Lipodystrophy Due to Adipose Tissue Specific Insulin Receptor Knockout Results in Progressive NAFLD

Samir Softic1,2,#, Jeremie Boucher1,3,#, Marie H. Solheim1,4, Shiho Fujisaka1, Max-Felix Haering1, Erica P. Homan1, Jonathon Winnay1, Antonio R. Perez-Atayde5, and C. Ronald Kahn1.

1 Section on Integrative Physiology and Metabolism, Joslin Diabetes Center and Department of Medicine, Harvard Medical School, Boston, MA
2 Division of Gastroenterology, Hepatology and Nutrition, Boston Children’s Hospital, Boston, MA
3 Cardiovascular and Metabolic Diseases iMed, AstraZeneca R&D, 431 83 Mölndal, Sweden (current address)
4 KG Jebsen Center for Diabetes Research, Department of Clinical Science, University of Bergen, Bergen, Norway
5 Department of Pathology, Boston Children’s Hospital, and Harvard Medical School, Boston, MA
# These authors contributed equally to this work.

Corresponding author:

C. Ronald Kahn, MD
Joslin Diabetes Center
One Joslin Place
Boston, MA 02215
Phone: (617)732-2635
Fax:(617)732-2487
E-mail: c.ronald.kahn@joslin.harvard.edu

Keywords: Insulin receptors, IGF-1 receptors, lipodystrophy, diabetes, dyslipidemia, fatty liver, liver tumor, NAFLD, NASH.

Running title: Lipodystrophic mice develop progressive NAFLD
SUMMARY

Ectopic lipid accumulation in the liver is an almost universal feature of human and rodent models of generalized lipodystrophy and also is a common feature of type 2 diabetes, obesity and metabolic syndrome. Here we explore the progression of fatty liver disease using a mouse model of lipodystrophy created by a fat-specific knockout of the insulin receptor (F-IRKO) or both IR and insulin-like growth factor-1 receptor (F-IR/IGF1RKO). These mice develop severe lipodystrophy, diabetes, hyperlipidemia and fatty liver disease within the first weeks of life. By 12 weeks of age, liver demonstrated increased ROS, lipid peroxidation, histological evidence of balloon degeneration and elevated serum ALT and AST levels. In these lipodystrophic mice, stored liver lipids can be utilized for energy production as indicated by a marked decrease in liver weight with fasting and increased liver FGF21 expression and intact ketogenesis. By 52 weeks of age, liver accounted for 25% of body weight and showed continued balloon degeneration in addition to inflammation, fibrosis, and highly dysplastic liver nodules. Progression of liver disease was associated with improvement in blood glucose levels with evidence of altered expression of both gluconeogenic and glycolytic enzymes. However, these mice were able to mobilize stored glycogen in response to glucagon. Feeding F-IRKO and F-IR/IGFRKO mice a HFD for 12 weeks accelerated the liver injury and normalization of blood glucose levels. Thus, severe fatty liver disease develops early in lipodystrophic mice and progresses to advanced NASH with highly dysplastic liver nodules. The liver injury is propagated by lipotoxicity and is associated with improved blood glucose levels.
INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is a common manifestation of diabetes, obesity, and metabolic syndrome. NAFLD also occurs in both human and rodent models of lipodystrophy (1-4). In the context of obesity and metabolic syndrome, NAFLD may progress to include liver inflammation or non-alcoholic steatohepatitis (NASH), fibrosis and occasionally hepatocellular carcinoma (5), whereas not much is known about natural history of liver disease in the context of lipodystrophy.

Metabolic syndrome is present in 80% of patients with lipodystrophy, almost universally manifesting as severe insulin resistance, profound hypertriglyceridemia, and ectopic lipid accumulation (6). One major difference between this form of metabolic syndrome and that associated with obesity is in the amount of adipose tissue, which is reduced in lipodystrophy, resulting in low levels of leptin (7), whereas obesity is associated with increased adiposity and high leptin levels. In regards to other metabolic outcomes, including ectopic fat accumulation in the liver, lipodystrophy resembles an extreme version of the obesity-associated metabolic syndrome (8). Whether lipid accumulation in the liver is a sign of a disease or a physiologic response in patients who have minimal capacity to store lipids in the adipose tissue is unknown. In lipodystrophic patients the liver fat decreases with leptin treatment (9; 10), suggesting that at least a portion of stored liver fat may be utilized for ketogenesis (11). It is unknown what processes mediate ketogenesis in the setting of minimal adipose tissue lipid stores, but it is interesting to note that serum levels of the ketogenic hormone, FGF21 are elevated with lipodystrophy in HIV-1 infected patients (12), and that the main source of elevated serum FGF21 levels in mice with lipodystrophy is the liver (13).
The liver disease in some patients with generalized lipodystrophy can progress to liver cirrhosis requiring liver transplantation (14). The most characteristic feature of fatty liver disease associated with lipodystrophy is prominent steatosis with hepatocyte balloon degeneration (8; 15), pointing to lipotoxicity as an important pathway of liver injury. Chronic liver injury resulting in cirrhosis and liver failure can be associated with reduced hepatic glucose production leading to hypoglycemia (16). What effects liver lipotoxicity may have on hepatic glucose homeostasis in the setting of lipodystrophy is unknown.

Here, we show that mice lacking IR or both IR and IGF1R in adipose tissue display a lipodystrophic phenotype associated with severe diabetes and NAFLD. The liver disease in these mice progresses to NASH, fibrosis and highly dysplastic liver nodules. Interestingly, this progressive liver disease is associated with improvement of hyperglycemia in these mice with age, despite the persistent insulin resistance and a normal ability to mobilize stored glycogen in response to glucagon. The liver injury in these lipodystrophic mice is propagated by lipotoxicity, and high fat diet (HFD) feeding accelerates the progression of liver disease, leading to normalization of blood glucose levels.
MATERIAL AND METHODS

Animals and diets

All protocols were approved by the IACUC of the Joslin Diabetes Center and were in accordance with NIH guidelines. Fat-specific IR, IGFR, and IR/IGFR knockout mice were generated as described (17). Mice were housed at 20-22°C on a 12 h light/dark cycle with ad libitum access to water, and food. For HFD experiments the mice were fed chow (Mouse Diet 9F, PharmaServ) diet until 8 weeks of age and then continued on chow or switched to 60% high fat diet (Research diets, D12492) for an additional 12 weeks.

Glucose tolerance test and glucagon response

Glucose tolerance tests were performed on overnight fasted mice injected with 2 mg/kg of dextrose IP. Glucagon response was assessed in overnight fasted mice injected with 1U/kg of glucagon (Sigma G2044) with blood glucose measured at 0, 15, 30, 60 and 120 minutes. Glucose levels were measured using a glucose meter (Infinity, US Diagnostics).

Tissue and serum analyses.

Tissues were stored frozen or fixed in formalin and sections were stained with Hematoxylin and Eosin (H&E) or Periodic acid-Schiff (PAS). mRNA extraction and quantification was performed as previously described (18). Serum parameters were determined by the Joslin’s DRC assay core using commercial kits. Hormones were assessed by ELISA, adipokines were measured by a multiplex assay and ALT and AST
were assessed by colorimetric assays. Triglycerides from liver samples were measured with a triglyceride quantification kit (Abnova) as described by Debosch et al. (19).

**Protein extraction and immunoblot analysis**

Tissues were homogenized in RIPA buffer (EMD Millipore) with protease and phosphatase inhibitor cocktail (Biotools). Proteins were separated using SDS-PAGE and transferred to PVDF membrane (Millipore). Immunoblotting was performed using the indicated antibodies: phospho-IR/IGFR (#3024) and p-PTEN (#9552) from Cell Signaling Technologies and IR (#SC-711), PTEN (#SC-9145) and actin (SC-1616) from Santa Cruz Biotechnology. Quantification of immunoblots was performed using ImageJ.

**Immunohistochemistry (IHC) and Immunofluorescence (IF)**

IHC was performed on formalin-fixed, and IF on frozen sections. In brief, paraffin embedded, formalin-fixed sections were deparaffinized and rehydrated. Antigen was recovered by boiling in citrate buffer (Vector Labs, H-3300), incubating in Triton-X and digested with proteinase K. Sections were blocked in goat serum and incubated overnight at 4°C for IHC with primary antibodies against beta catenin (Cell Signaling, D13A1), F4/80 (Abcam ab6640) or 4-HNE (Abcam, ab46545). Subsequently, slides were incubated with biotinylated secondary antibody, and the reaction was developed using avidin/streptavidin horseradish peroxidase (Vector Labs, PK-4001). For IF, slides were incubated overnight with primary antibody against Ki67 (BD, 556003) and PIP3 (Echelon, P-0008) followed by a secondary antibody labeled with Texas Red (Vector Labs) or Alexa Fluor (Invitrogen) and counterstained with DAPI mounting media (Vector
Labs, H-1500). DHE stain was performed on frozen liver sections fixed with 4% PFA and stained with 1:1500 dilution of DHE (10 ug/ml) dye for 10 min at 37°C. Slides were washed twice in PBS, coverslipped and images were captured using Olympus fluorescence microscope (Olympus BX60).

**In vivo glucose uptake**

Following 12 weeks of HFD, control, F-IRKO and F-IR/IGFRKO mice were injected IP with 2 mg/g of glucose (D20) in combination with 0.33 mCi [14C]2-deoxyglucose/g of body weight. After 15 min, [14C] levels in the liver and quadriceps muscle were determined as previously published (20).

**Statistical analyses**

All data are presented as mean ± SEM and analyzed by unpaired two-tailed Student’s *t*-test or analysis of variance (ANOVA) as appropriate. Significant difference noted with one star (*) represent a p value of <0.05, two stars (**) a p value of <0.01, and three stars (***) a p value of <0.001 throughout the figures and the paper.
RESULTS

Lipodystrophic mice develop fatty liver disease and lipotoxicity

Mice with fat-specific knockout of the insulin receptor (IR), the insulin-like growth factor-1 receptor (IGF1R) or both were generated by breeding mice with IR and IGF1R floxed alleles (18) and mice carrying a Cre-recombinase transgene driven by the adiponectin promoter (21). Whereas knockout of the IGF1R had minimal impact on white and brown fat development, F-IRKO and F-IR/IGFRKO mice displayed virtually from birth profound reduction in weights of both subcutaneous and visceral WAT depots (17). Lipodystrophy was associated with hyperglycemia (Figure 1A), and severe diabetes at 12 weeks of age. At this time, liver weights of both F-IRKO and F-IR/IGFRKO mice were increased 4- to 5-fold above the control, while the liver weight of F-IGFRKO mice remained normal (Figure 1B). Hepatomegaly in F-IRKO and F-IR/IGFRKO mice was associated with a 3- to 5-fold increase in liver triglyceride accumulation (Figure 1C and D). There was also 2- to 5-fold increases in expression of genes involved in de novo lipogenesis such as Acc1, Fas and Scd1 (Figure 1E). However, at this age, these mice did not display an increase in expression of Tnf-α, and F4/80, although there was some increase in the macrophage marker CD11c (Figure 1F). F-IRKO and F-IR/IGFRKO mice also did not show significant fibrosis as assessed by trichrome, sirius red and reticulin stains (Sup. Fig. 1), and mRNA levels of Tgf-β and αSma were not elevated. Coll1a1 was increased in F-IR/IGFRKO mice, however, in the absence of other markers of fibrosis, it may be a sign of stellate cell activation (22) (Figure 1G). Histological assessment of liver at 12 weeks of age confirmed that lipodystrophic F-IRKO and F-IR/IGFRKO mice exhibited micro- and macrovesicular steatosis and the presence of balloon degeneration,
with no significant inflammation or fibrosis (Table 1). Hepatocyte balloon degeneration is indicative of lipotoxicity and is associated with a progressive form of NAFLD (23; 24). By 12 weeks of age, ROS levels and lipid peroxidation, as assessed by dihydroethidium (DHE) and 4-hydroxynonenal (4-HNE) stains respectively, were also increased in livers of F-IR/IGFRKO mice (Figure 1H). F-IRKO and F-IR/IGFRKO mice exhibited decreased expression of one of the rate limiting gluconeogenic enzymes Pepck, while the expression of Fbp1 and Pc was actually increased in comparison to the controls (Figure 1I). In spite of a decrease in Pepck, it is likely that some gluconeogenic potential was preserved as these mice were hyperglycemic as compared to the controls. Thus, lipodystrophic F-IRKO and F-IR/IGFRKO mice had profound hepatic steatosis and minimal signs of liver injury which was primarily due to lipotoxicity. F-IGFRKO mice, which did not develop lipodystrophy, did not exhibit any of these changes.

**Lipodystrophic F-IRKO and F-IR/IGFRKO mice can mobilize stored liver fat**

In addition to marked hepatosteatosis, lipodystrophic F-IRKO and F-IR/IGFRKO mice at 12 weeks of age exhibited serum dyslipidemia with elevated circulating triglyceride, cholesterol and free fatty acid levels (17). To determine to what extent these lipids could be mobilized, F-IRKO mice were subjected to an overnight fasting and an 8-hour refeeding protocol. Fasting resulted in a decrease in blood glucose levels in all mice, although the F-IRKO mice remained hyperglycemic compared to the controls (135±13 vs. 87±8 mg/dl), and refeeding resulted in return to the marked hyperglycemic levels (Figure 2A). Liver weight of F-IRKO mice also decreased by 38% from 4.0±0.4 to 2.5±0.2 grams after fasting and returned to baseline (3.9±0.2g) after refeeding,
demonstrating the ability of lipodystrophic mice to store and mobilize fat in the liver (Figure 2B). Liver weight of control mice also decreased by fasting from 1.1±0.1 to 0.84±0.02 grams and increased to 0.94±0.07 grams with refeeding. Serum triglyceride levels, which were elevated in random fed F-IRKO mice (230±40 v. 96±10) decreased to levels even lower than control after an overnight fast (56±7 v. 108±21), but then returned to baseline after refeeding (Figure 2C). Free fatty acids (FFA) levels increased with fasting in control mice from 0.7±0.1 to 0.9±0.0 mEq/L, but they paradoxically decreased in F-IRKO mice during fasting (1.2±0.2 to 0.7±0.1), probably reflecting the lack of adipose tissue lipolysis and increased FFA utilization in these mice (Figure 2D). Insulin levels also decreased with fasting in both control mice and F-IRKO mice, indicating normal pancreatic responses to nutrient intake and blood glucose levels (Figure 2E). Likewise, beta-hydroxybutyrate levels significantly increased with fasting in both control and F-IRKO mice (Figure 2F), suggestive of normal ketogenesis in lipodystrophic mice, despite the almost complete absence of white adipose tissue. Liver FGF21 mRNA was elevated in F-IRKO and F-IR/IGFRKO mice at every age tested (Figure 2G).

**F-IRKO and F-IR/IGFRKO mice develop progressive NAFLD with aging.**

The profound hepatomegaly present at 12 weeks of age was already evident by 2.5 weeks of age and continued to advance throughout the lifetime. By 52 weeks of age livers in F-IRKO mice were 6.5-times heavier, and those in F-IR/IGFRKO mice were 9.2-times heavier than the controls (Figure 3A). In spite of almost complete reduction of white adipose tissue in F-IRKO and F-IR/IGFRKO mice, and as a result of the massive hepatomegaly, body weight was not significantly different from the controls at up to 3
months of age and was actually 20% greater than the controls at 52 weeks of age (Figure 3B). Hepatic triglycerides per milligram of tissue were also increased by 3- to 5-fold in F-IRKO and F-IR/IGFRKO mice as early as 2.5 weeks of age, compared to controls and remained elevated at 12 and 52 weeks of age (Figure 3C). In parallel, steatosis was observed in F-IRKO and F-IR/IGFRKO mice at 2.5 weeks of age, which persisted at 12 and 52 weeks of age (Sup. Fig. 2). This correlated with 2- to 3-fold increases in the expression of enzymes involved in de novo lipogenesis (Acc1, Fas, and Scd1) in the lipodystrophic mice compared to controls at 2.5, 12 and 52 weeks of age. (Figure 3D, E & F). Interestingly, the insulin-activated transcription factor Srebp1c, which regulates lipogenesis, was not elevated despite massive hyperinsulinemia (Sup. Fig. 3).

Excessive lipid accumulation in the liver was associated with increased serum alanine aminotransferase (ALT) in 5 and 12 week-old F-IRKO and F-IR/IGFRKO mice (Figure 3G). Serum aspartate aminotransferase (AST) levels were also elevated in F-IRKO mice at 5 and 12 weeks of age, and further increased in F-IRKO and F-IR/IGFRKO mice at 52 weeks of age (Figure 3H). F-IGFRKO mice did not develop hepatomegaly or increased levels of liver triglycerides, enzymes of de novo lipogenesis or serum ALT and AST levels.

One year old lipodystrophic mice develop liver inflammation and fibrosis

By one year of age, inflammatory infiltrates were evident in F-IRKO and F-IGFRKO livers on histological examination (Figure 4A, top panel), and this was confirmed by increased staining using antibody to the macrophage marker F4/80 (Figure 4A, bottom panel). mRNA expression of F4/80 and other macrophage and inflammatory
markers including CD11c and Tnf-α was also increased 3- to 8-fold in livers of one year old F-IRKO and F-IR/IGFRKO mice (Figure 4B), which was not observed at earlier time points (Sup. Fig. 4). Likewise, mRNA expression of fibrogenic genes aSma, Tgf-β and Coll1a1 was increased 2- to 8-fold in one year old F-IRKO and F-IR/IGFRKO mice (Figure 4C), which again was largely not increased at prior time points (Sup. Fig. 5).

At 52 weeks of age, the livers of all F-IRKO and F-IR/IGFRKO mice contained some hepatocytes showing balloon degeneration and most exhibited many ballooned hepatocytes; ballooning score 2.0 ± 0.0 and 1.3 ± 0.2, respectively (scale of 0-2, Table 1). At 52 weeks of age, F-IRKO and F-IR/IGFRKO mice showed increased inflammation score of 0.6 ± 0.2 and 1.5 ± 0.2 (scale 0-3, Table 1), respectively. The most striking histological difference between 12 and 52 week old mice was seen in the degree of fibrosis. Fibrosis was not present at 12 weeks of age (Table 1), however, at 52 weeks of age F-IRKO mice showed stage 1 fibrosis, consisting of both interstitial, and periportal fibrosis, while a more severe degree of interstitial fibrosis (stage 3 out of 4) was present in F-IR/IGFRKO mice (Figure 4D, and Table 1). F-IGFRKO mice did not develop liver inflammation or fibrosis at any age.

**One year old F-IRKO and F-IR/IGFRKO mice preserve some gluconeogenic potential.**

As lipodystrophic mice aged and developed progressive liver disease their blood glucose levels improved (17). We assessed whether impaired hepatic gluconeogenesis due to progressive liver disease could be responsible for the improved glucose levels in older mice. Indeed, mRNA levels of two rate-limiting enzymes of gluconeogenesis,
G6Pase and Pepck, were decreased in 1 year-old F-IRKO and F-IR/IGFRKO livers. The expression of other gluconeogenic enzymes, such as Fbp1 and Pc, on the other hand, were elevated (Figure 5A). Interestingly, the relative expression of gluconeogenic enzymes did not change between 12 and 52 weeks of age, except for G6pase which was elevated in F-IR/IGFRKO mice at 12 weeks but decreased in F-IRKO and F-IR/IGRKO mice at 52 weeks of age (Figure 5A). While reduction in G6pase and Pepck expression suggests impaired gluconeogenesis, some gluconeogenic potential was preserved as 52 week old F-IRKO and F-IR/IGFRKO mice did not develop hypoglycemia with fasting (Figure 5B). In addition, F-IRKO and F-IR/IGFRKO mice were able to mobilize stored glucose in response to glucagon and even showed an exaggerated response (Figure 5C). This is consistent with enhanced glycogen stores in the liver of lipodystrophic mice as assessed by PAS staining (Figure 5D). Improvement in glucose was also not due to failure of pancreatic alpha-cells, as serum glucagon levels were elevated in 52 week old F-IRKO and F-IR/IGFRKO mice (Figure 5E).

Liver tumors develop in lipodystrophic mice at one year of age

With aging, livers of both F-IRKO and F-IR/IGFRKO mice developed gross nodularity (Figure 6A, top); this occurred in the setting of fibrosis (Figure 6A, middle and Sup. Fig. 6). Histological sections of liver stained for Ki67 showed clusters of proliferating cells in F-IRKO and discrete proliferative nodules in F-IR/IGFRKO livers (Figure 6A, bottom). These nodules contained hepatocytes with increased mitotic activity and large, atypical nuclei, often with multiple, prominent nucleoli, indicative of severe cellular dysplasia (Sup. Fig. 7A). Livers of F-IR/IGFRKO mice also contained tumor-like
malformations of bile ducts, segments of bone with bone marrow elements and areas of extramedullary hematopoiesis (Sup. Fig. 7B, C and D), as well as increased expression of tumorigenic markers beta-catenin, Afp and cyclin D1 (Sup. Fig 8A, B and C). Expression of Pkm2, a rate limiting enzyme of glycolysis was also increased in F-IR/IGFRKO livers (Figure 6B). Pkm2 is normally found in tissues with high glycolytic activity, such as embryonic stem cells and tumor cells, but not in mature hepatocytes (24).

Whole body oxygen consumption (VO2) was significantly decreased by 30% in 1 year old F-IRKO and F-IR/IGFRKO mice due to, at least in part, reduced activity of the mice (Figure 6C and Sup. Fig. 9). Respiratory exchange ratio (RER) was also lower in these mice as compared to controls at 12 weeks of age, but by 52 weeks of age RER was above 0.9 in F-IRKO and F-IR/IGFRKO mice and actually significantly higher than controls (Figure 6C). The latter finding indicates a shift in favor of greater glucose than lipid utilization in the lipodystrophic mice, which is likely from the increased glycolytic activity associated with development of the dysplastic hepatic nodules. Insulin receptor levels were reduced in the livers of F-IR/IGFRKO mice (Figure 6D), probably as a result of sustained hyperinsulinemia (25). Also, there was increased serine/threonine phosphorylation of PTEN, decreased total levels of PTEN (Figure 6D) and increased staining of PIP3 (Figure 6E), all consistent with development of hepatic neoplasia.

**High fat feeding accelerates blood glucose normalization**

In order to test to what extent chronic lipotoxicity might be mediating the liver injury and improved glucose levels in aged mice, we placed a cohort of 8-week old control, F-IRKO and F-IR/IGFRKO mice on a high fat diet (60% fat by calories). Control
mice gained weight on HFD throughout the 12 week study period, while F-IR/IGFRKO mice gained body weight initially, but this began to level off after 8 weeks on the diet. F-IRKO mice also initially gained weight but then started losing body weight after 8 weeks on the diet (Figure 7A). Likewise, blood glucose levels at 8 weeks on the diet began trending downward, so that at 12 weeks blood glucose levels of F-IRKO and F-IR/IGFRKO mice were not different from the control mice (Figure 7B). After 12 weeks of HFD, glucose tolerance of lipodystrophic mice also improved and was not different from the control mice (Figure 7C). Liver histology showed marked steatosis and balloon degeneration in F-IRKO and F-IR/IGFRKO mice, while control mice did not develop significant steatosis (Figure 7D). Liver weight of F-IRKO and F-IR/IGFRKO mice was significantly increased as compared to the controls at 12 weeks on HFD (Figure 7E), and hepatomegaly was as profound as the liver weight of chow fed F-IRKO and F-IR/IGFRKO mice at 52 weeks of age.

The expression of gluconeogenic enzymes \textit{G6Pase} and \textit{Pepck} was again decreased in F-IRKO and F-IR/IGFRKO mice (Figure 7F), similar to the decreased expression of these enzymes at 52 weeks of age on chow diet. Conversely, the expression of \textit{Fbp1} was not significantly increased, while the mRNA levels of \textit{Pc}, \textit{Gapdh}, \textit{Enol1} and \textit{Aldo b} were increased in F-IRKO and F-IR/IGFRKO mice (Figure 7F and Sup. Fig. 10), suggestive of increased glucose flux. When \textsuperscript{14}C-2-deoxyglucose uptake was assessed \textit{in vivo}, uptake in the livers of F-IRKO and F-IR/IGFRKO mice tended to be higher, while glucose uptake was decreased in the muscle of F-IR/IGFRKO mice at 12 weeks on HFD (Sup. Fig. 11A). Again, normalization of blood glucose levels on HFD correlated with increased \textit{Pkml2} expression in F-IRKO and F-IR/IGFRKO mice (Figure 7G). Although,
these livers did not show gross nodularity, the liver parenchyma was heterogeneous in color (Sup. Fig. 11B). *FGF21* mRNA levels were also increased in lipodystrophic mice on HFD (Figure 7G), as was the expression of *Acc1*, *Fas* and *Scd1* (Figure 7H). The HFD-challenged lipodystrophic mice also developed signs of liver inflammation with elevated expression of *F4/80*, *Tnf-a*, *CD11c* (Figure 7I) and signs of liver fibrosis with elevated *αSma* and *Colla1* mRNA (Figure 7J).
DISCUSSION

In the present study we show that adipose-specific deletion of insulin receptor or combined deletion of IR and IGF1R induces a generalized lipodystrophy phenotype with profound hepatomegaly, marked steatosis and increased enzymes of de novo lipogenesis. Early on, this results in increased levels of reactive oxygen species in the liver, augmented lipid peroxidation and hepatocyte balloon degeneration - all indicative of lipotoxicity. At this age, these mice are able to mobilize stored liver lipids and utilize different substrates, such that blood glucose levels and liver weight decrease with fasting. Furthermore, lipodystrophic F-IRKO and F-IR/IGFRKO mice are able to robustly increase ketogenesis, perhaps due to increased liver FGF21 expression. Over time, however, lipotoxic effects accumulate, so that by 52 weeks of age lipodystrophic mice develop significant hepatic inflammation and fibrosis. Interestingly, blood glucose levels also normalize at this age, in part due to chronic lipotoxicity with altered expression of gluconeogenic enzymes and development of highly dysplastic liver nodules, resulting in greater glucose utilization by liver and a dramatic increase in whole body RER. Normalization of blood glucose levels can be accelerated by feeding mice HFD for 12 weeks.

The lipodystrophic syndrome observed in F-IRKO and F-IR/IGFRKO mice is similar to human generalized lipodystrophy in many ways. Both are characterized by low leptin levels, marked insulin resistance, hyperlipidemia and fatty liver disease (26; 27). Leptin replacement reduces blood glucose levels and hepatic steatosis in humans with lipodystrophy (8; 28), and leptin replacement also normalizes blood glucose levels in F-IRKO and F-IR/IGFRKO mice (17). The effects of leptin are, at least in part, secondary
to reduced food intake and can be mimicked in our lipodystrophic mice with fasting alone.

In humans with generalized lipodystrophy, NAFLD often progresses to non-alcoholic steatohepatitis (NASH) and cirrhosis (15), sometimes requiring liver transplantation (14). F-IRKO and F-IR/IGFRKO mice also develop profound and progressive fatty liver disease with massive hepatomegaly (liver weight up to ~25% of body weight) with inflammation, pericellular fibrosis, and an inversion of ALT to AST ratio. Indeed, by one year of age, the F-IRKO and F-IR/IGFRKO mice develop overt dysplastic hepatic nodules with severe large-cell dysplasia, frequent mitoses, as well as elevated tumor markers $Apf$ and $\beta$-catenin. This is associated with a decrease in liver PTEN levels and increased accumulation of PIP$_3$, changes often seen in liver cancer (29). Thus, the liver injury in our current lipodystrophic model describes a full spectrum of NAFLD progression including the development of severely dysplastic hepatic nodules.

The liver phenotype in these mice is in contrast to the liver phenotype in our previous study of fat specific IR and IGF1R deletion driven by aP2-cre promoter, which did not develop NAFLD unless challenged with HFD (18). The difference likely stems from the fact that deletion of IR or IR and IGF1R driven by aP2-cre, only results in moderately reduced adipose tissue mass, leading to improved glucose tolerance and protection from age-related and hypothalamic lesion-induced obesity (30). Lipodystrophic mice due to knockout of Srebp1c using aP2-Cre also develop NAFLD with progression to NASH, but have not been reported to develop tumors or dysplastic hepatic nodules (31). However, patients with lipodystrophy are known to develop liver adenomas (personal communication Dr. Rebecca Brown and Dr. Philip Gorden) and there is at least one
patient with acquired generalized lipodystrophy who developed hepatocellular carcinoma (32).

Our findings indicate that stored fat in the liver is a dynamic depot. Lipodystrophic mice lose about 40% of liver weight with fasting and gain 150% above fasted liver weight, following 8 hours of refeeding. Canonical thinking is that liver fat accumulation occurs over time and that additive insults lead to chronic liver injury (33). However, human studies also indicate the dynamic nature of liver fat. As an example, overfeeding for only 3 weeks can increase liver fat by 27 percent, while total body weight increases by only 2 percent (34). Furthermore, short-term (2 week) hypocaloric diets are used to reduce liver volume before bariatric surgery (35). In humans, caloric restriction to ~1,100 kcal/day for 48 hours can also markedly reduce liver triglyceride content, especially when fed a low carbohydrate diet (36). Stored liver fat in lipodystrophic mice is utilized for ketogenesis, which occurs in spite of almost complete absence of adipose tissue. This may be due to elevated liver expression of FGF21, a peptide which has been shown to regulate lipid metabolism (37) and hepatic ketogenesis (38; 39). Thus, liver can function as a fat depot, at least in conditions where adipose tissue does not develop. However, extensively relying on the liver for lipid storage does comes at a cost, as hepatic lipotoxicity is present even at a young age, and can be accelerated by HFD feeding.

One of the most unexpected aspects of the F-IRKO and F-IR/IGFRKO phenotype is the improvement in blood glucose levels with aging. The improvement in blood glucose is not due to regeneration of adipose tissue nor to a reduction in food intake (17). It is also not due to return of normal leptin levels or improved insulin sensitivity, as
insulin levels remained elevated and pancreatic islets continued to hypertrophy. Instead, the improvement parallels the progression of liver disease from NAFLD to NASH with inflammation, fibrosis and severely dysplastic nodules. At least three factors appear to contribute to the improved glycemia. The first is some impairment in gluconeogenesis due to chronic liver disease. *Pepck* and *G6Pase* were decreased in one-year old F-IRKO and F-IR/IGFRKO livers. Attempts to directly assess gluconeogenesis with a pyruvate challenge, however, resulted in death of the mice, so the extent of impairment is difficult to quantify. Mice did tolerate overnight fasting without development of hypoglycemia, and liver glycogen stores were increased. A second potential contributory factor could have been either a failure of pancreatic cells to secrete glucagon or a loss of glucagon receptors in the dysplastic liver. However, serum glucagon levels remained elevated with age, and F-IRKO and F-IR/IGFRKO mice showed a brisk glycemic response to glucagon injection.

The third, and perhaps most important, factor that could contribute to glucose normalization may be the chronic lipotoxicity and development of severely dysplastic hepatic nodules. Furthermore, expression of PKM2 in whole liver lysate was increased, contributing to increased glycolysis. This is consistent with the shift from fat to carbohydrate metabolism with aging, as exemplified by the increase in whole-body RER. Lipotoxicity likely mediates the liver injury as HFD feeding accelerates blood glucose normalization. This occurred even without gross evidence of tumor development, however, the key enzyme of glycolysis, PKM2, is profoundly increased in the lipodystrophic mice after only 12 weeks of HFD feeding. Hyperinsulinemia may directly
increase PKM2 levels (40) and thus may lead to glucose normalization in the absence of highly dysplastic liver nodules.

In summary, F-IRKO and F-IR/IGFRKO mice provide a unique new model to study the development and progression of fatty liver disease. Using this model, we show that lipodystrophic mice develop a full spectrum of NAFLD, which progresses to NASH, fibrosis and ultimately highly dysplastic liver nodules, which is associated with improvement in blood glucose levels. This liver injury can be accelerated by feeding mice a HFD. Taken together, these data indicate that lipotoxicity can play a major role in the development of liver disease. This can then contribute to altered whole-body metabolism, modifying disease pathogenesis in unexpected ways.
ACKNOWLEDGMENTS

The authors thank Joslin Diabetes and Endocrinology Research Center core laboratories (P30 DK036836). This work was also supported by NIH grants (R01 DK031036, and R01 DK082659) to C.R.K, and K12 HD000850 to S.S. The Sunstar Foundation supported S.F. and M.H.S. was supported in part by funds from the U of Bergen, KG Jebsen Foundation, Norwegian Society of Endocrinology, Eckbo’s Foundation, T. Wilhelmsen Foundation and the Norwegian Diabetes Association.

S.S. generated the data and wrote the manuscript. J.B. generated the data and wrote the manuscript. M.H.S., M.H., S.F., E.H.P., J.W., and A.R.P. generated the data and reviewed the manuscript. C.R.K. oversaw the project, contributed to discussion, and helped write the manuscript. C.R.K. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. There are no potential conflicts of interest relevant to this article by any of the authors.
REFERENCES


FIGURE LEGENDS

Figure 1: Fatty liver disease in 12 week old lipodystrophic mice
A) Blood glucose, B) liver weight and C) liver TG content in 12 week old, random-fed control, F-IGFRKO, F-IRKO and F-IR/IGFRKO mice. Results are mean ± SEM of 12 to 30 animals per group. D) H&E stained liver sections from the same mice. mRNA expression of genes involved in E) de novo lipogenesis, F) inflammation and G) fibrosis in the livers of 12 week old control, F-IGFRKO and F-IRKO mice. H) Dihydroethidium (DHE) and 4-hydroxynonenal (4-HNE) stained liver sections from control and F-IR/IGFRKO mice. I) Expression of gluconeogenic/glycolytic enzymes in the livers of control, F-IGFRKO, F-IRKO and F-IR/IGFRKO mice at 12 weeks of age fed chow diet. Results are mean ± SEM of 5 to 7 animals per group. *indicates a significant difference compared to controls (*, p<0.05; **, p<0.01; ***, p<0.001).

Figure 2: Lipodystrophic mice can mobilize stored liver fat.
A) Blood glucose, B) liver weight and C) serum TG livers D) Free Fatty Acids, E) Insulin and F) beta-hydroxybutyrate levels, in 12 week old random fed or overnight fasted control and F-IRKO mice. Results are mean ± SEM of 9 to 10 animals per group. G) FGF21 mRNA levels in the livers from 2.5, 12 and 52 week old control, F-IGFRKO, F-IRKO and F-IR/IGFRKO mice. Results are mean ± SEM of 5-8 mice per group. *indicates a significant difference compared to controls (*, p<0.05; **, p<0.01; ***, p<0.001). # indicates a significant difference from fed F-IRKO mice and § indicates a significant difference between adjacent groups.
Figure 3: F-IRKO and F-IR/IGFRKO mice develop progressive NAFLD with age
A) Liver weight and B) body weight of control, F-IGFRKO, F-IRKO and F-IR/IGFRKO mice at 2.5, 5, 8, 12 and 52 weeks of age. Results are mean ± SEM of 12 to 30 animals per group. C) TG content was measured in the livers from 2.5, 12 and 52 weeks of age. D) *Acc1*, E) *Fas* and F) *Scd1* mRNA expression in control, F-IGFRKO, F-IRKO and F-IR/IGFRKO mice at 2.5, 12 and 52 weeks of age graphed as a fold change over controls. Results are mean ± SEM of 5-8 animals per group. G) Serum ALT and H) AST levels measured in 2.5, 5, 12 and 52 week old chow fed control, F-IGFRKO, F-IRKO and F-IR/IGFRKO mice. *indicates a significant difference compared to controls (*, p<0.05; **, p<0.01; ***, p<0.001).

Figure 4: Liver inflammation is apparent at 52 weeks of life.
A) H&E stained liver sections from 52 week old control, F-IGFRKO, F-IRKO and F-IR/IGFRKO mice, 100x magnification, top panel. IHC for macrophage marker F4/80 in the same mice, 400 x magnification, bottom panel. One representative section from 3 mice per group is shown. Expression of genes involved in B) inflammation and C) fibrosis in chow fed control, F-IGFRKO, F-IRKO and F-IR/IGFRKO mice at 52 weeks of age. Results are mean ± SEM of 4 to 8 animals per group. *indicates a significant difference compared to controls (*, p<0.05; **, p<0.01; ***, p<0.001). D) Masson’s trichrome stained liver sections from the same mice at 52 weeks of age, 200x magnification. One representative section from 4-6 mice per group is shown.

Figure 5: Gluconeogenesis at 52 weeks of age.
A) mRNA expression of \textit{G6pase}, \textit{Pepck}, \textit{Fbp1} and \textit{Pc} in control, F-IGFRKO, F-IRKO and F-IR/IGFRKO mice at the indicated times from 2.5 to 52 weeks of age graphed as fold change over controls. Results are mean ± SEM of 5 to 6 animals/group. *indicates a significant difference compared to controls (*, p<0.05; **, p<0.01; ***, p<0.001) B) Random fed and overnight fasted blood glucose levels of chow fed control, F-IGFRKO, F-IRKO and F-IR/IGFRKO mice at 52 weeks of age. C) Blood glucose levels assessed over 90 minutes after IP glucagon challenge and serum glucagon levels in control, F-IRKO and F-IR/IGFRKO mice at indicated times. Results are mean ± SEM of 5-6 animals/group. E) Periodic Acid-Schiff stained liver sections from control, F-IGFRKO, F-IRKO and F-IR/IGFRKO mice at 52 weeks of age. One representative section from 5 mice per group is shown.

\textbf{Figure 6: Lipodystrophic mice develop liver tumors and increased glycolysis.}

A) Representative images of whole liver (upper panel), Masson’s Trichrome stain (middle panel) and Ki67 staining (lower panel) in 1 year old control, F-IGFRKO, F-IRKO and F-IR/IGFRKO mice. B) Ki67 and PKM2 mRNA levels in livers from 2.5, 12 and 52 week old mice. Results are mean ± SEM of 5 to 8 animals per group. C) VO\textsubscript{2} and RER measured in metabolic cages with control, F-IRKO and F-IR/IGFRKO mice at 12 and 52 weeks of age. Results are mean ± SEM of 5 to 11 mice per group. D) Western blot analysis and densitometric quantification of insulin signaling molecules from the livers of control, F-IRKO and F-IR/IGFRKO mice at 1 year of age. Results are mean ± SEM of 4 animals per group. E) Representative IHC images of PIP3 in livers of control, F-IRKO
and F-IR/IGFRKO mice at 1 year of age *indicates a significant difference compared to
controls (*, p<0.05; **, p<0.01; ***, p<0.001).

**Figure 7: HFD accelerates liver injury in lipodystrophic mice**
A) Body weight of control, F-IRKO and F-IR/IGFRKO mice assessed during 12 weeks
of HFD feeding. B) Blood glucose levels in the same mice assessed at indicated time
points. C) Glucose tolerance test of control, F-IRKO and F-IR/IGFRKO mice after 12
weeks of HFD. Results are mean ± SEM of 6 to 11 animals per group. D) H&E stained
liver sections from the same mice. One representative section from 6 mice per group is
shown. E) Liver weight from control, F-IRKO and F-IR/IGFRKO mice after 12 weeks
of HFD. F) mRNA expression of gluconeogenic enzymes and the expression of G) Pkm2
and Fgf21 mRNA after 12 weeks of HFD. mRNA levels of genes involved in H) de novo
lipogenesis, I) inflammation and J) fibrosis after 12 weeks of HFD. Results are mean ±
SEM of 4 to 6 animals per group. *indicates a significant difference compared to
controls (*, p<0.05; **, p<0.01; ***, p<0.001).
### Table 1

<table>
<thead>
<tr>
<th>Genotype</th>
<th>12 weeks</th>
<th></th>
<th></th>
<th></th>
<th>52 weeks</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WT</td>
<td>F-IGFRKO</td>
<td>F-IRKO</td>
<td>F-IR/IGFRKO</td>
<td>WT</td>
<td>F-IGFRKO</td>
<td>F-IRKO</td>
<td>F-IR/IGFRKO</td>
</tr>
<tr>
<td>Macrovesicular Steatosis, (0-3)</td>
<td>0</td>
<td>0</td>
<td>1.7 ± 0.4</td>
<td>2.6 ± 0.2</td>
<td>0.2 ± 0.2</td>
<td>0</td>
<td>2.8 ± 0.2</td>
<td>2.3 ± 0.2</td>
</tr>
<tr>
<td>Portal Inflammation, (0-3)</td>
<td>0</td>
<td>0</td>
<td>0.4 ± 0.2</td>
<td>0</td>
<td>0</td>
<td>1.0±0.0</td>
<td>0.6 ± 0.2</td>
<td>1.5 ± 0.2</td>
</tr>
<tr>
<td>Ballooning Degeneration, (0-2)</td>
<td>0</td>
<td>0</td>
<td>1.9±0.1</td>
<td>1.6 ± 0.2</td>
<td>0</td>
<td>0.2 ± 0.2</td>
<td>2.0±0.0</td>
<td>1.3 ± 0.2</td>
</tr>
<tr>
<td>Interstitial Fibrosis, Stage (0-4)</td>
<td>0</td>
<td>0</td>
<td>0.1 ± 0.1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.8 ± 0.4</td>
<td>2.7 ± 0.2</td>
</tr>
<tr>
<td>Fibrosis Present, % total</td>
<td>0</td>
<td>0</td>
<td>14</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>60</td>
<td>100</td>
</tr>
</tbody>
</table>

#### TABLE LEGEND

**Table 1: NAFLD Activity Scores (NAS)**

NAFLD activity scores were assessed in 12 and 52 week old mice utilizing H&E and Trichrome stained liver sections by a practicing clinical pathologist adopting a modified version of a previously published method (41). Results are mean ± SEM of 4-6 mice per group.
Figure 1

A. Blood glucose (mg/dl)
B. Liver weight (g)
C. TG (nmol/mg liver)

D. H&E staining of liver sections from different groups:
   - Control
   - F-IGFRKO
   - F-IRKO
   - F-IR/IGFRKO

E. Graph showing fold change in gene expression:
   - ACC1
   - FAS
   - SCD1

F. Graph showing fold change in gene expression:
   - TNFα
   - CD11c
   - F4/80

G. Graph showing fold change in gene expression:
   - TGFβ
   - αSMA
   - Col1α1

H. Immunofluorescence staining for DHE and 4-HNE:
   - Control
   - F-IR/IGFRKO

I. Graph showing fold change in gene expression:
   - G6Pase
   - PEPCK
   - FBP1
   - PC
Figure 3

A.

B.

C.

D.

E.

F.

G.

H.
**Figure 4**

A. Image panels showing histological sections labeled Control, F-IGFRKO, F-IRKO, and F-IR/IGFRKO.

B. Bar graphs showing fold change for TNFα, CD11c, F4/80, TGFβ, αSMA, and Col1a1. Significant differences are indicated with asterisks: * (p < 0.05), ** (p < 0.01), *** (p < 0.001).

C. Continued bar graphs.

D. Additional image panels for F-IGFRKO, F-IRKO, and F-IR/IGFRKO.
Figure 6

A. 

B. 

C. 

D. 

E.
Supplemental Figure 1: Reticulin, sirius red and trichrome stained liver sections from control, and F-IR/IGFRKO mice at 12 weeks of age. One representative section from 5 mice per group is shown.
Supplemental Figure 2: H&E stained liver sections from control, F-IGFRKO, F-IRKO and F-IR/IGFRKO mice at the indicated times from 2.5 to 52 weeks of age. One representative section from 5 mice per group is shown.
Supplemental Figure 3: mRNA expression levels of transcriptional factor SREBP1c regulating de novo lipogenesis in livers from 2.5, 12 and 52-week old control, F-IGFRKO, F-IRKO and F-IR/IGFRKO mice. Results are mean ± SEM of 5-6 animals/group.)
**Supplemental Figure 4:** mRNA expression levels of inflammatory markers in liver from 2.5, 12 and 52-week old control, F-IGFRKO, F-IRKO and F-IR/IGFRKO mice. Results are mean ± SEM of 5-6 animals/group. *indicates a significant difference compared to the Control group (*, p<0.05; **, p<0.01; ***, p<0.001)
Supplemental Figure 5: mRNA expression levels of pro-fibrogenic genes in liver from 2.5, 12 and 52-week old control, F4/IGFRKO, F4/IRKO and F4IR/IGFRKO mice. Results are mean ± SEM of 5-6 animals/group. *indicates a significant difference compared to the Control group (*, p<0.05; **, p<0.01; ***, p<0.001)
**Supplemental Figure 6:** Representative images of Masson’s Trichrome stained liver sections from control, F-IGFRKO, F-IRKO and F-IR/IGFRKO mice collected at 52 weeks of age. (one representative section from 5 mice per group is shown)
Supplemental Figure 7: A) H&E stained liver section from F-IR/IGFRKO mouse at 52 weeks of age shows a large tumor compressing surrounding hepatocytes, 4X mag. Inserts are 40X magnification, arrowheads show increased mitotic figures, while arrows point to atypical nuclei with condensed granular chromatin. Additional liver findings in 52 week old F-IR/IGFRKO mice show B) sites of extramedullary hematopodsis, arrows. C) Thick arrow points to bone and thin arrow points to bone marrow elements. D) Arrow points to von Meyenburg complex, a tumor like malformation of bile ducts.
Supplemental Figure 8: A) Beta-Catenin stained liver sections from control, F-IRKO and F-IR/IGFRKO mice collected at 1 year of age (one representative section from 3 mice per group is shown). Liver mRNA expression of B) alpha-fetoprotein and C) cyclin D1 from control, F-IGFRKO, F-IRKO and F-IR/IGFRKO mice at 2.5, 12 and 52 weeks of age. Results are mean ± SEM of 5-6 animals/group. * indicates a significant difference compared to the Control group (*, p<0.05; **, p<0.01; ***, p<0.001).
**Supplemental Figure 9:** Average activity measured in 3 month old and one year old control, F-IRKO and F-IR/IGFRKO mice in metabolic cages. Results are mean ± SEM of 5 to 11 mice per group.
**Supplemental Figure 10:** mRNA expression of glucogenetic enzymes after 12 weeks of HFD in the livers of control, F-IRKO and F-IR/IGFRKO mice. Results are mean ± SEM of 4 to 6 mice per group. *indicates a significant difference compared to the Control group (*, p<0.05; **, p<0.01; ***, p<0.001)
Supplemental Figure 11: A) Two deoxyglucose uptake in the liver and the muscle of control, F-IR and F-IR/IGFRKO mice. Results are mean ± SEM of 2 to 5 mice per group. * indicates a significant difference compared to the Control group (*, p<0.05). B) Gross pictures of, F-IRKO and F-IR/IGFRKO mice after 12 weeks on HFD. Arrows point to areas of liver discoloration indicating parenchymal heterogeneity.
Aldo b – Aldolase B
  For  GCTGGGCAATTTCAGAGAGC
  Rev  GAGGACTCTTCCCCCTTTTGCT

Afp - Alpha fetoprotein
  For  CAGCAGCCTGAGAGTCCATA
  Rev  GGCGATGGGTGTTTAGAAAG

αSma - alpha smooth muscle actin
  For  GTCAGTGGTGCTCCTCTGCA
  Rev  ACTGGGACGACATGGAAAG

Acc1 - Acetyl-CoA carboxylase 1
  For  AGCAGATCCGAGCTTGG
  Rev  ACCTCTGCTCGCTGAGTGC

CD11c
  For  CACTCAGTGACTGCACCAAAA
  Rev  CCTCAAGACAGGACATCGCT

Col1a1 - Collagen, type I, alpha 1
  For  TTGATCCAGAAGGACCTTGTTTG
  Rev  CCTCAGGGTATTGCTGGACAA

cyclin D1
  For  GGGTGGGTTGAATGAAAC
  Rev  TCCCTTCWAAATGCGCAAC

Enol1 – Enolase 1
  For  AGATCGACCTCAACAGTGGG
  Rev  CTAAAGCCTCTCCTCGGTGT

F4/80
  For  CTGGGATCCTACAGCTGCTC
  Rev  AGGAGCCTGGTACATTGGTG

Fas - Fatty acid synthase
  For  CCTCAAGCTTTAAACTCTCGGA
  Rev  CAGACATGCTGTGGATCTGG

Fbp1 - Fructose-bisphosphatase 1
  For  ACTTGATCCCCAGTCACATTG
  Rev  CGATCAAAGCCATCTCGTCT

FGF21 - Fibroblast growth factor 21
  For  CTCCAGCAGCAGTTCTCTGA
  Rev  CCTGGGTGTCAGCAGCCTCTA

G6Pase - Glucose 6-phosphatase
  For  GTGTCCAGGACCCACCAATA
  Rev  ACTGTGGGCATCAATCTCCT

Pepck - Phosphoenolpyruvate carboxykinase
  For  TGCTTTCATGAGGTGCCAG
  Rev  CTGGATGGAAGTTGTGCCC

Pc - Pyruvate carboxylase
  For  GGGATGCCACAGCTACT
  Rev  CATAGGGCGCAATCTTTTTGA

Pkm2 - Pyruvate kinase muscle isozyme
  For  TGTTTCCAGGAGGTGTCA
  Rev  GCTCTAGGTATCGCAGG

Scd1 - Stearoyl-CoA desaturase-1
For CAGCCGAGCCTTGTAAGTTTC
Rev GCTCTACACCTGCCTCTTCG

Srebp1c - Sterol regulatory element-binding protein 1
For TGGTTGTGTGAGCTGGAG
Rev GGCTCTGGAACAGACACTGG

Tnf-α - Tumor necrosis factor alpha
For ACGGCATGGATCTCAAAGAC
Rev AGATAGCAAATCGGCTGACG

Tgf-β - Transforming growth factor beta
For AAGTTGGCATGGTAGCCCTT
Rev GCCCTGGATACCAACTATTGC