Long non-coding RNA GAS5 inhibited hepatitis C virus replication by binding viral NS3 protein

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\textbf{A R T I C L E  I N F O}

Article history:
Received 22 December 2015
Returned to author for revisions
16 February 2016
Accepted 21 February 2016
Available online 21 March 2016

Keywords:
Long non-coding RNA
GAS5
Hepatitis C virus
NS3

\textbf{A B S T R A C T}

HCV infection has a complex and dynamic process which involves a large number of viral and host factors. Long non-coding RNA GAS5 inhibits liver fibrosis and liver tumor migration and invasion. However, the contribution of GAS5 on HCV infection remains unknown. In this study, GAS5 was gradually upregulated during HCV infection in Huh7 cells. In addition, GAS5 attenuated virus replication with its 5‘ end sequences, as confirmed by different GAS5 truncations. Moreover, this 5‘ end sequences showed RNA-protein interaction with HCV NS3 protein that could act as a decoy to inhibit its functions, which contributed to the suppression of HCV replication. Finally, the innate immune responses remained low in HCV infected Huh7 cells, ruling out the possibility of GAS5 to modulate innate immunity. Thus, HCV stimulated endogenous GAS5 can suppress HCV infection by acting as HCV NS3 protein decoy, providing a potential role of GAS5 as a diagnostic or therapeutic target.

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1. Introduction

Hepatitis C virus (HCV) infection is a major global public health problem, costing billions per year in medical expenses (Cox, 2015). Although effective anti-HCV drugs such as interferon alpha (IFN-\(\alpha\)), ribavirin and direct acting antivirals (DAAs) were developed and put into clinical use, the lack of preventive approaches and the recessive nature of the disease and its development result in a large number of patients who are diagnosed at late stage of HCV infection with progressed liver disorders or even hepatocellular carcinoma (Ippolito et al., 2015; Kohli et al., 2014). Thus, studying the underlying mechanism of host–virus interaction is vital to solve this problem.

During HCV infection, multiple host genes play important roles. The identification of these crucial genes could shed light on HCV studies and contribute to an effective gene therapy against this disease. However, to date most studies only include and decipher the roles of protein-coding genes or microRNAs (Xu et al., 2014), neglecting large amounts of long non-coding RNAs (lncRNAs) which, in recent years, are reported to play various important roles during both physiological conditions and disease progressions (Clark and Blackshaw, 2014).

LncRNAs are non-coding RNAs (ncRNAs) of 200 bp to 100 kb in length with highly conserved sequences (Mattick, 2005). They regulate gene expression at multiple molecular levels, including, but not limited to, transcriptional or post-transcriptional modulation, competing endogenous microRNA, and chromatin modification (Barrandon et al., 2008; Khalil et al., 2009; Sivasubbu, 2014). Nevertheless, the roles of IncRNAs in viral life cycle, especially during HCV infection, have been rarely discussed. Growth arrest-specific 5 (GAS5) is a single-strand IncRNA which has been identified to relate with proliferation, tumorigenesis and embryonic development (Coccia et al., 1992; Ma et al., 2015; Mourtada-Maarabouni and Williams, 2013). It is worth noting that GAS5 has been found to suppress liver fibrogenesis and inhibit the migration and invasiveness of some hepatoma cell lines, which represent severe complications that may take places after HCV infection (Hu et al., 2015; Yu et al., 2015). Whether this tumor suppressor IncRNA regulates HCV infection still remains an intriguing and meaningful research issue to be determined.

By screening lncRNA profiles during HCV infection, we found that all of GAS5 transcripts detected in the high-throughput sequencing data were upregulated in infected Huh7 cells (data not shown). Our results indicated that IncRNA GAS5 suppressed HCV infection by interacting with viral NS3 protein to decoy its function.
Fig. 1. GAS5 is upregulated by HCV infection. (A) A schematic picture of GAS5 variants. (B) Huh7 cells were infected with HCVcc of JFH-1 (MOI = 1), and lysed at 48 h after infection to detect the expression levels of different GAS5 variants by qPCR. Results were shown as relative RNA level of the respective variants. Meanwhile, the expression level of GAS5 were also analyzed by northern blot using Digoxigenin labeled GAS5 anti-sense probe. Each lane was loaded with 2 μg total RNA extracts from Huh7 cells lysed at indicated time points after infection (0 h, 24 h, and 48 h). β-actin was introduced as a loading control. (C) FISH analysis of GAS5 in HCVcc infected Huh7 cells compared with uninfected group (NC). The nuclei were stained with DAPI. The scale bar represented 50 μm. (D) Nuclear-plasma separation assay of GAS5 in HCVcc infected Huh7 cells compared with uninfected group (NC). (E) Huh7 cells were infected with JFH-1 HCVcc of different MOIs, and the expression levels of GAS5 were determined by qPCR. (F) Huh7 cells were infected with HCVcc of JFH-1 (MOI = 1), and lysed at indicated time points after infection. The expression levels of GAS5 were tested by qPCR. ** p < 0.01 compared with the uninfected group.
Fig. 2. Overexpression of GAS5 inhibited HCV infection. Huh7 cells were transfected with 1 μg/ml of the plasmid encoding GAS-FL (ENST00000450589). The upregulation of GAS5 were tested by both qPCR (A) and northern blot (B). β-actin was introduced as a loading control. (C) Huh7 cells were transfected with 1 μg/ml of GAS5-FL, and subsequently infected with JFH-1 HCVcc (MOI=1). HCV core protein expression levels were tested by western-blotting 48 h post-infection. (D) Huh7 cells were transfected with indicated concentrations of GAS5-FL, and subsequently infected with HCVcc of JFH-1 (MOI=1). HCV RNA levels were detected by qPCR 48 h post-infection. Huh7 cells were transfected with 1 μg/ml of GAS5-FL, and subsequently infected with JFH-1 HCVcc of indicated MOIs. HCV RNA levels were detected by qPCR. Blank indicated the group without transfection. NCOE indicated the group transfected with the empty pcDNA-3.1 vector as a negative control. ** p < 0.01 compared with the negative control.
2. Results

2.1. GAS5 upregulation during HCV infection over time.

GAS5 has many transcript variants (Fig. 1A), so we first determined the expression profile of all these variants during HCV infection using quantitative polymerase chain reaction (qPCR). Huh7 cells were infected with HCVcc and lysed at 48 h after viral infection to detect the expression level of GAS5. As shown in Fig. 1B, most of the GAS5 transcripts were upregulated after HCV infection. Among them, the 651 bp transcript of GAS5 (ENST00000450589) was the most increased, since its level was more than 5-fold 48 h after infection. Northern blot at different time points (0 h, 24 h and 48 h) detected this transcript and confirmed its remarkable upregulation. Therefore, this...
transcript was used in our subsequent studies on the role of lncRNA on HCV infection.

To gain insights into its expression change during infection, we examined its cellular localization after HCV infection using fluorescence in situ hybridization (FISH). The uninfected and infected Huh7 cells were fixed and hybridized with a full length anti-sense GAS5 probe labeled with Digoxigenin (DIG) for further observation. The results suggested that GAS5 was upregulated and predominantly localized in the cytoplasm after HCV infection compared with mock infected group (Fig. 1C). To verify and confirm this result, we performed nuclear-cytoplasmic separation assay, and the subsequent qPCR confirmed the localization of GAS5 in the cytoplasm, highly increased in the infected cells compared with the uninfected cells (Fig. 1D).

To further clarify this correlation between GAS5 and HCV infection, we tested the expression levels of GAS5 on HCV infection at different MOIs, as well as change trends overtime. The results showed that GAS5 elevated with increased MOIs (Fig. 1E) and the eminent upregulation was observed from 12 h after infection and onward (Fig. 1F). These data suggested that GAS5 was stimulated by HCV infection and predominantly enriched in the cytoplasm of Huh7 cells.

2.2. GAS5 overexpression inhibited HCV infection

In order to unveil the function of GAS5 in HCV infection, Huh7 cells were transfected with plasmids encoding GAS5. GAS5 overexpression after transfection is confirmed by both qPCR and northern blot (Fig. 2A and B). The cells were subsequently infected with HCVcc to test effect of GAS5 on it. The gain-of-function studies showed that GAS5 overexpression significantly inhibited viral core protein expression (Fig. 2C). Moreover, HCV infection was suppressed by GAS5 in a dose-dependent manner, manifesting as the reduced HCV RNA level along with the increased amount of GAS5 transfected plasmid (Fig. 2D). GAS5 also affected virus infection with various infective doses, which was confirmed by the qPCR results and NS5A immunofluorescent assay, since the inhibitory effect was in line with different MOIs used (Fig. 2E), and GAS5 overexpression decreased infected colony number in Huh7 cells significantly (Fig. 2F and G). Time course change of HCV RNA levels was also evaluated in GAS5 upregulated cells, confirming that antiviral activity of GAS5 was along with viral infection (Figs. 2H and S1A).

Besides, the inhibitory effect of GAS5 on HCV infection was consistent in HCVcc inoculation of both JFH-1 and J6/JFH1 strains (Fig. S1B), while upregulation of GAS5 did not affect dengue virus (DENV) or enterovirus 71 (EV71) infection in host cells (Fig. S1C). These results indicated that HCV infection was significantly inhibited in Huh7 cells through GAS5 gain-of-function specifically.

2.3. GAS5 knockdown promoted HCV infection.

As for the loss-of-function assay, small interfering RNAs (siRNAs) specifically targeting GAS5 transcript were used to test their efficiency to downregulate GAS5 (Fig. 3A). The two effective siRNAs were mixed and used for GAS5 knockdown in our further experiments. Huh7 cells were transfected with siRNAs, followed by HCVcc inoculation. Consistent with the previous results in the gain-of-function assay, GAS5 downregulation promoted virus infection to more than 2-fold as shown in core protein expression (Fig. 3B), and increased viral RNA levels significantly with the elevated transfected siRNAs (Fig. 3C). The facilitation of virus infection was also clear at different MOIs when GAS5 was knocked down (Fig. 3D). In addition, we found that the colony number in the GAS5 knockdown group was obviously higher compared with the control group at different MOIs (Fig. 3E and F). Time course of HCV infection assay suggested that reduced GAS5 promoted viral infection significantly after 24 h post-infection (Figs. 3G and S1A), and this effect was identical in both JFH-1 and J6/JFH1 HCV infection (Fig. S1B). However, GAS5 knockdown showed no effect on DENV or EV71 infection (Fig. S1C). Taken together, our results showed that GAS5 endogenous upregulation indeed protected Huh7 cells from HCV infection, ruling out a more general role for GAS5 in the antiviral response.

2.4. GAS5 did not affect virus entry, but suppressed HCV replication

Our next step was to understand at which level GAS5 protection against HCV infection was exerted. HCV pseudoparticles (HCVpp) were used to evaluate the effect of GAS5 on viral entry. Huh7 cells transfected with either full length GAS5 plasmid or GAS5 specific siRNA were treated with HCVpp to evaluate their ability to enter into the cells. Compared with the mock treated group, none of the treatments was able to efficiently block HCVpp entrance into the cells, indicating that GAS5’s inhibitory effect on HCV infection was not exerted during viral entry stage (Fig. 4A and B).

We further examined the role of GAS5 on virus replication. HCV replicon cells which only supported virus replication were used. As shown in Fig. 4C, GAS5 potently reduced HCV RNA levels in replicon cells in a GAS5 concentration-dependent manner, while its downregulation facilitated virus replication in a dose-dependent manner (Fig. 4D), indicating that GAS5 might play a vital role in HCV replication.

Since previous studies mainly showed the interactions of GAS5 with host factors (Kino et al., 2010; Pickard and Williams, 2015; Zhang et al., 2013b), we performed RNA-protein interaction on global RNA/DNA binding proteins using the catRAMP omics algorithm to predict GAS5’s potential targets when affecting HCV infection (Bellucci et al., 2011). We categorized the predicted host RNA/DNA binding proteins according to their functions using Gene Ontology analysis (Fig. S1D), and found that no HCV relevant GO terms were enriched in the analysis, suggesting that GAS5 might take effect by affecting replication-associated viral proteins directly.

2.5. GAS5 directly bound HCV NS3 protein with its 5' end sequences

It was previously reported that GAS5 competitively bound the DNA-binding domain of glucocorticoid receptor (GR) to modulate GR transcriptional activity (Kino et al., 2010; Lucofo et al., 2015). However, the increased GAS5 accumulation in the cytoplasm suppressed viral replication after HCV infection, phenomenon that also took place in the cytoplasm. We therefore wondered whether the cytoplasmic GAS5 would act as decoy of specific key viral proteins involved in HCV replication. Therefore, we constructed plasmids encoding full length (FL) or truncations of GAS5 (GAS5-451, GAS5-251), as well as a vector encoding GAS5 GR-binding sites (GAS5-GR, Fig. 5A), to discover the segment contributing to the HCV infection inhibition. Huh7 cells were transfected with the above GAS5 fragments and then infected with HCVcc. Our results showed that both the FL and GAS5 truncation fragments displayed a significant inhibitory effect on HCV infection by reducing HCV RNA level (Fig. 5B). However, GAS5-GR fragment did not contribute to the GAS5-mediated inhibition of HCV infection (Fig. 5B).

Next, to determine which site of GAS5 binds the viral protein and which viral protein GAS5 targets, we used again the RNA-protein interaction prediction algorithm to test GAS5 binding ability with the replication relative viral proteins. As shown in Figs. 5C and S1E, only the front 200 nucleotides of GAS5 possessed a relatively high interaction score with HCV NS3 and NS4 protein. Furthermore, the RNA structure analysis showed that the 1-
250 bp of GAS5 contained strong secondary structures which might represent the binding structure with the target protein (Fig. 5D).

In order to further confirm the binding of GAS5 to the viral protein, we performed the RNA immunoprecipitation (RIP) assay to investigate whether GAS5 directly bound NS3 or NS4. We constructed MS2 binding sequences (MS2bs) with GAS5 or its truncations or GR domain (MS2bs-FL, MS2bs-451, MS2bs-251, or MS2bs-GR), and they were co-transfected into the Huh7 cells which were subsequently infected with HCVcc with MS2 binding protein fused with YFP (MS2bp-YFP) expression vectors to pull down the transcript with an anti YFP antibody (Fig. 5E). The western-blotting analysis with specific viral protein antibodies suggested that NS3 protein is enriched in the GAS5 and its truncations’ pull down products compared with the control group, while GAS5-GR did not precipitate any product (Figs. 5F and S1F). These results indicated that GAS5 suppressed HCV replication probably acting as a decoy precluding the access of the key viral protease NS3 which was essential for viral replication with its 5’ end 200 sequences.

2.6. GAS5 5’ end sequences acted as a decoy on NS3 during HCV replication

To confirm the role of these sequences in suppressing HCV infection, we constructed GAS5 truncation which was lacking the 200 sequences (ΔGAS5). As shown in Fig. 6A, the qPCR analysis revealed that the truncation transcript lost the inhibitory effect on HCV infection compared with GAS5-FL group.

It is well known that HCV inhibits IFN pathway through NS3 and NS4 proteins to escape innate immunity (Cao et al., 2015; Cheng et al., 2006; Kang et al., 2013; Li et al., 2005). Thus, we tested the expression level of relevant factors involved in the IFN pathway, and we found that the GAS5-FL could increase the mRNA level of IRF-7, IPS-1, TRIF, while the ΔGAS5 did not have such effect (Fig. 6B). Nevertheless, the secreted level of IFN-α and IFN-λ1 still remained in a barely detectable level (Fig. 6C), ruling out the possibility of GAS5 to modulate innate immunity to block virus infection.

To determine the effect of GR inhibiting function of GAS5, we activate GR with dexamethasone (DEX). However, the increased dose of DEX did not affect HCV infection level as well as GAS5-GR.
Fig. 5. GAS5 binds HCV NS3 protein directly with its 5’ end sequences. (A) A schematic picture of GAS5 full length (GAS5-FL) or truncations (GAS5-451, GAS5-251), or a vector encoding GR-binding sites of GAS5 (GAS5-GR). (B) Huh7 cells were transfected with GAS5-FL or different truncations or GAS5-GR, and subsequently infected with HCVcc of JFH-1. HCV RNA levels were detected by qPCR. ** p < 0.01 compared with the negative group. The expression of full length and truncated GAS5 was confirmed by Northern blot analysis. (C) RNA-protein interaction prediction algorithm to test GAS5 binding ability with HCV NS3 by catRAPID omics algorithm. (D) The secondary structures of GAS5 from 1 to 250 bp. (E) A schematic picture of MS2 binding sequences (MS2bs) with GAS5 or its truncations or GR domain (MS2bs-FL, MS2bs-451, MS2bs-251, or MS2bs-GR). (F) RIP analysis of the GAS5 precipitated NS3.
Fig. 6. GAS5 5′ end sequences acted as a decoy on NS3. (A) A schematic picture of GAS5-FL and truncation which was lacking the 200 sequences (ΔGAS5) (above). Huh7 cells were transfected with GAS5-FL or ΔGAS5, and subsequently infected with HCVcc of JFH-1. HCV RNA level and mRNA levels of IFN pathway related proteins (IRF-3, IRF-7, IPS-1, TRIF) (B) were detected by qPCR. (C) The supernatants of the above cells were collected and the levels of IFN-α and IFN-λ1 were detected by ELISA (Biolegend). (D) Huh7 cells were transfected with GAS5-FL or GAS5-GR, and subsequently infected with HCVcc of JFH-1. Meanwhile, GR was activate with increased dose of DEX. HCV RNA level were detected by qPCR. (E) Cytotoxicity of GAS5 on Huh7 cells, HepG2 cells and primary human hepatocytes by CCK8 kit. (F) A summary of GAS5 function in HCV infection. ** p < 0.01 compared with the negative group.
overexpression (Fig. 6D), excluding the role of GAS5 GR binding domain in HCV infection.

We also examined the effect of GAS5 on the cleavage of HCV polyproteins. As shown in Fig. S1G, overexpression of GAS5-Fl or GAS5-251 reduced viral NS5A levels, while the polyproteins remained barely the same, suggesting that the cleavage of viral polyproteins was retarded probably due to the decay of GAS5 on NS3 protease function.

Finally, we evaluated the cytotoxicity of GAS5 on several hepatoma cell lines and primary hepatocytes. As shown in Fig. 6E, the transfection of GAS5 caused low toxic effect on Huh7 cells and HepG2 cells, while primary hepatocytes showed no toxic effect (PPHs). Besides, overexpression or downregulation of GAS5 did not affect cell proliferation (Fig. S1H). These results indicated that GAS5 might be of potential clinical significance in HCV infection.

Together, these results indicated HCV infection-stimulated GAS5 expression could in turn inhibit HCV replication by using its 5’ end sequence as NS3 binding decoy (Fig. 6F).

3. Discussion

HCV infection is a sophisticated pathological process which involves multiple viral and host factors to facilitate the infection. On the other hand, the host defense system utilizes the available defense strategies to combat and control the spread of the pathogen. LncRNAs have been considered to play important roles in regulating various aspects of biological or pathological processes including antiviral response, mainly by affecting protein abundance and localization at different molecular levels. Some lncRNAs are correlated to several virus infection, such as metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) upregulation in human papilloma virus (HPV) infection, human immunodeficiency virus (HIV)’s manipulation of nuclear paraspeckle assembly transcript 1 (NEAT1) and lincRNA-p21 in innate immunity (Barichievy et al., 2015; Jiang et al., 2014; Zhang et al., 2013a). GAS5 possesses a tumor suppressor property, and was downregulated in multiple cancers (Mourtada-Maarabouni et al., 2009; Shi et al., 2015; Sun et al., 2014; Tu et al., 2014). In addition, in recent years the molecular mechanism of GAS5 on gene expression or protein functioning has been elucidated (Pickard and Williams, 2015). GAS5 was thought to competitively bind to DNA-binding domains of certain steroid hormone receptors like GR and decoy the relevant function of the receptor (Hudson et al., 2014; Kino et al., 2010). Besides, GAS5 is able to sequestrate miR-21, affecting cell survival (Zhang et al., 2013b). However, the role of GAS5 in pathogen infection has never been discussed.

In our study, GAS5 expression was upregulated along with the progression of HCV infection. This lncRNA localized predominantly in the cytoplasm after HCV infection, and played an important role in viral replication which also took place in the cytoplasm. The RNA-protein prediction data suggested that less HCV relevant host factors were involved in the interaction with GAS5. We therefore considered the possibility of GAS5 to interact with viral proteins, especially those essential for viral replication. The prediction results suggested that GAS5 might bind to HCV NS3 or NS4 with the front 200 sequences, consistent with the discovery that all GAS5 truncations, including GAS5-251 exerted an anti-HCV activity. The RIP experiment mediated by MS2bp and the function loss on the truncation deprived of the 5’ end 200 sequences further demonstrated the role of GAS5-200 as an essential sequence binding HCV NS3 to exert an anti-HCV effect. Since the known GAS5 molecular mechanism is mainly referred to GR decoy and miR-21 sequestration during innate immune responses, we evaluated the role of IFN pathway which represents a key response of innate immunity during HCV infection after GAS5 overexpression.

Intriguingly, although the mRNA levels of IFN pathway related proteins was elevated, both IFN-α and IFN-λ levels in the supernatants of the above cells remained low, excluding the possibility for GAS5 to inhibit HCV infection by modulating innate immunity in Huh7 cells. We assumed that the inhibitory effect on mRNA levels of IFN pathway related proteins might be partially due to the decoy on HCV NS5 which inhibited IFN pathway. Nevertheless, GAS5 blocked HCV polyproteins processing, further verifying the decoy role of GAS5 on viral NS3 functions.

Further studies to better understand lncRNAs functions in diverse pathological processes including virus infection are of fundamental importance. GAS5 suppressed HCV infection working as an endogenous barrier against pathogen invasion, offering new insights for the clarification of HCV infection pathogenesis, therefore inspiring novel diagnoses or therapies against it. Besides, GAS5 inhibits liver fibrogenesis and migration or invasion of hepatoma cells (Hu et al., 2015; Tao et al., 2015; Yu et al., 2015), enhancing the research value of this lncRNA in HCV infection, progression and complications.

In summary, our findings suggested that lncRNA GAS5 elevated actively after HCV infection, and inhibited viral replication by decoying HCV NS3 protein. The GAS5 molecular mechanism against HCV infection that we described is novel and expanded the understanding of virus-host interaction. Our results also indicated the potential of lncRNAs as prospective novel biomarkers for diagnosis, therapeutic target and prognosis for HCV infection.

4. Materials and methods

4.1. Cells and reagents

Human hepatoma Huh7 and HepG2 cells, baby hamster Syrian kidney (BHK-21) cells and human rhabdomyosarcoma (RD) cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% Fetal bovine serum (FBS), 1 × non-essential amino acids (NAAs), 100 IU/ml of streptomycin and penicillin, 2 mM l-glutamine (GIBCO-Invitrogen, Carlsbad, CA). Primary human hepatocytes (PHHs) (#5200) were from ScienCell Research Laboratories (San Diego, CA) and cultured according to the manufacturer’s instructions (Nahmias et al., 2006). Dexamethasone was from Sigma-Aldrich (St. Louis, MO). 4,6-Diamidino-2-phenylindole (DAPI) was purchased from Invitrogen (Carlsbad, CA). Anti-HCV NS5A monoclonal antibody (mAb) from ViroGen Corporation (Watertown, MA), and anti-HCV NS5, NS4 and core antibody from Abcam (Cambridge, MA) was used in the study. Alexa 488- and horseradish peroxidase (HRP) conjugated anti-mouse IgG were obtained from Invitrogen (Carlsbad, CA).

4.2. Plasmids construction and transfection

The cDNA encoding full length and truncated transcripts of GAS5 was synthesized (Jie Li Biology, China) and subcloned into the Xho I and Eco R I sites of pcDNA3.1 vector (Invitrogen).

Transfection was performed with FuGene HD transfection reagent (Promega, Madison, WI) according to the manufacturer’s instructions. Briefly, 1 × 105 Huh7 cells were seeded in 12-well plates and transfected with indicated plasmids or siRNA (GenePharma Corporation, China).

4.3. Generation of cell culture-derived HCV (HCVcc) and infection assay

The plasmid encoding Japanese fulminant hepatitis type 1 (JFH-1) was obtained from T. Watanuki (National Institute of Infectious Diseases, Tokyo, Japan), and used to produce HCVcc of JFH-1 as...
described previously (Zhong et al., 2005). Briefly, the plasmid of JFH-1 was linearized and in vitro transcribed using MEGAscript kit (Promega, Madison, WI) to produce viral RNA. Huh7 cells were then transfected with pure HCV RNA by electroporation, and the supernatants were collected 5 days after transfection. The supernatants were further concentrated and purified in accordance with a previous protocol (Wakita et al., 2005). For infection assay, Huh7 cells were infected with HCVcc of JFH-1 at 37 °C for 6 h before the supernatants were changed with fresh medium. The immunofluorescent (IF) assay of infected cells were performed 48 h after virus inoculation using anti-NSSA antibody according to a previous study (Zhong et al., 2005).

4.4. Generation of DENV and EV71

DENV-2 New Guinea C strain was a gift from Jing An of Capital Medical University, and was propagated and conserved in our lab. The titers of DENV-2 were determined in BHK-21 cells by plaque assay according to a previous report (Mosso et al., 2008). EV71 was propagated in RD cells and the virus titers were tested by viral infectious plaque assay as described previously (Zhu et al., 2015).

4.5. Generation of HCV pseudoparticles (HCVpp) and entry assay

HCVpp bearing genotype 1a HCV E1/E2 (H77) were generated according to a previous study (Guan et al., 2012; Zhu et al., 2012). Briefly, the human embryonic kidney 293T cells were co-transfected with the plasmids of HCV envelope protein, Gag/Pol, Rev and p lentiv which contained the green fluorescent protein (GFP) gene. The cell supernatants were collected 48 h post-transfection and filtered through a 0.45 μm membrane. The plasmid encoding H77 envelope protein was provided by F.L. Cosset (INSERM U758, Lyon, France). For entry assay, Huh7 cells were incubated with HCVpp of H77 at 37 °C for 6 h before the supernatants were changed with fresh medium. The entry of HCVpp was determined by flow cytometry 72 h later as described previously (Bartosch et al., 2003).

4.6. Real time quantitative PCR (RT-qPCR) and northern blot

Total RNA was extracted using TRIzol Reagent (Invitrogen) followed the manufacturer’s instructions, and reverse transcribed with PrimeScript™ RT Master Mix Kit (Takara, China). RT-qPCR was performed with SYBR Premix Ex Taq™ (Takara, China), and GAPDH was utilized as mRNA endogenous control. See Supplementary data 1 for primer sequence.

Northern blot was performed with antisense probes labeled by Digoxigenin (DIG)-UTP (Roche, Indianapolis, Indiana) (Supplementary data 1). Total RNA was extracted by TRIzol Reagent (Invitrogen), and separated by 12% denaturing RNA PAGE which was subsequently transferred to a positively charged nylon membrane (GE Healthcare) through wet electro-transfer-blotting. The membrane was then crosslinked with ultraviolet for 3 min, prehybridized at 55 °C for 1 h, and hybridized with GASS complementary probes at 60 °C overnight. Next day, the membrane was washed with 2 × SSC 10 min for twice, and with 0.2 × SSC 10 min for once. Then it was incubated with AP-conjugated anti-DIG antibody, and exposed under Illuminator™ Chemiluminescent Detection System (Stratagene) using CDP-STAR (Roche, Indianapolis, Indiana).

4.7. Fluorescence in situ hybridization (FISH)

The FISH assay was conducted according to a protocol described previously (Wang et al., 2013). RNA probes for GASS were labeled with DIG-UTP (Roche, Indianapolis, Indiana) with mMMESSAGE T7 In Vitro Transcription Kit (Ambion) according to the manufacturer’s instructions.

4.8. RNA immunoprecipitation (RIP)

MS2bs-MS2bp related RIP was performed according to the protocol as described previously (Lee et al., 2010) by using the EZ-Magna RIP Kit (Millipore, Billerica, MA).

4.9. Prediction of RNA-protein interaction

CatRAPID (http://s.tartaglialab.com/) protein/RNA interaction algorithm was employed to predict the possibilities of GASS’s binding proteins. The NR_002578.2 transcript of GASS was used as RNA input, individual protein sequences were used as protein input, catRAPID fragment analysis was used to generate the score and graph. For predicting all possible binding proteins of GASS, we used the catRAPID omics analysis. Homo sapiens proteins were chose as input, and a list of possible binding proteins and a binding score was generated. Further function categorization of the predicted proteins were analyzed using David annotation analysis (https://david.ncifcrf.gov/home.jsp), and the enrichment of Gene Ontology terms were generated, only terms that has a p value lower than 0.01 were selected. See supplementary data 2 for detailed results.

4.10. Viability assay

Huh7 cells, HepG2 cells and PHHs were transfected with GASS, and viability assay was performed 24 h after transfection, using Cell Counting Kit-8 (CCK8) (Beyotime Biotechnology, China).

4.11. Statistical analysis

The bar and curve graphs showed mean and standard deviation of at least three independent experiments. The statistical analyses were performed by Statistical Product and Service Solutions (SPSS) 17.0. A p value which was less than 0.05 was considered as statistically significant.

Author contributions

Xijing Qian: Conception and design, collection of data, data analysis and interpretation, manuscript writing.
Chen Xu: Provision of study material, data analysis and interpretation, collection of data, manuscript writing.
Ping Zhao: Experiments and collection of data.
Zhongtian Qi: Conception and design, data analysis and interpretation, financial support, administrative support, final approval of manuscript.

Disclosure of potential conflicts of interest

The authors indicated no potential conflicts of interest.

Acknowledgments

We thank Prof. C. M. Rice, J. Zhong, F. L. Cosset and T. Wakita for gift of research materials. This study was supported by research grants from the National S&T Major Project for Infectious Diseases Control (2012ZX10002003-004-010), National Natural Science
Foundation of China (Nos. 81273557, 81302812 and 81521091), Shanghai Municipal Natural Science Foundation (13ZR1449300).

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.virol.2016.02.020.

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