RESEARCH REPORT
SWINE HEALTH

Title: Development of a Novel Self-Propagating PRSSV-VSV G Hybrid Replicon as a Vector for Inducing Broad PRRSV Protection - **NPB #13-186**

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Industry Summary:

Throughout successive cycles of ***Pork Check-off*** funding, our laboratories (Pattnaik's and Osorio's labs) have consistently produced new and fundamental information on: (i) the immunologic mechanisms that are important for protection against PRRSV infection, (ii) the structural basis for induction of PRRSV-neutralizing antibodies which are significant for conferring protective immunity, (iii) the possibility of producing a rationally-designed *new generation* DIVA vaccine that would offer more efficacious protection, and (iv) the presence of conserved B- and T-cell epitopes in the structural and nonstructural proteins (NSPs) of PRRSV.

In previous studies, we had identified important T-cell epitopes present in the NSP9 and NSP10 of PRRSV. The identified epitopes are highly conserved and we have shown that they are "seen" (recognized) by the immune system of the pig when infected by PRRSV (24). The degree of conservation of these epitopes suggests that they may be highly useful in the rational design of broadly efficacious vaccines against PRRSV. Furthermore, we have shown that a major component of protective immunity conferred by live vaccines is mediated by cell-mediated-immunity (CMI). Thus, if animals are preferentially immunized against conserved epitopes of NSPs, it is possible that such immunization could result in a highly "pan-strain specific" protective immunity which would establish protection against infection with a broader number of diverse PRRSV strains. **We rationalize that in order to design a broadly protective PRRSV vaccine, it is necessary to identify proteins and their well conserved epitopes derived from genomic regions that are unlikely to readily mutate due to functional and structural constraints.** Inducing CMI against the well conserved T cell epitopes should then control replication of a wide range of wt PRRSV strains.

In this NPB application (**#13-186**), we proposed to approach a rapid but thorough characterization of those genomic regions for cross (broad) protection by the development of a novel research tool: **a self-propagating (*def.* self-propagating = infectious) replicon (SPR) of PRRSV**. A replicon consists of a genetic construct that includes only the complete set of NSPs (i.e. the constituents of the replication

machinery) of PRRSV but none of the structural ones, and coupled to the glycoprotein (G) of vesicular stomatitis virus (VSV) that would provide the ability of the replicon to bud as an enveloped (infectious) particle as has been shown previously for Semliki Forest virus. We postulated that this **SPR**, when used to infect pigs, will be able replicate in swine cells in vivo and therefore expose the pig's immune system to the totality of non-structural antigens of PRRSV without causing any wt PRRSV infection or pathogenic effects **as no infectious PRRSV can be produced by this novel genetic construct.** The advantages of producing such **SPR** were multifold: (i) it would likely stimulate the immune system of the pig in a manner similar to the most effective platform: the modified live vaccines (MLVs); (ii) it would help elucidate and accurately quantify the level and broadness of protection contributed exclusively by the PRRSV NSPs in the absence of the structural proteins; (iii) the broad cell tropism of SPR would likely provide significant advantage for more robust immune response as the ability of this **SPR to infect different cell types in vivo** will be determined by the VSV G protein, thus significantly amplifying its tropism as opposed to the restricted tropism exhibited by the PRRSV MLVs; and (iv) it would provide a basic live, harmless immunogen to which we could later increase its immunogenic potential by inserting additional epitope(s) or individual structural antigen(s), eventually serving as a self-replicating virus-like immunogen that would stimulate further the immune system without producing any PRRSV infectious particle.

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Stated Objectives of the proposal:

Two objectives were proposed in the application. They are:

(1) Construct, generate, and characterize self-propagating replicons (SPR) of PRRSV encoding VSV G Protein

(2) Infect pigs with SPR to examine safety and pathogenic attributes of the SPR. Subsequently, examine immune response (CMI and humoral) in the animals.

Objective 1 was proposed to be completed in year 1 and objective 2 to be completed in year 2.

Complete report on research completed during the funding period:

OBJECTIVE 1

We carried out studies to construct and recover the SPR. In our interim report, we provided evidence of successful recovery of the SPR with the genomic organization as shown in Fig. 1A. This SPR (FL12GΔ2-4) lacked the PRRSV genomic regions encoding the PRRSV GP2 to GP4. In BHK-21 cells that are not normally susceptible to PRRSV infection, we were able to show that the SPR could replicate well. At early times (30 hpi) of infection with the SPR, several cells expressed the N protein of PRRSV but by 60 hpi, majority of the cells in the culture were expressing the N protein (Fig. 1B).

To determine if infectious SPR are produced from the BHK-21 cells transfected with FL12GΔ2-4 replicon RNA, culture supernatants from transfected cells were used to infect fresh BHK-21 cells and expression of VSV G protein as well as PRRSV N protein was examined. Both of these proteins were expressed in the infected cells (data not shown) indicating that the replicons containing particles were being produced from the originally transfected cells. These replicon containing particles (called SPR)

were then examined for their growth in BHK-21 cells and the proteins expressed in replicon

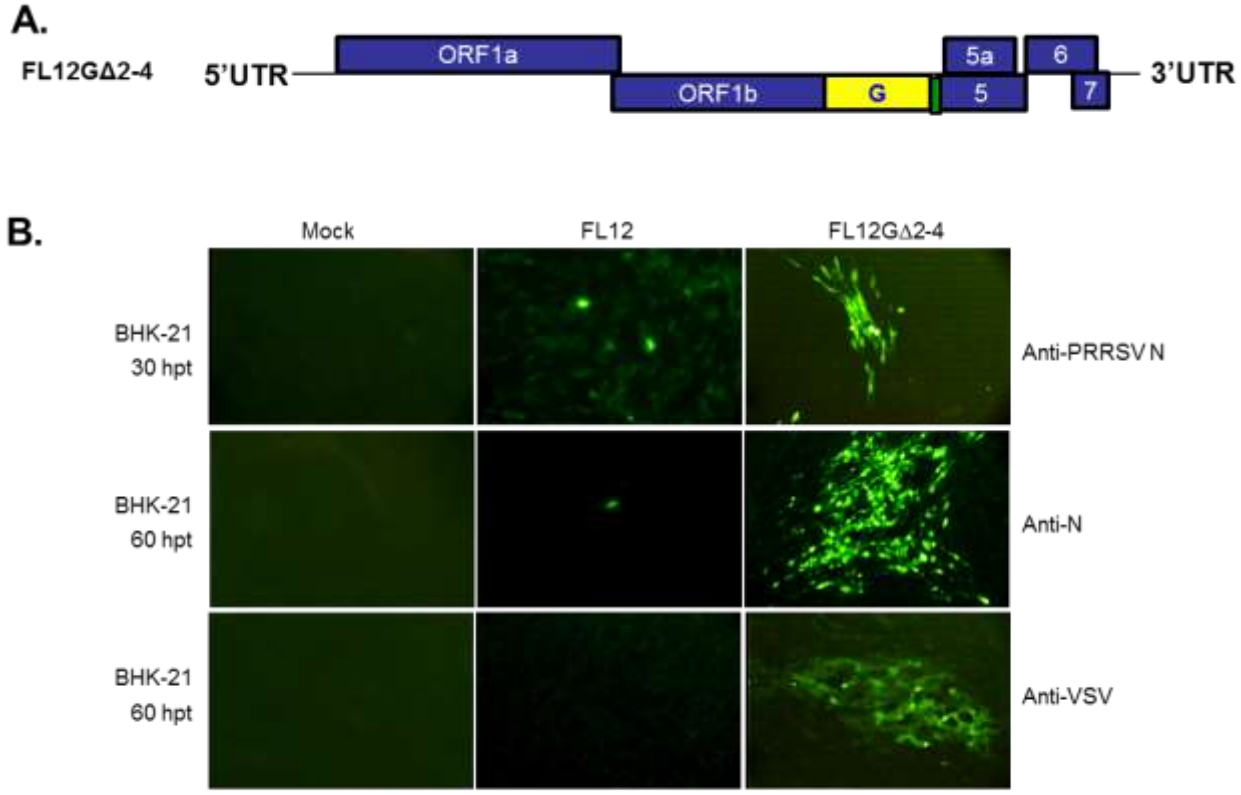

Figure 1

***Figure 1**, panel A above shows the construction of the SPR from which we were able to successfully recover the SPR, named here as FL12GΔ2-4. This is based on the genomic background of PRRSV in which the VSV G protein coding region was inserted immediately following the ORF1b and upstream of the ORF5 after deletion of PRRSV ORFS2 to 4. In BHK-21 cells transfected with the SPR, PRRSV N protein as well as VSV G protein could be readily detected even up to 60 h post-transfection as shown in panel B above. Unlike FL12GΔ2-4 replicon transfected BHK-21 cells, the PRRSV infectious clone FL12 RNA transfected cells did not express much PRRSV N protein after 60 h post-transfection and no VSV G protein was expressed in these cells (Fig. 1B).*

particle infected BHK-21 cells were also examined. As can be seen from Figure 2, the SPR particles grew to titers of about 10^5 infectious particles per ml (Fig. 2A) in BHK-21 cells. These particles also formed plaques in BHK-21 cells, whereas PRRSV FL12 virus did not (Fig. 2B). Furthermore, in BHK-21 cells infected with FL12GΔ2-4, VSV G protein as well as PRRSV nonstructural protein 1 (NSP1) and the PRRSV membrane protein (M) were readily synthesized (Fig. 2C and 2D), indicating that the replicons are capable of synthesizing all the proteins that are encoded in the replicon.

Figure 2. *The replicon containing particles were examined for their growth in BHK-21 cells and the proteins expressed in replicon particle infected BHK-21 cells were also examined. As can be seen from figure 2, the SPR particles grew to titers of at least 10^5 infectious particles per ml (Fig 2A) in BHK-21 cells. These particles also formed plaques in BHK-21 cells, whereas PRRSV FL12 virus did not (Fig. 2B). Furthermore, in BHK-21 cells infected with FL12GΔ2-4, VSV G protein as well as PRRSV nonstructural protein 1 (NSP1) and the PRRSV membrane protein (M) were readily synthesized (Fig. 2C and 2D), indicating that the replicons are capable of synthesizing all the proteins that are encoded in the replicon.*

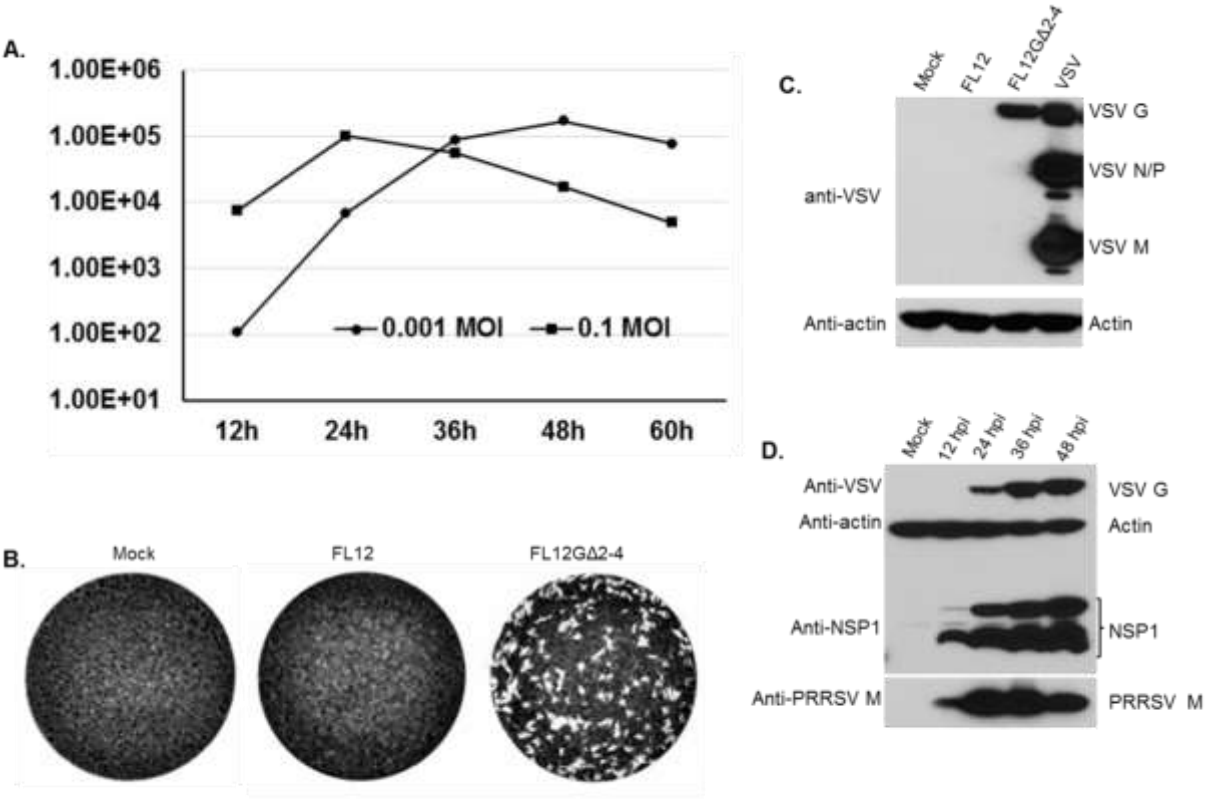

Figure 2

Thus, the result demonstrated that the SPR genome replicated in the infected cells, produced infectious particles and could be propagated in the culture resulting in infection of the vast majority of cells. In addition, a number of other PRRSV replicon constructs encoding VSV G protein were also generated and the corresponding SPRs were recovered from transfected cells. For example, we were able to recover an SPR containing ORF4-7 along with VSV G protein. All these PRRSV replicons have been grown in BHK-21 cells (non-permissive for PRRSV growth), their growth curve and overall virus titers have been determined. **Thus, the first objective was fully achieved.**

OBJECTIVE 2

In vitro growth characterization of the replicons in other mammalian cells

During the second year of the project, we further characterized various replicons produced from studies in objective 1. We conducted in-depth analysis for their ability to grow not only in BHK-21 cells but also in a variety of other mammalian cells. The results suggested that the replicons bearing VSV G protein on their envelope are capable of infecting many different cell types such as HeLa, HEK293T as well as Vero cells. Since the replicons grew to titers of about $2x10^5$ infectious units per ml of the culture supernatant in BHK-21 cells, our reason for examining the growth of the replicons in other cells lines was to determine the suitability of another mammalian cell that could support replication of the replicons to higher titers for in vivo inoculation studies in pigs. However, our results (Table 1) showed that BHK-21 cells supported highest level of replicon growth.

Table 1

Cell Line/Replicon	Infectious titers at 30 hpi	Infectious titers at 60 hpi
HeLa/FL12GΔ2-4	$7x10^4$/ml	$1.5x10^5$/ml
HeLa/FL12GΔ2-3	$6x10^4$/ml	$1x10^5$/ml
HEK293T/FL12GΔ2-4	$8x10^4$/ml	$1.2x10^5$/ml
HEK293T/FL12GΔ2-3	$8x10^4$/ml	$1x10^5$/ml
Vero/FL12GΔ2-4	$5x10^4$/ml	$1.2x10^5$/ml
Vero/FL12GΔ2-3	$7x10^4$/ml	$1.3x10^5$/ml

Animal experiments (inoculation of pigs with SPRs and examination of viremia and immune response)

Twenty-one-day old, recently weaned pigs were purchased from a specific-pathogen-free herd with a certified record of absence of PRRSV infection. Six pigs per group were infected with either FL12 wt PRRSV or with the two SPRs (FL12GΔ2-4 or FL12GΔ2-3). We used $1x10^5$ infectious units of FL12 or SPRs diluted in 2 ml. The virus or SPRs were administered intramuscularly in the neck. The rectal temperatures of the inoculated animals were monitored for 15 days post-inoculation (PI). Viremia was measured by regular isolation of FL12 virus on MARC-145 cells at days 4, 7, and 14 post-inoculation. For the SPR, we used BHK-21 cells and/or MARC-145 cells as we had found that SPRs can also be replicated in MARC-145 cells. Serum samples were drawn weekly for a total period of 45 days post-inoculation. The serum samples were used to detect homologous neutralization antibody titers (against FL12 virus) as well as against the SPRs. The titers of PRRSV-neutralizing antibodies in a serum samples were determined using the fluorescence focus neutralization assay. Serial dilutions of test sera were incubated for 60 min at 37ºC in the presence of 200 infectious units of the challenge virus, which consisted of either FL12 (wt PRRSV) or any of the SPRs in Dulbecco's modified Eagle's medium containing 5% FBS. The mixtures were added to 96-well microtitration plates containing confluent MARC-145 cells which had been seeded 48 hrs earlier. After incubation for 24 hrs at 37ºC in a humidified atmosphere containing 5% CO_2, the cells were fixed for 10 min with a solution of 50% methanol and 50% acetone. After extensive washing with PBS, the expression of N protein of PRRSV was detected with monoclonal antibody SDOW17 using a 1:500 dilution, followed by incubation with FITC-conjugated goat anti-mouse IgG (Sigma) at a 1:100 dilution. Neutralization titers were expressed as the reciprocal of the highest dilution that inhibited 90% of the foci present in the control wells. Since the SPRs contain VSV G protein on their envelope as well as in their genome, we anticipated that pigs infected with the SPRs will not only produce antibody against PRRSV but also against VSV G protein, which could potentially neutralize the SPRs.

We observed that the pigs inoculated with the SPRs did not establish viremia as judged from quantitative RT-PCR of the serum samples obtained from various times post-inoculation, although viremia could be readily detected in pigs infected with FL12 PRRSV. It is possible that the titer of the

SPRs used in these experiments were too low to allow detection of viremia in these time frames or that there are unknown or inherent difficulties in the SPRs for establishment of viremia in the infected pigs. Unsurprisingly, we could not detect any significant level of antibody titers in any of the animals infected with either of the SPRs.

To address the issue of low titers of SPRs in the inoculum, we prepared high titer stocks of FL12GΔ2-4 or FL12GΔ2-3 by initially generating the SPRs and then concentrating them by ultracentrifugation. In this manner, we were able to obtain titers of the two replicons at $2x10^6$ infectious units per ml.

Subsequently, we conducted animal inoculation experiments with the same number of animals in each group as described above and examined viremia and serum antibody titers. Again, we failed to detect viremia in the serum even after 35 days post-infection. These results suggest that even though the SPRs can be replication competent in cultured cells, it is likely that they are unable to replicate in the pigs or that the pigs mount a response against VSV G protein that helps to clear the SPRs from the infected pigs readily.

Undoubtedly, further work will be needed to provide more definitive answers for the use of SPRs as potential viral vaccines. However, it must be emphasized that unlike the wt PRRSV, which has very narrow host range in pigs or in cultured cells, the designed SPRs have significantly wider host range in cultured cells as they are capable of infecting and producing SPRs in several different mammalian host cells.