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Recombinant *Lactococcus lactis* NZ3900 expressing bioactive human FGF21 reduced body weight of Db/Db mice through the activity of brown adipose tissue

W.-Y. Cao[1,2#], M. Dong[2,3#], Z.-Y. Hu[1], J. Wu[1], Y.-C. Li[1*] and H.-D. Xu[1*]

[1]School of Pharmaceutical Sciences, Key Laboratory of State Ministry of Education, Key Laboratory of Henan province for Drug Quality Control and Evaluation, Collaborative Innovation Center of New Drug Research and Safety Evaluation, Zhengzhou University, 100 Ke xue Avenue, Zhengzhou, Henan 450001, China P.R.; [2]Key Laboratory of Animal Ecology and Conservation Biology Institute of Zoology, Chinese Academy of Sciences, Beijing 100101, China P.R.; [3]The University of the Chinese Academy of Sciences, Beijing 100049, China P.R.; ycli78@126.com; xhd1220@zzu.edu.cn; # these authors contributed equally to this work

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RESEARCH ARTICLE

Abstract

Fibroblast growth factor 21 (FGF21), a metabolism regulator, has an important effect on metabolic diseases, such as obesity and diabetes. It is also expressed in mice, and the murine source has high homology with human FGF21. Recently, it has been extensively studied and has become a potential drug target for the treatment of metabolic diseases. As it is a protein-based hormone, FGF21 cannot be easily and quickly absorbed into the blood through oral administration. Moreover, it has a 0-2 h half-life *in vivo,* as shown in a previous study, thus its efficacy lasts for a short period of time when used to treat metabolic diseases, limiting its clinical applications. To avoid these limitations, we used *Lactococcus lactis*, a food-grade bacterium, as the host to express FGF21. It could be used successfully for the expression and long-term effect of FGF21 *in vivo*. Instead of antibiotic resistance genes, the LacF gene was used as a selection marker in the NZ3900/PNZ8149 expression system, which is safe and could reduce the antibiotic resistance crisis. In this study, we a constructed human FGF21 expressing *L. lactis* strain and administered it to Db/Db mice by gavage. Compared with the control group, the body weight of mice in the experimental group was significantly reduced, and the overall homeostasis was improved in mice treated with human FGF21. Moreover, the activity of brown adipose tissue was enhanced. These results revealed that oral administration of FGF21 through heterologous expression in *L. lactis* appears to be an effective approach for its clinical application.

Keywords: animal models, *Lactococcus lactis* subsp. lactis, lactose, obesity, probiotics

1. Introduction

Obesity and type 2 diabetes have already become two of the major threats to public health due to the global changes in lifestyle and decreased quality of life (Bain *et al.*, 2016; Barthel *et al.*, 2011; Dejkhamron *et al.*, 2007; Schloot *et al.*, 2011; Zoka *et al.*, 2012). The general treatment of diabetes is a subcutaneous injection of insulin combined with diet and exercise. However, patients' adherence to the treatment is poor because of various factors. Pharmacotherapies mainly include Metformin, Glp-1 related drugs and Lorcaserin, which are often accompanied with side effects, such as mental problems, dizziness or headache (Baretic, 2013; Jones and Bloom, 2015; Nakazato, 2013). Regarding to a surgical treatment, there is a possibility of reoccurrence after surgery, since the potential hazards to the body are still not clear (Baretic, 2013; Jones and Bloom, 2015; Nakazato, 2013). Therefore, developing an easy and safe therapy is particularly essential to treat obesity as well as type 2 diabetes. Fibroblast growth factor 21 (FGF21) is a member of FGF family. It is composed of 209 amino acids and shares about 75% homology with mice (Seo and Kim, 2012). FGF21 has been recently described as a metabolic regulator and a candidate therapeutic agent for metabolic diseases, such as obesity and type 2 diabetes (Kharitonenkov *et al.*, 2005). It is expressed in multiple tissues, including liver, white or

brown adipose tissue as well as pancreas, among which, the liver is the major source of FGF21 in mice. Some studies showed that FGF21 is a key metabolic regulator of glucose and lipid homeostasis (Fisher and Maratos-Flier, 2016). In addition, FGF21 is known to function in the hypothalamus.

KLB (β-Klotho, the obligate co-receptor for FGF21) and fibroblast growth factor receptors (FGFRs) are both expressed in the hypothalamus and Agouti-related peptides are found to be released after FGF21 administration (Recinella *et al.*, 2017). Researchers have also revealed that FGF21 could inhibit diet-induced obesity (DIO) in mice (Kharitonenkov *et al.*, 2005). Giving external sources of FGF21 to the Ob/Ob, Db/Db and DIO mice led to weight reduction, a decrease in blood triglyceride level, and an improvement in blood glucose homeostasis (Coskun *et al.*, 2008; Giralt *et al.*, 2015; Hanssen *et al.*, 2015; Owen *et al.*, 2014). In recent years, brown adipose tissue (BAT) has been described as a key target tissue for FGF21, which is associated with cold-induced thermogenesis, and the BAT helps to consume fat in the form of heat to protect us against cold conditions (Hanssen *et al.*, 2015). Moreover, browning of the white adipose tissue (WAT) is also induced by FGF21 (Straub and Wolfrum, 2015). Other studies showed that FGF21 can go through the blood-brain barrier and combine with KLB to regulate blood glucose homeostasis and reduce weight (Owen *et al.*, 2014; Sarruf *et al.*, 2010), thus making it a potential candidate for treatment of type 2 diabetes mellitus (Ye *et al.*, 2013). However, there are some limitations, such as the just 0-2 h half-life *in vivo* and inability to be taken orally because of its peptide hormone essence which will be digested by proteases in the digestive system (Fisher and Maratos-Flier, 2016; Veniant *et al.*, 2012).

Considering the benefits of FGF21 and its limitations, finding a suitable protein expression system is especially important. In recent years, with the rapid development of genetic engineering technology and the accomplishment of whole genome sequencing of the model strains, a series of genetic transformation systems have been established by *Lactobacillus lactis*. *L. lactis* is generally recognised as safe (GRAS) as it is a part of the food fermentation since a long time, and is widely used in fermented dairy products (Ainsworth *et al.*, 2014). As a common inducible host bacterium, *L. lactis* has an important role in research and application. In other words, *L. lactis* is more beneficial than the other microorganisms in the dairy industry (Renye and Somkuti, 2010), and it has a greater value in the oral drug delivery system (Ainsworth *et al.*, 2014; Bahey-El-Din and Gahan, 2010; Dobson *et al.*, 2012; Osmanagaoglu *et al.*, 2010; Pontes *et al.*, 2011), as Ng and Sarkar (2011) have reported that *L. lactis* NZ9000 could secrete bioactive insulin analogs. Because of its potential in production of heterologous proteins of therapeutic or technological value (Pouwels and Leer, 1993), *L. lactis* is becoming increasingly popular, among which the nisin-controlled gene expression (NICE) system is the most commonly used (Mierau and Kleerebezem, 2005). In its combined form, *L. lactis* NZ3900/PNZ8149 is a crucial member of the NICE system (De Ruyter *et al.*, 1996b). The combination complies with Food and Drug Administration (FDA)'s safety regulation and reaches food-grade; thus, the system is an ideal heterologous protein expression system (Hugenholtz and Smid, 2002). The usp45 secretory signal peptide and LESS leading peptide sequence are two main *L. lactis* helpers for expression of extracellular protein (Van Asseldonk *et al.*, 1990), increasing the expression efficiency 1000 times, making oral dosage forms of human FGF21 possible.

In this study, we used the *L. lactis* NZ3900/PNZ8149 system to express the recombinant human FGF21 protein. To further investigate whether human FGF21 secreting *L. lactis* plays a role in obesity and type 2 diabetes, we treated Db/Db mice with the recombinant *L. lactis* by gavage. Our results suggested that oral administration of human FGF21 by food-grade recombinants, *L. lactis* NZ3900/PNZ8149, is efficient in combating obesity, thus providing a novel therapeutic approach to improve the obesity status and type 2 diabetes.

2. Materials and methods

Bacterial strains, growth conditions and the plasmid

The bacterial strains and plasmids used in this study are listed in Table 1. In this study, we chose the *L. lactis* NZ3900 (VS-ELS03900-01) strain as the host bacterium and the PNZ8149 plasmid was used. The vector had the lacF gene, which was used as a food grade selection marker. To enable selection of transformants, this vector needs a host strain devoid of the lactose operon, like *L. lactis* NZ3900. The vector and strain were bought from NIZO Food Research in the Netherlands. In addition, the strain was anaerobically cultured in Oxoid M17 broth (Oxoid Ltd, Basingstoke, UK) supplemented with 0.5% (w/v) lactose at 30 °C under static conditions.

The preparation of electrocompetent cells

L. lactis NZ3900 was first inoculated in 5 ml L-SGM17B and incubated at 30 °C overnight. After the incubation 12-14 h, 5 ml culture was diluted in 50 ml L-SGM17B and cultivated at 30 °C overnight. The 50 ml culture thus obtained was diluted in 400 ml L-SGM17B. The culture was then incubated until an OD_{600nm} of 0.2-0.3 was obtained (ca. 3 h). The cells were centrifuged for 20 min at 6,000×*g* and at 4 °C. The cells were washed with 400 ml of 0.5 M sucrose and 10% glycerol (4 °C), and again centrifuged at 6,000×*g*. It was then resuspended in 200 ml of 0.5 M sucrose, 10% glycerol and 50 mM EDTA (4 °C). The suspension was kept on ice for 15 min and then centrifuged. The cells were then washed with 100 ml of 0.5 M sucrose, 10% glycerol

Table 1. Bacterial strains and plasmids.

Strain or plasmid	Characteristics	Source or reference
Bacteria		
NZ3900	It is derived from strain NZ3000, which is a lacF deletion mutant of strain MG5267	De Ruyter *et al.* (1996a)
TOP10	Cloning host	Invitrogen
Electroporation-competent cells-NZ3900	The strain is used as plasmid for electrical transformation purposes	This study
Plasmids		
PNZ8149	It has the lacF gene as a food grade selection for growth on lactose	Charteris *et al.* (1998)
PUC57	PUC57 is a commonly used plasmid cloning vector in *Escherichia coli*	Sangon Biotech (Shanghai, China P.R.)
PNZ8149-Human FGF21	It is an artificially constructed recombinant vector that can express HumanFGF21 protein	This study

(4 °C) and spun down (6,000×*g*). Finally, the cells were resuspended in 4 ml 0.5 M sucrose and 10% glycerol (4 °C). The electrocompetent cells (40 µl cells per tube) were stored at -80 °C in sterile EP tubes.

Construction of a recombinant plasmid and electroporation

The gene sequence of human FGF21 (NM_019113.3) was obtained from GenBank, and the gene was synthesised and shipped by Sangon Biotech gene synthesis department (Shanghai, China P.R.). It was subcloned to remove the signal peptide and enhance its expression and production stability. The primer was ordered by Sangon Biotech primer synthesis department (Beijing, China P.R.). The primers used are listed in Table 2. The horizontal lines indicate the restriction enzymes sites present in the sequence. After the bridging PCR, the Nco1-HF and Xba1 restriction enzymes (New England Biolabs, Ipswich, MA, USA) were used to digest pNZ8149 and the PCR product. The reaction mixture was incubated at 37 °C for 2 h. It was then purified using the kit (Tiangen Biotech, Beijing, China P.R.). T4 ligase (New England Biolabs) was used for ligation overnight. The ligation product named PNZ8149-FGF21 was purified with 2.5 times the volume of anhydrous ethanol and 1/10 volume of 2.5 mol/l sodium acetate. It was incubated at -20 °C for l h and then centrifuged at 13,800×*g* for 5 min. After centrifugation, it was washed using 1 ml of 75% ethanol, then centrifuged at 13,800×*g* for 5 min and dried at room temperature for 20 min. Deionised water was used to elute the purified ligation product. Electroporation was carried out using the Gene Pulser (Bio-Rad Laboratories, Inc., Hercules, CA, USA) which was set with 2,500 V, 25 µF and 200 Ω. A pulse of 4.5-5 msec was used for transformation recombinant plasmid of PNZ8149-FGF21 into NZ3900. Elliker culture plates were used to screen positive clones

Table 2. Oligonucleotide primers used in this study.

Primers	Upstream sequence (5'-3')	Downstream sequence (5'-3')	Application
Ucp1	GGCAAAAACAGAAGGATTGC	TAAGCCGGCTGAGATCTTGT	QPCR
CyclophilinA	CAAATGCTGGACCAAACACA	GCCATCCAGCCATTCAGTCT	QPCR
Cidea	TGCTCTTCTGTATCGCCCAGT	GCCGTGTTAAGGAATCTGCTG	QPCR
ACADM	ACTCGAAAGCGGCTCACAA	ACGGGGATAATCTCCTCTCTGG	QPCR
Cebpβ	TGACGCAACACACGTGTAACTG	AACAACCCCGCAGGAACAT	QPCR
PGC1α	ACAGCTTTCTGGGTGGATT	TGAGGACCGCTAGCAAGTTT	QPCR
ATGL	ATATCCCACTTTAGCTCCAAGG	CAAGTTGTCTGAAATGCCGC	QPCR
FGF21	AGCGGTACCTCTACACAGAT	CTTTCAGCTGCAGGAGACTT	QPCR
HSL	CTGAGATTGAGGTGCTGTCG	CAAGGGAGGTGAGATGGTAAC	QPCR
Adiponectin	ACCAGACTAATGAGACCTGGCCAC	CGTCATCTTCGGCATGACTGGGC	QPCR
Fgf21-Nco1	CATGCCATGGGCATGAAAAAAAAGATTATCTCAG	TGGAGTCAGGGATGGGGTGCTTATCGTCAT CGTCGTGATGATGATGATGATG	Sub-clone primer
Fgf21-Xba1	CATCATCATCATCATCACGACGATGACGATAAGCA CCCCATCCCTGACTCCA	GCTCTAGATCAGGAAGCGTAGCTGGGGCTT	Sub-clone primer

which appeared yellow on the plate. Plasmid DNA was extracted from the positive clone and it was sequenced. The strain was successfully sequenced and was named Human-FGF21-PNZ8149/NZ3900. It was preserved in glycerin (1:3). NZ3900 was also electroporated with an empty plasmid-PNZ8149 to generate a vector control strain named PNZ8149/NZ3900.

Protein electrophoresis, silver stain and mass spectrometry analysis human FGF21

After obtaining the strain of our choice, which contained the human FGF21 gene, the culture was inoculated overnight. It was then sub-cultured into fresh medium, with a ratio of inoculation volume of 1 to 10. Thereafter it was cultured until an optical density at OD_{600nm} of 0.3-0.4 and induced with nisin (25 ng/ml) (Sigma SBR00021; Sigma-Aldrich, St. Louis, MO, USA). After induction, the culture was incubated for 5 h. The trichloroacetic acid (Aladdin OL436; Aladdin Bio-Chem Technology Co., Ltd., Shanghai, China P.R.) precipitation method (Link and LaBaer, 2011) was used to obtain the total protein content of the culture. The protein samples were prepared for analysis using 12% SDS-PAGE through electrophoretic separation of the protein. The SDS-PAGE gel was visualised using silver stain (Stochaj *et al.*, 2007). Then, LC-MS/MS sequencing was performed by the Institute of Biophysics, Chinese Academy of Sciences, Beijing, China P.R.

Oral administration of the recombinant human FGF21-PNZ8149/NZ3900 to the Db/Db mice

Obese C57BLKS/J-Lepr^{db}/Lepr^{db} (Db/Db) male mice (4-wk old) and the C57BL6/J (8-wk old) were purchased from the Model Animal Research Center of Nanjing University (Nanjing, China P.R.). The purchased mice were housed in the standard SPF animal house of the Institute of Animal Research, Chinese Academy of Sciences. The conditions of raising the animals required a temperature of 18~29 °C, a daily temperature difference ≤3 °C, a relative humidity up to 40~70%, fresh air ventilation times 10/Hr, an airflow speed ≤0.18m/s, pressure difference of 25 Pa, cleanliness level of 10,000, noise ≤60 dB, and illuminance of 150~300 Lux. One week later, the Db/Db mice were treated and the recombinant strain of Human FGF21-PNZ8149/NZ3900 was orally administered, i.e. 200 µl each day per mice (named FGF21), with a total number of colonies of 10^{13} cfu. The administration of bacteria lasted 13 weeks, and the number of mice each group was 8. At the same time, the control group mice (named PNZ) were treated in the same way but the strain used was PNZ8149/NZ3900. All animal studies were approved by the Institutional Animal Care and Use Committee of Institute of Zoology (IOZ20160050).

The measurement of human FGF21 in plasma

The blood samples of C57BL6/J mice were taken after an overnight fast. The mice were treated with the recombinant strain of Human-FGF21-PNZ8149/NZ3900 containing 10^{13} cfu by a one-time oral administration. At the same time, the inducer, Nisin (25 ng/ml) was administered. Post-induction, blood samples were taken at different time points in 0, 6, 12, 24, 48, 72 and 168 h. The blood samples were centrifuged at 1,580×*g* for 15 min and stored at -80 °C until further analysis. The concentration of Human FGF21 in plasma was measured using a commercially available enzyme-linked immunosorbent assay (ELISA) kit according to the protocol mentioned by the manufacturer (Nanjing Jian Cheng Bioengineering Institute, Nanjing, China P.R.). The sensitivity of Human FGF21 ELISA was as low as 5 ng/l. The intra-assay and inter-assay CV of the ELISA kit was <10 and <12%, respectively. The OD_{450nm} values were measured using the Microporous plate spectrophotometer (USA BioTek, Winooski, VT, USA). The software of ELISAcalc was used to calculate and apply the values to obtain a fitted logistic curve (four parameters).

RNA isolation and real-time quantitative PCR

The total RNA from tissues was extracted using the Trizol reagent (Thermo Fisher Scientific, Waltham, MA, USA). The total RNA (2 µg) was reverse transcribed with the high capacity cDNA reverse transcription kit (Promega, Madison, WI, USA). A real-time quantitative PCR was performed using the SYBR Green Master Mix (Promega). Cyclophilin A was used as an internal standard. The PCR reaction program was run in triplicates for each sample using a Prism VIIA7 real-time fluorescence quantitative PCR system (Thermo Fisher Scientific).

Western blot analysis

The western blot analyses were performed using total protein extracted from frozen tissues using RIPA lysis buffer containing protease inhibitors. The protein quantification was done with the enhanced BCA Protein Assay Kit (P009, Beyotime Biotechnology, Nanjing, China P.R.). Samples containing a fixed concentration of protein was loaded on a 10% SDS-PAGE gel after being heated at 55 °C for 10 min. The gel electrophoresis was carried out at 80 V for 30 min, post which it was carried out at 120 V for 1 h. The gel was then placed on a polyvinylidene fluoride (PVDF) membrane to enable the transfer of the protein onto the membrane. The transfer was carried out at 100 V for 1 h. After the transfer, the PVDF membrane was blocked with 5% skim milk (Oxoid) at room temperature for 1 h. The following antibodies were used: the monoclonal anti-oxidative phosphorylation (total anti-OXPHOS Cocktail, ab110413); the monoclonal anti-uncoupling protein-1 (anti-UCP1, ab155117); and the monoclonal anti-β-actin

(Sigma-Aldrich). The chemiluminescence technique was used to check the expression level of protein (Thermo Fisher Scientific) and the images were captured with an Chemiluminescent gel imager (Beijing Sage Creation Science Co, Beijng, China P.R.).

Histopathology

After the tissue removal from the mice, it was immediately divided and fixed by immersing it into 4% paraformaldehyde for at least 24 h. After the tissue was fixed, they were embedded in paraffin, and were sectioned to a thickness of 5 µm. After the histologic sectioning, the tissues were stained using haematoxylin-eosin (H-E) and incubated at 37 °C for 24 h. All images were captured with the BX51 system (Olympus, Tokyo, Japan).

Statistical analysis

Data are expressed as means ± standard error of the mean. Statistical significance was tested with one-way ANOVA followed by Tukey's post hoc test or a paired Student's t test. Statistical significance was set at $P<0.05$.

3. Results

Acquisition of recombinant vector and expression of human FGF21 in *Lactobacillus lactis* NZ3900

We modified the food-grade vector of PNZ8149 and constructed a recombinant vector named Human-FGF21-PNZ8149 (Figure 1A,B). It was modified and the USP45 signal peptide and LESS nucleotide sequence was incorporated into the vector for better expression of the target protein (Figure 1A). As the strain used had a thick cell wall compared to gram-negative bacteria, we chose electroporation to transform the recombinant vector into the *L. lactis* NZ3900. Sequencing of the gene of interest was performed by Sangon Biotech (Beijing). And the sequencing results are listed in Table 3. A positive recombinant strain of Human-FGF21-PNZ8149/NZ3900 was obtained. The NICE system was induced by nisin to express the protein of our choice (Mierau and Kleerebezem, 2005). The positive recombinant strain of Human-FGF21-PNZ8149/NZ3900 was cultured, induced and the protein obtained in the supernatant was separated by electrophoresis on a 12% SDS-PAGE gel. The gel was then stained by silver staining

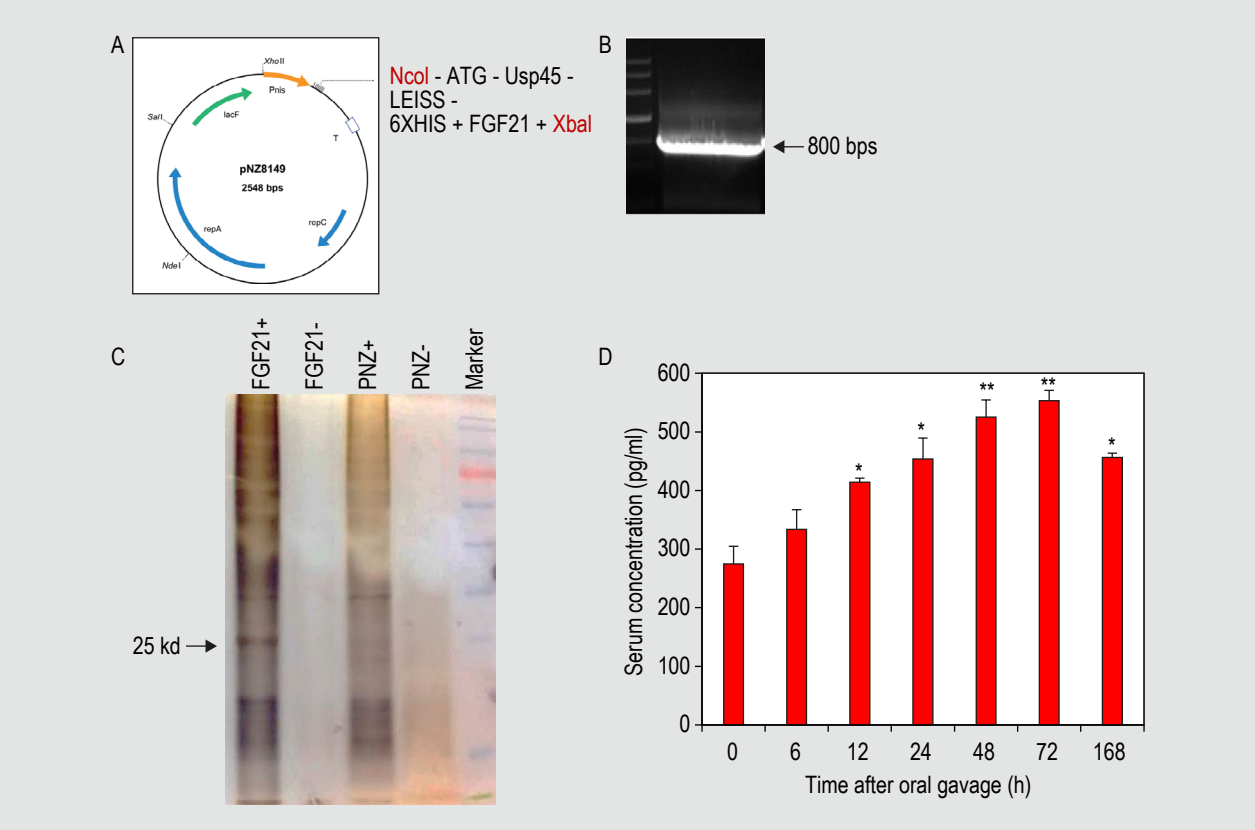

Figure 1. Expression of recombinant Human FGF21 by *Lactobacillus lactis* NZ3900. (A) The recombinant plasmid structure. (B) The AGE of the cloning production of the objective gene fragment. (C) The silver stained SDS-PAGE gel after induction of the recombinant Human FGF21-PNZ8149/NZ3900 and PNZ8149/NZ3900 strains. In PNZ- and FGF21-, the concentration of Nisin is 0 ng/ml; in PNZ+ and FGF21+, the concentration of Nisin is 25 ng/ml. (D) The serum of the human FGF21 ELISA of the c57 mice who were given orally by 1013 CUF in single dose (n=4).

Table 3. Gene sequencing results of recombinant vector.

NcoI-ATG-Usp45-6XHIS+LEISS+FGF21+XbaI

CATGCCATGGGCATGAAAAAAAAGATTATCTCAGCTATTTTAATGTCTACAGTGATACTTTCTGCTGCAGCCCCGTTGTCAGGTGTTTACGCTG
ATACTAATTCTGATTTGGAAATATCGTCGACTTGTGATGCTCATCATCATCATCATCACGACGATGACGATAAGCACCCCATCCCTGACTCCAG
TCCTCTCCTGCAATTCGGGGGCCAAGTCCGGCAGCGGTACCTCTACACAGATGATGCCCAGCAGACAGAAGCCCACCTGGAGATCAGGGA
GGATGGGACGGTGGGGGGCGCTGCTGACCAGAGCCCCGAAAGTCTCCTGCAGCTGAAAGCCTTGAAGCCGGGAGTTATTCAAATCTTGG
GAGTCAAGACATCCAGGTTCCTGTGCCAGCGGCCAGATGGGGCCCTGTATGGATCGCTCCACTTTGACCCTGAGGCCTGCAGCTTCCGGG
AGCTGCTTCTTGAGGACGGATACAATGTTTACCAGTCCGAAGCCCACGGCCTCCCGCTGCACCTGCCAGGGAACAAGTCCCCACACCGGG
ACCCTGCACCCCGAGGACCAGCTCGCTTCCTGCCACTACCAGGCCTGCCCCCCGCACTCCCGGAGCCACCCGGAATCCTGGCCCCCCAG
CCCCCCGATGTGGGCTCCTCGGACCCTCTGAGCATGGTGGGACCTTCCCAGGGCCGAAGCCCCAGCTACGCTTCCTGATCTAGAGC

and the objective location of 25 kD shown by specific bands on the gel (Figure 1C). This was further confirmed with the LC-MS/MS sequencing (Table 4). These data suggested that the recombinant strain could produce the target protein, Human FGF21.

The recombinant strain, human-FGF21-PNZ8149/NZ3900 expresses human FGF21 *in vivo*

To confirm the ability of the recombinant strain to express Human FGF21 *in vivo*, the concentration of Human FGF21 was detected *in vivo* after induction with Nisin. Moreover, *in vivo* human FGF21 has a short half-life (Kharitonenkov *et al.*, 2007), thus the NICE system was used to artificially control the protein expression (Mierau and Kleerebezem, 2005; Olejnik-Schmidt *et al.*, 2013) to achieve the ultimate goal of extending half-life *in vivo*. After an oral administration to C57B6/J mice, the Human FGF21 levels in plasma increased significantly at various time points compared with existing research (Veniant *et al.*, 2012), and its serum half-life increased significantly (Figure 1D). This suggested that the recombinant strain of Human-FGF21-PNZ8149/NZ3900 can efficiently express Human FGF21 *in vivo*.

Human FGF21 expressing strain can induce reduction in body weight in Db/Db mice

To study the function of human FGF21 in the Db/Db mice strain, we treated Db/Db mice with this recombinant strain through oral administration. During the 13-week gavage treatment, weight monitoring results showed that the weight of the FGF21 treated group reduced significantly compared to the PNZ group (Figure 2A). In the same batch of mice, the images of *in vitro* tissues also clearly showed that the volume of adipose tissue in the FGF21 treated group was lesser than the PNZ group (Figure 2B). Moreover, various tissues, such as the inguinal adipose tissue and liver tissue, also showed weight loss and their fat droplets decrease when compared to the PNZ group (Figure 2C, 3A,B). These results indicate that human FGF21 expressing strain can induce a weight loss in Db/Db mice, which could be due to the weight loss of adipose tissues.

Table 4. LC-MS/MS sequencing of human-FGF21.

Accession	Description	Score	Coverage	# Proteins	# Unique peptides[1]	calc. pI
Q9NSA1	Fibroblast growth factor 21 OS=Homo sapiens GN=FGF21 PE= 1 SV=1 - [FGF21_HUMAN]	271.12	49.28	1	7	5.20
A0A087WTA8	Collagen alpha-2(I) chain OS=Homo sapiens GN=COL1A2 PE= 1 SV=1 - [A0A087WTA8_HUMAN]	26.29	3.67	2	5	9.01
P81605	Dermcidin OS=Homo sapiens GN=DCD PE=1 SV=2 - [DCD_HUMAN]	15.20	22.73	2	3	6.54
P02452	Collagen alpha-1(I) chain OS=Homo sapiens GN=COL1A1 PE= 1 SV=5 - [CO1A1_HUMAN]	11.21	1.91	1	3	5.80
Q9H6N6	Putative uncharacterised protein MYH16 OS=Homo sapiens GN=MYH16 PE=1 SV=2 - [MYH16_HUMAN]	2.18	1.09	7	1	5.49
P15259	Phosphoglycerate mutase 2 OS=Homo sapiens GN=PGAM2 PE= 1 SV=3 - [PGAM2_HUMAN]	0.00	3.95	3	1	8.88

[1] # Unique peptides >3: represents a credible protein.

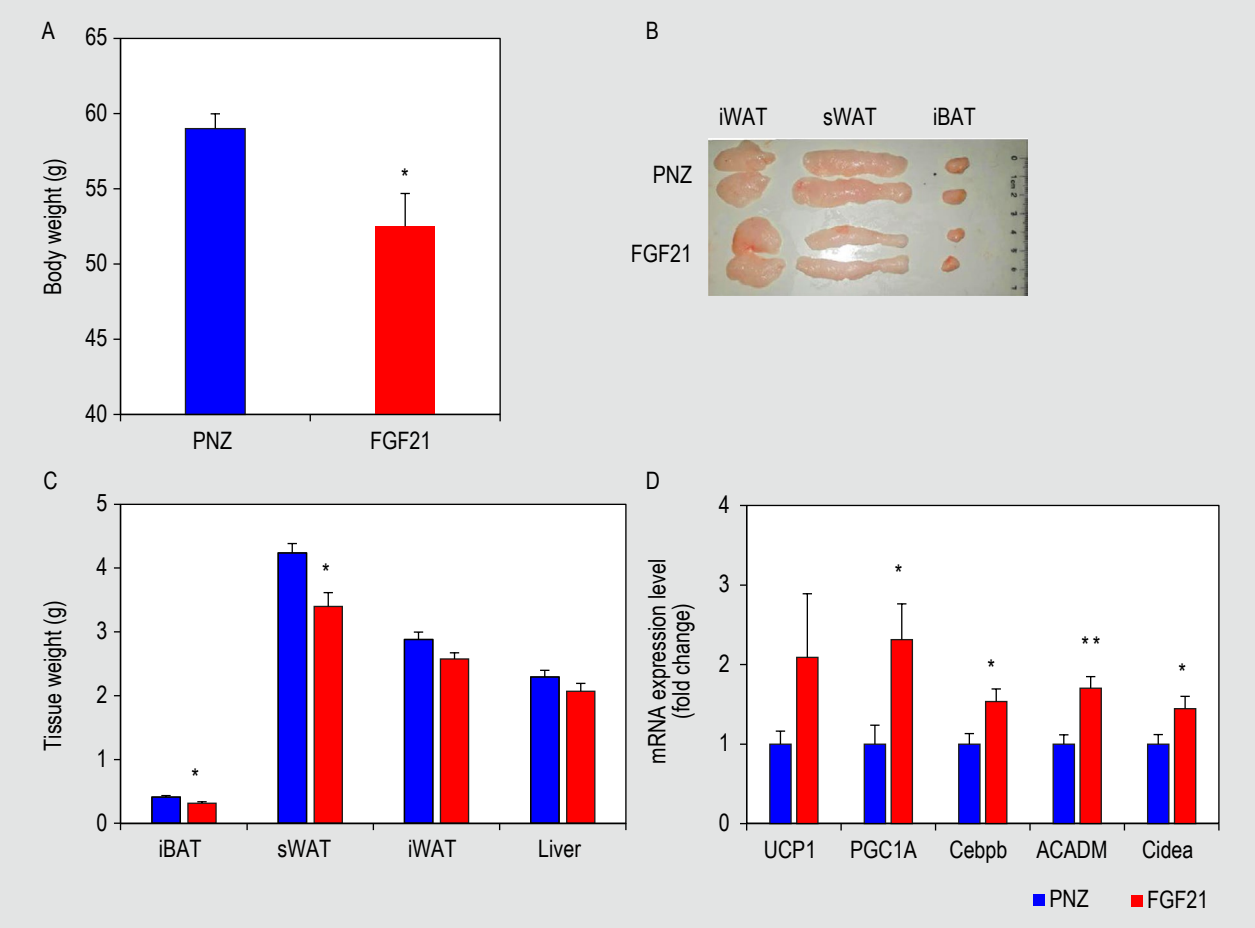

Figure 2. Human FGF21 expressing strain enables loss of body weight in Db/Db mice. (A) Weight of Db/Db mice who were orally administered with the recombinant strains for 13 weeks (n=8). (B,C) After the recombinant strains were given orally for 13 weeks, the tissues were removed, two groups of interscapular brown adipose tissue (iBAT), subcutaneous (sWAT), epididymis fat (iWAT) and the liver tissues were captured and the samples were weighed (n=8). (D) Real-time PCR of iBAT, after the recombinant strains were administered orally for 13 weeks (n=8).

Treatment with human FGF21 expressing strain can activate brown adipose tissue in Db/Db mice

In order to study the reasons for weight loss in the Db/Db mice induced by human FGF21 expressing strain, we analysed the marker gene in BAT, which is the main target of FGF21. Real-time PCR results of the genes in the BAT of the two groups of mice showed that the activity-related genes in BAT were up-regulated, such as UCP1 (uncoupling protein 1), PGC1α (peroxisome proliferator-activated receptor γ coactivator-1a), CEBPB (CCAAT/enhancer-binding protein beta), ACADM (acyl-coenzyme A dehydrogenase, C-4 to C-12 straight chain) and CIDE-A (cell death activator) (Figure 2D). The detection of protein levels in brown fat revealed that the oxidative phosphorylation-related proteins were up-regulated in the FGF21 treated group compared to the PNZ group of mice (Figure 4A,B). These results suggested that human FGF21 expressing strain can increase the activity of BAT by enhancing the transcription and translation levels of related genes.

Human FGF21 expressing strain can improve tissue status in Db/Db mice

To investigate the effect of human FGF21 expressing strain at the organ level in Db/Db mice, we performed HE-staining on the interscapular BAT and liver tissues of the two groups of Db/Db mice. The HE-staining results showed that the smaller lipid droplets of the interscapular BAT of Db/Db mice after treatment with human FGF21 expressing strain were more prominent than the control group (Figure 3A). At the same time, as the FGF21 induced fatty acid oxidation in liver (Fisher *et al.*, 2011; Villarroya *et al.*, 2013). Our results suggested that the fatty liver of Db/Db mice treated with human FGF21 expressing strain significantly improved compared with the control group (Figure 3B). Thus, our data showed that human FGF21 expressing strains can improve tissue status in Db/Db mice.

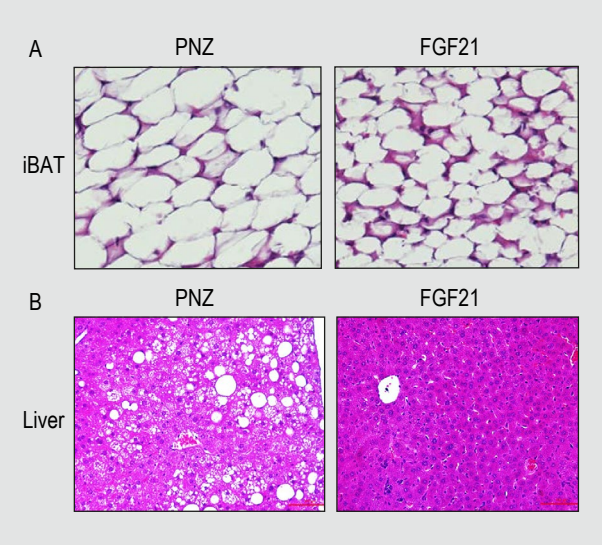

Figure 3. Human FGF21 expressing strain can improve organ status in Db/Db mice. (A) Interscapular brown adipose tissue (iBAT) histology using HE-staining. (B) Liver histology by HE-staining. PNZ = control Db/Db mice, orally given the PNZ8149/NZ3900 recombinant strain for 13 weeks; FGF21 = experimental group, orally given the Human-FGF21-PNZ8149/NZ3900 recombinant strain for 13 weeks.

4. Discussion

Our study is aimed at prolonging the biological half-life of human FGF21 by constructing a protein expression system (Huang *et al.*, 2013; Li and Tang, 2015; Mu *et al.*, 2012; Veniant *et al.*, 2012), which could also avoid the limitation of human FGF21. And on that basis, the recombinant human FGF21 was used to fight obesity and diabetes. Due to its inherent characteristics, FGF21 cannot pass through the digestive tract via oral administration (Li and Tang, 2015). Despite that (Itoh, 2014; Kharitonenkov and Adams, 2014; Kim and Lee, 2014; 2015), we successfully developed oral human FGF21 supplementation for the first time. The NICE protein expression system was applied, and the strain NZ3900 was chosen which is a food-grade safe lactic acid bacterium. Moreover, the nisin-induced expression system keeps the time and expression of human FGF21 under control. The NICE system has been widely used (Olejnik-Schmidt *et al.*, 2013) to express heterologous proteins, since the lactic acid bacteria are short-term intestinal parasites in the system (Alander *et al.*, 1999). Few researchers mainly focus on the expression of drugs which are effective only in the intestine (Yagnik *et al.*, 2016; Zamri *et al.*, 2012; Zhang *et al.*, 2009). We pioneered in using this protein expression system for producing human FGF21 and successfully detected human FGF21 protein in the plasma of mice.

We successfully constructed human FGF21 expressing strain named human FGF21-PNZ8149/NZ3900 and tested the expression of the recombinant strain *in vitro*. We identified the specificity of the recombinant strain by the LC-MS/MS sequencing method. A number of tests were performed in order to verify the functional activity of the target protein expressed in this system *in vivo*. If a drug works in the body, it should circulate through the bloodstream. Thus, we needed to focus on two questions: (1) whether the recombinant strain can successfully express human FGF21 protein *in vivo*; (2) whether human FGF21 protein could be absorbed into the blood.

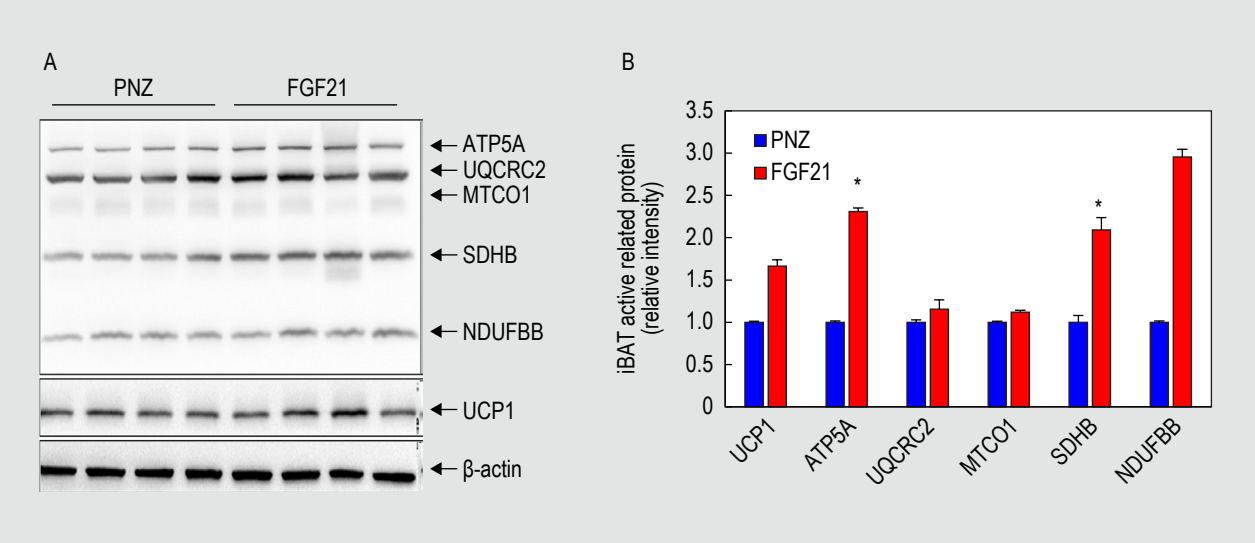

Figure 4. Human FGF21 expressing strain treatment can activate brown adipose tissue formation in Db/Db mice. (A) Representative western blot and (B) relative expression intensity from the interscapular brown adipose tissue (iBAT) from Db/Db mice orally treated for 13 weeks. PNZ8149/NZ3900 recombinant strain (PNZ) is the control; FGF21-PNZ8149/NZ3900 recombinant strain is the experimental group (FGF21).

Our results showed that the human FGF21 protein was successfully expressed in the serum of mice which have been administrated with human FGF21 expressing strain by gavage. As for the presence of basal values before gavage treatment, we consider that the homology of mouse FGF21 and human FGF21 is 75% (Seo and Kim, 2012). Surprisingly, the half-life of the protein was greatly extended compared to the one that has been previously reported as 0-2 h half-life (Angelin *et al.*, 2012; Kharitonenkov *et al.*, 2007; Veniant *et al.*, 2012). As the results showed that the peak concentration of human FGF21 in mice blood was 72 h after oral gavage treatment, the plasma half-life of the human FGF21 was significantly prolonged. This may be related to the short-term colonisation of the human FGF21 expressing strain in mice body, which is conducive to the continuous release of human FGF21.

Studies have shown that FGF21 plays an important role in metabolic diseases including obesity and type 2 diabetes. It also helps in weight loss, promoting blood glucose homeostasis as well as lipid metabolism, and reducing plasma triglyceride levels (Hondares *et al.*, 2011), thus improving homeostasis. FGF21 was mainly expressed in fat, liver, skeletal muscle and the brain. In this study, we focused on the role of BAT which takes part in the treatment of metabolic diseases. We detected changes in the expression levels of oxidative phosphorylation-related proteins in brown fat of mice in both groups by western blot. Our results showed that the expression level of oxidative phosphorylation related protein was increased in the human FGF21 expressing strain treated mice. According to existing studies, brown fat acts as the only tissue that plays a role in regulating non-shivering thermogenesis that occurs in the body (Boon and van Marken Lichtenbelt, 2016). The brown fat tissue is crucial in the regulation of energy metabolism (Poher *et al.*, 2015). It affects the partial oxidative phosphorylation process through the uncoupling action of BAT-specific thermogenic gene UCP1, so that the energy generated by the oxidation of substrate is released as heat rather being stored in the form of ATP (Cannon and Nedergaard, 2004; Oelkrug *et al.*, 2015). Recently, studies have shown that FGF21 promotes weight loss by increasing the thermogenic activity of mouse BAT and enhancing its thermogenesis (Coskun *et al.*, 2008; Hondares *et al.*, 2011; Kharitonenkov *et al.*, 2005; Xu *et al.*, 2009). Therefore, we checked if human FGF21 expressing strain could function *in vivo* by modulating BAT activity.

According to our experimental results, the body weight of mice treated with human FGF21 expressing strain significantly reduced, which was consistent with the previous reports of weight loss from FGF21 functioning (Giralt *et al.*, 2015). We found that the three kinds of adipose tissues were smaller and lighter than that of mice in the control group after sacrificing the Db/Db mice treated with human FGF21 expressing strain or the NZ3900/PNZ8149 recombinant strain, after 13 weeks.

Considering that the BAT plays a key role in weight loss (Saito, 2013), the morphology and activity of BAT was examined. The results of HE-staining showed that the lipid droplets of BAT in the mice treated with human FGF21 expressing strain were smaller than those in the control group. In addition, real-time PCR results revealed the transcriptional efficiency of the genes related to the activity of BAT was upregulated. We examined the expression of the oxidative phosphorylation related proteins which are in correlation with the activity of BAT. Moreover, western blot analysis displayed that these proteins were slightly upregulated after treatment with the human FGF21 expressing strain.

Since FGF21 is mainly expressed in the liver (Maratos-Flier, 2017), we observed the fatty liver status of the two groups of mice as well. HE-staining results showed that condition of the fatty liver of the mice that received the human FGF21 expressing strain treatment had greatly improved. However, compared with the dose measured in microgram in a previous report, our results estimated that the protein expression of human FGF21 recombinant strain could only be measured in nanograms, resulting in a slightly weak effect of human FGF21 expressing strain *in vivo* (Smith *et al.*, 2013). In the future, finding an efficient way to elevate the expression level of human FGF21 in a recombinant strain is indispensable for its clinical application. A very worthwhile approach is to change to a stronger promoter to increase its expression, and perhaps fewer cells' expression could meet the body's needs.

5. Conclusions

In summary, many studies have been done on FGF21 (Benedini and Luzi, 2016; Holmes, 2016; Marakova and Bazhan, 2016) and we successfully and innovatively applied the human FGF21 expressing strain through heterologous expression in *L. lactis*. The recombinant FGF21 expressing strain could express the human FGF21 well in the body and ameliorate obesity in Db/Db mice. Furthermore, the oral human FGF21 can enhance BAT activity and the liver fat in experimental group was improved. Last but not least, prolonged half-life of human FGF21 was achieved through oral gavage the human FGF21 expressing strain. Therefore, oral human FGF21 may open up a new avenue for the treatment of obesity and related diseases.

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