RESEARCH REPORT
SWINE HEALTH

Title: Identification of Type I Interferon Antagonists of PRRSV Viral Structural Proteins **– NPB #07-108**

Investigator: Kay S. Faaberg, PhD

Institution: USDA, ARS, National Animal Disease Center

Date Submitted: 8/3/2009

Industry Summary:

PRRSV is an important swine pathogen and infections caused by this virus have not been efficiently controlled. This is in part due to the lack of knowledge about the insufficient swine immune responses seen after infection with PRRSV. This proposal sought to pinpoint PRRSV structural proteins that may interfere with one arm of the swine immune system, called type I interferon. Type I interferon plays an extremely important role in early innate antiviral immune responses and the initiation of adaptive immune responses.

Individual PRRSV structural proteins of one viral strain were cloned and an attempt was made to express these clones as proteins. The results of this study aim were disappointing in that no PRRSV protein could be detected. Extensive research ensued in order to identify the experimental conditions needed to express the PRRSV proteins. PRRSV structural and nonstructural proteins have now been successfully expressed in Marc-145 cells, which was a critical step for further study in our laboratory. The initial PRRSV interferon antagonist screening method also did not perform as well as expected, providing inconclusive data. After much study, we concluded that the tools outlined in the proposal would not suffice. We are now collaborating with Dr. Laura Miller of The National Animal Disease Center, who had concurrently established an optimal method to identify type I interferons in infected cells. Screening of individual PRRSV proteins that may serve as Type I interferon antagonists, down-regulating the immune response, is currently underway.

Once the PRRSV protein(s) that act as type I interferon antagonists have been identified, we will pinpoint the actual polypeptides that down-regulate this important arm of the immune response and seek to mutate the protein region in our reverse genetic system to eliminate the antagonism. We will also seek to understand if other PRRSV strains act in a similar manner. This research will ultimately provide new strategies in designing new vaccines and control methods.

Kay Suzanne Faaberg, Ph.D.
Research Microbiologist USDA-ARS B-14
Virus and Prion Diseases of Livestock
National Animal Disease Center
2300 Dayton Avenue
Ames, IA 50010
Phone: (515) 663-7259
Fax: (515) 663-7458
Email: kay.faaberg@ars.usda.gov

Scientific Abstract:

PRRSV has been known to suppress type I interferon production, but the exact mechanism is still unknown. Type I interferons (INFα and INFβ) play an important role in early innate antiviral immune responses and initiation adaptive immune responses. This project was intended to identify PRRSV structural protein(s) that may counteract the immune response by serving as type I interferon antagonist(s). To achieve this goal, PRRSV structural proteins were initially cloned in pcDNA plasmid vectors that can support production of encoded proteins in eukaryotic cells, but no protein expression was detected. As a result, other plasmid vectors were investigated. One plasmid, pCI, was eventually identified as suitable for cloning and expression of PRRSV proteins. In addition, a Flag-tag was engineered at the C-terminal end of each protein to facilitate identification in transfected eukaryotic cells. Individual structural and nonstructural proteins of PRRSV have now been successfully expressed in Marc-145 cells. We also investigated several variations of the proposed screening method to be utilized in identification PRRSV proteins that may act as type I interferon antagonists, downregulating the robustness of the innate immune response. We concluded that the proposed method was not suitable for our purposes. Instead, we are now collaborating with Dr. Laura Miller, who has established confirmatory assays for the activity of type I interferon (IFN-α and IFN-β) at the National Animal Disease Center. Specifically, these tests include a bioassay based on interferon stimulation of Mx1 gene transcripts, interferon-alpha and interferon-beta (IFN-α and IFN-β) gene transcriptional assays (real-time RT-PCR), and immunoassays (ELISA) for IFN-α and IFN-β. This screening system for type I interferon has now been established with recombinant attenuated Newcastle disease virus with an incorporated gene for the green fluorescent protein (rNDV/GFP), a positive indicator of type I interferon induction. We are now testing the PRRSV protein transfected Marc-145 cell supernatants for interference with rNDV-GFP type I interferon induction.

Introduction:

PRRSV, an economically important pathogen in the US, causes reproductive failure in pregnant sows, respiratory infections in young pigs and is a problem for swine producers worldwide. The complexity of the virus has made standard control measures ineffective, and present vaccines do not provide sterilizing immunity for swine. In addition, PRRSV seems to induce a poor immune response, for reasons that are not well understood. This proposal sought to identify PRRSV proteins that may interfere with type I interferons, key components of an effective immune response against many viruses.

Type I interferons, i.e. IFN-α and IFN-β, are produced by most nucleated cells upon virus infection, and can act on cell surface receptors which will prime host cells to eliminate the invading virus by synthesizing hundreds of proteins, formally known as the innate immune response. Type I interferons are also involved in mediating acquired immune responses. The ability of many viruses to counteract type I interferon synthesis has been regarded as an important virulence factor. Some strains of PRRSV have been recently shown to inhibit type I interferon production both *in vitro* and *in vivo*, but the mechanism for PRRSV immune evasion has not been elucidated.

In this study, PRRSV structural proteins from North American (genotype 2) virulent strain MN184, were to be individually cloned onto a eukaryotic expression vector. Several variations using different commercial plasmids were tested, and one, pCI, proved effective. We also attempted to develop an effective screening method for type I interferon and potential inhibitors of interferon, but were unsuccessful. However, a recent collaboration with Dr. Laura Miller - who had established a bioassay for active type I interferon, qRT-PCR tests for IFN-α and IFN-β RNA expression, and assays for IFN-α and IFN-β protein expression - brought about effective analysis

tools for type I interferon antagonistic activity in cells transfected with plasmids expressing individual PRRSV proteins.

Objectives:

1. To evaluate whether any of the PRRSV MN184 strain structural proteins act as interferon antagonists *in vitro*.

2. To determine the viral proteins(s) with the most effective anti-IFN activity using different PRRSV strains

Materials & Methods:

1. To evaluate whether any of the PRRSV MN184 strain structural proteins act as interferon antagonists *in vitro*.

a. *Construction of PRRSV structural protein expressing plasmids:* Eukaryotic expression vectors, such as pcDNA3.0, pcDNA3.1/CT-GFP and pcDNA6.2/C-EmGFP (Invitrogen) and pCI (Promega) were used for cloning PRRSV MN184 structural proteins. Primers were designed to amplify the ORFs of each PRRSV structural proteins with Kozak sequence incorporated at the 3'-end of the forward primers. Restriction sites compatible with the vector multi-cloning site were also incorporated into the 5'-ends of the primer sequences to facilitate cloning of the PRRSV ORFs in frame with an ORF for GFP or FLAG-tag. The ORFs of the structural proteins were amplified from purified MN184 viral RNA through high fidelity RT-PCR. The PCR products of each ORFs were digested with compatible restriction enzymes and cloned onto the pre-digested plasmids with T4 ligase. Clones carrying the insert were selected using appropriate antibiotics and the inserted PRRSV ORF fused with either GFP or FLAG-tag were verified by PCR and sequencing. Plasmids with the correct PRRSV structural protein ORF-GFP/FLAG-tag fusions were propagated and purified using high-pure large scale plasmid purification kit (Invitrogen) and stored at – 20 °C.

b. *In vitro translation*: The use of rabbit reticulocyte lysates to translate encoded proteins is an effective measure of translatability. Our results using this method for the pcDNA clones of individual PRRSV proteins showed that these vectors did not produce any measurable amount of the encoded products. Later on, to streamline our expression studies, green fluorescent protein (GFP) or FLAG-TAG inserts were fused onto the C-terminus of each PRRSV protein ORF. Purified expression plasmids of each of the PRRSV structural proteins were used to transfect Marc-145 cells using Lipofectamine 2000 (Invitrogen) in 96-well and 24-well plates using the recommended protocol. Briefly, plasmid DNA and Lipofectamine 2000 were mixed in Opti-MEM medium in the ratio of 1:3 (µg:µl), and 0.3 and 1.0 µg DNA was used to transfect Marc-145 cells in 96-well and 24-well plates respectively. After 24 hr and 48 hr transfection, protein expression was tested either directly under fluorescence microscope (GFP) or through FLAG-tag immunofluorescent staining.

c. *Selection of the most IFN sensitive PRRSV strain on Marc-145 cells:* Eight PRRSV strains, MN184, VR-2332, Ingelvac PRRS MLV, pMN184, pVR-V7, pMLV, pMLVORF1/MN184 and pMN184ORF1/MLV were tested for sensitivity to a recombinant universal type I interferon. Marc-145 cells were plated onto 24-well dishes and incubated with 10-100 U/ml rIFN-α for 24 h before virus infection. IFN-α treated cells were then infected with the eight PRRSV strains at MOI of 0.01. After 48 h, each supernatant will be harvested and titrated for PRRSV infectivity using standard $TCID_{50}$ analysis. After obtaining inconclusive results, a recombinant Newcastle disease virus expressing GFP, an inducer of interferons, was also evaluated for use in type I interferon antagonist screening.

d. *Selection of the IFN antagonist*: Marc-145 cells were seeded onto a 96-well plate. Transfection of Marc-145 cells with PRRSV plasmid DNA and poly(I:C), an inducer of interferon, utilized Lipofectamine 2000 (Invitrogen) and was performed mainly as described by the manufacturer. Transfected cells were then infected with rNDV-GFP and fluorescence was recorded as an indicator of type I interferon reduction.

2. **To determine the viral proteins(s) with the most effective anti-IFN activity using different PRRSV strains** – not done

Results:

1. To evaluate whether any of the PRRSV MN184 strain structural proteins act as interferon antagonists in vitro.

a. *Construction and expression of PRRSV MN184 structural proteins in Marc-145 cells:* PRRSV MN 184 structural protein encoding sequences were amplified by RT-PCR and cloned under the control of the eukaryotic CMV promoter in the plasmid pcDNA3.0 (Invitrogen). To enhance protein expression, Kozak consensus sequences were incorporated into the forward primer sequences for each PRRSV structural protein. The cloned plasmids were amplified in *E. coli* and purified, then sequenced to confirm plasmid integrity.

b. *In vitro translation*: Initial *in vitro* translation results revealed that the PRRSV proteins were not suitably expressed as designed. We used an alternative method, immunofluorescent staining, to verify these negative results. Marc-145 cells were transfected using pcDNA3.0-ORF7 complexed with Lipofectamine 2000 (Invitrogen) at a ratio of 1: 3 (µg of DNA:µl of Lipofectamine). pMax-FP (Amaxa), a plasmid carrying GFP was included as a transfection control. After 24 hours, up to 30% of the pMax-FP transfected cells showed typical green fluorescence. Then, pcDNA3.0-ORF7 transfected cells were fixed with 4% paraformaldehyde in PBS for 30 minutes and permeabilized with 0.5% Triton-X 100 in PBS for 15 minutes at room temperature. The fixed cells were incubated with monoclonal antibody (MAb) SDOW-17. PRRSV strain MN184 infected Marc-145 cells for 48 hours was used as a control for immunofluorescent staining. PRRSV infected cells showed strong green fluorescence, but the pCDNA3.0-ORF7 transfected cells were negative for SDOW-17 MAb staining (Figure 1). Only pcDNA3.0-ORF7 was tested as the nucleocapsid protein, encoded by ORF7, is abundantly expressed in infected Marc-145 cells and is easily detected by monoclonal antibody SDOW-17.

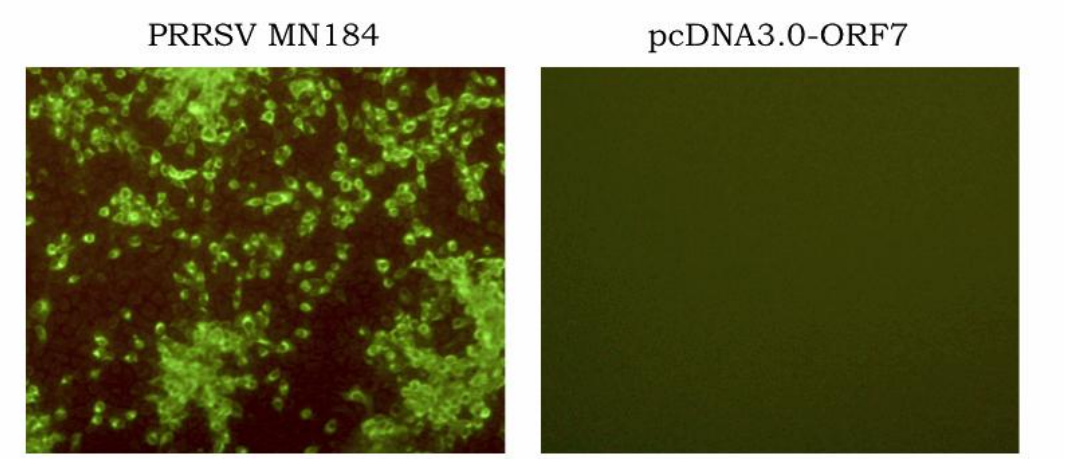

Figure 1. Marc-145 cells were infected with PRRSV MN184 or transfected with pcDNA3.0-ORF7 complexed with Lipofectamine 2000. After 48 hours of incubation at 37°C, the cells were stained by fluorescently-labeled SDOW-17 MAb.

We then investigated whether or not the pcDNA3.0 plasmid is suitable for PRRSV protein expression. The GFP gene of pMax-FP was cloned into pcDNA3.0, and transfections were performed at various conditions with Lipofectamine 2000 to optimize the transfection conditions, using pMax-FP as a positive control. Strong and consistent fluorescence from pMax-FP transfected Marc-145 cells could be detected, but there was no fluorescence detected in the

pcDNA3.0-GFP transfected cells. pcDNA-GFP was also evaluated for GFP expression on 293T and HeLa cells, which are more competent for transfection than Marc-145 cells. There was an increased transfection efficiency with pMax-FP, but none of the two cell types showed fluorescence when transfected with pcDNA3.0-GFP. Therefore, we concluded that pcDNA3.0 is not suitable for protein cloning for our studies. The CMV promoter region was sequenced in pcDNA3.0 to ensure vector integrity, and no sequence mutations were identified compared with the original sequence.

A newer version of pcDNA cloning plasmid, pcDNA6.2-GFP, was used to clone the PRRSV MN184 structural proteins. This plasmid carries GFP gene that allowed MN184 structural proteins to be fused with GFP at their C-terminal ends. The inserted structural protein encoding sequences in the plasmids were sequenced to make sure they were in frame with the GFP gene. Transfections of the pcDNA6.2-MN184 structural proteins into Marc-145 cells could not reliably demonstrate expression of inserted PRRSV protein sequence by direct microscopic examination or ORF7 SDOW-17 MAb staining (data not shown). The transfections were also performed in HeLa and 293T cells. No expression of any PRRSV proteins in these transfected cells was detected. pMax-FP and pcDNA6.2-GFP plasmids were used as controls. pcDNA6.2-GFP transfected cells showed only weak fluorescence, and the pMax-FP transfected cells showed strong and consistent fluorescence (Figure 2). We concluded that pcDNA6.2-GFP is also not suitable for PRRSV protein expression in our system.

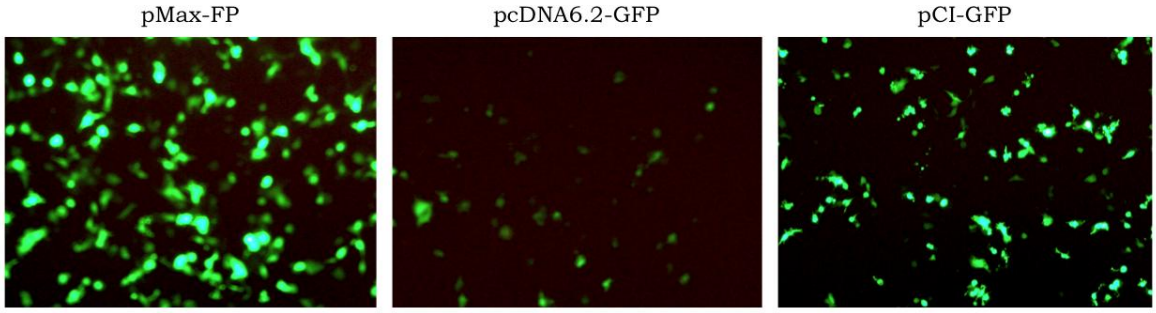

Figure 2. Marc-145 cells were transfected with pMax-FP (+ control), pcDNA6.2 and pCI eukaryotic expression vectors, each with a green fluorescence protein (GFP) insertion. pcDNA6.2 had the lowest performance in regards to GFP expression.

Next, we search for a plasmid suitable for transfection. Several other laboratories reported that the vector pCI (Promega) was suitable for abundant protein expression. The plasmid was tested initially by inserted the GFP gene from pMax-FP into pCI. pCI-GFP worked as well as pMax-FP for GFP expression, which indicated that the pCI plasmid was superior to pcDNA3.0 and pcDNA6.2 in protein expression in transfected cells (Figure 2). This finding was a major advancement in this study.

To facilitate the identification of PRRSV proteins in transfected cells, the nucleotide sequence encoding a FLAG-tag (DYKDDDDK) was incorporated into pCI (pCI-Flag). With this construct, each PRRSV protein was extended at the C-terminus with a FLAG-tag which could be detected by an anti-FLAG MAb. Initially, PRRSV strain MN184 ORF5 and ORF7 were cloned into pCI-Flag to test the performance for our protein expression studies. Our results suggested that this vector would express ample amounts of PRRSV protein. Eventually, all of the MN184 structural proteins and most of the nonstructural proteins were cloned with a Kozak consensus sequence at the initiation codon. Transfections of the pCI-structural/nonstructural protein-FLAG plasmids demonstrated that all of the structural proteins expressed well and so did the nonstructural proteins (Figure 3). Successful expression of PRRSV structural and nonstructural proteins laid a foundation for screening the antagonist proteins in PRRSV.

Translation of plasmids using the rabbit reticulocyte in vitro system (Promega) was intended for confirmation that the proteins expressed were full length. However, since there is now a FLAG-tag placed at the C-terminal end of the proteins, successful immunofluorescent staining was indicative of complete PRRSV protein production.

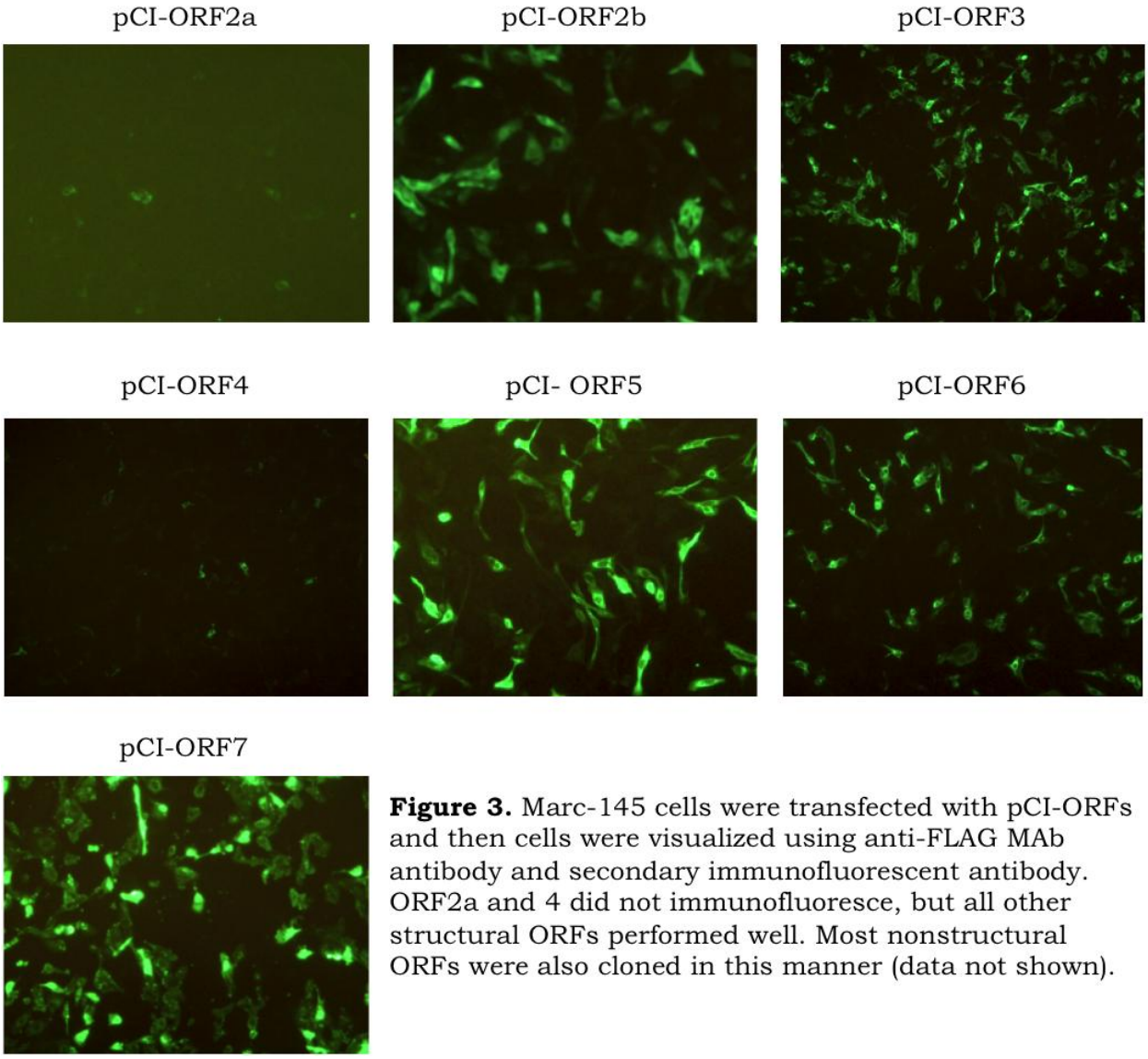

Figure 3. Marc-145 cells were transfected with pCI-ORFs and then cells were visualized using anti-FLAG MAb antibody and secondary immunofluorescent antibody. ORF2a and 4 did not immunofluoresce, but all other structural ORFs performed well. Most nonstructural ORFs were also cloned in this manner (data not shown).

c. *Selection of the most IFN sensitive PRRSV strain on Marc-145 cells:* PRRSV strain sensitivity detection on Marc-145 cells was performed to identify a virus that would be suitable for type I interferon antagonist screening. Sensitivity tests were performed on several PRRSV strains, including MN184, VR-2332, Ingelvac PRRS MLV, pMN184, pVR-V7, pMLV, pMLVORF1/MN184 and pMN184ORF1/MLV. Our results showed that the differences in interferon sensitivity among the viruses were inconsistent, indicating that the use of a PRRSV strain to screen for type I interferon antagonism was not going to be reliable (Table 1). Instead, a recombinant Newcastle disease virus expressing GFP was obtained (Dr. P. Palese, Mount Sinai School of Medicine). The rNDV-GFP can infect Marc-145 cells and NDV is sensitive to type I interferon. Test results were reproducible and thus rNDV-GFP was utilized for selection of the IFN antagonist (item “d” immediately below).

d. *Selection of the IFN antagonist*: Marc-145 cells were first transfected with individual PRRSV constructs in the presence of Lipofectamine overnight. The next day the transfected cells were infected with rNDV/GFP. After a subsequent overnight incubation, GFP fluorescence was monitored to screen for any reduction in rNDV-GFP replication. Control plasmids included pCI-FLAG (positive control for interferon) and pCI-GFP (positive control for transfection). It was expected that transfection of Marc-145 cells with pCI-FLAG would stimulate type I interferon production, which would in turn inhibit rNDV-GFP replication - a negative control for type I interferon antagonism. However, we found that Marc-145 cell transfection with pCI-FLAG did not reduce rNDV-GFP replication, which indicated that pCI-FLAG transfection induced very low levels (or does not induce) any type I interferon. We concluded that Marc-145 cells must be stimulated with another molecule (described below) to activate the type I interferon system in order to test for type I interferon antagonist effects that the PRRSV protein clones may possess.

Synthetic double stranded RNA, Poly(I:C), was ultimately used to stimulate type I interferon production in Marc-145 cells. Cells were transfected with poly(I:C) complexed with lipofectamine 2000, or treated with poly(I:C) in culture medium. Transfection with as low as 0.005 μg poly(I:C) in 96-well plate format can significantly reduce rNDV-GPF replication. Screening for potential type I interferon antagonists using the PRRSV structural protein clones is now in progress.

2. **To determine the viral proteins(s) with the most effective anti-IFN activity using different PRRSV strains** - not completed due to unforeseen technical difficulties detailed under Objective 1.

Table 1. TCID50 titration of supernatants of Marc-145 cells. Marc-145 cells were treated or untreated with IFN at concentrations of 10 of 100 units/ml overnight, then the cells were infected with different PRRSV strains at an MOI of 0.01. Supernatants were harvested 48 hrs post inoculation and titrated on Marc-145 cells to obtain $TCID_{50}$ values. Fold changes in TCID50 between treated and untreated groups were calculated and used as an indicator of IFN sensitivity. Rows highlighted in color have significant variation (pink>blue).

TCID50 assay on 2008-10-16

	Mean TCID50			Fold change	
Virus	**Untreated**	**10 units rIFN/ml**	**100 units rIFN/ml**	**10 units rIFN/ml**	**100 units rIFN/ml**
MN101/A1	1.00E+07	1.00E+06	1.00E+05	10.0	100.0
VR2332	1.00E+06	1.00E+06	3.16E+04	1.0	31.6
RespPRRS	4.68E+06	4.68E+06	1.00E+06	1.0	4.7
pMN184	4.68E+05	4.68E+05	3.16E+04	1.0	14.8
pMLV	1.00E+06	1.00E+06	1.00E+04	1.0	100.0
pVR-V7	3.16E+05	1.00E+05	1.00E+04	3.2	31.6
pMN184ORF1/MLV	2.14E+05	1.00E+06	4.68E+04	0.2	4.6
pMLVORF1/MN184	3.16E+06	2.14E+06	1.00E+05	1.5	31.6

TCID50 assay on 2008-11-3

	Mean TCID50			Fold change	
Virus	**Untreated**	**10 units rIFN/ml**	**100 units rIFN/ml**	**10 units rIFN/ml**	**100 units rIFN/ml**
MN101/A1	2.1E+05	1.0E+06	3.9E+04	0.2	5.3
VR2332	2.7E+04	3.9E+04	1.6E+04	0.7	1.7
RespPRRS	1.0E+07	1.0E+07	3.9E+06	1.0	2.6
pMN184	6.6E+04	6.6E+04	3.2E+04	1.0	2.1
pMLV	7.3E+06	7.3E+06	3.2E+06	1.0	2.3
pVR-V7	3.9E+04	3.9E+04	7.3E+02	1.0	53.3
pMN184ORF1/MLV	2.7E+05	2.8E+05	3.2E+04	0.9	8.4
pMLVORF1/MN184	7.3E+06	2.8E+06	2.8E+06	2.6	2.6

Discussion:

Type I interferon plays an important role in innate antiviral immunity and the initiation of adaptive response. PRRSV is known to suppress type I interferon secretion, but the mechanism is not yet understood. Understanding of this mechanism may help to define new strategies for vaccines and PRRSV control. We had hoped to identify type I interferon antagonists among the PRRSV structural proteins, leading to further studies on the remaining nonstructural regions of the virus. However, we found that the experiments, as designed, were not adventitious and thus had to reformulate our methods to allow for future streamlined exploration of the entire genome of PRRSV.

Several eukaryotic vectors were explored in order to clone and express PRRSV strain MN184 structural proteins, including pcDNA3.0, pcDNA6.2 and pCI. Through very time-consuming experiments, we demonstrated that only pCI could be used to properly express abundant amounts of PRRSV proteins in Marc-145 (and other) cells. pCI was also engineered to include an eight amino acid tag (Flag-tag) at the C-terminus of the PRRSV proteins in order to facilitate

expression identification. Structural proteins and also most of the nonstructural proteins of PRRSV strain MN184 were cloned into pCI-FLAG with abundant protein expression in Marc-145 cells. Furthermore, PRRSV and rNDV-GFP were tested for use in screening for type I interferon antagonists. It was shown that rNDV is a sensitive indicator of type I interferon secretion in cells and will be used in the screening of potential type I interferon antagonists using the pCI-Flag-PRRSV protein clones. Since cells transfected with the pCI construct does not induce type I interferon secretion as was expected, synthetic double stranded RNA (poly(I:C) was used to activate and induce type I interferon in Marc-145 cells. Currently, screening for type I interferon antagonists among the PRRSV structural proteins is in progress.

Cloning and expression of PRRSV proteins has been shown to be difficult. In this study, several plasmid vectors were tested for their suitability in PRRSV protein cloning and eukaryotic expression. Also, a screening system has been established using rNDV-GFP, which will prove advantageous in future studies. We are now collaborating with Dr. Laura Miller of our institute to utilize her recently derived analysis tools for interferon levels. These endeavors have thus laid useful foundations for further identification of PRRSV antagonist proteins.