ENHANCING THE INNATE IMMUNE RESPONSE IN PORCINE CELLS THROUGH GENETIC MANIPULATION OF THE IRF-7 5' UTR

A Thesis

by

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ABSTRACT

Viral epidemics in the pork industry continue to plague the agricultural industry, national food security, and human health. Current preventative measures are insufficient for deterrence as viruses rapidly mutate and reemerge despite vaccinations and biosecurity. In order to prevent viral outbreaks within and between pork facilities, alternative measures are needed.

The innate immune system is a host's first response to pathogens. It offers a rapid response; however, it does not have the specificity which is seen in the adaptive immune system. In its initial response, cytokines IFNα and IFNβ induce expression of interferon stimulated genes to inhibit viral replication within the cell. IRF-7 has been identified as the master regulator of type I IFN transcription and is tightly controlled by OASL and 4E-BP1, binding to the secondary structure of its 5' UTR. To enhance the innate immune system response to viruses, the DNA sequence for the 5' UTR of IRF-7 in porcine cells was modified using the CRISPR/Cas9 gene editing system. Following UTR modifications, 9 modified cell lines were produced and clonally selected, along with 2 control cell lines that were not modified. All cell lines were transfected with poly I:C to induce the innate immune response and transcript levels of genes associated with the IFN pathway analyzed by RT-qPCR.

Deletions induced by the CRISPR/Cas9 system in cell lines successfully altered the IRF-7 5' UTR DNA sequence in 82% of cells. RNA folding predictions of the two modified cell lines with a heightened immune response show drastic alterations to the

IRF-7 5’ UTR in regions hypothesized to be critical for IRF-7 translational regulation. Understanding the relationship of the 5’ UTR secondary structure and IRF-7 regulation allows for the possibility of enhanced innate immune responses in animal models with IRF-7 5’ UTR modifications or even in targeting the IRF-7 5’ UTR in humans using pharmacological substances.

DEDICATION

To my family and Sierra. Through the good, the bad, and the uncertainties, you have all continued to support my endeavors.

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Contributors

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NOMENCLATURE

PEDv	Porcine Epidemic Diarrhea virus
PRRSV	Porcine Reproductive and Respiratory Syndrome Virus
SIV	Swine Influenza Virus
PRCV	Porcine Respiratory Coronavirus
SFV	Classical Swine Fever Virus
FMDV	Foot and Mouth Disease Virus
PRR	Pathogen Recognition Receptors
TLR	Toll-like Receptors
TBK1	TANK Binding Kinase 1
IRF	Interferon Regulatory Factor
IFN	Interferon
JAK	Janus Kinase
TYK	Tyrosine Kinase
STAT	Signal Transducer and Activator of Transcription
ISGF3	Interferon Stimulated Gene Factor 3
ISG	Interferon Stimulate Genes
NK	Natural Killer
IL	Interleukin
PAMP	Pathogen Associated Molecular Patterns
LPS	Lipopolysaccharide

TRIF	TIR Domain-Containing Adaptor Protein Inducing IFNβ
RIG-I	RNA Helicase Retinoic Acid Inducible Gene I
MDA5	Melanoma Differentiation Associate Gene 5
DAI	DNA Dependent Activator of IRF
MAVS	Mitochondrial Antiviral Signaling Protein
NOD	NOD-Containing Protein
IKKε	IκB Kinase-ε
pDC	Plasmacytoid Dendritic Cells
MYD88	Myeloid Differentiation Primary Response Protein 88
dsRNA	Double Stranded RNA
TNFα	Tumor Necrosis Factor α
NCOR2	Nuclear Co-Repressor 2
HDAC3	Histone Deacetylase 3
eiF	Eukaryotic Initiation Factor
UTR	Untranslated Region
OASL	2'-5'-Oligoadenulate Synthetase Like 1
4E-BP	4E-Binding Protein
PI3K	Phosphoinositide 3-Kinase
mTOR	Mammalian Target of Rapamycin
EBV	Epstein-Barr Virus
LMP	Latent Membrane Protein
SLE	Systemic lupus Erythematosus

NF-κB	Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B Cells
ISRE	Interferon Stimulated Response Elements
GAS	Gamma Activated Sequences
miRNA	microRNA
MHC	Major Histocompatibility Class
MAPK	MAP Kinase
Mnk	MAPK Interacting Protein Kinase
PKR	Protein Kinase RNA-Activated
ssRNA	Single Strand RNA
MX	Myxovirus Resistant Protein
OAS	2'-5' Oligoadenylate Synthase
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
TALENS	Transcription Activator-Like Effector Nucleases
ZFN	Zinc Finger Nickases
RELA	REL Associated Protein
PAM	Protospacer Adjacent Motifs
crRNA	Precursor CRISPR RNA
DSB	Double Strand Break
NHEJ	Non Homologous End Joining
HDR	Homologous Directed Repair
gRNA	Guide RNA
EST	Expressed Sequence Tag

PCR	Polymerase Chain Reaction
NCBI	National Center for Biotechnology Information
SK6	Swine Kidney Cells
PIADC	Plum Island Animal Disease Center
DMEM	Dulbecco's Modified Minimum Essential Media
FBS	Fetal Bovine Serum
Poly I:C	Polyinosinic:Polycytidylic Acid
RT-qPCR	Reverse Transcription Quantitative PCR
UCSC	University of California Santa Cruz
ELISA	Enzyme Linked Immunosorbent Assay

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Impact of Disease in the Pork Industry

Agricultural sustainability and food security is a growing concern worldwide as the world population continues to increase at a tremendous rate. The world's population is estimated to grow to 9 billion by 2050 and there is a preexisting strain on the agricultural industry, as 1 out of 7 people do not have access to adequate protein or energy (Godfray et al., 2010). Worldwide demand for agricultural products is already rising past what the current international agricultural industry can supply. The increase in demand for animal protein is not only contributed to by the population rise, but by economic growth, rapid urbanization, and an increase in wealth (Godfray et al., 2010; Herrero and Thornton, 2013). Despite the increase in agricultural efficiency in the past few decades, the competition for land use is limiting the growth of both plant and animal crops resulting in the need for genetic research to continuously enhance crop and animal performance (Godfray et al., 2010). Recently the largest increase in livestock production efficiency has been witnessed in monogastric animals such as poultry and swine. Through years of selecting for high producing pigs and the vertical integration of the pork industry, pork production has become one of the most efficient sources of animal protein, a major contribution to the nation's and the world's food security. The nation's pork production has been growing approximately 3% each year but the maximization of the pork industry has also increased the risk of disease outbreaks (Herrero and Thornton,

2013). Although pork production is extremely efficient, its Achilles' heel is the continuous viral epidemics that effect the industry as well as the consumers.

Despite best efforts, the US pork industry is extremely vulnerable to future pathogenic outbreaks, such as Porcine Epidemic Diarrhea virus (PEDv), Porcine Reproductive and Respiratory Syndrome Virus (PRRSV), Swine Influenza Virus (SIV), Porcine Respiratory Coronavirus (PRCV), Classical Swine Fever Virus (SFV), as well as Foot and Mouth Disease Virus (FMDV), which could all cause significant economic loss. For example, in 2013, the United States pork industry was crippled by an outbreak of PEDv. Due to the rapid transmission of this virus, it quickly spread throughout the country and across international borders into Canada as well as Mexico. This resulted in the sudden loss of 7 million piglets, 10% of the United States' domestic pig population, within the year (Jung and Saif, 2015). Due to the massive loss in animals, the annual economic loss is estimated to be $900 million to $1.8 billion (Annamalai et al., 2015; Paarlberg, 2014). PEDv has contributed to viral outbreaks since the early 1970s, however, despite an increase in biosecurity of pork facilities and an intense vaccination protocol, the virus mutated and spread swiftly; most likely by transport trailers (Dee et al., 2014; Jung and Saif, 2015; Lowe et al., 2014). This is an extremely worrying example which illustrates that viral epidemics, such as was seen with PEDv, are able to infect animals despite vaccination and biosecurity protocols (Jung and Saif, 2015).

Another intriguing issue is the susceptibility of pigs to both avian and human influenza viruses, allowing for the creation of zoonotic diseases, threatening not only food security, but human health as well (Kida et al., 1994). The best example of this is

SIV, which is known to use pigs as a “mixing reservoir” to allow for these viruses, usually originating from an avian source, to mutate into transspecies viruses and affecting humans (Kothalawala et al., 2006). Although SIV is not known to be cause high mortality rates, the loss of productivity, withdrawal time, and threat to human health burdens the industry.

Functional mechanisms of the innate immune system

The first defense against pathogens in mammals is the innate immune system, expressed in almost every mammalian cell. Upon recognition of a viral infection, the porcine innate immune system immediately initiates several pathways to create an anti-viral state. The most important response to pathogens involves interferons, which are cytokines expressed in most cells with potent anti-viral and growth inhibitory effects (Platanias, 2005). Upon infection, pathogen recognition receptors (PRRs) on the cell membrane, in the cytosol, and on endosomal compartments, recognize bacterial and viral components, (McNab et al., 2015; Ning et al., 2011). The most important of these receptors are the Toll-like receptors (TLR) on the cell membrane. Upon recognition of viral infection, TLR receptors activate TANK Binding Kinase 1 (TBK1) which phosphorylate interferon regulatory factor (IRF) 3 and a small amount of IRF-7 which are then translocated to the nucleus, as seen in Figure 1 (McNab et al., 2015; Ning et al., 2011). The initial phosphorylation of IRF-3 and IRF-7 both allow for the transcription of Type I Interferons (IFNs) which, in turn, activates additional IRF-7 to create a positive feedback loop and increases Type I IFN production, (McNab et al., 2015). Type I IFNs are robust cytokines which inhibit the translation of viral mRNAs through many pathways in order to create an ant-viral state in the cell (Platanias, 2005). The Type I IFNs, most commonly IFNα and IFNβ, bind to IFNAR1 and IFNAR2 transmembrane receptors which ligate and activate Janus kinase I (JAK 1) and tyrosine kinase 2 (TYK 2). Upon autophosphorylation and activation of JAK 1 and TYK 2, several different immune pathways are induced, one being the signal transducer and activator of

transcription (STAT) 1 and 2 pathway. STAT 1 and STAT 2 bind to IRF-9 to form the interferon stimulated gene factor 3 (ISGF3) complex which initiates transcription of interferon stimulated genes (ISG) as well as increases the synthesis of more IRF-7. The transcription of ISGs cause the anti-viral state of the infected cell, as well as in neighboring cells, to limit the spread of the viral infection and continue production of IRF-7 which sustains the transcription of Type I IFNs, (McNab et al., 2015; Ning et al., 2011). Although only a few pathways to initiate IFN production have been mentioned, there are several others that contribute to IFN responses. The activation of multiple pathways simultaneously are required to trigger the production of IFN; one pathway alone is not sufficient, which minimizes IFN expression when a pathogen is not present, (Platanias, 2005). Efficient interferon production in an animal is critical for its survival against pathogens. Even more important than interferon production itself is the positive feedback loop between IRF-7 and IFN, which regulates the amount of interferon transcription, ISG transcription, and the amplitude of the animals' response to pathogens.

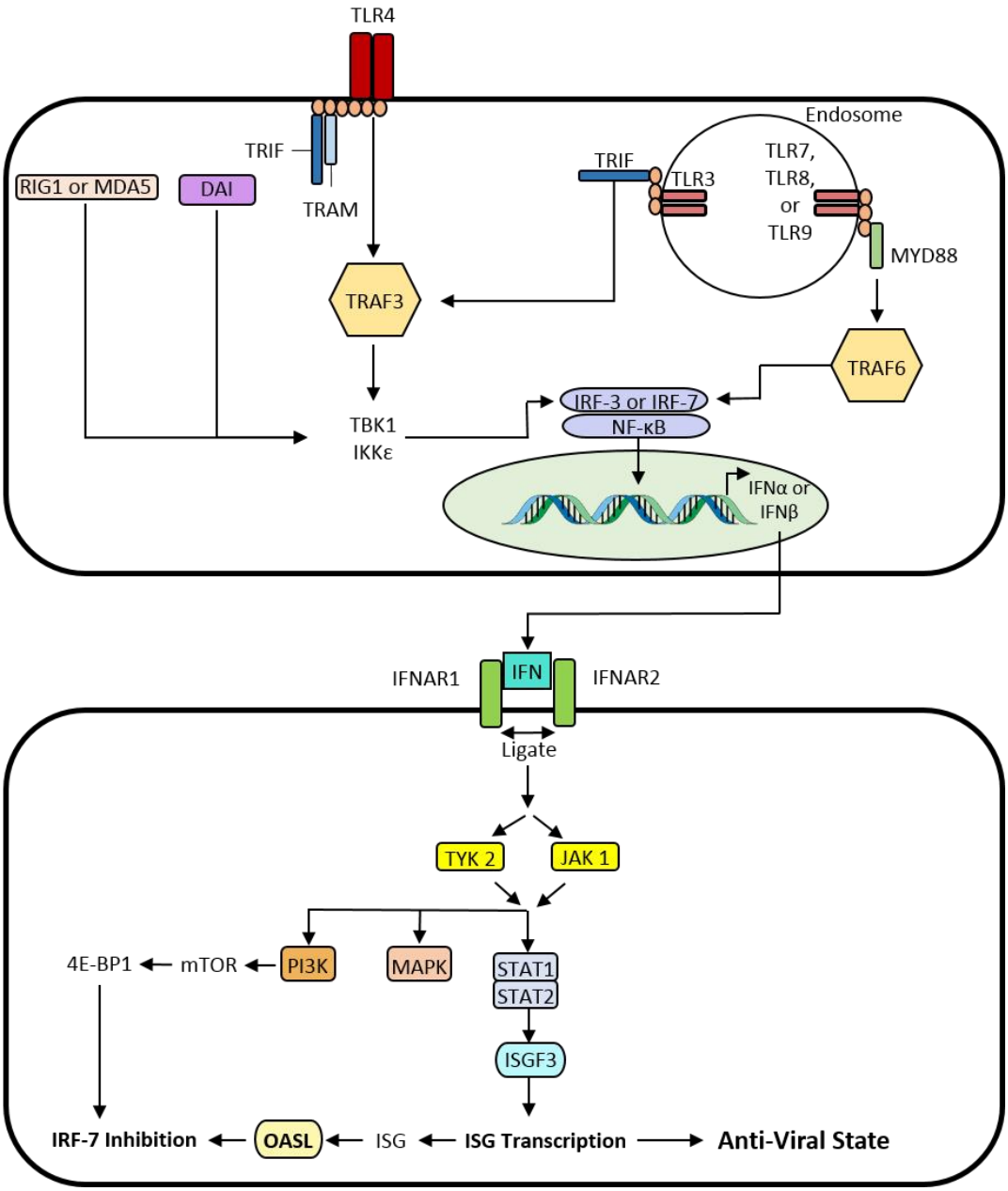

Figure 1: Overview of the innate immune response (McNab et al., 2015)

Upon recognition of viral pathogens, PRRs such as TLRs, DAI, MDA5, or RIG1 activate TBK1 and IKKε which phosphorylate IRF-3 and IRF-7. IRF-3 and IRF-7 translocate to the nuclease and, along with NF-κB, induce the transcription of type I IFNs. In a different pathway, TLR 7, TLR8, and TLR9 within the endosome may also recognize viral components and activate the MYD88 pathway which also activates IRF-3 and IRF-7. Following the induction of transcription, type I IFNs bind to its receptors on neighboring cells and initiates the JAK-STAT pathway which leads to ISG transcription. Adapted from (McNab et al., 2015).

Initiation of type I IFN production

Interferons are a very broad family of cytokines that are instrumental in innate and adaptive immunity. Type I IFNs, IFNα and IFNβ, are innate cytokines that initiate the antiviral state of the cell and work to prevent viral intracellular viral replication. There are other type I IFNs however, IFNα and IFNβ are the most potent and critical for proper innate immune system function. IFNγ is the only type II IFN but is an important cytokine, bridging the innate and adaptive immunity. IFNγ, produced by natural killer (NK) and T cells, is a potent activator of innate macrophages and has several other roles in the immune system beyond the innate system (Schoenborn and Wilson, 2007). Type III IFNs consist of IFN λ1-4 and were previously described as interferon-like cytokines and interleukin (IL) 28. IFN λ has similar functions to type I IFNs but are much more restricted (McNab et al., 2015). Although IFNs are all important to overall immune function, only type I IFNs, IFNα and IFNβ, will be discussed further.

Pathogens and their components are recognized by several different pathogen recognition receptors (PRRs) depending on the location of pathogen and what the nature of its pathogen associated molecular patterns (PAMP) are. TLRs, a family of PRRs, are present at several different locations of the cell such as in endosomes, in the cytosol, and on the cell surface. This broad range of receptor locations allows the cell to identify a wide range of pathogens, both intracellular and extracellular. As the cell is transporting broken down fragments of pathogens in endosomal compartments, the receptor within those compartments, TLR3, can recognize single and double stranded RNA as well as

DNA containing CpG islands to induce type I IFN expression (Der et al., 1998; McNab et al., 2015). Several extracellular pathogens present unique PAMPs which can be identified by PAMP specific PRRs, located on the cellular membrane, such as TLR4 recognizing lipopolysaccharides (LPS) from bacterial infections. The canonical signaling for type I IFN induction is usually initiated by TLR3 and TLR4, influencing the adaptor protein TIR domain-containing adaptor protein inducing IFNβ (TRIF) activation of TBK1(McNab et al., 2015). In addition to the TLRs, RNA helicase retinoic acid inducible gene I (RIG-I), melanoma differentiation associate gene 5 (MDA5), and DNA-dependent activator of IRF (DAI), which are located within the cytosol, mainly recognize intracellular viral RNA. These receptors utilize mitochondrial antiviral signaling protein (MAVS) to activate TBK1 (McNab et al., 2015; Paludan and Bowie; Takaoka et al., 2008). Also in the cytoplasm, NOD-containing protein (NOD) 1 and NOD2, which are molecular sensors, recognize several different ligands and foreign nucleic acids (McNab et al., 2015; Moreira and Zamboni, 2012). Following the activation of TBK1 by certain PRRs, kinase IκB kinase-ε (IKKε) along with TBK1, phosphorylate IRF-3 and IRF-7 (McNab et al., 2015). Plasmacytoid dendritic cells (pDCs) express TLR7 and TLR9 which, unlike the previous receptors, utilizes the myeloid differentiation primary response protein 99 (MYD88) signaling pathway, known as the MYD88 dependent pathway, instead of activating TBK1 for type I IFN induction (McNab et al., 2015; Moynagh, 2005). These signaling pathways are all unique and synchronous to facilitate proper type I IFN transcription and innate immunity

function. The phosphorylation of IRF-3 and IRF-7 by such pathways are key mediators in type I IFN transcription for the inhibition of intracellular viral replication.

IRF family and the induction of IFN transcription

The interferon regulatory factor (IRF) family are transcription factors with 9 members (IRF1-9) which frequently interact with ISGs, STATs, and other IRF family members in order to control type I IFN transcription, adaptive immune cell development, as well as regulate oncogenesis (Ning et al., 2011). Another member, IRF-10, has been identified but is only present in avian species (Nehyba et al., 2002). Genes encoding IRFs are highly conserved among vertebrates and originated in metazoans, directly associated with the formation of multi-cellular organisms (Nehyba et al., 2009). In most non-lymphatic cells, IRF-7 resides inactive in the cytoplasm until the recognition of a pathogen and the induction of type I IFNs by IRF-3.

IRF-7 has similar homology to IRF-3 and is stimulated with the same PRRs as IRF-3, however, IRF-7 translation can also be initiated by, TLR7, TLR8, and TLR9 (Takaoka et al., 2008). In the canonical pathway, the activation by phosphorylation of IRF-7 is the second phase of type I IFN transcription induction and is the most potent stimulation, leading to the identification of IRF-7 as the master regulatory of type I IFNs and their signaling pathways (Honda et al., 2005; Sato et al., 2000). However, IRF-7 translation can also be directly activated without the initial IFN burst induced by IRF-3, as discussed below. There are certain PAMPs that will cause the cellular receptors to act directly upon IRF-7 such as viral or bacterial double stranded RNA (dsRNA) with CpG islands, among others (Ning et al., 2011; Yang et al., 2003). In addition to direct

pathogen and type I IFN activation, IRF-7 translation can be induced by 12-o-tetradecanoylphonl-13-acetate, tumor necrosis factor α (TNFα), and LPS (Ning et al., 2011).

In the canonical pathway, following the recognition of a pathogen, type I IFN transcription is initiated biphasically by IRFs, Figure 2 (Sato et al., 2000). First, IRF-3, which is expressed constitutively in almost all cells, is activated by phosphorylated with the viral induction of PRRs such as TLR3, TLR4, RIG-1, MDA5, and DAI. In an MYD88 independent activation by TLR3 or TLR4, IRF-3 is phosphorylated by IKKε and TBK1, then translocated to the nucleus to induce type I IFNs, specifically IFNβ (Takaoka et al., 2008). However, in pDCs, IRF-7 may be phosphorylated by IKKα and IRAK1, however, more studies are needed to confirm this observation (Hoshino et al., 2006; Ning et al., 2011; Uematsu et al., 2005). Following phosphorylation, IRF-3 stimulates an initial burst of IFNβ transcription and is just enough to stimulate ISG expression for the second induction of type I IFNs. After the initial burst of IFNβ, IFN-activated transcription factor (ISGF3), which contains IRF-9 and STAT1/2, is activated and not only binds directly to inducible elements of IFNα and IFNβ, but also directly induces IRF-7 translation (Ivashkiv and Donlin, 2014; Sato et al., 1998). IRF-3 is an instrumental player in type I IFN transcription, however, it does not contribute to the pathway in the absence of IRF-7, suggesting that IRF-7 is the critical regulator (Honda et al., 2005).

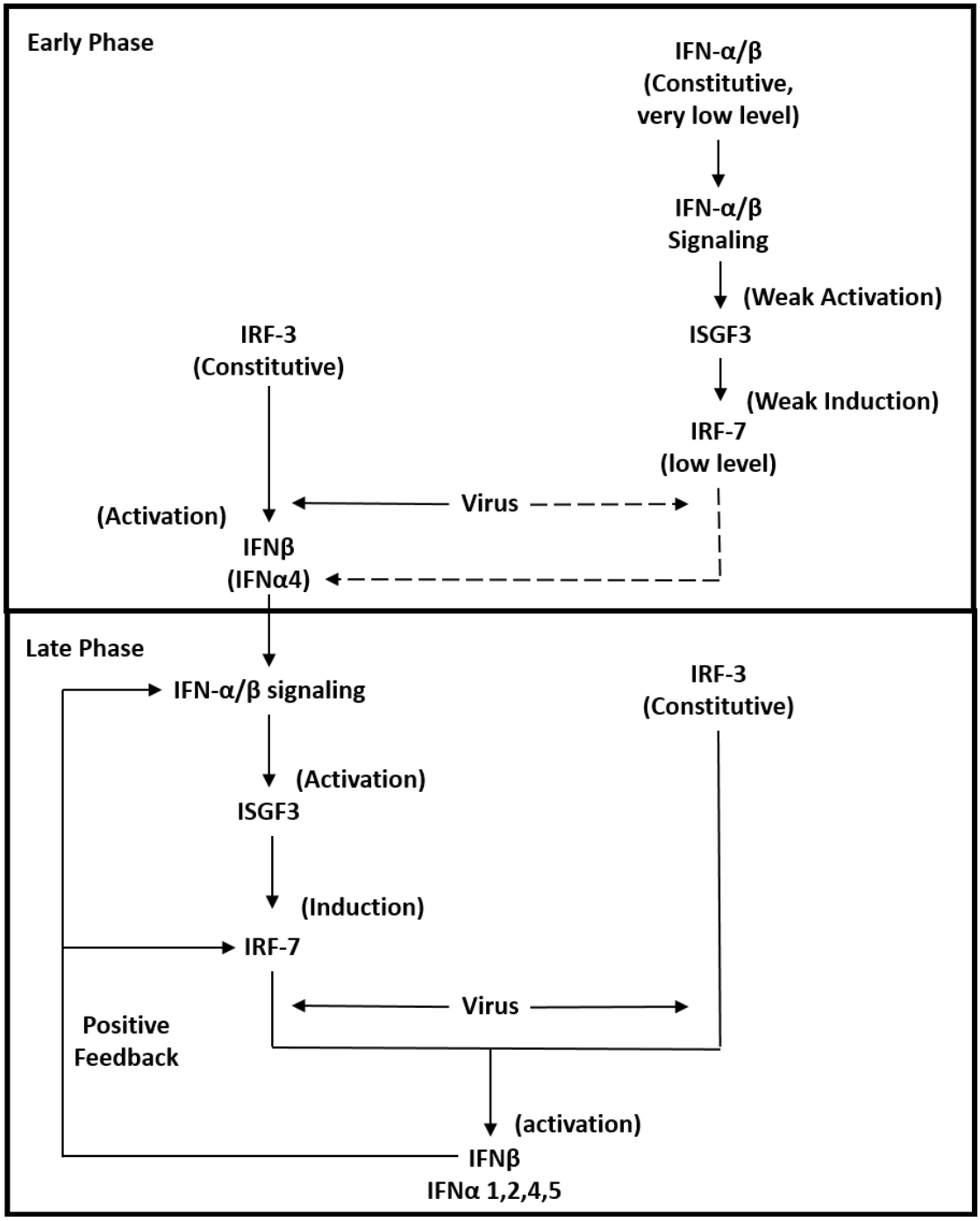

Figure 2: Biphasic activation of type I IFNs (Sato et al., 2000)

Type I IFNs require a biphasic activation. The early phase is critical for maintenance of IFN receptors and quick activation while the late phase is important for viral response. Modified from (Sato et al., 2000).

Upon activation, IRF-7 is translocated to the nucleus and forms complexes with other coactivators, such as IRF-3, NF-κB, C-Jun, activating transcription factor2, and p300/CREB-binding protein, to induce further transcription of type I IFNs (Honda et al., 2005; Ning et al., 2011). Once in the nucleus, IRF-7 protein forms heterodimeric or homodimeric complexes with IRF-3, known as the virus-activated factor, however, IRF-3 and IRF-7 act separately on type I IFN expression while complexed (Takaoka et al., 2008; Yang et al., 2003). IRF-3, as well as IRF-7, are known to potently induce transcription of IFNβ in almost all cells while IRF-7, alone, preferentially induces IFNα expression in pDCs. (Hiscott, 2007b; Takaoka et al., 2008). There are conflicting studies suggesting IRF-7 protein can activate IFN transcription alone, without forming complexes with co-activators, however if this is correct, IFN response will be much weaker (Ning et al., 2011; Yang et al., 2003). IRF-7 expression is maintained, after initial activation of type I IFNs, through a positive feedback loop between active type I IFNs and IRF-7 (Ning et al., 2011). This positive feedback loop allows for continuous type I IFN expression until translational factors inhibit IRF-7 translation, removing the transcriptional stimulation of IFN. Despite normally expressed IRF-7 having antitumor effects, when overexpressed, IRF-7 can exhibit oncogenic properties and contribute to other autoimmune disorders such as rheumatoid arthritis (Ning et al., 2011). Proper regulation of IRF-7 is key to its success in maintaining proper IFN expression and combatting pathogens through the innate immune system.

IRF-7 Regulation

IRF-7 expression initiates a robust cascade of IFN mediated responses and needs to be finely controlled by several unique and converging signals for proper activation and function. As the primary mediator of type I IFNs, a very restricted gene expression profile is critical for proper immune function. IRF-7 regulation can occur transcriptionally or translationally and is dependent on both viral infection and the presence of type I IFNs for proper activation (Hiscott, 2007a).

Transcriptional regulation of IRF-7 is not well known but a recent study has shown that some transcriptional regulation occurs through transcription factors, nuclear co-repressors, and histone deacetylases. The FOXO family of transcription factors are known to assist in maintaining cell homeostasis by regulating gene expression associated with cell cycle control (Dejean et al., 2009). Transcription factor FOXO3 specifically has function in the immune system and suppresses basal IRF-7 expression, however, it does not have an effect on stimulated IRF-7 levels. In FOXO3 null murine macrophages, the absence of FOXO3 correlated with an increase in IRF-7 histone acetylation and thus, an increase in basal levels of IRF-7, unveiling its transcriptional regulation. Additionally, in these macrophages, binding of nuclear co-repressor 2 (NCOR2) and histone deacetylase 3 (HDAC3) binding to the IRF-7 promoter was reduced suggesting that FOXO3 plays a role in enabling other transcriptional regulators to bind to the IRF-7 promoter (Litvak et al., 2012). Although transcriptional regulation of IRF-7 is understudied, it seems that translational regulation of this gene is more active and critical for proper function of IRF-7 in an anti-viral response.

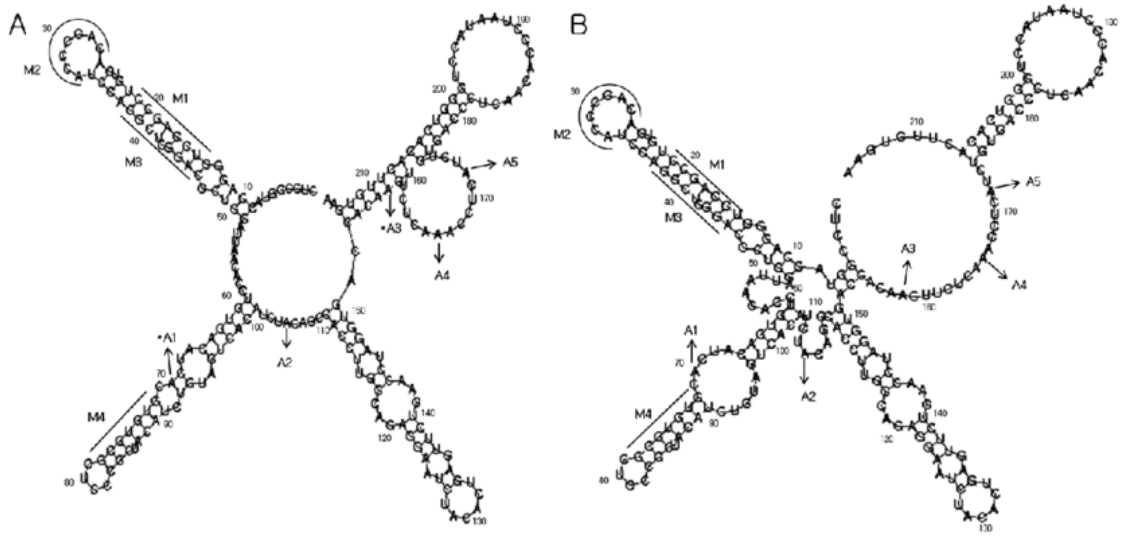

Figure 3: Predicted folding of the murine IRF-7 5' UTR (Kim et al., 2014)
A: Minimum free energy prediction of IRF-7 5' UTR mRNA folding. B: Centroid prediction of IRF-7 5' UTR mRNA folding. Four distinct stem loops are shown in both predictions which are hypothesized to be critical for translational factor binding (Kim et al., 2014).

The translation of mRNA to protein requires the formation of at least 9 eukaryotic initiation factors (eiF) within the cell's ribosome. These eiFs allow ribosomal subunits to attach to recycled post-termination complexes and form new complexes for translation, ultimately resulting in the 80S ribosomal initiation complex, which begins the translational elongation phase. Preceding the formation of the 80S complex, the mRNA is unwound at the cap region by the eiF4F complex, which activates the mRNA. Once the mRNA is activated, the 43S complex attaches to the activated mRNA and allows the 43S complex to scan the 5' UTR and recognize the initiation codon within the

mRNA. Following these initiation steps, additional subunits are added to the translational complexes to form the 80S complex and allow for the elongation of polypeptide strands (Jackson et al., 2010). The complexity of translation initiation provides an opportunity for gene expression to be regulated at this step. Regulation of translation allows for rapid expression or repression of genes when the cell is under stress (Sonenberg and Hinnebusch, 2009). During cell stress, global translation is inhibited and the cell's energy source goes into the rapid translation of genes needed to respond to such stress (Holcik and Sonenberg, 2005; Sonenberg and Hinnebusch, 2009). Cell stress caused by viruses complicate the cell's translational response to the infection, since most viruses target the host cell's translational mechanisms by either inhibiting translation or using the cell's translational mechanisms for its own replication (Sonenberg and Hinnebusch, 2009). In order to prevent the hijacking of the host cell's translational mechanisms by viruses, it is critical that the cell recognize and respond to the virus rapidly, usually through the initiation of IRF-7 translation.

IRF-7 is translationally regulated by features within its 5' untranslated region (UTR), upstream of the translational start site. Translational factors, 2'-5'-oligoadenylate synthetase like 1 (OASL1) and 4E-binding protein 1 (4E-BP1), are two known potent regulators of IRF-7 translation. OASL1 is a member of the OAS family as well as an interferon stimulated gene (ISG). Though not well understood, OASL1 is thought to be induced by type I IFNs, viral and bacterial infection, lipopolysaccharide (LPS) stimulation, and IRF-3 (Guo et al., 2012; McNab et al., 2015; Zhu et al., 2015; Zhu et al., 2014). Although OASL1 has been shown to have anti-viral effects itself, it is known

to inhibit the translation of IRF-7 by binding to its 5' UTR (Kim et al., 2014; Lee et al., 2013). The predicted folding of the 5' UTR RNA suggests 4 stem loops with 2 distinct single stranded regions which are assumed to be the binding sites of OASL1, Figure 3 (Kim et al., 2014). Studies suggest that the binding of OASL1 to the IRF-7 5' UTR inhibits the 43S complex scanning process from scanning the 5' UTR and identifying the start codon, thus inhibiting its translation (Lee et al., 2013). 4E-BP1 is also an inhibitor of IRF-7 and is regulated by the phosphoinositide 3-kinase (PI3K) / mammalian target of rapamycin (mTOR) pathway. During type I IFN stimulation, the PI3K and mTOR pathways are initiated which phosphorylates and inhibits 4E-BPs from binding to eIF4E, allowing the association of eIF4E and eIF4G to occur as well as the activation of the mRNA for translation (Colina et al., 2008; Ning et al., 2011; Piccirillo et al., 2014). In contrast to the OASL1 inhibition of the 43S complex scanning, 4E-BP1 prevents the 43S complex from loading onto mRNA, also inhibiting the IRF-7 translation (Lee et al., 2013; Sonenberg and Hinnebusch, 2009). The expression of 4E-BP1 is not affected by the innate immune response and therefore, its relative levels do not change during pathogen invasion. It is suggested that 4E-BP1 continuously inhibits IRF-7 translation during homeostatic conditions however, when the immune response stimuli becomes significantly heightened, the translational inhibition of 4E-BP1 is removed by its phosphorylation via mTORC1, allowing the dissociation of 4E-BP1 from the EIF4F complex, the association of eiF4E and eiF4G, the activation of mRNA, and thus, the translation of IRF-7 (Lee et al., 2013; Piccirillo et al., 2014). Together, OASL1 and 4E-BP1 regulate the duration of type I IFN expression. Once the type I IFN signaling

pathways are underway and the cell is in an anti-viral state, OASL1 inhibits IRF-7, lowering the type I IFN response without silencing it. Once the innate immune system is down regulated and returning to a normal quiescent state, 4E-BP1 returns to its hypophosphorylated state and along with OASL1, completely and directly inhibits IRF-7 translation and indirectly inhibits type I IFN transcription.

Several studies have suggested how and where on the IRF-7 5' UTR the translation inhibitory factor, OASL1, interacts. During previous studies in our laboratory, the porcine IRF-7 5' UTR was truncated at different locations and cloned into a luciferase plasmid. A 129 base pair region at the 3' end of the UTR was shown to be a critical regulatory element within the UTR as a whole, suggesting that the UTR RNA folding was crucial for IRF-7 regulation (Carvajal, 2014). In another study at Yonsei University, specific locations within the murine IRF-7 5' UTR were targeted and truncated, then cloned into luciferase plasmids to measure OASL1 suppression of IRF-7 (Lee et al., 2013). The authors suggested that OASL1 bound to stem loops present in the 5' end of the IRF-7 5' UTR (Lee et al., 2013). IRF-7 is proven to increase the innate immune response and its 5' UTR presents a unique target to enhance the innate immune response, however, unregulated overexpression of IRF-7 is not a viable option. The present work sought to better understand how IRF-7 is regulated by OASL1 in situ. Gene editing technologies were used to create modifications within the IRF-7 5' UTR. Subsequently, immune system stimulation, transcript and protein levels of immune regulated genes measured at different time points.

IRF-7 functions independent of IFNs

IRF-7 has several functions in addition to type I IFN induction, such as activating proteins for antiviral, inflammatory, and pro-apoptotic functions as well as regulating the cell cycle and tumor suppression. Although IRF-7 is mostly known for these functions in the innate immune system, it has shown important function in the adaptive immune system as well. Macrophages transduced with active IRF-7 displayed enhanced tumor suppression abilities as well as an IRF-7 induced long lasting up regulation of IFN α and IFN β, effectively utilizing both the innate and adaptive immune systems to ward off invading viruses and unregulated cell division (Hiscott, 2007b; Romieu-Mourez et al., 2006).

Another interesting link between innate and adaptive immunity is that seen in plasmacytoid dendritic cells (pDC). These specialized dendritic cells produce larger amounts of IFN α than conventional dendritic cells. Viruses such as vesicular stomatitis virus reside in the endosomal compartment following infection which, in pDCs, is where CpG-A, a TLR9 ligand, resides along with MyD88-IRF-7 complexes (Honda and Taniguchi, 2006). In conventional dendritic cells, CpG-A localizes in lysosomes and will not recognize these viruses. The localization of CpG-A in the endosome of pDCs, allows for the initiation of TLR9 signaling, IRF-7 translation, and finally, IFN α transcription and secretion (Gabriele and Ozato, 2007; Hiscott, 2007b; Honda and Taniguchi, 2006).

Recent studies have shown IRF-7 to suppress tumor formation through both the innate and adaptive mechanisms. In macrophages, IRF-7 can promote apoptosis in

macrophages through inducing the production of apoptotic ligands and subsequently assisting with programmed cell death of tumor cells (Huang et al., 2009; Ning et al., 2011). In another cascade, a tumor suppressor gene, BRCA1, can induce overexpression of IRF-7 which will inhibit the growth of certain breast cancer cells (Andrews et al., 2002; Ning et al., 2011).

Overexpression of IRF-7

Despite the positive effects of IRF-7 on the innate immune response, its expression must be tightly controlled by the mechanisms described above. IRF-7 overexpression has been shown to cause lymphomas when associated with Epstein-Barr virus (EBV) and its oncoprotein, latent membrane protein 1 (LMP1) (Ning et al., 2011; Zhang et al., 2004). Studies have suggested that LMP-1 and IRF-7 associate through a positive feedback loop where LMP induces IRF-7 activation and expression and the increase in active IRF-7 up regulates LMP-1 promoter activity, causing the formation of EBV associated tumors (Ning et al., 2011; Zhang et al., 2004).

Due to its potent regulation and activation of type I IFNs, IRF-7 is hypothesized to have roles in autoimmune disorders such as rheumatoid arthritis and systemic lupus erythematosus (SLE) (Fu et al., 2011; Heinig et al., 2010; Ning et al., 2011). Future studies are needed to further understand the effects of overexpressed IRF-7, nevertheless, evidence suggests that proper regulation and control of IRF-7 is critical for proper function in both the innate and adaptive immune systems.

Activated exogenous IRF-7 enhances immune response

The recent discovery of IRF-7 as a master regulator of the type I IFN response

has led to research in the use of IRF-7 as a therapeutic agent to assist the host's antiviral response. At the Plum Island Animal Disease Center, activated IRF-7/IRF-3 fusion protein in an adenovirus was used to inoculate pigs, which, after inoculation, were shown to be completely resistant to FMDV (Ramírez-Carvajal et al., 2016). In addition to using IRF-7/IRF-3 as protection against invading viruses, another study successfully used IRF-7/IRF-3 as adjuvants in vaccination. In an attempt to understand methods for plasmid DNA vaccinations, activated IRF-7/IRF-3 as adjuvants to a plasmid DNA vaccine were able to elicit protective immunity against *E. coli* β-galactosidase (Bramson et al., 2003). IRF-7 has been shown to be an effective agent in strengthening the host's immune response however, exogenous IRF-7 cannot be regulated by the host properly. The lack of regulation and the negative effects of IRF-7 overexpression limits its use as a therapeutic agent.

Proper IFN induction requires co-activator NF-κB

In addition to the activation of IRF-3 and IRF-7 through TBK1, TLRs also initiate the transcription factor, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB). This transcription factor, like IRF-7, has both MYD88 dependent signaling and MYD88 independent signaling. The MYD88 dependent pathway is triggered by the TLRs, with the exception of TLR3, recruiting MYD88 which promotes the activation of IKKα and IKKβ (Moynagh, 2005). These IKKs allow for the translocation of NF-κB to the nucleus where it stimulates the expression of co-stimulatory molecules and proinflammatory proteins (Moynagh, 2005). For the MYD88 independent pathway, TLR3 signaling activates NF-κB however, there are contradicting

studies about the mechanism by which NF-κB is activated in this pathway (Moynagh, 2005). Due to the extreme similarity between the activation of IRF-3, IRF-7, and NF-κB, these transcription factors together maximize the type I IFN response to viral invasion. In addition to their synergistic effects NF-κB can also act as a regulator during autoimmune disorders for example, the absence of NF-κB during IRF-7 overexpression events prevents type I IFN response from being at its maximal level, minimizing the possible negative consequences associated with unregulated IRF-7 translation.

Type I IFN Signaling

Type I IFNs, specifically IFNα and IFNβ, are critical cytokines of the innate immune response and function to induce anti-microbial states to minimize viral replication, to regulate the innate immune response in promoting antigen presentation, and to activate the adaptive immune system, providing a robust response against pathogen invasion (Ivashkiv and Donlin, 2014). Basal levels of IFNβ are maintained by most cells in homeostasis which allows for the maintenance of IFNAR, JAK, STAT1, and IRF9 expression (Ivashkiv and Donlin, 2014). This miniscule expression keeps the cells primed for a rapid and robust IFN response to any pathogen that may invade (Ivashkiv and Donlin, 2014). Following the recognition of a pathogen and type I IFN activation by IRF-3, IRF-7, and NF-κB, type I IFNs initiate the signaling pathway responsible for creating the anti-viral state within the cell. Both IFNα and IFNβ are transcribed and bind to a heterodimeric receptor with components IFNAR1 and IFNAR2. However, IFNAR expression, ligation, and responses can be altered by the host's environment, regulated by other signals outside the scope of this review (Ivashkiv

and Donlin, 2014). In the canonical pathway, upon the binding of type I IFNs, IFNAR1 and IFNAR2 temporarily bind together and allow for the autophosphorylation of Janus kinase 1 (JAK1) and tyrosine kinase 2 (TYK2), for activation. (Darnell et al., 1994; McNab et al., 2015; Platanias, 2005). These kinases individually associate with the specific IFN receptor components; JAK1 with IFNAR2 and TYK2 with IFNAR1 (Darnell et al., 1994; Platanias, 2005). JAK1 and TYK2 regulate the phosphorylation of signal transducer and activator of transcription (STAT) in the cytoplasm or in the nucleus, and when activated, the JAK-STAT signaling pathway is initiated (Platanias, 2005).

The activation of STAT is completely dependent on tyrosine phosphorylation and in this case, the IFN mediated dimerization of IFNAR allows for this to occur through autophosphorylated JAK (Darnell, 1997; Platanias, 2005). There are several STATs however, only STAT1, STAT2, STAT3, and STAT5 are directly affected by type I IFNs. STAT4 and STAT6 are activated in response to IFNα but only in lymphoid tissues (Platanias, 2005). In the classical pathway among non-lymphoid cells, STAT2 is the first to be phosphorylated in response to type I IFNS, following the activation of JAK, and STAT 1 activation is dependent on phosphorylated STAT2, resulting in a delayed stimulation of STAT1 (Darnell, 1997). STATI and STAT2 form DNA binding heterodimers, translocate to the nucleus, and can either directly initiate ISG transcription or form the ISGF3 complex with IRF-9 as mentioned earlier (Darnell, 1997; Platanias, 2005). The ISGF3 complex is able to directly initiate ISG transcription by binding to IFN-stimulated response elements (ISRE) on ISG promoter regions and will also assist

in IRF-7 induction to enhance the type I IFN response and antiviral response. Dissimilarly, most other cytokines induce the phosphorylation of STAT homodimers which bind to gamma activated sequences (GAS) to induce inflammatory responses (Ivashkiv and Donlin, 2014).

Important regulators of genes needed for proper cellular functions are microRNAs (miRNA) and are still undergoing novel studies to completely understand their roles in several biological processes. The innate immune system signaling pathway is no exception and recent studies have both suggested and shown the importance of miRNAs for proper immune function. These miRNAs can not only inhibit viral transcripts, but can also increase the transcription of type I IFN signaling molecules such as STAT1, STAT2, and even IFNβ (Ivashkiv and Donlin, 2014). These miRNAs are IFN stimulated but can also be stimulated by other IFN stimulated molecules such as PRRs and proinflammatory cytokines (David, 2010; Ivashkiv and Donlin, 2014). Promotion of the immune system by miRNAs is important for the potency of IFN signaling as well as the inhibition of viral replication.

Type I IFNs as effector molecules

Type I IFNs have some direct affects including the control of gene expression. IFNα and IFNβ have been shown to increase major histocompatibility (MHC) classes I and II, as well as costimulatory molecules, which are necessary for antigen presentation to T-cells (Khabar and Young, 2007). This increase in MHC due to type I IFNs leads to an increase in macrophage activation, increased natural killer cell activity, and increased cytotoxic T-cell activity, beginning the activation of the adaptive immune system

(Khabar and Young, 2007). In another post transcriptional control mechanism, IFN induces OAS expression which recognizes viral dsRNA and activates RNAse L for viral mRNA degradation (Khabar and Young, 2007).

The type I IFNs not only induce the transcription of ISGs, but they have direct effects on the innate and adaptive immune systems as well, effectively bridging the gap between the two for a regulated, robust response. The IFNs can promote the expression of chemokine receptors, assisting in the dendritic cell migration as well as indirectly promoting T-cell activation. Type I IFNs can also promote IFNγ production, affecting T helper I cell (CD4) differentiation, survival and clonal expansion of cytotoxic (CD8) T-cells (both effector and memory cells). Also through direct and indirect means, IFNα and IFNβ can promote NK cells as well as enhance B-cell responses (McNab et al., 2015).

Despite the importance of type I IFNs in the innate immune system, with certain infections, IFNs can have negative effects on the host and even help in pathogen survival. In chronic viral infections, type I IFNs can cause immunosuppression and exacerbate the inflammation responses, causing tissue damage (Davidson et al., 2014; McNab et al., 2015). In acute viral infections, IFNs produced by myeloid cells increase apoptotic receptor expression on epithelial cells, contributing to host morbidity and mortality (Davidson et al., 2014; McNab et al., 2015). IFNα and IFNβ can also have detrimental effects on the host in responses to bacteria, fungi, and parasitic infections. Additionally, overexpression of type I IFNs either by unregulated IRF-7 or other stimuli, contributes to several autoimmune disorders such as SLE, Sjogren's syndrome, systemic scleroses, myositis, and rheumatoid arthritis (Hall and Rosen, 2010; Ivashkiv and

Donlin, 2014). The contribution that IFNs have on these disease states stresses the powerful effects of IFNs when not regulated properly or when being overstimulated.

ISG activation

Type I IFN signaling is critical for inhibiting pathogen replication and translation however, IFNs are not the only active molecules that cause this inhibition. Once initiated, the IFN signaling cascade induces the transcription and the translation of ISGs, the main active molecules needed for pathogen inhibition and inducing the adaptive immune system.

Type I IFN mediated ISG transcription is controlled by both the ISGF3 complex and the MAP kinase (MAPK) pathways. As discussed earlier, ISGF3 is a major complex that allows for the transcription of ISGs as well as phosphorylated STATs which bind to GAS regions. In addition to ISGF3, Type I IFN and the JAK/STAT pathway activates MAPK which controls the transcription of ISGs (Katsoulidis et al., 2005). MAPK activates p38 and together, they induce ISG transcription however, the exact mechanism is not defined and it is hypothesized that MAPK/p38 could affect chromatin remodeling of ISGs or may have effects on coactivators (Katsoulidis et al., 2005).

In addition to transcriptional control of ISGs, IFNs also mediate translational regulatory pathways for ISGs. Phosphoinositide 3-kinase (PI3K)-AKT-mammalian target of rapamycin (mTOR) signaling is well-known for several other cellular functions however, in the innate immune system, this pathway inactivates translational suppressor eIF4E binding protein 1 complex as well as p70S6 kinase (Ivashkiv and Donlin, 2014; Lekmine et al., 2003). The p70S6 kinase phosphorylates 4E-BP1 which causes its

dissociation from eIF4E and inactivates 4E-BP1, allowing the translation of ISGs, specifically ISG15 and CXCL10, as well as IRF-7 (Joshi et al., 2010; Lekmine et al., 2003). At this point, eIF4E forms a complex with eIF4G and together, they form a translation initiation complex to further increase translation of ISGs (Kaur et al., 2007). Additionally, MAPK interacting protein kinases (Mnk) 1 and 2 assist the p38/MAPK as well as the PI3K-AKT-mTOR signaling cascades in the phosphorylation and control of eIF4E (Joshi et al., 2010). Another direct target of type I IFNs to control ISGs translation is protein kinase RNA-activated (PKR) which inactivates eukaryotic translation initiation factor 2A, allowing for ISG translation and the inhibition of viral protein synthesis (Ivashkiv and Donlin, 2014; Khabar and Young, 2007). Although the ISG name implies that these genes are only induced by type I IFN pathways, new research has revealed that ISGs can be induced by subviral particles, dsRNA, single strand RNA (ssRNA), or other cytokines as well (Terenzi et al., 2006).

ISG functions within the immune system

There are a certain ISGs that have a tremendous effect on the host's response to pathogens, especially viruses. ISG54 (IFIT2) and ISG56 (IFIT1) are very similar ISGs but have slightly different mechanisms. Both are induced by high levels of IFNβ, however, ISG56 is usually expressed in higher amounts and for longer periods of time than ISG54 (Terenzi et al., 2006). The protein products of ISG56 and ISG54, P56 and P54, bind to eiF3 to inhibit the translation of viral genes (Terenzi et al., 2006). ISG56 can also have a regulatory effect on IFN, blocking the cytoplasmic sensing of viral DNA and prevents IFN overexpression during an immune response (Yan and Chen, 2012).

Another important ISG, ISG15, is potently induced by type I IFNs, LPS, IRF-3, IRF-9, and NF-κB (Jeon et al., 2010). Once transcribed, ISG15 can regulate immune responses by inducing the secretion of IFNγ from B-cells and T-cells. It can also regulate signal transduction pathways, antiviral responses, as well as ubiquitination. ISG15 has an abundance of immune functions and when overexpressed, can also have oncogenic properties, so tight regulation must be in effect for ISG15 as well (Jeon et al., 2010).

Myxovirus resistant protein 1 (MX1 in mice, MXA in humans) is one of the few ISGs directly expressed in response to type I IFNs (Haller and Kochs, 2010). MX1 is potently expressed in response to IFNα binding to its IRES component (Yuan et al., 2015). MX1 is mostly known as a potent inhibitor of influenza viruses however, it is primarily an early block to viral replication of most viruses (Haller and Kochs, 2010).

The 2'-5' oligoadenylate synthase (OAS) family consists of 4 ISGs, OAS1, OAS2, OAS3, and OASL, however, OASL is also induced by IRF-3 (Kristiansen et al., 2010; Melchjorsen et al., 2009). OAS1 binds to dsRNA and ssRNA,, initiating OAS1 activation and causes RNA degradation by inducing the RNase L pathway which inhibits protein translation in infected cells and prevents viral replication of pathogens (Kristiansen et al., 2010). Although most of the OAS family research has been done on OAS1, it is believed that this is the same mechanism for OAS2 and OAS3. Uniquely, OASL1 has an inhibitory effect on type I IFN response by its binding and regulation of IRF-7 as discussed earlier. Together with 4E-BP1, OASL1 prevents unregulated amounts of IRF-7 from being translated and thus, prevents the overexpression of type I IFNs and its cascade.

Genetic Engineering Using the CRISPR/Cas System

In recent years, genetic editing technologies have become extremely popular among biologists and used to produce animals with targeted disease resistance. Although CRISPR is the most recent and popular tool currently, there have been animals produced with other technologies. In 2015, cattle with increased resistance to tuberculosis were produced using transcription activator-like effector nucleases (TALENs) (Wu et al., 2015). Other researchers have used zinc-finger nickases (ZFNickases) to produce cows that secrete lysostaphin in their milk, presenting the ability to kill *Staphylococcus aureus* in the milk (Liu et al., 2013). Another research group in 2013, utilized both zinc-finger nucleases (ZFN) and TALENs to target REL associated protein (RELA), an oncogene associated with the immune system, and successfully produced live pigs with disease resistance (Lillico et al., 2013). At the University of Missouri, CD 163, a gene utilized by the PRRS virus in pigs, has been knocked out using CRISPR/Cas9 which resulted in animals that are completely resistant to PRRSV (Whitworth et al., 2016). These are just a few examples of successful gene editing projects focusing on disease resistance in livestock, however, these examples are targeted towards genes that are crucial for specific viruses and their survival. In order to provide an enhanced resistance to a broader range of pathogens and pathogen mutations, the regulators of the host's immune system will need to be carefully and efficiently targeted by genetic editing mechanisms.

Bacteria and archaea exhibit an adaptive immune defense against foreign DNA using clustered regularly interspaced short palindromic repeats (CRISPR). This system mediates immunity through three stages: adaptation, expression, and interference (Jinek

et al., 2012; Makarova et al., 2011). The first stage, adaptation, consists of the host copying the invading DNA in a fragment approximately 30 base pairs in size, upstream of a 3 base pair region known as the protospacer adjacent motif (PAM). In the expression stage, the repeat spacer element is transcribed into precursor CRISPR RNA (pre-crRNA) which is then cleaved into mature, short crRNAs which are ready to bind to complementary DNA regions. During the last stage, interference, the crRNA binds to complementary DNA regions on the invading DNA and the Cas9nuclease, part of the type II CRISPR locus, will cleave the double stranded DNA upstream of the PAM sequence (Jinek et al., 2012; Makarova et al., 2011). Several researchers have modified this system for gene editing in mammalian cells as well as in animals and have had tremendous success. Cong et al. have identified wild-type spCas9 nuclease which requires a PAM sequence in NGG format and causes double strand breaks (DSB).These DSBs are repaired by the cell's DNA repair mechanism either by non-homologous end joining (NHEJ) or by homologous directed repair (HDR) (Cong et al., 2013). The current use of CRISPR for genetic editing in mammalian cells consists of producing a 20 base pair spacer or protospacer, complementary to the target DNA directly upstream of the PAM region. A matured crRNA or tracrRNA is added and together with the protospacer, makes up the guide RNA (gRNA) which will recruit the Cas9 nuclease to the region of interest for cleavage (Cong and Zhang, 2015). Following a double strand break in the DNA, the cell's repair machinery will effectively repair the break however, the CRISPR system will continue cleaving until a mutation within the guide targeted

region is generated and the gRNA can no longer bind, making this an extremely rapid and efficient gene editing system.

With such robust technology, the ability to finely edit the 5'UTR of IRF-7 is achievable and will enhance the host's innate immune response without causing overexpression issues. We hypothesize that finely editing the 5' UTR of IRF-7 using CRISPR/Cas9 in porcine cells will allow for an enhanced innate immune response as well as provide more insight on the regulation of IRF-7 by 4E-BP1 and OASL1. This hypothesis will be tested by systematically deleting unique regions of the IRF-7 5' UTR in SK6 cells and determine the effects on type I IFNs to pathogen recognition.

CHAPTER II

MATERIALS AND METHODS

CRISPR/Cas mediated modification of porcine IRF-7 5' UTR

IRF-7 RNA prediction and structure

The IRF-7 5' UTR sequence was determined by alignment to *Sus scrofa* expressed sequence tag (EST). The RNA folding predictions were performed using default folding parameters by an online RNA folding software, Vienna RNA Web Suite. Primers for polymerase chain reaction (PCR) were designed to target the porcine IRF-7 5'UTR (appendix table 1) and used to create an amplicon of 796 base pairs (appendix table 2). Several PCRs were performed using CloneAmp HiFi PCR Premix (Takara, Mountain View, CA) and purified using Qiagen PCR Purification (Qiagen, Germantown, MD). The amplicon was sequenced at the Texas A&M Laboratory for Plant and Genome Technologies then compared to the sus scrofa EST database using the National Center for Biotechnology Information (NCBI) Blast program (appendix table 2). Unfortunately, there are discrepancies between the NCBI annotation for the IRF-7 5' UTR and the EST identified IRF-7 5' UTR. RNA fold predictions were performed on both and gRNA design was based on homologous regions in both sequences.

Guide RNA design and construction

Guide sequences were designed in the IRF-7 5' UTR after identification of PAM sequences for wild type Cas9 using Benchling online software (Benchling San Francisco, CA). On target and off target scores are based on Doench et al. and executed using the Benchling online software (Doench et al., 2016). The guide sequences as well

as their complements were ordered (Thermo Fisher Scientific Waltham, MA) as custom oligos with BbsI cut sites at the 5' and 3' ends and the guides, along with their complements, were denatured at 95°C and cooled to room temperature to allow for proper annealing as described by the Zhang lab (Ran et al., 2013). Guide RNAs were then cloned into a linearized pSpCas9(BB)-2A-GFP (PX458) plasmid containing a GFP expression insert, a gift from Feng Zhang (Addgene plasmid # 48138). Guide RNA cloning was confirmed by DNA sequencing at the Texas A&M Laboratory for Plant and Genome Technology using the LKO 1.5 primer sequence. Guide RNA sequences and oligo sequences can be found in appendix table 3.

Cells and reagents

Immortalized swine kidney cells (SK6) were originally provided by APHIS at Plum Island Animal Disease Center (PIADC), Greenport, NY. Frozen cell stocks were thawed and grown in media containing Dulbecco's modified minimum essential media (DMEM) F12 and supplemented with 10% fetal bovine serum (FBS) (Atlanta Biologicals Flowery Branch, GA) and 1% antibiotic and antimitotic (Thermo Fisher Scientific Waltham, MA). Once cells were grown to approximately 90% confluency, cells were passaged using 1 X DPBS (Thermo Fisher Scientific Waltham, MA) and 0.25% trypsin-EDTA (Thermo Fisher Scientific Waltham, MA). Cells were maintained in culture at 37°C in a humidified atmosphere of 5% CO_2 and air.

Preliminary guide RNA transfections and efficiency determination

Cells were transfected with Cas9 plasmids, containing the appropriate target sequences, into 6 well tissue culture plates with Lipofectamine 3000 (Thermo Fisher

Scientific Waltham, MA) at 2.5 µg of DNA per well and incubated for 24 hours in normal tissue culture conditions. When needed, DNA was extracted from cells using a DNeasy blood and tissue kit (Qiagen Germantown, MD). To determine transfection efficiencies, GFP expression was visually analyzed 24 hours post transfection with a microscope equipped with UV fluorescence.

During guide RNA construction, preliminary transfections were performed to determine functionality of each gRNA. This was achieved using the T7 Endonuclease 1 assay (New England Biolabs Ipswich, MA) according to the manufacturer's protocol. For cleavage detection, products from this assay were run on a 1.5% agarose gel at 60V for 3 hours. Amplicons for this assay were amplified using primers in appendix table 1.

Primary gRNA transfections

Once gRNAs were determined functional, SK6 cells were transfected in three different groups as seen in figure 4. Treatment group 1 was transfected with 3 of the most 5' gRNAs. Treatment group 2 was transfected with 3 of the most 3' gRNAs. Treatment group 3 was transfected with the most 5' gRNA and the most 3' gRNA in attempt to create a knockout. Additionally, a group of cells were transfected with Cas9 plasmids which lacked the target sequence as unmodified controls. These unmodified cells underwent the same selection and handling processes as the cells transfected with target sequence containing plasmids, further known as unmodified-selected cells.

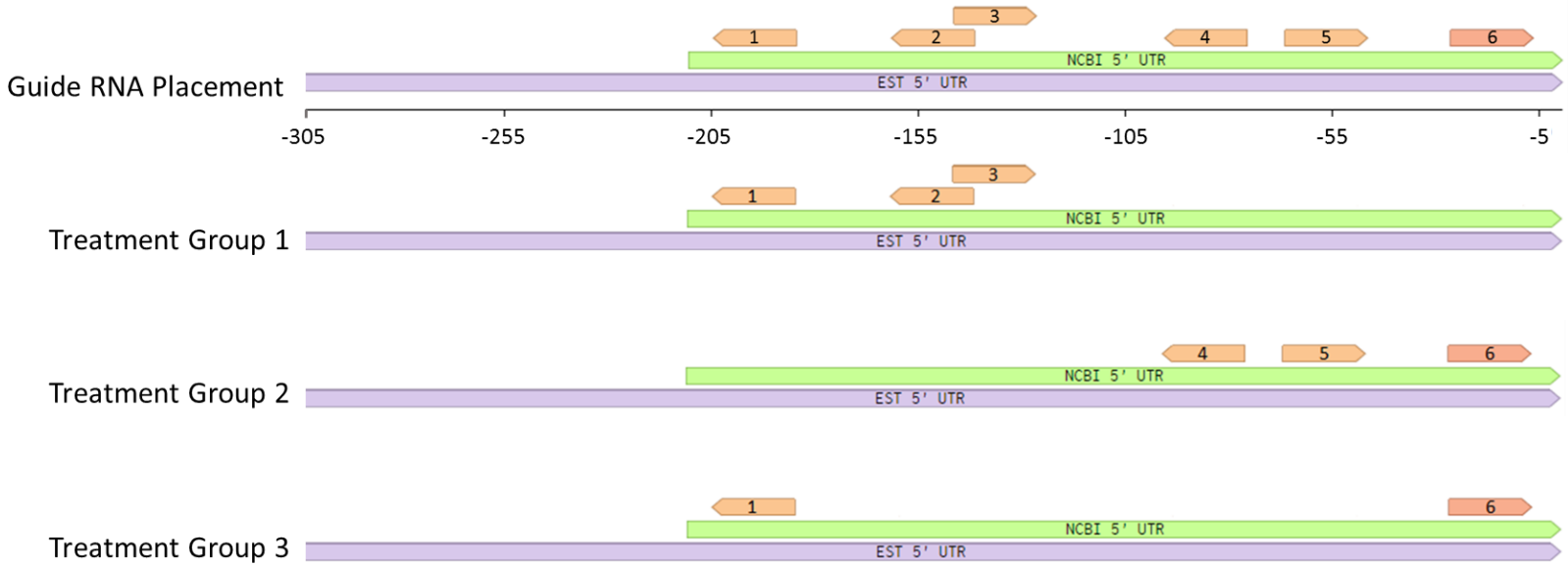

Figure 4: Transfection treatment groups for the development of modified cell lines

Illustration of the treatment groups used for the transfection of Cas9 plasmids containing target sequences (gRNAs) into SK6 cells. Multiple gRNAs were used in one treatment in attempt to produce several unique population modifications.

Cell selection and clonal propagation

Following primary transfections, cells were subjected to single cell sorting using the Texas A&M University Department of Pathobiology Flow Cytometry Core Laboratory. Cells were trypsinized as described previously and 1 x 10^6 cells were suspended in 1 mL of cold 1 X DPBS (Thermo Fisher Scientific Waltham, MA) supplemented with 10% FBS (Atlanta Biologicals Flowery Branch, GA), 1mM EDTA, and 1% antibiotic and antimicotic (Thermo Fisher Scientific Waltham, MA). Five µl of propidium iodide 1mg/mL (PI; final concentration 5 µg/ml) (Sigma-Aldrich St. Louis, MO) was added to the cell suspension. GFP positive and PI negative cells were sorted into 96 well plates with media containing 44% DMEM F12 (Thermo Fisher Scientific Waltham, MA), 45% FBS (Atlanta Biologicals Flowery Branch, GA), 10% conditioned media, and 1% antibiotic and antimitotic (Thermo Fisher Scientific Waltham, MA), further referred to as SK6 growth media. Sorted cells were incubated at 37º C in an atmosphere of 5% CO_2, 5% O_2, and 90% humidity.

Interferon and ISG response in cells containing modified IRF-7 5' UTR

Poly I:C transfection

Cells grown from single cell colonies and the non-clonal SK6 control were seeded in 12 well plates at a density of 0.2 x 10^6 cells per well. After 24 hours, cells were transfected with polyinosinic: polycytidylic acid (poly I:C) which is a synthetic analog of double stranded RNA (Invivogen, San Diego, CA) at 1 µg/mL using Lipofectamine 3000 (Thermo Fisher Scientific Waltham, MA), in SK6 growth media, for 8 hours at 37°C in atmospheric conditions of 5% CO_2 and air. Cells were harvested

immediately after poly I:C treatment for RNA extraction using the Qiagen RNeasy Plus Mini Kit (Qiagen Germantown, MD).

Analysis of mRNA expression by quantitative reverse transcription PCR

Following poly I:C stimulation, RNA was harvested from the cell lines as described above. RNA was DNase treated using the Quantabio Perfecta DNase I kit to remove residual genomic DNA (Quantabio Beverly, MA). RNA was then reverse transcribed using the Quantabio qScript cDNA Supermix according to manufacturer's protocol (Quantabio Beverly, MA). Reverse transcription PCR (RT-qPCR) was performed to evaluate expression of IRF-7, IRF-3, IFNα, IFNβ, 4E-BP1, OAS1, OAS2, OASL1, Mx1, ISG15, ISG54, ISG56 as well as GAPDH, HPRT, and TBP as reference genes for normalization, see appendix table 4 for primer sequences and efficiencies. Primer efficiencies were determined using standard curve quantification. Data analysis was performed using comparative quantification methods (Livak and Schmittgen, 2001).

Statistics and data analysis

Statistical analysis was performed using GraphPad Prism 6, version 6.02. Data distribution was tested for normality using Shapiro-Wilk normality test and the Kolmogorov-Smirnov goodness of fit test. Data was determined to be non-Gaussian and thus analyzed using the non-parametric Kruskal-Wallis test along with a post hoc Dunn's multiple comparisons test determine differences between each cell line including the unmodified cell lines. Alpha was set *a priori* at 0.05.

Due to variability among poly I:C stimulations and the magnitude of genes and cell lines originally tested, data was excluded from the results if any of the following

criterion was met: 1) Modified cell lines that did not consistently express Ct values below 32 for IFNβ under poly I:C stimulation and did not vary significantly from the unmodified selected cell line. 2) Biological replicates where the poly I:C stimulated Ct values for IFNβ in the unmodified-selected 1 cell line were 34 or above, which indicated a lack of stimulation. 3) The transcript levels of genes IFNα, 4E-BP1, ISG15, IRF-3, and IRF-7 expression did not differ significantly from the geometric mean of the reference genes therefore, they were not included in the reporting of the results.

CHAPTER III

RESULTS

CRISPR/Cas mediated modification of porcine IRF-7 5' UTR

5'UTR Sequencing

The NCBI sequence for porcine IRF-7 5' UTR was obtained through the University of California Santa Cruz (UCSC) Genome Browser. Primer sequences were designed to target the porcine IRF-7 5' UTR and several hundred base pairs upstream of the predicted 5' UTR. After a PCR was performed, the 796 base pair amplicon was sequenced and analyzed with the NCBI Blast program within the EST database. Due to an incomplete annotation of the porcine genome, the sequence obtained from the 796 bp amplicon allowed for the identification of the proper EST sequences associated with IRF-7. The sequence generated from the amplicon extends 140 base pairs upstream of the EST sequence and 196 base pairs downstream and into the coding sequence of IRF-7 leaving 305 base pairs in between, assumed to be the 5' UTR. The 305 base pair EST sequence that was obtained was aligned to the NCBI annotated 5' UTR which indicated that the 5' UTR EST was 94 base pairs larger than the NCBI annotated 5' UTR, figure 5. Individual sequences for the 796 base pair amplicon, the NCBI annotated sequence, and the EST sequence can be found in the appendix.

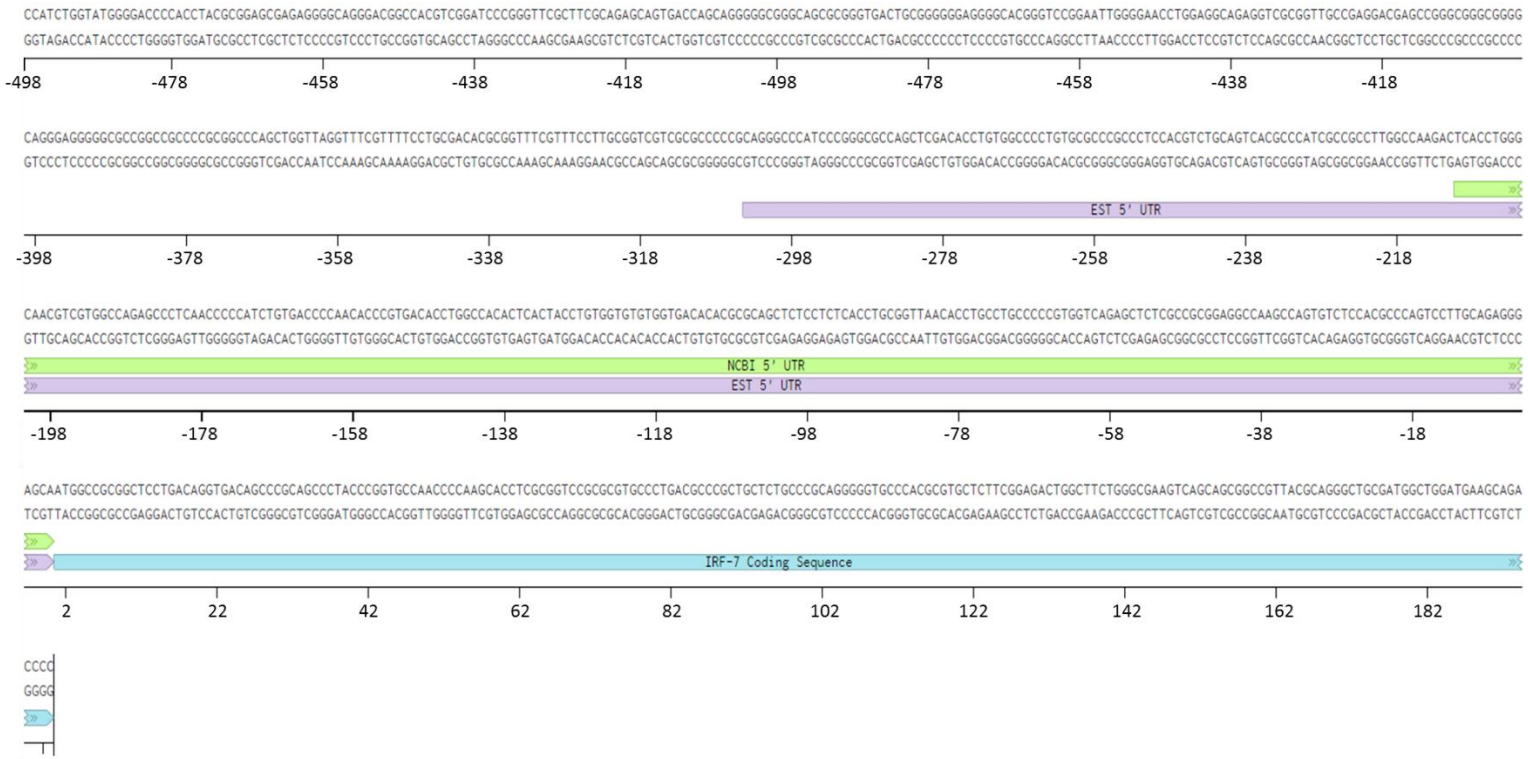

Figure 5: Amplified genomic sequence of porcine IRF-7

Illustration of the 796 base pair amplicon aligned with the previously known NCBI annotated 5' UTR and a matched EST sequence. The genomic amplicon extends beyond what is recorded in NCBI databases.

Guide RNA Design

Twice, gRNAs were synthesized to target 6 different regions throughout the porcine IRF-7 5' UTR which mapped to both the NCBI annotated sequence and the EST sequence. Twelve gRNAs were successfully cloned into the PX 458 plasmid and confirmed by DNA sequencing (figure 6 and appendix table 1).

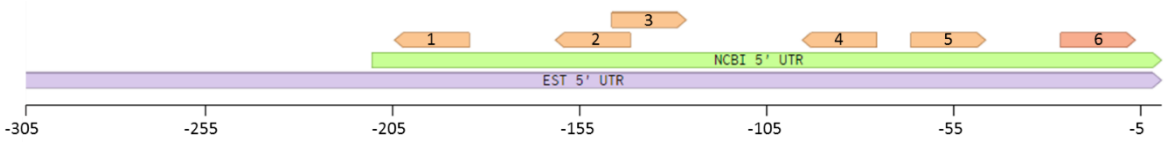

Figure 6: Guide RNAs designed to target 6 unique locations within the IRF-7 5' UTR

Guide RNAs were designed to target different regions among the whole NCBI 5' UTR sequence. The orange arrows indicate the location of the gRNAs to the 5' UTR sequences as well as the direction of the gRNA.

Guide RNA Subcloning

In preliminary experiments, SK6 cells were transfected with plasmids containing the gRNAs individually to determine guide functionality. The SK6 cell transfections resulted in a 60-70% transfection efficiency, as determined by visual assessment of GFP. Unknown issues caused the first gRNA design to fail, as discussed in the next section,

and 6 more guides were designed and subcloned into PX 458, their homologous locations in the 5' UTR are shown in figure 6.

Guide functionality and efficiency

Following successful transfections and cell harvesting, DNA was extracted from a mixed population of cells in a single treatment and the T7 Endonuclease 1 assay was performed. The first preliminary transfections and T7 Endonuclease 1 assay did not yield cleavage bands indicating that the first set of guides were either not functional or not efficient. The second preliminary transfections with new gRNA designs yielded successful transfections and T7 Endonuclease 1 assay, figure 7. Guides 2,3, and 6 were proven to be functional through the presence of cleavage bands as seen below, however, guide 1 may have created a large insertion, causing the multiple bands seen above and below the parent band. Although 2 gRNAs did not show cleavage bands, they may still be functional but with a low efficiency, therefore, all six guides were used for modified cell line development.

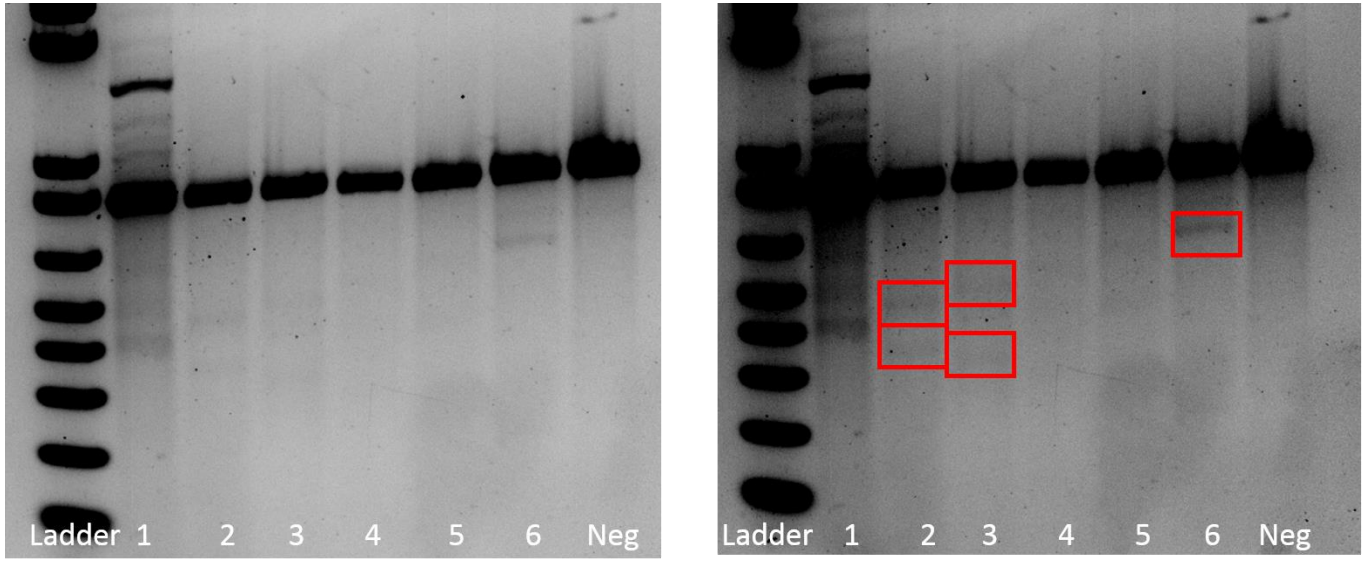

Figure 7: Four gRNAs caused cleavage bands in the gel electrophoresis of a T7 Endonuclease 1 assay

Guides 2,3, and 6 displayed cleavage bands indicating a positive modification to cells. Guide 1 may have caused a large insertion, causing the large band above the brighter parent band. Guide RNAs 3 and 4 did not produce cleavage bands but may have low efficiency causing the T7 endonuclease to not recognize mismatched DNA. Guide RNAs 1,3, and 4 were confirmed to produce deletions through single cell sorting and sequencing. Red boxes indicate the cleavage bands visible at high exposure.

Modified cell line development

SK6 cells were transfected with a combination of gRNAs to induce several unique mutations within a small region of the 5' UTR. The treatment groups consisted of cells transfected with gRNAs 1,2, and 3 (G1-3), gRNAs 4,5, and 6 (G4-6), and gRNAs 1 and 6 (G1&6). As a control for clonal cell selection, SK6 cells were transfected with a PX458 plasmid lacking a gRNA and were subjected to the same selection processes as the cells transfected with gRNAs. Twenty-four hours post transfection, the cells were sorted by GFP expression directly into 96-well plates to form single cell colonies. Cells were grown and expanded until they reached confluency in 48 well plates, which then, 12 cell lines per treatment group were selected for further culture and the rest were frozen and stored.

DNA was extracted from the cells selected for culture and each cell line was sequenced for mutation analysis. The transfection and selection process resulted in a total mutation rate of 82%, of which 41% of those mutations were unique.

Within the first treatment group, 8 out of 12 lines survived and 6 of those 8 cell lines were positive for a modification. Of the positive cell lines, 5 out of 6 had mixed populations of cells, with either a modified line mixed with a wild type line or with another modified line. These mixed population cells were diluted and re-grown from single cells in attempt to isolate individual mutations. In two of those diluted cell lines, modifications were identified, isolated, and analyzed. Altogether, 5 cell lines with modifications were isolated and only three unique deletions were observed.

All 12 cell lines within the second treatment group survived and yielded 7 modified lines, with only 1 line having a mixed population. The modification in the mixed population cell line could not be isolated. Altogether, 6 deletions were observed and 5 of those were unique.

The third treatment group yielded 11 surviving cell lines with 7 of those being modified. Two of those lines had mixed populations and 1 line was successfully isolated and its modification analyzed. Of the seven modified lines one line had a 360 base pair deletion that included part of the IRF-7 coding sequence. Another line resulted in a 191 base pair deletion, our closest modification to a knockout. This treatment yielded 6 deletions but also had one line with an insertion. A summary of each cell line's modification is listed in table 1.

Eight unique modifications were used for the quantification of innate immune response. With a total of 10 cell lines, 2 modifications were repeated in different cell lines to provide internal controls for clonal selection variation. These modifications are illustrated in figure 8.

Treatment	Colony ID	Population	Base Pair Change	Modification Type	Guides in Modification	Location
1: G1-G3	1A	Mixed			1,2,3	
	2A	Mixed				
	2.2	Single -from mixed	43 & 5	Deletion	1-2 and 3	-202 to -159 and -130 to -125
	2.5	Single -from mixed	28	Deletion	2,3	-160 to -132
	2.6	Single -from mixed	43 & 5	Deletion	1-2 and 3	-202 to -159 and -130 to -125
	6A	Single	Wild type			
	7A	Mixed				
	7.1	Single -from mixed	28	Deletion	2,3	-160 to -132
	8A	Mixed				
	9A	Single	72	Deletion	1,2,3	-202 to -130
	10A	Mixed				
2: G4-G6	1A	Mixed				
	2A	Single	83	Deletion	4,5,6	-94 to -11
	3A	Single	Wild type			
	4A	Mixed				
	5A	Single	83	Deletion	4,5,6	-94 to -11
	6A	Single	42	Deletion	4,5,6	-92 to -50
	7A	Single	40	Deletion	5,6	-50 to -10
	8A	Mixed				
	9A	Mixed				
	11A	Single	82	Deletion	4,5,6	-93 to -11
	12A	Single	82	Deletion	4,5,6	-93 to -11
3: G1 & G6	1A	Single	Wild type			
	2A	Single	191	Deletion	1,2,3,4,5,6	-203 to -12
	3A	Mixed				
	4A	Mixed				
	5A	Mixed				
	6A	Mixed				
	6.6	Single -from mixed	5	Deletion	6	-12 to -7
	7A	Single	Wild type			
	8A	Mixed			6	
	9A	Single	Wild Type			
	10A	Single	Wild Type			
	11A	Single	360	Deletion	1,2,3,4,5,6	-202 to +157

Table 1: CRISPR/Cas9 system induced modifications in 82% of sequenced cell lines

Population type, modification type, base pair changes, and their locations are listed for each cell line. Location is based on the translational start site as 0.

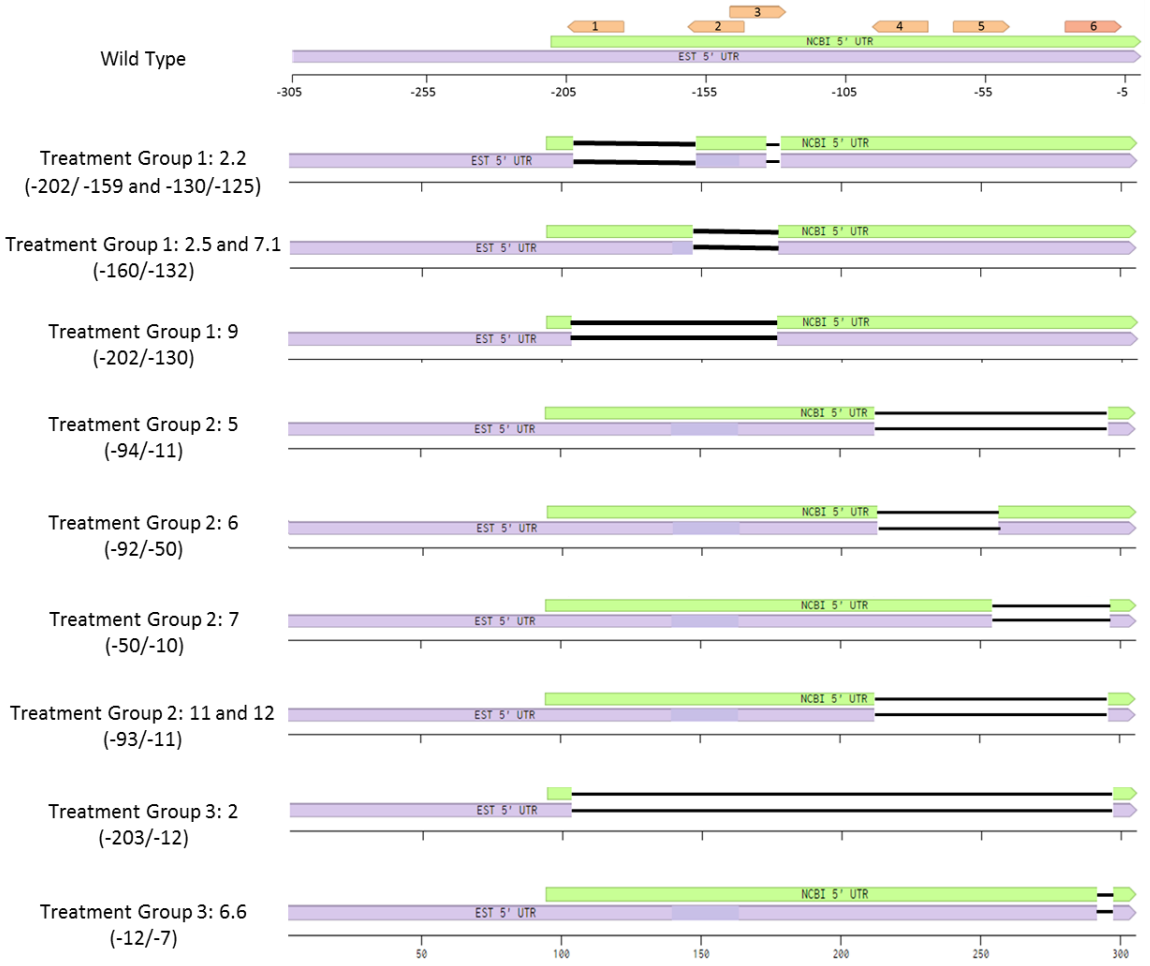

Figure 8: Deletions within the IRF-7 5' UTR ranged from 6 base pairs to 191 base pairs

The different treatment groups resulted in an array of unique deletions within the gRNA target region.

RNA Folding predictions of the modified cell lines' IRF-7 5' UTR

The IRF-7 5' UTR sequences for each modified cell line were entered into the Vienna Websuite RNAfold WebServer for prediction of RNA folding. Although the program predicts folding in both minimum free energy (MFE) and centroid algorithms, the folds presented here will be from the MFE algorithm. The wild type IRF-7 5' UTR structure yields four distinct stem loops as seen in tables 2A and 3A, similar to the murine model. Most of the modified cell lines maintained 4 stem loops with the exception of 4 lines. In treatment 1, SK -160/-132 (table 2C) had a reduced stem loop, bound to stem loop 3. Also SK -202/-130 (table 2D) completely lost a stem loop. SK -203/-12 (table 3F) seemed to have only two stem loops and is much smaller than the other structures.

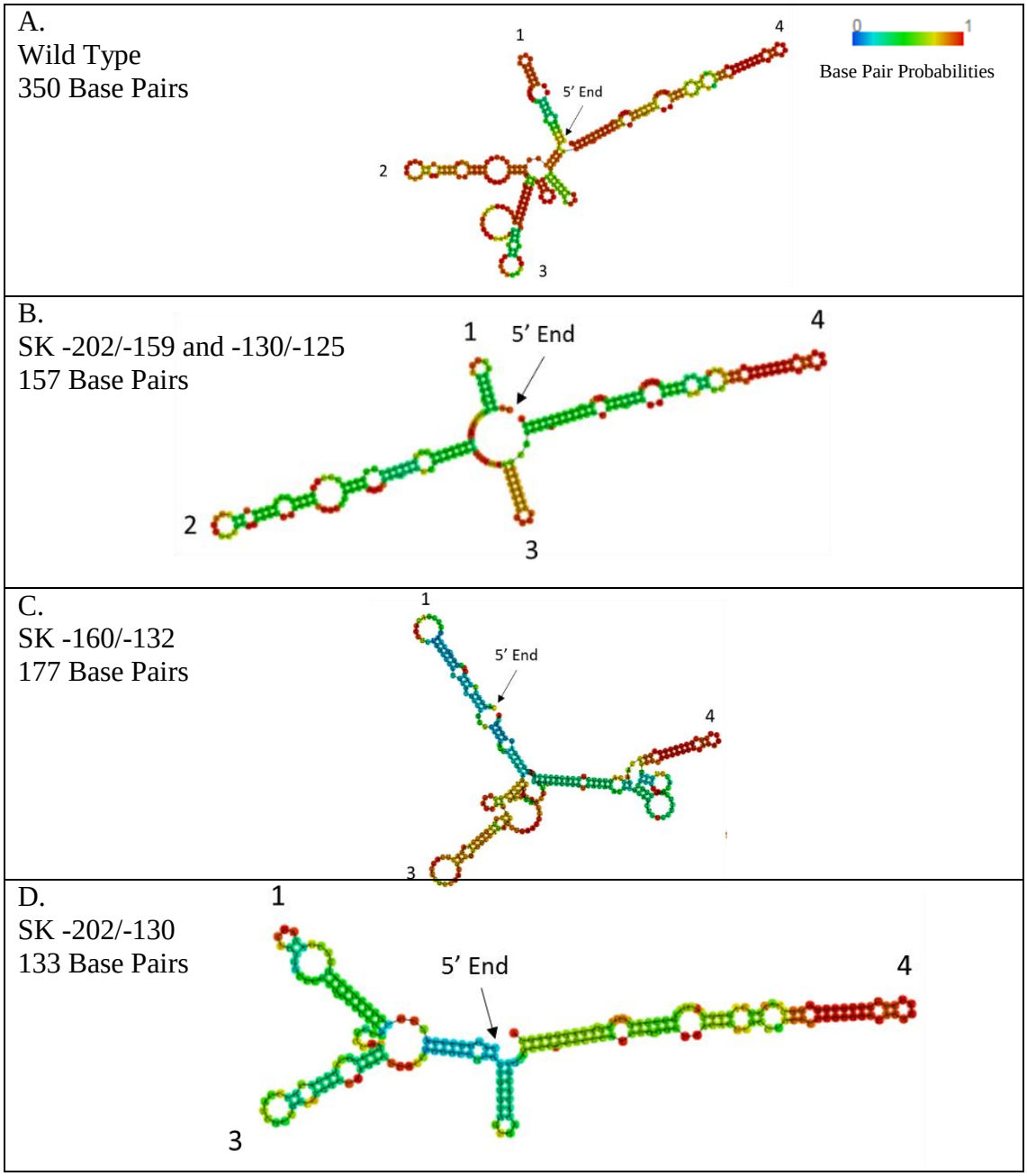

Figure 9: CRSPR/Cas9 induced deletions altered the predicted secondary structures of the IRF-7 5' UTR

The MFE algorithm was used to predict the RNA folding of the wild type 5' UTR and the 5' UTR of modified cell lines. Stem loops are numbered from the 5' end. In C and D, stem loop 2 is either diminished or missing.

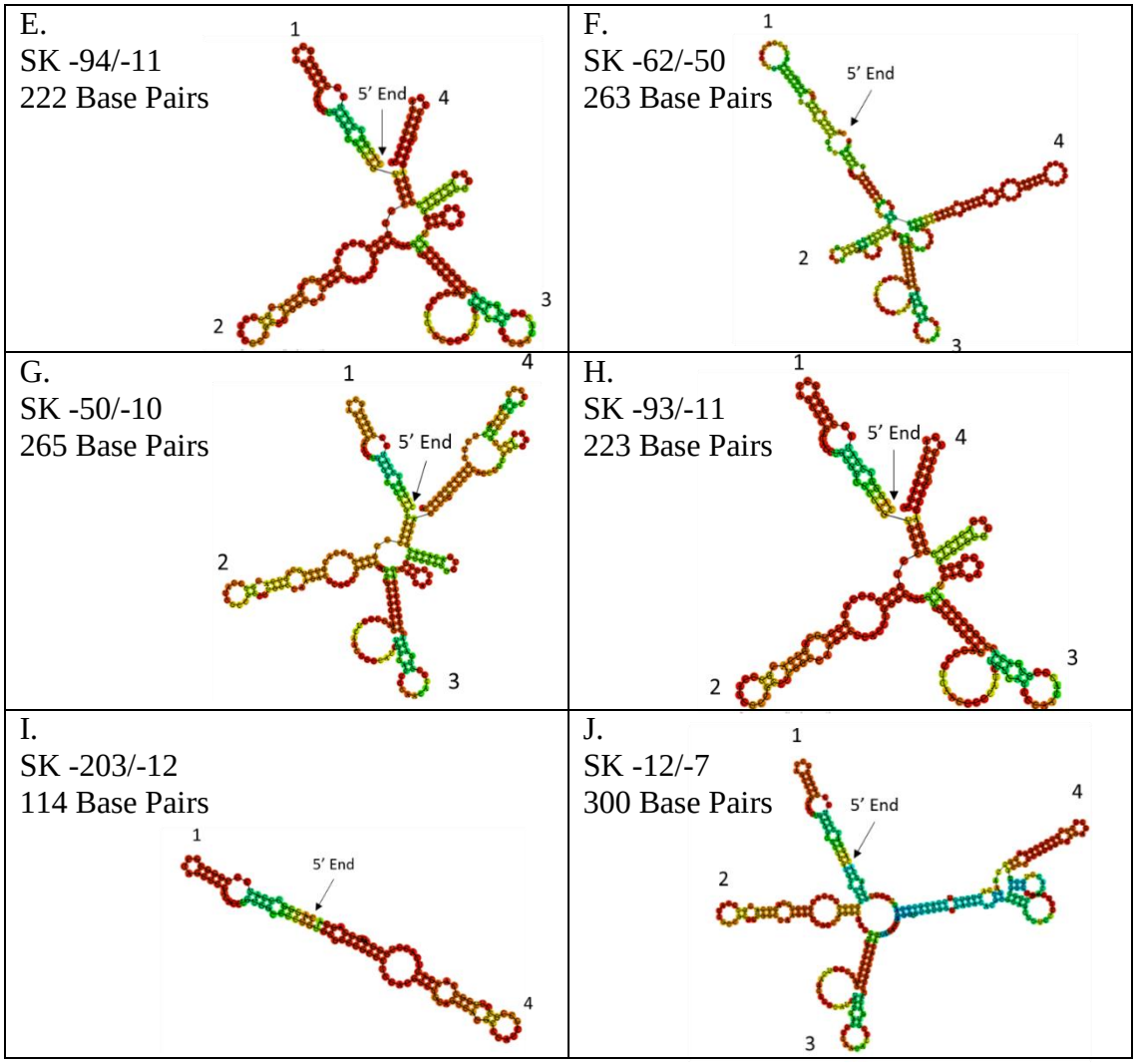

Figure 9 Continued

Modified cell line IFN stimulation

Poly I:C stimulation and gene expression

Originally, 13 cell lines were tested, 10 modified and 3 not modified. The 3 wild type cell lines consisted of 2 unmodified cell lines that were originally transfected with the empty Cas9 plasmid then subjected to selection and clonal expansion along with the

cell lines transfected with Cas9 plasmids containing target sequences, referred to as unmodified-selected 1 and 2. The third unmodified cell line originates from cells that were not transfected with a Cas9 plasmid nor were they subjected to selection or clonal expansion, referred to as non-selected.

After 3 preliminary biological replicates, modified cell lines that did not consistently express Ct values below 32 for IFNβ under poly I:C stimulation and did not vary significantly from the unmodified selected cell line, were excluded from statistical analysis and further biological replicates. Treatment 1 cell lines that fell under this exclusion were SK-202/-159 and SK-130/-125 and SK-160/-132. Modification -160/-130 was duplicated in two different cell line populations. One of these lines, was excluded from results. All of treatment 2 cell lines (SK-94/-11, SK-92/-50, SK-50/-10, SK-93/-12) were excluded along with cell line SK-203/-12. Additionally, IFNα, 4E-BP1, ISG15, IRF-3, and IRF-7 expression did not differ from the geometric mean of the reference genes (data not shown).

Due to variation in poly I:C stimulation between biological replicates, biological replicates where the poly I:C stimulated Ct values for IFNβ in the unmodified-selected 1 cell line did not go below 34, indicated a lack of stimulation and were excluded. Following the exclusions, 3 biological replicates, 3 modified cell lines, and 10 genes were used in analysis. Modified cell lines included were SK-160/-132 and SK-202/-130, as well as SK-12/-7. Genes that were included in that analysis were MX1, OAS1, OAS2, ISG54, ISG56, IFNβ, and OASL. Data gathered was tested for normality and was determined to be non-Gaussian, figure 9, therefore, the non-parametric Kruskal-Wallis

test along with a post hoc Dunn's multiple comparisons test was used for statistical analysis.

	Col. stats	A	B	C	D
		T1: 7.1	T1: 9	T3: 6.6	Unmod Sel1
		Y	Y	Y	Y
16	D'Agostino & Pearson omnibus normality test				
17	K2	25.95	27.95	27.58	27.59
18	P value	< 0.0001	< 0.0001	< 0.0001	< 0.0001
19	Passed normality test (alpha=0.05)?	No	No	No	No
20	P value summary	****	****	****	****
21					
22	Shapiro-Wilk normality test				
23	W	0.5297	0.4446	0.4601	0.4590
24	P value	< 0.0001	< 0.0001	< 0.0001	< 0.0001
25	Passed normality test (alpha=0.05)?	No	No	No	No
26	P value summary	****	****	****	****
27					
28	KS normality test				
29	KS distance	0.3682	0.4512	0.4422	0.4242
30	P value	0.0004	< 0.0001	< 0.0001	< 0.0001
31	Passed normality test (alpha=0.05)?	No	No	No	No
32	P value summary	***	****	****	****
33					
34	Coefficient of variation	203.73%	248.78%	238.86%	232.02%

Figure 10: Collected data failed tests for normality

Data from each cell line was determined to be non-Gaussian by the D'Agostine & Pearson omnibus normality test, the Shapiro-Wilk normality test, and the Kolmogorov-Smirnov goodness of fit test.

To understand variation due to selection and clonal expansion, the two unmodified-selected cell lines' immune response gene transcript levels were compared to the non-selected SK6 cells transcript levels and no differences were determined,

figure 9. Additionally, non-stimulated ΔCt values for ISGs and IFNβ were compared among cell lines to determine if cell line modifications altered baseline levels of interferon and interferon stimulated genes. Significant differences were not detected between cell lines indicating that the 5' UTR modifications did not alter baseline gene expression, figure 10.

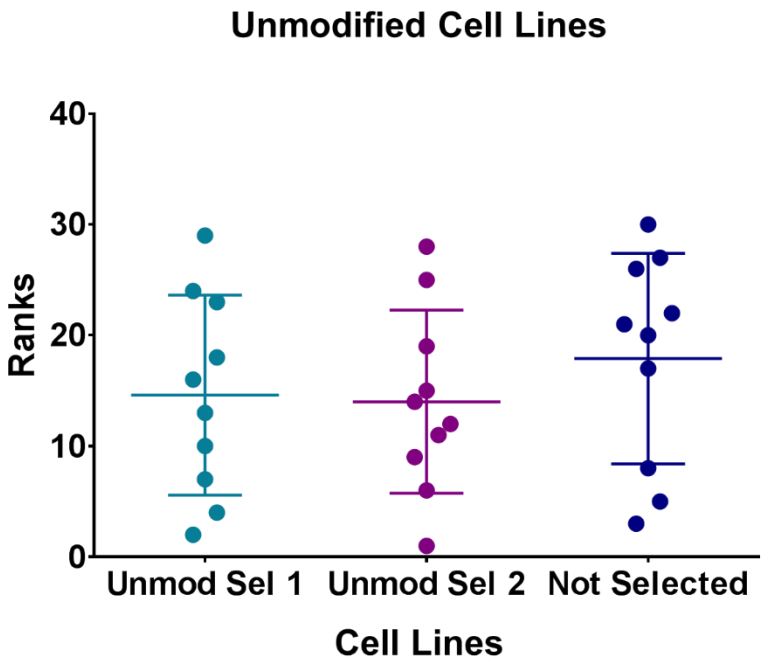

Figure 11: Selection and clonal propagation of SK6 cells does not alter gene expression profiles.

Selected and clonally expanded cell lines (Unmod Sel 1 and 2) were compared to not selected, wild type SK6 cells. No difference was observed in immune regulated gene expression between the unmodified selected cells and the wild type SK6 cells which indicates that there was not a difference in gene expression due to the selection and expansion process.

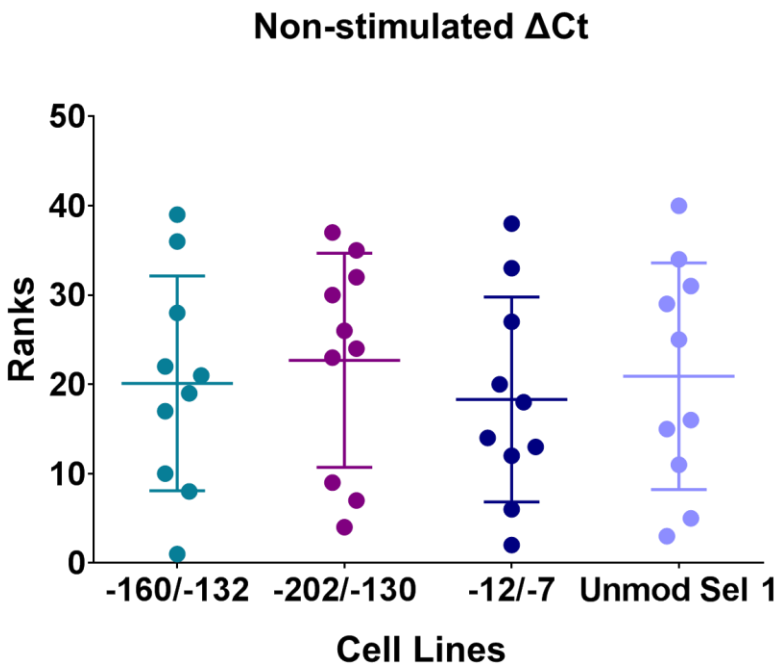

Figure 12: Genetic modification of the IRF-7 5' UTR does not alter unstimulated gene expression

Modified cell line baseline gene expression were compared to each other and an unmodified selected cell line. The lack of statistical significance indicates that the IRF-7 5' UTR modifications do not alter baseline gene expression when the immune system is not stimulated.

The fold change, ΔΔCt values, in gene expression following stimulation with poly I:C was compared in 3 modified cell lines and an unmodified control. SK -202/-120, had a consistent and significant heightened immune response than the unmodified selected cell line in all 7 genes, figure 11. SK -160/-132, only showed an increase in OASL expression ($p<0.05$), figure 11. These two cell lines indicate that the 5' UTR modifications do alter the immune responses to poly I:C stimulation. SK -12/-7 had some upregulation however, it was not significantly above the unmodified selected cell line, figure 11.

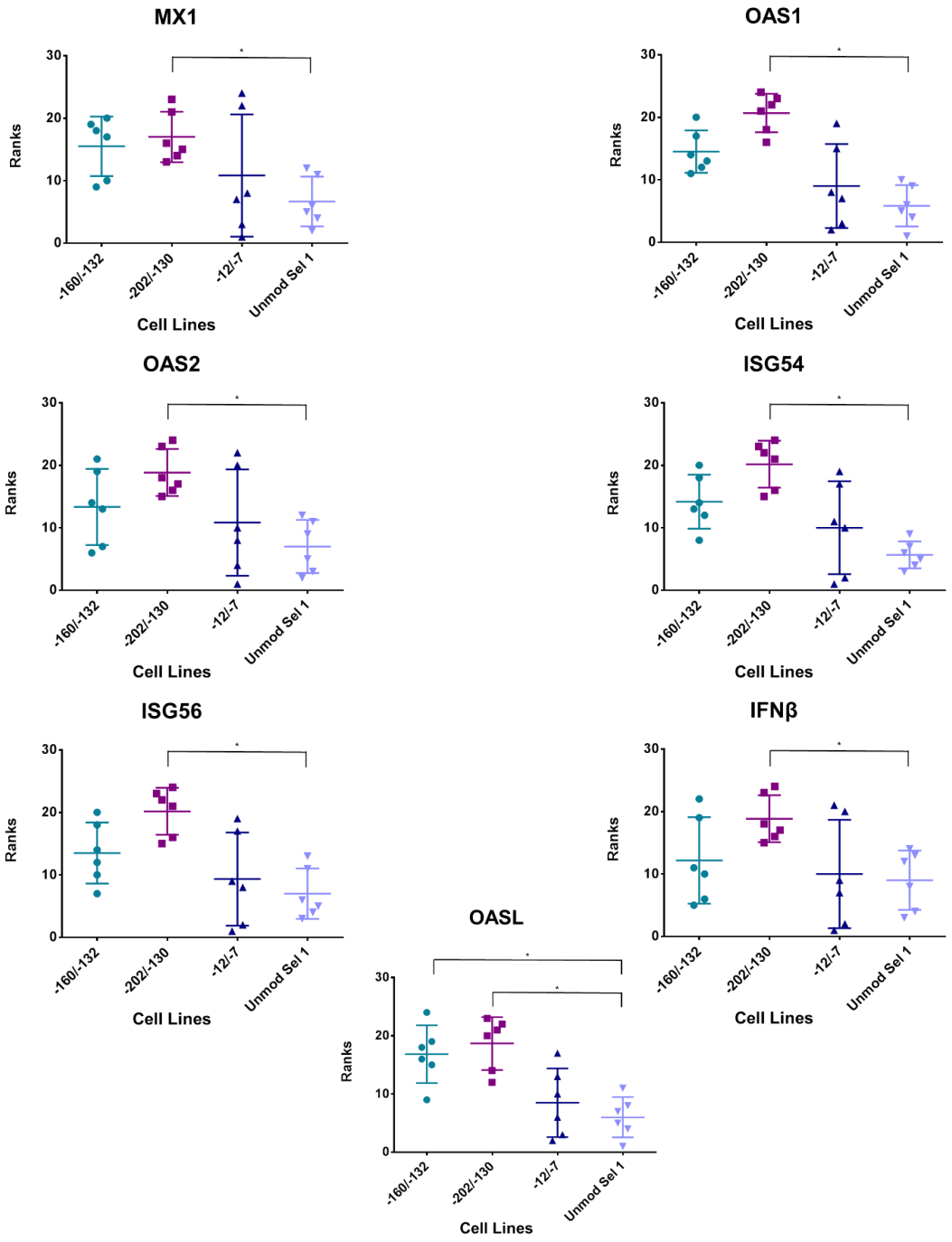

Figure 13: Specific cell lines have increased response to poly I:C.

SK -202/-130 gene expression was significantly increased compared to the unmodified selected cells among all 7 genes. SK -160/-132 exhibited a significant increase in only OASL expression. However, SK -160-132 tended to have an increased response in genes MX1, OAS1, and ISG54 ($p \leq 0.11$). SK -12/-7 was not different from unmodified selected cells. * $p < 0.05$

CHAPTER IV

DISCUSSION

The translational regulation of IRF-7 has been heavily studied since its discovery in 2005 as the master regulator of type I IFNs. Despite the numerous studies, the exact method and pathway for its regulation still remains a mystery. Each study has continued to enhance our knowledge of IRF-7 regulation in a very specific manner. Currently, two translational factors, OASL and 4E-BP1, are known to regulate IRF-7 through the secondary structure of its 5' UTR, however, the specific location of that regulation and how the secondary structure of the 5' UTR interacts with regulatory factors remains unknown. The discovery and optimization of the CRISPR/Cas9 gene editing system has now offered an opportunity to understand the location and structure needed for regulation.

The CRISPR/Cas9 system is known for its efficient and specific gene editing capabilities. The results from designing the gRNAs, testing gRNA efficiency, and from the development of the modified cell lines reinforces its use as a robust gene editing tool. Despite its efficiency, the CRISPR system is not perfect. Especially in cell lines, the system is known to produce off-target effects, where the gRNA initiates cleavage and possibly the modification of DNA regions outside of the region of interest. Current bioinformatics programs are not completely efficient at determining off-target effects and are still being optimized. Due to the incomplete and poorly annotated porcine genome, off target effects were not analyzed for this project.

Despite the limitation of determining off target effects, there were conflicting results from data gathered in this project. Two cell lines exhibited the exact same mutation -160/-132 but yielded different results. One cell line had a much more robust and potent response to poly I:C than the other. In fact, the low responding cell line was excluded from the results due its response not varying from the unmodified cell lines. Possible explanations for this could be that the low responding cell line had off target mutations, inhibiting its response to poly I:C. Another explanation could be that the selection and clonal expansion process induced an alteration in gene expression, which repressed response. Despite this issue, other cell lines revealed upregulation following stimulation of the innate immune response.

SK -160/-132 and SK -202/-130 resulted in surprising insights into the location of IRF-7 regulation. A study performed at our lab in 2014 using a luciferase plasmid and truncated IRF-7 5' UTR suggested that the most 3' region of the 5' UTR was critical for regulation however, the data from this study suggests otherwise (Carvajal, 2014). The most robust and significant upregulation of the immune response was obtained in SK -202/-130 which had its modification in the 5' region of the UTR and altered the predicted secondary structure by the elimination of one stem loop and the reduction stem loop size when compared to the wild type folding and cell lines with modifications in the more 3' region of the UTR. Although SK -160/-132 only demonstrated significant upregulation in OASL when compared to the unmodified-selected cells, it still tended to have an increase in response however, it was not as robust as SK -202/-130. OASL, a product of IFN stimulation, is evidence of an increase in the innate immune system.

When considering the 5’ UTR RNA folding, SK -160/-132 had a greatly reduced stem loop sizes and, similar to SK -202/-130, had 3 major stem loops. The reduction in stem loop size may have caused the upregulation of IRF-7 protein leading to a small increase in IFNβ causing a significant increase in OASL1 gene transcript levels in response to poly I:C stimulation. It cannot be speculated exactly what factor is regulating IRF-7 in SK -160/-132 but there does seem to be more translational regulation of IRF-7, possibly by translational factors 4E-BP1 and OASL1, in the cell line than in SK -202/-130. Interestingly, a 2013 study on the interaction between OASL and the IRF-7 5’ UTR, through dual luciferase assays, indicated that UTR modifications specifically altering the first or second stem loop altered the suppressive ability of OASL1, whereas modifications in the third and fourth stem loops retained OASL suppression (Lee et al., 2013). Although the current study did not target specific stem loops, the results confirm that the stem loops in the 5’ end of the IRF-7 5’ UTR may be more critical for translational factor regulation than stem loops in the 3’ region.

Two other modified cell lines resulted in the reduction of stem loop size. SK -202/-159 and -130/125 has stem loop size reduction in two stem loops, however, that cell line did not display an increased immune response. SK -203/-12 was the closest to a 5’ UTR knockout that was generated. Although its folding lacks 2 stem loops, it did not exhibit in increased expression of innate immune regulated genes either. The immune regulated gene transcript levels for this cell line did not differ from the unmodified cell lines, indicating that transcription was neither inhibited nor promoted. It is possible that the deletion resulted in the loss of a critical region for promoting translation of IRF-7,

resulting in no change to ISG and IFNβ transcript levels. However, since the ISG and IFNβ transcript levels were not significantly lower than in the unmodified cells lines, the -203/-12 modification did not completely inhibit expression of IRF-7.

Modifications in cell lines with modifications in the 3' region of the UTR did not alter the amount of stem loops. Minor modifications of the structure and its organization are seen however, that reorganization did not seem to have an effect on gene expression. This further solidifies the assumption that the translational factors do not bind to specific sequences, but to certain stem loops. Although increased immune responses were not shown with modifications to this region, it should be noted that this should remain a region of interest since deletions in this region may still reduce stem loop numbers.

Although there have been several studies to understand IRF-7 regulation, two translational factors have been found as key regulators, 4E-BP1 and OASL1. A study mentioned earlier by Lee et al. in 2013, sought to determine the role of OASL1 in IRF-7 regulation. The researchers produced fragments of the murine IRF-7 5' UTR and used a dual luciferase assay to determine where OASL1 repressed IRF-7 translation. By directly targeting sequences in the stem loop structures, they found that the removal of the first and second stem loops of the 5' UTR significantly reduced the suppressive abilities of OASL1 on IRF-7 translation (Lee et al., 2013). With this information, it can be hypothesized that the destruction of the wild type secondary structure in the present study inhibits OASL1 regulation. Additionally, the lack of change in baseline, non-stimulated immune response gene transcripts between modified cell lines and the unmodified selected cell line can be used to assume that regulation of IRF-7 translation,

while the innate immune system is in a quiescent state, is still occurring, possibly by 4E-BP1. The 4E-BP1 inhibition is removed from IRF-7 once there is a potent increase in IFN levels and it is assumed that the ability of OASL1 to down regulate the immune response is important for allowing 4E-BP1 to once again inhibit IRF-7 (Colina et al., 2008; Lee et al., 2013; Ning et al., 2011). If in fact the elimination of stem loops in the IRF-7 5' UTR secondary structure inhibits IRF-7 translational regulation by OASL1, it may be difficult for the cell to gain appropriate control of IRF-7 translation for 4E-BP1 to once again bind and inhibit IRF-7. This may lead to an overactive immune system, possibly causing autoimmune disorders, unless there is another inhibitor of IRF-7 that will assist in down regulating the immune response. Future experiments examining the chronological control of this pathway would help address this issue. More studies should be done measuring the immune response in modified cell lines at different time points after the removal of stimulus.

Overall, the data suggests that all four stem loops are critical for IRF-7 translational regulation. With more studies in these regulatory regions, a more in depth knowledge of IRF-7 translational regulation can be achieved. The current project will be continued and secreted IFNβ levels will be measured by enzyme linked immunosorbent assay (ELISA) and cellular IRF-7 protein levels, both phosphorylated and unphosphorylated, will be determined by western blot to further understand IRF-7 regulation.

More studies are needed for a complete understanding of this system. Future studies could use homologous directed repair (HDR) with CRISPR/Cas9 to finely edit

regions to reduce or eliminate stem loop structure. Although luciferase assays with modified IRF-7 5' UTRs have been done previously, using CRISPR and gene expression analysis will provide a more accurate representation of interactions between translational factors and the 5' UTR in vitro. Once the regions of regulation are determined, an animal model could be produced with IRF-7 5' UTR modifications specifically aimed towards the regulatory stem loops, which the animals can then be immunologically challenged and their immune responses analyzed. Such studies can lead to a deeper understanding of the IRF-7 gene and its regulation, which can be used to produce animals with more efficient immune responses to viruses and even unveil a genetic target for pharmacological substances to enhance the immune function in humans.

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APPENDIX

Primer Name	Primer Sequence
IRF-7 UTR Fw	ACGAAACTTCCTGCCCCG
IRF-7 UTR Rev	GGAACCGTGTTCTGGCTTCA

Appendix Table 1: Primers used for amplification of genomic DNA surrounding the porcine IRF-7 5' UTR region. Amplicon sequence is listed in appendix table 1.

Source	IRF-7 5' UTR Sequence
NCBI Annotated	TCACCTGGGCAACGTCGTGGCCAGAGCCCTCAACCCCCATCTGTGAC CCCAACACCCGTGACACCTGGCCACACTCACTACCTGTGGTGTGTGG TGACACACGCGCAGCTCTCCTCTCACCTGCGGTTAACACCTGCCTGC CCCCGTGGTCAGAGCTCTCGCCGCGGAGGCCAAGCCAGTGTCTCCAC GCCCAGTCCTTGCAGAGGGAGCA
Expressed Sequence Tag	CAGGGCCCATCCCGGGCGCCAGCTCGACACCTGTGGCCCCTGTGCGC CCGCCCTCCACGTCTGCAGTCACGCCCATCGCCGCCTTGGCCAAGAC TCACCTGGGCAACGTCGTGGCCAGAGCCCTCAACCCCCATCTGTGAC CCCAACACCCGTGACACCTGGCCACACTCACTACCTGTGGTGTGTGG TGACACACGCGCAGCTCTCCTCTCACCTGCGGTTAACACCTGCCTGC CCCCGTGGTCAGAGCTCTCGCCGCGGAGGCCAAGCCAGTGTCTCCAC GCCCAGTCCTTGCAGAGGGAGCA
796 Base Pair Amplicon	CCATCTGGTATGGGGACCCCACCTACGCGGAGCGAGAGGGGCAGGG ACGGCCACGTCGGATCCCGGGTTCGCTTCGCAGAGCAGTGACCAGCA GGGGGCGGGCAGCGCGGGTGACTGCGGGGGGAGGGGCACGGGTCC GGAATTGGGGAACCTGGAGGCAGAGGTCGCGGTTGCCGAGGACGAG CCGGGCGGGCGGGGCAGGGAGGGGGCGCCGGCCGCCCCGCGGCCCA GCTGGTTAGGTTTCGTTTTCCTGCGACACGCGGTTTCGTTTCCTTGCG GTCGTCGCGCCCCCGCAGGGCCCATCCCGGGCGCCAGCTCGACACCT GTGGCCCCTGTGCGCCCGCCCTCCACGTCTGCAGTCACGCCCATCGC CGCCTTGGCCAAGACTCACCTGGGCAACGTCGTGGCCAGAGCCCTCA ACCCCCATCTGTGACCCCAACACCCGTGACACCTGGCCACACTCACT ACCTGTGGTGTGTGGTGACACACGCGCAGCTCTCCTCTCACCTGCGG TTAACACCTGCCTGCCCCCGTGGTCAGAGCTCTCGCCGCGGAGGCCA AGCCAGTGTCTCCACGCCCAGTCCTTGCAGAGGGAGCAATGGCCGCG GCTCCTGACAGGTGACAGCCCGCAGCCCTACCCGGTGCCAACCCCAA GCACCTCGCGGTCCGCGCGTGCCCTGACGCCCGCTGCTCTGCCCGCA GGGGGTGCCCACGCGTGCTCTTCGGAGACTGGCTTCTGGGCGAAGTC AGCAGCGGCCGTTACGCAGGGCTGCGATGGCTGGATGAAGCAGACC CC

Appendix Table 2: IRF-7 5' UTR sequences

Guide RNA	Guide Sequence	Forward Oligo	Reverse Oligo
1	CTCTGGCCACGACGTTGCCC	CACCGctctgtccacgacgttgccc	aaacgggcaacgtcgtggacagagC
2	GCCAGGTGTCACGGGTGTTG	CACCgccaggtgtcacgggtgttg	aaaccaacacccgtgacacctggc
3	CTGGCCACACTCACTACCTG	CACCGctggccacactcactacctg	aaaccaggtagtgagtgtggccagC
4	AGGTGTTAACCGCAGGTGAG	CACCGaggtgttaaccgcaggtgag	aaacctcacctgcggttaacacctC
5	CGTGGTCAGAGCTCTCGCCG	CACCGcgtggtcagagctctcgccg	aaaccggcgagagctctgaccacgC
6	CCACGCCCAGTCCTTGCAGA	CACCGccacgcccagtccttgcaga	aaactctgcaaggactgggcgtggC

Appendix Table 3: Guide RNA sequences and oligo sequences.
Capitalization in oligos indicate added base pairs for restriction enzyme cut sites and for cloning optimization.

Gene	Direction	Sequence	Amplicon Size	Efficiency	R^2	Genbank Reference	Literature Reference
GAPDH	Fw	CAAGGAGTAAGAGCCCCTGG	123	92%	0.99	NM_001206359.1	
	Rev	AGTCAGGAGATGCTCGGTGT					
HPRT	Fw	GGACTTGAATCATGTTTGTG	91	100.5%	0.99	DQ845175	(Nygard et al., 2007)
	Rev	CAGATGTTTCCAAACTCAAC					
TBP	Fw	AACAGTTCAGTAGTTATGAGCCAGA	153	109.9%	0.99	DQ845178	(Nygard et al., 2007)
	Rev	AGATGTTCTCAAACGCTTCG					
OAS1	Fw	CTGTCGTTGGACGATGTATGCT	66	89.9%	0.99	AJ225090	(Diaz-San Segundo et al., 2010)
	Rev	CAGCCGGGTCCAGAATCA					
OAS2	Fw	CCCTGGACCTATCGAGACCT	132	137%	0.97	NM_001031796.1	
	Rev	TGAAACCGCCAGCTTTTCCT					
4E-BP1	Fw	TGTGACCAAAACGCCCCCGA	150	124%	0.97	NM_001244225.1	(Carvajal, 2014)
	Rev	AACTGTGACTCTTCACCGCCCG					
ISG15	Fw	CTGGAGGGTGGGGAGGGTAG	84	103%	0.95	ENSSSCT00000029764	(Wilkinson et al., 2015)
	Rev	GTCCCAGGGCCACCACATAG					
IRF-3	Fw	CATGGACTTGGCCTCGGATG	169	151%	0.95	NM_213770.1	
	Rev	GAACTCCCACTCGTCGTCAT					
IRF-7	Fw	TCGTGATGTTGCAGGACAACTC	110	104%	0.99	HQ026022.1	(Wang et al., 2010)
	Rev	CTCTTCCCCCGGGTTGAA					
ISG54	Fw	CTGGCAAAGAGCCCTAAGGA	104	118%	0.98	NM_001315658.1	(Wang et al., 2010)
	Rev	CTCAGAGGGTCAATGGAATTCC					
ISG56	Fw	AGAGGAGCCCATCCAGCTAA	74	95%	0.99	NM_001244363.1	
	Rev	AGCATTATTACTCATGGTTGCTGTG					
IFN α (4)	Fw	CCAACCTCAGCCTTCCTCAC	130	198%	0.97	AB369103.1	
	Rev	TCCTCATTTGTGCCAGGAGC					
IFN β	Fw	TGCATCCTCCAAATCGCTCT	180	128%	0.98	NM_001003923.1	
	Rev	ATTGAGGAGTCCCAGGCAAC					
OASL	Fw	CTAGGCTGCCCCCTCTCTAT	100	138%	0.97	NM_001031790.1	
	Rev	ACGAGGCCTCTGTCCAAATG					
MX1	Fw	GAGGTGGACCCCGAAGGA	57	115%	0.99	M65087	(Diaz-San Segundo et al., 2010)
	Rev	CACCAGATCCGGCTTCGT					

Appendix Table 4: RT-qPCR primer sequences, efficiencies, and references.