FGFR4 Prevents Hyperlipidemia and Insulin Resistance but Underlies High Fat Diet-Induced Fatty Liver

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Abstract

OBJECTIVE—Fibroblast growth factor (FGF) family signaling largely controls cellular homeostasis through short range inter-cell paracrine communication. Recently FGF15/19, 21 and 23 have been implicated in endocrine control of metabolic homeostasis. The identity and location of the FGF receptor isotypes that mediate these effects are unclear. The objective was to determine the role of FGFR4, an isotype that has been proposed to mediate an ileal FGF15/19 to hepatocyte FGFR4 axis in cholesterol homeostasis, in metabolic homeostasis in vivo.

RESEARCH DESIGN AND METHODS—FGFR4−/− mice, mice overexpressing constitutively active hepatic FGFR4, and FGFR4−/− with constitutively active hepatic FGFR4 restored in the liver were subjected to a normal and a chronic high fat diet sufficient to result in obesity. Systemic and liver-specific metabolic phenotypes were then characterized.

RESULTS—FGFR4-deficient mice on a normal diet exhibited features of metabolic syndrome that include increased mass of white adipose tissue, hyperlipidemia, glucose intolerance and insulin resistance in addition to hypercholesterolemia. Surprisingly, the FGFR4 deficiency alleviated high fat diet-induced fatty liver in obese mice that is also a correlate of metabolic syndrome. Restoration of FGFR4 specifically in hepatocytes of FGFR4-deficient mice decreased plasma lipid levels, restored the high fat diet-induced fatty liver, but failed to restore glucose tolerance and sensitivity to insulin.

CONCLUSIONS—FGFR4 plays essential roles in systemic lipid and glucose homeostasis. FGFR4 activity in hepatocytes that normally serves to prevent systemic hyperlipidemia paradoxically underlies the fatty liver disease associated with chronic high fat intake and obesity.

ACC1, acetyl-CoA carboxylase; CYP7A1, cholesterol 7α-hydroxylase; FAS, fatty acid synthase; FFA, free fatty acid; FGF, fibroblast growth factor; FGFR, FGF receptor; G6Pase, glucose-6-phosphatase; HS, heparan sulphate; MCAD, medium-chain acyl-CoA dehydrogenase; MTP, microsomal triglyceride transfer protein; NAFLD, non alcoholic fatty liver disease; PEPCK, phosphoenolpyruvate carboxykinase; PPARα, peroxisome proliferator-activated receptors α; PPARγ, peroxisome proliferator-activated receptors γ; SCD1, steroyl-CoA desaturase 1; SREBP1, sterol regulatory element-binding protein 1; TG, triglyceride.
Metabolic syndrome (also known as insulin resistance syndrome or syndrome X) is a multi-component disorder that is characterized by central body obesity, dyslipidemia, insulin resistance, glucose intolerance, and hypertension that are risk factors for numerous diseases including type 2 diabetes mellitus, cardiovascular diseases, neurodegenerative diseases, liver disease and cancer (1; 2). The hepatic manifestation of the metabolic syndrome is nonalcoholic fatty liver disease (NAFLD) that is evident from triglyceride accumulation in macroscopic fat droplets (3; 4; 5). NAFLD is the most common liver disease in developed countries. NAFLD may be an indicator of metabolic syndrome and risk for its associated diseases equal to body mass and shape, insulin resistance, blood triglycerides and HDL/LDL (6;7). The mechanisms underlying NAFLD and its relationship to the other components of metabolic syndrome are largely unknown.

The fibroblast growth factor (FGF) signaling system is a ubiquitous microenvironmental regulator of cell to cell communication in development and adult homeostasis (8-12). Recent developments indicate that specific members of the family may regulate metabolic homeostasis by endocrine mechanisms where FGF originates in one tissue and acts distally on FGFR in another. Administration of FGF19 (13;14) and FGF21 (15) or when expressed in the liver of transgenic animals impacts metabolic rate and multiple parameters associated with metabolic syndrome. Circulating FGF23 that resides in the same FGF subgroup as FGF15/19 and FGF21 based on sequence homology and affinity for heparan sulfate (16) regulates vitamin D and phosphate homeostasis (17;18). The regulation of expression and the tissue and cellular origin of FGF15/19, FGF21 and FGF23 as well as the isotype and location of the FGFR isotype underlying the metabolic effects of the three factors is not well resolved.

An ileal origin of FGF15/19 under control of the bile acid-activated farnesoid X receptor (FXR; NR1H4) that activates hepatocyte FGFR4 has been proposed that regulated cholesterol to bile acid metabolism (19). Hepatocyte FGFR4 regulates cholesterol to bile acid synthesis in the liver by transcriptional down-regulation of cholesterol 7a-hydroxylase (CYP7A1), the rate-limiting enzyme for classical bile acid synthesis (20;21). A gut to liver FGF15/19 to FGFR4 axis explains why only intestinal compared to portal or intravenous administration of bile acids represses hepatic cyp7a1 expression and bile acid synthesis (19). In contrast to FGF15/19 that is not expressed in liver (19), low level FGF21 expression that increases upon liver perturbation is relatively restricted to hepatocytes (22). When expressed in the hepatocyte, FGF21 improved glucose clearance and insulin sensitivity similar to systemic treatment of animals with FGF21 (15). We have shown that targeted expression of FGF21 in hepatocytes delays the appearance of DEN-induced liver adenoma. However, it has no effect on HCC incidence and burden. Although hepatocytes are a candidate for the autocrine action of FGF21, the most dramatic effects are on adipose tissue where neither
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FGF21 nor FGFR4 are expressed significantly (15;23). In adipocytes in vitro, FGF21 synergizes with the peroxisome-proliferator-activated receptor gamma (PPARγ) ligand and antidiabetic agent rosiglitazone to increase insulin-independent glucose uptake (23).

Because of the strong evidence that hepatocyte FGFR4 controls cholesterol to bile acid metabolism that occurs primarily in the liver, it is a strong candidate for the mediator either directly or indirectly of some of the effects of FGF15/19 on general metabolic homeostasis. In this report, we evaluated the consequences of a general ablation of FGFR4 and hepatocyte-specific FGFR4 restoration on features associated with metabolic syndrome. We show that FGFR4 plays a general role in maintenance of both lipid and glucose metabolism under normal dietary conditions in addition to its established role in cholesterol metabolism. Hepatocyte FGFR4 appears to exert a primary control on lipid metabolism. Effects on glucose metabolism could not be explained by activity of hepatocyte FGFR4 alone suggesting an additional role of FGFR4 at other organ sites. Ironically, hepatocyte FGFR4 that normally protects against hyperlipidemia and hypercholesterolemia underlies the fatty liver induced by high fat intake and obesity.

RESEARCH DESIGN AND METHODS

Animals and diets. Mice lacking FGFR4 (FGFR4-/-) and expressing constitutively activated human FGFR4 (Alb-caFGFR4) specifically in hepatocytes have been described (20;21). The FGFR4-/- mice were a mixed 129Sv-C57BL/6 background and Alb-caFGFR4 mice were a FVB background. FGFR4-/- and WT mice were produced from an FGFR4+/− mating. Alb-caFGFR4 and WT FVB mice were produced by mating heterozygous Alb-caFGFR4 transgenic males with WT FVB females. FGFR4-/- mice were crossed with Alb-caFGFR4 transgenic mice to obtain hybrids from the two strain backgrounds expressing caFGFR4 in the hepatocytes. Mice were maintained in 12 hr light/12 hr dark cycles with free access to food and water. Except where indicated, experimental animals were male.

The normal diet (Prolab Isopro RMH 3000, PMI Nutrition International LLC, Brentwood, MO) contained 3.46 kcal/gm of which 60 and 14 percent of the kcal were from carbohydrate and fat, respectively. Animals on a normal diet were analyzed at 6 months of age. Where indicated mice were presented with a high fat diet beginning at weaning over a period of 4 months to induce obesity. The high fat diet (D12451, Research Diets, New Brunswick, NJ) presented 4.73 kcal/gm of which 35 and 45 percent of kcal were from carbohydrate and fat, respectively. Animals were sacrificed, weighed, body fat depots were examined, and tissue was excised and weighed, and then subjected to analysis. All animal work was performed in accordance with the Institutional Animal Care and Use Committee at the Institute of Biosciences and Technology, Texas A&M Health Science Center.

Histochemistry. Tissues were fixed with Histochoice Tissue Fixative MB
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(Amresco, Solon, OH), and paraffin-embedded, serial sections were prepared, archived and then sections were stained for general pathological examination with hematoxylin and eosin (H&E). Lipid droplets were revealed by staining with Oil Red O. Livers were frozen in Neg-50 frozen section medium (Richard-Allan Scientific, Kalamazoo, MI). Ten micrometer frozen sections were prepared on glass slides which were then incubated with Oil Red O for 8 min at 60°C. After washing with 85% isopropanol, tissue was counterstained with hematoxylin.

Analysis of blood chemistries and tissue lipids. Blood was collected by retro-orbital puncture after anesthetization with 2,2,2-Tribromoethanol (avertin) (Sigma, St. Louis, MO). Serum was prepared by centrifugation of the clotted blood at 2000 X g for 10 min, frozen in aliquots and stored at -70°C for future analysis. Lipids were extracted from about 50 mg of tissue after homogenization in 1 ml PBS and incubation with 1 ml of chloroform/methanol (2:1) overnight at room temperature. After centrifugation of the homogenate at 12,000 X g for 15 min, the lower organic phase containing lipid was collected and evaporated under vacuum in a rotary evaporator. The lipid pellet was dissolved in 200 µl PBS containing 1% Triton X-100. Triglyceride, free fatty acids and cholesterol was measured enzymatically (Wako Pure Chemicals, Richmond, Virginia). Serum glucose was determined with the Glucometer Elite system (Bayer Corp., Elkhart, Indiana). Serum insulin, leptin and adiponectin levels were measured by ELISA (Linco Research, St. Charles, Missouri).

Glucose tolerance and insulin responsiveness. Conventional glucose and insulin tolerance tests were performed on mice fasted for 12h and 4h, respectively. Mice were injected intraperitoneally with either 1 g glucose per kg body weight or 0.4U or 0.6U recombinant human insulin per kg body weight (Eli Lilly Co., Indianapolis, IN). Blood was collected from the tail immediately before and 30, 60, 90 and 120 min after injection. Plasma glucose was measured as described above.

Analysis of gene expression. Steady-state mRNA levels were quantified by real-time polymerase chain reaction (PCR) analysis. Total RNA was prepared from tissues using the Ultraspec RNA isolation system (Biotex Laboratories, Houston, TX). Equal amounts of RNA from four to five mice were pooled and subjected to reverse transcription with Superscript II (Life Technologies, Grand Island, NY) and random primers according to protocols provided by the manufacturer. Oligonucleotide primer sequences are shown in supplemental Table 1 (available at http://diabetes.diabetesjournals.org).

Real-time PCR was performed using the Stratagene Mx 3000P QPCR system and SYBR Green JumpStart Taq Ready Mix (Sigma, St. Louis, Missouri). All reactions were done in triplicate and relative amounts of mRNA were calculated using the comparative threshold (CT) cycle method. Mouse β-actin was used as the internal control.
Fatty acid β-oxidation activity. Fatty acid oxidation activity was measured as described previously (24). Briefly, fresh livers were homogenized in 4 volumes of 0.25M sucrose containing 1 mM EDTA. About 1 mg homogenate was incubated in 0.2 mL assay medium (150 mM KCl, 10 mM HEPES (pH 7.2), 0.1 mM EDTA, 1mM potassium phosphate buffer (pH 7.2), 5 mM malonate, 10 mM MgCl₂, 1 mM carnitine, 0.5% BSA, 5mM ATP, and palmitic acid containing [9, 10 (n)-3H]palmitic acid). The reaction was run for 30 min at 25 °C and stopped by the addition of 0.2 mL of 0.6 N perchloric acid. The mixture was centrifuged at 2000 X g for 10 min, and the un-reacted fatty acid in the supernatant was removed with three extractions with 2 mL of n-hexane. Radioactive degradation products in the water phase were counted.

Liver triglyceride secretion. Liver triglyceride secretion rate was measured as described previously (25). Mice were fasted 4h prior to intraperitoneal injection with 1 mg/g body weight Poloxamer 407. Blood samples were collected retro orbitally immediately prior to injection and at 1, 2 and 4 h following injection. The triglyceride accumulation was linear during this time period. Hepatic triglyceride secretion rate was calculated from the slope of the curve and assuming a value of 0.071 mL plasma volume/g body weight (26).

Statistical analysis. Metabolic parameters were expressed as mean ± standard deviation (SD) from the numbers of replicates described in the text. Statistical significance was determined by Student’s t-test and p<0.05 was considered significant.

RESULTS
FGFR4−/− mice exhibit increased white adipose tissue (WAT) and hyperlipidemia. FGFR4−/− mice appeared normal in respect to feeding behavior and physical activity. The impact of ablation of FGFR4 on body, liver and adipose tissue mass in mice of both sexes on normal diets was examined over a 6 month period (Table 1). No significant changes in body mass between wildtype (WT) and FGFR4−/− males or females were noted. Liver mass was slightly higher in FGFR4−/− females and significantly higher in the FGFR4−/− males. Despite a similar body weight, the absence of FGFR4 caused a 1.5- and 2-fold increase respectively in mass of reproductive white adipose tissue in males and females (Table 1, Fig. 1A). The weight of subcutaneous and perirenal fat pads was also higher in the FGFR4−/− mice but less notable (data not shown). The mass of brown adipose tissue (BAT) was similar between the two genotypes (Table 1). A histological analysis of the reproductive white adipose tissue showed that the increase in mass was associated with an increase in size of adipocytes in the FGFR4−/− mice (Fig. 1B-E) and confirmed that there was no difference in brown adipose cell or tissue morphology (Fig. 1F-I). Although plasma leptin and adiponectin did not differ between FGFR4−/− and WT mice, triglycerides (TG), free fatty acids (FFA) and cholesterol were 30 to 40% higher in FGFR4−/− mice under normal dietary conditions (Fig. 2).
Both FGFR4⁻/⁻ and WT mice exhibited the expected increases in body and white adipose tissue mass when presented with only a high fat diet (HFD) over a 4 month period after weaning. No significant differences in the two parameters were noted between the two genotypes (Table 1, Fig. 1). WT mice on the high fat diet exhibited elevated levels of free fatty acids (p<0.05), cholesterol (p<0.001) and leptin (p<0.001), while plasma triglycerides and adiponectin remained constant (Fig. 2). Plasma leptin, adiponectin and free fatty acids did not differ between the two groups, but triglycerides and cholesterol were elevated by 1.4- (p<0.05) and 1.25- (p<0.001) fold, respectively, over WT levels in the FGFR4⁻/⁻ mice. These results indicate that FGFR4 plays a key role in maintenance of systemic lipid homeostasis.

**Hyperglycemia, glucose intolerance and insulin resistance in FGFR4⁻/⁻ mice.** To determine whether glucose metabolism was altered along with lipid metabolism and fat deposition in the FGFR4⁻/⁻ mice, we examined fasting plasma glucose and insulin levels. Although insulin levels were similar, plasma glucose in fasting FGFR4⁻/⁻ mice was about 1.3 times (p<0.05) that observed in WT mice (Fig. 3A,B). When subjected to the glucose tolerance test by administration of 1 g glucose per kg body weight, FGFR4⁻/⁻ mice exhibited elevated levels of glucose over WT mice at all times (183 ± 26 vs. 243 ± 33, WT vs. FGFR4⁻/⁻, p<0.001, 30 mins) after the infusion and levels were still elevated at 2 hr when levels had almost returned to normal (121 ± 15 vs. 180 ± 42, WT vs. FGFR4⁻/⁻, p<0.01) in WT mice (Fig. 3C). Administration of 0.4 U insulin per kg caused plasma glucose levels to drop to only 60% of normal in FGFR4⁻/⁻ mice compared to the 45% (p=0.06) observed in WT mice after 1 hr (Fig. 3D). At 90 and 120 min glucose levels were at 87 and 113 percent of normal, respectively, in the FGFR4⁻/⁻ mice while they remained depressed at 51 and 57 percent of normal in WT mice (p<0.01 and p<0.001, respectively). These results show that FGFR4⁻/⁻ mice exhibited reduced glucose tolerance concurrent with increased insulin resistance.

WT mice subjected to the chronic high fat diet exhibited a hyperinsulinemia (Fig. 3A) that was apparently sufficient to maintain similar fasting plasma glucose levels to animals on a normal diet in this strain of mouse (Fig. 3B). However, the high fat diet caused a reduced glucose tolerance and increased insulin resistance similar to the mice deficient in FGFR4 on the normal diet (Fig. 3C,D). No significant differences between WT and FGFR4⁻/⁻ mice subjected to the chronic high fat diet were detected. This suggests that FGFR4 deficiency or high fat diet causes glucose intolerance and insulin resistance. Any additional effects of the FGFR4 deficiency are overridden or masked by the high fat diet.

The FGFR4 deficiency reduces high fat diet-induced fatty liver. Livers of FGFR4⁻/⁻ mice on a normal diet exhibited no notable morphological differences coincident with the observed hyperlipidemia and insulin resistance. As expected, the chronic high fat diet induced severe fatty liver
in WT males and to a lesser extent in WT females (Fig. 4A,C). Surprisingly, fatty liver was dramatically reduced in FGFR4−/− males and undetectable in females (Fig. 4B,D). Oil Red O staining confirmed the reduction of lipid droplets caused by absence of FGFR4 in livers of mice on the high fat diet (Fig. 4E-H). A direct analysis further confirmed the effect of the FGFR4 deficiency on the elevated hepatic lipid content under the high fat dietary load (Fig. 4I-K). No difference was observed between WT and FGFR4−/− mice on a normal diet. The 71 percent reduction (p<0.001) in triglyceride content in FGFR4−/− mice was most dramatic (Fig. 4I). Cholesterol levels were also reduced by 36 percent (p<0.01) (Fig. 4J) while a reduction in free fatty acids was less significant (18.4 ± 4.2 vs. 14.9 ± 1.5, p=0.06) (Fig. 4K). Despite the reduction in lipid accumulation in the liver, no significant reduction of total liver weight was observed in the FGFR4−/− mice (Table 1) since lipid mass accounts for less than 10% of total mass (Fig. 4). Thus while FGFR4 maintains systemic glucose homeostasis, prevents plasma hyperlipidemia and fat accumulation in the white adipose tissue under normal dietary conditions, it underlies hepatic accumulation of lipid and the fatty liver that results from a chronic high fat dietary load.

The FGFR4 deficiency alters liver lipid metabolism but not glucose metabolism. Liver plays a key role in metabolic homeostasis of organisms by hormone and metabolite-responsive transcriptional level regulation of rate limiting enzymes in both synthetic and catabolic pathways in lipid and glucose metabolism (27;28). Therefore, steady-state levels of mRNA coding for key factors involved in hepatic lipid and glucose metabolism were examined in WT and FGFR4−/− mice subjected to normal and high fat diets (Fig. 5A). No change in expression of sterol regulatory element binding protein 1C (SREBP1C), a major regulator of lipogenesis (29;30), was observed. However, expression of lipogenic transcription factor PPARγ (31; 32) was elevated by 2.3- (p<0.01) and 1.7-fold (p<0.05) above WT levels in FGFR4−/− mice under normal and high fat dietary conditions, respectively. Under normal dietary conditions, expression of lipogenic genes involved in fatty acid synthesis and uptake was generally higher in the FGFR4−/− livers compared to WT. Most significant was the 2.5-fold increase (p<0.05) in stearoyl-CoA desaturase 1 (SCD1) that converts saturated to monounsaturated fatty acids and a 2.6-fold increase (p<0.01) in fatty acid translocase (CD36/FAT). Expression levels of fatty acid synthase (FAS), SCD1 and CD36, but not acetyl-CoA carboxylase (ACC1) increased 2- to 3.5-fold (p<0.01) after administration of the high fat diet in WT mice. However, no additional changes in the elevated levels were observed in the FGFR4−/− mice except a 2.6-fold increase of FAS (p<0.01). The increase in PPARγ, SCD1 and CD36 in the FGFR4−/− mice may contribute to the hyperlipidemia observed under normal dietary conditions.

In contrast to lipogenic transcripts PPARγ, SCD1 and CD36, expression of liver PPARα and its downstream targets, medium-chain acyl-CoA
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dehydrogenase (MCAD) that stimulates fatty acid oxidation (33), and microsomal triglyceride transfer protein (MTP) that is required for the assembly and secretion of apoB-containing lipoproteins (34) were unaffected by FGFR4 deficiency or the high fat dietary load (Fig. 5A). However levels of these genes were elevated by about 40 to 60 percent (p<0.05) in FGFR4−/− mice on the high fat diet. As we reported previously (20), hepatic FGFR4 is a negative regulator of expression of CYP7A, the rate-limiting enzyme for the canonical pathway of cholesterol to bile acid synthesis, under normal dietary conditions. Fig. 5A shows that the 3-fold elevation of CYP7A expression in FGFR4−/− mice relative to WT was also apparent in the obese mice under the high fat dietary load. Thus the reduction in liver cholesterol may also contribute to the alleviation of fatty liver in mice devoid of FGFR4.

We then determined rates of liver fatty acid oxidation and triglyceride secretion. Fatty acid oxidation was 1.43 (p<0.05) times higher in FGFR4−/− livers in mice on a high fat diet although they were similar to WT under normal dietary conditions (Fig. 5B). FGFR4−/− mice on a normal diet exhibited an insignificant 20% (p=0.08) increase in rate of hepatic triglyceride secretion. Similar to observations on obese ob/ob mice (35), the rate of secretion was reduced in the WT obese mice on the chronic high fat diet. The absence of FGFR4 abolished the resultant reduction in obese mice (Fig. 5C). Obese FGFR4−/− mice exhibited an 82% (p<0.01) increase in hepatic triglyceride secretion compared to WT littermates.

Lastly, we examined the effect of FGFR4 deficiency on expression of the two key transcriptionally-regulated regulators of hepatic gluconeogenesis, phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase). The FGFR4 deficiency had no effect on expression of either PEPCK or G6Pase under normal dietary conditions. The high fat dietary load caused about a 1.8-fold increase in G6Pase mRNA (p<0.05) that was reduced back to normal levels in the FGFR4−/− mice (Fig. 5A). We then determined whether insulin responsiveness was altered in livers of the FGFR4-deficient mice. Although insulin-stimulated phosphorylation of the insulin receptor and Akt were significantly decreased in obese compared to normal animals, no significant difference was observed between livers of WT and FGFR4−/− mice under either condition (Supplemental Fig. 1). This indicated normal insulin signaling in the FGFR4−/− mouse livers upstream through Akt. Together, these results suggest that lipid, but not glucose metabolism in the liver is impaired by the absence of germline FGFR4.

Hepatocyte FGFR4 is the determinant of plasma lipid levels and fatty liver. To determine the contribution of hepatocyte FGFR4 to lipid and glucose metabolism, we examined the impact of overexpression of FGFR4 in hepatocytes driven by the albumin promoter in FVB mice (21). A constitutively active FGFR4 (caFGFR4) mutant was employed to insure a sustained signal and bypass the need for an activating ligand. No significant changes relative to the WT
control in body, liver, white or brown adipose tissue weight was observed in the Alb-caFGFR4 mice expressing hyperactive FGFR4 in hepatocytes (data not shown). Basal fasting levels of plasma triglycerides, free fatty acids and cholesterol were higher in the WT FVB mice relative to the WT littermate control strain for the FGFR4+/− mice (Fig. 6A). However, in contrast to the elevation in FGFR4+/− mice relative to their WT control, the three parameters in Alb-caFGFR4 mice were decreased to about 80% (p<0.01), 70% (p<0.01) and 85% (p<0.05) , respectively, of the WT control (Fig. 6A).

To determine whether restoration of FGFR4 to the hepatocytes could rescue the metabolic phenotype of the FGFR4+/− mice, the two strains were crossed to produce a FGFR4+/−/Alb-caFGFR4 hybrid. Littermates from the cross were compared to minimize strain differences. The FGFR4+/−/Alb-caFGFR4 mice exhibited normal liver morphology although their weight was reduced compared to FGFR4+/− littermates (1.51 g ± 0.22 vs. 1.27 g ± 0.18, p<0.05). The hybrid mice exhibited a less significant 15 percent reduction in reproductive white adipose tissue. Plasma levels of triglyceride, free fatty acids, and cholesterol were also significantly reduced in the FGFR4+/−/Alb-caFGFR4 mice relative to their FGFR4+/− littermates (Fig. 6B). Except for a 30 percent reduction (p<0.05) in blood triglyceride levels in Alb-caFGFR4 mice, no differences in other parameters measured were observed when the mice were administrated a high fat diet (Fig. 6). This suggests an inability of FGFR4 activity to compensate for the increase in plasma lipids levels caused by the high fat diet.

We then determined whether restoration of FGFR4 to hepatocytes of the germline FGFR4-deficient mice that are resistant to high fat diet-induced fatty liver (Fig. 4) would restore the fatty liver condition. A fatty liver similar to WT mice on the chronic high fat dietary load was apparent in the FGFR4+/−/Alb-caFGFR4 hybrids (Fig. 7A-D). Quantification revealed that restored hepatocyte FGFR4 expression increased liver triglyceride levels (35.9 ± 8.69 vs. 108.6 ± 33.8, p<0.01). Analysis of gene expression revealed that restoration of hepatocyte FGFR4 largely reversed the altered gene expression related to lipid metabolism caused by FGFR4 deficiency, but as expected had no impact on genes related to glucose metabolism (Supplemental Fig. 2). These results show that specifically hepatocyte FGFR4 plays a major role in lipid metabolism in the liver. Its activity directly impacts plasma lipid homeostasis and underlies the fatty liver disease that results from a chronic high fat diet.

**Hepatocyte FGFR4 does not directly affect glucose metabolism.** We then determined whether hyperactive FGFR4 in hepatocytes or the restoration of hepatocyte FGFR4 to deficient livers would restore defects in glucose metabolism induced by the global absence of FGFR4 (Fig. 3). In contrast to FGFR4+/− mice that exhibited hyperglycemia and hyperinsulinemia neither Alb-caFGFR4 nor FGFR4+/−/Alb-caFGFR4 hybrids exhibited changes in these parameters relative to their appropriate WT or FGFR4+/−...
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littermate controls (Fig. 8A,B). Glucose and insulin tolerance tests further confirmed that the transgenic mice were exhibited similar glucose tolerance and insulin sensitivity to controls (Supplemental Fig. 3). These results suggest that the absence of hepatocyte FGFR4 activity is insufficient to explain the abnormalities in glucose homeostasis observed in FGFR4-/- mice. They suggest a potential role of FGFR4 on glucose homeostasis at another organ site.

DISCUSSION
In this study, we showed that FGFR4-/- mice displayed multiple elements of metabolic syndrome that included increased white adipose tissue, hyperlipidemia and insulin resistance. However, despite the beneficial effects of FGFR4 activity on plasma lipid and glucose homeostasis under normal dietary conditions, FGFR4 underlies development of fatty liver with the obesity that comes with a chronic high fat diet. The alleviation of fatty liver induced by the high fat dietary load by ablation of FGFR4 was associated with elevated plasma triglycerides without effect on an increase in body mass, adiposity, glucose intolerance and insulin resistance. Put another way the normal protection against hyperlipidemia mediated by hepatocyte FGFR4 is to the detriment of the liver under conditions of chronic high fat diet and obesity. Our observations revealed the physiological importance of FGFR4 signaling in normal lipid and glucose homeostasis in addition to cholesterol and bile acid metabolism. Hyperlipidemia and fatty liver are clinically associated with hyperglycemia and insulin resistance, all of which are part of metabolic syndrome. Type 2 diabetics with fatty liver are substantially more insulin resistant and have higher levels of plasma free fatty acids than those without it (6). Thus, reduced fatty liver in FGFR4-/- mice might explain the similar extent of insulin resistance of these mice under a high fat diet although FGFR4-/- mice on a normal diet were more insulin resistant.

Our findings that hepatocyte FGFR4 activity maintains systemic lipid homeostasis under normal dietary conditions, but underlies fatty liver in obese mice on a high fat diet indicate dramatically different roles of FGFR4 that are dependent on nutritional status. At the molecular level under normal dietary conditions, FGFR4 deficiency is associated with elevation of liver lipogenic genes PPARγ, SCD1 and CD36 with no change in catabolic factors which is consistent with the hyperlipidemia observed in FGFR4-/- mice. In contrast, on a high fat diet FGFR4 deficiency caused a net increase in PPARα and its downstream target genes MCAD and MTP, which is accompanied by elevated levels of fatty acid oxidation and hepatic triglyceride secretion. How high fat dietary overload causes the FGFR4 deficiency to increase gene expression associated with fatty acid oxidation and hepatic triglyceride secretion remains to be determined. Such dual and seemingly opposing effects dependent on nutritional status are not without precedent. When overexpressed in hepatocytes, transcriptional regulator LXRα which directly senses diverse lipid
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metabolites as ligands (38) elevates mouse serum lipid profiles on a normal diet, but improves high blood lipid profiles and protects from atherosclerosis in mice on a Western diet (39). It should also be noted that bile acids reduce high fat diet-induced hyperglycemia and triglyceride accumulation in liver (36). FGFR4-/- mice display increased bile acid levels (20).

An unresolved issue from our study is the contribution of other organs or tissues where FGFR4 is expressed other than liver for metabolic abnormalities in the FGFR4-deficient mice. We have shown that both overexpression and restoration of FGFR4 to specifically hepatocytes decreased plasma lipid levels, but failed to improve glucose tolerance and insulin sensitivity. Consistent with this, FGFR4 deficiency neither affects liver gluconeogenic enzymes nor hepatic insulin signaling. This indicates that hepatocyte FGFR4 plays a major role in control of hyperlipidemia, but not the hyperglycemia, glucose intolerance and insulin resistance caused by the general deficiency of FGFR4. Cholesterol synthesis and conversion to bile acids is limited to mature liver hepatocytes where FGFR4 is the only FGFR isotype (40). In contrast, lipid and glucose homeostasis is a partnership between liver and peripheral organs most significant of which are skeletal muscle and adipose tissue. This suggests that peripheral sites other than liver or the complex interaction of multiple sites are the determinant of hyperglycemia and insulin resistance in FGFR4-deficient mice. FGFR4 is not expressed in adipose tissue (13;15), but is functional in skeletal muscle and has been implicated in its cellular homeostasis during embryogenesis and regeneration (41;42). Compared with other obese mouse models which have a significantly increased body weight, the FGFR4-/- mice displayed an increase in the mass of reproductive white adipose tissue without a change in overall body weight and levels of two major adipokines, leptin and adiponectin. Apparently neither an increase in caloric intake nor obesity is the primary factor in determining the basal FGFR4-/- metabolic phenotype. One candidate for the obesity-independent upset in both systemic lipid and glucose metabolism due to FGFR4 deficiency is FXR that has been implicated in both systemic lipid and glucose metabolism (43-45). An enterohepatic FXR-FGF15-FGFR4 axis has been suggested for regulation of hepatic cholesterol to bile acid metabolism and this may also extend to hepatic lipid metabolism. Since hepatocyte FGFR4 does not play a major role in glucose metabolism and FGFR4 is not at play in adipose tissue (13;15), an FXR-regulated FGF15 to muscle FGFR4 axis that contributes to glucose metabolism is conceivable. Results not shown here indicated that skeletal muscle in FGFR4-/- mice exhibited elevated levels of lipid compared to wild type mice. Elevation of skeletal muscle lipids is associated with metabolic syndrome and insulin resistance (46; 47). Additional experiments using mice with muscle-specific alterations in FGFR4 should clarify the relative contributions of muscle FGFR4 to aberrant lipid and glucose metabolism, or whether
FGFR4 at still other organ sites is involved.

The opposite phenotypes between FGFR4-deficient mice on a normal diet and those caused by systemic administration or overexpression of FGF19, the human ortholog of mouse FGF15 (13;14), further suggest FGF15/19 as a candidate activator of FGFR4. However, both systemic administration or overexpression of FGF19 (13;14) and the FGFR4 deficiency reduced triglyceride content in the liver in obese mice. It has also been reported that mice with an FGFR4 gene deletion are still metabolically responsive to systemic FGF19 (13). Although it has been argued that FGF19 may be a specific FGF ligand for FGFR4 (48;49), this specificity has yet to be confirmed and particularly in tissues where FGFR4 has impact. Another candidate FGF ligand for FGFR4, particularly in hepatocytes is FGF21. Systemic administration or forced expression of FGF21 in hepatocytes results in reduced adiposity, improved glucose clearance and insulin sensitivity (15). FGF21 is expressed at low levels in hepatocytes and increases dramatically with liver perturbation (22;50). We have shown that constitutive expression of FGF21 in hepatocytes delays development of chemically-induced early hepatic adenomas in mice (22). The FGF21-dependent delay of adenoma development may suggest an internal autocrine activation of hepatocyte FGFR4 since resident hepatocyte FGFR4 exerts a suppressive effect on chemically-induced hepatomas (22, Huang et al., Resident hepatocyte FGFR4 limits while ectopic FGFR1 accelerates hepatocarcinogenesis, in preparation). However, the autocrine action of hepatocyte FGF21 on hepatocyte FGFR4 alone cannot explain the effects of systemic administration of FGF21 on systemic glucose metabolism. Hepatic glucose metabolism is insensitive to hepatic FGFR4 activity. From cell culture studies, Kharitonenkov and colleagues (15;23) have shown that FGF21 stimulates a PPARγ agonist-enhanced glucose uptake and metabolism in adipocytes that express other isoforms of FGFR kinase isoforms than FGFR4. An endocrine activity of hepatocyte FGF21 on adipose tissue under control of FXR-FGF15/19 activated hepatocyte FGFR4 is an attractive adjunct to FGF signal-mediated four-way communication among ileum, liver, skeletal muscle and adipose tissue in control of lipid and glucose homeostasis.

In summary, we have shown that in addition to cholesterol and bile acid homeostasis, FGFR4 plays an important role in systemic lipid and glucose homeostasis. Similar to bile acid metabolism, hepatocyte FGFR4 exerts major impact on lipid metabolism, but it appears that FGFR4 at other organ sites effects glucose homeostasis. Ironically, hepatocyte FGFR4 is also responsible for the fatty liver associated with obesity induced by chronic high fat intake. General agonists of FGFR4 may be beneficial in alleviation of elements and consequences of metabolic syndrome related to hyperglycemia and hyperlipidemia. However, in the obese under conditions of high caloric intake the benefits may be at the expense of aggravating fatty liver. General
antagonists of FGFR4 may relieve fatty liver in the obese, but aggravate the other consequences of metabolic syndrome. A complete knowledge of FGF ligand and tissue context specificity of FGFR4 signaling will be essential to design tissue-specific agonists and antagonists to alleviate all elements of metabolic syndrome in the obese.

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Table 1. Body and tissue mass in WT and FGFR4/- mice

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Male Normal Diet</th>
<th>Female Normal Diet</th>
<th>Male HF diet</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WT</td>
<td>FGFR4/-</td>
<td>WT</td>
</tr>
<tr>
<td>Body Mass (BM) (g)</td>
<td>27.90 ± 3.30</td>
<td>29.68 ± 4.41</td>
<td>24.22 ± 1.91</td>
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<tr>
<td>Liver Mass (LM) (g)</td>
<td>1.25 ± 0.19</td>
<td>1.44 ± 0.19*</td>
<td>1.13 ± 0.15</td>
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<tr>
<td>LM/BM (%)</td>
<td>3.89 ± 0.32</td>
<td>4.48 ± 0.75*</td>
<td>4.19 ± 0.55</td>
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<td>Reproductive WAT (g)</td>
<td>0.65 ± 0.15</td>
<td>0.96 ± 0.24*</td>
<td>0.86 ± 0.19</td>
</tr>
<tr>
<td>RWAT/BM (%)</td>
<td>2.25 ± 0.54</td>
<td>3.14 ± 0.45**</td>
<td>3.07 ± 0.52</td>
</tr>
<tr>
<td>BAT (g)</td>
<td>0.16 ± 0.05</td>
<td>0.15 ± 0.04</td>
<td>0.11 ± 0.01</td>
</tr>
</tbody>
</table>

Wildtype and FGFR4/- mice on a normal diet were examined at 6 months and mice on a high fat (HF) diet were examined after 4 months of exposure to it since weaning. WAT, white adipose tissue; BAT, brown adipose tissue. Data are mean ± SD (n=9-25 mice). *p<0.05, †p<0.01.
Figure Legends

FIG. 1. Increase in mass of white adipose tissue in FGFR4−/− mice. A: Reproductive white adipose tissue (WAT) in representative 6 month old WT and FGFR4−/− males. B-I: Adipocyte size increases in WAT, but not brown fat (BAT) in FGFR4−/− mice on normal diet. Sections were prepared from the respective type of adipose tissue from representative 6 month old males on a normal diet or males on a high fat diet 4 months since weaning. Sections were stained with H&E as described in Methods.

Fig. 2. Increased fasting plasma lipid levels in FGFR4−/− mice. A-E: Fasting plasma triglycerides (TG), free fatty acids (FFA), cholesterol, leptin and adiponectin levels of WT and FGFR4−/− mice on normal and high fat diets were measured. Data are expressed as mean ± SD (n = 8-18 mice). *p<0.05; ***p<0.001.

Fig. 3. Glucose intolerance and insulin resistance in FGFR4−/− mice. A,B: Fasting plasma insulin and glucose levels were assessed in mice with the indicated genotype on a normal and high fat diet. C: Plasma glucose levels were measured at the indicated times after intraperitoneal administration of 1 g glucose per kg body weight to fasting mice. D: Plasma glucose levels were measured at the indicated times following administration of 0.4 U insulin per kg body weight. N = normal diet; HF = high fat diet. Data are expressed as mean ± SD (n = 8-10 mice). *p<0.05

Fig. 4. FGFR4 deficiency alleviates high fat diet-induced fatty liver. A-D: Sections from livers of representative WT and FGFR4−/− mice of the indicated sex on the high fat diet were prepared, fixed and stained with H&E. E-H: Lipid in sections of livers from representative male WT and FGFR4−/− mice on a normal or high fat diet was stained with Oil Red O. Intense red indicates lipid droplets. I-K: Triglycerides (TG), cholesterol and free fatty acids (FFA) were measured in lipid extracts of livers of WT and FGFR4−/− males on the indicated diet (N = normal; HF = high fat). Data are expressed as mean ± SD (n = 7-12 mice). **p<0.01, ***p<0.001.

Fig. 5. FGFR4 deficiency alters liver lipid but not glucose metabolism. A: Expression of hepatic genes involved in lipid and glucose metabolism. Expression levels were determined in the indicated mice on the indicated diet by quantitative real-time PCR analysis of steady-state mRNA levels and normalized to β-actin expression. Values in WT mice on a normal diet was set to 1. Data are the mean ± SD of three independent experiments for each gene with 4 to 5 mice of each genotype on each dietary regimen. B: Hepatic fatty acid oxidation. C: Hepatic triglyceride secretion. Values are expressed relative to WT mice on a normal diet. Data are expressed as mean ± SD (n = 6-9 mice). *p<0.05, **p<0.01 relative to the corresponding WT mice.
Fig. 6. Hepatocyte-specific expression of activated FGFR4 reduces plasma lipid levels. **A**: Fasting plasma triglycerides (TG), free fatty acids (FFA) and cholesterol were determined in mice overexpressing constitutively active FGFR4 in hepatocytes (Alb-caFGFR4) and compared to the WT FVB strain on a normal (N) or high fat (HF) diet. **B**: Fasting plasma lipid levels were determined in the FGFR4**+/−** x Alb-caFGFR4 hybrid and compared to the FGFR4**+/−** littermates. Data are expressed as mean ± SD (n = 8-11 mice). *p<0.05, **p<0.01, ***p<0.001.

Fig. 7. Hepatocyte-specific restoration of FGFR4 in FGFR4**−/−** mice restores high fat diet-induced fatty liver. **A, B**: Representative livers from FGFR4**+/−** and hybrid FGFR4**+/−**/Alb-caFGFR4 mice on a high fat diet were processed and stained with H&E as described in Figure 4. **C, D**: Sections from the same livers were stained with Oil Red O.

Fig. 8. Hepatocyte FGFR4 does not affect glucose metabolism. **A, B**: Fasting plasma insulin and glucose levels were determined in the caFGFR4 and FGFR4-/-/Alb-caFGFR4 hybrids as described in Methods. Data are expressed as mean ± SD (n = 8-11 mice). N = normal diet; HF = high fat diet.
Figure 2

A: TG (mg/dL) comparison between WT and FGFR4+ in Normal and High Fat conditions.
B: FFA (mM/L) comparison between WT and FGFR4+ in Normal and High Fat conditions.
C: Cholesterol (mg/dL) comparison between WT and FGFR4+ in Normal and High Fat conditions.
D: Leptin (ng/mL) comparison between WT and FGFR4+ in Normal and High Fat conditions.
E: Adiponectin (ug/mL) comparison between WT and FGFR4+ in Normal and High Fat conditions.
FGFR4 regulation of metabolism

Figure 4
Figure 5

A. Lipogenesis/Uptake
- SREBP1C
- PPAR γ
- ACC1
- FAS
- SCD1
- CD36

B. Catabolism/Secretion
- PPAR α
- MCAD
- MTP
- PEPCK
- G6Pase
- CYP7A

C. Gluconeogenesis

D. Cholesterol

- WT Normal
- FGFR4−/− Normal
- WT High Fat
- FGFR4−/− High Fat
Figure 6

A

- TG (mg/dL)
- FFA (mM/L)
- Cholesterol (mg/dL)

WT | Alb-caFGFR4

N  | HF  | N  | HF  | N  | HF

** | *  | ** | ** | ** | *

B

- TG (mg/dL)
- FFA (mM/L)
- Cholesterol (mg/dL)

FGFR4−/− | FGFR4−/− X Alb-caFGFR4

N  | HF  | N  | HF  | N  | HF

*  |  | *** | *** | ** | **
Figure 7

FGFR4<sup>-/-</sup>  FGFR4<sup>-/-</sup> X Alb-caFGFR4

A  B

H&E

100 µm

C  D

Oil Red O

50 µm
Figure 8

A  

\[ \text{Insulin (ng/mL)} \]

\[ \begin{array}{c|c|c}
\text{WT} & \text{Alb-caFGFR4} \\
\hline
\text{N} & \text{HF} & \text{N} & \text{HF} \\
0 & 0 & 2.5 & 2.5 \\
0.5 & 1.5 & 2 & 2.5 \\
1 & 1.5 & 2 & 2.5 \\
2 & 2.5 & 2.5 & 2.5 \\
\end{array} \]

B  

\[ \text{FGFR4}^{-/-} \quad \text{FGFR4}^{-/-} \times \text{Alb-caFGFR4} \]

\[ \begin{array}{c|c|c}
\text{Insulin (ng/mL)} \]

\[ \begin{array}{c|c|c}
\text{N} & \text{HF} & \text{N} & \text{HF} \\
0 & 0 & 2.5 & 2.5 \\
0.5 & 1.5 & 2 & 2.5 \\
1 & 1.5 & 2 & 2.5 \\
2 & 2.5 & 2.5 & 2.5 \\
\end{array} \]

\[ \text{Glucose (mg/dL)} \]

\[ \begin{array}{c|c|c}
\text{N} & \text{HF} & \text{N} & \text{HF} \\
0 & 0 & 200 & 200 \\
0.5 & 1.5 & 160 & 160 \\
1 & 1.5 & 120 & 120 \\
2 & 2.5 & 80 & 80 \\
\end{array} \]