Alcoholic fatty liver is enhanced in CYP2A5 knockout mice: the role of the PPARα-FGF21 axis

Xue Chen[a], **Stephen C. Ward**[b], **Arthur I. Cederbaum**[c], **Huabao Xiong**[d], and **Yongke Lu**[a,c]

[a]Department of Health Sciences, College of Public Health, East Tennessee State University, Johnson City, Tennessee, United States of America

[b]Department of Pathology, Icahn School of Medicine at Mount Sinai, New York, New York, United States of America

[c]Department of Pharmacological Sciences, Icahn School of Medicine at Mount Sinai, New York, New York, United States of America

[d]Division of Immunology, Department of Medicine, Icahn School of Medicine at Mount Sinai, New York, New York, United States of America

Abstract

Background & Aims—Cytochrome P450 2A5 (CYP2A5) is induced by ethanol, and the ethanol induction of CYP2A5 is regulated by nuclear factor-erythroid 2-related factor 2 (NRF2). *Cyp2a5* knockout ($Cyp2a5^{-/-}$) mice develop more severe alcoholic fatty liver than $Cyp2a5^{+/+}$ mice. Fibroblast growth factor 21 (FGF21), a PPARα-regulated liver hormone, is involved in hepatic lipid metabolism. Alcoholic and non-alcoholic fatty liver are enhanced in *Ppara* knockout ($Ppara^{-/-}$) mice. This study investigates the relationship between the PPARα-FGF21 axis and the enhanced alcoholic fatty liver in $Cyp2a5^{-/-}$ mice.

Methods—Mice were fed the Lieber-Decarli ethanol diet to induce alcoholic fatty liver.

Results—More severe alcoholic fatty liver disease was developed in $Cyp2a5^{-/-}$ mice than in $Cyp2a5^{+/+}$ mice. Basal FGF21 levels were higher in $Cyp2a5^{-/-}$ mice than in $Cyp2a5^{+/+}$ mice, but ethanol did not further increase the elevated FGF21 levels in $Cyp2a5^{-/-}$ mice while FGF21 was induced by ethanol in $Cyp2a5^{+/+}$ mice. Basal levels of serum FGF21 were lower in $Ppara^{-/-}$ mice than in $Ppara^{+/+}$ mice; ethanol induced FGF21 in $Ppara^{+/+}$ mice but not in $Ppara^{-/-}$ mice, whereas ethanol induced hypertriglyceridemia in $Ppara^{-/-}$ mice but not in $Ppara^{+/+}$ mice. Administration of recombinant FGF21 normalized serum FGF21 and triglyceride in $Ppara^{-/-}$ mice. Alcoholic fatty liver was enhanced in liver-specific *Fgf21* knockout mice. *Ppara* and *Cyp2a5* double knockout ($Ppara^{-/-}/Cyp2a5^{-/-}$) mice developed more severe alcoholic fatty liver than $Ppara^{+/+}/Cyp2a5^{-/-}$ mice.

Correspondence: Yongke Lu, PhD, Department of Health Sciences, College of Public Health, East Tennessee State University, Johnson City, TN 37614, USA. luy004@etsu.edu; Phone: 423-439-4575; Fax: 423-439-4562.

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Conclusions—These results suggest that CYP2A5 protects against the development of alcoholic fatty liver disease, and the PPARα-FGF21 axis contributes to the protective effects of CYP2A5 on alcoholic fatty liver disease.

Keywords

CYP2A5; FGF21; PPARα; alcoholic fatty liver

INTRODUCTION

Alcoholic liver disease includes hepatosteatosis (fatty liver), hepatic inflammation, liver fibrosis, cirrhosis, and liver cancer. Fatty liver is a uniform and early response of the liver to alcohol consumption. Alcoholic fatty liver is generally benign, but it is more sensitive to liver injury induced by hepatotoxins such as lipopolysaccharide (Yang et al., 2001). Both cytochromes P450 2A6 (CYP2A6) and CYP2E1 were found to be increased in the liver sections from patients with alcoholic and non-alcoholic fatty liver diseases (Niemela et al., 2000). Using *Cyp2e1* knockout (*Cyp2e1$^{-/-}$*) mice and *Cyp2e1$^{-/-}$* mice reconstituted with human *CYP2E1* (humanized *CYP2E1* knock-in) mice, we confirmed the important role of CYP2E1 in the development of alcoholic fatty liver disease (Lu et al., 2008, 2010). But it is still unclear whether CYP2A6 also plays an important role in alcoholic fatty liver disease. Recently, we found that CYP2A5, a mouse orthologue of human CYP2A6, was also induced by ethanol in a CYP2E1-dependent manner (Lu et al., 2011). By comparing alcoholic fatty liver disease in *Cyp2a5* knockout (*Cyp2a5$^{-/-}$*) mice and the corresponding wild-type mice (*Cyp2a5$^{+/+}$*), we found that, in contrast to CYP2E1, CYP2A5 does not contribute to the development of alcoholic fatty liver disease but actually can be protective (Hong et al., 2015). CYP2A5 induction by ethanol is regulated by the redox sensitive transcriptional factor nuclear factor-erythroid 2-related factor 2 (NRF2) (Lu et al., 2012). NRF2 signaling usually protects against oxidative injury via regulating a panel of antioxidant genes (Cederbaum, 2009) and NRF2 protects against alcoholic fatty liver disease in mice (Lamlé et al., 2008). Therefore, CYP2A5 might be among the panel of NRF2-regulated antioxidants and its protective effect on alcoholic fatty liver disease might be associated with its antioxidant action. But further study is still needed to address the exact mechanisms by which alcoholic fatty liver disease is enhanced in *Cyp2a5$^{-/-}$* mice.

Peroxisome proliferator-activated receptor α (PPARα) is a major regulator for hepatic lipid metabolism. PPARα regulates genes of lipid oxidation by binding to promoters of specific genes (Kersten et al., 1999; Leone et al., 1999). After ethanol feeding, PPARα was upregulated in *Cyp2e1$^{-/-}$* mice, and consistently, *Cyp2e1$^{-/-}$* mice developed no or little alcoholic fatty liver (Lu et al., 2008). Very interestingly, ethanol feeding did not upregulate PPARα in the *Cyp2a5$^{-/-}$* mice although the basal level of PPARα was elevated in the *Cyp2a5$^{-/-}$* mice; consistently, alcoholic fatty liver disease was enhanced in the *Cyp2a5$^{-/-}$* mice (Hong et al., 2015). These results support the notion that PPARα protects against the development of alcoholic fatty liver. Indeed, *Ppara* knockout (*Ppara$^{-/-}$*) mice develop more severe alcoholic fatty liver disease (Nakajima et al., 2004; Okiyama et al., 2009). PPARα generally regulates expression of lipid metabolism genes encoding acyl-CoA oxidase, carnitine palmitoyltransferase I and short chain acyl-CoA dehydrogenase (Kersten et al.,

1999; Leone et al., 1999). After fasting, $Ppara^{-/-}$ mice develop more severe fatty liver due to impaired fat oxidation, but expression of carnitine palmitoyltransferase I and short chain acyl-CoA dehydrogenase is similar in fasted $Ppara^{-/-}$ and wild-type mice (Kersten et al., 1999). Alcoholic fatty liver disease was more pronounced in $Ppara^{-/-}$ mice, however, acyl-CoA oxidase levels were still comparable in $Ppara^{-/-}$ mice and wild-type mice (Okiyama et al., 2009). These results suggest that other PPARα-dependent mechanisms may occur and blunt fatty liver.

Fibroblast growth factor 21 (FGF21) is a novel metabolic regulator (Kharitonenkov et al., 2005). FGF21 exerts its effect via binding to FGF receptor 1 (FR1) (Luo and McKeehan, 2013). FGF21 can lower blood glucose levels and enhance insulin effects on blood glucose levels (Kharitonenkov et al., 2005). FGF21 can also attenuate hepatic steatosis by regulating hepatic lipid metabolism (Badman et al., 2007). FGF21 is mainly produced in liver and liver is a major source of serum FGF21 (Luo et al., 2013). In liver, FGF21 is regulated by PPARα (Badman et al., 2007; Inagaki et al., 2007). In this study, we hypothesize that PPARα protects against alcoholic fatty liver via FGF21; and that alcoholic fatty liver is enhanced in the $Cyp2a5^{-/-}$ mice because the PPARα-FGF21 axis cannot be upregulated in the $Cyp2a5^{-/-}$ mice.

1. MATERIALS AND METHODS

1.1 Animals and Treatments

The $Cyp2a5^{-/-}$ mice (C57BL/6 background) were created by crossing male $Cyp2a5^{-/-}$ mice (Zhou et al., 2010) (kindly provided by Dr. Xinxin Ding, SUNY College of Nanoscale Science and Engineering, Albany, NY, USA) and female C57BL/6 wild-type mice (purchased from Charles River Laboratory, MA, USA). Littermates ($Cyp2a5^{+/+}$) were bred as wild-type control. The $Ppara^{-/-}$ mice (B6.129S4 background; strain number 008154) were purchased from Jackson Laboratory. $Ppara$ and $Cyp2a5$ double knockout mice ($Ppara^{-/-}/Cyp2a5^{-/-}$) were created by crossing the $Ppara^{-/-}$ mice with $Cyp2a5^{-/-}$ mice. Littermates ($Ppara^{+/+}/Cyp2a5^{-/-}$) were used as wild-type control mice. The 6th generation of mice were used for the experiments. Liver specific $Fgf21$ knockout ($Fgf21^{alb-cre}$) mice and liver specific $Fr1$ knockout ($Fr1^{alb-cre}$) mice were created by crossing $Fgf21$ floxed mice (purchased from Jackson Laboratory; B6.129S6 background; strain number 022361) and $Fr1$ floxed mice (B6.129S4 background; generously provided by Dr. Philippe Soriano, Department of Development and Regenerative Biology, Icahn School of Medicine at Mount Sinai, NY, USA) with transgenic Alb-Cre recombinase (purchased from Jackson Laboratory; (C57BL/6 x DBA)F2 background; strain number 003574), respectively. The littermates that do not express Alb-Cre recombinase were used as control mice ($Fgf21^{fl/fl}$ and $Fr1^{fl/fl}$, respectively). The 6th generation of $Fgf21^{alb-cre}$ mice and $Fr1^{alb-cre}$ mice was used for the experiments. All mice were housed in temperature-controlled animal facilities with 12-hour light/12-hour dark cycles and were permitted consumption of tap water and Purina standard chow *ad libitum* until being fed the experimental diets. The mice received humane care, and experiments were carried out according to the criteria outlined in the Guide for the Care and Use of Laboratory Animals and with approval of the Mount Sinai Animal Care and Use Committee. $Nrf2$ knockout ($Nrf2^{-/-}$) mice were fed ethanol in the lab of Dr. Arndt Vogel in

the Department of Hepatology, Gastroenterology and Endocrinology, Hannover Medical School, Hannover, Germany as described in (Lamlé et al., 2008). The liver samples from $Nrf2^{-/-}$ mice were generously provided by Dr. Vogel.

In our lab, the mice were initially fed the control liquid dextrose diet (Bio-Serv, Frenchtown, NJ) for 3 days to acclimate them to the liquid diet. Afterward, the mice were fed the liquid ethanol diet (Bio-Serv, Frenchtown, NJ) as described by Lieber and DeCarli (Lieber and DeCarli, 1994). The content of ethanol was gradually increased from 10% of total calories to 20%, 25%, 30%, and finally 35% of total calories (about 6.2% [vol/vol]). The liquid dextrose diet were fed on an iso-energetic basis as a control. The ethanol-fed mice had access to their rations *ad libitum*. The total feeding duration was 3 weeks (21 days). The amount of food consumed by the different genetically mutated mice used in this study was approximately the same. Some mice were daily injected mouse recombinant FGF21 (rFGF21, purchased from Creative Biomart, Shirley, NY, USA) subcutaneously at 0.25 mg/kg body weight. The mice were fasted for 6 h and then sacrificed by cervical dislocation after blood was collected via the retro-orbital venous sinus under anesthesia by inhalation of isoflurane. The livers were rapidly excised into fragments and washed with cold saline. One piece of liver tissue was put in neutral Formalin solution for paraffin bedding. The other liver tissue aliquots were stored at −80°C for assays.

1.2 Liver Histology

Liver sections were stained with hematoxylin and eosin (H&E) for pathological evaluation as described before (Lu et al., 2010; Hong et al., 2015). Steatosis was quantified as the percentage of cells containing lipid droplets. Five fields (200×) per liver section were examined (one 200× field area contains about 200 cells). Steatosis scoring was based on the following criteria: 0, none; 1, <5%; 2, 5–33%; 3, 34–66%; 4, >67% of cells displaying fat accumulation.

1.3 Quantification of triglyceride (TG) and FGF21

Serum levels of TG were measured using commercially available kits (Pointe Scientific, Canton, MI, USA). Serum FGF21 was measured using a mouse FGF21 kit (BioVendor, Asheville, NC, USA). Liver homogenates were prepared in ice-cold 0.15 M potassium chloride (KCL) and liver TG content was measured as the same manner as serum TG.

1.4 Cytochrome P450 2E1 and 2A5 Activity

Hepatic microsomes were prepared from liver homogenates suspended in 0.15 M KCL. The homogenates were centrifuged at $9,000 \times g$ for 20 min, and then the resulting supernatant fractions were centrifuged at $105,000 \times g$ for 60 min. The resulting pellets (microsomes) were re-suspended in 0.15 M KCL. All procedures were carried out at 4 °C. CYP2E1 activity was measured by the rate of oxidation of 1 mM p-nitrophenol to p-nitrocatechol as described (Lu et al., 2005). Coumarin 7-hydroxylase (COH) is encoded by the mouse *Cyp2a5* gene and its human orthologue *Cyp2a6* gene, and COH activity is considered as a specific marker for catalytic activities of CYP2A5 and CYP2A6 (Su and Ding, 2004). CYP2A5 activity was measured by assessing COH with 100 μM coumarin as substrate (Kobliakov et al., 1993).

1.5 Western Blotting

Hepatic proteins were separated by 10% SDS-PAGE and transferred to nitrocellulose membranes. After 1 h of blocking with 2% fat-free milk, membranes were then incubated for overnight with primary antibodies followed by 1 h incubation with peroxidase secondary anti-rabbit, anti-chicken and anti-goat antibodies (Millipore), respectively. Calnexin or β-actin was detected as a protein loading control. Chemiluminescence was detected by Image Reader LAS-4000 (Fijifilm) after adding Pierce EC Western Blotting Substrate (Pointe Scientific, Canton, MI, USA). Anti-CYP2E1 IgG was a gift from Dr. Jerome Lasker, Hackensack Biomedical Research Institute, Hackensack, NJ, anti-CYP2A5 IgG was a gift from Dr. Risto Juvonen, Department of Pharmacology and Toxicology, University of Kuopio, Kuopio, Finland. Anti-PPARα and anti-FGF21 IgGs were from Abcam, Cambridge, MA, USA. The other IgGs were from Santa Cruz Biotechnology, CA, USA. The bands of proteins were quantified with the Automated Digitizing System (ImageJ gel programs, version 1.34S; National Institutes of Health, Bethesda, MD).

1.6 Statistics

Results are expressed as means ± SEMs. Statistical evaluation was carried out by using one-way analysis of variance (ANOVA) with subsequent the Student-Newman-Keuls post hoc test. $P<0.05$ was considered as statistical significance.

2. Results

2.1 Alcoholic fatty liver is more pronounced in *Cyp2a5*$^{-/-}$ mice than in *Cyp2a5*$^{+/+}$ mice

As shown in Fig 1A, ethanol feeding induced formation of lipid droplets in liver of both *Cyp2a5*$^{+/+}$ mice and *Cyp2a5*$^{-/-}$ mice, but the percentage of hepatocytes containing lipid droplets was higher in *Cyp2a5*$^{-/-}$ mice than in *Cyp2a5*$^{+/+}$ mice (Fig. 1B). Consistently, liver TG contents were increased in *Cyp2a5*$^{-/-}$ mice to a greater extent than in *Cyp2a5*$^{+/+}$ mice (Fig 1C **grey bars**). In a similar manner, serum levels of TG were increased in *Cyp2a5*$^{-/-}$ mice but not in *Cyp2a5*$^{+/+}$ mice (Fig 1C **black bars**). These results suggest that ablation of CYP2A5 promotes alcoholic fatty liver.

Alcoholic fatty liver is associated with insulin resistance (Zong et al., 2012). Indeed, insulin receptor (IR)-mediated signaling molecules PI3K/AKT and IRS-1 were decreased in response to ethanol feeding (Fig 1D, 1E). However, there were no differences in the decrease in these insulin signaling molecules between *Cyp2a5*$^{-/-}$ mice and *Cyp2a5*$^{+/+}$ mice (Fig 1D, E), suggesting that the ethanol-induced insulin resistance may not explain the difference in development of alcoholic fatty liver between *Cyp2a5*$^{-/-}$ mice and *Cyp2a5*$^{+/+}$ mice.

2.2 Ethanol feeding induces FGF21 in *Cyp2a5*$^{+/+}$ mice but not in *Cyp2a5*$^{-/-}$ mice

FGF21 is a novel metabolic regulator modulating glucose and lipid metabolism (Kharitonenkov et al., 2005). Ethanol feeding caused a 3-fold increase in serum FGF21 in *Cyp2a5*$^{+/+}$ mice, however, ethanol feeding had no effect on serum FGF21 in *Cyp2a5*$^{-/-}$ mice probably because basal levels of serum FGF21 in *Cyp2a5*$^{-/-}$ mice were already elevated (Fig 2A). Consistently, liver FGF21 expression was also induced by ethanol feeding in *Cyp2a5*$^{+/+}$ mice. In *Cyp2a5*$^{-/-}$ mice, however, ethanol feeding did not induce hepatic FGF21

expression although basal FGF21 expression in liver was already higher compared to *Cyp2a5*$^{+/+}$ mice (Fig 2B). Liver FGF21 is regulated by PPARα (Badman et al., 2007; Inagaki et al., 2007). Basal PPARα expression in *Cyp2a5*$^{-/-}$ mice was higher than that in *Cyp2a5*$^{+/+}$ mice although ethanol reduced PPARα expression in both the *Cyp2a5*$^{-/-}$ mice and *Cyp2a5*$^{+/+}$ mice (Fig 2B, C).

2.3 FGF21 is induced in *Nrf2*$^{-/-}$ mice but is not induced in *Ppara*$^{-/-}$ mice

Previously, we reported that ethanol induction of CYP2A5 was regulated by a NRF2-dependent pathway (Lu et al., 2012). Here, we found that CYP2A5 induction by ethanol was blunted in *Nrf2*$^{-/-}$ mice while CYP2E1 induction was not affected (Fig 3A, B). Basal FGF21 expression was undetectable in *Nrf2*$^{-/-}$ mice, which is consistent with the report that Nrf2 regulates FGF21 (Furusawa et al., 2014). However, ethanol was capable of inducing FGF21 in *Nrf2*$^{-/-}$ mice, although the FGF21 induction by ethanol was weaker compared with *Nrf2*$^{+/+}$ mice (Fig 3A, B). Interestingly, basal expression of PPARα, a major regulator of FGF21 in liver, was upregulated in *Nrf2*$^{-/-}$ mice (Fig 3A, B). To explore the vital role of PPARα in ethanol induction of FGF21, *Ppara*$^{-/-}$ mice and *Ppara*$^{+/+}$ mice were fed ethanol. As shown in Fig 4A, serum FGF21 was almost undetectable in *Ppara*$^{-/-}$ mice, which is consistent with the notion that FGF21 is regulated by PPARα (Badman et al., 2007; Inagaki et al., 2007). After ethanol feeding, while serum FGF21 was increased in *Ppara*$^{+/+}$ mice, serum FGF21 was still undetectable in *Ppara*$^{-/-}$ mice. Administration of rFGF21 completely restored serum FGF21 in *Ppara*$^{-/-}$ mice. These results suggest that ethanol induction of FGF21 is regulated by PPARα.

2.4 rFGF21 blunts ethanol-induced hypertriglyceridemia in *Ppara*$^{-/-}$ mice

PPARα plays an important role in the development of alcoholic fatty liver disease (Nakajima et al., 2004; Okiyama et al., 2009). Although a comparable increase in liver TG was observed in *Ppara*$^{-/-}$ mice and *Ppara*$^{+/+}$ mice (Fig 4B), ethanol-induced hypertriglyceridemia was observed in *Ppara*$^{-/-}$ mice rather than in *Ppara*$^{+/+}$ mice (Fig 4C). Administration of rFGF21 blunted the increase in liver TG in *Ppara*$^{+/+}$ mice (Fig 4B) and the increase in serum TG in *Ppara*$^{-/-}$ mice (Fig 4C). PPARα and rFGF21 had no effects on liver CYP2E1 and CYP2A5 activities (Fig 4D). These results suggest that the enhanced alcoholic fatty liver found in *Ppara*$^{-/-}$ mice is associated with the lower FGF21 levels.

2.5 Chronic ethanol-induced fatty liver is comparable in *Fr1*$^{alb-cre}$ mice but is more severe in *Fgf21*$^{alb-cre}$ mice

To further test the role of FGF21 in the development of alcoholic fatty liver, we created liver specific FGF21 knockout (*Fgf21*$^{alb-cre}$) mice and liver specific FR1 knockout (*Fr1*$^{alb-cre}$) mice and compared the effects of ethanol in these mice with the corresponding control mice, *Fgf21*$^{fl/fl}$ mice and *Fr1*$^{fl/fl}$ mice, respectively. Serum FGF21 was almost undetectable in the *Fgf21*$^{alb-cre}$ mice while it was unchanged in the *Fr1*$^{alb-cre}$ mice compared with the *Fr1*$^{fl/fl}$ mice; after ethanol feeding, the serum FGF21 was increased by 4-fold in the *Fr1*$^{alb-cre}$ mice but it was not induced in the *Fgf21*$^{alb-cre}$ mice (Fig 5A). Correspondingly, *Fgf21*$^{alb-cre}$ mice developed more severe alcoholic fatty liver than *Fgf21*$^{fl/fl}$ mice (Fig 5B, C), and liver TG accumulation was higher in *Fgf21*$^{alb-cre}$ mice than in *Fgf21*$^{fl/fl}$ mice (Fig 5D), suggesting that FGF21 plays an indispensible role in the development of alcoholic fatty liver. However,

alcoholic fatty liver was comparable in the *Fr1$^{alb-cre}$* mice and *Fr1$^{fl/fl}$* mice, suggesting that FGF21 does not exert its effects through liver FR1.

2.6 Pparα$^{-/-}$/Cyp2a5$^{-/-}$ mice develop more severe alcoholic fatty liver than Pparα$^{+/+}$/Cyp2a5$^{-/-}$ mice

To investigate whether the basal upregulation of PPARα-FGF21 in *Cyp2a5$^{-/-}$* mice protects against alcoholic fatty liver, we designed a model to abrogate the upregulation of PPARα in *Cyp2a5$^{-/-}$* mice by creating *Ppara$^{-/-}$/Cyp2a5$^{-/-}$* mice. A comparison between *Ppara$^{-/-}$/Cyp2a5$^{-/-}$* mice and *Ppara$^{+/+}$/Cyp2a5$^{-/-}$* mice would help to define the role of PPARα-FGF21 in the enhanced alcoholic fatty liver in *Cyp2a5$^{-/-}$* mice. As shown in Fig 6A, serum FGF21 was much lower in *Ppara$^{-/-}$/Cyp2a5$^{-/-}$* mice than in *Ppara$^{+/+}$/Cyp2a5$^{-/-}$* mice; ethanol feeding increased serum FGF21 in *Ppara$^{+/+}$/Cyp2a5$^{-/-}$* mice but did not in *Ppara$^{-/-}$/Cyp2a5$^{-/-}$* mice. Alcoholic fatty liver was more pronounced in *Ppara$^{-/-}$/Cyp2a5$^{-/-}$* mice than in *Ppara$^{+/+}$/Cyp2a5$^{-/-}$* mice (Fig 6B, C), and liver TG accumulation was also higher in *Ppara$^{-/-}$/Cyp2a5$^{-/-}$* mice than in *Ppara$^{+/+}$/Cyp2a5$^{-/-}$* mice (Fig 6D), suggesting that abrogating the upregulation of the PPARα-FGF21 axis enhances alcoholic fatty liver; and the difference in alcoholic fatty liver between *Cyp2a5$^{-/-}$* mice and *Cyp2a5$^{+/+}$* mice might be due to the inability of ethanol to upregulate the PPARα-FGF21 axis in *Cyp2a5$^{-/-}$* mice.

3. Discussion

Kirby et al first suggested that CYP2A5 may modulate hepatic lipid metabolism (Kirby et al., 2011). CYP2A6 upregulation was observed in patients with alcoholic and non-alcoholic fatty liver, and CYP2A6 co-localized with lipid droplets (Niemela et al., 2000). Previously, we reported that CYP2A5 protected against alcoholic liver injury (Hong et al., 2015), but the molecular mechanism for this was not clear. In the present study, we found that alcoholic fatty liver was more pronounced in *Cyp2a5$^{-/-}$* mice than in *Cyp2a5$^{+/+}$* mice, and that the alcoholic fatty liver was more pronounced in *Ppara$^{-/-}$/Cyp2a5$^{-/-}$* mice than in *Ppara$^{+/+}$/Cyp2a5$^{-/-}$* mice, suggesting that CYP2A5, together with PPARα, may participate in regulation of lipid metabolism and in the development of alcoholic fatty liver.

FGF21 is a recently-described lipid metabolism regulator (Kharitonenkov et al., 2005). Basal levels of serum FGF21 and liver FGF21 expression were elevated in *Cyp2a5$^{-/-}$* mice compared to *Cyp2a5$^{+/+}$* mice. However, ethanol did not further increase serum FGF21 or liver FGF21 expression in *Cyp2a5$^{-/-}$* mice while ethanol did elevate FGF21 in *Cyp2a5$^{+/+}$* mice. In liver, FGF21 is regulated by PPARα, a lipid metabolism regulator (Badman et al., 2007; Inagaki et al., 2007). Indeed, we found that serum FGF21 was almost undetectable in *Ppara$^{-/-}$* mice and ethanol feeding did not increase serum FGF21 but ethanol did induce hypertriglyceridemia in *Ppara$^{-/-}$* mice. In contrast, ethanol feeding induced serum FGF21 but did not cause an elevation of serum TG in *Ppara$^{+/+}$* mice. Administration of rFGF21 restored the blood FGF21 and normalized serum TG levels in ethanol-fed *Ppara$^{-/-}$* mice. These results suggest that FGF21 may act as a downstream mediator of the PPARα signaling pathway, resulting in a formation of a PPARα-FGF21 axis which protecte against the development of alcoholic fatty liver. The treatment with rFGF21 led to a decrease in liver

TG in $Ppara^{+/+}$ mice but did not in $Ppara^{-/-}$ mice. Alcoholic fatty liver is known to be enhanced in $Ppara^{-/-}$ mice (Nakajima et al., 2004). In our study, liver TG accumulation was not higher in $Ppara^{-/-}$ mice as compared with $Ppara^{+/+}$ mice, but hypertriglyceridemia was observed in the $Ppara^{-/-}$ mice but was not in the $Ppara^{+/+}$ mice. Administration of rFGF21 blunted the elevation of serum TG in the $Ppara^{-/-}$ mice, indicating that FGF21 is still protective in $Ppara^{-/-}$ mice. Although serum TG levels were lowered by rFGF21, liver TG accumulation was not decreased by rFGF21 in $Ppara^{-/-}$ mice. Possible reasons for why rFGF21 did not ameliorate liver fat accumulation in $Ppara^{-/-}$ mice may be: 1. PPARα regulates a panel of lipid metabolism genes, and FGF21 may need to cooperate with other PPARα-regulated genes to exert hepatic lowering effects; 2. While the rFGF21 dose (0.25mg/kg) was sufficient to exert actions in the $Ppara^{+/+}$ mice (where endogenous FGF21 is also produced), it may not be sufficient for $Ppara^{-/-}$ mice (where no or little endogenous FGF21 is produced); 3. The rFGF21 may be lowering non-hepatic secretion of TG into the serum e.g. adipose tissue lipolysis, thereby lowering hypertriglyceridemia but not hepatic TGF levels.

FGF21 exerts its effect via binding to and activating FR1 (Potthoff et al., 2012). Adipose tissues have comparatively high expression of FR1 (Fisher et al., 2011); FR1 expression in liver is only about 10% the level of expression detected in adipose tissue (Fisher et al., 2011), which leads to an expectation that FGF21 acts on adipose tissue to a greater extent than liver. We found that alcoholic fatty liver was comparable in the $Fr1^{alb-cre}$ mice and $Fr1^{fl/fl}$ mice, suggesting that FGF21 does not exert its lipid modulating effects through liver FR1. About 60% of hepatic fat is derived from adipose tissues (Donnelly et al., 2005), and adipose tissues play a very important role in the development of alcoholic fatty liver disease (Poggi and Di Luzio, 1964; Horning et al., 1960; Zhong et al., 2012). We plan to create adipose specific $Fr1$ knockout mice to address whether FGF21 exerts its effects through adipose FR1.

Both mouse CYP2A5 and human CYP2A6 are regulated by NRF2 (Lu et al., 2012; Yokota et al., 2011). Recently, we found that ethanol induction of CYP2A5 was lower in $Nrf2^{-/-}$ mice than in $Nrf2^{+/+}$ mice, suggesting that ethanol induction of CYP2A5 was also regulated, at least in part, by NRF2-dependent pathway (Lu et al., 2012). Redox sensitive NRF2 usually up-regulates a panel of antioxidant enzymes to antagonize oxidative stress (Cederbaum, 2009) and NRF2 deficiency enhanced alcoholic liver injury in mice (Lamlé et al., 2008), suggesting that NRF2 protects against alcoholic liver injury via its antioxidant promoting effects. Whether $Cyp2a5$ is among the panel of NRF2-regulated antioxidant genes is still unclear, but CYP2A5 may act as an antioxidant to protect against alcohol-induced oxidative liver injury (Hong et al., 2015). NRF2 can also regulate FGF21 expression (Furusawa et al., 2014). Does NRF2 also exert effects via FGF21? Here we found that ethanol induction of FGF21 was also lower in $Nrf2^{-/-}$ mice, which is consistent with the more severe alcoholic liver injury in $Nrf2^{-/-}$ mice (Lamlé et al., 2008). Thus, in addition to its antioxidant promoting effects, NRF2 may also regulate hepatic lipid metabolism through FGF21.

To directly address whether the PPARα-FGF21 axis is related to the enhanced fatty liver in $Cyp2a5^{-/-}$ mice, we developed $Ppara^{-/-}/Cyp2a5^{-/-}$ mice. In addition to liver, adipose tissue

also produce FGF21. In liver, FGF21 is regulated by PPARα, but in adipose tissues, FGF21 is regulated by PPARγ but not by PPARα (Dutchak et al., 2012). Thus, adipose FGF21 should not be affected in $Ppara^{-/-}/Cyp2a5^{-/-}$ mice which maintain PPARγ signaling. Similarly, to exclude the influence of adipose FGF21, liver-specific Fgf21 knockout ($Fgf21^{alb-cre}$) mice were applied instead of global FGF21 knockout mice to examine whether FGF21 plays a role in alcoholic fatty liver. $Fgf21^{alb-cre}$ mice developed more pronounced alcoholic fatty liver than $Fgf21^{fl/fl}$ mice, and most importantly, $Ppara^{-/-}/Cyp2a5^{-/-}$ mice developed a more severe alcoholic fatty liver than $Ppara^{+/+}/Cyp2a5^{-/-}$ mice. Serum FGF21 was much lower in $Ppara^{-/-}/Cyp2a5^{-/-}$ mice than in $Ppara^{+/+}/Cyp2a5^{-/-}$ mice. These results suggest that the PPARα-FGF21 axis regulates hepatic lipid metabolism, and it is due to the failed induction of the PPARα-FGF21 axis that $Cyp2a5^{-/-}$ mice develop more severe alcoholic fatty liver. In $Cyp2a5^{+/+}$ mice, ethanol induced a compensatory upregulation of the PPARα-FGF21 axis, which regulates hepatic lipid metabolism and blunts the development of fatty liver disease. When the PPARα-FGF21 axis was not upregulated as compensation, the fatty liver was more pronounced as seen in the $Cyp2a5^{-/-}$ mice.

In this study, we used several colonies of genetically mutated mice with different background. Alcohol-induced fatty liver in mice may be susceptible to genetic background. Therefore, the comparison was made among those mice with the same genetic background, and the comparison between those mice with different background was avoided. For example, $Ppara^{+/+}/Cyp2a5^{-/-}$ mice and $Ppara^{-/-}/Cyp2a5^{-/-}$ mice were litter mates sharing the same background, thus, the comparison between them leads to a conclusion that the enhanced alcoholic fatty liver in the $Cyp2a5^{-/-}$ mice is due to the failed upregulation of the PPARα-FGF21 axis by ethanol in $Cyp2a5^{-/-}$ mice. However, due to the different background, $Fgf21^{alb-cre}$ mice and $Fr1^{alb-cre}$ mice are not comparable. Although liver TG levels and steatosis scores were statistically higher in $Fgf21^{alb-cre}$ mice than in $Fr1^{alb-cre}$ mice (Fig. 5C, 5D), a conclusion that liver FGF21 plays a more important role in alcoholic fatty liver than liver FR1 was not from a direct comparison between $Fgf21^{alb-cre}$ mice and $Fr1^{alb-cre}$ mice. Instead, by comparing $Fgf21^{alb-cre}$ mice and $Fgf21^{fl/fl}$ mice, we know that liver FGF21 plays an important role in alcoholic fatty liver and by comparing $Fr1^{alb-cre}$ mice and $Fr1^{fl/fl}$ mice, we conclude that liver FR1 has no effect on alcoholic fatty liver. Thus, liver FGF21 is more important than liver FR1 in alcoholic fatty liver.

Mouse $Cyp2a5$ is an ortholog of human $Cyp2a6$, and the findings in this study may be extrapolated from mouse to human being. $Cyp2a6$ gene, which is highly polymorphic, has approximately 40 annotated allelic variants, and homozygosis for some of these alleles leads to a total lack of CYP2A6 activity. Individual difference in CYP2A6 expression and activity is due primarily to genetic polymorphisms in the $Cyp2a6$ gene (Raunio and Rahnasto-Rilla, 2012). There have been near 300 single nucleotide polymorphisms (SNPs) found in the $Cyp2a6$ upstream sequence, and polymorphism of CYP2A6 has been associated with smoking behavior, drug clearance and lung cancer risk (Di et al., 2009). Whether the polymorphisms of human $Cyp2a6$ affect sensitivity of patients to alcoholic fatty liver disease needs further studies.

In conclusion, CYP2A5 protect against the development of alcoholic fatty liver disease, and FGF21 acts as a downstream molecule of PPARα signaling pathway to regulate liver lipid metabolism and contribute to the CYP2A5 protective effects on alcoholic fatty liver disease.

Acknowledgments

Abbreviation

CYP2A6
Cytochrome P450 2A6

CYP2E1
Cytochrome P450 2E1

Cyp2e1^{−/−} **mice**
Cyp2e1 knockout mice

CYP2A5
Cytochrome P450 2A5

Cyp2a5^{−/−} **mice**
Cyp2a5 knockout mice

NRF2
nuclear factor-erythroid 2-related factor 2

Nrf2^{−/−} **mice**
Nrf2 knockout mice

PPARα
peroxisome proliferator-activated receptor α

Pparα^{−/−} **mice**
Pparα knockout mice

FGF21
fibroblast growth factor 21

Pparα^{−/−}/*Cyp2a5*^{−/−} **mice**
Pparα and *Cyp2a5* double knockout mice

Fgf21[alb-cre] **mice**
liver specific *Fgf21* knockout mice

FR1

FGF receptor 1

$Fr1^{alb-cre}$ mice

liver specific *Fr1* knockout mice

rFGF21

mouse recombinant FGF21

TG

triglyceride

KCL

potassium chloride

H&E

hematoxylin and eosin

COH

coumarin 7-hydroxylase

SDS-PAGE

sodium dodecyl sulfate-polyacrylamide gel electrophoresis

PI3K/AKT

phosphatidylinositol 3-kinase/Protein Kinase B

IRS-1

insulin receptor substrate 1

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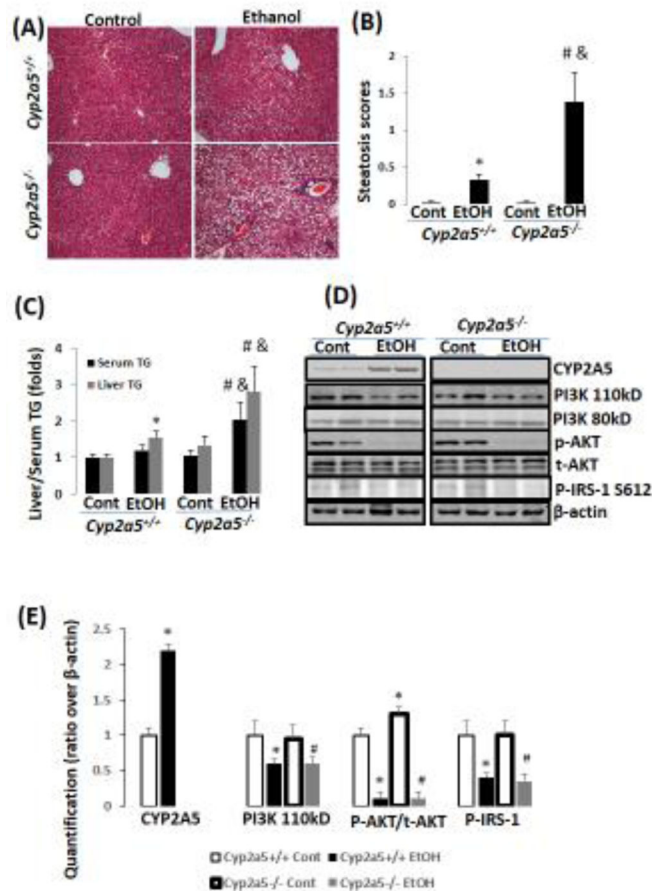
Figure 1.
Chronic ethanol-induced fatty liver is more pronounced in *Cyp2a5*$^{-/-}$ mice. (A) H&E staining. (B) Steatosis quantification (N=5). (C) Liver and serum TG (N=5). (D) Expression of CYP2A5, PI3K, AKT, and IRS by Western blotting analyses. (E) Quantitation of Western blots (N=4). *P<0.05, compared to *Cyp2a5*$^{+/+}$ Control Group; # P<0.05, compared to *Cyp2a5*$^{-/-}$ Control Group; & P<0.05, compared with *Cyp2a5*$^{+/+}$ Ethanol group. Cont, Control; EtOH, Ethanol.
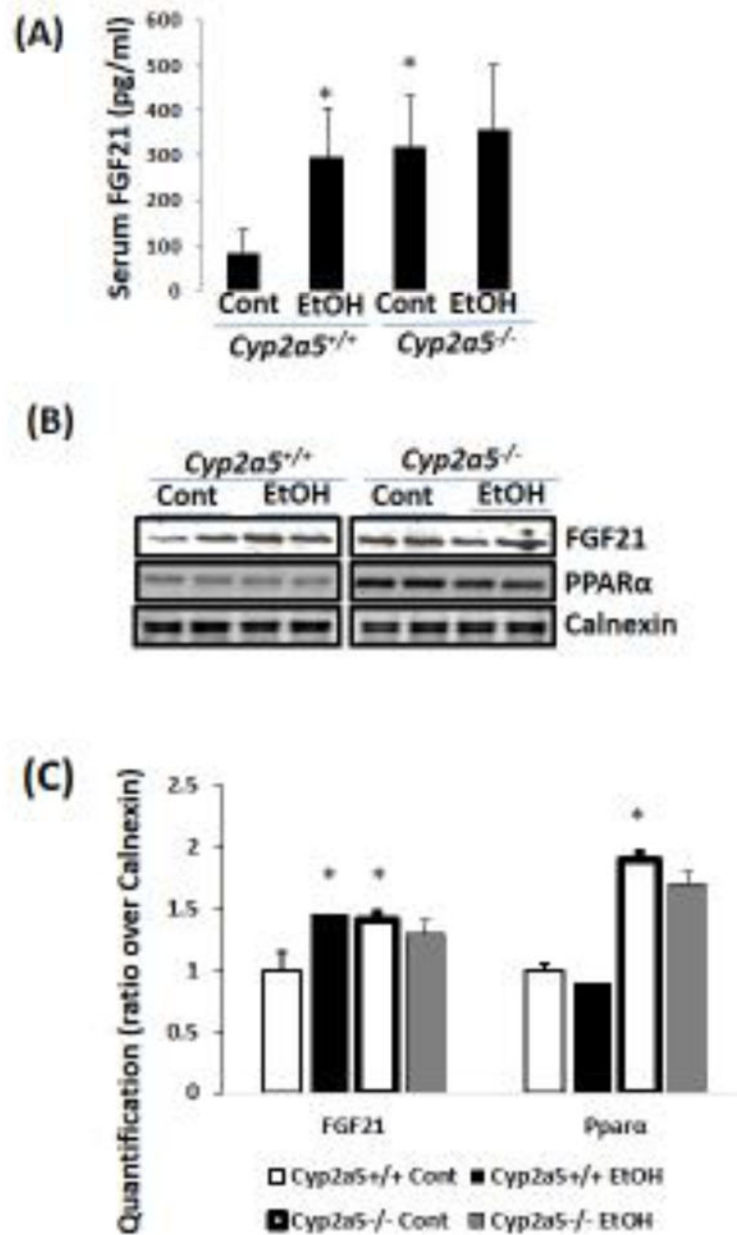
Figure 2.
Chronic ethanol induces FGF21 in *Cyp2a5$^{+/+}$* mice but does not in *Cyp2a5$^{-/-}$* mice. (A) Serum FGF21 (N=5). (B) Liver expression of FGF21 and PPARα by Western blotting analysis. (C) Quantitation of Western blots (N=4). *P<0.05, compared to *Cyp2a5$^{+/+}$* Control Group. Cont, Control; EtOH, Ethanol.

(A)

(B)
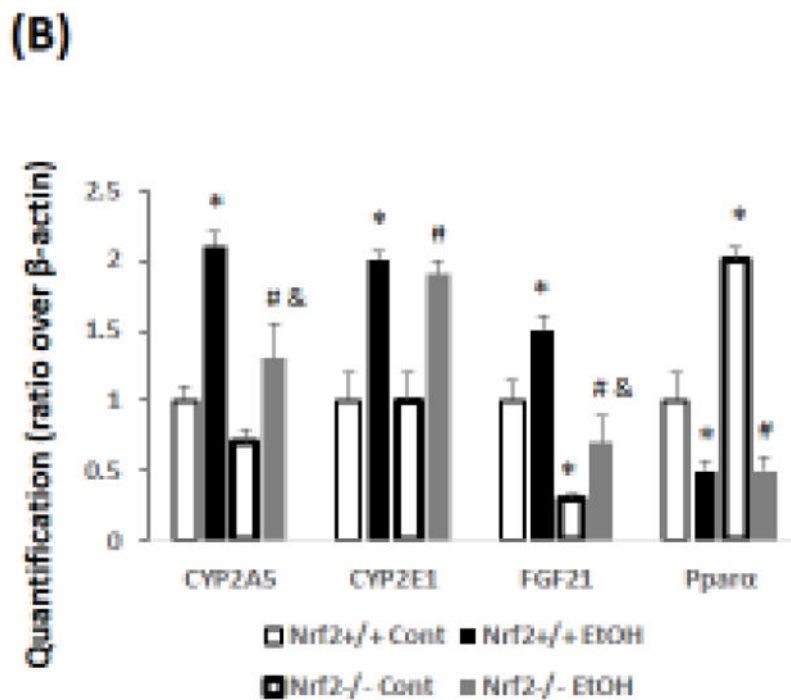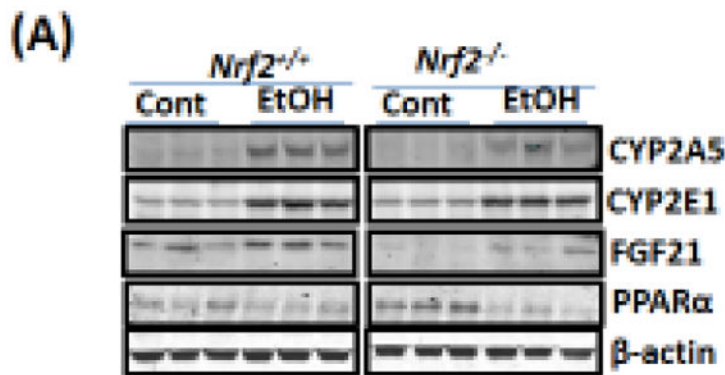
Figure 3.

Chronic ethanol induces FGF21 in $Nrf2^{+/+}$ mice more than in $Nrf2^{-/-}$ mice. (A) Western blots. (B) Quantitation of Western blots (N=3). *P<0.05, compared to $Nrf2^{+/+}$ Control Group; # P<0.05, compared to $Nrf2^{-/-}$ Control Group; & P<0.05, compared with $Nrf2^{+/+}$ Ethanol group. Cont, Control; EtOH, Ethanol.
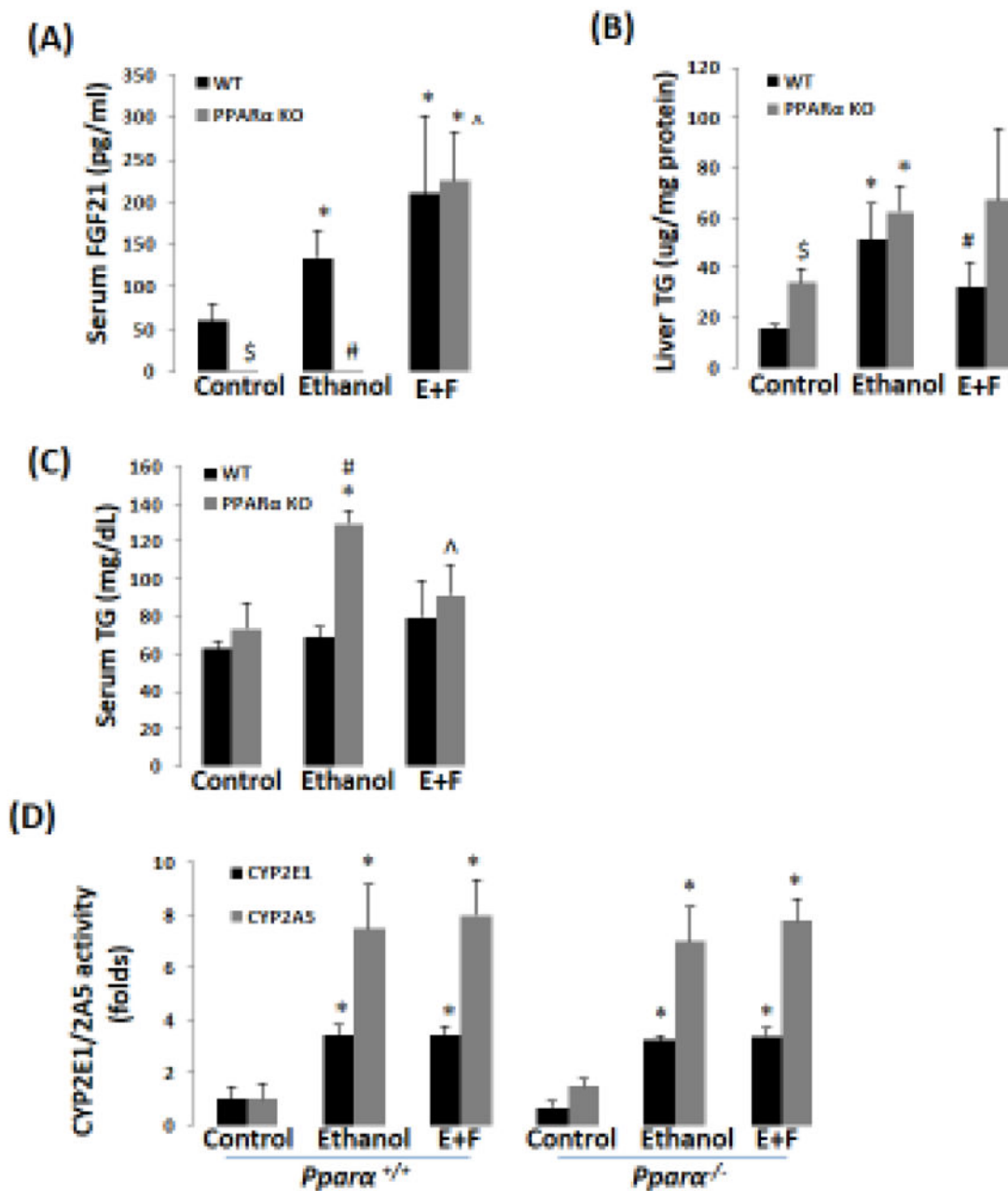
Figure 4.
Recombinant FGF21 blunts ethanol-induced hypertriglyceridemia (HTG) in *Ppara*$^{-/-}$ mice.
(A) Ethanol induction of FGF21 was blunted in *Ppara*$^{-/-}$ mice (N=5). (B) rFGF21 blunted ethanol-induced elevation of liver TG in WT mice but not in *Ppara*$^{-/-}$ mice (N=5). (C) rFGF21 blunted ethanol-induced HTG in *Ppara*$^{-/-}$ mice (N=5). (D) Ethanol induction of CYP2E1 and CYP2A5 was not affected in both *Ppara*$^{+/+}$ and *Ppara*$^{-/-}$ mice (N=5). *P<0.05, compared to Control Group. # P<0.05, compared to WT Ethanol Group. $ P<0.05, compared with WT Control Group. ^ P<0.05, compared with PPAR*a* KO Ethanol group. E+F, ethanol plus rFGF21; WT, wild-type; PPAR*a* KO, *Ppara*$^{-/-}$.
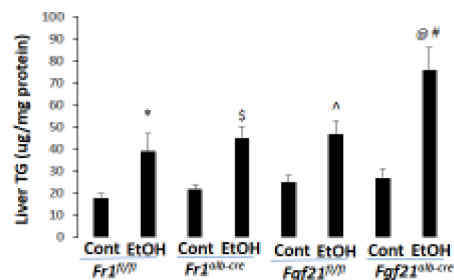
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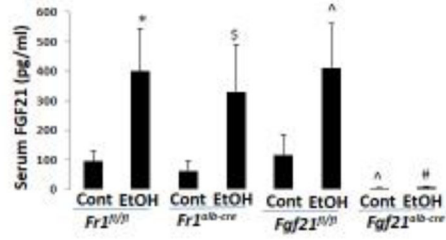
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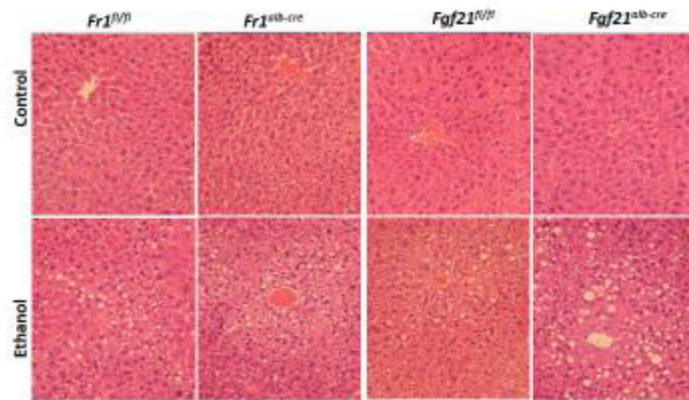
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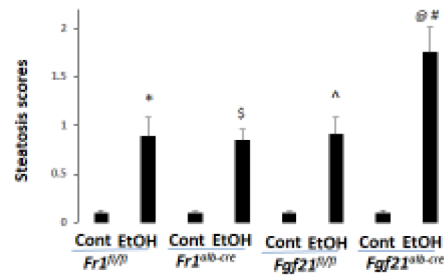
(D)

Figure 5.
Alcoholic fatty liver is enhanced in *Fgf21*$^{alb-cre}$ mice but is not in *Fr1*$^{alb-cre}$ mice. (A) Ethanol induction of FGF21 was blunted in *Fgf21*$^{alb-cre}$ mice but was not in *Fr1*$^{alb-cre}$ mice (N=5). (B) H&E staining in liver sections shows that alcoholic fatty liver was enhanced in *Fgf21*$^{alb-cre}$ mice but not in *Fr1*$^{alb-cre}$ mice. (C) Steatosis scores (N=5). (D) Liver TG contents (N=5). *P<0.05, compared to *Fr1*$^{fl/fl}$ Control Group. # P<0.05, compared to *Fgf21*$^{fl/fl}$ Ethanol Group. ^ P<0.05, compared with *Fgf21*$^{fl/fl}$ control group. $ P<0.05, compared to *Fr1*$^{alb-cre}$ Control Group. @ P<0.05, compared to *Fr1*$^{alb-cre}$ Control Group. Cont, Control; EtOH, Ethanol.

(C)

(D)
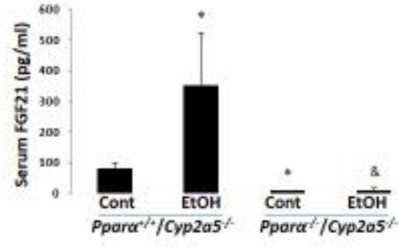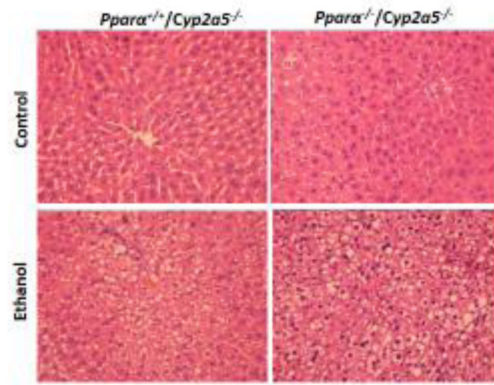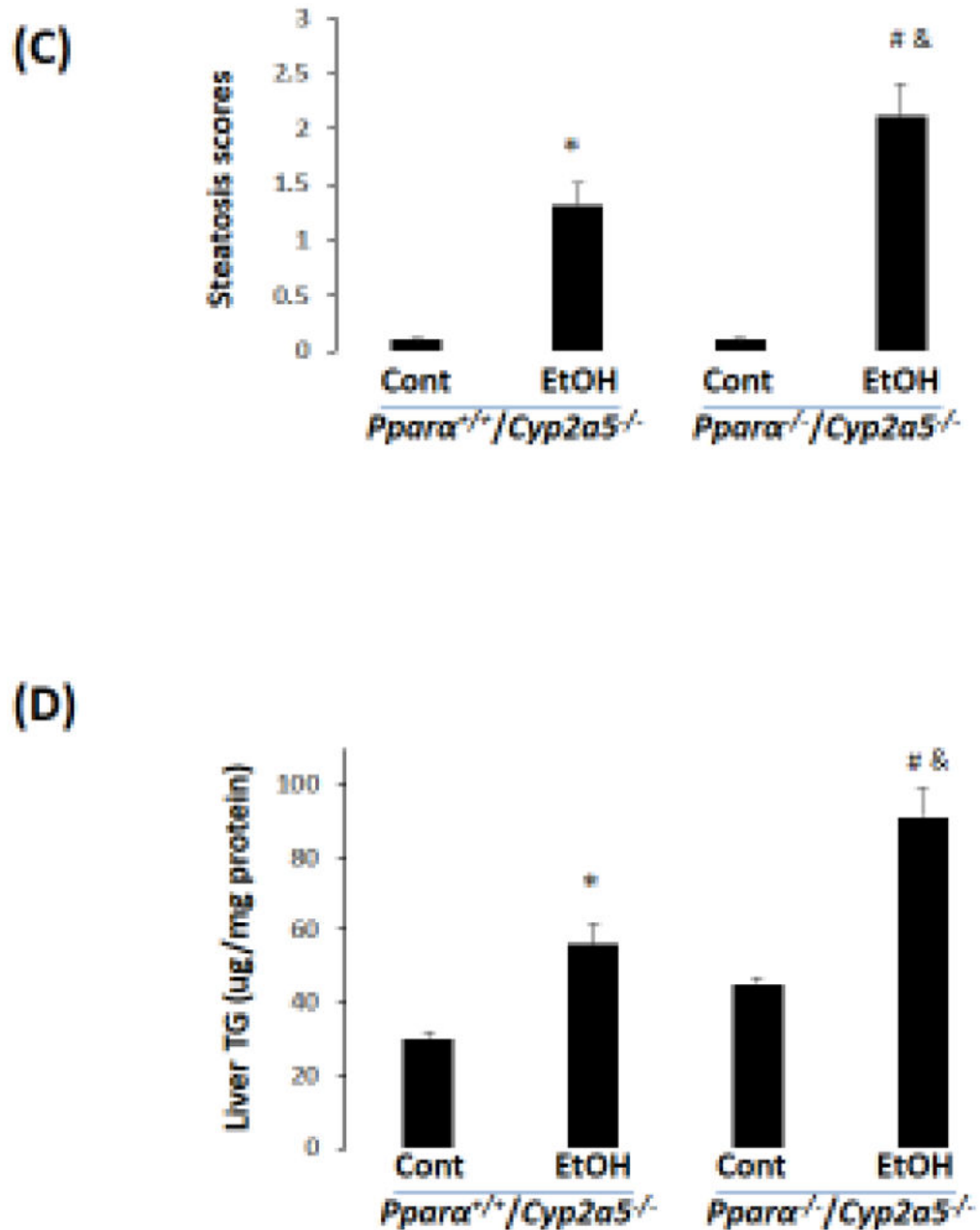
Figure 6.

Alcoholic fatty liver is more pronounced in $Ppara^{-/-}/Cyp2a5^{-/-}$ mice than in $Ppara^{+/+}/Cyp2a5^{-/-}$ mice. (A) Ethanol induction of FGF21 was blunted in $Ppara^{-/-}/Cyp2a5^{-/-}$ mice (N=4). (B) H&E staining in liver sections shows that alcoholic fatty liver was more pronounced in $Ppara^{-/-}/Cyp2a5^{-/-}$ mice than in $Ppara^{+/+}/Cyp2a5^{-/-}$ mice. (C) Steatosis scores (N=5). (D) Liver TG contents (N=5). *P<0.05, compared to $Ppara^{+/+}/Cyp2a5^{-/-}$ Control Group. # P<0.05, compared to $Ppara^{-/-}/Cyp2a5^{-/-}$ Control Group. & P<0.05, compared to $Ppara^{+/+}/Cyp2a5^{-/-}$ Ethanol Group. Cont, Control; EtOH, Ethanol.