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The AMPK-related kinase SNARK regulates hepatitis C virus replication and pathogenesis through enhancement of TGF-β signaling

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Background & Aims: Hepatitis C virus (HCV) is a major cause of chronic liver disease worldwide. The biological and therapeutic importance of host cellular cofactors for viral replication has been recently appreciated. Here we examined the roles of SNF1/AMP kinase-related kinase (SNARK) in HCV replication and pathogenesis.
Methods: The JFH1 infection system and the full-length HCV replicon OR6 cell line were used. Gene expression was knocked down by siRNAs. SNARK mutants were created by site-directed mutagenesis. Intracellular mRNA levels were measured by qRT-PCR. Endogenous and overexpressed proteins were detected by Western blot analysis and immunofluorescence. Transforming growth factor (TGF)-β signaling was monitored by a luciferase reporter construct. Liver biopsy samples from HCV-infected patients were analyzed for SNARK expression.
Results: Knockdown of SNARK impaired viral replication, which was rescued by wild type SNARK but not by unphosphorylated or kinase-deficient mutants. Knockdown and overexpression studies demonstrated that SNARK promoted TGF-β signaling in a manner dependent on both its phosphorylation and kinase activity. In turn, chronic HCV replication upregulated the expression of SNARK in patients. Further, the SNARK kinase inhibitor metformin suppressed both HCV replication and SNARK-mediated enhancement of TGF-β signaling.
Conclusions: Thus reciprocal regulation between HCV and SNARK promotes TGF-β signaling, a major driver of hepatic fibrogenesis. These findings suggest that SNARK will be an attractive target for the design of novel host-directed antiviral and antifibrotic drugs.

Keywords: SNARK; NUAK2; HCV; Metformin; Fibrosis; TGF-beta; SMAD; Kinase.
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Abbreviations: SNARK, sucrose-non-fermenting protein kinase 1/AMP-activated protein kinase-related protein kinase; TGF-β, transforming growth factor beta; JFH1, Japanese fulminant hepatitis 1; HTAs, host-targeting antivirals; HCC, hepatocellular carcinoma; SVR, sustained virological response; CsA, cyclosporin A.

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Introduction

Chronic infection with hepatitis C virus (HCV) is a major cause of chronic liver disease and hepatocellular carcinoma (HCC) and the leading reason for liver transplantation worldwide. HCV infects approximately 170 million individuals worldwide [1]. Current therapy with pegylated interferon (IFN)-α in combination with ribavirin produces sustained virological response (SVR) in fewer than half of the patients infected with genotype 1 HCV, and does so with high rates of often unacceptable side effects [2]. In recent years, it has become increasingly evident that HCV propagation is highly dependent on host cellular cofactors which in turn represent promising antiviral targets [3]. It is hoped that these strategies will lead to rational host-targeting antivirals (HTAs) [4]. Moreover, HCV interferes with host cellular signaling pathways causing pathogenic effects such as insulin resistance (IR), diabetes, and alterations in host lipids. Indeed, mounting evidence supports hepatitis C as a metabolic disorder [5]. Hence, intervention against key host cellular factors critical for both HCV replication and viral pathogenesis may yield anti-hepatitis C therapies that both halt replication and abrogate other pathogenic effects of HCV, which might be further thought of as host-directed antiviral and antipathogenic therapies. Based on this assumption, we have previously conducted a functional genomic screen for host cellular factors supporting HCV replication using an HCV replicon system [6]. Among positive hits in our original screen was sucrose-non-fermenting protein kinase 1 (SNF1)/AMP-activated protein kinase (AMPK)-related protein kinase (SNARK), the fourth member of 14 mammalian AMPK-related kinases [7], which has been consistently found in other screens [8,9]. Although SNARK function is not well understood, SNARK heterozygote knockout mice displayed elevated serum triglyceride concentrations, hyperinsulinemia, glucose intolerance [10], and impaired contraction-stimulated glucose transport [11], implying that SNARK operates to maintain glucose and lipid homeostasis in a

manner analogous to AMPK, which was recently described to inhibit HCV replication [12].

Transforming growth factor (TGF)-β is a pleiotropic cytokine partaking in cell proliferation, differentiation, apoptosis, migration [13], and the major cytokine responsible for fibrosis in tissues including the liver. In HCV-infected persons, levels of TGF-β are elevated [14], and TGF-β was exhibited to promote the viral replication in a replicon model and was correlated with accelerated liver fibrosis in an *in vivo* model [15,16]. Intriguingly, a prior high-throughput mapping study of protein-protein interaction (PPI) identified an association of SNARK with SMADs [17], implying a direct link of SNARK to TGF-β signaling. Therefore, we sought to examine the significance and potential of SNARK as a therapeutic target in HCV replication and pathogenesis and its contribution to TGF-β signaling. We report that the phosphorylation and phosphotransferase activities of SNARK are required for HCV replication. Furthermore SNARK was demonstrated to enhance TGF-β signaling, and finally chronic HCV infection upregulated the expression of SNARK in patients. SNARK has pleiotropic functions including pro-TGF-β signaling activities in addition to the previously described AMPK-like properties. The finding of a reciprocal regulation between HCV and SNARK suggests that SNARK could be an effective host cellular target not only for an antiviral but also antipathogenic strategy.

Materials and methods

Compounds, antibodies, cells, and viruses

Metformin, TGF-β, and CsA were purchased from EMD chemicals USA (Gibbstown, NJ), Fitzgerald (North Acton, MA), and Sigma-Aldrich (St. Louis, MO), respectively. Antibodies to SNARK, FLAG, and β-actin were obtained from Sigma-Aldrich, and antibodies to HCV NS5A and phosphothreonine were obtained from BioFront Technologies (Tallahassee, FL) and Cell Signaling Technology (Danvers, MA), respectively. HuH7.5.1 and OR6 replicon cells were cultured as described previously [18], and HeLa cells were cultured in DMEM with 10% FBS. JFH1 virus infection was performed as described previously [19].

Further Materials and methods are described in the Supplementary Material section.

Results

Functional SNARK enhances HCV replication

To assess the contribution of SNARK to HCV replication, we first knocked down endogenous SNARK expression (Supplementary Fig. 1) with siRNAs in the Japanese fulminant hepatitis 1 (JFH1) virus infection system. HuH7.5.1 cells were transfected with SNARK-targeted siRNAs, which was followed by JFH1 infection. Reduced levels of *SNARK* mRNA were associated with impaired viral replication (Fig. 1A). We then constructed plasmids encoding the siRNA-resistant *SNARK* open reading frame (ORF) bearing synonymous mutations that are not recognized by *SNARK* siRNAs. The over expression of these siRNA-resistant SNARK proteins successfully rescued *SNARK* RNAi-impaired HCV replication (Fig. 1A, rSN-1 and rSN-7). We also tested the effects of SNARK knockdown and overexpression in the genotype 1 OR6 replicon system, and found that the decreased level of HCV RNA replication was also rescued by overexpression of siRNA-resistant forms of SNARK (Fig. 1B). Thus, SNARK was demonstrated to specifically support HCV replication in both a *bona fide* infection system and replicon model.

Next we sought to identify the function(s) responsible for SNARK's contribution to HCV replication. We introduced single

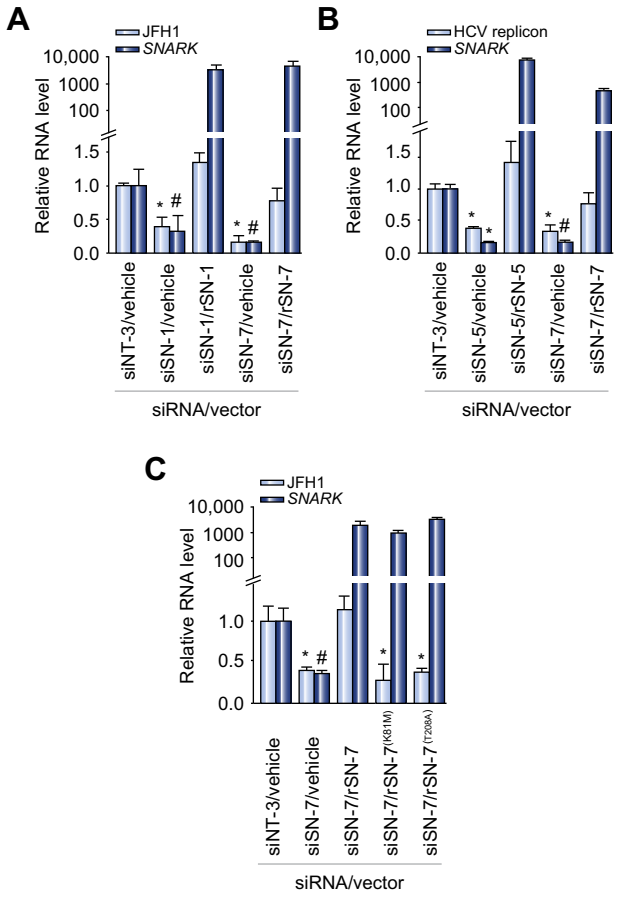


Fig. 1. SNARK supports HCV replication. (A) HuH7.5.1 cells were transfected with either non-targeting (siNT-3) or *SNARK*-targeted siRNAs (siSN-1 and siSN-7), followed by transfection of either empty or siRNA-resistant SNARK expression vectors (rSN-1 and rSN-7) 48 h later. The cells were infected with JFH1 on the next day and total RNA was harvested 48 h later. Relative JFH1 RNA and *SNARK* mRNA levels were quantified by real-time PCR analysis and normalized to *GAPDH*; $^{*}p$ <0.05 or $^{\#}p$ <0.01 *vs.* siNT-3 empty control. (B) OR6 replicon cells were transfected with siRNAs, followed by the transfection of empty or siRNA-resistant SNARK expression vectors 48 h later. Then total RNA was harvested 72 h later. Relative replicon RNA and *SNARK* mRNA levels were quantified by real-time PCR analysis and normalized to *GAPDH*. $^{*}p$ <0.01 or $^{\#}p$ <0.05 *vs.* siNT-3 empty control. (C) The rescue assay was conducted as described in (A). Here expression vectors of siRNA-resistant SNARK with either kinase-deficient mutation (rSN-7 K81M) or phosphorylation-deficient mutation (rSN-7 T208A) were used. Relative JFH1 RNA and *SNARK* mRNA levels were quantified by real-time PCR and normalized to *GAPDH*. $^{*}p$ <0.01 or $^{\#}p$ <0.05 *vs.* siNT-3 empty control.

mutations that abrogate either its phosphotransferase activity in the enzymatic pocket (K81M) or its phosphorylation at the phosphoacceptor site (T208A) in the siRNA-resistant *SNARK* ORF and overexpressed them in the rescue assay system used above with JFH1. In contrast to the rescue effects by wild type SNARK on viral replication, both functionally deficient mutants failed to recover impaired HCV replication by SNARK depletion (Fig. 1C and Supplementary Fig. 2). This result suggested that both the phosphorylation and kinase activities of SNARK are essential for its support of HCV replication.

SNARK phosphotransferase activity can be targeted

In a human hepatocarcinoma cell line, the kinase activity of SNARK was previously reported to be inhibited by metformin

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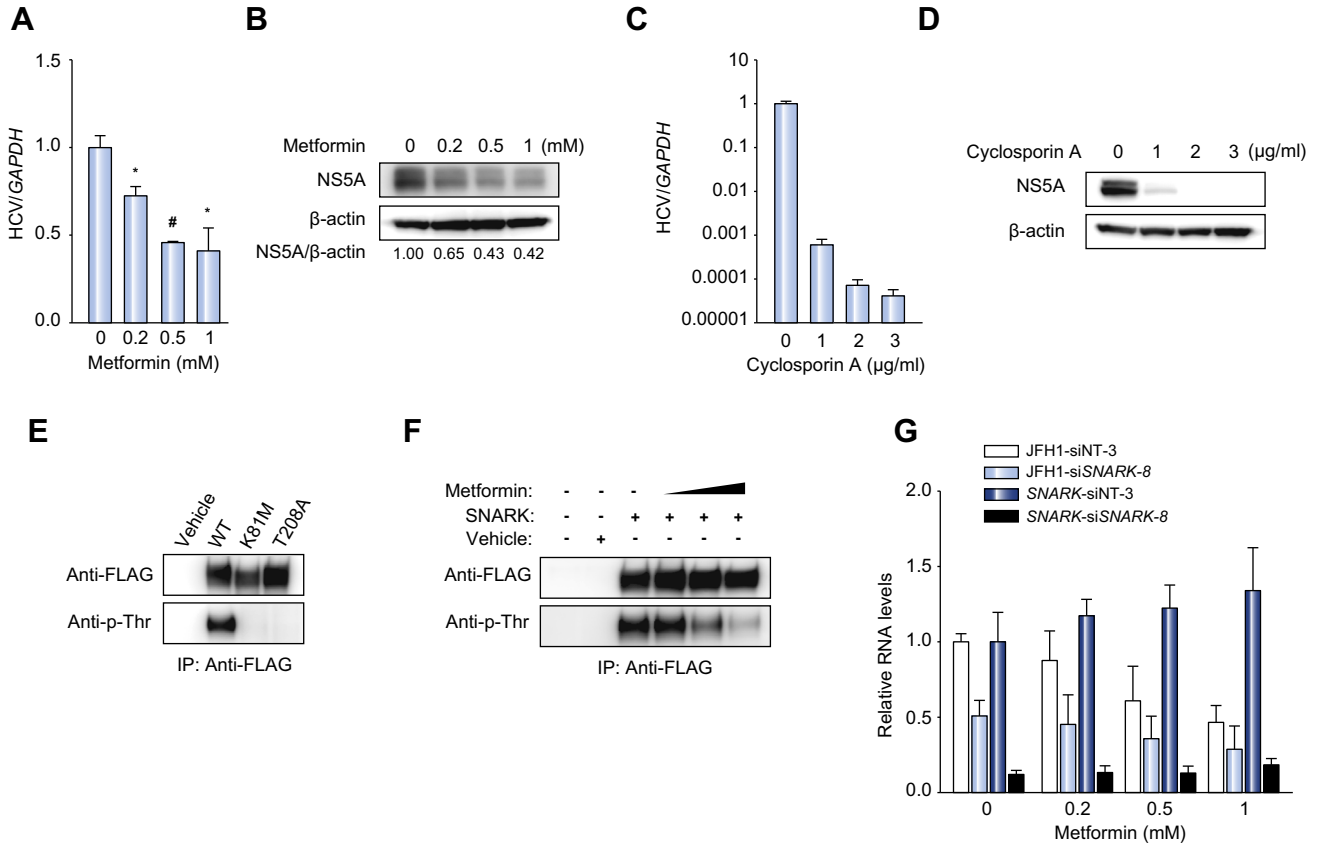


Fig. 2. Metformin suppressed HCV replication. HuH7.5.1 cells were infected with JFH1 and then treated with either metformin (A and B) or CsA (C and D) for 3 days. Densitometric values of NS5A normalized to those of β-actin were given as NS5A/β-actin. No significant cytotoxicity was observed by the compounds at the indicated concentrations. Relative HCV RNA level was quantified by real-time PCR and normalized to *GAPDH*; $^{*}p$ <0.05 or $^{\#}p$ <0.01 *vs.* untreated control. (E) In HuH7.5.1 cells either wild type or mutants (K81M or T208A) of FLAG-tagged SNARK were overexpressed and immunoprecipitated, followed by the detection with anti-FLAG or anti-phosphothreonine antibodies. (F) Wild type FLAG-tagged SNARK was overexpressed in the presence of metformin at 0.2, 0.5, and 1 mM in HuH7.5.1 cells and the levels of threonine phosphorylation were examined as indicated in (E). (G) HuH7.5.1 cells were transfected with either non-targeting (siNT-3) or *SNARK*-targeted (si*SNARK-8*) siRNAs. 48 hours later, cells were infected with JFH1 and treated with the indicated concentrations of metformin for 72 h. Relative mRNA levels for JFH1 or *SNARK* were quantified by real-time PCR and normalized to *GAPDH*.

[20], a well-known type 2 diabetes drug. In that setting, the kinase activity of SNARK was measured by incorporation of phosphate into SAMS peptide substrate, which was demonstrated to be significantly decreased by metformin treatment in the cell line. Here, we treated JFH1-infected HuH7.5.1 cells with metformin and observed moderate antiviral effects (Fig. 2A and B) with no cytotoxicity within the indicated dose range (data not shown). As a positive control, cyclosporin A (CsA) [21] strongly inhibited viral replication (Fig. 2C and D). To assess the phosphorylation level of SNARK subsequently, FLAG-tagged SNARK was overexpressed in HuH7.5.1 cells and immunoprecipitated for Western blot to monitor phosphothreonine levels [22]. While wild type SNARK was detected to be phosphorylated at threonine residue(s), neither K81M nor T208A mutant was (Fig. 2E), implying the autophosphorylation [23] and importance of threonine 208 as a phosphorylated site as reported [7]. Then we performed the assay using the wild type SNARK in the presence of metformin, which resulted in the reduced levels of phosphorylation dose-dependently (Fig. 2F). Here the data indicated that metformin suppressed SNARK phosphorylation, potentially interfering the phosphotransferase activity. The dose response of JFH1 to metformin was next examined when SNARK was knocked down by siRNAs. Metformin exerted dose-dependent antiviral effects in the cells transfected with non-targeting siRNAs, which was blunted by the siRNA-mediated reduction of SNARK expression (Fig. 2G). These data indicate that metformin's antiviral effect is mediated by inhibition of activated SNARK, bringing its full kinase activity, which may be a pharmacologic target for anti-HCV activity, and that metformin by itself could be a plausible component of a combination regimen targeting HCV.

SNARK is involved in TGF-β signaling

To investigate the possible roles of SNARK in viral pathogenesis based upon the induction of mRNA expression over the viral replication in cell culture (Supplementary Fig. 3), we also explored its involvement in downstream cellular signaling pathways. Intriguingly, SNARK appeared as an interactor with SMAD proteins in a high-throughput protein-protein interaction mapping study [17], which raised the distinct possibility that SNARK is involved in TGF-β signaling, the major profibrogenic pathway in the liver. Therefore, we first knocked down SNARK in HuH7.5.1 cells and assessed alterations in TGF-β signaling using an expression construct (PAI/L) encoding a luciferase reporter gene driven by promoter sequences of plasminogen activator inhibitor 1 (PAI-1), a transcriptional target of TGF-β [24]. siRNAs against *SNARK*

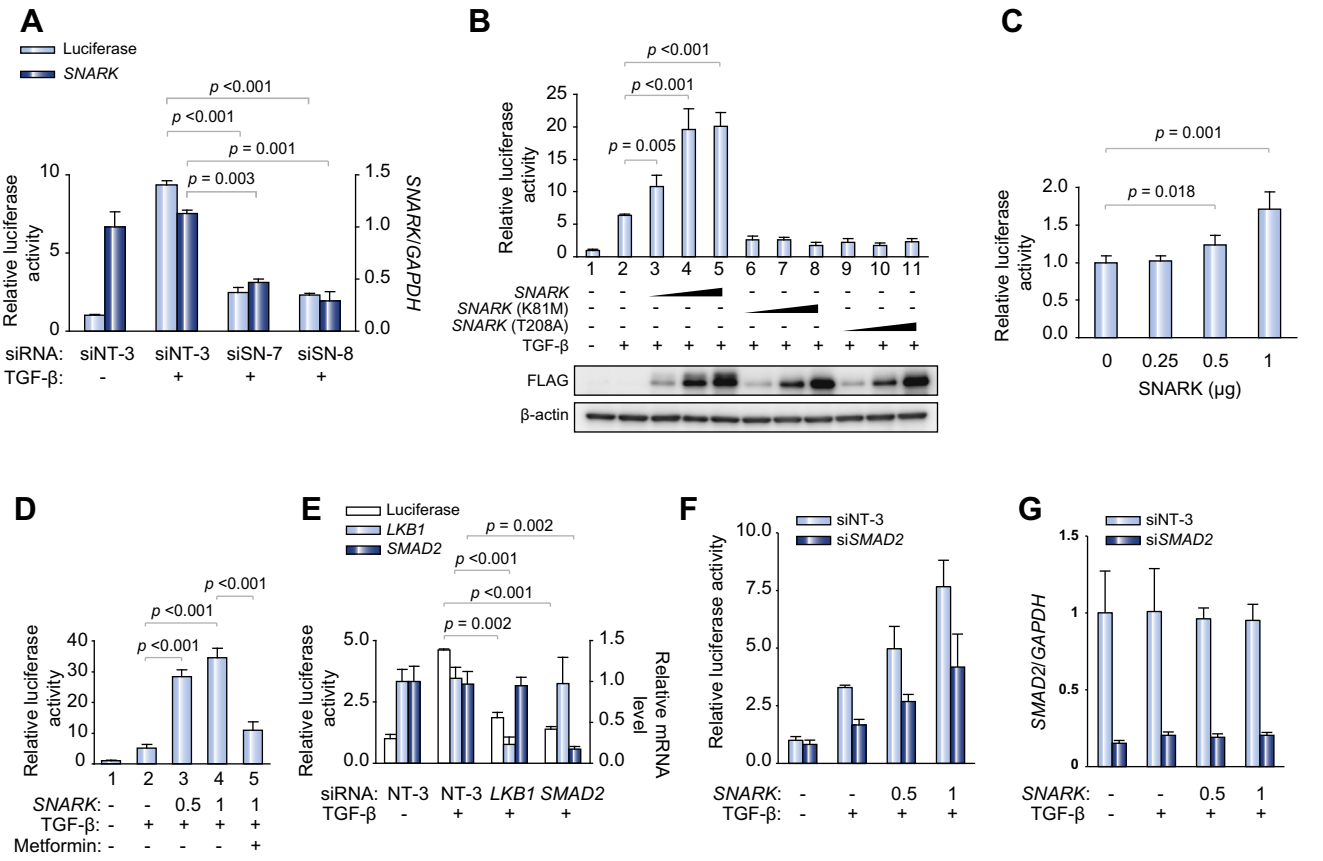


Fig. 3. SNARK accentuated TGF-β signaling. (A) HuH7.5.1 cells were transfected with siRNAs, which was followed by transfection of PAI/L reporter with pRL-TK 72 h later. On the next day, the cells were treated with TGF-β and lysed 24 hours later. The firefly and *Renilla* luciferase activities were measured and knockdown of *SNARK* was confirmed by real-time PCR with normalization to *GAPDH*. *P* Values were calculated as indicated. (B) Naïve HuH7.5.1 cells were transfected with either wild type (lanes 3–5) or mutant (lanes 6–11) SNARK expression plasmid at increasing doses (0.25, 0.5, and 1 μg) together with PAI/L reporter and pRL-TK. At 24 h post transfection, the cells were treated with TGF-β at 7 ng/ml for 20 h and lysed for dual luciferase assay. *P* Values were calculated as indicated. (C) HuH7.5.1 cells were transfected with increasing amount of wild type SNARK expression plasmid for dual luciferase assay as described in (A) without TGF-β. *P* Values were calculated as indicated. (D) HuH7.5.1 cells were transfected with increasing amount of SNARK expression plasmids (0.5 and 1 μg) together with PAI/L reporter and pRL-TK. On the next day, the cells were treated with TGF-β at 7 ng/ml and metformin at 1 mM (lane 5) for 20 h, and then lysed for dual luciferase assay. *P* Values were calculated as indicated. (E) HuH7.5.1 cells transfected with siRNAs against either *LKB1* or *SMAD2* for 72 h were transfected with PAI/L reporter and pRL-TK, which was followed by TGF-β treatment at 7 ng/ml for 20 h. Then the cells were lysed for dual luciferase assay and knockdown of targeted genes expression was confirmed by real-time PCR with normalization to *GAPDH*. *P* Values were calculated as indicated. (F) HuH7.5.1 cells transfected with siRNAs for 72 h were transfected with increasing amounts of wild type SNARK expression plasmid (0.5 and 1 μg) together with PAI/L reporter and pRL-TK. 24 h later, the cells were treated with TGF-β for 20 h, and then lysed for dual luciferase assay. (G) *SMAD2* knockdown levels were quantified by real-time PCR with normalization to *GAPDH*.

markedly reduced PAI/L luciferase activity and SNARK expression (Fig. 3A) in parallel, suggesting that SNARK is an important regulator of TGF-β signaling.

In order to elucidate the function of SNARK responsible for its contribution to TGF-β signaling, we assessed the effects of the overexpressed either wild type or mutant SNARK on TGF-β-stimulated PAI/L activity. In contrast to the dose-dependent increase of PAI/L activity by wild type SNARK, either kinase-dead K81M or unphosphorylated T208A mutant suppressed TGF-β-driven PAI/L activity (Fig. 3B). Moreover, the overexpression of SNARK in the absence of TGF-β moderately induced luciferase activity in HuH7.5.1 cells (Fig. 3C). These data demonstrate that both kinase activity and phosphorylation of SNARK are required for TGF-β signaling. Thereupon, in the same setting we treated HuH7.5.1 cells with metformin and found that SNARK-mediated stimulation of TGF-β signaling was inhibited by metformin (lane 5, Fig. 3D) though that was not the case in the absence of SNARK overexpression (Supplementary Fig. 5A) and the basal level of procollagen mRNA was not affected by metformin alone in HuH7.5.1 cells (Supplementary Fig. 5B), again underscoring that the kinase activity of SNARK is important for TGF-β signaling, and additionally raises the possibility that metformin may have utility as an anti-fibrotic agent in SNARK-facilitated pathogenesis.

We next examined regulators upstream and downstream of SNARK, depleting either liver kinase B1 (LKB1)/serine threonine kinase 11 (STK11), an upstream kinase of SNARK, or SMAD2, and assessed TGF-β-dependent PAI-1 luciferase activity. We found that knockdown of LKB1 abrogated PAI/L stimulation by TGF-β to the same extent as did SMAD2 knockdown (Fig. 3E). In addition, in contrast to luciferase activities in the presence of non-targeting siRNAs, overexpression of SNARK failed to rescue PAI/L activity in cells knocked down for either SMAD2 (Fig. 3F and G) or LKB1 (Supplementary Fig. 4). These data indicate that SNARK-mediated stimulation depends on SMAD2, and also that phosphorylation of SNARK by LKB1 and ensuing SNARK-mediated phosphorylation of downstream substrates, potentially in conjunction with SMAD2, are critical for TGF-β signaling.

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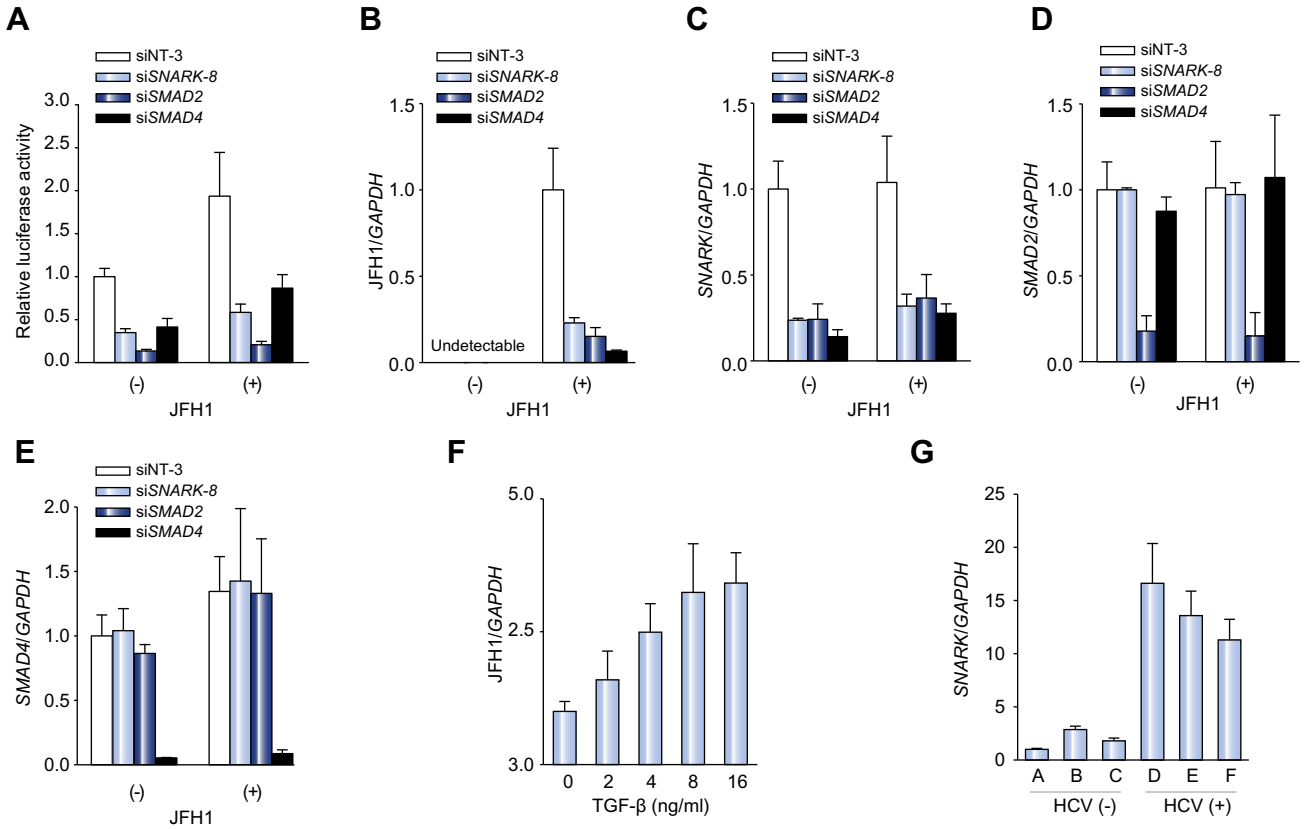


Fig. 4. Reciprocal regulation between SNARK and HCV in TGF-β signaling. (A) 48 hours after transfection of siRNAs, HuH7.5.1 cells were infected with JFH1, which was followed by transfection of PAI/L reporter 24 hours later and lysis 72 hours later for dual luciferase assay (A) and quantification of RNA levels for JFH1 (B), *SNARK* (C), *SMAD2* (D), and *SMAD4* (E) by real-time PCR with normalization to *GAPDH*. JFH1 RNA was not detected in the uninfected HuH7.5.1 cells (Undetectable, B). (F) 24 hours after the infection with JFH1, HuH7.5.1 cells were treated with TGF-β for 48 hours at indicated doses (0–16 ng/ml). Then cells were lysed and the viral RNA levels were measured by real-time PCR and normalized to *GAPDH*. (G) mRNA levels of *SNARK* in liver biopsies from patients were measured by real-time PCR and normalized to *GAPDH*. Patients A, B, and C were HCV-negative, and D, E, and F were infected with HCV. Details of patient characteristics are provided in Supplementary Table 2.

Reciprocal enhancement of TGF-β signaling and HCV infection via SNARK

Our data agree with the reported observation of TGF-β signaling elevated by HCV [14,25] and resultant proviral effects in a replicon cell line [15]. We therefore examined the effects of HCV replication on TGF-β signaling and immediate involvement of SNARK in the JFH1-HuH7.5.1 system. JFH1 replication (Fig. 4B) upregulated PAI/L luciferase activity, which was abolished by siRNA-mediated knockdown of SNARK (Fig. 4A and C), as was observed with the knockdown of either SMAD2 or SMAD4 (Fig. 4D and E). The data clearly demonstrate that SNARK expression plays a role in TGF-β signaling in JFH1 replication. Intriguingly, knockdown of either SMAD2 or SMAD4 led to the reduced expression of SNARK (Fig. 4C), implying converse regulation of SNARK by SMAD pathway. Next, the effects of enhanced TGF-β signaling on HCV replication were tested in this *bona fide* HCV infection system. JFH1-infected cells were treated with increasing quantities of TGF-β, and we observed that TGF-β enhanced viral replication in a dose-dependent fashion (Fig. 4F) with no cytotoxicity at the concentrations indicated (data not shown).

Lastly, SNARK expression was examined in human liver tissue to investigate its pathophysiological dynamics. The levels of *SNARK* mRNA were prominently elevated in HCV-infected patients in comparison to HCV-negative controls (Fig. 4G). These findings strongly suggest that HCV-mediated induction of SNARK facilitates both proviral and profibrogenic signaling of TGF-β, leading to reciprocal amplification of HCV and profibrogenic signals. Collectively, these factors could interact to accelerate hepatic fibrosis progression in HCV infection.

Discussion

SNARK is an AMPK-related kinase identified through our previous genome-wide RNAi screen as a host cellular cofactor for HCV replication [6]. Our present studies reveal an intersection of TGF-β-SMAD signaling, LKB1-AMPK-related kinase signaling, and viral replication, in which there is reciprocal stimulation. This convergence could well explain the relationship between HCV replication and its pathogenic effects observed *in vitro* and *in vivo*, providing strong support for the concept that the TGF-β signaling pathway is one of the key cellular pathways targeted by HCV to promote replication and may be therefore a future therapeutic target.

Growing numbers of host cellular cofactors for HCV replication have been discovered so far to support the viral lifecycle through interaction with viral proteins and alteration of host signaling pathways. Here we demonstrated that SNARK contributes to HCV replication through a reportedly proviral cytokine, TGF-β [15,26]. Simultaneously, the induction of SNARK by prolonged HCV replication in cell culture and patients demonstrates its reciprocal regulation by HCV. SNARK was transcriptionally upregulated in an NF-κB-dependent manner in a breast cancer cell line

[27] and was identified in a microarray analysis as the only kinase substantially induced in endothelial cells by tumor necrosis factor (TNF)-α, a well-known NF-κB activator [28]. Moreover, we and others reported that HCV infection activates NF-κB phosphorylation [29] and that NS5A stimulates NF-κB-dependent luciferase activity [30], respectively. Thus, SNARK expression may be transcriptionally induced by sustained HCV infection through virally-triggered NF-κB activation even though its basal expression may be quite low as illustrated by Western blot in HuH7.5.1 cells. Despite these low basal expression levels, the activation by infection of SNARK appears to enable further viral replication.

TGF-β is a pivotal cytokine and the central driver of hepatic fibrogenesis during HCV infection [31]. An increasing variety of proteins mediating TGF-β signaling have been described [32] and phosphorylation of SMAD2 and SMAD3 by non-TGF-βRI kinases such as Mps1 [33] and Rho/ROCK [34] also promoted TGF-β signaling. Therefore, SNARK associated with SMAD2 can be responsible for phosphorylation of SMAD2, enhancing SMAD-signaling. On the other hand, partial dependence of SNARK expression on SMADs observed in our knockdown experiments (Fig. 4C) may better explain reciprocal regulation between SNARK and TGF-β pathway. Possible modes of participation of SNARK in other phosphorylation-dependent processes in TGF-β signaling and TGF-β signaling-regulated SNARK expression, together with those in mediators in cross-talking pathways leading to epithelial-mesenchymal transition through cell dedifferentiation including Notch1, whose expression was mildly induced by SNARK overexpression in the presence of TGF-β in HuH7.5.1 cells (data not shown), remain an open subject for further investigation. On the other hand, SNARK alone was also capable of moderately inducing PAI-1 promoter-driven luciferase activity, underscoring its potential transcription-modulatory functions suggested by the nuclear localization of SNARK in HuH7.5.1 (Supplementary Fig. 1A and B), PLC/PRF/5, and HeLa cells [35], similarly to the HCV-activated transcription factor Elk1 [36], responsible for epidermal growth factor (EGF)-driven enhancement of PAI-1 expression [37].

Knockdown experiments in HuH7.5.1 cells demonstrate the importance of LKB1 in the TGF-β signaling pathway and in its promotion by SNARK, consistent with the decreased SMAD2 and TGF-β pathway activities in *Stk11*$^{-/-}$ mice, a model of Peutz-Jeghers syndrome [38]. It is tempting to conjecture critical roles of SNARK in HCV-induced liver disease in consideration of SNARK as a LKB1 signaling molecule directly activated by LKB1 in the TGF-β pathway. Currently, a wide variety of inhibitors of mediators in TGF-β/PAI-1 expression control are under clinical evaluation [39]. Thus SNARK, whose expression and activity are closely linked to TNF-α and TGF-β, activators of hepatic stellate cells [40], appears to be an attractive target for antifibrotic development.

Metformin has been widely used for the treatment of type 2 diabetes for 50 years [41], primarily decreasing hepatic glucose production [42] via AMPK activation [43]. Simultaneously, metformin was also revealed to exert multifaceted actions through AMPK-independent mechanisms targeting several kinases [44]. Indeed, the phosphotransferase activity of SNARK was inhibited by metformin in a human hepatocellular carcinoma cell line [20]. The phosphotransferase-dependent phosphorylation level of overexpressed SNARK was diminished by metformin in our immunoprecipitation assay as well, again indicating metformin-mediated inhibitory effects on SNARK phosphorylation partly via interference with its autophosphorylation. Hence it is rational that we observed an antiviral action of metformin against JFH1, and indeed a trial in Spain showed that the addition of metformin to standard anti-HCV treatment improved SVR [45], which suggests a possible productive application of metformin and SNARK inhibitors to the anti-HCV armamentarium. Furthermore, SNARK phosphotransferase activity-driven stimulation of TGF-β signaling in HuH7.5.1 cells allowed us to confirm the suppressive effects of metformin on TGF-β signaling accentuation by SNARK overexpression, implying SNARK as a target in HCV pathogenesis. Further studies to elucidate the mechanisms and consequences of inhibition of SNARK and metformin itself are warranted. Besides, since another pathogenic effect of HCV is insulin resistance, and type 2 diabetes is a risk factor for HCC [46], novel SNARK-mediated antiviral and antifibrotic properties of the antidiabetic metformin could offer an important and multifaceted agent for long term HCV disease management.

On the heels of development of anti-HCV agents targeting viral proteins, proviral host cellular cofactors have been discovered [3] and subsequent HTAs are emerging, best typified by cyclophilin (CyP) inhibitors [47], overcoming the drug resistance against virally-targeted inhibitors. In this study, we provide an example of a potential target for host-directed antiviral and antipathogenic therapies, which target a key host cellular cofactor involved not only in viral replication but also in viral pathogenesis. In fact, HCV modulates and depends on lipid metabolism enhancing lipogenesis for the establishment of efficient viral infection [48], and cholesterol-lowering statins were not only antiviral [49], but also effective in reducing steatosis and retarding fibrosis in viral and non-alcoholic fatty liver disease (NAFLD) patients [50], nicely exemplifying the notion above.

Taken together, our data overall suggest that SNARK is a novel host cellular factor for HCV replication and an additional mediator of TGF-β-SMAD signaling. Involvement of its activity as a kinase in proviral and pathogenic pathways positions SNARK as a potentially critical and druggable target for new therapies against hepatitis C.

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Conflict of interest

The authors who have taken part in this study declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jhep.2013. 06.025.

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Research Article

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