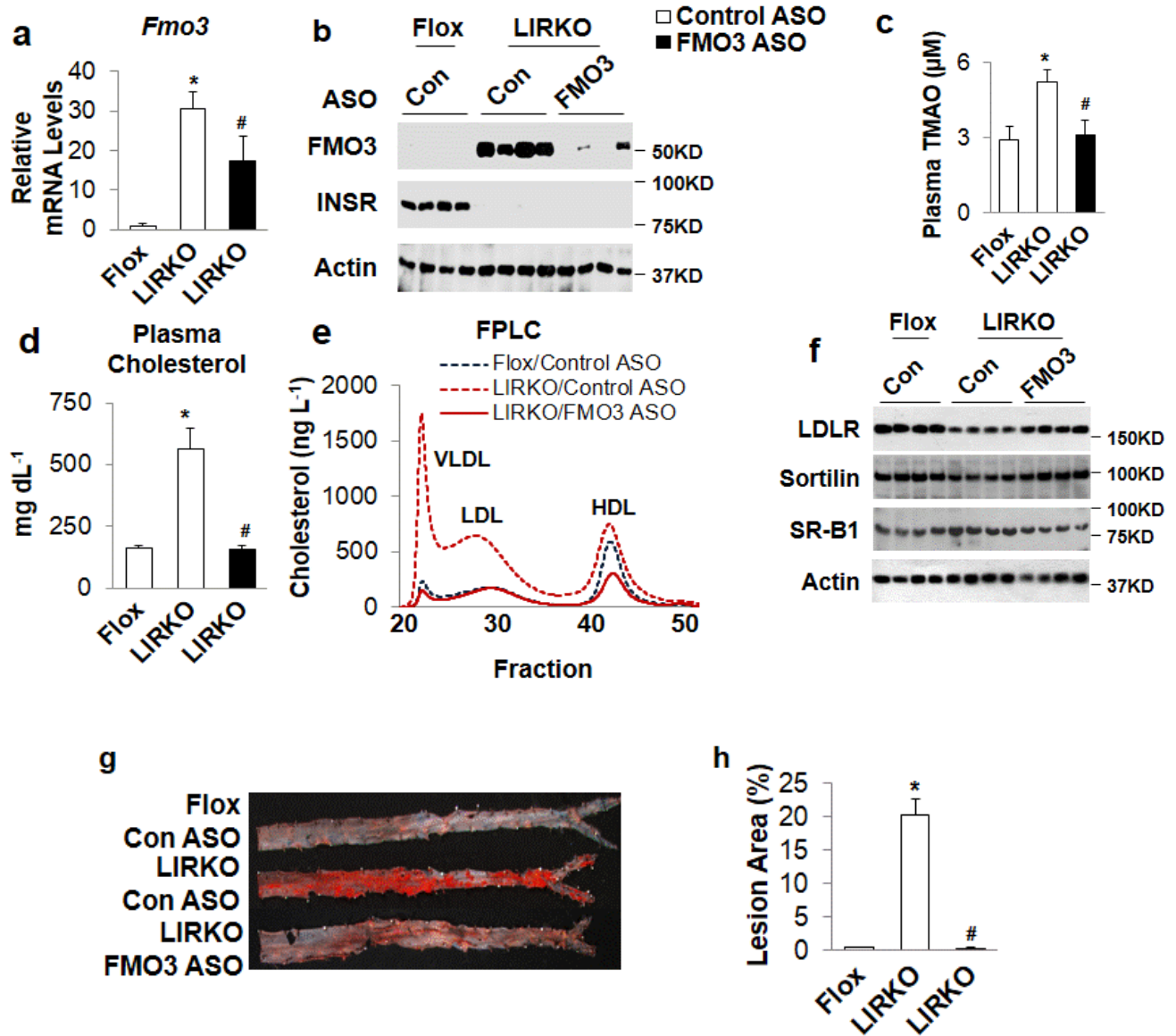


Figure 3.3 Knockdown of FMO3 prevents the development of atherosclerosis in LIRKO mice

At four to six weeks of age, male Flox and LIRKO mice were placed on an atherogenic Paigen diet, treated with control (Con) or FMO3 ASO for 16 weeks, and sacrificed in the non-fasted state. Hepatic gene expression (**a**) was measured by real-time PCR. Hepatic protein levels (**b, f**) were measured by western blotting whole cell lysates. (**c, e**) Plasma taken at the time of sacrifice was used to measure TMAO (**c**) or pooled ($n = 5 - 7$ mice per group) and subjected to FPLC analysis (**e**). Total cholesterol (**d**) was measured after 12 weeks of ASO treatment and a four-hour fast. (**g, h**) Abdominal aortas were dissected and stained with Oil-Red-O (representative images shown in **g**). Lesion area was quantified and expressed as a percentage of the whole aorta (**h**). In the above *in vivo* experiments, data represent the mean and SEM; $n = 5 - 13$; * $p < 0.05$ (Student's t-test) LIRKO mice treated with the control ASO versus Flox mice; # $p < 0.05$ (Student's t-test) control ASO versus FMO3 ASO treated LIRKO mice.

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In the presence of the Paigen diet, LIRKO mice developed severe hypercholesterolemia that was entirely prevented by the knockdown of FMO3 (Figure 3.3d). Similar results were obtained on a Western diet (21% fat, 0.2% cholesterol, 34% sucrose) which lacks cholic acid (Figure 3.4). In particular, knockdown of FMO3 decreased VLDL- and LDL-associated cholesterol in LIRKO mice (Figure 3.3d, e). This was associated with an increase in LDL receptor, which removes atherogenic lipoproteins from the plasma, and sortilin, which both promotes clearance and inhibits secretion of atherogenic lipoproteins (Strong et al., 2012) (Figure 3.3f). SR-B1, which participates in reverse cholesterol transport by removing HDL cholesterol from the serum (Zhang et al., 2005), was decreased; however, HDL was also decreased, suggesting a possible discordance between SR-B1 protein levels and activity. In any case, consistent with the prevention of hypercholesterolemia, FMO3 knockdown completely prevented the development of atherosclerosis in Paigen-fed LIRKO mice (Figure 3.3g, h).

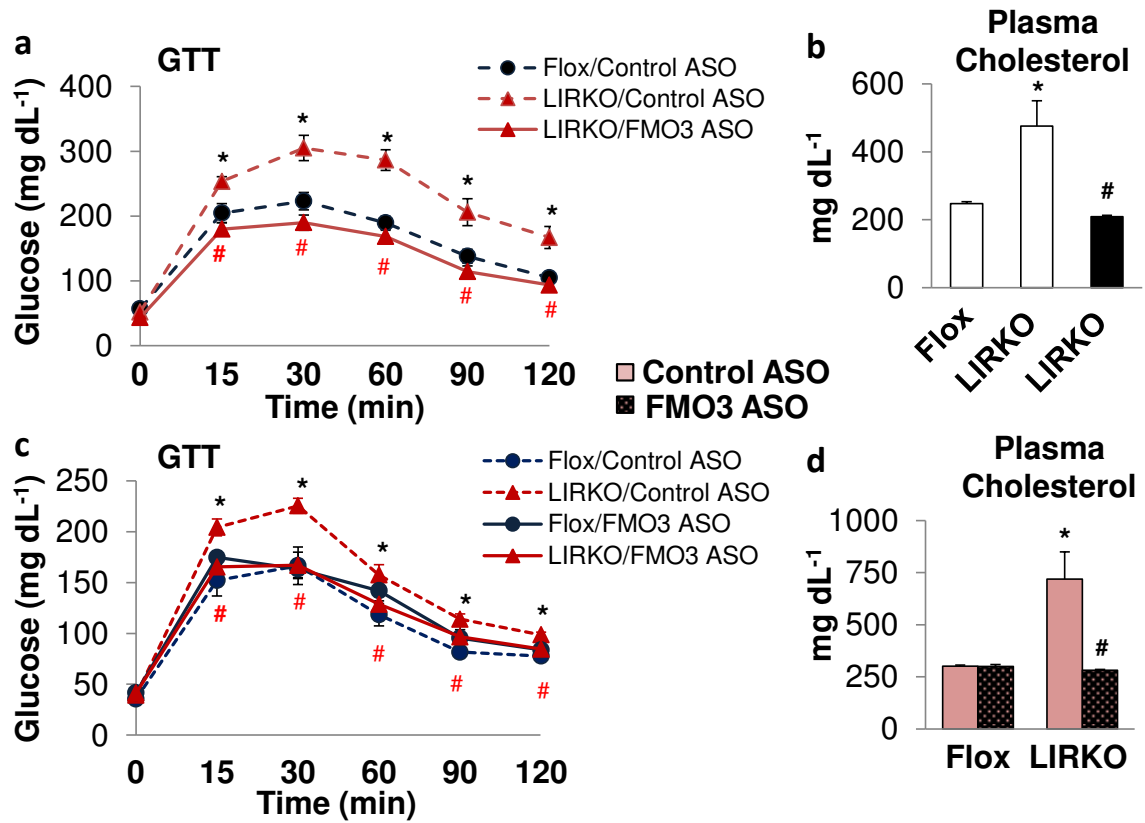


Figure 3.4 Knockdown of FMO3 in LIRKO mice on atherogenic diets.

(a to d) Four to six week old male (a, b) and female (c, d) Flox and LIRKO mice were placed on a Western diet and treated with control or FMO3 ASO for five weeks. Glucose tolerance testing was performed after four doses of ASO (a, c) and four-hour fasted plasma was collected for cholesterol measurement after five doses of ASO (b, d). Data represent the mean \pm SEM; n = 5; * p < 0.05 (Student's t-test) LIRKO versus Flox mice treated with the same ASO; # p < 0.05 (Student's t-test) control versus FMO3 ASO treated LIRKO mice.

Figure 3.5. FMO3 in obese/diabetic mice and humans.

(a to h) Eight to ten week old male mice were sacrificed in the non-fasted state. **(a to d)** Streptozotocin (STZ) treated mice were compared to their vehicle treated controls (Veh) and **(e to h)** *ob/ob* mice were compared to their wildtype controls (Con); all mice were sacrificed in the non-fasted state. **(i to m)** Six week old male *ob/ob* mice were treated with control (Con) or FMO3 ASO for four weeks and were sacrificed in the non-fasted state. Hepatic gene expression **(b, c, f, g, i, k, l)** was measured using real-time PCR, and protein levels **(d, h, j)** were measured by western blotting whole cell lysates. Glucose tolerance testing **(m)** was performed after three weeks of ASO treatment. Data represent the mean and SEM; n = 5 - 8; * p < 0.05 (Student's t-test) vehicle versus STZ treated **(a to d)**, control versus *ob/ob* mice (e to h), control ASO treated *ob/ob* mice versus FMO3 ASO treated *ob/ob* mice **(i to m)**. **(n to o)** Two to three month old male and female Flox and LIRKO mice were sacrificed in the non-fasted state. Hepatic gene expression **(n)** was measured using real-time PCR, and protein levels **(o)** were measured by western blotting whole cell lysates. **(p to s)** Four to six week old female Flox and LIRKO mice were treated with control (Con) or FMO3 ASO for seven weeks and sacrificed in the non-fasted state. Hepatic gene expression **(p, r)** was measured by real-time PCR, and protein levels were measured by western blotting whole cell lysates **(q)**. Glucose tolerance testing **(s)** was performed after three weeks of ASO treatment. Data represent the mean and SEM; n = 5; * p < 0.05 (Student's t-test) Flox versus LIRKO mice with control ASO treatment; # p < 0.05 (Student's t-test) control versus FMO3 ASO treated mice of the same genotype (blue # is for Flox mice and red # is for LIRKO mice). **(t, u)** Liver biopsies were taken from age and sex matched patients undergoing bariatric surgery (Obese) or other abdominal surgeries (Controls), and FMO3 expression was measured by real-time PCR **(u)**; biochemical and physiological data from these patients is shown **(t)**. Data represent the mean and SEM, as described in Methods. P-values were determined by the Mann-Whitney test for continuous variables and by the χ^2 test for categorical variables **(t)** or the Student's t-test **(u)**.

Figure 3.5 (Continued)

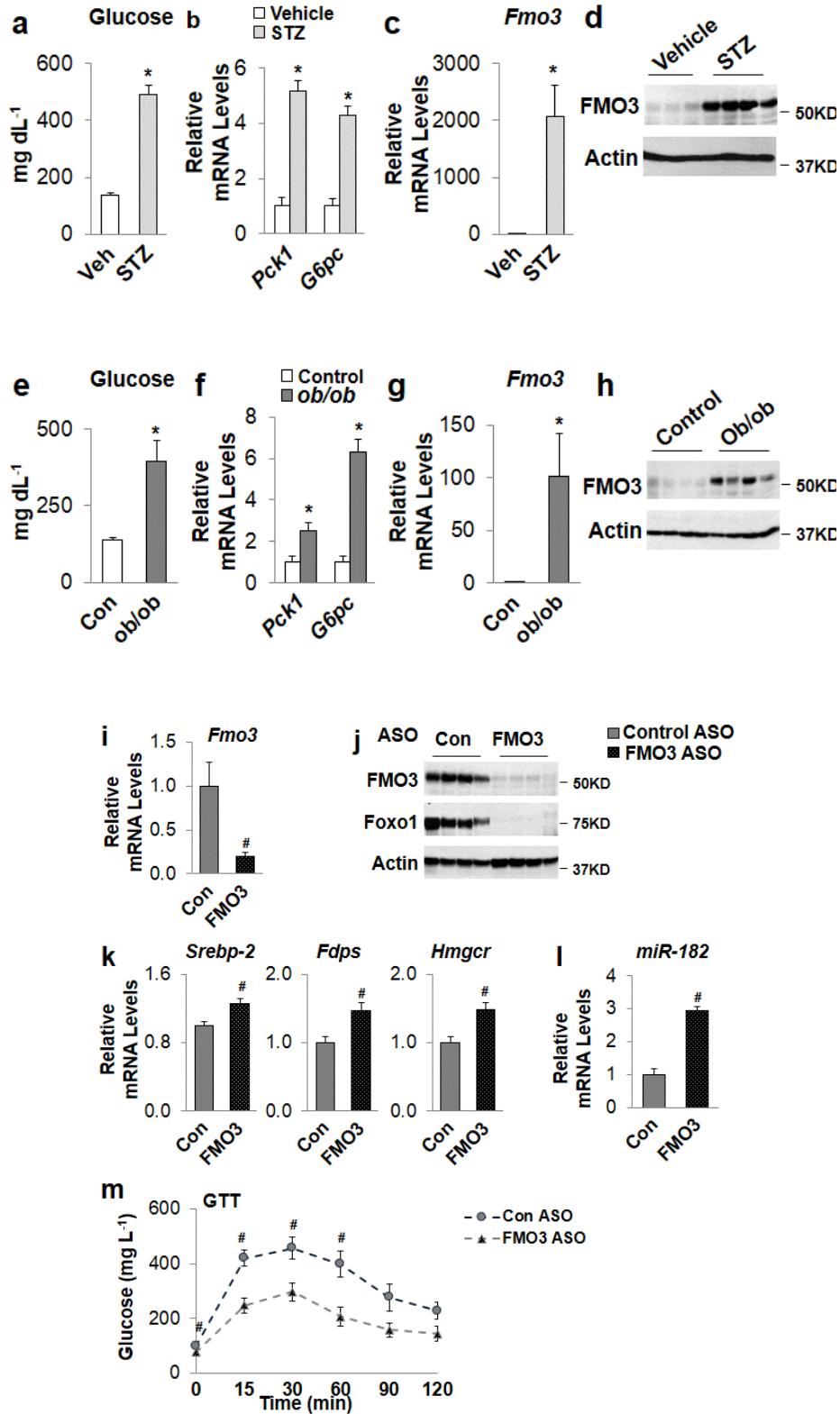
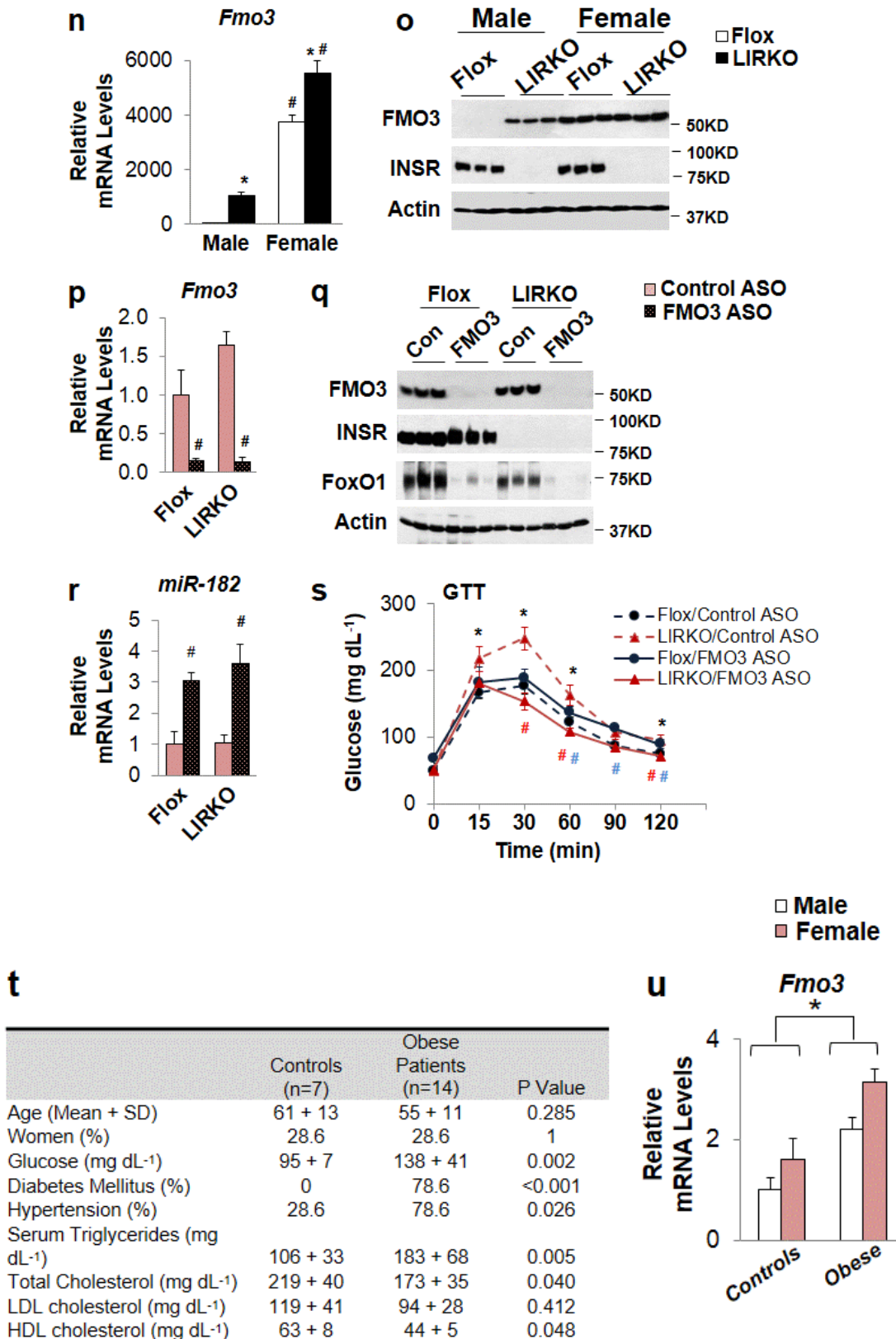


Figure 3.5 (Continued)



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We also examined FMO3 in other models of diabetes. Streptozotocin (STZ) treated mice are a model of Type 1 diabetes, as STZ treatment is toxic to the beta-cells of the pancreas, rendering STZ treated mice insulin deficient. As expected, male STZ mice were hyperglycemic with four- to five-fold increases in *Pck1* and *G6pc* expression in their livers relative to their untreated controls (**a, b**). *Fmo3* mRNA, however, was induced over 1000-fold, and FMO3 protein was markedly induced (Figure 3.5c, d).

Leptin deficient *ob/ob* mice are a commonly used model of obesity/Type 2 diabetes. Male *ob/ob* mice were hyperglycemic with increased hepatic expression of the gluconeogenic enzymes *Pck1* and *G6pc* relative to their lean controls (Figure 3.5e, f). FMO3 mRNA and protein were also markedly increased in the livers of *ob/ob* mice (Figure 3.5g, h). Blocking the effects of hyperinsulinemia in *ob/ob* mice by knocking down the insulin receptor further induced FMO3 protein, consistent with the notion that the signaling pathways utilized by insulin to suppress *Fmo3* become partially resistant to insulin in *ob/ob* mice.

Treatment with the FMO3 ASO markedly reduced FMO3 in *ob/ob* livers (Figure 3.5i, j). In parallel, it increased *Srebp-2*, the cholesterologenic enzymes, and miR-182 (Figure 3.5k-l). Again, FoxO1 protein levels were lowered to near undetectable levels, and glucose tolerance was significantly improved (Figure 3.5j, m). Thus, in *ob/ob* mice, as in LIRKO mice, the knockdown of FMO3 can activate SREBP-2/miR-182 and suppress FoxO1 and hyperglycemia.

Taken together, these data indicate that FMO3 expression, which is directly inhibited by insulin and induced by glucagon and corticosteroids, is increased in male mice with insulin deficiency/insulin resistance. However, FMO3 expression is sexually dimorphic, with regulation at the levels of transcription, translation/protein stability, and enzyme activity. (Bennett et al., 2013)

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Thus, FMO3 in female livers is more than 1000-fold higher at the mRNA level, and three-fold higher at the enzyme activity level. (Bennett et al., 2013)

We therefore examined the expression of FMO3 in LIRKO females. Flox females had 4000 times more *Fmo3* mRNA and markedly increased FMO3 protein than their male counterparts, as expected (Figure 3.5n, o). Though knockout of the insulin receptor increased *Fmo3* expression, the effects were modest and only reached significance in some cohorts. To determine whether FMO3 still regulated FoxO1 in female mice, we knocked down FMO3 in both Flox and LIRKO female mice (Figure 3.5p-s). Indeed, FMO3 knockdown increased expression of miR-182 (Figure 3.5r) and suppressed FoxO1 (Figure 3.5q) in females, just as it did in males. In LIRKO mice, this resulted in improved glucose tolerance (Figure 3.5s). In Flox mice, the reduction of FoxO1 protein was not associated with an improvement in glucose tolerance, consistent with prior studies showing that the effects of FoxO1 knockout on glucose metabolism are manifested primarily in the insulin-resistant state. (Wan et al., 2011)

The fact that FMO3 knockdown had similar effects in males and females suggests that the FMO3/TMAO pathway is important in metabolic control in both sexes, though its regulation is different. Such extreme sexual dimorphism of FMO3 is only observed in certain species of mice, such as *Mus musculus* and *Mus domesticus*. (Li et al., 2013) Even other rodent species, such as the mouse strain *Mus caroli* and *Rattus norvegicus*, do not show sexually dimorphic expression of FMO3 in their livers. (Li et al., 2013) In humans, the sex effect on TMAO/FMO3 is absent or much more modest, as TMAO levels are not significantly different between males and females (Wang et al., 2011), and hepatic *Fmo3* mRNA is increased only 50% to 3-fold in females. (Bennett et al., 2013)

To begin to explore the relationship between FMO3 and diabetes in humans, we examined *FMO3* expression in the livers of age and sex matched patients undergoing either bariatric surgery (Obese) or other abdominal surgeries (Controls). All of the individuals undergoing bariatric surgery

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had a BMI > 33.0 (with a mean BMI of 41.8), and 79% were diabetic. Consistent with their metabolic derangements, the obese group had higher levels of serum triglycerides and lower HDL than the controls (Figure 3.5t). *FM03* was significantly increased in the morbidly obese group (Figure 3.5u). However, the effect was more modest than seen in the mouse models, possibly because the diabetic patients were treated with insulin and/or other medications to improve insulin sensitivity, and because the liver biopsies were taken under the fasting conditions of surgery.

3.5 Discussion

Over the past four years, *FM03* and TMAO have emerged as key components of a complex axis integrating diet and the gut microbiome with atherosclerosis (Bennett et al., 2013; Koeth et al., 2013; Tang et al., 2013; Wang et al., 2011); and knockdown of *FM03* was recently shown in mouse models of hyperlipidemia to prevent atherosclerosis and improve the metabolic phenotype. (Shih et al.) Here, we have independently identified *FM03* and TMAO by performing non-biased profiling in male mice with diabetes associated atherosclerosis. Importantly, we find that *FM03* is required for expression of *FoxO1*, a key node within the cell, controlling growth, differentiation and metabolism. (Gross et al., 2009; Matsumoto et al., 2007) In addition to regulating glucose production, *FoxO1* regulates bile acid, lipoprotein, and fatty acid metabolism. (Cheng et al., 2009; Dong et al., 2008; Gross et al., 2008; Kamagate et al., 2008; Leavens et al., 2009; Lu et al., 2012; Samuel et al., 2006; Wan et al., 2011; Zhang et al., 2006b) It is clear that knockdown of *FoxO1* has profound effects, reversing most, but not all aspects of the insulin resistant state. (Dong et al., 2008; Wan et al., 2011)

In female mice, *FM03* is not strongly induced by insulin resistance and non-diabetic female mice have high levels of *FM03*/TMAO (Wang et al., 2011), but do not necessarily manifest hyperglycemia or atherosclerosis. In male mice, *FM03* is strongly induced by diabetes, but transient overexpression of *FM03* in lean male mice does not produce hyperglycemia (data not shown). In

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both male and female mice, FMO3 is required for the development of the diabetic phenotype. Taken together, these data suggest that FMO3, which is differentially regulated in male and female mice, is necessary but not sufficient for the development of the diabetic phenotype.

Several lines of evidence support the notion that the link between diabetes and FMO3/TMAO will be important in humans. First, our data in human liver samples show that FMO3 is increased in the livers of obese/insulin resistant individuals undergoing gastric bypass. Second, prior studies have shown that increased plasma levels of TMAO are associated with increased levels of serum glucose and diabetes in patients undergoing elective coronary angiography. (Tang et al., 2013) Finally, using Meta-Analysis of Glucose and Insulin-related traits Consortium data set (n = 15,234 nondiabetic individuals), we found several SNPs in the FMO3 locus to be associated with blood glucose levels ($p = 4 \times 10^{-5}$) (Saxena et al., 2010), though they did not reach genome-wide significance (Figure 3.6).

The results of this work suggest that therapies to reduce FMO3/TMAO to normal levels may be particularly helpful in the prevention of diabetes associated CVD. This may be through pharmacological manipulations to reduce FMO3 activity in diabetic patients, or by dietary interventions, such as the restriction of carnitine and/or choline, which serve as precursors of TMAO.

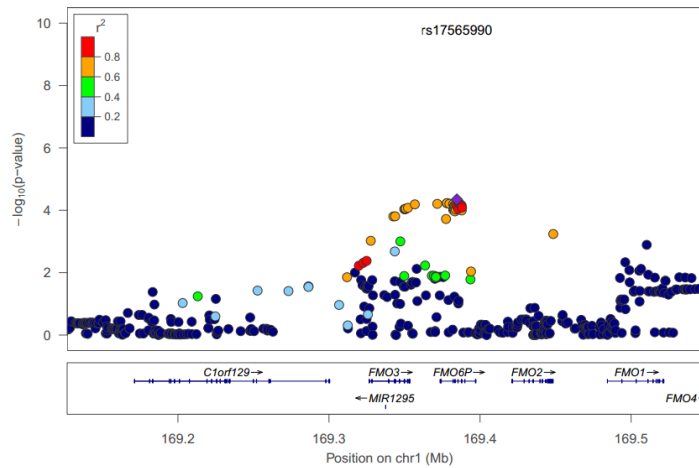


Figure 3.6 FMO3 and glucose metabolism

LocusZoom¹ was used to query the Meta-Analysis of Glucose and Insulin-related traits Consortium (MAGIC)² data set ($n = 15,234$ nondiabetic individuals) for associations between the FMO3 locus and glucose levels. Shown above are associations with glucose levels measured two hours after a glucose challenge and adjusted for body mass index. The purple diamond indicates the lead SNP ($p = 4.51 \times 10^{-5}$). Evaluated SNPs are colored based on their correlation with the lead SNP.

3.6 Attribution of Experiments in Chapter 3

This work was done in collaboration with Ji Miao. The experiments in Figures 2a-k, 3, 4, 5e-m were performed by Alisha Ling. Other contributors to this work include Sudha B. Biddinger, Praveen V. Mantheena, Mary E. Gearing, Mark J. Graham, Rosanne M. Crooke, Kevin J. Croce, Ryan M. Esquejo, Clary B. Clish, David Vicent and the Morbid Obesity Study Group, Morris F. White, Joseph Majzoub, John Cashman, Joel Haas, Yevgenia Tesmenitsky, Matthew Davis, Abhiruchi Mehta, and Jordan Grant. Data was generated by J.M., A.V.L., P.V.M., and M.E.G. R.M.E., M.J.G and R.M.C. generated reagents; C.B.C. generated the metabolomics data and TMAO quantification; K.J.C. contributed to the atherosclerosis studies; D.V. generated the human data; the Morbid Obesity Study Group acquired the liver biopsy specimens. J.M. and S.B.B. generated the hypothesis, designed the experiments, analyzed the data and wrote the manuscript; all authors contributed to the discussion. J.H. provided advice on microarray analysis. J.S. provided support with LC/MS measurements. Y.T. performed

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atherosclerosis measurements. M.D. performed FPLC analysis. A.M. and J.G. provided general technical assistance. M.F.W, J.M, and J.C. provided intellectual support.

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Chapter 4

Conclusions

The FoxO1 transcription factor is a key node of metabolic control. In insulin resistance, FoxO1 loses inhibition by insulin and becomes a potent driver of metabolic syndrome and potentially of cardiovascular disease. While many studies have investigated the role of FoxO1 in regulating glucose metabolism and driving hyperglycemia in the setting of insulin resistance, the role of FoxO1 in mediating lipid metabolism has remained unclear. Here we study the role of FoxO1 in lipid metabolism and in the development of diabetes-associated atherosclerosis.

In our first study, we established that FoxO1 suppresses the expression of cholesterol synthesis genes *in vivo* and activates their expression cell autonomously *in vitro*. We identified Cyp8b1 as a target of FoxO1, where FoxO1 was both necessary and sufficient for the expression of Cyp8b1. Importantly, the relationship between FoxO1 and Cyp8b1 was found to be conserved in a mouse model of metabolic syndrome, and in people with metabolic syndrome. The Cyp8b1 gene product, 12 α -hydroxylase, catalyzes the rate-limiting step of cholic acid synthesis, and so altered levels of Cyp8b1 due to FoxO1 changed the proportion of cholic acid and altered dietary cholesterol absorption from the gut lumen.

Taken together, these data indicate that hepatic FoxO1 regulates systemic cholesterol metabolism via Cyp8b1, cholic acid, and dietary cholesterol absorption. This further highlights FoxO1 as a potential therapeutic target for inhibition or knockdown in the clinical setting. Additionally, since Cyp8b1 is increased in humans with metabolic syndrome, these results suggest that Cyp8b1 inhibition or knockdown might have therapeutic benefit by decreasing cholesterol absorption.

In our second study, we used transcriptomic and metabolomic approaches to identify *Fmo3* and its product, TMAO, to be among the most increased in the livers of LIRKO mice. We demonstrated that *Fmo3* is suppressed by insulin *in vitro*, and increased in mouse models of obesity and insulin resistance, and in obese humans. *Fmo3* knockdown suppressed FoxO1 via *Srebp2* and miR-182, indicating that *Fmo3* is required for FoxO1 expression. Excitingly, *Fmo3* knockdown was sufficient to entirely prevent the development of hyperglycemia, hyperlipidemia and atherosclerosis in LIRKO mice. These results suggest that therapies to reduce FMO3 and TMAO to normal levels may be potent strategies in the prevention of diabetes-associated cardiovascular disease.

The identification of TMAO and *Fmo3* excitingly integrates diet and the gut microbiome in the risk and progression of cardiovascular disease. We now add insulin resistance as a potent amplifier of this pathway. Moving forward, an important line of inquiry will be to discover and specify the direct mechanisms linking *Fmo3* and TMAO to actual plaque formation. One recent study connected *Fmo3* to macrophages with increased scavenger receptor expression (Wang et al., 2011), another posits that *Fmo3* is a negative regulator of reverse cholesterol transport by macrophages (Warrier et al., 2015), and yet another puts forth PPAR α and Kruppel-like factor 15 as the pathways linking *Fmo3* to dyslipidemia and atherosclerosis. (Shih et al., 2015)

Given the dramatic level of FoxO1 suppression by Fmo3 knockdown, and our earlier findings of FoxO1's role in regulating lipid metabolism, there is the distinct possibility that hepatic FoxO1 suppression mediated not just the normalization of hyperglycemia but also the hyperlipidemia.

In conclusion, in examining the mechanistic links between insulin resistance and dyslipidemia and cardiovascular disease, we demonstrate that hepatic FoxO1 regulates systemic cholesterol metabolism through potent regulation of Cyp8b1 and cholic acid, and that knockdown of Fmo3 suppresses FoxO1 and entirely prevents the development of hyperglycemia, hyperlipidemia, and atherosclerosis. These results suggest that therapies to reduce Fmo3 and FoxO1 may be particularly helpful in the prevention of diabetes-associated cardiovascular disease.

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