Abstract

The proteasomal activator 28γ (PA28γ), frequently overexpressed in hepatocellular carcinoma, is believed to play several important roles in hepatitis C virus (HCV) replication and viral pathogenesis. However, the underlying mechanism for PA28γ overexpression in hepatocellular carcinoma and its role during HCV replication are still unclear. In the present study, we found that HCV core derived from either ectopic expression or HCV infection upregulates PA28γ levels in p53-positive human hepatocytes. For this effect, HCV core sequentially activated ataxia telangiectasia mutated and checkpoint kinase 2 via phosphorylation at Ser-1981 and Thr-68 residues, respectively, resulting in stabilization of p53 via phosphorylation at Ser-15 and Ser-20 residues and subsequent transcriptional activation of PA28γ expression. The elevated PA28γ in turn downregulated HCV core levels by either inducing its ubiquitination-dependent proteasomal degradation via upregulation of E6AP levels in the presence of p53 or activating an ubiquitin-independent proteasomal degradation pathway in the absence of p53, which ultimately led to a decrease in HCV propagation. HCV core modulates its own protein level via a negative feedback loop involving p53 and PA28γ to control HCV replication in p53-positive hepatocytes, which may help HCV evade immune responses and establish chronic infection.

INTRODUCTION

Hepatitis C virus (HCV) is a major cause of acute and chronic hepatitis, the latter of which frequently leads to liver cirrhosis and hepatocellular carcinoma (HCC) [1]. As a member of the family Flaviviridae, it contains a positive-stranded RNA genome of 9.5 kb, encoding a polyprotein processed into four structural proteins and six nonstructural proteins by cellular and viral proteases [2]. In addition to its role as a major component of the viral nucleocapsid, HCV core has been strongly implicated in viral pathogenesis and HCC development because it alters diverse signalling pathways, transcriptional activation, modulation of immune responses, apoptosis and lipid metabolism [3, 4]. Despite our increasing knowledge about HCV core in HCV pathogenesis, its role as a regulator of HCV replication is poorly understood.

Proteasomes are protein complexes involved in the degradation of unneeded or damaged proteins by proteolysis. Two distinct proteasomes differentially target proteins for degradation. The 26S proteasome, responsible for degradation of the majority of proteins through an ubiquitin (Ub)-dependent and ATP-dependent pathway, is formed by association of the 20S catalytic core, composed of α and β subunits, with the 19S regulator [5, 6]. Alternatively, the 20S proteasome which is responsible for Ub-independent and ATP-independent degradation of specific target substrates is generated by a combination of one 20S catalytic core and one proteasomal activator 28 (PA28) member [7, 8]. Of the three PA28 family members, PA28γ (also called REGγ, 11γ, PSME3 or Ki antigen) is implicated in tumorigenesis, as it regulates cell proliferation and apoptosis and predominantly exerts its function through nuclear proteolysis [9]. For example, PA28γ contributes to p53 turnover by facilitating interactions between MDM2 and p53, thereby inhibiting apoptosis as a result of DNA damage by limiting p53 accumulation [10, 11]. PA28γ also promotes degradation of several cell cycle regulatory proteins, including p14, p16 and p21 [12, 13]. In addition, PA28γ is frequently overexpressed...
in various human cancers, including HCC [14]. Moreover, PA28γ knock-out impairs development of both hepatic steatosis and HCC in HCV core transgenic mice [15], indicating that PA28γ plays a crucial role in the development of liver diseases induced by HCV infection.

Recent reports also have implicated PA28γ in the regulation of HCV propagation, although its exact role is controversial. According to initial studies, both the E6AP-mediated Ub-dependent pathway and the PA28γ-mediated Ub-independent pathway induce proteasomal degradation of HCV core [6, 16, 17]. Accordingly, knock-down of either E6AP or PA28γ results in stabilization of HCV core, indicating their roles as negative regulators of HCV replication. However, a completely opposite role of PA28γ also has been demonstrated. Morishii et al. [18] have shown that PA28γ knock-down in cells infected with the HCV JFH1 strain downregulates HCV core levels by enhancing its Ub-dependent proteasomal degradation and thereby impairs HCV replication. Based on this observation, they proposed that PA28γ positively regulates HCV replication by interfering with the E6AP-mediated Ub-dependent proteasomal degradation of HCV core. The exact role of PA28γ in the regulation of HCV core turnover and HCV propagation needs to be clarified.

We and others have reported that HCV core activates p53 in human hepatocytes [19, 20], although the underlying mechanism is still unclear. In addition, p53 is known to activate PA28γ transcription via p53 response elements (p53REs) located within its promoter [21]. Therefore, it is very challenging to investigate whether HCV core activates PA28γ expression by upregulating p53 levels to control its own protein level via a negative feedback loop. In the present study, we first examined whether HCV core activates PA28γ expression in a p53-dependent manner in both ectopic expression and HCV infection systems and then investigated the underlying mechanism involved in this process. Second, we investigated the mechanism by which HCV core activates p53 in human hepatocytes, focusing on its effects on two major pathways of p53 activation. Third, we investigated whether the activated PA28γ affects proteasomal degradation of HCV core to modulate its protein level. Finally, we attempted to elucidate a possible role of this regulatory circuit in HCV propagation.

RESULTS

HCV core upregulates PA28γ levels via activation of p53 in human hepatocytes

Initially, we investigated whether HCV core affects PA28γ expression in human hepatocytes. Either transient or stable expression of HCV core upregulated p53 and PA28γ levels in HepG2 and Huh7.5 cells (Fig. 1a). The effects of HCV core on p53 and PA28γ were also observed in HCV-infected Huh7.5 cells (Fig. 1b). In addition, according to the immunofluorescence assay (IFA), the stronger PA28γ signal was detected in the nucleus of the Huh7.5 cells expressing HCV core (Fig. 1c). In contrast, these effects were not observed in Hep3B cells, in which p53 was absent (Fig. 1a), indicating that p53 is required for the upregulation of PA28γ levels by HCV core.

To prove that HCV core upregulates PA28γ levels via activation of p53, we attempted to knock-down p53 in HCV-core-expressing HepG2 cells using a specific small hairpin RNA (shRNA). Indeed, p53 knock-down almost completely abolished the potential of HCV core to upregulate PA28γ levels in HepG2 cells (Fig. 1d). In addition, ectopic p53 expression in HepG2 and Hep3B cells upregulated PA28γ levels in the absence of HCV core (Fig. 1e, lanes 2 and 5), indicating that p53 upregulation itself is sufficient for the activation of PA28γ expression by HCV core. Moreover, HCV core also upregulated levels of ectopically expressed p53 in Hep3B cells, which led to further upregulation of PA28γ levels (Fig. 1e, lane 6). Taken together, we conclude that HCV core upregulates PA28γ levels via activation of p53 in human hepatocytes.

HCV core upregulates p53 levels via activation of the ataxia telangiectasia mutated (ATM)–checkpoint kinase 2 (CHK2) pathway

In general, the protein level of p53 is negatively regulated by an E3 Ub ligase, MDM2, which induces ubiquitination and subsequent proteasome degradation of p53 [22]. The protein level of MDM2 is also known to be regulated by Ub-dependent proteasomal degradation pathways [22, 23]. To investigate whether HCV core differentially affects Ub-dependent proteasomal degradation of MDM2 and p53, we introduced haemagglutinin (HA)-tagged Ub into HepG2 cells with or without HCV core expression and immunoprecipitated Ub-complexed products. As a result, more ubiquitinated forms of MDM2 were detected in HepG2-core cells, whereas p53 ubiquitination was impaired in these cells (Fig. 2a). These results suggest that HCV core upregulates p53 levels but downregulates MDM2 levels by oppositely affecting their Ub-dependent proteasomal degradation.

Next, we investigated the mechanism by which HCV core upregulates p53 levels. The p53 protein is usually accumulated in response to cellular stresses such as hyperproliferation and DNA damage, which lead to activation of p14 and ATM, respectively [24, 25]. Therefore, we first examined whether HCV core activates the p14–MDM2 pathway to upregulate p53 levels. Consistent to a previous report [20], HCV core induced promoter hypermethylation of p14 to downregulate its protein levels (Fig. 2b, c). These effects were almost completely abolished by treatment with a universal DNA methyltransferase inhibitor, 5-aza-2′-deoxycytidine (5-aza-2′dC) and ectopic p14 expression via DNA methylation. In spite of p14 downregulation, MDM2 levels were lower, and p53 levels were higher in HepG2-core cells compared to those in control cells (Fig. 2c). In addition, the potential of HCV core to modulate MDM2 and p53 levels was augmented by treatment with 5-aza-2′dC and ectopic p14 expression, which restored p14 levels in HepG2-core cells to those in control cells (Fig. 2c, d). These results suggest that the p14–MDM2 pathway is
The DNA damage sensors ATM and CHK2 (a direct downstream target of ATM) play central roles in response to DNA damage [25, 26]. In addition, HCV core has been suggested to be a potent reactive oxygen species (ROS) inducer, leading to DNA damage [27]. Therefore, we investigated whether HCV core upregulates p53 levels via activation of the ATM–CHK2 pathway. Indeed, HCV core induced phosphorylation of ATM at Ser-1981 residue without affecting its total protein levels, which led to phosphorylation of p53 at Ser-20 residue in HepG2-core cells. Phosphorylation of p53 at Ser-15 and Ser-20 residues is closely related to increased protein stability [25], as demonstrated by the higher p53 level in HepG2-core cells (Fig. 2e). The potential of HCV core to induce phosphorylation of CHK2 and p53 was almost completely abolished by treatment with an ATM inhibitor, KU-55933, which led to downregulation of p53 levels in HepG2-core cells. Phosphorylation of p53 at Ser-15 and Ser-20 residues is also believed to affect the interaction between p53 and MDM2 for p53 degradation [25]. Therefore, we investigated whether HCV core interferes with the interactions between MDM2 and p53.

![Diagram](image-url)

**Fig. 1.** HCV core upregulates PA28γ levels via activation of p53 in human hepatocytes. (a) Levels of the indicated proteins in HepG2-vector and HepG2-core were compared by Western blotting. HepG2, Huh7.5 and Hep3B cells were transiently transfected with an increasing concentration of HCV core expression plasmid. (b) Huh7.5 cells were either mock infected or infected with HCV at an m.o.i. of 1.0 for 24 h, followed by Western blotting. (c) Huh7.5 cells grown on coverslips were infected with HCV as in (b) and processed for double-label indirect IFA for HCV core and PA28γ. Immunofluorescence images show the intracellular localization of HCV core (green) and PA28γ (red). The nuclei stained with DAPI are shown in blue. (d) HepG2 cells were transiently transfected with either an empty vector or HCV core expression plasmid along with an increasing concentration of p53 shRNA plasmid for 48 h, followed by Western blotting. (e) HepG2 and Hep3B cells were transiently transfected with the indicated amount of HCV core expression plasmid with or without p53 expression plasmid for 48 h, followed by Western blotting.
According to a mammalian two-hybrid assay, the G5E1b-luc reporter activity derived from interactions between G4-MDM2 and p53-VP16 was significantly lower in the presence of HCV core (Fig. 2f). This effect was completely abolished by treatment with KU-55933, indicating that phosphorylation of p53 by the activated ATM and CHK2 interferes with the interactions between p53 and MDM2, contributing to the HCV-core-mediated p53 stabilization. Taken together, we conclude that HCV core upregulates p53 levels via activation of the ATM–CHK2 pathway.

**HCV core transcriptionally activates PA28γ expression via upregulation of p53 levels**

Next, we investigated the mechanism by which HCV core upregulates PA28γ protein levels. HCV core dose-dependently upregulated PA28γ RNA levels in HepG2 but not in Hep3B cells (Fig. 3a), suggesting that HCV core transcriptionally activates PA28γ expression via upregulation of p53 levels. The PA28γ promoter contains three p53REs (Fig. 3b), which are essential for the p53-mediated activation of PA28γ transcription [21]. Consistently, the PA28γ
promoter activity was dose-dependently elevated by HCV core in HepG2 cells but not in Hep3B cells (Fig. 3c, d). In addition, the reporter activity from the PA28γmp53RE-luc, in which p53REs were destroyed by point mutations (Fig. 3b), was minimally affected by HCV core (Fig. 3c). The potential of HCV core to upregulate PA28γ promoter activity was almost completely abolished by p53 knockdown in HepG2 cells, whereas ectopic expression of p53 restored the potential of HCV core to upregulate PA28γ promoter activity in Hep3B cells (Fig. 3d). Moreover, according to the chromatin immunoprecipitation (ChIP) assay, the binding of p53 to the PA28γ promoter was enhanced by HCV core in a dose-dependent manner (Fig. 3e). These results suggest that HCV core transcriptionally activates PA28γ expression via upregulation of p53 levels.

**PA28γ induces Ub-independent proteasomal degradation of HCV core**

Previous reports have demonstrated that PA28γ downregulates HCV core via an Ub-independent pathway [6, 16, 17]. Consistently, HCV core levels were downregulated when PA28γ levels were upregulated as a result of p53 inactivation (Figs 1d and 2e). In contrast, HCV core levels were upregulated when PA28γ levels were upregulated as a result of p53 activation (Figs 1e and 2c, d). To confirm the inverse correlation between PA28γ and HCV core, we compared their expression levels in HepG2 and Hep3B cells. For this purpose, HepG2 and Hep3B cells were transiently transfected with an equal amount of HCV core expression plasmid along with β-galactosidase (β-Gal) expression vector. The basal level of PA28γ was slightly higher in HepG2 cells (Fig. 4a), probably due to the action of p53. As

![Graph showing the relative luciferase activity with varying concentrations of Core and p53 shRNA](image-url)

**Fig. 3.** HCV core transcriptionally activates PA28γ expression by elevating p53 levels. (a) HepG2 and Hep3B cells transfected with an increasing concentration of HCV core expression plasmid for 48 h, followed by reverse transcription PCR to compare RNA levels of PA28γ, HCV core and GAPDH. (b) Schematic diagram of the PA28γ promoter containing three p53REs [21]. The sequences of wild-type p53REs and corresponding mutants are shown below. The consensus p53 binding sequences underlined in PA28γ-luc were point mutated in PA28γmp53RE-luc as shown in lower case letters. (c) HepG2 cells were transfected with either PA28γ-luc or PA28γmp53RE-luc along with an increasing concentration of HCV core expression plasmid for 48 h, followed by luciferase assay (n=4). (d) Either HepG2 or Hep3B cells were cotransfected with PA28γ-luc and the indicated amount of HCV core expression plasmid, p53 expression plasmid and p53 shRNA plasmid for 48 h, followed by luciferase assay (n=4). (e) ChIP assay was performed to determine levels of p53 bound to p53REs on the PA28γ promoter in HepG2 cells prepared as in (a). Positions of ChIP primers are shown as arrows in (b).
expected, HCV core upregulated both p53 and PA28γ levels in HepG2 cells but not in Hep3B cells. In addition, HCV core levels were lower in HepG2 cells, definitely not due to weaker transfection efficiency, as indicated by the β-Gal levels but due to higher PA28γ levels in these cells (Fig. 4a). These results suggest that both p53 and PA28γ act as negative regulators of HCV core.

Next, we investigated whether PA28γ induces proteasomal degradation of HCV core in a p53-dependent manner. Treatment with a universal proteasomal inhibitor, MG132, dramatically upregulated HCV core levels in HepG2 cells, resulting in further upregulation of p53 and PA28γ levels, whereas these effects were marginal or undetectable in Hep3B cells (Fig. 4a). As a consequence, HCV core levels in HepG2 and Hep3B cells were getting similar in the presence of MG132. These results indicate that PA28γ efficiently induces proteasomal degradation of HCV core in HepG2 but not in Hep3B cells, possibly due to differences in PA28γ levels between them.

We next investigated whether PA28γ alters ubiquitination of HCV core by affecting E6AP levels, as previously demonstrated [18]. HCV core downregulated E6AP levels in HepG2 cells (Fig. 4b), as demonstrated in a previous report [28], but not in Hep3B cells (Fig. 4c). In addition, the potential of HCV core to downregulate E6AP levels in HepG2 cells was abolished by p53 knock-down, whereas HCV core could downregulate E6AP levels in Hep3B cells expressing ectopic p53 (data not shown). These results suggest that HCV core represses E6AP expression via activation of p53. Consistently, PA28γ knock-down upregulated p53 levels via downregulation of MDM2 levels, as previously demonstrated [11], which led to further downregulation of E6AP levels in HepG2-core cells (Fig. 4b). Accordingly, PA28γ knock-down downregulated the ubiquitinated forms of HCV core (Fig. 4d), resulting in upregulation of intact HCV core in HepG2 cells (Fig. 4b). These results suggest that PA28γ induces Ub-dependent proteasomal degradation of HCV core via activation of E6AP expression in HepG2 cells. On the other hand, PA28γ overexpression did not affect E6AP expression and HCV core ubiquitination in the absence of p53, although it upregulated MDM2 levels in Hep3B cells (Fig. 4c, d). PA28γ overexpression, however, downregulated HCV core levels in Hep3B cells. These results suggest that PA28γ can induce Ub-independent proteasomal degradation of HCV core in Hep3B cells, as previously demonstrated [16].

PA28γ-mediated proteasomal degradation of HCV core restraints virus propagation

Next, we investigated whether HCV core can modulate its own protein levels via activation of PA28γ expression during HCV infection. Infection with the JFH-1 strain of HCV resulted in upregulation of p53 in Huh7.5 cells (Figs 1b and 5a). As a result, the PA28γ promoter activity as well as its RNA and protein levels were upregulated in the infected cells (Fig. 5a, b). These effects were almost completely abolished when p53 was knocked down in the infected cells (Fig. 5c). In addition, PA28γ knock-down upregulated p53 and HCV core levels in the infected cells. Other HCV proteins such as E2 and NS3 were similarly affected under the condition. Moreover, the IFA data clearly showed that PA28γ knock-down upregulated HCV core levels in the infected cells (Fig. 5d). Taken together, we conclude that HCV core activates PA28γ expression in a p53-dependent manner, which in turn lowers its own protein levels during HCV infection.

Finally, we investigated whether the proteasomal degradation of HCV core by PA28γ affects virus propagation. Knock-down of either p53 or PA28γ in the infected cells significantly enhanced HCV replication, as demonstrated by the higher levels of extracellular HCV RNA (Fig. 6a, b). We also attempted to provide evidence that PA28γ knock-down stimulates virus propagation by measuring the number of cells that are infected over time. HCV core was detected by IFA in approximately 12 and 24% of Huh7.5 cells after infection with HCV at an m.o.i. of 0.3 for 24 and 48 h, respectively (Fig. 6c). PA28γ knock-down increased the percentage of HCV-core-positive cells by roughly twofold under the corresponding condition, indicating that the PA28γ-mediated proteasomal degradation of HCV core negatively regulates HCV propagation in human hepatocytes. Taken together, we conclude that HCV core induces its own proteasomal degradation via upregulation of PA28γ expression to negatively regulate HCV replication in p53-positive hepatocytes.

DISCUSSION

HCV core is now considered a virulence factor responsible for HCV-induced metabolic diseases and HCC, since its expression alone can induce insulin resistance [29], liver steatosis [30] and HCC in transgenic mice [31]. Previous reports have demonstrated that PA28γ is closely implicated in the pathologic roles of HCV core. For example, HCV-core-induced insulin resistance results from upregulation of TNF-α [29], which is dependent on the presence of PA28γ [32]. HCV core also activates sterol regulatory element-binding protein-1c expression in a PA28γ-dependent manner, resulting in biosynthesis of triglycerides and subsequent accumulation of lipid droplets [15]. In addition, PA28γ appears to be required for HCV-core-induced overproduction of ROS [15], which can lead to the accumulation of genetic mutations associated with HCC development. Moreover, knock-out of PA28γ in HCV core transgenic mice invariably results in the loss of susceptibility to insulin resistance, liver steatosis and HCC [15, 32]. Therefore, elucidation of the mechanism by which PA28γ is activated in human hepatocytes might be crucial for understanding HCV-core-induced pathogenesis.

PA28γ overexpression is frequently detected in human cancers including HCC [14, 33–35]. According to previous reports, HCV core upregulates both the protein level and transcriptional activity of p53 in human hepatocytes [19, 36]. In addition, p53 binds to the promoter of PA28γ and...
activates expression of the gene [21]. Here, we provide several lines of evidence to suggest that HCV core upregulates PA28γ expression via activation of p53. First, the promoter activity and protein levels of PA28γ were upregulated by HCV core in HepG2 cells but not in p53-negative Hep3B cells (Figs 1a and 3d). Consistent with this finding, the PA28γ promoter with mutations in the p53REs was little affected by HCV core in HepG2 cells (Fig. 3c). In addition, HCV core increased p53 binding to the p53REs of PA28γ promoter in HepG2 cells (Fig. 3e). Accordingly, the potential of HCV core to activate PA28γ expression was almost completely abolished when p53 was knocked-down in HepG2 cells (Figs 1d and 3d). Moreover, HCV core could activate PA28γ expression in Hep3B cells expressing ectopic p53, whose level was also upregulated by HCV core (Figs 1e and 3d). Lastly, both p53 and PA28γ levels were also upregulated in Huh7.5 cells infected with the HCV JFH1 strain (Fig. 5), indicating that PA28γ upregulation is a general effect of HCV core in hepatocytes.

Two distinct proteasome pathways are known to be involved in the degradation of HCV core [5]. First, HCV core is ubiquitinated by an E3 ligase, E6AP, and degraded in an Ub/ATP-dependent pathway [17]. Therefore, knock-down of endogenous E6AP by shRNA increases the stability of HCV core and thereby enhances the production of infectious HCV particles, confirming the role of E6AP as a negative regulator of HCV propagation [17]. Alternatively, HCV core specifically interacts with PA28γ in the nucleus and is degraded through the PA28γ-mediated Ub-independent proteasome pathway [6, 16]. Knock-down of PA28γ also results in stabilization of HCV core [6, 16]. Interestingly, the role of PA28γ as a positive regulator of HCV core has also been suggested. Moriishi et al. [18] argued that PA28γ positively regulates HCV replication by interfering with E6AP-mediated Ub-dependent proteasomal degradation of HCV core. To clarify the discrepancy for the roles of PA28γ in the regulation of HCV core, we first examined whether the activated PA28γ in the presence of the core affects Ub-dependent proteasomal degradation of the
As a result, we found that PA28γ knock-down in HepG2 cells downregulated E6AP levels (Fig. 4b), resulting in a decrease in core ubiquitination (Fig. 4d), indicating that PA28γ induces Ub-dependent proteasomal degradation of HCV core via upregulation of E6AP levels.

However, PA28γ overexpression downregulated HCV core levels without affecting E6AP levels and core ubiquitination in Hep3B cells, indicating that PA28γ also induces Ub-independent proteasomal degradation of the core in the absence of p53. Therefore, it is likely that PA28γ can downregulate HCV core levels via either an Ub-dependent or Ub-independent pathway depending on the status of p53. Several pieces of evidence from the present study do not support the role of PA28γ as a positive regulator of HCV propagation.

First of all, PA28γ knock-down dramatically upregulated HCV core levels in HepG2 cells (Figs 4b and 5c), resulting in upregulation of HCV propagation (Fig. 6b, c). Under this condition, the ubiquitination of HCV core was also slightly impaired as p53 downregulated E6AP levels (Fig. 4b, d). In addition, ectopic expression of PA28γ in Hep3B cells downregulated HCV core levels without affecting E6AP levels and HCV core ubiquitination (Fig. 4c, d). The discrepancy in the regulation of HCV core by PA28γ might be attributable to different experimental conditions. More detailed studies may clarify the opposing roles of PA28γ in the regulation of HCV core turnover and HCV propagation.

Based on the present study and others, we propose a regulatory circuit involving HCV core, p53 and PA28γ (Fig. 7). According to this model, the circuit starts to work as HCV...
core upregulates p53 levels, although the underlying mechanism remains obscure. Several reports have proposed that HCV infection causes DNA breaks, increasing the mutation frequency of cellular genes [27, 37], and HCV core itself is a potent ROS inducer that can cause DNA damage [27], suggesting that HCV core activates p53 via modulation of DNA damage signalling pathways. Consistently, the present study showed that HCV core successively activated ATM and CHK2 via phosphorylation at Ser-1981 and Thr-68 residues, respectively, which led to stabilization of p53 via phosphorylation at Ser-15 and Ser-20 residues (Fig. 2e). The activated p53 protein then stimulates PA28γ expression to elevate its protein levels, which in turn induces proteasomal degradation of HCV core to downregulate core levels. The p53 protein is also subjected to PA28γ-mediated feedback inhibition, as elevated PA28γ induces p53 degradation by facilitating interactions between MDM2 and p53 [10, 11]. Both PA28γ-mediated stabilization and p53-induced transcriptional activation may accumulate MDM2 in HCV-core-expressing cells. In addition, HCV core directly upregulates MDM2 levels by inhibiting p14 expression via DNA methylation [20]. The negative feedback loop thus plays an important role in maintaining balance among HCV core, MDM2, p53 and PA28γ in infected cells.

According to the regulatory circuit shown in Fig. 7, HCV core induces its own degradation by upregulating its negative regulator PA28γ. Considering that HCV core is a major component of capsid assembly, its downregulation may inevitably lead to a decrease in virion production. The biological significance of this negative feedback regulation of
HCV core is unclear. It may allow HCV to evade immune responses by minimizing production of a highly antigenic viral protein and virus particles. It is also possible to speculate that it prevents accumulation of p53 to a level that can cause abortive HCV replication. The regulatory circuit is only effective in p53-positive cells. Accordingly, it is likely that the amount of HCV core is higher in p53-negative hepatocytes that express lower levels of PA28γ, as demonstrated in the present study (Fig. 4a). In addition, p53 knock-down significantly upregulated HCV core levels (Fig. 5a), thereby increasing the HCV propagation rate (Fig. 5b). Therefore, the regulatory circuit may strictly control HCV core and p53 levels via feedback inhibition, allowing HCV to evade host defences such as an immune response and programmed cell death that can lead to abortive infection.

**METHODS**

**Plasmids**

The HCV core expression plasmid, pCMV-3×HA1-core, encodes the full-length HCV core (genotype 1b) downstream of three copies of the influenza virus HA epitope [38]. The PA28γ promoter region from −1039 to +109, which contains three p53REs, was obtained by PCR amplification of genomic DNA from HepG2 cells using the primer set PA28γ−1039F (5′-GAG GAG CTG AGC TCT CTA ATA C-3′) and PA28γ+109R (5′-AGG GAC TCG AGA AAT CTC-3′) and then was subcloned into pGL2-basic (Promega) to generate the reporter plasmid PA28γ-luc. Subsequently, the three p53REs were point mutated from PA28γ-luc by PCR to generate PA28γmp53RE-luc, as previously described [21]. The PA28γ expression plasmid pCMV6 PSME3, encoding full-length human Myc-DDK-tagged PA28γ, was purchased from OriGene. Both p53 shRNA and PA28γ shRNA plasmids were purchased from Santa Cruz Biotechnology. Plasmid pCH110, encoding the *Escherichia coli* β-Gal gene under the control of the SV40 promoter, was obtained from Pharmacia. Plasmid pHA-Ub was kindly provided by Y. Xiong (University of North Carolina, Chapel Hill, NC). Plasmids pCMV p53-WT, G5E1b-luc, pG4-MDM2 and pCMVp53-VP16 were gifts from C. W. Lee (Sungkyunkwan University, Korea).

**Cell culture and luciferase assay**

HepG2 (KCLB no. 80065) and Hep3B (KCLB no. 88064) were purchased from the Korean Cell Line Bank. Huh7.5, an α-interferon-cured Huh7 derivative, is highly permissive for HCV infection [39]. For transient expression, 2×10⁵ cells per 60 mm dish were transfected with 2 μg of the appropriate plasmid(s), using WelFect-EX PLUS (WEGENE) according to the manufacturer’s instructions. Stable cell lines, HepG2-vector and HepG2-core, were established by transfection with pCMV-3×HA1 and pCMV-3×HA1-core, respectively, followed by selection with 500 μg ml⁻¹ G418 (Gibco) [40]. Cells were treated with MG132 (Sigma), if necessary, for 4 h before harvesting. For luciferase assay, 500 ng of a reporter plasmid was cotransfected with either an empty vector or an effector plasmid under the indicated conditions. One hundred nanograms of pCH110 was also included as an internal control. Forty-eight hours later, the luciferase assay was performed using Luciferase Reporter 1000 assay system (Promega), and values obtained were normalized to that of the β-Gal activity measured in the corresponding cell extracts.

**JFH1-based HCV infection system**

The plasmid pJFH-1, containing HCV cDNA from a Japanese patient with fulminant hepatitis behind a T7 promoter [41], was linearized at the 3′ end of the HCV cDNA by XbaI digestion. The linearized DNA was then used as a template for *in vitro* transcription (MEGAscript; Ambion). Ten micrograms of JFH-1 RNA was delivered to Huh7.5 cells by electroporation, and virus stocks were prepared as previously described [42]. Cells were either mock infected or infected with HCV at an m.o.i. of 1.0 for 24 h, as previously described [43].

**Western blot analysis**

Cells were lysed in buffer [50 mM Tris/HCl (pH 8.0), 150 mM NaCl, 0.1% SDS and 1% NP-40] supplemented with protease inhibitors. Cell extracts were separated by SDS-PAGE and transferred onto a nitrocellulose membrane (Amersham). Membranes were then incubated with antibodies against p14, pSer-1981 ATM, pSer-15 p53, pSer-20...
p53 and pThr-68 CHK2 (Cell Signaling); CHK2, HA, HCV E2, HCV NS3, MDM2, p53, PA28γ and Ub (Santa Cruz Biotechnology); E6AP and HCV core (Thermo Scientific); and γ-tubulin (Sigma) and subsequently with an HRP-conjugated secondary antibody, anti-mouse, anti-goat or anti-rabbit IgG (H+L)-HRP (Bio-Rad). An ECL kit (Amersham) was used to visualize protein bands, using the ChemiDoc XRS imaging system (Bio-Rad).

**Immunoprecipitation (IP)**

An IP assay was performed using a Classic Magnetic IP/Co-IP assay kit (Pierce) according to the manufacturer’s specifications. Cells were transfected with pHA-Ub for 48 h under the indicated conditions. The whole-cell lysates were incubated overnight at 4°C with an appropriate antibody. Protein A/G magnetic beads (Pierce) were then added, and the lysates were incubated for an additional 1 h. The beads were collected using a magnetic stand (Pierce), and the antigen/antibody complexes eluted were subjected to Western blotting using anti-HA antibody (Santa Cruz Biotechnology).

**ChIP assay**

ChIP assay was performed using a ChIP assay kit (Upstate Biotechnology), according to the manufacturer’s instructions. The sheared chromatin was immunoprecipitated with either anti-p53 antibody or human IgG (Santa Cruz Biotechnology). DNA released from the precipitated complexes was amplified to detect PA28γ promoter by PCR using a primer pair (forward, 5′-CGC ACT GGA TTT TGA AGA CTT-3′; reverse, 5′-CGA GGC TCA AGT GTT TAG GC-3′), as previously described [21].

**PCR**

For semi-quantitative reverse transcription PCR, total RNA (3 µg) extracted from either cells or culture supernatant, using an RNeasy Mini kit (Qiagen), was reverse transcribed with a corresponding reverse primer. One-quarter of the reverse transcribed RNA was amplified with Taq polymerase to determine HCV core and GAPDH RNA levels as previously described [38]. For the amplification of PA28γ RNA, the primer set PA28γ-rt-F (5′-TGA ATC TCC CAG TCC CTG-3′) and PA28γ-rt-R (5′-TCA ACA GCA GGA TCT CAG-3′) was designed from a conserved region of five different PA28γ transcription variants. Quantitative real-time RT-PCR of HCV RNA was performed using a Rotagen Q (Qiagen) with SYBR Green PCR master mix (Takara) as previously described [43].

**Methylation-specific PCR**

Genomic DNA (1 µg) denatured in 50 µl of 0.2 M NaOH was modified by treatment with 30 µl of 10 mM hydroquinone (Sigma) and 520 µl of 3 M sodium bisulfite (pH 5.0; Sigma) at 50°C for 16 h. For methylation-specific PCR, the modified DNA (100 ng) was amplified with Taq polymerase using both methylated and unmethylated primer pairs of p14, as previously described [44].

**Statistical analysis**

The values indicate means±SDs from at least four independent experiments. A two-tailed Student’s t-test was used for all statistical analyses. P<0.05 was considered statistically significant.

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**Conflicts of Interest**

The authors declare that there are no conflicts of interest.

**References**


