Effective treatment of steatosis and steatohepatitis by fibroblast growth factor 1 in mouse models of nonalcoholic fatty liver disease

Weilin Liu[a], Dicky Struik[a], Vera J. M. Nies[a], Angelika Jurdzinski[a], Liesbeth Harkema[b], Alain de Bruin[a,b], Henkjan J. Verkade[a], Michael Downes[c], Ronald M. Evans[c,1], Tim van Zutphen[a], and Johan W. Jonker[a,1]

[a]Center for Liver, Digestive and Metabolic Diseases, Department of Pediatrics, University of Groningen, University Medical Center Groningen, 9713 GZ Groningen, The Netherlands; [b]Dutch Molecular Pathology Center, Faculty of Veterinary Medicine, Utrecht University, 3584 CL Utrecht, The Netherlands; and [c]Gene Expression Laboratory, Salk Institute for Biological Studies, La Jolla, CA 92037

Nonalcoholic fatty liver disease (NAFLD) is the most common chronic liver disorder and is strongly associated with obesity and type 2 diabetes. Currently, there is no approved pharmacological treatment for this disease, but improvement of insulin resistance using peroxisome proliferator-activated receptor-γ (PPARγ) agonists, such as thiazolidinediones (TZDs), has been shown to reduce steatosis and steatohepatitis effectively and to improve liver function in patients with obesity-related NAFLD. However, this approach is limited by adverse effects of TZDs. Recently, we have identified fibroblast growth factor 1 (FGF1) as a target of nuclear receptor PPARγ in visceral adipose tissue and as a critical factor in adipose remodeling. Because FGF1 is situated downstream of PPARγ, it is likely that therapeutic targeting of the FGF1 pathway will eliminate some of the serious adverse effects associated with TZDs. Here we show that pharmacological administration of recombinant FGF1 (rFGF1) effectively improves hepatic inflammation and damage in leptin-deficient *ob/ob* mice and in choline-deficient mice, two etiologically different models of NAFLD. Hepatic steatosis was effectively reduced only in *ob/ob* mice, suggesting that rFGF1 stimulates hepatic lipid catabolism. Potentially adverse effects such as fibrosis or proliferation were not observed in these models. Because the anti-inflammatory effects were observed in both the presence and absence of the antisteatotic effects, our findings further suggest that the anti-inflammatory property of rFGF1 is independent of its effect on lipid catabolism. Our current findings indicate that, in addition to its potent glucose-lowering and insulin-sensitizing effects, rFGF1 could be therapeutically effective in the treatment of NAFLD.

FGF1 | steatosis | steatitis | NAFLD | inflammation

Nonalcoholic fatty liver disease (NAFLD) is the most common chronic liver disease in developed countries and is strongly associated with obesity and type 2 diabetes (1). NAFLD refers to a wide spectrum of liver disorders ranging from simple fatty liver (steatosis) to nonalcoholic steatohepatitis (NASH) with increased risk of developing progressive fibrosis, cirrhosis, and liver cancer (2).

Treatment options for NAFLD are limited and are directed mainly at weight loss or pharmacological improvement of insulin resistance (3). Although no pharmacologic therapy has been approved, the thiazolidinedione (TZD) class of insulin sensitizers has been demonstrated to improve steatosis, steatohepatitis, and liver function in mice and patients with NAFLD (1). TZDs improve insulin sensitivity through activation of nuclear receptor peroxisome proliferator-activated receptor-gamma (PPARγ), which reduces insulin resistance in adipose tissue, liver, and skeletal muscle (4). The exact mechanism by which PPARγ exerts its beneficial effects on NAFLD is not completely understood, but it is believed that improved hepatic insulin sensitivity enhances lipid oxidation and reduces hepatic lipogenesis, thereby reducing steatosis (5). In addition, increased peripheral insulin sensitivity may reduce lipolysis in white adipose tissue and thereby limit ectopic fat accretion.

PPARγ and its activators also have broad anti-inflammatory effects. On one hand, PPARγ has been shown to attenuate the expression and secretion of proinflammatory cytokines (including IL-1β and TNF-α) associated with M1 macrophages (6); on the other hand, it reduces macrophage activity via transrepression of NF-κB (7). Despite their efficacy in glycemic control and reduction of steatosis, TZDs are associated with various serious adverse side effects, including weight gain, fluid retention, osteoporosis, and cardiovascular toxicity, which have strongly limited their clinical use (4). These limitations highlight the need for novel approaches such as more selective PPARγ agonists or direct activation of downstream targets.

Recently we have identified fibroblast growth factor 1 (FGF1) as a target of PPARγ in visceral adipose tissue and as a critical factor in adipose remodeling (8). Mice with an FGF1 deficiency displayed a severe diabetic phenotype with increased inflammation

Significance

Fibroblast growth factor 1 (FGF1) is critical for adipose tissue remodeling under conditions of dietary stress. Pharmacological treatment with recombinant FGF1 (rFGF1) has potent glucose-lowering, insulin-sensitizing, and antisteatotic effects in hyperglycemic mouse models, but the mechanism is largely unknown. Here we characterize the effects of rFGF1 on nonalcoholic liver disease in two etiologically different mouse models. Strong antisteatotic effects of rFGF1 were observed in *ob/ob* mice but not in choline-deficient mice, suggesting that rFGF1 exerts its antisteatotic effect via processes specifically impaired in choline-deficient mice, such as lipid oxidation and lipoprotein secretion. In contrast, hepatic inflammation and alanine aminotransferase levels were reduced in both models, indicating that these effects are independent of the antisteatotic properties of rFGF1.

and fibrosis in adipose tissue. Conversely, pharmacological treatment with recombinant FGF1 (rFGF1) has a potent insulin-sensitizing effect at the systemic level, and in the liver it effectively reduces steatosis in *ob/ob* mice (9). It remains unclear, however, if and to what extent the hepatic effects of FGF1 are direct or indirect.

In this study we used two etiologically different models of NAFLD to determine the mechanism by which rFGF1 improves liver disease: leptin-deficient *ob/ob* mice, which develop steatosis primarily through excessive food intake, and mice with a dietary choline deficiency, which develop steatosis primarily as a result of a defect in hepatic lipid catabolism (10). Interestingly, we found that rFGF1 effectively reverses steatosis in *ob/ob* mice but not in mice with a dietary choline deficiency, suggesting that rFGF1 stimulates hepatic lipid catabolism. rFGF1 treatment improved steatohepatitis and plasma alanine transaminase activity (ALT) in both models, indicating that the effects of rFGF1 on hepatic inflammation and liver function are independent of its anti-steatotic properties. Together our results provide insight into the mechanism by which rFGF1 improves NAFLD and highlight its potential therapeutic value in the treatment of different aspects of liver disease.

Results

rFGF1 Has Potent Antisteatotic Effects in *ob/ob* Mice. To investigate the mechanism by which rFGF1 exerts its effects on NAFLD/NASH, we treated *ob/ob* mice for a period of 12 d with rFGF1 (0.5 mg/kg i.p. every 3 d). Twelve days of treatment significantly reduced hepatic levels of triglycerides and liver mass without affecting body weight (Fig. 1 A–C). Histological examination using H&E staining confirmed the antisteatotic effect of rFGF1 but also revealed that this reduction in hepatic lipids occurred in a zonated fashion (Fig. 1D). To further explore this zonation effect, we used the central vein marker glutamine synthetase (GS), which indicated pronounced reduction of steatosis in the periportal zone, but steatosis in the pericentral region was not significantly affected by rFGF1 treatment (Fig. 1 E–G).

rFGF1 Suppresses Hepatic Inflammation in *ob/ob* Mice. Hepatic steatosis can develop into NASH, which is more serious and is characterized by hepatic inflammation and fibrosis. In addition to its potent antisteatotic action, rFGF1 also suppressed hepatic inflammation in *ob/ob* mice as indicated by reduced mRNA expression of a range of hepatic inflammatory markers (Fig. 2 A–D). Twelve days of treatment with rFGF1 significantly reduced the expression of the proinflammatory M1 markers monocyte chemotactic protein 1 (MCP-1) and TNFα and the macrophage/Kupffer markers F4/80, CD68, and CD11c (Fig. 2A). After 5 wk of treatment with rFGF1 these markers were reduced even further, and significant reductions also were observed for the proinflammatory cytokine IL1β, the cell adhesion molecules E-selectin, intracellular adhesion molecule 1 (ICAM-1), and vascular cell adhesion molecule 1 (VCAM-1), which are activated by TNFα and IL-1β, and the macrophage marker CD11b (Fig. 2B). In contrast, hepatic expression of the anti-inflammatory M2 markers IL-10, CD163, and Arginase 1 (Arg1) was not affected by rFGF1 administration (except for CD206 in the mice treated for 5 wk) (Fig. S1 A–D), indicating that rFGF1 exerts its anti-inflammatory effect mainly by suppressing proinflammatory M1 markers in liver. Reduced hepatic inflammation also was observed by histopathological and protein analysis, indicating lower scores on lobular inflammation (Table S1) and reduced levels of TNF-α (Fig. 2 C–E).

rFGF1 Reduces Endothelial VCAM-1 Expression. To investigate how rFGF1 suppresses hepatic inflammation, we examined its potential to modulate cytokine- or endotoxin-induced inflammatory gene expression in several cell models representing different hepatic cell types (hepatocytes, macrophages, and endothelial cells). We did not find a role for hepatocytes, the major parenchymal cell type in liver, in the anti-inflammatory effect of rFGF1 because rFGF1 did not affect basal or slightly increased cytokine-
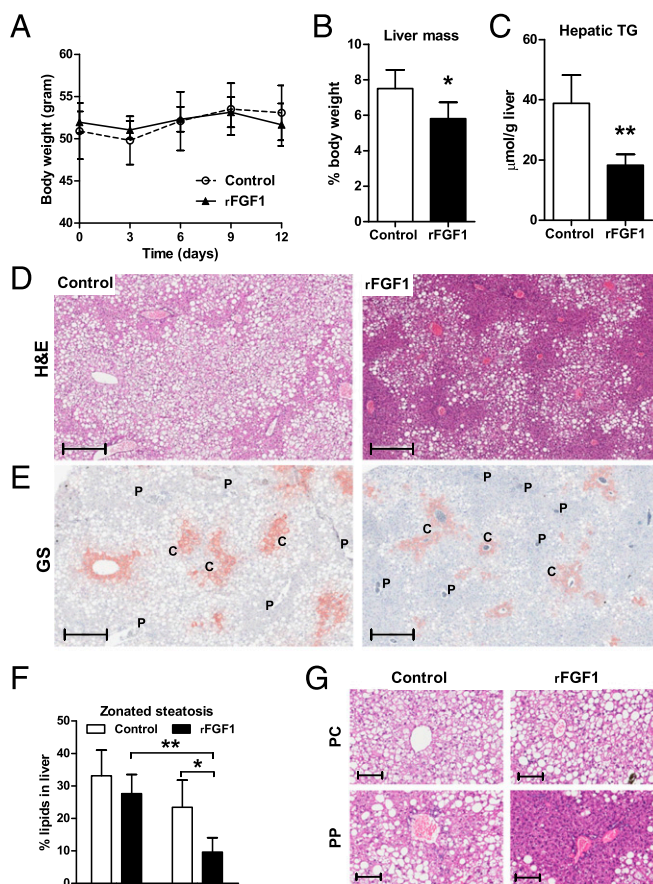

Fig. 1. rFGF1 reduces hepatic steatosis in *ob/ob* mice. (A–C) A 12-d rFGF1 treatment (0.5 mg/kg i.p. every 72 h) of *ob/ob* mice does not affect body weight (A) but does reduce liver mass (B) and hepatic triglyceride levels (C) (n = 6; unpaired t test with Welch's correction). (D and E) Histological visualization of steatosis in serial liver sections stained with H&E (D) and the central vein marker GS (E) combined with H&E. C, central vein; P, portal area. (Scale bars: 300 μm.) (F) Quantitation of zonal distribution of steatosis in liver (n = 6 slides per group; two-way ANOVA). PC, pericentral zone; PP, periportal zone. (G) High magnification H&E-stained liver sections show reduced steatosis at the periportal zone in rFGF1 treated animals (scale bars: 90 μM). *$P < 0.05$, **$P < 0.01$.

induced (i.e., TNFα/IL1β) inflammatory gene expression (Fig. S2). We next questioned if rFGF1 could mediate its anti-inflammatory effect through the modulation of endotoxin-induced activation of macrophages or endothelial cells. We examined the effect of rFGF1 preincubation on the activation of RAW264.7 macrophage cells and human umbilical vein endothelial cells (HUVECs) by LPS. In RAW264.7 cells, rFGF1 pretreatment did not interfere with basal or endotoxin-induced inflammatory gene expression (Fig. S2). In contrast, a significant reduction in the expression of VCAM-1 was observed in HUVECs in response to LPS (Fig. 3A). Basal and endotoxin-induced gene expression of MCP-1, ICAM-1, and E-selectin was unaffected by rFGF1 pretreatment in HUVECs (Fig. 3 B–D). Because VCAM-1 has been implicated in leukocyte recruitment, it is possible that the anti-inflammatory effects of rFGF1 are mediated through reduced endothelial VCAM-1 expression.

The Antisteatotic Effects of rFGF1 Are Absent in a Choline-Deficient Model of Steatosis. Steatosis results from an imbalance in hepatic lipid metabolism. Hepatic fatty acid synthesis and triglyceride accumulation occur predominantly in the pericentral zone, whereas fatty acid oxidation and secretion [very-low-density lipoprotein (VLDL) production] are associated more with the periportal

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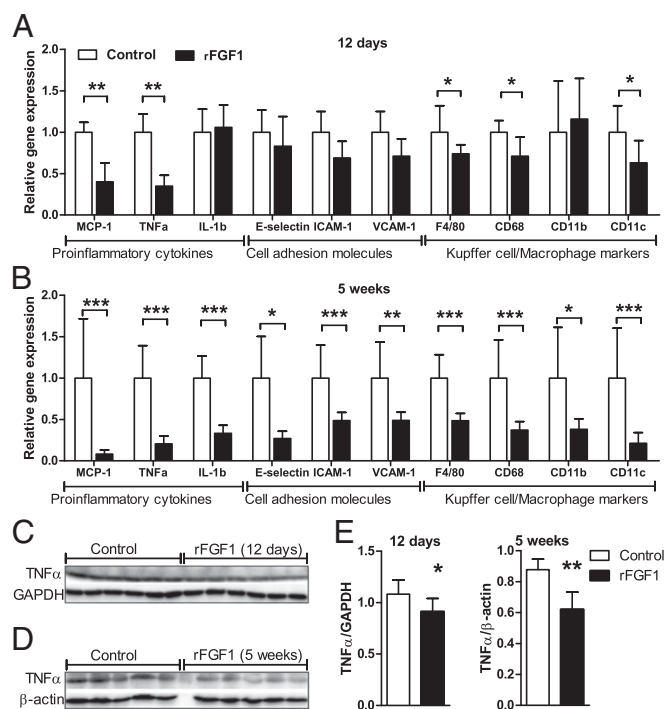
Fig. 2. rFGF1 suppresses hepatic inflammation in *ob/ob* mice. (*A* and *B*) Gene expression of inflammatory markers in livers of *ob/ob* mice treated with rFGF1 for 12 d (*A*) or for 5 wk (*B*). (*C*–*E*) Western blot analysis of TNF-α protein levels in the livers of rFGF1-treated *ob/ob* mice at 12 d (*C*) or 5 wk (*D*). (*E*) Quantitation of *C* and *D*. (*n* = 6–8; Mann–Whitney test.) *P < 0.05, **P < 0.01, ***P < 0.001.

zone (11). Our observation that rFGF1 primarily reduces steatosis in the periportal zone thus suggested that rFGF1 improves hepatic lipid catabolism (i.e., oxidation and/or secretion). To investigate further how rFGF1 exerts its antisteatotic effects, we used a choline-deficient, L-amino acid–defined (CDAA) diet, a commonly used rodent model for steatosis (10). In contrast to *ob/ob* mice and diet-induced obesity (DIO) models of steatosis, which have increased hepatic lipid accumulation through excessive food intake, choline deficiency causes a defective hepatic lipid catabolism, resulting in steatosis in the absence of obesity or insulin resistance (10).

Mice were challenged with the CDAA or control choline-supplemented, L-amino acid–defined (CSAA) diet for 3 or 6 wk, and the preventive effect of rFGF1 (0.5 mg/kg administered i.p. every 3 d) on the development of NAFLD/NASH was monitored. Body weights and white adipose mass of control and rFGF1-treated mice increased at similar rates and were not significantly different as compared with the dietary control group (Fig. 4 *A* and *B*). Liver mass, as a percentage of body weight, increased from ∼4% to ∼6% in the first 3 wk but remained stable at 6 wk (Fig. 4*C*). As expected, hepatic triglyceride levels in the CDAA-challenged mice increased progressively over time as compared with the CSAA control mice, but no effect of rFGF1 was observed on either liver mass or hepatic triglycerides (Fig. 4 *C* and *D*). These findings were supported by histological examination using H&E and Oil red O staining (Fig. 4 *E* and *F* and Figs. S3 and S4). GS staining further indicated a clear periportal localization of the steatosis in the CDAA model similar to previous reports (Fig. 4*G*) (12). Together, these results suggest that the antisteatotic effects of rFGF1 are dependent on the catabolic processes that are defective in the CDAA model.

rFGF1 Suppresses Hepatic Inflammation Independent of Its Antisteatotic Effects. Because rFGF1 did not affect steatosis in the CDAA model, this model allowed us to investigate whether the anti-inflammatory

properties of rFGF1 are dependent on its antisteatotic properties. After a 3-wk CDAA challenge, significant reductions in the mRNA expression of MCP-1, TNFα, ICAM-1, VCAM-1, and CD11c were observed in rFGF1-treated mice as compared with CDAA control mice (Fig. 5*A*). In addition, a trend toward decreased expression was observed for IL-1β and E-selectin in rFGF1-treated mice. Reduced hepatic inflammation was further confirmed by histopathological and protein analysis, indicating a reduction in the number of inflammatory foci in the liver (Fig. 5*B* and Table S2) and reduced levels of MCP-1 protein (Fig. 5*C*). These data show that rFGF1 exerts its anti-inflammatory effect in the liver independently from its antisteatotic effect. After a 6-wk CDAA challenge, however, mRNA expression of inflammatory markers were no longer reduced (and in the case of E-selectin were even increased) by rFGF1 treatment (Fig. S5). In addition, no effect of rFGF1 on anti-inflammatory M2 marker expression was observed (Fig. S1 *C and D*). Histopathological analysis further indicated that lobular inflammation was increased in the rFGF1-treated mice as compared with the CDAA control mice (Table S3).

Interestingly, rFGF1 also prevented the increase in plasma ALT activity, a marker for liver damage, after a 3 wk CDAA challenge, and a similar trend was seen after 6 wk (Fig. 5*D* and Fig. S5). rFGF1 may thus have hepato-protective properties beyond its antisteatotic and anti-inflammatory properties. In line with this finding, it has been reported previously that FGF1/FGF2 double-knockout mice exhibit increased levels of ALT after tetrachloride-induced hepatic injury (13). Together, our results show that in the CDAA model rFGF1 can prevent liver damage, as reflected by reduced plasma levels of ALT, and that it can delay but not prevent hepatic inflammation.

rFGF1 Does Not Induce Hepatic Fibrosis or Proliferation. Potential adverse effects of FGFs are fibrosis and proliferation. Previous studies have suggested that FGF1 has a role in promoting hepatic fibrosis. Increased expression of FGF1/FGFRc was observed in a rat model of experimental pulmonary fibrosis and in patients with idiopathic pulmonary fibrosis, respectively (14, 15). Conversely, loss of FGF1 and FGF2 in mice resulted in decreased liver fibrosis upon exposure to carbon tetrachloride (13). To assess the effect of rFGF1 on the development of hepatic fibrosis, liver samples from *ob/ob* mice treated with rFGF1 for 5 wk were
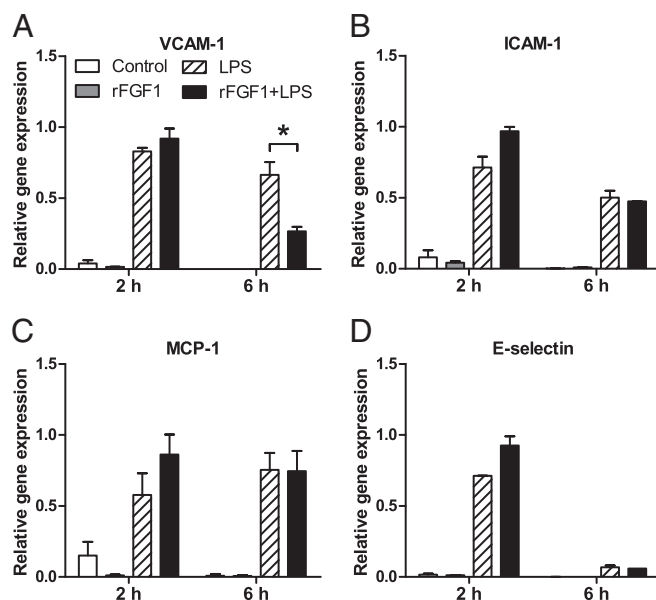
Fig. 3. Effect of rFGF1 on activation of HUVEC endothelial cells. The expression of VCAM-1 (*A*), ICAM-1 (*B*), MCP-1 (*C*), and E-selectin (*D*) in HUVECs after activation by LPS (*n* = 3; *P < 0.05; Mann–Whitney test).
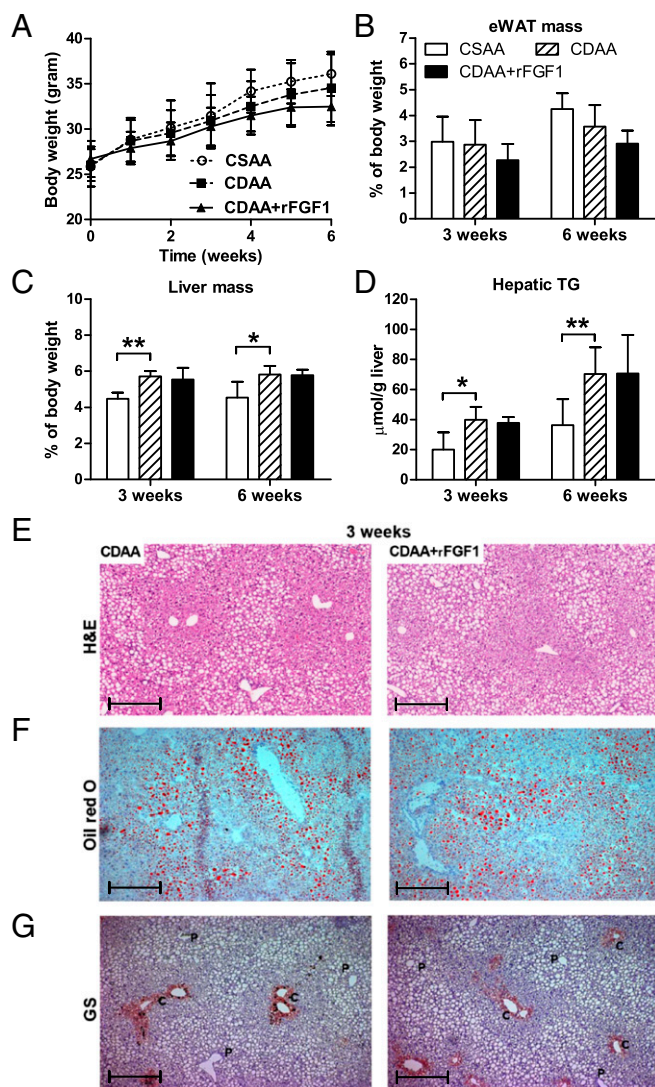
Fig. 4. rFGF1 does not reduce steatosis in mice with a dietary choline deficiency. (A–D) rFGF1 does not affect body weight (A), epididymal white adipose tissue (eWAT) mass (B), liver mass (C), or hepatic triglyceride (TG) levels (D) in mice challenged for 3 or 6 wk with a CDAA or on a control CSAA diet (n = 8 or 10 mice per group; one-way ANOVA). (E–G) Histological visualization of steatosis in serial liver sections after a 3-wk CDAA challenge, without (Left) or with (Right) rFGF1, stained with H&E (E), Oil red O (F), or the central vein marker GS combined with H&E (G). C, central vein; P, portal vein. (Scale bars: 300 μm.) *P < 0.05, **P < 0.01.

analyzed for the expression of fibrogenic maker genes. The expression of TGF-β1, which has been shown to accelerate liver fibrogenesis by promoting hepatic stellate cell transformation and activation of the expression of extracellular matrix genes (16), was significantly reduced in rFGF1-treated *ob/ob* mice as compared with control mice (Fig. 6A). The expression of collagen-α1, αSMA, and TIMP-1, however, was not significantly different. Also, no significant differences in the expression of fibrogenic genes or collagen deposition were observed between control and rFGF1-treated mice after a 3- or 6-wk CDAA challenge, respectively (Fig. 6 B–D and Fig. S6). Finally, we observed a significant reduction in the expression of the proliferation marker Ki-67 by rFGF1 after a 3-wk CDAA challenge, but no difference was observed after 6 wk (Fig. 6 E and F). These findings were supported by histopathological analyses (Table S4). Together, these results suggest that rFGF1 has no adverse effects on hepatic fibrosis or proliferation.

Discussion

Here we show that pharmacological administration of rFGF1 effectively improves obesity-related steatosis, hepatic inflammation, and hepatic damage. Our findings further suggest that these effects are at least partially independent, because the anti-inflammatory effects were observed in both the presence and absence of anti-steatotic effects.

Although no pharmacological treatment has currently been approved for NAFLD/NASH, insulin sensitizers and antioxidative treatment strategies with vitamin E are among the best-established approaches (1). However, both these approaches have long-term safety issues, and there is only limited evidence of improvement in cirrhotic patients (1, 17). Vitamin E treatment is associated with increased mortality, and TZDs have been associated with various adverse effects including weight gain, fluid retention, and osteoporosis, complicating their clinical use (4, 18). In addition, TZDs are contraindicated in patients with symptomatic chronic heart failure (19). Current strategies for novel PPARγ-based treatments therefore are directed at developing selective receptor modulators with reduced adverse effects or at activation of selective downstream targets (20).

Recently, we have identified FGF1 as a target of nuclear receptor PPARγ in visceral adipose tissue and as a critical factor in adipose function, insulin resistance, and the development of type 2 diabetes (8, 9). When challenged with a high-fat diet, mice lacking FGF1 display aberrant adipose expansion characterized by reduced angiogenesis and increased adipose inflammation and fibrosis, resulting in ectopic fat accumulation in the liver and in insulin resistance (8). Conversely, pharmacological administration of rFGF1 improved hyperglycemia, insulin sensitivity, and steatosis in mouse models of obesity (9).
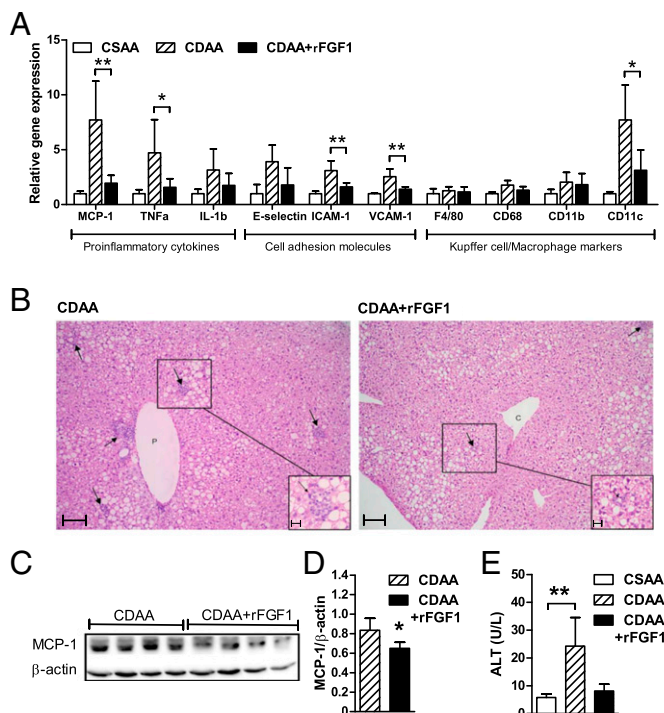
Fig. 5. rFGF1 suppresses hepatic inflammation in mice with a dietary choline deficiency. (A) Effect of rFGF1 on the expression of inflammatory genes in the livers of mice challenged for 3 wk with a CDAA diet or on a CSAA diet (n = 8 or 10; one-way ANOVA). (B) Histological visualization of inflammation in H&E-stained liver sections. Aggregations of lymphocytes, indicating lobular inflammation, are indicated by arrows. C, central vein; P, portal area. (C) Western blot analysis of MCP-1 protein levels. (D) Quantitation of C (unpaired t test with Welch's correction). (E) ALT activity (n = 3 or 5; one-way ANOVA). *P < 0.05, **P < 0.01.

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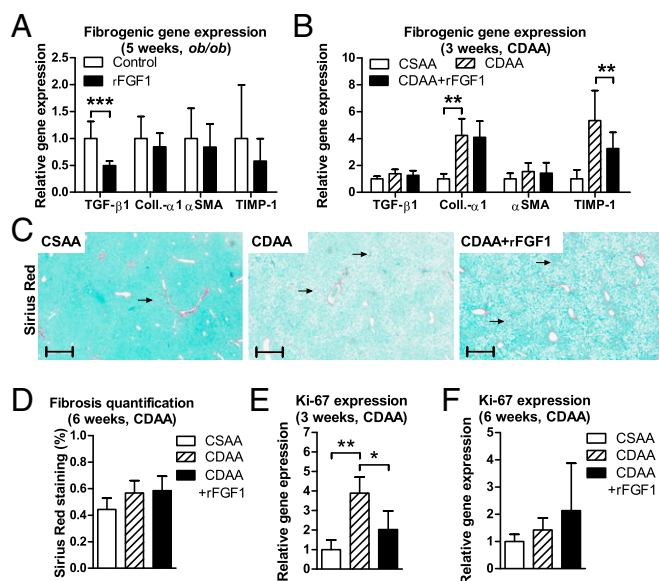
Fig. 6. FGF1 does not promote hepatic fibrosis and proliferation in *ob/ob* mice or in choline-deficient mice. (*A* and *B*) Fibrogenic gene expression in *ob/ob* mice treated for 5 wk with rFGF1 (*n* = 6 or 8; Mann–Whitney test) (*A*) and in mice challenged for 3 wk (*n* = 5 or 6) with a CDAA diet (*n* = 8–10) or on a CSAA (*n* = 5 or 6; one-way ANOVA) (*B*). (*C*) Histological visualization of fibrosis in liver sections stained with Sirius red from mice challenged for 6 wk with a CSAA diet or on a CDAA diet with or without rFGF1. Arrows indicate pericentral and hepatocyte collagen deposition. (Scale bars: 500 μm.) (*D*) Quantitation of fibrosis in liver sections from mice challenged for 6 wk with a CSAA diet or on a CDAA diet with or without rFGF1 (*n* = 5 slides per group; one-way ANOVA). (*E* and *F*) Expression of Ki-67 in mice fed the CDAA diet for 3 wk (*E*) or 6 wk (*F*) (*n* = 5 or 6). *P* < 0.05, **P* < 0.01, ***P* < 0.001.

Two other members of the FGF family, the endocrine hormones FGF15/19 and FGF21, also have been shown to improve hyperglycemia, insulin resistance, and steatosis (21). The effects of FGF15/19 are mediated directly through activation of FGF receptor 4 (FGFR4) and its coreceptor β-klotho in the liver (19, 20). FGF15/19 is produced in the ileum, where its expression is controlled by the bile acid-activated nuclear receptor FXR, and subsequently is secreted into the circulation (22, 23). In the liver, FGF15/19 suppresses bile acid synthesis and gluconeogenesis (22, 24, 25). Although we have not observed effects of FGF1 on bile acid homeostasis, it is possible that some of its metabolic effects are mediated directly through hepatic FGFR4 activation, because FGF1 acts as a universal ligand for all FGFRs. In contrast to FGF15/19, the glycemic effects of FGF1 and FGF21 are dependent on FGFR1 activation in adipose tissue (9, 26). FGF21 also can alleviate endoplasmic reticulum stress-induced hepatic steatosis by acting as a metabolic effector of the unfolded protein response (27). Whether these effects are directly mediated through FGFR activation in the liver and whether FGF1 and FGF15/19 act through the same pathway is not known.

In contrast to *ob/ob* mice and DIO models of steatosis, we did not observe an improvement in steatosis in the choline-deficient model. The difference in the etiology of steatosis in these models gives a clue to the mechanism of action of FGF1. The *ob/ob* mice and DIO mice have increased hepatic lipid accumulation caused by excessive food intake, but a choline deficiency causes defective hepatic β-oxidation and the production of VLDL, resulting in steatosis in the absence of obesity or insulin resistance (10, 28). These differences in the pathophysiology of steatosis were clearly reflected in the zonal distribution of lipids in these models. Steatosis in *ob/ob* mice was located primarily in the pericentral region but in the CDAA model was present mainly in the periportal region. Hepatic zonation plays an important role in the segregation of the different metabolic pathways in the liver (11, 29). Hepatic

fatty acid synthesis and triglyceride accumulation occur predominantly in the pericentral zone, whereas catabolic processes such as fatty acid oxidation and fatty acid secretion (VLDL production) are associated more with the periportal zone (11). Our observation that reduction of steatosis by rFGF1 is limited to the periportal zone thus suggests that rFGF1 acts by improving hepatic lipid catabolism (i.e., oxidation and/or secretion).

Hepatic lipid metabolism and inflammation are tightly linked processes, and both are known to exacerbate insulin resistance (30). The accumulation of toxic lipid species and their metabolites, such as saturated free fatty acids, free cholesterol, and the sphingolipid ceramide, has been shown to exert an inflammatory response by activating Bax protein translocation, which in turn triggers lysosomal and mitochondrial permeabilization, the production of reactive oxygen species, and apoptosis (31). This process, called "lipotoxicity," promotes the activation of Kupffer cells (specialized macrophages in the liver) and exacerbates insulin resistance and the progression of NASH (32). Our results show that rFGF1 effectively suppresses hepatic inflammation both in *ob/ob* mice and choline-deficient mice, as indicated by significant reductions in the expression of the proinflammatory M1 markers MCP-1 and TNFα. Interestingly, the anti-inflammatory effect of rFGF1 became more pronounced with prolonged (5-wk) treatment in *ob/ob* mice, as evidenced by the further suppression of M1 markers and also of cell adhesion markers (E-selectin, VCAM-1, ICAM-1) and macrophage/Kupffer cell markers (F4/80, CD68, CD11b, and CD11c). rFGF1 also suppressed hepatic inflammation after a 3-wk challenge with a choline-deficient diet, but this effect was no longer observed at 6 wk. It is possible that the anti-inflammatory effect of rFGF1 is achieved only in the presence of relatively low levels of hepatic lipids (e.g., 3-wk CDAA) and that when levels of hepatic lipids become progressively higher (e.g., 6-wk CDAA), the anti-inflammatory effect of rFGF1 is mitigated because of lipotoxicity.

Our in vitro data suggest that rFGF1 does not suppress inflammation through a direct effect on hepatocytes or through macrophage activation. However, we did find a strong suppression of VCAM-1 expression in HUVEC endothelial cells. Sinusoidal endothelial cells play a major role in hepatic inflammation through their involvement in adhesion molecule-mediated recruitment of leukocytes (33). It was shown previously that FGF1 suppresses transendothelial leukocyte migration by reducing the expression of several endothelial adhesion molecules, including VCAM-1 (34). Endothelial cells in normal liver express little or no VCAM-1, but VCAM-1 is highly induced during conditions of steatohepatitis (35). We speculate, based on these findings, that rFGF1 in vivo decreases leukocyte recruitment by reducing endothelial VCAM-1 and thereby suppresses hepatic inflammation.

Together, our findings show that FGF1 has therapeutic potential in the treatment of NAFLD and NASH. Because FGF1 is situated downstream of PPARγ, it is likely that therapeutic targeting of FGF1 will eliminate some of the adverse effects associated with TZDs that are mediated through direct activation of PPARγ.

Experimental Procedures

Animals. Mice were housed and handled according to institutional guidelines complying with Dutch legislation. All experiments were approved by the Ethical Committee for Animal Experiments, University of Groningen, Groningen, The Netherlands. Animals used in this study were male wild-type and *ob/ob* mice on a C57BL/6J genetic background (Charles River), between 8 and 12 wk of age. Animals were housed in a light- and temperature-controlled facility (lights on from 7:00 AM to 7:00 PM, 21 °C). All mice received a standard laboratory chow (RMH-B; Hope Farms) and acidified water ad libitum.

Animal Experiments. Choline deficiency was induced by a 3- or 6-wk challenge with a CDAA (518753; Dyets Inc.) or a CSAA control diet (518754; Dyets Inc.). Mice were treated with vehicle (PBS) or rFGF1 (0.5 mg/kg) (ProSpec) by i.p. injection starting 3 d before the dietary intervention and then every 72 h for 3 or 6 wk. Mice were killed by cardiac puncture after anesthesia with isoflurane. Terminal blood samples were collected in EDTA-coated tubes. Tissues were

collected and frozen in liquid nitrogen or were processed for histology. Hepatic lipids were extracted according to Bligh and Dyer (36). Triglycerides were determined using the Trig/GB kit (11877771; Roche) and absorption at 540 nm. Plasma was obtained by centrifugation at 2,000 × g for 10 min and was used for the determination of ALT activity using the spinreact GOT-GPT kit (1002500; Girona).

Histological Analysis and Immunohistochemistry. For microscopic examination, tissues were fixed in 4% (wt/vol) formaldehyde in PBS, embedded in paraffin, sectioned at 4 μm, and stained with H&E. Liver fibrosis was assessed by Sirius red staining. Liver steatosis was visualized by Oil red O staining of liver cryosections. To determine zonation, GS staining was used for central vein localization (37). Briefly, sections were deparaffinized in xylene and rehydrated in a series of graded alcohol-washing steps. Antigen retrieval was conducted by gently boiling of sections in 1 mM EDTA solution, pH 8.0, for 15 min. Endogenous HRP activity was blocked in 0.3% H_2O_2; 10% (vol/vol) normal goat serum in 1% BSA PBS solution was used to block nonspecific binding before antibody incubation. Anti-GS (mouse IgG$_{2a}$) (610518; BD Biosciences) primary antibody was incubated overnight at 4 °C, washed with PBS, followed by incubation with HRP-conjugated goat anti-mouse IgG (R40101; Invitrogen) for 1 h at room temperature. AEC (3-amino-9-ethyl-carbazole) reagent (Sigma) containing 0.3% H_2O_2 was used for visualization.

Hepatic steatosis and steatohepatitis were assessed in an unbiased manner by two board-certified pathologists (including A.d.B.). Hepatic steatosis and inflammation were graded in H&E-stained liver sections by using an adapted version of the NAFLD activity scoring system developed by Kleiner et al. (38). For quantitation of steatosis, H&E- and GS-stained sections (six slides for each group) were randomly selected. Next, three or four pericentral or periportal areas on each section were selected and quantified for steatosis using ImageJ.

Gene-Expression Analysis. Total RNA was isolated from the liver using Tri re-agent (Life Technologies) and was reverse transcribed into cDNA using Moloney murine leukemia virus, random primers, and dNTP according to standard procedures. For quantitative PCR (qPCR), cDNA was amplified using Hi-ROX SensiMix SYBR Green (Bioline) and the StepOnePlus Real-Time PCR System (Applied Biosystems). Primers used for qPCR are listed in Table S5. U36B4 was used as the house-keeping gene in all PCR analyses, and the ΔΔCt method was used for quantification.

Statistical Analysis. All values are given as means ± SD. The two-tailed unpaired Student's t test with Welch's correction, nonparametric Mann–Whitney test, or one-way or two-way ANOVA analysis with Holm–Sidak's multiple comparison test were used for statistical analysis. Significance was indicated as *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

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