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Porcine reproductive and respiratory syndrome virus counteracts type I interferon-induced early antiviral state by interfering IRF7 activity

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ABSTRACT

Porcine reproductive and respiratory syndrome (PRRS) is an economically important disease with a significant impact on the pig industry. It is caused by PRRS virus (PRRSV), which predominantly infects and replicates in porcine pulmonary alveolar macrophages (PAMs). We pretreated PAMs with porcine interferon (IFN)-α to induce an antiviral state within the cells and subsequently infected them with highly pathogenic PRRSV. Changes in global gene expression in IFN-α-pretreated PAMs in response to PRRSV infection were determined by RNA-sequence analysis and confirmed by real-time PCR. We found that IRF7 and other antiviral interferon stimulating genes (ISG)s were suppressed by PRRSV infection. Further studies demonstrated that PRRSV could down-regulate the expression of IRF7 by the non-structure protein 7 (nsp7). In conclusion, PRRSV infection had a strong immunosuppressive effect of IFN. PRRSV nsp7 inhibits the expression of IRF7, thereby down-regulating the expression of IFN and downstream ISGs and facilitated the virus to replicate.

1. Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV) is a positive-sense, single-stranded RNA virus of the family Arteriviridae, order Nidovirales. It is the causative agent of porcine reproductive and respiratory syndrome (PRRS), an infectious swine disease that causes respiratory disorders, abortion in pregnant sows, and variable mortality in piglets (Done et al., 1996). PRRSV first emerged in the USA in 1987 and subsequently spread worldwide, representing an economically important disease in the pig industry (Cho and Dee, 2006). In 2006, a highly pathogenic PRRSV (HP-PRRSV) strain emerged in China, responsible for HP-PRRS characterized by high and continuous fever, 50%–100% morbidity, and 20%–100% mortality (Li et al., 2011). HP-PRRS affected more than 2 million pigs with about 0.4 million fatalities in the first outbreak (Tian et al., 2007), and became the most important infectious disease in the pig industry in China.

PRRSV, including HP-PRRSV, predominantly infects and replicates in porcine pulmonary alveolar macrophages (PAMs), which are considered as the primary target cells during acute infection (Kimman et al., 2009). PAMs act as scavengers in the lung and play a crucial role in initiation of the host antiviral immune response against pulmonary infection. Infection of PAMs by PRRSV leads to their dysfunction and subsequent modulation of the host antiviral immune response, including a weakened cell-mediated immune response and low levels of type I interferons (IFNs) (Wang and Zhang, 2014). The immune response of PAMs to PRRSV infection has therefore attracted scientific interest as an in vitro model for exploring the interaction between PRRSV and PAMs. Several studies have analyzed changes in global gene expression in PRRSV-infected PAMs using high-throughput techniques such as microarray and RNA-sequence analysis, and have found that the type I IFN-mediated immune response plays a crucial role in restricting and eliminating PRRSV infection (Jiang et al., 2013; Wang and Zhang, 2014; Xiao et al., 2010; Xiao et al., 2015).

Pretreatment of PAMs with porcine type I IFNs significantly reduced PRRSV replication (Overend et al., 2007), while PRRSV in turn inhibited the production of type I IFNs (Sun et al., 2012a; Wang and Zhang, 2014). Treatment of cells with type I IFNs is known to stimulate the transcription of a broad range of IFN-stimulated genes (ISGs) with

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diverse biological functions, including antiviral and proapoptotic functions (Schoggins and Rice, 2011), which in turn establish an antiviral state within the cells to restrict and eliminate viral infection. However, viruses have evolved multiple mechanisms to counteract the type I IFN-induced antiviral state (Garcia-Sastre and Biron, 2006). This is due to the strong immunosuppressive capacity of PRRSV, and there are multiple immunosuppressive strategies against the interferon signaling pathway. For instance, PRRSV nsp1β and N proteins interfere IRF3 activation (Beura et al., 2010; Sagong and Lee, 2011), while nsp1β also inhibits JAK/STAT activity (Wang et al., 2013), nsp4 antagonize NF-kB transduction (Huang et al., 2014), and nsp2 directly binds to ISG15 and inhibits its antiviral function (Sun et al., 2012b). This demonstrates that IFN-mediated antiviral responses play an important role in preventing PRRSV infection.

In this study, we pretreated PAMs with porcine IFN-α to establish an antiviral state within the cells, and subsequently infected the PAMs with HP-PRRSV. We analyzed global gene expression in the PAMs by RNA-sequence analysis and compared changes in gene expression between infected and uninfected cells. We found that PRRSV suppressed a range of antiviral genes, including the important IFN regulatory factor IRF7. PRRSV utilizes nsp7 protein to down-regulate IRF7 expression, and IRF7 regulates the expression of IFN and ISGs. This study provides the basis for revealing the immunosuppressive mechanism of PRRSV for IFN.

2. Results

2.1. PRRSV antagonize IFN-α mediated antiviral function in early stage

Type I IFNs inhibit PRRSV replication in PAMs (Overend et al., 2007). We determined if the HP-PRRSV strain used in this study was sensitive to type I IFNs by pretreating PAMs with recombinant porcine IFN-α and then infecting them with HP-PRRSV. PRRSV replication was monitored by qRT-PCR at 4, 12, and 24 hpi. Low and similar levels of PRRSV replication were detected in both IFN-α- and mock-pretreated cells at 4 hpi, whereas significantly higher levels were observed in mock-pretreated cells at 12 and 24 hpi compared with IFN-α-pretreated cells, indicating that the HP-PRRSV strain was sensitive to type I IFNs (Fig. 1A). Notably, the level of PRRSV replication in IFN-α-pretreated cells at 12 hpi was similar to that in mock-pretreated cells at 4 hpi, suggesting that IFN-α treatment inhibited PRRSV replication during the early stage of viral replication.

ISG15 encoding the ISG15 ubiquitin-like modifier is an interferon-stimulated gene with anti-PRRSV activity (Sun et al., 2012b). PRRSV infection has been shown to inhibit ISG15 expression (Patel et al., 2010). ISG15 expression levels were significantly up-regulated in PAMs pretreated with porcine IFN-α compared with mock-pretreated control cells (Fig. 1B), suggesting that treatment of PAMs with IFN-α induced an antiviral state within the cells. However, PRRSV infection significantly reduced ISG15 expression up-regulated by IFN-α, suggesting that PRRSV was able to antagonize the antiviral state induced by porcine IFN-α treatment.

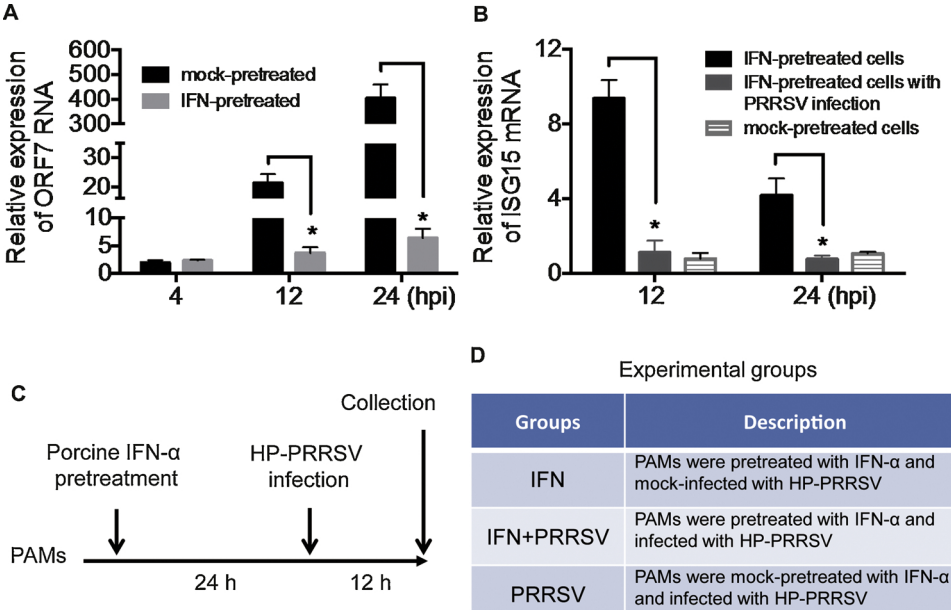
No remarkable replication of PRRSV was observed at 12 hpi in IFN-α-pretreated PAMs (Fig. 1A), but ISG15 expression was significantly inhibited by PRRSV infection at this time point (Fig. 1B). These observations suggest antagonism of the type I IFN-induced antiviral state by PRRSV at this time point. We therefore selected 12 hpi as the sampling time point for RNA-sequence analysis.

To investigate the basis of the antagonism of the type I IFN-induced antiviral state by PRRSV, PAMs were pretreated with porcine IFN-α for 24 h, infected with HP-PRRSV, and harvested at 12 hpi for RNA-sequence analysis (Fig. 1C). Global gene expression levels were determined by RNA-sequence analysis and compared among the IFN, IFN + PRRSV, PRRSV, and C groups (Fig. 1D).

2.2. Global analysis of significant DEGs

The objective of this study was to observe the early stage of interaction between PRRSV and the cellular IFN-α signaling pathway, thus the RNA-seq experiment was not consided to repeat for three times. RNA-seq experimental data were analyzed using EBseq method, which is specialized for RNA-seq data analysis without replicate. We also performed real-time PCR to validate the expression levels of genes involved in the antiviral immune response signaling pathway, and genes with consistent expression profiles between RNA-seq and real-time PCR were used in the subsequent experiments.

Unique reads for the four groups obtained from RNA-sequence data were 11,602,426–13,332,000, and the read coverage was



Groups	Description
IFN	PAMs were pretreated with IFN-α and mock-infected with HP-PRRSV
IFN+PRRSV	PAMs were pretreated with IFN-α and infected with HP-PRRSV
PRRSV	PAMs were mock-pretreated with IFN-α and infected with HP-PRRSV
C (control)	PAMs were mock-pretreated with IFN-α and mock-infected with HP-PRRSV

Fig. 1. Experimental design and preparation for PAM RNA-Seq.
PAMs were pretreated or mock-pretreated with porcine IFN-α for 24 h and infected with HP-PRRSV. The PAMs were collected at the indicated time points for analysis of gene expression. (A) Analysis of the expression of PRRSV ORF7 RNA by qRT-PCR. (B) Analysis of the expression of ISG15 mRNA by qRT-PCR. The data are means with ± standard error (SEM) from three independent experiments. *, $p < 0.01$ compared between groups. hpi, hours post-infection. (C) Experimental groups. The buffer dissolving IFN-α was used as mock in "mock-treated with IFN-α", while the buffer diluting HP-PRRSV functioned as mock in "mock-infected with HP-PRRSV". (D) PAMs were pre-cultured for 2 h and incubated with porcine IFN-α for 24 h. The PAMs were washed with PBS, inoculated with HP-PRRSV, and harvested at 12 h post-infection.

Table 1
Mapping summary.

Groups	All reads	UM[1]	UMR[2]	ER[3]	Transcripts[4]
IFN	17131748	13332000	77.8%	12111645	17053
IFN + PRRSV	14379355	11602426	80.7%	10380965	16963
PRRSV	16764745	13176440	78.6%	11707410	17012
C (control)	16588133	13022580	78.5%	11609458	16944

[1] Uniquely mapping reads.
[2] Uniquely mapping rate in all reads.
[3] Reads in exon.
[4] Annotated transcripts derived from gene build sus scrofa.

77.8%–80.7%. Around 17,000 transcripts were detected in the four groups (Table 1). Eight genes (C5 AR1, FTL, FN1, ATP6, GGH, ACADM, SGPL1, and STEAP3) were randomly selected to validate the expression data obtained from RNA-sequence analysis. Expression levels of the eight selected genes were determined by qRT-PCR and compared with the results of RNA-sequence analysis. Spearman's correlation coefficient (r) showed a significant correlation (r = 0.903) between the RNA-sequence and qRT-PCR data, suggesting that the changes in gene expression detected by RNA-sequence analysis were in agreement with those from qRT-PCR validation (S1 Table).

To screen out significant DEGs, gene expression levels in the IFN, IFN + PRRSV, and PRRSV groups were normalized to those in the C group. Significant DEGs were filtered out with FDR < 0.05 and FC > 1.5 or < 0.667. In comparison with C group, 828, 744, and 298 significant DEGs were identified in the IFN, IFN + PRRSV, and PRRSV groups, respectively (Fig. 2A). The intersection of the significant DEGs observed in the three groups is shown in Fig. 2B. GO enrichment analysis was performed to evaluate the biological importance of the significant DEGs. The significant DEGs in the IFN group were enriched in 28 GO terms, most of which belonged to the GO terms 'Response to stress', 'Immune system process', 'Defense response', 'Response to stimulus', 'Immune response', and 'Inflammatory response' (Fig. 2C; S2 Table), suggesting that porcine IFN-α established an antiviral state. The significant DEGs in the IFN + PRRSV group were enriched in 14 GO terms (Fig. 2C; S3 Table), most of which overlapped with the terms for the IFN group, but with higher p values, suggesting that the IFN-α-induced antiviral state was impaired by PRRSV infection. In contrast, no significant GO terms were filtered out using the Bonferroni-corrected p value and FDR < 0.05 in the PRRSV group (Fig. 2C; S4 Table), suggesting that the biological processes were disrupted by PRRSV infection.

Type I IFNs induce the expression of more than 300 ISGs with numerous biological functions, including antiviral and proapoptotic activities (Schoggins and Rice, 2011). Although hundreds of ISGs have been identified in humans and mice, information on porcine ISGs is relatively scarce. A total of 391 DEGs was significantly up-regulated in response to porcine IFN-α treatment (Fig. 2A). We therefore considered these 391 DEGs as potential porcine ISGs. Among these 391 DEGs, 346 were the identified porcine genes or showed high homology with their counterparts in humans, while 45 DEGs were uncharacterized genes. We compared the expression levels of the 346 up-regulated DEGs

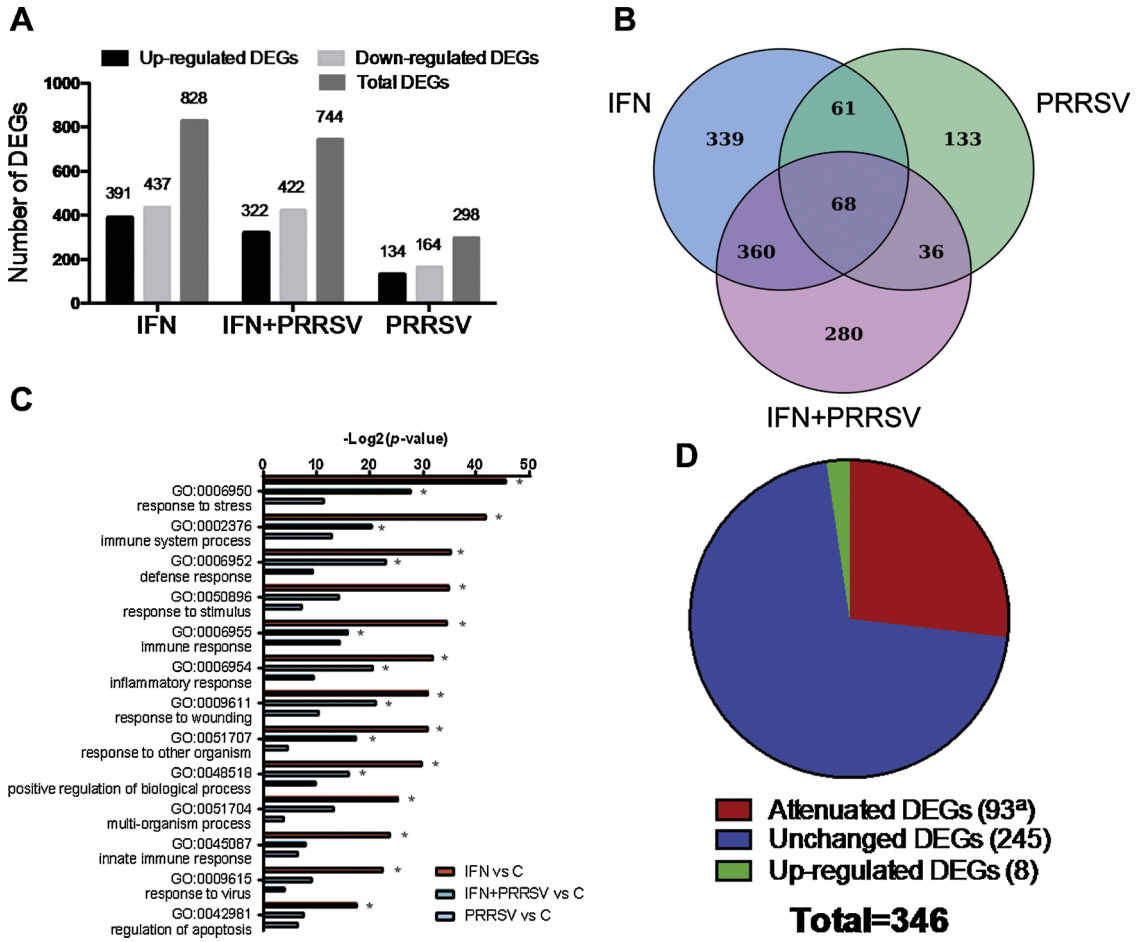


Fig. 2. Global analysis of significant DEGs.
(A) Summary of significant DEGs identified in IFN, IFN + PRRSV, and PRRSV groups. (B) Venn analysis of significant DEGs observed in the IFN, IFN + PRRSV, and PRRSV groups. (C) GO enrichment analysis of significant DEGs identified in the IFN, IFN + PRRSV, and PRRSV groups. *, the significantly enriched GO term compared to the control (C) group. (D) Sector graph illustrates the distribution of porcine IFN-α-induced DEGs in the IFN + PRRSV group.

between the IFN and IFN + PRRSV groups. 93 of them showed significantly attenuated expression levels, 245 showed similar expression levels, and eight DEGs showed higher expression levels in the IFN + PRRSV group compared with the IFN group (Fig. 2D; S5 Table).

All 346 up-regulated DEGs were analyzed by GO. These DEGs were enriched in 10 GO terms, of which the top three were 'Immune response', 'Immune system process', and 'Response to virus' (Supplement Fig. 1A; S6 Table). GO analysis of the 93 attenuated DEGs (Fig. 2D) in the IFN + PRRSV group showed that they were enriched in seven GO terms, of which the top one was 'Response to virus' (Supplement Fig. 1B; S7 Table). KEGG pathway analysis indicated that the 346 DEGs were enriched in the five KEGG terms 'RIG-I-like receptor signaling pathway', 'Toll-like receptor signaling pathway', 'Proteasome', 'NOD-like receptor signaling pathway', and 'Cell adhesion molecules' (Supplement Fig. 1C; S8 Table).

2.3. IRF7 is involved in IFN-α meditated antiviral stage

The RIG-I-like receptor (RLR) and Toll-like receptor (TLR) signaling pathways play key roles in initiating the immune response against RNA viruses (Thompson and Locarnini, 2007). Pretreatment of PAMs with porcine IFN-α led to a significant enrichment of DEGs in the RLR and TLR signaling pathways (Supplement Fig. 1C). Analysis of the genes involved in the RLR signaling pathway showed that the real-time PCR results were consistent with the RNA-Seq results (Fig. 3A; Fig. 3B). Among the seven DEGs enriched in the RLR signaling pathway, four showed significantly attenuated expression in the IFN + PRRSV group compared with the IFN group, including LOC100737466(DDX58), ISG15, IRF7, and CXCL10 (Fig. 3A; Fig. 3B). LOC100737466(DDX58) encodes retinoic-acid-inducible gene-I protein (RIG-I), an innate immune receptor, which functions as a cytoplasmic sensor recognizing RNA viruses and plays a major role in activating a cascade of antiviral responses in the RLR signaling pathway (Fujita et al., 2007). IRF7 encodes interferon regulatory factor 7 (IRF7), which is a key transcriptional regulator of type I IFN-dependent immune responses and plays a critical role in the innate immune response against virus infection (Ning et al., 2011). All the genes in the IFN + PRRSV and PRRSV groups were significantly differentially expressed from the IFN group ($p < 0.05$). (Fig. 3B). The attenuated expression of DEGs enriched in the RLR signaling pathway in the IFN + PRRSV group suggests that the RLR signaling pathway played a crucial role in restricting PRRSV infection, and that the RLR signaling pathway was targeted by PRRSV infection.

Analysis of the genes involved in the TLR signaling pathway showed that the real-time PCR results were consistent with the RNA-Seq results (Fig. 3C; Fig. 3D). Among the eight DEGs enriched in the TLR signaling pathway, five showed similar expression levels in the IFN and IFN + PRRSV groups, including TLR1, TLR3, and TLR7 (Fig. 3C; Fig. 3D). The remaining three DEGs showed significantly attenuated expression in the IFN + PRRSV group compared with the IFN group (Fig. 3C; Fig. 3D), including IRF7 and CXCL10, which overlapped with the RLR signaling pathway. Significant differences in expression of genes involving in RLR signaling pathways (e.g. DDX58, ISG15, IRF7, CXCL10, CASP8, DHX58, LOC100620995) were observed in both IFN + PRRSV vs IFN and PRRSV vs IFN (Fig. 3A; Fig. 3B). Overall, these results implied that RLR and TLR signaling pathways were involved in the innate immune response. Furthermore, IRF7 and CXCL10 were the antagonism target of PRRSV.

2.4. Impaired expression of antiviral genes by PRRSV infection

Certain ISGs possess antiviral activities (Schoggins and Rice, 2011). 16 antiviral DEGs were significantly up-regulated in response to porcine IFN-α treatment (Fig. 3E; Fig. 3F; S5 Table), among which 15 were significantly attenuated in the IFN + PRRSV group compared with the IFN group (asterisks are not marked because of graphical limitations): IRG6(RSAD2), IFI44, ISG20, PKR, LOC100738990(TRIM5), BST2, OAS1, IFIT1(ISG56), IRF7, IFIT2, ISG15, LOC100627004(IFITM1), ISG12(A)(IFI27), IFITM3, and IFIT5 (Fig. 3E; Fig. 3F). IRF7 is an upstream molecule that can activate IFN expression. It is also an ISGs molecule,which is regulated by interferon. Real-time PCR results confirmed that these 15 genes were suppressed by PRRSV. Specifically, the results showed that the expression levels of IRF7 gene in the PRRSV and IFN + PRRSV groups were significantly lower than that in the IFN-treated group. Notably, 4 antiviral DEGs, including PKR (encoding double-stranded RNA-dependent protein kinase) (Wang et al., 2016), OAS1 (encoding 2′-5′-oligoadenylate synthetase 1) (Wang and Zhang, 2014), IFIT1(ISG56) (encoding interferon-induced protein with tetratricopeptide repeats 1) (Sun et al., 2012a), and ISG15 (Sun et al., 2012b) have been demonstrated to inhibit PRRSV replication (Wang and Zhang, 2014). The remaining 12 antiviral DEGs have not been found to restrict PRRSV replication but were shown to inhibit the replication of other viruses (Schoggins and Rice, 2011). We examined the effects of the remaining 12 antiviral DEGs on PRRSV replication. PKR, OAS1, IFIT1(ISG56) and ISG15 were used as positive controls. The expression levels of these antiviral DEGs in PAMs were knocked down by RNA interference (RNAi) (Supplement Fig. 2) and the subsequent replication of PRRSV in the presence or absence of porcine IFN-α treatment was detected by qRT-PCR. Knockdown of IFI44, ISG20, BST2, IRF7, IFIT2, LOC100627004(IFITM1), IFITM3, IFIT5 and GBP1 expression resulted in significantly enhanced PRRSV replication in the PAMs mock-treated with IFN-α, suggesting that these DEGs were involved in the antiviral response against PRRSV replication. Comparison of the replication levels of PRRSV between the IFN-α-treated and the mock-treated PAMs revealed that knockdown of certain antiviral DEGs, including ISG20, PKR, BST2, IFIT2, LOC100627004(IFITM1), IFIT5, and GBP1, did not result in significantly enhanced PRRSV replication in the presence of IFN-α treatment compared with the absence of IFN-α treatment (Fig. 4A). A possible explanation was that knockdown of a single gene expression might have limited effect on the antiviral state, which was induced by IFN-α treatment and comprised multiple antiviral mechanisms. We further examined the anti-PRRSV effects of these antiviral DEGs by determining the dynamics of PRRSV replication in the siRNA-treated PAMs and observed similar results (Fig. 4B). Overall, these results demonstrate the roles of antiviral genes induced in PAMs by porcine IFN-α treatment in the inhibition of PRRSV replication. Furthermore, the impaired expression of these antiviral DEGs by PRRSV infection suggested that PRRSV was able to counteract most of these antiviral genes at the transcriptional level.

2.5. IRF7 regulates type I interferon antiviral response

PRRSV had significant immunosuppressive effects on IFN signaling pathway. IRF3, NF-kB and JAK/STAT were the targets of PRRSV viral proteins, which ultimately led to inhibition of ISGs gene expression. However, the proteins immunosuppressed by PRRSV (e.g. IRF3, NF-kB, JAK/STAT) did not regulate the expression of IRF7 directly. Our results demonstrated that the expression of IRF7 was inhibited by PRRSV, suggesting that PRRSV might have an additional suppressive mechanism for IRF7.

IRF7 mediate the response of IFN. To determine the regulatory role of IRF7 upon PRRSV infection, we used siRNA to interfere with IRF7 expression and subsequently infected the cells with PRRSV. The expression level of IFN was examined by using the treated cells. The IFN-α gene expression of cells in the IRF7 interference group was 2.92 times lower than that of the control cells group, and the IFN-β gene expression of cells in the IRF7 interference group was 2.60 times lower than that of the control cells group (Fig. 5A). Similar as mRNA results, the IFN-α protein level of cell supernatant in the IRF7 interference group was 3.62 times lower than that of the control cells group, and the IFN-β protein level of cell supernatant in the IRF7 interference group was 3.73 times lower than that of the control cells group (Fig. 5B). These results suggested that IRF7 regulates the expression of IFN-α and IFN-β. These

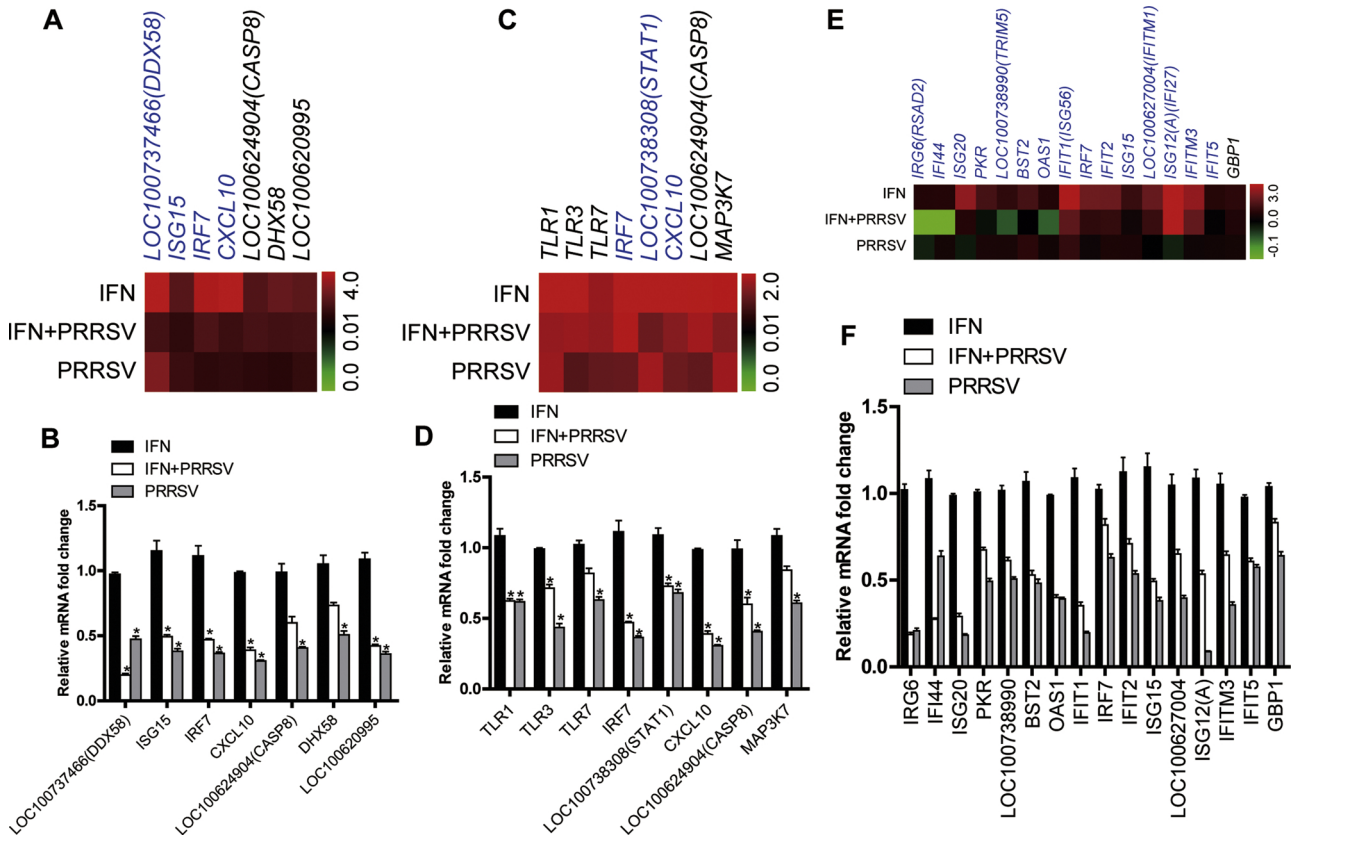


Fig. 3. Heatmaps and real-time PCR analysis of antiviral pathway.
(A) Heatmap analysis of DEGs enriched in the RIG-I-like receptor signaling pathway. (B) Validation of mRNA fold change in the RIG-I-like receptor signaling pathway. The mRNA was determined by SYBR real-time PCR and normalized with porcine GAPDH. Data are shown as the mean ± standard error (SEM). * , the significantly genes compared to the IFN group. The experiments were repeated at least three times. (C) Heatmap analysis of DEGs enriched in the Toll-like receptor signaling pathway. (D) Validation of mRNA fold change in the Toll-like receptor signaling pathway. The mRNA was determined by SYBR real-time PCR and normalized with porcine GAPDH. Data are shown as the mean ± standard error (SEM). * , the significantly genes compared to the IFN group. The experiments were repeated at least three times. (E) Heatmap of antiviral genes enriched in PRR pathway. Color scale illustrates the fold change values of genes. The up-regulated and down-regulated genes are highlighted in red and green, respectively. The names of genes with significant difference in gene expression between the IFN and IFN + PRRSV groups are highlighted in blue. (F) Validation of antiviral genes in PRR pathway fold change by real-time PCR. The mRNA was determined by SYBR real-time PCR and normalized with porcine GAPDH. Data are shown as the mean ± standard error (SEM). The experiments were repeated three times.

results of overexpression of IRF7 in PAM cells also proved the regulatory role of IRF7 in interferon signaling pathway. The mPNA (Fig. 5D) and protein (Fig. 5E) levels of IFN-α and IFN-β were significantly up-regulated in IRF7 overexpressed cells. The gene expression level of ISGs in the IRF7 interference group was 1.26–6.19 times lower than that in the control group (Fig. 5C), which further confirmed the regulation effects of IRF7 on IFN. Interference with IRF7 can down-regulate IFN gene expression (Fig. 5A; Fig. 5B) and increase PRRSV replication rate (Fig. 4A) indicated that IRF7 regulated the antiviral response during PRRSV infection.

2.6. PRRSV Nsp7 was responsible for inhibiting IRF7 transcription

To confirm that PRRSV infection transcriptionally inhibited IRF7 expression induced by porcine IFN-α treatment (Fig. 3). We examined the inhibitory effect of PRRSV on IRF7 expression at protein level. IRF7 protein expression was inhibited by PRRSV infection (Fig. 6A), confirming the inhibitory effect of PRRSV infection on IRF7 expression (Patel et al. , 2010). PRRSV inhibited the expression of IRF7 at both mRNA and protein levels. Then we screened the PRRSV proteins responsible for down-regulation of IRF7 expression. We evaluated the expressions of viral proteins by western blot (Fig. 6B). Subsequently, several non-structural proteins (nsps) of PRRSV, which played potential roles in the modulation of IFN signaling, were transiently expressed in PAMs. Overexpression of nsp7 significantly down-regulate the expression of IRF7 compared with Flag-vector (Fig. 6C), suggesting that PRRSV nsp7 inhibited IRF7 expression. Nsp5 also down-regulated IRF7 gene expression, but it was not statistically significant. Nsp11 up-regulated the expression of IRF7 significantly ($p < 0.001$), while IRF7 was not up-regulated significantly by Nsp4. This indicated that the nsp7 protein was the key protein that down-regulate the expression of IRF7. The inhibitory on IRF7 expression was confirmed at protein level. Flag-nsp7 remarkably down-regulated the IRF7 expression at protein level (Fig. 6D). Taken together, nsp7 of PRRSV was involved in the inhibition of IRF7 expression.

3. Discussion

PRRSV is sensitive to treatment with type I IFNs (Overend et al., 2007), but has evolved strategies to counteract the type I IFN-induced antiviral state (Sun et al., 2012a; Wang and Zhang, 2014). For example, PRRSV can facilitate its infection by up-regulates a host microRNA miR-30c, a negative regulator of the type I IFN signaling (Zhang et al., 2016). PRRSV blocks the nuclear translocation of STAT1/STAT2 heterodimer to inhibit the type I IFN signaling (Patel et al., 2010). The Nsp1β of PRRSV inhibits IFN-activated JAK/STAT signal transduction by inducing karyopherin-α1 degradation (Wang et al., 2013). A better knowledge of the mechanisms whereby PRRSV counteracts the type I IFN-induced antiviral state is essential for understanding the host antiviral immune response against PRRSV infection, as well as for

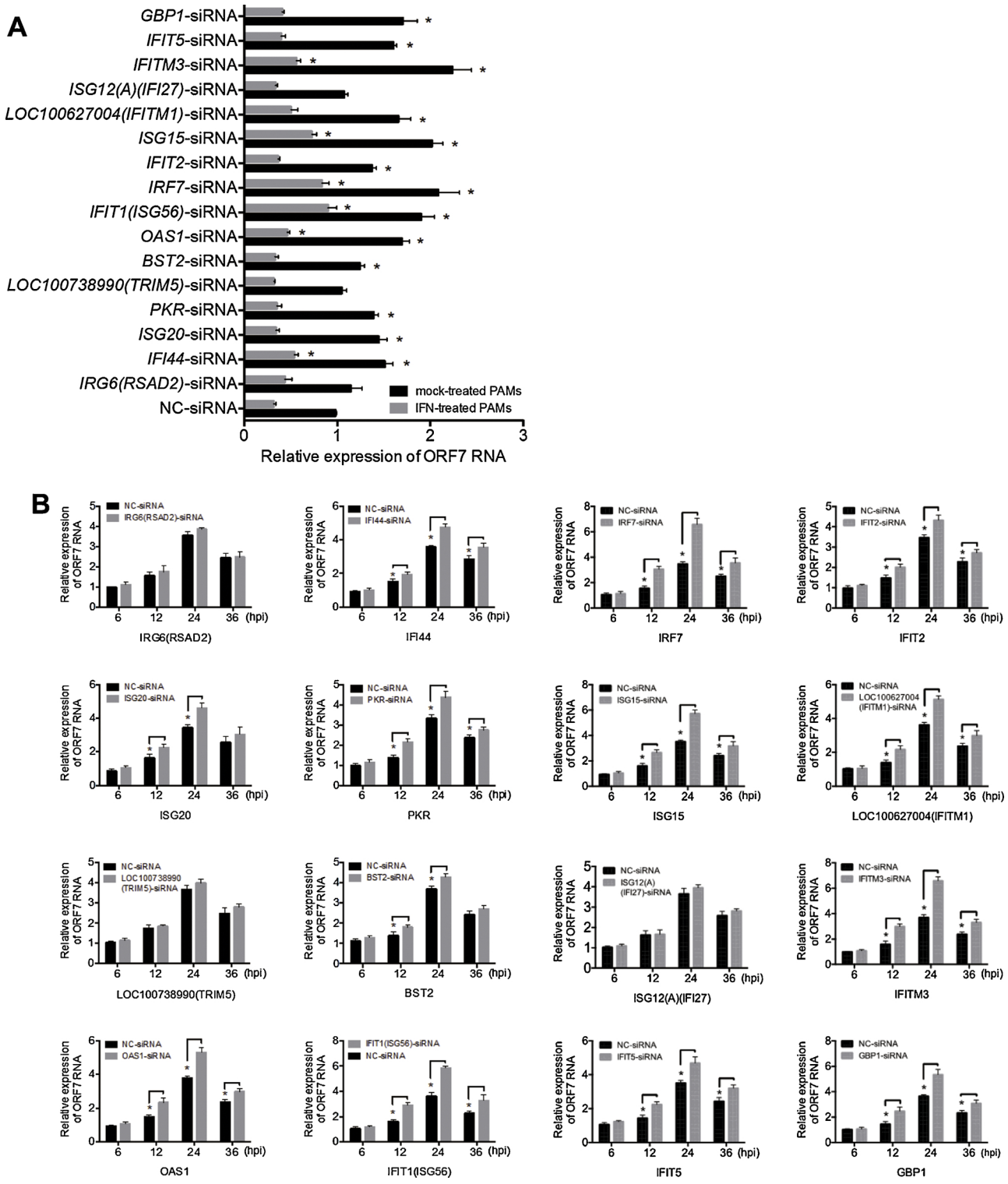


Fig. 4. Analysis of expression of antiviral genes.

(A) Heatmap of antiviral genes. Color scale illustrates the fold change values of genes. The up-regulated and down-regulated genes are highlighted in red and green, respectively. The names of genes with significant difference in gene expression between the IFN and IFN + PRRSV groups are highlighted in blue. (B) Validation of antiviral genes fold change by real-time PCR. The mRNA was determined by SYBR real-time PCR and normalized with porcine GAPDH. Data are shown as the mean ± standard error (SEM). The experiments were repeated three times. (C) PAMs were transfected with small interfering RNA (siRNA) to silence the indicated gene expression, or non-targeting small RNA (NC-siRNA), for 6 h and subsequently treated or mock-treated with porcine IFN-α at a final concentration of 100 ng/ml for 24 h. Following porcine IFN-α treatment, the cells were infected with HP-PRRSV. The expression of PRRSV ORF7 RNA in the transfectants was detected at 24 hpi by qRT-PCR. The data are means with SD from three independent experiments. *, $p < 0.05$ compared with NC-siRNA group. (D) PAMs were transfected with small interfering RNA (siRNA) targeting the indicated genes, or with non-targeting small RNA (NC-siRNA), for 24 h and subsequently infected with HP-PRRSV. The expression of PRRSV ORF7 RNA in the transfectants was detected at the indicated time points by qRT-PCR. The data are means with SE from three independent experiments. *, $p < 0.05$ compared between groups. hpi, hours post-infection. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

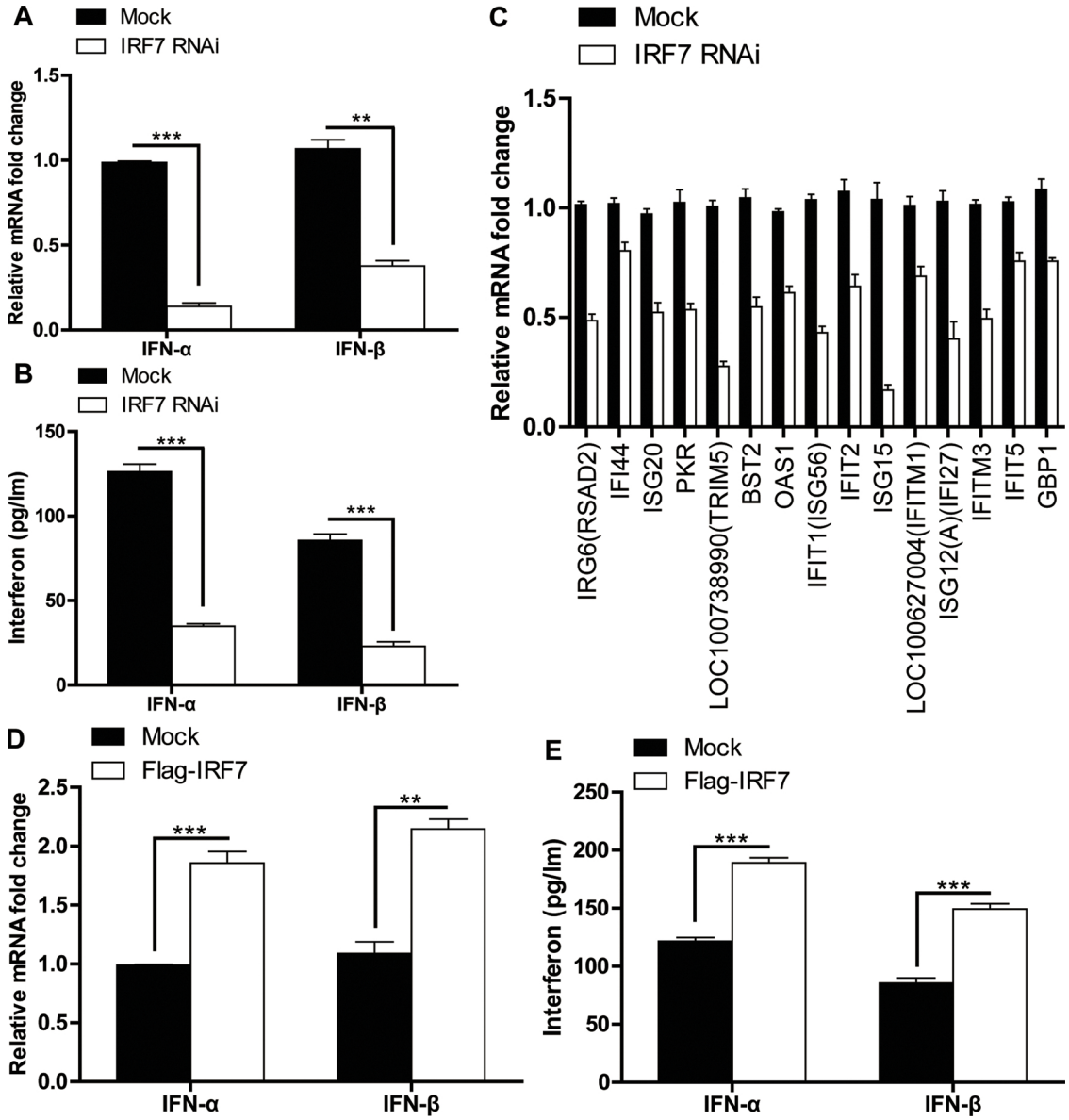


Fig. 5. IRF7 is responsible for type I interferon mediated anti-PRRSV in PAMs.
(A and B) IRF7 decrease IFN-α and IFN-β response in PAMs. (A) The fold change of IFN-α and IFN-β mRNA levels were measured by real-time PCR. IRF7 shRNA was added to cells. HP-PRRSV was inoculated after 24 h. The cells were collected after cultivation for another 24 h to evaluate gene expression. *, $p < 0.05$ compared between groups. Data are shown as the mean ± standard error (SEM). The experiments were repeated three times. (B) The change of IFN-α and IFN-β protein levels were measured by ELISA. IRF7 shRNA was added to cells. HP-PRRSV was inoculated after 24 h. The cellular supernatant samples were collected after cultivation for another 24 h to evaluate interferon level. *, $p < 0.05$ compared between groups. Data are shown as the mean ± standard error (SEM). The experiments were repeated three times. (C) IRF7 regulates interferon mediated ISGs response in PAMs. The fold change of ISGs were measured by real-time PCR. Data are shown as the mean ± standard error (SEM). The experiments were repeated three times. (D and E) IRF7 overexpression enhance IFN-α and IFN-β response in PAMs. (D) The fold change of IFN-α and IFN-β mRNA levels were measured by real-time PCR. IRF7 plasmid was transfected into cells. HP-PRRSV was inoculated after 24 h. The cells were collected after cultivation for another 24 h to evaluate gene expression. *, $p < 0.05$ compared between groups. Data are shown as the mean ± standard error (SEM). The experiments were repeated for three times. (E) The change of IFN-α and IFN-β protein levels were measured by ELISA. IRF7 plasmid was transfected into cells. HP-PRRSV was inoculated after 24 h. The cellular supernatant samples were collected after cultivation for another 24 h to evaluate interferon level.*, $p < 0.05$ compared between groups. Data are shown as the mean ± standard error (SE). The experiments were repeated for three times.

developing better control strategies. PAMs are the primary target cells of PRRSV infection (Kimman et al., 2009) and the immune response of PAMs to PRRSV infection has been extensively studied using high-throughput techniques (Jiang et al., 2013; Xiao et al., 2010; Xiao et al., 2015). In this study, we approached this problem by examining global gene expression in IFN-α-pretreated PAMs in response to infection of HP-PRRSV that is more virulent and triggers a stronger down-regulation of antiviral immune response than the classical PRRSV (Wang and Zhang, 2014). Three or more replications are important for RNA-seq study, especially for general gene expression and signal pathway analysis. However, this study mainly focused on the immunosuppression of PRRSV on immune system. With clinical phenomenon, published literatures and our previous work, we have known how key genes changed and how signal pathway functioned with PRRSV immunosuppression. Only the responses of a few genes were unclear. So, the aims of RNA-seq are: (1) to prove our previous results of PRRSV immunosuppressing IRF7; (2) to get a comprehensive result of how IRF7 and ISGs respond to PRRSV. Therefore, we think it's reasonable to conduct RNA-seq on one replication. After RNA-seq analysis, we checked the expression levels of all the IRF7-related genes by qRT-PCR

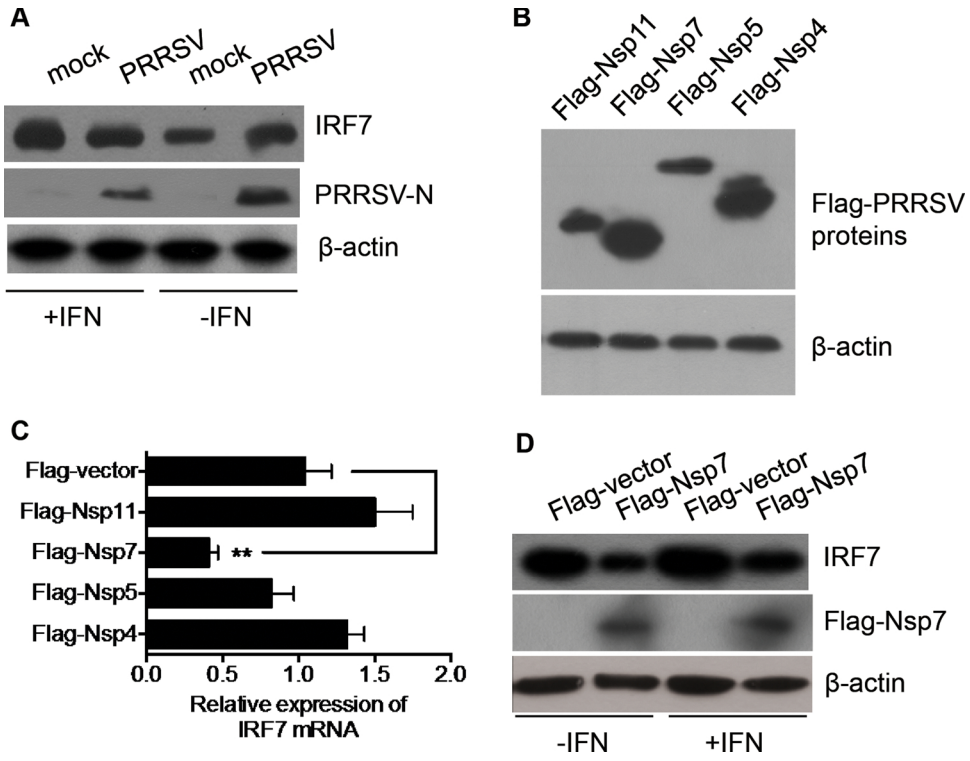


Fig. 6. PRRSV nsp7 down-regulated IRF7. (A) PRRSV infection impaired IRF7 protein expression. The infected or mock infected cells pre-treated with IFN or without IFN were collected, separated by SDS-PAGE, and incubated with rabbit anti-IRF7, rabbit anti-PRRSV N and mouse anti-actin antibodies. (B) PAM cells were transfected with PRRSV non-structural plasmids. The expression of Flag-tagged viral proteins in the transfectants was probed by Western blotting with anti-Flag antibody. (C) Porcine IRF7 mRNA expression was detected in viral proteins transfected PAMs. The relative expression of IRF7 mRNA was measured by real-time PCR. Data are shown as the mean ± standard error (SEM). The experiments were repeated three times. (D) PRRSV nsp7 impaired IRF7 protein expression. The transfected or mock transfected cells pre-treated with IFN or without IFN were collected, separated by SDS-PAGE, and incubated with rabbit anti-IRF7, mouse anti-Flag and mouse anti-actin antibodies.

with three biological replications. The results of qRT-PCR were in accordance with RNA-seq, indicating that the results of RNA-seq are reliable and stable. Additionally, the results of qRT-PCR also supported the results of GO and KEGG analysis on immune signaling pathway. The type I IFN-mediated immune response is known to confer an antiviral state within cells (Schoggins and Rice, 2011). Indeed, we identified 744 significant DEGs in the IFN + PRRSV group, which was 2.5 times higher as in the PRRSV group. These 744 significant DEGs in the IFN + PRRSV group were significantly enriched in 14 GO terms, whereas the 298 significant DEGs in the PRRSV group showed no significant enrichment in GO terms.

A total of 828 DEGs were up- or down-regulated in response to porcine IFN-α. In this study, we focused on the up-regulated DEGs because the products of ISGs play a critical role in the host antiviral immune response, such as antiviral and proapoptotic activities (Schoggins and Rice, 2011). A total of 346 DEGs, excluding 45 uncharacterized genes, was significantly up-regulated in response to IFN-α. We considered these as potential porcine ISGs. These up-regulated DEGs in the IFN group were enriched in ten GO and five KEGG terms that were remarkably related to host immune response. KEGG analysis showed that the two most-enriched terms were the RLR and TLR signaling pathways, which play key roles in the recognition of RNA viruses and subsequent initiation of an immune response (Fujita et al., 2007; Thompson and Locarnini, 2007). The expression levels of DEGs enriched in the RLR signaling pathway, such as LOC100737466(DDX58) and IRF7, were significantly attenuated in the IFN + PRRSV compared with the IFN group; Notably, both LOC100737466(DDX58) encoding the cytoplasmic viral RNA sensor RIG-I (Fujita et al., 2007) and IRF7 encoding the transcriptional regulator IRF7 of the type I IFN pathway (Ning et al., 2011), play crucial roles in the RLR signaling pathway. Previous studies described that the RLR signaling pathway was modulated by the infection of PAMs with North American genotype PRRSV (Jiang et al., 2013; Zhou et al., 2011) and more virulent eastern European genotype PRRSV (Badaoui et al., 2014). These observations together with our results suggest that the RLR signaling pathway may play a crucial role in restricting PRRSV infection and may be targeted by PRRSV infection which has been reported by a previous study (Luo et al., 2008).

Antiviral ISGs play crucial roles in restricting and eliminating viral infections (Schoggins and Rice, 2011). A total of 16 antiviral DEGs were up-regulated in response to IFN-α, and these were probably responsible for the observed inhibition of PRRSV replication. Among the 16 antiviral DEGs, four (PKR, OAS1, IFIT1(ISG56), and ISG15) have previously been demonstrated to inhibit PRRSV replication (Wang and Zhang, 2014). We examined the anti-PRRSV activity of the remaining 12 antiviral DEGs that have not been reported to restrict PRRSV replication, of which 9 DEGs including IFI44, ISG20, BST2, IRF7, IFIT2, LOC100627004(IFITM1), IFITM3, IFIT5, and GBP1 showed antiviral activity against PRRSV replication. Some of these DEGs including IRF7, IFIT2, LOC100627004(IFITM1), IFITM3, and GBP1 have been screened out using high-throughput techniques and considered as potential anti-PRRSV genes by previous studies (Badaoui et al., 2014; Xiao et al., 2010; Zhou et al., 2011). Our study confirmed the anti-PRRSV activity of these potential antiviral DEGs. Notably, IRF7 encoding the transcriptional regulator IRF7 of the type I IFN pathway (Ning et al., 2011) showed the anti-PRRSV activity. Aberrant expression of IRF7 has been observed during PRRSV infection (Badaoui et al., 2014; Sang et al., 2014; Xiao et al., 2010; Zhou et al., 2011). Our data suggested a crucial role of IRF7 in regulation of antiviral immune response against PRRSV replication. Comparison of the expression of these 16 antiviral DEGs between the IFN and IFN + PRRSV revealed that 15 (93.8%) were significantly attenuated in the IFN + PRRSV group compared with the IFN group, suggesting that the antiviral genes induced by porcine IFN-α were targeted by PRRSV. We also found that there were no remarkable changes in expression levels of these 16 DEGs in the PRRSV group, suggesting that PRRSV was able to counteract most of these antiviral genes at the transcriptional level. PRRSV infection is known to block the type I IFN signaling pathway and subsequently interfere with ISG expression (Patel et al., 2010; Wang and Zhang, 2014), and our results supported these earlier findings.

IRF3 and IRF7 are two important regulatory factors in the IFN signaling pathway. PRRSV can inhibit the activity of IRF3 through nsp1β and N proteins, but similar immunosuppressive effects have never been reported for IRF7. From our experimental results, IRF7 showed

significant regulation of IFN immune response and antiviral effects. IRF7 was able to inhibit PRRSV early replication. The expression of IRF7 was down-regulated in the IFN + PRRSV and PRRSV treated groups in comparison with the IFN group, which indicated that PRRSV perform inhibition effects on IRF7. Then we selected 4 PRRSV proteins that may interact with IRF7. Protein overexpression experiments demonstrated that nsp7 significantly down-regulated the expression of IRF7. However, nsp5, nsp11 and nsp4 did not down-regulate the expression of IRF7. Interestingly, nsp11 up-regulated the IRF7 expression with significantly difference. This indicated that 1) PRRSV nsp7 protein is the main effector protein that down-regulates IRF7; 2) Up- or down-regulation of IRF7 is a result of complex viral protein interactions. The specific mechanism of nsp7 role in down-regulating IRF7 expression remains to be further studied.

Though PRRSV could inhibit IRF7 production, it would induce the up-regulation of IRF7 (in comparison with uninfected cells) at the same time. It was caused by host immune responses and virus immunosuppressive effects, which was similar as PRRSV immunosuppressing ISG15 (Ref). Thereby, in the condition without interferon, IRF7 expresssion in PRRSV-infecting cells would be higher than uninfected cells only with interferon, we could observe virus immunosuppression. And the results of mRNA and protein tests were consistent. In conclusion, we analyzed changes in global gene expression in PAMs pretreated with porcine IFN-α and exposed to HP-PRRSV infection, to explore the mechanisms whereby PRRSV can counteract the type I IFN-induced antiviral state. A total of 346 DEGs were up-regulated by IFN-α. To the best of our knowledge, this study provides the first list of porcine ISGs induced by IFN-α. Among the 346 DEGs, 93 were attenuated in response to PRRSV infection and were enriched in immune-response-related GO/KEGG terms. The expression of 93.8% of antiviral genes and the genes encoding RIG-I, IRF7, STAT1, and TRAIL were significantly attenuated by PRRSV infection. These observations suggested that PRRSV counteracted the type I IFN-induced antiviral state by interfering with the expression of a range of genes involved in the host defense response. In addition, knockdown of antiviral DEGs expression by siRNA, including PKR, OAS1, IFIT1(ISG56), ISG15, IFI44, ISG20, BST2, IRF7, IFIT2, LOC100627004(IFITM1), IFITM3, IFIT5 and GBP1, resulted in significantly enhanced PRRSV replication, suggesting the involvement of these DEGs in the antiviral response against PRRSV replication. Notably, IRF7 encoding IRF7, a key transcriptional regulator of the type I IFN-dependent immune response, showed an anti-PRRSV activity and aberrant expression during PRRSV infection, demonstrating a crucial role of IRF7 in regulation of antiviral immune response against PRRSV replication.

4. Materials and methods

4.1. Ethics statement

All animal experiments were performed in compliance with the Guidelines on the Humane Treatment of Laboratory Animals (Ministry of Science and Technology of the People's Republic of China, Policy No. 2006 398) and were approved by the Institutional Animal Care and Use Committee at the Shanghai Veterinary Research Institute (IACUC No: Shvri-Pi-0124).

4.2. Cells and virus infection

Primary PAMs were isolated from 5-week-old clinically healthy crossbred pigs raised in the animal facility of Shanghai Academy of Agricultural Sciences, Shanghai, China. The pigs were tested negative for PRRSV antibody using a Reproductive and Respiratory Syndrome Virus Antibody Test Kit (HerdChek PRRS 2XR; IDEXX Laboratories, Westbrook, ME, USA), as well as for PRRSV, porcine circovirus type 2, classical swine fever virus, and swine influenza virus by real-time polymerase chain reaction (PCR). Isolation of PAMs was performed as described previously (Delputte and Nauwynck, 2004). Briefly, porcine lungs were collected immediately after euthanasia and were lavaged with ice-cold phosphate-buffered saline (PBS). Euthanasia was performed by intravenous injection of 100 mg/kg sodium pentobarbital. The lavage fluids were centrifuged at 1200 rpm for 10 min at 4 °C to spin down the cells, which were then re-suspended in RPMI-1640 medium containing 10% fetal bovine serum (FBS) (Thermo Fisher Scientific, Carlsbad, CA, USA). PAMs isolated from four pigs were pooled for PRRSV infection.

High pathogenic PRRSV strain (SH-PRRS01 strain) isolated in our laboratory was used to infect PAMs. PAMs were pre-cultured in RPMI-1640 medium containing 10% FBS at 37 °C for 2 h, washed with PBS, and then inoculated with HP-PRRSV at a multiplicity of infection (MOI) of 1, and incubated at 37 °C for 2 h. After inoculation, the inocula were removed and the cells were cultured in RPMI-1640 medium containing 2% FBS for the indicated times. Mock-infected cells were generated using PBS as the control inoculum.

4.3. Experimental groups and preparation of RNA samples for sequencing

PAMs were divided into four groups for RNA-sequence analysis (Fig. 1A): IFN (PAMs pretreated with porcine IFN-α and mock-infected with HP-PRRSV); IFN + PRRSV (PAMs pretreated with porcine IFN-α and infected with HP-PRRSV); PRRSV (PAMs mock-pretreated with porcine IFN-α and infected with HP-PRRSV); and C (control) (PAMs mock-pretreated with porcine IFN-α and mock-infected with HP-PRRSV). The buffer dissolving IFN-α was used as mock in "mock-treated with IFN-α", while the buffer diluting HP-PRRSV functioned as mock in "mock-infected with HP-PRRSV".

The preparation of the RNA samples is shown in Fig. 1B. The pooled PAMs were split into four groups as above, plated on 150 mm dishes (3 dishes per group), and cultured in RPMI-1640 medium containing 10% FBS at 37 °C for 2 h. For porcine IFN-α pretreatment, the PAMs were washed with PBS and re-cultured in RPMI-1640 medium containing 10% FBS and recombinant porcine IFN-α (PBI interferon source, NJ, USA) at a final concentration of 200 ng/ml (or indicated concentration) at 37 °C for 24 h, as described previously (Kim et al., 2012; Liu et al., 2013). Mock-pretreatment was performed in an identical fashion to porcine IFN-α pretreatment using bovine serum albumin as a control agent. Following pretreatment with porcine IFN-α, the culture media were removed and PAMs were washed with PBS and inoculated with HP-PRRSV at a MOI of 1, followed by removal of the inocula. The PAMs were cultured in RPMI-1640 medium containing 2% FBS at 37 °C for 12 h and harvested for RNA extraction. Total RNAs were extracted from the PAMs using TRIzol reagent (Thermo Fisher Scientific) according to the manufacturer's protocol and qualified using an Agilent 2100 bioanalyzer. RNAs isolated from three dishes of PAMs in each group were pooled and quantified. To minimize the influence of experimental variations, equal quantities of RNAs obtained from three independent experiments were combined into a single sample for construction of complementary DNA (cDNA) libraries, as described previously (Jiang et al., 2013).

4.4. RNA sequencing and read mapping

The combined RNA samples in each group were submitted to Novel Bio Co., Ltd (http://www.novelbio.com), Shanghai, China for construction of cDNA libraries, RNA sequencing, and read mapping. A total of four cDNA libraries for single-end sequencing were prepared with Ion Total RNA-Seq Kit v2.0 (Thermo Fisher Scientific) according to the manufacturer's instructions and subsequently subjected to Proton sequencing process. Briefly, samples were processed on an OneTouch 2 instrument (Thermo Fisher Scientific) and enriched on an OneTouch 2 ES station (Thermo Fisher Scientific) for preparing the template-positive Ion PI Ion Sphere Particles according to the instructions of Ion PI Template OT2 200 Kit v2.0 (Thermo Fisher Scientific). After

enrichment, the template-positive Ion PI Ion Sphere Particles of samples were loaded on to a P1v2 Proton Chip (Thermo Fisher Scientific) and sequenced on Proton Sequencers according to the instructions of Ion PI Sequencing 200 Kit v2.0 (Thermo Fisher Scientific). The obtained sequences were submitted to the Sequence Read Archive (SRA) at NCBI (SRP069059). For mapping of single-end reads, clean reads were obtained from raw reads by removing adaptor sequences, reads with > 5% ambiguous bases, and low-quality reads containing > 20% of bases with qualities of < 13. The clean reads were aligned to the pig reference genome (version: Sus scrofa Sscrofa10.2) and human reference genome (version: GRCh37/hg19) using the MapSplice program (v2.1.6).

4.5. Data analysis

Differentially expressed genes (DEGs) were identified using the EBSeq software (version 1.6) according to the EBseq's instructions of working without replicates (https://www.bioconductor.org/packages/3.3/bioc/vignettes/EBSeq/inst/doc/EBSeq_Vignette.pdf#search='EBSeq%3A+An+R+package+for+di'), as described previously (Miao et al., 2015). The EBSeq is an empirical Bayesian approach that models a number of features observed in RNA-sequence data (Leng et al., 2013). The EBSeq estimates the variance by pooling similar genes into a certain number of bins in the case of a data set with multiple biological conditions and no replicates. Genes with a false discovery rate (FDR) < 0.05 and fold change (FC) > 1.5 or < 0.667 were filtered out as significant DEGs between groups, as described previously (Badaoui et al., 2014). Gene ontology (GO) enrichment analysis was performed based on biological processes using DAVID Bioinformatics Resources 6.7 (http://david.abcc.ncifcrf.gov/) to evaluate the biological importance of the significant DEGs. GO terms showing a Bonferroni-corrected p value and FDR < 0.05 were considered significant. Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis was performed using DAVID Bioinformatics Resources 6.7 and KEGG terms showing a p value < 0.05 were considered significant.

4.6. Real-time PCR

PRRSV replication and gene expression were analyzed using total RNAs isolated from PRRSV-infected PAMs using TRIzol reagent (Thermo Fisher Scientific), according to the manufacturer's protocol. RNAs isolated for RNA-sequence analysis were subjected to real-time PCR to validate the RNA-sequence data. real-time PCR was performed as described previously(Zhu et al., 2013). A list of all primers is available upon request. Relative quantification of gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Data are presented as FC in gene expression normalized to the expression of endogenous glyceraldehyde-3-phosphate dehydrogenase and relative to the mock-infected/treated group. Significance was analyzed using Student's t-test. A value of $p < 0.05$ was considered significant.

4.7. RNA interference

Small interfering RNAs (siRNAs) targeting the indicated genes were synthesized chemically. A negative control siRNA without known target genes was synthesized in parallel. The siRNA sequences are shown in S1 Table. PAMs were pre-cultured in RPMI-1640 medium containing 10% FBS at 37 °C for 2 h, washed with PBS, and transfected with siRNA at a concentration of 10 nM using Lipofectamine 2000 (Thermo Fisher Scientific), according to the manufacturer's protocol. The transfectants were re-cultured at 37 °C for 24 h and subsequently inoculated with HP-PRRSV at a MOI of 1. PRRSV replication in the transfectants was determined by qRT-PCR at 24 h post-infection (hpi).

4.8. Transfection and Western blotting

IRF7 of pig and the gene encoding PRRSV non-structural protein were amplified by PCR. Then both fragments were ligated into the vector pFlag 7.1. The plasmid was confirmed by sequencing and transfected with liposome 3000 to freshly isolated PAMs cells of pig. The samples were collected after cultivation at 37℃ for 48 h. Protein expression was detected by western blotting.

Western blotting were performed as described previously (Qi et al., 2017). The antibodies used were anti-IRF7 rabbit polyclonal antibody (Aviva Systems Biology), anti-β actin monoclonal antibody (Abcam, Shanghai, China), anti-Flag monoclonal antibody (Sigma), and anti-PRRSV N protein rabbit polyclonal antibody.

Competing interests

The authors declare that they have no competing interests.

Author contributions

Conceived and designed the experiments: KL YQ ZM. Performed the experiments: KL GM. Analyzed the data: GM KL YQ ZM JW BL. Contributed reagents/materials/analysis tools: GM DS PQ YL YS BZ DM. Wrote the paper: KL GM ZM.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetmic.2018.12.015.

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