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Short communication

Endoribonuclease activities of porcine reproductive and respiratory syndrome virus nsp11 was essential for nsp11 to inhibit IFN-β induction

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ABSTRACT

Previous studies have shown that PRRSV nsp11, which was an endoribonuclease, was an interferon antagonist, however, the mechanism that nsp11 inhibited IFN-β production was unclear. To explore whether the endoribonuclease was required for nsp11 to disrupt the IFN-β production, substitutions of the presumed catalytic histidine and lysine residues of nsp11 were introduced into plasmid pcDNA 3.1-FLAG. The results showed that mutation that inactivated endoribonuclease made nsp11 lose its ability to inhibit Poly(I:C)-induced IFN-β promoter activity. In conclusion, our present work indicated that the endoribonuclease activity of nsp11 was essential for nsp11 to inhibit the IFN-β induction.

1. Introduction

IFN-β was the first key responder of innate immune response against viral infections (Muller et al., 1994; Weber et al., 2004). When virus infected, the virus RNA could be recognized by the pathogen-associated molecular patterns (PAMPs) such as membrane bound Toll-like receptors (TLRs), retinoic acid-inducible gene I (RIG-I) and the melanoma differentiation-associated gene 5 protein (MDA5). Among these receptors, TLRs recruited the adaptor molecule myeloid differentiation primary-response gene 88 (MyD88) and Toll/IL-1 receptor domain-containing adaptor inducing IFN (TRIF) while RIG-I and MDA-5 recruited virus-induced signaling adapter (VISA), to make TANK-binding kinase 1 (TBK1) or IκB kinase-ε (IKK-ε) phosphorylate IRF-3 and finally to induce IFN-β transcription (Bowie and Unterholzner, 2008). Then, IFN-β induced the IFN-regulated genes responsible for the antiviral response (Sadler and Williams, 2008). However, during the co-evolution with the host cells, many viruses encoded proteins that inhibited the interferon production, making it difficult for host cells to defeat viral infection (Bowie and Unterholzner, 2008; Spann et al., 2004; Weber et al., 2004).

PRRSV, a small enveloped virus including a single positive-stranded RNA genome, was a member of family *Arteriviridae* which, along with the *Coronaviridae*, were classified in the order *Nidovirales* (Cavanagh, 1997; Meulenberg, 2000). PRRSV shared many features with other members of the *Arteriviridae* viruses such as mouse lactate dehydrogenase-elevating virus (LDV), equine arteritis virus (EAV) and simian hemorrhagic fever virus (SHFV). Since the emergence of PRRSV in the United States in 1987 and in Europe in 1990, PRRSV has caused one of the most economically important diseases of swine which was characterized by severe reproductive failure in sows and respiratory distress in piglets and growing pigs (Rossow, 1998). Infection with PRRSV also subjected pigs to secondary infection by bacterial and viral pathogens, which may be due to the immunosuppression induced by the virus (Feng et al., 2001; Mateu and Diaz, 2008).

PRRSV genome had nine open reading frames (ORFs) composed of ORF1a, ORF1b, ORF2a, ORF2b, and ORF3-7. ORF1a and ORF1b could produce 14 nonstructural proteins (nsp1α, nsp1β, nsp2, nsp3, nsp4, nsp5, nsp6, nsp7α, nsp7β, nsp8, nsp9, nsp10, nsp11 and nap12) (den Boon et al., 1991; Fang and Snijder, 2010). Our previous study and others have found that PRRSV suppressed IFN-β production induced by Poly(I:C) (Luo et al., 2008) and that nsp1, nsp2 and nsp11 from PRRSV were the IFN antagonists (Beura et al., 2010; Shi et al., 2010). Sun et al. (2010) found that ubiquitin-deconjugating activity of nsp2 antagonized the type I interferon. Our recent study found that the papain-like cysteine protease of nsp1α was necessary for nsp1 as the interferon antagonist (Shi et al., unpublished results). However, up to now, the mechanism that nsp11 inhibited IFN-β production was still unclear.

PRRSV nsp11 possessed the nidovirus uridylate-specific endoribonuclease (NendoU) activity (Nedialkova et al., 2009), which was

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a major genetic marker that was unique to *Nidoviruses* (Ivanov et al., 2004). Our present work showed that nsp11 was an interferon antagonist and the endoribonuclease activity was essential for nsp11 to inhibit the IFN-β production.

2. Materials and methods

2.1. Cell, virus and primary antibodies

MARC-145 cell, a fetal green monkey fibroblast cell line derived from MA-104 (Kim et al., 1993), was maintained in Dulbecco's modified Eagle's medium (Gibco) supplemented with 10% fetal bovine serum (Hyclone). PRRSV strain BJ-4 was a kind gift from Dr. Hanchun Yang (China Agricultural University). Primary antibodies used for this study were anti-IRF-3, anti-serine 396-phosphorylated species of IRF-3 (pIRF-3) (Cell Signaling Technology), anti-actin (Beijing Zhongshan Goldenbridge Biotechnology Company, China) and anti-FLAG (Sigma).

2.2. Plasmids

The p-284 Luc (positions −284 and +19 in IFN-β gene prior to the luciferase report gene) and phRL-TK plasmids have already been described (Shi et al., 2010). pcDNA3.1-FLAG was constructed by using FLAG primers (Forward: CTAGCTAGCGCCACCATGGATTACAAGGATGACGA and Reverse: TCAGAAGCTTGCCTTATCGTCGTCATCCTTGTAATCCA). The PRRSV nsp11 was cloned from PRRSV RNA. PCR was amplified with the primers P1, CCAAGCTTCGCCACCATGGGGTCGAGCTCTC, and P2, GGAATTCTCATTCAAGTTGGAAATAGGCTGT. And the PCR product was cloned into pMD19-T vector (Takara) and then ligated into pcDNA3.1-FLAG. The catalytically inactive mutant of nsp11 (nsp11H129A) was generated by overlap PCR using the primers P3, GTCCCTCCCAGCCGCTTTCATTGGCGACG, and P4, ATGAAAGCGGCTGGGAGGGACGCAGCAACT. The catalytically inactive mutant of nsp11 (nsp11H144A) was generated by overlap PCR using the primers P5, AGGATGTGCCCATGTCACCTCCAGATACCT, and P6, GTGACATGGGCACATCCTCCAACGGTAGT. The catalytically inactive mutant of nsp11 (nsp11K173A) was generated by overlap PCR using the primers P7, AGCCGCGGCCGCATTGTGCACACTGA, and P8, ACAATGCGGCCGCGGCTTTTCCGGG. p55C1B Luc (Devaraj et al., 2007; Yoneyama et al., 1996, 2004), a firefly-luciferase reporter gene plasmid containing repetitive pIRF-3 binding sites, was kindly provided by Dr. Takashi Fujita. pcDNA3-FLAG-IKK-ε was kindly provided by Dr. Katherine A. Fitzgerald (Fitzgerald et al., 2003).

2.3. Transfection and reporter gene assay

All newly prepared plasmids were verified by sequencing. Transient transfection was performed by using Lipofectamine2000 (Invitrogen). Cells grown in 24-well plates were transfected in triplicate with the indicated reporter plasmid (200 ng), phRL-TK (80 ng), and expression vector (500 ng) or supplied with an equivalent control vector. At the appointed time, cells were harvested and the luciferase activity was measured using the dual luciferase reporter assay system (Promega) in MicroBeta® TriLux liquid scintillation and luminescence conters (Microbeta-1450) (Wallac).

2.4. Western blot

MARC-145 cells grown in 24-well plates were transfected in triplicate with the pcDNA3.1-FLAG (control vector) or pcDNA3.1-FLAG-nsp11 (1000 ng) or pcDNA3.1-FLAG-nsp11H129A (1000 ng), and 24 h later, transfected cells were mock-transfected or transfected with Poly(I:C) (1000 ng/well) by using Lipofectamine2000 for 6 h and lysed in lysing buffer (1% Nonidet P-40, 0.1% sodium deoxycholate, 0.1% SDS, 50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 2 mM Na_3VO_4, 2 mM NaF and a protease inhibitor cocktail). The detailed procedure for immunoblots has been described (Shi et al., 2008). Briefly, the total protein concentration was quantified with the Bradford protein assay (Biocolor Bioscience & Technology Company, Shanghai). Equal proteins were separated on SDS–PAGE gels and transferred to polyvinylidene difluoride membranes (Millipore Company), and then probed with appropriate antibodies. Proteins were detected by using an ECL detection system (Cell Signaling Technology).

2.5. Statistical analysis

Statistical analyses was performed by Student's *t*-test, and the comparisons were considered as statistical significance when $P < 0.05$.

3. Results and discussion

3.1. PRRSV nsp11 inhibited the activation of IFN-β promoter induced by Poly(I:C)

As shown in Fig. 1, nsp11 could inhibit the Poly(I:C) (a synthetic dsRNA analog)-induced activation of IFN-β promoter, which

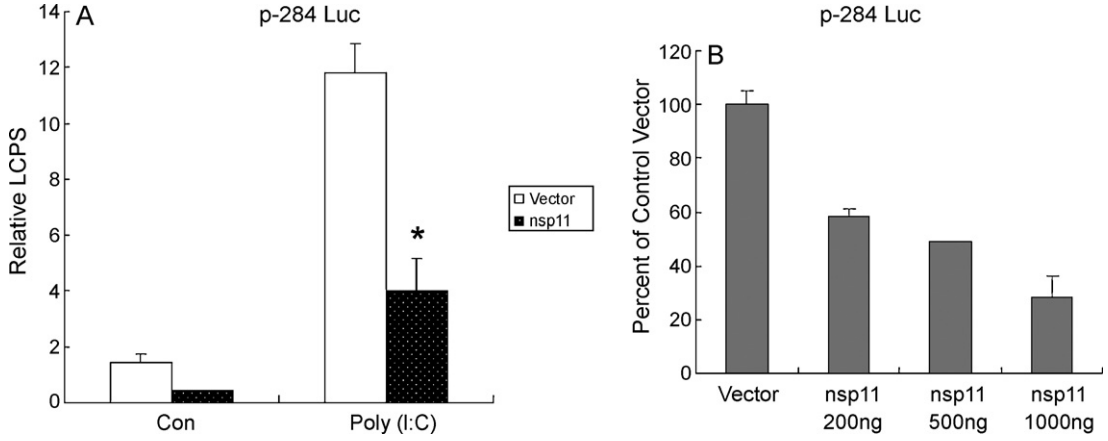

Fig. 1. nsp11 was an interferon antagonist. (A) MARC-145 cells were co-transfected with p-284 Luc, phRL-TK and pcDNA3.1-FLAG-nsp11 (nsp11). Twenty hours later, cells were either mock-transfected (Con) or transfected with Poly(I:C) (1000 ng/well) by using Lipofectamine2000 for 6 h, then the cells were harvested for dual luciferase reporter assay. (B) Increasing amounts of nsp11 coding plasmids were transfected, and luciferase activities were measured as described earlier. Vector: cells transfected with pcDNA3.1-FLAG. LCPS was the luminescence counts per second detected by luminescence counting on the Microbeta-1450. Bars showed relative luciferase activity (relative LCPS) normalized to phRL-TK activity (relative LCPS = LCPS of *firefly*-luciferase/LCPS of *Renilla*-luciferase). Data represented means of three replicates, and experiments were repeated three times. Error bars show the standard deviation (SD) for triplicate transfections. *$P < 0.05$ compared with the activity of the cells with the vector.

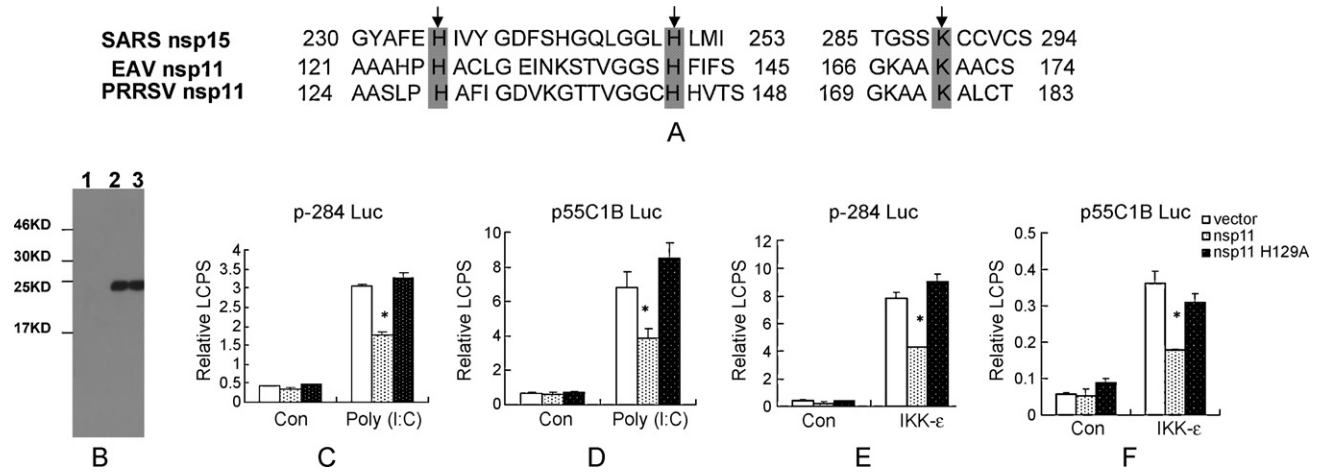

Fig. 2. His-129 was essential for PRRSV nsp11 to inhibit the Poly(I:C) or IKK-ε-induced activations of IFN-β promoter and pIRF-3 dependent promoter (p55C1B Luc). (A) Sequence alignment of PRRSV nsp1, EAV nsp11 and SARS-CoV nsp15 indicated that the sites (His-129, His-144 and Lys-173 in PRRSV nsp11) pointed by arrows were the catalytic residues of PRRSV nsp11. (B) Western blots analyzed the expressions of nsp11 and nsp11 H129A by anti-FLAG antibody in MARC-145 cells transfected with plasmid pcDNA3.1-FLAG (lane 1), pcDNA3.1-FLAG-nsp11 (lane 2), pcDNA3.1-FLAG-nsp11H129A (lane 3). MARC-145 cells were co-transfected with p-284 Luc (C) or p55C1B Luc (D), phRL-TK and different expression plasmids. Twenty hours later, cells were either mock-transfected (Con) or transfected with Poly(I:C) (1000 ng/well) by using Lipofectamine2000 for 6 h, then the cells were harvested for dual luciferase reporter assay. MARC-145 cells were co-transfected with p-284 Luc (E) or p55C1B Luc (F), phRL-TK, pcDNA3.1-IKK-ε (IKK-ε) and different expression plasmids. Twenty four hours later, the cells were harvested for dual luciferase reporter assay. Con: cells transfected with pcDNA3.1. Vector: cells transfected with pcDNA3.1-FLAG. nsp11: pcDNA3.1-FLAG-nsp11. nsp11H129A: pcDNA3.1-FLAG-nsp11H129A. Data represented means of three replicates, and experiments were repeated three times. Error bars show the standard deviation (SD) for triplicate transfections. $^{*}P < 0.05$ compared with the activity of the cells with the vector.

was consistent with the results in the previous study (Beura et al., 2010).

3.2. His-129 was essential for nsp11 as an interferon antagonist

Bioinformatics showed that the NendoU domain was highly conserved and belonged to a small protein family whose cellular branch was prototyped by XendoU, a *Xenopus laevis* endoribonuclease (Laneve et al., 2003; Nedialkova et al., 2009). The crystal structures of NendoU-containing nsp15 of several coronaviruses revealed that the active sites of NendoU included two histidines and a lysine (Ricagno et al., 2006; Xu et al., 2006). And site-directed mutagenesis studies showed that mutation of one of the three amino acids could abolish the nucleolytic activity (Guarino et al., 2005; Ivanov et al., 2004). Nedialkova's study showed that nsp11 from the arterivirus, EAV, also had the catalytic center (His-126, His-141 and Lys170) and mutating one of the three amino acids could abolish the endoribonuclease (NendoU) activity (Nedialkova et al., 2009). Aligning the protein sequence of NendoU domain among SARS-CoV nsp15, EAV nsp11 and PRRSV nsp11 (Fig. 2A), the active sites of PRRSV NendoU should be His-129, His-144 and Lys-173.

To investigate whether the endoribonuclease activity of PRRSV nsp11 were required for its disruption of IFN-β induction, we made single-amino acid substitution into nsp11 that disrupted the endoribonuclease activity [replacing His-129 with Ala (nsp11H129A)]. As shown in Fig. 2C, nsp11H129A failed to block Poly(I:C)-induced activation of the IFN-β promoter. These results suggested that the endoribonuclease activity of nsp11 was responsible for its inhibition to interferon induction.

Because phosphorylation of IRF-3 (pIRF-3) was a necessary component to the activation of IFN-β promoter, pIRF-3-dependent synthetic promoter, p55C1B-Luc (Devaraj et al., 2007; Yoneyama et al., 1998, 2004) was detected with luciferase reporter assays, after the Poly(I:C) treatment or the mock treatment. As shown in Fig. 2D, only nsp11 could inhibit the activation of p55C1B-Luc, while nsp11H29A could not, that is, the results in Fig. 2D confirmed that in Fig. 2C.

Poly(I:C), a double-stranded RNA, could be recognized by TLR3 (Yamamoto et al., 2003) and RIG-I (Kato et al., 2006). Then TLR3 recruited TRIF and RIG-I recruited VISA to activate IKK-ε and to phosphorylate IRF-3 and finally to activate IFN-β promoter (Bowie and Unterholzner, 2008). Over-expression of IKK-ε could induce the activation of IRF-3 and promote the activation of IFN-β promoter (Devaraj et al., 2007; Yoneyama et al., 2004; Zhong et al., 2008). As shown in Fig. 2E, PRRSV nsp11 suppressed the activation of IFN-β promoter induced by ectopic expression of IKK-ε, but nsp11H129A failed. Similar results were obtained when p55C1B Luc (Fig. 2F) was in place of p-284 Luc. These results indicated that nsp11 inhibited the IFN-β production induced by both TLR3 and RIG-I pathways.

3.3. His-129 was essential for nsp11 to inhibit the phosphorylation of IRF-3 induced by Poly(I:C)

Immunoblot analyzed the active and the latent of IRF-3. And the results showed that nsp11 inhibited the phosphorylation of IRF-3 induced by Poly(I:C) and did not influence the expression of IRF-3 (Fig. 3), but nsp1H129A could not inhibit the phosphorylation of IRF-3 induced by Poly(I:C).

3.4. His-144 and Lys-173 were essential for nsp11 as an interferon antagonist

We designed to probe whether the mutations of His-144 and Lys-173 could also influence the functions of nsp11 to antagonize IFN-β induction. Corresponding to the results of the mutation of His-129, both substitutions (nsp11H144A and nsp11K173A) caused the nsp11 to lose the function to inhibit the activations of IFN-β promoter (Fig. 4B and D) and the function to inhibit the activations of the p55C1B Luc (Fig. 4C and E).

PRRSV nsp11, along with EAV nsp11 and the nsp15 proteins from MHV-A59, SARS-CoV, all belonged to the XendoU family and possessed endoribonuclease activity (Snijder et al., 2003). A mutagenesis study of EAV nsp11 indicated that deleting parts of the NendoU domain from nsp11 of EAV was lethal and replacement

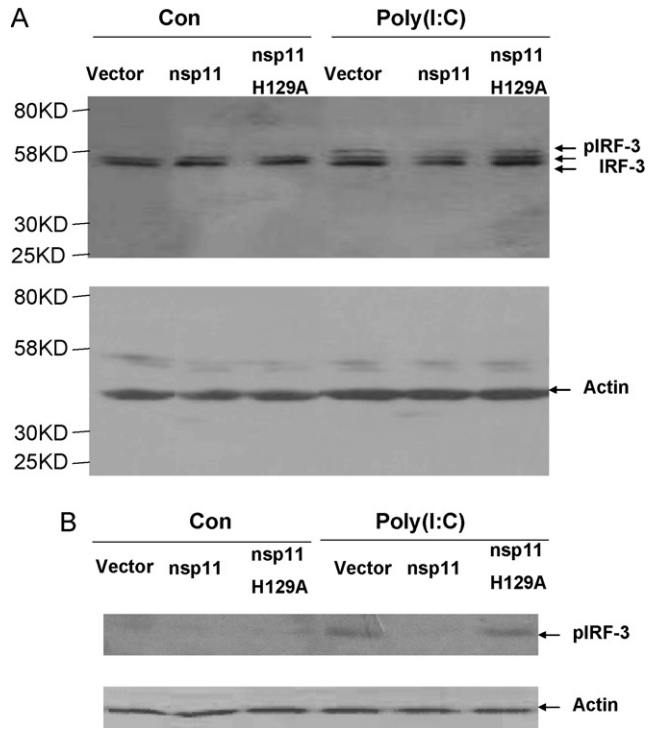

Fig. 3. His-129 was essential for PRRSV nsp11 to inhibit the phosphorylation of IRF-3 induced by Poly(I:C). MARC-145 cells were transfected with pcDNA3.1-FLAG (Vector), pcDNA3.1-FLAG-nsp11 (nsp11) or pCDNA3.1-FLAG-nsp11H129A (nsp11H129A). Twenty four hours later, the cells were either mock-transfected (Con) or transfected with Poly(I:C) (1000 ng/well) by using Lipofectamine2000 for 6 h. Then the cells were analyzed by immunoblots using the anti-IRF-3 antibody (A) or anti-pIRF-3 antibody (B).

of any of the three putative catalytic residues (His-126, His-141, or Lys-170) reduced plaque sizes and reduced yields of infectious progeny (Posthuma et al., 2006). And the in vitro study showed that replacement of any of the three putative catalytic residues (His-126, His-141, or Lys-170) reduced the EAV NendoU activity to background levels (Nedialkova et al., 2009), which was consistent with the results of inactivated endoribonuclease in other Nidovirales (Guarino et al., 2005; Ivanov et al., 2004; Xu et al., 2006). Mutation studies that inactivated the endoribonuclease activity in coronavirus indicated that endoribonuclease was required for optimal infection by virus (Ivanov et al., 2004; Kang et al., 2007). All of above indicated that nsp11 from PRRSV may also play an important role in the life cycle of PRRSV. Frieman et al. (2009) and Beura et al. (2010) found that SARS CoV nsp15 and PRRSV nsp11 were the interferon antagonists. Our represent study supplied the first evidence that endoribonuclease was essential for nsp11 as an IFN antagonist and indicated that endoribonuclease of PRRSV nsp11 may play an important role in opposing the defensive system of cell, and this needed further study. Coincidentally, our results were consistent with Yoo et al.'s (2010) and Sun et al.'s (2009) description that PRRSV nsp11 by virtue of its endoribonuclease activity inhibited the induction of IRF-3 and IFN-β. Our previous study found that PRRSV could weakly trigger the activation of IRF-3 as well as induce the activation of IFN-β promoter at 24 h p.i., but their activities were rapidly inhibited in following infection. That is, PRRSV could be sensed by professional beta interferon-producing system in MARC-145 cells and had mechanisms to inhibit this action in MARC-145 cells (Shi et al., 2010). So our present work supplied another evidence that the endoribonuclease of PRRSV nsp11 could antagonize the induction of IFN-β. Previous studies also showed the intriguing observations that recombinant IFN-β not only protected swine alveolar macrophages and MARC-145 cells from infection with PRRSV (Overend et al., 2007), but also could reduce the yield of PRRSV in vivo (Buddaert et al., 1998). All above results indicated that induction of IFN-β may be a useful method for control the PRRSV. And this merits the further study.

Our previous study and others have found that nsp1, nsp2 and nsp11 from PRRSV were the IFN antagonists (Beura et al., 2010; Shi et al., 2010). Sun et al. (2010) found that ubiquitin-deconjugating activity of nsp2 antagonized the type I interferon. Our recent study found that the papain-like cysteine protease of nsp1α was necessary for nsp1 as the interferon antagonist (Shi et al., unpublished results). And our present study showed that endoribonuclease of PRRSV nsp11 was essential for nsp11 to inhibit the induction of IFN-β. All of above results indicated that the protease activities played an important role for their functions of interferon antagonist. So exploring the special inhibitors to the papain-like cysteine protease of nsp1α, ubiquitin-deconjugating activity of nsp2 and endoribonuclease of PRRSV nsp11 may be an interesting issue for the treatment of PRRSV, that is, the protease activity of nsp1, nsp2 and nsp11 may be used as potential targets for exploiting new drags for PRRSV treatment or PRRSV vaccine.

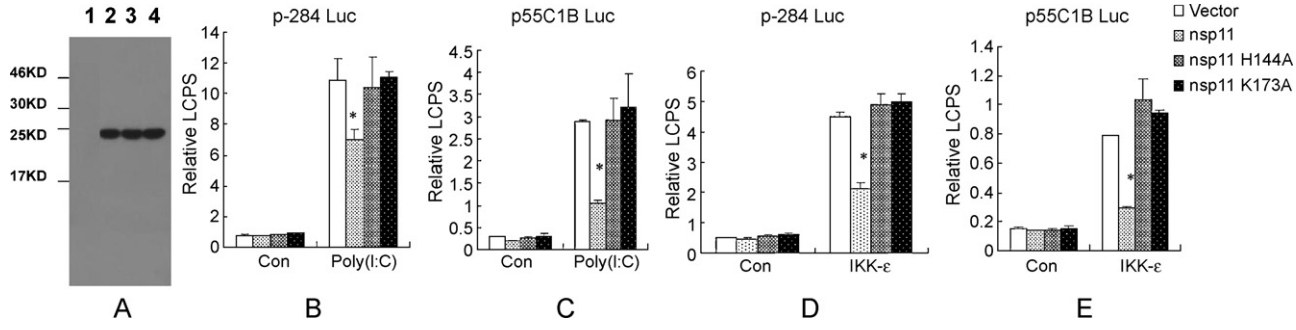

Fig. 4. His-144 and Lys-173 were essential for PRRSV nsp11 to inhibit the activities of IFN-β promoter and pIRF-3 dependent promoter (p55C1B Luc). (A) Western blots analyzed the expression of nsp11, nsp11H144A and nsp11K73A by anti-FLAG antibody in MARC-145 cells transfected with pcDNA3.1-FLAG (lane 1), pcDNA3.1-FLAG-nsp11 (lane 2), pcDNA3.1-FLAG-nsp11H144A (lane 3) or pcDNA3.1-FLAG-nsp11K173A (lane 4). MARC-145 cells were co-transfected with p-284 Luc (B) or p55C1B Luc (C), phRL-TK, and different expression plasmids. Twenty hours later, cells were either mock-transfected (Con) or transfected with Poly(I:C) (1000 ng/well) by using Lipofectamine2000 for 6 h, then the cells were harvested for dual luciferase reporter assay. MARC-145 cells were co-transfected with p-284 Luc (D) or p55C1B Luc (E), phRL-TK, pcDNA3.1-IKK-ε (IKK-ε) and different expression plasmids. Twenty four hours later, the cells were harvested for dual luciferase reporter assay. Data represented means of three replicates, and experiments were repeated three times. Con: cells transfected with pcDNA3.1. Vector: cells transfected with pcDNA3.1-FLAG. nsp11: pcDNA3.1-FLAG-nsp11. nsp11H144A: pcDNA3.1-FLAG-nsp11H144A. nsp11K173A: pcDNA3.1-FLAG-nsp11K173A. Error bars show the standard deviation (SD) for triplicate transfections. $^{*}P < 0.05$ compared with the activity of the cells with the vector.

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