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# Role of *IFNLR1* gene in PRRSV infection of PAM cells

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## ABSTRACT

**Background:** Interferon lambda receptor 1 (*IFNLR1*) is a type II cytokine receptor that clings to interleukins IL-28A, IL29B, and IL-29 referred to as type III IFNs (IFN-λs). IFN-λs act through the JAK-STAT signaling pathway to exert antiviral effects related to preventing and curing an infection. Although the immune function of IFN-λs in virus invasion has been described, the molecular mechanism of IFNLR1 in that process is unclear.
**Objectives:** The purpose of this study was to elucidate the role of IFNLR1 in the pathogenesis and treatment of porcine reproductive and respiratory syndrome virus (PRRSV).
**Methods:** The effects of IFNLR1 on the proliferation of porcine alveolar macrophages (PAMs) during PRRSV infection were investigated using interference and overexpression methods.
**Results:** In this study, the expressions of the *IFNLR1* gene in the liver, large intestine, small intestine, kidney, and lung tissues of Dapulian pigs were significantly higher than those in Landrace pigs. It was determined that porcine *IFNLR1* overexpression suppresses PRRSV replication. The qRT-PCR results revealed that overexpression of *IFNLR1* upregulated antiviral and IFN-stimulated genes. IFNLR1 overexpression inhibits the proliferation of PAMs and upregulation of p-STAT1. By contrast, knockdown of IFNLR1 expression promotes PAMs proliferation. The G0/G1 phase proportion in IFNLR1-overexpressing cells increased, and the opposite change was observed in IFNLR1-underexpressing cells. After inhibition of the JAK/STAT signaling pathway, the G2/M phase proportion in the IFNLR1-overexpressing cells showed a significant increasing trend. In conclusion, overexpression of IFNLR1 induces activation of the JAK/STAT pathway, thereby inhibiting the proliferation of PAMs infected with PRRSV.
**Conclusion:** Expression of the *IFNLR1* gene has an important regulatory role in PRRSV-infected PAMs, indicating it has potential as a molecular target in developing a new strategy for the treatment of PRRSV.

**Keywords:** IFNLR1; PRRSV; replication; proliferation; cell cycle

## INTRODUCTION

Porcine reproductive and respiratory syndrome virus (PRRSV) is an enveloped positive-stranded RNA virus within the family *Arteriviridae*, order *Nidovirales* [1]. The latest classification of the International Committee on Taxonomy of Viruses (ICTV) indicates

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**Conflict of Interest**

The authors declare no conflicts of interest.

**Author Contributions**

Conceptualization: Zeng Y, Qin M. Data curation: Li Z, Zhang Y. Formal analysis: Qin M. Funding acquisition: Zeng Y. Investigation: Ma L, Zhao J, Wang L. Methodology: Geng J, Wang L. Project administration: Chen W. Resources: Zeng Y. Software: Chen W. Supervision: Chen W, Zeng Y. Validation: Li Z, Ma L. Visualization: Qin M, Chen W. Writing - original draft: Qin M. Writing - review & editing: Chen W, Zeng Y.

that PRRSV is a porcine arterivirus (*Porartevirus*; https://talk.ictvonline.org/ictv-reports/ictv_online_report/). Pigs of different ages, breeds, and genders can be infected with PRRSV, with pregnant sows and piglets less than one month being the most susceptible. Porcine reproductive and respiratory syndrome (PRRS) is characterized by sow miscarriages, piglet dyspnea, and high mortality. Since the PRRSV was detected in wild boars in Europe and North America in 1987, it has resulted in substantial economic losses to the global pig industry [2]. Previous studies have shown that the persistence of the PRRSV within swine herds is often related to the recurrence of secondary infections [3,4]. PRRSV infection can elicit poor innate interferon and cytokine responses [5,6]. Therefore, PRRSV has been speculated to have immunosuppressive effects in pigs. In previous study, a large number of positive selection genes were identified in Dapulian pigs by performing specific-locus amplified fragment sequencing (SLAF) analysis. These genes are under positive selection pressure to adapt to environmental changes during species evolution [7]. Historically, Dapulian pigs are known for their resistance and immune properties [8]. The interferon lambda receptor 1 (*IFNLR1*) gene is one such positive selection gene and may have an important role in the disease resistance and adaptability of Dapulian pigs. The encoded protein product of this gene is a member of the type II cytokine receptor family [9]. Interferon (IFN)-λ identifies and combines with the dimer receptor complex formed by the IFNLR1 and IL10Rβ chains, subsequently activating the Janus kinase (JAK)- signal transducer and activator of transcription (STAT), phosphorylated (p)-protein kinase B, and mitogen-activated protein kinase/extracellular signal-regulated kinases signaling pathways, which exert antiviral and growth-suppressive activity *in vitro* and *in vivo* [10-12]. After IFN secretion activates the downstream signaling pathways, a variety of IFN-stimulated genes (ISGs) are transcribed to initiate the host defense response to the virus.

Interferon is an inducible protein. Normal cells generally do not produce interferon spontaneously, only having the potential to synthesize interferon, and the interferon gene is in a suppressed quiescent state. IFN-λs are usually induced in virus-infected cells but, like IFN-α and IFN-β, can also be induced by other mechanisms. IFN-λ is considered an epithelial cytokine, which is mainly expressed in antigen-presenting cells. Since the IFNLR1 gene is most abundantly expressed in epithelial-derived cells, this determines the tropism of the IFN-λ responses [13]. For example, PRRSV induces IFN-λ1 and IFN-λ3 expression at the mRNA level after the infection of porcine alveolar macrophages (PAMs) [14]. Recently, additional research has indicated that IFNLR1 is also widely expressed in immune cells. In viral infection or acute inflammation, IFN-λ can directly or indirectly act on immune cells to exert immunoregulatory functions [15]. Neutrophils can express IFNLR1 in mice infected with the influenza virus [16]. Over the past few years, IFNLR1 has been broadly investigated for its mutations associated with various diseases in humans [17,18]. It has been reported that West Nile virus (WNV) infection *in vivo* in the brain and spinal cord of IFNLR1(-/-) mice can replicate and spread quickly [19]. Although IFNLR1 is involved in biological activities induced by IFN-λs, including antiviral, antitumor, and immune regulation, research on these activities is still needed.

The anti-cell proliferation activity of IFN-λs varies greatly among different cell lines, and it has a close relationship with the expression of its IFNLR1 receptor. Cell proliferation is not only a cell life activity, but also is a necessary process for cell maintenance and for compensating for cell damage. Completion of this process is mainly accomplished by regulating the cell cycle. The cell cycle can be split into four phases: G1, S, G2, and mitosis (M), and cells that are no longer cycling are said to be quiescent or in phase G0. Mammalian cells regulate cell growth, genome replication, and cell division, all of which occur during the cell cycle [20]. The two

gap phases, G1 and G2, are separated by the S phase, during which DNA is replicated and are followed by mitosis, in which the DNA is divided between two new nuclei. After mitosis, the cell itself divides and each daughter cell repeats the cycle being at G1 or exits the cell cycle at G0. Under normal circumstances, the initiation and orderly progress of the cell cycle requires strict regulation by various regulatory factors. Cell-cycle proteins (cyclins), cyclin-dependent kinases (CDKs), and cyclin-dependent kinase inhibitors (CKIs) are the three main cell-cycle regulatory molecules. In addition, cell-cycle checkpoints are important in determining the transition of cells from one phase to the next [21]. So far, the effect of IFNLR1 on the cell cycle and its corresponding molecular mechanism have not been reported.

At present, there are few studies on the role of IFNLR1 in resisting PRRSV infection. After the PRRSV enters the pig body, it first invades PAMs, causing severe pneumonia. This study used interference and overexpression methods to analyze the effect of IFNLR1 on PAM proliferation. The purpose of the study was to elucidate the potential role of the *IFNLR1* gene in the prevention and treatment of PRRSV.

## MATERIALS AND METHODS

### Cell line and virus, tissue collection

Twelve two-month-old PRRSV-negative pigs, including six Dapulian pigs and six Landrace pigs, were purchased from the Jining breeding farm in Shandong province, China. All pigs were raised u the same environment and management conditions. After slaughter at the indicated time, tissue samples, including lung, liver, large intestine, spleen, lymph gland, kidney, heart, small intestine, tonsil, and muscle, were immediately frozen in liquid nitrogen and stored at −80°C. All animal care and treatment procedures were approved by the Animal Ethics Committee of Shandong Agricultural University, China and performed following that committee's guidelines and regulations (Approval No.: 2004006).

HP-PRRSV strain TA-12 (GenBank Accession No. HQ416720) was provided by Professor Yihong Xiao of Shandong Agricultural University. The PAMs were preserved in the laboratory.

### Tissue distribution

Total RNA was extracted with TRIzol reagent (TaKaRa, Dalian, China), and cDNA preparation was performed using a PrimeScript First Strand cDNA Synthesis Kit (TaKaRa, Dalian, China) according to the manufacturer's protocol. The mRNA expression of IFNLR1 was detected and analyzed by quantitative real-time PCR (qRT-PCR). HPRT1 was used as an internal control.

### qRT-PCR

After reverse transcription, qRT-PCR was performed on a Roche LightCycler96 machine using a 20 μL system and SYBR Green. The fold change values for changes in expression were obtained by applying the $2^{-\Delta\Delta CT}$ method. The primer sequences are listed in **Table 1**.

### Antibodies and reagents

The antibody against p-STAT1 (P42224 rabbit phospho-STAT1[S727] antibody) was purchased from Abcepta Biotech (Suzhou, China). Antibody against STAT1 (bs-9584R rabbit anti-STAT1 antibody) was purchased from Bioss Biotech (Beijing, China). Antibody against β-Actin (AF0003 β-Actin mouse monoclonal antibody) was purchased from Beyotime Biotech (Shanghai, China). Antibody against IFNLR1 (abs120780 rabbit anti-IL-28R polyclonal

**Table 1.** The gene-specific primer sequences used for quantitative real-time polymerase chain reaction analysis

| Gene | Primer sequences |
|---|---|
| IFNLR1-F | CCCAGTTTCTCTGCGGACAC |
| IFNLR1-R | ATGCCCCAGTGTCCTACCTC |
| IFN-λ1-F | ACATCCACGTCGAACTTCAGGCT |
| IFN-λ1-R | AGGCGGAAGAGGTTGAACATGAC |
| IFN-λ3-F | ACATCCACGTCGAACTTCAGGCT |
| IFN-λ3-R | GATGTGCAAGTCTCCACTGGT |
| IRF 7-F | TGCAAAGTCTACTGGGAGGTG |
| IRF 7-R | AAGGTGCCGAAGTCGAAGAT |
| ISG 15-F | AGTTCTGGCTGACTTTCGAG |
| ISG 15-R | AGGCGCAGATTCATATACACG |
| IFIT1-F | TGCAACAACACACCCACAGA |
| IFIT1-R | GCATTATTACTCATGGTTGCTGTGA |
| OAS1-F | TCCAGCAACTCAAGAAACCCA |
| OAS1-R | CCAGAGACCCATCCAGATTC |
| PKR-F | CAGCAAATACGTCAGAAAGCAG |
| PKR-R | GGGCGAGGTAAATGTAGGTG |
| MX1-F | CCACCTGAAGAAGGGCTAC |
| MX1-R | AACAGGGGCAGAGTTTTAC |
| PRRSV N-F | AGATCATCGCCCAACAAAAC |
| PRRSV N-R | GACACAATTGCCGCTCACTA |
| HPRT1-F | CATTATGCCGAGGATTTGG |
| HPRT1-R | CTTGAGCACACAGAGGGCTA |

antibody) was purchased from Absin Biotech (Shanghai, China). The RNA interference fragment was obtained from GenePharma (Shanghai, China). JAK inhibitor I (HY-50856) was purchased from MedChemExpress Biotech (Shanghai, China).

## Cell culture

PAMs were cultured in Hyclone1640 culture medium containing 10% fetal bovine serum (FBS), 1% penicillin-streptomycin, and 1% MEM nonessential amino acids solution, under $CO_2$ in a constant-temperature incubator at 37°C (5% $CO_2$ and 95% humidity). The medium was changed every day, and cells subcultured every other day.

## Virus infection

PAMs were infected and incubated with HP-PRRSV-12 at multiplicity of infection (MOI) levels of 0.05, 0.1, 0.5, and 1.0. Incubated cells were harvested at 0, 6, 24, and 48 h post-infection, and qRT-PCR was performed to detect *IFN-λ1*, *IFN-λ3*, and *IFNLR1* mRNA expression.

## Cell transfection and test grouping

Polymerase chain reaction (PCR) was carried out using *IFNLR1* primers (sequences are shown in **Table2**), and the products were cloned into the pcDNA3.1(+) vector and bidirectionally sequenced. The siRNA was designed according to porcine *IFNLR1*. The siRNA sequence of *IFNLR1* had the same composition as the siRNA of the negative control but had no homology. All interference fragments were synthesized by GenePharma (Shanghai GenePharma Co., Shanghai, China), and the sequences are listed in **Table 3**. When PAMs were at 50-70% confluence, 2500 ng pcDNA3.1(+), pcDNA3.1 (+)-IFNLR1 plasmid, SiNC, or 20 mL siRNA were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The results of western blot analysis showed that both the plasmid and interference fragment were successfully transfected. The treated group was infected with HP-PRRSV-12 at MOI = 0.5 for 48 h after transfection.

**Table 2.** The gene-specific primer sequences used for polymerase chain reaction analysis

| Gene | Primer sequences |
|---|---|
| IFNLR1-F0 | GGGAGGTGGGAACGGGT |
| IFNLR1-R0 | TCACCTGTGCAGTGTCATCC |
| IFNLR1-F1 | GAACGGGTCCCACCCTG |
| IFNLR1-R2 | AGGTCATAGGACAGGTGGCT |

**Table 3.** The primer sequences for the assessed siRNAs

| Gene | Primer sequences |
|---|---|
| Si-NC-F | UUCUCCGAACGUGUCACGUTT |
| Si-NC-R | ACGUGACACGUUCGGAGAATT |
| Si-IFNLR1-F | CCAAGCCUACCUGCUUCUUTT |
| Si-IFNRL1-R | AAGAAGCAGGUAGGCUUGGTT |

## Viral titer

Marc-145 cells were seeded into 96-well plates for culture overnight. After plasmid or siRNA transfection, the HP-PRRSV strain was diluted with DMEM 10 times to a series of concentrations, and cells from 10 wells were added to each dilution concentration. When the cells were inoculated with PRRSV for 48h, the number of cells with a cytopathic effect (CPE) was observed under an inverted microscope. Finally, the 50% tissue culture infected dose (TCID50) was determined using the Reed-Muench method.

## Cell proliferation assay

PAMs were seeded in 96-well plates at a density of $1 \times 10^4$ cells/well in 100 μL of growth medium. The test grouping and treatment methods are the same as above. Then, 10 μL of Cell Counting Kit 8 (CCK8) reagent (Beyotime Biotech, Beijing, China) were added to each well, and incubation continued for 3 h at 37°C. Finally, an automatic microplate reader (Molecular Devices, Sunnyvale) was used to determine the cell proliferation index at 450 nm, per the manufacturer's instructions.

## Western blot analysis

Protein extracts from cultured cells were prepared and underwent western blot analysis. Protein concentrations were determined using a BCA Protein Assay kit (Tiangen Biotech, Beijing, China). Total protein samples (30 μg) were separated by SDS-PAGE (12%) and transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, Shanghai, China). The membranes were incubated overnight with the indicated antibodies at 4°C. Then the secondary antibody was added, and the membranes incubated for 1.5 h. The membranes were then subjected to ECL reagents to detect immunoreactivities, and β-actin was used as an internal control.

## Cell cycle analysis

PAMs were cultured in 6-well plates ($1 \times 10^6$ cells/well) with 2 mL of growth medium. The cells were collected by conventional digestion centrifugation and washed with PBS solution before the cell concentration was adjusted to $1 \times 10^6$ $mL^{-1}$. Finally, cells were fixed in 70% ethanol overnight at −20°C before applying the Cell Cycle Analysis Kit (Beyotime Biotech, Beijing, China). Subsequently, the cells were analyzed by performing flow cytometry (BD LSRFortessa Flow Cytometer, BD Biosciences, USA). All data were analyzed by using FlowJo Software v10.0.7.


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### Statistical analysis

The data are expressed as means ± standard deviations (n = 3). Statistical testing was performed using one-way analysis of variance (ANOVA). The means were compared by using Duncan's multiple range test as contained in SAS 9.2 software (SAS Institute Inc., USA). Data were plotted using GraphPad Prism 5 and are presented as mean ± standard deviation values. Statistical significance ($p < 0.05$) of differences is indicated by the presence of a-d letters within a column; different letters within a column denote a significant difference between means.

## RESULTS

### Different IFNLR1 gene expressions in different tissues of Dapulian and Landrace pigs

In this study, qRT-PCR was used to detect the relative expression level of the *IFNLR1* gene in ten tissue types sampled from two-month-old Dapulian and Landrace pigs. The statistical analysis results are shown in **Fig. 1A**, which show that *IFNLR1* was expressed in all tissues of both breeds. Between the two pig breeds, the expressions of the liver, large intestine, small intestine, kidney, and lung tissues were significantly higher in Dapulian pigs than in Landrace pigs ($p < 0.05$). Furthermore, western blot analysis was used to examine the expression levels of IFNLR1 protein in the lung tissues of Dapulian and Landrace pigs (**Fig. 1B**). The results were consistent with the quantitative results, indicating the accuracy of the fluorescence-based quantitative results.

### Suppression of IFNLR1 expression by PRRSV infection

To investigate whether the expression of type III IFNs was affected by PRRSV inoculation, we utilized a PAM cell line shown to be infected by PRRSV. When PAMs were infected with PRRSV, the IFN-λ1 and IFN-λ3 mRNAs were upregulated, peaking at 48 h after inoculation, as shown by the qRT-PCR results (**Fig. 2A and B**). Based on that result, type III IFNs do affect PAMs, indicating that it is reasonable to use PAMs to explore the role of IFNLR1 in PRRSV inoculation. Subsequently, PAMs were collected at different MOI levels at 24 h post-infection. The results indicated that the *IFNLR1* mRNA level was significantly down-regulated by PRRSV infection ($p<0.01$), resulting in an approximately 2-fold decrease (**Fig. 2C**).

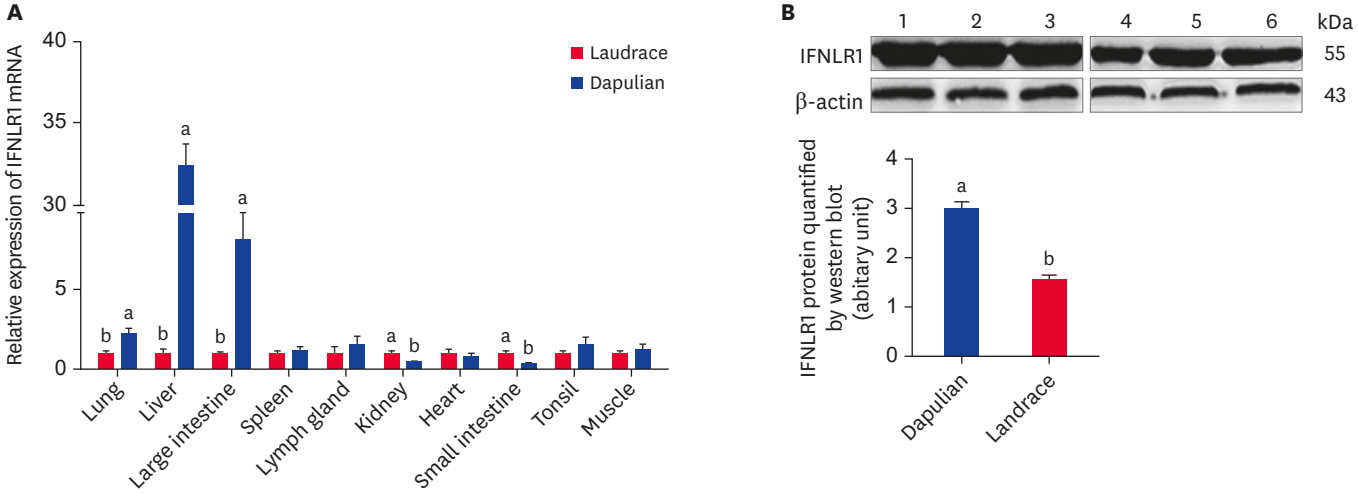


**Fig. 1.** Expression of IFNRL1 in various tissues of Dapulian and Landrace pigs. (A) Relative mRNA expression of IFNLR1 in lung, liver, large intestine, spleen, lymph gland, kidney, heart, small intestine, tonsil, and muscle of Dapulian and Landrace pigs. (B) Protein expression of IFNLR1 in lung tissue of Dapulian and Landrace pigs (1–3, Dapulian pigs; 4–6, Landrace pigs). Data represent mean ± SD values of at least three independent experiments. Significant differences are indicated with different letters, etc., a, b as determined by the Duncan's multiple range test ($p < 0.05$).


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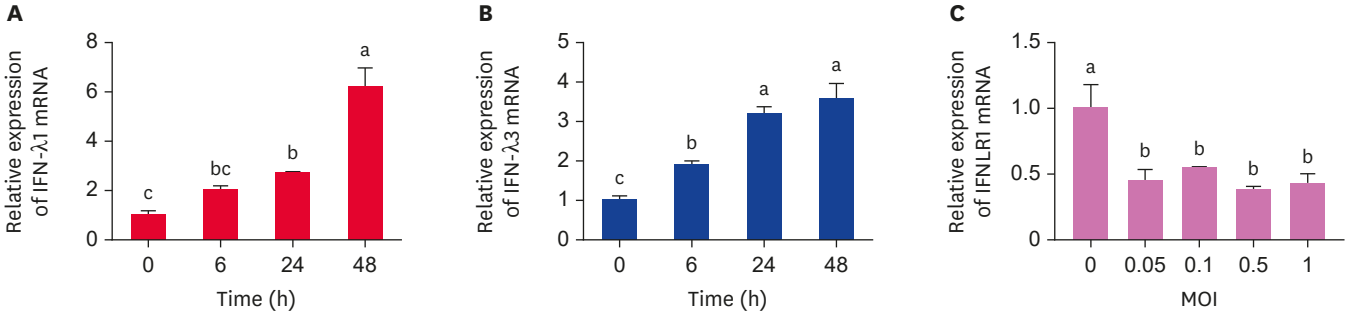


**Fig. 2.** The qRT-PCR assessment shows the effects at different times of PRSSV infection at MOI = 0.5 on the mRNA expression of IFN-λ1 (A) and IFN-λ3 (B). (C) qRT-PCR results show the effects of different PRRSV MOI levels on the mRNA expression of IFNLR1. Data represent mean ± SD values of at least three independent experiments. Significant differences are indicated with different letters, etc., a, b, c as determined by the Duncan's multiple range test ($p < 0.05$).
MOI, multiplicity of infection; qRT-PCR, quantitative real-time polymerase chain reaction.

## Suppression of PRRSV replication by IFNLR1 overexpression

The mRNA expression of *IFNLR1* was changed in PAMs after transfection by pcDNA3.1(+), pcDNA3.1(+)-IFNLR1, si-NC, and si-IFNLR1 plasmids. The qRT-PCR results indicated that the IFNLR1 knockdown efficiency was 62.55% (**Fig. 3A**). Compared with the pcDNA3.1(+) group, the expression of IFNRL1 in the pcDNA3.1(+)-IFNLR1 group was significantly higher ($p < 0.05$; **Fig. 3B**). Western blot analysis was performed to verify the presence of IFNLR1 expression (**Fig. 3C**). These results show that the overexpression plasmid was successfully transfected into PAMs, which was consistent with our expectations, indicating the PAMs could be used for further functional study of IFNLR1. After interference, the mRNA level of the PRRSV N protein was elevated significantly ($p < 0.05$; **Fig. 4A**). After overexpression, the $TCID_{50}$ was measured at 48 h post-infection, and the results showed that the control group virus titer was significantly higher than that of the pcDNA3.1(+)-IFNLR1 group ($p < 0.01$; **Fig. 4D**). In addition, the PRRSV N protein level changes were consistent with the viral titer changes (**Fig. 4E**).

## Activation of JAK/STAT signaling pathway by IFNLR1 overexpression

To investigate the effect of *IFNLR1* gene expression on the JAK/STAT signaling pathway, Western blot analysis was used to detect the STAT1 level in each treatment group. The results demonstrated that the level of STAT1 phosphorylation was higher in IFNLR1- overexpressing PAMs than in the control group PAMs. Conversely, the opposite result was obtained in the IFNLR1-knockdown PAMs (**Fig. 5**).

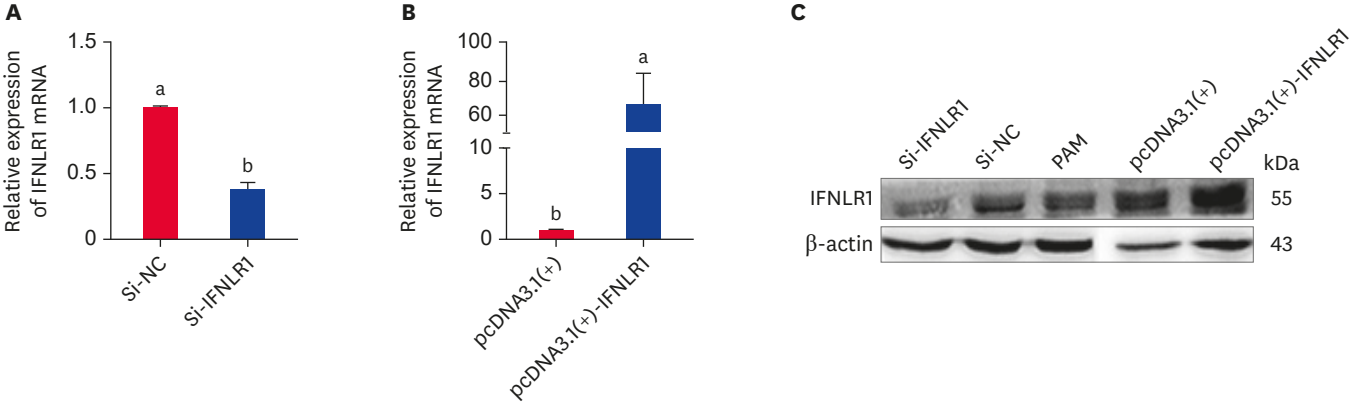


**Fig. 3.** IFNLR1 RNAi and overexpression plasmid were transfected into PAMs, and quantitative real-time polymerase chain reaction and western blot assessments were used to determine the level of expression of IFNLR1 in the cells. Data represent mean ± SD values of at least three independent experiments. Significant differences are indicated with different letters, etc., a, b as determined by the Duncan's multiple range test ($p < 0.05$).


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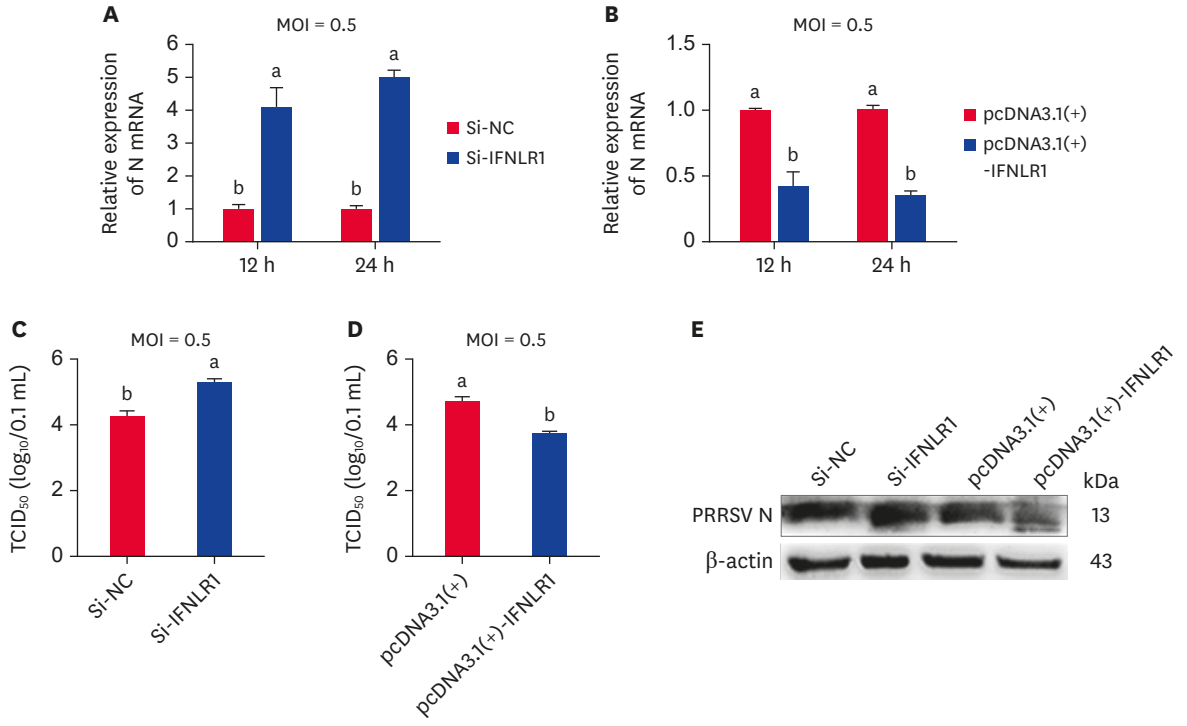


**Fig. 4.** (A, B) After transfection of IFNLR1 and RNAi, the PAMs were infected with PRRSV at MOI = 0.5 at 12 h and 24 h post-infection. The mRNA expression level of the PRRSV N protein was detected with qRT-PCR. (C, D) The virus titer was counted as TCID50 at 24 h post-transfection. (E) Western blot analysis was used to detect the expression of the PRRSV N protein. Data represent mean ± SD values of at least three independent experiments. Significant differences are indicated with different letters, etc., a, b as determined by the Duncan's multiple range test ($p < 0.05$).
MOI, multiplicity of infection.

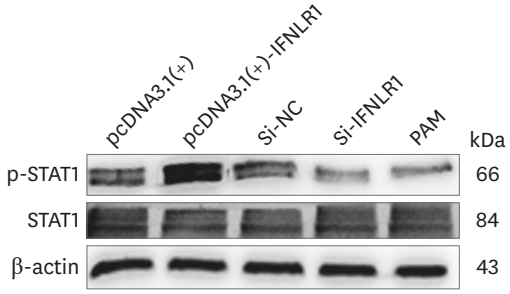


**Fig. 5.** PAMs were transfected with IFNLR1 RNAi and the overexpression plasmid. Total cellular proteins were extracted, and the protein expressions of p-STAT1 and STAT1 were detected by performing western blot analysis.

## The relative expression of IFN-stimulated genes and antiviral genes by IFNLR1

In order to further clarify the changes in the IFN signal pathway, the mRNA expressions of interferon regulator factor 7 (*IRF7*), interferon-induced protein with tetratricopeptide repeats 1 (*IFIT1*), and interferon-stimulated gene 15 (*ISG15*) in each treatment group were assessed. Compared with the control and pcDNA3.1(+)groups, the expression levels of the three IFN-stimulated genes were markedly increased ($p < 0.01$; **Fig. 6A**); to the contrary, IFNLR1-silencing yielded opposite results. To explore the effect of the IFNLR1 on the mRNA expression levels of the antiviral genes 2′-5′-oligoadenylate synthetase 1 (*OAS1*), protein kinase R (*PKR*), and myxoma resistance protein 1 (*MX1*), qRT-PCR analysis was performed. As **Fig. 6B** shows, the mRNA level of *PKR* and *MX1* in the IFNLR1 overexpression group was significantly higher than those in the other three treatment groups and the non-transfected control group


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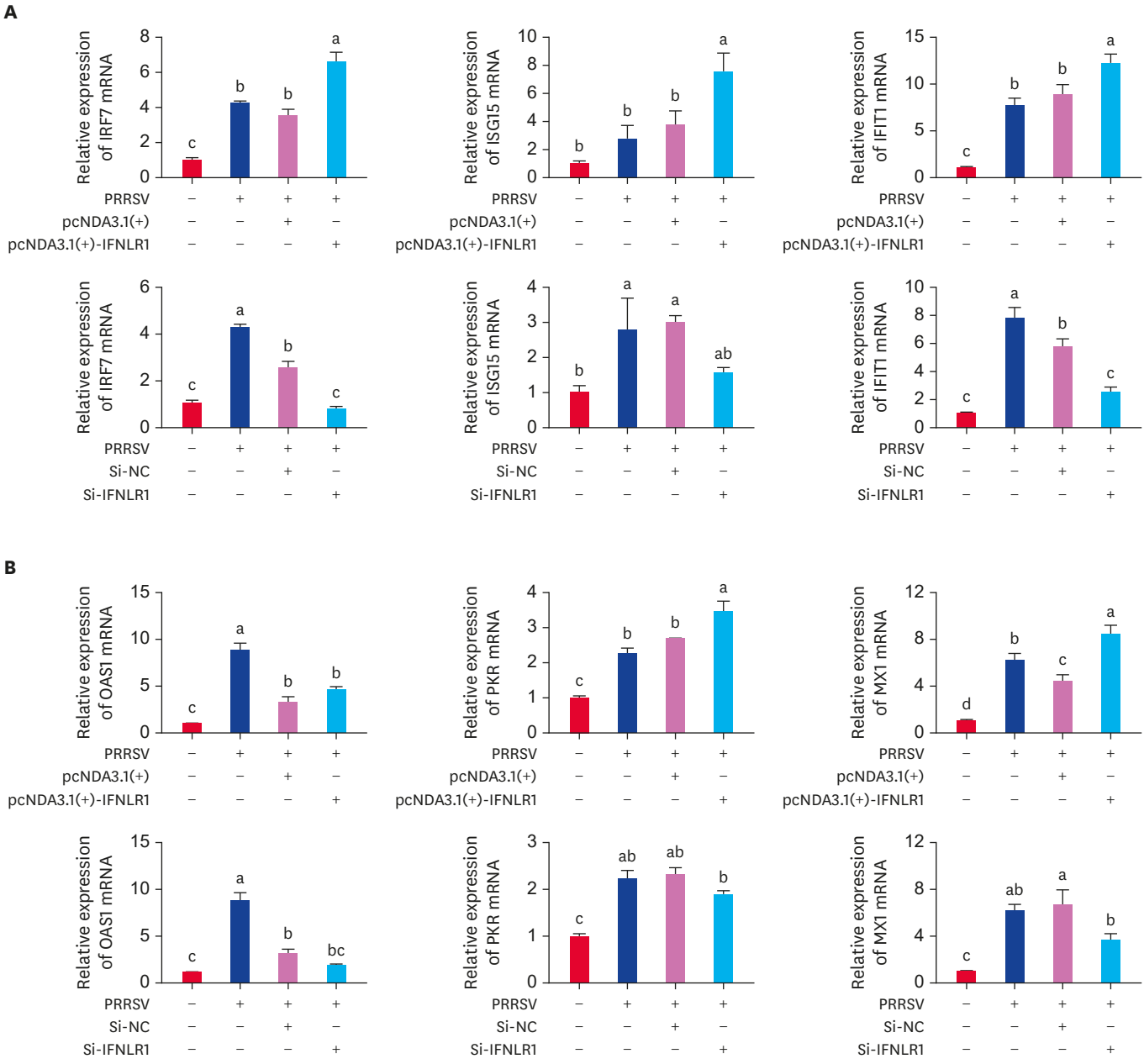


**Fig. 6.** After transfection with IFNLR1 and RNAi, and the mRNA expressions of (A) interferon-related genes (IRF7, ISG15, IFIT1) and (B) antiviral proteins (OAS1, PKR, MX1) were measured by performing quantitative real-time polymerase chain reaction. Data represent mean ± SD values of at least three independent experiments. Significant differences are indicated with different letters, etc., a, b, c as determined by the Duncan's multiple range test ($p < 0.05$).

($p < 0.01$; **Fig. 6B**). In contrast, there was no statistically significant difference in *OAS* gene expression between the pcDNA3.1(+) and pcDNA3.1(+)-IFNLR1 groups ($p > 0.05$).

## Inhibition of PRRSV-infected PAM proliferation by IFNLR1 overexpression

Absorption values were detected by microplate reader at a 450 nm wavelength in each group after incubation for 24 h and 48 h. The results are represented in **Fig. 7**. Compared with the corresponding control group, the proliferation ability of the IFNLR1 overexpression and knockdown groups had not changed significantly at 24 h. However, at 48 h, PAM proliferation had decreased significantly in the IFNRL1 overexpression group compared to that in the


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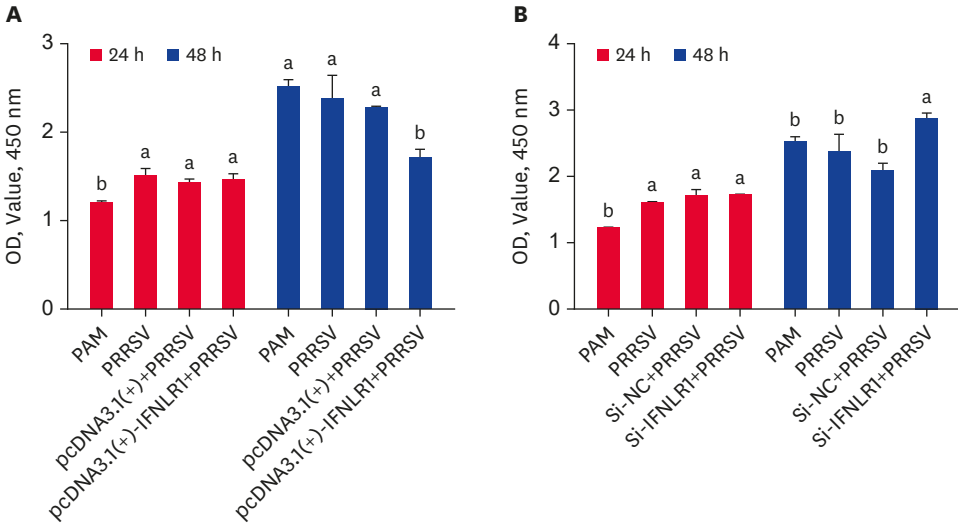


**Fig. 7.** The effects of IFNLR1 on PAMs infected with PRRSV as assessed by CCK8 assay. After transfection of (A) IFNLR1 plasmid and (B) RNAi, the PAMs were infected with PRRSV at MOI = 0.5 at 24 h and 48 h post-infection; cell proliferation was assessed by CCK8 assay. Data are presented as means ± SD of three independent experiments. Data represent mean ± SD values of at least three independent experiments. Significant differences are indicated with different letters, etc., a, b as determined by the Duncan's multiple range test ($p < 0.05$).

pcDNA3.1(+) group ($p < 0.01$; **Fig. 7A**). On the contrary, the proliferation ability of the IFNLR1 knockdown PAMs had significantly increased ($p < 0.01$; **Fig. 7B**).

## Effect of IFNLR1 on cell-cycle transitions in PRRSV-infected PAMs

To investigate the effects of PRRSV on cell proliferation, cell-cycle phase changes were detected by performing flow cytometry. PRRSV infection disrupted the normal PAM cell cycle. At 48 h, the proportion of PAMs in the G0/G1 phase was reduced, while the PAMs at the S and G2/M phases increased significantly. The results demonstrated that the G0/G1 phase (56.67%) and S phase (19.53%) PAMs in the transfected IFNLR1 group were significantly greater than those in the transfected pcDNA3.1(+) group (51.60% and 15.50%, respectively; $p < 0.05$). In the transfected IFNLR1 group, 10.01% of the PAMs were at the G2/M phase compared to 17.70% in the transfected pcDNA3.1 group, a notable difference between the two groups ($p < 0.01$). In contrast, the main cell-cycle phase change in the IFNLR1 knockout group was an increase of PAMs at the G2/M phase ($p < 0.01$; **Fig. 8A and B**).

## Effect of inhibiting JAK/STAT pathway on proliferation and cell cycle of PRRSV-infected PAMs

When JAK inhibitor I (800 nM), an inhibitor of the JAK/STAT pathway, was added to pcDNA3.1(+) PAMs and IFNLR1 overexpression PAMs, the phosphorylation level of STAT1 was obviously decreased (**Fig. 9A**). Furthermore, a CCK8-based assessment was performed to determine the level of PAM proliferation in each group. The results showed that proliferation ability increased after JAKI was added, suggesting that the inhibition of PAM proliferation by IFNLR1 was reduced (**Fig. 9B**). When the JAK/STAT pathway inIFNLR1-overexpressing PAMs was inhibited, the percentage of PAMs at the G2/M phase increased (**Fig. 10**).


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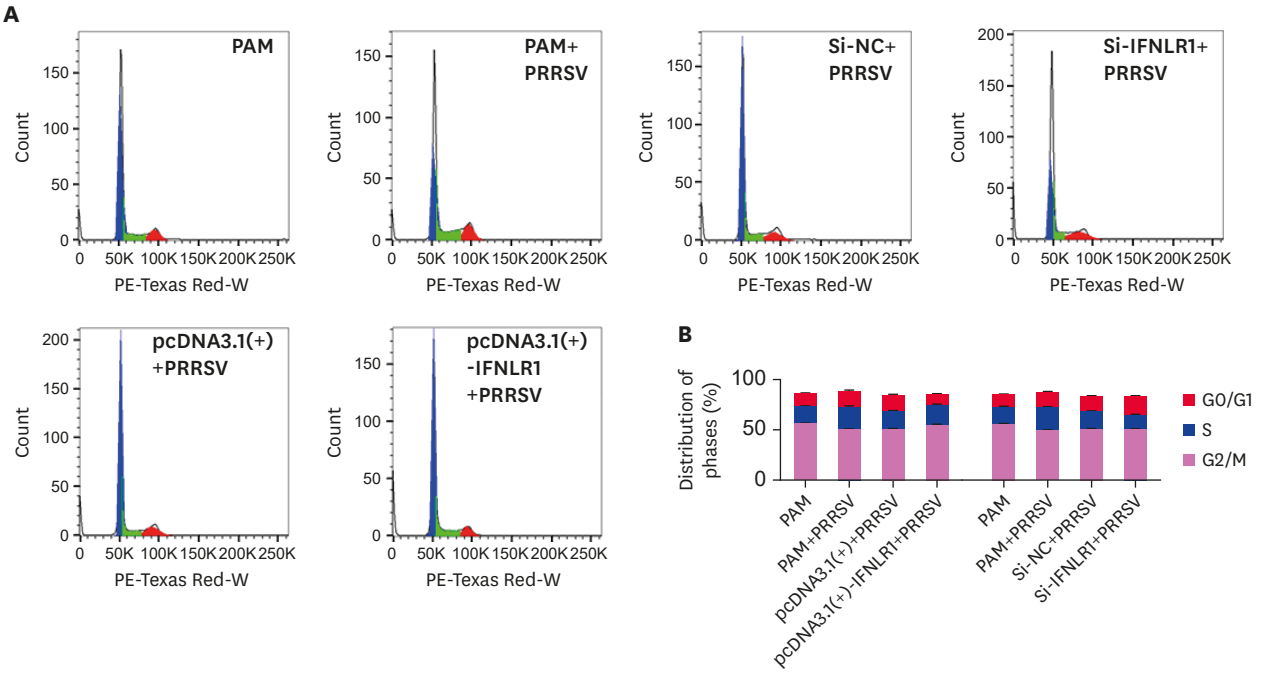


**Fig. 8.** The influence on cell cycle in IFNLR1-overexpressed and -knockdown PAMs. (A) Flow cytometry analysis was performed to analyze the cell-cycle phase distribution of the transfected cell lines. (B) The proportions of cells in different cycle phases.

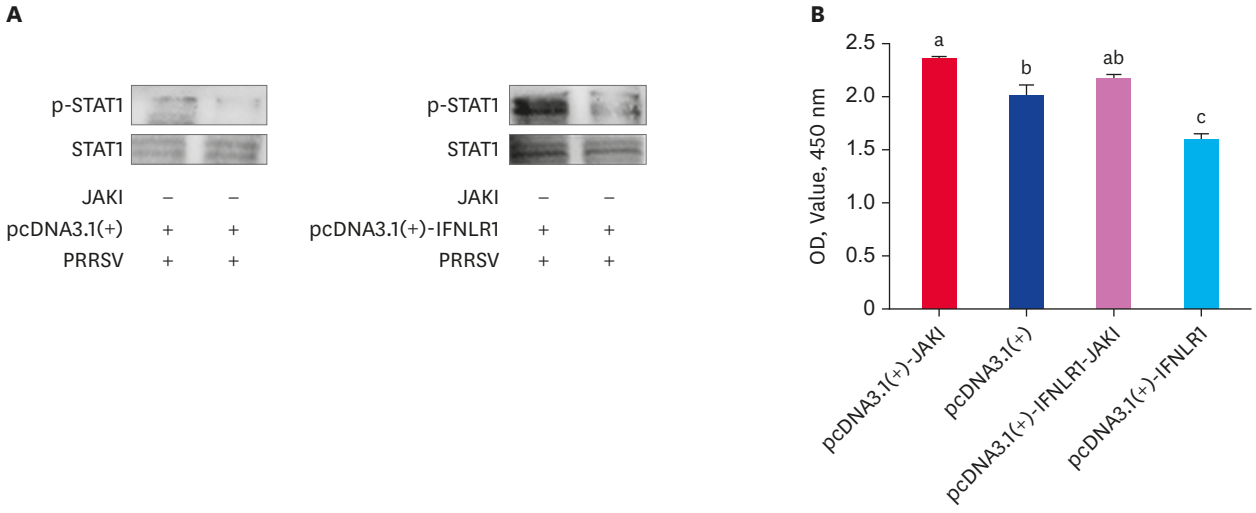


**Fig. 9.** PAMs signal via the JAK/STAT pathway. (A) JAKI was added to PAMs transfected with the pcDNA3.1(+) plasmid or pcDNA3.1(+)-IFNLR1 plasmid for 1 h and then infected with PRRSV for 48 h. The phosphorylation level of STAT1 was detected by performing western blot analysis. (B) The CCK8 assay was used to detect changes in PAM proliferation ability after adding JAKI. Data represent mean ± SD values of at least three independent experiments. Significant differences are indicated with different letters, etc., a, b, c as determined by the Duncan's multiple range test ($p < 0.05$).

## DISCUSSION

A positive selection gene *IFNLR1* was previously identified in Dapulian pigs by performing high-throughput sequencing [7]. Historically, Dapulian pigs have been known for their infection resistance and immunity characteristics. This study explored the role of the *IFNLR1* gene in the process of antiviral infection in pigs. Previous research on the gene functions of *IFNLR1* has been mainly based on the mouse model. So far, it has been reported that *IFNLR1* is involved


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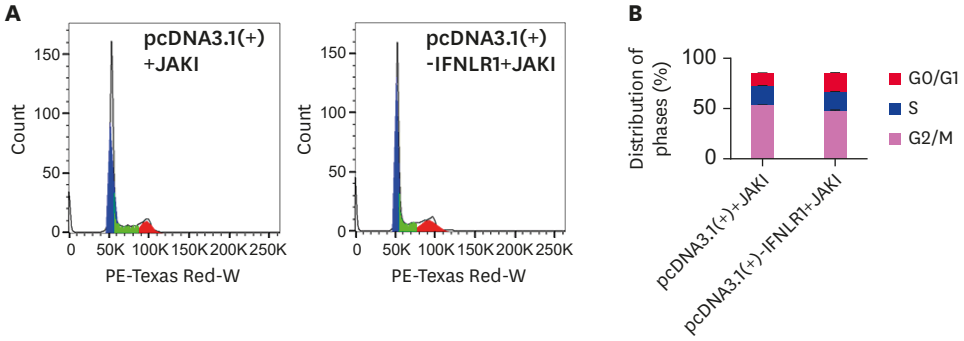


**Fig. 10.** JAKI was added to PAMs transfected with the pcDNA3.1(+) plasmid orpcDNA3.1(+)-IFNLR1 plasmid for 1 h and then infected with PRRSV for 48 h. Cell-cycle phase distribution changed after adding JAKI.

in biological activities, including antivirus, antitumor, and immunomodulation activities. However, little has been reported about the expression pattern and immune reactions of porcine *IFNLR1* in response to PRRSV stimulation *in vitro*. The current research revealed for the first time that the *IFNLR1* gene has a vital role in defending against PRRSV invasion.

The study results confirming the tissue distribution of porcine IFNLR1 were useful in revealing the recognition, induction, and inflammatory reaction of the host to the pathogen. The results showed an abundance of IFNLR1 in lung and lymph gland tissues both Dapulian and Landrace pigs, although the abundance was higher in the Dapulian pigs. Those results indicate that the *IFNLR1* gene has a crucial role in the immune system of both pig breeds. This could be due to the stimulation from viruses or bacteria in the external environment, which subsequently promotes activation of the type III interferon signaling pathway. The lung is one of the most important organs of the animal body, and its primary function is to exchange gas with the outside world. Because lungs are directly connected to the outside world, it is an organ that is very vulnerable to external invasion. Previous studies have shown that when PRRSV invades the body, it first attacks macrophages in alveoli [6,22]. These results indicate that the *IFNLR1* gene has a significant role in the antiviral infection-related processes in porcine lungs.

PRRSV infection promotes the expression of type III interferon but inhibits *IFNLR1* gene expression, further indicating the IFN signal pathway feature of *IFNLR1*. After knockout, the virus titer increases, which may be due to the specific binding of IFNλs that participate in signal transduction, which reduces antiviral and antitumor efficacy. The response of IFN-λs *in vitro* depends on the expression of its receptor, IFNLR1. Prior research displayed that overexpression of IL-28Ra, a response of non-responding cells to IFN-λ, was restored [23]. Our results are similar to those in the following research studies. Taniguchi et al. [24] constructed type III IFN receptor knockout H358 cells by adding interference fragments and compared the Measles virus (MeV) proliferation between normal and knockout cells. In all cases, virus titers of IFNLR1 knockout cells (IFNLR1 KO1 and KO2 clones) were significantly higher than those in control cells.

IFNs do not have a direct antiviral effect; instead, they initiate a series of signal transduction cascades to induce the expression of ISGs to exert their antiviral effects [25]. *ISG15*, *IFIT1*, and *IRF7* are important ISGs that can be rapidly induced and expressed by IFNs. Their expression products have direct antiviral activity and have an immunomodulatory role during viral infection. For example, ISG15 is a ubiquitination-like protein that can modify the ISG target


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protein and inhibit the virus [26]. IFIT1 is generally expressed in IFN-dependent inducible expression in cells and has been reported to affect virus replication. Correct addition of the mRNA cap structure during the infection inhibits virus transcription and protein synthesis [27]. This study showed that *IFNLR1* overexpression could induce upregulation of *ISG15*, *IFIT1,* and *IRF7* in PAMs. This further indicates that the downstream signal pathway activated by the binding of the *IFNLR1* gene and the interferon ligand has an important role in inhibiting PRRSV infection. Several studies have shown that *OAS*1, *PRK*, and *MX1* proteins have natural resistances to viruses and can act directly on viruses [28-30]. Our results show that *IFNLR1* induces significant upregulation of *PKR* and *MX1* mRNA levels, but a change in *OAS* gene expression was not noticeable. We speculate that this may be due to the type III interferon signaling pathway mainly relying on *PRK* and *MX1* antiviral proteins to produce an anti-infective effect after being stimulated by PRRSV. Also, there may be synergy or antagonism among them, but follow-up experiments are needed to investigate that suggestion.

As further elucidation of the therapeutic mechanism of IFNLR1, our results demonstrated that overexpression of IFNLR1 could inhibit the proliferation of PAMs infected with PRRSV, whereas IFNLR1 knockdown promoted PAM proliferation. In addition, overexpression of IFNLR1 significantly increased the proportion of G0/G1 and S phase PAMs and decreased the proportion at the G2/M phase. Notably, the opposite result was observed in IFNLR1 knockdown PAMs. Finally, western blot analysis was used to show that *IFNLR1* inhibited PAM proliferation by activating the JAK/STAT signaling pathway. Based on these results, it is concluded that the high expression of the *IFNLR1* gene in Dapulian pigs may be related to the strength of their disease resistance.

Previous studies have shown that PRRSV replication causes host immune regulation, leading to weakened adaptive immunity and innate immune responses [31,32]. During the PRRSV infection process, the virus mainly infects the lungs and lymphatic organs, and it can replicate and proliferate in those organs. The main cell types infected by PRRSV *in vivo* are monocyte cells, especially PAMs, which are the principal target of PRRSV infection. In support of that observation, the titer of PRRSV is reportedly the highest in lung tissue [33]. It is noteworthy that some Chinese indigenous pig breeds display resistance to PRRSV infection, such as Dapulian [34] and Tongcheng [35] pigs. Hu described the whole blood gene expression profiles of Dapulian and Landrace pigs treated with CpG ODN [36]. Subsequently, transcriptome profiling of PAMs from PRRSV-infected Tongcheng and Large White pigs was performed using RNA-sequencing [37]. We compared their results and noted that Tongcheng and Dapulian pigs share several common immune-relevant differentially expressed genes, such as the *CXCR6* and *CCR5* genes. This similarity may form a theoretical basis for explaining the strong disease resistance of these two breeds. In this study, we observed that the expression of the *IFNLR1* gene in lung tissue of Dapulian pigs was significantly higher than that in Landrace pigs, illustrating that *IFNLR1* could be a key candidate gene for use in determining the pathogenesis and treatment of PRRSV.

Type III IFN, also known as IFN-λ, is a member of the IL-10-related cytokine family. At present, the function of IFN-λs has not been not fully described. Initial studies show that IFN-λs exhibit antiviral, antiproliferative immunoregulatory functions similar to those of IFN types I and II. Various cell cultures and *in vivo* experiments on humans have confirmed that IFN-λs have activity against multiple viruses, including respirovirus and rotavirus [38,39]. In pigs, IFNλs can inhibit the replication of porcine epidemic diarrhea virus (PEDV) in epithelial cells [40]. Similarly, recombinant IFN-λ1 has been shown to enhance the immune response

of the PRRSV DNA vaccine [41]. Part of the IFN-λ function is controlled by a set of cellular genes, which are rapidly induced by the binding of IFN-λs to specific receptors. The IFN-λs receptor complex consists of a unique ligand-binding chain, IFNLR1 (also known as IL28RA), and an accessory chain, IL10R2, that is shared with the IL10 receptor [9]. The low expression of IFNLR1 in Western commercial pigs appears to be an important factor that makes them more susceptible to PRRSV.

The results of this study have demonstrated that IFNLR1 overexpression can suppress PAM proliferation in PRRSV-infected cells. This is consistent with prior studies that reported IL28RA overexpression could markedly inhibit the proliferation of HaCat cells *in vitro* [42]. Flow cytometry showed that IFNLR1 overexpression down-regulated the percentage of PAMs at the G2/M phase, indicating that the cell cycle was arrested in the S phase; a contrasting result was obtained in IFNLR1 knockdown PAMs. These results indicate that IFNLR1 inhibits cell proliferation by affecting the cell-cycle process. We speculate that this is an important way for IFNLR1 to participate in the antiviral function activated by IFN-λs. Several researchers have shown that many viruses and their related proteins can perturb the cell cycle and induce cell-cycle arrest during infection [21,43]. Inhibition at the G2/M phase of the host cell cycle is a well-known strategy used by RNA and DNA viruses [44]. Song demonstrated that the p53/p21 pathway is involved in the G2/M cell-cycle arrest of PRRSV-infected MARC-145 cells [45]. Furthermore, experiments with small molecule inhibitors have shown that cell-cycle arrest at the G2/M phase can benefit the early stages of the human immunodeficiency virus (HIV) life cycle by increasing the number of integrated proviruses [46]. Dove reported that infectious bronchitis virus (IBV) could induce G2/M phase cell-cycle arrest in infected cells, thereby promoting favorable conditions for viral replication [47]. Our results are consistent with these reports. The proportion of PAMs in the G2/M phase increased significantly after PRRSV infection. We further detected cell cycle changes after transfection with the pcDNA3.1(+)-IFNLR1 plasmid. The results indicate that, compared to the control group, the G2/M percentage was markedly decreased in IFNLR1-overexpressing cells. Interestingly, cyclins are a class of proteins that can precisely regulate the cell cycle, and they have an important role in regulating cell proliferation and division. Therefore, the effect of IFNLR1 on cyclin function needs to be studied.

In order to evade the host's innate immune system, PRRSV has evolved a variety of strategies to protect itself while fighting with the host, including interfering with the host's JAK/STAT signaling pathway. Previous study has shown that PRRSV infection can inhibit JAK/STAT signal transduction and ISGs expression induced by type I interferon [48]. Recently, it was reported that PRRSV nsp1β inhibits the activity of JAK/STAT signaling pathway by inhibiting the expression of karyopherin-α1 (KPNA1) and preventing the IFN-stimulated gene factor (ISGF3) complex from entering the nucleus [49]. These results strongly suggest that the JAK/STAT pathway can have an immunomodulatory role in PRRSV infection. Among the signaling pathways associated with IFNLR1, the JAK/STAT pathway is the most classic [50]. After IFN-λs bind to specific receptors, Jak1 and Tyk2 protein kinases are activated through tyrosine phosphorylation, and STAT1 and STAT2 are phosphorylated by the activated JAK protein, promoting the expression of IFN-stimulated genes (ISGs). Researchers have previously reported that activation of the IFNLR1 signaling pathway can increase the phosphorylation level of STAT protein in different cell types [51,52]. Moreover, the anti-growth effect of IFN-λs is inseparable from the expression of JAK1 and STAT1. In our study, after transfection of the pcDNA3.1(+)-IFNLR1 plasmid, the phosphorylation level of STAT1 was significantly increased, and immediately after the JAK inhibitor was added, the *IFNLR1* gene inhibition

of cell proliferation weakened. These results indicate that the inhibitory effect of IFNLR1 on PAM proliferation was partly dependent on the activation of STAT1 phosphorylation. IFNLR1 and its signaling pathway induce the expression of antiviral proteins (such as MX1 and PKR) and promote the release of proinflammatory cytokines and chemokines (such as CXCL10 and CXCL11) for participation in antiviral immune responses. However, an excessive inflammatory response due to a large number of inflammatory factors is usually fatal. Therefore, the molecular mechanisms of IFNLR1 and the associated signaling pathway involved in regulating the expression of inflammatory factors need to be further explored.

In summary, overexpression of IFNLR1 inhibits the proliferation of PRRSV-infected PAMs by changing cell-cycle progression. We speculate that *IFNLR1* is an important target gene involved in the pathogenesis of PRRSV and may have potential in its treatment.

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