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Hepatitis Virus Infection Affects DNA Methylation in Mice With Humanized Livers

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BACKGROUND & AIMS: Cells of tumors associated with chronic inflammation frequently have altered patterns of DNA methylation, including hepatocellular carcinomas. Chronic hepatitis has also been associated with aberrant DNA methylation, but little is known about their relationship. **METHODS:** Pyrosequencing was used to determine the methylation status of cultured Huh7.5.1 hepatoma cells after hepatitis C virus (HCV) infection. We also studied mice with severe combined immunodeficiency carrying the urokinase-type plasminogen activator transgene controlled by an albumin promoter (urokinase-type plasminogen activator/severe combined immunodeficient mice), in which up to 85% of hepatocytes were replaced by human hepatocytes (chimeric mice). Mice were given intravenous injections of hepatitis B virus (HBV) or HCV, liver tissues were collected, and DNA methylation profiles were determined at different time points after infection. We also compared methylation patterns between paired samples of hepatocellular carcinomas and adjacent nontumor liver tissues from patients. **RESULTS:** No reproducible changes in DNA methylation were observed after infection of Huh7.5.1 cells with HCV. Livers from HBV- and HCV-infected mice had genome-wide, time-dependent changes in DNA methylation, compared with uninfected urokinase-type plasminogen activator/severe combined immunodeficient mice. There were changes in 160 ± 63 genes in HBV-infected and 237 ± 110 genes in HCV-infected mice. Methylation of 149 common genes increased in HBV- and HCV-infected mice; methylation of some of these genes also increased in hepatocellular carcinoma samples from patients compared with nontumor tissues. Expression of *Ifng*, which is expressed by natural killer cells, increased significantly in chimeric livers, in concordance with induction of DNA methylation, after infection with HBV or HCV. Induction of *Ifng* was reduced after administration of an inhibitor of natural killer cell function (anti–asialo GM1). **CONCLUSIONS:** In chimeric mice with humanized livers, infection with HBV and HCV appears to activate a natural kill cell–dependent innate immune response. This contributes to the induction and accumulation of aberrant DNA methylation in human hepatocytes.

Keywords: Epigenetic; Inflammatory Response; Liver Cancer; Gene Regulation.

BASIC AND TRANSLATIONAL LIVER

The majority of hepatocellular carcinomas (HCCs) occur as a consequence of chronic hepatitis and liver cirrhosis, particularly after infection with hepatitis B virus (HBV) or hepatitis C virus (HCV).[1] Aberrant DNA methylation in the promoter CpG islands has been described in many types of human cancers, including HCCs. This epigenetic alteration, sometimes together with point mutations and deletions, serves as a mechanism that leads to inactivation of cancer-related genes connected with essential tumor properties, such as tumor cell proliferation, anti-apoptosis, neoangiogenesis, and chemotherapy resistance.[2,3]

In earlier studies, we and other groups demonstrated that aberrant DNA methylation was detected even in precancerous liver tissues, such as chronic hepatitis, liver cirrhosis, or dysplastic nodules, suggesting that DNA methylation is an early and ubiquitous event during HCC development.[4–8] Intriguingly, these studies consistently showed that certain

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Abbreviations used in this paper: **ESR1, estrogen receptor 1; HBV, hepatitis B virus; HBx, hepatitis B virus X; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HOXA6, homeobox A6; IFN, interferon; MCAM, methylated CpG island amplification microarray; NK, natural killer; PCNA, proliferating cell nuclear antigen; RASSF1A, Ras association domain family 1 isoform A; ROS, reactive oxygen species; SCID, severe combined immunodeficient.**

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genes, such as the tumor suppressor Ras association domain family 1 isoform A (*RASSF1A*), are frequently methylated in precancerous tissues as well as cancerous tissues, regardless of the type of hepatitis virus infection. This suggests that DNA methylation of certain genes might reflect the clinical course of persistent inflammation, and a subset of cells that have acquired aberrant DNA methylation in their promoters could be prone to cancer formation.

Regarding the cause of aberrant DNA methylation, a connection between chronic inflammation and DNA methylation has been suggested for a long time, however, the key factors linking these 2 processes are not completely understood. Several studies have shown that hepatitis B virus X protein, as well as HCV protein, could induce regional hypermethylation of specific tumor suppressor genes.[9-12] These findings indicate the role of the hepatitis B virus X protein and HCV protein as important players in hepatitis virus–induced epigenetic aberrations. However, a previous genome-wide DNA methylation analysis revealed that CpG island promoters were methylated in different patterns during progression of the disease, suggesting that multiple mechanisms were involved in the acquisition of epigenetic changes during the development of HCCs.[8]

Recent technological advances have enabled the development of severe combined immunodeficient (SCID) mouse carrying a urokinase-type plasminogen activator transgene controlled by an albumin promoter (urokinase-type plasminogen activator/SCID mouse), in which the liver is repopulated with human hepatocytes (human hepatocyte chimeric mouse).[13,14] This mouse model shows severe combined immunodeficiency due to lack of both T- and B-cell activities, but possesses normal macrophages and natural killer (NK) cell activity, which are important components of the innate immune system. Because both HBV and HCV can infect human hepatocytes, but not murine hepatocytes, this model is a useful tool for mimicking and unraveling hepatitis virus–host interactions in vivo.[14-16] Using this model, we demonstrated here that DNA methylation was induced in human hepatocytes after HBV and HCV infections, and that induction of DNA methylation was closely associated with NK cell activity.

Materials and Methods

Tissue Samples

Paired samples of adjacent noncancerous liver tissue and cancerous tissue were obtained from 34 patients with HCC who underwent surgical resection at the Aichi Cancer Center Hospital in accordance with institutional policies (Supplementary Table 1, Supplementary Methods). All patients provided written informed consent. In addition, samples of normal liver tissue were also obtained from 8 patients without HBV or HCV infection who underwent partial hepatectomy for liver metastasis of primary colon cancer.

Cell Lines and Culture Conditions

The hepatoma cell line, Huh7.5.1 (a gift from Dr Francis V. Chisari), was grown in Dulbecco's modified Eagle medium (Invitrogen, Carlsbad, CA) with 10% fetal bovine serum in plastic tissue culture plates in a humidified atmosphere containing 5% CO_2 at 37°C. An efficient tissue culture-based HCV infection system in Huh7.5.1 cells was conducted using the HCV-JFH1 strain.[17] Infection of HCV-JFH1 was confirmed by reverse transcription polymerase chain reaction at 7, 9, 10, 21, and 26 weeks after initial infection (Supplementary Figure 1).

Establishment of HBV or HCV Infection in Human Hepatocyte Chimeric Mouse

The chimeric mice, in which up to 85% of hepatocytes in the liver were repopulated by human hepatocytes, were obtained from Phoenix Bio Co, Ltd (Hiroshima, Japan). Human hepatocytes were derived from 7 different individuals (2 to 28 years old, 3 females and 4 males). No significant difference in basal level of DNA methylation was observed between the transplanted hepatocytes of each mouse. Forty-seven chimeric mice were intravenously injected with either HBV or HCV (1.0×10^4 copies, respectively) and successfully infected (HBV, 23 mice; HCV, 24 mice; Supplementary Table 2). In order to inhibit the mouse NK cell activity, 10 chimeric mice were treated with 20 μL (0.02 mg/body) anti–asialo GM1 (#986-10001; Wako Pure Chemical Industries, Osaka, Japan) 2 times per week from 1 day before the hepatitis virus infection until they were sacrificed. Two mice were treated with the nonspecific rabbit polyclonal antibody (#AB-105-C; R&D Systems, Minneapolis, MN) as a control for the anti–asialo GM1 treatment. Because the levels of cytokines, reactive oxygen species (ROS) induction, and cell proliferation in these 2 control mice were concordant with those in mice without nonspecific rabbit polyclonal antibody treatment, we combined the data of mice with and without treatment of nonspecific rabbit polyclonal antibody for use as control data. Five chimeric mice were treated with 0.2 mg of anti-mouse interferon (IFN)-gamma–neutralizing rat monoclonal antibody (Clone XMG1.2, 40-7311; TONBO, San Diego, CA) 2 times per week from 1 day before hepatitis virus infection.[18] In order to evaluate hepatocyte proliferation, bromodeoxyuridine (550891; BD, Franklin Lakes, NJ) was injected 2 hours before the sacrifice (Supplementary Table 2).

Bisulfite-Pyrosequencing for DNA Methylation Analysis

DNA methylation levels were measured by pyrosequencing technology using species-specific primer sets, which can discriminately amplify either mouse genes or human genes (Supplementary Table 3, Supplementary Methods).

Methylated CpG Island Amplification-Microarray

Methylated CpG island amplification-microarray (MCAM) was carried out to analyze the methylation status of 34 cancerous tissues from HCC patients, Huh7.5.1 cells infected with HCV-JFH1, and samples from 20 human hepatocyte chimeric mice (Supplementary Figure 2, Supplementary Methods). A detailed protocol of MCAM is described in the Supplementary Methods.

Measurement of ROS Production

ROS production was evaluated by staining with dihydroethidium (Invitrogen).[16] In the presence of ROS, dihydroethidium is oxidized to ethidium bromide that stains nuclei bright red by means of intercalation into DNA. Intracellular ROS level of Huh7.5.1 was evaluated using an ROS/reactive nitrogen species detection kit (Enzo Life Sciences, Plymouth Meeting, PA) according to manufacturer's protocol. The fluorescent signals were analyzed using an AF7000 fluorescent microscope (Leica Microsystems, Wetzlar, Germany).

Statistical Analysis

All statistical analyses were performed using JMP statistical software version 10. Fisher's exact test was used to determine nonrandom associations between 2 categorical variables.

Kruskal-Wallis analysis was used to evaluate the extent of differences among more than 3 groups. All reported P values are 2-sided, with $P < .05$ considered statistically significant.

Results

DNA Methylation Analysis in HCC Samples

We initially assessed the genome-wide DNA methylation status in clinical HCCs. MCAM was performed in 34 HCC samples (Supplementary Table 1). DNA methylation was most frequently observed in cancerous tissues from the patients with liver cirrhosis background and HCV infection in comparison with the other types of histological or viral status (mean 599 ± 131 genes; $P = .0034$; Figure 1*A* and *B*). These data indicated that a long period of chronic HCV infection was closely associated with accumulation of aberrant DNA methylation, as reported previously.[4,8,19]

Among the frequent methylation target genes, which were methylated in >25% of the cases in each group, many genes were commonly methylated in both HBV-associated (85 of 212 genes [40%]) and HCV-associated (85 of 289 genes [29%]) adjacent noncancerous tissues (Figure 1*C*). This was more obvious in cancerous tissues; 338 genes were commonly methylated in HBV-associated (407 genes [83%]) and HCV-associated (657 genes [51%]) HCCs. The majority of the methylated genes in HBV-associated cancerous tissues were also methylated in HCV-associated cancerous tissues, and HCV-associated cancerous tissues had a number of specifically methylated genes (319 of 657 genes [49%]). These data suggest that there is a common mechanism for the induction of aberrant DNA methylation between HBV and HCV infection and that HCV might play a role in accelerating the methylation process.[19]

HCV Infection In Vitro Did Not Induce Aberrant DNA Methylation in a Hepatocellular Carcinoma Cell Line

The clinical data argued that HCV infection can enhance the induction of DNA methylation in hepatocytes. To address this possibility, we evaluated for DNA methylation using a tissue culture–based HCV-infection system. HCV-JFH1 clone was used to infect a Huh-7–derived cell line (Huh7.5.1) that allowed the production and efficient propagation of virus in tissue culture.[20] First, we assessed for genome-wide DNA methylation status and found no reproducible gain of DNA methylation in the HCV-infected Huh7.5.1 cells in comparison with the uninfected cells (Figure 1*D*). Consistently, quantitative DNA methylation analysis revealed that no significant difference in the DNA methylation level of *LINE1*, which represents overall methylation status,[21] was observed between HCV-infected Huh7.5.1 cells and the uninfected cells at 26 weeks (Figure 1*E*, Supplementary Table 5). The methylation level of the GRAM domain containing 3 gene, which is frequently methylated in clinical HCC samples, but not in uninfected Huh7.5.1 cells, remained unmethylated in the HCV-infected Huh7.5.1 cells at 26 weeks (Figure 1*E*). Notably, in vitro HCV infection did not enhance the proliferation of Huh7.5.1 and moderately increased ROS production (Supplementary Figure 5). Our findings demonstrated that HCV infection itself did not induce DNA methylation in this in vitro HCV infection system.

DNA Methylation Analysis in Human Hepatocyte Chimeric Mice With HBV or HCV Infection

In order to unravel the effects of virus–host interactions, especially their impact on DNA methylation in vivo, we examined DNA methylation status in human hepatocyte chimeric mice after infection with HBV (HBV mice) or HCV (HCV mice) (Supplementary Table 2). Both HBV mice (n = 10) and HCV mice (n = 10) were sacrificed at different time points and analyzed for genome-wide DNA methylation status using MCAM. The number of methylated genes in HCV mice was similar to or slightly more than that in HBV mice (Figure 2*A*, mean 237 ± 110 genes vs 160 ± 63 genes, respectively; Supplementary Table 4). More genes were methylated in long-term HBV- or HCV-infected mice (≥16 weeks) than in short to middle-term infected mice (<16 weeks), suggesting that aberrant DNA methylation propagated in a time-dependent manner after viral infection (Figure 2*A*).

The majority of methylated genes in HBV mice were also methylated in HCV mice (149 of 214 genes [70%]), and about half of the methylated genes in HCV mice were specific to these mice (146 of 295 genes [50%]; Figure 2*B*). These data obtained in the mouse model were consistent with the clinical data of HCCs (Figure 1*C*). In addition, a number of methylated genes in human clinical samples were also methylated in HBV mice (Figure 2*C*; 78 of 214 genes [36%]) and HCV mice (134 of 295 genes [45%]). The human hepatocyte chimeric mouse with either HBV or HCV infection appeared to represent the virus–host interactions in human liver and can be an appropriate model to study the mechanisms of induction of aberrant DNA methylation by viral infection in vivo.

Time-Dependent Alterations in DNA Methylation After HBV and HCV Infections

We then evaluated the precise changes in DNA methylation at different time points after viral infection by

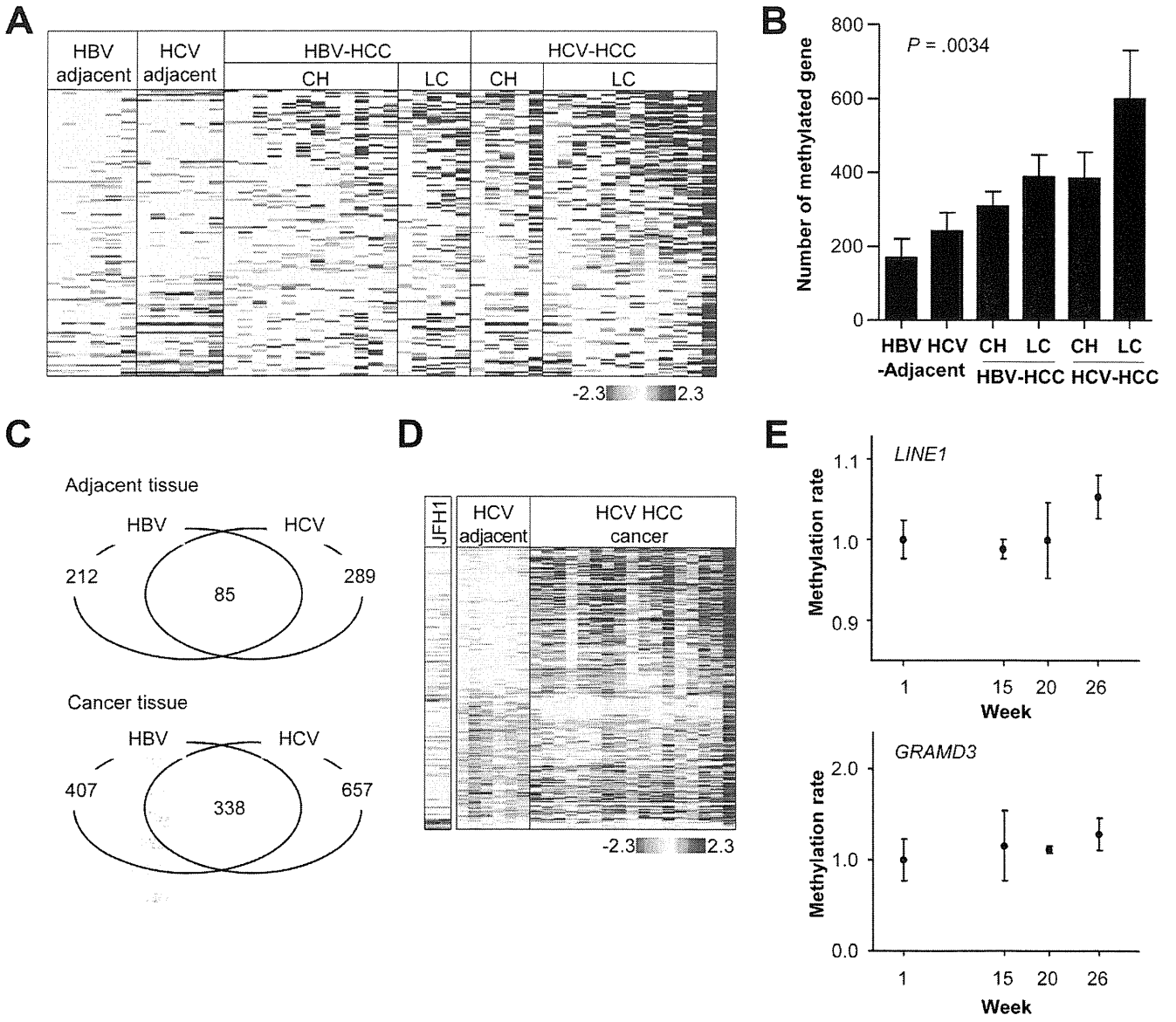

Figure 1. DNA methylation analysis in HCCs. (*A*) Heatmap overview of 940 significantly methylated genes in cancerous tissues (17 cases each of samples associated with HBV and HCV infections) from chronic hepatitis (CH) patients and liver cirrhosis (LC) patients, and corresponding adjacent noncancerous tissues (6 cases each of samples associated with HBV and HCV infections). *Red* and *blue* in cells reflect high and low methylation levels, respectively, as indicated in the scale bar (log2-transformed scale). (*B*) Mean number of hypermethylated genes in clinical samples. Kruskal-Wallis analysis was used to evaluate the extent of differences among the groups ($P = .0034$). (*C*) Number of DNA methylation target genes in HBV-associated and HCV-associated HCCs are shown by Venn diagram (*upper panel*, adjacent noncancerous tissues; *lower panel*, cancerous tissues). (*D*) Heatmap overview of 880 methylated genes in Huh7.5.1 cells infected with HCV-JFH1 (at 26 weeks after infection, duplicated results), and 6 adjacent noncancerous and 17 cancerous tissues from HCV-HCC patients. (*E*) DNA methylation levels of *LINE1* and GRAM domain containing 3 in Huh7.5.1 cells after HCV-JFH1 infection at 1 week, 15 weeks, 20 weeks, and 26 weeks after infection. Y-axis indicates relative values of DNA methylation level in HCV-JFH1–infected Huh7.5.1 to that of uninfected Huh7.5.1 cells. GRAMD3, GRAM domain containing 3.

pyrosequencing analysis in 16 HBV mice and 16 HCV mice (Supplementary Table 2). Methylation of the *LINE1* gene as an indicator of global methylation status has been shown to be inversely associated with tumor transformation.[3,21] The level of *LINE1* methylation in long-term HCV-infected mice was significantly lower than in uninfected control mice (Figure 3*A*; $P = .037$). A similar tendency was also observed in long-term HBV-infected mice, although the decreased methylation level was not statistically significant. Next, 5 genes were selected from the list of MCAM data. Estrogen receptor 1 (*ESR1*) and homeobox A6 (*HOXA6*) were the most frequently methylated in both clinical samples and the mouse models as assessed by MCAM analysis. Zinc finger protein 385A and ELOVL fatty acid elongase 3 genes were less frequently methylated as compared with *ESR1* and *HOXA6* in MCAM analysis. *RASSF1A* is a well-known tumor

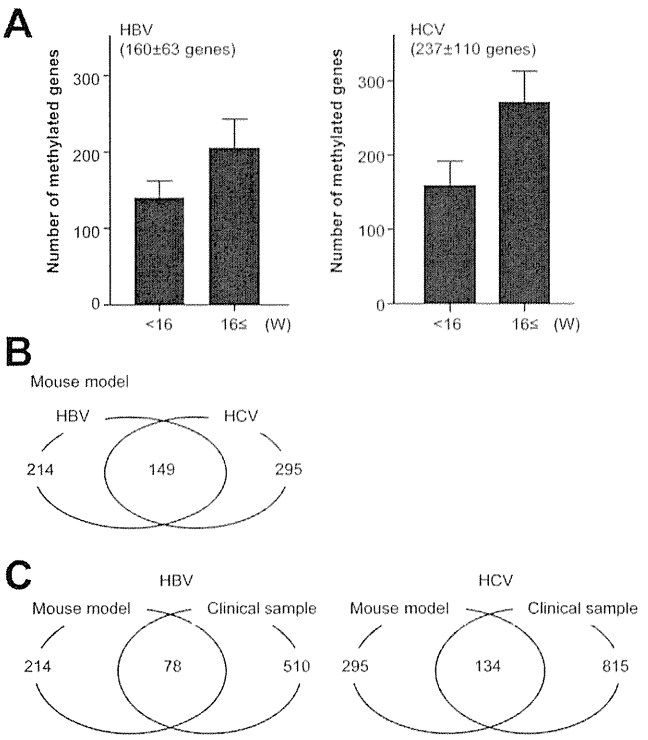

Figure 2. DNA methylation analysis in human hepatocyte chimeric mice with HBV or HCV infection. (*A*) The number of DNA methylation target genes in HBV mice and HCV mice by MCAM analysis. Error bars denote standard deviations. (*B*) The number of DNA methylation target genes in HBV mice and HCV mice is shown by Venn diagram. (*C*) The number of common DNA methylation target genes in the mouse model and clinical samples is shown by Venn diagram (*left*, HBV mice and HBV-associated clinical samples; *right*, HCV mice and HCV-associated clinical samples).

suppressor gene, DNA methylation of which has been shown to accumulate according to HCC progression.[8] Four (*RASSF1A*, *HOXA6*, *ESR1*, and *zinc finger protein 385A*) of 5 genes were moderately to highly methylated in the hepatocellular carcinoma cell line, Huh7.5.1 (Supplementary Table 5). Most of the 5 genes showed increased DNA methylation in a time-dependent manner during HBV or HCV infection (Figure 3*B*). Intriguingly, the DNA methylation level in 3 genes, *RASSF1A*, *ESR1*, and ELOVL fatty acid elongase 3, in uninfected mice (≥16 weeks) was slightly or moderately increased (13%, 29%, and 14%, respectively, Supplementary Table 5) as compared with unmethylated control DNA (normal lymphocyte DNA, 8%, 9%, and 7%, respectively). This was consistent with the MCAM data showing that the average signal intensity of Cy3 (DNA from uninfected mice) of the commonly methylated 149 genes in both HBV mice and HCV mice (Figure 2*B*) was significantly higher than that of other genes (Supplementary Figure 3). Given the principle of the MCAM technique, which allows for efficient polymerase chain reaction amplification of methylated CpG islands,[22] these data suggested that there were some genes that were methylated at low levels, even in normal tissues (eg, age-related DNA methylation) and were prone to DNA methylation in response to continuous viral infection regardless of viral types.[23] Conceivably, increased DNA methylation level in this model might not be due simply to the increased expression level of DNA methyltransferases or decreased level of methylcytosine dioxygenases, such as ten-eleven translocation 1 and 2, which are associated with DNA demethylation (Supplementary Figure 6)

Expression of Inflammation-Related Genes in Human Hepatocyte Chimeric Mice With HBV or HCV Infection

Our data in human hepatocyte chimeric mice suggested a link between activated host immune system by viral infection and induction of DNA methylation. We examined the kinetics of several inflammation-related genes, *Ifng*, *Il1b*, *Il6*, *Il12b*, *Tnf*, and *Cxcl2* using either mouse- or human-specific primer sets; among them IFN-gamma production was attributed to NK cells of SCID mice, and interleukin (IL)-1β, IL-6, IL-12, CXCL2, and tumor necrosis factor–α were produced by the other types of cells, such as Kupffer cells and hepatocytes.[24,25] In addition, the protein level of IFN-gamma from mouse-derived cells was evaluated by mouse-specific anti–IFN-gamma (Supplementary Figure 8*E*). The levels of these inflammation-related genes produced from human-derived cells were much lower than those produced from mouse-derived cells, indicating the predominant role of the mouse innate immune system against HBV or HCV infection in this mouse model (Supplementary Figure 4). Uninfected mice showed very low expression of the *Ifng* and *Il12b* genes in liver tissues, while *Il1b*, *Il6*, *Tnf*, and *Cxcl2* were substantially expressed. Expression levels of *Ifng* and *Tnf* significantly increased in both HBV- and HCV-infected livers ($P < .05$, Figure 4*A*). Expression level of *Il12b* was significantly increased in HCV-infected liver, and also tended to increase in HBV-infected liver, although the difference was not statistically significant. By contrast, expression levels of the 3 other genes, *Il1b*, *Il6*, and *Cxcl2*, did not significantly increase after viral infection. It appears that DNA methylation was not involved in the regulation of expression of those cytokines, because no aberrant DNA methylation was detected in the promoter regions of the corresponding genes (Supplementary Table 5). The induction of *Ifng* and *Il12b* genes was almost completely abolished by administration of a specific inhibitor of NK cell activity, anti–asialo GM-1 treatment.[26] However, the treatment of anti–asialo GM-1 did not significantly affect *Tnf* expression in HCV mice. In addition, this treatment showed no effect on virus levels in both HBV and HCV mice (Supplementary Figure 7).

Notably, IFN-gamma is known to enhance the production of ROS in inflammatory cells, such as activated Kupffer cells.[27,28] ROS production was evaluated by dihydroethidium staining (Figure 4*B*).[16] Quantification of fluorescence intensity in liver specimens revealed that the level of ROS production was significantly increased in both HBV and HCV mice in comparison with uninfected mice (Figure 4*C*; $P < .05$). Intriguingly, induction of ROS was abolished by anti–asialo GM-1 treatment, which confirmed that ROS

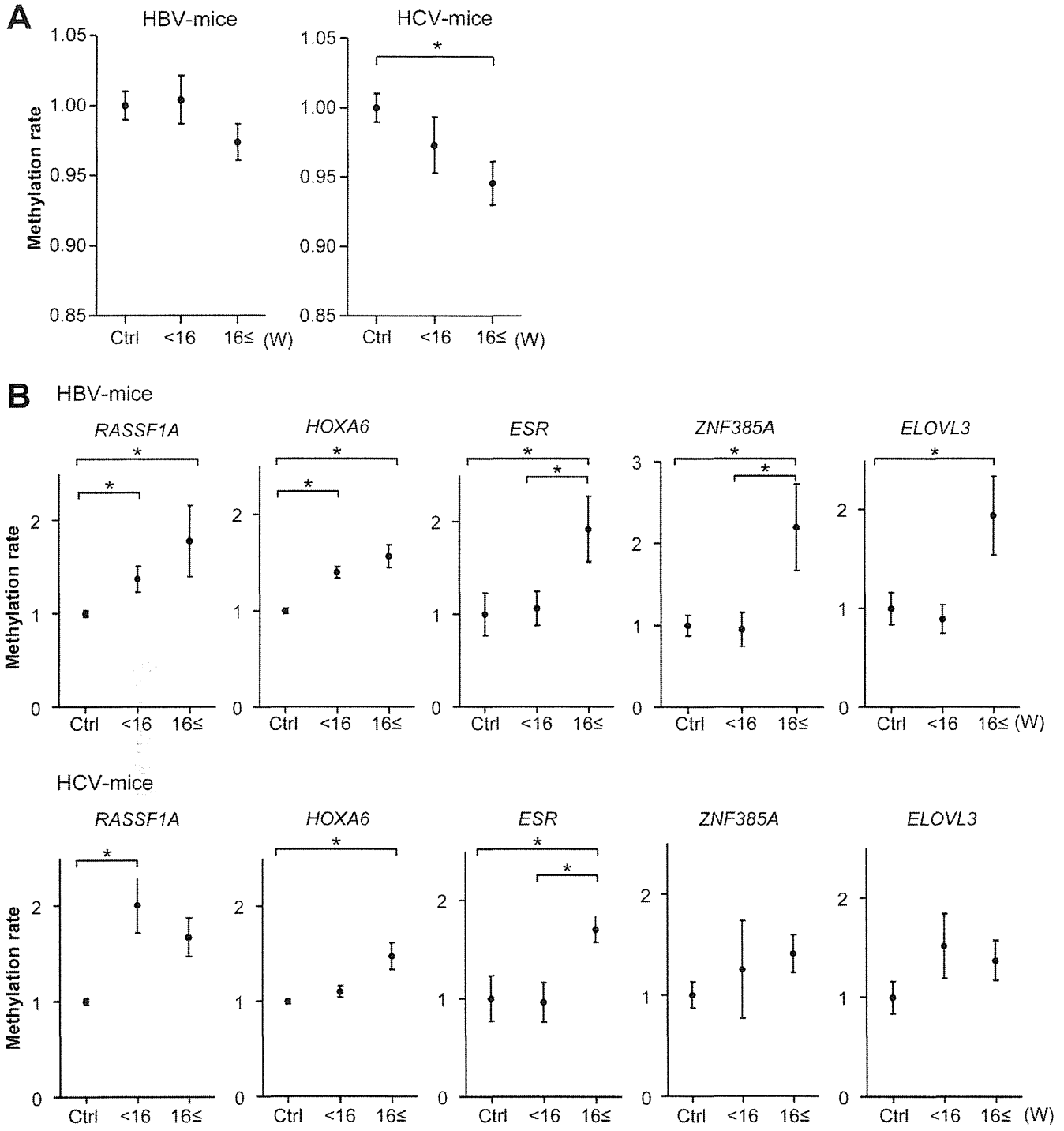

Figure 3. Time-dependent alterations in DNA methylation after HBV and HCV infections. DNA methylation levels of *LINE1* (*A*) and 5 target genes (*B*) in HBV mice and HCV mice by pyrosequencing analysis. Y-axis indicates relative values of DNA methylation level in HBV or HCV mice to that of uninfected mice. Error bars denote standard deviations. *$P < .05$. Ctrl, control; ELOVL3, ELOVL fatty acid elongase 3; ZNF385A, zinc finger protein 385A.

production after virus infection was dependent on NK cell activity. These dynamic changes in cytokine level and ROS production appeared to be reflected by the alanine aminotransferase level, especially in HBV mice (Supplementary Figure 7).

Evaluation of Cell Proliferation in Human Hepatocytes With Ki-67 and Proliferating Cell Nuclear Antigen After Viral Infection

When virus-infected hepatocytes are destroyed, hepatocyte regeneration is activated, resulting in continual

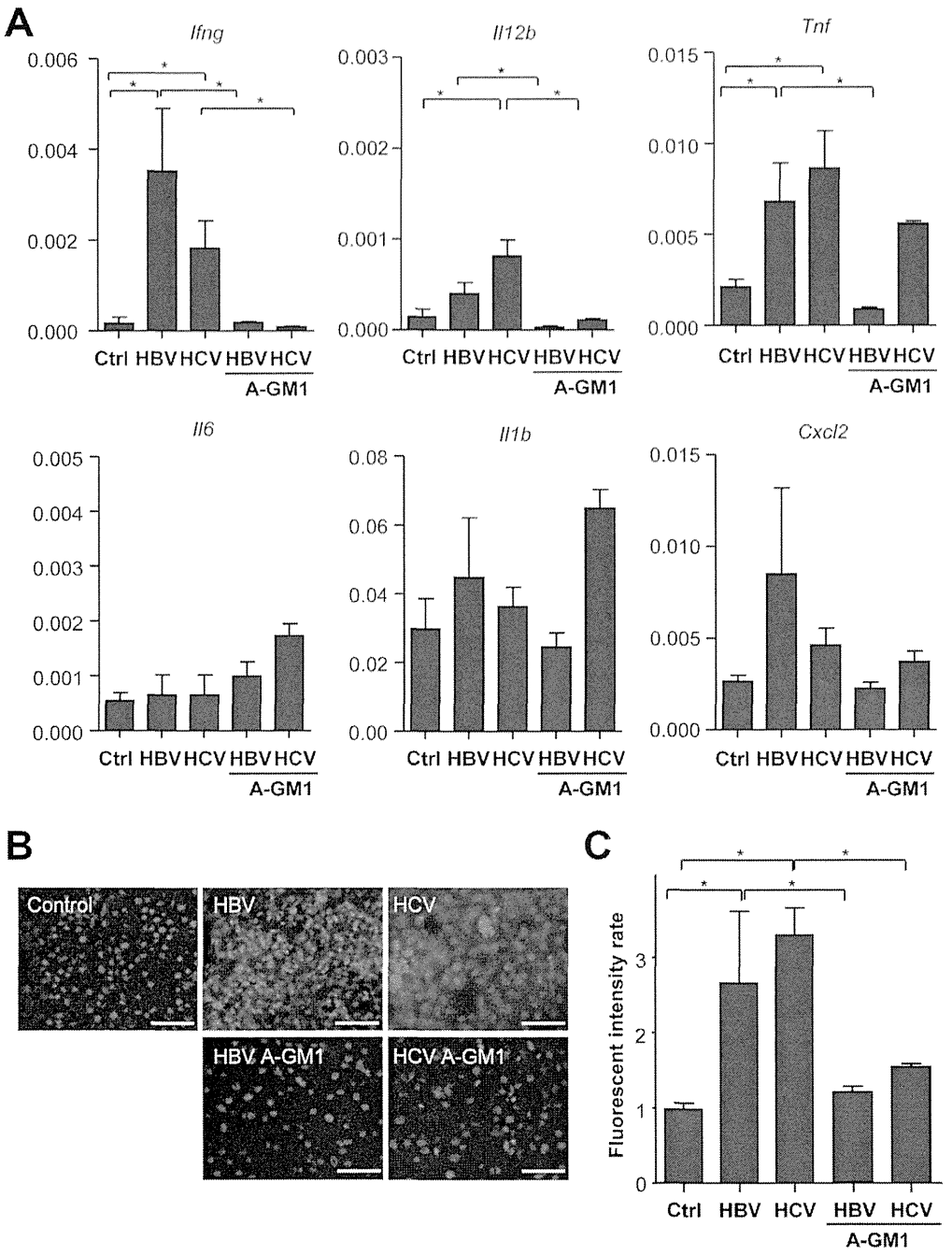

Figure 4. Expression analysis of inflammation-related genes in HBV and HCV mice. (*A*) Gene expression of *Ifng*, *Il12b*, *Tnf*, *Il1b*, *Il6*, and *Cxcl2* was measured by quantitative reverse transcription polymerase chain reaction in HBV and HCV mice with and without treatment of anti–asialo GM-1 (A-GM1) using mouse-specific primers. Relative values of messenger RNA expression for each gene normalized to *glyceraldehyde-3-phosphate dehydrogenase* are shown in Y-axis. Error bars denote standard deviations. $^*P < .05$. (*B*) Production of ROS was analyzed in liver sections of HBV and HCV mice with and without treatment of anti–asialo GM-1 by fluorescence microscopy. Bar = 50 μm. (*C*) Fluorescence intensity in different areas (at least 5 areas) of each image was quantified. Relative values of averaged intensities in HBV and HCV mice to uninfected control (Ctrl) are shown in Y-axis. Error bars denote standard deviations. $^*P < .05$.

proliferation of hepatocytes and constant liver regeneration.[29,30] In response to viral infection and activation of the innate immune system, expression of *NFKB1*, which enhances the proliferation of hepatocytes, was increased in human hepatocytes (Figure 5*A*). This induction was abolished by anti–asialo GM-1 treatment. We analyzed the Ki-67 index as a proliferation marker and found that it was increased in both HBV-infected and HCV-infected liver tissues as compared with uninfected control (P =.03, P =.006, respectively; Figure 5*B*). Consistently, a similar tendency was observed in the expression of another proliferation marker, proliferating cell nuclear antigen *(PCNA)* and in the bromodeoxyuridine uptake analysis as well (Figure 5*C* and *D*). The induction of *PCNA* or bromodeoxyuridine incorporation was also abolished by anti–asialo GM-1 treatment.

Inhibition of NK Cell Function by Anti–Asialo GM1 Attenuated the Induction of DNA Methylation in HBV and HCV Mice

NK cell activity can be almost completely abolished by administration of anti–asialo GM-1 treatment. To clarify

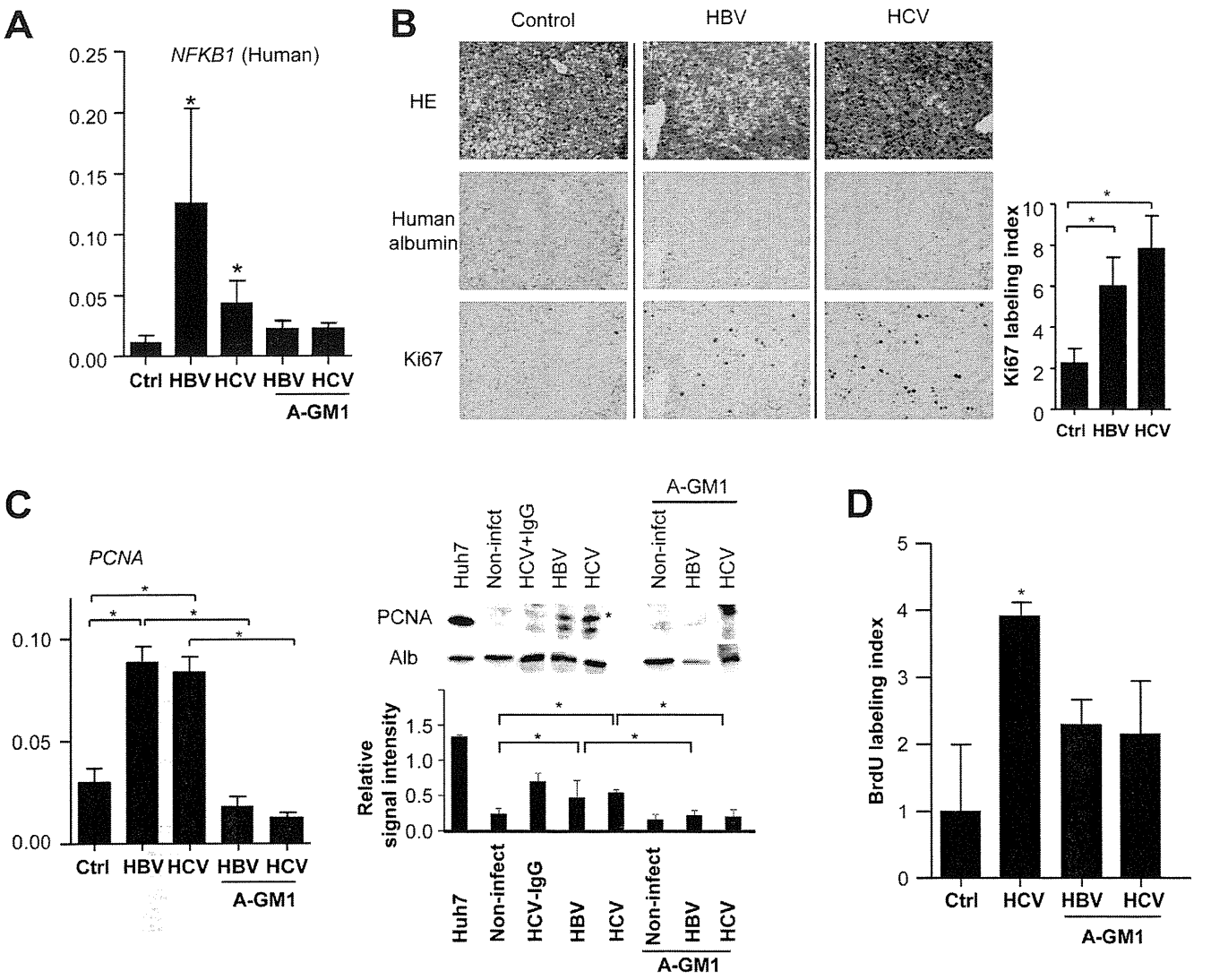

Figure 5. Evaluation of hepatocyte proliferation in HBV and HCV mice. (*A*) Expression of *NFKB1* was analyzed in HBV and HCV mice using human-specific primers. Expression of *NFKB1* in HBV and HCV mice without anti–asialo GM-1 treatment (A-GM1) was significantly higher than that in HBV and HCV mice with A-GM1 treatment (*$P < .05$). Error bars denote standard deviations. (*B*) Histologic comparison among uninfected, HBV, and HCV mice. Liver specimens stained with H&E, anti-human albumin, and anti-human Ki-67 are shown (*left panel*). Ki-67 labeling index was calculated and is shown in *bar graph* (*right panel*). (*C*) Expression of PCNA was analyzed by quantitative reverse transcription polymerase chain reaction using human-specific primers (*left panel*). *$P < .05$. Protein level of PCNA was examined by Western blotting (*right upper panel*) and band intensities were quantified (*right bottom panel*). IgG, nonspecific rabbit polyclonal antibody treatment. *Nonspecific bands are observed around 40 kDa. (*D*) Bromodeoxyuridine (BrdU) labeling index was calculated and is shown in *bar graph*.

whether attenuated NK cell activity affected DNA methylation status, we examined HBV and HCV mice with and without anti–asialo GM-1 treatment (Figure 6). In the context of global DNA methylation, the level of *LINE1*-methylation was decreased in HBV and HCV mice, and anti–asialo GM-1–treated mice showed sustained levels of *LINE*1 methylation. Consistently, anti–asialo GM-1 treatment attenuated the induction of DNA methylation in *RASSF1A*, *HOXA6*, and *ESR1* genes in HBV and HCV mice as well ($P < .05$, respectively).

In order to confirm whether NK cell activity via IFN-gamma is a major cause of induction of DNA methylation, we neutralized IFN-gamma activity using an anti-mouse IFN-gamma in the chimeric mice with and without virus infection (Supplementary Figure 8). Concordant with the anti–asialo GM-1 treatment, anti-mouse IFN-gamma blocked the induction of DNA methylation together with suppression of ROS production and hepatocyte cell proliferation, although the effects of anti–IFN-gamma were a little milder than those of anti–asialo GM-1 treatment.

Taken together, these data demonstrated that NK cell activity and its associated immune reaction were incriminated in induction of aberrant DNA methylation in human hepatocytes after viral infection.

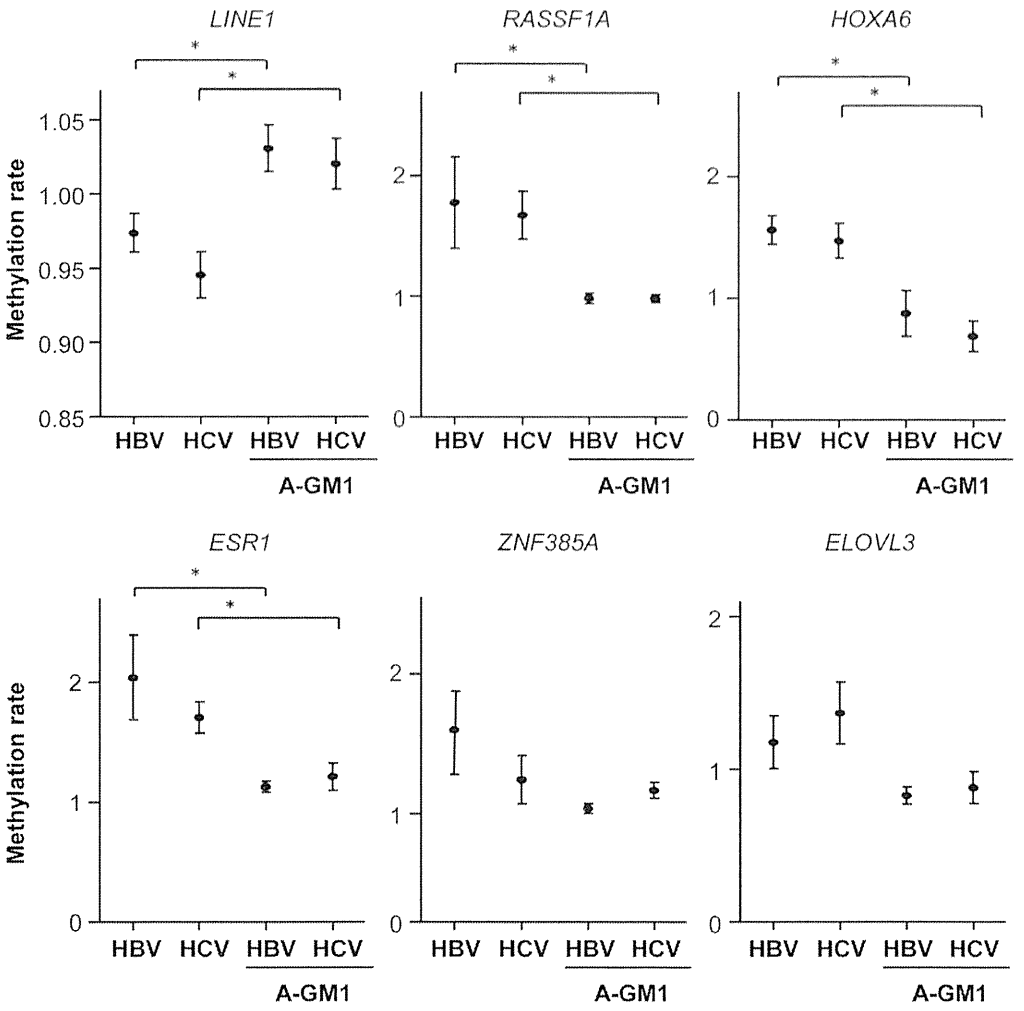

Figure 6. Levels of DNA methylation in HBV and HCV mice with and without treatment of anti–asialo GM1 (A-GM1). DNA methylation levels of *LINE1*, *RASSF1A*, *HOXA6*, *ESR1*, zinc finger protein 385A (*ZNF385A*), and ELOVL fatty acid elongase 3 (*ELOVL3*) genes in HBV and HCV mice with and without treatment of A-GM1 were analyzed by pyrosequencing analysis. Y-axis indicates the same as in Figure 3. *$P < .05$.

BASIC AND TRANSLATIONAL LIVER

Discussion

In the current study, we examined the dynamics of DNA methylation after initial infection with HBV or HCV in hepatocytes using an in vivo human hepatocyte chimeric mouse model.[13,14,16] Using this model, we reproducibly found that genome-wide DNA methylation changes were induced in a time-dependent manner after viral infection, and no significant alterations in DNA methylation were induced in a tissue culture–based HCV infection model. Conceivably, the minimum effects on cell proliferation and moderate ROS production after in vitro HCV infection enabled induction of DNA methylation in Huh7.5.1 cells, in which the basal level of DNA methylation is already elevated.

Some of the DNA methylation target genes in human hepatocytes in HBV and HCV mice were comprised of well-known tumor suppressor genes (eg, *RASSF1A*), which were also methylated in clinical HCC cases, suggesting clinical relevance of this mouse model to study dynamics of DNA methylation after hepatitis virus infection.

Among the examined cytokine-associated genes, *Ifng* was significantly up-regulated in response to HBV and HCV infections. Typically, IFN-gamma is produced by NK cells and plays an important role in attenuating HBV and HCV pathogenesis during initial infection.[24,31] IFN-gamma potentially stimulates Kupffer cells and dendritic cells to produce an abundance of cytokines, including IL-12.[32,33] In addition to producing cytokines, Kupffer cells activated by IFN-gamma are the major source of ROS in the liver.[27,28] Intriguingly, IL-12 is a potent activator of NK cell effector function. This feedback loop can efficiently function to activate the innate immune system and induce NK cell–mediated liver damage. In our model, up-regulation of mouse-specific *Ifng* and *Il12b* was almost completely attenuated by treatment with anti–asialo GM1, together with sustained DNA methylation in several genes. These data indicated that in our mouse model, NK cell function was a key player in inducing aberrant DNA methylation in human hepatocytes after initial HBV and HCV infections. In line with our study, several studies have demonstrated that inflammation-associated mechanisms can induce aberrant DNA methylation in mouse epithelial cells of the stomach and colon in vivo.[34–36] In addition, a recent in vitro study showed that oxidative damage induces recruitment of DNA

methyltransferase in certain loci to promote aberrant DNA methylation.[37,38] In addition to these models, we propose here a possibility that aberrant DNA methylation accumulates via cell-cycle activities, which induce inappropriately accelerated aging in hepatocytes. A few lines of evidence support our hypothesis. When virus-infected hepatocytes are destroyed by immune reaction, such as by activated Kupffer cells, hepatocyte regeneration is generally activated; repeated cycles of immune-mediated clearance of virus-infected hepatocytes cause continual proliferation of hepatocytes and constant liver regeneration, which is modulated by nuclear factor–κB function.[29] We detected significantly increased cell proliferation coupled with nuclear factor–κB induction in HBV- and HCV-infected liver specimens in the mice. These phenomena might be connected with the replication-linked stochastic error model that predicts that variability first arises during aging and that cancers could accentuate variability.[23,39] Given that it can be seen even in normal aging tissues, this variation and the consequent natural selection are probably more obvious in highly proliferative conditions (eg, chronic hepatitis), resulting in their contribution to focal proliferative lesions, such as preneoplastic tumors. Large numbers of the DNA methylated genes after viral infection in the mouse model appeared to be age-related DNA methylation target genes. We propose that continuous inflammation and induction of certain types of cytokines in the liver can induce aberrant DNA methylation via increased cell turnover. Compellingly, recent clinical reports showing that the presence of hepatitis viruses, especially HCV, could play a role in accelerating the methylation process (age-related) that is involved in HCC development.[19] Although there is a weak inverse correlation between DNA methylation level and gene expression in the 5 genes examined, none of them were silenced (Supplementary Figure 6), suggesting that the accumulated DNA methylation level of each gene during the period of viral infection examined in this study was still not high enough to inactivate the gene expression.

In conclusion, this is the first study to show the initial dynamics of DNA methylation after HBV and HCV infections in human hepatocytes in an in vivo model. Multiple machineries, including DNA methyltransferase and oxidative stress, can induce DNA methylation; however, the current model convincingly showed that NK cell function and consequent mechanisms were the key factors for induction of DNA methylation. This model probably did not develop HCC due to limited observation time. Given the critical roles of epigenetic alterations in hepatocarcinogenesis after viral infection, control of inflammation, as well as key molecules such as IFN-gamma, are good potential targets for prevention of inflammation-associated cancer development.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at http://dx.doi.org/10.1053/j.gastro.2013.10.056.

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