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Hepatitis C virus regulates proprotein convertase subtilisin/kexin type 9 promoter activity

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ABSTRACT

Proprotein convertase subtilisin/kexin type 9 (PCSK9) is a secretory serine protease mainly expressed in liver. Although PCSK9 has been shown to inhibit hepatitis C virus (HCV) entry and replication, whether HCV regulates PCSK9 transcription has not been well studied. PCSK9 promoter activity is modulated by numerous transcription factors including sterol-regulatory element binding protein (SREBP)-1a, -1c, −2, hepatocyte nuclear factor-1 (HNF-1), and forkhead box O3 (FoxO3). Since they are differently regulated by HCV, we studied the effects of these transcription factors on PCSK9 promoter activity in the context of HCV infection and replication. We demonstrated that PCSK9 promoter activity was up-regulated after HCV infection and in HCV genomic replicon cells. We also studied the effects of HCV proteins on the PCSK9 promoter activity. While HCV structural proteins core, E1, and E2 had no effect, NS2, NS3, NS3-4A, NS5A and NS5B enhanced, and p7 and NS4B decreased PCSK9 promoter activity. Furthermore, we showed that transcription factors SREBP-1c, HNF-1α and specificity protein 1 increased PCSK9 promoter activity in HCV replicon cells, whereas SREBP-1a, HNF-1β and FoxO3 had an inhibitory effect. These results demonstrated the molecular mechanisms of how HCV modulates PCSK9 promoter activity and advanced our understanding on the mutual interactions between HCV and PCSK9.

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1. Introduction

Proprotein convertase subtilisin/kexin type 9 (PCSK9), a secretory serine protease in the proprotein convertase family, is mainly expressed in liver, but also found in intestine, kidney and brain [33,34]. Since it enhances lysosome-mediated degradation of low-density lipoprotein receptor (LDLR), it indirectly up-regulates low-density lipoprotein cholesterol (LDLC) in plasma and is associated with hypercholesterolemia [19,44]. PCSK9 is also involved in regulating lipoprotein synthesis, glucose metabolism, neurological function, inflammation and infection [21,32]. The level of PCSK9 is regulated by intracellular cholesterol concentration [22]. Accordingly, numerous transcription factors involved in maintaining lipid homeostasis can regulate PCSK9 promoter activity. For example, sterol-regulatory element binding protein (SREBP)-1a, -1c, −2 and hepatocyte nuclear factor-1α (HNF-1α) have been shown to differentially regulate PCSK9 transcription [6,7,15]. Other transcription factors, such as forkhead box O3 (FoxO3) and specificity protein 1 (Sp1), are also involved in regulating PCSK9 promoter activity [4,12,40].

Hepatitis C virus (HCV) is a single-stranded RNA virus that affects about 2−3% world population and causes severe liver damage [17,41]. It has seven genotypes and its positive-sense genome encodes a polyprotein. The polyprotein is processed to generate structural proteins core, envelope proteins E1 and E2, and non-structural proteins p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B [9,20,38]. A complex interplay between HCV and host lipid metabolism has been identified during the HCV life cycle [26,28]. Accordingly, numerous viral proteins have been reported to be able to regulate transcriptional factors involved in maintaining host lipid homeostasis. HCV-3a core, 1a NS2, 1b, 2a and 3a NS4B, and 3a NS5A are reported to activate SREBP-1 [11,23,25,47,50]. HCV-2a infection can induce SREBP-1, -2 and FoxO3 [2,45,47].

Abbreviations: DN, dominant negative; FoxO3, forkhead box O3; HCV, hepatitis C virus; HCVcc, cell culture-derived HCV; HNF, hepatocyte nuclear factor; LDLC, low-density lipoprotein cholesterol; LDLR, low-density lipoprotein receptor; PCSK9, proprotein convertase subtilisin/kexin type 9; Sp1, specificity protein 1; SRE, sterol-regulatory element; SREBP, sterol-regulatory element binding protein.

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Interestingly, different HCV genotypes show different regulatory effects on HNF-1. Qadri et al. demonstrated an up-regulation of HNF-1 by HCV-1b [29], whereas Matsui et al. reported a down-regulation of HNF-1α by HCV-2a [18]. Taken together, these studies demonstrate that HCV utilizes multiple means to regulate transcription factors involved in host lipid metabolism.

We and others have demonstrated that PCSK9 can inhibit HCV entry and replication [14,16,39]. However, the effect of HCV on PCSK9, especially on its promoter activity, has not been well characterized. As such, we studied the effect of HCV-2a on the PCSK9 promoter activity.

2. Material and methods

2.1. Plasmids

Human PCSK9 -440 promoter (−440 to −94)-luciferase reporter, wild-type or with sterol-regulatory element (SRE) or HNF-1 binding sequence mutations were received from Dr. Liu [15]. PCSK9 -440 promoter-luciferase reporters containing Sp1 binding site mutations were received from Dr. Park [12]. To generate plasmids expressing HCV-2a proteins or GST with a Flag tag, the respective coding sequences were amplified by PCR using plasmid pFLneo-J6/JFH-1(p7-rLuc-2A) provided by Dr. Rice [13] or pGEX-5X-1 (GE Healthcare Life Sciences) as the templates and cloned into the pEF vector. Plasmids expressing Flag-tagged SREBP-1a, -1c, −2 and dominant negative (DN) SREBP-1 were described previously [36]. Plasmids expressing GST, GST-DN Sp1, or full-length Sp1 were received from Drs. Thiel and Suske, respectively [27,31,42]. Plasmid expressing Myc-tagged Sp1 was generated by cloning the Sp1 cDNA with a Myc-tag into pcDNA3.1 vector (Thermo Fisher Scientific). FR_HNF-1α [35] and FR_HNF-1β [43] were gifts from Dr. Ryffel (Addgene plasmids #31104 and #31101). The coding sequences of HNF-1α and HNF-1β were cloned into the p3xFlag CMV7.1 vector (Sigma-Aldrich), resulting in plasmids expressing Flag-tagged HNF-1α and HNF-1β. A DN HNF-1α-expressing plasmid was generated by cloning the coding sequence of aa. 1–280 [48] into the p3xFlag CMV7.1 vector. Flag-FoxO3a was a gift from Dr. Greenberg (Addgene plasmid #8360) [3]. Sequences encoding full-length or aa. 1–304 (DN) [24,30,46] FoxO3 were also cloned into the p3xFlag CMV7.1 vector.

2.2. Cell lines, transfection, HCV infection

Huh-7 cells were cultured in Dulbecco's Modified Eagle Medium (Sigma-Aldrich) with 10% (v/v) fetal bovine serum (Sigma-Aldrich) at 37 °C and 5% CO_2. Huh-7 replicon cells with HCV-2a J6/JFH-1(p7-RLuc2A) or HCV-2a J6 core^{Flag}/JFH-1(p7-RLuc-2A) replicating RNAs were described previously [10,49]. DNA transfection was carried out using calcium phosphate or the jetPEI reagent (Polyplus) according to the manufacturer's protocol. Huh-7 cells were infected with cell-culture derived HCV-2a J6 core^{Flag}/JFH-1(p7-RLuc-2A) (HCVcc) as previously described [49]. Mithramycin A (Sigma-Aldrich) was used as previously described [50].

2.3. Luciferase assay and western blotting

Cells were lysed in Passive Lysis Buffer (Promega) or SDS sample buffer 48 h after transfection. Luciferase assay and Western blotting were performed as previously described [49]. The antibodies used were: GST (Cell Signaling Technology, CST), Myc (CST), β-actin (CST), Flag (Sigma-Aldrich), and secondary antibodies IRDye 800CW goat anti-mouse IgG and IRDye 680RD goat anti-rabbit IgG (Li-Cor Biosciences).

2.4. Statistical analysis

All experiments were performed in triplicate. The luciferase assay data were analyzed using GraphPad Prism 7. Statistical differences were determined by Student's *t*-test or one-way ANOVA, and indicated as * if $p < 0.05$, ** if $p < 0.01$, or *NS* for not significant.

3. Results and discussion

3.1. HCV up-regulates PCSK9 promoter activity

To determine the effect of HCV infection on the PCSK9 promoter activity, Huh-7 cells were transfected with PCSK9 -440 promoter-luciferase reporter and then infected with HCVcc at 8 h after transfection. Luciferase assay performed at 48 h after infection demonstrated significantly higher luciferase activities conferred by the PCSK9 -440 promoter after HCV infection in comparison to mock infection (Fig. 1A). This result indicates that HCV infection up-regulates PCSK9 promoter activity.

To determine whether HCV replication is sufficient to activate PCSK9 promoter, we transfected Huh-7-HCV-2a J6/JFH-1(p7-RLuc2A) genomic replicon cells with the PCSK9 promoter-luciferase reporter plasmid. Huh-7 cells were also transfected as a control. Fig. 1B showed that PCSK9 -440 promoter activity was elevated in HCV replicon cells compared to that in Huh-7 cells, indicating PCSK9 promoter activation by HCV replication.

Then we studied the effects of HCV proteins on the PCSK9 promoter activity. For this purpose, we cloned the coding sequences for individual HCV proteins in an expression vector. While the expression of all the other HCV proteins could be readily demonstrated by Western blotting after transfection, the expression of the E1 protein could only be detected after deleting the C-terminal transmembrane domain (ΔTM, Δaa. 159–192) [8] (Fig. 1D and data not shown). To determine the effects of HCV proteins on PCSK9 promoter activity, Huh-7 cells were co-transfected with plasmids expressing HCV proteins and the PCSK9 -440 promoter-luciferase reporter. Plasmid vector and GST-expressing plasmid were used as controls. Fig. 1C showed that the PCSK9 promoter activity increased when co-transfected with NS2, NS3, NS3-4A, NS5A or NS5B plasmids, while p7 or NS4B expression inhibited PCSK9 promoter activity. The three structural proteins had no effect. These results indicate that HCV proteins differently regulate PCSK9 promoter activity. Future studies are required to elucidate the underlying mechanisms by investigating the regulatory effects of HCV proteins on the transcription factors involved in PCSK9 transcription.

In summary, HCV infection and replication could up-regulate PCSK9 promoter activity with the involvement of several HCV proteins.

3.2. The role of SREBPs in PCSK9 promoter regulation by HCV

The PCSK9 -440 promoter contains an SRE (−345 to −337) sequence and SREBPs have been shown to regulate PCSK9 promoter activity [12]. Given the fact that HCV can up-regulate the activity of SREBPs, we hypothesized that SREBPs were involved in PCSK9 promoter activation by HCV. To test this, we used a mutant PCSK9 -440 promoter without the SRE sequence for transfection into HCV replicon cells. As shown in Fig. 2A, luciferase activity conferred by the PCSK9 -440 mSRE promoter decreased significantly than the wild-type, suggesting that the SRE sequence is essential.

There are three SREBP proteins: SREBP-1a, SREBP-1c and SREBP-2 [37]. SREBP-1a and -1c only differ in the N-terminal transactivation domain, whereas SREBP-2 is a separate protein [37,51]. To determine the effect of individual SREBPs on PCSK9 promoter

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