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Molecular Immunology

Tumor suppressor p53 inhibits porcine epidemic diarrhea virus infection via interferon-mediated antiviral immunity

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ABSTRACT

p53 is a tumor suppressor gene that can be activated in many contexts, such as DNA damage or stressful conditions. p53 has also been shown to be important for responses to certain viral infections. Porcine epidemic diarrhea virus (PEDV) is a major enteric pathogen of the coronavirus family that causes extensive mortality among piglets. The involvement of p53 during PEDV infection has not previously been investigated. In this study, we detected p53 upregulation in response to PEDV infection. Treatment with a p53 specific activator or p53 overexpression markedly decreased viral replication, and we showed that there was more viral progeny produced in p53 knock-out cells than in p53 wild-type cells. Finally, we demonstrated that inhibition of viral infection by p53 was mediated via p53-dependent IFN signaling, leading to IFN-stimulated response element (ISRE) activation, as well as the upregulation of IFN-stimulated genes (ISGs) and IFN-β released from infected cells. These findings demonstrate that p53 suppresses PEDV infection, offering a novel therapeutic strategy for combatting this deadly disease in piglets.

1. Introduction

Porcine epidemic diarrhea (PED) is a serious and extremely contagious enteric infection that causes extensive death in sucking piglets, thereby having a major adverse economic impact on the global swine industry (Pensaert and Martelli, 2016). PED was first identified in the 1970s in England, but it has become more prominent and lethal in Asia over the past decade, devastating local economies (Wood, 1977; Zhou et al., 2012). This disease is caused by the coronavirus porcine epidemic diarrhea virus (PEDV) (Lee, 2015). This virus is a single-stranded, positive-sense RNA virus with an envelope and a 28,000 bp genome encoding a total of 7 known open reading frames (ORFs): replicase polyproteins 1a and 1b, ORF3, spike (S), envelope (E), membrane (M), and nucleocapsid (N) (Duarte et al., 1993). Accumulated evidence indicates that PEDV could manipulate the immune responses of its hosts to benefit its own replication. Sun et al. reported that the CH/SXYL/2016 strain of PEDV could cause cell cycle arrest in Vero cells at the G0/G1 phase, and synchronization of the cells at the G0/G1 phase could increase the proportion of infected cells (Sun et al., 2018); the same results were also observed in transmissible gastroenteritis virus (TGEV)-infected PK-15 cells (Ding et al., 2013). In addition, an

increasing number of studies have demonstrated that viruses encode defensive mechanisms to evade the antiviral activities of host cells (Xing et al., 2013; Yang et al., 2018). However, hosts have many mechanisms aimed at detecting and disrupting PEDV replication and spread (Cao et al., 2015; Kong et al., 2016). An improved understanding of how this pathogen interacts with the host immune system is required to enhance efforts to control PEDV spread and to develop efficacious PEDV vaccines.

Many aspects of the viral life cycle can be mediated through a variety of cellular defense mechanisms (Katze et al., 2002). p53, the best studied tumor suppressor gene (Oren, 2003), has been shown to play different roles in virus replication (Dharel et al., 2008; Muñoz-Fontela et al., 2011); upon viral infection, cells could activate the p53 signaling pathway to cause different cell fate (Ding et al., 2014; Huang et al., 2013; Purvis et al., 2012). p53 is also activated in other stressful contexts, such as DNA damage, hypoxia, altered metabolism, and oncogene activation (Vousden and Lu, 2002; Zhang et al., 2011). Induction of p53 can trigger responses such as DNA repair, cell cycle arrest, or even cell death (Sherr, 2004; Vogelstein et al., 2000). p53 is hence termed the "guardian of the genome". p53 has now also been described as a central regulator of the innate immune response (Rivas et al.,

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2010). It was also reported that p53 can activate ISGs, thereby giving it the ability to amplify the intracellular interferon (IFN) response (Muñoz-Fontela et al., 2011; Takaoka et al., 2003). However, exactly what if any role is played by p53 during PEDV infection is unknown. In this study, we reveal that there is a direct role for p53 in mediating host innate immune responses to PEDV infection.

In our previous study, we have shown that human embryonic kidney 293 (HEK293) cells are susceptible to infection with the CV777 PEDV vaccine strain with similar growth kinetics to those seen in Vero cells (Zhang et al., 2017). Vero cells are the most common cell line used to study PEDV infection (Jarvis et al., 2016). Vero cells, however, do not produce type I IFN when infected with viruses due to loss of the type I IFN gene cluster (Desmyter et al., 1968; Emeny and Morgan, 1979). As such, Vero cells are a poor model of innate antiviral immunity, leading us to use HEK293 cells to characterize the molecular mechanisms underlying responses to PEDV.

In this study, we demonstrated the increased expression and activation of p53 upon PEDV infection in HEK293 cells. Using a p53-specific activator or overexpression of p53 could effectively inhibit PEDV replication. Additionally, decreasing p53 activity using CRISPR/Cas9 could increase viral replication and dissemination by inhibiting the interferon pathway in HEK293 cells, suggesting that higher viral titers may be caused by a reduction in the subsequent interferon response.

2. Materials and methods

2.1. Cells and viral culture

HEK293 (human embryonic kidney 293; ATCC) and Vero E6 (African green monkey kidney epithelial cells; ATCC) were cultured in DMEM (Dulbecco's modified Eagle's medium (Gibco, Waltham, USA)) with 10% heat-inactivated fetal calf serum (FCS) (Gibco, Waltham, USA), 2 mM Ultra Glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. All cells were grown at 37 °C in a humidified 5% CO2 incubator. PEDV strain CV777 (GeneBank accession number KT323979), which was maintained at the Harbin Veterinary Research Institute of the Chinese Academy of Agricultural Sciences, was amplified and titrated in Vero E6 cells.

2.2. Plasmids, antibodies, and reagents

The human p53 gene containing a His-tag was amplified from the genome of HEK293 cells and then cloned into the pCDNA3.1(+) vector (Invitrogen, Waltham, USA) with the BamHI and XhoI restriction enzymes (Thermo Scientific, Waltham, USA). The interferon stimulated response element (ISRE) / luciferase (pISRE-Luc) reporter plasmid was kept in our laboratory. The internal control plasmid encoding Renilla luciferase (phRL-TK) and the Dual-Luciferase Reporter Assay System were purchased from Promega Corporation (Madison, USA). The p53 activator Nutlin-3 was purchased from Selleck (Houston, USA). Antibodies against p53 (1C12) and the His-tag (27E8) were purchased from Cell Signaling Technology (Danvers, USA). Antibody against β-actin (AC-74) was purchased from Sigma–Aldrich Corporation (St. Louis, USA). Mouse anti-PEDV spike protein (PEDV-S) monoclonal antibody (3F12) was purchased from Median Diagnostics Inc (Chuncheon, South Korea). Mouse polyclonal antibody against PEDV nucleocapsid protein (PEDV-N) was prepared by our team in our previous study (Shi et al., 2014). The human IFN-beta ELISA Kit was purchased from R&D (Minneapolis, USA).

2.3. CRISPR/cas9 KO cell

p53 gene targeting sites were designed using appropriate guide selection software (http://www.genome-engineering.org/crispr/). Target sequence oligonucleotide pairs were annealed and then cloned into the single guide RNA (sgRNA) expression plasmid pMD18-T-sgRNA (modified by our team) and cotransfected into HEK293 cells with the Cas9 fused with GFP expression plasmid pMJ920 (Addgene: 42234). Forty-eight hours after transfection, the cells were trypsinized and resuspended to form a single cell suspension in 500 μl of PBS. Then, ten cells per well of GFP-positive cells were divided into 96-well plates by FACS (optical path/laser used = 488 nm) (Beckman Coulter, Brea, USA). After 14 days, GFP-positive cells were analyzed by Western blot using the p53 antibody. After three rounds of subcloning, stable p53-deficient cell lines were obtained.

2.4. Immunofluorescence assay (IFA)

The HEK293 monolayers were grown to 100% confluency in 24-well plates and incubated with dimethyl sulfoxide (DMSO) or with different concentrations of the p53 activator Nutlin-3 (10, 2.5 μM). After 24 h, HEK293 cells were infected with PEDV at a multiplicity of infection (MOI) of 1. At 24 hpi, cells were fixed with 33.3% acetone and stained with PEDV-S antibody (1:200 dilution), followed by a FITC-labeled secondary goat anti-mouse IgG (1:400 dilution) (Jackson Immunoresearch, West Grove, USA). An Olympus IX53 inverted fluorescence microscope was used for visualization.

2.5. Western blot assay

Protein samples were run on 12% polyacrylamide gels prior to polyvinylidene fluoride (PVDF) membrane transfer. Membranes were blocked and incubated overnight in a solution of primary antibody at 4 °C, including p53 antibody (1:1000 dilution), PEDV-N antibody (1:500 dilution) or β-actin antibody (1:5000 dilution). A secondary IRDye-conjugated antibody (1:4000 dilution) (LI-COR Biosciences, Lincoln, USA) was then added, and the Odyssey infrared imaging system was used for visualization. Odyssey 2.1 software was used for quantification of results.

2.6. TCID$_{50}$ assay

The method of Reed and Muench was used to perform the TCID50 assay in Vero E6 cells as previously described (Chua et al., 2008). Briefly, cell monolayers were inoculated with each group of virus samples and incubated for 5 days prior to observation of the presence of a cytopathic effect (CPE).

2.7. Real-time quantitative PCR

Total RNA from each group was extracted from cell lysates with the RNeasy Mini Kit (Qiagen Science, Hilden, Germany) based on provided protocols, and a DNase treatment of the isolated RNA was performed before RNA reverse transcription. A PrimeScript™ II 1st Strand cDNA Synthesis Kit (TaKaRa, Dalian, China) was used for RNA reverse transcription, prior to quantitative RT-PCR in a LightCycler 480 II (Roche, Shanghai, China) using the SYBR Advantage qPCR Premix (TaKaRa, Dalian, China). All the results from each group were normalized to those of the control housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH Real-time quantitative PCR), whose expression does not change with treatment. The LightCycler 480 software 1.5 was used for result analysis via the cycle threshold ($\Delta\Delta C_T$) approach (Livak and Schmittgen, 2001). Oligo 6 software was used to create primers, which are shown in Table 1.

2.8. Dual-luciferase reporter assay

p53 wild-type or p53 knock-out cells were transfected with 0.2 μg of pISRE-Luc together with 0.05 μg of phRL-TK luciferase plasmid and infected with PEDV at an MOI of 1 or mock-infected. PhRL-TK Renilla served as an internal control. After 24 h, lysates were collected, and this

Table 1
Real-time PCR primers used for quantification of host cell gene expression.

Gene name	Forward (5`-3`)	Reverse (5`-3`)
RIG-I	CTGGACCCTACCTACATCCTG	GGCATCCAAAAAGCCACGG
IRF-9	CCCGAAAACTCCGGAACTG	CAGCACACTCCGGGAAA
ISG56	TCATCAGGTCAAGGATAGTCTG	GGTGTTTCACATAGGCTAGTAG
PKR	AGAGTAACCGTTGGTGACATAACCT	GCAGCCTCTGCAGCTCTATGTT
OAS-1	AGAAGGCAGCTCACGAAACC	CCACCACCCAAGTTTCCTGTA
MxA	GCCGGCTGTGGATATGCTA	TTTATCGAAACATCTGTGAAAGCAA
IFN-β	CACGACAGCTCTTTCCATGA	AGCCAGTGCTCGATGAATCT
GAPDH	CGGGAAGCTTGTGATCAATGG	GGCAGTGATGGCATGGACTG

Real-time PCR primers used for quantification of host cell gene expression.

reporter assay was conducted based on appropriate protocols, with luciferase activities quantified via luminometer (PE Envision, Shanghai, China).

2.9. Measurement of IFN-β by ELISA

To analyze IFN-β release from p53 wild-type or p53 knock-out HEK293 cells, two groups of these cells were PEDV infected at an MOI of 1 or mock-infected for 24 h or 48 h. Then, secreted IFN-β levels were assessed in the supernatant using the human IFN-β ELISA Kit (R&D, Minneapolis, USA) based on provided protocols.

2.10. Cell viability assay

A total of 1×10^4 cells per well of p53 wild-type or p53 knock-out HEK293 cells were seeded into 96-well plates. After 24 h or 48 h, cell viability was measured with the CCK-8 system (Toyobo, Shanghai, China). Briefly, at each time point, CCK-8 solution (10 μl/100 μl medium) was added, the plates were incubated at 37 °C for 4 h, and the absorbance of each well was read at 450 nm by a microplate spectrophotometer (Bio-Rad, Hercules, USA).

2.11. Statistical analysis

Student's *t*-tests were used after checking the normal distribution of the data, and all statistical data were expressed as the means and standard deviations (SD) of three independent experiments. P < 0.05 was the threshold for statistical significance.

3. Results

3.1. PEDV infection activates p53 in HEK293 cells

p53 is known to be important both for preserving genomic integrity and for perpetuating antiviral responses. The expression of p53 was first investigated to examine the effects of PEDV infection. HEK293 cells were PEDV infected (strain CV777) or mock infected, and p53 transcription was then assessed via quantitative reverse transcription-PCR (RT-qPCR). As expected, p53 transcription was increased in HEK293 cells infected with PEDV (MOI = 1) after 12, 24, and 48 hpi (Fig. 1A). Moreover, PEDV infection could also upregulate p53 expression at the protein level in a time-dependent fashion (Fig. 1B and C); however, p53 expression was lower at 48 hpi than at 12 or 24 hpi. These results demonstrated that PEDV infection could stimulate p53 transactivation.

3.2. Loss of p53 promotes viral replication

To examine the antiviral effects of p53 on PEDV infection, CRISPR/Cas9 was used to mutate the p53 gene and assess its role in PEDV infection. To avoid off-target mutagenesis, a sgRNA was designed with mutation target sites of low homology to other regions of the genome. SgRNA158 and sgRNA334 were targeted to the N-terminal domain of
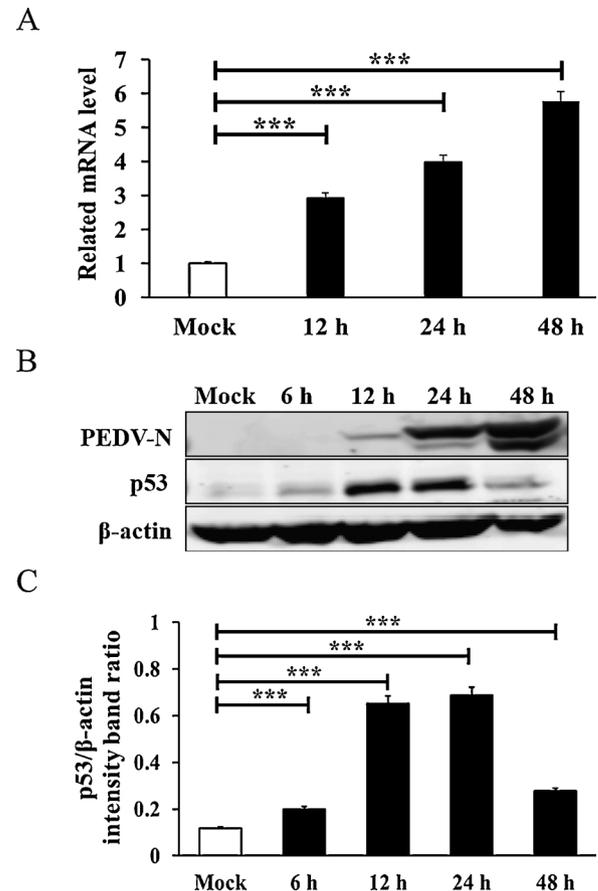
Fig. 1. p53 activation by PEDV. (A) HEK293 cells were mock or PEDV infected (MOI = 1) for 12, 24 or 48 h, and p53 mRNA levels were detected by qRT-PCR. (B) HEK293 cells were mock or PEDV infected (MOI = 1) prior to total protein Western blot analysis. (C) The intensity band ratio of p53 to β-actin was analyzed by using ImageJ software. Data are the mean ± SD of three independent experiments. P-values were estimated with Student's t-tests (***p < 0.05).

p53 (Fig. 2A). GFP-positive cells were selected by FACS sorting and were propagated prior to Western blot analysis. The sgRNA334 clone showed complete p53 deletion, but the clone generated by sgRNA158 did not decrease p53 expression (Fig. 2B). Thus, the clone generated by sgRNA334 was used to investigate the influence of p53 on PEDV replication. The cell growth and viability of p53 knock-out cells were indistinguishable from those of the parent HEK293 cells (Fig. S1). Next, the p53 knock-out HEK293 cells (p53-/-) and wild-type HEK293 cells (WT) were infected with PEDV for different times, and the data showed that the susceptibility of p53 knock-out HEK293 cells to PEDV infection was significantly higher than that of p53 wild-type cells as determined by detection of PEDV-N protein expression (Fig. 2C). This enhanced viral proliferation was also confirmed via assessing the viral 50% tissue

A

### Schematic Target p53 Genomic Structure

D

B

C
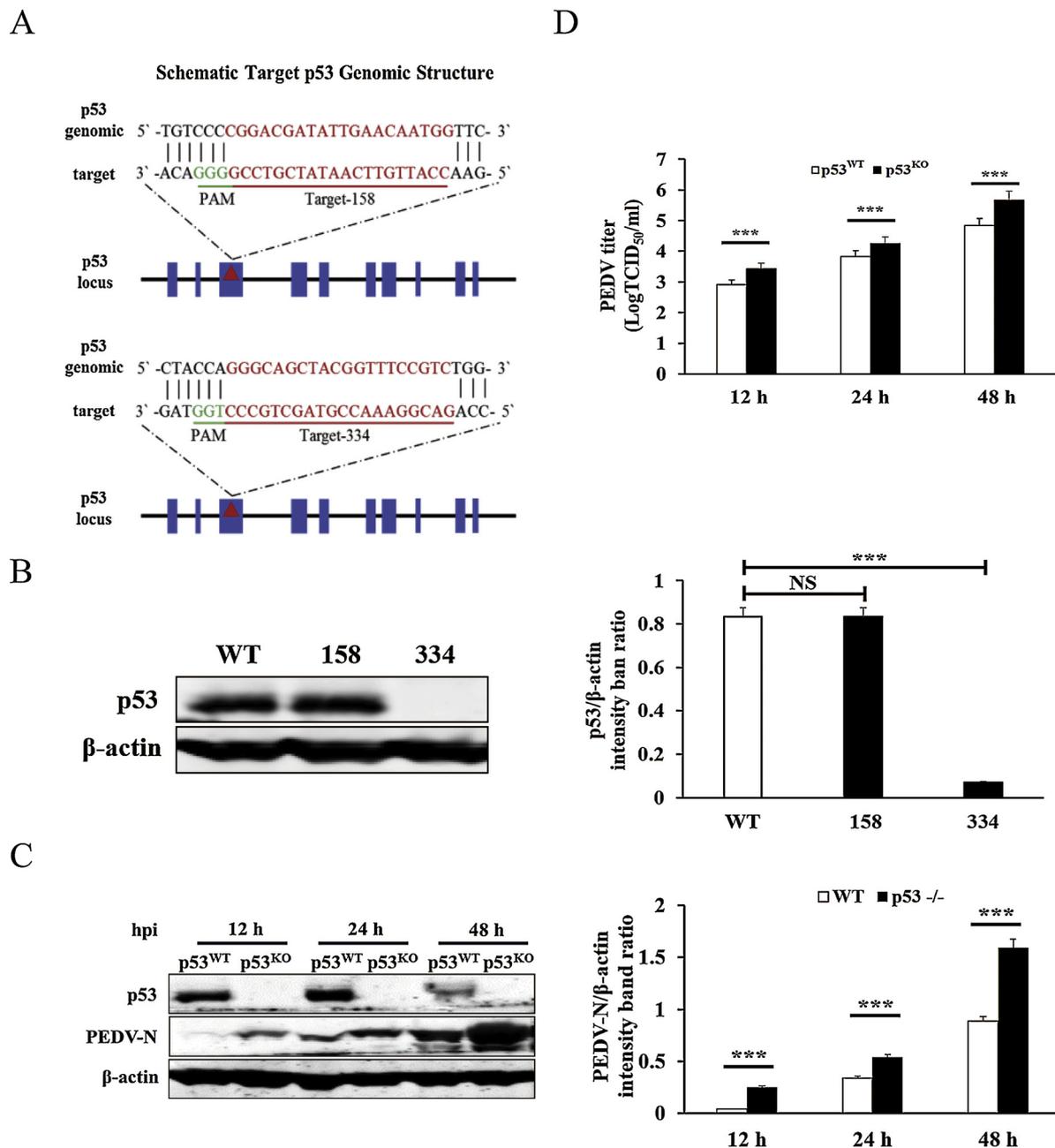
Fig. 2. Loss of p53 expression enhances virus replication. (A) Schematic illustration of the p53 targeting sgRNA genomic region, with underlined PAM sequences in green and guide target sites in red. (B) The cells were cotransfected with pMJ920 and pMD18-T-sgRNA158 (158) or pMD18-T-sgRNA 334 (334). The cells were selected by FACS, and the GFP-positive HEK293 cells (WT) were analyzed using Western blots. In (C) and (D), p53 wild-type (WT) or p53 knock-out (p53-/-) HEK293 cells were infected with PEDV (MOI = 1 at 12, 24 or 48 h), p53 and PEDV-N levels were assessed (C), and viral titers were measured in supernatants (D). Data are the means ± SD of three independent experiments. P-values were estimated with Student's t-tests (***p < 0.05) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

culture infective dose (TCID50) (Fig. 2D). These results together show that knockout of p53 increases PEDV infection susceptibility, suggesting that p53 plays an important role against PEDV infection.

3.3. Activation of p53 decreases PEDV infection

To explore the importance of p53 during PEDV infection, the p53 specific activator Nutlin-3 was used to determine how p53 affects PEDV replication. After treatment with increasing doses of Nutlin-3 for 24 h, HEK293 cells were infected with PEDV (MOI = 1), and the infection was confirmed by IFA detection of PEDV spike (S) protein. The results showed that Nutlin-3 pretreatment potently inhibited PEDV replication

compared with the DMSO treatment. Additionally, 10 μM Nutlin-3 resulted in more than 60% inhibition of PEDV infection (Fig. 3A). Levels of PEDV-N proteins were additionally assessed by Western blot, further confirming the inhibition of PEDV replication by Nutlin-3 (Fig. 3B). Next, the effect of Nutlin-3 on PEDV replication was examined via assessment of the TCID50. This examination confirmed lower viral yields in the Nutlin-3 treated HEK293 cells relative to the control cells (Fig. 3C). Finally, we tested whether overexpression of p53 in HEK293 cells would reduce how readily these cells could be infected by PEDV, transfecting cells with various doses of His-tag p53 and then infecting them with mock or PEDV. The Western blot results demonstrated that PEDV infectivity in p53 overexpressing HEK293 cells was significantly

A

B

C

D
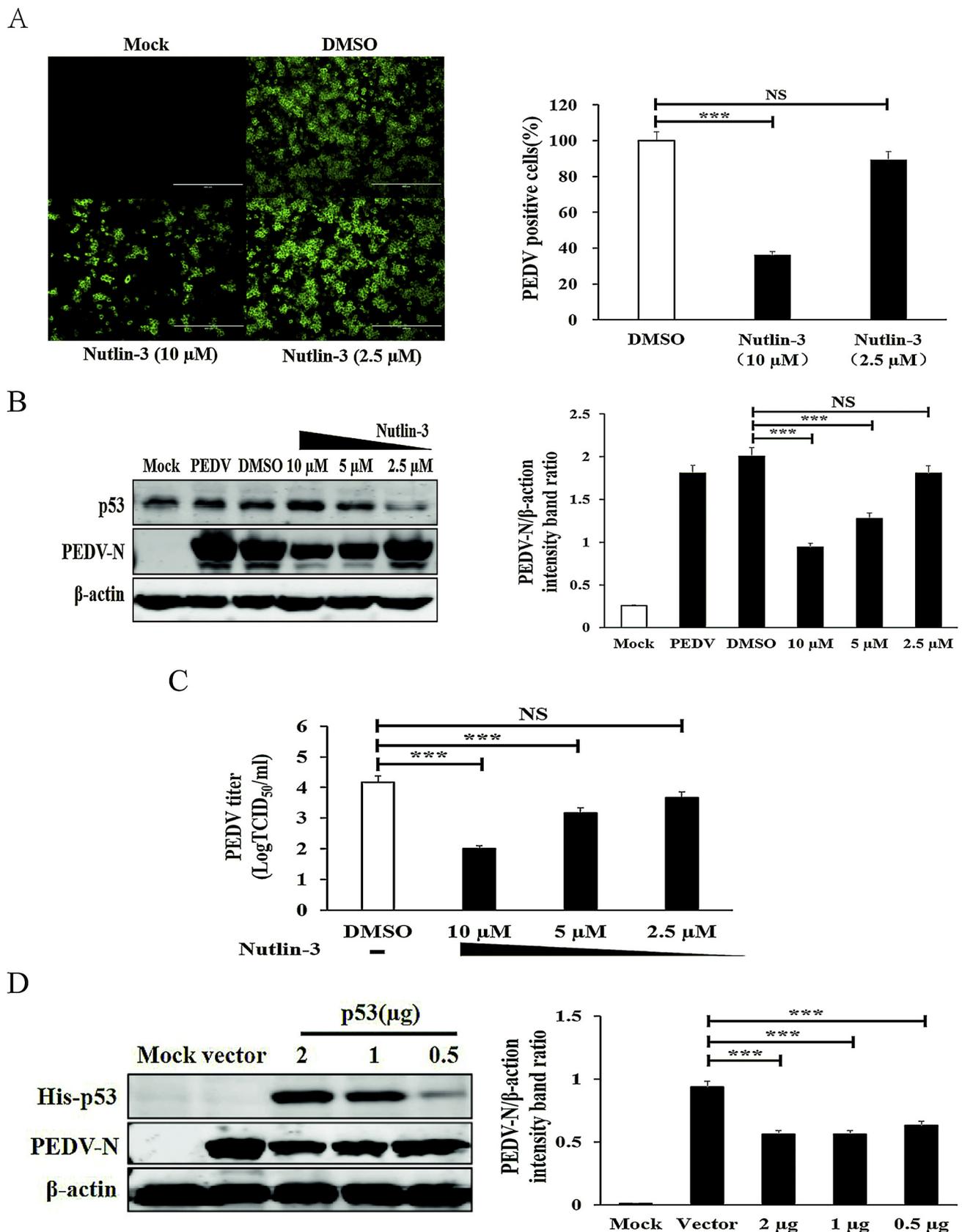
Fig. 3. p53 inhibits PEDV growth. (A) HEK293 cells were treated with Nutlin-3 or DMSO for 24 h before PEDV infection (MOI = 1) in the presence of drug for 24 h. Immunofluorescent anti-PEDV spike (S) staining was used to assess infected cell frequency. The number of PEDV-positive cells was calculated by using ImageJ software. In (B) and (C), HEK293 cells were treated with Nutlin-3 or DMSO for 24 h and infected with PEDV at an MOI of 1 in the presence of drug for 48 h. p53 and PEDV-N levels were assessed via Western blotting (B), and viral titers were measured in supernatants (C). (D) HEK293 cells transfected with pHis-p53 or empty vector were infected with PEDV (MOI = 1). Twenty-four hours after infection, His-p53 and PEDV-N expression were examined by Western blot. Data are the means ± SD of three independent experiments. P-values were estimated with Student's t-tests (***p < 0.05).

A

B

C
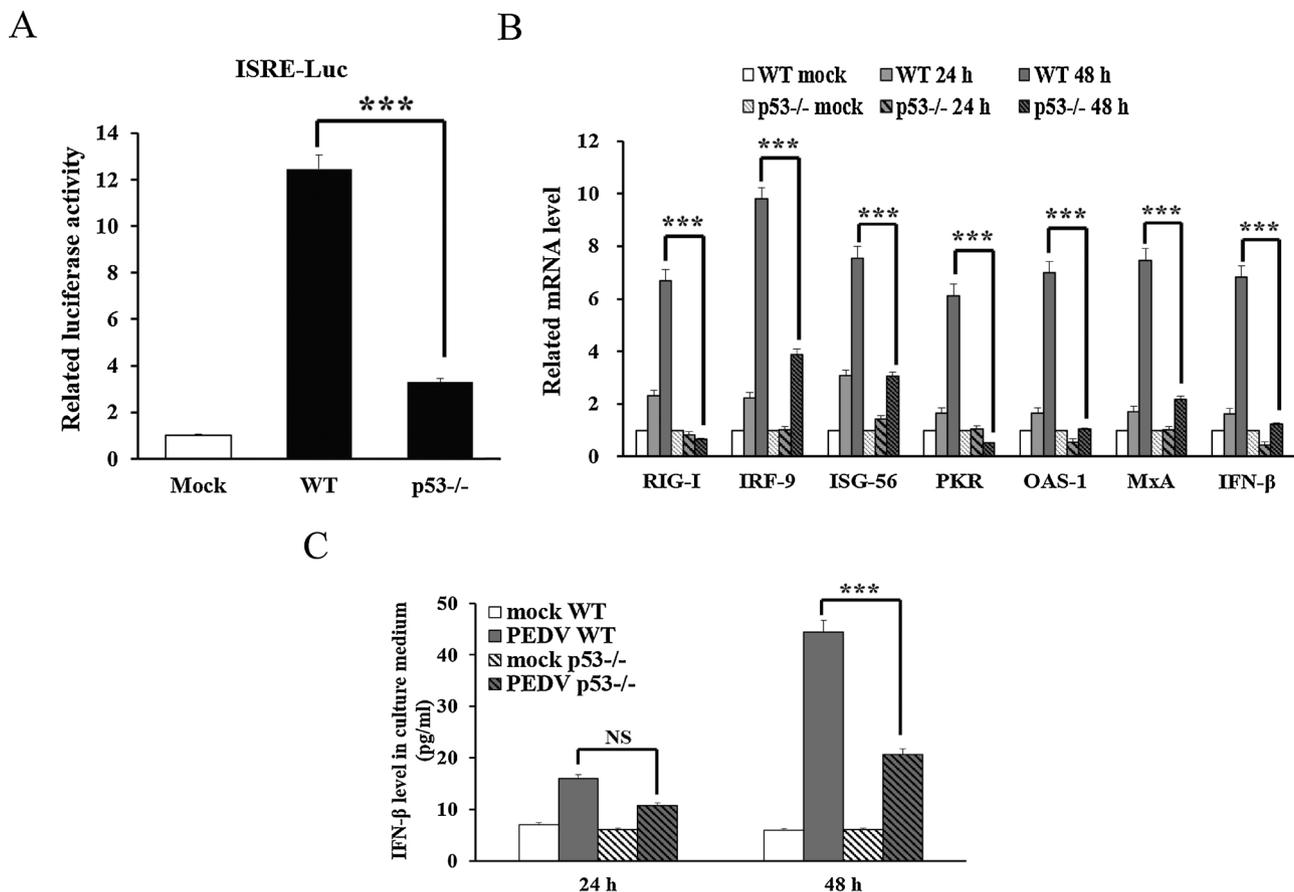
Fig. 4. p53 enhances IFN-mediated antiviral immunity. (A) p53 wild-type (WT) or p53 knock-out (p53-/-) cells were cotransfected with the pISRE-Luc and phRL-TK plasmids and infected with PEDV (MOI = 1) or mock-infected. Cells were harvested at 24 h, and the lysates were analyzed via the dual-luciferase assay. Renilla luciferase activity served as a normalizing control. Fold change relative to mock-infected cells is shown. In (B) and (C), p53 wild-type (WT) or p53 knock-out (p53-/-) cells were infected with PEDV (MOI = 1) or mock-infected at 24 h or 48 h. Total cellular RNA was used for qRT-PCR, with GAPDH as a normalization control (B), and supernatant IFN-β levels were measured (C). Data are the means ± SD of three independent experiments. P-values were estimated with Student's t-tests (***p < 0.05) relative to mock-infection controls.

reduced relative to that in parental controls (Fig. 3D). This demonstrated that p53 expression reduces target susceptibility to PEDV infection, suggesting that p53 acts as a major antiviral mediator during PEDV infection.

3.4. p53 enhances IFN-mediated antiviral protection

p53 is important for the apoptotic response to viral infection – a pathway that is vital to the immune control of many viral pathogens. However, p53 is also vital for the type I IFN response upon viral infection. To assess whether p53 plays an IFN signaling-dependent role in the control of PEDV infection, we assessed the ability of p53 to induce interferon-stimulated response element (ISRE) transcription. The pISRE-Luc vector was used to transfect wild-type and p53 knock-out HEK293 cells with an ISRE-luciferase reporter construct, and cells were infected with PEDV for 24 h, after which luciferase activity was measured. Although PEDV infection significantly activated ISRE in p53 wild-type cells, this activation was substantially reduced in p53 knock-out cells (Fig. 4A). Indeed, there was an over 6-fold increase in ISRE transactivation upon PEDV infection in wild-type p53 cells relative to that in p53 knock-out cells. To determine whether ISRE-dependent ISGs were involved in p53 antiviral function, we infected wild-type and p53 knock-out cells with PEDV and assessed ISG expression at the RNA level. The basal transcript levels of RIG-I, IRF-9, ISG56, PKR, MxA, OAS-1 and IFN-β were significantly upregulated in p53 wild-type HEK293 cells relative to those in p53 knock-out HEK293 cells (Fig. 4B). We next explored how p53 affects IFN-β secretion by infected cells. Supernatants

from p53 wild-type cells had greater amounts of IFN-β upon PEDV infection relative to those from p53 knock-out cells. This observation confirmed the p53-dependent secretion of IFNs during PEDV infection (Fig. 4C). Taken together, these results demonstrate the mechanism by which p53 enhances IFN signaling and secretion of IFN-β during PEDV infection.

4. Discussion

As a major cause of mortality in suckling piglets, PED contributes to extensive annual economic damage to the swine industry, making the control of the causative agent, PEDV, vital (Scott et al., 2016). The genome of PEDV contains many genes that act to subvert host innate immunity (Yang et al., 2018). Moreover, upon virus infection, the host must have a quick response to the invading virus to avoid infection and prevent viral transmission to nearby cells. By better understanding the host responses important for the control of viruses such as PEDV, it may be possible to better design therapies aimed at PEDV control. In this study, we revealed a role for the tumor suppressor protein p53 as a regulator of PEDV infection, with multiple experiments highlighting the contribution of this gene to viral control.

Through the use of p53 knock-out HEK293 cells, we found that p53 knock-out HEK293 cells are highly susceptible to PEDV infection, indicating that p53 is a vital regulator of PEDV replication. Moreover, overexpression of p53 or use of a p53 specific activator effectively suppressed PEDV replication and dissemination in HEK293 cells, clearly showing that p53 activation is important for the inhibition of PEDV

replication.

Previous studies have suggested links between p53 and the IFN antiviral signaling system (Takaoka et al., 2003). p53 can help perpetuate IFN signaling via ISG-dependent positive feedback loops that contribute to robust antiviral immunity (Miciak and Bunz, 2016). Our present study demonstrates that p53 contributes to the activation of ISREs and ISG expression during PEDV infection. Moreover, we found that p53 increased both IFN signaling and secretion during PEDV infection, further perpetuating an effective antiviral state in HEK293 cells. Together, these findings suggest a clear link between p53 and IFN signaling during PEDV infection, with this gene modulating IFN-dependent defense mechanisms.

Viral infection has the potential to affect myriad signaling pathways, including that of p53, which plays an important role in the antiviral response. A previous report showed that p53 inhibits HIV-1 transcript elongation by disrupting phosphorylation of serine 2 of the pol II CTD, which impairs HIV-1 transcription and replication (Mukerjee et al., 2010). Influenza virus-infected A549 cells trigger the activation of the p53 pathway leading to apoptosis, and blocking p53 increases viral titers (Turpin et al., 2005). Similarly, in this study, we observed that PEDV infection enhanced p53 expression in HEK293 cells at 6–24 hpi but not after 48 hpi. This observation suggests that PEDV could antagonize the p53 antiviral response by some unknown mechanism. Some reports indicate that coronavirus papain-like proteinase (PLP) can adversely impact p53 stability via increasing its ubiquitination and consequent export from the nucleus (Yuan et al., 2015). The precise molecular mechanism through which PEDV viral proteins regulate p53 degradation requires further investigation. Taking our findings together, we conclude that p53 was activated early during PEDV infection to promote antiviral functions, but it was degraded at later stages of infection by PEDV to benefit the replication of the virus.

In conclusion, our results demonstrate the importance of p53, a protein normally studied as a tumor suppressor, in inducing strong antiviral defenses against PEDV infection via the upregulation of IFNs and other antiviral defensive genes. Given its key role in protecting cells against PEDV infection, these findings may have therapeutic implications for the control of this economically important pathogen.

Conflict of interest

The authors declare no conflicts of interest related to this work.

Acknowledgments

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.molimm.2019.02.005.

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