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A functional genomic screen reveals novel host genes that mediate interferon-alpha's effects against hepatitis C virus

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Abstract

Background & Aims—The precise mechanisms by which IFN exerts its antiviral effect against HCV have not yet been elucidated. We sought to identify host genes that mediate the antiviral effect of IFN- α by conducting a whole-genome siRNA library screen.

Methods—High throughput screening was performed using an HCV genotype 1b replicon, pRep-Feo. Those pools with replicate robust Z scores ≥ 2.0 entered secondary validation in full-length OR6 replicon cells. Huh7.5.1 cells infected with JFH1 were then used to validate the rescue efficacy of selected genes for HCV replication under IFN- α treatment.

Results—We identified and confirmed 93 human genes involved in the IFN- α anti-HCV effect using a whole-genome siRNA library. Gene ontology analysis revealed that mRNA processing (23 genes, $P=2.756e-22$), translation initiation (9 genes, $P=2.42e-6$), and IFN signaling (5 genes, $P=1.00e-3$) were the most enriched functional groups. Nine genes were components of U4/U6.U5 tri-snRNP. We confirmed that silencing squamous cell carcinoma antigen recognized by T cells (SART1), a specific factor of tri-snRNP, abrogates IFN- α 's suppressive effects against HCV in both replicon cells and JFH1 infectious cells. We further found that SART1 was not an IFN- α inducible, and its anti-HCV effector in the JFH1 infectious model was through regulation of interferon stimulated genes (ISGs) with or without IFN- α .

Conclusions—We identified 93 genes that mediate the anti-HCV effect of IFN- α through genome-wide siRNA screening; 23 and 9 genes were involved in mRNA processing and

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translation initiation, respectively. These findings reveal an unexpected role for mRNA processing in generation of the antiviral state, and suggest a new avenue for therapeutic development in HCV.

Keywords

Hepatitis C Virus, HCV; Interferon- α , IFN- α ; Small interfering RNA, siRNA; Squamous cell carcinoma Antigen Recognized by T cells, SART1; U4/U6.U5 tri-small nuclear ribonucleoproteins, U4/U6.U5 tri-snRNP

Introduction

There are 170 million persons worldwide and nearly 4 million persons in the United States chronically infected by the hepatitis C virus (HCV) [1, 2]. Interferon alpha (IFN- α) – based therapy is currently the standard of care for chronic hepatitis C, but only about 50% of patients are able to achieve sustained virologic response (SVR), and therapy is laden with significant toxicity [3]. There is thus a great need for alternative approaches to current therapy.

IFN- α is a broadly acting antiviral agent with pleiotropic effects and has been used for HCV treatment for over two decades [4, 5]. Exogenous IFN- α binds to the IFN- α receptor (IFNAR) complex at the cell surface, leading to activation of the Janus kinase/signal transducers and activators of transcription (JAK-STAT) pathway, which subsequently induces the expression of over 300 IFN-stimulated genes (ISGs) [6, 7]. These ISGs, which have not been completely characterized, encode antiviral effector molecules that also directly affect protein synthesis, cell growth/survival, and apoptosis functions. For instance, ISG 15 (IFN-stimulated protein of 15 kDa), the GTPase Mx1 (myxovirus resistance 1), ribonuclease L (RNase L), and protein kinase R (PKR) have been well-characterized antiviral effectors [8] for other viruses. Apart from the JAK/STAT signaling pathway, the p38 mitogen-activated protein kinase (MAPK) [9] and phosphoinositide 3 kinases (PI3K) [10] pathways have been found to be critical for alternative responses to IFN- α .

The precise effectors that mediate the antiviral effects of IFN- α against HCV remain unknown. Identifying these interferon effector genes will be critical for elucidating the mechanism of the anti-HCV effect of IFN- α , and, it is hoped, sparing unwanted systemic effects of IFN- α . In this study, we identified host genes that mediate the anti-HCV effect of IFN- α using a functional genomic approach in hepatocyte-derived cell lines.

Materials and Methods

Cell culture

The genotype 1b Huh7/Rep-Feo subgenomic HCV replicon (Feo) cells encode firefly luciferase, which can maintain a stable luminescence signal for over 25 minutes [11]. Feo cells have been previously demonstrated to be particularly well suited to high-throughput small molecule and siRNA library screening studies [12]. For secondary validation, we used the genotype 1b OR6 full-length HCV replicon cell line [13]. These cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/mL of penicillin, 100 μ g/mL of streptomycin, and 500 μ g/mL G418 and cultured at 37°C, 5% CO₂.

Huh7.5.1 cells (human hepatocellular carcinoma) were grown in DMEM supplemented with 10% FBS. The cell culture infectious genotype 2a JFH1 HCV plasmid was used [14] and infection performed as previously described [12, 15].

Pilot Experiments

Two thousand Huh7/Rep-Feo cells (or 3000 OR6 cells) were seeded in each well of a 384-well low flange white flat bottom microplate (Corning, #3570) (or 96-well flat clear bottom white microplate (Corning, #3610) for OR6 cells). After 72 hours incubation, the cells were treated with varying doses of PEG-IFN α -2b (PEG-IFN α -2b, Schering-Plough Corporation, Kenilworth, NJ) for 24 hours. Firefly (or *Renilla* for OR6) luciferase activities were detected by the Bright-Glo assay system (or *Renilla* Luciferase assay system) (Promega, Madison, WI) according to the manufacturer's instructions. Cell viability (cellular ATP content) was measured in replicate plates by using CellTiter-Glo reagent (Promega, Madison, WI) according to the manufacturer's instructions.

Small interfering RNA (siRNA) targeting IFNAR1 (Dharmacon SMART pool M-020209-00) and non-targeting siRNA (AllStars negative control siRNA, QIAGEN; Valencia, CA) were reverse transfected at a 50 nM final concentration into 2000 Huh7/Rep-Feo (or 3000 OR6) replicon cells per well in 384-well (or 96-well) microplates by using HiPerFect Transfection Reagent (QIAGEN; Valencia, CA) according to the manufacturer's instructions. After 72 hours incubation, Huh7/Rep-Feo (or OR6) cells were treated with 5 IU/mL (or 30 IU/mL for OR6) PEG-IFN α -2b in 5 μ L/well (or 10 μ L/well for OR6) DMEM, or an equal volume of culture medium alone. Luciferase activity and cell viability were measured after 24 hours.

Primary screening

High throughput screening was performed at the Institute of Chemistry and Cell Biology (ICCB) at Harvard Medical School. The siARRAY Human Genome siRNA Library (Dharmacon; Lafayette, CO) targets 21,094 human genes in the NCBI RefSeq database. siRNAs were reverse transfected into 2000 Huh7/Rep-Feo cells/well at a 50nM final concentration by using HiPerFect Transfection Reagent according to the manufacturer's instructions in quadruplicate 384-well microplates. A positive control siRNA SmartPool against IFNAR1 and non-targeting siRNA were added to empty wells on each plate. After 72 hours incubation, PEG-IFN α -2b was added to 2 screening plates to a final concentration of 5 IU/mL, while the other 2 plates were treated with culture media. Firefly luciferase activity was measured 24 hours later using Bright-Glo assay reagent (Promega, Madison, WI).

Hit selection

Rescue efficacy for each gene was calculated by dividing the luciferase activity of each IFN treated siRNA pool by the mean value of untreated wells. Each gene's rescue efficacy was then divided by the rescue efficacy of the non-targeting control, yielding the fold rescue value. Robust Z scores were then calculated by dividing the difference between the fold rescue of each gene and the median fold rescue of the experimental wells by the median absolute deviation (MAD) of the plate [16]. siRNA pools with replicate robust Z scores ≥ 2.0 were chosen for the first round of validation.

In the first round of validation, the 4 individual siRNA duplexes of each selected siRNA pool were individually tested following the primary screening protocol.

Functional enrichment

Candidate genes were tested for functional enrichment of biological processes and pathways using GeneGo's MetaCore system [17], a manually curated set of non-redundant Gene Ontology categories and biological pathway maps. In addition, individual gene annotations from the Gene Ontology 'biological process' category were retrieved from the DAVID knowledgebase [18] and filtered for their enrichment in the gene signature using all human

genes as background set (default parameters, $p \leq 0.05$). Identified GO terms were clustered using Revigo [19], ('medium' stringency), annotated manually for redundancy and visualized in Cytoscape [20].

Luciferase reporter assay

Huh7.5.1 cells were reverse transfected in 96-well plates with the indicated siRNA SmartPool 24 hours before plasmids transfection. Interferon stimulated response element (ISRE)-mediated IFN signaling was monitored by dual-luciferase reporter assay system after co-transfecting the plasmids pISRE-luc expressing firefly luciferase and pRL-TK expressing *Renilla* Luciferase as previously described [21]. [22]. Forty eight hours after p-ISRE transfection, PEG-IFN α -2b was added at 100 IU/mL and incubated for 8 hours. Relative luciferase activity was assessed by the Promega dual-luciferase reporter assay system (Promega, Madison, WI). Relative luciferase unit (RLU) was calculated by dividing the firefly luciferase value by the *Renilla* luciferase value.

Plasmid transfection

Squamous cell carcinoma antigen recognized by T cells (SART1) with Flag tagged expression plasmid (p-SART1) was kindly provided by Prof. Mei Yee Koh (Department of Experimental Therapeutics, M.D. Anderson Cancer Center, Houston, Texas) [23]. The empty vector, p3xFLAG-CMV-14, was purchased from Sigma-Aldrich (St. Louis, MO). Plasmids were transfected into Huh7.5.1 cells using FuGene HD at a ratio of 6:3. After 48 hours' transfection, cells were infected with JFH1 at ~0.2 moi for 6 hours, washed with medium and incubated for an extra 42 hours. Alternatively, the cells were treated with 30 IU/mL PEG-IFN α -2b post JFH1 infection 6 hours and then lysed 42 hour later.

Western blot

Proteins were obtained, separated, and transferred to PVDF membranes as reported in our previously works [15, 22]. The primary antibodies used in this paper were mouse anti-SART1 (Novus Biologicals, Littleton, CO), mouse anti-HCV core (Affinity BioReagents Inc., Golden, CO), mouse anti-actin (Sigma Life Science and Biochemicals, St. Louis, MO).

Quantitative PCR

Total cellular RNA isolation, reversed transcription to cDNA, and q-PCR were done following the protocol in our previously works [15, 22]. Human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a control for basal RNA levels. Primers are listed in Table S3.

Statistics

Data analysis was performed using a 2-tailed Student's t-test. Data are expressed as mean $\pm$ SD of at least three sample replicates, unless stated otherwise.

Results

Preliminary screens reveal that silencing IFNAR1 rescues HCV replication

We conducted initial IFN inhibitory studies to establish optimal conditions for high throughput screening. We found that 5 IU/mL and 30 IU/mL PEG-IFN α -2b inhibited nearly 80% of HCV replication in Huh7/Rep-Feo (Figure 1A) and OR6 replicon cells (Figure 1B), respectively.

In Huh7/Rep-Feo cells transfected with non-targeting siRNA or transfection buffer (Mock), 5 IU/mL of PEG-IFN- α 2b inhibited HCV replication by 80%, compared with 52%

inhibition of HCV replication after silencing IFNAR1. This indicated that silencing IFNAR1 rescued 48% of HCV replication in the presence of 5 IU/mL of PEG-IFN- α 2b, which was 2.36 fold higher than that of non-targeting siRNA (Figure 1C). In OR6 cells, 30 IU/mL PEG-IFN- α -2b inhibited 84% HCV replication following transfection with non-targeting siRNA and mock. The inhibition efficacy of PEG-IFN- α -2b was significantly suppressed after silencing IFNAR1 (Only 27%, Figure 1D), which indicated that silencing IFNAR1 significantly rescued HCV replication in OR6 replicon cells.

These preliminary studies confirmed that, as expected, silencing the IFNAR1 gene expression rescues HCV replication in IFN- α -treated Huh7/Rep-Feo and OR6 replicon cells. We sought to identify additional genes that modulate IFN- α signaling or that act as anti-HCV effectors using a whole-genome RNAi screening approach. We chose siRNA targeting IFNAR1 as the positive control for subsequent experiments, since it induced at least 2-fold rescue efficacy over non-targeting siRNA in both replicon systems.

Whole genome siRNA screening identifies 93 candidate genes that mediate IFN- α 's antiviral activity against HCV

The primary screen results are summarized in Supplemental Table 1 (Table S1). The distribution of robust Z scores for the whole genome siRNA library screen in Huh7/Rep-Feo replicon cells is shown in Figure 1E. Each point represents the robust Z scores of the same gene from 2 plates. Three hundred sixty one genes (1.71% of the whole library) produced robust Z scores ≥ 2.0 in both plates (upper right corner of Figure 1E), which entered the first round of validation.

The 4 individual siRNA duplexes that comprised the siRNA pool were evaluated by following the identical protocol as in the primary screen. For 206 of 361 pools (57.06%), at least 1 siRNA induced the same phenotype observed in the primary screen. Among these 206 pools, 93 pools (45.15%) were confirmed to have at least 2 siRNAs rescuing HCV replication by 2 MAD compared to the non-targeting control (Table S2). As expected, IFNAR 1, JAK 1, TYK 2, STAT 2, and IRF 9 were each among these 93 pools.

Gene ontology analysis

Functional enrichment of biological processes and pathways of the 93 candidate genes were obtained using GeneGo's MetaCore system. An additional analysis of biological processes using Gene Ontology (GO) terms assigned 92 partially redundant processes to the candidate genes, which could be clustered into 45 functional groups. As expected, the IFN signaling and innate immune response to RNA viral infection ranked high and were found at the 3rd and 5th rank of these 45 groups ($P = 1.00 \times 10^{-3}$ and 0.02, respectively). Unexpectedly, the two strongest functional enrichment groups were mRNA processing ($P = 2.76 \times 10^{-22}$) and translation initiation ($P = 2.42 \times 10^{-6}$) (Figure 2A, upper 5 bars in blue, and Table S4). As expected, the most significantly enriched pathway of the 93 host genes were IFN α/β signaling pathway ($P = 3.55 \times 10^{-10}$), antiviral actions of IFN ($P = 1.68 \times 10^{-6}$), and Interleukine-15 (IL-15) signaling via JAK-STAT cascade ($P = 7.92 \times 10^{-6}$) (Figure 2A, lower 5 bars in green, and Table S4). Further analysis by using DAVID illustrated more detailed GO biological processes and the gene overlap between processes (Figure 2B and Table S4). Remarkably, there were 17 genes involved in the spliceosome; 9 of them were components of U4/U6.U5 tri-snRNP.

Secondary validation in the full-length HCV replicon system

We performed further evaluation of candidate genes in full-length genome OR6 replicon cells. The initial screen used pools of 4 individual siRNA duplexes targeting a certain gene. We identified 31 siRNA pools with at least 3 of 4 duplexes rescuing HCV replication by 2

MAD compared to the non-targeting siRNA. These 31 genes were further evaluated in the secondary validation, except the unavailable one (RPL14L) from the manufacturer. Twelve genes were involved in mRNA processing.

None of the siRNAs targeting those 30 genes exhibited significant cell toxicity since cellular ATP content was comparable to that of non-targeting siRNA. Knock-down of most selected genes reduced the HCV replication in the absence of IFN, except IFNAR1, TYK2, WBP11, and ZC3HAV1. These data indicated that most of these selected genes are antiviral in the OR6 HCV replicon model (Fig 3A). Silencing GDI 2 did not rescue HCV from IFN's effects (rescue fold was 1.16, resembling non-targeting siRNA). Silencing TYK2, HCFC1, and SMU1 showed moderate rescue efficacy compared to non-targeting siRNA (rescue fold was 1.77, 1.58, and 1.51, respectively). Knockdown of the other 26 genes was confirmed to rescue HCV from IFN effects, as HCV replication during each knockdown was at least 2 fold higher than that of non-targeting siRNA (Figure 3A-B).

All 12 genes involved in mRNA processing rescued HCV replication from IFN- α in OR6 replicon cells. Four out of 12 genes were components of U4/U6.U5 tri-snRNP. Among these 4 genes, SART1 (squamous cell carcinoma antigen recognized by T cells, Gene ID: 9092) was reported as a specific factor of the tri-snRNP in human major spliceosome [24], and had the strongest IFN- α rescue effect in our screen (Figure 1 and Suppl table 1). We then sought to further evaluate the effect of SART1 on IFN- α anti-HCV action.

SART1 is downstream of or independent of the IFN-stimulated response element

We sought to determine the intersection of SART1 with the IFN signaling pathway by using a luciferase reporter system driven by the IFN-stimulated response element (ISRE), as previously described [22]. As expected, the ISRE-driven luciferase activity of non-targeting siRNA increased more than 12-fold upon IFN- α stimulation in the presence of non-targeting siRNA. Knockdown of OAS and ISG15 (well-known IFN pathway downstream genes), and SART1 had no to minimal effects on ISRE signaling, comparable to non-targeting siRNA (Fig 4A). These data indicate that knockdown of SART1 did not significantly affect ISRE-induced signaling, implying that its activity is independent of ISRE.

SART1 exerts its anti-HCV effect is through regulation of interferon stimulated genes (ISGs)—Transfection of siRNA targeting SART1 into Huh7.5.1 cells significantly abolished SART1 expression, as confirmed by qPCR for mRNA level (Fig 4D) and immunoblot for protein level (Figure 4E). Three individual oligo siRNAs from the original SmartPool duplexes had the same effect in Huh7.5.1 cells (Fig 4E). This was consistent with results from deconvolution of the SmartPools used for the primary screen in Huh7/Rep-Feo replicon cells. We found that SART1 knock-down increased JFH1 HCV replication in Huh7.5.1 cells (Fig 4C), but similar effects were not observed in OR6 replicon cells. We speculate that this effect is cell-type specific. In IFN-treated JFH1 infected Huh7.5.1 cells, HCV replication levels were rescued by siRNA SART1 (Fig 4B-C), indicating that silencing SART1 diminished the IFN- α 's anti-HCV effect. We further found that SART1 siRNA reduced ISG mRNA levels, including MxA, OAS, and PKR, either in the presence or absence of IFN- α (Table 1). These findings indicate that SART1 may exert basal antiviral effects against HCV through regulation of ISG expression with or without IFN- α . Nonetheless, SART1 may still be required for ISG expression or function. We further assessed the effect of IFN- α dose and time on SART1 expression. We found that IFN- α has minimal effect on SART1 mRNA expression in Huh7.5.1 and JFH1 cells (Table 2). These results suggest that SART1 expression is not itself induced by IFN- α . However, we found that HCV replication reduced SART1 mRNA in JFH1 cells 48 hrs post infection (Table 2).

We then evaluated the effect of SART1 itself on HCV infection. Huh7.5.1 cells were transfected with a SART1 expression plasmid or empty vector and then infected with JFH1, followed by IFN- α treatment. HCV core protein levels were diminished in the setting of SART1 overexpression (Figure 4F). Consistently, HCV RNA levels were increased moderately when we silenced SART1 using specific siRNA (Figure 4C). These findings indicate that SART1 may exert basal antiviral effect against HCV through regulation of ISGs expression with or without IFN- α . SART1 may be required for ISGs expression or function.

Discussion

Interferon alpha is the current backbone of effective therapy for HCV. Despite its clear antiviral effects against HCV, the precise repertoire of ISGs that suppress HCV have not yet to be characterized. To assess the host repertoire of genes that participate in IFN- α 's antiviral effects in an unbiased manner, we performed a high throughput whole genome siRNA screen. Our screen identified 93 candidate host genes participating IFN- α anti-HCV effect. In addition to well known ISGs, we found several new genes involved in mRNA processing and translation initiation. Among them were 9 genes comprising U4/U6.U5 tri-snRNP, which is the major component of human spliceosome complex B and C [24]. SART1 is a U4/U6.U5 tri-snRNP specific factor, which we confirmed is necessary for IFN- α anti-HCV effect in 2 replicon cell lines and the JFH1 infectious system. We further found that SART1 was not IFN- α inducible and exerted its anti-HCV effects in the JFH1 infectious system through reduction of ISG expression, either in the presence or absence of exogenous IFN- α . Bioinformatics analysis strongly indicated that the 93 host genes passed the first validation involved in IFN- α anti-HCV effect (Figure 2 and Table S4).

Of the 93 host genes that could rescue HCV replication from IFN- α , the IFN signaling ($P = 1.00 \times 10^{-3}$) and innate immune response to RNA viral infection ($P = 0.02$) were among the top 5 strongest functional enrichment gene groups. Further analysis in pathway mapping showed that the IFN α/β signaling pathway ($P = 3.55 \times 10^{-10}$), antiviral actions of IFN ($P = 1.68 \times 10^{-6}$), and IL-15 signaling via JAK-STAT cascade ($P = 7.92 \times 10^{-6}$) were the most enriched pathways. These pathways and biological functions were all reported as essential for IFN- α antiviral activity [5, 8].

Our results strongly indicate that mRNA processing plays an important role in IFN- α 's anti-HCV effect. We found that 23 out of 93 candidate genes (24.7%) rescuing HCV replication from IFN- α are involved in mRNA processing with significant P value ($P = 2.76 \times 10^{-22}$). Our lab previously found that host genes involved in HCV replication were enriched in Golgi vesicle binding and vesicle organization and biogenesis [12]. In this study, we used IFN- α to treat the same cell line and siRNA library as in our prior study. The only change was adding IFN- α . We then speculated that IFN- α through interfering mRNA processing to fulfill its anti-HCV effect. Additional mechanistic studies are currently underway.

A growing literature supports that alternative splicing is an important player in the host response to viral infection. A recent study found that among the 10 isoforms of the human OAS gene family, OAS1 p42/p46 and OAS3 p100 were identified as the effectors of IFN against dengue virus infection. All 10 of these isoforms were generated through alternative splicing [25]. The IFN- α induced 76KDa MxA showed antiviral activity against herpes simplex virus 1 (HSV-1), while a variant MxA (produced from an alternatively spliced transcript) propagated HSV-1 [26]. Alternative splicing variants in occludin 1 determined the susceptibility to HCV infection and may be associated with the outcome of the infection [27].

SART1 is a U4/U6.U5 tri-snRNP specific factor of the human major spliceosome [24]. SART1 protein is not required for the stability of the U4/U6 U5 tri-snRNP but is essential for mature spliceosome assembly [28]. SART1 interacts with PRPF3, PRPF6, and SNRNP200 [29]. Combined with our results, there would appear to be two plausible explanations for the participation of SART1 in the interaction between IFN- α and HCV. First, SART1 strengthened IFN- α 's antiviral effects through mRNA processing. IFN- α did not significantly induce endogenous SART1 expression (Table 2), and silencing SART1 abrogates the anti-HCV effect of IFN- α (Figure 4B, C). Secondly, SART1 itself has a direct anti-HCV effect when it is highly overexpressed (Figure 4F), while silencing SART1 increases HCV RNA replication in the absence of IFN- α (Figure 4C). The relationship between SART1 and HCV replication has not been reported. Since siRNA SART1 significantly reduced expression of ISGs including MxA, OAS, and PKR with or without exogenous IFN- α (Table 1), these data suggest that SART1's antiviral actions on HCV replication do not absolutely depend on IFN. However, SART1 does appear to be a critical factor for ISG expression and/or function, and its abrogation diminishes IFN's antiviral effects. It exerts its actions on ISGs largely independent of ISRE stimulation. Further study of the precise mechanism of SART1 function on ISGs and HCV replication is warranted.

The membrane receptor complex-JAK-STAT-IRF9-ISRE-ISGs signaling pathway is the best known mechanism of IFN- α 's antiviral activity [5]. We validated that silencing IFNAR1, JAK1, TYK2, STAT2, and IRF9 did rescue HCV replication from IFN- α in sub-genomic and full-length HCV replicon cell lines (Figure 3, Table S1). All of these 5 genes were upstream of ISRE. We did not find the classical IFN-inducible antiviral effectors including PKR, Mx, and OAS [8], which sit downstream of ISRE. One possibility was the weak strength of each signal one of these ISRE downstream ISGs, silencing only one single gene in the downstream of ISRE could not impaired the integrity of the whole pathway. Further research in combination of some selected ISGs is warranted.

In conclusion, we have performed a high throughput functional genomic screen to identify host factors that mediate the anti-HCV effects of interferon alpha. We confirmed 93 human genes that involved in IFN- α anti-HCV effect which enriched in mRNA processing, translation initiation, and IFN signaling. We further confirmed that silencing SART1 abrogates IFN- α 's suppressive effects against HCV in both replicon cells and JFH1 infectious cells. This unexpected and multiply confirmed finding opens a potentially promising new area of investigation in the HCV field, and suggests a completely novel approach to interrupting HCV infection.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Robust Z scores of 2 replicates from the primary whole genome siRNA screen in Huh7/pRepFeo cells

Peginterferon α -2b efficiently inhibits HCV replication in Huh7/Rep-Feo (A) and OR6 replicon cells (B) in a dosage-dependent manner. Huh7/Rep-Feo (2000/well) or OR6 cells (3000/well) were seeded in a 384-well or 96-well microplate. After 72 hours incubation, the cells were treated with different dosage of Peginterferon α -2b (PEG-IFN α -2b) for an additional 24 hours. Firefly (or *Renilla*) luciferase activity and cell viability were detected simultaneously. Five IU/mL and 30 IU/mL PEG-IFN α -2b inhibited nearly 80% of the HCV replication in Huh7/Rep-Feo and the OR6 replicon cells, respectively. Small interfering RNA (siRNA) targeting IFNAR1 rescued HCV replication in the presence of PEG-IFN α -2b

in Huh7/Rep-Feo (C) and OR6 replicon cells (D). siRNAs were reverse transfected into Huh7/Rep-Feo (2000/well in a 384-well plate) and OR6 cells (3000/well in 96-well plate) at 50nM final concentration. After 72 hours incubation, cells were treated with or without PEG-IFN α -2b (5 IU/mL for Huh7/Rep-Feo, 30 IU/mL for OR6 cell) for an extra 24 hours. Silencing IFNAR1 induced at least 2 fold higher rescue effect over non-targeting siRNA in both replicon systems. Luminescence activity and cell viability were obtained from quadruplicate assays and expressed as mean $\pm$ SD. (E) Distribution of robust Z scores of host genes from primary screening. Each point corresponds to the robust Z score of the same siRNA pool in plate A and B.

Figure 2. Bioinformatic analysis of primary screening data

(A) Strongest functional enrichment of 93 candidate genes in GeneGo processes (blue) and pathways (green), with the orange line indicating a significance threshold of $p=0.05$. (B) Gene Ontology processes significantly enriched in the candidate gene list compared to a background distribution of all human genes according to an analysis by DAVID. Redundant terms were filtered automatically with Revigo and curated manually before visualization with Cytoscape. Node color reflects significance of process enrichment, size indicates the number of gene mapped to this process and edge width shows gene overlap between processes.

Figure 3. Secondary validation in OR6 full-length replicon cells

OR6 cells (3000/well) were reverse transfected with the indicate siRNA pools at 50nM in 96-well microplates. After 72 hours incubation, the cells were treated with or without 30 IU/mL PEG-IFN α -2b for an extra 24 hours. *Renilla* luciferase unit and cell viability unit were obtained from quadruplicate assays in three independent experiments. Relative Luciferase Unit (RLU) was obtained by calculating *Renilla* luciferase unit/cell viability unit. The Normalized Luciferase Unit (NLU) of each siRNA pool was obtained by normalizing each RLU to non-targeting siRNA without IFN treatment (set as 1) (Figure 3A). The IFN Rescue Efficacy Unit (IREU) of each siRNA target gene was derived from NLU of IFN treated/ No-IFN treatment. Each siRNA target gene IFN Rescue Unit (fold) was calculated by normalizing each IREU to non-target siRNA control (Fig 3B).

Figure 4. SART1 is essential for IFN- α 's antiviral activity against HCV in JFH1 infected Huh7.5.1 cells

(A) Huh7.5.1 cells were reverse transfected with the indicated siRNA 24 hours before p-ISRE and pRL-TK co-transfection in 96-well plates. PEG-IFN α -2b 100 IU/mL was added 48 hours later. Relative luminescence activity (RLA) was measured 8 hours later. Knockdown of SART1 using siRNA did not markedly inhibit ISRE-mediated signaling in Huh7.5.1 cells. Data were from quadruplicate assays in three independent experiments and expressed as mean $\pm$ SEM. (B) Knockdown of SART1 protein expression by siRNA rescues HCV core expression. Huh7.5.1 cells (30 000 cells per well) were reverse transfected in 24-well plate with the indicated siRNA targeting SART1 at 50 nM. Cells were infected with

JFH1 at ~0.2 moi after 48 hours transfection. PEG-IFN α -2b was added at 30 IU/mL 24 hours post infection. Whole cell lysates were harvested 48 hours after treatment. Levels of HCV core protein were restored in the presence of siRNA targeting SART1. (C) HCV RNA levels were rescued by siRNA targeting SART1. The results were obtained from five independent experiments and expressed as mean $\pm$ SEM. (D) SART1 mRNA level confirmed that SART1 knocked down by SART1 specific siRNA. (E) SART1 protein expression was significantly decreased using a SART1 siRNA SmartPool (lane 2) and 3 of 4 individual oligo siRNAs (lanes 3-5) compared with non-targeting siRNA (lane 1). (F) Overexpression of SART1 inhibited HCV core expression. Huh7.5.1 cells (50 000 cells per well) were transfected with the SART1 expression plasmid p-SART1 or empty vector alone for 48 hours and then infected with JFH1 for 6 hours. Cells were untreated or incubated with 30 IU/mL PEG-IFN α -2b for 24 hours. HCV core protein was decreased in the presence of SART1 overexpression (lanes 5 and 6).

Table 1
siRNA against SART1 reduced interferon stimulated genes (ISGs) MXA, OAS, and PKR mRNA expression in Huh7.5.1 and JFH1-infected Huh7.5.1 (JFH1) cells with or without IFN- α

The non-targeting negative control and SART1 siRNAs were transfected into Huh7.5.1 cells using HiPer-Fect Transfection Reagent (Qiagen, Valencia, CA) as previous described. JFH1 virus (1:10 final dilution) was introduced to the appropriate wells to infect Huh7.5.1 cells after 48 hr of siRNA transfection. IFN- α (final concentration 30 IU/ml) was added to the appropriate wells after 6 hr of HCV infection. Total RNA was harvested by RNeasy kit (Qiagen, Valencia, CA) after 72 hrs siRNA, 24 hr of IFN treatment for Q-PCR to measure ISGs and GAPDH mRNA level. We found that siRNA SART1 inhibited mRNA expression of several ISGs, including MxA, OAS, and PKR, in both Huh7.5.1 and JFH1 cells, with or without IFN- α .

MxA/GAPDH Arbitrary Unit (AVE $\pm$ STD)		
Treatment	Neg siRNA	SART1 siRNA
Huh7.5.1	1.00 $\pm$ 0.09	0.49 $\pm$ 0.07
JFH1	0.78 $\pm$ 0.10	0.42 $\pm$ 0.03
Huh7.5.1+IFN	123.6 $\pm$ 16.4	50.0 $\pm$ 4.0
JFH1+IFN	79.0 $\pm$ 5.4	24.8 $\pm$ 2.8

OAS/GAPDH Arbitrary Unit (AVE $\pm$ STD)		
Treatment	Neg siRNA	SART1 siRNA
Huh7.5.1	1.00 $\pm$ 0.10	0.52 $\pm$ 0.06
JFH1	0.55 $\pm$ 0.03	0.42 $\pm$ 0.07
Huh7.5.1+IFN	9.04 $\pm$ 0.68	3.27 $\pm$ 0.48
JFH1+IFN	5.45 $\pm$ 0.30	2.77 $\pm$ 0.27

PKR/GAPDH Arbitrary Unit (AVE $\pm$ STD)		
Treatment	Neg siRNA	SART1 siRNA
Huh7.5.1	1.00 $\pm$ 0.12	0.55 $\pm$ 0.03
JFH1	0.57 $\pm$ 0.02	0.52 $\pm$ 0.08
Huh7.5.1+IFN	5.98 $\pm$ 0.81	3.25 $\pm$ 0.21
JFH1+IFN	4.60 $\pm$ 0.50	3.67 $\pm$ 0.20

IFN- α dose and time course effects on HCV replication (A) and SART1 mRNA expression (B) in Huh7.5.1 and JFH1-infected Huh7.5.1 (JFH1) cells

Table 2

Huh7.5.1 cells (100,000/1 ml/well) were seeded in 24-well plate and incubated overnight. JFH1 virus (1:10 final dilution) was introduced to the appropriate wells to infect Huh7.5.1 cells the next day. IFN- α (final concentrations of 0, 10, 30, and 100 IU/ml) was added to the appropriate wells after 6 hr of HCV infection. Total RNA was harvested by RNeasy kit (Qiagen, Valencia, CA) after 1, 8, 24, and 48 hr of IFN treatment for Q-PCR to measure SART1, JFH1 HCV, and GAPDH mRNA levels. HCV RNA levels were higher at 1 hr (15033 $\pm$ 2905) $\pm$ 8 hr (6115 $\pm$ 138) $\pm$ 24 hr (4030 $\pm$ 653) post infection, indicating that JFH1-infected cells did not release new virus before 24 hrs after infection. However, JFH1 RNA levels were significantly higher at 48 hr (36479 $\pm$ 4684), compared to 1 hr of infection, indicating replication of HCV in JFH1-infected cells. JFH1 virus slightly increased SART1 mRNA levels at 1 hr and 8 hr after viral infection. However, SART1 mRNA levels were significantly lower at 48 hr (0.56 $\pm$ 0.09), compared to uninfected Huh7.5.1 cell (1.01 $\pm$ 0.19), indicating that HCV replication inhibited SART1 expression. IFN- α slightly increased SART1 expression in Huh7.5.1 cells at 1, 8, and 24 hr, but not in JFH1 cells.

A.					
1 hr post IFN α treatment			8 hr post IFN α treatment		
	IFN (IU/ml)	JFH1/GAPDH Arbitrary Unit		IFN (IU/ml)	JFH1/GAPDH Arbitrary Unit
Huh7.5.1	0	AVE $\pm$ STD	Huh7.5.1	0	AVE $\pm$ STD
Huh7.5.1	10	1.01 $\pm$ 0.19	Huh7.5.1	10	1.01 $\pm$ 0.15
Huh7.5.1	30	1.17 $\pm$ 0.19	Huh7.5.1	30	1.01 $\pm$ 0.17
Huh7.5.1	100	0.99 $\pm$ 0.15	Huh7.5.1	100	1.07 $\pm$ 0.14
JFH1	0	1.26 $\pm$ 0.20	JFH1	0	1.14 $\pm$ 0.13
JFH1	10	15033 $\pm$ 2905	JFH1	10	6115 $\pm$ 138
JFH1	30	12281 $\pm$ 1573	JFH1	30	5878 $\pm$ 377
JFH1	100	11825 $\pm$ 1951	JFH1	100	5266 $\pm$ 974
		11494 $\pm$ 1206			4566 $\pm$ 219

B.					
24 hr post IFN α treatment			48 hr post IFN α treatment		
	IFN (IU/ml)	JFH1/GAPDH Arbitrary Unit		IFN (IU/ml)	JFH1/GAPDH Arbitrary Unit
Huh7.5.1	0	AVE $\pm$ STD	Huh7.5.1	0	AVE $\pm$ STD
Huh7.5.1	10	1.01 $\pm$ 0.17	Huh7.5.1	10	1.01 $\pm$ 0.19
Huh7.5.1	30	1.24 $\pm$ 0.17	Huh7.5.1	30	0.98 $\pm$ 0.18
Huh7.5.1	100	1.21 $\pm$ 0.11	Huh7.5.1	100	0.98 $\pm$ 0.17
		1.14 $\pm$ 0.21			1.18 $\pm$ 0.17

24 hr post IFN α treatment			48 hr post IFN α treatment		
	IFN (IU/ml)	JFH1/GAPDH Arbitrary Unit		IFN (IU/ml)	JFH1/GAPDH Arbitrary Unit
		AVE $\pm$ STD			AVE $\pm$ STD
JFH1	0	4030 $\pm$ 653	JFH1	0	36479 $\pm$ 4684
JFH1	10	1719 $\pm$ 229	JFH1	10	2732 $\pm$ 500
JFH1	30	1321 $\pm$ 186	JFH1	30	1834 $\pm$ 235
JFH1	100	1233 $\pm$ 100	JFH1	100	1602 $\pm$ 159

B.						
1 hr post IFN α treatment				8 hr post IFN α treatment		
		IFN (IU/ml)	SART1/GAPDH Arbitrary Unit		IFN (IU/ml)	SART1/GAPDH Arbitrary Unit
			AVE $\pm$ STD			AVE $\pm$ STD
Huh7.5.1	0.00		1.00 $\pm$ 0.10	Huh7.5.1	0.00	1.01 $\pm$ 0.10
Huh7.5.1	10.00		1.35 $\pm$ 0.24	Huh7.5.1	10.00	1.18 $\pm$ 0.04
Huh7.5.1	30.00		1.20 $\pm$ 0.16	Huh7.5.1	30.00	1.27 $\pm$ 0.07
Huh7.5.1	100.00		1.30 $\pm$ 0.23	Huh7.5.1	100.00	1.03 $\pm$ 0.09
JFH1	0.00		1.37 $\pm$ 0.22	JFH1	0.00	1.35 $\pm$ 0.10
JFH1	10.00		1.21 $\pm$ 0.07	JFH1	10.00	1.49 $\pm$ 0.06
JFH1	30.00		1.05 $\pm$ 0.17	JFH1	30.00	1.10 $\pm$ 0.17
JFH1	100.00		1.00 $\pm$ 0.04	JFH1	100.00	0.98 $\pm$ 0.10

24 hr post IFN α treatment			48 hr post IFN α treatment			
		IFN (IU/ml)	SART1/GAPDH Arbitrary Unit		SART1/GAPDH Arbitrary Unit	
			AVE $\pm$ STD		AVE $\pm$ STD	
Huh7.5.1	0.00		1.00 $\pm$ 0.11	Huh7.5.1	0.00	1.01 $\pm$ 0.13
Huh7.5.1	10.00		1.21 $\pm$ 0.16	Huh7.5.1	10.00	1.16 $\pm$ 0.21
Huh7.5.1	30.00		1.40 $\pm$ 0.15	Huh7.5.1	30.00	1.13 $\pm$ 0.20
Huh7.5.1	100.00		1.29 $\pm$ 0.19	Huh7.5.1	100.00	1.06 $\pm$ 0.13
JFH1	0.00		1.14 $\pm$ 0.20	JFH1	0.00	0.56 $\pm$ 0.09
JFH1	10.00		1.17 $\pm$ 0.14	JFH1	10.00	0.41 $\pm$ 0.05
JFH1	30.00		1.26 $\pm$ 0.13	JFH1	30.00	0.46 $\pm$ 0.06
JFH1	100.00		1.06 $\pm$ 0.19	JFH1	100.00	0.60 $\pm$ 0.04