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Poly(I:C) inhibits porcine reproductive and respiratory syndrome virus replication in MARC-145 cells via activation of IFIT3

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ABSTRACT

Porcine reproductive and respiratory syndrome virus (PRRSV) is a major cause of heavy economic losses in many swine-producing regions. Current vaccination strategies and antiviral drugs provide only limited protection. Interferon (IFN)-induced protein with tetratricopeptide repeats 3 (IFIT3) has been characterized as the product of a novel antiviral gene and as an important modulator in innate immunity. However, the role of IFIT3 in PRRSV infection is scarcely understood. In this study, polyinosinic–polycytidylic acid (poly(I:C)) inhibited PRRSV replication in MARC-145 cells, following the appearance of increased IFIT3. Overexpression of porcine IFIT3 resulted in a decrease of PRRSV. Knockdown of IFIT3 in MARC-145 cells increased PRRSV replication and impaired the antiviral activity mediated by poly(I:C). Moreover, in the presence or absence of IFIT3, poly(I:C)-induced IFN-β promoter activity was significantly boosted or crippled, respectively. IFIT3, TBK1 and phosphorylation of IRF3 were activated in poly(I:C)-transfected MARC-145 cells. It demonstrated that IFIT3 plays an important role in IFN-β induction in MARC-145 cells, and, when activated, it can inhibit PRRSV replication.

1. Introduction

Porcine reproductive and respiratory syndrome (PRRS) is one of the most devastating and costly diseases to the swine industry worldwide, causing abortion in pregnant sows and gilts, and respiratory distress with increased susceptibility to secondary infection in piglets and growing pigs (Christianson et al., 1992; Rossow et al., 1994). However, current vaccination strategies and antiviral drugs provide only limited protection (Dwivedi et al., 2012). The etiological agent, PRRS virus (PRRSV), belongs to the family *Arteriviridae* in the order *Nidovirales* (Meulenberg et al., 1993). The viral genome is approximately 15 kb in length and consists of ten open reading frames (ORFs), designated ORF1a, ORF1b, and ORF3 though 7, including ORF2a and ORF5a (Firth et al., 2011; Johnson et al., 2011; Snijder and Meulenberg, 1998). Two large ORFs, 1a and 1b, are located at the 5′ terminal end of the genome and encode a large polyprotein, which are cleaved into 14 small non-structural protein (nsp) products: nsp1α, nsp1β, and nsp2 through 12 including nsp7α and nsp7β, in order from the N-terminus (Beerens et al., 2007; Fang and Snijder, 2010; Snijder and Meulenberg, 1998; van Aken et al., 2006a,b). The remaining ORFs in the 3′-terminal region code for structural GP2, small envelope (E), GP3, GP4, 5a, GP5, membrane (M), and nucleocapsid (N) proteins (Firth et al., 2011; Johnson et al., 2011; Meulenberg et al., 1995; Wu et al., 2001).

Secretion of type I IFN (IFN-α/β) is a key step in the innate immune response to viral infection (Kawai and Akira, 2006; Takaoka and Yanai, 2006). Initially, the pathogen-associated molecular pattern in dsRNA is recognized by multiple pattern recognition receptors (PRRs), including Toll-like receptor 3 (TLR3), and cytoplasmic RNA helicases, such as retinoic acid-inducible protein I (RIG-I) or the product of the melanoma differentiation-associated gene 5 (MDA5) (Yoneyama et al., 2004). The RIG-I or MDA5 serves as an intracellular dsRNA receptor via DexD/H-box helicase domains. Caspase recruitment domains (CARDs) of RIG-I or MDA5 interact with the counterpart domains of IFN-β promoter stimulator 1 (IPS-1, also known as MAVS/VISA/Cardif) (Kawai et al., 2005). Despite utilization of the different adaptors, both pathways converge to activate the two downstream kinases, Tank-binding kinase 1 (TBK1) and inhibitor of κB kinase ε (IKKε), resulting in the phosphorylation and activation of transcription factors, including IFN regulatory factor 3 (IRF3), NF-κB, and AP-1 (Fitzgerald et al., 2003). The activated IRF3 induce transcription of type I IFN genes (Akira et al., 2006; Hiscott, 2007; Honda et al., 2006). PRRSV is proven to inhibit IFN induction and downstream JAK/STAT signaling (Albina et al., 1998; Calzada-Nova et al., 2011; Li et al., 2010; Miller et al., 2004; Patel et al., 2010), whereas there are a few strains known to induce IFN, such as A2MC2 (Nan et al., 2012).

The IFN-induced protein with tetratricopeptide repeats (IFIT) family of genes is clustered on human chromosome 10, and

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includes IFIT1, IFIT2, IFIT3/4 and IFIT5. All are robustly induced by IFNs, viral infection and Lipopolysaccharide (LPSs) (Fensterl and Sen, 2011). Orthologs of the IFIT family are evolutionarily conserved from amphibians to mammals. The IFIT family proteins are characterized by the unique helix-turn-helix motifs called tetratrico-peptide repeats (TPRs), which mediate a variety of protein–protein interactions (D'Andrea and Regan, 2003). Recently, it was demonstrated that IFIT3 was associated with antiviral activity as an IFN-α-induced protein (Miettinen et al., 2012; Schmeisser et al., 2010). In addition, IFIT3 is an important adaptor bridging TBK1 to MVAS on the mitochondrion (Liu et al., 2011). Evidence has shown that PRRSV infection can inhibit the induction of type I interferon genes (Chand et al., 2012). Pre-treatment with the dsRNA suppressed PRRSV infection in pulmonary alveolar macrophages (PAMs) *in vitro* (Miller et al., 2009; Sang et al., 2011). Our previous study demonstrated that the expression of IFIT3 was correlated with PRRSV-infection by iTRAQ technology (Lu et al., 2012). In the present study, we first found that IFIT3 is responsible for mediating the dsRNA-induced production of IFN-β in MARC-145 cells. IFIT3, when activated, could inhibit PRRSV replication in MARC-145 cells. This finding should be helpful in the future development of novel antiviral therapies against PRRSV infection.

2. Materials and methods

2.1. Cells and virus

The HEK-293A and MARC-145 cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum, and were maintained at 37 °C with 5% CO_2. The highly pathogenic PRRSV strain SY0608 (GenBank accession number EU144079) was used throughout the study.

2.2. Construction and production of recombinant adenovirus

To construct a recombinant adenovirus expressing the porcine IFIT3 gene (GenBank No. NM_001204395), the amplicon of IFIT3 was digested with *Kpn* I and *Xho* I and cloned into the transfer vector pShuttle–CMV by the introduction of the pAdEasy-1 system (Qbiogene, CA, USA). The recombinant shuttle vector was linearized with *Pme* I and cotransformed with the pAdEasy-1 into *Escherichia coli* BJ5183 by electroporation. A positive clone was selected and confirmed by making DNA minipreps and performing *Pac* I digestion. The resulting adenoviral plasmid was linearized with *Pac* I, purified by ethanol precipitation, and transfected into HEK-293A cells using TransFastTM Transfection Reagent (Promega, Madison, WI, USA) for virus rescue. The recombinant adenovirus was propagated in HEK-293A cells and named rAd-IFIT3. The adenoviral titer, passaged three times, was determined by evaluation of the tissue culture infectious dose 50 ($TCID_{50}$).

2.3. Treatment of poly(I:C)

To assess the capacity of poly(I:C) on IFIT3 expression, MARC-145 cells were seeded onto 24-well plates and incubated overnight at 37 °C with 5% CO_2. When the cells reached 80% confluence, they were treated with four concentrations of poly(I:C) (0, 0.25, 2.5, 25 μg/ml, respectively). Twenty-four hours later, the supernatants of MARC-145 cells were discarded and the cells were challenge with PRRSV at 10 MOI. Fourty-eight hours post challenge (hpc), virus yield titration and Western blotting were used to detect the effect of poly(I:C) on IFIT3 and PRRSV. Meanwhile, Western blotting were used to detect the effect of poly(I:C) on IFIT3 in MARC-145 treated with 25 μg/ml of poly(I:C) at the indicated time points.

2.4. Viral challenge

To assess the capacity of rAd-IFIT3 to inhibit PRRSV replication in MARC-145 cells, which are susceptible to rAd5 infection but do not permit productive replication, the cells were seeded and incubated overnight at 37 °C with 5% CO_2. When the cells reached 80–90% confluence, they were inoculated with rAd-IFIT3 or rAd-Wt (control) in DMEM with 2% fetal bovine serum. At 0–36 h post-inoculation (hpi), the cells were challenged with PRRSV at 10 multiplicity of infection (MOI). One hour later, DMEM containing 2% fetal bovine serum was added into wells. At indicated time point post challenge, the cells lysate samples were collected and the effect of overexpressed IFIT3 on PRRSV replication were detected by indirect immunofluorescence assay (IFA), real-time PCR, virus yield titration and Western blotting.

2.5. Western blotting assay

The MARC-145 cells, plated in 24-well plates, were harvested in protein extraction reagent (Thermo Fisher Scientific, Rockford, IL, USA) at the indicated time points. Protein concentration was determined with the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific). Equivalent protein amounts of cell lysates were subjected to 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) followed by blotting onto nitrocellulose (NC) membrane (Pall Co., Ann Arbor, MI, USA). The membrane was incubated with anti-N monoclonal antibody (mAb), anti-β-actin mAb (Abmart, Shanghai, China), anti-IFIT3 (ProteinTech Group, Inc., Chicago, IL, USA), anti-TBK1 (Santa Cruz Biotechnology, CA, USA) and anti-phospho-IRF3 (Cell signaling technology, Boston, MA, USA). After washing three times with 0.05% PBST, the membranes were incubated at 37 °C for 1 h with horseradish peroxidase-conjugated goat anti-mouse IgG (Boster Bio-Tech Co. Ltd., Wuhan, China) or goat anti-rabbit IgG (Boster). Detection was performed using chemiluminescence luminol reagents (Thermo Fisher Scientific). Protein spot levels were determined by using ImageJ quantification software.

2.6. Quantitative RT-PCR assays

Total RNA was extracted from the cellular samples using TRIzol reagent (Invitrogen). Reverse transcription was carried out using M-MLV Reverse Transcriptase (Promega) according to the manufacturer's instructions, as described previously (Li et al., 2009a). Two microliters of the RT reaction mixture was submitted to quantitative RT-PCR (Q-PCR) using IFIT3 specific primers (sense: 5′-GGC AGT TTT TTC CTG TCA GCA T-3′; antisense: 5′-CGT CCT GGC CCA TTT CCT-3′), or PRRSV ORF7-specific primers (sense: 5′-AAT AAC AAC GGC AAG CAG CAG-3′; antisense: 5′-CCT CTG GAC TGG TTT TGC TGA-3′), and SYBR Green Real-time PCR Master Mix (Toyobo Co., Ltd., Osaka, Japan), according to the manufacturer's recommendations. The reaction procedure was 95 °C for 30 s, followed by 40 cycles at 95 °C for 5 s and 60 °C for 31 s. The β-actin gene served as an internal reference (Li et al., 2007). Cycle times of the internal reference that varied by >1.0 unit in triplicate were discarded. The relative amount of target gene mRNA was normalized to that of β-actin mRNA in the same sample. To confirm specific amplification, melting curve analysis of the RT-PCR products was performed according to the manufacturer's protocol. The Q-PCR was performed in an ABI PRISM 7300 sequence detection system and analyzed with ABI PRISM 7300_{SDS} software (Applied Biosystems).

2.7. Indirect immunofluorescence assay (IFA)

MARC-145 cells plated in 96-well plates were inoculated with 500 MOI rAd-IFIT3, or rAd-Wt as control. At 24 h post-inoculation

(hpi), the cells were challenged with 10 MOI PRRSV. After 48 h, the infected cells were washed with PBS, fixed with cold acetone/methanol (1/1 v/v) for 20 min at −20 °C, and air-dried. The fixed cells were incubated with monoclonal antibody (McAb) against the N protein (made in our laboratory) for 1 h at 37 °C. Unbound antibody was washed three times with PBS containing 0.1% Tween-20 (PBST). Fluorescein-conjugated goat anti-mouse antibody (Boster) was added and incubated for 1 h at 37 °C. After three washes with PBST, infected cells were analyzed by Zeiss LSM510 laser confocal microscopy. Fluorescence in a whole well was measured at 495 nm for excitation and 525 nm for emission with a fluorescence plate reader (TECAN infinite M200, TECAN).

2.8. siRNA knockdown

All siRNAs (Invitrogen) were transfected with Lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturer's instructions. MARC-145 cells were plated in 24-well plates in antibiotic-free DMEM. At 60% confluence, 60 pmol siRNA was transfected into the cells. To determine the efficiency of protein knockdown, at 48 h post-transfection, the cells were lysed in protein extraction reagent (Thermo Fisher Scientific) and immunoblotted with the polyclonal anti-IFIT3 Ab. GenBank accession number of monkey IFIT3 is XM_001086192. The siRNA sequences were as follows: IFIT3–1 siRNA monkey (S1), 5′-GGG ACU GAA UCC UCU GGA U-3′; IFIT3–2 siRNA monkey (S2), 5′-GGA UGC GUA CUC CGA UCU U-3′; nonspecific control (NC), 5′-UUC UCC GAA CGU GUC ACG U-3′ (disordered S1 oligonucleotides).

2.9. Luciferase reporter assays

Lipofectamine 2000 transfection reagent (Invitrogen) was used for transient transfection following the manufacturer's protocol. MARC-145 cells cultured in 24-well plates were cotransfected with 900 ng of IFN-β-Luc reporter plasmid and 100 ng of *Renilla* luciferase construct phRL-TK (Promega), which served as an internal control. At the indicated time, cells were further transfected with poly(I:C) (2.0 μg) or not. Luciferase activities were measured at 24 h after poly(I:C) transfection. Cell lysates were collected for dual-luciferase assays (Promega). Relative firefly luciferase activity was normalized to *Renilla* luciferase activity.

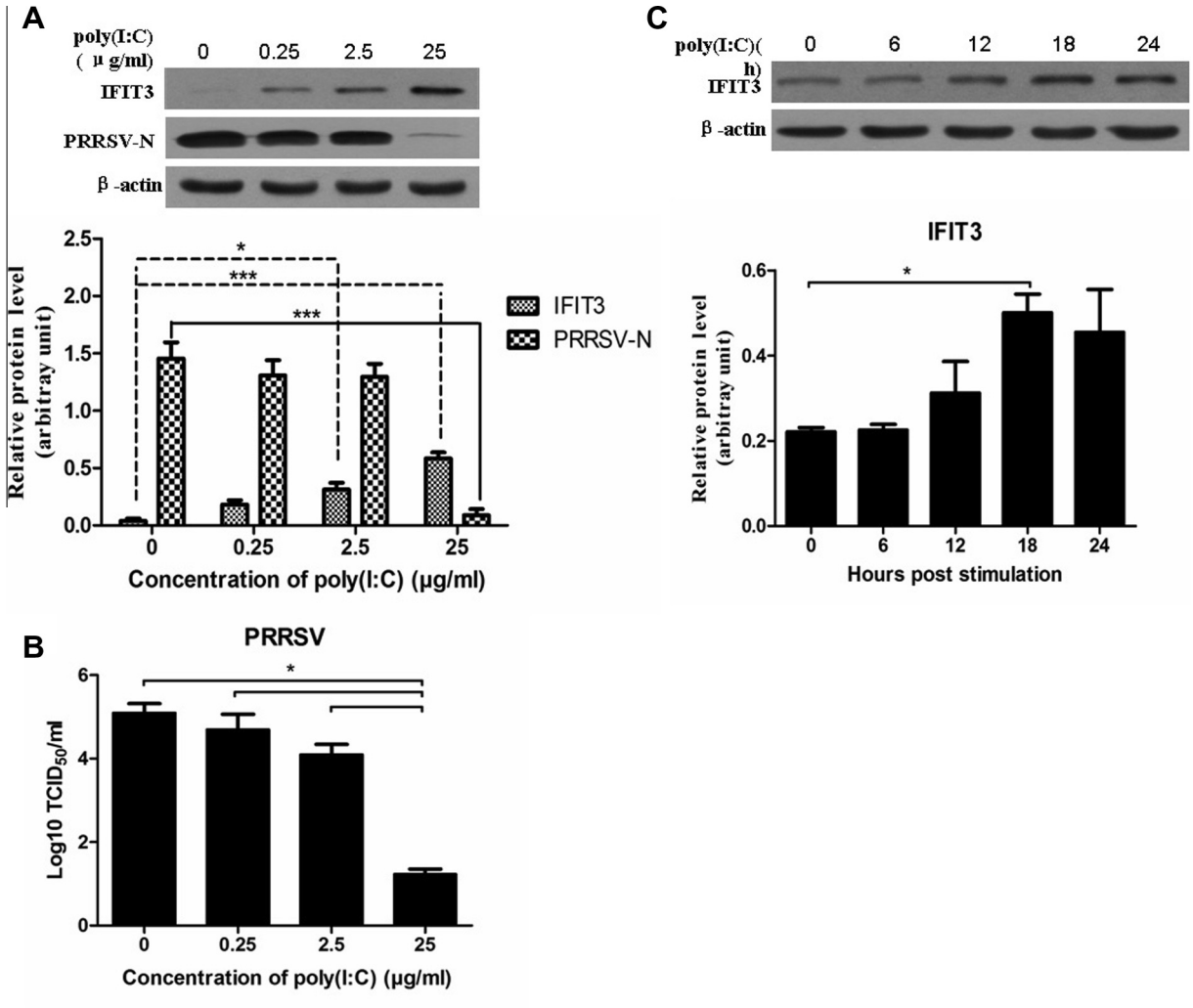

Fig. 1. Induction of IFIT3 by poly(I:C) in MARC-145 cells was dose- and time-dependent. (A) MARC-145 cells were stimulated with poly(I:C) (0.25–25 μg/ml); 24 h later, cells were challenged with PRRSV SY0608 (at 10 MOI). At 48 hpc, cells were harvested for Western blotting analysis with anti-N, anti-IFIT3 and anti-β-actin antibodies. The levels of IFIT3 and PRRSV N protein were quantified by immunoblot scanning and normalized with respect to the amount of β-actin (lower panel). Meanwhile, the culture supernatants were collected and PRRSV yields were titrated for $TCID_{50}$ (B). (C) MARC-145 cells were stimulated with poly(I:C) (25 μg/ml). At the indicated time points, cells were harvested for Western blotting analysis with anti-IFIT3 and anti-β-actin antibodies. The level of IFIT3 was quantified by immunoblot scanning and normalized with respect to the amount of β-actin (lower panel). The data shown here were analyzed statistically for three independent experiments. (*; **; ***) indicate significant difference ($P < 0.05$; $P < 0.01$; $P < 0.001$), respectively.

2.10. Statistical analysis

The results were analyzed for significance by one-way or two-way ANOVA using GraphPad PRIM software (version 5.02 for Windows; GraphPad software Inc.). Significant difference between two groups should be <0.05.

3. Results

3.1. Induction of IFIT3 by poly(I:C) is dose- and time-dependent

Recently, it was reported that IFIT3 is a potential regulator of MAVS antiviral signaling (Liu et al., 2011). Therefore, we wondered whether IFIT3 plays a key role in the procedure by which poly(I:C) inhibits PRRSV replication in MARC-145 cells. As expected, a high concentration of poly(I:C) significantly inhibited PRRSV replication in MARC-145 cells, based on the results of Western blotting and virus titers ($P < 0.05$). Meanwhile, the expression of IFIT3 was robustly induced by poly(I:C) stimulation in MARC-145 cells, which was dose- and time-dependent (Fig. 1).

Meanwhile, the expression levels of IFIT3 in MARC-145 cells were detected by Western-blotting, at different time points after infection with PRRSV at 1 MOI, 10 MOI and 100 MOI. The results showed that the IFIT3 level increased after PRRSV infection at 12 hpc, but no significant change at other time points tested (Fig. 2).

3.2. Replication of PRRSV is inhibited by overexpression of IFIT3

To detect the dose–effect relationship of IFIT3 on PRRSV replication, MARC-145 cells were first inoculated with the rAd-IFIT3 and rAd-Wt at increasing amounts (100, 200, 500 MOI), individually, followed by PRRSV challenge at 24 hpi. At 48 hpc, the cells were lysed and analyzed by Western blotting; meanwhile, the supernatant and cells were collected for Q-PCR analysis and virus yield titration. The results showed that overexpression of porcine IFIT3 inhibited PRRSV replication in a dose-dependent manner (Fig. 3).

To investigate the time-dependent suppression of viral replication, MARC-145 cells seeded in 24-well plates were inoculated with rAd5 (500 MOI) individually, followed by PRRSV challenge at 0, 12, 24 and 36 hpi. At 48 hpc, the cells were lysed and analyzed by Western blotting, while PRRSV yield in the supernatant was titrated for $TCID_{50}$. As shown in Fig. 4A and B, the longer the cells were inoculated with rAd-IFIT3, the higher the inhibitory effect we observed.

The IFA was also used to detect the effect of IFIT3 on PRRSV replication. MARC-145 cells seeded in 96-well plates were inoculated with rAd5 (500 MOI), and challenged with PRRSV at 24 hpi. At 48 hpc, the cells were collected for IFA analysis, and the fluorescence was measured. The results showed that the number of infected cells in the rAd-IFIT3 inoculated group was lower than that in rAd-Wt inoculated group (Fig. 4C). The results of three independent experiments clearly showed that rAd-IFIT3 treatment for 24 h significantly ($P < 0.05$) reduced the amount of PRRSV-infected cells (Fig. 4D).

3.3. Knockdown of IFIT3 increases PRRSV replication and impairs the antiviral activity mediated by poly(I:C)

In addition, we used the knockdown approach to investigate the function of IFIT3 in MARC-145 cells via Western blotting and Q-PCR. We designed two siRNAs, S1 and S2, and a negative control (namely NC) targeting the IFIT3 gene (Fig. 5A). Initially, we analyzed the effect of IFIT3 knockdown on the replication of PRRSV in MARC-145 cells via Western blotting, virus yield titration and Q-PCR. The results showed that knockdown of IFIT3, especially by siRNA S1, significantly ($P < 0.05$) increased the replication of PRRSV (Fig. 5A–C). Consistently, knockdown of IFIT3, especially by S1, significantly attenuated poly(I:C)-induced antiviral activity ($P < 0.001$) (Fig. 5D–F).

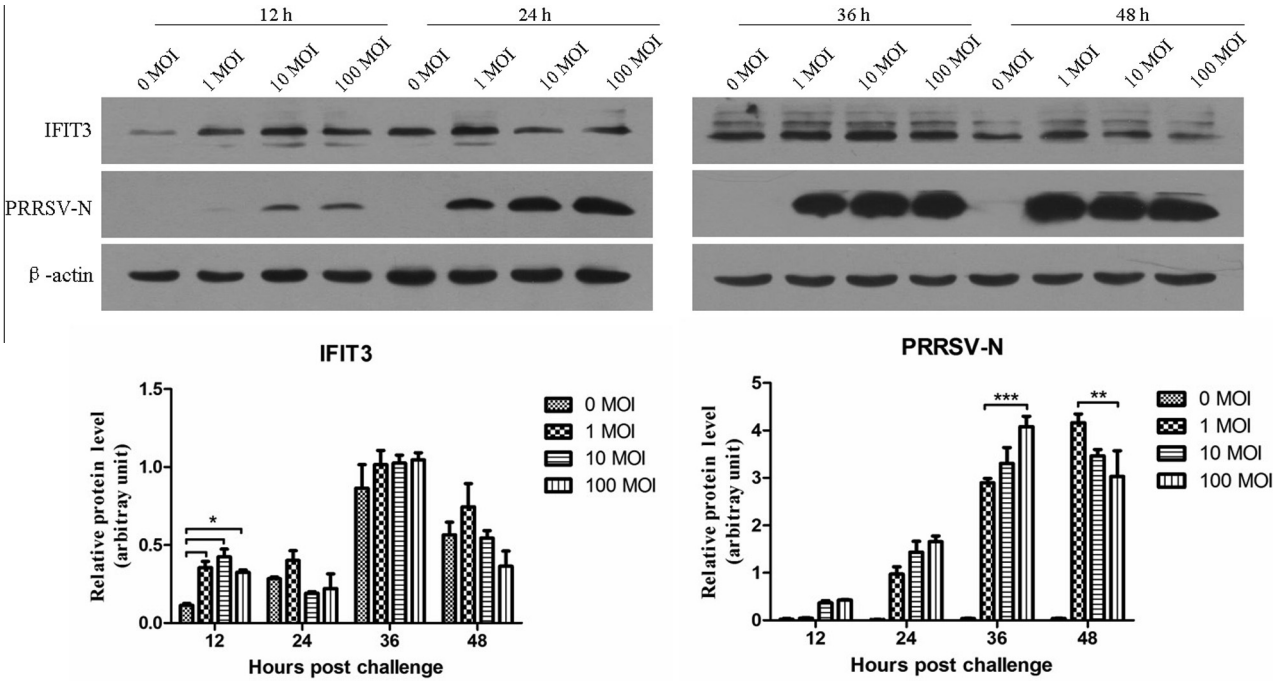

Fig. 2. Kinetics of the level of IFIT3 in MARC-145 cells, by Western blot analysis, at different time points after infection with three different doses of PRRSV. β-actin was used as an internal control to normalize the quantitative data. The level of IFIT3 and N protein were quantified by immunoblot scanning and normalized with respect to the amount of β-actin (lower panel). (*; **; ***) indicate significant difference ($P < 0.05$; $P < 0.01$; $P < 0.001$), respectively.

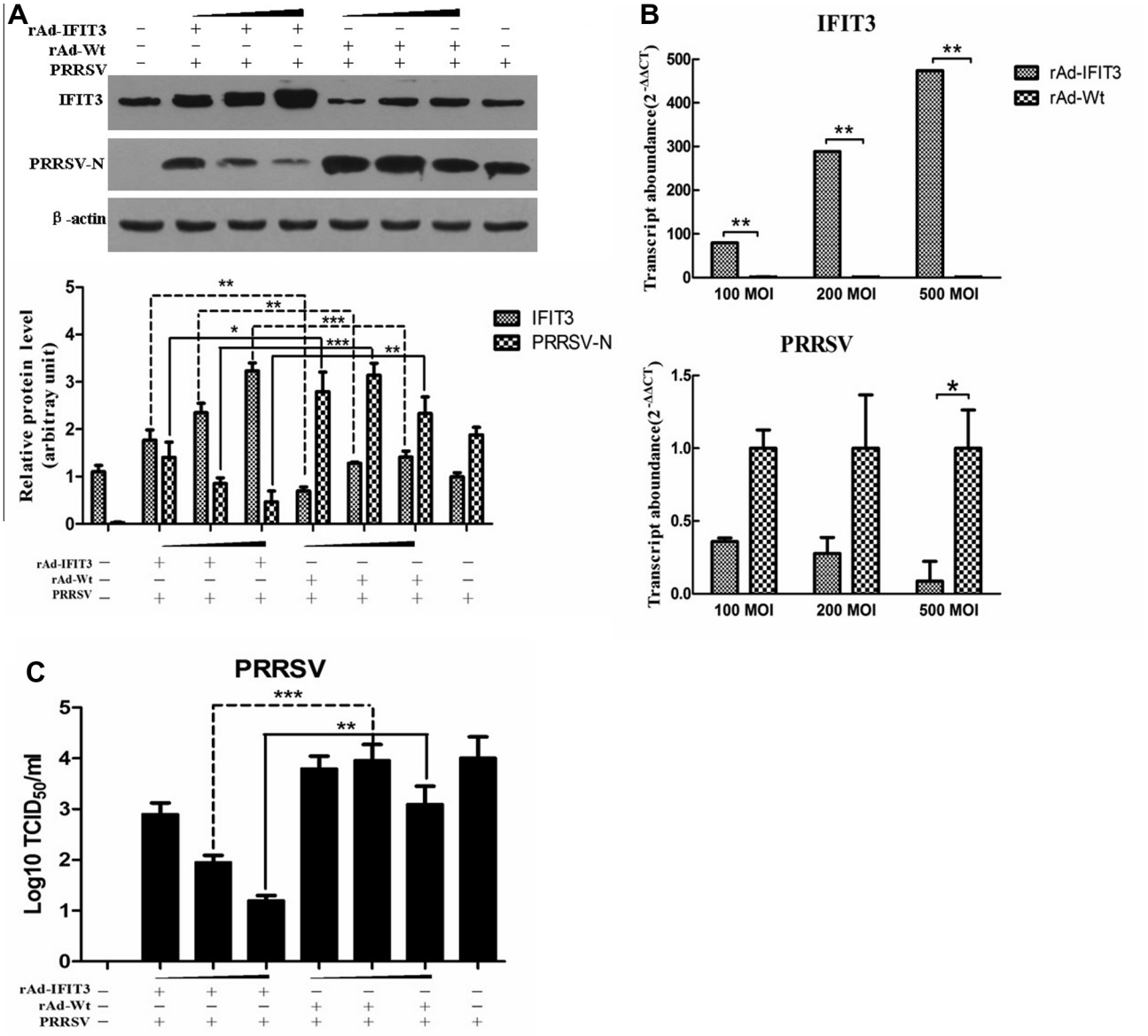

Fig. 3. Overexpression of porcine IFIT3 inhibited PRRSV replication in a dose-dependent manner. MARC-145 cells were inoculated with 100, 200 and 500 MOI of rAd-IFIT3 or rAd-Wt individually. After incubation for 24 h, the cells were subsequently challenged with 10 MOI of PRRSV SY0608. At 48 hpc, the cells were harvested for Western blotting analysis with anti-N, anti-IFIT3 and anti-β-actin antibodies. The levels of IFIT3 and N protein were quantified by immunoblot scanning and normalized with respect to the amount of β-actin (lower panel) (A). In addition, the total RNA was extracted for the determination of PRRSV ORF7 and porcine IFIT3 mRNA levels by quantitative RT-PCR. The levels of PRRSV-ORF7 and IFIT3 mRNA were normalized to the level of β-actin mRNA in the same sample (B). Meanwhile, the culture supernatants were collected and virus yields were detected by $TCID_{50}$ (C). Values represent means ± SEM; (*; **; ***) indicate significant difference ($P < 0.05$; $P < 0.01$; $P < 0.001$), respectively.

Moreover, after interference of the IFIT3, MARC-145 cells were challenged with PRRSV, then inoculated with rAd-IFIT3 or rAd-Wt. The results showed that overexpression of IFIT3 could rescue the antiviral activity in IFIT3 silence MARC-145 cells (Fig. 6).

3.4. IFIT3 enhances poly(I:C)-induced IFN-β promoter activation, whereas knockdown of IFIT3 has the opposite effect

It is well known that the induction of IFN-β is a hallmark of host antiviral responses. MARC-145 cells were cotransfected with the IFN-β-Luc reporter plasmid and phRL-TK; 24 h later, the cells were inoculated with rAd5. Twenty-four hours post-inoculation, the cells were treated with poly(I:C), and the cells were harvested and subjected to a dual luciferase reporter assay system after another 24 h. The results showed that rAd-IFIT3 significantly activated the IFN-β promoter ($P < 0.01$) (Fig. 7A). In contrast, knockdown of endogenous IFIT3 by the siRNA S1 and S2 significantly inhibited the IFN-β promoter ($P < 0.001$) (Fig. 7B), which is consistent with the findng that knockdown of IFIT3 significantly impaired the antiviral activity mediated by poly(I:C) ($P < 0.001$) (Fig. 5D–F).

3.5. The IFIT3–TBK1–pIRF3 axis is activated in MARC-145 cells after poly(I:C) treatment

Given that IFIT3 has been identified as an important adaptor bridging TBK1 to MAVS on the mitochondrion (Liu et al., 2011), we initially investigated whether TBK1 is activated in poly(I:C)-transfected MARC-145 cells. As expected, TBK1 was detected in the cellular fraction of MARC-145 cells transfected with poly(I:C) (Fig. 8). We analyzed subsequently the activation status represented by phosphorylation of IRF3 (pIRF3) that is considered crucial for IFN-β induction (Sato et al., 2000). The pIRF3 was readily detected in whole extracts prepared from MARC-145 cells treated

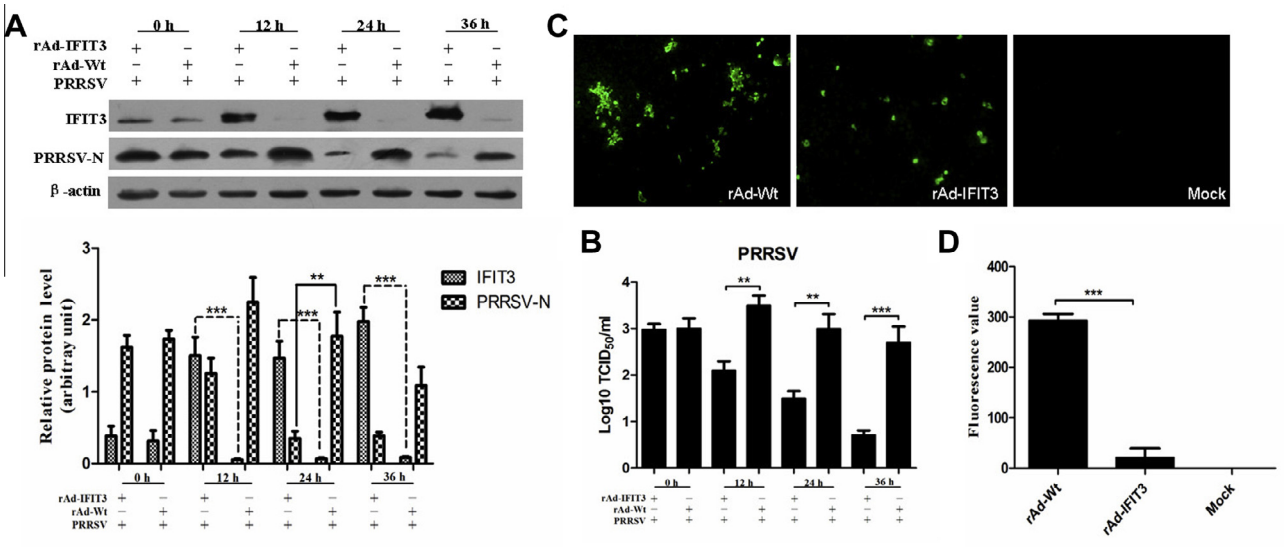

Fig. 4. Overexpression of porcine IFIT3 inhibited PRRSV replication in a time-dependent manner. MARC-145 cells were inoculated with 500 MOI of rAd-IFIT3 or rAd-Wt. After incubation for the indicated number of hours, the cells were challenged with 10 MOI of PRRSV SY0608. At 48 hpc, cells were harvested for Western blotting analysis with anti-N, anti-IFIT3 and anti-β-actin antibodies. (A) The levels of IFIT3 and N protein were quantified by immunoblot scanning and normalized with respect to the amount of β-actin (lower panel), while PRRSV yield in the supernatant was titrated for $TCID_{50}$ (B). In addition, the cells in the group incubated for 24 h, after being inoculated with 500 MOI of rAd-IFIT3 and challenged with 10 MOI of PRRSV SY0608, were fixed and PRRSV were detected by IFA with anti-N antibody (C) (Magnification, 100×). The fluorescence in each well was measured at 495 nm for excitation and 525 nm for emission with a fluorescence plate reader (D). Values represent means ± SEM; (*; **; ***) indicate significant difference ($P < 0.05$; $P < 0.01$; $P < 0.001$), respectively.

with poly(I:C) (Fig. 8). This demonstrated that IFIT3, TBK1 and pIRF3 are rapidly activated in the cells treated with poly(I:C) and play critical roles in antiviral gene transcription.

4. Discussion

Type I IFNs, such as IFN-α and -β, are critical to innate immunity against viral infection and contribute to the modulation of adaptive immunity (Takaoka and Yanai, 2006). Activation of the TLR or RIG-1-like receptors (RLRs) pathways eventually leads to the secretion of type I IFNs. The binding of type I IFNs to their receptors activates the Janus kinase (JAK)-signal transducer and the activator of transcription (STAT) pathway, which induces expression of IFN-stimulated genes (ISGs) and results in the establishment of an antiviral state (Darnell et al., 1994; Schindler and Darnell, 1995). Among the ISGs, there is a subfamily named IFITs. IFIT3 is an essential adaptor to bridge TBK1 to MAVS on mitochondria (Liu et al., 2011). PRRSV activates the transcription of type I IFN in its host cells (Wang and Christopher-Hennings, 2012; Zhang et al., 2012). However, the role of IFIT3 in PRRSV infection is scarcely understood. In this study, poly(I:C) stimulation inhibited PRRSV replication in MARC-145 cells, following the appearance of increased IFIT3. The expression of IFIT3 was robustly induced by poly(I:C) stimulation in MARC-145 cells in a dose- and time-dependent manner. Overexpression of porcine IFIT3 significantly increased the poly(I:C)-induced IFN-β promoter, whereas knockdown of endogenous IFIT3 had the opposite effect. Overexpression of porcine IFIT3 inhibited PRRSV replication, whereas knockdown of endogenous IFIT3 reversed poly(I:C)-induced inhibition of PRRSV replication. Furthermore, TBK1 and pIRF3 were readily detected in whole extracts prepared from MARC-145 cells transfected with poly(I:C). These results suggest that IFIT3 regulates positively the poly(I:C)-triggered IFN-β induction and cellular antiviral responses. IFIT3 is a mediator of positive-feedback regulation of cellular antiviral responses through activation of the MAVS–IFIT3–TBK1 axis in MARC-145 cells.

Viruses have evolved many strategies to counteract host immune responses. It has been reported that many IFIT family members (IFIT3, IFIT2, ISG54, etc.) are induced by Sendai virus, dsRNA and type I IFN (Liu et al., 2011; Schmeisser et al., 2010). They play important roles in antiviral defense during infections with hepatitis C virus (Metz et al., 2012), vesicular stomatitis virus (VSV) (Fensterl et al., 2012) and human respiratory syncytial virus (Hastie et al., 2012). Our previous study demonstrated that the expression of IFIT3 was increased in PRRSV-infected PAMs by using iTRAQ technology, which is time-dependent (Lu et al., 2012). However, our other experimental results indicated that the induction of IFIT3 was only increased in PRRSV-infected MARC-145 cells at 12 hpc (Fig. 2). Miller et al. reported that stimulation of MARC-145 cells with exogenous double-stranded RNA resulted in a significant increase in type I IFN mRNA expression (Miller et al., 2004). However, in the presence of PRRSV infection, the production of type I IFN was significantly inhibited (Shi et al., 2010). Beura et al. (2010) first reported that various nsp, such as nsp1, nsp2, nsp4 and nsp11, were able to suppress dsRNA-induced IFN-β promoter activation. Recently, it was reported that the PRRSV N protein also inhibited dsRNA-induced IFN-β induction (Sagong and Lee, 2011). Whether PRRSV replication inhibits the expression of IFIT3 to subvert or prevent IFN induction and response needs to be analyzed in more detail in the future.

Adenoviruses can infect many host cells. The viral vector expression cassettes can deliver genes easily into cells with high efficiency and they may persist for a long duration. However, rAd itself was found to activate the IFN-β promoter, which is consistent with previous studies (Cavanaugh et al., 1998; Shiver et al., 2002). In this study, since MARC-145 cells are relatively hard to transfect, we used adenovirus to transfect IFIT3 to the cells. Meanwhile, we also used wild type adenovirus as negative control. The level of the recombinant adenovirus rAd-IFIT3 replication was almost the same as that of wild type adenovirus by using a real-time PCR (data not shown here). The recombinant adenovirus expressing the porcine IFIT3 gene could be used to model the role of IFIT3 in PAMs and *in vivo* in future studies of PRRSV infection.

The TPR motif is critical for a multitude of cellular and viral regulatory processes, such as protein transport, translational initiation, cell migration and proliferation, antiviral signaling, and virus

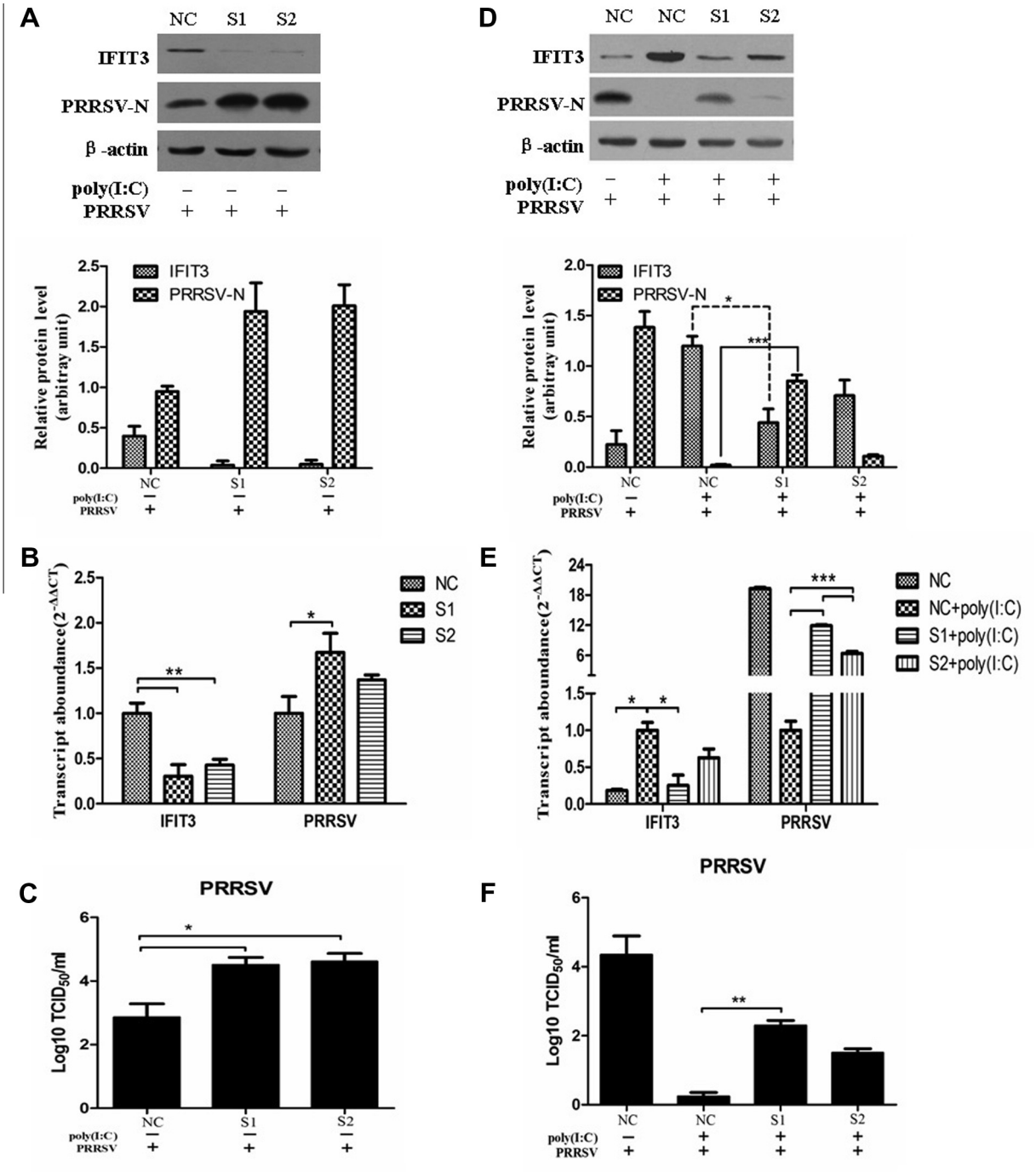

Fig. 5. Knockdown of IFIT3 increased PRRSV replication and impaired the poly(I:C)-mediated antiviral activity. MARC-145 cells were transfected with siRNA S1 and S2 and nonspecific control (NC) targeting the IFIT3 gene, and incubated for 24 h. The cells were challenged with 10 MOI of PRRSV. At 48 hpc, the cells were harvested for Western blotting analysis with anti-N, anti-IFIT3 and anti-β-actin antibodies. The levels of IFIT3 and N protein were quantified by immunoblot scanning and normalized with respect to the amount of β-actin (lower panel) (A). The PRRSV ORF7 and mIFIT3 mRNA were detected by quantitative RT-PCR. The levels of ORF7 and mIFIT3 mRNA were normalized to the level of β-actin mRNA in the same sample (B). Meanwhile, PRRSV yield in the supernatant was titrated for $TCID_{50}$ (C). In another experiment, MARC-145 cells were transfected with the siRNA and 24 h later the cells were treated with poly(I:C). After 24 h of treatment, these cells were challenged with 10 MOI of PRRSV. At 48 hpc, the cells and culture supernatants were harvested, and the levels of mIFIT3 and N protein were also detected by Western blotting with anti-N, anti-IFIT3 and anti-β-actin antibodies (D); PRRSV ORF7 and mIFIT3 mRNA were also detected by quantitative RT-PCR (E), and PRRSV yields were titrated for by $TCID_{50}$ (F). Values represent means ± SEM; (*; **; ***) indicate significant difference ($P < 0.05$; $P < 0.01$; $P < 0.001$), respectively.

replication (D'Andrea and Regan, 2003; Daffis et al., 2010; Fensterl and Sen, 2011; Li et al., 2009b; Wu and Sha, 2006). IFIT3 has been reported to interact with TBK1, IRF3 and other IFIT family members, leading to enhanced antiviral signaling (Liu et al., 2011; Pichlmair et al., 2011), which raises a question of whether it is a direct or an indirect inhibitor of PRRSV replication. In order to determine whether the PRRSV is colocalized with cellular IFIT3 in MARC-145 cells, we did confocal imaging and immunoprecipitation assay with anti-N and anti-IFIT3 antibodies. The results showed that the interaction between the N protein and IFIT3 in infected cells could not be detected by the immunoprecipitation assay, even though the colocalization of IFIT3 with the PRRSV N

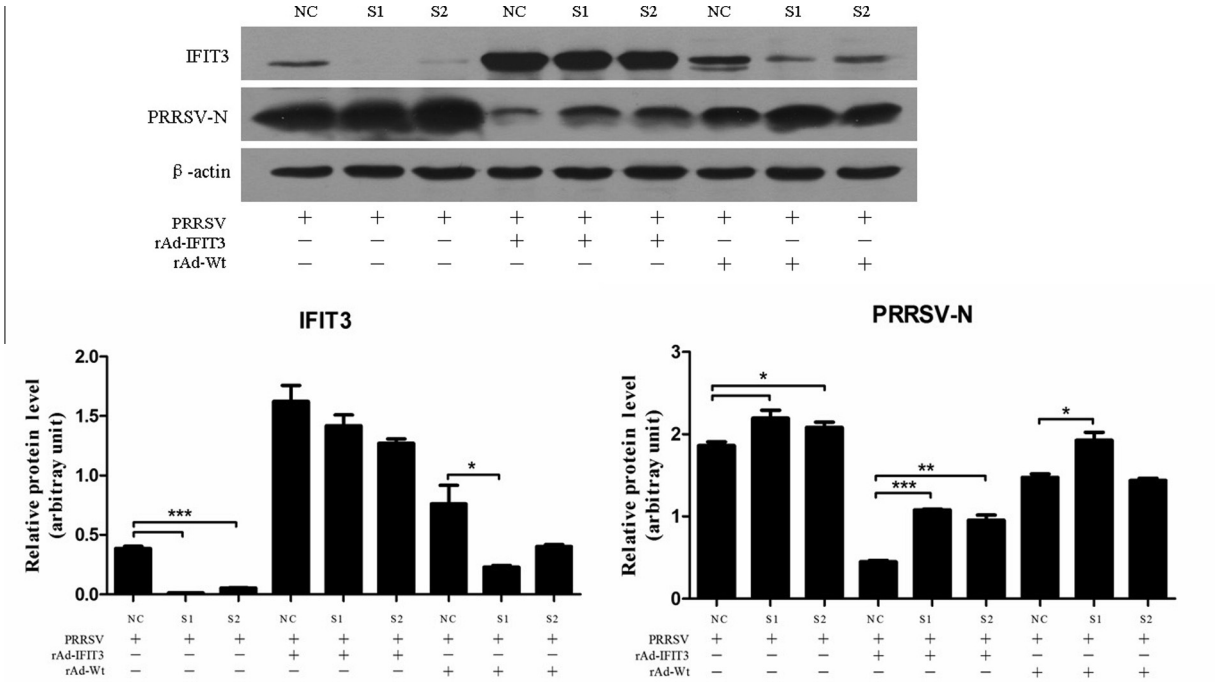

Fig. 6. MARC-145 cells were transfected with siRNA S1 and S2 and nonspecific control (NC) targeting the IFIT3 gene, and incubated for 24 h. The cells were challenged with 10 MOI of PRRSV. At 36 hpc, the cells were inoculated with 500 MOI of rAd-IFIT3 or rAd-Wt. At 24 hpi, the cells were harvested for Western blotting analysis with anti-N, anti-IFIT3 and anti-β-actin antibodies. The levels of IFIT3 and N protein were quantified by immunoblot scanning and normalized with respect to the amount of β-actin (lower panel). (*; **; ***) indicate significant difference ($P < 0.05$; $P < 0.01$; $P < 0.001$), respectively.

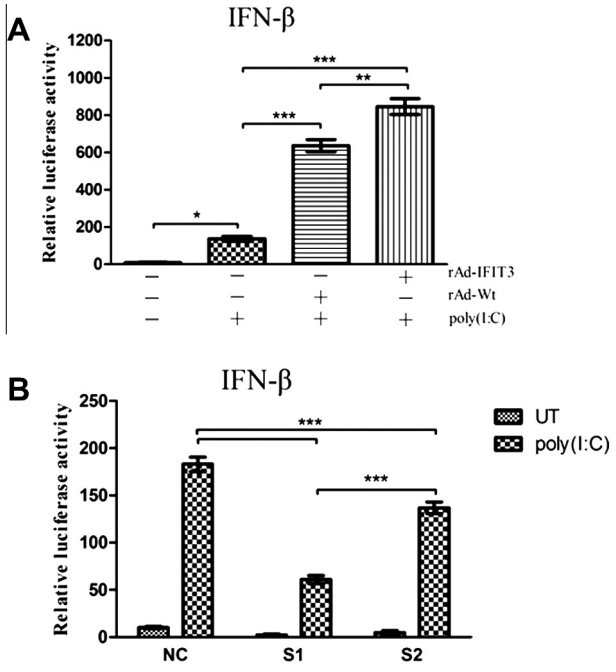

Fig. 7. IFIT3 enhanced poly(I:C)-induced IFN-β promoter activation, whereas knockdown of IFIT3 had the opposite effect. (A) MARC-145 cells were cotransfected with phRL-TK and pIFN-β-Luc/pGL3. After 24 h the cells were inoculated with rAd-IFIT3 or rAd-Wt at 100 MOI. Another 24 h later, the cells were transfected with 2 μg poly(I:C). (B) MARC-145 cells were transfected with the siRNA S1 and S2 and negative control NC siRNA targeting the IFIT3 gene. Twenty-four hours later, cells were cotransfected with phRL-TK and pIFN-β-Luc/pGL3. Another 24 h later, cells were transfected with 2 μg poly(I:C). After incubation for 24 h, the cell lysates were collected for dual-luciferase assays. The results were expressed as relative luciferase activity (RLU), which was presented in comparison with the RLU of untreated pGL3-basic-transfected cells. Values represent means ± SEM; (*; **; ***) indicate significant difference ($P < 0.05$; $P < 0.01$; $P < 0.001$), respectively. The data shown here are from one of three experiments.

protein was observed by the confocal imaging(data not shown here). However, it still could not exclude the possibility that other proteins of PRRSV might interact with cellular IFIT3 in MARC-145 cells, which requires further investigation in the future. In this study, we analyzed the homology of IFIT3 between porcine and monkey and designed the primers in the conservative region. Throughout the experiment, we "mixed" used and detected the porcine and monkey IFIT3. In addition, it should be noted that MARC-145 cells are not of porcine origin and are of monkey origin. The dynamics of host cell-PRRSV interaction should be study in porcine cells permission for PRRSV in the future. By the way, poly(I:C) can affect expression of many genes. It also needs to look at some downstream genes of MAV signaling, such as USP18 (Bellecave et al., 2010).

In summary, poly(I:C) triggers unique innate mechanisms within MARC-145 cells that limit PRRSV replication, which is largely

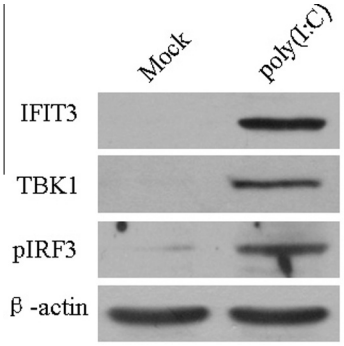

Fig. 8. Activation of IFIT3, TBK1 and phosphorylation of IRF3 (pIRF3) in poly(I:C)-transfected MARC-145 cells. The MARC-145 cells were transfected with 2 μg poly(I:C) and cultured for 24 h. Subsequently, the cells were harvested, lysed and subjected to Western blotting to determination the presence of IFIT3, TBK1, pIRF3 and β-actin. The data shown here are from one of three experiments.

mediated through type I IFNs. The appearance of IFIT3 is a part of poly(I:C)-mediated activation and correlates with the resistance of MARC-145 cells to PRRSV replication. This finding should be helpful in the future development of novel antiviral therapies.

Acknowledgements

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