Molecular Mechanism Underlying the Suppression of CPB2 Expression Caused by Persistent Hepatitis C Virus RNA Replication

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The mechanisms of hepatitis C virus (HCV)-associated hepatocarcinogenesis and disease progression are unclear. We previously observed that the expression level of carboxypeptidase B2 (CPB2) gene was remarkably suppressed by persistent HCV RNA replication in human hepatoma cell line Li23-derived cells. The results of the present study demonstrated that the CPB2 expression in patients with chronic hepatitis C was inversely correlated with several risk factors of hepatic fibrosis or steatosis, although ectopic CPB2 expression did not suppress the expression of fibrogenic or lipogenic genes. The suppressed CPB2 expression was restored by treatment with 5-azacytidine. To clarify the mechanism underlying this phenomenon, we analyzed the CPB2 promoter, and the results revealed that (1) hepatocyte nuclear factor 1 (HNF1), especially HNF1\textsubscript{a}, was essential for the CPB2 promoter, and (2) CPB2 promoter was not methylated by persistent HCV RNA replication. The expression levels of HNF1\textsubscript{a} and HNF1\textsubscript{b} were also not changed by persistent HCV RNA replication. These results suggest the existence of 5-azacytidine-inducible or -reducible unknown factor(s) that can control the CPB2 expression. To evaluate this idea we performed a microarray analysis, and several gene candidates corresponding to the suggested factor(s) were identified.

Key words: persistent hepatitis C virus replication, carboxypeptidase B2, suppression mechanism of CPB2 expression, DNA methylation, hepatocyte nuclear factor 1

Hepatitis C virus (HCV), an enveloped positive single-stranded RNA (9.6-kb) virus belonging to the Flaviviridae family, is a causative agent of chronic hepatitis C, which frequently progresses to liver cirrhosis and hepatocellular carcinoma (HCC) \cite{1-3}. The understanding of the life cycle of HCV has greatly progressed \cite{4} since the development of a cell-based HCV replicon \cite{5}, genome-length HCV RNA replication \cite{6}, and infectious HCV production \cite{7} systems, whereas the mechanisms of HCV-associated hepatocarcinogenesis and disease progression are still unclear.

We developed an HCV replicon \cite{8} and genome-length HCV RNA replication \cite{9} systems. Since most of the existing HCV replication systems were devel-

Conflict of Interest Disclosures: No potential conflict of interest relevant to this article was reported.
oped using a human hepatoma cell line, *i.e.*, HuH-7-derived cells, we developed our HCV replicon and genome-length HCV RNA replication system using a new human hepatoma cell line, Li23-derived cells [10], whose gene expression profile is considerably distinct from that of HuH-7-derived cells [11]. In that study [10], we established Li23-derived genome-length HCV RNA (O strain of genotype 1b)-replicating cell lines, *i.e.*, OL (polyclonal), OL8 (monoclonal), OL11 (monoclonal), and OL14 (monoclonal) cell lines. Using these cell lines, we recently demonstrated that long-term (4 years) cultured HCV RNA-replicating cells can be useful for analyses of evolutionary dynamics and variations of HCV and for drug resistance analyses [12].

During the course of the study, we speculated that the long-term replication of HCV RNA might cause irreversible changes in the expression profiles of particular genes in host cells. To test this speculation, we carried out several cDNA microarray analyses using OL, OL8, OL11, and OL14 cells and their long-term (>2 years) cultured cells, and we observed that the expression level of carboxypeptidase B2 (*CPB2*) gene was irreversibly downregulated by long-term persistent HCV RNA replication [13]. No significant changes in the expression level of *CPB2* in the long-term (>2 years) cultured cured cells, from which the HCV RNA had been eliminated by interferon treatment, were confirmed [13]. In addition, no significant changes in the expression levels of these genes by the ectopic expression of HCV proteins were also confirmed [13].

*CPB2*, known as thrombin-activatable fibrinolysis inhibitor [14], is produced mainly by the liver and circulates in plasma as a plasminogen-bound zymogen [15]. It has been reported that *CPB2* also has an anti-inflammatory function based on its cleavage of several proinflammatory mediators such as C5a and osteopontin [16, 17]. Since C5 is known to be a quantitative trait gene that modifies liver fibrogenesis in humans [18], we suspected that the suppression of *CPB2* expression observed in our earlier study might be involved in liver fibrogenesis. In fact, the suppression of *CPB2* expression has been reported in patients with liver cirrhosis, although it was not an HCV-associated phenomenon [19].

In addition, it was reported that *CPB2* expression is regulated by several transcription factors including hepatocyte nuclear factor 1 homeobox A (*HNF1α*) [20]. *HNF1α* is an important transcription factor for the hepatocyte-specific expression of albumin, insulin-like growth factor 1 and so on [21]. To the best of our knowledge, there is no report regarding the relationship between the level of *HNF1α* expression and HCV-associated diseases. Therefore, in the present study we focused on the relationship between *CPB2* expression and *HNF1α*. We report a novel mechanism underlying the suppression of *CPB2* expression that occurs by persistent HCV RNA replication.

### Materials and Methods

**Cell culture.** Li23-derived OL8 and OL11 cells harboring genome-length HCV RNA were cultured with medium in the presence of 0.3 mg/ml of G418 (Gentric, Invitrogen, Carlsbad, CA, USA) as described [13]. In the present study, these cells were renamed according to the length of the culture period; *i.e.*, OL8(0Y), OL8(0.5Y), OL8(1Y), OL8(1.5Y), and OL8(2Y) cells were OL8 cells cultured for 0, 0.5, 1, 1.5, and 2 years, respectively. Cured cells (OL8c(0Y) cells) were also cultured with medium in the absence of G418 as described [13]. Two-year cultures of OL8c(0Y) cells were designated as OL8c(2Y).

**Reagents.** 5-azacytidine (5-azaC) and 4-phenylbutyric acid (4-PBA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Blasticidin S was purchased from Funakoshi (Tokyo).

**Quantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis.** Total RNAs from the cells were prepared using an RNeasy extraction kit (Qiagen, Hilden, Germany). The quantitative RT-PCR analysis for mRNA was performed as described [13]. Briefly, total RNA (1 μg) was reverse-transcribed with M-MLV reverse transcriptase (Invitrogen) using oligo dT primer (Invitrogen). One-tenth of the cDNA was used for the quantitative PCR analysis using a real-time LightCycler PCR (Roche Diagnostics, Basel, Switzerland).

The following primer sets were used for the quantitative PCR: collagen type I, alpha 1 (*COL1A1*: fibrogenic gene), 5'-AGAGCTCGTTGATCCCCACTCAG-3' and 5'-TAGGCCACGCTTTCCTGGCAGTG-3'; collagen type IV, alpha 1 (*COL4A1*: fibrogenic gene), 5'-AGCACAATGGCCCTTCCCTTCTG-3' and
5'-TGGCGCActTtCtaACTctcctcAG-3'; acetyl-CoA carboxylase alpha (ACCl: lipogenic gene), 5'-CATCCACtTTgCtGaCGATTTGG-3' and 5'-GTTTtCCTCTATtaCGaGTAaACaC-3'; acetyl-CoA carboxylase beta (ACC2: lipogenic gene), 5'-CGaACTCTGTCTCTaAgACCaCATaCAG-3' and 5'-TGgTaAcAGGAGGaGTaAC-G3'; fatty acid synthase (FASV: lipogenic gene), 5'-GAaACtGCaGgAGCTGTG-3' and 5'-CaCGGAGTtTGAGGCaGCAT-3'; stearoyl-CoA desaturase 1 (SCD1: lipogenic gene), 5'-GTTTtACtTTGaGCTGTG-3' and 5'-TTgAtGTGCaAGGtGCTACT-3'; HNF1a, 5'-CTtCCACaCGGCaGCATaCTaCAG-3' and 5'-GTGGAGaTAGGCTCtGaGAc-3'; HNF1 homeobox B (HNF1b), 5'-AGtGCaGACGCtCCCAaCCaG-3' and 5'-GAaACaTAGGTtGTGtGaCT-3'; actin, beta (β-actin), 5'-GAaAGAGCaGCaGCTGCtGAC-3' and 5'-GTGAtCTCtCtCTCtG CATCtCGTC-3'.

The primer sets for CPB2 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were as described [13]. Endogenous CPB2 mRNA was quantified using the same forward primer as described above and a reverse primer (5'-ACGGAACaGaATGATAAAA TCaG-3') arranged from the 3' untranslated region of CPB2 mRNA. GAPDH mRNA was quantified as a loading control except for the experiments of 5-azaC treatment, in which β-actin mRNA was quantified as a loading control. Experiments were done in triplicate.

Western blot analysis. The preparation of cell lysates, the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and the immunoblotting analysis with a PVDF membrane were as described [8]. The antibodies used in this study were sheep anti-CPB2 antibody (SATAFI-AP; Affinity Biologics, Ancaster, ON, Canada), rabbit polyclonal anti-HNF1α antibody (GeneTex, Hsinchu City, Taiwan), rabbit polyclonal anti-HNF1β antibody (Proteintech, Chicago, IL, USA), and mouse monoclonal anti-β-actin antibody (AC-15; Sigma-Aldrich). β-actin antibody was used for the loading control.

5-azaC and/or 4-PBA treatment. OL8(0Y), OL8(2Y), OL8(3.5Y), or OL8(4Y) cells were treated with 5-azaC (2.5 or 10 μM) or 4-PBA (1mM), or a combination of 5-azaC (2.5 or 10 μM) and 4-PBA (1mM). Total RNAs prepared from the treated cells were subjected to quantitative RT-PCR for CPB2 mRNA and β-actin mRNA as a control.

Gene promoter analysis. Genomic DNAs from OL8(0Y) cells were prepared using DNeasy (Qiagen) as described [22]. According to the putative transcriptional start site [20], we used PCR to amplify various lengths of 5' flanking region containing the 5' end (21 bp) of CPB2 (NT_024524.14; −2700, −989, −609, −200, −150, −91, −75 or −52 to +21), using KOD-plus DNA polymerase (Toyobo, Osaka, Japan). The PCR products were inserted into the Neol-HindIII site on pGL4.10-luc2 (Promega, Madison, WI, USA). pGL4.10-luc2 possessing serially truncated CPB2 promoter and pRL-SV40 (internal control) were cotransfected into OL8(0Y) or OL8(2Y) cells by FuGENE HD (Promega). Two days later, a dual luciferase assay was performed as described [22, 23]. Experiments were done in triplicate.

Bisulphite sequencing analysis. We conducted a bisulphite treatment of genomic DNAs from OL8(0Y), OL8(2Y), or OL8(4Y) cells using a MethylEasy™ Xceed Rapid DNA Bisulphite Modification Kit (Takara Bio, Otsu, Japan). A set of primers (5'-GGTTGTtGGTtGtATTGTaAATTAAaAaCtG-3' and 5'-TTGATtGTGCaAGGCtGcACT-3') was used to amplify the CPB2 promoter region (307 bp) using TaKaRa EpiTel HS (for bisulphite-treated DNA; Takara Bio). The PCR product was inserted into T-vector pMD19(Simple) and then subjected to the sequencing analysis as described [12].

Site direct mutagenesis. Using QuickChange mutagenesis (Stratagene, La Jolla, CA, USA) [6], we introduced 3 types of mutation into the plasmids that were made for the analysis of CPB2 promoter as described above.

RNA interference. Small interfering RNA (siRNA) duplexes targeting HNF1α (M-008215-01-0005; GE Dharmacon, Lafayette, CO, USA) and HNF1β (M-009721-01-0005; GE Dharmacon) were chemically synthesized. Non-targeting siRNA (D-001206-13-20) and siRNA targeting Rab18 (M-010824-00-0005; Thermo Fisher Scientific, Waltham, MA, USA) were used as controls [24]. OL8(0Y) cells were transfected with the siRNA duplexes using RNAiMAX (Invitrogen).

Ectopic gene expression. The open reading frame (ORF) of CPB2 (NM_001872.3) was amplified by PCR using KOD-plus DNA polymerase, and then the PCR product was inserted into MluI–NolI site of pCX4bser. Retroviral transfer to OL8(2Y) cells and
the selection by blasticidin S were performed as described [25]. The ORFs of aldehyde dehydrogenase 1 family, member A1 (ALDH1A1; NM_000689.4), albumin (ALB; NM_000477.5), annexin A1 (ANXA1; NM_000700.1), fibrinogen gamma chain (FGG; NM_000509.4), fibrinogen beta chain (FGB; NM_005141.4), tissue factor pathway inhibitor (TFPI; NM_006287.4), fibrinogen-like 1 (FGL1; NM_004467.3), and alpha-2-HS-glycoprotein (AHSG; NM_001622.2) were also amplified by PCR and inserted into pCX4bsr as described above.

The ORFs of glutathione peroxidase 2 (GPX2; NM_002083.3), variable charge, X-linked 2 (VCX2; NM_016378.3), and anterior gradient 2 (AGR2; NM_006408.3) were amplified by PCR, and then each PCR product was inserted into the EcoRI-MluI site of pCX4bsr. The ORFs of synovial sarcoma, X breakpoint 4 (SSX4; NM_005636.3) and zinc finger with KRAB and SCAN domains 7 (ZKSCAN7; NM_018651.3) were also amplified by PCR, and then each PCR product was inserted into the EcoRI-NotI site of pCX4bsr. The constructed plasmid possessing GPX2 ORF was transfected into OL8(0Y) cells using FuGENE HD. The other constructed plasmids were also transfected into OL8(2Y) cells using FuGENE HD. After culturing for 48h, total RNAs prepared from the cells were subjected to a quantitative RT-PCR analysis for CPB2 mRNA.

**Immunofluorescence analysis.** The intracellular localization of HNF1α was visualized and photographed under a confocal microscope as described [24].

**mRNA and miRNA microarray analyses.** Total RNAs from OL8(2Y) and those from OL8c(2Y) cells treated with or without 5-azaC (10μM) for 1 week were prepared using an RNeasy kit for the cDNA microarray analysis, and total RNAs from OL8(0Y), OL8(2Y), OL11(0Y), and OL11(2Y) cells were prepared using the miRNeasy mini kit (Qiagen) for the microRNA microarray analysis. As described [10, 22], both of the array analyses were carried out by the Dragon Genomics Center of Takara Bio through an authorized Affymetrix and Agilent Certified service provider, using the GeneChip Human Genome U133 Plus 2.0 Array or the Agilent Human miRNA microarray (Rel. 16.0).

**Statistical analysis.** The significance of differences among groups was determined using Student’s t-test with a two-sided test. P-values < 0.05 were considered significant.

**Results**

**The expression level of CPB2, suppressed by persistent HCV RNA replication, was inversely correlated with the status of hepatic diseases.** In our earlier study we observed that the CPB2 expression was greatly suppressed by the 2-year persistent HCV RNA replication in Li23-derived cells [13]. Here we first investigated the time point during the 2 years at which the CPB2 expression was suppressed. Our quantification of CPB2 mRNA in OL8(0Y), OL8(0.5Y), OL8(1Y), OL8(1.5Y), and OL8(2Y) cells revealed that the CPB2 expression was significantly suppressed between 6 months and 1 year of HCV RNA replication (Fig. 1). This result suggests that something occurs in the transcriptional control mechanism of the host cells by the HCV RNA replication for more than 6 months. Such a persistent HCV RNA replication suggested a state of chronic hepatitis C, and using the clinical data and the gene expression profiles of 91 patients with chronic hepatitis C [26], we therefore next examined the relationship between several of the patients’ clinical factors and their hepatic expression levels of CPB2.

![Fig. 1](image_url) The downregulation of CPB2 expression occurred mainly between 6 months and 1 year of persistent HCV RNA replication. We quantified the expression level of CPB2 mRNA in OL8(0Y), OL8(0.5Y), OL8(1Y), OL8(1.5Y), and OL8(2Y) cells using a real-time LightCycler PCR. Experiments were performed in triplicate. **p < 0.01.
The analysis revealed that the expression level of CPB2 was significantly and inversely correlated with the patients' fibrotic stages, which indicate F1 as mild hepatic fibrosis to F4 as cirrhosis (Fig. 2A). We also observed that the expression level of CPB2 was low in the patients with a body mass index (BMI) value > 25, which is a risk value for the development of hepatic diseases including steatosis (Fig. 2B). The results of our analysis demonstrated that the expression level of CPB2 was correlated with the number of platelets (Fig. 2C) and inversely correlated with the markers of the status of hepatic damage: aspartate transaminase (AST; Fig. 2D), alanine transaminase (ALT; Fig. 2E), and gamma-glutamyl transferase (γ-GTP; Fig. 2F). These results suggest that the suppression of CPB2 expression is involved in the hepatic fibrosis or steatosis.

However, the ectopic expression of CPB2 in the OL8(2Y) cells (Fig. 3A) did not affect the expression levels of fibrogenic genes such as COL1A1 and COL4A1 (Fig. 3B) or lipogenic genes such as ACC1, ACC2, FASN, and SCD1 (Fig. 3C), suggesting that the expression levels of these genes are not regulated only by CPB2.

**The suppression of CPB2 expression in long-term cultured OL8(0Y) cells was cancelled by 5-azaC treatment.** Since the marked suppression of CPB2 expression occurred between 6 months and 1 year of culture, we first suspected that a somatic mutation in the CPB2 locus caused the transcriptional suppression. However, such a mutation was not detected at the 2-kb upstream from the transcription initiation site of CPB2 derived from OL8(2Y) cells (data not shown). In addition, no mutation was detected in the ORF of CPB2 including both the 5'- and 3' untranslated regions derived from OL8(2Y) cells (data not shown). We therefore speculated that an epigenetic alteration such as DNA methylation occurred during the long-term culture of OL8(0Y) cells.

To test this speculation, we examined the effect of 5-azaC treatment on the CPB2 expression. Using the OL8(0Y), OL8(2Y), and OL8(4Y) cells treated with 5-azaC for 1 week, we quantified the level of CPB2 mRNA in these cells by a quantitative RT-PCR analysis, which revealed that the expression levels of CPB2 in OL8(2Y) and OL8(4Y) cells were recovered to the same level as that of OL8(0Y) cells (Fig. 4A). We also observed that the level of CPB2 expression in OL8(0Y) cells was not further elevated by 5-azaC treatment (Fig. 4A). These results suggest that persistent HCV RNA replication suppresses CPB2 expression through DNA methylation.

We further examined the epigenetic effect on the expression level of CPB2 using OL8(0Y), OL8(2Y), and OL8(3.5Y) cells treated with 5-azaC and/or 4-PBA (histone deacetylase inhibitor). The results revealed that the treatment with 4-PBA or its combination with 5-azaC did not affect the expression level of CPB2 in these cells (Fig. 4B, C). These results suggest that histone deacetylation is not involved in the suppression of CPB2 expression.

**The CPB2 promoter was not methylated by persistent HCV RNA replication.** Before the detailed analysis of the methylation state of CPB2 promoter, we attempted to identify the essential domain for CPB2 promoter activity. Using luciferase reporter plasmids containing CPB2 promoters systematically truncated from −2700 (Fig. 5A), we carried out the promoter assay in OL8(0Y) cells. The results revealed that the region from −150 to −52 was a main domain for CPB2 promoter activity (Fig. 5A). Similar results of the promoter assay were also obtained using OL8(2Y) and OL8c(0Y) cells (data not shown).

These findings revealed that the number of CpG sites in the region from −150 to −52 was only one (C is located at −82). To clarify whether the methylation of this CpG site caused the suppression of CPB2 expression, we examined the methylation status of this CpG site in OL8(0Y), OL8(2Y), and OL8(4Y) cells by the bisulphite sequencing method. We obtained the unexpected result that the methylation frequencies for C at −82 in the OL8(2Y) and OL8(4Y) cells were only 4% and 8%, respectively (Fig. 5B), whereas that in the OL8(0Y) cells was 0%. Taken together, these results and those described above suggest that the suppression of CPB2 expression by the persistent HCV RNA replication is caused by a DNA methylation event other than the methylation of CPB2 promoter.

**HNF1 was essential for CPB2 expression, but it was not a cause of the suppression of CPB2 expression by persistent HCV RNA replication.** In light of the above-described findings, we considered the possibility that the expression of tran-
The expression level of CPB2 was inversely correlated to F-stage, body mass index (BMI), and biomarkers of liver health. The hepatic expression levels of CPB2 mRNA in 91 patients with chronic hepatitis C were compared with the patients' F-stages (A), BMI (B), number of platelets (C), and AST (D), ALT (E), and γ-GTP (F) levels.

To determine whether the wt sequence functions as a main domain for the promoter activity, we modified a wild type of reporter plasmid (−91 wt) containing the region from −91 to +21 of CPB2 promoter, and we created 3 types of mutants (−91mt1 and −91mt2, which would diminish HNF1 binding, and −91mt3, which is the same as the consensus sequence) (Fig. 6A).
Fig. 3  The effects of ectopic CPB2 expression on the expression levels of hepatic fibrogenic or lipogenic genes. (A) The ectopic CPB2 expression in the OL8(2Y) cells. CPB2 gene was stably and ectopically expressed in OL8(2Y) cells by pCX4bsr retroviral transfer. The Western blot analysis of CPB2 was performed as described in the Materials and Methods. (B) Quantitative RT-PCR analysis results of fibrogenic COL1A1 and COL4A1 mRNAs in OL8(2Y) cells (Control) and OL8(2Y) cells expressing CPB2. Each mRNA level was calculated relative to the level in OL8(2Y) cells (Control), which was assigned a value of 100%. Experiments were performed in triplicate. (C) Results of the quantitative RT-PCR analysis of lipogenic ACC1, ACC2, FASN, and SCD1 mRNAs in OL8(2Y) cells (Control) and OL8(2Y) cells expressing CPB2. The experiments were performed as described in (B).

Fig. 4  The suppression of CPB2 expression in long-term cultured cells was cancelled by 5-azaC treatment. (A) The suppression of CPB2 mRNAs in OL8(2Y) and OL8(4Y) cells were restored by 1-week 5-azaC treatment. OL8(2Y) and OL8(4Y) cells were treated with or without 2.5 or 10μM of 5-azaC for 1 week. OL8(0Y) cells were used as a control. A quantitative RT-PCR analysis for CPB2 mRNA was carried out as described in the Fig. 1 legend. The levels of CPB2 mRNA were normalized to the levels of β-actin mRNA. Experiments were performed in triplicate. *p < 0.05. (B) Effect of 4-PBA on the CPB2 expression in OL8(3.5Y) cells. OL8(0Y) and OL8(3.5Y) cells were treated with 5-azaC, 4-PBA, or 5-azaC and 4-PBA for 2 days. A quantitative RT-PCR analysis for CPB2 mRNA was performed as described in (A). The experiments were performed in triplicate. (C) The effect of 4-PBA on the CPB2 expression in OL8(2Y) cells. OL8(0Y) and OL8(2Y) cells were treated with 5-azaC, 4-PBA, or 5-azaC and 4-PBA for 2 days after a 1-week treatment with 5-azaC (10μM) only. A quantitative RT-PCR analysis for CPB2 mRNA was performed as described in (A). The averages of duplicate experiments are shown.
Using these modified plasmids with a wild type (−91 wt) and 2 truncated (−75 and −52) plasmids, we performed the promoter assay in OL8(0Y) cells.

The results revealed that the promoter activities of −91mt1 and −91mt2 were completely diminished, as was observed in the other plasmids (−75 and −52), whereas the activity of −91mt3 was higher than that of −91 wt (Fig. 6B). We obtained similar results using a reporter plasmid (−2700 wt) containing the region from −2700 to +21 and 3 types of modified plasmids (−2700mt1, −2700mt2, and −2700mt3) (Fig. 6C). These results indicate that the HNF1 binding site-like sequence (from −81 to −69) was essential for the CPB2 promoter activity.

Since the activity of the −91mt3 or −2700mt3 increased to approximately twice that of the −91 wt or −2700 wt (Fig. 6B, C), we suspected that HNF1 was truly bound to our identified region (−89 to −69) of the CPB2 promoter. To further investigate whether HNF1 controls the CPB2 promoter, we first created the HNF1α- and HNF1β-double-knockdown OL8(0Y) cells, because HNF1α and HNF1β were known, and then we performed the promoter assay using the wild type and modified plasmids in the knockdown cells. The results revealed that the promoter activity of −91 wt or −2700 wt was greatly decreased in the double-knockdown cells (Fig. 6C), demonstrating that HNF1 controls the CPB2 promoter.

In this analysis, we noticed an additional interesting phenomenon: the promoter activity of −2700mt3 was not decreased in the double-knockdown cells (Fig. 6C), suggesting the existence of another HNF1-like transcription factor which is able to bind to the HNF1 consensus sequence in the CPB2 promoter. In a series of promoter assays using these siRNAs, we found that HNF1α or HNF1α and HNF1β, but not HNF1β, controlled the CPB2 promoter (Fig. 6D). Similar results were obtained by the quantitative RT-PCR analysis for CPB2 mRNA in the HNF1α and/or HNF1β-knockdown OL8(0Y) cells (Fig. 6E). In that analysis, we
observed that the CPB2 mRNA level was significantly but weakly decreased in the HNF1β-knockdown cells, although no suppression of CPB2 mRNA was observed in the Rab18 (the component required for HCV assembly [24])-knockdown cells used as a control (Fig. 6E).

The quantitative RT-PCR analysis for HNF1α, HNF1β, or Rab18 mRNA demonstrated an effective knockdown of each gene’s expression in the OL8(0Y) cells transfected with the corresponding siRNA(s) (data not shown). Taking this finding together with our other results, we conclude that HNF1α acts more preferentially than HNF1β on the CPB2 expression, although it is regulated by not only HNF1α but also HNF1β. We therefore hypothesized that HNF1 function is suppressed by persistent HCV RNA replication. However, at both the mRNA and protein levels, HNF1α and HNF1β were not suppressed in OL8(0Y) or OL8(4Y) cells in comparison with OL8(0Y) cells (Fig. 7A, B). The subcellular localization of HNF1 was also not different between OL8(0Y) cells and OL8(2Y) or OL8(4Y) cells (Fig. 7C). These results suggest that 5-azaC-induced or -reduced some factor(s) other than HNF1 suppressed or induced by persistent HCV RNA replication causes the marked suppression of CPB2 expression.

Selection of candidate genes underlying the 5-azaC-induced restoration of suppressed CPB2 expression. To identify the gene(s) underlying the restoration of suppressed CPB2 expression by the treatment with 5-azaC, we performed a cDNA microarray analysis using OL8(2Y) cells treated with or without 5-azaC for 1 week. As a control analysis, OL8c(2Y) cells treated with or without 5-azaC were also used. In these analyses, we hypothesized that there were 2 types of candidate genes. The first type of candidate is the gene(s) which act as a suppressor of CPB2 expression and whose expression is reduced by 5-azaC treatment. The expression level of this type of gene should be relatively high in OL8(0Y), OL8c(0Y), OL8c(2Y), 5-azaC-treated OL8(2Y), and 5-azaC-treated OL8c(2Y) cells in comparison with OL8(2Y) cells. As a result of our analyses, 13 genes were identified as candidate genes in the second category (Table 1). As a matter of course, CPB2 was also identified as a member of the second category (Table 1).

To further evaluate these 14 candidate genes, we examined the effects of the transient ectopic expression of the genes on CPB2 expression. Each candidate gene was ectopically and transiently expressed in OL8(2Y) cells or OL8(0Y) cells (only GPX2), and then we checked whether the CPB2 expression was induced or suppressed (only GPX2) by performing a quantitative RT-PCR analysis. We confirmed the ectopic expression of each candidate gene in OL8(2Y) or OL8(0Y) cells by a Western blot analysis, although we failed to detect FGG and GPX2 (data not shown). We were unfortunately not able to identify the gene that significantly influenced the CPB2 expression (Fig. 8A, 8B). These results suggest that plural genes, not a single gene, are required for the restoration of suppressed CPB2 expression by 5-azaC treatment.

Discussion

The results of the present study demonstrated that the expression of CPB2 that was severely suppressed by persistent HCV RNA replication was restored by treatment with a demethylating agent, 5-azaC. Our findings also revealed that the methylation of the CPB2 promoter was not a cause of the suppression of CPB2 expression. Although our data demonstrated that HNF1 (preferentially HNF1α rather than HNF1β) was essential for CPB2 expression, HNF1 was also not a cause of the suppression of CPB2 expression. These results suggest that the suppression of CPB2 expression was caused by the methylation of certain gene(s) other than HNF1. Microarray analyses based on this hypothesis identified several gene candidates, although the identification of the actual gene(s) was not achieved.

Because the remarkable suppression of CPB2 expression occurred between 6 months and 1 year of HCV RNA replication, we were interested in the expression level of hepatic CPB2 in patients with chronic hepatitis C. Our findings indicated that the expression level of CPB2 was inversely correlated with
several risk factors for hepatic fibrosis or steatosis. However, we did not observe that \textit{CPB2} expression suppressed the expression levels of fibrogenic or lipogenic genes. These results suggest that the expression of these genes is not controlled by single factor like \textit{CPB2}, and that several factors including \textit{CPB2} participate in their transcriptional regulation.

We also observed that the hepatic \textit{CPB2} expression
decreased with the number of platelets. Although it is known that CPB2 is also expressed in platelets [27] and the number of platelets decreases according to the progress of HCV-associated fibrosis [28], the suppression of hepatic CPB2 expression would not be dependent on the decrease in the number of platelets.

As we originally hypothesized, we demonstrated that CPB2 expression was suppressed by persistent HCV RNA replication through DNA methylation, which caused an epigenetic alteration of gene expression. Nevertheless, our findings unexpectedly demonstrated that the essential region of CPB2 promoter was not methylated. Based on our findings, we considered a novel mechanism in which the suppression of CPB2 expression was caused by unknown factor(s) whose promoter region was methylated by persistent HCV RNA replication. However, in this study we observed that HNF1, especially HNF1α, controlled the CPB2 expression. This finding is consistent with the previous report suggesting the participation of HNF1α in the control of CPB2 promoter activity in HepG2 cells [20]. However, our results clarified that HNF1α or HNF1β was not our suggested factor(s).

In this context, we obtained an interesting result in the analysis of CPB2 promoter using the reporter possessing an HNF1 consensus sequence (−2700mt3 in Fig. 6C). The activity of this reporter did not decrease in either the HNF1α- or HNF1β-knockdown
Table 1  The candidate genes which may regulate CPB2 expression

<table>
<thead>
<tr>
<th>Downregulated gene</th>
<th>OL8(2Y)</th>
<th>OL8c(2Y)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GPX2</td>
<td>833</td>
<td>178</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Upregulated genes</th>
<th>OL8(2Y)</th>
<th>OL8c(2Y)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALDH1A1</td>
<td>4</td>
<td>1,197</td>
</tr>
<tr>
<td>AGR2</td>
<td>78</td>
<td>8,755</td>
</tr>
<tr>
<td>VCX2</td>
<td>42</td>
<td>4,176</td>
</tr>
<tr>
<td>ALB</td>
<td>460</td>
<td>13,181</td>
</tr>
<tr>
<td>SSX4</td>
<td>24</td>
<td>476</td>
</tr>
<tr>
<td>ANXA1</td>
<td>27</td>
<td>508</td>
</tr>
<tr>
<td>FGG</td>
<td>51</td>
<td>955</td>
</tr>
<tr>
<td>FGB</td>
<td>46</td>
<td>796</td>
</tr>
<tr>
<td>TFF1</td>
<td>48</td>
<td>594</td>
</tr>
<tr>
<td>FGL1</td>
<td>103</td>
<td>1,243</td>
</tr>
<tr>
<td>AHS1G</td>
<td>604</td>
<td>4,333</td>
</tr>
<tr>
<td>CPB2</td>
<td>688</td>
<td>4,021</td>
</tr>
<tr>
<td>ZKSCAN7</td>
<td>39</td>
<td>207</td>
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</tbody>
</table>

The signal intensity of candidate genes in human genome U133 Plus 2.0 array. The gray box means that it was judged as no expression.

Fig. 8  The effect of the ectopic expression of 14 candidate genes on the CPB2 expression. (A) The effect of the ectopic expression of 5-azaC-suppressed GPX2 on CPB2 expression. The ORF of GPX2 was inserted into pCX4bsr encoding HA-tagged protein. The plasmid was then transfected into OL8(0Y) cells as described in the Materials and Methods. The quantitative RT-PCR analysis for CPB2 mRNA was carried out as described in the Fig. 1 legend. The expression level of CPB2 mRNA was normalized to the levels of GAPDH mRNA. Experiments were performed in triplicate. (B) The effect of the ectopic expression of 13 5-azaC-induced genes on CPB2 expression. The preparation of expressing plasmid for each candidate gene, except for ANXA1 and CPB2, and the ectopic expression of each gene in OL8(2Y) cells were carried out as described in the Materials and Methods. The quantitative RT-PCR analysis for CPB2 mRNA was carried out as described in the Fig. 1 legend. The ORF of ANXA1 and CPB2 was inserted into pCX4bsr without HA-tag. The endogenous CPB2 mRNA expression in CPB2-expressed OL8(2Y) cells was compared with that in OL8(2Y) cells as described in the Materials and Methods.
cells, suggesting that some other HNF1-like transcription factor binds to the HNF1 consensus sequence of −2700mt3 and enhances the promoter activity. However, such an HNF1-like factor would not be involved in the suppression of CPB2 expression, because this factor should not be able to bind to the wild-type sequence (Fig. 6A) of CPB2 promoter. We therefore speculate that the suppression of CPB2 expression by persistent HCV RNA replication is caused by an unexpected or complex mechanism.

Our present findings showed that the suppression of CPB2 expression by persistent HCV RNA replication required more than 6 months, which suggests that some accumulations of changes in the host factor(s) must be important for the suppression. With the goal of finding the host factor(s) involved in the suppression of CPB2 expression, we carried out a microarray analysis using total RNAs prepared from the cells treated with or without 5-azaC, and we were able to identify several candidate genes. Although the objective factor was not revealed by our experiments using the individual candidate genes, we suspect that multiple factors are involved in the HCV-induced suppression of CPB2 expression.

Since it is likely that there are objective genes among the candidate genes identified in this study, further analysis is needed to clarify this speculation. As an alternative possibility, we speculate that certain miRNA(s) participate in the suppression of CPB2 expression. In this context, we checked the results of the miRNA microarray analysis in the case of OL8(0Y) cells versus OL8(2Y) cells and OL11(0Y) cells versus OL11(2Y) cells, because the severe suppression of CPB2 expression had been observed in OL11(2Y) cells in addition to OL8(2Y) cells [13]. Although we found several miRNA species whose expression levels were altered by a 2-year replication of HCV RNA (Table 2), we could not find the miRNA candidates that may control CPB2 or HNF1. In addition, the results of the microarray analysis revealed that the expression levels of most of the commonly upregulated miRNAs seemed to be too low to affect other gene expressions (Table 2).

Although we were unable to completely elucidate the mechanism underlying the suppression of CPB2 expression, the present clarification of the mechanism contributes to the understanding of the mechanism by which persistent HCV replication irreversibly changes the gene expression profile of host cells. Our findings will also contribute to the elucidation of the mechanism of HCV-induced hepatocarcinogenesis.

Table 2  MicroRNAs whose expression levels were commonly altered both comparisons of OL8(0Y) versus OL8(2Y) cells and OL11(0Y) versus OL11(2Y) cells

<table>
<thead>
<tr>
<th>miRNA expression</th>
<th>OL8 series</th>
<th>OL11 series</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OL8(0Y)</td>
<td>OL8(2Y)</td>
</tr>
<tr>
<td><strong>Upregulated miRNAs</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>miR-1914*</td>
<td>0.1</td>
<td>7.5</td>
</tr>
<tr>
<td>miR-4298</td>
<td>0.1</td>
<td>5.1</td>
</tr>
<tr>
<td>miR-4270</td>
<td>0.1</td>
<td>3.4</td>
</tr>
<tr>
<td>miR-3679-5p</td>
<td>0.1</td>
<td>3.7</td>
</tr>
<tr>
<td>miR-378</td>
<td>0.1</td>
<td>5.6</td>
</tr>
<tr>
<td>miR-2116*</td>
<td>0.1</td>
<td>5.0</td>
</tr>
<tr>
<td>miR-1246</td>
<td>102</td>
<td>162</td>
</tr>
<tr>
<td>miR-1268</td>
<td>5.2</td>
<td>9.1</td>
</tr>
<tr>
<td>miR-2861</td>
<td>15</td>
<td>24</td>
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<tr>
<td><strong>Downregulated miRNAs</strong></td>
<td></td>
<td></td>
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<tr>
<td>miR-34a</td>
<td>32</td>
<td>15</td>
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<tr>
<td>miR-22</td>
<td>190</td>
<td>89</td>
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</tbody>
</table>

The signal intensity in Human miRNA Rel16.0 array of miRNAs, whose expression levels were commonly upregulated at a ratio of more than 1.5 or commonly downregulated at a ratio of less than 0.5, were listed. The gray box means that it was judged as no expression.
Acknowledgments. We thank Takashi Nakamura for his technical assistance. We also thank Drs. Kyoko Mori and Youki Ueda for their helpful suggestions. This research was supported in part by the Practical Research Program on Hepatitis from the Japan Agency for Medical Research and Development, (AMED) and by JSPS KAKENHI Grant No. 25293110.

References


