Hepatitis C Virus Induces Epithelial-Mesenchymal Transition in Primary Human Hepatocytes

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Hepatitis C virus (HCV)-mediated liver disease progression may reflect distinct molecular mechanisms for increased hepatocyte growth and hepatic stellate cell activation. In this study, we have observed that primary human hepatocytes, when infected *in vitro* with cell culture-grown HCV genotype 1a or 2a, display viral RNA and protein expression. Infected hepatocytes displayed a fibroblast-like shape and an extended life span. To understand the changes at the molecular level, we examined epithelial-mesenchymal transition (EMT) markers. Increased mRNA and protein expression levels of vimentin, snail, slug, and twist and a loss of the epithelial cell marker E-cadherin were observed. Snail and twist, when examined separately, were upregulated in chronically HCV-infected liver biopsy specimens, indicating an onset of an active EMT state in the infected liver. An increased expression level of fibroblast-specific protein 1 (FSP-1) in the infected hepatocytes was also evident, indicating a type 2 EMT state. Infected hepatocytes had significantly increased levels of phosphorylated β -catenin (Ser⁵⁵²) as an EMT mediator, which translocated into the nucleus and activated Akt. The phosphorylation level of β -catenin at Thr⁴¹/Ser⁴⁵ moieties was specifically higher in control than in HCV-infected hepatocytes, implicating an inactivation of β -catenin. Together, these results suggested that primary human hepatocytes infected with cell culture-grown HCV display EMT via the activation of the Akt/ β -catenin signaling pathway. This observation may have implications for liver disease progression and therapeutic intervention strategies using inhibitory molecules.

Over 200 million people are estimated to be infected with hepatitis C virus (HCV) worldwide, reflecting the unique capacity of this virus to establish long-standing, persistent infection. HCV infection is the leading cause of liver fibrosis and cirrhosis and is an increasingly important factor in the etiology of hepatocellular carcinoma (HCC) within the United States (6, 8). Fibrotic liver disease is characterized by changes in tissue architecture and extracellular matrix composition that ultimately compromise organ function. The aberrant expression of E-cadherin and the activation of β -catenin are associated with disorders of fibrosis resulting from an epithelial-mesenchymal transition (EMT) and a wide variety of human malignancies. Results from several recent studies suggested that EMT may be an important mechanism for HCC metastasis (15, 33, 40, 49).

EMT is a biological process that allows a polarized epithelial cell, which normally interacts with the basement membrane via its basal surface, to undergo multiple biochemical changes to assume a mesenchymal-cell phenotype. These cells exhibit an enhanced migratory capacity, invasiveness, elevated resistance to apoptosis, and a greatly increased production of extracellular matrix (ECM) components (22). A number of distinct molecular processes are engaged in initiating EMT and enabling it to reach completion. These processes include the activation of transcription factors, the expression of specific cell surface proteins, the reorganization and expression of cytoskeletal proteins, the production of ECM-degrading enzymes, and changes in the expression levels of specific microRNAs. In many cases, the involved factors are also used as biomarkers to demonstrate the passage of a cell through an EMT (23).

EMT is encountered in three distinct biological settings that carry very different functional consequences. While the specific signals that delineate the different types of EMT are not yet clear, it is now well accepted that functional distinctions are apparent (23). Type 1 EMT is associated with implantation and embryonic

gastrulation, giving rise to the mesoderm and endoderm and to mobile neural crest cells. The EMTs associated with wound healing, tissue regeneration, and organ fibrosis are of type 2. In the setting of organ fibrosis, type 2 EMTs can continue to respond to ongoing inflammation, leading eventually to organ destruction. Type 3 EMTs occur in neoplastic cells that enable invasion and metastasis. A major distinction between EMT involving primitive epithelial cells and that involving secondary epithelial cells is that type 1 EMT during embryogenesis produces mesenchymal cells, whereas type 2 EMT in adult or maturing tissues such as the liver results in fibroblasts (47).

β -Catenin is a key downstream effector in the Wnt signaling pathway (32). β -Catenin binds directly to the intracellular domain of E-cadherin and α -catenin, which connects the adherens junction complex with the actin cytoskeleton (1). During EMT, β -catenin is released from E-cadherin complexes into the cytoplasm, where it interacts with other proteins, raising the possibility that β -catenin signaling contributes to EMT (25). The level of cytoplasmic β -catenin is tightly controlled by glycogen synthase kinase 3 β (GSK-3 β) phosphorylation, which triggers its degradation through the ubiquitin pathway via interactions with Axin, adenomatous polyposis coli (APC), and beta-transducin repeat-containing proteins (1, 27). Several previous studies suggested that the β -catenin/LEF-1 transcription factor complex may play a role in EMT. Novak et al. (34) suggested previously that the down-regulation of E-cadherin, which releases free β -catenin, and the

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upregulation of LEF-1 correlate with EMT in colon epithelial cells overexpressing integrin-linked kinase (ILK). Several upregulated target genes of the Wnt/ β -catenin signaling pathway, such as fibronectin (18) and matrix metalloproteinase 7 (MMP-7) (12), are correlated with the mesenchymal phenotype and invasiveness.

The relationship between HCV infection and EMT in primary human hepatocytes remains unknown. In this study, we have shown that HCV infection of primary human hepatocytes induces EMT, as observed by the marked phenotypic changes and altered marker genes. The results further suggested that HCV induces EMT in primary human hepatocytes by modulating β -catenin phosphorylation via Akt activation in driving EMT processes.

MATERIALS AND METHODS

Generation of cell culture-grown HCV. HCV genotype 1a (clone H77) was grown in immortalized human hepatocytes (IHH), or HCV genotype 2a (clone JFH1) was grown in IHH or Huh7.5 cells, as previously described (24). Virus released into cell culture supernatants was filtered through a 0.45- μ m-pore-size cellulose acetate membrane (Nalgene, Rochester, NY). HCV RNA from infected-cell culture supernatants was quantified by real-time PCR (with an ABI Prism 7000 real-time thermocycler), using HCV analyte-specific reagents (ASRs; Abbott Molecular) (Department of Pathology, Saint Louis University). Virus growth from cell culture supernatants was measured by using a fluorescent focus-forming assay. The average HCV titer was calculated to be $\sim 10^4$ to 10^5 focus-forming units/ml.

Primary human hepatocyte culture and infection. Primary human hepatocytes from 5 independent donors were procured from a commercially available source (Lonza, MD). Donors tested negative for hepatitis B virus (HBV), HCV, cytomegalovirus (CMV), Epstein-Barr virus (EBV), and HIV infection. Cells (1.4×10^6 cells per well of a 6-well plate) were positive for albumin secretion and were maintained in SABM medium (Lonza, MD) supplemented with 5% heat-inactivated fetal calf serum at 37°C in a humidified 5% CO₂ incubator. Cells were infected with 0.25 to 0.5 ml of cell culture-grown HCV genotype 1a (5×10^4 focus-forming units [FFU]/ml) or HCV genotype 2a (5×10^5 FFU/ml). The HCV inoculum was incubated with primary human hepatocytes for ~ 8 h. After virus adsorption, hepatocytes were washed and incubated with fresh medium. Cell culture medium was replaced at 24-h intervals for 1 week and subsequently replaced every alternate day. Cell viability was determined by trypan blue exclusion, when necessary.

Patient materials. Liver biopsy specimens from 10 randomly selected, chronically HCV-infected patients were used. RNA was prepared from liver biopsy specimens by using TRIzol (Invitrogen), as previously described (7). Three commercially available control liver RNAs were procured (Clontech, CA, and Lonza, NJ) and used in this study.

EMT array. RNA was extracted from mock-treated control and HCV-infected primary human hepatocytes with TRIzol, and an EMT pathway-specific array was performed according to the manufacturer's instructions (SA Biosciences).

Antibodies. Commercially available antibodies to E-cadherin, vimentin, snail, slug, twist, low-density lipoprotein (LDL) receptor-related protein 6 (Lrp-6), phosphorylated Lrp-6 (phospho-Lrp-6), β -catenin, phospho- β -catenin (Ser⁵⁵² and Thr⁴¹/Ser⁴⁵), and smad2/3 (Cell Signaling Technology, Danvers, MA); phosphor-specific antibodies to Akt (Ser⁴⁷³ and Thr³⁰⁸) and antibody to total Akt (Santa Cruz Biotechnology); and antibody to fibroblast-specific protein 1 (FSP-1) (Millipore, MA) and horseradish peroxidase (HRP)-conjugated antibody to actin (Sigma-Aldrich, St. Louis, MO) were procured. An anti-HCV NS5A monoclonal antibody was kindly provided by Chen Liu. A rabbit antiserum to the HCV core antigen was kindly provided by Arvind Patel (University of Glasgow, United Kingdom).

Real-time PCR analysis. Cellular RNA was isolated with TRIzol (Invitrogen, Carlsbad, CA). cDNA synthesis was carried out by using random

hexamers and ThermoScript II RNase H reverse transcriptase (Invitrogen). The presence of HCV RNA was determined by real-time PCR (Applied Biosystems, Foster City, CA), using specific oligonucleotide primers (HCV primer targeted toward the 5' untranslated region [UTR]) (assay identification number AI6Q1G; Invitrogen). The mRNA statuses of snail and twist in HCV-infected liver samples were determined by using specific oligonucleotide primers (assay identification numbers Hs00195591_m1 for snail and Hs01675818_s1 for twist; Invitrogen). The results were normalized for to 18S rRNA values. All reactions were performed in triplicate with an ABI Prism 7500 Fast analyzer.

Western blot analysis. Proteins from cell lysates in sample-reducing buffer were resolved by SDS-PAGE and transferred onto a nitrocellulose membrane, and the blot was blocked with 3% nonfat dry milk. The membrane was incubated with a primary antibody followed by a secondary antibody coupled to horseradish peroxidase to detect protein bands by chemiluminescence (Amersham, Piscataway, NJ). Cellular actin was detected, using a specific antibody, for comparisons of the protein loads in each lane.

Immunofluorescence (IF). Primary human hepatocytes infected with HCV genotype 1a or 2a were grown in an 8-well chamber slide to about 60% confluence. Cells were fixed with formaldehyde (3.7%) and permeabilized by using 0.2% Triton X-100. Cells were stained for β -catenin with a rabbit monoclonal antibody and a secondary antibody conjugated with Alexa Flour 488 (Molecular Probes, CA). Cells were treated with ProLong Gold antifade reagent with 4',6-diamidino-2-phenylindole (DAPI; Invitrogen, Carlsbad, CA) for fluorescence microscopy (Olympus FV1000). The HCV NS5A protein was stained by using a mouse monoclonal antibody (kindly provided by Chen Liu, University of Florida, Gainesville, FL) and a secondary antibody conjugated with Alexa Flour 594 (Molecular Probes, CA).

β -Galactosidase staining for cellular senescence. A Senescence β -galactosidase staining kit (Cell Signaling) was used to determine the expression level of β -galactosidase, which serves as a marker of senescence. Cells were stained according to the protocol provided by the manufacturer.

RESULTS

HCV infection induces phenotypic changes and extends the life span of primary human hepatocytes.

Primary human hepatocytes from independent donors were infected with cell culture-grown HCV genotype 1a or 2a. Uninfected primary human hepatocytes were maintained as a control (Fig. 1A). Control hepatocytes displayed cell death within 3 weeks, as determined by trypan blue exclusion. Figure 1E shows mock-infected control primary human hepatocytes at about 2 weeks. HCV-infected hepatocytes grew slowly and developed distinct phenotypic changes after about 1 to 2 weeks of infection (Fig. 1B and F). Infected hepatocytes adopted a spindle-like shape (Fig. 1C and G) and grew in a number of discrete areas on the plate (after 3 to 5 weeks in different donor hepatocytes). Hepatocytes stopped growth at around 8 to 12 weeks after infection and entered into a senescence phase (Fig. 1D and H). We examined the entry of infected hepatocytes into the senescent stage by staining for β -galactosidase activity. A majority of cells infected with HCV genotype 1a or 2a stained blue (Fig. 1L), as a marker of β -galactosidase activity.

Infected hepatocytes displaying EMT were examined for the detection of HCV RNA by real-time PCR using primers directed toward the 5' UTR of the HCV genomic sequence at early (3 days) and late (4 weeks) times postinfection (Fig. 1I). Infections with both genotypes of HCV displayed similar increases in viral RNA levels at day 3 after infection, which decreased to about half of those levels after 28 days. A typical HCV RNA profile with HCV genotype 2a infection is shown in Fig. 1I. HCV RNA was undetectable when hepatocytes ceased proliferation and failed to sur-

FIG 1 Primary human hepatocytes infected with HCV display marked phenotypic changes and extended life span. Primary human hepatocytes were grown on collagen-coated plates. Mock-infected control hepatocytes displayed an epithelial shape in culture (A) and progressed to senescence and cell death after 3 weeks in culture (E). Hepatocytes infected with cell culture-grown HCV genotype 1a or 2a displayed phenotypic changes in a number of areas on the plates and cell growth after 1 week (B and F). Cells growing in infected plates exhibited a fibroblast-like shape after ~3 weeks (C and G) of infection. Hepatocytes displayed a granular appearance on the cell surface and death at around 4 months (D and H). HCV RNA was detected by using a specific probe against the 5' UTR from mock-treated control and HCV genotype 2a-infected hepatocytes at early (3 days) and late (28 days) stages postinfection (I). HCV RNA was undetectable in mock-infected control cells, as expected, in an early (3 days) or late (15 days) culture. The results were normalized to results for an endogenous 18S rRNA control. Immunofluorescence with the mock-infected control (J) and HCV genotype 2a-infected cells (K) displayed a perinuclear localization of the HCV NS5a protein after 28 days. β -Galactosidase activity as a senescence marker in HCV genotype 2a-infected hepatocytes after 3 months of infection is shown (L).

vive (after ~4 months), indicating that HCV replication is necessary for EMT to occur (data not shown). HCV protein expression in infected human hepatocytes after 4 weeks was verified by IF using an NS5a-specific antibody. Infections with both genotypes of HCV displayed similar results. The results are shown for HCV genotype 2a-infected Huh7 cells (Fig. 1J and K). The results from this study suggested that HCV replication may be necessary for hepatocyte growth progression.

HCV infection of primary human hepatocytes induces EMT marker gene expression. The altered morphology of HCV-infected primary human hepatocytes led us to examine the induction of EMT signaling. For this, an EMT pathway-specific PCR array was performed by using total cellular RNA from mock-infected control and HCV-infected primary human hepatocytes (at 4 weeks postinfection). A differential gene expression of EMT markers was observed (Fig. 2A and B). The majority of the mesenchymal cell markers, including snail, slug, vimentin, and twist,

were upregulated in HCV genotype 1a- or 2a-infected primary human hepatocytes compared to the mock-infected control (Fig. 2A). On the other hand, epithelial cell markers, including E-cadherin, occludin, and desmoplakin, were downregulated in infected hepatocytes (Fig. 2B).

Since we observed a downregulation of epithelial cell marker genes and an upregulation of EMT gene expression at the mRNA level, a selection of key EMT markers was validated at the protein level by Western blot analysis. HCV genotype 1a or 2a infection of primary human hepatocytes downregulated the expression of E-cadherin, a well-known epithelial cell marker (Fig. 2C). On the other hand, HCV infection of hepatocytes upregulated several mesenchymal cell markers, including vimentin, snail, slug, and twist (Fig. 2D to G). All of these experiments were done at 4 weeks postinfection. Reports in the literature suggested that fibroblasts are derived from hepatocytes via EMT during liver fibrosis (48). All fibroblasts express FSP-1 and are likely to appear through type

FIG 2 Differential EMT gene expression in primary human hepatocytes following HCV infection. (A) Clustergram of the mesenchymal genes differentially regulated in HCV genotype 1a- or 2a-infected primary human hepatocytes compared to mock-infected control cells. (B) Similar analysis for epithelial cell marker genes differentially regulated in HCV-infected hepatocytes compared to control cells. (C to H) Protein expression statuses of selected EMT genes, such as the epithelial cell marker E-cadherin (C) and the mesenchymal cell markers vimentin (D), snail (E), slug (F), twist (G), and FSP-1 (H), were analyzed by Western blotting using specific antibodies. The expression level of actin in each lane was determined as a loading control for comparison.

2 EMT (38). This transition was also demonstrated by previous lineage-tracing studies during the formation of fibroblasts in renal tissues (21) and was later confirmed for other organs, including liver, lung, and heart (26, 46, 48). Thus, we wanted to determine if HCV-infected primary human hepatocytes displaying a fibroblast-like morphology from our study express FSP-1. Western blot analysis suggested that these infected cells expressed FSP-1, while FSP-1 was absent in mock-infected control cells (Fig. 2H). Interestingly, albumin expression in hepatocyte lysates or culture supernatants was undetectable by Western blotting at day 28 postinfection. Taken together, the results indicated that HCV infection induces hepatocytes to enter into a transition state, where decreases in the expression levels of epithelial cell markers and increases in the expression levels of mesenchymal cell markers and FSP-1 occur.

We also determined the expression statuses of selected transcription factors, snail and twist, as EMT markers in liver biopsy specimens from HCV-infected patients by real-time PCR (Fig. 3A and B). Interestingly, both snail and twist were upregulated in HCV-infected liver samples ($n = 10$), compared to levels in normal control livers ($n = 3$), suggesting a progressive EMT state in HCV-infected liver specimens.

β -Catenin-mediated regulation of EMT markers in HCV-infected primary human hepatocytes. β -Catenin is a key downstream effector of the Wnt signaling pathway (32) and is known to play an important role in EMT progression. The phosphorylation

FIG 3 Statuses of snail and twist transcription factors in chronically HCV-infected human liver biopsy specimens. Shown are results from real-time PCR analyses displaying expression statuses of snail (A) and twist (B) mRNAs in HCV-infected liver biopsy specimens ($n = 10$), compared to non-HCV-infected control liver tissues ($n = 3$). The results are presented as box plots and are normalized to values for endogenous 18S rRNA. The difference between experimental and control values was statistically significant by a one-way analysis of variance ($P < 0.0001$).

FIG 4 β -Catenin activation in HCV-infected primary human hepatocytes. (A and B) Mock-infected and HCV genotype 1a- or 2a-infected primary human hepatocytes were analyzed for the phosphorylation statuses of Ser⁵⁵² (A) and Thr⁴¹/Ser⁴⁵ (B) in β -catenin. (C) The total β -catenin levels from same experiments are shown separately. (D to I) Immunofluorescence showing nuclear localization of activated β -catenin in HCV genotype 1a (D to F)- or 2a (G to I)-infected primary human hepatocytes. Cells were stained with an antibody specific for β -catenin (green) (D and G). The cell nucleus was stained with DAPI (blue) (E and H), and the image was merged with the image of β -catenin (F and I).

of β -catenin by Akt at Ser⁵⁵² causes its disassociation from cell-cell contacts and accumulation in both the cytosol and nucleus and increases its transcriptional activity (16). We determined the Ser⁵⁵² phosphorylation status of β -catenin. A significant upregulation of the Ser⁵⁵² phosphorylation status of β -catenin in HCV genotype 1a- or 2a-infected primary human hepatocytes compared to mock-infected control cells was observed by Western blot analysis (Fig. 4A). The phosphorylation of β -catenin at Thr⁴¹ and Ser⁴⁵ is important for degradation by the ubiquitin proteasome pathway (43). We observed a significant downregulation of the Thr⁴¹/Ser⁴⁵ phosphorylation status of β -catenin in HCV genotype 1a- or 2a-infected primary human hepatocytes compared to mock-infected control cells (Fig. 4B). However, we did not observe degradation in the control cells, as the total β -catenin expression statuses were similar for infected and mock-infected primary human hepatocytes (Fig. 4C). The results indicated that HCV infection of primary human hepatocytes modulates the phosphorylation of β -catenin in increasing the transcriptional activity necessary for EMT progression.

Activated β -catenin acts as a transcription factor which migrates from the cell cytoplasm into the nucleus, where it promotes the transcription of genes necessary for EMT progression. The phosphorylation of β -catenin at Ser⁵⁵² induces the translocation of the protein into the nucleus and increases its transcriptional activity. As we observed the phosphorylation of Ser⁵⁵² in β -catenin, we examined whether β -catenin localizes to the nucleus of HCV-infected hepatocytes. The localization of β -catenin in the nucleus of HCV genotype 1a- or 2a-infected primary human hepatocytes was apparent (Fig. 4D to I). The result implies

the role of β -catenin as a transcription factor for the promotion of snail, slug, and twist for EMT progression.

EMT-induced HCV-infected hepatocytes display Akt activation. β -Catenin activation by phosphorylation at Ser⁵⁵² is mediated by Akt (16). Since we observed a significant upregulation of the Ser⁵⁵² phosphorylation of β -catenin in HCV-infected primary human hepatocytes, we examined the phosphorylation status of Akt. Western blot analysis suggested a significant upregulation of phosphorylation at both Thr³⁰⁸ and Ser⁴⁷³ of Akt, essential for Akt activation in HCV-infected hepatocytes, compared to uninfected controls. On the other hand, the total Akt levels were similar in HCV-infected and mock-infected control cells (Fig. 5A). The Akt-mediated phosphorylation of β -catenin causes the disassociation of cell-cell contacts and leads to accumulation in both the cytosol and the nucleus, increasing the transcriptional activity of β -catenin (16). Here, we have shown that HCV induces the Ser⁵⁵² phosphorylation of β -catenin and activates Akt. Thus, the Akt-mediated activation of β -catenin appears to be promoting its transcriptional activity for the induction of snail, slug, and twist.

Wnt signaling is a major activator of β -catenin. The phosphorylation of LDL receptor-related protein 6 (Lrp-6) is essential to initiate the Wnt signaling pathway (39). We examined whether the Wnt signaling pathway is active in HCV-infected primary human hepatocytes. Western blot analysis suggested that the total levels of Lrp-6 (a coreceptor of Frizzled) were similar while its phosphorylated form was undetectable in mock-infected control and HCV-infected primary human hepatocytes. This result indicated that the Wnt signaling pathway is not activated. (Fig. 5B). The transforming growth factor β (TGF- β)–Smad signaling path-

FIG 5 Activation of Akt in EMT-induced HCV-infected primary human hepatocytes. (A) The expression statuses of the Thr³⁰⁸/Ser⁴⁷³ phosphorylation of Akt and total Akt in mock-infected and HCV genotype 1a- or 2a-infected primary human hepatocytes were determined. The actin level in each lane was determined as a loading control. (B) The Lrp-6 phosphorylation status was determined by Western blot analysis as a measure of the activation of the Wnt signaling pathway. (C) The expression level of the Smad2/3 protein was determined by Western blotting as a measure of the activation of the TGF- β signaling pathway.

way plays an important role in mediating EMT. However, TGF- β was not upregulated in the EMT array, and the Smad protein was undetectable in HCV-infected hepatocytes by Western blotting (Fig. 5C). These results suggest that EMT progression is mediated via the Akt/ β -catenin signaling pathway in HCV-infected primary human hepatocytes.

DISCUSSION

In this study, we have shown that primary human hepatocytes infected with cell culture-grown HCV display EMT via the activation of the Akt/ β -catenin signaling pathway, which may have profound long-term implications for the progression of end-stage liver disease. We have observed distinct morphological changes typical of EMT in primary human hepatocytes infected with HCV genotype 1a or 2a. The upregulation of important mesenchymal genes and the downregulation of key epithelial genes, which are hallmarks of EMT, were observed at the RNA and protein levels. E-cadherin, a typical epithelial cell marker, was downregulated in HCV-infected primary human hepatocytes compared to mock-treated control cells. A change in the E-cadherin expression level is the prototypical epithelial cell marker for EMT (19, 22). A mesenchymal cell marker, vimentin, was upregulated in HCV-infected hepatocytes. We also observed enhancements in the levels of the transcription factors snail, slug, and twist in HCV-infected hepatocytes. These three proteins are also recognized as mesenchymal cell markers upregulated in cells undergoing EMT (9, 11, 45). The upregulated mesenchymal cell markers may play a role in repressing the expression of E-cadherin, and other epithelial tight-junction proteins, for promoting a mesenchymal phenotype. Previous studies have shown that snail and slug bind to E boxes present in the E-cadherin promoter, consequently repressing E-cadherin transcription (10, 11, 14). Snail and slug also induce the loss of tight-junction integral membrane proteins, such as the claudins and occluding, by a similar mechanism (20). Twist is a master regulator of EMT, and the activation of this protein results in the loss of E-cadherin-mediated cell-cell adhesion, the activation of mesenchymal cell markers, and the induction of cell motility (45). Our results indicate that hepatocytes enter a transition state early after cell culture infection with HCV, and the upregulation of transcription factors such as snail, slug, and twist results in the downregulation of epithelial cell markers such as E-cadherin, desmoplakin, and occludin.

The HBVx protein was similarly implicated in the downregulation of E-cadherin, which might represent a mechanism for the

pathogenesis of chronic liver disease and HCC associated with chronic hepatitis B virus infection (5). A recent report by Akkari et al. (3) described that HCV NS5a induces EMT in a mouse cell line derived as primary bipotential mouse embryonic liver cells. Although those authors used HCV-infected primary human hepatocytes to substantiate the physiological significance of NS5a as a trigger for EMT by studying only Twist2 mRNA, they clearly stated in the discussion that the molecular mechanism linking NS5a to the regulation of Twist2 remains to be established. Previous studies have shown that the HCV core protein plays a down-regulatory role in the E-cadherin promoter, altering the methylation status and resulting in reduced levels of protein expression (4, 37). We previously reported the generation of immortalized human hepatocytes (IHH) upon the introduction of the HCV core gene following a brief transition into a fibroblast-like appearance (36). We examined IHH at a very early passage level and did not observe a decrease in the expression level of E-cadherin, and the Ser⁵⁵² phosphorylation of β -catenin was undetectable. The results implied that the HCV core protein may not be involved in the induction of EMT. Further study is necessary to verify the transition from EMT to an immortalized or transformed phenotype. A different report (42) suggested that the E-cadherin level is reduced in HCV glycoprotein-expressing HepG2 cells, and HCV infection promotes snail and twist expression in both Huh7.5 cells and primary human hepatocytes. However, those authors failed to observe any morphological fibroblast features of HCV-infected or glycoprotein-expressing hepatoma cells, possibly due to the partial de-differentiation process *in vitro*.

The EMT is encountered in 3 different settings. Type 1 EMT produces mesenchymal cells during embryogenesis, while type 2 EMT in adult or maturing tissues results in a fibroblast-like state (47). Type 3 EMT is a process that uses this mechanism to transition and progress to developing cancer cells capable of migration and invasion (44). Much interest has been focused on how to better detect epithelial cells undergoing type 2 EMT in different culture systems and tissues. One reliable marker for type 2 EMT is FSP-1 (fibroblast-specific protein 1), which is expressed by all fibroblasts and likely to appear during type 2 EMT (21, 38). During liver fibrosis, fibroblasts are derived from hepatocytes via EMT (48). As indicated for type 2 EMT, we observed the expression of FSP-1 in HCV-infected cells, which was not apparent in the control cells. This result further suggests that HCV infection of primary human hepatocytes induces a type 2 EMT state, resulting in the development of fibroblast-like growth.

TGF- β -Smad signaling is often implicated in the progression of EMT (41). Thus, we investigated whether this pathway is active in HCV-infected primary human hepatocytes. TGF- β was undetectable at the mRNA level, and Smad proteins were undetectable by Western blot analysis. β -Catenin binds directly to the intracellular domain of E-cadherin. It also binds to α -catenin, which connects the adheren junction complex with the actin cytoskeleton (2). β -Catenin is released from the E-cadherin complex into the cytoplasm during EMT, where it interacts with other proteins, raising the possibility that β -catenin signaling contributes to EMT (25). The role of the Wnt/ β -catenin signal transduction pathway in understanding the molecular basis of hepatocellular carcinoma was suggested previously (30, 31). HCV genotype 1b core-transfected Huh7 cells upregulate Wnt-1 and WISP-2 transcription levels, indicating a role for the HCV core protein in activating the Wnt pathway (17). A recent report by Liu et al. (28) also documented the role of the HCV core protein in the enhancement of canonical Wnt/ β -catenin signaling in hepatoma cell lines and its involvement in HCV-associated carcinogenesis. The involvement of HCV NS5a in the activation of Wnt/ β -catenin signaling was also suggested by previous studies using human hepatoma cell lines (35). Interestingly, our observations indicate that HCV genotype 1a or 2a infection of primary human hepatocytes induces EMT by the activation of β -catenin without involving the Wnt signaling pathway (Fig. 5B). Therefore, a distinct difference was observed between the changes in primary human hepatocytes and the transformed human hepatoma cells used in other studies with respect to changes in β -catenin. Our results suggest that β -catenin activation occurs via Akt (Fig. 5A). We also observed that HCV core protein alone did not induce EMT, implying that another viral protein(s) may be involved in the induction of EMT. The phosphorylation of β -catenin by Akt at the Ser⁵⁵² residue causes its dissociation from cell-cell contacts and accumulation in both the cytosol and nucleus and increases its transcriptional activity (16). Here, we have shown that HCV induces the Ser⁵⁵² phosphorylation of β -catenin and activates Akt. Thus, the Akt-mediated activation of β -catenin appears to be promoting its transcriptional activity for the induction of snail, slug, and twist. On the other hand, it is also possible that snail and slug promote EMT through the transcriptional activation of β -catenin (29). Furthermore, Lrp-6 was not phosphorylated in HCV-infected primary human hepatocytes, indicating that the canonical Wnt signaling pathway was not active. These results suggest that HCV promotes EMT in primary human hepatocytes via the activation of Akt/ β -catenin signaling. A simplified diagram showing the activation of EMT genes via the Akt/ β -catenin pathway mediated by HCV is shown in Fig. 6.

Primary hepatocytes entered senescence ~3 to 4 months after infection with HCV. β -Galactosidase staining at a later stage implied that the hepatocytes were in a senescent phase and the cells were unable to grow. The time periods for the initial appearance of fibroblast-like growth after HCV infection, and entry into senescence, varied to some extent among the donor hepatocytes. HCV RNA was undetectable in hepatocytes at a later stage when senescence was apparent. It is possible that the clearance of HCV from hepatocytes may occur due to the natural innate immune response. However, chronic infection may increase senescence and promote a transformed phenotype in a selected hepatocyte population as an underlying trait of disease progression to hepatocellular carcinoma, and future studies should unravel this possibility.

FIG 6 Schematic diagram showing the HCV-induced activation of EMT genes via the Akt/ β -catenin pathway. HCV activates Akt via the phosphorylation of Thr³⁰⁸ and Ser⁴⁷³. Activated Akt in turn activates β -catenin via the phosphorylation of Ser⁵⁵². Activated β -catenin migrates from the cytoplasm to the nucleus and acts as a transcription factor to induce the transcription of EMT genes, such as vimentin, snail, slug, and twist, and mediates the down-regulation of the epithelial cell marker E-cadherin.

Dysfunctional telomeres trigger senescence through the p53 pathway. However, cells may undergo replicative senescence independent of telomere shortening, and the nature of this response is poorly understood. These processes increase p16 expression levels and engage the p16-retinoblastoma protein (pRB) pathway (13). However, both p53 and pRB were undetectable in the late stage of infection of primary human hepatocytes, indicating that some other mechanisms are involved in hepatocyte death.

In summary, we have identified a link between HCV infection and EMT, a novel causative mechanism for the growth potential of primary human hepatocytes during chronic virus infection. The mechanisms involved in EMT appear to be integrated in concert with liver disease progression, including cirrhosis and oncogenic pathways. Future studies directed at the identification of the factors which initiate, modulate, and effectuate EMT signatures may provide strategies to impair HCV-related liver disease progression. The targeting of critical orchestrators of EMT signaling pathways by inhibitory molecules may provide novel therapeutic modalities against liver disease progression.

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REFERENCES

1. Aberle H, Bauer A, Stappert J, Kispert A, Kemler R. 1997. β -Catenin is a target for the ubiquitin-proteasome pathway. *EMBO J.* 16:3797–3804.
2. Aberle H, et al. 1994. Assembly of the cadherin-catenin complex in vitro with recombinant proteins. *J. Cell Sci.* 107:3655–3663.
3. Akkari L, et al. 2012. Hepatitis C viral protein NS5A induces EMT and participates in oncogenic transformation of primary hepatocyte precursors. *J. Hepatol.* 57:1021–1028.
4. Arora P, Kim EO, Jung JK, Jang KL. 2008. Hepatitis C virus core protein downregulates E-cadherin expression via activation of DNA methyltransferase 1 and 3b. *Cancer Lett.* 261:244–252.
5. Arzumanyan A, et al. 2012. Epigenetic repression of E-cadherin expression by hepatitis B virus x antigen in liver cancer. *Oncogene* 31:563–572.

6. Baldo V, Baldovin T, Trivello R, Floreani A. 2008. Epidemiology of HCV infection. *Curr. Pharm. Des.* 14:1646–1654.
7. Banerjee A, et al. 2011. Transcriptional repression of C4 complement by hepatitis C virus proteins. *J. Virol.* 85:4157–4166.
8. Banerjee A, Ray RB, Ray R. 2010. Oncogenic potential of hepatitis C virus proteins. *Viruses* 2:2108–2133.
9. Barrallo-Gimeno A, Nieto MA. 2005. The snail genes as inducers of cell movement and survival: implications in development and cancer. *Development* 132:3151–3161.
10. Battle E, et al. 2000. The transcription factor snail is a repressor of E-cadherin gene expression in epithelial tumour cells. *Nat. Cell Biol.* 2:84–89.
11. Bolós V, et al. 2003. The transcription factor slug represses E-cadherin expression and induces epithelial to mesenchymal transitions: a comparison with snail and E47 repressors. *J. Cell Sci.* 116:499–511.
12. Brabletz T, Jung A, Dag S, Hlubek F, Kirchner T. 1999. Beta-catenin regulates the expression of the matrix metalloproteinase-7 in human colorectal cancer. *Am. J. Pathol.* 155:1033–1038.
13. Campisi J, d'Adda di Fagnana F. 2007. Cellular senescence: when bad things happen to good cells. *Nat. Rev. Mol. Cell Biol.* 8:729–740.
14. Cano A, et al. 2000. The transcription factor snail controls epithelial-mesenchymal transitions by repressing E-cadherin expression. *Nat. Cell Biol.* 2:76–83.
15. Dang H, Ding W, Emerson D, Rountree CB. 2011. Snail1 induces epithelial-to-mesenchymal transition and tumor initiating stem cell characteristics. *BMC Cancer* 11:396. doi:10.1186/1471-2407-11-396.
16. Fang D, et al. 2007. Phosphorylation of β -catenin by Akt promotes β -catenin transcriptional activity. *J. Biol. Chem.* 282:11221–11229.
17. Fukutomi T, et al. 2005. Hepatitis C virus core protein stimulates hepatocyte growth: correlation with upregulation of wnt-1 expression. *Hepatology* 41:1096–1105.
18. Gradl D, Kuhl M, Wedlich D. 1999. The Wnt/Wg signal transducer beta-catenin controls fibronectin expression. *Mol. Cell. Biol.* 19:5576–5587.
19. Huber MA, Kraut N, Beug H. 2005. Molecular requirements for epithelial-mesenchymal transition during tumor progression. *Curr. Opin. Cell Biol.* 17:548–558.
20. Ikenouchi J, Matsuda M, Furuse M, Tsukita S. 2003. Regulation of tight junctions during the epithelium-mesenchyme transition: direct repression of the gene expression of claudins/occludin by snail. *J. Cell Sci.* 116:1959–1967.
21. Iwano M, et al. 2002. Evidence that fibroblasts derive from epithelium during tissue fibrosis. *J. Clin. Invest.* 110:341–350.
22. Kalluri R, Neilson EG. 2003. Epithelial-mesenchymal transition and its implications for fibrosis. *J. Clin. Invest.* 112:1776–1784.
23. Kalluri R, Weinberger RA. 2009. The basics of epithelial-mesenchymal transition. *J. Clin. Invest.* 119:1420–1428.
24. Kanda T, et al. 2006. Generation of infectious hepatitis C virus in immortalized human hepatocytes. *J. Virol.* 80:4633–4639.
25. Kim K, Daniels KJ, Hay ED. 1998. Tissue-specific expression of beta-catenin in normal mesenchyme and uveal melanomas and its effect on invasiveness. *Exp. Cell Res.* 245:79–90.
26. Kim KK, et al. 2006. Alveolar epithelial cell mesenchymal transition develops in vivo during pulmonary fibrosis and is regulated by the extracellular matrix. *Proc. Natl. Acad. Sci. U. S. A.* 103:13180–13185.
27. Liu C, et al. 1999. β -Trcp couples beta-catenin phosphorylation degradation and regulates Xenopus axis formation. *Proc. Natl. Acad. Sci. U. S. A.* 96:6273–6278.
28. Liu J, et al. 2011. Enhancement of canonical Wnt/ β -catenin signaling activity by HCV core protein promotes cell growth of hepatocellular carcinoma cells. *PLoS One* 6:e27496. doi:10.1371/journal.pone.0027496.
29. Medici D, Hay ED, Olsen BR. 2008. Snail and Slug promote epithelial-mesenchymal transition through beta-catenin-T-cell factor-4-dependent expression of transforming growth factor-beta3. *Mol. Biol. Cell* 19:4875–4887.
30. Merle P, et al. 2004. Functional consequences of frizzled-7 receptor overexpression in human hepatocellular carcinoma. *Gastroenterology* 127:1110–1122.
31. Merle P, et al. 2005. Oncogenic role of the frizzled-7/beta-catenin pathway in hepatocellular carcinoma. *J. Hepatol.* 43:854–862.
32. Moon RT, Kohn AD, De Ferrari GV, Kaykas A. 2004. WNT and β -catenin signalling: diseases and therapies. *Nat. Rev. Genet.* 5:691–701.
33. Na DC, et al. 2011. Invasion and EMT-associated genes are up-regulated in B viral hepatocellular carcinoma with high expression of CD133-human and cell culture study. *Exp. Mol. Pathol.* 90:66–73.
34. Novak A, et al. 1998. Cell adhesion and the integrin-linked kinase regulate the LEF-1 and beta-catenin signaling pathways. *Proc. Natl. Acad. Sci. U. S. A.* 95:4374–4379.
35. Park CY, et al. 2009. Nonstructural 5A protein activates beta-catenin signaling cascades: implication of hepatitis C virus-induced liver pathogenesis. *J. Hepatol.* 51:853–864.
36. Ray RB, Meyer K, Ray R. 2000. Hepatitis C virus core protein promotes immortalization of primary human hepatocytes. *Virology* 271:197–204.
37. Ripoli M, et al. 2011. Hypermethylated levels of E-cadherin promoter in Huh-7 cells expressing the HCV core protein. *Virus Res.* 160:74–81.
38. Strutz F, et al. 1995. Identification and characterization of a fibroblast marker: FSP1. *J. Cell Biol.* 130:393–405.
39. Tamai K, et al. 2000. LDL-receptor-related proteins in Wnt signal transduction. *Nature* 407:530–535.
40. Van Zijl F, et al. 2009. Epithelial-mesenchymal transition in hepatocellular carcinoma. *Future Oncol.* 5:1169–1179.
41. Willis BC, Borok Z. 2007. TGF-beta-induced EMT: mechanisms and implications for fibrotic lung disease. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 293:L525–L534. doi:10.1152/ajplung.00163.2007.
42. Wilson GK, et al. 2012. A dual role for hypoxia inducible factor-1 α in the hepatitis C virus lifecycle and hepatoma migration. *J. Hepatol.* 56:803–809.
43. Wu G, He X. 2006. Threonine 41 in beta-catenin serves as a key phosphorylation relay residue in beta-catenin degradation. *Biochemistry* 45:5319–5323.
44. Xue C, Plieth D, Venkov C, Xu C, Neilson EG. 2003. The gatekeeper effect of epithelial-mesenchymal transition regulates the frequency of breast cancer metastasis. *Cancer Res.* 63:3386–3394.
45. Yang J, et al. 2004. Twist, a master regulator of morphogenesis, plays an essential role in tumor metastasis. *Cell* 117:927–939.
46. Zeisberg EM, et al. 2007. Endothelial-to-mesenchymal transition contributes to cardiac fibrosis. *Nat. Med.* 13:952–961.
47. Zeisberg M, Neilson EG. 2009. Biomarkers for epithelial-mesenchymal transitions. *J. Clin. Invest.* 119:1429–1437.
48. Zeisberg M, et al. 2007. Fibroblasts derive from hepatocytes in liver fibrosis via epithelial to mesenchymal transition. *J. Biol. Chem.* 282:23337–23347.
49. Zhao XL, et al. 2011. Promotion of hepatocellular carcinoma metastasis through matrix metalloproteinase activation by epithelial-mesenchymal transition regulator Twist1. *J. Cell Mol. Med.* 15:691–700.