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Research paper

Liver-derived fibroblast growth factor 21 mediates effects of glucagon-like peptide-1 in attenuating hepatic glucose output

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ABSTRACT

Background: Glucagon-like peptide-1 (GLP-1) and its based agents improve glycemic control. Although their attenuating effect on hepatic glucose output has drawn our attention for decades, the potential mechanisms remain unclear.

Methods: Cytokine array kit was used to assess cytokine profiles in *db*/*db* mice and mouse primary hepatocytes treated with exenatide (exendin-4). Two diabetic mouse models (*db*/*db* and *Pax6*$^{m/+}$) were treated with a GLP-1 analog exenatide or liraglutide. The expression and secretion of fibroblast growth factor 21 (FGF21) in the livers of diabetic mice, primary mouse and human hepatocytes, and the human hepatic cell line HepG2 treated with or without GLP-1 analog were measured. Blockage of FGF21 with neutralizing antibody or siRNA, or hepatocytes isolated from *Fgf21* knockout mice were used, and the expression and activity of key enzymes in gluconeogenesis were analyzed. Serum FGF21 level was evaluated in patients with type 2 diabetes (T2D) receiving exenatide treatment.

Findings: Utilizing the cytokine array, we identified that FGF21 secretion was upregulated by exenatide (exendin-4). Similarly, FGF21 production in hepatocytes was stimulated by exenatide or liraglutide. FGF21 blockage attenuated the inhibitory effects of the GLP-1 analogs on hepatic glucose output. Similar results were also observed in primary hepatocytes from *Fgf21* knockout mice. Furthermore, exenatide treatment increased serum FGF21 level in patients with T2D, particularly in those with better glucose control.

Interpretation: We identify that function of GLP-1 in inhibiting hepatic glucose output is mediated *via* the liver hormone FGF21. Thus, we provide a new extra-pancreatic mechanism by which GLP-1 regulates glucose homeostasis.

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1. Introduction

Glucagon-like peptide-1 (GLP-1) enhances glucose-dependent insulin secretion in pancreatic β cells and suppresses glucagon secretion in α cells [1]. GLP-1 also exerts protective effects on β cell *via* promoting its proliferation and neogenesis and inhibiting its apoptosis [2]. Except for these pancreatic effects, GLP-1 can also reduce food intake [3], slow gastric emptying [4], decrease body weight [5], and improve insulin sensitivity [6] in multiple tissues including muscle [7], adipose tissue [8] and liver [9]. All these effects contribute to the regulation of glucose homeostasis. In addition to its glucose-lowering effect, GLP-1 can display favorable actions on several systems such as cardiovascular [10], nervous [11] and bone [12] systems. Currently, GLP-1-based agents, including GLP-1 analogs and dipeptidyl peptidase 4 inhibitors, have become new therapeutic options for patients with type 2 diabetes (T2D).

Traditionally, GLP-1 mainly aims at lowering postprandial blood glucose since this incretin hormone is predominantly released from intestinal L-cells in response to nutrient ingestion [13]. However, GLP-1 analogs can also lower fasting blood glucose (FBG) in patients with

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Research in context

Evidence before this study

Glucagon-like peptide-1 (GLP-1)-based agents, including GLP-1 analogs and dipeptidyl peptidase 4 inhibitors, have become new therapeutic options for patients with type 2 diabetes (T2D). GLP-1 analogs exert their glucose-lowering effect *via* multiple mechanisms. Several reports showed that GLP-1-based agents down-regulated hepatic glucose output. However, the potential mechanism remains unclear.

Added value of this study

We identified that FGF21, a liver hormone, was upregulated in the GLP-1 analog-treated mice, humans, and primary mouse and human hepatocytes. GLP-1 analogs inhibited hepatic glucose output *in vivo* and *in vitro*, while blockage of FGF21 attenuated the effects of GLP-1 analogs. In addition, the increment of serum FGF21 level resulted from exenatide treatment was more significant in T2D patients with better glucose control, suggesting that FGF21 might be involved in the GLP-1 analog-mediated improvement of blood glucose control.

Implications of all the available evidence

GLP-1 stimulates hepatocytes to produce FGF21, and the liver-derived FGF21 inhibits hepatic glucose output. Our results provide a new mechanism by which GLP-1 regulates glucose homeostasis.

T2D. In randomized clinical trials, treatment with exenatide or liraglutide resulted in a significant reduction in FBG, glycated hemoglobin A1c (HbA1c) and body weight in patients with T2D [14–17]. Interestingly, the maximal effect of liraglutide on FBG was evident after the first week of treatment [17]. There are several possible explanations for the GLP-1 analog-induced FBG reduction, including suppression of glucagon secretion, weight loss, improvement in insulin sensitivity [18], activation of glucokinase [19,20] and inhibition of hepatic glucose output [21,22]. However, the effect and mechanism of GLP-1 analogs on hepatic gluconeogenesis have not been addressed clearly.

In this study, we uncovered a novel role of fibroblast growth factor 21 (FGF21) in the GLP-1-mediated glucose metabolism regulation in hepatocytes. We showed that GLP-1 analogs could stimulate hepatic FGF21 production, which served as a key regulator of inhibition of gluconeogenesis by GLP-1 analogs in hepatocytes both *in vivo* and *in vitro*.

2. Materials and methods

2.1. Animals, treatment and tests

The animal care and experimental procedures were approved by Peking University Animal Ethics Committee. Male *db*/*db* and *db*/*m* mice were purchased from Vital River Animal Center (Beijing, China). After 1-week acclimatization, *db*/*db* mice were randomized into three groups (eight mice per group), two of which were treated for 2 weeks with exenatide (AstraZeneca, Cambridge, UK) twice daily at a dose of 100 nmol/kg body weight *via* subcutaneous injection, and the third group was treated with phosphate-buffered saline (PBS). The *db*/*m* mice treated with PBS served as control (n = 8). To antagonize FGF21 activity, half of the exenatide-treated mice were given a single intraperitoneal injection with an FGF21 neutralizing antibody (Cat: 12180, Antibody & Immunoassay Services, Hong Kong, China) at 8 μg per mouse at the end of the 2-week treatment. Six hours later, an intraperitoneal glucose tolerance test (IPGTT), an insulin tolerance test (ITT) and a pyruvate tolerance test (PTT) were performed.

Male *Pax6* heterozygous R266Stop mutant (*Pax6*$^{m/+}$) mice were used as an early-stage diabetic model as previously described by our group [23,24]. The diabetic *Pax6*$^{m/+}$ mice were randomized into two groups (three mice per group), which were injected subcutaneously with either PBS or liraglutide (Novo Nordisk, Bagsvaerd, Denmark) twice daily at a dose of 0.2 mg/kg body weight for 2 weeks. Age-matched male C57BL/6 wild-type mice (Vital River Animal Center) treated with PBS were used as a normal control (n = 3). At the end of the 2-week treatment period, an IPGTT and an ITT were performed.

The IPGTT and ITT were performed as detailed previously [24]. The PTT was performed with an intraperitoneal injection of pyruvate (2 g/kg body weight) after 15 h fasting. Blood glucose levels were measured at the specified time points with a One Touch Ultra glucometer (LifeScan, Chesterbrook, PA). Insulin levels were measured using a mouse insulin enzyme-linked immunosorbent assays (ELISA) kit (Cat: EZRMI-13 K, Millipore, Billerica, MA).

2.2. Cell culture and treatment

HepG2, a human hepatic cell line purchased from ATCC, was kindly gifted by Department of Immunology, Peking University Health Science Center, Beijing, China. Cells were cultured in DMEM medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS; Thermo Fisher Scientific, Waltham, MA) and 1× GlutaMax (Invitrogen). Cells were incubated with a GLP-1 analog exendin-4 (Cat: E7144, Sigma, St. Louis, MO) or liraglutide (both at 0.1, 1, 10 and 100 nM) for 24 h. To silence *FGF21* gene, HepG2 cells were transfected with siRNAs (synthesized by RiboBio, Guangzhou, China) using Lipofectamine RNAiMAX reagent (Invitrogen). After transfection for 48 h, cells were incubated with exendin-4 or liraglutide (100 nM) for 24 h. The culture supernatants were collected for ELISA, and cells were lysed for mRNA or protein analysis.

Male liver-specific *Fgf21* knockout (KO) mice (from Jackson laboratory [25]) were kindly gifted by Prof. Lirui Wang, China Pharmaceutical University, Nanjing, China. Mouse primary hepatocytes from *Fgf21* KO or C57BL/6 wild-type mice were isolated by nonrecirculating collagenase perfusion through the portal vein as previously described [26]. The hepatocytes were plated on dishes coated with rat collagen type I and were then cultured in RPMI 1640 (Invitrogen) containing 10% FBS. The cells were incubated for 24 h with exendin-4 or liraglutide (100 nM) in the presence or absence of FGF21 neutralizing antibody (5 μg/mL). The culture supernatants were collected for ELISA, and cells were lysed for protein analysis.

Human primary hepatocytes were purchased from ScienCell Research Laboratories (Cat: 5200, Carlsbad, CA) and were cultured following the manufacturer's instructions in Hepatocyte Medium (ScienCell). The cells were incubated with or without liraglutide (100 nM). The culture supernatants were collected for ELISA, and cells were lysed for protein analysis.

2.3. Proteome profiler array for mouse cytokines

The plasma samples from *db*/*db* mice and supernatants from cultured primary mouse hepatocytes in the exenatide (exendin-4) or PBS treatment groups were analyzed with a Mouse XL Cytokine Array Kit (Cat: ARY028, R&D Systems, Minneapolis, MN) according to the manufacturer's instructions.

2.4. Quantitative real-time RT-PCR

Total RNA was extracted with TRIzol (Thermo Fisher Scientific) and reverse-transcribed into cDNA using a First Strand cDNA Synthesis kit (Cat: K1622, Thermo Fisher Scientific) according to the manufacturer's instructions. Real-time PCR was performed in triplicate using the SYBR

Green PCR Master Mix (Applied Biosystems, Foster City, CA) with QuantStudio 5 Real-Time PCR System (Thermo Fisher Scientific). The sample input was normalized against the cycle threshold value of the housekeeping gene GAPDH. The primer sequences are summarized in Table S1.

2.5. Western blot analysis

Denatured proteins were separated by 12% (wt/vol) SDS-PAGE and transferred to a nitrocellulose membrane. The membranes were incubated overnight at 4 °C with the following primary antibodies (all at 1:1,000 dilution): rabbit anti-FGF21 (Cat: ab171941, Abcam, Cambridge, UK), rabbit anti-glucose 6 phosphatase (G6Pase; Cat: ab83690, Abcam), rabbit anti-phosphoenolpyruvate carboxykinase (PEPCK; Cat: 16754-1-AP, Proteintech Group, Rosemont, IL) and mouse anti-GAPDH (Cat: TA-08, Zhongshan Biotechnology, Beijing, China). After three washes, the blots were incubated with IRDye 800CW-conjugated goat anti-rabbit IgG or goat anti-mouse IgG (both at 1:10,000 dilutions; LI-COR Biosciences, Lincoln, NE) for 1 h. Protein bands were visualized with an Odyssey 290 infrared imaging system (LI-COR Biosciences). GAPDH was used as a loading control.

2.6. Immunohistochemical staining

Mouse liver tissues were collected for preparation of 5 μm thick paraffin sections. The sections were treated with 3% hydrogen peroxide for 10 min to block endogenous peroxidase activity and incubated in 10% (*v*/*v*) normal goat serum (Zhongshan Biotechnology, Beijing, China) to prevent the nonspecific binding of antibody. The sections were then incubated with rabbit anti-FGF21 antibody (1:250; Abcam) overnight at 4 °C and subjected to immunohistochemical analysis as reported previously [27].

2.7. Assay of FGF21 secretion

Mouse and human FGF21 levels were determined using ELISA kits (Cat: MF2100 and DF2100, R&D Systems) in mouse plasma and cell culture supernatant. The supernatant FGF21 level in each group was normalized to the total protein content.

2.8. Assays of G6Pase or PEPCK activity and glycogen content

The activity of G6Pase and PEPCK in mouse liver tissues was determined with the G6Pase and PEPCK assay kits (Cat: BC3325 and BC3315, Solarbio LIFE SCIENCES, Beijing, China) following the manufacturer's instructions. For estimation of hepatic glycogen content, the liver tissue samples were prepared and detected using a glycogen content assay kit (Cat: BC0345, Solarbio LIFE SCIENCES) according to the manufacturer's instructions.

2.9. Subjects

All participants were recruited from the Outpatient Clinic at seven tertiary hospitals in China. Subjects enrolled in our trial were 20–70 years old with body mass index (BMI) ranging from 22 to 40 kg/m^2, a diagnosis of T2D ≥ 1 year, and inadequate glycemic control (FBG ranging from 7.0 to 13.9 mM and HbA1c ranging from 7.0% to 10.0%) by metformin and an insulin secretagogue alone or in combination at a stable dosage for at least 3 months. The exclusion criteria were as follows: histories of diabetic ketoacidosis or diabetic hyperosmolar syndrome, severe hypoglycemia or frequent hypoglycemia (≥1 times/week) within 3 months, acute or chronic pancreatitis, pancreatic or stomach surgery, inflammatory bowel disease, any treatment for weight loss or weight change ≥5 kg for the last 3 months, and diseases that affected the accomplishment of the study or the judgment of the results. Subjects with the following conditions were also excluded: progressive proliferative diabetic retinopathy or macular edema, untreated or poorly controlled hypertension (systolic blood pressure ≥ 180 mmHg and/or diastolic blood pressure ≥100 mmHg), severe comorbidities (including the diseases of liver, kidney, cardiovascular, nervous and endocrine systems), pregnancy or lactation, and abnormalities in laboratory tests (*e.g.*, blood amylase and/or lipase >3 times the upper limit of normal ranges, hemoglobin <110 g/L, neutrophil granulocytes $<1.5 \times 10^9$/L, platelets $<100 \times 10^9$/L, and serum creatinine ≥133 μM for male or ≥124 μM for female).

2.10. Trial design

This multiple-center interventional trial was approved by the Ethics Committee of Peking University Third Hospital. The trial was registered at www.chictr.org.cn (registration No.: ChiCTR-IPR-15006558). All the eligible participants signed informed consent. Thereafter, all participants received exenatide twice daily for 16 weeks. Exenatide was given by subcutaneous injection initially at 5 μg twice daily for 4 weeks, followed by 10 μg twice a day for an additional 12 weeks (if not tolerated, the primary dosage was maintained).

This study was conducted in a subgroup of the enrolled participants. Forty-four patients whose T2D was inadequately controlled by metformin monotherapy received an exenatide add-on therapy. Additionally, 31 age-matched individuals with normal FBG and HbA1c levels were recruited as healthy controls. Fasting blood samples were collected for clinical biochemistry analysis and serum FGF21 detection. The levels of FBG, lipid profiles and renal function were measured by standard procedures. HbA1c was measured by high-performance liquid chromatography (Tosoh, Tokyo, Japan). Serum FGF21 level was determined by ELISA.

2.11. Statistical analysis

At least 3 independent experiments were performed in the animal and *in vitro* studies. Data are expressed as means ± S.D. Statistical differences were assessed by Student's *t*-test or one-way ANOVA (followed by the *post hoc* Tukey-Kramer test), as appropriate. Statistical analyses were performed by Prism 7.0 (GraphPad Software, La Jolla, CA). For the clinical study, data are presented as means ± S.D., number or median (interquartile range), and Student's *t*-test, χ^2 test or Mann-Whitney *U* test was used for statistical analysis, as appropriate. Statistical analyses were carried out using SPSS 16.0J for Windows (SPSS Japan, Tokyo, Japan). A value of $P < 0.05$ (two-tailed) was considered statistically significant.

3. Results

3.1. Hepatic FGF21 production is upregulated by exenatide (exendin-4) in diabetic db/db mice and cultured hepatocytes

The effects of the GLP-1 analog exenatide (a synthetic form of exendin-4) on metabolic parameters were first examined in *db*/*db* mice. After 2 weeks of exenatide treatment, body weight was significantly decreased when compared with PBS-treated controls (Fig. S1a). Notably, exenatide administration resulted in a marked decrease in FBG at week 1 and 2 after the treatment (Fig. 1a). We hence aimed to explore the possible mechanisms underlying the reduction. GLP-1 and its analogs have been suggested to improve functions of various organs and cell lineages including adipose tissues [28], endothelial cells [29] and pancreatic β cells [30] by counteracting the deleterious effects of pro-inflammatory cytokines. Utilizing a cytokine array kit to assess plasmic cytokine profiles, we identified that 7 cytokines were upregulated >2.5-fold in *db*/*db* mice received 2-week exenatide treatment. Among them, FGF21 was the cytokine with the highest fold increase (Fig. 1b and c; Table S2). Therefore, we chose FGF21 for our further studies.

In our animal studies, plasma FGF21 level was significantly increased in *db*/*db* mice compared with *db*/*m* mice and was further augmented by exenatide treatment (Fig. 1d). The levels of FGF21 mRNA and protein in liver tissues were higher in *db*/*db* mice than those in *db*/*m* mice and were further upregulated by exenatide treatment (Fig. 1e and f). Meanwhile, hepatic FGF21 levels were correlated with plasma FGF21 concentrations ($r = 0.884$, $P < 0.0001$) (Fig. 1g), suggesting that circulating FGF21 might be mainly released from liver. In addition, the mean integrated optical density of FGF21 staining detected by immunohistochemistry was consistent with the results determined by RT-PCR and western blot (Fig. 1h and i).

The changes of cytokines in plasma may be due to the direct effects of the GLP-1 analog on the specific tissues, or be caused by its indirect effects secondary to the lowering of blood glucose and/or improvement of other metabolic parameters. Besides, the cytokines in the plasma are not only derived from hepatocytes, but also from many other cell types, including adipocytes, myocytes and immunocytes [31]. In order to clarify whether there was a direct effect of GLP-1 on the liver, we conducted the *in vitro* experiments with mouse primary hepatocytes. As shown in Fig. 2a–c and Table S3, the levels of 7 cytokines were elevated >2.5-fold after a 24-h incubation with exendin-4, among which FGF21 ranked the second. In line with this observation, exendin-4 also upregulated the levels of FGF21 mRNA and protein in HepG2 cells, and increased the levels of FGF21 in their culture supernatants in a dose-dependent manner (Fig. 2d–f). Since 100 nM exendin-4 had an optimal effect in stimulating the expression and secretion of FGF21, this concentration was used for subsequent experiments. Similarly, the upregulating effects of exendin-4 on FGF21 expression and secretion were also observed in cultured mouse primary hepatocytes (Fig. 2g and h).

3.2. Hepatic FGF21 production is upregulated by liraglutide in diabetic *Pax6*$^{m/+}$ mice and cultured hepatocytes

The *Pax6*$^{m/+}$ mice established in our lab exhibits a decreased level of prohormone convertase 1/3 and a defective proinsulin processing in pancreatic β cells [23]. Such *Pax6*$^{m/+}$ genetic background combined with a high-fat diet consumption can promote the early onset of diabetes [24,32]. To verify whether the effects of exenatide on FGF21 production in *db*/*db* mice were reproducible, we performed similar intervention studies in *Pax6*$^{m/+}$ mice. After a 2-week treatment with liraglutide (another GLP-1 analog), body weight (Fig. S1b), FBG (Fig. 3a) and postload blood glucose during an IPGTT (Fig. S1c,d) were significantly reduced in *Pax6*$^{m/+}$ mice. However, liraglutide treatment did not affect insulin sensitivity, as assessed by an ITT (Fig. S1e).

When compared with C57BL/6 wild-type mice, *Pax6*$^{m/+}$ mice showed increased plasma FGF21 level and elevated hepatic FGF21 production at both mRNA and protein levels. Liraglutide treatment, however, further increased the elevation in *Pax6*$^{m/+}$ mice (Fig. 3b–d). The upregulation of FGF21 expression detected by RT-PCR and western blot was confirmed by immunohistochemistry analysis (Fig. 3e and f). Likewise, liraglutide dose-dependently upregulated FGF21 expression in HepG2 cells and increased FGF21 level in their culture supernatants (Fig. 3g–i). Since 100 nM liraglutide displayed an optimal effect on the expression and secretion of FGF21, this concentration was used for

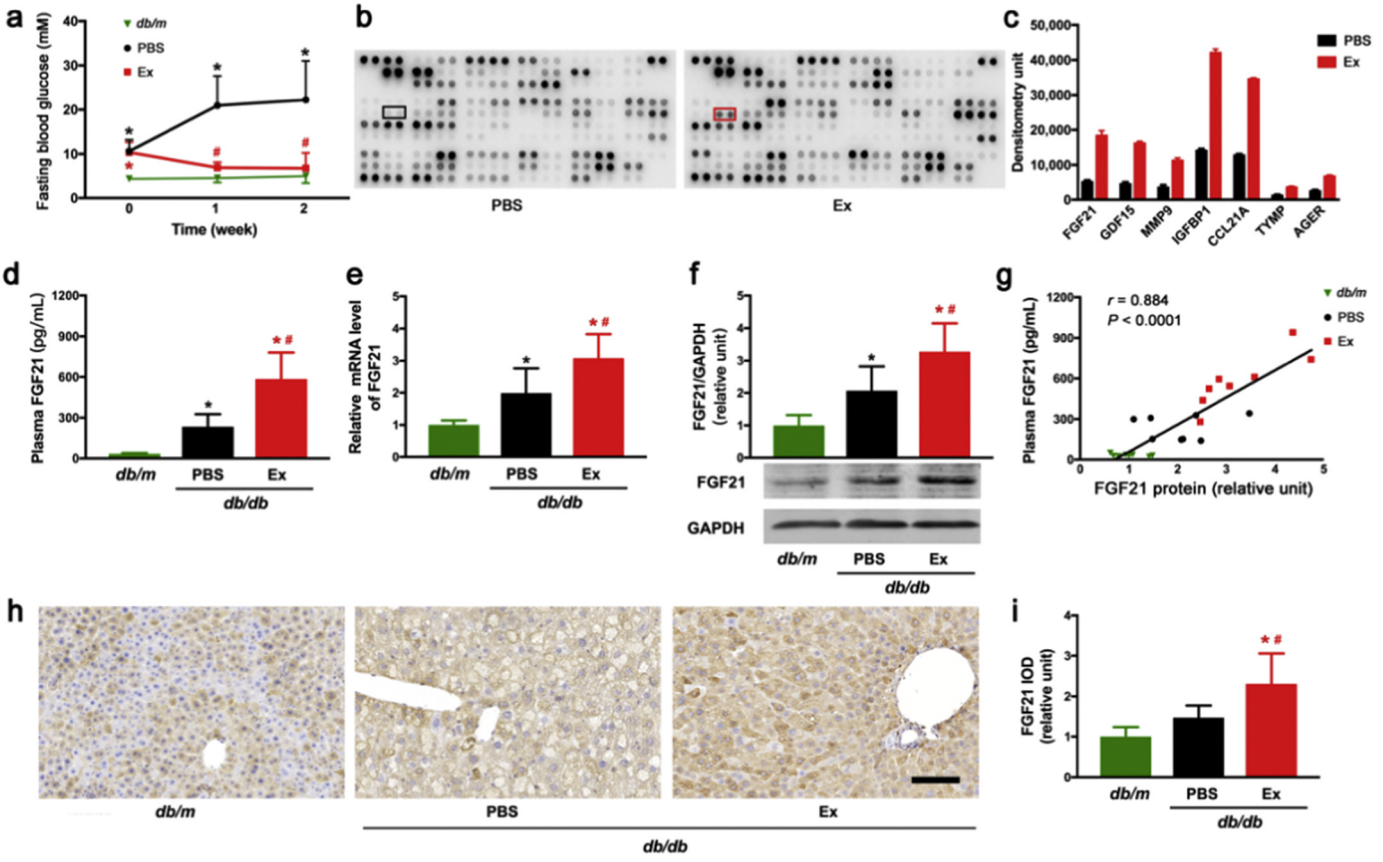

Fig. 1. FGF21 production is upregulated by diabetes and/or exenatide treatment in the liver of diabetic *db*/*db* mice. (a) Eight-week-old male diabetic *db*/*db* mice were treated for 2 weeks with the GLP-1 analog exenatide (Ex, 100 nmol/kg) or PBS (as vehicle control) *via* subcutaneous injection twice daily. Age-matched male heterozygous *db*/*m* mice treated with PBS were used as a normal control. Fasting blood glucose was monitored weekly (n = 8). (b) Cytokine profile changes in the plasma of *db*/*db* mice (n = 2). The boxes indicate the blots of FGF21. (c) Quantification of the mean densitometry unit of the specified cytokines with >2.5-fold upregulation (n = 2). (d-f) Fasting plasma FGF21 (d), and liver FGF21 mRNA (e) and protein (f) levels in the mice treated with Ex or PBS (n = 8). (g) Correlation of plasma FGF21 concentrations with hepatic FGF21 protein levels. (h) Representative images of hepatic FGF21 immunohistochemistry. Scale bar, 100 μm. (i) Quantitation of mean integrated optical density (IOD) of FGF21 immunohistochemical staining (n = 8). Data are shown as means ± S.D. One-way ANOVA, followed by the *post hoc* Tukey-Kramer test, was used for statistical analysis. $^{*}P < 0.05$ (*vs. db*/*m*); $^{\#}P < 0.05$ (*vs. db*/*db* PBS).

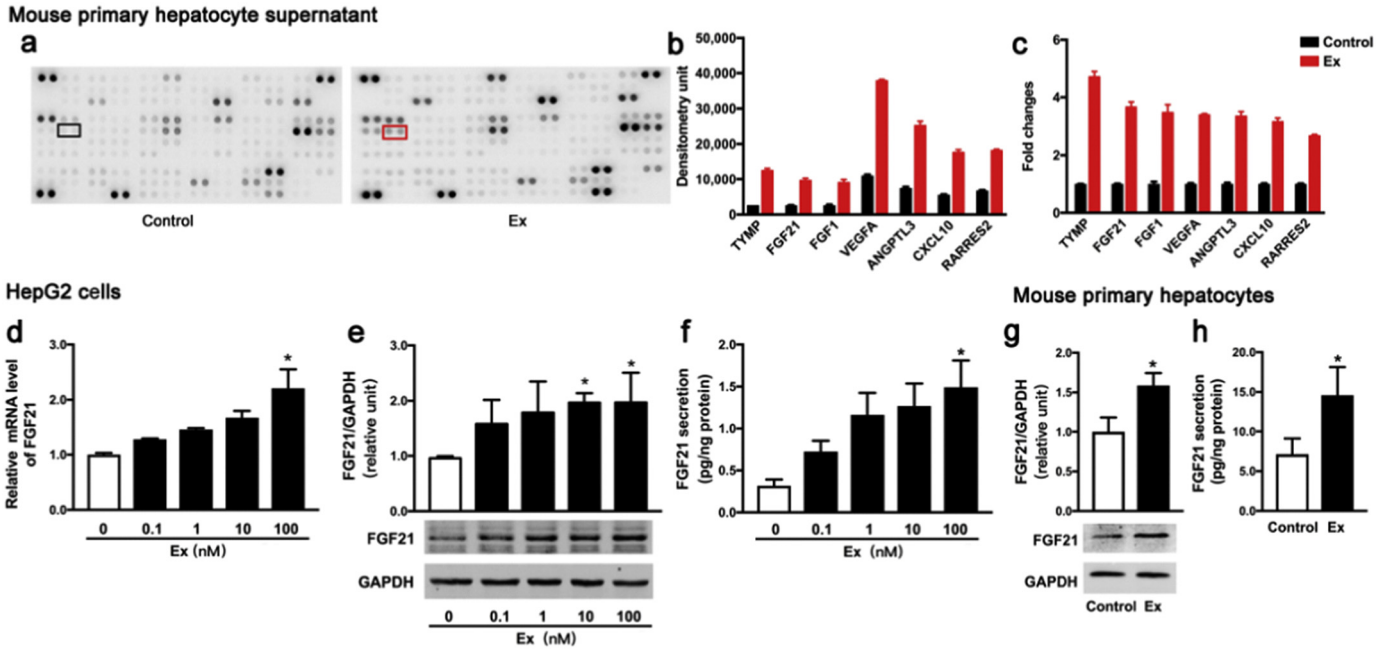

Fig. 2. FGF21 production is upregulated by exendin-4 in cultured hepatocytes. (a) Mouse primary hepatocytes were incubated with exendin-4 (Ex, 100 nM) or PBS for 24 h. Cytokine profile changes in the supernatant of mouse primary hepatocytes (n = 2). The boxes indicate the blots of FGF21. (b,c) Quantification of the mean densitometry unit (b) and fold changes (c) of the specified cytokines with >2.5-fold upregulation (n = 2). (d-f) HepG2 cells were incubated with various concentrations of Ex for 24 h. The expression levels of FGF21 mRNA (d) and protein (e) in the cells, and the level of FGF21 in their culture supernatant (f) were detected (n = 3). (g,h) Mouse primary hepatocytes were incubated with Ex (100 nM) or PBS for 24 h. Intracellular (g) and supernatant (h) levels of FGF21 protein were determined (n = 6). Data are shown as means ± S.D. One-way ANOVA (followed by the *post hoc* Tukey-Kramer test) or unpaired two-tailed Student's *t*-test was used for statistical analysis. $^{*}P < 0.05$ (*vs.* control).

subsequent experiments. We found that liraglutide caused a marked increase in FGF21 expression and secretion in cultured mouse primary hepatocytes (Fig. 3j and k), which were similar to those observed with exendin-4.

3.3. FGF21 is a key mediator of inhibition of G6Pase and PEPCK levels and activity by GLP-1 analogs in hepatocytes

After 2 weeks of exenatide treatment, fasting and postload hyperglycemia during IPGTT were significantly improved in *db/db* mice compared with PBS treatment. When circulating FGF21 was neutralized by an FGF21 antibody, the glucose-lowering effect of exenatide was diminished (Fig. 4a and b). The early-phase (30 min) insulin secretion was slightly restored after exenatide treatment (Fig. 4c). Exenatide treatment did not attenuate insulin resistance that was assessed by ITT in *db/db* mice, despite the presence of the FGF21 antibody (Fig. 4d). In response to pyruvate injection, the increase in blood glucose in *db/db* mice was attenuated by exenatide treatment. Notably, the FGF21 antibody exhibited a tendency to diminish the effect of exenatide ($P = 0.068$) (Fig. 4e and f). In *db/db* mice, 2-week exenatide treatment downregulated the hepatic mRNA and protein levels of G6Pase and PEPCK, two key gluconeogenic enzymes, as well as the activity of these two enzymes. The downregulation, however, could be partially attenuated by the FGF21 antibody (Fig. 4g–l). Similarly, liraglutide treatment also downregulated the levels and activity of G6Pase and PEPCK protein in the liver tissues of *Pax6*$^{m/+}$ mice (Fig. 4m–p). Besides, hepatic glycogen content was significantly higher in the GLP-1 analog-treated group than those in the PBS-treated group, either in *db/db* mice or in *Pax6*$^{m/+}$ mice (Fig. S2).

To determine whether GLP-1 analogs could directly regulate FGF21-mediated hepatic glucose output, we further performed a series of *in vitro* experiments. After a 24-h incubation, both exendin-4 and liraglutide downregulated the protein levels of G6Pase and PEPCK in mouse primary hepatocytes. These effects were diminished by a FGF21 neutralizing antibody (Fig. 5a–d). We also conducted siRNA-mediated *FGF21* knockdown. Among three *FGF21* siRNAs, siRNA#1 demonstrated a significant decrease in FGF21 at both mRNA and protein levels in HepG2 cells (Fig. S3). With the utilization of siRNA#1, we identified that *FGF21* knockdown attenuated the exendin-4- or liraglutide-mediated downregulation of G6Pase and PEPCK at both mRNA (Fig. 5e–h, upper panels) and protein (Fig. 5e–h, lower panels) levels. Likewise, in primary hepatocytes isolated from the *Fgf21* KO mice, the exendin-4- or liraglutide-mediated downregulation of G6Pase and PEPCK at both mRNA (Fig. 5i–l, upper panels) and protein (Fig. 5i–l, lower panels) levels were partially diminished. These results suggested that FGF21 was directly involved in GLP-1 analog-mediated inhibition of hepatic glucose output.

3.4. Liraglutide stimulates FGF21 production and downregulates G6Pase and PEPCK levels in human primary hepatocytes

We also performed a series of experiments in isolated human primary hepatocytes. We observed that liraglutide had a tendency to upregulate FGF21 protein level in the cells and significantly increased the level of FGF21 in their culture supernatant (Fig. 6a and b). Meanwhile, liraglutide caused a marked decrease in G6Pase protein level and appeared to downregulate PEPCK protein level in human primary hepatocytes (Fig. 6c and d). These data were in agreement with our findings in mouse primary hepatocytes and HepG2 cells.

3.5. Upregulation of FGF21 production by exenatide is recapitulated in patients with T2D

A total of 44 patients whose T2D was inadequately controlled by metformin monotherapy and 31 age-matched healthy control subjects were recruited for this study. The clinical characteristics and metabolic parameters of these two groups are summarized in Table S4. T2D subjects exhibited higher BMI, FBG, HbA1c, serum total cholesterol and

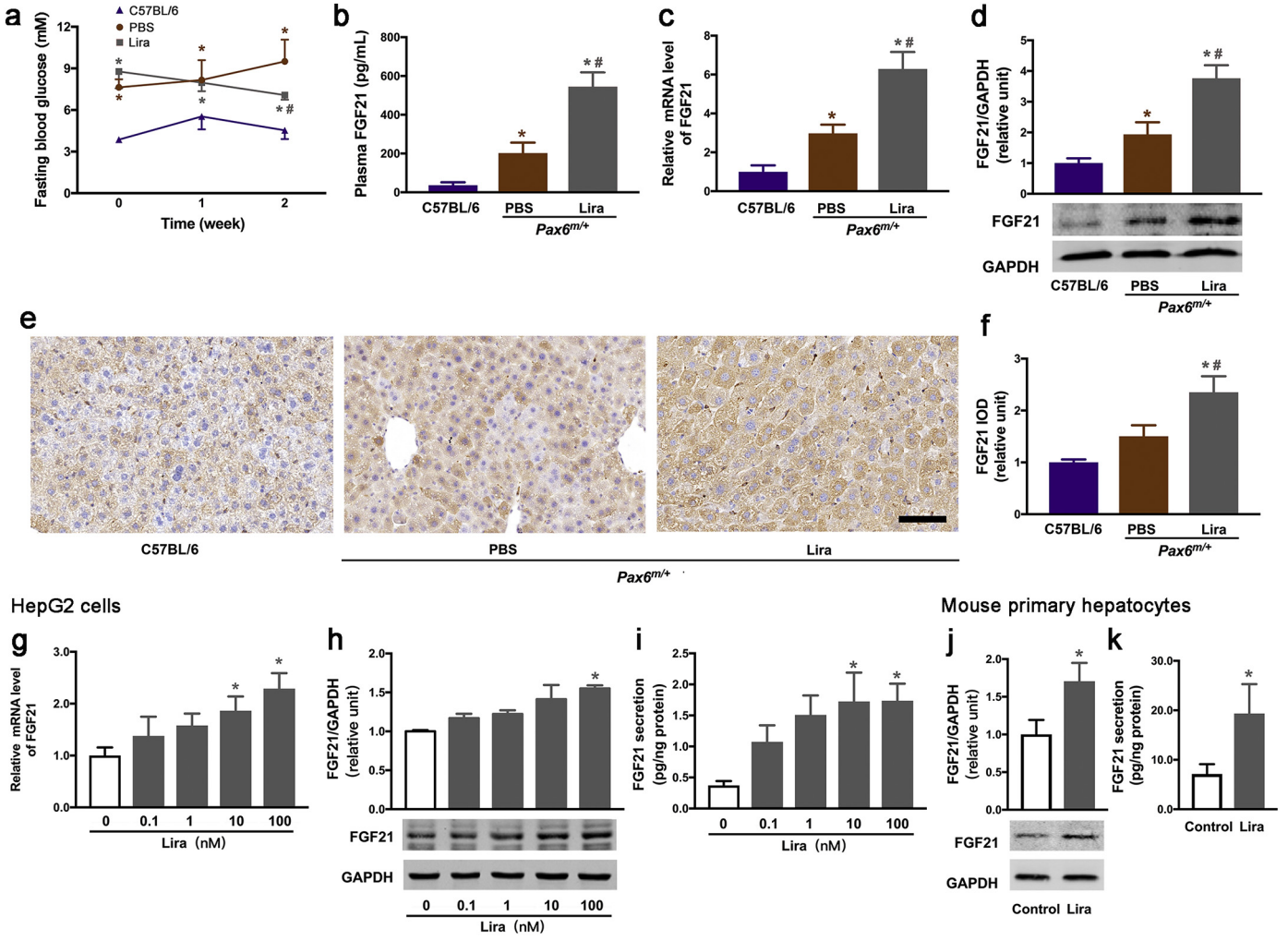

Fig. 3. FGF21 production is upregulated by diabetes and/or liraglutide treatment in the liver of diabetic *Pax6*$^{m/+}$ mice and cultured hepatocytes. (a) Three-week-old male *Pax6* heterozygous mutant (*Pax6*$^{m/+}$) mice were fed on a high-fat diet for 10 weeks to generate an early-stage diabetic model. The diabetic *Pax6*$^{m/+}$ mice were treated for 2 weeks with a GLP-1 analog liraglutide (Lira, 0.2 mg/kg) or PBS *via* subcutaneous injection twice daily. Age-matched male C57BL/6 wild-type mice treated with PBS were used as a normal control. Fasting blood glucose was monitored weekly (n = 3). (b-d) Fasting plasma FGF21 (b), and liver FGF21 mRNA (c) and protein (d) levels in *Pax6*$^{m/+}$ mice treated with Lira or PBS (n = 3). (e) Representative images of hepatic FGF21 immunohistochemistry. Scale bar, 100 μm. (f) Quantitation of mean integrated optical density (IOD) of FGF21 immunohistochemical staining (n = 3). (g-i) HepG2 cells were cultured with various concentrations of Lira for 24 h. The expression levels of FGF21 mRNA (g) and protein (h) in the cells, and the level or FGF21 in their culture supernatant (i) were detected (n = 4). (j,k) Mouse primary hepatocytes were incubated with Lira (100 nM) or PBS for 24 h. Intracellular (j) and supernatant (k) levels of FGF21 protein were determined (n = 6). Data are shown as means ± S.D. Statistical analysis was carried out using one-way ANOVA (followed by the *post hoc* Tukey-Kramer test) or unpaired two-tailed Student's *t*-test, as appropriate. Data in a-f, $^{*}P < 0.05$ (*vs.* C57BL/6); $^{\#}P < 0.05$ (*vs. Pax6*$^{m/+}$ PBS). Data in g-k, $^{*}P < 0.05$ (*vs.* control).

triglyceride levels than control subjects. As shown in Fig. 6e and Table S4, serum FGF21 level in the T2D group was higher than that in the control group [132.6 (102.2, 205.0) pg/mL *vs.* 108.0 (74.8, 147.2) pg/mL, $P = 0.043$].

The 44 patients with T2D were assigned to an add-on therapy study to evaluate the efficacy and safety of exenatide. Clinical characteristics obtained before and after 16 weeks of treatment with exenatide are presented in Table 1. Compared with the baseline, exenatide treatment significantly reduced BMI, FPG, postprandial blood glucose at 2 h, HbA1c and serum total cholesterol levels ($P < 0.001$). As shown in Table 1 and Fig. 6f, serum FGF21 level was significantly increased after the exenatide treatment compared with the baseline [163.1 (116.5, 280.8) pg/mL *vs.* 132.6 (102.2, 205.0) pg/mL, $P = 0.001$]. In the subgroup analysis stratified by median HbA1c reduction from the baseline, serum FGF21 level after the treatment showed a more significant increase in the patients with HbA1c reduction ≥1.4% than those with an HbA1c reduction <1.4%, which meant that the increase in serum FGF21 level was associated with an improvement in blood glucose control (Fig. 6g and h).

4. Discussion

In this study, we found that GLP-1 analogs downregulated the hepatic levels and activity of G6Pase and PEPCK in diabetic *db/db* and *Pax6*$^{m/+}$ mice, and in mouse and human primary hepatocytes, as well as HepG2 cells. Importantly, we identified that GLP-1 analogs upregulated FGF21 levels in the plasma and liver of diabetic *db/db* and *Pax6*$^{m/+}$ mice, in mouse and human primary hepatocytes, and in HepG2 cells. Blockage of FGF21 by a specific antibody or siRNA diminished the inhibitory effects of GLP-1 analogs on the two key enzymes of hepatic gluconeogenesis in *db/db* mice or cultured hepatocytes. Similar results were also observed in primary hepatocytes isolated from the *Fgf21* KO mice. These results suggested that GLP-1 analogs could directly stimulate liver FGF21 production, which contributed to the inhibitory effects of GLP-1 analogs on hepatic glucose output.

GLP-1 exerts its glucose-lowering effect through multiple actions, such as enhancing glucose-stimulated insulin secretion, suppressing glucagon secretion, slowing gastric emptying and reducing food intake

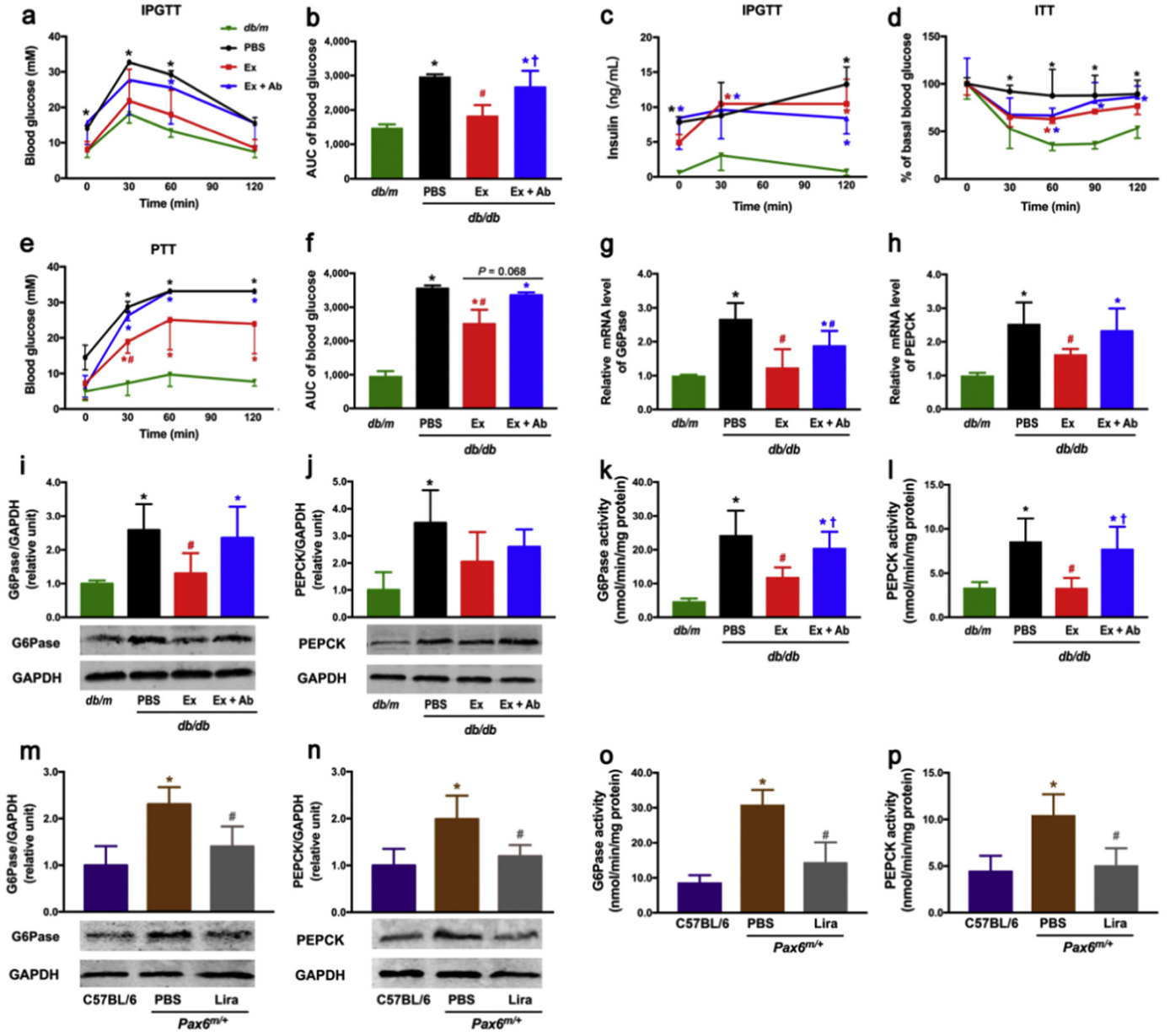

Fig. 4. FGF21 is a key mediator of inhibition of glucose output by GLP-1 analogs in the liver of diabetic mice. (a-f) Eight-week-old male *db/db* mice were treated with exenatide (Ex) or PBS for 2 weeks, and half of the Ex-treated mice were given a single intraperitoneal injection of 8 μg FGF21 neutralizing antibody (Ab) before being subjected to the intraperitoneal glucose tolerance test (IPGTT), insulin tolerance test (ITT) and pyruvate tolerance test (PTT). Blood glucose levels (a) and their areas under curve (AUC) (b), and plasma insulin levels (c) during the IPGTT are shown. Blood glucose levels during ITT (d) and PTT (e), and the AUC of blood glucose during PTT (f) are shown. Age-matched male *db/m* mice treated with PBS were included as a normal control. n = 8 per group. (g-l) The mRNA (g,h), protein (i,j) levels and activity (k,l) of glucose 6 phosphatase (G6Pase) and phosphoenolpyruvate carboxykinase (PEPCK) in the liver tissues of *db/db* mice were measured (n = 8). (m-p) Thirteen-week-old male diabetic *Pax6*$^{m/+}$ mice were treated for 2 weeks with liraglutide (Lira, 0.2 mg/kg) or PBS *via* subcutaneous injection twice daily. Age-matched male C57BL/6 mice treated with PBS were used as a normal control. The protein levels (m,n) and activity (o,p) of G6Pase and PEPCK in the liver tissues were measured (n = 3). Data are shown as means ± S.D. One-way ANOVA, followed by the *post hoc* Tukey-Kramer test, was used for statistical analysis. Data in a-l, $^{*}P < 0.05$ (*vs. db/m*); $^{\#}P < 0.05$ (*vs. db/db* PBS); $^{\dagger}P < 0.05$ (*vs. db/db* Ex). Data in m-p, $^{*}P < 0.05$ (*vs.* C57BL/6); $^{\#}P < 0.05$ (*vs. Pax6*$^{m/+}$ PBS).

[33]. In our animal studies, GLP-1 analog treatment lowered body weight and blood glucose. These findings are consistent with previous reports on T2D patient studies [34]. Notably, we also noted that exenatide significantly decreased FBG after only 1 week of treatment in *db/db* mice. Numerous hormones, substrates and intracellular regulatory factors contribute to the regulation of hepatic fuel homeostasis [35,36]. However, there may be some undiscovered regulators. We screened cytokines both *in vivo* and *in vitro*, attempting to identify novel regulators of hepatic glucose metabolism. Since FGF21, a hepatokine secreted mainly from the liver [37], was the cytokine with the highest fold increase in the plasma of *db/db* mice after exenatide treatment in our first cytokine array analysis, we used the second cytokine array in primary mouse hepatocytes to clarify whether the GLP-1 analog has a direct effect on liver FGF21 production. Again, the array analysis revealed that FGF21 was one of the cytokines with the highest fold increase induced by exendin-4 (a native form of exenatide). We further showed that FGF21 production in the livers of diabetic mice, primary mouse and human hepatocytes, and HepG2 cells was upregulated by both exenatide and liraglutide. Similarly, several previous studies demonstrated that GLP-1 analogs could upregulate FGF21 expression and secretion in animal models of obesity, hepatic steatosis and insulin resistance [38–40]. These observations confirmed that GLP-1 analogs could stimulate hepatic FGF21 production.

FGF21 is considered as a hormone that plays a critical role in metabolic regulation [41]. It is predominantly produced in liver [37], although it is also expressed in adipose tissues [42], pancreatic β cells

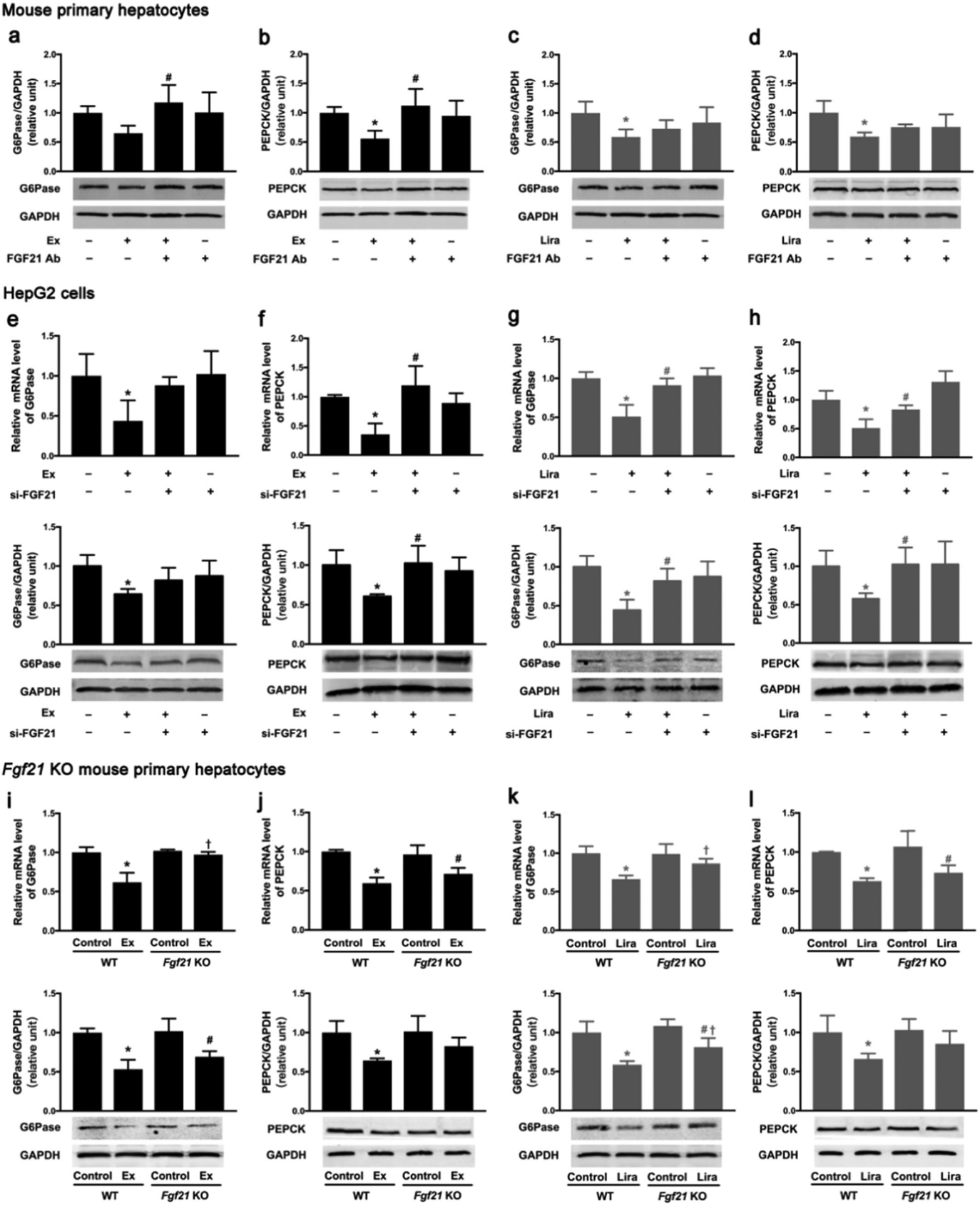

Fig. 5. FGF21 is a key mediator of inhibition of gluconeogenesis by GLP-1 analogs in cultured hepatocytes. (a-d) Effect of FGF21 neutralizing antibody (Ab, 5 μg/mL) on glucose 6 phosphatase (G6Pase) and phosphoenolpyruvate carboxykinase (PEPCK) protein levels in mouse primary hepatocytes incubated with exendin-4 (Ex) (a,b) or liraglutide (Lira) (c,d). (e-h) HepG2 cells were transfected with *FGF21* siRNA (si-FGF21) for 48 h and then cultured with Ex (e,f) or Lira (g,h) for an additional 24 h. The mRNA (upper panel) and protein (lower panel) levels of G6Pase and PEPCK were detected. (i-l) Primary hepatocytes isolated from *Fgf21* knockout (KO) and wild-type (WT) mice were cultured with Ex (i,j) or Lira (k,l). The mRNA (upper panel) and protein (lower panel) levels of G6Pase and PEPCK were measured. Data are shown as means ± S.D. n = 4. One-way ANOVA, followed by the *post hoc* Tukey-Kramer test, was used for statistical analysis. Data in a-h, $^{*}P < 0.05$ (*vs.* control); $^{\#}P < 0.05$ (*vs.* Ex or Lira). Data in i-l, $^{*}P < 0.05$ (*vs.* WT control); $^{\#}P < 0.05$ (*vs. Fgf21* KO control); $^{\dagger}P < 0.05$ (*vs.* WT Ex or Lira).

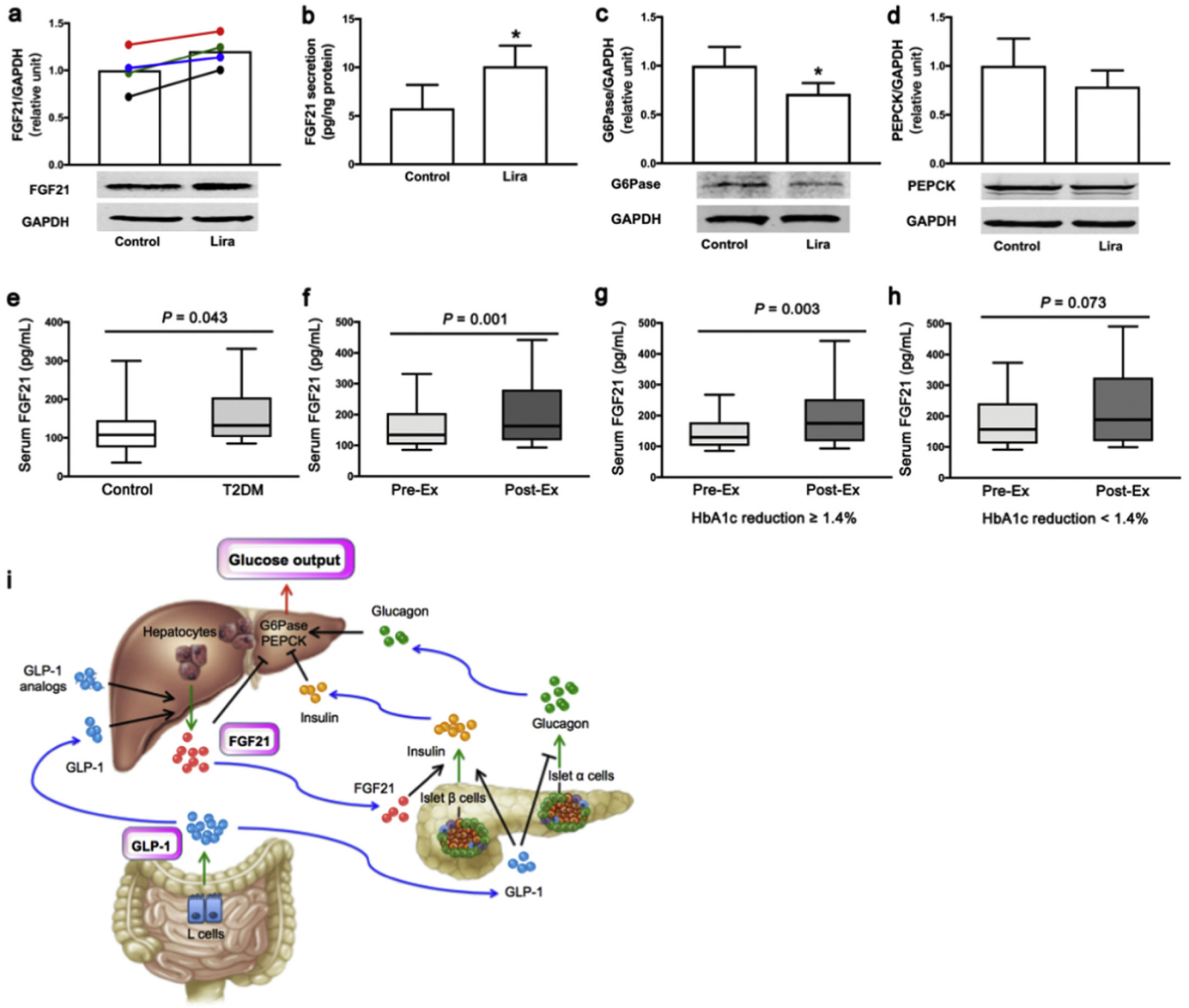

Fig. 6. Upregulation of FGF21 production by GLP-1 analogs is recapitulated in isolated human hepatocytes and in patients with type 2 diabetes (T2D). (a-d) Human primary hepatocytes were cultured with or without liraglutide (Lira, 100 nM) for 24 h. Intracellular (a) and supernatant (b) levels of FGF21 protein were examined. The protein levels of glucose 6 phosphatase (G6Pase) (c) and phosphoenolpyruvate carboxykinase (PEPCK) (d) were detected. Lines with each color represent different batches (a). Data in b-d are shown as means ± S.D. Statistical analysis was carried out using unpaired two-tailed Student's *t*-test. $^{*}P < 0.05$ (*vs.* control). n = 4. (e) Serum FGF21 levels were assayed by ELISA in healthy control subjects (n = 31) and patients with T2D (n = 44). (f) Among patients with T2D receiving the 16-week exenatide (Ex) treatment, serum FGF21 levels were analyzed before (Pre-Ex) and after the Ex treatment (Post-Ex) (n = 44). (g,h) Differences in serum FGF21 levels between Pre-Ex and Post-Ex were further analyzed in subgroups of patients with glycated hemoglobin A1c (HbA1c) reduction ≥1.4% (g) or < 1.4% (h). Data in e-h are expressed as medians (interquartile range). Mann-Whitney *U* test was used for statistical analysis. (i) A proposed scheme for the role of FGF21 in the inhibition of hepatic glucose output by GLP-1 and its analogs. GLP-1, an incretin hormone predominantly released from intestinal L-cells, potentiates glucose-stimulated insulin secretion from pancreatic β cells and suppresses glucagon secretion from α cells. Insulin can act on G6Pase and PEPCK to inhibit hepatic glucose output, while glucagon exerts exactly the opposite effect. Interestingly, we uncover that GLP-1 directly stimulates hepatic FGF21 production to participate in the inhibitory effects of GLP-1 on hepatic glucose output, which represents a new glucose-lowering mechanism of GLP-1. In addition, FGF21 also enhances insulin secretion from β cells. Therefore, hormones secreted from intestine (GLP-1), pancreatic islets (insulin and glucagon) and liver (FGF21) can coordinate to regulate hepatic glucose output. The coordination of the intestine-islet-liver axis plays an important role in maintaining glucose homeostasis.

[43] and skeletal muscles [44]. Increasing evidence indicates that FGF21 has beneficial metabolic effects on glucose and fat metabolism when administered to obese mice [45], diabetic mice [46] or nonhuman diabetic primates [47]. Treatment with exogenous FGF21 could reduce body weight and improve glycemic control [45–47]. In this study, blockage of circulating FGF21 activity by a specific FGF21 antibody diminished the hypoglycemic effects of exenatide during IPGTT and PTT in *db*/*db* mice. Furthermore, FGF21 neutralizing antibody partially attenuated the inhibitory effects of exenatide or liraglutide on G6Pase and PEPCK expression in the livers of *db*/*db* mice and cultured mouse primary hepatocytes. Similar observations were made in HepG2 cells by application of *FGF21* siRNA and in primary hepatocytes isolated from the *Fgf21* KO mice. These results suggested that FGF21 was a key mediator of inhibition of hepatic glucose output by GLP-1 analogs. In isolated human primary hepatocytes, our findings supported this conclusion. Notably, in our clinical study, exenatide treatment increased serum FGF21 level in patients with T2D, which was particularly the case in subgroups of patients with better glucose control. These results suggested that GLP-1 analogs might also improve glucose metabolism *via* upregulating FGF21 production in humans.

Our study showed that FGF21 level was significantly increased in the plasma and liver tissues of diabetic mice and in the serum of patients

Table 1
Clinical characteristics and serum FGF21 level before and after 16 weeks of treatment with exenatide.

Parameters	Pretreatment	Posttreatment	Statistical value	*P* value
Age, yr	47.5 ± 10.5			
Sex, male/female	29/15			
Weight, kg	79.0 ± 14.7	77.2 ± 14.1	2.390	0.021
BMI, kg/m^2	27.6 ± 3.6	27.0 ± 3.4	2.506	0.016
SBP, mmHg	123.1 ± 11.1	122.5 ± 12.1	0.360	0.721
DBP, mmHg	77.6 ± 8.5	75.4 ± 6.7	1.890	0.066
FBG, mM	9.40 ± 1.85	7.80 ± 2.03	5.437	<0.001
PBG 2 h, mM	16.0 ± 3.2	11.9 ± 3.9	6.644	<0.001
HbA1c, %	8.18 ± 0.86	6.85 ± 0.93	8.303	<0.001
TC, mM	5.03 ± 1.01	4.68 ± 0.90	2.424	0.020
LDL-C, mM	3.06 ± 0.82	2.83 ± 0.72	1.212	0.232
HDL-C, mM	1.22 (0.95, 1.48)	1.13 (0.99, 1.47)	−1.034	0.301
TG, mM	1.77 (1.26, 2.86)	1.55 (1.28, 2.38)	−1.389	0.165
UA, μM	309.8 ± 77.5	312.9 ± 65.7	−0.326	0.746
FGF21, pg/mL	132.6 (102.2, 205.0)	163.1 (116.5, 280.8)	−3.233	0.001

Data are presented as means ± S.D., number or median (interquartile range), as appropriate. n = 44. Differences were analyzed by paired Student's *t*-test, χ^2 test or Mann-Whitney *U* test, as appropriate.
Abbreviations: FGF21, fibroblast growth factor 21; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; FBG, fasting blood glucose; PBG 2 h, postprandial blood glucose at 2 h; HbA1c, glycated hemoglobin A1c; TC, total cholesterol; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; TG, triglyceride; UA, uric acid.

with T2D, suggesting the presence of FGF21 compensation for a condition of so-called FGF21 resistance [48,49]. These findings are in line with the data from previous prospective cohort studies [50,51]. However, the effects of GLP-1 analogs on circulating FGF21 levels were rarely reported in patients with T2D. A recent observational study showed that exenatide treatment decreased plasma FGF21 levels compared with the baseline in patients with newly-diagnosed T2D [52]. The reasons for the inconsistency with our findings might be due to the difference in duration of diabetes and exenatide treatment as well as other concomitant medications.

Our previous studies demonstrated that GLP-1 analogs could exert their beneficial effects on endothelial cells [27,29] and pancreatic β cells [24] *via* activation of GLP-1 receptor (GLP-1R). Although several studies reported that functional GLP-1R was presented in isolated human and rat hepatocytes, HepG2 cells, and human liver tissues [53,54], the current mainstream opinion states that the GLP-1R is not expressed in hepatocytes. Researchers failed to detect GLP-1R mRNA transcripts by PCR analysis using primers that span nearly the entire coding region in isolated mouse hepatocytes [55] and by southern blot analysis in rat liver tissues [56]. Notably, GLP-1 in the liver has a direct inhibitory effect on hepatic lipogenesis, thereby reversing hepatic steatosis [57,58]. Moreover, GLP-1 (9–36) amide metabolite, which functions without binding to GLP-1R, could suppress glucose production in isolated mouse hepatocytes [59]. These observations suggest that GLP-1 may have a direct role in hepatocytes *via* a GLP-1R-independent mechanism, just like in the myocardial and endothelial cells [60]. Our studies showed that GLP-1 analogs could stimulate FGF21 production and downregulate the expression and activity of the two key enzymes of hepatic gluconeogenesis in mouse and human primary hepatocytes as well as in HepG2 cells, suggesting a GLP-1R-independent hepatic function of GLP-1.

It is known that organs such as intestine, pancreas and liver have a coordinated effect in regulating blood glucose homeostasis [61]. GLP-1, an incretin hormone secreted by intestinal L-cells, may exerts its glucose-lowering effects *via* multiple mechanisms, including stimulation of insulin release, increase of insulin-independent glucose disposal, enhancement of insulin sensitivity, activation of glucokinase [19] or direct suppression of hepatic glucose production [62]. As is known, GLP-1 potentiates glucose-stimulated insulin secretion in pancreatic β cells and suppresses glucagon secretion in α cells [33]. Insulin inhibits the activity of G6Pase and PEPCK to suppress hepatic glucose output, while glucagon exerts the opposite effect [63]. In this study, we identified that GLP-1 could stimulate hepatic FGF21 production, which further participated in the inhibitory effects of GLP-1 on G6Pase and PEPCK activity and led to decreased hepatic glucose output. These data highlight a previously unappreciated role of FGF21 in regulating GLP-1-mediated hypoglycemic actions. In addition, FGF21 has been reported to promote insulin secretion from β cells [43]. Collectively, hormones from intestine (GLP-1), pancreas (insulin and glucagon) and liver (FGF21) can coordinate to regulate hepatic glucose output (Fig. 6i).

There are some limitations in our study. First, *in vivo* deletion of FGF21, particularly in a liver-specific *Fgf21* KO mouse model, was of great importance for evaluating the role of FGF21 in the glucose-lowering effect of GLP-1 analogs. However, our study adopted two FGF21-blocking strategies, specific antibody- and siRNA-mediated blockage of FGF21, and was also testified in isolated primary hepatocytes from the *Fgf21* KO mice. The data from both *in vivo* and *in vitro* studies were consistent, indicating that FGF21 was indeed involved in the GLP-1 analog-induced inhibition of hepatic glucose output. Second, the sample size was relatively small in the clinical study. Nevertheless, even based on the data from any large-scale randomized clinical trial, we were unable to make the conclusion that exenatide has a direct effect on hepatic FGF21 production, owing to the complicated regulation *in vivo*. Therefore, we performed the *in vitro* experiment to verify this direct hepatic effect. Third, the molecular mechanisms of the GLP-1 analog-induced hepatic FGF21 production must be clarified in the future.

In summary, treatment with GLP-1 analogs significantly increased circulating FGF21 level and improved glucose control in diabetic mice and humans. Furthermore, GLP-1 analogs suppressed hepatic glucose output in diabetic mice, which was accounted for at least partially by the GLP-1 analog-induced FGF21 production in hepatocytes. Thus, our study provides a novel mechanism for the glucose-lowering effect of GLP-1-based agents.

Declaration of interests

The authors declare no conflicts of interest that pertain to this work

Author contributions

R.W. and T.H. designed research. J.L. K.Y., Y.L., F.Y., L.G., and S.L. performed experiments. J.L., K.Y., J.Y., Q.T., R.W. and T.H. analyzed data. J.L. and R.W. wrote the paper. W.X., T.J. and T.H. reviewed/edited the manuscript. T.H. is the guarantor of this work and had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ebiom.2019.02.037.

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