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Hepatocyte-specific fibroblast growth factor 21 overexpression ameliorates high-fat diet-induced obesity and liver steatosis in mice

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Fibroblast growth factor (FGF) 21 is an endocrine growth factor mainly secreted by the liver in response to a ketogenic diet and alcohol consumption. FGF21 signaling requires co-receptor β-klotho (KLB) co-acting with FGF receptors, which has pleiotropic metabolic effects, including induced hepatic fatty acid oxidation and ketogenesis, in human and animal models of obesity. We examined the hepatocyte-specific enhancer/promoter of FGF21 expression plasmids in high-fat diet-fed mice for 12 weeks. Hydrodynamic injection for FGF21 delivery every 6 weeks sustained high circulating levels of FGF21, resulting in marked reductions in body weight, epididymal fat mass, insulin resistance, and liver steatosis. FGF21-induced lipolysis in the adipose tissue enabled the liver to be flooded with fat-derived FFAs. The hepatic expression of Glut2 and Bdh1 was upregulated, whereas that of gluconeogenesis-related genes, G6p and Pepck, and lipogenesis-related genes, Srebp-1 and Srebp-2, was significantly suppressed. FGF21 induced the phosphorylation of AMPK at Thr172 and Raptor at ser792 and suppressed that of mTOR at ser2448, which downregulated mTORC1 signaling and reduced IRS-1 phosphorylation at ser1101. Finally, in the skeletal muscle, FGF21 increased Glut4 and Mct2, a membrane protein that acts as a carrier for ketone bodies. Enzymes for ketone body catabolism (Scot) and citrate cycle (Cs, Idh3a), and a marker of regenerating muscle (myogenin) were also upregulated via increased KLB expression. Thus, FGF21-induced lipolysis was continuously induced by a high-fat diet and fat-derived FFAs might cause liver damage. Hepatic fatty acid oxidation and ketone body synthesis may act as hepatic FFAs' disposal mechanisms and contribute to improved liver steatosis. Liver-derived ketone bodies might be used for energy in the skeletal muscle. The potential FGF21-related crosstalk between the liver and extraliver organs is a promising strategy to prevent and treat metabolic syndrome-related nonalcoholic steatohepatitis.

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INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is one of the most common liver diseases worldwide; it is significantly associated with metabolic syndromes, including obesity, dyslipidemia, and insulin resistance[1,2]. Emerging evidence indicates that obesity-related metabolic inflammation is a key process in the development of NAFLD[3,4]. Several metabolic and inflammatory pathways and mediators between the liver and various extraliver organs have been reported to associate with NAFLD, leading to type 2 diabetes mellitus, cardiovascular disease, and chronic kidney disease[5–7]. However, currently available pharmacotherapeutic options for the treatment of patients with NAFLD remain limited in the heterogeneous population; hence, novel treatments are needed.

FGF21, which has an increased expression in obesity and NAFLD, has been identified as a promising therapeutic target in obesity-related metabolic disorders[8,9]. FGF21 is a member of the hormone-like subgroup within the FGF superfamily that also contains FGF19 and FGF23, which are critical for maintaining whole-body homeostasis[10,11]. FGF21 expression is increased by peroxisome proliferator-activated receptor α and activating transcription factor 4 via the exposure to excess endoplasmic reticulum stress, oxidative stress, and amino acid deprivation[12,13]. The C-terminus of FGF21 binds with high affinity to KLB, which is present as a complex with FGF receptors (FGFRs) on the surface of cells[14–16]. FGF21 is known to exert antidiabetic, lipid-lowering, and weight-reducing effects when administered at pharmacological concentrations in rodents[17]. Its functions are deemed vital in the lipolysis of peripheral/abdominal adipose tissue and stimulation of adiponectin production, which, in turn, enhances insulin sensitivity in the liver[18,19].

The mechanism through which FGF21 mediates metabolic activity in the liver remains largely unknown. KLB is required for FGF21 to activate two specific FGFR subtypes—FGFR1c and FGFR3c. FGFRs are widely expressed in adult tissues, although their relative levels differ among the various organ systems[14–16]. Under normal conditions, hepatocytes express high levels of KLB and FGFR3c; however, the expression level of FGFR1c is quite low[20]. Obesity increases the expressions of FGF21, KLB, FGFR1c, and FGFR3c in the liver[21,22]. Recently, FGF21 has been reported to

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repress the mammalian target of rapamycin complex 1 (mTORC1) and improve insulin sensitivity and glycogen storage in a hepatocyte-autonomous manner, indicating an autocrine function of FGF21 in the liver[23].

Given the numerous metabolic functions of FGF21, FGF21-based pharmacotherapies are currently being explored as potential antiobesity/NAFLD drugs and are under evaluation in clinical trials[24–26]. In addition, recent studies on long-acting FGF21 analogs in obese patients with type 2 diabetes have demonstrated improvements in serum lipid profiles and weight loss. A glyco-engineered long-acting FGF21 variant with optimal pharmaceutical and pharmacokinetic properties enabled weekly to twice-monthly subcutaneous dosing[27–29].

Because FGF21 stimulates lipolysis in white adipose tissue (WAT) and the oxidation of fatty acids and production of ketone bodies in the liver, the pharmacological effects of sustained FGF21 treatment in humans with NAFLD as well as in rodent NAFLD models include direct and indirect consequences induced by FGF21[30–34]. In this study, we aimed to determine the beneficial effects of sustained FGF21 levels by the hepatocyte-specific overexpression of FGF21 on the progression or reversal of NAFLD in diet-induced obese mice. High-fat diet (HFD)-fed C57BL/6 mice were intravenously injected with *pLIVE* plasmid expressing mouse FGF*21* under the control of an albumin promoter and α-fetoprotein enhancer[35]. Hepatocyte-derived FGF21 has a higher physiological activity than recombinant FGF21 from *Escherichia coli* because the endocrine FGF signaling through FGFRs requires klothos, which are cell-surface proteins that possess tandem glycosidase domains[27]. In this present study, FGF21 therapy was demonstrated to protect against diet-induced obesity and NAFLD and systemically improve insulin resistance via lipolysis in WAT.

Insulin activities in the adipose tissue regulate the delivery of fatty acids from blood to the liver, following the lipolysis of triglyceride in the adipose tissue. Mechanistically, FGF21 reduced intrahepatic lipid accumulation not by enhancing fatty acid oxidation but by inhibiting lipogenesis via the suppression of sterol regulatory element-binding proteins (Srebps). Moreover, FGF21 increased 3-hydroxybutyrate dehydrogenase (Bdh) 1 expression, which catalyzes the interconversion of acetoacetate and 3-hydroxybutyric acid (3-OHBA) during fatty acid catabolism[36,37]. Despite being the source of ketone bodies, the liver cannot use them for energy. Liver-derived ketone bodies could be used in the skeletal muscle in this NAFLD mouse model. Furthermore, FGF21 overexpression activated adenosine monophosphate-activated protein kinase (AMPK) signaling, suppressed mTOR signaling, and improved insulin sensitivity by decreasing the gluconeogenesis genes, namely, glucose-6-phosphate (G6p) and phosphoenolpyruvate carboxylase (Pepck), in the liver. Our data suggest that the hepatocyte-specific expression of FGF21 prevents and reverses NAFLD. This FGF21-related potential crosstalk between the liver and extraliver organs is a promising strategy for NAFLD therapy.

MATERIALS/SUBJECTS AND METHODS

Animals and treatment

A mouse model of diet-induced obesity and NAFLD was used in this study. Eight-week-old male C57/BL6J mice were purchased from Japan Charles River. Eleven mice were fed a control chow diet and ten mice were fed an HFD (D12492, Research Diet Inc., Tokyo, Japan) for 12 weeks. Half of the chow- or HFD-fed mice were hydrodynamically injected with *pLIVE-mouse fgf21* plasmids every 6 weeks, whereas the other half of each group were injected with *pLIVE-control* plasmids. At the end of the treatment, livers, epididymal fats, skeletal muscles, and blood were isolated from each animal. The study protocol was conducted in accordance with the guidelines for the care and use of laboratory animals set by the Kyoto Prefectural University of Medicine (Kyoto, Japan) and was approved by the Committee on the Ethics of Animal Experiments of the same institution. Animals were housed under conventional conditions with controlled temperature, humidity, and light (12-h light–dark cycle), were provided with food and water ad libitum, and were housed in transparent polymer X (TPX) cages (CL-0104-2, CLEA Japan Inc., Tokyo, Japan) with a maximum of eight mice per cage.

Hydrodynamic tail vein injections of fgf21 plasmid

The wild-type mouse *fgf21* cDNA constructs obtained by PCR amplification were sub-cloned into the *pLIVE* vector (Mirus Bio, Madison, WI). Plasmid DNA constructs were delivered by hydrodynamic tail vein injection[38]. The tail vein injections were performed with 40 μg of each plasmid in a solution of TransIT-EE® according to the manufacturer's instructions (Mirus Bio LLC, Madison, WI, USA).

Analysis of liver architecture

As previously described, liver sections were stained with hematoxylin and eosin or Oil Red O utilizing standard techniques[39].

Two-step real-time polymerase chain reaction

PCR was performed as described below[39]. Briefly, the total RNA was extracted from whole livers using RNeasy kits (Qiagen, Valencia, CA, USA), which were reverse-transcribed using a random primer and Superscript RNase H-reverse transcriptase (Invitrogen, Carlsbad, CA, USA). Specificity was confirmed for all primer pairs (Supplemental material, Table 1) by sequencing the PCR products. Target gene levels were then presented as a ratio of levels in the treated versus corresponding control groups. Fold changes were determined by using point and interval estimates.

Immunoblot assay

Proteins isolated from whole fats, livers, and muscles were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes. Membranes were then probed with anti-AMPK, anti-phospho-AMPK at Thr172, anti-insulin receptor substrate (IRS)-1, anti-phospho-IRS-1 at Ser1101, anti-regulatory associated protein of mammalian target of rapamycin (Raptor), anti-phospho-Raptor at Ser792, anti-mTOR, anti-phospho-mTOR at ser2448, anti-p70S6 kinase, anti-phospho-p70S6K at Thr389, anti-S6 ribosomal protein (S6RP), anti-phospho-S6RP at ser235/236, anti-adipose triglyceride lipase (ATGL), anti-hormone-sensitive lipase (HSL), anti-phospho-HSL at ser660 (Cell Signaling Technology Inc., Beverly, MA, USA), anti-myogenin (F5D, Santa Cruz, Dallas, TX, USA. sc-12732), anti-Srebp-1 (C-20, Santa Cruz, Dallas, TX, USA. sc-366) or anti-actin (Sigma-Aldrich, St. Louis, MO, USA), followed by incubation with horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG (Invitrogen, Carlsbad, CA, USA). Antigens were then visualized by electrochemiluminescence (GE Healthcare, Chicago, IL, USA). Immunoblots were scanned, and band intensities were quantified by Image J (NIH) densitometry analysis.

Tissue and plasma biochemical measurements

Serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), total cholesterol, triglyceride and FFA levels were measured at SRL, Inc. (Tokyo, Japan). Serum FGF21, adiponectin, leptin, insulin and 3-OHBA were measured using a Mouse FGF21 ELISA Kit (Arigo Biolaboratories Corp., Hsinchu, Taiwan), Mouse Adiponectin/Acrp30 Quantikine ELISA kit (R&D Systems, Inc., Minneapolis, MN, USA), Mouse Leptin AssayMax ELISA Kit (Assaypro, St Charles, MO, USA), Morinaga Mouse/Rat Insulin ELISA Kit (Morinaga, Yokohama, Japan) and β-Hydroxybutyrate, Ketone Body, Assay Kit (Cayman Chemical, Ann Arbor, MI, USA). Meanwhile, tissue triglyceride levels were determined by the commercial assay kits in accordance with the manufacturer's instructions (Triglycerides-

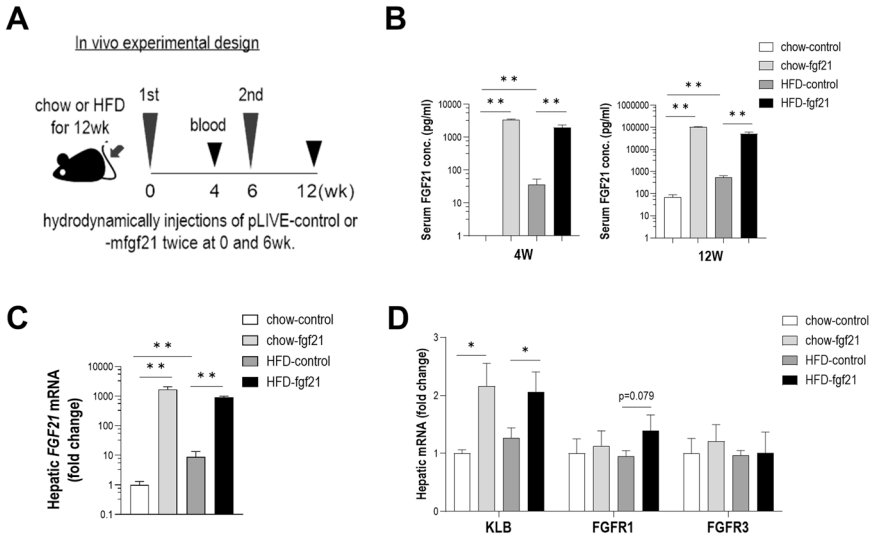

Fig. 1 Hydrodynamically injection of *pLIVE-mouse(m)fgf21* plasmids. A In vivo experimental design. **B** Serum FGF21 levels were determined at 4 weeks and end of the treatment ($n = 5$–6/group). **C** Hepatic mRNA levels of FGF21 were determined by quantitative real-time PCR analysis. **D** Hepatic mRNA levels of KLB, FGFR1 and FGFR3 expression were determined. Results were normalized to glucuronidase expression. Means data are displayed as fold changes relative to chow-fed and *pLIVE-control* plasmid-injected mice (*$p < 0.05$, **$p < 0.01$).

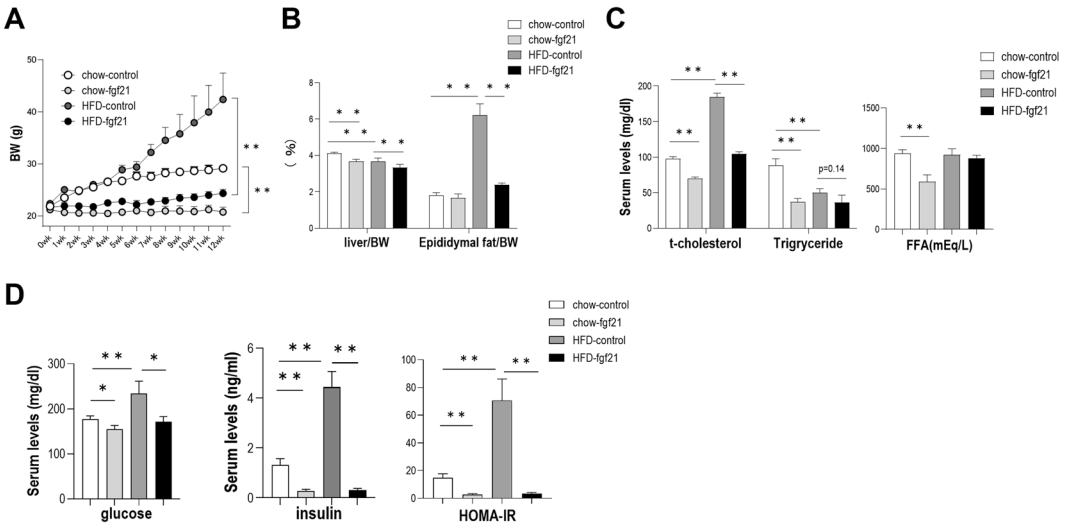

Fig. 2 The effect of FGF21 overexpression. A Body weight changes during 12-week treatment are plotted. **B** Liver/body weight (BW) and epididymal fat/BW ratio (%) were assessed at 12 weeks. **C** Serum total cholesterol, triglyceride, and FFA were determined. **D** Serum glucose, insulin and HOMA-IR were determined. Means data from each group ($n = 5$–6/group) are displayed (*$p < 0.05$, **$p < 0.01$).

E-test; Wako Pure Chemical Industries, Tokyo, Japan). Homeostasis model assessment-insulin resistance (HOMA-IR) was calculated as (fasting glucose mM × fasting insulin microunits/ml)/22.5.

Statistical analysis

Results are presented as the mean ± SEM. Significance was established using the Student's *t* test and analysis of variance, when appropriate. Differences were considered significant if $p < 0.05$.

RESULTS

Sustained high circulating levels of FGF21 with the hydrodynamic injection system for FGF21 delivery every 6 weeks

In total, 21 B6 mice that were fed a normal chow ($n = 11$) or HFD ($n = 10$) for 12 weeks and were transfected with *pLIVE-control* or *pLIVE-mfgf21* every 6 weeks were examined (Fig. 1A). To confirm that the circulating levels of serum FGF21 were sustained throughout the treatment duration, blood tests measuring the FGF21 level were performed at 4 weeks of treatment (Fig. 1B). Serum FGF21 levels were increased in the HFD-fed mice and were much higher in all mice that were transfected with *pLIVE-mfgf21*, regardless of the diet (Fig. 1B). At the end of the treatments, serum FGF21 levels and hepatic FGF21 mRNA were assessed. Serum FGF21 levels and hepatic FGF21 expressions were significantly elevated in mice that were transfected with *pLIVE-mfgf21* compared with the control mice (Fig. 1B, C). Although the FGF21 treatment had no effect on the expression of hepatic FGFR1 and FGFR3, the hepatic mRNA levels of KLB expression were noted to increase in mice fed with chow and HFD (Fig. 1D).

Improved obesity, dyslipidemia, and insulin resistance under FGF21 treatment

We assessed the body weight (BW) during treatment as well as the liver and epididymal fat weight/BW ratio at the end of the 12-

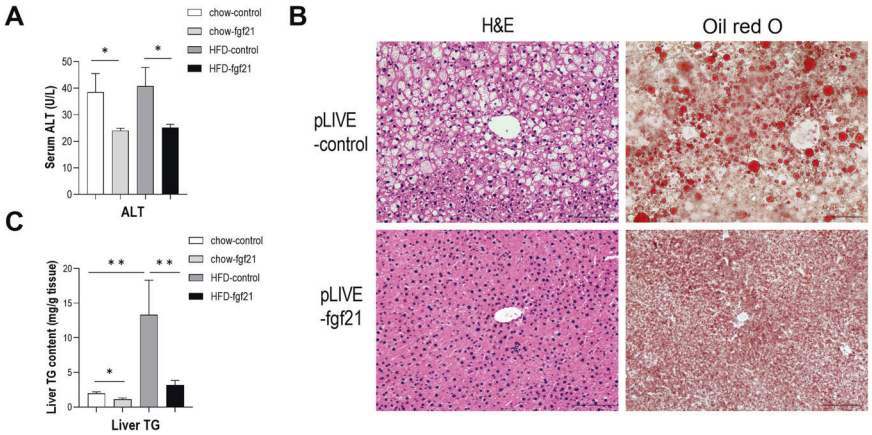

Fig. 3 The effect of FGF21 overexpression on liver steatosis. A Serum ALT values were determined. **B** Hematoxylin and eosin (H&E), and Oil red O staining of liver sections from representative HFD-fed mice injected by *pLIVE-control* or *-mfgf21*. **C** Hepatic triglyceride (TG) contents were measured. Means data from each group ($n = 5$–6/group) are displayed (*$p < 0.05$, **$p < 0.01$).

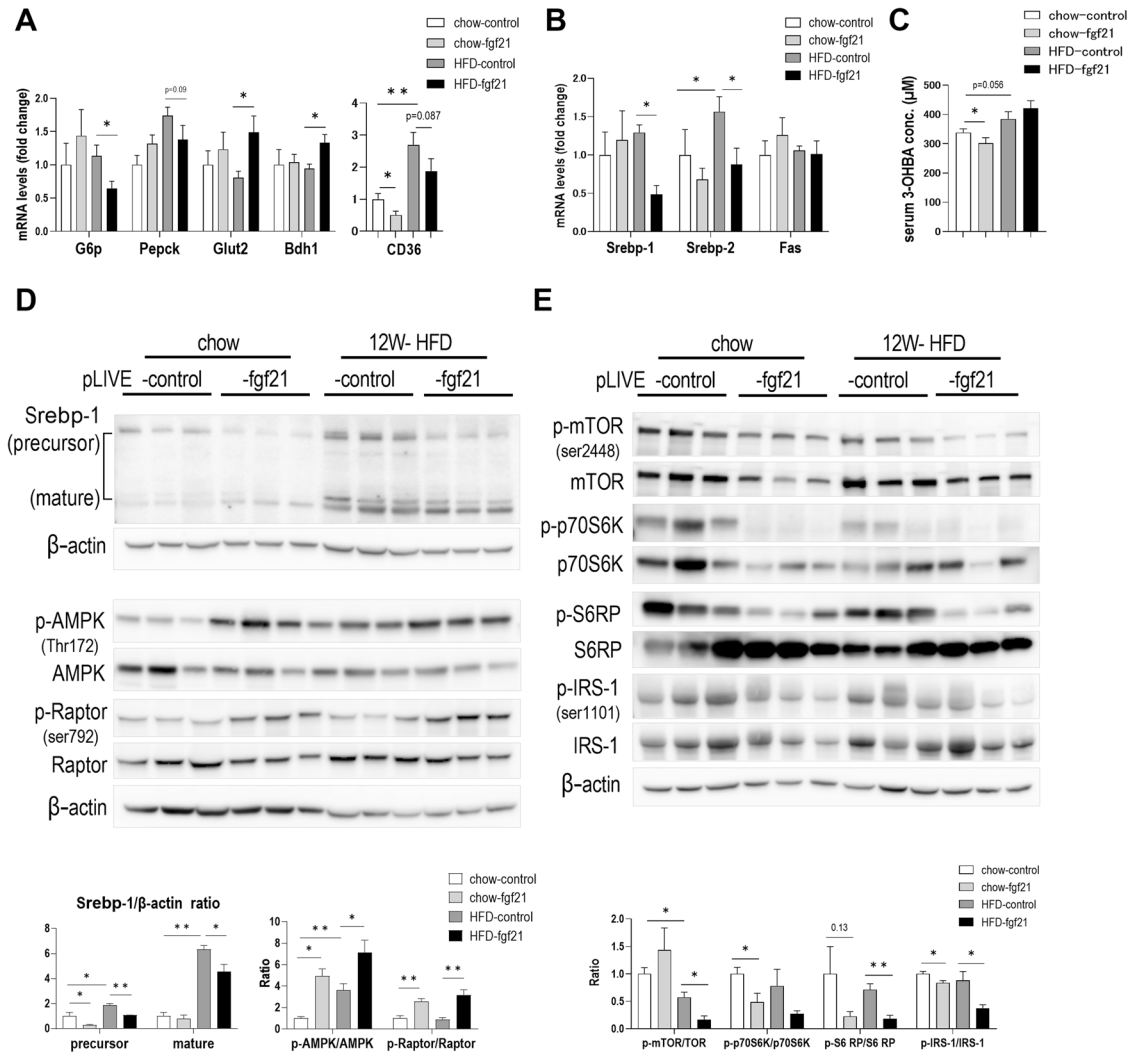

Fig. 4 The actions of FGF21 overexpression in liver. A Hepatic mRNA levels of G6p, Pepck, Glut2, Bdh1, CD36 **B** Srebp-1, -2 and fatty acid synthase (Fas) were determined by quantitative real-time PCR analysis ($n = 5$–6/group). **C** Serum 3-OHBA levels were determined in each group ($n = 5$-6/group). Means data from each group ($n = 5$-6/group) are displayed (*$p < 0.05$, **$p < 0.01$). **D** Srebp-1 (precursor and mature), p-AMPK, AMPK, p-Raptor and Raptor levels were evaluated by immunoblot analysis. To control for loading, the blot was stripped and re-probed for β-actin, a housekeeping gene. **E** p-mTOR, mTOR, p-p70S6K, p70S6K, p-S6RP, S6RP, p-IRS, and IRS-1 levels were evaluated by immunoblot analysis of livers from three mice/group. Immunoblots were scanned, and band intensities were quantified by Image J (NIH) densitometry analysis. Each ratio was also expressed as fold change of controls. (*$p < 0.05$, **$p < 0.01$).

week treatment period. Although HFD-fed mice gained BW and became obese, FGF21 treatment reduced BW gains even in chow-fed mice (Fig. 2A). The liver/BW ratio was decreased in both FGF21 treatment groups, and the increased epididymal fat/BW ratio due to the HFD was greatly suppressed by FGF21 treatment despite the average intake of a similar amount of food (Fig. 2B, Supplementary Fig. S2a). Furthermore, feeding the mice with HFD for 12 weeks elevated serum total cholesterol levels but decreased triglyceride levels with no change in FFAs (Fig. 2C). FGF21 treatment decreased the total serum cholesterol levels in both chow- and HFD-fed mice and decreased triglyceride levels only in the chow group (Fig. 2C). Although FGF21 treatment reduced serum FFAs only in chow-fed mice, which was a similar effect as that observed regarding serum triglyceride levels, FGF21 had no effect on these levels in HFD-fed mice (Fig. 2C). HFD-fed mice exhibited high blood glucose, insulin levels, and homeostatic model assessment-insulin resistance index, which were then ameliorated by FGF21 treatment (Fig. 2D). Consequently, FGF21 improved HFD-induced obesity, hypertrophy of epididymal fats, hyperlipidemia, and systemic insulin resistance but had no effect on serum FFA levels.

Hepatic steatosis under FGF21 treatment

The serum ALT levels were decreased to approximately half in both chow- and HFD-fed mice injected with FGF21 (Fig. 3A). The amelioration of liver steatosis was confirmed by hematoxylin and eosin staining, Oil Red O staining, and the liver triglyceride content measurement (Fig. 3B, C). FGF21-treated mice showed a clear reversal of HFD-induced intrahepatic lipid accumulation and liver triglyceride contents. Thus, FGF21 induced a marked reduction in liver steatosis.

Autocrine activities in the liver under FGF21 treatment

To explore the molecular mechanisms underlying the FGF21 effect in the regulation of insulin and glucose homeostasis in the liver, we assessed hepatic gluconeogenesis-, lipogenesis-, fatty acid oxidation- and ketogenesis-related gene expressions and insulin signaling-related molecules such as AMPK and mTORC1. When hepatic gluconeogenesis-related genes (G6p and Pepck), hepatic lipogenesis-related genes (Srebp-1 and -2), and hepatic fatty acid transporter CD36 were underexpressed in HFD-fed mice, FGF21 treatment significantly increased the mRNA levels of hepatic Glut2 and Bdh1 (Fig. 4A, B). FGF21 treatment reduced the protein levels of both the Srebp-1 precursor (molecular weight, 125 kDa) and mature soluble fragment (molecular weight, 68 kDa) in the nucleus, which were induced by HFD (Fig. 4D, Supplementary Fig. S2e). Microsomal triglyceride transfer protein 1 and, unexpectedly, hepatic mitochondrial β-oxidation-related genes, carnitine palmitoyltransferase-1, -2, and long-chain acyl-CoA dehydrogenase were not upregulated (Supplementary Fig. S2b). We also evaluated the mRNA levels of hepatic bile acid synthesis-related genes (cytochrome P (Cyp)7a1, Cyp8b1, Cyp27a1, Cyp7b1, farnesoid X receptor, and small heterodimer partner) and transport genes (bile salt export pump, sodium taurocholate cotransporting polypeptide, ATP binding cassette subfamily B member (Abcb) 4, Abcg5, and Abcg8) (Supplementary Figs. S2c, d). Although bile acid transport-related genes (both import and export) were mostly enhanced in HFD-fed mice, FGF21 treatment had minimal effect on hepatic bile acid synthesis for example cyp7a1, which was consistent with the findings of previous studies[40,41]. However, the effects of short- or long-term FGF21 treatment on bile acid synthesis have not been fully elucidated yet. These results revealed that the effects of FGF21 on the liver suppress gluconeogenesis and lipid synthesis without obvious increases in fatty acid oxidation and partly induce the synthesis of ketone bodies for the disposal of excess acetyl-CoA derived from FFAs. Slightly increased serum levels of 3-OHBA due to HFD ($p = 0.056$) did not differ between the control and FGF21-treated mice (Fig. 4C). Furthermore, in HFD-fed mouse livers, FGF21 upregulated the phosphorylation of Raptor at ser792 via AMPK activation (Fig. 4D) and directly downregulated the tuberous sclerosis complex 1/2-mediated phosphorylation of mTOR at ser2448 (Fig. 4E). As a result, p70S6 kinase at thr389, S6 ribosomal protein at ser235/236, and IRS-1 at ser1101 were clearly suppressed by FGF21 treatment (Fig. 4E). Consistent with the findings of a previous study[23], these observations indicated that FGF21 restored insulin signaling by suppressing the mTOR signaling-mediated phosphorylation of IRS-1 at ser1101.

FGF21-induced lipolysis in the adipose tissue

Despite an increase in the serum adiponectin levels of chow-fed mice, which was inconsistent with previous study findings, FGF21 treatment had little effect on these levels of HFD-fed mice (Fig. 5A). On the other hand, serum leptin levels that would have

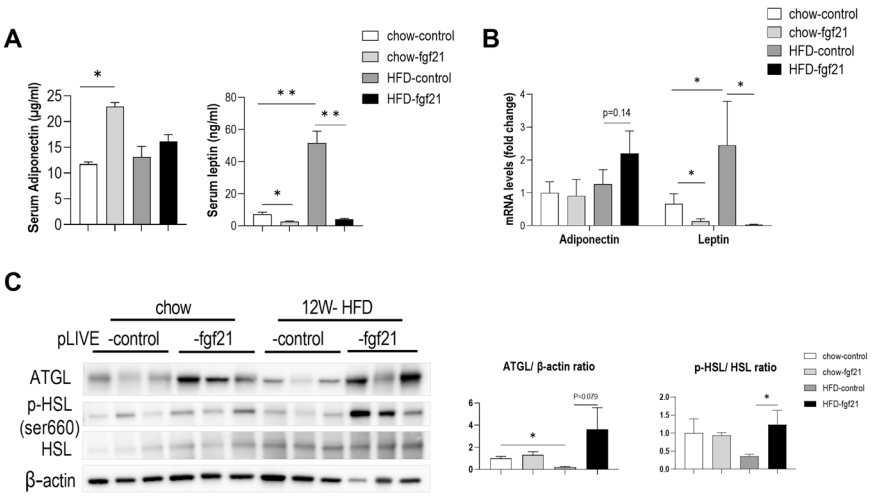

Fig. 5 The effect of FGF21 overexpression on fat tissue. A Serum adiponectin and leptin levels were determined in each group ($n = 5$-6/group). Data are presented as Means. **B** mRNA levels of adiponectin and leptin in fat tissues were determined by quantitative real-time PCR analysis. Results were normalized to GUS expression and then expressed as fold changes relative to gene expression in control plasmid-injected and chow-fed mice. Means data from each group ($n = 5$–6/group) (*$p < 0.05$, **$p < 0.01$). **C** ATGL, p-HSL at Ser660 and HSL levels were evaluated by immunoblot analysis of fat tissues from three mice/group. Immunoblots were scanned, and band intensities were quantified by Image J (NIH) densitometry analysis. Each ratio was also expressed as fold change of controls. (*$p < 0.05$).

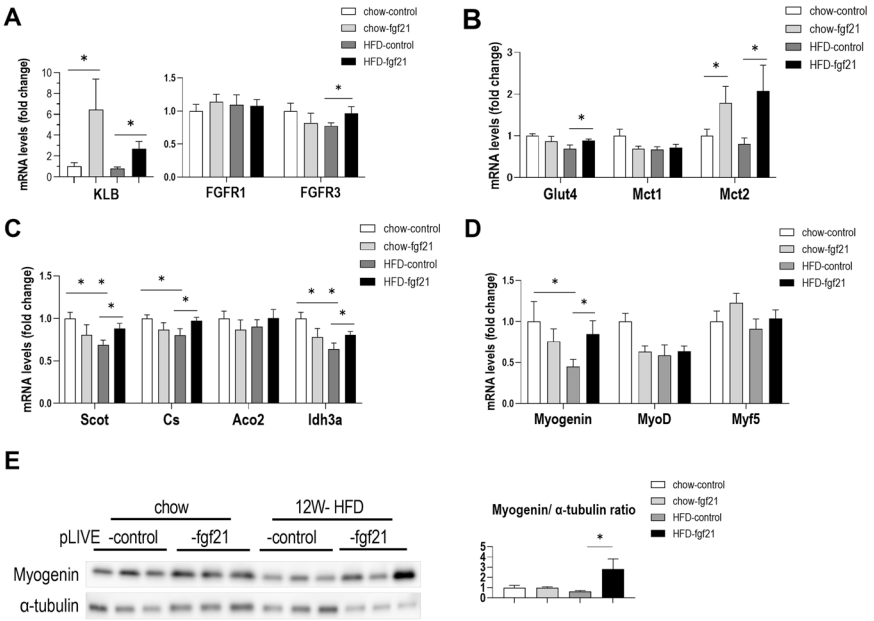

Fig. 6 The effect of FGF21 overexpression on skeletal muscle. A mRNA levels of KLB, FGFR1, FGFR3 (**B**) Glut4, Mct1, Mct2 (**C**) Scot, Cs, Aco2, Idh3α, **D** Myogenic, Myoid and Myf5 in skeletal muscles were determined by quantitative real-time PCR analysis ($n = 5$-6/group). **E** Myogenin levels were evaluated by immunoblot analysis of skeletal muscles from three mice/group. Immunoblots were scanned, and band intensities were quantified by Image J (NIH) densitometry analysis. Each ratio was also expressed as fold change of controls. (*$p < 0.05$).

been elevated by HFD were clearly decreased in mice transfected with *pLIVE-mfgf21*. FGF21 treatment suppressed the mRNA expression levels of leptin, but not adiponectin, in both groups of FGF21-treated mice (Fig. 5B). Although FGF21 improved liver steatosis with a marked loss of epididymal fats, serum FFA levels were found to be similar among the mice in HFD-fed groups (Figs. 2B, C, and 3). To confirm the mechanism underlying fat loss and blood FFAs, we examined lipolysis-related molecules and found that the phosphorylation of ATGL and HSL at ser660 was upregulated by FGF21 treatment (Fig. 5C). The lipolysis of the adipose tissue by FGF21 produces FFAs. Therefore, continuous lipolysis during FGF21 treatment may lead to hepatic steatosis, but the disposal system of FFAs can overcome this phenomenon.

FGF21-induced glucose and ketone body uptake for energy in the skeletal muscle

FGF21 treatment decreased gluconeogenesis and lipogenesis and had negligible effect on fatty acid oxidation and the export of very-low-density lipoproteins in the liver (Supplementary Fig. S2b). To explore the disposal system for excess FFAs derived from lipolysis, we assessed the role of skeletal muscle in glucose and ketone body metabolism. FGF21 treatment increased the KLB mRNA level in the skeletal muscle of both chow- and HFD-fed mice (Fig. 6A). Interestingly, FGFR3 was significantly upregulated by FGF21 in HFD-fed mice (Fig. 6A). In these mice, the mRNA levels of Glut4, which is a transporter for glucose uptake, and monocarboxylate transporter 2 (Mct2), which is essential for ketone body uptake, were significantly elevated after FGF21 treatment (Fig. 6B). Moreover, to confirm the optimal utilization of acetyl-CoA from glucose and ketone bodies, a key enzyme for ketone body catabolism, succinyl-CoA: 3-ketoacid CoA transferase (Scot), and the enzymes of the TCA cycle, citrate synthetase (Cs), aconitase 2, and isocitrate dehydrogenase (NAD+) 3 catalytic subunit alpha (Idh3α) were examined. The mRNA levels of these TCA cycle enzymes, which were significantly decreased by HFD, were increased in HFD-fed mice under FGF21 treatment. Finally, we analyzed the expression of muscle regeneration-related genes, such as myoblast determination protein 1, myogenic factor 5, and myogenin. The mRNA and protein levels of myogenin expression suppressed by HFD were restored by FGF21 treatment (Fig. 6D, E).

DISCUSSION

Our data suggest that the hepatocyte-specific expression of FGF21 mediated by a nonviral vector can achieve long-term sustained levels of FGF21 in blood and potential FGF21-related crosstalk between the liver and extraliver organs, which makes it an attractive target for obesity-related NAFLD therapy. Because liver processes were continuously induced by HFD- and fat-derived FFAs because of FGF21-induced lipolysis, hepatic fatty acid oxidation and ketone body synthesis could act as a hepatic FFAs disposal system and improve liver steatosis. Moreover, liver-derived ketone bodies could be utilized for energy in the skeletal muscle in this model.

The levels of hepatic FGF21 mRNA and serum FGF21 sustained for a long period (>3 months) were not harmful and did not worsen the levels of serum AST, ALT, and other biomarkers. Preliminarily, we confirmed that hepatic FGF21 was rapidly released into the culture medium from Hep3B and HepG2 cells transfected with FGF21 expression plasmids and was not detected in the immunoblotting experiment with these cell lysates (Supplementary Fig. S1a–c). Owing to the low cell binding affinity of FGF21, FGF21 signaling was regulated by the expression of its receptor complex, FGFR1, FGFR3, and in particular, KLB in the target organ[14–16]. Technically, intravascular hydrodynamic gene delivery is a safe and efficient in vivo procedure for gene transfer in small animal models. The clinical translation of this approach requires the prevention of hemodynamic side effects. Direct injection of plasmid DNA into the target liver segment using a catheter with a balloon has been recently demonstrated to restrict the perfusion area[38]. FGF21, a secreted protein that can be expressed long-term using the *pLIVE* vector, is a suitable candidate for this purpose. On the other hand, various long-acting FGF21 analogs, including LY2405319, PF-05231023, and AKR-001, have already been tested in clinical trials for people with obesity[26,28,29,42]. Moreover, pegbelfermin (BMS-986036) is a PEGylated FGF21 analog that is currently being investigated for

the treatment of nonalcoholic steatohepatitis[43]. In a phase IIa trial, the subcutaneous injection of 10-mg pegbelfermin once a day or 20-mg pegbelfermin once a week has shown promising improvements in several nonalcoholic steatohepatitis-related outcomes. Therefore, gene transfer using *pLIVE* vectors is an excellent experimental model, which is, however, somewhat invasive from the perspective of clinical applicability.

FGF21 not only stimulates lipolysis in WAT but also activates brown adipose tissue and induces the browning of WAT[44]. The hallmarks of browning include the appearance of uncoupling protein-1-expressing multilocular and mitochondria-rich adipocytes in WAT. However, we found that the mRNA levels of uncoupling protein-1 expression did not increase by FGF21 treatment (data not shown), whereas lipolysis was induced (Fig. 5C). The lipolysis of the adipose tissue produces glycerol and FFAs that serve as energy sources during the fasting state. This lipolysis is tightly regulated under normal conditions. Conversely, in the context of obesity and insulin resistance, excessive lipolysis causes hepatic steatosis as FFAs released from peripheral adipose depots constitute a major source of triglycerides in the liver of patients with NAFLD[36].

Mitochondrial β-oxidation is determined as the dominant oxidative pathway for the disposal of FFAs in liver[45]. Fatty acid β-oxidation is the process of breaking down long-chain acyl-CoA into acetyl-CoA, which is used in a myriad of biochemical pathways. For example, it may be used as a starting material for the biosynthesis of lipids such as triglycerides, phospholipids, or cholesterol[36]. Most importantly, for energy generation, acetyl-CoA may enter the citric acid cycle and be oxidized to produce energy, if oxygen is available. Acetyl-CoA also enters the ketogenic pathway to form 3-OHBA. Ketones cannot be oxidized in the liver but are exported and utilized by peripheral tissues, with little impact on hepatic energetics in a normal state[46]. In people with obesity as well as the murine model used in the present study, the liver is continuously exposed to the postabsorptive and fat-derived circulating FFAs. Although acetyl-CoA disposal is primarily associated with the TCA cycle, ketone synthesis plays a critical role in the removal of excess acetyl-CoA and is impaired in human fatty liver with increased acetyl-CoA and hyperglycemia[46]. Bdh1 is the last enzyme of hepatic ketogenesis and the first enzyme of ketolysis in extraliver organs. Bdh1 deficiency is well-tolerated under normal diet conditions but is exacerbated during fasting, with a marked increase in the AcAc/3HB ratio and hepatic steatosis[47]. As has been recently reported, FGF21 regulates lipolysis in adipocytes in response to the metabolic state but is not required for ketogenesis[48]. At least, the phenotypes of *fgf21* transgenic mice demonstrate that FGF21 stimulates hepatic ketogenesis[12,37]. In our diet-induced obesity model, the high levels of serum FGF21 induced lipolysis and FFAs production from the peripheral adipose tissue (Fig. 5C). Ketone body production as a secondary system for hepatic FFA disposal might improve liver steatosis.

Ketone bodies are a major oxidative fuel source for muscles during starvation and in diabetes and attenuate wasting in models of atrophy[49]. These are important vectors of energy transfer. The relative capacity of tissues to utilize ketone bodies for energy is considered to be determined by the levels of the ketolytic enzyme Scot, which is often expressed in the myocardium, brain, kidney, and skeletal muscle[50]. Although fatty acids, glucose, and 3-OHBA may compete for acetyl-CoA in the TCA cycle, 3-OHBA is utilized for energy by diabetic muscles under FGF21 treatment[51]. We found that the transporter of ketone bodies, Mct2 and Scot, and the TCA cycle enzymes Cs and Idh3α were upregulated by FGF21 treatment (Fig. 6A–C). Furthermore, as recently reported concerning the metabolic functions of FGF21 in extraliver organs, FGF21 enhances the neuronal ability to use ketone bodies by upregulating the expression of MCTs and inducing myogenic differentiation in C2C12 cells[52–55]. We found that FGF21 treatment increased the mRNA and protein levels of myogenin expression, thereby indicating the protective function of FGF21 in muscle atrophy in people with obesity.

Recently, the sodium/glucose transporter (SGLT) 2 inhibitor has been reported to induce transcriptional reprogramming to activate catabolic pathways, increase fatty acid oxidation, reduce hepatic steatosis, and increase hepatic and plasma levels of FGF21[51]. SGLT2 inhibitors are antidiabetic drugs that induce urinary glucose loss. As a result, plasma glucose levels reduce and overall glycemic control improves[51]. Intriguingly, SGLT2 inhibitors reduce the risk of cardiovascular disease and mortality in type-2 diabetes and may improve hepatic steatosis and NAFLD[56]. SGLT2 inhibitors act in an insulin-independent manner, which leads to modest weight loss and induced fatty acid oxidation and ketogenesis[57]. FGF21 is an important coordinator of fasting-induced metabolic responses and reduction in adiposity by increasing lipolysis, hepatic fatty acid oxidation, and ketogenesis. FGF21 is not essential for a metabolic switch to a fasting-like catabolic state but is required to induce lipolysis and reduction in adiposity in response to SGLT2 inhibitors[57].

The present study has several limitations. First, tail vein injection was performed using 40 μg plasmid DNA but the amount of DNA could have been reduced. The serum FGF21 levels were much higher than the controls because of the long-term, continuous expression of exogenous FGF21 in the liver due to the *pLIVE* vector. These levels were sufficient compared to the previous in vivo FGF21 administration model to produce an effect[58]. Therefore, a direct injection of the plasmid DNA to a small liver segment may be enough to achieve sustained serum levels of FGF21. Second, the study findings were not sufficient in explaining FGF21-induced ketone production and utility. Ketones are produced as a fuel source when glucose is not available to cells. This condition is most frequently noted in patients with uncontrolled type 1 diabetes and can lead to a medical emergency. In the present study, the administration of FGF21 did not increase serum 3-OHBA levels in HFD-fed mice, suggesting that the produced 3-OHBA was immediately utilized and compensated for by the muscles and brain. Further studies using ketogenic diet models and describing a comparison with single-injection models of FGF21 analogs are warranted. Considering the results of the present study and those of previous studies, we conclude that FGF21 treatment can prevent the progression of obesity-related NAFLD via FGF21-related potential crosstalk and direct/indirect consequences between the liver and extraliver organs. Although FGFs, including FGF19, induce cell proliferation, FGF21 is required to limit the progression from NAFLD to hepatocellular carcinoma in response to prolonged exposure to an obesogenic diet[59]. The induction of hepatic FGF21 in response to the high-fat, high-sucrose obesogenic diet plays an important role in limiting the progression of liver pathology from NAFLD to hepatocellular carcinoma[60,61]. The lack of FGF21 induces nonalcoholic steatohepatitis–hepatocellular carcinoma transition via hepatocyte-TLR4-IL-17A signaling[62]. To determine an effective treatment for nonalcoholic steatohepatitis–hepatocellular carcinoma transition and obesity-related complications such as sarcopenia, future studies on the therapeutic use of FGF21, including their related drugs, are needed.

DATA AVAILABILITY

The data that support the findings of this study are available from the corresponding author, Kya, upon reasonable request.

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AUTHOR CONTRIBUTIONS

Kyan, Kya and Y.I. planned experiments. Kyan, Kya and Y.I. wrote the manuscript. Kyan and Kya performed experiments. N.T., S.O., and Y.L. assisted with the animal experiments. Kyan and Kya performed the histological experiments. Kyan, Kya, S.O., A.T., S.K., K.O., Y.S., A.U., M.M., T.O. and Y.I. read and commented on the manuscript.

COMPETING INTERESTS

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