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Deletions of Fstl3 and/or Fst Isoforms 303 and 315 Results in Hepatic Steatosis

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DELETION OF FSTL3 AND/OR FST ISOFORMS FST303 AND 315 RESULTS IN HEPATIC STEATOSIS

A Thesis Presented

by

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ABSTRACT

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HEPATIC STEATOSIS

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TGFβ ligands, activin and myostatin have been shown to stimulate insulin production

and secretion. Antagonists, Follistatin (FST) and Follistatin like 3 (FSTL3) were partially

and fully ablated, respectively, creating hyperinsulinemic mice with fatty liver. Much

research has surfaced on the connection between hepatic steatosis and hepatic insulin

resistance. We present two different models, each with a different mechanism behind

the development of fatty liver. FST288-only mice have increased synthesis of mRNA

and proteins responsible for hepatic triglyceride (TG) uptake, while our double mutants

have increased synthesis of mRNA and proteins responsible for TG synthesis. This

alteration was likely independent of hepatic insulin resistance as livers from both mouse

lines were insulin sensitive. Experiments conducted in this study to realize the causal

factor of hepatic steatosis can be performed on adipose and muscle tissues in the future

to better characterize the phenotype.

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CHAPTER 1

INTRODUCTION

Type II Diabetes Mellitus is a common disease affecting an increased proportion of the population each year. Twenty three million people in the United States have been diagnosed with the disease with another 57 million considered to be pre-diabetic[1]. Insulin, produced by beta cells of the pancreas, regulates a variety of metabolic systems including glucose and fat uptake and storage and protein synthesis. Cells become insulin resistant in many different ways. From a macroscopic level, target cells become less responsive to insulin over time, requiring more insulin for the same effect. The mechanisms by which this occurs will be described in more detail below. The organs relevant to this study which are known to become insulin resistant are muscle, adipose, and liver.

For many years, the term insulin resistance has been used to describe impaired glucose control with respect to the entire body. However, in the early portion of this century tissue specific insulin receptor knockouts were generated. Comparing the phenotype of different tissue specific insulin receptor knockouts (Table 1), it became clear that different tissues can contribute uniquely to the diabetes syndrome[2]. A liver insulin receptor knockout (LIRKO) mouse exhibited extreme postprandial hyperglycemia, extreme hyperinsulinemia (20 fold higher serum insulin levels), β-cell hypertrophy, and decreased circulating free fatty acids and triglycerides[3] . The muscle insulin receptor knockout (MIRKO) showed normal blood glucose and insulin levels[4],

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53% increase in epididymal fat pad weight, and a 38% increase in whole-body fat content[5],[6]. They also have a 16% increase in circulating free fatty acids and a 43% increase in serum triglycerides[3]. The fat insulin receptor knockout (FIRKO) shows fasting insulin levels are decreased by 45%, decreased serum triglycerides, and sustained glucose tolerance and insulin sensitivity to an older age than control mice. This shows that liver insulin resistance has the most pathological effect, while adipose insulin resistance appears to ameliorate the diabetic phenotype.

The insulin receptor models characterize the effects of a complete interruption in signaling. While this is helpful in determining the effect insulin resistance on each organ has on the entire body, it is not a perfect representation of type 2 diabetes. After observance of diabetic mice with impaired insulin stimulated gluconeogenesis but with enhanced insulin stimulated lipogenesis, Brown and Goldstein published a model of postreceptor insulin resistance[7]. The lipogenic pathway begins with the same insulin receptor and splits off the glucose regulatory pathway downstream, allowing for the possibility of different levels of response sensitivity.

Non-Alcoholic Fatty Liver Disease (NAFLD) is one of the most common liver diseases in the world today[8]. The relationship between insulin resistance, type 2 diabetes mellitus (TIIDM), and Non-Alcoholic Fatty Liver Disease is a complex one. Most cases of NAFLD are strongly associated with hepatic insulin resistance[9],[10] and it affects around 20 million patients in the United States alone[11]. Type 2 diabetics who undergo weight loss end up lowering their liver TGs and impoving their hepatic insulin sensitivity[12]. The accumulation of lipids in liver tissue causes hepatic steatosis, which

progresses into non alcoholic steatohepatitis (cirrhosis of liver tissue). The liver is a major regulator of lipid uptake and storage as well as cleavage and metabolism. NAFLD is a result of a disparity in the uptake + production versus the secretion + oxidation of free fatty acids and triglycerides. Hepatic lipid uptake can be linked to peripheral adipose tissue lipid secretion as well as postprandial lipid absorption. De novo lipogenesis (the synthesis of lipids from glucose precursors), beta oxidation, and triglyceride secretion in the form of VLDLs take place in the hepatocytes themselves, and are regulated by insulin to some extent.

Activin and myostatin appear to have important metabolic functions, but have been somewhat understudied for this role. Two known antagonists of activin and myostatin are follistatin (FST)[13] and follistatin like protein 3 (FSTL3)[14]. A knockout of FST and FSTL3 allows for the enhanced activity of activin and myostatin. The complete FST knockout is not viable so the contribution of FST to energy homeostasis is impossible to discern from this model. FSTL3 knockout animals survive and are born in normal mendelian ratios and are thus useful for this application. An FSTL3 knockout mouse demonstrated an increase in pancreatic islet number and size, β-cell hyperplasia, decreased visceral fat mass, improved glucose tolerance, and enhanced insulin sensitivity[15]. Other then the hepatic steatosis, the phenotype appeared to be anti-diabetic.

Alternative splicing results in two different mRNAs produced from the *fst* gene, one of which undergoes post-translational processing resulting in a total of three FST isoforms, FST288, FST303, and FST315[16]. FST288 has the highest affinity for heparan sulfate proteoglycans and is therefore suggested to be a tissue-bound protein. FST303,

the intermediate isoform, is also found bound to tissue, but not as strongly[17] and found

primarily in gonadal fluids. FST315 is considered the circulating isoform due to its weak

binding of heparin sulfate proteoglycans[18]. In order to study the effects of FST ablation,

it was necessary to leave the FST 288 isoform intact, which is the apparent isoform

required for development. FST288-only mice were created by altering the *fst* gene to

delete alternative splicing[19] Now with two different genetically modified mouse lines

(FST288-only, FSTL3 KO), each one having a complementary antagonist rendered

inactive, the next step was to mate these mice to create a double mutant mouse line

(DM). The DM (FST303-/-, FST315-/-, FSTL3-/-), is as close as we can come to a

complete ablation of the antagonists of myostatin and activin. The present study

focuses on the comparison of these three genetically modified mice lines.

Mice of each genotype and their wildtype littermates were analyzed over a 2 year

period[23]. The unpublished data helped give direction to this liver study and is

summarized in Table 1. Total fat composition in FST288-only and FSTL3 KO was

similar to WT levels but DM mice displayed a 150% increase in percent body fat. All

three genotypes had a 33% reduction in visceral fat pad mass. This could be due to an

attenuated insulin response in their visceral fat since visceral adipose tissue is known to

be more insulin resistant than subcutaneous fat[20]. Insulin inhibits lipolysis[21], explaining

how insulin resistant adipose tissue can diminish in size; more triglycerides will be

cleaved and released in this state.

Mice over 6 months of age showed no hyperglycemia with random and fasting

glucose tests. Only DM had a significant (2-fold) increase in random serum insulin

compared to either WT. During GTTs, DM mice were more glucose intolerant and

insulin resistant than single mutant FST288-only mice or WT littermates. FST288-only mice have normal GTT, ITT, and body fat percentages and do not appear diabetic based on these physiological tests. FSTL3 KOs appear to be particularly anti-diabetic. They are glucose tolerant, insulin sensitive, and have high serum insulin levels[22]. This could indicate that FSTL3 has a greater antagonistic activity than FST, with partial FST ablation producing only undetectable effects. Furthermore upon combination of the anti-diabetic FSTL3 KO and normal FST288-only, the result is a diabetic mouse. Based on the physiologic study alone, the DM genotype appeared to be insulin resistant, while the other genotypes seemed to maintain their insulin sensitivity even with enhanced islet function. Because the liver has a direct role in the maintenance of metabolite homeostasis, a defect in the liver insulin response was expected.

HEPATIC INSULIN RESISTANCE: GLUCOSE REGULATION

Hepatic insulin resistance can have very serious diabetic implications. Following fasting, the liver is the major source of plasma glucose, and hepatic glucose output is tightly regulated by insulin[23]. A healthy liver responds to insulin signaling by reducing gluconeogenesis and glycogenolysis, and secondarily by increasing glucose storage via glycogenesis. In insulin resistant states, the liver will still secrete glucose postprandially. If β-cells are properly functioning, the excess circulating glucose will cause hyperinsulinemia. High insulin levels can downregulate their own signal response pathways as a form of negative feedback, resulting in insulin resistance.

Insulin acts on many different tissues and it is unlikely that every tissue develops resistance at the same rate. In addition, multiple forks in signal cascades can make it difficult to pinpoint which component of the pathway is responding irregularly. Insulin signal transduction begins with the circulating hormone binding to the extracellular α-domains of the heterodimeric insulin receptor (IR). Once bound, the intermembranal β-domains of the receptor exhibit tyrosine kinase activity[24] and phosphorylate the insulin receptor substrate proteins (IRS1, IRS2, IRS3, IRS4). These eventually activate PI3 kinase, which then phosphorylates AKT at the serine residues, activating this kinase. These are considered the main intermediate steps in the signal cascade and eventually produce the known insulin response. One way insulin inhibits gluconeogenesis is by repressing the transcription of phosphoenolpyruvate carboxykinase (PEPCK) [25] and glucose-6-phosphatase (G6Pase)[26]. PEPCK in its enzymatic form is responsible for the conversion of oxaloacetate into phosphoenolpyruvate, the rate limiting step in gluconeogenesis. G6Pase catalyzes the hydrolysis of glucose-6-phosphate into glucose[27], a required final step in both gluconeogenesis and glycogenolysis. Hepatic glucokinase (GCK) is an important enzyme for glucose storage and its mRNA expression is known to be depressed in diabetic states and restored to normal with insulin treatment[28]. Glucokinase is responsible for the phosphorylation of glucose to glucose-6-phosphate, the opposite function of G6Pase. IRS1 and IRS2 are understood to be the two most relevant of the IRS isoforms. IRS1/IRS2 are downregulated in diabetic patients[29] and insufficient levels of IRS1/IRS2 will impair insulin response at all downstream targets. To test for the sensitivity of insulin regulation at the transcriptional

level, we decided to quantify the mRNA levels of G6Pase, PEPCK, GCK, IRS1, and IRS2.

Glycogenesis is the conversion of glucose into its more efficient storage form, glycogen. Glycogen is a large branched polymer of glucose molecules. Fatty acids pack more energy per unit mass than glycogen, but glycogen can be readily broken down into glucose in the presence or absence of oxygen. This makes it the best functioning buffer for glucose levels. One of the better known results of insulin signaling in the liver is the upregulation of the glycogenic pathway. In insulin resistant liver tissue, we would expect glycogen stores to be lower, so we examined glycogen stores in the liver.

HEPATIC INSULIN RESISTANCE: LIPID REGULATION

The relevant insulin response can be broken down into two pathways: Glucose regulation and lipid regulation. The convergence of these two pathways can explain the connection between insulin resistance and fatty liver. Insulin has a direct and indirect role in lipid regulation. Insulin directly activates components of the lipogenic pathway. Insulin also causes glucose uptake into cells, causing the preferential burning of glucose for energy, thus saving the fat. Lastly, in one published diabetic mouse model, there is evidence of a "sensitivity split" where the glucose response sensitivity diminishes and lipid response pathway remains sensitive to insulin[30]. This is a possible explanation for the DM mice which have drastically increased serum insulin levels. To examine the severe hepatic steatosis found in our mice, mRNA and protein expression studies were first performed on components of the lipogenic pathway. The study

included determining mRNA levels of both PPARγ and SREBP1c, as well as protein levels of SREBP1. Sterol regulatory element binding protein 1c (SREBP1c) is an important lipogenic transcription factor known to promote the expression of many lipogenic enzymes. Insulin stimulates SREBP1c activity[31] even in insulin resistant states[32]. PPARγ is a transcription factor that promotes the expression of many genes involved in fatty acid uptake[33].

Because lipids are hydrophobic, they need to be carried through the blood by hydrophilic molecules. Low density lipoproteins are composed of cholesterols, triglycerides, and protein. In humans, at least 75% of circulating LDL is taken up by the liver via LDLR[34]. When overexpressed in mice, liver LDL receptor causes total serum cholesterol levels to be reduced to less than 50%[35]. Overexpressed lipoprotein lipase (LPL) in the liver results in steatosis[36]. We hypothesized that the genes involved in lipid uptake and lipogenesis would be upregulated in the livers of all of our knockout mice.

HEPATIC STEATOSIS

Some studies suggest that circulating fatty acids due to peripheral insulin resistance is the cause of fatty liver[37]. Blood is supplied to the liver through the portal vein and through the hepatic artery. The hepatic artery brings in oxygenated blood, and the portal vein brings in deoxygenated blood with nutrients and hormones (including insulin) from the GI tract. The two blood supplies combine and enter the sinusoids, the capillaries of the liver. Blood flows through the sinusoids, and finally exits through the

central vein. The hepatic artery, portal vein, and bile ducts form the "hepatic triads". The

hepatic triads form the vertices of the hexagonal lobule, the structural unit of the liver.

DIRECT EFFECT MEDIATED BY THE TGFB LIGANDS

The TGFβ ligands likely to have the highest bioactivity in the absence of FST303,

FST315, and FSTL3 are myostatin and activin. Homozygous myostatin knockout mice

were glucose tolerant and protected against insulin resistance[38]. Myostatin is primarily

expressed in muscle and adipose tissue. Activin A has been found to enhance glucose

stimulated insulin secretion of rat and human islets in culture[39], as well as increase β-

cell proliferation[40]. While Activin A inhibits hepatocyte proliferation[41], myostatin has no

known direct effects on the liver. To determine if myostatin and activin had direct

metabolic altering effects on the liver, mRNA expression data was collected.

CHAPTER 2

METHODS

Histology

Liver tissues were isolated from mice and fixed in 4% paraformaldehyde overnight. The tissues were then processed for paraffin embedding. 6 μm microtome sections were stained with PAS reagent (for glycogen) and counterstained with Hematoxylin. Adjacent sections were stained with Hematoxylin for structure determination. The localization of lipids were examined with H&E staining.

Glycogen Extraction

Glycogen extraction was performed as previously described[42]. Liver tissues were harvested and 100 mg were homogenized in 3mL of 10% perchloric acid. Homogenates were centrifuged at 2500 x g for 15 minutes. Supernatant was separated and 5 mL of 100% EtOH was added to each sample. Samples were left in the freezer overnight and centrifuged the following day at 2000 x g. Supernatent was discarded and glycogen pellet was reconstituted in 5 mL of ddH20.

Glycogen Quantification

Glycogen was quantified as previously described[22]. Samples and standards (Glycogen powder from Sigma) were diluted appropriately. 1 mL of 5% phenol was added to 1 mL of diluted sample and vortexed. 5 mL of concentrated sulfuric acid was then added to each sample. Each sample was shaken vigorously and measured with a spectrophotometer at 490 nm.

RT and QPCR

Total RNA was extracted from flash frozen liver tissues with TRIzol (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. RNA was treated with DNase I (Invitrogen) for 15 minutes at room temperature. 1 µg of total RNA was reverse-transcribed to cDNA with Superscript III First-Strand Synthesis SuperMix (Invitrogen) according to manufacturer's instructions. cDNA was amplified using SYBR green and complementary reagents from Stratagene (La Jolla, CA) on a Stratagene MX3000. A standard, made from the cDNA of 25 different livers was run in each PCR for each target, and the quantification of each gene was normalized to mouse ribosomal protein RPL19 to control for RNA quality.

Immunoblotting

Insulin injected mice were sacrificed 10 minutes after injection. Flash frozen liver tissue was homogenized in Cell Lysis buffer (Sigma) containing a protease inhibitor and Phosphatase Inhibitor Cocktail 1 & 2 (Sigma). After the removal of the pellet, protein concentrations were determined colorimetrically using Bradford reagent (Bio-Rad). 30 ug of protein from each lysate were loaded onto 10% precast NuPage mini-gels (Invitrogen). Gels were electrophoresed for 50 minutes at 200V. Proteins were transferred to PVDF membrane via wet electrophoresis for 1 hour at 60V. Membrane was blocked in 10% milk, washed, and incubated with primary antibody overnight at 4 degrees C. Membrane was then washed and incubated in HRP-conjugated secondary antibody for 1 hour. Membrane was washed again, and incubated in luminal hrp-substrate reagent (Bio-Rad) for 3 minutes, and exposed to film. Relative densities were

calculated using UltraQuant density calculation. Mouse monoclonal IRB (1:1000), rabbit polyclonal Ser473 Phospho-Akt (1:1000), rabbit polyclonal Akt (1:1000), rabbit polyclonal B-Actin (1:2000) were purchased from Cell Signaling. Mouse monoclonal Srebp1 (1:100) was purchased from AbCam.

Triglyceride Measurements

50mg of flash frozen liver was homogenized in 2:1 chloroform methanol solution. The organic layer was separated and triglyceride content was measured colorimetrically with enzymatic Triglyceride kit (Sigma). Serum measurements were performed as described above.

CHAPTER 3

RESULTS

Histological analysis of PAS stained liver sections showed different storage patterns in knockout and wildtype mice (Figure 1). WT mice had evenly spread glycogen stores. In FST288-only, DM, and FSTL3 KO mice, the glycogen was stored heterogeneously. Some portions of the liver had heavy stores of glycogen, while others had little to none. There was no obvious pattern as to where glycogen was preferentially stored. Upon glycogen extraction, it was clear that there were no significant differences in glycogen concentrations between any of the genotypes (Figure 3).

PEPCK mRNA levels were not significantly higher in any of the knockout animals (Figure 4). Comparing DM to each background of WT shows ~2 fold increases in G6Pase mRNA levels (Figure 4). FSTL3 KO and 288-only mice have no significant increase, meaning that there is a compensatory effect in each of the single mutant mice, and ablation of both antagonists is necessary for the partial insulin resistant state. IRS1 mRNA levels were about 1.5 fold higher in 288-only mice (Figure 4). It is interesting to note that DM mice had IRS1 levels equivalent to WT. It is also important to point out that there were no significant differences in IRS2 mRNA expression between any of the knockouts and their wildtype littermates (Figure 4). In FSTL3 KO and 288 only mice, GCK mRNA expression is slightly lower than their respective wildtypes (Figure 5), suggesting insulin resistance at this level, and potentially allowing glucose to reenter the bloodstream.

Insulin Receptor (IR) protein levels were significantly elevated in FST288-only mice (Figure 6). Akt is an important signaling molecule which mediates the metabolic actions of insulin [43]. Akt is phosphorylated and activated downstream of IR and IRS1/IRS2. Akt protein levels were similar for FST288-only, DM, and WT (Figure 7). However, the pAkt/Akt levels of 288-only mice were significantly higher than FST WT. Sterol regulatory element binding protein 1c (SREBP1c) mRNA levels were not different between the genotypes (Figure 8).

DM mice had PPARγ mRNA expression at about 50% the level of their wt littermates, which was an unexpected result due to their steatotic livers (Figure 8). Lipase C and LDLR mRNA levels displayed similar patterns across the genotype panel, with 288-only mice exhibiting significantly higher expression levels for both genes (Figure 9). LDLR mRNA expression was ~5 fold higher in 288-only mice than FST WT. Given this information, it is possible that 288-only mice have increased LDL uptake in the liver, causing the steatosis.

To determine if our mice had hepatic steatosis and to what extent, lipid extraction was performed and measured colorimetrically (Figure 10). FSTL3 KO, 288 only, and DM mice each had significantly higher triglyceride concentrations in their livers than WT mice. Next, to investigate hepatic triglyceride localization, livers were sectioned and stained with Hematoxylin and Eosin (Figure 11). All three mutant lines had a similar hexagonal pattern of TG accumulation. The liver sections show the TG accumulation primarily taking place at the perimeter of the hepatic lobule. This is where all the blood enters the liver, thus suggesting that excess fat is initially coming in through the blood.

To test this, a random serum triglyceride assay was performed. No significant differences were found between the genotypes (Figure 12).

There were no detectable levels of myostatin mRNA in the liver (Figure 13). This could be due to levels being lower than the lower detection limits of a qPCR, or because myostatin is not made in the liver. Activin A is slightly elevated in FSTL3 KO, but slightly decreased in 288-only. Activin B expression was slightly decreased in DM mice.

CHAPTER 4

DISCUSSION

Hepatic glycogen is a good physiological marker for insulin sensitivity but it was rather difficult to quantify glycogen stores based on the histology. Apparent amounts differed drastically, even on the same liver section (Figure 2). It is possible that the triglyceride deposits may have shifted glycogen stores to peripheral areas, but we are not certain that this caused the pattern. The difficulty in analyzing heterogeneous tissue made it necessary to extract the glycogen out of the liver and quantify it colorimetrically. We purchased an enzymatic glycogen quantification kit from BioVision, but did not get reproducible results. A sulfuric acid-phenol quantification eventually proved to be the most reliable, with a consistent correlation coefficient of .999 for the standard curve. After quantification it was evident that glycogen concentrations in the knockout livers were not significantly different from wildtype. This was in part due to a large variability between each mouse in the fed state (Table 3).

FSTL3 KO mice appeared to be insulin sensitive. They had normal levels of PEPCK, G6Pase, IRS1, and IRS2 mRNA expression. FST288-only mice appeared to be even more insulin sensitive than WT mice, expressing normal levels of PEPCK, G6Pase, and IRS2, but increased levels of IRS1 mRNA. FST288-only mice also had increased IR (insulin's role in the downregulation of its own receptor is well established[44]) and pAkt/Akt protein levels. The DM mouse also appeared to be relatively insulin sensitive. They expressed normal levels of PEPCK, IRS1, and IRS2, but increased G6Pase, a sign of resistance. However, because IR and pAkt/Akt levels

were normal as well, DM livers still appear to be sensitive to insulin. The hepatic steatosis does not appear to originate with hepatic insulin resistance in the glucose regulatory pathway.

An important point to realize is that the DM is a combination of both backgrounds. This can disguise certain results. For example, the FSTL3 background had ~2.5 fold higher IRS2 levels than the FST background. DM had levels consistent with the FST background. This shows that the FST background has a dominant effect on IRS2 gene transcription when combined with the FSTL3 background. Until further research is done, it is impossible to infer that DM had reduced IRS2 expression because of the comparison between DM and FSTL3 WT.

SREBP1 protein measurements were difficult as the primary antibody was a mouse monoclonal and required an anti mouse HRP-conjugated secondary antibody. The anti mouse secondary antibody was detected in no primary loading controls at ~115 kDa and 55 kDa sizes when gel was run in non-reducing conditions. When run in reducing conditions, the upper band disappeared, allowing for the detection of the 125 kDa SREBP1 band.

SREBPs are translated as precursors which are bound to the endoplasmic reticulum and the nuclear membrane. Upon activation, they are cleaved and able to enter the nucleas where they activate target gene transcription[45]. The mRNA expression of SREBP1c is increased in isolated rat hepatocytes exposed to insulin[46], indicating that insulin should have an effect on transcription. SREBP1c mRNA levels were not elevated in any knockout mouse. However, SREBP1 *protein* levels were elevated in DM mice, meaning that lipogenesis likely had a role in the accumulation of hepatic TG in these

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mice. FST288-only mice had normal SREBP1 protein levels, but increased LDLR and Lipase C mRNA expression. Thus, DM appear to have increased lipogenesis and FST288-only seem to have increased lipid uptake.

The pattern of lipid deposits suggested that the cause for fatty liver begins with the incoming blood. Serum TG measurements indicated that at any given time, there was no increase. It is possible that the FSTL3 KO, 288-only, and DM mice have already accounted for the high peripheral release of lipids via hepatic storage. This would suggest that the liver is working as a buffer for serum TG levels. Visceral fat pad mass is reduced by 33% in each knockout but total body fat remains the same (FSTL3 KO, 288-only), or increases (DM) (Unpublished data) indicating that there is some systemic alteration in lipid storage. It is unclear as to if there was initially a normal size fat pad and it just deteriorated, or if fat was never stored there. The former would be worth testing to determine if adipose insulin resistance caused the fatty liver.

Myostatin primers worked well with muscle tissue, showing that the reagents were functional. The assay was repeated, and the lack of myostatin found in the liver was consistent. The differences in TGFβ ligand mRNA expression were minor. Activin A expression was not significantly different between knockout and wildtype mice. While there are differences, there is no evidence that these ligands directly act on liver tissue to change the metabolic profile of the mouse. All indications are that these ligands act peripherally to induce the metabolic phenotype. Even though activin B was reduced in DM mice, more research needs to be done to determine what effect it had on metabolism. In the future we will culture hepatocytes and treat with TGFβ ligands.

CONCLUSIONS

Activin was discovered as a reproductive hormone with important functions as a stimulator of FSH secretion in the pituitary. Follistatin (FST), named for its inhibitory effect on FSH secretion, was found to be an extracellular antagonist of activin irreversibly binding to activin and preventing it from binding to its receptor. FST is produced in many tissues, with the highest expression being in ovarian tissue[34]. Also part of the TGFβ family is myostatin, an inhibitor of muscle growth. FST also binds to myostatin, rendering it inactive. FSTL3 is similar in structure to FST, made in many tissues, but highest expression occurs in testes and placenta[47]. FSTL3 also blocks myostatin and activin activity in the same manner and like FST, is made to balance the potent effects of these TGFβ ligands. Activin, although vital as a reproductive hormone, was also found to have metabolic functions. The FSTL3 knockout mouse was generated in order to better characterize activin effects on glucose homeostasis. Because the deletion of FSTL3 and/or FST increases the activity of both activin and myostatin, they both were considered in this study.

Activin and myostatin appear to increase islet function in vivo. The higher serum insulin levels in response to similar serum glucose levels in the DM mouse are an indication that beta cells are overproducing. Further evidence for increased beta cell function is an increase in beta cell area and mass, and a higher level of pancreatic intracellular insulin content. The single knockouts also demonstrate increased insulin output, with DM > FSTL3 KO > 288 only > wildtype in regards to serum levels.

When peripheral tissues become resistant to insulin over time, beta cells detect more glucose in the blood supply prompting the secretion of more insulin. This is the beginning of the pathological condition, ultimately resulting in type 2 diabetes when beta cells cannot keep up with the demands of downregulated insulin response pathways. A good indication of insulin resistance is normal glucose levels complemented by high insulin levels. Higher insulin levels should promote lower glucose in the blood unless the response to insulin is attenuated somehow.

DM mice had a reduced ability to clear high levels of glucose from the blood as well as reduced glucose clearance in response to insulin injection. FSTL3 KO mice had normal glucose, increased insulin, but did not turn out to be insulin resistant. In fact, FSTL3 KO had glucose tolerant and insulin sensitive results. Deleting only FSTL3 had a less potent effect on islet function, with peripheral tissues remaining sensitive to insulin. When both antagonists are removed, the effect is a giant increase in islet function and large quantities of insulin are secreted. It is possible that insulin is secreted in excess so dramatic that it causes peripheral tissues such as liver, muscle and/or adipose tissue to eventually decrease response. The activity of myostatin can be postulated to be higher in DM mice, not only because of the genetic background, but because of muscle data. The single deletion mice have no change in muscle mass, while the DM mouse has a 40% reduction. Myostatin is known to be a potent muscle growth inhibitor[48]. The activity of activin is also likely to be higher due to the double mutant genotype. The phenotype also could be a secondary effect of the enhanced islets producing and secreting too much insulin. Their serum insulin levels were higher than WT and the single deletion mice, while their serum glucose was normal. This means that more insulin was

necessary to clear the same amount of glucose. It could also indicate that elevated insulin is the primary change and high insulin causes the necessity for the mice to eat more to balance glucose levels. To investigate further, a GTT was run. The GTT showed that blood glucose levels remained higher for longer after a glucose injection. An ITT was run to determine if their tissues were insulin sensitive or not. This showed that given the same amount of insulin, they cleared less glucose (unpublished data). All results in the DM mouse point to highly functioning islets with peripheral insulin resistance as a secondary effect. This shows that the ablation of the two main antagonists of myostatin and activin had a synergistic effect.

Hepatic steatosis was found in all three genetically modified mice. This is a disorder heavily correlated with obesity, metabolic syndrome, and type 2 diabetes. Hepatic triglyceride accumulation in addition to the aforementioned insulin increase prompted the hypothesis that insulin resistance caused the steatotic livers. Hepatic specific insulin resistance was tested. FSTL3 KO mice appeared to be insulin sensitive. They had normal levels of PEPCK, G6Pase, IRS1, and IRS2 mRNA expression. FST288-only mice appeared to be more insulin sensitive than WT mice, expressing normal levels of PEPCK, G6Pase, and IRS2, but increased levels of IRS1 mRNA. FST288-only mice also had increased insulin receptor (insulin's role in the downregulation of its own receptor is well established[49]) and pAkt/Akt protein levels. The DM mouse also appeared to be relatively insulin sensitive. They expressed normal levels of PEPCK, IRS1, and IRS2, but increased G6Pase, a sign of resistance. But because insulin receptor and pAkt/Akt levels were normal as well, DM mice still appear to be sensitive to insulin in their liver. Histological examination of glycogen was

inconclusive. In the knockout mice, it was difficult to determine whether there were higher levels of glycogen or not because of the heterogeneous deposits. Liver glycogen extractions showed no significant differences between the genotypes, including DM. Future research will include testing more mRNA and protein targets from the insulin signaling pathway to be certain of the hepatic insulin sensitivity. It will also include radiolabeling metabolites and following them as they are incorporated or broken down in the liver. Lastly, it will include testing adipose tissue for insulin resistance, because this is where fat should be stored; not the liver. It does not appear as though hepatic steatosis causes hepatic insulin resistance in these mice.

Increased SREBP1 protein levels suggested that lipogenesis was the primary cause of fatty liver in the DM. The increased LDLR and Lipase C mRNA suggested that increased lipid uptake caused the fatty liver in FST288-only mice. The histology of the liver tissues of all three genotypes shows that fat accumulation occurred preferentially surrounding the areas where blood first enters the liver. It makes sense that as hepatocytes clear lipids from the blood, the reduction in blood lipids causes less to be taken up by the areas furthest from the entry point. To explain the DM, insulin directly activates SREBP1c mediated lipogenic action and insulin enters the liver from the same blood supply as lipids. Hepatocytes near the entry point would be affected the most. Thus, the cause for the hepatic steatosis was not confirmed. The interesting thing is that the mice present different models for fatty liver. None of them include hepatic insulin resistance as a cause, disagreeing with both popular beliefs: 1) Hepatic insulin resistance causes steatosis and 2) Steatosis causes hepatic insulin resistance.

Table 1. Comparison of Tissue Specific Insulin Receptor Knockout Mice

	Circ glucose	Circ Insulin	Circ FFA, TG
LIRKO	High	High	Low
MIRKO	Normal	Normal	High
FIRKO	Normal	Low	Low

Comparison of tissue-specific insulin receptor knockouts.

Table 2. Metabolic Comparison of FSTL3 KO, FST288-Only, DM Mice

	BW	Visceral Fat Pad	% Body Fat	Muscle Mass	Serum Glucose	Serum Insulin	GTT	ITT
FSTL3 KO[50]	Reduced	Reduced	Normal	Normal	Normal	Slightly Increased	Tolerant	Sensitive
FST288 -only	Reduced	Reduced	Normal	Normal	Normal	Normal	Normal	Normal
DM	Normal	Reduced	Increased	Reduced	Normal	Increased	Intolerant	Insensitive

Metabolic comparison of each of the genetically modified mice. Results relative to respective WT, or both WTs in the case of DM

Table 3. Variability of Liver Glycogen Concentrations Between Mice

mg Glycogen/ g Liver Tissue in each mouse				
fstl3 wt	fstl3 ko	fst wt	288-only	DM
51.08	20.63	45.92	39.61	73.09
17.54	38.99	43.05	50.95	42.57
46.82	51.91	18.11	43.77	36.76
32.20	40.53	8.21	11.51	34.51
24.25	29.15	6.26	16.53	29.46
12.38	13.63		18.60	30.44
	28.94			30.26

Hepatic glycogen concentrations for each sample, demonstrating the large variability.

Figure 1. Histologic Comparison of Liver Tissue Glycogen
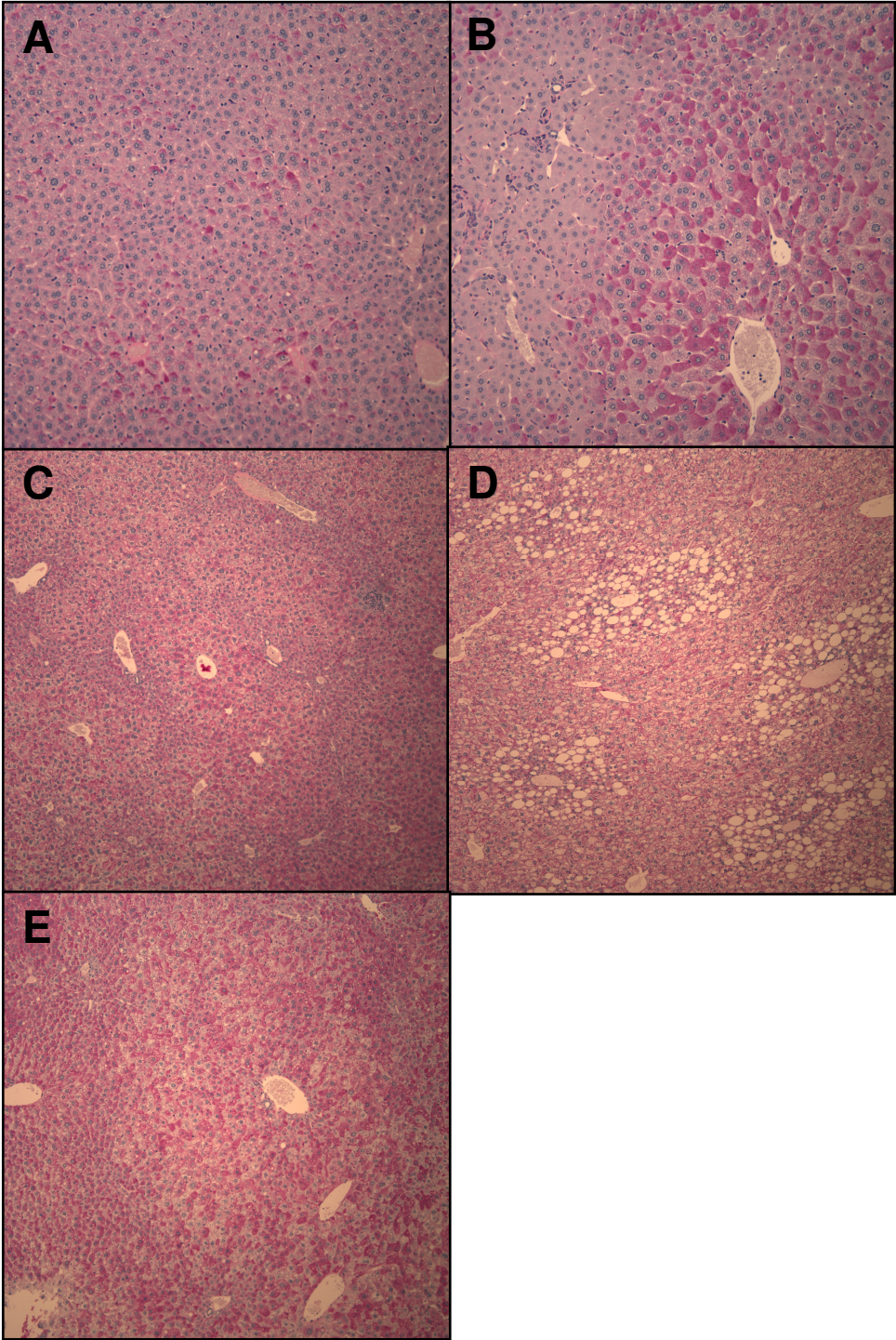
Fstl3 wt (A), fstl3 ko (B), fst wt (C), fst288-only (D), and dm (E) liver sections stained with Periodic acid-Schiff's stain. Glycogen stains magenta. (Magnification: x10)

Figure 2. Intrahepatic Variability in Glycogen Storage
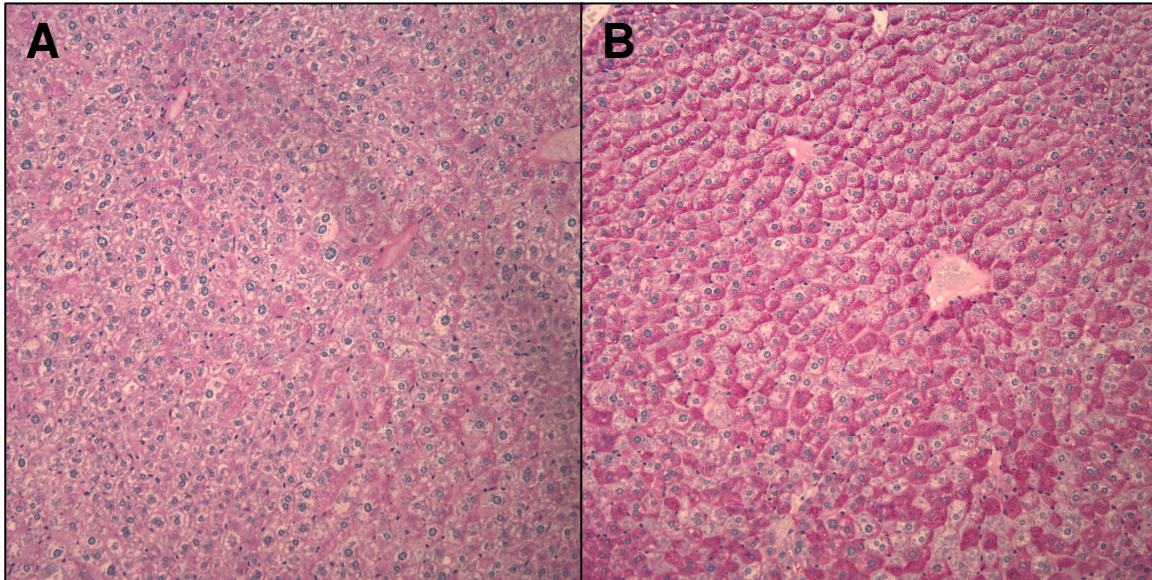
Liver sections stained with Periodic acid-Schiff's stain. Micrographs taken from the same section of fstl3 ko tissue (A, B). Glycogen stains magenta. (Magnification: x10)

Figure 3. Biochemical Glycogen Analysis
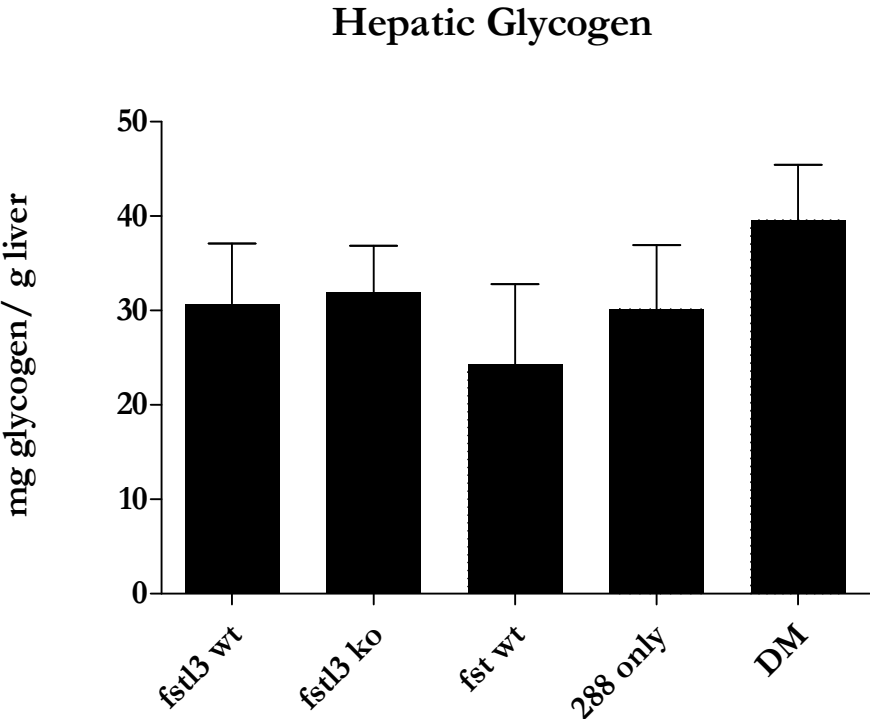
Hepatic Glycogen

Hepatic glycogen concentrations measured via extraction and colorimetric quantification.

Figure 4. mRNA Expression of Insulin Response Components
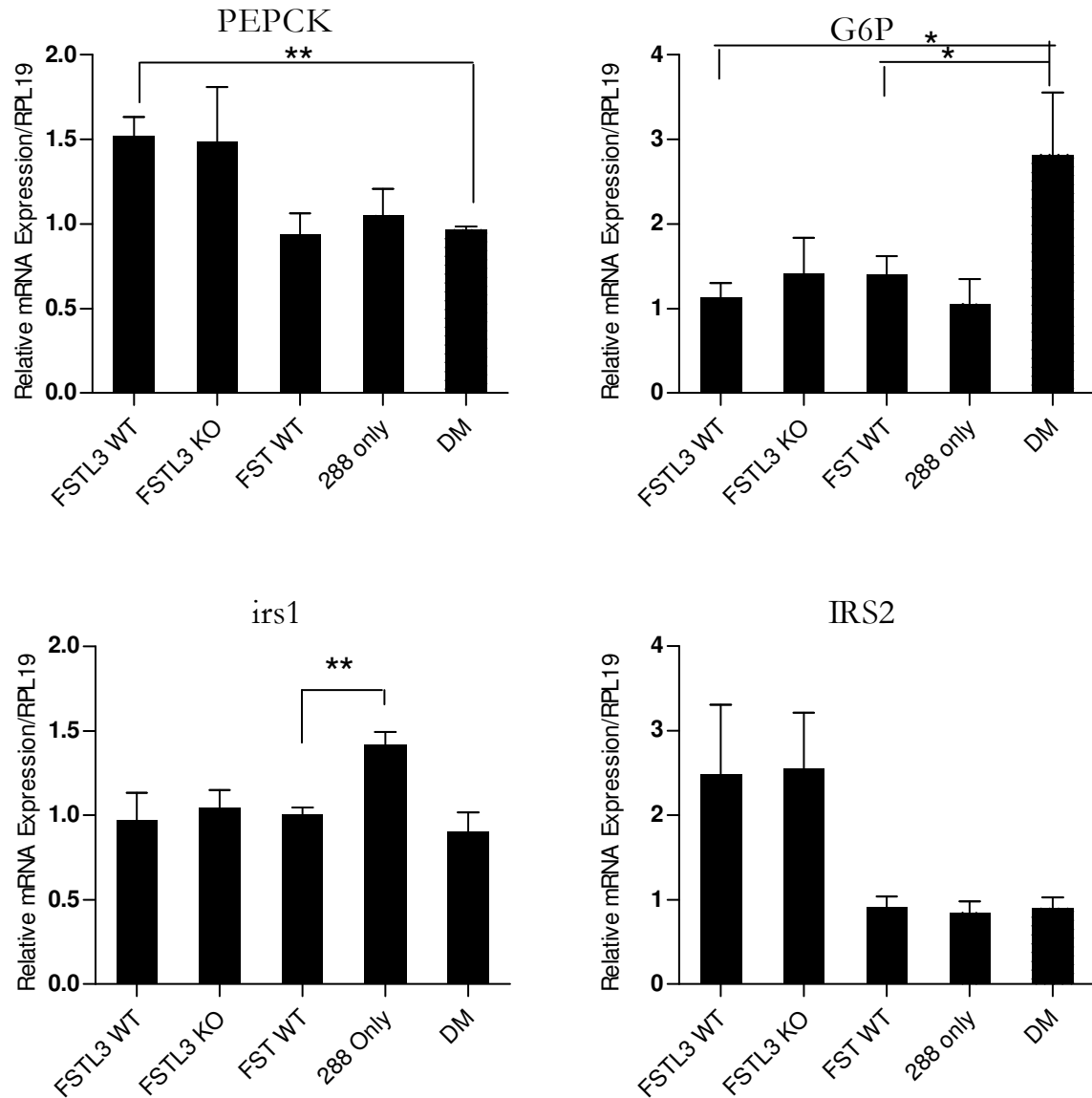
mRNA expression levels of gluconeogenic genes PEPCK and G6P, and insulin receptor substrates 1 & 2 (IRS1, IRS2). Significance marked by * ($p < .05$) and ** ($p < .01$).

Figure 5. Gck mRNA expression
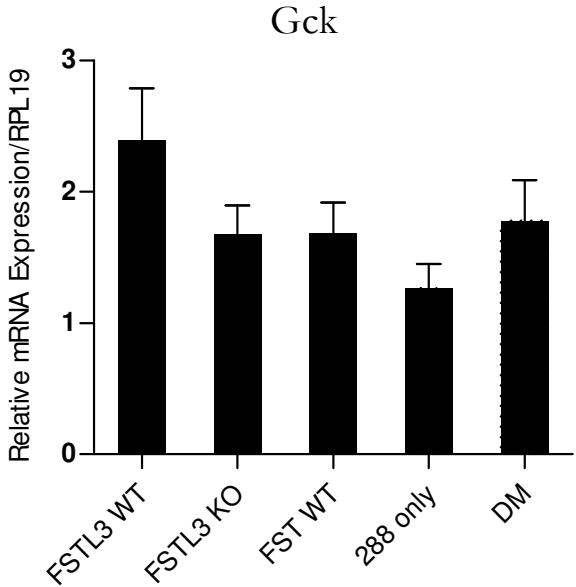
mRNA expression levels of glucokinase (GCK), Significance marked by * (p < .05) and ** (p < .01).

Figure 6. Relative IRβ Protein Levels
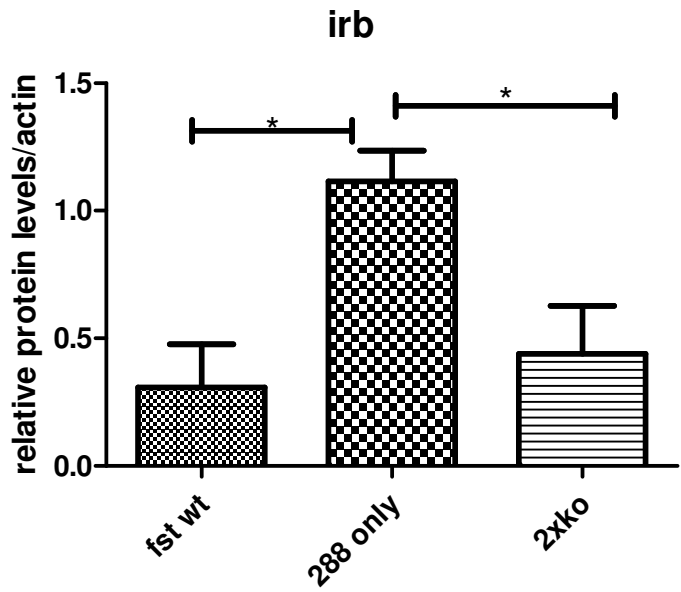
Western blot; protein expression levels of IRβ normalized to β-Actin. Significance marked by * (p < .05) and ** (p < .01).

Figure 7. Relative pAkt Levels
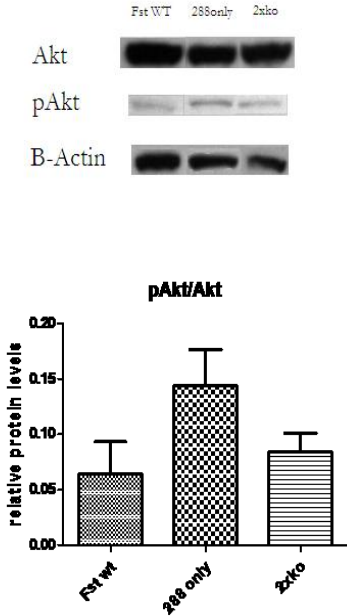
Western blot; phosphorylation levels of Akt. Significance marked by * (p < .05) and ** (p < .01).
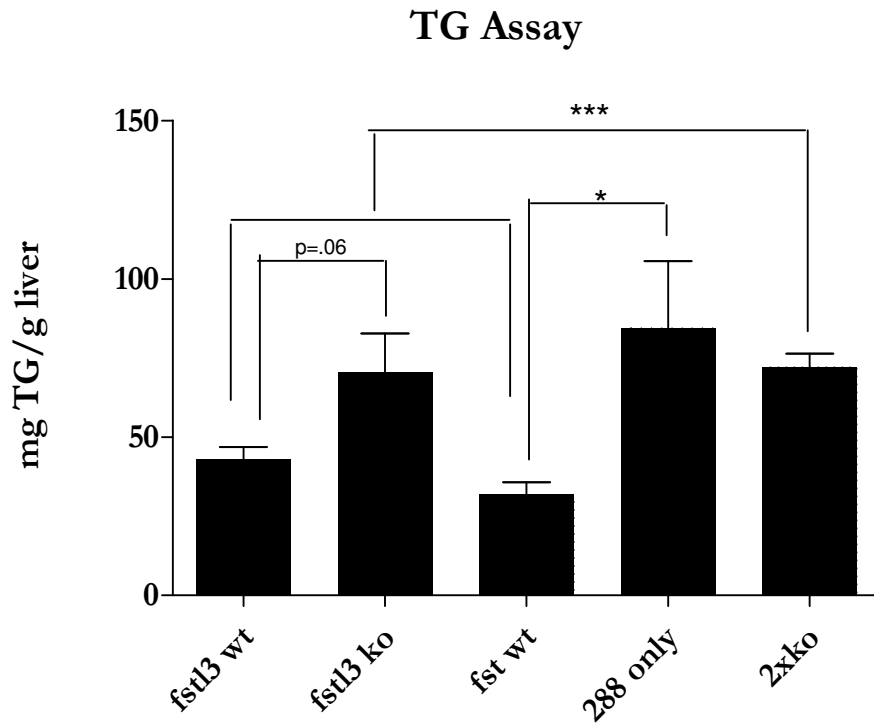
Figure 8. mRNA Expression of SREBP1 and PPARy
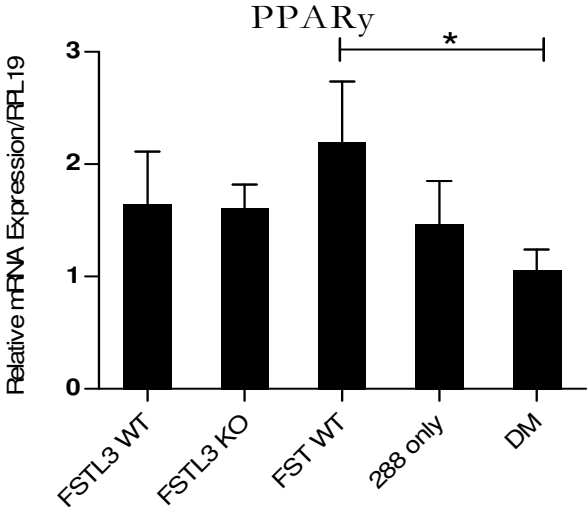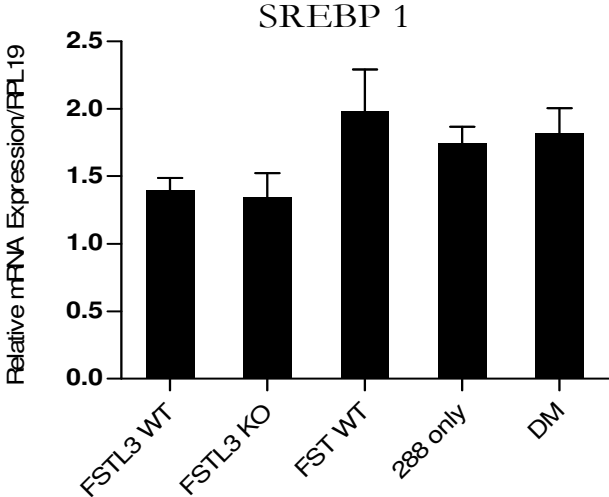
mRNA expression levels of SREBP1 and PPARγ. Significance marked by * (p < .05) and ** (p < .01).

Figure 9. mRNA Expression of Lipase C and LDLR
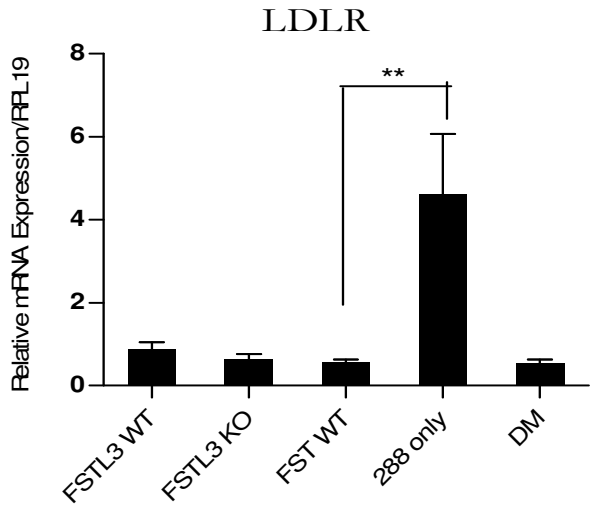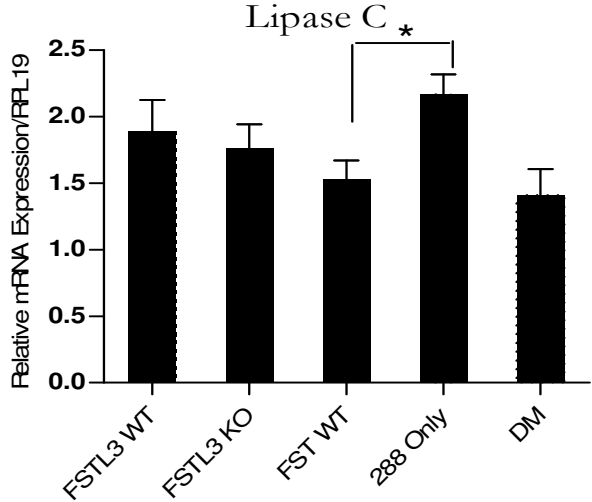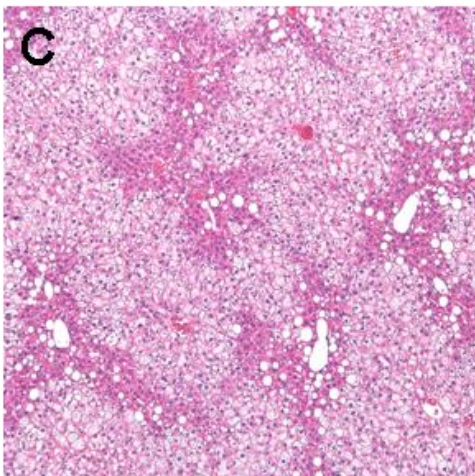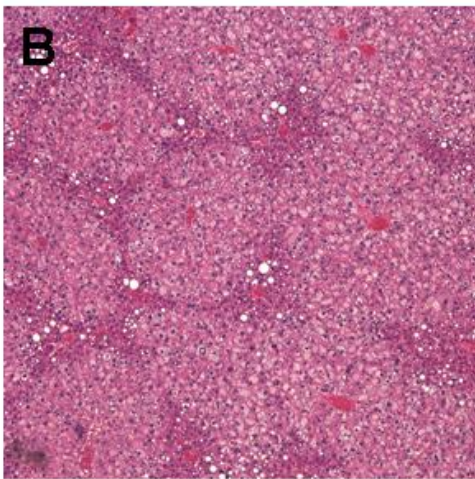

mRNA expression levels of Lipase C and LDLR. Significance marked by * (p < .05) and ** (p < .01).

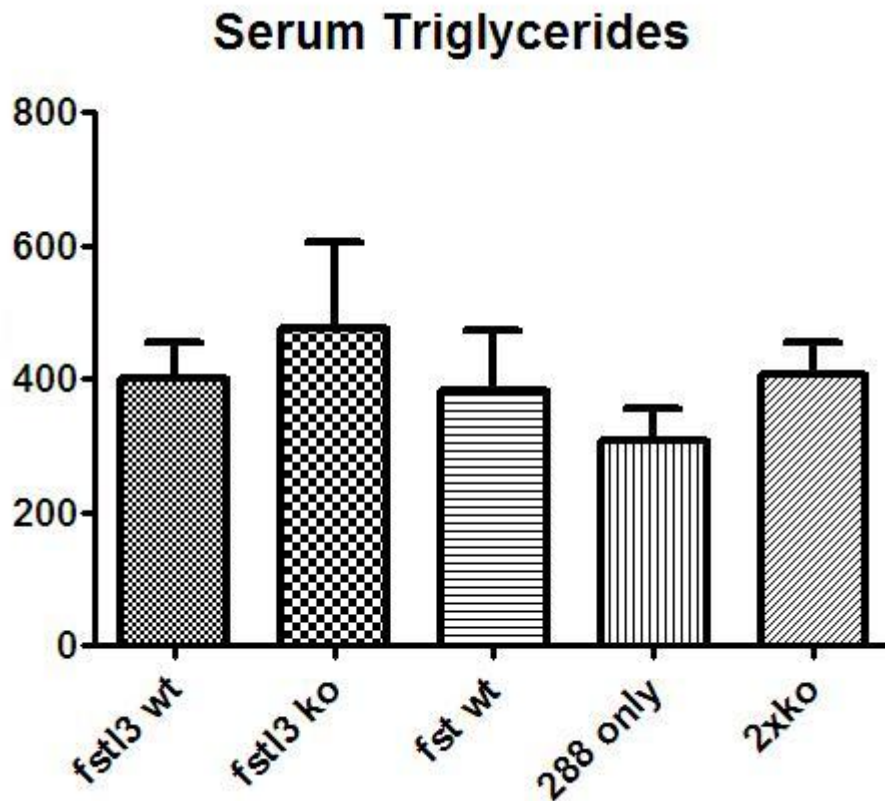**Figure 10. Hepatic Triglyceride Concentrations**

TG Assay

Liver triglyceride concentrations normalized to weight of tissue. Significance marked by * ($p < .05$), ** ($p < .01$), and *** ($p < .001$).

Figure 11. Fatty Liver Patterns
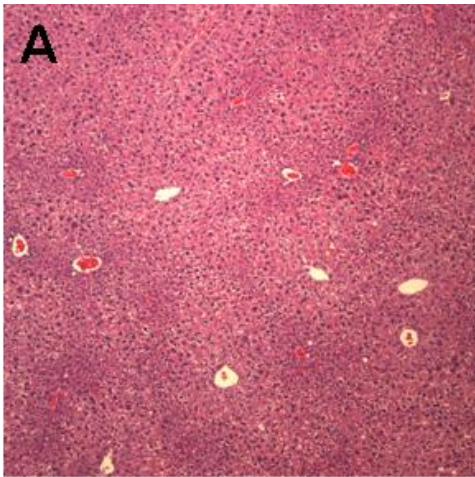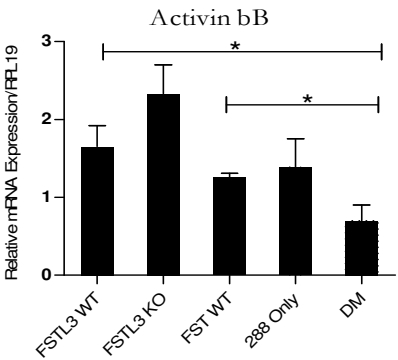

Liver sections stained with H&E. Triglycerides appear as clear deposits. (Magnification: x10)

Figure 12. Serum Triglyceride Levels

Serum triglyceride concentrations. Significance marked by * (p < .05), ** (p < .01), and *** (p < .001).

Figure 13. mRNA Expression Levels of TGFβ Ligands

MSTN

BA
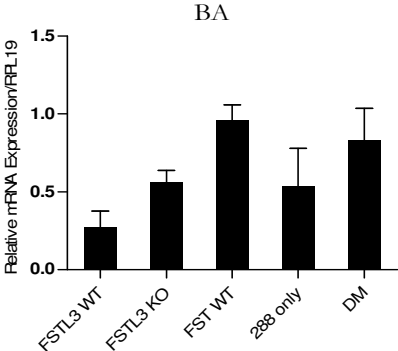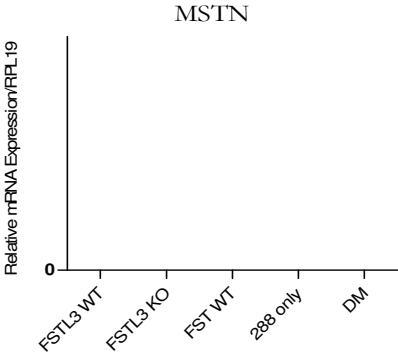
Activin bB

mRNA expression levels of myostatin, activin bA, and activin bB. Significance marked by * (p < .05) and ** (p < .01).

Figure 14. Structure of Hepatic Lobule

Liver Lobule

Detail of Lobule

- Hepatic venule
- Branch of hepatic artery
- Hepatocytes
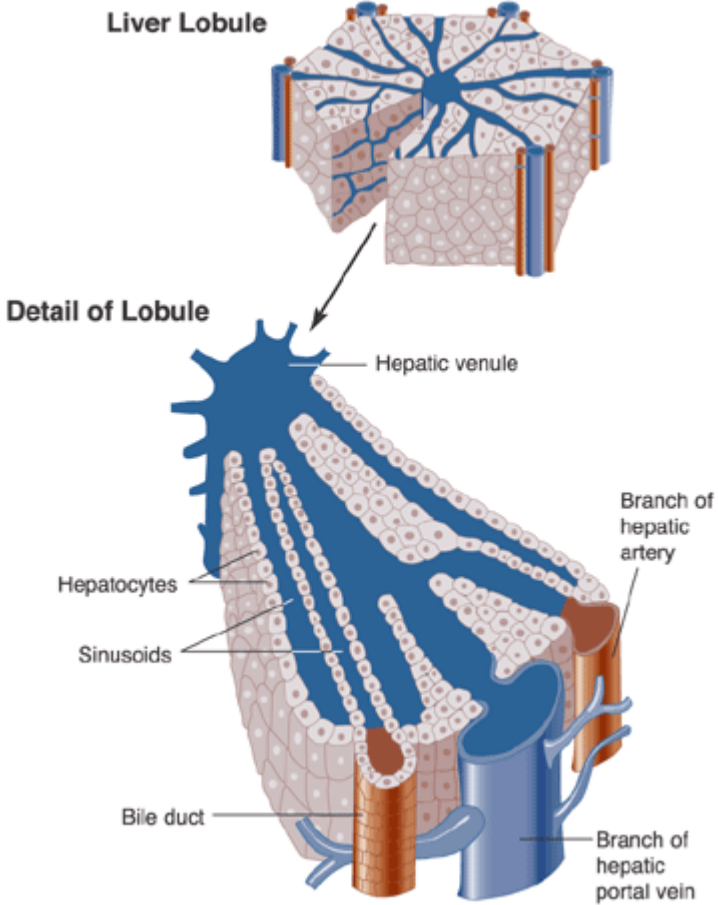- Sinusoids
- Bile duct
- Branch of hepatic portal vein

Diagram of functional unit of hepatic lobule (Cunningham, C.C., and Van Horn, C.G. Energy availability and alcohol-related liver pathology. *Alcohol Research & Health*27(4):281–299, 2003.)

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