

Original Article

Lack of fibroblast growth factor 21 accelerates metabolic liver injury characterized by steatohepatitis in mice

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Abstract: Fibroblast growth factor 21 (FGF21) concentrations are increased in human subjects who either have type 2 diabetes or nonalcoholic fatty liver disease (NAFLD). While excessive fat in the liver promotes the release of pro-inflammatory cytokines, NAFLD progresses from steatosis to non alcoholic steatohepatitis (NASH), a more aggressive form of hepatic damage, and lastly toward cirrhosis and HCC. In our previous study, loss of FGF21 is associated with hyper-proliferation, aberrant p53, and HCC development in diabetes mice. In this study, we proposed to investigate the liver metabolic disorders by diabetes and the potential roles of FGF21 played in NASH and potential carcinogenetic transformation of HCC. NASH was induced in FGF21 knockout (FGF21KO) mice by streptozotocin administration or fed with high fat diet (HFD). The pathological transformation of steatohepatitis as well as parameters of inflammation, lipid metabolism, cellular events, mesenchymal-epithelial transition (MET) and Wnt/ β -catenin signaling was determined in the FGF21 KO diabetic mice and HFD fed mice. We found that mice lacking the FGF21 gene are more prone to develop NASH. A compromised microenvironment of NASH, which could facilitate the HCC carcinogenetic transformation, was found in FGF21 KO mice under metabolic disorders by diabetes and HFD feeding. This study provided further evidence that lack of FGF21 worsened the metabolic disorders in NASH and could render a tumor microenvironment for HCC initiation and progression in the liver of diabetes mice.

Keywords: Fibroblast growth factor 21, steatohepatitis, diabetes, hepatocellular carcinoma

Introduction

It has been found that fibroblast growth factor 21 (FGF21) concentrations are increased in human subjects who either have type 2 diabetes or nonalcoholic fatty liver disease (NAFLD) [1, 2]. In a population-based study including 14% of the United States population, diabetes conferred a 3-fold risk of hepatocellular carcinoma (HCC) [3]. More than 70% of diabetic individuals suffer from NAFLD, which is known to be a hepatic manifestation of metabolic syndrome [4]. While excessive fat in the liver promotes the release of pro-inflammatory cytokines, NAFLD progresses from steatosis to non alcoholic steatohepatitis (NASH), a more

aggressive form of hepatic damage, and lastly toward cirrhosis and HCC [5]. With systemic potent insulin-sensitizing actions, FGF21 has been well addressed to control the metabolic disorder such as hyperglycemia and hyperlipidemia [6, 7]. Because FGF21 is synthesized mainly in the hepatocytes, it is natural to consider that FGF21 may play an important role during pathological progression of steatohepatitis [8] and even carcinogenetic transformation [9] in diabetic mice.

FGF21 is a protein consisting of 210 amino acids in mouse, and 209 amino acids in human [6]. As an endocrine FGF, FGF21 is synthesized in liver and secreted into blood [10]. Circulating

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FGF21 binds to a plasma membrane receptor complex, mainly FGF receptor 1 and β -Klotho, and enhances expression of glucose transporter 1 to promote glucose uptake in extra-hepatic tissues such as adipose tissues [10]. Adipose-derived inflammation [11] and insulin resistance [12] were the underline mechanisms linked to the NASH that might accelerate the development of HCC. The following important findings call our attention on the potential protective roles of FGF21 in NASH and HCC. 1) Administration of recombinant FGF21 lowered plasma glucose, increased insulin sensitivity, and reversed hepatic steatosis and obesity in a range of diabetes/obesity animal models [7, 13, 14], indicating the hepatoprotective effects of FGF21 on metabolic disorders. 2) Increased liver injury by lipopolysaccharide has been found in FGF21-null mice, while treatment of recombinant FGF21 can improve their survival [15], indicating the hepatoprotective effects of FGF21 on inflammation; 3) Overexpression of FGF21 delays initiation of chemically induced hepatocarcinogenesis [16], implying the potential anti-cancer properties of FGF21. Therefore, FGF21 could play a critical hepatoprotective role during the pathogenetic changes of NASH and HCC. However, the mechanism by which FGF21 protection against NASH and HCC is largely not known.

In our previous study, increased hepatic FGF21 protein levels were found in diabetic mice with steatohepatitis, but decreased FGF21 protein levels in the cancerous liver tissues when the diabetic mice treated with carcinogen [17]. The loss of FGF21 protein in liver was associated with hyper-proliferation, aberrant p53 and TGF- β /Smad signaling during the development of hepatocellular carcinoma (HCC) [17]. As a modulator of glucose/lipid metabolism, the induction of FGF21 derived from the liver was likely due to elevated hepatic lipid and carbohydrate levels in diabetic condition. However consequences of loss of FGF21 during NASH progression and carcinogenetic transformation in diabetic mice remain unknown. Metabolic disorders have been recognized as major risk factors for the development of certain types of human malignancies, including HCC [3]. The increase of hepatic FGF21 in diabetic mice with NASH could be just compensatory adaptation, while loss of hepatic FGF21 might further worsen metabolic disorders and the compromised

microenvironment of diabetes-NASH liver could facilitate the initiation of HCC.

In this study, we proposed to further investigate the liver metabolic disorders by diabetes and the potential roles of FGF21 played in NASH and potential carcinogenetic transformation of HCC. NASH was induced in FGF21 knockout (FGF21KO) mice by STZ administration or fed with high fat diet (HFD). The pathological transformation of steatohepatitis as well as parameters of inflammation, lipid metabolism, cellular events, mesenchymal-epithelial transition (MET) and Wnt/ β -catenin signaling was determined in FGF21 KO mice with diabetes or HFD fed mice. We found that lacking FGF21 gene in diabetic mice was more prone to develop NASH. A compromised microenvironment with metabolic disorders, which could facilitate the HCC carcinogenetic transformation, was found in FGF21 KO mice with diabetic and HFD feeding.

Materials and methods

Animals and treatment

Male FGF21KO mice with C57 BL/6J background were generously granted by Dr. Steve Kliewer (University of Texas Southwestern Medical Center). Wild-type (WT) C57 BL/6J mice obtained from Jackson Laboratory (Bar Harbor, ME). Both FGF21KO and C57 BL/6J mice were housed four per cage, given commercial chow and tap water, and maintained at 22°C on a 12-hour light/dark cycle. 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 body weight (BW) daily. This protocol of low dose at 60 mg/kg and multiple injections for 5 times is used to reduce the potential toxic side effects of STZ and the mortality. The control mice received same amount citrate buffer alone (i.p.). 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). The STZ treated mice with blood glucose around 350-450 mg/dl were selected as diabetic mice. The diabetic mice (DM) along with the non-diabetes controls (CT) were assigned into the study groups, including WT-CT, WT-DM, FGF21KO-CT (KO-CT)

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and KO-DM. Blood glucose and body weight were monitored every two weeks. The diabetic animals as well as the control animals were sacrificed at month 2, month 4, and month 6. In addition to the diabetic mouse model, a high fat diet (HFD) mouse model was also established. Both FGF21KO and C57 BL/6J mice were fed with HFD (Rodent Diet with 60% kcal% fat, D12492, Research Diets, Inc., New Brunswick, NJ) or Control Diet (CD) with 10% kcal% fat (D12450B, Research Diets, Inc., New Brunswick, NJ). The HFD fed animals and CD fed animals were sacrificed at month 8. At respective time points for sacrifice, mice liver weights and tibia length were measured. Serum plasma and hepatic tissues were harvested from two models for further analysis. Animal procedures were approved by the Institutional Animal Care and Use Committee of University of Louisville, which is certified by the American Association for Accreditation of Laboratory Animal Care.

Hematoxylin-and-eosin staining and Oil Red O staining

Hepatic tissues were fixed in 10% neutral phosphate buffered formalin. Tissues were embedded in paraffin and sectioned to a thickness of 5 μ m for histopathological examination. Hematoxylin-and-eosin (H&E) stained sections were evaluated microscopically. Oil Red O staining for lipid accumulation in the liver tissues was performed in Optimal Cutting Temperature medium (O.C.T.)-embedded frozen tissue. Cryosections (10 μ m thick) from OCT-embedded liver tissues were fixed in 4% buffered formalin for 5 minutes at room temperature, and stained with Oil Red O dye for 1 hour. All the images were reviewed and analyzed under microscope at 20x magnification.

Biochemical analysis

To analyze the liver injury and metabolic abnormalities in the liver, serum plasma alanine aminotransferase (ALT), serum glucose, serum insulin, serum and liver triglyceride (TG) were determined. The serum ALT measured using an ALT infinity enzymatic assay kit (Thermo Fisher Scientific Inc., Waltham, MA), according to the instruction provided. Serum glucose assay was performed using a Sigma assay kit (Sigma-Aldrich Company, MI). TG assay was performed

with TG assay kit (Cayman Chemical Company, CA).

Immunohistochemical analysis

Immunohistochemical staining was also performed on the paraffin embedded tissue sections. Endogenous peroxidase was blocked with 3% hydrogen peroxide, and then with 5% animal serum for 30 min to block non-specific reaction. These tissue sections were incubated with primary antibodies, F4/80 (Abcam San Francisco, CA) and proliferating cell nuclear antigen (PCNA, Invitrogen, Camarillo, CA). Tissue sections were incubated with horseradish peroxidase-conjugated secondary antibody (1:300-400 dilutions with PBS) for 2 hours in room temperature, and then incubated with peroxidase substrate DAB kit (Vector Laboratories, Inc., Burlingame, CA) to develop brown color. The counterstaining was performed using hematoxylin. The computer image-analysis was performed to determine F4/80 index and PCNA index (positive stained cells per 100 cells) under microscope at 20x magnification.

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay

TUNEL staining was performed using an Apop-Tag Peroxidase In Situ Apoptosis Detection Kit (Chemicon, Billerica, CA). Briefly, each slide was deparaffinized, rehydrated, and treated with proteinase K (20 mg/L) for 15 min. The slide was incubated with terminal deoxynucleotidyl transferase (TdT) and digoxigenin-11-dUTP for 1 hr at 37°C. Anti-digoxigenin antibody conjugated with horseradish peroxidase (HRP) along with the substrate (DAB-H₂O₂) was used for visualization. Apoptotic cell death was quantitatively analyzed by counting the TUNEL positive cells in ten fields for each section at 20X magnification. The apoptotic index was presented as TUNEL positive cells per 100 cells.

Western blot assay

Protein levels for the biomarkers were semi-quantified by Western blot analysis as described previously [36]. Electrophoresis was performed on 12% SDS-PAGE gel and the proteins were transferred to nitrocellulose membranes. Membranes were incubated with primary antibodies overnight at 4°C and with secondary antibody for 1 hr at room temperature. The antigen-anti-

body complexes were then visualized using ECL (Amersham, Piscataway, NJ). Primary antibodies used include those raised against interleukin 6 (IL-6), transforming growth factor β 1 (TGF β 1), Phospho smad3 (P-smad3), Peroxisome proliferator-activated receptor alpha (PPAR- α) from Cell Signaling Technology, Danvers, MA, and fatty acid synthase (FASN), E-Cadherin, Vimentin, Fibronectin, Epithelial cell adhesion molecule (EpCAM), Total p53 (T-p53), Phospho p53 (P-p53), β -catenin, glycogen synthase kinase 3 β (GSK-3 β), glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and β -actin from Santa Cruz, CA. The protein bands were quantified by densitometry analysis and protein expression was presented pixel ratio of target protein vs endogenous reference, GAPDH or β -actin.

Real-time RT-PCR (qPCR)

Total RNA was extracted using the TRIzol reagent (Invitrogen, CA). First-strand complementary DNA (cDNA) was synthesized from total RNA according to the manufacturer's protocol for the RNA PCR kit (Promega, Madison, WI). Quantitative PCR was carried out using the ABI 7300 real-time PCR system (Applied Biosystems, Carlsbad, CA). FGF21 expression was quantified and β -actin was used as an endogenous reference. Results were expressed as fold change in gene expression.

Statistical analysis

Collected data from repeated experiments were presented as mean \pm SD. One-way ANOVA was used to determine if differences existed. If so, a post hoc Tukey's test was used for analysis of any differences between groups (Origin 8 laboratory data analysis and graphing software).

Results

Liver injury in FGF21 KO diabetic mice

Liver injuries were evaluated by histology in H&E stained tissue sections and serum ALT levels. The pathological changes of steatohepatitis were reviewed in a blind manner by two pathologists. The results from WT mice are consistent to our previous finding, there was no obvious morphological abnormality in the diabetic liver of WT mice at earlier stages of month 2 and month 4, but steatohepatitis character-

ized by inflammatory infiltration and lipid drops were found at later stage of month 6 in the WT mice. However, FGF21 KO mice showed very different histological pattern. Severe steatohepatitis were found at month 4, and more severe steatohepatitis were found at month 6 in diabetic FGF21 KO-mice. In addition, steatohepatitis were also found in non diabetes FGF21 KO-mice at month 6 (**Figure 1A**). The levels of serum ALT were significantly increased in the diabetic mice both WT and FGF21 KO. Interestingly, the serum ALT levels were also increased in the non diabetes FGF21 KO mice. The ALT level from FGF21 KO diabetic mice reached a highest level at 6 months and the increased ALT activity is consistent with the severity of steatohepatitis in the diabetic mice at 6 months (**Figure 1B**). FGF 21 expression was further determined in the liver tissues. Increased FGF 21 mRNA levels were detected in WT diabetic mice at 6 months (**Figure 1B**). The results indicated that liver injuries was induced at later stages of diabetes in the WT, and increased FGF 21 levels associated to the steatohepatitis development [17]. Lack of FGF 21, diabetes causes liver injury was even worse than WT diabetic mice, indicating that FGF21 protected liver from the diabetes induced injury.

Metabolic abnormalities in FGF21 KO diabetic mice

As we know, FGF21 is an important regulator of glucose and lipid metabolism. Hepatic manifestation of metabolic syndrome by diabetes is presented as steatohepatitis. Therefore, the parameters of metabolic disorder contributing to steatohepatitis were further evaluated. The body weights were significantly decreased ($p < 0.05$) in diabetic mice both WT and FGF21 KO compared to the respective non-diabetic controls. The liver weights in WT diabetic mice were significantly increased ($p < 0.05$) compared to non-diabetic WT controls. The liver weights in FGF21 KO diabetic mice were slightly decreased but not reach statistic significance ($p > 0.05$) compared to non-diabetic FGF21 KO controls. The blood glucose levels in all diabetic mice were significantly increased and around 400-500mg/dl from month 2 to month 6. There was no significant difference of blood glucose levels between the diabetic WT and diabetic FGF21 KO mice. The plasma TG levels were significantly increased in the diabetic mice of both WT

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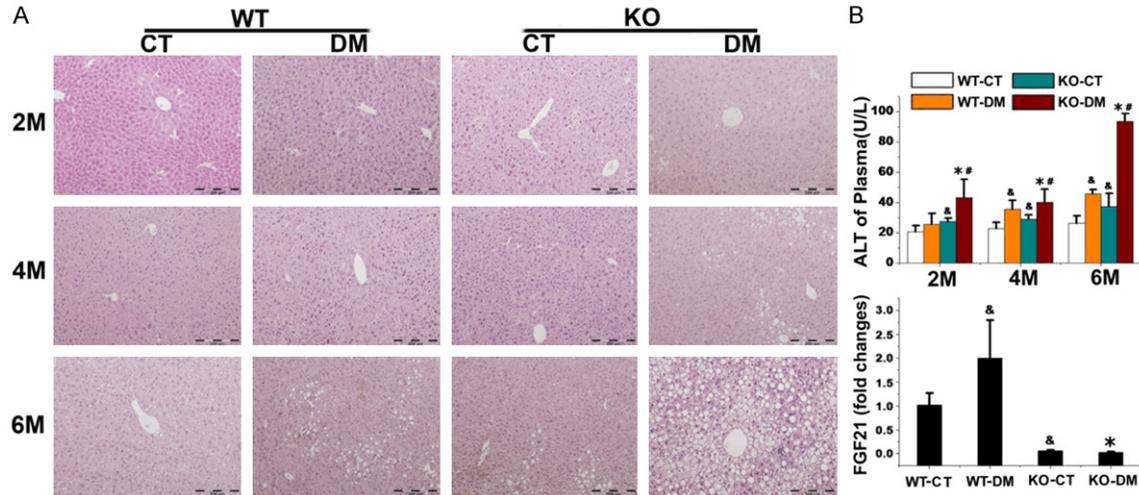


Figure 1. A: Representative histological changes from all 4 experimental groups in time-course study. Histological changes of NASH were identified in tissue sections by H&E staining. NASH was found typically affect the liver parenchyma with the occurring of macrovesicular presented in a predominantly perivenular distribution. In addition to fatty change, many hepatocytes in acinar showed ballooning and contain Mallory's hyaline, and mixed infiltrate of inflammatory cells. B: Upper, serum ALT levels in all 4 experimental groups. Lower, the FGF21 mRNA fold changes in the hepatic tissues from all 4 experimental groups at month 6. WT: wild type mice; FGF21 KO: FGF21 knockout mice; CT: control; DM: Diabetes Mellitus; M: month. * $p < 0.05$ vs WT-DM; # $p < 0.05$ vs KO-CT; & $p < 0.05$ vs WT-CT.

and FGF21 KO, beginning to increase at month 2, and continuing to increase at month 4 and month 6. There was no significantly difference of the plasma TG levels between diabetes WT and diabetic FGF21 KO mice, from month 2 and month 4. However, the plasma TG levels significantly increased ($p < 0.05$) in diabetic FGF21 KO mice at month 6 compared to the same age diabetic WT mice (**Figure 2A**). The hepatic lipid accumulation was further confirmed by Oil Red O staining in hepatic tissue. As shown in the **Figure 2B**, the positive staining was found in the diabetic mice at month 4 and month 6. Interestingly, the non diabetic FGF21 KO mice at month 6 also showed widely distributions of positive staining, even though their plasma TG did not increased. The trend of hepatic lipid accumulating is consisted to plasma TG levels, implying dynamic increases of metabolic disorders in diabetes mice, especially in diabetic FGF21 KO, from month 4 to month 6.

Macrophages and cytokins in FGF21 KO diabetic mice

As ectopic intracellular lipid accumulation in hepatocytes, subsequent recruitment of inflammatory cells and activations of proinflammatory cytokines lead to further liver injury. We further evaluated the inflammatory cells and pro-

inflammatory cytokins in FGF21 KO diabetic mice. Because macrophage-associated inflammation are characteristics of NASH [18], we evaluated macrophages by immunohistochemical staining in liver tissues. As shown in **Figure 3A**, increased F4/80 positive macrophages were found in the in the diabetic mice of both WT and FGF21 KO at month 6. Interestingly, the non diabetic FGF21 KO mice also showed widely distributions of positive F4/80 positive macrophages in the liver tissue. IL-6 has been reported to attenuate lipid accumulation in hepatocytes thereby to protect liver from [19, 20]. One the other hand, as a non-cellular component of the microenvironment, IL-6 is also one of the best-characterized pro-tumorigenic cytokines [21] and IL-6 is associated with rapid progression from viral hepatitis to HCC [22, 23]. Our results indicated that increased IL-6 protein levels in the diabetic FGF21 KO and non diabetic FGF21 KO mice at month 6, but not WT mice. As we know, lipid peroxides generated by hepatocytes could initiate hepatic fibrosis which linked cirrhosis and HCC. TGF β 1 is a potent pro-fibrogenic cytokine and TGF β 1/Smad signaling activation has been found in hepatic fibrosis and HCC of humans [24, 25]. In our study, significantly increased protein levels of TGF β 1 were found in the diabetic FGF21 KO mice and non diabetic FGF21 KO mice.

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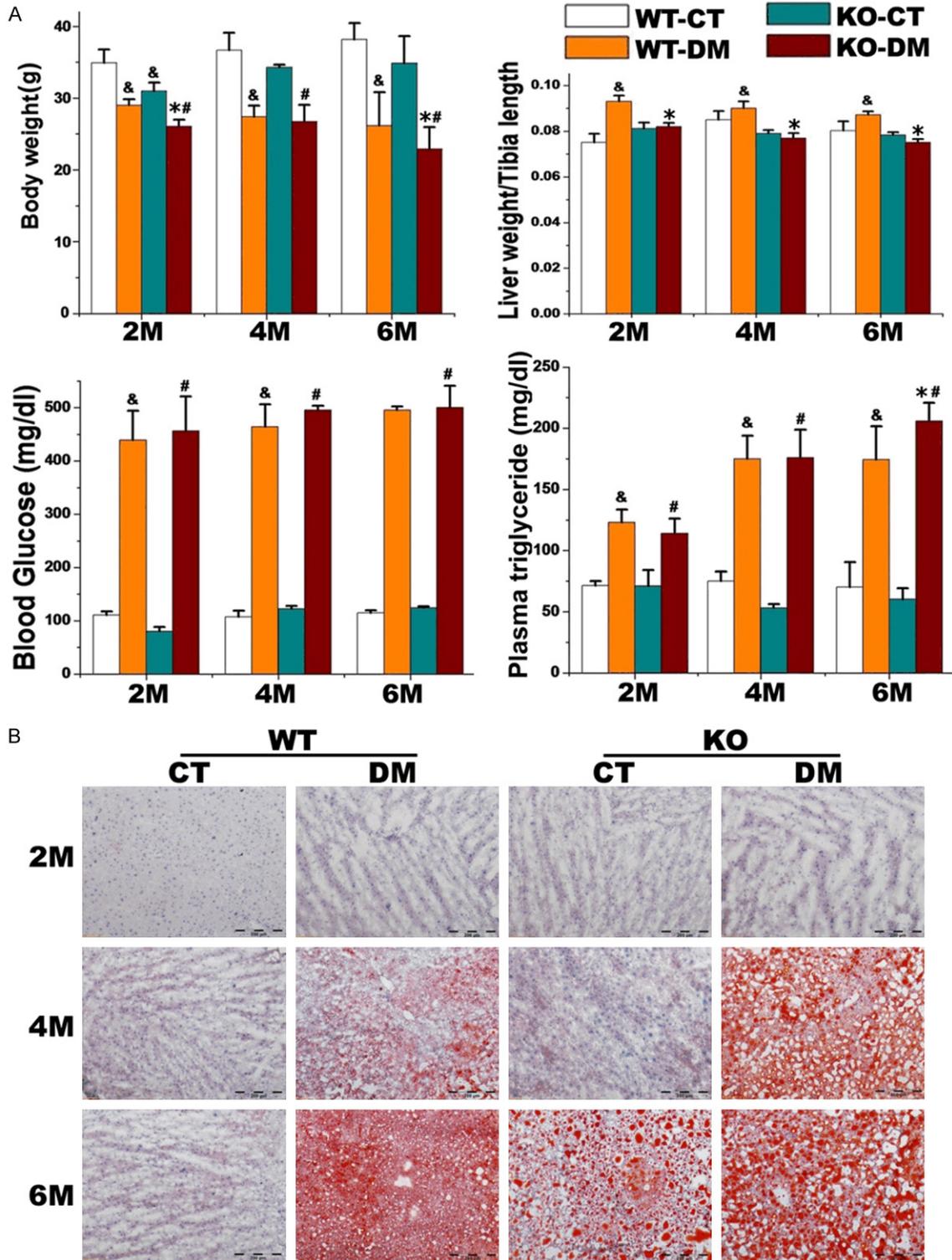


Figure 2. A: The body weights, liver weights, blood glucose and plasma triglyceride levels in all 4 experimental groups. B: Comparison of lipid accumulation in liver parenchyma from all 4 experimental groups in time-course study. Lipid drops were identified in tissue sections by Oil red O staining. WT: wild type mice; FGF21 KO: FGF21 knockout mice; CT: control; DM: Diabetes Mellitus; M: month. * $p < 0.05$ vs WT-DM; # $p < 0.05$ vs KO-CT; & $p < 0.05$ vs WT-CT.

Increased P-Smad3 expression was found in FGF21 KO mice and diabetic WT mice. The pro-

tein levels of TGF β 1 and P-Smad3 in FGF21 KO mice were much higher than that in the diabetic

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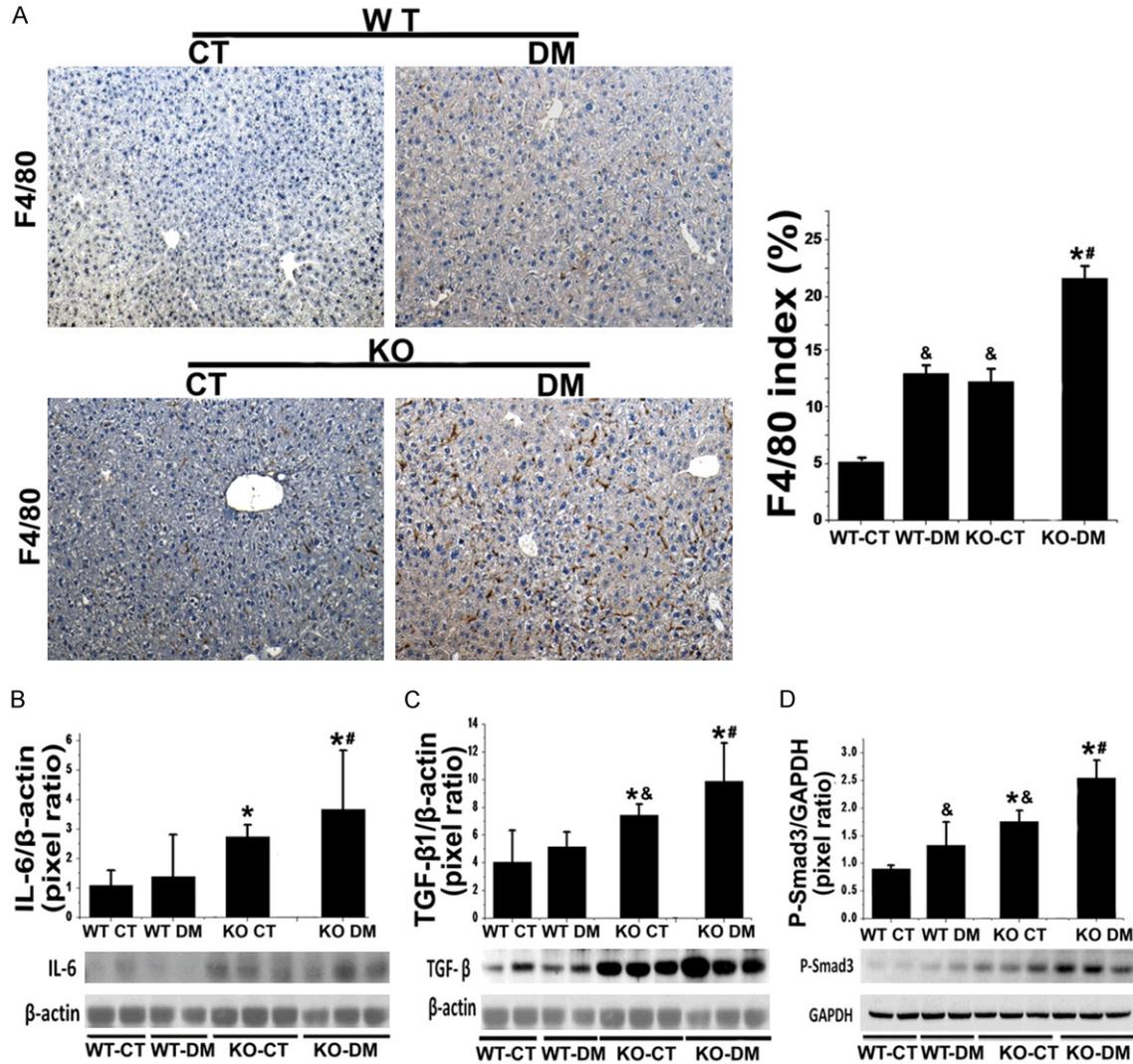


Figure 3. A: Representative images of macrophages infiltration in liver parenchyma in all 4 experimental groups at month 6. Macrophages were identified by F4/80 immunohistochemical staining. The positive stained cells were calculated by computer-image analysis and presented as index. B-D: The protein levels of caspase-8, the protein levels of TGF- β , and the protein levels of P-Smad3 in liver tissues by Western blot analysis. The protein levels were quantified by image analysis and presented as pixel ratio over the controls either β -actin or GAPDH. WT: wild type mice; FGF21 KO: FGF21 knockout mice; CT: control; DM: Diabetes Mellitus; M: month. * $p < 0.05$ vs WT-DM; # $p < 0.05$ vs KO-CT; & $p < 0.05$ vs WT-CT.

WT mice. Our results indicated that lack of FGF21 could suffer more severe liver injury which linked cirrhosis and HCC.

Lipid metabolism and Cellular events in FGF21 KO diabetic mice

We further investigated whether lack of FGF21 could affect the lipid metabolism and *de novo* fatty acid biosynthesis in the liver, PPAR- α and FASN were measured. PPAR- α is predominantly expressed in the liver and regulated hepatic lipid accumulation [26]. FASN catalyzes the last

step in fatty acid (FA) biosynthesis to generate FA by *de novo* lipogenesis in liver [27]. The results indicated that the protein levels of PPAR α and FASN were increased in the liver of WT diabetes mice as well as diabetic FGF21 KO and non diabetic FGF21 KO mice. The highest levels of PPAR α and FASN were found in the FGF21 KO mice with diabetes at month 6 (**Figure 4A**). All the abnormalities of metabolism and macrophage-associated inflammation encouraged us to further investigate two important cellular events, cell death and cell proliferation, to evaluate if the liver of FGF21 KO dia-

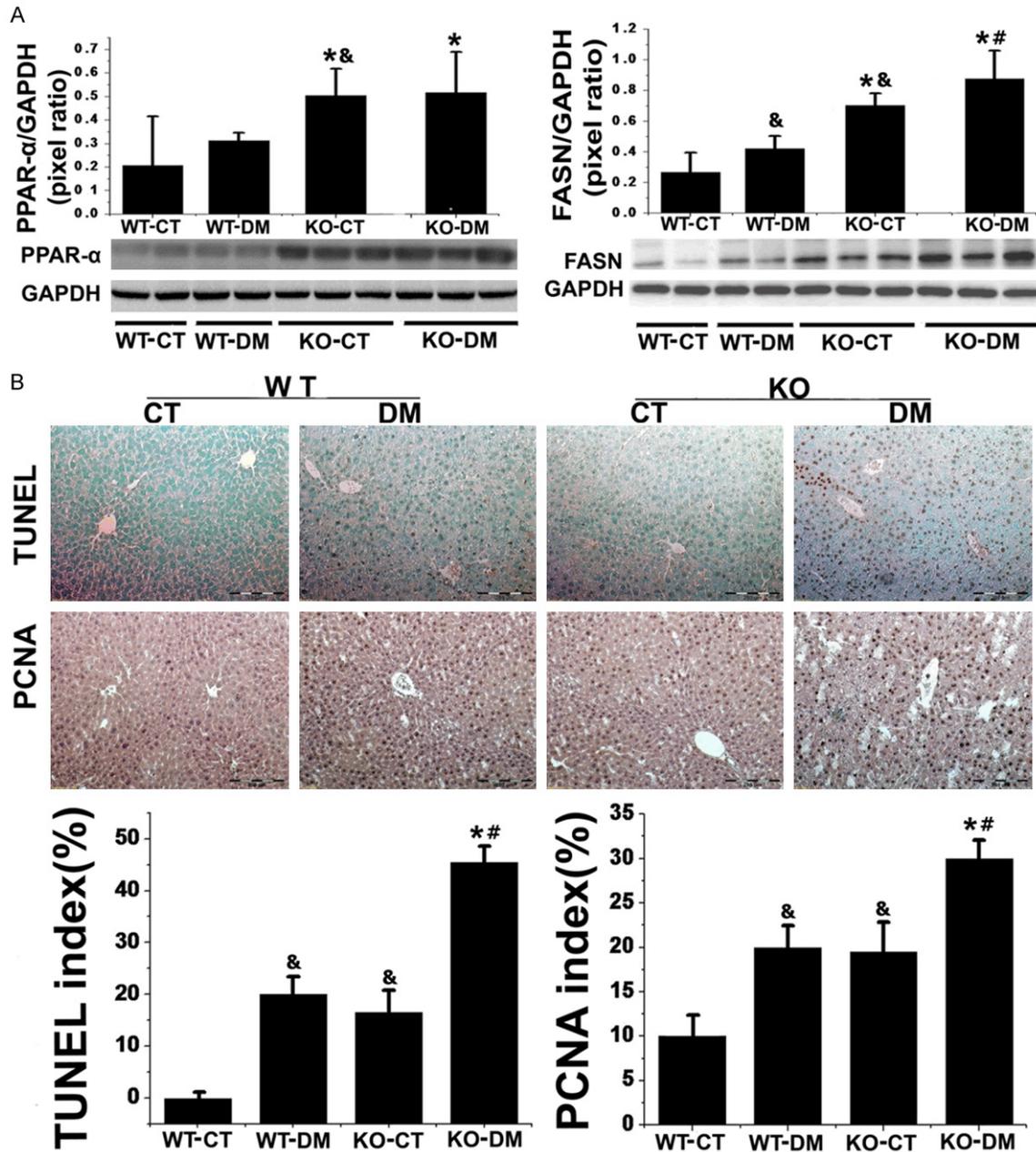


Figure 4. A: The protein levels of PPAR- α and FASN in liver tissues by Western blot analysis. The protein levels were quantified by image analysis and presented as pixel ratio over the control GAPDH. B: Representative images of apoptosis and proliferation by TUNEL staining and PCNA immunohistochemical staining in liver parenchyma in all 4 experimental groups at month 6. The apoptotic index and proliferation index were quantified by image-analysis of the positive TUNEL cells and positive PCNA cells. WT: wild type mice; FGF21 KO: FGF21 knockout mice; CT: control; DM: Diabetes Mellitus; M: month. * $p < 0.05$ vs WT-DM; # $p < 0.05$ vs KO-CT; & $p < 0.05$ vs WT-CT.

abetic mice rendered to a compromised micro-environment in the liver. Increased apoptotic index by TUNEL assay was found in the diabetic mice both WT and FGF21 KO, while increased proliferative index by PCAN staining was found in the diabetic mice both WT and FGF21 as well non diabetic FGF21 KO mice (Figure 4B).

Epithelial-mesenchymal transition (EMT), p53 and Wnt/ β -catenin signaling in FGF21 KO diabetic mice

NASH stage is aggressive and disastrous. About 80% NASH patients with diabetic background having fibrosis progression may advan-

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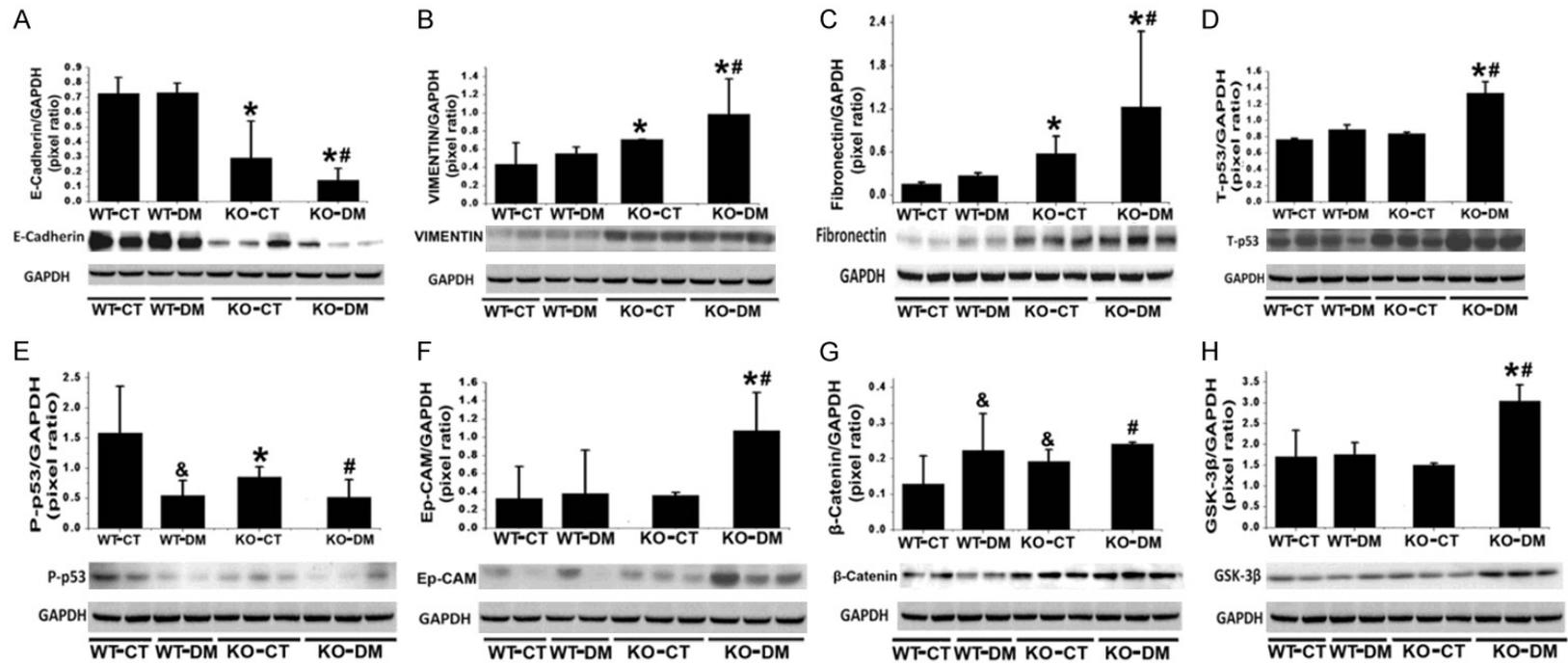


Figure 5. Aberrant protein expression of EMT and Wnt/ β -catenin signaling by Western blot analysis in liver parenchyma in all 4 experimental groups at month 6. A: E-cadherin; B: Vimentin; C: Fibronectin; D: EpCAM; E: T-p53; F: P-p53; G: β -catenin; and H: GSK-3 β . The protein levels were quantified by image analysis and presented as pixel ratio over the controls either β -actin or GAPDH. WT: wild type mice; FGF21 KO: FGF21 knockout mice; CT: control; DM: Diabetes Mellitus; M: month. * p <0.05 vs WT-DM; # p <0.05 vs KO-CT; & p <0.05 vs WT-CT.

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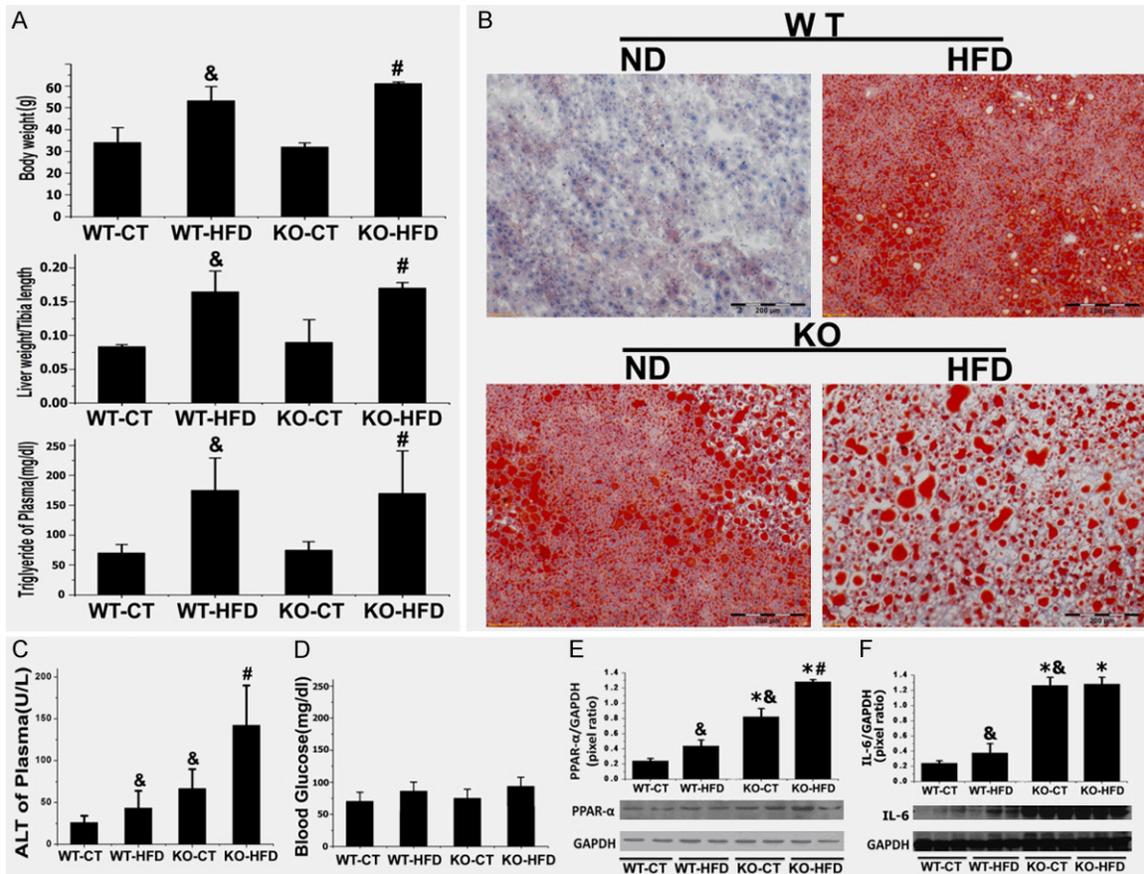


Figure 6. A: The body weights, liver weights, and plasma triglyceride levels in HFD fed for 8 months from WT and FGF21 KO mice as well as controls. B: Comparison of lipid accumulation in liver parenchyma from in HFD fed for 8 months from WT and FGF21 KO mice as well as controls. Lipid drops were identified in tissue sections by Oil red O staining. C: Serum ALT levels; D: Blood glucose; E, F: The protein levels of PPAR- α and T-P53 in liver tissues by Western blot analysis. The protein levels were quantified by image analysis and presented as pixel ratio over the controls of GAPDH. WT: wild type mice; FGF21 KO: FGF21 knockout mice; CT: control; HFD: high fat diet. * $p < 0.05$ vs WT-HFD; # $p < 0.05$ vs KO-CT; & $p < 0.05$ vs WT-CT.

ce to cirrhosis and are at risk for complications of end-stage liver disease-HCC [28]. Our previous data showed that decreased FGF21 protein levels were associated with hyper-proliferation, aberrant p53 and TGF- β /Smad signaling during the development of HCC [17]. The aberrant lipid metabolism and viperous cellular events in the liver diabetic FGF21 KO mice might render a tumor microenvironment in liver. Therefore, we further evaluated the molecular events including EMT, the gate keeper gene p53, and Wnt/ β -catenin signaling, which were closely linked to carcinogenetic transformation. Loss of E-cadherin, the epithelial cell type, was found in the FGF21 KO mice with or without diabetes but not the diabetic WT mice or WT control mice. Significant decrease of E-cadherin protein level was found in diabetic FGF21 KO

mice. Gain of vimentin and fibronectin, the mesenchymal cell type, were found in the FGF21 KO mice with or without diabetes but not the diabetic WT mice or WT control mice, while significant increases of vimentin and fibronectin protein levels were found in diabetic FGF21 KO mice (**Figure 5A-C**). T-p53 was up-regulated in diabetic FGF21 KO mice, however P-p53 was down regulated diabetic mice both WT and FGF21 KO, and non diabetic FGF21 KO mice (**Figure 5D, 5E**). EpCAM, a CSC surface marker, was significantly increased in diabetic FGF21 KO mice. Increased β -catenin expression was found in diabetic WT mice, diabetic FGF21 KO mice and non diabetic FGF21 KO mice. GSK-3 β , an important regulator of β -catenin, was significantly increased in diabetic FGF21 KO but not in other groups (**Figure 5F-H**).

Metabolic abnormalities in FGF21 KO NASH mice

The results from diabetic FGF21 KO mice indicated that lack of FGF21 worsened lipid metabolism and rendered a compromised microenvironment in the liver. However blood glucose in diabetic FGF21 KO mice was diabetic WT mice, but the worst hepatic lipid accumulation was found not only in diabetic FGF21 KO mice. It is wondered if, without overt hyperglycemia, overload of HFD per se can cause a compromised microenvironment in the liver of FGF21 KO mice. To address this issue, we further studied the HFD fed FGF21 KO mice for 8 months. The results indicated that body weights and liver weights were significantly increased ($p > 0.05$) in mice fed with HFD. There were no statistical differences of the body weight and liver weight between the HFD fed WT mice and HFD fed FGF21 KO mice. The plasma TG levels were also significantly increased in the HFD fed mice, and there were no significant differences of the plasma TG levels between HFD fed WT mice and HFD fed FGF21 KO mice (**Figure 6A**). The hepatic lipid accumulation was detected by Oil Red O staining. HFD fed mice showed positive staining. Consistent to the finding in diabetes study, plasma TG level was not increased but hepatic lipid accumulation was detected in FGF21 KO mice aged at 8 months (**Figure 6B**). The serum ALT levels were increased in the HFD fed mice as well as CD fed FGF21 KO mice. The HFD fed FGF21 KO mice showed much higher compared to HFD fed WT mice and CD fed FGF21 KO mice level (**Figure 6C**). The blood glucose levels were normal in all four groups (**Figure 6D**), implying that the hepatic lipid accumulation contributed to the liver injury. We further evaluated PPAR α protein level in hepatic tissues. As expected, increased PPAR α protein levels were consistent to the respective groups' ALT levels. Because intracellular lipid accumulation in hepatocytes can activate proinflammatory cytokines lead to further liver injury, we further evaluated the IL-6 protein levels in liver tissues. Significant increased IL-6 protein levels were found in FGF21 KO mice with either CD feeding or HFD feeding, however IL-6 protein level was much lower in HFD fed WT mice (**Figure 6E, 6F**), implying that lack of FGF21 protein could be a critical factor for the severity of steatohepatitis.

Discussion

Hepatic FGF21 was suggested as a key mediator of hepatic lipid metabolism [29]. Our previous study showed that adaptive increase of FGF21 associated with the severity of steatohepatitis in diabetic mice [17]. However the exact role of FGF21 play in the diabetes caused hepatic metabolic disorders was unknown. We also found decreased FGF21 protein levels associated with hyper-proliferation, aberrant p53 and TGF- β /Smad signaling during the development of HCC [17]. The relevant experiments to study the metabolic disorders linking to the carcinogenetic transformation in diabetic FGF21 knockout animals are unavailable. FGF21 knockdown in mice was reported caused increases of hepatic and plasma triglyceride when the mice fed with ketogenic diet [29], it is reasonable to speculate that lack of FGF21 could also make the hepatic metabolism worsen in diabetes condition. In this study, we investigated, for the first time, the pathogenesis of metabolic liver injury in the FGF21 KO diabetic mice. The results indicated that pronounced liver damages characterized by steatohepatitis were found in the diabetic FGF21 KO mice. Interestingly, the severe metabolic abnormalities were associated to the compromised tumor suppress signaling such as p-p53 and the aberrant carcinogenetic signaling such as EMT and Wnt/ β -catenin components in hepatic tissues of diabetic FGF21 KO mice.

Evidence from both animal and clinical studies supported a role for FGF21 as a liver safeguard [30]. The hepatoprotective effect of FGF21 regarding the metabolic disorders was well addressed previously, for examples, recombinant FGF21 and its variant LY2405319 markedly improve dyslipidemia in both diabetic rhesus monkeys [14, 31], and obese humans with type 2 diabetes [32]. In this study, there was no difference of blood glucose between diabetic FGF21 KO mice and diabetic WT mice, but the worst hepatic lipid accumulation was found not only in diabetic FGF21 KO mice as but also non diabetic FGF21 KO mice. Extensive liver damages associated hepatic lipid accumulation were observed in diabetic FGF21 KO mice. The extensive liver damages in diabetic FGF21 KO mice could be from either loss of the systemic action or paracrine action of the FGF21. As we

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know, with the systemic action, hepatic FGF21 elicits metabolic benefits by targeting adipocytes of the peripheral adipose tissue through the transmembrane FGFR1- co-factor β Klotho complex [33, 34]. In the diabetic FGF21 KO mice, they lost bodyweight significantly, and the bodyweight lost is mainly from the adipose tissue lost. Because FGF-21 stimulated uptake glucose was adipocyte specific, other than other type of cells such as hepatocytes, myocytes and fibroblasts [6], lack of FGF21 did not affect the blood glucose in the diabetic FGF21 KO mice because of less adipocytes available. However, lack of FGF21 is important for lipid metabolism contributing to steatohepatitis and this was supported by the followings: 1) adipogenesis-related genes are increased in hepatic tissues of FGF21 KO mice because these genes can be suppressed by FGF21 [35]; 2) plasma free fatty acids (FFAs) and hepatic FFAs uptake, which were suppressed by FGF21 [36], are increased in FGF21 KO mice. 3) in this current study, the HFD fed FGF21 KO mice showed that HFD induced steatohepatitis in the non diabetic FGF21 KO mice without overt hyperglycemia.

NASH characterized mainly by hepatic lipotoxicity, inflammation and varying stages of fibrosis. Beside the effect to control metabolic disorders, FGF21 was also suggested to have a beneficial action on inflammation [37, 38]. Therefore, lack of FGF21 could worsen inflammation in NASH. In the current study, extensive distribution of macrophages as well as increased cytokines such as IL-6 and TGF- β 1 was found in the diabetic FGF21 KO mice. Our results agreed with a recent finding in which the lipotoxic hepatocyte-derived extracellular vesicles (EV), but not normal hepatocyte EV, release proinflammatory signals in the form of EV that can induce macrophage activation and release of cytokines such as IL-1 β and IL-6 from macrophages [39]. In current study, increased TGF- β signaling is also observed in the diabetic FGF21 KO mice, and this result is strongly supported by our previous data [17] and a recent study in which FGF-21 treatment can down-regulate the expression of TGF- β 1 and phosphorylation levels of smad2/3 [40]. In addition to the pro-inflammatory signals, increase of fibrogenesis is also an important biomarker to adjust the NASH stage aggressive and disastrous. Our result indicates an increase fibronectin protein levels in diabetic FGF21 KO mice. It has been

reported that FGF-21 can attenuate hepatic fibrogenesis and inhibit the activation of hepatic stellate cell [40], therefore, lack of FGF21 make it unable to control the hepatic fibrogenesis and hepatic stellate cell activation thereby causes the increased fibronectin in diabetic FGF21 KO mice.

Pro-inflammatory cytokines/chemokines play a critical role in the progression of NASH to more advanced stages of liver damage [41]. Our previous study showed that loss of FGF21 protein level associated with cancerous hyperproliferation as well as aberrant p53 and TGF- β /Smad signaling in diabetic mice [17]. In this study, aberrant cellular events were also found when lack of FGF21, both apoptotic index and proliferation index increased in diabetic FGF21 KO mice. In NASH, hepatocyte lipoapoptosis mediated by death receptor signaling was reported [42], therefore extensive hepatocyte death via lipoapoptosis in the liver of diabetic FGF21 KO mice because of lipid accumulation in hepatocyte. While the increase of proliferation could be compensatory for the massive cell death, which needed to be further confirmed. Nevertheless, the increased rates of both cell death and proliferation implied repetitive damages in the liver of diabetic FGF21 KO mice. The lipid accumulation, inflammation, fibrogenesis, lipoapoptosis and regenerative proliferation of hepatocytes could provide a compromised microenvironment to initiate HCC. As we further studied, EMT and Wnt/ β -catenin signaling were activated abnormally in the liver of diabetic FGF21 KO mice. Although the molecular mechanisms linking NASH to hepatocellular carcinoma are not well defined, there is growing evidence to suggest that compromised hepatic microenvironment might serve as a critical modulator in carcinogenesis, and severe hepatitis promoted hepatocellular carcinoma via NF- κ B mediated EMT [43]. For this reason, exist of aberrant EMT and dysregulated Wnt/ β -catenin signaling in the liver of diabetic FGF21 KO mice could be deleterious. It has been shown that the aberrant Wnt/ β -catenin signaling was frequently occurring in earlier HCC suggested that patients with "MET-high" phenotype [44]. Unfortunately, the gate keeper gene p53 was also not function well in the diabetic FGF21 KO mice, implying the diabetic mice could be at high risk to develop liver

cancer when they lost the liver safeguard-FGF21.

In conclusion, knockout of FGF21 caused extensive liver damages and worsened metabolic disorders contributing to the aberrant cellular events and molecular events in diabetic mice. This study provided further evidence that FGF21 played a critical role in lipid metabolism and prevention to steatohepatitis. Lack of FGF21 could render a compromised microenvironment in liver for HCC initiation.

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Disclosure of conflict of interest

None.

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