

# **Liver-specific glucocorticoid action in alcoholic liver disease**

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### **Author Contributions**

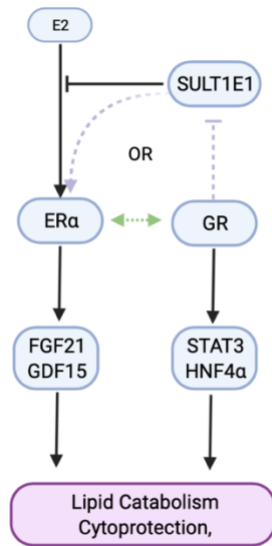
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**Abstract:**

The number of deaths due to alcoholic liver disease is increasing every year. Glucocorticoids (GCs) are the only first-line drugs for alcoholic hepatitis (AH) treatment but have limited efficacy. Long-term high-dose GC use can cause various side effects on extrahepatic tissues, such as immunosuppression and neuromuscular side effects, which



may be a limiting factor for GC treatment of AH. Therefore, liver-specific GC-targeted therapy may have multiple advantages compared with systemic GC for AH. This research explored the role of liver-specific deficiency of glucocorticoid receptor (GR) in AH induced by a high-fat diet (HFD) plus ethanol binge. Females are less prone to AH induced by HFD plus acute binge drinking, likely due to sex differences in estrogen (E2) signaling. We found that hepatic GR deficiency worsened steatosis in both genders of AH mice but only

aggravated the liver injury in male AH mice. Multiple signaling pathways were dysregulated in GR knockout AH mice. Interestingly, hepatic expression of estrogen receptor (ER $\alpha$ ) was induced, and the E2-inactivating enzyme was markedly down-regulated in GR knockout AH mice, suggesting enhanced E2 signaling in these mice. Our data mining found marked dysregulation of many GR-target genes important for lipid catabolism, cytoprotection, and inflammation in patients with severe AH. These key GR-target genes were similarly induced or down-regulated by our liver-targeting GC prodrugs and the parent drug at 1 $\mu$ M in primary human hepatocytes. In contrast, GC prodrugs had

much weaker inhibitory effects than the parent drug on LPS-induction of IL-1B in mouse macrophages, suggesting a good liver selectivity of our liver-targeting GC prodrugs.

The ultimate goal of this study is to determine the mechanistic role of GR in alcoholic fatty liver disease and develop targeted drug therapies to treat alcoholic hepatitis.

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## List of Abbreviations

<b>Abbreviation</b>	<b>Full Word</b>
ACLY	ATP citrate synthase
AH	alcoholic hepatitis
ALB	Albumin
ALD	alcoholic liver disease
ALT	serum alanine aminotransferase
AP-1	activating protein-1
Apoa4	apolipoproteins Apo-AI
ATF3	activating transcription factor 3
BA	bile-acid
CA	cholic acid
CB	zwitterionic carboxy
CHO	total cholesterol
Cxcl10	C-X-C motif chemokine ligand 10
Cyp7b1	cytochrome P450 7b1
DEX	dexamethasone
DUSP1	dual specificity protein phosphatase 1
E2	estrogen
EREs	estrogen response elements
ERK	Extracellular signal-regulated kinase
ERRFI1	ERBB receptor feedback inhibitor 1
ER $\alpha$	estrogen receptor

FAO	fatty acid oxidation
FAT10	HLA-F-adjacent transcript 10
FGF21	fibroblast growth factor 21
G0S2	G0/G1 switch gene 2
G6PC	glucose-6-phosphatase catalytic-subunit
GADD45B	growth arrest DNA damage-inducible gene 45 $\beta$
GCs	glucocorticoids
GDF15	growth differentiation factor 15
GILZ/TSC22D3	leucin zipper
GNMT	glycine N-methyltransferase
GR	glucocorticoid receptor
GRE	glucocorticoid response element
HAO2	hydroxy acid oxidase 2
HCC	hepatocellular carcinoma
HDAC2	histone deacetylase 2
HFD	high-fat diet
HNF4 $\alpha$	hepatocyte nuclear factor-4 alpha
HSP70	heat shock protein 70
HSP90	heat shock protein 90
IACUC	Institutional Animal Care and Use Committee
KLF15	Kruppel-like factor 15
LEPR	leptin receptor
LHet	liver-specific heterozygote

LKO	liver-specific knockout
Lpin1	Lipin-1
LPS	lipopolysaccharide
Mt1	Metallothionein 1
MT1X	metallothionein 1X
mTOR	mammalian target of rapamycin
NAFLD	non-alcoholic fatty liver disease
NAMPT	nicotinamide phosphoribosyl-transferase
NASH	non-alcoholic steatohepatitis
NF- $\kappa$ B	nuclear factor-kappa B
NQO1	quinone oxidoreductase 1
p-4E-BP1	phosphorylation of eIF4E-binding protein 1
p-mTORC1	Phosphorylation of mammalian target of rapamycin complex 1
p-mTORC2	Phosphorylation of mammalian target of rapamycin complex 1
PDK4	pyruvate dehydrogenase kinase 4
Pfkfb3	6-Phosphofructo-2-Kinase/Fructose-2,6-Biphosphatase 3
PFKFB3	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3
PGC1 $\alpha$	peroxisome proliferator activated receptor gamma coactivator 1 $\alpha$
PHH	primary human hepatocytes
RNA-seq	RNA-sequencing
RORA	retinoic acid receptor-related orphan receptor
SCD1	stearoyl-CoA desaturase

SE	standard error
SIRT1	Sirtuin 1
SREBP-1c	sterol regulatory element-binding protein 1
STAT3	signal transducer and activator of transcription 3
SULT1E1	sulfotransferase 1E1
SYVN1	synoviolin
TG	Triglycerides
VEH	vehicle
WT	wildtype

## **Introduction**

According to the World Health Organization: there are about 15 ~ 20 million people worldwide who abuse alcohol, of which 10-20% (1.5 to 4 million) have varying degrees of alcoholic liver disease (ALD). Statistics show that 40% to 90% of the 26,000 people who die from cirrhosis each year are associated with ALD<sup>1</sup>. Alcohol abuse can lead to three kinds of liver injury, and they usually develop in the following order: fatty liver or hepatic steatosis, alcoholic hepatitis (AH), and finally, cirrhosis. Cirrhosis may further progress to hepatocellular carcinoma (HCC). AH patients have severe inflammation and cholestatic liver<sup>2</sup>, and severe AH has a high mortality rate<sup>3</sup>. Corticosteroids (GCs) drug prednisolone is the only available drug therapy for AH<sup>4</sup>, however, it only marginally reduces 28-day mortality without long-term improvement<sup>5</sup>. Dissecting the benefits of GC therapy's detrimental actions is a significant challenge in basic research, drug development, and personalized medicine.

The marked decrease of urea synthesis in AH is associated with the disease severity and hepatic encephalopathy<sup>6,7</sup>. Literature suggests hepatic GR deficiency in NAFLD and AH<sup>8,9</sup>. GR controls the urea cycle in the liver<sup>10</sup>, and prednisolone restores urea synthesis in survivors of severe AH<sup>11</sup>. Additionally, cholestasis correlates highly with malnutrition and the severity of AH<sup>12-15</sup>. GR in hepatocytes is anti-apoptotic and anti-inflammatory<sup>16,17</sup>, and activation of GR protects against cholestatic liver injury and steatohepatitis in patients<sup>18</sup> and mice<sup>19</sup>. For the first time, our data identified a novel critical role of hepatic GR in protecting male mice from AH. All these hepatoprotective and anti-inflammatory effects of GR activation on hepatocytes contribute to the beneficial effects of current GC therapy

in severe AH. Additionally, hepatic GR is essential in protecting against liver failure and mortality in sepsis<sup>20</sup>.

Conversely, GR activation in extrahepatic tissues promotes alcohol consumption and psychiatric problems<sup>21,22</sup>, adipose lipolysis<sup>23</sup>, intestinal bile acid (BA) reabsorption and cholestasis and gastrointestinal bleeding<sup>24,25</sup>, and skeletal muscle wasting<sup>26</sup>. Importantly, GC can exacerbate alcoholic liver injury and delays liver regeneration by inhibiting macrophage-mediated phagocytic and hepatic regenerative functions<sup>27-30</sup>. In contrast, GR deficiency in hepatocytes impairs liver regeneration<sup>31</sup>.

Long-term treatment with GCs can cause a series of adverse effects and is the main reason for limiting AH treatment, the severity of which is proportional to the dose and duration of GC administration<sup>32</sup>. GCs have a negative impact on extrahepatic tissues. GC can cause steroidal diabetes (or exacerbation of existing diabetes), which is caused by GCs that accelerate glucose synthesis in the body and reduce the body's consumption of glucose, resulting in elevated blood glucose<sup>33</sup>. GCs may induce or aggravate gastroduodenal ulcers because GCs can promote the secretion of gastric acid and pepsin, and at the same time destroy the barrier on the stomach wall to resist gastric acid digestion of the stomach wall, resulting in the occurrence or aggravation of ulcers, and in severe cases, gastrointestinal hemorrhage or perforation<sup>34</sup>. Studies have shown that GCs contribute to neuromuscular side effects due to the interaction between GCs and neuromuscular blocking agents. The principle is that a deleterious interaction between GCs and denervation treatment in skeletal muscle is responsible for pre-translational defects, and the resulting selective depletion of myosin heavy chains leads to severe muscle atrophy<sup>35</sup>. Importantly, GC can exacerbate the alcoholic liver injury and delays liver regeneration by inhibiting macrophage-mediated

phagocytic and hepatic regenerative functions<sup>27-30</sup>. The development of these side effects can counteract GC's beneficial effects in AH treatment.

GC diffuses across the cell membrane, where they bind with GR in the cytoplasm. The binding of the GC causes a release of inhibitory proteins such as heat shock protein 90 (HSP90), allowing the GC-bound GR to diffuse across the nuclear membrane where it binds to the glucocorticoid response element (GRE). The GRE is responsible for transcribing anti-inflammatory proteins. Additionally, the binding of GC to GR results in the recruitment of histone deacetylase 2 (HDAC2), which is responsible for deacetylating GR, permitting its binding to nuclear factor-kappa B (NF- $\kappa$ B) and activating protein-1 (AP-1). Upon GR binding, these transcription factors are deactivated, thereby inhibiting the transcription of pro-inflammatory proteins.

E2 signaling is mediated primarily via the nuclear hormone receptors ER $\alpha$  and ER $\beta$ . E2 binding leads to dimerization of ER, then ER translocate into the nucleus and interacts with transcriptional coactivators and/or co-repressor and binds to genomic DNA at specific sequences known as estrogen response elements (EREs) to activate or repress the transcription of specific genes. In female mammals, ER $\alpha$  is highly expressed in the liver, where it acts as a sensor of the nutritional status and adapts the metabolism to the reproductive needs. Impaired ER $\alpha$  function is associated with obesity and metabolic dysfunction in humans and rodents<sup>36</sup>. E2 induces ER $\alpha$  in periportal hepatocytes, ameliorates liver injury, and promotes liver regeneration. Acute binge alcohol studies indicate female protection from acute AH<sup>37-39</sup>. ER $\alpha$ -target genes growth differentiation factor 15 (GDF15) and fibroblast growth factor 21 (FGF21) are involved in ALD. GDF15 maintains mitochondrial function and protects against ALD. FGF21 is a liver-derived

metabolic regulator which protects against NAFLD and ALD by reducing ethanol/sugar intake and promoting fatty acid oxidation (FAO)<sup>40</sup>. Sulfotransferase 1E1 (SULT1E1) is a key enzyme for the inactivation of E2. E2/ER $\alpha$  signaling also inhibits hepatic lipogenesis<sup>41</sup>. Importantly, hepatic ER $\alpha$  levels correlate negatively with ALD severity<sup>42-44</sup>. E2 is largely decreased after menopause which occurs earlier in alcoholic women<sup>45,46</sup>, and menopause increases risks of NAFLD and ALD<sup>47</sup>. ER $\alpha$  also regulates lipid metabolism in males<sup>48,49</sup>. ER $\alpha$  has recently been recognized as a relevant molecular target for the prevention of non-alcoholic fatty liver disease (NAFLD). However, its role in AH remains poorly understood. Previous studies have shown that ER $\alpha$  levels in normal liver do not differ significantly from ER $\alpha$  levels in patients with non-alcoholic cirrhosis, ER $\alpha$  levels are elevated in hepatitis patients who abuse alcohol, and that hepatic ER $\alpha$  levels in alcoholic cirrhotic patients who abstain from alcohol are similar to those in normal liver<sup>50</sup>. In patients with severe AH, ER $\alpha$  levels dropped dramatically<sup>51,52</sup>. This suggests that elevated ER $\alpha$  levels in the early stage of AH may help ameliorate liver injury, whereas down-regulation of ER $\alpha$  in the late stage of AH, particularly in postmenopausal women, may play an important role in the pathogenesis of severe AH in these patients.

Although GCs are the only first-line drugs recommended for the treatment of severe alcoholic hepatitis, the role of hepatocellular GR in alcoholic hepatitis has not yet been studied. Mice with the whole-body knockout of GR are embryonic lethal. Knockout of GR specifically in perinatal hepatocytes, using the *Alfp-cre*, results in the death of ~50% of the knockout mice within 48 h after birth, and the survived knockout mice have postnatal growth retardation<sup>53,54</sup>. GR plays a key role in early-life programming<sup>55-57</sup>. These ~50% survived knockout mice<sup>53,54</sup> most likely already undergo marked genetic reprogramming

to survive the perinatal loss of GR, and thus results from the study of conventional liver-specific GR knockout mice may obscure the true role of GR in adult liver. The purpose of this study was to use our novel model of mice with tamoxifen-inducible liver-specific knockout of GR to determine the effects of hepatic deficiency of GR alcoholic hepatitis induced by high-fat diet (HFD) feeding plus binge alcohol and the alterations of ER $\alpha$ -target genes in AH mice with GR deficiency. Additionally, we characterized the novel liver-targeting GC prodrug developed in our laboratory by comparing the differential effects of our liver-targeting GC prodrug and the parent drug on primary human hepatocytes (PHH) and mouse macrophages.

## **Materials and Methods**

### **Cell culture and drug treatment**

Treatment of PHH with dexamethasone (DEX) and prodrugs: PHH (Liver Tissue Cell Distribution System, Pittsburg) was replaced with RPCD1 medium (without DEX) in the afternoon and cultured overnight. DEX and prodrugs (CA-CB-DEX and DEX at 1  $\mu$ M or 100nM in 50% DMSO) were added in the next morning (final DMSO concentration 0.1%). 0.5 ml of medium in each 6-well plate, and the corresponding 12-well plate was taken 6 h after drug treatment (6 samples per group) for future analysis of LDH and secreted proteins. After taking the 0.5 ml medium, all the medium was removed 6 h after drug treatment, and PHH was stored at -80 C for future analysis.

Comparative study on effects of DEX and bile-acid (BA) conjugates of DEX (DEX-BAs) on macrophage activation by lipopolysaccharide (LPS): Mouse macrophage RAW 264.7 cells were seeded in 12-well plates ( $5 \times 10^5$  cells/well) and incubated at 37 °C for 18 h (overnight). Cells were co-treated with DEX, DEX-COOH, or the three DEX-BA conjugates (100 nM and 1  $\mu$ M) and LPS (200 ng/mL). After 6h incubation periods, cells were collected for total RNA isolation and real-time PCR analysis of TNF and IL1b, normalized to beta-actin.

### **Animals and treatments**

We generated the mice with inducible liver-specific knockout (LKO) of GR by crossing the GR floxed mice (#021021, Jackson Laboratory) with the SA<sup>+CreERT2</sup> mice, which carry a tamoxifen-inducible ER $\alpha$ -fused Cre under the control of an albumin promoter<sup>58,59</sup>. Adult male and female wildtype (WT) (Cre<sup>-/-</sup>), GR liver-specific heterozygote (LHet) (GR fl/+),

Cre/+), and GR LKO (GR fl/fl, Cre/+) mice (in C57BL/6 background) were injected tamoxifen 5 mg/kg once daily for 2 d to activate the Cre-ERT2. Ten days after tamoxifen injection, all mice were fed the HFD (#D12492, Research Diets) for 3 weeks. Three weeks after HFD feeding, mice had intragastric administration of ethanol 5 g/kg (50% 10 ml/kg) or isocaloric maltose (VEH) 9 g/kg (0.45 g/ml 20 ml/kg) in the morning. All mice were sacrificed 9 h after ethanol treatment to collect blood and tissues<sup>60</sup>. Liver tissues were snap-frozen in liquid nitrogen upon collection and stored at  $-80^{\circ}\text{C}$  until use. Mouse blood samples were taken by orbital bleeding and then centrifuged at 8000 rpm for 10 min. The resultant supernatants were collected as serum samples and stored at  $-80^{\circ}\text{C}$  until future use. All animals received humane care and all animal procedures in this study were approved by the Institutional Animal Care and Use Committee (IACUC) of the SUNY Upstate Medical University.

#### **Determination of blood chemistry as well as lipids in mouse liver samples**

Lipids of frozen mouse liver samples were extracted with chloroform: methanol (2:1) and concentrated by vacuum<sup>61</sup>. The lipid pellets were dissolved in a mixture of 270  $\mu\text{l}$  of isopropanol and 30  $\mu\text{l}$  of Triton X-100. Triglycerides (TG) and total cholesterol (CHO) in the liver and serum were determined using commercial triglyceride and cholesterol analytical kits with standards (Pointe Scientific, Canton, MI). Serum alanine aminotransferase (ALT), bilirubin, and albumin were determined using commercial ALT, bilirubin, and albumin analytical kits with standards (Pointe Scientific, Canton, MI).

### **Quantification of serum corticosterone by ELISA**

The DetectX® Corticosterone Enzyme Immunoassay Kit (K014-H1, Arbor Assays LLC, Ann Arbor, MI, USA) was used to determine serum corticosterone levels. Serum samples were prepared and analyzed following the manufacturer's protocol. The online tools from MyAssays provided by Arbor Assays LLC were used to calculate the data.

### **RNA isolation and real-time PCR quantification of mRNA**

Total RNA from liver tissues was extracted using RNA STAT-60 (Tel-Test, Friendswood, TX, USA). According to the manufacturer's instructions, cDNA was produced using a cDNA Synthesis kit (iScript™ cDNA Synthesis Kit, Bio-Rad, Hercules, CA, USA). The diluted cDNA was used for real-time PCR quantification of mRNA using iTaq SYBR® Green Supermix (Bio-Rad, Hercules, CA, USA) and CFX Real-Time PCR Detection System (Bio-Rad). The data were analyzed by CFX Maestro qPCR Analysis Software, and the amounts of mRNA were calculated using the CQ value, normalized to an endogenous reference, phosphoglycerate kinase 1 (Pgk1)<sup>62</sup>.

### **Western blot quantification of liver proteins**

Briefly, the mouse liver was homogenized in hypotonic buffer (20mM HEPES.KOH (pH 7.8), 5 mM potassium acetate, 0.5 mM MgCl<sub>2</sub>, 1X protease inhibitor, and phosphatase inhibitor). After centrifugation at 1500 g for 5 min (4 °C), the supernatant was removed (as cytosol), and the rest of the supernatant and most of the upper lipid layer were removed and discarded. The cytosol was centrifuged again 14000 g at 4 degrees for 5 min to remove additional lipid/nucleus. The nuclei pellets were washed with hypotonic buffer and

centrifuged again at 1500 g, and the resultant pelleted nuclei were incubated at 4 °C for 1 h in RIPA buffer (10 mM Tris-HCL (pH 8.0), 1 mM EDTA, 0.5 mM EGTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, 140 mM NaCl and phosphatase inhibitor). After centrifugation of the nuclear/RIPA samples at maximum speed for 10 min (4 °C), the supernatants were collected as nuclear protein samples.

Western blot quantification of ATP citrate lyase (ACLY), 70-kDa heat shock protein (HSP70), phosphorylated eukaryotic translation initiation factor 4E (eIF4E)-binding protein 1 (4E-BP1) (P-4EBP1), histone deacetylase 6 (HDAC6), HSP90, extracellular signal-regulated kinase 1/2 (ERK1/2), ER $\alpha$ , and GR in liver nuclear and cytosol extracts was carried out with the primary antibodies as follows: ACLY (D1X6P) (#13390), HSP70 (#4872), p-4E-BP1 (Thr37/46) (236B4) (#2855), HDAC6 (D21B10) (#7612), HSP90 (C45G5) (#4877), Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (D13.14.4E) XP® (#4370), and GR (D6H2L) XP® (#12041) from Cell Signaling Technologies (Danvers, MA, USA). The ER $\alpha$  rabbit pAb (A0296) was purchased from Abclonal (Wobum, MA). Primary antibodies were revealed with HRP-conjugated secondary antibodies (anti-rabbit IgG #7074, Cell Signaling) and ECL Western Blotting Substrate (1705061, Bio-Rad). Image Lab software (Bio-Rad) was used to capture signals and determine signal intensities (**Supplement Figure1**).

### **Statistical analysis**

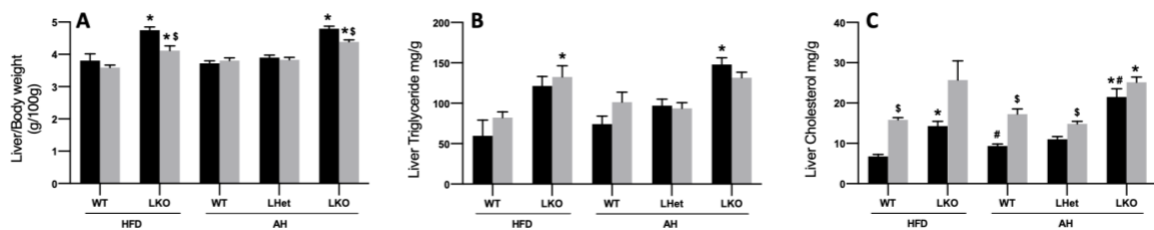
Data are presented as mean  $\pm$  standard error (SE) (pooled samples for female HFD groups in real-time PCR). The student's t-test was used to determine the statistical difference

between WT and KO group, HFD and AH group, as well as the Male and Female group (Prism 8). Statistical significance was set at \*  $p < 0.05$  vs. corresponding WT group; #  $p < 0.05$  vs. corresponding HFD group; \$  $p < 0.05$  vs. corresponding male group.

## Results

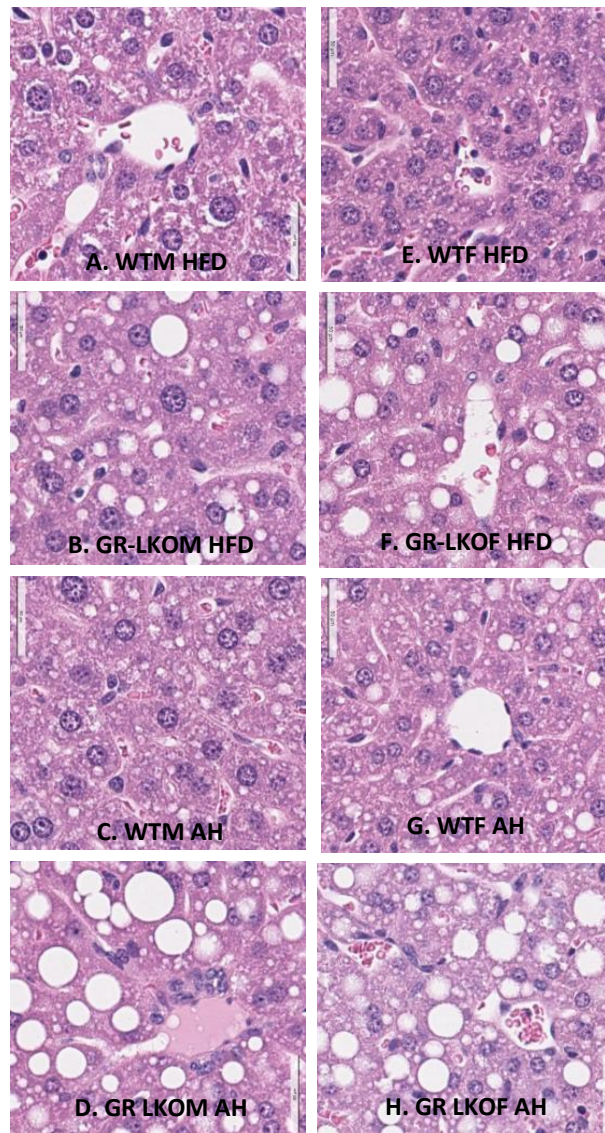
### GR LKO mice had more liver injury than WT mice in AH

We attempted to explore the hepatoprotective mechanisms of GR and the adaptive changes in GR LKO mice. Data showed that LKO of GR increased liver/body weight ratio, and GR-LKO males (P=0.01) had more increase in the liver/body weight ratio than GR-LKO females (P=0.03) (**Fig. 1A**). Both male and female HFD-fed GR-LKO mice had elevated hepatic triglycerides (TG) and cholesterol compared to the HFD-fed WT mice (**Fig. 1B, C**). Thus, hepatic GR deficiency led to the accumulation of TG and cholesterol in the liver, indicating an important role of hepatic GR in protecting against HFD-induced NAFLD. Ethanol treatment caused marked increases in hepatic TG and cholesterol only in WT and GR-LKO male mice but not in females which had higher basal hepatic lipids when fed the HFD but were resistant to the ethanol-induced increase of hepatic lipids and liver injury.



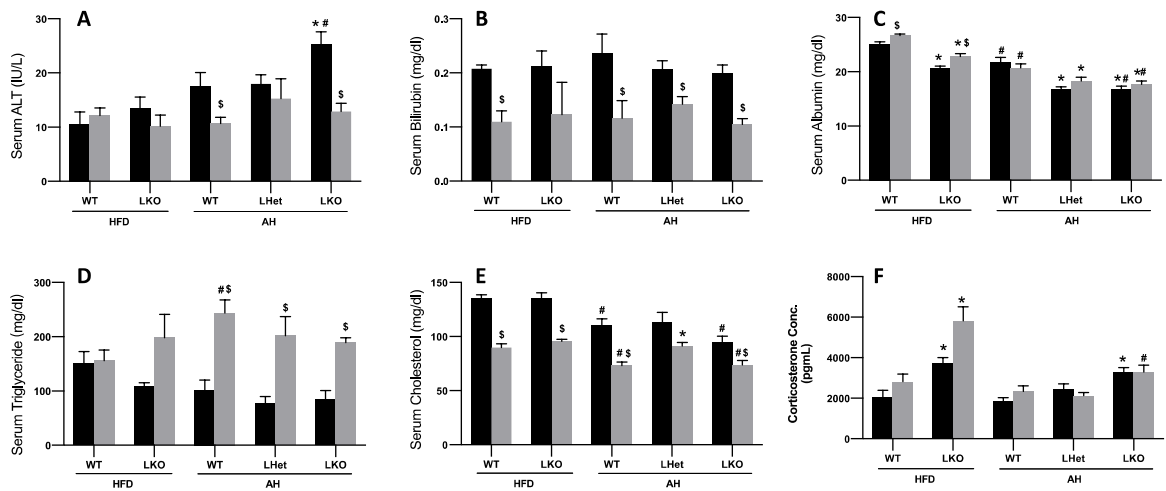
**Figure 1: Liver/body weight ratio (A), hepatic triglycerides (B), and hepatic cholesterol (C) in male and female wildtype (WT), GR liver-specific heterozygous (GR-LHet) and GR liver-specific knockout (GR-LKO) HFD-fed and AH mice. N=3-7 per group, Mean ± SE (pooled samples for F). \* p<0.05 vs corresponding WT group; # p<0.05 vs corresponding HFD group; \$ p<0.05 vs corresponding male group**

Histology analysis (H&E staining) found mainly microvesicular steatosis in WT AH mice (**Fig. 2C, 2G**), whereas massive increases in macrovesicular and microvesicular steatosis were found in both the HFD-fed (**Fig. 2B, 2F**) and AH (**Fig. 2D, 2H**) GR-LKO males and females compared to the corresponding WT males (**Fig. 2C**) and females (**Fig. 2G**). There was no marked difference in histology between the male and female GR-LKO AH mice.



**Figure 2. Histology (H&E staining) of livers in wildtype (WT) and GR liver-specific knockout (LKO) male (top) and female (bottom) HFD-fed and AH mice.**

Consistent with literature<sup>37-39</sup>, we found less liver injury in female AH mice than male AH mice, manifested by the lack of elevation of blood levels of ALT in WT and GR-LKO AH female mice (**Fig. 3A**). Compared to WT HFD-fed mice, ALT tended to be increased in ethanol-treated WT males ( $p = 0.08$ ) and was significantly elevated in GR-LHet and GR-LKO AH male mice. The serum bilirubin levels were not altered by GR deficiency or ethanol treatment, although the bilirubin levels were higher in male mice than in female mice (**Fig. 3B**). Regarding hepatocellular synthetic function, the serum albumin levels were significantly lower in alcohol-treated AH mice. Interestingly, serum albumin levels were diminished in both the GR-LHet and GR-LKO mice compared to WT mice, indicating a critical role of hepatic GR in maintaining blood levels of albumin (**Fig. 3C**). Blood levels of TG were similar in HFD-fed male and female WT mice, whereas blood TG was increased in the AH WT females but tended to be decreased in the AH WT males, resulting in 1.5-fold higher blood TG in the AH WT females than the AH WT males (**Fig. 3D, E**). These suggest that a shift of TG distribution from blood to the liver may contribute to the steatosis in the AH WT males. In contrast to the higher blood TG in the females than the males, blood cholesterol levels were lower in the females than in the males. Besides, serum cholesterol levels were reduced in both ethanol-treated WT and GR-LKO mice, which may contribute to the increases of hepatic lipids in these mice. Similar to a previous study<sup>16</sup>, circulating corticosteroid markedly increased in the HFD-fed GR LKO mice (**Fig. 3F**). Alcohol treatment had no effect on circulating corticosteroids in WT mice but decreased circulating corticosteroids in the GR LKO females.



**Figure 3: serum ALT (A), bilirubin (B), albumin (C), triglyceride (D), cholesterol (E), and corticosteroid (F) in male and female wildtype (WT), GR liver-specific heterozygous (GR-LHet) and GR liver-specific knockout (GR-LKO) HFD-fed and AH mice. N=3-7 per group, Mean  $\pm$  SE (pooled samples for F). \*  $p < 0.05$  vs corresponding WT group; #  $p < 0.05$  vs corresponding HFD group; \$  $p < 0.05$  vs corresponding male group**

### **Changes in hepatic mRNA expression in GR LKO mice**

The present study clearly demonstrates a crucial role of hepatic GR in the prevention of fatty liver induced by HFD and alcohol binge. However, we found only moderately increased liver injury in GR LKO AH mice compared to WT AH mice. Some adaptive protective mechanisms may help ameliorate liver injury in the GR LKO AH mice. Thus, we determined hepatic mRNA expression using RNA-sequencing (RNA-seq) of pooled samples, followed by qPCR verification with individual samples.

Albumin (ALB) is exclusively produced by the liver; Low albumin levels can indicate a problem with the liver or kidneys. Consistent with the serum albumin data, Alb mRNA expression was higher in females than males ( $P < 0.001$ ), Alb expression was decreased in both the GR LHet and LKO mice ( $P < 0.001$ ), indicating a critical role of hepatic GR in maintaining hepatic Alb expression and production (**Fig. 4A**). Hepatic GR deficiency caused gene-dosage-dependent down-regulation of a group of key GR-target cytoprotective genes such as 6-Phosphofructo-2-Kinase/Fructose-2,6-Biphosphatase 3 (Pfkfb3) and Metallothionein 1 (Mt1), which is consistent with the marked down-regulation of these two genes in patients with severe AH (**Fig. 4B, C**). Hepatic expression of the bile-acid synthetic genes cytochrome P450 7b1 (Cyp7b1) and Cyp39a1 were male- and female-predominant, respectively. Cyp7b1 was down-regulated in the male ( $P = 0.002$ ), but not female GR LHet and LKO AH mice, whereas Cyp39a1 was highly gene-dosage-dependently down-regulated in both the male and female GR LHet and LKO AH mice (**Fig. 4D, E**). Activation of sterol regulatory element-binding protein 1 (SREBP-1c) enhances lipid synthesis via induction of critical lipogenic enzymes such as stearyl-CoA desaturase (SCD1) and ATP citrate synthase (ACLY). Unsaturated FAs potently down-regulate

SREBP-1C to feedback inhibit lipogenesis<sup>63</sup>. Consistently, hepatic Srebp-1c was down-regulated in the WT AH mice; however, such feedback down-regulation of Srebp-1c was impaired in the GR LHet and LKO AH mice. The induction of Srebp-1c in the GR LHet and LKO AH mice was associated with the induction of the lipogenic enzymes Scd1 and Acly in these mice (**Fig. 4F-H**).

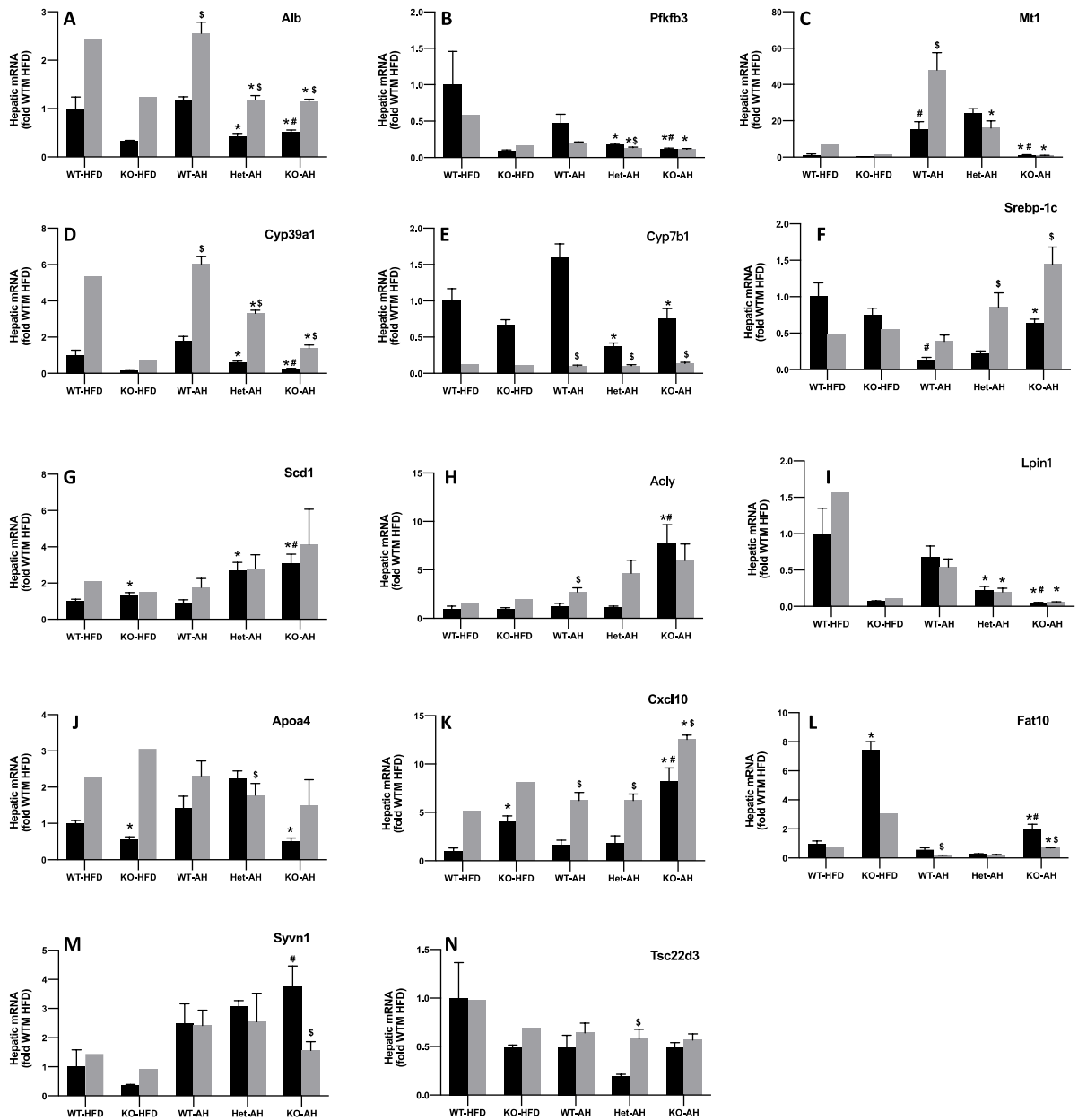
Lipin-1 (Lpin1) deficiency in mice causes lipodystrophy, characterized by impaired adipose tissue development and insulin resistance, and GC is the stimulator of Lpin1 expression in adipocyte differentiation<sup>64</sup>. GR binds to the GRE in the Lpin1 promoter and leads to transcriptional activation of Lpin1 in adipocytes and hepatocytes<sup>64</sup>. Liver-specific lpin1 deficiency exacerbates experimental alcohol-induced steatohepatitis in mice<sup>65</sup>. We found a gene-dosage-dependent down-regulation of Lpin1 in GR LHet and LKO AH mice (**Fig. 4I**).

The apolipoproteins Apo-AI (Apoa4) promotes hepatic secretion and the expansion of larger very-low-density lipoproteins that are thought to have less cardiovascular risk<sup>66</sup>. WT female mice had higher Apoa4 mRNAs than the WT males. Hepatic Apoa4 mRNA was down-regulated in GR-LKO AH males, but little changed in GR-LKO AH females, which may contribute to the lower blood levels of TG but more increases in hepatic lipids in GR-LKO AH males than GR-LKO AH females (**Fig. 4J**).

GC can inhibit the expression of pro-inflammatory factors, such as C-X-C motif chemokine ligand 10 (Cxcl10), a small chemokine<sup>67</sup>. WT females had higher hepatic Cxcl10 expression than the WT males. Cxcl10 was strongly induced in the HFD-fed and AH GR-LKO males but only moderately increased in the GR-LKO AH females (**Fig. 4K**).

HLA-F-adjacent transcript 10 (FAT10), a ubiquitin-like modifier, is implicated as a key contributor to AH<sup>68</sup>. FAT10 mediates the ubiquitin-independent proteasomal degradation and NF- $\kappa$ B activation, and FAT10 is markedly induced in diverse liver diseases. Fat10 has a key role in the regulation of lipid metabolism, manifested by the marked decreased adipogenesis in the Fat10 knockout mice<sup>69</sup>. We found strong induction of Fat10 in HFD-fed GR-LKO mice (**Fig. 4L**). Acute alcohol treatment down-regulates FAT10 in hepatocytes<sup>68</sup>. Likewise, ethanol treatment attenuated hepatic induction of Fat10 in GR-LKO mice. Interestingly, Fat10 expression was much higher in AH males than females, suggesting a role of estrogen in preventing hepatic induction of Fat10. In addition to Fat10, synoviolin (SYVN1) is another E3 ligase that promotes adipogenesis<sup>70</sup>. Moreover, SYVN1 suppresses Nrf2-mediated cellular protection during liver cirrhosis via enhanced Nrf2 ubiquitylation and degradation<sup>71</sup>. Interestingly, Syvn1 tended to be down-regulated in the HFD-fed GR-LKO males but upregulated in the AH GR-LKO males, resulting in a higher expression of Syvn1 in the GR-LKO AH males than GR-LKO AH females (**Fig.4M**). Syvn1 is induced by ER stress<sup>71</sup>. The higher SYVN1 expression in the GR-LKO AH males than females may be due to elevated ER stress in the males. Glucocorticoid-induced leucine zipper (GILZ/TSC22D3) is a prototypical GR-target gene that plays a key role in mediating the anti-inflammatory response of GCs. Gilz was down-regulated by ethanol treatment in the WT AH mice (**Fig. 4N**). Surprisingly, Gilz tended to be further down-regulated in the GR LHet AH males but not in the GR-LKO AH males, suggesting that some compensatory pathway(s) may prevent the further down-regulation of GILZ in the GR-LKO AH mice.

These data strongly support a key role of GR in protecting against fatty liver and liver injury in AH.

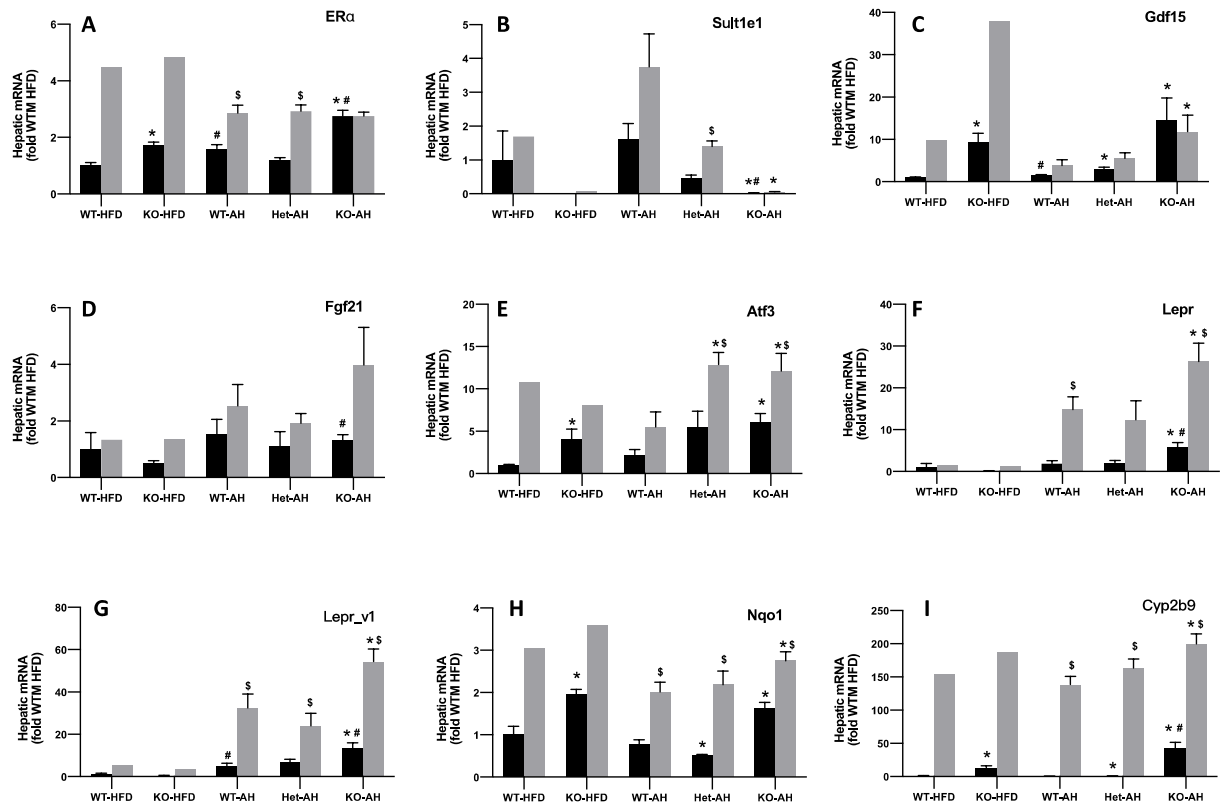


**Figure 4. Hepatic mRNA expression in male and female wildtype (WT), GR liver-specific heterozygous (GR-LHet) and GR liver-specific knockout (GR-LKO) HFD-fed and AH mice.** N=3-7 per group, Mean  $\pm$  SE (pooled samples for F). \*  $p < 0.05$  vs corresponding WT group; #  $p < 0.05$  vs corresponding HFD group; \$  $p < 0.05$  vs corresponding male group.

### **Alteration of hepatic ER $\alpha$ signaling in GR-LKO AH mice**

The lack of marked elevation of blood levels of ALT and bilirubin suggests that certain adaptive changes in GR LKO AH mice may help ameliorate severe liver injury in these mice. Since GR-LKO female AH mice were protected from liver injury, we explored changes in hepatic estrogen signaling. Hepatic ER $\alpha$  was female predominant and induced by GR deficiency and ethanol treatment in males (**Fig. 5A**). In contrast, hepatic expression of the E2-inactivating enzyme Sult1e1 was highly gene-dosage-dependent on GR in both genders (**Fig. 5B**). Our data mining found that human severe AH had induction of GDF15 ( $\uparrow 66\%$ ) but marked down-regulation of both ER $\alpha$  ( $\downarrow 74\%$ ), SULT1E1 ( $\downarrow 76\%$ ), and the ER $\alpha$ -target genes leptin receptor (LEPR) ( $\downarrow 80\%$ ) and activating transcription factor 3 (ATF3) ( $\downarrow 91\%$ )<sup>72,73</sup>. Studies of HFD plus acute binge drinking have shown that females are less prone to AH, likely due to sex differences in E2 signaling. The ER $\alpha$ -target growth differentiation factor 15 (GDF15) maintains mitochondrial function and protects against NAFLD and ALD<sup>74</sup>. We found gene-dosage-dependent induction of Gdf15 in the GR LHet and GR LKO AH mice (**Fig. 5C**). Loss of the ER $\alpha$ -target stress-responsive ATF3 exacerbates liver damage via the deficiency of NRF2/heme oxygenase-1 and activation of mTOR/p70S6K/HIF-1 $\alpha$  signaling pathways in inflammatory liver injury<sup>75,76</sup>. Hepatic Atf3 expression was female-predominant and increased in the GR LHet and LKO AH mice (**Fig. 5E**). Hepatic induction of ATF3 may help ameliorate liver injury in these AH mice. Leptin deficiency enhances the sensitivity of rats to alcoholic steatohepatitis<sup>77</sup>. The ER $\alpha$ -target gene leptin receptor (Lepr) was strongly induced in the female, but not male, WT AH mice (**Fig. 5F, G**). In contrast, Lepr was highly induced in both genders of GR-LKO AH mice.

The active long isoform of the LEPR (Lepr-V1/Ob-RB) was similarly induced like the total Lepr, indicating that the functional Ob-RB accounts for the majority of Lepr induced in the GR LKO mice. The antioxidative enzyme quinone oxidoreductase 1 (NQO1) protects obese mice through improvements in glucose and lipid metabolism. Hepatic expression of the ER $\alpha$ -target Nqo1 in female mice was twice as high as those of male mice (**Fig. 5H**). Nqo1 expression remains unchanged in the WT AH mice but was induced in both genders of GR-LKO AH mice, suggesting that induction of the antioxidative enzyme NQO1 may help ameliorate oxidative liver injury in these mice. CYP2B family enzymes are important for the catabolism of unsaturated fatty acids<sup>78</sup>. Cyp2b9, a well-established female-specific ER $\alpha$ -target gene<sup>79</sup>, was highly induced in the male HFD-fed and AH GR LKO mice (**Fig. 5I**), which may help the clearance of unsaturated fatty acids in these mice. In summary, these data strongly suggest that adaptive induction and activation of ER $\alpha$  and ER $\alpha$ -target genes may play a key role in protecting against liver injury in GR-LKO AH mice and early AH in humans.



**Figure 5. Hepatic mRNA expression of ER $\alpha$  and ER $\alpha$ -target genes in male and female wildtype (WT), GR liver-specific heterozygous (GR-LHet) and GR liver-specific knockout (GR-LKO) HFD-fed and AH mice. N=3-7 per group, Mean  $\pm$  SE (pooled samples for F). \* p<0.05 vs corresponding WT group; # p<0.05 vs corresponding HFD group; \$ p<0.05 vs corresponding male group.**

### **Changes in hepatic protein expression in AH mice**

As expected, hepatic nuclear (**Fig. 7A male; Fig. 11A female**) and cytosolic (**Fig. 9A**) GR proteins were gene-dosage-dependently decreased in GR LHet and LKO AH mice, demonstrating the highly efficient deletion of GR in hepatocytes in our inducible GR LHet and LKO mice. Hepatocyte nuclear factor-4 alpha (HNF4 $\alpha$ ) is a master regulator of liver development and function whose deficiency has been implicated to play a key role in the progression of AH in humans<sup>80</sup>. HNF4 $\alpha$  controls liver metabolism and lipid homeostasis via crosstalk with diverse signaling pathways to regulate hepatic nutrient metabolism<sup>81,82</sup>. Interestingly, HNF4 $\alpha$  protein levels were strongly increased in the nucleus (**Fig. 7B male; Fig. 11A female**) and cytosol (**Fig. 9B**) of WT mice in this acute AH model, whereas the increase of nuclear HNF4 $\alpha$  proteins by acute ethanol treatment was lost in both the GR LHet and LKO AH mice, indicating a critical role of GR in maintaining hepatic protein levels of HNF4 $\alpha$  in AH. Consistent with the induction of *Acly* and down-regulation of *Pfkfb3* mRNAs, nuclear levels of ACLY (**Fig. 7C; Fig. 11C female**) and PFKFB3 (**Fig. 7D**) proteins were elevated and decreased in HFD-fed and AH GR-LKO mice, respectively. Compared to the nucleus, cytosolic levels of ACLY proteins (**Fig. 9D**) increased to a less degree in the GR-LKO AH males, whereas cytosolic PFKFB3 proteins (**Fig. 9D**) were similarly elevated in the WT and GR-LKO AH males. An increase of heat shock protein 70 (HSP70) by E<sub>2</sub> is hepatoprotective during ischemia-reperfusion injury<sup>83</sup>. HSP70 proteins were increased in the nucleus (**Fig. 7E**), but not cytosol of GR-LKO AH mice (**Fig. 9E**), which may help ameliorate liver injury in the male GR-LKO AH mice. In contrast, hepatic expression of HSP90 remained unchanged in all the HFD-fed and AH mice (**Fig. 7F**).

Alcohol causes mammalian target of rapamycin complex 1 (mTORC1) activation, which increases lipogenesis, and decreases autophagy. Phosphorylation of mTORC1 (p-mTORC1) and p-mTORC2 were increased in both the nucleus (**Fig. 7G-I**) and cytosol (**Fig. 9G-I**) of the WT male AH mice. Compared to male WT AH mice, p-mTORC1 and p-mTORC2 in the nucleus, but not the cytosol, of GR-LKO AH mice were decreased. The contribution of the selective inhibition of nuclear mTOR activation to the pathology in the GR-LKO AH mice warrants further investigation. Phosphorylation of eIF4E-binding protein 1 (4E-BP1), a repressor of mRNA translation, by mTOR on Thr-37 and Thr-46 serves as a priming event for subsequent phosphorylation and inactivation of 4E-BP1<sup>84</sup>. Surprisingly, Thr-37 and Thr-46 p-4E-BP1 were increased in GR-LKO AH mice (**(male: Fig. 7J nucleus; Fig. 9I cytosol), (female Fig. 11D)**), despite decreased p-mTORC1 and p-mTORC2 in these mice. In addition to mTORC1/C2, 4E-BP1 can also be phosphorylated by other kinases such as glycogen synthase kinase 3 $\beta$  and p38<sup>85</sup>.

Extracellular signal-regulated kinase (ERK), a type of serine/threonine protein kinase, is a signal transduction protein that transmits mitogen signals. ERK is generally located in the cytoplasm; after activation, ERK enters the nucleus and regulates transcription factor activity and gene expression. In the GR-LKO AH mice, nuclear (**Fig. 7K male; Fig 11E female**) and cytosolic (**Fig. 9J**) p-ERK was increased to less degrees than in the WT AH mice, which indicates that GR regulates hepatic activation of MAPK/ERK signaling pathway. Activation of signal transducer and activator of transcription 3 (STAT3) by interleukin-6 protects against HFD-fed-induced fatty liver and alcoholic hepatitis<sup>86</sup>.

Hepatic protein expression of STAT3 was moderately increased (**Fig. 7L nucleus; 9K cytosol**), whereas p-STAT3, the active form, was dramatically increased by ethanol treatment in the nucleus (**Fig. 7M**) and cytosol (**Fig. 9L**) of WT AH mice. GR deficiency attenuated the increase of hepatic STAT3 phosphorylation by AH, which indicates less liver protective effects by STAT3 in the GR-LKO AH mice. After activation by tumor necrosis factor, nuclear factor kappa B (NF-kB) translocate to the nucleus to promote cytokine production and cell survival. Nuclear NF-kB levels were similarly increased moderately in the WT and GR-LKO AH mice (**Fig. 7N**). This is consistent with the previous study that GR inhibits the transcriptional activity of NF-kB by disturbing the interaction of p65 with the basal transcription machinery without affecting the protein levels of p65<sup>87</sup>.

Consistent with the mRNA results (**Fig. 5A**), in nuclear proteins, the expression of ER $\alpha$  rises in male AH mice (**Fig. 7O**), but the change was less prominent in females (**Fig. 11F**). This may be due to gender differences, with higher hepatic expression content of ER $\alpha$  in females than in males.

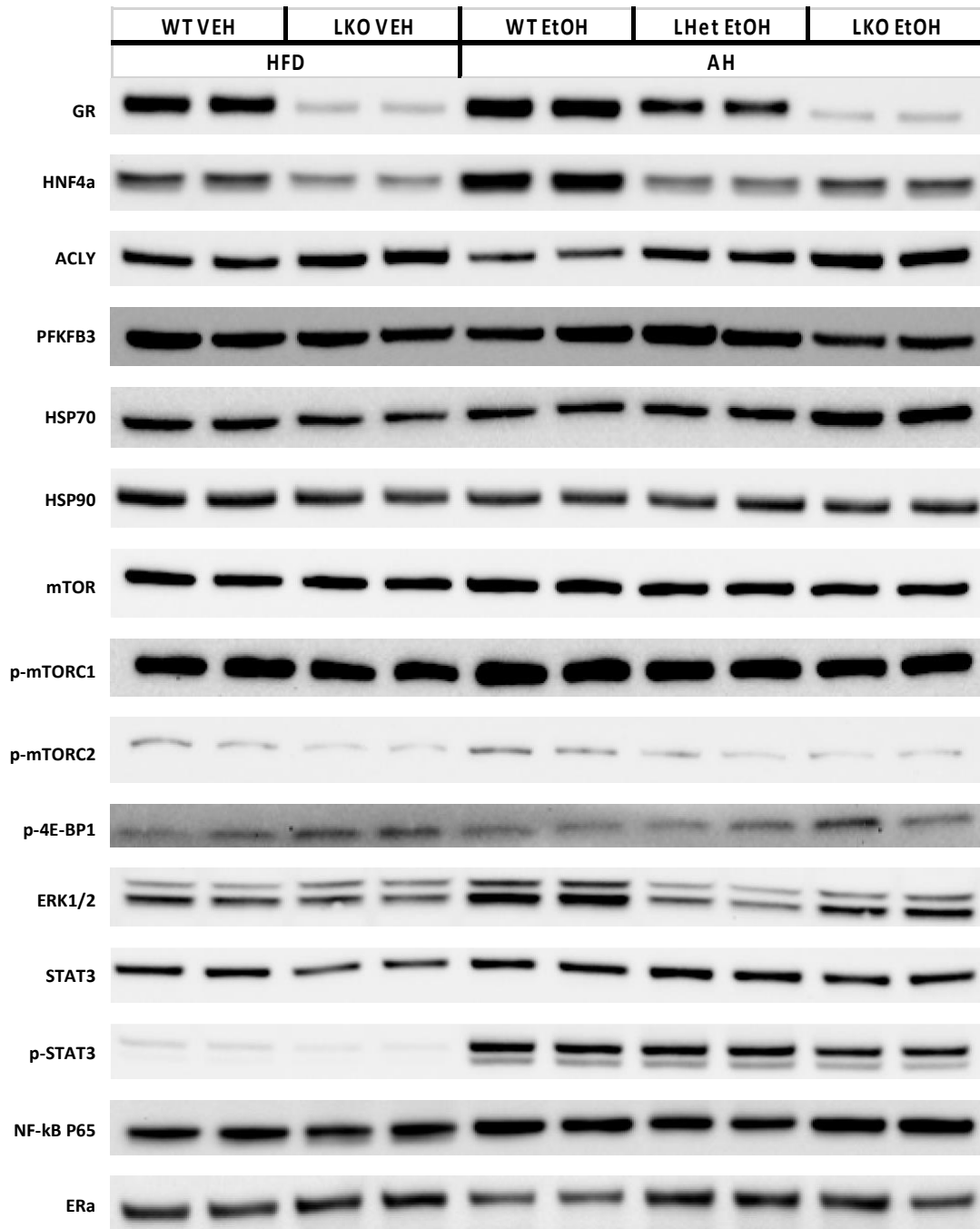
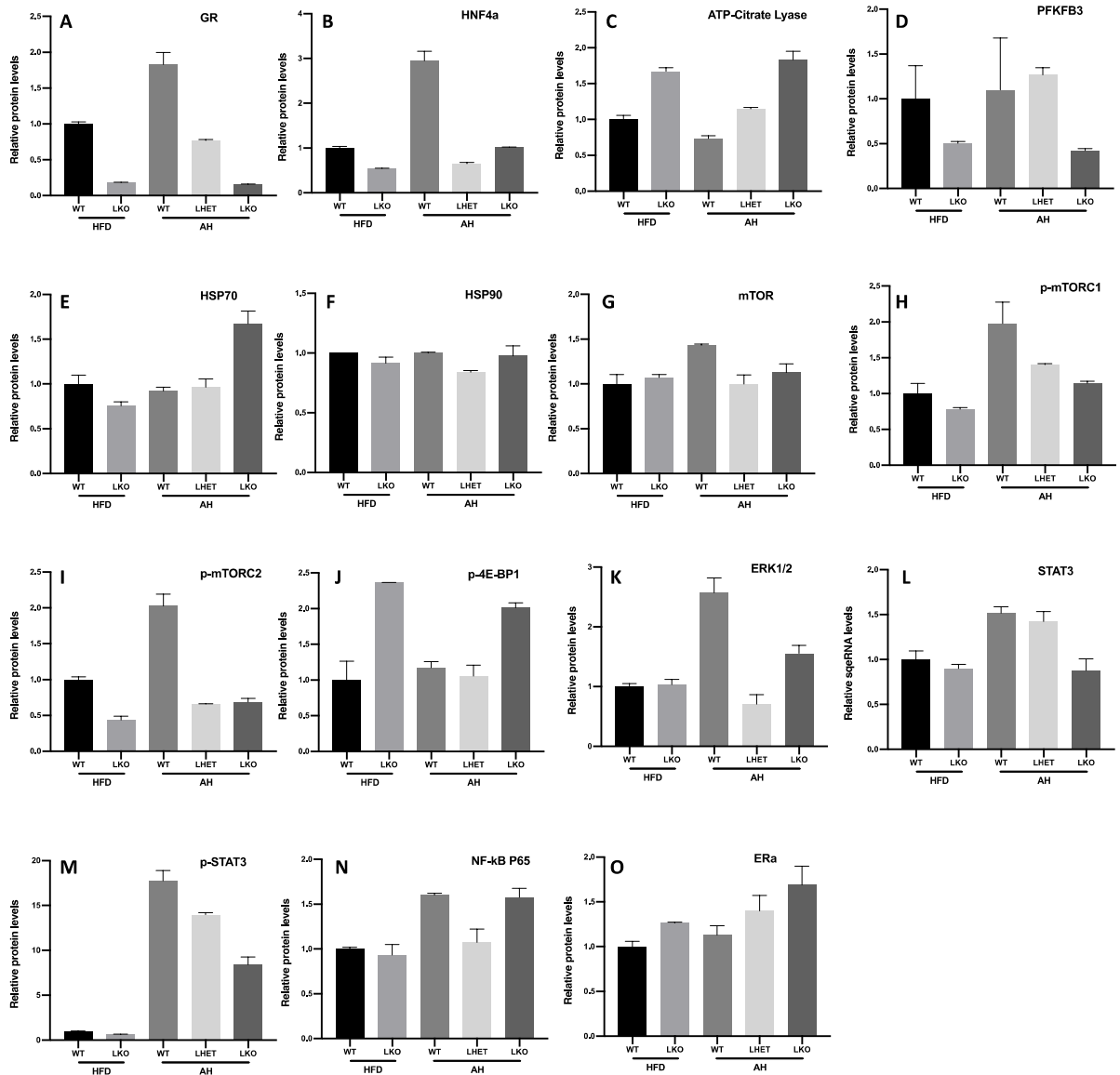


Figure 6. Western blot of proteins in pooled liver nuclear extracts from male wildtype (WT), GR liver-specific heterozygous (GR-LHet) and GR liver-specific knockout (GR-LKO) HFD-fed and AH mice.



**Figure 7. Quantification of Western blot of proteins in pooled liver nuclear extracts from male wildtype (WT), GR liver-specific heterozygous (GR-LHET) and GR liver-specific knockout (GR-LKO) HFD-fed and AH mice.**

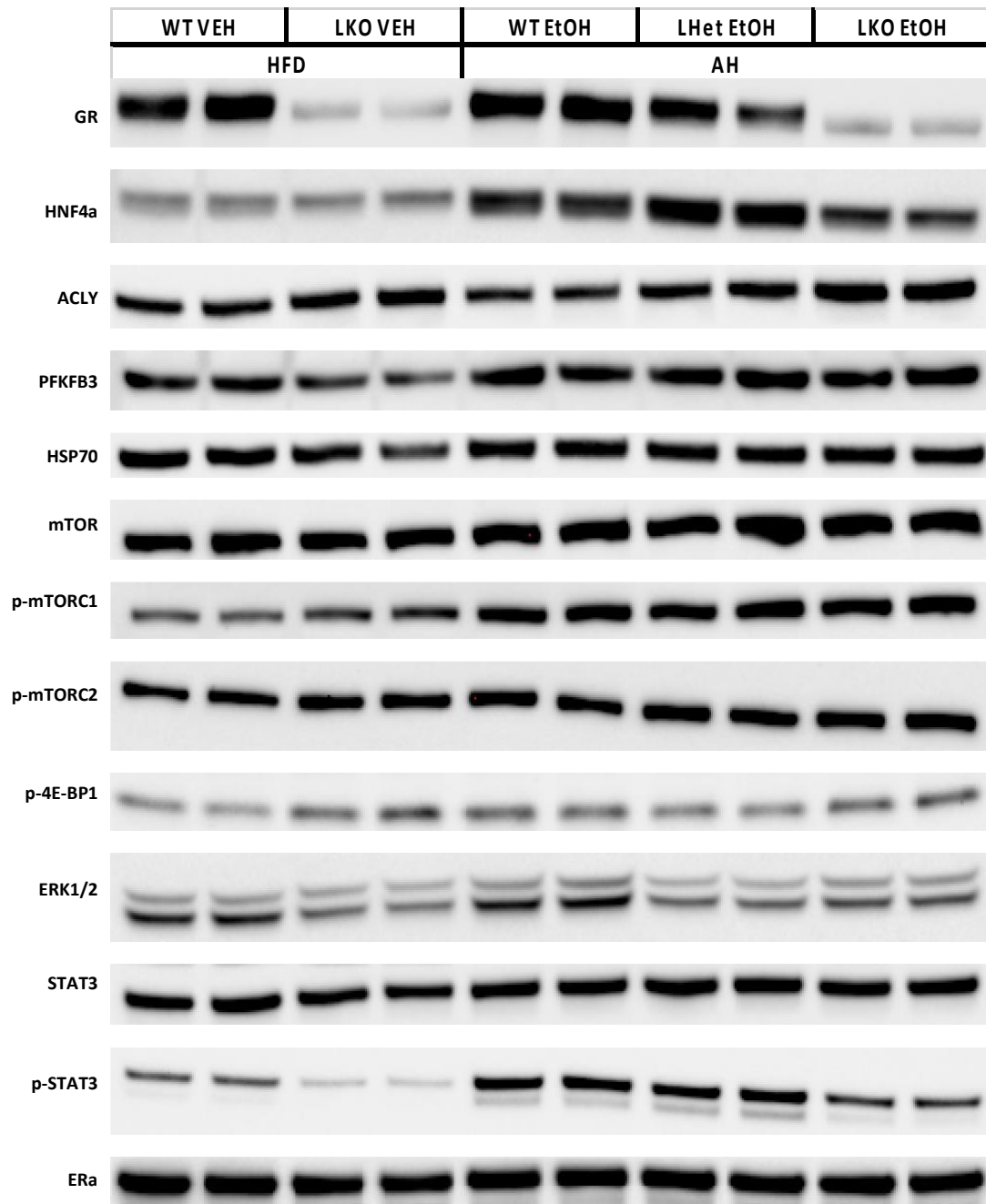
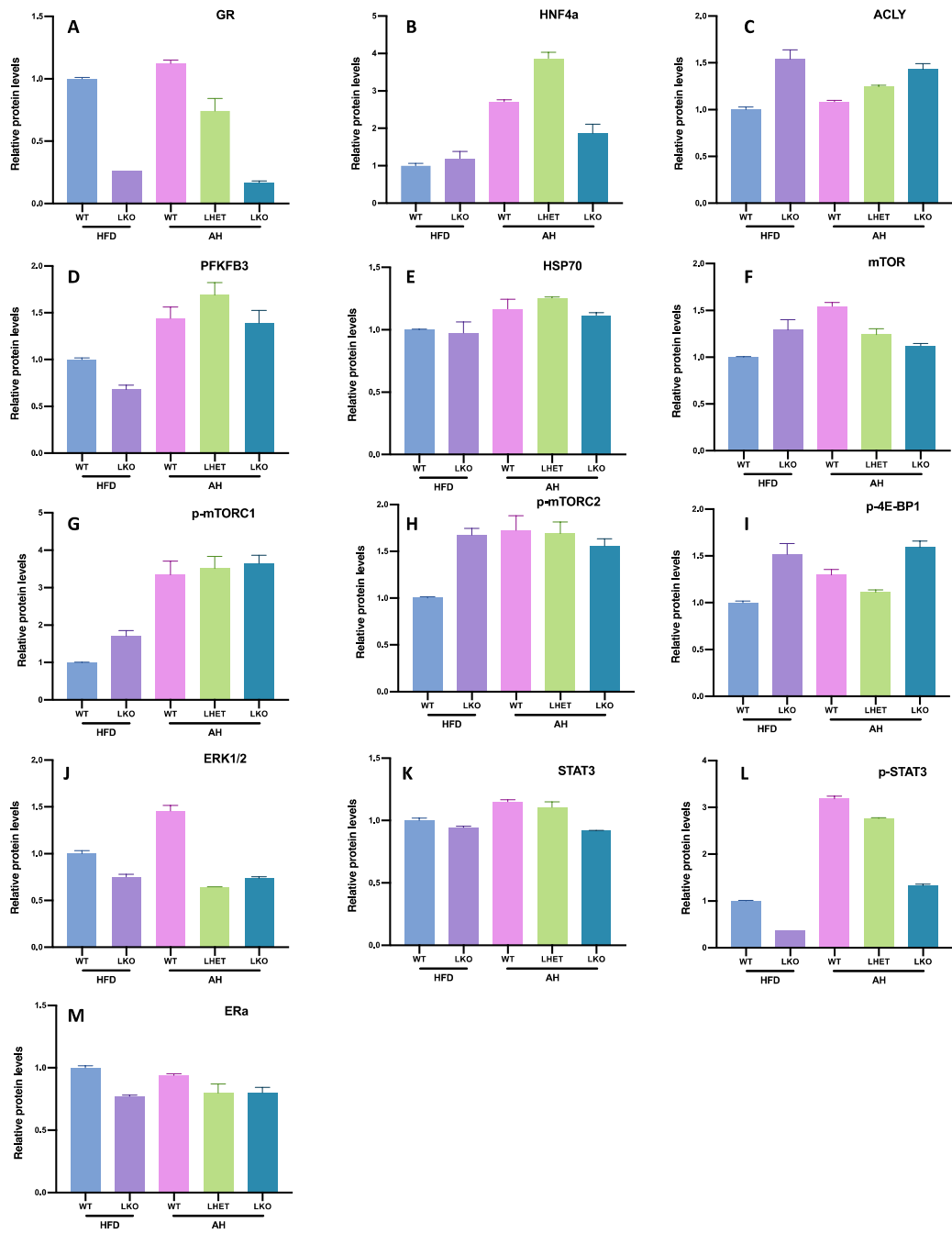


Figure 8. Western blot of proteins in pooled liver cytosol extracts from male wildtype (WT), GR liver-specific heterozygous (GR-LHet) and GR liver-specific knockout (GR-LKO) HFD-fed and AH mice.



**Figure 9. Quantification of Western blot of proteins in pooled liver cytosol extracts from male wildtype (WT), GR liver-specific heterozygous (GR-LHet) and GR liver-specific knockout (GR-LKO) HFD-fed and AH mice.**

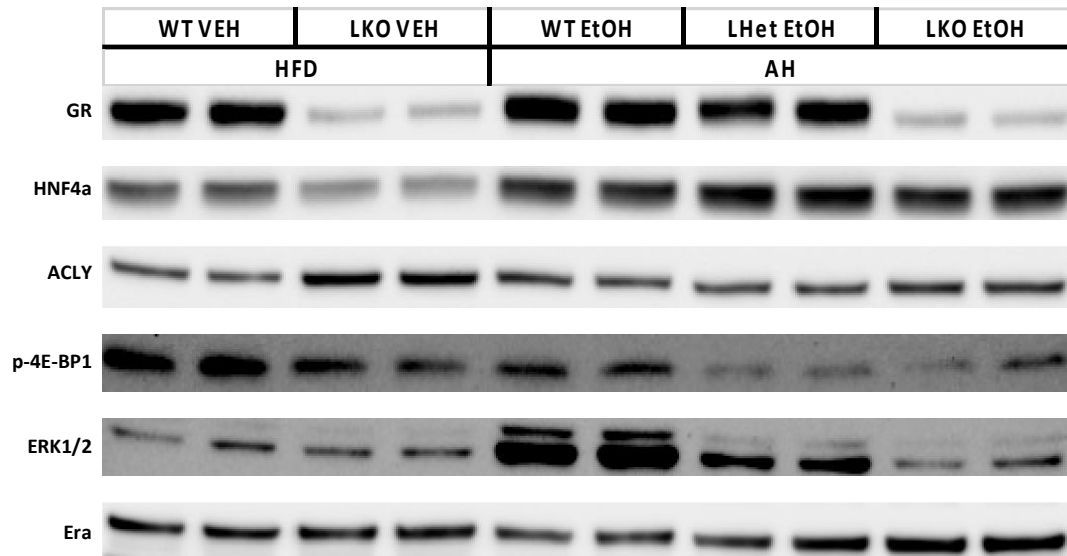
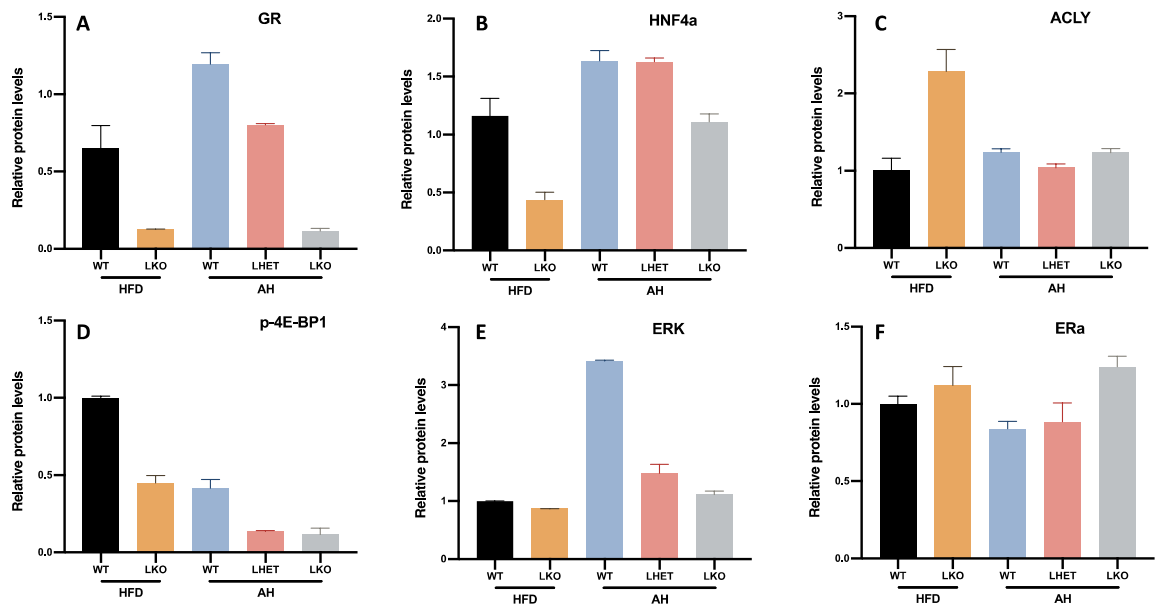


Figure 10. Western blot of proteins in pooled liver nuclear extracts from female wildtype (WT), GR liver-specific heterozygous (GR-LHet) and GR liver-specific knockout (GR-LKO) HFD-fed and AH mice.



**Figure 11. Quantification of Western blot of proteins in pooled liver nuclear extracts from female wildtype (WT), GR liver-specific heterozygous (GR-LHET) and GR liver-specific knockout (GR-LKO) HFD-fed and AH mice.**

### **Down-regulation of known GR-target genes in severe human AH (GSE28619, data mining)<sup>88</sup>**

Compared to normal liver, liver from severe AH (Maddrey's discriminant function >32) had moderate down-regulation of GR and GR-target Na(+)/taurocholate transport protein (NTCP)<sup>89</sup> (**Fig. 12A**), consistent with cholestasis in AH patients<sup>90</sup>. Moreover, severe AH liver had marked down-regulation of known GR-target genes HNF4A (↓69%)<sup>91</sup>, Kruppel-like factor 15 (KLF15, ↓69%)<sup>92</sup>, GILZ (↓87%)<sup>93</sup>, dual specificity protein phosphatase 1 (DUSP1, ↓92%)<sup>94,95</sup>, growth arrest DNA damage-inducible gene 45β (GADD45B, ↓81%)<sup>96</sup>, peroxisome proliferator activated receptor gamma coactivator 1α (PGC1α, ↓65%)<sup>97</sup>, glucose-6-phosphatase catalytic-subunit (G6PC, ↓82%), pyruvate dehydrogenase kinase 4 (PDK4, ↓85%)<sup>98</sup>, glycine N-methyltransferase (GNMT, ↓88%)<sup>99</sup>, and metallothionein 1X (MT1X, ↓60%)<sup>100</sup> (**Fig. 12A**). Hepatic KLF15 enables rapid switch between lipogenesis and gluconeogenesis to ameliorate hyper-triglyceridemia<sup>101</sup>. DUSP1 is essential in protecting against TNFα-induced inflammation in liver<sup>95</sup>. GADD45β is a key hepatoprotective gene by inhibiting the JNK signaling<sup>102-104</sup>. The PGC1α is a master regulator of mitochondria biogenesis<sup>105</sup>. Hepatic deficiency of the key gluconeogenic enzyme G6PC aggravates HFD-induced steatosis and liver injury<sup>106</sup>. Hepatic PDK4 is critical in FAO<sup>107</sup>, and loss of PDK4 switches the hepatic NF-κB pathway from pro-survival to pro-apoptosis<sup>108</sup>. GNMT maintains DNA methylation and protects against steatohepatitis and cholestatic liver injury<sup>109</sup>. Metallothionein protects against NAFLD and ALD by inhibiting oxidative stress<sup>110,111</sup>. Thus, marked down-regulation of these key known GR-target genes likely play important roles in steatohepatitis and cholestatic liver injury in severe AH patients.

### **Dysregulation of novel GR-target genes in severe AH**

We found that severe AH liver had marked dysregulation of a group of novel GR-target genes (**Fig. 12A**), including the retinoic acid receptor-related orphan receptor (RORA, ↓76%), ERBB receptor feedback inhibitor 1 (ERRFI1, ↓80%), 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 (PFKFB3, ↓74%), hydroxy acid oxidase 2 (HAO2, ↓83%), nicotinamide phosphoribosyl-transferase (NAMPT, ↓93%), and G0/G1 switch gene 2 (G0S2, ↑105%). The orphan receptor ROR $\alpha$  protects against NASH by inhibiting lipogenesis and inflammation<sup>112-114</sup>. ERRFI1, a negative EGFR regulator, protects against fatty liver and insulin resistance<sup>115,116</sup>. Increased glycolysis can provide the energy and intermediate metabolites to permit the survival of hypoxic hepatocytes<sup>117</sup>. The rate-limiting glycolytic enzyme PFKFB3 activates the AMP kinase to promote glycolysis and cell survival and inhibit lipogenesis<sup>118,119</sup>. The peroxisomal enzyme HAO2 promotes lipid catabolism to eliminate lipid accumulation<sup>120</sup>. NAMPT is a rate-limiting enzyme for the biosynthesis of NAD<sup>+</sup><sup>121</sup> that is depleted in AH. NAMPT is down-regulated in human AH and ethanol-fed mice, whereas NAMPT overexpression ameliorates alcoholic liver injury by restoring NAD<sup>+</sup> level and activity of Sirtuin 1 (SIRT1)<sup>122</sup>, a key mitochondrial biogenesis regulator that protects against AH<sup>123</sup>. Conversely, G0S2 is a potent inhibitor of lipolysis and lipid droplet degradation<sup>124</sup>. Thus, dysregulation of these novel GR-target genes also plays an important role in steatohepatitis and cholestatic liver injury in AH patients.

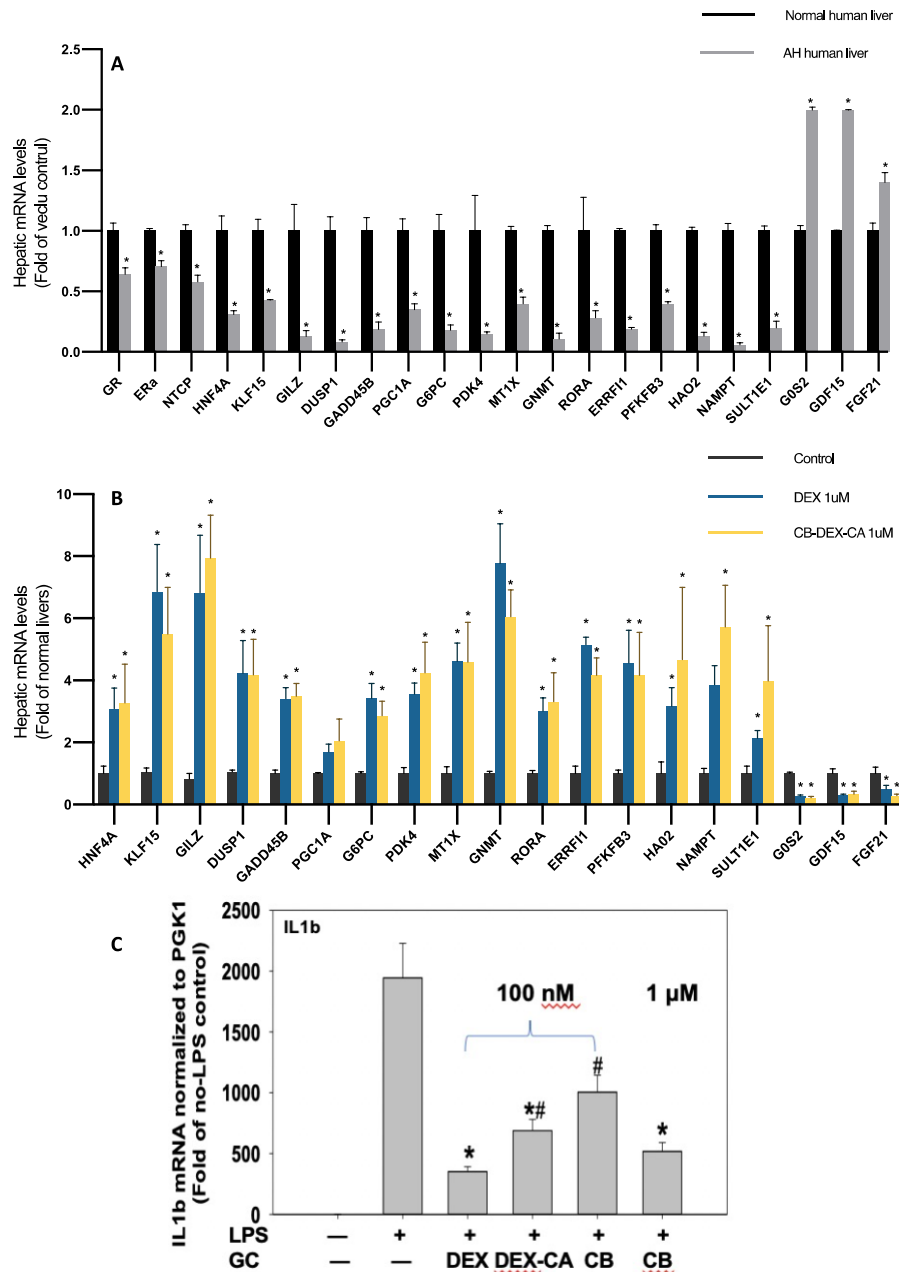
### **Effects of liver-targeting GC prodrugs on hepatocytes and macrophages**

For the GC prodrug, we are collaborating with Dr. Juntao Luo's lab. In contrast to GR, activation of mineralocorticoid receptor (MR) by GCs worsens non-alcoholic steatohepatitis (NASH) and liver fibrosis<sup>125</sup>. Many natural glucocorticoids and synthetic corticosteroid hormones can activate both the GR and the MR, and we want to avoid the deleterious hepatic MR side effects. So, we used dexamethasone (DEX), a highly potent and selective agonist of GR, over MR. We generated the prodrug with DEX joined by a linker to a molecule of cholic acid (CA). DEX alone is hydrophobic, so it is easily absorbed by a variety of cells; by adding a hydrophilic, flexible linker and/or a zwitterionic carboxy betaine (CB), the prodrug becomes hydrophilic to minimize cellular uptake. CA molecule allows the prodrug to be absorbed into hepatocytes by NTCP, a liver-specific bile acid transport protein. This bridge of CA and DEX to generate DEX-CA and CA-CB-DEX through solid-phase peptide chemistry allows the prodrug to be absorbed by the liver but minimizes absorption by other cell types. DEX-CA was moderately water-soluble, whereas the zwitterionic CA-CB-DEX was highly water-soluble. We then studied the regulation of GR-target genes in PHH by GC treatments.

## **Normalization of GR-target genes in primary human hepatocytes (PHH) by GC treatments**

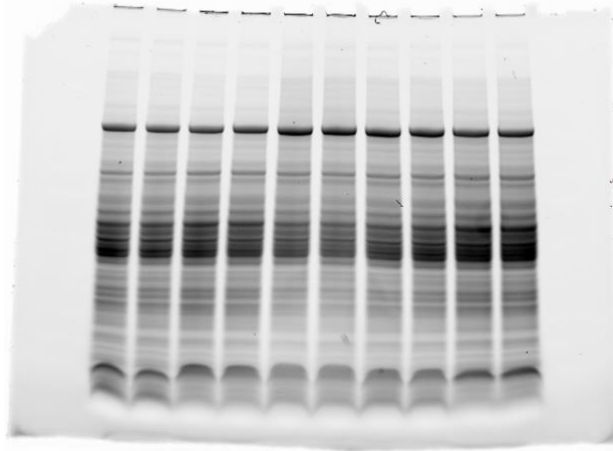
PHH (Liver Tissue Cell Distribution System, Pittsburg) were cultured overnight in the serum-free medium for PHH<sup>126</sup> without GC, and then treated with CA-CB-DEX and DEX at 1  $\mu$ M for 6 h to determine the direct effect of GR activation on transcriptome by RNA-sequencing, followed by qPCR verification (DEX at 1  $\mu$ M does not activate the human pregnane X receptor<sup>127</sup>). Excitingly, we found that treatment of PHH with 1  $\mu$ M DEX and CA-CB-DEX for 6 h caused similarly rapid and marked down-regulation of G0S2 and strong induction of all those known and novel GR-target genes down-regulated in severe AH (**Fig. 12B**). Taken together, hepatic GR signaling is markedly impaired in severe AH patients, and GR in hepatocytes protects against AH via anti-inflammatory, anti-apoptotic, lipid-catabolic, and anti-cholestatic effects. These data strongly support the development of our liver-targeting GC prodrug as a novel improved therapy for severe AH.

To test whether the prodrug was liver-specific and effective, we conducted a comparative study using DEX and DEX-BAs (100 nM any one 1  $\mu$ M) to treat macrophage RAW264.7 cells activated by LPS (200 ng/mL). Compared to Vehicle (VEH), LPS treatment caused a dramatic induction of Interleukin-1-beta (IL1b). DEX at 100 nM largely inhibited IL1b induction by LPS. DEX-CA had weaker, and CA-CB-DEX had much weaker inhibition compared to DEX at 100 nM. Even at 1  $\mu$ M of CA-CB-DEX, its inhibitor effect was still weaker than the parent drug at 100 nM (**Fig. 12C**). In conclusion, our novel highly hydrophilic CA-CB-DEX displayed good selectivity toward hepatocytes over the macrophages which means the design idea of the prodrug is feasible.



**Figure 12. (A)**Microarray analysis of mRNAs in humans with severe AH (normalized to p-actin). Mean  $\pm$  SE, N=7-15. **(B)** qPCR analysis of mRNAs in primary human hepatocytes (PHH) treated with 1 M DEX or CA-CB-DEX for 6 h (normalized to GAPDH). Mean  $\pm$  SE, N=3. \* $p$  < 0.05 vs normal livers (A) or control PHH (B). **(C)** Effects of CA-CB-DEX and DEX on IL1b mRNA in mouse RAW264.7 cells stimulated with lipopolysaccharides (LPS); N=3, mean  $\pm$  SE. \*  $p$  < 0.05 vs LPS, #  $p$  < 0.05 vs DEX;

A



B



**Supplement Figure1. Weston Blot for mouse liver protein.**

All WBs in this experiment used Mini-PROTEAN TGX Stain-Free Precast Gels from Bio-Rad. The advantages of using stain-free gel are obvious.

**A. Stain free gel.** Avoiding the tedious experimental steps, Bio-Rad's Stain-Free™ precast gels are preloaded with tryptophan-binding "trihalo" fluorescent dyes, so they can be imaged within 5 minutes after electrophoresis without staining and with comparable sensitivity to Coomassie Brilliant Blue dyes.

**B. Stain free blot and use Image Lab software to analyze target protein.** Analyze target proteins using total protein normalization. Using Stain-Free total protein measurements as a loading control, we can ensure that the target protein and loading control are measured within a linear dynamic range typical of western blotting experiments, allowing users to obtain truly quantitative western blotting data by normalizing the bands in each lane to the total protein. This eliminates the problems inherent in using housekeeping proteins as controls.

## Discussion

In this study, we find that hepatic GR deficiency worsened steatosis in both genders of AH mice but only aggravated the liver injury in male AH mice. Certain key GR-target genes important for cytoprotectant and lipid metabolism were deregulated in GR LKO AH mice. For the first time, our data identified a novel important role of hepatic GR in protecting male mice from AH. Interestingly, hepatic expression of ER $\alpha$  was induced, and the key E2-inactivating enzyme SULT1E1 was markedly down-regulated in GR knockout AH mice, suggesting enhanced E2 signaling in these mice. Multiple ER $\alpha$ -target cytoprotective genes were induced in GR LKO AH mice, which may help ameliorate liver injury in these mice. The findings of this study will help us determine the mechanistic role of GR in alcoholic and non-alcoholic fatty liver disease and to develop targeted drug therapies to treat alcoholic/non-alcoholic steatohepatitis.

The aggravation of steatosis and liver injury in the GR-LKO AH males clearly indicates an important role of hepatocellular GR in the protection against alcoholic hepatitis and thus strongly supports the current usage of glucocorticoids as the first-line drugs to treat severe alcoholic hepatitis. Our data mining demonstrates the downregulation of GR and key GR-target genes in patients with severe AH. Likewise, certain key GR-target genes were gene-dosage-dependently down-regulated in the GR LHet and GR LKO mice. Changes in GR levels at different stages in AH patients are also a point of interest. GR appears to be activated in the liver of WT AH mice (**Fig.7A, 9A, 11A**) despite the lack of increase in the circulating corticosteroid. In this regard, ethanol treatment has been shown to induce the GR-target gene GILZ in the cultured human lung epithelial cells via increasing nuclear

translocation of GR<sup>128</sup>. Despite activation of GR, hepatic expression of the prototypical GR-target gene GILZ tended to be down-regulated in the WT AH mice. It has been shown that the induction of GILZ by GR requires the transcription factor FOXO3<sup>129</sup>. We found that Foxo3 was down-regulated by ethanol in the WT AH mice, which may contribute to the trend of down-regulation of GILZ in WT AH mice.

Despite the severe steatosis developed in the GR-LKO AH mice, the moderate increase of ALT and lack of hyperbilirubinemia is distinct from those in the patients with severe AH. It is well known that many severe liver diseases require a second hit. Studies of HFD plus acute binge drinking have shown that females are less prone to AH, likely due to sex differences in E2 signaling. Hepatic ER $\alpha$  protects against fatty liver disease and promotes liver regeneration. In the early stage of AH, the hepatic ER $\alpha$  increases, which may help ameliorate AH. E2 is inactivated by hepatic SULT1E1. In addition to being a critical transactivator of SULT1E1, GR inhibits ER $\alpha$ 's transcriptional activity via direct physical interaction. We found certain adaptive changes in the GR-LKO AH mice that may help ameliorate liver injury in these mice, among which the induction of ER $\alpha$  and ER-target genes such as LEPR, ATF3, GDF15, NQO1, and CYP2B9 was the most striking changes adaptive to GR deficiency in these mice. The data suggest that in the early stages of AH, to protect the liver cells, GR and ER $\alpha$  levels rise. However, in the later stages with increases of liver damage, neutrophil infiltration, and fibrosis, GR and ER $\alpha$  contents decrease in severe human AH. The role of hepatocellular ER $\alpha$  in the protection against liver injury in the GR-LKO AH mice warrants further investigation

In this experiment, eight-week-old mice were fed HFD for 3 weeks, followed by binge alcohol to induce AH in these mice. The alteration of liver function in wildtype mice is moderate, and the knockout of GR significantly worsened steatosis in both genders and liver injury in the male mice. However, the severity of AH in these GR-LKO mice was not as bad as in patients with late-stage AH. We think that a major cause of severe AH is aging. Aging humans and rodents have markedly increased alcoholic liver injury and decreased liver regeneration<sup>123</sup>. The majority of severe AH patients are middle-aged (mean age 53 years)<sup>4</sup>. Mice reach postmenopausal at 12 months of age<sup>130</sup>. Thus, 12-month-old mouse liver will be similar to middle-aged human liver. Interestingly, long-term HFD feeding alone causes liver injury in middle-aged mice(12 months)<sup>131</sup>. Thus, we will use the 12-month-old GR-LKO mice in the future study to test the hypothesis that the middle-aged GR-LKO mice will develop more severe AH than the young GR-LKO mice due to the loss of protection by estrogens and other hormones.

Literature shows that GCs partially inhibit the activation of ER $\alpha$ -target genes by E2. E2 induces ER $\alpha$  in periportal hepatocytes, ameliorates liver injury, and promotes liver regeneration<sup>132,133</sup>. We would like to elucidate the major mechanism by which GR inhibits the ER $\alpha$  signaling pathway. We have two hypotheses, namely GR inhibits the ER $\alpha$  signaling pathway mainly via induction of SULT1E1 or via GR- ER $\alpha$  physical interaction. Study shows that GR and ER $\alpha$  physically interact with each other<sup>134</sup>. Importantly, in future clinical applications, if we can activate the ER $\alpha$  downstream genes, it will reduce liver damage and accelerate liver regeneration. ER $\alpha$  expression is regulated by estrogen and is positively correlated with estrogen levels. However, after menopause in women, the

secretion of estrogen decreases dramatically, which will lead to a decrease of ER $\alpha$  in the liver. We plan to use PHH cells to determine the effects of GC-E2 crosstalk on the hepatic transcriptome. If GR mainly inhibits the ER $\alpha$  signaling pathway by inducing SULT1E1, then in E2+DEX+ triclosan (SULT1E1 inhibitor) treatment group, ER $\alpha$ -target genes like GDF15 will have higher level compared to the E2+DEX treatment group. If SULT1E1 induction dominates the GR- ER $\alpha$  interaction, we can consider introducing SULT1E1 inhibitor in the GC treatment of AH. On the other hand, if the GR- ER $\alpha$  physical interaction dominates, we can consider the development of antagonists to interrupt the GR-ER $\alpha$  interaction.

In the future, we plan to determine the role of ER $\alpha$  in AH using ER $\alpha$  LKO and ER $\alpha$  -GR double LKO mice. We will test the hypothesis that hepatic deficiency of both GR and ER $\alpha$  causes severe liver injury in the late stage of AH. Moreover, we will develop liver-targeting E2 prodrugs to improve GC therapy of severe AH. Studies of HFD plus acute binge drinking have shown that females are less prone to AH, likely due to sex differences in E2 signaling. Hepatic ER $\alpha$  protects against fatty liver disease and promotes liver regeneration. Our preliminary data showed that SULT1E1 mRNA was dramatically down-regulated, and specific ER $\alpha$ -target genes were markedly induced in GR LKO mice, suggesting that the activation of ER $\alpha$  helps ameliorate the AH-induced liver injury in GR LKO mice. The liver targeting E2 prodrug will improve GC's long-term efficacy in severe AH.

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