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Hepatitis C virus represses E-cadherin expression via DNA methylation to induce epithelial to mesenchymal transition in human hepatocytes

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ABSTRACT

Hepatitis C virus (HCV) core protein is known to induce promoter hypermethylation of tumor suppressor genes including E-cadherin to repress their expression when overexpressed in human hepatocytes; however, its actual role during HCV infection is still unknown. Here, we report that infection with HCV derived from pJFH-1 replicon system that mimics natural infection elevates protein levels of DNA methyltransferase 1 and 3b to enhance DNMT activity in human hepatocytes. As a consequence, HCV induced promoter hypermethylation of E-cadherin, resulting in repression of its expression. In addition down-regulation of E-cadherin by HCV led to epithelial–mesenchymal transition that is known to be a critical event during the late stage of tumorigenesis.

1. Introduction

Hepatocellular carcinoma (HCC) is caused by both genetic and epigenetic alterations of several tumor-associated genes [1,2]. In particular, DNA methylation of tumor suppressor genes has been described as one of the major epigenetic alterations in HCC [1,2]. Aberrant promoter hypermethylation of tumor suppressor genes (TSGs) involved in the cell proliferation, apoptosis, cell adhesion, DNA repair, and detoxification is frequently detected in HCC, resulting in loss of the corresponding gene function [1,2]. In addition, up-regulation of DNMT1, 3a and 3b has been detected in HCC compared to non-neoplastic or normal liver [5,6], indicating a close correlation between DNMT up-regulation and promoter hypermethylation-mediated inactivation of TSGs. However, the mechanism and its biological significance are largely unknown.

Hepatitis C virus (HCV) is a major cause of non-A and non-B acute and chronic hepatitis, which frequently leads to liver cirrhosis and HCC [3]. Interestingly, in comparison with normal liver, the incidence of promoter methylation in some tumor suppressor genes like SOCS-1, GSTP, APC, and p16 is significantly higher in HCV-positive ones [4,5]. In addition, it has been demonstrated that HCV Core protein up-regulates levels of DNMT1 and 3b and induces promoter hypermethylation of tumor suppressor genes like E-cadherin and p16, resulting in down-regulation of their expression [6–8]. These observations suggest that HCV Core is responsible for the DNA methylation-mediated inactivation of TSGs in HCV-associated HCC. However, these studies were performed using Core overexpression systems, which may not properly reflect the actual HCV replication situation *in vivo*. The present study thus aimed to verify that Core expressed from the viral genome during virus replication actually induces DNA methylation of TSGs. For this purpose, we first examined whether infection with HCV derived from pJFH-1 replicon system that mimics natural infection up-regulates both protein levels and enzyme activity of DNMTs. In addition, it was investigated whether the increased DNMT activity induces DNA methylation of E-cadherin and down-regulates its expression during HCV infection. Lastly, we attempted to provide biological significance of the DNA methylation-mediated down-regulation of E-cadherin expression in the HCV-infected cells.

2. Materials and methods

2.1. HCV replicon system

The plasmid pJFH-1 containing HCV cDNA from a Japanese patient with fulminant hepatitis behind a T7 promoter [9] was linearized at the 3′ end of the HCV cDNA by XbaI digestion. The linearized DNA was then used as a template for *in vitro* transcription (MEGAscript; Ambion). 10 μg of JFH-1 RNA was delivered to Huh7.5 cells by electroporation and virus stocks were prepared as described by Zhong et al. [10]. Real-time RT-PCR analysis was performed as described before [11] to determine HCV RNA levels relative to a standard curve comprised of serial dilutions of plasmid containing the HCV JFH-1 cDNA.

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2.2. Transient transfection and luciferase assay

For transient expression, 2×10^5 cells per 60-mm dish were transfected with 1 μg of appropriate plasmid(s) using WelFect-EX-™PLUS (WelGENE) following the manufacturer's instructions. The reporter plasmids, DNMT1-luc [12] and Ecad-luc [13] containing the promoter region of DNMT1 and E-cadherin, respectively, in front of a firefly luciferase gene in pGL2-Basic Vector (Promega) were described before. 0.1 μg of pCH110 (Pharmacia) containing the *Escherichia coli* lacZ gene under the control of the SV40 promoter was cotransfected as an internal control. At 48 h after transfection, luciferase assay was performed using a luciferase assay system (Promega) and the value obtained was normalized to the β-galactosidase activity measured in the corresponding cell extracts. Each experiment was repeated at least three times prepared in triplicate.

2.3. DNMT activity assay

2×10^5 cells per 60-mm diameter plate were either mock-infected or infected with HCV at 0.3 MOI for 24 h. DNMT activity in the cell lysates was measured using EpiQuick DNMT Activity/Inhibition Assay Ultra Kit (Epigentek) following the manufacturer's instructions.

2.4. RNA interference

The SirenCircle RNAi system (Allele Biotech.), a plasmid-based RNA interference system that uses U6 RNA-based polymerase III promoter and modified terminator for high level siRNA in a hairpin format inside target cells was employed to knock-down specific gene expression. Based on the target sequences of DNMT1 (5′-CCA TGC AGA CCG TTC TCC-3′) [14] and DNMT3b (5′-GAA TTT GAG CAG CCC AGG TTG-3′) [15], siRNA inserts composed of both sense and antisense sequences separated by a central loop sequence were designed.

2.5. Western blot analysis

Cells were lysed in buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 0.1% SDS, 1% NP-40) supplemented with protease inhibitors. Cell extracts were separated by SDS–PAGE and transferred onto a nitrocellulose membrane (Hybond PVDF, Amersham). Membranes were then incubated with antibodies to DNMT1, DNMT2, DNMT3a, DNMT3b, E-cadherin, Fibronectin, c-Jun, phosphor-c-Jun and Vimentin (Santa Cruz Biotechnology), to Plakoglobin (BD Biosciences), to Core (HCV positive human patient sera), and to γ-tubulin (Sigma) and subsequently with an appropriate horseradish peroxidase-conjugated secondary antibody: anti-mouse, anti-goat, anti-human, or anti-rabbit IgG (H+L)-HRP (Bio-Rad). The ECL kit (Amersham) was used to visualize protein bands via the ChemiDoc XRS imaging system (Bio-Rad).

2.6. Polymerase chain reaction (PCR)

For semi-quantitative RT-PCR, total RNA (3 μg) extracted using RNeasy mini kit (Qiagen) was reverse transcribed with the corresponding antisense primer. One-quarter of the reverse-transcribed RNA was amplified with *Taq* polymerase to detect levels of E-cadherin [13], Core [7], and GAPDH [13] as described before.

2.7. Methylation-specific PCR (MSP) and bisulfite DNA sequencing

Genomic DNA (1 μg) denatured in 50 μl of 0.2 N NaOH was modified by treatment with 30 μl of 10 mM hydroquinone (Sigma) and 520 μl of 3 M sodium bisulfite (pH 5.0; Sigma) at 50 °C for 16 h. For MSP, the modified DNA (100 ng) was amplified with *Taq* polymerase using both methylated and unmethylated primer pairs of E-cadherin as described before [16]. For bisulfite DNA sequencing, modified genomic DNA was amplified by PCR using a primer pair to detect the GpC-rich region of E-cadherin promoter (−179 to +30) as described before [17]. The PCR products were subcloned into the pGEM-T Easy vector (Promega) and their nucleotide sequences were determined.

2.8. Chromatin immunoprecipitation (ChIP) assay

ChIP assay was performed using a ChIP assay kit (Upstate Biotechnology) according to the manufacturer's instruction. The sheared chromatin was immunoprecipitated with an antibody against Sp1 (Santa Cruz Biotechnology) and a negative control rabbit IgG (Santa Cruz Biotechnology). DNA released from the precipitated complexes was amplified by PCR using a pair of primers for the detection of E-cadherin promoter as described before [18].

2.9. Immunofluorescence analysis

Cells grown to confluence on coverslips were fixed in 4% formaldehyde (15 min, room temperature) and then perforated in methanol (10 min, −20 °C), followed by incubation with a primary antibody against E-cadherin (Calbiochem; overnight, −4 °C) and then anti-mouse IgG-FITC (Santa Cruz Biotechnology; 1 h, room temperature). Slides were prepared on UltraCruz mounting medium (Santa Cruz Biotechnology) and visualized using Axioscope fluorescence microscope (Carl Zeiss).

2.10. Cell aggregation assay

Fast aggregation assay was performed as described previously [19]. Cells were dissociated with Hank's balanced salt solution (HBSS) with 0.01% trypsin and 1 mM $CaCl_2$ and washed twice in Ca^{2+}-free HBSS. The resulting cells (1×10^5) resuspended in 2 ml of HBSS containing 1 mM $CaCl_2$ were incubated for 30 min at 37 °C on a gyratory shaker. After incubation, the total particle number (single cells plus cell clusters) in each cell suspension was counted. The degree of aggregation is represented by the aggregation index N_t/N_0, where N_0 is the total particle number before incubation and N_t is the total particle number after incubation for t min.

2.11. Wound-healing assay

For the determination of cell migration rate, the classical scratch wound-healing assay was performed as described before [20]. Briefly, 1×10^6 cells were plated and cultured to create a confluent monolayer in 60-mm dish. After gently scratching with a pipette tip to produce a wound, cells were either mock-infected or infected with HCV in the presence or absence of 5 μM 5-Aza-2′dC for 0, 12, and 24 h. Wound healing rate was determined by measuring closure rate of the wound.

2.12. Statistical analysis

The values indicate means ± S.D. from at least three independent experiments prepared in duplicate. Two-tailed student's *t*-test was used for all statistical analyses; a P value of <0.05 was considered statistically significant.

3. Results

3.1. HCV elevates levels of DNMT1 and 3b to enhance DNMT activity

The HCV genotype 2a replicon, JFH-1, replicates efficiently in Huh-7 cells [21,22] and thus mimics natural infection in cell culture. Therefore, we first examined whether infection with HCV derived from this replicon system up-regulates both protein levels and enzyme activity of DNMTs. The progression of HCV infection was monitored by measuring levels of HCV genomic RNA isolated from the infected cells (Fig. 1A). In addition, HCV Core was detected only in the infected cells (Fig. 1B). Levels of DNMT1 and 3b were up-regulated in the infected cells whilst those of DNMT2 and 3b were little affected (Fig. 1B). In addition, the infected cells exhibited about twofold higher DNMT activity (Fig. 1C), resulting in promoter hypermethylation of E-cadherin (Fig. 1D).

To determine the enzyme responsible for the promoter hypermethylation of E-cadherin in the HCV-infected cells, we conducted RNA interference experiments using siRNA specific for each enzyme. When either DNMT1 or 3b was specifically knocked-down in the HCV-infected cells, the effect on DNA methylation of E-cadherin was almost completely abolished (Fig. 1D). Therefore, up-regulation of both DNMT1 and 3b appears to be required for the promoter hypermethylation of E-cadherin in the HCV-infected cells.

3.2. HCV activates DNMT1 expression via activation of AP1 activity

Although both DNMT1 and 3b appears to be required for the HCV-medicated promoter hypermethylation of E-cadherin, levels of DNMT1 were more dramatically up-regulated in the infected cells (Fig. 1b), suggesting its critical role in this process. Therefore, we next investigated the mechanism by which HCV up-regulates levels of DNMT1. According to previous reports, Core activates AP-1 transcriptional activity [23] and DNMT1 promoter contains the binding motifs of the AP-1 complex [24]. Furthermore, it has been demonstrated that Core activates DNMT1 via activation of AP-1 [8]. Consistently, treatment with a specific JNK inhibitor, SP600125 [25], not only dose-dependently inhibited the phosphorylation and expression of c-Jun in the infected cells (Fig. 2A) but also almost completely abolished the potentials of HCV to up-regulate the promoter activity and protein level of DNMT1 (Fig. 2A and B), suggesting that HCV stimulates expression of DNMT1 via up-regulation of AP-1 activity. In contrast, levels of DNMT3b were little affected under the condition, indicating that HCV up-regulates levels of DNMT1 and DNMT3b via different mechanisms.

3.3. HCV represses E-cadherin expression via DNA methylation

According to previous reports, Core represses E-cadherin expression via DNA methylation in human hepatocytes [6,7]. Consistently, infection with HCV in Huh7.5 cells induced promoter

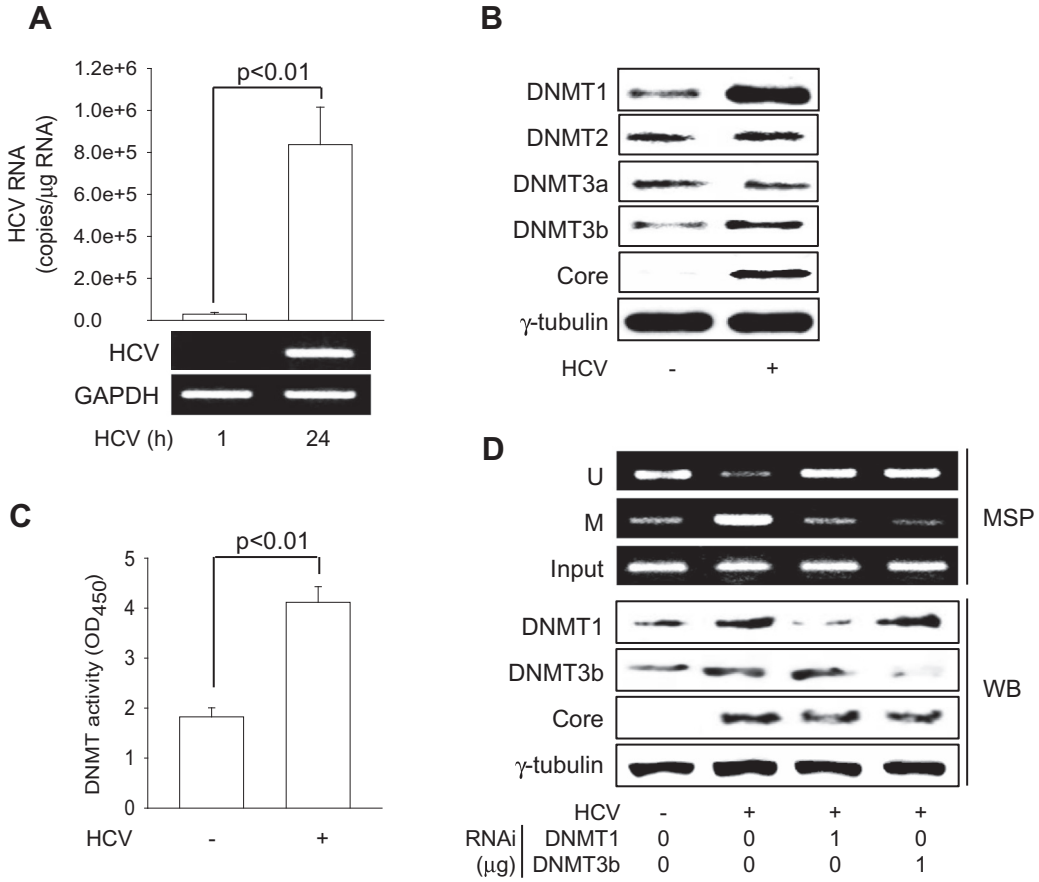

Fig. 1. HCV up-regulates levels of DNMT1 and 3b to enhance DNMT activity. Huh7.5 cells were infected with HCV at 0.3 MOI for the indicated period (A). Huh7.5 cells were either mock-infected or infected with HCV at 0.3 MOI for 24 h (B–D). (A) Levels of HCV RNA in the infected cells were determined by both real-time and conventional RT-PCR to determine HCV RNA levels. (B) Levels of the indicated proteins were determined by Western blots. (C) DNMT activity in the cell extracts prepared from the infected cells was determined. (D) The methylation status of E-cadherin promoter was analyzed by MSP (upper panel). Levels of DNMT1 and DNMT3b were measured by Western blots (lower panel). For lanes 3 and 4, the indicated amount of RNAi plasmid specific to either DNMT1 or DNMT3b was transfected into Huh7.5 cells 24 h before HCV infection.

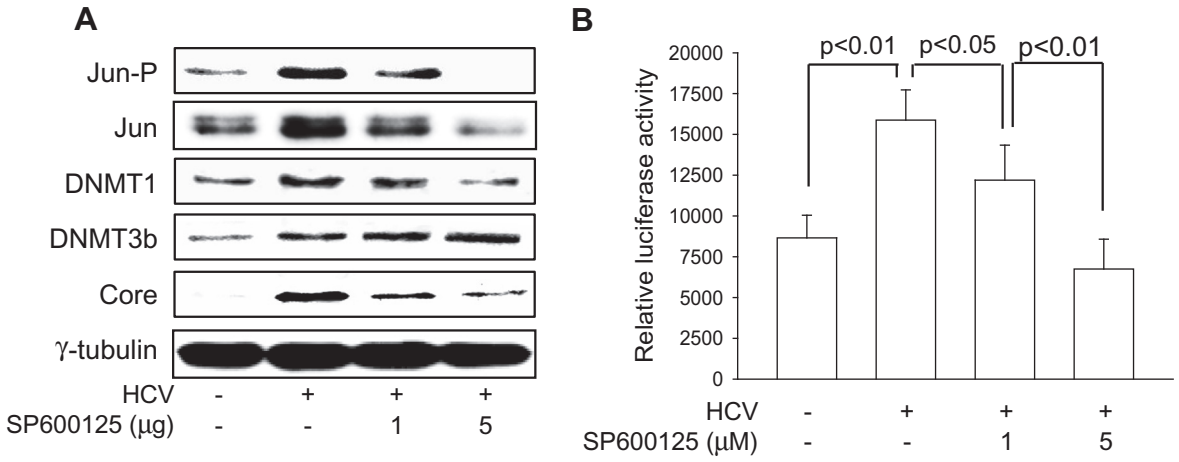

Fig. 2. HCV activates DNMT1 expression via activation of AP1. (A) Huh7.5 cells were either mock-infected or infected with HCV at 0.3 MOI for 24 h in the presence or absence of SP600125. Protein levels of phosphorylated c-Jun (Jun-P), c-Jun, DNMT1, DNMT3b, Core, and γ-tubulin were determined by Western blots. (B) Huh7.5 cells were transfected with DNMT1-luc for 24 h and then infected with HCV as above followed by luciferase assay.

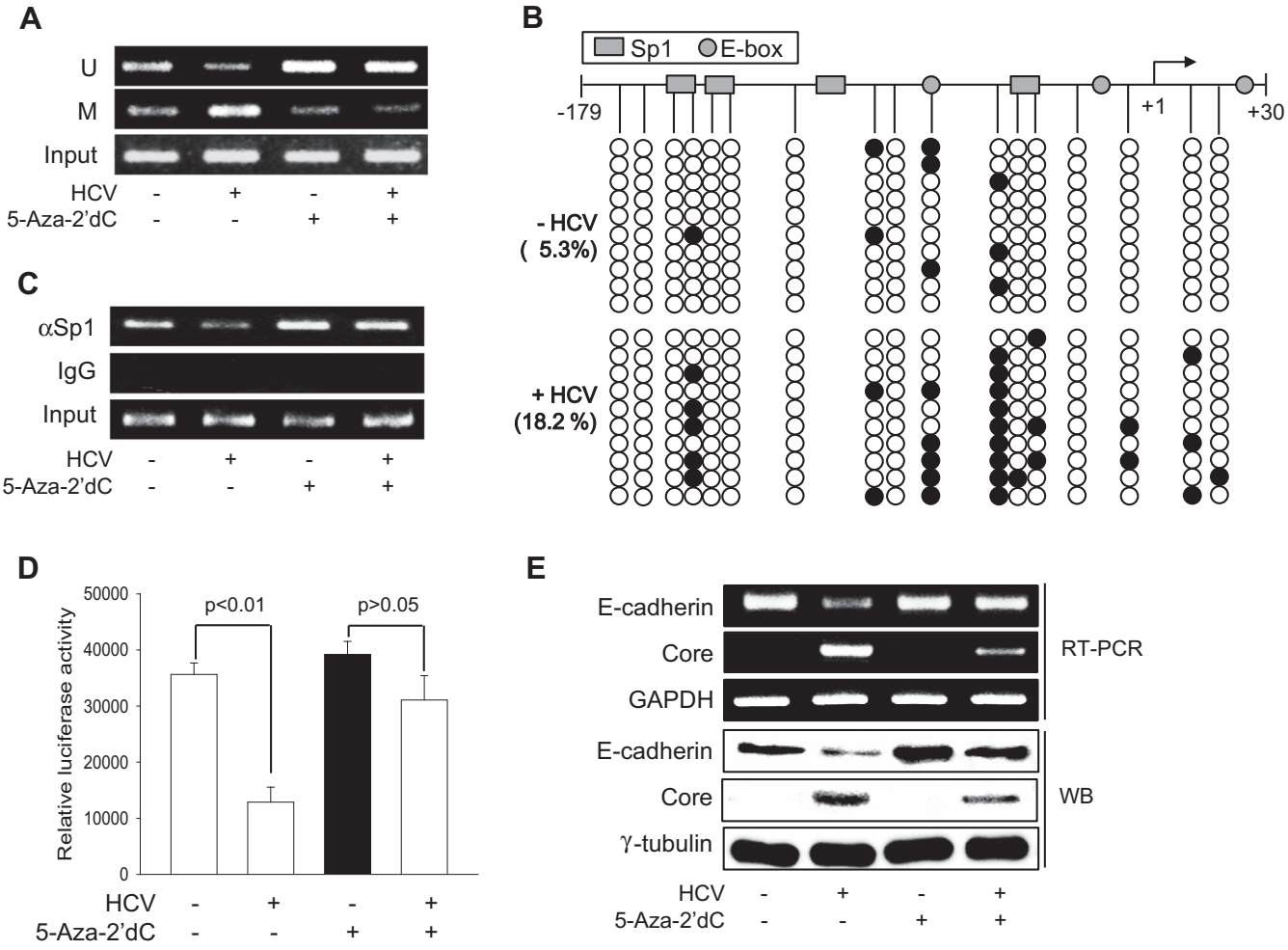

Fig. 3. HCV represses E-cadherin expression via DNA methylation. Huh7.5 cells were either mock-infected or infected with HCV at 0.3 MOI for 24 h in the presence or absence of 5 μM 5-Aza-2′dC. (A) Genomic DNA purified from Huh7.5 cells prepared as above was modified by treatment with sodium bisulfite as described in Methods. MSP analysis was performed to determine whether the CpG sites within the E-cadherin promoter is methylated (M) or unmethylated (U). (B) Bisulfite sequencing of the E-cadherin promoter region in Huh7.5 cells with or without HCV infection. The CpG sites in a 209 bp region (−179 to +30) of the E-cadherin promoter from ten different clones are shown as unmethylated (open circles) or methylated (filled circles). The positions of the Sp1 binding sites, E-boxes, and transcription initiation site are indicated. (C) ChIP assay was performed to determine the level of Sp1 bound to the positions of E-cadherin promoter in the Huh7.5 cells under the indicated conditions. (D) Huh7.5 cells were transiently transfected with E-cad-luc for 24 h and then either mock-infected or infected with HCV at 0.3 MOI for 24 h in the presence or absence of 5 μM 5-Aza-2′dC for 24 h followed by luciferase assay. (E) RNA and protein levels of the indicated genes were determined by RT-PCR and Western blots, respectively.

hypermethylation of E-cadherin (Figs. 1D and 3A). To confirm that HCV actually induces DNA methylation of E-cadherin, we performed bisulfite DNA sequencing analysis of genomic DNA isolated from Huh7.5 cells either mock-infected or infected with HCV (Fig. 3B). As a result, DNA methylation was detected in a few CpG sites (5.3%) of the E-cadherin promoter in the mock-infected cells whilst it was much higher in the HCV-infected ones (18.2%).

We next examined whether the promoter hypermethylation induced by HCV infection affects Sp1 binding on the E-cadherin promoter. According to ChIP analysis, infection with HCV dramatically down-regulated levels of Sp1 bound on the E-cadherin promoter (Fig. 3C), indicating that HCV-induced DNA methylation prevents Sp1 recruitment to the E-cadherin promoter. As a consequence, the infected cells showed significantly lower E-cadherin promoter activity (Fig. 3D), which led to the down-regulation of its RNA and protein levels (Fig. 3E). The potentials of HCV to induce DNA methylation of E-cadherin promoter (Fig. 3A), to prevent Sp1 binding on the promoter (Fig. 3C), to inhibit E-cadherin promoter activity (Fig. 3D), and finally to down-regulate both RNA and protein levels of E-cadherin (Fig. 3E) were almost completely abolished by treatment with a universal DNMT inhibitor, 5-Aza-2′dC. Therefore, we conclude that HCV induces represses E-cadherin expression via DNA methylation.

3.4. HCV induces epithelial to mesenchymal transition of Huh7.5 cells

E-cadherin is a cell–cell adhesion molecule and the loss of its expression is a hallmark of epithelial–mesenchymal transition (EMT). Therefore, we finally investigated whether the down-regulation of E-cadherin by HCV induces EMT of Huh7.5 cells. Levels of mesenchymal markers such as fibronectin and vimentin were up-regulated whilst those of epithelial markers including E-cadherin and Plakoglobin were down-regulated in the HCV infected cells (Fig. 4A and B). In addition, the infected cells showed decreased cell-to-cell interactions, as demonstrated by their lower cell aggregation ability (Fig. 4C). Furthermore, according to wound healing assay, the infected cells showed a relatively fast migratory behavior, almost completely colonizing the wound surface 24 h after the wound was made, whereas the uninfected cells at this time left 68% of the incised surface uncovered (Fig. 4D). Treatment with 5-Aza-2′dC almost completely abolished the potentials of HCV to modulate levels of EMT and MET markers (Fig. 4A and B), to decrease cell to cell interactions (Fig. 4C), and to increase cell migration (Fig. 4D), and taken together, we conclude that HCV induces EMT of Huh7.5 cells by down-regulating E-cadherin expression via DNA methylation.

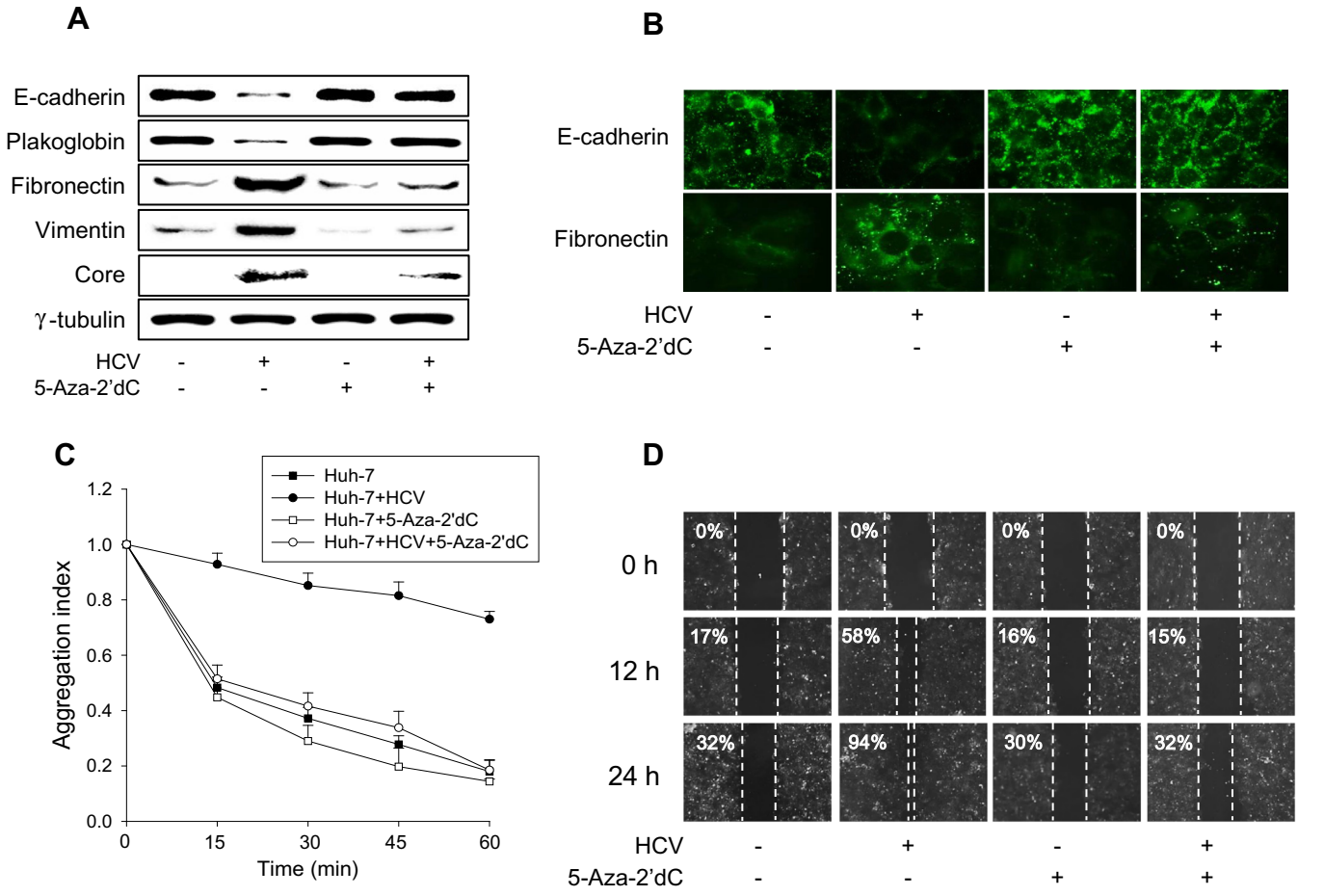

Fig. 4. HCV induces epithelial-mesenchymal transition of Huh7.5 cells. Huh7.5 cells were either mock-infected or infected with HCV at 0.3 MOI for 24 h in the presence or absence of 5 μM 5-Aza-2′dC. (A) Western blot analysis was performed to measure levels of the indicated proteins. (B) Immunofluorescent images of Huh7.5 cells showing the localization of E-cadherin and Fibronectin. (C) Fast aggregation assay of HCT116 cells was performed as described in Methods. (D) The migratory behavior of Huh7.5 cells prepared as above was analyzed in an *in vitro* wound model. Photographs of the cultures were taken immediately after the incision (0 h) and after 12 and 24 h in culture. The dotted lines indicate the boundary of cells moved to wound areas. The wound-healing rate indicates the closure rate of the wound, i.e. the percentage of recovered area at 12 and 24 h postincision compared to the incision area at 0 h.

4. Discussion

Infection with HCV is strongly associated with the development of HCC [3]. Despite current molecular evidence suggesting that Core plays an important role during HCV-mediated hepatocarcinogenesis, the detailed mechanism is still controversial [3]. Recently, we and others have provided a new action mechanism of Core involving inactivation of TSGs via DNA methylation [6–8]. However, most of the studies on the Core-mediated DNA methylation so far were based on the overexpression systems that can result in artifacts. The present study clearly shows that infection with HCV up-regulates levels of DNMT1 and 3b and elevates their enzyme activity. In addition, infection with HCV induces promoter hypermethylation of E-cadherin and represses its expression during HCV infection.

DNA methylation in mammalian cells is catalyzed by DNMT1, 3a, and 3b enzymes, which use S-adenosylmethionine as the methyl donor [26]. Overexpression of these enzymes has been associated with promoter hypermethylation of TSGs in various human tumors, including HCC [1,2,27]. According to previous reports [7,8], HCV Core up-regulates levels of DNMT1 and 3b but not DNMT2 and 3a to induce DNA methylation of TSGs. Consistently, the present study shows that HCV up-regulates levels of DNMT1 and DNMT3b, both of which are essential for E-cadherin promoter hypermethylation by core, as demonstrated with RNA interference experiments. In addition, the present study shows that treatment with a specific JNK inhibitor, SP600125 [25], almost completely abolished the potentials of HCV to up-regulate the promoter activity and protein level of DNMT1 (Fig. 2A and B), which is consistent to our previous data obtained with Core overexpression system [8].

E-cadherin as a member of the cadherin superfamily of calcium-dependent transmembrane glycoproteins plays an essential role in normal physiologic processes such as development, cell polarity, and tissue morphology [28,29]. Several studies have provided consistent evidence for a role of E-cadherin as a tumor suppressor [30]. Loss or alteration of E-cadherin expression during tumor development was observed in a variety of different tumor types, including HCC [3,4,28]. In addition, transfection of E-cadherin cDNA into invasive carcinoma cells leads to significant reduction of their invasive capacity *in vitro* [31], and activation of E-cadherin results in growth retardation of tumor cell lines [32]. In general, aberrant cellular distribution of E-cadherin or repression of its expression is accompanied during EMT, an essential component of cancer progression to more aggressive phenotypes characterized by tumor dedifferentiation, infiltration, and metastasis [33,34]. Therefore, knowledge of the molecular mechanism that represses its expression or function is of prime importance in understanding the process of tumor invasion. E-cadherin expression is known to be negatively regulated at the transcription level by transcriptional repressors. For example, the zinc-finger transcription factor Snail functions as a negative regulator by binding to the E-box motifs of E-cadherin promoter and recruits a transcriptional repressor complex containing mSin3A/HDAC [36]. However, E-cadherin expression in most human cancers seems to be down-regulated primarily via DNA methylation [36]. Consistently, the present study demonstrates that HCV induces promoter hypermethylation of E-cadherin gene and represses Sp1 binding to its recognition sites, resulting in inactivation of E-cadherin expression.

Physical interaction in tumor cells mediated by E-cadherin acts to restrain cell migration and its loss is associated with decrease in cellular adhesion and altered cellular morphology [19,28]. Indeed, the present study was observed that the reduced E-cadherin expression in infected with HCV cells aggregate poorly in suspension culture, reflecting their altered intercellular interactions. The biological significance was further demonstrated by the colonizing the wound surface of infected with HCV cells. Based on these observations, this study proposes that HCV plays a role during hepatocellular carcinogenesis by favoring cell detachment from the surrounding matrix and migration outside of the primary tumor site.

Conflict of interest

None declared.

Acknowledgments

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