Elevation of Plasminogen Activator Inhibitor-1 Promotes Differentiation of Cancer Stem-Like Cell State by Hepatitis C Virus Infection

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ABSTRACT Plasminogen activator inhibitor-1 (PAI-1) is a critical factor that regulates protein synthesis and degradation. Increased PAI-1 levels are detectable in the serum of patients with chronic hepatitis C virus (HCV) liver disease. The differentiation state and motility of HCV-induced cancer stem-like cells (CSCs) play a major role in severe liver disease progression. However, the role of PAI-1 in the pathological process of chronic liver diseases remains unknown. In this study, we determined how PAI-1 affects the differentiation of CSC state in hepatocytes upon HCV infection. We found that HCV infection induced the expression of PAI-1 while decreasing miR-30c expression in Huh7.5.1 cells. Similar results were obtained from isolated hepatocytes from humanized-liver mice after HCV infection. Moreover, decreased miR-30c expression in HCV-infected hepatocytes was associated with the increased levels of PAI-1 mRNA and protein. Notably, the increased PAI-1 levels resulted in the activation of protein kinase B/AKT, a major mediator of cell proliferation in HCV-infected hepatocytes, along with the increased expression of CSC markers such as human differentiated protein (CD) 133, epithelial cell adhesion molecule (EpCAM), octamer 4 (Oct4), Nanog, cyclin D1, and MYC. Moreover, blockade of PAI-1 activity by miR-30c mimic and anti-PAI-1 monoclonal antibody (Mab) abrogated the AKT activation with decreased expression of CSC markers. Our findings suggest that HCV infection induces the CSC state via PAI-1-mediated AKT activation in hepatocytes. This finding implies that manipulation of PAI-1 activity could provide potential therapeutics to prevent the development of HCV-associated chronic liver diseases.

IMPORTANCE The progression of chronic liver disease by HCV infection is considered a major risk factor for hepatocellular carcinoma (HCC), one of the major causes of death from cancer. Recent studies have demonstrated that increased CSC properties in HCV-infected hepatocytes are associated with the progression of HCC. Since protein and microRNA (miRNA) production by HCV-infected hepatocytes can play various roles in physiological processes, investigating these factors can potentially lead to new therapeutic targets. However, the mechanism of HCV-associated progression of hepatocytes to CSC remains unclear. Here, we identify the roles of PAI-1 and miR-30c in the progression of CSC during HCV infection in hepatocytes. Our data show that increased secretion of PAI-1 following HCV infection promotes this CSC state and activation of AKT. We report that the inhibition of PAI-1 by an miR-30c mimic reduces HCV-associated CSC properties in hepatocytes. Taken together, targeting this interaction of secreted PAI-1 and miR-30c in HCV-infected hepatocytes may provide a potential therapeutic intervention against the progression to chronic liver diseases and HCC.

KEYWORDS HCV, PAI-1, CSC, miRNAs, miR-30c

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Hepatitis C virus (HCV) infection is a major cause of chronic hepatitis, liver fibrosis, cirrhosis, and hepatocellular carcinoma (HCC) (1). Chronic liver disease caused by HCV infection is characterized by excessive accumulation of extracellular matrix (ECM) proteins and increased cell mobility, leading to severe liver failure if there is no therapeutic intervention. The progression of liver disease depends on the rates of collagen synthesis and cellular proteolytic activities by the urokinase-type plasminogen activator and tissue-type plasminogen activator (uPA/tPA) system (2).

Plasminogen activator inhibitor-1 (PAI-1) is a member of the serine protease inhibitor gene family and a major physiologic inhibitor of the serine proteases urokinase-type plasminogen activator (uPA) and tissue plasminogen activator (tPA) (3). Under normal physiological conditions, PAI-1 regulates collagen synthesis and ECM protein homeostasis while assisting in wound healing (4, 5). However, persistent activities of PAI-1 can result in excessive protein accumulation in a variety of tissues, which leads to an epithelial-mesenchymal transition (EMT) state and an increase in cell invasiveness properties (6). Recent studies report cancer stem-like cell (CSC) properties of HCV-infected hepatocytes with increased EMT (7, 8). CSCs are known to be cancer-propagating cells involved in tumor metastasis, as they exhibit properties such as self-renewal, cell proliferation, and cell survival (9, 10). CSCs have been identified in several tumors, such as liver, lung, breast, and pancreatic tumors (11). Especially, recent studies have identified the role of the CSC, as well as their specific markers in the liver disease progression (12). A clear elucidation of the mechanisms of the CSC state can contribute to the development of new biomarkers for precise disease diagnosis.

A microRNA (miRNA) is a small, noncoding RNA molecule (about 18 to 22 nucleotides) known as small regulatory RNAs, which can control mRNA life span and translation through complementary pairing with the 3' untranslated region (UTR) of the target mRNA. miRNAs play various roles in physiological processes, including growth, differentiation, proliferation, and apoptosis (13–15). In particular, the miRNA miR-30c downregulates PAI-1 expression by binding directly to the 3' UTR. Also, miR-30c has been shown to inhibit ventricular collagen synthesis in cardiac fibroblasts (16, 17). Moreover, transforming growth factor beta (TGF- β) downregulates miR-30c expression in endothelial cells and hepatic stellate cells, a process that was linked to several pathological conditions, including fibrosis (18, 19). These studies provide a critical pathological role for PAI-1, such that the regulation of PAI-1 and miR-30c may be responsible for the progression of chronic liver disease caused by HCV infection. However, the role of PAI-1 and miR-30c produced by HCV-infected hepatocytes during disease progression has yet to be elucidated.

In this report, we demonstrate that HCV-associated elevation of PAI-1 levels results in AKT activation and the upregulation of factors associated with the CSC state in hepatocytes. Importantly, inhibition of PAI-1 expression by an miR-30c mimic or PAI-1 neutralizing antibody interfered with the progression of the CSC state and AKT activation in HCV-infected hepatocytes. These results suggest that HCV infection in hepatocytes increases cell invasiveness and mobility by stimulating AKT activation through PAI-1 upregulation.

RESULTS

HCV infection in hepatocytes increases PAI-1 mRNA and protein levels. PAI-1 is a risk factor in patients with cirrhosis and hepatocellular carcinoma (20). PAI-1 is significantly elevated in plasma from patients with cirrhosis and hepatocellular carcinoma following HCV infection compared to that of uninfected healthy donors (21–23). Moreover, recent studies reported increased CSC properties in HCV-infected hepatocytes (10, 11). These findings prompted us to determine the effect of PAI-1 on the progression of the CSC state following HCV infection. HCV RNA levels increase until 5 days postinfection (dpi) with HCV and begin to decline at 6 dpi in Huh7.5.1 cells. Huh7.5.1 cells showed stable viability by day 5 post HCV infection (Fig. 1A). Notably, PAI-1 mRNA levels continue to increase up to 6 dpi of viral infection, but the level of miR-30c expression declines during HCV infection (Fig. 1B). Intracellular PAI-1 is upregulated by

FIG 1 HCV infection in hepatocytes increases the level of PAI-1. Huh7.5.1 cells were infected with hepatitis C virus (HCV) at 0.5 multiplicity of infection (MOI). (A) Cell survival rate was measured every day for 6 days using an MTT assay and cell morphology changes were observed every day for 6 days by microscopy. (B) mRNA levels of HCV, PAI-1, and miR-30c were analyzed every day for 6 days by qRT-PCR. HPRT was used as an endogenous control for normalization. (C) At 5 days postinfection (dpi), the cell lysates and cell culture medium (CM) were collected for analysis. The intracellular PAI-1 protein was analyzed by confocal microscopy. Confocal microscopic images of HCV core (red) and PAI-1 (green) expression in Huh7.5.1 cells at 5 dpi; nuclei were stained with DAPI. Scale bar, 50 μ m. Extracellular PAI-1 protein in cell culture medium was analyzed by Western blotting. Data are expressed as mean $\pm$ standard deviation (SD) ($n=3$). *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$ were considered statistically significant, as assessed using an unpaired Student's t test.

stimuli such as HCV infection, inducing cell damage and increasing cell mobility. In addition, secreted extracellular PAI-1 binds to the uPA/tPA receptor complex present on the cell surface, promoting ECM protein deposition through reduced plasmin (24). To determine the protein level of PAI-1 in HCV-infected hepatocytes, we performed confocal microscopy for intracellular PAI-1 and Western blot analysis for secreted PAI-1 protein in culture medium from HCV-infected Huh7.5.1 cells at 5 dpi. Intracellular and extracellular protein levels of PAI-1 significantly increased in HCV-infected Huh7.5.1 cells and culture medium (CM) in comparison to uninfected Huh7.5.1 cells (Fig. 1C).

HCV infection decreases miR-30c levels, and miR-30c mimic inhibits PAI-1 in hepatocytes. Previous studies have reported that the serum levels of miR-30c are significantly decreased in patients with HCC and chronic HCV infection (25). To determine whether miR-30c directly regulates PAI-1 mRNA expression, the PAI-1/luciferase construct was generated by cloning the PCR product of PAI-1 3' UTR mRNA at the 3' end of the luciferase reporter gene and was used for miR-30c binding studies. An miR-30c mimic/inhibitor (miR20000244) significantly affected the relative luciferase activity in PAI-1-3' UTR compared to cotransfection with miR-NC (26). These results support that miR-30c is able to regulate PAI-1 expression by binding directly to its 3' UTR. In this study, to determine whether PAI-1 activity can be regulated by miR-30c in HCV-infected Huh7.5.1 cells, we measured the level of miR-30c present during HCV infection. We found a significant downregulation of miR-30c levels in HCV-infected Huh7.5.1 cells at 5 dpi (Fig. 2A). Then we examined the effect of miR-30c on PAI-1 levels in HCV-infected Huh7.5.1 cells by using mimic or inhibitor miR-30c transfection (miR20000244). To clarify the activity of miR-30c, we examined the effect of mimic or inhibitor miR-30c at different concentrations on uninfected and HCV-infected Huh7.5.1 cells. When we tested three different doses of mimic or inhibitor miR-30c, there was the highest efficiency at 5 nM mimic and 50 nM inhibitor miR-30c, which was indicated in the manufacturer's protocol (Fig. 2B). Transfection of miR-30c mimic on HCV-infected Huh7.5.1 cells significantly decreased PAI-1 levels compared with control mimic-treated HCV-infected Huh7.5.1 cells. Inversely, HCV-infected Huh7.5.1 cells transfected with miR-30c inhibitor showed significantly increased level of PAI-1 compared with control inhibitor-treated HCV-infected Huh7.5.1 cells (Fig. 2B). In addition, we found intracellular and secreted PAI-1 increased in HCV-infected Huh7.5.1 cells using immunofluorescence microscopy and Western blotting (Fig. 2C). However, intracellular and secreted PAI-1 significantly decreased in HCV-infected Huh7.5.1 cells transfected with miR-30c mimic (Fig. 2C). This suggests that miR-30c regulates PAI-1 expression, and that these factors negatively correlate with PAI-1 level in HCV-infected hepatocytes.

HCV infection induces differentiation to CSC with increased CSC markers in hepatocytes via AKT activation. AKT is a key regulator of the proliferation, differentiation, and metastasis of cancer cells. Activated AKT (phospho-AKT [pAKT]) is associated with several types of human cancer (27). AKT is phosphorylated at Thr308 and Ser473 for full activation. AKT phosphorylation at Ser473 enhances the subsequent AKT phosphorylation of Thr308 by phosphatidylinositol-dependent kinase 1 (PDK1) (28). AKT phosphorylation on Ser473 residue has been especially observed during EMT in various solid cancers, and previous studies have shown that increased pAKT(Ser473) activity is involved in the non-small-cell lung cancer stem-like cell metastasis pathway through subsequent phosphorylation of AKT downstream transcription factors (29, 30). Moreover, the AKT pathway is considered an important step related to the progression to the CSC state in chronic liver disease (31). CD133 and EpCAM serve as representative markers for the CSC state in liver cancer. CD133 and EpCAM are transmembrane glycoproteins and are overexpressed in tumor cells. They are involved in increasing cell proliferation properties during progression of various cancers (32–34).

We hypothesized that changes in PAI-1 can affect the CSC state in HCV-infected hepatocytes through AKT activation. To verify this, we first determined the change in pAKT(Ser473) and CSC-related markers at 5 dpi. We found a significant increase in transcripts for CSC-related markers, including Oct4, Nanog, cyclin D1, and MYC in HCV-infected cells in comparison to uninfected Huh7.5.1 cells (Fig. 3A). In addition, there was a significant increase in protein levels for CD133, EpCAM, Cyclin D1, and pAKT (Ser473) in HCV-infected cells compared to uninfected Huh7.5.1 cells (Fig. 3B). Conversely, the expression of PAI-1 and CSC markers was decreased in HCV-infected Huh7.5.1 cells treated with a direct acting antiviral (DAA) agent targeting NS5B polymerase (sofosbuvir) (Fig. 3C). To investigate whether pAKT(Ser473) has an impact on PAI-1 expression, we determined the change in PAI-1 after AKT(Ser473) kinase inhibitor (Sigma) treatment in HCV-infected hepatocytes. AKT inhibitor effectively suppressed the expression of pAKT(Ser473) but did not affect the mRNA expression of PAI-1 in HCV-infected hepatocytes (Fig. 3D). AKT inhibitor did not make any significant changes

FIG 2 HCV infection decreases miR-30c levels, and miR-30c mimic inhibits PAI-1 in hepatocytes. Huh7.5.1 cells were infected with HCV at 0.5 MOI. (A) At 5 dpi, PAI-1 and miR-30c expression were analyzed by qRT-PCR. Total RNA, including miRNA, was extracted (Continued on next page)

in the protein levels of intracellular or extracellular PAI-1 in HCV-infected hepatocytes (Fig. 3E). However, there was a significant decrease in transcripts for CSC-related markers (Oct4, Nanog, cyclin D1, and MYC) in HCV-infected cells treated with AKT inhibitor compared to HCV-infected cells without AKT inhibitor (Fig. 3F). Taken together, these results suggest that HCV infection can activate AKT, leading to the progression of the CSC state in hepatocytes.

miR-30c mimic decreases PAI-1 levels, leading to the inhibition of AKT activation and CSC differentiation in HCV-infected hepatocytes. Next, we used miR-30c mimic transfection to confirm the impact of PAI-1 on AKT activation and the CSC state in HCV-infected hepatocytes. After 3 days of HCV infection (0.5 multiplicity of infection [MOI]) in Huh7.5.1 cells, miR-30c mimic was transfected into HCV-infected cells. After 48 h (5 dpi), we collected cell lysates for quantitative PCR (qPCR) and Western blot analysis of factors associated with the CSC state. There was a significant decrease in Oct4, Nanog, cyclin D1, and MYC in HCV-infected Huh7.5.1 cells with the miR-30c mimic compared to HCV-infected Huh7.5.1 cells treated with mimic control (Fig. 4A). Additionally, we found a significant decrease in protein levels for pAKT (Ser473) in HCV-infected Huh7.5.1 cells with the miR-30c mimic compared to the HCV-infected Huh7.5.1 cells with mimic control (Fig. 4B). Then, we checked the protein levels related to the CSC state using Western blotting. We found a significant decrease in CD133, EpCAM, and cyclin D1 in miR-30c mimic-treated HCV-infected Huh7.5.1 cells compared with mimic control-treated HCV-infected Huh7.5.1 cells (Fig. 4C). These results suggest that abrogating PAI-1 by miR-30c mimic reduces AKT activation and CSC markers in HCV-infected hepatocytes.

PAI-1 gene silencing and neutralizing anti-PAI-1 Mab inhibits AKT activation and CSC markers in HCV-infected hepatocytes. We determined the action of extracellular PAI-1 on AKT activation in HCV-infected hepatocytes and the CSC state by using neutralizing PAI-1 antibodies. We infected Huh7.5.1 cells with HCV at 0.5 MOI. At 3 dpi, we added 0.5 μ g/ml of neutralizing anti-PAI-1. At 48 h after antibody treatment, we collected and analyzed cell lysates. We compared mRNA levels of markers related to the CSC state and found a significant decrease in Oct4, Nanog, cyclin D1, and MYC in HCV-infected Huh7.5.1 cells with anti-PAI-1 compared to HCV-infected Huh7.5.1 cells without antibody treatment (Fig. 5A). We confirmed the levels of pAKT(Ser473) in HCV-infected hepatocytes with or without anti-PAI-1 using Western blotting, and found that a significant decrease in pAKT(Ser473) in HCV-infected Huh7.5.1 cells with anti-PAI-1 compared with HCV-infected Huh7.5.1 cells. In addition, CD133, EpCAM, and cyclin D1 protein levels were lower in HCV-infected Huh7.5.1 cells with anti-PAI-1 compared with HCV-infected Huh7.5.1 cells without antibody (Fig. 5B). Next, to clarify the functional role of PAI-1, we decreased PAI-1 gene expression in Huh 7.5.1 cells by transfection of PAI-1 short interfering RNAs (siPAI-1). As shown in Fig. 5C, siPAI-1 transfection inhibited the mRNA levels of Oct4, Nanog, cyclin D1, and MYC, as well as protein levels of pAKT(Ser473), PAI-1, CD133, and EpCAM compared with HCV-infected Huh7.5.1 cells absent interfering RNA. These data suggest that extracellular PAI-1 levels have a role in AKT activation and upregulation of CSC markers in HCV-infected hepatocytes.

Inhibition of PAI-1 activity results in decreasing cell proliferative responses. Ki67 staining serves as an indicator of proliferative tumor cells and was reported to increase the possibility of cancer metastasis (35). In addition, high Ki67 expression in chronic liver disease patients is positively correlated with HCC progression and poor

FIG 2 Legend (Continued)

using the miRNeasy minikit. HPRT was used as an endogenous control for normalization. (B) At 3 dpi, HCV-infected Huh7.5.1 cells were transfected with 3 different concentrations of miR-30c mimic or miR-30c inhibitor. At 5 dpi, cell lysates were collected for analysis. (C) At 3 dpi, HCV-infected Huh7.5.1 cells were transfected with miR-30c mimic (5 nM). After 48 h, cell lysates and cell culture medium were collected for analysis. Intracellular and extracellular PAI-1 protein levels were analyzed by confocal microscopy and Western blotting. Confocal microscopic images of HCV core (red) and PAI-1 (green) expression in Huh7.5.1 cells at 5 dpi; nuclei were stained with DAPI. Scale bar, 20 μ m. Data are expressed as mean $\pm$ SD ($n=3$). *, $P<0.05$; **, $P<0.01$; and ***, $P<0.001$ were considered statistically significant, as assessed using an unpaired Student's t test.

FIG 3 HCV infection induces differentiation of CSC with increased CSC markers in hepatocytes via AKT activation. Huh7.5.1 cells were infected with HCV at 0.5 MOI. (A) At 5 dpi, cell lysates were collected for analysis. mRNA was extracted using TRIzol and mRNA expression was analyzed (Continued on next page)

prognosis (36). We determined the profile of Ki67 staining as well as the effect of PAI-1 on Ki67 expression in HCV-infected hepatocytes by flow cytometry and confocal microscopy. As shown in Fig. 6A, HCV-infected Huh7.5.1 cells showed an increase in the population of Ki67-positive cells. In contrast, miR-30c mimic- or anti-PAI-1-treated HCV-infected Huh7.5.1 cells showed a significant decrease in the Ki67-positive population in comparison to untreated HCV-infected Huh7.5.1 cells (Fig. 6A). Similar results were found by confocal microscopy. The number of green foci (Ki67 positive) per blue nucleus was calculated by the ImageJ software. While 28 spots were found among 31 nuclei (average of 0.9/cell) in uninfected Huh7.5.1 cells, HCV-infected Huh7.5.1 cells had 50 spots among 35 nuclei (average of 1.4/cell). Moreover, HCV-infected Huh7.5.1 cells with miR-30c mimic or anti-PAI-1 treatment showed a significant decrease in the Ki67-positive spots (average of 0.82/cell and 0.85/cell, respectively) in comparison to HCV-infected Huh7.5.1 cells (Fig. 6B). These results suggest that HCV infection leads to an increase in the Ki67-positive population in hepatocytes, while inhibiting PAI-1 by miR-30c mimic or anti-PAI-1 reversed this effect. Taken together, expression of PAI-1 by HCV infection leads to increased CSC properties in hepatocytes, which indicates that PAI-1 plays an important role in HCC progression. Moreover, our data indicate that miR-30c can control the progression of HCC through the inhibition of PAI-1 expression.

Increased PAI-1 expression in HCV-infected humanized-liver mice. To confirm the change in PAI-1 levels following HCV infection *in vivo*, we used the *Fah*^{-/-} *Rag2*^{-/-} *Il2rg*^{-/-} (FRGN) mouse model, transplanted with human hepatocytes. This humanized-liver mouse model is known to be well suited for studies of HCV infection and liver disease (37, 38). We injected humanized-liver mice with HCV diluted in PBS into the tail vein. At 7 dpi, we harvested mouse liver tissues and isolated hepatocytes (Fig. 7A). First, we confirmed HCV RNA replication and HCV core protein expression in isolated hepatocytes from humanized-liver mice. As shown in Fig. 7B, mice with HCV infection showed a significant increase in HCV RNA replication and HCV core protein levels compared to PBS-injected mice. These results indicate that mice transplanted with human hepatocytes are successfully infected with HCV. Furthermore, we observed a significant increase in PAI-1 levels, a decrease in miR-30c (Fig. 7B), and increases in the CSC marker proteins CD133 and EpCAM (Fig. 7C) in isolated hepatocytes from HCV-infected groups compared to the PBS group.

DISCUSSION

PAI-1 is a serine protease inhibitor that plays an important role in tissue protein synthesis and degradation and is synthesized by various tissues, including the liver. Increased PAI-1 has been associated with poor prognosis in some cancers, including HCC (21, 22, 39). Notably, there was shown to be a significant increase in PAI-1 expression in HCV-infected Huh7.5.1 cells and chronic HCV patients with HCC/cirrhosis (40). Direct-acting antiviral therapy resulted in a significant decrease in serum PAI-1 from patients with HCV-related chronic liver disease compared to chronic HCV patients without treatment (22). These studies indicate that the increase in PAI-1 levels plays a pivotal role in the progression of severe liver diseases such as HCC/cirrhosis. PAI-1 not only contributes to the fibrinolysis process, but also triggers signal transduction for cell transition and cell migration in liver tissue (41, 42). Specifically, PAI-1 reduces fibrinolysis through tissue plasminogen activator (tPA) inhibition and can promote invasion

FIG 3 Legend (Continued)

by qRT-PCR. HPRT was used as an endogenous control for normalization in cells. (B) Protein levels of pAKT(Ser473), AKT, cyclin D1, CD133, EpCAM, and HCV core in cell lysates were analyzed by Western blotting. (C) At 2 dpi, a direct-acting antiviral agent sofosbuvir (250 nM) was added and incubated for 72 h; cells were then collected for analysis. (D and E) Huh7.5.1 cells were infected with HCV at 0.5 MOI and then AKT inhibitors were added. At 5 dpi, cell lysates were collected for analysis. Cell culture mediums were collected to analyze extracellular PAI-1 levels by Western blotting. Intracellular PAI-1 protein levels were analyzed by confocal microscopy. Confocal microscopic images of HCV core (red) and PAI-1 (green) expression in Huh7.5.1 cells at 5 dpi; nuclei were stained with DAPI. Scale bar, 50 μ m. (F) mRNA was extracted using TRIzol and mRNA expression was analyzed by qRT-PCR. HPRT was used as an endogenous control for normalization in cells. Protein levels of CD133, cyclin D1, and EpCAM in cell lysates were analyzed by Western blotting. Data are expressed as mean $\pm$ SD ($n=3$). *, $P<0.05$; **, $P<0.01$; and ***, $P<0.001$ were considered statistically significant, as assessed using an unpaired Student's *t* test.

FIG 4 miR-30c mimic decreases PAI-1 levels, leading to the inhibition of AKT activation and CSC markers in HCV-infected hepatocytes. Huh7.5.1 cells were infected with HCV at 0.5 MOI. At 3 dpi, HCV-infected Huh7.5.1 cells were transfected with miR-30c mimic (5 nM). After 48 h (5 dpi), cell lysates were collected for analysis. (A) mRNA was extracted using TRIzol, and mRNA expression was analyzed by qRT-PCR. HPRT was used as an endogenous control for normalization in cells. (B) Protein levels of pAKT(Ser473), AKT, (C) cyclin D1, CD133, EpCAM, and HCV core in cell lysates were analyzed by Western blotting. Relative protein expression was quantified by ImageJ software by reference to internal standards as follows: CD133/GAPDH, EpCAM/GAPDH, cyclin D1/GAPDH, and pAKT/AKT. Data are expressed as mean $\pm$ SD ($n=3$). *, $P<0.05$; **, $P<0.01$; and ***, $P<0.001$ were considered statistically significant, as assessed using an unpaired Student's t test.

and metastasis of cancer cells. Recent studies have reported that increased PAI-1 plays a crucial role in angiogenesis and tumor growth induced by transforming growth factor β (TGF- β) stimulation in the endothelium (18). In addition, PAI-1 deficiency reduces liver fibrosis after bile duct ligation in mice through increased tPA activity (43). In the present study, we found that HCV infection not only upregulates PAI-1, but also

FIG 5 PAI-1 gene silencing and neutralizing anti-PAI-1 Mab inhibits AKT activation and CSC markers in HCV-infected hepatocytes. Huh7.5.1 cells were infected with HCV at 0.5 MOI. At 3 dpi, neutralizing anti-PAI-1 (0.5 μ g/ml) was added. After 48 h (5 dpi), cell lysates were collected for analysis. (A) mRNA was extracted using TRIzol, and mRNA expression was analyzed by qRT-PCR. HPRT was used as an endogenous control for normalization in cells. (B) Protein levels of pAKT (Ser473), AKT, cyclin D1, CD133, and EpCAM in cell lysates was analyzed by Western blotting. Relative protein expression was quantified by ImageJ software with reference to internal standards as follows: CD133/GAPDH, EpCAM/GAPDH, cyclin D1/GAPDH, and pAKT/AKT. (C) Huh 7.5.1 cells were infected with HCV at 0.5 MOI. At 1 day postinfection, Huh 7.5.1 cells were transfected with AllStars negative control siRNA (5 nM) (Qiagen) or PAI-1 siRNA (20pM) (Sigma) using Lipofectamine 2000 transfection reagent (Thermo Fisher Scientific), following the manufacturer's protocol. At 5 dpi, cell lysates were collected for analysis. Data are expressed as mean $\pm$ SD ($n=3$). *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$ were considered statistically significant, as assessed using an unpaired Student's t test.

FIG 6 Inhibition of PAI-1 activity results in decreasing cell proliferative responses. Huh7.5.1 cells were infected with HCV at 0.5 MOI. At 3 dpi, HCV-infected Huh7.5.1 cells were transfected with miR-30c mimic (5 nM) or neutralizing anti-PAI-1 (0.5 μ g/ml). After 48 h (5 dpi), cell lysates were collected for analysis. (A) Ki67 protein was measured from cell lysates. Cells were stained with Ki67-Alexa-fluor 488 and analyzed by flow cytometry. (B) Confocal microscopic images of HCV core (red) and Ki67 (green) expression in Huh7.5.1 cells at 5 dpi. Nuclei were stained with DAPI. Scale bar, 20 μ m. The number of green foci (Ki67 positive) per blue nucleus was calculated by the ImageJ software. Data are expressed as mean $\pm$ SD ($n=3$). *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$ were considered statistically significant, as assessed using an unpaired Student's t test.

induces the proliferation of hepatocytes by 5 dpi (Fig. 1, Fig. 6). These results provide evidence for the role of PAI-1 in the differentiation of hepatocytes to the CSC phenotype during HCV infection.

Our studies identifying the role of PAI-1 in hepatocyte differentiation are limited to using HCV-infected Huh7.5.1 cells. However, the human hepatoma-derived Huh7 cell line and its derivatives (Huh7.5 and Huh7.5.1) have been widely used as an experimental model for isolated hepatocytes. In particular, Huh7.5.1 cells are well known host cells suitable for propagating HCV *in vitro*, and, since they have a higher density of viral receptors, provide efficient cell adhesion enabling higher virus infection than Huh7.5 cells (44, 45). Previous studies by other labs have shown increased proliferation rate and viral replication up to 5 days after HCV infection in Huh7.5.1 cells (46), and the HCV core promotes cell proliferation in Huh7 cells (47). These results are consistent with our results showing increased HCV replication and cell viability by day 5 after HCV infection (Fig. 1B). These studies clearly

(A)

HCV infection in Humanized liver mice

(B)

(C)

FIG 7 Increased PAI-1 expression in HCV-infected humanized-liver mice. (A) Humanized-liver mice were injected into the tail vein with 1×10^4 and 1×10^5 FFU/100 μ l of JFH-1 (genotype 2a) Huh7.5.1 cell culture supernatants diluted in PBS. Liver tissues were harvested at 7 dpi and hepatocytes were isolated. (B and C) Isolated hepatocytes were used for protein and mRNA extraction. mRNA was extracted using TRIzol and mRNA expression was analyzed by qRT-PCR. Protein levels of PAI-1, HCV core, CD133, and EpCAM were analyzed by Western blotting. There were three mice per group. Data are expressed as mean $\pm$ SD ($n=3$). *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$ were considered statistically significant, as assessed using an unpaired Student's t test.

FIG 8 Schematic diagram showing the possible role of plasminogen activator-1 in differentiation of cancer stem-like cell state by hepatitis C virus infection in hepatocytes.

show changes in hepatoma cells during HCV infection. It indicates that the pathological condition induced by HCV infection affects changes in the characteristics of Huh7.5.1 cells during cellular survival.

Recent studies define CSC as specific cell populations that play a key role in tumor growth (9). CSCs are known to promote cell proliferation, self-renewability, and are responsible for tumor initiation and metastasis. In particular, the regenerative capacity of hepatocytes provides clues to the origin of HCC, supporting the role of hepatocytes as the origin of CSC (38). There are reports of increased CSCs in Huh7 cells transduced with HCV core or nonstructural protein NS5A (7, 8, 48), and several studies for the CSC differentiation and characteristics in liver disease are currently ongoing. In particular, human differentiated protein 133 (CD133), EpCAM, CD44, and CD90 are known as valid markers of a CSC state in chronic liver disease, including HCC (49, 50). CD133 is a glycoprotein with five transmembrane single chain domains and is well known for its role as a CSC marker in various tumor tissues, including liver cancer (51). CD133⁺ liver cancer cells show more powerful capacity for proliferation and self-renewal than CD133⁻ liver cancer cells. In addition, higher levels of pAKT were detectable in CD133⁺ cancer cells than in CD133⁻ cancer cells (52–54). However, the mechanism for a CSC-related AKT pathway is not yet clear. One possibility is that the PI3K/AKT pathway can be directly activated through the interaction between Src (proto-oncogene tyrosine-protein kinase) of CD133 and P85 (55, 56). Moreover, epithelial cell adhesion molecule (EpCAM), a single transmembrane glycoprotein, is another representative marker of the CSC state in liver. EpCAM is also known as one of the cancer markers, due to its overexpression in various tumor types (57, 58). EpCAM induces cell plasticity within epithelial tissues by weakening cadherin-mediated cell-to-cell adhesion, which can promote cell proliferation and motility during tumor progression (59). Moreover, excessive EpCAM expression induces cell proliferation as well as c-MYC, which is known as a carcinogenic transcription factor. The cell cycle protein cyclin D1 and pluripotency markers, including octamer 4 (Oct4) and Nanog are also positively correlated with EpCAM (60, 61). Notably, EpCAM⁺ cells isolated from HCC patients exhibited characteristics of liver cancer stem-like cells, such as differentiation and self-renewal (49, 62).

In addition, AKT signaling pathways are well known to correlate with the survival and proliferation of tumor cells in various cancers, including HCC. Activated AKT induces cell survival proteins containing pluripotent cell markers such as cyclin D1, MYC, and Oct4 (63–66). In this study, we confirmed that HCV infection not only induces the expression of pAKT(Ser473) and PAI-1, but also increases the representative hepatic CSC markers, including CD133, EpCAM, cyclin D1, and MYC. Further, inhibition of viral RNA replication by the NS5B polymerase inhibitor sofosbuvir resulted in reduced expression of PAI-1 and CSC markers in HCV-infected Huh7.5.1 cells (Fig. 3C). The

inhibition of pAKT(Ser473) in HCV-infected Huh7.5.1 cells did not affect PAI-1 expression but resulted in significantly reduced levels of CSC markers (Fig. 3F). Importantly, PAI-1 inhibition by miR-30c mimic or anti-PAI-1/siPAI-1 resulted in reduced pAKT (Ser473) as well as CSC and EMT markers in the HCV-infected Huh 7.5.1 cells. Taken together, these results suggest that increased PAI-1 by HCV infection affects progression to the CSC state through AKT activation in hepatocytes (Fig. 8).

Ki67 staining is strongly related to tumor cell growth. Previous studies show that inhibiting PAI-1 leads to a decrease in the Ki67-positive cell population (67). Ki67 is required for maintaining the CSC state in certain human cancer cell lines (68). As mentioned earlier, extracellular PAI-1 induces ECM protein accumulation, whereas intracellular PAI-1 can regulate cell adhesion/migration and induces tumor proliferation by activating intracellular signaling. Our study reveals that the suppression of PAI-1 activity by miR-30c mimic or anti-PAI-1 significantly lowers Ki67-positive cell populations in HCV-infected Huh 7.5.1 cells. These results support our hypothesis that upregulation of PAI-1 expression induced by HCV plays a key role in the progression to the CSC state, and regulating PAI-1 may delay the progression of liver disease through inhibition of the CSC differentiation state.

HCV studies using animals are subject to ethical, economic, and time constraints due to their limited host range natures. In this study, we used a humanized-liver mouse model suitable for HCV study. These mice are FGRN immunodeficient mice with human hepatocyte transplantation. We found that HCV infection induces expression of PAI-1 while miR-30c decreased in the HCV-infected group compared with the PBS-injected group. However, there was no significant change in the CSC markers in humanized-liver mice hepatocytes at 7 days after infection (Fig. 7). This might be due to a limitation in evaluating the CSC state and markers of chronic liver disease progression after HCV infection in this short time period. Previous studies have identified significant increases in fibrotic regions and HCV viral load in the liver at 8 to 10 weeks postinfection in humanized-liver mouse models (69, 70). Our present study shows overexpression of PAI-1 during the early phase of HCV infection, which may further stimulate CSC state in hepatocytes at a later time of infection. Therefore, further studies are required to evaluate the effects of PAI-1 and miR-30c on the CSC state during the progression to chronic liver disease in HCV-infected humanized-liver mice.

Depending on the pathological condition, miRNAs can play a unique role and serve as potential biomarkers for the diagnosis and prognosis of disease. miRNAs play a specific role in liver diseases, including fibrosis and HCC (71). Circulating miR-125b, miR-199, miR-221, and miR-122 are known as markers for early and advanced stage diagnosis, prognosis, and therapy of HCC patients (72). Despite numerous studies on the role of miRNA in liver disease, little is understood regarding the role of miR-30c and their mechanisms in liver disease progression. The miR-30 family has been shown to be involved in the pathogenesis of various diseases, such as cardiovascular disease, breast cancer, fibrosis, and liver disease (13, 17, 71, 72). PAI-1 is one of the target proteins regulated by miR-30c. McCann et al. demonstrated that miR-30c suppresses tumor growth via inhibition of PAI-1 induced by TGF- β in endothelial cells, and Wen et al., suggested that the role of miR-30c is via PAI-1 repression in hepatic stellated cell activation (18, 73). In our present study, we found that HCV infection reduces miR-30c levels, leading to inhibition of the expression of PAI-1 in HCV-infected hepatocytes. Also we determined the effect of miR-30c inhibition on the expression of PAI-1, pAKT, and CSC markers in uninfected cells. There were no significant changes in PAI-1 or CSC marker levels in uninfected Huh7.5.1 cells with inhibitor miR-30c. In addition, there was no significant change in the expression level of mTOR, which is well known to be activated by AKT and is involved in cell survival (data not shown). These results suggest that the pathological condition resulting from HCV infection plays a significant role in the change of miR-30c, PAI-1, and the CSC state in hepatocytes. Therefore, we believe that miR-30c is not only a potential biomarker for the prognosis of chronic liver

disease, but also has potential as a therapeutic target for controlling HCV-induced liver disease progression.

HCV proteins, including structural and nonstructural forms, are known to induce hepatic fibrosis and HCC in human hepatoma cells through various signaling pathways. HCV core, NS3, NS5A, and HCV subgenomic replicon have been reported to stimulate TGF- β 1 pathway activation through reactive oxygen species (ROS) induction (74–76), and DAA combinations targeting nonstructural proteins (NS3, NS5A, and NS5B) have shown remarkable efficacy for cures in patients (77). This suggests that a comprehensive response that occurs after HCV infection, rather than a single protein action on a specific pathway, leads to a change in pathological condition. Our results showed that increased PAI-1 and CSC-related markers after HCV infection were attenuated by NS5B polymerase inhibitor (Fig. 3C). However, this result cannot prove the direct relationship between PAI-1 expression and NS5B action, and further studies will be needed as to whether PAI-1 expression is affected by specific viral factors of HCV.

In summary, we identified the role of PAI-1 during HCV infection in hepatocytes. We found that PAI-1 induces the CSC state and AKT activation in HCV-infected hepatocytes. Furthermore, we confirmed that progression to the CSC state during HCV infection can be regulated through inhibiting PAI-1. In particular, the inhibitory effects of miR-30c on PAI-1 are noteworthy and show potential for new targets in HCV-related disease progression. Future studies are warranted to investigate the role of the PAI-1 and miR-30c signaling axis in regulating hepatic stellate cell activation. These studies would help elucidate the role and associated mechanisms of the various molecules released from HCV-infected hepatocytes during liver disease progression.

MATERIALS AND METHODS

Reagents. Antibodies: HCV core (1:1,000, Invitrogen, MA1-080); PAI-1, CD133, EpCAM, cyclin D1, and Ki67 (1:1,000, Abcam); AKT, pAKT(Ser473), GAPDH (1:1,000, Cell Signaling); and neutralizing anti-PAI-1 human Serpin E1/PAI-1 (0.5 μ g/ml, R&D Systems, MAB1786). Inhibitors: sofosbuvir, NS5B polymerase inhibitor (250 nM, Med Chem Express, PSI-7977), AKT 1/2 inhibitor (Sigma, A6730), PAI-1 siRNA (20 pM, Sigma, EHU016891), AllStars negative control siRNA (5 nM, Qiagen), miScript miRNA inhibitor, anti-hsa-miR-30c-5p (human) (50 nM, Qiagen, MIN0000244), miScript miRNA mimic, Syn-hsa-miR-30c-5p (human) (5 nM, Qiagen, MSY0000244).

Cell culture and preparations of HCV virus stock. The human hepatoma cell line Huh7.5.1 was obtained from the American Type Culture Collection (ATCC). Huh7.5.1 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 100 mg/liter penicillin-streptomycin, and 2 mmol/liter glutamine. Cells were maintained at 37°C under a humidified atmosphere of 5% CO₂. Huh7.5.1 cells were infected with HCV (JFH-1 strain, genotype 2a) at a multiplicity of infection (MOI) of 0.5. After 5 days of infection, the supernatant was collected and centrifuged at 1,500 rpm for 5 min to pellet dead cells and debris. Finally, the supernatant was collected and frozen at –80°C in cryotubes at 1 ml/vial.

Ethics statement. All animal experiments were conducted following NIH guidelines for housing and care of laboratory animals and in accordance with protocols approved by The University of Virginia's Institutional Animal Care and Use Committee, protocol number 2720.

Mice and HCV inoculation. Human hepatocytes repopulated Fah^(–/–) Rag1^(–/–) IL2rgnull on NOD (FRGN) mice were provided by Yecuris (Tualatin, OR, USA). Male, 4 to 5 month-old male humanized-liver mice were group housed in cages of up to 3 and maintained under a 12 h light/dark cycle with *ad libitum* access to food and water. The mice were initially maintained on 2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexedione (NTBC) for 5 days, then removed from NBTC during the infection experiment (7 days) according to the Yecuris protocol. FRGN mice were injected into the tail vein with 1×10^4 and 1×10^5 FFU/100 μ l of JFH-1 (genotype 2a) Huh7.5.1 cell culture supernatants diluted in PBS. Mice liver tissues were harvested on 7 days after virus inoculation.

Isolation of humanized-liver mice hepatocytes. Harvested liver tissue was collected on ice and washed with Hank's balanced salt solution (HBSS) to remove blood. Liver tissue was minced with scalpels or scissors until it was a slurry form (<3 mm). Minced liver tissue was transferred to 50-ml tubes containing prewarmed EGTA buffer (HBSS, 0.5 mM EGTA, 0.5% bovine serum albumin [BSA]) and agitated (100 rpm) in a water bath with shaking for 10 min at 37°C. Tissue was then washed 3 times in HBSS and placed in prewarmed digestion buffer (HBSS, 0.05% collagenase IV, 0.5% BSA, 10 mM CaCl₂) and agitated (100 rpm) in a water bath with shaking for 30 min at 37°C. Digested tissue was passed through a metal strainer and the supernatant was collected and filtered through a 100- μ m cell strainer. Cell suspensions were pooled to 50-ml tubes and centrifuged ($80 \times g$ for 5 min at 4°C) and supernatant discarded. After suspending in culture medium (Williams E with supplements), cells were counted and viability was assessed using trypan blue. For purification of hepatocytes, 5 ml of cell suspension was gently

mounted over a cushion of 15 ml percoll suspension and centrifuged ($1,470 \times g$ for 20 min at 4°C). After aspiration of the supernatant, cell pellets were used for further analysis.

siRNA transfection. Huh 7.5.1 cells were seeded at a density of 2×10^5 cells per well in 6-well culture plates. At 1 day postinfection, Huh 7.5.1 cells were transfected with AllStars negative control siRNA (5 nM) (Qiagen) or PAI-1 siRNA (20pM) (Sigma) using Lipofectamine 2000 transfection reagent (Thermo Fisher Scientific), following the manufacturer's protocol, and cell lysates were used for analysis at 5 days postinfection.

RNA extraction and quantitative real-time reverse transcription PCR (qRT-PCR) analysis. Huh7.5.1 cells were seeded at a density of 5×10^5 on 6-well plates and stabilized for up to 16 h. Then, Huh7.5.1 cells were infected with HCV (strain JFH-1) at 0.5 MOI. After 3 days, HCV-infected Huh7.5.1 cells were transfected with miR-30c mimic (5 nM) or miR-30c inhibitor (50 nM) (Qiagen) or added neutralizing anti-PAI-1 (0.5 $\mu\text{g}/\text{ml}$) (R&D system). After 48 h, cell lysates were used for analysis. HCV-infected Huh7.5.1 cells were treated with NS5B polymerase inhibitor sofosbuvir (250 nM, Med Chem Express) for 3 days. Huh7.5.1 cells and isolated hepatocytes were lysed in the presence of TRIzol. Total RNA, including miRNAs, was extracted from the tissues or isolated hepatocytes using a miRNeasy minikit, according to the manufacturer's protocols (Qiagen). RNA concentration and quality were determined using a Nanodrop 2000 (Thermo Fisher). Only samples showing a 260/280 nm ratio between 1.8 and 2.1 were selected for cDNA transcription. A total of 1 μg of RNA, or 10 ng of RNA for the miRNA assay, was used for cDNA synthesis. Complementary DNA (cDNA) was then synthesized using High-Capacity cDNA reverse transcription kits (Thermo Fisher Scientific) for the mRNA assay or TaqMan MicroRNA reverse transcription kit and TaqMan microRNA assay (Thermo Fisher Scientific) for the miRNA assay. Real-time PCR (Applied Biosystems) was performed on triplicate samples using 1 μl of cDNA with Fast SYBR Green Master Mix (Thermo Fisher Scientific) for mRNA assay or 1.33 μl with the TaqMan Universal PCR Master Mix, no AmpErase UNG (Thermo Fisher Scientific). The cDNA for mRNA assays was amplified for 40 cycles of denaturation (95°C for 3 s) and annealing and extension (60°C for 30 s) using the following primers: HPRT (fwd 5'-CCT GGC GTC GTG ATT AGT GA-3', rev 5'-CGA GCA AGA T CAG TCC T-3'); PAI-1 (fwd 5'-GCA ACG T TTT TCT CAC CC-3', rev 5'-CCG GAA CAG CCT GAA GAA GT-3'); HCV (JFH-1) (fwd 5'-CGT AGA CCG TGC ACC ATG AG-3', rev 5'-ATA CTC CGC CAA CGA TCT GG-3'); cyclin D1 (fwd 5'-CAA TGA CCC CGC ACG ATT TC-3', rev 5'-CAA TGA CCC CGC ACG ATT TC-3'); MYC (fwd 5'-CAT CAG CAC AAC TAC GCA GC-3', rev 5'-ACT CAG CCA AGG TTG TGA GG-3'); OCT4 (fwd 5'-GGC CAC ACG TAG GTT CTT GA-3', rev 5'-ATA CTT TCC CAA ATA GAA CCC C-3'); Nanog (fwd 5'-GGA GCC TAA TCA GCG AGG TT-3', rev 5'-GCC AGA GAC GGC TTC TAT CA-3'); mTOR (fwd 5'-CTT AGA GGA CAG CGG GGA AG-3', rev 5'-TCC AAG CAT CTT GCC CTG AG-3'); miR-30c TaqMan microRNA assays hsa-miR-30c (000419). The cDNA for miRNA assays was amplified for 40 cycles of denaturation (95°C for 15 s) and annealing and extension (60°C for 60 s) using a specific TaqMan microRNA assay (Thermo Fisher Scientific). The reaction was performed using StepOne real-time PCR systems (Applied Biosystems). Hypoxanthine phosphoribosyltransferase (HPRT) was used as an endogenous control for normalization in Huh7.5.1 cells and primary hepatocytes.

Western blotting. Huh7.5.1 cells were seeded at a density of 5×10^5 on 6-well plates and stabilized for up to 16 h. Then, Huh7.5.1 cells were infected with HCV (strain JFH-1) at 0.5 MOI. After 3 days, HCV-infected Huh7.5.1 cells were transfected with miR-30c mimic (5 nM) (Qiagen) or added neutralizing anti-PAI-1 (0.5 $\mu\text{g}/\text{ml}$). After 48 h, cell lysates were used for analysis. Huh7.5.1 cells and isolated hepatocytes were lysed in a radioimmunoprecipitation assay (RIPA) lysis buffer system (Santa Cruz Biotechnology), and centrifuged at $12,000 \times g$ for 20 min at 4°C . The protein content of the clear lysates was estimated using a Pierce BCA protein assay kit according to the manufacturer's protocols (Thermo Fisher Scientific). Total protein (60 μg) was dissolved in NuPAGE LDS sample buffer (4 $\times$; Life Technologies). Protein samples were separated on a 10% SDS-polyacrylamide gel and then transferred to a nitrocellulose membrane (Bio-Rad Laboratories). Membranes were incubated for 1 h in a blocking solution containing 5% nonfat milk in Tris-buffered saline, and then incubated for 12 h at 4°C with antibodies recognizing PAI-1, CD133, EpCAM, Cyclin D1 (Abcam), pAKT(Ser473), AKT, and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) (Cell Signaling Technology). After incubation with the primary antibody, the membranes were incubated with a secondary antibody (anti-rabbit IgG horseradish peroxidase [HRP]-linked antibody, 1:5,000) (Cell Signaling Technology, Inc.) for 1 h at room temperature. The immunoreactive protein bands were then developed and protein expression was quantified by ImageJ software.

Immunofluorescence microscopy. Huh7.5.1 cells were seeded at a density of 1×10^5 on 2-well glass chamber slides (Nunc Lab-Tek) and stabilized for up to 16 h. Then, Huh7.5.1 cells were infected with HCV (strain JFH-1) at 0.5 MOI and, at 3 dpi, Huh7.5.1 cells were transfected with miR-30c mimic (5 nM) or added neutralizing anti-PAI-1 (0.5 $\mu\text{g}/\text{ml}$). After 48 h, cells were fixed using 4% paraformaldehyde in PBS for 15 min, and then washed. Cells were blocked and permeabilized using 3% bovine serum albumin (BSA), 10% FBS, and 0.3% Triton X-100 in PBS overnight. Then, cells were incubated for 12 h at 4°C with primary antibodies recognizing PAI-1, Ki67 (Abcam), and HCV (Invitrogen) monoclonal antibody. After incubation with the primary antibody, cells were incubated with a secondary antibody (Alexa Fluor 488, and Alexa Fluor 555, Invitrogen) at a final dilution of 1:1,000 for 1 h at room temperature. The cells were then washed using PBS and overlaid with 4',6-diamidino-2-phenylindole (DAPI) (Sigma). Images were captured using a LSM700 (Carl Zeiss, Oberkochen, Germany) confocal microscope.

Flow cytometry analysis. Huh7.5.1 cells were seeded at a density of 5×10^5 on 6-well plates and stabilized for up to 16 h. Then, Huh7.5.1 cells were infected with HCV (strain JFH-1) at 0.5 MOI. At 3 dpi, HCV-infected Huh7.5.1 cells were transfected with miR-30c mimic (5 nM) or added neutralizing anti-PAI-1 (0.5 $\mu\text{g}/\text{ml}$). After 48 h, cell lysates were fixed using 4% paraformaldehyde in PBS for 5 min, and then permeabilized using 0.3% Triton X-100 in PBS for 15 min. The cell lysates were washed and centrifuged

with PBS three times and then stained with Ki67-Alexa-fluor 488 (Abcam), and then washed and measured using a flow cytometer (Attune NxT Flow Cytometer, Life Technologies).

MTT assay. In a 96-well plate, 5,000 Huh 7.5.1 cells were seeded in 100 μ l of complete medium per well. The next day, cells were infected with HCV (strain JFH-1) at 0.5 MOI and cells were incubated at 37°C, and cell survival rate was measured every day for 6 days. MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) was prepared at 5 mg/ml in PBS and 10 μ l of MTT solution was added to cells, which were then incubated for 4 h at 37°C. Following incubation, the supernatant was carefully removed from each well. Then, 100 μ l of dimethyl sulfoxide (DMSO) was added to each well and the plate was lightly shaken for 20 min at 37°C. Cell viability was measured using a microplate reader (Bio-Tek) at 540 nm.

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