

ABSTRACT

DORT, MATTHEW CARTER. Identification of Host MicroRNAs Associated with Marek's Disease Virus Challenge. (Under the direction of Dr. Sunny Liu).

The causative agent of Marek's disease, a common lymphoproliferative disorder in chickens, is Marek's disease virus serotype 1 (MDV-1). Current MDV vaccination strategies include the use of non-pathogenic serotypes, such as herpesvirus of Turkey (HVT). Understanding alterations in host gene expression upon infection will provide a better understanding of both MDV pathogenesis and aid in the improvement of preventative strategies. MicroRNAs (miRNAs) are ~18-23 nucleotide small RNA molecules that post-transcriptionally regulate the expression of target mRNAs. The objective of this study was to identify chicken miRNAs associated with the response to MDV infection.

Total RNA was isolated from the spleens of 3 MDV-1 infected birds and 3 controls birds at 42dpi. Small RNA populations were enriched and subjected to deep sequencing using an Illumina GAII platform. Sequences were then searched against miRBase and Rfam to determine sequence reads representing known miRNAs and other types of small RNA, using two server-based data analysis programs, DSAP and miRanalyzer. Differential expression analysis revealed an increased expression of *miR-146b*, *miR-21*, and *miR-10b*, and decreased expression of *miR-30d*, *miR-128*, and *miR-92* in MDV-1 infected spleens. Further target prediction and target validation experiments were performed to identify immunologically-relevant pathways affected by differential expression of *miR-128* and *miR-147*.

In silico target prediction identified 475 targets for *miR-128* and 147 targets for *miR-147*. Dual luciferase assay further validated 8 targets for *miR-147* in the 3'UTRs of target mRNA. *TUBB* and *RAB22A* are two validated *miR-147* targets involved in viral particle assembly and egress. Upregulation of *miR-147* at the tumor stage of MD may reduce adaptive immune responses by delaying MHC-mediated processing and presentation of viral antigen. DAVID Pathway analysis of predicted *miR-128* and *miR-147* targets identified a number of pathway clusters in immunologically-relevant signaling networks. This work implicates differential expression of *miR-128* and *miR-147* in the pathogenesis of MDV-1.

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Identification of Host MicroRNAs Associated with Marek's Disease Virus Challenge

by
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A thesis submitted to the Graduate Faculty of
North Carolina State University
in partial fulfillment of the
requirements for the Degree of
Master of Science

Animal Science

Raleigh, North Carolina

2013

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DEDICATION

This work is dedicated to my parents. Without their support and guidance, I may have ended up in competitive eating rather than the field of molecular biology.

BIOGRAPHY

Matthew Dort was born and raised in Columbus, OH. He graduated from Pickerington High School North in 2005 where he was a part of the men's swim team, volleyball team, pep band, jazz band, symphonic band, and marching band. He received a B.S. majoring in Toxicology and minoring in Biology and Chemistry from Ashland University in Ashland, OH in May 2009 where he was a member of the University's men's swim team. He then worked as a chemist at Calgon Carbon Corporation in Columbus, OH for a year before being accepted to a Master's program in the Animal Science Department at North Carolina State University in 2010. Upon completion of his Master's degree, he plans to find work as a researcher in an agricultural, biomedical, or pharmaceutical company.

ACKNOWLEDGEMENTS

I would like to thank my mentor Dr. HC Sunny Liu for her guidance throughout my graduate career. Without her help I would not have made it this far. I would also like to thank my committee members, Dr. Lin Xi and Dr. Matthew Koci, for taking time out of their schedules to help me. I would also like to extend my gratitude to all the members of the Liu Lab for their help and friendship. Last, but certainly not least I would like to thank my family for their support. Without their support and guidance I would not be where I am today.

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CHAPTER 1: LITERATURE REVIEW

1.01) Marek's Disease

Marek's disease (MD) is a lymphoproliferative disease in chickens caused by the oncogenic alphaherpesvirus MD virus (MDV). MD is characterized by the formation of solid tumors in the viscera, immunosuppression, and polyneuritis, which leads to paralysis (Nair and Kung 2004). See table 1-1 for a comparison of MDV infection to that of two other oncogenic avian viruses, avian leukosis virus (ALV) and reticuloendotheliosis virus (REV). The first written record of symptoms characteristic of Marek's disease was made by József Marek as "fowl paralysis" or "polyneuritis" in 1907 while he was a lecturer and director of the Internal Medicine Department in the Royal Veterinary College at the Royal Veterinary Institute in Budapest. Since its initial discovery, countless hours of research have contributed to how MD is understood today.

After Marek's initial publication in the *Deutsche Tierärztliche Wochenschrift* of what he termed multiple neuritis (polyneuritis) in chickens, there was a drop in interest in the subject until the 1920s, where another study group further characterized the condition as a distinct clinical entity, calling it "neurolymphomatosis gallinarum" (Pappenheimer et al. 1926). In the 1940s, the disease became particularly difficult to differentiate from another lymphoproliferative disease in chickens called lymphatic leukosis (LL). Both were characterized by the presence of visceral tumors, and the two conditions were often misdiagnosed until a clearer classification of the two diseases was presented and accepted at the World Veterinary Poultry Association in 1960 following the presentation of a paper by Biggs (Biggs 1961). Biggs' paper suggested that MD should be differentiated from LL by using

criteria based on the organs and tissues affected, age group affected, and histo-pathogenesis. Biggs further indicated that MD began as early inflammatory lesions that only became neoplastic later in life. Both Biggs and another proponent of nomenclature reclassification (Campbell and Biggs 1961) presented at this meeting. As a result of these two papers, the disease once known as polyneuritis and neurolymphomatosis gallinarum was reclassified under the name of Marek's disease.

MD became an even greater problem in the 1960s. Infection with MDV was first demonstrated with cell-culture-propagated virus in whole blood (Sevoian et al. 1962) and tumor cells (Biggs and Payne 1963). From the 1960s to 1970s, particular efforts were being made to identify resistant and susceptible strains of chickens. It was conceivable that trait-selection of MD-resistant chickens would be more cost-efficient, despite the potential reduction in other agriculturally-favorable traits.

In the late '60s and throughout the '70s, the first successful vaccination strategy to MD was demonstrated (Churchill et al. 1969a and 1969b), and the notion of selecting for MD-resistance was abandoned for the more cost-effective method of vaccination. This vaccine was actually the first case of a neoplastic disease model successfully controlled by vaccination. The early MD vaccination strategies were highly effective in providing protective immunity to chickens, protecting them not from the infection by oncogenic strains of MDV, but instead protecting from the transformation process that leads to tumor formation.

In the 1980s, it became apparent that the MDV strains responsible for causing MD were becoming more and more virulent (Witter 1997). This increased virulence is in part

attributed to the widespread use of Churchill's and later Witter's (Witter et al. 1969) and Okazaki's (Okasaki et al. 1970) use of herpesvirus of turkeys (HVT, meleagrid herpesvirus, MeHV) as a vaccines. As more and more effective vaccines were developed and implemented commercially, MDV virulence failed to plateau. Even today, researchers are making an effort to use epidemiological parameters to quantify strain-specific viral shedding and host mortality in hopes of making a more objective scale for virulence (Atkins et al. 2011).

MD is undoubtedly one of the most pressing concerns in the world of avian diseases, along with avian influenza, infectious bursal disease, *Ascaridia galli*, Newcastle disease, and salmonella infection. Economic losses due to MD-induced mortality, reduced egg production, and meat contamination are an international problem (Davies et al. 2009). The destructive potential of MD is difficult to quantify. However, a more conservative estimate of global losses due to MD is estimated at around \$1-2 billion annually (Morrow and Fehler 2004).

1.02) The Marek's Disease Virus

Marek's disease virus (MDV) is a double-stranded DNA virus (Group I) in the family herpesviridae. The herpesviridae family is further divided into three subfamilies: alpha-, beta-, and gamma-herpesvirinae. Originally, MDV was classified as a gammaherpesvirus along with Epstein Barr virus (EBV) and Kaposi Sarcoma-Associated Herpesvirus (KSHV), based on its lymphotropic biological nature. However, later studies indicated that its genomic composition more closely matched other alphaherpesvirinae like herpes simplex

virus-1 and -2 (HSV-1 and -2), Varicella zoster virus (VZV), and Herpes B virus (Buckmaster et al. 1988 and Camp et al. 1991). Additionally, MDV's replication cycle is notably similar to that of VZV (Calnek 2001, Arvin et al. 2010). Both viruses primarily infect the epithelial cells in the respiratory tract. Furthermore, this infection jumps from epithelial cells to lymphocytes, which migrate to the skin, where fully-formed virions are shed from epithelial skin cells into the surrounding environment. This new genomic evidence, combined with the VZV-MDV similarities in replication cycle led the International Committee on the Taxonomy of Viruses (ICTV) to reclassify MDV to the alphaherpesvirinae subfamily.

MDV is divided further within alphaherpesvirinae to the genus mardivirus, which is further split into four species by the ICTV, including gallid herpesvirus 2 (GaHV-2, MDV-1), gallid herpesvirus 3 (GaHV-3, MDV-2), meleagrid herpesvirus-1 (MeHV-1, HVT), and columbid herpesvirus 1 (CoHV-1). Based on sequence data and infection patterns, there are three serotypes of MDV including MDV-1, MDV-2, and HVT. Table 1-2 shows a list of common MDV strains with corresponding pathogenicity.

MDV-1 is the only oncogenic serotype, leading to the transformation of T-lymphocytes. These transformed T-lymphocytes migrate to the spleen and other visceral organs, generating solid tumors. The pleomorphic lymphoid cells also infiltrate peripheral nerves, most notably the sciatic nerve, which leads to the classic paralysis/ataxia symptom characteristic of MD.

MDV-2 and HVT are both non-pathogenic, and the differences between oncogenic MDV-1 strains and non-pathogenic MDV-2 and HVT present interesting subjects for

research. Those sequences that are either present in MDV-1 but not the other two serotypes or on the other hand are lacking in MDV-1 but are present in the other serotypes offer clues to what it is in MDV-1 that leads to transformation and tumor formation. MDV-2 and HVT are actually useful as vaccines, protecting against oncogenesis in the case of infection with MDV-1.

One of the classical characteristics of herpesviruses is their potential to shift between a productive (lytic) and a non-productive (latent) phase of infection. A lytic phase is marked by cell “breaking” or cell death, which releases cellular contents into the surrounding space. This cellular content includes virus particles which further infect other cell types; in the case of MDV these include B-cells, T-cells, and feather follicle epithelia (FFE). The latent phase often occurs in T-cells, and is essentially a “dormant” phase in a cell with minimal gene expression. As with the other herpesviruses, these latent cells can reactivate, like in the case of VZV, which leads to the condition known as shingles in humans. In the case of MDV-1, this reactivation leads to oncogenic transformation and lysis.

1.03) MDV Genome

All members of the herpesviridae family are double-stranded DNA viruses that range in size from 108-230 kbp. Within herpesviridae, there are six classes (A through F). Of those six classes, only D and E are contained in the alphaherpesvirinae subfamily. MDV-1, MDV-2, and HVT are all in Class E. Class E alphaherpesvirinae genome structure typically contains two unique sequences called unique long (U_L) and unique short (U_S)--each of which is flanked by inverted internal (IR_L , IR_S) repeats and terminal repeats (TR_L , TR_S)

All three members of the mardivirus genus have been sequenced in genome assembly projects. From these projects, 103 protein-coding genes were found for MDV-1, MDV-2 has 102, and HVT has 99. The majority of the ORFs in these three genomes are homologous to those in the other alphaherpesvirinae.

There are a number of serotype-1-specific transcripts which contribute to MDV-1 pathogenesis. Figure 1-1 shows some of the more notable transcripts within MDV-1. Annotations in figure 1-1 were made based on the genome assembly of the Md5 MDV-1 strain (Tulman et al. 2000), and was adapted from data presented in recent publications. (Parcells et al. 2003, Zhao et al. 2011). MDV-1 has ORFs for *Meq* (MDV EcoRI-Q) and interleukin-8 (*IL8*), while there is no such homologue for these genes in MDV-2 or HVT. Phosphorylated polypeptide *pp38* is also present in MDV-1 genome but absent in MDV-2 and truncated in HVT (Smith et al. 1995).

Another important quality of herpesviruses is that they have a temporal regulation of viral gene expression. A more in-depth description of the genes involved in MDV's infection cycle will be discussed later in this chapter, but there are some regulatory commonalities in all herpesviruses. The ORFs of herpesviruses are classified as either immediate early (IE, α), early (E, β), or late (L, γ) genes. The α -genes are expressed first. There is often a tegument protein called *VPI6* (α -*TIF*, α -trans-inducing factor) carried in with the invading virion which activates transcription of these α -genes. Alpha-gene transcripts are exported to the cytoplasm, where they are translated into autoregulatory proteins. These genes can be considered autoregulatory because they tend to inhibit transcription of more α -genes, in addition to activating transcription of β and γ genes.

Beta-genes are expressed before replication of viral genome, as many of these genes code for proteins that tend to play a role in the replication process (ie. a dsDNA binding protein, or a DNAPol). Many β -genes carry nuclear localization signal motifs which bind with transport proteins that carry them back to the nucleus. Nuclear β proteins promote the transcription of γ_1 (late) and γ_2 (true late) genes which are all activated after the start of viral DNA replication. Gamma-genes generally repress transcription of α - and β - genes. Many γ -genes code for virion structural proteins (ie. nucleocapsid, envelope spike proteins, and VP16/other tegument proteins). These γ -gene products are trafficked to a number of locations within the cell, where they await assembly into mature virions.

As stated earlier, there are more than 100 ORFs in MDV-1. A vast majority of these ORFs have homologues in MDV-2 and HVT strains. It is logical to assume that those genes that are present in the oncogenic serotype (MDV-1) but which are missing in non-oncogenic serotypes may play a role in oncogenesis. In fact, a number of MDV-1-specific genes have been associated with oncogenesis. *Meq*, *vIL-8*, and *pp38* are all only present in serotype-1 MDV isolates (although there is a truncated ORF of *pp38* in HVT), and are all considered to play a role in oncogenesis.

Meq is in a family of transcriptional activators called *Jun/Fos* proteins. *Meq* is a 339-amino acid protein which occurs as a homo-/hetero-dimer that binds to AP-1 activator sites. *Meq*'s structure (figure 1-2) contains an N-terminal bZIP (basic leucine zipper) domain resembling the *c-Jun* oncogene and a proline-rich transactivation domain homologous to the Wilms' tumor suppressor gene (*WT-1*) near its C-terminus. The bZIP domain is the site of *Meq* dimerization resulting in homodimers. The bZIP domain is also involved in the

association of Meq with other proteins in the *Jun/Fos* family like *c-Jun* (the primary dimerization partner), *JunB*, *ATF2*, or *c-Fos*. *Meq* was also shown to bind to *p53* (Deng et al. 2010), inhibiting downstream *p53*-mediated apoptotic and cell cycle arrest signaling pathways. The WT-1-like transactivation domain has been shown to bind at Gal4 DNA binding motifs (Qian et al. 1995), cellular *IL-2* promoters, and AP-1 activation sites (Levy et al. 2003) on both chicken and viral genomes. There are a number of chicken genes induced by *Meq*, including *chIL-2*. There are also very important binding sites in the MDV lytic origin of replication (*ori_{lyt}*) and in the promoters for *Meq* and *ICP4*. These viral binding sites may explain *Meq*'s association with ending latency and initiating transformation of T-cells, as the *ori_{lyt}* is the site where DNA replication machinery binds for genome replication and *ICP4* is a lytic gene.

Viral *IL-8* (*vIL-8*) was originally identified as a CXC chemokine and was named *vIL-8* because of its high homology to chicken *IL-8* (*chIL-8*). Cellular *IL-8* is characterized as a strong chemoattractant and is produced by activated neutrophils (Leung et al. 2001) and macrophages as part of a TLR signaling pathway. MDV-1 *vIL-8* is normally a 3-exon transcript, although splice variants have been identified including variants where exons II and III are fused to *Meq*, *RLORF4a*, or *RLORF5*. A recent study found *chIL-8* to be downregulated in response to MDV infection (Heidari et al. 2010) while other expression analyses show that *vIL-8* is expressed freely (Xu et al. 2011, Engel et al. 2012). Infection and subsequent production of the late gene *vIL-8* functions as a chemoattractant for other lymphocytes. Attraction of B- and T-cell subsets increases the likelihood of infecting the

attracted lymphocytes, and an in-depth analysis suggests that *vIL-8* is involved in lymphoma formation via activation of T-lymphocytes (Engel et al. 2012).

Phosphorylated polypeptide *pp38* is a lytic gene located in the IRL region of MDV-1. It is expressed with early kinetics in the infection cycle, along with its inverted repeat, *pp24*, in the TR_L region. *pp38/24* share 55 common amino acids residues at their N-termini, and have been shown to form a heteropolymer. The *pp38/pp24* heteropolymer was further shown to enhance expression at a bi-directional promoter upstream of *pp38* (Ding et al. 2008), resulting in the production of both *pp38* and a 1.8kb mRNA transcript with 132bp repeats which is also associated with oncogenesis (Chen et al. 2009).

Meq, *vIL-8*, and *pp38/pp24* are not the only MDV genes linked to oncogenesis. The MDV-encoded viral telomerase RNA subunit (*vTR*), *ICP4* homologues/latency-associated transcripts (*LAT*), and microRNA (miRNA) clusters have all been experimentally shown to play a part in oncogenesis.

The MDV1-specific *vTR* is contained in the long repeat regions and is thought to play a role in oncogenesis. Telomerase is a ribonucleoprotein responsible for extending telomeres. This enzyme is essential in normal eukaryotic cells to prevent excessive chromosomal degradation. Abnormally-shortened telomeres are associated with aging, and one of the classical features of neoplastic cells is the overextension of telomeres (Tümpel et al. 2012). Further analysis suggests that expression of *vTR* may play a role in MDV1-mediated oncogenesis (Fragnet et al. 2003, Robinson et al. 2010). Deletion analysis suggests *vTR* is crucial for lytic replication and MDV-induced lymphomagenesis (Trapp et al. 2006). Recent studies suggest that *vTR* may facilitate viral genome integration during latency into

chicken telomeres (Kaufer et al. 2011), which strengthens the idea that *vTR* contributes to oncogenesis.

Latency-associated transcripts (*LATs*) are antisense to *ICP4* and share a clear association with establishing latency. *ICP4* is an immediate early gene that functions as a transactivating factor which induces expression of other early viral genes essential for initiation of the lytic cycle. Expression of virally-encoded *LAT* creates transcripts that can bind to *ICP4* transcripts, thereby reducing *ICP4* expression. Studies show that null-*LAT* virus reconstituted from BACs is unable to form tumors or suppress the immune system in the RB-1B strain of MDV-1 (Cantello et al. 1997).

One of the more recent connections with oncogenesis is the MDV1-specific miRNA clusters. There are three miRNA clusters located in the repeat regions. Clusters 1 and 2 flank the Meq ORF, while cluster 3 is at the 5' end of the *LAT* ORF. Studies have shown these clusters are crucial to oncogenesis. One study shows that a deletion of all miRNAs abrogates oncogenesis (Zhao et al. 2011). The same study also indicated that deletion of only *mdv1-miR-M4* (a *gga-miR-155* ortholog) also results in lack of tumor formation. Another miRNA (*mdv1-miR-M7-5p*) in the 5' end of *LAT* was shown to target immediate-early genes *ICP4* and *ICP27* (Strassheim et al. 2012), which may contribute to the establishment and/or maintenance of latency.

1.04) Pathogenesis of Marek's Disease

The natural course of MDV-1 infection begins with inhalation of cell-associated virion in skin dander shed from FFE of previously-infected chickens (Jarosinski et al. 2006). The virus infects pulmonary epithelium, and primarily macrophages, which carry the virus to lymphoid tissues, where it infects B-cells. Primary cytolytic replication occurs first in B lymphocytes (2-10dpi), and these new virus particles infect T-cells. After the B-cell lytic phase, MDV enters a latent phase in activated CD4+ T-cells (10-14dpi). MDV in these activated T-cells can undergo reactivation (14-18dpi), transforming lymphocytes into highly proliferative T-cell lymphomas (18-21dpi), depending on the viral strain and host genotype. Some T-cells never experience transformation, and instead undergo a secondary cytolytic phase after reactivation (18-21dpi).

Transformed lymphocytes migrate to the skin (21-42dpi), where FFE cells are infected. This leads to the production of cell-associated infectious virus particles, which are then shed in skin dander.

Oncogenically-transformed T cells can migrate to peripheral nerves, especially the sciatic nerves. Infiltration of sciatic nerves by oncogenically-transformed T cells brings about peripheral nerve inflammation. This leads to paralysis of chickens, a hallmark of MD. See Figure 1-3 for further details on the time-scale of MDV pathogenesis.

Latency is one of the classical biological properties common to herpesviruses. During latency, the viral genome is generally thought to exist as a circular episome in the nucleus of infected cells. Interestingly, some herpesvirus genomes, including EBV, human herpesvirus 6 (HHV6), and MDV-1, have been shown to actually integrate into the telomeres

of host chromosomes (see Morissette and Flamand 2010 for review). The earliest work on MDV genome integration (Delecluse and Hammerschmidt 1993, Delecluse et al. 1993) found that the MDV genome was commonly integrated into host telomeric regions. This integration process was identified in the oncogenic serotype 1 (Robinson et al. 2010) during latency and was suggested to be mediated in part by the virally-encoded telomerase RNA (vTR) subunit only found in the oncogenic serotype (Kaufer et al. 2011).

1.05) Host Immune Response to MDV Infection

Animal immune systems respond to viral infections in an efficient manner. One may think that because MDV is immunosuppressive it completely evades the immune system, but this is not the case. There are many host defenses at play, actively responding to MDV infection.

The innate immune response classically involves an initial recognition of non-self antigen, to which secretion of pro-inflammatory cytokines, chemokines, nitric oxide (NO), and antimicrobial peptides (AMPs) is common. Induction of the toll-like receptor (TLR) signaling pathways is also expected in innate immune responses. There are a number of cell types involved in an immune response, and there is often overlap between a single cell type's involvement in innate/adaptive immune function. However, there are some classical effector cell types involved in the innate/adaptive immune response. Monocytes/macrophages, granulocytes (neutrophils, eosinophils, and basophils), mast cells, and natural killer (NK) cells are all commonly associated with the non-specific innate immune response.

Each of these cell types has a number of functions but it can be said that each cell type has a main defensive strategy. Macrophages are activated/mature monocytes. Both monocytes and macrophages are considered phagocytic cell types which secrete cell-signaling cytokines and NO to destroy infected and tumor cells. After infecting lung epithelial cells, macrophages are considered one of the first cell types infected by MDV (Calnek et al. 1970). In the course of MDV's pathogenesis, as in other viral infiltrations of lymphoid cells, MDV antigens are processed and presented on cell surface of antigen presenting cells (APCs). Macrophages are one such APC class that has been found to carry MDV antigen to primary lymphoid tissues like the bursa of Fabricius early in the infection cycle (Abdul-Careem et al. 2008) where they infect B-lymphocytes as part of the primary cytolytic stage of pathogenesis.

NK cells mediate cell death of virus-infected or tumor cells with perforin (a transmembrane pore-forming complex) and granzyme (a serine protease). In the case of MDV infection, expression of perforin and granzyme A and NK lysine is up-regulated in 4 and 7dpi in infected birds (Sarson et al. 2008). Secretion of α - and β -interferon (IFN- α / β) is also a potent antiviral cytokine, as it activates NK cells, enhances MHC-I mediated antigen presentation, and acts to block translation of various transcripts. This translational block reduces the overall replicative capacity of infected cells.

TLR pathway activation by viral peptides or dsDNA motifs also brings about innate immune responses in a number of cell types. Binding of lipopolysaccharide (LPS) and oligodeoxynucleotides (which are common antigens produced by and contained within the MDV virion) were found to activate chicken TLR (*chTLR*) pathways commonly associated

with the human TLR orthologues *TLR-4* and *TLR-9* (Schwarz et al. 2007). This TLR activation induces production of type I IFNs (*IFN- α* and *IFN- β*) and interleukin-6 (*IL-6*) in chicken splenocytes.

The antigen-specific, adaptive immune responses are divided into cell-mediated (T-lymphocytes) and humoral (B-lymphocytes) response mechanisms. Both cell-mediated and humoral responses depend on antigen presenting cells (APCs) such as monocytes/macrophages and dendritic cells (DCs) to engulf, process, and present exogenous antigen in order for T-cell receptors (TCRs) and B-cell receptors (BCRs) to bind and activate those B-/T-cells to perform effector functions. Because MDV is a highly cell-associated virus, the humoral responses of neutralization and antibody-dependent cell cytotoxicity (ADCC) are considered less-effective against the viral infection than are the cell-mediated defense mechanisms (Parvizi et al. 2010).

Among T-cells, there are several subsets including CD4⁺ helper T cells (T_H), regulatory T cells (T_{reg}), and CD8⁺ cytotoxic T lymphocytes (CTLs). A single naïve T cell has the potential to differentiate into any of these subsets after activation by an antigen-carrying APC. There have been many studies looking at acquired immune responses to herpesvirus infections (Mester and Rouse 1991, Uni et al. 1994, Schat and Xing 2000, Markowski-Grimsrud and Schat 2002). Activated CD8⁺ CTL-mediated responses are documented particularly well.

It has been known for quite some time that activated lymphocytes have been shown to limit the replicative potential of MDV in cell culture (Ross 1977). More recently, effort has been devoted to elucidating the specific role of CTLs in MDV infection. Although CTLs are

antigen-specific immune responders, their cytotoxic effector mechanisms are similar to those of NK cells in that CTLs also secrete perforin, granzyme and Fas ligand in order to kill infected cells. Cell death via these effector cytotoxins is messy, releasing all manner of cellular debris. This principle of CTL-mediated cell lysis can and has been utilized in chromium- (^{51}Cr) release assays to look at the effectiveness of CTLs in MDV-1, -2, and -3 infected effector cells (Uni et al. 1994). In these ^{51}Cr -release assays, transformed target cells are labeled with ^{51}Cr and transfected with various MDV1-specific coding sequences (*pp38*, *gB*, *meq*, and *ICP4*). When target cells are destroyed by cytotoxic effector cells, ^{51}Cr is released and measured. Substantial CTL response was observed in MDV1-infected target cells and interestingly, *meq* was shown to have the weakest CTL-mediated lysis response in all three infection groups. *Meq* is a transactivating protein with homology to endogenous *Jun/Fos* proteins and it is most-well-characterized in its capacity to transform T-cells. This lesser response to *Meq* protein is one explanation for how MDV-1 evades the adaptive immune response.

It is clearly established that host immune responses have evolved alongside viral infection. When a virus experiences a mutation, insertion, deletion or recombination event that increases its virulence, the host must have a mechanism to compensate for the new viral trait. And it is important to remember that these compensatory host mechanisms are not always purely immunological. Oftentimes there are genetic or epigenetic mechanisms at work, limiting the viral infection cycle in some way.

1.06) Vaccines for Marek's Disease

One of the more lucrative topics in the field of MD research is vaccine development. It is important to note that current vaccines do not prevent infection by virulent strains of MDV-1, instead they work by preventing oncogenesis. There are three main vaccination strategies for MD: attenuated MDV-1, non-pathogenic MDV-2, or non-pathogenic HVT. Table 1-3 shows a list of common MD vaccines. The first effective vaccine against MD was developed in 1969 via attenuation of MDV-1 strain HPRS-16 (Churchill et al. 1969a). This marked the first neoplastic model controlled by vaccination. After attenuated HPRS-16 came the non-pathogenic HVT, and then today's golden standard: another attenuated MDV-1 strain called Rispens/CVI988 (Rispens et al. 1972).

Automated *in ovo* vaccination at E17-E19 is currently the most popular method of vaccination, as it is a vastly more time-efficient process compared to manual vaccine injections 1-2 days post-hatch. Commercially, the most common vaccination strategy consists of using a combination of the two or three of the most popular vaccine types as either a bivalent or trivalent MD vaccine (Witter et al. 1985). However, MDV has been increasing in virulence since the 1970s (Table 1-4), and as a result there is a greater need for novel, more effective vaccines.

1.07) RNA Interference

RNA interference (RNAi) utilizes small regulatory RNAs, which bind to complementary sites in target RNAs thereby reducing translation or replication of that target RNA. Structural and functional research has identified several classes of small RNAs (Figure 1-4). There are currently two main regulatory mechanisms that employ RNAi: short interfering RNA (siRNA) and microRNA (miRNA). Both siRNA and miRNA involve a dicer ribonuclease III to sequentially cleave precursor molecules into mature RNA-silencing complexes. Mature siRNA and miRNA molecules form a RNA-induced silencing complex (RISC) with a member of the Argonaut protein family. SiRNA is often associated with viral infection or transposon repression. MiRNA functions as a post-transcriptional gene silencing mechanism. Figure 1-5 shows the most common mechanism for miRNA biogenesis and gene silencing.

The other two RNAi mechanisms involve dicer-independent RNA biogenesis, and rely on PIWI rather than the AGO Argonaute RNA-binding/nuclease proteins. Piwi-interacting RNAs (piRNAs) and repeat-associated short interfering RNAs (rasiRNAs) are relatively new classes of RNA, and their characterization is limited due to their recent discovery. A first class of so-called pre-pachytene-like piRNAs (pachytene referring to the meiotic-1 pachytene stage of spermatogenesis) have been found to be associated with transposon (mobile genetic elements) silencing (Grimson et al. 2008). A second class of piRNAs (pachytene piRNAs) is even less-studied, but may be associated with silencing mRNAs by interacting with an mRNA cap-binding complex (Grivna et al. 2006). RasiRNA

is another very recently-discovered RNA class thought to silence germline transposable elements (Vagin et al. 2006).

1.08) MicroRNA

MicroRNAs are 18-23 nucleotide small regulatory RNA that execute post-transcriptional gene silencing. The biogenesis of microRNA (miRNA) is shown below and is generally thought of as starting out as part of a larger, primary RNA transcript called pri-miRNA. The pri-miRNA is then cleaved post-transcriptionally into the short 18-23 nt mature miRNA. The 1-7 or 2-8 nucleotides on the 5' end of the miRNA (called the seed sequence) have sequence complementarity to the 3' untranslated region (UTR) on target mRNA. The miRNA associates with RISC, which promotes formation of a miRNA-mRNA duplex (dsRNA) between 5' seed sequence of mature miRNA and 3'UTR of target mRNA. Seed sequence and other context determinants (Crimson et al. 2007) govern duplex formation, and are the major parameters in predictive target site algorithms. The mode of context-dependent post-transcriptional gene silencing is dependent on miRNA-mRNA base complementarity (Doench and Sharp 2004). Full sequence-3'UTR base complementarity triggers degradation via dsRNA-specific RNase. Partial base complementarity can result in translational repression.

MiRNA have been found in many organisms, from viruses and single-celled green algae (Molnar et al. 2007) to multi-cellular organisms such as the nematode *C. elegans* (Lee and Ambros 2001), birds and mammals. To date, 26 mature miRNAs have been identified in the MDV viral genome, 368 in *C. elegans*, 791 in chickens, and 2042 in *Homo sapiens*,

according to mirBase (an international online repository for miRNA sequence information) (www.mirbase.org). The initial discovery and first functional roles of miRNA were elucidated in *C. elegans* in 1993 (Lee et al. 1993). Since their discovery by the Ambros group, miRNAs have been studied extensively due to their involvement in the regulation of gene expression. MiRNA-mediated gene regulation is involved in a range of biological processes, including development, metabolism, and homeostasis. Genes that play a role in immune function are frequent targets for miRNAs. Among the miRNAs involved in regulating immune gene expression are *miR-221*, *miR-222* (Zhang et al. 2010), and *miR-155* (Thai et al. 2007). *MiR-221* and *miR-222* target a number of mRNAs involved in immunological pathways, including *STAT1* and *STAT2* (both are core proteins in the IFN- α signaling pathway). Increased expression of the *miR-221/222* cluster is associated with the development of glioblastoma. *MiR-155* null lines of mice exhibit dysfunctional immune responses, as *miR-155* targets genes crucial to the normal differentiation of helper T-cells.

Another oncomir involved in tumor formation is *miR-21* (see Selcuklu et al. 2009 for review). *MiR-21* is strongly evolutionally conserved in vertebrate species like mammals, fish, and birds, and was among the first identified in humans (Mourelatos et al. 2002). Overexpression of *miR-21* has been linked with glioblastomas (Gabriely et al. 2008), gastric cancers (Zhang et al. 2008), prostate cancer (Reis et al. 2012), and a variety of other cancers in large part due to targeting expression of the major matrix metalloproteinase regulator, *RECK*. These studies demonstrate that miR-mediated silencing of *RECK* expression leads to reduced apoptosis, and increased potential for tumor cell invasion. *MiR-21* knock-down in MCF-7 and MDA-MB-231 human breast cancer cells resulted in reduced cell proliferation *in*

vitro and reduced tumor growth *in vivo* (Yan et al. 2011). A recent publication also found that *miR-21* is overexpressed in chickens with Marek's disease virus infection (Stik et al. 2012). Given the involvement of *miR-21* in various tumor types, it follows that host *miR-21* over-expression transactivated by MDV-encoded latent phase protein *Meq* contributes to Marek's disease's characteristic lymphomagenesis. It is also logical to assume that other differentially-expressed host miRNAs may also contribute to tumorigenesis, immunosuppression, and transient paralysis in the case of MD.

There have also been a number of studies that have found differential host miRNA expression in response to viral infection. For example, the expression of *miR-129**, *miR-129-3p*, and *miR-130a* is decreased in the brain tissue of patients with HIV-induced encephalitis (HIVE) (Noorbakhsh et al. 2010). These miRNAs were shown to target caspase 6, a protein crucial to apoptotic pathways. It was further demonstrated that increased expression of caspase 6 transcripts in astrocytes is associated with neuro-degeneration in transgenic mice astrocytes modeling HIVE. Another study showed differential expression of 48 miRNAs in human hepatoma cells infected with the oncogenic hepatitis C virus (HCV) (Steuerwald et al. 2010). These kinds of studies have shown miRNAs to play a key role in regulating gene expression in response to viral infection.

1.09) MiRNAs Associated with Marek's Disease

Infection with MDV is similar to other viral infections in that cellular miRNAs with immunologically-relevant and other target genes are differentially expressed upon infection. The idea that miRNAs are differentially expressed in response to pathogenic infection is

well-established. Viruses have arguably been around as long as any other organism (see Koonin and Martin 2005), and the pressure of constant host-virus interactions have led to evolution of both host and viral genomes. As a species evolves a mechanism to fight a virus, viruses tend to find a way to subvert that adaptation, in part due to very short replicative cycles. As a result, infection-induced gene expression patterns are likely to favor pathways that aid both host defense/adaptability and viral evasion. Virus-induced differential expression of miRNAs is found in the case of MDV infections. Differential miRNA expression has also been characterized as a common response many viral infections. Research on herpesvirus-induced differential miRNA expression is gaining momentum, although only a few herpesviruses have been studied in this manner.

Epstein-Barr virus (EBV) is a lymphomagenic gammaherpesvirus that has been found to induce differential miRNA expression in human hosts. One study found reduced expression of the miR-200 family of miRNAs in latently EBV-infected B-cells (Lin et al. 2010). The miR-200 family of miRNAs has been shown to suppress many genes, including *ZEB1* and *ZEB2* (putative tumor suppressors), both of which are involved in suppressing the reactivation of EBV and subsequent transformation.

MiRNA expression profiles have clearly been shown to contribute to viral pathogenicity. However, there are oftentimes very closely related viruses where one strain/serotype is highly pathogenic while another strain is nonpathogenic. Finding exactly which miRNAs contribute to pathogenicity can be difficult to determine. Thanks to modern genetic analysis techniques, genetic comparisons can be made between related viruses.

For example, each year a new trivalent inactivated virus vaccine (“flu shot”) and live-attenuated virus vaccine (“nasal spray”) is formulated, manufactured, and distributed due to antigenic drift of circulating influenza subtypes. One of the first antigens on the influenza virus particle encountered by host immune system is the spike proteins, hemagglutinin (HA) and neuraminidase (NA). Influenza A and influenza B viruses are known for accumulating mutations in HA and NA leading to amino acid changes, which lead to weakened specific antibody-antigen interaction. Mutants that have undergone this antigenic drift have a selective advantage under host immune system pressure and can out-compete parental strains. This antigenic drift leads to weakened antibody-antigen interaction, which results in lesser antigen recognition signaling cascades and weakened cellular gene expression of genes involved in immune pathways. This kind of cellular gene expression pattern has been shown in a comparison of expression profiling between reconstructed highly virulent H1N1 1918 influenza virus (r1918) infection compared to mice infected with a less-virulent H1N1 strain isolated in 1991 (Tx91) (Kash et al. 2006). Another study involving r1918 infection in macaques also implicated dysregulation of antiviral response in r1918’s increased virulence (Kobasa et al. 2007). Further analysis found that differential miRNA expression strongly contributed to the greater virulence of r1918 (Li et al. 2010). It is also common for researchers to compare gene expression profiles between infected versus mock-infected controls. Differential gene expression derived from this experimental setup would identify all genes involved in host response, instead of highlighting only the host gene expression patterns associated with pathogenicity. Profiling gene (and miRNA) expression in a more

virulent vs. a less virulent strain normalizes the general host response and selectively identifies differentially expressed genes attributed to pathogenicity.

As covered earlier in this chapter, there are three serotypes of MDV. MDV-1 is the oncogenic serotype while MDV-2 and HVT (MDV-3) are non-oncogenic. Like the work done on influenza and many other viral infections, cellular gene expression patterns associated with infection with the pathogenic relative to non-pathogenic (or mock-infected) strains have been well-characterized (Ramaroson et al. 2008, Lu et al. 2010, Chen et al. 2011). But just as in the case of influenza, the exact mechanism(s) regulating gene expression are poorly-characterized.

To address this lack of knowledge, some of the most recent studies look at miRNA-induced differential gene expression associated with MDV infection *in vitro* and *in vivo*. As mentioned before, the *miR-221* and *miR-222* cluster have been associated with regulating immune-response genes, and dysregulated immune genes expression is associated with cancer (Zhang et al. 2010). One study using the MSB-1 MDV-transformed lymphoblastoid cell line found significant upregulation of *miR-221* and *miR-222* (Lambeth et al. 2009). The Lambeth group also found binding sites in the 3'UTR of *p27(Kip1)* mRNA. P27(Kip1) is a protein involved in regulation of the cell cycle, and aberrant p27(Kip1) expression contributes to progression of MDV-induced T-cell lymphoma. Another study looked at the global miRNA expression profile in MSB-1 MDV-transformed cell line versus retrovirus-transformed AVOL-1 cell line and found *miR-155* to be downregulated specifically in the MDV-transformed cells, whereas *miR-150* and *miR-223* are downregulated in both

transformed lines (Yao et al. 2009). This work suggests that differential expression of *miR-155* has a role in MD-specific expression pathways.

MDV infection is similar to HCV, influenza virus, and other viral infections in that the host responds with differential expression of miRNAs (Steuerwald et al. 2010, Meliopoulos et al. 2012). However, MDV is different in that MDV and other herpesvirus genomes encode their own miRNAs. Viral genome evolution selects for genes which support viral replication and transmission, so it is common to see expression of miRNAs that modulate the host immune response. These virally-encoded miRNAs often have sequence homology to host miRNAs, thereby hijacking endogenous miRNA regulatory pathways.

The notion that a virus has miRNA homologous to those miRNA endogenous to the host has profound implications. It's been known that MDV-1 suppresses the immune system following the latent period after T cell infection, but it is somewhat unclear as to what regulatory processes are suppressing host immune response.

MiRNA serve as a perfectly viable means of suppressing host immune response, especially if MDV-1 miRNAs are found to target host mRNAs that code for immunologically-relevant genes. It may also be interesting to see if the paralysis characteristic of end-stage MD may be due to MDV-1 miRNA that target mRNAs involved in neuromuscular processes.

Early observations by Burnside and Morgan identified several potential oncomirs (miRNAs involved in oncogenesis) specific to MDV-1 and infectious laryngotracheitis virus (ILTV) (Burnside and Morgan 2011). In particular, there was a virulence-associated polymorphism in the putative promoter of the MDV-1 miRNAs upstream of the meq

oncogene with increased expression in tumor tissue relative to control. Furthermore, MDV-1 and HVT encode homologs of *gga-miR-121*, a host miRNA that has been shown to target mRNAs involved in cell cycle regulation. MDV-1 also encodes *mdv1-miR-M4*, which has the same seed sequence as *gga-miR-155*, which targets mRNAs involved in immune function.

The state of research on differential miRNA expression and target pathway analysis in MDV-infected chickens is still in its infancy and requires further study. A better understanding of the regulatory mechanisms involved in virus-induced oncogenesis will contribute directly to vaccine development and trait selection in breeding. More research in this field will also translate over to our understanding of virus-induced cancers in humans.

1.10) Approaches to MiRNA Profiling

1.10.1) Historical Perspective

Since the initial observation of miRNAs in 1993, a number of methods have been developed to create a quantitative “profile” of miRNAs present in biological samples. These samples can be single cell types, specific tissue, or even extracellular fluid like blood plasma (Mitchell et al. 2008), urine (Wang et al. 2010), or saliva (Park et al. 2009).

The first reliable profiling experiments were initially performed on custom-built microarray platforms containing small miRNA libraries (Liu et al. 2004). This process was quickly improved upon and commercialized, increasing the number of miRNA to a “genome-wide” analysis, and increasing the reproducibility of results. Then a bead-based flow cytometric miRNA expression profiling method was developed (Lu et al. 2005) and quickly

surpassed in popularity by the superior high throughput deep sequencing platforms. Before these methods were developed, researchers used the less-quantitative and more-qualitative northern blotting technique to profile miRNA expression. Northern blotting and/or qRT-PCR are often used as validating methods to check the results in a microarray or sequencing experiment. The work in this thesis utilized the Illumina sequencing platform for miRNA profiling.

1.10.2) Sequencing

Since the discovery of the double-helical structure of DNA, molecular biologists have made efforts to find the sequence of nucleotides that give DNA its inherited expression patterns. In the 1960's, various sequencing methods were used to find the genome sequences of RNA viruses. Sequencing by these first few methods was laborious, slow, and involved hazardous reagents. It wasn't until the mid 1970's when sequencing technology evolved into a standard molecular technique. Here, the development of Sanger and Maxam-Gilbert sequencing became the mainstream sequencing methodologies.

The Sanger method utilized chain terminating fluorescent dideoxynucleotide chemistry to elucidate nucleotide sequence. The Maxam-Gilbert method was initially more popular, utilizing specific base cleavage and incorporation of gamma-³²P ATP. However, the Sanger methodology soon saw improvements that made it more reproducible in a practical and manufacturable manner. Development of the PCR technique in early 1980s helped streamline sample preparation, and automation of the Sanger method made it the go-to sequencing technology for genomic research in the early 90's.

As with all technologies, there became a need for a cheaper, higher-throughput and speedier sequencing tech. Parallel sequencing was the market's response to this need. In the early 90's, as Sanger was at the peak of its popularity, massively parallel signature sequencing (MPSS) came on the scene. MPSS, a technique similar to SAGE analysis, allowed a much greater throughput by parallelizing the data collection process, running many more samples in the same run. This set the stage for the so-called “second generation” sequencing technologies, which all focus on high throughput through parallel sample runs.

In 2005, Polony (soon to become SOLiD) sequencing became the first high-throughput sequencing (HTS) methodology to meet genetics researchers demands, sequencing the *E. coli* genome. SOLiD utilized ligation-based chemistry, whereby base calls were made based on a ligation event (or lack thereof). 454 Life Sciences (later Roche 454) developed sequencing by synthesis chemistry based on the principle of pyrosequencing. As a nucleotide is added by DNA polymerase complementary to a template DNA strand base, a pyrophosphate is released, which activates luciferase, thereby releasing light of a specific, recordable wavelength. Solexa (later Illumina) responded with their own sequencing by synthesis technique. The Illumina method capitalized on a cluster formation by bridge amplification chemistry, whereby chain-terminating fluorodyes were incorporated into a growing strand of DNA by DNA polymerase.

In the present project, miRNA profiling will require a preparation of small RNA libraries and subsequent sequencing on an Illumina (Solexa) platform. There are specific advantages for this miRNA profiling project in utilizing Illumina over competing sequencing platforms. Illumina has shorter reads and relatively greater base call errors inherent to the

technology, but has a significantly higher throughput. The higher throughput essentially negates the base call error, and generates a greater number of high-quality reads.

Annotation of sequenced RNAs can be split up into different categories. Such categories will include, and are not limited to: non-coding RNA (ncRNA), repeat-associated RNA, miRNA, mRNA associated, and unclassified. The miRNAs can be split up into known and novel varieties. Sequence alignment comparison and bioinformatics software will do the brunt of the data analysis on a program called miRtools.

1.10.3) In Silico Target Prediction

In early miRNA expression profiling experiments, researchers contributed substantial resources to finding differential expression in one tissue sample relative to a healthy control. This was enough to associate a particular miRNA(s) expression pattern with a disorder. Although investigators could go further to assess target mRNAs in *in vitro* experiments, these procedures tend to be very time consuming and expensive. Modern bioinformatics and structural biochemistry research have paved the way for an impressive array of *in silico* or computational molecular modeling programs. These *in silico* predictive programs are cheap and quick and many are open-sourced. They operate on a number of empirically-derived biochemical rules and some of the more popular algorithms predict probable inter-/intra-molecular protein-protein, protein-DNA, protein-RNA, and RNA-RNA interactions.

In miRNA association studies, it is now common to use these *in silico* approaches to predict which mRNAs are targeted by a particular miRNA. Some of the more popular miRNA target prediction algorithms along with other miRNA-related *in silico* prediction algorithms are reviewed by Watanabe and coworkers (Watanabe et al. 2007). MiRanda was

initially developed to predict miRNA targets in *drosophila* (Enright et al. 2003), but has since been adapted for prediction in humans and other vertebrate species (John et al. 2004).

TargetScan is another such algorithm originally written to predict target mRNA of mammalian miRNA (Lewis et al. 2003), and has since been expanded to predict regulatory targets of *C. elegans* (Jan et al. 2011), zebrafish (Ulitsky et al. 2012), and *drosophila* (Ruby et al. 2007a, Ruby et al. 2007b) miRNAs. There are a number of other target predictor algorithms designed for individual or multiple species, including miRU for identification of miRNA-mRNA hybridization sites in various plant species (Zhang 2005). It should be noted that miRNA target prediction in plants is slightly different, requiring near-perfect or perfect miRNA complementarity among other things for transcriptional/translational repression to occur, while animal miRNA mechanisms involve complementarity of a short “seed sequence.” Because the work in this thesis involved miRNA expression profiling in an animal species, any further discussion will focus on the animal target prediction rules.

As stated above, all of these programs work on a set of empirically-derived biochemical rules. Newer research has identified various accessory context determinants, which may or may not be included in predictive algorithms (Crimson et al. 2007), but the most basic and commonly-accepted rule governing miRNA-mRNA hybridization is the complementarity between the miRNA seed sequence (the 1-8 or 2-7 nucleotides at the 5' end of the mature miRNA) and the mRNA target site (6-8 nucleotides in the 3'UTR of the target mRNA). In one of the simplest target prediction pipelines, a researcher could select alignment score threshold and a minimum free energy (ΔG), and set a threshold for either perfect or 1-mismatch seed sequence-target site complementarity.

It is important to note that these predictive algorithms are just that: predictive. *In silico* predictions can be very powerful tools to help investigators narrow down the list of potential miRNA targets, but there is always the potential for false-positive predictions. Predicted targets must be validated experimentally. Target validations are typically involve *in vitro* analysis of the signal-to-noise ratio of a given miRNA hybridizing with predicted target relative to a negative control. This has been done via reporter-gene assays (Kirjakidou et al. 2004, Trakooljul et al. 2010) relative to scrambled 3'UTR controls or the process can be scaled up for a genome-wide high-throughput analysis via microarray (Wang and Wang 2006).

1.10.4) Pathway Analysis

The final step in modern miRNA expression profiling experiments is conducting a pathway analysis of the targeted gene groups. A single miRNA can have multiple sites on the same 3'UTR or can target multiple 3'UTRs. This means a single miRNA can regulate a number of genes. These miRNA-regulated genes are involved in functional pathways, and because there are generally multiple target genes, there are multiple pathways affected by differential expression of a single miRNA.

There are pathway analysis programs that perform statistical analyses on a given list of genes to assess the biological impact of that list of genes by finding enriched clusters of similar functional pathways that gene list is involved in. In short, a researcher can input a list of predicted or experimentally-validated targets of a differentially-expressed miRNA(s) and generate a list of functional processes regulated by that miRNA(s). These pathway analysis programs can be open-source like the DAVID database (Huang et al. 2009a, Huang et al.

2009b) or commercially-available like the Ingenuity Pathway Analysis (IPA) software (Ingenuity Systems, Inc.), but they are common in that they integrate multiple gene function/pathway databases like Gene Ontology, KEGG pathways, OMIM, RefSeq, TarBase, and TargetScan to identify biologically-enriched functional pathways associated a gene list.

1.11) Specific Aims of this Project

For the most evolutionarily successful pathogens, a balance must be struck between virulence and host immune response. There must be sufficient time for any given pathogen to infect its host, run through its infection cycle, and pass to the next host/reservoir/carrier. Throughout the infection cycle, pathogens need to replicate in preparation, but as the population of pathogen increases, so does the host immune response.

As will other pathogens, in the case of MDV infection, it is valuable to observe how gene expression is affected throughout the infection cycle, as gene expression brings about proper immune response. The objective of the current project is to catalogue and quantify relative abundances of cellular miRNAs in the spleens of MDV-1 (strain Md5) infected chickens as at 42 days post infection (dpi),and perform target analysis. This study will be a two-part process involving:

- 1) Identification of host miRNA expression differences between MDV-infected and control spleens at 42dpi using Illumina deep sequencing.
- 2) Identification of target genes regulated by miRNA *miR-128* and *miR-147* and pathway analysis of their specific target genes.

1.12) Tables and Figures

Table 1-1. Features characteristic of MDV, ALV, and REV infections. Adapted from table in chapter 2.3.13 of OIE Terrestrial Manual 2010 by World Assembly of Delegates of the OIE.

Feature	Marek's Disease Virus	Avian Lymphoid Leukosis Virus	Reticuloendotheliosis Virus (Bursal lymphoma syndrome)
Age	Usually 6+ weeks	> 16 weeks	> 16 weeks
Signs	Paralysis	Non-specific	Non-specific
Incidence	> 5% in unvaccinated flocks. Rare in vaccinated flocks	Rarely > 5%	Rare
Macroscopic lesions			
	Neural Involvement	Frequent	Absent
	Bursa	Diffuse enlargement or atrophy	Nodular tumors
	Tumors in skin, muscle, and proventriculus, "grey eye"	May be present	Usually absent
Microscopic lesions			
	Neural involvement	Yes	No
	Liver tumors	Often perivascular	Focal or diffuse
	Spleen	Diffuse	Often focal
	Bursa	Interfollicular tumor and/or atrophy of follicles	Intrafollicular tumor
	Central nervous system	Yes	No
	Lymphoid proliferation in skin and feather follicles	Yes	No
Cytology of tumors	Pleomorphic lymphoid cells, including lymphoblasts, small medium and large lymphocytes and reticulum cells. Rarely can be only lymphoblasts	Lymphoblasts	Lymphoblasts
Category of neoplastic lymphoid cell	T cell	B cell	B cell

Table 1-2. Common MDV strains with corresponding pathogenicity.

Serotype	Strain/Isolate	Virulence
MDV-1	HPRS-B14	v
MDV-1	GA/22	v
MDV-1	Md5	vv
MDV-1	RB-1B	vv
MDV-1	645	vv+
MDV-1	648A	vv+
MDV-1	686	vv+
MDV-2	SB-1	apathogenic
MDV-3	FC-126 (HVT)	apathogenic

Table 1-3. Common MD vaccines.

Original Serotype	Vaccine Name	Preparation Method
MDV-1	HPRS-16/att	Attenuated
MDV-1	CVI988/Rispens	Attenuated
MDV-1	rMd5ΔMeq	Recombinant
MDV-1	rMd5ΔMeq-50	Recombinant, Attenuated
MDV-1	RB-1BΔRLORF4	Recombinant
MDV-1	BACdelMEQ	Recombinant
MDV-2	SB-1	Non-oncogenic serotype
MDV-3	HVT	Non-oncogenic serotype

Table 1-4. Oncogenic MDV-1 isolates show a trend of increasing virulence over time.

Isolate	Virulence	Year of Initial Publication
HPRS-B14	v	1967
GA/22	v	1971
Md5	vv	1977
RB-1B	vv	1981
645	vv+	1994
648A	vv+	1994
686	vv+	2004

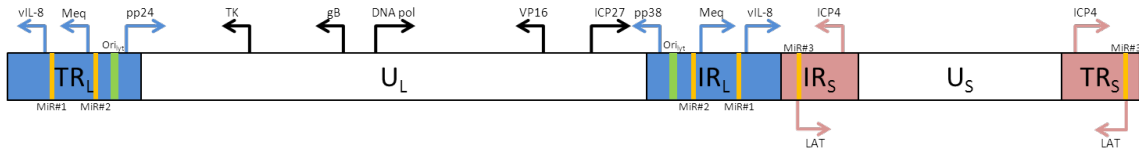


Figure 1-1. MDV-1 (Gallid herpesvirus 2) genome structure with important transcripts. Transcript locations are drawn in their respective repeat or unique region in proper order and are not drawn to scale.

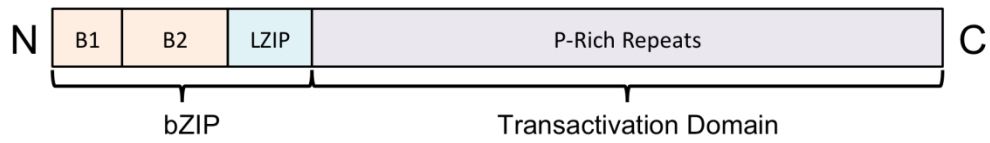


Figure 1-2. Linearized *Meq* protein structure. B1 and B2 are basic regions 1 and 2. LZIP is the leucine zipper motif. The B1+B2+LZIP regions make up a bZIP dimerization domain. The P-rich repeat region is the DNA-binding transactivation domain.

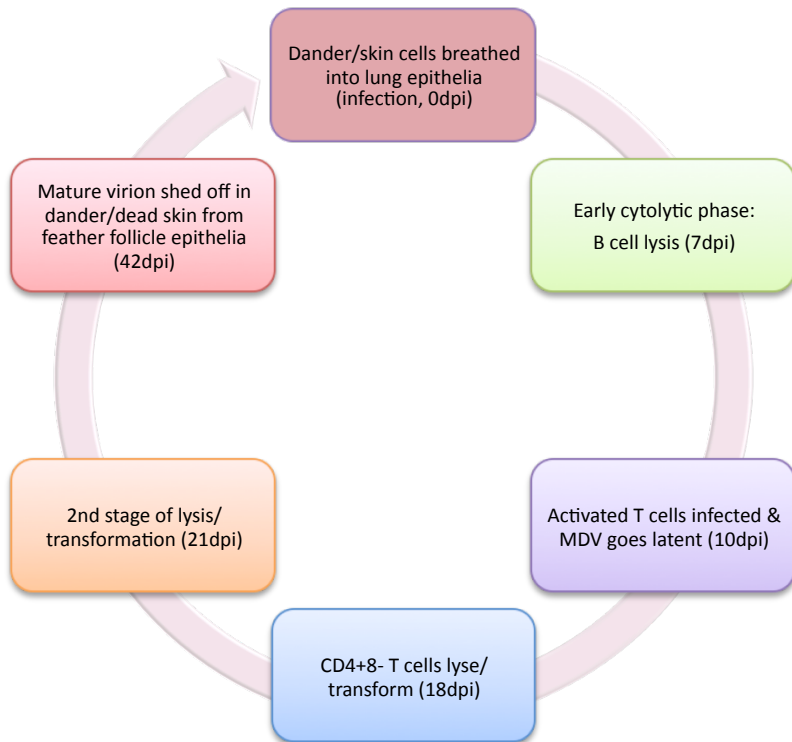


Figure 1-3. MDV pathogenesis.

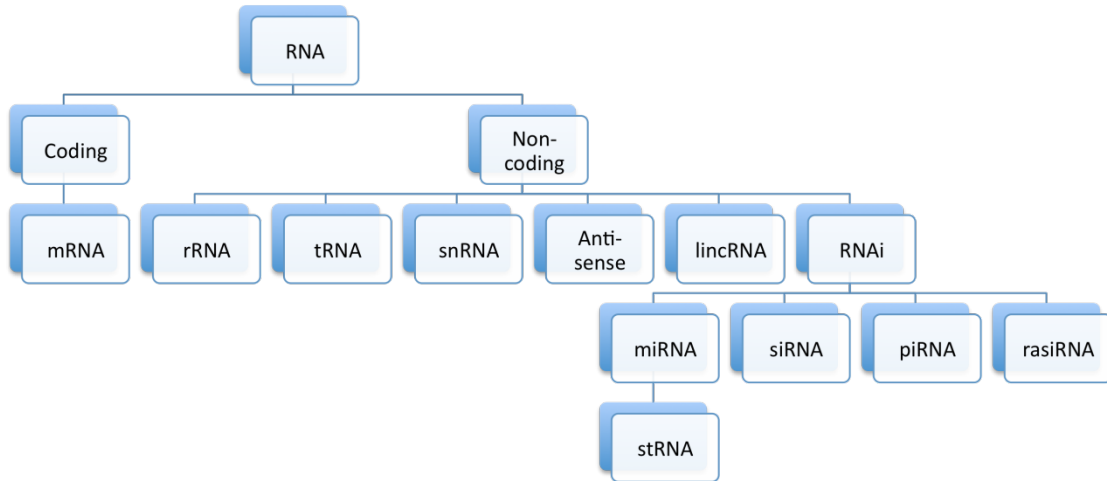


Figure 1-4. A list of naturally-occurring coding and non-coding RNAs. snRNA: small nuclear RNA; lincRNA: long noncoding RNA; RNAi: RNA interference; stRNA: short temporal RNA; miRNA: microRNA; siRNA: short interfering RNA; piRNA: piwi-interacting RNA; rasiRNA: repeat associated small interfering RNA

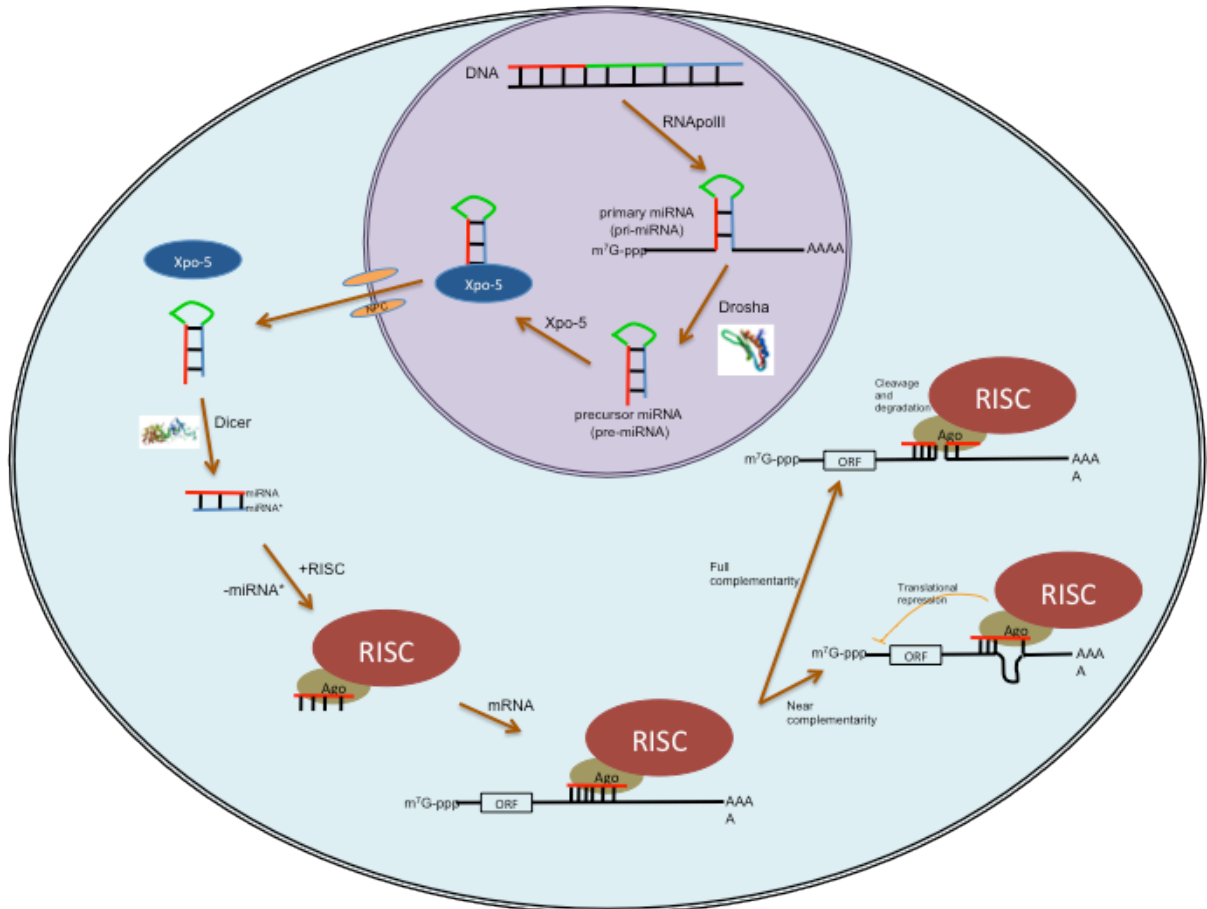


Figure 1-5. Biogenesis of miRNA and gene silencing mechanism.

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CHAPTER 2: PROFILING DIFFERENTIALLY-EXPRESSED microRNAs IN MDV INDUCED TUMORS USING A DEEP SEQUENCING APPROACH

2.1) Introduction

Transcriptomes and proteomes have been sequenced in an effort to fully-characterize the incidence of differential gene expression. However, transcriptome and proteome profiling experiments do not necessarily provide a full picture of differential gene expression. These experiments are qualitative insofar as they indicate the state of differential gene expression, but they fail to identify the underlying regulatory mechanisms associated with differential gene expression. MiRNA profiling research addresses this problem.

It is well-known that miRNAs serve to regulate gene expression via post-transcriptional gene silencing. Mature miRNA bind to target mRNA sequences. The miRNA associates with a RNA-inducing silencing complex (RISC complex), and based on seed-region base complementarity, RISC will either cleave the mature miRNA:mRNA complex, or block the ribosomal complex from translating the mRNA. In either case, protein translation is reduced in a very specific way. As a result, when the expression of a particular miRNA decreases, the expression of its target mRNA(s) increases and vice versa.

Genes coding for miRNAs have been characterized in red and green algae, animals, slime molds, plants, and some viruses (www.mirbase.org). MiRNA biogenesis has been characterized in each of these taxonomical groups (except viruses, which can only produce miRNAs while infecting their host cell). Furthermore, it has been shown that miRNAs are differentially expressed during organism development, homeostasis and throughout different disease states (Ambros 2004; Bartel 2004; Schneider 2012; Abdellatif 2012).

It has been demonstrated that chickens undergo differential gene expression in response to MDV infection. It is expected that a portion of those genes modulated under infection are regulated by miRNAs. As a result, we can expect not just differential gene expression, but differential gene expression due, at least in part, to differential expression of miRNAs.

Studies have shown associations between differential miRNA expression and the immune response. Lipopolysaccharide (LPS), a molecule commonly found in the outer membrane of gram-negative bacteria has been found to modulate both *miR-146* and *miR-155* expression (Schulte et al. 2012). LPS is a common activator of toll-like receptor (TLR) innate immune response pathways, and the initial immune response to bacterial infection such as inflammation and cytokine secretion. Both *miR-146* and *miR-155* were shown to target transcripts associated with inflammatory pathways, thereby playing a part in limiting the innate inflammatory response.

Differential cellular miRNA expression is also characterized in the case of viral infections. MiRNA expression in viral infection varies greatly depending on which virus is involved. HIV, HCV, and human papillomavirus (HPV), and other viruses have been found to elicit differential expression of miRNAs (Noorbakhsh et al. 2010, Steuerwald et al. 2010, Dreher et al. 2011). In fact, differential miRNA expression has been found to be associated with both host defenses and viral evasion. This highlights the co-evolution of host and virus, as host evolves a new defense to fight a virus, a virus evolves a compensatory mechanism to survive the host's new defense. A recent study found reduced expression of multiple members of the miR-200 family in the latent phase of Epstein-Barr virus (EBV) infection

(Lin et al. 2010). It was found that the miR-200 family of miRNAs suppressed putative tumor suppressor genes ZEB1 and ZEB2. Because of their targets, the reduced miRNA expression acts as a host response, blocking EBV reactivation and tumor formation (Lin et al. 2010). EBV is an oncogenic herpesvirus like MDV, so observations made regarding regulatory expression mechanisms in EBV may be translational to MDV and vice versa.

There is a range of cancer models in the toolbox of cancer research, including oncogenic viruses. There are a number of naturally-oncogenic viruses in various families including hepatitis C virus (HCV), HPV, and multiple herpesviruses, including MDV (Ramaroson et al. 2008, Lu et al. 2010, Chen et al. 2011), EBV, and Kaposi's sarcoma-associated herpesvirus (KSHV, HHV-8). Despite extensive research into the pathogenesis and host immunological responses involved in the aforementioned infections, the involvement of miRNA-mediated regulation of gene expression remains poorly understood in both oncogenic virus infections and in cancers with other causative agents.

Recent studies have made an effort to characterize the involvement of miRNA expression associated with MDV infections (Burnside et al. 2008, Tian et al. 2012). A study by Tian and colleagues looked at miRNA expression profile associated with host resistance and susceptibility to MD (Tian et al. 2012), by identifying differentially expressed miRNA in MD-susceptible chickens (7₂) relative to MD-resistant (6₃) chickens. They found reduced expression of *gga-miR-15b* in susceptible chickens and increased expression of its putative target mRNA for *ATF-2*, a DNA-binding peptide known to regulate apoptosis and cell proliferation pathways.

Early work by the Burnside group looked at differential expression of miRNAs in MDV-infected chicken embryonic fibroblasts (CEFs) relative to uninfected control CEFs (Burnside et al. 2008). This *in vitro* experiment found that MDV did not significantly perturb miRNA expression relative to uninfected controls. The investigators attributed the minor miRNA expression change to the fact that CEFs are not an *in vivo* target cell, as MDV is a lymphotropic virus. The group stated that miRNA-mediated regulation may be more pronounced in CD4+ T-cell subsets. Despite the limited extent of differential miRNA expression observed, they did find that the *gga-miR-221/-222* cluster and putative oncomir *miR-21* were upregulated, and *let-7* was downregulated. As mentioned before, the *miR-221* and *miR-222* cluster has been associated with targeting immune-response genes in the STAT signaling pathway, potentially contributing to cancer development (Zhang et al. 2010). Another study found significant *miR-221/-222* cluster up-regulation in an MDV-transformed T-cell line (MSB-1) and subsequent reduced expression of the putative target mRNA p27(Kip1) (Lambeth et al. 2009).

There are other observations of up-regulated *miR-21* expression in MDV-infected tumor tissue (Stik et al. 2012). Furthermore, increased *miR-21* expression is not only associated with MDV-induced cancers. *MiR-21* is considered a classical oncomir, as it has been shown in a variety of studies to be associated with tumorigenesis. Overexpression of *miR-21* has been linked with glioblastomas (Gabriely et al. 2008), gastric cancers (Zhang et al. 2008), prostate cancer (Reis et al. 2012), and a variety of other cancers in large part due to targeting expression of the major matrix metalloproteinase regulator, *RECK*. These studies

found that miR-mediated silencing of RECK expression is associated with reduced apoptosis, and increased potential for tumor cell invasion.

2.2) Specific Aims

Recent advances in cellular and molecular biology technology/methodology allow researchers to look past the symptoms of a disease, to the root cause of diseases. Despite these advances, there is a void of scientific knowledge in the study of Marek's disease and conditions resulting in cancer as to what role cellular miRNA-mediated gene silencing plays in tumorigenesis. In order to more completely understand host-virus interactions, it's becoming increasingly important to investigate differential expression of miRNA in various disease states. A limited number of studies have made a case for miRNA involvement in the development of cancer.

The present study aims to identify those cellular miRNAs that are differentially expressed in MDV-infected chickens by using a second generation deep sequencing platform. This study will go further by computationally predict the mRNA targets of those differentially expressed miRNA. Server-based and local programs will be used to predict target genes and look at the pathway maps of the affected genes.

2.3) Objectives

- 1) Identify host miRNAs expression differences between MDV-infected and control spleens at 42dpi using Illumina deep sequencing.
- 2) Computationally predict mRNA targets of differentially-expressed miRNA with using miRanda and perform a brief pathway analysis of targets with DAVID to find biologically-enriched functional terms affected by differentially-expressed miRNAs.

2.4) Materials and Methods

2.4.1) Tissue Collection.

Three white leghorn chickens (SunRise Farms) were infected (1,000 pfus) with MDV-1 strain Md5 (obtained from USDA-ARS ADOL in East Lansing, MI) at five days of age and three chickens served as a non-infected control group. Spleens were collected at 42dpi, snap-frozen and stored at -80 °C until further analysis.

2.4.2) RNA Isolation.

Tissues were lysed with QIAzol Lysis Reagent (Qiagen) by a process adapted from the manufacturer's protocol. About 200 mg of tissue were resuspended in 2 ml of QIAzol reagent and homogenized. The 2 ml of QIAzol + tissue solution was split into two 1 ml aliquots. A total of 0.2 ml chloroform was added for every 1 ml QIAzol reagent. The samples were vortexed for 15 s, and left on the benchtop for 10 min. The samples were centrifuged at 12,000 x g for 15 min at 4 °C. After centrifugation, the aqueous layer was transferred to a fresh tube and 0.8 ml of 100% isopropanol was added to the aqueous RNA-containing solution. Total RNA was precipitated in a -20 °C freezer overnight.

The RNA was spun down at 13,000 x g for 30 min at 4 °C. Supernatant was removed and the RNA was washed with 1 ml of 75% ethanol. The RNA pellet was spun down at

13,000 x g for 30 minutes at 4 °C. Supernatant was removed and another 75% ethanol wash was performed, spinning down for 15 min. Supernatant was removed and the pellets were resuspended in 50µl of DEPC-treated water. RNA concentration was checked on a NanoDrop ND-1000 spectrophotometer (Thermo Scientific) and RNA quality was checked on a 1% agarose gel with ethidium bromide.

2.4.3) MicroRNA Isolation and Library Construction.

A mirVana miRNA isolation kit (# AM1560; Ambion) was used to collect the small RNA-enriched fraction (<200 nt) from total RNA. The miRNA library was constructed using the proprietary TruSeq Small RNA Sample Preparation Kit (# RS-200-0012; Illumina), which allowed for a multiplexed sequencing method by pooling six libraries into one flow cell lane.

The libraries were constructed by the following protocol. Isolated small RNA samples first were ligated to 3' and 5' adapter sequences in 200 µl PCR tubes. The adapter ligation reactions were carried out sequentially. 1 µl of RNA 3' adapter was added to 5 µl of RNA (1 µg) + nuclease-free water. This mixture was pipetted up and down to mix. The reaction was incubated at 70 °C for 2 min then placed on ice. The thermocycler was then pre-heated to 28 °C for a later incubation period. A ligation mix was then made in a separate nuclease-free 200 µl tube on ice containing 2 µl 5X HM ligation buffer (HML), 1 µl of RNase inhibitor, and 1 µl T4 RNA ligase 2 (truncated). This volume was pipetted up and down to mix. Four microliters of the ligation mix was added to the reaction tube and pipetted again to mix. This amounted to a total volume of 10 µl. This ligation reaction was incubated at 28 °C for 1 hour. A 1 µl stop solution (STP) was added to the reaction tube while still on the

thermocycler. This new mix was allowed to incubate at 28 °C for 15 min, and then placed on ice.

The 5' adapter ligation reaction had a similar setup. The thermocycler was pre-heated to 70 °C, and 1.1 x N ul of the RNA 5' adapter into a fresh 200 ul tube, with N = number of samples for the day's experiment. The adapter was pre-heated at 70 °C for 2 min then placed on ice. Again, icing the adapter mixture prevents secondary structure formation. The thermocycler was then reset to 28 °C. 1.1 X N ul of 10mM ATP was added to the aliquoted RNA 5' adapter tube. The solution was pipetted up and down to mix. A total of 1.1 X N ul of T4 ligase was added to the 5' adapter tube, and pipetted to mix. Three microliters of the aliquoted RNA 5' adapter tube was added to the product of the Ligase 3' Adapter reactions. This solution was pipetted to mix at a final volume of 14 ul. This reaction tube was incubated at 28 °C for 1 hour and placed on ice.

The samples were then reverse transcribed to create complementary DNA (cDNA) from the adapter-ligated small RNA. First, 0.5 ul 25 mM dNTP stock solution was diluted to 12.5 mM with 0.5 ul Ultra Pure Water, pipetted to mix, and placed on ice. Six microliters of 3'+5' adapter-ligated RNA was added to 1 ul of RNA RT primer (RTP), amounting to 7 ul, in a 200 ul tube. This tube was incubated on a thermocycler at 70 °C for 2 min and placed on ice. Thermocycler was reset to 50 °C for a later incubation period. Two microliters 5X first strand buffer, 0.5 ul 12.5 mM dNTP mix, 1 ul 100 mM DTT, 1 ul RNase inhibitor, and 1 ul SuperScript II Reverse Transcriptase were combined in a 200 ul tube and placed on ice before mixed by pipetting and centrifuged. Next, 5.5 ul of this mix was added to the adapter-

ligated RNA + RTP mix currently on ice. This solution was mixed by pipetting and centrifuged. This 12.5 ul mix was incubated at 50°C for 1 hour and placed on ice.

A PCR master mix was created 1.1 X N vol from 22.5 ul ultra pure water, 10 ul 5X Phusion HF Buffer, 2 ul RNA PCR Primer, 2 ul RNA PCR primer index, 0.5 ul 25 mM dNTP, and 0.5 ul Phusion DNA polymerase amounting to 37.5 ul, which was placed on ice. Properly indexed samples can be pooled later in a single flow cell lane, as to avoid inter-lane variation of sequencing conditions. A total of 37.5 ul of PCR master mix was added to the product from the Reverse Transcription reactions, pipetted to mix, and placed on ice. The tubes were then amplified in the thermal cycler using the PCR program in table 2-1.

Library quality was checked on a High Sensitivity DNA Chip with a 2100 Bioanalyzer. Each library was compared to a pre-prepared sample of human brain total RNA to evaluate the trace of amplicons. The PCR product is gel-purified to limit cluster formation by bridge amplification to only intended cDNA.

2.4.4) Data Analysis

The Illumina GAI sequencing platform generates raw data in the FASTQ format. There were six libraries, each with a unique index sequence, which means six FASTQ files were made. FASTQ is a text-based format designed to pair nucleotide sequence with a Phred quality score at each base call. These Phred scores are indicated in the FASTQ file as one of the 128 ASCII characters. For this project, Q20 was selected, which means 1 in every 100 base calls are incorrect. Quality score selection was checked using the FastQC program.

All six FASTQ files were converted to FASTA format (Blankenberg D, Gordon A, Von Kuster G, Coraor N, Taylor J, Nekrutenko A; Galaxy Team. Manipulation of FASTQ

data with Galaxy. *Bioinformatics*. 2010 Jul 15;26(14):1783-5.) with a local instance of Galaxy (<http://main.g2.bx.psu.edu/>). It was necessary to convert to FASTA for use on other downstream analyses. These FASTQ files were collapsed using a tool based on the FASTX-toolkit by Assaf Gordon. Normal FASTA files included all the reads from the sample flow cell lane, which included every read without meaning. The collapse function sorted through the list of all sequences and grouped like sequences to a unique identifier coupled to a read count.

Each library/collapsed file was then subjected to two web-based small RNA analysis pipelines (DSAP and miRanalyzer) designed to annotate small RNA generated by the Illumina/Solexa platform. This required the collapsed files to be converted with MS Excel to the proper tab-delimited input format. Deep Sequencing Small RNA Analysis Pipeline (DSAP, <http://dsap.cgu.edu.tw/index.htm>) and miRanalyzer version 0.2 (<http://bioinfo2.ugr.es/miRanalyzer/miRanalyzer.php>) were the two analysis pipelines used.

DSAP output assigned the input sequences into one of three groups (known miRNA, Rfam (other types of regulatory RNAs, and other). Known miRNA were the sequences that were referenced through miRBase (<http://mirbase.org/>) and aligned to experimentally-validated *Gallus gallus* miRNA. Rfam were the sequences that were identified by the Rfam database (<http://rfam.sanger.ac.uk/>). The “other” category is unmatched unique sequence clusters (USC). Unmatched means the USC from the input file was not present in the Rfam database. In an effort to identify homologous sequences currently not present in the chicken miRNA the original “other” output file was re-run through DSAP a second time. This second DSAP

run was made without the *Gallus gallus*-specific miRNA condition on the initial analysis parameters.

Statistical analysis was performed using the edgeR package (Robinson et al. 2010), using four analytical methods. Each of the two miRNA annotation files (combined “known miRNA” + homologous miRNA files from DSAP and combined mature_unique.txt and maturestarunobs_unique.txt files from mirAnalyzer) were subjected to analysis by two statistical methods (common dispersion and tagwise dispersion), resulting in four edgeR output files. Differential expression here was indicated by a logarithmic expression of fold change (logFC), a representation of the fold change of expression of miRNA.

After the edgeR analysis yielded significantly differential expression ($p \leq 0.05$ and $FDR < 0.1$) of certain miRNA, target mRNA were computationally predicted using the miRanda (John et al. 2005) application (version 3.3a), which utilized the miRanda miRNA target prediction algorithm (Enright et al, 2003). A local instance of the application can be installed on either MacOS or Windows operating system, and the miRanda analysis script language for analysis is identical. Target mRNAs were identified using the most current *Gallus gallus* mRNA library from the Unigene FTP (<http://www.ncbi.nlm.nih.gov/guide/all/#downloads>). For target prediction, the score threshold was set at 140, minimum free energy (ΔG) was -18kcal/mol. The miRanda generates an output file that has a wide range of targets. An in-house filter perl script was developed in the Liu lab to sort through the miRanda output with even more stringency. This script allowed either a 1-8nt seed match or a 2-7nt and 13-16nt match, with wobble base-pairing (A-U) tolerated.

Only the 3'UTR targets file will be considered for further study, as the seed regions of miRNAs in animals tend to be complementary (and therefore target) the 3'UTR of mRNAs. Enriched gene sets (those mRNAs that had biological functions similar to each other) were grouped in functional annotation categories by Database for Annotation, Visualization and Integrated Discovery (DAVID v6.7, <http://david.abcc.ncifcrf.gov/>). DAVID then gives useful information in the terms of KEGG pathway maps, gene-disease associations, identification of enriched biological themes (including GO terms) and identification of enriched functionally-related gene groups.

2.5) Results

2.5.1) Illumina Sequencing of miRNA-derived cDNA Libraries from MDV-Infected Versus Control Spleen Samples at 42dpi

The deep sequencing experiment with the Illumina (Solexa) sequencing platform yielded a total of 21,721,036 reads from all six cDNA libraries. 13,003,122 (~59%) were from the three control libraries and 8,717,914 (~40%) reads were from the three MDV-infected libraries. The variance here will not be a problem in statistical analysis after read count normalization as part of the edgeR analysis.

Analysis with DSAP small RNA analysis pipeline (table 2-2) indicated that 2,632,988 (~12%) reads matched to entries in Rfam. 9,131,368 (~42%) reads were matched to entries in miRBase. 9,916,247 reads were left unidentified in the “other” category from the first DSAP run. A second run of this “other” category through DSAP indicated 5,737,237

(~26%) reads qualified as homologous miRNAs, and 4,179,010 (~19%) reads were still unmatched/unknown sequences.

The miRanalyzer analysis pipeline (table 2-3) resulted in a similar % miRNA (~54%) composition of the sequenced sample compared to the DSAP % miRNA (~68%).

MiRanalyzer also did not require a second analysis run, as the run parameters were loose enough to include what DSAP would consider “homologous miRNAs” in the initial miRanalyzer results. MiRanalyzer may catch these homologous miRNAs in the first run, but its algorithm fails to align sequence tags through Rfam. This means miRanalyzer, while it quickly identifies miRNAs in the sequencing data, does not identify whether there are any mRNA fragments, tRNA, rRNA, snRNA, mtRNA, or other ncRNAs in the raw sequencing data. It is clear that neither program is perfect, but an analysis of the sequencing data with both programs gives a better composite of each cDNA library.

After running the raw sequencing data through DSAP and miRanalyzer, it is necessary to apply common and tagwise dispersion analyses on the normalized output of both DSAP and miRanalyzer. This was done using the edgeR bioconductor package (Robinson, 2010). The result of the edgeR analysis is seen in table 2-4. EdgeR identified 33 miRNAs with significant ($p \leq 0.05$) differential expression between healthy and MDV-infected birds.

The p values (≤ 0.05) were acceptable for all 33 miRNAs, but the logConc (an expression of the density of any particular miRNA in the overall cDNA library) was quite low for some miRNAs. The most statistically- and biologically-relevant differentially expressed miRNA (from most over-expressed to most under-expressed) were *gga-miR-146b*

(logFC = 1.91), *gga-miR-21* (logFC = 1.65), *gga-miR-10b* (logFC = 1.38), *gga-miR-146a* (logFC = 1.24), *gga-miR-147* (logFC = 1.06), *gga-miR-92* (logFC = -0.82), *gga-miR-128* (logFC = -0.83), and *gga-miR-30d* (logFC = -1.94). These eight miRNA were selected for further investigation.

Initial target predictions with miRanda revealed that there were a wide range of immunologically-relevant targets for each of the eight selected miRNA. MiRanda identified 475 potential mRNA target 3'UTRs for *miR-128*. 280 targets for *miR-146a*, 282 targets for *miR-146b*, and 147 targets for *miR-147* were found.

DAVID analysis of these target mRNAs suggested enrichment of biological themes. Some of the most biologically-enriched functional terms were immunological, neuromuscular, and phosphorylation pathways. See table 2-5 and table 2-6 for a condensed list of putative mRNA targets for *gga-miR-128* and *gga-miR-147* suggested by DAVID analysis.

2.6) Discussion

In the course of the present study, we were able to support the hypothesis that there is differential host miRNA expression in MDV-infected birds relative to healthy controls. We identified a total of 21,721,036 sequence reads from three healthy and three MDV-infected chickens. With the use of the DSAP and miRanalyzer server-based small RNA analysis pipelines for Illumina (Solexa) sequencing data, we were able to match ~68% (for DSAP data) and ~53% (for miRanalyzer data) of the total sequence reads to miRNAs present in the mirbase (www.mirbase.org) database. The fact that both methods began with 21,721,036

reads and resulted in the same range of % known miRNA suggests that both core algorithms are able to parse through a tremendous amount of sequencing data and come to a common conclusion. In fact, nearly all miRNAs identified in the DSAP analysis were also identified by the miRanalyzer.

Our analysis with edgeR further elucidated (within a $p \leq 0.05$) that 33 miRNAs were differentially expressed. At least 11 of these 33 miRNA (*gga-miR-10b*, *gga-miR-128*, *gga-miR-1458*, *gga-miR-146a*, *gga-miR-146b*, *gga-miR-147*, *gga-miR-21*, *gga-miR-30a-5p*, *gga-miR-30d*, *gga-miR-455-5p*, and *gga-miR-92*) were shown to have mRNA targets with immunologically- and neuromuscularly-relevant biological GO terms, as shown by our DAVID analysis of filtered miRanda files.

Data from other studies link aberrant *miR-128* expression to a variety of cancers in humans (Roth et al. 2011, Shi et al. 2012, Papagiannakopoulos et al. 2012, Qian et al. 2012). *MiR-128* has been shown to act as a tumor suppressor by targeting various anti-apoptotic and receptor tyrosine kinase (RTK) oncogenes. *MiR-128* has also been shown to repress the process of nonsense mediated decay (Bruno et al. 2011) by targeting a subunit of the exon junction complex (EJC) and UPF1 both proteins are responsible for the degradation of nonsense transcripts. Given the tumor suppressor function of *miR-128* in various human cancers, the reduced expression of *miR-128* we observed in Marek's disease tumors is likely a regulatory mechanism involved in transforming infected T lymphocytes.

Our edgeR analysis found *miR-147* is also differentially expressed in MDV-infected spleens ($\log_{2}FC = 1.06$). The implications of up-regulation of *miR-147* are unclear because only a limited number of studies have found an association between *miR-147* expression and

disease. However, a few recent studies have found a link between up-regulated expression of *miR-147* and innate immune signaling and human cancers (Liu et al. 2009, Yao et al. 2009). Work by Yao and colleagues found a two-fold increase in *miR-147* expression in gastric cancer (GC) tumor tissue ($p \leq 0.05$). This experiment only found an association between *miR-147* and GC patients, failing to conduct a target analysis. Another study observed conflicting results, qualifying *miR-147* as a tumor suppressor that targets multiple proteins in the EGFR cell cycle protein network in breast cancer cells. Given these contradictions, we intend to further investigate the role of *miR-147* expression in MDV-infected spleens exhibiting tumor formation. Additional study is certainly warranted, given the number of immunologically-relevant predicted target genes generated by our miRanda analysis.

A growing pool of evidence is piquing interest in *miR-146* as an immunologically-relevant miRNA (Wang et al. 2009; Taganov et al. 2006). Taganov's group found that *miR-146a/b* expression was induced in response to a mix of microbial components (like lipopolysaccharide) and various proinflammatory cytokines. And that same study predicted seed complementarity with the 3' UTRs of the TNF receptor-associated factor 6 and IL-1 receptor-associated kinase 1 genes, two targets that were also predicted by our filtered miRanda analysis.

The obvious next step for this project is to focus on certain differentially-expressed miRNA and investigate the functional impact of modulated expression of those miRNA. The next chapter will feature the use of target prediction/validation experiments and pathway analysis. MiRanda and DAVID analysis will predict miRNA target genes and the functional

pathways affected, but we will also experimentally validate the miRNA:target mRNA interaction with a dual luciferase assay.

2.7) Tables

Table 2-1. PCR enrichment of indexed, adapter-ligated small RNA libraries.

Step	Temp.	Function	Time
1)	98 °C	Initial Denaturation...	30sec
2)	98 °C	Denaturation.....	10sec
3)	60 °C	Annealing.....	30sec
4)	72 °C	Extension.....	15sec
5)		Go to step 2.....	11X
6)	72 °C	Final Elongation.....	10min
7)	4 °C	Temporary Storage..	∞ (00:00:00)

Table 2-2. DSAP result summaries for all six miRNA libraries, controls alone, and MDV-infected alone.

JOB_ID	all 6 libraries	controls	MDV-treated
No. of total reads	21721036	13003122	8717914
No. of reads in cleaned sequence tags	21680603	4326239	2900629
No. of total reads matched to Rfam	2632988	541844	335819
Avg % of reads matched to Rfam	12.14	12.53	11.50
No. of total reads matched to miRBase	9131368	1896845	1146944
Avg % of reads matched to miRNA	42.12	43.82	39.07
No. of other reads	9916247	1887550	1417866
No. of other reads qualified as homologous miRNAs	5737237	1212435	699978
Avg % of other reads qualified as homologous miRNAs	26.46	28.06	24.16
No. of other reads still "unknown"	4179010	675115	717888
Avg % of still unmatched reads	19.28	15.59	25.28

Table 2-3. MiRanalyzer miRNA result summaries for all six miRNA libraries.

Job_ID	all 6	controls (average)	MDV-treated (average)
Tot. read count	21721036	13003122	8717914
Tot. known miRNA read count	11860517	7463477	4397040
avg % reads map to known miRNA	53.62	57.40	49.85
No. other reads	5539645	5539645	4320874
% reads map to other	46.38	42.60	50.15

Table 2-4. Moderated tagwise (tgw) dispersion of normalized miranalyzer data. Color coding indicates how many (1-4) statistical methods suggested differential expression of a particular miRNA. Red = 4 methods agreed. Orange = 3 methods. Green = 2 methods. Blue = only tgw dispersion of miRanalyzer data indicated differential expression. Yellow highlighted the miRNAs picked for further investigation. Positive fold change (logFC) values indicate overexpression and negative indicates underexpression in MDV-infected spleen samples.

miR	logConc	logFC	p.value
gga-miR-375	-34.68	30.67	4.88E-02
gga-miR-1684	-34.89	30.25	3.91E-02
gga-miR-1458	-18.24	2.69	3.56E-02
gga-miR-144	-11.35	2.26	3.35E-02
gga-miR-429	-19.09	2.24	3.05E-02
gga-miR-146b	-8.37	1.91	3.03E-02
gga-miR-1b	-16.30	1.87	2.86E-02
gga-miR-21	-4.06	1.65	2.62E-02
gga-miR-215	-11.77	1.55	2.54E-02
gga-miR-3538	-13.05	1.46	1.93E-02
gga-miR-10b	-9.58	1.38	1.92E-02
gga-miR-146a	-8.11	1.24	1.67E-02
gga-miR-1674	-16.36	1.12	1.63E-02
gga-miR-147	-14.56	1.06	1.31E-02
gga-mir-21*	-13.63	1.04	1.24E-02
gga-miR-3536	-16.33	1.04	9.82E-03
gga-miR-203	-16.71	0.98	6.77E-03
gga-miR-146c	-5.09	0.85	5.88E-03
gga-mir-16*	-15.50	0.80	4.81E-03
gga-miR-125b	-9.15	-0.68	4.37E-03
gga-miR-15c	-9.99	-0.69	2.37E-03
gga-miR-30a-5p	-10.47	-0.77	1.92E-03
gga-miR-92	-4.92	-0.82	1.62E-03
gga-miR-128	-9.84	-0.83	1.06E-03
gga-miR-455-5p	-11.08	-0.92	3.52E-04
gga-miR-218	-16.30	-0.96	1.82E-04
gga-miR-1456	-15.34	-1.24	1.01E-04
gga-miR-458	-15.41	-1.31	5.32E-05
gga-miR-2954	-14.89	-1.48	3.69E-05
gga-miR-1677	-18.73	-1.67	2.28E-06
gga-miR-30d	-7.82	-1.94	2.24E-07
gga-miR-1677*	-19.89	-2.49	1.41E-07
gga-miR-122	-16.44	-2.52	4.44E-08

Table 2-5. Condensed list of putative targets of gga-miR-128 after DAVID analysis.

# Targets ^a on mRNA	gb accession #	Symbol	Gene name/description
1	AF121963	ERBB4	v-erb-a erythroblastic leukemia viral oncogene homolog 4
1	AJ719297	ADCK1	aarF domain containing kinase 1
1	AJ719357	ELK3	ELK3, ETS-domain protein (SRF accessory protein 2)
1	AJ719385	SRRM1	serine/arginine repetitive matrix 1
1	AJ719493	PRKAR1A	protein kinase, cAMP-dependent, regulatory, type I, alpha (tissue specific extinguisher 1)
1	AJ719814	CD79B	CD79b molecule, immunoglobulin-associated beta
1	AJ719822	ARL6IP1	ADP-ribosylation factor-like 6 interacting protein 1; hypothetical LOC416603
1	AJ719860	VAMP7	synaptobrevin-like 1
1	AJ719931	GCLM	glutamate-cysteine ligase, modifier subunit
1	AJ719990	INTS2	integrator complex subunit 2
1	AJ720037	SLC25A36	solute carrier family 25, member 36
1	AJ720044	ST3GAL6	ST3 beta-galactoside alpha-2,3-sialyltransferase 6
1	AJ720064	DTNBP1	dystrobrevin binding protein 1
1	AJ720079	ODF2	outer dense fiber of sperm tails 2
1	AJ720109	MFN1	similar to mitofusin 1; mitofusin 1
1	AJ720113	CLTB	clathrin, light chain (Lcb)
1	AJ720161	SASH3	chromosome X open reading frame 9
1	AJ720169	PCSK7	proprotein convertase subtilisin/kexin type 7
1	AJ720239	MCCC1	methylcrotonoyl-Coenzyme A carboxylase 1 (alpha)
1	AJ720351	PPT1	palmitoyl-protein thioesterase 1 (ceroid-lipofuscinosis, neuronal 1, infantile)
1	AJ720429	LCORL	ligand dependent nuclear receptor corepressor-like
1	AJ720543	SYK	spleen tyrosine kinase
1	AJ720598	PLK1	polo-like kinase 1 (Drosophila)
1	AJ720650	SBDS	Shwachman-Bodian-Diamond syndrome
1	AJ720686	KATNB1	katanin p80 (WD repeat containing) subunit B 1
1	AJ720705	SHISA5	shisa homolog 5 (Xenopus laevis)
1	AJ720830	SLC25A13	solute carrier family 25, member 13 (citrin)
1	AJ720849	USP28	ubiquitin specific peptidase 28
1	AJ721122	MAP2K5	mitogen-activated protein kinase 5
1	AJ851437	SNRNP200	activating signal cointegrator 1 complex subunit 3-like 1
1	AJ851444	PTBP1	polypyrimidine tract binding protein 1
1	AJ851455	CLK3	CDC-like kinase 3
1	AJ851528	GTF2H4	general transcription factor IIH, polypeptide 4, 52kDa

Table 2-5 Continued

1	AJ851539	CLN8	ceroid-lipofuscinosis, neuronal 8 (epilepsy, progressive with mental retardation)
1	AJ851557	USP12P1	ubiquitin specific peptidase 12 pseudogene 1
1	AJ851558	RIPK1	receptor (TNFRSF)-interacting serine-threonine kinase 1
1	AJ851606	PIK3CD	phosphoinositide-3-kinase, catalytic, delta polypeptide
1	AJ851640	MYD88	myeloid differentiation primary response gene (88)
1	AJ851693	HK1	hexokinase 1
1	AJ851698	MYSM1	myb-like, SWIRM and MPN domains 1
1	AJ851709	GTF2A1	general transcription factor IIA, 1, 19/37kDa
1	AJ851725	ERLIN1	ER lipid raft associated 1
1	AJ851762	APBA2	amyloid beta (A4) precursor protein-binding, family A, member 2 binding protein
1	AJ851776	FAM129A	family with sequence similarity 129, member A
2	AJ851798	NRAS	neuroblastoma RAS viral (v-ras) oncogene homolog
1	AJ851819	STAT3	signal transducer and activator of transcription 3 (acute-phase response factor)
2	AY729886	RAP2B	RAP2B, member of RAS oncogene family
3	EF692644	FSTL4	similar to follistatin-like 4; follistatin-like 4
2	NM_001004387	EPHB6	EPH receptor B6
1	NM_001006201	CDK9	cyclin-dependent kinase 9
1	NM_001079714	COL1A2	collagen, type I, alpha 2
1	NM_001167765	CCNC	cyclin C
1	NM_204118	AHR	aryl hydrocarbon receptor
1	NM_204256	CDKN1B	cyclin-dependent kinase inhibitor 1B (p27, Kip1)
1	NM_204297	HIF1A	hypoxia-inducible factor 1, alpha subunit (basic helix-loop-helix transcription factor)
1	NM_204299	IRF4	interferon regulatory factor 4
1	NM_204381	THY1	Thy-1 cell surface antigen
1	NM_204390	SGMS1	sphingomyelin synthase 1
2	NM_204396	CIP1	cdk inhibitor CIP1 (p21)
1	NM_204926	USP2	ubiquitin specific peptidase 2
1	NM_205027	TMOD1	tropomodulin 1
2	NM_205115	LIMK2	LIM domain kinase 2
1	NM_205155	FASN	fatty acid synthase
1	NM_205206	INHBB	activin beta B
1	NM_205239	CCNB3	cyclin B3
1	NM_205240	ST6GALNAC1	ST6 (alpha-N-acetyl-neuraminy-2,3-beta-galactosyl-1,3)-N-acetylgalactosaminide alpha-2,6-sialyltransferase 1
1	NM_205303	CAPN1	calpain 1, (mu/I) large subunit
1	NM_205367	ACVR2A	activin A receptor, type IIA

Table 2-5 Continued

3	NM_205470	PCK2	phosphoenolpyruvate carboxykinase
1	NM_206863	VAV3	vav 3 oncogene
1	NM_206984	GJA4	gap junction protein, alpha 4, 37kDa
1	NM_207178	SLC2A2	solute carrier family 2 (facilitated glucose transporter), member 2
1	X03578	HRAS	v-Ha-ras Harvey rat sarcoma viral oncogene homolog
1	X72218	RB1	retinoblastoma 1 (including osteosarcoma)
3	XM_001232074	CABLES1	Cdk5 and Abl enzyme substrate 1
1	XM_001232308	PRPF4B	PRP4 pre-mRNA processing factor 4 homolog B (yeast)
1	XM_001233543	CHERP	calcium homeostasis endoplasmic reticulum protein
1	XM_414998	HNRPLL	heterogeneous nuclear ribonucleoprotein L-like
1	XM_415091	ULK1	unc-51-like kinase 1 (C. elegans)
1	XM_415266	POP5	processing of precursor 5, ribonuclease P/MRP subunit (S. cerevisiae)
1	XM_416310	NUAK1	NUAK family, SNF1-like kinase, 1
1	XM_416445	CYB5R3	similar to cytochrome b-5 reductase
1	XM_416551	GTF2E1	hypothetical protein LOC769761; general transcription factor IIE, polypeptide 1, alpha 56kDa
1	XM_416587	SLC35A5	solute carrier family 35, member A5
1	XM_416621	CBLB	Cas-Br-M (murine) ecotropic retroviral transforming sequence b
1	XM_417640	MFN2	similar to CPRP1; mitofusin 2
1	XM_417993	ATP5F1	ATP synthase, H ⁺ transporting, mitochondrial F0 complex, subunit B1
1	XM_421129	PPP1R14D	protein phosphatase 1, regulatory (inhibitor) subunit 14D
1	XM_424462	NEDD4L	NEDD4L; hypothetical protein LOC776508; neural precursor cell expressed, developmentally down-regulated 4-like
1	XM_424684	SLC25A42	solute carrier family 25, member 42
1	XM_424734	MAP3K1	mitogen-activated protein kinase kinase kinase 1
1	XM_424739	PLK2	polo-like kinase 2 (Drosophila)
1	XM_424759	PIK3R1	phosphoinositide-3-kinase, regulatory subunit 1 (p85 alpha)
1	XM_425424	RELN	reelin

^aNumber of targets on each mRNA (gene) were established by searching how many times the accession number occurred in the 128filteredLow.txt filtered miranda file. Target genes in red are of particular interest, due to the multiple target sites on the mRNA, the particularly strong affinity of the seed region to 3'UTR binding site, or the protein's involvement in the pathogenesis of Marek's disease/immune response.

Table 2-6. Condensed list of putative targets of gga-miR-147 after DAVID analysis.

# Targets ^a on mRNA	gb accession #	Symbol	Gene name/description
1	XM_413874	AKAP13	A kinase (PRKA) anchor protein 13
1	AJ720308	ABCE1	ATP-binding cassette, sub-family E (OABP), member 1
1	XM_424388	BAG4	BCL2-associated athanogene 4
1	XM_001233716	BAZ1B	bromodomain adjacent to zinc finger domain, 1B
1	NM_001039258	CDH1	cadherin 1, type 1, E-cadherin (epithelial)
1	NM_206859	CEBPG	CCAAT/enhancer binding protein (C/EBP), gamma
1	AJ851659	CD80	CD80 molecule
1	AM050135	CD86	CD86 molecule
1	NM_204605	F2	coagulation factor II (thrombin)
1	AJ720910	DHX30	DEAH (Asp-Glu-Ala-His) box polypeptide 30
1	AJ851526	EFTUD2	elongation factor Tu GTP binding domain containing 2
1	AJ719654	XPO7	exportin 7
1	NM_204885	EZR	ezrin
1	AJ890143	FASLG	Fas ligand (TNF superfamily, member 6)
1	AJ719529	FEM1B	fem-1 homolog b (C. elegans)
1	AJ721059	FOXN2	forkhead box N2
1	XM_001234504	FMNL1	formin-like 1
1	AJ851452	GPR89B	G protein-coupled receptor 89B
1	XM_415415	GPSM1	G-protein signaling modulator 1 (AGS3-like, C. elegans)
2	NM_204619	HOXA11	homeobox A11
1	XM_420813	HTRA3	HtrA serine peptidase 3
1	NM_204131	LIMK1	LIM domain kinase 1
1	AB260848	MMP16	matrix metalloproteinase 16 (membrane-inserted)
1	AJ720147	MOV10	Mov10, Moloney leukemia virus 10, homolog (mouse)
1	NM_205116	MYBPC3	myosin binding protein C, cardiac
1	NM_001167728	NAGA	N-acetylgalactosaminidase, alpha-paired related homeobox 2
1	XM_415476	PRRX2	phosphatidylinositol-5-phosphate 4-kinase, type II, alpha
1	AJ851794	PIP4K2A	phosphatidylinositol-5-phosphate 4-kinase, type II, alpha
1	DQ340396	PRKAA2	protein kinase, AMP-activated, alpha 2 catalytic subunit
1	AJ719816	RAB22A	RAB22A, member RAS oncogene family
1	AJ851430	RAB8A	RAB8A, member RAS oncogene family
1	AJ720845	RASGRP3	RAS guanyl releasing protein 3 (calcium and DAG-regulated)
1	AJ719758	RASGEF1A	RasGEF domain family, member 1A
1	AJ851738	RNF166	ring finger protein 166
1	XM_419000	MYO10	similar to myosin X; myosin X
1	NM_204964	SLC11A1	solute carrier family 11 (proton-coupled divalent metal ion transporters), member 1

Table 2-6 Continued

1	AJ720559	SLC9A8	solute carrier family 9 (sodium/hydrogen exchanger), member 8
1	AJ851535	SUV39H2	suppressor of variegation 3-9 homolog 2 (Drosophila)
1	AJ719860	VAMP7	synaptobrevin-like 1
1	NM_204940	T	T, brachyury homolog (mouse)
1	NM_204219	TRAF5	TNF receptor-associated factor 5
1	U72641	TCF15	transcription factor 15 (basic helix-loop-helix)
3	NM_205315	TUBB2A	tubulin, beta 2A
1	AJ851443	WBP4	WW domain binding protein 4 (formin binding protein 21)

^aNumber of targets on each mRNA (gene) were established by searching how many times the accession number occurred in the 147filteredLow.txt filtered miranda file. Target genes in red are of particular interest, due to the multiple target sites on the mRNA, the particularly strong affinity of the seed region to 3'UTR binding site, or the protein's involvement in the pathogenesis of Marek's disease/immune response.

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CHAPTER 3: CHARACTERIZATION OF microRNAs ASSOCIATED WITH MDV-1 VIRAL CHALLENGE

3.1) Introduction

After identifying multiple differentially-expressed cellular miRNAs in tumorigenic spleens induced by MDV infection, the next step is to further characterize the downstream effects of differentially-expressed miRNAs. The infection cycle of any virus brings about a host immune response, which involves differential expression of genes involved in immunological pathways. Studies have found that protein expression changes often occur over the course of viral infections (e.g. Levy et al. 2011, Hughes et al. 2012, Zhou and Zhang 2012).

This temporal change in gene expression is due to both host and viral regulatory factors. MiRNA is one such factor regulating gene expression in response to viral infections (Ghosh et al. 2009, Forte et al. 2011). We observed reduced expression of *miR-128* and increased expression of *miR-147* in MDV-induced tumorigenic spleens relative to non-infected control spleens. As discussed in chapter 2, there have been several reports associating down-regulation of *miR-128* with cancer development (Roth et al. 2011, Shi et al. 2012, Papagiannakopoulos et al. 2012, Qian et al. 2012).

There have also been conflicting reports as to whether increased *miR-147* expression contributes to tumor formation (Yao et al. 2009) or acts as a tumor suppressor (Uhlmann et al. 2012). Yao et al. posit that reduced or no differential expression of *miR-147* at the transformation stage of infection supports the idea that *miR-147* acts as an oncomir. On the other hand, Uhlmann et al. identified tumor suppressor functionality of *miR-147* in breast

cancer pathogenesis, stating that up-regulation of *miR-147* was associated with limiting cell cycle progression by targeting the epidermal growth factor receptor (*EGFR*, *HER1*). To further clarify the involvement of *miR-128* and *miR-147* in MDV pathogenesis we utilized standard target prediction/validation assays and pathway analysis to identify biologically-enriched functional pathways affected by differential expression of miRNAs.

3.2) Objectives

- 1) *In silico* prediction and *in vitro* validation of target mRNAs of *miR-128* and *miR-147*.
- 2) Perform pathway analysis of predicted/validated targets of *miR-128* and *miR-147*.

3.3) Materials and Methods

3.3.1) *miRNA Target Prediction and Pathway analysis*

The target prediction algorithm miRanda (v3.3, John et al. 2005) was used to identify potential target genes of *miR-128* and *miR-147*. This program utilized the miRanda miRNA target prediction algorithm (Enright et al, 2003). A local instance of the application can be installed on either MacOS or Windows operating system, and the miRanda analysis script language for analysis is identical. A common example miRanda analysis script (using Terminal for MacOS, or using a bash emulator on the Windows command line interface) is as follows:

miranda *file1 file2* [*options ...*]

'file1' is a .fasta file with a microRNA query and 'file2' is a FASTA file containing the sequence(s) to be scanned. In our analysis, 'file 1' would be any FASTA-formatted mature

miRNA sequence of interest, downloaded from www.mirbase.org. ‘File 2’ was always Gga.seq.uniq, which was the most current *Gallus gallus* mRNA library from the Unigene FTP (http://www.ncbi.nlm.nih.gov/guide/all/#downloads_). A more specific example of one of our analysis is as follows:

```
miranda file1 file2 [-sc score] [-en energy] [-out fileout]
```

where score threshold was set at 140, minimum free energy (ΔG) was -18kcal/mol, and the output file would be named something like ‘-out gga-mir-128.txt’. The miRanda algorithm generates an output file that has a wide range of targets. An in-house filter perl script was developed in the Liu lab to sort through the miRanda output with even more stringency. A common filter script is as follows:

```
$ perl filterLow.pl filein [-o fileout1 fileout2]
```

where filterLow.pl is the perl script, ‘filein’ is the miRanda output .txt file (this is now an input file for the filter), ‘fileout1’ is an output file of targets in the 3’UTR region of the mRNA sequence and ‘fileout2’ is an output file of targets in the 5’UTR (seed region matches to the coding sequence (cds) region of the mRNA were also considered as potential targets in ‘fileout2’). Additionally, the filteredLow.pl script allowed either a 1-8nt seed match or a 2-7nt and 13-16nt match, with wobble base-pairing (A-U) tolerated. Here’s an actual use of the script:

```
$ perl filterLow.pl gga-mir-128.txt -o 128filteredLow1.txt  
128filteredLow2.txt
```

A DAVID pathway analysis was performed on the list of mRNA targets of the differentially-expressed miRNAs. Only the filtered 3’UTR targets file was considered for

further study, as the seed regions of miRNAs in animals tend to be complementary (and therefore target) the 3'UTR of mRNAs. Enriched gene sets (those mRNAs that had biological functions similar to each other) were grouped in functional annotation categories by Database for Annotation, Visualization and Integrated Discovery (DAVID v6.7, <http://david.abcc.ncifcrf.gov/>). DAVID analysis gives useful information in terms of KEGG pathway maps, gene-disease associations, identification of enriched biological themes (including GO terms) and identification of enriched functionally-related gene groups.

3.3.2) Dual luciferase assay vector construction

For each predicted target gene 20-22nt forward and reverse primers were designed (Table 3-1) flanking each predicted target site in the 3'UTRs of selected target mRNAs using Vector NTI Advance (v11.5, Invitrogen). PCR was performed using the conditions in Table 3-2, with annealing temperatures ranging from 53-57°C (optimization required for each primer set) in order to amplify *miR-147* target site-containing sequences in Red Jungle fowl genomic DNA (gDNA). One microliter of each 10uM primer (forward and reverse) was mixed with 1ul gDNA (50ng/ul). The products of this PCR reaction were purified using an UltraClean PCR Clean-Up Kit (MO BIO Laboratories, Cat# 12500-100). For target site cloning, 1ug psiCHECK-2 vector (Promega, Cat# C8021) and 1ug of PCR products were digested with XhoI and NotI-HF (New England Biolabs) for 2.5hr at 37°C subjected to agarose electrophoresis and gel purification using a QIAquick Gel Extraction Kit (QIAGEN, Cat# 28706). Ligation reactions were carried overnight at 14°C using T4 DNA ligase (New England Biolabs). Ligated products were then transformed into chemically competent DH5 α bacteria. Colony PCR was performed on 3 colonies on each plate (tables 3-7 and 3-8). A

single positive colony (one that was positive for the 3'UTR insert) was selected and plasmid DNA was purified with a Wizard Plus SV Minipreps DNA Purification System (Promega, Part#9FB004), eluting off a final volume of 30ul DNA in nuclease free water. A total of 1µg of purified plasmid was digested with XhoI and NotI-HF (New England Biolabs Table 3-5) to confirm the presence of miRNA target region and was then subjected to DNA sequencing (Operon)

The preparation of the recombinant RCASBP(A)miRNA virus was based on work done by the Dodgson group at Michigan State University (Chen et al. 2007) with a replication-competent retrovirus. Forward (68-nt) and reverse (76-nt) primers were synthesized and PAGE purified by Invitrogen . To generate a short double-stranded DNA fragment, the primers were mixed at a final concentration of 1 µM, denatured at 95 °C for 20 sec and annealed at RT. The DNA fragment was then cloned into the pENTR3C-miR-SphNgo vector at the SphI and NgoMIV restriction sites. MiR-147, and miR-SC (a scrambled control hairpin structure) were each cloned into the RCASBP(A)-YDV gateway vector using a LR clonase kit acco(Invitrogen). The RCASBP(A)-miRNA vectors were transfected into the DF-1 chicken embryonic fibroblast cell line using FuGENE-6 following the manufacturer's instructions (Promega). Virus was collected from DF-1 media and titer was determined uisng immunofluorescence staining with a mouse-α RSV/ALV gag protein (coded for in the RCAS vector) and FITC-conjugated goat anti-mouse IgG (Invitrogen).

DF-1 cells were infected with the RCAS-miR-147 or RCAS-miR-SC (m.o.i. = 1) and incubated at 37°C with ~5% CO₂ for 6 days in RPMI 1640 with 1% FBS, penicillin (100 U/ml), streptomycin (100ug/ml), fungisone (4 ug/ml), and L-glutamine. Forty-eight hours

before the luciferase assay, 100ng psiCHECK2 target-containing vector was transfected (in triplicate) into RCAS-infected DF-1 cells on a 96-well plate using Fugene-6. Cells were washed with 1X PBS. Cells were lysed with 30ul 1X passive lysis buffer (Promega) per well. Plates were rocked for 30 minutes. Next, firefly and *Renilla* luciferase activities were then assessed using 10µl of cell lysate and the Dual-Luciferase Reporter Assay System (Promega) and a VictorLight 1420 luminescence counter (PerkinElmer). Two-tailed t-test was performed to qualify target validation.

3.4) Results

3.4.1) Computational target prediction

After screening the 11 most interesting differentially expressed miRNA identified through edgeR statistical analysis, two (*miR-128* and *miR-147*) were selected for more in-depth downstream analysis. In addition to finding differential expression of these miRNAs, a computational target prediction was performed using the miRanda algorithm. Potential *miR-128* and *miR-147* regulated genes identified in the *Gallus gallus* Unigene database based on a seed sequence match to the 3'UTR region of a target mRNA. An in-house perl script was then used to filter down to the most likely target mRNA. It is interesting to note that both *miR-128* and *miR-147* had multiple target sites on the same mRNA. For example, the 5' 2-7 (or 2-8) seed region nucleotides on the mature sequence of *miR-128* had multiple predicted target sites on the following eight mRNAs: NRAS (2 sites), RAP2B (2), FSTL4 (3), EPHB6 (2), CIP1 (2), LIMK2 (2), PCK2 (3), and CABLES1 (3). Additionally, *miR-147* has multiple

predicted target sites in the 3'UTRs of HOXA11 (2sites) and TUBB2A (3). *MiR-128* and *miR-147* also had a common predicted target, VAMP7.

3.4.2) *In vitro* target validation on miR-147 with dual luciferase assay

Fourteen predicted targets for *miR-147* were selected for use in an *in vitro* target validation experiment using a dual luciferase assay. These targets and their miRanda-generated target prediction profiles are shown in table 3-6. The luciferase assay results for *miR-147* target validation are shown in figure 3-1. In all, six of the fourteen predicted target sites were validated. Three target sites were validated in the 3'UTR of TUBB2A mRNA as evidenced by the fact that when all three sites were included luciferase activity was repressed to a greater extent than when one or two sites were used (figure 3-1). The luciferase assay also validated single target sites in TRAF5, RAB22A, HOXA11, and CEBPG.

3.4.3) *Pathway analysis*

The list of filtered miRanda targets were run through DAVID for each miRNA to find the biologically-enriched pathways which may be affected by the differential expression of *miR-128* and *miR-147*. Some of the most immunologically-/neuromuscularly-relevant target mRNAs are shown in tables 3-11 and 3-12.

Further analysis with DAVID revealed instances where multiple predicted targets were involved in a single KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway. These instances are termed *pathway clusters*. *MiR-128* targets are involved with three pathway clusters (table 3-9), including NK cell-mediated cytotoxicity, ErbB signaling pathway, and actin cytoskeleton regulation. *MiR-147* targets are involved with two pathway clusters (table 3-10): cell adhesion molecules, and the intestinal immune network for IgA.

3.5) Discussion

In chapter 2, 33 miRNAs were found to be differentially expressed in MDV-infected versus a control group. This sequencing experiment found that miR-128 was down-regulated and *miR-147* was up-regulated at the tumor stage. The 42dpi time point, as indicated in chapter 2, was chosen to reflect miRNA expression during the tumor phase. Reduced expression of *miR-128* at the tumor phase in MDV-infected birds (from the sequencing experiment results) suggests that *miR-128* may target oncogenes. By combining our observations from the sequencing experiment and the target prediction analysis, we can conclude that reduced expression of *miR-128* may be associated with tumor formation, and increased expression may be associated with preventing tumor formation.

It is likely that reduced *miR-128* expression is linked with some generalized immune response to viral infection. This conclusion is further supported by multiple predicted immunologically-relevant targets (NRAS, HRAS, SYK, VAV3) involved with in the NK cell-mediated cytotoxicity, ErbB signaling pathway, and actin cytoskeleton regulation pathway clusters (Table 3-9). Vav3 and Syk are both involved in B-cell receptor (BCR) signaling cascades, which lead to the production of antiviral antibodies (Fujikawa et al. 2003, van Oers and Weiss 1995). It could be said that *miR-128* limits these pathways. This line of reasoning is supported by how reduction of *miR-128* expression as the chick matures is linked with tumor formation. Our observations agree with a number of other studies (Roth et al. 2011, Shi et al. 2012, Papagiannakopoulos et al. 2012) that link reduced expression of *miR-128* with tumorigenesis in patients with various gliomas. It is possible that these evaluations of the functional significance of *miR-128* were incomplete, as they focused on

evaluating single *miR-128* targets while our analysis looked for involvement of many predicted targets in biologically-enriched pathway clusters. Another recent study looked at multiple target genes and found an association between reduced *miR-128* expression and breast cancer (Qian et al. 2012). Further study of the functional significance of *miR-128* is needed, but our initial observations are exciting as they link increased *miR-128* expression with preventing tumor formation. MiRNAs have recently been found to have therapeutic value (reviewed in Broderick and Zamore 2011), and a number of companies are actively assessing the viability of miRNA-mimic therapies as cancer treatments, and our results indicate *miR-128* may be an attractive candidate for future research.

Our sequencing experiment also identified up-regulation of *miR-147* at 42dpi (tumor stage) in MDV-infected birds from our deep sequencing analysis. This suggest that *miR-147* may act as an oncomir. Predicted *miR-147* targets (CD80, CD86, and CDH1) are involved with two pathway clusters involved in immune response (table 3-10): cell adhesion molecules, and the intestinal immune network for IgA. CD80 and CD86 are both B7 family co-stimulatory ligands on the surface of APCs and are required for the activation of T-cells. If primary adhesion ligand:receptor binding and antigen specific recognition occur, but B7 co-stimulatory signal is not present, the T-cell experiences anergy, an inactivated state where the T-cell is hypo-responsive/tolerant of the antigen presented (Schwartz 2003). Likewise, if these co-stimulatory molecules are up-regulated via reduced *miR-147* expression early during pathogenesis, T-cell anergy is less likely to occur, which may result in a more robust host immune response. Our target validation experiment further supports the argument that increased expression of *miR-147* prevents tumor formation.

We validated two *miR-147* targets (TUBB and RAB22A) involved in virion assembly and egress via dual luciferase assay. Understanding how reduced expression of *miR-147* may contribute to a more robust immune response requires a basic understanding of herpesvirus virion assembly and egress, and of how TUBB and RAB22A are involved in this process. MDV-1 is a herpesvirus, and as such go through virion assembly and egress mechanisms similar to that of other model herpesviruses (reviewed in Mettenleiter et al. 2009), which is described below.

In the lytic phase of herpesvirus replication, nucleocapsid assembly occurs in the nucleus of the cell. Primary envelopment of nucleocapsids occurs at the inner nuclear membrane. Genomic DNA-containing nucleocapsid leaves the nuclear membrane, entering the cytoplasm, where it binds tegument. Mature enveloped virions bud into lumen of trans-Golgi-derived exocytic vesicles that have embedded mature virion glycosylated spike proteins (Johnson and Spear 1982). Mature enveloped virions accumulate inside exocytic vesicles until cell lysis or vesicles fuse with plasma membrane to release mature virion. TUBB is one of the two tubulin subunits that compose protofilaments that make up microtubules. These tubule networks are cytoskeletal “tracks” along which vesicles and organelles are transported. A number of recent studies have found microtubules to be involved in virus transport for a range of viruses (Carter et al. 2003, Lakadamyali et al. 2003, McDonald et al. 2002, and reviewed in Greber and Way 2006). In the process of virion assembly and egress, TUBB is involved in transporting nucleocapsid along microtubules from nucleus through tegumentation in cytoplasm. TUBB is also required for transportation of tegumented nucleocapsid to trans-Golgi-derived exocytic vesicles.

RAB22A is also involved in this process. The Rab proteins are a family of small GTPases involved in the fusion event between vesicle-Golgi and vesicle-plasma membrane. Reduced expression of *miR-147* means more TUBB and RAB22A protein will likely be translated. Removing TUBB-limiting *miR-147* removes a restriction on tubulin production, which may result in greater intracellular transport of herpesvirus nucleocapsid on its path to virion assembly and maturation. Similarly, over-expression of RAB22A has been linked with promoting endosome fusion (Mesa et al. 2005) a process required for production of mature vaccine-strain HVT in a cell. RAB22A was also linked to MHC-I recycling (Weigert et al. 2004), and an increased rate of MHC recycling rather than ubiquitination and lysosomal degradation may contribute to a more robust processing and presentation of viral antigen.

The association of reduced *miR-147* expression with reduced incidence of cancer is not limited to our observations. *MiR-147* expression increased in response to TLR-2, TLR-3, and TLR-4 activation, and is part of the normal innate response in murine macrophages (Liu et al. 2009). It was further postulated by Liu and colleagues that *miR-147* acted to limit excessive inflammatory signaling at the site of infection. Further study into the functional significance of *miR-147* is necessary, as very few reports have linked abnormal *miR-147* expression to prevention of tumor formation. In fact, another investigation into the functional significance of *miR-147* made conclusions that conflict with our own. This group found that increased miR-147 expression was found in patients with gastric cancer (Yao et al. 2009).

A number of studies have investigated the transcriptome and proteome profiles in chickens infected with MDV strains (Sarson et al. 2006, Liu et al. 2006, Sarson et al. 2008,

Heidari et al. 2010). These studies identified a number of differentially-expressed genes throughout MDV pathogenesis. Two studies found CD80 to be down-regulated in at 14dpi and 15dpi, relative to 7dpi and 5dpi, respectively (Sarson et al. 2006 and Heidari et al. 2010). Our target prediction identified CD80 as a target of *miR-147*. It follows that the observation of reduced CD80 expression in the Sarson and Heidari experiments may be due to increased expression of *miR-147*.

One of the few investigations into differential gene expression in vaccinated chickens found that CD79B was down-regulated in CVI988-vaccinated chickens at 21dpi relative to unvaccinated chickens (Kano et al. 2009). Our analysis found that CD79B is a computationally-predicted target of *miR-128*.

3.6) Tables and Figures

Table 3-1. Primer sets used in miR-147 target cloning.

Oligo Name	Sequence (5' to 3')	GenBank accession #
VAMP7-both-F	CTCGAGTACTTCCTCTGTAGTCTGAA	AJ719860
VAMP7-both-R	GCGGCCGCTGTACAGTAACTTTATTTA	
CDH1-miR-147-F	CTCGAGTCAGCTCCCAGTGCCCCAAT	NM_001039258
CDH1-miR-147-R	GCGGCCGCACACTTAGGCAATCGCCGGC	
CEBPG-miR-147-F	CTCGAGGTCAGCAAGGAGCTTACTAT	NM_206859
CEBPG-miR-147-R	GCGGCCGCTGTGCAAGCCAGGTTAACAT	
CD80-miR-147-F	CTCGAGTTCTCCCCTAAAGAGGCAGT	AJ851659
CD80-miR-147-R	GCGGCCGCAGCAGATGTAGGAAGCAAGG	
CD86-miR-147-F	CTCGAGTGCCGCTAGTCATAGAGATA	AM050135
CD86-miR-147-R	GCGGCCGCGGTGAACACTGACAATCGTT	
TNFSF6-miR-147-F	CTCGAGTAGGGCAGGAGAGCAAGCCA	AJ890143
TNFSF6-miR-147-R	GCGGCCGCGCATGTCCCTGGTGTGCGGGG	
FOXN2-miR-147-F	CTCGAGCTCCAGGCTCCACATAGCGC	AJ721059
FOXN2-miR-147-R	GCGGCCGCTTGCCAAAAGATGTGCGGG	
HOXA11-miR-147-F1	CTCGAGGAGAGGCGGAGGCGGCCGGAG	NM_204619
HOXA11-miR-147-F2	CTCGAGTGTCTATATATTTACGTACCA	
HOXA11-miR-147-R1	GCGGCCGCTGGTACGTAATATATAGACA	
HOXA11-miR-147-R2	GCGGCCGCCGCTCGCGGTGCCGGTGCCCA	
MOV10-miR-147-F	CTCGAGCTGCTGATCGTGGTGGTAACG	AJ720147
MOV10-miR-147-R	GCGGCCGCAAGGTAGGTGTGCTGCACGTGG	
MYBPC3-miR-147-F	CTCGAGCCAAATACCGCATGTTTCAGT	NM_205116
MYBPC3-miR-147-R	GCGGCCGCTTGCCAGTAAAAGCAGAAGG	
RAB22A-miR-147-F	CTCGAGGGCCATCTGCTTTCATTCTG	AJ719816
RAB22A-miR-147-R	GCGGCCGCCTTAGGCGGCACATTGAAGT	
RASGRP3-miR-147-F	CTCGAGCAGCACATCCTGGAGCACAA	AJ720845
RASGRP3-miR-147-R	GCGGCCGCATGGTTGGCTTCTGTCCCTCG	
TRAF5-miR-147-F	CTCGAGGGACTCATGAGTAGTGCAGCTT	NM_204219
TRAF5-miR-147-R	GCGGCCGCTTATAGCTTGGGGTCTGTGC	
TUBB-miR-147-F1	CTCGAGTCGTCCCCACGCTGACGTTG	NM_205315
TUBB-miR-147-F2	CTCGAGTTGGCCGCCATCGCTGTCCCTT	
TUBB-miR-147-F3	CTCGAGTCGCTGTCCCTTCAGCAGTGT	
TUBB-miR-147-R1	GCGGCCGCAAGGACAGCGATGGCGGCCAA	
TUBB-miR-147-R2	GCGGCCGCACACTGCTGAAGGACAGCGA	
TUBB-miR-147-R3	GCGGCCGCCATGAGGGGACACCGTTGGC	

Table 3-2. Thermocycler program for amplification of target sites from Red Junglefowl gDNA.

Step	Temp.	Function	Time
1)	95 °C	Initial Denaturation.....	2min
2)	95 °C	Denaturation.....	30sec
3)	55 °C	Annealing.....	30sec
4)	72 °C	Extension.....	1min
5)		Go to step 2.....	34X
6)	72 °C	Final Elongation.....	2min
7)	10 °C	Temporary Storage...	∞ (00:00:00)

Table 3-3. A mastermix (MM) is made for each gene, then added to the boiled water:bacteria solution.

Reagent	1 rxn (µl)	x4
5X buffer (w/ dye)	5	20
MgCl ₂	2.5	10
dNTPs	0.5	2
F. primer (10µM)	1	4
R. primer (10µM)	1	4
taq (added last)	0.2	0.8
H ₂ O	14.8	-
total	25	

Table 3-4. Thermocycler conditions for colony PCR.

1)	95 °C	Initial Denaturation...	2min 30sec
2)	94 °C	Denaturation.....	30sec
3)	55 °C	Annealing.....	1min
4)	72 °C	Extension.....	1min 15sec
5)		Go to step 2.....	5X
6)	94 °C	Denaturation	30sec
7)	53 °C	Annealing	1min
8)	72 °C	Extension	1min 15sec
9)		Go to step 6	26X
10)	72 °C	Final Elongation.....	10min
11)	10 °C	Temporary Storage..	∞ (00:00:00)
12)		END	

Table 3-5. Target vector digestion check (post-3' UTR ligation).

Reagent	TV-A	TV-C	TV-G
Buffer #4 (NEB, 10X)	2	2	2
10X BSA	2	2	2
psiCHECK2-GeneName-MyGeneID vector (1μg)	1.4	2.1	1.2
XhoI enzyme (1μl is enough for 1μg DNA)	1	1	1
NotI-HF enzyme (1μl is enough for 1μg DNA)	1	1	1
Autoclaved DI H ₂ O	12.6	11.9	112.8
total=	20μl	20μl	20μl

Table 3-6. Computationally-predicted target sites for gga-miR-147. These target sites were cloned into target vectors for target validation via dual luciferase assay. Red text is from miRNA sequence. Blue text is from 3'UTR of target mRNA. Alignment score and ΔG calculated by miRanda algorithm.

Target mRNA/ gb#	miRNA:mRNA (3'UTR) duplex	Alignment Score/ ΔG (kcal/mol)	Binding Site
VAMP7/ AJ719860	3' cgUCUUCGUA AAAGGCGUGu 5' : : 5' caGGAAGGG --GCCGCACAg 3'	143.00/- 19.85	2239-2256
CDH1/ NM_0010392 58	3' cgucUUCGUA AAAGGCGUGu 5' : : 5' gggcGATCGCTCCGCACAc 3'	148.00/- 19.72	3206-3225
CEBPG/ NM_206859	3' cgUCUUCGUA AAAGGCGUGu 5' : : 5' acAGAGCAATTCCTGCACAg 3'	146.00/- 22.72	1303-1322
CD80/ AJ851659	3' cgUCUUCGUA -AAGGCGUGu 5' : : 5' acAGGGGCATACCTCGCACAg 3'	141.00/- 25.21	2080-2100
CD86/ AM050135	3' cgUCUUCGUA AAAGGCGUGu 5' : : 5' tgGGAACCATTTCTGCACAt 3'	162.00/- 22.50	940-959
TNFSF6/ AJ890143	3' cgUCUUCGUA AAAGGCGUGu 5' : : 5' ctGGCAGCA -CCCCGCACAc 3'	148.00/- 20.69	795-813
FOXN2/ AJ721059	3' cgucucUCGUA AAAGGCGUGu 5' : 5' tccttTAGAGCTCCGCACAt 3'	147.00/- 18.54	2820-2839
HOXA11/ NM_204619	3' cgucUCUUCGUA AAAGGCGUGu 5' : 5' cccGAGGT-TTTCGTACAt 3' 3' cgucucucGUA AAAGGCGUGu 5' : 5' tcggtttgATTTTCGCACAc 3'	147.00/- 20.94 144.00/- 21.89	1038-1056 1124-1143
MOV10/ AJ720147	3' cgucucguaaa GGGCGUGu 5' 5' gctgacctcagcCCGCACAc 3'	140.00/- 19.29	3304-3323
MYBPC3/ NM_205116	3' cgucUUCGUA AAAGGCGUGu 5' : 5' acctAAG-AGTTCTGCACAt 3'	142.00/- 18.06	4042-4060
RAB22A/ AJ719816	3' cgUCUUCG -UAAAGGCGUGu 5' : : : 5' agGGAAGTGATTGCTGCACAA 3'	149.00/- 18.14	4177-4197
RASGRP3/ AJ720845	3' cgucucGUA AAAGGCGUGu 5' : : 5' cactattTATTTTCGCACAc 3'	145.00/- 19.30	2431-2450
TRAF5/ NM_204219	3' cgUCUUCGUA AAAGGCGUGu 5' : : 5' atGGAAGTAATTTTCGCACAc 3'	142.00/- 22.16	3084-3103
TUBB2A/ NM_205315	3' cgUCUUCGUA AAAGGCGUGu 5' : 5' tcAGCAGTGTACCCGCGCAg 3' 3' cgUCUUCGUA AAAGGCGUGu 5' : 5' tcAGCAGTGTACCCGCGCAg 3' 3' cgUCUUCGUA AAAGGCGUGu 5' : 5' tcAGCAGTGTACCCGCGCAg 3'	142.00/- 19.37 142.00/- 19.37 142.00/- 19.37	2024-2043 2067-2086 2153-2172

Table 3-7. Condensed list of putative immunologically-relevant targets of gga-miR-128 after DAVID analysis.

# Targets ^a on mRNA	gb accession #	Symbol	Gene name/description
1	AF121963	ERBB4	v-erb-a erythroblastic leukemia viral oncogene homolog 4
1	AJ719297	ADCK1	aarF domain containing kinase 1
1	AJ719357	ELK3	ELK3, ETS-domain protein (SRF accessory protein 2)
1	AJ719385	SRRM1	serine/arginine repetitive matrix 1
1	AJ719493	PRKAR1A	protein kinase, cAMP-dependent, regulatory, type I, alpha (tissue specific extinguisher 1)
1	AJ719814	CD79B	CD79b molecule, immunoglobulin-associated beta
1	AJ719822	ARL6IP1	ADP-ribosylation factor-like 6 interacting protein 1; hypothetical LOC416603
1	AJ719860	VAMP7	synaptobrevin-like 1
1	AJ719931	GCLM	glutamate-cysteine ligase, modifier subunit
1	AJ719990	INTS2	integrator complex subunit 2
1	AJ720037	SLC25A36	solute carrier family 25, member 36
1	AJ720044	ST3GAL6	ST3 beta-galactoside alpha-2,3-sialyltransferase 6
1	AJ720064	DTNBP1	dystrobrevin binding protein 1
1	AJ720079	ODF2	outer dense fiber of sperm tails 2
1	AJ720109	MFN1	similar to mitofusin 1; mitofusin 1
1	AJ720113	CLTB	clathrin, light chain (Lcb)
1	AJ720161	SASH3	chromosome X open reading frame 9
1	AJ720169	PCSK7	proprotein convertase subtilisin/kexin type 7
1	AJ720239	MCCC1	methylcrotonoyl-Coenzyme A carboxylase 1 (alpha)
1	AJ720351	PPT1	palmitoyl-protein thioesterase 1 (ceroid-lipofuscinosis, neuronal 1, infantile)
1	AJ720429	LCORL	ligand dependent nuclear receptor corepressor-like
1	AJ720543	SYK	spleen tyrosine kinase
1	AJ720598	PLK1	polo-like kinase 1 (Drosophila)
1	AJ720650	SBDS	Shwachman-Bodian-Diamond syndrome
1	AJ720686	KATNB1	katanin p80 (WD repeat containing) subunit B 1
1	AJ720705	SHISA5	shisa homolog 5 (Xenopus laevis)
1	AJ720830	SLC25A13	solute carrier family 25, member 13 (citrin)
1	AJ720849	USP28	ubiquitin specific peptidase 28
1	AJ721122	MAP2K5	mitogen-activated protein kinase kinase 5
1	AJ851437	SNRNP200	activating signal cointegrator 1 complex subunit 3-like 1
1	AJ851444	PTBP1	polypyrimidine tract binding protein 1
1	AJ851455	CLK3	CDC-like kinase 3
1	AJ851528	GTF2H4	general transcription factor IIH, polypeptide 4, 52kDa
1	AJ851539	CLN8	ceroid-lipofuscinosis, neuronal 8 (epilepsy, progressive with mental retardation)
1	AJ851557	USP12P1	ubiquitin specific peptidase 12 pseudogene 1
1	AJ851558	RIPK1	receptor (TNFRSF)-interacting serine-threonine kinase 1
1	AJ851606	PIK3CD	phosphoinositide-3-kinase, catalytic, delta polypeptide
1	AJ851640	MYD88	myeloid differentiation primary response gene (88)
1	AJ851693	HK1	hexokinase 1

Table 3-7 Continued

1	AJ851698	MYSM1	myb-like, SWIRM and MPN domains 1
1	AJ851709	GTF2A1	general transcription factor IIA, 1, 19/37kDa
1	AJ851725	ERLIN1	ER lipid raft associated 1
1	AJ851762	APBA2	amyloid beta (A4) precursor protein-binding, family A, member 2 binding protein
1	AJ851776	FAM129A	family with sequence similarity 129, member A
2	AJ851798	NRAS	neuroblastoma RAS viral (v-ras) oncogene homolog
1	AJ851819	STAT3	signal transducer and activator of transcription 3 (acute-phase response factor)
2	AY729886	RAP2B	RAP2B, member of RAS oncogene family
3	EF692644	FSTL4	similar to follistatin-like 4; follistatin-like 4
2	NM_001004387	EPHB6	EPH receptor B6
1	NM_001006201	CDK9	cyclin-dependent kinase 9
1	NM_001079714	COL1A2	collagen, type I, alpha 2
1	NM_001167765	CCNC	cyclin C
1	NM_204118	AHR	aryl hydrocarbon receptor
1	NM_204256	CDKN1B	cyclin-dependent kinase inhibitor 1B (p27, Kip1)
1	NM_204297	HIF1A	hypoxia-inducible factor 1, alpha subunit (basic helix-loop-helix transcription factor)
1	NM_204299	IRF4	interferon regulatory factor 4
1	NM_204381	THY1	Thy-1 cell surface antigen
1	NM_204390	SGMS1	sphingomyelin synthase 1
2	NM_204396	CIP1	cdk inhibitor CIP1 (p21)
1	NM_204926	USP2	ubiquitin specific peptidase 2
1	NM_205027	TMOD1	tropomodulin 1
2	NM_205115	LIMK2	LIM domain kinase 2
1	NM_205155	FASN	fatty acid synthase
1	NM_205206	INHBB	activin beta B
1	NM_205239	CCNB3	cyclin B3
1	NM_205240	ST6GAL NAC1	ST6 (alpha-N-acetyl-neuraminy-2,3-beta-galactosyl-1,3)-N-acetylgalactosaminide alpha-2,6-sialyltransferase 1
1	NM_205303	CAPN1	calpain 1, (mu/I) large subunit
1	NM_205367	ACVR2A	activin A receptor, type IIA
3	NM_205470	PCK2	phosphoenolpyruvate carboxykinase
1	NM_206863	VAV3	vav 3 oncogene
1	NM_206984	GJA4	gap junction protein, alpha 4, 37kDa
1	NM_207178	SLC2A2	solute carrier family 2 (facilitated glucose transporter), member 2
1	X03578	HRAS	v-Ha-ras Harvey rat sarcoma viral oncogene homolog
1	X72218	RB1	retinoblastoma 1 (including osteosarcoma)
3	XM_001232074	CABLES1	Cdk5 and Abl enzyme substrate 1
1	XM_001232308	PRPF4B	PRP4 pre-mRNA processing factor 4 homolog B (yeast)
1	XM_001233543	CHERP	calcium homeostasis endoplasmic reticulum protein
1	XM_414998	HNRPLL	heterogeneous nuclear ribonucleoprotein L-like
1	XM_415091	ULK1	unc-51-like kinase 1 (C. elegans)
1	XM_415266	POP5	processing of precursor 5, ribonuclease P/MRP subunit (S. cerevisiae)

Table 3-7 Continued

1	XM_416310	NUAK1	NUAK family, SNF1-like kinase, 1
1	XM_416445	CYB5R3	similar to cytochrome b-5 reductase
1	XM_416551	GTF2E1	hypothetical protein LOC769761; general transcription factor IIE, polypeptide 1, alpha 56kDa
1	XM_416587	SLC35A5	solute carrier family 35, member A5
1	XM_416621	CBLB	Cas-Br-M (murine) ecotropic retroviral transforming sequence b
1	XM_417640	MFN2	similar to CPRP1; mitofusin 2
1	XM_417993	ATP5F1	ATP synthase, H ⁺ transporting, mitochondrial F0 complex, subunit B1
1	XM_421129	PPP1R14D	protein phosphatase 1, regulatory (inhibitor) subunit 14D
1	XM_424462	NEDD4L	NEDD4L; hypothetical protein LOC776508; neural precursor cell expressed, developmentally down-regulated 4-like
1	XM_424684	SLC25A42	solute carrier family 25, member 42
1	XM_424734	MAP3K1	mitogen-activated protein kinase kinase kinase 1
1	XM_424739	PLK2	polo-like kinase 2 (Drosophila)
1	XM_424759	PIK3R1	phosphoinositide-3-kinase, regulatory subunit 1 (p85 alpha)
1	XM_425424	RELN	reelin

^aNumber of targets on each mRNA (gene) were established by searching how many times the accession number occurred in the 128filteredLow.txt filtered miranda file. Target genes in red are of particular interest, due to the multiple target sites on the mRNA, the particularly strong affinity of the seed region to 3'UTR binding site, or the protein's involvement in the pathogenesis of Marek's disease/ immune response.

Table 3-8. Condensed list of putative targets of gga-miR-147 after DAVID analysis.

# Targets ^a on mRNA	gb accession #	Symbol	Gene name/description
1	XM_413874	AKAP13	A kinase (PRKA) anchor protein 13
1	AJ720308	ABCE1	ATP-binding cassette, sub-family E (OABP), member 1
1	XM_424388	BAG4	BCL2-associated athanogene 4
1	XM_001233716	BAZ1B	bromodomain adjacent to zinc finger domain, 1B
1	NM_001039258	CDH1	cadherin 1, type 1, E-cadherin (epithelial)
1	NM_206859	CEBPG	CCAAT/enhancer binding protein (C/EBP), gamma
1	AJ851659	CD80	CD80 molecule
1	AM050135	CD86	CD86 molecule
1	NM_204605	F2	coagulation factor II (thrombin)
1	AJ720910	DHX30	DEAH (Asp-Glu-Ala-His) box polypeptide 30
1	AJ851526	EFTUD2	elongation factor Tu GTP binding domain containing 2
1	AJ719654	XPO7	exportin 7
1	NM_204885	EZR	ezrin
1	AJ890143	FASLG	Fas ligand (TNF superfamily, member 6)
1	AJ719529	FEM1B	fem-1 homolog b (C. elegans)
1	AJ721059	FOXN2	forkhead box N2
1	XM_001234504	FMNL1	formin-like 1
1	AJ851452	GPR89B	G protein-coupled receptor 89B
1	XM_415415	GPSM1	G-protein signaling modulator 1 (AGS3-like, C. elegans)
2	NM_204619	HOXA11	homeobox A11
1	XM_420813	HTRA3	HtrA serine peptidase 3
1	NM_204131	LIMK1	LIM domain kinase 1
1	AB260848	MMP16	matrix metalloproteinase 16 (membrane-inserted)
1	AJ720147	MOV10	Mov10, Moloney leukemia virus 10, homolog (mouse)
1	NM_205116	MYBPC3	myosin binding protein C, cardiac
1	NM_001167728	NAGA	N-acetylgalactosaminidase, alpha-
1	XM_415476	PRRX2	paired related homeobox 2
1	AJ851794	PIP4K2A	phosphatidylinositol-5-phosphate 4-kinase, type II, alpha
1	DQ340396	PRKAA2	protein kinase, AMP-activated, alpha 2 catalytic subunit
1	AJ719816	RAB22A	RAB22A, member RAS oncogene family
1	AJ851430	RAB8A	RAB8A, member RAS oncogene family
1	AJ720845	RASGRP3	RAS guanyl releasing protein 3 (calcium and DAG-regulated)
1	AJ719758	RASGEF1A	RasGEF domain family, member 1A
1	AJ851738	RNF166	ring finger protein 166
1	XM_419000	MYO10	similar to myosin X; myosin X
1	NM_204964	SLC11A1	solute carrier family 11 (proton-coupled divalent metal ion transporters), member 1
1	AJ720559	SLC9A8	solute carrier family 9 (sodium/hydrogen exchanger), member 8
1	AJ851535	SUV39H2	suppressor of variegation 3-9 homolog 2 (Drosophila)
1	AJ719860	VAMP7	synaptobrevin-like 1
1	NM_204940	T	T, brachyury homolog (mouse)
1	NM_204219	TRAF5	TNF receptor-associated factor 5

Table 3-8 Continued

1	U72641	TCF15	transcription factor 15 (basic helix-loop-helix)
3	NM_205315	TUBB2A	tubulin, beta 2A
1	AJ851443	WBP4	WW domain binding protein 4 (formin binding protein 21)

^aNumber of targets on each mRNA (gene) were established by searching how many times the accession number occurred in the 147filteredLow.txt filtered miranda file. Target genes in red are of particular interest, due to the multiple target sites on the mRNA, the particularly strong affinity of the seed region to 3'UTR binding site, or the protein's involvement in the pathogenesis of Marek's disease/ immune response.

Table 3-9. Three functional annotation clusters of predicted miR-128 target pathways

Pathway Cluster	gb#	Target mRNA	Gene Name
Natural killer cell mediated cytotoxicity	AJ851798	NRAS	neuroblastoma RAS viral (v-ras) oncogene homolog
	AJ720543	SYK	spleen tyrosine kinase
	X03578	HRAS	v-Ha-ras Harvey rat sarcoma viral oncogene homolog
	NM_206863	VAV3	vav 3 oncogene
ErbB signaling pathway	NM_204396	CIP1	cdk inhibitor CIP1 (p21)
	AJ851798	NRAS	neuroblastoma RAS viral (v-ras) oncogene homolog
	X03578	HRAS	v-Ha-ras Harvey rat sarcoma viral oncogene homolog
Regulation of actin cytoskeleton	AJ851798	NRAS	neuroblastoma RAS viral (v-ras) oncogene homolog
	X03578	HRAS	v-Ha-ras Harvey rat sarcoma viral oncogene homolog
	NM_206863	VAV3	vav 3 oncogene

Table 3-10. Two Functional annotation clusters of predicted miR-147 target pathways

Pathway Cluster	gb#	Gene Name
Cell adhesion molecules (CAMs)	AJ851659	CD80 CD80 molecule
	AM050135	CD86 CD86 molecule
	NM_001039258	CDH1 cadherin 1, type 1, E-cadherin (epithelial)
Intestinal immune network for IgA production	AJ851659	CD80 CD80 molecule
	AM050135	CD86 CD86 molecule

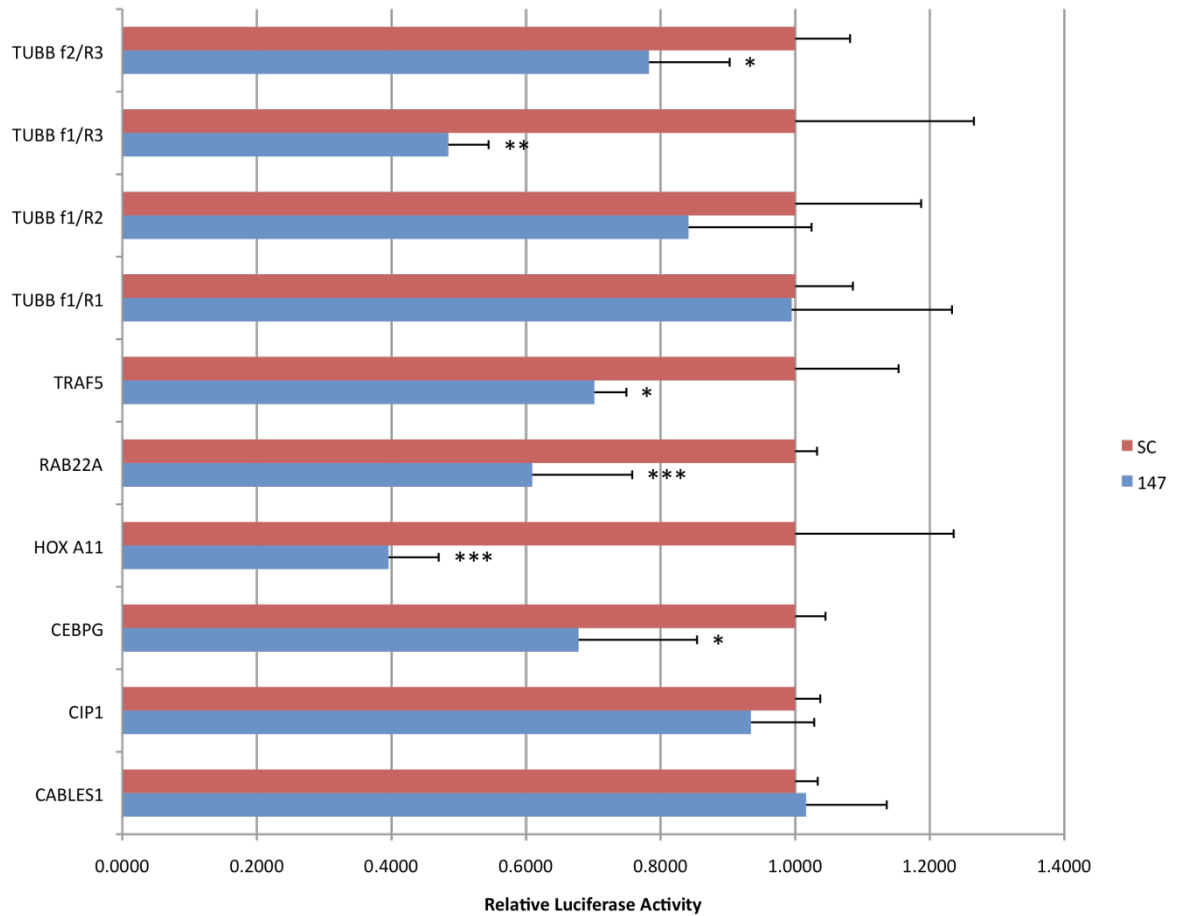


Figure 3-1. MiR-147 target validation results from dual luciferase assay. CABLES1 and CIP1 served as negative controls as these genes contained no predicted miR-147 binding sites. Scrambled control (SC) renilla/firefly ratios are shown in red. MiR-147 renilla/firefly ratios are shown in blue. TUBB f2/R3 contained only the third miR-147 binding site, TUBB f1/R3 contained all three binding sites, while TUBB f1/R2 contained the first two binding sites and TUBB f1/R1 contained only the first binding site. *: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$

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CHAPTER 4: FUTURE DIRECTIONS

The work presented here adds to the global understanding of host response to MDV infection. The state of Marek's disease research revolves around the notion that newer, more virulent strains are emerging, and we need to develop more effective methods to reduce the financial loss due to MD. The most recent efforts in Marek's disease research focus on this trend of increased MDV virulence. Of the most recent MD research, MDV pathology, candidate vaccines, and molecular and mathematical modeling are some of the more popular research areas. These topics reflect the international push to better understand MDV pathogenesis and by improving the effectiveness of protective measures like vaccines and selective breeding based on genotype.

In the field of MDV pathology, some of the more recent literature is looking at the problem of emergence of novel and more virulent field isolates of MDV-1. In addition to simply finding MDV-1 in ducks and geese as reservoir and carrier populations (Murata et al. 2012), new field isolates of MDV-1 are continually emerging (Tian et al. 2011, Wozniakowski et al. 2010, Wozniakowski et al. 2011) throughout the world. New isolates are expected, as all organisms collect mutations that result in a change in phenotype, and bacteria and viruses commonly evolve by collecting mutations that modulate the pathogen's virulence and survivability. In the last five decades, more virulent strains of MDV-1, characterized by increased incidence of neurologic lesions and high transmission rates, have emerged (Witter 1997, Shamblin et al. 2004). This presents a problem for farmers and researchers alike, as these more virulent strains are often more resistant to conventional vaccines (Tavlarides-Hontz et al. 2009).

The most recent publication on epidemiological modeling of MDV opened the door for comparative studies between novel and well-established vaccination strategies by using the contemporary parameters for infection in conjunction with the more established calculations for vaccine efficacy (Sharma and Burmester 1982). There are a myriad of new candidate vaccines for MDV being developed. Cell culture attenuated strains of MDV-1, recombinant viruses of MDV-1 strains, non-oncogenic MDV-2 strains, non-oncogenic MDV-3 (HVT), and bivalent vaccines like HVT + a MDV-2 strain like SB-1 (Calnek et al. 1983) are the currently the most popular vaccination strategies for protecting against MDV-1 transformation and subsequent oncogenesis.

In work by Chang et al. (Chang et al. 2011), the protective index (PI) and total Marek's disease (TMD, term which included any challenged chicken with nerve enlargement or visceral tumors or both, or challenged chickens dying more than 7 days post infection but before the end of the study) was calculated for the rMd5 Δ Meq, a recombinant form of the serotype 1 strain Md5 inserted into a cosmid with the Meq gene removed. The PI of rMd5 Δ Meq was compared in this paper to the PI and TMD of the CVI988/Rispens vaccine, a conventional vaccine attenuated from a MDV-1 strain (Rispens et al. 1972). This study showed that the recombinant vaccine was more effective than Rispens, except the rMd5 Δ Meq resulted in atrophy of lymphoid organs like the thymus and bursa of Fabricius. The results of this study are very exciting, but as with most novel/candidate vaccines, there is a downside, the bursal and thymic atrophy (BTA). However, this line of study is a great example of how continued research and development of a candidate vaccine can lead to a safe, effective, and commercially-viable vaccine. In fact, an even more recent study (Lee et

al. 2012) looked at the attenuation of rMd5ΔMeq in cell culture, expecting a change in the extent of BTA. The authors found that vaccinated/infected the MDV-susceptible (7₂) birds from the 15I₅ x 7₁ line of chickens which were negative for maternally-derived MDV-antibodies (MAB-), are particularly susceptible to BTA. In rMd5ΔMeq passaged from 40-50 times in primary duck embryonic fibroblasts (DEF), body weight and bursal and thymic weights were restored in MAB- chickens. Viremia titer (# of PFUs) and MDV-antibodies decreased as passage increased from 19 to 50. Additionally, MD-derived tumor incidence was lower in the rMd5ΔMeq/att versus the CVI988/Rispens relative to unvaccinated chickens. As stated in the Lee et al. paper, this observation “facilitates commercialization and licensing by vaccine manufacturers.” Furthermore, the authors noted that future studies should lead to a better understanding for the gene(s) responsible for BTA and *in vivo* lymphocyte replication.

Transcriptional and proteomic profiling of host gene expression in chickens infected with oncogenic MDV is perhaps one of the most promising areas of Marek’s disease research. Many studies of this nature have been performed recently (Lu et al. 2010, MacEachern et al. 2011, and Gimeno and Cortes 2011). Our group has done research on infectious diseases in chicken and pigs, looking at genetic and epigenetic factors associated with disease. The lab has recently focused on publishing studies on proteomic expression differences due to MDV infection (Liu et al. 2006, Ramarosan et al. 2008, and Chien et al. 2011) as well as investigation of miRNA expression in chickens (Hicks et al. 2008, Hicks et al. 2009, Trakooljul et al. 2010, Hicks et al. 2010, and Trakooljul et al. 2012). Marek’s disease virus, presents an appropriate bridge between these two lines of research, as it is the

causative agent for Marek's disease and the virus elicits differential protein expression (in part due to differential miRNA expression). Furthermore, the MDV genome codes for its own biologically-active miRNA. The work performed for this thesis unites the work done on the infectious diseases with miRNA expression in chickens. This work indicated that *miR-128* and *miR-147* were both differentially expressed under MDV-1 challenge. This conclusion brings about further questions. Does infection with vaccine strains of MDV bring about another miRNA expression profile?

Another logical question is whether the miRNA profile under infection with MDV-1 is specific to the spleen? Or is the expression profile similar in the other immune organs? The hypothesis of differential gene expression should be tested with a vaccinated treatment group and in more tissue types; at least in the bursa, thymus, and liver if not also in the brain and classically-inflamed peripheral nerves.

Another subject this research will logically lead to is an analysis of viral miRNA expression. The MDV genome codes for its own miRNAs—most of which are in the unique long and unique short regions. These viral miRNAs modulate endogenous mRNA translation and have furthermore been implicated in establishing latency or initiating the virus' transformation phase. Efforts are being made to elucidate the function of MDV-encoded miRNA in host-virus interactions (Morgan and Burnside 2011). Interestingly, some viral miRNA are homologous to chicken miRNA. For example, *mdv1-miR-M4* has an identical seed sequence to *gga-miR-155*, which targets mRNAs involved in immune function. Our group has acquired additional sequencing data in the course of completing this thesis that

will be analyzed and applied to this line of research and is likely to contribute to a better understanding of host-pathogen interactions and the herpesvirus infection cycle.

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