



Published in final edited form as:

Hepatology. 2016 August ; 64(2): 425–438. doi:10.1002/hep.28523.

Fibroblast Growth Factor 21 Improves Hepatic Insulin Sensitivity by Inhibiting Mammalian Target of Rapamycin Complex 1 in Mice

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Abstract

Among the 22 fibroblast growth factors (FGFs), FGF21 has now emerged as a key metabolic regulator. However, the mechanism whereby FGF21 mediates its metabolic actions *per se* remains largely unknown. Here, we show that FGF21 represses mammalian target of rapamycin complex 1 (mTORC1) and improves insulin sensitivity and glycogen storage in a hepatocyte-autonomous manner. Administration of FGF21 in mice inhibits mTORC1 in the liver, whereas FGF21-deficient mice display pronounced insulin-stimulated mTORC1 activation and exacerbated hepatic insulin

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Additional Supporting Information may be found in the online version of this article at <http://onlinelibrary.wiley.com/doi/10.1002/hep.28523/supinfo>.

Potential conflict of interest: Nothing to report.

Author names in bold designate shared co-first authorship.

Supporting Information

Additional Supporting Information may be found in the online version of this article at <http://onlinelibrary.wiley.com/doi/10.1002/hep.28523/supinfo>.

resistance (IR). FGF21 inhibits insulin- or nutrient-stimulated activation of mTORC1 to enhance phosphorylation of Akt in HepG2 cells at both normal and IR condition. TSC1 deficiency abrogates FGF21-mediated inhibition of mTORC1 and augmentation of insulin signaling and glycogen synthesis. Strikingly, hepatic β Klotho knockdown or hepatic hyperactivation of mTORC1/ribosomal protein S6 kinase 1 abrogates hepatic insulin-sensitizing and glycemic-control effects of FGF21 in diet-induced insulin-resistant mice. Moreover, FGF21 improves methionine- and choline-deficient diet-induced steatohepatitis. *Conclusions:* FGF21 acts as an inhibitor of mTORC1 to control hepatic insulin action and maintain glucose homeostasis, and mTORC1 inhibition by FGF21 has the therapeutic potential for treating IR and type 2 diabetes.

Fibroblast growth factor 21 (FGF21) has increasingly gained attention as a novel metabolic regulator. FGF21 is physiologically induced by prolonged fasting and feeding of a ketogenic diet⁽¹⁾ in rodents and functions as a hormone to regulate carbohydrate and lipid metabolism.⁽²⁾ FGF21 binds to FGF receptor (FGFR) and the scaffold protein, β Klotho (KLB),⁽³⁾ and activates FGFR substrate 2a and extracellular signal-regulated kinase 1 and 2 (ERK1/2).⁽⁴⁾ Whereas FGFRs are widely expressed in most tissues, tissue specificity of FGF21 actions is determined by distribution of the coreceptor β Klotho.⁽⁵⁾ The liver, where β Klotho is abundantly expressed,⁽⁶⁾ is the major tissue mediating FGF21's beneficial effects. FGF21 inhibits diet- and fasting-induced hepatic steatosis (HS),^(1,7,8) induces ketogenesis,^(1,9,10) enhances hepatic fatty acid oxidation and tricarboxylic acid cycle flux through peroxisome proliferator-activated receptor gamma coactivator 1 alpha,^(3,7,11) and protects against methionine- and choline-deficient (MCD) diet-induced lipotoxicity.⁽¹¹⁾

Pharmacological administration or transgenic overexpression of FGF21 has been shown to ameliorate hyperglycemia, hyperinsulinemia, and insulin resistance (IR) in obese rodents and monkeys.⁽³⁾ Several lines of evidence support FGF21's effects on improving insulin sensitivity in the liver. FGF21 gain of function protects against hepatic IR in diet-induced obese (DIO) mice.⁽⁸⁾ Hyperinsulinemic clamp studies, the gold standard for assessing insulin action *in vivo*, have demonstrated that FGF21 improves hepatic insulin sensitivity and promotes glucose disposal in various rodent models, such as ob/ob mice, DIO mice, and Zucker fatty rats, in which FGF21 appears to have no effects on glucose uptake in adipose tissue or muscle.^(12,13) Moreover, whole-body FGF21-deficient mice are prone to late onset of glucose intolerance,⁽¹⁴⁾ and liver-specific FGF21 knockout mice show impaired insulin sensitivity.⁽¹⁵⁾ However, it is currently unclear how FGF21 regulates hepatic and systemic glucose homeostasis.

Although adipose-produced hormone adiponectin has been previously shown to mediate some metabolic effects of FGF21, it is important to note that glycemic effects of acute administration of FGF21 remain largely intact in adiponectin^{-/-} mice,⁽¹⁶⁾ suggesting an adiponectin-independent mechanism that may contribute to glucose-lowering effects of FGF21. Recently, the mammalian target of rapamycin complex 1 (mTORC1) and its downstream effector, ribosomal protein S6 (S6) kinase 1 (S6K1) have been revealed as critical regulators for nutrient overload-induced pathogenesis of IR and type 2 diabetes (T2D).⁽¹⁷⁾ S6K1 phosphorylates insulin receptor substrate 1 (IRS-1) at Ser1101⁽¹⁸⁾ and Ser302.⁽¹⁹⁾ Phosphorylation of IRS-1 on serine residues causes a disruption of

phosphatidylinositol 3-kinase and has emerged as a key molecular basis underlying induction of IR. However, whether mTORC1 mediates effects of FGF21 on insulin sensitivity remains unknown.

Our recent studies demonstrate that sirtuin 1 (SIRT1) and retinoic acid receptor beta act as potent upstream regulators of FGF21.^(7,10) In this study, we utilized pharmacological and genetic approaches to characterize that the mTORC1 complex serves as a novel downstream target of FGF21. These *in vivo* and *in vitro* studies illustrate that (1) FGF21 inhibits nutrient- and hormonal-induced hepatic mTORC1 activity; (2) hepatic β Klotho is necessary for FGF21 to inhibit mTORC1 and enhance insulin sensitivity; (3) FGF21 down-regulates mTORC1 in a tuberous sclerosis complex (TSC)-dependent manner; and (4) FGF21 enhances hepatic insulin sensitivity through mTORC1 inhibition.

Materials and Methods

ANIMALS

FGF21 knockout mice were described.⁽¹⁶⁾ Tamoxifen-inducible TSC1 knockout mice were generated by crossing Cre-ER recombinase transgenic mice with floxed TSC1 mice⁽²⁰⁾ (The Jackson Laboratory, Bar Harbor, ME). Male C57BL/6 mice at 8 weeks of age were purchased from Shanghai Laboratory Animal Co. (Shanghai, China). All animal experimental protocols were approved by the institutional animal care and use committee at Institute for Nutritional Sciences, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (Shanghai, China).

LIVER HISTOLOGICAL ANALYSIS

Livers were fixed in 10% phosphate-buffered formalin acetate at 48C overnight and embedded in paraffin wax. Paraffin sections (5 μ m) were cut and mounted on glass slides for hematoxylin and eosin (H&E) staining as described⁽²¹⁾ and for Periodic Acid-Schiff (PAS) staining and Sirius Red staining according to the manufacturer's instructions (Maixin Biotech, Fujian, China).

SHORT-HAIRPIN RNA KNOCKDOWN

Adenoviruses (Ad) expressing short-hairpin RNAs (shRNAs) targeting β Klotho (shKLB) and negative control (shNC) were purchased from GenePharma (Shanghai, China). The following shRNA targeting sequences were used: shKLB-17, GCGACTACCCTGAGTTCATGA; shKLB-18, GCAATCTGTCCAAAGTTAACA; shKLB-19, GCTCTGGATCACCATCAATGA; shKLB-20, GGAATACGATGACCCTCAAAT; shNC, TTCTCCGAACGTGTCACGT.

IN VIVO ADENOVIRAL GENE TRANSFER

Adenovirus-mediated gene transfer in livers of C57/BL/6 mice was accomplished as described.^(7,10) Adenoviruses (5×10^9 plaque-forming units [PFU] $\sim 1 \times 10^{10}$ PFU/mouse) were delivered into mice by tail vein injection. Two weeks postinjection, animals were killed and tissues were rapidly collected.

STATISTICAL ANALYSIS

Data are expressed as mean \pm SEM. Statistical significance was evaluated using the unpaired two-tailed Student *t* test and among more than two groups by analysis of one-way analysis of variance. Differences were considered significant at a *P* value <0.05 .

Additional materials and methods are detailed in the Supporting Information.

Results

HEPATIC KNOCKDOWN OF β KLOTHO ABROGATES FGF21'S EFFECTS ON IMPROVING INSULIN SENSITIVITY AND GLYCOGEN STORAGE IN LIVERS OF HIGH-FAT, HIGH-SUCROSE DIET-FED MICE

To investigate the relative contribution of FGF21 to insulin signaling and glucose homeostasis, hepatic knockdown of β Klotho using adenovirus-mediated shRNA (Ad-shKLB) was performed. shKLB-18, which produced the greatest knockdown efficiency, was chosen for the following *in vivo* experiments (Supporting Fig. S1A,B). We next administered FGF21 to Ad-shKLB-injected mice fed on a type 2 diabetogenic diet composed of high fat, high sucrose (HFHS).⁽²¹⁾ Strikingly, as shown in Fig. 1A, Ad-shKLB largely abrogated insulin-sensitizing effects of FGF21, including fasting glucose, plasma insulin levels, and the calculated value for the homeostasis model assessment of IR (HOMA-IR). Importantly, the stimulatory effect of FGF21 on hepatic glycogen, as determined by either biochemical measurements or PAS staining, was diminished by Ad-shKLB (Fig. 1B,C), which is consistent with increased glycogen content by FGF21 in ob/ob mice.⁽¹³⁾ These results suggest a critical role of hepatic β Klotho in mediating insulin-sensitizing effects of FGF21. Notably, hepatic knockdown efficiency of β Klotho was evidenced (Fig. 1E and Supporting Fig. S1C,D). Consistent with the effects of FGF21 in DIO mice,⁽⁸⁾ FGF21 treatment caused a significant reduction of body weight, likely through increased energy expenditure,^(7,22) which was not obviously affected by Ad-KLB. Similar to previous studies,⁽¹⁶⁾ FGF21 treatment stimulated messenger RNA (mRNA) levels of adiponectin in the white adipose tissue (Supporting Fig. S1F), suggesting a potential role of adiponectin in mediating some of FGF21's hepatic actions, such as partial reduction of liver triglyceride levels as shown in (Supporting Fig. S1E). Moreover, FGF21's beneficial effects on glucose tolerance and insulin sensitivity in HFHS diet-fed mice as measured by glucose tolerance tests (GTTs) and insulin tolerance tests (ITTs) were largely abrogated by Ad-shKLB (Fig. 1F), further supporting the essential role of hepatic β Klotho in mediating FGF21 effects on insulin sensitivity.

HEPATIC β KLOTHO IS NECESSARY FOR FGF21 TO INHIBIT mTORC1 ACTIVITY TO IMPROVE HEPATIC INSULIN SENSITIVITY IN HFHS DIET-FED MICE

Our recent study demonstrated that FGF21 is up-regulated by the nutrient sensor, SIRT1,⁽⁷⁾ and SIRT1 ameliorates hepatic IR in diabetic obese mice through suppression of mTORC1.⁽²³⁾ These studies suggest a potential link between FGF21 and mTORC1. To investigate the causal association between FGF21-mediated mTORC1 inhibition and insulin-sensitizing effects, immunoblotting analysis was performed using mouse livers. Administration of FGF21 significantly decreased mTORC1 kinase activity toward S6K, S6,

and 4E-BP1, resulting in decreased phosphorylation of IRS-1 at Ser1101 that is critical for development of hepatic IR,⁽¹⁸⁾ whereas phosphorylation of Akt (protein kinase B) was increased, suggesting improved hepatic insulin sensitivity (Fig. 2A,B). The effect of FGF21 on hepatic glycogen synthesis was further determined. Glycogen synthesis is controlled by glycogen synthase (GS). Phosphorylation of glycogen synthase kinase 3 (GSK-3) inhibits its own kinase activity, which prevents its inhibition of GS and leads to increased glycogen synthesis.⁽²⁴⁾ Notably, FGF21 increased phosphorylation of GSK-3 β and reduced phosphorylation of GS, suggesting enhanced hepatic glycogen synthesis. Strikingly, the ability of FGF21 to attenuate mTORC1 activity and promote hepatic insulin sensitivity and glycogen synthesis was abrogated by Ad-shKLB.

FGF21 IS SUFFICIENT TO INHIBIT mTORC1 ACTIVATION AND ENHANCE HEPATIC INSULIN SIGNALING IN MICE

To determine the effects of FGF21 on insulin-stimulated mTOR/S6K activity *in vivo*, adenovirus-mediated overexpression of FGF21 (Ad-FGF21) was performed in mice injected without or with insulin. Overexpression of FGF21 was confirmed by increased hepatic FGF21 mRNAs and elevated plasma levels of FGF21 and β -hydroxybutyrate (Fig. 3A and Supporting Fig. S2A,B). No obvious changes in body weight and plasma lipid levels were evident (Supporting Fig. S2C–E). Strikingly, FGF21 caused a robust reduction in mTORC1 activity and serine phosphorylation of IRS-1 in livers of insulin-injected mice, resulting in increased phosphorylation of Akt and GSK-3 β and decreased phosphorylation of GS (Fig. 3B,C). Therefore, these results suggest that FGF21 inhibits mTORC1 and acts as a hepatic insulin sensitizer, which may explain the beneficial effects of FGF21 on systemic glucose homeostasis.

HEPATIC mTORC1 ACTIVITY AND INSULIN SIGNALING ARE ALTERED IN LIVERS OF FGF21 KNOCKOUT MICE

We next elucidated a causal relationship between FGF21 and mTORC1 complex and their effects on insulin signaling using FGF21 knockout mice. FGF21 deficiency was confirmed by hepatic FGF21 mRNA levels (Fig. 4A). No obvious changes in plasma lipid levels were evident (Supporting Fig. S3), which is consistent with early studies of FGF21-deficient mice fed on a chow diet.⁽¹⁴⁾ In contrast to FGF21 overexpression, FGF21 deletion caused a profound activation of insulin-induced mTORC1 and phosphorylation of IRS-1, leading to decreased phosphorylation of Akt and GSK-3 β and increased phosphorylation of GS (Fig. 4B,C). These studies indicate that FGF21 is necessary for suppression of mTORC1 and improvement in hepatic insulin sensitivity *in vivo*.

FGF21 INHIBITS mTORC1 ACTIVATION TO STIMULATE INSULIN ACTION AND PROMOTE GLYCOGEN SYNTHESIS *IN VITRO*

To investigate whether FGF21 is a driving force to inhibit hepatic mTORC1 and improve insulin sensitivity *in vitro*, effects of FGF21 overexpression were rigorously assessed in multiple hepatocytes. Compared with Ad-GFP, adenovirus-mediated overexpression of FGF21 potently inhibited insulin-stimulated mTORC1 activity (Fig. 5A and Supporting Fig. S4A). Notably, mTORC1 activity was blocked by rapamycin, an inhibitor of the mTORC1 protein kinase complex.⁽²⁵⁾ Consistent with *in vivo* results, FGF21 suppressed insulin-

stimulated phosphorylation of IRS-1, resulting in an induction of phosphorylation of Akt and GSK-3 β . Effects of FGF21 on mTORC1 activity and insulin signaling were confirmed in nutrient- and amino acid-stimulated HepG2 cells (Fig. 5B and Supporting Figs. S4B and S5A), in palmitate/BSA (bovine serum albumin)-induced insulin-resistant HepG2 cells (Fig. 5C) and further in human Huh7 hepatocytes (Supporting Fig. S5B). Notably, media levels of FGF21 were around 4,000 pg/mL in cells expressing Ad-FGF21 (Fig. 5D), suggesting that adenovirus-delivered expression of FGF21 may inhibit mTORC1 by hormonal stimulus.

To further test the functional consequence of FGF21-mTORC1 on insulin sensitivity, glycogen synthesis activities were measured in primary hepatocytes. Hepatic glycogen synthesis was induced by insulin and synergistically induced by cotreatment with FGF21 (Fig. 5E). Likewise, inhibition of mTORC1 activity, using an acute and low dose of shows similar effects. Intriguingly, compared with FGF21 or rapamycin alone, cotreatment with FGF21 and rapamycin did not cause additional stimulation of glycogen synthesis, suggesting that FGF21 and rapamycin may promote glycogen synthesis through a similar mechanism of inhibiting mTORC1 in hepatocytes.

FGF21 IMPROVES HEPATIC INSULIN SENSITIVITY THROUGH INHIBITING mTORC1 ACTIVITY *IN VIVO* AND *IN VITRO*

Next, the downstream signaling in mediating FGF21's inhibition of mTORC1 was vigorously explored. FGF21 attenuated insulin-stimulated phosphorylation of mTORC1 activity toward S6K and increased phosphorylation of Akt in TSC1^{1/1} hepatocytes, whereas these effects were abrogated by TSC1 deficiency-induced hyperactivation of mTORC1 (Fig. 6A,B). Likewise, FGF21-induced glycogen synthesis was diminished in TSC1^{-/-} cells (Fig. 6C), suggesting that FGF21 may act upstream of the TSC complex to inhibit mTORC1 and improve insulin action. Moreover, treatment with lentivirus encoding a constitutively active Rag mutant, RagA^{Q66L}, potently stimulated phosphorylation of S6K in amino acid-free (-AA) media, consistent with a positive role of Rags in mediating amino acid-stimulated mTOR activation under nutrient-poor conditions (Fig. 6D,E).⁽²⁶⁾ Interestingly, treatment of FGF21 persistently reduced phosphorylation of S6K. Furthermore, FGF21's inhibition of phosphorylation of S6K remained intact in the presence or absence of a selective ERK inhibitor (U0126)⁽²⁷⁾ (Fig. 6F). These results suggest that FGF21 inhibits mTORC1 in a Rag GTPase- or ERK-independent manner.

To further demonstrate the causal link between FGF21's inhibition of mTORC1 and augmentation of insulin sensitivity, *in vitro* and *in vivo* assays were performed. Constitutive activation of mTORC1's downstream effector, S6K1 (Ad-S6K1), which has been shown to induce hepatic IR,⁽¹⁷⁾ was confirmed by increased phosphorylation of S6 in HepG2 cells (Supporting Fig. S6A,B). Importantly, FGF21-stimulated phosphorylation of Akt and GSK-3 β in the presence of insulin was abrogated by Ad-S6K1. Notably, phosphorylation of ERK1/2 was comparable between Ad-S6K1 and green fluorescent protein (GFP) treatment. Next, effects of mTOR/S6K activation on FGF21 actions were further investigated in diet-induced mice. Strikingly, Ad-S6K1 abrogated FGF21's improvements in metabolic parameters in livers of HFHS diet-induced mice, such as fasting glucose, plasma insulin levels, HOMA-IR, hepatic glycogen levels, GTT, and ITT (Fig. 7A-C and Supporting Fig.

S7). These studies suggest that FGF21's effects on hepatic mTORC1 inhibition and insulin sensitization are causally linked.

ADMINISTRATION OF FGF21 ATTENUATES THE DEVELOPMENT OF NONALCOHOLIC STEATOHEPATITIS

To investigate the role of FGF21 on nonalcoholic steatohepatitis (NASH), an MCD diet-induced mouse model⁽¹¹⁾ was used. Administration of FGF21 showed decreased pathological features of NASH in mice fed with MCD diet, including decreased HS and hepatocellular ballooning, and reduced perisinusoidal fibrosis as evidenced by Sirius Red staining (Fig. 8A–C). FGF21 caused a significant reduction of the NASH Clinical Research Network (NASH CRN) scores, a semiquantification analysis of NASH.⁽²⁹⁾ Notably, plasma alanine aminotransferase (ALT) levels were decreased by FGF21, suggesting decreased hepatocyte damage. These results are consistent with a previous observation showing that FGF21 protects against NASH in mice.⁽¹¹⁾ Intriguingly, FGF21 treatment decreased mTORC1 activity toward S6K (Fig. 8D). Further investigation is needed to examine whether mTORC1 inhibition mediates FGF21's improvements in NASH.

Discussion

This study demonstrates that the hepatocyte-derived hormone, FGF21, acts as a novel inhibitor of the nutrient sensor mTORC1 complex and functions as an autocrine/paracrine modulator of hepatic insulin sensitivity and glucose homeostasis. Although pharmacological and physiological studies have demonstrated beneficial functions of FGF21 in the liver, the downstream signaling pathways mediating these activities remain largely unknown. We demonstrate, for the first time, that the FGF21- β Klotho pathway is sufficient and necessary to repress mTOR/S6K activity. Moreover, FGF21's inhibition of mTORC1 and augmentation of hepatic insulin sensitivity are causally linked. FGF21-mediated inhibition of mTORC1 may represent a molecular mechanism by which pharmacological and genetic manipulation of FGF21 ameliorate hepatic insulin resistance, hyperglycemia, and T2D.

THE LIVER IS A DIRECT TARGET OF FGF21 ACTIONS TO IMPROVE HEPATIC INSULIN SENSITIVITY AND GLUCOSE HOMEOSTASIS

The present study utilizes *in vivo* and *in vitro* approaches to demonstrate previously unrecognized mechanisms of FGF21 directly in the liver. First, hepatic knockdown of β Klotho exacerbates hepatic IR and abolishes glucose-lowering effects of FGF21 in diet-induced IR mice. These results are consistent with recent findings that β Klotho is essential for FGF21 activity^(2,30) and that FGF21 deficiency in the liver, instead of adipose tissue, impairs insulin sensitivity and glucose homeostasis in DIO mice.⁽¹⁵⁾ Second, the present study utilizes cell-based assays, such as mouse primary hepatocytes and human HepG2 and Huh7 hepatocytes, to rigorously demonstrate the downstream signaling of FGF21, which circumvents FGF21's endocrine actions in other tissues. These data demonstrate that FGF21 inhibits mTORC1 activity to improve insulin signaling in a hepatocyte-autonomous manner. Third, this study identifies, for the first time, that administration of FGF21 induces hepatic glycogen synthesis both *in vivo* and *in vitro*, which is consistent with increased glycogen storage in FGF21 transgenic mice⁽³¹⁾ and ob/ob mice treated with recombinant FGF21.⁽¹³⁾

This study defines FGF21 as a potent activator of hepatic glycogen synthesis. Although these data appear to contradict the previous study showing that FGF21 improves hyperglycemia in hepatic IR liver-specific insulin receptor-deficient mice,⁽³²⁾ the intact IRS in the mice that mediates FGF21-mTORC1 action on normalization of hyperglycemia may be a contributing factor to this difference. Taken together, in addition to regulation of fatty acid oxidation, FGF21 may promote glucose utilization and control peripheral glucose homeostasis by stimulating hepatic glycogen storage.

FGF21 SERVES AS A NEGATIVE REGULATOR OF mTORC1 KINASE COMPLEX

The present study establishes the nutrient sensor mTORC1 complex as a bona-fide downstream target of FGF21. FGF21 gain- and loss-of-function approaches have demonstrated the inhibitory action of FGF21 on mTORC1 in mouse livers, primary hepatocytes, and multiple liver cell lines in response to insulin-resistant challenges. These findings are consistent with our previous observation that SIRT1, a potent upstream inducer of FGF21,⁽⁷⁾ inhibits mTORC1 in liver of insulin-resistant mice.⁽²³⁾ Further investigation is needed to examine whether FGF21 inhibition of mTORC1 mediates the insulin-sensitizing effects of SIRT1. In obese animals as well as T2D and nonalcoholic fatty liver disease patients, hepatic and circulating levels of FGF21 are elevated,⁽³³⁾ whereas mTOR/ S6K activity is overactivated in obesity and insulin-resistant rodents.⁽¹⁸⁾ There are several reasons that may account for the seeming discrepancy between FGF21 levels and mTORC1 activity in obesity and T2D. First, activation of the mTOR/S6K pathway by nutrient excess may induce production and secretion of FGF21 in the liver, which is consistent with the recent findings that hepatic mTORC1 induces FGF21.⁽³⁴⁾ Second, FGF21 resistance in obesity⁽³⁵⁾ may account for compensatory overproduction of FGF21 in the circulation. Therefore, it is likely that the ability of FGF21 to repress hepatic mTORC1 is diminished because of FGF21 resistance in obesity.

One of the most important findings is that FGF21 regulates mTORC1, at least in part, through TSC1/2. The fact that FGF21-mediated inhibition of mTORC1 is abolished in TSC1-deficient hepatocytes suggests the presence of an inhibitory site upstream of TSC1/2. Moreover, FGF21 is sufficient to inhibit mTORC1 in HepG2 cells in the presence of the constitutive active Rag GTPase, RagA^{Q66L}, and the selective ERK inhibitor, U0126, suggesting a Rag- or ERK-independent mechanism in mediating FGF21's inhibition of mTORC1. These results are consistent with previous findings that FGF21's beneficial effects are independent of ERK.⁽¹⁶⁾ Taken together, this study demonstrates an essential role of TSC1/2 in mediating FGF21's inhibition of mTORC1, although the molecular link between FGF21- β Klotho and TSC1/2 needs further investigation.

mTORC1 INHIBITION MEDIATES EFFECTS OF FGF21 ON IMPROVING HEPATIC INSULIN SENSITIVITY

There are several reasons that FGF21's inhibition of mTORC1 and augmentation of insulin sensitivity are causally linked. First, hepatic overexpression of S6K1 abolishes metabolic effects of FGF21 in diet-induced insulin-resistant mice and in hepatocytes. Second, mTORC1 activation by TSC1 deficiency abrogates FGF21-stimulated glycogen synthesis in hepatocytes. Moreover, compared with treatment of FGF21 or rapamycin alone, cotreatment

with FGF21 and rapamycin does not cause additional stimulation of glycogen synthesis in hepatocytes, suggesting that FGF21 may repress mTORC1 to induce glycogen synthesis through a mechanism similar to rapamycin.

Interestingly, the recent study showed that activation of mTORC1 by TSC1 deficiency induces FGF21 under starvation.⁽³⁴⁾ Our studies may provide a mechanistic insight into a role of FGF21 in counteracting mTORC1-induced desensitization of IRS and IR in obesity and T2D (Fig. 8E). The interplay between FGF21 and mTORC1 may provide a finely tuned mechanism for insulin signaling in response to nutrient availability. It is conceivable that hepatic insulin sensitivity is likely controlled by the precise physiological context, which may be determined by net effects of the negative feedback loop of mTORC1 activity toward S6K-IRS and the positive feedback loop through inducing FGF21. The implication that FGF21 inhibits mTORC1 also provides a possible explanation for the opposite metabolic effects of FGF21 and mTORC1 on energy expenditure and growth.^(22,25,36,37) Collectively, the present study indicates that FGF21 enhances insulin sensitivity through inhibition of mTORC1 in livers. It may also represent a mechanism in the regulation of metabolism in other tissues to control whole-body physiology, which would be of interest and is to be further determined.

In summary, the present study has identified a central role of mTORC1 in mediating therapeutic actions of FGF21 on hepatic and systemic IR. Moreover, it is intriguing that FGF21 is sufficient to ameliorate MCD diet-induced NASH. These findings provide a novel mechanism by which FGF21 regulates metabolic effects by its actions in hepatocytes and livers. The FGF21-mTORC1 axis may represent a novel drug target to combat hyperglycemia, T2D, and potentially the development of steatohepatitis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We appreciate Qiwei Zhai (Institute for Nutritional Sciences, Shanghai, INS) for helpful discussion. We are grateful to Leping Cheng (Institute of Neuroscience, Shanghai, ION), Jiawei Zhou (ION), Shu Zhuo (INS), Yifan Bu (INS), Huating Li (The University of Hong Kong, China, HKU), Pengchen Zhou (HKU), and Hao Zhong (Jinhai, China) for technical assistance and Zhonghui Weng (INS) for animal studies.

This work is supported by grants from the National Natural Science Foundation of China (nos. 81270930 and 31471129) and the Hundred Talents Program of the Chinese Academy of Sciences (2013OHTP04) to Y.L. This work was also supported by the Research Grants Council of Hong Kong (C7055-14G and HKU783413) to A.X.

Abbreviations

4E-BP1	eIF4E-binding protein 1
Ad	adenovirus
Akt	protein kinase B
ALT	alanine aminotransferase

BSA	bovine serum albumin
DIO	diet-induced obese
ERK	extracellular signal-regulated kinase
FGF21	fibroblast growth factor 21
FGFR	FGF receptor
GFP	green fluorescent protein
GS	glycogen synthase
GSK-3	glycogen synthase kinase 3
GTT	glucose tolerance test
H&E	hematoxylin and eosin
HFHS	high fat, high sucrose
HOMA-IR	homeostasis model assessment of IR
HS	hepatic steatosis
IR	insulin resistance
IRS-1	insulin receptor substrate 1
ITT	insulin tolerance test
KLB	β Klotho
MCD	methionine and choline deficient
mRNA	messenger RNA
mTORC1	mammalian target of rapamycin complex 1
NASH	nonalcoholic steatohepatitis
NASH CRN	NASH Clinical Research Network
NC	negative control
PAS	Periodic Acid-Schiff
PBS	phosphate-buffered saline
PFU	plaque-forming units
S6	ribosomal protein S6
S6K1	ribosomal protein S6 kinase 1
shRNAs	short-hairpin RNAs

SIRT1	sirtuin 1
TSC	tuberous sclerosis complex
Rag GTPase	Ras-related GTP-binding protein GTPase
T2D	type 2 diabetes

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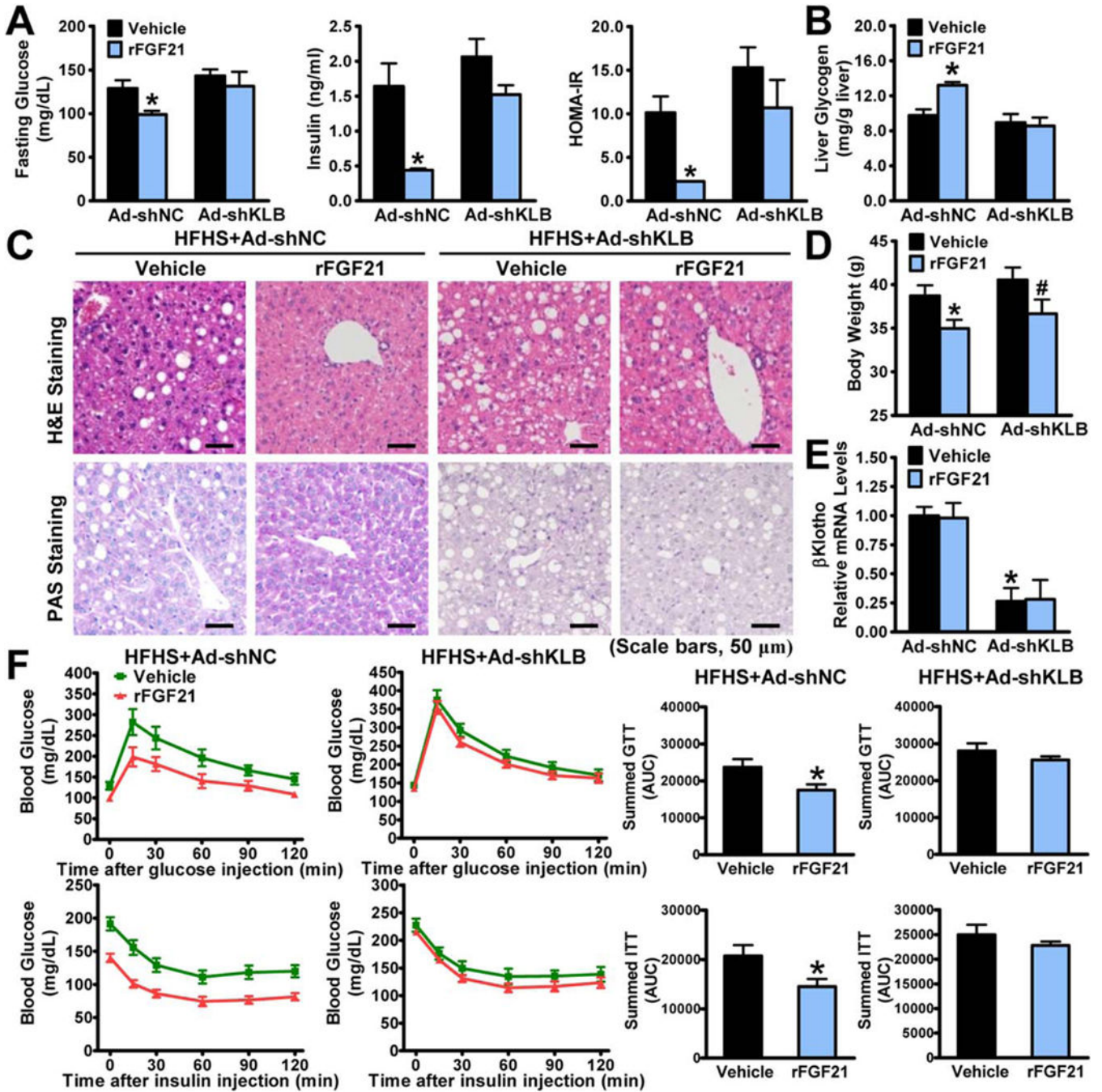


FIG. 1.

Beneficial effects of FGF21 on insulin sensitivity and hepatic glycogen storage are compromised by hepatic knockdown of β Klotho in HFHS diet-fed mice. Eight-week-old male C57BL/6 mice were fed on a HFHS diet for 14 weeks, followed by treatment with adenoviruses encoding shRNAs targeting β Klotho (shKLB) or control shNC by tail vein injection, and then treated with recombinant FGF21 (0.4 mg/kg/day) or vehicle (phosphate-buffered saline) by subcutaneous injection once-daily for 10 days. (A) Blood glucose, plasma insulin levels, and calculated HOMA-IR were assessed. (B) Liver glycogen content

was determined. (C) Representative H&E staining and PAS staining of liver sections (scale bars, 50 μ m). (D) FGF21 reduced body weight in both shNC- and shKLB-treated mice. (E) mRNA amounts of β Klotho were decreased by shKLB in livers. (F) GTTs (1 g/kg) or ITTs (1 U/kg) were performed. Data are presented as the mean \pm SEM; n = 5–7. * P < 0.05 versus shNC and vehicle; # P < 0.05 versus shKLB and vehicle.

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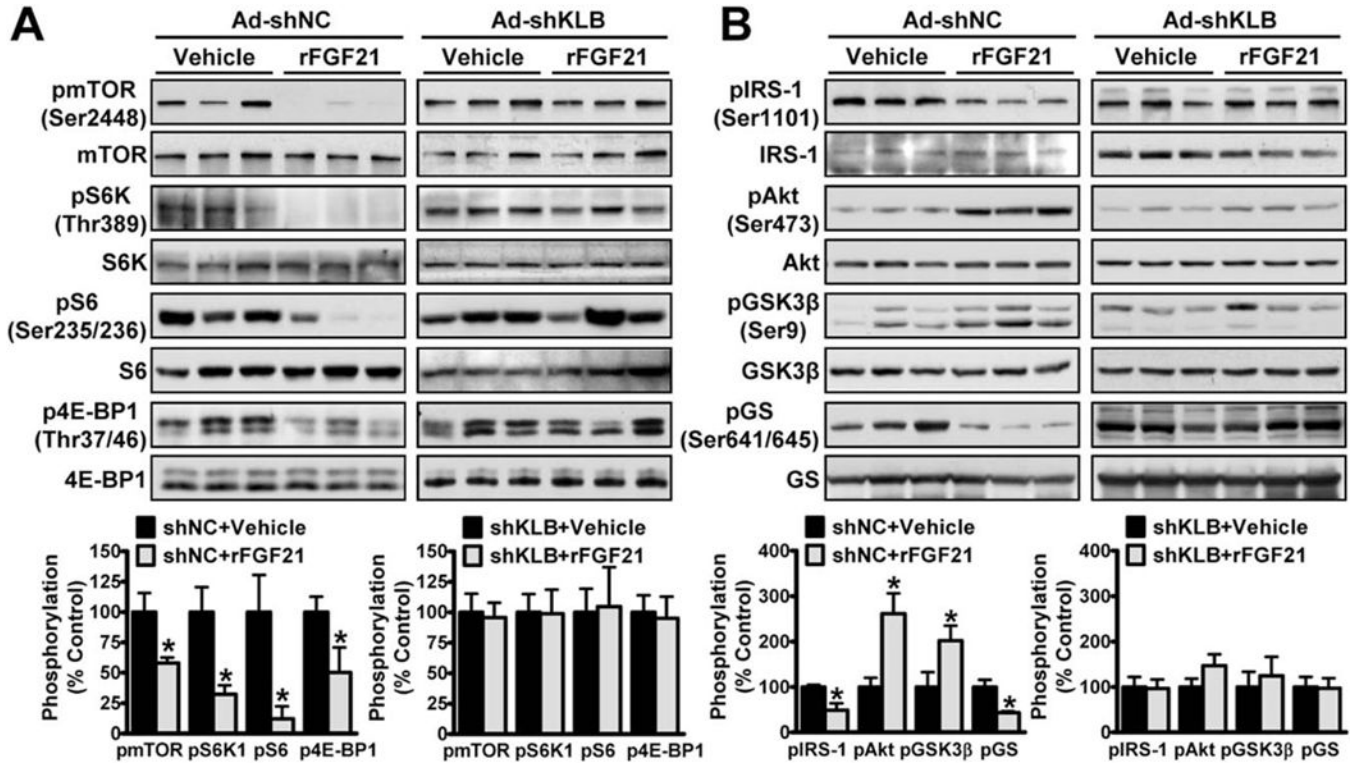


FIG. 2.

Administration of FGF21 inhibits mTORC1 activity to improve hepatic insulin sensitivity in a β Klotho-dependent manner in HFHS diet-fed mice. (A,B) FGF21 inhibited mTORC1 activity (A) and improved insulin sensitivity (B) in liver, and the effects were eliminated by Ad-KLB. Data are presented as the mean \pm SEM; n = 4–6. * P < 0.05 versus Ad-shNC and vehicle.

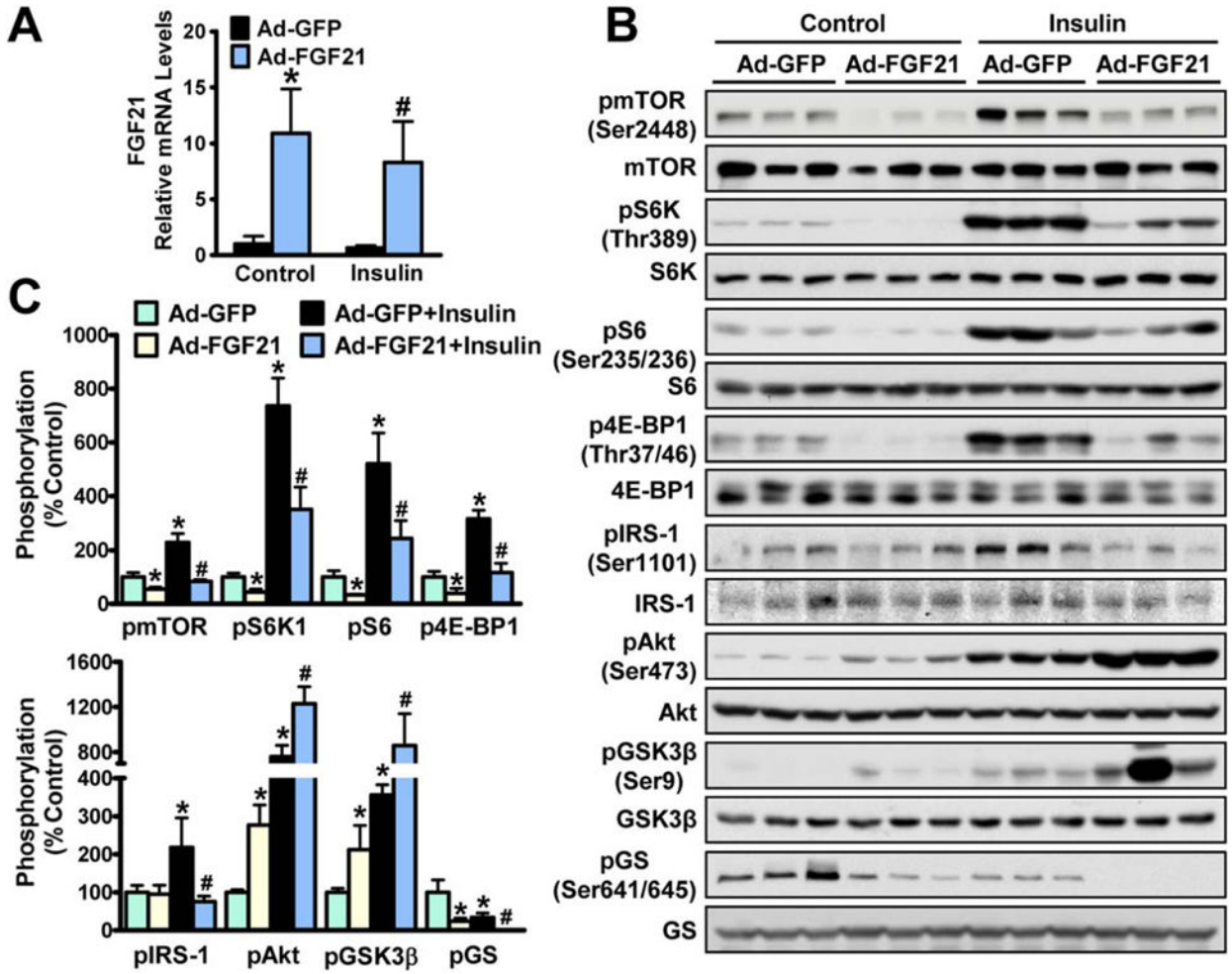


FIG. 3. Activation of FGF21 is sufficient to repress mTORC1 activity and enhance insulin sensitivity in mice. Eight-week-old male C57BL/6 mice were treated with FGF21 or GFP adenoviruses by tail vein injection for 10 days, followed by intraperitoneal injection with insulin (1 U/Kg) or control (PBS) for 10 minutes; animals were killed and livers were collected. (A) Hepatic gene expression of FGF21. (B,C) Adenovirus-mediated overexpression of FGF21 potentially attenuated insulin-induced activation of mTORC1 activity and stimulated insulin signaling in livers. Data are presented as the mean \pm SEM; n = 6–7. * P < 0.05 versus Ad-GFP; # P < 0.05 versus Ad-GFP and insulin.

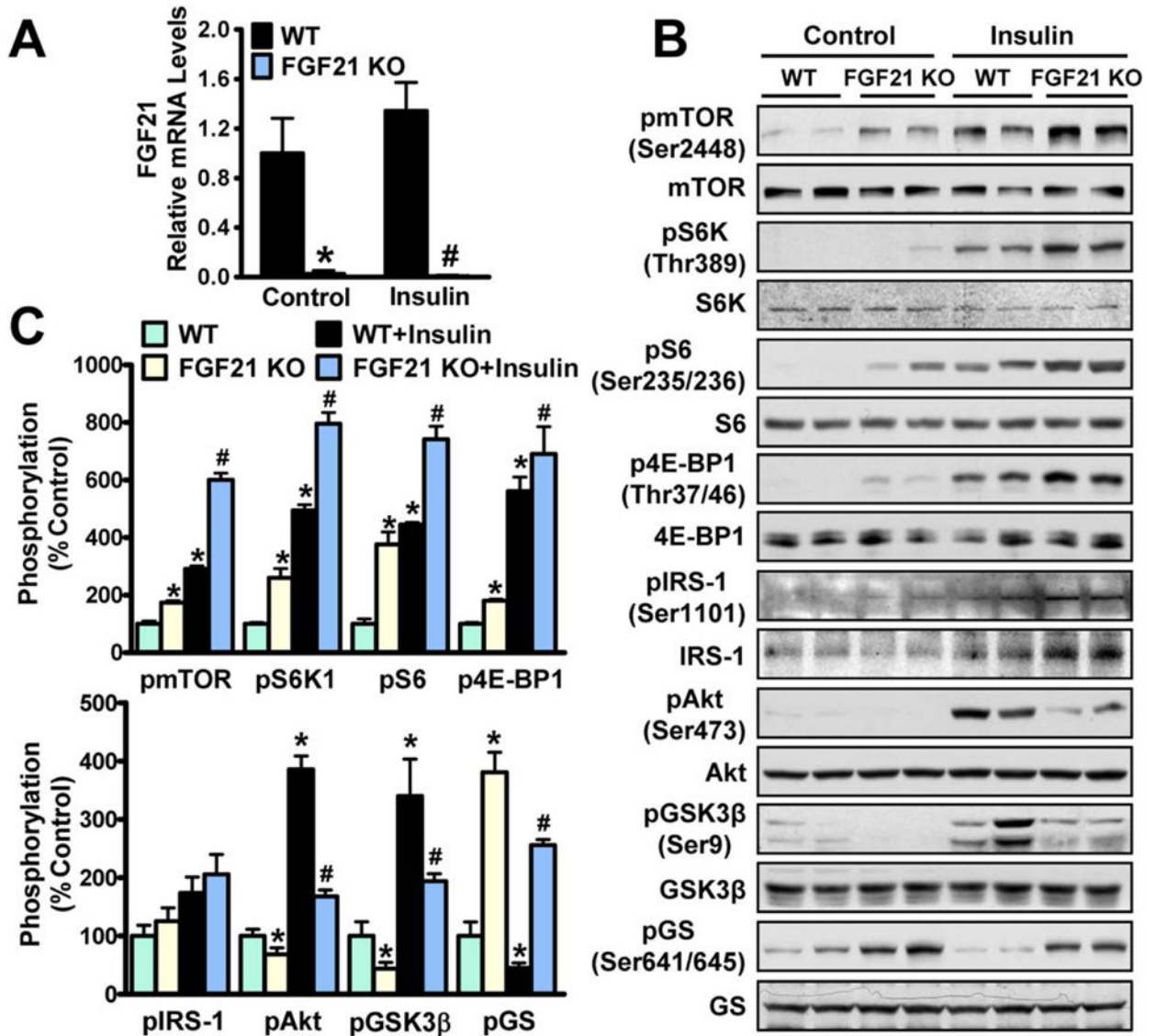


FIG. 4.

FGF21 deficiency results in activation of mTORC1 and hepatic IR in mice. FGF21 KO and WT mice at 5–6 months of age were injected with insulin (1 U/Kg) or control (PBS) intraperitoneally for 10 minutes, and then animals were killed. (A) Hepatic gene expression of FGF21. (B,C) Loss of FGF21 increased mTOR/S6K activity and suppressed hepatic insulin signaling. Data are presented as the mean \pm SEM; $n = 3$. * $P < 0.05$ versus WT mice; # $P < 0.05$ versus WT mice treated with insulin. Abbreviations: KO, knockout; WT, wild type.

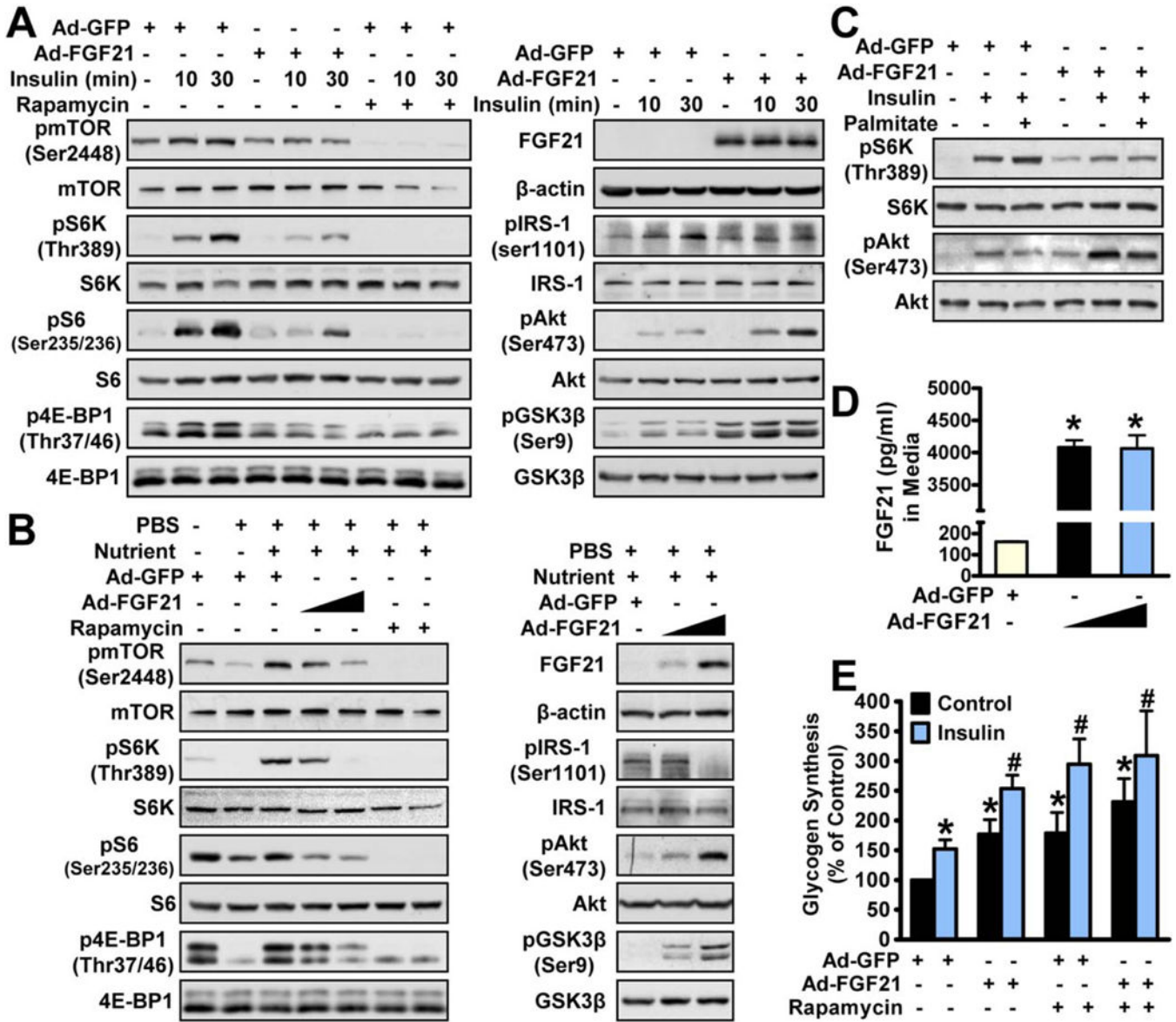
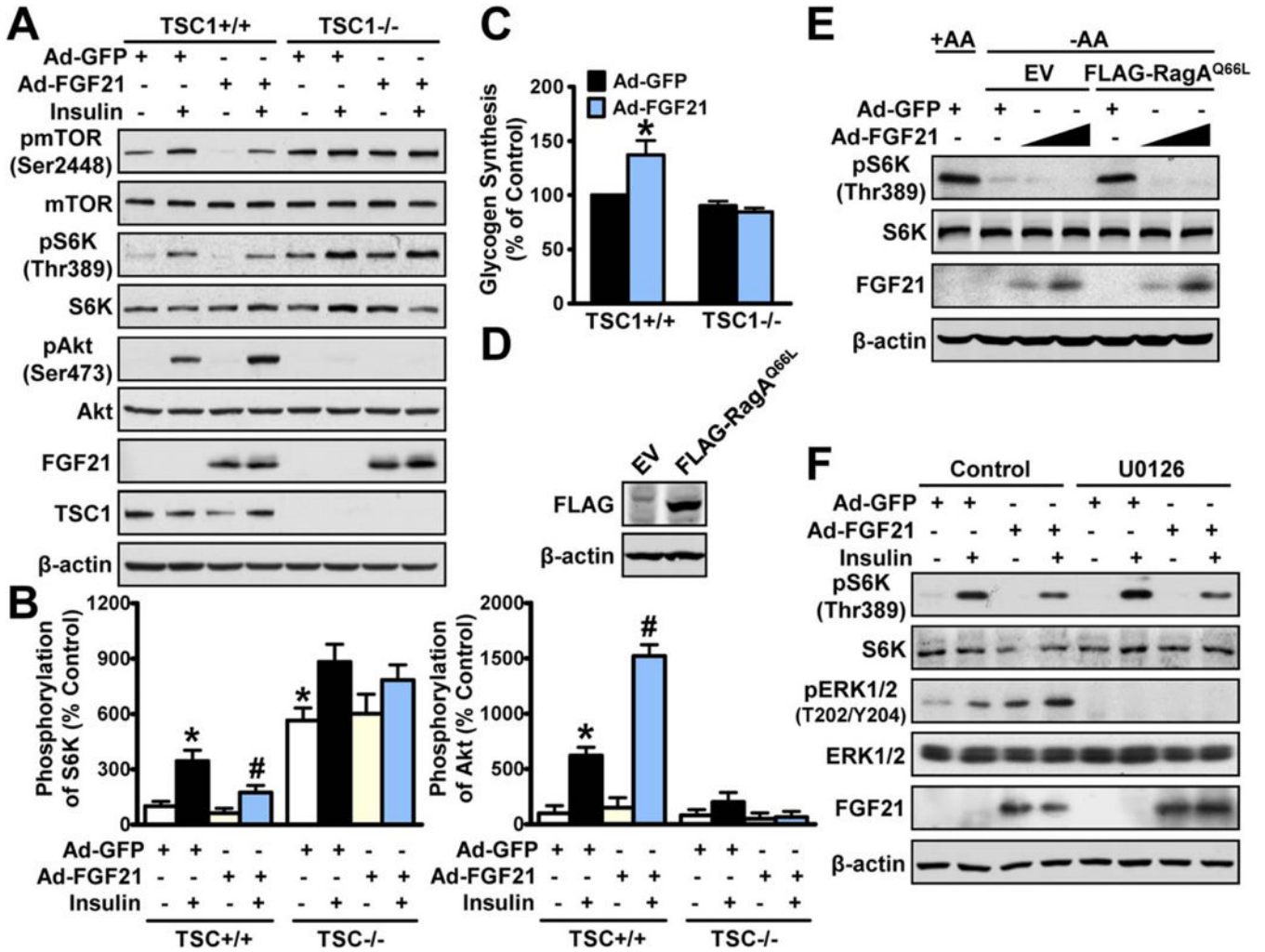


FIG. 5. FGF21 stimulates hepatic insulin action and glycogen synthesis by repressing mTORC1 activation in hepatocytes. (A,B) Overexpression of FGF21 inhibited insulin-stimulated (A) and nutrient-stimulated (B) activation of mTORC1 to enhance phosphorylation of Akt in HepG2 cells. (C) FGF21 overexpression inhibited palmitate/BSA-induced mTORC1 activation and IR in HepG2 cells. (D) Media FGF21 levels were increased in HepG2 cells treated with Ad-FGF21. (E) Effects of FGF21 and rapamycin on insulin-induced glycogen synthesis in primary mouse hepatocytes. Cells were infected with adenoviruses for 48 hours and then incubated in serum-free medium overnight, followed by incubation with rapamycin (5 nM) or insulin (100 nM) for 3 hours as indicated. Data are represented as the mean ± SEM; n = 4–5. **P* < 0.05 versus Ad-GFP; #*P* < 0.05 versus Ad-GFP and insulin.

**FIG. 6.**

TSC mediates hepatic insulin-sensitizing effects of FGF21 in hepatocytes. (A,B) TSC1 deficiency abrogated FGF21-mediated inhibition of mTORC1 and augmentation of insulin sensitivity in hepatocytes ($n = 3-4$). (C) FGF21-stimulated glycogen synthesis in response to insulin was abrogated in TSC^{-/-} hepatocytes ($n = 4-5$). Data are represented as the mean \pm SEM. * $P < 0.05$ versus Ad-GFP; # $P < 0.05$ versus Ad-GFP and insulin. (D,E) FGF21-mediated inhibition of mTORC1 is independent of Rag GTPases in HepG2 cells. Cells expressing FLAG-tagged RagA^{Q66L} or empty vector (EV) were infected with adenoviruses for 72 hours in DMEM (1AA), followed by incubation in amino acid-free DMEM (-AA) for 50 minutes. Overexpression of lentivirus encoding FLAG-RagA^{Q66L} in HEK293T cells was confirmed by immunoblottings. (F) FGF21-mediated inhibition of mTORC1 remained intact in HepG2 cells treated with U0126 (10 μ M). Cells were treated as indicated and representative immunoblottings are shown. Abbreviation: DMEM, Dulbecco's modified Eagle's medium.

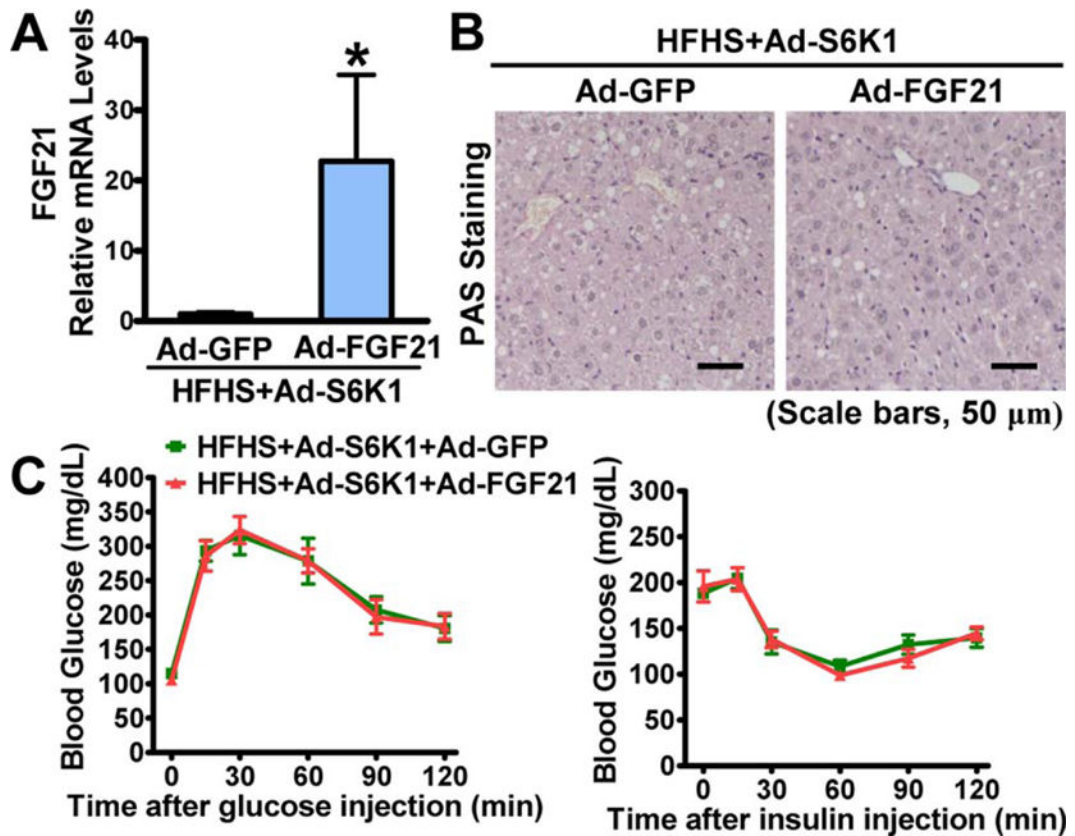
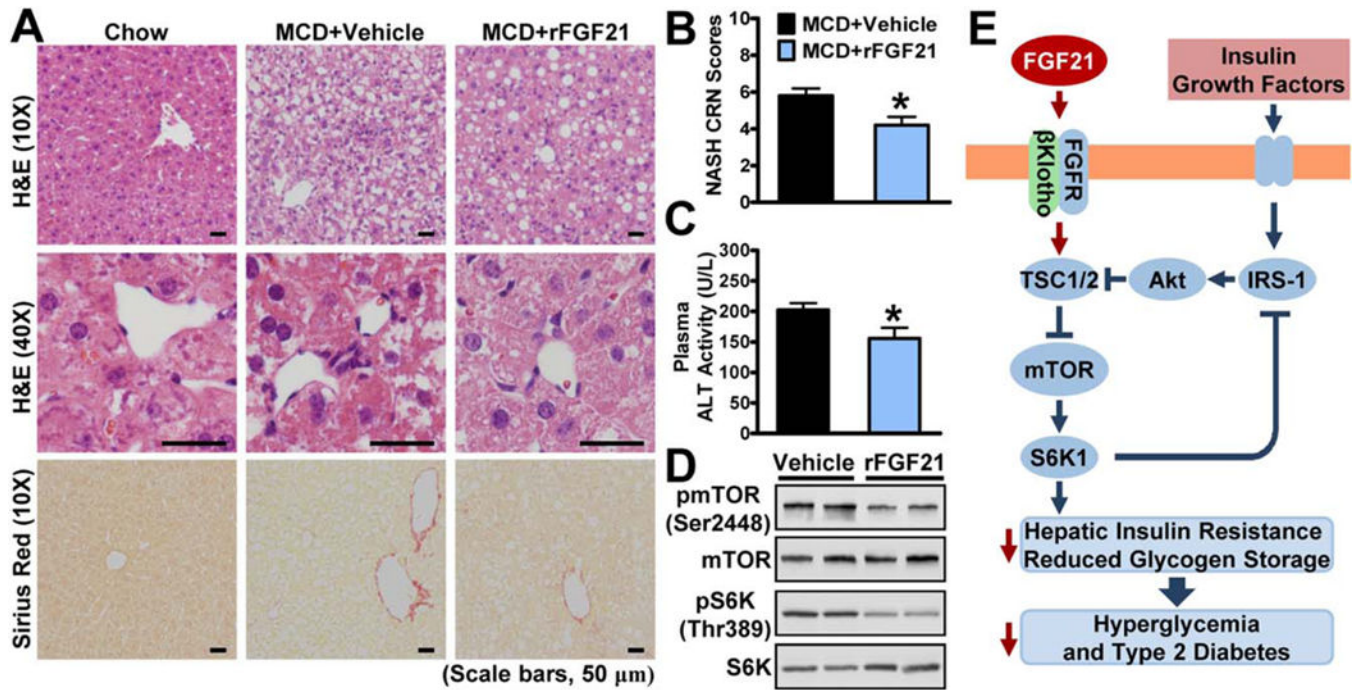


FIG. 7.

Hepatic overexpression of S6K1 abolishes hepatic insulin-sensitizing effects of FGF21 in HFHS diet-fed mice. Eight-week-old male C57BL/6 mice were fed on an HFHS diet for 11 weeks, followed by treatment with S6K1, FGF21, or GFP adenoviruses by tail vein injection for 10 days. (A) Hepatic gene expression of FGF21. (B) A representative PAS staining of liver sections is shown (scale bars, 50 μ m). (C) GTTs (1 g/kg) or ITTs (1 U/kg) were performed. Data are represented as the mean \pm SEM; n = 4. * P < 0.05 versus Ad-GFP.

**FIG. 8.**

Administration of FGF21 improves MCD diet-induced steatohepatitis in mice. Eleven-week-old male C57BL/6 mice were fed MCD diet for 3 weeks and then treated with recombinant FGF21 (0.6 mg/kg/day) or vehicle (PBS) by subcutaneous injection once-daily for 10 days. (A) Representative H&E and Sirius Red stainings of liver sections (scale bars, 50 μ m). (B,C) Reduction of histological NASH CRN scores (B) and ALT levels (C) in mice treated with FGF21. Data are represented as the mean \pm SEM; n = 5–7. * P < 0.05 versus MCD and vehicle. (D) FGF21 inhibited mTORC1 activity in livers, and representative immunoblottings are shown. (E) The proposed model for regulation of hepatic insulin sensitivity by FGF21- β Klotho-mediated inhibition of mTORC1 activation. Excessive nutrient- and hormonal-dependent activation of mTORC1 causes insulin resistance through a negative feedback loop by mediating IRS-1 serine phosphorylation, whereas administration of FGF21 represses mTORC1 and serine phosphorylation of IRS-1, leading to improved hepatic insulin sensitivity and glucose homeostasis. The FGF21-mTORC1 axis may represent a novel drug target for treating hepatic IR and T2D.