Interferon type I response in porcine reproductive and respiratory syndrome virus-infected MARC-145 cells

Brief Report

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Summary. Infection by porcine reproductive and respiratory syndrome virus (PRRSV) results in a weak induction of the innate immune response. There are many genes that collectively comprise this response and the extent to which each gene responds to PRRSV infection is unclear and warrants further investigation. To this end, we have utilized real-time PCR using SYBR Green I dye-based detection to quantify transcript abundance of the type I interferons (IFN-α and -β) and IFN-β transcriptional enhanceasome genes. In MARC-145 cells, both IFN-α and -β transcript abundance were unaffected by PRRSV infection. However, stimulation of MARC-145 cells by exogenous double-stranded RNA, resulted in significant increases in transcript abundance of both IFN-α and -β as well as IFN-β enhanceasome components, indicating that a type I IFN response could be induced in these cells. The double-stranded RNA induction of type I IFN transcription was significantly inhibited by dual-exposure with PRRSV. These results suggest that PRRSV infection directly interferes with type I IFN transcriptional activation early in its pathway, at the level of IFN-β gene transcription.

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Porcine reproductive and respiratory syndrome virus (PRRSV), the causative agent of porcine reproductive and respiratory syndrome (PRRS) in swine, is a member of the family *Arteriviridae* in the order *Nidovirales*. Initially described in the U.S.A. in 1987 and called "mystery swine disease" or "blue-ear disease," PRRS is a major problem for swine production and is endemic in most swine-producing countries, with the majority of herds persistently infected [23, 25]. PRRS results in both reproductive failure (late-term abortions and stillbirths) in pregnant

sows and respiratory disease (pneumonia) in nursery and grower/finishing pigs [36].

The primary cellular target of PRRSV is the pulmonary alveolar macrophage (PAM) [31, 33]. Other cell types permissive for PRRSV are peritoneal macrophages, pulmonary intravascular macrophages, type II pneumocytes, and testicular germ cells [9, 22, 26, 27]. The MARC-145 cell line, derived from MA-104 cells, supports replication of PRRSV and is widely used in diagnostic and research applications [14].

Type I interferons (IFN-α and -β) are synthesized and secreted by eukaryotic cells in response to viral infections [6]. Initiation of IFN synthesis typically involves binding of the IFN-β gene promoter's positive regulatory domain by specific transcription factors that collectively comprise the transcriptional enhanceasome [6, 18]. These transcription factors include ATF-2/c-Jun (AP-1), NF-κB, and members of the IFN regulatory factors (IRF) family, such as IRF-3 [12, 17, 32, 35]. Viral infection leads to the activation of these transcription factors and subsequently to the synthesis and secretion of IFN-β. This process triggers a cascade that leads to the secretion of a small subset of IFN-α gene products [18]. IFN secretion now primes cells through IFN activated signaling pathways to express IFN-inducible genes, including different members of the IRF family, such as IRF-3 and -7 [3, 11, 34].

Previous studies have shown that only low levels, if any, of IFN-α are recovered from PRRSV-infected pig lungs or *in vitro*-infected alveolar macrophages [1, 4, 29]. Albina et al. described PRRSV suppression of IFN-α production by transmissible gastroenteritis coronavirus (TGEV)-infected alveolar macrophages [2]. In addition, PRRSV replication was shown to be inhibited by the addition of IFN-α [2]. Collectively, these studies suggest that PRRSV does not induce, and perhaps actively suppresses, IFN-α expression. In this study, we investigated the effect of PRRSV infection on type I IFN gene expression in MARC-145 cells by examining directly the transcript abundance of both IFN-α and -β as well as IFN-β transcriptional enhanceasome genes. The transcript abundance for each gene was measured for both PRRSV-infected cells and PRRSV-infected cells that had the type I IFN genes up-regulated by exposure to exogenous double-stranded RNA.

Throughout these studies, MARC-145 cells were infected using a multiplicity of infection of 10 to ensure exposure of all cells to virus and resulted in the maximal number of infected cells. PRRSV isolate 16244 b was applied to confluent monolayers in chilled MEM media and permitted to adhere at $4\,°C$ for 1 h, then pre-warmed MEM media with 5% fetal calf serum (FCS) was added and the cells were placed at $37\,°C$ with 5% CO_2 and humidification. For each experiment, triplicate samples were utilized under the given experimental condition to minimize variability observed among samples. The cells were harvested at 16 and 24 h post-infection by a quick rinse with PBS followed by lysis in guanidium isothiocyanate (GITC) buffer containing β-mercaptoethanol. The supernatants from multiple experiments were pooled and placed at $-80\,°C$ until RNA purification was performed. Total RNA was purified from the GITC lysates using

the RNeasy Mini Kit (Qiagen, Valencia, CA, U.S.A.). Following extraction, the RNA preparation was precipitated with sodium acetate/ethanol using RNase-free glycogen as a non-specific carrier. RNA samples were treated with DNA-free™ (Ambion, Austin, TX, U.S.A.) to ensure that all DNA had been removed. The RNA preparations were quantified and purity assessed using spectrophotometry and electrophoresis.

Primers directed against the human sequence of the genes listed in Table 1 were designed using Primer Express® software v1.0 (Applied Biosystems, Foster City, CA, U.S.A.) to generate an amplicon of 300 to 350 bp from MARC-145 genomic DNA. The amplicons were purified and sequenced using capillary fluorescence based sequencing methods [10]. The identity of each gene was confirmed by sequence homology against the known human sequence and "nested" sequence-specific primers were designed using Primer Express® software v1.0 and synthesized (Integrated DNA Technologies, Inc., Coralville, IA, U.S.A.) to generate optimal sized (100 to 150 bp) amplicons for real-time reverse transcription-polymerase chain reaction (RT-PCR) (Table 1). Primers were designed that flanked

Table 1. Oligonucleotide sequences designed for real-time RT-PCR

GenBank accession no.	Gene specificity (region of cDNA sequence amplified)	Annealing acquisition temperature (°C)	Oligonucleotide sequences (5′ → 3′)
NM_006347	Cyclophilin (207–307)	58; 79	GAAGCACCTTCCACAGGGTC (forward) AAATGGCCCCCGGTAAATAC (reverse)
V00549	Interferon α_2 (70–191)	58; 80	ACCTTTGCTTTACTGGTGGCC (forward) ATCTGTGCCAGGAGCATCAAG (reverse)
V00546	Interferon β_1 (39–147)	58; 79	TAGGCGACACTGTTCGTGTTG (forward) CCAAGCAAGTTGTAGCTCATGG (reverse)
BE616960	Activating transcription factor-2 (218–335)	56; 76	GACAGATGACCCCCAATTGAA (forward) GACTGCAACTCGGTTTTCCAG (reverse)
AI417972	v-jun avian sarcoma virus 17 oncogene homolog (105–219)	58; 79	TCTCCGTCGCAACTTGTCAAG (forward) GCTAACGCAGCAGTTGCAAAC (reverse)
M58603	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (p105) (577–677)	58; 79	ATGTGAAGGCCCATCCCAT (forward) TTGCTGGTCCCACATAGTTGC (reverse)
NM_001571	Interferon regulatory factor 3 (177–294)	60; 81	AGGATGCACAGCAGGAGGATT (forward) GCAGAGCGGAAATTCCTCTTC (reverse)
AF046869	PRRSV isolate 16244b (Michelle) ORF7 14889–15260 positive strand (11–111)	60; 81	ACAACGGCAAGCAGCAGAA (forward) TCTGGACTGGTTTTGCTGAGC (reverse)
AF046869	PRRSV isolate 16244b (Michelle) ORF7 14889–15260 negative strand (260–366)	59; 81	CCTCTGGACTGGTTTTGCTGA (forward) AATAACAACGGCAAGCAGCAG (reverse)

splice sites to eliminate signal from potential genomic DNA contamination. An initial annealing temperature was selected for each primer pair based on their computed T_m (Table 1).

RT-PCR amplifications were performed using the MessageSensor^{TM} (Ambion) real-time RT-PCR kit as per the manufacturer's instructions with the following modifications. Briefly, a master mixture was prepared for a one-step RT-PCR reaction containing RT-PCR grade water, RT-PCR buffer containing 3 mM MgCl$_2$, SYBR Green I (final concentration of 1:40,000, Molecular Probes, Inc., Eugene, OR, U.S.A.), 8% glycerol, dNTPs (2.5 mM each), RNase inhibitor (10 U/µl), ROX internal reference dye (250 µM, Ambion), Super*Taq* polymerase (5 U/µl), Moloney murine leukemia virus (M-MLV) reverse transcriptase, gene specific RT-PCR primer mix (1 mM each of the gene-specific forward and reverse primers), and 50 ng RNA template. Triplicate reactions for each gene were prepared along with a "no-template" negative control (NTC) and a "no-amplification" control (NAC). The real-time RT-PCR reaction was performed using the ABI Prism 7700 Sequence Detection System (Applied Biosystems) in either 8-well strips, or 96-well plates. The cycling conditions were as follows: RT for 15 min at 42 °C, denaturation for 5 min at 95 °C, amplification for 40 cycles, with denaturation for 15 sec at 95 °C, annealing for 30 sec at 60 °C, extension for 50 sec at 76 °C, and detection after 2 sec at 76 °C. A melting curve analysis was performed from 60 °C to 95 °C post-amplification. The temperature transition rate for all segments of the amplification cycles and the melting curve cycle were set at 20 °C/sec, except for product melting curve analysis which was set at 0.2 °C/sec.

To quantify RNA abundance, the comparative threshold cycle (C_T) method was used which compares the relative amount of target sequence to any of the reference values chosen and the result is presented as relative to the reference value (expression level of mock-infected MARC-145 cells). The comparative C_T method ($\Delta\Delta C_T$) is the most practical method if the target and reference have similar dynamic ranges [21]. Calculations for target transcript quantitation begin with the difference (ΔC_T) between the C_T values of the target and the reference: $\Delta C_T = C_T \text{(target)} - C_T \text{(reference)}$. This is followed by transforming these values to absolute values, using the formula: Comparative expression level $= 2^{-\Delta\Delta C_T}$.

All statistical analyses were performed using SYSTAT 9.0 (SPSS, Inc., Chicago, IL, U.S.A.). Natural log transformed comparative expression data were evaluated by analysis of variance, and post hoc differences between treatment groups compared using least significant difference pairwise multiple comparison test.

To ensure that successful virus infection was achieved in each of the experiments performed, the amount of virus present was measured using real-time RT-PCR to detect the amount of PRRSV ORF7 positive- and negative-strand RNA (Table 1). In PRRSV-infected cells, the average relative RNA abundance of positive- and negative-strand ORF7 to cyclophilin in mock-infected cells was 18- to 60-fold at 16 h and 436- to 109-fold at 24 h, respectively. The change in positive- and negative-strand PRRSV ORF7 RNA between 16 and 24 h was 24- and 2-fold, respectively. These results were consistent with what one would expect

in a positive-stranded RNA infection [19] and allowed us to ensure that each set of experiments were equivalently infected (data not shown).

Previous work in our laboratory indicated that cyclophilin transcript abundance was unchanged in mock compared to infected MARC-145 cells (unpublished data) and, thus, would provide a good control gene for internal normalization. Primers were designed for the cyclophilin gene (Table 1) and an assay developed. The amplification conditions of the cyclophilin gene were optimized on the ABI 7700 and a single specific PCR product during both melting curve and electrophoretic analysis was observed. The fidelity of the real-time PCR assay is dependent upon NAC and no-template NTC controls, as well as the experimentally determined coefficients of variation present in the assay. Cyclophilin was used to determine the inter- and intra-assay variability present in the real-time RT-PCR assays by aliquoting cyclophilin-specific primers into six sample wells, three for mock-infected template and three for PRRSV-infected template. Each of the six samples was assayed through 40 cycles and the C_T of each reaction determined. The mean intra-assay coefficient of variation for mock-infected samples was $3.53\% \pm 0.03$ and for infected samples was $2.48\% \pm 0.01$. The assays were performed on three separate days and inter-assay coefficient of variation was determined to be 5.21% and 5.41% for mock-infected and infected cyclophilin mRNA abundance, respectively. The results of these experiments provided confidence that our assays would allow the accurate detection of mRNA abundance changes of genes examined. It also demonstrated that cyclophilin mRNA levels were invariant between mock- and PRRSV-infected cells with a calculated relative expression level of 0.98 between the two samples. In addition, cyclophilin levels were analyzed for each experiment performed and found to vary from 0.98 to 1.05, relative to the mock-treated cells. Thus, cyclophilin levels were unchanged as a result of PRRSV infection or exposure of the cells to dsRNA.

The effect of PRRSV infection of MARC-145 cells on type I IFN (IFN-α and -β) mRNA abundance was examined. The relative change in levels of mRNA between mock- and PRRSV-infected cells for IFN-α and -β genes at 24 h post-infection are shown in Fig. 1A. PRRSV did not significantly alter the abundance of either IFN-α or -β in the infected cells. This was surprising since replication of PRRSV results in the generation of dsRNA at levels expected to induce an IFN response. The abundance of IFN-α and -β mRNA at 16 h post-infection was measured as well (data not shown), and were similar to that observed at 24 h p.i. This assured that the lack of increase in IFN-α and -β mRNA abundance at 24 h p.i. was not due to examining the cells belatedly in infection and further confirmed that PRRSV infection was not inducing expression of these genes.

Since mRNA levels of both IFN-α and -β were not changed as a result of virus infection, the mRNA abundance of the enhanceasome component genes were examined. Alterations in the transcript abundance of these genes could influence IFN-β expression directly and IFN-α expression indirectly. Similar to the IFN genes, the mRNA abundance for all of the enhanceasome components did not significantly change as a result of PRRSV infection at 24 h post-infection (Fig. 1A). The abundance of these genes at 16 h post-infection were also examined
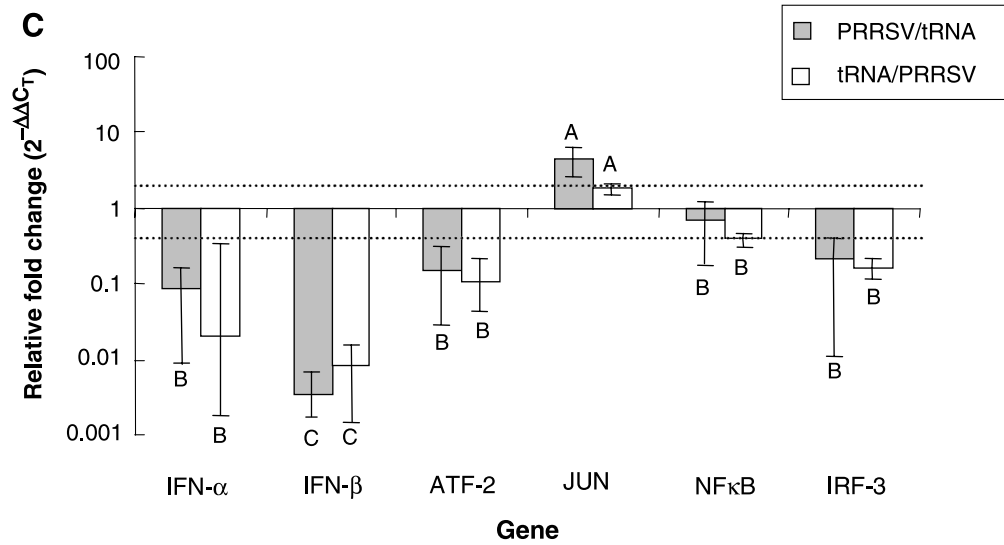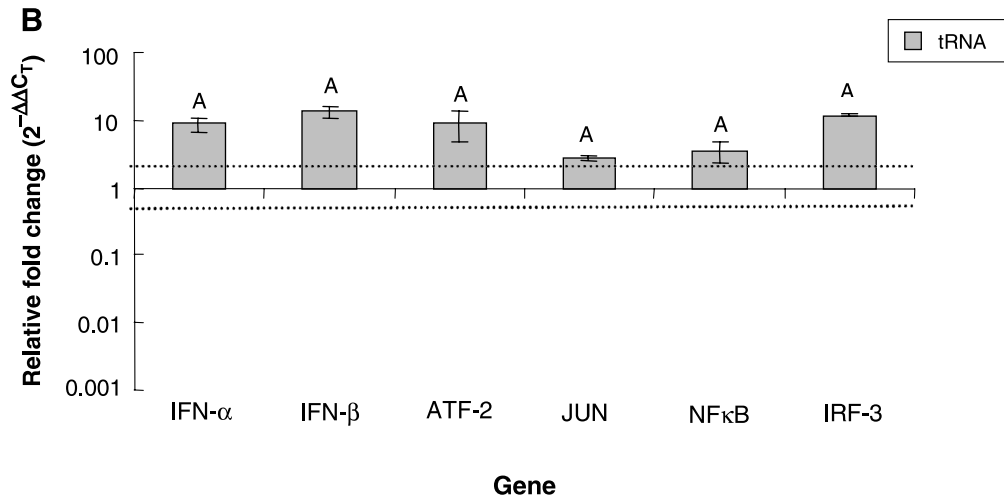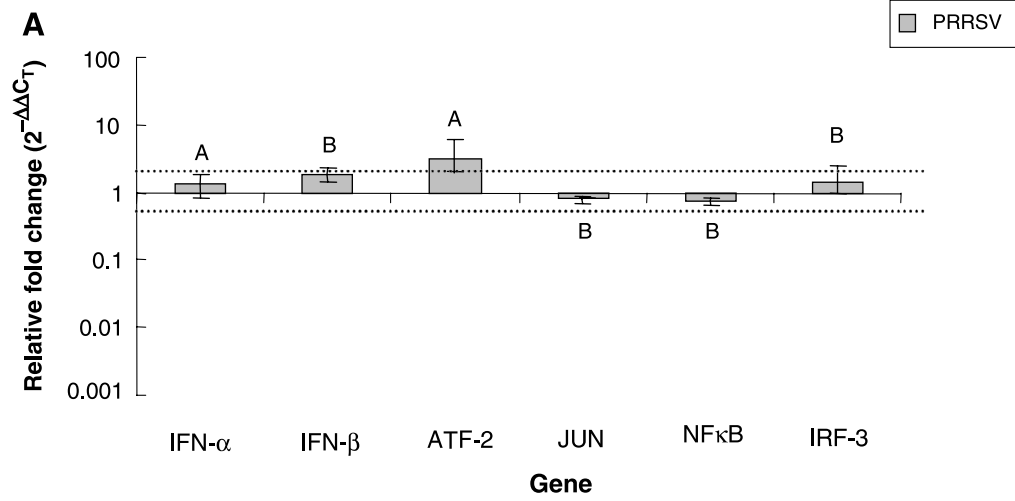
and found to have relative expression levels similar to that observed at 24 h post-infection. These results indicated that PRRSV did not specifically alter the expression of the enhanceasome components and, thus, the lack of type I IFN gene induction was unlikely attributed to a deficiency in enhanceasome gene product availability as a result of changes in transcript abundance.

Double-stranded RNA is formed during the multiplication of most viruses and is an important trigger for type I IFN synthesis [5, 16, 30]. It is known that yeast tRNA, a double-stranded RNA, stimulates the production of IFN-α and -β in exposed cells [30]. Therefore, yeast tRNA was utilized to determine if MARC-145 cells were capable of inducing an IFN response upon exposure to double-stranded RNA. MARC-145 cells were treated with phenylalanine-specific transfer RNA (tRNA) from brewer's yeast (Sigma-Aldrich, St. Louis, MO, U.S.A.) at a concentration of 50 μg/ml in serum-free MEM for 2 h at 37 °C before adding pre-warmed MEM with 5% FCS, and incubating at 37 °C with 5% CO_2 and humidification for 24 h and RNA extraction was performed. MARC-145 cells exposed to dsRNA showed a marked increase in expression of both IFN-α and -β with 8.8- and 13.2-fold increases, respectively (Fig. 1B). In addition, components of the IFN-β enhanceasome, ATF-2, Jun, NFκB, and IRF-3, were significantly increased in comparison to mock-infected cells (Fig. 1B). These results demonstrated that yeast tRNA is capable of inducing type I IFN expression in MARC-145 cells in a manner similar to that expected upon viral infection.

These experiments led to the conclusion that PRRSV infection does not result in the induction of the type I IFN genes or the corresponding enhanceasome genes. However, they did not distinguish between the lack of induction or, alternatively, specific interference with induction of these genes. To determine which of these two alternatives were occurring, MARC-145 cells were exposed to both yeast tRNA and PRRSV, and mRNA levels were measured as previously described. The experiments were performed in two ways, first exposing cells to yeast tRNA and then infecting them with PRRSV 2 h later (tRNA-PRRSV), or infecting cells with PRRSV and then exposing them to yeast tRNA 2 h later (PRRSV-tRNA). The results were similar regardless of the order in which the cells were treated (Fig. 1C). IFN-α and -β mRNA levels were decreased significantly in comparison to both the dsRNA-exposed cells and the PRRSV-infected cells. Similarly, the abundance of ATF-2 and IRF-3 transcripts were significantly lower

Fig. 1. Type I IFN response in MARC-145 cells after 24 h treatment. Data is represented as relative fold change in mRNA abundance in relation to mock-treated cells. The treatment groups include PRRSV (**A**), yeast tRNA (**B**), PRRSV followed by tRNA (filled box) and tRNA followed by PRRSV (open box) (**C**). Cyclophilin mRNA abundance from mock-infected MARC-145 cells was used to normalize treatment groups. Dashed lines indicate significant fold change from the reference gene, cyclophilin, set at a value of 2.0. Statistical analyses were performed on all of the data simultaneously as a group. Letters *A*, *B*, and *C* indicate statistically significant differences by ANOVA (p = 0.001), where series assigned the same letter are not significantly different

when compared to the dsRNA-exposed cells. The enhanceasome components JUN and NFκB were not significantly altered as a result of the dual-exposure to dsRNA and PRRSV (Fig. 1C). These results suggested that PRRSV infection specifically interfered with the dsRNA-induction of the type I IFN genes and the corresponding enhanceasome component genes. The mechanism by which PRRSV inhibits induction of IFN-α and -β, ATF-2, and IRF-3 in dsRNA-treated is unknown.

Dual exposure to dsRNA and PRRSV resulted in the down-regulation of four of the six genes analyzed when compared to mock-exposed cells. This was surprising given that PRRSV infection alone did not result in the down-regulation of these same genes. One possible explanation is that the PRRSV IFN interference mechanism is over-activated in these cells due to the continuous induction of type I IFN genes by dsRNA. This overcompensation could result in a decreased abundance of these transcripts. A second explanation is that our assay sensitivity does not permit an accurate quantitation of transcripts when present at extremely low levels. It would be expected that the type I IFN genes would be expressed at low levels, if at all, in un-induced cells. Given that our data is relative to mock-infected cells, which would have low levels of these transcripts present, slight changes in transcript abundance could have large effects if below the assays sensitivity. Thus, the fold down-regulation isn't as important as the fact that there wasn't an up-regulation that would be expected by the dsRNA induction.

Many viruses have evolved to actively interfere with type I IFN expression and activity. Viruses have been documented to inhibit IFN synthesis, bind and inactivate secreted IFN molecules, block IFN-activated signaling, and disturb the action of IFN-induced antiviral proteins [15, 24]. Among the best characterized evasion strategies are those of the influenza A viruses, which use the NS1 protein [7, 8] to block phosphorylation and nuclear transport of IRF-3 [28]. In addition, these viruses also interfere with the proper post-transcriptional processing of cellular antiviral pre-mRNAs [13]. The present study suggests that PRRSV can be added to the list of viruses that actively interfere with the induction of type I IFN gene expression.

Our results agree with previous studies showing minimal, if any, IFN-α induction by PRRSV infection *in vivo* [1, 4, 29]. Examining gene expression responses within pigs, or in alveolar macrophages harvested from pigs, presents many challenges. Factors such as inhaled debris, age of pigs, immune status, and the presence of other infectious agents make it difficult to decipher between those effects resulting from PRRSV infection and those resulting from the "other" factors. Thus, MARC-145 cells offered a feasible approach to identify PRRSV-responsive (or non-responsive) genes in a controlled, high throughput manner. With this information, we are initiating experiments to examine the type I IFN expression in PRRSV-infected porcine macrophages in a manner that will permit us to distinguish between PRRSV-specific and non-specific (non-PRRSV) responses.

As shown by many examples, interference with the IFN response may contribute significantly to the success of viruses in the interaction with their hosts [15, 24]. Further studies examining additional genes in the type I IFN pathway as

well as examining the expressed proteins are necessary to fully elucidate the mechanism of PRRSV interference. Experiments employing reverse genetics using the newly developed PRRSV infectious clone [20] will enable the identification of PRRSV proteins involved directly in modulating the host IFN response. Because many cellular factors are involved in IFN signaling, there are multiple locations in the pathway in which a virus can interfere. Identification of these locations and characterization of the interference mechanisms will provide needed information to develop novel strategies to better control PRRSV. One such strategy may be the development of vaccines that, upon delivery, induce a better type I IFN response.

Acknowledgements

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