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REVIEW

Research progress in the development of porcine reproductive and respiratory syndrome virus as a viral vector for foreign gene expression and delivery

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ABSTRACT

Introduction: Porcine reproductive and respiratory syndrome (PRRS) is an infectious disease of swine characterized by respiratory disorders in growing and finishing pigs and reproductive failure in pregnant sows. PRRSV has been recognized as one of the most economically significant pathogens affecting the global pig industry.

Areas covered: Currently, commercially available vaccines, including traditional killed virus (KV) vaccines and modified live virus (MLV) vaccines, are the cardinal approaches to prevent and control porcine reproductive and respiratory syndrome virus (PRRSV) infection. However, the protective efficacy of these vaccines is not satisfactory, resulting in the continuous evolution and recurrent appearance of the virus as well as the emergence of new variants. A safe and effective vaccine against PRRSV is in dire need. Here, we review the research progress in recent years in the development and use of PRRSV as a viral vector to express foreign genes, and their potential application in gene delivery and vaccine development.

Expert opinion: The potential of using PRRSV-based vectors to express multiple antigens would be particularly instrumental for the development of a new generation of multivalent vaccines against PRRSV and other porcine viruses.

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KEYWORDS
Porcine reproductive and respiratory syndrome virus; infectious clone; foreign genes; vaccine; viral vector

1. Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV) is one of the most economically important viral pathogens worldwide. PRRSV causes persistent outbreaks and has led to tremendous economic losses to the swine industry, since it was initially identified in the United States in 1987 and Europe in 1990, respectively. Within a few years, the virus spreads extensively, becoming a pandemic in most pig-producing countries [1]. PRRSV has strict host specificity and restricted tissue tropisms. It mainly infects macrophages and dendritic cells in the lungs, causing acute or persistent infection. Porcine alveolar macrophages (PAMs) are the primary target cells for PRRSV replication during in vivo respiratory infection [2,3]. The genetic and antigenic diversity of PRRSV, along with the immune suppression and persistence of PRRSV infection, poses a great challenge to the development of effective vaccines. Nevertheless, recent progress on PRRSV reverse genetics has rapidly advanced PRRSV vaccine research, opening the potential of using PRRSV as a viral vector for expressing heterologous genes of other porcine viruses. By exploiting the unique transcription and translation of multiple sub-genomic RNAs (sgRNAs) during the replication of PRRSV, it is feasible to construct recombinant PRRSVs expressing multiple foreign genes. In addition, due to its unique cell and tissue tropisms, PRRSV-based vector vaccines would efficiently deliver foreign antigens from other swine pathogens to the immune system, thereby eliciting a strong immune response in pigs.

PRRSV belongs to the genus Porarterivirus, together with the other four genera, Equarterivirus, Simarterivirus, Diparterivirus, Nesarterivirus, placing within the family Arteriviridae of the order Nidovirales [4]. This family includes other members, such as lactate dehydrogenase-elevating virus of mice (LDV), simian hemorrhagic fever virus (SHFV), equine arterivirus (EAV) and the recently described wobbly possum disease virus (WPDV) [1,5,6]. According to the latest classification system, the traditional two genotypes of PRRSV, European (Type 1) and North American (Type 2), can be reclassified into two different species, betaarterivirus suid 1 species for PRRSV 1 and betaarterivirus suid 2 for PRRSV 2, sharing less than 70% sequence identity [7].

As illustrated in Figure 1, PRRSV is a single-stranded, positive-sense (+) enveloped RNA virus with a genome of about 15 kb. The RNA genome includes the cap structure and the untranslated region (UTR) at the 5′-end, UTR and poly (A) tail at the 3′-end. It encodes at least 10 open reading frames (ORFs) between the 5′- and the 3′-ends, designated ORF1a, ORF1b, ORF2a, ORF2b, ORF3–7 and ORF5a [8–10]. ORF1a and ORF1b, account for three-quarters of the viral genome, mainly encode large replicase polyproteins pp1a, pp1a-nsp2N, pp1a-nsp2TF, and pp1ab via ribosomal frameshift (RFS). These polyproteins are proteolytically processed into nearly 16 functional

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Article Highlights

- An overview of PRRSV biology and pathogenesis, including genome replication, transcription and translation, and tissue/cell tropisms.
- Constructing PRRSV infectious clones by transfection of in vitro transcribed full-length genomic RNA or full-length cDNA genome driven by a cellular RNA PolII promoter.
- Using chimeric arteriviruses to investigate functional complementation of viral proteins, cell tropism, virulence determinants, antigenic epitopes and cross-protection.
- Engineering PRRSV genome to express multiple foreign antigens and construction of epitope-missing marker vaccines by deleting dominant antigenic epitopes.
- Pros and cons of sites in the PRRSV genome for foreign gene insertion and the potential to develop multivalent vaccines against diverse porcine viruses.

nonstructural proteins (nsps), including nsp1α, nsp1β, nsp2TF, nsp2N, nsp2–6, nsp7α, nsp7β and nsp8–12, functionally responsible for the viral genome replication and transcription [5,11].

ORF2a, ORF2b, ORFs3–7 and the recently discovered ORF5a encode the structural proteins GP2, E, GP3, GP4, GP5, the matrix protein (M), the nucleocapsid protein (N) and GP5a, respectively, which all constitute the PRRSV virion [12]. GP2, GP3 and GP4, as minor envelope proteins, are reported to interact with each other to form a heterotrimer along with E, which is responsible for the viral infectivity and receptor binding [10,13]. The non-glycosylated E protein is required for viral uncoating, presumably due to its ion channel properties [10,13]. The non-glycosylated envelope protein GP5a may be required for virus viability, but its function needs further study [14]. GP5, as the major glycosylated and most variable envelope protein, acts as a key target for PRRSV neutralizing antibodies [15,16]. M protein, as the non-glycosylated and most highly conserved membrane protein, interacts with GP5 to form heterodimers in the virus particles, plays a significant role in virus attachment and assembly, and is indispensable for viral particle formation [14,17–19]. N protein is the most abundant protein in the PRRSV virion and interacts with the viral RNA to form the nucleocapsid [20].

The structural proteins of PRRSV are encoded by a nested series of sgRNAs. Two key elements, the leader transcription regulatory sequence (TRS) in the 5′-UTR and a downstream body TRS preceding the respective structural gene, are required for the transcription of these sgRNAs. The leader TRS, including the hexanucleotide sequence UUAACC, is highly conserved in PRRSV. In contrast, the body TRS, encompassing the conserved hexanucleotide motif similar to that of the leader TRS and highly variable flanking sequences, has been reported to be diverse in different PRRSV isolates [21]. These two TRSs interact by base-pairing between the leader TRS and the negative-sense body TRSs in the minus-strand viral RNA in a site-specific manner, playing vital roles in the discontinuous transcription and regulation of protein expression [9,21–24]. Resembling other nidoviruses, such as coronaviruses and toroviruses, PRRSV adopts a unique co-terminal

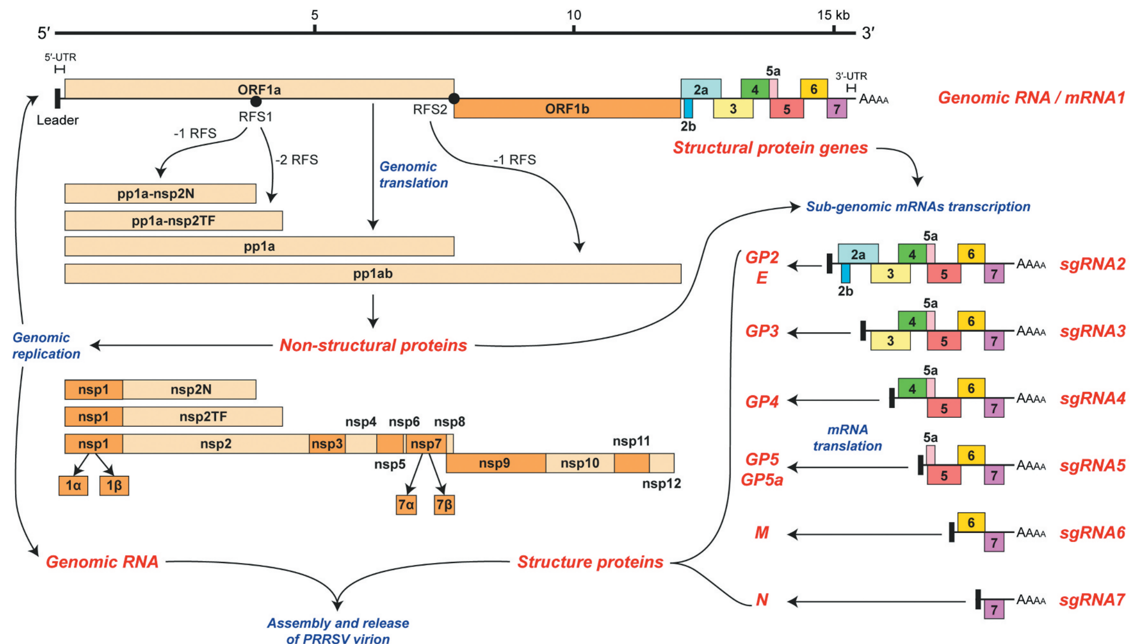

Figure 1. Schematic representation of the genome organization, replication cycle, transcription and translation strategies of PRRSV. The regions of the genome specifying the leader sequence, the two large replicase ORFs (ORFs 1a and 1b), and the structural genes are indicated. PRRSV replication progresses via a set of genetic and protein regulatory mechanisms. The nested set of seven PRRSV mRNAs (mRNA1 and sgRNAs 2–6) is depicted below. ORF1a/b codes for large polyproteins (pp1a, pp1a-nsp2N, pp1a-nsp2TF, pp1ab) through two documented programmed RFS (black round dot). Polyproteins are co- and post-translationally processed into at least 16 distinct functional nonstructural proteins (nsps), including nsp1α, nsp1β, nsp2TF, nsp2N, nsp2–6, nsp7α, nsp7β and nsp8–12. Structural proteins (GP2, E, GP3, GP4, GP5, GP5a, M and N) are expressed exclusively by a set of sub-genomic RNAs (sgRNA 2–7) via a co-terminal discontinuous transcription strategy through a negative-strand intermediate.

discontinuous transcription mechanism to synthesize a nested set of at least six sgRNAs from negative-strand intermediates. For PRRSV, sgRNA2 encodes ORF2a/2b to generate GP2 and E; sgRNA5 encodes ORF5/5a to produce GP5 and GP5a; sgRNA3/4/6/7 encode the corresponding ORF 3/4/6/7 to yield GP3, GP4, M and N, respectively. Besides sgRNA7, other sgRNAs are genetically polycistronic, presumably functioning as monocistronic or bicistronic [5]. Also, these sgRNAs share the same 3′- and 5′- UTR with the genomic RNA, but only the 5′-terminal ORF(s) can be expressed for each sgRNA [25,26]. The genomic organization and associated expression profiles of PRRSV are depicted in Figure 1.

To date, vaccine control and prevention of PRRSV have not been fully and effectively realized. Currently available commercial PRRSV vaccines include KV vaccines and MLV vaccines. Although KV vaccines are relatively safer, their protective efficacy is lower than MLV vaccines, partly because KV vaccines do not effectively stimulate PRRSV-specific antibodies [27] and cell-mediated immune (CMI) responses [28,29]. MLV vaccines offer effective protection against homologous strains, but only partial or no protection against heterologous strains [3,30,31]. Furthermore, there is a potential risk of MLV shedding, persistent infection and reversion to virulence [3,30,31]. It was also reported that recombination between wild-type PRRSV and MLV or between different MLVs may occur, resulting in the emergence of vaccine-like PRRSV strains [32]. These conventional vaccines from a single specific strain are unable to provide adequate protection against genetically diverse PRRSV strains, while multi-strain vaccines may render more severe clinical symptoms in pigs due to the heterogeneity among different strains [33,34]. Therefore, development of a safe and effective genetically engineered vaccine is the focus of current research. These studies also provide a feasible way to develop vectored vaccines based on PRRSV infectious clones, allowing for the expression of foreign genes including marker genes and other viral antigens, as well as the construction of recombinant PRRSV vaccines, gene-deleted vaccines, and so on. The feasibility of a new generation of PRRSV-vectored vaccines expressing multiple foreign genes is discussed in the following sections.

2. Current strategies used to construct infectious clone systems for PRRSV

The development of reverse genetics paves the way for the rescue of RNA viruses in vitro. A series of steps are involved in the construction of infectious clone systems, including the assembly of the genome-length cDNA, preparation of RNA transcripts and introduction of RNAs into host cells. This technology makes it possible to manipulate viral genomes at the molecular level. It provides an effective way for developing PRRSV as a viral vector for the expression of foreign genes and the construction of genetically modified vaccines [35].

In 1981, Racaniello and Baltimore successfully constructed the full-length cDNA clone for poliovirus type I, and produced the recombinant virus for the first time by transfecting a plasmid containing the infectious full-length cDNA into host cells [36]. Since then, reverse genetics systems have been extensively developed and applied for RNA viruses, especially for positive-sense RNA viruses with infectious genomes. In 1998, the first infectious cDNA clone of PRRSV was developed for the European-type representative strain Lelystad virus (LV) [35], and subsequently, an infectious cDNA clone for the North American-type isolate VR-2332 was established successfully [37].

The key to establish a PRRSV infectious cloning system is to construct the full-length cDNA, which commonly needs to be divided into several short segments, via restriction digestion and ligation of DNA fragments covering the viral genome step by step. In order to drive the expression of the viral genome, a promoter sequence, such as the bacteriophage T7 promoter or the eukaryotic cytomegalovirus (CMV) immediate-early promoter, is introduced to the immediate 5′-terminus of the genome. The subsequent steps for the full-length construct with the T7 promoter are in vitro transcription and transfection of the transcribed RNA into cells. On the other hand, plasmid DNA containing the full-length construct with the CMV promoter is transfected directly into cells and transcribed within the cells (Figure 2). In the full-length cDNA clones of LV and VR-2332, the T7 promoter was fused to the genomic cDNA, which was assembled in the plasmid vector pOK12. The viral RNA was synthesized in vitro by T7 RNA polymerase using the linearized full-length cDNA clone plasmid as the template and then transfected into BHK-21 cells to initiate the formation of viable virus particles [37]. Using the supernatants from the transfected cells to infect MARC-145 cells, infectious progeny virus was recovered. Alternatively, a full-length infectious cDNA clone plasmid containing the CMV promoter can be directly transfected into MARC-145/BHK-21 cells, which triggers the transcription of the genomic RNA in cells by the host RNA polymerase II and initiates an infection cycle for positive-strand RNA viruses [35,38].

In addition, there are also some complementary methods to promote the rescue efficiency of the recombinant virus and to avoid the tedious and sometimes inefficient in vitro transcription of the full-length transcripts. These include inoculating a recombinant vaccinia virus expressing the T7 RNA polymerase to facilitate the synthesis of viral RNA in cells for an infectious PRRSV clone driven by the T7 promoter [39] or transfecting the full-length infectious clone plasmid containing the T7 promoter into a stable cell line expressing the T7 RNA polymerase to allow the transcription of viral RNA in cells [40]. Taken together, infectious cloning systems for PRRSV make it possible to engineer the viral genome with point mutation, insertion, deletion, replacement or recombination at the DNA level, which will be conducive to the research in viral protein structure and function, pathogenesis and development of vaccine vectors.

3. Homologous substitution of genes between different members of the family Arteriviridae

It is very common to construct chimeric strains by homologous gene replacement between different arteriviruses. Chimeric viruses can be constructed to inquire into the

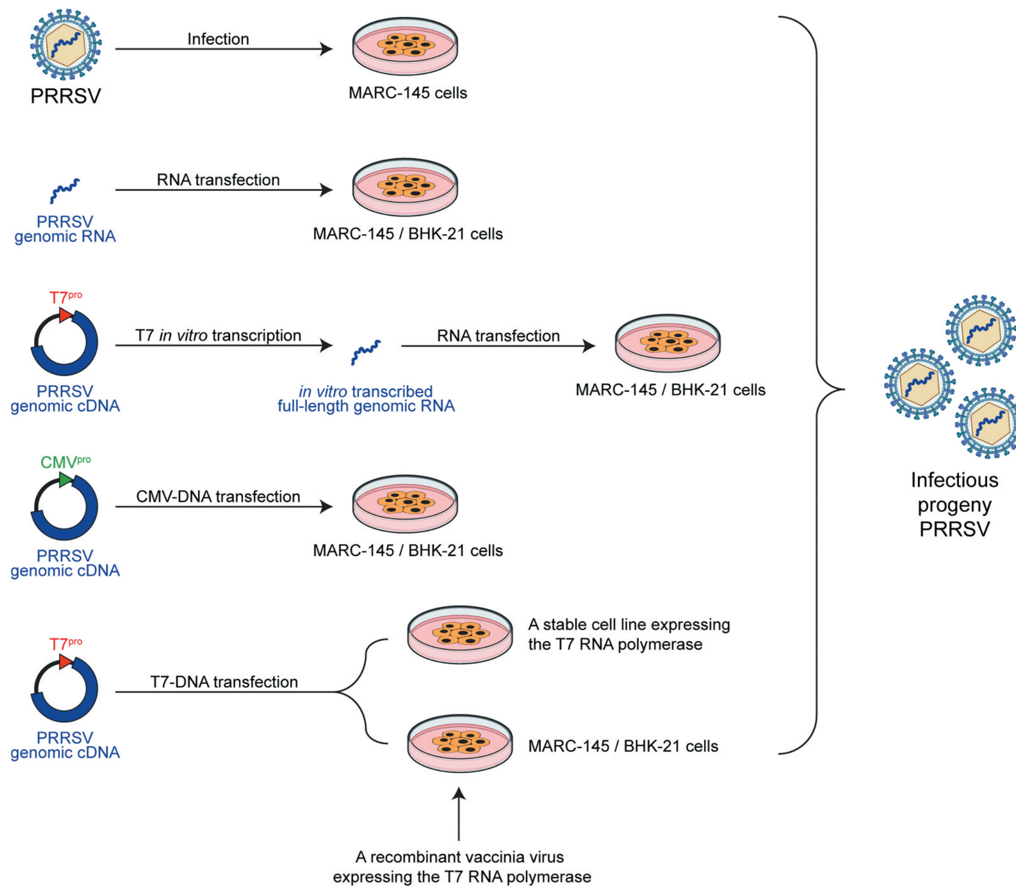

Figure 2. The infectivity of the PRRSV genomic RNA and rescue of PRRSV from full-length infectious cDNA clones. The genome-length cDNA is typically assembled in a plasmid under the control of the bacteriophage T7 promoter or the eukaryotic cytomegalovirus (CMV) intermediate-early promoter. For the full-length infectious cDNA clone plasmid driven by the T7 promoter, the full-length viral RNA is transcribed in vitro, and then transfected into MARC-145 and BHK-21 cells to initiate the formation of viable virus particles. Alternatively, the full-length infectious cDNA clone driven by the CMV promoter can directly be transfected into cells, and the PRRSV genomic RNA is synthesized in cells by cellular Pol II and initiates an infection cycle. In addition, other complementary methods to rescue the virus, such as inoculating a recombinant vaccinia virus expressing the T7 RNA polymerase to facilitate the synthesis of viral RNA in cells for the infectious clone of PRRSV driven by T7 promoter or transfecting the full-length infectious clone plasmid containing the T7 promoter into a stable cell line expressing the T7 RNA polymerase for the transcription of viral RNA in cells.

functional complementation of viral proteins, cell tropism, virulence determinants and antigenic epitopes. To investigate the targeting functions of PRRSV, some viable recombinant viruses were obtained by replacing the ectodomain of M protein (ORF6) with the corresponding sequence of EAV, LDV and North American-type isolate VR-2332 based on the infectious cDNA clone of European prototype LV (Figure 3). The rescued PRRSV chimeric viruses remained infectious to pig cells, but could not infect BHK-21 cells. These findings suggest that the extracellular domain of M protein is not involved in determining the cell tropism of artertivirus [41]. The same strategy had been applied to the LDV vector to prove that the ectodomain of GP5 is not the main determinant of cell tropism of artertivirus [42]. Based on the full-length infectious clone pAPRRS, it was reported that chimeric progeny viruses, constructed by replacing single or several envelope protein gene(s) of vAPRRS (PRRSV 1) with that of vSHE (PRRSV 2), remained stably viable and exhibited similar replication levels

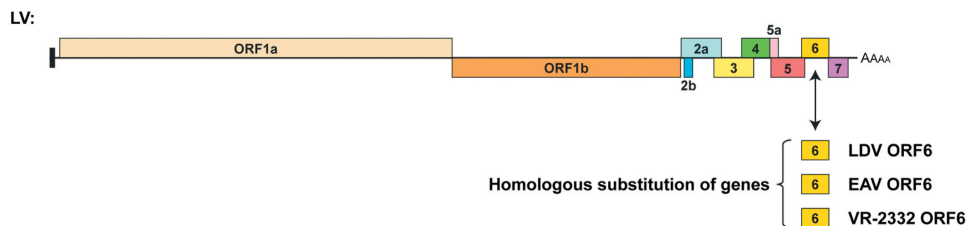

Figure 3. Homologous substitution of genes between different members of the family Arteriviridae. Using an infectious cDNA clone of LV, some viable recombinant viruses were obtained by replacing the ectodomain of M protein (ORF6) with the corresponding sequence from EAV, LDV and North American-type isolate VR-2332, respectively.

as the parental strain [10]. Furthermore, a series of chimeric clones were constructed by exchanging reciprocal ORFs or UTR between the full-length cDNA clones of a virulent isolate and a vaccine strain of PRRSV, in order to study the effect of attenuation and anti-heterologous strains, further developing potentially and broadly cross-protective vaccines [18,31,43–46].

In an attempt to make an effective and cross-protective vaccine against different PRRSV genotypes, a chimeric PRRSV was constructed by substituting ORFs 2–4 from European strain SD01–08 with the corresponding genes from the North American strain FL [14]. The chimeric virus was able to express and deliver the major envelope proteins (GP5-M) of a PRRSV-1 strain and the minor glycoproteins (GP2–4) of a PRRSV-2 strain, inducing cross-protective neutralizing antibodies and cellular immune responses against homologous and heterologous PRRSV strains [14].

4. Insertion of foreign genes into the nonessential region in nsp2

The nsp2-encoding region is highly diverse and variable in the PRRSV genome, where substitutions, deletions and insertions usually occur naturally [47]. In particular, gene deletions in the nsp2 hypervariable region often appeared in the field isolates or in vitro cultures of PRRSV, such as the representative PRRSV isolates NADC30-like, Em2007 and GDHY from China, and MN184 from the United States [4,32,48–50]. These isolates contain an over 30-amino acid (aa)-deletion in the nsp2 region relative to the North American prototype strain VR-2332 [4,32,-32,48–50]. In addition, the attenuated PRRSV strain TJM containing a unique 120 aa deletion in nsp2 was successfully obtained by serial passages of the parental, highly pathogenic (HP) strain TJ on MARC-145 cells, thus becoming a good candidate for MLV and marker vaccines against HP-PRRSV [51]. Previous studies on deletions in the nsp2 region with mutant viruses derived from the infectious clone of type 2 strain VR-2332 indicated that deletion up to 403 aa in the hypervariable region did not affect the rescue of infectious virus in vitro [52]. On the other hand, the predicted nsp2 region of strain SP contains an insertion with 36 or 155 aa, compared to the nsp2 gene of VR-2332 and LV [53], suggesting that the nsp2 region of PRRSV can tolerate the insertion of foreign genes. In addition, nsp2 is an immunodominant protein with strong immunogenicity. Pigs inoculated with PRRSV can induce strong antibody responses against nsp2 within 2 weeks [54]. Deletion of some antigenic epitopes in the non-essential region of nsp2 would be an attractive approach for the development of marker vaccines.

Based on these observations, efforts have been made to construct recombinant viruses with insertions/deletions in the nsp2-coding region. In a previous study, a recombinant green fluorescent protein (GFP)-tagged PRRSV was constructed by inserting GFP into a unique site containing a 17 aa deletion relative to LV, based on the full-length cDNA infectious clone of the type 1 PRRSV isolate SD01–08 [55]. GFP was initially expressed by the rescued recombinant virus until passage 7, but a subpopulation of the non-GFP-expressing virus began to appear due to the deletion of the GFP gene and became dominated in the subsequent cell cultures. Based on information from this spontaneous GFP-deleted mutant virus, another gene-deleted recombinant virus expressing GFP(SD01–08-GFP /ΔES4) was constructed, using the infectious clone of SD01–08-GFP [55]. In SD01–08-GFP/ΔES4, the B-cell epitope ES4, located downstream of GFP, was deleted (Figure 4). This recombinant virus with the GFP insertion and ES4 deletion retained genetically stable in vitro for at least 10 serial passages without deletion of the GFP gene [55], suggesting that deletion of the ES4 epitope region may enhance the stability of the inserted GFP gene to some extent. Yet, it has been reported that some mutations in GFP (such as the R97C mutation) probably occur during sequential passages of the recombinant virus, leading to the loss of the GFP fluorescence. In spite of this, the combination of the positive marker GFP and the negative marker ΔES4 in the recombinant virus makes it distinguishable from the field virus by ELISA, opening the feasibility for developing a potential marker vaccine [56].

In another study, a recombinant PRRSV was constructed by replacing 25 aa (aa 508–532) in the nonessential region of nsp2 with a 49aa immunodominant B-cell epitope from the Newcastle disease virus (NDV) nucleoprotein (NP) (NDV-NP49), based on an infectious cDNA clone from the attenuated vaccine strain HuN4-F112 [57]. The viable recombinant virus was continuously cultured in MARC-145 cells for up to 20 passages, stably expressing the inserted NDV-NP49, as identified by indirect immunofluorescence assay (IFA) and genomic

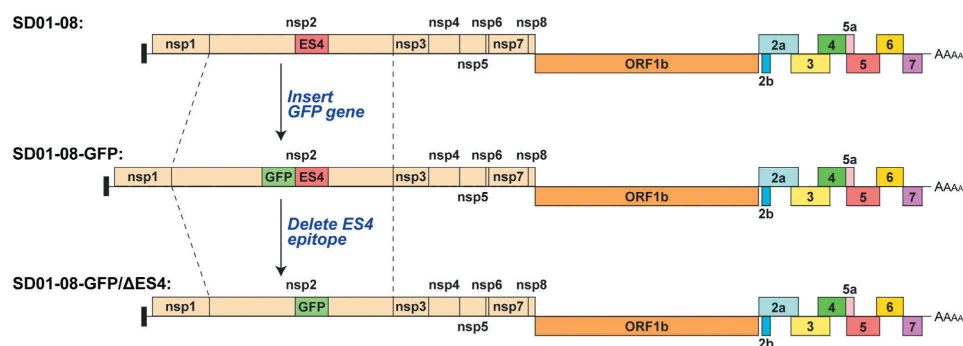

Figure 4. Schematic diagram showing the genome organization of the recombinant viruses SD01-08-GFP and SD01-08-GFP/ΔES4. The GFP gene was inserted into the Nsp2 deletion region (aa 733 and 734 of pp1a), and an immunogenic B-cell epitope, ES4, downstream of GFP (aa 736–790) was deleted from the full-length infectious cDNA clone of SD01-08 virus.

sequencing [57]. Pigs infected with the rescued recombinant virus elicited specific antibody responses against the inserted NDV-NP49 antigen, while no specific antibody response was detected against the deleted 25 aa epitope [57]. This study indicated that the recombinant vaccine strain could be used as a potential marker vaccine.

Some nonessential regions in the hypervariable region in PRRSV nsp2 appeared to be tolerable for gene deletion or insertion, and have become an ideal insertion site for foreign genes. Furthermore, this protein contains several antigenic epitopes with strong immunogenicity, and some of them could be deleted and replaced, making nsp2 an ideal target for the construction of gene-deleted vaccines. However, several studies have shown that recombinant viruses with GFP inserted into the highly variable region of nsp2 tend to be unstable and gradually losing the GFP gene during the viral replication. It appears that size, inserted position or the heterogeneity of the foreign genes may dictate the stability of the recombinant viruses [57]. This would pose a challenge to the development of these recombinant viruses as marker vaccines. More systematic studies would be required to solve this obstacle.

5. Insertion of foreign genes between ORF1b and ORF2a

The presence of a certain number of overlapping sequences between adjacent ORFs is a characteristic of the PRRSV genome. For example, ORF3 and ORF4 overlap by more than 200 nt. This increases the difficulty of genetic manipulation of PRRSV. However, there is no overlapping sequence between ORF1b and ORF2a. The two ORFs are separated by one nucleotide, making this position a suitable site for foreign gene insertion.

Based on the infectious cDNA clone of North American strain P129, two unique restriction enzyme sites and a copy of TRS6 were inserted between ORF1b and ORF2a. Subsequently, the GFP gene or porcine circovirus type 2 (PCV2) capsid gene was introduced between the restriction enzyme site and TRS6 by restriction enzyme digestion and ligation, so that GFP or PCV2 capsid gene could be transcribed and expressed from a separate sgRNA (Figure 5). The rescued recombinant PRRSV expressing GFP or PCV2 capsid was phenotypically stable for at least 37 passages in cell culture. Specific antibodies against the inserted GFP or PCV2 capsid antigen were detectable by ELISA in pigs inoculated with the rescued recombinant virus at 5 weeks post-inoculation [58]. These results demonstrated that the inserted foreign genes are efficiently expressed by the recombinant PRRSVs and have good immunogenicity to stimulate a strong immune response in the host. These recombinant viruses thus can be potentially developed as multivalent vaccines against swine diseases.

Using the same infectious clone of strain P129, a series of 'indicator' genes, including Renilla luciferase (Rluc), firefly luciferase (FLuc), green and red fluorescent proteins (GFP and DsRed) and type I interferons (IFNs), were inserted between ORF1b and ORF2a [59]. The PRRSV clone vector carrying the FLuc gene (pCMV-P129-FLuc) failed to rescue a viable virus expressing FLuc, while the others were efficiently expressed and produced recombinant progeny viruses. These results suggested that the PRRSV clone vector may have a limited capacity for the large size or heterogeneity of exogenous genes. Also, recombinant PRRSVs expressing type I IFNs produced active IFNs and induced anti-PRRSV activity, thus becoming promising candidates for MLV vaccines [59].

In addition, PRRSV could be attenuated by introducing an additional transcription unit between ORF1b and ORF2a. After inserting a GFP gene and an extra TRS element between ORF1b and ORF2a based on the infectious cDNA clone of a HP-PRRSV strain, the rescued recombinant virus replicated more slowly in cell culture, compared with the parental virus, but pigs infected with the recombinant virus showed mild or even no clinical symptoms [60]. In another study, the granulocyte-macrophage colony stimulating factor (GM-CSF) gene and the synthesized TRS6 sequence were inserted between ORF1b and ORF2 of the HuN4-F112 vaccine strain by overlapping PCR. The recombinant virus displayed a similar level of replication as the parental

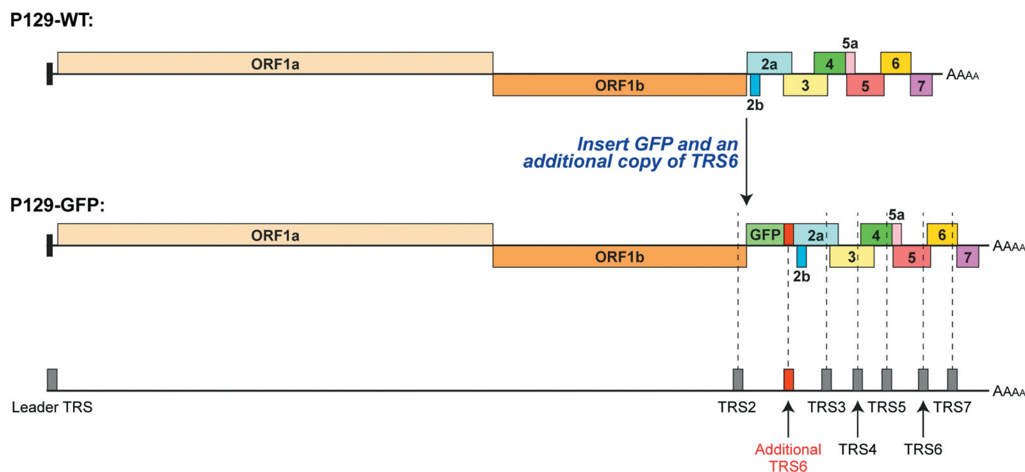

Figure 5. Schematic diagram showing the genome organization of the recombinant virus P129-GFP and the positions of the leader and body TRSs. A copy of TRS6 and the GFP gene were introduced between ORF1b and ORF2a, based on the infectious cDNA clone of North American PRRSV strain P129. The black and gray boxes in the genomic RNA indicate the positions of leader and body TRSs (TRS2, additional TRS6, TRS3, TRS4, TRS5, TRS6, TRS7), respectively. The red box indicates the position of an additional TRS6.

strain in vitro and stably expressed GM-CSF for 20 passages in MARC-145 cells. GM-CSF expressed by the recombinant virus could enhance the activation of bone marrow-derived dendritic cells (BMDCs) and promote the secretion of several cytokines, leading to stimulation of stronger immune responses and enhanced vaccine efficacy against PRRSV infection [61]. These results indicated that rHuN4-GM-CSF has the potential to be developed as a more effective vaccine candidate.

To sum up, recombinant PRRSV vectors carrying a foreign gene driven by the viral body TRS2 and an additional synthetic TRS controlling the transcription of ORFs 2a and 2b can be rescued, and the foreign genes can be stably expressed. As a result, the location between ORF1b and ORF2a is suitable for the insertion of a foreign gene, due to the genetic stability of the recombinant virus and the high expression level of the inserted foreign gene with well-maintained biological activity and antigenicity. However, it was also reported that when the same method was used to insert the same exogenous gene into this site of a different PRRSV strain, the rescued recombinant viruses were unstable and lost the inserted gene during serial passages. It is uncertain if this is caused by the heterogeneity of different PRRSV strains.

6. Insertion of foreign genes into ORF7 and between ORF7 and the 3′-UTR

ORF7, next to the 3′-UTR, encodes the N protein of PRRSV. It has been reported that 40 nucleotides in the 3′-UTR immediately downstream of the stop codon of ORF7 are not essential for the virus viability [62]. In addition, deletion of the N-terminal residues 5–13, the internal residues (39–42 or 48–52), or the C-terminal last four residues of the N protein did not affect the rescue of type 2 PRRSV in vitro [63]. These results indicate that ORF7 and the 3′-UTR can tolerate the deletion of a certain number of nucleotides, and some dispensable regions can be used as insertion sites for foreign tag expression.

Recombinant PRRSVs with a 9-aa hemagglutinin (HA) tag from human influenza A virus directly fused to the N-terminus or the C-terminus of the N protein were constructed based on the infectious cDNA clone of LV. However, those recombinant viruses expressing the HA epitope tended to be unstable and lose the inserted epitope simultaneously after subsequent cell passages. To solve the problem, additional sequences encoding the auto-protease 2A from the foot-and-mouth disease virus (FMDV) were inserted in-frame with the HA and ORF7 sequences. HA epitope could be expressed from a cleavable N protein precursor by the self-cleaving 2A protease [64]. This strategy not only successfully rescued the virus, but also controlled the stable expression of the HA tag [64]. Hence, ORF7 can be selected for the insertion of a foreign gene, but the size limit for foreign genes remains to be further studied.

In previous studies, a recombinant HP-PRRSV/SD16 expressing enhanced GFP (EGFP) with an additional TRS-ORF cassette as a separate transcription unit inserted between ORF7 and the 3′-UTR was stable for at least 10 passages [21,65] (Figure 6). Based on the infectious clone of PRRSV vaccine strain CH-1 R, inserting an extra TRS6 and GM-CSF or interleukin 4 (IL-4) gene between ORF7 and the 3′-UTR, the rescued recombinant virus showed similar growth properties as the parental virus and the foreign gene was stably expressed for at least 10 passages in cell culture [66,67]. Pigs immunized with the recombinant virus induced higher levels of CD4+ CD8+ double-positive T cells and IFN-γ in peripheral blood than those infected with the parental virus, although the protective efficacy was not significantly improved [66,67]. From these studies, the site between ORF7 and the 3′-UTR is ideal for the insertion of foreign genes, but further studies are required to determine the maximal size of a foreign fragment this site can accommodate and the biological activity of the expressed foreign proteins.

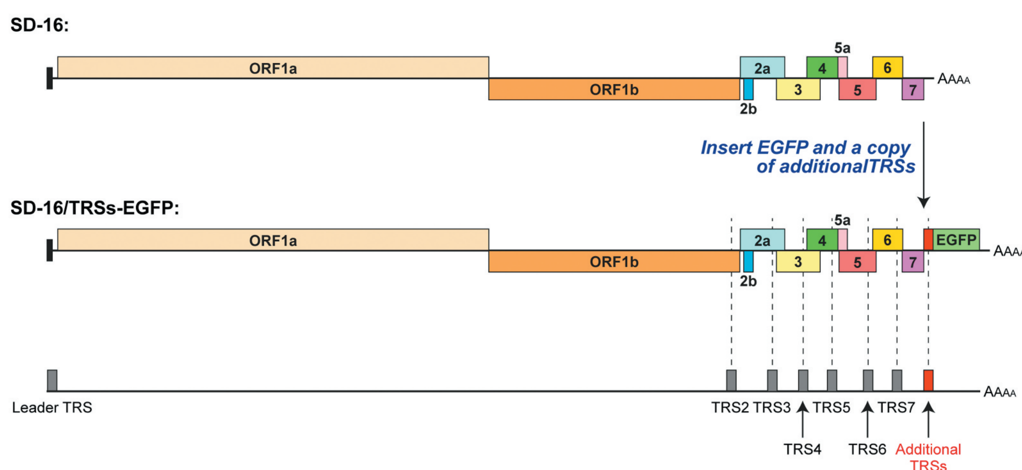

Figure 6. Schematic diagram showing the genome organization of recombinant virus SD-16/TRSs-EGFP and the positions of the leader and body TRSs. A copy of TRS and the EGFP gene were introduced between ORF7 and the 3 ‘UTR, based on the infectious cDNA clone of the HP-PRRSV strain SD16. The black and gray boxes in the genomic RNA indicate the positions of the leader TRS and body TRSs (TRS2, TRS3, TRS4, TRS5, TRS6, TRS7, a copy of additional TRSs), respectively. The red box indicates the position of a copy of additional TRSs (TRS2-7).

7. Other insertion sites to be studied

PRRSV genome contains some overlapping regions, especially the adjacent ORFs that encode the structural proteins. This property poses problems for the insertion of foreign genes into the overlapping sequences. Direct insertions in the overlapping regions cause mutations in the two adjacent structural proteins, thereby potentially affecting the assembly and rescue of viable viral particles. Fortunately, it is possible to separate some of these overlapping ORFs by inserting additional nucleotides between the two adjacent ORFs to maintain the original amino acid sequences of the two encoded proteins, without affecting the viral viability [9]. Therefore, recombinant viruses can be produced by first separating the two overlapping genes between the adjacent ORFs, so that there is no overlap between the two ORFs. Foreign genes as an additional TRS-ORF cassette can then be inserted between the two ORFs, such as the region between ORF 5 and 6, or the region between ORF 6 and 7 [64]. Further studies would be required to determine if other sites in the nonessential regions of nsps or structural proteins in the PRRSV genome can tolerate the insertion of foreign genes. A summary of the common sites for insertion of foreign genes based on PRRSV infectious clones and their benefits or disadvantages was listed in Table 1.

Table 1. Summary of common sites for insertion of foreign genes based on PRRSV infectious clones.

Insertion Site	Foreign Gene	Benefit/Disadvantage
Replacing the corresponding ORF or gene region	*Homologous genes from different members of the family Arteriviridae*	(1) *Sites are extensive* (2) *Very common to construct chimeric strains for further developing potentially and broadly cross-protective vaccines* (3) *Restricted to genes from some members of the family Arteriviridae*
Nsp2	*GFP, NDV nucleoprotein (NP)*	*1. Suitable for the construction of gene-deleted vaccines or marker vaccines, 2. Recombinant virus may be unstable and tend to delete the inserted foreign gene(s)*
Between ORF1b and ORF2a	*GFP and a series of 'indicator' genes, PCV2 capsid, type I interferons and GM-CSF*	*1. Suitable for the insertion of foreign genes 2. Retaining genetic stability of the recombinant virus for a high number of passages, and good biological activity of the expressed foreign proteins 3. Recombinant viruses with High potential to be developed as multivalent vaccines 4. Some recombinant viruses are prone to become unstable and appear some mutations in the target gene in the process of serial passages*
ORF7-coding region and between ORF7 and the 3′-UTR	*HA epitope, EGFP, GM-CSF and IL-4*	*1. Ideal for foreign genes insertion 2. Retaining genetic stability of the recombinant virus in a certain number of passages 3. The toleration capacity and the size of the foreign genes is limited 4. Some recombinant viruses tend to be instable and lose the inserted foreign gene(s) in the process of serial passages*
Other adjacent ORFs or in the nonessential regions of viral proteins	*To be studied*	*To be studied*

8. Conclusion and future prospective

Vaccines for PRRSV, including KV vaccines and MLV vaccines, are commercially available. These vaccines are usually generated based on a single virus strain. KV vaccines failed to induce detectable neutralizing antibodies and cell-mediated immune responses against PRRSV, while MLV vaccines are unable to offer adequate protection against heterologous strains. The problems of reversion to virulence and recombination between MLV vaccines and field strains have also been reported frequently. Furthermore, current MLV vaccines could not be distinguished from wild strains, making it difficult to establish a PRRS-free status regionally or in a country. The constant evolution of PRRSV not only leads to the emergence of new genetic and antigenic variants, but also results in numerous outbreaks of PRRS, posing tremendous challenges to the development of a new generation of safe and effective vaccines.

As summarized in this review article, the current efforts in the development of PRRSV as viral vectors expressing foreign genes by reverse genetic manipulation would have significant implications in developing a new generation of safe and effective PRRSV vaccines. Firstly, a number of chimeric viruses have been constructed by homologous substitution of genes between different members of the family Arteriviridae (LDV, EAV and most divergent PRRSV isolates), based on the genetic backbone of a PRRSV infectious clone. By identifying and characterizing these chimeras with improved protective efficacies against homologous and heterologous challenges, it is potentially feasible to develop a potent and universal vaccine against diverse PRRSV strains. Secondly, regions such as nsp2, between ORF1b and ORF2a, ORF7 and a site between ORF7 and the 3′-UTR of the PRRSV genome are commonly used as the insertion sites for foreign genes. For example, some recombinant viruses with the foreign gene inserted into the nonessential region of nsp2 are appropriate for the construction of a new generation of gene-deleted vaccines or marker vaccines. Sites between ORF1b and ORF2a and between ORF7 and the 3′-UTR are ideal and convenient for the insertion of various foreign genes, including a series of 'indicators' and

antigenic epitopes. Furthermore, recombinant PRRSVs expressing foreign genes with an additional TRS-ORF cassette as a separate transcription unit inserted between ORF1b and ORF2a and between ORF7 and the 3′-UTR can retain genetic stability for a certain number of cell culture passages, stably expressing the foreign gene(s). This would make it possible to develop multivalent vaccines against PRRS and other significant swine diseases. Finally, replacement of the virulence determinants of PRRSV with foreign genes and/or the introduction of an additional transcription unit into the genome of a HP-PRRSV would attenuate the virus and contribute to the rapid development of a genetically stable vaccine candidate.

In brief, by studying a series of sites in the PRRSV genome for insertion of foreign genes without disrupting viral gene expression and RNA replication, PRRSV can be developed as a stable and efficient viral vector expressing multiple foreign genes. This would open a new avenue for developing PRRSV-based multivalent vaccines against PRRSV and other swine diseases.

9. Expert opinion

Current vaccination of pigs with both KV and MLV PRRSV vaccines faces a number of challenges, including the failure to induce detectable neutralizing antibodies and cell-mediated immune responses against PRRSV infection by KV vaccines, inadequate protection against heterologous strains by MLV vaccines, potential reversion of MLV to virulence, recombination between MLV vaccines and field strains, and the absence of a marker MLV vaccine that is distinguishable from wild strains. The constant evolution of PRRSV leading to the emergence of new genetic and antigenic variants and numerous outbreaks of PRRS calls for urgent development of a new generation of safe and more effective vaccines. Current efforts in the construction of chimeric PRRSV to improve protective efficacies against homologous and heterologous PRRSV strains and in the development of PRRSV as viral vectors expressing foreign genes by the reverse genetic manipulation would have significant implications in the development of such new PRRSV vaccines.

Multiple sgRNAs are produced during the replication of PRRSV. Together with the presence of multiple potential insertion sites in the genome, this unique replication mechanism would be exploited to construct recombinant PRRSV expressing more than one heterologous antigen, based on infectious clones from genetically stable vaccine strains. As the production of a specific sgRNA can be regulated by the complementarity of the leader and body TRS sequences, it would be feasible to fine-tune the expression efficiency of an inserted foreign antigen. This would be potentially useful if adjusting the ratio of individual antigen components in a multivalent vaccine preparation is required. Generation of such stable recombinant PRRSV-based multivalent vaccines would reduce the number of vaccinations and render simultaneous protection against various diseases. Further studies by constructing stable recombinant PRRSV with the insertion of foreign antigens at different positions of the genome under the control of various TRS sequences would be required to address this possibility.

Another useful trait of a PRRSV-based recombinant vaccine would be the host cell-specificity of PRRSV. Similar to other known porcine viruses, such as porcine circovirus (PCV) and African swine fever virus (ASFV), PRRSV primarily infects immune cells of swine, particularly those involved in antigen presentation. PRRSV-based vector would deliver antigens from these pathogens to the similar tissues/cells as in the infected host, eliciting similar local and systematic immune responses and rendering optimal protection against diseases caused by these pathogens. As no effective vaccine currently available against ASFV infection, it would be particularly interesting to test if a PRRSV vectored vaccine expressing multiple protective antigens from ASFV would render protection against this devastating porcine disease.

Despite their application potential, current PRRSV-based vectors suffer from two main drawbacks. First, some recombinant PRRSVs are not genetically stable, and the inserted genes(s) may be partially or completely lost during viral propagation in cultured cells. The genetic stability of a recombinant virus would be determined by multiple factors, including the size of the inserted fragment, the genomic position of the fragment inserted, and the biochemical and biophysical features of the products encoded by the inserted genes. Alternatively, it may reflect an intrinsic nature of the viral genome: get rid of any additional sequence in order to safeguard the integrity of their original compact genome. Second, the size limit for a heterologous sequence has not been systemically determined. Previous studies have shown that recombinant PRRSVs with large insertions are either non-viable or unstable, but size limits may also vary for different insertion sites and different PRRSV strains under consideration.

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Author contributions

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