RESEARCH REPORT
SWINE HEALTH

Title: Enhancement of *in vitro* replication efficiency of Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) in MARC-145 cell line - **NPB #06-161**

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Industrial summary

Porcine Reproductive and Respiratory Syndrome (PRRS) affects porcine industry globally and brings a significant economic loss in the agricultural sector. It is reported that both European and North American strains of PRRSV circulate in respective local pig farms. Although several PRRS vaccines, including both modified Lelystad virus and killed virus, are currently available in the market, the efficacy of these vaccines is mainly based on the genetically diverged field strains of PRRSV and do not confer protection against a virulent heterologous strain. Specific vaccine against designated strains maybe a solution, but it is found that some strains of PRRSV isolated from field failed to propagate in cell culture making the vaccine production difficult. In this project a PRRSV-susceptible cell-line derived from African green monkey kidney cell, MARC-145, are modified to enhance the susceptibility to various strains of PRRSV and thus the propagation efficiency of the virus. The cells were transfected with porcine sialoadhesin, recently shown to be a putative PRRSV receptor. These modified cells were infected with locally isolated PRRSV strains and the replication efficiency will be assessed and evaluated. Both EU and NA PRRSV strains were employed in the experiment. The result shows that expression of porcine sialoadhesin in MARC-145 cells did not facilitate a higher proliferation rate than in the wild-type MARC-145 cells. The reason may be due to that the virus strains used in the experiments propagate well in the MARC-145, therefore the enhancement brought by the addition of sialoadhesin may not be significantly shown. To further characterize the effect of porcine sialoadhesin in MARC-145, a virus strain that cannot grow well in MARC-145 should be isolated and employed in this experiment. Nevertheless, successful transformant may still provide an insight into vaccine production against specific PRRSV strains.

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Scientific abstract

Porcine Reproductive and Respiratory Syndrome (PRRS) is one of the most serious problems in the global porcine agricultural industry. Although several PRRS vaccines, including both modified Lelystad virus and killed virus, are currently available in the market, the efficacy of these vaccines is mainly based on the genetically diverged field strains of PRRSV and do not confer protection against a virulent heterologous strain. Specific vaccine against designated strains maybe a solution, but it is found that some strains of PRRSV isolated from field fail to propagate in cell culture, which make the vaccine production difficult. In this project, a PRRSV-susceptible cell-line derived from African green monkey kidney cell, MARC-145, was modified to enhance the susceptibility to various strains of PRRSV. Sialoadhesin (CD169) is a lectin-like receptor and is restrictedly expressed in subsets of tissue and inflammatory macrophages. It is shown that sialoadhesin mediates endocytosis and internalization of bound PRRSV particle into porcine alveolar macrophage. Transcripts of sialoadhesin were isolated from the porcine alveolar macrophage and a 5.4kb DNA fragment was obtained by RT-PCR. The fragment was cloned into expression vector to form a pcDNA3.1-sialo expression construct. Wild-type MARC-145 cells were then transfected with the construct and extrinsic sialoadhesin expression was detected with real-time RT-PCR assay. Both EU and NA PRRSV strains were inoculated into modified MARC-145, wild-type MARC-145 and MARC-145 transfected with vector only. ORF5 specific real-time PCR reveals that the expression of extrinsic sialoadhesin did not enhance the virus replication rate of both PRRSV strains. The reason may be due to that both these two virus strains used in the experiment have been already adapted to the wild-type MARC-145 cells, therefore these viruses are able to replicate efficiently and grow well in the MARC-145 cells. As these strains may use the intrinsic putative PRRSV receptor in the MARC-145 cells efficiently, the enhancing effect of the extrinsic sialoadhesin may be hampered and therefore there was no significant increase in the rate of replication. In order to further characterize the function of this extrinsic sialoadhesin, virus strains that replicate poorly in the wild-type MARC-145 should be isolated and screened for this experiment.

Nevertheless, this approach may take advantages from both PAM and MARC-145 cells and provide an alternative method of continuous *in vitro* PRRSV propagation.

Introduction

Killed and live-attenuated vaccines are the common strategies employed in PRRSV vaccination. Production of such vaccines depends on an efficient line of continuous cell culture. Porcine alveolar macrophages (PAM) are the most susceptible culture system for both European and North American PRRSV, and they are commonly used for virus isolation purpose. Nevertheless, there is variability among batches of macrophages in terms of their susceptibility to PRRS viruses. Moreover, as macrophage culture is a comparatively unstable culture, it is not suitable to be used in large scale propagation of PRRSV. While MARC-145, a derivative of African green monkey kidney cells MA-104, is also susceptible to PRRSV infection and can be used for virus propagation. However, it is found that not all PRRSV strains are able to propagate in this cell line. One reason for such propagation failure may be due to the incompetence of cell surface receptors to specific strains of virus. In this project, PRRSV receptor, porcine sialoadhesin, were cloned and expressed *in vitro* on the surface of MARC-145 cells. As sialoadhesin is the natural PRRSV receptor in the host, virus binds to this molecule more specifically than to the putative receptors on MARC-145 cells. Specific binding may in turns facilitate the entry process of the virus. This approach takes advantages from both PAM and MARC-145 cells and provides an alternative method of continuous *in vitro* PRRSV propagation.

Objective

Sialoadhesin (CD169) is a lectin-like receptor and is restrictedly expressed in subsets of tissue and inflammatory macrophages. It is shown that sialoadhesin mediates endocytosis and internalization of bound PRRSV particle into porcine alveolar macrophage. Expression of sialoadhesin on MARC-145 cell may increase the binding of virus onto cell monolayer and hence facilitate the entry process.

Materials and methods

Molecular Cloning of porcine sialoadhesin

A fresh pig lung was obtained from a local slaughterhouse and the tissue was kept on ice just after slaughtering and immediately transported to laboratory. Alveolar macrophages were isolated by larvaging the lung with approximately 500ml of sterile cold phosphate-buffered saline (pH7.4). The recovered larvage solution was centrifuged at 240 ×g at 4°C for 15 min. The cell pellet was re-suspended in sterile PBS. Total RNA from approximately 1×10^7 cell was extracted with 1ml TRIzol reagent according to manufacturer's instruction. After then mRNA was purified from the pool of total RNA with Straight A's mRNA Isolation System (Novagen). Briefly, 500μg total RNA sample was mixed with 1ml of Magnetight oligo-dT solution. The mixture was allowed to incubate at room temperature for 5mins. The RNA-Magnetight particle was then captured by Separation stand. The particle was then washed twice with wash buffer and the mRNA was eluted with 100μl nuclease-free water. A total of 3.5μg mRNA was then used from 1st strand cDNA synthesis. Briefly, reverse-transcription was carried out with 200 U of Superscript II reverse transcriptase (Invitrogen, CA) in a 20 μl reaction containing 500ng of oligo-dT primers, 1 × RT buffer, 10mM dithiothreitol (DTT) and 0.5 mM deoxynucleotide triphosphates (dNTPs). Reaction was carried out in Peltier Thermal Cycler (MJ Research, CA) for 50 min at 42 °C followed by 15 min at 70 °C. The synthesized cDNA was subjected to RNase H treatment prior to PCR amplification.

Specific primers are designed according to sequence of *Sus scrofa* sialoadhesin mRNA (GenBank accession no. DQ176853). The sense primers overlap the start ATG codon and include a Kozak motif (5'-GCCGCCGCC-3') immediately upstream of the start codon to facilitate the translation of mRNA in eukaryotic cells. The antisense primers overlap the translation termination codon and, both primers include restriction sites that facilitate directional cloning into expression vector. Conventional PCR and gel electrophoresis was carried out. Briefly, 1 μl of cDNA from clinical samples was amplified with 1U ThermalAce DNA polymerase (Invitrogen, CA) in a 25 μl reaction containing 1 × PCR buffer, 1 × dNTPs and 0.5 pmol of each forward and reverse primer. Reaction was performed in Peltier Thermal

Cycler (MJ Research, MA) for 3 min at 95 °C, followed by 30 cycles of 95 °C for 30 sec, at 56 °C for 30 sec, at 74 °C for 5mins, and for a 10-min final extension step at 74 °C. Amplicons were analyzed with 1 % agarose gel electrophoresis and positive samples were subjected to cycle sequencing reaction for sequence confirmation. The amplicon was then purified and cloned into pcDNA3.1 expression vector via *Nhe*I and *EcoR*I sites.

Expression of porcine sialoadhesin in MARC-145 cells

MARC-145 cells were seeded into 6-well culture plate and grown into 80% confluent. The cells were transfected with pcDNA3.1-sialo and pcDNA3.1 vector control respectively. Mock transfection control was also included. These cells were then selected for Geneticin resistance (1mg/ml). Three weeks after transfection a population of Geneticin-resistant MARC-145 was obtained and this population of cells was used in this study .Expression of sialoadhesin was ascertained with real-time RT-PCR. Briefly, at 48hrs after transfection, the cells were rinsed once with 1×PBS. Total RNA was extracted with TRIzol reagent followed by DNase I digestion. First strand cDNA was synthesized with random primers. PCR was then conducted with cDNA from cells transfected with pcDNA3.1-sialo, pcDNA3.1 vector control and from non-transfected cells. DNase I treated RNA was also tested to avoid artifact from the plasmid construct.

Preparation of virus

Two strains of PRRSV representing both European (EU) and North American (NA) types were isolated in local pig farm in 2005. The viruses were propagated and titrated in MARC-145 cells. Virus titer was estimated in both $TCID_{50}$.

Evaluation of replication efficiencies of PRRSV in wild-type and modified MARC-145 cells

The cells transfected with pcDNA3.1-sialo, pcDNA3.1 vector control, and non-transfected MARC-145 cells were inoculated with 0.1/ 1.0/ 5.0 m.o.i. of EU or NA type of PRRSV respectively. Medium was harvested from the corresponding wells at 12, 24, 48, 72 and 96hrs post-inoculation. RNA extraction and 1st strand cDNA synthesis was carried out with 200μl harvested medium. Real-time PCR was performed with primers specific to ORF5 of EU and NA PRRSV respectively. Briefly, In a 25 μl reaction, 1μl cDNA template was amplified with 0.625 unit *Taq* DNA polymerase in present of 1 × Sybr® Green I Buffer, 0.2 mM dNTPs (with dUTP), 3 mM $MgCl_2$ and 0.5 pmol of each forward and reverse primer. Reaction volume was then adjusted to 25 μl, and the reactions were performed in the iCycler iQ Real-Time PCR Detection System (Bio-Rad Laboratories, CA) for 10 min at 94 °C, followed by 50 cycles of 94 °C for 10 sec, at 55 °C for 10 sec, at 72 °C for 10 sec. Fluorescence signals (FAM, excitation = 490 nm, emission = 530 nm) were collected at the end of each extension step during the PCR cycles. Threshold cycle (Ct) of each sample was determined using maximum curvature approach, and melting curve analysis was performed after PCR cycles. PCR amplification was performed in triplicate.

Result

Molecular Cloning of porcine sialoadhesin

A 5.4kb DNA fragment was amplified from the mRNA extracted from porcine alveolar macrophage (Fig.1). The result shows that only the cDNA synthesized with mRNA gave positive amplicon in the PCR amplification. The amplicon was then cloned into the pcDNA3.1 eukaryotic expression vector via *Nhe*I and *EcoR*I sites (Fig.2). Sequence analysis reveals that this porcine sialoadhesin fragment showed high homology (>95%) to the other porcine sialoadhesin sequence published in the Genbank.

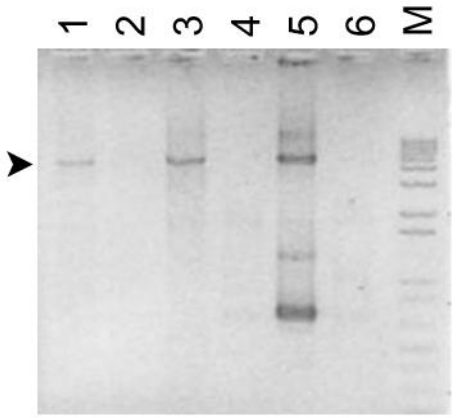


Figure 1. PCR amplification of porcine sialoadhesin. Both the total RNA and purified mRNA were employed as the starting materials for cDNA synthesis. Lane 1, 3 and 5: cDNA synthesized with mRNA; lane 2, 4 and 6: cDNA synthesized with total RNA. M = 1kb marker.

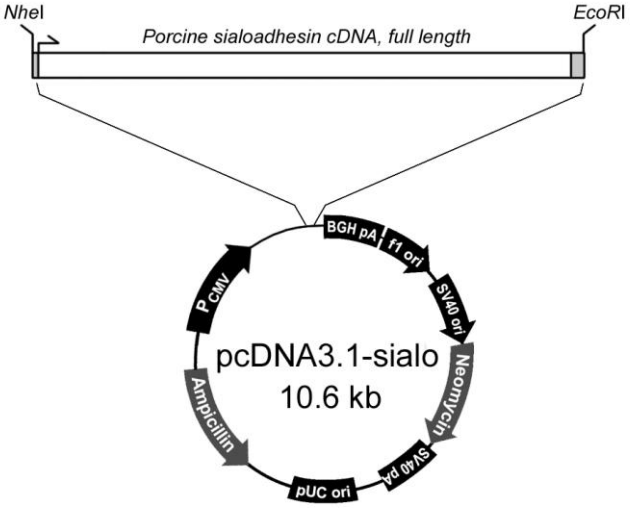
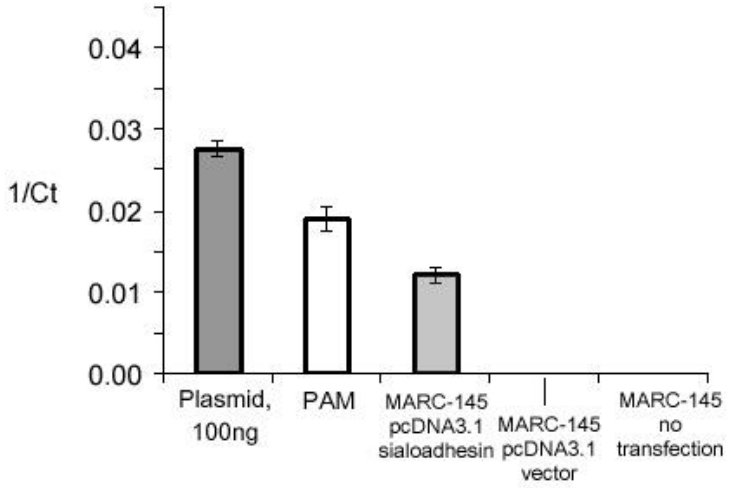


Figure 2. Expression construct of porcine sialoadhesin. The sequence of the open reading frame was confirmed prior to transfection to avoid frame-shifting and mis-sense codons.

<u>Expression of porcine sialoadhesin in MARC-145 cells</u>

The expression of porcine sialoadhesin in MARC-145 cells was confirmed by real-time RT-PCR assay (Fig.3). The result reveals that sialoadhesin transcript was detected in both porcine alveolar macrophage and the cell transfected with pcDNA3.1-sialo. Between them the alveolar macrophage shows higher sialoadhesin expression level than that in the transfected MARC-145 cells. No detectable sialoadhesin transcript was observed in the cell transfected with pcDNA3.1 vector only and the wild-type PRRSV cells.

Figure 3. Expression of sialoadhesin in MARC-145 cells. Sybr-green based real-time RT-PCR was employed to estimate the sialoadhesin level in various cell types. Among four cell types, native PAM (porcine alveolar macrophage) showed highest sialoadhesin transcript level, while the MARC-145 transfected with pcDNA3.1-sialo also expressed the molecule at a detectable level. In contrast, no sialoadhesin transcript was detected in the wild-type and vector transfected MARC-145 cells. Total RNA samples were DNase I-treated to avoid artifacts.



Evaluation of replication efficiencies of PRRSV in wild-type and modified MARC-145 cells

Virus replication efficiencies of EU and NA PRRSV strains in MARC-145 cells were evaluated with real-time PCR. The virus titer was estimated by quantifying the PRRSV ORF5 copy number in the infected cells. The result shows that upon infection, the virus titers of EU and NA strains increased along with time within 96 hrs. At 0.1 m.o.i., NA PRRSV replicated at the same rate in the three cell types. While at 1.0 m.o.i., the virus replicated at a higher rate and achieved a higher titer in the cells transfected with pcDNA3.1-sialo than that in the cell transfected with vector only or the wild-type PRRSV. However, at a higher virus titer, i.e., 2.0 m.o.i., the replication rate resumed to be similar to those transfected with vector.

On the other hand, the transfection of pcDNA3.1-sialo showed no enhancement for EU PRRSV replication in MARC-145 cells. At 0.1 and 2.0 m.o.i, the virus replicated at the highest rate in the wild-type PRRSV (no transfection). While at 1.0 m.o.i, the replication rate and virus titer achieved the highest level in the cell transfected with vector only.

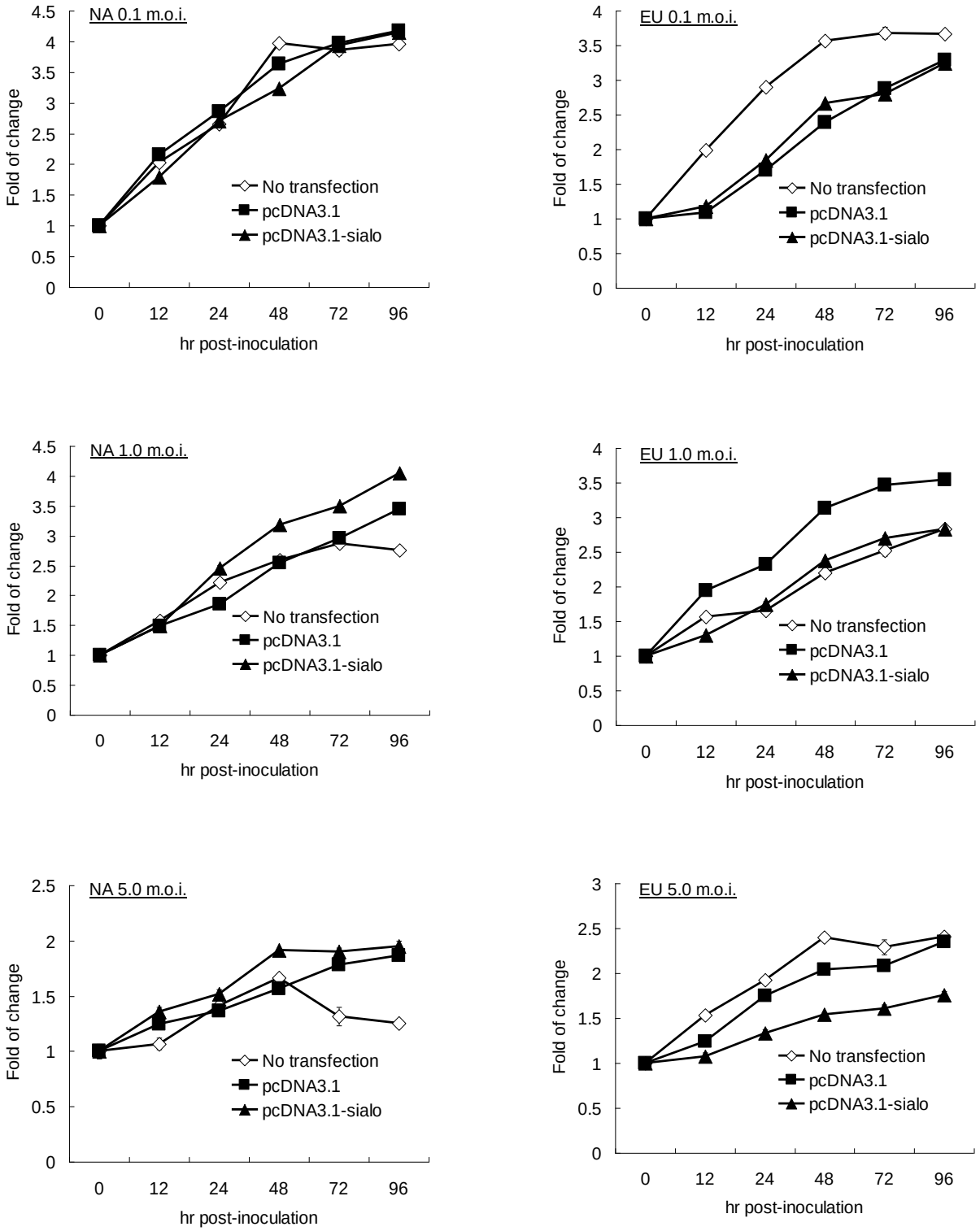


Figure 4. EU and NA PRRSV replication efficiencies in MARC-145 cells.

Discussion

In this project, we hypothesized that the extrinsic porcine sialoadhesin expression in the MARC-145 cells which are commonly used for PRRSV propagation may facilitated the entry process of the virus strains that poorly replicate in wild-type MARC-145 cells. The sialoadhesin transcript was firstly isolated from porcine alveolar macrophage. Previous report revealed that the amplification of full-length sialoadhesin fragment was hampered and therefore we proposed to construct the full-length expression clone by joining three amplicon segments. The experiment was then re-performed with using mRNA instead of total RNA as the starting material for the cDNA synthesis. Besides, the *Taq* DNA polymerase was replaced by the ThermalAce DNA polymerase system which was able to give a longer amplicon size. Eventually a 5.4kb fragment was obtained and was then cloned into pcDNA3.1 eukaryotic expression vector. In order to avoid truncation and frame-shifting, the entire sequence of the clone was analysed. The result shows that the sequence was with high homology (>95%) to the porcine sialoadhesin sequence published in the Genbank and the open reading frame was also confirmed.

After sequence confirmation, the expression construct was transfected into MARC-145 cells. The vector control was also transfected with the same condition in order to assess the effect of the transfection process on the virus replication. DNase I was used to remove the residual DNA plasmid sample in order to prevent PCR artifacts. By this treatment, there was no positive amplicon observed in the pcDNA3.1-sialo transfected MARC-145 RNA sample (data not shown). The real-time RT PCR result shows that the sialoadhesin transcript level in PAM was higher in the MARC-145 transfected with pcDNA3.1-sialo construct. PAM was the natural primary replication site for the PRRSV. It is believed that the virus mainly utilizes the sialoadhesin molecules exposed on the PAM surface for the entry purpose. Therefore, the expression of sialoadhesin should be significant. On the other hand, it is also expected that the transfection of the pcDNA3.1-sialo may lead into an over-expression of sialoadhesin within the MARC-145. However, the level of transcript was lower than that in the PAM. The reason may be due to that the transfection process affected the protein synthesis machinery in the MARC-145 cells, or the condition of transfection, for example, the ratio of DNA to lipofectamine, was not at its optimum. Nevertheless, the present of extrinsic sialoadhesin molecule may be sufficient for the downstream virus replication analysis

The virus replication efficiency in the MARC-145 cells was assessed with real-time PCR specific to the viral ORF5 subgenomic transcript. This ORF5 subgenomic transcript increased stably during the course of virus replication and it copy number was directly proportional to the virus titer (data not shown). It is expected the the expression of extrinsic sialoadhesin molecule in the MARC-145 cells may increase the rate of virus replication. However, the result reveals that there was no significant difference among the wild-type cells and those transfected with the pcDNA3.1-sialo construct. From our hypothesis, we supposed that the viruses may utilize sialoadhesin in addition to the intrinsic putative PRRSV receptor in the MARC-145 cells. As the chance of entry increases, the rate of replication may increase accordingly. On the contrary, no significant increase on the rate of both EU and NA PRRSV was observed in the cell transfected with MARC-145. The reason may be due to that both these two virus strains used in the experiment have been already adapted to the wild-type MARC-145 cells, therefore these viruses are able to replicate efficiently and grow well in the MARC-145 cells. As these strains may use the intrinsic putative PRRSV receptor in the MARC-145 cells efficiently, the enhancing effect of the extrinsic sialoadhesin may be minimized and therefore there was no significant increase in the rate of replication.

In order to further characterize the function of this extrinsic sialoadhesin, virus strains that replicate poorly in the wild-type MARC-145 should be isolated and screened for this experiment.

Notwithstanding the virus replication rate was not enhanced in this experiment, this approach may take advantages from both PAM and MARC-145 cells and provide an alternative method of continuous *in vitro* PRRSV propagation.