HCBP6 upregulates human SREBP1c expression by binding to C/EBPβ-binding site in the SREBP1c promoter

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Sterol regulatory element-binding protein-1c (SREBP1c) plays an important role in triglyceride (TG) homeostasis. Although our previous study showed that hepatitis C virus core-binding protein 6 (HCBP6) regulates SREBP1c expression to maintain intracellular TG homeostasis, the mechanism underlying this regulation is unclear. In the present study, we found that HCBP6 increased intracellular TG levels by upregulating SREBP1c expression. HCBP6 increased SREBP1c transcription by directly binding to the SREBP1c promoter (at the −139- to +359-bp region). Moreover, we observed that HCBP6 interacted with C/EBPβ-binding site in the SREBP1c promoter both in vitro and in vivo. These results indicate that HCBP6 upregulates human SREBP1c expression by binding to the C/EBPβ-binding site in the SREBP1c promoter. [BMB Reports 2018; 51(1): 33-38]

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is one of the most common liver disorders worldwide and includes a series of disorders from liver steatosis to non-alcoholic steatohepatitis that eventually lead to liver cirrhosis and in some cases hepatocellular carcinoma (HCC) (1). In liver steatosis, triglycerides (TGs) accumulate in lipid droplets formed in the cytoplasm of hepatocytes, which leads to NAFLD (2). Thus, serum TG levels increase during NAFLD development (3).

Sterol regulatory element-binding proteins (SREBPs) contain approximately 1150 amino acids. SREBP precursors are bound to the endoplasmic reticulum (ER) (4). Upon activation, they release their active domain and translocate into the nucleus (5, 6). After entering the nucleus, SREBPs promote the transcription of various genes involved in lipogenesis and cholestero-gensis. Mammalian SREBP family contains three proteins, namely, SREBP1a, SREBP1c, and SREBP2, that function as important transcription factors. SREBP2 regulates cholesterol synthesis, SREBP1c regulates TG synthesis, and SREBP1a regulates constant cholesterol and TG synthesis (7-9). SREBPs are regulated through three major processes, i.e., (1) transcription, (2) proteolytic cleavage of SREBP precursors, and (3) post-translational modification. Various evidences suggest that SREBP1c is mainly regulated at the transcriptional level. Moreover, some studies have shown that NF-Y, LXR, Sp1, C/EBPβ, and E-box induce SREBP1c transcription (10-12).

We previously identified hepatitis C virus (HCV) core-binding protein 6 (HCBP6) (also called FUNDC2, HCC3, DC44, or PD03104) by performing yeast two-hybrid assay (13). Although our previous study showed that HCBP6 participates in SREBP1c-FASN-mediated TG accumulation, it is unclear whether HCBP6 interacts with the SREBP1c promoter (14).

The present study is the first to provide information on the mechanism underlying SREBP1c-mediated TG accumulation. In the present study, we analyzed HCBP6-induced activation of the SREBP1c promoter in HepG2 cells. Thus, our findings provide new insights on the regulation of SREBP1c expression by HCBP6.

RESULTS

HCBP6 upregulates SREBP1c expression and promotes TG synthesis in HepG2 cells

To investigate whether HCBP6 promoted SREBP1c expression, HepG2 cells were transfected with pcDNA-HCBP6 expression plasmid. Results of qRT-PCR and western blotting showed that pcDNA-HCBP6-transfected cells showed significant HCBP6 overexpression (P < 0.01; Fig. 1A, C). Furthermore, results of qRT-PCR showed that HCBP6 overexpression increased the
expression of SREBP1c and FASN (P < 0.05; Fig. 1B), and results of western blotting showed that HCBP6 overexpression significantly promoted expression of SREBP1c, p-ACC1, and FASN (P < 0.01; Fig. 1C). We next examined whether HCBP6 overexpression induced TG synthesis in HepG2 cells. As expected, HCBP6-overexpressing HepG2 cells showed TG synthesis (Fig. 1D, E), as indicated by increased intracellular TG content and results of oil red O staining.

HCBP6 silencing decreases SREBP1c expression and suppresses TG synthesis in HepG2 cells

To examine whether HCBP6 knockdown affected SREBP1c expression, we transfected HepG2 cells with a specific siRNA against HCBP6 (si-HCBP6) to silence HCBP6 expression. The efficacy of siRNA-induced HCBP6 silencing is shown in Fig. 2. HCBP6 mRNA and protein expression levels significantly decreased in si-HCBP6-transfected cells (Fig. 2A, C). Moreover, results of qRT-PCR and western blotting showed that SREBP1c, ACC1, p-ACC1, and FASN expression decreased significantly in si-HCBP6-transfected cells, which was consistent with the decrease in HCBP6 expression (P < 0.05; Fig. 2B, C). Moreover, intracellular TG levels decreased significantly in si-HCBP6-transfected cells compared with that in negative control siRNA (si-NC)-transfected cells (Fig. 2D). Results of oil red O staining showed that si-HCBP6 transfection significantly decreased lipid droplet accumulation (Fig. 2E) after 48 h. These results indicate that HCBP6 silencing decreases SREBP1c expression and suppresses lipid synthesis.

HCBP6 upregulates the transcriptional activity of the SREBP1c promoter in HepG2 cells

Deletion derivatives of the SREBP1c promoter named P-P6 (Fig. 3A, left) and these derivatives were respectively transiently transfected in HepG2 cells. The luciferase activity
of P was 40.36-fold higher than that of the pGL4.10-Basic vector (P = 0.015), and the luciferase activity of P2 was 23.80-fold higher than that of the pGL4.10-Basic vector (P = 0.015). Analysis of the luciferase activity showed that the SREBP1c promoter fragment containing these mutant promoter fragments. The basal activity of the mutation P2 promoter fragment was significantly lower than that of the wild-type P2 promoter fragment in absence or presence of 100-fold excess of the unlabeled probe or mutant biotin-labeled probe (left and right, respectively) were incubated with biotin-labeled probes. Results indicate that HCBP6 and C/EBPβ interacted with the CEBPβ-binding site in the SREBP1c promoter in vitro. (D) ChIP assay was performed using the EZ-Enzyme Chromatin prep kit and EZ-Chip kit. Chromatin solution was immunoprecipitated using 5 μg anti-His antibody, anti-C/EBPβ antibody or 5 μg normal anti-IgG antibody, followed by overnight incubation with 20 μl protein A agarose beads at 4°C. Next, the immunoprecipitated DNA was amplified by performing qRT-PCR targeting −236- to −37-bp region of the SREBP1c promoter. Total chromatin was used as a positive control, and normal rabbit IgG was used as a negative control. Results of the ChIP assay showed that both C/EBPβ and HCBP6 interacted with the SREBP1c promoter in HepG2 cells. Data are expressed as mean ± SEM. Statistically significant difference between the wild-type P2 and mut-C/EBPβ promoter fragments in absence or presence of pcDNA-C/EBPβ as determined by performing Student’s t-test (n = 3).

designated as +1).

Co-transfection with pcDNA-HCBP6 significantly increased the transcriptional activity of the P and P2 derivatives of the SREBP1c promoter (Fig. 3B). Analysis by using Genomatix software predicted the presence of several putative cis-acting elements in the P2 fragment of the SREBP1c promoter containing NF-κB, C/EBPβ, E-box, Sp1, and GATA-binding sites. We constructed a series of SREBP1c promoter fragments with mutations in these binding sites by performing site-directed mutagenesis and transfected HepG2 cells with vectors containing these mutant promoter fragments. The basal activity
of the mutant promoter fragments decreased to different degrees (Fig. 3C). However, cotransfection with pcDNA-HCBP6 increased the activity of all the mutant promoter fragments, except that of mut-C/EBPβ promoter fragment (Fig. 3D). These results indicate that HCBP6 overexpression increased the activity of the SREBP1c promoter by binding to the region from −109 to −106 bp, which is almost similar to the binding region for C/EBPβ.

**HCBP6 directly binds to the C/EBPβ-binding site in the SREBP1c promoter**

C/EBPβ enhanced the activity of the SREBP1c promoter. Moreover, introduction of specific mutations in the C/EBPβ-binding site inhibited the ability of C/EBPβ to stimulate the transcriptional activity of the SREBP1c promoter (Fig. 4A).

Furthermore, both C/EBPβ and HCBP6 were conserved among mammals (Fig. 4B). Next, we performed electrophoretic mobility shift assay (EMSA) by using nuclear protein extracts (NPS) of HepG2 cells to determine whether HCBP6 interacted with the −109- to −106-bp region of the SREBP1c promoter in vitro. A biotin-labeled probe led to the formation of a DNA-protein complex. The presence of supershifted bands when an anti-His or anti-C/EBPβ antibody was added confirmed the specificity of interaction (Fig. 4C). The overexpression of HCBP6 protein group produced the largest amount of complexes (Fig. 4D, left, lane 5) and non-transfected protein group came second (Fig. 4D, left, lane 3), and si-HCBP6 protein group came the least complex (Fig. 4D, left, lane 4). To assess the specificity of this protein-DNA interaction, cross-competition using a 100-fold molar excess of unlabeled probe (Fig. 4D, left, lane 2) and mutant biotin-labeled probe (Fig. 4D, left, lane 6) containing the C/EBPβ-binding site from SREBP1c promoter was performed, resulting in reduction amounts of complex formation. Importantly, these results are consistent with C/EBPβ-binding site from SREBP1c promoter spanning −109- to −106-bp (Fig. 4D, right).

Chromatin immunoprecipitation (ChIP) assay to determine whether HCBP6 interacted with the −109- to −106-bp region of the SREBP1c promoter in vivo in HepG2 cells. Chromatin was immunoprecipitated using anti-His antibody, anti-C/EBPβ antibody or 5 μg normal anti-IgG antibody, and DNA fragments of expected size were used as templates for performing qRT-PCR (Supplementary Table 1). Results of the ChIP assay showed that both HCBP6 and C/EBPβ interacted with the −109- to −106-bp region of the SREBP1c promoter (Fig. 4E).

**DISCUSSION**

The HCV core protein performs different functions. To elucidate its biological role, we previously screened cellular proteins that directly interacted with the HCV core protein by using a human liver cDNA library (13). We found that many proteins interacted with the HCV core protein, including apolipoprotein A1, apolipoprotein A2, and translin (15). Based on these findings, we focused on the role of HCV infection in the development of liver steatosis (16, 17). We found that the HCV core protein modulated cholesterol homeostasis through SREBP2 pathway (18). Moreover, several studies have shown that the HCV core protein plays an important role in the development of HCV-induced liver steatosis (19-21). In our previous study, we performed the yeast two-hybrid assay to determine the role of new genes, particularly HCBP6, in the development of liver steatosis; however, biological functions of proteins encoded by these genes are unclear. Our previous study showed that HCBP6 participated in cell proliferation, signal transduction, growth, and differentiation (22). Therefore, we have validated that HCBP6, an HCV core-binding protein, affected lipid metabolism.

In the present study, we overexpressed and knocked down HCBP6 in HepG2 cells to determine whether HCBP6 promoted intracellular lipogenesis through SREBP1c pathway (Fig. 1 and 2). We found that HCBP6 promotes lipogenesis and that SREBP1c is a potential target and is positively regulated by HCBP6. Next, we identified HCBP6-binding site in the SREBP1c promoter. Bioinformatics analysis showed that HCBP6 interacted with the C/EBPβ-binding site in the SREBP1c promoter (Fig. 3C). We confirmed the interaction between HCBP6 and the C/EBPβ-binding site in the SREBP1c promoter by isolating the 5'-flanking region of SREBP1c from the DNA of HepG2 cells and by performing a series of experiments, including the luciferase reporter assay, site-directed mutagenesis, EMSA, and ChIP assay (Fig. 3 and 4).

SREBP1c plays a key role in energy homeostasis by regulating lipogenesis in the liver (23, 24). SREBP1c regulation in the liver has been extensively studied, and factors such as LXR, Sp1, NF-Y, and E-box are known to induce SREBP1c in hepatocytes (11, 25-27). In the present study, we performed site-directed mutagenesis to confirm that C/EBPβ interacted with the −109- to −106-bp region of the SREBP1c promoter to modulate SREBP1c transcription (Fig. 4A). Results of EMSA and ChIP assay performed in the present study indicate that HCBP6-related factors perform a direct key role in the regulation of SREBP1c expression by binding to the C/EBPβ-binding site in the SREBP1c promoter (Fig. 4D, E). Previous studies have shown that HCBP6 is located in the nucleus (28, 29). Thus, the results of the present study suggest that HCBP6 functions as a transcription factor to regulate SREBP1c expression both in vitro and in vivo. Moreover, we found that both C/EBPβ and HCBP6 are a part of a protein complex that binds to the SREBP1c promoter.

C/EBPβ belongs to CCAAT/enhancer-binding protein family that includes basic leucine zipper transcription factors that regulate lipid metabolism in the liver by regulating transcription and translation (30, 31). In addition, C/EBPβ is a pioneer transcription factor that binds to the PPARY promoter and induces PPARY expression to activate liver steatosis and inflammation (32, 33). In addition to its role in inducing the PPARY promoter to activate lipogenesis in the liver, C/EBPβ
binds upstream of 368 nucleotides in the SREBP1c promoter (34). We also found that C/EBPβ interacted with the SREBP1c promoter to induce TG accumulation. Particularly, we observed that mutation of the C/EBPβ-binding site in the SREBP1c promoter, overexpression C/EBPβ can still enhance SREBP1c promoter activity, but the ability to enhance SREBP1c promoter activity has been decreased (Fig. 4A). Thus, our results suggest that C/EBPβ is important for regulating SREBP1c transcription and there is more than one of C/EBPβ-binding-sites in the SREBP1c promoter.

While our previous study showed that HCBP6 inhibited the SREBP1c pathway to reduce TG level, results of the present study indicate that HCBP6 promotes SREBP1c transcription to increase lipid accumulation (14). This finding indicates that HCBP6 and SREBP1c are a part of a complex inter-regulatory network and not a fixed canonical cascade and are activated by factors such as extracellular signals and differentiation status of involved cells (30).

In summary, the present study is the first to show that HCBP6 induces SREBP1c expression to increase TG levels in HepG2 cells by directly interacting with the C/EBPβ-binding site in the SREBP1c promoter. These results also suggest that the effect of HCBP6 on lipogenesis in the liver is partially mediated by the increase in SREBP1c transcription. However, further studies are required to explore a systematic and comprehensive study that aims to demonstrate the regulatory mechanism by C/EBPβ and HCBP6 on same region of SREBP1c promoter.

MATERIALS AND METHODS
Detailed information is in the Supplementary Material.

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CONFLICTS OF INTEREST
The authors have no conflicting interests.

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