IMPROVING INNATE IMMUNITY THROUGH GENETIC EDITING OF THE 5' UNTRANSLATED REGION OF INTERFERON REGULATORY FACTOR 7

An Undergraduate Research Scholars Thesis

by

MAYRA GUZMAN and MATTHEW LEWIS

Submitted to the Undergraduate Research Scholars program at
Texas A&M University
in partial fulfillment of the requirements for the designation as an

UNDERGRADUATE RESEARCH SCHOLAR

Approved by Research Advisor: Dr. Charles Long

May 2017

Major: Genetics
Animal Science

TABLE OF CONTENTS

ABSTRACT

Improving Innate Immunity Through Editing of the 5' Untranslated Region of Interferon Regulatory Factor 7

Mayra Guzman
Department of Biochemistry and Biophysics
Texas A&M University

Matthew Lewis
Department of Animal Science
Texas A&M University

Research Advisor: Dr. Charles Long
Department of Veterinary of Physiology and Pharmacology
Texas A&M University

Disease decreases production efficiency and kills millions of animals across all types of livestock operations every year. This is severely detrimental to both animal producers and consumers from an economic and overall food supply standpoint. Unfortunately, many current options for disease control prove to be impractical in various situations. Vaccines are costly to administer, can require several boosters over extensive periods of time to become effective, can become ineffective due to rapid viral mutations, and some diseases still do not have effective treatments or vaccines. Therefore, alternative methods of disease control must be implemented in order to better protect livestock and the agricultural industry.

Type I interferons (IFN) are the first line of defense against viral pathogen invasions in animals and humans. Type I IFNs are activated by the master regulator, interferon regulatory factor 7 (IRF-7). In turn, IRF-7 can be inhibited by 2'-5'-oligoadenylate synthase-like protein 1 (OASL-1), eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1), and eukaryotic translation initiation factor 4E-binding protein 2 (4E-BP2), (Honda, Yanai et al. 2005). OASL-1,

4E-BP, and 4E-BP2 negatively regulate IRF-7 translation and therefore, the transcription of type I IFN stimulation (Lee, Kim et al. 2013). Translational regulation of IRF-7 by OASL-1, 4E-BP1, and 4E-BP2 occurs at the complex secondary structure of the 5' untranslated region (UTR) upstream of the translational start site. The IRF-7 5' UTR forms several stem loops, allowing for the binding of these inhibitory proteins, therefore, reducing the number of IRF-7 5' UTR stem loop structures may alter the binding of negative regulators and decrease IRF-7 inhibition.

Genomic editing using the clustered regularly interspaced short palindromic repeats (CRISPR) CRISPR/Cas9 system will be used to alter the IRF-7 5' UTR structure via the stem loop sequence. We hypothesize that these DNA sequence modifications will inhibit the binding of translational factors and lead to increased IRF-7 expression; ultimately causing an increased interferon response, resulting in improved resistance to viruses. This technology may also potentially increase resistance against other pathogens, creating genetically engineered animals with improved immune systems.

ACKNOWLEDGEMENTS

We would like to thank Dr. Charles Long and Hanah Georges for the guidance and unending support provided throughout this research project. This project was made possible with support from the Reproductive Sciences Lab and the Texas A&M University Department of Physiology and Pharmacology.

Mayra Guzman would like to thank the encouragement, support, and love received from mother, Cristina Cruz, father, Roberto Guzman, and sister, Vanessa Cruz.

Matthew Lewis would like to personally thank his fiancé, Chandler Staton, father, Jeff Lewis, Mother, Stephanie Lewis, and brother, Scott Lewis for their unwavering support in his life.

Life is full of challenges, successes and failures, but when there is a family full of encouragement to fall back on, the failures in life only result in improvement and the successes become more and more abundant.

CHAPTER I

INTRODUCTION

Disease control and prevention is a substantial expense for almost any current livestock operation. The cost of routine veterinary and preventative care per animal is not normally an astronomical number, but it is a substantial part of the operating expenses. However, in larger commercial hog operations, disease can spread rapidly throughout the herd and not only affect the company financially, but will ultimately hurt the nation's pork supply. Tremendous amounts of money, time, and labor are spent on the prevention of these outbreaks, but inevitably, through negligence or viral mutation, an infection will spread through the entire facility's animal population and possibly to neighboring facilities. For example, in 2013, the Porcine Epidemic Diarrhea Virus (PEDV) epidemic resulted in large economic and animal losses worldwide, but it was exceptionally detrimental to the United States' economy, export market, and food supply, ("Study on Swine", 2015). A foot and mouth disease epidemic in the United Kingdom in 2001 resulted in the slaughter of more than 6 million animals, and other countries were also forced to kill millions of animals between 2001 and 2010 including, Uruguay, Japan and South Korea, among others, ("The costs of animal disease", 2012). A faster more effective response to these viral outbreaks must be implemented, and one promising solution to this problem would be the production of livestock with heightened immune responses to viruses.

Disease Control Through Genome Editing

There have been multiple studies focused on disease resistance that targets a specific gene of interest that is related to only one type of viral infection or disease. One such study

conducted at the University of Missouri confirmed that cluster of differentiation 163 gene (CD163) knockout pigs are resistant to the type 1 and 2 porcine reproductive and respiratory syndrome virus (PRRSV), (Wells, 2016). Another study took this a step further, and generated pigs with a deletion in Exon 7 of the CD163 gene. These pigs showed no adverse effects during growth and were completely resistant to type 1, sub types 1, 2, and 3, and type 2 PRRSV, (Burkhard, 2016). This advancement in immunology could have a substantial effect on the swine industry in terms of saving money and keeping animals alive. Similar work was done at Texas A&M University and Plum Island where experiments focused on preventing foot and mouth disease (FMD) in pigs. With the knowledge that the FMD virus (FMDV) can cause inhibition of IFN,IRF-7, and IRF-3 expression, a fused vector of porcine IRF7 and IRF-3 (5D) was created and used to successfully inoculate pigs and mice against FMDV infection, (Ramirez, 2014). However, resistance to one virus will not be enough to prevent other diseases from plaguing livestock. An all-encompassing measure is needed in order to effectively replace current disease control measures that simply cannot act quickly or effectively enough. Instead of targeting signaling cascades specific to one virus, the upregulation of a gene critical for the induction of the immune response in response to pathogens may become an effective resistance measure to multiple viruses.

IRF-7 and Translational Regulation

During a foreign nucleic acid invasion of a host, one of the first defense mechanisms to activate is an assembly of cytokines, especially IFNα and IFNβ, from the Type 1 IFN family. The production of IFNα and IFNβ, begins with pattern recognition receptors (PRR) on the cell's surface recognizing foreign bodies as pathogen-associated molecular patterns (PAMPs), (McNab, Mayer-Barber et al. 2015). Microbial products can be recognized by a multitude of

these receptors, but toll-like receptor 4 (TLR4) initiates a powerful pathway for Type 1 IFN induction. TLR4 begins this process by using a protein adaptor, TNF receptor-associated factor 3 (TRAF3), to allow translation of tank binding kinase 1 (TBK1), a protein coding gene, which phosphorylates and activates interferon regulatory factor 3 (IRF3), (McNab, Mayer-Barber et al. 2015). IRF3 expression creates a flood of IFN transcription, producing IFNβ and IFNα4, which prompts the transcription of yet another interferon regulatory transcription factor, IRF-7, (McNab, Mayer-Barber et al. 2015). IRF-7, known as the master regulator of Type 1 IFN, induces the transcription of IFNα and IFNβ, creating a positive feedback loop.

Although other proteins may activate the Type 1 IFN response, IRF-7 has proven critical in the production of Type 1 IFNs, as shown in a study with IRF-7 deficient mice where type I IFN transcription was down regulated or completely inhibited, (Honda, Yanai et al. 2005).Therefore, IRF-7 is critical in inducing and maintaining interferon expression. Despite the importance of IRF-7 in IFN transcription, an unregulated increase in Type 1 IFN will not increase the efficiency of a host's immune system, but instead, create damage such as cancers or autoimmune disorders, (McNab, Mayer-Barber et al. 2015). Thus, tight regulation of IRF-7 must be in effect, which is done naturally with regulatory proteins. Several laboratories have heavily researched IRF-7 in attempt to understand the regulatory mechanisms, particularly those that inhibit its translation.

Currently, the most researched inhibitors of IRF-7 are OASL-1, and the translational repressors 4E-BP1 and 4E-BP2. These negative regulatory proteins inhibit the translation of IRF-7 by different mechanisms. 4E-BP inhibits an elF4F complex that has the ability to recognize the 5' cap structure in all nuclear transcribed eukaryotic mRNAs, which facilitates ribosome recruitment during the pairing of mRNA with ribosomal unit 40S (Colina, Costa-Mattioli et al.

2008). Unit 40S is a part of a preinitiation complex that binds the mRNA which allows it to scan the 5' untranslated region of IRF-7 and bind to it, effectively inhibiting translation of IRF-7, (Nahum Soneberg, A. G. H. 2009). The exact region of the IRF-7 5' UTR which these regulatory proteins bind is unknown, (Choi, Oh et al. 2014). However, in a study done at Texas A&M University, certain regions in the stem loop structures of IRF-7 5'UTR are elemental in OASL-1 mediated inhibition and they may also be for 4E-BP, (Choi, 2016). As the 5' UTR of IRF-7 is truncated incrementally from the 3' end, negative regulation is reduced, IFN production increases and the stem loop structure of the IRF-7 5'UTR RNA is altered. This confirmed that the complex secondary structure of the 5' UTR is instrumental in the binding of repressors and inhibition of IRF-7 translation, and consequently lead to the knowledge of the sequence important for IRF-7 translation, (Ramirez Dissertation, 2014).

4E-BP and 4E-BP2

A series of studies illustrated that 4E-BP1 and 4E-BP2 have the ability to regulate IFN activity through translational control of IRF7. In these studies, 4E-BP1and 4E-BP2 deficient mice were exposed to viral infections and were found with lower viral loads and enhanced survival rates. Later, the target of the translational repressor was found to be IRF7 with a 12-fold increase in 4E-BP1and 4E-BP2 deficient phenotypic cells, (Colina, R. et al. 2008). Also, luciferase reporter gene assays on 4E-BP1and 4E-BP2 deficient cells produced an enhanced expression of the 5'UTR-IRF7 luciferase, concluding that possible IRF-7 regulation occurred at the RNA secondary structure, (Erickson, Andrea K. et al. 2008). As stated, the 4E-BP1and 4E-BP2 translational repressors inhibit the elF4F complex, negatively regulating IRF7. These repressors bind tightly to the complex when hypophosphorylated and some viral infections have the capability of controlling their phosphorylation, (Erickson, Andrea K. et al. 2008). Also at

Texas A&M University, the CRISPR/Cas9 system was utilized and porcine cells were engineered to have a knockout of 4E-BP1. As a translational inhibitor of IRF-7, the knockout of 4E-BP1 showed increased expression of IFNα and IFNβ, (Ramirez, 2015). This work highlighted an enhanced antiviral response in porcine cells through the use of the CRISPR/Cas9 system, and it also inspired future work with the IRF-7 gene. In order to continue work towards a prevention method that will be effective against several livestock viruses, this study focuses specifically on inhibiting the binding of the repressors 4E-BP1 and OASL-1 to the 5' UTR of the IRF-7 gene. Therefore, controlling the immune response against viral infection through the IRF-7 5' UTR which binds 4E-BP1 and 4E-BP2 may be promising.

OASL-1

It is known that OASL-1 has multiple cellular effects, besides inhibiting translation of the 5'UTR of IRF-7. OASL-1 is an allosteric activator of RNase L, a Type I interferon induced ribonuclease, that destroys all RNA in a cell when activated, inhibiting protein synthesis, and causing apoptosis, (Clemens, Michael J. 2005). This pathway is meant to place the cell in an antiviral state, but it has the ability to regulate translation of a cell even when uninfected by a virus, in response to physiological stress, effectively eliminating healthy cells that may have been essential in fighting off viral disease, (Clemens, Michael J. 2005). To understand the inhibitory effects of OASL-1, one study used OASL-1 as an antiviral target. These studies showed OASL-1 deficient mice had a higher IFN response illustrating its negative regulation on the innate immune response, (Lee, 2013). After the induction of type I IFNs by IRF-7, several interferon stimulated genes are activated including OASL-1. OASL-1 then down regulates the immune response by inhibiting the translation of IRF-7, effectively shutting down the innate

immune response. The elimination of this regulation can aid in an increase in IRF-7 and thus an increase in IFN, making it a suitable therapeutic antiviral target.

Considering the inhibitory proteins, OASL-1 and 4E-BP, and their effects on the secondary structure of the IRF-7 5' UTR, to decrease inhibition of IRF7 expression, the stem loop structure in the 5'UTR of IRF7 will be modified. Through targeted genome editing with CRISPR and Cas9 nuclease, the IRF-7 5' UTR sequence will be altered, resulting in a change in the structural loops necessary for negative regulation. We hypothesize that this will decrease inhibition of IRF-7, and increase the host's innate immune response against viruses without the negative consequences of IRF-7 over expression.

CHAPTER II

METHODS

Plasmid Construction

The porcine IRF-7 5' UTR sequence (GenBank: HQ026022, GenBank: FD634523.1 1-338(EST)) was used to synthesize 6 guide RNA (gRNA) sequences. Due to the poor annotation of the porcine genome among known databases, primers were designed outside of the annotated region to allow for proper sequencing and amplification of the 5' UTR. The gRNA sequences were then cloned at the BbsI sites of pSpCas9(BB)-2A-GFP (PX458), a gift from Feng Zhang, (Addgene plasmid # 48138), a plasmid containing Cas9 nuclease and the green fluorescence protein (GFP) genes. A PX458 plasmid without a cloned guide RNA sequence was used as a negative control. The gRNA sequences are presented in appendix 1.

Cells and Reagents

Swine kidney cells (SK6) were used for plasmid transfections. Cells were grown in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F-12) (Gibco, Pittsburg, Kansas) supplemented with 10% Fetal Bovine Serum (FBS) (Atlanta Biologics, Flowery Branch, GA) and 1% Antibiotic-Antimycotic (Thermo Fisher Scientific, Waltham, MA). The SK6 cells were seeded in 6-well plates, at a density close to $1x10^5$, 24 hours before transfection. The cells were transfected with the constructed plasmids described above, using Lipofectamine 3000 (Thermo Fisher Scientific, Waltham, MA). The following day, fluorescence microscopy was used to view GFP expression and analyze transfection efficiencies.

T7 Endonuclease I Assay

The SK6 cells were harvested 24 hours post-transfection and used in the T7 Endonuclease I Assay Kit (New England, Biolabs, Ipswich, MA) to determine the genome targeting efficiency of the synthesized gRNA's. A polymerase chain reaction (PCR) was run with forward and reverse primers that cover 756 bp of sequence. The resulting amplicon was purified with the QlAquick PCR Purification Kit (QIAGEN, Hilden, Germany)and used in the T7 endonuclease I Assay according to the manufacturer's protocol. The cleavage bands produced by the detection kit were analyzed using gel electrophoresis with a 1.5% agarose gel run for 3 hours at 90 volts.

Treatment Transfections

After the gRNAs were tested for functionality, they were utilized in three different treatment groups that contained multiple gRNA's per transfection to allow several gRNAs to target the UTR at the same time. This was set up to produce a wide variety of unique mutations in cells within the colony. The first treatment group contained gRNAs 1-3 (represented as G:1-3 in the results), the second group contained gRNAs 4-6 (represented as G: 4-6 in the results), and the last treatment contained only gRNAs 1 and 6 (represented as G: 1&6 in the results). The cells were plated into 6 wells with the treatment groups described above using Lipofectamine 3000 (Life Technologies, Carlsbad, CA). The following day, fluorescence microscopy was used to view GFP expression in the colonies.

Flow Cytometry and Clonal Propagation of SK6 Cells

Twenty four hours post transfections, cells in each treatment group were harvested and sorted by GFP expression and propidium iodide (PI) staining using flow cytometry (Flow Cytometry Core Laboratory at Veterinary Medicine and Biomedical Sciences, Texas A&M University). The living cells that were positive for GFP expression and negative for PI were sorted singularly into 96 well plates that were then allowed to replicate into larger colonies originating from that single cell. The colonies that survived were passed into successively larger plates until they were healthy enough to be harvested and either cryogenically preserved or grown for further analysis.

DNA Sequencing of Modified SK6 IRF-7 5'UTR

Selected cells were harvested and DNA was extracted using the QlAquick DNeasy Blood and Tissue kit (QIAGEN, Hilden, Germany) and sent for Sanger sequencing (Laboratory for Genome Technology, Texas A&M University). The sequencing data was aligned to the wild type 5'UTR sequences using the molecular biology suite of benchling.com, and the modifications that were found in the sequence were analyzed. Any colonies that represented a consistent mutation were matched with specific gRNA's and any sequences that expressed data that could be attributed to a mixed population of cells within the colony, were diluted out in hopes of isolating the modified cells.

CHAPTER III

RESULTS

T7 Endonuclease I Assay

In order to gain insight into the functionality and efficiency of the gRNAs, the T7 Endonuclease I Assay was performed 24 hours post successful transfection of the constructed CRISPR/Cas9 plasmids into SK6 cells. The transfections had a 50-70% efficiency determined visually by the expression of GFP. Out of the 6 gRNAs, the T7 endonuclease detected heteroduplex DNA in guides 1, 2, 3, and 6, determined by the presence of faint cleavage bands, illustrating their functionality (Figure 1). Guide RNA 1 showed several cleavage bands, gRNA 2 showed 2 cleavage bands, and gRNA 6 showed 1 cleavage band. Due to the faintness bands from

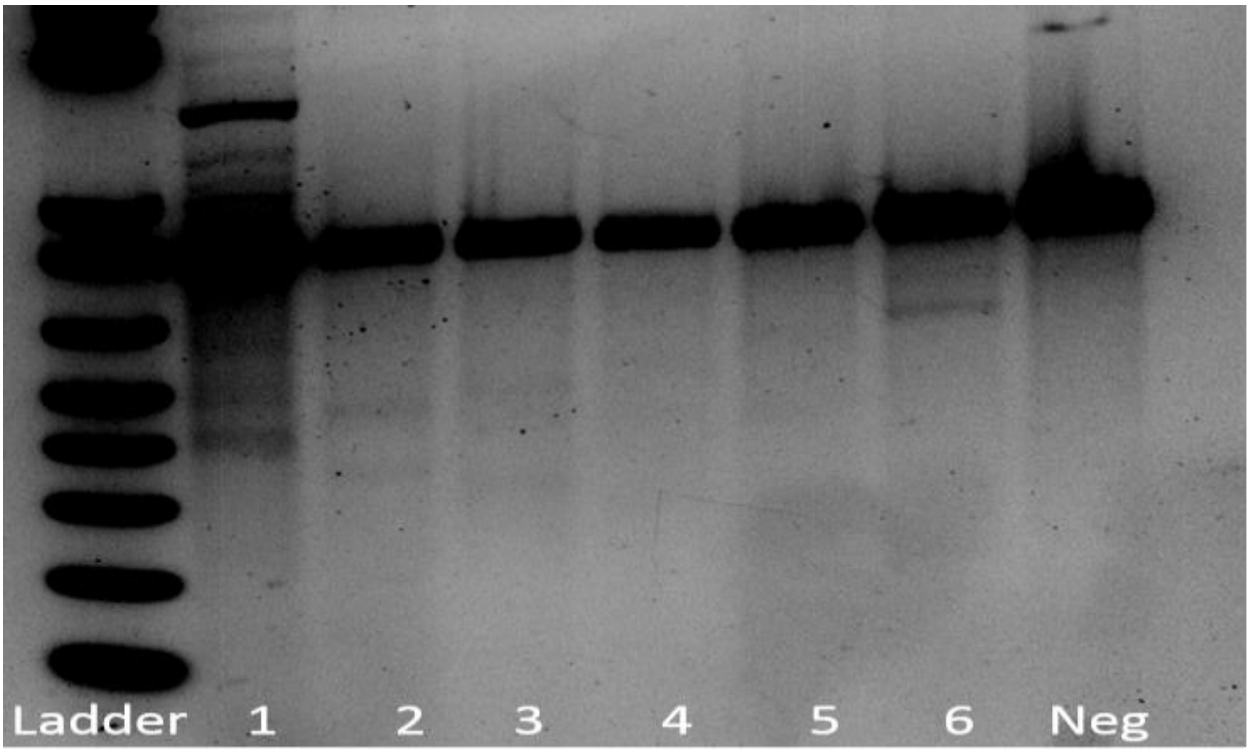


Figure1. T7 endonuclease 1 assay: Genomic PCR was prepared and tested for CRISPR/CAS 9 induced mutations by the T7 endonuclease assay. Target site and primers are found in the index.

guides 1,2,3 and 6, guides 4 and 5 were deemed possibly functional. As stated in the methods section, the negative control is a PX458 plasmid without a gRNA sequence, and is clearly absent of a cleavage band, which ensured that the assay worked properly.

RNA folding predictions from DNA Sequencing of Modified SK6 IRF-7 5'UTR Cells

DNA sequencing demonstrated a mutation rate of 54% of all selected GFP positive colonies. DNA sequences from transfected cells were aligned to wildtype IRF-7 and those with modifications are shown in Figure 2 which also identifies the location of the 6 gRNAs within the sequence. RNA folding predictions of the modified IRF-7 5' UTRs were performed using Vienna Websuite. Two databases were used for the UTR sequences, the National Center for Biotechnology Information (NCBI) mRNA sequences as well as the Expressed Sequence Tag (EST), also provided by the NCBI database. The mRNA sequence is approximately 104 base pairs shorter than the EST sequence which showed a fault in the annotation of the 5' UTR. Folding predictions of the cell modifications and wildtype IRF-7 for both NCBI and EST sequences are included in figure 2.

Colony 1 for treatment G1-3 had a 72 base pair deletion between guides 1,2 and 3Colony 1 for treatment G:1 and 6 had a 191 base pair deletion between guides 1 and 6. Colony 2 for the same treatment had a 3 base pair deletion near guide 6. Colony 3 had various insertions of different lengths between guides 4, 5 and 6. Colony 4 had a 360 base pair deletion. Colony 1 for treatment G: 4-6 had an 83 base pair deletion between guides 4, 5 and 6. Colony 2 had a 72 base pair deletion between 4, 5, and 6. Colony 3 had a 42 base pair deletion between 4, 5, and 6. Colony 4 had a 4 base pair deletion between 4 and 5. Colony 5 had a 83 base pair deletion between 4, 5, and 6. Similarly colony 6 and 7 had a 82 base pair deletion between 4, 5, and 6. Figure 2 depicts where the modifications lie on the wildtype 5'UTR IRF-7. Colony 1 and 3 from

treatment G:1 and 6 represent knockout modifications, showing large deletions that effectively eliminated majority of the IRF-7 5'UTR as show in table 1. All of these modifications effectively altered the folding structures. These folding predictions along with the sequence alignments will give insight into the important sequences and/or structures for proper binding of proteins OASL-1 and 4E-BP1 to the IRF-7 5' UTR.

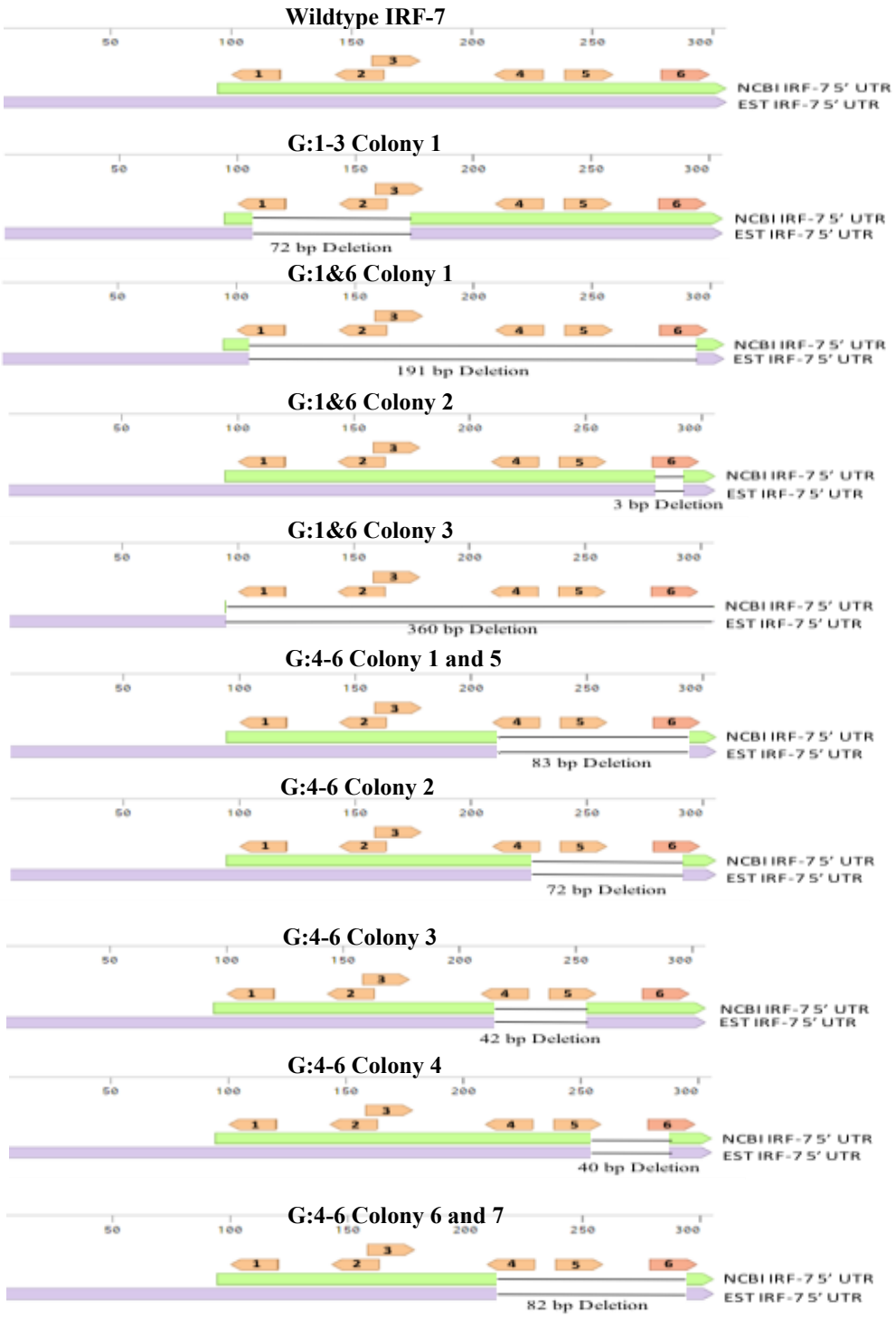


Figure 2. Guide RNA targets in the IRF-7 NCBI and EST sequence for wildtype IRF-7 and treatment groups G:1-3, G:4-6, and G:1&6. The NCBI sequence is from a database collecting DNA information. The expressed sequence (EST), database is a collection of sequences from cDNA information. Both sequences contain the sequence specific for IRF-7 translation, however the NCBI database contains this sequence only, while the EST database shows a more complete picture of the IRF-7 sequence.

Table 1. Model of RNA folding for the wildtype and modified 5'UTR of IRF-7, generated by Vieana Websuite.

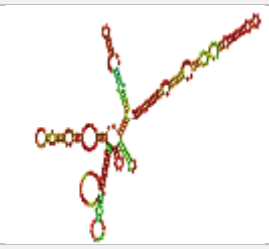
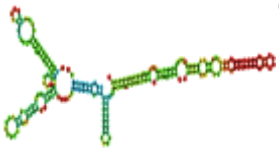
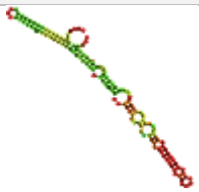
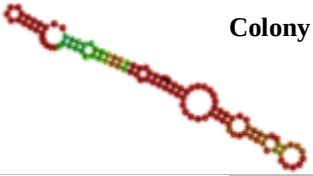
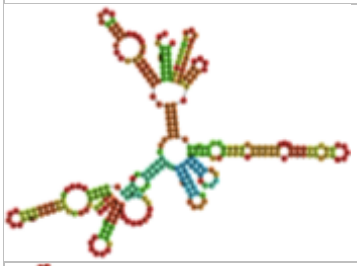
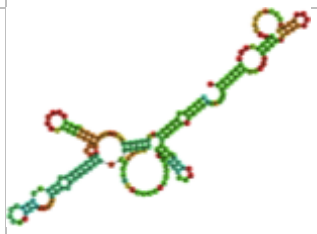
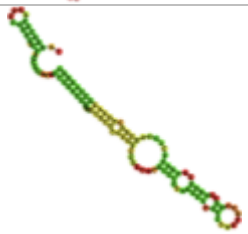
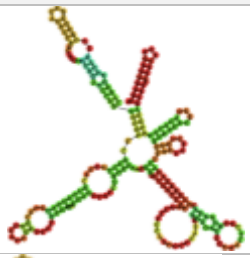
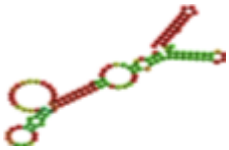
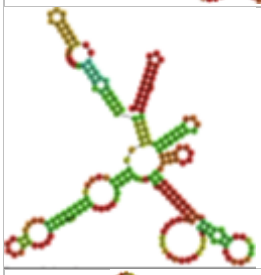
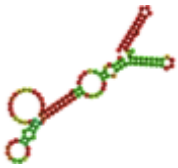
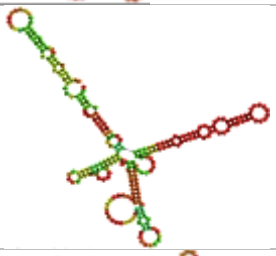
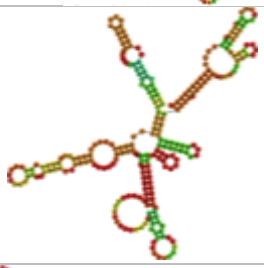
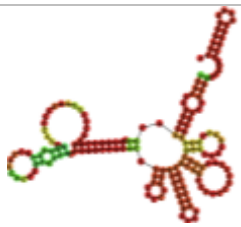
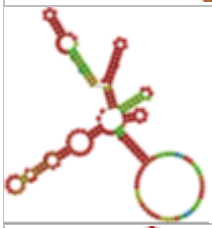
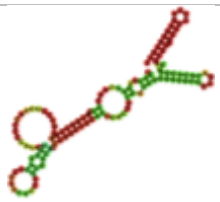
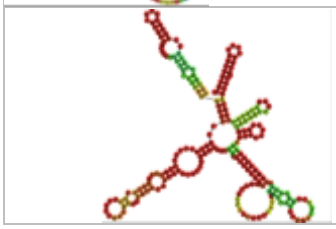
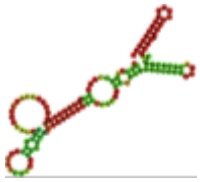
EST	NCBI
Wildtype 5'UTR IRF-7	
	
Treatment G 1-3:	
Colony 1: 3bp D 	**Colony 1**: 3 bp D 
Treatment G 1& 6:	
Colony 1: 191 bp D 	**Colony 1: 191 bp D** 
Colony 2: 3bp D 	**Colony 2**: 3bp D 
Colony 4: 360 bp D 	**Colony 4:** 360 bp D 

Table 1. Model of RNA folding for the wildtype and modified 5'UTR of IRF-7, generated by Vieana Websuite(contd.)

EST	NCBI
Treatment G 4-6:	
 **Colony 1&5:** 83 bp D	 **Colony 1&5:** 83 bp
 **Colony 2: 72 bp D**	 **Colony 2: 72 bp D**
 **Colony 3:**42 bp D	 **Colony 3:**42 bp D
 **Colony 4:**40 bp D	 **Colony 4:**40 bp D
 **Colony 6:** 82 bp D	 **Colony 6:** 82 bp D
 **Colony 6:** 82 bp D	 **Colony 6:** 82 bp D

CHAPTER IV

CONCLUSION

CRISPR/Cas9 is an efficient system for inducing genetic modifications in numerous cell lines including SK6 cells. The induced modifications produced in the 5'UTR of IRF-7 are predicted to alter the secondary stem loop structure of the mRNA, and thus alter any negative regulation from 4EB-P1, OASL-1, or any other unknown binding proteins, allowing for increased IRF-7 expression and an enhanced immune response through heightened production of IFNα and IFNβ.

Ongoing studies on this project will evaluate each of the cell lines' response to viral challenges and measure changes in type I IFNs, IRF-3, IRF-7, 4EBP, OASL-1, and several other interferon stimulated genes that may give insight into how the innate immune system will be affected by these modifications. Significant changes in IRF-7 and type I IFN transcript levels will be attributed to deletions in specific sites of the sequence, specific stem loop alterations, or a combination of the two. Any changes in other immune system gene transcript levels will be attributed to an increase in IRF-7 or type I IFNs if present.

In addition to producing multiple, unique modifications within the 5' UTR, treatment G1&6 was used in hopes of creating a maximal deletion event. G1&6 colony 2 was our expected result and will hopefully give an example of unregulated IRF-7 translation and its effects on the immune system. Without any repressors inhibiting the translation of IRF-7, there should be extremely heightened levels of IFN I response providing a benchmark for unregulated IRF-7 production. Furthermore, modifications in colonies G1-3 and G4-6 should lead to a more controlled increase in IRF-7 expression. Thus, we expect the Type IFN I response to increase

due to the alteration of both the gene sequence and the modification the secondary structure. Granted, there are more drastic modifications seen in the sequence and number of stem loop structures in colonies G1-3 than in G4-6, but according to a study at Texas A&M University, the truncation of the 5' UTR from the 3' end of the sequence , where the initial 129 base pair deletion of the UTR sequence immediately upstream of the translational start site resulted in the largest increase in response, the treatment group G4-6 targets that region and is expected to have a much larger effect on the Type 1 IFN , (Ramirez dissertation, 2014).

Considering these alterations of the 5'UTR secondary structure, 4EB-P1, 4EB-P2, and OASL-1 will not be able to bind to the modified IRF-7 5' UTR at a normal level because of the change in the sequence and consequently, the stem loop structure. This, in turn, should decrease their repressive effects on the IRF-7 translation and type I IFN response, increasing IRF-7 and type I IFN expression. IRF-3 is also expected to increase since it is critical for the initial induction of type I IFNs.

REFERENCES

1. Akira, S., S. Uematsu, and O. Takeuchi (2016).“Pathogen recognition and innate immunity.” *Cell,* 124(4), 783-801.

2. Erickson, Erickson K., Gale, Michael (2008). “Regulation of interferon production and innate antiviral immunity through translational control of IRF-7.” *Cell Research*, 18(4), 433-436.

3. Choi, W.-Y., et al. (2016) “Secondary structure of the Irf7 5'-UTR, analyzed using SHAPE (selective 2'-hydroxyl acylation analyzed by primer extension).” *BMB Reports*, 47(10), 558-562.

4. Clemens, Michael J. (2005) “Translational control in virus-infected cells: Models for cellular stress responses.” *Cell & Developmental Biology*, 16, 13-20.

5. Colina, R., et al. (2008). "Translational control of the innate immune response through IRF-7." *Nature,* 452(7185), 323-328.

6. Honda, K., et al. (2005). "IRF-7 is the master regulator of type-I interferon-dependent immune responses." *Nature*, 434(7034), 772-777.

7. Lee, M., et al. (2013). "OASL1 inhibits translation of the type I interferon-regulating transcription factor IRF7." *Nature Immunology*, 14(4), 346-355.

8. McNab, F., et al. (2015). “Type I interferons in infectious disease.” *Nature Reviews Immunology*, 15(2), 87-103.

9. Nahum Soneberg, A. G. H. (2009). "Regulation of Translation Initiation in Eukaryotes: Mechanisms and Biological Targets." *Cell*, 136(4), 731-745.

10. Ning, S., J.S. Pagano, and G.N. Barber (2011). “IRF7: activation, regulation, modification and function.” *Genes Immunology*, 12(6), 399-414.

11. Ramírez-Carvajal, L., et al. (2014). “Expression of Porcine Fusion Protein IRF7/3(5D) Efficiently Controls Foot-and-Mouth Disease Virus Replication.” *Journal of Virology*, 88(19), 11140-11153.

12. Ramirez-Carvajal, L. (2014). “Enhancement of Innate Immunity to Control Viral Infections in Livestock Species.” Doctoral Dissertation, Texas A&M University.

13. Ramírez-Carvajal, L., et al. (2015). "Depletion of elongation initiation factor 4E binding proteins by CRISPR/Cas9 enhances the antiviral response in porcine cells." *Antiviral Research*, 125, 8-13.

14. Ramirez-Carvajal, L., et al. (2016). “Constitutively active IRF7/IRF3 fusion protein completely protects swine against food-and-mouth disease.” Journal of Virology, 90 (19), 8809-8819.

15. Sato, M., et al (1998). “Positive feedback regulation of type I IFN genes by the IFN-inducible transcription factor IRF-7.” *FEBS letters*, 441(1), 106-110.

16. Sato, M., et al (2000).”Distinct and essential roles of transcription factors IRF-3 and IRF-7 in response to viruses for IFN-alpha/beta gene induction.” *Immunity*, 13(4), 539-548.

17. Takaoka, A., T. Tamura, and T. Taniguchi (2008). “Interferon regulatory factor family of transcription factors and regulation of oncogenesis.” *Cancer science*, 99(3), 467-478.

18. “The costs of animal disease: A report produced for the International Federation for Animal Health.”(2012) Oxford Analytica, 1-36.

19. “Study on swine catastrophic disease.”(2015) Agralytica consulting, 1-78.

APPENDIX

Reverse Primer: GGAACCGTGTTCTGGCTTCA

Forward Primer: CCGGGGAGACGAAACTTCC

Guide 1: 5’ CTCTGGCCACGACGTTGCCC 3’

Guide 2: 5’ GCCAGGTGTCACGGGTGTTG 3’

Guide 3: 5’ CTGGCCACACTCACTACCTG 3’

Guide 4: 5’ AGGTGTTAACCGCAGGTGAG 3’

Guide 5: 5’ CGTGGTCAGAGCTCTCGCCG 3’

Guide 6: CCACGCCCAGTCCTTGCAGA