DIFFERENTIAL EXPRESSION OF TYPE I INTERFERONS IN FETAL TISSUES AND THE MATERNAL-FETAL INTERFACE IN RESPONSE TO PRRSV INFECTION

by

WENJING SANG

B.S., Anhui Agriculture University, 2010

A THESIS

submitted in partial fulfillment of the requirements for the degree

MASTER OF SCIENCE

Department of Diagnostic Medicine/Pathobiology
College of Veterinary Medicine

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2012

Approved by:

Major Professor
Raymond R. R. Rowland

Copyright

Abstract

Interferons (IFNs) comprise a group of antiviral cytokines; however, their expression at the porcine maternal-fetal interface and in fetal tissues has not previously been investigated. The purpose of this study was to analyze the expression of type I IFNs and their receptors in maternal and fetal tissues from sows infected with PRRSV. The approach was to use real-time RT-PCR to identify the expression of different subtypes of type I IFN genes. The results show that the constitutive gene expression of some subtypes including IFN-α and IFN-αω were detected in fetal lymphoid nodes (IFN-αω), placenta (several IFN-α subtypes and IFN-ω5) and particularly, thymus (multiple IFN-α, IFN-δ and IFN-ω5).The results demonstrate that porcine type I IFNs are differentially expressed at the maternal-fetal interface and in fetal tissues in response to PRRSV infection.

Table of Contents

List of Figures

List of Tables

Acknowledgements

After receiving a research assistantship at the end of 2010, I joined the laboratory of Dr. Raymond R. R. Rowland as a graduate research assistant for my master's degree. First, I appreciate for the financial support from Dr. Rowland. During the period of my graduate study, I would like to thank my major professor Dr. Rowland for his expertise, great help and especially his patience.

Thanks to my committee members, Drs. Frank Blecha, Wenjun Ma and Yongming Sang for their help and insightful advices in my research and thesis writing.

Thanks to Maureen Kerrigan for her excellent technical assistance. Thanks to all the other members in Dr. Rowland and Dr. Blecha's lab for their help!

Finally, thanks to my family and parents. It is their constant love and support that encouraged me to complete my overseas study.

Chapter 1 - PRRSV literature review

Introduction

Porcine reproductive and respiratory syndrome (PRRS) is one of the most economically important swine diseases. Infection with PRRS virus (PRRSV), the etiological agent of PRRS, results in reproductive failure in sows or gilts, respiratory distress, poor growth performance, and increased mortality in growing pigs. PRRSV strains can be classified into one of two genotypes, European genotype (EU or type I) and North American (NA or type II). Innate immunity is the first line of defense against virus infections. Interferons (IFNs) are a group of innate immune effectors critical in regulation of antiviral responses. Three families, type I, II and III IFNs, have been identified, with type I IFNs containing the greatest number of subtypes and isotypes. Pigs have nearly 40 different type I IFNs. Although quite a few type I IFNs have already been identified, their expression at the maternal-fetal interface and fetal tissues during the later stages of fetal development have not been investigated. The purpose of this study was to make a detailed analysis of the expression of IFNs and their receptors in maternal and fetal tissues of the late gestation fetus during infection with PRRSV. The results provide a platform for investigating maternal-fetal immunity and for testing antiviral therapies.

The Arteriviradae Family

The Arteriviradae family was first established in 1996, and consists of four enveloped, positive-stranded RNA viruses, which include lactate dehydrogenase elevating virus (LDV) of mice, equine arteritis virus (EAV), porcine reproductive and respiratory syndrome virus (PRRSV) and simian hemorrhagic fever virus (SHFV). Three viruses were first characterized in the 1950s/1960s, while PRRSV was first identified in the early1990s. The consequences of infection

with arteriviruses range from asymptomatic infection to lethal disease. When PRRSV first appeared, it caused vast epidemics of reproductive and respiratory disease in both North American and European countries. Later, after molecular analysis of isolates revealed that NA and EU viruses share only ~60% genetic identity. The two PRRSV genotypes might have evolved separately, but from a common ancestor (Knipe et al. 2007).

Porcine reproductive and respiratory syndrome virus (PRRSV)

A new disease appeared in the late 1980's, which was associated with reproductive failure and respiratory problems of piglets and growing pigs. The disease was called "mystery swine disease" in the US and "blue ear disease" in Europe. Later on, the North American and European investigators named the disease "porcine reproductive and respiratory syndrome" or PRRS. The causative agent was identified as a small enveloped, RNA virus (Lelystad Virus) (Wensvoort, et al. 1991). Almost simultaneously, a similar viral isolate, VR-2332, was identified in the US (Benfield, et al. 1992; Collins, et al. 1992). Lelystad-like isolates were placed in a group called type-I genotype viruses; whereas, isolates similar to VR-2322 were placed into the type-II genotype group.

Under the electron microscope, PRRSV appears as a round particle with a relatively smooth surface (Snijder and Meulenberg 1998).The average diameter is about 55 nm without large surface projections commonly observed for other viruses (Dokland, 2010). The envelope contains seven proteins: two major (M, GP5) and five minor species (GP2a, 2b, GP3, GP4, 5a). The envelope surrounds a nucleocapsid core, which contains the 15 kb RNA genome associated with the nucleocapsid protein (N) (Figure 1.2). All major and minor envelope proteins are required for the production of infectious virions (Molenkamp, et al. 2000; Knipe et al. 2007; Dokland, 2010).

The PRRSV genome is a 15 kb single-strand, positive-sense RNA [ss(+)RNA] flanked by a 5' leader and a 3' untranslated region followed by a poly-A tail (Figure 1.1). The major structural proteins, GP5, 5a, matrix (M) and nucleocapsid (N) are derived from ORFs 5, 6 and 7, respectively. GP2a, 2b, GP3 and GP4 are translated from ORFs2, 2, 3 and 4, respectively. Polyproteins, pp1a and pp1ab are encoded by ORF1a and ORF1b, which comprise 75% of the whole genome. PP1ab is proteolytically cleaved into at least 13 nonstructural proteins (Snijder and Meulenberg 1998). Autocatalytic processing is due to the three accessory cysteine proteinases, nsp1α, nsp1β and nsp2 and a serine protease, nsp4 (den Boon, et al. 1995; Ziebuhr, et al. 2000). The viral RNA-dependent RNA polymerase, helicase (Bautista et al., 2002) and uridylate-specific endoribonuclease are encoded by ORF1b and represent nsp's 9-11, respectively (Nedialkova , et al. 2009). In addition to involvement in virus replication, there is convincing evidence to show that nsp1α, nsp1β, nsp2, nsp4 and nsp11 can attenuate the type I IFN response (Beura, et al. 2010).

The major proteins, M and GP5, comprise at least half of the viral proteins (Mardassi, et al. 1996; Dea, et al. 2000; Dokland 2010). The M protein is a non-glycosylated 174 amino acid (173 for type-I viruses) protein containing a short 16-residue N-terminal signal peptide, a three transmembrane (TM) region and an 84-residue C-terminal endodomain ((Dokland 2010). The M protein is the most conserved structural protein of PRRSV (Meng, et al. 1995). GP5 is a glycosylated 200-residue (201 for type-I) protein. It is the most variable structural protein of PRRSV, with only 51-55% peptide sequence identity between type 1 and type II viruses (Murtaugh, et al. 1995; Kapur, et al. 1996). It contains a N-terminal signal sequence, which after cleavage, leaves a very short, but heavily glycosylated, 30 amino acid ectodomain. The central hydrophobic region is assumed to span the membrane three times and is followed by a

cytoplasmic domain, which is about 50 to 75 amino acids long. Presumably, glycosylation functions to shield variable and conserved B cell epitopes. Recently, an additional AUG-initiated ORF was identified which overlaps the 5' end of ORF5 and was named ORF5a. It codes for a 43-64 amino acid protein and is the eighth structural protein of arteriviruses (Firth, et al. 2011).

The 14 kDa N protein, derived from ORF7, is small (12-15kDa) and dominated by basic residues. It is believed that the N protein interacts with genomic RNA in its N-terminal domain during nucleocapsid assembly (Knipe et al. 2007). The protein is phosphorylated with phosphoserines identified in both RNA-binding and capsid-forming domains (Wootton, et al. 2002; Knipe et al. 2007).

There are four minor structural proteins on the PRRS virion. GP2 is 253 amino acid residues for type-I virus and 256 for type-II virus. GP2 contains a N-terminal signal sequence between residues 1-37 in type –I (1-40 in type-II), followed by a 168-residue ectodomain. GP2 has two conserved glycosylation sites; however, glycosylation is not a prerequisite for viral infectivity (Meulenberg, et al. 1995; Wissink, et al. 2004; Dokland 2010). The small E or 2b protein is derived from ORF2b, which is fully embedded within ORF2 for type-II viruses (Wu, et al. 2001). E is non-glycosylated and 70-73 amino acids in length (Wu, et al. 2001; Wu, et al. 2005). E consists of a single transmembrane helix and is predicted to form an oligomeric ion channel (Lee and Yoo 2006). The function of the ion channel is to lower the internal PH of the virus during fusion in the endosomal compartment (Gonzalez and Carrasco 2003).

GP3 is a glycosylated integral membrane protein, which contains an uncleaved N-terminal signal sequence and a hydrophobic C-terminal domain (Knipe et al. 2007). The overall sequence identity between type-I and type-II genotypes is only about 58%, and the highest divergence appears in the C-terminal 30-50 amino acid residues. It is predicted that GP3 contains

six glycosylation in a putative ectodomain region (Dokland 2010). All six glycosylation sites are used, which add about 16 kDa to its mass (Gonin, et al. 1998).

The size of GP4 ranges from 178 (type-II) to 183 (type-I) residues (Meulenberg, et al. 1997). It contains a predicted signal peptide, which cleaves the first 21 amino acids. Among the four glycosylation sites, at least three have been confirmed to possess glycans (Dokland 2010).

GP2, GP3 and GP4 interact to form a multimeric complex within the envelope of the virus (both type-I and type-II) (Wissink, et al. 2005). GP2, GP3 and GP4 are disulfide linked in EAV, but this phenomenon has not been identified in PRRSV (Wieringa, et al. 2003; Wieringa, et al. 2004). All the minor proteins are required for the viral infectivity, but not all of them are required for the formation of the virus particle (Wissink, et al. 2005; Dokland 2010).

PRRSV replication

Several cellular factors are required for the attachment and binding of the virus. Early investigation revealed that PRRSV matrix (M) protein binds heparin sulfate proteoglycans and heparin-like molecules on porcine alveolar macrophages (Jusa, et al. 1997). Virions are internalized via clathrin-coated vesicles and the genome RNA released following the acidification of vesicles (Nauwynck, et al. 1999). CD169 (procine sialoadhesin), a membrane glycoprotein that belongs to the siglec family of sialic acid binding immunoglobulin-like lectins, facilitates the internalization of virions (Vanderheijden, et al. 2003). This interaction between sialoadhesin (CD169) and PRRS virion is mediated through GP5/M. Recently, CD163, which belongs to the SRCR protein superfamily and interacts with PRRSV in the early endosome, plays a role in the uncoating and release of the virus genome (Welch and Calvert 2010).

After entry into the cytoplasm, the genome is directly translated into pp1a and pp1ab polyproteins, which are cleaved into polypeptides with protease, replicase and scaffolding

functions (Molenkamp, et al. 2000). Replication complexes, formed in the perinuclear region of the cell are used to generate genome length minus strands (antigenomes), which generates positive sense genomes and subgenomic (sg) mRNAs.

A nested set of sg mRNAs, which encode the structural proteins, share a common leader and polyA tail. The fusion of the leader, which is derived from the 5' end of the genome, to the body incorporates a discontinuous RNA synthesis mechanism (Knipe et al. 2007). Conserved transcriptional regulatory sequences (TRSs) are present at the 3' end of the leader sequence and at the 5' end of the each transcriptional sg mRNA segment. PRRSV acquires its envelope by budding of preformed nucleocapsids from the ER and Golgi complex (Magnusson, et al. 1970). After budding, the virions stay in intracellular vehicles, which are transported to the plasma membrane for release of virus progeny (Figure 1.3).

Adaptive immunity

The humoral immune response following PRRSV infection is characterized by a robust production of non-neutralizing antibodies followed by the delayed production of a relatively weak neutralizing antibody response. PRRSV-specific Ab is detected in some pigs as early as 5-7 days post-infection (DPI) and in all the pigs by 14 DPI (Yoon , et al. 1992). PRRSV-specific IgM reaches its peak by 14 DPI and then declines; whereas, IgG reaches its peak by 21-49 PI (Vezina, et al. 1996). Neutralizing activity appears at about 4 weeks after infection. The largest Ab response is against N and nsp2, which explains the weak neutralizing response (Mateu and Diaz 2008). A variety of studies involving type I and type II isolates identify neutralizing

epitopes in GP5, GP4, M and GP3 (Gonin, et al. 1999; Cancel-Tirado, et al. 2004; Mateu and Diaz 2008).

Cell-mediated immunity following PRRSV infection is characterized by a robust production of IFN-γ and to lesser extent IL-2 (Fuertes, et al. 1999). After vaccination of pigs with a type-II PRRSV strain, PRRSV-specific IFN-γ secreting cells first appear at about 3 weeks after infection. PRRSV-specific IFN-γ secreting cells are mainly CD4+CD8+ T cells, together with a small portion of CD8+ cytotoxic T cells (Meier, et al. 2003; Mateu and Diaz 2008). In addition to, IFN-γ, TNF-α and IL-12 can also be detected in alveolar macrophages and dendritic cells, along with increased IL-10 (Yoo, et al. 2010).

Vaccines

Live PRRSV vaccines are available for use in the USA. Generally, live attenuated vaccines can depress the severity of PRRS clinical signs and reduce viremia (Kimman, et al. 2009). Protection is greater when the vaccine and challenge viruses are homologous (Kimman, et al. 2009; Meng 2000). Inactivated virus vaccines are generally considered non-protective (Kimman, et al., 2009; Zuckermann, et al. 2007; Kimman, et al. 2009). The greatest challenges for vaccine design is to provide protection in the face of the large amount of antigenic variability offered by field strains and reduce the naturally occurring immune dysregulation. For example live vaccines generate non-neutralizing antibodies, which are directed against the N protein (Thanawongnuwech and Suradhat 2010).

Innate immunity

The term "interferon" (IFN) was first used more than 50 years ago to describe a factor secreted by virus-infected cells that could protect neighboring cells from infection. The IFN family is composed of three classes, type I, II and III IFNs. Type I and type III IFNs consist of multiple members and are important for antiviral innate immunity; whereas type II IFN has only one member, IFN-γ, and is mostly associated with adaptive immunity. The porcine type I IFN genome locus contains at least 39 functional genes distributed along chromosomes 1 (SSC1) with one exception of IFN-κ on chromosome 10 (Sang, et al. 2010). Porcine type I IFNs contain multiple subclasses including 17 IFN-α subtypes, 11 IFN-δ subtypes, 7 IFN-ω subtypes, and single IFN-αω, IFN-β, IFN-ε, and IFN-κ (Pestka 2007). Among all identified type I IFNs, IFN-κ has the longest open reading frame encoding a 208-amino acid protein. All other type I IFNs encode 153-193 amino acid proteins (Sang, et al. 2010).

Type I IFN production is initiated when single-stranded RNA is recognized by TLR8/9, which leads to the dimerization of receptors and recruitment of MyD88 into endosomes. The formation of signaling complexes involve the activation of the transcription factors, AP-1, Interferon regulatory factor 3/7 and NF-κB, which are translocated to the nucleus together with CBP. The results are the transcription of IFNα and IFNβ genes. IFNα and IFNβ bind to the type I IFN receptor, which consists of two units, IFNAR1 and IFNAR2. The result is phosphorylation of Jak1 kinase, which phosphorylates Signal Transducers and Activators of Transcription (STAT) 1 and STAT2. STAT1 and STAT2 detach from the receptor and form a complex with ISG3 and IRF9. ISG3 translocates to the nucleus and binds ISRE to initiate the transcription of hundreds of ISGs (Sun, et al. 2012). Five major ISGs, MxA, OAS–1/RNaseL, RIG-I/MDA5, ISG15, and PKR, are responsible for suppression of viral infection.

Type I IFNs during PRRSV infection

Although PRRSV is highly sensitive to IFN-α, the virus has the ability to suppress the expression of type I IFNs. Suppression of IFN-β production has also been observed (Kimman, et al. 2009). One action of PRRSV is to interfere with the activation of IFN-β promoter stimulator-1 (IPS-1) in the retinoic acid-inducible gene I (RIG-I)(Luo, et al. 2008; Kimman, et al. 2009). PRRSV infection also inhibits the activation of interferon regulator factor 3 (IRF3), which plays a critical role in IFN-β transcription (Loving, et al. 2007; Genini, et al. 2008; Luo , et al. 2008). The ability of PRRSV to suppress type I IFN production is virus strain dependent (Lee, et al. 2004; Yoo, et al. 2010).

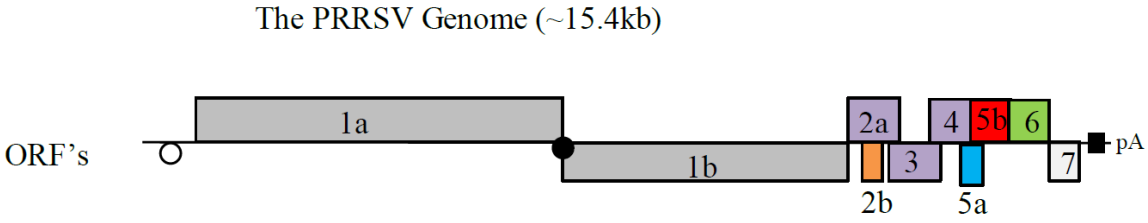
IFN production at the maternal-fetal interface influences fetal development and immunity. For example, a single injection of IL-2 and IFN γ into certain strains of mice can induce fetal resorption (Lala, 1990). Therefore, the induction of antiviral immunity presents an interesting paradox for the infected fetus. Cytokines that can protect the fetus from viral infection have a negative influence on fetal development. Three levels of IFN regulation control virus infection during pregancy: (1) induction of maternal-specific IFNs and adaptive responses for control of virus replication on the maternal side, (2) induction of placenta-specific IFNs to protect the fetuses, but proinflammatory responses may lead to fetal rejection, and (3) induction of fetal IFNs and adaptive responses, which control virus replication at the level of the fetus. Understanding the family-wide expression of porcine type I IFNs at maternal-fetal interface and in fetal tissues will provide important information on how the fetus responds to viral infection during the late gestation period.

A Pig model for the study of the maternal-fetal antiviral response

As an experimental model of human disease pigs have many advantages. For example, pigs are physiologically and anatomically closer to humans. Many viruses that infect humans can also infect pigs. The gestation period and time to adulthood of pigs are relatively short (112 to 114 days of gestation and about 6 months to sex maturity). Pig and human fetuses become immunocompetent during the last third of pregnancy. In this study, the expression of porcine type I IFNs was extensively analyzed in the immunocompetent pig fetus. Because there is no transfer of passive antibodies from mother to fetuses, PRRS virus isolation and serology can be used to estimate if the fetuses are infected in utero. The reproductive form of PRRS can result in severe consequences including increased regular and delayed returns to estrus, decreased conception, abortions, mummified fetuses, stillbirths and weak-born neonates. In this study, we use a real-time RT-PCR to examine the gene expression profile of type I IFNs to demonstrate the complex pattern of type I IFNs antiviral responses.

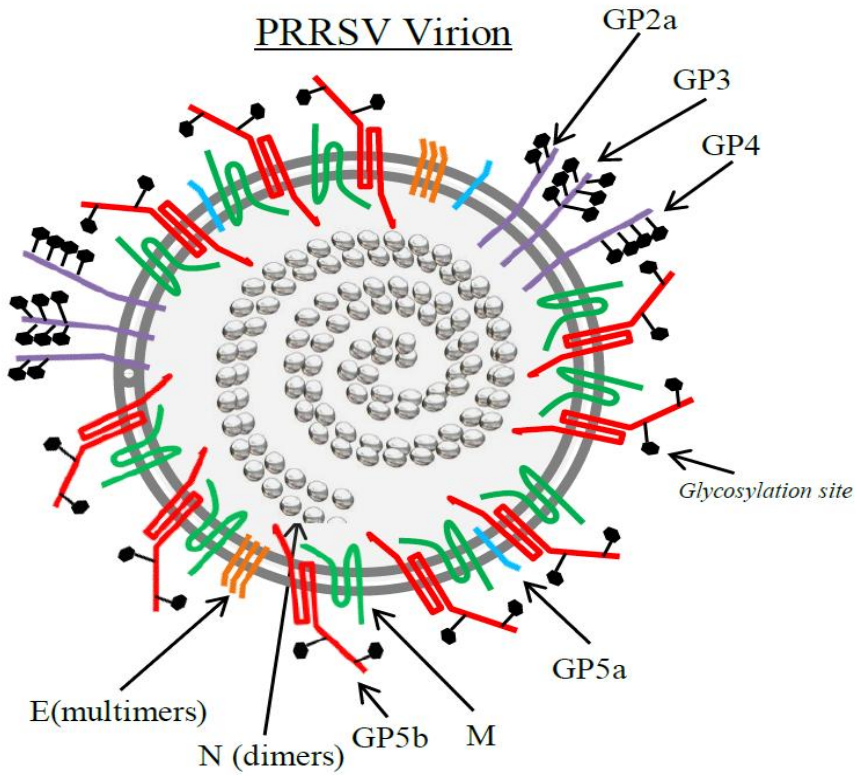
Figures and Tables

Figure 1.1 Diagram of gene composition of PRRSV genome.



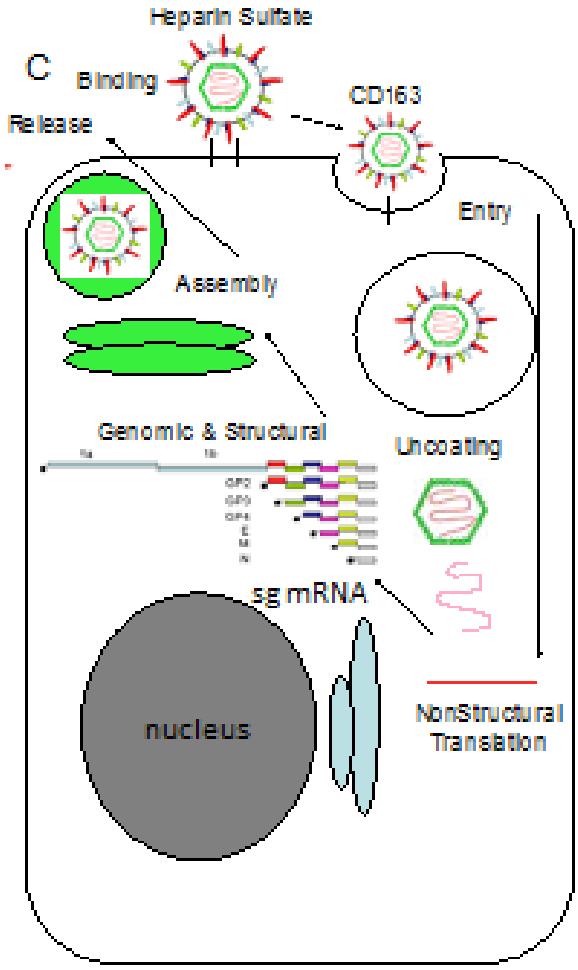
Overview of PRRSV genome organization. The replicase open reading frames (ORFs) 1a and 1b are followed by the gene encoding the 2b protein, three genes (ORFs 5,6,7) encoding major structural proteins GP5, M and N, and the genes (ORF 2a, 2b, 3, 4) for the minor proteins GP2a, GP2b, GP3 and GP4.

Figure 1.2 The structure of the PRRS virion



(Provided by Benjamin Trible)

Figure 1.3 PRRSV life cycle.



(Adopted from John Patton)

Table 1.1 RT-PCR primer sequences for porcine type I, type III interferons and type-I interferon receptors

Subtype	**Primer name and sequence (5' to 3')**		**Product size (bp)**
IFNα1	Sense	GGCTCTGGTGCATGAGATGT	337
	Antisense	GCCTTCTTCCTGAATCTGTCTTA	
IFNα2	Sense	CAACCTCAGCCTTCCTCACA	549
	Antisense	GAATCTGTCTCACAGGTTTC	
IFNα3	Sense	TGAGGAGAATCTCCCCT	432
	Antisense	CTTCCTGAATCTGTCTCA	
IFNα4	Sense	TCTCTGGGCTGCGACCCGGT	502
	Antisense	CCTCCTGAGTCTGTCTTG	
IFNα5/6	Sense	GCACAAATGAGGAGAATATCT	437
	Antisense	CCTCCTGAGTCTGTCTTG	
IFNα7/11	Sense	GGGACTTTGGATCCCCTCAT	369
	Antisense	GTGGAGGAAGAGAAGGATG	
IFNα8	Sense	AACCTCAGCCTTCCTCACG	546
	Antisense	GTCTGTCTTGCAGGTTTG	
IFNα9	Sense	GTGCTGCTCAGCTGCAAG	384
	Antisense	AGTCCTCCTCCAGCAGGGGC	
IFNα10	Sense	TCTCTGGGCTGCGACCCGGT	502
	Antisense	CTTCCTGAATCTGTCTCA	
IFNα12	Sense	CCTCAGCCTTCCTCACGGT	509

	Antisense	CTCATGACTTCTGCCCTGAT	
IFNα13/14	Sense Antisense	TCCCCTCATGAGGCCTTTG CAGGTGTCTGTCACTCCTTC	401
IFNαω	Sense Antisense	AGATCTTCCGCCTCTTCAGCACAA TTCTGGTTTCCACCCTGACAACCT	261
IFNAR1	Sense Antisense	ACCACAGTGAAACATCACCTGCCT TGTTGATGACGGGAGGAAACAGGA	349
IFNAR2	Sense Antisense	TCAACGGGAATCAGAGTCGTCAGA TCAGGAAATACCCAGGCGGACAAT	180
IFNβ	Sense Antisense	ATGTCAGAAGCTCCTGGGACAGTT AGGTCATCCATCTGCCCATCAAGT	246
IFNδ1	Sense Antisense	TATAAGCTTCTGGCAGGAGT AGCCTTGAGTCATCTTGT	205
IFNδ3/4/5	Sense Antisense	AGAACTTGTCTGCTGTCCATT TTTGGAGAAGACACCGGA	209
IFNδ8/9	Sense Antisense	ATGCTCTGCTCCACTCCTGC GTGCCTTGAGTCATCTGGATTGG	194
IFNε	Sense Antisense	TTGGTACTGCTGGCTTCTTCCACT AACTGCCCTGAAGAGGCTGAAGAT	255
IFNκ	Sense Antisense	GCAGAATGAGCCATTCGTTTCCCA TCCTCTTCCTCCTGCAAGCATTGT	259
IFNω1	Sense Antisense	TGGTGCTTCTGCGTCAGATG CTCACCTGCACCAAGCAGGAC	265

IFNω3	Sense Antisense	TGTGTTCTCTCTACTGACGGTC CAGATGTCTGCTCTCCCATC	389
IFNω5	Sense Antisense	TCATGCTCTCTCTACTGACAGC TGGAGCTTGTCCAGGAGGA	300
IFNL3	Sense Antisense	AAGAGGGCCAAGGATGCCTTTGAA AGGCGGAAGAGGTTGAACATGACA	374
SCMHI	Sense Antisense	CACGGAGGCGCAGTCGTCTC GTACCTGGCGTGGCCGTGAG	177

Table 1.2 Porcine type-I and type-III IFN family members and the their functions

Subtype	Isotype	Receptor	Function	Anti-PRRSV-activity
IFNα	17	IFNAR1 IFNAR2	IFNα and IFNβ stimulate both NK cells, macrophages and some other cell types to elicit an antiviral response	IFNα provide nearly full protection against PRRSV infection
IFNβ	1			IFNβ shows effective protection (60%~80%)
IFN αω	1		IFN αω is an unique porcine IFN subtype, exerting antiviral protection	IFNαω, a unique porcine IFN subtype, also found anti-PRRSV activity (~40%)
IFN δ	11		IFN δ has high antiproliferative activity	IFN δ shows low-anti-PRRSV activity (20%~60%)
IFN ε	1		IFN ε mediates TNF-alpha induced STAT1 phosphorylation and induction of RIG1	IFN ε shows little antiviral activity against PRRSV (<20%)
IFN ω	7		IFN ω to date the exact biological activities and the physiological role of this interferon are unknown	IFN ω shows low-anti-PRRSV activity (20%~60%)
IFN κ	1		IFN-κ induces TNF-α, IL-10 and MCP-1 production by monocytes	IFN κ shows little antiviral activity against PRRSV (20%)
IFN λ3	1	IL28a	IFN λ3 has antiviral activity and up-regulates of MHC class I expression	IFN λ3 also has anti-PRRSV activity

(Modified from Chang et al (2006), Takaoka and Yanai (2006), Sang et al (2010a, b).

Chapter 2 - Materials and methods

Virus and infection:

Experiments involving animals were approved by the Kansas State University IACU Committee. Two PRRSV strains, the low-pathogenic KS483 and the high-pathogenic 72109, were used for infection of sows. The high-pathogenic 72109 was isolated during am abortion storm in East Kansas. Pregnant dams were infected with PRRSV at 90 days of gestation. Fetuses were collected at 112 days of gestation. The methods for the preparation of the PRRSV inoculum on MARC-145 cells and infection of pigs are described in Rowland et al. (2003). Briefly, virus was cultivated on MARC-145 cells in MEM supplemented with 7% FBS and antibiotics. Dams, at 90 days gestation were challenged with approximately 10^5 TCID50 of virus diluted in 5ml of culture medium. One half of the inoculum was administered by intramuscular injection in the neck. The remaining dose was administered intranasally. As comparative controls, one mock-infected dam was administered MARC-145 culture medium. Between 109 and 112 days of gestation of the 114 days gestation period, dams were sacrificed. For the collection of fetuses, the uterine horns were immediately removed after euthanasia. Beginning at the tip of each uterine horn, individual fetuses with intact placenta were carefully removed and immediately necropsied. A sample of amniotic fluid was collected prior to removal. Tissue samples were collected and stored in RNAlater (Ambion) for RT-PCR of cytokines mRNAs. PRRSV specific antibodies were measured using a commercial ELISA performed at the Kansas State University Veterinary Diagnostic Laboratory. Serology results were reported as a sample/positive (S/P) ratio. A ratio greater than 0.4 was considered positive for PRRSV-specific antibody.

RT-PCR to detect virus (Ct value):

The PRRS virus load in serum or thoracic fluid was assayed using a commercial semi-quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR) kit (ABI, Foster City, California, USA), performed by the Kansas State Veterinary Diagnostic Laboratory (KSVDL). A series of sample buffer containing known concentrations of cDNA template were included as a standard. PCR reactions were considered positive if the cycle threshold (Ct) level was <40 cycles. The results were reported as the number of templates per 50 µl reaction, which is approximately equal to the number of templates per ml of serum or thoracic fluid.

RT-PCR for detection the expression of type I IFN genes:

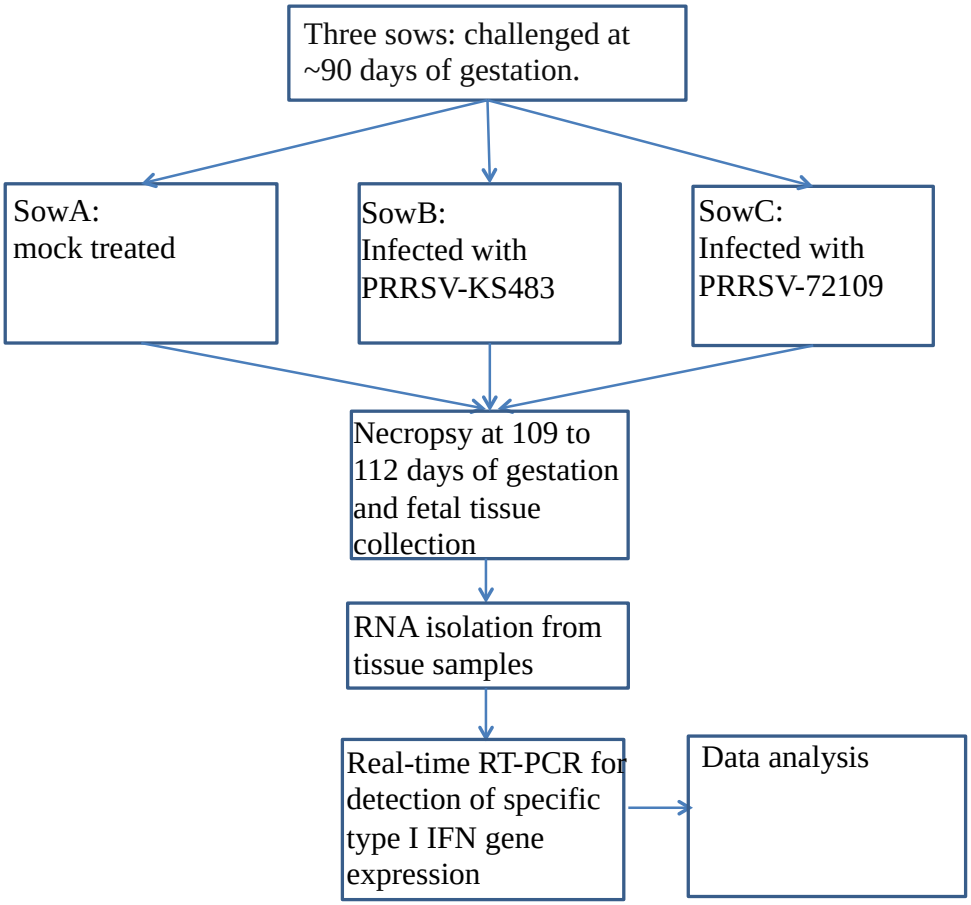
Tissues samples for RT-PCR were immediately placed in RNAlater (Ambion) and stored at -80°. Total RNAs were extracted from the tissues (lung, lymph node, placenta and thymus). Following the protocol for RNA extraction using TRI reagent (Sigmaalderich, St. Louis). In brief, about 500 mg of tissues were homogenized in 1 ml of TRI reagent until it reached a liquid phase. Homogenates were transfered to DNAse/RNnase free 1.5 ml tubes. After standing at room temperature for 5-30 min, the homogenate was centrifuge at 12,000 g for 10 min. The 500 µl upper aqueous phase containing RNA were collected into new tubes and mixed with 50 ul of (1/10 of the TRI reagent) of 1-bromo-3-chloropropane. The mixtures were shaken vigorously for 15 sec and stood for 2~15 min at room temperature. After centrifuge at 12,000g for 15 min at 4 °C, the RNA in the aqueous phase was precipitated with addition of 500 ul of 2-propanol and collected by centrifugation. The RNA pellets were washed once with 70% alcohol and processed for treatment with 5 U RQ DNase (1/100 ul) to remove potential trace amounts of contaminated genomic DNA. The purified RNA was determined by a Nano-spectrometer, gel-electrophoresis

and BioAnalyser (BioLegend). The qualified RNA samples were stored at -80 °C until use as templates for RT-PCR detection.

Real-Time RT-PCR analysis of IFN gene expression:

Gene-specific primers were designed based on multiple alignments of related IFN sequences. Real-time RT-PCR arrays were performed in 96-well microplates. Reactions were conducted with a SYBR Green RT-PCR system (Qiagen, Valencia, CA) with 200 ng of total RNA in a 20ul reaction mixture and RT-PCR conditions described as following. The reverse transcription step was set at 50° for 10 min, following with PCR initial activation step at 95° for 5min, the two-step cycling was set at 95° for 10s, 60° for 30s with 40 cycles. Critical threshold (Ct) values and melt curves were monitored and collected with StepOne Plus software (Applied Biosystems). Relative gene expression data in different tissues (lung, lymph node, thymus, placenta) were normalized against Ct values of the housekeeping gene (SCMHI), and the relative expression index ($2^{-\Delta\Delta Ct}$) was determined in order to get the log2 value compared with the average expression levels of control samples.

Figure 2.1 Experimental design



Chapter 3 - Results

Fetal map

A map showing the location and infection status of individual fetuses is shown in Figure 3.1. In total, 14, 17 and 12 fetuses were obtained from Sow A, Sow B and Sow C, respectively. All the fetuses from Sow A Group were confirmed to be uninfected. Tissues from fetuses 1, 3, 4, 5, 6, 7, 8 and 9 were used for analysis. For Sow B Group, 10 fetuses were found to be RT-PCR positive and seronegative, 4 fetuses were RT-PCR negative and seronegative, 1 fetus was RT-PCR positive and seropositive and 2 fetuses were dead. Tissues for IFN analysis were obtained from fetuses 1, 4, 5, 11, 12, 14 and 15. For Sow C, 8 fetuses were found to be RT-PCR positive and seronegative, two fetuses were RT-PCR positive and seropositive, 1 fetus was dead and 1 fetus was uninfected. Tissues from fetuses 2, 3, 4, 5, 7, 9, 12 were chosen for IFN expression analysis.

Type-I IFN gene expression in tissues of uninfected fetuses

The first study was to examine the expression of type I IFNs in the lung, lymph node, placenta and thymus from 8 uninfected fetuses. All IFNs were detected in all tissues. Mean values for IFN gene expression for the different tissues are shown in the Figure 3.2. There was variation in gene expression between subtypes. The lowest amounts of mRNA were found for IFNA3, IFNA10 and IFNL3. The highest amount of RNA was obtained for IFNW5, and we can observe that the gene expression of type I IFNs shows viability among different IFN subtypes and different tissues. Differences were also observed between tissues. Statistically different levels of expression were observed for IFNAR2 in thymus versus placenta ($p=0.001$), and for IFNB between lymph node and placenta ($p=0.04$).

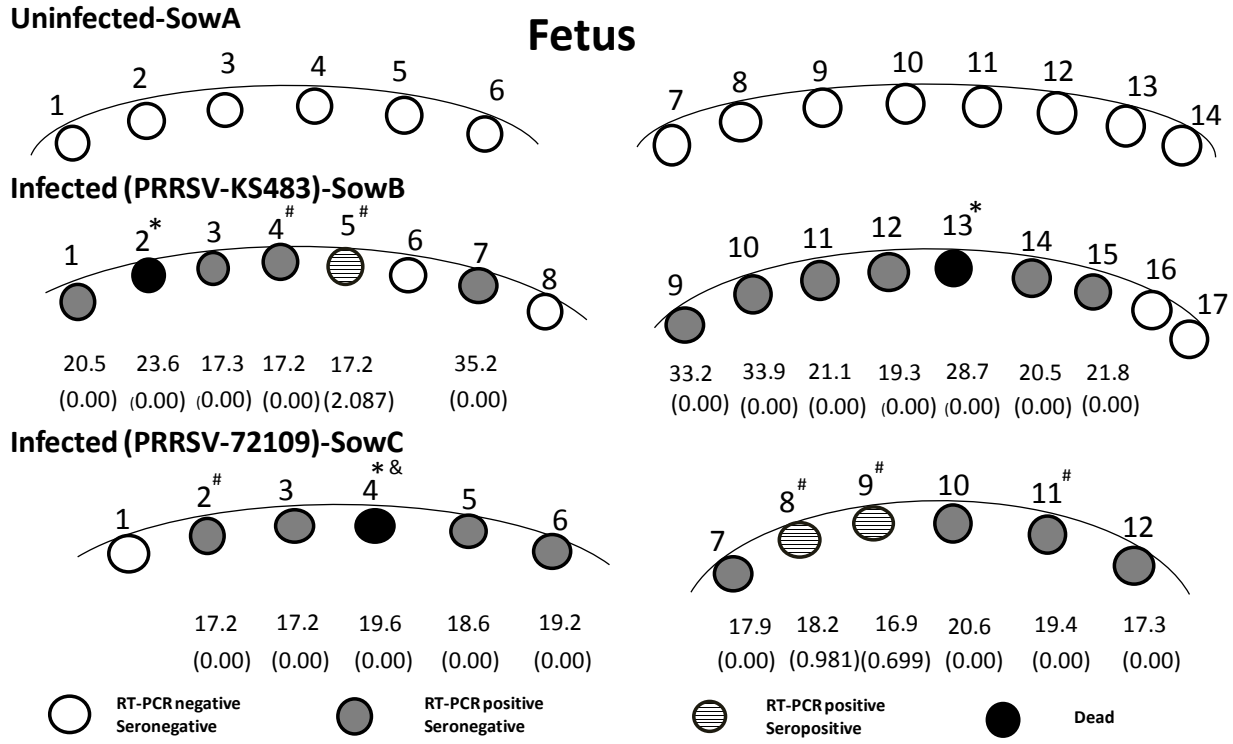
IFN gene expression during PRRSV infection.

Fetuses were infected with two different PRRSV strains, KS483, a historical PRRSV isolate, and KS72109, a more contemporary virus isolated during an abortion storm. Figure 3.3 shows results for IFN gene expression in infected fetuses. The data are presented as the level of expression relative to the control fetuses. Differences between the two viruses were evident for lung and lymph node. Overall, the trend was a relative decrease in IFNA isotype expression in fetuses infected with KS72109. For the lung, the only statistically significant differences were increased IFNE and IFNW1 in KS72109 fetuses. In contrast, the KS72109 fetuses showed increased expression in IFNA. The only significant difference was increased IFNB in the KS72109 fetuses.

For placenta and thymus, there was an overall downregulation of IFN gene expression. Significant downregulation was observed for IFNA13/14 and IFNAR2 in thymus of fetuses infected with KS72109.

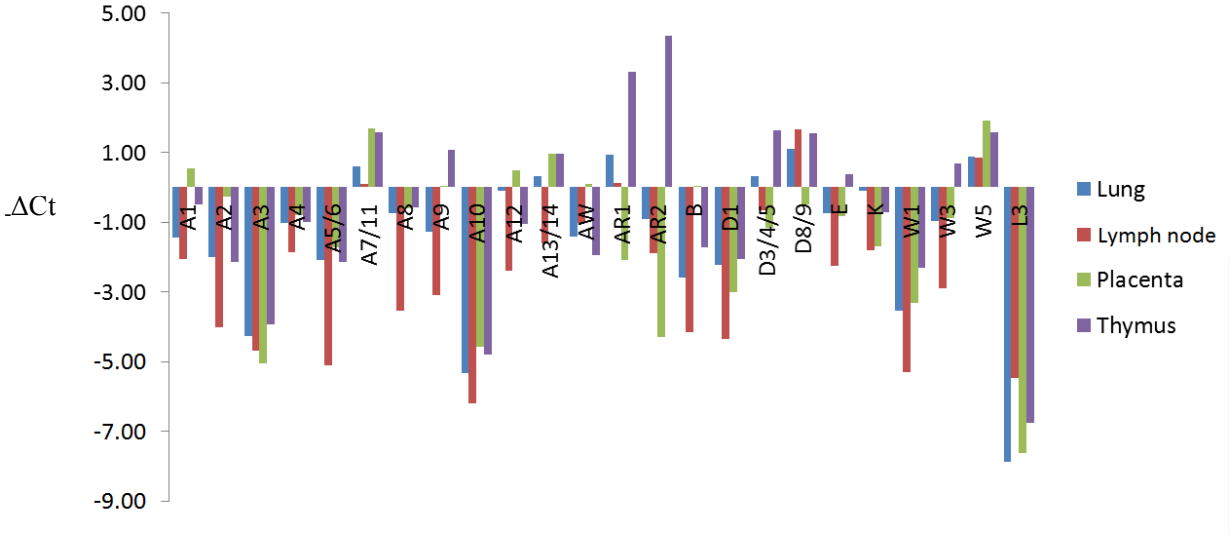
Figures and Tables

Figure 3.1 Fetuses map



Status of fetus from infected dams. Circles represent the relative locations of each fetus in the left and right uterine horns. The number above each fetus identifies the order of removal from the uterus. The CT Value of RT-PCR is below each infected fetus. Serology results, shown in parentheses, are presented as S/P ratio. S/P ratios greater than 0.4 are considered positive for PRRSV antibody.

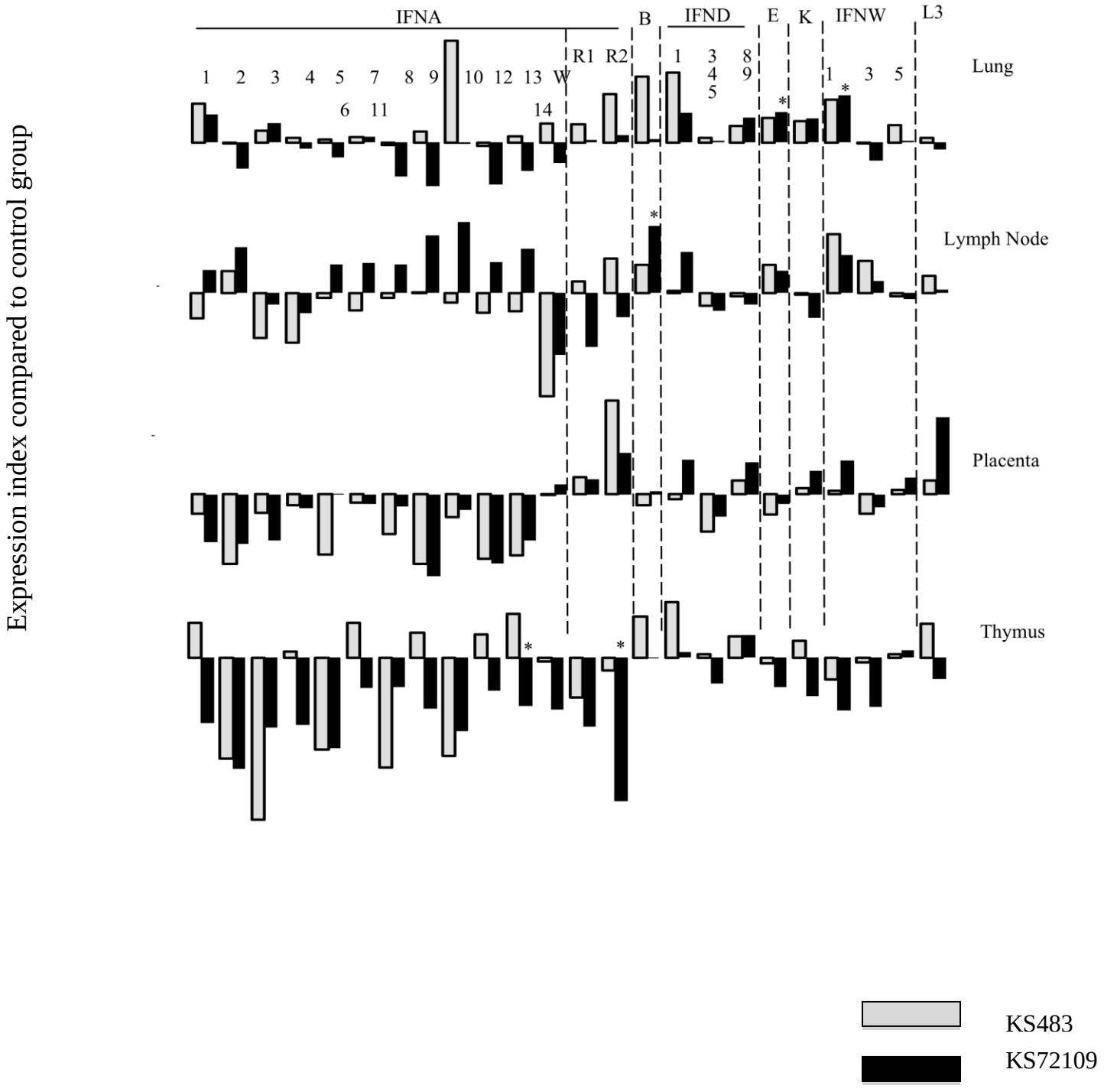
Figure 3.2 Expression of porcine type I IFNs in uninfected fetuses



IFN subtype

Figure 3.3 Differential expression of porcine type I IFNs and type III IFN in infected fetuses.

Gene expression is shown for different tissues from KS483-infected (grey bars) and KS72109-infected (black bars) fetuses. The levels of expression are relative to the mean value obtained for control fetuses.



Chapter 4 - Discussion

Type I IFNs represent a critical group of antiviral molecules showing evolutionary gene expansion. Each mammalian species could possess 15-60 functional genes encoding multiple IFN protein, which are involved in developmental processes and antimicrobial activities (Sang Y, 2010). Pigs possess at least 39 functional type I IFN genes. In this study, the expression of all IFN genes was detected in all tissues. As shown in Figure 3.2, there were differences in the level of expression between tissues. Not all members of the IFNA subtype were expressed to the same levels. Compared to the expression of the housekeeping gene, IFNA 7/11 were expressed at high levels than IFNA-10.

PRRSV infection resulted in alterations of the expression pattern. In tissues, such as lung and lymph node, there were distinct differences in the patterns of the two viruses. The KS72109 virus showed an overall reduction in IFNA isotypes compared to uninfected fetuses. However, the same virus produced increased levels of IFNA in lymph nodes. IFNAW, the subtype only found in pigs and cattle, was down-regulated in lymph nodes of fetuses during PRRSV infection. The most consistent observation was the down-regulation of IFNA isotypes in placenta. Almost all IFNs were down-regulated in the thymus.

PRRSV infection showed less effect in suppression of type I IFNs in lymph nodes. Notably, PRRSV infection also significantly suppressed the expression of IFNAR1 and IFNAR2 genes in the fetal thymus, indicating porcine thymus in addition to the lung may be another major target for PRRSV exerting immune subversion through targeting suppression both type I IFN production and action. In the fetal lungs, most members of IFNA subtype, as well as in the thymus, showed different levels of suppression; in contrast, most other subtypes of IFND, IFNE, INFK and IFNW were up-regulated in the PRRSV infected tissues. This tissue- and IFN

subtype-dependent differential expression as shown in the fetal thymus and lung was further verified in the lymph nodes and placenta. Interestingly, IFNAR2 gene was more up-regulated compared to other subtypes in the placenta. Located at the maternal-fetal interface, placenta is an immune privilege site to limit over-proinflammatory responses to protect fetal development. Therefore, the upregulation of IFNAR gene expression other than increase of proinflammatory IFN ligands could be a feature of placenta in response to viral infection. Indeed, recent studies showed that IFNAR density is a key factor for the modulatory activity of type I IFNs (Moraga et al., 2009) in different cell types, implying that IFNAR expression level should be also considered for modulating antiviral activity of type I IFNs, particularly in fetuses or at maternal-fetal interface.

PRRSV have evolved multiple mechanisms targeting porcine type I IFN system including the components in IFN production and action signaling. The suppression of IFNA and IFNB subtypes by PRRSV has been demonstrated in both porcine alveolar macrophages and peripheral pDCs infected in vitro as well as in pigs infected in vivo (Sun, 2012). It was reported that by inactivating IPS-1 which is a molecule involved in the RIG-1 pathway, PRRSV can interfere the nuclear translocation of IRF-3. Studies have also shown that the over expressed of nonstructural proteins (Nsp) 1, 2, 4, 11 can interfere the nuclear translocation of IRF-3 and the promoter activity of IFN β (Wang, 2012).

The data presented here demonstrated for the first time about the differential expression of family-wide porcine type I IFNs in response to PRRSV infection in the fetal tissues and the placenta. Firstly, in comparison to the uninfected fetuses, PRRSV infection in general showed significant suppression of some IFNA subtypes in the fetal thymus. However, PRRSV infection

showed less effect in suppression of type I IFNs in lymph nodes and lung. The result indicates that the type I IFN responses in the fetal tissues were dependent on the different isolates.

In conclusion, these results demonstrate that (1) type-I IFNs shows differential expression profile in different kinds of tissues; (2) porcine type I IFNs and their subtypes are differentially expressed in fetal tissues upon PRRSV infection during the third phase of gestation; (3) the differential expression of type I IFN genes upon PRRSV infection is dependent on the pathogenicity of viral isolates.

References

Aaronson, D. S. and C. M. Horvath (2002). "A road map for those who don't know JAK-STAT." Science **296**(5573): 1653-1655.

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

Basurko, C., G. Carles, et al. (2009). "Maternal and fetal consequences of dengue fever during pregnancy." Eur J Obstet Gynecol Reprod Biol **147**(1): 29-32.

Benfield, D. A., E. Nelson, et al. (1992). "Characterization of swine infertility and respiratory syndrome (SIRS) virus (isolate ATCC VR-2332)." J Vet Diagn Invest **4**(2): 127-133.

Beura, L. K., S. N. Sarkar, et al. (2010). "Porcine Reproductive and Respiratory Syndrome Virus Nonstructural Protein 1 beta Modulates Host Innate Immune Response by Antagonizing IRF3 Activation." J Virol **84**(3): 1574-1584.

Blaha, T. (2000). "The "colorful" epidemiology of PRRS." Vet Res **31**(1): 77-83.

Buddaert, W., K. Van Reeth, et al. (1998). "In vivo and in vitro interferon (IFN) studies with the porcine reproductive and respiratory syndrome virus (PRRSV)." Adv Exp Med Biol **440**: 461-467.

Bush, E. J., B. Corso, et al. (1999). "Update on the acute PRRS investigative study." Swine Health and Production **7**(4): 179-180.

Burrone ORRF, Gilmore D, Milstein CKefford. (1985). Stimulation of HLA-A,B,C by IFN-alpha. The derivation of Molt 4 variants and the differential expression of HLA-A,B,C subsets. EMBO J, 1985 Nov;4(11):2855-60.

Cancel-Tirado, S. M., R. B. Evans, et al. (2004). "Monoclonal antibody analysis of porcine reproductive and respiratory syndrome virus epitopes associated with antibody-dependent

enhancement and neutralization of virus infection." Vet Immunol Immunopathol **102**(3): 249-262.

Collins, J. E., D. A. Benfield, et al. (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." J Vet Diagn Invest **4**(2): 117-126.

Dea, S., C. A. Gagnon, et al. (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., N. Sawyer, et al. (1995). "Ultrastructural characteristics and morphogenesis of porcine reproductive and respiratory syndrome virus propagated in the highly permissive MARC-145 cell clone." Adv Exp Med Biol **380**: 95-98.

Delputte, P. L., P. Meerts, et al. (2004). "Effect of virus-specific antibodies on attachment, internalization and infection of porcine reproductive and respiratory syndrome virus in primary macrophages." Vet Immunol Immunopathol **102**(3): 179-188.

den Boon, J. A., K. S. Faaberg, et al. (1995). "Processing and evolution of the N-terminal region of the arterivirus replicase ORF1a protein: identification of two papainlike cysteine proteases." J Virol **69**(7): 4500-4505.

Dokland, T. (2010). "The structural biology of PRRSV." Virus Res **154**(1-2): 86-97.

Fields, B. N., D. M. Knipe, et al. (2007). Fields virology. Philadelphia, Wolters Kluwer Health/Lippincott Williams & Wilkins.

Genini, S., P. L. Delputte, et al. (2008). "Genome-wide transcriptional response of primary alveolar macrophages following infection with porcine reproductive and respiratory syndrome virus." J Gen Virol **89**(Pt 10): 2550-2564.

Gonin, P., H. Mardassi, et al. (1998). "A nonstructural and antigenic glycoprotein is encoded by ORF3 of the IAF-Klop strain of porcine reproductive and respiratory syndrome virus." Arch Virol **143**(10): 1927-1940.

Gonin, P., B. Pirzadeh, et al. (1999). "Seroneutralization of porcine reproductive and respiratory syndrome virus correlates with antibody response to the GP5 major envelope glycoprotein." J Vet Diagn Invest **11**(1): 20-26.

Gonzalez, M. E. and L. Carrasco (2003). "Viroporins." FEBS Lett **552**(1): 28-34.

Halbur, P. G. and E. Bush (1997). "Update on abortion storms and sow mortality." Swine Health and Production **5**(2): 73-73.

Han, J., Y. Wang, et al. (2006). "Complete genome analysis of RFLP 184 isolates of porcine reproductive and respiratory syndrome virus." Virus Res **122**(1-2): 175-182.

Hu, H., X. Li, et al. (2009). "Porcine reproductive and respiratory syndrome viruses predominant in southeastern China from 2004 to 2007 were from a common source and underwent further divergence." Arch Virol **154**(3): 391-398.

Joyce MMJR, Burqhardt RC, Hooper RN, Bazer FW, Johnson GABurqhardt. (2008). Uterine MHC class I molecules and beta 2-microglobulin are regulated by progesterone and conceptus interferons during pig pregnancy. J Immunol., Aug 15;181(4):2494-505.

Kapur, V., M. R. Elam, et al. (1996). "Genetic variation in porcine reproductive and respiratory syndrome virus isolates in the midwestern United States." J Gen Virol **77 (Pt 6)**: 1271-1276.

Key, K. F., G. Haqshenas, et al. (2001). "Genetic variation and phylogenetic analyses of the ORF5 gene of acute porcine reproductive and respiratory syndrome virus isolates." Vet Microbiol **83**(3): 249-263.

Kimman, T. G., L. A. Cornelissen, et al. (2009). "Challenges for porcine reproductive and respiratory syndrome virus (PRRSV) vaccinology." Vaccine **27**(28): 3704-3718.

Lala, P. K. (1990). "Interruption of murine pregnancy by activation of antigen-non-specific killer cells in the endometrium with indomethacin, high dose IL-2 or a combination." Res Immunol **141**(2): 159-164.

Lager KM, M. W. (2003). Strain predominance following exposure of vaccinated and naive pregnant gilts to multiple strains of porcine reproductive and respiratory syndrome virus. Can J Vet Res , May;67(2):121-7.

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

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

Lemke, C. D., J. S. Haynes, et al. (2004). "Lymphoid hyperplasia resulting in immune dysregulation is caused by porcine reproductive and respiratory syndrome virus infection in neonatal pigs." J Immunol **172**(3): 1916-1925.

Lopez Fuertes, L., N. Domenech, et al. (1999). "Analysis of cellular immune response in pigs recovered from porcine respiratory and reproductive syndrome infection." Virus Res **64**(1): 33-42.

Loving, C. L., S. L. Brockmeier, et al. (2007). "Differential type I interferon activation and susceptibility of dendritic cell populations to porcine arterivirus." Immunology **120**(2): 217-229.

Luo, R., S. Xiao, et al. (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.

Magnusson, P., B. Hyllseth, et al. (1970). "Morphological studies on equine arteritis virus." Arch Gesamte Virusforsch **30**(2): 105-112.

Mardassi, H., R. Athanassious, et al. (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., B. Massie, et al. (1996). "Intracellular synthesis, processing, and transport of proteins encoded by ORFs 5 to 7 of porcine reproductive and respiratory syndrome virus." Virology **221**(1): 98-112.

Mateu, E. and I. Diaz (2008). "The challenge of PRRS immunology." Vet J **177**(3): 345-351.

Meager, A. (2006). The interferons : characterization and application. Weinheim, Wiley-VCH.

Meier, W. A., J. Galeota, et al. (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.

Meng, X. J., P. S. Paul, et al. (1995). "Sequence comparison of open reading frames 2 to 5 of low and high virulence United States isolates of porcine reproductive and respiratory syndrome virus." J Gen Virol **76 (Pt 12)**: 3181-3188.

Meulenberg, J. J., A. Petersen-den Besten, et al. (1995). "Characterization of proteins encoded by

ORFs 2 to 7 of Lelystad virus." Virology **206**(1): 155-163.

Meulenberg, J. J., A. P. van Nieuwstadt, et al. (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.

Molenkamp, R., H. van Tol, et al. (2000). "The arterivirus replicase is the only viral protein required for genome replication and subgenomic mRNA transcription." Journal of General Virology **81**: 2491-2496.

Murtaugh, M. P., M. R. Elam, et al. (1995). "Comparison of the structural protein coding sequences of the VR-2332 and Lelystad virus strains of the PRRS virus." Arch Virol **140**(8): 1451-1460.

Nedialkova, D. D., R. Ulferts, et al. (2009). "Biochemical characterization of arterivirus nonstructural protein 11 reveals the nidovirus-wide conservation of a replicative endoribonuclease." J Virol **83**(11): 5671-5682.

Nelson, E. A., J. Christopher-Hennings, et al. (1994). "Serum immune responses to the proteins of porcine reproductive and respiratory syndrome (PRRS) virus." J Vet Diagn Invest **6**(4): 410-415.

Osorio, F. A., J. A. Galeota, et al. (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.

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

Pirzadeh, B. and S. Dea (1997). "Monoclonal antibodies to the ORF5 product of porcine

reproductive and respiratory syndrome virus define linear neutralizing determinants." J Gen Virol **78 (Pt 8)**: 1867-1873.

RR, R. (2010). The interaction between PRRSV and the late gestation pig fetus. Virus Res , Dec;154(1-2):114-22.

Sang, Y., R. R. Rowland, et al. (2010). "Differential expression and activity of the porcine type I interferon family." Physiol Genomics **42**(2): 248-258.

Shi, M., T. T. Lam, et al. (2010). "Molecular epidemiology of PRRSV: a phylogenetic perspective." Virus Res **154**(1-2): 7-17.

Shi, M., T. T. Lam, et al. (2010). "Phylogeny-based evolutionary, demographical, and geographical dissection of North American type 2 porcine reproductive and respiratory syndrome viruses." J Virol **84**(17): 8700-8711.

Snijder, E. J. and J. J. Meulenberg (1998). "The molecular biology of arteriviruses." J Gen Virol **79 (Pt 5)**: 961-979.

Sun Y, H. M. (2012). Interplay between interferon-mediated innate immunity and porcine reproductive and respiratory syndrome virus. Viruses , 4(4):424-46

Thanawongnuwech, R. and S. Suradhat (2010). "Taming PRRSV: revisiting the control strategies and vaccine design." Virus Res **154**(1-2): 133-140.

Tian, K., X. Yu, et al. (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.

Vanderheijden, N., P. L. Delputte, et al. (2003). "Involvement of sialoadhesin in entry of porcine reproductive and respiratory syndrome virus into porcine alveolar macrophages." J Virol **77**(15): 8207-8215.

Vezina, S. A., H. Loemba, et al. (1996). "Antibody production and blastogenic response in pigs experimentally infected with porcine reproductive and respiratory syndrome virus." Can J Vet Res **60**(2): 94-99.

Wang, X. (2012). Post-Transcriptional Control of Type I Interferon Induction by Porcine Reproductive and Respiratory Syndrome Virus in Its Natural Host Cells. *Viruses* , 725–733.

Wensvoort, G. (1993). "Lelystad virus and the porcine epidemic abortion and respiratory syndrome." Vet Res **24**(2): 117-124.

Wensvoort, G., C. Terpstra, et al. (1991). "['Lelystad agent'--the cause of abortus blauw (mystery swine disease)]." Tijdschr Diergeneeskd **116**(13): 675-676.

Wieringa, R., A. A. De Vries, et al. (2003). "Intra- and intermolecular disulfide bonds of the GP2b glycoprotein of equine arteritis virus: relevance for virus assembly and infectivity." J Virol **77**(24): 12996-13004.

Wieringa, R., A. A. de Vries, et al. (2004). "Structural protein requirements in equine arteritis virus assembly." J Virol **78**(23): 13019-13027.

Wieringa, R., A. A. F. de Vries, et al. (2004). "Structural protein requirements in equine arteritis virus assembly." J Virol **78**(23): 13019-13027.

Wissink, E. H., M. V. Kroese, et al. (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.

Wissink, E. H., M. V. Kroese, et al. (2005). "Envelope protein requirements for the assembly of infectious virions of porcine reproductive and respiratory syndrome virus." J Virol **79**(19): 12495-12506.

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

Wu, W. H., Y. Fang, et al. (2001). "A 10-kDa structural protein of porcine reproductive and respiratory syndrome virus encoded by ORF2b." Virology **287**(1): 183-191.

Wu, W. H., Y. Fang, et al. (2005). "The 2b protein as a minor structural component of PRRSV." Virus Res **114**(1-2): 177-181.

Yan Sun 1Han 1, Chiyong Kim 1, Jay G. Calvert 2 and Dongwan YooMingyuan. (2012). Interplay between Interferon-Mediated Innate Immunity and Porcine Reproductive and Respiratory Syndrome Virus. Viruses, 4(4), 424-446.

Yoo, D., C. Song, et al. (2010). "Modulation of host cell responses and evasion strategies for porcine reproductive and respiratory syndrome virus." Virus Res **154**(1-2): 48-60.

Yoon, I. J., H. S. Joo, et al. (1992). "An indirect fluorescent antibody test for the detection of antibody to swine infertility and respiratory syndrome virus in swine sera." J Vet Diagn Invest **4**(2): 144-147.

Yoon, I. J., H. S. Joo, et al. (1994). "A modified serum neutralization test for the detection of antibody to porcine reproductive and respiratory syndrome virus in swine sera." J Vet Diagn Invest **6**(3): 289-292.

Ziebuhr, J., E. J. Snijder, et al. (2000). "Virus-encoded proteinases and proteolytic processing in the Nidovirales." J Gen Virol **81**(Pt 4): 853-879.

Zuckermann, F. A., E. A. Garcia, et al. (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.