ABSTRACT

Title of Dissertation:	A NOVEL INTERFERON-INDUCING PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS STRAIN: CHARACTERIZATION AND VACCINE DEVELOPMENT
	Zexu Ma, Doctor of Philosophy, 2018
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Porcine reproductive and respiratory syndrome virus (PRRSV) causes a swine infectious disease characterized by severe reproductive failure in sows and respiratory disease in pigs of all ages. Despite substantial efforts to control PRRS, no production or vaccination regimen has demonstrated sustaining success. Type I interferons (IFNs) are critical to the innate immunity against viral infections and play an important role in activation of the adaptive immune response. PRRSV appears to antagonize induction of type I IFNs. Fortunately, we discovered an atypical PRRSV strain, A2MC2, which induces type I IFNs in cultured cells. A2MC2 elicits earlier onset and higher levels of virus-neutralizing antibodies than the Ingelvac PRRS® MLV in pigs. However, moderate virulence of A2MC2 was observed in infected piglets. The objective of this project was to characterize A2MC2 and explore this unique strain for the development of an improved vaccine against PRRS. First, I attenuated this strain by serial passaging in MARC-145 cells for 90 consecutive

passages. The passage 90 virus (A2MC2-P90) was avirulent and retained the capability of IFN induction. The A2MC2-P90 virus induced higher level virus-neutralizing antibodies in pigs. Secondly, I constructed an infectious cDNA clone of A2MC2. The recovered virus from the infectious clone was similar to the parental strain in growth properties and IFN induction. Gene fragment swapping demonstrated that the middle half genome of A2MC2 was essential for its IFN induction. Thirdly, I conducted studies to exam the genetic source of A2MC2 in IFN induction. Comparison of A2MC2 and other closely relevant PRRSV strain identifies five unique non-synonymous nucleotides. These five nucleotides remained unchanged in the A2MC2-P90 virus. Site-directed mutagenesis indicated that one unique nucleotide in A2MC2 genome was critical in the IFN induction as mutation of this nucleotide led to the loss of IFN induction. Together, our data demonstrate that A2MC2 is a novel strain that is worth further exploration for an improved vaccine against PRRS. The infectious clone of A2MC2 will be useful for the development of a marker vaccine by insertion of a marker sequence into the A2MC2 genome.

A NOVEL INTERFERON-INDUCING PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS STRAIN: CHARACTERIZATION AND VACCINE DEVELOPMENT

by

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Dissertation submitted to the Faculty of the Graduate School of the
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Preface

I worked in UK as a project coordinator for a biotech company before I applied for admission to the University of Maryland to pursue graduate studies. I liked the job to interact with scientists in research laboratories. My girlfriend (now my wife) studied in the University of California, Davis as a Ph.D. student. I visited her occasionally. I noted the difference of lifestyle in Europe and the U.S. though I heard of it before. I had a dilemma in mind with the slower lifestyle in UK as on one way it is good for life with more leisure and on the other it would minimize my chance for further studies like my wife. It was not an easy decision to give up the good job and easy life in UK and started applying for graduate studies in the United States. Under the encouragement of my then girlfriend, I resigned from the company and came to the U.S. to study for a Ph.D. degree. Interestingly, time spent in the Department of Veterinary Medicine at the University of Maryland, College Park has become the most momentous chapter in my life. I am so proud for the decision I made six years ago. Doing Ph.D. research is a challenge, but it still deserves my efforts and endeavor because I learned to be independent and have critical thinking when facing different opinions. It also provides me with the opportunity to work with diverse people and learn from multidisciplinary projects. The knowledge, techniques, experience, and courage I have gained from my Ph.D. study and life here in Maryland are precious and will help me move forward on a fruitful career.

Dedication

This dissertation is dedicated to my family. Thanks to my father Shihong Ma and my mother Xuehong Hu for raising me up and always supporting me during my hard times. They supported my decision to pursue a Ph.D. degree in the U.S. and encouraged me at hard times during this journey. I would also like to dedicate this achievement to my wife, Man Zhang for her love, inspiration and sacrifice. Her encouragement, endless support and love brought me here and keep me move forward. She is my best friend, life instructor and the most important one in my life.

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List of Abbreviations

ANXA2	Annexin A2
APCs	Antigen presenting cells
ATCC	American Type Culture Collection
BEI	Binary ethylenimine
BPL	β-propiolactone
CPE	Cytopathic effect
DMEM	Dulbecco's Modified Eagle Medium
DMV	Double membrane vesicles
DMSO	Dimethyl sulfoxide
dsDNA	Double-stranded DNA
dsRNA	Double-stranded RNA
EAV	Equine Arteritis Virus
ELISA	Enzyme-linked immunosorbent assay
EMCV	Encephalomyocarditis virus
GAGs	Glycosaminoglycans
GASs	Gamma-IFN-activated sequences
GFP	Green fluorescent protein
GP	Glycoproteins
HP-PRRSV	Highly pathogenic PRRSV
IFA	Immunofluorescence assay
IFN	Interferon
IFNR	Interferon receptor
ISG	Interferon stimulated gene

ISGF3	IFN-stimulated gene factor 3
ISRE	Interferon-stimulated response element
JAK/STAT	Janus kinase/signal transducer and activator of transcription
KPNA1	Karyopherin-α1
KV	Killed virus
LDV	Lactate dehydrogenase-elevating virus
LGP2	Laboratory of Genetics and Physiology 2
LV	Lelystad Virus
LUBAC	Linear ubiquitin chain assembly complex
MAbs	Monoclonal antibodies
MAVS	Mitochondrial antiviral signaling protein
MDA5	Melanoma differentiation-associated gene 5
MLV	Modified live virus
MyD88	Myeloid differentiation primary response gene 88
N	Nucleocapsid
NDV	Newcastle disease virus
NendoU	Nidovirus-specific uridylate-specific endonuclease
NLS	Nuclear localization signal
NSP	Non-structural protein
ORF	Open reading frame
OTU	Ovarian tumor domain
PAM	Pulmonary alveolar macrophages
PAMP	Pathogen-associated molecular patterns

PCR	Polymerase chain reaction
pDC	Plasmacytoid dendritic cells
PEV	Porcine enterovirus
PKR	Protein kinase K
PLP1α	Papain-like cysteine proteinases 1α
PLP1β	Papain-like cysteine proteinases 1 β
PLP2	Papain-like cysteine proteinases 2
polyI:C	Polyinosinic:polycytidylic acid
PPV	Porcine parvovirus
pRb	Retinoblastoma protein
PRCV	Porcine respiratory coronavirus
PRR	Pattern recognition receptors
PRRS	Porcine reproductive and respiratory syndrome
PRRSV	Porcine reproductive and respiratory syndrome virus
PRV	Pseudorabies virus
RFS	Ribosomal frame shift
RdRp	RNA dependent RNA polymerase
RLRs	Retinoic acid-inducible gene-I-like receptors
RNaseL	Ribonuclease L
RTC	Replication and transcription complex
RT-PCR	Reverse transcription polymerase chain reaction
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SIRS	Swine infertility and respiratory syndrome

SIV	Swine influenza virus
sgRNA	Subgenomic RNA
SP	Serine proteinase
Sn	Sialoadhesin
SRCR	Scavenger receptor cysteine-rich
SRRS	Swine reproductive and respiratory syndrome
TGEV	Swine transmissible gastroenteritis virus
TIR	Toll-IL-1R
TIRAP	TIR-containing adaptor protein
TLRs	Toll-like receptors
TRAM	TRIF-related adaptor molecule 2
TRIF	TIR containing adaptor inducing interferon-β
UTR	Untranslated regions
ZF	Zinc-finger

Chapter 1: Porcine Reproductive and Respiratory Syndrome Virus – Literature Review

1.1 Porcine Reproductive and Respiratory Syndrome

1.1.1 Discovery and negative economic impact

Porcine reproductive and respiratory syndrome (PRRS) outbreak was first reported in 1987 in the U.S.A. By 1990, the disease spread in North America and in Europe (Goyal, 1993). Initially, PRRS was termed as "mystery reproductive syndrome", "mystery pig disease" and "Blue ear disease" (Paton, Brown, Edwards, & Wensvoort, 1991; Wensvoort, Terpstra, Pol, ter Laak, Bloemraad, de Kluyver, Kragten, van Buiten, den Besten, Wagenaar, et al., 1991). Since there was no confirmed cause for this syndrome, various names were given to describe the disease. In the U.S.A., it was called pig plague 89, disease 89, swine reproductive failure syndrome, swine infertility and respiratory syndrome (SIRS), swine reproductive and respiratory syndrome (SRRS), and SMEDI-like syndrome (Keffaber, 1989). In Germany, this disease was called Epidemisch Spatabort der Schweine (ESS; enzootic late abortion of pigs) and Seuchenhafter Spatabort der Schweine (SSS; infectious late abortion of pigs) (Goyal, 1993). In 1992, at the First International Symposium on SIRS/PRRS held at St. Paul, Minnesota, U.S.A., an official name, PRRS, was given to this "mystery pig disease" (Meredith, 1992; Morrison, Bautista, Goyal, Collins, & Annelli, 1992).

Despite a global PRRS-related research for almost 30 years, PRRS remains an economically significant viral disease to swine industry worldwide as assessed by its adverse effect on productivity and substantial financial cost to producers. It causes a loss of estimated

$664 million annually in the US swine industry alone found in a study in 2011 (Holtkamp et al., 2013).

1.1.2 PRRS clinical signs

Clinical signs are variable and are influenced by several factors including age of the pigs infected, the strain of the virus, the concurrent infections and the immune status of the herd and management (S. Dee, Joo, & Pijoan, 1995; S. A. Dee & Joo, 1994; Halbur et al., 1995; McCaw; Park, Yoon, & Joo, 1996).

Clinical signs in sows: late term abortions are often the first signs to be noted. An increase in stillborn or weak piglets and mummified fetuses often observed. In addition to reproductive failure, other clinical signs may show on sows and gilts: anorexia, fever, lethargy, pneumonia, agalactica, skin lesions such as blue discoloration of the ears, subcutaneous and hind limb edema, nervous sign and delayed return to oestrus after weaning (Hopper, White, & Twiddy, 1992; Keffaber, 1989; Loula, 1991; Rossow, 1998).

Clinical signs in neonates: severe respiratory disease in pigs less than two weeks of age is often the first sign. Severely affected pigs will exhibit dyspnea and tachypnea (Rossow, 1998). Other clinical signs reported in neonates include loss of appetite and lethargy, diarrhea, edema around the eyes, obvious failure to thrive, conjunctivitis, blue discoloration of ears, blotchy reddening of the skin, rough hair coats and profuse bleeding post-injection (Rossow, 1998). Preweaning mortality is high, which can reach 75%.

Clinical signs in young, growing and finishing pigs: primary clinical signs among young pigs are fever, depression, lethargy, and pneumonia (Keffaber, 1989). Reduced growth rates are frequently observed, and increased number of smaller sized pigs occur (Keffaber, 1989). The peak age for the respiratory disease is four to ten weeks of old (Zimmerman et al., 2006).

Clinical signs in boars: boars shows clinical disease similar to sows other than abortion, although the severity and the percentage of boars exhibiting clinical signs may be lower than sows (Goyal, 1993). In addition to clinical signs, boars show loss of libido, lowered fertility, poor litter sizes and lowered sperm output (Feitsma, Grooten, Schie, & Colenbrander, 1992).

1.2 PRRSV Virology

1.2.1 Etiology of PRRS

Initially, a variety of causative agents for PRRS were suspected, including encephalomyocarditis virus (EMCV), swine influenza virus (SIV), porcine parvovirus (PPV), classical swine fever (hog cholera) virus, porcine enterovirus (PEV), porcine enterovirus (PEV), pseudorabies virus (PRV, Aujeszky's disease), hemagglutinating encephalomyelitis virus (HEV), an antigenic variant of type A influenza virus, *L. Pomona*, *Leptospira interrogans serovar Bratislava*, *Chlamydia psittaci*, and mycotoxins (Bane & Hall; Daniels, 1990; Elazhary, Weber, Bikour, Morin, & Girard, 1991; Joo, 1988; Joo, Christianson, & Kim; Quaife, 1989; Reotutar, 1989). However, none of these agents was proven to be the cause of PRRS because none of them was able to fulfill the Koch's postulates (Goyal, 1993; Walker, LeVine, & Jucker, 2006). The causative agent was first identified in 1991 when Netherlands scientists isolated a virus from piglets named Lelystad virus (LV) and fulfilled Koch's postulates with it (Terpstra, Wensvoort, & Pol, 1991; Wensvoort, Terpstra, Pol, Ter Laak, Bloemraad, De Kluyver, Kragten, Van Buiten, Den Besten, & Wagenaar, 1991). In the same year, the virus was also isolated in the U.S., which was designated as the American Type Culture Collection VR-2332 (ATCC VR-2332) (J. E. Collins, 1991; James E. Collins et al., 1992). The PRRS were successfully reproduced with both virus isolates in Europe and in the

U.S. under experimental conditions (James E. Collins et al., 1992; Terpstra et al., 1991). The PRRS virus (PRRSV) is now known as the etiological agent of PRRS worldwide.

1.2.2 PRRSV genotypes and virion structure

PRRSV identified in Europe (Type 1) and North America (Type 2) have significant sequence differences (James E. Collins et al., 1992; Wensvoort, Terpstra, Pol, Ter Laak, Bloemraad, De Kluyver, Kragten, Van Buiten, Den Besten, & Wagenaar, 1991). They are two related but antigenically and genetically distinguishable genotypes: Type 1 (prototype Lelystad virus, predominating in Europe) and Type 2 (prototype VR-2332, predominating in North America), which share only about 55% nucleotide identity (Lunney et al., 2016). Based on 2016 ICTV Virus Taxonomy, the two conventional genotypes of PRRSV: Type 1 and Type 2 are reassigned to the two new species *PRRSV-1* and *PRRSV-2*, respectively, in the genus *Porartevirus*, the family *Arteriviridae* (Adams et al., 2016; Kuhn et al., 2016). The family *Arteriviridae* also expands from one genus to five genera, including *Dipartevirus*, *Equartevirus*, *Nesartevirus*, *Porartevirus,* and *Simartevirus*. In addition to PRRSV, the *Porartevirus* genus also includes lactate dehydrogenase-elevating virus (LDV) of mice and Rat arterivirus 1.

In the 1990s, based on the techniques of negative stain and thin section electron microscopy, PRRSV virion structure was described roughly as spherical or oval particles of 60 nm in diameter with a smooth outer appearance (Dea, Sawyer, Alain, & Athanassious, 1995; Mardassi, Athanassious, Mounir, & Dea, 1994). Since then, with the development of cryo-EM, Cryo-electron tomography and X-ray crystallography, more detailed structural information of PRRSV virions has been revealed (Fig.1.1A) (Spilman, Welbon, Nelson, & Dokland, 2009). The virion is round to oval in shape with a diameter of 54 nm in average. Few PRRSV particles are larger than 60 nm. The smooth outer lipid bilayer is around 4.5 nm of thickness and is embedded with envelope proteins. Between internal core and envelope

outer layer, a 2-3 nm gap can be observed. The diameter of virion double-layered internal core is around 39 nm. Previous studies show PRRSV virion has an isometric core (Spilman et al., 2009). However, with the three-dimensional information obtained from Cryo-electron tomographic studies, the PRRSV viral nucleocapsid has an asymmetric, linear arrangement, rather than isometric. A schematic representation of the PRRS virion is depicted in Fig.1.1B. The viral genomic RNA is enclosed by nucleocapsid protein homodimers. Surrounding the nucleocapsid, structural proteins are embedded into the smooth lipid-bilayered envelope to form the virion particles. Among all the structural proteins, GP5 and M forms a heterodimer, and are the major component of virion envelope (Verheije, Welting, Jansen, Rottier, & Meulenberg, 2002). In contrast, GP2, GP3, GP4, and E form a multimeric complex and are the minor component of the envelope (Das et al., 2010; Wissink et al., 2005).

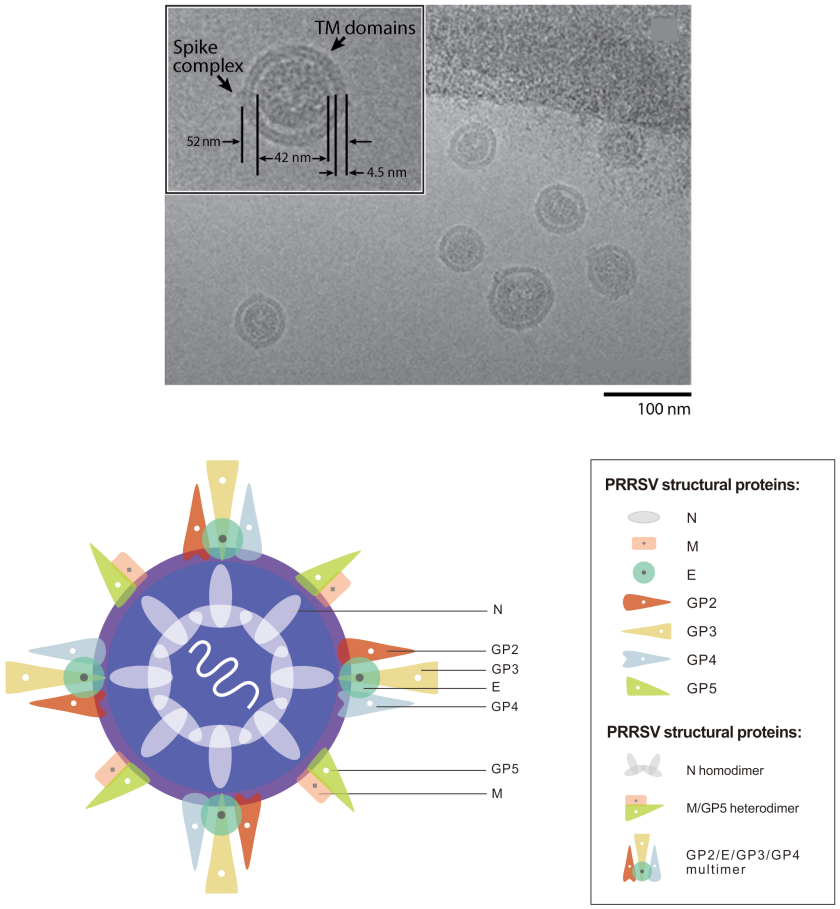

Fig.1. 1 PRRSV virions

A. Cryo-EM image of PRRSV virions. Inset shows a typical particle with pertinent dimensions indicated (Dokland, 2010). B. Schematic illustration of the PRRSV virion.

1.2.3 PRRSV genome organization and associated expression

PRRSV is a single-stranded, non-segmented, positive-sense RNA virus. The genome size of PRRSV is ranging from 14.9 Kb to 15.5 Kb in length. From 5′ to 3′ direction of the genome, it contains 5′ methylated cap structure, 5′ proximal noncoding element (5′-untranslated region; 5′ UTR), 11 known open reading frames (ORFs), 3′ untranslated region

and 3′-polyadenylated tail. The 11 ORFs are ORF1a, ORF1a′ -TF, ORF1b, ORF2a, ORF2b, ORF3, ORF4, ORF5, ORF5a, ORF6, and ORF7 (Lunney et al., 2016). ORF1a and ORF1b occupy two-thirds of the genome and locate at the 5' proximal end of the genome. ORF1a encodes polyprotein pp1a, and ORF1b encodes pp1ab through −1 ribosomal frame shift (RFS) signal in the ORF1a/ORF1b overlap region. The newly discovered ORF1a -TF encodes pp1a-nsp2N and pp1a-nsp2TF depending on a −1/−2 programmed RFS signal in the central region of ORF1a, respectively (Fang et al., 2012; Yanhua Li et al., 2014). These four polyproteins are synthesized from genomic RNA template, and post-translationally processed into at least 16 nonstructural proteins (nsps) that are needed for the viral replication by four viral proteases including papain-like cysteine proteinases 1α (PLP1α; nsp1α), PLP1β (nsp1β) and PLP2 (nsp2), and the main serine proteinase (SP; nsp4) (Kappes & Faaberg, 2015). The ORFs 2-7, occupies the rest of the viral genome, encode structural proteins including glycoproteins (GP2a, GP3, GP4, and GP5), unglycosylated membrane proteins (E, ORF5a, and M), and a nucleocapsid protein (N). All the structural proteins are translated from a 3'-terminal nested set of subgenomic RNAs (sgRNA2-7), which are generated through a negative-strand intermediate (van Marle et al., 1999). The genomic organization and associated expression profiles are schematically shown in Fig. 1.2.

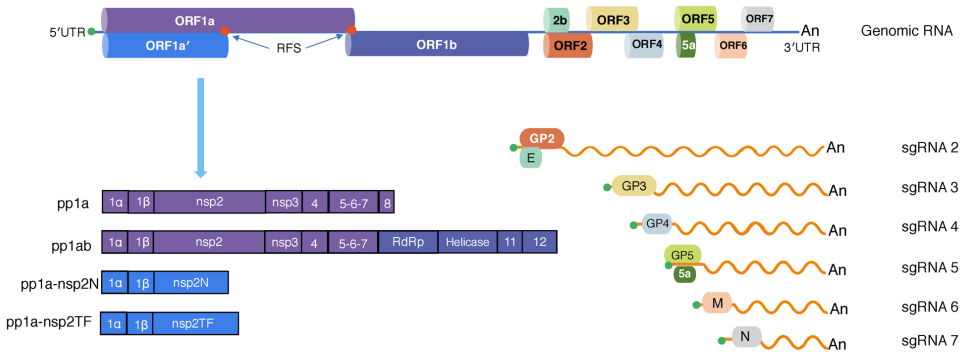

Fig.1. 2 PRRSV genome illustration

1.3 Functions of individual PRRSV proteins

1.3.1 PRRSV nsps from ORF1a, ORF1a-TF, ORF1ab Polyproteins

According to the *PRRSV-2* prototype strain VR-2332 (GenBank accession number U87392), ORF1a is 7512nt in length and encodes polyprotein pp1a (2503aa). ORF1ab includes ORF1a and ORF1b (4374nt in length) and encodes pp1ab (3960aa). And the newly discovered ORF1a-TF encodes pp1a-nsp2N (1234aa) and pp1a-nsp2TF (1403aa) (Fang et al., 2012; Yanhua Li et al., 2014). The polyprotein pp1a is further processed into nsp1 to nsp8, the pp1ab is cleaved into nsp1 to nsp12, and the pp1a-nsp2N and pp1a-nsp2TF are processed into nsp1 to nsp2N and nsp1 to nsp2TF, respectively. All these four polyproteins are post-translationally processed by four viral proteases including PLP1α, PLP1β, PLP2, and SP. PLP1α is responsible for cleaving the nsp1α↓nsp1β junction. The cleavage site of PLP1β proceeded is the nsp1β↓nsp2 junction. PLP2 cleaves nsp2↓nsp3 junction, and all remaining nsps are processed by SP (Chen et al., 2010; Kappes & Faaberg, 2015; Nelsen, Murtaugh, & Faaberg, 1999; Snijder, Kikkert, & Fang, 2013). The functions of all viral nsps are reviewed in the following section.

Nsp1α and nsp1β.

The nsp1 is auto-cleaved by PLP1α yielding two subunits, nsp1α and nsp1β (Chen et al., 2010). The nsp1α and nsp1β proteins are around 180aa and 205aa, respectively, in length. The crystal structure analysis of nsp1α reveals that it contains a zinc-finger (ZF) domain, a papain-like cysteine protease (PLP) domain, and a carboxyl-terminal extension. In *PRRSV-2*, the nsp1α↓nsp1β junction cleavage site is Cys-Ala-Met180↓Ala-Asp-Val (Y. Sun et al., 2009). The nsp1β contains an N-terminal nuclease domain, C-terminal PLP domain, a linker domain (LKD) that connects the NTD and the PCP domain, and a C-terminal extension (Xue et al., 2010). The Gly383↓Ala384 in *PRRSV-2* has also been confirmed as PLP1β cleavage site of the nsp1β↓nsp2 junction (Chen et al., 2010).

The nsp1α is involved in subgenomic mRNA synthesis, but not in genome replication. In contrast, nsp1β is required for genome replication (Kroese et al., 2008). Nsp1β blocks host mRNA nuclear export to the cytoplasm to enhance the PRRSV genome replication (M. Han, Ke, Zhang, & Yoo, 2017). Furthermore, nsp1α can strongly inhibit type I interferon production, whereas nsp1β can block both the type I interferon synthesis and downstream signaling pathway by inducing Karyopherin-α1 (KPNA1) degradation to inhibit nuclear translocation of STAT1 (Chen et al., 2010; R. Wang, Nan, Yu, & Zhang, 2013). Recently, nsp1α was identified to inhibit NF-κB activation by impairing the linear ubiquitin chain assembly complex (LUBAC) (Jing et al., 2017).

Nsp2, nsp2TF and nsp2N

The largest nonstructural protein of PRRSV is nsp2, which consists of N-terminal highly conserved chymotrypsin-like cysteine protease (PL2) domain, C-terminal three to four putative transmembrane domains and the large hypervariable domain in the middle region (J. Han, Wang, & Faaberg, 2006; McGuffin, Bryson, & Jones, 2000). Insertions and deletions are common in the nsp2 hypervariable domain, which influences nsp2 protein in two ways

(Z. Ma et al., 2016; Ni, Huang, Cao, Opriessnig, & Meng, 2011; F. X. Wang et al., 2013). One is the variable length of nsp2 among different strains; the other one is the variable homology of nsp2. The length of nsp2 protein in *PRRSV-1* and *PRRSV-2* is around 1078aa and 1196aa, respectively (Fang & Snijder, 2010). Sequence alignment of nsp2 suggests that the homology of nsp2 of *PRRSV-2* strains is approximately 80%, whereas nsp2 of *PRRSV-1* strains only share 40% similarity (F. X. Wang et al., 2013). In *PRRSV-2*, the cleavage site of the nsp2 ↓ nsp3 junction is Gly1446 ↓ Ala1447 by PL2, which is one amino acid downstream from the previously predicted Gly1445 ↓ Gly1446 site (Fang & Snijder, 2010; Y. H. Li, Tas, Sun, Snijder, & Fang, 2015; Ziebuhr, Snijder, & Gorbalenya, 2000).

The nsp2 protein has multiple functions during the PRRSV life cycle. First, the N-terminal PL2 domain of the nsp2 contains both cis- and trans-cleavage domain, which cleaves the nsp2 and nsp3 junction, and helps the nsp2 release from the polyprotein pp1a/pp1ab (J. Han, Rutherford, & Faaberg, 2009; Snijder, Wassenaar, Spaan, & Gorbalenya, 1995). Secondly, this domain was identified as deubiquitinating enzyme, belongs to the ovarian tumor domain (OTU) family, which is an interferon (IFN) antagonist by inducing the deconjugation of IFN-stimulated gene 15 (ISG15) (Frias-Staheli et al., 2007; Makarova, Aravind, & Koonin, 2000). Thirdly, during the virus replication, nsp2 with the collaboration of nsp3 involves in the formation of perinuclear double membrane vesicles (DMVs), which are associated with virus replication and transcription complex (RTC) (Knoops et al., 2012; Snijder, van Tol, Roos, & Pedersen, 2001). Fourthly, the nsp2 protein is identified to be incorporated into the virions in multiple isoforms, which suggests that nsp2 contains the potential property of a structural protein (Kappes, Miller, & Faaberg, 2013).

Nsp2TF and nsp2N are two recently discovered virus proteins (Fang et al., 2012; Yanhua Li et al., 2014). Both of the proteins are the products of a newly identified short transframe (TF) ORF in the central region of ORF1a via the method of −1/−2 programmed

RFS. Nsp2TF is yielded from −2RFS, which has the occurrence rate of approximately 20% (Fang et al., 2012); whereas nsp2N is through −1RFS mechanism at the same shift site, and the occurrence rate is approximately 7% (Yanhua Li et al., 2014). The size of nsp2TF is 902aa (*PRRSV-1*), 1019aa (*PRRSV-2*); and nsp2N is 733aa (*PRRSV-1*), 850aa (*PRRSV-2*). Both −2 and −1 RFS have to be transactivated by PRRSV nsp1β to produce nsp2TF and nsp2N. The exact functions of nsp2TF and nsp2N are still unclear, however, both of the proteins contain the PL2 domain, which indicates these two proteins may have the properties of PL2.

Nsp3

Nsp3 is recognized as a putative transmembrane protein with the length of 230aa in both *PRRSV-1* and *PRRSV-2*. It interacts tightly with nsp2 to form the DMVs during virus replication (Snijder et al., 2001).

Nsp4

Nsp4 is the 3C-like serine proteinase (SP) with the length of 203aa and 204aa in *PRRSV-1* and *PRRSV-2*, respectively (Snijder et al., 2013). The crystal structure analysis indicates that nsp4 is composed of three domains. Domains I and II are two chymotrypsin-like β-barrels domains, and the domain III located between domains I and II is an extra C-terminal α/β domain, which is essential for polyprotein proteolysis (X. Tian et al., 2009). It is responsible for the cleavage of nsp3-12 (Ziebuhr et al., 2000). In PRRSV-infected cells, nsp4 is able to inhibit the IFN-β induction via cleaving the NEMO, a subunit of the IκB kinase complex, to downregulate the NF-κB signaling pathway (Huang et al., 2014). Besides as an IFN antagonist, nsp4 has the ability to activate caspase 3, 8 and 9, which can lead to apoptosis (Z. Ma et al., 2013).

Nsp5

Nsp5 is another protein contains the transmembrane domain with the length of 170aa in both *PRRSV-1* and *PRRSV-2* (Kappes & Faaberg, 2015). Therefore, nsp5 may involve in the formation of membrane-bound viral RTCs during virus replication (Y. H. Li et al., 2015). Most recently, nsp5 was identified as an antagonist of STAT3 signaling to increase the degradation of STAT3 via the ubiquitin-proteasomal pathway (L. P. Yang et al., 2017).

Nsp6, nsp7α, nsp7β and nsp8

Nsp6 to nsp8 are not well studied. The functions and structures of these proteins are still unknown. The lengths of these proteins are: nsp6 is 16aa (*PRRSV-1* and *PRRSV-2*), nsp7α is 149aa (*PRRSV-1* and *PRRSV-2*), nsp7β is 120aa (*PRRSV-1*) and 110aa (*PRRSV-2*), and nsp8 is 45aa (*PRRSV-1* and *PRRSV-2*) (Lunney et al., 2016). Nsp7α and nsp7β are the products of nsp7 internally cleaved (Y. H. Li et al., 2015). It has been reported that nsp3-nsp8 is the determinants of PRRSV virulence (Kwon, Ansari, Pattnaik, & Osorio, 2008). Among these proteins, the recombinant nsp7 is highly antigenic (H. Li et al., 2017), which makes nsp7 a good candidate for development of diagnostic assays (Brown et al., 2009; H. Wang et al., 2017). Recently, nsp7α nuclear magnetic resonance (NMR) structure analysis showed that nsp7α contains three α-helices and five β-strands, and a predominant proline-rich region (PRR) in the C-terminus. Furthermore, nsp7α is able to interact with nsp9, the RNA dependent RNA polymerase (RdRp) of PRRSV, which is consistent with a previous study suggesting that nsp7α is essential for the viral replication and viral RNA synthesis (Manolaridis et al., 2011; M. Zhang et al., 2013) (J. Chen, X. Xu, et al., 2017).

Nsp9 and nsp10

Nsp9 and nsp10 are two most conserved proteins between PRRSV genotypes (Gorbalenya, Enjuanes, Ziebuhr, & Snijder, 2006). Nsp9 is a product of pp1ab via a programmed -1RFS with 685aa in length, which encodes RdRp (Nelsen et al., 1999; van Dinten, Wassenaar, Gorbalenya, Spaan, & Snijder, 1996). During the virus replication, nsp9

can interact with several viral proteins or host cellular proteins to regulate the virus replication, which include nsp7α, nucleocapsid protein, Annexin A2 (ANXA2), cellular retinoblastoma protein (pRb), and DEAD-box RNA helicase 5 (DDX5) (Dong et al., 2014; J. N. Li et al., 2014; L. Liu et al., 2016; Zhao et al., 2015).

Nsp10 is an RNA NTPase/helicase with the length around 441aa, which has a putative zinc-binding motif. It is responsible for the double-stranded RNA (dsRNA) unwinding (van Dinten et al., 1996). A recent study showed that nsp10 could interact with host cellular protein DEAD-box RNA helicase 18 (DDX18) to promote virus replication (Jin et al., 2017). It was also involved in CD83 signaling pathway by inducing CD83 production through NF-κB and Sp1 signaling pathways (X. Chen et al., 2017). Both nsp9 and nsp10 contribute to the virulence of a highly pathogenic PRRSV in China (Y. Li et al., 2014).

Nsp11 and nsp12

Nsp11 is also a conserved protein with the length of 224aa, which is nidovirus-specific uridylate-specific endonuclease (NendoU) (Kappes & Faaberg, 2015). The crystal structure of nsp11 reveals that nsp11 is composed by N-terminal domain (NTD), linker domain (LKD) and C-terminal catalytic domain. NTD contains six β-strands and two α-helices, whereas catalytic domain contains three α-helices and nine β-strands. Nsp11 mainly exists as a dimer, and the endoribonuclease function needs the nsp11 dimerization (Y. J. Shi et al., 2016). In the meanwhile, nsp11 plays an important role in several signaling pathways including interferon pathways, NF-κB signaling, and NLRP3 inflammasome pathway. It is an interferon antagonist, which inhibits the synthesis of MAVS and RIG-I (X. B. Shi et al., 2011; Y. Sun, H. Z. Ke, et al., 2016). In NF-κB signaling pathway, nsp11 shows a unique deubiquitinating activity on lysine 48 (K48)-linked polyubiquitin to inhibit NF-κB activation (D. Wang et al., 2015).

Nsp12 is a protein of 153aa in length. The structure and function of this protein remain unclear. Nsp12 is shown to activate STAT1 via serine 727 phosphorylation in an IFN-independent way (Y. Yu, Wang, Nan, Zhang, & Zhang, 2013). Ingelvac PRRS® MLV, a vaccine strain, has a minimal effect on serine 727 phosphorylation. Compared to MLV-infected cells, VR-2385, a moderate virulent strain, caused significantly higher level of proinflammatory cytokines, including interleukin 1 beta (IL-1beta) and IL-8. The STAT1-S727 and cytokine expression depend on p38 mitogen-activated protein kinase (MAPK) pathway. Nsp12 has also been shown to induce elevation of KPNA6, an importin in nucleocytoplasmic trafficking, via blocking its degradation (L. Yang et al., 2018). It is demonstrated that KPNA6 plays an important role in replication of PRRSV and Zika virus.

1.3.2 ORF2a/b

ORF2a and ORF2b are encoded by sgRNA2, which is translated into glycoprotein 2 (GP2) and a small hydrophobic unglycosylated envelope protein (E), respectively (Lunney et al., 2016). The size of GP2 is 249aa and 256aa in *PRRSV-1* and *PRRSV-2*, respectively. GP2 is one of the minor structural proteins and forms a disulphide-linked heterotrimeric complex with GP3 and GP4 in virus particles (Das et al., 2010) (Das et al., 2010; de Vries, Chirnside, Horzinek, & Rottier, 1992). It is a putative class I integral membrane protein with two conserved N-glycosylation sites at residues Asn 173 and Asn 179 in *PRRSV-1* or Asn 178 and Asn 184 in *PRRSV-2* (Dea, Gagnon, Mardassi, Pirzadeh, & Rogan, 2000; Wissink et al., 2004). These two N-glycosylation sites do not influence the infectivity in *PRRSV-1*, whereas the site N184 in GP2 *PRRSV-1* is needed for virus infection (Das et al., 2011). GP2 is composed of four domains: a cleaved N-terminal signal sequence, a 168aa ectodomain, a single transmembrane (TM) helix and a 20aa endodomain (Wissink et al., 2004). Furthermore, GP2 has been proved to interact with CD163 as viral attachment protein to mediate the virus entry to the host cells (Das et al., 2010). A recent study also revealed

another potential role of GP2 as an anti-apoptotic factor during the virus infection (Pujhari, Baig, & Zakhartchouk, 2014).

The unglycosylated envelope protein (E) is also the product of sgRNA2 with the length 70aa in *PRRSV-1* and 73aa in *PRRSV-2*. It contains three putative domains: a ~25aa N-terminal domain, a ~20aa central hydrophobic transmembrane helix domain, and a ~25aa hydrophilic, polybasic C-terminal domain (Snijder, van Tol, Pedersen, Raamsman, & de Vries, 1999). E protein colocalizes with ER and Golgi markers, which indicates that E protein is located at ER and various regions of the Golgi (C. Lee & Yoo, 2006; Snijder et al., 1999; Thaa et al., 2009; M. R. Yu et al., 2010). The E protein of PRRSV is suggested to possess an ion channel-like function, which could help uncoating of the virions and promote the viral genome release into the cytosol (C. Lee & Yoo, 2006). The deletion of the E gene in infectious cDNA clone leads to the failure of virus recovery, further confirming that E protein is essential during the virus entry (C. Lee & Yoo, 2006). The interaction of E protein with the GP2/3/4 complex is also essential for virus incorporation of GP2/3/4 into the virion (Wieringa et al., 2004; Wissink et al., 2005). Recent studies have shown that E protein could interact with some host proteins like tubulin-α, microtubules and mitochondrial proteins (Pujhari & Zakhartchouk, 2016; M. D. Zhang & Zakhartchouk, 2017). The interaction between E and mitochondrial proteins ATP5A could induce host cell apoptosis via caspase 3 activation (Pujhari & Zakhartchouk, 2016). Whereas the interaction between protein E and tubulin-α leads to microtubules depolymerization at the late phase of the virus infection (M. D. Zhang & Zakhartchouk, 2017).

1.3.3 ORF3

GP3 is translated from ORF3 with the length of 265aa in *PRRSV-1* (254aa in *PRRSV-2*) (Lunney et al., 2016). GP3 is the second most heterogenous protein of PRRSV as the homology between *PPRSV-1* and *PRRSV-2* of the GP3 is only around 54-60% (Dea et al.,

2000). The mature protein is composed of an N-terminal signal peptide, an ectodomain containing seven putative N-glycosylation sites, a hydrophobic region at C-terminus (*PRRSV-2*) or a C-terminus hydrophilic domain (*PRRSV-1*) (Veit, Matczuk, Sinhadri, Krause, & Thaa, 2014). The GP3 is the most N-glycosylated protein among all PRRSV proteins, and the seven potential N-glycosylation sites are N42, N50, N130, N151, N159, and N 194 in *PRRSV-1*; and N29, N42, N50, N131, N152, N160, and N196 in *PRRSV-2* (Dea et al., 2000). The function of the GP3 heavily relies on these glycosylation sites. N42, N50, and N131 of GP3 are believed essential for PRRSV-2 virus infection as individual mutation of these sites in infectious clone FL-12 leads to failure of virus recovery (Das et al., 2011). Simultaneously mutation of the residues N29, N152, and N160 impairs the virus growth (Das et al., 2011). Variable structure of the GP3 makes it highly antigenic, and several epitopes of GP3 induce antibodies (de Lima, Pattnaik, Flores, & Osorio, 2006; Oleksiewicz, Botner, & Normann, 2002; Oleksiewicz, Botner, Toft, Normann, & Storgaard, 2001). One study also indicates it may also be involved in viral neutralization (Vu et al., 2011). Initially, GP3 was not considered as a structural protein as some GP3 could be secreted to cell medium as a non-virion-associated soluble protein during *PRRSV-2* infection (Mardassi, Gonin, Gagnon, Massie, & Dea, 1998). The secreted subset of GP3 may exert the function to distract the antibodies against PRRSV (Veit et al., 2014). Later studies prove that GP3 is a minor structural protein in both *PRRSV-1* and *PRRSV-2* viruses (de Lima et al., 2009; Wissink et al., 2005).

1.3.4 ORF4

GP4 is encoded by ORF4 with the length of 183aa in *PRRSV-1* (178aa in PRRSV-2) (Lunney et al., 2016). It is composed of an N-terminal signal peptide, an ectodomain, one short hydrophobic transmembrane region (class I integral membrane proteins) (Veit et al., 2014). There are four conserved N-glycosylation sites in the GP4 sequence, which are N37,

N88, N124, and N134 in *PRRSV-1*, and N37, N84, N120, and N130 in *PRRSV-2* (Das et al., 2011). Several studies showed that these four glycosylation sites related to the virus replication. A single mutation of the sites does not influence the virus replication, whereas mutations of more than two sites are lethal for the virus (Das et al., 2011; Wei, Tian, et al., 2012). The highly variable ectodomain may have the epitope to induce neutralizing antibodies (Costers et al., 2010; Meulenberg, van Nieuwstadt, van Essen-Zandbergen, & Langeveld, 1997; Vanhee et al., 2010). As the neutralizing site of GP4 is highly variable, the site may not be essential during the virus cell entry process.

The three minor envelope glycoproteins GP2, GP3, and GP4 form multimeric complexes to embed into the PRRSV lipid envelopes and to exert their functions (Veit et al., 2014). GP2 and GP4 preform as a dimer before associating with GP3, and all three proteins are disulfide-linked (Wieringa, de Vries, & Rottier, 2003). E protein is assumed as part of the GP2/3/4 complex to help the complex incorporate into the virion at least in *PRRSV-1* (Wieringa et al., 2004). Also, the existence of GP5 is essential for the GP2/3/4 complex formation, and the interaction between GP5 and GP4 may help the complex fold into a suitable conformation (Das et al., 2010). During the virus infection, the complex plays an important role in virus entry. GP2 and GP4 could be co-immunoprecipitated with the CD163 receptor, which suggests the complex mediate the receptor binding (Das et al., 2010). However, whether the complex mediates the membrane fusion is still unknown.

1.3.5 ORF5a

The sgRNA5 encode ORF5a and ORF5, which are translated into ORF5a protein (pORF5a) and GP5 protein, respectively, in different frames. pORF5a is the latest structural protein of PRRSV that was identified in 2011 (Firth et al., 2011; Johnson, Griggs, Gnanandarajah, & Murtaugh, 2011). pORR5a is an unglycosylated protein with the length of 43aa in *PRRSV-1* and 51aa in PRRSV-2 and is incorporated into the virion as a multimeric

complex with GP2/3/4 (Lunney et al., 2016). It is putatively considered as a type III membrane protein, which is composed of a central hydrophobic transmembrane domain, a short N-terminal ER-luminal ectodomain and a long C-terminal endodomain (Firth et al., 2011; Johnson et al., 2011). The conserved RQ-motif (arginine/glutamine-rich sequence) in pORF5a was predicted to involve in RNA-binding (Johnson et al., 2011). The function of pORF5a is still unclear, but it is essential for the virus replication as the inactivation of ORF5a severely impair the virus viability (Firth et al., 2011; L. Sun et al., 2013). pORF5a could induce a specific antibody in infected pigs, whereas the antibodies are unable to neutralize the virus, and also fail to protect against challenge infection (Robinson, Figueiredo, Abrahante, & Murtaugh, 2013).

1.3.6 ORF5

As a major component of the PRRSV virions, GP5 is encoded by ORF5 with the length of ~200aa. It is the most abundant and most variable structural protein of PRRSV (Meng, 2000; Music & Gagnon, 2010). GP5 of *PRRSV-1* and PRRSV-2 share only ~50% amino acid identity. GP5 is composed of the following domains: N-terminal cleavable signal peptide; ~30aa length ectodomain containing several putative N-glycosylation sites; hydrophobic transmembrane region, and hydrophilic C-terminal part (Dokland, 2010). Among several N-glycosylation sites of GP5, two are highly conserved: N46 and N53 in *PRRSV-1*, N44 and N51 in *PRRSV-2*. These two sites are tightly correlated with the virus replication since mutations in N46 (*PRRSV-1*) or N44 (*PRRSV-2*) impair the virus growth (Ansari, Kwon, Osorio, & Pattnaik, 2006; Wissink et al., 2004). In addition to these two conserved sites, there are several variable potential glycosylation sites in the hypervariable region of GP5. During the virus infection and passaging, the loss or the addition of the glycosylation to these variable sites could appear (Chang et al., 2002; Kwon et al., 2008). Several studies show that these flexible glycosylation sites may influence protein folding, and

also have the effect on antigenicity of GP5. Deletion of glycosylation sites at N34 and N51 in *PRRSV-1* and N51 in *PRRSV-2* can enhance the production of neutralizing antibodies against PRRSV in experimentally-infected pigs compared to wild type PRRSV (Vu et al., 2011; Wei, Lin, et al., 2012).

GP5 is assumed to be the most relevant protein in PRRSV for eliciting virus-neutralizing antibodies (Lopez & Osorio, 2004). It harbors two epitopes in *PRRSV-2*, one at the residues 27-31, which elicits strong non-neutralizing antibody at the early stage of infection; the other one at the residues 37-44, which induce neutralizing antibodies at the late stage. According to the "decoy hypothesis," the epitope at the residues 27-31 acts as a decoy to distract the humoral immune response, and, as a result, causes the delay in the recognition of the epitope at the residues 37-44 (Lopez & Osorio, 2004). Although GP5 is usually depicted as the main antigen for PRRSV eliciting neutralizing antibodies, GP5 ectodomain-specific antibodies from PRRSV-neutralizing pig serum do not have neutralizing activity (J. Li & Murtaugh, 2012). Therefore, the surface protein(s) of PRRSV that are the antigens to elicit neutralizing antibody is still an open question.

1.3.7 ORF6

M protein is translated by ORF6 with the length of 173aa in *PRRSV-1* and 174aa in *PRRSV-2* (Lunney et al., 2016). M protein is unglycosylated and is the most conserved membrane protein of PRRSV. It consists of an N-terminal short ectodomain, three putative transmembrane domains, and a long, hydrophilic cytoplasmic tail (de Vries et al., 1992).

The heterodimers of M protein and GP5 is disulfide-linked between the conserved cysteine residue at the ectodomain of M and the cysteine residue at ectodomain of GP5 (Snijder, Dobbe, & Spaan, 2003). Only the GP5/M heterodimers can be transported from ER to Golgi apparatus, and eventually inserted into the virion. The mutation of the conserved cysteine residue can abolish the virus production, which indicates the proper assembly of

GP5/M complex is vital for the virus (Snijder et al., 2003). Several studies suggest that the main function of the GP5/M complex is for virus assembly and budding. The complex drives the membrane curvature to form a bud (Derganc, Antonny, & Copic, 2013; Doan & Dokland, 2003; Garoff, Hewson, & Opstelten, 1998; Nam et al., 2013; Wieringa et al., 2004). Besides the function of the GP5/M dimer for virus assembly and budding, the complex is also involved in virus entry process. It is revealed that the GP5/M complex initiates the interaction between PRRSV and two cell receptors, heparan sulphate glycosaminoglycans and CD169, and the conformation of the complex is crucial for this interaction (Wander Van Breedam et al., 2010).

1.3.8 ORF7

ORF7 is expressed from sgRNA7, resulting in the generation of the nucleocapsid (N) protein. N protein is an unglycosylated and phosphorylated structural protein with 128aa in *PRRSV-1* and 123aa in *PRRSV-2* in length and is the only structural protein without transmembrane domain and ectodomain (Lunney et al., 2016; Wootton, Rowland, & Yoo, 2002). The sequence of the N protein is well conserved within the same genotype, whereas N proteins between the *PRRSV-1* and *PRRSV-2* only share 56% identity (Doan & Dokland, 2003; Wootton & Yoo, 2003). It is the sole component of the viral capsid, which forms disulfide-linked, double-layered homodimers to wrap the viral genomic RNA within the PRRSV virion (Spilman et al., 2009). Cryo-electron tomography analysis reveals that the capsid core is separated from the lipid envelope by a gap of 2-3 nm (Spilman et al., 2009). Although PRRSV replicates in the cytoplasm, a fraction of the N protein is identified in the nucleolus during the virus infection (Rowland, Kervin, Kuckleburg, Sperlich, & Benfield, 1999). Sequence analysis indicates two nuclear localization signal (NLS) in N protein. NLS-1 (or the cryptic NLS) is located at aa residues 10 to 13; NLS-2 (or the functional NLS) is located at aa residues 41 to 47 of the N protein (Rowland et al., 2003).

N is a multifunctional protein and plays an important role in viral infection and pathogenesis besides as a scaffold protein for viral RNA. It was reported that N protein may interact with replication complex membranes to induce the double-membrane vesicles formation and virion assembly (Knoops et al., 2012). The Src homology 3 (SH3) motif within N protein interacts with host cellular signaling proteins including STAMI, TXK, Fyn, Hck, and cortactin (Kenney & Meng, 2015). Meanwhile, N protein could induce interleukin-10 (IL-10) and increase regulatory T-lymphocytes in PRRSV-infected pigs (Wongyanin et al., 2012). It also modulates the IFN and NF-κB signal pathways during the virus infection (J. Chen, X. Shi, et al., 2017; R. Luo et al., 2011).

N protein is highly immunogenic, making it a suitable candidate for the detection of virus-specific antibodies. Five immune reactive domains are identified in N protein and are located among the residues 30 to 52, 37 to 52, 69 to 112, and 112 to 123 (Music & Gagnon, 2010). Based on these sites, plenty of monoclonal antibodies (MAbs) have been generated, whereas none of them are neutralizing (Diaz, Darwich, Pappaterra, Pujols, & Mateu, 2006).

1.4 PRRSV replication cycle

1.4.1 Attachment and entry into host cells

Pigs are the only natural host for PRRSV (Lawson, Rossow, Collins, Benfield, & Rowland, 1997). PRRSV infection is highly restricted to cells of the monocyte-macrophage lineage, and the main target cells for PRRSV are pulmonary alveolar macrophages (PAMs). However, PRRSV propagation *in vitro* is generally conducted in MARC-145 or CL2621 cells, which are epithelial-derived cells from African green monkey kidney (H. S. Kim, Kwang, Yoon, Joo, & Frey, 1993).

Although PRRSV infection is highly restricted, transfection of the viral genomic RNA to non-permissive cells can also lead to PRRSV replication (Meulenberg, Bos-de

Ruijter, van de Graaf, Wensvoort, & Moormann, 1998). This indicates that the PRRSV entry to the cell is dependent on the cell surface molecules (Kreutz, 1998). Several mediators have been identified to facilitate the viral infection, three of them are well studied: heparan sulphate, sialoadhesin and CD163(Calvert et al., 2007) (Calvert et al., 2007; Delputte, Vanderheijden, Nauwynck, & Pensaert, 2002; Vanderheijden et al., 2003).

Heparan sulphate is the first identified entry mediator for the virus entry in 1997 on MARC-145 cells (Jusa, Inaba, Kouno, & Hirose, 1997). Incubation of the PRRSV with heparan sulphate or treatment of the cells with heparinase before virus infection could block the virus infection greatly (Jusa et al., 1997). Further experiments discovered the precise molecules interact with the PRRSV was heparan sulphate glycosaminoglycans (GAGs) (Delputte et al., 2002). Heparan sulphate GAGs is not macrophage specific, which can be found in several animal tissues. This explains why non-permissive cells could bind to PRRSV but not allow the virus entry and replication in the cells (Delputte, Costers, & Nauwynck, 2005). Therefore, the function of heparan sulphate GAGs on the macrophage surface is binding the PRRSV to concentrate the virions on the cell surface, which improves the efficiency of the infection.

Sialoadhesin is a macrophage-restricted type 1 transmembrane glycoprotein, which was first postulated as a PRRSV entry mediator in 1998 (Duan, Nauwynck, Favoreel, & Pensaert, 1998). Later studies confirmed that porcine sialoadhesin (pSn or CD169) binding to PRRSV envelope protein M/GP5 complex to facilitate the virus entry and internalization (Delputte et al., 2007; Vanderheijden et al., 2003). Furthermore, N-terminal V-set domain of pSn and sialic acid present on the virion surface are essential for the pSn and M/GP5 complex interaction (Delputte et al., 2007))(W. Van Breedam et al., 2010). Both heparan sulphate and pSn are the main attachment and internalization mediators for PRRSV infection, however, these two factors are not enough to release the virus genome into the cytoplasm. The protein

CD163 is the critical factor for the genome release process (Van Gorp, Van Breedam, Delputte, & Nauwynck, 2008).

CD163 is a scavenger receptor around 130kDa in size, which is mainly expressed in monocyte/macrophage cells (Sanchez et al., 1999). It belongs to the scavenger receptor cysteine-rich (SRCR) superfamily class B, which consists of an N-terminal extracellular domain, a single transmembrane domain and a cytoplasmic domain (Onofre, Kolackova, Jankovicova, & Krejsek, 2009). There are nine SRCR domains in CD163 extracellular domain. It was first identified as a potential PRRSV entry mediator in 2007 (Calvert et al., 2007). The study shows transfection of CD163 are able to confer the PRRSV susceptibility to non-permissive cell lines (Calvert et al., 2007). The exact mechanism of CD163 facilitation of the virus genome release is still unknown. However, some studies show the fifth SRCR domain in N-terminal CD163 is crucial for virus infection (Van Gorp, Van Breedam, Van Doorsselaere, Delputte, & Nauwynck, 2010). Most recently, macrophages from pigs with CD163 SRCR5 domain knockout through CRISPR/Cas9 technology were generated by researchers from the University of Edinburgh. Theses genome-edited pigs are fully resistant to PRRSV of both genotypes (Burkard et al., 2017). The minor PRRSV glycoproteins GP2 and GP4 have been identified to interact with CD163 and may thus have a role in viral uncoating (Das et al., 2010).

PRRSV enters host cells through conventional receptor-mediated clathrin-dependent endocytosis. Upon virus contact to the cell membrane, heparan sulphate GAGs binding to M protein to initiate the entry process (Delputte et al., 2002). The interaction between heparan sulphate GAGs and M protein concentrates the virions on the cell surface to help pSn recognize and bind to M/GP5 complex (Delputte et al., 2005). Following the attachment, the pSn binds to the sialic acid residues on the viral GP5 protein to start virus internalization via clathrin-mediated endocytosis (Wander Van Breedam et al., 2010; W. Van Breedam et al.,

2010). After entry into the cells, the virion uncoating process occurs in early endosome following the endosome acidification. The interaction between CD163 and virus GP2 and GP4 is believed to be involved in the uncoating and viral genome release (Chen et al., 2010; Kappes & Faaberg, 2015; Nelsen et al., 1999; Snijder et al., 2013; Snijder & Meulenberg, 1998).

1.4.2 Replication and translation

ER derived perinuclear DMVs are the site of the viral replication, where almost all the nonstructural proteins at the DMVs are involved in the establishment of RTC. Though little is known about the DMVs and RTC formation, it is believed that protein-protein interaction among nsps and host membrane rearrangement may be involved (Knoops et al., 2012; Snijder et al., 2001).

The translation mechanisms between the nonstructural and the structural proteins are different, though both processes are directed by RTC (Fang & Snijder, 2010; Pasternak, Spaan, & Snijder, 2004). The structural proteins are produced from a set of nested sgmRNAs (sg mRNA2-7), which are 5' and 3'-coterminal with genomic RNA, through a non-continuous negative-strand intermediate RNA synthesis (van Marle et al., 1999). Six sgRNAs encode eight structural proteins that constitute the infectious virion.

Following the genomic RNA and structural protein synthesis, virus life cycle comes to the late stage. Nucleocapsid complex is formed by multiple N protein homodimers binding with newly generated genomic RNA, which indicates the start of virion assembly (Yun & Lee, 2013). The nucleocapsid complex acquires the rest structural proteins during the transportation from smooth ER to Golgi to assemble an intact virion (Dea et al., 1995; Pol, Wagenaar, & Reus, 1997). Finally, the progeny virions bud into intracellular membrane

compartments and then are released into the extracellular space through exocytosis (Dea et al., 1995).

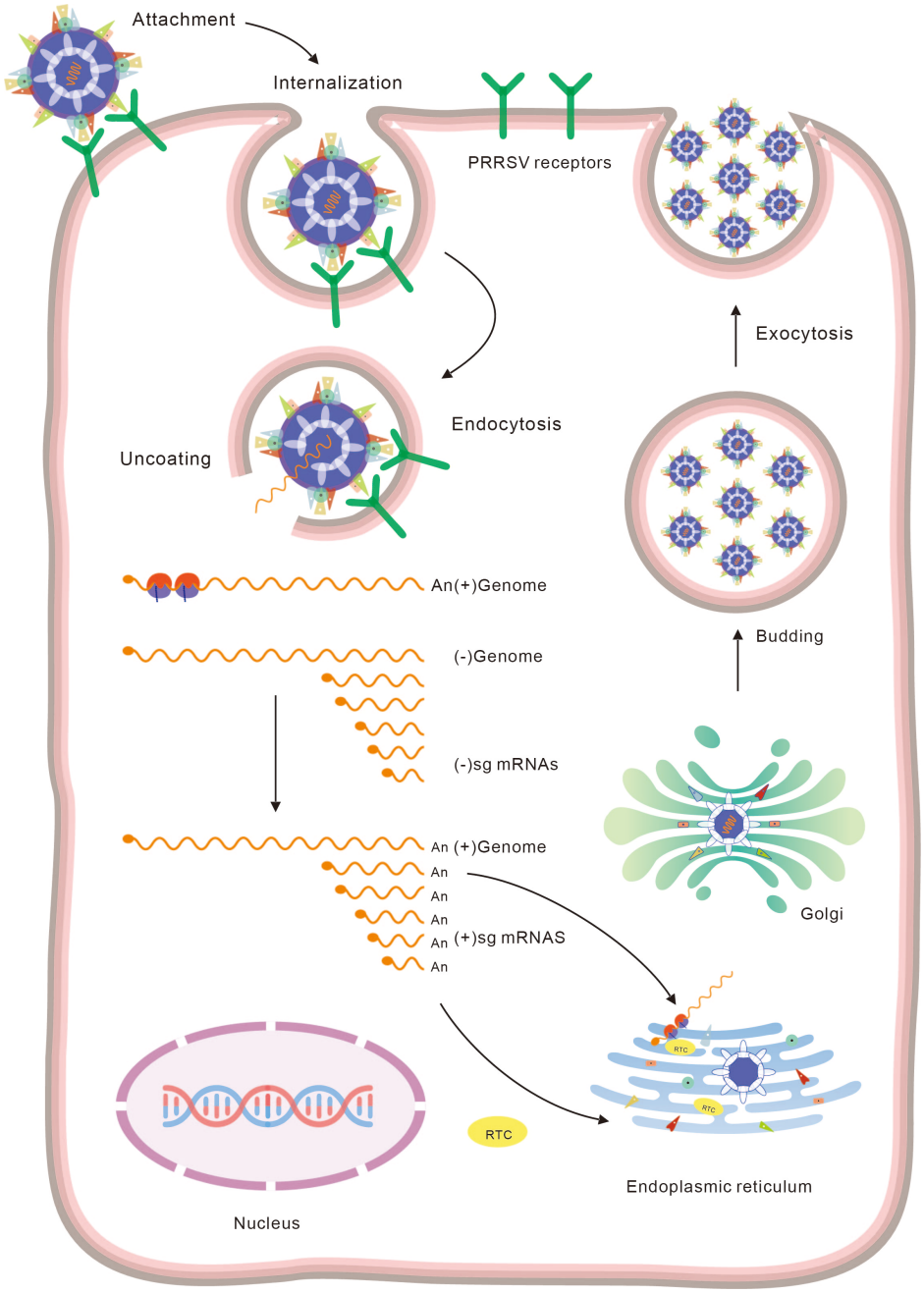

Fig.1. 3 Schematic illustration of PRRSV life cycle

Chapter 2: PRRSV Vaccine Development

2.1 Introduction

Vaccines have been explored to prevent PRRS since the early 1990s. There are two types commercial PRRSV vaccines including modified live virus (MLV) and inactivated/killed virus (KV). Among them, PRRSV-MLV is the most effective and widely used in the field. The first commercial PRRS vaccine, Ingelvac PRRS® MLV, is based on a *PRRSV-2* strain and was marketed in 1994. And the first marketed *PRRSV-1* vaccine, Suvaxyn (Cyblue®), is a KV vaccine and was also launched in 1994 (Renukaradhya, Meng, Calvert, Roof, & Lager, 2015a, 2015b).

Although there have been substantial efforts to prevent and control PRRS, no vaccination regimen has demonstrated sustaining success. The PRRS-MLV has been widely used over two decades, however, the efficacy of the vaccine and its potential to revert to virulence are always a hot topic. Due to both antigenic and genomic variations among PRRSV strains, the vaccine provides poor cross-protection. Consequently, there are frequent transmissions between pig populations and persistence of the virus in infected populations. The prevalence of PRRSV infection in pig herds remains high. Unfortunately, in 2006, a highly pathogenic PRRSV (HP-PRRSV) outbreak swept Asian countries, including China, India, Vietnam, Bhutan, Cambodia, Laos, Malaysia, Myanmar, the Philippines, Thailand, and Singapore (An, Tian, Leng, Peng, & Tong, 2011; Rajkhowa, Jagan Mohanarao, Gogoi, Hauhnar, & Isaac, 2015; K. Tian et al., 2007). The mortality rate caused by HP-PRRSV can reach 20-100% in sows. In addition to HP-PRRSV, other virulent strains like MN184 have also isolated after the initial identification of PRRSV. Several studies showed the constantly evolving PRRSV strains are more virulent than those encountered in the past.

Therefore, an improved PRRSV vaccine is needed. Ideally, the next generation vaccine should overcome the drawbacks of both MLV and KV vaccines, like the safety concern of MLV and low protection efficacy of KV. In 2007, experts on PRRSV in academia and industry gathered together to propose an ideal vaccine: rapid induction of host immunity, protection against most currently prevalent PRRSV strains, no adverse outcomes to swine health and ability to differentiate vaccinated from infected animals (DIVA) (Rock, 2007).

Later in 2010, the PoRRSCon project - New tools and approaches to control PRRS in the EU and Asia - was launched by the scientists from 15 partners from Europe and Asia to characterize PRRSV strains in Europe and Asia. A similar conclusion was drawn by the PoRRSCon project with the emphasis on setting up control measures by using marker vaccines with DIVA assays. Unfortunately, till now there is no one vaccine on the market could meet all of the above expectations. In this chapter, progress and challenges of current PRRSV vaccines, and perspectives to develop better PRRS vaccines are discussed.

2.2 MLV Vaccines against PRRSV

Since the first MLV vaccine was marketed in 1994, more than 20 commercial MLV vaccines against *PRRSV-1* and *PRRSV-2* have been licensed in different countries. Among them, some are widely used in North America, Europe, and Asia, including Ingelvac® PRRSFLEX EU (Boehringer Ingelheim), ReproCyc® PRRS EU (Boehringer Ingelheim), PORCILIS® PRRS (Merck) and Suvaxyn® PRRS MLV (Zoetis) against *PRRSV-1*; and Ingelvac® PRRS MLV (Boehringer Ingelheim), FLEXMycoPRRS™ (Boehringer Ingelheim), Fostera® PRRS (Zoetis) against *PRRSV-2* (CFSPH, 2018; Murtaugh & Genzow, 2011). All the commercial MLV vaccines are generated from repetitive passages in the cell lines derived from the monkey kidney MA-104 cell line, like MARC-145 (Renukaradhya et al., 2015b).

Although not all PRRSV-MLV vaccines from various manufacturers have been assessed side by side for efficacy, they have common properties and concerns.

2.2.1 Efficacy concern of MLV

Generally, the efficacy of PRRSV-MLV is considered better than killed virus vaccines. However, studies on comparison and evaluation of most commercial PRRSV-MLVs found two efficacy concerns that need attention (Diaz et al., 2006; Zuckermann et al., 2007). On one hand, PRRSV-MLV induces relative low adaptive immunity in the host, including both humoral and cell-mediated immune (CMI) responses. PRRSV specific neutralizing antibodies (NA) develop slowly (28DPI) and usually with low titer (Diaz et al., 2006). And virus-specific gamma-interferon-secreting cells (IFN-γ-SC) frequencies, an indicator for cell-mediated immunity, are also low compared to other pig viral infections (Meier et al., 2003). On the other hand, PRRSV-MLV provides incomplete cross-protection, which means PRRSV-MLV can confer effective protection against infection of homologous strains, whereas efficacy against heterologous infection is variable (Roca et al., 2012).

2.2.2 Safety concern of MLV

Another serious concern with the use of PRRSV-MLV is the risk of reversion to virulence through genetic mutations of the vaccine virus and/or recombination with field virulent virus. Ingelvac PRRS® MLV has been widely used for over two decades. Outbreaks of PRRS resulting from viral strains nearly identical in sequence to the vaccine strain have been reported (Botner et al., 1997; Opriessnig et al., 2002; Sorensen, Botner, Madsen, Strandbygaard, & Nielsen, 1997).

Till now, the PRRSV-MLV is still the first choice to control PRRSV in swine farms. However, outbreaks of atypical or acute PRRS in vaccinated pigs have raised concerns about

the efficacy and safety of current MLV vaccines (Madsen et al., 1998; Renukaradhya et al., 2015b). This all indicates that an improved vaccine is needed to control PRRS.

2.3 Inactivated PRRSV Vaccines

Similar to PRRSV-MLV, the first inactivated/KV vaccine was marketed in 1994. Since then, more than 14 commercially available KV vaccines have been licensed in different countries to combat *PRRSV-1* and *PRRSV-2* based on the prevalence of the virus in a licensed country. However, due to its low efficacy, the KV vaccines have been pulled out of the market from the United States since 2005 (Charerntantanakul, 2012). Considering the safety concern of PRRSV-MLV, development of KV vaccines is warrantied for the control and eradication of PRRS. There are several types of inactivated vaccines including inactivated preparation of attenuated PRRSV strains [e.g., Progressis® (Merial)], inactivated preparation of virulent isolates from the field for use in the same herd (autogenous vaccines) and inactivated preparation of multiple virulent isolates to enrich viral antigens [e.g. MJPRRS (MJ Biologics)].

Compared to PRRSV-MLV, KV vaccines are considered safe and could help PRRSV-positive pigs reduce levels of viremia (Zuckermann et al., 2007). However, several studies have revealed that KV vaccines cannot induce detectable PRRSV antigen-specific cellular and antibody responses (neither neutralizing nor non-neutralizing antibody response) even against homologous virus challenge (Bassaganya-Riera et al., 2004; H. Kim et al., 2011; Piras et al., 2005). This indicates that current KV vaccines are inadequate to control PRRS.

To improve the efficacy of KV vaccines, several promising attempts in developing innovative killed PRRSV vaccines have been reported recently. Incorporation of the KV vaccines with suitable adjuvants is a promising way to improve efficacy. Enhanced VN titers and reduced viremia were reported in several studies when pigs were vaccinated with adjuvant-containing vaccines that were prepared with various inactivation methods including

β-propiolactone (BPL) inactivation, UV-radiation inactivation, and binary ethylenimine (BEI) inactivation (Dwivedi, Manickam, Binjawadagi, Joyappa, & Renukaradhya, 2012; Misinzo, Delputte, Meerts, Drexler, & Nauwynck, 2006; Vanhee, Delputte, Delrue, Geldhof, & Nauwynck, 2009). In addition, a nanotechnology-based novel vaccine delivery system has been tested (Binjawadagi, Dwivedi, Manickam, Ouyang, Torrelles, et al., 2014; Binjawadagi, Dwivedi, Manickam, Ouyang, Wu, et al., 2014). By using this delivery system, vaccine antigens entrapped in nanoparticles promote the process of antigen presenting cells (APCs) taking up the antigens. Therefore, the CMI response can be increased significantly (Heit, Schmitz, Haas, Busch, & Wagner, 2007; Schliehe et al., 2011; Smith, Simon, & Baker, 2013). In summary, novel adjuvants in combination with advanced antigen delivery system may enhance the efficacy of KV vaccines. However, they need to be pursued further to improve their heterologous efficacy and cost-effectiveness before considered for commercialization.

2.4 Subunit, DNA, and Virus-Vectored PRRSV Vaccines

Aside from MLV and KV, other approaches have been tried, such as subunit vaccines, DNA vaccines or virus-vector based vaccines. Unfortunately, most of these attempts cannot fulfill the expectation of an ideal PRRSV vaccine, and none of them can provide better protection than PRRS MLVs on the market. For example, virus-vectored vaccine systems using baculovirus (Binjawadagi et al., 2016), adenovirus (Cai et al., 2010; W. Jiang et al., 2008), fowlpox virus (Shen et al., 2007; Q. Zheng et al., 2007) and pseudorabies virus (Y. Jiang et al., 2007; Qiu et al., 2005), incorporating several PRRSV proteins including GP5, M, and/or GP3, have been studied and evaluated against PRRSV. In some of the studies, animals vaccinated with virus-vectored vaccines could develop virus-specific neutralizing antibodies and cell mediated immune response (Cai et al., 2010; W. Jiang et al., 2008). Some of these vaccines could provide protection against homologous virus

challenge, whereas none of them can protect the animals against heterologous virus challenge (Y. Jiang et al., 2007; Qiu et al., 2005). In summary, new approaches to develop PRRSV vaccines still need to be further studied before practical administration.

2.5 Application of reverse genetics in PRRSV vaccine development

2.5.1 Infectious PRRSV cDNA clones

Reverse genetics is an approach to discover functions or phenotypes of a gene sequence, which is opposite to the study of the genetic basis of a phenotype in classic genetics. The first reverse genetics system was constructed for poliovirus, a positive-strand RNA virus in 1981 (Racaniello & Baltimore, 1981a, 1981b). Over the past three decades, the reverse genetics systems have been widely used for many RNA viruses, and assisted our understanding in many aspects of virology, including viral pathogenesis, viral replication, virus-host interactions, and vaccine development. Reverse genetics has been used in PRRSV research. As PRRSV is an RNA virus, reverse transcription is needed to generate its cDNA for genetic engineering. Full-length cDNA of the PRRSV genome is cloned into a vector for genetic manipulation and virus recovery. Transcripts of the cDNA would initiate replication once introduced into permissible cells. Such cDNA clones are known as infectious cDNA clones.

Infectious clones have been constructed for at least 21 PRRSV strains, and this number is still increasing (Ellingson et al., 2010; Fang et al., 2008; Fang et al., 2006; J. Han, Liu, Wang, & Faaberg, 2007; M. Han & Yoo, 2014a; Kwon, Ansari, Osorio, & Pattnaik, 2006; Kwon et al., 2008; Z. Ma et al., 2017; Meulenberg et al., 1998; Ni et al., 2011; Nielsen et al., 2003; Pei et al., 2009; Truong et al., 2004; Y. Wang et al., 2008; Yoo, Welch, Lee, & Calvert, 2004). The first PRRSV cDNA infectious clone is generated from Lelystad virus, the prototype of *PRRSV-1*, by inserting the virus genome cDNA sequence into a plasmid with T7

promoter in 1998 (Meulenberg et al., 1998). Five years later, the infectious clone of VR-2332, the prototype of *PRRSV-2* was reported (Nielsen et al., 2003).

The reverse genetics technology allows for alteration of the cDNA of PRRSV genome and generation of mutant viruses to examine viral virulence traits, attenuate virulent strains and promote vaccine development. The full-length infectious cDNA clones have enabled understanding of the molecular basis of virulence and attenuation of PRRSV.

2.5.2 Reverse Genetics-Based Chimeric PRRSV Strains

An infectious cDNA clone of Prime Pac, an attenuated vaccine strain was constructed by replacing the genomic fragments of FL12, a virulent strain, with the corresponding regions from Prime Pac (Kwon et al., 2006). The virus rescued from the Prime Pac cDNA clone had similar growth properties and *in vivo* phenotypes in a sow PRRSV model to its parental virus. Infectious chimeric cDNA clones were constructed from different combinations of the vaccine strain MLV and a virulent strain MN184 (Ellingson et al., 2010) (Y. Wang et al., 2008). The chimeric viruses were tested in a respiratory challenge model in young pigs. The exchange of ORF5-6 region of strain MN184 can protect against challenge with another strain. The reverse genetic based chimeric PRRSV infectious clones can not only improve the PRRSV MLV vaccine efficacy but also be a good tool to study the viral virulence factors. With similar methods, Kwon et al. constructed a series of chimeric viruses by using virulent strain FL12 as the backbone to swap specific genes from an attenuated vaccine strain (Kwon et al., 2008). Compare to parental virus inoculation group, pigs inoculated with the chimeric virus containing nsp3-8 region from the vaccine strain showed a significantly improved piglet survival rate, which therefore suggests that nsp3-nsp8 region is crucial for the virus virulence.

2.5.3 PRRSV as a vector in marker vaccine development

Based on the reverse genetics technology and characterization of PRRSV genome, PRRSV infectious clone has been used for marker vaccine development. There are two types of markers employed in the marker vaccine. The first type is DIVA (Differentiating Infected from Vaccinated Animals) vaccines, also referred as negatively marked vaccines, which are generated by deletion of a part of genomic sequence. These epitope sequences present in field virus strain but are absent in the vaccine strain. Therefore, the DIVA assays can be utilized to differentiate vaccinated animals from naturally infected animals by detecting the serum antibody response to the deleted genetic marker. The other type is positively marked vaccines, also referred as compliance marked vaccine, which is created by insertion of foreign antigen sequences. Contrary to the DIVA vaccines, positively marked vaccines elicit production of marker antibodies in vaccinated animals, which indicates that vaccine is indeed administered to a given animal.

Several research groups have generated marker vaccine candidates for PRRSV (Groot Bramel-Verheije, Rottier, & Meulenberg, 2000; Lin et al., 2012; Ni et al., 2011; Pei et al., 2009; Welch et al., 2004). The majority of them choose the green fluorescent protein (GFP) as a marker gene for insertion into PRRSV infectious clone, since GFP expression can be readily observed in live cells under a fluorescent microscope. Several groups inserted the GFP into the central region of the nsp2 sequence (de Lima et al., 2008; Fang et al., 2008; Fang et al., 2006; Lin et al., 2012). However, there is stability issue for these marker viruses. Fortunately, another group uses a new approach to insert the GFP gene into PRRSV infectious clone (Pei et al., 2009). An additional transcription unit with a dedicated subgenomic RNA was generated by insertion of GFP gene between ORF1b and ORF2a, followed by a copy of TRS6 to drive ORF2a/b transcription. The recovered chimeric virus

was subjected to serial passages *in vitro*, and it was stable for at least 37 passages (Pei et al., 2009).

Chapter 3: Interplay between Interferons and PRRSV

3.1 Interferons and production

3.1.1 Introduction

Host innate immune response is the front line of host defense, which plays a key role against viral infection. Interferons (IFN) are major components of the innate immunity and have diverse biological functions including antiviral activity, antiproliferative activity, stimulation of T cell cytotoxic activity and activation of adaptive immune response (Pestka, 2007). It was discovered in 1957 by Isaacs and Lindenmann when studying the phenomenon of influenza virus interference (Isaacs & Lindenmann, 1957). However, the first purified interferon was reported 20 years later (Friesen et al., 1981). Since then, the interferon chemical, biological, and immunological studies have been conducted.

There are three types of interferons. Type I IFNs include IFN-α, IFN-β, IFN-ε, IFN-κ, and IFN-ω in humans (Fensterl & Sen, 2009; Pestka, Krause, & Walter, 2004; Uze, Schreiber, Piehler, & Pellegrini, 2007). In swine, type I IFNs include seven subclasses, which are IFN-α, IFN-αω, IFN-β, IFN-ε, IFN-κ, IFN-δ, and IFN-ω (Sang, Rowland, & Blecha, 2011). Almost all cell types are capable of producing IFN-α/β, however, plasmacytoid dendritic cells (pDC) are considered to be the major source of IFN-α production during viral infection (Y. J. Liu, 2005; Siegal et al., 1999). Type I IFNs are critical to the innate immunity against viral infections and play an important role in the control of the adaptive immune response (Gonzalez-Navajas, Lee, David, & Raz, 2012; Takaoka & Yanai, 2006).

Unlike type I IFNs, IFN-γ is the sole member of type II interferon. IFN-γ is mainly produced by activated T cells and natural killer cells. Some studies showed that with specific stimulation, macrophages and monocytes can also produce IFN-γ (Darwich et al., 2009;

Kraaij et al., 2014). IFN-γ, a 17kDa cytokine, exerts its function by forming an anti-parallel homodimer, which engages the heterodimeric IFN-γ receptor (IFNGR) (Fensterl & Sen, 2009). Initially, IFN-γ was described as an antiviral factor. However, with the identification of IFNGR expressing nearly on all cell types, IFN-γ has been known to play significant roles in establishing cell-mediated immune response (Teixeira, Fonseca, Barboza, & Viola, 2005); activating macrophages and inducing expression of class II major histocompatibility complex (MHC) molecules (Giroux, Schmidt, & Descoteaux, 2003; Martinez & Gordon, 2014); promoting NK cell activity (Aquino-Lopez, Senyukov, Vlasic, Kleinerman, & Lee, 2017; Jaczewska et al., 2014).

Type III IFNs are newly classified to interferon family and consist of four members: IFN-λ1, IFN-λ2, IFN-λ3 (also termed as interleukin (IL)-29, IL-28A, and IL28B, respectively) and IFN-λ4 (Kotenko et al., 2003; O'Brien, Prokunina-Olsson, & Donnelly, 2014). Although type III IFNs termed as "interferon-like cytokines" initially due to its structurally and functionally related to the type I IFNs, these cytokines have distinct chromosomal locations, gene sequences, and binding receptors. The genes for type III IFNs are located on chromosome 19, and the binding receptor complex for these three members are consisting of IL10R2 and IL28RA (Kotenko et al., 2003). Plasmacytoid dendritic cells (pDC) are considered to be the major source of IFN-λ production during viral infection (Reid et al., 2016; Yin et al., 2012). Enhancement of MHC class I antigen expression and increment of 2', 5'-oligoadenylate synthetase and MX1 expression are the functions of IFN-λ. Type III IFNs has since been demonstrated to contribute to resistance to virus infection in the host cells.

Considering the major roles by type I IFNs in the antiviral response and more studies on type I IFNs than other types, we focus on type I IFNs and discuss the PRRSV-mediated interference with their production and signaling.

3.1.2 The induction of Type I IFN synthesis

Pathogen-associated molecular patterns (PAMP) of viral infections can be recognized by host pattern recognition receptors (PRR), which are located on the host cell surface, in the cytoplasm, or endosomes. PRR recognize PAMP to sense the presence of the virus and subsequently induce the production of type I IFNs. Most of the virus PAMP are viral nucleic acids including single-stranded (ss)RNA, double-stranded (ds)RNA or dsDNA, or their synthetic analogs such as synthetic polyinosinic:polycytidylic acid (polyI:C). PRR of host cells include Toll-like receptors (TLRs) and RIG-I-like receptors (retinoic acid-inducible gene-I-like receptors, or RLRs) (Fig3.1) (Koyama, Ishii, Coban, & Akira, 2008). In the following section, IFNs induced by TLRs and RLRs are discussed in detail.

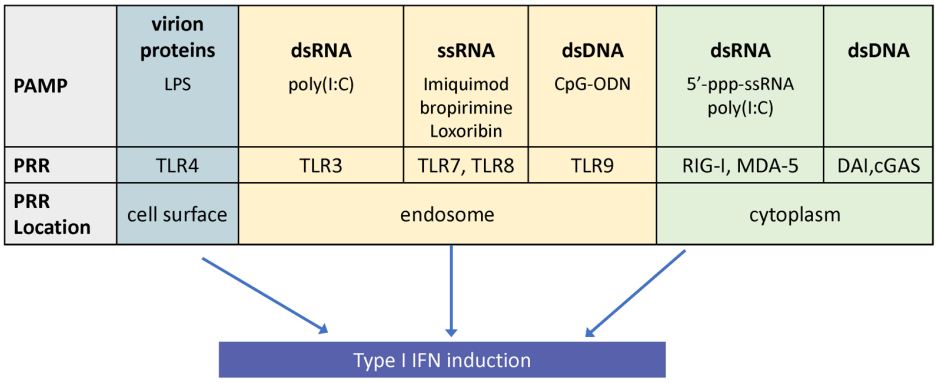

Fig.3. 1 Viral PAMP and host PRR

Activation of TLR signaling pathway leads to the production of type I IFNs. After TLR binds to the PAMP, the Toll-IL-1R (TIR) domain of TLR will be activated, followed by recruitment of adaptor protein through homophilic interaction. The adaptors include myeloid differentiation primary response gene 88 (MyD88), TIR-containing adaptor protein/ (TIRAP), TIR containing adaptor inducing interferon-β (TRIF) and TRIF-related adaptor molecule 2 (TRAM). Among the TLRs, TLR7, TLR8, and TLR9 use MyD88; TLR3 employs TRIF; and

TLR4 uses all the adaptors above to activate different signaling cascades (Fig3.2) (Kawai & Akira, 2010).

Once TLR7 and TLR8 activate MyD88, MyD88 interacts with IRAK1 and IRAK4

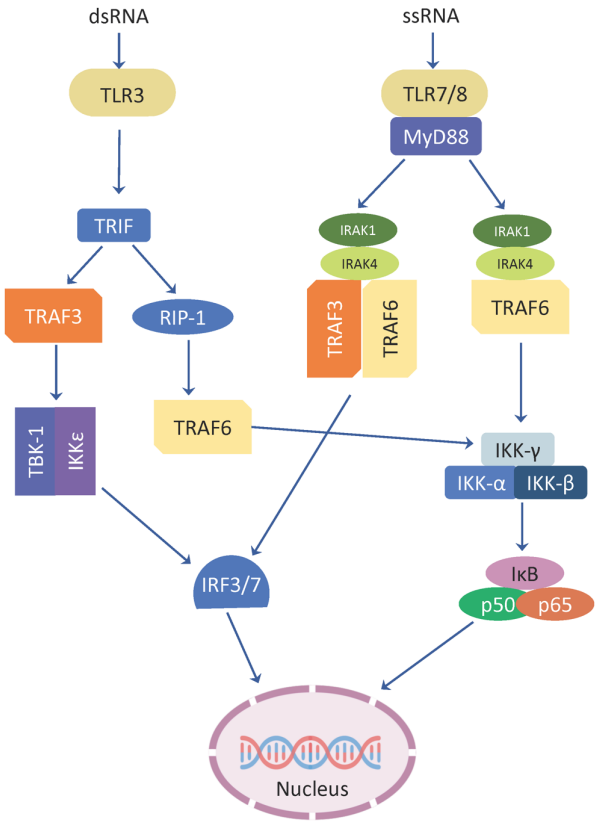

Fig.3. 2 TLR3, TLR7, TLR8 signaling pathways

(IL-1 receptor-associated kinase), which results in the phosphorylation of IRAK1 and IRAK4. Subsequently, phosphorylated IRAK1 and IRAK4 interact with TRAF6 or TRAF3/TRAF6 complex, leading to activation of subsequent signaling pathways. If IRAK1 and IRAK4 bind to TRAF6, the NF-κB signaling pathway is activated to induce the expression of inflammatory cytokines (Moynagh, 2005). If IRAK1 and IRAK4 bind to TRAF3/TRAF6 complex, interferon regulatory factor 7 (IRF7) is phosphorylated and translocated to the nucleus to activate the IFN induction (Kawai et al., 2004). Unlike TLR7,

TLR3 interacts directly with TRIF instead of MyD88. Activated TRIF can also transduce two distinct signaling pathways. If TRIF binds TRAF6, it activates NF-κB pathway and induces expression of inflammatory cytokines. If TRIF binds TBK1 and IKKε, IRF-3 and IRF-7 are phosphorylated and activated, thereby leading to the production of type I IFNs (Sharma et al., 2003).

Activation of RLR signaling pathway induces type I IFNs. RLRs currently have three members: RIG-I, melanoma differentiation-associated gene 5 (MDA5) and Laboratory of Genetics and Physiology 2 (LGP2) (Loo & Gale, 2011). All three PRRs are cytoplasmic proteins that sense viral RNAs derived from viral genomes or virus replication in the cytosol. However, LGP2 lacks CARD domain, and its effect on downstream signaling seems to depend on interaction with other RLRs (Bruns & Horvath, 2015). The adaptor protein for RLRs is mitochondrial antiviral signaling protein (MAVS, also known as IPS-1, VISA, and Cardif). The binding of viral RNA by RIG-I or MDA5 results in conformational change of the RLRs, leading to exposure of their N-terminal CARD domains. The RLR CARD domains interact with the MAVS CARD domain and eventually cause MAVS conformational change to form a prion-like aggregate (Hou et al., 2011). Activated MAVS recruits and activates IKKs and TBK1, leading to activation of IRF-3, IRF-7, and NF-kB, and consequently the production of type I IFNs and inflammatory cytokines.

3.2 Type I interferon-activated signaling

3.2.1 JAK/STAT pathway

All IFNs exert their effects by binding to specific cell-surface receptors to activate downstream signaling and induce expression of a large number of interferon-stimulated genes (ISGs), resulting in antiviral and other functions.

The Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway is the first characterized in the type I IFN response (Darnell, Kerr, & Stark, 1994). The components of the canonical type I IFN-induced signaling pathway are: 1) heterodimeric IFNα receptor (IFNAR), which consists of IFNAR1 and IFNAR2; 2) Receptor-associated kinases: Janus kinase 1 (Jak1) and tyrosine kinases 2 (Tyk2), which belong to a four-membered JAK family; 3) STAT1 and STAT2; and 4) IRF9 (Nan, Wu, & Zhang, 2017). JAK proteins are composed of a C-terminal catalytic domain and a kinase-like domain. There are seven members in STAT family: STAT1, 2, 3, 4, 5A, 5B and 6. All these STATs share the similar structure: an N-terminal domain (ND), a coiled-coil domain (CCD), a DNA-binding domain (DBD), a linker domain (LD), an Src homology 2 (SH2) domain, and a transactivation domain (TAD) (Steen & Gamero, 2013).

Upon the binding of IFNα or IFNβ to IFNAR heterodimeric receptor complex of IFNAR1 and IFNAR2, the type I IFN signaling transduction initiates. IFNAR1 and IFNAR2 subunits are associated with Tyk2 and JAK1, respectively. The IFN binding to the receptor activates Tyk2 and JAK1, which phosphorylate the latent cytoplasmic transcription factors STAT1 and STAT2 on a tyrosine residue (Levy & Darnell, 2002). Subsequently, the phosphorylated STAT1 and STAT2 form a heterodimer, followed by assembly with IRF9 to form a ternary complex termed IFN-stimulated gene factor 3 (ISGF3). The ISGF3 translocates to the nucleus and directly activates the transcription of ISGs by binding to the interferon-stimulated response element (ISRE) in the promoters of ISGs (Platanias, 2005).

In addition to the canonical type I IFN signaling pathway mediated by STAT1 and STAT2 heterodimer, other STATs complex could also mediate type I IFN signaling pathway, which could lead to the induction of several hundred distinct cytokines. For example, STAT1 homodimers bind to gamma-IFN-activated sequences (GASs) to induce pro-inflammatory genes. STAT3 homodimers indirectly suppress pro-inflammatory gene expression. These

complexes include STAT1, STAT3, STAT4, STAT5 and STAT6 homodimers, along with STAT1-STAT3, STAT1-STAT4, STAT1-STAT5, STAT2-STAT3 and STAT5-STAT6 heterodimers (Nan et al., 2017; Platanias, 2005).

3.2.2 IFN-stimulated genes and their functions

Over 1000 IFN-stimulated genes (ISGs) can be induced by type I IFNs signaling cascade (Hertzog, Forster, & Samarajiwa, 2011; Rusinova et al., 2013) (de Veer et al., 2001). The quantity of ISGs guarantees their functional diversity, which further confirms that IFNs play multiple roles in the host immune system.

Depending on the major functions, ISGs can be classified into two broad categories. The first category is restraining pathogens invading into the host cells. The antiviral effects may be achieved through several mechanisms, including inhibition of viral entry, blocking viral gene transcription, modification of post-transcriptional regulation, degradation of viral nucleic acids and alteration of cellular lipid metabolism (MacMicking, 2012). The activation of this group ISGs not only eliminate the virus in the infected cells but also help the neighboring cells to establish an "antiviral state". Moreover, one ISG protein is often in concert with the other ISGs to inhibit different viruses since they do not have virus-specificity. In this group of the ISGs, ISG15, ISG56, 2'-5' oligoadenylate synthetase (OAS) family, protein kinase R (PKR), myxovirus resistance A (MxA), and ribonuclease L (RNase L) are the most extensively studied ISGs (Y. Sun, Han, Kim, Calvert, & Yoo, 2012).

Another category of the ISGs, such as MyD88, STAT family, and NF-κB proteins, are related to host cell signaling. TLRs, RLRs and their adaptors all belong to this category. The induction of these proteins forms a type I IFN pathway amplification loop, resulting in increased IFN production and ISG expression (Kane et al., 2016) (Leaman et al., 2003).

3.3 PRRSV interference with IFN pathway

3.3.1 PRRSV interference with IFN synthesis

PRRSV elicits poor innate and adaptive immunity in infected pigs (Lunney et al., 2016; Xiao, Batista, Dee, Halbur, & Murtaugh, 2004). PRRSV infected pigs do not show typical type I IFN response, though PRRSV is highly sensitive to type I IFNs (Albina, Piriou, Hutet, Cariolet, & L'Hospitalier, 1998). Compared to other lung targeting pig viruses, like swine influenza virus (SIV) and porcine respiratory coronavirus (PRCV), PRRSV induces undetectable IFN-α level in lung (Van Reeth, Labarque, Nauwynck, & Pensaert, 1999). Similar inhibition of IFN synthesis is also shown in MARC-145 and PAM cells in vitro (Buddaert, Van Reeth, & Pensaert, 1998; Luo et al., 2008; Miller, Laegreid, Bono, Chitko-McKown, & Fox, 2004). All these studies suggest that PRRSV suppresses IFN production.

Since the discovery of IFN inhibition by PRRSV, the mechanism behind this phenomenon has been an interesting topic. PRRSV infection of PAM cells reduces TLR3 and TLR7 expression in comparison to Poly(I:C) control (Chaung, Chen, Hsieh, & Chung, 2010). And blocking TLR3 signaling can result in an increase of the PRRSV replication. Thus, TLR3-mediated IFN induction may be inhibited by PRRSV. All PRRSV nsps have been screened for type I IFN suppression in the IFN-β reporter assay. Nsp1α, nsp1β, nsp2, nsp4, and nsp11 inhibit the IFN-β promoter activity (O. Kim, Sun, Lai, Song, & Yoo, 2010). Further investigation demonstrates that nsp1 inhibits the IFN production through disruption enhanceosome assembly by degradation of its essential protein CREB (cyclic AMP responsive element-binding)-binding protein (CBP) in the nucleus (O. Kim et al., 2010). As discussed in chapter 1, the PL2 domain of nsp2 contains deubiquitinating activity and belongs to ovarian tumor (OTU) family. Therefore, nsp2 inhibits IFN production by blocking the

polyubiquitination process of phosphorylated IκB through the OTU domain (Z. Sun, Chen, Lawson, & Fang, 2010). The mechanism of nsp11 suppression of IFN-β production is related to its NendoU domain, which contains endoribonuclease activity. It was shown that overexpressed nsp11 could reduce MAVS expression, specifically degrading its mRNA (Y. Sun, H. Ke, et al., 2016). Therefore, this degradation leads to the block of MAVS-mediated downstream signaling and IFN production.

3.3.2 PRRSV interference with IFN-activated signaling

PRRSV interferes with not only RLR or TLR-activated IFN induction, but also IFN-activated downstream signaling. PRRSV inhibits the IFN-activated JAK/STAT signal transduction and ISG expression in both MARC-l45 and PAM cells (Patel et al., 2010; R. Wang, Nan, Yu, Yang, & Zhang, 2013; R. Wang, Y. Nan, Y. Yu, & Y. J. Zhang, 2013). PRRSV replication in MARC-145 cells suppresses JAK/STAT signaling stimulated by IFN-α (Patel et al., 2010). PRRSV infection of PAM cells also blocks IFN-activated JAK/STAT signaling, while the vaccine strain MLV has little effect (Patel et al., 2010). PRRSV nsp1β inhibits the JAK/STAT signaling via inducing the degradation of karyopherin-αl (KPNA1, also called importin-α5), which is known to mediate the nuclear import of STAT1 (R. Wang, Y. Nan, Y. Yu, & Y. J. Zhang, 2013). PRRSV infection of MARC-145 cells also reduces KPNA1 level. Besides nsp1β, other PRRSV proteins including nsp7, nsp12, GP3, and N have also been identified to inhibit IFN signaling (R. Wang, Y. Nan, Y. Yu, Z. Yang, et al., 2013). N inhibits IFN-activated STAT1 nuclear translocation, albeit less effective than nsp1β (R. Wang, Y. Nan, Y. Yu, Z. Yang, et al., 2013).

3.3.3 Atypical IFN-inducing PRRSV strains

Two atypical PRRSV strains that induce type I IFNs production have been identified by two independent groups (Nan et al., 2012; H. Sun, Pattnaik, Osorio, & Vu, 2016). A novel

strain, A2MC2, induces type I IFNs in both MARC-145 and PAM cells and the virus replication is needed for the IFN induction (Nan et al., 2012). Type 1 IFNs and ISGs are detected in A2MC2-infected cells. A2MC2 has no detectable inhibitory effect on the ability of IFN-alpha to induce an antiviral response in MARC-145 or PAM cells. Sequence analysis indicates that A2MC2 is closely related to VR-2332 and the MLV vaccine strain with an identity of 99.8% at the nucleotide level. A2MC2 induces earlier onset and significantly higher levels of PRRSV neutralizing antibodies than the MLV (R. Wang, Y. Xiao, et al., 2013).

Another IFN induction PRRSV strain is PRRSV-Con, which is recovered from the artificially synthesized infectious clone based on 59 wild-type PRRSV sequences (H. Sun et al., 2016). And the first 3.3kb of this virus is believed to be responsible for the IFN induction. As these two strains are able to induce IFNs, further exploration for vaccine development is warranted.

3.4 Objectives of this dissertation research

Despite substantial efforts to control PRRS, no production or vaccination regimen has demonstrated sustaining success. Further research on PRRSV virology, pathogenesis, immune responses and vaccines are much needed. In our laboratory, we discovered that PRRSV strain A2MC2 induces type I IFNs in the cultured cells, and virus replication is required for the IFN induction, whereas other PRRSV strains, including VR-2332, Ingelvac PRRS® MLV, and VR-2385, do not induce IFNs. Inoculation of pigs with A2MC2 virus led to earlier onset and higher virus-neutralizing antibodies than the MLV vaccine strain. The overall goal of this research is to characterize the A2MC2 strain, explore it for a better vaccine against PRRS, and identify the genetic source of A2MC2 in the interferon induction. In this study, there were three objectives: a). attenuate A2MC2 by serial passaging and

characterize the high-passage A2MC2; b). establish A2MC2 infectious cDNA clone and characterize the recovered virus, c). identify the A2MC2 genomic source for IFN induction by gene fragment swapping and site-directed mutagenesis.

To fulfill the three objectives, I conducted research and gathered data that are described in the following three chapters. Data in chapter 4 and 5 have been published in *Scientific Reports* and *Journal of General Virology*, respectively. Chapter 6 describes the current progress of the study and will be submitted for publication once it is completed. At the end of the dissertation, conclusion and perspectives are provided.

Chapter 4: Sustaining Interferon Induction in a High-Passage A2MC2 Virus

4.1-ABSTRACT

Porcine reproductive and respiratory syndrome virus (PRRSV) strain A2MC2 induces type I interferons in cultured cells. The objective of this study was to attenuate this strain by serial passaging in MARC-145 cells and assess its virulence and immunogenicity in pigs. The A2MC2 serially passaged 90 times (A2MC2-P90) retains the feature of interferon induction. The A2MC2-P90 replicates faster with a higher virus yield than wild type A2MC2 virus. Infection of primary pulmonary alveolar macrophages (PAMs) also induces interferons. Sequence analysis showed that the A2MC2-P90 has genomic nucleic acid identity of 99.8% to the wild type but has a deletion of 543 nucleotides in nsp2. The deletion occurred in passage 60. The A2MC2-P90 genome has a total of 35 nucleotide variations from the wild type, leading to 26 amino acid differences. Inoculation of three-week-old piglets showed that A2MC2-P90 is avirulent and elicits immune response. Compared with Ingelvac PRRS® MLV strain, A2MC2-P90 elicits higher virus neutralizing antibodies. The attenuated IFN-inducing A2MC2-P90 should be useful for development of an improved PRRSV vaccine.

4.2-Introduction

Porcine reproductive and respiratory syndrome (PRRS) is an economically important swine contagious disease across the world, which has resulted in an estimated $664 million loss per year to the swine industry in the United States alone (Holtkamp et al., 2013). The causative agent of the contagious disease is PRRS virus (PRRSV), a positive-sense single-

stranded RNA virus of the family Arteriviridae (Meulenberg, 2000) (Faaberg et al., 2012). The main target cells for PRRSV infection of pigs are pulmonary alveolar macrophages (PAMs) (Rossow et al., 1995). PRRSV propagation *in vitro* is generally conducted in MARC-145 cells, derived from MA-104, a kidney cell line of an African green monkey (H. S. Kim et al., 1993).

PRRSV appears to inhibit synthesis of type I interferons (IFNs) in pigs, whereas swine transmissible gastroenteritis virus (TGEV) and porcine respiratory coronavirus (PRCV) induce high levels of IFN-α (Albina, Carrat, & Charley, 1998; Buddaert et al., 1998; R. Wang & Zhang, 2014). PRRSV antagonizes induction of type I IFNs in both PAMs and MARC-145 cells as infection of the cells *in vitro* leads to very low level interferon-α (IFN-α) expression (Albina, Carrat, et al., 1998; S. M. Lee, Schommer, & Kleiboeker, 2004; Miller et al., 2004). Type I IFNs are critical to the innate immunity against viral infections and play an important role in activation of the adaptive immune response (Gonzalez-Navajas et al., 2012; Takaoka & Yanai, 2006). Adenovirus-mediated expression of IFN-α in pigs leads to reduction in disease signs when the animals were challenged with PRRSV (Brockmeier et al., 2009). Presence of the exogenous IFN-α at the time of PRRSV infection alters innate and adaptive immune responses by increasing IFN-γ secreting cells and changing cytokine profile in the lung 14 days post-infection (Brockmeier et al., 2012).

An atypical type 2 PRRSV strain A2MC2 induces synthesis of type I IFNs in the cultured cells and replication of A2MC2 is needed for the IFN induction, whereas PRRSV strains VR-2332, Ingelvac PRRS® MLV, NVSL 97-7895 and VR-2385 do not induce detectable IFNs (Nan et al., 2012). Experimental infection of pigs with the A2MC2 strain leads to earlier onset and higher levels of virus-neutralizing antibodies than the Ingelvac PRRS® MLV vaccine strain (R. Wang, Y. Xiao, et al., 2013). Virus neutralizing antibodies against PRRSV confer protection of pigs against challenge with virulent strain (Osorio et al.,

2002). Passive transfer of PRRSV-neutralizing antibodies in pregnant sows confers sterilizing immunity against reproductive failure induced by virulent strain challenge. Passive transfer with PRRSV-neutralizing antibodies to young weaned pigs blocks PRRSV viremia from challenge (Lopez et al., 2007)

Despite substantial efforts to control PRRS, no production or vaccination regimen has demonstrated sustaining success (Renukaradhya et al., 2015a, 2015b). This is likely in part due to biosecurity challenges and both antigenic and genomic variations among PRRSV isolates, allowing for frequent transmission between pig populations and persistence of the virus in infected pigs (Lunney et al., 2016). Attenuated live virus vaccines have been commercially available for over two decades, however, PRRS remains one of the top challenges for swine producers and outbreaks of PRRS. Therefore, an improved vaccine is needed to prevent and control PRRS.

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4.3-Materials and methods

4.3.1 Cells and viruses

MARC-145 (H. S. Kim et al., 1993) and Vero (ATCC CCL-81) cells were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum

(FBS). CRL-2843 (porcine macrophages, ATCC) were cultured in RPMI1640 medium supplemented with 10% FBS. Primary PAM cells were prepared from 4-8-week-old piglets and cultured in RPMI1640 medium supplemented with 10% FBS (Patel et al., 2008).

PRRSV strain A2MC2, VR-2385 and Ingelvac PRRS® MLV were propagated and titrated in MARC-145 cells. Virus yields were titrated by 10-fold serial dilutions and presented as the median tissue culture infectious dose (TCID50) (Y. J. Zhang et al., 2006). Newcastle disease virus (NDV) strain LaSota carrying the gene of green fluorescence protein (NDV-GFP) was propagated and titrated in Vero cells (S. H. Kim & Samal, 2010).

4.3.2 Interferon bioassay

Detection of presence of IFNs in culture supernatant from PRRSV-infected MARC-145 cells was done as previously described (Nan et al., 2012). Briefly, the supernatant was diluted in DMEM and used to treat Vero cells in 96-well plates overnight, followed by inoculation with NDV-GFP. Fluorescence microscopy was conducted 24 h after NDV inoculation to observe GFP-positive cells.

4.3.3 Immunofluorescence assay (IFA)

PRRSV propagation in MARC-145 cells was detected with IFA using an N-specific monoclonal antibody EF11 (Y. Zhang, Sharma, & Paul, 1998). The infected cells in 96-well plate were fixed and rinsed with phosphate-buffered saline (PBS) pH7.2 before addition of the EF11 antibody. DyLight™ 488 conjugated goat anti-mouse IgG (Rockland Immunochemicals Inc., Limerick, PA) was used to detect the EF11 binding to the N protein in the infected cells. Observation of N-positive cells was conducted under fluorescence microscopy.

4.3.4 Western blotting

Total proteins in cell lysate samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by transfer to nitrocellulose membrane (Y. J. Zhang et al., 2007). Blotting of the membrane with antibodies against RIG-I (Santa Cruz Biotechnology, Inc., Dallas, TX) and tubulin (Sigma-Aldrich Corp, St. Louis, MO) was conducted. Horseradish peroxidase-conjugated secondary antibodies (Rockland Immunochemicals Inc.) and chemiluminescence substrate were used to reveal specific reactions by the primary antibodies. Chemi-Doc Imaging System (Bio-Rad, Hercules, CA) was used to capture the luminescence signal.

4.3.5 RNA isolation, reverse transcription, and real-time PCR

Total RNA was isolated with the TRIzol Reagent (Life Technologies Corporation, Carlsbad, CA) following the manufacturer's instructions. Reverse transcription followed by PCR (RT-PCR) and real-time PCR were conducted to amplify target PRRSV sequences or to determine PRRSV RNA level (Patel et al., 2008; Patel, Stein, & Zhang, 2009). Detection of ribosomal protein L32 (RPL32) expression in the same sample was conducted to normalize the total input RNA. Primers of real-time PCR in this study previously described (Patel et al., 2010) and analysis of relative transcript levels were performed by normalization of RPL32 in comparison with controls.

For RT-PCR to determine possible deletions in the nsp2 region of the A2MC2 genome during high passages, primers 85nspF3 (5'CTCGACGAACTCAAAGACC3') and 32nsp2R2 (5'CTGCGGACGGAGCTGATGTGC3') were used to amplify the target fragment with Phusion Flash High-Fidelity PCR Master Mix (Fisher Scientific, Pittsburgh, PA).

4.3.6 Plaque assay

A plaque assay in MARC-145 cells was done to compare the growth property of A2MC2 high passage with the wild type virus (Nan et al., 2012). Briefly, PRRSV A2MC2 was diluted to 10 and 100 TCID50 per ml and added to the monolayer cells in 6-well plates at 1 ml per well. After 2 h incubation at 37°C, the inoculum was removed and 3 ml 0.5% agarose overlay containing the complete growth medium was added. The cells were stained at 72 h after incubation by addition of 2 ml neutral red mixture with agarose and observed for plaques after further overnight incubation.

4.3.7 Virus neutralization assay

Virus neutralization assay was performed on MARC-145 cells to determine PRRSV-neutralizing antibodies in pig serum samples (R. Wang, Y. Xiao, et al., 2013). VR-2332, the prototype of type 2 PRRSV with nucleic acid identity of 99.8% to A2MC2 (Nan et al., 2012), was used as target virus in the assay at 100 TCID50 for each reaction. The starting dilution of serum samples was 1:8, followed by 2-fold serial dilutions. IFA with N-specific monoclonal antibody EF11 was conducted 24 h after inoculation of the cells. Compared to serum samples from mock-infected pigs, the reciprocal of the highest serum dilution that reduced 50% PRRSV replication was counted as the VN titer.

4.3.8 Sequencing

RNA isolation from A2MC2 virions was done for reverse transcription, PCR amplification and DNA sequencing by chain-termination method using ABI Genetic Analyzer 3130 (ThermoFisher Scientific, Waltham, MA) (Nan et al., 2012). Sequence assembly and analysis was done with LaserGene Core Suite (DNASTAR Inc., Madison, WI). The cDNA sequence of the full-length A2MC2-P90 genome has been deposited in GenBank (accession number: KU318406).

4.3.9 Animal study

Two animal studies were conducted after approval by Institutional Animal Care and Use Committees (IACUC) of the University of Maryland and Iowa State University according to relevant guidelines and policies for the care and use of laboratory animals. The first animal study was to determine the virulence of the high passages of A2MC2 virus. Three-week-old PRRSV-negative piglets weighing from 3.2 to 7.5 kg were randomly divided into five groups with 4 pigs in each group. The piglets in groups 1 to 4 were inoculated with 1 ml of PRRSV strains A2MC2-P9, A2MC2-P75, A2MC2-P90, and Ingelvac PRRS® MLV, respectively, at 5 x 105 TCID50/ml via intranasal (I.N.) inoculation, while group 5 was mock-infected with PBS pH7.2. The I.N. inoculation was used as PRRSV transmits via respiratory route. The pigs in each group were humanely euthanized on day 14 post infection (DPI14) by pentobarbital overdose (FATAL-PLUS, Vortech Pharmaceuticals, LTD. Dearborn, MI). Visible macroscopic lung lesions and histopathology were scored and recorded as previously described (Halbur et al., 1995; Halbur et al., 1996). The level of interstitial pneumonia was scored ranging from 0 (absent) to 6 (severe diffuse interstitial pneumonia). Scoring of macroscopic and microscopic lung pathology was done in a treatment status-blinded fashion independently by two veterinary pathologists (TO, PGH). If results disagreed, they were combined and the average was used for further analysis.

The second animal study was conducted to assess the immunogenicity of high passages of A2MC2. Three-week-old PRRSV-negative piglets were randomly divided into four groups with 4 pigs in each group. The piglets in groups 1 to 4 were inoculated with 1 ml of PRRSV A2MC2-P9, A2MC2-P90, and Ingelvac PRRS® MLV, respectively, at 5 x 105 TCID50/ml via intramuscular (I.M.) inoculation, while group 4 was mock-infected with PBS pH7.2. The I.M. route is generally used for porcine vaccination. Blood samples were collected weekly. The pigs were euthanized on DPI48. To assess the antibody response

against PRRSV in the pigs, serum samples of DPI35 was tested with a commercial PRRSV ELISA kit (IDEXX PRRS X3 Ab Test; IDEXX Inc., Westbrook, MA, USA) according to the manufacturer's instructions. A sample-to-positive (S/P) ratio greater than 0.4 was considered positive.

4.3.10 Statistical analysis

Differences between treatment samples and control were assessed by the Student t-test. Differences between two groups for VN antibody titers of individual pigs were analyzed using analysis of variance (ANOVA). A two-tailed P-value of less than 0.05 was considered significant.

4.4-Results

4.4.1 Serial passaging of A2MC2 in MARC-145 cells

PRRSV strain A2MC2 was subjected to serial passaging in MARC-145 cells to minimize previously observed moderate virulence (R. Wang, Y. Xiao, et al., 2013). The

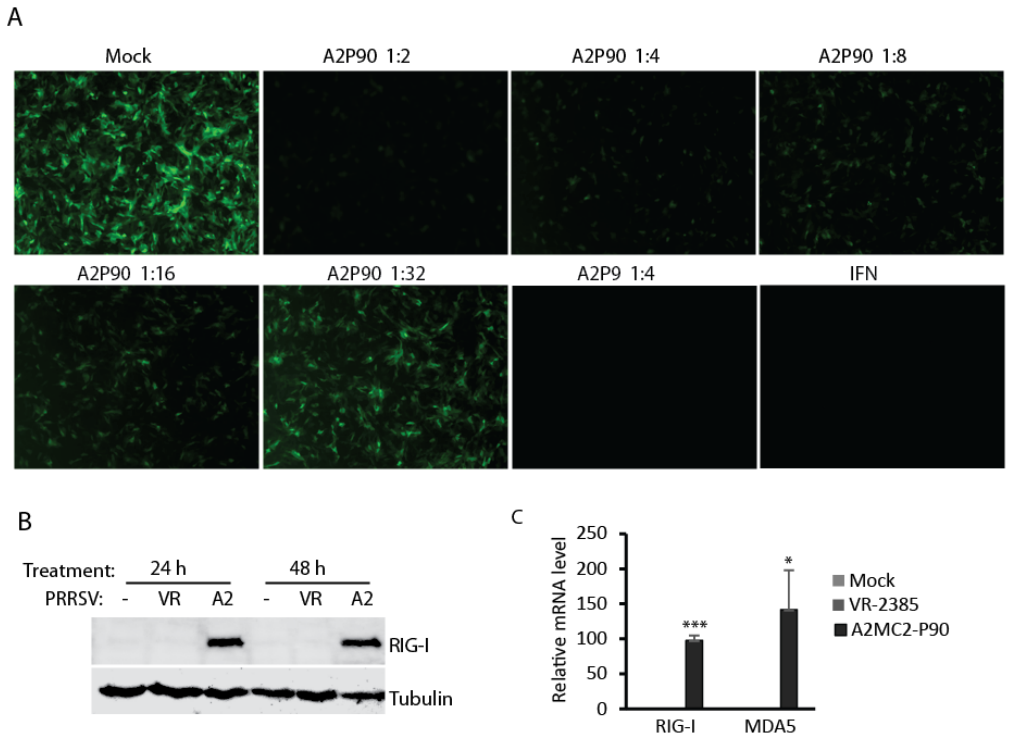

Fig.4. 1 A2MC2-P90 induces synthesis of interferons

A. Interferon bioassay in Vero cells. Dilutions of cell culture supernatant of MARC-145 cells infected with A2MC2-P90 (A2P90) or A2MC2-P9 (A2P9) were used to treat Vero cells. Treatment with 1000 U IFN-α was included as a control. At 12 h after the treatment, the Vero cells were inoculated with NDV-GFP. At 24 h post-inoculation of NDV, the cells were observed under fluorescence microscopy. B. Elevation of RIG-I in Vero cells treated with A2MC2-P90 supernatant. The Vero cells were treated with culture supernatant from MARC-145 cells infected with VR-2385 (VR) or A2MC2-P90 (A2) and harvested 24 and 48 h post treatment for immunoblotting. C. Treatment with A2MC2-P90 supernatant leads to elevation of RIG-I and MDA5 expression in Vero cells detected by real-time PCR. Relative levels of transcripts are shown in folds in comparison to treatment with supernatant from mock-infected MARC-145 cells. Significant difference from treatment with supernatant of mock-infected cells is denoted by "*", which indicates $P < 0.05$ and "***" $P < 0.001$.

A2MC2 virus was passaged in MARC-145 cells for 90 consecutive passages. For each passage, the cells were frozen and thawed three times when cytopathic effect (CPE) occurred over 50% of the cells. Virus samples were collected for each passage. IFN bioassay results showed that treatment of Vero cells with the supernatant of A2MC2 passage 90 (A2MC2-

P90) even at a dilution of 1 to 16 inhibited the replication of NDV-GFP (Fig. 4.1A). This suggested that A2MC2-P90 retains the capacity of IFN induction of the wild type A2MC2.

To confirm IFN induction by strain A2MC2-P90, we performed immunoblotting detection of RIG-I, which is upregulated by type I IFNs (Indraccolo et al., 2007). Result showed that the RIG-I level increased in the Vero cells treated with culture supernatant from A2MC2-P90-infected MARC-145 cells, whereas no change in RIG-I level was observed in cells treated with supernatant from VR-2385-infected cells (Fig.4.1B). Compared with treatment of mock-infected cells, the treatment with supernatant from A2MC2-P90 infected cells led to 97.8 and 141.5-fold higher RIG-I and MDA5 transcript levels, respectively, whereas treatment with supernatant from VR-2385-infected cells had only 0.9 and 0.7-fold of RIG-I and MDA5 transcript levels, respectively (Fig. 4.1C).

4.4.2 Growth property determination and plaque assay

A2MC2-P90 was tested in MARC-145 cells for growth properties, including a multi-step growth curve and a plaque assay as described previously (Nan et al., 2012). The virus yields reached its peak at 72 hours post inoculation (hpi) and were 7.7, 7.9 and 8.0 Log10/ml in TCID50 for the cells with inoculum at an MOI (multiplicity of infection) of 0.01, 0.1 and 1, respectively (Fig. 4.2A). The virus yields for the cells inoculated with A2MC2-P90 at an MOI of 0.1 and harvested at 24, 48, 72, 96 and 120 hpi were 6.7, 7.3, 7.8, 7.6 and 7.2 Log10/ml, respectively, which were significantly higher than the yields from the cells inoculated with wild type A2MC2 at an MOI of 0.1: 5.0, 5.7, 5.5, 4.6, and 4.1, respectively. Similar trends and titers of virus yields were observed for the samples harvested from the cells with the three different amounts of A2MC2-P90 inoculation.

A plaque assay was done for A2MC2-P90 and compared with wild type A2MC2. The plaque sizes of A2MC2-P90 were 8-10 mm in diameter, much bigger than the wild type

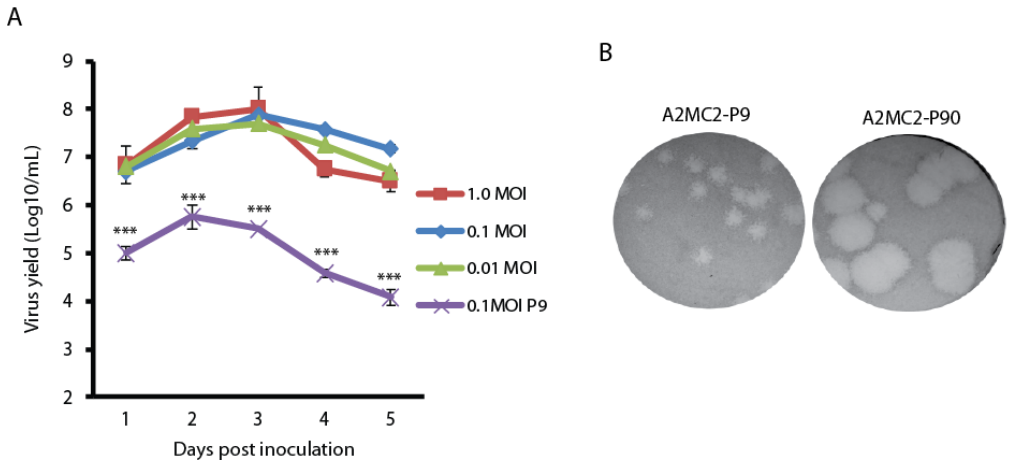

Fig.4. 2 Growth properties of A2MC2-P90 in MARC-145 cells

A. Multi-step growth curve of A2MC2-P90 in MARC-145 cells. The cells were inoculated with A2MC2 virus at a multiplicity of infection of 0.01, 0.1 or 1. Inoculation of the cells with A2MC2-P9 at an MOI of 0.1 (0.1 MOI P9) was included as a control. Virus yields at different time points after inoculation were titrated by an immunofluorescence assay. Error bars represent variation of three repeated experiments. B. Plaque assay in MARC-145 cells. The cells were infected with diluted A2MC2-P9 and A2MC2-P90 and overlaid with agarose. Plaques were observed 4 days post-infection and photographed for comparison.

A2MC2 plaques, which were generally 3-4 mm in diameter (Fig. 4.2B). The larger size of plaques produced by A2MC2-P90 is consistent with its higher yield compared to the wild type virus.

4.4.3 Sequencing of cDNA of A2MC2-P90 genomic RNA

The virions of A2MC2-P90 were used for RNA isolation and RT-PCR. DNA sequencing of the PCR products was done and compared with sequences of wild type A2MC2 virus. Variations of nucleotides and derived amino acids in comparison with wild type A2MC2, VR-2332 and Ingelvac PRRS® MLV were identified (Supplemental Table 4.1). The locations of the differences in genomic RNA are illustrated in Fig. 4.3. The A2MC2-P90 genome has a deletion of 543 nucleotides (2994-3536) in ORF1a in comparison with wild

type A2MC2 virus, leading to a deletion of 181 amino acid residues in hypervariable region of nsp2. Moreover, compared to the wild type, the A2MC2-P90 has 35 nucleotide mutations, among which 26 are non-synonymous, leading to 26 amino acid changes (Table 4.1). Interestingly, among the 15 unique nucleotides in A2MC2 genome compared with Ingelvac

Fig.4. 3 Illustration of sequence variation of A2MC2-P90 in comparison to VR-2332, Ingelvac PRRS® MLV and WT A2MC2

Illustration of sequence variation of A2MC2-P90 (GenBank accession number: KU318406) in comparison to VR-2332 (GenBank accession number: U87392), Ingelvac PRRS® MLV (GenBank accession number: AF066183) and wild type A2MC2 (GenBank accession number: JQ087873). The top line indicates the genomic sequence of VR-2332 and the numbers above the line indicate nucleotide positions in the genome. The nucleotide variations in comparison with VR-2332 are indicated by vertical bars. The wide bars indicate the unique nucleotides identified earlier for A2MC2 in comparison with VR-2332 and MLV. Deletion (Del) in A2MC2-P90 from nt2994 to 3536 is indicated. For a list of non-synonymous nucleotide variations in A2MC2-P90 genome compared to wild type A2MC2, see Table 1. For a full list of all nucleotide variations in A2MC2-P90 genome compared to wild type A2MC2, VR-2332 and Ingelvac PRRS® MLV, see supplemental Table 1

PRRS® MLV and VR-2332 (Nan et al., 2012), 14 remained the same in the A2MC2-P90 genome (Fig. 4.3). As a result, 5 of the 6 unique amino acid residues of A2MC2 compared to the MLV and VR-2332 remained the same in A2MC2-P90. The conserved five nucleotides leading to unique amino acids in A2MC2 are nt7621, 9655, 12012, 12972 and 12975 and the five unique residues are Ser20 in nsp8/9, Leu13 in nsp10, Gly135 in nsp12, and Val93 and Val94 in GP3. This result indicates that the 14 nucleotides in A2MC2 are highly conserved

and sustained during the 90 serial passages. It also suggests that these 14 nucleotides or their related RNA structures might correlate with the feature of A2MC2 in IFN induction.

4.4.4 The deletion in ORF1a occurs in passage 60 of A2MC2

Having noticed the deletion in nsp2 of A2MC2-P90, we wondered at which passage

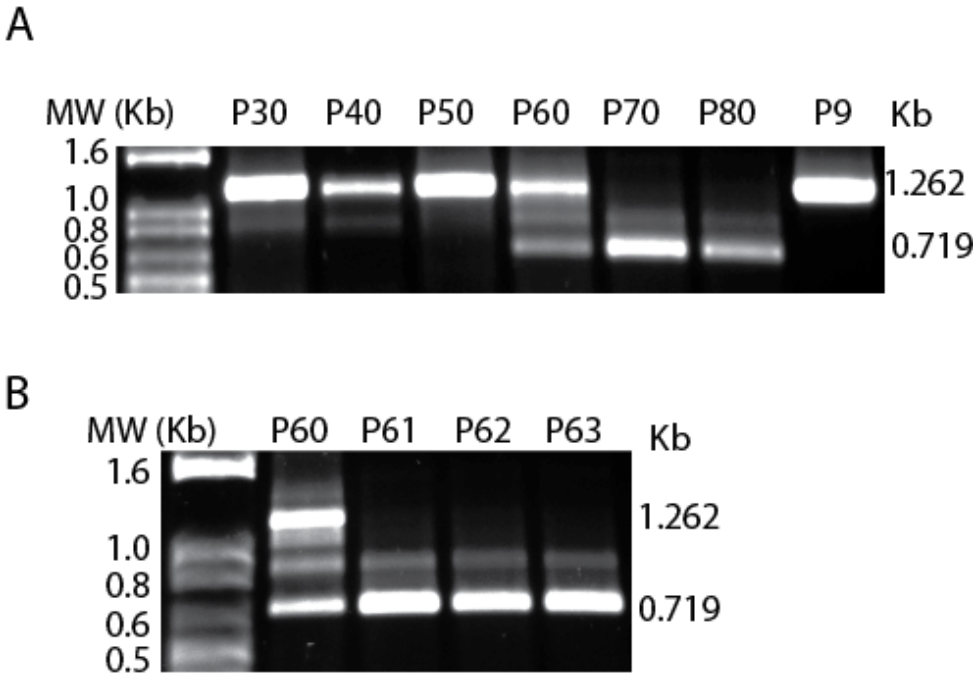

Fig.4. 4 Identification of the initial A2MC2 passage that carries the nsp2 deletion in genome

A. PCR detection of the nsp2 deletion in A2MC2 passages. The passages P30 to P80 were tested. Wild type A2MC2 (P9) was included as a control. The PCR product from genome without deletion is 1.262 kb and it is 0.719 kb from genome with the nsp2 deletion. B. Identification of the initial A2MC2 passage that has the nsp2 deletion. The passages P60 to P63 were tested.

the deletion occurred. RT-PCR was conducted to amplify a fragment spanning the deletion area. The expected sizes of the PCR product are 719 bp for A2MC2-P90 and 1262 bp for wild type A2MC2. The PCR products for passage 30, 40 and 50 are the same size as wild type A2MC2, while the sizes of passage 70 and 80 are the same as A2MC2-P90 (Fig. 4.4A).

There were two main bands in PCR products of passage 60. Therefore, the deletion likely occurred around passage 60. PCR amplification of passage 60 through 63 showed that the size shift from 1262 bp to 719 bp likely occurred from passage 60 to 61 (Fig. 4.4B). The size shift suggests that mutant virus with the spontaneous deletion appeared to become the main virus quickly.

4.4.5 A2MC2-P90 induces interferons in PAM cells

PAMs are the major target cells for PRRSV infection *in vivo* (Rossow et al., 1995).

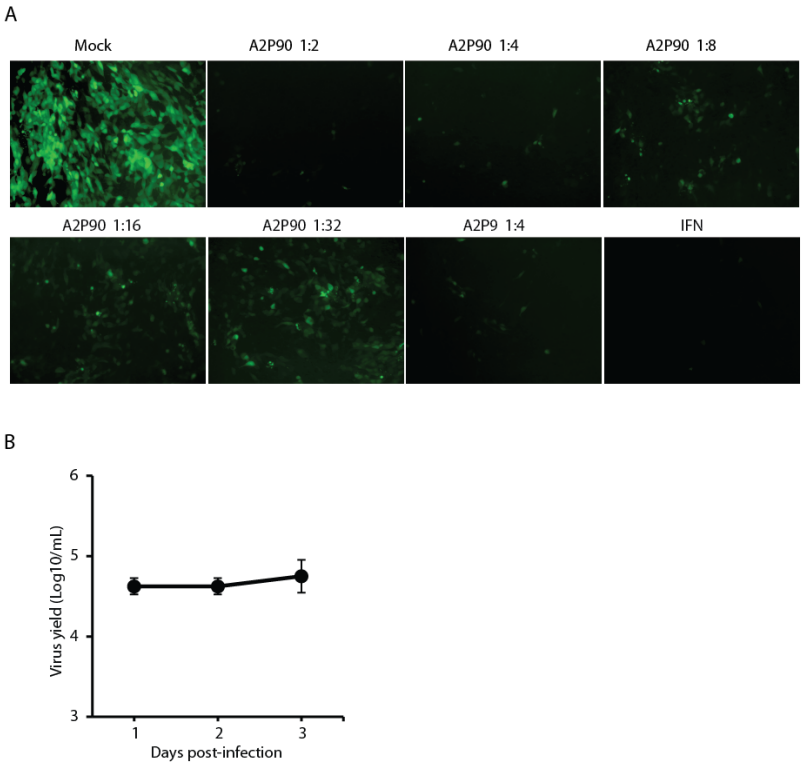

Fig.4. 5 A2MC2-P90 induces IFN synthesis in PAM cells

A. IFN bioassay in CRL2843 cells, in comparison with wild type A2MC2. IFN-α was included as a positive control. B. Multi-step growth curve in PAM cells. The cells were inoculated with A2MC2-P90 at an MOI of 0.5. Virus yields were titrated on MARC-145 cells. Error bars represent variation of three repeated experiments.

To determine if A2MC2-P90 can infect PAMs and induce interferons, we inoculated PAMs

with the high-passage virus at an MOI of 3. Wild type A2MC2 was included as a control. Interferon bioassay was conducted on CRL-2843 cells, immortalized porcine macrophages that are not susceptible to PRRSV, as reported (Nan et al., 2012). Results showed that the supernatant of the A2MC2-P90 infected PAMs induced an antiviral effect in CRL-2843 cells by blocking the replication of NDV-GFP (Fig. 4.5A). The supernatant dilutions at 1 to 32 still induced inhibition of NDV-GFP.

A multi-step growth curve was also done to determine the propagation of A2MC2-P90 in PAM cells. The virus yields of PAMs inoculated at an MOI of 0.5 were 4.6, 4.6 and 4.8 Log10/ml 24, 48 and 72 hpi, respectively (Fig. 4.5B). A2MC2-P90 appears to be able to replicate in the primary cells though at low level.

4.4.6 Non-virulence of A2MC2-P90 *in vivo*

The objective of the serial passaging of A2MC2 was to attenuate the strain. To determine the degree of attenuation of the A2MC2-P90, we conducted an animal study by inoculating 3-week-old PRRSV-negative piglets. A2MC2-P9, A2MC2-P75, and Ingelvac PRRS® MLV were included in the animal study for control. Compared with pigs inoculated with A2MC2-P9, the pigs infected with A2MC2-P75 and A2MC2-P90 had significantly lower macroscopic lung lesion scores, like the MLV-infected pigs and the mock-infected control in magnitude (Fig. 4.6A).

Microscopically, the interstitial pneumonia scores of the pigs infected with A2MC2-P75 and A2MC2-P90 were significantly lower than pigs infected with A2MC2-P9 (Fig. 4.6B). Both A2MC2-P75 and A2MC2-P90 groups had pathology scores similar to the MLV-

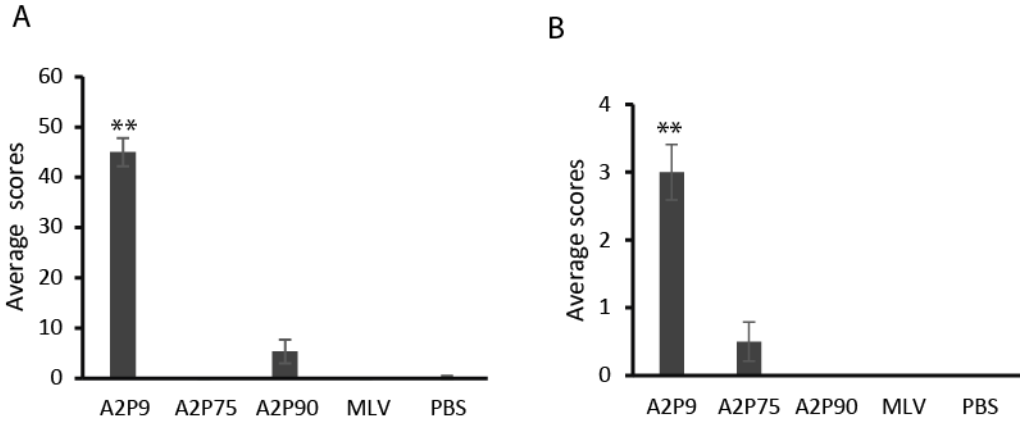

Fig.4. 6 Lung lesions in pigs infected with A2MC2-P9, A2MC2-P75, A2MC2-P90 and MLV at 14 DPI

Four pigs from each group were necropsied. Mock-infected pigs (PBS) were included as controls. A. Average macroscopic lung lesion scores. Error bars represent standard errors of the scores among the four pigs in each group. A2: A2MC2. B. Average microscopic lung lesion scores. Significant differences between the group of A2MC2-P9-infected pigs and each of the rest groups are denoted by "**", which indicates $P < 0.01$.

infected or mock-infected pigs. All the groups except for A2MC2-P9 had no significant difference from the mock-infected control group. These results suggest that under the study conditions, A2MC2-P75 and A2MC2-P90 are avirulent in pigs, like the MLV strain.

4.4.7 A2MC2-P90 elicits higher level virus-neutralizing antibodies than the MLV strain

To assess the immunogenicity of A2MC2-P90, we conducted an animal study by

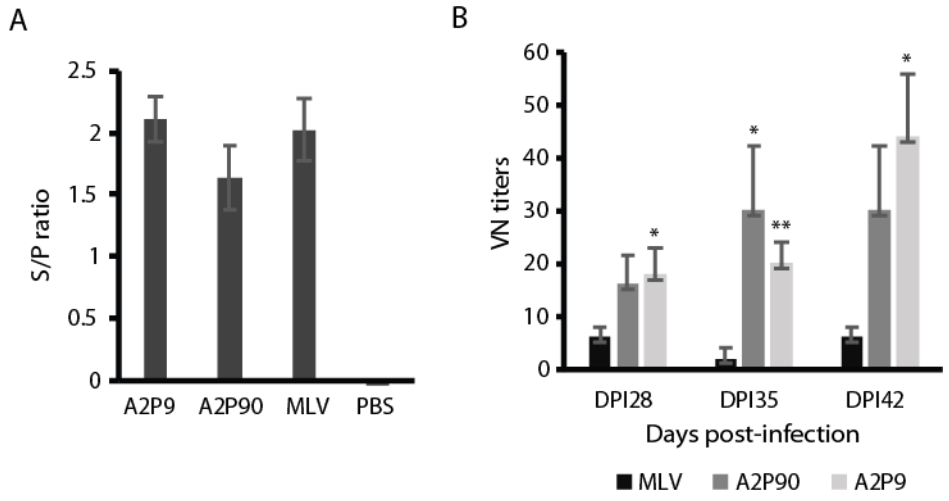

Fig.4. 7 Serological testing of serum samples from pig studies

Four pigs from each group were infected. Mock-infected pigs (PBS) were included as controls. A. ELISA of PRRSV antibodies in serum samples of 35 days post-infection (DPI). The S/P ratio above 0.4 is considered positive. Error bars represent standard errors of the scores among the four pigs in each group. A2: A2MC2. B. Virus-neutralization assay against PRRSV VR-2332. The virus neutralization (VN) titers are shown as reciprocal of serum dilutions shown VN activity. Significant differences between the group of MLV-infected pigs are denoted by * and **, which indicate $P < 0.05$ and $P < 0.01$, respectively

inoculating 3-week-old PRRSV-negative piglets with A2MC2-P9, A2MC2-P90, and Ingelvac PRRS® MLV viruses. ELISA result showed that all pigs that were inoculated with the PRRSV viruses developed specific antibodies by DPI35, whereas the pigs of mock-infected group were all negative (Fig. 4.7A). The average S/P ratios for the virus-infected groups were over 1.6 for all groups without much difference.

Virus-neutralizing antibody assay was conducted for serum samples of DPI28 to DPI42 based on our previous study showing the appearance of VN antibody at DPI28 (R.

Wang, Y. Xiao, et al., 2013). All pigs in groups of A2MC2-P9 and A2MC2-P90, and three of four in MLV group had detectable VN antibodies at DPI28 (Fig. 4.7B). The average VN titers of the A2MC2-P90 and A2MC2-P9 groups were higher than those in MLV group for DPI28, DPI35 and DPI42 samples. The results show that similar to the wild type A2MC2 virus, A2MC2-P90 elicits higher VN antibodies than the MLV strain in this study.

4.5 Discussion

Interferon induction is a unique characteristic of PRRSV strain A2MC2 as PRRSV strains generally antagonize interferon synthesis (Nan et al., 2012; R. Wang & Zhang, 2014). Considering the importance of interferons in activating the adaptive immune response, this feature may be desired in vaccine development against PRRS. Remarkably, the capability of strain A2MC2 to induce interferons is sustained after 90 serial passages in MARC-145 cells. Like the wild type virus, the high-passage virus also induces interferons in PAM cells. Moreover, the A2MC2-P90 is attenuated shown by its non-virulence in pigs and elicits higher virus-neutralizing antibodies.

Sequence comparison showed that 14 of 15 unique nucleotides of A2MC2 in comparison with both VR-2332 and MLV (Nan et al., 2012) are conserved in the A2MC2-P90 genome. Among the six unique amino acid residues, five are identical in both wild type A2MC2 and A2MC2-P90 and are located in nsp8/9, nsp10, nsp12 and GP3. These residues do not correlate with virulence as the A2MC2-P90 is avirulent. The nsp8 has unknown functions. The nsp9 is the RNA-dependent RNA polymerase; nsp10 is the helicase and GP3 is a structural glycoprotein (de Lima et al., 2009; Fang & Snijder, 2010). The nsp12 induces STAT1 phosphorylation at Ser727 and may contribute to expression of inflammatory genes (Y. Yu et al., 2013). None of these genes are known to be involved in the PRRSV antagonizing feature of interferon induction. It is not known whether these proteins or the

nucleotide-related RNA structures play a role in inducing interferon induction. Further studies are needed to address the question.

Compared to the wild type virus, A2MC2-P90 genome has a deletion of 543 nucleotides in nsp2. The deletion likely occurred around passage 60 as there were both 719 and 1262 bp bands in the PCR of passage 60, but the 1262 bp band disappeared in passage 61. It appears that the virus with deletion grows faster and quickly became the main virus in passage 61. A2MC2-P90 propagates faster with higher yield in MARC-145 cells than the wild type A2MC2. Our results are consistent with an earlier report that the spontaneous nsp2 deletion contributes to the faster virus propagation in a different strain *in vitro* (Ni et al., 2011). However, the nsp2 deletion does not have an effect on PRRSV virulence for that strain *in vivo*.

In addition, A2MC2-P90 has 35 nucleotide differences compared to the wild type virus, scattered throughout the genome. Nsp9 and nsp10 were found to contribute to fatal virulence of high pathogenic PRRSV strains in China (Y. Li et al., 2014). There are one and three different amino acid residues in nsp9 and nsp10, respectively, between A2MC2-P90 and the wild type virus (Table 1). Compared with moderate virulent VR-2332 and its derived avirulent MLV, these residues in the wild type A2MC2 are not unique (supplemental Table 1), suggesting that their correlation with virulence is unlikely. There are no unique synonymous mutations in nsp9 and nsp10 in A2MC2-P90. It is thus unknown which nucleotide mutations contribute to the attenuation of A2MC2, possibly a combined effect of the multiple mutations has to be considered. Among the 35 nucleotide mutations of A2MC2-P90 in comparison with the wild type A2MC2, only nt13011 is the same as in the MLV but different from VR-2332, leading to serine in both A2MC2-P90 and the MLV, and glycine in strains A2MC2 and VR-2332. The significance of this one amino acid variation between both

moderate virulent strains and their avirulent descendants is not known and may need to be investigated.

A2MC2-P90 virus replicates faster in MARC-145 cells by inducing larger plaques and having higher titer of virus yield. It appears that A2MC2-P90 virus is less sensitive to the interferons it induces, as it replicates well when the cells are inoculated at an MOI of 1, while the wild type replicates poorly at this amount of inoculum (Nan et al., 2012). This indicates that A2MC2-P90 virus has been adapted to the cells and may gain the ability to dampen the interferon-activated antiviral response.

The animal studies demonstrate that A2MC2-P90 is avirulent and elicits better adaptive immune response than the MLV strain. The ELISA result showed that all PRRSV-infected pigs were seroconverted by DPI35. The VN test result showed most infected pigs had detectable VN antibodies by DPI28. The pigs infected with A2MC2-P90 had higher VN titers than the MLV group. The results indicate that the high passage of A2MC2 carries similar immunogenicity as the wild type virus.

In conclusion, the attenuation of A2MC2 was accomplished by serial passaging in MARC-145 cells. The unique feature of interferon induction in both MARC-145 and PAM cells sustains the 90 serial passaging. A2MC2-P90 propagates more rigorously in MARC-145 cells than wild type A2MC2. A2MC2-P90 is avirulent in pigs. Sequence analysis shows A2MC2-P90 has a 543-nucleotide deletion in nsp2 and 35 nucleotide mutations throughout the genome in comparison to the wild type virus. A2MC2-P90 is avirulent and elicits higher VN antibodies than the Ingelvac PRRS® MLV strain. Further characterization of the attenuated virus is warranted for development of an improved vaccine against PRRS.

4.6 TABLES

Table 4. 1 List of non-synonymous nucleotide mutations and their derived amino acids in A2MC2-P90 genome compared to the wild type A2MC2[a]

Position[b]	Nucleotide[c]		Amino acid[d]		Protein[e]
	A2MC2-P90	A2MC2	A2MC2-P90	A2MC2	
1414	G	A	A	T	nsp2/TF/N
1568	G	A	G	E	nsp2/TF/N
3706	T	C	S	P	nsp2/TF/N
5369	C	T	T	I	nsp3
6520	A	G	T	A	nsp5
7168	A	G	I	V	nsp7a
7171	C	G	H	D	nsp7a
7606	A	G	I	V	nsp8/nsp9
9729	G	A	A	T	nsp10
10122	G	A	V	I	nsp10
11197	T	A	F	Y	nsp11
12361	G	A	V	M	GP2a
12613	G	A	V	I	GP2a
13011	A	G	S	G	GP3
13264	T	C	L	S	GP3
13367	G	A	G	S	GP4
13409	A	G	N	D	GP4
13475	A	G	I	V	GP4
13798	T	A	I	K	GP5
			D	E	GP5a
14026	T	G	V	G	GP5
14344	T	C	V	A	GP5
15219	G	A	A	T	N

a. GenBank accession numbers: A2MC2 (GenBank ID: JQ087873) and A2MC2-P90 (GenBank ID: KU318406). Some nucleotides locate in ORF overlap regions and result in different amino acids in the corresponding ORF.

b. Nucleotide positions are indicated on the left column based on A2MC2 genomic sequence.

c. Nucleotides at the indicated genomic sequence positions are listed.

d. Amino acids derived from the codon of indicated nucleotides are listed.

e. PRRSV viral proteins corresponding to the amino acids derived from the codon of indicated nucleotide positions are listed on the right column.

Supplemental Table 4. 1 List of nucleotide and amino acid variations in A2MC2-P90 in comparison with A2MC2, VR-2332, and MLV strains[a]

	Nucleotide[c]				Amino acid[d]				
Position[b]	A2MC2-P90	A2MC2	MLV	VR-2332	A2MC2-P90	A2MC2	MLV	VR-2332	Protein[e]
102	G	A	A	A	-	-	-	-	-[f]
784	G	G	A	G	V	V	I	V	nsp1β
1027	T	C	C	C	L	L	L	L	nsp1β
1181	C	C	T	C	S	S	F	S	nsp1β
1414	G	A	A	A	A	T	T	T	nsp2/TF/N
1568	G	A	A	A	G	E	E	E	nsp2/TF/N
1998	T	C	C	C	D	D	D	D	nsp2/TF/N
2192	C	C	T	C	S	S	F	S	nsp2/TF/N
2658	G	A	A	A	K	K	K	K	nsp2/TF/N
3040	-	G	A	G	-	D	N	D	nsp2/TF/N

3433	-	G	G	G	-	V	V	V	nsp2/TF/N
3457	-	G	A	G	-	D	N	D	nsp2/TF/N
3706	T	C	C	C	S	P	P	P	nsp2/TF/N
4086	T	C	C	C	G	G	G	G	nsp2/TF
4681	G	G	G	T	A	A	A	S	nsp3
4944	C	C	T	T	A	A	A	A	nsp3
5097	G	G	A	G	R	R	R	R	nsp3
5369	C	T	T	T	T	I	I	I	nsp3
5448	C	C	T	T	V	V	V	V	nsp3
5781	G	A	A	A	Q	Q	Q	Q	nsp4
6345	A	A	T	A	P	P	P	P	nsp5
6519	G	G	G	G	A	A	A	A	nsp5
6520	A	G	G	G	T	A	A	A	nsp5
6674	T	T	T	C	L	L	L	P	nsp5
7168	A	G	G	G	I	V	V	V	nsp7a
7171	C	G	G	G	H	D	D	D	nsp7a
7449	G	G	A	A	G	G	G	G	nsp7b
7554	C	C	T	T	V	V	V	V	nsp7b
7606	A	G	G	G	I	V	V	V	nsp8/nsp9
7621	T	T	A	A	S	S	T	T	nsp8/nsp9
7754	G	G	A	A	T	T	T	T	nsp9
9617	A	A	G	G	E	E	E	E	nsp9
9627	T	G	T	T	S	A	S	S	nsp10
9655	T	T	C	C	L	L	P	P	nsp10
9729	G	A	A	A	A	T	T	T	nsp10
9918	T	T	C	T	L	L	L	L	nsp10
9958	A	A	A	G	E	E	E	G	nsp10
10037	C	C	T	T	N	N	N	N	nsp10
10122	G	A	A	A	V	I	I	I	nsp10
10533	T	T	C	T	Y	Y	H	Y	nsp10
10697	C	C	C	T	A	A	A	A	nsp10
10781	A	A	A	G	T	T	T	T	nsp10
10803	C	C	C	T	R	R	R	C	nsp10
10895	C	C	T	C	D	D	D	D	nsp10
11055	A	A	A	T	T	T	T	S	nsp11
11081	A	A	A	G	P	P	P	P	nsp11
11169	T	C	C	C	L	L	L	L	nsp11
11197	T	A	A	A	F	Y	Y	Y	nsp11
11221	A	A	A	G	E	E	E	G	nsp11
11229	G	G	T	G	V	V	L	V	nsp11

11329	C	C	C	G	A	A	A	G	nsp11
11450	G	A	A	A	K	K	K	K	nsp11
11666	T	T	T	C	P	P	P	P	nsp12
11681	A	G	G	G	V	V	V	V	nsp12
12012	G	G	A	A	G	G	S	S	nsp12
12102	G	G	T	G	L	L	F	L	GP2a
					D	D	Y	D	GP2b
12261	T	C	C	C	A	A	A	A	GP2a
					S	P	P	P	GP2b
12330	C	T	T	T	I	I	I	I	GP2a
12361	G	A	A	A	V	M	M	M	GP2a
12600	G	G	T	G	G	G	G	G	GP2a
12613	G	A	A	A	V	I	I	I	GP2a
12943	G	G	A	G	G	G	E	G	GP3
12950	C	C	T	C	D	D	D	D	GP3
12972	G	G	A	A	V	V	M	M	GP3
12975	G	G	A	A	V	V	I	I	GP3
13011	A	G	A	G	S	G	S	G	GP3
13264	T	C	C	C	L	S	S	S	GP3
					L	L	L	L	GP4
13367	G	A	A	A	Q	Q	Q	Q	GP3
					G	S	S	S	GP4
13409	A	G	G	G	A	A	A	A	GP3
					N	D	D	D	GP4
13475	A	G	G	G	I	V	V	V	GP4
13654	T	T	C	C	V	V	V	V	GP4
13798	T	A	A	A	I	K	K	K	GP5
					D	E	E	E	GP5a
13825	G	G	A	G	R	R	Q	R	GP5
					A	A	A	A	GP5a
14026	T	G	G	G	V	G	G	G	GP5
14238	A	A	G	A	R	R	G	R	GP5
14344	T	C	C	C	V	A	A	A	GP5
14420	C	C	G	C	Q	Q	E	Q	M
14446	C	T	T	T	I	I	I	I	M
14735	G	G	G	C	G	G	G	R	M
14737	C	C	C	G	G	G	G	R	M
14903	T	T	C	C	N	N	N	N	N
15219	G	A	A	A	A	T	T	T	N

a. GenBank accession numbers: VR-2332 (GenBank ID: U87392), MLV (GenBank ID: AF066183), A2MC2 (GenBank ID: JQ087873) and A2MC2-P90 (GenBank ID: KU318406). Some nucleotides locate in ORF overlap regions and result in different amino acid in corresponding ORF.

b. Nucleotide positions are indicated on the left column based on VR-2332 genomic sequence.

c. Nucleotides at the indicated genomic sequence positions are listed. The residues highlighted in blue color indicate those different from A2MC2.

d. Amino acids derived from the codon of indicated nucleotides are listed. The residues highlighted in red color indicate those different from A2MC2.

e. PRRSV viral proteins corresponding to the amino acids derived from the codon of indicated nucleotide positions are listed on the right column.

f. The nt102 locates in 5' UTR.

Chapter 5: The Middle Half Genome of Interferon-Inducing PRRSV Strain A2MC2 Is Essential for Interferon Induction

5.1 Abstract

Porcine reproductive and respiratory syndrome virus (PRRSV) is known to antagonize the innate immune response. An atypical PRRSV strain A2MC2 is capable of inducing synthesis of type I interferons (IFNs) in cultured cells. Here, we show that the middle half of the A2MC2 genome is needed for triggering the IFN synthesis. First, a cDNA infectious clone of this atypical strain was constructed as a DNA-launched version. Virus recovery was achieved from the infectious clone and the recovered virus, rA2MC2, was characterized. The rA2MC2 retained the feature of interferon induction in cultured cells. Infection of pigs with the rA2MC2 virus caused viremia similar to that of the wild type virus. Chimeric infectious clones were constructed by swapping genomic fragments with a cDNA clone of a moderately virulent strain VR-2385 that antagonizes IFN induction. Analysis of the rescued chimeric viruses demonstrated that the middle two fragments, ranging from nt4545 to nt12709 of the A2MC2 genome, were needed for the IFN induction, whereas the chimeric viruses containing any one of the two A2MC2 fragments failed to do so. The results and the cDNA infectious clone of the IFN-inducing A2MC2 will facilitate further study of its biology, ultimately leading towards the development of an improved vaccine against PRRS.

5.2 INTRODUCTION

Porcine reproductive and respiratory syndrome virus (PRRSV) infection of pigs has a high economic impact on swine production across the world, and has resulted in an estimated

$664 million loss per year in the United States alone (Holtkamp et al., 2013; Lunney et al., 2016). PRRSV is a single-stranded positive-sense RNA virus belonging to the family Arteriviridae, order Nidovirales (Faaberg et al., 2012). There are two PRRSV genotypes: Type 1 (European) and Type 2 (North American), which are classified into two species in the genus Porartevirus: *PRRSV-1* and *PRRSV-2* in the new taxonomy (Adams et al., 2016; Kuhn et al., 2016). The genome of PRRSV is around 15 kb in length and contains at least ten open reading frames (ORFs). ORF1a and ORF1b occupy two thirds of the viral genome and encode non-structural proteins that are needed for viral replication, while ORFs 2-7 encode structural proteins. PRRSV mainly targets pulmonary alveolar macrophages (PAMs) during acute infection of pigs (Rossow et al., 1995). MARC-145 cells, derived from a monkey kidney, are generally used for PRRSV propagation *in vitro*(H. S. Kim et al., 1993).

Host innate immune responses play a critical role against early viral infection. The pattern recognition receptors (PRR) for RNA viruses include RIG (retinoic-acid-inducible gene)-I-like receptors (RLRs) and Toll-like receptors (TLRs) (Heil et al., 2003; Kawai & Akira, 2006). Stimulation of RLR and TLR signaling pathways leads to activation of IFN regulatory factor 3 (IRF-3), IRF-7 and NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells), followed by induction of type I interferons (IFNs) (i.e. IFN-α and β) and expression of inflammatory cytokines. Type I IFNs are critical to innate immunity against viral infections and play an important role in activation of the adaptive immune response (Gonzalez-Navajas et al., 2012; Takaoka & Yanai, 2006).

PRRSV appears to inhibit synthesis of type I IFNs *in vivo* (Albina, Carrat, et al., 1998; Buddaert et al., 1998; R. Wang & Zhang, 2014) and *in vitro* (Albina, Carrat, et al., 1998; S. M. Lee et al., 2004; Miller et al., 2004). Recovered virus from an infectious clone of a high-pathogenic PRRSV isolate was reported to induce IFNs in infected pigs, which may be because of its significant higher-level replication (100-1000 fold) and induced more severe

inflammatory response than VR-2332 (Guo et al., 2013). An atypical PRRSV strain A2MC2 was discovered to induce high level IFNs in cultured cells, whereas other strains tested, including PRRSV VR-2332, Ingelvac® PRRS MLV (Boehringer Ingelheim, Inc; hereinafter referred as MLV vaccine strain), and VR-2385, antagonize IFN induction (Nan et al., 2012). Inoculation of pigs with the A2MC2 virus leads to earlier onset and higher virus-neutralizing antibodies than the MLV vaccine strain (R. Wang, Y. Xiao, et al., 2013). Neutralizing antibodies against PRRSV confer protection of pregnant sows against reproductive failure induced by virulent strain challenge (Osorio et al., 2002). Passive transfer of PRRSV-neutralizing antibodies to young weaned pigs blocks PRRSV viremia after challenge (Lopez et al., 2007).

The interferon induction by strain A2MC2 sustains serial passaging of the virus in MARC-145 cells for 90 passages for attenuation, as the A2MC2-P90 induces IFNs similarly to the wild type virus (Z. Ma et al., 2016). This high-passaged virus, A2MC2-P90, is avirulent and induces higher virus-neutralizing antibodies than the MLV vaccine strain. The interferon induction of A2MC2 and its ability to induce high levels of neutralizing antibodies indicate that this virus carries unique feature in its genomic sequence, which might correspond to pathogen-associated molecular patterns (PAMP). The PAMP is prone to be recognized by host PRR and remains intact in the avirulent A2MC2-P90 virus.

In the present study, the objective was to construct a cDNA infectious clone of A2MC2 and to study the genomic source of the interferon induction. A DNA-launched infectious clone was constructed, and virus recovery was achieved. The recovered virus maintained the feature of IFN induction in cultured cells. When the recovered virus was tested in the pig model, it caused pathology similar to that of the wild type virus. Chimeric clones of A2MC2 with moderately virulent PRRSV strain VR-2385 that does not induce

IFNs were constructed. Analysis of the recovered chimeric viruses demonstrated that the middle half of the A2MC2 genome is essential for the interferon induction.

5.3 RESULTS

5.3.1 Construction of cDNA infectious clone of atypical PRRSV strain A2MC2 and determination of the growth property of the recovered virus

A cDNA infectious clone of strain A2MC2 was constructed as a DNA-launched

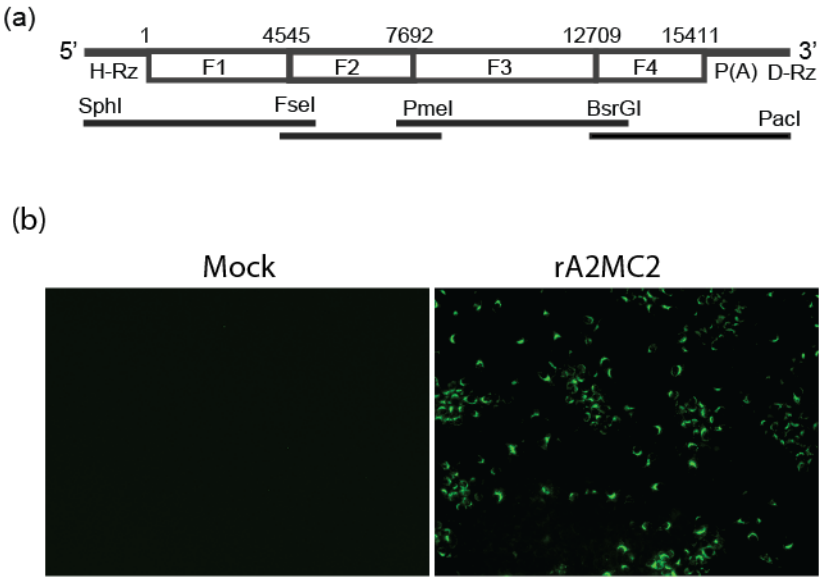

Fig.5. 1 Construction of a cDNA infectious clone of PRRSV strain A2MC2

(a) Schematic illustration of the strategy for the cloning of A2MC2 cDNA into pCAGEN vector. F1, F2, F3, and F4 denote four fragments amplified from cDNA of A2MC2. The numbers 4545, 7692, and 12709 above the insert indicate nucleotide positions of the restriction enzymes FseI, PmeI and BsrGI in the cDNA of A2MC2 (GenBank accession number JQ087873). The lower four lines indicate PCR amplified fragments. The five restriction enzymes used to assemble the full-length cDNA are indicated above each fragment. H-Rz: hammerhead ribozyme. D-Rz: hepatitis delta virus ribozyme. (b) Immunofluorescence assay (IFA) of MARC-145 cells infected with recovered virus from the cDNA infectious clone of A2MC2 (rA2MC2).

version (Fig. 5.1a). Sequences of hammerhead ribozyme and hepatitis delta virus ribozyme

were added at the 5' and 3' terminus, respectively, of the cDNA of A2MC2. The full-length

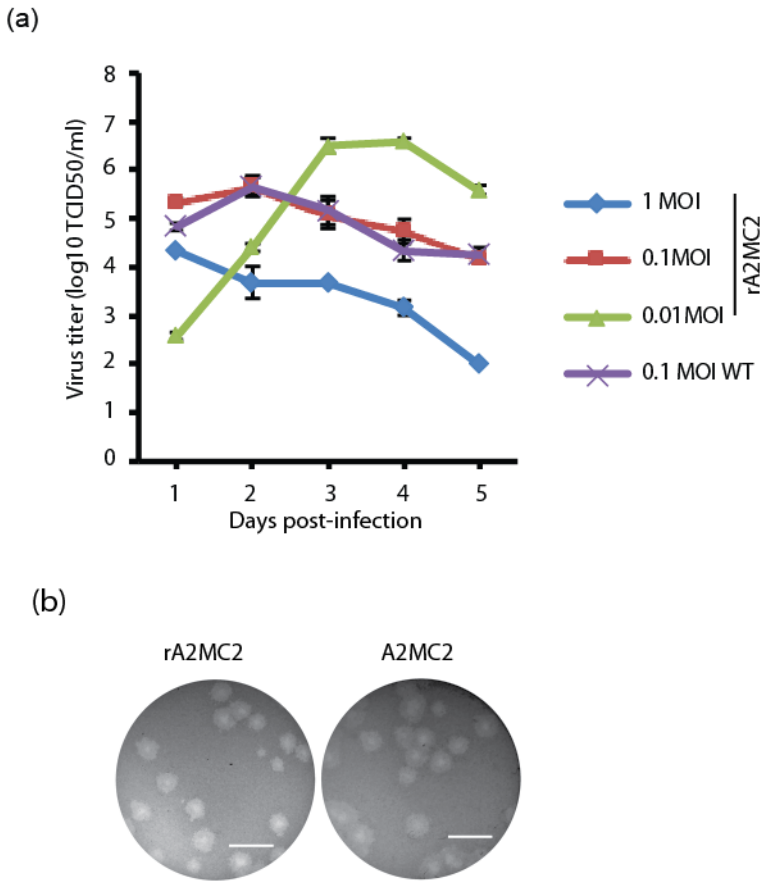

Fig.5. 2 Growth property of the rA2MC2 virus

(a) Multi-step growth curve of the rA2MC2 virus. MARC-145 cells were infected with rA2MC2 virus at an MOI of 1, 0.1, and 0.01, respectively. Culture supernatants were collected daily for virus yield titration. The virus titers (log10 TCID50/ml) were determined in MARC-145 cells. Error bars represent standard errors of three repeated experiments. Wild type parental A2MC2 virus was included as a control (0.1 MOI WT). (b) Plaque assay of rA2MC2 and parental A2MC2 virus in MARC-145 cells. The bars in the images correspond to 10 mm

PRRSV sequence in the resulting plasmid pCAGEN-A2MC2-Rz was confirmed by DNA sequencing. MARC-145 cells were transfected with the pCAGEN-A2MC2-Rz plasmid to recover virus. The transfected cells were harvested four days after transfection and

supernatant of the cell lysate was passaged in fresh cells. Typical cytopathic effect of PRRSV was visible 48 h post inoculation (hpi), and the virus proliferation was verified by IFA with an N-specific monoclonal antibody (Fig. 5.1b). Partial DNA sequencing of the progeny virus confirmed they were derived from the infectious clone.

The rA2MC2 virus was propagated in MARC-145 cells for a multi-step growth curve and a plaque assay. The virus yields for the cells with inoculum at an MOI (multiplicity of infection) of 0.01 peaked at 72 hpi (Fig. 5.2a). The virus yields for the cells inoculated with an MOI of 0.01 were higher than the cells inoculated with an MOI of 0.1 and 1.0. The virus yields for the cells inoculated with an MOI of 1.0 decreased along with time extension. The virus yields for the cells inoculated with an MOI of 0.1 were similar to those of parental wild type A2MC2 virus in the same amount of inoculum (Fig. 5.2a).

A plaque assay was conducted in MARC-145 cells to compare the rA2MC2 and its parental A2MC2 virus. Both parental A2MC2 and rA2MC2 had similar plaque sizes, 3-4 mm in diameter (Fig. 5.2b). The results indicate that the rA2MC2 virus had a growth property similar to its parental virus.

5.3.2 The recovered A2MC2 virus induces interferon synthesis

As the wild type A2MC2 induces interferon production in cultured cells (Nan et al.,

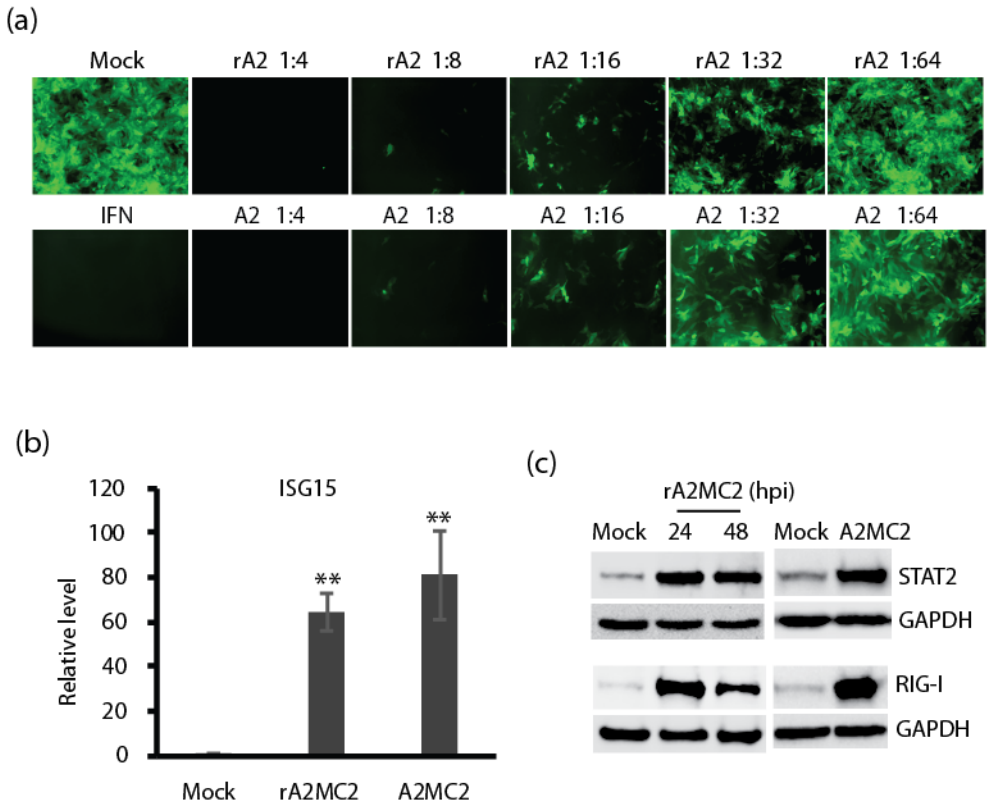

Fig.5. 3 The rA2MC2 virus induces type I interferons in MARC-145 cells

(a) Interferon bioassay in Vero cells. Cell culture supernatants from rA2MC2-infected MARC-145 cells collected at 24 hpi were diluted and added to Vero cells. 12 h later, the cells were infected with NDV-GFP. Fluorescence microscopy was conducted 24 h after NDV-GFP inoculation. Culture supernatant from A2MC2-infected cells and IFN-α at 1000 U/ml were included as controls. (b) Activation of ISG15 expression in infected MARC-145 cells (24 hpi) detected by real-time PCR. "**" denotes a significant difference compared to the mock-infected cells ($P < 0.01$). (c) Increase of STAT2 and RIG-I protein levels in rA2MC2-infected MARC-145 cells determined by Western blotting

2012), we tested whether the rA2MC2 virus kept the feature of IFN induction. The supernatant of rA2MC2-infected MARC-145 cells was used for an interferon bioassay in Vero cells. Results showed that NDV-GFP replication was inhibited in the Vero cells treated

with the rA2MC2 supernatant diluted up to 1:16, similar to the wild type A2MC2 (Fig. 5.3a). This suggests that rA2MC2 induced production of interferons, which led to the suppression of NDV replication in the Vero cells, in a similar capacity to the wild type A2MC2 virus.

Expression of interferon-stimulated genes (ISG) was also determined to confirm the effect of the treatment of Vero cells. Compared with the mock-treated cells, the cells treated with supernatant samples of rA2MC2 and parental wild type A2MC2 at 24 hpi had ISG15 transcript level increased by 64 and 81-fold, respectively (Fig. 5.3b). There is no significant difference between the ISG15 levels in cells treated with rA2MC2 or wild type A2MC2 supernatants. STAT2 and RIG-I are also known to be upregulated by type I IFNs (Z. Ma et al., 2016; Nan et al., 2012). The protein levels of STAT2 and RIG-I in the rA2MC2-infected MARC-145 cells at both 24 and 48 hpi were upregulated (Fig. 5.3c). The result demonstrated that rA2MC2 kept the feature of IFN induction.

PAMs are the major target cells for PRRSV infection *in vivo*. We inoculated primary porcine PAMs with the rA2MC2 virus and found that the virus replicated in PAMs and induced IFNs (data not shown). This indicates the recovered virus is similar to the wild type A2MC2 virus in inducing IFN synthesis in the cultured cells.

5.3.3 Pig study

An animal experiment was conducted by inoculating 3-week-old PRRSV-negative piglets with the rA2MC2 virus. The pigs were euthanized at 14 days post-infection (DPI). Compared with the pigs inoculated with parental A2MC2 virus, the pigs infected with the rA2MC2 virus had similar lesion scores, though there were some low responders (Fig. 5.4a).

Real-time PCR was conducted to determine PRRSV RNA in serum samples of 14 DPI.

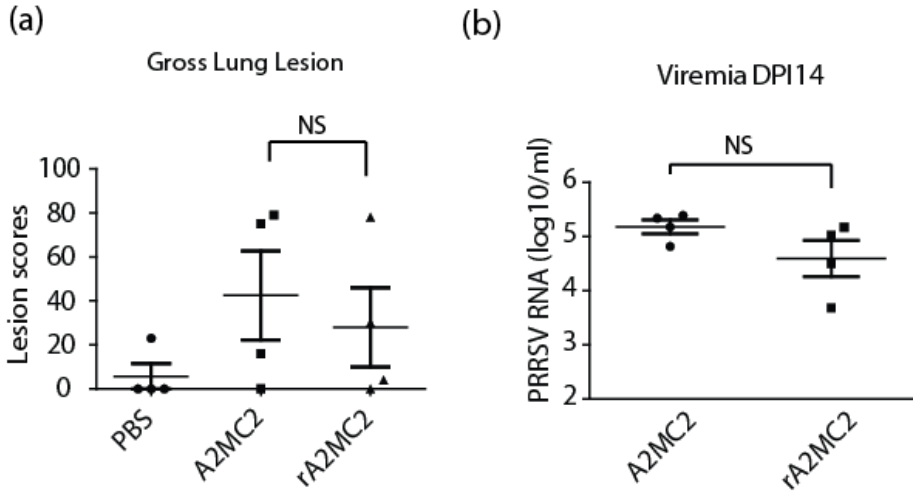

Fig.5. 4 Pig test of the rA2MC2 and its parent virus

Four pigs from each group were euthanized at 14 days post infection (DPI). Mock-infected pigs (PBS) were included as controls. (a) Gross lung lesion scores. Median values are shown. Error bars represent standard errors of the scores among the four pigs in each group. NS: no significant difference. (b). Viremia on DPI14 detected by RT-qPCR

The average PRRSV RNA levels for the pigs infected with the rA2MC2 and the parental A2MC2 virus were 4.55 and 4.46 log10 copies/ml, respectively (Fig. 5.4b). There was no significant difference in the PRRSV RNA levels between the two groups. These results suggest that the rA2MC2 virus has similar virulence and replication kinetics in pigs compared to its parental A2MC2 virus.

5.3.4-Construction of chimeric cDNA infectious clones and determination of the growth property of the recovered chimeric virus

To determine the genetic source of A2MC2 virus for interferon induction, we constructed three chimeric A2MC2 infectious clones by genomic fragment swapping with pIR-VR2385 (Fig. 5a). VR-2385 does not induce IFNs (Nan et al., 2012) and the pIR-

VR2385 was robust in virus recovery (Ni et al., 2011). The cDNA sequence of the viral

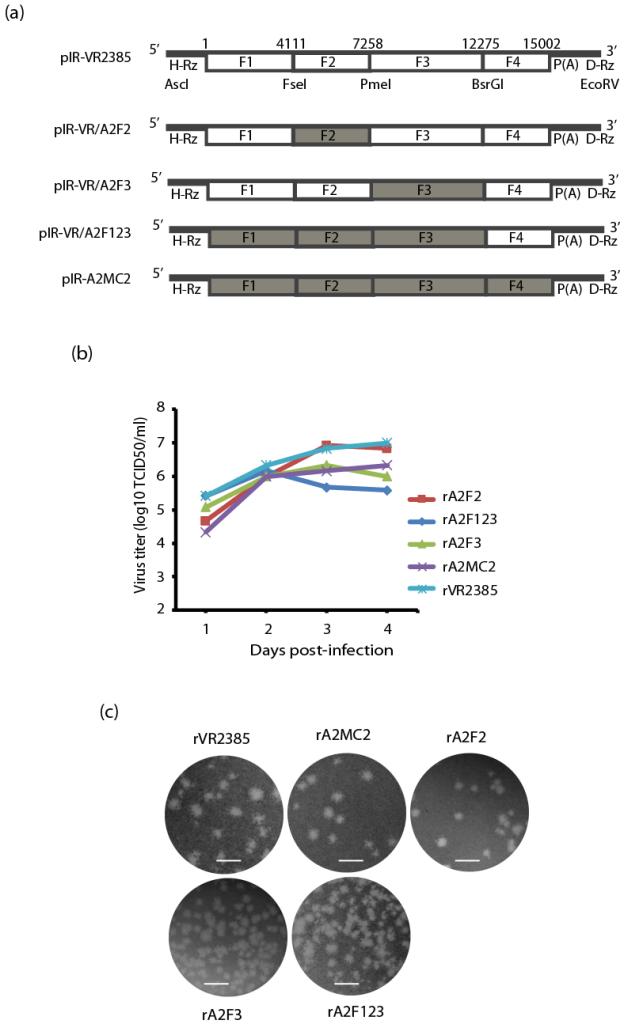

Fig.5. 5 Chimeric cDNA infectious clones and growth property of the chimeric viruses

(a) Schematic illustration of the strategy for the construction of chimeric cDNA infectious clones via fragment swapping between PRRSV strains VR-2385 and A2MC2. The numbers above pIR-VR2385 denote nucleotide positions in the VR-2385 genome. The restriction enzymes used for the fragment swapping are indicated below the pIR-VR2385 box. The shaded boxes indicate fragments from strain A2MC2. H-RZ: hammerhead ribozyme; F1 to F4: fragment 1 to 4; P(A): poly(A); D-RZ: hepatitis delta virus ribozyme; pIR-VR/A2F2: pIR-VR2385/A2F2. (b) Multi-step growth curve in MARC-145 cells infected with the virus at an MOI of 0.01. The virus titers (log10 TCID50/ml) were determined in MARC-145 cells. rVR2385: recovered virus from the cells transfected with pIR-VR2385; rA2F2: recovered chimeric virus from pIR-VR/A2F2; rA2F3: recovered chimeric virus from pIR-VR/A2F3; rA2F123: recovered chimeric virus from pIR-VR/A2F123; rA2MC2: recovered virus from pIR-A2MC2. (c) Plaque assay in MARC-145 cells. The bars in the images correspond to 10 mm.

genome in pIR-VR2385 is shorter than the A2MC2 sequence due to deletion in nsp2 (Ni et al., 2011). So the nucleotide positions of the three restriction enzymes are different from A2MC2 cDNA (Fig. 5.5a). The F2, F3, and F1-F3 fragments in pIR-VR2385 were replaced with their counterparts of A2MC2, resulting in chimeric clones: pIR-VR2385/A2F2, pIR-VR2385/A2F3, and pIR-VR2385/A2F123. MARC-145 cells were transfected with these chimeric infectious clones as well as pIR-VR2385 and pIR-A2MC2 plasmids for virus recovery separately. The virus recovery was successful from all the clones.

The chimeric viruses were subjected to growth property assay in MARC-145 cells. The virus yields for the cells inoculated with the rA2F2 and rVR2385 from 48 to 96 hpi were similar (Fig. 5.5b). The virus yields for the cells inoculated with the rA2MC2 and rA2F3 were similar, approximately 0.5-1 log lower than rA2F2 and rVR2385 at 72 and 96 hpi. All the viruses had similar yields at 48 hpi. The yields for the rA2F123 virus at 72 and 96 hpi were lower than all the other four viruses. The results indicate that the chimera rA2F2 is similar to rVR2385 in replication rate, chimera rA2F3 is similar to rA2MC2, and chimera rA2F123 has a lower replication rate than both rVR2385 and rA2MC2.

A plaque assay was conducted in MARC-145 cells to compare the chimeric viruses. All the chimeric viruses had similar plaque sizes, 2-4 mm in diameter (Fig. 5.5c).

Subsection 5.3.5 The middle half of the strain A2MC2 genome is essential for interferon induction

For interferon bioassay, the culture supernatants of MARC-145 cells infected with the chimeric viruses were used to treat Vero cells. Results showed that NDV-GFP replication was inhibited in the Vero cells treated with the culture supernatants of rVR/A2F123 and rA2MC2 virus in a dilution up to 1:16 (Fig. 5.6a). However, treatment with the culture supernatants of rVR/A2F2, rVR/A2F3, and rVR2385 viruses had a minimal effect on the NDV-GFP replication when compared to the mock-treated cells. A2MC2 has the same

sequence in fragment 1 (nt1-4544) as VR-2332, and the latter does not induce IFNs in MARC-145 cells (Nan et al., 2012). So, both fragments 2 and 3 (nt4545-12709) are critical for the IFN induction.

To confirm this observation, immunoblotting to determine the RIG-I protein level in

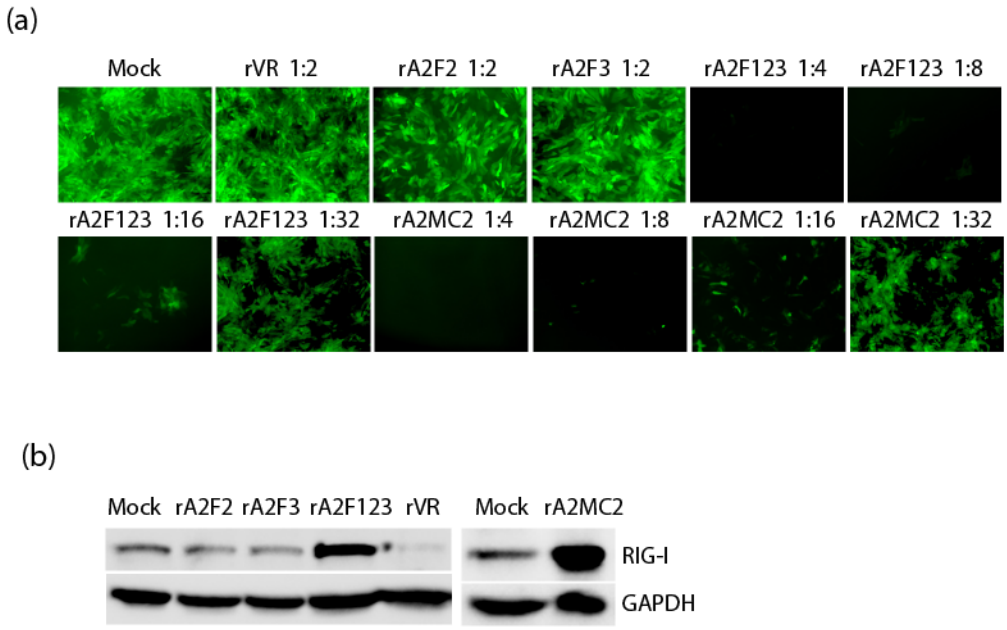

Fig.5. 6 The middle half of A2MC2 genome is essential for the interferon induction

(a) Interferon bioassay in Vero cells. Cell culture supernatants from MARC-145 cells infected with the chimeric viruses were collected and used to treat Vero cells at indicated dilutions. rVR: rVR2385. (b) Increase of RIG-I protein level in MARC-145 cells infected with rA2F123 chimeric virus determined by Western blotting.

MARC-145 infected with the chimeric viruses was conducted. Compared with the mock-infected cells, the MARC-145 cells infected with rVR/A2F123 chimeric virus at 48 hpi had higher RIG-I protein level, whereas the cells infected with rVR/A2F2 and rVR/A2F3 chimeric viruses had a slight reduction (Fig. 5.6b). The rVR2385-infected cells had lower RIG-I level than the mock-infected cells. This confirmed that the rVR/A2F123 chimeric virus

induced interferon production in MARC-145 cells and the presence of both fragments 2 and 3 of the A2MC2 genome is required for the sensing by host pattern recognition receptors.

5.4 DISCUSSION

Although it has been nearly 30 years since the first reports of PRRS, broadly effective vaccines against PRRSV infection are still not available due to the genetic diversity of PRRSV isolates and apparent lack of cross-protection between isolates (Lunney et al., 2016). It is known that PRRSV strains generally antagonize interferon synthesis (Nan et al., 2012; Y. Sun et al., 2012; R. Wang & Zhang, 2014). The effect of PRRSV replication on IFN induction appears to be variable among different strains and different cell types. PRRSV field isolates have variable suppressive effect on IFN-α induction in cultured PAM cells (S. M. Lee et al., 2004). The interferon induction by PRRSV strain A2MC2 is a unique feature of this virus. Considering the importance of interferons in activating the adaptive immune response (Gonzalez-Navajas et al., 2012; Iwasaki & Medzhitov, 2015), this feature is desirable in vaccine development against PRRSV. Type I IFNs have an important role in the differentiation of CD4+ and CD8+ T cells (Gonzalez-Navajas et al., 2012; Iwasaki & Medzhitov, 2015). This study identified that the middle half of the A2MC2 genome is pivotal for IFN induction by chimeric infectious clone analyses. The rescued A2MC2 virus has the capability of IFN induction in cultured cells like its parental strain. Treatment of Vero cells with the culture supernatants from rA2MC2-infected cells induced expression of ISG15, RIG-I, and STAT2, which indicates the activation of IFN signaling. The expression of the interferon-activated genes was confirmed at both the RNA and the protein levels. These results suggest that the genetic feature of A2MC2 is maintained in the cDNA infectious clone.

The recovered rA2MC2 virus maintained the feature of IFN induction in cultured

cells. The rA2MC2 virus has growth properties in terms of multi-step growth and plaque formation similar to its parental virus. The cells infected with rA2MC2 inoculum at an MOI of 0.01 had higher viral yields than the cells inoculated with an MOI of 0.1 and 1.0, which indicates less inoculum leads to more efficient virus replication. This result is also consistent with the feature of IFN induction. The lower the inoculum, the less IFN induction in the cells and the weaker the antiviral response the cells would mount. The virus in the cells at low level would have a better chance to replicate to high level without triggering a rigorous antiviral response than high level of virus in the initial inoculum.

The rA2MC2 virus led to pathology and viremia in pigs similar to its parental virus, though the lung lesion scores were numerically variable among the pigs infected. This is possible due to the variation of individual pigs used in this study, some of which were low responders. This is also indicated by their similar viremia levels. The rA2MC2 virions are expected to be a more homogenous population than the wild type virus though the latter was plaque purified (Nan et al., 2012).

The chimeric cDNA clones of A2MC2 and VR-2385 were constructed. Similar growth trend and yields from 48 to 96 hpi were observed between the rA2F2 and rVR2385, as well as between the rA2F3 and rA2MC2. All the five recovered viruses have similar yields at 48 hpi. The rA2F123 has similar yields to rVR2385, but lower yields than the other four viruses at 72 and 96 hpi, which may be because rA2F123 induces IFNs. The yields of the rA2MC2 virus was slightly higher than rA2F123 in the two late time points when the cells were inoculated at this amount of inoculum. It is not known whether the fragment 4 that are different between these two recovered IFN-inducing viruses contributes to the variation in late virus replication.

The chimeric clones of A2MC2 and VR-2385 were used to study the genomic source of the IFN induction. Since the nucleotide sequence of A2MC2 fragment 1 (nt1-4544) is the

same as VR-2332 and the latter antagonizes IFN induction (Nan et al., 2012), the fragment 1 has no pivotal role in the A2MC2 induction of IFNs. Thus, the genomic source for the IFN induction must be from the middle two fragments. The results from analyzing the chimeric viruses demonstrated that both fragment 2 and 3 (nt4545-12709) are required for IFN induction. The sequence of nt4545-12709 in the A2MC2 genome encodes proteins of C-terminal 126 amino acid residues of nsp2, nsps 3-12, and N-terminal 213 residues of GP2a. Among these PRRSV nsps, nsp2, nsp4, and nsp11 are reported to inhibit IFN induction (Beura et al., 2010; R. Wang & Zhang, 2014). A2MC2 has the identical nsp2, nsp4, and nsp11 as VR-2332 or MLV (Nan et al., 2012). Thus, lack of inhibition of IFN induction by these proteins is not possible.

Our results indicate that the middle half genome of A2MC2 contributes to the PAMP recognition by host PRR. RIG-I and MDA5 are the PRR to sense viral RNA in the cytoplasm. RIG-I preferentially recognizes the 5' terminus of the virus RNA, including both the genomic and subgenomic RNA for PRRSV. A prerequisite for RIG-I recognition is the presence of 5' diphosphate or triphosphate (Goubau et al., 2014; D. Luo et al., 2011). Conversely, MDA5 recognizes an internal duplex structure of long double-stranded RNA (dsRNA) (Wu et al., 2013; J. Zheng et al., 2015). RIG-I was previously reported to be the PRR interfered by PRRSV to antagonize IFN induction (Luo et al., 2008). For IFN induction by strain A2MC2, the role of RIG-I is unknown. Presumably, dsRNA is formed during PRRSV replication, and the middle half of the A2MC2 genome confers the internal duplex structure for MDA5 recognition. This is consistent with our observation that A2MC2 replication is needed for the IFN induction as UV-inactivated A2MC2 virus cannot trigger IFNs (Nan et al., 2012). We speculate that many PRRSV strains like VR-2332 and VR-2385 that antagonize IFN induction may escape the MDA5 recognition by formation of a different internal duplex structure.

Our data indicate that both fragments 2 and 3 (nt4545 to nt12709) of A2MC2 are pivotal for the possible internal duplex structure for MDA5 recognition. Replacement of either F2 or F3 in the cDNA clone of VR-2385 with the corresponding fragment of A2MC2 failed to confer the chimeric viruses the capability to induce IFNs. In addition, the chimeric viruses from the F2 or F3 chimeric clones were unable to evoke elevation of RIG-I. In contrast, the chimeric virus rA2F123 containing both the F2 and F3 of A2MC2 induced IFNs and triggered the elevation of RIG-I protein level. The result indicates that the A2MC2 fragment 4 (nt12710-3'terminus) covering ORFs 3-7 appears not to play a decisive role in the IFN induction. A2MC2 ORF2 (nt12073-12843) overlaps with the 3'terminus of F3 and the 5'terminus of fragment 4. It is not known whether the ORF2 sequence contributes to the potential internal duplex structure for MDA5 recognition. The requirement of both fragment 2 and 3 in IFN induction might be coincidental. Further study is needed to define the minimum sequence and/or nucleotides that are required for the IFN induction.

Though the fragment 1 is assumed to have no pivotal role in the IFN induction by strain A2MC2, other studies have shown three proteins encoded by this part of the genome, nsp1α, nsp1β, and nsp2, involve in PRRSV inhibition of IFN induction (M. Han & Yoo, 2014b; Y. Sun et al., 2012; R. Wang & Zhang, 2014). Reversal of the inhibition leads to improvement of IFN induction. Site-directed mutagenesis of R128 and R129 of nsp1β reduced its inhibition of IFN induction and led to improvement of the innate and adaptive immune responses by the mutant virus (Y. Li et al., 2016). A synthetic PRRSV strain that was prepared on the basis of a consensus genome from alignment of 59 full-length genomes is shown to induce IFNs (H. Sun et al., 2016) and elicits heterologous protection (Vu et al., 2015). The IFN induction phenotype of the synthetic PRRSV was mapped to the 3.3 kb genome encoding nsp1α, nsp1β, and N-terminal part of nsp2 (H. Sun et al., 2016). Our data indicates that A2MC2 has a unique mechanism to trigger IFN synthesis, which is presumed to

begin before the nsps are able to mount sensible suppression. This presumption is in consistent with our result that replication kinetics of A2MC2 is inverse proportional to the inoculum MOI. This mechanism is stable during serial passaging for 90 times in cultured cells since the A2MC2-P90 maintains the feature of IFN induction (Z. Ma et al., 2016).

In conclusion, the middle half genome of strain A2MC2 is pivotal for its IFN induction. The DNA-launched cDNA infectious clone of A2MC2 was constructed, and the recovered virus carries the unique feature of interferon induction in cultured cells. Further study using the A2MC2 cDNA infectious clone is warranted for examination of the precise mechanism of IFN induction and development of an improved vaccine against PRRS.

5.5 MATERIALS AND METHODS

5.5.1 Cells and viruses

Vero (ATCC CCL-81) and MARC-145 (H. S. Kim et al., 1993) cells were grown in DMEM (Dulbecco's Modified Eagle's Medium) supplemented with 10% fetal bovine serum (FBS). CRL2843 cells (porcine macrophages, ATCC) were cultured in RPMI1640 medium supplemented with 10% FBS. Primary PAM cells were prepared from 4-8-week-old piglets and cultured in RPMI1640 medium supplemented with 10% FBS (Patel et al., 2008).

PRRSV strains VR-2385 (passage 15) and A2MC2 (passage 10) were propagated and titrated in MARC-145 cells. Virus yields were titrated by 10-fold serial dilutions and presented as the median tissue culture infectious dose (TCID50) (Y. J. Zhang et al., 2006). Newcastle disease virus strain LaSota carrying the gene of green fluorescence protein (NDV-GFP) was propagated in embryonated eggs and titrated in Vero cells (S. H. Kim & Samal, 2010).

5.5.2 RNA isolation, reverse transcription, PCR and real-time PCR

Total RNA was isolated using the TRIzol® LS Reagent (Thermo Fisher Scientific Inc., Waltham, MA) following the manufacturer's instructions. Reverse transcription and PCR (RT-PCR) and real-time PCR (RT-qPCR) were conducted to amplify cDNA for cloning or to determine PRRSV RNA levels (Patel et al., 2008; Patel et al., 2009). To normalize the total input RNA, ribosomal protein L32 (RPL32) RNA was measured. Specifically, the analysis of the relative transcript levels was performed by normalization of RPL32 in comparison with controls (Patel et al., 2010).

5.5.3 Construction of cDNA clone of PRRSV strain A2MC2

The strategy to construct the cDNA clone of A2MC2 (passage 10) is illustrated in Fig. 1a (Nielsen et al., 2003). PCR was done to amplify four fragments spanning the full-length cDNA of strain A2MC2 genomic RNA. Primers used in the PCR are listed in Table 1. The unique restriction sites FseI (nt4545), PmeI (nt7692) and BsrGI (nt12709) in the A2MC2 genome were used to assemble the cDNA clone. SphI and PacI were also used to clone the cDNA into the target vector pCAGEN, which was a gift from Connie Cepko (Addgene plasmid # 11160) (Matsuda & Cepko, 2004). A stuffer sequence containing these restriction sites was designed and ligated into pCAGEN at EcoRI/XhoI sites to generate the pCAGEN-Stuffer. The PCR products digested with the restriction enzymes were ligated into the pCAGEN-Stuffer vector in the following order: F1, F4, F3 and F2 to generate pCAGEN-A2MC2. DNA sequencing was done to confirm the cloned fragments. For insertion of a hepatitis delta virus ribozyme to the 3'end cDNA of the virus genome, two oligos A2-3endRiboF and A2-3endRiboR were annealed and digested with PacI before ligation into pCAGEN-A2MC2 that was prepared with PacI digestion. DNA sequencing was done to confirm the addition, orientation and correct sequence of the two ribozymes in the recombinant pCAGEN-A2MC2-Rz.

Correction of point mutations in the cDNA clone was done with Thermo Scientific™ Phusion Site-Directed Mutagenesis Kit (Thermo Fisher Scientific). The cDNA clone of PRRSV VR-2385, pIR-VR2385-CA, was a gift from Xing-Jin Meng (Ni et al., 2011) and used for fragment swapping with A2MC2 cDNA to generate chimeric clones and the pIR-A2MC2 plasmid. The rA2MC2 virus (passage 6) was used in experiments in this study.

5.5.4 Interferon bioassay

The presence of interferons in the culture supernatant samples was determined as described previously (Nan et al., 2012). Briefly, culture supernatant samples from PRRSV-infected MARC-145 cells were diluted and added to Vero cells. After overnight incubation, the Vero cells were inoculated with NDV-GFP, as it is sensitive to IFN-induced antiviral effect. Fluorescence microscopy was conducted 24 h after NDV inoculation to observe GFP-positive cells.

5.5.5 Immunofluorescence assay (IFA)

PRRSV propagation in MARC-145 cells on coverglass was detected with IFA using an N-specific monoclonal antibody EF11 (Y. Zhang et al., 1998). DyLight™ 488 conjugated goat anti-mouse IgG (Rockland Immunochemicals Inc., Limerick, PA) was used to detect the EF11 binding to the N protein in the infected cells. SlowFade Gold antifade reagent containing 4'6-diamidino-2-phenylindole (DAPI) (Thermo Fisher Scientific) was used for mounting the coverglass onto the slide before observation under fluorescence microscopy.

5.5.6 Western blotting

Total proteins in the cell lysate samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by Western blotting as described previously (Y. J. Zhang et al., 2007). Antibodies against STAT2, RIG-I, and GAPDH (Santa

Cruz Biotechnology, Inc., Dallas, TX) and horseradish peroxidase-conjugated secondary antibodies (Rockland Immunochemicals Inc.) were used in this study.

5.5.7 Plaque assay

Plaque assay in MARC-145 cells was done to compare the recovered virus with its parental virus (Z. Ma et al., 2016; Nan et al., 2012). PRRSV was diluted to 10 and 100 TCID50 per ml and added to the monolayer cells. After 2 h incubation, the inoculum was removed and 0.5% agarose overlay containing complete growth medium was added onto the cells. Plaques were stained with neutral red and observed after overnight incubation.

5.5.8 DNA sequencing

DNA sequencing was performed with ABI Prism 3130 Genetic Analyzer (Thermo Fisher Scientific). Sequence assembly and analysis was done with LaserGene Core Suite (DNASTAR Inc., Madison, WI). The GenBank accession number of the cDNA sequence of A2MC2 genomic RNA is JQ087873.

5.5.9 Animal study

The animal study was approved by the Institutional Animal Care and Use Committees (IACUC) of Iowa State University and the University of Maryland and according to relevant guidelines and policies for the care and use of laboratory animals. Three-week-old PRRSV-negative piglets were randomly divided into three groups with 4 pigs in each group. The piglets in groups 1 and 2 were inoculated with 1 ml of wild type A2MC2 (passage 10) and rA2MC2 (passage 6), respectively, at 105 TCID50/ml via intranasal inoculation (I.N.), while group 3 was mock-infected with phosphate-buffered saline (PBS) pH7.2. The pigs were observed daily. Blood samples were collected prior to inoculation and at 14-day post-infection (DPI).

The pigs were euthanized on DPI14 by pentobarbital overdose (FATAL-PLUS, Vortech Pharmaceuticals, LTD. Dearborn, MI). Visible macroscopic lung lesions were scored and recorded as previously described (Halbur et al., 1995; Halbur et al., 1996).

5.5.10 Statistical analysis

Differences between treatment and control samples were assessed by the t-test (Mann-Whitney test (nonparametric)) in GraphPad Prism. A two-tailed P-value of less than 0.05 was considered statistically significant.

Chapter 6: Single Nucleotide in Interferon-Inducing PRRSV Strain A2MC2 Is Essential for Interferon Induction

6.1 ABSTRACT

An atypical porcine reproductive and respiratory syndrome virus (PRRSV) strain, A2MC2, induces type I interferons (IFN). The middle half of the virus genome is involved in the IFN induction. The objective of this study was to further identify the genetic source of the A2MC2 induction of IFN. We conducted site-directed mutagenesis of the highly conserved unique nucleotides of A2MC2 after sequence analysis. Here, we demonstrate that nucleotide 12012 of the virus is pivotal for triggering the IFN synthesis. First, we constructed three mutant infectious clones, each of which contained one nucleotide mutation at nt7621, nt9655 or nt12012 of the genome. These three nucleotides were changed to those corresponding ones present in VR-2332. Mutant virus was recovered, and growth property was determined. The results showed similar growth kinetics and plaque sizes of all three mutant viruses compared to the wild-type virus. The IFN induction of these mutant viruses was assessed via IFN bioassay. Only the mutant virus with the mutation at nt12012 failed to induce IFN synthesis. The results indicate that nt12012 is critical for A2MC2 induction of IFN. Further study of the virus biology is warranted to facilitate the development of an improved vaccine against PRRS.

6.2 INTRODUCTION

PRRSV infection causes mild to severe infertility and respiratory syndrome in all age of pigs. Despite a global PRRS related research for over three decades, PRRS remains an

economically significant viral disease to swine industry worldwide as shown by its adverse effect on productivity and substantial financial cost to producers. PRRSV identified in Europe (Type 1, prototype Lelystad Virus) and North America (Type 2, prototype VR-2332) have significant sequence differences (James E. Collins et al., 1992; Wensvoort, Terpstra, Pol, Ter Laak, Bloemraad, De Kluyver, Kragten, Van Buiten, Den Besten, & Wagenaar, 1991). Based on 2016 ICTV Virus Taxonomy, the two conventional genotypes of PRRSV: Type 1 and Type 2 are reassigned to the two new species *PRRSV-1* and *PRRSV-2*, respectively, in the genus *Porartevirus*, the family *Arteriviridae* (Adams et al., 2016; Kuhn et al., 2016). PRRSV is enveloped single-stranded positive-sense RNA virus with the genome approximately 15 kb in length, which encodes 11 open reading frames (ORFs). Pulmonary alveolar macrophages (PAMs) of pigs are the main target cells of PRRSV during infection. An epithelial-derived cell line from a monkey kidney, MARC-145, is mostly used in *in vitro* research (H. S. Kim et al., 1993).

Host innate immune response is the front line of host defense, which plays a key role against viral infection. Pathogen-associated molecular patterns (PAMP), produced by the virus or associated with virus, are recognized by host pattern recognition receptors (PRR) on the cell membrane or in the cytoplasm. RIG (retinoic-acid-inducible gene)-I-like receptors (RLRs) and Toll-like receptors (TLRs) are common PRR for RNA virus (Heil et al., 2003; Kawai & Akira, 2006). Virus-activated RLR and TLR signaling pathways result in the production of type I interferons (IFNs) and subsequently trigger the IFNs related signaling cascade, which exerts profound antiviral effects (Gonzalez-Navajas et al., 2012; Takaoka & Yanai, 2006).

Typical PRRSV infection elicits weak IFN production in swine (Albina, Carrat, et al., 1998; Buddaert et al., 1998; R. Wang & Zhang, 2014) and in cultured cells (Albina, Carrat, et al., 1998; S. M. Lee et al., 2004; Miller et al., 2004). Furthermore, PRRSV also inhibits the

IFN-activated JAK/STAT signal transduction and ISG expression in both MARC-l45 and PAM cells (Patel et al., 2010; R. Wang, Y. Nan, Y. Yu, Z. Yang, et al., 2013; R. Wang, Y. Nan, Y. Yu, & Y. J. Zhang, 2013). However, an atypical PRRSV strain, A2MC2, induces production of type I IFNs in cultured MARC-145 and PAM cells (Nan et al., 2012). Type 1 IFNs and ISGs are detected in A2MC2-infected cells. A2MC2 has no detectable inhibitory effect on the ability of IFN-alpha to induce an antiviral response in MARC-145 or PAM cells. Sequence analysis indicates that A2MC2 is closely related to VR-2332 and the MLV vaccine strain with an identity of 99.8% at the nucleotide level. A2MC2 induces earlier onset and significantly higher levels of PRRSV neutralizing antibodies than the MLV. Moreover, the A2MC2-induced neutralizing antibodies are capable of neutralizing VR-2385, a heterologous strain (R. Wang, Y. Xiao, et al., 2013). After serial passaging of the A2MC2 in MARC-145 cells for 90 passages for attenuation, the virus maintains the IFN induction ability and elicits higher virus-neutralizing antibodies than the MLV (Z. Ma et al., 2016). Construction of A2MC2 chimeric infectious clones and studying recovered virus revealed that the middle half of the A2MC2 genome, ranging from nt4545 to nt12709, is essential for interferon induction (Z. Ma et al., 2017).

The objective of this study was to further dissect the genetic source of A2MC2 in the interferon induction. Based on earlier sequence analysis of A2MC2 (Z. Ma et al., 2016, 2017; Nan et al., 2012), we selected three highly conserved unique nucleotides in the middle-half genome and conducted site-directed mutagenesis in the A2MC2 infectious cDNA clone. Analysis of the recovered mutant virus demonstrated that a single nucleotide at nt12012 was critical for the A2MC2 induction of IFNs. The results provide insights of the virus biology.

6.3 MATERIALS AND METHODS

6.3.1 Cells and viruses

MARC-145 (H. S. Kim et al., 1993) and Vero (ATCC CCL-81) cells were routinely maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10 % FBS (Atlanta) (Patel et al., 2008).

PRRSV strain A2MC2 and VR-2385 were propagated as previously described and titrated in MARC-145 cells by limiting dilution (Zexu Ma, Yang, & Zhang, 2018). Virus yields were calculated as the median tissue culture infectious dose ($TCID_{50}$) (Y. J. Zhang et al., 2006). Newcastle disease virus (NDV) strain LaSota inserted with the gene of green fluorescence protein (NDV-GFP) was propagated and titrated in Vero cells (S. H. Kim & Samal, 2010).

6.3.2 Site-directed mutagenesis of A2MC2 infectious cDNA clone

Based on previously constructed A2MC2 infectious clone, pIR-A2MC2, point mutations in the cDNA clone was done as illustrated in Fig6.1A with Thermo Scientific™ Phusion Site-Directed Mutagenesis Kit (Thermo Fisher Scientific). The parallel overlapped PCR was performed. Primers used in the PCR are listed in Table 6.1. Unique enzyme restriction sites FseI (nt4545), PmeI (nt7692) and BsrGI (nt12709) in the A2MC2 genome were employed for replacement of the fragments in A2MC2 cDNA clone.

6.3.3 Interferon bioassay

The bioassay of type I IFNs in a set of samples was performed as previously described (Nan et al., 2012). Briefly, MARC-145 cells were infected by PRRSV for 24 hours, and the supernatant was harvested, followed by 1/2 series dilution in DMEM and treatment of Vero cells in a 96-well plate overnight. NDV-GFP, which is sensitive to IFN, was inoculated

to the Vero cells. NDV-GFP positive cells were observed under fluorescence microscopy 24 h after NDV inoculation.

6.3.4 Immunofluorescence assay (IFA)

PRRSV replication in MARC-145 cells was detected with IFA on cover glass and 96-well plate by using an N-specific monoclonal antibody (Y. Zhang et al., 1998). PRRSV infected cells were fixed with Acetone/Methanol fixative for 15 minutes and rinsed with phosphate-buffered saline (PBS) pH7.2. Before the addition of the antibody, the cells were blocked with 1% Bovine Serum Albumin (BSA) for 1 hour at 37 °C. The antibody binding to the N protein in the infected cells was detected by DyLight™ 488 conjugated goat anti-mouse IgG (Rockland Immunochemicals Inc., Limerick, PA). Fluorescence microscopy was conducted to observe PRRSV N-positive cells.

6.3.5 Western blotting

Cell lysates samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting. Briefly, total proteins were separated by SDS-PAGE and transfected to nitrocellulose membrane (Y. J. Zhang et al., 2007). Before probing the membrane with antibodies against STAT2 and GAPDH (Santa Cruz Biotechnology, Inc., Dallas, TX), the membrane was blocked with 5% BSA buffer for 1 hour at room temperature. Horseradish peroxidase-conjugated secondary antibodies (Rockland Immunochemicals Inc.) and chemiluminescence substrate were used to probe the primary antibodies. Blots were imaged using a Chemi-Doc Imaging System (Bio-Rad, Hercules, CA).

6.3.6 Plaque assay

PRRSV virus plaque assay was performed in MARC-145 cells for comparison of the growth properties between A2MC2 mutated virus and the wild-type virus as previously described (Z. Ma et al., 2016; Nan et al., 2012). Briefly, the confluent MARC-145 cells in 6-

well plates were inoculated with either 10 or 100 $TCID_{50}$ per ml. The plate was incubated for 2 h at 37°C. The inoculum in each well was discarded before adding 3 ml complete growth medium containing 0.5% agarose. The plate was left in the biosafety hood for 15 minutes at room temperature to allow the agar overlay to turn to solid. Subsequently, the plate was inverted and incubated in a humidified incubator at 37°C for 72 hours. The plate was stained with 2 ml complete growth medium which containing neutral red and 0.5% agarose. The plaques were observed after overnight incubation.

6.3.7 DNA Sequencing

The cDNA plasmids and rescued PRRSV were sequenced by ABI Prism 3130 Genetic Analyzer (Thermo Fisher Scientific). LaserGene Core Suite (DNASTAR Inc., Madison, WI) was used for sequence assembly and analysis.

6.4 RESULTS

6.4.1 Site-directed mutagenesis of A2MC2 infectious cDNA clone

Sequence analysis of A2MC2 genome identified five unique nucleotides (nt7621, nt9655, nt12012, nt12972, nt12975) compared to closely related VR-2332 and Ingelvac PRRS® MLV strains (Nan et al., 2012). These five nucleotides lead to non–synonymous mutations compared to MLV and VR-2332. These five nucleotides remain unchanged when A2MC2 is serially passaged for 90 times, and the A2MC2-P90 virus maintained the IFN induction capacity (Z. Ma et al., 2016). Gene swapping of A2MC2 with another strain that does not induce IFNs indicates that the middle half of the genome (nt4545-12709) is essential for A2MC2 induction of IFNs (Z. Ma et al., 2017). Among the five highly conserved unique nucleotides, three are located in the middle half of the genome. Therefore, we speculated that these nucleotides might correlate with the feature of A2MC2 IFN induction. To determine

their roles in the A2MC2 induction of IFNs, we selected these nucleotides to do site-directed mutagenesis of the A2MC2 cDNA clone.

Three mutant A2MC2 cDNA infectious clones were constructed as a DNA-launched version based on previously constructed pIR-A2MC2 plasmid (Fig. 6.1A) (Z. Ma et al.,

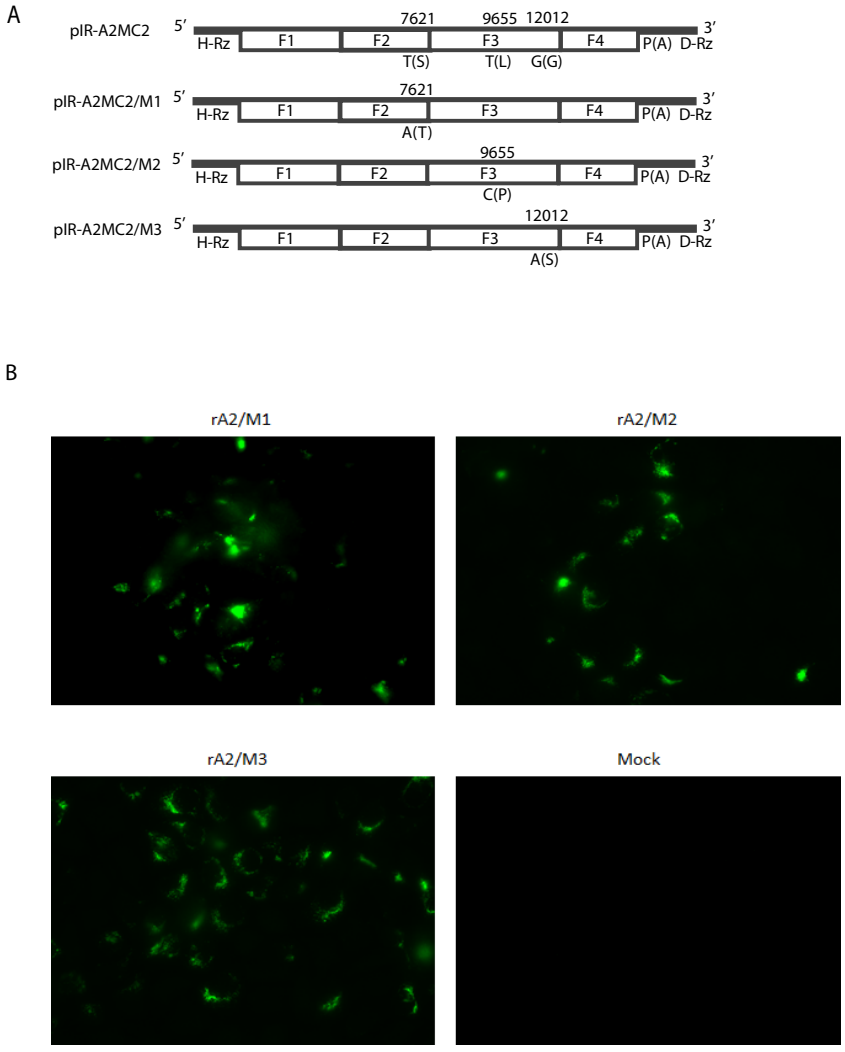

Fig.6. 1 Construction of mutant A2MC2 cDNA infectious clones

A. Strategy of site-directed mutagenesis in pIR-A2MC2: nt7621 (T to A, aa Ser to The), nt9655 (T to C, aa L to P), nt12012 (G to A, aa G to S). B. IFA of MARC-145 cells infected with recovered virus from the cDNA infectious clone of A2MC2-T7621A (rA2/M1), A2MC2-T9655C (rA2/M2), A2MC2-G12012A (rA2/M3).

2017). The three mutant clones contained T7621A, T9655C or G12012A mutations, respectively, and, as a result, had the same nucleotide in the corresponding position as VR-2332, the prototype of *PRRSV-2* that does not induce IFN. The T7621A, T9655C and G12012A mutant infectious clones were named as A2/M1, A2/M2, and A2/M3 respectively. The mutation in the plasmids was confirmed by DNA sequencing.

Transfection of MARC-145 cells with the plasmids of pIR-A2m1, pIR-A2m2, and pIR-A2m3 was done to recover the mutant virus. Passaging of the supernatant from lysate of the transfected cells in fresh MARC-145 cells resulted in successful recovery of the viruses. Typical PRRSV CPE was observed 48 h post inoculation (hpi). An N-specific monoclonal antibody of PRRSV was used to confirm the virus proliferation by IFA (Fig. 6.1B). The recovered viruses were also subjected to DNA sequencing and confirmed they were derived from the mutant infectious clones.

6.4.2 Determination of the growth property of the recovered virus

To compare the growth properties of the recovered A2MC2 mutant viruses (rA2/M1,

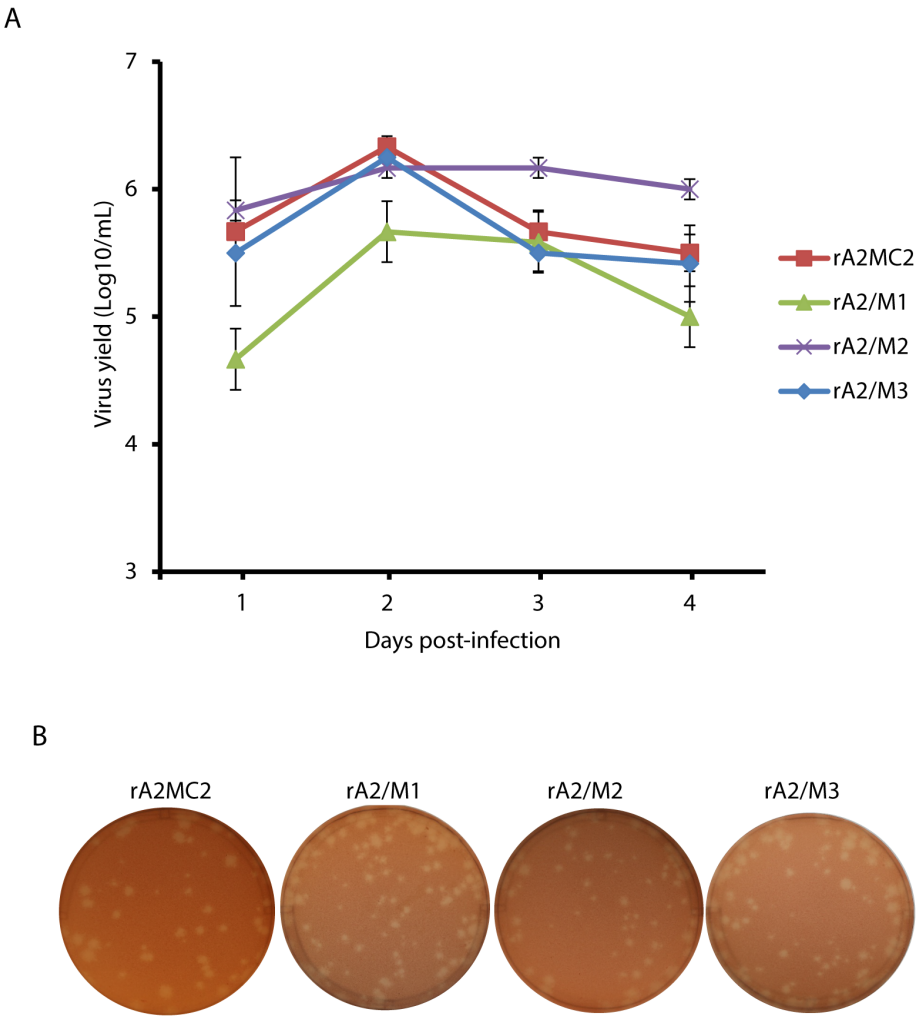

Fig.6. 2 Growth property of A2MC2 mutant viruses

A. Multi-step growth curve of A2MC2 mutant viruses. MARC-145 cells were infected with rA2MC2, rA2/M1, rA2/M2 and rA2/M3 viruses at an MOI of 0.1. Culture supernatant samples were daily collected for virus yield titration. The virus titers are shown as Log10 TCID50/ml. Error bars represent variations of three repeated experiments. B. Plaque assay of A2MC2 mutant viruses and rA2MC2 virus in MARC-145 cells.

rA2/M2, and rA2/M3) with the wild-type virus, the growth kinetics and plaque assay were

performed in MARC-145 cells. The cells were inoculated at an MOI (multiplicity of infection) of 0.1. All these viruses reached peak titer ($TCID_{50}$/mL) at 48 hpi, ranging from $10^{5.5}$ to $10^{6.5}$ $TCID_{50}$/ml (Fig. 6.2A). The growth kinetic patterns of the mutant rA2/M1, rA2/M2, and rA2/M3 were similar to the parental wild-type A2MC2 virus. However, the rA2/M1 yield was lower than the other mutants and wild-type A2MC2 virus at 24 and 48 hpi.

Plaque assay of rA2/M1, rA2/M2, rA2/M3 and wild-type A2MC2 virus was conducted in MARC-145 cells. Four days post infection, all the viruses developed plaques with the similar sizes around 3-4 mm in diameter (Fig. 2B). The results suggest that the rA2/M1, rA2/M2 and rA2/M3 mutant viruses have a similar growth property to the wild-type virus.

6.4.3 The nt12012 of the A2MC2 genome is essential for IFN induction

After recovery of these mutant rA2/M1, rA2/M2 and rA2/M3 virus, interferon

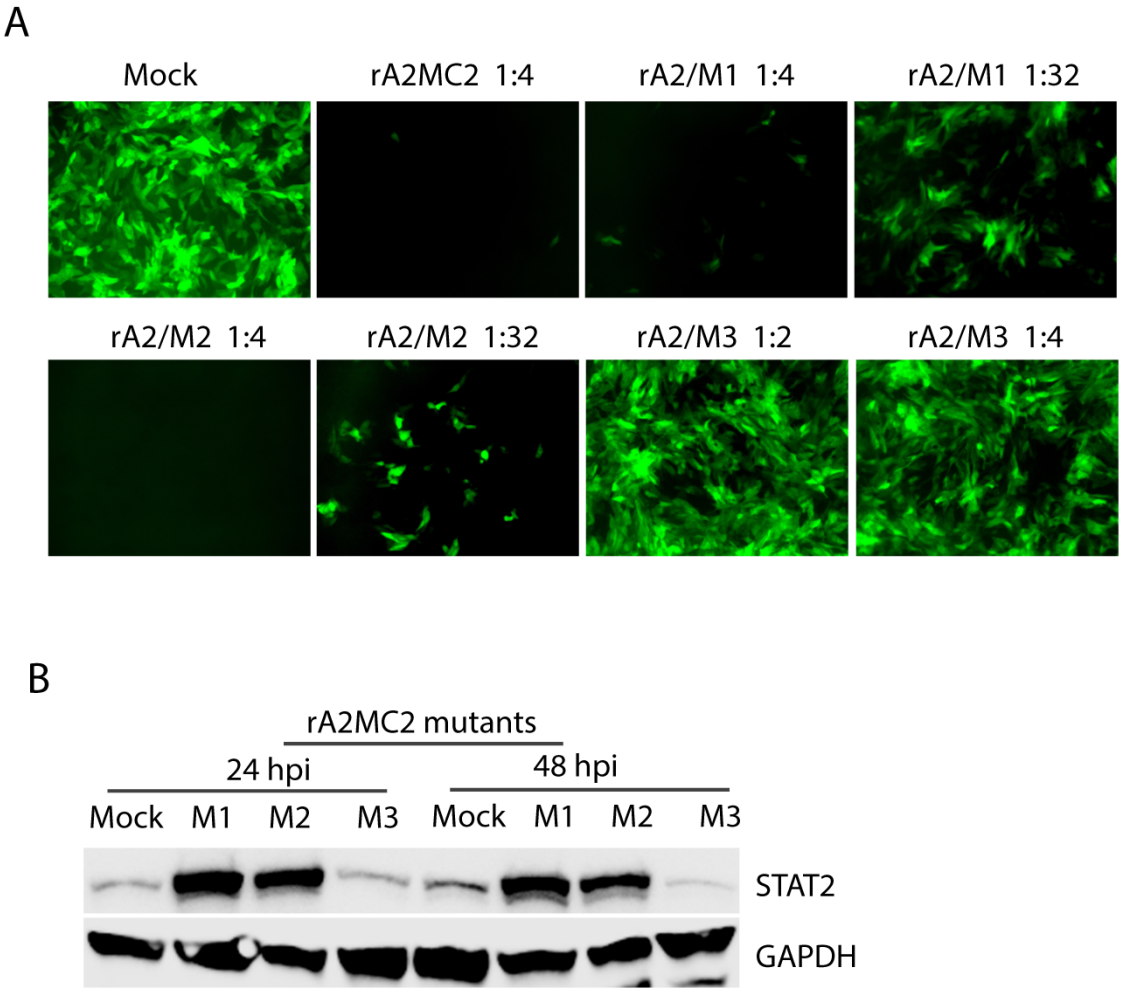

Fig.6. 3 The nt12012 of A2MC2 is the critical nucleotide for IFN induction.

A. Interferon bioassay in Vero cells. Cell culture supernatants from MARC-145 cells infected with recovered A2MC2 mutant viruses were used to treat Vero cells at dilutions indicated. Fluorescence microscopy was conducted at 24 h after NDV-GFP inoculation. The reduction of GFP-positive cells indicates inhibition of NDV-GFP replication. B. STAT2 protein level in MARC-145 cells infected with recovered mutant A2MC2 viruses.

bioassay was conducted to determine their capacity of IFN induction in comparison with the wild-type A2MC2 virus. Supernatant from MARC-145 cells infected with these mutant

viruses was used to treat Vero cells, which are not susceptible to PRRSV infection. On next day, Vero cells were inoculated with NDV-GFP that is sensitive to IFNs and used as an indicator for the presence of IFN in the MARC-145 culture supernatant samples. Results showed that NDV-GFP failed to grow in the Vero cells treated with the supernatant of rA2/M1 and rA2/M2 diluted up to 1/32, which is similar to the wild-type A2MC2 (Fig. 6.3A). In contrast, NDV-GFP replicated well in the Vero cells treated with the supernatant of rA2/M3 even at 1/2 dilution (Fig. 6.3A). This indicates that rA2/M3 lost the capacity to induce the production of type I IFNs, and consequently, its culture supernatant failed to induce the antiviral state of Vero cells to suppress NDV replication.

To confirm the observation, we determined the protein level of STAT2, an interferon-stimulated gene, in infected MARC-145 cells by Western blotting. The results showed that the STAT2 level increased in rA2/M1 and rA2/M2-infected MARC-145 cells compared to mock-infected cells. In contrast, the rA2/M3-infected cells had a similar STAT2 level to the mock-infected cells. This suggests that rA2/M3 did not induce IFN production in MARC-145 cells. Together, our data indicate that the nt12012 in A2MC2 is crucial for its IFN induction.

6.5 DISCUSSION

PRRSV is known to antagonize the innate immune response mainly through suppressing IFN production and IFN related signaling pathways (Albina, Carrat, et al., 1998; S. M. Lee et al., 2004; Miller et al., 2004). Only two strains of PRRSV have been reported to induce IFN production. A high-pathogenic PRRSV strain was reported to induce IFNs in infected pigs due to its remarkable replication rate and induction of exuberant inflammatory response (Guo et al., 2013). It is not known whether it can induce IFNs in the cultured cells. Another strain inducing IFN is generated from a synthetic infectious clone based on 59 wild-

type PRRSV sequences (Vu et al., 2015). It is believed that this IFN induction ability is related to first 3.3 kb of the genome (H. Sun et al., 2016). A2MC2 is an atypical virus with the unique feature of IFN induction (Nan et al., 2012). However, the mechanism of A2MC2 IFN induction is very different with other two strains. The middle half of the A2MC2 genome is essential for interferon induction (Z. Ma et al., 2017). Considering the importance of interferons in the innate and adaptive immunity and the possibility this feature may be useful in vaccine development against PRRS, this study on the mechanism of A2MC2 induction of IFNs provides informational results.

In this study, we constructed three mutant A2MC2 infectious clones and successfully recovered the mutant virus. Our results indicate that the mutant rA2/M3 virus with G12012A mutation lost the ability to induce IFNs and consequently, supernatant from its infected MARC-145 cells failed to induce the antiviral state in Vero cells to suppress NDV-GFP replication. Furthermore, rA2/M3 mutant virus was unable to increase the protein level of STAT2. All the data indicate that the nt12012 of the A2MC2 genome is crucial for its IFN induction.

Our finding of this single nucleotide mutation in A2MC2 abolishes its capacity of IFN induction is interesting and intriguing. The mechanism for this mutation-induced loss of IFN induction is unknown. A preliminary co-immunoprecipitation indicates that A2MC2 dsRNA co-precipitated with MDA5, whereas VR-2385 failed to do so (data not shown). The caveat of this experiment was that A2MC2 induces MDA5 elevation as A2MC2 induces IFN and MDA5 is an ISG, while VR-2385 inhibits IFN induction and IFN-activated signaling. We speculate that A2MC2 induces IFN via biding MDA5, not RIG-I. The possible reasons for the mutant A2MC2-induced feature change are that the single nucleotide mutation leads to change in A2MC2 RNA secondary or tertiary structure, and consequently, the host PRR fails to bind to the viral RNA; or that there was difference in the nsp12 protein (nt12012 is located

at nsp12 sequence) between A2MC2 and VR-2332, and, as a result, mutant A2MC2 nsp12 gains function to inhibit IFN induction. The first speculation makes more sense as MDA5 recognizes the viral dsRNA and the mutation potentially results in disruption of the interaction. The possibility of the second speculation is small as we compared the nsp12 proteins from A2MC2 and VR-2385 in IFN-β reporter assay and did not observe any differences. Further work is needed to elucidate the mechanism of A2MC2 induction of IFN synthesis, which might be unique.

For rA2/M1, it had similar plaque size to others but had lower virus yield at the first two days. This discrepancy is unexpected and needs further work to confirm. The lower virus yield of rA2/M1 might be possibly due to the mutation of T7621A or other unidentified mutation in the genome. The first speculation might be plausible as VR-2332 grows slower than A2MC2. If it were true, the result would suggest that nt7621 might be important for the virus growth. Further work is needed to test this speculation.

In conclusion, the A2MC2 ability to induce IFN is tightly correlated to nt12012, not nt7621 or nt9615, in A2MC2 genome. The mutation at nt12012 might cause a change in A2MC2 RNA structure that is unable to be recognized by host PRR. The results from this study provide insights to the mechanism of the intriguing IFN induction feature of A2MC2. Further study is needed to define the mechanism of the IFN induction including the PRR stimulated and the PAMP presented. This feature of IFN induction is worth to be further explored for the development of an improved vaccine against PRRS.

6.6 TABLE

Table 6. 1 Primers used in Chapter 6

Primer*	Sequence (5’ to 3’)
A2-4095F	TGGCTTTTGCTGTTGGTCTGTTC
A2-8077R	GAGCGTCGCCGCGCCTAATGTC
A2-7255F	GGATGAGGACCGTTTGAATAAG
9627R	GCGTACGGGGCCGGGGCCCCGCAGTACCCGCACACTCTCGACTTCTTCCCTTC
9627F	GAAGGGAAGAAGTCGAGAGTGTGCGGGTACTGCGGGGCCCcGGCCCCGTACG C
A2-13200R	CCGCCGTCGACTTGATGTTGGTAA
9627F	GAAGGGAAGAAGTCGAGAGTGTGCGGGTACTGCGGGGCCCcGGCCCCGTACG C
9627R	GCGTACGGGGCCGGGGCCCCGCAGTACCCGCACACTCTCGACTTCTTCCCTTC

*The primers were designed on the basis of the PRRSV A2MC2 cDNA sequence (GenBank accession number JQ087873). F, forward; R, reverse.

Chapter 7: Conclusions and Perspectives

It has been over three decades since PRRS was first reported. PRRS is still the top challenge to the global swine industry, although PRRSV virology, pathogenesis, and immune responses have been extensively studied and numerous vaccines are marketed or under testing/development. A better vaccine is much needed for PRRS control and elimination. The main focus of this dissertation research has been on vaccine development based on A2MC2, an atypical IFN-inducing PRRSV strain, and on studying its genetic source for IFN induction. Serial passaging of A2MC2 leads to attenuation of this virus, as shown in Chapter 4. The data from this study demonstrate that the high passaged A2MC2 (A2MC2-P90) has successfully been attenuated as indicated by experimental pig infection. However, more importantly, A2MC2-P90 retains the feature of interferon induction and elicits higher virus neutralizing antibodies than Ingelvac PRRS® MLV strain. The A2MC2-P90 propagates much faster with a higher yield and larger plaque size than the wild type A2MC2 virus. Sequencing comparison reveals that A2MC2-P90 contains a deletion of 543 nucleotides in the nsp2 hypervariable domain. The spontaneous deletion occurs around passage 60. The five unique amino acid residues in A2MC2 remain unchanged in A2MC2-P90.

Moreover, a DNA-launched A2MC2 cDNA infectious clone has been constructed to explore the mechanism of IFN synthesis induction by A2MC2. The recovered virus (rA2MC2) retains the feature of IFN induction and has similar growth kinetics and plaque sizes to the parental virus. The rA2MC2 has similar virulence and replication kinetics in pigs compared to its parental A2MC2 virus. Following the A2MC2 infectious clone construction, a set of chimeric A2MC2/VR2385 infectious clones were also constructed through genome swapping to further explore the genetic sources of IFN induction. Although all three chimeric viruses (rA2F2, rA2F123 and rA2F3) share similar growth trend, virus titer, and plaque sizes,

they have variable IFN induction ability. Only rA2F123 induces the IFN synthesis, whereas rA2F2 and rA2F3 fail to do so. Thus, the genomic source for the IFN induction must be from the middle half of the genome (nt4545-12709).

To further define the genomic source for the IFN induction, more detailed studies with site-directed mutagenesis were carried out in chapter 6. All three mutant viruses have similar growth kinetics and plaque sizes compared to the wild-type A2MC2 virus. Interestingly, only the nt12012 mutant virus loses the capacity to induce IFN synthesis, whereas other two mutant viruses stimulate IFN production and similar STAT2 level compared to the wild-type virus. The results indicate that the single nucleotide nt12012 is pivotal for A2MC2 induction of IFN.

A future study regarding A2MC2 can be conducted in two aspects. One aspect is about A2MC2 characterization and interaction with host PRR. Our results indicate that the middle half genome and nt12012 of A2MC2 contribute to the PAMP recognition by host PRR. Yet, there are many unanswered questions, including: What are the PRR for A2MC2, RIG-I or MDA5? RIG-I was previously reported to be the PRR interfered by PRRSV to antagonize IFN induction. However, for strain A2MC2, the role of RIG-I is unknown. What is the real PAMP for A2MC2 inducing IFN, viral protein or RNA? Mutations occurred in high-passage A2MC2-P90 compared with the wild-type virus. What are their roles in the higher rate replication of A2MC2-P90? Does A2MC2-P90 use cellular genes more efficiently for better replication? What are these genes and their potential roles in the viral replication? Addressing these questions will be future research directions.

Another aspect for A2MC2 research is vaccine development. Many factors may determine whether a vaccine works or not. Until now no PRRSV vaccine can meet all expectations proposed by scientists back in 2007. However, DIVA and marker vaccines are favorite choices for swine producers. The unique feature of A2MC2-P90 makes it as a DIVA

vaccine candidate. Nsp2 deletion part in A2MC2-P90 will be explored as a DIVA feature to differentiate vaccinated and infected animals. Furthermore, a foreign gene will be inserted between ORF1b and ORF2a for marker vaccine development. Combined with the IFN induction of A2MC2-P90, the foreign gene can be either fluoresce protein gene, like GFP for easy live cell observation, or a host cytokine gene, such as IFN-γ or IL-12, to enhance the virus capacity to elicit protective immune response. In a word, further research on the promising A2MC2-P90 virus is warranted for an improved vaccine against PRRS.

Bibliography

Adams, M. J., Lefkowitz, E. J., King, A. M., Harrach, B., Harrison, R. L., Knowles, N. J., . . . Davison, A. J. (2016). Ratification vote on taxonomic proposals to the International Committee on Taxonomy of Viruses (2016). *Arch Virol, 161*(10), 2921-2949. doi:10.1007/s00705-016-2977-6

Albina, E., Carrat, C., & Charley, B. (1998). Interferon-alpha response to swine arterivirus (PoAV), the porcine reproductive and respiratory syndrome virus. *J Interferon Cytokine Res, 18*(7), 485-490.

Albina, E., Piriou, L., Hutet, E., Cariolet, R., & L'Hospitalier, R. (1998). Immune responses in pigs infected with porcine reproductive and respiratory syndrome virus (PRRSV). *Vet Immunol Immunopathol, 61*(1), 49-66.

An, T. Q., Tian, Z. J., Leng, C. L., Peng, J. M., & Tong, G. Z. (2011). Highly pathogenic porcine reproductive and respiratory syndrome virus, Asia. *Emerg Infect Dis, 17*(9), 1782-1784. doi:10.3201/eid1709.110411

Ansari, I. H., Kwon, B., Osorio, F. A., & Pattnaik, A. K. (2006). Influence of N-linked glycosylation of porcine reproductive and respiratory syndrome virus GP5 on virus infectivity, antigenicity, and ability to induce neutralizing antibodies. *J Virol, 80*(8), 3994-4004. doi:10.1128/JVI.80.8.3994-4004.2006

Aquino-Lopez, A., Senyukov, V. V., Vlasic, Z., Kleinerman, E. S., & Lee, D. A. (2017). Interferon Gamma Induces Changes in Natural Killer (NK) Cell Ligand Expression and Alters NK Cell-Mediated Lysis of Pediatric Cancer Cell Lines. *Front Immunol, 8*, 391. doi:10.3389/fimmu.2017.00391

Bane, D. P., & Hall, W. F. (1990, 1990). *Fumonisin as a predisposing factor for "Mystery Swine Disease."*.

Bassaganya-Riera, J., Thacker, B. J., Yu, S., Strait, E., Wannemuehler, M. J., & Thacker, E. L. (2004). Impact of immunizations with porcine reproductive and respiratory syndrome virus on lymphoproliferative recall responses of CD8+ T cells. *Viral Immunol, 17*(1), 25-37. doi:10.1089/088282404322875430

Beura, L. K., Sarkar, S. N., Kwon, B., Subramaniam, S., Jones, C., Pattnaik, A. K., & Osorio, F. A. (2010). Porcine reproductive and respiratory syndrome virus nonstructural protein 1beta modulates host innate immune response by antagonizing IRF3 activation. *J Virol, 84*(3), 1574-1584. doi:10.1128/JVI.01326-09

Binjawadagi, B., Dwivedi, V., Manickam, C., Ouyang, K., Torrelles, J. B., & Renukaradhya, G. J. (2014). An innovative approach to induce cross-protective immunity against porcine reproductive and respiratory syndrome virus in the lungs of pigs through adjuvanted nanotechnology-based vaccination. *Int J Nanomedicine, 9*, 1519-1535. doi:10.2147/IJN.S59924

Binjawadagi, B., Dwivedi, V., Manickam, C., Ouyang, K., Wu, Y., Lee, L. J., . . . Renukaradhya, G. J. (2014). Adjuvanted poly(lactic-co-glycolic) acid nanoparticle-entrapped inactivated porcine reproductive and respiratory syndrome virus vaccine elicits cross-protective immune response in pigs. *Int J Nanomedicine, 9*, 679-694. doi:10.2147/IJN.S56127

Binjawadagi, B., Lakshmanappa, Y. S., Longchao, Z., Dhakal, S., Hiremath, J., Ouyang, K., . . . Renukaradhya, G. J. (2016). Development of a porcine reproductive and respiratory syndrome virus-like-particle-based vaccine and evaluation of its immunogenicity in pigs. *Arch Virol, 161*(6), 1579-1589. doi:10.1007/s00705-016-2812-0

Botner, A., Strandbygaard, B., Sorensen, K. J., Have, P., Madsen, K. G., Madsen, E. S., & Alexandersen, S. (1997). Appearance of acute PRRS-like symptoms in sow herds after vaccination with a modified live PRRS vaccine. *Vet Rec, 141*(19), 497-499.

Brockmeier, S. L., Lager, K. M., Grubman, M. J., Brough, D. E., Ettyreddy, D., Sacco, R. E., . . . Lehmkuhl, H. D. (2009). Adenovirus-mediated expression of interferon-alpha delays viral replication and reduces disease signs in swine challenged with porcine reproductive and respiratory syndrome virus. *Viral Immunol, 22*(3), 173-180. doi:10.1089/vim.2008.0075

Brockmeier, S. L., Loving, C. L., Nelson, E. A., Miller, L. C., Nicholson, T. L., Register, K. B., . . . Kehrli, M. E., Jr. (2012). The presence of alpha interferon at the time of infection alters the innate and adaptive immune responses to porcine reproductive and respiratory syndrome virus. *Clin Vaccine Immunol, 19*(4), 508-514. doi:10.1128/CVI.05490-11

Brown, E., Lawson, S., Welbon, C., Gnanandarajah, J., Li, J., Murtaugh, M. P., . . . Fang, Y. (2009). Antibody response to porcine reproductive and respiratory syndrome virus (PRRSV) nonstructural proteins and implications for diagnostic detection and differentiation of PRRSV types I and II. *Clin Vaccine Immunol, 16*(5), 628-635. doi:10.1128/CVI.00483-08

Bruns, A. M., & Horvath, C. M. (2015). LGP2 synergy with MDA5 in RLR-mediated RNA recognition and antiviral signaling. *Cytokine, 74*(2), 198-206. doi:10.1016/j.cyto.2015.02.010

Buddaert, W., Van Reeth, K., & Pensaert, M. (1998). In vivo and in vitro interferon (IFN) studies with the porcine reproductive and respiratory syndrome virus (PRRSV). *Advaced Experimental Medical Biology, 440*, 461-467.

Burkard, C., Lillico, S. G., Reid, E., Jackson, B., Mileham, A. J., Ait-Ali, T., . . . Archibald, A. L. (2017). Precision engineering for PRRSV resistance in pigs: Macrophages from genome edited pigs lacking CD163 SRCR5 domain are fully resistant to both PRRSV genotypes while maintaining biological function. *PLoS Pathog, 13*(2), 28. doi:10.1371/journal.ppat.1006206

Cai, J., Ma, Y., Li, J., Yan, C., Hu, R., & Zhang, J. (2010). Construction and characterization of a recombinant canine adenovirus expressing GP5 and M proteins of porcine reproductive and respiratory syndrome virus. *J Vet Med Sci, 72*(8), 1035-1040.

Calvert, J. G., Slade, D. E., Shields, S. L., Jolie, R., Mannan, R. M., Ankenbauer, R. G., & Welch, S. K. (2007). CD163 expression confers susceptibility to porcine reproductive and respiratory syndrome viruses. *J Virol, 81*(14), 7371-7379. doi:10.1128/JVI.00513-07

CFSPH. (2018). Vaccines : Porcine Reproductive Respiratory Syndrome (PRRS). Retrieved from

http://www.cfsph.iastate.edu/Vaccines/disease_list.php?disease=porcine-reproductive-respiratory-syndrome&lang=en

Chang, C. C., Yoon, K. J., Zimmerman, J. J., Harmon, K. M., Dixon, P. M., Dvorak, C. M., & Murtaugh, M. P. (2002). Evolution of porcine reproductive and respiratory syndrome virus during sequential passages in pigs. *J Virol, 76*(10), 4750-4763.

Charerntantanakul, W. (2012). Porcine reproductive and respiratory syndrome virus vaccines: Immunogenicity, efficacy and safety aspects. *World J Virol, 1*(1), 23-30. doi:10.5501/wjv.v1.i1.23

Chaung, H. C., Chen, C. W., Hsieh, B. L., & Chung, W. B. (2010). Toll-Like Receptor expressions in porcine alveolar macrophages and Dendritic Cells in responding to poly IC stimulation and porcine reproductive and respiratory syndrome virus (PRRSV) infection. *Comp Immunol Microbiol Infect Dis, 33*(3), 197-213. doi:10.1016/j.cimid.2008.10.001

Chen, J., Shi, X., Zhang, X., Wang, A., Wang, L., Yang, Y., . . . Zhang, G. P. (2017). MicroRNA 373 Facilitates the Replication of Porcine Reproductive and Respiratory Syndrome Virus by Its Negative Regulation of Type I Interferon Induction. *J Virol, 91*(3). doi:10.1128/JVI.01311-16

Chen, J., Xu, X., Tao, H., Li, Y., Nan, H., Wang, Y., . . . Chen, H. (2017). Structural Analysis of Porcine Reproductive and Respiratory Syndrome Virus Non-structural Protein 7alpha (NSP7alpha) and Identification of Its Interaction with NSP9. *Front Microbiol, 8*, 853. doi:10.3389/fmicb.2017.00853

Chen, X., Zhang, Q. Y., Bai, J., Zhao, Y. X., Wang, X. W., Wang, H. Y., & Jiang, P. (2017). The Nucleocapsid Protein and Nonstructural Protein 10 of Highly Pathogenic Porcine Reproductive and Respiratory Syndrome Virus Enhance CD83 Production via NF-kappa B and Sp1 Signaling Pathways. *Journal of virology, 91*(18), 26. doi:10.1128/jvi.00986-17

Chen, Z., Lawson, S., Sun, Z., Zhou, X., Guan, X., Christopher-Hennings, J., . . . Fang, Y. (2010). Identification of two auto-cleavage products of nonstructural protein 1 (nsp1) in porcine reproductive and respiratory syndrome virus infected cells: nsp1 function as interferon antagonist. *Virology, 398*(1), 87-97. doi:10.1016/j.virol.2009.11.033

Collins, J. E. (1991). Newly recognized respiratory syndromes in North American swine herds. *American Association of Swine Practitioners Newsletter, 3*(7), 7.

Collins, J. E., Benfield, D. A., Christianson, W. T., Harris, L., Hennings, J. C., Shaw, D. P., . . . Joo, H. S. (1992). Isolation of swine infertility and respiratory syndrome virus (isolate ATCC VR-2332) in North America and experimental reproduction of the disease in gnotobiotic pigs. *Journal of Veterinary Diagnostic Investigation, 4*(2), 117-126.

Costers, S., Vanhee, M., Van Breedam, W., Van Doorsselaere, J., Geldhof, M., & Nauwynck, H. J. (2010). GP4-specific neutralizing antibodies might be a driving force in PRRSV evolution. *Virus Res, 154*(1-2), 104-113. doi:10.1016/j.virusres.2010.08.026

Daniels, E. D. (1990, 1990). *Chlamydia proteins found in swine reproductive failure cases*.

Darnell, J. E., Jr., Kerr, I. M., & Stark, G. R. (1994). Jak-STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins. *Science, 264*(5164), 1415-1421.

Darwich, L., Coma, G., Pena, R., Bellido, R., Blanco, E. J., Este, J. A., . . . Bofill, M. (2009). Secretion of interferon-gamma by human macrophages demonstrated at the single-cell level after costimulation with interleukin (IL)-12 plus IL-18. *Immunology, 126*(3), 386-393. doi:10.1111/j.1365-2567.2008.02905.x

Das, P. B., Dinh, P. X., Ansari, I. H., de Lima, M., Osorio, F. A., & Pattnaik, A. K. (2010). The Minor Envelope Glycoproteins GP2a and GP4 of Porcine Reproductive and Respiratory Syndrome Virus Interact with the Receptor CD163. *Journal of virology, 84*(4), 1731-1740. doi:10.1128/jvi.01774-09

Das, P. B., Vu, H. L., Dinh, P. X., Cooney, J. L., Kwon, B., Osorio, F. A., & Pattnaik, A. K. (2011). Glycosylation of minor envelope glycoproteins of porcine reproductive and respiratory syndrome virus in infectious virus recovery, receptor interaction, and immune response. *Virology, 410*(2), 385-394. doi:10.1016/j.virol.2010.12.002

de Lima, M., Ansari, I. H., Das, P. B., Ku, B. J., Martinez-Lobo, F. J., Pattnaik, A. K., & Osorio, F. A. (2009). GP3 is a structural component of the PRRSV type II (US) virion. *Virology, 390*(1), 31-36. doi:10.1016/j.virol.2009.04.017

de Lima, M., Kwon, B., Ansari, I. H., Pattnaik, A. K., Flores, E. F., & Osorio, F. A. (2008). Development of a porcine reproductive and respiratory syndrome virus differentiable (DIVA) strain through deletion of specific immunodominant epitopes. *Vaccine, 26*(29-30), 3594-3600. doi:10.1016/j.vaccine.2008.04.078

de Lima, M., Pattnaik, A. K., Flores, E. F., & Osorio, F. A. (2006). Serologic marker candidates identified among B-cell linear epitopes of Nsp2 and structural proteins of a North American strain of porcine reproductive and respiratory syndrome virus. *Virology, 353*(2), 410-421. doi:10.1016/j.virol.2006.05.036

de Veer, M. J., Holko, M., Frevel, M., Walker, E., Der, S., Paranjape, J. M., . . . Williams, B. R. (2001). Functional classification of interferon-stimulated genes identified using microarrays. *J Leukoc Biol, 69*(6), 912-920.

de Vries, A. A., Chirnside, E. D., Horzinek, M. C., & Rottier, P. J. (1992). Structural proteins of equine arteritis virus. *J Virol, 66*(11), 6294-6303.

Dea, S., Gagnon, C. A., Mardassi, H., Pirzadeh, B., & Rogan, D. (2000). Current knowledge on the structural proteins of porcine reproductive and respiratory syndrome (PRRS) virus: comparison of the North American and European isolates. *Arch Virol, 145*(4), 659-688.

Dea, S., Sawyer, N., Alain, R., & Athanassious, R. (1995). Ultrastructural characteristics and morphogenesis of porcine reproductive and respiratory syndrome virus propagated in the highly permissive MARC-145 cell clone. In *Corona-and Related Viruses* (pp. 95-98): Springer.

Dee, S., Joo, H. S., & Pijoan, C. (1995). Controlling the spread of PRRS virus in the breeding herd through management of the gilt pool. *Swine health and production: the official journal of the American Association of Swine Practitioners (USA)*.

Dee, S. A., & Joo, H. S. (1994). Prevention of the spread of porcine reproductive and respiratory syndrome virus in endemically infected pig herds by nursery depopulation. *Vet Rec, 135*(1), 6-9.

Delputte, P. L., Costers, S., & Nauwynck, H. J. (2005). Analysis of porcine reproductive and respiratory syndrome virus attachment and internalization: distinctive roles for heparan sulphate and sialoadhesin. *J Gen Virol, 86*(Pt 5), 1441-1445. doi:10.1099/vir.0.80675-0

Delputte, P. L., Van Breedam, W., Delrue, I., Oetke, C., Crocker, P. R., & Nauwynck, H. J. (2007). Porcine arterivirus attachment to the macrophage-specific receptor sialoadhesin is dependent on the sialic acid-binding activity of the N-terminal immunoglobulin domain of sialoadhesin. *J Virol, 81*(17), 9546-9550. doi:10.1128/JVI.00569-07

Delputte, P. L., Vanderheijden, N., Nauwynck, H. J., & Pensaert, M. B. (2002). Involvement of the matrix protein in attachment of porcine reproductive and respiratory syndrome virus to a heparinlike receptor on porcine alveolar macrophages. *J Virol, 76*(9), 4312-4320.

Derganc, J., Antonny, B., & Copic, A. (2013). Membrane bending: the power of protein imbalance. *Trends Biochem Sci, 38*(11), 576-584. doi:10.1016/j.tibs.2013.08.006

Diaz, I., Darwich, L., Pappaterra, G., Pujols, J., & Mateu, E. (2006). Different European-type vaccines against porcine reproductive and respiratory syndrome virus have different immunological properties and confer different protection to pigs. *Virology, 351*(2), 249-259. doi:10.1016/j.virol.2006.03.046

Doan, D. N., & Dokland, T. (2003). Structure of the nucleocapsid protein of porcine reproductive and respiratory syndrome virus. *Structure, 11*(11), 1445-1451.

Dokland, T. (2010). The structural biology of PRRSV. *Virus Res, 154*(1-2), 86-97. doi:10.1016/j.virusres.2010.07.029

Dong, J. G., Zhang, N., Ge, X. N., Zhou, L., Guo, X., & Yang, H. C. (2014). The interaction of nonstructural protein 9 with retinoblastoma protein benefits the replication of genotype 2 porcine reproductive and respiratory syndrome virus in vitro. *Virology, 464*, 432-440. doi:10.1016/j.virol.2014.07.036

Duan, X., Nauwynck, H. J., Favoreel, H., & Pensaert, M. B. (1998). Porcine reproductive and respiratory syndrome virus infection of alveolar macrophages can be blocked by monoclonal antibodies against cell surface antigens. *Adv Exp Med Biol, 440*, 81-88.

Dwivedi, V., Manickam, C., Binjawadagi, B., Joyappa, D., & Renukaradhya, G. J. (2012). Biodegradable nanoparticle-entrapped vaccine induces cross-protective immune response against a virulent heterologous respiratory viral infection in pigs. *PLoS One, 7*(12), e51794. doi:10.1371/journal.pone.0051794

Elazhary, Y., Weber, J., Bikour, H., Morin, M., & Girard, C. (1991). 'Mystery swine disease'in Canada. *Veterinary record, 129*(22), 495-496.

Ellingson, J. S., Wang, Y., Layton, S., Ciacci-Zanella, J., Roof, M. B., & Faaberg, K. S. (2010). Vaccine efficacy of porcine reproductive and respiratory syndrome virus chimeras. *Vaccine, 28*(14), 2679-2686. doi:10.1016/j.vaccine.2009.12.073

Faaberg, K. S., Balasuriya, U. B., Brinton, M. A., Gorbalenya, A. E., Leung, F. C.-C., Nauwynck, H., . . . Yoo, D. (2012). Family Arteriviridae. In A. M. Q. King, M. J. Adams, E. B. Carstens, & E. J. Lefkowitz (Eds.), *Virus taxonomy: classification and nomenclature of viruses: Ninth Report of the International Committee on Taxonomy of Viruses* (pp. 796-805). San Diego: Elsevier Academic Press.

Fang, Y., Christopher-Hennings, J., Brown, E., Liu, H., Chen, Z., Lawson, S. R., . . . Nelson, E. (2008). Development of genetic markers in the non-structural protein 2 region of a US type 1 porcine reproductive and respiratory syndrome virus: implications for future recombinant marker vaccine development. *J Gen Virol, 89*(Pt 12), 3086-3096. doi:10.1099/vir.0.2008/003426-0

Fang, Y., Rowland, R. R., Roof, M., Lunney, J. K., Christopher-Hennings, J., & Nelson, E. A. (2006). A full-length cDNA infectious clone of North American type 1 porcine reproductive and respiratory syndrome virus: expression of green fluorescent protein in the Nsp2 region. *J Virol, 80*(23), 11447-11455. doi:10.1128/JVI.01032-06

Fang, Y., & Snijder, E. J. (2010). The PRRSV replicase: exploring the multifunctionality of an intriguing set of nonstructural proteins. *Virus Res, 154*(1-2), 61-76. doi:10.1016/j.virusres.2010.07.030

Fang, Y., Treffers, E. E., Li, Y., Tas, A., Sun, Z., van der Meer, Y., . . . Firth, A. E. (2012). Efficient -2 frameshifting by mammalian ribosomes to synthesize an additional arterivirus protein. *Proc Natl Acad Sci U S A, 109*(43), E2920-2928. doi:10.1073/pnas.1211145109

Feitsma, H., Grooten, H. J., Schie, F. W. V., & Colenbrander, B. (1992). The effect of porcine epidemic abortion and respiratory syndrome (PEARS) on sperm production. *PrOf 12th Int Congr Anim Reprod*, 1710-1712.

Fensterl, V., & Sen, G. C. (2009). Interferons and viral infections. *Biofactors, 35*(1), 14-20. doi:10.1002/biof.6

Firth, A. E., Zevenhoven-Dobbe, J. C., Wills, N. M., Go, Y. Y., Balasuriya, U. B., Atkins, J. F., . . . Posthuma, C. C. (2011). Discovery of a small arterivirus gene that overlaps the GP5 coding sequence and is important for virus production. *J Gen Virol, 92*(Pt 5), 1097-1106. doi:10.1099/vir.0.029264-0

Frias-Staheli, N., Giannakopoulos, N. V., Kikkert, M., Taylor, S. L., Bridgen, A., Paragas, J., . . . Virgin, H. W. t. (2007). Ovarian tumor domain-containing viral proteases evade ubiquitin- and ISG15-dependent innate immune responses. *Cell Host Microbe, 2*(6), 404-416. doi:10.1016/j.chom.2007.09.014

Friesen, H. J., Stein, S., Evinger, M., Familletti, P. C., Moschera, J., Meienhofer, J., . . . Pestka, S. (1981). Purification and molecular characterization of human fibroblast interferon. *Arch Biochem Biophys, 206*(2), 432-450.

Garoff, H., Hewson, R., & Opstelten, D. J. (1998). Virus maturation by budding. *Microbiol Mol Biol Rev, 62*(4), 1171-1190.

Giroux, M., Schmidt, M., & Descoteaux, A. (2003). IFN-gamma-induced MHC class II expression: transactivation of class II transactivator promoter IV by IFN regulatory factor-1 is regulated by protein kinase C-alpha. *J Immunol, 171*(8), 4187-4194.

Gonzalez-Navajas, J. M., Lee, J., David, M., & Raz, E. (2012). Immunomodulatory functions of type I interferons. *Nat Rev Immunol, 12*(2), 125-135. doi:10.1038/nri3133

Gorbalenya, A. E., Enjuanes, L., Ziebuhr, J., & Snijder, E. J. (2006). Nidovirales: evolving the largest RNA virus genome. *Virus Res, 117*(1), 17-37. doi:10.1016/j.virusres.2006.01.017

Goubau, D., Schlee, M., Deddouche, S., Pruijssers, A. J., Zillinger, T., Goldeck, M., . . . Reis e Sousa, C. (2014). Antiviral immunity via RIG-I-mediated recognition of RNA bearing 5'-diphosphates. *Nature, 514*(7522), 372-375. doi:10.1038/nature13590

Goyal, S. M. (1993). Porcine Reproductive and Respiratory Syndrome. *Journal of Veterinary Diagnostic Investigation, 5*(4), 656-664. doi:10.1177/104063879300500435

Groot Bramel-Verheije, M. H., Rottier, P. J., & Meulenberg, J. J. (2000). Expression of a foreign epitope by porcine reproductive and respiratory syndrome virus. *Virology, 278*(2), 380-389. doi:10.1006/viro.2000.0525

Guo, B., Lager, K. M., Henningson, J. N., Miller, L. C., Schlink, S. N., Kappes, M. A., . . . Faaberg, K. S. (2013). Experimental infection of United States swine with a Chinese highly pathogenic strain of porcine reproductive and respiratory syndrome virus. *Virology, 435*(2), 372-384. doi:10.1016/j.virol.2012.09.013

Halbur, P. G., Paul, P. S., Frey, M. L., Landgraf, J., Eernisse, K., Meng, X. J., . . . Rathje, J. A. (1995). Comparison of the pathogenicity of two US porcine reproductive and respiratory syndrome virus isolates with that of the Lelystad virus. *Vet Pathol, 32*(6), 648-660. doi:10.1177/030098589503200606

Halbur, P. G., Paul, P. S., Meng, X. J., Lum, M. A., Andrews, J. J., & Rathje, J. A. (1996). Comparative pathogenicity of nine US porcine reproductive and respiratory syndrome virus (PRRSV) isolates in a five-week-old cesarean-derived, colostrum-deprived pig model. *Journal of Veterinary Diagnostic Investigation, 8*(1), 11-20.

Han, J., Liu, G., Wang, Y., & Faaberg, K. S. (2007). Identification of nonessential regions of the nsp2 replicase protein of porcine reproductive and respiratory syndrome virus strain VR-2332 for replication in cell culture. *J Virol, 81*(18), 9878-9890. doi:10.1128/JVI.00562-07

Han, J., Rutherford, M. S., & Faaberg, K. S. (2009). The porcine reproductive and respiratory syndrome virus nsp2 cysteine protease domain possesses both trans- and cis-cleavage activities. *J Virol, 83*(18), 9449-9463. doi:10.1128/JVI.00834-09

Han, J., Wang, Y., & Faaberg, K. S. (2006). Complete genome analysis of RFLP 184 isolates of porcine reproductive and respiratory syndrome virus. *Virus Res, 122*(1-2), 175-182. doi:10.1016/j.virusres.2006.06.003

Han, M., Ke, H., Zhang, Q., & Yoo, D. (2017). Nuclear imprisonment of host cellular mRNA by nsp1beta protein of porcine reproductive and respiratory syndrome virus. *Virology, 505*, 42-55. doi:10.1016/j.virol.2017.02.004

Han, M., & Yoo, D. (2014a). Engineering the PRRS virus genome: updates and perspectives. *Vet Microbiol, 174*(3-4), 279-295. doi:10.1016/j.vetmic.2014.10.007

Han, M., & Yoo, D. (2014b). Modulation of innate immune signaling by nonstructural protein 1 (nsp1) in the family Arteriviridae. *Virus Res, 194*, 100-109. doi:10.1016/j.virusres.2014.09.007

Heil, F., Ahmad-Nejad, P., Hemmi, H., Hochrein, H., Ampenberger, F., Gellert, T., . . . Bauer, S. (2003). The Toll-like receptor 7 (TLR7)-specific stimulus loxoribine uncovers a strong relationship within the TLR7, 8 and 9 subfamily. *Eur J Immunol, 33*(11), 2987-2997. doi:10.1002/eji.200324238

Heit, A., Schmitz, F., Haas, T., Busch, D. H., & Wagner, H. (2007). Antigen co-encapsulated with adjuvants efficiently drive protective T cell immunity. *Eur J Immunol, 37*(8), 2063-2074. doi:10.1002/eji.200737169

Hertzog, P., Forster, S., & Samarajiwa, S. (2011). Systems biology of interferon responses. *J Interferon Cytokine Res, 31*(1), 5-11. doi:10.1089/jir.2010.0126

Holtkamp, D. J., Kliebenstein, J. B., Neumann, E. J., Zimmerman, J. J., Rotto, H. F., Yoder, T. K., . . . Haley, C. A. (2013). Assessment of the economic impact of porcine reproductive and respiratory syndrome virus on United States pork producers. *Journal of Swine Health and Production, 21*(2), 72-84.

Hopper, S. A., White, M. E., & Twiddy, N. (1992). An outbreak of blue-eared pig disease (porcine reproductive and respiratory syndrome) in four pig herds in Great Britain. *The Veterinary Record, 131*(7), 140-144.

Hou, F., Sun, L., Zheng, H., Skaug, B., Jiang, Q. X., & Chen, Z. J. (2011). MAVS forms functional prion-like aggregates to activate and propagate antiviral innate immune response. *Cell, 146*(3), 448-461. doi:10.1016/j.cell.2011.06.041

Huang, C., Zhang, Q., Guo, X. K., Yu, Z. B., Xu, A. T., Tang, J., & Feng, W. H. (2014). Porcine reproductive and respiratory syndrome virus nonstructural protein 4 antagonizes beta interferon expression by targeting the NF-kappaB essential modulator. *J Virol, 88*(18), 10934-10945. doi:10.1128/JVI.01396-14

Indraccolo, S., Pfeffer, U., Minuzzo, S., Esposito, G., Roni, V., Mandruzzato, S., . . . Amadori, A. (2007). Identification of genes selectively regulated by IFNs in endothelial cells. *J Immunol, 178*(2), 1122-1135.

Isaacs, A., & Lindenmann, J. (1957). Virus interference. I. The interferon. *Proc R Soc Lond B Biol Sci, 147*(927), 258-267.

Iwasaki, A., & Medzhitov, R. (2015). Control of adaptive immunity by the innate immune system. *Nat Immunol, 16*(4), 343-353. doi:10.1038/ni.3123

Jaczewska, J., Abdulreda, M. H., Yau, C. Y., Schmitt, M. M., Schubert, I., Berggren, P. O., . . . Wojcikiewicz, E. P. (2014). TNF-alpha and IFN-gamma promote lymphocyte adhesion to endothelial junctional regions facilitating transendothelial migration. *J Leukoc Biol, 95*(2), 265-274. doi:10.1189/jlb.0412205

Jiang, W., Jiang, P., Wang, X., Li, Y., Du, Y., & Wang, X. (2008). Enhanced immune responses of mice inoculated recombinant adenoviruses expressing GP5 by fusion with GP3 and/or GP4 of PRRS virus. *Virus Res, 136*(1-2), 50-57. doi:10.1016/j.virusres.2008.04.016

Jiang, Y., Fang, L., Xiao, S., Zhang, H., Pan, Y., Luo, R., . . . Chen, H. (2007). Immunogenicity and protective efficacy of recombinant pseudorabies virus expressing the two major membrane-associated proteins of porcine reproductive and respiratory syndrome virus. *Vaccine, 25*(3), 547-560. doi:10.1016/j.vaccine.2006.07.032

Jin, H., Zhou, L., Ge, X. N., Zhang, H., Zhang, R. M., Wang, C., . . . Guo, X. (2017). Cellular DEAD-box RNA helicase 18 (DDX18) Promotes the PRRSV Replication via Interaction with Virus nsp2 and nsp10. *Virus Research, 238*, 204-212. doi:10.1016/j.virusres.2017.05.028

Jing, H., Fang, L., Ding, Z., Wang, D., Hao, W., Gao, L., . . . Xiao, S. (2017). Porcine reproductive and respiratory syndrome virus nsp1α inhibits NF-κB activation by targeting the linear ubiquitin chain assembly complex. *Journal of virology, 91*(3), e01911-01916.

Johnson, C. R., Griggs, T. F., Gnanandarajah, J., & Murtaugh, M. P. (2011). Novel structural protein in porcine reproductive and respiratory syndrome virus encoded by an alternative ORF5 present in all arteriviruses. *J Gen Virol, 92*(Pt 5), 1107-1116. doi:10.1099/vir.0.030213-0

Joo, H. S. (1988). EMC virus causes poor reproduction. *Int Pigletter, 8*, 3-4.

Joo, H. S., Christianson, B., & Kim, H. (1990, 1990). *Encephalomyocarditis virus as a potential cause for "Mystery swine disease."*.

Jusa, E. R., Inaba, Y., Kouno, M., & Hirose, O. (1997). Effect of heparin on infection of cells by porcine reproductive and respiratory syndrome virus. *Am J Vet Res, 58*(5), 488-491.

Kane, M., Zang, T. M., Rihn, S. J., Zhang, F., Kueck, T., Alim, M., . . . Bieniasz, P. D. (2016). Identification of Interferon-Stimulated Genes with Antiretroviral Activity. *Cell Host Microbe, 20*(3), 392-405. doi:10.1016/j.chom.2016.08.005

Kappes, M. A., & Faaberg, K. S. (2015). PRRSV structure, replication and recombination: Origin of phenotype and genotype diversity. *Virology, 479-480*, 475-486. doi:10.1016/j.virol.2015.02.012

Kappes, M. A., Miller, C. L., & Faaberg, K. S. (2013). Highly divergent strains of porcine reproductive and respiratory syndrome virus incorporate multiple isoforms of nonstructural protein 2 into virions. *J Virol, 87*(24), 13456-13465. doi:10.1128/JVI.02435-13

Kawai, T., & Akira, S. (2006). Innate immune recognition of viral infection. *Nat Immunol, 7*(2), 131-137. doi:10.1038/ni1303

Kawai, T., & Akira, S. (2010). The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. *Nat Immunol, 11*(5), 373-384. doi:10.1038/ni.1863

Kawai, T., Sato, S., Ishii, K. J., Coban, C., Hemmi, H., Yamamoto, M., . . . Akira, S. (2004). Interferon-alpha induction through Toll-like receptors involves a direct interaction of IRF7 with MyD88 and TRAF6. *Nat Immunol, 5*(10), 1061-1068. doi:10.1038/ni1118

Keffaber, K. K. (1989). Reproductive failure of unknown etiol-ogy. *Am. Assoc. Swine Pract. Newsl, 1*, 1-9.

Kenney, S. P., & Meng, X. J. (2015). An SH3 binding motif within the nucleocapsid protein of porcine reproductive and respiratory syndrome virus interacts with

the host cellular signaling proteins STAMI, TXK, Fyn, Hck, and cortactin. *Virus Res, 204*, 31-39. doi:10.1016/j.virusres.2015.04.004

Kim, H., Kim, H. K., Jung, J. H., Choi, Y. J., Kim, J., Um, C. G., . . . Song, D. S. (2011). The assessment of efficacy of porcine reproductive respiratory syndrome virus inactivated vaccine based on the viral quantity and inactivation methods. *Virol J, 8*, 323. doi:10.1186/1743-422X-8-323

Kim, H. S., Kwang, J., Yoon, I. J., Joo, H. S., & Frey, M. L. (1993). Enhanced replication of porcine reproductive and respiratory syndrome (PRRS) virus in a homogeneous subpopulation of MA-104 cell line. *Arch Virol, 133*(3-4), 477-483.

Kim, O., Sun, Y., Lai, F. W., Song, C., & Yoo, D. (2010). Modulation of type I interferon induction by porcine reproductive and respiratory syndrome virus and degradation of CREB-binding protein by non-structural protein 1 in MARC-145 and HeLa cells. *Virology, 402*(2), 315-326. doi:10.1016/j.virol.2010.03.039

Kim, S. H., & Samal, S. K. (2010). Role of Untranslated Regions in Regulation of Gene Expression, Replication, and Pathogenicity of Newcastle Disease Virus Expressing Green Fluorescent Protein. *Journal of Virology, 84*(5), 2629-2634.

Knoops, K., Barcena, M., Limpens, R. W., Koster, A. J., Mommaas, A. M., & Snijder, E. J. (2012). Ultrastructural characterization of arterivirus replication structures: reshaping the endoplasmic reticulum to accommodate viral RNA synthesis. *J Virol, 86*(5), 2474-2487. doi:10.1128/JVI.06677-11

Kotenko, S. V., Gallagher, G., Baurin, V. V., Lewis-Antes, A., Shen, M., Shah, N. K., . . . Donnelly, R. P. (2003). IFN-lambdas mediate antiviral protection through a distinct class II cytokine receptor complex. *Nat Immunol, 4*(1), 69-77. doi:10.1038/ni875

Koyama, S., Ishii, K. J., Coban, C., & Akira, S. (2008). Innate immune response to viral infection. *Cytokine, 43*(3), 336-341. doi:10.1016/j.cyto.2008.07.009

Kraaij, M. D., Vereyken, E. J., Leenen, P. J., van den Bosch, T. P., Rezaee, F., Betjes, M. G., . . . Rowshani, A. T. (2014). Human monocytes produce interferon-gamma upon stimulation with LPS. *Cytokine, 67*(1), 7-12. doi:10.1016/j.cyto.2014.02.001

Kreutz, L. C. (1998). Cellular membrane factors are the major determinants of porcine reproductive and respiratory syndrome virus tropism. *Virus Res, 53*(2), 121-128.

Kroese, M. V., Zevenhoven-Dobbe, J. C., Bos-de Ruijter, J. N., Peeters, B. P., Meulenberg, J. J., Cornelissen, L. A., & Snijder, E. J. (2008). The nsp1alpha and nsp1 papain-like autoproteinases are essential for porcine reproductive and respiratory syndrome virus RNA synthesis. *J Gen Virol, 89*(Pt 2), 494-499. doi:10.1099/vir.0.83253-0

Kuhn, J. H., Lauck, M., Bailey, A. L., Shchetinin, A. M., Vishnevskaya, T. V., Bao, Y., . . . Goldberg, T. L. (2016). Reorganization and expansion of the nidoviral family Arteriviridae. *Arch Virol, 161*(3), 755-768. doi:10.1007/s00705-015-2672-z

Kwon, B., Ansari, I. H., Osorio, F. A., & Pattnaik, A. K. (2006). Infectious clone-derived viruses from virulent and vaccine strains of porcine reproductive and

respiratory syndrome virus mimic biological properties of their parental viruses in a pregnant sow model. *Vaccine, 24*(49-50), 7071-7080. doi:10.1016/j.vaccine.2006.07.010

Kwon, B., Ansari, I. H., Pattnaik, A. K., & Osorio, F. A. (2008). Identification of virulence determinants of porcine reproductive and respiratory syndrome virus through construction of chimeric clones. *Virology, 380*(2), 371-378. doi:10.1016/j.virol.2008.07.030

Lawson, S. R., Rossow, K. D., Collins, J. E., Benfield, D. A., & Rowland, R. R. (1997). Porcine reproductive and respiratory syndrome virus infection of gnotobiotic pigs: sites of virus replication and co-localization with MAC-387 staining at 21 days post-infection. *Virus Res, 51*(2), 105-113.

Leaman, D. W., Chawla-Sarkar, M., Jacobs, B., Vyas, K., Sun, Y., Ozdemir, A., . . . Borden, E. C. (2003). Novel growth and death related interferon-stimulated genes (ISGs) in melanoma: greater potency of IFN-beta compared with IFN-alpha2. *J Interferon Cytokine Res, 23*(12), 745-756. doi:10.1089/107999003772084860

Lee, C., & Yoo, D. (2006). The small envelope protein of porcine reproductive and respiratory syndrome virus possesses ion channel protein-like properties. *Virology, 355*(1), 30-43. doi:10.1016/j.virol.2006.07.013

Lee, S. M., Schommer, S. K., & Kleiboeker, S. B. (2004). Porcine reproductive and respiratory syndrome virus field isolates differ in in vitro interferon phenotypes. *Vet Immunol Immunopathol, 102*(3), 217-231.

Levy, D. E., & Darnell, J. E., Jr. (2002). Stats: transcriptional control and biological impact. *Nat Rev Mol Cell Biol, 3*(9), 651-662. doi:10.1038/nrm909

Li, H., Yang, J., Bao, D., Hou, J., Zhi, Y., Yang, Y., . . . Zhang, G. (2017). Development of an immunochromatographic strip for detection of antibodies against porcine reproductive and respiratory syndrome virus. *J Vet Sci, 18*(3), 307-316. doi:10.4142/jvs.2017.18.3.307

Li, J., & Murtaugh, M. P. (2012). Dissociation of porcine reproductive and respiratory syndrome virus neutralization from antibodies specific to major envelope protein surface epitopes. *Virology, 433*(2), 367-376. doi:10.1016/j.virol.2012.08.026

Li, J. N., Guo, D. W., Huang, L., Yin, M. M., Liu, Q. F., Wang, Y., . . . Weng, C. J. (2014). The interaction between host Annexin A2 and viral Nsp9 is beneficial for replication of porcine reproductive and respiratory syndrome virus. *Virus Research, 189*, 106-113. doi:10.1016/j.virusres.2014.05.015

Li, Y., Shyu, D. L., Shang, P., Bai, J., Ouyang, K., Dhakal, S., . . . Fang, Y. (2016). Mutations in a Highly Conserved Motif of nsp1beta Protein Attenuate the Innate Immune Suppression Function of Porcine Reproductive and Respiratory Syndrome Virus. *Journal of Virology, 90*(7), 3584-3599. doi:10.1128/JVI.03069-15

Li, Y., Treffers, E. E., Napthine, S., Tas, A., Zhu, L., Sun, Z., . . . van Hemert, M. J. (2014). Transactivation of programmed ribosomal frameshifting by a viral protein. *proceedings of the National Academy of Sciences, 111*(21), E2172-E2181.

Li, Y., Zhou, L., Zhang, J., Ge, X., Zhou, R., Zheng, H., . . . Yang, H. (2014). Nsp9 and Nsp10 contribute to the fatal virulence of highly pathogenic porcine reproductive and respiratory syndrome virus emerging in China. *PLoS Pathog, 10*(7), e1004216. doi:10.1371/journal.ppat.1004216

Li, Y. H., Tas, A., Sun, Z., Snijder, E. J., & Fang, Y. (2015). Proteolytic processing of the porcine reproductive and respiratory syndrome virus replicase. *Virus Research, 202*, 48-59. doi:10.1016/j.virusres.2014.12.027

Lin, T., Li, X., Yao, H., Wei, Z., Tan, F., Liu, R., . . . Yuan, S. (2012). Use of reverse genetics to develop a novel marker porcine reproductive and respiratory syndrome virus. *Virus Genes, 45*(3), 548-555. doi:10.1007/s11262-012-0812-z

Liu, L., Tian, J., Nan, H., Tian, M. M., Li, Y., Xu, X. D., . . . Chen, H. Y. (2016). Porcine Reproductive and Respiratory Syndrome Virus Nucleocapsid Protein Interacts with Nsp9 and Cellular DHX9 To Regulate Viral RNA Synthesis. *Journal of virology, 90*(11), 5384-5398. doi:10.1128/jvi.03216-15

Liu, Y. J. (2005). IPC: professional type 1 interferon-producing cells and plasmacytoid dendritic cell precursors. *Annu Rev Immunol, 23*, 275-306. doi:10.1146/annurev.immunol.23.021704.115633

Loo, Y. M., & Gale, M., Jr. (2011). Immune signaling by RIG-I-like receptors. *Immunity, 34*(5), 680-692. doi:10.1016/j.immuni.2011.05.003

Lopez, O. J., Oliveira, M. F., Garcia, E. A., Kwon, B. J., Doster, A., & Osorio, F. A. (2007). Protection against porcine reproductive and respiratory syndrome virus (PRRSV) infection through passive transfer of PRRSV-neutralizing antibodies is dose dependent. *Clin Vaccine Immunol, 14*(3), 269-275.

Lopez, O. J., & Osorio, F. A. (2004). Role of neutralizing antibodies in PRRSV protective immunity. *Vet Immunol Immunopathol, 102*(3), 155-163. doi:10.1016/j.vetimm.2004.09.005

Loula, T. (1991). Mystery pig disease. *Agri-practice, 12*(1), 23-34.

Lunney, J. K., Fang, Y., Ladinig, A., Chen, N., Li, Y., Rowland, B., & Renukaradhya, G. J. (2016). Porcine Reproductive and Respiratory Syndrome Virus (PRRSV): Pathogenesis and Interaction with the Immune System. *Annu Rev Anim Biosci, 4*, 129-154. doi:10.1146/annurev-animal-022114-111025

Luo, D., Ding, S. C., Vela, A., Kohlway, A., Lindenbach, B. D., & Pyle, A. M. (2011). Structural insights into RNA recognition by RIG-I. *Cell, 147*(2), 409-422. doi:10.1016/j.cell.2011.09.023

Luo, R., Fang, L., Jiang, Y., Jin, H., Wang, Y., Wang, D., . . . Xiao, S. (2011). Activation of NF-kappaB by nucleocapsid protein of the porcine reproductive and respiratory syndrome virus. *Virus Genes, 42*(1), 76-81. doi:10.1007/s11262-010-0548-6

Luo, R., Xiao, S., Jiang, Y., Jin, H., Wang, D., Liu, M., . . . Fang, L. (2008). Porcine reproductive and respiratory syndrome virus (PRRSV) suppresses interferon-beta production by interfering with the RIG-I signaling pathway. *Mol Immunol, 45*(10), 2839-2846.

Ma, Z., Wang, Y., Zhao, H., Xu, A. T., Wang, Y., Tang, J., & Feng, W. H. (2013). Porcine reproductive and respiratory syndrome virus nonstructural protein 4

induces apoptosis dependent on its 3C-like serine protease activity. *PLoS One, 8*(7), e69387. doi:10.1371/journal.pone.0069387
Ma, Z., Yang, L., & Zhang, Y. J. (2018). Porcine Reproductive and Respiratory Syndrome Virus: Propagation and Quantification. *Current protocols in microbiology*, 15M. 11.11-15M. 11.14.
Ma, Z., Yu, Y., Xiao, Y., Opriessnig, T., Wang, R., Yang, L., . . . Zhang, Y. J. (2016). Sustaining Interferon Induction by a High-Passage Atypical Porcine Reproductive and Respiratory Syndrome Virus Strain. *Sci Rep, 6*, 36312. doi:10.1038/srep36312
Ma, Z., Yu, Y., Xiao, Y., Opriessnig, T., Wang, R., Yang, L., . . . Zhang, Y. J. (2017). The middle half genome of interferon-inducing porcine reproductive and respiratory syndrome virus strain A2MC2 is essential for interferon induction. *J Gen Virol, 98*(7), 1720-1729. doi:10.1099/jgv.0.000819
MacMicking, J. D. (2012). Interferon-inducible effector mechanisms in cell-autonomous immunity. *Nat Rev Immunol, 12*(5), 367-382. doi:10.1038/nri3210
Madsen, K. G., Hansen, C. M., Madsen, E. S., Strandbygaard, B., Botner, A., & Sorensen, K. J. (1998). Sequence analysis of porcine reproductive and respiratory syndrome virus of the American type collected from Danish swine herds. *Arch Virol, 143*(9), 1683-1700.
Makarova, K. S., Aravind, L., & Koonin, E. V. (2000). A novel superfamily of predicted cysteine proteases from eukaryotes, viruses and Chlamydia pneumoniae. *Trends Biochem Sci, 25*(2), 50-52.
Manolaridis, I., Gaudin, C., Posthuma, C. C., Zevenhoven-Dobbe, J. C., Imbert, I., Canard, B., . . . Snijder, E. J. (2011). Structure and genetic analysis of the arterivirus nonstructural protein 7alpha. *J Virol, 85*(14), 7449-7453. doi:10.1128/JVI.00255-11
Mardassi, H., Athanassious, R., Mounir, S., & Dea, S. (1994). Porcine reproductive and respiratory syndrome virus: morphological, biochemical and serological characteristics of Quebec isolates associated with acute and chronic outbreaks of porcine reproductive and respiratory syndrome. *Can J Vet Res, 58*(1), 55-64.
Mardassi, H., Gonin, P., Gagnon, C. A., Massie, B., & Dea, S. (1998). A subset of porcine reproductive and respiratory syndrome virus GP3 glycoprotein is released into the culture medium of cells as a non-virion-associated and membrane-free (soluble) form. *J Virol, 72*(8), 6298-6306.
Martinez, F. O., & Gordon, S. (2014). The M1 and M2 paradigm of macrophage activation: time for reassessment. *F1000Prime Rep, 6*, 13. doi:10.12703/P6-13
Matsuda, T., & Cepko, C. L. (2004). Electroporation and RNA interference in the rodent retina in vivo and in vitro. *Proc Natl Acad Sci U S A, 101*(1), 16-22. doi:10.1073/pnas.2235688100
McCaw, M. B. (1995, 1995). *MCREBEL PRRS: Management procedures for PRRS Control in large herd nurseries*.
McGuffin, L. J., Bryson, K., & Jones, D. T. (2000). The PSIPRED protein structure prediction server. *Bioinformatics, 16*(4), 404-405.

Meier, W. A., Galeota, J., Osorio, F. A., Husmann, R. J., Schnitzlein, W. M., & Zuckermann, F. A. (2003). Gradual development of the interferon-gamma response of swine to porcine reproductive and respiratory syndrome virus infection or vaccination. *Virology, 309*(1), 18-31.

Meng, X. J. (2000). Heterogeneity of porcine reproductive and respiratory syndrome virus: implications for current vaccine efficacy and future vaccine development. *Vet Microbiol, 74*(4), 309-329.

Meredith, M. J. (1992). Review of porcine reproductive and respiratory syndrome.

Meulenberg, J. J. (2000). PRRSV, the virus. *Vet Res, 31*(1), 11-21. doi:10.1051/vetres:2000103

Meulenberg, J. J., Bos-de Ruijter, J. N., van de Graaf, R., Wensvoort, G., & Moormann, R. J. (1998). Infectious transcripts from cloned genome-length cDNA of porcine reproductive and respiratory syndrome virus. *J Virol, 72*(1), 380-387.

Meulenberg, J. J., van Nieuwstadt, A. P., van Essen-Zandbergen, A., & Langeveld, J. P. (1997). Posttranslational processing and identification of a neutralization domain of the GP4 protein encoded by ORF4 of Lelystad virus. *J Virol, 71*(8), 6061-6067.

Miller, L. C., Laegreid, W. W., Bono, J. L., Chitko-McKown, C. G., & Fox, J. M. (2004). Interferon type I response in porcine reproductive and respiratory syndrome virus-infected MARC-145 cells. *Arch Virol, 149*(12), 2453-2463. doi:10.1007/s00705-004-0377-9

Misinzo, G., Delputte, P. L., Meerts, P., Drexler, C., & Nauwynck, H. J. (2006). Efficacy of an inactivated PRRSV vaccine: induction of virus-neutralizing antibodies and partial virological protection upon challenge. *Adv Exp Med Biol, 581*, 449-454. doi:10.1007/978-0-387-33012-9_81

Morrison, R., Bautista, E., Goyal, S., Collins, J., & Annelli, J. (1992). Seroprevalence of SIRS virus in the United States in 1990. *AASP Newsletter, 4*(4), 47.

Moynagh, P. N. (2005). TLR signalling and activation of IRFs: revisiting old friends from the NF-kappaB pathway. *Trends Immunol, 26*(9), 469-476. doi:10.1016/j.it.2005.06.009

Murtaugh, M. P., & Genzow, M. (2011). Immunological solutions for treatment and prevention of porcine reproductive and respiratory syndrome (PRRS). *Vaccine, 29*(46), 8192-8204. doi:10.1016/j.vaccine.2011.09.013

Music, N., & Gagnon, C. A. (2010). The role of porcine reproductive and respiratory syndrome (PRRS) virus structural and non-structural proteins in virus pathogenesis. *Anim Health Res Rev, 11*(2), 135-163. doi:10.1017/S1466252310000034

Nam, H. M., Chae, K. S., Song, Y. J., Lee, N. H., Lee, J. B., Park, S. Y., . . . Choi, I. S. (2013). Immune responses in mice vaccinated with virus-like particles composed of the GP5 and M proteins of porcine reproductive and respiratory syndrome virus. *Arch Virol, 158*(6), 1275-1285. doi:10.1007/s00705-013-1612-z

Nan, Y., Wang, R., Shen, M., Faaberg, K. S., Samal, S. K., & Zhang, Y. J. (2012). Induction of type I interferons by a novel porcine reproductive and respiratory

syndrome virus isolate. *Virology, 432*(2), 261-270. doi:10.1016/j.virol.2012.05.015

Nan, Y., Wu, C., & Zhang, Y. J. (2017). Interplay between Janus Kinase/Signal Transducer and Activator of Transcription Signaling Activated by Type I Interferons and Viral Antagonism. *Front Immunol, 8*, 1758. doi:10.3389/fimmu.2017.01758

Nelsen, C. J., Murtaugh, M. P., & Faaberg, K. S. (1999). Porcine reproductive and respiratory syndrome virus comparison: divergent evolution on two continents. *J Virol, 73*(1), 270-280.

Ni, Y. Y., Huang, Y. W., Cao, D., Opriessnig, T., & Meng, X. J. (2011). Establishment of a DNA-launched infectious clone for a highly pneumovirulent strain of type 2 porcine reproductive and respiratory syndrome virus: identification and in vitro and in vivo characterization of a large spontaneous deletion in the nsp2 region. *Virus Res, 160*(1-2), 264-273. doi:10.1016/j.virusres.2011.06.027

Nielsen, H. S., Liu, G., Nielsen, J., Oleksiewicz, M. B., Botner, A., Storgaard, T., & Faaberg, K. S. (2003). Generation of an infectious clone of VR-2332, a highly virulent North American-type isolate of porcine reproductive and respiratory syndrome virus. *Journal of Virology, 77*(6), 3702-3711.

O'Brien, T. R., Prokunina-Olsson, L., & Donnelly, R. P. (2014). IFN-lambda4: the paradoxical new member of the interferon lambda family. *J Interferon Cytokine Res, 34*(11), 829-838. doi:10.1089/jir.2013.0136

Oleksiewicz, M. B., Botner, A., & Normann, P. (2002). Porcine B-cells recognize epitopes that are conserved between the structural proteins of American- and European-type porcine reproductive and respiratory syndrome virus. *J Gen Virol, 83*(Pt 6), 1407-1418. doi:10.1099/0022-1317-83-6-1407

Oleksiewicz, M. B., Botner, A., Toft, P., Normann, P., & Storgaard, T. (2001). Epitope mapping porcine reproductive and respiratory syndrome virus by phage display: the nsp2 fragment of the replicase polyprotein contains a cluster of B-cell epitopes. *J Virol, 75*(7), 3277-3290. doi:10.1128/JVI.75.7.3277-3290.2001

Onofre, G., Kolackova, M., Jankovicova, K., & Krejsek, J. (2009). Scavenger receptor CD163 and its biological functions. *Acta Medica (Hradec Kralove), 52*(2), 57-61.

Opriessnig, T., Halbur, P. G., Yoon, K. J., Pogranichniy, R. M., Harmon, K. M., Evans, R., . . . Meng, X. J. (2002). Comparison of molecular and biological characteristics of a modified live porcine reproductive and respiratory syndrome virus (PRRSV) vaccine (ingelvac PRRS MLV), the parent strain of the vaccine (ATCC VR2332), ATCC VR2385, and two recent field isolates of PRRSV. *J Virol, 76*(23), 11837-11844.

Osorio, F. A., Galeota, J. A., Nelson, E., Brodersen, B., Doster, A., Wills, R., . . . Laegreid, W. W. (2002). Passive transfer of virus-specific antibodies confers protection against reproductive failure induced by a virulent strain of porcine reproductive and respiratory syndrome virus and establishes sterilizing immunity. *Virology, 302*(1), 9-20.

Park, B. K., Yoon, I. J., & Joo, H. S. (1996). Pathogenesis of plaque variants of porcine reproductive and respiratory syndrome virus in pregnant sows. *Am J Vet Res, 57*(3), 320-323.

Pasternak, A. O., Spaan, W. J., & Snijder, E. J. (2004). Regulation of relative abundance of arterivirus subgenomic mRNAs. *J Virol, 78*(15), 8102-8113. doi:10.1128/JVI.78.15.8102-8113.2004

Patel, D., Nan, Y., Shen, M., Ritthipichai, K., Zhu, X., & Zhang, Y. J. (2010). Porcine reproductive and respiratory syndrome virus inhibits type I interferon signaling by blocking STAT1/STAT2 nuclear translocation. *J Virol, 84*(21), 11045-11055. doi:10.1128/JVI.00655-10

Patel, D., Opriessnig, T., Stein, D. A., Halbur, P. G., Meng, X. J., Iversen, P. L., & Zhang, Y. J. (2008). Peptide-conjugated morpholino oligomers inhibit porcine reproductive and respiratory syndrome virus replication. *Antiviral Res, 77*(2), 95-107.

Patel, D., Stein, D. A., & Zhang, Y. J. (2009). Morpholino oligomer-mediated protection of porcine pulmonary alveolar macrophages from arterivirus-induced cell death. *Antivir Ther, 14*(7), 899-909. doi:10.3851/IMP1409

Paton, D. J., Brown, I. H., Edwards, S., & Wensvoort, G. (1991). 'Blue ear'disease of pigs. *Veterinary record, 128*(26), 617-617.

Pei, Y., Hodgins, D. C., Wu, J., Welch, S. K., Calvert, J. G., Li, G., . . . Yoo, D. (2009). Porcine reproductive and respiratory syndrome virus as a vector: immunogenicity of green fluorescent protein and porcine circovirus type 2 capsid expressed from dedicated subgenomic RNAs. *Virology, 389*(1-2), 91-99. doi:10.1016/j.virol.2009.03.036

Pestka, S. (2007). The interferons: 50 years after their discovery, there is much more to learn. *J Biol Chem, 282*(28), 20047-20051. doi:10.1074/jbc.R700004200

Pestka, S., Krause, C. D., & Walter, M. R. (2004). Interferons, interferon-like cytokines, and their receptors. *Immunological Reviews, 202*, 8-32. doi:10.1111/j.0105-2896.2004.00204.x

Piras, F., Bollard, S., Laval, F., Joisel, F., Reynaud, G., Charreyre, C., . . . Juillard, V. (2005). Porcine reproductive and respiratory syndrome (PRRS) virus-specific interferon-gamma(+) T-cell responses after PRRS virus infection or vaccination with an inactivated PRRS vaccine. *Viral Immunol, 18*(2), 381-389. doi:10.1089/vim.2005.18.381

Platanias, L. C. (2005). Mechanisms of type-I- and type-II-interferon-mediated signalling. *Nat Rev Immunol, 5*(5), 375-386. doi:10.1038/nri1604

Pol, J. M., Wagenaar, F., & Reus, J. E. (1997). Comparative morphogenesis of three PRRS virus strains. *Vet Microbiol, 55*(1-4), 203-208.

Pujhari, S., Baig, T. T., & Zakhartchouk, A. N. (2014). Potential Role of Porcine Reproductive and Respiratory Syndrome Virus Structural Protein GP2 in Apoptosis Inhibition. *Biomed Research International*, 7. doi:10.1155/2014/160505

Pujhari, S., & Zakhartchouk, A. N. (2016). Porcine reproductive and respiratory syndrome virus envelope (E) protein interacts with mitochondrial proteins and induces apoptosis. *Arch Virol, 161*(7), 1821-1830. doi:10.1007/s00705-016-2845-4

Qiu, H. J., Tian, Z. J., Tong, G. Z., Zhou, Y. J., Ni, J. Q., Luo, Y. Z., & Cai, X. H. (2005). Protective immunity induced by a recombinant pseudorabies virus expressing the GP5 of porcine reproductive and respiratory syndrome virus in piglets. *Vet Immunol Immunopathol, 106*(3-4), 309-319. doi:10.1016/j.vetimm.2005.03.008

Quaife, T. (1989). Scramble is on to solve mystery disease. *Swine Pract, July*, 5-10.

Racaniello, V. R., & Baltimore, D. (1981a). CLONED POLIOVIRUS COMPLEMENTARY-DNA IS INFECTIOUS IN MAMMALIAN-CELLS. *Science, 214*(4523), 916-919. doi:10.1126/science.6272391

Racaniello, V. R., & Baltimore, D. (1981b). MOLECULAR-CLONING OF POLIOVIRUS CDNA AND DETERMINATION OF THE COMPLETE NUCLEOTIDE-SEQUENCE OF THE VIRAL GENOME. *Proceedings of the National Academy of Sciences of the United States of America-Biological Sciences, 78*(8), 4887-4891. doi:10.1073/pnas.78.8.4887

Rajkhowa, T. K., Jagan Mohanarao, G., Gogoi, A., Hauhnar, L., & Isaac, L. (2015). Porcine reproductive and respiratory syndrome virus (PRRSV) from the first outbreak of India shows close relationship with the highly pathogenic variant of China. *Vet Q, 35*(4), 186-193. doi:10.1080/01652176.2015.1066043

Reid, E., Juleff, N., Windsor, M., Gubbins, S., Roberts, L., Morgan, S., . . . Seago, J. (2016). Type I and III IFNs Produced by Plasmacytoid Dendritic Cells in Response to a Member of the Flaviviridae Suppress Cellular Immune Responses. *J Immunol, 196*(10), 4214-4226. doi:10.4049/jimmunol.1600049

Renukaradhya, G. J., Meng, X. J., Calvert, J. G., Roof, M., & Lager, K. M. (2015a). Inactivated and subunit vaccines against porcine reproductive and respiratory syndrome: Current status and future direction. *Vaccine, 33*(27), 3065-3072. doi:10.1016/j.vaccine.2015.04.102

Renukaradhya, G. J., Meng, X. J., Calvert, J. G., Roof, M., & Lager, K. M. (2015b). Live porcine reproductive and respiratory syndrome virus vaccines: Current status and future direction. *Vaccine, 33*(33), 4069-4080. doi:10.1016/j.vaccine.2015.06.092

Reotutar, R. (1989). Swine reproductive failure syndrome mystifies scientists. *J Am Vet Med Assoc, 195*, 425-428.

Robinson, S. R., Figueiredo, M. C., Abrahante, J. E., & Murtaugh, M. P. (2013). Immune response to ORF5a protein immunization is not protective against porcine reproductive and respiratory syndrome virus infection. *Vet Microbiol, 164*(3-4), 281-285. doi:10.1016/j.vetmic.2013.03.006

Roca, M., Gimeno, M., Bruguera, S., Segales, J., Diaz, I., Galindo-Cardiel, I. J., . . . Mateu, E. (2012). Effects of challenge with a virulent genotype II strain of porcine reproductive and respiratory syndrome virus on piglets vaccinated with an attenuated genotype I strain vaccine. *Vet J, 193*(1), 92-96. doi:10.1016/j.tvjl.2011.11.019

Rock, D. (2007). Report: Colloquium on Prospects for Development of an Effective PRRS Virus Vaccine. *Perry, IA: American Association of Swine Veterinarians*.

Rossow, K. D. (1998). Porcine reproductive and respiratory syndrome. *Vet Pathol, 35*(1), 1-20. doi:10.1177/030098589803500101

Rossow, K. D., Collins, J. E., Goyal, S. M., Nelson, E. A., Christopher Hennings, J., & Benfield, D. A. (1995). Pathogenesis of porcine reproductive and respiratory syndrome virus infection in gnotobiotic pigs. *Veterinary Pathology, 32*(4), 361-373.

Rowland, R. R., Kervin, R., Kuckleburg, C., Sperlich, A., & Benfield, D. A. (1999). The localization of porcine reproductive and respiratory syndrome virus nucleocapsid protein to the nucleolus of infected cells and identification of a potential nucleolar localization signal sequence. *Virus Res, 64*(1), 1-12.

Rowland, R. R., Schneider, P., Fang, Y., Wootton, S., Yoo, D., & Benfield, D. A. (2003). Peptide domains involved in the localization of the porcine reproductive and respiratory syndrome virus nucleocapsid protein to the nucleolus. *Virology, 316*(1), 135-145.

Rusinova, I., Forster, S., Yu, S., Kannan, A., Masse, M., Cumming, H., . . . Hertzog, P. J. (2013). Interferome v2.0: an updated database of annotated interferon-regulated genes. *Nucleic Acids Res, 41*(Database issue), D1040-1046. doi:10.1093/nar/gks1215

Sanchez, C., Domenech, N., Vazquez, J., Alonso, F., Ezquerra, A., & Dominguez, J. (1999). The porcine 2A10 antigen is homologous to human CD163 and related to macrophage differentiation. *J Immunol, 162*(9), 5230-5237.

Sang, Y., Rowland, R. R., & Blecha, F. (2011). Porcine type I interferons: polymorphic sequences and activity against PRRSV. *BMC Proc, 5 Suppl 4*, S8. doi:10.1186/1753-6561-5-S4-S8

Schliehe, C., Redaelli, C., Engelhardt, S., Fehlings, M., Mueller, M., van Rooijen, N., . . . Groettrup, M. (2011). CD8- dendritic cells and macrophages cross-present poly(D,L-lactate-co-glycolate) acid microsphere-encapsulated antigen in vivo. *J Immunol, 187*(5), 2112-2121. doi:10.4049/jimmunol.1002084

Sharma, S., tenOever, B. R., Grandvaux, N., Zhou, G. P., Lin, R., & Hiscott, J. (2003). Triggering the interferon antiviral response through an IKK-related pathway. *Science, 300*(5622), 1148-1151. doi:10.1126/science.1081315

Shen, G., Jin, N., Ma, M., Jin, K., Zheng, M., Zhuang, T., . . . Jin, M. (2007). Immune responses of pigs inoculated with a recombinant fowlpox virus coexpressing GP5/GP3 of porcine reproductive and respiratory syndrome virus and swine IL-18. *Vaccine, 25*(21), 4193-4202. doi:10.1016/j.vaccine.2007.03.010

Shi, X. B., Wang, L., Li, X. W., Zhang, G. P., Guo, J. Q., Zhao, D., . . . Deng, R. G. (2011). Endoribonuclease activities of porcine reproductive and respiratory syndrome virus nsp11 was essential for nsp11 to inhibit IFN-beta induction. *Mol Immunol, 48*(12-13), 1568-1572. doi:10.1016/j.molimm.2011.03.004

Shi, Y. J., Li, Y. W., Lei, Y. Y., Ye, G., Shen, Z., Sun, L. M., . . . Peng, G. Q. (2016). A Dimerization-Dependent Mechanism Drives the Endoribonuclease Function of Porcine Reproductive and Respiratory Syndrome Virus nsp11. *Journal of virology, 90*(9), 4579-4592. doi:10.1128/jvi.03065-15

Siegal, F. P., Kadowaki, N., Shodell, M., Fitzgerald-Bocarsly, P. A., Shah, K., Ho, S., . . . Liu, Y. J. (1999). The nature of the principal type 1 interferon-producing cells in human blood. *Science, 284*(5421), 1835-1837.

Smith, D. M., Simon, J. K., & Baker, J. R., Jr. (2013). Applications of nanotechnology for immunology. *Nat Rev Immunol, 13*(8), 592-605. doi:10.1038/nri3488

Snijder, E. J., Dobbe, J. C., & Spaan, W. J. (2003). Heterodimerization of the two major envelope proteins is essential for arterivirus infectivity. *J Virol, 77*(1), 97-104.

Snijder, E. J., Kikkert, M., & Fang, Y. (2013). Arterivirus molecular biology and pathogenesis. *J Gen Virol, 94*(Pt 10), 2141-2163. doi:10.1099/vir.0.056341-0

Snijder, E. J., & Meulenberg, J. J. (1998). The molecular biology of arteriviruses. *J Gen Virol, 79 (Pt 5)*, 961-979. doi:10.1099/0022-1317-79-5-961

Snijder, E. J., van Tol, H., Pedersen, K. W., Raamsman, M. J., & de Vries, A. A. (1999). Identification of a novel structural protein of arteriviruses. *J Virol, 73*(8), 6335-6345.

Snijder, E. J., van Tol, H., Roos, N., & Pedersen, K. W. (2001). Non-structural proteins 2 and 3 interact to modify host cell membranes during the formation of the arterivirus replication complex. *J Gen Virol, 82*(Pt 5), 985-994. doi:10.1099/0022-1317-82-5-985

Snijder, E. J., Wassenaar, A. L., Spaan, W. J., & Gorbalenya, A. E. (1995). The arterivirus Nsp2 protease. An unusual cysteine protease with primary structure similarities to both papain-like and chymotrypsin-like proteases. *J Biol Chem, 270*(28), 16671-16676.

Sorensen, K. J., Botner, A., Madsen, E. S., Strandbygaard, B., & Nielsen, J. (1997). Evaluation of a blocking Elisa for screening of antibodies against porcine reproductive and respiratory syndrome (PRRS) virus. *Vet Microbiol, 56*(1-2), 1-8.

Spilman, M. S., Welbon, C., Nelson, E., & Dokland, T. (2009). Cryo-electron tomography of porcine reproductive and respiratory syndrome virus: organization of the nucleocapsid. *J Gen Virol, 90*(Pt 3), 527-535. doi:10.1099/vir.0.007674-0

Steen, H. C., & Gamero, A. M. (2013). STAT2 phosphorylation and signaling. *JAKSTAT, 2*(4), e25790. doi:10.4161/jkst.25790

Sun, H., Pattnaik, A. K., Osorio, F. A., & Vu, H. L. X. (2016). Identification of viral genes associated with the interferon-inducing phenotype of a synthetic porcine reproductive and respiratory syndrome virus strain. *Virology, 499*, 313-321. doi:10.1016/j.virol.2016.09.018

Sun, L., Li, Y., Liu, R., Wang, X., Gao, F., Lin, T., . . . Yuan, S. (2013). Porcine reproductive and respiratory syndrome virus ORF5a protein is essential for virus viability. *Virus Res, 171*(1), 178-185. doi:10.1016/j.virusres.2012.11.005

Sun, Y., Han, M., Kim, C., Calvert, J. G., & Yoo, D. (2012). Interplay between interferon-mediated innate immunity and porcine reproductive and respiratory syndrome virus. *Viruses, 4*(4), 424-446. doi:10.3390/v4040424

Sun, Y., Ke, H., Han, M., Chen, N., Fang, W., & Yoo, D. (2016). Nonstructural Protein 11 of Porcine Reproductive and Respiratory Syndrome Virus Suppresses Both MAVS and RIG-I Expression as One of the Mechanisms to Antagonize Type I Interferon Production. *PLoS One, 11*(12), e0168314. doi:10.1371/journal.pone.0168314

Sun, Y., Ke, H. Z., Han, M. Y., Chen, N., Fang, W. H., & Yoo, D. (2016). Nonstructural Protein 11 of Porcine Reproductive and Respiratory Syndrome Virus Suppresses Both MAVS and RIG-I Expression as One of the Mechanisms to Antagonize Type I Interferon Production. *PLoS One, 11*(12), 23. doi:10.1371/journal.pone.0168314

Sun, Y., Xue, F., Guo, Y., Ma, M., Hao, N., Zhang, X. C., . . . Rao, Z. (2009). Crystal structure of porcine reproductive and respiratory syndrome virus leader protease Nsp1alpha. *J Virol, 83*(21), 10931-10940. doi:10.1128/JVI.02579-08

Sun, Z., Chen, Z., Lawson, S. R., & Fang, Y. (2010). The cysteine protease domain of porcine reproductive and respiratory syndrome virus nonstructural protein 2 possesses deubiquitinating and interferon antagonism functions. *J Virol, 84*(15), 7832-7846. doi:10.1128/JVI.00217-10

Takaoka, A., & Yanai, H. (2006). Interferon signalling network in innate defence. *Cell Microbiol, 8*(6), 907-922. doi:10.1111/j.1462-5822.2006.00716.x

Teixeira, L. K., Fonseca, B. P., Barboza, B. A., & Viola, J. P. (2005). The role of interferon-gamma on immune and allergic responses. *Memorias do Instituto Oswaldo Cruz, 100*, 137-144.

Terpstra, C., Wensvoort, G., & Pol, J. M. A. (1991). Experimental reproduction of porcine epidemic abortion and respiratory syndrome (mystery swine disease) by infection with Lelystad vims: Koch's postulates fulfilled. *Veterinary Quarterly, 13*(3), 131-136.

Thaa, B., Kabatek, A., Zevenhoven-Dobbe, J. C., Snijder, E. J., Herrmann, A., & Veit, M. (2009). Myristoylation of the arterivirus E protein: the fatty acid modification is not essential for membrane association but contributes significantly to virus infectivity. *J Gen Virol, 90*(Pt 11), 2704-2712. doi:10.1099/vir.0.011957-0

Tian, K., Yu, X., Zhao, T., Feng, Y., Cao, Z., Wang, C., . . . Gao, G. F. (2007). Emergence of fatal PRRSV variants: unparalleled outbreaks of atypical PRRS in China and molecular dissection of the unique hallmark. *PLoS One, 2*(6), e526. doi:10.1371/journal.pone.0000526

Tian, X., Lu, G., Gao, F., Peng, H., Feng, Y., Ma, G., . . . Gao, G. F. (2009). Structure and cleavage specificity of the chymotrypsin-like serine protease (3CLSP/nsp4) of Porcine Reproductive and Respiratory Syndrome Virus (PRRSV). *J Mol Biol, 392*(4), 977-993. doi:10.1016/j.jmb.2009.07.062

Truong, H. M., Lu, Z., Kutish, G. F., Galeota, J., Osorio, F. A., & Pattnaik, A. K. (2004). A highly pathogenic porcine reproductive and respiratory syndrome virus generated from an infectious cDNA clone retains the in vivo virulence and transmissibility properties of the parental virus. *Virology, 325*(2), 308-319. doi:10.1016/j.virol.2004.04.046

Uze, G., Schreiber, G., Piehler, J., & Pellegrini, S. (2007). The receptor of the type I interferon family. *Curr Top Microbiol Immunol, 316*, 71-95.

Van Breedam, W., Delputte, P. L., Van Gorp, H., Misinzo, G., Vanderheijden, N., Duan, X., & Nauwynck, H. J. (2010). Porcine reproductive and respiratory syndrome virus entry into the porcine macrophage. *Journal of General Virology, 91*(7), 1659-1667.

Van Breedam, W., Van Gorp, H., Zhang, J. Q., Crocker, P. R., Delputte, P. L., & Nauwynck, H. J. (2010). The M/GP(5) glycoprotein complex of porcine reproductive and respiratory syndrome virus binds the sialoadhesin receptor in a sialic acid-dependent manner. *PLoS Pathog, 6*(1), e1000730. doi:10.1371/journal.ppat.1000730

van Dinten, L. C., Wassenaar, A. L., Gorbalenya, A. E., Spaan, W. J., & Snijder, E. J. (1996). Processing of the equine arteritis virus replicase ORF1b protein: identification of cleavage products containing the putative viral polymerase and helicase domains. *J Virol, 70*(10), 6625-6633.

Van Gorp, H., Van Breedam, W., Delputte, P. L., & Nauwynck, H. J. (2008). Sialoadhesin and CD163 join forces during entry of the porcine reproductive and respiratory syndrome virus. *J Gen Virol, 89*(Pt 12), 2943-2953. doi:10.1099/vir.0.2008/005009-0

Van Gorp, H., Van Breedam, W., Van Doorsselaere, J., Delputte, P. L., & Nauwynck, H. J. (2010). Identification of the CD163 protein domains involved in infection of the porcine reproductive and respiratory syndrome virus. *J Virol, 84*(6), 3101-3105. doi:10.1128/JVI.02093-09

van Marle, G., Dobbe, J. C., Gultyaev, A. P., Luytjes, W., Spaan, W. J., & Snijder, E. J. (1999). Arterivirus discontinuous mRNA transcription is guided by base pairing between sense and antisense transcription-regulating sequences. *Proc Natl Acad Sci U S A, 96*(21), 12056-12061.

Van Reeth, K., Labarque, G., Nauwynck, H., & Pensaert, M. (1999). Differential production of proinflammatory cytokines in the pig lung during different respiratory virus infections: correlations with pathogenicity. *Res Vet Sci, 67*(1), 47-52. doi:10.1053/rvsc.1998.0277

Vanderheijden, N., Delputte, P. L., Favoreel, H. W., Vandekerckhove, J., Van Damme, J., van Woensel, P. A., & Nauwynck, H. J. (2003). Involvement of sialoadhesin in entry of porcine reproductive and respiratory syndrome virus into porcine alveolar macrophages. *J Virol, 77*(15), 8207-8215.

Vanhee, M., Costers, S., Van Breedam, W., Geldhof, M. F., Van Doorsselaere, J., & Nauwynck, H. J. (2010). A variable region in GP4 of European-type porcine reproductive and respiratory syndrome virus induces neutralizing antibodies against homologous but not heterologous virus strains. *Viral Immunol, 23*(4), 403-413. doi:10.1089/vim.2010.0025

Vanhee, M., Delputte, P. L., Delrue, I., Geldhof, M. F., & Nauwynck, H. J. (2009). Development of an experimental inactivated PRRSV vaccine that induces virus-neutralizing antibodies. *Vet Res, 40*(6), 63. doi:10.1051/vetres/2009046

Veit, M., Matczuk, A. K., Sinhadri, B. C., Krause, E., & Thaa, B. (2014). Membrane proteins of arterivirus particles: structure, topology, processing and function. *Virus Res, 194*, 16-36. doi:10.1016/j.virusres.2014.09.010

Verheije, M. H., Welting, T. J. M., Jansen, H. T., Rottier, P. J. M., & Meulenberg, J. J. M. (2002). Chimeric arteriviruses generated by swapping of the M protein ectodomain rule out a role of this domain in viral targeting. *Virology, 303*(2), 364-373.

Vu, H. L., Kwon, B., Yoon, K. J., Laegreid, W. W., Pattnaik, A. K., & Osorio, F. A. (2011). Immune evasion of porcine reproductive and respiratory syndrome

virus through glycan shielding involves both glycoprotein 5 as well as glycoprotein 3. *J Virol, 85*(11), 5555-5564. doi:10.1128/JVI.00189-11

Vu, H. L., Ma, F., Laegreid, W. W., Pattnaik, A. K., Steffen, D., Doster, A. R., & Osorio, F. A. (2015). A Synthetic Porcine Reproductive and Respiratory Syndrome Virus Strain Confers Unprecedented Levels of Heterologous Protection. *J Virol, 89*(23), 12070-12083. doi:10.1128/JVI.01657-15

Walker, L., LeVine, H., & Jucker, M. (2006). Koch's postulates and infectious proteins. *Acta neuropathologica, 112*(1), 1.

Wang, D., Fan, J. X., Fang, L. R., Luo, R., Ouyang, H. P., Ouyang, C., . . . Xiao, S. B. (2015). The nonstructural protein 11 of porcine reproductive and respiratory syndrome virus inhibits NF-kappa B signaling by means of its deubiquitinating activity. *Mol Immunol, 68*(2), 357-366. doi:10.1016/j.molimm.2015.08.011

Wang, F. X., Song, N., Chen, L. Z., Cheng, S. P., Wu, H., & Wen, Y. J. (2013). Non-structural protein 2 of the porcine reproductive and respiratory syndrome (PRRS) virus: a crucial protein in viral pathogenesis, immunity and diagnosis. *Res Vet Sci, 95*(1), 1-7. doi:10.1016/j.rvsc.2013.03.015

Wang, H., Liu, R., Zhang, W., Sun, L., Ning, Z., Ji, F., . . . Zhang, G. (2017). Identification of epitopes on nonstructural protein 7 of porcine reproductive and respiratory syndrome virus recognized by monoclonal antibodies using phage-display technology. *Virus Genes, 53*(4), 623-635. doi:10.1007/s11262-017-1472-9

Wang, R., Nan, Y., Yu, Y., Yang, Z., & Zhang, Y. J. (2013). Variable interference with interferon signal transduction by different strains of porcine reproductive and respiratory syndrome virus. *Vet Microbiol, 166*(3-4), 493-503. doi:10.1016/j.vetmic.2013.07.022

Wang, R., Nan, Y., Yu, Y., & Zhang, Y. J. (2013). Porcine reproductive and respiratory syndrome virus Nsp1beta inhibits interferon-activated JAK/STAT signal transduction by inducing karyopherin-alpha1 degradation. *J Virol, 87*(9), 5219-5228. doi:10.1128/JVI.02643-12

Wang, R., Xiao, Y., Opriessnig, T., Ding, Y., Yu, Y., Nan, Y., . . . Zhang, Y. J. (2013). Enhancing neutralizing antibody production by an interferon-inducing porcine reproductive and respiratory syndrome virus strain. *Vaccine, 31*(47), 5537-5543. doi:10.1016/j.vaccine.2013.09.023

Wang, R., & Zhang, Y. J. (2014). Antagonizing interferon-mediated immune response by porcine reproductive and respiratory syndrome virus. *Biomed Res Int, 2014*, 315470. doi:10.1155/2014/315470

Wang, Y., Liang, Y., Han, J., Burkhart, K. M., Vaughn, E. M., Roof, M. B., & Faaberg, K. S. (2008). Attenuation of porcine reproductive and respiratory syndrome virus strain MN184 using chimeric construction with vaccine sequence. *Virology, 371*(2), 418-429. doi:10.1016/j.virol.2007.09.032

Wei, Z., Lin, T., Sun, L., Li, Y., Wang, X., Gao, F., . . . Yuan, S. (2012). N-linked glycosylation of GP5 of porcine reproductive and respiratory syndrome virus is critically important for virus replication in vivo. *J Virol, 86*(18), 9941-9951. doi:10.1128/JVI.07067-11

Wei, Z., Tian, D., Sun, L., Lin, T., Gao, F., Liu, R., . . . Yuan, S. (2012). Influence of N-linked glycosylation of minor proteins of porcine reproductive and respiratory syndrome virus on infectious virus recovery and receptor interaction. *Virology, 429*(1), 1-11. doi:10.1016/j.virol.2012.03.010

Welch, S. K., Jolie, R., Pearce, D. S., Koertje, W. D., Fuog, E., Shields, S. L., . . . Calvert, J. G. (2004). Construction and evaluation of genetically engineered replication-defective porcine reproductive and respiratory syndrome virus vaccine candidates. *Vet Immunol Immunopathol, 102*(3), 277-290. doi:10.1016/j.vetimm.2004.09.022

Wensvoort, G., Terpstra, C., Pol, J. M., ter Laak, E. A., Bloemraad, M., de Kluyver, E. P., . . . et al. (1991). Mystery swine disease in The Netherlands: the isolation of Lelystad virus. *Vet Q, 13*(3), 121-130. doi:10.1080/01652176.1991.9694296

Wensvoort, G., Terpstra, C., Pol, J. M. A., Ter Laak, E. A., Bloemraad, M., De Kluyver, E. P., . . . Wagenaar, F. (1991). Mystery swine disease in The Netherlands: the isolation of Lelystad virus. *Veterinary Quarterly, 13*(3), 121-130.

Wieringa, R., de Vries, A. A., & Rottier, P. J. (2003). Formation of disulfide-linked complexes between the three minor envelope glycoproteins (GP2b, GP3, and GP4) of equine arteritis virus. *J Virol, 77*(11), 6216-6226.

Wieringa, R., de Vries, A. A., van der Meulen, J., Godeke, G. J., Onderwater, J. J., van Tol, H., . . . Rottier, P. J. (2004). Structural protein requirements in equine arteritis virus assembly. *J Virol, 78*(23), 13019-13027. doi:10.1128/JVI.78.23.13019-13027.2004

Wissink, E. H., Kroese, M. V., Maneschijn-Bonsing, J. G., Meulenberg, J. J., van Rijn, P. A., Rijsewijk, F. A., & Rottier, P. J. (2004). Significance of the oligosaccharides of the porcine reproductive and respiratory syndrome virus glycoproteins GP2a and GP5 for infectious virus production. *J Gen Virol, 85*(Pt 12), 3715-3723. doi:10.1099/vir.0.80402-0

Wissink, E. H., Kroese, M. V., van Wijk, H. A., Rijsewijk, F. A., Meulenberg, J. J., & Rottier, P. J. (2005). Envelope protein requirements for the assembly of infectious virions of porcine reproductive and respiratory syndrome virus. *J Virol, 79*(19), 12495-12506. doi:10.1128/JVI.79.19.12495-12506.2005

Wongyanin, P., Buranapraditkul, S., Yoo, D., Thanawongnuwech, R., Roth, J. A., & Suradhat, S. (2012). Role of porcine reproductive and respiratory syndrome virus nucleocapsid protein in induction of interleukin-10 and regulatory T-lymphocytes (Treg). *J Gen Virol, 93*(Pt 6), 1236-1246. doi:10.1099/vir.0.040287-0

Wootton, S. K., Rowland, R. R., & Yoo, D. (2002). Phosphorylation of the porcine reproductive and respiratory syndrome virus nucleocapsid protein. *J Virol, 76*(20), 10569-10576.

Wootton, S. K., & Yoo, D. (2003). Homo-oligomerization of the porcine reproductive and respiratory syndrome virus nucleocapsid protein and the role of disulfide linkages. *J Virol, 77*(8), 4546-4557.

Wu, B., Peisley, A., Richards, C., Yao, H., Zeng, X., Lin, C., . . . Hur, S. (2013). Structural basis for dsRNA recognition, filament formation, and antiviral

signal activation by MDA5. *Cell, 152*(1-2), 276-289. doi:10.1016/j.cell.2012.11.048

Xiao, Z., Batista, L., Dee, S., Halbur, P., & Murtaugh, M. P. (2004). The level of virus-specific T-cell and macrophage recruitment in porcine reproductive and respiratory syndrome virus infection in pigs is independent of virus load. *J Virol, 78*(11), 5923-5933. doi:10.1128/JVI.78.11.5923-5933.2004

Xue, F., Sun, Y., Yan, L., Zhao, C., Chen, J., Bartlam, M., . . . Rao, Z. (2010). The crystal structure of porcine reproductive and respiratory syndrome virus nonstructural protein Nsp1beta reveals a novel metal-dependent nuclease. *J Virol, 84*(13), 6461-6471. doi:10.1128/JVI.00301-10

Yang, L., Wang, R., Yang, S., Ma, Z., Lin, S., Nan, Y., . . . Zhang, Y. J. (2018). Karyopherin alpha6 Is Required for the Replication of Porcine Reproductive and Respiratory Syndrome Virus and Zika Virus. *J Virol*. doi:10.1128/JVI.00072-18

Yang, L. P., Wang, R., Ma, Z. X., Xiao, Y. Q., Nan, Y. C., Wang, Y., . . . Zhang, Y. J. (2017). Porcine Reproductive and Respiratory Syndrome Virus Antagonizes JAK/STAT3 Signaling via nsp5, Which Induces STAT3 Degradation. *Journal of virology, 91*(3), 14. doi:10.1128/jvi.02087-16

Yin, Z., Dai, J., Deng, J., Sheikh, F., Natalia, M., Shih, T., . . . Fitzgerald-Bocarsly, P. (2012). Type III IFNs are produced by and stimulate human plasmacytoid dendritic cells. *J Immunol, 189*(6), 2735-2745. doi:10.4049/jimmunol.1102038

Yoo, D., Welch, S. K., Lee, C., & Calvert, J. G. (2004). Infectious cDNA clones of porcine reproductive and respiratory syndrome virus and their potential as vaccine vectors. *Vet Immunol Immunopathol, 102*(3), 143-154. doi:10.1016/j.vetimm.2004.09.019

Yu, M. R., Liu, X. L., Sun, L., Chen, C. W., Ma, G. P., Kitamura, Y., . . . Liu, W. J. (2010). Subcellular localization and topology of porcine reproductive and respiratory syndrome virus E protein. *Virus Research, 152*(1-2), 104-114. doi:10.1016/j.virusres.2010.06.012

Yu, Y., Wang, R., Nan, Y., Zhang, L., & Zhang, Y. (2013). Induction of STAT1 phosphorylation at serine 727 and expression of proinflammatory cytokines by porcine reproductive and respiratory syndrome virus. *PLoS One, 8*(4), e61967. doi:10.1371/journal.pone.0061967

Yun, S. I., & Lee, Y. M. (2013). Overview: Replication of porcine reproductive and respiratory syndrome virus. *J Microbiol, 51*(6), 711-723. doi:10.1007/s12275-013-3431-z

Zhang, M., Cao, Z., Xie, J., Zhu, W., Zhou, P., Gu, H., . . . Zhang, G. (2013). Mutagenesis analysis of porcine reproductive and respiratory syndrome virus nonstructural protein 7. *Virus Genes, 47*(3), 467-477. doi:10.1007/s11262-013-0957-4

Zhang, M. D., & Zakhartchouk, A. (2017). Porcine reproductive and respiratory syndrome virus envelope (E) protein interacts with tubulin. *Veterinary Microbiology, 211*, 51-57. doi:10.1016/j.vetmic.2017.10.002

Zhang, Y., Sharma, R. D., & Paul, P. S. (1998). Monoclonal antibodies against conformationally dependent epitopes on porcine reproductive and respiratory syndrome virus. *Veterinary Microbiology, 63*(2-4), 125-136.

Zhang, Y. J., Stein, D. A., Fan, S. M., Wang, K. Y., Kroeker, A. D., Meng, X. J., . . . Matson, D. O. (2006). Suppression of porcine reproductive and respiratory syndrome virus replication by morpholino antisense oligomers. *Veterinary Microbiology, 117*, 117-129.

Zhang, Y. J., Wang, K. Y., Stein, D. A., Patel, D., Watkins, R., Moulton, H. M., . . . Matson, D. O. (2007). Inhibition of replication and transcription activator and latency-associated nuclear antigen of Kaposi's sarcoma-associated herpesvirus by morpholino oligomers. *Antiviral Res, 73*(1), 12-23.

Zhao, S., Ge, X., Wang, X., Liu, A., Guo, X., Zhou, L., . . . Yang, H. (2015). The DEAD-box RNA helicase 5 positively regulates the replication of porcine reproductive and respiratory syndrome virus by interacting with viral Nsp9 in vitro. *Virus Research, 195*, 217-224. doi:10.1016/j.virusres.2014.10.021

Zheng, J., Yong, H. Y., Panutdaporn, N., Liu, C., Tang, K., & Luo, D. (2015). High-resolution HDX-MS reveals distinct mechanisms of RNA recognition and activation by RIG-I and MDA5. *Nucleic Acids Res, 43*(2), 1216-1230. doi:10.1093/nar/gku1329

Zheng, Q., Chen, D., Li, P., Bi, Z., Cao, R., Zhou, B., & Chen, P. (2007). Co-expressing GP5 and M proteins under different promoters in recombinant modified vaccinia virus ankara (rMVA)-based vaccine vector enhanced the humoral and cellular immune responses of porcine reproductive and respiratory syndrome virus (PRRSV). *Virus Genes, 35*(3), 585-595. doi:10.1007/s11262-007-0161-5

Ziebuhr, J., Snijder, E. J., & Gorbalenya, A. E. (2000). Virus-encoded proteinases and proteolytic processing in the Nidovirales. *J Gen Virol, 81*(Pt 4), 853-879. doi:10.1099/0022-1317-81-4-853

Zimmerman, J., Benfield, D. A., Murtaugh, M. P., Osorio, F., Stevenson, G. W., & Torremorell, M. (2006). Porcine reproductive and respiratory syndrome virus (porcine arterivirus). *Diseases of swine, 9*, 387-418.

Zuckermann, F. A., Garcia, E. A., Luque, I. D., Christopher-Hennings, J., Doster, A., Brito, M., & Osorio, F. (2007). Assessment of the efficacy of commercial porcine reproductive and respiratory syndrome virus (PRRSV) vaccines based on measurement of serologic response, frequency of gamma-IFN-producing cells and virological parameters of protection upon challenge. *Vet Microbiol, 123*(1-3), 69-85. doi:10.1016/j.vetmic.2007.02.009