Mechanism of Hepatitis C Virus (HCV)-induced Osteopontin and Its Role in Epithelial to Mesenchymal Transition of Hepatocytes

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Background: The mechanism underlying HCV-induced OPN remains elusive.

Results: HCV-induced Ca\(^{2+}\) signaling, oxidative stress, and activation of AP-1 and Sp1 play a critical role in OPN activation.

Conclusion: OPN plays important roles in EMT and cell migration induced by HCV through the activation of Akt, GSK-3\(\beta\), and \(\beta\)-catenin.

Significance: Our findings provide a novel implication of OPN in HCV-induced HCC.

Osteopontin (OPN) is a secreted phosphoprotein, originally characterized in bone and then found in a number of other normal tissues and in a variety of malignant tumors. OPN is involved in the interactions of malignant-transformed epithelial cells, and is overexpressed in tumor metastasis of several tumors and is overexpressed in hepatocellular carcinoma (HCC). OPN is required for epithelial-mesenchymal transition, a critical process characterizing in malignant-transformed epithelial cells. OPN is involved in the interactions of malignant-transformed epithelial cells. OPN is overexpressed in tumor metastasis of several tumors and is overexpressed in hepatocellular carcinoma (HCC). OPN is required for epithelial-mesenchymal transition, a critical process characterizing in malignant-transformed epithelial cells.

Hepatitis C virus (HCV)\(^2\) infection leads to chronic hepatitis, which may progress to liver fibrosis, cirrhosis, and hepatocellular carcinoma (HCC)\(^1\). HCV is an enveloped single-stranded, positive sense RNA virus, which is a generic model for a number of diseases including infectious hepatitis and chronic hepatitis C. Chronic HCV infection is a major cause of cirrhosis and HCC. HCC is one of the most common cancers, which is mainly associated with chronic hepatitis B or C. The mechanism underlying HCV-induced OPN remains elusive. This article has been withdrawn by Jawed Iqbal, Steven McRae, Krishna Banaudha, and Gulam Waris. Thi Mai could not be reached. The image of Huh7.5 + NS5A (merge) was reused in Iqbal et al. (2014) PLOS One 9, e87464, representing similar experimental conditions. The p-GSK-3\(\beta\) immunoblot from Fig. 9 was reused as NLRP3 in Fig. 1D of McRae et al. (2016) J. Biol. Chem. 291, 3254-3267. The paper, with confirmatory data supporting the results, can be obtained by contacting the withdrawing authors. The withdrawing authors sincerely apologize to the readers.

The abbreviations used are: HCV, hepatitis C virus; OPN, osteopontin; EMT, epithelial to mesenchymal transition; HCC, hepatocellular carcinoma; ROS, reactive oxygen species; ER, endoplasmic reticulum; BAPTA-AM, 1,2-bis(2-aminophenoxy)ethane-N,N',N''-tetraacetic acid tetrakis(acetoxymethyl ester); PHH, primary human hepatocyte; NAC, N-acetylcysteine.
Osteopontin (OPN) is linked to tumor progression and metastasis in a variety of cancers, including HCC (15, 16). Recent studies have shown the up-regulation of OPN in tumorigenesis and angiogenesis and in response to inflammation and liver injury (15, 16). Recent studies have shown the correlation of serum OPN levels with hepatic inflammation and fibrosis in association with alcohol intake (17). Several viruses such as murine polyoma virus middle T antigen, HBV X protein, and HIV have been shown to induce OPN, which can lead to cell invasion and metastasis (18–20). OPN can induce autocrine and paracrine signaling by binding to the cell surface receptors such as integrins αVβ3, αVβ3, αVβ3, α4β1, α5β1, αβ1, and CD44 and transduces cell-matrix signaling directed to increased motility, invasion, and angiogenesis (15, 16).

Tumor progression and metastasis are closely related to the signaling cascade. Transcription factors are end points of signaling pathways and affect the cellular gene expression (21). OPN has been shown to be regulated by several transcription factors, including AP-1, Sp1, Myc, Oct-1, and c/EBPβ. AP-1 and Sp1 in OPN promoter activation. Furthermore, we investigated the role of AP-1 and Sp1 in OPN promoter activation. Collectively, these observations provide a novel role of HCV-induced OPN in EMT and migration of hepatocytes.

**EXPERIMENTAL PROCEDURES**

**Plasmid, Antibodies, and Reagents**—The infectious J6/JFH-1 cDNA (HCV genotype 2a) along with the replication-defective JFH-1/GND construct were obtained from Dr. Charles Rice (Rockefeller University, New York). The wild-type and various deletion constructs of the OPN promoter-luciferase reporter were provided by Dr. J. H. Chen (Taiwan University, Taiwan) (29). The full-length FLAG-OPN DNA (pDest490-OPN-a) was purchased from Addgene, Cambridge, MA.

The following antibodies were used according to the manufacturer’s protocols: HCV NS3 and HCV NS5A (Virogen, Watertown, MA); actin, β-tubulin, and anti-FLAG M2 (Sigma); OPN (human) (R & D Systems, Inc., Minneapolis, MN); E-cadherin, N-cadherin, integrin β3, p-Akt, β-catenin, phospho-β-catenin, GSK-3β, phospho-GSK-3β, c-Jun, c-Fos, and Sp1 (Cell Signaling Technology, Danvers, MA); anti-CD44 (anti-HCAM) (Santa Cruz Biotechnology, Dallas, TX); and antiserum albumin (Thermo Scientific Inc., Rockford, IL).

Inhibitors of p38 MAPK (SB203580), JNK (SP600125), PI3K (LY294002), MyD88 (Calbiochem, La Jolla, CA), NAC, Ca2⁺ chelators (BAPTA/AM and EGTA), an inhibitor of caspase-3 (Z-VAD-FMK), caspase-3 inhibitor I, caspase-9 inhibitor II (Ac-DEVD-CHO), and calpain II inhibitors (ALLM), ALLN, and calpain I inhibitors (ALL) were purchased from Calbiochem (La Jolla, CA). The inhibitors of transcription factors AP-1 (Tanshinone IIA) (Enzo, Farmingdale, NY), Sp1 (mithramycin A) (Cell Signaling Technology), and Stat3 inhibitor V (Statick) were obtained from Santa Cruz Biotechnology, Dallas, TX.

The human hepatoma cell line Huh7.5 (obtained from Dr. J. H. Chen, Taiwan University, Taiwan) was grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum, 100 units of penicillin/ml, and 100 μg of streptomycin sulfate/ml. The cells were incubated at 37 °C in a 5% CO₂ incubator. The study of HCV-mediated liver disease progression is difficult due to the lack of a convenient small animal model susceptible to virus infection. The cell culture system using the human hepatoma cell line Huh-7/Huh7.5 is widely used to study HCV-mediated disease pathogenesis in the HCV field.

**HCV Cell Culture Infection System**—The J6/JFH-1 RNA was transcribed and delivered into Huh7.5 cells by electroporation (26). The cell-free virus was propagated in Huh7.5 cell culture supernatant at the highest dilutions as described previously (4). The expressions of HCV protein in HCV-infected cells were analyzed using Western blot. The HCV cell culture supernatant was collected at appropriate time points and was used to infect naive Huh7.5 cells at a multiplicity of infection of 1 for 5–6 h at 37 °C and 5% CO₂ (4, 23, 26). The viral titer in cell culture supernatant was expressed as focus forming unit ml⁻¹, which was determined by the average number of HCV-NSSA-positive foci detected at the highest dilutions as described previously (4). The cell culture supernatant collected from Huh7.5 cells expressing JFH-1/GND (replication defective virus) was used as a negative control. In most of the experiments, HCV-infected cells were serum-starved for 4 h before harvesting. Most of the experiments were performed at day 6 HCV post-infection, and we observed significant OPN induction and secretion.
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TABLE 1

| Target genes | Sense primers | Antisense primers |
|--------------|---------------|-------------------|
| OPN          | 5′-CGAGGATTTGATAGTTGTTGCGA-3′ | 5′-TTTATGTATGTGTATGTTGTC-3′ |
| 18 S rRNA    | 5′-ACATCGAGAACAACGACAGA-3′ | 5′-TTGATGTTGTTGTTGTTGTTG-3′ |
| HCV          | 5′-CGGAGACCAAGTGGTGC3′ | 5′-TTTATGTATGTGTATGTTGTC-3′ |
| HCV TaqMan probe | 5′-6-FAM-CCTGGAGAGCCATAGTGG-3′ | 5′-TTTATGTATGTGTATGTTGTC-3′ |
| Sp1          | 5′-CCTGCGCTTCTCTGTCCT-3′ | 5′-TTTATGTATGTGTATGTTGTC-3′ |
| AP-1*        | 5′-GCTGCTGTTGTTGTTGTTGTTG-3′ | 5′-TTTATGTATGTGTATGTTGTC-3′ |

Wild-type and site-directed mutagenesis primers

| AP-1 (WT)     | 5′-GGCAGAAGACCTCCTGAGCACAAGTCC-3′ | 5′-GAGATTTGCTGTTGTTGTTGTC-3′ |
| AP-1 (mut)    | 5′-GGCAGAAGACCTCCTGAGCACAAGTCC-3′ | 5′-GAGATTTGCTGTTGTTGTTGTC-3′ |
| Sp1 (WT)      | 5′-CAACTCCGCCGCTCCCTGTTGTTG-3′ | 5′-CAACTCCGCCGCTCCCTGTTGTTG-3′ |
| Sp1 (mut)     | 5′-CAACTCCGCCGCTCCCTGTTGTTG-3′ | 5′-CAACTCCGCCGCTCCCTGTTGTTG-3′ |

* Primers used in ChIP assay are WT and mutated (mut). The AP-1 primers in our experiments amplify the AP-1-binding sites either immunoprecipitated with anti-c-Jun or anti-c-Fos.

For proteolytic processing of OPN, we used HCV days 7–8 post-infection because HCV has the ability to process all cleaved forms significantly at later time points (data not shown).

Hepatocyte Co-culture System—The primary human hepatocytes were obtained from Dr. Ajit Kumar (George Washington University, Washington, D.C.). Briefly, the hepatic stellate cell line (CFSC-8B) was used as a feeder cell layer, and a freshly isolated human hepatocyte suspension (Cambrex, Walkersville, MD) was seeded over the feeder cell line in a hepatocyte-defined medium, as described previously (30). Primary hepatocyte cultures form spherical masses after 30 days. The hepatocyte cultures containing spheroids were then infected with HCV at a multiplicity of infection of 1, was harvested at day 8 post-infection because HCV has the ability to process all cleaved forms significantly at later time points (data not shown).

Liver specimens from non-humanized Mice Liver Tissue—Liver specimens from normal and human hepatocyte-engrafted MUP-uPA/SCID/bg mice infected with HCV were received from Dr. Ajit Kumar (George Washington University). Frozen samples were washed twice with cold PBS and thawed in RIPA buffer (as described above) and gently crushed with a glass rod, followed by sonication and incubation on ice for 30 min. Samples were centrifuged at 4 °C (13,400 × g) for 5 min, and the supernatant was collected, and the status of OPN was analyzed by Western blotting as described by us earlier (25).

Western Blot Analysis—Mock (Huh7.5) and HCV-infected cells were harvested, and cellular lysates were prepared by incubating in RIPA buffer for 30 min on ice. Supernatants from mock- and HCV-infected cells were concentrated using centrifugal filter units (Millipore, MA). Equal amounts of protein from lysates or cell culture supernatants were subjected to SDS-PAGE. Gels were transferred onto nitrocellulose membrane (Thermo Scientific, IL) and blocked in 5% dry milk, probed with primary antibody for 1 h at room temperature (RT), and washed twice for 5 min with blocking buffer (5% dry milk). Membranes were incubated for 1 h at room temperature in blocking buffer (5% dry milk) with the appropriate secondary antibody for 1 h at room temperature. Membranes were then washed twice for 5 min with blocking buffer (5% dry milk) and incubated with secondary antibody for 1 h at RT. Blots were visualized using the Odyssey automated system, and amplification plots were generated. To monitor during every PCR cycle at the annealing step. At the termination of each PCR run, the data were analyzed by the manufacturer.

Quantitative Real Time RT-PCR—Total RNA was extracted from liver tissues using TRIzol (Invitrogen) and DNase treated with RQ1 RNase-free DNase prior to cDNA synthesis. The cDNA was reversetranscribed from 1 µg of total RNA using a reverse transcription kit (Applied Biosystems). Quantitative RT-PCR was carried out using SYBR Green Master Mix (Applied Biosystems) and specific primer sets (Table 1). 18 S rRNA was used as an internal control. Amplification reactions were performed under the following conditions: 2 min at 50 °C, 10 min at 95 °C, 40 cycles for 10 s at 95 °C, and 1 min at 60 °C. Relative transcript levels were calculated using ΔΔCt method as specified by the manufacturer.
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determine the HCV RNA copy number, standards ranging from 10¹ to 10⁸ copies/μg were used for comparison.

Site-directed Mutagenesis—The base substitution mutations of AP-1- and Sp1-binding sites on the OPN promoter luciferase-reporter were carried out using oligonucleotide-mediated mutagenesis as described previously (26). The PCR reactions were performed with AP-1, Sp1, wild-type, and mutated primers (Table 1) according to the manufacturer’s protocol (AccuPrime PfX, Invitrogen). Briefly, the reaction buffer (5 μl), consisting of 10× reaction mix, 1 μl (50 ng) of dsDNA template, 1.5 μl (10 μM) of each oligonucleotide primer, 1 μl of PfX DNA polymerase (2.5 units/μl), and 40 μl of double distilled H₂O, was added to a final volume of 50 μl. PCR program was as follows: initial denaturation at 95 °C for 2 min; denaturation at 95 °C for 15 s; annealing at 56 °C for 30 s, and extension at 68 °C for 5 min for 35 cycles. DpnI digestion was performed, and samples were transformed using 1 μl of DpnI-treated DNA from control and sample reaction into separate 25-μl aliquots of DH5α-competent cells (Invitrogen). Clones were tested by restriction digestion, and positive clones were confirmed by DNA sequencing.

Chromatin Immunoprecipitation (ChIP) Assay—The ChIP assay was performed using Simple Chip Enzymatic Chromatin IP kit (Cell Signaling, Danvers, MA) as described previously (26). Briefly, mock- and HCV-infected cells (5 × 10⁷ cells) were fixed in 1% formaldehyde for 10 min to cross-link the DNA-protein complexes. The reaction was quenched using 125 mM glycine for 5 min, and cells were washed twice with ice-cold buffer B + DTT. The nuclei were pelleted by centrifugation at 3000 rpm for 5 min at 4 °C and suspended in ice-cold buffer A containing DTT, PMSF, and protease inhibitor mixture. The nuclei were further sonicated for 30 s using Qsonica Q700. The sonicated lysates were centrifuged at 10,000 rpm for 5 min to remove cell debris. To amplify the AP-1 (c-Jun and c-Fos) and Sp1 (c-Fos, c-Jun, and Sp1) binding sites on the immunoprecipitated DNA, the PCR reactions were performed with AP-1 or Sp1 primers against the AP-1 or Sp1 sites (c-Jun and c-Fos). The DNA-protein interactions were reversed by heating to 65 °C for 12 h. The AP-1 or Sp1-binding sites on the immunoprecipitated DNA were analyzed by quantitative RT-PCR using primers against AP-1 (c-Jun and c-Fos) and Sp1 (Table 1). The PCR products were further visualized onto 1% agarose gel stained with 0.5 μg/ml ethidium bromide.

Luciferase Assay—Mock- and HCV-infected cells were transfected with OPN promoter-luciferase reporter plasmids. At 48 h post-transfection, cells were serum-starved for 4 h, and cellular lysates were analyzed for luciferase activity using the Dual-Luciferase reporter assay kit (Promega, Madison, WI). All transfections included a Renilla expression vector as an internal control.

RNA Interference—Mock- and HCV-infected cells at day 4 were transfected with GFP siRNA (siGFP), siOPN, siCD44, and siβ3 according to the manufacturer’s protocols (Santa Cruz Biotechnology). Each siRNA consists of pools of three to five target-specific 19–25-nucleotide siRNA designed to knock down the target gene expression. For siGFP, siOPN, siCD44, and siβ3 transfections, two solutions were prepared. Solution A, containing 60 pmol of siRNA duplex, was mixed with 100 μl of siRNA transfection medium. Solution B, containing 6 μl of transfection reagent, was added to 100 μl of siRNA transfection medium. Solutions A and B were allowed to incubate at RT for 20 min. After 20 min, solutions A and B were combined and incubated again for 20 min at RT. The combined solutions were then added to the cells and incubated for 5–7 h at 37 °C and 5% CO₂, and the transfection solution was replaced with 2 ml of complete DMEM growth media.

Cell Migration Assay—Mock- and HCV-infected cells were plated in 6-well culture plates, incubated overnight, and then followed by scratching using a sterile pipette tip across the monolayer. Images were captured after wounding at 0 and 48 h post-wounding. The percent migration distance was scored according to the following formula: percent migration distance = percent wound width at time 0 − percent wound width at 48 h. Statistics: Data are shown as mean ± standard deviations of the mean of at least three independent trials. Two-tailed Student’s t-tests were applied to compare the experimental conditions. Differences were considered statistically significant at a significance level of p < 0.05.

HCV Activates OPN via Ca²⁺ Signaling and Elevation of ROS—Our previous studies have shown that HCV-induced Ca²⁺ signaling and elevation of ROS play a key role in the cell signaling cascade (12, 23, 25, 26). To determine whether HCV-induced Ca²⁺ signaling and induction of ROS activate OPN, mock- and HCV-infected cells were incubated with nontoxic doses of antioxidant (NAC), an inhibitor of mitochondrial Ca²⁺ uptake.
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Ru360, intracellular Ca^{2+} chelator (BAPTA-AM), and extracellular Ca^{2+} chelator (EGTA). Cellular lysates as well as cell culture supernatants were immunoblotted with anti-OPN antibody. The results show an increased OPN expression and secretion in HCV-infected cells, which was reduced in the presence of NAC, Ru360, and BAPTA-AM (Fig. 2, A and B) but not with EGTA, which serves as a negative control.

To demonstrate if HCV-induced Ca^{2+} signaling and induction of ROS also activate endogenous OPN mRNA, mock- and HCV-infected cells were treated with the above inhibitors, and OPN mRNA was analyzed by quantitative RT-PCR. We observed increased OPN mRNA expression (∼3-fold) in HCV-infected cells compared with mock cells, which was reduced in the presence of NAC, Ru360, and BAPTA-AM but not with EGTA, which serves as a negative control (Fig. 2C).

To evaluate if HCV-induced Ca^{2+} signaling and induction of ROS stimulate OPN promoter induction, mock- and HCV-infected cells were transfected with the OPN promoter (∼500/20)-luciferase reporter followed by treatment with the above inhibitors and subjected to Dual-Luciferase assay. The results show increased OPN promoter (∼500/20) luciferase activity (∼2.7-fold) in HCV-infected cells compared with mock cells, which was reduced in the presence of NAC, Ru360, and BAPTA-AM (Fig. 2D). In contrast, we did not observe any effect of EGTA (extracellular Ca^{2+} chelator) (Fig. 2D). No significant cytotoxicity was observed in mock- and HCV-infected cells treated with these inhibitors as shown by us earlier (12). Taken together, these results suggest that HCV-mediated altered Ca^{2+} signaling in the ER and elevation of ROS production in the mitochondria are critical for OPN induction as well as secretion.

In addition, our ongoing studies suggest that HCV core, E1/E2, and NS3/4A induced OPN activation in hepatoma cells (data not shown). These proteins are also known to induce ROS in human hepatoma cells (31–33).

HCV Infection Induces OPN via Activation of Cellular Kinases—Previously, we have shown that HCV-induced Ca^{2+} signaling and elevation of ROS activate several cellular kinases (23, 25). To investigate the role of HCV-activated cellular kinases on OPN induction and secretion, mock- and HCV-infected cells were incubated with inhibitors of p38 MAPK (SB203580), JNK (SP600125), PI3-kinase (LY294002), MEK1/2 (U0126), and PKC (Go6976). Cellular lysates and cell culture supernatants were subjected to Western blot analysis using anti-OPN antibody. Increased expression and secretion of OPN were observed in HCV-infected cells, which were abrogated in the presence of above kinase inhibitors; however, no inhibition was observed with PKC inhibitor (Go6976), which serves as a control (Fig. 3, A and B).

To determine the role of HCV-activated cellular kinases on endogenous OPN mRNA expression, total cellular RNA was extracted from mock- and HCV-infected cells treated with the
above inhibitors, and the OPN mRNA level was analyzed by SYBR Green RT-PCR. We observed increased OPN mRNA expression (~3-fold) in HCV-infected cells compared with mock cells, which was reduced in the presence of above kinase inhibitors except PKC inhibitor (Go6976) (Fig. 3C).

To further strengthen the role of HCV-induced cellular kinases on OPN promoter activation, mock- and HCV-infected cells were transfected with wild-type OPN promoter luciferase-reporter, followed by treatment with the above kinase inhibitors and subjected to Dual-Luciferase assay. The results show increased OPN promoter luciferase activity (~3-fold) in HCV-infected cells compared with mock cells, which was abrogated in the presence of above kinase inhibitors (Fig. 3D). In contrast, we did not observe any effect by PKC inhibitor (Go6976) (Fig. 3D). Collectively, these results demonstrate that HCV infection stimulates the induction of OPN via activation of cellular kinases. No significant cytotoxicity was observed in mock- and HCV-infected cells treated with these inhibitors as shown by us recently (26).

Effect of HCV Infection on OPN Promoter Activity—OPN promoter contains binding sites of several transcription factors such as Oct-1/Oct-2, Ets, Sp1, AP-1, c-Myc, E2A, E4TF-1, AML-1/C/EBP, Myb, TCF-1, SIF, AP-2, GATA, and TCF-1 (29, 34). To determine whether HCV activates the OPN promoter via activating these transcription factors, mock- and HCV-infected cells were transfected with wild-type and various deletion constructs of OPN promoter-luciferase reporter (~500/20, ~267/20, ~220/20, ~170/20, ~127/20, ~107/20, and ~79/20) (Fig. 4A). The results show ~3-~, ~2.9-~, and ~2.7-fold increased luciferase activity of the wild-type OPN construct (~500/20) and deletion constructs (~97/20) in HCV-infected cells, respectively, but not with other deletion constructs (Fig. 4B). These results suggest that transcription factors AP-1 and Sp1 may be playing a role in OPN promoter activity as the deletion constructs (~79/20 and ~97/20) contain binding sites of AP-1 and Sp1. These results also indicate the role of the negative regulatory region from ~107 to ~97.

To further demonstrate the role of AP-1 and Sp1 on OPN promoter activation, mock- and HCV-infected cells were transfected with wild-type OPN promoter-luciferase reporter (~500/20) and treated with nontoxic doses of the inhibitors of AP-1 (tanshinone IIA) and Sp1 (mithramycin A), and Dual-Luciferase assay was performed. The results showed ~3-fold increased luciferase activity by HCV that was reduced in the presence of the above inhibitors, suggesting that AP-1 and Sp1
are critical for OPN promoter activation (Fig. 4C). However, Stat3 inhibitor V (Stattic), used as negative control, did not show any effect on OPN promoter activation.

To confirm the role of AP-1- and Sp1-binding sequences in OPN promoter activity, wild-type OPN promoter-luciferase reporter and those with mutated AP-1- and Sp1-binding sites were transfected into mock- and HCV-infected cells and subjected to Dual-Luciferase assay. The results showed 2.6- and 3-fold increased luciferase activity of wild-type AP-1 and Sp1 OPN promoter in HCV-infected cells compared with mock cells (Fig. 4D). In contrast, the OPN promoter containing mutated AP-1- or Sp1-binding sites showed reduced luciferase activity in HCV-infected cells (Fig. 4D). These results confirm the role of HCV-induced AP-1 and Sp1 in OPN promoter activation. We observed no significant cytotoxicity using tenshione II A and mithramycin A inhibitors as shown by us recently (26). The mutated bases of AP-1- and Sp1-binding sites on OPN promoter are shown in red in Fig. 4E.

In Vivo Interaction of HCV-induced AP-1 and Sp1 on OPN Promoter—To demonstrate if AP-1 and Sp1 interact with the OPN promoter in vivo in HCV-infected cells, chromatin immunoprecipitation (ChIP) assay was performed using c-Jun, c-Fos, and Sp1 antibodies as per the manufacturer's instruction (SimpleChIP Enzymatic Chromatin IP kit, Cell Signaling Technology). The DNA quantitative RT-PCR analysis showed that c-Jun-, c-Fos-, and Sp1-specific antibodies immunoprecipitated chromatin from HCV-infected cells (Fig. 5A). However, immunoprecipitation with nonspecific antibody (normal rabbit IgG) did not amplify the DNA fragments. The PCR amplification of input chromatin before immunoprecipitation was served as a positive control. The amplified DNA fragments were further confirmed by agarose gel electrophoresis (Fig. 5B). These results indicate that AP-1 and Sp1 form a protein-DNA transcriptional regulatory complex by binding to the OPN promoter in HCV-infected cells.

HCV Induces Proteolytic Processing of OPN—To determine whether HCV infection induces proteolytic cleavage of precursor OPN, cellular lysates from mock- and HCV-infected cells at day 8 post-infection were subjected to Western blot analysis using anti-OPN antibody. We observed increased expression of the precursor form of OPN (~75 kDa) followed by its cleavage into various forms of OPN (~55, ~42, and ~36 kDa) in HCV-infected cells compared with mock-infected cells (Fig. 6A, lane 2). Furthermore, cellular RNA from above mock- and HCV-infected cells were amplified using OPN-specific primers by semi-quantitative RT-PCR. We observed a single OPN band that corresponds to full-length OPN mRNA, but no splice variants were observed (Fig. 6B).
To determine whether the activation of OPN was specific to HCV replication, Huh7.5 cells were electroporated with J6/JFH-1 (wild-type) and JFH-1/GND (replication defective mutant) RNA. We observed the expression of OPN precursor and cleaved forms in J6/JFH-1 RNA-electroporated Huh7.5 cells. In contrast, we did not observe any expression of OPN in JFH-1/GND RNA-electroporated cells (Fig. 6C).

To further demonstrate whether proteolytic processing of OPN is specific to HCV, mock- and HCV-infected Huh7.5 cells were transiently transfected with full-length FLAG-OPN expression vector. The immunoblot results show the expression of precursor (full-length) OPN (~75 kDa) and one cleaved form of OPN (~36 kDa) in HCV-infected cells using anti-FLAG antibody (Fig. 6D, lane 4), but we did not observe the cleavage of FLAG-OPN in Huh7.5 cells (Fig. 6D, lane 2). However, we observed precursor and three cleaved products (55, 42, and 36 kDa) of OPN in the above lysates (Fig. 6D, lanes 3 and 4) when incubated using anti-OPN antibody (Fig. 6E, lane 2). These results suggest that the cleavage site of ~36-kDa OPN might be close to FLAG sequences at the C terminus, and anti-FLAG was able to detect only precursor and 36-kDa cleaved product. However, anti-OPN antibody was able to detect both exogenous and endogenous OPN (Fig. 6E).

Previously, it has been shown that hydrogen peroxide (H$_2$O$_2$) enhances OPN expression (35). To demonstrate if H$_2$O$_2$ has a
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A role in the proteolysis of OPN, Huh7.5 cells were treated with H₂O₂ (500 μM) followed by Western blot analysis using anti-OPN antibody. The results show the increased expression of precursor OPN (75 kDa) in H₂O₂-treated mock cells compared with untreated cells (Fig. 6F, lane 2). However, we did not observe any cleavage of OPN in Huh7.5 cells treated with H₂O₂ (Fig. 6F, lane 2). Collectively, these results suggest that the OPN processing occurs in HCV-infected cells.

To verify the OPN cleavage in primary human hepatocytes (HPP), cellular lysates from PHH- and HCV-infected PHH were subjected to Western blot analysis using anti-OPN antibody. Similar cleaved products of OPN (~55, ~42, and ~36 kDa) were observed (Fig. 6G, lane 2) as described above. To further strengthen our results, equal amounts of cellular lysates from normal and HCV-infected human hepatocyte-engrafted MUP-uPA/SCID/bg mice were subjected to Western blot analysis using anti-human OPN. We observed increased expression of precursor OPN (~75 kDa) in HCV-infected samples followed by its cleavage into 55 and 42 kDa (Fig. 6H, lane 2). However, we could not detect the 36-kDa cleaved form of OPN in HCV-infected human hepatocyte-engrafted MUP-uPA/SCID/bg mouse liver tissue samples (Fig. 6H, lane 2). These results suggest that at later time points HCV has the ability to cleave OPN into various forms.

Previously, full-length OPN has been shown to be cleaved into various forms by thrombin and matrix metalloproteases (15, 36, 37). To investigate if protease plays an important role in HCV-induced OPN cleavage, mock- and HCV-infected cells were treated with nontoxic doses of various inhibitors against calpain II inhibitor (ALLM), calpain I inhibitor (ALLN), caspase-3 inhibitor I, and proteosome inhibitor (MG-132), and cellular lysates were immunoblotted using anti-OPN antibody. The results show that the cleavage of OPN in HCV-infected cells was significantly blocked by ALLM and ALLN, leading to stabilization of the precursor form of OPN (Fig. 6I, lanes 3 and 4). In contrast, we did not observe any effect of caspase-3 and MG-132 inhibitors in the abrogation of OPN cleavage (Fig. 6I, lanes 5 and 6). In addition, we also used MMP-2/MMP-9 inhibitor V (Calbiochem) and did not observe the stabilization of precursor OPN (data not shown). Moreover, no cytotoxicity was observed in mock- and HCV-infected cells treated with these inhibitors as shown by us (38). Collectively, these results indicate the involvement of calpain proteases in the post-translational processing of HCV-induced OPN.

HCV Induces EMT—To determine whether HCV induces EMT, cellular lysates from mock- (Huh7.5 cells and PHH) and HCV-infected cells were subjected to Western blot analysis using antibodies against E-cadherin (epithelial marker) and N-cadherin (mesenchymal marker). The results show decreased expression of E-cadherin and increased expression of N-cadherin in HCV-infected Huh7.5 cells as well as PHH compared with mock cells (Fig. 7, A and B).
To demonstrate if HCV-activated OPN induces EMT, HCV-infected cells were transfected with siGFP (control siRNA), siOPN, siCD44, and siβ3. Cellular lysates were subjected to Western blot analysis using anti-OPN, anti-CD44, and anti-β3 antibody. The results show significant knockdown of OPN, CD44, and β3 expression in HCV-infected cells transfected with the above siRNA (Fig. 7C). The above cellular lysates were immunoblotted with anti-E-cadherin and anti-N-cadherin. The results show decreased expression of E-cadherin in HCV-infected cells compared with mock-infected cells (Fig. 7D, lane 2), which reappeared in HCV-infected cells transfected with siGFP, siCD44, and siβ3 (lanes 4–6) but not with siGFP (lane 3).
However, the expression of N-cadherin was increased in HCV-infected cells compared with mock-infected cells (Fig. 7D, lane 2), which was abrogated in cells transfected with siOPN, siCD44, and siβ3 (lanes 4–6) but not with siGFP (lane 3). Collectively, these results suggest that HCV has the ability to induce EMT via OPN, including cell surface receptors CD44 and αVβ3.

To determine the level of HCV infection, HCV-infected PHH and Huh7.5 cells were immunostained using anti-HCV NS5A antibody. The results showed about >95% of the cells were infected with HCV as observed by immunofluorescence microscopy (Fig. 7, E and F).

**OPN Induces Hepatoma Cells Migration**—To determine whether HCV-induced OPN plays a role in human hepatoma cell migration, HCV-infected cells (from the same pool of Fig. 7F) were transfected with siGFP, siOPN, siCD44, and siβ3 and were subjected to wound healing assay. The results show increased cell migration in HCV-infected cells at 48 h compared with mock cells, which was reduced in HCV-infected cells transfected with siOPN, siCD44, and siβ3 but not with siGFP (control siRNA) (Fig. 8A). The increased migration depth at 48 h was recorded as ~85% in HCV-infected cells that was reduced to ~39, 67, and 54% in HCV-infected cells transfected with siOPN, siCD44, and siβ3, respectively (Fig. 8B), suggesting the critical role of HCV-induced OPN in wound healing.

**OPN-induced β-Catenin-mediated Signaling Cascade in HCV-infected Cells**—β-Catenin is known to be a downstream effector of Wnt signaling cascades and plays a critical role in EMT progression (28). Similarly, OPN and cell surface receptors, αVβ3 and CD44, have also been known to be critical for EMT in various cancers cells (15, 16, 38). To determine whether HCV-activated OPN induces EMT via Akt, GSK-3β, and β-catenin phosphorylation, the cellular lysates from siRNA-transfected cells (Fig. 7C) were immunoblotted using anti-Akt, GSK-3β, and anti-β-catenin (Ser33/37/Thr41). The results show increased phosphorylation of Akt, GSK-3β, and β-catenin in HCV-infected cells compared with mock cells, which was reduced in HCV-infected cells transfected with siOPN, siCD44, and siβ3 but not with siGFP.
In contrast, we did not observe any significant change in the total expression of GSK-3β and β-catenin (Fig. 9). Taken together, these results suggest that HCV-induced OPN is critical for Akt-, GSK-3β-, and β-catenin-mediated EMT.

DISCUSSION

HCC represents an extremely poor prognostic cancer that remains one of the most common and aggressive human malignancies worldwide (5, 6). Current therapies are inefficient, mainly due to usually late diagnosis and high recurrence rate within the remaining cirrhotic liver after surgical resection. It is a multistep process involving different genetic alterations that ultimately lead to malignant transformation of hepatocytes (39). HBV and HCV chronic infections account for 75% of HCCs, whereas nonviral etiologies such as alcohol and genetic or metabolic disorders represent less than 25% of cases. HCV-induced chronic liver injury can lead to progressive fibrosis, cirrhosis, and eventually HCC (40). HCV-associated HCC has been reported to promote tumor growth and metastasis due to an increased recurrence after liver resection (8). In addition, expression of HCV structural proteins in transgenic mouse liver may contribute to the development of hepatic steatosis, fibrosis, and HCC (41, 42). However, the underlying mechanisms responsible for the metastatic spread of HCV-induced HCC are not fully elucidated.

Previous studies have demonstrated that OPN is involved in tumor metastasis and has been detected not only in numerous cancers but also in the plasma of HCV-related HCC patients, suggesting a correlation between high levels of OPN expression and malignant invasion (16, 43). Recently, α-fetoprotein has been shown to be a valid serological marker for HCV-related HCC, but α-fetoprotein alone is not sufficient unless ultrasound is available (43, 44). OPN is mainly expressed in transformed malignant epithelial cells and has been suggested as an additional biomarker for HCC screening due to inadequacy of α-fetoprotein (43, 44). However, the role of OPN in HCV-induced HCC remains unclear.

In this study, we determined the molecular mechanism of OPN induction and secretion by HCV infection. Our results...
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FIGURE 9. Role of HCV-induced OPN in cell signaling cascade. Mock- and HCV-infected cells were transfected with siGFP, siOPN, siCD44, and siβ3 as described under “Experimental Procedures.” Equal amounts of cellular lysates were subjected to Western blot analysis using anti-p-Akt, anti-p-GSK-3β, anti-p-β-catenin, anti-GSK-3β, and anti-β-catenin antibodies. Actin serves as a protein loading control.

suggest that HCV infection induces OPN via calcium homeostasis in the ER and elevation of ROS in mitochondria (Fig. 2). Previously, Ca\(^{2+}\)-mediated liver disease pathogenesis has been suggested to play a role in HCV-induced liver disease (45). Interestingly, the HCV core has also been shown to increase Ca\(^{2+}\)-dependent mitochondrial dysfunction (46). Moreover, an increased expression of OPN is associated with migration and metastasis of cancer cells through the Ca\(^{2+}\)-dependent binding of S100A4, a member of the S100 family encoding an EF-hand calcium-binding protein (47). In cancer cells, mild oxidative stress induces the cell signaling cascade such as proliferation, migration, and invasion, but high oxidative stress can induce cell death (48). Our results are consistent with previous studies where ROS up-regulated OPN and antioxidants prevented this effect in the mouse model (49).

In our findings, we observed that the induction of OPN in HCV-infected cells is mediated by the activation of cellular kinases such as p38 MAPK, JNK, PI3K, and MEK1/2 (Fig. 3). Interestingly, several kinases such as MAPK (JNK and p38), ERK, and PI3K are known to be involved in transcriptional regulation of metastasis-related genes leading to EMT, cell migration, and tumor progression (50). It is well established that HCV activates several oncogenic transcription factors via phosphorylation by these cellular kinases (23–25). The OPN promoter is known to be regulated by the binding of several transcription factors (16, 29, 34). Our data suggest that AP-1 and Sp1 are responsible for OPN gene expression, which is consistent with transactivation of the OPN promoter by the human T-cell leukemia virus type 1-encoded Tax protein via AP-1 (51). In addition, we also observed ∼3-fold luciferase activity in the wild-type OPN promoter and ∼2-fold in the OPN deletion construct −267/20 (Fig. 4B), indicating the roles of the AP-2-, GATA-1-, and TCF-1-binding site on OPN promoter activation, which are our future plans of study.

Previously, it has been reported that full-length OPN is composed of ∼314 amino acid residues, which may exist as functionally important cleaved products as well as the occasional splice variants (16, 37, 43). In HCV-infected hepatoma cells, we could observe only the expression of the full-length OPN mRNA (Fig. 6B), indicating that HCV infection does not induce the formation of OPN splice variants. In contrast, recent studies have shown the formation of two splice variants of OPN in HCC (43). This could be due to different cell types and the source of HCC tissue samples used in those studies. We observed the induction of full-length OPN polypeptide (∼75 kDa), which is cleaved into ∼55, ∼42, and ∼36 kDa. The most intriguing finding of our studies is the involvement of calcium-activated calpain proteases in HCV-induced proteolytic processing of OPN. Calpain is activated by calcium binding to its catalytic domain, which stimulates its protease activity (52). In HCV-infected cells, calpain inhibitors (ALLN) completely blocked the proteolytic cleavage of OPN (Fig. 6I), suggesting the role of calcium-activated calpain proteases in the cleavage of OPN.

α/β-catenin signaling and CAPE-mediated OPN cleavage is confirmed by in vivo studies using transgenic mice expressing OPN in hepatocytes, suggesting that extrahepatic manifestation could occur in other organs due to Th1 immune reaction provoked by circulation of OPN (55).

β-Catenin is a proto-oncogene, a key downstream target of GSK-3β. Aberrant activation of Wnt/β-catenin signaling results in enhanced cell growth and malignant cellular transformation. The hallmark of canonical Wnt signaling activation is the stabilization and nuclear translocation of β-catenin. In HCV-infected cells, we observed increased phosphorylation of GSK-3β, which can lead to the activation of β-catenin followed by increased transcriptional activity critical for EMT progression as described in recent studies (56). Our results also suggest that the activation of β-catenin is mediated by HCV-induced OPN via α/β3 and CD44 (Fig. 9). Our findings are consistent with previous reports where authors have concluded that GSK-3β and β-catenin activity can be modulated by virus-encoded proteins such as the latent membrane protein 2A of Epstein-Barr virus and hepatitis B virus X protein (57, 58). Recently, the HCV core and NSSA have been shown to inactivate GSK3-β activity and to subsequently increase nuclear
accumulation of β-catenin in human hepatoma cells (59, 60). Furthermore, our findings suggest the critical involvement of HCV-induced OPN on the phosphorylation of Akt via Ṽ3 and CD44 (Fig. 9). These results are consistent with the previous studies showing OPN-mediated phosphorylation of Akt via Ṽ3 and CD44 (15, 43). It has been shown that Akt-mediated phosphorylation of β-catenin leads to increased transcriptional activity in the nucleus (61). Previously, we and others have reported that HCV can stimulate the phosphorylation of Akt, a downstream target of OPN, followed by phosphorylation of GSK-3β, and results in β-catenin-mediated EMT and cell migration (23, 43, 61, 62).

The epithelial marker, E-cadherin, is primarily expressed in epithelia at the sites of cell-cell contacts, whereas in most cancers of epithelial origin, E-cadherin-mediated cell-cell adhesion is lost concomitantly with progression toward tumor malignancy (63). Loss of E-cadherin promotes the progression from a benign tumor to carcinoma. Our results demonstrate the loss of E-cadherin and gain of N-cadherin expression in HCV-infected cells (Fig. 7), suggesting that HCV infection has the ability to induce EMT of hepatoma cells, which are consistent with HCV infection and HCV glycoproteins (E1 and E2) involved in hepatoma cell migration through the activation of EMT markers Twist and Snail (56, 64). In our observations, OPN binding with integrin αVβ3 and CD44 increased induction of N-cadherin leading to EMT and cell migration in HCV-infected hepatoma cells (Figs. 7D and 8) (15, 43). Moreover, studies have also shown that HCV core protein expression induces EMT in the cholangiocarcinoma cell line, but in cell culture or in transgenic mice it led to the development of steatosis, a risk factor that contributes to HCC (65, 66).

In summary, our studies demonstrate the mechanism of HCV-induced OPN activation, which involves altered Ca2+ homeostasis, elevation of ROS, and activation of various cellular kinases, followed by activation of transcription factors AP-1 and Sp1. We also showed that OPN proteolytic processing in HCV-infected cells is dependent on calpain proteases. Furthermore, we investigated that HCV infection induces EMT and cell migration via OPN through αVβ3 and CD44 receptors. In addition, we also demonstrated that HCV infection induces OPN-dependent increased phosphorylation of Akt, GSK-3β, and β-catenin, which can lead to tumor progression and EMT of human hepatoma cells (Fig. 10). Our results provide novel insight into the mechanisms of HCV-induced OPN activation leading to hepatoma cell migration and HCC.

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