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Activation of farnesoid X receptor induces RECK expression in mouse liver

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ABSTRACT

Farnesoid X receptor (FXR) belongs to the ligand-activated nuclear receptor superfamily, and functions as a transcription factor regulating the transcription of numerous genes involved in bile acid homeostasis, lipoprotein and glucose metabolism. In the present study, we identified RECK, a membrane-anchored inhibitor of matrix metalloproteinases, as a novel target gene of FXR in mouse liver. We found that FXR agonist substantially augmented hepatic RECK mRNA and protein expression *in vivo* and *in vitro*. FXR regulated the transcription of RECK through directly binding to FXR response element located within intron 1 of the mouse RECK gene. Moreover, FXR agonist reversed the down-regulation of RECK in the livers from mice fed a methionine and choline deficient diet. In summary, our data suggest that RECK is a novel transcriptional target of FXR in mouse liver, and provide clues to better understanding the function of FXR in liver.

1. Introduction

Farnesoid X receptor (FXR, NR1H4) belongs to the ligand-activated nuclear receptor superfamily, and functions as a transcription factor regulating the transcription of numerous genes involved in bile acid homeostasis, lipoprotein and glucose metabolism [1,2]. FXR is primarily expressed in liver, small intestine, kidneys, and adrenal glands [3]. Similar to many other non-steroid nuclear receptors, FXR heterodimerizes with the retinoid X receptor α (RXRα, NR2B1) and binds to specific DNA response elements (FXRE) at target genes [4,5]. The most common FXRE is an inverted repeat of the canonical AGGTCA half sites spaced by one nucleotide (IR-1), and this motif has been identified within many known FXR target genes [6]. FXR is proved to promote transcriptional expression of small heterodimer partner (SHP), which negatively regulates synthesis and hepatic intake process of bile acid [7]. Recent reports have also reported that FXR is essential to maintain lipid and carbohydrate homeostasis by regulating genes such as sterol regulatory element-binding protein-1c (SREBP-1c) and phosphoenolpyruvate carboxykinase (PEPCK) [8,9]. As a consequence, FXR-null mice display abnormal bile salts, triglyceride levels and impaired insulin-sensitivity [10,11]. Activation of FXR by synthetic agonists results in significant protection from cholestasis [12], atherosclerosis [13], liver fibrosis and inflammation [14,15].

Reversion-inducing cysteine rich protein with Kazal motifs (RECK) is recognized as the membrane-anchored protease regulator and negatively regulates the activity of matrix metalloproteinases (MMPs) [16,17]. RECK is essential for organic growth and development. $RECK^{-/-}$ embryos show elevated MMP activity and develop disrupted mesenchymal tissues [17]. In multiple human malignancies, RECK is proved to limit tumor development and metastasis by inhibiting MMPs post-transcriptionally [18]. The Sp1 site and hypoxia-responsive element (HRE) in RECK promoter region have been reported to modulate the transcription of RECK [19,20].

In the present study, we identified RECK as a novel transcriptional target gene of FXR. We showed that FXR-deficient mice exhibited lower RECK mRNA level in livers compared to wild-type mice, and synthetic FXR agonists induced RECK expression at both mRNA and protein levels in mouse liver. Furthermore, FXR agonist

Abbreviations: FXR, farnesoid X receptor; RXRα, retinoid X receptor α; SHP, small heterodimer partner; SREBP-1c, sterol regulatory element-binding protein-1c; PEPCK, phosphoenolpyruvate carboxykinase; RECK, reversion-inducing cysteine rich protein with Kazal motifs; MCD, methionine and choline deficient; MMPs, matrix metalloproteinases; FBS, fetal bovine serum.

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reversed the reduction of RECK in mice fed a methionine and choline deficient (MCD) diet. Our data provide clues to better understanding the role of FXR in liver.

2. Materials and methods

2.1. Antibodies and reagents

Rabbit anti-RECK antibody and DRAQ5 were purchased from Cell Signaling Technology. Anti-FXR, anti-GAPDH and normal rabbit IgG antibody were obtained from Santa Cruz Biotechnology. The secondary antibody Alexa Fluor® 488-conjugated goat anti-rabbit IgG was from Jackson ImmunoResearch. GW4064, leupeptin, aprotinin and phenylmethylsulfonylfluoride were purchased from Sigma–Aldrich Inc. WAY-362450 was obtained from Selleck chemicals. Other reagents were commercially available in China.

2.2. Animals and treatments

All procedures involving animals were performed according to the criteria outlined in the Guide for the Care and Use of Laboratory Animals and approved by the ethics committee of Fudan University. $FXR^{-/-}$ mice on a C57BL/6 background were obtained from Jackson Laboratories. 8-week-old male wild-type C57BL/6 or $FXR^{-/-}$ mice were treated with vehicle or WAY-362450 (30 mg/kg, IG) daily. In methionine and choline deficient (MCD) diet models, male C57BL/6 mice were divided into 3 experimental groups, fed and treated as previously described [15]. At sacrifice, mice were euthanized under anesthesia, and livers were collected for subsequent analysis.

2.3. Cell culture

HEK293T cells were obtained from the Institute of Cell Biology, Academic Sinica (Shanghai, China) and maintained in Dulbecco's modified Eagle's medium (Sigma–Aldrich) containing 10% fetal bovine serum (FBS) and 100 U/ml penicillin and 0.1 mg/ml streptomycin at 37 °C in 5% CO_2. AML12 cells (ATCC), an immortal mouse hepatocyte cell line, were cultured in a 1:1 mixture of Dulbecco's modified Eagle's medium and F-12 (Gibco) supplemented with 10% FBS, 5 μg/ml insulin, 5 μg/ml transferrin, 5 ng/ml selenium and 40 ng/ml dexamethasone at 37 °C with 5% CO_2. Mouse primary hepatocytes (MPH) were isolated and cultured as previously described [21].

2.4. RNA isolation and real-time PCR

Total RNA was isolated from mouse livers or hepatocytes with Trizol reagent (Invitrogen). Complementary DNA (cDNA) was synthesized using PrimeScript™ RT reagent kit (Takara). Real-time PCR was performed on ABI 7500 Sequence Detection system (Applied Biosystems). The relative mRNA levels were normalized to GAPDH expression and the fold changes were determined by $2^{-\Delta\Delta CT}$ method.

2.5. Western blot and immunofluorescence assay

Protein extraction from liver tissues or cultured cells and Western blot analysis were performed as previously described [22]. For immunofluorescence assay, frozen sections of mouse livers were fixed with cold acetone for 10 min and incubated with anti-RECK antibody for 2 h at room temperature. Alexa Fluor® 488 anti-rabbit IgG was used as the secondary antibody, and the nucleus was stained with DRAQ5. Fluorescent signals were visualized with a Leica TCS SP5 confocal microscope (Leica Microsystems).

2.6. Plasmid construction and luciferase reporter assay

Full length mouse FXR and RXRα were amplified from mouse cDNA and cloned into pcDNA3.1 and pcDNA3.0 (Invitrogen), respectively. Four copies of the IR1-type FXR response element from mouse SHP gene was cloned into the pGL3-promoter vector (Promega) resulting in pGL3-FXRE*4. A 167 bp DNA fragment containing the putative IR1-type FXR response element from mouse RECK gene (AGGTCACTGACCC) was cloned into pGL3-promoter vector (RECK FXRE). The DNA fragment containing mutated RECK IR-1 sequence (AAATCACTGATTC) was also cloned to generate the RECK mutFXRE reporter plasmid [23]. Efficient insertion was confirmed by sequencing.

For the luciferase reporter assay, HEK293T cells were transfected with FXR, RXRα, pRL-CMV (Promega) and pGL3-promoter plasmids using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. After treatments, cells were applied to luminescence assay using a Lumat LB 9507 luminometer (Perkin-Elmer). Each experiment was performed in triplicate.

2.7. Chromatin immunoprecipitation (ChIP) assay

ChIP assay with primary hepatocytes from WAY-362450-treated mice was performed according to the manufacturer's protocol (Millipore). Briefly, sonicated chromatin samples were precleared using protein A beads and then incubated with the antibody against FXR (Santa Cruz Biotechnology) or control IgG (Santa Cruz Biotechnology) for immunoprecipitation. After DNA purification, real-time PCR was used to analyze ChIP results. Final results of each sample were normalized to the inputs.

2.8. Statistical analysis

All results are presented as the means ± SEM. Differences between groups were calculated using two-tailed Student's *t*-test. $P < 0.05$ was considered statistically significant.

3. Results

3.1. RECK expression is induced by FXR agonist in wild-type instead of FXR-deficient mice

FXR functions as a transcription factor regulating the transcription of numerous genes involved in bile acid homeostasis, lipoprotein and glucose metabolism. To explore novel FXR target genes, wild-type mice were treated with specific synthetic FXR agonist WAY-362450 [24], and liver tissues were applied to microarray analysis. Results suggest FXR agonist stimulated the expression of RECK at mRNA level (data not shown). Real-time PCR analysis further confirmed that FXR-deficient ($FXR^{-/-}$) mice exhibited significantly lower mRNA level of RECK in livers compare to wild-type (WT) mice, and FXR agonist remarkably induced RECK mRNA and protein expression in the livers from WT mice (Fig. 1A–C). Moreover, treatment with WAY-362450 induced the increase of RECK mRNA level in WT mice but not in $FXR^{-/-}$ mice, further suggesting the regulation of RECK expression by FXR (Fig. 1D). Immunostaining for RECK in mouse liver tissues revealed that RECK was predominantly anchored to the hepatocytes membrane, and RECK protein level was dramatically increased upon WAY-362450 administration (Fig. 1E). Taken together, these results suggest that FXR agonist modulates the expression of RECK in mouse liver *in vivo*.

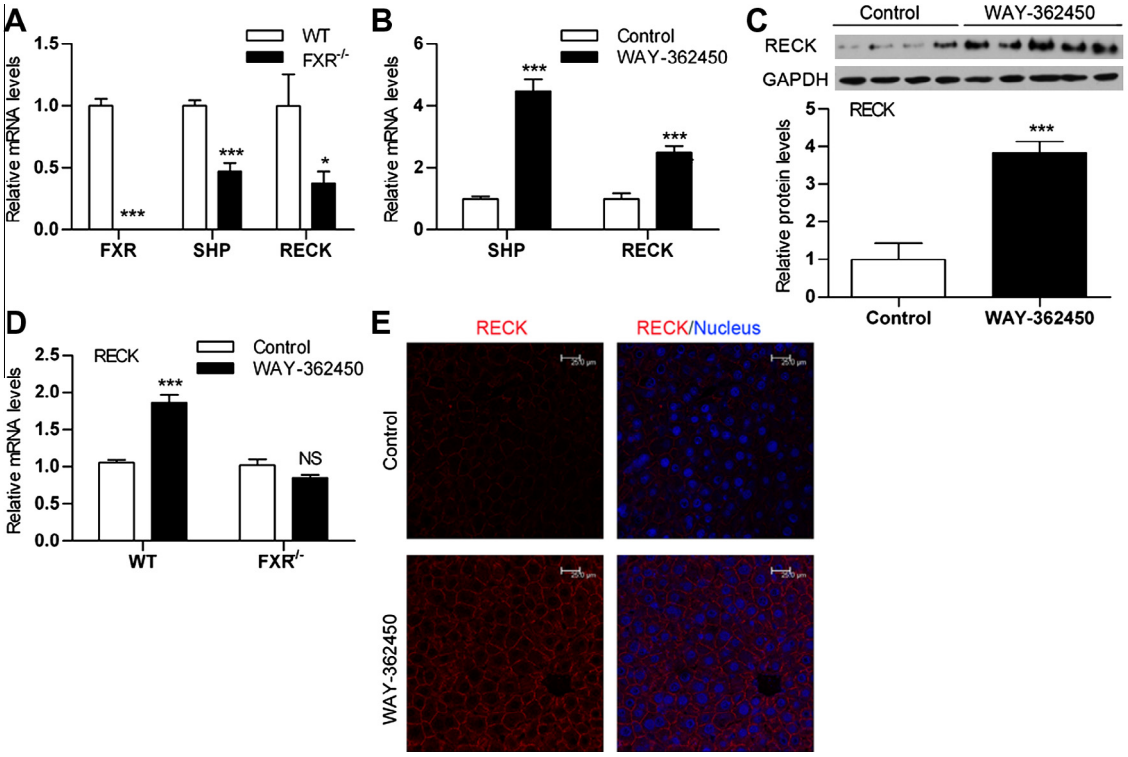

Fig. 1. RECK expression is induced by WAY-362450 in wild-type mice instead of FXR-deficient mice. (A) Quantitative RT-PCR analysis was applied to detect the mRNA levels of FXR, SHP and RECK in liver obtained from wild-type (WT) or FXR-deficient ($FXR^{-/-}$) mice ($n = 5$). (B and C) Wild-type mice were treated with vehicle (control) or WAY-362450, and liver samples were collected for measurements of RECK mRNA (B) and protein (C) levels ($n = 4$ in control group, $n = 5$ in WAY-362450 group). (D) Effects of WAY-362450 treatment on hepatic RECK mRNA levels in WT and $FXR^{-/-}$ mice ($n = 5$). (E) Wild-type mice were treated as in (B), and frozen sections of liver samples were stained with anti-RECK antibody (rhodamine-conjugated, red) and DRAQ5 for nuclear (blue). Representative images were obtained by confocal microscopy. Magnifications, 200×. In (A–D), results were normalized to GAPDH expression. $^{*}P < 0.05$, $^{***}P < 0.001$. NS, not statistically significant. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.2. FXR agonists promote RECK expression in mouse hepatocytes

We next examined whether RECK expression was regulated by FXR in mouse hepatocytes *in vitro*. In mouse primary hepatocytes, treatment with FXR agonist GW4064 [25] or WAY-362450 resulted in an increase in RECK mRNA level (1.8-fold and 2.4-fold, respectively) (Fig. 2A). FXR agonists also increased RECK mRNA and protein levels in AML12 cells (Fig. 2B and C). Moreover, the induction of RECK expression by FXR agonist was in a time- and dose-dependent manner (Fig. 2D and E). These results suggest that activation of FXR induces RECK expression in mouse hepatocytes *in vitro*.

3.3. FXR regulates RECK transcription through binding to an IR-1 element

FXR regulates target genes preferentially through binding to FXR response element (FXRE) [3], such as an inverted repeat separated by a single nucleotide (IR-1) [26]. We employed a bioinformatics-driven approach to analyze RECK gene for potential FXR binding sites. As analyzed, RECK harbours a potential IR-1 type FXRE (AGGTCACTGACCC, at nucleotides +2797/2809 in mouse) within the first intron, which is conserved in mouse, human, orang and rat (Fig. 3A). Luciferase reporter assay with plasmid constructs containing the IR-1 element demonstrated that the luciferase activities were increased upon FXR agonists treatment, and the increment was in an agonist dose-dependent manner (Fig. 3B and C). However, the induction of luciferase activity by FXR agonist GW4064 was abolished by the mutation within the IR-1 motif (RECK mutFXRE) (Fig. 3D). ChIP-qPCR assays also confirmed the direct association of FXR with the IR-1 region within intron 1 of RECK gene (Fig. 3E). These results suggest that FXR directly regulates RECK transcription through binding to an IR-1 element within intron 1 of RECK gene.

3.4. FXR agonist reverses the down-regulation of RECK in mouse MCD dietary model

To explore the regulation of RECK by FXR at pathological condition, mice were fed with MCD diet to induce non-alcoholic steatohepatitis (NASH) [27]. Previous report demonstrated that FXR mRNA level was suppressed in the liver tissue of mouse MCD model [28]. As shown in Fig. 4, RECK expression was also down-regulated both at mRNA and protein levels in the liver of MCD mice, and administration of WAY-362450 reversed the reduction of RECK expression in MCD diet-fed mice. Furthermore, treatment of WAY-362450 also significantly repressed MMP-9 activity in the liver of MCD mice (Supplementary Fig. 1). Together, these data provide evidence to better understanding the anti-inflammation effects of FXR in liver diseases.

4. Discussion

Our data identified RECK as a novel transcriptional target of FXR in mouse liver under both physiological and pathological conditions. We found that FXR regulated the transcription of RECK by directly binding to the IR-1 element within intron 1 of RECK gene (Fig. 3). FXR binding motifs are usually located in the proximal promoters of target genes, yet there are several examples in which the response elements are found in the introns of FXR target genes [6,29,30]. Though the consensus IR-1 sequence has been shown

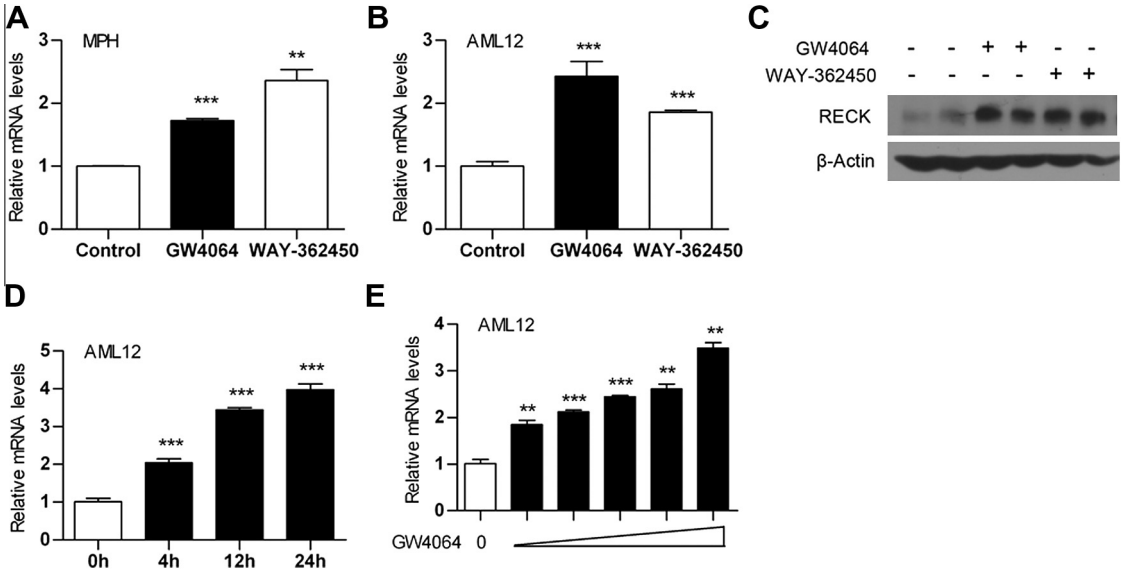

Fig. 2. FXR agonists increase RECK expression in mouse hepatocytes *in vitro*. (A and B) Primary mouse hepatocytes (A) and AML12 (B) were treated with DMSO, GW4064 (2 μM), or WAY-362450 (2 μM) for 4 h. RECK mRNA expression was detected by quantitative RT-PCR. (C) AML12 cells were treated for 48 h as indicated, and cell lysates were applied to Western blot analysis to detect RECK expression. (D and E) AML12 cells were treated with GW4064 for different time periods (D) or doses (E), and RECK mRNA level was analyzed. $^{**}P < 0.01$, $^{***}P < 0.001$.

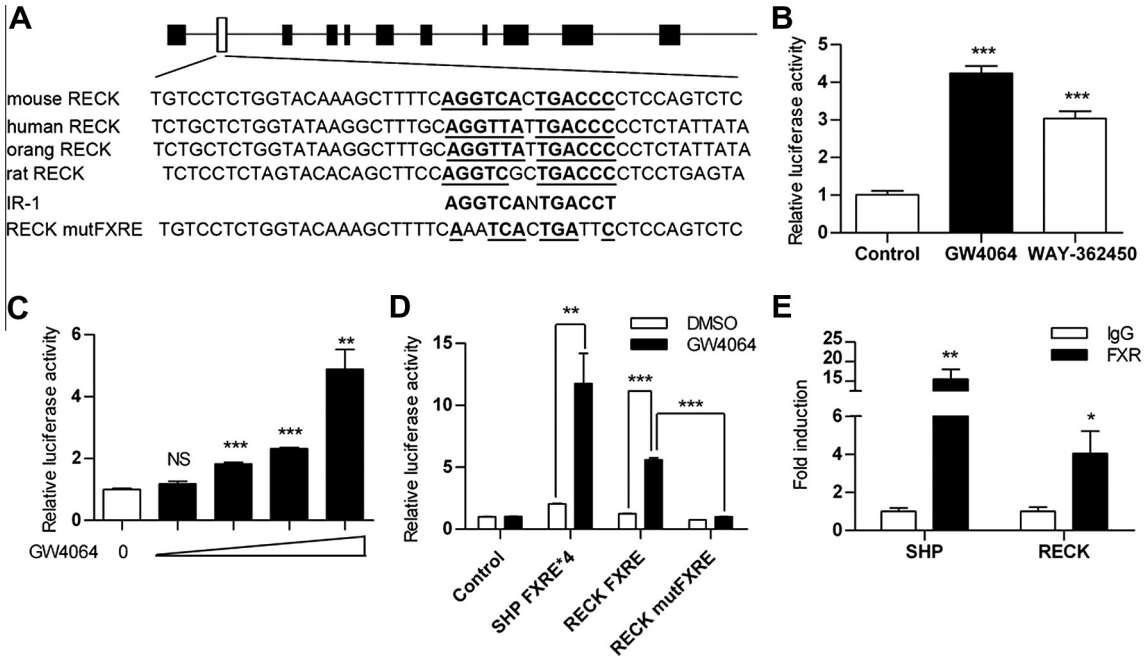

Fig. 3. FXR regulates RECK gene expression through an IR-1 element located in the first intron of mouse RECK gene. (A) Schematic representation of the RECK gene with the IR-1 site harboured in the first intron. The consensus IR-1 elements are shown in bold. (B–C) The effects of FXR agonist GW4064 or WAY-362450 on the activity of RECK FXRE were determined using luciferase report assay. (D) The effects of FXR agonist GW4064 on the activity of SHP FXRE*4, RECK FXRE or RECK mutFXRE were determined using luciferase report assay. (E) ChIP assay was performed using primary hepatocytes isolated from WAY-362450-treated mice, followed by quantitative RT-PCR analysis. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. NS, not statistically significant.

as the most common element that FXR can bind to, other elements, such as IR0 [31], ER8 [32], DR1 [33], DR8 [34], are also capable of conferring responsiveness on FXR-target genes. However, the IR-1 element was found to be the only conserved FXR-responsive element of best match within the RECK gene by using bioinformatics-driven approach.

Our results also indicated that the expression of RECK was decreased in livers from MCD mice, and treatment of WAY-362450 reversed the reduction of RECK mRNA and protein expression (Fig. 4). Previous report demonstrated that FXR mRNA level was suppressed in the liver tissue of mouse MCD model [28]. Therefore, the decreased expression and activity of FXR may contribute to the down-regulation of RECK in the liver of MCD mice. In addition, a previous study also reported that binding of transcription factor Sp1 on RECK promoter results in the down-regulation of RECK [18]. Since Sp1 has been shown to be activated in the liver of MCD mice and responsible for inflammatory response and fibrogenesis in the development of NASH [35,36], the inactivation of

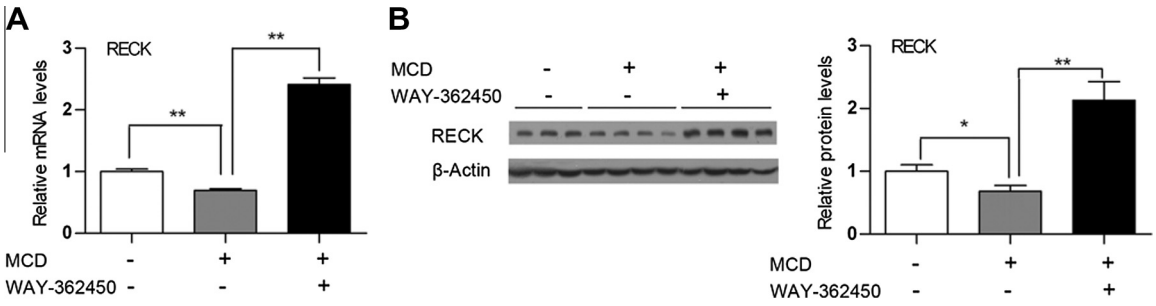

Fig. 4. FXR agonist reverses the reduction of RECK in MCD diet-fed mice. Wild-type mice were treated as indicated for 4 weeks, and liver samples were isolated to detect relative expression of RECK mRNA (A) and protein (B) ($n = 6$). $^{*}P < 0.05$, $^{**}P < 0.01$.

FXR and activation of Sp1 may be both involved in the down-regulation of RECK in the mouse model of NASH.

FXR is a well-known metabolic regulator and hepatoprotective factor. Activation of FXR could provide protection against cholestatic liver damage in rat cholestasis models [12]. Normal liver regeneration was proved to be regulated by and dependent on FXR [37]. $FXR^{-/-}$ mice display pronounced inflammation and spontaneously developed liver tumors at 15 months of age while no tumors were found in wild-type mice [38]. In addition, a previous report demonstrated that FXR agonist WAY-362450 attenuates hepatic inflammation and fibrosis in the mouse model of NASH induced by MCD diet [15]. However, the underlying mechanism for the anti-inflammation effects of FXR is not fully understood. In our study, we found that FXR agonist suppressed the activity of MMP-9, which has been reported to be up-regulated in the hamster model of NASH [39]. Since RECK negatively regulates the transcription and secretion of MMP-9 and directly inhibits its enzymatic activity [16,40], it is possible that FXR agonist may suppress the activity of MMP-9 through promoting the expression of RECK. And whether FXR agonist attenuates hepatic inflammation and fibrosis in mouse NASH model through FXR–RECK–MMP-9 cascade needs further investigation.

In summary, our study demonstrates that activation of FXR induced RECK expression *in vivo* and *in vitro*, through directly binding to FXR response element located within intron 1 of the mouse RECK gene. FXR agonist reversed the down-regulation of RECK in the livers from mice fed a methionine and choline deficient diet. Our data suggest that RECK is a novel transcriptional target of FXR in mouse liver, and provide clues to better understanding the anti-inflammation effects of FXR.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.11.082.

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