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ORF5a Protein of Porcine Reproductive and Respiratory Syndrome Virus is Indispensable for Virus Replication

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In this study, a DNA-launched reverse genetics system was developed from a type 2 porcine reproductive and respiratory syndrome virus (PRRSV) strain, KNU-12. The complete genome of 15,412 nucleotides was assembled as a single cDNA clone and placed under the eukaryotic CMV promoter. Upon transfection of BHK-tailless pCD163 cells with a full-length cDNA clone, viable and infectious type 2 progeny PRRSV were rescued. The reconstituted virus was found to maintain growth properties similar to those of the parental virus in porcine alveolar macrophage (PAM) cells. With the availability of this type 2 PRRSV infectious clone, we first explored the biological relevance of ORF5a in the PRRSV replication cycle. Therefore, we used a PRRSV reverse genetics system to generate an ORF5a knockout mutant clone by changing the ORF5a translation start codon and introducing a stop codon at the 7th codon of ORF5a. The ORF5a knockout mutant was found to exhibit a lack of infectivity in both BHK-tailless pCD163 and PAM-pCD163 cells, suggesting that inactivation of ORF5a expression is lethal for infectious virus production. In order to restore the ORF5a gene-deleted PRRSV, complementing cell lines were established to stably express the ORF5a protein of PRRSV. ORF5a-expressing cells were capable of supporting the production of the replication-defective virus, indicating complementation of the impaired ORF5a gene function of PRRSV *in trans*.

Keywords: PRRSV, reverse genetics, ORF5a, complementation

Introduction

Porcine reproductive and respiratory syndrome (PRRS) was first recognized in 1987 in the United States and shortly thereafter in Europe [12, 35]. The disease has since continued to plague nearly all pig-producing countries, causing severe economic losses in the global swine industry [1, 26]. The etiological agent of PRRS, the PRRS virus (PRRSV), was isolated almost simultaneously in Europe and North America in the early 1990s [3, 6, 36]. PRRSV is a member of the family *Arteriviridae* including equine arteritis virus (EAV), lactate dehydrogenase-elevating virus of mice, and simian hemorrhagic fever virus, which forms the order *Nidovirales* along with the *Coronaviridae* family [5, 19, 31]. Since the emergence, PRRSV has evolved divergently on the two continents and consequently, consists of two major genotypes, European (type 1) and North American (type 2) [9, 10, 23, 28]. The two genotypes exhibits antigenic and genetic variations, sharing only about 60% sequence identity at the genome level [24, 18].

PRRSV is a small enveloped virus with a single-stranded, positive-sense RNA genome of ~15 kb in size. The PRRSV genome possesses the 5' cap structure and 3' polyadenylated tail and constitutes the 5' untranslated region (UTR), ten open reading frames (ORF1a, ORF1b, ORF2a, ORF2b, and ORFs 3 through 7 including ORF5a), and the 3' UTR [8, 11, 22, 30, 36]. The two large ORF1a and 1b occupying the 5' two-third of the genome encode the ORF1a and ORF1ab polyproteins by a ribosome frame-shifting mechanism that are translated directly from the genomic RNA. The polyproteins are then autocleaved into 14 protease and replicase-associated nonstructural proteins

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(nsp1α, nsp1β, nsp2 to nsp6, nsp7α, nsp7β, and nsp8 to nsp12) [2, 14, 32, 34, 38]. The remaining ORF2a through 7 in the 3' terminal region code for structural GP2a, small envelop (E), GP3, GP4, ORF5a, GP5, membrane (M), and nucleocapsid (N) proteins that are expressed from a nested set of 3'-coterminal subgenomic (sg) mRNAs [8, 7, 36].

Several infectious cDNA clones have been developed for both type 1 and type 2 PRRSV isolates so that the viral RNA genome is manipulated to introduce alterations at specific sites or regions and to create respective mutant viruses [17, 20, 25, 33, 37]. In the present study, we described an infectious clone of type 2 PRRSV and exploited this tool to generate an ORF5a gene-deleted mutant clone. DNA transfection with the ORF5a-knockout genomic clone showed the absence of virus replication. In addition, the non-viability of the ORF5a-deleted replication-defective virus was rescued by functional complementation *in trans* from cells stably expressing the ORF5a protein. Our findings indicated that the PRRSV ORF5a protein is essential for virus replication.

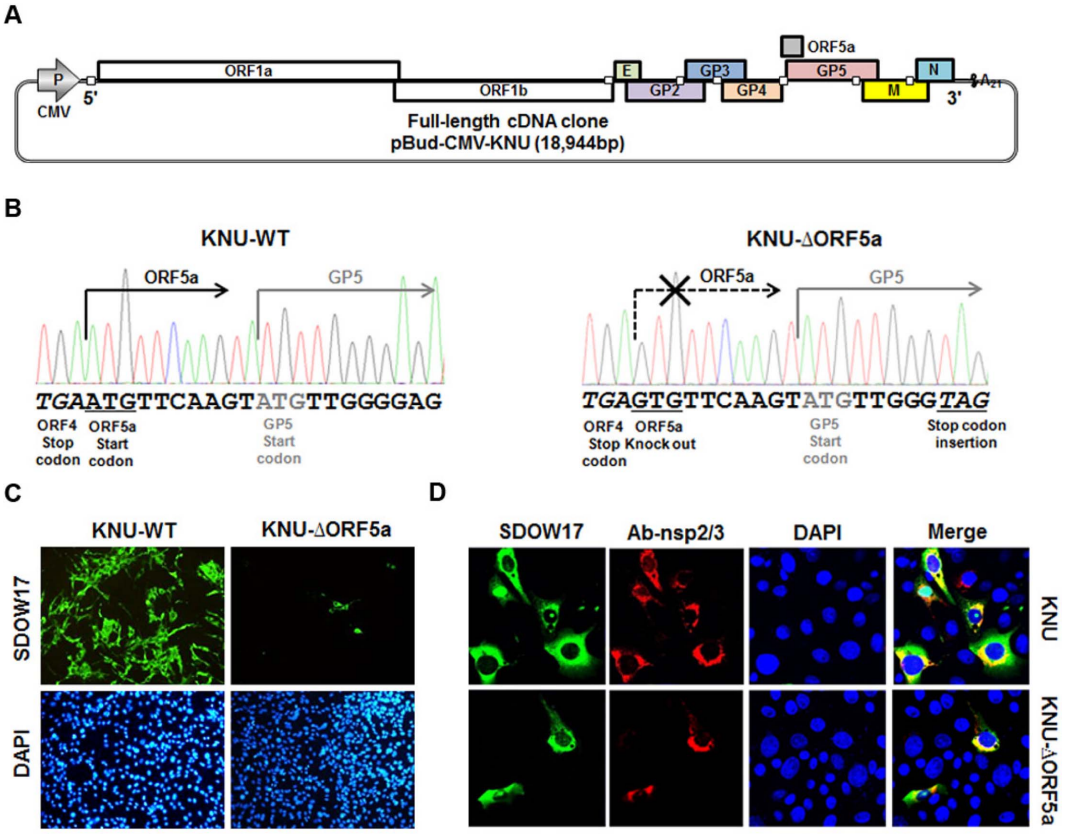

Fig. 1. Construction of an ORF5a knock-out mutant clone using reverse genetics. (A) Genome organization of a full-length KNU-12 infectious cDNA clone. The fully assembled KNU-12 genomic cDNA was cloned into the pBud-CMV plasmid containing the human cytomegalovirus (CMV) immediate early promoter (P). Both 5' and 3' untranslated regions (UTRs) are indicated and downstream of the 3' UTR, A_{21} indicates a poly(A) tail of 21 A's. (B) Generation of a ORF5a knock-out mutant full-length clone. The ORF5a gene starts immediately after the ORF4 stop codon. The ORF4 stop codon was indicated as italic font, whereas the ORF5a start codon was underlined. To abolish the ORF5a gene expression, 'ATG' for translation initiation of the ORF5a gene was changed to 'GTG' underlined in right panel. The stop codon was introduced at the 7th codon of ORF5a gene by changing GAG to TAG indicated in boldface. These mutations do not affect GP5 amino acid sequences (right panel). (C) Absence of infectivity of the ORF5a gene-knockout full-length clone for PRRSV, KNU-12-ΔORF5a. BHK-tailless cells were transfected with the full-length cDNA genomic clone of KNU-12-WT or KNU-12-ΔORF5a and incubated for 48 h. For immunofluorescence, cells were fixed with 4% formaldehyde at 48 h post-transfection and incubated with the N-specific MAb SDOW-17 (upper panels). (D) Double staining for N (green) and nsp2/3 (red) proteins for KNU-12-WT (upper panels) or KNU-12-ΔORF5a (lower panels). BHK-tailless cells transfected with KNU-12-WT or KNU-12-ΔORF5a plasmid DNA were fixed at 48 h post-transfection and co-stained with nsp2/3-specific rabbit antiserum and N-specific MAb SDOW17. Yellow indicates merged images where both N and nsp2/3 are co-localized.

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Materials and Methods

Cells, virus, and antibodies

PAM-pCD163 [15] and BHK-tailless pCD163 [16] cells were cultivated as described previously. HEK-293T cells were cultured in Dulbecco's modified Eagle medium (DMEM) with high glucose (Invitrogen, Carlsbad, CA) with 10% fetal bovine serum (FBS; Invitrogen) and antibiotic-antimycotic solutions (100×; Invitrogen). The cells were maintained at 37°C with 5% CO_2. A type 2 PRRSV strain KNU-12 was used to prepare virus stocks as described previously [15]. The virus stock then served as a parental virus for the construction of a full-length genomic cDNA clone. A monoclonal antibody (MAb) against the PRRSV N protein was purchased from MEDIAN Diagnostics (Chuncheon, Republic of Korea). Rabbit polyclonal antiserum against PRRSV nsp2/3 was a kind gift of Eric Snijder of Leiden University Medical Center, Leiden, The Netherlands [13]. *E. coli* strains DH5α and XL1-Blue (RBC Bioscience, Taiwan) was used as the host for general cloning and site-directed mutagenesis, respectively.

Assembly of full-length cDNA clone

A eukaryotic expression pBudCE4.1 vector (Invitrogen) containing the human cytomegalovirus (hCMV) immediate early promoter was modified by removing the EF-1 promoter region and inserting a linker fragment prepared as a double-stranded synthetic adapter consisting of the unique restriction enzyme sites (*Asc*I, *Mlu*I, *Pme*I, *EcoR*V, and *Pac*I) between *Sac*I and *Hind*III sites (Fig. 1A). The resulting vector plasmid was designated pBud-CMV. Four overlapping cDNA fragments covering the entire KNU-12 genome were RT-PCR amplified using gene-specific primer sets (Table 1) and inserted in the pCR-XL-TOPO plasmid as described previously [22]. Each of the viral fragments was excised from the corresponding pCR-XL-TOPO plasmid and assembled into a single clone using available restriction sites in the pBud-CMV plasmid, generating the full-length cDNA construct pBud-CMV-KNU-12 (Fig. 1A). DNA manipulation and cloning were performed according to standard procedures [29].

Construction of genetically engineered ORF5a knock-out full-length cDNA clone

To introduce specific modifications to the full-length genomic clone, the shuttle plasmid was constructed. The fourth fragment (KNU-12-F4) covering the 3 most 3.3 kb region of the KNU-12 genome was subcloned into the pBud-CMV vector to create pBud-shuttle-KNU-12. To construct the ORF5a gene-knockout mutant clone, the translational initiation codon and the 7th codon of PRRSV ORF5a were modified. PCR-directed mutagenesis was conducted simultaneously to change the ATG start codon and the 7th codon of the ORF5a gene to GTG and TAG at genomic nucleotide positions 13,780 to 13,782 and 13,798 to 13,800, respectively, using pBud-shttle-KNU-12 with the following primer; for A13780G/G13798T mutation, ORF5a-KO-Fwd (5'-CTTACTGGCAATTTGAgTGTTCAAGTATGT

Table 1. Primers used for RT-PCR to synthesize four fragments of cDNA clones for KNU-12 virus.

Primer name	Nucleotide sequences[1)]	Primer location in KNU-12 genome (nt)
Fragment 1 (4445)		
KNU-12-F1-Fwd	5'-GCCGGCGCGCCACCATGACGTATAGGTGTTG-3'	1-17
KNU-12-F1-Rev	5'-GGCACGCGTAAAAGGGAACACGTTGAAGGC-3'	4,419-4,445
Fragment 2 (3251)		
KNU-12-F2-Fwd	5'-GCCACGCGTGCGACCAGGTCGTCACTCATC-3'	4,440-4,466
KNU-12-F2-Fwd	5'-GGCGTTTAAACACTGCTCCTTAGTCAGGCC-3'	7,671-7,697
Fragment 3 (4472)		
KNU-12-F3-Fwd	5'-GCCGTTTAAACTGCTAGCCGCCAGCGGC-3'	7,690-7,714
KNU-12-F3-Fwd	5'-GGCGATATCAACAATGGACACCAAAAATTC-3'	12,136-12,166
Fragment 4 (3252)		
KNU-12-F4-Fwd	5'-GCCGATATCATCATATTTTTGGCCATTTTG-3'	12,161-12187
KNU-12-F4-Rev	5'-GGCTTAATTAAT^{21}AATTTCGGCCGCATGG-3'	15,397-15,412

[1)]Restriction enzyme sites in the primers are underlined.

TGGGtAGATGCTTGACC-3') and ORF5a-KO-Rev (5'-GGTCAAGCATCTaCCCAACATACTTGAACAcTCAAATT GCCAGT AAG-3'), where lowercase letters indicate mutated nucleotides. The G13798T mutation was translationally silent with respect to ORF5 encoding the GP5 protein. The modified shuttle plasmid was digested with *EcoR*V and *Pac*I, and a 3252-bp fragment was purified. The wild type full-length genomic clone was digested with the same enzymes, and the *EcoR*V-*Pac*I fragment was replaced with the corresponding fragment obtained from the shuttle plasmid carrying the ORF5a-knockout mutation. The resulting mutant clone was designated pBud-CMV-KNU-12-ΔORF5a and the construct was verified by nucleotide sequencing.

Production of infectious virus from full-length cDNA clones

BHK-tailless pCD163 cells were grown at 5.0×10^5 cells in 6-well tissue culture plates for 24 h. The cells were transfected with the full-length cDNA plasmid using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocols (Invitrogen). The culture supernatants were harvested at 4 days post-transfection and designated 'passage-1'. The passage-1 virus was used to inoculate fresh PAM-pCD163 cells and the 4-day harvest was designated 'passage-2'. The 'passage-3' virus was prepared in the same way as for passage-2. Each passage virus was aliquoted and stored at −80°C until use. Titers of each stock virus were measured by limiting dilution on PAM-pCD163 cells through immunofluorescence assay (IFA) as described below and quantified as the 50% tissue culture infectious dose ($TCID_{50}$).

Immunofluorescence assay (IFA)

PAM-pCD163 or BHK-tailless pCD163 cells were seeded on microscope coverslips in 6-well tissue culture plates for 24 h and transfected with the full-length cDNA using Lipofectamine 2000 or infected with the cloned passaged virus. At 48 h post-transfection or post-infection, cell monolayers were fixed with 4% paraformaldehyde for 10 min at room temperature (RT) and permeabilized with 0.2% Triton X-100 in PBS at RT for 10 min. The cells were blocked using 1% bovine serum albumin (BSA) in PBS for 30 min at RT and then incubated with incubated with the nsp2/3-specific rabbit antiserum or N-specific MAb for 2 h. After washing five times in PBS, the cells were incubated for 1 h at RT with goat anti-rabbit secondary antibody or anti-mouse secondary antibody conjugated with Alexa green dye (Molecular Probes, Carlsbad, CA). For dual immunofluorescence, the cells were co-stained with nsp2/3-specific rabbit antiserum and N-specific MAb, followed by staining with goat anti-rabbit antibody conjugated with Alexa green and goat anti-mouse antibody conjugated with Texas red (Molecular Probes). The cells were finally counterstained with 4',6-diamidino-2-phenylindole (DAPI; Sigma, St. Louis, MO) and the coverslips were washed five times in PBS and mounted on microscope glass slides in mounting buffer (60% glycerol and 0.1% sodium azide in PBS). Cell staining was visualized by a fluorescent Leica DM IL LED microscope (Leica, Wetzlar, Germany) or Confocal Laser Scanning microscope (Carl Zeiss, Oberkochen, Germany) using an excitation wavelength in the range of 450-500 nm and an emission wavelength in the range of 515-565 nm.

Generation of complementing cell lines

RT-PCR was performed using the extracted viral RNA to amplify the ORF5a gene with the following primer pairs: ORF5a-F (5'-GCCGTCGACACCATGTTCAAGTATG-3') and ORF5a-R (5-GCCGGATCCCATAGCGTCAAGTTG-3'), where underlines indicate the *Sal*I and *Bam*HI restriction enzyme sequence. The PCR amplicon was initially inserted into a pBudCE4.1 vector that contains six repetitive histidine codons and the resulting plasmid, pBud-ORF5a, was verified by nucleotide sequencing. The ORF5a cDNA fragment obtained from the plasmid described above was then subcloned into a pFB-Neo retroviral vector (Stratagene, La Jolla, CA) using *Sal*I and *Eco*RI restriction sites to construct the retroviral gene transfer plasmids, thereby producing respective His-tagged fusion proteins. The retrovirus-mediated gene expression system (Stratagene) was applied to generate PAM or BHK cell lines constitutively expressing the ORF5a gene as described elsewhere [15, 21, 27]. The retroviral culture supernatant was collected and used to infect target PAM-pCD163 or BHK-tailless pCD163 cells. Antibiotics-resistant cell clones were examined by RT-PCR, IFA, western blot to determine the presence of the corresponding gene as described previously [15, 21, 27]. Each of the positive cell clones (PAM-pCD163-ORF5a and BHK-tailless pCD163-ORF5a) was amplified for subsequent analyses. The complementing cells were transfected with the gene-knock out mutant cDNA plasmid and at 48 h post-transfection, its ability to complement was

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examined by IFA. The cloned ORF5a-KO virus was prepared in complementing cells and its infectivity was tested by IFA. Titers of the replication-defective cloned virus were measured by limiting dilution on the appropriate complementing cells as well as non-complementing PAM-pCD163 cells as described above.

Results and Discussion

The cDNA fragments were assembled into a single clone from four overlapping cDNA fragments (designated F1, F2, F3 and F4) using the restriction sites *Asc*I at nucleotide position 1, *Mlu*I at position 4,440, *Pme*I at position 7,690, *EcoR*V at 12,161, and *Pac*I at 15,443 derived from the modified pBud-CMV plasmid (Fig. 1A). The final construct pBud-CMV-KNU-12 is comprised of cDNA representing the entire genome and a 21-residue synthetic polyadenosine tail, positioned behind the CMV promoter. Upon transfection, infectivity of the full-length cDNA clone pBud-CMV-KNU-12 was first determined in BHK-tailless pCD163 cells by immunofluorescence using the N-specific MAb (Fig. 1C, left panels). The cloned virus was further passaged using the culture supernatant and each of the passaged viruses was designated 'passage-1', 'passage-2', and 'passage-3', respectively. Many clusters of cells infected with the cloned virus showed bright fluorescence, indicating the infection and spread of virus to neighboring cells. These data demonstrates that PRRSV infection can be initiated directly from the plasmid pBud-CMV-KNU-12.

The growth characteristics of the reconstituted virus were determined by virus titration assay. The titer of passage-1 virus was determined to be 1×10^3 $TCID_{50}$/ml. The cloned virus was amplified by subsequent passages in PAM-pCD163 and the peak titer of passage-3 virus increased to 5.7×10^5 $TCID_{50}$/ml similar to that of the parental virus. The growth kinetics of the reconstituted virus was compared to the parental virus by one-step growth curve using the passage-3 virus. The parental virus and the cloned virus reached the peak titer within 2 days post-infection, showing indistinguishable growth rates in PAM-pCD163 cells (data not shown).

We next explored whether the novel ORF5a gene is dispensable for the replication of PRRSV. To address this issue, our reverse genetics system was applied to construct a mutant clone by changing the start codon of ORF5a to GTG at genomic positions 13,780 to 13,782. This A13780G mutation did not alter the amino acid sequence in ORF5 encoding GP5 protein (Fig. 1B). The infectivity of the mutant genomic clone pBud-CMV-KNU-12-ΔORF5a was examined by transfection of BHK-tailless pCD163 cells followed by subsequent passages in PAM-pCD163 cells. A few single cells displayed N-specific fluorescence, and these cells represent individually transfected cells with KNU-12-ΔORF5a (Fig. 1C, right panels). However, no N-specific staining was detectable with serial passages of KNU-12-ΔORF5a virus up to five passages in PAM-pCD163 cells, indicating the lack of infectivity (data not shown). The transcription ability of KNU-12-ΔORF5a was evaluated by double staining of transfected cells using nsp2/3-specific antiserum and N-specific MAb. Dual-staining of nsp2/3 (green) and N (red) was observed, demonstrating the expression of nsp2/3 and N proteins in cells transfected with KNU-12-ΔORF5a cDNA clone (Fig. 1D, lower panels). These results indicated the synthesis of both nonstructural and structural proteins, which in turn suggests that the PRRSV genome replication and mRNA transcription occurred upon transfection of KNU-12-ΔORF5a cDNA clone. Taken together, our data indicated that the novel ORF5a gene is essential for PRRSV infection.

We sought to restore the infectivity of KNU-12-ΔORF5a by provision of the ORF5a protein *in trans*. Thus, complementing PAM or BHK cell lines stably expressing the ORF5a protein were generated by the use of the retroviral gene transfer system. The constitutive expression of the corresponding protein was demonstrated in their respective stable PAM or BHK cell lines by IFA (Fig. 2B, second panels). To determine whether KNU-12-ΔORF5a in the complementing cells leads to the production of infectious progeny virus, the appropriate complementing BHK cells were transfected with KNU-12-ΔORF5a DNA. The virus rescued from transfection of cDNA clones with complementing cells was designated KNU-12-ORF5a virus and further passaged three times in the complementing PAM cells. The infectivity of individual passage-3 KNU-12-ORF5a virus was examined by immunofluorescent staining with N-specific MAb in complementing cells and non-complementing PAM cells. Bright N-specific fluorescent signal was clearly observed in clusters of PAM-pCD163-ORF5a cells infected with KNU-12-ΔORF5a virus, indicating that the ORF5a-deleted virus replication was rescued by trans-complementation of the respective protein (Fig. 2B. fourth panels). In contrast, ORF5a-deleted mutant virus was

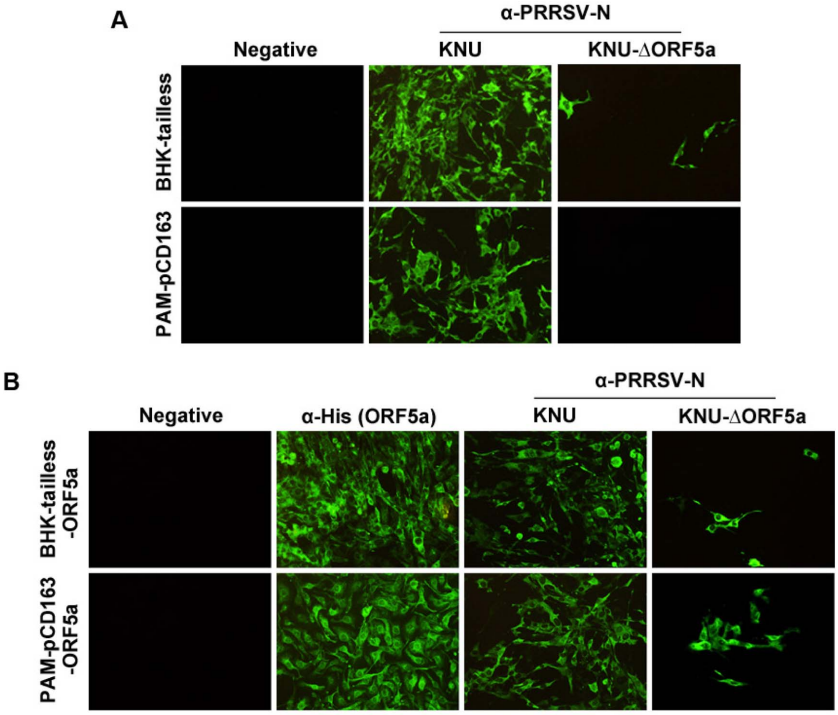

Fig. 2. Complementation of a ORF5a knockout mutant virus by expression of wild-type ORF5a protein *in trans*. (A) Detection of N protein expression in BHK-tailless cells by IFA. BHK-tailless cells were transfected individually with wild-type (WT) and ORF5a knockout mutant clones that fixed at 48 h post-transfection (upper panels). PAM-pCD163 cells were inoculated with supernatants collected from BHK-tailless cells and fixed then stained at 48 h post-infection. Viral replication of ORF5a knockout mutant clone was not detected in PAM-pCD163 cells compared with KNU-12 WT clone (lower panels). (B) Complementation of the ORF5a knockout mutant by stable expression of ORF5a protein in BHK-tailless and PAM-pCD163 cells. Intracellular expression levels of ORF5a protein was detected by anti-His tag antibody (second panels). BHK-tailless-ORF5a cells were transfected with KNU-12 clone or ORF5a knockout mutant clone. The supernatants were harvested to inoculate PAM-pCD163-ORF5a cells. Each virus replication was confirmed by IFA at 48 h post-infection (third and fourth panels).

found to be replication-defective in non-complementing PAM-pCD163, as shown by only single stained cells (Fig. 2A, right panels). This result is indicative of the abortive single round replication that is capable of initial infection but incapable of dissemination. Our data revealed functional complementation and rescue of KNU-12-ΔORF5a virus by the provision of the ORF5a protein *in trans*.

Reverse genetics allows manipulation of the viral genome in order to create genetically-engineered recombinant viruses. This molecular tool thus provides a useful platform for studying virus replication, pathogenesis, virus-host interactions, and function(s) of each viral protein as well as for developing viral vectors and vaccines [4]. In the present study, we generated a full-length infectious cDNA clone for the type 2 PRRSV strain KNU-12 and subsequently constructed an ORF5a-defective mutant clone to investigate the role of the ORF5a protein in PRRSV replication. Recently, it was shown that ORF5a protein is a novel structural protein in PRRSV, which is encoded by an alternative ORF5a that in present in all arteriviruses [11]. Furthermore, inactivation of ORF5a expression in EAV mutant revealed that ORF5a protein is dispensable, but it is important for EAV viability [8].

Our reverse genetics approach was used to modify the translation initiation and induced stop translation of ORF5a gene, so that the modified genome was unable to express the ORF5a protein. In this experiment, we showed that inactivation of ORF5a gene expression in context of type 2 PRRSV KNU-12 full-length cDNA clone was lethal for the production of viable virus. However, repressed KNU-12-ΔORF5a mutant virus viability was rescued by expressing ORF5a protein *in trans*. These data indicate that the PRRSV ORF5a protein is essential structural component for infectivity. Although the role of ORF5a in viral replication

remains unknown, our previous proteomics study using ORF5a protein expressing PAM cells indicated that the PRRSV ORF5a protein particularly modulates host cytoskeleton networks and hnRNPs family-associated proteins [27]. These responses to the ORF5a protein suggest that ORF5a protein may manipulate the host cytoskeleton network for effective PRRSV infection and replication. In the absence of ORF5a protein during PRRSV replication, effective viral RNA processing and packaging regulated by hnRNPs may be harmful, and consequently, ORF5a-defective PRRSV appears to lose the virus viability.

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국문초록

PRRS 바이러스 ORF5a 단백질의 기능학적 역할

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돼지생식기호흡기증후군(porcine reproductive and respiratory syndrome; PRRS) 바이러스의 ORF5a 단백질이 바이러스 생장에 필수적인 단백질인지 확인하기 위해서 PRRS 바이러스 감염성 클론을 이용하여 ORF5a 단백질 유전자를 결손시킨 변이 클론을 제작하였다. 야생형 PRRS 바이러스 감염성 클론과 ORF5a 단백질이 결손된 변이 클론을 BHK-tailless pCD163 세포에 transfection시킨 결과 변이 클론에서 감염성 있는 바이러스가 숙주세포로부터 만들어지지 않았다. 이 결과가 ORF5a 단백질 발현의 부재 때문인지 검증하기 위해서 BHK-tailless pCD163-tailless 세포에 ORF5a 단백질을 안정적으로 발현하는 세포주를 제작하였고 이 세포주에 동일한 transfection 실험을 한 결과 세포에서 공급되는 ORF5a 단백질 발현에 의해 감염성 있는 바이러스가 만들어지는 것을 확인하였다. 이로써 ORF5a 단백질이 PRRS 바이러스가 생장하는데 있어서 필수적인 단백질임을 확인할 수 있었다.

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