Electronic Supplementary Material (ESM) Methods

Animal experiments

All animal experiments were carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocols were approved by the Animal Policy and Welfare Committee of Wenzhou Medical University and the Institutional Animal Care and Use Committee of the University of Louisville. All surgeries were performed under anesthesia induced by intraperitoneal injection of 1.2% 2,2,2-Tribromoethanol (avertin) at the dose of 240 mg/kg body weight and all efforts were made to minimize suffering.

Nine-week old male mice were used in this study. FGF21-KO and genetic background matched wild type (WT) C57BL/6J mice were gifted by Dr. Steve Kliewer, University of Texas Southwestern Medical Center [1]. FVB mice were purchased from Jackson Laboratory (Bar Harbor, Maine). Mice were housed at 22°C with a 12:12-h light-dark cycle and free access to rodent chow and tap water. Five groups of animal studies were performed.

For the first study, FVB mice were given multiple injections (intraperitoneal) of either saline for non-diabetic group or low doses of STZ at 50 mg/kg body weight daily for 5 days for diabetic group. Whole blood glucose obtained from the mouse tail vein was measured by using a Life-style blood glucose meter (American Diabetes Wholesale, Pompano Beach, FL) [2, 3]. Hyperglycemia was considered when the whole blood glucose levels were >250 mg/dl. Hyperglycemic and control mice (n=8 for each group) were sacrificed at 2, 4 and 6 months of diabetes for measurement of cardiac FGF21 mRNA expression.

The second study was designed to directly asses cardiac lipotoxicity *in vivo*, FVB mice were given daily intraperitoneal injection of long-chain NEFA bovine serum albumin ([NEFA(+)BSA], Sigma, Catalog no. A4503) or NEFA free BSA ([NEFA(-)BSA], Sigma, Catalog no. A6003) at 0.1g /10g

body weight or sham with the same volume of saline (n≥8 for each group). The NEFA is mainly comprised of oleic acid (18:1ω-9), stearic acid (18:0), palmitic acid (16:0) and linoleic acid (18:2ω-6). Albumin solutions were prepared with sterile saline (150 mmol/l NaCl) [4] at the final concentration of 33% with pH 6.5 for the NEFA(+)BSA solution and pH 6.9 for the NEFA(-)BSA solution [5]. The BSA preparations tested negative for endotoxin [6]. One group of control mice and NEFA-infused mice were treated with FGF21 at the dose of 100 μg/kg body weight per day (BAS/NEFA/FGF21) as previous reports [7-9].

For the third study, type 1 diabetes was induced in FVB mice with one intraperitoneal injection of STZ at 150 mg/kg since the acute β cell damage rapidly increased cardiac lipid accumulation due to inactivation of AMP-activated protein kinase (AMPK) [10]. Hyperglycemia was considered when the whole blood glucose levels were >250 mg/dl in the blood obtained from the mouse tail vein using a Life-style meter (American Diabetes Wholesale, Pompano Beach, FL) [2, 3]. One group of diabetic mice (DM) were treated with FGF21 (DM/FGF21) or FGF21 and extracellular signal-regulated kinases1/2 (Erk1/2) inhibitor PD98059 (DM/FGF21/PD) for 10 days (n=8 for each group). Dose of PD98059 were10 mg/kg body weight, and FGF21 was given at 100 μg/kg body weight per day as described in the first study.

For the fourth study, FGF21-KO and their WT control mice were divided into control (Con), diabetes (DM), and diabetes treated with FGF21 (DM/FGF21) only in FGF21-KO group (n=8 for each group). After induction of hyperglycemia with single dose of STZ (200 mg/kg body weight) as described above, some of diabetic mice were given FGF21 at the same dose as mentioned above for the third study. Mice were then sacrificed at 10 days after FGF21 treatment, and heart tissues were harvested.

For the fifth study (chronic model), FGF21-KO and WT mice were divided into control (Con), diabetes (DM), and DM/FGF21. There was one subset of DM/FGF21 treated with ERK inhibitor, PD98059 (DM/FGF21/PD) only in WT mice (n=8 for each group). These mice were kept until 2 months after diabetes onset, and then sacrificed for the cardiac histological and biomedical measurements after cardiac function assay by echocardiography (Echo, see below description).

Cardiac function and blood pressure assay

To assess cardiac function, transthoracic echocardiograph (echo) was performed on mice using a Visual Sonics Vevo 770 high-resolution imaging system (Visual Sonics, Toronto, ON, Canada) and equipped with a RMV 707B High-Frame-Rate Scanhead (focal length 12.7 mm, frequency 30 MHz), as described before.[11] Under sedation with Avertin (240 mg/kg IP), mice were placed in a supine position on a heating pad to maintain body temperature at 36 to 37 °C that was continuously monitored using a rectal thermometer. Under these conditions, the animal's heart rate ranged between 400 and 550 beats per min. Two-dimensional and M-mode echocardiography were used to assess wall motion, chamber dimensions and cardiac function. The indices directly measured included left ventricle (LV) cavitary dimensions in diastole (LVID, d) and systole (LVID, s), LV posterior wall thickness in diastole (LVPW;d) and systole (LVPW;s), and interventricular septum thickness in diastole (IVS,d) and systole (IVS,s). LV fractional shortening (FS) %= [(LVIDd − LVIDs)/LVIDd] × 100; LV ejection fraction (EF) %= [(LV end-diastolic volume − LV end-systolic volume)/ LV end-diastolic volume] × 100.

Blood pressure (BP) was measured by tail-cuff manometry using a CODA^{TM} non-invasive BP monitoring system (Kent Scientific, Torrington, CT). The mice were restrained in a plastic tube restrainer. Occlusion and volume-pressure recording (VPR) cuffs were placed over the tail, and the

mice were allowed to adapt to the restrainer for 5 minutes prior to starting BP measurement. After a 5 minute adaptation period, BP was measured for 10 acclimation cycles followed by 20 measurement cycles. Mice were warmed by heating pads during the acclimation cycles to ensure sufficient blood flow to the tail. The animals were monitored closely throughout the measurement protocol, and removed from restraint as soon as possible upon completing the measurement protocol [12]. After three days of training for the BP measurement, formal measurements were performed and BP data were collected.

Biochemical and histochemical assay

Plasma triacylglycerol concentrations were measured by using triacylglycerol assay kit (Cayman Chemicals, Ann Arbor, MI) following the manufacture's instruction.

Cardiac cell death was detected by Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining as described previously [12].

Cardiac fibrosis was examined by Sirius-red Staining with 0.1% Sirius-red F3BA and 0.25% Fast green FCF for the collagen accumulation, as described in our previous study [11]. The collagen content was determined by quantitative analysis of Sirius-red positive area using Image Pro software (Media Cybernetics, Silver Spring, Maryland).

The expression and/ or phosphorylation of target proteins were detected by Western blot. Total proteins from H9c2 cells, adult mouse cardiomyocytes and heart tissue, were fractionated on 10% SDS-PAGE gels and transferred to a nitrocellulose membrane. The membrane was blocked with a 5% non-fat, dried milk for 1 h, and incubated overnight at 4 °C with the following antibodies: anti-cleaved caspase 3, anti-phospho-p44/42 MAPK (Erk1/2, Tyr202/ Phe204), anti-p44/ 42 MAPK (Erk1/2), anti-phospho-p38- MAPK(Thr180/Tyr182), anti-p38 MAPK, anti-phospho-AMPKα (Thr172),

anti-AMPKα, anti-phospho-PTEN (Ser380/Tyr382), anti-PTEN (1:1,000, Cell Signaling, MA), anti-connective tissue growth factor (CTGF) and β-actin (1:1,000, Santa Cruz Biotechnology, CA). After the unbound antibodies were removed with Tris-buffered saline (pH 7.2) containing 0.05% Tween20, membranes were incubated with the secondary antibody for 1 h at room temperature. Antigen-antibody complexes were visualized with enhanced chemiluminescence detection kit (Thermo scientific, IL). To determine loading, blots were stripped using stripping buffer (SignaGen Laboratories, MD) and reprobed for β-actin. Quantitative densitometry was performed on the identified bands by using a computer-based measurement system, as performed for previous studies.

The expression of gene at mRNA level was examined by real-time qPCR as previous report [13]

Isolation of adult mouse cardiomyocytes, cell culture, palmitate and FGF21 treatments, and siRNA transfection

The whole procedure was carried out as described previously [14, 15]. Mice aged 2 months were anesthetized with ketamine (43.5 mg/kg), acepromazine (1.5 mg/kg) and xylazine (1.7 mg/kg), and given heparin (100U/ml). After median sternotomy, the heart was rapidly excised and rinsed with physiological saline. The aortic lumen was tied to a 18-g cannula and perfused with tyrode bicarbonate buffer at 37°C for 5 min. The perfusate was then changed to 50 ml of recirculating Liberase Blendzyme type 1 (Roche) digestion buffer for 12–15 min. The heart tissue was removed, shredded and filtered through a 140 μm nylon mesh. The supernatant was transferred to another tube and then $CaCl_2$ was added in a graded fashion at 4-min intervals (five total steps) to sequentially increase the Ca^{2+} concentration to 500 μmol/l. The suspension was then plated on laminin-coated culture dishes overnight at 37°C in a 5% CO_2 incubator. The media was replaced before experimentation to wash away unattached cells and ensure that only rod-shaped myocytes were used for subsequent studies.

Rat cardiac cell line H9c2 purchased from ATCC (CRL-1446, MD) were maintained in high glucose Dulbecco's modified Eagle's medium (hDMEM, 4.5g/l) supplemented with 10% fetal bovine serum (FBS) and antibiotics (50 U/ml penicillin and 50 μg/ml streptomycin) at 37 °C in an atmosphere of 95% air and 5% CO_2.

Palmitate (Pal, Sigma Aldrich, MO) was prepared as described before [16]. The recombinant FGF21 was dissolved in phosphate buffered saline (PBS) to the required concentration. In apoptotic protection study, H9c2 cells and/ or adult mouse cardiomyocytes were pre-treated with different dose of FGF21 followed by Pal treatment for15 h. For Pal treatment, the regular medium was replaced by FBS free hDMEN with 2% BSA.

The cell death detection ELISA kit (Roche Life Sciences, IN) was used to measure histone-bound DNA fragments, as described before [16]. Erk1/2 inhibitor (PD98059), p38 mitogen-activated protein kinases (P38-MAPK) inhibitor (SB203580), and AMPK inhibitor (Compound C), purchased form Sigma (St. Louis, MO) were dissolved in pure DMSO to the required concentration. For mechanistic study, H9c2 cells were pre-treated by FGF21 with or without one of the above three inhibitors or specific siRNAs against *Erk1/2*, *p38 Mapk* and *Ampk*, respectively, followed by palmitate treatment for 15 h. For time-course study, H9c2 cells were treated by FGF21 at the dose of 50 ng/ml for different times (1, 3, 6, 9, 12, 15 h) with or without inhibitors pre-treatment.

The siRNA transfections in H9c2 cardiac cells and adult mouse cardiomyocytes were performed using Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. Briefly, 1) dilute 8.0 μg siRNA in 0.5 ml of MEM Medium without serum and mix gently; 2) mix Lipofectamine™ 2000 gently before use, then dilute 20 μl lipofectamine 2000 in 0.5 ml of MEM medium and incubate for 5 min at room temperature; 3) after 5 min incubation, combine the diluted siRNA with diluted Lipofectamine™ 2000 (total volume = 1 ml), and mix gently and incubate for 20

min at room temperature; 4) add the 1ml of complexes to each well containing cells and medium and mix gently by rocking the plate back and forth; 5) after incubation cells at 37°C in a CO_2 incubator for 4 h, the transfection medium was replaced by MEM culture medium with 10% serum, and the cells were continued to be incubated for 44 h for H9c2 cells or 32 h for adult mouse cardiomyocytes prior to testing for transgene expression.

Specifically, in order to knock down Erk1/2, P38 MAPK, AMPK and PTEN in H9c2 cells, H9c2 cells were transfected with rat non-targeting Stealth RNAi™ siRNA (120 nmol/l) along with the corresponding non-specific control siRNA (120 nmol/l) (Invitrogen, Carlsbad, CA). After 32 h transfection of siRNA, H9c2 cells were pre-treated with FGF21 (50 ng/ml) for 1 h followed by co-treatment with palmitate (Pal, 62.5 μmol/L) for another 15 h as indicated in each figure. The transfection efficiency was assessed by Western blot analysis for Erk1/2, P38 MAPK, AMPK and PTEN protein expression;

In order to knock down Erk1/2, P38 MAPK and AMPK expression in adult mouse cardiomyocytes, the primary cardiomyocytes were transfected with mouse non-targeting Stealth RNAi™ siRNA (120 nmol/l) along with the corresponding negative control siRNAs (120 nmol/l) (Invitrogen, Carlsbad, CA). After 20 h transfection of siRNA, the primary cardiomyocytes were treated with FGF21 (50 ng/ml) for 1 h, followed by co-treatment with palmitate (Pal, 62.5 μmol/L) for another 15 h. The transfection efficiency was assessed by Western blot for the target protein expression.

The selection of transfection for total 36 h as described above was based on the pilot time-course study for the transfection efficiency with P38 MAPK siRNA transfection in adult mouse cardiomyocytes (ESM Fig. 1). From this pilot study, we found that most of the cardiomyocytes were still in rod-shape at 36 h after transfection, and both expression and phosphorylation of P38 MAPK were significantly down-regulated at 24 and 36 h after transfection. Therefore, transfection for 36 h

was followed for Erk1/2, P38 MAPK and AMPK siRNAs transfection in the formal study in adult mouse cardiomyocytes described above.

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