Hepatitis C virus down-regulates SERPINE1/PAI-1 expression to facilitate its replication

Chee-Hing Yang,† Hui-Chun Li,‡ Tzu-Shan Ku,³ Pi-Ching Wu,³ Yung-Ju Yeh,⁴ Ju-Chien Cheng,⁴ Teng-Yi Lin⁵ and Shih-Yen Lo¹,3,5,*

Abstract

Identification of host factors involved in viral replication is critical for understanding the molecular mechanism of viral replication and pathogenesis. Genes differentially expressed in HuH-7 cells with or without a hepatitis C virus (HCV) sub-genomic replicon were screened by microarray analysis. SERPINE1/PAI-1 was found to be down-regulated after HCV infection in this analysis. Down-regulation of SERPINE1/PAI-1 expression at the transcriptional level was verified by the real-time reverse transcriptase (RT)-PCR assay. Reduced SERPINE1/PAI-1 protein secretion was detected in the supernatant of HCV replicon cells and in sera from HCV-infected patients. SERPINE1 gene expression was down-regulated by HCV NS3/4A and NS5A proteins through the transforming growth factor-β (TGF-β) signalling pathway at the transcriptional level. Down-regulated genes in HCV replicon cells could be the factors supressing HCV replication. Indeed, over-expressed PAI-1 inhibited HCV replication but the mechanism is unknown. It has been demonstrated that HCV induces the expression of TGF-β, and TGF-β enhances HCV replication by a not-yet-defined mechanism. SERPINE1/PAI-1 is also known to be potently induced by TGF-β at the transcriptional level through both Smad-dependent and Smad-independent pathways. The exogenously expressed SERPINE1/PAI-1 suppressed the expression of the endogenous SERPINE1 gene at the transcriptional level through the TGF-β signalling but not the Smad pathway. Thus, SERPINE1/PAI-1 could suppress HCV replication possibly by negatively regulating TGF-β signalling. A model is proposed for the interplay between the TGF-β signalling pathway, HCV and SERPINE1/PAI-1 to keep the homeostasis of the cells.

INTRODUCTION

Infection with hepatitis C virus (HCV) is a major cause of chronic hepatitis, liver cirrhosis and hepatocellular carcinoma. Approximately 170 million individuals are infected by HCV worldwide (www.who.int). HCV belongs to the genus Hepacivirus in the family Flaviviridae. More than two decades of study has provided a better understanding of the HCV life cycle [1, 2]. The HCV genome is a single, positive-stranded RNA with a nucleotide length of about 9.6 kb, encoding a large polyprotein precursor which is processed into NH2-C(21Kd)-E1(31Kd)-E2(70Kd)-p7-NS2(23Kd)-NS3(70Kd)-NS4A(8Kd)-NS4B(27Kd)-NS5A(58Kd)-NS5B(68Kd)-COOH. Due to the development of novel direct-acting anti-viral agents, new all-oral, interferon-free, pan-genotyping anti-HCV therapy has become available [3–6]. However, HCV replication and pathogenesis are still not completely understood. HCV propagation depends on host factors. Identification of host factors involved in viral replication is critical for understanding the molecular mechanism of the viral life cycle and pathogenesis. Many cellular factors important for HCV replication have been identified previously [7, 8].

The evolutionarily conserved transforming growth factor-β (TGF-β) proteins are distributed ubiquitously throughout the body. The TGF-β protein has multiple and diverse roles in regulating cell proliferation, differentiation and migration [9]. It has been demonstrated that HCV induces TGF-β expression [10, 11] and the TGF-β signalling pathway enhances HCV replication [12]. However, how TGF-β signalling modulates HCV replication is largely unknown.

There are several ways to activate the TGF-β pathway [13]. Two groups of TGF-β receptors are known. The activated type I receptor initiates the Smads signalling pathway.
(Smad2 and Smad3 become phosphorylated and form heteromeric complexes with Smad4), ending with the translocation of Smad4 into the nucleus to regulate the genes with Smad-binding elements (SBEs). Among these TGF-β regulated genes, SERPINE1 (7q22.1) gene (serine protease inhibitor E1; also called PAI-1, for human plasminogen activator inhibitor-1) is potently induced by TGF-β at the transcriptional level [14, 15]. In general, PAI-1 is often used for the protein encoded from the SERPINE1 gene (http://www.genecards.org).

Initially, we screened the genes differentially expressed in HuH-7 cells with or without HCV sub-genomic replicon by microarray analysis. The SERPINE1 mRNA level was found to be lower in the HCV replicon cells than in the HuH-7 cells. This result was unexpected as it had been reported that the TGF-β signalling pathway enhanced HCV replication and up-regulated SERPINE1 gene expression [12, 14]. In this study, we demonstrated that HCV down-regulates SERPINE1 gene expression at the transcriptional level through both NS3/4A and NS5A proteins. Moreover, PAI-1 was able to suppress HCV replication, possibly through suppression of TGF-β signalling but not the Smad pathway.

**RESULTS**

**HCV down-regulates SERPINE1/PAI-1 expression**

Genes differentially expressed in HuH-7 cells with or without HCV sub-genomic replicon were screened by microarray analysis. In this screen, the SERPINE1 mRNA level was found to be lower in the HCV replicon cells than in the HuH-7 cells (data not shown; also see below). To verify the microarray results, the SERPINE1 mRNA level in HuH-7 cells with or without the HCV sub-genomic replicon was determined by real-time reverse transcriptase (RT)-PCR. Indeed, the SERPINE1 mRNA level was lower in HCV replicon cells than in HuH-7 cells (left panel in Fig. 1a). To check the expression at the protein level, the secreted PAI-1 was quantified by ELISA while the intracellular PAI-1 was analysed by Western blotting. A lower PAI-1 level was detected in HCV replicon cells than in HuH-7 cells both intracellularly (Fig. 1b) and extracellularly (right panel in Fig. 1a).

**Fig. 1.** HCV down-regulated SERPINE1/PAI-1 expression. (a) (Left panel) Real-time RT-PCR analysis of SERPINE1 mRNA level used samples from HuH-7 and HCV replicon cells. (Right panel) Relative secreted PAI-1 level detected by ELISA using the culture medium from HuH-7 and HCV replicon cells. (b) Western blotting analysis of NS5A (as an indicator of the presence of HCV) and PAI-1 in HuH-7 and HCV replicon cells. (c) HuH-7.5 cells were either mock-infected or infected with infectious HCV (m.o.i.=0.5). At 3 or 5 days after infection, protein samples derived from these cells were analysed by Western blotting. The un-glycosylated PAI-1 protein is marked by a thin line. In general, the endogenous un-glycosylated but not glycosylated PAI-1 protein is often detected. The presence of HCV in the cells was demonstrated by the detection of core protein. (d) ELISA was used to detect the amount of PAI-1 in the plasma or sera of the same subjects (left panel) or in sera from subjects who were anti-HCV (–), anti-HCV (+) plus HCV RNA (+), and anti-HCV (+) but HCV RNA (–) (right panel).
plasma than in the sera of the same subjects (left panel in Fig. 1d). Next, the PAI-1 level in the sera of three groups (anti-HCV negative; both HCV RNA and anti-HCV positive; only anti-HCV positive) was determined. The amount of PAI-1 in the sera of HCV-infected patients was lower than that of control subjects (right panel in Fig. 1d).

To further confirm that HCV down-regulated SERPINE1 expression, IFN-α was used to remove HCV sub-genomic RNA from the HCV replicon cells. PAI-1 expression was only slightly elevated in IFN-α-treated HuH-7 cells (Fig. 2a). On the other hand, after removing HCV sub-genomic RNA (demonstrated by the NS5A protein amount), PAI-1 expression was enhanced significantly (Fig. 2a). To determine which HCV viral protein(s) suppressed SERPINE1/PAI-1 expression, the intracellular PAI-1 was analysed using cells stably expressing various HCV proteins. HCV NS3/4A and NS5A proteins were found to down-regulate SERPINE1/PAI-1 expression at the protein level (Fig. 2b), and also at the mRNA level (Fig. 2c).
Plasmid construction for analyzing SERPINE1 promoter

Fig. 3. HCV NS5A protein suppressed SERPINE1/PAI-1 gene expression at the transcriptional level. (a, b) Western blotting analysis (a) or real-time RT-PCR results (b) for SERPINE1/PAI-1 in HuH-7 cells transiently transfected with different amount of the plasmids expressing HCV NS5A-V5 protein. (c) Plasmid construction for the analysis of SERPINE1 gene promoter activity. Possible SBEs are marked. (d) The relative luciferase activities of different SERPINE1 gene promoter segments. (e) Luciferase assay was used to detect
Therefore, down-regulation of the SERPINE1/PAI-1 expression by HCV was demonstrated in HCV replicon cells, in cells infected by HCV virions and in the sera of HCV-infected patients.

**HCV NS5A down-regulates SERPINE1/PAI-1 expression at the transcriptional level**

To further prove the effect of NS5A protein on SERPINE1/PAI-1 expression, the transient transfection system was used. As expected, SERPINE1/PAI-1 expression was down-regulated by NS5A protein dose-dependently at both the protein and the mRNA level (Fig. 3a, b). These results suggested that HCV NS5A down-regulates SERPINE1 expression at the transcriptional level.

SERPINE1/PAI-1 is potently induced by TGF-β at the transcriptional level [14, 16, 17]. The SERPINE1 promoter has also previously been characterized [18]. Reporters with different lengths of SERPINE1 promoter region were constructed (Fig. 3c) and relative SERPINE1 promoter activity was determined (Fig. 3d). Within these reporters, NS5A suppressed the reporters with the SERPINE1 promoter region containing the three Smad-binding sites from −200 to −800 bp (Fig. 3e). It has been demonstrated that NS5A suppresses the TGF-β signalling pathway through interacting with the type I TGF-β receptor [19]. Indeed, NS5A did not suppress SERPINE1 promoter activity when one Smad-binding site (SBE3) was mutated (Fig. 3f). It has previously been reported that NS5A binds to the TGF-β receptor and, in turn, reduces Smad2 phosphorylation [19]. As expected, the phosphorylated Smad2 level was reduced in the cells expressing NS5A protein compared with those expressing GFP protein (Fig. 3g).

Therefore, HCV NS5A protein was able to down-regulate SERPINE1/PAI-1 expression at the transcriptional level through reducing the Smad2 phosphorylation.

**HCV NS3/4A down-regulates SERPINE1/PAI-1 expression at the transcriptional level**

Effect of NS3/4A protein on the SERPINE1/PAI-1 expression was also detected using the transient transfection system. As expected, SERPINE1/PAI-1 expression was down-regulated by NS3/4A protein dose-dependently when analysed at both the protein and mRNA levels (Fig. 4a, b). To determine which region of the NS3 protein is responsible for the reduced SERPINE1/PAI-1 expression, plasmids with different NS3 domains were constructed. The NS3 N-terminal protease domain alone (NS3P), but not the NS3 protein without the protease domain (NS3M), suppressed SERPINE1/PAI-1 expression (Fig. 4c, d). However, functional protease activity is not essential for the down-regulation because mutation at the active site still suppressed SERPINE1/PAI-1 expression (Fig. 4e).

Similar to NS5A, NS3/4A also suppressed the SERPINE1 promoter region containing the three Smad-binding sites from −200 to −800 bp (Fig. 4f). It has also been demonstrated that NS3/4A suppresses TGF-β signalling pathway through interacting with Smad protein [20]. Indeed, NS3/4A did not suppress the SERPINE1 promoter activity when one Smad-binding site (SBE3) was mutated (Fig. 4g). As expected, NS3/4A protein interacted with Smad3 protein as demonstrated by the co-immunoprecipitation assay (Fig. 4h). Furthermore, the suppressive effect of NS3/4A protein on SERPINE1/PAI-1 expression was reversed by the over-expressed Smad3 protein (Fig. 4i).

Therefore, HCV NS3/4A protein could down-regulate SERPINE1/PAI-1 expression at the transcriptional level through interacting with Smad3 protein.

**Exogenously expressed SERPINE1/PAI-1 inhibits HCV replication**

Genes up-regulated in HCV replicon cells may facilitate HCV replication while down-regulated genes may suppress HCV replication. Indeed, HCV replicon cells stably expressing PAI-1 protein had lower levels of HCV replication than those expressing control GFP, as shown by the level of either viral NS5A protein (left panel in Fig. 5a) or HCV subgenomic RNA (right panel in Fig. 5a). Furthermore, HCV replication based on viral NS5A protein amount was also inhibited by exogenously transient expression of PAI-1 protein in a dose-dependent manner (Fig. 5b).

HCV has been demonstrated to enhance TGF-β expression [10, 11], and TGF-β reciprocally enhances HCV replication by a not-yet-defined mechanism [12]. We also found that TGF-β could simultaneously facilitate HCV replication and up-regulate SERPINE1/PAI-1 expression in the HCV replicon cells (Fig. 5c). SERPINE1/PAI-1 is also known to be potently induced by TGF-β at the transcriptional level [14]. Indeed, TGF-β significantly up-regulated SERPINE1/PAI-1 expression (Fig. S1, available with the online Supplementary Material). Moreover, this modulation occurred at the transcriptional level as TGF-β significantly turned on the reporter under the control of SERPINE1 promoter (Fig. S2). Thus, it is unexpected to see that PAI-1 protein inhibits HCV replication (Fig. 5a, b).

**Intracellular PAI-1 inhibits HCV replication not through its protease-inhibiting activity**

PAI-1 is a serine protease inhibitor that functions as the principal inhibitor of tissue plasminogen activator and urokinase [21, 22]. To determine whether the signal peptide of PAI-1 protein is important for its suppressive effect on HCV replication, PAI-1 without signal peptide was expressed in HCV replicon cells and its suppressive effect was retained (Fig. 5d). Extracellular PAI-1 protein can enter the cells through the
LDL receptor–related protein [23, 24]. To determine whether the secreted or intracellular form of PAI-1 suppresses HCV replication, the PAI-1 protein with or without signal peptide was expressed in 293 T cells (left panel in Fig. 5e). Culture media from these transfected cells, containing the secreted PAI-1 proteins with or without the signal peptide, were added to HCV replicon cells. Both extracellularly applied PAI-1 proteins entered the cells but failed to inhibit HCV replication, based on viral NS5A protein amount (right panel in Fig. 5e).

HCV NS3 protein is a multi-function protein containing serine protease activity [25]. To determine whether PAI-1 protein inhibits NS3 protease activity, the reporter containing the NS3 cleavage site (i.e. the peptide between the NS5A and NS5B proteins) was used. However, PAI-1 protein did not inhibit NS3 protease activity (Fig. 5f). Furthermore, mutation of the PAI-1 active site as a serine protease inhibitor [26] did not affect its suppressive effect on HCV replication (Fig. 5g). Taken together, PAI-1 inhibited HCV

![Image](image_url)

**Fig. 4.** HCV NS3/4A protein suppressed SERPINE1/PAI-1 gene expression at the transcriptional level. (a, b) Western blotting analysis (a) or real-time RT-PCR results (b) for SERPINE1/PAI-1 in HuH-7 cells transiently transfected with different amount of the plasmids expressing HCV NS3/NS4A protein with a myc tag. (c–e) Western blotting analysis of PAI-1 in HuH-7 cells transiently transfected with different amounts of the plasmids expressing the V5-tagged HCV NS3 protease domain (c), without the protease domain (d), and with a mutation (S139A) in the protease domain (e). (f) Luciferase assay was used to detect the effect of NS3/4A on different promoter regions of the SERPINE1 gene. (g) Luciferase assay was used to detect the effect of NS3/4A on the SERPINE1 gene promoter with different SBE mutations. (h) Co-immunoprecipitation of NS3 and Smad3 proteins. At 48 h after transfection of HuH-7 cells with different plasmids as indicated, cell lysates from these cells were directly analysed by Western blotting (input) for expression control or immunoprecipitated with the anti-Myc antibody prior to Western blotting (bottom panel). Smad3-V5 protein is marked by an arrow. (i) The suppressive effect of NS3/4A protein on SERPINE1/PAI-1 expression was reversed by the over-expressed Smad3 protein. After the establishment of HuH-7 cells stably with GFP or HA-NS3/4A protein, cells with HA-NS3/4A protein were transfected with different amounts of Smad3-expressing plasmids. At 48 h after transfection, protein samples derived from these cells were Western-blotted.
replication intracellularly and did not function as a traditional serine protease inhibitor.

If exogenously over-expressed PAI-1 protein could inhibit HCV replication, reduction of PAI-1 expression should enhance HCV replication. Indeed, compared with control shRNA, knockdown of PAI-1 expression by shRNAs did enhance HCV replication (Fig. 5h).

Therefore, over-expressed PAI-1 protein could intracellularly suppress HCV replication, and reduction of PAI-1 expression could increase HCV replication.

**Exogenously expressed SERPINE1/PAI-1 suppresses the expression of endogenous SERPINE1 gene**

Compound C has been demonstrated to enhance HCV replication through the inhibition of AMPK phosphorylation [27, 28]. It would be interesting to know whether PAI-1 inhibited HCV replication through the activation of AMPK phosphorylation. However, PAI-1 did not activate AMPK phosphorylation (Fig. S3). In fact, two activators of AMPK phosphorylation, AICAR and metformin, inhibited SERPINE1/PAI-1 expression (Fig. S4), while compound C increased SERPINE1/PAI-1 expression, although not in a dose-dependent manner (Fig. S5). Therefore, PAI-1 did not suppress HCV replication through the AMPK pathway.

On the other hand, exogenously expressed SERPINE1/PAI-1 was found to inhibit the expression of endogenous SERPINE1 gene in both transiently transfected and stably expressed systems (Fig. 6a). To study whether PAI-1 protein affects its own promoter, HuH-7 cells stably expressed with control GFP or PAI-1 protein were used. The inhibitory effect of PAI-1 protein on its own expression is probably at the transcriptional level because the exogenously expressed PAI-1 protein inhibited the expression of the reporter under the control of SERPINE1 promoter (left panel of Fig. 6b). The inhibitory effect was overcome by TGF-β treatment (right panel of Fig. 6b). Indeed, TGF-β counteracted the effect of the exogenous PAI-1 protein to up-regulate endogenous SERPINE1/PAI-1 expression and...
Fig. 5. The PAI-1 without protease inhibitor function inhibited HCV replication intracellularly. (a) Western blotting results (left panel, the exogenously expressed un-glycosylated and glycosylated PAI-1 proteins are marked by thin and thick arrows respectively) or real-time RT-PCR results for the amount of HCV 5'UTR (right panel) using samples from HCV replicon cells stably with GFP or PAI-1 protein. (b) Western blot of NS5A in HCV replicon cells transiently transfected with different amounts of plasmids expressing PAI-1 protein with a V5 tag. (c) Western blotting analysis of NS3 and PAI-1 in the HCV replicon cells treated with different amounts of TGF-β. (d) Western blot of NS5A in HCV replicon cells transiently transfected with different amount of the plasmids expressing PAI-1 protein without its signal peptide. (e) Extracellular PAI-1 did not affect HCV replication. (Left panel) Western blot of proteins from cell lysates (top) or medium (bottom) of 293T cells either mock-transfected or transfected with V5-tagged intact PAI-1 or PAI-1 without signal peptide (aa 24–402). (Right panel) HCV replicon cells were cultured with media from 293T cells either mock-transfected or transfected with V5 tagged intact PAI-1 or PAI-1 without signal peptide (aa 24–402). After 8 or 20 h, protein samples derived from these replicon cells were Western blotted. (f) PAI-1 did not affect NS3 protease activity. At 48 h after transfection of HuH-7 cells with different
expressing plasmids as indicated, protein samples from these cells were Western-blotted to detect GFP (for the intact RFP-GFP fusion protein, marked with the thin dotted arrows, or cleaved GFP protein processed by the NS3, marked with the thick dotted arrow) or V5 (for the exogenous PAI-1). (g) Western blot of NS3 in HCV replicon cells transiently transfected with different amounts of the plasmids expressing PAI-1 protein with a T356R mutation. (h) Western blotting analysis of NS3 (to reflect the HCV amount) and PAI-1 in HCV replicon cells with control shRNA (scramble) or two shRNAs targeting SERPINE1.

at the same time enhance HCV replication in HCV sub-genomic replicon cells (Fig. 6c).

The relative SERPINE1 promoter activities of wild-type or the mutant without Smad-binding sites (MB3) were analysed without (left panel of Fig. 6d) or with (right panel of Fig. 6d) TGF-β treatment. It is well known that TGF-β activates the SERPINE1 promoter through the Smad pathway [14]. Indeed, TGF-β treatment activated the wild-type promoter significantly (from 132-fold to 200-fold; Fig. 6d). Interestingly, TGF-β even activated the SERPINE1 promoter without Smad-binding sites, although to a lesser extent (from 95-fold to 106-fold; Fig. 6d). Therefore, in agreement with a previous report [29], these results suggested that TGF-β activated the SERPINE1 promoter through both Smad-dependent and Smad-independent pathways. On the other hand, compared to GFP protein, the exogenously expressed PAI-1 protein suppressed not only the wild-type SERPINE1 promoter (left panel of Fig. 6b) but also the SERPINE1 promoter without Smad-binding sites (left panel of Fig. 6e). Moreover, this suppressive effect on the SERPINE1 promoter without Smad-binding sites was not overcome by the TGF-β treatment (right panel of Fig. 6e). In addition, the exogenously expressed PAI-1 protein affected neither the expression of Smad nor the phosphorylation of Smad2 (data not shown). These results suggested that the exogenously expressed PAI-1 suppressed the endogenous SERPINE1/PAI-1 gene expression at the transcriptional level through TGF-β signalling independent of the Smad pathway.

DISCUSSION

Down-regulation of the SERPINE1/PAI-1 expression by HCV was demonstrated in HCV replicon cells, in cells infected by HCV virions and in the sera of HCV-infected patients (Fig. 1). A previous report also showed that HCV sub-genomic replicon inhibits TGF-β inducing signalling pathways [20]. Suppression of SERPINE1/PAI-1 expression by HCV seems to be reversible in this cell model because the removal of HCV sub-genomic RNA by IFN-α enhanced SERPINE1/PAI-1 expression (Fig. 2a). However, similar to that of HCV RNA(+) group, a lower PAI-1 level was detected in the group of HCV RNA(−) plus anti-HCV(+) than in the control subjects (Fig. 1d). This may be due to tiny amount of HCV, which is not detectable by RT-PCR but is still enough to down-regulate the SERPINE1/PAI-1 expression.

In agreement with the previous report regarding HCV NS5A protein interacts with TGF-β receptor I and blocks TGF-β signalling [19], HCV NS5A protein was shown to suppress SERPINE1/PAI-1 expression through Smad signalling in this study (Fig. 3).

A previous report showed that HCV NS3 protein interacts with Smad3 and blocks TGF-β signalling [20]. In this study, HCV NS3 protein was also shown to suppress SERPINE1/PAI-1 expression through interacting with Smad 3. Furthermore, the N-terminal protease domain of NS3 but not its protease activity is responsible for this activity (Fig. 4). NS3 protein of some HCV genotypes could further auto-cleave itself to generate this N-terminal product [30]. It would be interesting to know whether these HCV genotypes could down-regulate SERPINE/PAI-1 expression more than the other genotypes.

TGF-β signalling has been demonstrated to enhance HCV production in the infectious virion system [12]. In this study, TGF-β signalling has also been shown to enhance HCV replication in the sub-genomic system (Fig. 5c). Therefore, TGF-β signalling may directly enhance HCV RNA synthesis.

PAI-1 is a serine protease inhibitor that functions as the principal inhibitor of tissue plasminogen activator and urokinase [21, 31]. Therefore, PAI-1 is known to function extracellularly. Indeed, PAI-1 was recently demonstrated to target extracellular airway proteases, thereby reducing the infectivity of influenza A viruses [32]. However, the intracellular role of PAI-1 is unknown. Both PAI-1 with or without the signal peptide were secreted from 293T cells and entered HCV replicon cells (right panel in Fig. 5e). However, unlike the intracellularly expressed PAI-1, the extracellular PAI-1 (intact or truncated), after entering into the cells, did not inhibit HCV replication, as demonstrated by the unchanged level of NS5A protein (right panel in Fig. 5e). Therefore, the intracellular but not the extracellular PAI-1 inhibits HCV replication. Suppression of HCV replication by PAI-1 is also independent of its protease inhibitor role (Fig. 5g). This would be a novel function for PAI-1 protein. Interestingly, a recent report demonstrated that serpin antithrombin III inhibited HCV replication through stimulating a novel innate host cell defence [33].

The TGF-β protein has diverse and sometimes contradicting biological roles in regulating different cellular functions [34]. It has been reported that PAI-1 inhibits the activation of TGF-β [35]. However, how PAI-1 regulates TGF-β signalling is not known. It has been demonstrated that TGF-β induces SERPINE1/PAI-1 expression through Smad-dependent or Smad-independent pathways [2, 29]. Indeed, results from this report agreed with previous studies (Fig. 6d). PAI-1 could suppress its own expression and this suppression
could be reversed by TGF-β treatment (Fig. 6c). This reversal is through the Smad-dependent but not the Smad-independent pathway (Fig. 6b, e). These results suggest that PAI-1 suppressed one of the TGF-β signalling pathways, which is Smad-independent. The suppressive effect of PAI-1 on HCV replication (Fig. 5a) is much more significant than on its own expression (Fig. 6a), possibly due to the existing TGF-β in the cultured HuH-7 cells, which induced SERPINE1/PAI-1 expression through Smad-dependent pathway. This is supported by the results that TGF-β overcame the effect of PAI-1 on the reporter with wild-type SERPINE1 promoter but not with mutations in the Smad-binding sites (Fig. 6b, e).

A model was proposed to explain the results from previous studies and this one (Fig. 7). The TGF-β signalling pathway...
induces the expression of many genes (including the \textit{SERPINE1} gene) to execute its various regulatory roles (including enhancement of HCV replication). Some of these TGF-β induced genes (e.g. \textit{SERPINE1}) negatively regulate the TGF-β signal, and in turn inhibit its regulatory functions (including the modulation of HCV replication), to maintain homeostasis of the cells (feedback inhibition).

**METHODS**

**Cell culture and viruses**

HuH-7 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS), 100 U ml\(^{-1}\) penicillin and 100 µg ml\(^{-1}\) streptomycin (GibcoA). HCV sub-genomic replicon cells were cultured in DMEM with 10% FBS, 100 U ml\(^{-1}\) penicillin, 100 µg ml\(^{-1}\) streptomycin and 400 µg ml\(^{-1}\) G418. All cultured cells were maintained at 37°C with 5% CO\(_2\).

Generation of infectious HCV particles (HCVcc) and infectivity assay in HuH-7.5 cells were performed as described previously [36].

**Plasmid construction and DNA transfection**

The expression plasmids used in this study were constructed using standard protocols as described in our previous studies [37]. The primers used for the cloning are listed in Table S1. These expression plasmids were all verified by sequencing. The PEI (polyethylenimine, linear, MW 25 000) used to transfect DNA (e.g. pcDNA3.1-SERPINE1) into HuH-7 cells was commercially available (Polysciences Inc.).

**RNA extraction and real-time RT-PCR**

Total RNAs, extracted from the cells using TRIZol reagent (Invitrogen, Thermo Fisher Scientific) following the manufacturer’s instructions, were converted into cDNAs using oligo-dT or random hexamer as the primer. The High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, ThermoFisher Scientific) was used for the reverse transcription. LabStar SYBR qPCR Kit (TAIGEN Bioscience Corporation) was used for the real-time PCR. The primers used for the real-time RT-PCR are listed in Table S2. In these reactions, β-actin or TFRC mRNA was used as the internal control. All experiments were repeated at least three times, and the real-time RT-PCR procedures followed the minimum information for publication of quantitative real-time PCR experiments (MIQE) guide [38].

**Western blotting analysis**

Our previous procedures were followed for Western blotting analysis [39]. The primary antibodies used for the analyses in this study were antibodies against HCV NS5A protein (Meridian Life Science), against HCV NS3 protein (Abcama), against Myc tag clone 4A6 (Merck Millipore), against V5 tag (Bio-Rad), against beta-actin and SerpinE1/PAI-1 (Genetex), against HA tag, GFP and ERK-2 protein (Santa Cruz BiotechnologyA), and against Smad2, pSmad2, Smad3, Smad4, Smad6, Smad7, and TGF-β receptor.
Smad3, AMPK, and pAMPK (Cell Signaling Technology).

In this assay, actin or ERK-2 protein was used as the loading control.

**ELISA assay**

The Human PAI1 ELISA Kit (SERPINE1) (Abcam) was used for the analysis of PAI-1 following the manufacturer’s instructions. Serum samples were collected from Tzu Chi hospital (approved by IRB 101–75), including 70 anti-HCV negative control samples, 72 samples with both HCV RNA and anti-HCV positive and 25 samples with only anti-HCV positive. In addition, nine paired samples of healthy individuals (both plasma and serum samples from the same subjects) were used for comparison.

**The shRNA knockdown and stably over-expressed experiments**

The shRNA knockdown (e.g. SERPINE1) and stably over-expressed (e.g. SERPINE1, HCV NS3/4A, HCV NS5A) experiments were performed using the lentiviral expressing system (http://rnai.genmed.sinica.edu.tw), following the manufacturer’s instructions and our previous procedures [40]. The shRNA knockdown reagents were obtained from the National RNAi Core Facility located at the Institute of Molecular Biology/Genomic Research Center, Academia Sinica, Taiwan.

**Co-immunoprecipitation assay**

Co-immunoprecipitation assay conducted in HuH-7 cells was following our previous procedures [41]. In this experiment, expression plasmids for pcDNA3-myc-NS3/4A and/or pcDNA3.1-Smad2 (or pcDNA3.1-Smad3) were used.

**Luciferase assay**

HuH-7 cells were cultured in DMEM supplemented with 10% FBS. 1×10^5 cells were transfected with the reporter pGL3Basic/pGL3-SerpinE1P, the internal control plasmid pRL-TK (Promega) and different expressing plasmids. Cells were harvested 72 h after transfection. The dual-luciferase assay system (Promega) was used for the luciferase assays following the manufacturer’s instructions and our previous procedures [41]. In each experiment, triplicate samples were analysed. The results shown were the average of three different experiments. ANOVA was used to compare the means among groups. The post-hoc Tukey test was used to test the means between two groups.

**Chemical/drugs**

he recombinant Human TGF-beta 1 Protein used in this study was purchased from R & D Systems. Chemicals (AICAR and metformin hydrochloride) were commercially available (Sigma-Aldrich).

**Acknowledgements**

We thank Dr J.-H. Ou for providing the HCV sub-genomic RNA cells, Dr Charles M. Rice for providing the p90/HCVFL-long pU plasmid, HuH-7.5 cells and pFL-J6/JFH (with the permission of Apath) to generate HCVcc and Dr K.-S. Jeng for the expression plasmids of various HCV proteins. The RNAi reagents were obtained from the National RNAi Core Facility located at the Institute of Molecular Biology/Genomic Research Center, Academia Sinica, supported by grants from the NSC National Research Program for Genomic Medicine (NSC 94–3112-B–001–003 and NSC 94–3112-B–018–Y).

**Conflicts of interest**

The authors declare that there are no conflicts of interest.

**References**


24. Olson D, Pöll


