Journal of Virological Methods 274 (2019) 113727

Contents lists available at ScienceDirect

Journal of Virological Methods

journal homepage: www.elsevier.com/locate/jviromet

A simple and efficient method for the generation of a porcine alveolar macrophage cell line for high-efficiency *Porcine reproductive and respiratory syndrome virus* 2 infection

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ARTICLE INFO

Keywords:
PiggyBac
CD163
PRRSV
3D4/21/CD163
Susceptibility

ABSTRACT

CD163 is a cellular receptor for *Porcine reproductive and respiratory syndrome virus* (PRRSV). Transgenic expression of CD163 can predispose a variety of PRRSV non-permissive cells to PRRSV infection. These resulting cells can then be used for PRRSV production and the study of PRRSV biology. The PiggyBac (PB) transposon is a non-viral, plasmid-based mobile genetic element that can be used for gene delivery into mammalian cells. In this study, a simple and efficient method for the transfection of the porcine CD163 transgene into an immortalized porcine alveolar macrophage cell line (3D4/21), a non-permissive cell line to PRRSV infection, by PB transposition was demonstrated. The resultant stably transformed 3D4/21/CD163 cells expressed CD163 constitutively and were shown to be fully permissive for PRRSV-2 strains and yielded an excess of 10^6 $TCID_{50}$/mL of progeny virus. The PRRSV replicated more efficiently in the 3D4/21/CD163 cells than in Marc-145 cells, and the titers of the progeny PRRSV produced in the 3D4/21/CD163 cells were higher than those produced in Marc-145 cells. This simplified PB transposon-generated PRRSV-2 permissive 3D4/21/CD163 cell line could facilitate PRRSV production and accelerate the study of virus-host interactions *in vitro*.

1. Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV) is the causative agent of porcine reproductive and respiratory syndrome (PRRS) which is one of the most significant viral diseases in the swine industry. PRRSV is an enveloped, single-stranded positive-sense RNA virus that belongs to the family *Arteriviridae* (Cavanagh, 1997). Due to the high degree of genetic diversity, PRRSV was recently categorized into two species: PRRSV-1 (formerly European genotype 1) and PRRSV-2 (formerly North American genotype 2) (Adams et al., 2016; Kuhn et al., 2016; Adams et al., 2017). The two species cause reproductive failure in sows, high mortality rates among nursery pigs and respiratory disease in pigs of all ages. PRRSV has a highly restricted cell tropism both *in vitro* and *in vivo*. The fully differentiated primary porcine alveolar macrophage (PAM) cells are the predominant natural host cells for PRRSV proliferation. Previous reports have indicated that the restricted tropism of PRRSV is determined by the interaction of the host cell-specific surface receptor(s) with the virus surface protein(s) (Kreutz, 1998; Meulenberg et al., 1998; Nauwynck et al., 1999; Vanderheijden et al., 2003). PRRSV infects primary PAM cells by binding to heparan sulphate (Delputte et al., 2002) and is then internalized by siglec-1 or siglec-10 (Vanderheijden et al., 2003; Xie et al., 2017, 2018) and followed by CD163 disassembling (Van Gorp et al., 2008). Transgenic expression of CD163 results in a variety of non-permissive cells to become susceptible to PRRSV infection (Calvert et al., 2007), indicating that CD163 has a crucial role in determining PRRSV infection.

For virus isolation, PRRSV can be grown on primary PAM cells, which are derived from the lungs of PRRSV-free pigs (Duan et al., 1997). Besides primary PAM cells, other cell types permissive for PRRSV replication are the immortalized monkey kidney cell line MA-104 and its derivatives, such as Marc-145 cells and CL-2621 cells (Kim

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https://doi.org/10.1016/j.jviromet.2019.113727
Received 14 November 2018; Received in revised form 3 September 2019; Accepted 3 September 2019
Available online 04 September 2019
0166-0934/

et al., 1993). Currently, the Marc-145 cells are the most widely used cells for PRRSV production. However, this cell line is not of swine origin and therefore is limited to swine-related research *in vitro*. Additionally, PRRSV infects Marc-145 cells by a different pathway of entry compared to that of the primary PAM cells, and may result in an alteration of the epitopes associated with PRRSV entry upon growth on Marc-145 cells (Delrue et al., 2010). Moreover, not all PRRSV strains can grow directly on Marc-145 cells. Only some PRRSV-2 strains can directly grow on Marc-145 cells. Marc-145 cells rarely support the spontaneous growth of PRRSV-1 strains (Kim et al., 1993). Unfortunately, the use of the primary PAM for PRRSV production is limited by its high production costs, batch variation, difficult standardization, risk of contamination with other pathogens and the ethical cost of sacrificing animals to harvest the cells (Delrue et al., 2010). Also, a previous immortalized PAM cell line did not support PRRSV replication (Weingartl et al., 2002), because this cell line lost the expression of the PRRSV cellular receptor CD163 (Lee et al., 2010). One way of overcoming all these problems associated with PRRSV production is to generate a porcine cell line for PRRSV cultivation.

The PiggyBac (PB) transposon system is a promising and efficient non-viral vehicle for gene delivery. Because of the PB's ability to mediate stable gene transfer into a variety of mammalian cells, including those derived from humans and mice (Ding et al., 2005), there is a great deal of interest in its potential use for gene transfer into the immortalized PAM cells (Wang et al., 2013). In this study, the PB transposon system was applied to deliver the CD163 gene into immortalized PAM cells, demonstrating a simple and efficient method of generating a porcine alveolar macrophage cell line susceptible to PRRSV-2 strains. The PB transposon system revealed advantageous properties in mediating gene transfer into immortalized PAM cells and this PRRSV susceptible cell line could be an alternative tool for PRRSV production and PRRSV biology study.

2. Material and methods

2.1. Cells and viruses

Primary PAM cells were collected from 5-week-old PRRSV-negative pigs. The immortalized PAM cell line 3D4/21 was purchased from the American Type Culture Collection (ATCC cell Number: CRL-2843) and was propagated and maintained in RPMI 1640 medium (Invitrogen, Grand Island, USA) supplemented with 10% fetal bovine serum (FBS) (Invitrogen, Auckland, New Zealand), 1% non-essential amino acids and 1% antibiotic/antimycotic (Invitrogen, Grand Island, USA). The Marc-145 cells were obtained from Chinese Center for Type Culture Collection (Wuhan, China) and were cultured in Dulbecco's modified eagle's medium (DMEM) supplemented with 10% FBS and 1% antibiotic/antimycotic. The PRRSV strain VR-2332 (GenBank: EF536003.1) was that described by Wang et al. (2013) and was maintained on Marc-145 cells; the PRRSV vaccine strain CH-1R (GenBank: EU807840.1) was purchased from Yebio Bioengineering Co. Ltd., Qingdao, China, and was maintained on Marc-145 cells). A natural, highly pathogenic PRRSV HN strain was sourced from the serum of an infected farmed pig in Henan province and was maintained on primary PAM cells (see Section 2.5 for details). All these viruses are of PRRSV-2 strains.

2.2. pPB-CD163 donor plasmid construction and its propagation

The PB transposon dual-vector system used in this study was purchased from System Bioscience Company (Mountain View, USA). The PB donor vector (pPB) (Catalog No. PB513B-1), containing ampicillin resistance for bacterial selection, has two expression cassettes, one for expressing the transgene of interest and the other for expressing the green fluorescence protein (GFP) and the puromycin resistance gene for selection of the transposed cells. The entire twin-cassette is flanked by genomic insulator elements for stabilized transgene expression and PB inverted terminal repeat sequences (ITRs) for transposase mobilization and integration. The PB helper vector (Catalog No. PB200PA-1) contains a codon-optimized PB transposase gene to express the PB transposase. During transposition, the PB transposase recognizes the ITRs on the PB donor vector and moves the contents between the ITRs and integrates them into TTAA chromosomal sites.

To construct the pPB-CD163 donor plasmid (PB donor vector containing the CD-163 open reading frame (ORF)), the CD163 ORF was amplified from total cellular RNA extracted from primary PAM cells by RT-PCR as described previously using the primer pair CD163-F (5′-GCTCTAGAATGGTGCTACTTGAAGACTCTGG-3′, XbaI site underlined) and CD163-R (5′-CGGGATCCTCATTGTACTTCAGAGTGGTCT CCT-3′, BamHI site underlined) (Calvert et al., 2007). The porcine CD163 ORF was cloned into the pPB vector using the following procedures: First, the pPB vector and the amplified CD163 cDNA fragment were respectively double-digested with XbaI (New England Biolabs, Ipswich, UK) and BamHI (New England Biolabs, Ipswich, UK) restriction endonuclease at 37 °C for 4 h. Then, the CD163 fragment was ligated into the digested pPB vector using T4 DNA ligase (New England Biolabs, Ipswich, UK) at 16 °C for 4 h and the resultant reaction mixture was transformed into *E. coli* DH5α competent cells. *E. coli* transformed with the pPB-CD163 plasmid were selected by ampicillin resistance by growing on agar plates with Luria Bertani (LB) medium and ampicillin (100 μg/mL). Several ampicillin-resistant bacteria colonies were picked and separately cultured in 5 mL LB medium containing ampicillin at 37 °C with vigorous shaking for 16 h. The pPB-CD163 plasmids were extracted from the bacterial cells using a plasmid purification kit (Tiangen Biotech, Beijing, China) according to the manufacturer's recommended protocol. The identity of the pPB-CD163 plasmid was confirmed by XbaI and BamHI endonuclease enzyme analysis and CD163 sequence analysis (Sangon Biotech, Shanghai, China).

2.3. DNA transfection and stable cell line generation

DNA transfection was performed as described in a previous report (Wang et al., 2013). Twenty-four hours before transfection, the 3D4/21 cells were seeded into a six-well plate at a density of 2×10^5 cells/mL in RPMI 1640 medium. Lipofectamine 2000™ reagent (Invitrogen, Carlsbad, USA) was used as the transfection reagent. On the day of transfection, the existing cell growth medium was removed and fresh growth medium was added into the wells and then the cells (about 8×10^5 cells per well) were co-transfected with 3.0 μg of the pPB-CD163 plasmid and 0.6 μg of the PB helper vector plasmid. At 48 h post-transfection, the cell medium was removed and the transformed 3D4/21 cells were selected with fresh growth medium containing 10 μg/mL of puromycin (Merck, Darmstadt, Germany). As described in a previous report (Wang et al., 2013), the selection medium was changed every two days until GFP-positive and puromycin-resistant cell colonies appeared in the six-well plate. A GFP-positive and puromycin-resistant transfected 3D4/21 cell colony with a diameter of more than 0.5 mm was selected for further study and was passaged in 96-well plates. When the cells reached confluency after ten days, they were multiplied in six-well plates in the same medium and stored as cell stocks.

2.4. RT-PCR, immunofluorescence assay and Western blot

Cellular RNA was extracted from the pBP-CD163-transfected cells using a RNA isolation kit (Tiangen Biotech, Beijing, China) and tested for the presence of CD163 transcripts by RT-PCR (Wang et al., 2013). To detect for CD163 protein expression in the resultant CD163 transcript-positive cells (designated as 3D4/21/CD163 cells) by immunofluorescence assays, the 3D4/21/CD163 cells and the negative control 3D4/21 cells, grown in a 96-well plate, were first washed with PBS and then fixed with 4% paraformaldehyde. After being permeabilized with 0.5% Triton X-100, the cells were stained with a primary monoclonal antibody against CD163 (AbD Serotech, Kidlington, UK) at a

concentration of 1.0 μg/mL. Rhodamine (TRITC)-conjugated goat polyclonal anti-mouse IgG antibodies (Jackson, West Grove, USA), at a dilution of 1:500, were used as a second antibody. The cells were counterstained with 4′,6′-diamidino-2-phenylindole (DAPI, Sigma-Aldrich, St. Louis, USA) at a concentration of 0.01 μg/mL and visualized using a fluorescence microscope (Leica Microsystems, Heidelberg, Germany) at 200× magnification. Similarly, to ascertain the expression of CD163 protein by Western blotting, the cells were washed with PBS and lyzed in RIPA lysis buffer (Beyotime, Nantong, China), and total protein was quantified using a BCA protein assay kit (Beyotime, Nantong, China). The proteins (10 μg per lane) were separated by 10% SDS-PAGE and then transferred to a PVDF membrane (Millipore, Billerica, USA). The membrane was blocked with 5% non-fat dry milk dissolved in PBST (0.05% Tween-20 in PBS) and then incubated in 5 mL of PBS, containing either the anti-CD163 monoclonal antibody at a concentration of 0.5 μg/mL, or containing an anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) monoclonal antibody (Sigma-Aldrich, St. Louis, USA) as an internal control at a concentration of 0.01 μg/mL. Horseradish peroxidase (HRP)-conjugated goat polyclonal anti-mouse IgG antibodies (Jackson, West Grove, USA), at a dilution of 1:5000, were then used as the second antibody. The CD163-specific protein bands were detected using the ECL™ western blot analysis system (Beijing CoWin Bioscience, Beijing, China).

2.5. Analysis of the PB insertion site by inverse PCR

Five 3D4/21/CD163 colonies were analyzed by inverse PCR to confirm the PB integration sites in the 3D4/21/CD163 cells (Wilson et al., 2007). Briefly, Genomic DNA was isolated from the 3D4/21/CD163 cells using the genomic DNA purification kit (Thermo Scientific, Pittsburgh, USA) and five micrograms of genomic DNA were digested with MspI (New England Biolabs, Ipswich, UK), followed by ligation with T4 DNA ligase at 16 °C. The DNA was self-ligation after ligation with T4 DNA ligase and the ligated DNA was used as a template for inverse PCR amplification with PrimeSTAR HS DNA polymerase (Takara, Dalian, China). The primers pair, designed to bind to the 5′ ITR sequences region of the PB donor vector, had the following sequences: 5′-ATGTAATTACGTCCCTCCC-3′ and 5′-GGCTGTCCCTCATAAAAGT-3′. The PCR parameters were as follows: 30 cycles, each comprising of 98 °C for 10 s, 55 °C for 15 s and 72 °C for 5 min, was followed by one final cycle at 72 °C for 10 min. The amplified PCR products were sequenced after they were cloned into the pJET1.2 blunt cloning vector (Thermo Scientific, Pittsburgh, USA).

2.6. Study of PRRSV infection in 3D4/21/CD163 cells

The 3D4/21/CD163 and the 3D4/21 cells (a PRRSV non-permissive cell line used as a negative control for virus infection), both grown in RPMI 1640 medium, and the Marc-145 cells (a PRRSV permissive cell line used as the positive control for virus infection), grown in DMEM medium, were seeded at 2×10^5 cells/mL in 24-well plates and used for PRRSV infectivity studies. The cells were inoculated 24 h later with each of the VR-2332, HN and CH-1R PRRSV strains at a multiplicity of infection (MOI) of 0.1 at 37 °C for 1 h as described previously (Wang et al., 2013). Mock infection control cells were treated the same way but without PRRSV in the inoculum. The development of cytopathic effect (CPE) was monitored daily using an inverted microscope. At 24 h post-infection, total RNA was extracted from the infected cells using a RNA isolation kit (Roche, Mannheim, Germany) for RT-PCR to amplify the PRRSV N gene from the infected cells using the following primer pair: 5′-ATGCCAAATACCGGCAAGCAGC-3′ and 5′-TCATGCTGAGGGT GATGCTGT G-3′ as described previously (Wang et al., 2013). Immunofluorescence and Western blot assays to detect for the presence of the PRRSV nucleocapsid (N) proteins, were performed as described above using 1.0 μg/mL of a primary mouse anti-PRRSV-nucleocapsid antibody (GeneTex, Irvine, USA) and the same second antibodies as mentioned in 2.4.

To demonstrate that the 3D4/21/CD163 cell line can support the replication of a field isolate of PRRSV, 3D4/21/CD163 and Marc-145 cells prepared as described above were inoculated with a natural, highly pathogenic PPRV HN strain using the serum of a naturally-infected swine as the inoculum. Corresponding uninoculated control cells were treated the same way. After inoculation at 37 °C for 1 h, the virus-containing serum was removed and the growth of the 3D4/21/CD163 cells was continued in RPMI 1640 medium supplemented with 3% fetal bovine serum while the Marc-145 cells were maintained in DMEM medium supplemented with 3% fetal bovine serum. At 48 h post-infection, the infection of the cells by the HN strain was assessed by immunofluorescence assay and RT-PCR as described above. Finally, to further confirm that the natural HN strain could be isolated from the 3D4/21/CD163 cells, the intracellular and extracellular HN virus titers were determined at 72 h post-infection. The cells were lysed by three cycles of freezing and thawing for intracellular virus titer determination. To determine the titer of the extracellular virus, the supernatant of the culture medium was collected and centrifuged for 20 min at 300 × g at 4 °C to remove cell debris. Virus titer measurements, calculated as $TCID_{50}$/mL, were performed on Marc-145 cells according to the standard protocol (Labarque et al., 2000).

2.7. Assessment of PRRSV production in 3D4/21/CD163 cells

The efficiency of PRRSV production of the 3D4/21/CD163 cells was assessed by, firstly, growing the cells and inoculating them with each of the PRRSV strains VR-2332, HN and CH-1R at a MOI of 0.1 as described above, and then passaging the virus in the supernatants of the culture media of the infected cells, three times for strains VR-2332 and HN strains and five times for the CH-1R strain, on fresh 3D4/21/CD163 cells. For each passage, the supernatants were collected at various times (24 h, 48 h, 72 h, 96 h and 120 h) post-inoculation for virus titer determination until the virus titer of each virus passage reached a stable level. The virus titers in the supernatants were determined by centrifuging the collected culture media for 20 min at 300 × g at 4 °C and then stored at -70 °C until being titrated. Virus titer measurements, calculated as $TCID_{50}$/mL, were performed on Marc-145 cells according to the standard protocol (Labarque et al., 2000).

3. Results

3.1. Construction and propagation of the pPB-CD163 plasmid

The porcine CD163 cDNA sequence was amplified from the primary PAM cellular RNA preparation by RT-PCR and cloned into the pPB vector to produce the pPB-CD163 vector. Sequencing of the cloned CD163 cDNA revealed a 3333 bp open reading frame (ORF) whose sequence was identical to that of the GenBank ID: JX292263.1 sequence (results not shown). The ORF, from the initial ATG to the final TGA, codes for a 1110-amino acid protein with a deduced molecular weight of 130 kDa. Sequence analysis of the pPB-CD163 vector showed that the CD163 ORF sequence was located downstream of the constitutively active CMV (Cytomegalovirus) promoter in pPB (results not shown).

3.2. Production of 3D4/21/CD163 cells and detection of CD163 gene expression

The pPB-CD163 plasmid, together with the PB helper plasmid, were successfully introduced into 3D4/21 cells to produce the 3D4/21/CD163 cells. The puromycin-resistant and GFP-positive 3D4/21/CD163 cell colonies were sufficiently large, with diameters > 0.5 mm, to be picked after 10 days under puromycin selection. One cell colony was used for further studies. RT-PCR of the cellular RNA of the 3D4/21/CD163 cells showed that the CD163 mRNA was expressed in these cells, while no CD163 mRNA was expressed in the 3D4/21 cells (Fig. 1A).

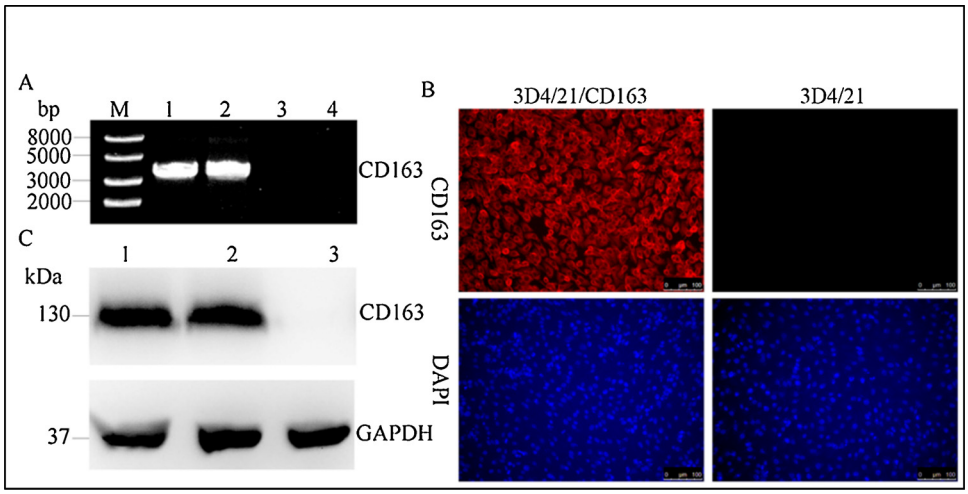

Fig. 1. Detection of CD163 gene expression in 3D4/21/CD163 cells. (A) Analysis of the products from RT-PCR assays of CD163 transcripts in a 0.8% agarose gel. M, DNA markers. Lanes 1 and lane 2, RT-PCR products from 3D4/21/CD163 cells, showing the amplification of a 3333 bp fragment corresponding to the CD163 gene (duplicated assays); lane 3, corresponding RT-PCR products from 3D4/21 cells; lane 4, RT-PCR negative control (the template was water). (B) Detection of CD163 protein expression in 3D4/21/CD163 cells and 3D4/21 cells by immunofluorescence staining. CD163, both cells incubated with anti-CD163 antibody. DAPI, both cells incubated with 4′,6′-diamidino-2-phenylindole. (C) Western blot analysis of 3D4/21/CD163 cells, showing the expression of a 130 kDa protein detected by the anti-CD163 antibody (upper panel), using anti-GAPDH antibody as a control (lower panel). Lane 1 and lane 2 (duplicated assays), cell lysates of the 3D4/21/CD163 cells; lane 3, cell lysates of the 3D4/21 cells.

Indirect immunofluorescence assays showed that the CD163 protein was expressed in the 3D4/21/CD163 cells and not in the 3D4/21 cells (Fig. 1B). Similarly, Western blot analysis showed that the CD163 protein expressed in the 3D4/21/CD163 cells had the expected molecular weight of 130 kDa (Fig. 1C).

3.3. Insertion site determination

To confirm that the CD163 expression observed in the 3D4/21/CD163 cells was generated by the transposition of the cloned gene and not by recombination, inverse PCR was performed to determine the PB integration site(s) in the cell genome and to analyze the integration flanking sequences. During transposition, the PB transposon is excised from the PB donor vector plasmid and then integrated into TTAA chromosomal sites. As shown in Table 1, the PB element in each of five colonies analysed was inserted adjacent to a TTAA site. These results showed that the chromosomal integration of the CD163 gene in the 3D4/21 cells transfected with the pPB-CD163 plasmid was mediated by a true transposition event.

3.4. Confirmation of PRRSV replication in 3D4/21/CD163 cells

Comparison of the susceptibility of the 3D4/21/CD163 cells to the PRRSV strains, VR-2332, HN and CH-1R, with those of 3D4/21 and Marc-145 cells showed overt CPE in the 3D4/21/CD163 cells infected by each of the three PRRSV strains by 48 h post-infection (Fig. 2A). No CPE appeared in the mock-infected 3D4/21/CD163 cells (Fig. 2A) and the correspondingly inoculated CD163-negative control 3D4/21 cells (results not shown). The specificity of the CPE in the 3D4/21/CD163 cells was validated by indirect immunofluorescence assay with the anti-PRRSV-nucleocapsid (N) antibody which resulted in PRRSV N protein-specific staining in many cell clusters, similar to those observed in the parallelly infected Marc-145 cells, indicating PRRSV infection in these cells. In contrast, the inoculated 3D4/21 cells did not show any PRRSV N protein-specific staining (Fig. 2A). Infection of the 3D4/21/CD163 cells by all three PRRSV strains was further confirmed by RT-PCR amplification of the N gene from the infected cells, in which a 372 bp product was produced in the 3D4/21/CD163 cells and not in the corresponding 3D4/21 cells (Fig. 2B). In addition, PRRSV infectivity in the 3D4/21/CD163 cells was demonstrated by Western blot analysis in which the PRRSV N protein was detected in the 3D4/21/CD163 cells infected with each of the three PRRSV strains and not in the mock-infected 3D4/21/CD163 cells (Fig. 2C).

Infection of the 3D4/21/CD163 cell line by the natural HN strain was confirmed by immunofluorescence assay (Fig. 3A) and RT-PCR (Fig. 3B) of the infected cells. The intracellular and extracellular HN virus titers in the infected 3D4/21/CD163 cells were $10^{4.6 \pm 0.4}$ $TCID_{50}$/mL and $10^{4.3 \pm 0.3}$ $TCID_{50}$/mL respectively, indicating that the 3D4/21/CD163 cells could be a useful for the isolation of natural PRRSV isolates.

Table 1
Analysis of transposon-chromosomal junctions mediated by pPB-CD163 in 3D4/21/CD163 cells[a].

Independent isolated clone	Chromosomal insertion site	Flanking chromosomal sequence (5'→3')
3D4/21/CD163-A	TTAA	TGGTACTTCTTTGAGAGA
3D4/21/CD163-B	TTAA	ATCTATCATCTTTTCTAT
3D4/21/CD163-C	TTAA	ACATCAAGAATACTAACA
3D4/21/CD163-D	TTAA	TCTAAAAGGCAGTAGATG
3D4/21/CD163-E	TTAA	TATTAGCTTTCTGAAATG

[a] Five 3D4/21/CD163 colonies were analyzed, and all showed that the PB transposon mediated CD163 integration into a TTAA site.

3.5. Efficiency of PRRSV production in 3D4/21/CD163 cells

The efficiency of PRRSV production of the 3D4/21/CD163 cells was established after infections with the strains VR-2332, HN and CH-1R at a MOI of 0.1 were serially passage-adapted through the cell line. As shown in Supplementary Table 1, when the VR-2332 and HN strains were passaged three times and the CH-1R strain was passaged five times on 3D4/21/CD163 cells, the virus titers of each of the PRRSV strains increased with each passage to reach a stable level. Peak titers of the progeny VR-2332 and HN strains, harvested from the 3D4/21/CD163 cells at 72 h post-infection, and of the CH-1R strain harvested at 96 h after inoculation, were $10^{6.8 \pm 0.5}$, $10^{6.5 \pm 0.3}$ and $10^{6.6 \pm 0.3}$ $TCID_{50}$/mL, respectively, while corresponding peak titers of $10^{5.8 \pm 0.4}$, $10^{6.0 \pm 0.3}$ and $10^{5.6 \pm 0.3}$ were achieved from the Marc-145 cells infected with the VR-2332, HN and CH-1R strains, respectively (results not shown).

4. Discussion

PRRSV shows a highly restricted tropism for cells of the monocyte-macrophage lineage *in vivo* (Nauwynck et al., 1999). The restricted tropism of PRRSV for these host cells was attributed to the presence of specific cellular receptors (Adams et al., 2016; Zhang and Yoo, 2015).

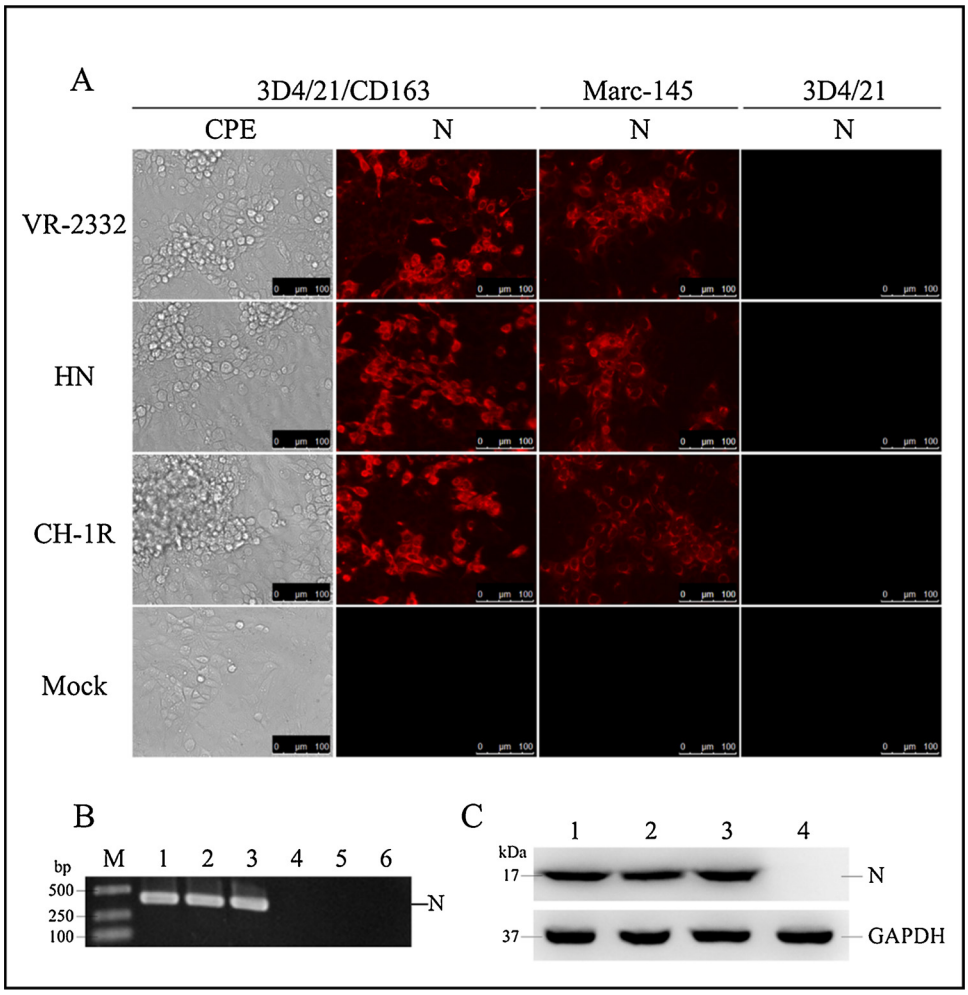

Fig. 2. Analysis of PRRSV replication in 3D4/21/CD163 cells. (A) Confirmation of PRRSV infection of 3D4/21/CD163 cells, showing the development of PRRSV-specific CPE and the detection of virus-specific immunofluorescence against the nucleocapsid protein (N) in the infected cells (200× magnification) 2 days post-infection, using correspondingly infected Marc-145 cells as positive controls and correspondingly infected 3D4/21 cells as negative controls. The panels from left to right show: PRRSV-specific CPE and immunofluorescence in 3D4/21/CD163 cells infected with each of the PRRSV strain (VR-2332, HN and CH-1R) and the corresponding mock-infected (Mock) controls; virus-specific immunofluorescence in infected Marc-145 cells and the mock control; and no virus-specific immunofluorescence in the inoculated 3D4/21 cells and the mock control. (B) Detection of PRRSV-specific RNA in the various infected cell lines by RT-PCR, showing the amplification of a 372 bp fragment of the PRRSV nucleocapsid protein gene (N) detected in a 0.8% agarose gel. M, DNA marker. Lane 1, VR-2332-infected 3D4/21/CD163 cells; lane 2, HN-infected 3D4/21/CD163 cells; lane 3, CH-1R-infected 3D4/21/CD163 cells; lane 4, VR-2332-inoculated 3D4/21 cells; lane 5, HN-inoculated 3D4/21 cells; lane 6, CH-1R-inoculated 3D4/21 cells. (C) Detection of PRRSV-specific protein synthesis in the infected cell lines by Western blot assay of the PRRSV nucleocapsid protein (N) (upper panel) and using GAPDH as control (lower panel). Lane 1, VR-2332-infected 3D4/21/CD163 cells; lane 2, HN-infected 3D4/21/CD163 cells; lane 3, CH-1R-infected 3D4/21/CD163 cells; lane 4, mock-infected 3D4/21/CD163 cells.

Therefore, the development of a monocyte-macrophage cell line of swine origin that is both highly susceptible to PRRSV and yielding high titers of the progeny virus is crucial to the study of PRRSV biology as well as the reliable propagation of the virus.

Although PRRSV-susceptible monocyte-macrophage cell lines suitable for PRRSV production *in vitro* were generated, including the Marc-145 cell line that stably expressed CD163 (Wu et al., 2018) and the murine macrophage MH-S^{CD163} cell line (Li et al., 2017), these cell lines were not of swine origin and thus their application to swine-related studies are limited. Similarly, CD163 transgenic PRRSV-susceptible cell lines of swine origin, such as the porcine endometrial endothelial (PEE) cell line and the PK-15^{CD163} cell line (Wang et al., 2013), were established, but they were not from the monocyte-macrophage lineage. In contrast, primary PAM cells are the natural host cells of PRRSV and thus

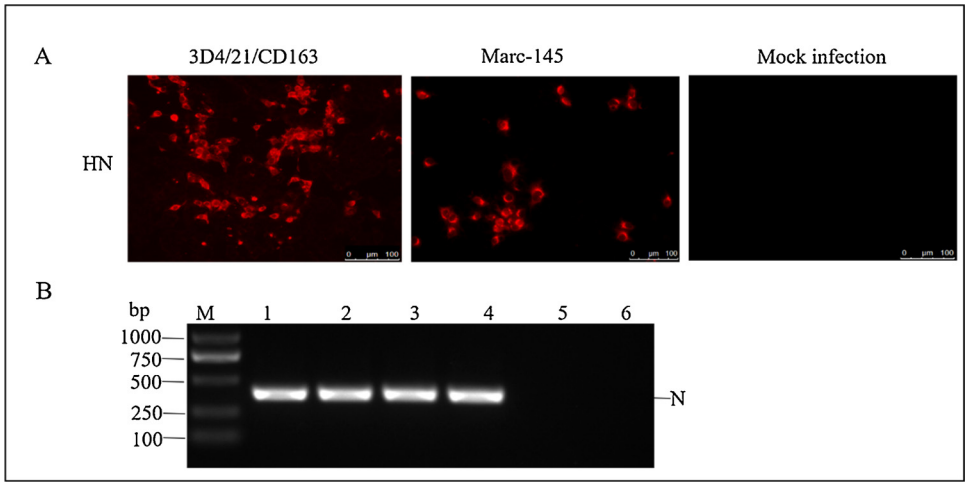

Fig. 3. Analysis of a natural source of PRRSV HN strain isolation in 3D4/21/CD163 cells. (A) Confirmation of HN isolation in 3D4/21/CD163 cells by virus-specific immunofluorescence against the PRRSV nucleocapsid protein gene (N) protein in the infected cells (200× magnification) at 48 h post infection, using correspondingly infected Marc-145 cells as positive control and 3D4/21/CD163 cells as mock infection control. The panel from left to right shows: PRRSV-specific immunofluorescence in 3D4/21/CD163 cells infected with HN strain prepared from the serum of infected swine; HN-specific immunofluorescence in infected Marc-145 cells; Mock infection control performed in 3D4/21/CD163 cells. (B) Detection of HN-specific RNA in 3D4/21/CD163 cells by RT-PCR, showing the amplification of a 372 bp fragment of the PRRSV N gene detected in a 0.8% agarose gel. M, DNA marker. Lane 1 and lane 2 (duplicated assay), HN-infected 3D4/21/CD163 cells; lane 3 and lane 4 (duplicated assay), HN-infected Marc-145 cells; Lane 5, HN mock-infected 3D4/21/CD163 cells; Lane 6, RT-PCR negative control (the template was water).

are the ideal cells for the study of PRRSV pathogenesis and virus production, since they are likely to replicate the processes of disease development in pigs upon PRRSV infection. However, the primary PAM cells are difficult to standardize and cannot be continuously passaged and the immortalized PAM cell lines (3D4/2, 3D4/21 and 3D4/31) established (Weingartl et al., 2002) were not permissive for PRRSV infection due the lack of the CD163 receptor. Although retroviral gene transfer was used to efficiently transfect the receptor CD163 into the immortalized 3D4/21 cells to make it susceptible to PRRSV (Lee et al., 2010), there are certain obstacles against its practical application due to safety and manufacturing challenges (Di Matteo et al., 2012). Therefore, a non-viral gene transfer vehicle that allows for the simple and highly efficient exogenous gene integration into the host genome was needed.

The PiggyBac transposon-based method meets the criteria of simplicity and high efficiency in generating stable cell lines and, in the present study, it was successfully used to transfer the CD163 gene for expression in an immortalized PAM cell line. This was an advancement over the use of viral vectors for transgenic cell line construction. Firstly, the PB transposon method is technically simple and does not require the production of high-titer virus stocks which are of limited shelf-life (Woltjen et al., 2009). Secondly, the PB transposon has a very large cargo capacity for the size of the transgene that can be cloned into the vector. It was reported that the PB transposon could deliver DNA fragments of up to 100 kb into the cell genome (Li et al., 2011) and that the transposition efficiency was not affected by the cargo size, while viral vectors have a limit on the size of the transgene that can be incorporated. Thirdly, the PB transposon integrates the transgene into the cell genome, which allows for the long-term and stable expression of the transgene. Fourthly, the transgene can be removed by transposase expression (Mitra et al., 2008; Wang et al., 2008). Thus, based on these four characteristics, the PB transposon method could be readily applied to other cell types to generate stable cell lines.

The susceptibility of the 3D4/21/CD163 cells to PRRSV infection was tested using three different PRRSV-2 strains. All the strains grew well on the 3D4/21/CD163 cells, indicating that the cells would be a useful tool for PRRSV-2 production and isolation. In addition, the progeny PRRSV titers produced in the 3D4/21/CD163 cells were higher than those produced in Marc-145 cells, indicating that the virus replicated more efficiently in the 3D4/21/CD163 cells than in the Marc-145 cells. Moreover, a higher titer of the vaccine strain CH-1R was produced in the 3D4/21/CD163 cells than in Marc-145 cells, indicating that the 3D4/21/CD163 cells could be used for vaccine production. However, this study did not test whether the 3D4/21/CD163 cells are susceptible to PRRSV-1 strains. Further research is necessary to determine whether PRRSV-1 strains, and PRRSV-1 and PRRSV-2 strains that do not grow on Marc-145 cells, can be propagated in the 3D4/21/CD163 cells.

5. Conclusions

In conclusion, the current study demonstrated the dispensability of using a viral vector for transgenic cell line generation. Instead, a significant step towards using non-viral vectors to generate stable transgenic cell lines was demonstrated and, in this study, the 3D4/21/CD163 cell line was efficiently generated by the PB transposon system. Further, the 3D4/21/CD163 cell line was used successfully for the isolation and production of certain PRRSV-2 strains and, potentially, for use in the study of PRRSV-host cell interactions *in vitro*. Therefore, the PB transposon system represents an alternative and an attractive tool for the construction of recombinant pig cell lines for the production and study of other porcine viruses.

Declaration of Competing Interest

There was no conflict of interest among the authors.

Acknowledgements

This study was supported by the Natural Science Foundation of Henan Province [grant number 162300410211] and the National Natural Science Foundation of China [grant number 81802837] and Key Scientific Research Project of Colleges and Universities in Henan Province [grant number 18B310019].

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jviromet.2019.113727.

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