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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*.

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