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Original article

The roles and interaction of FXR and PPARs in the pathogenesis of nonalcoholic fatty liver disease

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ABSTRACT

Background and study aims: To identify the roles and interaction of farnesoid X receptor (*FXR*) and peroxisome proliferator activated receptors (*PPARs*) in Non-alcoholic fatty liver disease (NAFLD) pathogenesis.
Material and Methods: 16 C57/BL male *FXR* knockout (KO) mice and sex- and age-matched C57/BL wild type mice were received either standard rodent chow or high-fat and sucrose diet (Blank control, NAFLD, *FXR* KO and *FXR* KO NAFLD) for 8 weeks. After that, all mice were sacrificed. Liver tissues and blood samples were collected for laboratory and RT-PCR examination.
Results: NAFLD, *FXR* KO and *FXR* KO NAFLD mouse models were successful established. Compared with blank control, *FXR* and *PPAR-α* mRNA expression decreased significantly ($P < 0.05$), *PPAR-β* expression increased slightly ($P > 0.05$), *PPAR-γ* expression increased significantly in NAFLD ($P < 0.05$). Slight increased *PPAR-α* mRNA expression ($P > 0.05$) and markedly decreased *PPAR-β* and *PPAR-γ* expression ($P < 0.05$) were found in *FXR* KO. Compared with *FXR* KO group, there was a slight increase in *PPAR-α*and *PPAR-β*mRNA expression ($P > 0.05$) and significant increase in *PPAR-γ* expression ($P < 0.05$) in *FXR* KO NAFLD group. Comparison with NAFLD, *PPAR-α* mRNA expression increased slightly ($P > 0.05$), *PPAR-β* and *PPAR-γ* expression decreased significantly ($P < 0.05$) in *FXR* KO NAFLD.
Conclusion: *FXR* and *PPARs* interaction may play important roles in NAFLD pathogenesis.

Introduction

Non-alcoholic fatty liver disease (NAFLD) is the most common cause of chronic liver disease worldwide with a prevalence ranging from 6% to 33% in various populations [1,2]. Recent studies estimate the prevalence ranged between 30 and 40% in the USA [3]. Our previous survey in 2007 reported a prevalence of 15% in southern China [4]. In recent years, due to alterations of lifestyle and dietary habits, an increasing trend of NAFLD prevalence was acknowledged in Asia-Pacific region [5]. A *meta*-analysis in 2014 revealed a 20.1% NAFLD prevalence in China [6]. NAFLD refers to a pathological spectrum of liver disorders ranging from simple nonalcoholic steatosis to non-alcoholic steatohepatitis (NASH), and then cirrhosis [7]. The natural course of NAFLD is comparatively benign. After a median follow-up of 4 years, there are only 22.2% patients progressed in our earlier report [8]. In addition, NAFLD is strongly associated with metabolic syndrome (MS) and associated obesity, type 2 diabetes, hypertension and dyslipidemia, which insulin resistance (IR) is the major mechanism [5,9].

The molecular mechanisms of NAFLD have not been fully understood, and no administration approved drug has been recommended for NAFLD/NASH therapy [3]. Currently, the researches in nuclear receptors (NRs) have shed light on NAFLD pathogenesis. In humans, there are 48 nuclear receptors categorized in 7 subfamilies designated as NR0–NR6. Nuclear receptors are ligand-dependent factors regulating epigenetic changes through cellular machinery to control transcription. Peroxisome proliferator activated receptors (*PPARs*), retinoid X receptor (*RXR*), farnesoid X receptor (*FXR*) and liver X receptor (*LXR*) in NR1 subfamily are particularly important in adaption of lipid homeostasis and development of NAFLD [10,11]. *FXR* and *PPARs* are mainly involved in nutrient acquisition from the gut and distribution through the liver to peripheral tissues including adipose and muscle. *PPARs* contain three isotypes encoded by the *PPAR-α, PPAR-β/δ*

Abbreviations: NAFLD, Non-alcoholic fatty liver disease; FXR, farnesoid X receptor; *PPARs*, peroxisome proliferator activated receptors; KO, knockout; NASH, nonalcoholic steatosis to non-alcoholic steatohepatitis; *LXR*, liver X receptor; TG, triglyceride; TC, total cholesterol; ALT, alanine aminotransferase.

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(or *PPAR-β)* and *PPAR-γ* genes. *PPAR-α* is mainly present in the liver, *PPAR-β* in skeletal muscle, adipose tissue and skin, *PPAR-γ* in adipose tissue. All isotypes of *PPARs* regulate gene expression related to energy metabolism and participate in NAFLD development. The balance of *PPARs* activity is associated with various mechanisms i.e. induction of gene expression in decreasing oxidative stress, increasing the secretion of beneficial adipokines and anti-inflammatory factors [12,13]. *FXR,* a bile acid receptor, is highly expressed in liver, kidney, adrenal glands and intestine. *FXR* regulates a variety of gene involved in regulation of lipid homeostasis and inflammation, and play important roles in hepatic steatosis, hyperglycemia and hyperlipidemia. Genetic activation of *FXR* might reverse hepatic steatosis [10,11,14,15]. A few studies have demonstrated the interaction among *FXR* and the three *PPAR* isotypes. [16] Bile acids induce the expression of *PPAR-α* gene via activation of the *FXR* in humans [16]. Cross-talk between *FXR* and *PPAR-γ* contributes to the antifibrotic activity of FXR ligands in rodent liver cirrhosis models [17]. However, the detail mechanisms remain poorly defined.

So far, the agonists of *PPAR-α, PPAR-β, PPAR-γ* and *FXR* have attracted pharmaceutical attention and stimulated research. The agonists of *PPAR-α* (such as fibrates), *PPAR-β* (GW501516), dual *PPARα/β* (elafibranor), *PPAR-γ* (thiazolidinediones), dual *PPARα/γ* (saroglitazar) and *FXR* (obeticholic acid) have been proposed being therapeutic agents for NAFLD and MS. However, widespread use of these agonists is limited by their adverse effects [12,13,18,19]. Our previous study revealed down-expression of *PPAR-α* mRNA and protein in NAFLD rat model after DNA methylation [20], and *PPAR-γ* gene polymorphism was involved in the NAFLD development in humans [21]. Putting above information together and based on our experience in animal models of NAFLD [20], the present study investigated the roles and interaction of *FXR* and the three *PPAR* isotypes in NAFLD pathogenesis.

Material and Methods

Mouse use and animal ethics

Healthy 8-week-old male C57/BL wild type mice (n = 16) were purchased from Guangdong medical laboratory animal center, Guangzhou, China, and *FXR* knockout (KO) C57/BL male mice (n = 16) were obtained from Shanghai University of Traditional Chinese Medicine, Shanghai, China. All mice were housed (ten mice per cage) in Experimental Animal Center of Guangzhou medical university, Guangzhou, China at a room temperature of 20 ± 3℃, humidity of 60 ± 6%, maintained in 12-h light/dark cycles (8 am to 8 pm) with unlimited access to food and water. Before the experiment, mice received standard rodent chow for one week for acclimation. Subsequently, they were randomly divided into four groups using drawing label method: group A-blank control group, group B-NAFLD group, group C-*FXR* KO group and group D-*FXR* KO NAFLD group (n = 8 for each group) (Fig. 1). The mice in blank control and FXR KO groups continuously received standard rodent chow, whereas the mice in NAFLD and FXR KO NAFLD groups were fed with a self-prescribed high-fat and sucrose diet (HFSD) containing 82.5% basic feed, 10% lard, 5% sucrose, 0.3% cholate, 2% cholesterol and 0.2% propylthiouracil, NAFLD mouse models were established according to Zeng description [22]. The animal experiments were performed in compliance with the laboratory animal regulations of the Ministry of Science and Technology, China and were approved by the Ethics Committee of the hospital. The animal experiments complied with the ARRIVE guidelines and were performed in accordance with the U.K. Animals Act,1986.

Sample collection and related index detection

The body weight of the mice was measured once a week. After 8 weeks, all the mice were fasted, weighed and sacrificed by cervical dislocation. Blood samples were immediately collected from the postcava and centrifugated. Serum levels of triglyceride (TG), total cholesterol (TC) and alanine aminotransferase (ALT) were determined by a biochemistry autoanalyzer (Olympus, Tokyo, Japan). The livers of the mice were rapidly excised and weighed with scale. The left lobe of the liver were fixed in 10% formalin, embedded in paraffin, cut into 3–5-μm-thick sections, stained with hematoxylin and eosin (HE), observed with light microscopy (Nikon, Tokyo, Japan). The remainder was immediately snap-frozen in liquid nitrogen and stored at − 80 °C until use. Liver index was calculated as liver weight divided by body weight. In each group, three out of 10 microscopic fields (×200) were randomly

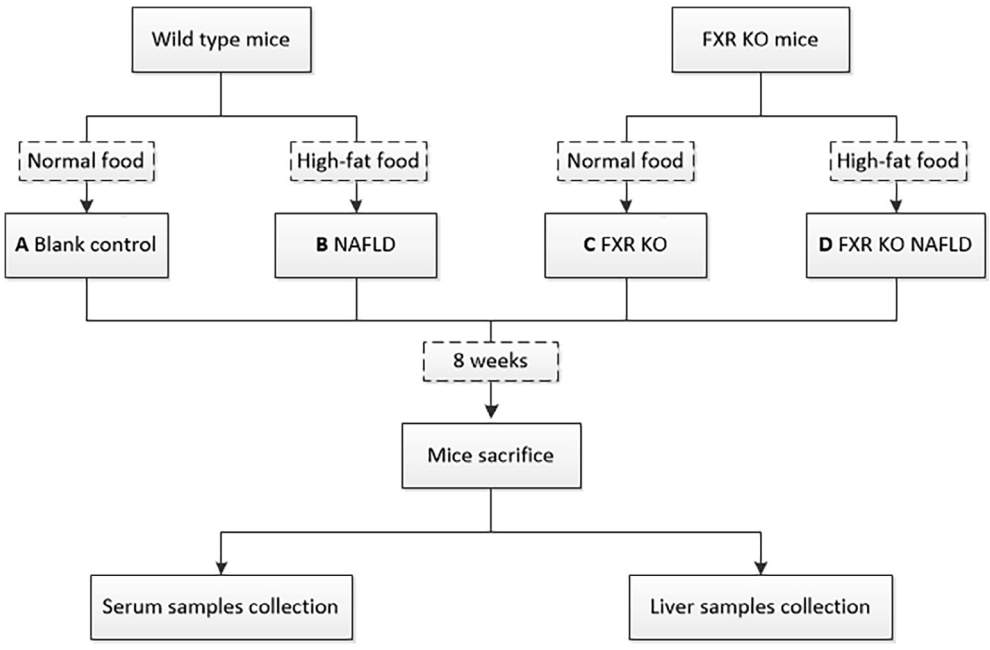

Fig. 1. Establishment of four groups (n = 8 for each group). NAFLD, nonalcoholic fatty liver disease; *FXR*, Farnesoid X receptor; KO, knockout.

chosen for observation. Lipid droplets in each field were evaluated by counting the droplets in the microscopic fields.

Real-time fluorescent quantitative polymerase chain reaction (RT-PCR)

RT-PCR was used to determine mRNA expression levels in *FXR, PPAR-α, PPAR-β* and *PPAR-γ*. Briefly, total RNA from mice liver was isolated using TRIzol reagent (Invitrogen, Shanghai, China) according to the manufacture's protocol. The concentration and purity of RNA were determined by Nanodrop2000 (Thermo Scientific, Waltham, MA, USA). RNA integrity was verified using formaldehyde degeneration agarose gel electrophoresis. Then the total RNA was reversely transcribed to complementary DNA (cDNA) using a Prime- Script™ RT reagent kit (TaKaRa Biotechology Co., Ltd., Dalian, Liaoning Province, China) following the manufacturer's instructions. cDNA was then applied as a template to the standard PCR reaction with a SYBR Green PCR Master Mix (Fermentas, Vilnius, Lithuania). The primer sequences were designed by Primer Premier 5.0 software and synthesized by Invitrogen (Shanghai, China) (Table 1). The level of *FXR* and *PPARs* expression was normalized to β-actin. The reaction conditions of RT-PCR were as follows: preheating at 95℃ for 5 min, degeneration at 95℃ for 5 s, renaturation at 50.5℃ for 30 s and extension at 72℃ for 30 s for 40 cycles, and a final extension at 72℃ for 30 min. The $2^{\triangle\triangle CT}$ CT method was applied to calculate relative gene expression

Table 1
The primer sequences.

Genes		Sequences
FXR	forward	5′-GCTTGATGTGCTACAAAAGCTG-3′
	reverse	5′-CGTGGTGATGGTTGAATGTCC-3′
PPAR-α	forward	5′-CCTGGAAAGTCCCTTATCT-3′
	reverse	5′-GCCCTTACAGCCTTCACAT-3′
PPAR-β	forward	5′-TCACCAGCAGCCTAAAAGCA-3′
	reverse	5′-AGGCCAGGCTTCTTGGAAAG-3′
PPAR-γ	forward	5′-AGACCACTCGCATTCCTTTG-3′
	reverse	5′-CATTGGGTCAGCTCTTGTGA-3′
β-actin	forward	5′-GGACTCCTATGTGGGTGACGAGG-3′
	reverse	5′-GGGAGAGCATAGCCCTCGTAGAT-3′

Statistical analysis

Statistical analyses were carried out by SPSS 20.0 software package for Windows (IBM, Armonk, NY, USA). Continuous variables were expressed as mean ± standard deviation (SD) and analyzed using the Student's *t*-test. Categorical variables were expressed as numbers and percentages and analyzed using the x^2 test. A two-tailed *P* value of < 0.05 was considered statistically significant.

Results

Histological findings and laboratory results

Macroscopic observation showed that in blank control the livers had regular and clear borderline, but in NAFLD mice, the livers enlarged with yellow color, in *FXR* KO mice, the livers decreased with red color, and in *FXR* KO NAFLD the livers had blunt borderline with yellow color, the size were similar to that of *FXR* KO mice. Under light microscopy, hepatic steatosis, hepatocyte ballooning degeneration (lipid accumulation), lobular lymphocyte infiltration were obviously increased and the nuclei were compressed to the border of the cells by intracellular lipid droplets in NAFLD compared with those in blank control. In *FXR* KO mice, liver steatosis, sinusoidal foam cells, ballooning degeneration and lymphocyte

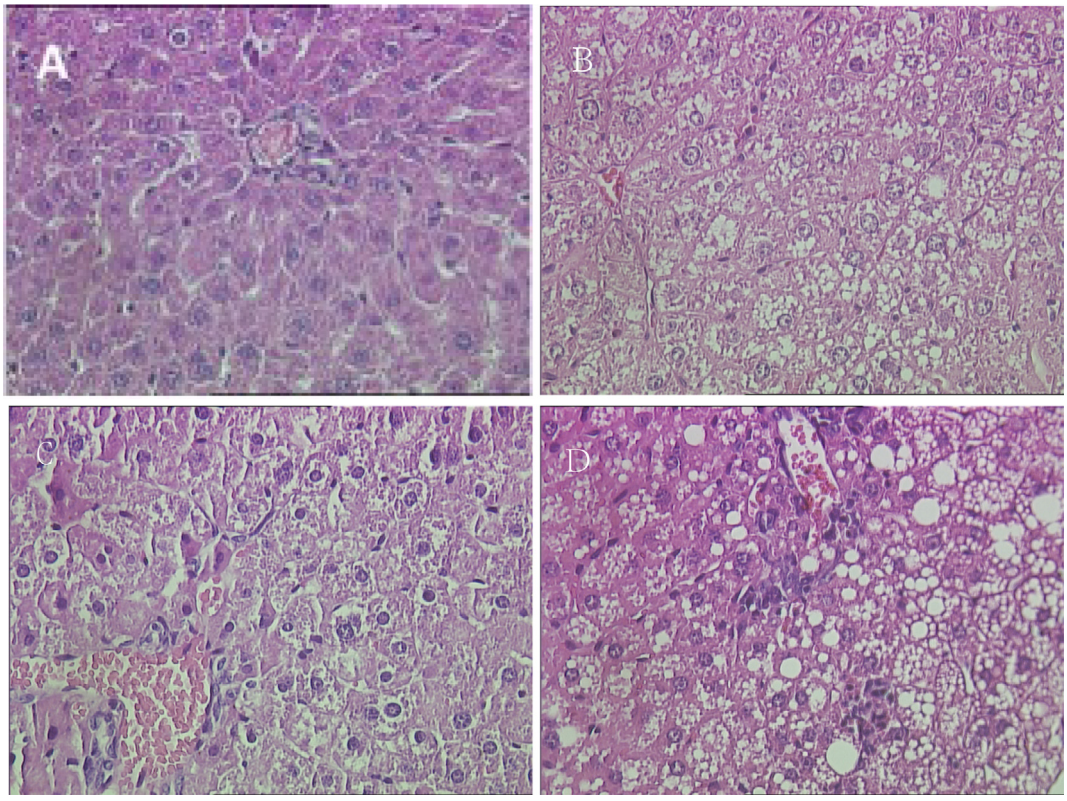

Fig. 2. Microscopic features of the mice livers in four groups. A Blank control; B, NAFLD; C, *FXR* KO; D, *FXR* KO NAFLD ; NAFLD, non-alcoholic fatty liver disease; *FXR*, Farnesoid X receptor; KO, knockout.

infiltration were found compared with those in blank control. In *FXR* KO NAFLD mice, more abundant lipid accumulation and lobular inflammation were observed compared with those in *FXR* KO mice (Fig. 2). The above findings revealed *FXR* deficiency might result in liver steatosis.

The liver weight and biochemical variables of the four groups were shown in Table 2 and Fig. 3. The body weight of the mice at baseline did not differ among the four groups. After 8-week administration, the body weight of the mice was higher in NAFLD group than that in blank control group, but difference was not significant ($P > 0.05$). A similar trend of body weight was seen in *FXR* KO NAFLD group compared with *FXR* KO group. The body weight was significantly lower in *FXR* KO group than that in blank control group ($P < 0.05$). The liver index in NAFLD, *FXR* KO and *FXR* KO NAFLD groups were significantly higher than that in blank control group ($P < 0.05$). And the liver index in *FXR* KO NAFLD groups was significantly higher than that in NAFLD group. Serum levers of ALT, TC and TG in NAFLD, *FXR* KO and *FXR* KO NAFLD groups were all significantly higher than those in blank control group ($P < 0.05$). There was a slight increase in serum levels of ALT ($P > 0.05$), TC ($P < 0.05$) and TG ($P > 0.05$) in *FXR* KO NAFLD group compared with those in *FXR* KO group. These results including the trend of body weight reduction and liver indexes increase in the NAFLD were consistent with the results in most literatures [21,23] and confirmed the successful establishment of the NAFLD, *FXR* KO and *FXR* KO NAFLD mouse models as descripted in literature.

Hepatic FXR and PPARs mRNA expression NAFLD mice

In comparison with blank control group, *FXR* and *PPAR-α* mRNA expression in NAFLD group decreased significantly($P < 0.05$), *PPAR-β* expression increased slightly($P > 0.05$), whereas, *PPAR-γ* expression increased significantly ($P < 0.05$). In comparison with blank control group, *PPAR-α* mRNA expression in *FXR* KO group increased slightly ($P > 0.05$), whereas, *PPAR-β* and *PPAR-γ* expression decreased significantly ($P < 0.05$). In comparison with *FXR* KO group, *PPAR-α* and *PPAR-β* mRNA expression in *FXR* KO NAFLD group increased slightly ($P > 0.05$); and *PPAR-γ* expression increased significantly ($P < 0.05$). In comparison with NAFLD group, *PPAR-α* mRNA expression in *FXR* KO NAFLD group increased slightly ($P > 0.05$), whereas *PPAR-β* and *PPAR-γ* expression decreased significantly ($P < 0.05$). Finally, compared with blank control group, *PPAR-α* mRNA expression in *FXR* KO NAFLD group

Table 2
Liver weight and biochemical variables among the four groups (n = 8 in each group).

	Blank control	NAFLD	*FXR* KO	*FXR* KO NAFLD
Weight(g)	30.80 ± 2.04	32.16 ± 0.90	24.83 ± 2.40*	26.74 ± 3.47
Liver index	0.042 ± 0.002	0.060 ± 0.00*	0.059 ± 0.01*	0.070 ± 0.01* †
ALT (mmol/L)	55.66 ± 15.30	180.25 ± 69.24*	209.16 ± 213.89*	362.10 ± 162.92* †
TC (mmol/L)	3.18 ± 0.14	4.46 ± 0.59*	4.41 ± 1.03*	10.86 ± 3.80* †
TG (mmol/L)	0.75 ± 0.14	0.91 ± 0.17*	1.76 ± 0.46*	2.02 ± 0.24*

*$P < 0.05$ compared with the blank control group; †$P < 0.05$ compared with the NAFLD group
NAFLD, non-alcoholic fatty liver disease; *FXR*, farnesoid X receptor; KO, knockout; ALT, alanine aminotransferase; AST, aspartate aminotransferase; TC, total cholesterol; TG, triglyceride

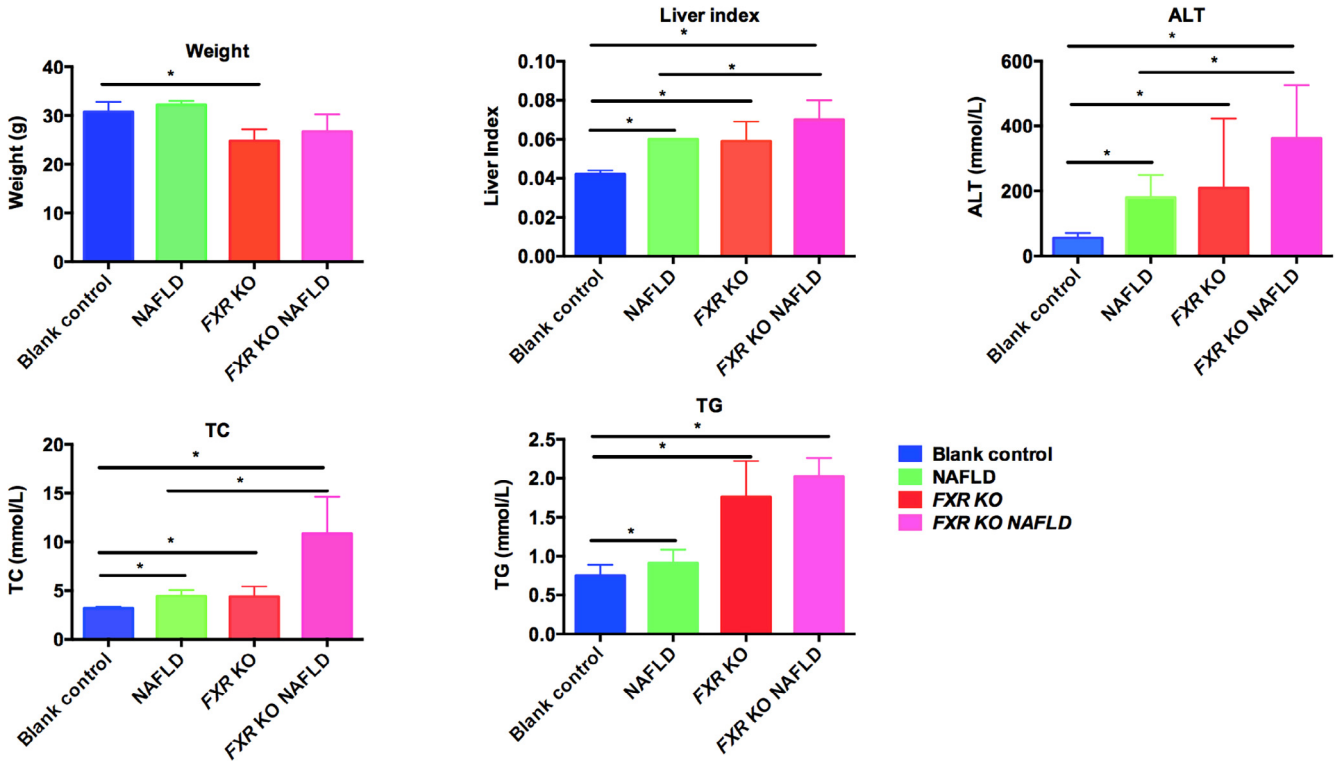

Fig. 3. Liver weight and biochemical variables among the four groups (n = 8 in each group) *$P < 0.05$ compared between groups. NAFLD, non-alcoholic fatty liver disease; *FXR*, Farnesoid X receptor; KO, knockout; ALT, alanine aminotransferase; AST, aspartate aminotransferase; TC, total cholesterol; TG, triglyceride.

Table 3
FXR and *PPARs* mRNA expression in the four groups.

	FXR	PPAR-α	PPAR-β	PPAR-γ
Blank control	1.06 ± 0.38	1.52 ± 1.16	1.53 ± 1.46	1.33 ± 0.96
NAFLD	0.35 ± 0.11*	0.82 ± 0.48*	2.78 ± 1.81	3.41 ± 1.54*
FXR KO	0.00 ± 0.00	1.83 ± 1.45	0.21 ± 0.05*	0.30 ± 0.06*
FXR KO NAFLD	0.00 ± 0.00	2.69 ± 1.91*	0.33 ± 0.22*+	0.53 ± 0.14*+#

*P < 0.05 compared with blank control group; +P < 0.05 compared with NAFLD group; # P < 0.05 compared with *FXR* KO group.NAFLD, nonalcoholic fatty liver disease; *FXR*, farnesoid X receptor; *PPAR*, peroxisome proliferator activated receptor; KO, knockout.

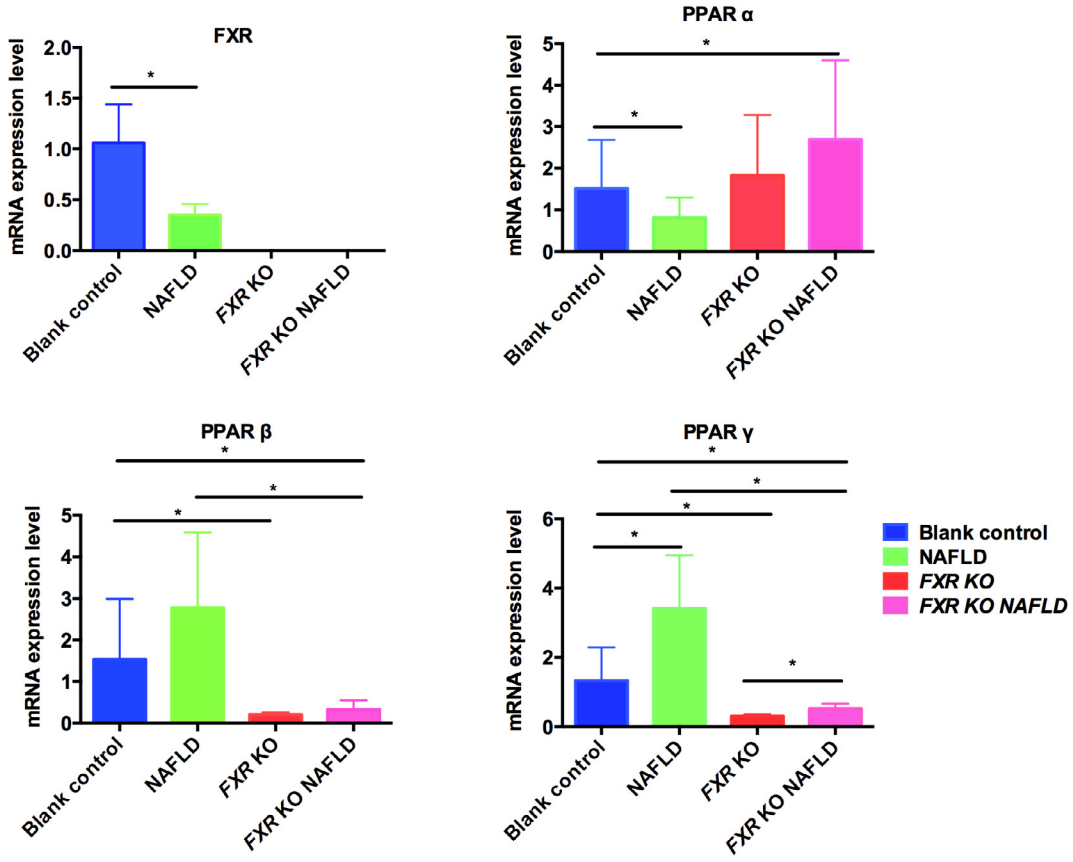

Fig. 4. Hepatic *FXR* and *PPARs* mRNA expressions in the four groups. * P < 0.05 compared between groups; NAFLD, nonalcoholic fatty liver disease; *FXR*, Farnesoid X receptor; *PPAR*, Peroxisome proliferator activated receptor; KO, knockout.

increased markedly (P < 0.05), while *PPAR-β* and *PPAR-γ* expression decreased significantly (P < 0.05) (Table3 and Fig. 4).

Discussion

FXR and *PPARs* are ligand-regulated transcription factors that perform multiple functions including lipid homeostasis. It has been well documented that *FXR* and the three *PPAR* isotypes play important roles in the development of NAFLD [13,24,25], and that their agonists have been used in NAFLD treatment [12,13,18,19]. However, few studies have been performed to investigate these four nuclear receptors in NAFLD. The present study investigated the cross-talk of these four nuclear receptors in NAFLD pathogenesis using a *FXR* KO mouse model.

We first demonstrated that hepatic *FXR* and *PPAR-α* expression decreased, while *PPAR-γ* expression increased in NAFLD mice compared with blank control. As the expression of *FXR* and *PPARs* is animal and time-dependent in NAFLD progression [26], the literature results were controversial. The findings in the present study were consistent with most previous studies [13,24,25,27]. Indeed, previous studies showed that hepatic expression of *PPAR-α* was decreased in humans with NAFLD, which can be reversed with improved NAFLD histology after lifestyle intervention or bariatric surgery [27]. *PPAR-α* down-regulation might facilitate the activity of hepatic pro-inflammatory cytokines and hepatic steatosis in humans [28]. Hepatic *PPAR-γ* expression was up-regulated in NAFLD patients and experimental animal models [29,30]. In addition, *PPAR-γ* deletion in mouse hepatocytes was a protective factor against NAFLD development [31,32], whereas increased *PPAR-γ* activity in liver increased lipid storage [13]. Coordination between *PPAR-α* and *PPAR-γ* activity was required for maintaining the synthesis of fatty acids [33], and the effect of *PPAR-α* down-regulation on NAFLD was aggravated by *PPAR-γ* up-regulation [24,25]. The suppression of *FXR* in NAFLD mice in the present study was also consistent with the findings in NAFLD patients. *FXR* activation effectively prevented hepatic TG accumulation [10,34,35]. However, our findings disagreed with the paper showing *PPAR-α* up-regulation and *PPAR-β* down-regulation during the development of hepatic steatosis in mice [26].

Yet, only few *FXR* KO mouse studies have been reported so far. The findings of hepatic steatosis and hyperlipidemia in *FXR* KO mice in the present study agreed with other previously reported results in the literature. Indeed, it has been shown that *FXR* deficiency may result in hepatic steatosis and activation of *FXR* gene might reverse NAFLD [14,15,23]. In this regard, *FXR* deficient mice fed with ethanol diet were more likely to develop liver injury i.e. alcoholic fatty-liver disease (ALD), alcoholic steatohepatitis (ASH) and hepatic fibrosis [36]. The present study revealed a trend of *PPAR-α* upregulation and *PPAR-β/γ* downregulation in *FXR* KO mice compared to wild-type control mice. This trend was maintained in *FXR* KO NAFLD mice compared to NAFLD mice. These novel findings that *FXR* deficiency resulted in *PPAR-α* induction and *PPAR-β/-γ* downregulation in the studied conditions suggest an *FXR/PPARs* cross-regulation on hepatocyte. Indeed, *FXR* regulated the responsiveness of *PPAR-γ* gene [37] to adipose, and *PPAR-γ* coactivator-1α regulated triglyceride metabolism by activation of *FXR* [38,39].

The trend of *PPAR-β/γ* over-expression in NAFLD mice compared with wild type mice was reserved in *FXR* KO NAFLD, compared with *FXR* KO mice. However, the trend of *PPAR-α* downregulation in NAFLD mice was inversed by *FXR* KO. These results provide a novel information for understanding the coordination of FXR and *PPARs* in NAFLD pathogenesis.

Finally, the present study revealed that hepatic expression of *PPAR-β* was similar to that of *PPAR-γ*, but different from *PPAR-α* in hepatic steatosis, although the change of *PPAR-γ* was more obvious than that of *PPAR-β* in most situations.

We have identified the correlation between FXR and the three PPAR nuclear receptors in hepatic steatosis. Several studies have suggested that both *PPAR-α* and *FXR* act on common metabolic pathways [16,24,25,27,33]; however, the information was inconclusive and hard to interpret. To our knowledge, the present study is the first to evaluate *PPARs* expression in *FXR* deficient mice, where we showed that *FXR* could be a key nuclear receptor related to *PPARs* in NAFLD pathogenesis. However, there are several limitations in the present study. First, we did not test protein expression to confirm the mRNA expression results. Second, we did not expand our study to humans to prove our findings. Indeed, the results in mice do not necessarily represent human expression and mechanisms. Nevertheless, our study contributes novel data to the interaction between *FXR* and *PPARs* and their role in NAFLD pathogenesis. In conclusion: *FXR* and *PPARs* interaction may play important roles in NAFLD pathogenesis.

Declaration of competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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