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RIG-I Is a Key Antiviral Interferon-Stimulated Gene Against Hepatitis E Virus Regardless of Interferon Production

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Interferons (IFNs) are broad antiviral cytokines that exert their function by inducing the transcription of hundreds of IFN-stimulated genes (ISGs). However, little is known about the antiviral potential of these cellular effectors on hepatitis E virus (HEV) infection, the leading cause of acute hepatitis globally. In this study, we profiled the antiviral potential of a panel of important human ISGs on HEV replication in cell culture models by overexpression of an individual ISG. The mechanism of action of the key anti-HEV ISG was further studied. We identified retinoic acid–inducible gene I (RIG-I), melanoma differentiation–associated protein 5, and IFN regulatory factor 1 (IRF1) as the key anti-HEV ISGs. We found that basal expression of RIG-I restricts HEV infection. Pharmacological activation of the RIG-I pathway by its natural ligand 5′-triphosphate RNA potently inhibits HEV replication. Overexpression of RIG-I activates the transcription of a wide range of ISGs. RIG-I also mediates but does not overlap with IFN-α-initiated ISG transcription. Although it is classically recognized that RIG-I exerts antiviral activity through the induction of IFN production by IRF3 and IRF7, we reveal an IFN-independent antiviral mechanism of RIG-I in combating HEV infection. We found that activation of RIG-I stimulates an antiviral response independent of IRF3 and IRF7 and regardless of IFN production. However, it is partially through activation of the Janus kinase (JAK)–signal transducer and activator of transcription (STAT) cascade of IFN signaling. RIG-I activated two distinct categories of ISGs, one JAK-STAT-dependent and the other JAK-STAT-independent, which coordinately contribute to the anti-HEV activity. *Conclusion*: We identified RIG-I as an important anti-HEV ISG that can be pharmacologically activated; activation of RIG-I stimulates the cellular innate immunity against HEV regardless of IFN production but partially through the JAK-STAT cascade of IFN signaling. (HEPATOLOGY 2017;65:1823-1839).

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Hepatitis E virus (HEV) infection is the most common cause of acute viral hepatitis worldwide.[1] As a single-strand RNA virus, HEV has been divided into four genotypes.[2] Although acute HEV infections are mostly self-limiting, genotype 1 HEV infection during pregnancy may lead to up to 30% mortality.[3] In immunosuppressed patients, such as organ transplant recipients, genotype 3 HEV infection can cause chronic hepatitis.[2] For these chronic patients, monotherapy or the combination of ribavirin and/or pegylated interferon-α (IFN-α) have been used as off-label treatment.[1] The observation that different

Abbreviations: Fluc, Photinus pyralis *luciferase; GFP, green fluorescent protein; HCV, hepatitis C virus; HEV, hepatitis E virus; IFN, interferon; IRF, IFN regulatory factor; ISG, interferon-stimulated gene; ISRE, IFN-stimulated response element; JAK, Janus kinase; MAVS, mitochondrial antiviral signaling (protein); MDA5, melanoma differentiation–associated protein 5; MEF, mouse embryonic fibroblast; mIFN, mouse IFN; NF-κB, nuclear factor kappa B; pppRNA, triphosphate RNA; RANTES, regulated upon activation, normal T cell expressed, and secreted; RIG-I, retinoic acid–inducible gene I; STAT, signal transducer and activator of transcription; WT, wild type.*

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populations with different immune system states have distinct outcomes of HEV infection highlights the importance of studying HEV–host interactions.

The innate immune response plays an essential role in defending against viral infections. Patients with genetic deficiencies in the innate immune system are often prone to viral infection and develop more severe symptoms.[4,5] In response to viral infection, host cells produce virus-induced cytokines including IFNs, particularly type 1 IFNs (IFN-α and IFN-β), which have potent antiviral activity against a broad spectrum of viruses.[6] Type 1 IFNs promote an antiviral state in an autocrine or paracrine manner by transcriptional induction of hundreds of IFN-stimulated genes (ISGs).[7] However, excessive accumulation of type 1 IFNs may evoke pathological effects to the organism.[8] Recently, emerging studies have described the independent innate antiviral defenses of type 1 IFNs.[9,10] These IFN-independent antiviral mechanisms including the production of alternative antiviral cytokines (e.g., IFN-λ or interleukin 22) and the basal expression of direct antiviral ISGs.[10] The basal expression of these ISGs may be attributed to tonic IFN signaling, which establishes a cell-autonomous antiviral status of the host, independent of virus-triggered IFN production. Therefore, ISGs play important roles in both IFN-dependent and IFN-independent antiviral mechanisms.

As the ultimate antiviral effectors, ISGs are transcriptionally induced through the Janus kinase (JAK)–signal transducer and activator of transcription (STAT) pathway by tonic or exogenous IFNs. In previous studies, more than 380 individual human ISGs have been tested for their antiviral effects on a wide array of virus species including many important human and animal viruses.[11,12] Surprisingly, only small subsets of ISGs exert antiviral activities against either a specific virus or a broad spectrum of viruses. Unexpectedly, a few ISGs even promote the replication of certain viruses.[11,12] IFN-α has anti-HEV activity *in vitro* and is probably also effective in chronic patients,[13-15] strongly suggesting that ISGs may play a vital role in IFN-mediated HEV clearance. Furthermore, genome-wide transcriptome profiling has identified the up-regulation of 30 genes in blood cells of chronic HEV patients, of which 25 are ISGs.[16]

Because the function of ISGs during HEV infection remains largely elusive, we have profiled the effects of a panel of ISGs that are known to have antiviral or proviral effects on certain viruses.[11,12] We found that whereas most of these ISGs have only minor effects, some have potent anti-HEV effects. Among these ISGs, retinoic acid–inducible gene I (RIG-I) is a key member that effectively restricts HEV replication. Furthermore, biological or pharmacological activation of RIG-I exerts potent anti-HEV effects. Mechanistically, it robustly activates the innate cellular antiviral response. This activation unexpectedly occurs regardless of IFN production, but requires the key elements of JAK-STAT signaling.

Materials and Methods

HEV CELL CULTURE MODELS AND LENTIVIRAL ISG VECTORS

A plasmid containing the full-length HEV genome (Kernow-C1 p6; GenBank accession JQ679013) and a construct containing a subgenomic HEV sequence in

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which open reading frame 2 was replaced with *Gaussia princeps* luciferase reporter gene (p6-Luc) were used to generate HEV cell culture models as described.[17,18] pTRIP.CMV.IVSb.ISG.ires.TagRFP-based ISG overexpression vectors were kind gifts from Prof. Charles M. Rice (Rockefeller University).[11] Lentiviral pseudoparticles were generated and transduced as described.[11] More details are provided in the Supporting Information.

Results

IDENTIFICATION OF ANTIVIRAL ISGs AGAINST HEV REPLICATION

To identify key ISGs that regulate HEV replication, 25 important human ISGs which are known to have antiviral or proviral effects on certain viruses[11] were tested in two Huh7.5 cell–based HEV models (Huh7.5-p6-Luc and Huh7.5-p6). Huh7.5 is an RIG-I-defective hepatoma cell line derived from Huh7 cells, which was widely used for supporting viral infections (Supporting Fig. S1A).[19] These ISGs include MAP3K14, IFI44L, RIG-I (also known as DDX58), HPSE, RTP4, NAMPT (also known as PBEF1), IFN regulatory factor 1 (IRF1), IFITM1, IFITM2, IFITM3, C6orf150 (also known as cGAS), UNC84B (also known as SUN2), IRF2, IRF7, IRF9, IFI6, OASL, DDX60, MOV10, TREX1, melanoma differentiation–associated protein 5 (MDA5; also known as IFIH1), ADAR, FAM46C, LY6E, and MCOLN2.[11]

Ectopic overexpression of each ISG was delivered by a bicistronic lentiviral vector coexpressing the ISG and a red fluorescent protein (TagRFP). Two vectors that express a *Photinus pyralis* luciferase (Fluc) gene or a green fluorescent protein (GFP) gene were used as controls.[11] The successful overexpression of each ISG was confirmed by flow-cytometric analysis to measure the expression of the red fluorescent protein (Supporting Fig. S1B-F). Transient transfection was used to overexpress ISG when lentiviral stocks failed to achieve high-level transduction.[11] Before profiling on HEV, each ISG was first tested for the ability to inhibit hepatitis C virus (HCV) replication in the Huh7.5 cell–based HCV luciferase replicon model.[11] Similar to the previous study, most ISGs inhibit HCV replication to some extent, whereas several genes (IRF1, IRF2, and RIG-I) have strong anti-HCV effects (Supporting Fig. S1G). Next, all ISGs were tested for their anti-HEV ability in a subgenomic HEV model (Huh7.5-p6-Luc). At 24 hours after lentivirus transduction, most genes inhibited HEV-related luciferase activity to some extent (Fig. 1A). One gene, RIG-I, was found to have strong anti-HEV activity at 48 hours post-transduction (Fig. 1B). More anti-HEV ISGs were identified 72 hours after transduction, including IRF1, MDA5, and RIG-I (Fig. 1C). These genes could inhibit HEV-related luciferase activity by almost 50% compared to control. To further validate the antiviral ability, those ISGs were also tested in the full-length infectious HEV model (Huh7.5-p6). A similar inhibition pattern was obtained in this model (Fig. 1D). At 48 hours after transduction, IRF1, MDA5, and RIG-I potently decreased HEV viral RNA level, whereas most of the other genes showed minor effects.

RIG-I IS A KEY ANTI-HEV ISG

Among the three potent anti-HEV ISGs, we have demonstrated that IRF1 inhibits HEV replication by stimulating antiviral ISG expression.[17] Both RIG-I and MDA5 are pattern recognition receptors that sense viral RNA in the cytoplasm.[20] In this study, we mainly focused on the antiviral potential of RIG-I. To validate the anti-HEV activity of RIG-I, we performed additional independent experiments in two Huh7.5-based HEV models (Huh7.5-p6 and Huh7.5-p6-Luc). Lentiviral transduced RIG-I overexpression was confirmed in Huh7.5-p6 cells by quantitative RT-PCR and immunoblotting (Fig. 2A). In both models, RIG-I overexpression inhibited HEV replication to an extent similar to a high dose of IFN-α treatment (Fig. 2B). Next, we confirmed the anti-HEV ability of RIG-I in different cell models: a human lung epithelial cell line, A549, that is widely used for HEV propagation and a human hepatic progenitor cell–derived cell line, HepaRG. Both are capable of supporting long-term HEV replication (A549-p6 and HepaRG-p6).[17] RIG-I overexpression also significantly inhibited HEV replication in both HepaRG-based and A549-based HEV models (Fig. 2C,D).

To further explore the role of basal RIG-I in constraining HEV infection, an RNA interference approach was used to silence RIG-I gene expression. The HEV RNA level was significantly increased in RIG-I-silenced A549 cells (Fig. 2E,F). As expected, in RIG-I-defective Huh7.5 cells, RIG-I knockdown did not affect HEV infection (Supporting Fig. S2A, B), and we indeed observed that RIG-I-defective Huh7.5 cell are more permissive for supporting HEV

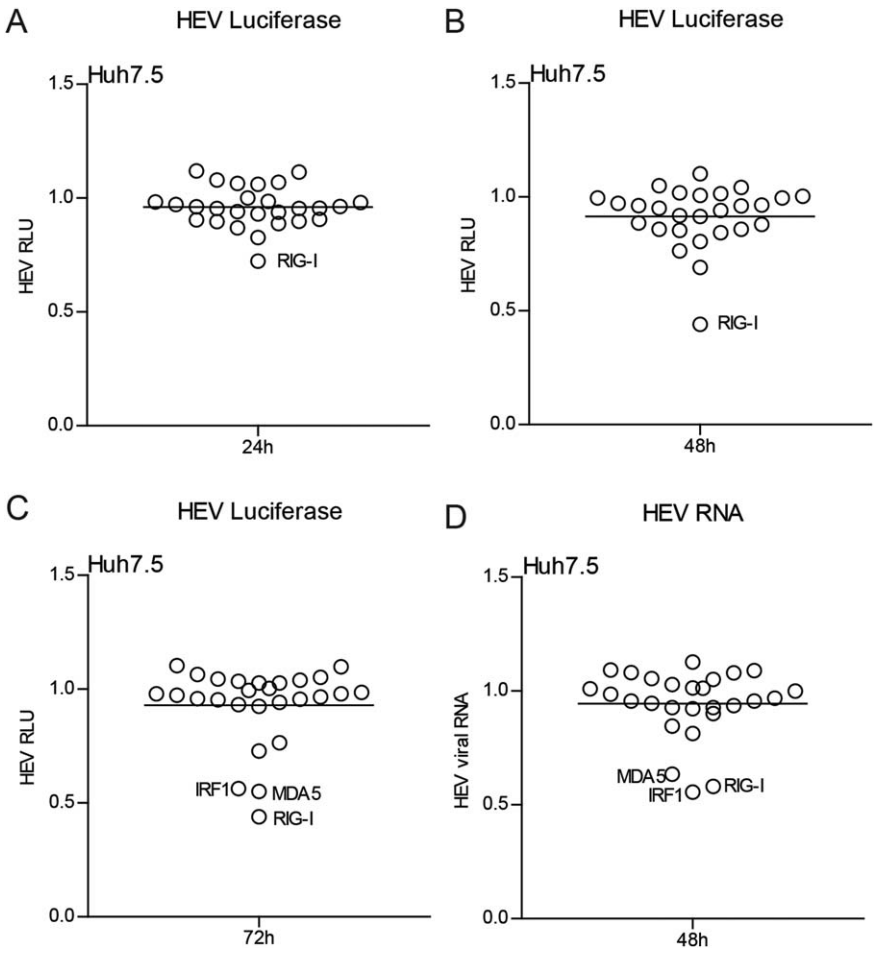

FIG. 1. Identification of ISGs that inhibit HEV replication. Luciferase activity analysis of HEV-related *Gaussia* luciferase activity in Huh7.5-p6-Luc cells transduced with ISG overexpression or Fluc vector for 24 hours (A), 48 hours (B), or 72 hours (C) (n = 4 independent experiments with each of 2 replicates). (D) quantitative RT-PCR analysis of HEV viral RNA level in Huh7.5-p6 cells transduced with ISG overexpression or Fluc vector for 48 hours (n = 4). Data were normalized to the Fluc control (set as 1) and are presented in dot plots. Abbreviation: RLU, relative luciferase unit.

infection (Supporting Fig. S2C). These results demonstrate that RIG-I has potent anti-HEV ability and that the basal expression of RIG-I plays an important role in defending HEV infection.

PHARMACOLOGICAL ACTIVATION OF RIG-I STIMULATES AN ANTIVIRAL RESPONSE THAT INHIBITS HEV REPLICATION

To explore the antiviral response induced by the RIG-I pathway, the natural ligand of RIG-I, 5′-triphosphate RNA (5′-pppRNA), was used to activate RIG-I signaling.[21] Different concentrations of RIG-I agonist were used to induce an antiviral response in A549 cells, a model with functional RIG-I expression (Supporting Fig. S1A). Gene expression of type 1 IFN (IFN-β) and type 3 IFN (IFN-λ) was significantly induced by RIG-I agonist 48 or 72 hours after treatment (Fig. 3A). Concurrently, the expression of many ISGs—including STAT1; IFIH1; PKR; tumor necrosis factor–related apoptosis-inducing ligand TRAIL; regulated upon activation, normal T cell expressed, and secreted (RANTES); and RIG-I—was also significantly induced by 5′-pppRNA treatment (Fig. 3A). Correspondingly, treatment with RIG-I agonist resulted in a significant reduction of HEV replication in A549-p6 cells. With 1,000 ng/mL 5′-pppRNA treatment, HEV viral RNA were inhibited by 67.8 ± 8.1% (mean ± standard error of the mean) (n = 7, $P < 0.01$), 90.0 ± 8.6% (n = 6, $P < 0.01$) at 48 and 72 hours after treatment, respectively (Fig. 3B). Because Huh7.5 cells are RIG-I-defective cells (Supporting Fig. S1A), RIG-I agonist was unable to induce any antiviral response, as expected (Supporting Fig. S2D).

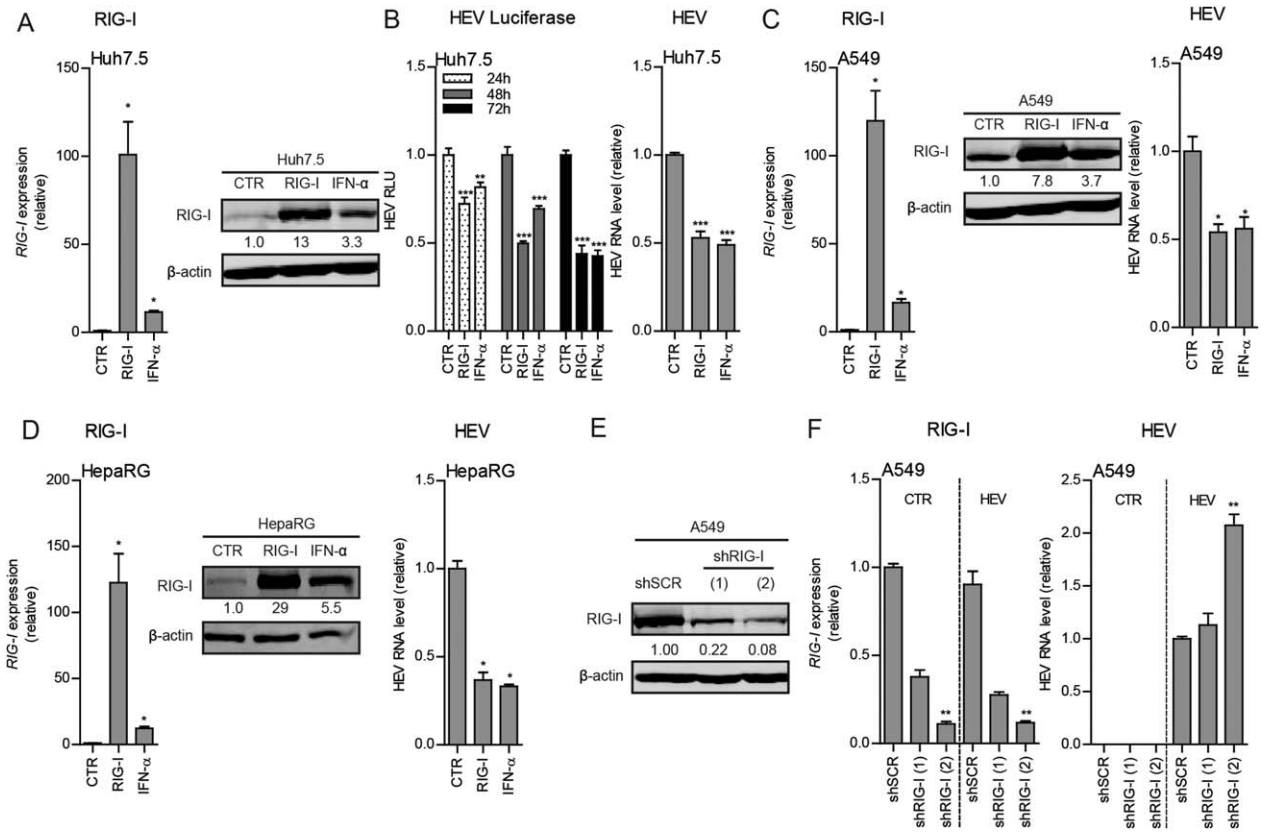

FIG. 2. RIG-I inhibits HEV replication in multiple cell models. Quantitative RT-PCR analysis and immunoblot analysis of RIG-I expression in Huh7.5-p6 cells (A), A549-p6 cells (C), and HepaRG-p6 cells (D) transduced with RIG-I or Fluc vector or treated with IFN-α (1,000 IU/mL) for 48 hours (n = 4). (B) Analysis of HEV-related *Gaussia* luciferase activity in Huh7.5-p6-Luc cells transduced with RIG-I or Fluc vector or treated with IFN-α (1,000 IU/mL) for 24, 48, or 72 hours (n = 4 independent experiments with each of 3-4 replicates) and quantitative RT-PCR analysis of HEV viral RNA level in Huh7.5-p6 cells transduced with RIG-I vector or treated with IFN-α (1,000 IU/mL) for 48 hours (n = 8). Quantitative RT-PCR analysis of HEV viral RNA level in A549-p6 cells (C) and HepaRG-p6 cells (D) transduced with RIG-I or Fluc vector or treated with IFN-α (1,000 IU/mL) for 48 hours (n = 4). (E) Immunoblot analysis of RIG-I expression in A549 cells transduced with lentiviral short hairpin RNA vector targeting RIG-I or scrambled control. Stable RIG-I knockdown or scrambled control A549 cells were infected with HEV. RIG-I expression level and HEV viral RNA level (F) were analyzed by quantitative RT-PCR 72 hours after HEV infection. Data were normalized to the Fluc control (set as 1, A-D) or to the scrambled control (set as 1, F). Data are means ± standard error of the mean. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. For immunoblot results (A, C-E), the band intensity of each lane was quantified by Odyssey software. Immunoblot quantification results were normalized to β-actin expression, and control was set as 1. Abbreviations: CTR, control; RLU, relative luciferase unit; SCR, scrambled control; sh, short hairpin.

To clarify whether the antiviral activity of 5′-pppRNA exclusively relies on the RIG-I pathway, we employed wild-type (WT) mouse embryonic fibroblast (MEF) cells (RIG-I$^{+/+}$) and RIG-I-deficient MEF cells (RIG-I$^{-/-}$). These two MEF cell lines were transfected with different concentrations of 5′-pppRNA. Treatment with 1,000 ng/mL 5′-pppRNA induced mouse IFN genes (mouse IFN-β [mIFN-β], mIFN-λ) more than 1,000-fold at 24 hours after stimulation in RIG-I$^{+/+}$ MEFs (Supporting Fig. S3A). In contrast, no IFN gene was induced in RIG-I$^{-/-}$ MEFs, indicating that this is exclusively dependent on RIG-I signaling (Supporting Fig. S3A). Meanwhile, 5′-pppRNA activated the expression of many mouse ISGs, including mMX1, mIRF9, mIFIH1, mSTAT1, mIRF1, mPML, mXAF, mIRF7, mISG15, and mRIG-I in RIG-I$^{+/+}$ MEF cells (Supporting Fig. S3A). After 48-hour treatment, IFN gene and ISG expression was significantly induced by 5′-pppRNA, although to a lesser extent compared to 24 hours after treatment (Supporting Fig. S3B). Hence, the ISG induction activity of 5′-pppRNA was specifically through RIG-I.

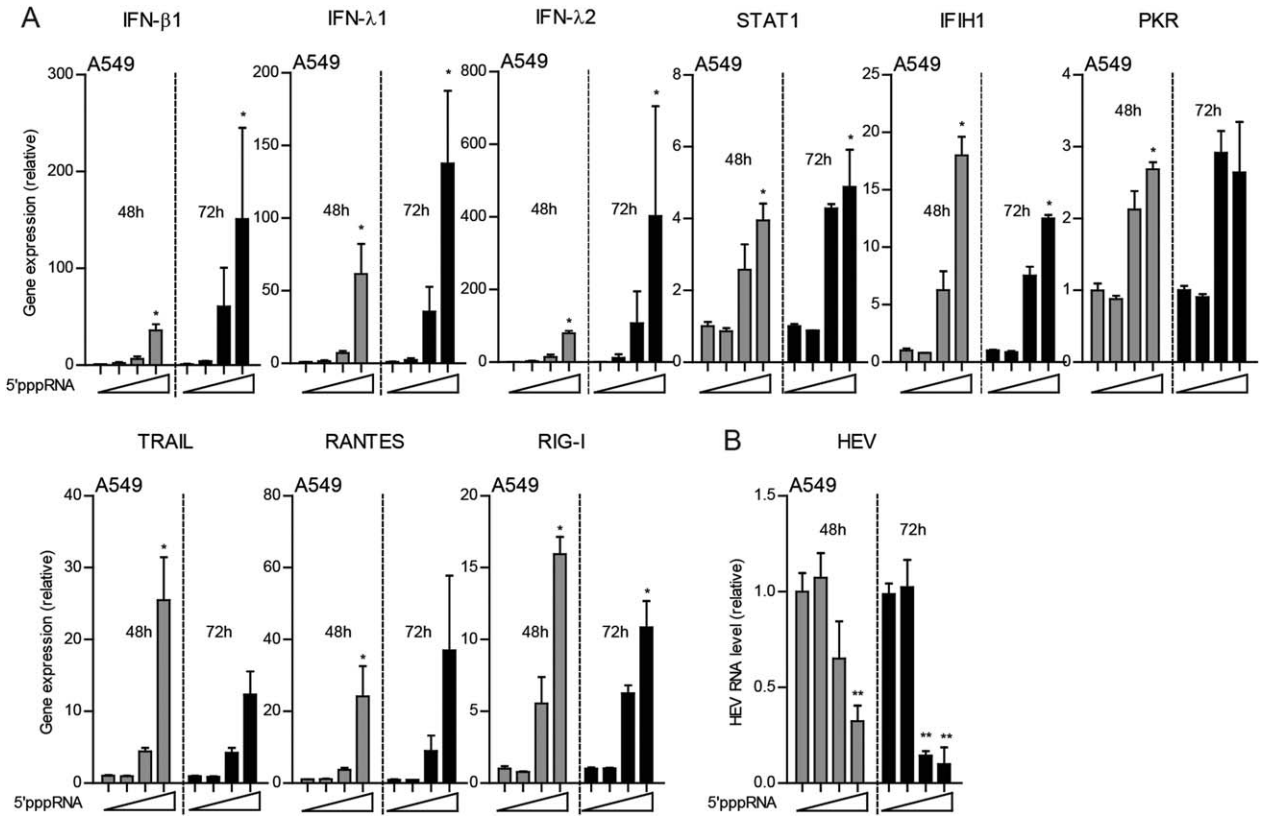

FIG. 3. 5′-pppRNA stimulates an antiviral response that inhibits HEV. A549-p6 cells were transfected with various concentrations of 5′-pppRNA (10 ng/mL, 100 ng/mL, and 1,000 ng/mL), and IFN gene mRNA levels, ISG mRNA levels (A), and HEV viral RNA levels (B) were analyzed by real-time quantitative RT-PCR 48 or 72 hours after transfection (A, n = 3-5; B, n = 6-8). Data were normalized to a control that was transfected with PEI-Mix but without 5′-pppRNA at each time point (48 and 72 hours, both set as 1), respectively. Data are means ± standard error of the mean. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

RIG-I ACTIVATES THE TRANSCRIPTION OF A WIDE RANGE OF ISGs

RIG-I has been shown to trigger STAT1 activation and ISG expression.[22] In general, activation of STAT1 leads to the formation and nuclear translocation of ISG factor 3. This complex further binds to the IFN-stimulated response element (ISRE) motifs in the genome DNA and drives the transcription of ISGs. A recent study has reported that RIG-I overexpression stimulates ISRE promoter activity.[23] Thus, we employed a transcriptional reporter system that mimics the IFN response with a reporter luciferase gene that was driven by multiple ISREs (ISRE-Luc). RIG-I significantly increased ISRE-related luciferase activity (Fig. 4A). Activation of ISRE usually leads to the transcription of ISGs that contain this element in their promoter regions. Therefore, we measured gene expression of a wide range of important antiviral ISGs. RIG-I overexpression stimulated the expression of a large number of ISGs in Huh7.5, A549, and HepaRG cells (Fig. 4B-D). Interestingly, the ISG expression pattern induced by RIG-I was different from that induced by IFN-α treatment (Supporting Fig. S4A-C). The induction of some important ISGs was further confirmed by immunoblotting at the protein level (Fig. 4E,F).

RIG-I MEDIATES IFN-α-INDUCED ANTIVIRAL ISG TRANSCRIPTION

Gene expression profile analysis in the previous study revealed that 5′-pppRNA treatment induced a distinct transcriptome compared to IFN-α treatment.[21] Besides, as a nucleic acid sensor, RIG-I is also an ISG that can be induced by IFN-α treatment; and we already demonstrated that RIG-I activates the

expression of many ISGs (Fig. 4). Therefore, we hypothesized that RIG-I may reinforce the IFN-α-initiated ISG induction. We thus investigated the association of RIG-I expression with the response to IFN-α treatment. Indeed, in RIG-I-overexpressed Huh7.5 cells, IFN-α-induced ISG expression was significantly enhanced (Fig. 5A). Similarly, IFN-α-induced ISG expression was also enhanced in RIG-I-transduced A549 cells (Fig. 5B).

To further determine the role of RIG-I in IFN-α-activated cell signaling, the RIG-I knockdown A549 cell line and the RIG-I-deficient MEF cell line (RIG-I$^{-/-}$) were employed. In A549 cells, RIG-I deficiency significantly attenuated the ISG induction ability of IFN-α (Fig. 5C). Consistently, in RIG-I-deficient MEF cells (RIG-I$^{-/-}$), the ISG induction ability of mIFN-α was also significantly reduced (Fig. 5D). In contrast, the ISG induction ability of mIFN-α was not affected in IRF3/7$^{-/-}$ or nuclear factor kappa B–deficient (NF-κB$^{-/-}$) MEF cells (Supporting Fig. S5A,B). Taken together, these results demonstrated that RIG-I functionally contributes to the antiviral ISG induction ability of IFN-α.

RIG-I ACTIVATES THE INNATE ANTI-HEV IMMUNE RESPONSE REGARDLESS OF IFN PRODUCTION

RIG-I is a cytosolic nucleic acid sensor, and the binding of viral RNA to RIG-I leads to the activation of downstream pathways that eventually triggers IFN gene expression through IRF3 and IRF7.[20] In turn, the secreted IFNs establish an antiviral response in infected and surrounding cells by stimulating the expression of ISGs. To further study the ISG induction ability of RIG-I pathways, the activator of RIG-I, 5′pppRNA, was transfected in IRF3/7 double knockout MEF cells (IRF3/7$^{-/-}$). Indeed, 5′-pppRNA failed to induce IFN-β expression in IRF3/7$^{-/-}$ cells (Fig. 6A). Strikingly, 5′-pppRNA is still capable of inducing ISGs in the absence of IRF3/7 and IFN-β expression (Fig. 6B). These results indicate that RIG-I could also stimulate ISG transcription in an IFN-independent manner. Next, we determined the mRNA expression levels of IFN genes in the RIG-I-overexpressed Huh7.5 cell line, a cell line that is unable to produce any IFNs.[24] We found that the mRNA expression level of IFN genes was very low and that no IFN gene (IFN-α, IFN-β, and IFN-λ) was induced by RIG-I overexpression in Huh7.5-p6 cells (Fig. 6C; Supporting Fig. S6A). Furthermore, no IFN gene was up-regulated in HepaRG-p6 cells by RIG-I overexpression (Supporting Fig. S6B,C). To confirm the lack of IFN production in these RIG-I-overexpressed cells, conditioned medium (supernatant) from RIG-I-transduced Huh7.5-p6 cells was collected (Fig. 6D). Two IFN-sensitive assays were performed: an IFN functional assay and an HCV replicon–based bioassay. The IFN functional assay was based on a transcriptional reporter system that mimics the IFN response, as above (Fig. 4A). Conditioned medium collected from RIG-I-overexpressed Huh7.5-p6 cells was not able to induce ISRE activation (Fig. 6E). Furthermore, this conditioned medium did not affect HCV-related luciferase activity (Fig. 6F). Similarly, conditioned medium collected from HepaRG cells also failed to activate ISRE-related luciferase activity (Supporting Fig. S6D). These results suggest that ectopic overexpression of RIG-I did not trigger IFN expression and production. Thus, we demonstrated that RIG-I could activate the innate immune response regardless of IFN production.

RIG-I MEDIATED ISG TRANSCRIPTION AND ANTI-HEV ACTIVITY PARTIALLY THROUGH ACTIVATION OF THE JAK-STAT PATHWAY

It has been demonstrated that RIG-I can augment STAT1 activation, which is a key element of the JAK-STAT cascade within the IFN pathway.[22] Consistent with these results, we also observed that RIG-I overexpression induced the phosphorylation of STAT1 at the 701 site in Huh7.5-p6 cells, which is an indispensable marker of JAK-STAT pathway activation (Fig. 7A).

To elucidate whether the ISG induction and anti-HEV abilities of RIG-I are the result of STAT1 activation, we used a JAK inhibitor, CP-690550 (tofacitinib), to pharmacologically block the JAK-STAT pathway. In Huh7.5-p6 cells, RIG-I-induced STAT1 phosphorylation and IFN-α-induced STAT1 phosphorylation were totally blocked by CP-690550 (Fig. 7B). Meanwhile, lentivirus-delivered RIG-I overexpression was not affected by this inhibitor (Fig. 7B,C). Surprisingly, we found that RIG-I-induced ISRE activation was not totally diminished by this inhibitor, whereas IFN-α-triggered ISRE activation was totally blocked (Fig. 7C). These results suggest that RIG-I-

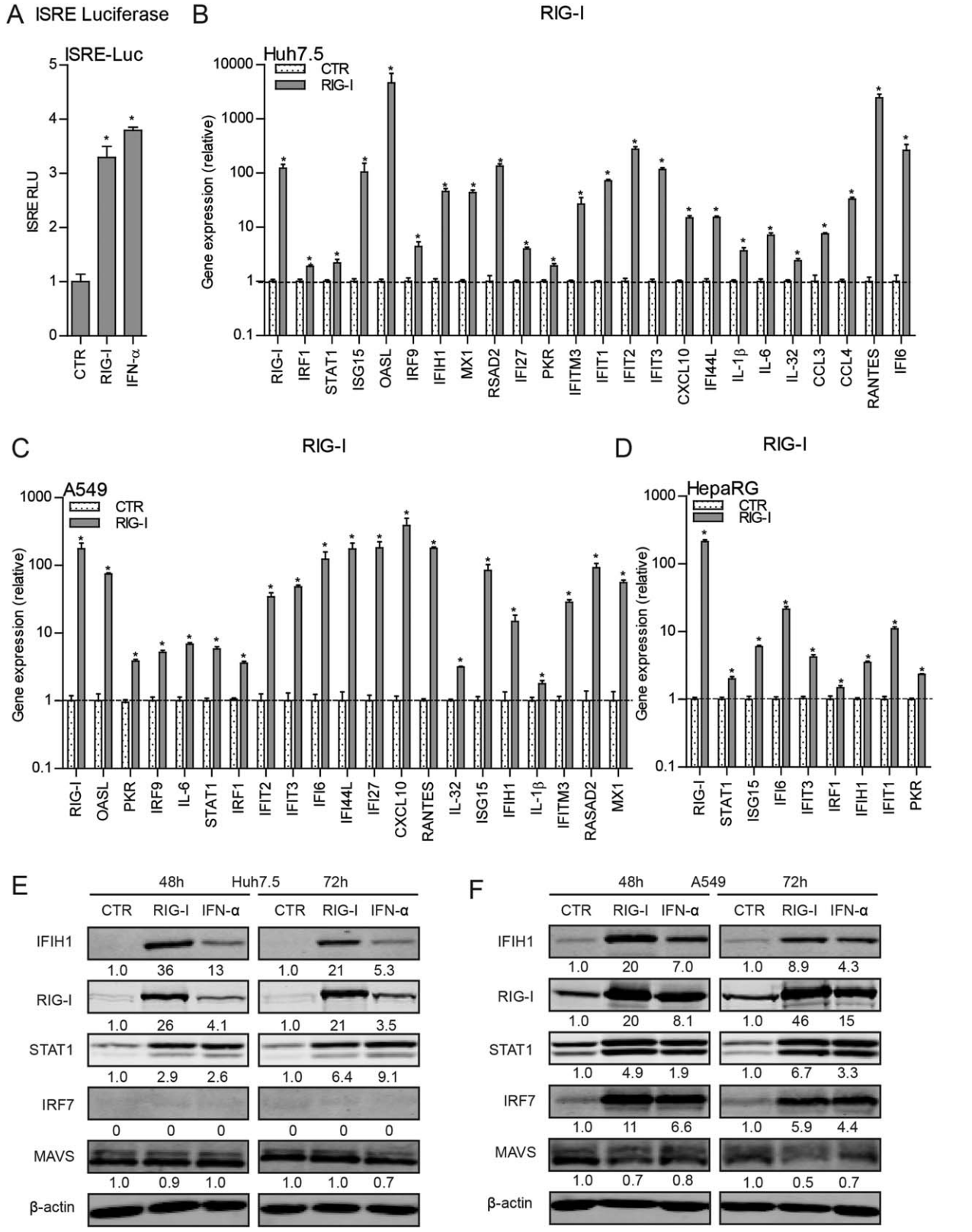
A ISRE Luciferase
ISRE-Luc
ISRE RLU
CTR
RIG-I
IFN-α
B
RIG-I
Huh7.5
CTR
RIG-I
Gene expression (relative)
RIG-I IRF1 STAT1 ISG15 OASL IRF9 IFIH1 MX1 RSAD2 IFI27 PKR IFITM3 IFIT1 IFIT2 IFIT3 CXCL10 IFI44L IL-1β IL-6 IL-32 CCL3 CCL4 RANTES IFI6
C
RIG-I
A549
CTR
RIG-I
Gene expression (relative)
RIG-I OASL PKR IRF9 IL-6 STAT1 IRF1 IFIT2 IFIT3 IFI6 IFI44L IFI27 CXCL10 RANTES IL-32 ISG15 IFIH1 IL-1β IFITM3 RASAD2 MX1
D
RIG-I
HepaRG
CTR
RIG-I
Gene expression (relative)
RIG-I STAT1 ISG15 IFI6 IFIT3 IRF1 IFIH1 IFIT1 PKR
E
48h Huh7.5 72h
CTR RIG-I IFN-α CTR RIG-I IFN-α
IFIH1 1.0 36 13 1.0 21 5.3
RIG-I 1.0 26 4.1 1.0 21 3.5
STAT1 1.0 2.9 2.6 1.0 6.4 9.1
IRF7 0 0 0 0 0 0
MAVS 1.0 0.9 1.0 1.0 1.0 0.7
β-actin
F
48h A549 72h
CTR RIG-I IFN-α CTR RIG-I IFN-α
IFIH1 1.0 20 7.0 1.0 8.9 4.3
RIG-I 1.0 20 8.1 1.0 46 15
STAT1 1.0 4.9 1.9 1.0 6.7 3.3
IRF7 1.0 11 6.6 1.0 5.9 4.4
MAVS 1.0 0.7 0.8 1.0 0.5 0.7
β-actin

FIG. 4

induced ISRE activation is partially independent of its STAT1 phosphorylation ability. Next, we tested the mRNA expression level of 23 RIG-I-inducible ISGs in RIG-I-overexpressed Huh7.5-p6 cells treated with the JAK inhibitor CP-690550. Surprisingly, among these 23 tested RIG-I-inducible ISGs, only 10 genes were affected by CP-690550 treatment, and the others were not affected at all (Fig. 7D,E). As a positive control, the expression level of all of the 23 genes that induced by IFN-α was totally diminished by this inhibitor (Supporting Fig. S7A,B). Consequently, the anti-HEV ability of RIG-I was only partially blocked by CP-690550, whereas the anti-HEV effects of IFN-α were totally abolished (Fig. 7F). To further confirm these results, another JAK inhibitor, named JAK inhibitor 1, was used to treat RIG-I-transduced Huh7.5-p6 cells. As expected, similar results were obtained (Supporting Fig. S8). To explore whether this is a common mechanism in different cell lines, we also used JAK inhibitor 1 to treat RIG-I-overexpressed HepaRG-p6 cells, and similar results were obtained (Supporting Fig. S9).

To further confirm that the ISG induction ability of RIG-I is not totally dependent on the JAK-STAT pathway, we overexpressed RIG-I in STAT1-deficient human ($STAT1^{-/-}$) fibrosarcoma cells.[25] In $STAT1^{-/-}$ cells, RIG-I failed to induce STAT1 phosphorylation (Fig. 8A). A similar ISG induction pattern was also observed in RIG-I-overexpressed $STAT1^{-/-}$ cells. Some genes, such as STAT1, IRF9, and IFI6, can only be induced in WT cells but not in $STAT1^{-/-}$ cells by RIG-I, although the RIG-I overexpression level was similar in both cell lines (Fig. 8B,C). Meanwhile, another group includes genes that can be activated in both WT and $STAT1^{-/-}$ cells such as IFIT1, RANTES, and chemokine (C-X-C motif) ligand 10 (CXCL10) (Fig. 8D). As a control, IFN-α-induced ISG transcription was totally abolished in $STAT1^{-/-}$ cells (Supporting Fig. S10A). Together, these results demonstrated that RIG-I activates ISG transcription and exerts its anti-HEV activity partially through activation of the JAK-STAT pathway.

Discussion

Currently, IFNs (in particular IFN-α) have been approved for treating viral infections including chronic hepatitis B and HCV infections.[7] In some cases, IFN-α has been used as an off-label drug to treat chronic HEV infection.[13,26] *In vitro* study also showed the inhibition of HEV replication by IFN-α treatment.[14,15,27] IFN-α exerts its antiviral ability through the induction of ISGs, but how these ISGs affect HEV replication is still largely unknown. This study comprehensively profiled the antiviral ability of many important human ISGs described previously.[11] We found that most of these ISGs showed minor anti-HEV effects. In contrast, several reported broad antiviral ISGs including MDA5, IRF1, and RIG-I were identified as strong anti-HEV ISGs. We have demonstrated that IRF1 inhibits HEV replication by activating antiviral ISGs.[17] RIG-I is a pattern recognition receptor, and numerous studies have demonstrated that it plays important roles in defending against a wide spectrum of viral infections such as HCV,[11,21] hepatitis B virus,[28] and influenza virus.[29] In this study we comprehensively investigated the antiviral potential of RIG-I and its mechanism of action.

RIG-I, as a pattern recognition receptor, senses the viral RNA in the cytoplasm during infection. Binding of RIG-I with its ligand such as 5′-pppRNA activates the downstream signaling pathway through the adaptor protein mitochondrial antiviral signaling protein (MAVS). Classically, the aggregation of MAVS in mitochondria subsequently leads to the production of type 1 and 3 IFNs through the phosphorylation of IRF3 and IRF7. The produced IFN proteins subsequently activate ISG expression in infected and bystander cells to eradicate the virus and prevent further infections. In this study, we observed overexpression of RIG-I-activated transcription and expression of a wide range of ISGs, and many of them are known to have strong antiviral activities.[11,12,17,21,30-33]

Although working as a cytosolic nucleic acid sensor that triggers IFN production, RIG-I itself is also an

FIG. 4. RIG-I activates the transcription of a wide range of ISGs. (A) Analysis of ISRE-related *firefly* luciferase activity in Huh7-ISRE-Luc cells transduced with RIG-I vector or treated with IFN-α (1,000 IU/mL) for 48 hours (n = 3 independent experiments with each of 1-2 replicates). Quantitative RT-PCR analysis of ISG mRNA levels in Huh7.5-p6 cells (B), A549-p6 cells (C), and HepaRG-p6 cells (D) transduced with RIG-I or Fluc vector for 48 hours (n = 6). Immunoblot analysis of ISG protein levels in Huh7.5-p6 cells (E) and A549-p6 cells (F) transduced with RIG-I or Fluc vector for 48 or 72 hours. Date in (A) were normalized to the untreated Fluc control (set as 1). Data in (B-D) were normalized to the Fluc control (set as 1). Data are means ± standard error of the mean. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. For immunoblot results (E and F), the band intensity of each lane was quantified by Odyssey software. Immunoblot quantification results were normalized to β-actin expression, and control was set as 1. Abbreviations: CTR, control; RLU, relative luciferase unit.

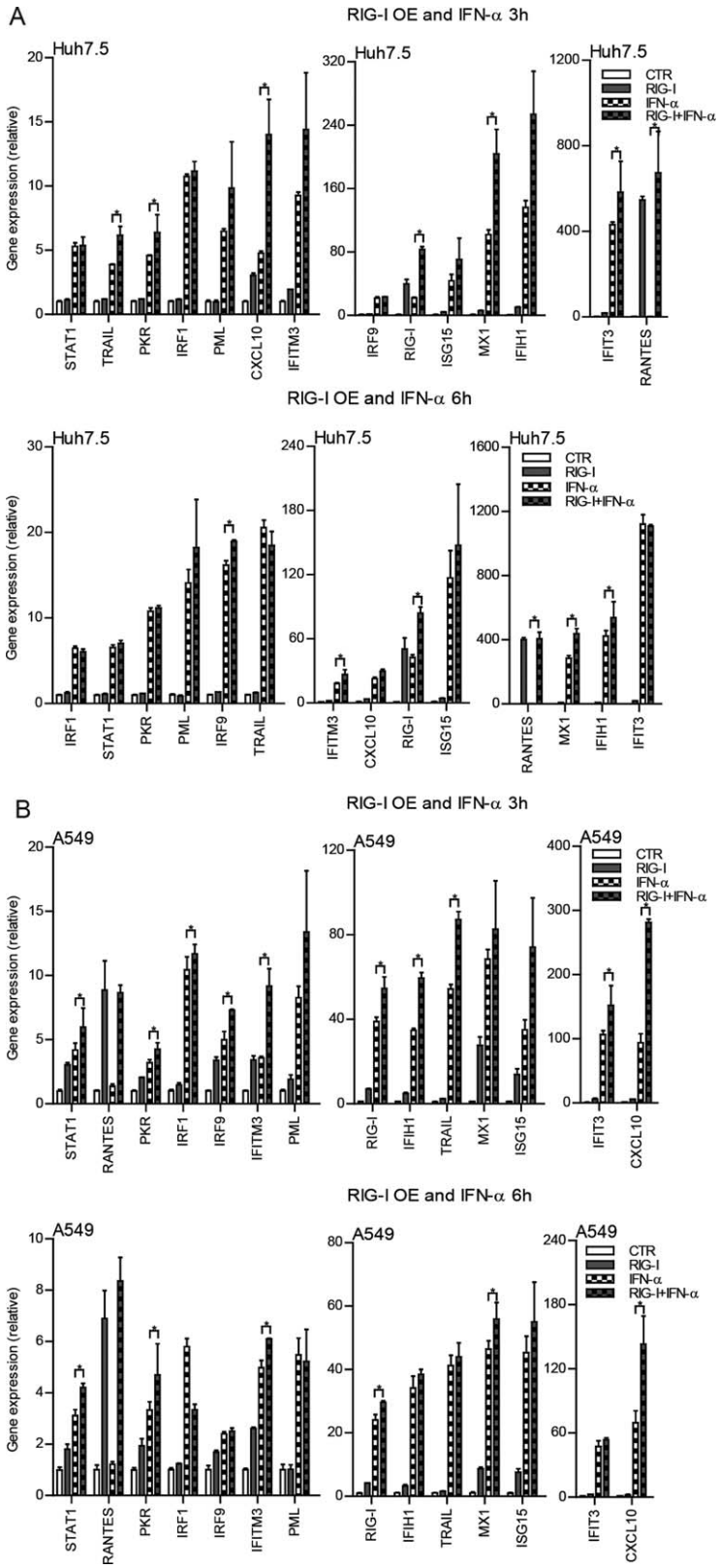

FIG. 5. RIG-I mediates IFN-α-induced antiviral ISG transcription. Quantitative RT-PCR analysis of ISG mRNA expression levels. Huh7.5 cells were transduced with RIG-I or Fluc vector, and at 48 hours posttransduction cells were treated with IFN-α (1,000 IU/mL) for 3 or 6 hours (A) (n = 4). A549 cells were transduced with RIG-I or Fluc vector, and at 48 hours posttransduction cells were treated with IFN-α (1,000 IU/mL) for 3 or 6 hours (B) (n = 4). Data in (A) and (B) were normalized to untreated Fluc control (set as 1). A549 cells were transduced with lentiviral short hairpin RNA vectors targeting RIG-I or scrambled control. Stable RIG-I knockdown and control A549 cells were treated with IFN-α (1,000 IU/mL) for 3 or 6 hours (C). ISG mRNA levels were analyzed by quantitative RT-PCR (n = 6). Quantitative RT-PCR analysis of mouse ISG mRNA levels in WT and RIG-I$^{-/-}$ MEF cells treated with mIFN-α (1,000 IU/mL) for 3 or 6 hours (D) (n = 6). Data in (C) were normalized to the untreated scrambled control (set as 1). Data in (D) were normalized to untreated WT MEF cells (set as 1). Data are means ± standard error of the mean. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Abbreviations: CTR, control; RLU, relative luciferase unit; SCR, scrambled control; sh, short hairpin.

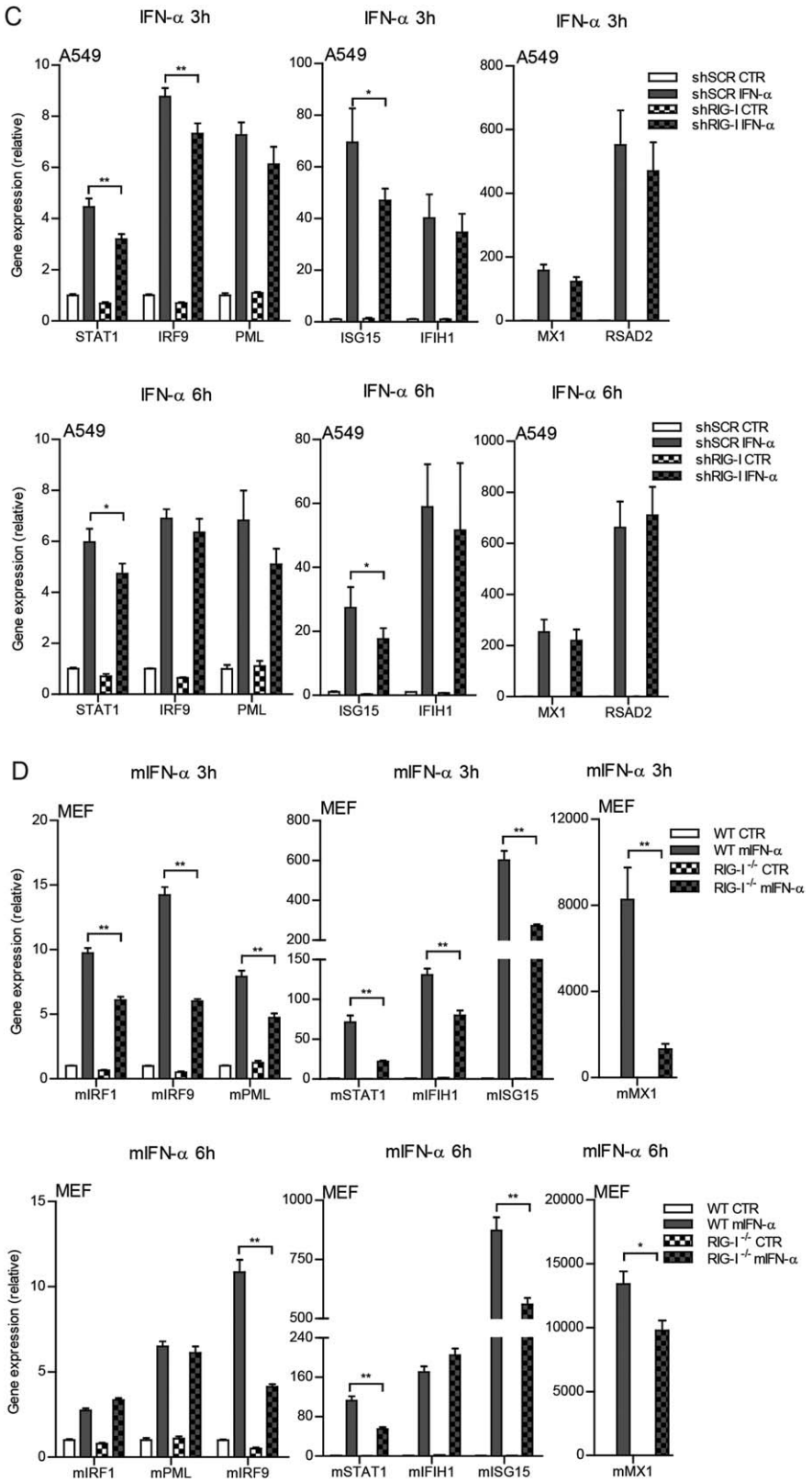

FIG. 5. Continued

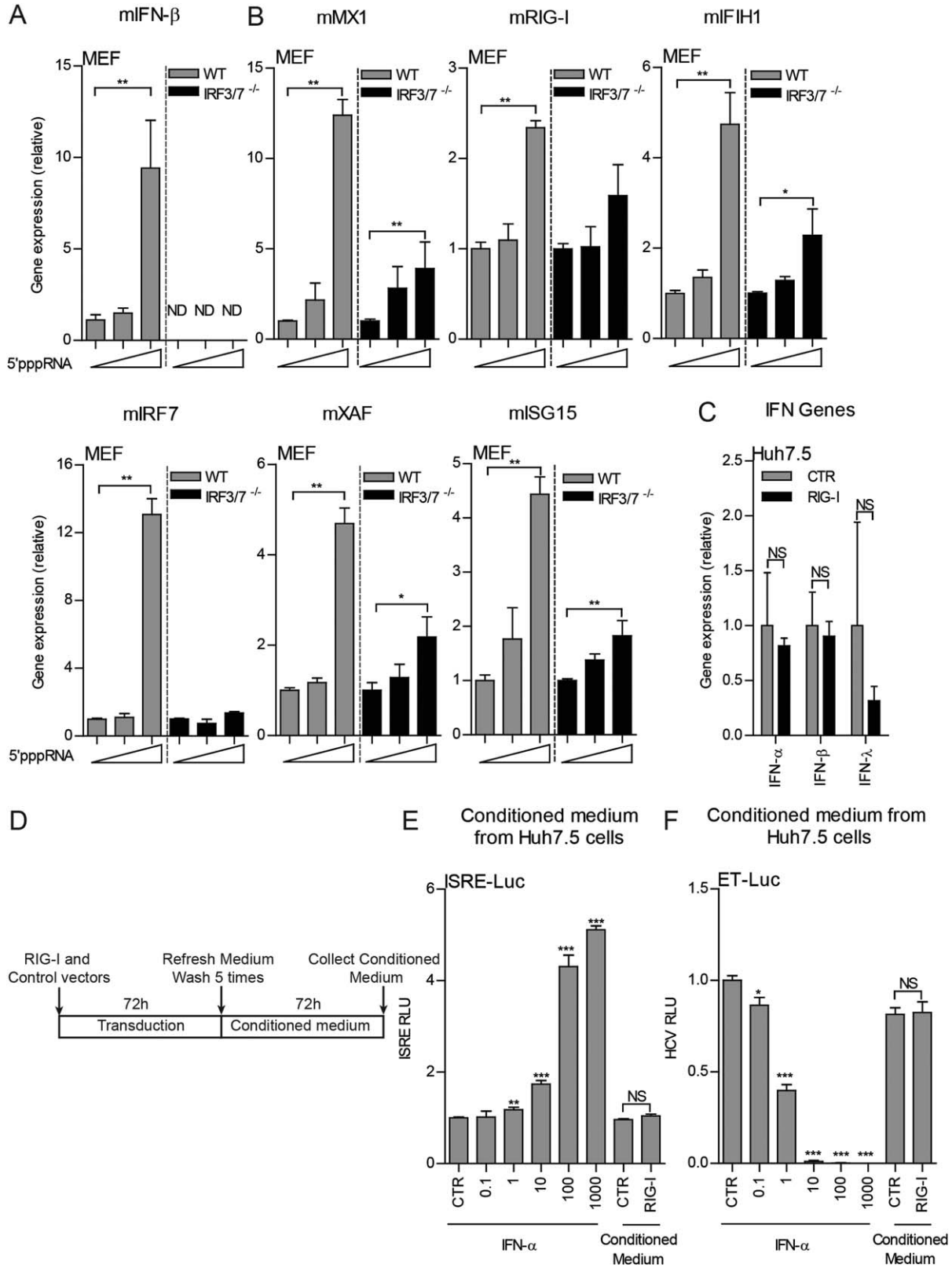
A
mIFN-β
B
mMX1
mRIG-I
mIFIH1
MEF
WT
IRF3/7 -/-
Gene expression (relative)
ND ND ND
5'pppRNA
mIRF7
mXAF
mISG15
C
IFN Genes
Huh7.5
CTR
RIG-I
NS
IFN-α
IFN-β
IFN-λ
D
RIG-I and Control vectors
Refresh Medium Wash 5 times
Collect Conditioned Medium
72h
Transduction
Conditioned medium
E
Conditioned medium from Huh7.5 cells
ISRE-Luc
ISRE RLU
CTR
0.1
1
10
100
1000
RIG-I
IFN-α
Conditioned Medium
F
Conditioned medium from Huh7.5 cells
ET-Luc
HCV RLU

FIG. 6

ISG regulated by the IFN-initiated JAK-STAT cascade. This feedback amplification loop is able to enhance the responsiveness of host cells to infection. It has been shown that 5′-pppRNA treatment induced a broader transcriptome compared to IFN-α treatment. Consistently, we found a combinatorial action of IFN-α treatment and RIG-I overexpression in ISG induction and anti-HEV activity. Conversely, IFN-α-induced ISG transcription was attenuated when RIG-I was deleted (Fig. 5). These results suggest that RIG-I partially mediates the ISG transcription activity of IFN-α.

Classically, the main antiviral function of RIG-I is believed to be from the induction of IFN production upon sensing cytosolic viral nucleic acid. However, we found that RIG-I overexpression in our models does not induce the production of IFNs but triggers the transcription of a wide range of genes including ISGs, chemokines, and cytokines, without the activation of MAVS at the protein level (Fig. 4E,F). In fact, emerging recent studies have proposed additional antiviral mechanisms of RIG-I that are partially dependent or independent of IFNs.[28,29,34]

It has been demonstrated that induction of ISGs and antiviral ability by RIG-I activation were only partially reduced in the absence of IFN-α/β receptors.[21] Additionally, RIG-I can exert broad antiviral activity in the models that have defective IFN signaling.[11] We now observed that in cells that lack the key downstream components of the RIG-I pathway (IRF3 and IRF7), the RIG-I activator 5′-pppRNA is still able to induce ISG expression (Fig. 6A) without activating IFN-β gene expression. In line with this, we also observed that in Huh7.5 cells (a cell line which could not produce any IFN protein) RIG-I overexpression induces ISG transcription and exerts potent anti-HEV activity (Fig. 2). Together with recent reports, our observations strongly support that RIG-I can also execute its antiviral action through IFN-independent mechanisms. However, these IFN-independent actions are diverse, and their exact mechanisms remain largely elusive.

Typically, the activation of RIG-I will trigger the activation of IRF3/7 and NF-κB through MAVS antiviral signaling.[21,35] Activation of the NF-κB pathway leads to the transcription of many proinflammatory genes including IFN genes. Our previous study also revealed that the NF-κB complex can directly bind to ISRE and drive its transcription of some ISGs.[36] Interestingly, many of the proinflammatory genes are regulated by both the NF-κB and JAK-STAT pathways. For instance, the transcription of chemokine (C-X-C motif) ligand 10 (CXCL10) is positively regulated by ISRE and NF-κB during viral infection.[37] It has been reported that MEF cells with a defective NF-κB pathway were more sensitive to the antiviral action of type 1 IFN.[38] We also observed that the IFN-induced expression of some ISGs was enhanced in NF-κB knockout MEF cells, whereas the expression of other genes was lower or unaffected (Supporting Fig. S5). Furthermore, a subset of RIG-I-induced ISGs was unaffected when the JAK-STAT pathway was blocked (Fig 7E), indicating the involvement of additional regulatory mechanisms. Thus, RIG-I and its downstream pathways form a large, complex web. Besides the JAK-STAT cascade, other pathways such as NF-κB may also involve the regulation of RIG-I-mediated ISG induction and antiviral activity.

RIG-I overexpression has been demonstrated to trigger STAT1 activation and ISG expression independent of its canonical MAVS pathway.[22] We now confirm the overexpression of RIG-I-activated STAT1 phosphorylation at the 701 site (Fig. 7A) without the involvement of functional IFNs. We further addressed the contribution of STAT1 phosphorylation to the anti-HEV action of RIG-I. Using pharmacological inhibitors to block the JAK-STAT pathway, we demonstrated that RIG-I-induced ISG transcription and anti-HEV activity are only partially

FIG. 6. RIG-I activates an immune response regardless of IFN production. WT and IRF3 and IRF7 double-deficient (IRF3/7$^{-/-}$) MEF cells were transfected with various concentrations of 5′-pppRNA (100 ng/mL and 1,000 ng/mL), and mIFN-β (A) and mISG (B) mRNA levels were analyzed by quantitative RT-PCR 24 hours after transfection (n = 6). (C) Quantitative RT-PCR analysis of IFN gene mRNA levels in Huh7.5-p6 cells transduced with RIG-I or Fluc vector for 48 hours (n = 4). (D) Production of conditioned medium (supernatant). Cells were transduced with RIG-I or GFP (control) vector for 72 hours; then, the cells were washed five times, and medium was refreshed. Cells were cultured for another 72 hours, and supernatant was collected as conditioned medium. Analysis of ISRE-related *firefly* luciferase activity in Huh7-ISRE-Luc cells (E) or HCV-related *firefly* luciferase activity in Huh7.5-ET-Luc cells (F) treated with conditioned medium from Huh7.5 cells or various concentrations of IFN-α for 48 hours (n = 3 independent experiments with each of 3-4 replicates). Data in (A) and (B) were normalized to a control that was transfected with PEI-Mix but without 5′-pppRNA in each cell line (WT and IRF3/7$^{-/-}$, both set as 1), respectively. Data in (C) were normalized to the Fluc control (set as 1). Data in (E) and (F) were normalized to the untreated GFP control (set as 1). Data are means ± standard error of the mean. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Abbreviations: CTR, control; ND, not determined; NS, not significant; RLU, relative luciferase unit.

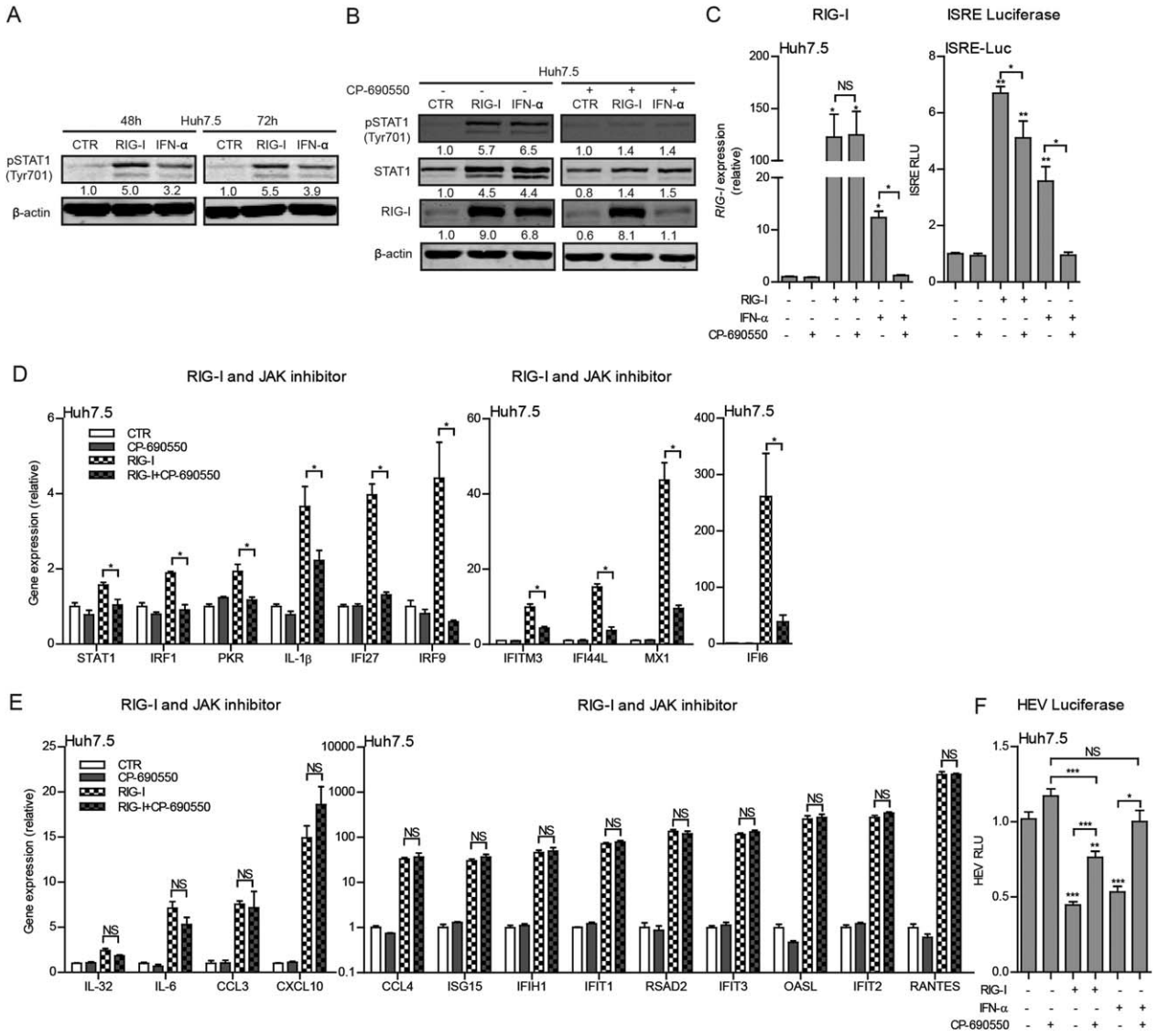

FIG. 7. JAK inhibitor CP-690550 partially diminishes RIG-I-induced ISG transcription and anti-HEV activity. (A) Immunoblot analysis of p-STAT1 (Tyr701) expression in Huh7.5 cells transduced with RIG-I vector or treated with IFN-α (1,000 IU/mL) for 48 or 72 hours. (B) Immunoblot analysis of ISG protein levels in Huh7.5 cells transduced with RIG-I vector or treated with IFN-α (1,000 IU/mL) or CP-690550 (1000 ng/mL) for 48 hours. Quantitative RT-PCR analysis of RIG-I mRNA level in Huh7.5-p6 cells and analysis of ISRE-related *firefly* luciferase activity in Huh7-ISRE-Luc cells (C) transduced with RIG-I vector or treated with IFN-α (1,000 IU/mL) or CP-690550 (1,000 ng/mL) for 48 hours (quantitative RT-PCR, n = 4; ISRE, n = 3 independent experiments with each of 2 replicates). (D,E) Quantitative RT-PCR analysis of ISG mRNA levels in Huh7.5 cells transduced with RIG-I vector or treated with CP-690550 (1,000 ng/mL) for 48 hours (n = 5-6). (F) Analysis of HEV-related *Gaussia* luciferase activity in Huh7-p6-Luc cells transduced with RIG-I vector or treated with IFN-α (1,000 IU/mL) or CP-690550 (1,000 ng/mL) for 72 hours (n = 4 independent experiments with each of 3-4 replicates). For immunoblot results (A,B), the band intensity of each lane was quantified by Odyssey software. Immunoblot quantification results were normalized to β-actin expression, and control was set as 1. Data in (C left panel, D and E) were normalized to the untreated Fluc control (set as 1). Data in (C right panel and F) were normalized to the untreated GFP control (set as 1). Data are means ± standard error of the mean. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Abbreviations: CTR, control; NS, not significant; RLU, relative luciferase unit.

dependent on this cascade, whereas the IFN-α-mediated effect is totally dependent on it. Furthermore, in a JAK-STAT-deficient cell model, only a small proportion of RIG-I-inducible ISGs were affected in response to RIG-I overexpression. We thus classified these RIG-I-inducible ISGs into two categories.

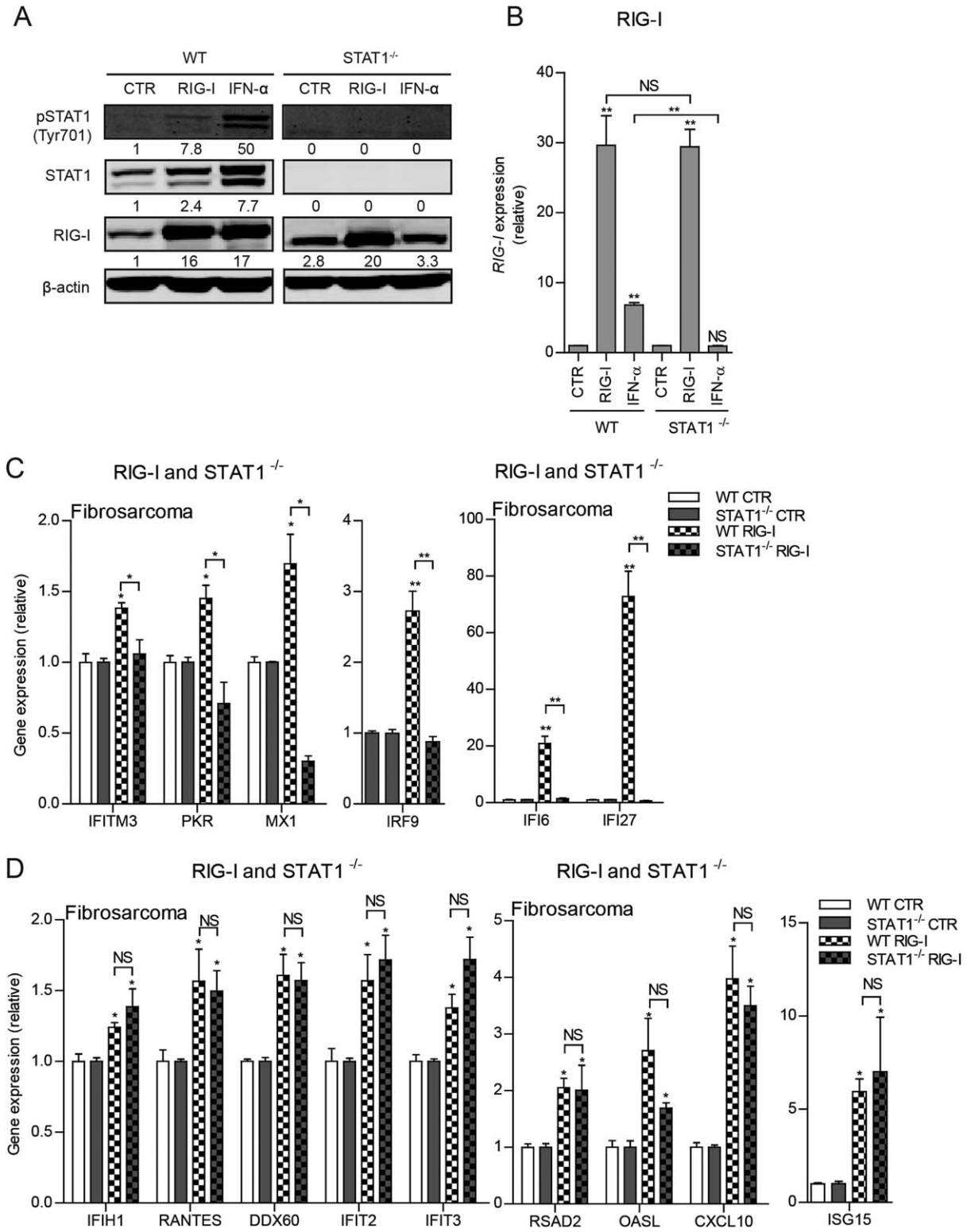

FIG. 8. The ISG induction ability of RIG-I is partially diminished in STAT1-deficient cells. (A) Immunoblot analysis of ISG protein levels in WT and STAT1-deficient ($STAT1^{-/-}$) fibrosarcoma cells transduced with RIG-I vector or treated with IFN-α (1,000 IU/mL) for 48 hours. (B) Quantitative RT-PCR analysis of RIG-I mRNA level in WT and $STAT1^{-/-}$ cells transduced with RIG-I vector or treated with IFN-α (1,000 IU/mL) for 48 hours (n = 4-5). (C,D) Quantitative RT-PCR analysis of ISG mRNA levels in WT and $STAT1^{-/-}$ cells transduced with RIG-I or Fluc vector for 48 hours (n = 4-5). For immunoblot results (A), the band intensity of each lane was quantified by Odyssey software. Immunoblot quantification results were normalized to β-actin expression, and control was set as 1. Data in (C) were normalized to the untreated Fluc control (set as 1). Date in (D) and (E) were normalized to untreated WT and $STAT1^{-/-}$ cells, respectively (both set as 1). Data are means ± standard error of the mean. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Abbreviations: CTR, control; NS, not significant.

One group, including STAT1, IRF1, and IRF9, is completely dependent on RIG-I-induced STAT1 phosphorylation. The other group, including IFIT1, IFIH1, and RANTES, is induced independently of STAT1 activation at the 701 site. Of note, both subsets may contribute to the anti-HEV ability of RIG-I as the anti-HEV action of RIG-I was only partially attenuated by blocking the JAK-STAT pathway (Fig. 7E). Recently, accumulating evidence has revealed direct antiviral action of RIG-I independent of its downstream IFN production effect.[28,29,34] However, whether RIG-I has a direct anti-HEV effect is still unknown and needs further investigation.

IFN-α and ribavirin have been used as monotherapy or in combination for treating chronic HEV patients. Ribavirin monotherapy appears effective in many patients but failed in a substantial proportion of cases, probably due to the development of drug-resistance mutations in the viral genome.[39-41] IFN-α seems also effective but is associated with organ rejection as most chronic HEV patients are immunocompromised organ recipients.[42] It is also well established that excessive exposure to IFNs can result in pathogenesis to the host, and treatment with IFN-α is associated with various severe side effects in patients.[43] Therefore, dissecting the antiviral and the pathogenic mechanisms is necessary for developing specific antiviral strategies while avoiding unnecessary side effects. Our identification that RIG-I is a key anti-HEV ISG and that activating it by the natural ligand 5′-pppRNA exerts potent anti-HEV activity provide proof of concept for designing such a specific anti-HEV approach. Several RIG-I agonists (ImOl-100, Rigontec; MCT-465, Multicell Technologies; SB-9200, Spring Bank Pharmaceuticals) are at various stages of preclinical or clinical development for treating viral infections.[44] Thus, the possibility of using these RIG-I agonists in treating HEV infection deserves further evaluation.

In conclusion, we have identified RIG-I as a key anti-HEV ISG that inhibits HEV replication. Biological or pharmacological activation of the RIG-I pathway potently inhibits HEV replication. We further observed that ectopic overexpression of RIG-I activated the transcription of many antiviral ISGs to establish an anti-HEV status. This occurred regardless of IFN production but partially through activation of the JAK-STAT cascade.

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