FGF21 deletion exacerbates diabetic cardiomyopathy by aggravating cardiac lipid accumulation

Xiaoqing Yan [a, b], Jun Chen [a, b, c], Chi Zhang [a], Shanshan Zhou [b, d], Zhiguo Zhang [b, d], Jing Chen [b], Wenke Feng [e], Xiaokun Li [a], Yi Tan [a, b, e, *]

[a] Chinese-American Research Institute for Diabetic Complications at the Wenzhou Medical University, Wenzhou, China
[b] Kosair Children's Hospital Research Institute, The Department of Pediatrics of the University of Louisville,
School of Medicine, Louisville, USA
[c] Scool of Nursing, Wenzhou Medical University, Wenzhou, China
[d] Department of Cardiovascular Disorders of the First Hospital of Jilin University, Changchun, China
[e] Department of Pharmacology and Toxicology of the University of Louisville School of Medicine, Louisville, USA

Abstract

Fibroblast growth factor 21 (FGF21) plays an important role in energy homoeostasis. The unaddressed question of FGF21's effect on the development and progression of diabetic cardiomyopathy (DCM) is investigated here with FGF21 knockout (FGF21KO) diabetic mice. Type 1 diabetes was induced in both FGF21KO and C57BL/6J wild-type (WT) mice *via* streptozotocin. At 1, 2 and 4 months after diabetes onset, the plasma FGF21 levels were significantly decreased in WT diabetic mice compared to controls. There was no significant difference between FGF21KO and WT diabetic mice in blood glucose and triglyceride levels. FGF21KO diabetic mice showed earlier and more severe cardiac dysfunction, remodelling and oxidative stress, as well as greater increase in cardiac lipid accumulation than WT diabetic mice. Western blots showed that increased cardiac lipid accumulation was accompanied by further increases in the expression of nuclear factor (erythroid-derived 2)-like 2 (Nrf2) and its target protein CD36, along with decreases in the phosphorylation of AMP-activated protein kinase and the expression of hexokinase II and peroxisome proliferator-activated receptor gamma co-activator 1α in the heart of FGF21KO diabetic mice compared to WT diabetic mice. Our results demonstrate that FGF21 deletion-aggravated cardiac lipid accumulation is likely mediated by cardiac Nrf2-driven CD36 up-regulation, which may contribute to the increased cardiac oxidative stress and remodelling, and the eventual development of DCM. These findings suggest that FGF21 may be a therapeutic target for the treatment of DCM.

Keywords: fibroblast growth factor 21 ● diabetic cardiomyopathy ● CD36 ● cardiac lipotoxicity

Introduction

Diabetic cardiomyopathy (DCM) is one of the most severe diabetic complications. It has been defined as ventricular dysfunction that occurs independently of coronary artery disease and hypertension [1]. The pathogenesis of DCM is multifactorial [2]. Altered myocardial substrate and energy metabolism has emerged as an important contributor to the development of DCM [3]. Despite an increase in fatty acid use in diabetic hearts, fatty acid uptake likely exceeds its oxidation rate, thereby resulting in cardiac lipid accumulation that promotes cardiac lipotoxicity [4]. Thus, targeting to correct diabetes-induced abnormal substrate metabolism in the heart will potentially lower the prevalence of DCM, thereby improve long-term survival of the patients with diabetes.

Fibroblast growth factor 21 (FGF21), a novel member of the FGF family, encoded by the *fgf21* gene located in chromosome 19 in human [5] and chromosome 7 in mice, has been identified as a potent metabolic regulator with specific effects on glucose and lipid metabolism [6]. FGF21 is preferentially expressed in the liver [5]. But other tissues, such as pancreas [7], white [8] and brown [9] adipose

*Correspondence to: Dr. Yi TAN, The Chinese-American Research Institute for Diabetic Complications at the Wenzhou Medical University, Wenzhou, China; or The Kosair Children's Hospital Research Institute, the Department of Pediatrics of the University of Louisville, School of Medicine, Louisville, KY, USA.
Tel.: 86-577-86689983
Fax: 86-577-86699350
E-mail: y0tan002@louisville.edu

tissues, skeletal muscle [10] and heart [7] also express FGF21. FGF21 stimulates glucose uptake in adipocytes *via* the induction of glucose transporter-1, which is additive and independent of insulin [11]. Under hypothermic conditions, FGF21 can induce browning of white adipose tissues to up-regulate thermogenic activity, which could, at least in part, lead to a greater clearance of glucose [12]. Moreover, FGF21 has shown beneficial effects on lipid profiles in animal models [13, 14]. FGF21 can also regulate lipolysis in adipocytes in response to fasting [15]. Treatment with FGF21 enhances the expression and secretion of downstream effector adiponectin in adipocytes which in turn further improves fatty acid oxidation and lipid clearance in the liver and skeletal muscle [16].

Since its benefits in regulating glucose and lipid metabolism, FGF21 has shown therapeutic potential in treating diabetes [17]. FGF21 has insulin-sensitizing ability [16] and can ameliorate glucose tolerance [18] by reducing hepatic glucose production and stimulating glucose uptake in adipocytes. Acute FGF21 treatment suppressed hepatic glucose production, increased liver glycogen, lowered glucagon and improved glucose clearance in *ob/+* mice, while chronic FGF21 treatment ameliorated fasting hyperglycaemia in *ob/ob* mice *via* increased glucose disposal and improved hepatic insulin sensitivity [19]. Besides those insulin-mimetic properties, FGF21 does not induce mitogenicity, hypoglycaemia or weight gain at any dose tested in diabetic or healthy animals or when overexpressed in transgenic mice [13]; therefore, FGF21 shows a promise as an effective treatment of diabetes.

To date, the function of FGF21 has been extensively investigated, but most studies focused on the liver, adipose tissue [20] and skeletal muscle [10, 16]. The effect of FGF21 on the heart has been neglected. FGF21 activity depends on its binding to the fibroblast growth factor receptor 1 (FGFR1), especially FGFR1c, and co-factor β-Klotho [21]. The existence of FGFR1c, β-Klotho [22] and FGF21 [7] in the heart suggests that FGF21 may play certain roles in the physiological and pathophysiological aspects of the heart. Recently, FGF21 was found to have cardio-protective effects against myocardial ischaemia/reperfusion injury [23] and isoproterenol-induced cardiac hypertrophy [24]. However, its effect on DCM has not been characterized. In this study, we investigated the effect of FGF21 deletion on the development of DCM in a FGF21 knockout (FGF21KO) mouse model. We found that mice lacking the *fgf21* gene are more prone to develop DCM, which is likely because of the overexpression of CD36-mediated cardiac lipid accumulation.

Materials and methods

Ethics statement

This study was 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 protocol was 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 anaesthesia induced by

intraperitoneal injection of 1.2% 2,2,2-Tribromoethanol (avertin) at the dose of 300 mg/kg bw and all efforts were made to minimize suffering.

Animal model

Male FGF21KO mice with C57 BL/6J background (gift from Dr. Steve Kliewer, University of Texas Southwestern Medical Center) and wild-type (WT) C57 BL/6J mice purchased from Jackson Laboratory (Bar Harbor, ME, USA) were used in this study. Type 1 diabetic mouse model was induced in 10 week-old male FGF21KO mice and age-matched WT mice by intraperitoneal (i.p.) injection of six doses of streptozotocin (STZ, Sigma-Aldrich, St. Louis, MO, USA in 10 mM sodium citrate buffer, pH 4.5) at 60 mg/kg bw daily. Control group (Ctrl) of FGF21KO and WT mice received citrate buffer alone. Seven days after the last STZ injection, whole blood glucose obtained from the mouse tail vein was detected using a SureStep complete blood glucose monitor (LifeScan, Milpitas, CA, USA), and animals with blood glucose levels greater than 250 mg/dl were considered diabetic. At 1, 2 and 4 months after diabetes onset, heart function and blood pressure were measured and mice were then killed.

Echocardiography

At 1, 2 and 4 months after diabetes onset, heart function was evaluated by transthoracic echocardiography (ECHO). 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 30.0 MHz), as described previously [25]. Under sedation with avertin (300 mg/kg bw), mice were placed in a supine position on a heating pad to maintain body temperature at 36–37°C that was continuously monitored using a rectal thermometer probe. Under these conditions, the animal's heart rate ranged between 400 and 550 beats/min. Two-dimensional and M-mode ECHO were used to assess wall motion, chamber dimensions and cardiac function.

Blood pressure measurement

Blood pressure was measured using a CODA™ mouse/rat tail-cuff system (Kent scientific, Torrington, CT, USA) following our previous published protocol [25].

Plasma FGF21 assay

Whole blood was collected in a lithium heparin tube (BD, Franklin Lakes, NJ, USA), and centrifuged at 2000 r.p.m. for 20 min. Then, plasma was collected for FGF21 assay using a FGF21 Quantikine Elisa kit (R&D systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

Plasma and cardiac triglyceride assay

Triglyceride concentrations were measured using a triglyceride assay kit (Cayman Chemicals, Ann Arbor, MI, USA) according to the

manufacture's protocol. For plasma triglyceride assay, plasma from diabetic mice was diluted 1:1 with standard diluent assay reagent, while the plasma from control mice was not diluted. For cardiac triglyceride assay, 10–20 mg heart tissue was minced into small pieces and then homogenized in standard diluent assay reagent (10 μl/mg tissue). Tissue homogenate was centrifuged at 10,000 × g for 10 min. at 4°C, and 10 μl supernatant was used for triglyceride assay.

Histopathological examination

Paraffin sections (5 μm) from cardiac tissue dissected from mice were stained with haematoxylin and eosin and observed under light microscopy as described before [26].

Oil Red O staining

Lipid accumulation was evaluated by Oil Red O staining as described previously [27]. Cryosections (10 μm thick) from heart tissue embedded in optimal cutting temperature medium (Tissue-Tek® O.C.T™ Compound, Sakura, Torrance, CA, USA) were fixed in 10% buffered formalin for 30 min. at room temperature and stained with Oil Red O for 1 hr. After washing with 60% isopropanol, the sections were then counterstained with haematoxylin (DAKO, Carpinteria, CA, USA) for 30 sec. A Nikon Eclipse E600 microscope (Nikon, Melville, NY, USA) was used to capture the Oil Red O-stained tissue sections at 40× magnification.

Sirius Red staining

Sirius Red staining for collagen deposition was used to determine cardiac fibrosis as described previously [25]. Briefly, 5 μm paraffin-embedded heart tissue sections were stained with 0.1% Sirius Red F3BA and 0.25% Fast Green FCF. The proportion of collagen in Sirius Red-stained sections was then evaluated using a Nikon Eclipse E600 microscopy system.

Real-time quantitative polymerase chain reaction (RT-qPCR)

Total RNA was extracted from heart tissue using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). After quantified using a Nanodrop ND-1000 spectrophotometer, 1 μg total RNA was used to synthesize first-strand complimentary DNA (cDNA) using reverse transcription kit (Promega, WI, USA) as described before [28]. RT-qPCR was carried out with the ABI 7300 real-time PCR system (Applied Biosystems, Grand Island, NY, USA) in a 20 μl reaction system containing 10 μl of TaqMan Universal PCR Master Mix, 1 μl of primers, 6 μl ddH$_2$O and 3 μl of cDNA (1:4 dilution with nuclease-free water). Primers for mouse glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and FGFR1 (Invitrogen) were used for RT-qPCR assay.

Western blot

Total proteins from heart tissue were fractionated on 10% SDS-PAGE gels and transferred to a nitrocellulose membrane. The membrane was blocked with 5% non-fat milk for 1 hr, and incubated overnight at 4°C on a rocking platform with the following primary antibodies: anti-phosphor-AMP-activated protein kinase (AMPK)α (Thr172), anti-AMPKα (Cell Signaling, Danvers, MA, USA), anti-CD36, anti-peroxisome proliferator-activated receptor gamma co-activator 1alpha (PGC1α; Abcam, MA, USA), anti-connective tissue growth factor (CTGF), anti-hexokinase II (HKII), anti-nuclear factor (erythroid-derived 2)-like 2 (Nrf2) and anti-GAPDH (Santa Cruz Biotechnology, Dallas, TX, USA). After unbound antibodies were washed out with tris-buffered saline (pH 7.2) containing 0.05% Tween20, membranes were incubated with corresponding secondary antibody for 1 hr at room temperature. Antigen-antibody complexes were visualized with an enhanced chemiluminescence detection kit (Thermo Scientific, Waltham, MA, USA). Quantitative densitometry was performed on the identified bands by using a computer-based measurement system as performed in previous studies [25, 29].

Statistical analysis

Data were collected from five or more mice per group and presented as mean ± SD. One-way ANOVA was used to determine general difference, followed by a *post hoc* Turkey's test for the difference between groups, using Origin 7.5 laboratory data analysis and graphing software. Statistical significance was considered as $P < 0.05$.

Results

FGF21 expression decreased under type 1 diabetes conditions

Wild-type and FGF21KO diabetic mice showed similar, persistent increases in whole blood glucose and plasma triglyceride levels up until organ harvested 4 months after STZ-induced diabetes onset (Fig. 1A and B). To elucidate the relationship between FGF21 and DCM, we measured the plasma levels of FGF21 and the cardiac mRNA levels of its preferred receptor FGFR1 in both WT and FGF21KO mice under diabetic and non-diabetic conditions. Plasma FGF21 level in WT diabetic mice significantly decreased at 1, 2 and 4 months after diabetes onset (Fig. 1C), which was accompanied by obvious trend of cardiac FGFR1 mRNA level up-regulation at 1 and 2 months after DM onset (Fig. 1D), indicating that type 1 diabetes systematically down-regulation of FGF21 resulted in its receptor compensatory up-regulation in cardiac tissue. No obvious FGF21 expression was observed under diabetic and non-diabetic conditions in FGF21KO mice, as expected (Fig. 1C). The deletion of FGF21 was accompanied by a slight compensatory up-regulation of cardiac FGFR1 mRNA under basal conditions, which was significantly amplified by diabetes in FGF21KO mice (Fig. 1D).

FGF21 deletion-aggravated diabetes-induced cardiac dysfunction

The role of FGF21 in the development of DCM was investigated by determining the effect of FGF21 deletion on cardiac structure and
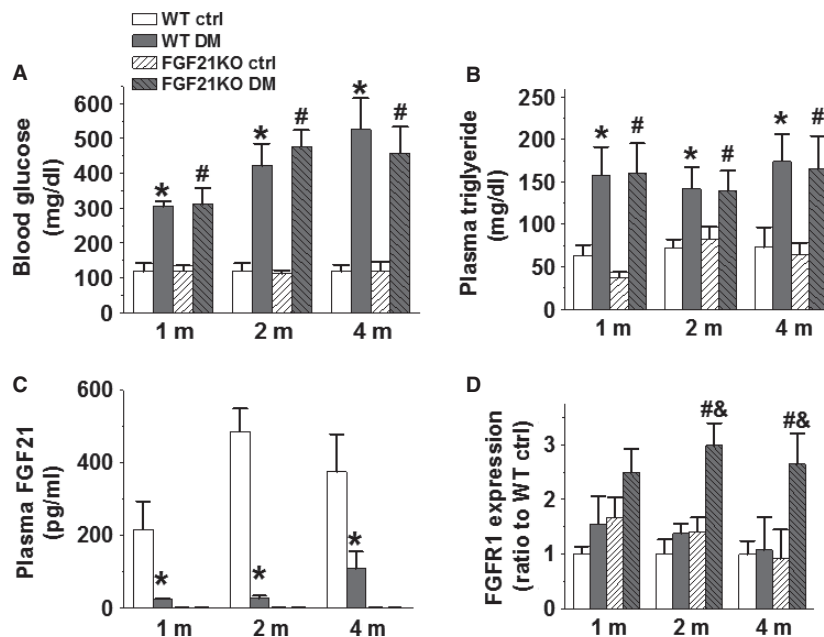
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Fig. 1 Diabetes-induced high blood glucose and triglyceride levels, and FGF21 deficiency. At indicated time-points after diabetes onset, fasting blood glucose was detected using a SureStep complete blood glucose monitor (**A**), plasma triglyceride level was measured using a triglyceride assay kit (**B**), and plasma FGF21 was assayed using a FGF21 Quantikine Elisa kit (**C**). The expression of cardiac FGFR1 mRNA was detected by RT-qPCR and GAPDH was used as the loading control. Three duplications were set for each sample (**D**). Data are presented as means $\pm$ SD ($n \geq 5$ for each group). *$P < 0.05$ *versus* WT Ctrl group; #$P < 0.05$ *versus* FGF21KO Ctrl group; &$P < 0.05$ *versus* WT DM group. Ctrl: control; DM: diabetes mellitus; WT: wild-type; FGF21KO: FGF21 knockout; m: month(s). [Correction added on 12 May 2015 after first online publication: Panel D was inserted in Figure 1]

function in diabetic mice. Under basal conditions, FGF21KO mice did not show marked alterations in heart weight (Fig. S1), cardiac structure and function (Fig. 2 and Table S1). However, both WT and FGF21KO diabetic mice showed heart weight decrease at 1, 2, and 4 months after diabetes onset (Fig. S1). WT diabetic mice did not exhibit cardiac dysfunction until 4 months after diabetes, reflected by decreased ejection fraction and fraction shortening (Fig. 2). FGF21KO diabetic mice developed cardiac dysfunction at 2 months after diabetes onset; at 4 months, FGF21KO diabetic mice showed more severe cardiac functional impairment than WT diabetic mice (Fig. 2). Parameters of cardiac structure showed similar patterns of change (Table S1). At 4 months, WT diabetic mice showed decrease in diastolic and systolic LVPW and systolic LVID, and an increase in systolic LV volume compared to WT control mice, and FGF21KO diabetic mice showed decrease in systolic and diastolic IVS and LVPW, and an increase in diastolic LVID and systolic LVID. Moreover, FGF21KO diabetic mice had dramatically lower systolic IVS and diastolic and systolic LVPW, and higher systolic LVID and diastolic and systolic LV volume than WT diabetic mice (Table S1). These results demonstrate that the cardiac dysfunction developed in FGF21KO diabetic mice was earlier and more severe than that in WT diabetic mice, indicating that deletion of FGF21 aggravated the development of DCM.

Histological examination revealed severe pathological changes in the hearts of FGF21KO diabetic mice in comparison with that of FGF21KO control and WT diabetic mice. As shown in Figure 2C, both

FGF21KO and WT control hearts had regular and intact myocardial arrangements and clearly visible nuclei, and WT diabetic hearts showed certain irregularity of the myocardial fibres, especially at 4 months after diabetes onset, while FGF21KO diabetic hearts exhibited large areas of irregular myocardial arrangements, myofibrillar discontinuation and cell death (karyorrhexis, pyknosis and/or karyolysis).

Blood pressure was detected using a CODA™ mouse tail-cuff system, and no significant changes in systolic and diastolic pressure were observed in both WT and FGF21KO mice under diabetic and non-diabetic conditions (Fig. S2), which indicated that FGF21 deletion did not affect blood pressure under basal and experimental diabetic conditions. The above-mentioned diabetes-induced cardiac dysfunction was independent of blood pressure changes.

FGF21 deletion accelerated diabetes-induced cardiac remodelling

FGF21KO mice did not show marked cardiac fibrosis compared to WT mice under normal conditions, but diabetes-induced significant cardiac remodelling. Collagen accumulation (Fig. 3A) and expression of fibrotic mediator CTGF (Fig. 3B) were significantly increased in the heart of WT diabetic mice from 2 months, and FGF21KO diabetic mice from 1 month after diabetes onset. The elevation of collagen
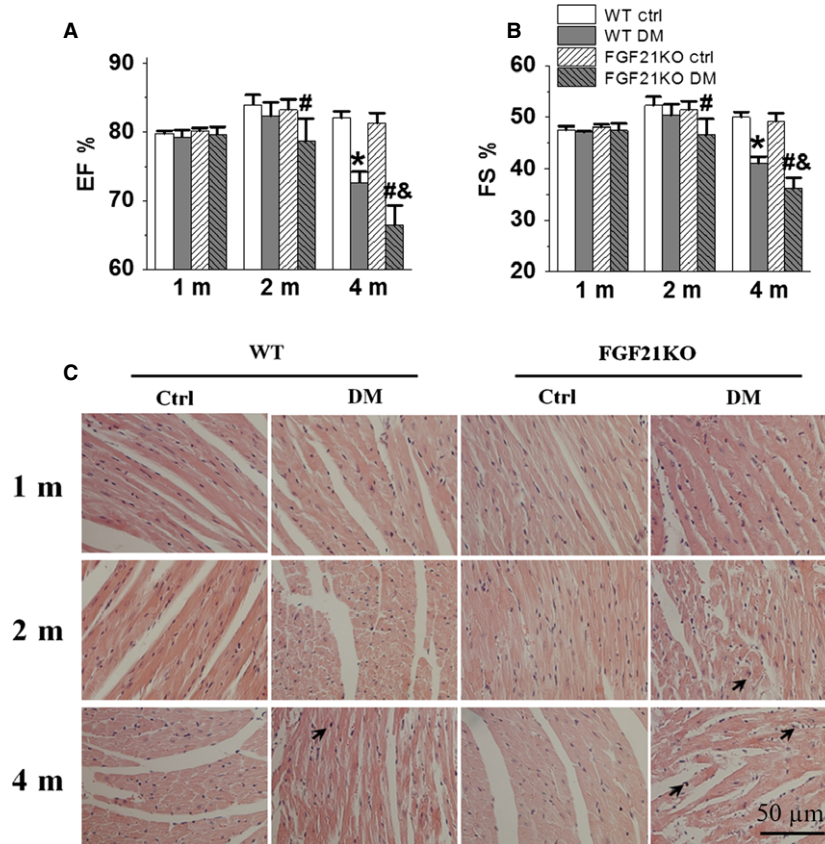
Fig. 2 FGF21 deletion-aggravated diabetes-induced cardiac dysfunction. At indicated time-points after diabetes onset, cardiac function was evaluated by transthoracic echocardiography and expressed as LV ejection fraction (EF%, **A**) and fraction shortening (FS%, **B**). Myocardial structural damage indicated by haematoxylin and eosin staining (**C**). Paraffin-embedded heart tissue was stained with haematoxylin and eosin and examined under light microscope. Arrows indicate cardiac cell death with karyorrhexis, pyknosis and/or karyolysis. Magnification = 400 × . Data are presented as means ± SD ($n \geq 5$ for each group). *$P < 0.05$ *versus* WT Ctrl group; #$P < 0.05$ *versus* FGF21KO Ctrl group; &$P < 0.05$ *versus* WT DM group. The abbreviations are same as in Figure 1.

accumulation and CTGF expression was higher in FGF21KO diabetic mice than that in WT diabetic mice at 2 months after diabetes (Fig. 3A and B), indicating that FGF21 deletion accelerated and aggravated diabetes-induced cardiac fibrotic remodelling.

FGF21 deletion-exacerbated diabetes-induced cardiac oxidative stress

Diabetic hyperglycaemia- and hyperlipidaemia-induced oxidative stress plays a critical role in the development of DCM [29]. Consequently, cardiac oxidative status was evaluated by the expression of 3-NT and 4-HNE as in our previous report [30]. The expression of 3-NT remained unchanged in FGF21KO mice under basal conditions, but was elevated under diabetic conditions (Fig. 4A). It was elevated only at 4 months in WT diabetic mice, but at both 2 and 4 months after diabetes onset in FGF21KO diabetic mice (Fig. 4A). Moreover,

the 3-NT expression in FGF21KO diabetic mice was significantly higher than that of WT diabetic mice at 2 and 4 months after diabetes onset (Fig. 4A). The expression of 4-HNE showed similar change pattern with that of 3-NT, both FGF21KO and WT diabetic mice exhibited higher 4-HNE expression than their controls at 2 and 4 months after diabetes onset, but FGF21KO diabetic mice had higher 4-HNE expression than WT diabetic mice at 4 months after diabetes onset (Fig. 4B).

FGF21 deletion-aggravated diabetes-induced cardiac lipid accumulation

Fibroblast growth factor 21 was identified as a potent metabolic regulator for lipid metabolism in several organs [6]. We therefore quantified cardiac lipid accumulation by Oil Red O staining (Fig. 5A) and triglyceride assay (Fig. 5B). No obvious cardiac lipid accumulation
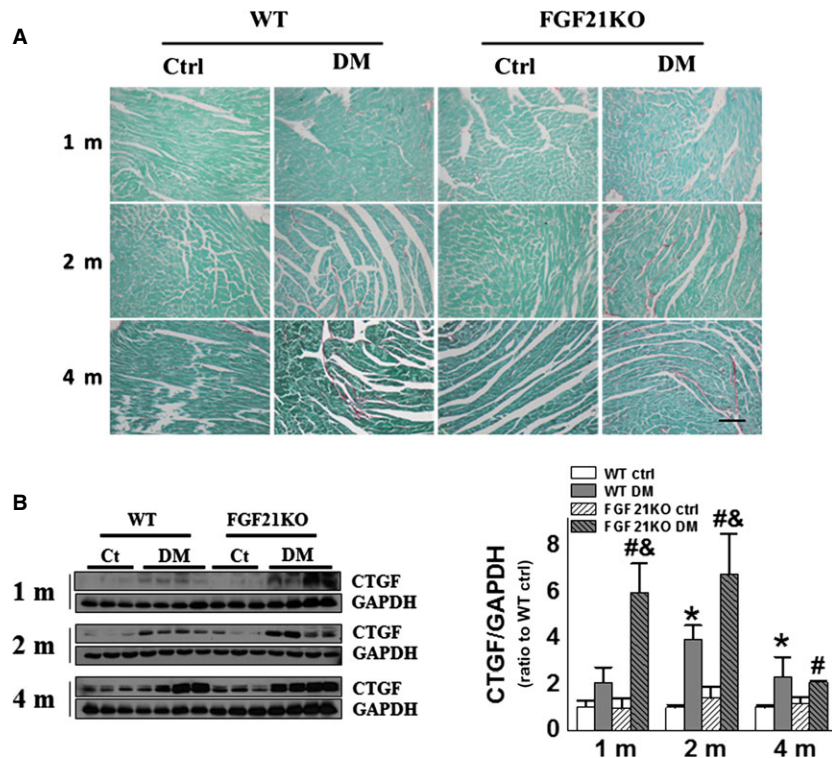
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Fig. 3 FGF21 deletion accelerated diabetes-induced cardiac remodelling. At indicated time-points after diabetes onset, cardiac fibrosis was evaluated by Sirius Red staining of collagen accumulation (**A**) and Western blot of CTGF expression (**B**). Data are presented as means ± SD ($n \geq 5$ for each group). *$P < 0.05$ *versus* WT Ctrl group; #$P < 0.05$ *versus* FGF21KO Ctrl group; &$P < 0.05$ *versus* WT DM group; bar = 100 μm. The abbreviations are same as in Figure 1.
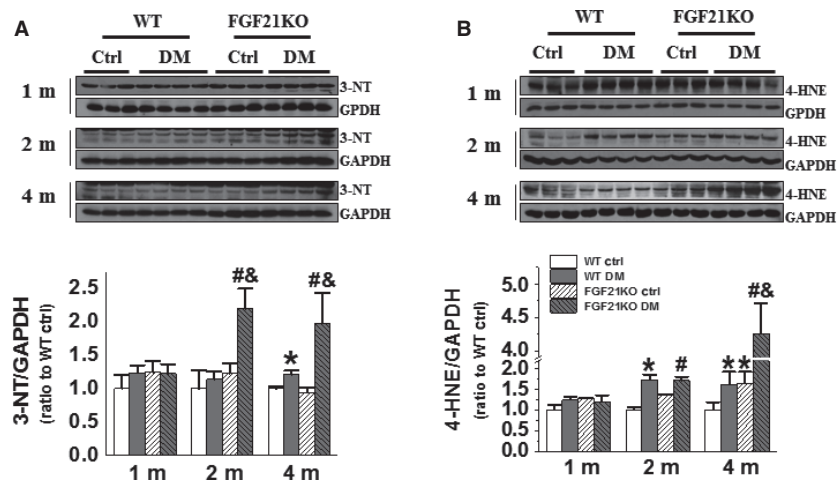

Fig. 4 FGF21 deletion-exacerbated diabetes-induced cardiac oxidative stress. At indicated time-points after diabetes onset, the markers of cardiac oxidative stress including 3-NT (**A**) and 4-HNE (**B**) were evaluated by Western blot. Data are presented as means ± SD ($n \geq 5$ for each group). *$P < 0.05$ *versus* WT Ctrl group; #$P < 0.05$ *versus* FGF21KO Ctrl group; &$P < 0.05$ *versus* WT DM group. The abbreviations are same as in Figure 1.

was observed in both WT and FGF21KO mice under basal conditions (Fig. 5A and B). Diabetes-induced significant cardiac lipid accumulation in WT mice only at 4 months, but in the FGF21KO mice starting from 1 month until 4 months after diabetes onset (Fig. 5A and B). Moreover, FGF21KO diabetic mice exhibited more severe cardiac lipid accumulation at 2 and 4 months after diabetes onset than WT diabetic mice (Fig. 5A and B). These results demonstrate that FGF21 deletion accelerated and exacerbated cardiac lipid accumulation under diabetic conditions.

FGF21 deletion accelerated diabetes-induced cardiac CD36 and Nrf2 up-regulation and PGC1α down-regulation

To uncover how FGF21 deletion affects cardiac lipid accumulation, the expression of CD36, a critical regulator of fatty acid transport [31], was detected by Western blot. FGF21 deletion had no obvious effects on cardiac CD36 expression under basal conditions, but diabetes significantly up-regulated CD36 expression in both WT and FGF21KO hearts (Fig. 6A). Cardiac CD36 expression was elevated from 2 months in WT diabetic mice and from 1 month in FGF21KO diabetic mice after diabetes onset. At 4 months after diabetes onset, cardiac CD36 expression in FGF21KO diabetic mice was significantly higher than that of WT diabetic mice (Fig. 6A). Reportedly, Nrf2-mediated CD36 up-regulation plays critical role in lipid metabolism in macrophage, aorta and liver tissues [32–34]. So, we detected cardiac Nrf2 expression by Western blot (Fig. 6B). We found that cardiac Nrf2 expression showed no difference between FGF21KO mice and WT mice under basal conditions (Fig. 6B). Under diabetic conditions, Nrf2 expression was slightly, but not significantly, elevated in WT mice; but progressively increased from 1 to 4 months after diabetes onset in FGF21KO mice (Fig. 6B). These results indicate that FGF21 deletion-exacerbated diabetes-induced cardiac Nrf2 and CD36 up-regulation.

PGC1α, an essential regulator of fatty acid oxidation [35], was also detected by Western blot. The cardiac PGC1α expression was not affected by FGF21 deletion under basal conditions, but was significantly attenuated by diabetes in FGF21KO diabetic mice at 4 months after diabetes onset (Fig. 6C). No significant changes of cardiac PGC1α expression were observed under either diabetic or non-diabetic conditions in WT mice (Fig. 6C). The elevation of Nrf2 and CD36, and decline of PGC1α expression imply that FGF21 deletion increased lipid uptake and decreased lipid oxidation leading to suboptimal cardiac lipid metabolism under diabetic conditions, which resulted in aggravated cardiac lipid accumulation in FGF21KO diabetic mice.

FGF21 deletion accelerated diabetes-induced cardiac glucose metabolism impairment

In addition to lipid, glucose metabolism is another important source of energy for heart contraction. Reportedly, FGF-21 is also a potent glucose metabolic regulator in several organs including the heart [6], and AMPK is a sensor of energy homoeostasis and a regulator of glucose uptake and fatty acid β-oxidation [36]. So, glucose metabolism in cardiac tissue was also investigated to evaluate the effect of FGF21 deletion. FGF21KO mice did not show marked alterations in phosphorylation of AMPK under basal conditions (Fig. 7A). Significant down-regulation of cardiac AMPK phosphorylation was observed from 2 months in WT diabetic mice, and from 1 month after diabetes onset in FGF21KO diabetic mice. Moreover, the cardiac AMPK phosphorylation in FGF21KO diabetic mice was significantly lower than that of WT diabetic mice at 1 and 4 months after diabetes onset (Fig. 7A). The expression of HKII, an enzyme that catalyses the first step of glycolysis by conversion of glucose to glucose-6-phosphate, was also detected. The cardiac HKII expression was not affected by FGF21 deletion under basal conditions, but was significantly impaired by diabetes in FGF21KO diabetic mice at 2 and 4 months after diabetes onset (Fig. 7B). No significant changes of cardiac HKII expression were observed under both diabetic and non-diabetic conditions in WT mice (Fig. 7B). These results indicate that FGF21 deletion-aggravated diabetes-induced impairment of cardiac glucose metabolism and cardiac energy metabolic balance in FGF21KO diabetic mice.
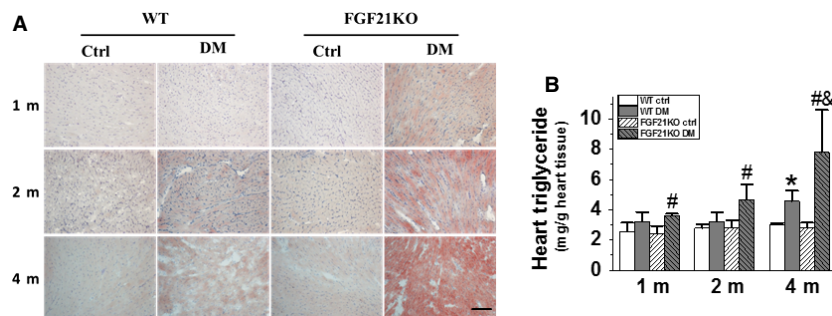

Fig. 5 FGF21 deletion-aggravated diabetes-induced cardiac lipid accumulation. At indicated time-points after diabetes onset, cardiac lipid accumulation was evaluated by Oil Red O staining (**A**) and triglyceride content (**B**). Data are presented as means ± SD ($n \geq 5$ for each group). *$P < 0.05$ *versus* WT Ctrl group; #$P < 0.05$ *versus* FGF21KO Ctrl group; &$P < 0.05$ *versus* WT DM group; bar = 100 μm. The abbreviations are same as in Figure 1.
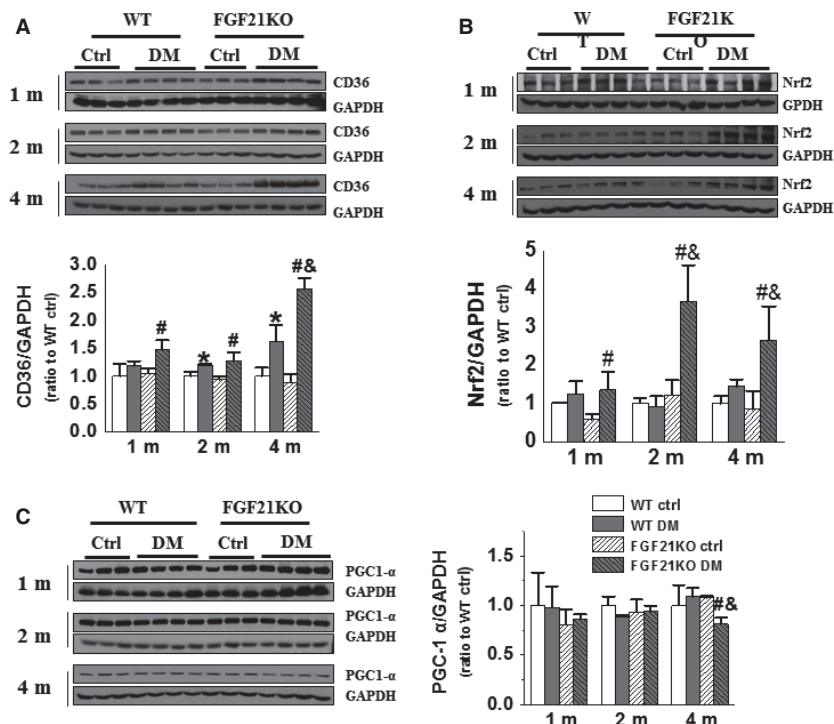
Fig. 6 FGF21 deletion accelerated diabetes-induced cardiac CD36 and Nrf2 up-regulation and PGC1α down-regulation. At indicated time-points after diabetes onset, the expression of CD36 (**A**), Nrf2 (**B**) and PGC1α (**C**) were detected by Western blot. Data are presented as means ± SD ($n \geq 5$ for each group). *$P < 0.05$ *versus* WT Ctrl group; #$P < 0.05$ *versus* FGF21KO Ctrl group; &$P < 0.05$ *versus* WT DM group. The abbreviations are same as in Figure 1.

Discussion

Fibroblast growth factor 21 is a newly discovered metabolic hormone. In addition to its essential roles in regulating glucose and lipid metabolism through pleiotropic actions in liver and adipocyte tissues, FGF21 also plays critical role in cardiac pathogenesis. In this study, for the first time we established that FGF21 deletion is susceptible to develop DCM in STZ-induced type 1 diabetic mice, which is predominantly attributed to the exacerbated cardiac lipid accumulation *via* Nrf2 up-regulation of CD36-mediated cardiac fatty acid accumulation.

In STZ- induced type 1 diabetes in this study, circulating FGF21 reduced from 1 month to the experimental termination at 4 months after diabetes onset (Fig. 1C), which was consistent with a previous report that serum FGF21 levels were significantly lower in type 1 diabetic patients than that of control subjects [37]. It was also found that decreased FGF21 levels were accompanied by significant cardiac dysfunction, remodelling and oxidative stress at 4 months after diabetes onset in WT diabetic mice, while FGF21 complete deletion significantly accelerated and aggravated the above-mentioned cardiac structural, functional and oxidative stress changes in FGF21KO diabetic mice (Figs 2–4 and Table S1), which indicate that FGF21 plays a critical role in protecting the heart against the development of DCM under experimental type 1 diabetic conditions.

Since diabetic down-regulation of plasma FGF21 levels (Fig. 1C) was accompanied by an obvious trend of compensatory up-regulation of cardiac FGFR1 mRNA levels in WT mice (Fig. 1D), while the deletion of FGF21 was also accompanied by a slight compensatory up-regulation of cardiac FGFR1 mRNA levels under basal conditions, which was significantly amplified under diabetic condition in FGF21KO mice (Fig. 1D). These results imply that the exacerbation of DCM by the *fgf21* gene deletion might directly attribute to the dysfunction of FGF21/FGFR1 axis.

Fibroblast growth factor 21 was reported to have anti-hyperglycemic and anti-hyperlipidemic properties in diabetic rodent [13] and monkey [14] models, and hyperglycaemia and hyperlipidaemia were thought to be the major contributors to DCM [2]. Thus, we assumed that FGF21 deletion might further elevate plasma glucose and triglyceride levels, contributing to the accelerated and aggravated development of DCM in FGF21KO diabetic mice. Unexpectedly, both FGF21KO and WT diabetic mice have typically diabetic hyperglycaemia and hyperlipidaemia, no significant differences were observed in plasma glucose (Fig. 1A) and triglyceride (Fig. 1B) levels between FGF21KO and WT diabetic mice. This implies that the dramatic diabetes-induced down-regulation of plasma FGF21 levels in WT type 1 diabetic mice (Fig. 1C) were comparable to FGF21 deletion in FGF21KO diabetic mice with respect to the whole body glucose and
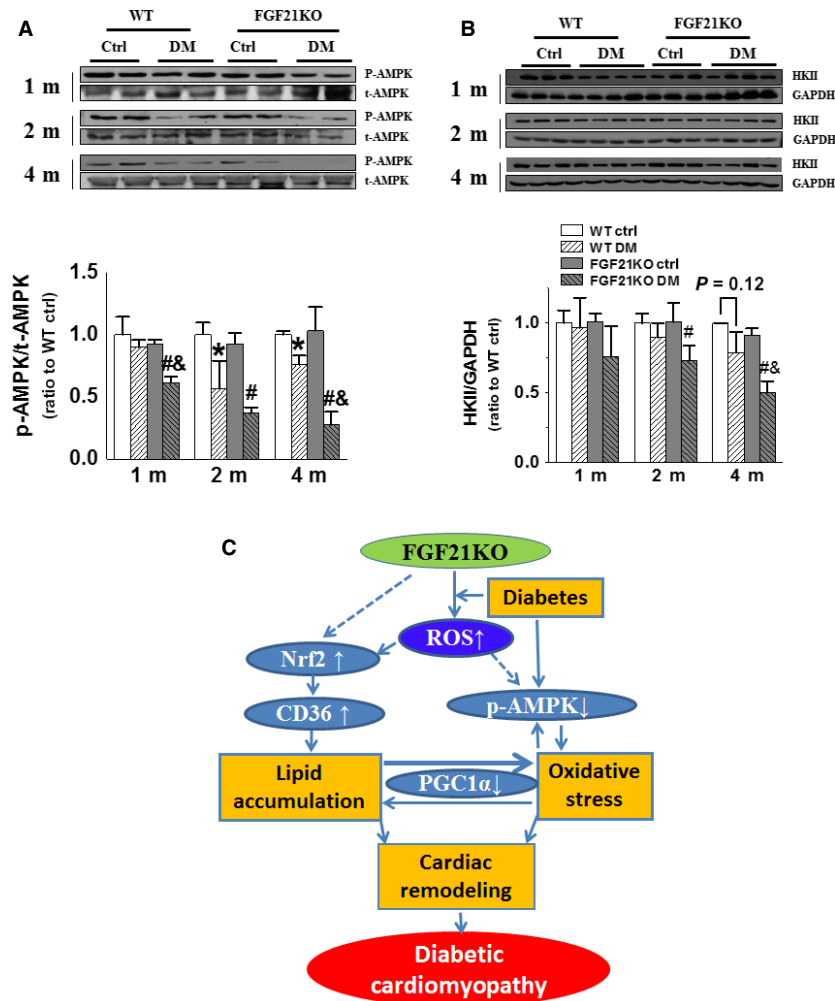
Fig. 7 FGF21 deletion accelerated diabetes-induced cardiac glucose metabolism impairment. At indicated time-points after diabetes onset, cardiac AMPK phosphorylation (**A**) and HKII expression (**B**) were detected by Western blot. Data are presented as means ± SD ($n \geq 5$ for each group). *$P < 0.05$ *versus* WT Ctrl group; #$P < 0.05$ *versus* FGF21KO Ctrl group; &$P < 0.05$ *versus* WTDM group. The proposed mechanism of FGF21 deletion leading to aggravated DCM is schematically described (**C**): FGF21 deletion aggravates the ROS accumulation induced by diabetes, which in turn increases Nrf2 expression. Nrf2 up-regulation elevates CD36 expression, and induces fatty acid over-uptake and cardiac lipid accumulation. In addition, FGF21 deletion-mediated diabetic ROS accumulation attenuates the phosphorylation of AMPK, which leads to impairment of cardiac glucose metabolism and cardiac energy metabolic balance and further aggravates cardiac oxidative stress. The elevated cardiac lipid accumulation and oxidative stress synergistically down-regulate PGC1α function and induce cardiac remodelling, and eventually accelerate the development of DCM.

lipid metabolic regulation by FGF21. The elevated serum FGF21 was reported to associate with hypertension [38]. But in the present study, FGF21KO mice did not exhibit alteration in blood pressure under either basal or diabetic conditions (Fig. S2).

Cardiac lipid accumulation plays a causative role in the development of DCM [39]. FGF21 was also reported to regulate lipid homoeostasis in liver [40], adipose tissue [41] and kidney [27]. In the present study, we observed that FGF21 deletion significantly aggravated cardiac lipid accumulation (Fig. 5), which was time-dependently associated with diabetes-accelerated cardiac dysfunction (Fig. 2) and remodelling (Fig. 3) in FGF21KO diabetic mice, indicating that FGF21

plays a critical role in cardiac protection from the development of DCM by regulation of cardiac lipid metabolism under type 1 diabetic conditions.

CD36 is a pivotal lipid transport protein that mediates fatty acid transport and utilization in the heart [42]. CD36 is believed to play a critical role in intramyocardial lipid accumulation, fatty acid and glucose oxidation and in the subsequent deterioration in cardiac ATP supply in age-induced cardiomyopathy in mice [43]. Under physiological conditions, increased cardiac CD36 expression can compensate for the decreased supply of long-chain fatty acid [44]; but under diabetic conditions, the elevated cardiac CD36 expression

mediates excess uptake of fatty acid leading to cardiac lipid accumulation [31]. In the present study, cardiac CD36 expression was further up-regulated in FGF21KO diabetic mice (Fig. 6A), which was time-dependently associated with excess cardiac lipid accumulation (Fig. 5A and B). Meanwhile, cardiac AMPK phosphorylation (Fig. 7A), an indicator of energy homoeostasis, and HKII expression (Fig. 7B), an indicator of glucose utilization, were also time-dependently further decreased in FGF21KO diabetic mice. Moreover, PGC1α, a critical regulator of fatty acid β-oxidation and a key mediator of FGF21 regulation of lipid metabolism [45] was significantly decreased only in the heart of FGF21KO diabetic mice at the late stage of DCM (Fig. 6C). These results imply that FGF21 deletion-induced excess cardiac lipid uptake and lipid accumulation might impair cardiac lipid and glucose utilization and energetic balance, which further exacerbated lipid accumulation and impaired lipid β-oxidation, contributing to the accelerated DCM in FGF21KO diabetic mice.

CD36 expression was strictly regulated in the heart tissue [46]. Accumulating evidence indicate that Nrf2 up-regulation of CD36-mediated lipid uptake and excess accumulation in macrophages and smooth muscle cells play critical roles in the development of atherosclerosis [32–34]. Consistent with these previous studies, cardiac Nrf2 expression was also found to be largely elevated in FGF21KO diabetic mice, but not in WT diabetic mice (Fig. 6B), which were time-dependently associated with the up-regulation of cardiac CD36 expression (Fig. 6A) and cardiac lipid accumulation (Fig. 5A and B) in the present study. Even though there was one report that has identified Nrf2 as a novel regulator repressing FGF21 expression in liver and adipose tissue under long-term high-fat diet-induced obese conditions [47], further study to dissect the mechanism of FGF21 deletion-mediated up-regulation of cardiac Nrf2-driven CD36 expression and lipid accumulation under type 1 diabetic conditions is warranted.

Among diabetic patients, 90–95% suffer from type 2 diabetes [48, 49]. A limitation of the present study is the lack of the relevance to type 2 diabetes. It has been generally accepted that DCM has similar pathological mechanisms in both type 1 and type 2 diabetes [50], but FGF21 has been demonstrated to have different changing patterns of serum level increase in type 2 diabetes and decrease in type 1 diabetes [37]. To ensure a greater clinical relevance, whether FGF21 plays a similar role in the development of DCM in type 1 and type 2 diabetes really needs to be comparatively studied in the future.

In conclusion, FGF21 deletion up-regulation of Nrf2-driven CD36 expression exacerbates cardiac lipid uptake and accumulation, which in turn impairs cardiac lipid and glucose utilization and cardiac energy balance, and aggravates cardiac oxidative stress, eventually accelerating the development of DCM (Fig. 7C). FGF21 deletion-aggravated DCM indicates that FGF21 may be a therapeutic target for the treatment of diabetic cardiovascular complications.

Acknowledgements

Conflicts of interest

None.

Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Heart weight

Fig. S2. Blood pressure

Table S1. Heart function

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