Effect of mdv1-miR-M4 and Related MicroRNAs On Expression of Innate Immune Response Genes In Chicken Embryo Fibroblasts

by

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A thesis submitted to the Faculty of the University of Delaware in partial fulfillment of the requirements for the degree of Bachelor of Science in Biological Sciences with a Concentration in Cell & Molecular Biology and Genetics with Distinction

Spring 2017

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ACKNOWLEDGMENTS

I would like to thank Dr. Robin Morgan for allowing me the opportunity to work in her lab while I have been at the University of Delaware. I have received much support while working in Dr. Morgan's lab and for that I am truly grateful. I thank Erin Bernberg for answering all of my questions, reading my thesis, and providing insight throughout my thesis writing process. I would like to thank Amy Anderson who prepared the recombinant HVT and helped me with general lab practices and data analysis. I extend my gratitude to Nazanin Sarpoulaki who helped me throughout the duration of the project by helping run experiments and put together and analyze data. Thanks to Grace Isaacs and Natalie Stevenson for introducing me to lab work. The primers used were obtained from Dr. Mark Parcells lab and I thank Upendra Katneni for preparing the primers and allowing our lab to use them. I thank my thesis committee of Dr. Robin Morgan, Dr. Mark Parcells, and Dr. Gary Laverty for taking the time to read my thesis and being present for my defense. Thank you to everyone who took part in supporting me and being patient with my many questions. My time at the University of Delaware would not have been so wonderful without all the guidance and kindness I have received from so many over the past four years.

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ABSTRACT

MicroRNAs (miRNAs) have been shown to post-transcriptionally regulate gene expression. Past studies have shown that Marek's disease virus (MDV) has multiple miRNAs in its genome. MDV1-miR-M4 is a particularly important miRNA found in MDV serotype 1 and this miRNA has been implicated in T cell lymphoma formation caused by MDV. MDV1-miR-M4 shares a seed sequence with miR-155, which is another oncogenic miRNA. MiR-155 is found across phylogeny. We inserted mdv1-miR-M4, the *meq* miRNA cluster, as well as miR-155 into the genome of herpesvirus of turkeys (HVT) in order to study the functions of these miRNAs since the non-recombinant parent HVT does not express them. The expression levels of interferons (IFNs), including IFN-alpha, IFN-beta, and IFN-lambda were examined post-infection. IFN-alpha and IFN-beta were not induced under the experimental conditions, and IFN-lambda was induced at approximately 48 hours post-infection. We also examined expression of IFN-inducible genes. Expression of the IFN-inducible genes MX-1 and OAS-3 was elevated in some instances under the experimental conditions. Expression of MX-1 and OAS-3 is typically type I IFN dependent, but in this case elevation was observed in the absence of induction of IFN-alpha and/or IFN-beta. Inhibition of MX-1 and OAS-3 induction by *meq* miRNAs and miR-155 when compared to infection with parent HVT was seen at 30 minutes, and at 3, 6, 15, 24, and 48 hours post-infection The results suggest that *meq* miRNAs, including MDV1-miR-M4 and the MDV1-miR-M4 analog, oncogenic miR-155, contribute to inhibition of innate immune responses.

INTRODUCTION

1.1 Introduction to Marek's Disease

Poultry is particularly important to the state of Delaware and especially to its economy. Marek's disease (MD) not only presents a threat to Delaware's poultry farms, but also presents a global threat to the poultry industry. Marek's disease (MD) is the most common clinical neoplastic condition of any organism, including man, and occurs in poultry-producing countries throughout the world (Payne, 1985). MD is caused by a herpesvirus, Marek's disease virus (MDV), and clinical signs of the disease vary. Infection of susceptible chickens with oncogenic MDV results in the formation of tumors that consist of T-cell lymphomas present in a variety of organs such as the lungs, spleen, kidney, liver, heart, and intestines. Classic MD disrupts nerve function and common signs of infection are leg paralysis, torticollis, and weight loss. MDV and related viruses are grouped into three serotypes. Serotype 1 includes all oncogenic types of the virus and their attenuated derivatives. Serotype 2 includes all naturally occurring non-oncogenic strains that infect chickens. Serotype 3 is a related, nononcogenic poultry herpesvirus that is found naturally in turkeys (Hirai, 2001). A serious MD outbreak can cause catastrophe to the poultry industry. Currently there is no cure for MD, and prevention of this disease by vaccination of susceptible chickens remains the primary means of control.

1.1.1 Pathogenesis

MDV is an alphaherpesvirus that is atypical in that it targets lymphocytes and eventually establishes latency within T-cells. Examples of alphaherpesviruses in humans are herpes simplex viruses 1 and 2, which cause herpesvirus infections, and varicella-zoster virus, which causes chicken pox and can reactivate to cause shingles later on in life. MDV infection results in productive and latent infections. Productive infection occurs in epithelial cells and in B-lymphocytes, while latent infection occurs in T lymphocytes and in transformed cells (Hirai, 2001).

MDV is a highly contagious disease and can easily spread among entire flocks. The disease is usually transmitted among chickens by inhalation of feather dander present in the environment (Hirai, 2001). Virus associated with feathers and dander is infectious, and contaminated poultry house dust remains infectious for at least several months at 20-25 C and for years at 4 C (Saif, 2003). After susceptible birds inhale particles infected with virus, cytolytic infection can be detected in the spleen, thymus, and other tissues. The primary target cells in the infected organs are B cells, although some activated T cells become infected and undergo degeneration as well (Saif, 2003).

After about one week from the onset of infection, the virus enters a latency phase within activated T cells. MDV preferentially targets CD4+ T cells, resulting in viral latency and immune evasion. Hereafter, MDV in the latency phase can no longer be readily detected by the host immune system while it continues to replicate inside the lymphocytes (Boodhoo et al, 2016). The length of the latency period can vary, and latency may not result in tumor formation. The latent infection can last the lifetime of the bird and some asymptomatic birds will continue to shed MDV-infected feathers into the environment and spread the viral infection throughout the flock.

1.2 MicroRNAs

MicroRNAs (miRNAs) are ~22-nucleotide noncoding RNAs that regulate gene expression post-transcriptionally. RNA polymerase II transcribes miRNAs to produce a primary miRNA (pri-miRNA). The pri-mRNA is then capped and polyadenylated and later processed into pre-miRNA by Drosha, which is a ribonuclease-like enzyme. Another ribonuclease-like enzyme, Dicer, then processes the pre-miRNA into double-stranded RNA that resembles a hairpin structure. One of the miRNA strands is used as the template for the RNA-induced silencing complex (RISC) to recognize complementary mRNA sequences while the second strand is typically degraded. RISC is a multi-protein complex of which a key component is an Argonaute (Ago) protein. Ago-loaded miRNAs (miRISC) typically bind to target transcripts and repress gene expression (Kincaid and Sullivan, 2012). Gene silencing is based upon complementarity between the miRNA bound to the Ago protein and mRNA. Ago proteins have endonuclease activity and are able to splice the mRNA, destabilize the mRNA, or repress translation.

MicroRNAs are being widely studied and many of their functions are still being identified. One miRNA, miR-155, is involved in the differentiation of B- and T-lymphocytes, and is found across phylogeny. Several oncogenic viruses have the ability to upregulate miR-155. An MDV-encoded version of miR-155, MDV1-miR-M4, is active in the pathogenesis of MD and has been implicated in MD tumor formation (Rodriguez, 2007) (Zhao, 2008). MDV1-miR-M4 and miR-155 share the same seed sequence (Morgan et al., 2008) (Zhao, 2009). MDV1-miR-M4 has been found to be highly expressed in solid tumors formed by the T. King pathogenic strain of MDV, which suggests that MDV1-miR-M4 plays a role in tumor formation or growth (Morgan et al., 2008). Further research has implicated MDV1-miR-M4 in

MDV oncogenicity using miRNA deletion mutants, with the deletion of miR-M4 greatly diminishing lymphoma formation (Zhao et. al, 2011)(Yu et al., 2014).

The *meq* gene is closely associated with a cluster of miRNAs in MDV as shown in Figure 1. The Meq protein is the strongest candidate oncoprotein described so far for MDV (Hirai, 2001). MDV1-miR-M4 is one of the seven miRNAs that are located immediately upstream of *meq*.

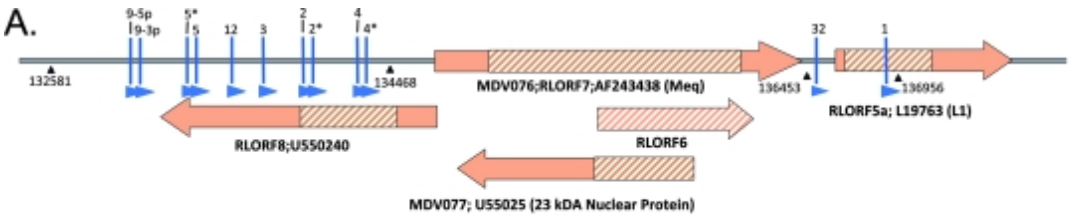

Figure 1: MicroRNA map of Marek's Disease Virus

Many viruses utilize miRNAs to aid in the survival and production of virus particles. Virus-encoded miRNAs can be grouped into two classes; i.e., those that are analogs of host miRNAs and those that are virus specific (Kincaid and Sullivan, 2012). Analogs of host miRNAs are similar in structure to host miRNAs whereas the other class is specific to the virus. The miRNAs modulate cellular regulation to ensure that the needs of the virus are met. Virus-encoded miRNAs help a virus evade the host immune response and inhibit apoptosis to allow for virus survival. Avoiding cellular apoptosis will allow for maximum viral production. Inhibition of apoptosis is likely to be a key factor in MD tumor formation.

1.3 Interferons and IFN Inducible Genes

MDV targets CD4+ cells, which are crucial to an organism's immune response against viral infections. CD4+ cells aid the immune system by releasing cytokines, such as interferons (IFNs). The IFN family of cytokines is now recognized as a key component of the innate immune response and the first line of defense against virus infection (Sadler and Williams, 2008). IFNs respond to the presence of pathogens, such as viruses, to help protect the organism, and in particular, to interfere with viral replication in cells. IFN alpha and IFN beta are type I IFNs, and IFN lambda is a type III IFN. Type III IFN was recently identified in chickens, and testing is still being done to discover its biological effects.

Many viruses have evolved ways of averting the host's immune response. HSV-1 infection is known to block the signaling effects of IFNs -alpha and -beta by reversing the effects of the double-stranded RNA-activated protein kinase (Melroe et. al, 2004). If the effects of IFNs are blocked, then IFN-stimulated genes (ISGs) may not be activated. IFN-stimulated genes (ISGs) are crucial components of the IFN responses as they set up the antiviral, antiproliferative, and immunoregulatory state in the host cells (Costa et al, 2012). When ISG expression is blocked, suppression of the host's immune response can occur.

Mx-1 (see below) and OAS-3 (see below) are two well-known antiviral genes. Myxovirus-resistance (Mx) proteins are large GTPases that interact directly with viral constituents (Ewald et al., 2011). Mx proteins are produced by host cells in response to IFN and have been shown to have the capability of limiting viral replication. Mx proteins have a well-characterized antiviral role and show a strict dependence on type I and type III IFN for their expression (Sadler and Williams, 2008). Chicken OAS-L is related to OAS-3 by sequence, but its function has not been fully delineated.

Nevertheless, OAS-3 is well understood. 2'-5'-oligoadenylate synthetase 3 (OAS-3) is a protein-coding gene. OAS-3 is stimulated by dsRNA and produces 2'-5'-linked oligoadenylates, which bind to RNase L, resulting in its dimerization and activation (Masuda et. al, 2012). Activated RNase L cleaves viral RNA transcripts as well as host RNAs. The activity of RNase L has the capability of inhibiting protein synthesis and therefore terminating viral replication. It has recently become apparent that the OAS proteins have additional antiviral functions that are independent of the RNase L activity but the mechanism remains unclear (Sadler and Williams, 2008). Masuda et al found that the mRNA expression of Mx and OAS in CEFs increased when treated with type III IFN, as well as when treated with type I IFN, in a dose-dependent manner (Masuda et al., 2012), suggesting that type III IFN does have antiviral capability.

We were interested in seeing how IFN-inducible genes OAS-3 and MX-1 expression was affected by *meq* miRNAs and miR-155. MiRNAs that flank *meq* do not differ between the MDV1 strains with different virulence levels, but expression of these miRNAs is much greater in more virulent strains (Morgan et al., 2008). We have hypothesized that increased expression of these miRNAs results in subversion of innate immunity and facilitates viral infections. Our approach to addressing this hypothesis was to use recombinant HVT strains that express MDV1 *meq* miRNAs and seeing if this expression affected induction of IFN-inducible genes OAS-3 and/or MX-1.

Chapter 2

MATERIALS AND METHODS

2.1 Chickens

Fertile specific pathogen-free eggs were obtained from Sunrise Farms for the first and second experimental sets and from Charles River Laboratories for the third experimental set. For all three experimental sets, eggs were incubated at the Delaware Biotechnology Institute.

2.2 Recombinant Preparation

The constructs of rHVT-M4, rHVT-155, and rHVT-meqmirs, which were used during infection, were prepared by Amy Anderson. rHVT-M4 expresses just mdv1-miR-M4, rHVT-meqmirs expresses all the upstream meq miRNAs, and rHVT-155 expresses gga-miR-155. The coding sequences for mdv1-miR-M4 and mdv1-meqmirs regions were amplified from MDV1 strain RBIB using PCR. The resulting amplicons were inserted into a specialized transfer vector, pVEC48, provided by Intervet International (Boxmeer, NL). The non-essential region US10 in pVEC48 can be used to insert the mdv1-miR-M4 and mdv1-meqmirs into the HVT genome (Amy Anderson, personal communication). A DNA segment containing the chicken miR-155 was removed from plasmid pBIC (Bolisetty et. al, 2009) with restriction enzymes and cloned into the US10 region of pVEC48.

2.3 Viral Cultures

Primary chicken embryo fibroblasts (CEFs) were prepared and incubated and then used to prepare secondary CEFs. Secondary CEFs were infected 24 hours after plating with parent HVT or one of the HVT recombinants. Each T75 flask received approximately 131,500 pfu/mL (total pfu/flask) of each virus. Titers were confirmed post plating. Uninfected CEFs were used as the control for all of the infected samples.

2.4 RNA Isolation

Total RNA was isolated from the cultures at 3, 6, and 48 hours post infection for experimental set 1 and at 30 minutes and 3, 6, 15, 24, and 48 hours post infection for experimental sets 2 and 3. RNA isolation was performed using RNeasy® (Qiagen). Genomic DNA from all samples was removed according to kit instructions. Isolation of RNA was confirmed by performing gel electrophoresis.

2.5 Polymerization Chain Reaction

Complementary DNA (cDNA) was synthesized from the isolated RNA via reverse transcription PCR using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). To determine the expression levels of gene products, quantitative real time PCR (qRT-PCR) was performed using gene-specific primers and SYBR PCR master mix (Qiagen). PCR was done using the 7500 Real Time PCR machine and each reaction was done in triplicate. Deaminase primers (Table 1) were used to amplify a reference gene such that CT values could be normalized. In addition, a specific primer (070; Table 1) was used to ensure equal infection across all samples. The CT threshold for all samples was set at 160,000 for data analysis. Fold changes were calculated using the ddCT method (ABI PRISM®) after CT values were obtained from the qRT-PCR.

Table 1: qPCR Primer Sequences

Primer Name	Primer Sequence
Deaminase F	5’-GCC AGG GAA AGC ACC AAA-3’
Deaminase R	5’-CCA GTG TCT CCC ACT GTC CA-3’
070 F	5’-AGC AAT ACG ACG ACG GAC AGT-3’
070 R	5’-GGT CGC GAT AGC AAT TTT GG-3’
IFN-alpha F	5’-CCA GCA CCT CGA GCA AT-3’
IFN-alpha R	5’-GGC GCT GTA ATC GTT GTC T-3’
IFN-beta F	5’-CCT CAA CCA GAT CCA GCA TT-3’
IFN-beta R	5’-GGA TGA GGC TGT GAG AGG AG-3’
IFN-lambda F	5’-ATC GGA AGT GGG ACA TAG-3’
IFN-lambda R	5’-GTC TCT GCC AGT CTC TG-3’
MX-1 F	5’-ATG GGC AAA TGG ACT TCT GCA ACG-3’
MX-1 R	5’-TGC CAG ATG TGG GAT AGT AGC CTT-3’
OAS-3 F	5’-ACA TCC TCG CCA TCA TCG A-3’
OAS-3 R	5’-GCG GAC TGG TGA TGC TGA CT-3’

Chapter 3

RESULTS

3.1 Quantitative Real Time PCR

Secondary CEFs were infected with HVT parent, recombinant HVT containing mdv1-miR-M4 (rHVT-M4), recombinant HVT containing miR-155 (rHVT-155), or recombinant HVT containing all of the *meq* microRNAs (rHVT-all). A control group of uninfected secondary CEF was utilized for each time point for comparison to the infected CEF. The initial experimental set included the time points of 3, 6, and 48 hours. The second and third experimental sets included the time points of 30 minutes and of 3, 6, 15, 24, and 48 hours.

3.1.1 Interferon-Inducible Gene Expression

MX-1 and OAS-3 genes were significantly induced in experimental sets 1 and 2 early on upon infection with HVT, indicating that HVT infection readily stimulates an immune response in CEFs. However, induction of MX-1 and OAS-3 expression was markedly lessened when HVT containing the *meq* miRNAs (rHVT-all or rHVT-M4) was used as the infecting virus. Infection with rHVT-155, which contains the analog of mdv1-miR-M4, also showed decreased induction of MX-1 and OAS-3 compared to the parent HVT. These results support our hypothesis that *meq* miRNAs inhibit innate immune responses, as the CEFs infected with the HVT recombinants were impaired in their ability to induce the OAS-3 and Mx-1 IFN-inducible genes, indicating that miRNAs can subvert immune responses. For experimental set 3, the

results were different, and neither parent HVT nor any of the recombinant derivatives induce MX-1 and OAS-3 to the extent seen using experimental sets 1 and 2. We do not have a definitive explanation for this discrepancy at this time (see discussion). The fold change values for Mx-1 are shown in Table 2 and graphically represented in Figures 2, 3, and 4. The fold change values for OAS-3 are shown in Table 3 and graphically represented in Figures 5, 6, and 7. Note that Figures 4 and 7 are on an adjusted y-axis scale to better see expression levels.

Table 2: MX-1 Fold Change Results

Experimental Set 1		
Time Point	**Sample**	**Fold Change**
3 hours	un CEF	1.00
	HVT	99.23
	rHVT-M4	6.88
	rHVT-155	34.23
	rHVT-all	10.84
6 hours	un CEF	1.00
	HVT	277.05
	rHVT-M4	47.67
	rHVT-155	140.48
	rHVT-all	80.46
48 hours	un CEF	1.00
	HVT	10.62
	rHVT-M4	2.27
	rHVT-155	2.16
	rHVT-all	3.65
Experimental Set 2		
30 min	un CEF	1.00
	HVT	214.60
	rHVT-M4	8.05
	rHVT-155	85.56
	rHVT-all	7.98
3 hours	un CEF	1.00
	HVT	92.26

	rHVT-M4	4.46
	rHVT-155	36.65
	rHVT-all	15.24
6 hours	un CEF	1.00
	HVT	562.06
	rHVT-M4	163.39
	rHVT-155	265.07
	rHVT-all	121.68
15 hours	un CEF	1.00
	HVT	496.62
	rHVT-M4	78.55
	rHVT-155	57.41
	rHVT-all	71.53
24 hours	un CEF	1.00
	HVT	24.51
	rHVT-M4	36.32
	rHVT-155	15.25
	rHVT-all	27.06
48 hours	un CEF	1.00
	HVT	22.68
	rHVT-M4	30.17
	rHVT-155	7.58
	rHVT-all	22.21
Experimental Set 3		
30 min	un CEF	1.00
	HVT	0.93
	rHVT-M4	1.02
	rHVT-155	4.97
	rHVT-all	0.85
3 hours	un CEF	1.00
	HVT	1.35
	rHVT-M4	1.17
	rHVT-155	8.14
	rHVT-all	1.38
6 hours	un CEF	1.00
	HVT	2.51
	rHVT-M4	2.62
	rHVT-155	14.42
	rHVT-all	3.27
15 hours	un CEF	1.00
	HVT	1.84

	rHVT-M4	4.74
	rHVT-155	5.01
	rHVT-all	5.58
24 hours	un CEF	1.00
	HVT	1.08
	rHVT-M4	2.56
	rHVT-155	2.75
	rHVT-all	1.89
48 hours	un CEF	1.00
	HVT	2.67
	rHVT-M4	4.02
	rHVT-155	3.62
	rHVT-all	3.76

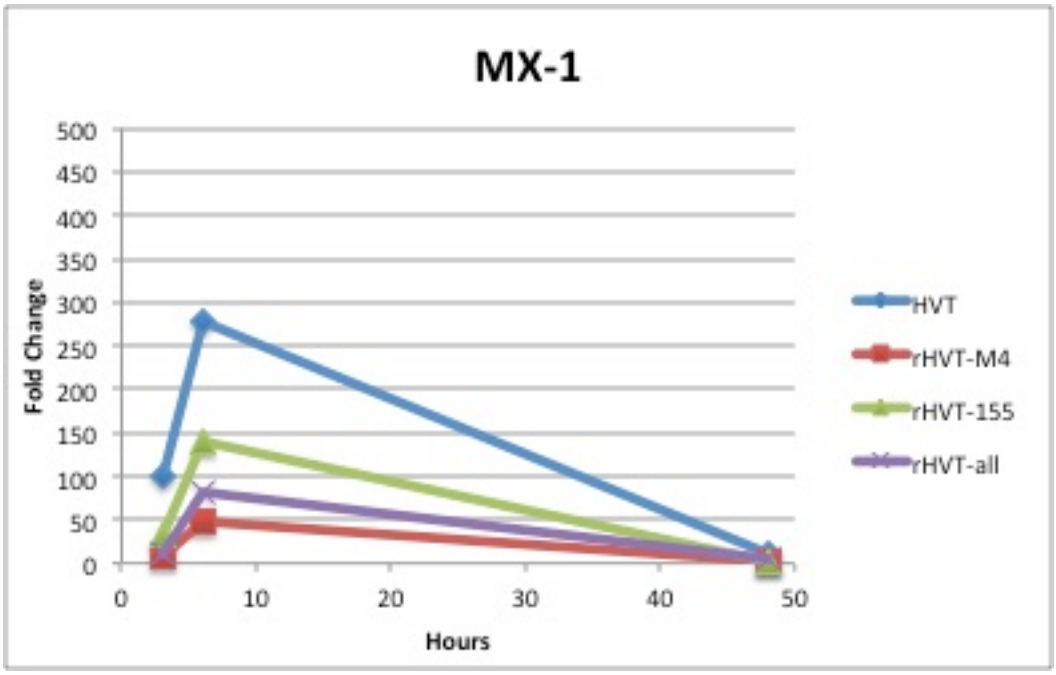

Figure 2: Experimental Set 1 MX-1 Compared to un CEF Fold Change Graph

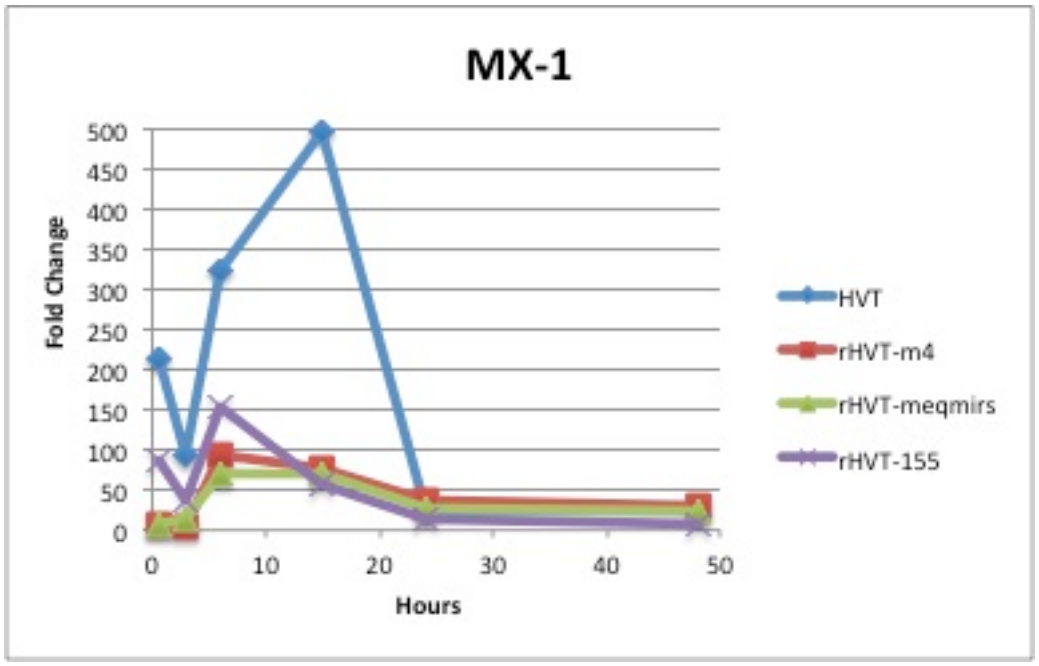

Figure 3: Experimental Set 2 MX-1 Compared to un CEF Fold Change Graph

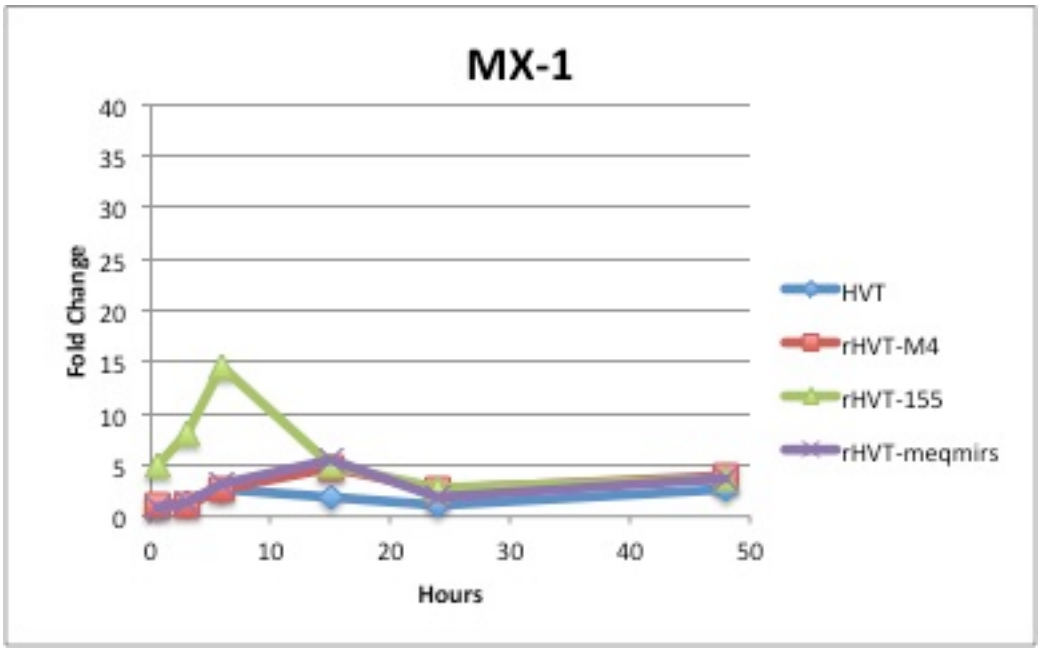

Figure 4: Experimental Set 3 MX-1 Compared to un CEF Fold Change Graph (note adjusted Y-axis)

Table 3: OAS-3 Fold Change Results

Experimental Set 1		
Time Point	**Sample**	**Fold Change**
3 hours	un CEF	1.00
	HVT	32.45
	rHVT-M4	3.42
	rHVT-155	7.54
	rHVT-all	3.12
6 hours	un CEF	1.00
	HVT	150.23
	rHVT-M4	33.64
	rHVT-155	48.34
	rHVT-all	21.69
48 hours	un CEF	1.00
	HVT	6.43
	rHVT-M4	2.00
	rHVT-155	2.88
	rHVT-all	1.76
Experimental Set 2		
30 min	un CEF	1.00
	HVT	23.52
	rHVT-M4	6.55
	rHVT-155	17.42
	rHVT-all	1.79
3 hours	un CEF	1.00
	HVT	33.25
	rHVT-M4	3.44
	rHVT-155	6.51
	rHVT-all	4.94
6 hours	un CEF	1.00
	HVT	112.31
	rHVT-M4	81.41
	rHVT-155	64.77
	rHVT-all	24.42
15 hours	un CEF	1.00
	HVT	52.76
	rHVT-M4	15.67
	rHVT-155	12.81
	rHVT-all	13.67
24 hours	un CEF	1.00
	HVT	10.21

	rHVT-M4	5.37
	rHVT-155	3.18
	rHVT-all	5.59
48 hours	un CEF	1.00
	HVT	13.28
	rHVT-M4	10.00
	rHVT-155	4.77
	rHVT-all	7.29
Experimental Set 3		
30 min	un CEF	1.00
	HVT	1.27
	rHVT-M4	1.96
	rHVT-155	5.04
	rHVT-all	1.78
3 hours	un CEF	1.00
	HVT	1.60
	rHVT-M4	2.76
	rHVT-155	8.35
	rHVT-all	3.10
6 hours	un CEF	1.00
	HVT	2.43
	rHVT-M4	4.00
	rHVT-155	13.95
	rHVT-all	4.93
15 hours	un CEF	1.00
	HVT	1.92
	rHVT-M4	3.25
	rHVT-155	3.45
	rHVT-all	4.05
24 hours	un CEF	1.00
	HVT	1.98
	rHVT-M4	2.82
	rHVT-155	2.53
	rHVT-all	2.71
48 hours	un CEF	1.00
	HVT	11.06
	rHVT-M4	15.62
	rHVT-155	9.59
	rHVT-all	17.01

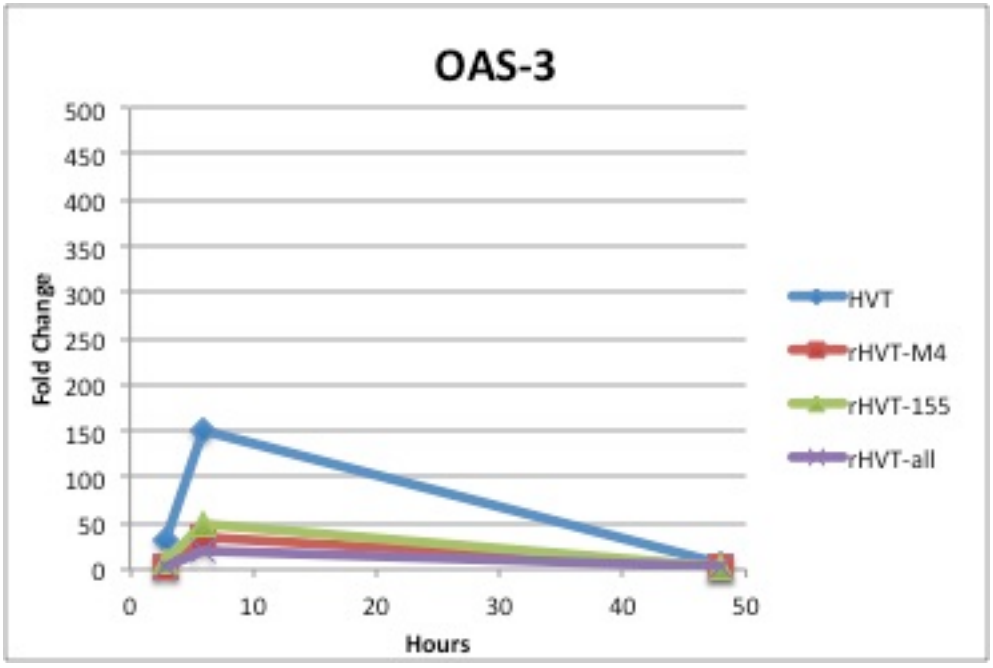

Figure 5: Experimental Set 1 OAS-3 Compared to un CEF Fold Change Graph

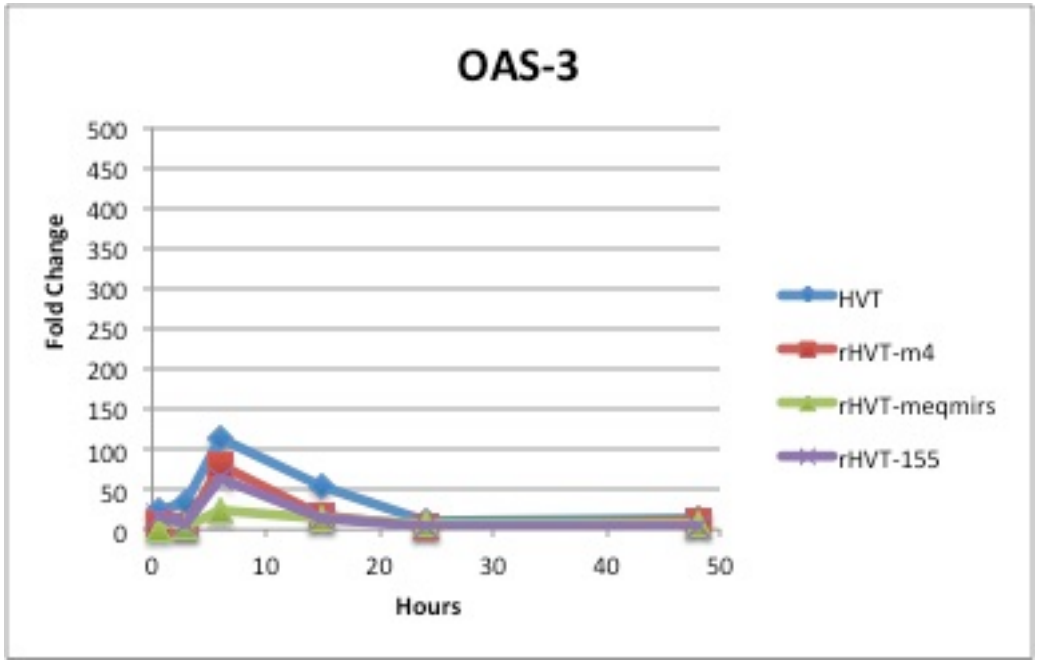

Figure 6: Experimental Set 2 OAS-3 Compared to un CEF Fold Change Graph

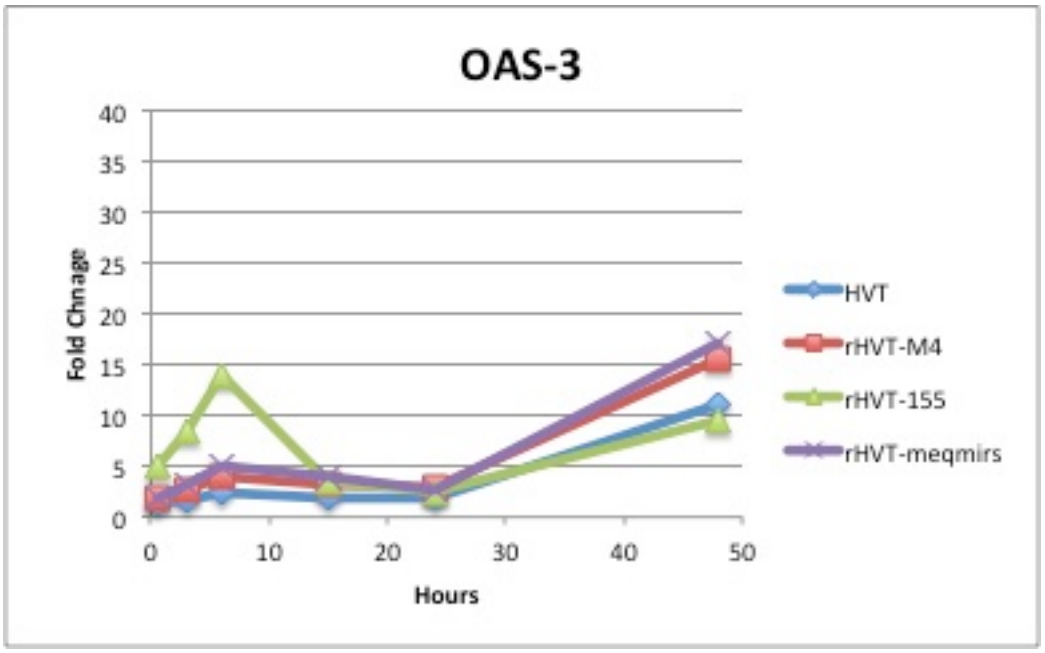

Figure 7: Experimental Set 3 OAS-3 Compared to un CEF Fold Change Graph (note adjusted Y-axis)

As mentioned above, when analyzing the CT values for the IFN-inducible genes, MX-1 and OAS-3, the results obtained with experimental set 3 were different from those obtained with experimental sets 1 and 2. Table 4 (below) compares the actual average CT values for all samples across the three experimental sets for the three common time points used among them. In some cases, the uninfected CEF CT value in experimental sets 1 and 2 (3 and 6 hour time points for the MX-1 data), but this was not a consistent finding across the time points.

Table 4: Mean CT values for MX-1 and OAS-3 for Common Time Points for Experimental Sets 1, 2, and 3

Time Point	Experimental Set #	Sample	Mean CT (MX-1)	Mean CT (OAS-3)

3 hr	Set 1	un CEF	34.08	29.48
		HVT	28.44	25.45
		rHVT-M4	31.73	28.14
		rHVT-155	28.99	26.57
		rHVT-all	31.50	28.70
	Set 2	un CEF	34.65	30.77
		HVT	28.73	26.32
		rHVT-M4	33.23	29.73
		rHVT-155	30.63	29.24
		rHVT-all	31.82	29.57
	Set 3	un CEF	32.28	30.35
		HVT	32.01	29.84
		rHVT-M4	32.75	29.59
		rHVT-155	30.04	28.08
		rHVT-all	32.17	29.08
6 hr	Set 1	un CEF	34.37	30.20
		HVT	25.95	22.67
		rHVT-M4	29.10	25.43
		rHVT-155	27.52	24.89
		rHVT-all	28.38	26.10
	Set 2	un CEF	36.11	31.12
		HVT	28.09	25.43
		rHVT-M4	30.60	26.61
		rHVT-155	29.33	26.37
		rHVT-all	29.41	26.74
	Set 3	un CEF	33.70	31.27
		HVT	31.54	29.15
		rHVT-M4	31.00	27.96
		rHVT-155	29.54	27.15
		rHVT-all	31.05	28.03
48 hr	Set 1	un CEF	30.73	28.02
		HVT	27.68	25.68
		rHVT-M4	29.79	27.25
		rHVT-155	30.40	27.27
		rHVT-all	29.41	27.75
	Set 2	un CEF	33.39	30.74
		HVT	29.41	27.54
		rHVT-M4	29.58	28.52
		rHVT-155	30.76	28.79
		rHVT-all	29.55	28.51
	Set 3	un CEF	34.14	31.80

		HVT	33.31	28.92
		rHVT-M4	32.41	28.12
		rHVT-155	31.97	28.23
		rHVT-all	32.71	28.20

3.1.2 Interferon Expression

Type I IFN (IFN alpha and IFN beta) was not robustly induced in any of the experimental sets following infection of CEF with HVT or recombinant derivatives. This result is consistent with known inhibition of type I IFN by other herpesviruses. The fold change did not exceed 7.52 for IFN-beta for any of the samples and did not exceed 5.47 for IFN-alpha. Both of these elevations of type I IFN occurred approximately 30 minutes post-infection, which appears reasonable as IFN repression takes time. IFN-alpha fold change values are shown in Table 4 and graphically represented in Figures 8, 9, and 10. IFN-beta fold change values are shown in Table 5 and graphically represented in Figures 11, 12, and 13. Type III IFN (IFN lambda) was not induced early post-infection but was induced relatively late at around 48 hours post-infection. The highest fold change when compared to uninfected CEF among the three experimental sets for HVT-infected CEF was 7.98, 14.52 for rHVT-M4, 6.66 for rHVT-155, and 11.92 for rHVT-all. IFN lambda induction does not appear to be inhibited by *meq* miRNAs any more so than with parent HVT or miR-155. IFN-lambda fold change values are shown in Table 6 and graphically represented in Figures 14, 15, and 16.

Table 5: IFN-alpha Fold Change Results

Experimental Set 1		
Time Point	**Sample**	**Fold Change**

3 hours	un CEF	1.00
	HVT	1.06
	rHVT-M4	1.39
	rHVT-155	0.47
	rHVT-all	0.54
6 hours	un CEF	1.00
	HVT	0.42
	rHVT-M4	1.52
	rHVT-155	0.48
	rHVT-all	0.36
48 hours	un CEF	1.00
	HVT	0.75
	rHVT-M4	0.76
	rHVT-155	0.54
	rHVT-all	0.42
Experimental Set 2		
30 min	un CEF	1.00
	HVT	2.73
	rHVT-M4	5.47
	rHVT-155	5.00
	rHVT-all	1.18
3 hours	un CEF	1.00
	HVT	2.16
	rHVT-M4	2.08
	rHVT-155	2.17
	rHVT-all	1.93
6 hours	un CEF	1.00
	HVT	1.29
	rHVT-M4	1.73
	rHVT-155	2.11
	rHVT-all	0.76
15 hours	un CEF	1.00
	HVT	2.25
	rHVT-M4	3.29
	rHVT-155	2.03
	rHVT-all	2.65
24 hours	un CEF	1.00
	HVT	1.28
	rHVT-M4	1.45
	rHVT-155	0.69
	rHVT-all	1.12

48 hours	un CEF	1.00
	HVT	1.67
	rHVT-M4	1.00
	rHVT-155	0.91
	rHVT-all	0.70
Experimental Set 3		
30 min	un CEF	1.00
	HVT	1.25
	rHVT-M4	1.77
	rHVT-155	1.46
	rHVT-all	1.25
3 hours	un CEF	1.00
	HVT	0.93
	rHVT-M4	1.08
	rHVT-155	2.04
	rHVT-all	1.20
6 hours	un CEF	1.00
	HVT	1.95
	rHVT-M4	1.57
	rHVT-155	0.97
	rHVT-all	1.32
15 hours	un CEF	1.00
	HVT	1.04
	rHVT-M4	0.75
	rHVT-155	1.06
	rHVT-all	0.85
24 hours	un CEF	1.00
	HVT	0.76
	rHVT-M4	1.13
	rHVT-155	0.94
	rHVT-all	1.17
48 hours	un CEF	1.00
	HVT	0.83
	rHVT-M4	1.43
	rHVT-155	0.95
	rHVT-all	1.12

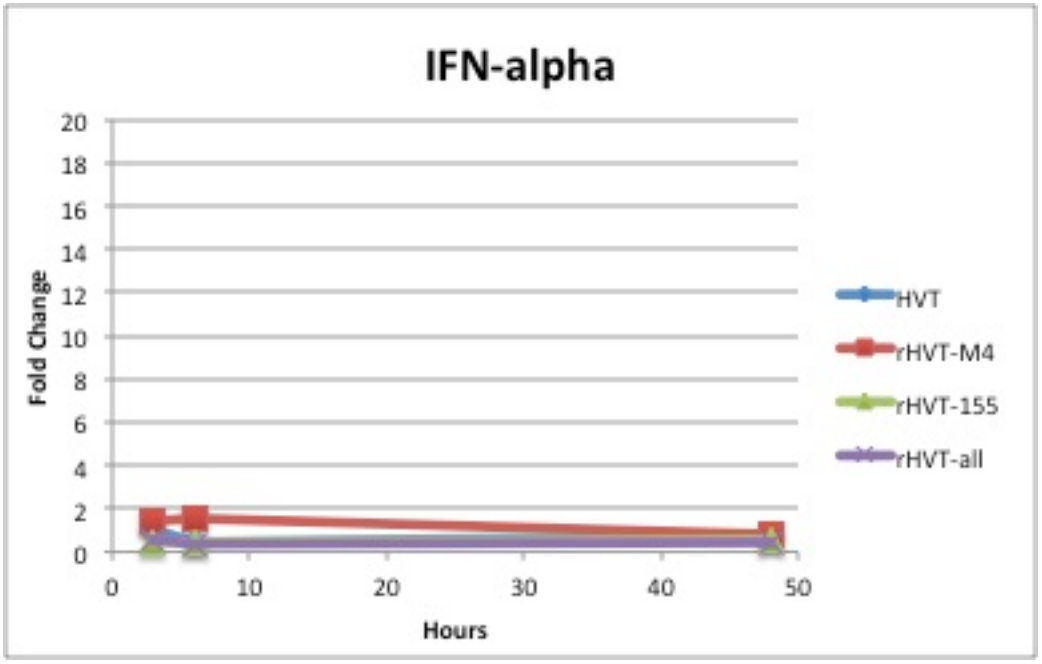

Figure 8: Experimental Set 1 IFN-alpha Compared to Un CEF Fold Change Graph

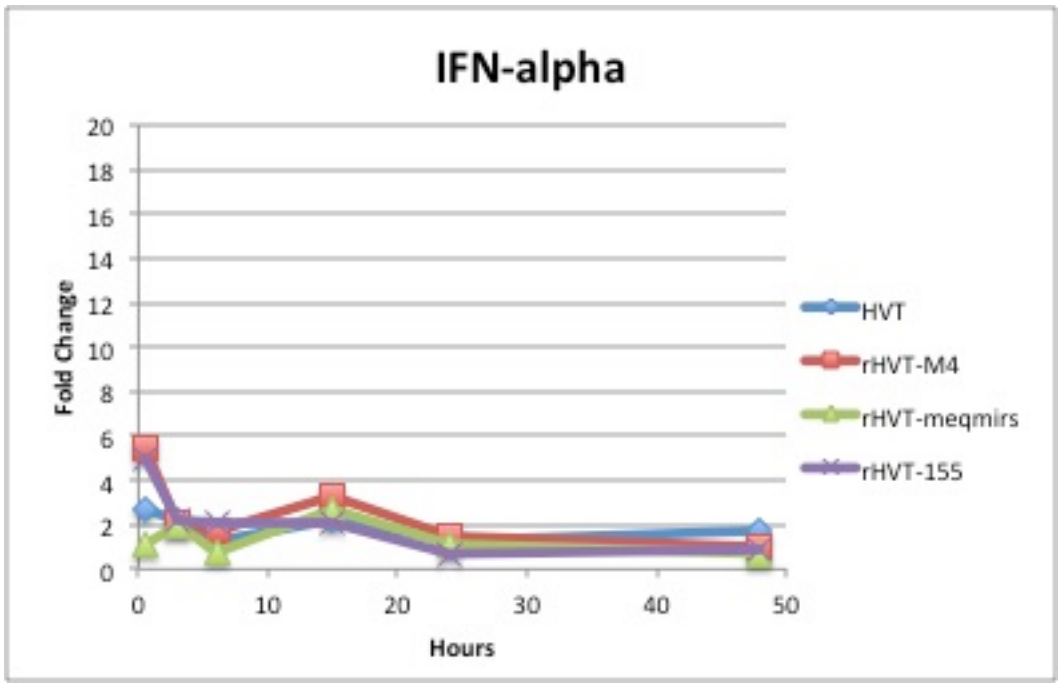

Figure 9: Experimental Set 2 IFN-alpha Compared to un CEF Fold Change Graph

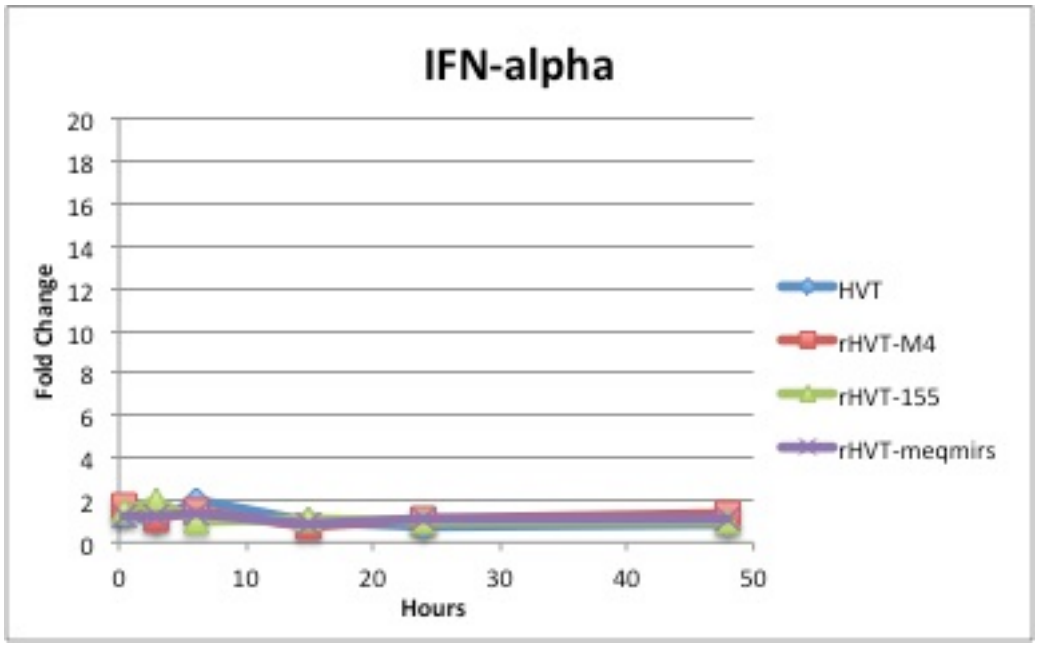

Figure 10: Experimental Set 3 IFN-alpha Compared to un CEF Fold Change Graph

Table 6: IFN-beta Fold Change Results

Experimental Set 1		
Time Point	**Sample**	**Fold Change**
3 hours	un CEF	1.00
	HVT	1.19
	rHVT-M4	1.22
	rHVT-155	0.50
	rHVT-all	0.44
6 hours	un CEF	1.00
	HVT	0.72
	rHVT-M4	1.21
	rHVT-155	0.48
	rHVT-all	0.49
48 hours	un CEF	1.00
	HVT	0.60

	rHVT-M4	0.58
	rHVT-155	0.43
	rHVT-all	0.30
Experimental Set 2		
30 min	un CEF	1.00
	HVT	3.39
	rHVT-M4	6.59
	rHVT-155	7.52
	rHVT-all	2.37
3 hours	un CEF	1.00
	HVT	2.37
	rHVT-M4	3.19
	rHVT-155	3.66
	rHVT-all	3.60
6 hours	un CEF	1.00
	HVT	1.93
	rHVT-M4	3.25
	rHVT-155	3.83
	rHVT-all	1.16
15 hours	un CEF	1.00
	HVT	2.59
	rHVT-M4	3.96
	rHVT-155	2.46
	rHVT-all	3.05
24 hours	un CEF	1.00
	HVT	1.47
	rHVT-M4	1.89
	rHVT-155	0.97
	rHVT-all	1.61
48 hours	un CEF	1.00
	HVT	2.21
	rHVT-M4	1.29
	rHVT-155	1.40
	rHVT-all	1.19
Experimental Set 3		
30 min	un CEF	1.00
	HVT	1.18
	rHVT-M4	1.87
	rHVT-155	1.47
	rHVT-all	1.09
3 hours	un CEF	1.00

	HVT	1.08
	rHVT-M4	1.00
	rHVT-155	1.98
	rHVT-all	1.09
6 hours	un CEF	1.00
	HVT	1.70
	rHVT-M4	1.29
	rHVT-155	0.69
	rHVT-all	0.98
15 hours	un CEF	1.00
	HVT	1.16
	rHVT-M4	0.74
	rHVT-155	0.81
	rHVT-all	0.74
24 hours	un CEF	1.00
	HVT	0.76
	rHVT-M4	0.84
	rHVT-155	0.78
	rHVT-all	1.02
48 hours	un CEF	1.00
	HVT	0.78
	rHVT-M4	1.17
	rHVT-155	0.58
	rHVT-all	0.87

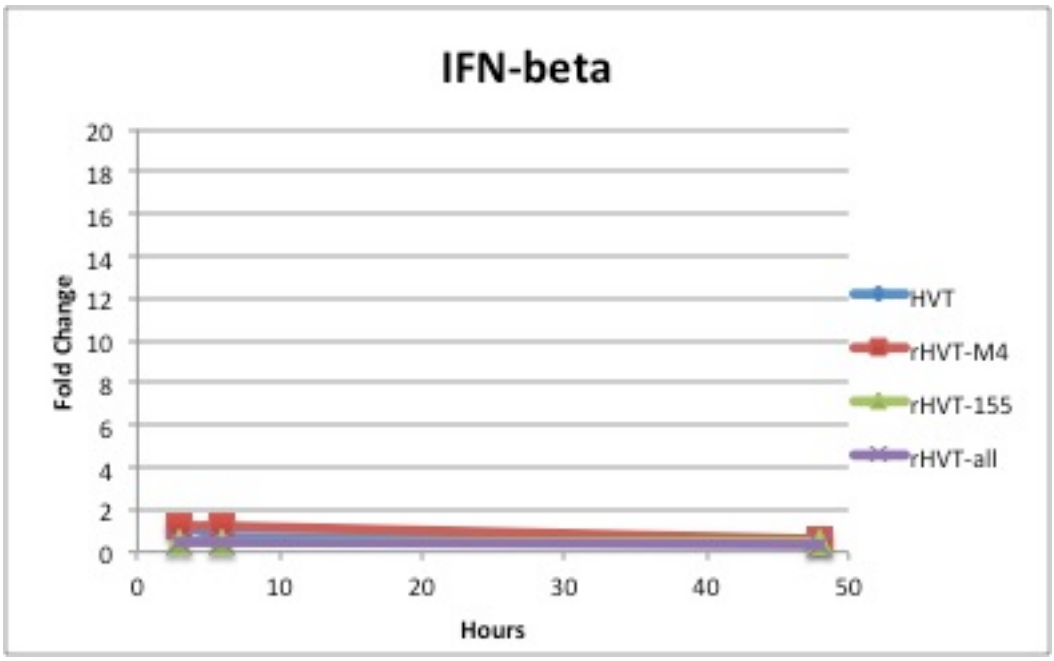

Figure 11: Experimental Set 1 IFN-beta Compared to un CEF Fold Change Graph

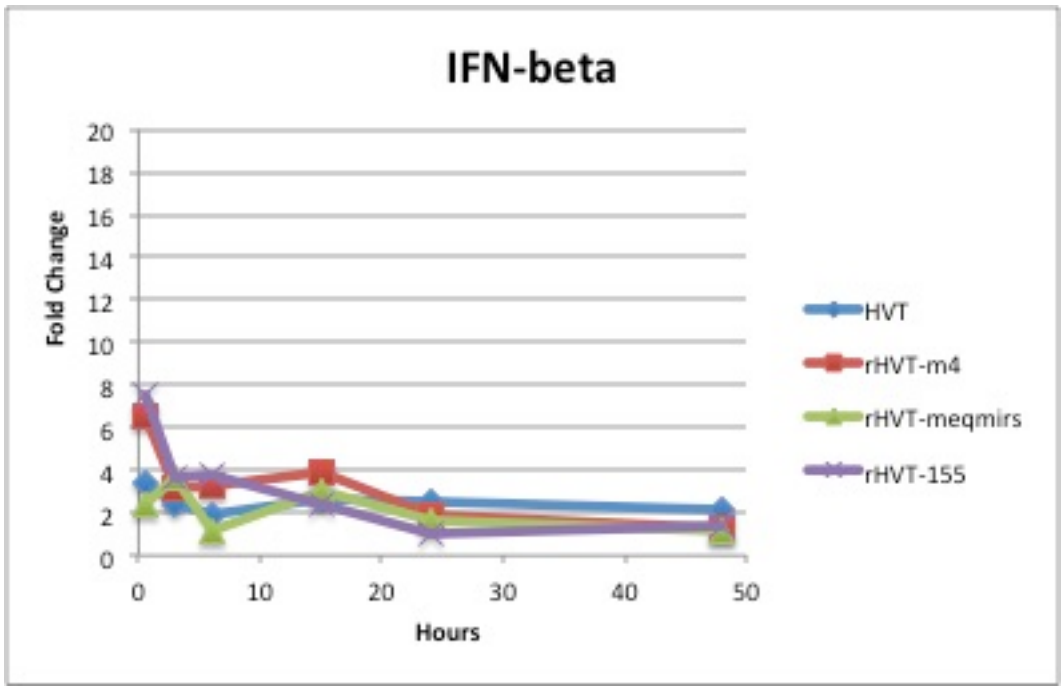

Figure 12: Experimental Set 2 IFN-beta Compared to un CEF Fold Change Graph

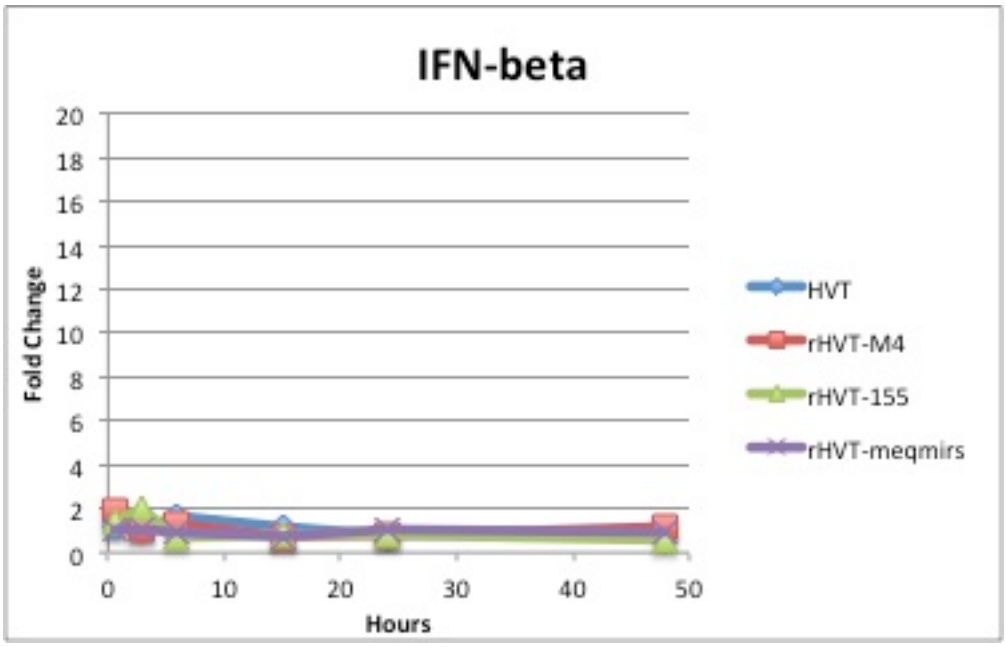

Figure 13: Experimental Set 3 IFN-beta Compared to un CEF Fold Change Graph

Table 7: IFN-lambda Fold Change Results

Experimental Set 1		
Time Point	**Sample**	**Fold Change**
3 hours	un CEF	1.00
	HVT	2.77
	rHVT-M4	2.04
	rHVT-155	1.60
	rHVT-all	0.84
6 hours	un CEF	1.00
	HVT	1.31
	rHVT-M4	2.53
	rHVT-155	1.58
	rHVT-all	0.88
48 hours	un CEF	1.00
	HVT	6.00
	rHVT-M4	9.05
	rHVT-155	6.66
	rHVT-all	11.92
Experimental Set 2		
30 min	un CEF	1.00
	HVT	4.77
	rHVT-M4	2.51
	rHVT-155	3.56
	rHVT-all	1.34
3 hours	un CEF	1.00
	HVT	2.15
	rHVT-M4	1.33
	rHVT-155	2.48
	rHVT-all	1.81
6 hours	un CEF	1.00
	HVT	1.74
	rHVT-M4	0.57
	rHVT-155	1.38
	rHVT-all	1.22
15 hours	un CEF	1.00
	HVT	0.47
	rHVT-M4	0.37
	rHVT-155	0.31
	rHVT-all	0.23
24 hours	un CEF	1.00
	HVT	1.58

	rHVT-M4	1.33
	rHVT-155	1.25
	rHVT-all	1.13
48 hours	un CEF	1.00
	HVT	2.41
	rHVT-M4	2.35
	rHVT-155	3.22
	rHVT-all	1.41
Experimental Set 3		
30 min	un CEF	1.00
	HVT	1.04
	rHVT-M4	1.58
	rHVT-155	0.99
	rHVT-all	1.36
3 hours	un CEF	1.00
	HVT	0.99
	rHVT-M4	1.09
	rHVT-155	1.95
	rHVT-all	1.76
6 hours	un CEF	1.00
	HVT	2.18
	rHVT-M4	1.57
	rHVT-155	1.79
	rHVT-all	1.87
15 hours	un CEF	1.00
	HVT	0.91
	rHVT-M4	0.71
	rHVT-155	0.88
	rHVT-all	1.06
24 hours	un CEF	1.00
	HVT	2.04
	rHVT-M4	2.19
	rHVT-155	1.80
	rHVT-all	2.50
48 hours	un CEF	1.00
	HVT	7.98
	rHVT-M4	14.52
	rHVT-155	5.72
	rHVT-all	10.26

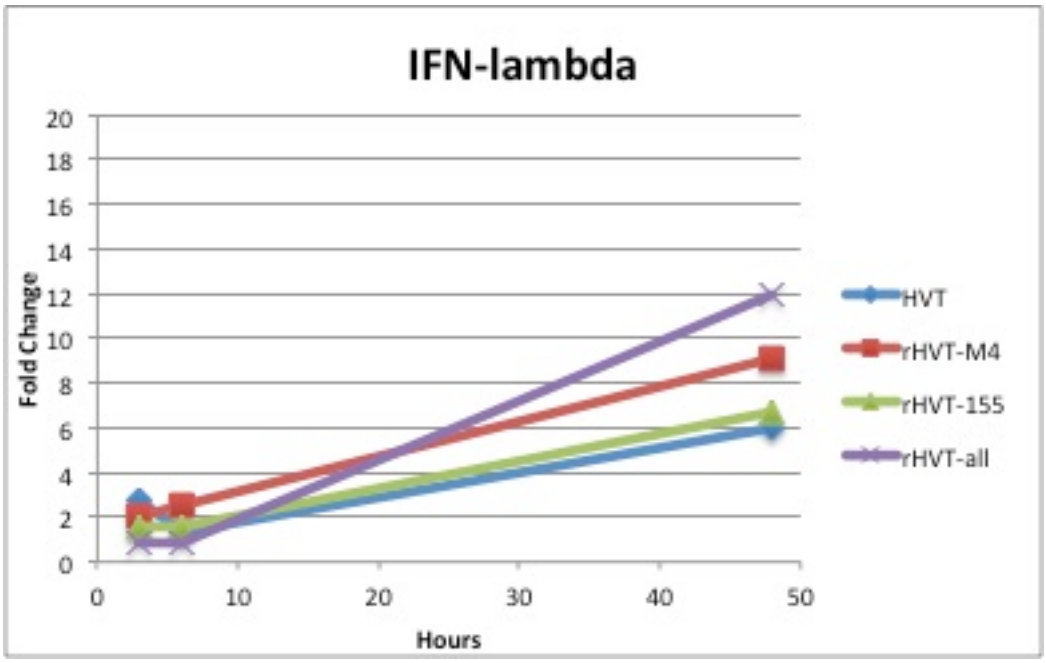

Figure 14: Experimental Set 1 IFN-lambda Compared to un CEF Fold Change Graph

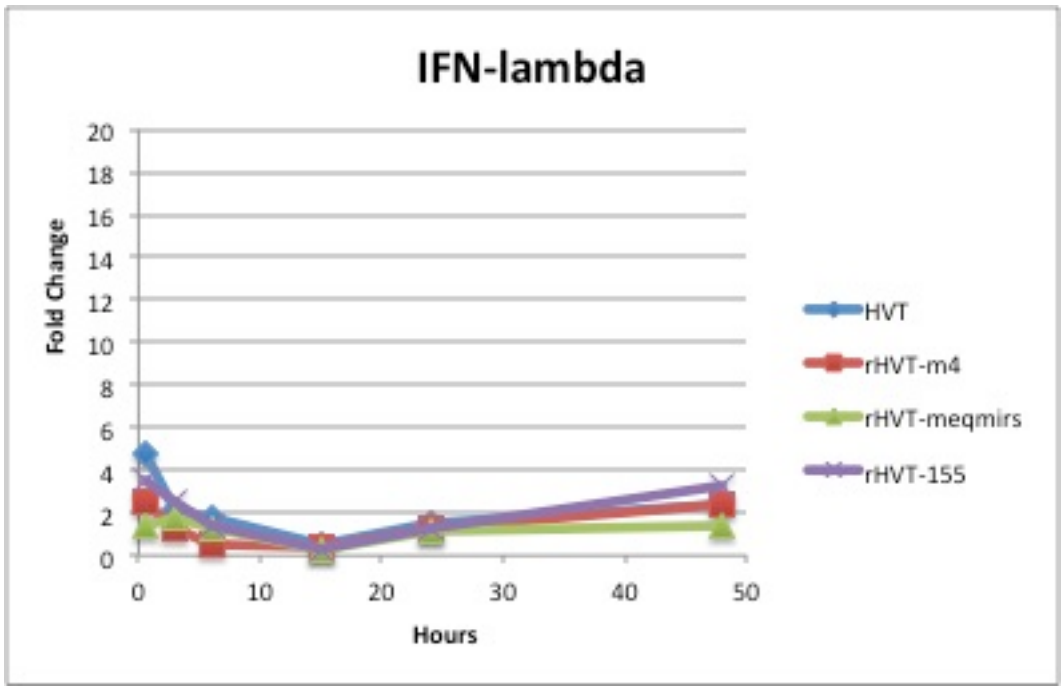

Figure 15: Experimental Set 2 IFN-lambda Compared to un CEF Fold Change Graph

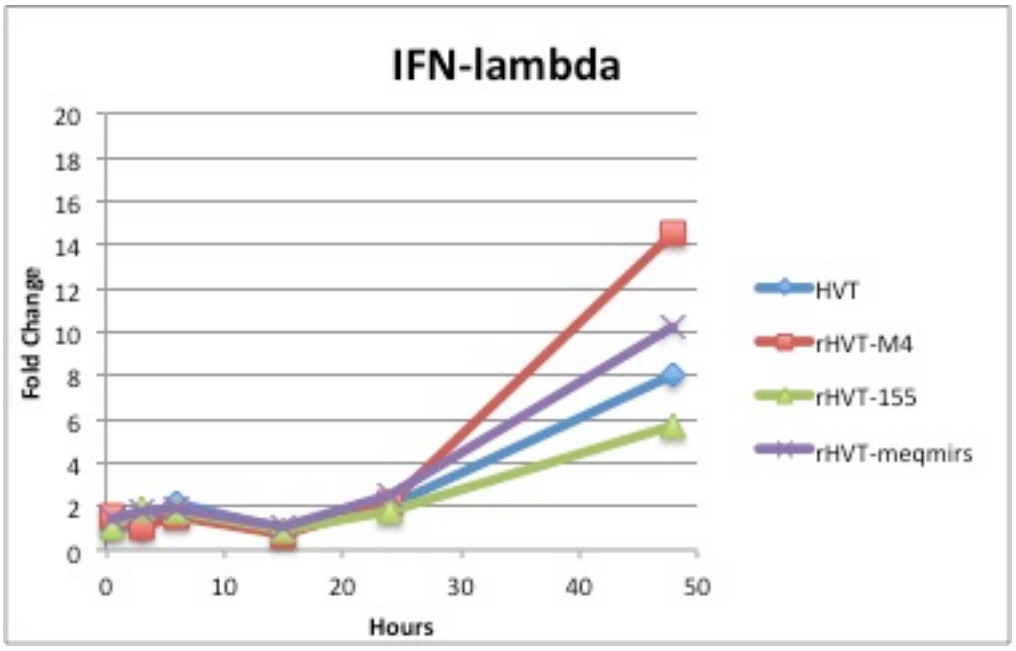

Figure 16: Experimental Set 3 IFN-lambda Compared to un CEF Fold Change Graph

Chapter 4

DISCUSSION

MicroRNAs (miRNAs) can be variable in their functions and we examined a subset of mdv1 miRNAs and one analog to evaluate the effects of these miRNAs on genes involved in innate immune responses in chickens. Mdv1-miR-M4 is one of the miRNAs that flank the mdv1 *meq* gene, and miR-155 is an analog of mdv1-miR-M4. Upon analysis of Mx-1 and OAS-3 expression by qRT-PCR in three biological replicate samples, the results for experimental sets 1 and 2 were similar, but these varied from results obtained using experimental set 3. In other words, the fold changes for experimental set 3 were very different when compared to experimental sets 1 and 2.

We can think of two possible explanations for these results with OAS-3 and Mx-1 expression in experimental set 3. First, experimental sets 1 and 2 were done using eggs from Sunrise Farms, while experimental set 3 was done using eggs from Charles River Laboratories. These eggs were from chickens with different genetic backgrounds. It is possible that throughout the course of commercial breeding of chicken lines over many decades, selection for increased innate immunity has occurred. We know that the Mx gene is highly polymorphic in chickens (Ko et al., 2002), and it is possible that polymorphisms in these genes would be contributing to the differing levels of background expression in uninfected CEF. Unfortunately, eggs from Sunrise Farms are no longer available, so it is not possible to directly compare the background level of expression of OAS-3 and Mx-1 between CEF prepared from Sunrise versus Charles River eggs. However, we could obtain eggs from a variety of

legacy and modern chicken breeds and compare the background levels of expression of these IFN-inducible genes.

A second possible explanation for the difference among the experimental sets concerns an incubator malfunction that occurred in the laboratory at about the time that experimental set 3 was being prepared. After experiencing some challenges with other experiments, we learned that the CO2 sensor in our incubator was defective. It is possible that the sub-optimal CO2 level or the inconsistencies in CO2 levels due to the defective sensor may have stressed the CEF cultures used for experimental set 3. This could be addressed by redoing several of the key time points using Charles River eggs but making certain that the incubator is functioning optimally. The 070 primer was used to ensure equal infection among all of the samples and the deaminase primer was used to check that the RNA levels in each sample was equal across samples so this cannot account for the differences in CT values between sets.

In two of our three biological replicates, expression of OAS-3 and Mx-1 appears to be robustly induced by infection of CEFs with parent HVT. However, in these samples, induction of Mx-1 and OAS-3 genes was repressed when *meq* miRNAs were present during the infection. Mx-1 and OAS-3 induction was also inhibited when miR-155 was delivered during infection. Both Mx-1 and OAS-3 encode antiviral proteins, therefore inhibition of their expression should suppress a host's innate immune responses and enable the virus to better establish infection and produce progeny. In other words, Mx-1 and OAS-3 suppression should decrease the ability of the host to limit and terminate viral replication. We have more work to do in order to clarify what happened with our third experimental sample set, but at least two of the

sample sets yielded results consistent with the notion the mdv1-miR-M4 and its analog miR-155 can suppress induction of OAS-3 and Mx-1 following HVT infection.

In the initial experimental data set with the three time points, the earliest time point sampled was 6 hours post infection. In order to assess IFN expression earlier during infection, the 30-minute time point was added to the second and third experimental samples. IFN repression due to viral infection takes time, therefore utilizing the earlier time points allowed us to see if the IFNs were activated earlier post-infection. However, in these experimental samples, IFN expression was not highly elevated for any of the experimental sets with parent HVT or recombinant viruses, indicating that IFN expression is suppressed very early post-infection. The IFN-inducible genes Mx-1 and OAS-3 were induced in two of the experimental sets despite apparent lack of robust IFN induction with HVT or recombinant HVT infection. This suggests that these genes may have a type I IFN independent pathway for expression. It is possible that the timing remains an issue, and IFN induction occurs much earlier than 30 minutes post-infection. Given the challenges of MDV infections in CEFs, it is not feasible to prepare samples earlier than about 30 minutes post infection. Immune pathways are highly complex, and so it is not surprising that the IFN-inducible genes and the IFN expression levels varied. Cells are likely to have evolved alternate routes to fight viruses besides the classically established pathways or the known steps that link IFN and IFN-inducible gene expression.

In conclusion, we have shown that expression of mdv1-miR-M4 likely plays a role in augmenting inhibition of the innate immune response. We have shown that typically infection with the mdv1-miR-M4 recombinant decreases expression of IFN-inducible genes OAS-3 and Mx-1 post-infection. It has been shown that infection with

the miR-155 recombinant inhibits IFN-inducible gene expression about as well as mdv1-miR-M4 and *meq* miRNAs and likely has a function similar to that of mdv1-miR-M4. Some of these findings, however, may be species-specific, and more research will need to be done to confirm them.

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