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Bile acids override steatosis in farnesoid X receptor deficient mice in a model of non-alcoholic steatohepatitis

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ABSTRACT

Non-alcoholic fatty liver disease (NAFLD) is one of the most common liver diseases, and the pathogenesis is still not well known. The farnesoid X receptor (FXR) is a member of the nuclear hormone receptor superfamily and plays an essential role in maintaining bile acid and lipid homeostasis. In this study, we study the role of FXR in the pathogenesis of NFALD. We found that FXR deficient ($FXR^{-/-}$) mice fed methionine- and choline-deficient (MCD) diet had higher serum ALT and AST activities and lower hepatic triglyceride levels than wild-type (WT) mice fed MCD diet. Expression of genes involved in inflammation (*VCAM-1*) and fibrosis (*α-SMA*) was increased in $FXR^{-/-}$ mice fed MCD diet ($FXR^{-/-}$/MCD) compared to WT mice fed MCD diet (WT/MCD). Although MCD diet significantly induced hepatic fibrosis in terms of liver histology, $FXR^{-/-}$/MCD mice showed less degree of hepatic steatosis than WT/MCD mice. Moreover, FXR deficiency synergistically potentiated the elevation effects of MCD diet on serum and hepatic bile acids levels. The super-physiological concentrations of hepatic bile acids in $FXR^{-/-}$/MCD mice inhibited the expression of genes involved in fatty acid uptake and triglyceride accumulation, which may be an explanation for less steatosis in $FXR^{-/-}$/MCD mice in contrast to WT/MCD mice. These results suggest that hepatic bile acids accumulation could override simple steatosis in hepatic injury during the progression of NAFLD and further emphasize the role of FXR in maintaining hepatic bile acid homeostasis in liver disorders and in hepatic protection.

1. Introduction

Non-alcoholic fatty liver disease (NAFLD) is one of the most common liver diseases in the world, which refers to a spectrum of liver disorders encompassing hepatic steatosis, non-alcoholic steatohepatitis (NASH), and fibrosis [1,2]. Patients with NASH progress to cirrhosis with high incidence and even to end-stages, such as liver failure and hepatocellular carcinoma. Although the prevalence of NASH increases gradually, few treatments have been proven effective for NASH, making the full understanding of its pathogenesis imperative. The pathogenesis of NAFLD and the mechanisms of progression of the disease remain elusive. It was proposed that multiple factors would be involved. The "two hit" hypothesis is a commonly accepted pathway by which fatty liver progresses to NASH [3]. The first hit involves hepatic steatosis due to excessive lipid accumulation. Accumulation of triglycerides in hepatocytes is the hallmark of NAFLD. The second hit induces liver damage and promote inflammation with neutrophil infiltration. Other evidence supported that steatosis is not mandatory for

Abbreviations: *ACAA1a*, acetyl-CoA acyltransferase 1; *ACADM*, acyl-coenzyme A dehydrogenase; *ACC1*, acetyl-CoA carboxylase 1; *Acox1*, acyl-CoA oxidase 1; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BAs, bile acids; *BSEP*, bile salt export pump; CDCA, chenodeoxycholic acid; *CPT1a*, carnitine palmitoyltransferase 1a; *CYP7A1*, cholesterol 7α-hydroxylase; *CYP8B1*, sterol 12α-hydroxylase; *DGAT*, diacylglycerol acyltransferases; *FABP1*, fatty acid binding protein 1; *FABP4*, fatty acid binding protein 4; *FASN*, fatty acid synthase; *FAT*, fatty acid translocase; $FXR^{-/-}$, FXR deficient; FXR, farnesoid X receptor; GPCR, G protein-coupled receptor; *LIPC*, hepatic lipase; *LRP1*, low-density lipoprotein receptor related protein-1; *LPL*, lipoprotein lipase; MCD, methionine- and choline-deficient; *MCP-1*, monocyte chemotactic protein-1; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; *SHP*, small heterodimer partner; *SREBP-1c*, sterol regulatory element binding protein-1c; *SCD1*, stearoyl-CoA desaturase 1; *SLC27a1*, solute carrier family 27 member 1; *VCAM-1*, vascular cell adhesion molecule-1.

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progression toward hepatic inflammation in the mouse models. In fact, others have postulated triglycerides accumulation in the liver may even serve as a protective mechanism against inflammation development by acting as a reservoir for harmful free fatty acid [4].

The farnesoid X receptor (FXR; NR1H4) is a member of the nuclear receptor superfamily and a ligand-activated transcriptional factor. FXR is mainly expressed in the liver, gastrointestinal tract, kidneys and adrenal gland [5,6]. FXR regulates transcription of genes through binding to FXR response element (FXRE) on target gene [7]. Both endogenous bile acids and synthetic ligands activate FXR. FXR plays a central role in bile acid homeostasis by regulating genes involved in bile acid synthesis, secretion and re-absorption, including small heterodimer partner (*SHP*), cholesterol 7α-hydroxylase (*CYP7A1*), sterol 12α-hydroxylase (*CYP8B1*), and bile salt export pump (*BSEP*) [8–10]. FXR agonist has been shown to protect against cholestatic liver injury and fibrosis in rat models of extrahepatic and intrahepatic cholestasis [11].

Our previous study demonstrated that WAY-362450, a synthetic potent FXR agonist, protects against hepatic inflammation and fibrosis without inhibiting hepatic triglyceride accumulation in mice fed a methionine- and choline-deficient (MCD) diet [12]. To further understand the role of steatosis and FXR in the pathogenesis of NASH, we explored the role of FXR in MCD diet-induced NASH model. Surprisingly, we found FXR deficient mice fed MCD diet showed less steatosis but higher ALT and AST level compared with their WT counterparts.

2. Materials and methods

2.1. Animals and treatments

$FXR^{-/-}$ mice on a C57BL/6J background were obtained from the Jackson Laboratory (BarHarbor, ME). Wild-type mice (C57BL/6J) were from the Shanghai Laboratory Animal Center (Chinese Academy of Sciences, Shanghai, China). All animal procedures were performed according to the criteria outlined in the guide for the *Care and Use of Laboratory Animals* and with Approval of the Animal Care and Use Committee of Fudan University. Wild-type and $FXR^{-/-}$ mice were fed either a MCD diet (Harlan Teklad, TD90262) or a control diet (Harlan Teklad, TD94149) for 4 weeks as previously described [12]. All animals had free access to the diet throughout the experimental period. At the end of the experiment, animals were fasted for 4 h, anesthetized with sodium pentobarbital (75 mg/kg, intraperitoneal injection). Blood was collected from the *vena cava* just prior to sacrifice by exsanguination and plasma was stored at −80 °C until further analysis. Portions of liver tissue were either frozen immediately in liquid nitrogen, fixed in 10% neutral buffered formalin, or frozen-fixed in OCT mounting media (Tissue Tek, Hatfield, PA) for subsequent sectioning.

2.2. Serum and tissue biochemical assay

Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities were measured according to manufacturer's instruction (DiaSys Diagnostic Systems, Holzheim, Germany). Hepatic triglyceride was extracted and measured using a commercially available kit (Biovision Inc., Mountain View, CA). Total bile acid in livers was extracted using 75% ethanol for 2 h and measured using a kit from DiaSys Diagnostic Systems, and serum bile acid level was measured using the same kit.

2.3. Liver histopathological examination

In all groups, formalin-fixed and paraffin embedded liver tissues were processed for hematoxylin and eosin staining (H&E), and OCT-embedded liver tissues were stained with Oil Red O (Sigma, St. Louis, MO) to estimate the degree of hepatic steatosis as previously described [13]. Siruid red staining was applied to assess the degree of fibrosis as previously described [12].

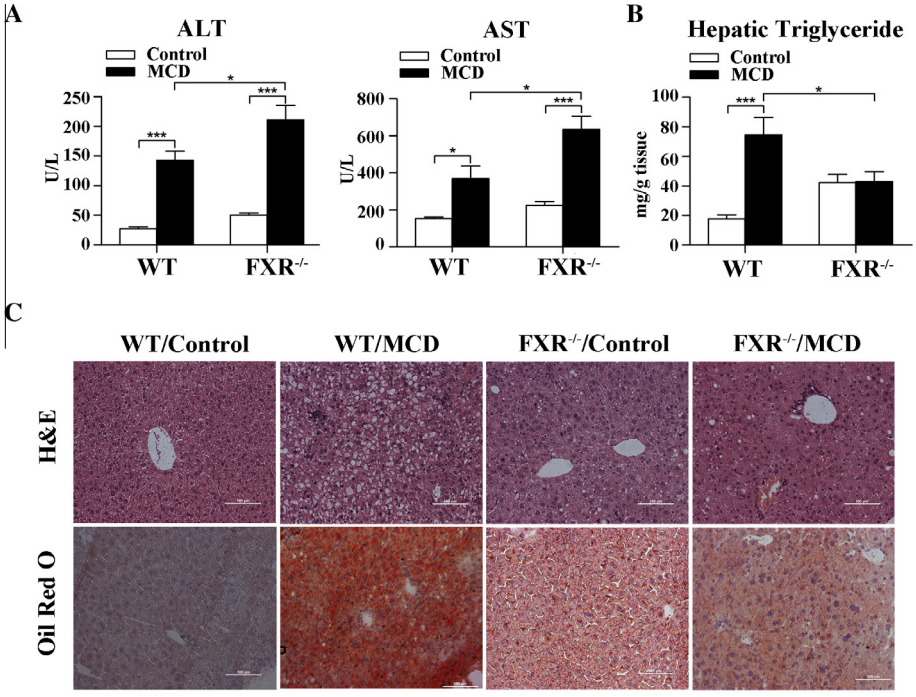

Fig. 1. FXR deficient mice fed MCD diet developed more severe liver injury but lower degree of steatosis. Wild-type and $FXR^{-/-}$ mice were fed with MCD diet or control diet for 4 weeks. (A) Serum ALT and AST activities were determined. (B) Hepatic triglyceride content was determined. (C) Liver sections were stained with H&E or Oil Red O (ORO) (magnification, 200×). ($n = 10$ each group, and $^{*}p < 0.05$, $^{***}p < 0.001$.)

2.4. Total RNA isolation and quantitative RT-PCR assay

Total RNA was extracted using Trizol reagent according to the manufacturer's instruction (Invitrogen, Carlsbad, CA). RNA extracts were reverse-transcribed using a commercial kit (Takara Biotech, Dalian, China). Quantitative RT-PCR was performed for assessment of mRNA expression on an ABI Prism 7500 Sequence Detection system (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol. Primer sequences are listed in Supplementary Table 1.

2.5. Statistics

Data are shown as means ± S.E.M. Statistical analysis was determined by Student's *t* test. A *p* value less than 0.05 was considered significantly different.

3. Results

3.1. FXR deficient mice fed MCD diet developed more severe liver injury but lower degree of steatosis

Our previous report demonstrated that activation of FXR by WAY-362450, a synthetic potent FXR agonist, protected against hepatic inflammation and fibrosis without attenuating hepatic steatosis in mice fed a MCD diet [12]. To better understand the role of FXR in NASH, we fed WT and FXR$^{-/-}$ mice with MCD diet for 4 weeks. As shown in Fig. 1A, After MCD diet feeding, FXR$^{-/-}$ mice had higher serum level of ALT and AST than WT mice, indicating more severe liver injury. However, FXR$^{-/-}$ mice showed a lower level of hepatic triglyceride accumulation compared to WT mice after 4-week feeding with MCD diet (Fig. 1B). Histopathological examination also confirmed lower degree steatosis in FXR$^{-/-}$ mice upon MCD diet feeding (Fig. 1C). Taken together, our results suggest FXR$^{-/-}$ mice fed MCD diet developed more severe liver injury but lower degree of steatosis than WT mice fed MCD diet.

3.2. FXR deficient mice fed MCD diet developed severe intra-hepatic cholestasis

To further understand the effects of FXR loss on the bile acids (BAs) content in mice fed MCD diet, we evaluated the serum and hepatic total BAs in WT and FXR$^{-/-}$ mice. MCD diet or FXR deficiency could increase serum bile acid levels, respectively. Moreover, FXR deficiency synergistically potentiated the elevation effects of MCD diet on serum bile acid levels (Fig. 2A). A similar synergistic pattern was noticed when hepatic bile acids were

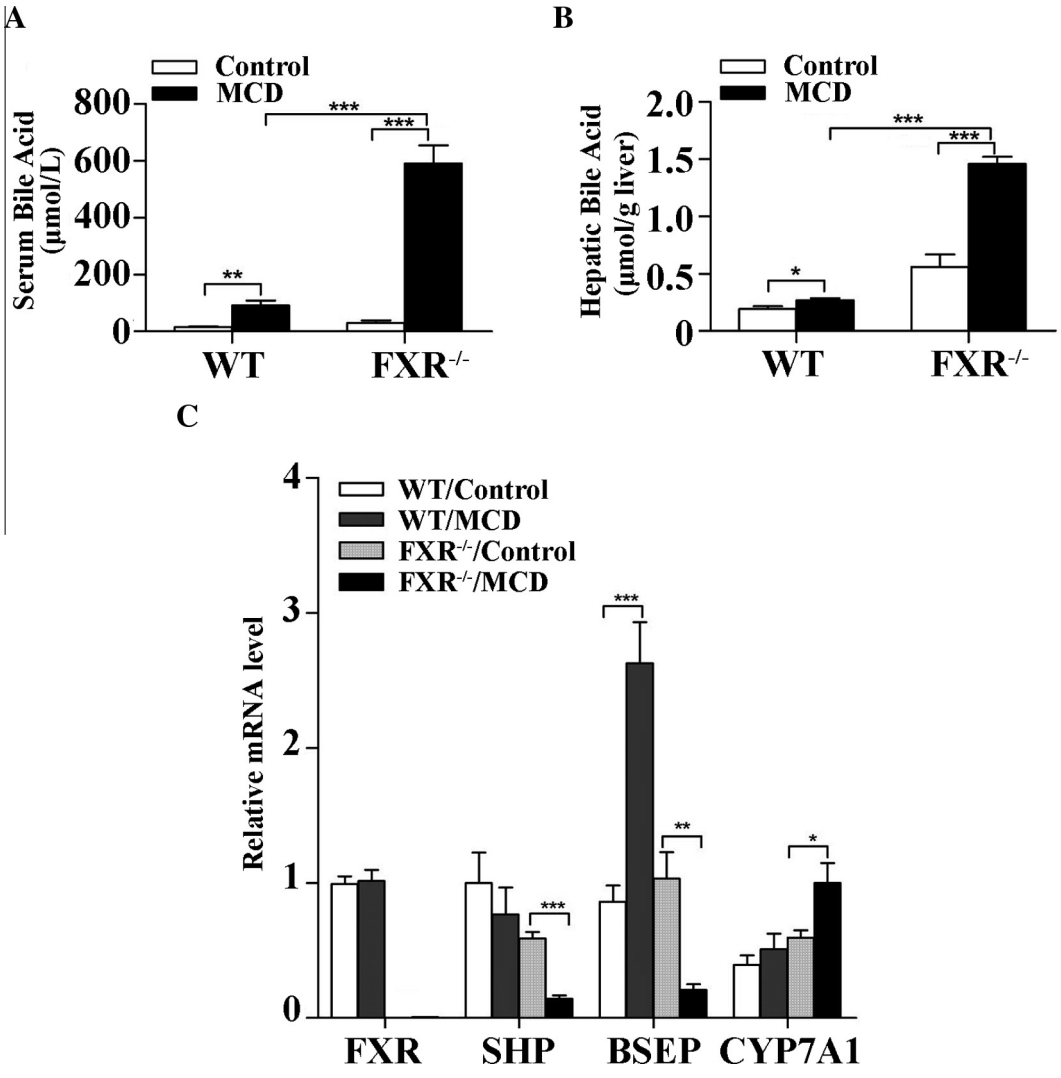

Fig. 2. Intrahepatic cholestasis of FXR deficient mice after MCD diet feeding. Wild-type and FXR$^{-/-}$ mice were treated as indicated. Total bile acid levels in the serum (A) and the liver tissues (B) were determined. (C) Total RNA from liver tissue was subjected to real-time RT-PCR for determination of *FXR*, *SHP*, *BSEP* and *CYP7A1* mRNA levels. ($n = 10$ each group, and $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.)

detected (Fig. 2B). To test whether there was difference in expression of the major bile acids transporter and synthesis enzymes, we performed quantitative real-time PCR for bile acids export gene *BSEP* and bile acids synthesis gene *CYP7A1*. MCD diet elevated *BSEP* expression in WT mice, but decrease its expression in FXR$^{-/-}$ mice. MCD diet increased *CYP7A1* expression in FXR$^{-/-}$ mice rather than WT mice. *SHP*, an orphan nuclear receptor and transcriptional corepressor, is the major down-stream target gene of FXR. MCD diet decreased *SHP* expression in FXR$^{-/-}$ mice rather than WT mice (Fig. 2C).

3.3. Evaluation of hepatic fatty acid and triglyceride metabolism genes expression

We evaluated the expression of major genes involved in fatty acid and triglyceride biosynthesis, fatty acid oxidation, fatty acid uptake and triglyceride accumulation. The expression of fatty acid and triglyceride biosynthesis genes including fatty acid synthase (*FASN*), sterol regulatory element binding protein-1c (*SREBP-1c*), acetyl-CoA carboxylase 1 (*ACC1*), triglyceride synthesis coding genes diacylglycerol acyltransferases (*DGAT1* and *DGAT2*) were significantly higher in FXR$^{-/-}$ mice than WT mice. Interestingly, MCD diet increased the expression of these genes in WT mice, but decreased their expression in FXR$^{-/-}$ mice. As reported previously, expression of stearoyl-CoA desaturase 1 (*SCD1*) was almost completely absent in the WT/MCD diet–fed mice [14]. However, SCD1 was slightly decreased in FXR$^{-/-}$/MCD mice (Fig. 3A). On the fatty acid oxidation genes, MCD diet did not change the expression of carnitine palmitoyltransferase 1a (*CPT1a*) and acyl-coenzyme A dehydrogenase (*ACADM*) in WT mice, but repressed their expression in FXR$^{-/-}$ mice. MCD diet also decreased the expression of acyl-CoA oxidase 1 (*Acox1*) and acetyl-CoA acyltransferase 1 (*ACAA1a*) in both WT and FXR$^{-/-}$ mice (Fig. 3B). Differential expression of fatty acid uptake and triglyceride accumulation genes was also noted. As shown in Fig. 3C, MCD diet significantly repressed the expressions of liver fatty acid binding protein (*FABP1*) and solute carrier family 27 member 1 (*SLC27a1*) in both WT and FXR$^{-/-}$ mice, but increased the expression of fatty acid translocase (*FAT/CD36*) and lipoprotein lipase (*LPL*) in both groups. MCD diet did not change the expression of fatty acid binding protein 4 (*FABP4*) in FXR$^{-/-}$ mice, but increased its expression in WT mice. In contrast, MCD diet did not change the expression of low-density lipoprotein receptor related protein-1 (*LRP1*) and hepatic lipase (*LIPC*) in WT mice, but greatly decreased their expression in FXR$^{-/-}$ mice.

3.4. Effect of FXR deficiency on liver inflammation and fibrosis

Since MCD diet induced hepatic inflammation and fibrosis progression in mice, as important pathological features of NASH, we next examined the expression of hepatic inflammation and fibrosis genes. In WT and FXR$^{-/-}$ mice, both vascular cell adhesion molecule-1 (*VCAM-1*) and monocyte chemotactic protein-1 (*MCP-1*) gene expression increased in response to the MCD diet (Fig. 4A and B). Additionally, the MCD diet elevated the mRNA levels of hepatic fibrosis genes (*TIMP-1* and *α-SMA*) in both WT and FXR$^{-/-}$

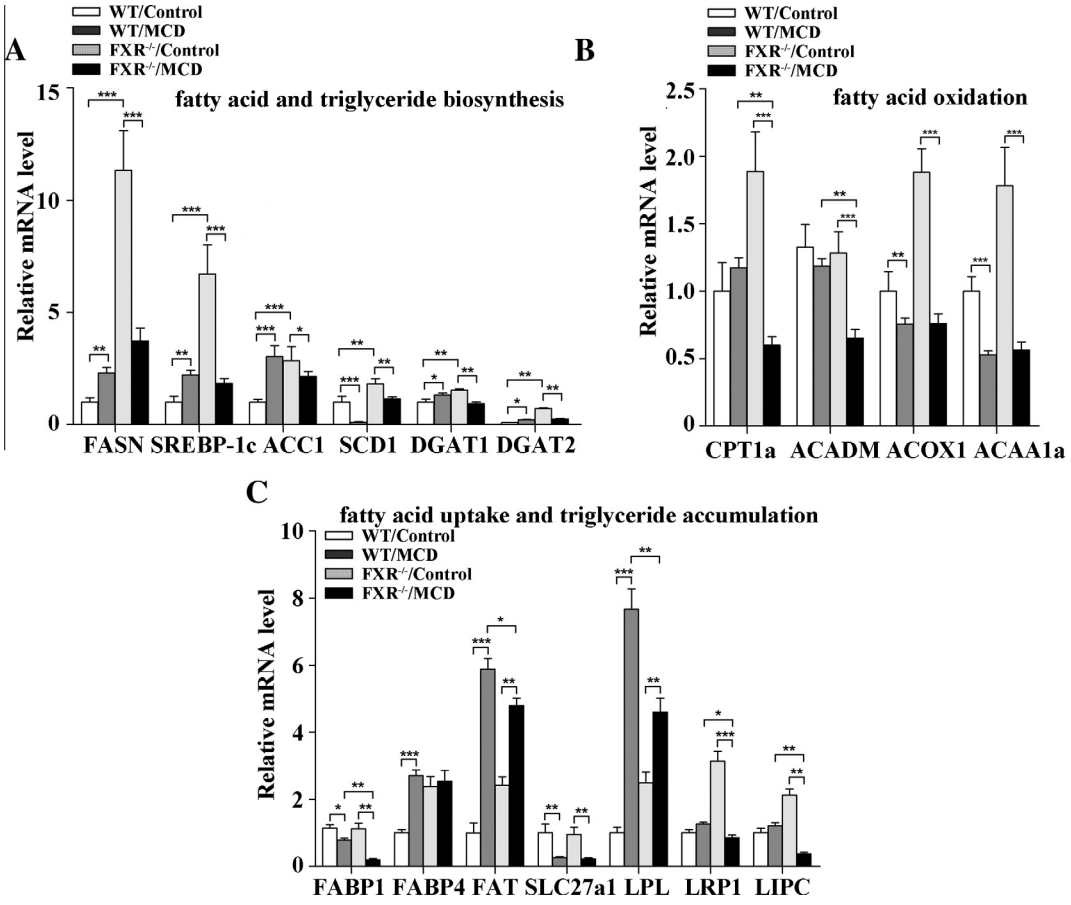

Fig. 3. Evaluation of genes expression involved in hepatic fatty acid and triglyceride metabolism. Wild-type and FXR$^{-/-}$ mice were treated as indicated. Liver tissue was subjected to real-time RT-PCR for determination of mRNA levels of genes involved in fatty acid and triglyceride biosynthesis (A), fatty acid oxidation (B), fatty acid uptake and triglyceride accumulation (C). (n = 10 each group, and $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.)

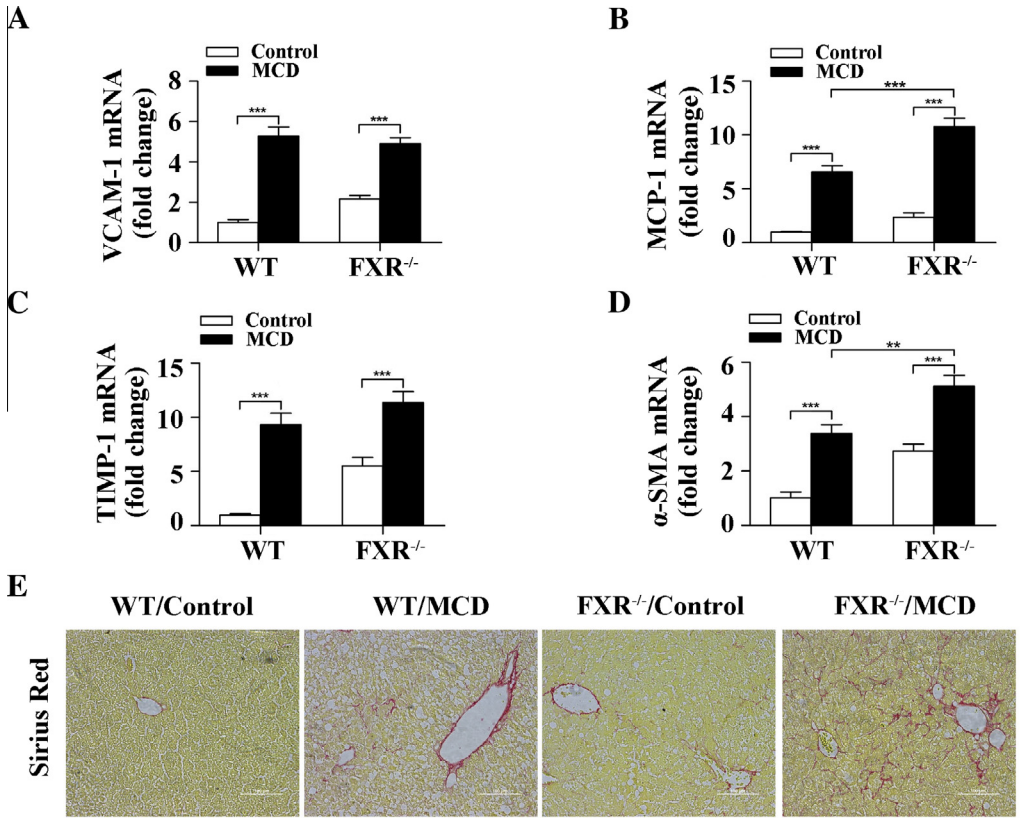

Fig. 4. Effect of FXR deficiency on liver inflammation and fibrosis. Wild-type and $FXR^{-/-}$ mice were treated as indicated. Total RNA from liver tissue was subjected to real-time RT-PCR for determination of VCAM-1 (A), MCP-1 (B), TIMP-1 (C) and α-SMA (D) mRNA levels. (n = 10 each group, and $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$). (E) Liver sections were stained with Sirius red for staining collagen (magnification, ×200).

mice (Fig. 4C and D). Interestingly, FXR deficiency enhanced the induction effect of MCD diet on *MCP-1* and *α-SMA* expression (Fig. 4B and D). Histological examination of the liver sections by Sirius Red staining confirmed the deteriorative effect of FXR deficiency on hepatic fibrosis. The MCD diet caused extensive fibrosis in 4 weeks, evidenced by the Sirius Red staining, which were more abundant in $FXR^{-/-}$ mice (Fig. 4E). Therefore, $FXR^{-/-}$ mice on MCD diet still developed inflammation and fibrosis notwithstanding lower level of liver triglyceride accumulation.

4. Discussion

Bile acids are essential constituents of bile that facilitate digestion and absorption of lipids as well as regulate cholesterol homeostasis. In recent years, however, it has proved that bile acids are also an important biological signaling molecule. Bile acids can activate nuclear receptors and G protein-coupled receptor (GPCR) signaling to regulate some important physiological function such as lipid, glucose and energy homeostasis [15]. Importantly, bile acids are critical for preventing accumulation of cholesterol, triglycerides, toxic metabolites, and injury in the liver and intestine [16]. Although toxic bile acids could induce inflammation, apoptosis and cell death, bile acids-activated nuclear and GPCR signaling improves inflammation and detoxification [17]. Our previous report showed that activation of FXR in liver protected against MCD diet-induced liver damage, inflammatory cell infiltration and liver fibrosis, without any improvement on hepatic triglyceride accumulation [12]. In this study, we demonstrated that FXR deficient mice fed MCD diet showed more severe liver injury, but less triglycerides accumulation in liver compared with wild-type mice fed MCD diet.

The role of FXR in the regulation lipid metabolism is multifaceted. It was known to repress SREBP-1c expression through an indirect mechanism by induction of SHP [18]. FXR could also repress FAS and MTP expression, which plays an important role in VLDL secretion [19,20]. It has been known that CYP7A1 is the limited rate enzyme for bile acid synthesis. But CYP7A1 is repressed by bile acids. Previous studies proved that FXR–SHP axis that mediated bile acid feedback is responsible for the inhibition of *CYP7A1* expression [21,22]. However, FXR–SHP axis may be not the only pathway regulating the expression of *CYP7A1*, because MCD diet increased the expression of hepatic *CYP7A1* and elevated serum and hepatic bile acid levels in $FXR^{-/-}$ mice. Owing to loss of FXR–SHP mediated feedback inhibition of bile acid synthesis, FXR deficient mice fed MCD diet show dramatically elevated bile acid level than wild-type mice fed MCD diet. Our data suggest that MCD diet can provoke bile acid liver accumulation, which could be exaggerated by FXR deficiency, and the super-physiological concentrations of hepatic bile acids may repress triglyceride (TG) accumulation in the FXR-independent way.

Bile acids have been known to regulate TG homeostasis. Bile acid chenodeoxycholic acid (CDCA) was reported to reduce hypertriglyceridemia in the patient with cholesterol gallstones [17]. The underlying mechanism may be related to the repression of hepatic triglyceride production/secretion and stimulation of serum triglyceride clearance by bile acids. Previous studies also indicated that bile acids lowered serum and liver TG levels by the FXR–SHP–SREBP-1c regulatory cascade [23]. However, we found that the expression of fatty acid synthase gene *SREBP-1c* was not so dramatically changed in $FXR^{-/-}$/MCD mice as in WT/MCD mice. We believe that bile acids may have additional effects on TG homeostasis in FXR–SHP–SREBP-1c independent way. Chronic elevation of the bile acid pool in $FXR^{-/-}$/MCD mice led to the attenuation of

liver steatosis or TG retention. The possible explanations are that super-physiological concentrations of bile acids in $FXR^{-/-}$/MCD mice can inhibit fatty acid uptake and TG accumulation through suppressing the *FABP1*, *FAT*, *LPL*, *LRP1* and *LIPC* expression. Indeed, there is a significant difference of expression of all these genes between WT/MCD mice and $FXR^{-/-}$/MCD mice.

Some specific components in bile acids have been shown to protect against liver cell apoptosis or stimulate hepatobiliary secretion and antioxidant activity [24–26]. Moreover, bile acids function as signaling molecule promoting liver regeneration and nutrition uptake [27,28]. Several studies have characterized the cellular and molecular mechanisms of liver injury induced by accumulation of hydrophobic bile acids [29,30]. Studies have demonstrated that treatment of hepatocytes with bile acids did not directly induce cell toxicity but caused the explosion of numerous proinflammatory responses and immune cell infiltration [31]. Furthermore, bile acids promote hepatic stellate cell proliferation via activation of the epidermal growth factor receptor [32]. Our data show that MCD diet exaggerates hepatic inflammation and fibrosis progression in $FXR^{-/-}$ mice. Meanwhile, bile acid accumulation in liver overrides steatosis in MCD/$FXR^{-/-}$ mice.

Taken together, these results provide a perspective that hepatic bile acid accumulation could override simple steatosis in hepatic injury during the progression of NAFLD.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.04.048.

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