



Orphan nuclear receptor ERR γ regulates hepatic TGF- β 2 expression and fibrogenic response in CCl $_4$ -induced acute liver injury

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Abstract

Acute liver injury results from the complex interactions of various pathological processes. The TGF- β superfamily plays a crucial role in orchestrating fibrogenic response. In contrast to TGF- β 1, a role of TGF- β 2 in hepatic fibrogenic response has not been fully investigated. In this study, we showed that TGF- β 2 gene expression and secretion are induced in the liver of CCl $_4$ (1 ml/kg)-treated WT mice. Studies with hepatocyte specific ERR γ knockout mice or treatment with an ERR γ -specific inverse agonist, GSK5182 (40 mg/kg), indicated that CCl $_4$ -induced hepatic TGF- β 2 production is ERR γ dependent. Moreover, IL6 was found as upstream signal to induce hepatic ERR γ and TGF- β 2 gene expression in CCl $_4$ -mediated acute toxicity model. Over-expression of ERR γ was sufficient to induce hepatic TGF- β 2 expression, whereas ERR γ depletion markedly reduces IL6-induced TGF- β 2 gene expression and secretion in vitro and in vivo. Promoter assays showed that ERR γ directly binds to an ERR response element in the TGF- β 2 promoter to induce TGF- β 2 transcription. Finally, GSK5182 diminished CCl $_4$ -induced fibrogenic response through inhibition of ERR γ -mediated TGF- β 2 production. Taken together, these results firstly demonstrate that ERR γ can regulate the TGF- β 2-mediated fibrogenic response in a mouse model of CCl $_4$ -induced acute liver injury.

Keywords CCl $_4$ · IL6 · TGF- β 2 · ERR γ · GSK5182

Introduction

Liver disease is a global health problem, especially acute liver injury is associated with high mortality. The causes of acute liver injury are known to include all different liver cell types and complex interactions of oxidative stress,

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apoptosis, autophagy and necrosis (Jaeschke et al. 2012; Shi et al. 2017). The mouse model of carbon tetrachloride (CCl₄)-induced acute liver injury is widely used to delineate mechanisms of injury and repair, aiming at identifying potential treatment strategies, since it is comparable to chemical toxicity during acute liver injury in human (Chen et al. 2014; Shi et al. 2017; Torres et al. 2016; Zhang et al. 2017; Zhu et al. 2010). CCl₄, like paracetamol, is metabolized by cytochrome P450 enzymes to form reactive intermediates, such as trichloro methyl-free radicals and peroxy radicals, then initiate lipid peroxidation and cell injury (Slater et al. 1985). Previous studies suggested that CCl₄-injured hepatocytes secrete high mobility group box 1 as damage-associated molecular pattern, which activates hepatic stellate cells (HSCs), among others via autophagy induction (He et al. 2015), therewith initiating a fibrogenic response (Chen et al. 2014). In CCl₄ toxicity, production of several cytokines, including interleukin-6 (IL6), is evident as a trigger of liver regeneration, including extra cellular matrix (ECM) production from activated HSCs (Dai et al. 2018).

The transforming growth factor β (TGF- β) family of multifunctional cytokines comprises three different mammalian isoforms (TGF- β 1 to 3), which function through the same receptor signaling pathways. All three isoforms are expressed in the liver with cell type specific expression patterns, and play key roles in the regulation of various cellular processes like apoptosis, proliferation, differentiation and migration (Bissell et al. 1995; Dropmann et al. 2016; Leask and Abraham 2004; Massagué 1998). Among the TGF- β family ligands, TGF- β 1 has been vastly studied in liver diseases, whereas the role of TGF- β 2 has been less investigated. TGF- β 2 is expressed in different cell types in the liver (De Bleser et al. 1997; Milani et al. 1991), and its dysfunction leads to developmental defects and facilitates fibrotic diseases in mice and human (Coker et al. 1997; Dropmann et al. 2020; Pinzani and Rombouts 2004; Serini and Gabiana 1996). Recently, we showed that TGF- β 2 silencing using antisense oligonucleotides ameliorated hepatic fibrogenesis in multidrug resistance gene 2-deficient mice without adverse effects on healthy livers (Dropmann et al. 2020). Additionally, TGF- β 2 is important for regulation of immune tolerance (de Martin et al. 1987; Maheshwari et al. 2011; Zhang et al. 2008). In this aspect, recombinant TGF- β 2 was found more effective than TGF- β 1 and TGF- β 3 in suppressing the macrophage inflammatory response in neonatal mice with inflammatory bowel necrosis (Maheshwari et al. 2011). Although the transcription factor cyclic AMP-responsive element-binding protein H (CREBH, encoded by Creb3l3) was reported to regulate TGF- β 2 gene expression in hepatitis C virus infected hepatocytes (Chida et al. 2017), further regulatory mechanisms of TGF- β 2 gene expression in liver parenchymal cells, to our knowledge have not been described yet.

Estrogen-related receptors (ERRs) are orphan nuclear receptors composed of ERR α (NR3B1), ERR β (NR3B2) and ERR γ (NR3B3) (Giguère 2008). ERRs bind to classic estrogen response elements (ERE) as dimers, or to extended half-site core sequences (TCAAGGTCA) as monomers (Giguère et al. 1988; Luo et al. 2003; Razzaque et al. 2004). ERR γ , a third member of the ERR subfamily (Audet-Walsh and Giguère 2015; Huss et al. 2015), is found expressed in several organs, especially in key metabolic tissues such as heart, brain, skeletal muscle, adipose tissue and liver (Alaynick et al. 2007; Bookout et al. 2006), and its expression levels show dynamic changes, e.g., by peripheral circadian clock or various stimuli (Jung et al. 2016; Kim et al. 2012; Yang et al. 2006). ERR γ -dependent transactivation of target genes relies on interaction with co-activators or co-repressors, which enhance or inhibit the binding of ERR γ to target gene promoter sequences. Even though ERR γ is constitutively active in the absence of an endogenous ligand, transcriptional activity of ERR γ can be regulated by various synthetic compounds like, 4-hydroxytamoxifen (4-OHT) (Giguère 2008). GSK5182, a 4-OHT analogue, acts as a specific inverse agonist of ERR γ by enhancing the interaction of ERR γ with its co-repressor SHP-interacting leucine zipper protein (SMILE), and thereby inhibits the binding of ERR γ to its target gene promoter (Hentschke et al. 2002; Herzog et al. 2007; Hong et al. 1999). Upon bacterial infections, the pro-inflammatory cytokine IL6 activates the Janus kinase (JAK)/signal transducer and activator of transcription 3 (STAT3) signaling, which increases ERR γ gene expression in hepatocytes. Further, we recently reported that ERR γ mediates IL6-dependent transcriptional regulation of bone morphogenetic protein 6 and fibroblast growth factor 23 in liver (Radhakrishnan et al. 2020, 2021), therewith contributing to the regulation of iron homeostasis and mediating kidney liver communication in acute kidney injury. Besides this, ERR γ is involved in the regulation of glucose- (Kim et al. 2012; Misra et al. 2016), alcohol- (Jung et al. 2020; Kim et al. 2013), cholesterol- and lipid- (Kim et al. 2019) metabolism. However, a potential role of ERR γ upon chemical liver toxicity and its repair response remains to be elucidated.

In this study, we for the first time revealed a role of ERR γ in the hepatic fibrogenic response via upregulating TGF- β 2 gene expression and secretion from hepatocytes in the CCl₄-evoked acute liver injury mouse model.

Materials and methods

Chemicals

GSK5182 was synthesized as described previously (Chao et al. 2006) and used at a concentration of 40 mg/kg for

in vivo experiments and 10 μM for in vitro experiments. Recombinant human IL6 (Cat # cyt-213; Prospec Protein Specialists, East Brunswick, NJ) was used at a concentration of 2.5 $\mu\text{g}/\text{kg}$ for in vivo experiments and 20 ng/ml for in vitro experiments. Recombinant human tumor necrosis factor- α (TNF α) was used at a concentration of 20 ng/ml for in vitro experiments (R&D Systems, Minneapolis, MN, USA). CCl_4 (Sigma-Aldrich Chemical Co., St Louis, MO, USA) was dissolved in corn oil to a 10% solution and injected as 1 ml/kg of body weight for in vivo experiments.

Animal studies

Eight-week-old male C57BL/6 J mice were used for all experiments. C57BL/6 J wild-type (WT) mice were obtained from Korea Research Institute of Biosciences and Biotechnology (KRIBB, Daejeon, Korea). C57BL/6 J mice containing floxed ERR γ exon 2 (ERR γ *f/f*) were obtained from PHENOMIN-iCS, PHENOMIN, the French National Infrastructure in Biology and Health (Illkirch, France). To produce the hepatocyte-specific ERR γ knock-out line (ERR γ -LKO), ERR γ *f/f* animals were crossbred with C57BL/6 J-*Alb*-Cre transgenic mice, which express Cre recombinase in hepatocytes under the control of the albumin promoter (Jackson Laboratories, Bar Harbor, ME, USA) (Radhakrishnan et al. 2020, 2021). Prior to the experiments, mice were acclimatized to a 12 h light/dark cycle at 22 ± 2 °C for 2 weeks with unlimited food and water in a specific pathogen-free facility. WT or ERR γ -LKO mice were treated with a single dose of CCl_4 (10% CCl_4 dissolved in corn oil as 1 ml/kg of body weight) or IL6 (2.5 $\mu\text{g}/\text{kg}$ in PBS) via intraperitoneal injection for the indicated time points. Where indicated, GSK5182 (40 mg/kg in 30% PEG400) was administered intraperitoneally (i.p) into mice. Ad-GFP or Ad-ERR γ were injected into WT mice (3×10^9 PFU/mouse) via the tail vein, and mice were sacrificed 5 days after adenovirus injection. Ad-US and Ad-shERR γ were injected into WT mice (5×10^9 PFU/mouse) via the tail vein for the indicated time points, and IL6 (2.5 $\mu\text{g}/\text{kg}$) was given one time by intraperitoneal injection 3 h before sacrifice. All animal procedures were approved by the Institutional Animal Care and Use Committee of KRIBB (KRIBB-AEC-20135). All animal experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health.

Histopathology and immunohistochemistry

Liver samples were fixed in 10% neutral buffered formalin, embedded in paraffin, cut into 5- μm -thick sections and stained with hematoxylin and eosin (H&E) staining. To detect expression of fibrogenic factor, liver sections were stained with anti-alpha smooth muscle actin (αSMA)

antibody (ab5694; Abcam, Cambridge, MA, USA) and visualized using 3,3'-diaminobenzidine (SK4100; Vector Lab, Burlingame, CA, USA). Images were captured using a light microscope (BX51; Olympus Corporation, Tokyo, Japan).

Blood analysis

Plasma alanine aminotransferase (ALT) and aspartate transaminase (AST) were determined using an automated blood chemistry analyzer (AU480; Beckman Coulter, Krefeld, Germany).

Plasmids and DNA constructs

The promoters of mouse TGF- β 2 (−2904 bp/+93 bp) was cloned into the SacI/BglIII site of the PGL3-basic vector. TGF- β 2 ERR response element mut-Luc (−1686bpTTAGG TCAGC−1676 bp to −763 bp TTAATCAGC−753 bp) was generated using the QuikChange II site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA). FLAG-ERR α , FLAG-ERR β , and FLAG-ERR γ constructs were described previously (Sanyal et al. 2002). All plasmids used were confirmed by complete sequence analysis.

Recombinant adenoviruses

Ad-GFP and Ad-US were described previously (Kim et al. 2012). In brief, the cDNA encoding ERR γ was cloned into a pAd-YC2 shuttle vector, containing a bovine growth hormone polyadenylation signal sequence under the control of the cytomegalovirus (CMV) promoter. Adenovirus short hairpin ERR γ (Ad-shERR γ) was generated with the pAd-easy system. The target sequence of shERR γ is GAACGG ACTGGACTCGCCACCTCTCTA (Jeong et al. 2009). All virus constructs were purified by cesium chloride density gradient ultracentrifugation. Ad-GFP and Ad-FLAG-ERR γ were used to infect cells (1×10^8 PFU) and mice (3×10^9 PFU/mouse). For ERR γ knockdown experiments, cells were infected with Ad-US and Ad-shERR γ (1×10^8 PFU) for 48 h, and mice were infected with Ad-shERR γ (5×10^9 PFU/mouse) for 5 days, before the indicated treatment protocol.

Cell culture and transient transfection assays

Huh7 (human hepatoma cells), 293 T (human embryonic kidney cells) and AML12 (mouse immortalized hepatocytes) cells were obtained as described previously (Radhakrishnan et al. 2020). The cells were maintained in a humidified atmosphere containing 5% CO_2 at 37 °C and used for experiments at 75% confluence. Transient transfections were performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The cells were treated with 10 μM

GSK5182 unless noted otherwise. After 48 h of transfection, the cells were harvested, and luciferase activity was measured and normalized to β -galactosidase activity. All cell culture experiments were performed as three independent biological replicates.

RNA isolation and analysis

Total RNA was isolated using the TRIzol reagent (Invitrogen, CA, USA) according to the manufacturer's instructions, and used as template to synthesize cDNA with TOPscript RT DryMix (dT18 plus; Enzymomics, Daejeon, Korea). Reverse transcription was performed with temperature set as 37 °C for 5 min, 45 °C for 60 min and 95 °C for 5 min on a thermocycler (TaKaRa, Shiga, Japan). cDNAs were analyzed by the Applied Biosystems StepOnePlus real-time PCR system (Applied Biosystems, Foster City, CA, USA) using Power SYBR Green PCR Master Mix (Applied Biosystems). From the obtained CT values, mRNA expressions were calculated by the $2^{-\Delta\Delta CT}$ method. The following primers were used in this study: ERR γ (mouse/human), 5'-AAGATCGACACATTGATTCCAGC-3' (Forward) and 5'-CATGGTTGAACTGAATTCCCAC-3' (Reverse); TGF- β 2 (mouse), 5'-CTTCGACGTGACAGACGCT-3' (Forward) and 5'-GCAGGGCAGTGTAACCTTATT-3' (Reverse); TGF- β 2 (human), 5'-CAGCACACTCGATATGGACCA-3' (Forward) and 5'-CCTCGGGCTCAGGATAGTCT-3' (Reverse); IL6 (mouse), 5'-CTGCAAGAGACTCCATCCAG-3' (Forward) and 5'-AGTGGTATAGACAGGTCTGTTGG-3' (Reverse); α SMA (mouse), 5'-AACGCCTTCCGCTGCC-3' (Forward) and 5'-CGATGCCCGCTGACTCC-3' (Reverse); COL1A1 (mouse), 5'-GCTCCTCTTAGGGGCCACT-3' (Forward) and 5'-CCACGTCTCACCATTTGGG-3' (Reverse); PDGFR (mouse) 5'-TTCAGGAGTGATACCAGCTT-3' (Forward) and 5'-AGGGGGCGTGACTAGG-3' (Reverse); TIMP1 (mouse), 5'-GCAACTCGGACCTGGTCATAA-3' (Forward) and 5'-CGGCCCGTGATGAGAACT-3' (Reverse). Relative target gene expression levels in adenovirus infected mice and cells were normalized to L32 (mouse/human) 5'-TCTGGTGAAGCCCAAGATGG-3' (Forward) and 5'-CTCTGGGTTTCCGCCAGT-3' (Reverse). Relative target gene expression levels in CCl₄-treated mice were normalized to GUSB (mouse) 5'-GGCTGGTGACCTACTGGATTT-3' (Forward) and 5'-GGCACTGGGAACCTGAAGT-3' (Reverse) expression. Relative target gene expression levels in IL6-treated mice and cells were normalized to HPRT1 (human) 5'-CCTGGCGTCGTGATTAGTGAT-3' (Forward) and 5'-AGACGTTTCAGTCCGTCCATAA-3' (Reverse); HPRT1 (mouse) 5'-TCAGTCAACGGGGGACATAAA-3' (Forward) and 5'-GGGGCTGTACTGCTTAACCAG-3' (Reverse) expression.

Western blot analysis

Mouse tissues were lysed with RIPA buffer (Elpis-Biotech, Daejeon, Korea) and subjected to immunoblot analysis as described previously (Lee et al. 2008). Proteins from liver, brain and heart lysates were separated by 10% SDS-PAGE and transferred to nitrocellulose membranes. Membranes were probed with anti- β -actin (AbFrontier, Seoul, Korea; diluted 1:5000) (Kim et al. 2013), anti-ERR γ (Cell Signaling Technology, Danvers, MA, USA; diluted 1:1000) (Jung et al. 2020) and anti- α -tubulin (AbFrontier; diluted 1:5000). Immunoreactive proteins were visualized using an Amersham ECL kit (GE Healthcare, Piscataway, NJ, USA), or using an iBright CL1000 imaging system (Invitrogen) according to the manufacturer's instructions.

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) assays were performed according to the manufacturer's protocol of SimpleChIP Plus Enzymatic Chromatin IP kit (Upstate Biotechnology, Lake Placid, NY, USA). Immunoprecipitation was performed using Primary antibodies used include mouse monoclonal anti-ERR γ (Clone # H6812, Cat # PP-H6812-00; Perseus Proteomics, Tokyo, Japan) or IgG as a negative control. After recovery of DNA, real-time quantitative PCR analysis was performed using primers encompassing the TGF- β 2 promoter region. The primers used for PCR were as follows: TGF- β 2 -2.8 kb/-2.6 kb, 5'-AAGAAAATTTACAAAAGATT-3' (Forward) and 5'-GCTGCAGAATAATCTACTGC-3' (Reverse); and -1.7 kb/-1.5 kb, 5'-TGTGGAATCCCGGAGAACTC-3' (Forward) and 5'-CCCTGAGTCCCTCCTGTGTT-3' (Reverse).

Measurement of TGF- β 2 levels

TGF- β 2 levels from mice plasma and cell culture media were determined using an enzyme-linked immunosorbent assay (ELISA) kit, according to the manufacturer's protocol (MBS703270 for mouse plasma and medium; MyBioSource, San Diego, CA, USA; DB250 for human plasma, R&D Systems) (Takahashi et al. 2019).

Statistical analysis

Data were analyzed with Prism 8 (GraphPad Software, La Jolla, CA, USA) and presented as mean \pm SD. Comparison between two groups was performed using the two-tailed Student's *t*-test, whereas comparison between multiple groups was performed by ordinary one-way ANOVA with

Tukey's multiple comparison test. Differences were considered statistically significant at $p < 0.05$.

Results

CCl₄ toxicity increases ERRγ to regulate TGF-β2 gene expression and secretion in mouse liver

We treated WT mice with CCl₄ for different time periods, as indicated and confirmed acute liver injury, as evidenced by elevated plasma levels of liver damage markers (ALT and AST) and increased parenchymal degeneration in histopathological analysis of liver sections (Supplementary Fig. 1). In CCl₄-injected mouse liver, ERRγ, TGF-β1 and TGF-β2, but not TGF-β3 mRNA levels were increased at

3 h, reaching maximum levels at 6 h (Fig. 1A). Moreover, ERRγ protein levels were induced at 6 h after CCl₄ injection (Fig. 1B), and plasma levels of TGF-β2 were increased at the 3 h time point, reaching maximum levels after 6 h (Fig. 1C). We hypothesized that ERRγ is an upstream regulator of CCl₄-induced TGF-β1 and TGF-β2 expression. Therefore, we generated hepatocyte-specific ERRγ knockout (ERRγ-LKO) mice (Supplementary Fig. 2) (Radhakrishnan et al. 2020), and treated those with CCl₄ for 6 h for a comparative investigation. As expected, ERRγ protein levels were only increased in CCl₄-injected (6 h) WT mice, but not in ERRγ-LKO mice (Fig. 1E). In WT mice, CCl₄ treatment for 6 h increased hepatic TGF-β1 and TGF-β2 mRNA levels, whereas TGF-β2, but not TGF-β1 mRNA levels were significantly reduced in ERRγ-LKO mice (Fig. 1D). TGF-β2 plasma levels were

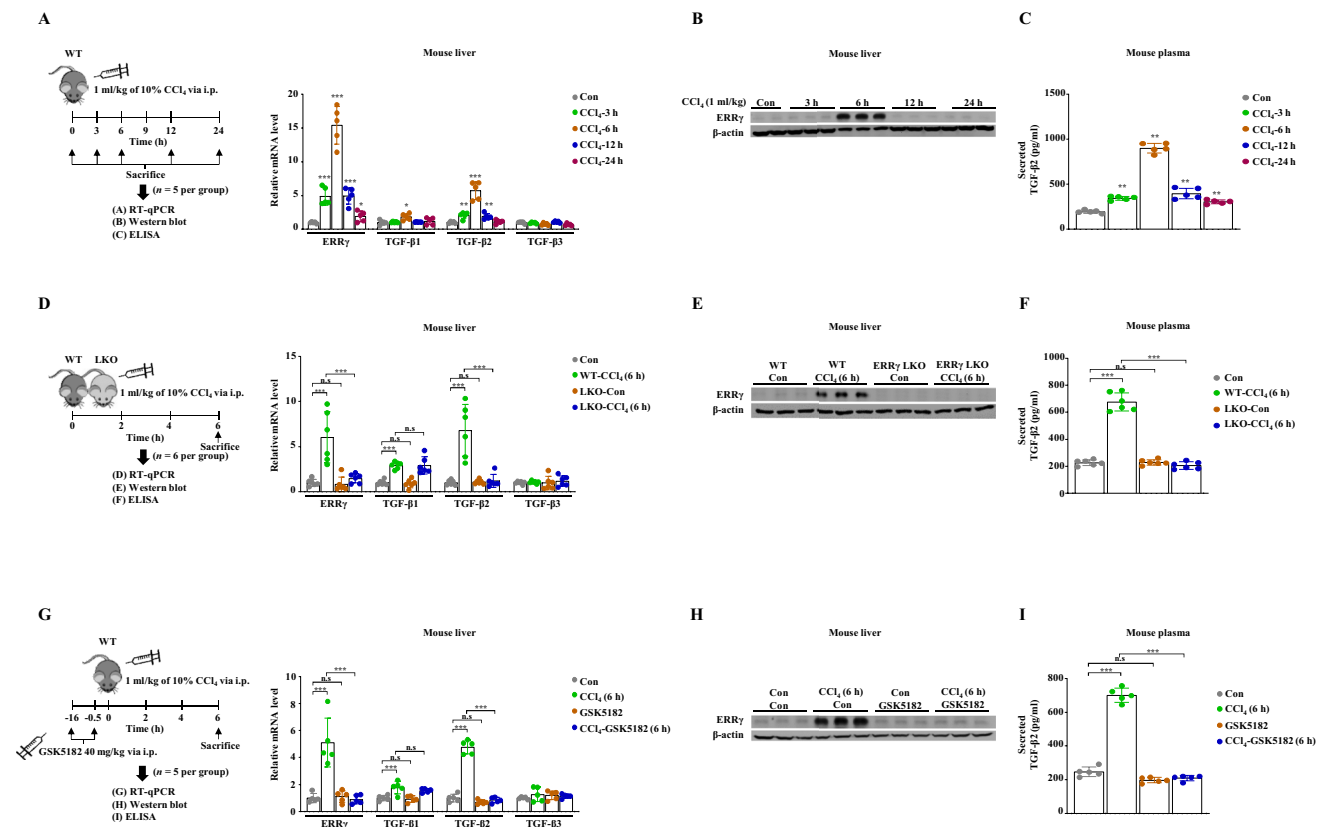


Fig. 1 CCl₄-induced ERRγ regulates TGF-β2 gene expression and secretion. **A–C** WT mice were treated with CCl₄ for different time intervals as indicated. **A** Schematic representation of the treatment protocol (left panel) and RT-qPCR analysis of total RNA isolated from mouse livers (right panel) ($n=5$ per group). **B** Western blot analysis of liver tissues (control $n=2$ and CCl₄ $n=3$). **C** ELISA measurement of plasma TGF-β2 levels ($n=5$ per group). **D–F** WT and ERRγ-LKO mice were treated with CCl₄ and sacrificed after 6 h. **D** Schematic representation of the treatment protocol (left panel) and RT-qPCR analysis of total RNA isolated from mouse livers (right panel) ($n=6$ per group). **E** Western blot analysis of liver tissues ($n=3$ per group). **F** ELISA measurement of plasma TGF-β2 levels

($n=6$ per group). **G–I** WT mice were treated with CCl₄ in presence or absence of GSK5182 and sacrificed after 6 h. **G** Schematic representation of the treatment protocol (left panel) and RT-qPCR analysis of total RNA isolated from mouse livers (right panel) ($n=5$ per group). **H** Western blot analysis of total protein isolated from liver tissues ($n=3$ per group). **I** ELISA measurement of plasma TGF-β2 levels ($n=5$ per group). Data represent mean \pm S.D. Data in A and C were analyzed by two-tailed Student *t*-test as * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ compared with the control group. Data in D, F, G and I were analyzed by ordinary one-way ANOVA with Tukey's multiple comparisons test as *** $p < 0.001$; not significant (n.s)

also significantly reduced after CCl₄ treatment (6 h) in ERR γ -LKO compared to WT mice (Fig. 1F). We further verified the role of ERR γ in CCl₄-mediated hepatic TGF- β 2 gene upregulation using GSK5182, an ERR γ -specific inverse agonist. GSK5182 treatment specifically and significantly inhibited TGF- β 2 mRNA levels and ERR γ protein levels in CCl₄-treated (6 h) mouse liver (Fig. 1G and H). Plasma levels of TGF- β 2 was increased by CCl₄ treatment, whereas significantly inhibited in the GSK5182 administration group (Fig. 1I). These results suggest that ERR γ is the key regulator for TGF- β 2, but not TGF- β 1 or TGF- β 3 gene expression, in CCl₄-damaged mouse livers.

IL6 is an upstream regulator of hepatic ERR γ and TGF- β 2 gene expression

A previous study suggests that IL6 and TNF α levels are increased in CCl₄-induced acute liver injury (Dai et al. 2018), and we previously reported that IL6 can induce ERR γ gene expression in the liver (Kim et al. 2014; Radhakrishnan et al. 2020). Therefore, we hypothesized that IL6 (or TNF α) may be upstream signal to induce hepatic ERR γ and TGF- β 2 gene expression in response to CCl₄ toxicity. To test our hypothesis, we firstly treated AML12 cells in vitro with TNF α for different time points as indicated, and found that ERR γ and TGF- β 2 mRNA levels were unchanged (Fig. 2A), suggesting that TNF α does not modulate ERR γ and TGF- β 2

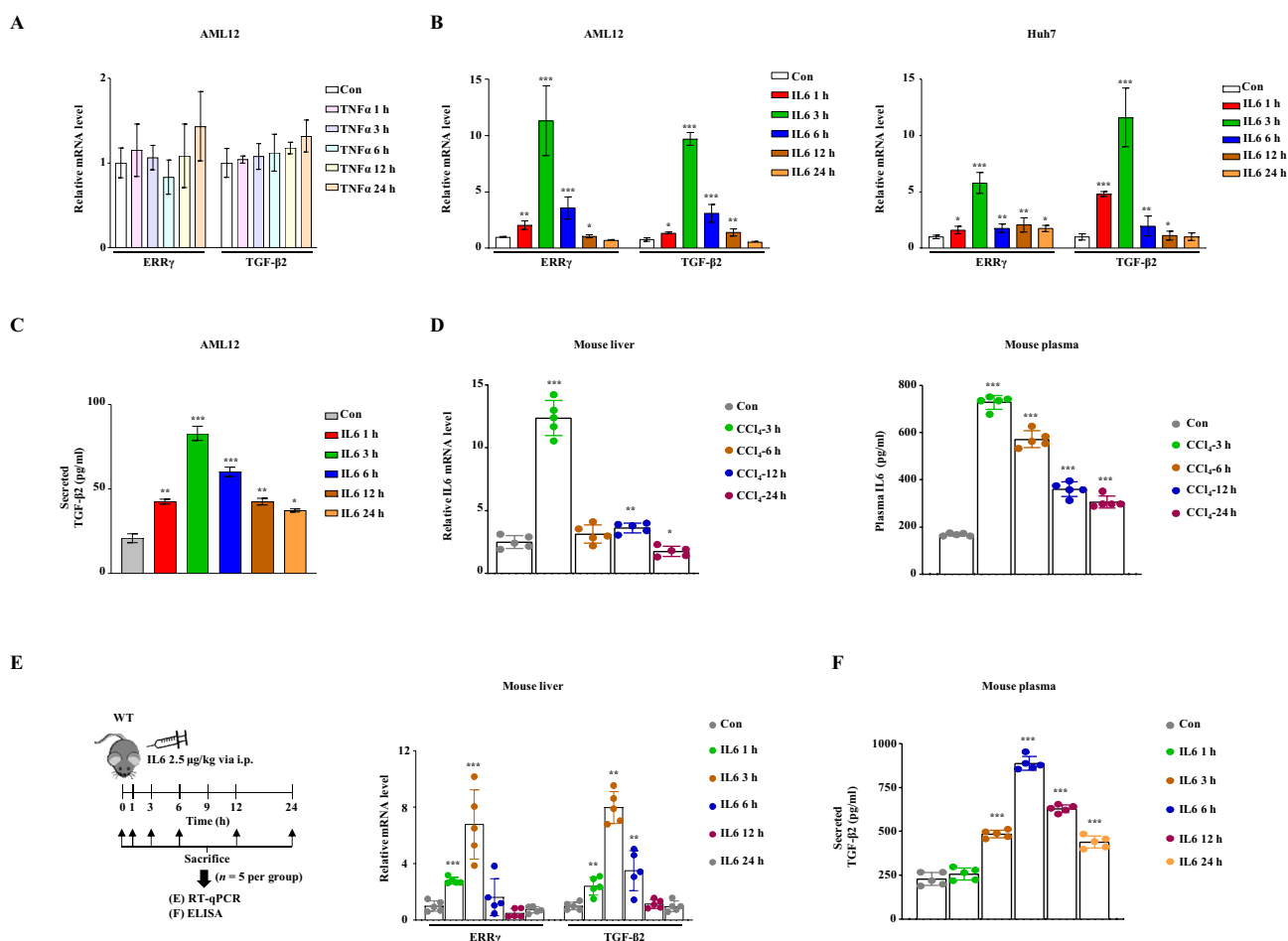


Fig. 2 IL6-induced ERR γ regulates TGF- β 2 gene expression. **A** RT-qPCR analysis of total RNA isolated from AML12 cells treated with TNF α for different time intervals as indicated. **B** RT-qPCR analysis of total RNA isolated from AML12 and Huh7 cells treated with IL6 for different time intervals as indicated. **C** Measurement of TGF- β 2 levels in culture supernatant IL6-treated AML12 cells. **D** RT-qPCR analysis of total RNA isolated from mouse livers (left panel), and measurement of plasma IL6 levels from mice treated with vehicle or CCl₄ for the indicated times (right panel) ($n=5$ per group). **E**,

F WT mice were treated with IL6 for different times as indicated ($n=5$ per group). **E** Schematic representation of the treatment protocol (left panel), and RT-qPCR analysis of total RNA isolated from mouse livers (right panel). **F** ELISA measurement of plasma TGF- β 2 levels. All cell culture experiments were performed as three independent biological replicates. Data represent mean \pm SD. All data were analyzed by two-tailed Student's t -test as * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ compared with control groups

expression in hepatocytes. Next, we treated AML12 cells with recombinant IL6 in a time-dependent manner. Intriguingly, IL6 increased $ERR\gamma$ and TGF- β 2 mRNA levels that both reached maximum levels at 3 h. These data could also be reproduced in Huh7 cells (Fig. 2B). Moreover, TGF- β 2 secretion was significantly increased in AML12 cells, reaching maximum levels after 3 h of IL6 treatment (Fig. 2C). To confirm this result in vivo, we firstly measured mRNA and secretion levels of IL6 in CCl₄-treated WT mice, and found that both, IL6 mRNA and secreted levels were significantly increased and reached maximum levels at 3 h CCl₄ treatment (Fig. 2D). Next, we injected recombinant IL6 for into WT mice and analyzed the mice at different time points as indicated. In accordance with in vitro results, IL6 treatment significantly increased hepatic $ERR\gamma$ and TGF- β 2 mRNA levels at the 1 h time point, reaching maximum levels at

3 h (Fig. 2E). Finally, plasma TGF- β 2 levels were markedly increased 3 h after IL6 injection, and reached maximum levels at 6 h (Fig. 2F). These results indicate that IL6 is the upstream regulator of $ERR\gamma$ induced hepatic TGF- β 2 gene expression and secretion in mice.

$ERR\gamma$ directly regulates TGF- β 2 gene expression

As we found $ERR\gamma$ required for CCl₄-mediated upregulation of hepatic TGF- β 2 gene expression and secretion (Fig. 1), we intended to test whether $ERR\gamma$ is sufficient to increase TGF- β 2 expression. For this purpose, we overexpressed $ERR\gamma$ using adenovirus (Ad- $ERR\gamma$) in AML12 and Huh7 cells, which induces TGF- β 2 mRNA expression in both cell types (Fig. 3A). Upregulated TGF- β 2 secretion was also shown in the supernatant of $ERR\gamma$ overexpressing AML12

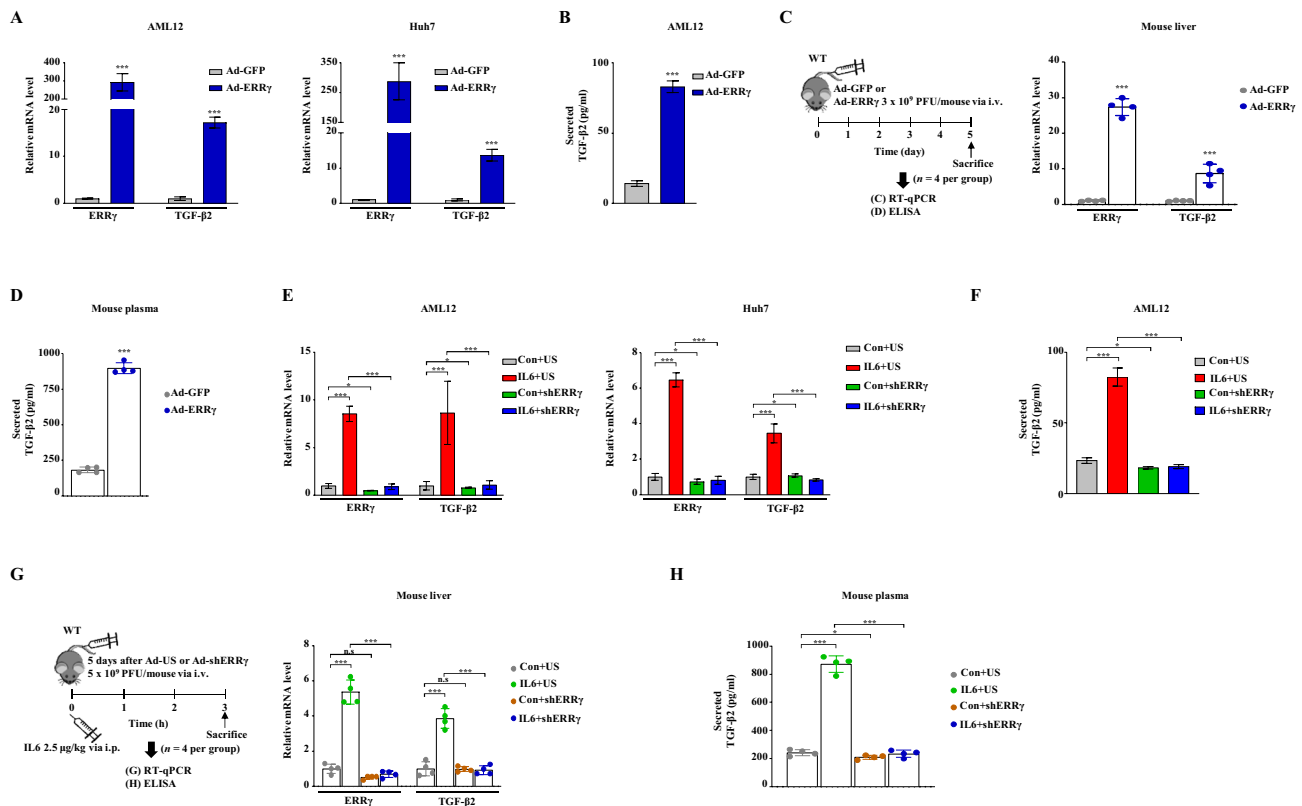


Fig. 3 $ERR\gamma$ directly regulates TGF- β 2 gene expression. **A** RT-qPCR analysis of total RNA isolated from AML12 and Huh7 cells infected with Ad-GFP or Ad- $ERR\gamma$. **B** Measurement of TGF- β 2 levels in Ad-GFP- or Ad- $ERR\gamma$ -infected AML12 cell supernatant. **C**, **D** WT mice were infected with Ad-GFP or Ad- $ERR\gamma$ and sacrificed after 5 days ($n=4$ per group). **C** Schematic representation of the treatment protocol (left panel) and RT-qPCR analysis of total RNA isolated from mouse livers (right panel). **D** ELISA measurement of plasma TGF- β 2 levels. **E** RT-qPCR analysis of total RNA isolated from AML12 and Huh7 cells infected with Ad-US or Ad-sh $ERR\gamma$ for 36 h, and then treated with IL6 for 3 h. **F** Measurement of TGF- β 2 levels in the supernatant of AML12 cells infected with Ad-US or Ad-sh $ERR\gamma$

for 36 h, and then treated with IL6 for 3 h. **G**, **H** WT mice were infected with Ad-US or Ad-sh $ERR\gamma$ for 5 days, and then treated with IL6 for 3 h ($n=4$ per group). **G** Schematic representation of the treatment protocol (left panel), and RT-qPCR analysis of total RNA isolated from mouse livers (right panel). **H** ELISA measurement of plasma TGF- β 2 levels. All cell culture experiments were performed as three independent biological replicates. Data represent mean \pm SD. Data in **A–D** were analyzed by two-tailed Student's *t*-test as *** $p < 0.001$. Data in **E–H** were analyzed by ordinary one-way ANOVA with Tukey's multiple comparison test as * $p < 0.05$; *** $p < 0.001$; not significant (n.s)

cells (Fig. 3B). To confirm this result *in vivo*, we injected Ad-ERR γ via the tail-vein into WT mice and sacrificed these after 5 days. TGF- β 2 mRNA levels were significantly increased in Ad-ERR γ infected mice compared to the Ad-GFP infected control group (Fig. 3C), and ERR γ overexpression also increased plasma TGF- β 2 levels. (Fig. 3D). The participation of ERR γ in IL6-induced hepatic TGF- β 2 mRNA expression and secretion was examined by knockout experiments using adenoviral shERR γ (Ad-shERR γ) infection. IL6 (3 h)-induced TGF- β 2 mRNA levels were significantly reduced in Ad-shERR γ -infected AML12 and Huh7 cells, compared to Ad-US infected cells (Fig. 3E). Moreover, depletion of ERR γ expression in AML12 cells significantly inhibited IL6-induced TGF- β 2, as measured in the supernatant (Fig. 3F). Next, we infected WT mice with Ad-US or Ad-shERR γ for 5 days, and then treated them or not with IL6 for 3 h. IL6-induced hepatic TGF- β 2 mRNA and plasma TGF- β 2 levels were significantly reduced in Ad-shERR γ

infected mice (Fig. 3G and H). These results confirm that (1) overexpression of ERR γ is sufficient to increase hepatic TGF- β 2 gene expression and (2) ERR γ is required for IL6-induced TGF- β 2 gene expression and secretion.

ERR γ activates the TGF- β 2 gene promoter

To explore the molecular mechanism of ERR γ regulated TGF- β 2 gene expression, we performed gene promoter studies after modulating ERR γ expression. Knockdown of ERR γ by Ad-shERR γ infection decreased IL6-induced TGF- β 2 promoter activity in AML12 cells (Fig. 4A). Overexpression of ERR γ , but not ERR α or ERR β , enhanced TGF- β 2 promoter activity in 293 T cells (Fig. 4B). As mentioned above, *in silico* sequence analysis suggested a putative ERR γ binding site (AGGTCA) in the TGF- β 2 gene promoter, which we mutated by site directed mutagenesis. ERR γ overexpression mediated upregulation of TGF- β 2 promoter activity was

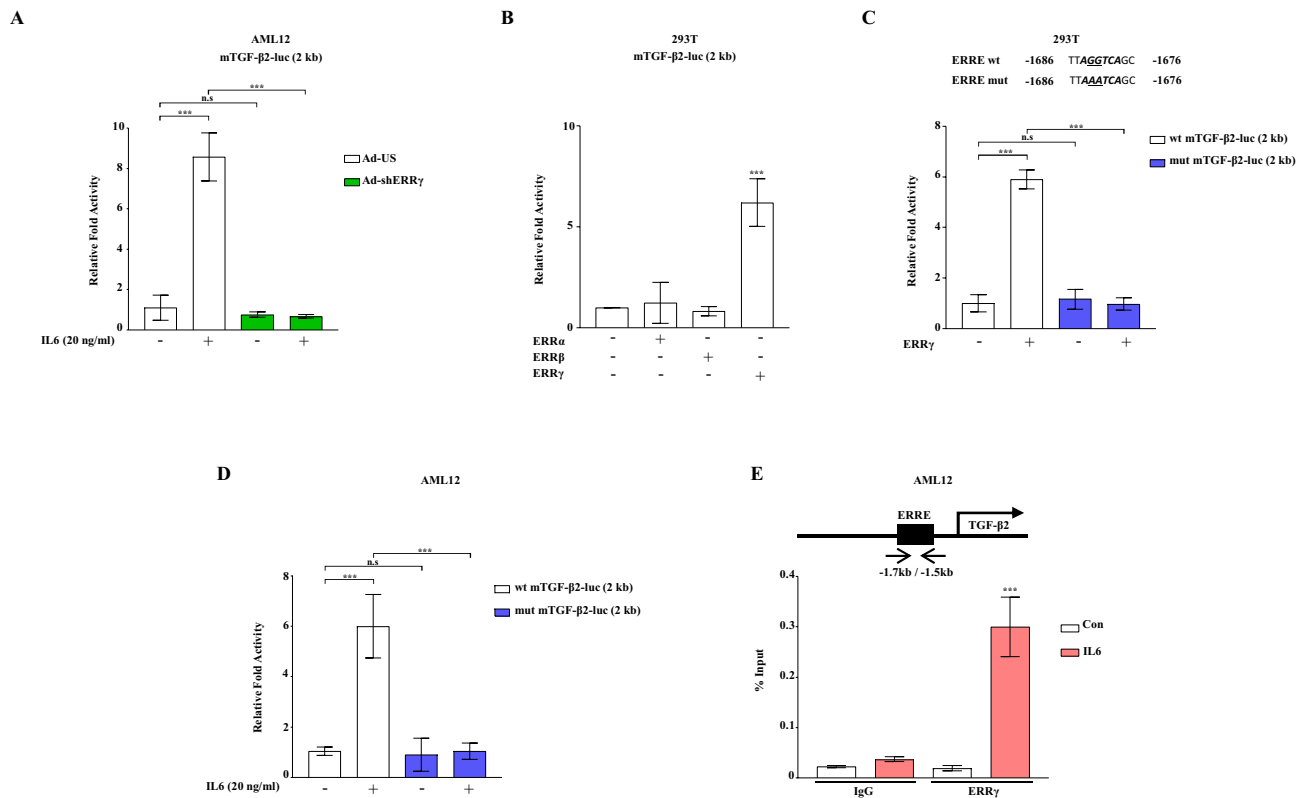


Fig. 4 ERR γ activates mouse TGF- β 2 gene promoter activity. **A** AML12 cells were transfected with a mouse TGF- β 2 promoter luciferase construct and stimulated with IL6 for 3 h. **B** 293 T cells were transfected with the mouse TGF- β 2 promoter luciferase construct, along with expression vectors for ERR α , ERR β or ERR γ . **C** The alignment of potential ERRE sequences in the mouse TGF- β 2 promoter is shown. 293 T cells were co-transfected with vectors expressing WT or ERRE mutant TGF- β 2 promoter luciferase constructs and ERR γ overexpression plasmids. **D** AML12 cells were transfected with WT or ERRE mutant TGF- β 2 promoter luciferase constructs

and stimulated with IL6 for 3 h. **E** ChIP assays, showing binding of ERR γ to the TGF- β 2 gene promoter. AML12 cells were treated with IL6 for 3 h, and soluble chromatin was immunoprecipitated with an ERR γ antibody. Purified DNA samples were used for PCR with primers that are embedding the ERREs in the TGF- β 2 gene promoter (-1.7 kb to -1.5 kb). All cell culture experiments were performed as three independent biological replicates. Data represent mean \pm SD. All data were analyzed by ordinary one-way ANOVA with Tukey's multiple comparison test as *** $p < 0.001$; not significant (n.s)

significantly decreased, when analyzing the construct with the TGF- β 2 promoter ERR γ binding site mutation (Fig. 4C). Moreover, IL6 treatment increased WT TGF- β 2 promoter activity, but failed to activate the ERR γ binding site mutant TGF- β 2 promoter in AML12 cells (Fig. 4D). To confirm binding of ERR γ to the endogenous TGF- β 2 promoter in AML12 cells, we performed ChIP assays. IL6-induced endogenous ERR γ was recruited to the ERR γ consensus binding site in the TGF- β 2 promoter (Fig. 4E). Collectively, these results indicate that ERR γ directly binds and activates the TGF- β 2 promoter in response to IL6.

An inverse agonist of ERR γ abolishes IL6-induced hepatic TGF- β 2 gene expression and protein secretion

GSK5182, an ERR γ -specific inverse agonist, suppresses ERR γ target gene expression by inhibiting ERR γ transactivation (Chao et al. 2006; Kim et al. 2012). In the present study, IL6-mediated TGF- β 2 promoter activity was significantly inhibited by GSK5182 in AML12 cells (Fig. 5A). In line, AML12 and Huh7 cells treated with IL6 in presence or absence of GSK5182, both showed dramatically decreased IL6-stimulated TGF- β 2 mRNA levels in the GSK5182

setting (Fig. 5B). GSK5182 also decreased IL6 mediated TGF- β 2 secretion into the supernatant of AML12 cell (Fig. 5C). To test the effect of GSK5182 in vivo, we treated WT mice with IL6 (3 h) with or without GSK5182 pretreatment. GSK5182 markedly decreased IL6-induced hepatic TGF- β 2 mRNA and plasma TGF- β 2 levels (Fig. 5D and E). These results suggest that inhibition of ERR γ transcriptional activity by GSK5182 administration represses IL6-mediated hepatic TGF- β 2 mRNA expression and plasma TGF- β 2 levels.

Hepatic ERR γ expression is required for the fibrogenic response in CCl₄-induced acute liver injury

We examined the effect of GSK5182 and liver-specific ERR γ knockout on expression of fibrogenic factors in CCl₄-induced acute liver injury. WT or ERR γ -LKO mice were treated with CCl₄ for 72 h, where a maximum fibrogenic tissue response including activation of HSC is evident (data not shown). CCl₄-mediated upregulation of plasma TGF- β 2 levels are significantly decreased in ERR γ -LKO mice, compared to WT mice (Fig. 6A). Gene expression levels of all tested fibrogenic factors (α SMA, COL1a1, PDGFR and TIMP1)

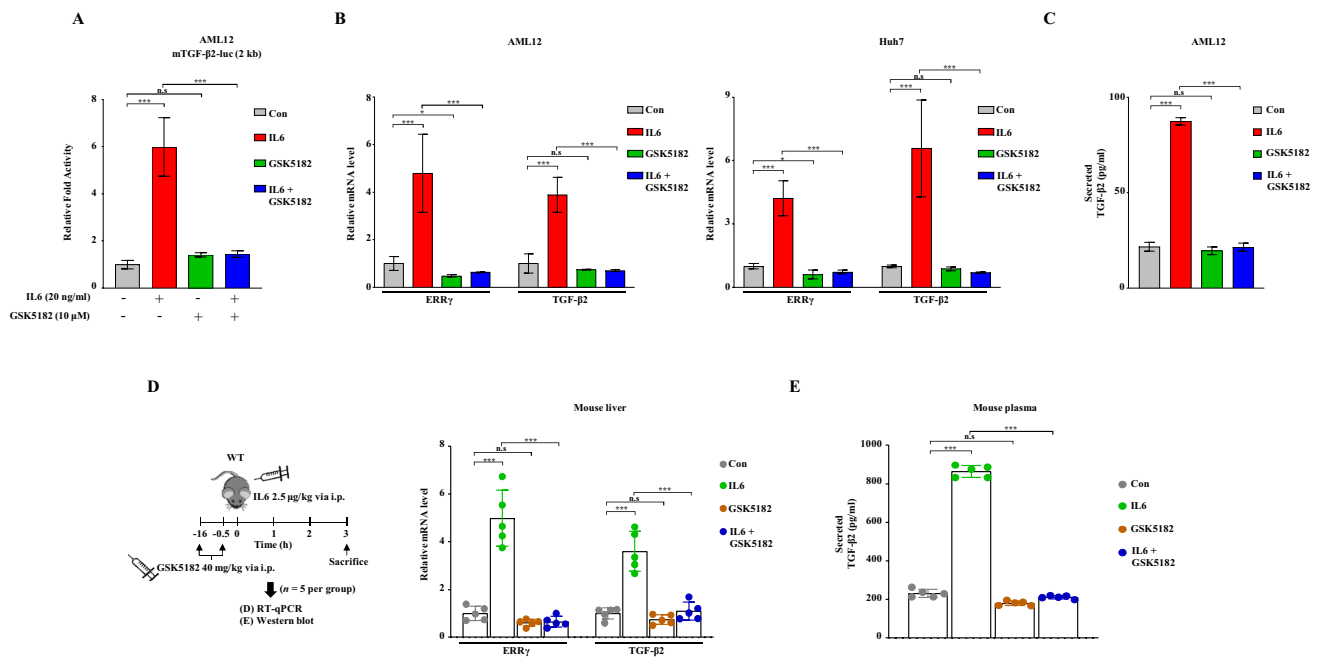


Fig. 5 An inverse agonist of ERR γ inhibits IL6-induced TGF- β 2 gene expression. **A** AML12 cells were transfected with the mouse TGF- β 2 promoter luciferase construct and stimulated with IL6 for 3 h, in presence or absence of GSK5182. **B** RT-qPCR analysis of total RNA isolated from AML12 and Huh7 cells treated with IL6 for 3 h with or without GSK5182. **C** Measurement of TGF- β 2 levels in the supernatant of AML12 cells stimulated with IL6 for 3 h in presence or absence of GSK5182. **D**, **E** WT mice were treated with IL6 in

presence or absence of GSK5182 and sacrificed after 3 h ($n=5$ per groups). **D** Schematic representation of the treatment protocol (left panel), and RT-qPCR analysis of total RNA isolated from mouse livers (right panel). **E** ELISA measurement of plasma TGF- β 2 levels. All cell culture experiments were performed as three independent biological replicates. Data represent mean \pm SD. All data were analyzed by ordinary one-way ANOVA with Tukey's multiple comparison test as * $p < 0.05$; *** $p < 0.001$; not significant (n.s)

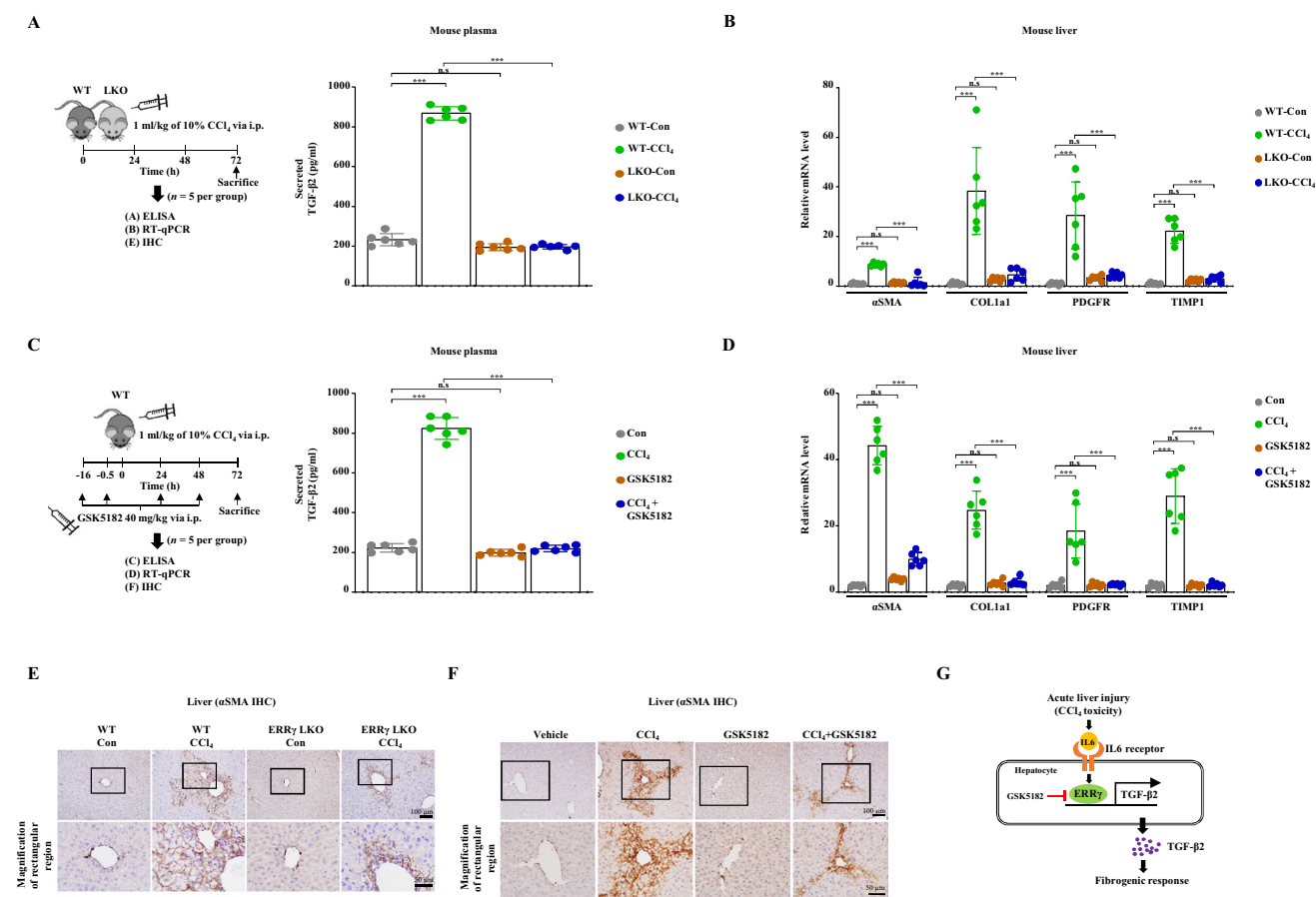


Fig. 6 An inverse agonist of ERR inhibits expression of fibrogenic factors and HSC activation in CCl₄-induced acute liver injury. **A**, **B** and **E** WT and ERR_γ-LKO mice were treated with CCl₄ and sacrificed after 72 h ($n=5$ per groups). **A** Schematic representation of the treatment protocol (left panel) and ELISA measurement of plasma TGF-β2 levels (right panel). **B** RT-qPCR analysis of total RNAs isolated from mouse livers. **C**, **D** and **F** WT mice were treated with CCl₄ in the presence or absence of GSK5182 and sacrificed after 72 h ($n=5$ per groups). **C** Schematic representation of the treatment

protocol (left panel) and ELISA measurement of plasma TGF-β2 levels (right panel). **D** RT-qPCR analysis of total RNAs isolated from mouse livers. **E**, **F** Representative images of αSMA immunohistochemical analysis in liver sections. Data represent mean ± S.D. All data analyzed by one-way ANOVA with Tukey's multiple comparisons test as *** $p < 0.001$; not significant (n.s). **G** Schematic representation of CCl₄-IL6 axis-induced fibrogenic response through ERR_γ regulated TGF-β2 gene upregulation

were also markedly reduced in liver of CCl₄-injected ERR_γ-LKO mice compared to WT mice (Fig. 6B). Consistent with mRNA levels, αSMA protein levels were reduced as a result of the hepatocyte-specific ERR_γ knockout (Fig. 6E). Next, we treated WT mice with CCl₄ for 72 h in presence or absence of GSK5182. Plasma TGF-β2 levels are increased after 72 h of CCl₄ intoxication, whereas GSK5182 treatment effectively inhibited CCl₄-mediated upregulation of TGF-β2 plasma levels (Fig. 6C). Elevated mRNA levels of fibrogenic factors (αSMA, COL1a1, PDGFR and TIMP1) after CCl₄ administration were also significantly reduced upon GSK5182 treatment (Fig. 6D). In line with gene expression results, immunohistochemistry of CCl₄-treated mouse liver sections shows that αSMA expression was drastically reduced in the GSK5182 co-treatment group compared to the CCl₄ only group, indicating a significant reducing effect

towards HSC activation (Fig. 6F). These results propose that ERR_γ, besides other signals, is a modulator of the fibrogenic response by inducing TGF-β2 expression and secretion from hepatocytes upon CCl₄-acute toxicity in mice.

Discussion

As a results of this study, we suggest that ERR_γ mediated induction of hepatic TGF-β2 gene expression contributes to the fibrogenic response in the CCl₄-mediated acute liver injury in mice. We have evidence from effects of GSK5182, a selective ERR_γ inverse agonist, and from data obtained in hepatocyte specific ERR_γ knockout mice, which both lead to attenuated TGF-β2 gene expression and fibrogenic response in CCl₄-treated mice.

Previous reports suggest that $ERR\gamma$ gene expression is increased by fasting (via glucagon receptor) (Kim et al. 2012), upon alcohol consumption (via endocannabinoid 1receptor) (Kim et al. 2013), during inflammation (via IL6 receptor) (Kim et al. 2014). We previously reported that $ERR\gamma$ upregulates the transcription of a number of target genes by direct binding at ERRE regions and thereby plays an important role in various biological processes, like glucose (Kim et al. 2012) and lipid metabolism (Kim et al. 2019) or iron homeostasis (Kim et al. 2014). Studies with knockout mice showed that the general depletion leads to lethality during the first week of life (Alaynick et al. 2007). To overcome this dramatic phenotype, we generated hepatocyte specific $ERR\gamma$ -deficient mice to perform depletion studies and found that $ERR\gamma$ is a key mediator for IL6-induced hepatic BMP6 gene expression (Radhakrishnan et al. 2020). In this study, we also used hepatocyte-specific $ERR\gamma$ -deficient mice to examine the role of $ERR\gamma$ in regulating CCl_4 -induced hepatic TGF- β 2 expression.

Previous studies dealing with TGF- β 2 regulation suggest that TGF- β 2 gene expression is induced by cyclic AMP in the human androgen-independent prostate carcinoma cell line PC-3 (Bang et al. 1992), or by CREBH in hepatitis C virus infected mouse liver (Chida et al. 2017). Moreover, miR-30b modulates TGF- β 2 production via regulating the activating transcription factor (ATF) 2 activity (Howe et al. 2017). However, other membrane receptor signaling pathways regulating hepatic TGF- β 2 gene expression remain elusive. In this study, we provide evidence for the transcriptional upregulation of hepatic TGF- β 2 gene expression through $ERR\gamma$ by direct binding at the ERRE motif in the TGF- β 2 gene promoter. Indeed, TGF- β 2 gene expression is upregulated in various pathological conditions, including developmental defects and fibrotic diseases in mice (Coker et al. 1997; Pinzani and Rombouts 2004; Serini and Gabbiana 1996). We have recently shown that TGF- β 2 is the predominant driver of liver fibrogenesis in biliary liver disease (Dropmann et al. 2020). Here, we show that $ERR\gamma$ upregulates hepatic TGF- β 2 gene expression and secretion, as well as increases the expression of fibrogenic factors in CCl_4 -treated mice. It is known that in CCl_4 -mediated chronic liver damage with multiple CCl_4 injections, TGF- β 1 gene expression is upregulated, mediating fibrogenesis (Shrestha et al. 2016). We now found that TGF- β 2 mRNA levels are increased much stronger compared to TGF- β 1 mRNA levels upon $1 \times CCl_4$ treatment, after 6 h (Fig. 1A). CCl_4 -mediated TGF- β 2 mRNA levels, but not those of TGF- β 1, were significantly reduced in $ERR\gamma$ -LKO mice and in a setting of GSK5182 pretreatment, an inverse agonist of $ERR\gamma$ (Fig. 1D and 1G). Therefore, we suggest that TGF- β 2, induced by CCl_4 -via $ERR\gamma$, plays an important role for the fibrogenic response in acute liver injury.

Previous studies reported that IL6 signaling cascades are activated during the liver response on acute injury, both by CCl_4 injection or partial hepatectomy (PHx), and the importance in liver regeneration was shown (Trautwein et al. 1992, 1994; Zhang et al. 1996). IL6 serum levels are increased early time while hepatocyte proliferation begins after 24 h of liver resection in mice. Moreover, targeted disruption of IL6 results in impaired liver regeneration, as characterized by necrosis in PHx mice (Cressman et al. 1996). CCl_4 toxicity involves an inflammatory response and upregulates several pro-inflammatory cytokines, whereby in particular plasma IL6 levels were significantly increased 6 h after CCl_4 injection (Dai et al. 2018). Moreover, helicid treatment (4-formylphenyl-O- β -d-allopyranoside, a main active constituent from seeds of the Chinese herb *Helicium nilagirica*) protected CCl_4 -induced acute liver injury in mice including anti-inflammatory and anti-oxidative effects (Chen et al. 2020). Here, we identified IL6 as upstream signal to induce hepatic $ERR\gamma$ expression, which in turn upregulates TGF- β 2 gene expression in response to CCl_4 toxicity. IL6 mRNA levels are increased already at 1 h in mouse liver upon LPS injection. In addition, FGF23 mRNA levels and secretion are maximally increased after 3 h in LPS-treated mice (Agoro et al. 2021). We confirmed gene expression and protein secretion of IL6 early in CCl_4 -treated mice, and showed subsequent $ERR\gamma$ and TGF- β 2 expression at the 6-h time point, all in all a fast tissue response upon CCl_4 intoxication. Treatment with GSK5182 or hepatocyte-specific $ERR\gamma$ depletion significantly reduced CCl_4 -induced TGF- β 2 plasma levels at least for 3 days after CCl_4 injection (Fig. 6A–D). In line, the fibrogenic response at day 3 is also blunted, as evident from reduced expression of fibrogenic factors, including α SMA, COL1a1, PDGFR and TIMP1, or significantly decreased numbers of activated HSC/ α SMA staining (Fig. 6B, D, E, and F). A previous study suggests that TGF- β 2 contributes to induce TGF- β 1/2 gene expression and secretion in an autocrine manner through its own signaling pathway (Chida et al. 2017), which may have impact on initiating the above described TGF- β 1 mediated fibrogenic response in the chronic setting. Taken together, upregulation of TGF- β 2 gene expression is a fast tissue response after CCl_4 intoxication, acting as fibrogenic hepatokine, upon induction by IL6 via $ERR\gamma$.

In conclusion, we showed a novel mechanism of $ERR\gamma$ -mediated TGF- β 2 gene upregulation in hepatocytes, and demonstrated that hepatic $ERR\gamma$ could modulate the fibrogenic response in CCl_4 -intoxicated mice.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s00204-021-03112-1>.

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Declarations

Conflicts of interest The authors declare that they have no conflicts of interest to declare.

Ethical standards The manuscript does not contain clinical studies or patient data.

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