Front. Med. 2019, 13(1): 104–111
https://doi.org/10.1007/s11684-017-0568-x

RESEARCH ARTICLE

Rdh13 deficiency weakens carbon tetrachloride-induced liver injury by regulating Spot14 and Cyp2e1 expression levels

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Abstract **Mitochondrion-localized retinol dehydrogenase 13 (Rdh13) is a short-chain dehydrogenase/reductase involved in vitamin A metabolism in both humans and mice. We previously generated *Rdh13* knockout mice and showed that Rdh13 deficiency causes severe acute retinal light damage. In this study, considering that Rdh13 is highly expressed in mouse liver, we further evaluated the potential effect of Rdh13 on liver injury induced by carbon tetrachloride (CCl_4). Although Rdh13 deficiency showed no significant effect on liver histology and physiological functions under regular culture, the *Rdh13*$^{-/-}$ mice displayed an attenuated response to CCl_4-induced liver injury. Their livers also exhibited less histological changes and contained lower levels of liver-related metabolism enzymes compared with the livers of wild-type (WT) mice. Furthermore, the *Rdh13*$^{-/-}$ mice had Rdh13 deficiency and thus their liver cells were protected from apoptosis, and the quantity of their proliferative cells became lower than that in WT after CCl_4 exposure. The ablation of *Rdh13* gene decreased the expression levels of thyroid hormone-inducible nuclear protein 14 (Spot14) and cytochrome P450 (Cyp2e1) in the liver, especially after CCl_4 treatment for 48 h. These data suggested that the alleviated liver damage induced by CCl_4 in *Rdh13*$^{-/-}$ mice was caused by Cyp2e1 enzymes, which promoted reductive CCl_4 metabolism by altering the status of thyroxine metabolism. This result further implicated Rdh13 as a potential drug target in preventing chemically induced liver injury.**

Keywords retinol dehydrogenase 13; carbon tetrachloride; acute liver injury; Cyp2e1; Spot14

Introduction

Mitochondrion-localized retinol dehydrogenase 13 (Rdh13) is a member of short-chain dehydrogenase/reductase (SDR) participating in vitamin A metabolism in both humans and mice [1,2]. SDR belongs to a large family of nicotinamide adenine dinucleotide/triphosphopyridine nucleotide phosphate (NAD(P)H)-dependent oxidoreductase [3]. The members of SDR share the same cofactors and substrates, although they exist in different organelles, such as the mitochondria, peroxisomes, and endoplasmic reticulum [4,5]. As a member of the SDR superfamily, Rdh13 displays high sequence similarity to Rdh11, Rdh12, and Rdh14 and belongs to short-chain-retinol dehydrogenases with dual-substrate specificity (RDH family) [6–10]. The concentrate on the inner mitochondrial membrane of Rdh13 suggests that it plays different roles in contrast to Rdh11, Rdh12, and Rdh14 localized in the endoplasmic reticulum [11]. SDRs play significant roles in the metabolism of lipid, protein, carbohydrate, and chemical compounds. In the human genome database, at least 63 gene members have been identified, but the cellular functions of most oxidoreductases, including Rdh13, are not fully understood [4,5]. Previously, we generated an *Rdh13* knockout mouse model and found the important roles of Rdh13 in protecting the retina from light damage; this finding was also observed in *Rdh11* and *Rdh12* mutant mice [6–10]. Furthermore, Rdh13 is highly expressed in mouse liver such that its

Received November 15, 2016; accepted June 2, 2017
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amount in the liver is 3–10 times greater than that observed in other tissues, suggesting that the Rdh13 will play significant roles in liver metabolism. The liver can renew by itself after being attacked by toxic substances, hepatitis virus, and surgery, and liver parenchymal cells in G_0 phase initiate proliferation upon these stimuli [12–15]. Both hepatocytes and hepatic stellate cells are indispensable in retinoid metabolism [16]. Numerous chemical compounds, such carbon tetrachloride (CCl_4), are normally used to generate animal models in studying the progress and mechanism of liver injury and regeneration [12,13,16]. Free trichloromethyl radical, the metabolites of CCl_4 catalyzed by Cyp2e1, can bind to macromolecules, such as proteins, nucleic acids, and lipids, to prevent them from eliciting functions that damages the liver [17]. Acute liver damage caused by CCl_4 is full of inflammation, apoptosis, hepatocyte necrosis, and then is completely restored by liver regenered [18,19]. The progress and mechanism of acute liver damage are relatively well characterized for histological, biochemical, and molecular alteration [20]. Substantial evidence reveal that vitamin A and its metabolites, such as retinoic acid, are involved in liver injury, regeneration, fibrosis, and tumorigenesis [21,22].

In the present study, we evaluate the potential effect of Rdh13 in liver metabolism, especially during CCl_4-induced liver injury and regeneration by utilizing *Rdh13*$^{-/-}$ mouse model. No remarkable difference is observed between wild-type (WT) and *Rdh13*$^{-/-}$ mice with respect to liver enzymes and liver histology. However, after CCl_4 treatment, the *Rdh13*$^{-/-}$ mice display attenuated liver injury in contrast to the WT controls. Rdh13 deficiency protects liver cells from apoptosis. Furthermore, proliferative cells decrease after CCl_4 exposure. Rdh13 deficiency decreases the expression levels of Spot14 and cytochrome P450 (Cyp2e1). This result suggests that alleviated liver damage induced by CCl_4 in *Rdh13*$^{-/-}$ mice is due to Cyp2e1 enzyme, which promotes reductive CCl_4 metabolism by changing status of thyroxine metabolism, further implicating Rdh13 as a potential drug target in preventing chemically induced liver injury.

Materials and methods

Animals

Rdh13$^{-/-}$ mice were obtained as previously reported [9]. WT and *Rdh13*$^{-/-}$ mice (8–10 weeks old) with mixed C57BL/6 and 129/Sv background were used in the study. The food and water intake were provided freely, and the mice were raised under specific pathogen-free conditions at a constant room temperature of 22–24 °C at 12 h light/dark cycle. All animal experiments were authorized by the Animal Care and Use Committee of Shanghai Jiao Tong University School of Medicine.

CCl_4 induced acute liver injury

CCl_4 was used to induce acute liver injury. WT and *Rdh13*$^{-/-}$ mice with the same ratio of male and female were intraperitoneally injected with a single dosage of CCl_4 (1.0 mL/kg body weight of 20% CCl_4 diluted in olive oil) [4,5,11]. Four mice in each cohort were killed at 0, 3, 6, 12, 24, 48, and 72 h after CCl_4 treatment. Two hours before sacrificing, single dosage of bromodeoxyuridine (Brdu) was intraperitoneally injected at the dosage of 50 mg/kg mouse weight (0.2% ddH_2O solution).

Histological analysis

Formalin-fixed liver samples were processed overnight and then paraffin-embedded according to standard procedure. Liver sections were examined by hematoxylin and eosin (H&E) staining and subsequently analyzed under a light microscope (Nikon i90).

Serum biochemical analyses

The serum alanine aminotransferase (ALT), alkaline phosphatase (ALP), aspartate aminotransferase (AST), and other parameters were detected by the automatic biochemical analyzer according to the manufacturers' instructions.

Terminal deoxynucleotidyl transferase-mediated uDP nick-end labeling (TUNEL) assay

We used the TUNEL assay (Cell Death Detection Kit, Promega) to assess apoptosis. Quiescent and DNase I pretreated WT livers were used as negative and positive controls, respectively. TUNEL-stained liver sections in each group at each time point after CCl_4 injection were examined as previously described [16]. The average percentage of apoptotic hepatocytes was compared between the two genotypes.

Immunohistochemistry

Immunohistochemistry analysis was performed by following the manufacturers' instructions of VECTASTAIN®ABC system (Vector Laboratories). Mouse antiBrdu (Sigma), rabbit antiCyclin D1 (Cell Signaling Technology), and rabbit antiCaspase-3 (Abcam) were used. By counting Brdu labeled and total hepatocytes in 10 high-power fields (400×), Brdu incorporation of hepatocytes was calculated for each liver sample.

RNA isolation and quantitative real-time reverse transcription polymerase chain reaction (PCR)

Total RNA was prepared from mouse liver tissues using

Trizol Reagent (Invitrogen) according to the manufacturer's instructions. Quantitative PCR was carried out with SYBR Green real-time PCR Master Mix (Takara). The reactions were performed on an Eppendorf Mastercycler system, and the cycling parameters were reported previously [13]. Samples were run in triplicate, and the expression of specific transcript was normalized against β-actin. The primers used are listed in Supplementary Table S1.

Western blot analysis

Extraction and quantitative analysis of protein were performed as previously reported [9]. The primary antibody of Cyp2e1 (Sigma), caspase-3 (Abcam), Cyclin D1 (Cell Signaling Technology), Spot14 (Abcam), Bcl-2 (Abcam), and Bax (Abcam) were used in Western blot analysis, and glyceraldehyde 3-phosphate dehydrogenase (Cell Signaling Technology) serves as a loading control.

Statistical analysis

All arguments investigated are presented as mean ± standard error of the mean (S.E.M.). Comparisons between the two groups were performed by two-tailed unpaired Student's *t*-test. *P* values less than 0.05 were considered statistically significant.

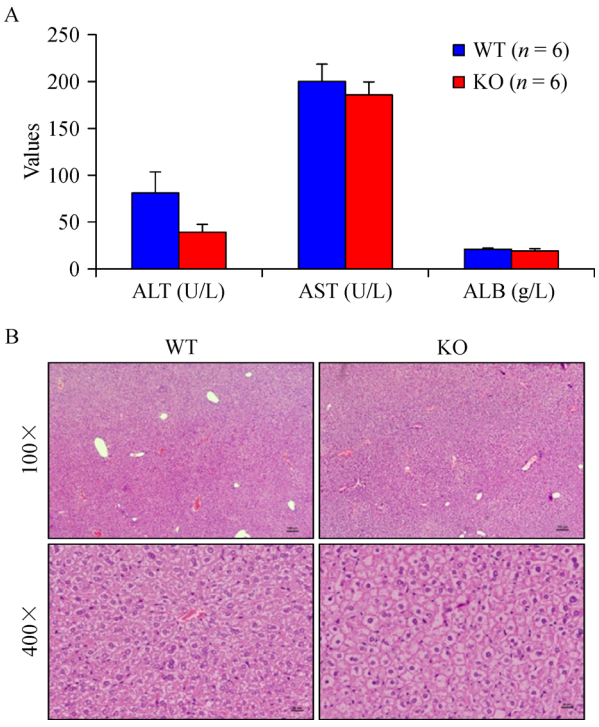

Fig. 1 Liver functions and histology remain normal in *Rdh13*$^{-/-}$ mice. (A) Serum levels of alanine aminotransferase, aspartate aminotransferase, and albumin are shown as mean values ± S.E.M. from sex- and age-matched wild type and *Rdh13*$^{-/-}$ mice (n = 6, $P > 0.05$). (B) Histological analyses of liver sections stained with H&E are shown at original magnifications of 100× and 400×, correspondingly.

Results

Rdh13 deficiency exhibited no marked effect on liver functions and histology

We performed quantitative reverse transcription polymerase chain reaction (qRT-PCR) on major mouse tissues to examine the expression profile of *Rdh13* in mice. We found that *Rdh13* is widely expressed, and the expression levels vary considerably in different tissues. Meanwhile, the highest level was found in liver tissue, as shown in Supplementary Fig. 1, suggesting an essential functional compartment of Rdh13 in mice. Therefore, we first determined the biochemical parameters of liver functions in WT and *Rdh13*$^{-/-}$ mice, which have been generated previously [9]. Unexpectedly, although *Rdh13*$^{-/-}$ mice showed decreased level of liver function albeit ALT, no significant variation was observed in biochemical parameters including ALT, AST, and albumin (ALB) compared with WT mouse (Fig. 1A). In addition, liver histology displayed no discernable difference between *Rdh13*$^{-/-}$ and WT mice (Fig. 1B). These data suggested an indispensable role of Rdh13 in liver biochemistry or histology of mice at basal condition.

Targeted deletion of *Rdh13* alleviates CCl_4-induced hepatic injury

Given that Rdh13 deficiency showed no marked effect on basal liver biochemistry and histology, we decided to challenge the mutant mice with CCl_4 administration intraperitoneally because CCl_4-induced acute liver injury model is widely used to evaluate the progress and mechanism of liver injury and regeneration. As expected, all biochemical parameters tested, such as ALT, ALP, ALB, globulin, total cholesterol, AST, and triglyceride levels, respond to CCl_4 treatment to some degree. However, no significant difference was found between WT and *Rdh13*$^{-/-}$ mice except for ALT (Fig. S2). *Rdh13*$^{-/-}$ mice showed significantly lower levels of ALT after CCl_4 administration compared with WT mice. The serum ALT level in both genotypes peaked at 24 h and decreased at 48 h to 200 U/L in *Rdh13*$^{-/-}$ mice, whereas serum ALT level was 1800 U/L in WT mice (Fig. 2A). Meanwhile, liver histology displayed marked vacuolization, necrosis, inflammation, and sinusoidal dilatations upon CCl_4 administration in both genotypes. However, these histological changes were milder in *Rdh13*$^{-/-}$ mice than those

observed in the WT controls (Fig. 2B). These data suggested that Rdh13 is involved in regulating the responses to CCl_4-induced acute liver injury.

Reduced hepatocyte apoptosis in *Rdh13*$^{-/-}$ liver after CCl_4 challenge

The hepatic apoptosis and following cell proliferation are essential responses to CCl_4 treatment in mouse model. Therefore, we further assessed the hepatic apoptosis in WT and *Rdh13*$^{-/-}$ mice upon CCl_4 treatment. We found that the amount of apoptotic hepatocytes decrease at 24, 48, and 72 h after CCl_4 exposure in *Rdh13*$^{-/-}$ mice and lower than that in WT mice, as indicated by the results of TUNEL analysis (Fig. 3A and 3B). Caspase-3 is an evolutionary conserved cysteine protease and plays a central role in apoptotic cell death pathways [23,24]. We determined the expression level of caspase-3 by immunohistochemistry and Western blot. The results indicated that the amount of caspase-3 immune-reactive cells are significantly lesser in *Rdh13*$^{-/-}$ mice compared with that in WT mice 24, 48, and 72 h after CCl_4 treatment (Fig. 3C). Consistent with this finding, caspase-3 protein levels in the liver showed a weak response to CCl_4 in the absence of Rdh13. Furthermore, antiapoptotic protein Bcl-2 increased and apoptotic protein Bax decreased in *Rdh13*$^{-/-}$ livers relative to WT controls during CCl_4-induced liver injury (Fig. 3D). These results suggested that Rdh13 deficiency exhibited a protective effect against CCl_4-induced hepatocyte apoptosis.

Reduced compensatory response in hepatocellular proliferation post CCl_4-exposure in *Rdh13*$^{-/-}$ mice

After CCl_4-induced injury, liver mass is rapidly replenished [12]. The effect of Rdh13 on liver regeneration after acute toxic liver injury was evaluated. Liver/body weight ratio reflects the liver proliferation status. However, no gross divergence was observed between WT and *Rdh13*$^{-/-}$ mice (Fig. S3). Hepatocyte compensatory proliferation was further assessed by Brdu incorporation. Compared with WT mice, the *Rdh13*$^{-/-}$ mice had lower degree of Brdu incorporation during liver regeneration. DNA synthesis peaked at 48 h post-CCl_4 exposure. More than 60% of the hepatocytes were Brdu positive in the WT liver, whereas less than 45% of the hepatocytes in *Rdh13*$^{-/-}$ livers were Brdu positive (Fig. 4A and 4B). Cyclin D1 is a key molecule regulating the progress of G_1 phase in cell cycle [25–27]. We determined whether reduced cell proliferation is due to the altered expression of Cyclin D1 in the liver of *Rdh13*$^{-/-}$ mice. The results showed that the expression levels of Cyclin D1 mRNA by qRT-PCR (Fig. 4C) and protein detected by either immunohistochemistry (Fig. 4D)

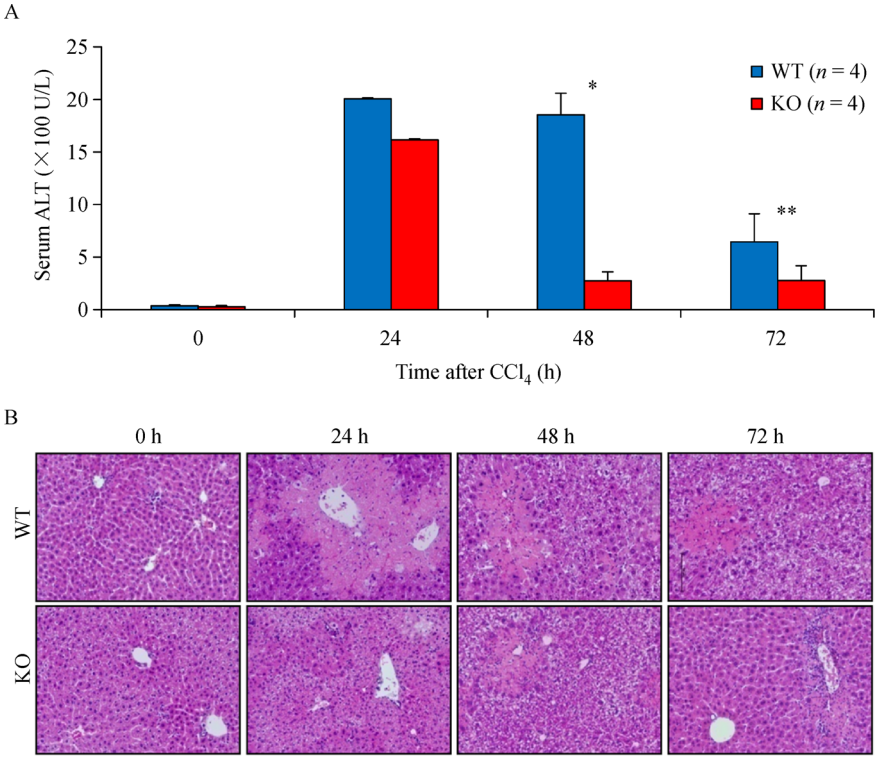

Fig. 2 Acute liver injury induced by CCl_4 is alleviated in *Rdh13*$^{-/-}$ mice. (A) Serum ALT levels in WT and *Rdh13*$^{-/-}$ mice at the indicated times post-CCl_4 exposure are expressed as mean ± S.E.M. (n = 4, $^*P < 0.01$, $^{**}P < 0.05$). (B) Representative H&E-stained liver sections of WT and *Rdh13*$^{-/-}$ mice at the indicated times post-CCl_4 exposure are shown at an original magnification of 100×.

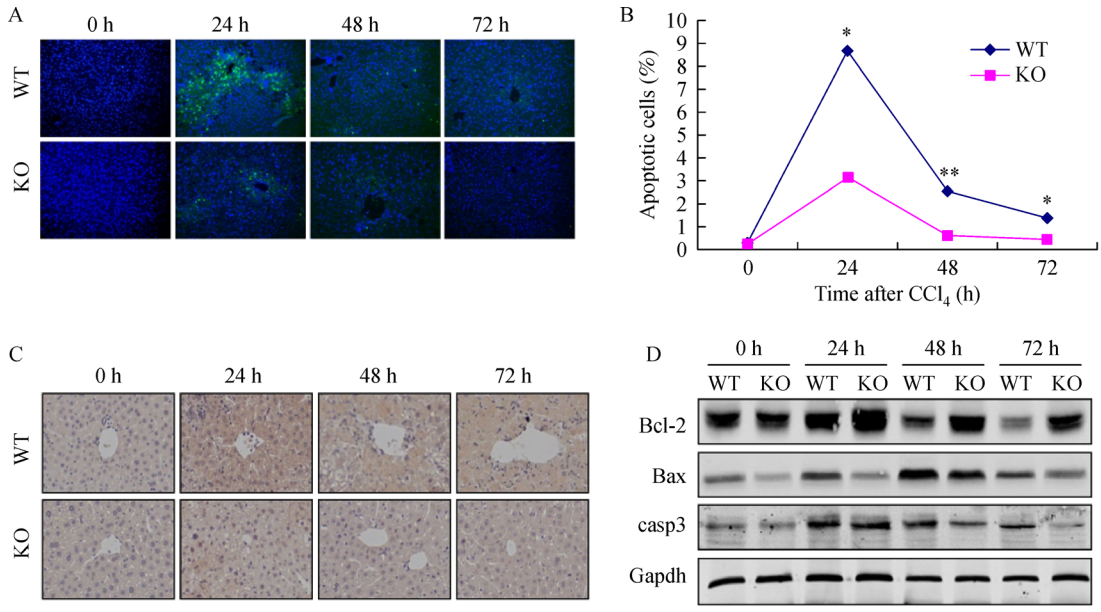

Fig. 3 Apoptosis induced by CCl_4 treatment is reduced in *Rdh13*$^{-/-}$ livers. (A) *In situ* fluorescence TUNEL assay on liver frozen sections shows hepatocyte nuclei (blue, 4′,6-diamidino-2-phenylindole) and apoptotic cells (green, fluorescein isothiocyanate). (B) Quantitative analysis on apoptotic cells in the liver upon CCl_4 treatment for the indicated time points (*$P < 0.05$, **$P < 0.01$). (C) Caspase-3 expression by immunohistochemistry in the liver upon CCl_4 treatment for the indicated time points. (D) Western blot analyses for the indicated proteins in the livers of mice exposed to CCl_4 for the indicated time points. Glyceraldehyde 3-phosphate dehydrogenase is shown as loading control.

or Western blot (Fig. 4E) were lower in *Rdh13*$^{-/-}$ liver than those in WT controls after the CCl_4 treatment. Extracellular signal-regulated kinase (ERK) is also a significant factor for Cyclin D1 induction in mid-late G_1 phase [28,29]. Hepatocyte DNA replication and Cyclin D1 accumulation are mainly associated with ERK activation [30]. Our result indicated that ERK activation in the absence of Rdh13 was reduced and lower compared with that in WT livers upon CCl_4 administration (Fig. 4F). All these data indicated the reduced compensatory response in hepatocellular proliferation post-CCl_4 exposure in *Rdh13*$^{-/-}$ mice, at least in part, is due to the attenuated activation of ERK and reduced expression of Cyclin D1.

Expression levels of Cyp2e1 and Spot14 decreased in *Rdh13*$^{-/-}$ livers after CCl_4 administration

CCl_4 toxicity is caused by the metabolite (trichloromethyl free radical) of CCl_4 by Cyp2e1 in the liver. We detected the expression levels of mRNA and protein of Cyp2e1 in both WT and *Rdh13*$^{-/-}$ livers. As shown in Fig. 5A and 5B, the amounts of Cyp2e1 mRNA by qRT-PCR and protein detected by Western blot were reduced in Rdh13-deficient mice, especially at 48 h post-CCl_4 exposure, and lower compared with those in WT mice. Cyp2e1 regulation is particularly complex. As reported previously, insulin can suppress Cyp2e1 expression, whereas triiodothyronine (T_3) can upregulate Cyp2e1 expression [30]. We first determined the blood glucose levels of the WT and *Rdh13*$^{-/-}$ mice before and after CCl_4 treatment to exclude the possibility that altered insulin level affects Cyp2e1 expression. However, no significant difference was observed between the blood glucose levels of the WT and *Rdh13*$^{-/-}$ mice (Fig. S4). Spot14 (thyroid hormone-inducible nuclear protein Spot14) is one of the T_3 downstream target genes [31]. Thus, we assessed the expression level of Spot14, indirectly representing the thyroxine metabolic status [32]. We found that either Spot14 mRNA or protein levels in the livers of WT and *Rdh13*$^{-/-}$ mice were induced after CCl_4 treatment. However, the induction of Spot14 in *Rdh13*$^{-/-}$ mice was compromised at transcription (Fig. 5C) and protein (Fig. 5D) levels when compared with WT controls. Based on these data, the compromised response in Spot14 expression to CCl_4 may contribute to reduced Cyp2e1 expression levels in the absence of Rdh13.

Discussion

Rdh13 belongs to the SDR family and is localized in the mitochondria. As previously reported, Rdh13 is involved in vitamin A metabolism of humans and mice [1,2]. Structurally, Rdh13 shows high-sequence similarity to Rdh11, Rdh12, and Rdh14, which are the members of the short-chain RDH family with dual-substrate specificity [6–8]. All the members of the RDH family are express in the mammalian retina and essential for the normal

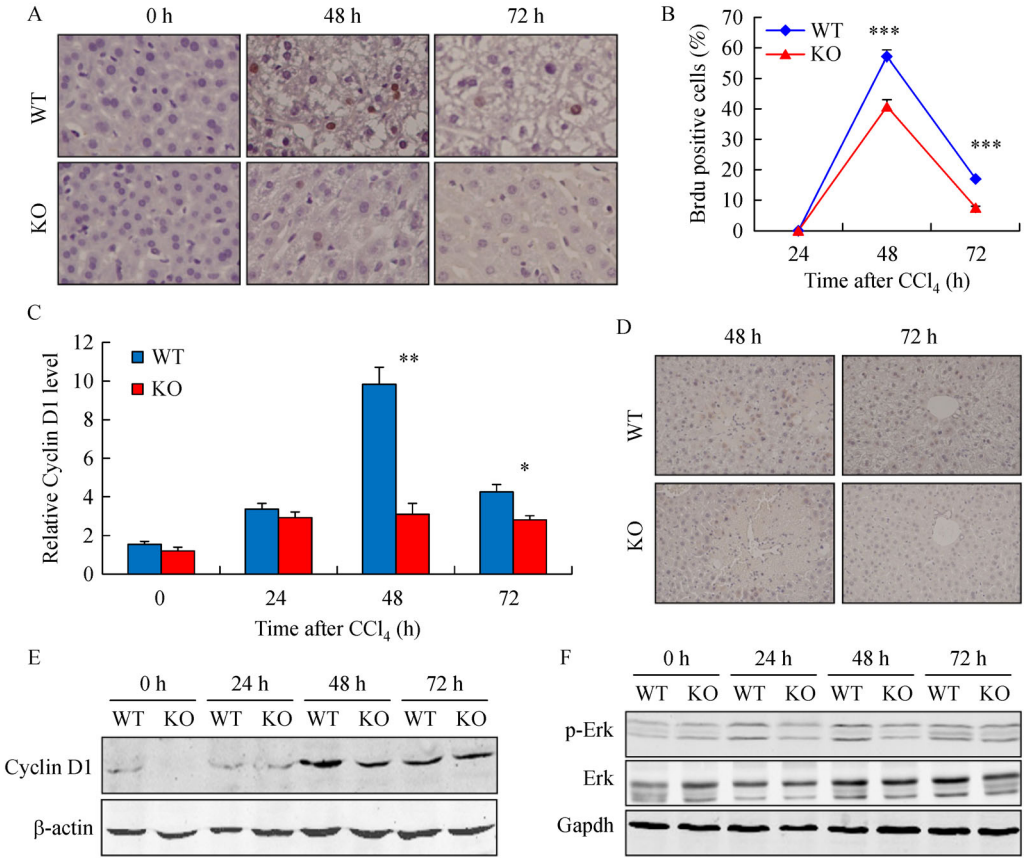

Fig. 4 Rdh13 deficiency reduced hepatocyte compensatory proliferation after CCl_4-induced liver damage in mice. (A) *In vivo* Brdu incorporation assayed by immunohistochemistry in the liver upon CCl_4 treatment for the indicated time points. (B) Percentages of Brdu-positive cells in the livers are shown as mean ± S.E.M. (***$P < 0.001$). (C) Cyclin D1 mRNA expression at the indicated time points in the livers by real-time RT-PCR is shown as mean ± S.E.M. (*$P < 0.05$, ** $P < 0.01$). Protein levels of cyclin D1 in the livers after CCl_4 treatment are shown by immunohistochemistry (D) and Western blot (E). (F) Western blot for p-ERK1/2 and total ERK1/2 in the livers of WT and *Rdh13*$^{-/-}$ mice after CCl_4 treatment is shown.

functioning of the retina [12,13]. In our previous study, by using *Rdh13* knockout mouse model, we found that Rdh13 deficiency protects the retina from light damage [9]. Considering that Rdh13 is highly expressed in mouse liver, we explored the potential roles of Rdh13 in liver function. Unexpectedly, Rdh13 deficiency displayed no significant effect on the biochemical parameters of liver function and histological structure, suggesting a dispensable role of Rdh13 in the regulation of liver functions in mice. A compensative mechanism by other members of RDH family is triggered in the absence of Rdh13.

CCl_4-induced acute liver injury in mice opens a window to investigate the biological processes of liver injury and subsequent liver regeneration. Upon CCl_4 challenge, *Rdh13*$^{-/-}$ mice were resistant to CCl_4 treatment, as suggested by its mildly elevated liver enzyme and less liver histological changes when compared with WT mice. The results of *in situ* TUNEL for apoptotic hepatocytes and immunostaining for caspase-3 expression also revealed that CCl_4-induced apoptosis cell is lesser in *Rdh13*$^{-/-}$ mice than that in WT controls. In line with this data, the expression levels of antiapoptotic protein Bcl-2 and apoptotic protein Bax displayed downregulation and upregulation in the livers while lacking Rdh13 after CCl_4 treatment. We further evaluated the ability of hepatocyte compensatory proliferation following CCl_4-induced acute liver injury by Brdu incorporation. As expected, the percentage of Brdu-positive hepatocytes in *Rdh13*$^{-/-}$ liver was lower than that in WT mice. Moreover, we showed that *Rdh13*$^{-/-}$ mice displayed a weak response to Cyclin D1 expression, as well as ERK phosphorylation to CCl_4 treatment, implicating the involvement of Rdh13 in regulating ERK signaling.

Rdh13 may be involved in the metabolic conversion of CCl_4 by Cyp2e1 to CCl_3O and/or CCl_3OO radicals, which are toxic to mouse hepatocytes [33]. This association may explain why *Rdh13*$^{-/-}$ mice are resistant against CCl_4 toxicity. To this end, we investigated the mRNA and

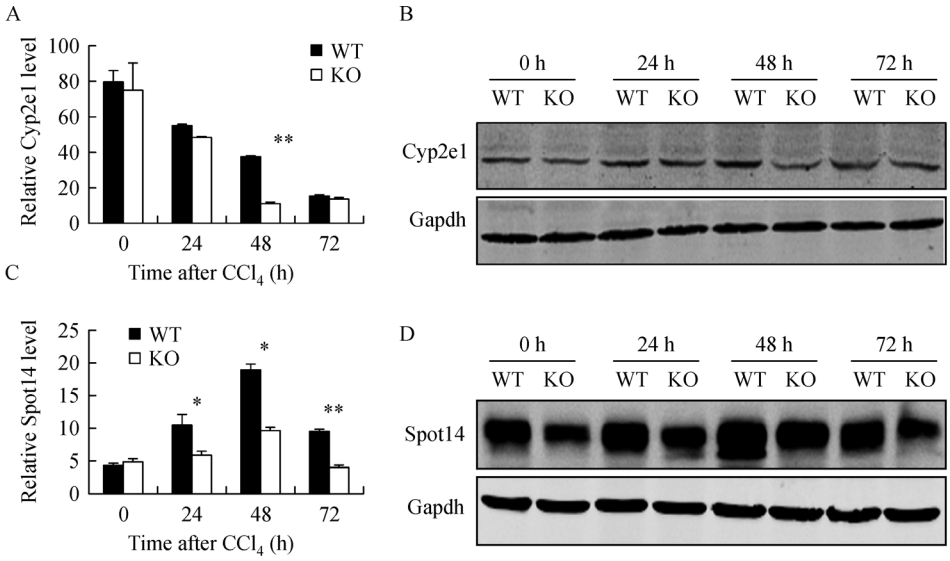

Fig. 5 Decreased expression levels of Cyp2e1 and Spot14 in the livers of *Rdh13*$^{-/-}$ mice after CCl_4 treatment. (A) Expression levels of Cyp2e1 mRNA and protein by real-time PCR (mean ± S.E.M., ** $P < 0.01$) and Western blot (B) show reduced Cyp2e1 expression levels in *Rdh13*$^{-/-}$ livers 24 h after CCl_4 exposure. The expression levels of Spot14 in the livers after CCl_4 treatment are detected by quantitative real-time PCR (C, mean ± S.E.M., * $P < 0.05$, ** $P < 0.01$) and Western blot (D).

protein expression levels of Cyp2e1 and Spot14, indirectly reflecting the thyroxine metabolic status, because T_3 regulates Cyp2e1 expression *in vivo* [34]. Our data indicated that Rdh13 deficiency reduced the expression levels of Cyp2e1 mRNA and protein in the liver tissues, especially at 48 h after CCl_4 treatment. Spot14 induction was also compromised in *Rdh13*$^{-/-}$ livers to a greater degree than that in WT mice. Basing on these data, we concluded that Rdh13 participates in the regulation of thyroxine metabolism and contributes to the lowering of Cyp2e1 expression. These findings shed a light on the function of Rdh13 in liver disease *in vivo* and implicated Rdh13 as a potential new therapeutic target for the prevention and treatment of chemically induced liver injury.

Acknowledgements

This work was supported by grants from the National Natural Science Foundation of China (No. 81430028), the Ministry of Science and Technology of China (No. 2011BAI15B02), the grants from the Science and Technology Commission of Shanghai Municipality (Nos.13DZ2280600 and 15DZ2290800), and the grant from Shanghai First People's Hospital Affiliated to Shanghai Jiao Tong University (No. 81300776).

Compliance with ethics guidelines

Xiaofang Cui, Benting Ma, Yan Wang, Yan Chen, Chunling Shen, Ying Kuang, Jian Fei, Lungen Lu, and Zhugang Wang declare no conflicts of interests. All institutional and national guidelines for the care and use of laboratory animals were followed.

Electronic Supplementary Material Supplementary material is available in the online version of this article at https://doi.org/10.1007/s11684-017-0568-x and is accessible for authorized users.

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