Acta Med. Okayama, 2016
Vol. 70, No. 2, pp. 75-88
Copyright© 2016 by Okayama University Medical School.

Acta Medica Okayama
http://escholarship.lib.okayama-u.ac.jp/amo/

Original Article

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

Hiroe Sejima[a], Shinya Satoh[a], Hiromichi Dansako[a], Masao Honda[b], Shuichi Kaneko[b], Masanori Ikeda[c], and Nobuyuki Kato[a]*

[a]*Department of Tumor Virology, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama 700-8558, Japan,* [b]*Department of Gastroenterology, Kanazawa University Graduate School of Medicine, Kanazawa 920-8641, Japan, and* [c]*Department of Persistent and Oncogenic Viruses, Kagoshima University Graduate School of Medical and Dental Sciences, Kagoshima 890-8544, Japan*

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*α*, was essential for the *CPB2* promoter, and (2) *CPB2* promoter was not methylated by persistent HCV RNA replication. The expression levels of HNF1*α* and HNF1*β* 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) [1-3]. The understanding of the life cycle of HCV has greatly progressed [4] since the development of a cell-based HCV replicon [5], genome-length HCV RNA replication [6], and infectious HCV production [7] systems, whereas the mechanisms of HCV-associated hepatocarcinogenesis and disease progression are still unclear.

We developed an HCV replicon [8] and genome-length HCV RNA replication [9] systems. Since most of the existing HCV replication systems were devel-

Received September 9, 2015; accepted November 12, 2015.
*Corresponding author. Phone:+81-86-235-7385; Fax:+81-86-235-7392
E-mail:nkato@md.okayama-u.ac.jp (N. Kato)

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. Li-23-derived OL8 and OL11 cells harboring genome-length HCV RNA were cultured with medium in the presence of 0.3 mg/ml of G418 (Genticin, 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(OY) 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'-AGACCTGCGTGTACCCCACT CAG-3' and 5'-TAGGCCACGCTGTTCTTGCAGTG-3'; collagen type IV, alpha 1 (*COL4A1*: fibrogenic gene), 5'-AGCACAATGCCCTTCCTGTTCTG-3' and

5'-TGGCGCACTTCTAAACTCCTCCAG-3'; acetyl-CoA carboxylase alpha (*ACC1*: lipogenic gene), 5'-CATCCACTTGGCTGAGCGATTGG-3' and 5'-GTTTTCCTCTATTACCGAGTGAACAC-3'; acetyl-CoA carboxylase beta (*ACC2*: lipogenic gene), 5'-CGACTCTGTCCTCAAGACCAT-3' and 5'-TGG TAGACAGCAGGTGAACG-3'; fatty acid synthase (*FASN*: lipogenic gene), 5'-GAAACTGCAGGAGCT GTC-3' and 5'-CACGGAGTTGAGGCGCAT-3'; stearoyl-CoA desaturase 1 (*SCD1*: lipogenic gene), 5'-GGTTTCACTTGGAGCTGTGG-3' and 5'-TTGATGTGCCAGCGGTACT-3'; *HNF1α*, 5'-CTTCACACGCCGGCATCTCAG-3' and 5'-GTGGAGATGAAGGTCTCGATGAC-3'; HNF1 homeobox B (*HNF1β*), 5'-AGCTGCACAGCCCTCA CCAG-3' and 5'-GAAGACATGTTGGTGAGTGTA CTG-3'; actin, beta (*β*-actin), 5'-GAAGAGCTACG AGCTGCCTGAC-3' and 5'-GTGATCTCCTTCTG CATCCTGTC-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'-ACGGAAGCAGAATGATAAAA 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 Biologicals, Ancaster, ON, Canada), rabbit polyclonal anti-HNF1*α* antibody (GeneTex, Hsinchu City, Taiwan), rabbit polyclonal anti-HNF1*β* 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 (1 mM), or a combination of 5-azaC (2.5 or 10 μM) and 4-PBA (1 mM). 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 *Nhe*I-*Hind*III 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'-GTTGATTGATTTATAGGAATAAGA GG-3' and 5'-TTACCATTTTATCCACCAACTC-3') was used to amplify the *CPB2* promoter region (307 bp) using TaKaRa EpiTaq HS (for bisulfite-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 flame (ORF) of *CPB2* (NM_001872.3) was amplified by PCR using KOD-plus DNA polymerase, and then the PCR product was inserted into *Mlu*I-*Not*I site of pCX4bsr. 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 pCX-4bsr 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 *Eco*RI–*Mlu*I 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 *Eco*RI–*Not*I 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 48 h, 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 (10 μM) 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*.

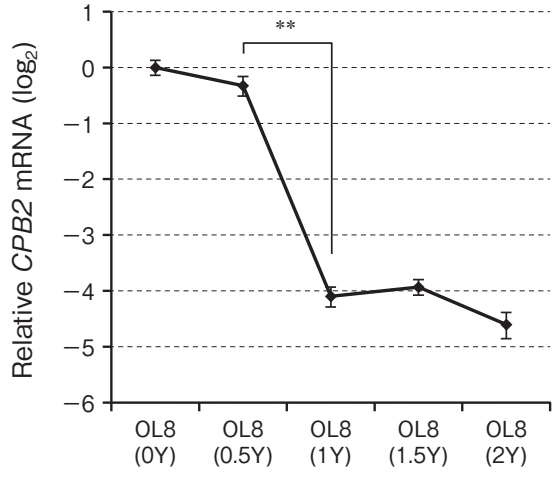

Fig. 1 The downregulation of *CPB2* expression occurred mainly between 6 months and 1 year of persistent HCV RNA replication. We quantified the expression level of *CPB2* mRNA in OL8(0Y), OL8(0.5Y), OL8(1Y), OL8(1.5Y), and OL8(2Y) cells using a real-time LightCycler PCR. Experiments were performed in 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-

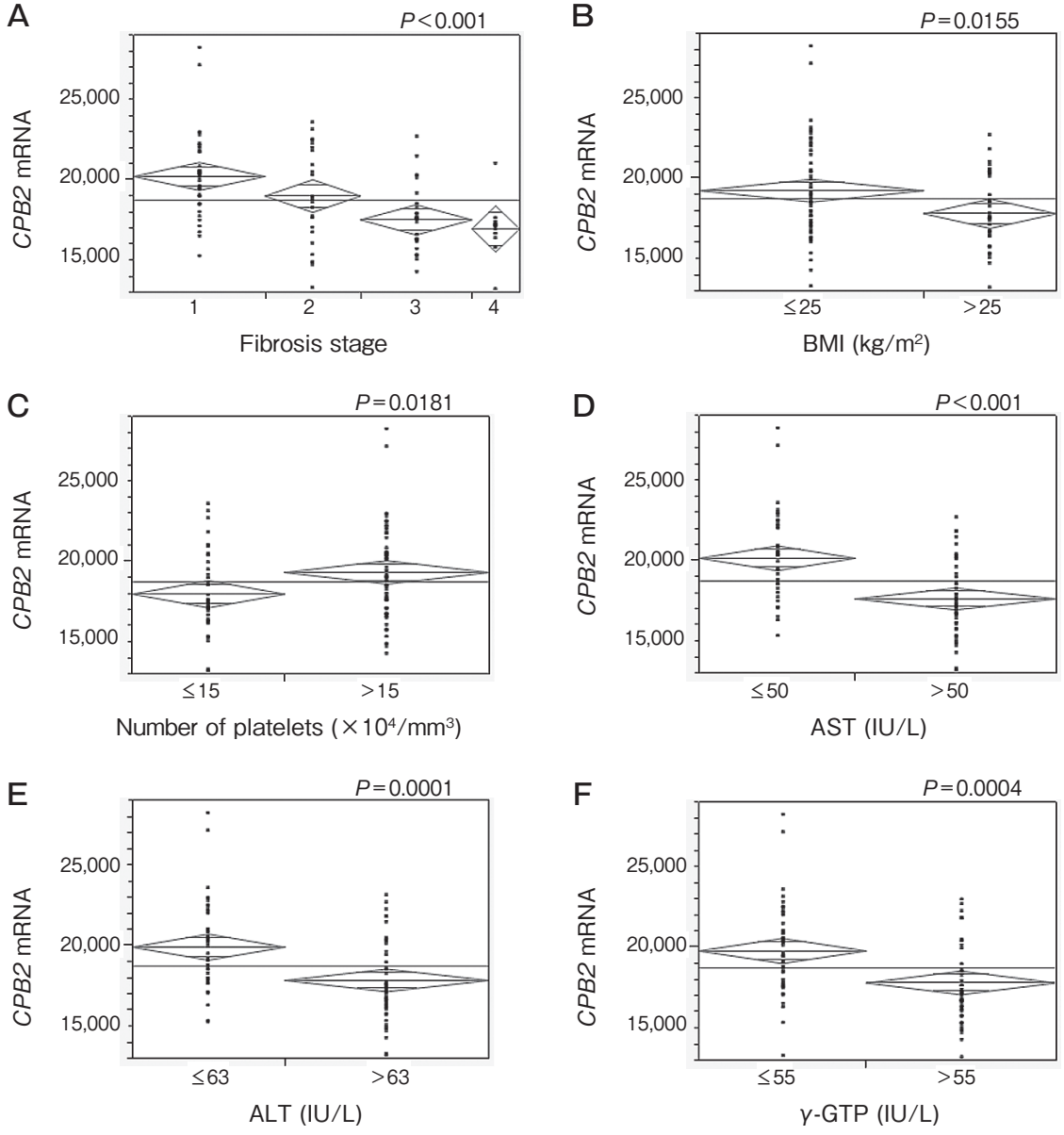

Fig. 2 The expression level of *CPB2* was inversely correlated to F-stage, body mass index (BMI), and biomarkers of liver health. The hepatic expression levels of *CPB2* mRNA in 91 patients with chronic hepatitis C were compared with the patients' F-stages (**A**), BMI (**B**), number of platelets (**C**), and AST (**D**), ALT (**E**), and γ-GTP (**F**) levels.

scription factor, which positively controls the *CPB2* expression, was suppressed by DNA methylation. In this context, we noticed the HNF1 binding site-like sequence in the region from −81 to −69, which is included within our identified main domain (−150 to −52) for *CPB2* promoter (Fig. 6A). This sequence (wt) shows the difference in 2 nucleotides from the consensus sequences for the HNF1 binding site (Fig. 6A).

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 (−91 mt1 and −91 mt2, which would diminish HNF1 binding, and −91 mt3, which is the same as the consensus sequence) (Fig.

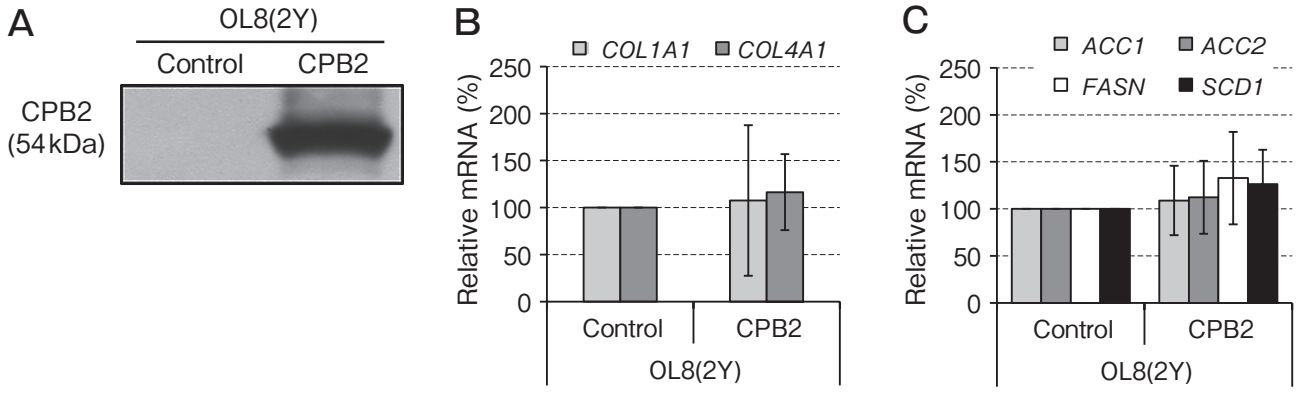

Fig. 3 The effects of ectopic *CPB2* expression on the expression levels of hepatic fibrogenic or lipogenic genes. (**A**) The ectopic *CPB2* expression in the OL8(2Y) cells. *CPB2* gene was stably and ectopically expressed in OL8(2Y) cells by pCX4bsr retroviral transfer. The Western blot analysis of 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**).

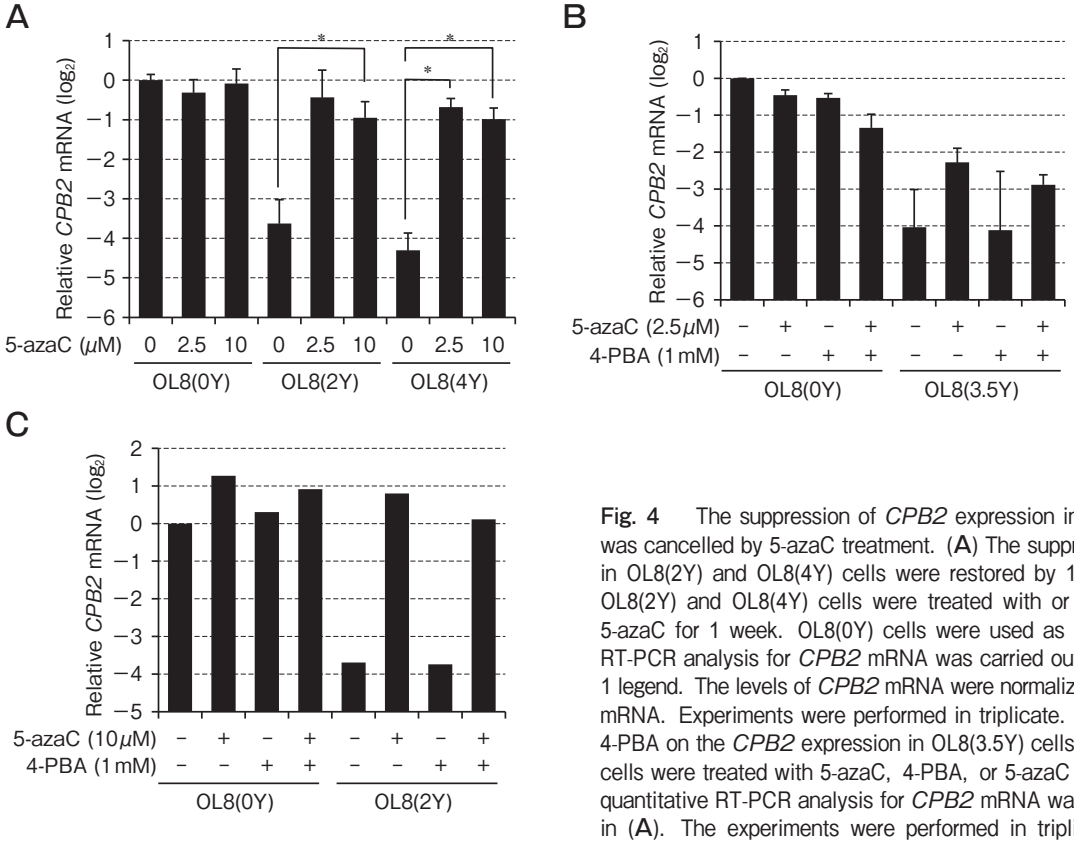

Fig. 4 The suppression of *CPB2* expression in long-term cultured cells was cancelled by 5-azaC treatment. (**A**) The suppression of *CPB2* mRNAs in OL8(2Y) and OL8(4Y) cells were restored by 1-week 5-azaC treatment. OL8(2Y) and OL8(4Y) cells were treated with or without 2.5 or 10 μM of 5-azaC for 1 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 (10 μM) only. A quantitative RT-PCR analysis for *CPB2* mRNA was performed as described in (**A**). The averages of duplicate experiments are shown.

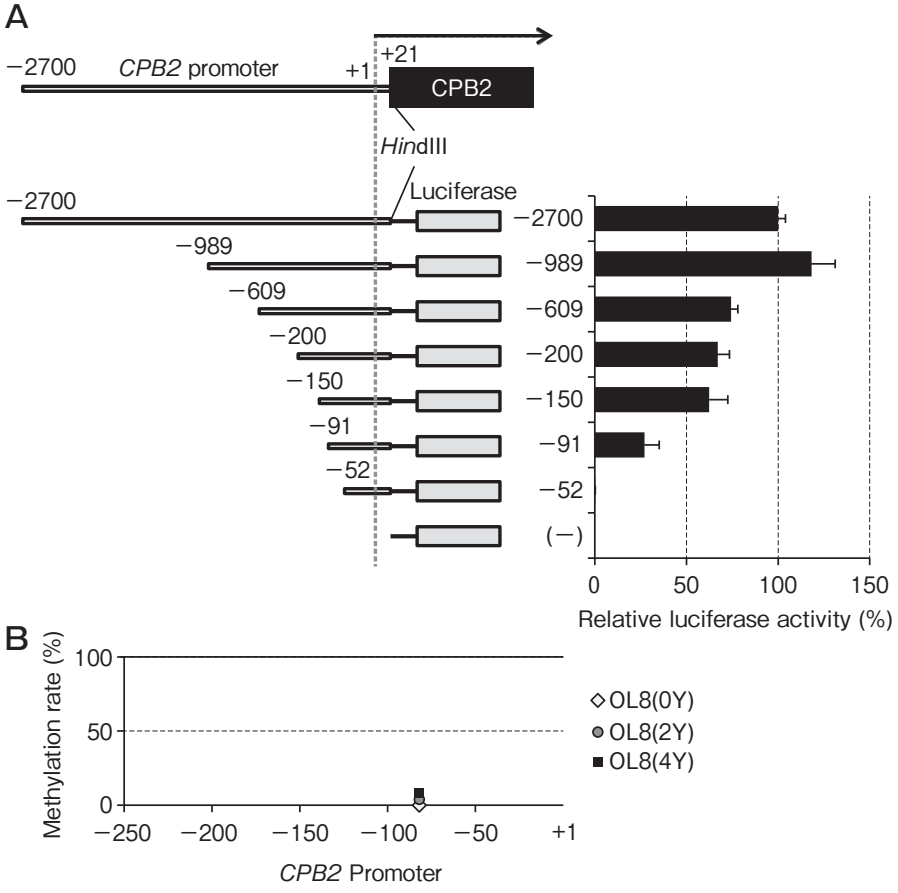

Fig. 5 *CPB2* promoter in OL8(0Y), OL8(2Y), and OL8(4Y) cells was not methylated. (**A**) Results of the *CPB2* promoter analysis using a luciferase reporter assay. DNA fragments of various lengths (−2700, −989, −609, −200, −150, −91, or −52 to +21) of the *CPB2* promoter region were inserted upstream of a firefly luciferase gene in pGL4.10-luc2. DNA transfection and dual luciferase assays using OL8(0Y) cells were performed as described in the Materials and Methods. The results of the relative luciferase activity calculated using the level of luciferase activity in the longest (−2700 to +21) plasmid assigned as 100% are shown. The lysate of cells transfected with pGL4.10-luc2 was used as a control (−). Experiments were done in triplicate. (**B**) DNA methylation analysis of the *CPB2* promoter region. The bisulphite sequencing of the *CPB2* promoter region derived from OL8(0Y), OL8(2Y), and OL8(4Y) cells was performed as described in the Materials and Methods.

6A). 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 −91 mt1 and −91 mt2 were completely diminished, as was observed in the other plasmids (−75 and −52), whereas the activity of −91 mt3 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 (−2700 mt1, −2700 mt2, and −2700 mt3) (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 −91 mt3 or −2700 mt3 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 −2700 mt3 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(2Y) 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 low 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 these analyses, only one gene, *GPX2*, was revealed as a candidate gene in this first category (Table 1).

The second type of candidate is the gene(s) that act as a trap agent of the suppressor for *CPB2* expression and whose expression is induced 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

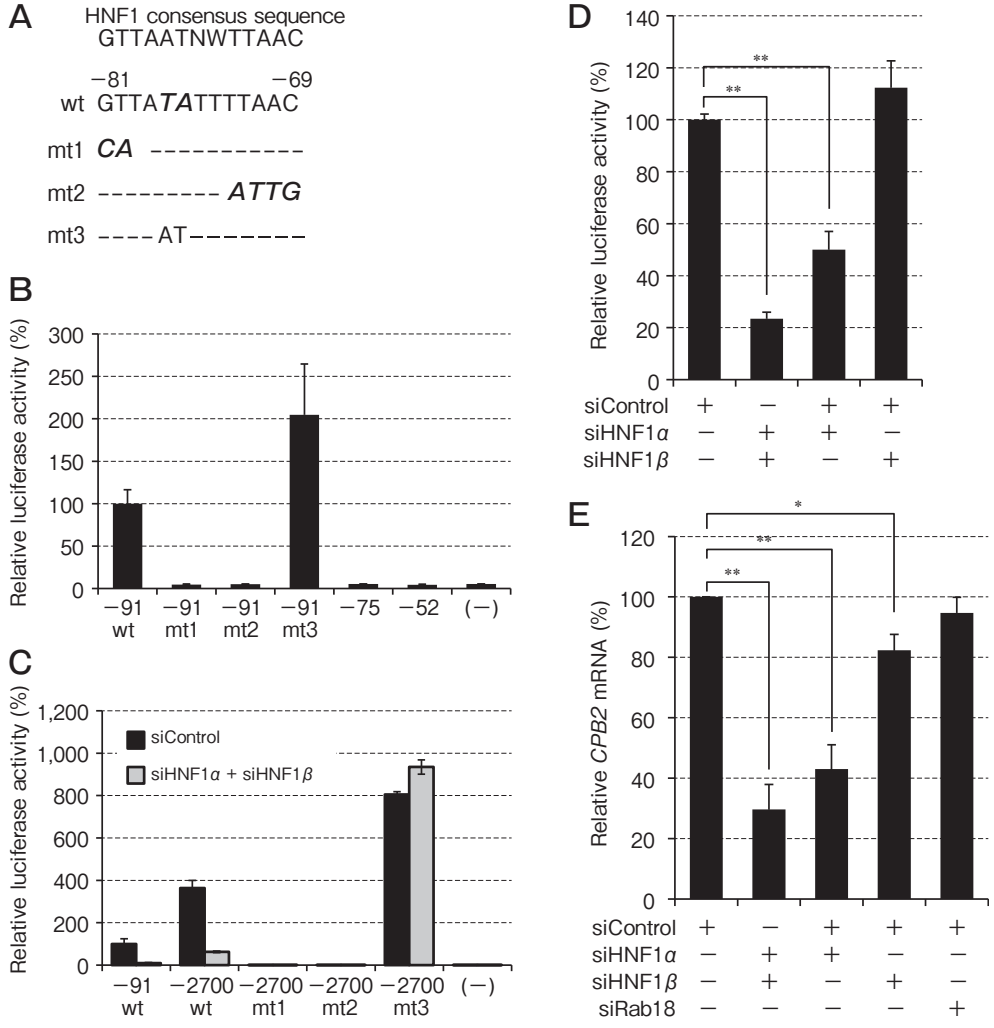

Fig. 6 HNF1 was essential for *CPB2* promoter activity. (**A**) Nucleotide sequence alignment of the HNF1-binding site in the reporter plasmids (wt, mt1, mt2, and mt3) used in this study. Nucleotides differing from the consensus sequences are shown. N is equivalent to A, G, C, or T, and W is equivalent to A or T. (**B**) Luciferase reporter assay using *CPB2* mutant promoters. The assay was performed as described in the Fig. 5A legend. The results of the relative luciferase activity calculated using the level of luciferase activity in the plasmid −91 wt assigned as 100% are shown. Experiments were done in triplicate. (**C**) Luciferase reporter assay using HNF1-knockdown OL8(0Y) cells. OL8(0Y) cells were treated with 5 nM of siRNAs targeting HNF1α and HNF1β at 1 day before the transfection of reporter plasmids containing the *CPB2* promoter (−91 wt and −2700 to +21 with wt, mt1, mt2, or mt3). The luciferase assay was performed as described in the Fig. 5A legend. The relative luciferase activity was calculated using the level of luciferase activity in the non-targeting siRNA-treated cells (siControl) transfected by plasmid −91 wt assigned as 100%. Experiments were done in triplicate. (**D**) Luciferase *CPB2* promoter assay using HNF1α- or/and HNF1β-knockdown OL8(0Y) cells. The luciferase reporter assay using the reporter plasmid containing the *CPB2* promoter (−91 to +21 with wt) was performed as described in (**C**). $^{**}p<0.01$. (**E**) Effects of siRNAs targeting HNF1α or/and HNF1β on the expression level of *CPB2*. Non-targeting and Rab18-targeting siRNAs were also used for the transfection as controls. Three days after the transfection, the expression level of *CPB2* was measured by quantitative RT-PCR. $^{*}p<0.05$, $^{**}p<0.01$.

several risk factors for hepatic fibrosis or steatosis. However, we did not observe that *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 CPB2, and that several factors including CPB2 participate in their transcriptional regulation.

We also observed that the hepatic *CPB2* expression

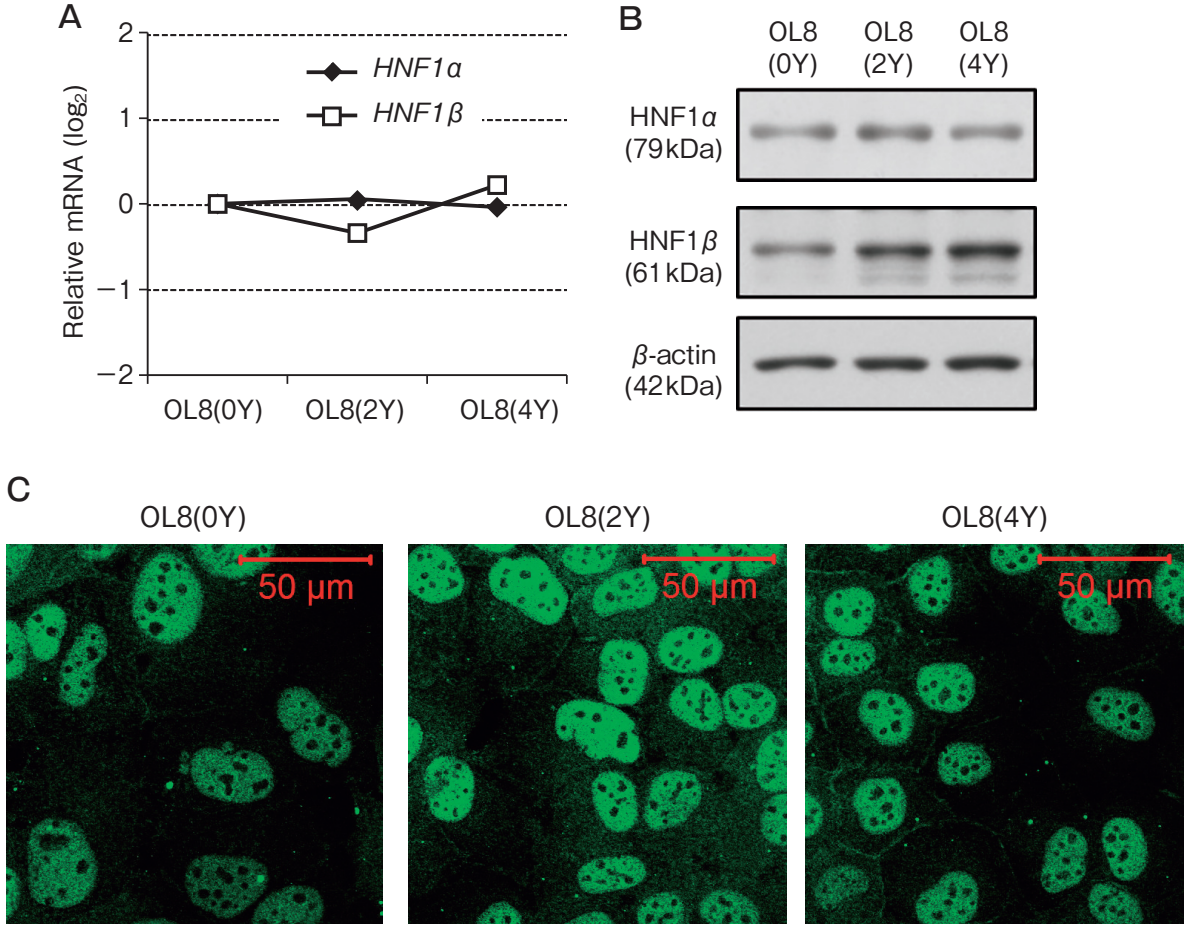

Fig. 7 The *HNF1* expression was not affected by persistent HCV RNA replication. (**A**) The expression levels of *HNF1α* and *HNF1β* mRNAs in OL8(0Y), OL8(2Y), and OL8(4Y) cells. Quantitative RT-PCR was performed as described in the Fig. 1 legend. (**B**) The expression levels of HNF1α and HNF1β in OL8(0Y), OL8(2Y), and OL8(4Y) cells. The Western blot analysis was performed as described in the Materials and Methods. β-actin was included as a loading control. (**C**) The cellular localization of HNF1α in OL8(0Y), OL8(2Y), and OL8(4Y) cells. The immunofluorescence analysis using anti-HNF1α antibody was carried out as described in the Materials and Methods.

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 (−2700 mt3 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

	mRNA expression					
	OL8(2Y)			OL8c(2Y)		
	–	5-azaC	fold	–	5-azaC	fold
Downregulated gene						
GPX2	833	178	0.21	223	13	0.06
Upregulated genes						
ALDH1A1	4	1,197	340	1,792	3,186	1.8
AGR2	78	8,755	110	757	5,981	7.9
VCX2	42	4,176	100	999	5,676	5.7
ALB	460	13,181	29	8,209	18,533	2.3
SSX4	24	476	20	931	1,183	1.3
ANXA1	27	508	19	940	1,814	1.9
FGG	51	955	19	515	1,343	2.6
FGB	46	796	17	370	1,173	3.2
TFPI	48	594	14	684	2,730	4.0
FGL1	103	1,243	12	1,562	3,820	2.4
AHSG	604	4,333	7.2	4,719	5,274	1.1
CPB2	686	4,021	5.9	3,377	4,165	1.2
ZKSCAN7	39	207	5.3	281	319	1.1

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.

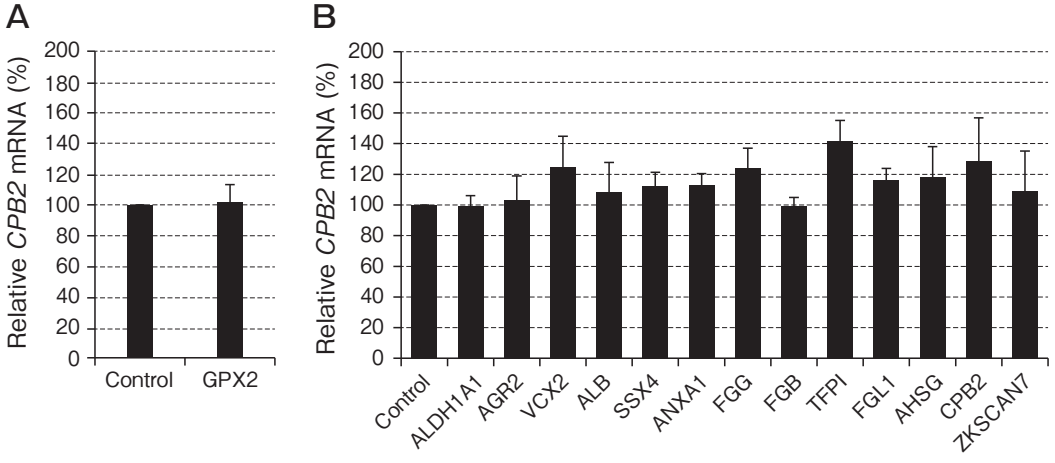

Fig. 8 The effect of the ectopic expression of 14 candidate genes on the *CPB2* expression. (**A**) The effect of the ectopic expression of 5-azaC-suppressed *GPX2* on *CPB2* expression. The ORF of *GPX2* was inserted into pCX4bsr encoding HA-tagged protein. The plasmid was then transfected into OL8(0Y) 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 be not 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

	miRNA expression					
	OL8 series			OL11 series		
	OL8(0Y)	OL8(2Y)	fold	OL11(0Y)	OL11(2Y)	fold
Upregulated miRNAs						
*miR-1914**	0.1	7.5	75	0.1	11	110
miR-4298	0.1	5.1	51	0.1	12	120
miR-4270	0.1	3.4	34	0.1	8.5	85
miR-3679-5p	0.1	3.7	37	0.1	8.5	80
miR-378	0.1	5.6	56	0.1	3.6	36
*miR-2116**	0.1	5.0	50	0.1	2.3	23
miR-1246	102	162	1.6	50	166	3.1
miR-1268	5.2	9.1	1.7	4.4	13	3.0
miR-2861	15	24	1.6	14	29	2.1
Downregulated miRNAs						
miR-34a	32	15	0.46	35	17	0.49
miR-22	190	89	0.47	223	108	0.49

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

1. Kato N, Hijikata M, Ootsuyama Y, Nakagawa M, Ohkoshi S, Sugimura T and Shimotohno K: Molecular cloning of the human hepatitis C virus genome from Japanese patients with non-A, non-B hepatitis. Proc Natl Acad Sci USA (1990) 87: 9524–9528.
2. Saito I, Miyamura T, Ohbayashi A, Harada H, Katayama T, Kikuchi S, Watanabe Y, Koi S, Onji M, Ohta Y, Choo Q, Houghton M and Kuo G: Hepatitis C virus infection is associated with the development of hepatocellular carcinoma. Proc Natl Acad Sci USA (1990) 87: 6547–6549.
3. Kato N: Molecular virology of hepatitis C virus. Acta Med Okayama (2001) 55: 133–159.
4. Scheel TK and Rice CM: Understanding the hepatitis C virus life cycle paves the way for highly effective therapies. Nat Med (2013) 19: 837–849.
5. Lohmann V, Korner F, Koch JO, Herian U, Theilmann L and Bartenschlager R: Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. Science (1999) 285: 110–113.
6. Ikeda M, Yi M, Li K and Lemon SM: Selectable subgenomic and genome-length dicistronic RNAs derived from an infectious molecular clone of the HCV-N strain of hepatitis C virus replicate efficiently in cultured Huh7 cells. J Virol (2002) 76: 2997–3006.
7. Wakita T, Pietschmann T, Kato T, Date T, Miyamoto M, Zhao Z, Murthy K, Habermann A, Krausslich HG, Mizokami M, Bartenschlager R and Liang TJ: Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. Nat Med (2005) 11: 791–796.
8. Kato N, Sugiyama K, Namba K, Dansako H, Nakamura T, Takami M, Naka K, Nozaki A and Shimotohno K: Establishment of a hepatitis C virus subgenomic replicon derived from human hepatocytes infected in vitro. Biochem Biophys Res Commun (2003) 306: 756–766.
9. Ikeda M, Abe K, Dansako H, Nakamura T, Naka K and Kato N: Efficient replication of a full-length hepatitis C virus genome, strain O, in cell culture, and development of a luciferase reporter system. Biochem Biophys Res Commun (2005) 329: 1350–1359.
10. Kato N, Mori K, Abe K, Dansako H, Kuroki M, Ariumi Y, Wakita T and Ikeda M: Efficient replication systems for hepatitis C virus using a new human hepatoma cell line. Virus Res (2009) 146: 41–50.
11. Mori K, Ikeda M, Ariumi Y and Kato N: Gene expression profile of Li23, a new human hepatoma cell line that enables robust hepatitis C virus replication: Comparison with HuH-7 and other hepatic cell lines. Hepatol Res (2010) 40: 1248–1253.
12. Kato N, Sejima H, Ueda Y, Mori K, Satoh S, Dansako H and Ikeda M: Genetic characterization of hepatitis C virus in long-term RNA replication using Li23 cell culture systems. PLoS One (2014) 9: e91156.
13. Sejima H, Mori K, Ariumi Y, Ikeda M and Kato N: Identification of host genes showing differential expression profiles with cell-based long-term replication of hepatitis C virus RNA. Virus Res (2012) 167: 74–85.
14. Redlitz A, Tan AK, Eaton DL and Plow EF: 1995. Plasma carboxypeptidases as regulators of the plasminogen system. J Clin Invest (1995) 96: 2534–2538.
15. Wang W, Hendriks DF and Scharpe SS: Carboxypeptidase U, a plasma carboxypeptidase with high affinity for plasminogen. J Biol Chem (1994) 269: 15937–15944.
16. Sharif SA, Du X, Myles T, Song JJ, Price E, Lee DM, Goodman SB, Nagashima M, Morser J, Robinson WH and Leung LLK: Thrombin-activatable carboxypeptidase B cleavage of osteopontin regulates neutrophil survival and synoviocyte binding in rheumatoid arthritis. Arthritis Rheum (2009) 60: 2902–2912.
17. Song JJ, Hwang I, Cho KH, Garcia MA, Kim AJ, Wang TH, Lindstrom TM, Lee AT, Nishimura T, Zhao L, Morser J, Nesheim M, Goodman SB, Lee DM, Bridges Jr, SL, Gregersen PK, Leung LL and Robinson WH: Plasma carboxypeptidase B downregulates inflammatory responses in autoimmune arthritis. J Clin Invest (2011) 121: 3517–3527.
18. Hillebrandt S, Wasmuth HE, Weiskirchen R, Hellerbrand C, Keppeler H, Werth A, Schirin-Sokhan R, Wilkens G, Geier A, Lorenzen J, Koehl J, Gressner AM, Matern S and Lammert F: Complement factor 5 is a quantitative trait gene that modifies liver fibrogenesis in mice and humans. Nat Genet (2005) 37: 835–843.
19. Lisman T, Leebeek FW, Mosnier LO, Bouma BN, Meijers JC, Janssen HL, Nieuwenhuis HK and De Groot PG: Thrombin-activatable fibrinolysis inhibitor deficiency in cirrhosis is not associated with increased plasma fibrinolysis. Gastroenterology (2001) 121: 131–139.
20. Garand M, Bastajian N, Nesheim ME, Boffa MB and Koschinsky ML: Molecular analysis of the human thrombin-activatable fibrinolysis inhibitor gene promoter. Br J Haematol (2007) 138: 231–244.
21. Armendariz AD and Krauss RM: Hepatic nuclear factor 1-alpha: inflammation, genetics, and atherosclerosis. Curr Opin Lipidol (2009) 20: 106–111.
22. Mori K, Hiraoka O, Ikeda M, Ariumi Y, Hiramoto A, Wataya Y and Kato N: Adenosine kinase is a key determinant for the anti-HCV activity of ribavirin. Hepatology (2013) 58: 1236–1244.
23. Naka K, Dansako H, Kobayashi N, Ikeda M and Kato N: Hepatitis C virus NS5B delays cell cycle progression by inducing interferon-beta via Toll-like receptor 3 signaling pathway without replicating viral genomes. Virology (2006) 346: 348–362.
24. Dansako H, Hiramoto H, Ikeda M, Wakita T and Kato N: Rab18 is required for viral assembly of hepatitis C virus through trafficking of the core protein to lipid droplets. Virology (2014) 462–463: 166–174.
25. Dansako H, Ikeda M and Kato N: Limited suppression of the interferon-beta production by hepatitis C virus serine protease in cultured human hepatocytes. FEBS J (2007) 274: 4161–4176.
26. Honda M, Sakai A, Yamashita T, Nakamoto Y, Mizukoshi E, Sakai Y, Yamashita T, Nakamura M, Shirasaki T, Horimoto K, Tanaka Y, Tokunaga K, Mizokami M, Kaneko S and Hokuriku Liver Study Group: Hepatic ISG expression is associated with genetic variation in interleukin 28B and the outcome of IFN therapy for chronic hepatitis C. Gastroenterology (2010) 139: 499–509.
27. Lin JH, Garand M, Zagorac B, Schadinger SL, Scipione C, Koschinsky ML and Boffa MB: Identification of human thrombin-activatable fibrinolysis inhibitor in vascular and inflammatory cells. Thromb Haemost (2011) 105: 999–1009.
28. Nagamine T, Ohtuka T, Takehara K, Arai T, Takagi H and Mori M: Thrombocytopenia associated with hepatitis C viral infection. J Hepatol (1996) 24: 135–140.