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The DEAD-box RNA helicase 5 positively regulates the replication of porcine reproductive and respiratory syndrome virus by interacting with viral Nsp9 *in vitro*

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ABSTRACT

The nonstructural protein 9 (Nsp9) of porcine reproductive and respiratory syndrome virus (PRRSV) has been recognized to play important roles in viral replication. The present study first screened that the DEAD-box RNA helicase 5 (DDX5) was a cellular protein interacting with the Nsp9 of PRRSV by a yeast two-hybrid method in a pulmonary alveolar macrophages (PAMs) cDNA library. Next, DDX5 was shown to interact with viral Nsp9 in the co-transfected HEK293 cells with the DDX5- and Nsp9-expressing plasmids, and the interaction between endogenous DDX5 and Nsp9 was also confirmed in MARC-145 cells infected with the Nsp9-expressing lentiviruses. Then, the interacting domains between DDX5 and Nsp9 were determined to be the DEXDc and HELICc domains in DDX5 and the RdRp domain in Nsp9, respectively. Moreover, in the HEK293 cells, MARC-145 cells and PAM cell lines co-transfected with the DDX5- and Nsp9-expressing plasmids, Nsp9 was shown to co-localize with DDX5 in the cytoplasm with a perinuclear pattern, and meanwhile in PRRSV-infected MARC-145 cells and PAMs, endogenous DDX5 was also found to co-localize with Nsp9. Finally, silencing the DDX5 gene in MARC-145 cells significantly impacted the replication of PRRSV, and while the over-expression of DDX5 could slightly enhance viral replication. These findings indicate that DDX5 positively regulates the replication of PRRSV *via* its interaction with viral Nsp9 *in vitro*.

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1. Introduction

Porcine reproductive and respiratory syndrome (PRRS) is the most significant swine viral disease impacting the pig industry worldwide (Albina, 1997; Cho and Dee, 2006; Neumann et al., 2005; Pejsak et al., 1997; Zhou and Yang, 2010). The PRRS virus (PRRSV), the causative agent, is classified in the genus *Arterivirus* of the family *Arteriviridae* (Cavanagh, 1997), and has antigenically and genetically distinct two types—European genotype (type 1) and North American genotype (type 2) (Meng et al., 1995; Nelson et al., 1993). The genome of PRRSV is a single-strand, positive-sense RNA of approximately 15 kb in length, which contains at

least ten open reading frames (ORFs) (Conzelmann et al., 1993; Johnson et al., 2011; Snijder and Meulenberg, 1998). The ORF1a and ORF1b occupying 80% of the genome encode two viral replicase polyproteins—pp1a and pp1ab. The pp1a is processed into ten nonstructural proteins (Nsps) including Nsp1 α , Nsp1 β , Nsp2 to Nsp6, Nsp7 α , Nsp7 β and Nsp8, while the pp1ab is cleaved into Nsp9, Nsp10, Nsp11 and Nsp12 which are recognized to be involved in viral genome transcription and replication (Bautista et al., 2002; den Boon et al., 1995; Fang and Snijder, 2010; Snijder and Meulenberg, 1998; van Dinten et al., 1996).

Among the putative Nsps encoded by the ORF1b region of PRRSV, the Nsp9 is considered to possess RNA-dependent RNA polymerase (RdRp) activity that plays an important role in viral replication. This protein contains a RdRp domain in its C-terminal portion that is critical for its polymerase activity and viral genome transcription (Beerens et al., 2007; Fang and Snijder, 2010). Our recent studies uncovered that Nsp9 and Nsp10 together not only affect the Chinese highly pathogenic PRRSV (HP-PRRSV) replication and growth *in vitro* and *in vivo*, but also contribute to its fatal

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Table 1
Primers used in this study.

Primers ^a	Sequence (5'–3') ^b	Product size (bp)	Use
1F	GGTTTAAACTAGCCGCCAGCGGCTTGACC (<i>Dra I</i>)	1920	Nsp9 gene amplification
1R	GGAAGCTTCTCATGATTGGACCTGAGTTT (<i>HindIII</i>)		
2F	GGCATATGATGTCGAGTTATCAAGTGAC (<i>Nde I</i>)	1845	DDX5 gene amplification
2R	GCGGATCCTTATTGAGAATATCCTGTTGG (<i>BamH I</i>)		
3F	GGTTTAAACTAGCCGCCAGCGGCTTGACC (<i>Sal I</i>)		pCMV-HA-Nsp9 construction
3R	GGAAGCTTCTCATGATTGGACCTGAGTTT (<i>Kpn I</i>)		
4F	GGCATATGATGTCGAGTTATCAAGTGAC (<i>Sal I</i>)		pCMV-Myc-DDX5 construction
4R	GCGGATCCTTATTGAGAATATCCTGTTGG (<i>Kpn I</i>)		
5F	GCGTTTAAACCTAGCCGCCAGCGGCTTGACC (<i>Pme I</i>)		pWPXL-Nsp9 construction
5R	GCACGCGTCTCATGATTGGACCTGAGTTT (<i>Mlu I</i>)		
6F	GCGTTTAAACATGTCGAGTTATCAAGTGAC (<i>Pme I</i>)		pWPXL-DDX5 construction
6R	GCACGCGTCTTATTGAGAATATCCTGTTGG (<i>Mlu I</i>)		
7F	CGGTCCGACCTAGCCGCCAGCGGCTTGACC (<i>Sal I</i>)		pCMV-HA-Nsp9-N construction
7R	CGGCGGCCGCTAGGACCGGGCTTGAGTCT (<i>Not I</i>)		
8F	CCGTCCGACCGCAGGTGCCTTGAAGCTGAT (<i>Sal I</i>)		pCMV-HA-Nsp9-C construction
8R	CCGCGGCCGCTCATGATTGGACCTGAGTTT (<i>Not I</i>)		
9F	GCAGATCTCCATGTCGAGTTATCAAGTGAC (<i>Bgl II</i>)		pCMV-Myc-DDX5-N construction
9R	GCGCGGCCGCTACTCGGGCAGTTCCTCC (<i>Not I</i>)		
10F	GCGAATTCGGAAAACAGGCACAGCATACACT (<i>EcoR I</i>)		pCMV-Myc-DDX5-C construction
10R	GCGGTACCTTATTGAGAATATCCTGTTGGC (<i>Kpn I</i>)		

^a F denotes forward PCR primer; R denotes reverse PCR primer.

^b Restriction sites are underlined.

virulence for piglets (Y. Li et al., 2014). Obviously, the Nsp9 is the most important protein contributing to the replication and virulence of PRRSV. To date, limited evidence is known about the roles of Nsp9 in the regulation of PRRSV replication. A recent literature indicated that the interaction of Nsp9 with endogenous cellular annexin A2 is beneficial for PRRSV replication in MARC-145 cells (J. Li et al., 2014). Our latest work showed that the interaction of Nsp9 with cellular retinoblastoma protein (pRb) contributes the replication of genotype 2 PRRSV *in vitro* (Dong et al., 2014). Therefore, further exploring the interaction of Nsp9 with host cellular proteins is necessary for fully understanding the mechanisms associated with the replication regulation and pathogenesis of PRRSV.

The DEAD-box RNA helicases belonging to superfamily 2 of cellular RNA helicases have been shown to be essential for different processes of RNA metabolism, including pre-mRNA processing, RNA decay, mRNA export, translation initiation and transcription regulation (Li et al., 2013; Linder and Fuller-Pace, 2013; Mazurek et al., 2012). These proteins share eight conserved motifs (I, Ia, Ib, II, III, IV, V and VI) in the DExD/H helicase family (Choi and Lee, 2012; Zonta et al., 2013), which are considered to be involved in RNA binding and decay, ATP binding and hydrolysis. The DEAD-box RNA helicase 5 (DDX5) has been shown to affect viral RNA replication by interacting with viral helicase in hepatitis C virus (HCV) (Goh et al., 2004), and severe acute respiratory syndrome coronavirus (SARS-CoV) (Chen et al., 2009). In the present study, we described the interaction of DDX5 with the Nsp9 of the Chinese HP-PRRSV and analyzed the role of this interaction in regulating viral replication *in vitro*.

2. Materials and methods

2.1. Cells, virus, and antibodies

MARC-145 cells and human embryonic kidney (HEK) 293 cells were cultured in Dulbecco's modified Eagles medium (DMEM) (Invitrogen, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS) (Hyclone Laboratories Inc., South Logan, UT, USA) at 37 °C under 5% CO₂. Primary pulmonary alveolar macrophages (PAMs) were prepared as previously described (Zhang et al., 2009). PAM cell line 3D4/21 (CRL-2843) was purchased from the American Type Culture Collection (ATCC) and

was grown in RPMI-1640 medium (Invitrogen) supplemented with 10% FBS. The stock of HP-PRRSV JXwn06 with a titer of 10⁶ TCID₅₀/ml was employed in this study (Zhou et al., 2009). Mouse anti-HA monoclonal antibody (mAb) (H3663), rabbit anti-Myc antibody (C3956), mouse anti-β-actin mAb (A5441) and rabbit anti-DDX5 antibody (D1070) were all purchased from Sigma-Aldrich (St. Louis, MO, USA). Mouse anti-DDX5 mAb (sc-166167) and mouse anti-GFP mAb (sc-390394) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Mouse anti-Nsp9 mAb was prepared in our laboratory. The secondary antibodies including horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (ZB-2301), HRP-conjugated goat anti-mouse IgG (ZB-2305), fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (ZF-0312) and tetramethyl rhodamine isothiocyanate (TRITC)-conjugated goat anti-rabbit IgG (ZF-0316) were purchased from ZSGB-BIO (Beijing, China).

2.2. Plasmid construction

The Nsp9 gene of PRRSV JXwn06 was amplified by PCR with the primers 1F/1R using the plasmid pWSK-JXwn as template (Zhou et al., 2009). The amplified fragment was then cloned into the vector pGBKT7 (Clontech, Mountain View, CA, USA) via *Dra I*/*Hind III* sites to generate a plasmid pGBKT7-Nsp9, which was served as a bait protein in yeast hybrid screening. The cDNA encoding DDX5 was amplified from PAMs using the primer 2F/2R, and the amplified fragment was then cloned into the vector pGADT7 (Clontech) via *Nde I*/*BamH I* sites to construct a recombinant plasmid pGADT7-DDX5. Meanwhile, the Nsp9 gene was inserted into the vector pCMV-HA (Clontech) via *Sal I*/*Kpn I* sites with the primers 3F/3R to construct a pCMV-HA-Nsp9, and the DDX5 gene was sub-cloned into pCMV-Myc (Clontech) via *Sal I*/*Kpn I* sites with the primers 4F/4R to generate a pCMV-Myc-DDX5. Additionally, the Nsp9 gene and DDX5 gene were inserted into the vector pWPXL with the primers 5F/5R and 6F/6R via *Pme I*/*Mlu I* sites to generate pWPXL-Nsp9/pWPXL-DDX5, respectively.

To identify the interacting region in Nsp9 and DDX5, truncated constructs of Nsp9 and DDX5 were generated by PCR with respective primers. Two fragments of Nsp9 gene were cloned into the vector pCMV-HA, generating recombinant plasmids pCMV-HA-Nsp9-N (aa1-385) and pCMV-HA-Nsp9-C (aa386-640). Two

fragments of DDX5 gene were cloned into the vector pCMV-Myc, generating plasmids pCMV-Myc-DDX5-N (aa1-436) and pCMV-Myc-DDX5-C (aa437-615). All constructs were further confirmed by sequencing. All the primers used for amplification and plasmid construction were listed in Table 1.

2.3. Yeast two-hybrid screening

Yeast two-hybrid screening was performed using pGBKT7-Nsp9 as a bait to screen a cDNA library from PAM cells as previously described (Wang et al., 2012). Briefly, the Matchmaker™ Gold Yeast Two-Hybrid System (Clontech) was used according to the manufacturer's instructions. The yeast strain Y2HGold was transformed with pGBKT7-Nsp9, and was then mating with Y187 and selected quadruple dropout (QDO) plates for one week. The positive colonies were re-selected on high-stringency QDO plates containing 0.04 mg/ml 5-bromo-4-chloro-3-indolyl- α -D-galactopyranoside (X- α -Gal) and 0.07 μ g/ml Aureobasidin A (ABA), and further identified using PCR and sequencing.

2.4. The expression of target proteins in mammalian cells and by lentiviral expression system

HEK293 cells were co-transfected with pCMV-HA-Nsp9 and pCMV-Myc-DDX5 using Lipofectamine LTX and plus reagents (Invitrogen) according to the manufacturer's protocol. Meanwhile, the vector pCMV-HA and pCMV-Myc were served as negative controls.

The lentiviral expression system containing pWPXL, PMD2.G and psPAX2 was purchased from Addgene (Cambridge, MA, USA). The process of target protein expression mediated by lentiviruses was performed according to the manufacturer's protocol. Briefly, the packaging plasmid pWPXL-Nsp9/pWPXL-DDX5, PMD2.G and psPAX2 were mixed with appropriate proportion and were co-transfected into HEK 293FT cells with FuGENE® HD Transfection Reagent (Promega, Madison, WI, USA). The cells were then cultured in DMEM at 37 °C under 5% CO₂. The supernatants were harvested after a number of syncytias appeared and filtered with a 0.45 filter (Pall Corporation, Port Washington, NY, USA) and were then enriched by Amicon ultra-100 centrifuge tubes (Mick Millipore, Billerica, MA, USA). The titers of lentiviruses were measured by a QuickTiter™ Lentiviral Titer Kit (Lentivirus-Associated HIV p24) (Cell Biolabs, San Diego, CA, USA) and the lentiviruses were stored for use at –80 °C. MARC-145 cells were infected with the lentiviruses in the presence of 8 μ g/ml polybrene (Sigma). At 24 h post-transfection, the inoculums were removed and the GFP-Nsp9- or GFP-DDX5-expressing cells were maintained in DMEM with 10% FBS, respectively.

2.5. Co-immunoprecipitation and Western blotting

Co-immunoprecipitation (Co-IP) and Western blotting analyses were performed to confirm the interaction between Nsp9 and DDX5. At 36 h post-transfection, the HEK293 cells co-transfected with pCMV-HA-Nsp9 and pCMV-Myc-DDX5 were harvested and then lysed with cell lysis buffer (Beyotime, Shanghai, China). Meanwhile, MARC-145 cells infected with the lentiviruses were harvested at 24 h post-infection. The total cell lysates were clarified by centrifugation at 12,000 rpm for 15 min and the supernatants were precipitated with a mouse anti-HA mAb or mouse anti-GFP mAb in conjunction with protein A Sepharose beads (Sigma). After incubation for 2 h, the precipitates were collected by centrifugation and the beads were washed five times with cell lysis buffer. The proteins isolated from the beads were subjected to SDS-PAGE and transferred onto a polyvinylidene difluoride membrane (PVDF) (Sigma). The membrane was further probed using

a rabbit anti-Myc antibody or mouse anti-DDX5 mAb, and the horseradish peroxidase conjugated goat anti-rabbit IgG or goat anti-mouse IgG, and detected by using a chemiluminescence detection kit (Thermo Scientific, Waltham, MA, USA) and finally exposed to a chemiluminescence apparatus (ProteinSimple, Santa Clara, CA, USA).

2.6. Confocal immunofluorescence assay

HEK293 cells, MARC-145 cells or PAM cell lines grown in 24-well plates (Corning Inc., Corning, NY, USA) were co-transfected with pCMV-HA-Nsp9 and pCMV-Myc-DDX5 using Lipofectamine™ LTX and PLUS™ Reagents (Invitrogen) according to the manufacturer's protocol. Meanwhile, MARC-145 cells and PAMs grown in 24-well cell culture plates were infected with PRRSV JXwn06 at MOI of 0.01. Thirty-six hours after transfection or infection, the cells were fixed with 100% pre-cooled ethyl alcohol and permeabilized for 20 min, and were then incubated with respective primary antibodies. After being washed with phosphate buffered saline (PBS) three times, the cells were incubated with FITC-conjugated anti-mouse IgG or TRITC-conjugated anti-rabbit IgG for 2 h at 37 °C. Finally, the images were visualized under an Olympus confocal microscope.

2.7. Small RNA interfering assay

To analyze the effect of DDX5 knockdown on the replication of PRRSV, a small RNA interfering (siRNA) assay was performed by using the siRNA targeting for DDX5 gene (siDDX5) and control siRNA (siCon) which were synthesized by GenePharma (Suzhou, China). Briefly, MARC-145 cells grown up to 80% confluence in 6-well plates were transfected with the siRNA at the final concentration of 30 pmol with Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's protocol. Forty-eight hours after transfection, the siRNA-transfected cells were infected with the PRRSV JXwn06 at MOI of 0.01, and the cells were incubated for additional 72 h. The viral titers in the supernatants were determined by using a microtitration infectivity assay. The cell viability of the transfected MARC-145 cells was detected by Cell Titer 96 Aqueous One Solution Cell Proliferation Assay (Promega) according to the manufacturer's protocol.

2.8. The over-expression of DDX5 in MARC-145 cells

To check the influence of the DDX5 over-expression on the replication of PRRSV, MARC-145 cells that were stably expressing DDX5 were established by lentiviral packing system according to the manufacturer's protocol.

2.9. Virus infection and titration

MARC-145 cells grown up to 80% confluence in 96-well plates were infected with the PRRSV JXwn06 at MOI of 0.01. Viral titers were titrated by a microtitration infectivity assay as previously described (Zhou et al., 2009).

2.10. Statistical analysis

The data were represented as means \pm standard deviations (SD). The Graph Prism (version 5.0) software was used to determine the significance of the variability among different groups by Two-way ANOVA test of variance. *P* values of less than 0.05 were considered statistically significant.

Table 2

The predicted cellular proteins interacting with Nsp9 by the yeast two-hybrid screening.

Targeting proteins	Number of positive colonies	GenBank accession no.
Sus scrofa clone rptbr0137.p18.y1.abd	4	AY610505
Sus scrofa lactate dehydrogenase B (LDHB)	2	NM.001113287.1
Sus scrofa BMWN10003B11 DEAD (Asp-Glu-Ala-Asp) box helicase 5 (DDX5)	1	AK399667.1

3. Results

3.1. Cellular DDX5 interacted with the Nsp9 of PRRSV

A cDNA library derived from the PAMs was used for searching the cellular proteins interacting with the Nsp9 of PRRSV. Approximately 1.0×10^6 cDNA library clones were screened using the Nsp9 as a bait protein. The results showed that the cellular DDX5 was a potential interacting partner with the PRRSV Nsp9 (Table 2), which was selected for further study. In addition to DDX5, other two cellular proteins interacting with Nsp9 were also screened in this study.

Co-IP assay was performed to confirm the interaction between DDX5 and Nsp9 in HEK293 cells. HEK293 cells were co-transfected with pCMV-HA-Nsp9 and pCMV-Myc-DDX5 or empty vectors as negative controls. The HA- or Myc-tagged protein was detected with an anti-HA mAb or anti-Myc antibody by Western blotting, respectively. The cell lysates were precipitated with an anti-HA mAb in conjunction with protein A Sepharose, and then detected using a rabbit anti-Myc antibody. The results showed that HA-Nsp9 could be co-precipitated with Myc-DDX5 (Fig. 1A). To exclude the involvement of cellular DNA or RNA in the interaction between DDX5 and Nsp9, the cell lysates were treated with RNase of

100 $\mu\text{g/ml}$ and DNase of 100 $\mu\text{g/ml}$ and were subjected to Co-IP. The results indicated that the interaction between DDX5 and Nsp9 remained to be found (Fig. 1B), suggesting this interaction was not mediated by cellular DNA or RNA.

3.2. Endogenous DDX5 in MARC-145 cells interacted with the PRRSV Nsp9

Co-IP was performed to verify the interaction of endogenous DDX5 with the PRRSV Nsp9 in MARC-145 cells. MARC-145 cells were infected with the GFP-Nsp9- or GFP-expressing lentiviruses, and the cell lysates were subjected to Western blotting analyses. Meanwhile the cell lysates were precipitated with an anti-GFP mAb, and cellular DDX5 was detected by Western blotting with an anti-DDX5 mAb. The results showed that the GFP-Nsp9 could be detected in MARC-145 cells infected with the GFP-Nsp9-expressing lentiviruses (Fig. 2A), and DDX5 could be examined only in cells infected with the GFP-Nsp9-expressing lentiviruses, not in GFP-mock-infected cells (Fig. 2B), indicating that the endogenous DDX5 in MARC-145 cells could interact with the Nsp9 of PRRSV.

3.3. Determination of the binding regions of Nsp9-DDX5 interaction

To determine the interacting regions in Nsp9 and DDX5, two truncated Nsp9, Nsp9-N (aa1-385) and Nsp9-C (aa386-640), were constructed (Fig. 3A). Each domain interacting with DDX5 in Nsp9 was examined using Co-IP. The results revealed that Nsp9-C was able to interact with DDX5, while Nsp9-N did not (Fig. 3B and C), indicating that the RdRp domain (aa386-640) of Nsp9 is the region binding to DDX5. Similarly, to further ascertain the region interacting with Nsp9 in DDX5, two truncated DDX5 were constructed (Fig. 4A). DDX5-N was shown to interact with Nsp9 (Fig. 4B), while DDX5-C did not (Fig. 4C), indicating that the DEXDc and HELICc

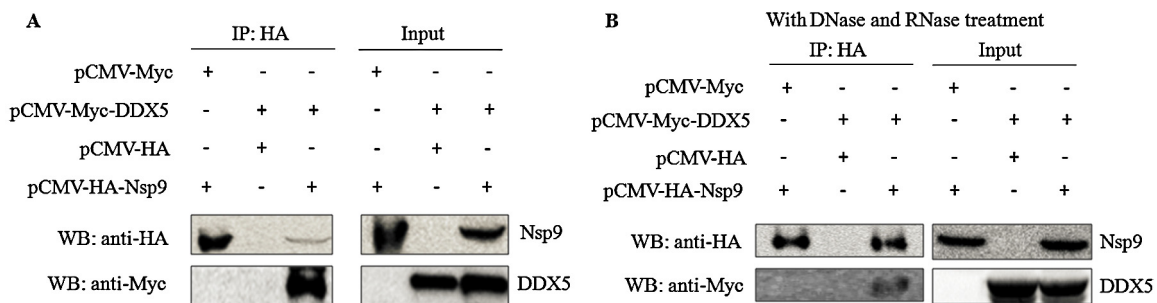


Fig. 1. Identification of the interaction between DDX5 and the Nsp9 of PRRSV by Co-IP. (A) Shown is the interaction of DDX5 with Nsp9 by Co-IP. HEK293 cells were transfected or co-transfected with the indicated plasmids. Thirty-six hours after transfection, the cell lysates were precipitated with an anti-HA mAb in conjunction with protein A Sepharose, and further detected by Western blotting with an anti-HA mAb and an anti-Myc antibody, respectively. (B) Shown is the interaction of DDX5 with Nsp9 with the presence of DNase and RNase. The cell lysates were treated with 100 $\mu\text{g/ml}$ DNase and 100 $\mu\text{g/ml}$ RNase. The interaction was then confirmed by Co-IP as above described.

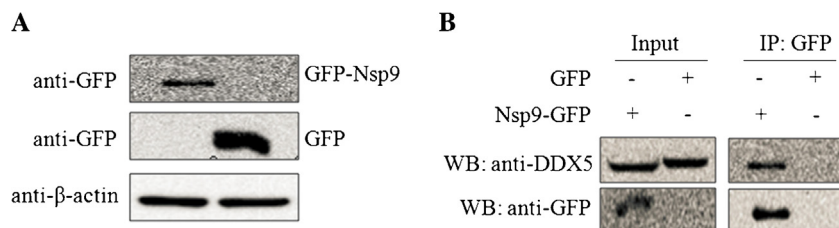


Fig. 2. Interaction of endogenous DDX5 with Nsp9 in MARC-145 cells. (A) Shown is the GFP-Nsp9 expression in MARC-145 cells. MARC-145 cells were infected with the GFP-Nsp9- or GFP-expressing lentiviruses, and the cell lysates were then subjected to Western blotting. The GFP-Nsp9 and GFP were detected with a mouse anti-GFP mAb. β -actin was detected as an internal control using an anti- β -actin mAb. (B) Shown is the interaction of endogenous DDX5 with Nsp9 in MARC-145 cells. MARC-145 cells were infected with the GFP-Nsp9-expressing lentiviruses; the cell lysates were then precipitated with a mouse anti-GFP mAb and further detected by Western blotting with a mouse anti-DDX5 mAb.

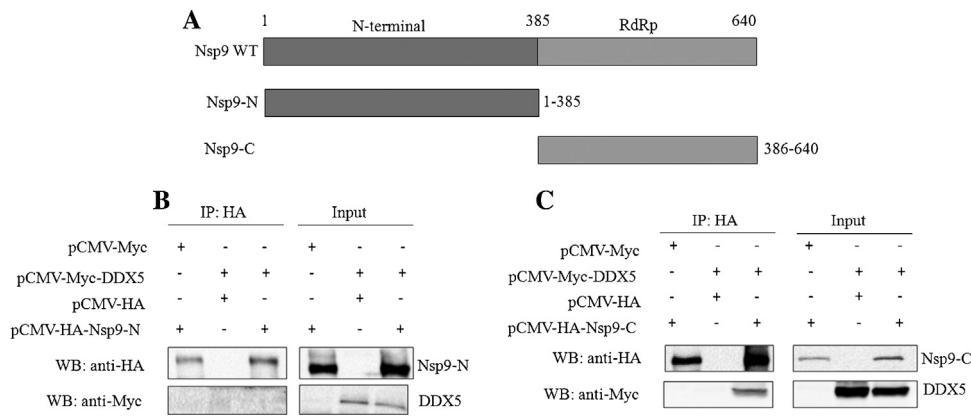


Fig. 3. Determination of the binding regions of Nsp9 with DDX5. (A) The schematic diagrams of Nsp9 (wt), N-terminal fragment (Nsp9-N, aa1–385) and C-terminal fragment (Nsp9-C, aa386–640). (B) Shown is the no interaction between Nsp9-N and DDX5 by Co-IP. (C) Shown is the interaction of Nsp9-C with DDX5 by Co-IP. HEK293 cells were co-transfected with pCMV-Myc-DDX5 and pCMV-HA-Nsp9-N or pCMV-HA-Nsp9-C, the cell lysates were subjected to Western blotting with a mouse anti-HA and a rabbit anti-Myc antibody, respectively.

domains (aa1–436) of DDX5 are the binding regions with the Nsp9 of PRRSV.

3.4. DDX5 mainly co-localized with the PRRSV Nsp9 in the cytoplasm

To analyze whether DDX5 co-localized with viral Nsp9, the cells were co-transfected with pCMV-HA-Nsp9 and pCMV-Myc-DDX5, and were examined at 36 h post-transfection with confocal immunofluorescence microscopy. The results showed that Nsp9 and DDX5 co-localized in the cytoplasm with a perinuclear pattern in HEK293 cells (Fig. 5A), MARC-145 cells (Fig. 5B) and PAM cell line (Fig. 5C). Moreover, the co-localization between Nsp9 and endogenous DDX5 was also observed in the cytoplasm of PRRSV-infected MARC-145 cells (Fig. 5D) and PAMs (Fig. 5E), whereas DDX5 mainly localized in the nucleus in mock-infected cells (Fig. 5D and E).

3.5. Silencing of DDX5 by siRNA impacted the replication of PRRSV in MARC-145 cells

To characterize the effect of the interaction of DDX5 with Nsp9 on the PRRSV replication, a small RNA interfering assay for silencing DDX5 gene was performed. We first examined the expression level of DDX5 in MARC-145 cells by using Western blotting analysis after the cells were treated with different concentrations of three

siRNAs (Sus-681, Sus-189 and Sus-147) targeting for DDX5 gene. The results showed that the DDX5 expression could be inhibited obviously by the three siRNAs compared with the cells treated with control (Fig. 6A). The cell viability examination of the MARC-145 cells transfected with Sus-147 demonstrated that the siRNAs transfection had no effects on the cell viability (Fig. 6B). A siRNA (Sus-147) concentration of 30 pmol was used for subsequent experiments.

The MARC-145 cells transfected with the siRNA were infected with the PRRSV, and the virus titers in the supernatants were assayed at different time points post-infection. The results showed that the virus titers in the supernatants of the siRNA-transfected cells were significantly lower than those of control and normal cells at each time points post-infection, and in particular, with a decrease of more than 10-fold ($P < 0.001$) at 60 h and 72 h post-infection (Fig. 6C), indicating that silencing of DDX5 by siRNA obviously impacts the replication of PRRSV in MARC-145 cells.

3.6. The over-expression of DDX5 slightly enhanced the replication of PRRSV in MARC-145 cells

Western blotting was first carried out to examine the GFP-DDX5 or GFP expression level in MARC-145 cells infected with GFP-DDX5- or GFP-expressing lentiviruses. As shown in Fig. 7A, the expression of GFP-DDX5 could be observed, with a similar level to GFP.

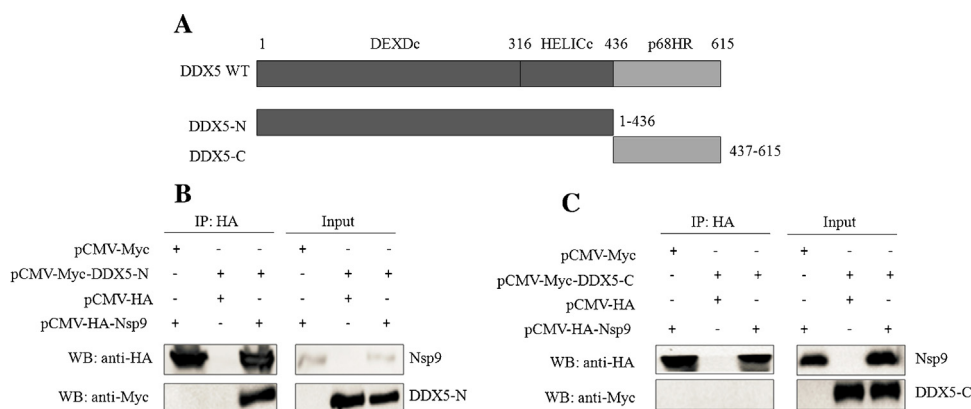


Fig. 4. Determination of the binding regions of DDX5 with Nsp9. (A) The schematic diagrams of DDX5 (wt), N-terminal fragment (DDX5-N, aa1–436) and C-terminal fragment (DDX5-C, aa437–615). (B) Shown is the interaction of DDX5-N with Nsp9 by Co-IP. (C) Shown is the no interaction of DDX5-C with Nsp9 by Co-IP. HEK293 cells were co-transfected with pCMV-HA-Nsp9 and pCMV-Myc-DDX5-N or pCMV-Myc-DDX5-C, and the cell lysates were then subjected to Western blotting with a mouse anti-HA mAb and a rabbit anti-Myc antibody, respectively.

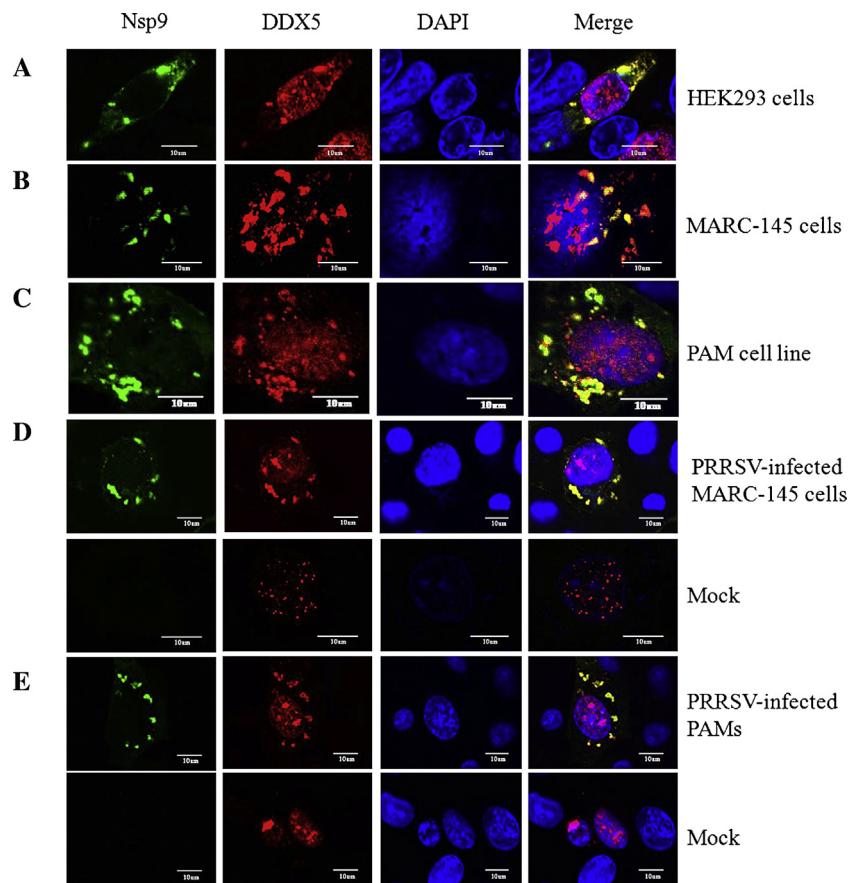


Fig. 5. Co-localization of Nsp9 with DDX5 in cells. HEK293 cells (A), MARC-145 cells (B) and PAM cell lines (C) were co-transfected with pCMV-Myc-DDX5 and pCMV-HA-Nsp9. The cells at 36 h post-transfection were then fixed and double-stained with a mouse anti-HA mAb and a rabbit anti-Myc antibody, and followed by FITC-conjugated anti-mouse IgG (green) and TRITC-conjugated anti-rabbit IgG (red). Co-localization of Nsp9 with endogenous DDX5 in PRRSV- or mock-infected MARC-145 cells (D) and PAMs (E). The cells were infected with PRRSV JXwn06 at MOI of 0.01, and were then fixed and double-stained at 36 h post-infection with a mouse anti-Nsp9 mAb and a rabbit anti-DDX5 antibody, and followed by FITC-conjugated anti-mouse IgG (green) and TRITC-conjugated anti-rabbit IgG (red). Nuclei were stained with DAPI (blue).

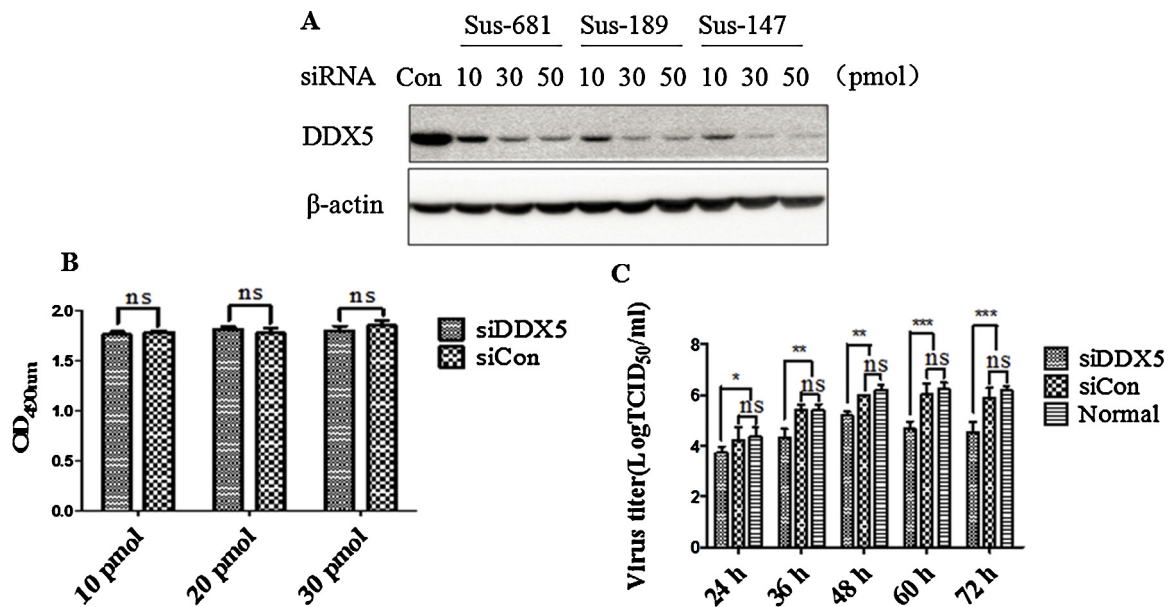


Fig. 6. Inhibition of PRRSV replication by silencing DDX5 gene in MARC-145 cells. (A) Silencing efficiency of DDX5 gene with different concentrations of three siRNAs. MARC-145 cells were transfected with each siRNA (Sus-681, Sus-189 and Sus-147) specific for DDX5 at a final concentration of 10 pmol, 30 pmol and 50 pmol or 50 pmol of control siRNA, respectively. The expression level of DDX5 was examined at 48 h post-transfection by Western blotting with a mouse anti-DDX5 mAb. β-actin was detected as an internal control using a mouse anti-β-actin mAb. (B) The cell viability analyses of the MARC-145 cells transfected with Sus-147 and control siRNA. The cells were examined at 48 h post-transfection by Cell Titer 96 Aqueous One Solution Cell Proliferation Assay. (C) PRRSV titers in the supernatants of DDX5 gene-silenced MARC-145 cells. MARC-145 cells were transfected with Sus-147 or control siRNA at a final concentration of 30 pmol, and were then infected at 48 h post-transfection with PRRSV JXwn06 at MOI of 0.01. Normal MARC-145 cells were also infected with the virus as a control. The virus titers in the supernatants were assayed at the indicated time points. Data are shown as means + SD of three independent experiments (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; ns, no significant).

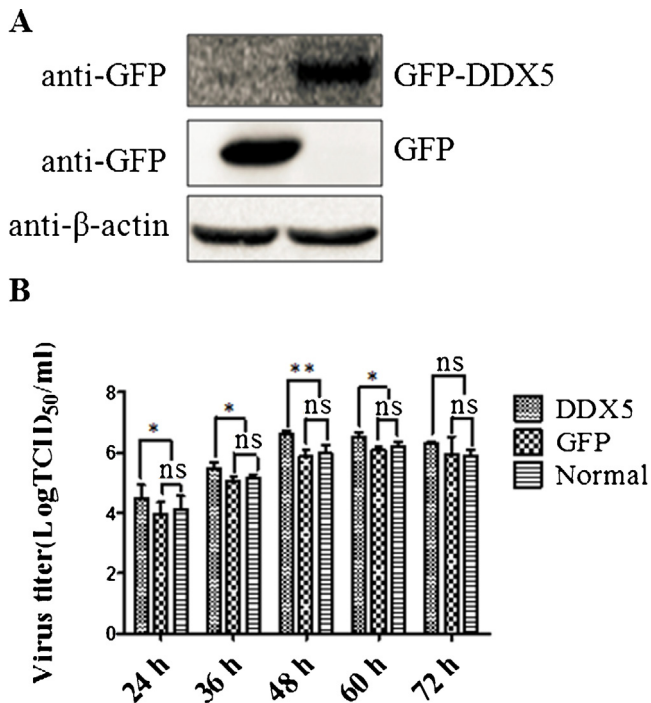


Fig. 7. Enhancement of PRRSV replication by DDX5 over-expression in MARC-145 cells. (A) The expression of GFP-DDX5 and GFP in MARC-145 cells. MARC-145 cells were infected with GFP-DDX5- or GFP-expressing lentiviruses. The GFP-DDX5 and GFP were detected with a mouse anti-GFP mAb. β-Actin was detected as an internal control using a mouse anti-β-actin mAb. (B) PRRSV titers in MARC-145 cells with DDX5 over-expression. MARC-145 cells were infected with GFP-DDX5- or GFP-expressing lentiviruses, and were then infected at 48 h post-transduction with PRRSV JXwn06 at MOI of 0.01. Normal MARC-145 cells were also infected with the virus as a control. PRRSV titers in the supernatants were assayed. Data are shown as means ± SD of three independent experiments (* $p < 0.05$; ** $p < 0.01$; ns, no significant).

MARC-145 cells that were expressing GFP-DDX5 or GFP by lentiviral packaging system were then infected with the PRRSV, and the virus titers in the supernatants were assayed at different time points post-infection. The results showed that the virus titers in the cells with DDX5 over-expression were statistically higher than those in the cells with only GFP over-expression at 24–48 h post-infection ($P < 0.01$ or $P < 0.05$) (Fig. 7B), indicating that the over-expression of DDX5 slightly enhances the replication of PRRSV in MARC-145 cells.

4. Discussion

Host cellular proteins have been shown to be required for efficient activity of influenza virus polymerases contributing to the efficiency of viral RNA synthesis and to disease severity *via* interacting with viral proteins (Bortz et al., 2011). The interactions between viral RdRp and host cellular proteins have been recognized in some viruses (Chen et al., 2009; Goh et al., 2004; McBride et al., 1996). A previous study on equine arteritis virus (EAV) showed that a cytosolic host protein factor with a molecular mass in the range of 59–70 kDa is essential for the RNA-dependent RNA polymerase (RdRp) activity of viral replication/transcription complex (RTC) (van Hemert et al., 2008). As an important RdRp of PRRSV, the Nsp9 is involved in the replication and transcription of viral genomic and subgenomic RNAs. Recent studies have indicated the role of Nsp9 in the replication of PRRSV by interacting with host cellular proteins (J. Li et al., 2014; Dong et al., 2014). However, more studies are required to uncover a possible network of host cellular proteins interacting with this nonstructural protein in the PRRSV replication.

As the largest family of cellular RNA helicase, the DEAD-box RNA helicases have been proven to be involved in RNA metabolism (Chahar et al., 2013; Choi and Lee, 2012; Clark et al., 2008). Several members including DDX1, DDX3, DDX5 and DDX6 in this family are shown to interact with viral proteins in several viruses (Li et al., 2013; Xu et al., 2010; Yasuda-Inoue et al., 2013; Yu et al., 2011). In the present study, we first screened that porcine cellular DDX5 interacted with the Nsp9 of PRRSV by using the yeast two-hybrid method, and further confirmed this interaction in both HEK293 cells and MARC-145 cells by Co-IP assay. Our results implicate that DDX5, as a typical RNA helicase, might provide additional activities to facilitate the accomplishment of viral genomic replication and transcription although PRRSV can encode own RNA helicase, the Nsp10 with helicase activity. Recently, DDX5 has been shown to interact with human immunodeficiency virus (HIV) Rev protein that plays a part in the late phase of HIV-1 replication and to facilitate the export of unspliced viral mRNAs from nucleus to cytoplasm in infected cells (Zhou et al., 2013).

The Nsp9 of PRRSV contains different domains. In our study, we determined that the binding region with DDX5 in Nsp9 located in the C-terminal portion with an RdRp domain (aa386–640), and the DEXDc and HELICc domains (aa1–436) of DDX5 are the binding regions with the Nsp9 of PRRSV. Recent studies have demonstrated that DDX5 could interact with the core protein, NS3 and NS5 of Japanese B encephalitis virus (JEV) during viral infection. Since NS3 and NS5 of JEV can bind to 3'UTR of viral RNA that may be involved in the first round of viral protein translation and RNA replication, further research indicated that DDX5 binds to the JEV 3'UTR to enhance viral replication but not viral protein translation (Li et al., 2013). Thus, we propose that the interaction between DDX5 and Nsp9 might regulate the replication of PRRSV *via* forming a complex binding to the 3'UTR of viral genome, which needs to be further confirmed.

The interaction of viral proteins with host cellular proteins and the subsequent re-localization of those cellular proteins may play important roles in regulating the viral life cycle, especially in the replication of viruses. The endogenous DDX1 interacting with the Nsp14 of infectious bronchitis virus (IBV) has been found to be re-localized from the nucleus to the cytoplasm in virus-infected cells (Xu et al., 2010). Our results showed that DDX5 co-localized with Nsp9 in the cytoplasm with a perinuclear pattern in HEK293 cells, MARC-145 cells and PAM cell line, and meanwhile it co-localized with Nsp9 in PRRSV-infected MARC-145 cells and PAMs, suggesting that endogenous DDX5 might diffuse from the nucleus to the cytoplasm in PRRSV-infected MARC-145 cells. These findings lead us to postulate that cellular DDX5 with helicase activity might help to unwind the viral RNA to make the RdRp transcribe the RNA more efficiently during the PRRSV infection.

In this study, we further analyzed the effect of the interaction between cellular DDX5 and viral Nsp9 on the replication of PRRSV. Our results indicated that silencing of DDX5 expression in MARC-145 cells could inhibit significantly the replication of PRRSV, and while the over-expression of DDX5 in MARC-145 cells displayed an enhancement effect on the replication of PRRSV, suggesting that DDX5 might function as a cellular cofactor that positively regulates the PRRSV replication. The exact mechanism concerning this role, however, needs to be further explored. Recent studies have revealed that DDX1 and DDX3 are co-factors of HIV Rev protein to export HIV-1 transcripts (Edgcomb et al., 2012; Robertson-Anderson et al., 2011; Yasuda-Inoue et al., 2013). DDX5, as a new cellular co-factor of Rev protein, is shown to be beneficial for HIV-1 replication (Zhou et al., 2013). Thus, the recruitment of DDX5 by Nsp9 would have important significances for understanding the genomic replication and transcription of PRRSV. Further study on this issue will help to reveal new insights into the molecular mechanisms of PRRSV replication.

In summary, our present study first confirmed that the DEAD-box RNA helicase DDX5 derived from PAMs could interact with the Nsp9 of PRRSV, and then found that the endogenous DDX5 co-localized with Nsp9 in PRRSV-infected MARC-145 cells and PAMs. Moreover the knockdown of DDX5 could significantly inhibit the replication of PRRSV, while the over-expression of DDX5 slightly enhanced viral replication in MARC-145 cells. These findings indicate that DDX5 positively regulates the replication of PRRSV by interacting with viral Nsp9 *in vitro*, providing more knowledge for understanding the mechanisms associated with the replication regulation and pathogenesis of PRRSV.

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