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

Junling Liu a,b,1, Kun Yang a,1, Jin Yang a,b, Wenhua Xiao a, Yunyi Le a, Fei Yu a,b, Liangbiao Gu a,b, Shan Lang a,b, Qing Tian a, Tianru Jin c,d, Rui Wei a,b,* , Tianpei Hong a,b,*

a Department of Endocrinology and Metabolism, Peking University Third Hospital, Beijing, China
b Clinical Stem Cell Research Center, Peking University Third Hospital, Beijing, China
c Department of Physiology, University of Toronto, Toronto, Ontario, Canada
d Banting and Best Diabetes Center, University of Toronto, Toronto, Ontario, Canada

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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−/−) 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 cells 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...
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 glycogenolysis 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 glucose output 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 and db/m 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+/−) mice were used as an early-stage diabetic model as previously described by our group [23,24]. The diabetic Pax6+/− 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 1x 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 tissue 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/m², 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 (≥ 2 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 co-morbidities (including the diseases of liver, kidney, cardiovascular, nervous and endocrine systems), pregnancy and 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 × 10⁹/L, platelets <100 × 10⁹/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, χ² 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].

![Fig. 1. FGF21 production is upregulated by diabetes and/or exenatide treatment in the liver of diabetic db/db mice.](image)

(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 levels. (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.

### 3.2. Hepatic FGF21 production is upregulated by liraglutide in diabetic Pax6m/+ mice and cultured hepatocytes

The Pax6m/+ 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 Pax6m/+ 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 Pax6m/+ 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 Pax6m/+ mice. However, liraglutide treatment did not affect insulin sensitivity, as assessed by an ITT (Fig. S1e).

When compared with C57BL/6 wild-type mice, Pax6m/+ 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 Pax6m/+ 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
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 exenatide-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 significantly higher in the GLP-1 analog-treated group than PBS treatment. When circulating FGF21 was neutralized by the FGF21 antibody (Fig. 4g–l). Similarly, liraglutide treatment also attenuated the exendin-4- or liraglutide-mediated downregulation of G6Pase and PEPCK protein level in the cells and significantly increased the level of 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. S5e–h, upper panels) and protein (Fig. S5e–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. S5i–l, upper panels) and protein (Fig. S5i–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
triglyceride levels than control subjects. As shown in Fig. 6 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 \( \geq 1.4\% \) 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 \( \text{db/db} \) and \( \text{Pax6}^+\text{m/+} \) 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 \( \text{db/db} \) mice, and 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 \( \text{db/db} \) mice or cultured hepatocytes. Similar results were also observed in primary hepatocytes isolated from the \( \text{Fgf21} \) 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.
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. 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 Pax6m/+ 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); †P < 0.05 (vs. db/db Ex). Data in m-p, *P < 0.05 (vs. C57BL/6); †P < 0.05 (vs. Pax6m/+ PBS).
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); †P < 0.05 (vs. WT Ex or Lira).
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, blockade 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...
GLP-1, an incretin hormone secreted by intestinal L-cells, may exert coordinated effects in regulating blood glucose homeostasis [61].

Independent hepatic function of GLP-1.

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-

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 tested 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., L.G., and S.L. performed experiments. J.L., K.Y., J.Y., 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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