Anti-human fibroblast growth factor-21 monoclonal antibody preparation, characterization and analysis of *in vitro* bioactivity

LIANGJUN DING[1*], ZHICHAO HAO[2*], QINGYAN YUAN[1], PENGFEI XU[1], YINHANG YU[1] and DESHAN LI[1]

[1]Biopharmaceutical Department, College of Life Science, Northeast Agricultural University, Harbin, Heilongjiang 150030; [2]State Key Laboratory of Veterinary Biotechnology, Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences, Harbin, Heilongjiang 150069, P.R. China

Received June 9, 2015; Accepted August 5, 2016

DOI: 10.3892/etm.2016.3753

Abstract. Human fibroblast growth factor 21 (hFGF-21) is involved in numerous metabolic processes and elevated hFGF-21 levels are associated with many metabolic diseases. However, the role hFGF-21 serves in the metabolic system is not fully understood. A humanized anti-hFGF-21 monoclonal antibody (mAb) would provide a novel method for further investigations into the role hFGF-21 serves in the metabolic system and related diseases, which may reveal therapeutic targets for future treatment of these diseases. The present study aimed to prepare an anti-hFGF-21 mAb, followed by identification of its characteristics and bioactivity *in vitro*. The results of the present study identified that the anti-hFGF-21 mAb (clone 2D8) produced had good specificity, had an immunoglobulin isotype of IgG2b and a titer of 1:1.024x10^6. hFGF-21 was screened for epitopes using fluorescence-activated cell sorting, which revealed a specific 15 amino acid sequence (YQSEAHGLPLHLPGN) that the anti-hFGF-21 mAb recognized. *In vitro* bioactivity of anti-hFGF-21 was determined using a glucose uptake assay and by measuring the expression of glucose transporter 1 (GLUT1) messenger RNA (mRNA) in 3T3-L1 adipocytes. This revealed that hFGF-21-dependent glucose uptake and GLUT1 mRNA expression were negatively correlated with increasing levels of the anti-hFGF-21 mAb tested, and that hFGF-21 activity could be overcome by increasing concentrations of the mAb, demonstrating that the mAb has hFGF-21-neutralizing activity *in vitro*.

Correspondence to: Professor Deshan Li, Biopharmaceutical Department, College of Life Science, Northeast Agricultural University, 59 Mucai Street, Harbin, Heilongjiang 150030, P.R. China
E-mail: deshanli@163.com

*Contributed equally

Key words: characterization, epitope, glucose uptake, bioactivity

Introduction

Human fibroblast growth factor 21 (hFGF-21), which is a member of the FGF family, is known to be a regulator of the metabolic system and can increase glucose uptake and GLUT1 mRNA expression (1,2). For example, there is a weak cause-effect relationship between hFGF-21 and ketogenesis in humans (3,4). In addition, previous studies reported that hFGF-21 regulates glucose and lipid metabolism via its interactions with the peroxisome proliferator-activated receptor alpha and gamma pathways (5-7). However, the role of hFGF-21 in the metabolic system remains unclear. In the present study, an anti-hFGF-21 monoclonal antibody (mAb) was prepared and characterized, in order to provide a novel method for researching the molecular mechanisms by which hFGF-21 acts in the metabolic system.

Previous studies identified that elevated serum levels of hFGF-21 were associated with numerous diseases, including diabetes (8-10). In addition, a previous study revealed an association between hFGF-21 serum levels and long term diabetic complications, such as nephropathy and carotid atheromatosis (11). Furthermore, increased serum levels of hFGF-21 have been found to be associated with obesity (12). Additionally, elevated serum hFGF-21 levels were discovered in patients with ischemic heart disease (1). Conversely, improvement in the symptoms of patients with diseasesassociated with elevated hFGF-21 serum levels is typically accompanied with reduced serum levels of hFGF-21, for example, weight loss was accompanied by a decrease in hFGF-21 levels in humans with obesity-related metabolic complications (13). and hFGF-21 levels decreased in diabetic patients following therapy with insulin or oral agents (14). In the current study, anti-hFGF-21 mAb (clone 2D8) was produced, purified and characterized *in vitro*. Then, new lipoprotein A (NlpA) -based bacterial display library technology was used for epitope screening. In addition, the *in vitro* bioactivity of the mAb was determined using a glucose uptake assay and by measuring glucose transporter 1 (GLUT1) mRNA expression. There is a lack of relevant previous studies on the anti-hFGF-21 mAb and its bioactivity. The present study identified that the mAb prepared could specifically detect serum levels of hFGF-21 and thus has potential as a prognostic factor to indicate the development of hFGF-21-related diseases. In addition, it could be used for

future research into hFGF-21, which may identify therapeutic targets for the treatment of hFGF-21-associated diseases.

Materials and methods

Ethics statement. All experiments in the present study were approved by the Northeast Agricultural University Provincial Experimental Animal Management Committee (Harbin, China) and were performed in accordance with the guidelines of this committee.

Chemicals and reagents. Freund's adjuvant (complete), incomplete Freund's adjuvant, bovine serum albumin (BSA) and 3,3',5,5'-tetramethylbenzidine (TMB) were purchased from Sigma-Aldrich (Merck Millipore, Darmstadt, Germany). Horseradish peroxidase (HRP)-conjugated goat anti-mouse secondary antibody (A21010) was purchased from Abbikine, Inc. (Redlands, CA, USA). SAB Clonotyping System-HRP (5300-05) was purchased from SouthernBiotech (Birmingham, AL, USA). Fluorescein isothiocyanate (FITC) Antibody Labeling Kit (53027) was purchased from Thermo Fisher Scientific, Inc., (Waltham, MA, USA). Glucose Assay Kit (0105102), which utilzizes the GOD-PAP method was purchased from Sichuan Maccura Biotechnology Co., Ltd (Chengdu, China). Other reagent grade chemicals were purchased from Sigma-Aldrich (Merck Millipore). DNA $maker_{2000}$, $\lambda EcoT_{14}$ DNA Marker, and prestained protein MW Marker were purchased from Fermentas (Thermo Fisher Scientific, Inc.). All polymerase chain reaction (PCR) primers (Tables I and II) were synthesized by Invitrogen (Thermo Fisher Scientific, Inc.).

Cell culture. Sp2/0 lymphocytes, 3T3-L1 adipocytes and DH5α *Escherichia coli* were lab stocks. DH5α (MLCC3002) was purchased from Miaolingbio Bioscience & Technology Co., Ltd., (Wuhan, China; -80˚C). Sp2/0 (CC-Y2093), which were cultured in RPMI-1640 supplemented with 10% fetal bovine serum (FBS) at 37˚C in an atmosphere containing 5% CO_2, and 3T3-L1 adipocytes (CC-Y2002), which were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% FBS at 37˚C, 5% CO_2, were both purchased from Enzyme Research Biotechnology Co., Ltd., (Beijing, China). RPMI-1640 (CM0302) was purchased from You Kang Biotechnology Co., Ltd., (Beijing, China). DMEM (PM150310) was purchased from Procell (Wuhan, China).

hFGF-21 expression and purification. Whole hFGF-21 protein was expressed and purified during previous studies conducted in our laboratory (2).

Experimental animals. Six female and six male BALB/c mice (age, 6-8 weeks old; weight, 11-13 g) were purchased from Harbin Veterinary Research Institute (Harbin, China), and housed in starter batteries with access to water and commercial feed.

Anti-hFGF-21 mAb (clone 2D8) production. BALB/c mice (female, n=3) were immunized with 100 μg hFGF-21 (as 400 ml of 1:1 hFGF-21: Freund's adjuvant), after 2-weeks of feeding, followed by second immunization with 100 μg hFGF-21 (400 ml of 1:1 hFGF-21: incomplete Freund's adjuvant), third immunization was the same as the second immunization and was performed 2-weeks later. Prior to hybridoma production, the mice received a booster immunization of 100 μg hFGF-21 in phosphate-buffered saline (PBS; pH 7.5), and separated eyeball blood samples as positive serum. BALB/c mice (female, n=3) under the same rearing conditions were used to obtain negative serum by separating eyeball blood sampling. The establishment methods of hybridoma were performed according to previously described methods (15).

Indirect ELISA was performed to screen for individual clones secreting hFGF-21 mAb. Prior to cell plating, the 96-well plates were coated with 20 μg/ml hFGF-21 (100 μl) and incubated at 4˚C overnight. Then, plates were washed three times with washing buffer (0.05% Tween-20 in PBS), blocked with 5% skimmed milk in PBS for 2 h at 37˚C, followed by washing (as previously described). Hybridomas cultures (100 μl) as primary antibodies (positive serum as positive control, negative serum as negative control) were added to the wells and incubated for 1 h at 37˚C. Following washing (as described above), plates were incubated with HRP-conjugated goat anti-mouse secondary antibody (1:15,000; 100 μl) for 1 h at 37˚C, followed by washing (as previously described). The chromogenic reagent TMB (100 μl) was added to the wells and following 20 min the reaction was stopped with 2 mol/l H_2SO_4 (50 μl), and the plates spectroscopically analyzed at 450 nm using a microplate reader (Bio-Rad, Hercules, CA, USA). hFGF-21-positive clones were then subcloned and rescreened (as previously described).

Purification, classification and SDS-PAGE analysis of anti-hFGF-21 mAb. For large-scale production of the hFGF-21 mAb, a single highly positive subclone hybridoma cells (2D8) were injected into the peritoneal cavity of BALB/c mice (male, n=3) and allowed to proliferate prior to collection of fluid from ascites, the Sp2/0 cells were injected into the peritoneal cavity of BALB/c mice (male, n=3) and allowed to proliferate prior to collection of fluid from ascites as negative control. The fluid was centrifuged (12,000 x *g*, 10 min; 4˚C) and the supernatant purified using a Protein A-Sepharose (GalaxyBio, Beijing, China). The concentration of purified mAb was measured by a spectrophotometer, and it was used as the source of the 2D8 hFGF-21 mAb used in the present study. The mAb was further analyzed using SDS-PAGE (resolving gel, 15%; stacking gel, 5%; 1 h at 200 V; 10 μl sample; 10 ng). The class and subclass of the mAb was determined using the mouse mAb isotyping kit.

Titer measurements. With the exception of to the purified mAbs (gradient dilution from $250x2^0$-$250x2^{13}$; 100 μl) replaced with hybridomas cultures (100 μl) as primary antibodies, (purified Sp2/0 ascites replaced hybridomas cultures as negative control), the other operating methods were the same as indirect ELISA, as previously described. Each sample was tested in triplicate and the results presented as the mean ± standard deviation. The maximum dilution ratio of the positive well value and negative control greater than or equal to 2.1 is the titer of the mAb.

Western blot analysis. The hFGF-21 protein and negative control (BSA) were used analyzed by western blot. Once the quality of hFGF-21 and BSA were consistent, samples (10 ng) were loaded onto a 12% gel, subjected to SDS-PAGE

and blotted on to a polyvinylidene difluoride membrane. The membrane was blocked with 5% skimmed milk in tris-buffered saline with Tween-20 (TBST; 10 mmol/l pH 7.5 Tris-HCl; 150 mmol/l NaCl) at 4˚C overnight. Following washing three times for 15 min in TBST, the membrane was incubated with the mAb (1:500 dilution) for 1 h at room temperature. Following washing (as described above), the membrane was incubated with HRP-conjugated goat anti-mouse secondary antibody (1:7,500 dilution) at room temperature for 1 h. The membrane was then washed again (as described above) and blots were visualized by Super Signal West Femto Maximum Sensitivity Substrate (34095; Thermo Fisher Scientific, Inc.) and SmartChem™ II image analysis system (SG2011060; Sagecreation, China).

Analysis of cross-reactivity. Indirect ELISA was performed to determine the cross-reactivity between hFGF-21 and mouse FGF-21 (mFGF-21) produced in our laboratory (16). In the ELISA, 20 μg/ml of hFGF-21, mFGF-21 or BSA (negative control) were used as the coating antigen and 2.5 μg/ml of the mAb (100 μl) was used as the primary antibody. All other steps of the ELISA were as previously described. All assays were performed in triplicate and the results presented as the mean ± standard deviation.

APEx bacterial display analysis to identify the anti-hFGF-21 mAb epitope. Anchored periplasmic expression (APEx) bacterial display vectors containing the new lipoprotein A (NlpA) sequence (CDQSSS) plus 6 amino acids, obtained via previous studies published by our laboratory (17-19). hFGF-21 sequence was obtained from our previous study (2).

For the first round of screening, the hFGF-21 polypeptide sequence was divided into 2 segments that included polymerase chain reaction upper sequence (PU: 1-91 amino acids upstream of hFGF-21) and polymerase chain reaction downstream sequence (PD: 92-181 amino acids downstream of hFGF-21), and then cloned (primers described in Table I) into the APEx . The cloning vector was constructed from the secretory expression vector pET27b(+) as previously described (20). The vectors formed (APEx-PU and APEx-PD) were analyzed using the restriction enzyme *Sfi* I, and 1% gel analysis was performed to determine the size of PU and PD.

Vectors were transformed into DH5α *E. coli*, which were cultured in LB media (Amp 100 μg/ml; 37˚C for 4 h). When the optical density (OD) reading of the culture at 600 nm (OD_{600}) reached between 0.3 and 0.4, 0.25 mmol/l of isopropyl-β-D-thiogalactoside (IPTG) was added. The *E. coli* were grown at 37˚C for 4 h to induce the expression of PU or PD. Then, bacterial cells (1 ml) were collected, centriguged (12,000 x *g*; 5 min; 4˚C), washed twice with PBS and resuspended in 350 μl of ice-cold suspension solution (0.75 mol/l sucrose, 0.1 mol/l Tris-HCl pH 8.0) supplemented with 10 mg/ml lysozyme (35 μl; Shanghai Yi Sheng Biotechnology Co., Ltd., Shanghai, China). Cells were treated with 1 mmol ice-cold EDTA (700 μl) and 0.5 mol $MgCl_2$ (50 μl), and centrifuged again as previously described. Following this, cell pellets were centrifuged as previously described, resuspended in 90 μl PBS, and incubated with 2 mg/ml FITC-labeled anti-hFGF-21 mAb (4 μl) and 10 μl 1% BSA at 4˚C for 1 h. Then, the cells were washed three times with 0.5% PBS-Tween and resuspended in 500 μl of PBS for fluorescence-activated cell sorting (FACS) analysis at 488 nm. The negative control was treated the same, except that it was not incubated with the FITC-labeled mAb.

For the second round of screening, the PD was divided into 5 overlapping segments, which included PD_1 (amino acids 92-121 of hFGF-21), PD_2 (amino acids 122-151 of hFGF-21), PD_3 (amino acids 152-181 of hFGF-21), PD_4 (amino acids 107-136 of hFGF-21) and PD_5 (amino acids 137-166 of hFGF-21). The 5 segments were cloned and cloned into the APEx bacterial display vector to produce APEx-PD_1, APEx-PD_2, APEx-PD_3, APEx-PD_4 and APEx-PD_5. The steps described in the first screening were then performed.

3T3-L1 stimulation with hFGF-21 and anti-hFGF-21 mAb. 3T3-L1 adipocytes ($1x10^6$ per ml) were seeded into 96-well plates and incubated at 37˚C in a 5% CO_2, and 95% humidified atmosphere overnight. Then, the cells were starved for 12 h in serum-free DMEM, followed by stimulation with hFGF-21 (1,000 nmol/l) and anti-hFGF-21 mAb (0.0, 0.075, 0.15, 0.3, 0.6 or 1.2 μg/ml) for 24 h. Controls were not stimulated.

Glucose uptake assay. Glucose uptake of 3T3-L1 adipocytes was measured following stimulation using the glucose uptake assay kit, according to the manufacturer's instructions. Absorbance was recorded at 490 nm and the glucose consumption rate was calculated. Each sample was tested in triplicate and the results presented as the mean ± standard deviation.

Measuring GLUT1 mRNA expression

RNA isolation and reverse transcription (RT). Following stimulation of 3T3-L1 adipocytes (as described above), total RNA was isolated using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions. Total RNA was subsequently converted to cDNA using 10 μl oligo-dT primers (500 μg/ml) and 10 μl dNTP (10 mmol/l each) in 100 μl ddH_2O at 65˚C for 5 min, followed by cycling at 42˚C for 50 min with 40 μl 5X first-strand buffer, 20 μl 0.1 MDTT, 10 μl RNase inhibitor (40 U/μl) and 10 μl M-MLV reverse transcriptase (200 U/μl). The reaction was terminated by heating at 70˚C for 15 min.

Quantitative PCR (qPCR). qPCR was performed using the Thermal Cycler Dice Real Time PCR System (Takara Biotechnology Co., Ltd., Dalian, China), according to the manufacturer's instructions. The PCR reaction (20 μl final volume) contained 10 μl SYBR Premix Ex Taq (2X), 2 μl cDNA (as described above), 0.4 μl of each primer (Table II, 10 μmol/l), 0.4 μl ROX Reference Dye II (50X) 25 μmol and 6.8 μl of double distilled H_2O. PCR was then performed as follows: 95˚C for 10 sec; 40 cycles of 95˚C for 5 sec; and 60˚C for 34 sec. Each sample was tested in triplicate. The mean cycle quantification (Cq) was calculated for GLUT1 and β-actin. The quantity of GLUT1 mRNA copies was normalized to β-actin [ΔCq = Cq (GLUT1) - Cq (β-actin)]. The ΔΔCq value was calculated as: ΔΔCq = ΔCq (stimulated sample) - ΔCq (non-stimulated sample). The relative quantity of GLUT1 mRNA copies in the stimulated sample compared with the non-stimulated sample was calculated using the $2^{-\Delta\Delta Cq}$ method (21). The results were presented as the mean ± standard deviation.

Table I. Primer sequences for polymerase chain reaction amplification of different hFGF-21 segments.

Segment	Primer sequence (5'-3')
PU	F: GGCCCAGCCGGCCCACCCCATCCCTGACTCC
	R: GGCCCCCGAGGCCTTACTCAGGGTCAAAGTGGAGCGAT
PD	F: GGCCCAGCCGGCCGCCTGCAGCTTCCGGGAG
	R: GGCCCCCGAGGCCTCAGGAAGCGTAGCTGGGGC
PD_1	F: GGCCCAGCCGGCCGCCTGCAGCTTCCGGGAG
	R: GGCCCCCGAGGCCTTAGTTCCCTGGCAGGTGCAGC
PD_2	F: GGCCCAGCCGGCCAAGTCCCCACACCGGG
	R: GGCCCCCGAGGCCTTATCCGGGTGGCTCCGGGAG
PD_3	F: GGCCCAGCCGGCCATCCTGGCCCCCCAGCC
	R: GGCCCCCGAGGCCTCAGGAAGCGTAGCTGGGGC
PD_4	F: GGCCCAGCCGGCCTACCAGTCCGAAGCCCACG
	R: GGCCCCCGAGGCCTTAGAAGCGAGCTGGTCCTC
PD_5	F: GGCCCAGCCGGCCCTGCCACTACCAGGCCTGC
	R: GGCCCCCGAGGCCTTACAGAGGGTCCGAGGAGC

Underlining indicates the *Sfi* I restriction sites. F, forward; R, reverse; PU, polymerase chain reaction upper sequence; PD, polymerase chain reaction downstream sequence.

Table II. Primer sequences for the quantitative polymerase chain reaction.

Gene name	Primer sequences (5'-3')
β-actin	F: GAGACCTTCAACACCCC
	R: GTGGTGGTGAAGCTGTAGCC
GLUT1	F: CCATCCACCACACTCACCAC
	R: GCCCAGGATCAGCATCTCAA

GLUT1, glucose transporter 1; F, forward; R, reverse.

Statistical analysis. Data was analyzed using a two-tailed Student's t-test. All analyses were performed using SPSS software (version 13.0; SPSS, Inc., Chicago, IL, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Preparation and characterization of the anti-hFGF-21 mAb (clone 2D8). Secretory hybridoma cells were cloned, followed by recloning three times to increase monoclonality of the produced immunoglobulins (Igs). One stable hybridoma, designated 2D8 was obtained. The concentration of the mAbs produced following purification was 2.3 mg/ml. As expected, the heavy chain of the mAbs was 50 ku, and light chain was 25 ku (Fig. 1A). The isotype of the mAb was determined to be IgG2b. The titer of the mAb was $1:1.024x10^6$ (Fig. 1B). Western blot analysis revealed that hFGF-21 could be detected by the mAb, whereas the negative control (BSA) had no reaction with the mAb, indicating good specificity (Fig. 1C). Cross-reactivity analysis identified that compared with the negative control (BSA), the mAb reacted significantly with mFGF-21 and hFGF-21 ($P<0.01$; Fig. 1D), which have ~80% amino acid sequence homology (16). The reaction with hFGF-21 reaction was stronger compared with the mFGF-21 reaction (Fig. 1D), which is consistent with previous published data (16).

Identification of the anti-hFGF-21 mAb (clone 2D8) epitope. Clones of different hFGF-21 segments showed that PU and PD were both 273 base pairs (bp) in length (Fig. 2A), and that the 5 different segments of PD were all 90 bp (Fig. 2B). The results of restriction enzyme analysis identified that each segment was successfully cloned into the APEx bacterial display vector (Fig. 2C and D). The first round of FACS (Fig. 3A-C) showed that, compared with the negative control (Fig. 3A), the mAb could not bind to the PU segment (Fig. 3B), whereas it could strongly bind to the PD segment (Fig. 3C), which suggests that the epitope the mAbs recognizes is in the PD segment. The second round of FACS (Fig. 3D-I) determined that, compared with negative control (Fig. 3D), the mAb could not bind to PD_2, PD_3 or PD_5 (Fig. 3F, G and I, respectively), whereas it could strongly bind to PD_1 and PD_4 (Fig. 3E and H). This revealed that the epitope that the mAb binds to was in the region overlapping the PD_1 and PD_4 segments, a specific fifteen amino acid sequence (YQSEAHGLPLHLPGN).

Glucose uptake assay. In the glucose uptake assay a control group (without hFGF-21 and the mAb) and 6 groups stimulated with hFGF-21 (1,000 nmol/l) and different concentrations of the mAb were tested. Results of the assays determined that, compared with the control, the mAb (0.0 μg/ml) and hFGF-21 group had significantly increased glucose uptake ($P<0.01$; Fig. 4A), which is consistent with the results of a previous study (1). The mAb (0.075 μg/ml) and hFGF-21 group also had a significantly increased glucose uptake compared with the control ($P<0.05$; Fig. 4A). However, the mAb (0.15, 0.3, 0.6 or 1.2 μg/ml) and hFGF-21 groups did not have increased glucose uptake compared with the mAb(0) group (Fig. 4A),

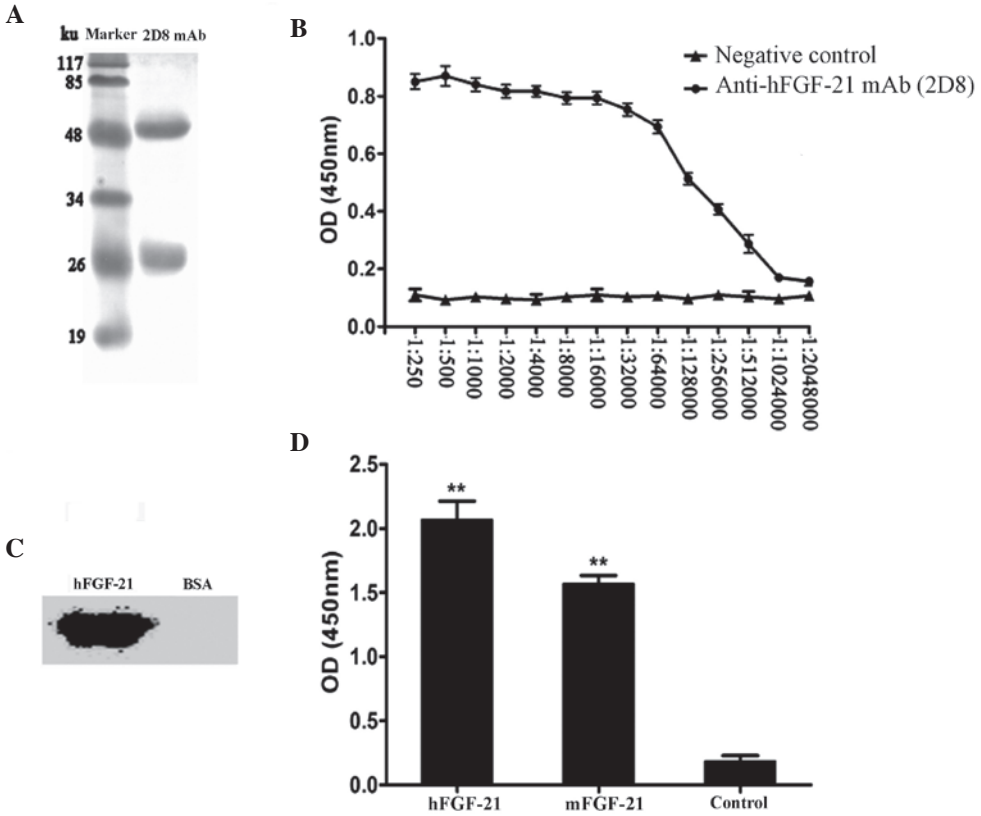

Figure 1. Characterization of anti-hFGF-2 mAb (2D8). (A) SDS-PAGE analysis. (B) Titer measurements, showing the mAb titer was $1:1.024x10^6$ (negative control, BSA). (C) Western blotting. (D) Cross-reactivity reaction results (negative control, BSA). $^{**}P<0.01$ vs. the control group. mAb, monoclonal antibody; OD, optical density; hFGF-21, human fibroblast growth factor-21; BSA, bovine serum albumin; mFGF-21, mouse fibroblast growth factor-21.

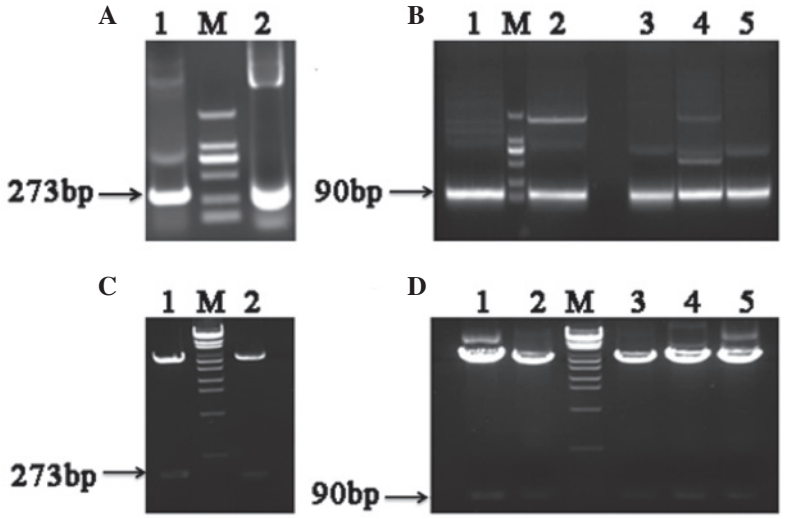

Figure 2. Electrophoretic analysis of polymerase chain reaction (PCR) human fibroblast growth factor-21 (hFGF-21) segment products and restriction enzyme analysis of anchored periplasmic expression (APEx) bacterial display vectors. (A) Electrophoretic analysis of PCR products. 1, polymerase chain reaction upper sequence PCR product; M, DNA marker; 2, polymerase chain reaction downstream sequence PCR product. (B) Electrophoretic analysis of PCR products. 1, PD_1 product; M, DNA marker; 2, PD_2 product; 3, PD_3 product; 4, PD_4 product; 5, PD_5 product. (C) Restriction enzyme analysis. 1, APEx-PU; M, $\lambda EcoT_{14}$ DNA marker; 2, APEx-PD. (D) Restriction enzyme analysis. 1, APEx-PD_1; 2, APEx-PD_2; M, $\lambda EcoT_{14}$ DNA marker; 3, APEx-PD_3; 4, APEx-PD_4; 5, APEx-PD_5. PU, 1-91 amino acids upstream of hFGF-21; PD, 92-181 amino acids downstream of hFGF-21. Bp, base pairs.

which indicates that the mAb reduces hFGF-21-dependent glucose uptake *in vitro*.

GLUT1 mRNA expression. GLUT1 mRNA expression was quantified in a control group (without hFGF-21 and the mAb) and in 6 groups stimulated with hFGF-21 (1,000 nmol/l) and different concentrations of the mAb. The results of qPCR analysis identified that, compared with the control, the mAb (0.0 μg/ml) and hFGF-21 group had significantly increased GLUT1 mRNA expression ($P<0.01$; Fig. 4B), which is consistent

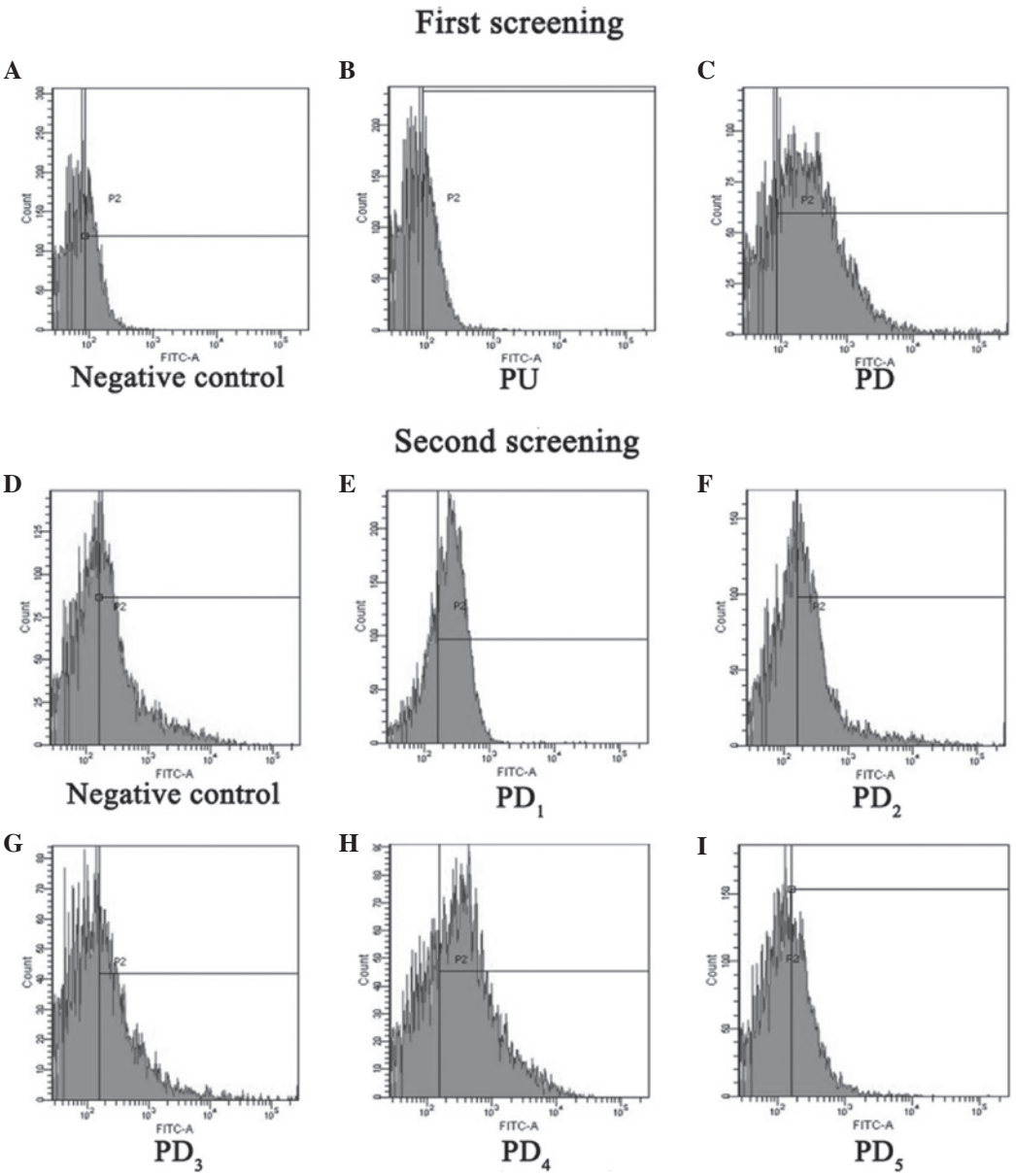

Figure 3. Epitope screening by fluorescence-activated cell sorting analysis. The first screening included the a (A) negative control, (B) PU and (C) PD. The second screening included a (D) negative control, (E) PD_1, (F) PD_2, (G) PD_3, (H) PD_4 and (I) PD_5. PU, 1-91 amino acids upstream of hFGF-21; PD, 92-181 amino acids downstream of hFGF-21; FITC, fluorescein isothiocyanate; PU, polymerase chain reaction upper sequence; PD, polymerase chain reaction downstream sequence.

with the results of a previous study (1). The mAb (0.075 μg/ml) and hFGF-21 group also had significantly increased GLUT1 mRNA expression ($P<0.05$; Fig. 4B). However, mAb (0.15, 0.3, 0.6 or 1.2 μg/ml) and hFGF-21 groups did not have increased GLUT1 mRNA expression (Fig. 4B). This indicates that the mAb reduces hFGF-21-dependent GLUT1 mRNA expression *in vitro* compared with the Ab(0) group.

Discussion

An anti-hFGF-21 2D8 mAb was prepared and purified in the present study. Bacterial display technology was used to screen for the epitope recognized by the mAb. Harvey *et al* (20) first reported the NlpA-based bacterial display technology. Our APEx bacterial display vectors were obtained from our laboratory previous studies (17-19). Different hFGF-21 segments were anchored to the inner membrane of the host cell, in order to allow screening for the epitope that anti-hFGF-21 mAb (2D8) recognizes. Bacterial cells are the host of choice for expression of recombinant proteins, due to their fast expression foreign genes, rapid growth rate and ease of genetic manipulation (20,22-26). APEx-FACS enables real-time visualization of mAb-peptide binding, which allowed identification of the epitope that the anti-hFGF-21 mAb recognized. The anti-hFGF-21 mAb-binding ability of different hFGF-21 segments could be visualized via the FITC-label on the mAb during FACS. In addition, the intensity of fluorescence during FACS reflected the binding affinity. The segments PD_1 and PD_4 showed high mAb-binding ability, therefore, the anti-hFGF-21 mAb epitope was located in the region overlapping both segments. Compared with traditional method, the APEx-FACS reduced the time taken to screen for epitopes, reduced costs

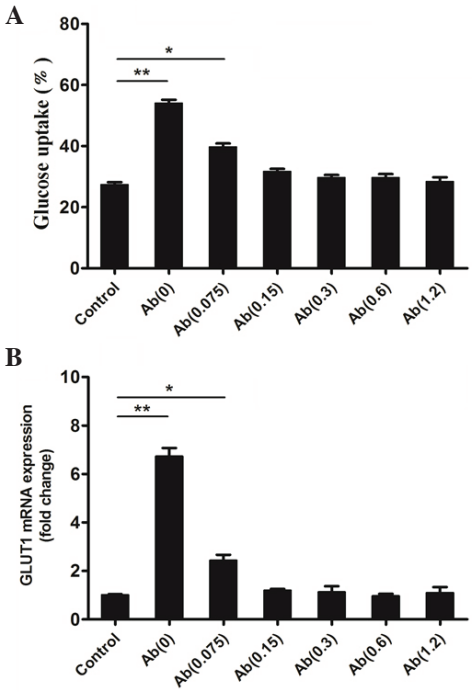

Figure 4. Anti-human fibroblast growth factor-21 (anti-hFGF-21) monoclonal antibody (mAb) (2D8) bioactivity assays in 3T3-L1 adipocytes. (A) Effect of the mAb on hFGF-21-dependent glucose uptake. Glucose uptake rate (%) = $[(C_{blank} - C_{sample}) / C_{blank}]$ x 100%; whereas C_{blank} refers to the glucose concentration in culture medium without 3T3-L1 adipocytes and C_{sample} refers to the glucose concentration in culture medium with 3T3-L1 adipocytes. (B) Effect of the mAb on hFGF-21-dependent GLUT1 mRNA expression (compared with the control). $^{*}P<0.05$ and $^{**}P<0.01$ vs. control group (no hFGF-21 or mAb stimulation). Ab, anti-hFGF-21 mAb (2D8); GLUT1, glucose transporter 1.

and was thus more efficient (17). Numerous previous studies have shown that hFGF-21 serves an important role in metabolism, for example, hFGF-21 has been shown to activate the coreceptor β-Klotho (27-29). The present study identified that the epitope of the anti-hFGF-21 mAb was close to the binding sites of the coreceptor β-Klotho and hFGF-21. Therefore, the results of the current study suggest an interaction between hFGF-21 and the coreceptor β-Klotho.

The results of the *in vitro* glucose uptake bioactivity of the mAb indicated that the mAb decreased hFGF-21-dependent glucose uptake compared with the Ab(0) in 3T3-L1 adipocytes. To determine a potential molecular mechanism for this effect of the mAb, its effect on hFGF-21-dependent GLUT1 mRNA expression in 3T3-L1 adipocytes was investigated. As expected, compared with the Ab(0) hFGF-21-dependent GLUT1 upregulation was reduced with increasing concentrations of the mAb, suggesting that this is the mechanism through which the mAb decreased glucose uptake. These assays provided evidence that the mAb had hFGF-21-neutralizing activity.

Therapeutic antibodies, including those for cancer, autoimmune diseases and viral infections, are widespread. Therapeutic effect of the humanized mAb (Palivizumab) to treat respiratory syncytial virus infection was confirmed a decade ago (30). The anti-hFGF-21 mAb (clone 2D8) prepared and analyzed in the present study has potential as a novel clinical treatment for hFGF-21-related diseases. Therefore, the current study provides a solid foundation for future research and development of the humanized anti-FGF-21 mAb (clone 2D8).

In conclusion, the present study prepared and characterized an anti-hFGF-21 mAb (2D8), which will provide a novel tool for future research investigating the role that hFGF-21 serves in metabolism. The epitope of the mAb was identified as a specific fifteen amino acid sequence (YQSEAHGLPLHLPGN). In addition, compared with the Ab(0) the mAb was found to reduce hFGF-21-dependent glucose uptake by decreasing hFGF-21 dependent-GLUT1 mRNA expression in 3T3-L1 adipocytes, which demonstrated that the mAb had hFGF-21-neutralizing activity. This study, to the best of our knowledge, is the first to identify the epitope of anti-hFGF-21 mAb (2D8) and study its bioactivity *in vitro*. The 2D8 anti-hFGF mAb described in the present study has potential as a therapeutic agent and further research should be conducted to investigate the this application.

Acknowledgements

The present study was supported by The National Natural Science Foundation of China Youth Science Project Fund (grant no. 31001084).

References

1. Kharitonenkov A, Shiyanova TL, Koester A, Ford AM, Micanovic R, Galbreath EJ, Sandusky GE, Hammond LJ, Moyers JS, Owens RA, *et al*: FGF-21 as a novel metabolic regulator. J Clin Invest 115: 1627-1635, 2005.
2. Hou YT, Li JN, Ren GP, Liu MY, Sun GP, Wang WF and Li DS: Cloning, expression and glucose regulation activity of human FGF-21. Yi Chuan 32: 583-587, 2010 (In Chinese).
3. Adams AC and Kharitonenkov A: FGF21: The center of a transcriptional nexus in metabolic regulation. Curr Diabetes Rev 8: 285-293, 2012.
4. Gälman C, Lundåsen T, Kharitonenkov A, Bina HA, Eriksson M, Hafström I, Dahlin M, Amark P, Angelin B and Rudling M: The circulating metabolic regulator FGF21 is induced by prolonged fasting and PPARalpha activation in man. Cell Metab 8: 169-174, 2008.
5. Badman MK, Pissios P, Kennedy AR, Koukos G, Flier JS and Maratos-Flier E: Hepatic fibroblast growth factor 21 is regulated by PPARalpha and is a key mediator of hepatic lipid metabolism in ketotic states. Cell Metab 5: 426-437, 2007.
6. Inagaki T, Dutchak P, Zhao G, Ding X, Gautron L, Parameswara V, Li Y, Goetz R, Mohammadi M, Esser V, *et al*: Endocrine regulation of the fasting response by PPARalpha-mediated induction of fibroblast growth factor 21. Cell Metab 5: 415-425, 2007.
7. Moyers JS, Shiyanova TL, Mehrbod F, Dunbar JD, Noblitt TW, Otto KA, Reifel-Miller A and Kharitonenkov A: Molecular determinants of FGF-21 activity-synergy and cross-talk with PPARgamma signaling. J Cell Physiol 210: 1-6, 2007.
8. Xiao Y, Xu A, Law LS, Chen C, Li H, Li X, Yang L, Liu S, Zhou Z and Lam KS: Distinct changes in serum fibroblast growth factor 21 levels in different subtypes of diabetes. J Clin Endocrinol Metab 97: E54-E58, 2012.
9. Chen WW, Li L, Yang GY, Li K, Qi XY, Zhu W, Tang Y, Liu H and Boden G: Circulating FGF-21 levels in normal subjects and in newly diagnose patients with Type 2 diabetes mellitus. Exp Clin Endocrinol Diabetes 116: 65-68, 2008.
10. Mashili FL, Austin RL, Deshmukh AS, Fritz T, Caidahl K, Bergdahl K, Zierath JR, Chibalin AV, Moller DE, Kharitonenkov A and Krook A: Direct effects of FGF21 on glucose uptake in human skeletal muscle: Implications for type 2 diabetes and obesity. Diabetes Metab Res Rev 27: 286-297, 2011.
11. Stein S, Bachmann A, Lössner U, Kratzsch J, Blüher M, Stumvoll M and Fasshauer M: Serum levels of the adipokine FGF21 depend on renal function. Diabetes care 32: 126-128, 2009.
12. Dushay J, Chui PC, Gopalakrishnan GS, Varela-Rey M, Crawley M, Fisher FM, Badman MK, Martinez-Chantar ML and Maratos-Flier E: Increased fibroblast growth factor 21 in obesity and nonalcoholic fatty liver disease. Gastroenterology 139: 456-463, 2010.

13. Zhang X, Yeung DC, Karpisek M, Stejskal D, Zhou ZG, Liu F, Wong RL, Chow WS, Tso AW, Lam KS and Xu A: Serum FGF21 levels are increased in obesity and are independently associated with the metabolic syndrome in humans. Diabetes 57: 1246-1253, 2008.
14. Li L, Yang G, Ning H, Yang M, Liu H and Chen W: Plasma FGF-21 levels in type 2 diabetic patients with ketosis. Diabetes Res Clin Pract 82: 209-213, 2008.
15. Ding LJ, Li JC, Fu R, Zhou YJ and Huo GC: Preparation and characteristic identification of monoclonal antibody against sulfamethazine. Journal of Northeast Agricultural University (English edition) 13: 145-148, 2006.
16. Jiang YY, Liu MY, Ren GP, Wang WF, Liu XM and Li DS: Cloning, expression and purification of mouse fibroblast growth factor-21 and its function in adipocyte glucose metabolism. Sheng Wu Hua Xue Yu Sheng Wu Wu Li Jin Zhan Bian Ji Bu 36: 157-164, 2009 (In Chinese).
17. Guo M, Xu LM, Zhou B, Yin JC, Ren GP and Li DS: A novel efficient method for B cell epitopes mapping base on bacterial display. Zhongguo Mianyixue Zazhi 30: 366-372, 2014 (In Chinese).
18. Xu WJ, Zhang YJ, Li QQ, Wu Q, Yu XF, Guo XC, Yin JC, Ren GP and Li DS; Biopharmaceutical Lab, College of Life Science, Northeast Agricultural University: Systematic mapping the linear antigenic domains of the porcine circovirus 2b Cap protein by bacterial display technology. Zhong Guo Yu Fang Shou Yi Xue Bao Bian Ji Bu 38: 398-402, 2016 (In Chinese).
19. Xu LM, Yin CK, Ren GP, Tian H, Wang XQ, Ding LJ and Li DS: Establishment of bacterial display technology for fab antibody library screening. Xi Bao Yu Fen Zi Mian Yi Xue Za Zhi 27: 1090-1093, 2011 (In Chinese).
20. Harvey BR, Georgiou G, Hayhurst A, Jeong KJ, Iverson BL and Rogers GK: Anchored periplasmic expression, a versatile technology for the isolation of high-affinity antibodies from *Escherichia coli*-expressed libraries. Proc Natl Acad Sci USA 101: 9193-9198, 2004.
21. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25: 402-408, 2001.
22. Wingfield PT: Overview of the purification of recombinant proteins produced in *Escherichia coli*. Curr Protoc Protein Sci Chapter 6: Unit 6.1, 2003.
23. Georgiou G: Analysis of large libraries of protein mutants using flow cytometry. Adv Protein Chem 55: 293-315, 2000.
24. Daugherty PS, Olsen MJ, Iverson BL and Georgiou G: Development of an optimized expression system for the screening of antibody libraries displayed on the *Escherichia coli* surface. Protein Eng 12: 613-621, 1999.
25. Daugherty PS: Protein engineering with bacterial display. Curr Opin Struct Biol 17: 474-480, 2007.
26. Rice JJ, Schohn A, Bessette PH, Boulware KT and Daugherty PS: Bacterial display using circularly permuted outer membrane protein OmpX yields high affinity peptide ligands. Protein Sci 15: 825-836, 2006.
27. Kurosu H, Choi M, Ogawa Y, Dickson AS, Goetz R, Eliseenkova AV, Mohammadi M, Rosenblatt KP, Kliewer SA and Kuro-o M: Tissue-specific expression of betaKlotho and fibroblast growth factor (FGF) receptor isoforms determines metabolic activity of FGF19 and FGF21. J Biol Chem 282: 26687-26695, 2007.
28. Ogawa Y, Kurosu H, Yamamoto M, Nandi A, Rosenblatt KP, Goetz R, Eliseenkova AV, Mohammadi M and Kuro-o M: BetaKlotho is required for metabolic activity of fibroblast growth factor 21. Proc Natl Acad Sci USA 104: 7432-7437, 2007.
29. Kharitonenkov A, Dunbar JD, Bina HA, Bright S, Moyers JS, Zhang C, Ding L, Micanovic R, Mehrbod SF, Knierman MD, *et al*: FGF-21/FGF-21 receptor interaction and activation is determined by betaKlotho. J Cell Physiol 215: 1-7, 2008.
30. Wang D, Cummins C, Bayliss S, Sandercock J and Burls A: Immunoprophylaxis against respiratory syncytial virus (RSV) with palivizumab in children: A systematic review and economic evaluation. Health Technol Assess 12: iii, ix-x, 1-86, 2008.