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Oral administration of human FGF21 expressed by mycelium of *Cordyceps militaris* improves blood glucose and lipid in type II diabetes mellitus

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ABSTRACT

The purpose of this study was to express human FGF21 (hFGF21) using *Cordyceps militaris* (*C. militaris*) as a bioreactor and to observe its hypoglycemic and lipid-lowering effects on type II diabetes. The recombinant plasmid pCB130-hFGF21 was transformed into *C. militaris* to form recombinant recombinant *C. militaris* (RhFGF21), the stability of RhFGF21 *in vitro* and *in vivo* was analyzed. RhFGF21 significantly promoted glucose uptake in a dose-dependent manner in adipocytes and increased the levels of p-PLCγ, p-FRS2 and p-ERK, which was consistent with the commercial hFGF21. In animal experiments, oral RhFGF21 obviously reduced the levels of glucose, insulin, TG, T-CHO, NEFA, and LDL-C in the blood, the contents of ALT, AST, TNF-α, MCP-1, F4/80, CD68 and CD11b in the fatty liver, and the apoptosis of pancreatic cells. *C. militaris* is an excellent carrier that can stabilize the expression of hFGF21 and protect the biological activity of hFGF21 during oral administration, which provides a theoretical basis for the development of hFGF21 oral preparations for type II diabetes.

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Introduction

Diabetes is a metabolic disease characterized by hyperglycemia, which can cause chronic damage and dysfunction of various tissues, especially the eyes, kidneys, heart, blood vessels and nerves(1-3). Type II diabetes is caused by insulin resistance in the skeletal muscle, fat, and liver, and absolute or relative deficiency of insulin secretion due to islet β cell dysfunction, and accounts for 85% -95% of all diabetes cases(4). Its early symptoms are not obvious, only mild fatigue and thirst, so macrovascular and microvascular complications often occur before a clear diagnosis, which poses great risks to people's health.

Fibroblast growth factor 21 (FGF21) is a promising drug candidate for diabetes treatment that has positive effects on anti-hyperglycemia, anti-hyperlipidemia and increasing insulin sensitivity(5, 6). FGF21 is mainly produced by the liver and acts on adipose tissue, the liver and the pancreas(7). It has been reported that FGF21 can inhibit hepatic glycogen output by activating the signaling pathways of pancreatic exocrine cells and hepatocytes(8). Koh confirmed that FGF21 was beneficial to lipid metabolism in animal models by reducing the serum levels of total lipid, total cholesterol, and triglyceride phospholipid(9). At present, FGF21 is mainly administered by injection, and patients are required to be injected once a day because of its relatively short half-life. Repeated injections of FGF21 severely increase the suffering of patients, and bring a heavy burden on patients, their families and even society. In order to effectively avoid the fear of needle injection and reduce the pain caused by injection, it is an urgent problem to use bioreactor system to efficiently express FGF21 and develop oral administration dosage.

Fungal bioreactor systems have received widespread attention for their powerful protein secretion capabilities, low production costs, high yields, and biological safety. *Cordyceps militaris* (*C. militaris*) is an important edible and medicinal fungus in China, which has a variety of pharmacological and biological activities, such as anti-tumor, anti-oxidation, blood lipid-lowering and blood sugar lowering(10, 11). Because *C. militaris* itself has the advantage of being edible orally, it is considered as a good receptor material for expressing foreign proteins. With the completion of C. militaris sequencing, the research on its molecular biology and genetics has become more in-depth(12). *C. militaris* has a short growth cycle, good safety and mature mycelial fermentation technology with a simple method and fast speed. Therefore, if combined with the excellent properties of FGF21 and *C. militaris*, an oral preparation of *C. militaris* with high FGF21 content can be developed, which has a very good application prospect.

In this study, *C. militaris* was chosen as a bioreactor to efficiently express FGF21, to investigate the hypoglycemic and lipid-lowering effects of recombinant *C. militaris* on type II diabetes by oral administration, thereby providing a theoretical basis for the development of a green, safe and effective new hypoglycemic drug dosage forms.

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Materials and Methods

Materials

C. militaris strain (CM-X06) and mouseembryonic fibroblasts (3T3-L1 cells, ATCCnumber: BNCC100294) were obtained from the Engineering Research Center of Ministry of Education for Edible and Medicinal Fungi, Jilin Agricultural University. Plasmid (pCB130-NG) was purchased by Ministry of Education Engineering Research Center of Bioreactor and Pharmaceutical Development, Jilin Agricultural University and recombinant plasmid (pCB130-hFGF21) was constructed by Beijing jinweizhi Biotechnology Co., Ltd.

BALB/c mice (male, 4 weeks, 18-22 g) were purchased from Changchun yisi laboratory animal technology Co., Ltd, and type II diabetes model db/db mice (male, 4 weeks) were purchased from Nanjing Junke Biotechnology Co., Ltd. The animals were domesticated for one week and housed in standard-sized cages for a 12-hour light-dark cycle in a temperature and humidity-controlled, non-pathogenic room with free access to food and water. The mice were fasted for 12 hours (with water) before the experiment. All animal experiments were approved by the Ethics Committee of Jilin Agricultural University.

Establishment and identification of recombinant *C. militaris*

The mass of wild-type *C. militaris* (CM-X06, 0.5 cm×0.5 cm) with good growth on PDA solid medium was inoculated into a shaker containing 150 mL PDB culture medium and shaking cultured at 130 rpm and 25°C in a dark environment for 4 d, resulting in the formation of many mycelium pellets. The mycelium pellets with a net weight of 200 mg was weighed into a 2 mL centrifuge tube and 1.2 mL mixed enzyme solution (10 mL mannitol buffer [0.8 M, PH=4.5] containing 0.15 g lywallzyme and 0.15 g snail enzyme) was added, which was incubated at 42°C for 4 h, then a syringe containing cotton was used to filter the bacteria solution. After centrifugation at 4000 rpm for 10 min, the precipitation was dissolved with 0.8M mannitol and counted with blood counting chamber, making the obtained protoplast of *C. militaris* diluted to 10^7 protoplasts/mL. Then 30 μg recombinant plasmid pCB130-hFGF21was added to the protoplast solution of *C. militaris*, which was bathed in ice for 5 min. After adding 50 μL 25% PEG4000 buffer and ice bath for 10 min, the solution was added with 250 μL 25% PEG4000 buffer, placed for 20 min at room temperature and centrifuged at 4500 rpm for 5min. The supernatant was discarded. The precipitation was recovered by 200 μL MYG liquid medium and uniformly coated in the MYG regeneration medium containing 450 mg/L concentrations of hygromycin B and dark cultured for 10 d in a 25°C incubator. The single colonies grown in the medium were randomly selected and transferred to the new PDA solid medium with dark culture in the 25°C incubators for 7 d, then the recombinant *C. militaris* would grow to the entire medium.

PCR

The genome of the recombinant *C. militaris* was extracted using the mycelium genome extraction kit (Solarbio, Beijing, China). According to the genome DNA, the primers were designed by Primer 5.0 software. The PCR condition was as follows: (i) 94°C for 5 min, 1 cycle; (ii) 94°C for 30 s, 62°C for 30 s, 72°C for 30 s, 30 cycles; (iii) 72 °C for 7 min, 1 cycle; (4) finished at 4°C. The sequences of primers were used as follows: forward, 5'-ACTCCGGCCTCTGGGTTT-3' and reverse, 5'-CGGGTGGTTCTGGTAAAGC-3'. PCR products were detected by 1% agarose gel electrophoresis and observed by gel imager.

SDS-PAGE and Western blot

Mycelia of recombinant *C. militaris* were randomly selected from PDA solid medium and inoculated into a PDB liquid medium with a shaking culture at 130 rpm and a constant temperature of 25°C in a dark environment. After 4 d of culture, the culture medium was discarded. The mycelium pellets were collected and lyophilized in a lyophilizer. Total proteins of the lyophilized powder were extracted using a filamentous fungal protein extraction kit (BestBio, Shanghai, China) and loaded to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). At the end of the electrophoresis, the gel was placed in the Coomassie Blue R250 Staining Solution (Solarbio, Beijing, China), shaken for 2 h, and decolorized for 4 h, then the gel was observed by gel imager. Besides, the gel was transferred onto PVDF membranes (Millipore, Bedford, MA, USA). The membrane was blocked with 5% skim milk at room temperature and shaken for 30-60 min, then incubated overnight at 4 °C with FGF21 antibody (Abcam, Cambridge, MA, USA). GAPDH served as a loading control. After being washed with TBST three times, an anti-rabbit secondary antibody (Bioss, Beijing, China) was added. The mixture was incubated for 1 h at room temperature and stained with DAB solution (Solarbio, Beijing, China).

Genetic stability of recombinant *C. militaris*

The recombinant *C. militaris* with the most obvious stripe in western blot results was named as X06-49 and used for follow-up experiments. X06-49 was cultured in a PDB medium containing 450 mg/L of hygromycin B, while the wild-type *C. militaris* was cultured in a PDB liquid medium without hygromycin B. After 7 d of culture, the samples were taken at the same time, and the morphological structure was observed under 200 x electron microscope.

Mass of X06-49 (0.5 cm × 0.5 cm) from the PDA solid medium was inoculated into a PDB liquid medium containing 450 mg/L hygromycin B, and dark cultured at 130 rpm for 10 d in a 25°C incubator. From the 3rd day to the 10th day, the mycelia were regularly collected every day and lyophilized in a lyophilizer. 100 mg of lyophilized powder was used to detect the RhFGF21 expression by hFGF21 ELISA Kit (Renjiebio, Shanghai, China). At the same time, the mycelia on the 7th day were subcultured into a new PDB liquid medium containing 450 mg/L hygromycin B, and the culture was continued for 7 days, and so on, for a total of 11 generations. The mycelia in the 1st, 3rd, 5th, 7th, 9th, and 11th generation cultures were collected and lyophilized. The genomic DNA and Soluble protein of 100 mg of lyophilized powder were examined by PCR and Western blot.

Purification of RhFGF21

X06-49 was inoculated into a PDB liquid medium containing 450 mg/L hygromycin B, and dark cultured at 130 rpm for 7 d in a 25°C incubator. Then, the myce-

lia were lyophilized and the total proteins were extracted using a filamentous fungal protein extraction kit. The purification of total proteins was applied to a Ni–NTA sepharose column (3.0×30 cm, 100 mL of bed volume) and AKATA purification apparatus. The concentration of total proteins was determined by NanoDrop ultramicro spectrophotometer.

Induced differentiation of 3T3-L1 cells

Mice 3T3-L1 preadipocytes were seeded at 200,000 cells/well density on six-well plates containing Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin, and cultured at 37°C and 5% CO_2. The culture medium was replaced every 2 days. After the cells had grown to a complete confluence, they began to induce differentiation (day 0). The medium was changed to a complete medium containing 0.5 mmol/L IBMX, 0.25 μmol/L dexamethasone and 10 μg/mL or 2 μmol/L insulin. After 48 h of culture, the medium was changed to a complete medium containing 10 μg/mL insulin. After 48 h of culture, the medium was changed to a complete medium without any inducer, and the culture medium was replaced every 2 days until more than 95% of the cells had differentiated into mature adipocytes.

The effect of RhFGF21 on the glucose absorption

Purified RhFGF21 (0.6 mg/mL) and commercial hFGF21 were diluted with cell culture medium to a final concentration of 0, 25, 50 and 100 μg/mL. Mature adipocytes were starved with 0.5% FBS high glucose DMEM for 12 h and then added with different concentrations of purified RhFGF21 and commercial hFGF21. After 24 h, the relative absorption values of glucose by adipocytes in the medium were determined by the glucose oxidase and peroxidase (GOD-POD) method.

The effect of RhFGF21 on the glucose absorption signaling pathway

Mature adipocytes were treated with purified RhFGF21 and commercial hFGF21 at concentrations of 25, 50, and 100 μg/mL, respectively. After removing the culture medium, Mature adipocytes were collected, washed with PBS, physiological saline or serum-free medium, and added lysate (150-250 μL/cell) was in a 6-well plate. Then the plate was centrifuged at 10000-14000 rpm for 3-5 min., and the supernatant was collected. The proteins were separated using SDS-PAGE and then detected by a western blot according to the method described above. The primary antibodies were the p-PLCγ antibody, p-FRS2 antibody and p-ERK antibody. GAPDH served as a loading control.

***In vitro* activity stability of RhFGF21**

Lyophilized powders of recombinant *C. militaris* and commercial hFGF21 were stored at -20, 4 and 37 °C, respectively. After storage for 1, 3, 6, 12 and 24 months, the RhFGF21 protein of mycelium was extracted and purified. The *in vitro* glucose absorption activity was compared between RhFGF21 and commercial hFGF21.

***In vivo* activity stability of RhFGF21**

Three BALB/c mice were injected with RhFGF21 (0.5 mg/kg) and commercial hFGF21 (0.5 mg/kg) via tail vein. At 10, 20, 30, 40, 60, 80, 100, 120, 140 min after injection, blood was collected from the tail vein and the content of FGF21 in the blood was quantified by human blood FGF21 ELISA kit (Renjiebio, Shanghai, China).

Effects of RhFGF21 on blood glucose and blood fat in type II diabetic mice

Totally 30 db/db mice (type II diabetic model**)** were divided into 5 groups (n=6): control group (normal feed), commercial hFGF21 group (oral commercial FGF21 dry powder, 200 μg/kg/d), RhFGF21 group (oral recombinant *C. militaris* dry powder, 0.25 g/kg/d), wild type *C. militaris* group (oral wild type *C. militaris* dry powder, 0.25 g/kg/d) and metformin group (oral metformin, 200 mg/kg/d). Mice were administered orally once a day. Blood was collected from the tail vein of mice at 10, 20, 30, 40, 50, and 60 d after administration, and the glucose content in blood was measured using a serum glucose assay kit (Nanjing Jiancheng Bioengineering Research Institute, Nanjing, China). At the end of the drug administration experiment, 3 mice were randomly selected from each group. After overnight fasting of 10 h, mice were intraperitoneally injected with glucose solution (1 g/kg), and blood was collected from the caudal vein at 0, 15, 30, 60, 90, 120 and 180 min after the glucose injection. The glucose content in the blood was detected by the serum glucose assay kit. Then the mouse eye blood was drawn, and the insulin content in the blood was measured using the insulin measurement kit (Nanjing Jiancheng Bioengineering Research Institute, Nanjing, China). Besides, tail vein blood was drawn from three other mice in each group, and the contents of triglyceride (TG), total cholesterol (T-CHO), low-density lipoprotein cholesterol (LDL-C), nonesterified fatty acid (NEFA), high-density lipoprotein cholesterol (HDL-C), alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were determined by TG assay kit, T-CHO assay kit, LDL-C assay kit, NEFA assay kit, HDL-C assay kit, ALT content detection kit and AST content detection kit (Nanjing Jiancheng Bioengineering Research Institute, Nanjing, China). Then, the mice were sacrificed, and liver and pancreas tissues were removed for subsequent testing.

Quantitative RT-PCR

Fresh liver tissues of mice were added with liquid nitrogen and ground into a powder. Total RNA was isolated by using TRIzol reagent (Takara Bio Inc, Beijing, China), and cDNA was synthesized by using the reverse transcription kit (Takara Bio Inc, Beijing, China) according to the manufacturer's protocol. The prepared cDNA was subjected to PCR amplification, and the PCR primers were synthesized by Suzhou Jinweizhi Biotechnology Co., Ltd (Suzhou, China) (Table 1). β-actin was used as an internal control.

HE staining and oil red staining

The liver tissues of mice were quickly severed and fixed with paraformaldehyde. Then the liver tissues were dehydrated with gradient ethanol, embedded in paraffin and cut into thin sections for HE staining and oil-red staining. The morphological changes were observed by electron microscope.

Table 1. Genes and primer sequences of identification for RT-PCR.

Gene	Sequences (Forward/Reverse 5'-3')	
TNF-α	AGGGGACATTCCTGTGTTCC	TTACCCTGTTTCCCCATTCC
MCP-1	CCAACCACCAGGCTACAGG	GCGTCACACTCAAGCTCTG
F4/80	TTGTACGTGCAACTCAGGACT	GATCCCAGAGTGTTGATGCAA
CD68	TGTCTGATCTTGCTAGGACCG	GAGAGTAACGGCCTTTTTGTGA
CD11b	ATGGACGCTGATGGCAATACC	TCCCCATTCACGTCTCCCA
β-actin	GTGACGTTGACATCCGTAAAGA	GCCGGACTCATCGTACTCC

Immunohistochemistry

The pancreas was fixed with formalin solution for 24 h, embedded in paraffin and cut into thin sections. Paraffin sections were de-waxed to the water, incubated with 0.01M Sodium citrate buffer for 2 min, and sealed with 5% BSA at 37°C for 10 min. Then the antibodies were added to incubate at 4°C overnight, and biotinylated sheep anti-rabbit IgG secondary antibody was added at 37°C for 1 h, followed by DAB staining. Then the reaction time was controlled under the microscope and the sections were washed with distilled water or PBS, re-dyed with hematoxylin, dehydrated, sealed after being transparent, and examined under the microscope.

Statistical analysis

The data were presented as mean ± SD and performed using the SPSS version 20.0. The data was analyzed by one-way ANOVA and pairwise comparison by LSD. $P<0.05$ indicated that the difference was statistically significant.

Results

RhFGF21 is successfully expressed in the protoplast of *C. militaris*

In order to screen the protoplast of *C. militaris* with positive recombinant hFGF21 (RhFGF21) expression, a totally 65 single colonies of *C. militaris* mycelium were randomly selected from a PDA medium with a concentration of 450 mg/L hygromycin B, and their genomic DNA were detected using PCR. The PCR verification of the RhFGF21 gene is shown in Figure 1A. There were 22 single colonies with a single specific band between 500-750 bp, which was consistent with the theoretical PCR length. Then, SDS-PAGE and western blot were performed on these 22 single colonies. In the single colonies, there was a significant difference in the target band at 25-35 kD between recombinant *C. militaris* and wild *C. militaris* (Figure 1B), which was consistent with the result of the western blot (Figure 1C). The above results indicated that RhFGF21 was successfully expressed in recombinant *C. militari* and the conversion efficiency was about 12.3%.

RhFGF21 can be hereditarily stable in the protoplast of *C. militaris*

Mycelium of X06-49 and wild-type *C. militaris* were cultured for 7 d and sampled at the same time, and the morphology and structure were observed under a 200 x electron microscope. As shown in Figure 2A, there was no significant difference in the shape of Mycelium between X06-49 and wild-type *C. militari*, indicating that the exogenous gene RhFGF21 has no effect on the morphology of *C. militaris*. Besides, the RhFGF21expression of

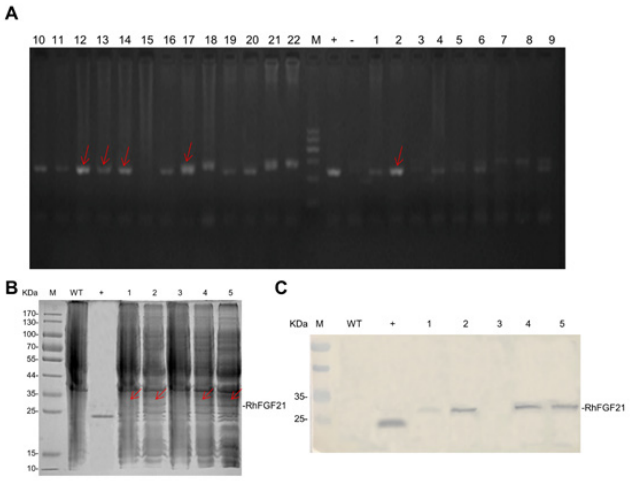

Figure 1. RhFGF21 is successfully expressed in the mycelium of *C. militaris*. (A) PCR analysis of RhFGF21 gene in the recombinant *C. militaris*. M: DNA Marker (DL 2000); +: Positive plasmid; -: Negative control; 1-22: 22 single colonies. (B-C) RhFGF21 protein level of recombinant *C. militaris* was detected by (B) SDS-PAGE and (C) Western blotting. M: protein marker; WT: wild type *C. militari*; 1-5: 5 single colonies.

X06-49 under different culture times was examined by the ELISA method. The results showed that the expression levels of RhFGF21 increased notably with the growth of culture time at the beginning, while it did not increase significantly with the extension of culture time after 7 d. Therefore, the 7th day could be used as the best culture time for *C. militaris*, and expressed RhFGF21 concentration could reach 176 μg/mL, which was converted to *C. militaris* mycelium content of 0.88 mg/g mycelium (Figure 2B). Through the subculture of X06-49, it was found that RhFGF21 had an obvious expression level before 5 times of generation, which decreased from the 7th generation and few expressed in the 11th generation (Figure 2C and D).

RhFGF21 promotes glucose absorption by activating the glucose absorption signaling pathway

To evaluate the bioactivity of RhFGF21, the effect of RhFGF21 on glucose uptake assay was performed using 3T3-L1 adipocytes. The results showed that both RhFGF21 and commercial hFGF21 increased the glucose absorption capacity of adipocytes with the increase of the concentration of FGF21, while there was no significant difference between the two groups (Figure 3A). Then, the effect of RhFGF21 on the glucose absorption signaling pathway was analyzed. As shown in Figure 3B, the protein expressions of p-PLCγ, p-FRS2 and p-ERK was increased with the increase of FGF21 concentration both in RhFGF21 and commercial hFGF21 groups, indica-

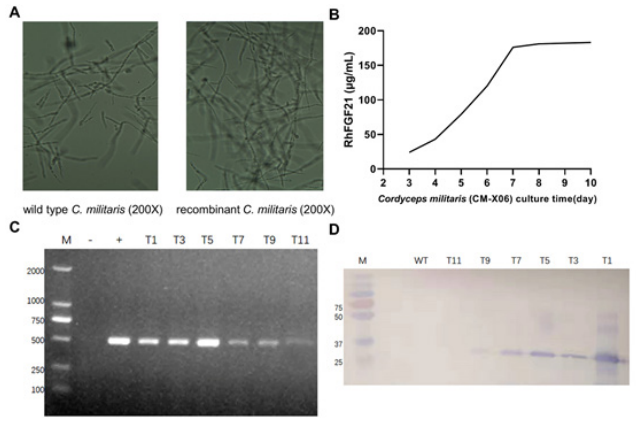

Figure 2. RhFGF21 can be hereditarily stable in the protoplast of *C. militaris*. (A) Morphology of wild type *C. militaris* and X06-49. (B) The expression of RhFGF21 in X06-49 at culture times of 3, 4, 5, 6, 7, 8, 9, 10 days. (C-D) RhFGF21 expression level of X06-49 was detected by (C) Genomic PCR and (D) Western blotting. M: DL2000/protein marker; -: negative control; +: positive plasmid; WT: wild type *C. militari*; T1-T11: different generations.

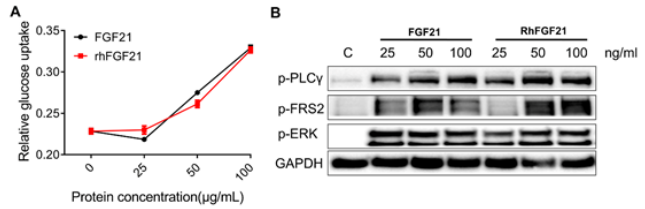

Figure 3. RhFGF21 promotes glucose absorption by activating the glucose absorption signaling pathway. (A) *In vitro* glucose uptake activity of 3T3-L1 adipocytes treated with RhFGF21 and commercial hFGF21 at concentrations of 0, 25, 50, and 100 μg/mL. (B) The protein expressions of p-PLCγ, p-FRS2 and p-ERK in the adipocytes were treated with RhFGF21 and commercial hFGF21 at concentrations of 0, 25, 50, and 100 μg/mL.

ting that RhFGF21 and commercial FGF21 had the same mechanism of promoting glucose absorption, which was by binding to FGFR and activating phosphorylation of downstream ERK pathway-related proteins.

RhFGF21 is stable *in vitro* and *in vivo*

At -20°C, there was no significant difference in the relative absorption activity of glucose after storage for 24 months between RhFGF21 and commercial FGF21 groups, and the relative activities could reach 95% (Figure 4A). As shown in Figure 4B, at 4°C, the relative activities of RhFGF21 and commercial FGF21 began to decline after 3 months. After 12 months of storage, the relative activity of commercial FGF21 was close to 50%, while the relative activity of RhFGF21 was close to 75%, which is significantly higher than that of commercial FGF21. At 37°C, the relative activity of commercial FGF21 decreased to 0 from the third month, while the relative activity of RhFGF21 still had a relative activity of nearly 50% after 24 months of storage, though it also decreased obviously (Figure 4C). These results suggested that the mycelium of *C. militaris* had a good protective effect on the biological activity of RhFGF21 and increased the stability of RhFGF21 *in vitro*, which had obvious advantages compared with commercial FGF21. In addition, as shown in Figure 4D, there was no significant difference in the changes in the blood concentration of hFGF21 in the mice between RhFGF21 and commercial FGF21 groups, which proved the stability of RhFGF21 is consistent with that of commercial FGF21.

RhFGF21 can reduce blood glucose in type II diabetic mice

After daily oral administration of 5 groups of db/db mice, the tail vein blood of the mice was taken at 10, 20, 30, 40, 50, 60d, respectively, and the glucose content in the blood was measured with a blood glucose concentration determination kit. Mice in the control group maintained high blood glucose levels during the 60-day feeding period; the metformin group had significant blood glucose lowering effect ($P<0.001$), which was close to that of normal mice at the 20th day; commercial hFGF21 group had no significant effect on blood glucose; the wild-type *C. militaris* group had no effect on blood glucose in mice in a short period, while the blood glucose began to decrease at the 50th day, and it was significantly different with the control group after 60 days of oral administration($0.01<P<0.05$); the RhFGF21 group decreased the blood glucose obviously and reach the blood glucose level of normal mice after oral administration for 60 days, which was significantly different with the control group ($P<0.001$), while the effect on the blood glucose did not as quickly as the metformin group (Figure 5A). These results indicated that recombinant *C. militaris* could reduce blood glucose levels in type II diabetic mice. Although the effect was not as rapid as metformin, long-term experimental results can still have significant hypoglycemic effects.

After the drug administration experiment, an intraperitoneal glucose tolerance test (IGTT) and insulin tolerance test (ITT) were performed. The results of IGTT showed that the metformin group and RhFGF21 group significantly reduced the initial blood glucose level and quickly promoted the glucose absorption of blood in mice. The commercial hFGF21 group and control group had high initial blood glucose levels, and their blood glucose concentrations were basically the same after 180 minutes (Figure 5B). Then, the area under the curve in Figure 6B was calculated by GraphPad software, which reflected the total value of glucose in the IGTT. There was no significant

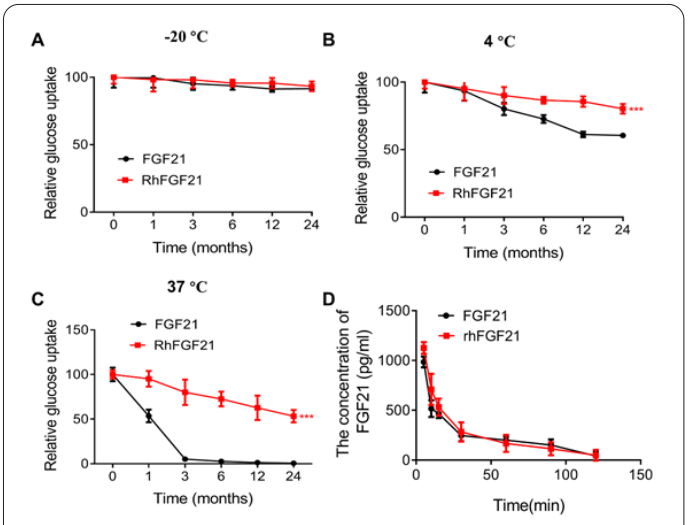

Figure 4. RhFGF21 is stable *in vitro* and *in vivo*. (A-C) The relative value of glucose uptake of commercial FGF21 and RhFGF21 under storage time of 0, 1, 3, 6, 12, 24 months at (A) -20°C, (B) 4°C or (C) 37°C. Compared with the FGF21 group, ***$P<0.001$.

difference in the area between the commercial hFGF21 and control groups, while the data of the metformin and RhFGF21 group were significantly different than that of the control group (P<0.05) (Figure 5C). The result of ITT is shown in Figure 5D. The content of insulin in wild-type *C. militaris*, RhFGF21 and metformin groups were significantly lower than that of the control group, while there was no significant difference in the content of insulin between commercial hFGF21 and control groups.

RhFGF21 regulates lipid metabolism disorders

After the mice were treated with commercial FGF21, recombinant *C. militaris*, wild type *C. militaris* and metformin, their blood lipid indexes were detected. Compared with the control group, the contents of TG, T-CHO, NEFA and LDL-C in the RhFGF21 group were significantly decreased, while the content of HDL-C was obviously increased, which was basically close to the metformin group. However, there was no significant difference in the contents of TG, T-CHO, NEFA, HDL-C and LDL-C between commercial hFGF21 and control groups (Figure 6A-E).

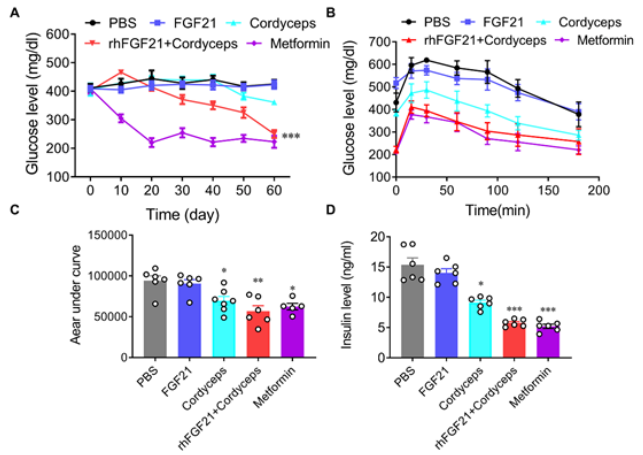

Figure 5. RhFGF21 can reduce blood glucose in type II diabetic mice. (A) The blood glucose content was measured at 10, 20, 30, 40, 50, 60 d after oral administration of normal feed, commercial hFGF21, wild-type *C. militaris*, recombinant *C. militaris* and metformin in db/db mice, respectively. (B) The blood glucose level was detected at 0, 15, 30, 60, 90, 120 and 180 min after the mice were intraperitoneally injected with glucose solution. (C-D) The serum levels of (C) glucose and (D) insulin were detected at 180 min after the mice were intraperitoneally injected with glucose solution. Compared with the PBS group, *P<0.05, **P<0.01, ***P<0.001.

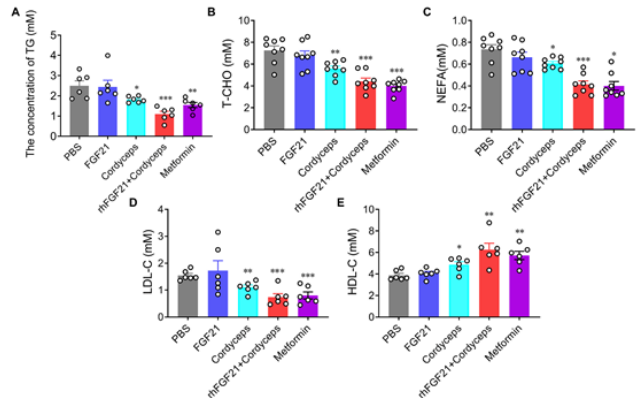

Figure 6. RhFGF21 regulates lipid metabolism disorders. The contents of (A) triglyceride (TG), (B) total cholesterol (T-CHO), (C) nonesterified fatty acid (NEFA), (D) low-density lipoprotein cholesterol (LDL-C), (E) high-density lipoprotein cholesterol (HDL-C) were examined at 60 d after oral administration of normal feed, commercial hFGF21, wild-type *C. militaris*, recombinant *C. militaris* and metformin in db/db mice, respectively. Compared with the PBS group, *P<0.05, **P<0.01, ***P<0.001.

RhFGF21 improves fatty liver

After the drug administration experiment, the AST and ALT levels in the tail vein blood drawn from the mice were measured. As shown in Figure 7A, B, the AST and ALT levels in the RhFGF21 and wild-type *C. militaris* groups were significantly decreased compared with the control group, while those in the metformin group were notably higher than those in the control group. There was no significant difference in the AST and ALT levels between commercial hFGF21 and control groups. In addition, the quantitative RT-PCR detection of inflammatory factors in fatty liver tissue showed that the expression of TNF-α, MCP-1, F4/80, CD68 and CD11b in RhFGF21 and wild-type *C. militaris* groups was obviously reduced compared with the control group, while those in metformin and commercial hFGF21 groups were higher than those in the control group (Figure 7C-G). As shown in Figure 8H, compared with the control group, RhFGF21 and wild-type *C. militaris* significantly reduced the adipocytes in fatty liver. However, there was no significant difference among metformin, commercial hFGF21 and control groups.

RhFGF21 reduces the apoptosis of pancreatic cells

From Figure 8, there were a large number of apoptotic pancreatic cells in the control group. RhFGF21 and metformin group significantly reduced the apoptosis of pancreatic cells, while the commercial hFGF21 group had no effect on pancreatic cells and the wild-type *C. militaris* group had a small effect of reducing apoptosis.

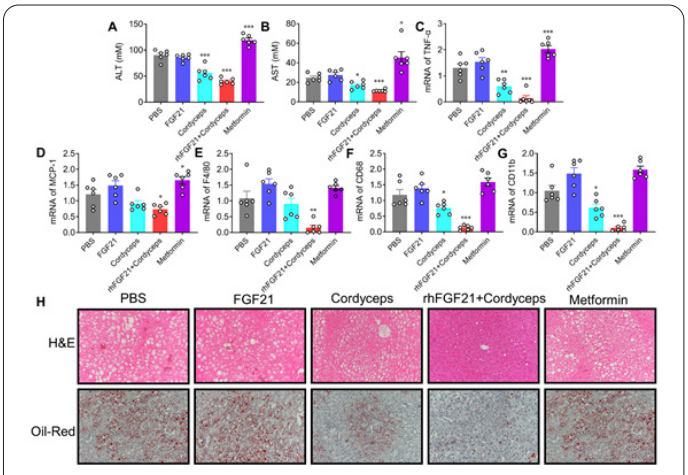

Figure 7. RhFGF21 improves fatty liver. (A-B) The contents of (A) alanine aminotransferase (ALT) and (B) aspartate aminotransferase (AST) in the fatty liver were determined after drug administration experiment. (C-G) the expression of (C) TNF-α, (D) MCP-1, (E) F4/80, (F) CD68 and (G) CD11b in the fatty liver were detected by quantitative RT-PCR. (H) The morphological changes were observed after HE staining and oil red staining of liver tissues in db/db mice after oral administration of normal feed, commercial hFGF21, wild-type *C. militaris*, recombinant *C. militaris* and metformin, respectively, magnification: 100 x. Compared with the PBS group, *P<0.05, **P<0.01, ***P<0.001.

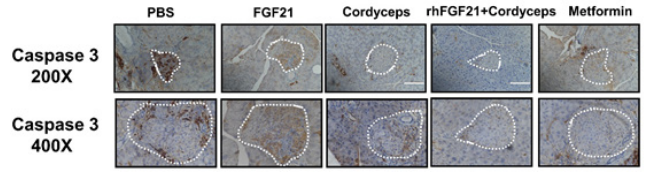

Figure 8. RhFGF21 reduces the apoptosis of pancreatic cells. Immunohistochemical analysis of pancreatic tissues in the db/db mice after oral administration of normal feed, commercial hFGF21, wild-type *C. militaris*, recombinant *C. militaris* and metformin, respectively.

Discussion

In this study, a PEG-mediated *C. militaris* transformation system was established to successfully construct the expression vector pCB130-RhFGF21 containing the FGF21 gene. After being screened with hygromycin B, followed by verification of PCR and WB methods, 8 single colonies with high expression of RhFGF21 were obtained, and the conversion rate was 12.3%. In addition, the lyophilized powder extract of *C. militaris* mycelium confirmed that hFGF21 was a soluble expression. After several generations of fungus, it will cause the loss of the foreign gene, which is called fungus degradation. It is heritable and can affect the fungus quality and cause cultivation failure, which brings great losses to mass production. Therefore, in the mass production of *C. militaris*, fungus degradation is the most common core technical problem. In order to investigate the genetic stability of RhFGF21 in *C. militaris* mycelium, X06-49 was successively passaged for 11 generations, and the results showed that the recombinant *C. militaris* can be stably inherited for 7 generations and has good genetic stability.

FGF21 plays an important role in regulating energy metabolism, including improving glucose and lipid metabolism under pathological conditions such as obesity and diabetes(13), promoting liver fatty acid oxidation and ketone body production(14), and increasing the ability of adipocytes on glucose ingestion and sensitivity of insulin (15, 16). In order to prove whether RhFGF21 expressed by recombinant *C. militaris* has the pharmacological activity of hFGF21, *in vitro* glucose absorption experiments on RhFGF21 were conducted, and the results showed that the RhFGF21 promoted glucose uptake in a dose-dependent manner in adipocytes, which was consistent with the function of commercial hFGF21. In addition, the detection of the known signaling protein index of FGF21 in promoting glucose absorption of 3T3-L1 cells, such as p-PLCγ, p-FRS2 and p-ERK, found that the signaling pathways activated by RhFGF21 and commercial hFGF21 in promoting glucose absorption were consistent, and both activated the phosphorylation of proteins related to the downstream ERK pathway after FGFR binding, thus exerting the ability to promote sugar absorption. The absorption effect of hFGF21 glucose requires the activation of the glucose transporter GLUT1, which is not consistent with the traditional way that insulin acts to utilize GLUT4, suggesting that hFGF21 was independent of insulin in promoting glucose absorption.

Compared with *in vitro* cell experiments, the *in vivo* environment is more complicated. In order to investigate whether RhFGF21 will still have corresponding pharmacological activities *in vivo*, lyophilized powder of X06-49 was fed orally to diabetes mice, and blood glucose, insulin, lipids, fatty liver and other indicators were measured to study the hypoglycemic and lipid-lowering effects of RhFGF21 on diabetic mice. The results showed that RhFGF21, although slower than metformin, effectively reduced blood glucose in type II diabetic mice by improving insulin sensitivity. In addition, RhFGF21 decreased the levels of TG, T-CHO, NEFA, and LDL-C in the blood and increased the level of HDL-C, which effectively improved the disorder of lipid metabolism associated with type II diabetes. It has been reported that *C. militaris* has a variety of biological activities, such as inhibiting tumor growth, reducing blood lipids and blood glucose, and enhancing sexual function(17). In this study, it was found that long-term use of wild-type *C. militaris* had certain effects on lowering blood glucose and improving insulin resistance and dyslipidemia. However, it was not as effective as recombinant *C. militaris*. However, the commercial FGF21 had almost no hypoglycemic and lipid-lowering effects, which may be due to the consumption of gastric acid during oral administration, reflecting that *C. militaris* was a suitable carrier that could effectively maintain the biological activity of hFGF21.

Type II diabetes is common in obese patients, and obese patients are usually accompanied by fatty liver symptoms(18). An important biological function of FGF21 is to improve non-alcoholic fatty liver(19). ALT and AST are important indicators of liver function. When the liver is damaged, the content of ALT and AST will increase. In this experiment, the serum levels of ALT and AST were analyzed to evaluate the improving effect of RhFGF21 on liver function. RhFGF21 significantly reduced the contents of ALT and AST, suggesting that it had a good relief effect on fatty liver in type II diabetic mice. Besides, the occurrence of fatty liver is often accompanied by an increase in fatty inflammatory factors. RT-PCR was used to detect fatty liver-related inflammatory factors in each group of mice, and to analyze the improvement of liver inflammation indicators. The results suggested that RhFGF21 significantly reduced the expression of inflammation indicators of fatty liver in type II diabetic mice and restored the normal liver function of type II diabetic mice. HE staining and oil-red staining of fatty liver tissues proved that RhFGF21 decreased the content of adipocytes in fatty liver. At the same time, the results of immunohistochemistry demonstrated that RhFGF21 protected pancreatic tissues by reducing apoptosis of pancreatic cells. Therefore, RhFGF21 not only had hypoglycemic and lipid-lowering effects but also protected the liver and pancreatic tissues from type II diabetes mellitus. Metformin is the first-line drug for the treatment of type II diabetes that can regulate blood lipids, reduce blood viscosity, and stimulate β-cells to release insulin(20-24). In this study, metformin reduced blood glucose and blood lipids, but it caused significant damage to the fatty liver. In addition, oral administration of commercial FGF21 had no effect on the fatty liver and pancreas of type II diabetic mice, further indicating that *C. militaris* as a host protected the biological activity of the exogenous gene hFGF21.

The results of *in vitro* stability analysis demonstrated that compared with commercial hFGF21, recombinant mycelium lyophilized powder had obvious advantages under different temperature storage conditions. RhFGF21 maintained a relative biological activity of 50% even when

stored for 24 months at a relatively harsh 37°C. This might be because the cell wall of mycelium had a protective effect on the intracellular environment, effectively reducing the effect of the extracellular environment on the structure or activity of intracellular biological macromolecules, thereby maintaining the biological activity of RhFGF21. This provides great convenience for the subsequent preservation and transportation of *C. militaris* FGF21 oral preparation.

In conclusion, *C. militaris* is an excellent carrier that can stabilize the expression of the inherited foreign gene hFGF21 and protect the biological activity of hFGF21 during oral administration, which provides a theoretical basis for the development of hFGF21 oral preparations for type II diabetes.

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Ethics approval
Ethics approval was obtained from the ethics committee of Jilin Medical University.

Availability of data and materials
The data used in this article are available from the corresponding author on reasonable request.

Competing interests
The authors declare no competing interests.

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Authors contributions
All authors contributed to the intellectual content of this manuscript and approved the final manuscript as submitted. Xiaomei Zhang drafted the initial manuscript. Xin Sun contributed to the conception and design of the research. Weidong Qiang contributed to the experiment and analysis of the data. Yongxin Guo contributed to the analysis and interpretation of the data. Di Wu revised the article critically.

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