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PCNA promotes PRRSV replication by increasing the synthesis of viral genome

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ABSTRACT

Porcine reproductive and respiratory syndrome virus (PRRSV) is an RNA virus belonging to the *Arteriviridae* family. Currently, the strain has undergone numerous mutations, bringing massive losses to the swine industry worldwide. Despite several studies had been conducted on PRRSV, the molecular mechanisms by which it causes infection remain unclear. Proliferating cell nuclear antigen (PCNA) is a sign of DNA damage and it participates in DNA replication and repair. Therefore, in this study, we investigated the potential role of PCNA in PRRSV infection. We observed that PCNA expression was stable after PRRSV infection in vitro; however, PCNA was translocated from the nucleus to the cytoplasm. Notably, we found the redistribution of PCNA from the nucleus to the cytoplasm in cells transfected with the N protein. PCNA silencing inhibited PRRSV replication and the synthesis of PRRSV shorter subgenomic RNA (sgmRNA) and genomic RNA (gRNA), while PCNA overexpression promoted virus replication and PRRSV shorter sgmRNA and gRNA synthesis. By performing immunoprecipitation and immunofluorescence colocalization, we confirmed that PCNA interacted with replication-related proteins, namely NSP9, NSP12, and N, but not with NSP10 and NSP11. Domain III of the N protein (41–72 aa) interacted with the IDCL domain of PCNA (118–135 aa). Therefore, we propose cytoplasmic transport of PCNA and its subsequent influence on PRRSV RNA synthesis could be a viral strategy for manipulating cell function, thus PCNA is a potential target to prevent and control PRRSV infection.

1. Introduction

A highly prevalent disease, porcine reproductive and respiratory syndrome (PRRS) is caused by PRRSV and leads to miscarriage and stillbirth in sows and respiratory infections in piglets (Rossow, 1998). PRRS first emerged in the United States. Since then, it has spread globally, causing massive economic losses to the swine industry worldwide (Neumann et al., 2005). In recent years, PRRSV continues to undergo new mutations, thereby it is more difficult to prevent and control the disease.

PRRSV is a single and positive-strand RNA virus belonging to the *Arteriviridae* family. The gene length of PRRSV is approximately 15 kb. The virus genome contains 10 open reading frames (ORFs), among which ORF1a and ORF1b mainly encode viral replicases and account for approximately 80% of the viral genome. The replicases encoded by ORF1 can be further hydrolyzed into 16 nonstructural proteins. ORF2–7 encode the structural proteins (Kappes and Faaberg, 2015).

Replication and transcription complexes (RTCs), which primarily were viral nonstructural proteins (NSPs), directed the viral RNA synthesis (Yan et al., 2020; Snijder et al., 2016). PRRSV RNA synthesis

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includes gRNA and sgmRNA and its template is a negative-strand RNA strand (Wang et al., 2020). NSP9 is, an RNA-dependent RNA polymerase (RdRp), important for genome replication and sgmRNA synthesis and plays a crucial role in viral replication (Liu et al., 2016). NSP10 is an RNA helicase. During the process of viral replication, NSP10 must hydrolyze the intermediate double-stranded RNA (dsRNA) to complete the synthesis of viral RNA (Shi et al., 2020). NSP11 shows NendoU activity and plays an important role in the viral life cycle. NSP11 cleaves single-stranded RNA (ssRNA) and dsRNA at 3′-uridylate-specific sites (Nedialkova et al., 2009; Zhang et al., 2017). NSP12 is an important component of RTCs that is only involved in the synthesis of plus-strand and minus-strand sgmRNAs (Wang et al., 2019). The N protein is encoded by ORF7 and is most abundant viral protein. The basic function of the N protein is to form the viral capsids and package the viral genome (Yoo et al., 2003).

Proliferating cell nuclear antigen (PCNA) is an essential factor in DNA replication and repair, and involved in regulating the cell cycle. It surrounds double-stranded DNA to form a circular clip and can be enriched in the viral genome. PCNA Residues 1–117 form the N-terminal domain, while residues 135–258 form the C-terminal domain and the IDCL domain (residues 118–134). It has been reported that PCNA directly binds to up to 200 proteins, and the main site of interaction with most proteins is the front of PCNA, which forms a hydrophobic pocket consisting of the IDCL region, the C-terminal tail (254–257 aa), and the central ring (41–44 aa). PCNA is localized exclusively in the nucleus, however, previous studies have shown that it can enter the cytoplasm. PCNA also helps to maintain cytoskeletal integrity and binds to cytoplasmic proteins (Bhardwaj and Purohit, 2020; Choe et al., 2016; Strzalka and Ziemienowicz, 2011). A previous study revealed that PCNA could enter the cytoplasm and bind directly to BaMV replication complex, thereby reducing the viral replication (Lee et al., 2019). PCNA also interacts with the SARS-CoV-2 M protein, and M protein influences the nuclear translocation of PCNA to promote viral replication (Zambalde et al., 2022).

In our study, we investigated the potential role of PCNA in PRRSV infection to know the mechanisms by which PCNA facilitates viral replication.

2. Materials and methods

2.1. Cells and virus

Marc-145 and HEK-293 T cells were maintained in our laboratory. PAMs were isolated from specific pathogen-free piglets. The PRRSV strain XH-GD (GenBank accession no. EU624117.1) was maintained in our laboratory.

2.2. Antibodies and reagents

PRRSV anti-N-protein monoclonal antibodies (SDOW17, Korea), anti-HA antibodies (3724 S), and anti-PCNA antibodies (13110) were obtained from Cell Signaling Technology (MA, USA), and anti-PCNA antibodies (AF1363), anti-FLAG antibodies (AF519), and anti-GST antibodies (AF0174) were obtained from Beyotime (Shanghai, China). BeyoGold™ GST-tag purification resin (P2251) was obtained from Beyotime (Shanghai, China). Glutathione Sepharose 4B (17075601) was purchased from Cytiva (SF, USA). Protein A/G PLUS-Agarose (sc-2003) was obtained from Santa Cruz (CA, USA). PCNA-I1 (20454; 5 mg) was purchased from Cayman (Ann Arbor, Michigan).

2.3. Plasmid construction and transfection

PCNA cDNA was amplified from PAMs and cloned into pCAGGS-HA/mCherry-C1 expression vector. The PRRSV XH-GD N/NSP9/NSP10/NSP11/NSP12 genes were cloned into pCAGGS-FLAG/EGFP-C1 expression vector. Lipofectamine 3000 (CA, USA) or PEI reagent were used to transfect expression vectors in accordance with the manufacturer's instructions.

2.4. RNA interference

Small interfering RNAs (siRNAs) against PCNA and siRNA negative control (NC) were designed by RIB-BIO. Marc-145 cells were inoculated onto 6-well plates at the cell density of 2×10^6 cells/well, and adherent cells were transfected with siRNAs at the final concentration of 50 nM for 24 h by using RFect in accordance with the manufacturer's instructions.

2.5. GST-tagged protein expression and purification

An expression vector pGEX-4 T was used to clone and express the N gene in *Escherichia coli* BL21 cells (DE3). The proteins were induced with 0.1 mM IPTG at 16 °C under shaking condition at 200 rpm for 10 h. After centrifugation, the bacterials were resuspended in an equilibrated buffer and lysed using an ultrasonic cell crusher. GST-N and GST proteins were purified by GST-agarose in accordance with the manufacturer's instructions.

2.6. Western blotting assay

Cell samples were lysed in a cell lysis buffer (Beyotime, China) containing phenylmethyl sulfonyl fluoride (PMSF) (Beyotime, China) and a phosphatase inhibitor. The proteins were isolated by SDS-PAGE (12.5%) and transferred onto nitrocellulose membranes (Millipore, USA). After blocking the membranes with 5% skimmed milk powder (BD, USA), and the indicated antibodies were incubated overnight at 4 °C. Next, the corresponding secondary antibody was incubated for 1 h at room temperature. The membrane was analyzed by the Odyssey system and visualized the proteins.

2.7. Immunofluorescence assay

Sterilized glass coverslips were placed on each well of a 12-well plate. Marc-145 cells were cultured in DMEM containing 10% fetal bovine serum. The plasmid was transfected into Marc-145 cells for 24 h, each well was washed with PBS, and fixed with 4% ice-cold paraformaldehyde for 30 min. The cells were then permeated with 0.1% Triton X-100 for 30 min, blocked with 5% BSA for 60 min, and incubated overnight at 4 °C with the indicated antibodies. After washing with PBS, each well added the solution containing the secondary antibodies (1:1000). The wells were again washed three times with PBS, stained with DAPI for 5 min, and then washed three times with PBS. Subsequently, the glass coverslips were removed from the wells with forceps, and 10 μL antifluorescence quenching sealing solution was added to the slide. Fluorescence images were obtained using an inverted fluorescence microscope or a confocal laser scanning microscope.

2.8. Immunoprecipitation assay

For anti-FLAG/EGFP immunoprecipitation assay, HEK-293 T cells were cultured in 100-mm-diameter dishes and transfected plasmids. After 36 h, the cells were washed twice with PBS. The cells were collected and centrifuged at 14,000 × *g* for 5 min. We collected the supernatants and performed protein quantification using the BCA protein assay kit. The samples were gently stirred with 30 μL agarose-conjugated beads and 2 μL FLAG/EGFP-tagged antibodies at 4 °C. Subsequently, we washed the beads with 800 μL ice-cold PBS and added 60 μL 1 × SDS loading buffer and heated for 10 min. The supernatants were collected and stored at − 20 °C for western blotting assay.

Table 1
Primer sequence for RT-qPCR.

Primer name	Sequebce (5 ' -3 ')	Remark
Leader-F	CACCTTGCTTCCGGAGTTG	sgmRNA1–7
sgmRNA1-R	GAGAGACCGTGCACTGAGACATC	gRNA
sgmRNA2-R	CAGCCAACCGGCGATTGTGAA	sgmRNA2
sgmRNA3-R	GCAAAGCGGGCATACCGTGT	sgmRNA3
sgmRNA4-R	ACGAAGTCTGATGCTGCGGTG	sgmRNA4
sgmRNA5-R	CTGGCGTTGACGAGCACAGCA	sgmRNA5
sgmRNA6-R	CATCACTGGCGTGTAGGTAATGGA	sgmRNA6
sgmRNA7-R	GGCTTCTCCGGGTTTTTCTTCCTA	sgmRNA7
Monkey GAPDH-F	TGATGACATCAAGAAGGTGGTGAAG	Internal control
Monkey GAPDH-R	TCCTTGGAGGCCATGTGGGCCAT	Internal control
Sus-swine GAPDH-F	CCTTCCGTGTCCCTACTGCCAAC	Internal control
Sus-swine GAPDH-R	GACGCCTGCTTCACCACCTTCT	Internal control

2.9. In vitro antiviral efficacy assay

Marc-145 cells were grown as monolayers in 12-well plates and inoculated by PRRSV (MOI=0.1) for 2 h. We discarded the supernatant, and added fresh DMEM containing different concentrations of PCNA-I1. Cell proliferation was measured by MTT assay at the specified time period. Cells were collected for subsequent RT-qPCR and western blotting assay.

2.10. Nuclear and cytoplasmic extraction assay

For cells, harvest with trypsin-EDTA and then centrifuge at 500g for 5 min. Wash cells by with PBS and transfer cells to a 1.5 mL microcentrifuge tube, pellet by centrifugation at 500g for 2–3 min. Use a pipette to carefully remove and discard the supernatant, leaving the cell pellet as dry as possible and add ice-cold CER I to the cell pellet, Vortex

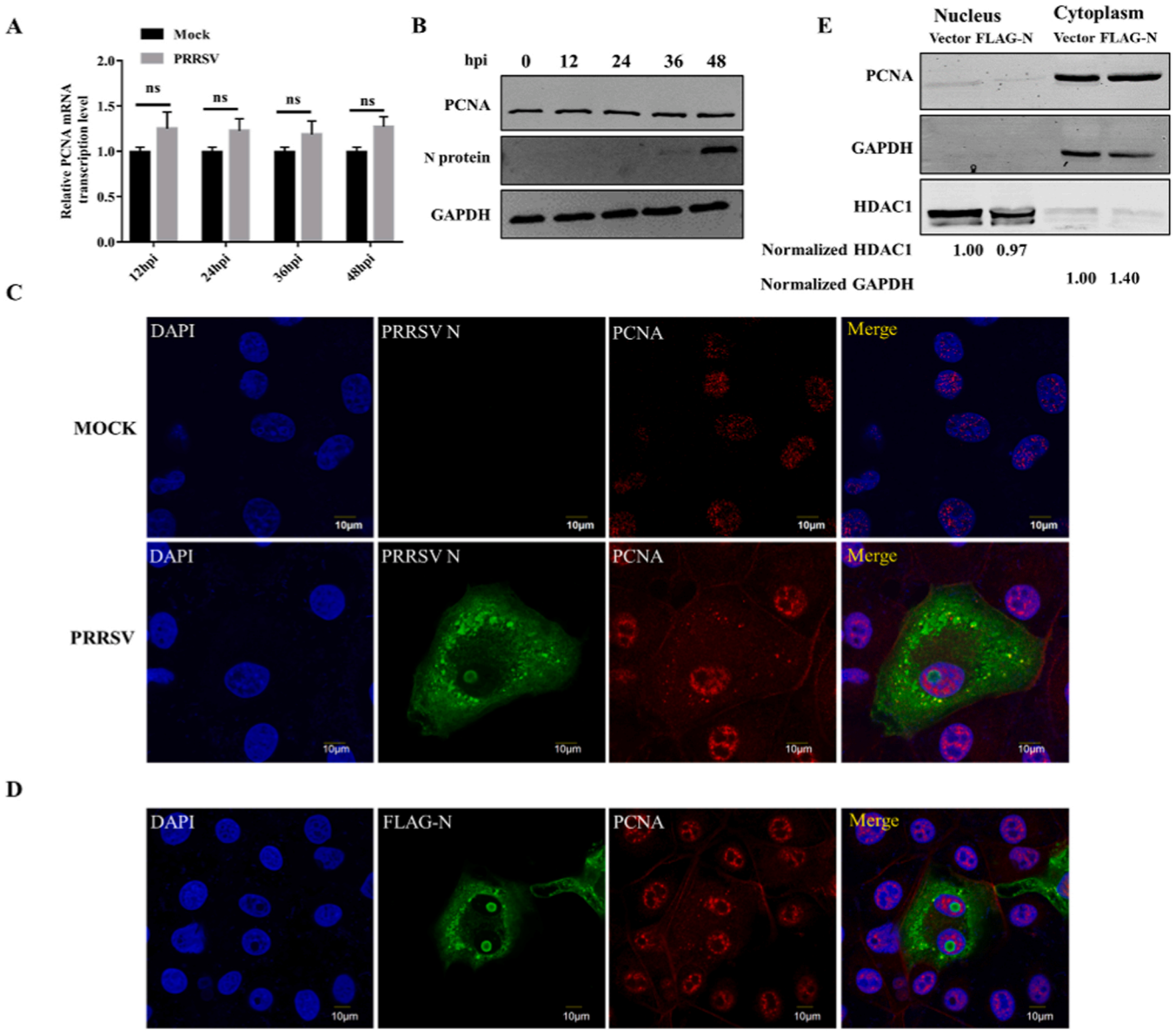

Fig. 1. PCNA expression maintain stable during PRRSV infection. PAMs were infected with mock infected or PRRSV (MOI = 0.1), and cells were collected at the specified time points. (A) The relative abundance of PCNA mRNA was analyzed by RT-qPCR. (B) The endogenous PCNA protein expression level was detected by western blotting assay. (C) Marc-145 cells were infected with PRRSV (MOI = 0.1) and the confocal assay was performed to observe the localization of PCNA. (D) HEK-293 T cells were transfected with an empty vector/FLAG-tagged N protein, and nuclear and cytoplasmic extraction was performed after 48 h. Western blotting assay was performed to analyze the protein expression level. GAPDH and HADC1 served as cytoplasmic and nuclear protein controls, respectively.

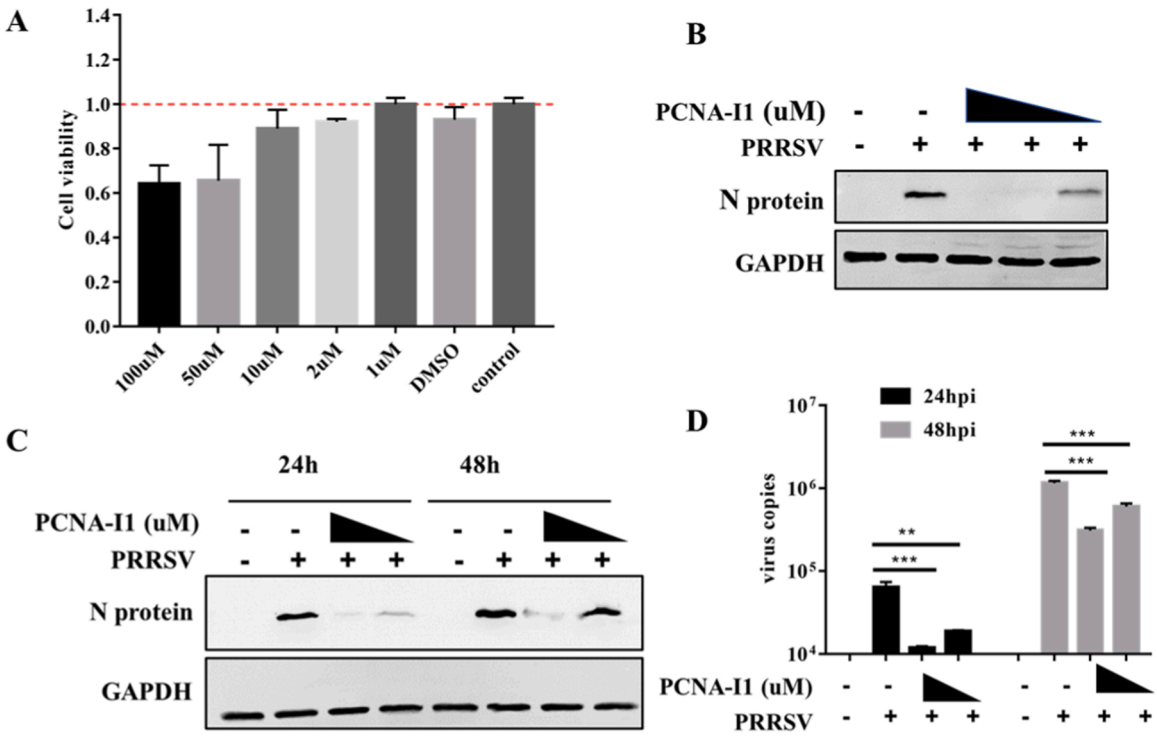

Fig. 2. PCNA-I1 inhibits PRRSV replication. (A) The safe concentration of PCNA-I1 was determined by CCK-8 assay. (B) Marc-145 cells were infected with PRRSV (MOI=0.1) for 2 h at 37 °C and then treated with varying concentrations of PCNA-I1. At 36 hpi, the inhibitory effect of different concentrations of PCNA-I1 on PRRSV was determined by western blotting assay. (C) Marc-145 cells were infected with PRRSV for 2 h, and PCNA-I1 (1 and 2 μM) was then added. The cell samples were collected at the indicated time point. Western blotting assay was performed to analyzed N protein expression level. (D) Viral load was analyzed by RT-qPCR. The results were confirmed by three independent experiments. Significant differences were indicated as follows: * $P < 0.05$, * * $P < 0.01$, and * ** $P < 0.001$.

the tube vigorously to fully suspend the cell pellet and incubate the tube on ice for 10 min. Add ice-cold CER II to the tube and vortex the tube for 5 s. Incubate tube on ice for 1 min. and vortex the tube for 5 s. Centrifuge the tube for 5 min at maximum speed in amicrocentrifuge (~16,000g). Immediately transfer the supernatant (cytoplasmic extract) to a clean pre-chilled tube. Suspend the insoluble (pellet) fraction in ice-cold NER, which contains nuclei. Vortex on the highest setting for 15 s. Place the sample on ice and continue vortexing for 15 s every 10 min, for a total of 40 min.Centrifuge the tube at maximum speed (~16,000g) in a micro-centrifuge for 10 min.Immediately transfer the supernatant (nuclear extract) fraction to a clean pre-chilled tube.

2.11. Quantitative real-time PCR assay

We extracted total RNA using a total RNA rapid extraction kit (Fastagen, Shanghai, China) and converted into cDNA by the Reverse Transcriptase of MLV. RT-qPCR was performed using Pro Taq HS Probe Premix E (Probe qPCR) and ChamQ Universal SYBR qPCR Master Mix in a CFX96 real-time system (Bio-Rad, USA). The mRNA expression levels were calculated using standard curves and the $2^{-\Delta\Delta CT}$ method. All primers for experiment were listed in Table 1.

2.12. Statistical analysis

All experiments were performed in triplicates. Data was expressed as mean ± standard deviation (SD). Statistical analysis was performed using ANOVA followed by Tukey's t-test with GraphPad Prism 7.0. A $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. PRRSV infection does not affect PCNA expression but induces its translocation to the cytoplasm

Proliferating cell nuclear antigen (PCNA) is a highly conserved protein that controls several important cellular processes (Strzalka and Ziemienowicz, 2011). Therefore, we investigated the importance of PCNA in PRRSV replication. First, to understand how PCNA is affected during PRRSV infection. PAMs were subjected to PRRSV infection or mock infection, the PCNA expression levels were analyzed by RT-qPCR and western blotting assay at the specified time periods. The results verified that the mRNA transcription and protein levels of PCNA in PRRSV-infected cells were not significantly different from those in mock-infected cells (Figs. 1A, 1B). We also found that some endogenous PCNA proteins were significantly redistributed into the cytoplasm after PRRSV infection (Fig. 1C). In order to understand more clearly which viral proteins are involved in this process, and in the experiment, N protein antibodies represent viral infection. The N protein was expressed and transfected into Marc-145 cells. The fluorescence was then assessed by wide-field microscopy. We observed that the transfected cells showed more PCNA expression in the cytoplasm than the nontransfected cells. Subsequently, we transfected empty plasmids and the N protein for nucleo-cytoplasmic separation and observed that PCNA was distributed more in the cytoplasm than control group (Fig. 1D).

3.2. Stabilization of the PCNA trimer by PCNA-I1 inhibits PRRSV replication

Our results suggested that PCNA was redistributed to the cytoplasm after PRRSV infection. We analyzed the role of this redistribution in the viral replication cycle, a transport inhibitor was used to block PCNA migration into the cytoplasm and to determine its possible antiviral

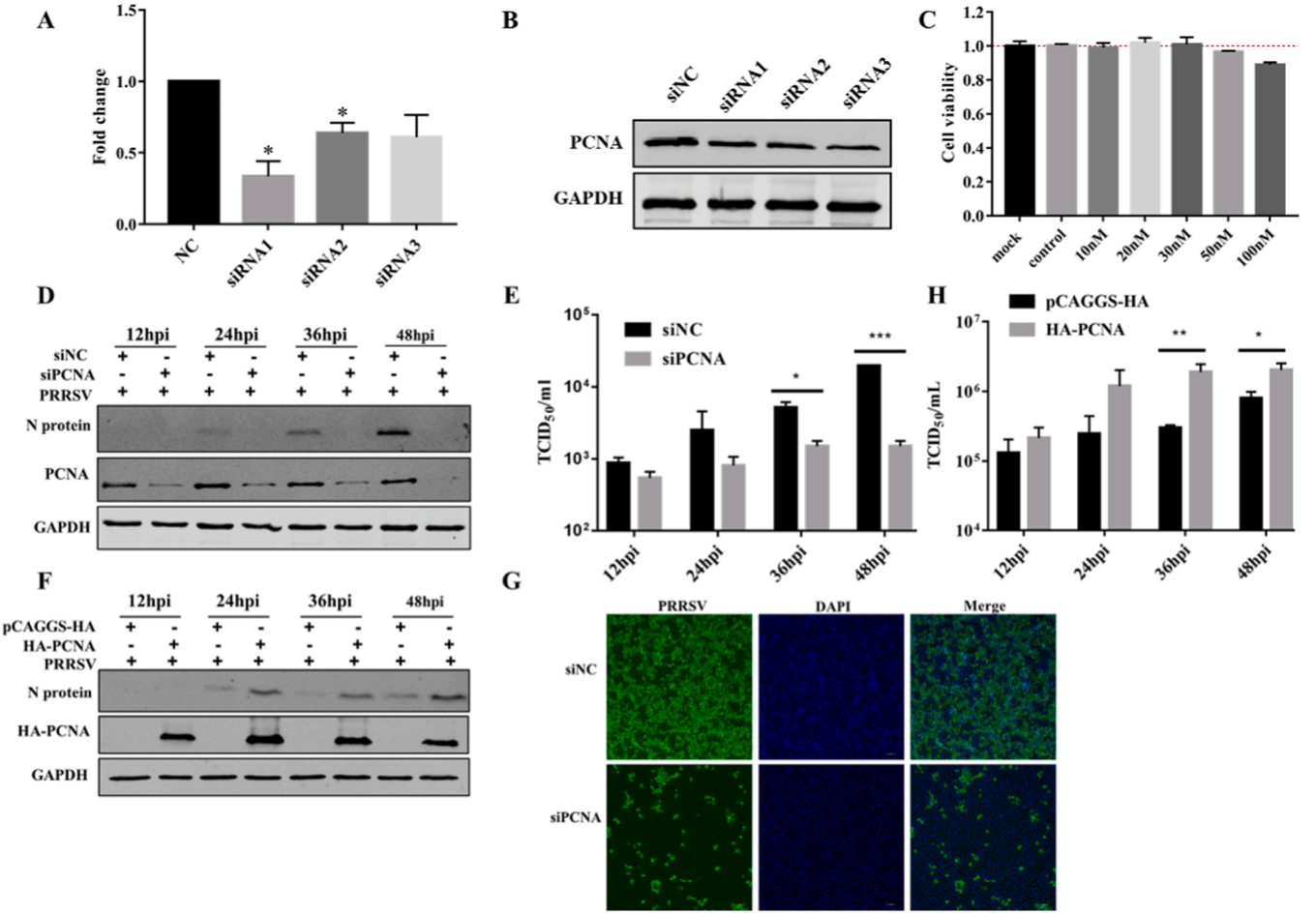

Fig. 3. PCNA contributes to PRRSV replication. (A) siNC or three siPCNA were used to transfect Marc-145 cells for 24 h, RT-qPCR analysis showed the expression level of the PCNA gene. (B) the PCNA protein expression level was analyzed by western blotting assay. (C) Assay of the activity of Marc-145 cells treated with varying concentrations of siRNA. (D) Marc-145 cells were transfected with siNC or siPCNA for 24 h and then infected with PRRSV (MOI = 0.1) at the indicated time point. Protein levels are analyzed by western blotting assay. (E) Cell supernatants after infection were collected for virus titration method. (F) Marc-145 cells were processed as described above and performed immunofluorescence assay (scale bar: 200 μm). (G) Marc-145 cells were transfected with vector or HA-tagged PCNA for 24 h and then infected with PRRSV (MOI = 0.1) for the indicated time period. Protein levels were detected by western blotting assay. (H) Virus production in Marc-145 cells was measured by virus titration method. Significant differences are indicated as follows: * $P < 0.05$, * * $P < 0.01$, and * ** $P < 0.001$.

activity. PCNA-I1 is a novel class of small-molecule inhibitors that can directly bind to the PCNA trimer, stabilize the trimer structure, reduce PCNA binding to the chromatin, inhibit DNA replication, and selectively inhibit tumor cell growth (Lu and Dong, 2019; Dillehay et al., 2014). Toxicity tests of PCNA-I1 were performed in Marc-145 cells, and the results showed that 0.1–10 μM concentration of PCNA-I1 did not decrease cell viability. (Fig. 2A). We tested the effects of different concentrations of PCNA-I1 on viral replication. The results showed that PCNA-I1 reduced PRRSV replication in a dose-dependent manner (Fig. 2B). To determine the optimal period during which PCNA-I1 affected viral replication, the cells were infected before treatment with the inhibitor, and cells were collected at the indicated time points for western blotting assay and RT-qPCR analysis. The results showed that the PRRSV N protein levels (Fig. 2C) and virus copy number (Fig. 2D) were consistently reduced after the treatment of cells with PCNA-I1. These results revealed that, in vitro, PCNA-I1 continuously reduced PRRSV infection in a dose-dependent manner.

3.3. PCNA promotes PRRSV infection

To gain insights into how endogenous PCNA plays a role in PRRSV infection, three siRNAs were designed to reduce PCNA expression. siRNA2 can significantly reduce the mRNA and protein levels of PCNA by RT-qPCR and western blotting assay. (Figs. 3A, 3B), it was selected for the subsequent experiments, and the cellular activity of siRNA at different concentrations was examined (Fig. 3C). PCNA silencing significantly reduced N protein levels (Fig. 3D). The measurement of viral titers at different time points after PRRSV infection showed that PCNA silencing reduced lower virus load at 36 and 48 hpi (Fig. 3E). Immunofluorescence assay also found that PCNA silencing significantly inhibited viral replication in Marc-145 cells (Fig. 3F). Next, PCNA was overexpressed in Marc-145 cells. We found that PCNA overexpression promoted N protein expression (Fig. 3G). Moreover, PCNA overexpression significantly increased virus production in Marc-145 cells as compared to that in controls (Fig. 3H). In conclusion, PCNA promotes PRRSV replication.

3.4. PCNA facilitates the synthesis of viral shorter sgmRNA and gRNA

To analyze the reason through which PCNA promotes PRRSV replication, PCNA expression was downregulated in Marc-145 cells by transfecting an siRNA targeting the PCNA gene (siPCNA), cell samples were collected at 36 h. Western blotting assay showed that siPCNA significantly decreased the protein level of PCNA (Fig. 4A). PRRSV gRNA synthesis level and some short sgmRNAs was significantly decreased after silencing PCNA expression (Fig. 4B). This result

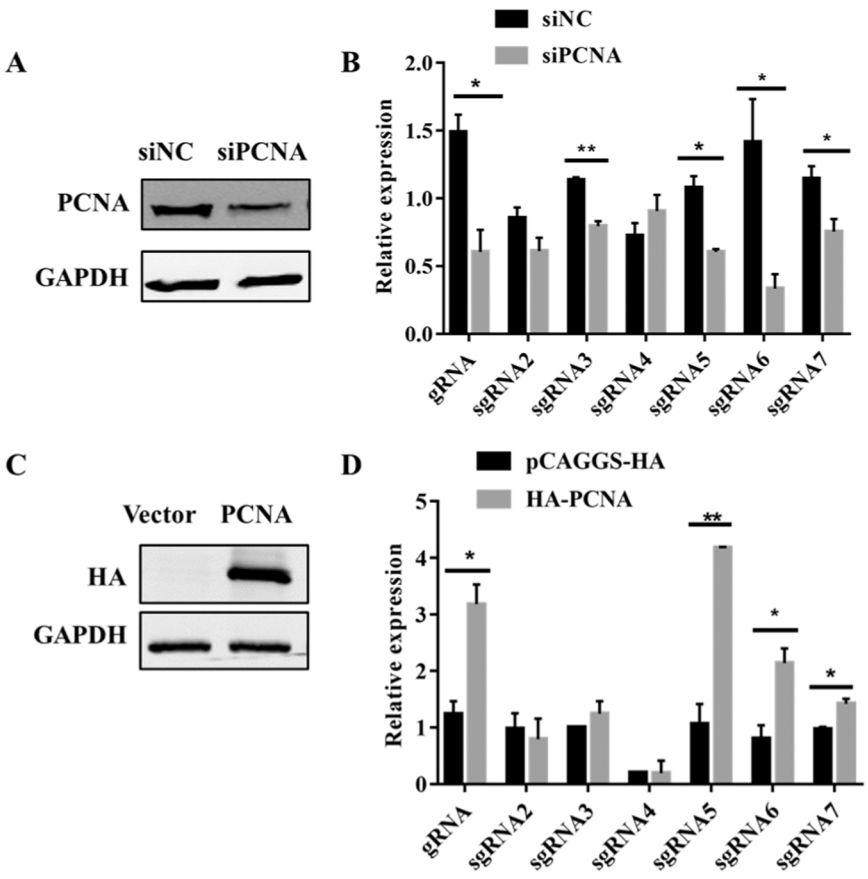

Fig. 4. PCNA affects viral RNA synthesis. Marc-145 cells were transfected with siNC or siPCNA for 24 h and then infected with PRRSV (MOI = 0.1). (A) The expression level of the PCNA protein was analyzed by western blotting assay. (B) The viral RNA level was determined by RT-qPCR and normalized to 1 for control group. Marc-145 cells were infected with PRRSV (MOI = 0.1) after transfection with plasmids. (C) Western blotting assay was performed to analyze the PCNA protein expression level. (D) The viral RNA level was monitored by RT-qPCR and normalized to 1 for the empty vector treatment. The data shown are the average of the results of three independent experiments, with error lines indicating standard deviations. Significant differences were indicated as follows: * $P < 0.05$, * * $P < 0.01$, * ** $P < 0.001$.

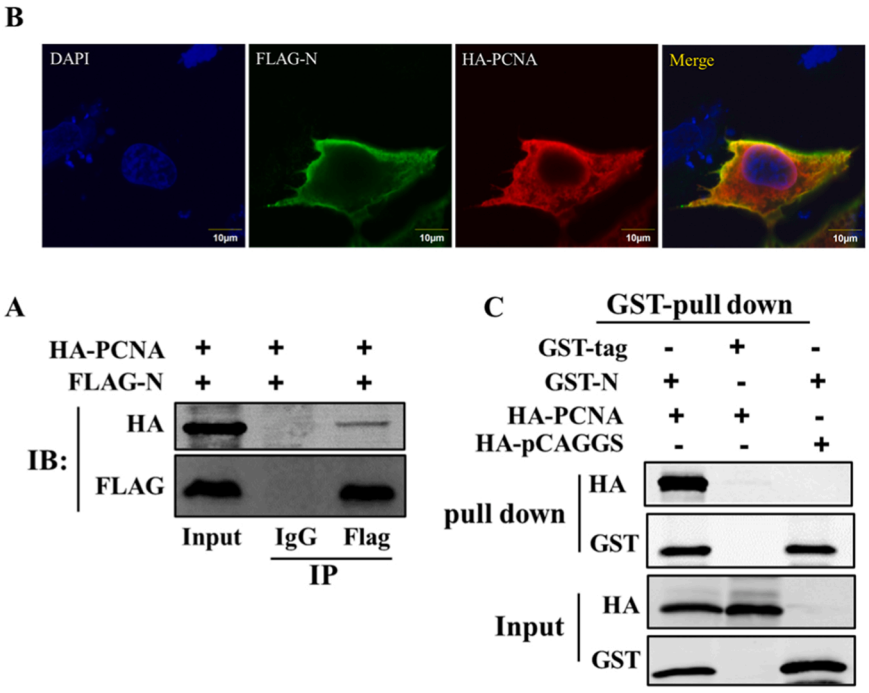

Fig. 5. PRRSV N proteins interacts with PCNA. (A) HEK-293 T cells were transfected with FLAG-tagged N protein and HA-tagged PCNA, Anti-FLAG antibodies were used for immunoprecipitation assay. (B) The successfully constructed plasmids were transfected into Marc-145 cells, and confocal assay was performed after 24 h to analyze the colocalization between them. (C) A pull-down assay was performed using the purified protein. The purified fusion protein GST/GST-N and the PCNA protein transfected HEK-293 T cells were incubated at 4 °C for 6 h. The protein mixtures were bound to GST agarose gels. Proteins were eluted and then detected by western blotting assay.

preliminarily indicated that the cellular protein PCNA facilitated viral RNA synthesis. Next, we transfected HA-tagged PCNA plasmid prior to PRRSV infection, Western blotting assay was performed to confirm PCNA overexpression level (Fig. 4C). We observed the synthesis levels of short sgmRNAs and gRNA were significantly increased but the synthesis levels of sgmRNA2, sgmRNA3, and sgmRNA4 were no significant changes compared with the control group. (Fig. 4D). These results indicated that PCNA facilitated the synthesis of PRRSV viral gRNA and

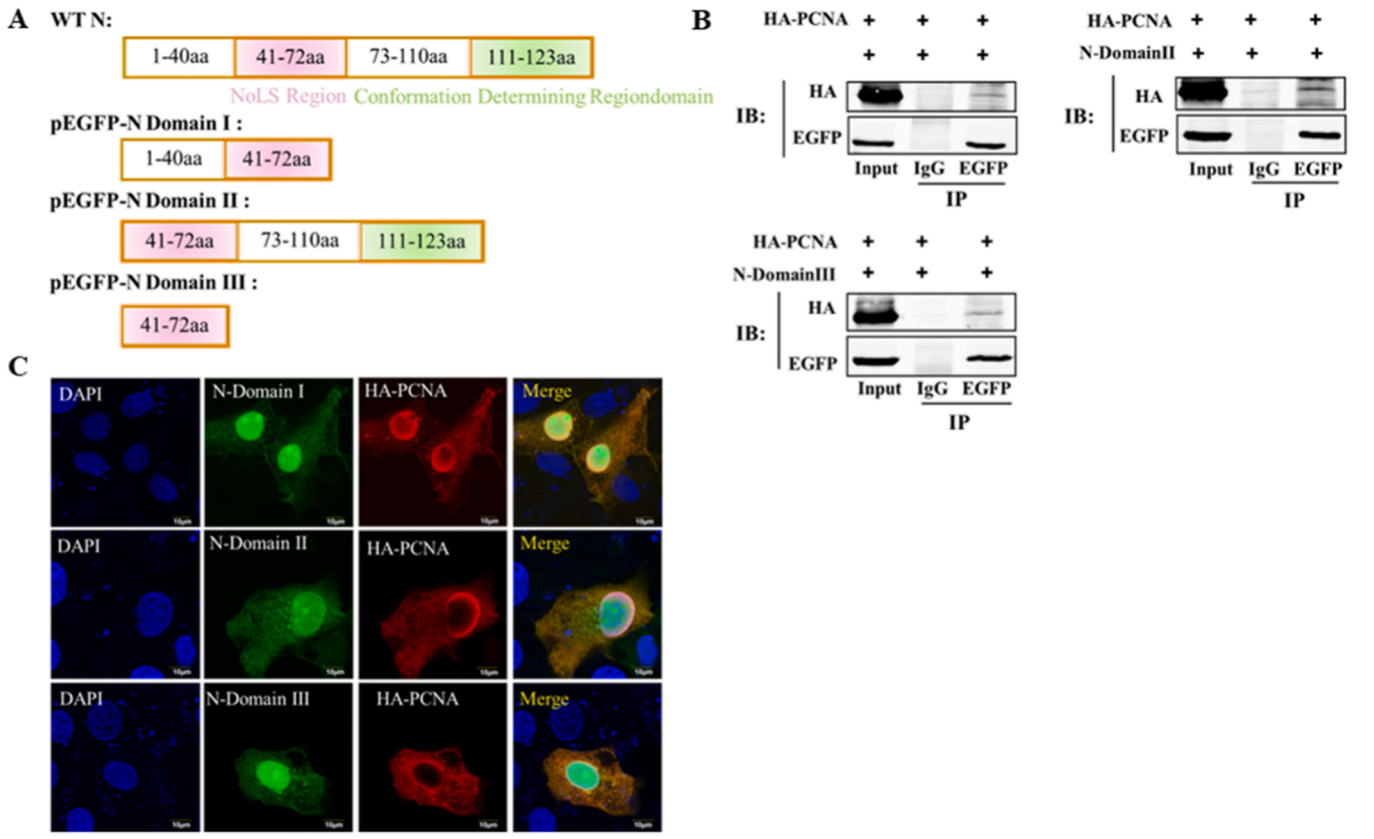

Fig. 6. Domain III of the N protein interacts with PCNA. (A) Diagram of N protein truncations. (B) HA-tagged PCNA and EGFP-tagged domain I/domain II/domain III proteins were co-transfected into HEK-293 T cells. The cells were collected at 36 h and immunoprecipitated with anti-EGFP antibodies. (C) Marc-145 cells were transfected with plasmids, and confocal assay was performed after 24 h to analyze the colocalization between them. The nuclei were stained with DAPI (blue) (scale bar: 10 μm).

short sgmRNAs.

3.5. PRRSV N proteins interact with PCNA

The N protein binds to full-length gRNA and all six sgRNAs, but it has a strong affinity for gRNA (Kappes and Faaberg, 2015), The N protein could recruits PCNA proteins to transfer from the nucleus to the cytoplasm, we investigated the interaction of N proteins with PCNA. First, the plasmids were transfected into 293 T cells, we collected cells for immunoprecipitation with anti-FLAG antibodies. Immunoprecipitation assay confirmed the N protein interacted PCNA (Fig. 5A). We also found that PCNA was colocalized with the N protein by confocal assay (Fig. 5B). Next, we expressed the recombinant protein GST-N in *Escherichia coli* (BL21) and purified the GST-N protein for pull-down assays. We found that the N protein could interact directly with PCNA (Fig. 5C). These results indicated that PCNA could interact with PRRSV N protein.

3.6. N protein domain III interacts with PCNA

The N protein is divided into the N-terminal RNA-binding domain and the C-terminal dimerization domain. Several domains of the PRRSV N protein are involved in the nucleo-cytoplasmic shuttle, including the covert nuclear localization signal (NLS) called NRS-1, functional NLS (NRS-2), nucleolus localization sequence (NoLS), and possible nuclear export signal (NES) (Rowland and Yoo, 2003). To identify the major sequences required for N-PCNA interactions, three N-fusion structures were constructed based on the structure and function of the N protein (Fig. 6A). The domain I/domain II/domain III and PCNA were co-transfected into HEK-293 T cells, and the cell lysate was immunoprecipitated with anti-EGFP antibodies. Domain III was targeted to bind PCNA (Fig. 6B). Subsequently, EGFP-tagged fusion fragments and the PCNA protein were expressed in Marc-145 cells, and confocal assay revealed that domain III of the N protein was ultimately colocalized with PCNA (Fig. 6C). These results confirmed that domain III of the N protein interacted with PCNA.

3.7. The IDCL region of PCNA interacts with the N protein

The main site of interaction with most partner proteins is the frontal side of PCNA, which consists of the IDCL region, a C-terminal tail (254–257 aa), and a central ring (41–44 aa) (Bhardwaj and Purohit, 2020). We assessed which domain of PCNA is responsible for its interaction with the N protein. Based on this structure, we first expressed the protein as domain I and domain II (Fig. 7A) and then co-transfected HA-tagged domain I/domain II and FLAG-tagged N into HEK-293 T cells. We observed that domain I/domain II coimmunoprecipitated with the N protein by Immunoprecipitation assay (Fig. 7B). Subsequently, we truncated the 81–135 region to domain III and domain IV. We confirmed the interaction between them by coimmunoprecipitation assay. Finally, we found that domain IV could specifically bind to the N protein but not domain III (Fig. 7B). We then co-transfected HA-tagged domain I/domain II/domain III/domain IV and FLAG-tagged N into Marc-145 cells. Confocal assay revealed that domain I/domain II/domain IV colocalized with the N protein (Fig. 7C). These results confirmed that the IDCL region of PCNA (118–135 aa) interacted with the N protein.

3.8. PRRSV RTCs interact with PCNA

The assembly of RTCs is a key step in viral RNA synthesis. The core

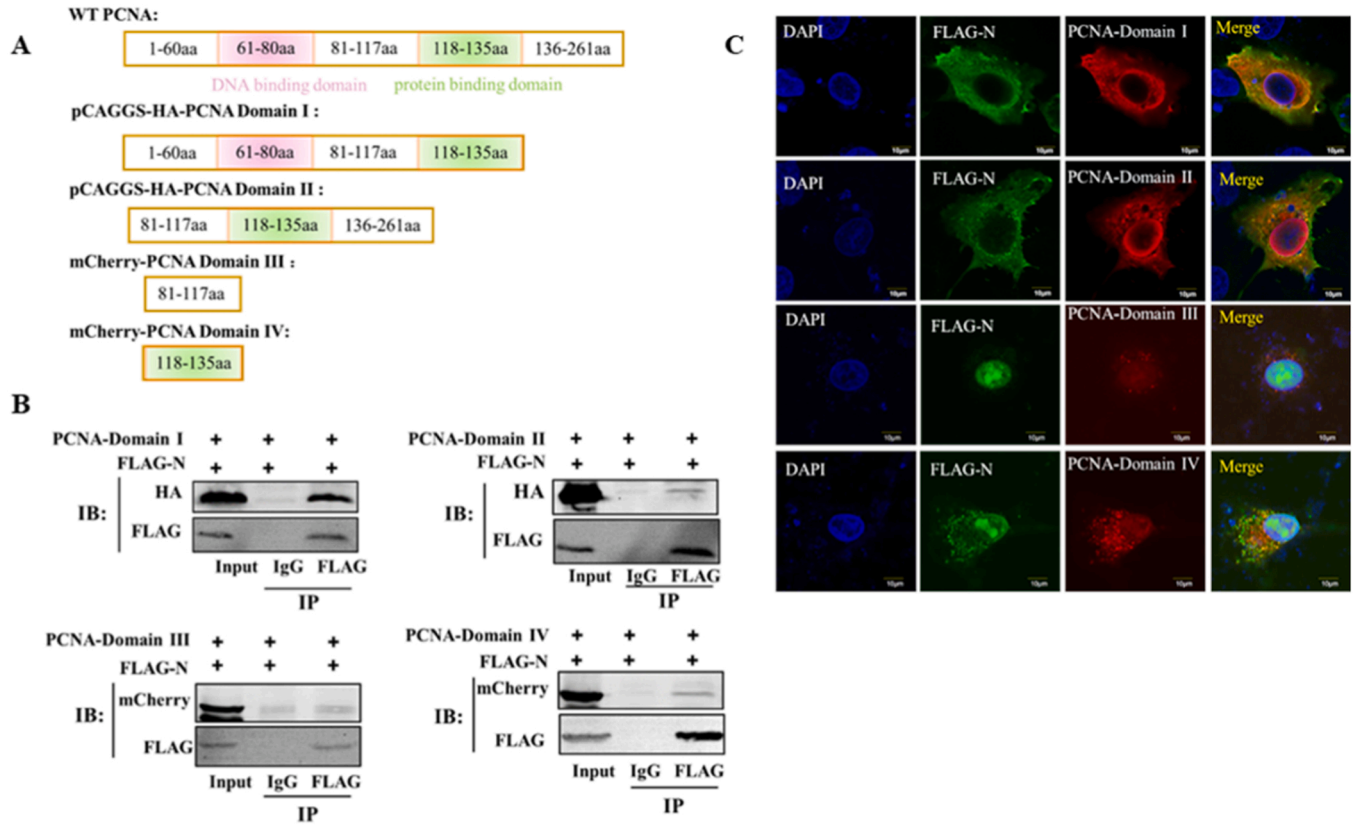

Fig. 7. The IDCL region of PCNA interacts with the N protein. (A) Diagram of PCNA truncations. (B) HEK-293 T cells were co-transfected with HA-tagged domain I/domain II and mCherry-C1-tagged domain III/domain IV and FLAG-tagged N protein. The cells were collected at 36 and immunoprecipitated with anti-FLAG antibodies. (C) Plasmids were transfected into Marc-145 cells, and confocal assay was performed after 24 h to analyze the colocalization between them. The nuclei were stained with DAPI (blue) (scale bar: 10 μm).

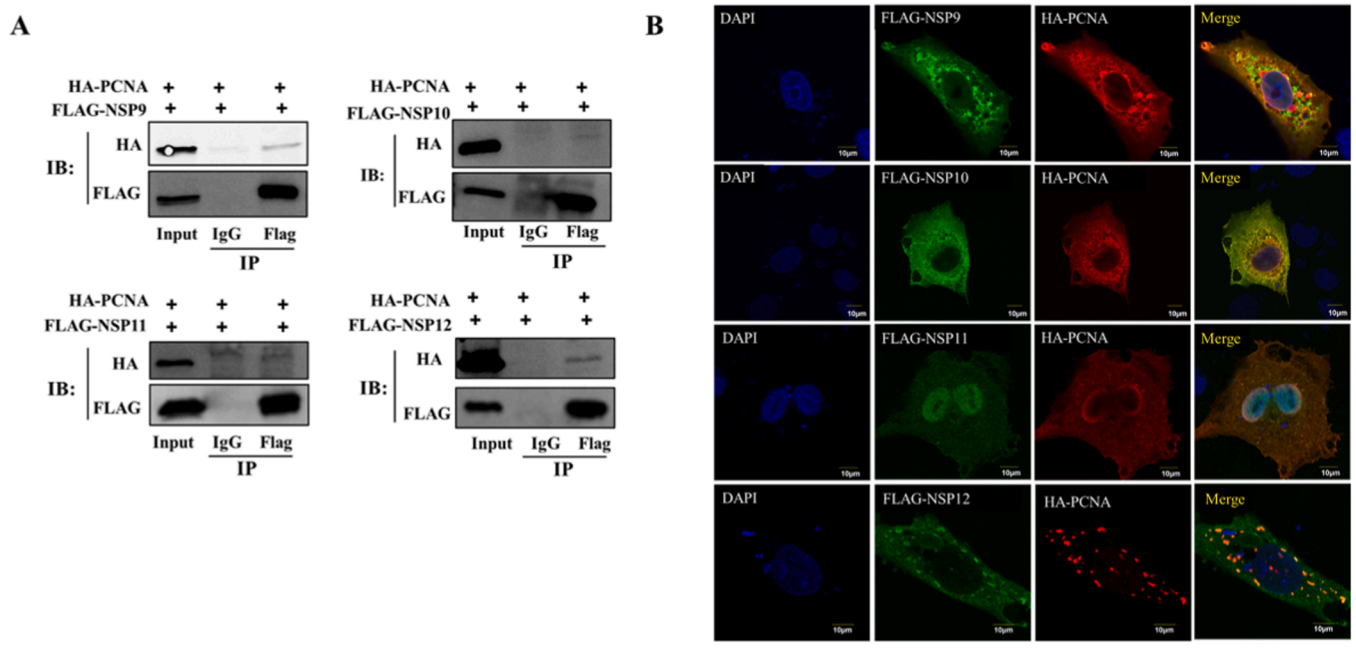

Fig. 8. PRRSV RTCs interact with PCNA. (A) HEK-293 T cells were co-transfected with HA-tagged PCNA and FLAG-tagged RTC protein. The cells were collected at 36 and immunoprecipitated with anti-FLAG antibodies. (B) The successfully constructed plasmids were transfected into Marc-145 cells, and confocal assay was performed after 24 h to analyze the colocalization between them.Nuclei were stained with DAPI (blue), (scale bar: 10 μm).

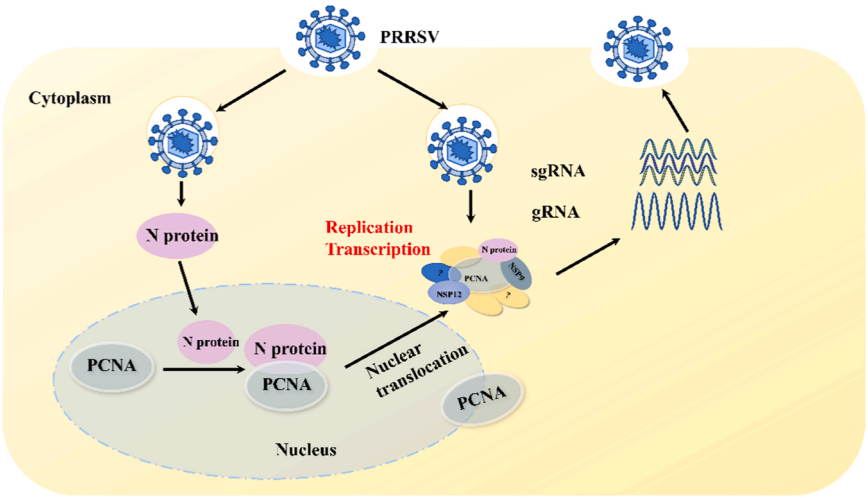

Fig. 9. A model diagram describing the role of PCNA in PRRSV infection. PCNA was recruited into the cytoplasm by the N protein and interacted with the PRRSV virus protein to further contribute to PRRSV RNA synthesis.

components of PRRSV RTCs mainly include some NSPS encoded by ORF1b (Nan et al., 2018). Based on the above-mentioned results, PCNA could affect viral RNA synthesis. We explored the interaction between PCNA and viral RTC. We found that NSP9 and NSP12 were specifically immunoprecipitated by PCNA (Fig. 8A). We also found that PCNA was colocalized with the NSP9 and NSP12 not NSP10 and NSP11 protein by confocal assay (Fig. 8B). These results confirmed that the PCNA interacted with the NSP9 and NSP12 protein.

On the basis of the above results, we proposed a model describing the function of PCNA in PRRSV infection (Fig. 9). As a novel protein, PCNA may interacted with the viral protein and facilitate PRRSV RNA synthesis.

4. Discussion

In recent years, with the continuous variation in the structure of PRRSV strains, it is necessary to investigate the underlying mechanism of PRRSV infection. The N protein is a multifunctional protein and may encapsulate the viral genomic RNA (Yoo et al., 2003). The N protein of equine arteritis virus (EAV) colocalizes with the viral replication complex in early infection (Dokland, 2010). The assembly of RTCs occurs on intracellular membranes where the transcription and replication cascades can coordinate. Consequently, it is an essential and rate-limiting step to assemble correct and efficient RTC for efficient replication (Knoops et al., 2012).

As an important host factor, PCNA interacts with a variety of viruses. For example, PCNA can interacted with agnoprotein and inhibit the effect of the agnoprotein on DNA synthesis, it may switch off the assembly of replicated genomes (Hu et al., 2021). PCNA significantly enhances hepatitis B virus (HBV) replication and accelerates hepatocellular carcinoma (HCC) growth (Gerits et al., 2015). Therefore, PCNA was selected for further investigation in our study. We confirmed that PCNA promoted PRRSV infection through knockdown and overexpression of PCNA level (Fig. 3). Next, we investigated whether PRRSV affected the PCNA expression level for their own benefit. Interestingly, PCNA mRNA and protein levels is stable in PRRSV-infected or mock-infected cells. However, after PRRSV infection, PCNA translocated more to the cytoplasm (Fig. 1). Similar to our results, a previous study on SARS-CoV-2 reported that PCNA translocated from the nucleus to the cytoplasm, which may regulate the metabolism of the infected cells to maintain viral replication(Zambalde et al., 2022).

Previous studies on plant RNA viruses reported that PCNA had a binding affinity to BaMV genomic RNA, this PCNA-RNA interaction might inhibit BaMV replication (Lee et al., 2019). Overexpression PCNA increased the accumulation of TBSV replicator RNA in Saccharomyces cerevisiae(Shah et al., 2012). PCNA might be immunoprecipitated the BMRF1 replication protein of Epstein-Barr virus through the viral DNA genome. Moreover, PCNA loading might trigger the transfer of a series of host MMR proteins to viral DNA synthesis sites (Daikoku et al., 2006). In our study, we found that overexpression PCNA promoted the synthesis of viral gRNA and sgmRNAs, while PCNA knockdown had the opposite effect (Fig. 4). Therefore, we speculate that PCNA promotes viral replication by promoting viral RNA synthesis.

PCNA can bind with many replication-related proteins to regulate DNA replication. The discovery of the interaction of five SIRV2 proteins with PCNA provides insights into the recruitment of host replication proteins for viral DNA replication (Gardner et al., 2014). Some PCNA molecules were found to interact with the BaMV replication complex in the cytoplasm and participated in the assembly of the viral replication complex (Liu et al., 2016). Here, we found that PCNA interacts with PRRSV replication-related proteins, namely NSP9, NSP12 and N by confocal, GST-pull down, and immunoprecipitation assays. The N protein coordinates protein function during viral infection and recruits them to promote viral replication (Yoo et al., 2010). For example, the N protein may recruit PARP-1, which is beneficial to viral RNA replication and transcription processes (Liu et al., 2015). During PRRSV infection, the N protein may recruit DHX9 to regulate viral RNA synthesis(Liu et al., 2016). In our study, we observed that PCNA interacted with the N protein (Fig. 5), and the redistribution of the endogenous PCNA protein from the nucleus to the cytoplasm was observed in N protein-transfected cells, thus suggesting that the PCNA protein may be recruited by the N protein to the RTCs to regulate viral RNA synthesis (Fig. 1). NSP9 is a key enzyme for RNA synthesis, DDX5 may interact with NSP9 in vitro and promote PRRSV replication (Zhao et al., 2015).

At present, it is few to know the interaction between cellular proteins and NSP12. Previous studies have shown that NSP12 is essential for viral sgmRNA synthesis (Wang et al., 2019). Cellular proteins can be recruited by NSP12 such as HSP70 to maintain its own stability and promote viral replication (Dong et al., 2016). In our study, we found that PCNA may interact with NSP9 and NSP12 in vitro. This implies that PCNA may be assembled into RTCs.

The present study has some limitations. First, some PRRSV NSPs show unstable expression, and we cannot rule out their interaction with PCNA. Second, it needs to be determined whether PCNA affects viral RNA synthesis by interacting directly with PRRSV-replication proteins. Finally, it is essential to determine how PCNA-I1, an inhibitor that targets the PCNA protein, affects viral replication.

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Declaration of Competing Interest

The authors declare no conflict of interest.

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