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The interaction of nonstructural protein 9 with retinoblastoma protein benefits the replication of genotype 2 porcine reproductive and respiratory syndrome virus in vitro

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The nonstructural protein 9 (Nsp9) of porcine reproductive and respiratory syndrome virus (PRRSV) is a RNA-dependent RNA polymerase (RdRp) that plays a vital role in viral replication. This study first demonstrated that the Nsp9 of genotype 2 PRRSV interacted with cellular retinoblastoma protein (pRb), and Nsp9 co-localized with pRb in the cytoplasm of PRRSV-infected MARC-145 cells and pulmonary alveolar macrophages (PAMs). Next, the overexpression of truncated pRb was shown to inhibit the PRRSV replication and silencing the pRb gene could facilitate the PRRSV replication in MARC-145 cells. Finally, the pRb level was confirmed to be down-regulated in PRRSV-infected MARC-145 cells, and Nsp9 was shown to promote the pRb degradation by proteasome pathway. These findings indicate that the interaction of Nsp 9 with pRb benefits the replication of genotype 2 PRRSV in vitro, helping to understand the roles of Nsp9 in the replication and pathogenesis of PRRSV.

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Introduction

Porcine reproductive and respiratory syndrome (PRRS) is an important viral disease that brings about great economic losses to the pig industry worldwide (Garner et al., 2001; Neumann et al., 2005; Pejsak et al., 1997). The causative agent, PRRS virus (PRRSV), belongs to the Arterivirus genus of the Arteriviridae family, together with equine arteritis virus (EAV), lactate dehydrogenase-elevating virus (LDV) and simian hemorrhagic fever virus (SHFV) (Cavanagh, 1997). The PRRSV is divided into two major genotypes, genotype 1 (European) and genotype 2 (North American) (Nelson et al., 1993). Clinically, the viruses of the two genotypes cause a similar syndrome although they share only approximately 60% nucleotide identity of the genome (Meng et al., 1995; Nelson et al., 1993). In China, a highly pathogenic PRRSV (HP-PRRSV) of genotype 2 was recognized to cause atypical PRRS outbreaks in the pig-producing areas in 2006 (Tian et al., 2007; Zhou and Yang, 2010). A recent investigation indicated that the HP-PRRSV has become the dominating virus circulating in pig farms during the past years (Zhou et al., 2014).

The PRRSV genome is a single strand positive RNA with approximately 15 kb in length, which encodes at least ten open reading frames (ORFs) comprising of ORF1a, ORF1b, ORF2a, ORF2b, ORFs3–7 and the newly discovered ORF5a (Conzelmann et al., 1993; Firth et al., 2011; Johnson et al., 2011; Snijder and Meulenberg, 1998). ORF1a and ORF1b, the replicase-associated genes, encode two polyproteins pp1a and pp1ab, respectively. The pp1a is predicated to be cleaved into ten non-structural proteins (NspS) including Nsp1α, Nsp1β, Nsp2 to Nsp6, Nsp7α, Nsp7β and Nsp8 (den Boon et al., 1995; Fang and Snijder, 2010; Snijder and Meulenberg, 1998; van Aken et al., 2006), whereas the pp1ab can be cleaved into four NspS, Nsp9, Nsp10, Nsp11 and Nsp12, which are considered to be involved in viral replication and genomic transcription (Snijder and Meulenberg, 1998; van Dinten et al., 1996; Wassenaar et al., 1997). The transframe fusion (TF) in Nsp2-coding region was recently discovered (Fang et al., 2012). In recent years, a growing number of studies have paid much attention to the pathogenesis involved by the NspS of PRRSV. The Nsp1, Nsp2, Nsp4 and Nsp11 of PRRSV have been shown to inhibit the induction of type I interferon and modulate host innate immune response (Beura et al., 2010; Han et al., 2013; Kim et al., 2010; Sun et al., 2010, 2012). Moreover, the Nsp1β of PRRSV was shown to interact with the cellular poly(C)-binding protein (PCBP1) 1 and 2, and this interaction could facilitate viral replication and RNA transcription (Beura et al., 2011; Wang et al., 2012). A latest study indicated that the PRRSV Nsp4 could induce apoptosis dependent on its 3C-like serine protease activity (Tripp et al., 2013).
The Nsp9 of PRRSV, a RNA-dependent RNA polymerase (RdRp), is considered to be a major component of membrane-associated viral replication and transcription complex (RTC) that is important for virus replication based on the studies of the equine arteritis virus (EAV) and comparative sequence analysis (Pedersen et al., 1999). The SDD motif of Nsp9 has been recognized to be critical for its polymerase activity and virus transcription (Zhou et al., 2011). In addition to its polymerase activity, the interaction between Nsp9 and the host cellular proteins that affect viral replication is poorly understood although a recent study revealed that Nsp9 could interact with endogenous annexin A2 in PRRSV-infected MARC-145 cells (J. Li et al., 2014). Moreover, our recent publication has shown that Nsp9 and Nsp10 together not only affected the HP-PRRSV replication and growth in vitro and in vivo, but also contributed to its fatal virulence for piglets (Y. Li et al., 2014). Thus, further exploring the interaction of Nsp9 with host cellular protein(s) is essential for understanding the roles of Nsp9 in the replication and pathogenesis of HP-PRRSV.

The retinoblastoma protein (pRb) encoded by the retinoblastoma tumor suppressor gene has been shown to modulate cell cycle and suppress human tumors (Khrid and Chen, 2006; Weinberg, 1995). The proteins that bind to pRb have been recognized to share a conserved motif—LxCxE (Dahiya et al., 2000), which was first identified in the oncoproteins of DNA tumor viruses (Boyer et al., 1996; White and Khalili, 2006; Whyte et al., 1988). Some proteins of RNA viruses have been shown to bind to pRb through this motif. The interaction between the Rubella virus Nsp9 and pRb could affect viral replication (Forng and Atreya, 1999). In coronavirus, the endoribonuclelease Nsp15 could bind to pRb, resulting in the acceleration of cell cycle progression (Bhardwaj et al., 2012). Interestingly, the NS5B of hepatitis C virus (HCV), an RdRp, could interact with pRb and down-regulated the pRb level (Munakata et al., 2005, 2007). In the present study, we explored the interaction between the Nsp9 of genotype 2 PRRSV and pRb and analyzed the effect of this interaction on viral replication in vitro.

Results

The Nsp9 of genotype 2 PRRSV interacted with pRb

The conserved LxCxE motif is a key feature for viral proteins that bind to pRb (Dahiya et al., 2000). We aligned the amino acid sequences of the Nsp9 among 17 representative strains of genotype 2 PRRSV and found that the Nsp9 of all the analyzed viruses shared an identical LxCxE motif—LACAE (Table 2). To analyze whether the Nsp9 of genotype 2 PRRSV interacts with pRb through the LACAE motif, the recombinant lentiviruses that were expressing Nsp9-GFP, Nsp9 (L415A/C417A)—GFP and Nsp9 (E419K)—GFP were constructed. MARC-145 cells were transduced with the lentiviruses and the cell lysates were immunoprecipitated with an anti-GFP mAb, and were then detected with an anti-pRb mAb by Co-IP assay. The GFP-expressing lentiviruses were used as the control. As shown in Fig. 1A, the endogenous pRb could be probed in the cells that were expressing Nsp9-GFP and Nsp9 (L415A/C417A)—GFP, whereas it could not be examined in the cells that were expressing Nsp9 (E419K)—GFP, indicating that Nsp9 interacts with pRb and the third conserved residue (E) of the LACAE motif plays a crucial role in sustaining the interaction between Nsp9 and pRb. To further confirm the interaction of Nsp9 with the exogenous pRb, HEK 293T cells were co-transfected with the Myc-pRb-tru-expressing plasmid together with the HA-Nsp9-, HA-Nsp9 (L415A/C417A)— or HA-Nsp9 (E419K)-expressing plasmids, and Co-IP assays were performed. The similar results were obtained (Fig. 1B). Our results suggest that the LACAE motif indeed plays a critical role in the interaction between the viral Nsp9 and pRb.

The Nsp9 of genotype 2 PRRSV co-localized with pRb in the cytoplasm

To analyze the co-localization of Nsp9 and pRb, MARC-145 cells were co-transfected with pCMV-HA-Nsp9 and pCMV-Myc-pRb-tru. The confocal immunofluorescence assays showed that Nsp9 primarily co-localized with pRb-tru in the cytoplasm (Fig. 2A), and the endogenous pRb also co-localized with Nsp9 in the cytoplasm of PRRSV-infected MARC-145 cells (Fig. 2B) and PAMs (Fig. 2C); whereas the endogenous pRb localized in the nucleus of mock-infected MARC-145 cells and PAMs (Figs. 2D and E).

The pRb could inhibit the replication of genotype 2 PRRSV in vitro

To investigate the effect of pRb on the replication of PRRSV, we first analyzed the effect of the exogenous pRb overexpression on the replication of PRRSV. MARC-145 cells were transduced with the pRb-tru-expressing lentiviruses, and were then infected with the PRRSV JXwn06. The virus titers were assayed. As shown in Fig. 3A, the virus titers in the pRb-tru-overexpressing group significantly reduced at the different time points compared with the control group (p < 0.001), indicating that the overexpression of pRb-tru can inhibit the replication of PRRSV in vitro. To further confirm this effect, MARC-145 cells were individually transfected with three different siRNAs specific for the pRb gene. Western blotting analysis showed that the pRb level in MARC-145 cells was effectively down-regulated by these siRNAs compared with the cells transfected with the control (siCon) siRNA (Figs. 3B and C). One pair of siRNA (siRb2) was used for subsequent experiment.

Table 1

| Primer | Sequence(5’–3’)* | Product length (bp) | Use |
|--------|------------------|---------------------|-----|
| 1F     | AGCTTGTATTAAAACGACATGCTCTTGGAGATTTG(Pmel) | 1914 | pRb-tru fragment amplification and clone |
| 1R     | CCGCGAGCGCTAACTTCTCTCTTGCCTC(Mlu) | 1914 | pRb-tru fragment amplification and clone |
| 2F     | CCGGAATTCGCTCTTGGAGATTTGACCTC(TcEcoRI) | 1920 | Nsp9 amplification and clone |
| 2R     | CGCGTACCTCTTCCTCTCTCCGTTT(NotI) | 1920 | Nsp9 amplification and clone |
| 3F     | AGCTTTCTTAAACGACATGCTCTTGGAGATTTG(Pmel) | 1920 | Nsp9 amplification and clone |
| 3R     | CCGCGGACCACCTACTCTCGATTCCTGACCT(Mlu) | 1920 | Nsp9 amplification and clone |
| 4F     | TACGAAACATACCTTCAGAACGCGAACGTCTTCAGAC(NotI) | 1920 | Nsp9 amplification and clone |
| 4R     | TACGAAACATACCTTCAGAACGCGAACGTCTTCAGAC(NotI) | 1920 | Nsp9 amplification and clone |
| 5F     | AATCTCCATTAGACCGCGAACGTCTCGACGACCTAC | 5Mlu | Mutagenesis of Nsp9 (L415A/C417A) |
| 5R     | GTATGATCTCTGACCGCGAACGTCTCGACGACCTAC | 5Mlu | Mutagenesis of Nsp9 (E419K) |

* F denotes forward PCR primer; R denotes reverse PCR primer.

* Restriction sites are underlined. Mutated nucleotides are shown as italics.
The MARC-145 cells silenced with sipRb2 were infected with the virus and the virus titers were measured. The results showed that the virus titers of the sipRb2-silenced group were significantly higher than those of the control (siCon) group (p < 0.001 or p < 0.01) (Fig. 3D), indicating that silencing the pRb gene in MARC-145 cells facilitates the viral replication. The above findings suggest that pRb can inhibit the replication of genotype 2 PRRSV.

**Genotype 2 PRRSV infection down-regulated the pRb level**

The change of the pRb abundance in MARC-145 cells was analyzed following the PRRSV infection. MARC-145 cells were infected with the PRRSV, and the cell lysates were subjected to western blotting analysis. As shown in Fig. 4, the pRb abundance in PRRSV-infected MARC-145 cells reduced significantly from 36 h to 60 h post-infection compared with mock-infected cells, indicating that the PRRSV infection can down-regulate the pRb level in MARC-145 cells.

The Nsp9 of genotype 2 PRRSV could promote the degradation of pRb by ubiquitin proteasome pathway

Several studies have shown that many viral proteins that interact with pRb promoted the pRb degradation by ubiquitin proteasome pathway (Bhardwaj et al., 2012; Kalejta et al., 2003; Kehn et al., 2004; Munakata et al., 2007). The fact that the Nsp9 of genotype 2 PRRSV interacts with pRb and the PRRSV infection reduces the pRb level suggests that Nsp9 likely modulates the pRb level by ubiquitin proteasome pathway. To confirm this finding, MARC-145 cells were transduced with the Nsp9-expressing lentivirus and the pRb level was examined by western blotting. Compared with the GFP-expressing cells, the pRb level was significantly down-regulated in the Nsp9-expressing cells, whereas it recovered when the cells were treated with the proteasome inhibitor MG132 (Fig. 5A), indicating that the Nsp9 of genotype 2 PRRSV promotes the degradation of pRb. To consolidate this finding, HEK 293T cells were co-transfected with a dose-dependent increasing Nsp9 and dose-constant pRb-tru, and the pRb-tru level was examined by western blotting. The results showed that the pRb-tru level reduced gradually as the Nsp9 level increased, and it recovered in the cells treated with MG132 (Fig. 5B), indicating that the Nsp9 of genotype 2 PRRSV can down-regulate the pRb abundance by ubiquitin proteasome pathway.

To determine whether Nsp9 promotes the pRb ubiquitination leading to the pRb degradation, HEK 293T cells were co-transfected with pCMV-Myc-pRb-tru and pCMV-HA-Nsp9, and meanwhile, HEK 293T cells were co-transfected with pCMV-Myc-pRb-tru and pCMV-HA as control. The pRb-tru proteins were immunoprecipitated with an anti-Myc antibody and were then detected with an anti-ubiquitin mAb. Compared with the control group, a clear high-molecular-weight smear of the pRb-tru proteins was observed in the presence of the expressed Nsp9, showing that Nsp9 can mediate the ubiquitinization of pRb-tru (Fig. 5C).

**Discussion**

A number of viruses have been shown to directly or indirectly interact with host cellular proteins to modulate the normal cellular life activities and to facilitate their replications. In this study, we described that the Nsp9 of genotype 2 PRRSV interacted with endogenous and exogenous pRb, one of the key cell-cycle regulating proteins. A conserved motif–cycle regulating proteins. LAR protease to modulate in the Nsp9-overexpressing cells by RNA-dependent RNA polymerases (Bhardwaj et al., 2012; Corbeil and Branton, 1994; Munakata et al., 2005, 2007; Ran et al., 2013). We found the presence of a conserved motif—LACAE in the Nsp9 of genotype 2 PRRSV by aligning the amino acid sequences of the Nsp9 among 17 representative strains of genotype 2 PRRSV. Our results confirmed that the mutation of the conserved residue (E) at the position 419 in this motif fully impacted the interaction between Nsp9 and pRb, whereas the substitution of the conserved residues (L and C) at the position 415 and 417 did not affect this interaction, suggesting that the third conserved residue (E) of the LACAE motif plays an essential role in sustaining the interaction between the Nsp9 of genotype 2 PRRSV and pRb. We found no identical or similar LxCxE motif in the Nsp9 of genotype 1 PRRSV. The pRb has shown interaction with histone deacetylase3 (HDAC3) which lacks the LxCxE-like motif (Dahiya et al., 2000). Thus, whether the Nsp9 of genotype 1 PRRSV interacts with pRb or it shares other motif that binds to pRb needs to be further explored.

A study on mouse hepatitis virus (MHV) showed that the viral protein production was delayed at the presence of the pRb over-expression (Bhardwaj et al., 2012). Our results found that the overexpression of exogenous pRb-tru significantly decreased the replication of genotype 2 PRRSV in MARC-145 cells, and silencing the pRb gene obviously increased the viral replication in MARC-145 cells, suggesting that pRb might function as a restriction factor that inhibits the replication of genotype 2 PRRSV. Similar to HCV (Munakata et al., 2007), our data indicated that the pRb had a reduced level in PRRSV-infected MARC-145 cells, inferring that the decrease of the pRb abundance is likely required for the replication of PRRSV. The pRb has been shown to regulate the cell cycle and the pRb–E2F pathway plays a vital role in cell cycle progression. Previous studies in some RNA viruses have shown that the interaction of viral proteins with pRb could alter the cell cycle progression to create favorable cellular environment that is beneficial for viral replication (Nelson and Tang, 2006; Pietschmann et al., 2001; Scholle et al., 2004). Thus, we propose that the interaction between the Nsp9 of genotype 2 PRRSV and pRb might modulate the pRb–E2F pathway and altered the cell cycle progression which affects the viral replication. Further investigation for this issue will be required.

Viruses can encode some proteins to degrade the restrictive factors of host and to counteract their inhibition activities on viruses by utilizing ubiquitin proteasome pathway. The E6 of human papillomavirus has been shown to degrade the tumor suppressor p53 through ubiquitin proteasome pathway (Freedman and Levine, 1998). The Vif of human immunodeficiency virus-1 (HIV-1) and the Vpx of HIV and simian immunodeficiency virus (SIV) have been indicated to target APOBEC3G and HD domain-containing protein-1 (SAMHD1) for proteasomal degradation, separately (Ahn et al., 2012; Hrecka et al., 2011; Kobayashi, 2005). Our results clearly showed that the Nsp9 of PRRSV could promote the degradation of pRb by the ubiquitin–proteasome pathway. Thus, we suggest that the ubiquitinational degradation of pRb might be an important mechanism for the PRRSV Nsp9 to counteract the pRb function. The E3 ubiquitin ligase has been recognized as an important component of ubiquitin–proteasome pathway (Ciechanover, 1994). Similar to HCV NS5B and CoV-SARS Nsp15 (Bhardwaj et al., 2012; Munakata et al., 2007), we observed that pRb altered its distribution and co-localization with Nsp9 of PRRSV in the cytoplasm following PRRSV infection. Thus, we propose a model that Nsp9 recruited pRb to E3 ligases and modulated E3 ligases dependent degradation of pRb by proteasome pathway in the cytoplasm. It needs to be done to clarify the exact mechanism of the pRb degradation.

The pRb interacts with many cellular regulatory proteins and it participates in cell life activities, including the G1/S transition of cell cycle (Nevins, 1992), NF-IL6 and IL-8 expression during immune response (Chen et al., 1996; Zhang et al., 2000) and cell apoptosis (Morgenbesser et al., 1994; Tsai et al., 1998). A further
exploration of the mechanisms concerning how the PRRSV Nsp9 affects the pRb function will be necessary to obtain a more detailed understanding of the relationship between viral replication and pRb loss in vitro.

Taken as a whole, our findings are the first to indicate that the Nsp9 of genotype 2 PRRSV interacted with pRb, and this interaction promoted the pRb degradation by ubiquitin proteasome pathway in the cytoplasm and benefited viral replication in vitro, providing a novel insight for understanding the roles of Nsp9 in the replication and pathogenesis of PRRSV.

Materials and methods

Cells, virus and antibodies

Porcine pulmonary alveolar macrophages (PAMs) were prepared as previously described (Zhang et al., 2009) and maintained in RPMI-1640 medium (Invitrogen, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) (Hyclone Laboratories Inc., South Logan, UT). MARC-145 cells and human embryonic kidney (HEK) 293 T cells were maintained in Dulbecco’s modified Eagle medium (DME) (Invitrogen) supplemented with 10% FBS at 37 °C. The stock of PRRSV JXwn06 was used in this study (Zhou et al., 2009). Mouse anti-HA monoclonal antibody (mAb) (H3663), mouse anti-Myc mAb (M4439), rabbit anti-Myc mAb (C9596), mouse anti-β-actin mAb (A5441) and MG132 (M7449) were all purchased from Sigma-Aldrich (St. Louis, MO). Mouse anti-pRb mAb (#9309) was purchased from Cell Signaling Technology (Danvers, MA). Rabbit anti-pRb polyclonal antibody (sc-7505) and mouse anti-ubiquitin mAb (P4D1) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse anti-GFP mAb (66002-1-lg) and rabbit anti-GFP polyclonal antibody (50430-2-AP) were purchased from Proteintech (Chicago, IL).

Plasmid construction

A 1914-nt cDNA fragment of truncated porcine pRb gene (pRb-tru) was amplified by PAMs by RT-PCR with the designed primers 1F/1R (Table 1) according to the porcine pRb gene sequence available in the GenBank database (accession no. XM_001926853.4). The truncated porcine pRb gene was deduced to encode 638 amino acids (aa301–938) with an LxCxE-binding motif. The amplified product was confirmed by sequencing and then subcloned into a lentiviral expression vector pWPXL (Addgene, Cambridge, MA) to generate the pWPXL-pRb-tru vector. The pWPXL-pRb-tru was constructed using pCMV-Myc (Clontech Laboratories Inc.), generating the pWPXL-pRb-tru-GFP by PmeI/MluI sites. The pCMV-Myc-pRb-tru expression vector was amplified by PCR using the plasmid pWSK-JXwn as template (Zhou et al., 2009) and cloned into the pWPXL vector. The amplified fragments were subcloned into pWPXL and pCMV-HA (Clontech Laboratories Inc.) by PmeI/Mlu and BglI/NsiI sites to generate the pWPXL-hpRb-tru vector. The pWPXL-hpRb-tru vector was subcloned into the expression vector pWPXL, respectively. The mutants of the Nsp9 LACAE motif were generated by using the primers 5F/5R with lysine substitution of L415 (Table 1). The pWPXL-Nsp9 (E419K)–HA plasmids were constructed by using the primers 5F/5R with alanine substitution of L415 and C417 (Table 1).

Preparation of Nsp9-GFP, Nsp9 (E419K)/GFP vector was used for transfection. The pWPXL-Nsp9 (E419K)–GFP and pCMV-Nsp9 (E419K)–HA plasmids were generated by using the primers 6F/6R with lysine substitution of E419

Table 2

| PRRSV strain | GenBank accession no | LxCxE motif |
|--------------|----------------------|-------------|
| VR-2332      | AP0496869.1          | 40NLNLYLELACEHELPS* |
| NVSL-97-7985 IA 1-4-2 | AF325691.1 | 40NLNLYLELACEHELPS |
| P129         | AF494042.1           | 40NLNLYLELACEHELPS |
| JA142        | AY424277.1           | 40NLNLYLELACEHELPS |
| BJ-4         | AF331831.1           | 40NLNLYLELACEHELPS |
| HB-1(sh):2002 | AY15012.1        | 40NLNLYLELACEHELPS |
| CH-1a        | AY032626.1           | 40NLNLYLELACEHELPS |
| NB/04        | FJ36165.1            | 40NLNLYLELACEHELPS |
| JXA1-780     | FJ384883.1           | 40NLNLYLELACEHELPS |
| MLY Resp/PRRS/Repro | AF559149.1 | 40NLNLYLELACEHELPS |
| Prime pac    | DQ779791.1           | 40NLNLYLELACEHELPS |
| Ingelvac AT   | DQ988080.1           | 40NLNLYLELACEHELPS |
| SP           | AF814212.1           | 40NLNLYLELACEHELPS |
| TJ           | EU60248.1            | 40NLNLYLELACEHELPS |
| JXA1         | EF12445.1            | 40NLNLYLELACEHELPS |
| HuN4         | EF635008.1           | 40NLNLYLELACEHELPS |
| JXwn06       | EF641008.1           | 40NLNLYLELACEHELPS |

* The bold characters denote the motif.

Fig. 1. The interaction between the Nsp9 of genotype 2 PRRSV and pRb. (A) Interaction of Nsp9 with the endogenous pRb. MARC-145 cells were transduced with the lentiviruses expressing GFP, Nsp9, Nsp9 (L415A/C417A) and Nsp9 (E419K) individually. The cell lysates were immunoprecipitated with an anti-GFP mAb and followed by western blotting analysis with anti-pRb and anti-GFP antibodies. The left figure shows the Co-IP analyses of GFP, Nsp9-GFP, Nsp9 (L415A/C417A)-GFP and Nsp9 (E419K)-GFP from cell lysates and the right figure indicates the identification of GFP, Nsp9-GFP, Nsp9 (L415A/C417A)-GFP and Nsp9 (E419K)-GFP expressed in cell lysates. (B) Interaction of Nsp9 with the exogenous truncated pRb (pRb-tru). HEK 293T cells were co-transfected with the Myc-pRb-tru-expressing plasmids and the HA-Nsp9, HA-Nsp9 (L415A/C417A)–HA or HA-Nsp9 (E419K)–expressing plasmids. The cell lysates were immunoprecipitated with an anti-HA mAb and probed with anti-HA and anti-Myc antibodies. The left figure shows the Co-IP analyses of HA-Nsp9, HA-Nsp9 (L415A/C417A) and HA-Nsp9 (E419K) from cell lysates and the right figure indicates the identification of HA-Nsp9, HA-Nsp9 (L415A/C417A) and HA-Nsp9 (E419K) expressed in cell lysates.

A lentiviral packaging system including pWPXL (12257), pMD2.G (12259) and psPAX2 (12260) was available from Addgene. The lentivirus-mediated protein expression was performed according to the standard protocol. Briefly, each fragment of Nsp9, Nsp9 (L415A/C417A), Nsp9 (E419K) and pRb-tru was subcloned into the expression vector pWPXL, respectively. The plasmid pWPXL-Nsp9, pWPXL-Nsp9 (L415A/C417A), pWPXL-Nsp9 (E419K) and pWPXL-pRb-tru were mixed with pMD2.G and psPAX2 with appropriate proportion, respectively and were then co-transfected into 80% confluent of HEK 293FT cells using the FuGENE HD Transfection Reagents (Roche Applied Science, Indianapolis, IN). The cells were incubated at 37 °C. After a number of syncytias appeared, the
For the Co-IP assay of the Nsp9 and exogenous pRb-tru, HEK 293T cells were co-transfected with pCMV-HA-Nsp9 and pCMV-Myc-pRb-tru, pCMV-HA-Nsp9 (L415A/C417A) and pCMV-Myc-pRb-tru, pCMV-HA-Nsp9 (E419K) and pCMV-Myc-pRb-tru, pCMV-HA-Nsp9 and empty vector, or empty vector and pCMV-Myc-pRb-tru, respectively, using a Lipofectamine™ LTX and PLUS™ Reagents (Invitrogen). Thirty-six hours after the transfection, the cells were harvested and lysed in pre-cooled Co-IP lysis buffer. The eluted proteins from the beads were subjected to western blotting analysis using an anti-pRb or anti-GFP mAb. To distinguish light chain and GFP, an anti-GFP polyclonal antibody was used.

For the Co-IP of the Nsp9 and exogenous pRb-tru, MARC-145 cells were co-transfected with pCMV-HA-Nsp9 and pCMV-Myc-pRb-tru. Twenty-four hours after the transfection, the cells were harvested and lysed in pre-cooled Co-IP lysis buffer. The eluted proteins from the beads were subjected to western blotting analysis using an anti-pRb or anti-GFP mAb.

Confocal immunofluorescence assay

For the co-localization analysis of the Nsp9 and exogenous pRb-tru, MARC-145 cells seeded on coverslips in 24-well plates (Corning Inc., Corning, NY) were co-transfected with pCMV-HA-Nsp9 and pCMV-Myc-pRb-tru using the Lipofectamine™ LTX and PLUS™ Reagents when they were grown to approximately 70% confluence. Thirty-six hours after the transfection, the cells were fixed with 100% pre-cooled ethyl alcohol for 15 min at room temperature, and were then washed with PBS for 3 min, and incubated for 1 h at 37 °C with an anti-HA mAb and anti-Myc polyclonal antibody. After washing three times with PBS, the cells were incubated with TRITC-conjugated goat anti-mouse IgG or FITC-conjugated goat anti-rabbit IgG for 1 h at 37 °C. After three washes with PBS, the cells were stained for nuclei with DAPI (Beyotime) for 5 min at room temperature and visualized under a Nikon TE-2000E confocal fluorescence microscope (Nikon Instruments, Inc., Melville, NY).

Infection of MARC-145 cells that were expressing pRb-tru with PRRSV

MARC-145 cells that were seeded into 24-well culture plates were transduced with the lentiviruses expressing pRb-tru. Twenty-four hours after the transfection, the inoculums were removed and the cells were harvested at 24 h, 36 h and 48 h post-infection and processed by immunostaining with a mouse anti-HA mAb and anti-Myc polyclonal antibody. After washing three times with PBS, the cells were incubated with TRITC-conjugated goat anti-mouse IgG or FITC-conjugated goat anti-rabbit IgG for 1 h at 37 °C. After three washes with PBS, the cells were stained with DAPI for 5 min at room temperature and visualized under a Nikon TE-2000E confocal fluorescence microscope.

Infection of pRb-tru expression with PRRSV

For the infection of MARC-145 cells that were expressing pRb-tru with PRRSV JXwn06 at a multiplicity of infection (MOI) of 0.01. Thirty-six hours after the infection, the cells were fixed with 100% pre-cooled ethyl alcohol, and then washed with PBS for 3 min. The cells were probed with an anti-Nsp9 mAb prepared in our laboratory or anti-pRb polyclonal antibody, followed by TRITC-conjugated goat anti-mouse IgG or FITC-conjugated goat anti-rabbit IgG for 1 h at 37 °C. After three washes with PBS, the nuclei were stained with DAPI for 5 min at room temperature and visualized under a Nikon TE-2000E confocal fluorescence microscope.
Silencing pRb gene with siRNA

The siRNA for silencing pRb gene were purchased from Shanghai GenePharma (Shanghai, China). MARC-145 cells were treated with 30 pmol of siRNA using the Lipofectamine RNAiMax (Invitrogen) according to the manufacturer’s instructions. Forty-eight hours after the treatment, the cell lysates were subjected to western blotting analysis using an anti-pRb antibody. The siRNA target sequences were as follows: sipRb1 (sense), 5’-GGACAUGUGAACUUAUAUATT-3’; sipRb2 (sense), 5’-GCUCUCAGAUUCACCUUUATT-3’; sipRb3 (sense), 5’-CCAGAAGCAUUGAAAUCUTT-3’; the control siCon (sense), 5’-UUGCGGGUCUAAUCCACGATT-3’.

Infection of MARC-145 cells with PRRSV

MARC-145 cells seeded into 6-well culture plates (Corning Inc.) were infected with the PRRSV at MOI of 0.01 in DMEM with 5% FBS. The cells were harvested at 12 h, 24 h, 36 h, 48 h, and 60 h post-infection. The cell lysates were subjected to western blotting analysis using an anti-pRb antibody. Meanwhile, the lysates of mock-infected cells were served as the control.

pRb degradation assay

For the endogenous pRb degradation assay, the MARC-145 cells seeded into 6-well culture plates were transduced with the lentiviruses that were expressing Nsp9 or GFP in the presence of 8 μg/mL polybrene. After 24 h, the inoculums were removed and replaced with DMEM containing 10% FBS. The cells were treated with DMSO or 10 μM MG132 for 12 h. The cell lysates were then harvested and subjected to western blotting analysis using an anti-pRb and anti-GFP antibody.

For the exogenous pRb degradation assay, HEK 293T cells seeded into 6-well culture plates were co-transfected with the
constant pCMV-Myc-pRb-tru (0.5 μg) and dose-dependent pCMV-HA-Nsp9 (0 μg, 0.5 μg, 1 μg, 1.5 μg, 1.5 μg). The cells were then harvested and subjected to western blotting analysis using an anti-HA antibody.

The pRb ubiquitination assay

HEK 293T cells seeded into 20 cm² cell culture dish (Corning Inc.) were co-transfected with 3 μg of pCMV-Myc-pRb-tru and 9 μg of pCMV-HA-Nsp9 or empty vector. The cells were treated with 10 μM MG132 for 12 h. The cell lysates were then precipitated with an anti-Myc mAb in conjunction with Protein A Sepharose over-night at 4 °C with gentle rotation. The beads were washed three times with the lysing buffer, and were then boiled with 5 × SDS loading buffer for 5 min. The eluted proteins were used for western blotting analysis using an anti-ubiquitin mAb.

Statistical analysis

The data were expressed as means ± standard deviations (SD). Statistical significance among different groups was determined by Two-way ANOVA test of variance using the GraphPad Prism software (version 5.0). A P < 0.05 was considered to be statistically significant.

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