2016

# Development of Inducible Anti-influenza Therapies

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# Development of Inducible
# Anti-influenza Therapies

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**Dissertation submitted to the School of Medicine
at West Virginia University in partial fulfillment
of the requirements for the degree of**

**Doctor of Philosophy
in
Immunology and Microbial Pathogenesis**

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2016**

**Keywords: influenza virus, antiviral therapy, conserved promoter, inducible, RNA interference (RNAi), nonfunctional neuraminidase, universal, antigenic variation, antigenic shift, pandemic**

**Abstract**

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Cynthia Marie McMillen

Influenza viruses continue to cause significant morbidity and mortality each year despite the development of vaccines and antiviral therapies targeting these viruses. The inherent ability of influenza viruses to accumulate mutations over time has led to the emergence of strains resistant to antiviral therapies. Furthermore, genetic reassortment creates antigenically diverse viruses, making it difficult to develop vaccines that yield broad protection. The objective of the following research studies is to develop two alternative approaches to current methods of antiviral therapeutics.

Six new siRNAs targeting influenza protein expression by RNA interference (RNAi) were characterized. Three siRNAs (M747, M776, M832) knocked down the expression of matrix protein 2 and attenuated influenza infection to a similar degree as MDCK cells treated with a previously published siRNA, M950.  The three siRNAs (NS570, NS595, NS615) that target the nonstructural protein 1 and 2 genes promoted the expression of type I interferons, but were unable to attenuate the production of infectious virus. However NS595- and NS615-siRNAs promoted the production of defective interfering viruses. Another siRNA, M331, was able knock down the expression matrix 1 and matrix 2 and attenuate viral replication. Combination siRNA treatment was found to attenuate 20.9% more infectious virus than M950-siRNA treatment alone. Treatment with a single siRNA (M331, NS570, NS595, or NS615) that targets two protein coding sequences was able to knock down the expression of two proteins, thus enhancing the utilities of the siRNAs.

To further take advantage of RNAi as a mechanism to attenuate influenza infection, we developed an inducible anti-influenza therapy containing the influenza conserved promoter that expresses asRNAs only after influenza infection or in the presence of the influenza virus RNA-dependent

RNA polymerase (RdRP). asRNA expression was restricted to pM950, pM776, pNS595, or pNA105 treated cells containing the RdRP. The asRNAs expressed from the inducible asRNA expression vectors (pM776 or pNS595) were 84- to 343-fold below the concentration needed to reduce influenza virus infection by RNAi, thus illustrating the need for improved expression kinetics. Limiting expression of asRNAs within influenza infected cells could potentially reduce the adverse effects and limitation of RNAi therapeutics.

In an attempt to reverse antigenic variation and attenuate influenza titer, we developed additional inducible anti-influenza therapies (pUC57 NF-NA and pUC57 F-NA), similar to the inducible asRNA expression vector, which express nonfunctional or functional neuraminidases (NF-NA or F-NA) upon influenza infection. The presence of vector expressed RdRP or influenza infection induced the expression of NF-NA and F-NA. Overexpression of NF-NA was originally hypothesized to attenuate influenza titer; however, NF-NA regained its sialidase activity after RdRP-mediated transcription. pUC57 NF-NA or F-NA transfected cells produced an RNA-intermediate regardless of the presence of the RdRP, whereas the polymerase was required for NF-NA mRNA and protein expression. Interestingly, reinfection of MDCK cells with the supernatant from pUC57 NF-NA or F-NA treated and influenza (N1 subtype) infected cells revealed that the naïve MDCK cells generated N2 subtype viruses, indicating the induced N2 viral RNA could be packaged into progeny viruses forcing the N1 virus to become an N2 virus.

These studies demonstrate that RNAi can be an effective means to attenuate influenza infection. Furthermore, incorporation of the influenza conserved promoter into asRNA or neuraminidase expression vectors can be exploited to promote influenza infected cell-specific expression of anti-influenza molecules. This approach may impact the design and advancement of antiviral therapeutics by overcoming the limitations associated with RNAi and allow for current vaccines to protect against influenza infection by forcing influenza viruses to converge into a single subtype.

# Acknowledgements

*"Alone we can do so little; together we can do so much" – Helen Keller*

Without the support and encouragement from those listed below, I would not have made it through the long and demanding task of obtaining my doctorate. It means so much to me that all of you stood alongside of me and pushed me to become the best scientist and person I could be.

First, I would like to express my sincere gratitude to my mentors Dr. John Noti and Dr. Donald Beezhold, for their continued support of my Ph.D. research. Both of you have molded me into the scientist I am today. Thank you for sharing your expertise and thoughtful advice (scientific, professional and personal) throughout this whole process. Your willingness to let me think independently and critically from the get-go has allowed me to gain the confidence needed for my future endeavors.

My committee members, Dr. Chris Cuff, Dr. Richard Dey, Dr. Rosana Schafer, and Dr. David Weissman, have been a tremendous help. Your insight, feedback, and advice have been instrumental during the completion of my thesis research. I am truly humbled by your willingness to guide and help me through my graduate career and beyond.

I would like to thank the members of the "flu lab", your camaraderie and scientific guidance has been invaluable. Dr. Sreekumar Othumpangat, thank you for your help and guidance over the past few years. Bob Thewlis, thank you for providing the lab with your chipper attitude. I have learned many admirable qualities from Bahar Noorbakhsh, M.S., including strength, endurance and steadfast kindness. Dr. Bill Lindsley, there aren't enough pies in the world to thank you for your technical assistance and wonderful friendship. Francoise Blachere, M.S., words can't even begin to express how much I have appreciated our friendship over the years. You have not only taught me how to be a quality scientist by showing me how to always try my best, pay attention to detail, and never take shortcuts, but you have

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taught me how to be a kind, selfless and thoughtful person. For these reasons, you have truly been a great role model.

To those at the Office of Research and Graduate Education and in the Microbiology, Immunology and Cell Biology Department thank you for giving me the opportunity to continue my education at West Virginia University. I would like to thank Dr. Slawomir Lukomski for the time he has invested in my education and for treating me like one of his own graduate students. Brooke Phillips, thank you for always lending a helping hand while I try to balance work at NIOSH and WVU. Admittance into the Biomedical Sciences Graduate Program wouldn't have been possible without the help of Dr. Rita Rio, Dr. Kevin Lee, and Dr. Alvin Moss whose training and encouragement prepared me for a career in the biomedical sciences.

I am very appreciative of the many friends and colleagues I have met over the last few years at West Virginia University, CDC/NIOSH and outside of my schooling. Kathy Kelley, thank you for always being available to help schedule meetings and travel arrangements throughout my research endeavors, I will miss our late night talks. Breanne Farris, it has been a pleasuring trekking through graduate school with you. I am immensely grateful for your insight and friendship.

My family has been a solid rock to which I have always been able to depend on. Mom and dad, thank you so much for your unfailing love and devotion that continuously gives me the strength to conquer any challenge. Watching your hard-work, dedication and perseverance throughout my childhood has inspired me to achieve my many aspirations. To my brother, thank you for always believing in me and cheering me on throughout this process. Finally, I would like to thank Vijay Manickam for always lending me a listening ear, reminding me of my strengths and giving me continued inspiration to keep going. Your love and support has been unwavering and for that, I am truly grateful.

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# List of Abbreviations

| | |
|---|---|
| **4-MU** | 4-methylumbelliferone |
| **AGO2** | Arogonaute 2 |
| **ANOVA** | Analysis of variance |
| **asRNA** | Anti-sense RNA |
| **ATCC** | American Type Culture Collection |
| **BCA** | Bicinchoninic acid assay |
| **BM2** | Influenza B matrix protein 2 |
| **BSA** | Bovine serum albumin |
| **CAT** | Chloramphenicol acetyltransferase |
| **CDC** | Centers for Disease Control and Prevention |
| **cDNA** | complementary DNA |
| **CM1** | Influenza C matrix protein 1 |
| **CM2** | Influenza C matrix protein 2 |
| **CMV** | Cytomegalovirus |
| **cRNA** | Complementary RNA |
| **DEAE** | Diethylaminoethyl |
| **DGCR8** | DiGeorge Syndrome Critical Region Gene 8 |
| **DNA** | Deoxyribonucleic acid |
| **EDTA** | Ethylenediaminetetraacetic acid |
| **eIF2** | Eukaryotic initiation factor 2 |
| **eIF4F** | Eukaryotic initiation factor 4F |
| **EMEM** | Eagle's Minimum Essential Medium |
| **ERK** | Extracellular signal-regulator kinase |
| **F-NA** | Functional neuraminidase |
| **FAM** | 6-carboxyfluorescein |
| **FDA** | Food and Drug Administration |
| **GAPDH** | Glyceraldehyde 3-phosphate dehydrogenase |
| **GISRS** | Global Influenza Surveillance and Response System |
| **GU** | Guanine-uracil |
| **HA** | Hemagglutinin |
| **HBc** | Hepatitis B virus core protein |
| **HCV** | Hepatitis C virus |
| **HEF** | Hemagglutinin-esterase-fusion protein |
| **HEPES** | 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid |
| **IFNα** | Interferon-alpha |
| **IFNβ** | Interferon-beta |
| **K9TI** | Canine polymerase-I termination |
| **M1** | Matrix protein 1 |

| | |
|---|---|
| **M2** | Matrix protein 2 |
| **M2e** | Extracellular domain of matrix protein 2 |
| **MAPK** | Mitogen-activated protein kinase |
| **MDCK** | Madin-Darby canine kidney |
| **MEK** | MAPK/ERK kinase |
| **MGB-NFQ** | Minor groove binder-non-fluorescent quencher |
| **MHC** | Major histocompatibility complex |
| **miRNA** | Micro RNA |
| **MOI** | Multiplicity of infection |
| **mRNA** | Messenger RNA |
| **MUNANA** | 2'-(4-methylumbelliferyl)-$\alpha$-D-N-acetylneuraminic acid |
| **N1** | Neuraminidase subtype 1 |
| **N2** | Neuraminidase subtype 2 |
| **NA** | Neuraminidase |
| **NCR** | Noncoding region |
| **NEP** | Nuclear export protein |
| **NES** | Nuclear export protein |
| **NF-NA** | Nonfunctional neuraminidase |
| **NI** | No infection |
| **NLS** | Nuclear localization signal |
| **NMDAR** | N-methyl-D-aspartate receptor |
| **NMR** | Nuclear magnetic resonance |
| **NP** | Nucleoprotein |
| **NS1** | Nonstructural protein 1 |
| **NS2** | Nonstructural protein 2 |
| **OAS** | Oligoadenylate synthetase |
| **PA** | Polymerase acidic protein |
| **PB1** | Polymerase basic protein 1 |
| **PB1-F2** | Polymerase basic protein 1 frame 2 |
| **PB2** | Polymerase basic protein 2 |
| **PBS** | Phosphate buffered saline |
| **PCR** | Polymerase chain reaction |
| **PFU** | Plaque forming units |
| **pk9POL-I** | Canine polymerase-I promoter |
| **PKR** | Protein kinase R |
| **qPCR** | Quantitative polymerase chain reaction |
| **RdRP** | RNA-dependent RNA polymerase |
| **RIG-I** | Retinoic acid-inducible gene-I |
| **RIPA** | Radioimmunoprecipitation assay |
| **RISC** | RNA-induced silencing complex |
| **RNP** | Ribonucleoprotein |

| | |
|---|---|
| **RLU** | Relative luminescence units |
| **RNA** | Ribonucleic acid |
| **RNAi** | RNA interference |
| **SDS-PAGE** | Sodium dodecyl sulfate polyacrylamide gel electrophoresis |
| **shRNA** | Short-hairpin RNA |
| **siRNA** | Small interfering RNA |
| **T-705** | Favipiravir |
| **TBS** | Tris-buffered saline |
| **VPA** | Viral plaque assay |
| **vRNA** | Viral RNA |
| **WHO** | World Health Organization |

# Chapter 1 – Introduction & Specific Aims

## 1.1 Overview

Throughout history, influenza virus pandemics and epidemics have been responsible for millions of illnesses and deaths. Influenza virus infections account for 3-5 million cases of severe illnesses and between 3,000 to 49,000 deaths each year in the United States alone (CDC, 2012). Even with the development of antiviral therapies and vaccines targeting influenza A and B viruses, mortality associated with influenza infection still persists. The high error rate of the viral RNA-dependent RNA polymerase (RdRP) yields genetically diverse influenza viruses through a process called antigenic drift (Drake, 1993). Antigenic drift, or the gradual accumulation of mutations over time, makes it difficult to develop antiviral therapies that can target a diverse array of viruses. In addition, the segmented genome of these viruses and broad host range of influenza A viruses allows for genetic reassortment (Nelson et al., 2008; Schweiger et al., 2006). Genetic reassortment causes a shift in viral antigenicity and produces new subtypes of influenza viruses. With the emergence of novel influenza strains due to antigenic drift, alternative forms of antiviral therapies must be developed to address the increased resistance against antiviral therapeutics. Likewise, new methods of vaccination are needed in order to address the lack of protection generated by conventional vaccines due to antigenic variation. Consequently, the objective of the studies included in this dissertation is to develop an alternative to the standard methods of antiviral therapeutics.

## 1.2 Strategies for targeting influenza viruses

The influenza virus can be targeted at different stages of its 'lifecycle' in order to prevent replication and spread of disease, but each of the existing approaches to antiviral therapies has limitations. The development of neutralizing antibodies targeting the hemagglutinin surface glycoprotein (Itoh et al., 2014; Wei et al., 2008) and sialic-acidic mimics (Matsubara et al., 2010) have

been investigated to prevent the virus from binding to the host cell. However, antigenic drift causes gradual changes in the hemagglutinin antigenicity. This restricts the use of neutralizing antibodies targeting this protein, as new neutralizing antibodies must be developed with the emergence of new strains (Both et al., 1983; Smith et al., 2004; Yewdell et al., 1986). Adamantanes (i.e. amantadine and rimantadine) are a class of antiviral drugs approved by the United States Food and Drug Administration (FDA) that inhibit viral uncoating by specifically targeting the matrix protein 2. During the 2005-2006 flu season pyrosequencing revealed that 96% of the influenza A H3N2 isolates were resistant to the adamantane class of antiviral therapies (Deyde et al., 2007); resistance to adamantanes still persists and their use is no longer recommended by the World Health Organization (WHO) (WHO, 2010). Replication can be inhibited by using nucleoside analogs such as ribavirin (Pauly and Lauring, 2015) or the RNA polymerase inhibitor favipiravir (T-705) (Sleeman et al., 2010). Resistance toward nucleoside analogs has been reported for treatment against human immunodeficiency virus and hepatitis C virus and is likely to occur for treatment against influenza viruses (Lam et al., 2011; Menendez-Arias, 2008; Rezende and Prasad, 2004). In order to form a mature virion, the viral ribonucleoprotein (vRNP) must be exported from the nucleus into the cytoplasm where it is packaged (Shapiro et al., 1987). Pleschka et al. (2001) showed that treatment of influenza-infected cells with the MEK-specific inhibitor, U1026, prevents the nuclear-cytoplasmic translocation of vRNPs. Raf/MEK/ERK signaling pathways regulate cell proliferation, apoptosis and cell cycle arrest which are essential function in all cells. Broad inhibition of the Raf/MEK/ERK signaling pathway might lead to cellular toxicity or cancer development; therefore, localized or cell specific delivery would be essential for the use of U0126 as an anti-influenza therapy (McCubrey et al., 2007). FDA approved neuraminidase inhibitors such as oseltamavir and zanamivir inhibit the sialidase activity of neuraminidases which prevents the release of mature virions from the host cell (Hayden et al., 1997; Hayden et al., 1999). Oseltamivir resistance has been observed in influenza

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A (H5N1) strains, and 15% of influenza A (H1N1) strains isolated during the last quarter of 2007 to the first quarter of 2008 were reported to be resistant to oseltamivir (de Jong et al., 2005; WHO, 2008). (refer to Chapter 2.7)

**1.3 RNA interference as an alternative method of antiviral treatment**

A majority of antiviral therapies today rely on the use of protein inhibitors or nucleoside analogs to inhibit viral replication. One alternative approach that has not yet been explored is the use of a genetics-based approach, such as RNA interference (RNAi), to prevent viral infection. RNAi is a genetic mechanism that uses small RNA molecules to inhibit protein expression, typically by causing the destruction of specific transcripts. Small RNA molecules that are involved in RNAi include microRNA (miRNA), small interfering RNA (siRNA), or anti-sense RNA (asRNA). RNAi was first reported by Fire et al. (1998) after observing that injection of double-stranded RNA molecules into the nematode *Caenorhabditis elegans* resulted in effective gene-specific silencing. Since then, research studies have analyzed the use of RNAi for a myriad of applications such as antiviral therapies (Ketzinel-Gilad et al., 2006), cancer therapies (Mansoori et al., 2014), and the production of transgenic plants (Ali et al., 2010). The use of RNAi has been studied *in vitro* and *in vivo* using higher order mammals such as zebrafish (Andrews et al., 2014), rats (Hasuwa et al., 2002), and macaques (Zimmermann et al., 2006).

miRNAs are derived from non-coding primary miRNA (pri-miRNA) transcribed by the host RNA polymerase and are predominantly involved with endogenous gene regulation (Lee et al., 2002). The pri-miRNA is at least 1000 nucleotides in length with a hairpin structure that contains the sequence for a mature miRNA (Lim et al., 2003; Saini et al., 2007). The microprocessor complex (Drosha, a class 2 RNase III enzyme & DGCR8, a RNA binding protein) modifies the pri-miRNA into precursor miRNA (pre-miRNA) by cleaving the RNA at the base of the hairpin stem, leaving behind the hairpin structure of about 70-80

nucleotides in length (Lee et al., 2002). The pre-miRNA is then transported into the host cytoplasm by exportin-5 where it is further modified into mature miRNA by another RNase III enzyme, Dicer (Bernstein et al., 2001; Zeng and Cullen, 2004). Dicer removes the loop found in the center of the hairpin complex, and leaves behind a mature miRNA consisting of a double-stranded RNA of about 22 to 25 nucleotides in length with two nucleotide overhangs at each 3' terminus (Elbashir et al., 2001a). The mature miRNA is loaded into an Argonaute protein family complex, with the help of Dicer and TRBP, to form the RNA-induced silencing complex (RISC). Either strand of the mature miRNA can be incorporated into Argonaute. However, studies have shown that the RNA strand with the least stable 5' end, the guide strand, is integrated into the Argonaute protein whereas the complementary RNA strand with the stable 5' end, the passenger strand, is completely degraded by the Argonaute protein (Hibio et al., 2012; Kawamata et al., 2009). The RISC, containing the Argonaute and guide strand, is available to mediate gene silencing in the host cytoplasm.

siRNAs are associated with gene silencing in response to foreign nucleic acids during viral infection or to destructive nucleic acids such as transposable elements. Growing oocytes were found to express endogenous double-stranded RNA of about 21-27 nucleotides in length which regulate retrotransposon and protein expression (Dalmay et al., 2000; Watanabe et al., 2008). In plants, virus-induced expression of small double stranded RNA can protect the plant from virus infection (Lindbo and Dougherty, 1992). Similar to miRNA, the siRNA must be processed in the cytoplasm. Endogenously expressed longer double-stranded RNAs must be processed into small double stranded RNA (21-25 nucleotides with 3' overhang) before the passenger RNA strand can be loaded into the Argonaute protein of the RISC.

Rivas et al. (2005) observed that asRNA can be incorporate into Argonaute 2 and form a RISC that can mediate gene specific degradation *in vitro*, without undergoing the same cytoplasmic processing needed for miRNA and siRNA maturation. Moreover, delivery of asRNA into mice by subcutaneous or intravenous injection was able to cause Argonaute RISC-associated gene silencing, without the presence of the passenger strand (Lima et al., 2012).

Gene silencing via miRNA, siRNA, and asRNA occurs by two routes: direct mRNA degradation or translational repression. Nucleotides 2 through 7 of the guide strand, called the seed region, initiate binding of the guide strand to the transcript. When the miRNA or siRNA binds with 100% complementarity of the seed region to the mRNA transcript recognized in the cytoplasm, the Argonaute protein of the RISC complex facilitates endonuclease cleavage of the host mRNA between the $10^{th}$ and $11^{th}$ nucleotide relative the 5' end of the guide strand (Elbashir et al., 2001b; Lima et al., 2012). The mRNA that was once protected from exonuclease cleavage at the 5' and 3' end of the transcript by 5' 7-methylguanosine cap and poly-(A)-tail then becomes susceptible to complete degradation by the exonucleases, thus inhibiting gene expression (Orban and Izaurralde, 2005; Souret et al., 2004). Translational repression, on the other hand, occurs when the guide strand doesn't bind with 100% complementarity to the mRNA transcript. The RISC complex binds to the mRNA transcripts and inhibits protein translation by preventing the ribosome from reading the transcript. The exposed mRNA degrades by the natural process of mRNA decay (Garneau et al., 2007). Many studies have identified that the complementarity of the seed region to mRNA , but not necessarily the rest of the small RNA molecule, accounts for non-specific gene silencing because the complementarity of the seed region seems to dictate the route of RNAi (Kamola et al., 2015).

## 1.4 Shortcomings of RNA interference treatment

Although RNAi has the potential to be an effective technique to treat viral infection, only one small RNA molecule that promotes RNAi has been approved by the FDA. Mipomersen is a miRNA inhibitor that specifically silences the activity of the liver-specific miRNA, miR122. miR122 is known to promote hepatitis C virus (HCV) translation. By inhibiting the activity of miR122, Mipomersen was shown to prevent HCV replication during a phase 2a clinical trial (Henke et al., 2008; Janssen et al., 2013). The lack of FDA-approved RNAi therapies is due to a number of limitations associated with RNAi such as their short half-life, potential for causing off-target gene silencing, and difficulty in delivering the small RNA molecules to the site of interest.

In order for miRNA, siRNA, or asRNA to successfully execute their regulatory effects, the most efficacious dose must be delivered to the target tissue. Unfortunately, physical barriers can prevent efficient drug delivery (Hickerson et al., 2008; Layzer et al., 2004). A study by Eder et al. (1991) showed that siRNAs and asRNAs are highly susceptible to 3' nuclease cleavage in human, mouse, and rat plasma. siRNA are also readily cleared from the blood through the kidneys (van de Water et al., 2006). Up to 40 times more siRNA can be found in the kidneys compared to other organs after intravenous delivery of siRNA. Delivery of siRNA by intravenous injection can limit the amount of siRNA that is able to reach the target tissue and requires higher doses of siRNA to be delivered in order to reach the effective siRNA concentration. Treatment with double-stranded siRNA (Sioud, 2005) and guanine- uracil (GU)-rich single-stranded RNA (Heil et al., 2004) can activate toll-like receptors 7 & 8 that stimulate the expression of type I interferons and IL-6 (Hornung et al., 2005; Judge et al., 2005). Over-activation of pro-inflammatory cytokines can cause adverse effects such as unnecessary inflammation, fever, chills, etc. (Judge and MacLachlan, 2008). Chemical modifications of siRNA must be performed in order to extend the siRNA or

asRNA half-life, prevent induction of the innate immune response, and promote gene specific silencing (Broering et al., 2014; Eder et al., 1991; Layzer et al., 2004). Taken together, although RNAi treatment can be an effective method to treat diseases, small RNA molecules are highly susceptible to inactivation and clearance, thus making it difficult to design, deliver and safely utilize RNAi molecules as a therapeutic. One was to avoid this problem would be to restrict expression of the regulatory RNA molecules to the target cells. By doing so, off-target effects can potentiall be restricted to localized tissue without causing harm to surrounding tissue.

## 1.5 Influenza viruses' inherent ability to resist antiviral therapies and vaccines

Influenza viruses are negative-sense segmented viruses that rely on an RNA-dependent RNA polymerase (RdRP) for replication and transcription. During replication and transcription, the influenza RdRP must transcribe the negative sense viral RNA into positive-sense complementary RNA or mRNA, respectively. Polymerases that are involved in replication, such as DNA polymerases, typically have proofreading capabilities to prevent mutations (Kunkel and Mosbaugh, 1989). However the influenza RdRP does not have such capabilities. Instead, the RdRP is associated with a high error rate that yields genetically diverse influenza viruses through a process called antigenic drift (Drake, 1993). On average, the RdRP makes one new mutation with each round of replication. Antigenic drift creates new strains of influenza viruses that are no longer susceptible to both classes of antiviral therapies, neuraminidase inhibitors (WHO, 2008) and adamantanes (Sheu et al., 2008). Constant immune surveillance selects for continued circulation of mutant viruses because they have become unrecognizable by an immune system previously primed against another variant while the previously circulating variant is cleared (Webster et al., 1982). For this reason, a new seasonal influenza vaccine must be created each year in order to target the influenza strains that are circulating at that time (Barr et al., 2014; WHO, 2014).

The segmented composition of the influenza genome also allows for antigenic shift to occur, which makes the design of effective long-lasting vaccines difficult. Antigenic shift is a process in which one influenza virus acquires a new segment from another influenza virus that has infected the same cell (Webster et al., 1977). For instance, in 1957, 1968 and 2009 influenza viruses underwent antigenic shift which led to the emergence of new influenza subtypes. The lack of immunological protection against these antigenically novel influenza subtypes led to pandemics that accounted for a large number of fatalities (Taubenberger, 2006). Current research efforts have focused on the development of a universal vaccine that can protect against all strains and subtypes of influenza viruses, regardless of antigenic drift or shift. Many researchers have dedicated their studies to identify conserved antigens, such as the extracellular domain of matrix protein 2 (M2e) or hemagglutinin stalk, that can be used to elicit specific immune responses against a broad range of influenza viruses (refer to Chapter 2.8) (Gong et al., 2016; Kang et al., 2011; Kolpe et al., 2016; Neirynck et al., 1999). Although a universal vaccine is essential for the eradication of this disease, a universal antiviral therapy should also be considered in order to control infection and aid in disease eradication.

## 1.6 How to use the viruses' machinery and resistance mechanisms against itself

Each segment of influenza viruses A, B and C contain 12 3' terminal noncoding nucleotides and 13 5' terminal noncoding nucleotides that are conserved for all influenza segments, strains, and subtypes (Robertson, 1979; Skehel and Hay, 1978). These noncoding regions have partial and inverted complementarity which forms a double-stranded conserved promoter (Desselberger et al., 1980) that is specifically recognized by the influenza virus RdRP (Huang et al., 1990). Mutational analysis of the influenza promoter revealed that certain mutations or the addition of nucleotides on 3' noncoding region prevents recognition of the viral RNA by the influenza promoter, emphasizing the importance of

the promoter composition (Enami et al., 1990; Li and Palese, 1992). Since the discovery of the influenza conserved promoter and the specificity of the RdRP for the promoter, they have been used to develop reverse genetics systems and diagnostic tools for influenza research (de Wit et al., 2007; Enami and Enami, 2000; Lutz et al., 2005). Hossain et al. (2010) designed a reporter assay that uses the influenza conserved promoter in order to express the reporter protein luciferase. They showed that plasmid expressed RNA-dependent RNA polymerases and influenza A or B infection specifically induced luciferase expression. This assay was able to be used for the detection of influenza infection or to test neutralizing antibodies or antiviral molecules.

The conserved promoter of influenza viruses could potentially be exploited in order to develop an antiviral therapy that is induced by the virus itself. By doing so, the antiviral molecule that is expressed will be limited to cells that are infected with influenza. Limiting expression to influenza-infected cells can restrict the adverse effects associated with RNAi. Because the conserved promoter is recognized by the RdRP of all influenza viruses, it can be used to generate a universal antiviral therapy that is activated upon exposure to any influenza strain or subtype.

A second approach would be to reduce the rate of antigenic variation. By delivering influenza viral RNA encoding for a particular subtype (i.e. subtype 2) of neuraminidase into a cell that becomes infected with influenza, it may be possible to promote genetic reassortment between the neuraminidase vRNA encoded by the infectious virus and the delivered vRNA. In addition, expression of the new neuraminidase to influenza-infected cells can be attained by expressing the neuraminidase under the control of the influenza conserved promoter. This will allow all subtypes of influenza to elicit the expression of the neuraminidase, promote genetic reassortment and cause various subtypes of influenza to express neuraminidase subtype 2.

This method of reverting the influenza virus back to a single subtype can also be extended to expressing a neuraminidase that is unable to promote the release of progeny viruses. The neuraminidase protein is essential for the release of new viruses by cleaving sialic acid residues off of the surface of the host cell and influenza viruses (Palese and Compans, 1976). Removing sialic acid residues from the surface of influenza viruses, prevents viral aggregation and promotes infection of surrounding cells (Palese et al., 1974). Therefore, expression of an inactive neuraminidase might aid in viral attenuation.

## 1.7 Objectives, hypotheses, and specific aims

The overall objective of this research project is to develop antiviral therapies that utilize an alternative approach to deliver antiviral molecules (such as asRNA or a protein) that can inhibit the replication and release of various strains and subtypes of influenza viruses and overcome the limitations and resistance toward current forms of anti-influenza therapies. It is hypothesized that delivery of an expression vector encoding asRNA specific for influenza mRNA flanked by the conserved promoter of influenza can produce an effective therapy against all influenza viruses. The asRNA can mediate RNAi and reduce the production of specific influenza proteins, thus attenuating viral replication. Moreover, as an alternative to an RNAi-mediated antiviral approach, delivery of an expression vector encoding non-functional neuraminidases under the control of the influenza conserved promoter is hypothesized to be an effective therapy against all influenza viruses. Oversaturation of influenza-infected cells with non-functional neuraminidases (NF-NA) is postulated to prevent the release of infectious virus and promote the aggregation of viruses, making them noninfectious. The influenza-targeting molecule (asRNA or NF-NA) will only be expressed after the delivery of influenza virus' RNA-dependent-RNA polymerase, whether via natural infection or delivery of plasmids expressing the proteins that make up the RNA-dependent-RNA polymerase. In addition, it is hypothesized that the neuraminidase encoding viral RNA

(vRNA) that is encoded by the influenza RNA-dependent RNA polymerase is able to be incorporated into progeny virus. To test these hypotheses, the two following specific aims were studied:

**Specific Aim 1:** *Develop and characterize inducible anti-influenza therapies utilizing RNA interference (RNAi).*

**Specific Aim 1.1:** Design and characterize new influenza-targeting siRNA that can perform better than previously published siRNA.

I hypothesized that treatment with siRNA targeting both the primary and secondary transcripts of the matrix or nonstructural proteins will result in decreased expression of both the matrix proteins 1 and 2 and nonstructural proteins 1 and 2, respectively. As a result, a reduction in the expression of influenza proteins will lead to an attenuation of viral titer. Additionally, treatment of influenza-infected cells with a combination of siRNA is hypothesized to be a more effective means to inhibit viral replication than treatment with a single siRNA.

**Specific Aim 1.2:** Design an asRNA expression vector that is induced by influenza infection.

By designing a plasmid encoding for influenza-targeting asRNA flanked by the conserved promoter of influenza viruses, it is hypothesized that this antiviral therapy will only be induced after influenza infection or the presence of the influenza RNA-dependent RNA polymerase. Ideally, antisense RNA that is reverse transcribed by the influenza polymerase will bind to complementary viral mRNA and promote endonuclease degradation, thus inhibiting translation and viral replication.

**Specific Aim 2:** *Develop and characterize an inducible anti-influenza therapy expressing a conserved non-functional protein (NF-NA).*

Similar to specific aim 1, it is hypothesized that an expression vector encoding for the NF-NA that is under the control of the influenza conserved promoter will express NF-NA only after influenza infection or when in the presence of the influenza RNA-dependent RNA polymerase. Because the neuraminidase protein, which is essential for the release of progeny viruses from the host cell, is not functional, it is hypothesized that oversaturation of the non-functional neuraminidase protein during influenza infection will reduce the production of infectious progeny viruses. In addition, the neuraminidase encoding vRNA that is recognized by the RNA-dependent RNA polymerase is hypothesized to be incorporated into the newly formed viruses.

Developing an antiviral therapy that is triggered by influenza-specific infection or by command (i.e. using RdRP expression vectors) could be beneficial as 1) it limits the expression of asRNA and neuraminidase antigen until after influenza infection, thus reducing the non-specific asRNA mediated RNA interference and immune activation that is sometimes associated with RNAi treatment or expression of foreign antigens (Jackson et al., 2003); 2) specialized delivery to the host cells and expression of asRNA in the cells can bypass clearance through the kidneys which typically occurs during intravenous delivery of RNAi-mediating molecules (van de Water et al., 2006); and 3) as long as the therapy and virus are within the same cell, asRNA or NF-NA can be regenerated over time.

The innovative design of this antiviral therapy, which relies on the recognition of the influenza conserved promoter by the viral RdRP, has the potential to replace current anti-influenza therapies as it can be used for different strains and subtypes of influenza viruses. Moreover, a molecular-based antiviral

therapy can be easily designed and quickly synthesized in order to keep up with the actively mutating or reassorted influenza genome. The mechanism of action of this antiviral therapy may be applied to the treatment of other negative-sense RNA viruses that utilize a similar conserved promoter – RNA-dependent RNA polymerase mediated method of replication.

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# Chapter 2 - Literature Review

## 2.1 Introduction

The first recorded influenza pandemic occurred in 1918 and caused a high rate of morbidity and mortality. Since then, four pandemics have occurred, the Asian (A H2N2) flu of 1957 (A H2N2), the Hong Kong (A H3N2) flu of 1968, the Russian (A H1N1) flu of 1977 and the 2009 (H1N1) pandemic flu, which have ranged in mortality and morbidity. Since the first clinical influenza isolate was collected in 1933 (Smith et al., 1933), major advancements in the field of influenza research have aided in the prevention and treatment of influenza infection. The first influenza vaccine for civilian distribution was approved by the FDA in 1943 (Francis, 1953). Since then, various forms of antiviral therapies and vaccines have been developed. However, the inherent ability of influenza viruses to mutate and undergo antigenic variation has made it difficult to develop effective antiviral therapies and vaccines against the multiple variants of the virus. Therefore, the development of antiviral therapies that overcome the emergence of antiviral resistance and the production of vaccines that generate broad-range protection against all strains and subtypes of influenza viruses have been the forefront of influenza research.

This chapter explains the structure, replication, and life cycle of influenza viruses in order to give a better understanding of how and why influenza viruses continuously undergo antigenic variation and have the potential to cause pandemics. The existing antiviral therapies and vaccinations that target influenzas viruses and their limitations will be discussed, followed by an explanation of approaches that are being used to develop a universal influenza vaccine.

**2.2 History**

Evidence of influenza virus infection was first observed in 1878 when poultry suffered from a high rate of mortality (Lupiani and Reddy, 2009; Perroncito, 1878). This infection was originally termed the "fowl plague". In 1901, filterable virus was isolated from chickens with fowl plague by Centanni and Savonuzzi (1901), though it wasn't until 1955 that the etiological agent that caused fowl plague was identified as an avian influenza A virus (Schäfer, 1955). Influenza viruses have been isolated from more than 105 species of aquatic wild bird species (Olsen et al., 2006) . Waterbirds within the Anseirformes (i.e. geese, swans, and ducks) and Charadriiformes (i.e. terns and gulls) orders are most commonly associated with influenza infection (Olsen et al., 2006). The first clinically identified swine influenza was isolated in 1931 and shortly thereafter, the first influenza A isolate was obtained in 1933 (Dorset et al., 1922; Shope, 1931).

The most widely recognized influenza pandemic was caused by the 1918 Spanish influenza, which had a case fatality rate of about 2.5-3.5% (Collins et al., 1930; Johnson and Mueller, 2002) and resulted in an estimated 25-40 million deaths worldwide (Patterson and Pyle, 1991). The disease spread in three distinct waves. The first began in March 1918 throughout the United States, Europe and Asia. A second, stronger wave that caused higher mortality occurred from September to December 1918 and was followed by a third wave that reached Australia around February 1919 (Jordan, 1927; Raoult and Drancourt, 2008; Taubenberger and Morens, 2010). After two years, around 1920, the infection rate weakened and the virus only reappeared during seasonal epidemics.

The 1918 pandemic virus was initially speculated to be an H1N1 subtype closely resembling swine influenza strains after genomic sequencing of formalin-fixed paraffin-embedded tissue from U.S. servicemen killed during the 1918 pandemic (Taubenberger et al., 1997). However more recent

phylogenetic analyses have indicated that the 1918 Spanish influenza derived from an avian lineage (Taubenberger et al., 2005; Worobey et al., 2014).

In February 1957, a new pandemic virus influenza A H2N2 emerged in East Asia and spread worldwide within a matter of nine months (Cox and Subbarao, 2000; Rogers, 2016a). The virus caused an estimated 1-2 million deaths worldwide, which is substantially lower than the mortality caused by the 1918 Spanish influenza. This virus was a descendent of the 1918 H1N1 pandemic strain, but had acquired three new gene segments through genetic reassortment (also known as antigenic shift) (Steel and Lowen, 2014). The donor virus from which the 1957 pandemic virus obtained the new gene segments is unknown. The 1957 pandemic virus was identified as a new subtype (H2N2) because it obtained an avian-like hemagglutinin, subtype H2, and neuraminidase, subtype N2 (Scholtissek et al., 1978).  The virus also obtained a new segment 2, which encodes for the basic protein 1 (PB1; Kawaoka et al., 1989).

The third pandemic to occur during the 20$^{th}$ century began in July 1968 in Hong Kong, China and caused an estimated 1-4 million deaths (Rogers, 2016c). Scholtissek (1994) discovered that the virus emerged from genetic reassortment between a human and an avian influenza virus. The resulting virus was derived from the 1957 pandemic virus, as it retained the neuraminidase (N2) subtype, but the virus obtained a novel hemagglutinin (H3) subtype (Scholtissek et al., 1978) and PB1 segment (Kawaoka et al., 1989). Scientists speculate that the case mortality rate was low during this pandemic because the virus still retained the neuraminidase (N2) antigen, allowing for immunological memory against this antigen in subjects previously infected with the 1957 pandemic virus (Gill et al., 1971; Kilbourne, 1997; Raoult and Drancourt, 2008). A study by Marine et al. (1969) evaluated sera collected in 1964 (prior to the 1968 pandemic) from 145 nursing home residents born before the 1918 pandemic; they found a high prevalence of antibodies to the Hong Kong influenza. These data, along with archeological data and

recordings of illnesses similar to influenza virus infection prior to the 20th century, suggest the presence of influenza pandemics in 1889 and 1900. (Enserink, 2006; Raoult and Drancourt, 2008).

In November 1977, an influenza A H1N1 outbreak occurred in children in the Soviet Union. Later that year, in May, it had spread to northeastern China. Infection was restricted to those who were born after 1957, when H2N2 viruses seemed to become the predominant strain, presumably due to the lack of immunological memory against the H1N1 subtype among those less than 20 years old (Kilbourne, 2006).

The first case of "swine" flu was identified in La Floria, Veracruz, Mexico in February 2009, which led to the 2009 influenza A H1N1 pandemic (pH1N1) (Rogers, 2016b). The virus emerged by genetic reassortment and obtained genetic material from multiple sources of swine influenza (Smith et al., 2009), thus the name "swine" flu. Although the virus was referred to as the "swine" flu, only one zoonotic episode from swine to human seemed to occur, which was followed by transmission between humans (Smith et al., 2009). From Mexico, the virus spread to northern North America, Europe, and then New Zealand. In June 2009, the WHO declared a pandemic. During the first year of circulation, 151,700 - 575,400 deaths were estimated due to infection with pH1N1 (Dawood et al., 2012). A vaccine targeting the pH1N1 virus was quickly produced by Sinovac Biotech, Ltd., in China, in order to control a second wave of pH1N1 (Rogers, 2016b).

Since 2009, influenza A H1N1, influenza A H3N2, and influenza B viruses continue to circulate and cause seasonal epidemics in the United States (CDC, 2016e). However, because of genetic reassortment, pandemic strains can emerge at any time. Thus, research efforts have focused on developing a universal vaccine that can account for the emergence of unforeseen viral strains and subtypes and prevent the spread of infection and high rates of mortality and morbidity associated with pandemics.

## 2.3 Classification

Influenza A, B and C viruses are of the *Orthomyxoviridae* genera within the *Orthomyxoviridae* family of viruses along with three other genera: *Thogotovirus*, *Isavirus*, and *Quaranjavirus* (Fields et al., 2007). The name *Orthomyxoviridae* originates from the Greek words *orthós*, meaning "straight, upright or correct" and *mýxa*, meaning "mucus or slime", relating to the genetic structure of these viruses and the disease state associated with infection (Fields et al., 2007). Viruses within the *Orthomyxoviridae* family have genomes that are segmented, negative-sense, ribonucleic acids (RNA) and are contained within an enveloped virion (Fields et al., 2007). The Baltimore Classification System identifies Orthomyxoviruses as a "group V" virus based on their genetic makeup and method of replication. These viruses depend on the expression of their own RNA-dependent RNA polymerase to mediate replication and transcription.

All viruses belonging to the *Orthomyxoviridae* family can readily exchange their segmented genome with the genome of a similar virus in a process known as genetic reassortment (Steel and Lowen, 2014). Genetic reassortment appears to be restricted to viruses of the same genera, as reassortment between different genera have not been described nor reassortment between different influenza types. This constraint may be explained, in part, due to the lack of compatible or matching packaging signals necessary for packaging of the reassorted segments into newly created virions (Baker et al., 2014).

The three influenza types, A, B and C, were first differentiated using complement fixation, where the antisera that recognizes the antigen of a specific influenza type did not cross-react with the antisera of another influenza type (Hayden F.G., 2009; Hoyle, 1948). Nucleotide analyses are currently used to distinguish between different influenza types (CDC, 2015) and have identified that all three influenza

viruses, A, B and C originate from a common ancestor (Desselberger et al., 1980; Palese and Young, 1982).

Influenza A viruses have been characterized into subtypes based on serological cross-reactivity of the virus surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA) (Hayden F.G., 2009). More recently, sequence analyses are used to identify the various influenza subtypes (CDC, 2015). Sequencing analyses have identified 18 different HA and 11 NA that can mix and match to form different influenza subtypes (Castrucci et al., 1993; Steel and Lowen, 2014). A list of identified influenza A virus subtypes is shown in Table 2.1.

**Table 2.1: List of influenza A subtypes that have or are currently circulating in avian, swine, human and bats.** Hemagglutinin (H) and neuraminidase (N) subtypes that are bolded indicate avian influenza subtypes that have been isolated in humans (Bright et al., 2005; Lupiani and Reddy, 2009; Mehle, 2014; Webster et al., 1992).

| Hemagglutinin (HA) | Neuraminidase (NA) | Subtype | Host |
|---|---|---|---|
| H1 - H16 | N1 – N9 | H1N1, N1N8, N2N9, N3N8, H3N2, H4N3, H4N6, H5N1H5N3, H5N4, H5N9, H6N1, H6N2, H6N5, H6N6, H6N8, H7N1, H7N3, H7N9, H8N4, H9N2, H9N6, H9N7, N9N8, H10N8, H11N6, H11N9, H12N5, H13N6, H14N4, H15N9 | Avian |
| H1, H3 | N1, N2 | H1N1, H2N1, H2N2, H2N3, H3N1, H3N2 | Swine |
| H1, H3, **H5, H7, H9** | N1, **N2, N9** | H1N1, H3N2, **H5N1, H7, N9 H9N2** | Human |
| H17, H18 | N10, N11 | | Bat |

Influenza B and C viruses are not characterized into subtypes, but sequencing (Biere et al., 2010) and phylogenetic analyses (Rota et al., 1990) have identified two distinct lineages of influenza B viruses,

B/Yamagata/16/88 and B/Victoria/2/87. Phylogenetic analyses showed that the lineages, Yamagata and Victoria, diverged from a common ancestor, B/Singapore/222/79, and diverged from one another around the 1970s (Hay et al., 2001). Influenza virus lineages and subtypes are further classified into strains which identify variants in each lineage or subtype over time.

In 1979 the World Health Organization (WHO) adopted an updated naming convention that is now used worldwide (WHO, 1980) to identify various strains of influenza A, B, and C viruses. Each influenza virus strain is to be identified by the following distinguishing elements:

- The antigenic type (A, B or C)

- The host of origin (i.e. swine, duck, equine, etc). If the host of origin is human, then no host of origin is designated; rather, a human origin is understood if no designation is given.

- Geographical location of isolate

- Strain number, which indicates the order of isolates obtained from that strain, in sequential order

- Year of isolation

Each of these components is listed in sequential order and separated by a forward-slash. For example, strains of influenza viruses can be named influenza B/Yamagata/16/88 or B/Yamagata/16/1988. Influenza A viruses are identified using an additional distinguishing feature, its subtype, which is listed after the strain designation in parentheses. For example, two influenza A virus strains are influenza A/turkey/Ontario/6632/1966 (H5N9) or influenza A/California/7/2009 (H1N1), which is of human origin.

## 2.4 Virion structure and morphology

*Influenza A viruses*

Influenza A viruses are contained in a lipid envelope which is obtained from the host's cell membrane when the virus is released from the host cell. The viral envelope also includes three proteins, two surface-expressed glycoproteins (hemagglutinin (HA) and neuraminidase (NA)), and one integral membrane protein, the matrix protein 2 (M2). Both the HA and NA are attached to the lipid membrane by a hydrophobic domain (Bilsel et al., 1993; Chang et al., 2008; Kundu et al., 1996) and protrude from the lipid membrane like spikes (Laver and Valentine, 1969). The HA protein forms a homotrimer and resembles a rod-like structure when analyzed by electron microscopy (Laver and Valentine, 1969). The HA protein is essential for binding of the virus to the host receptor protein and viral entry (Skehel et al., 1995). The NA protein, which forms a homotetramer resembling a mushroom-like structure (Laver and Valentine, 1969), is needed for the release of progeny virus from the host cell (Lentz et al., 1987; Palese et al., 1974). The M2 protein homotetramer spans the lipid membrane as an integral membrane protein in order to form an ion channel (Ciampor et al., 1992; Lamb et al., 1985). The ion channel activity of the M2 proteins acidifies the interior of the virus, resulting in the release of the viral genome into the host cytoplasm (Bui et al., 1996).

Directly beneath the lipid envelope is the viral capsid that is made of a layer of matrix protein 1 (Ruigrok et al., 1989). This capsid serves as a protective barrier that houses the eight segmented single-stranded RNAs that make up the influenza genome. Each viral RNA (vRNA) segment is associated with four proteins: the polymerase basic protein 1 (PB1), polymerase basic protein 2 (PB2), polymerase acidic protein (PA) and nucleoprotein (NP). Each vRNA contains one PB1, PB2 and PA, and one NP that binds to the vRNA every twenty-four nucleotides (Albo et al., 1995; Eisfeld et al., 2015). The PB1, PB2, and PA

make up the influenza RNA-dependent RNA polymerase (RdRP), which mediates viral replication and transcription (Huang et al., 1990). The NP is involved in viral replication and transcription, in addition to the transport of the vRNA into the host nucleus (Elton et al., 2001; O'Neill et al., 1995). The RdRP, vRNA, and NPs form a complex that is referred to as the viral ribonucleoprotein (vRNP) complex.

The nonstructural protein 2 (NS2) is found within the virion, but only in low amounts (Richardson and Akkina, 1991). Its presence within the virus is not needed for the virus to be infectious. This protein is also called the nuclear export protein (NEP) because of its role in transporting the vRNP out of the nucleus and into the host's cytoplasm where the vRNP is packaged into new virions (O'Neill et al., 1998). The nonstructural protein 1 (NS1) and polymerase basic protein – frame 2 (PB1-F2) proteins are encoded by the vRNA, but are not needed to form the structure of infectious influenza virions. A study infecting interferon-deficient cells with an NS1-null strain of influenza resulted in infectious virus, indicating that the NS1 protein is not essential for viral replication (Garcia-Sastre et al., 1998). However, NS1 is an important immunomodulatory molecule as it is involved in inhibiting anti-influenza molecules such as the protein kinase R (PKR) and oligoadenylate synthetase (OAS; Garcia-Sastre, 2011). Both PKR, OAS and NS1 contain double-stranded RNA (dsRNA) binding domains. PKR is activated by binding of dsRNA, which then promotes the phosphorylation of eukaryotic initiation factor 2 (eIF2; Li et al., 2006). Phosphorylation of eIF2, inhibits translation, thus preventing translation of both host and viral proteins. NS1 competes with PKR for dsRNA binding, which inhibits PKR activation. Similarly, NS1 competes with OAS for dsRNA binding, which inhibits the activation of OAS and downstream activation and expression of RNase L (Min and Krug, 2006). RNase L destroys viral RNA, thus reducing influenza infection. PB1-F2 also has immunomodulatory functions, as it promotes apoptosis of macrophages by entering the mitochondria and promoting the release of cytochrome c that induces apoptosis (Zamarin et al., 2005).

**Table 2.2: List of proteins encoded by the influenza A viruses and the corresponding segments in which the virus gene sequence is located.** Segments 2, 7 and 8 each encoded for two different proteins. The NS2 is also referred to as the nuclear export protein (NEP).

| Influenza A Virus | | |
|---|---|---|
| **Segment** | **Encoded Protein** | |
| 1 | Polymerase Basic Protein 2 | PB2 |
| 2 | Polymerase Basic Protein 1<br>Polymerase Basic Protein 1 – Frame 2 | PB1/PB1-F2 |
| 3 | Polymerase Acidic Protein | PA |
| 4 | Hemagglutinin | HA |
| 5 | Nucleoprotein | NP |
| 6 | Neuraminidase | NA |
| 7 | Matrix Protein 1<br>Matrix Protein 2 | M1/M2 |
| 8 | Nonstructural Protein 1<br>Nonstructural Protein 2 | NS1/NS2(NEP) |

*Influenza B viruses*

Influenza B viruses have a similar structure to influenza A viruses, but the M2 integral membrane protein with ion channel activity is known as the BM2 protein in influenza B viruses (Mould et al., 2003). Although the two proteins have the same function, they share limited sequence homology, except for a Histidine-X-X-X-Tryptophan domain that is responsible for the ion channel function (Paterson et al., 2003; Pielak and Chou, 2011). Structural mapping of the influenza A and B proteins indicate that the pore-lining residues within the influenza BM2 protein are hydrophilic, polar serines, which prevents the virus from being susceptible to matrix inhibitors (Davies et al., 1964; Pinto and Lamb, 2006). Influenza B viruses also express an NB that is a type III integral membrane protein found within the viral envelope

(Brassard et al., 1996). The NB protein is believed to be an ion channel, but the exact role of this protein has yet to be identified (Betakova et al., 1996).

**Table 2.3: List of proteins encoded by the influenza B viruses and the corresponding segments in which the virus gene sequence is located.** Segments 6, 7 and 8 each encoded for two different proteins. The nonstructural protein 2 (NS2) is also referred to as the nuclear export protein (NEP).

| Influenza B Virus | | |
|---|---|---|
| **Segment** | **Encoded Protein** | |
| 1 | Polymerase Basic Protein 2 | PB2 |
| 2 | Polymerase Basic Protein 1 | PB1 |
| 3 | Polymerase Acidic Protein | PA |
| 4 | Hemagglutinin | HA |
| 5 | Nucleoprotein | NP |
| 6 | Neuraminidase | NA/NB |
| 7 | Matrix Protein 1<br>Matrix Protein 2 | M1/BM2 |
| 8 | Nonstructural Protein 1<br>Nonstructural Protein 2 | NS1/NS2(NEP) |

*Influenza C viruses*

Like the M1 and M2 proteins of influenza A viruses, the CM1 protein of influenza C forms the viral capsid (Zhirnov and Grigoriev, 1994) and the CM2 protein acts as an ion channel (Hongo et al., 2004; Hongo et al., 1994). Influenza C viruses have a slightly different structure than influenza A and B viruses, in that the hemagglutinin-esterase-fusion protein (HEF) is expressed in influenza C viruses instead of HA and NA proteins. The HEF protein has the ability to perform functions similar to both the HA and NA proteins (Herrler et al., 1985; Rogers et al., 1986).

**Table 2.4: List of proteins encoded by the influenza C viruses and the corresponding segments in which the virus gene sequence is located.** Segments 6 and 7 each encoded for two different proteins. The nonstructural protein 2 (NS2) is also referred to as the nuclear export protein (NEP).

| Influenza C Virus | | |
|---|---|---|
| **Segment** | **Encoded Protein** | |
| 1 | Polymerase Basic Protein 2 | PB2 |
| 2 | Polymerase Basic Protein 1 | PB1 |
| 3 | Polymerase Protein 3 | P3 |
| 4 | Hemagglutinin-esterase-fusion Protein | HEF |
| 5 | Nucleoprotein | NP |
| 6 | C Matrix Protein 1<br>C Matrix Protein 2 | CM1/CM2 |
| 7 | Nonstructural Protein 1<br>Nonstructural Protein 2 | NS1/NS2(NEP) |

*Morphology*

Influenza viruses are pleomorphic viruses that can be found in a spherical, filamentous, or irregular form (Mosley and Wyckoff, 1946; Roberts et al., 1998) and virion size can range from approximately 80-120 nm in diameter (Hayden F.G., 2009; Noda, 2011; Stanley, 1944). Newly isolated strains are typically found in a filamentous morphology, whereas adapted strains, such as influenza A/Puerto Rico/8/34 (H1N1), are predominately found with a spherical morphology (Choppin et al., 1960; Chu et al., 1949). Reverse genetics experiments have shown that the M1 and M2 are important factors in determining the spherical or filamentous shape of influenza virions (Muraki et al., 2007; Roberts et al., 1998; Rossman et al., 2010). The cytoplasmic tail of two surface glycoproteins, HA and NA, have been identified as possible determinants of virion morphology, as an irregular morphology has been observed in their absence (Jin et al., 1997).

**2.5 Genome**

Influenza A, B and C viruses contain segmented, negative-sense, single-stranded RNA genomes that encode for various proteins. The viral RdRP facilitates transcription of the vRNA to generate positive-sense RNA that can be translated by the host ribosomes into protein (Samji, 2009). The viral RdRP also facilitates replication by performing two rounds of transcription; the first round of transcription generates complementary positive-sense RNA (cRNA) that is then used as a template for the second round of transcription that generates more vRNA.

All eight (influenza A and B viruses) or 7 (influenza C viruses) vRNA contain noncoding regions at the 3' and 5' terminal nucleotides that include packaging signals. The 12 3' terminal noncoding nucleotides and 13 5' terminal noncoding nucleotides are conserved for all influenza segments, strains, and subtypes (Robertson, 1979; Skehel and Hay, 1978). Because these noncoding regions have partial and inverted complementarity to one another, they form a double-stranded promoter (Desselberger et al., 1980) that is specifically recognized by the influenza virus RdRP (Huang et al., 1990). Transcription and replication is initiated by the binding of the RdRP to the influenza promoter (Huang et al., 1990). A study by Li and Palese (1992) showed that the addition of an extra nucleotide to the 3' terminus of the vRNA decreased gene expression, thus emphasizing the importance of the conserved promoter for viral replication.

Segments 7 and 8 of influenza A and B each encode for two proteins. Segment 7 encodes for M1 and M2, whereas segment 8 encodes for NS1 and NS2. Transcription of either of these segments by the RdRP generates an mRNA that is then either directly translated to express M1 or NS1 or spliced to form a secondary transcript that encodes for M2 or NS2 (Fields et al., 2007; Hayden F.G., 2009; Lamb and Lai, 1980; Shih et al., 1995). Segment 6 of influenza C viruses encode for two proteins matrix protein 1 (MC1)

and matrix protein 2 (MC2). The transcript is either directly translated to express CM1 or spliced to express CM2 (Yamashita et al., 1988) Influenza A virus PB1-F2 and Influenza B NB proteins, however, are expressed by an alternative ribosome initiation start site (Shaw et al., 1983; Wise et al., 2009).

## 2.6 Viral life cycle

*Attachment and Entry*

Influenza viruses attach to the surface of the host cell by interactions between the viral hemagglutinin and sialic acid receptors. The precursor hemagglutinin HA0 located on the surface of the virus protein is cleaved by extracellular proteases, which creates two disulfide-linked subunits, HA1 and HA2 (Taubenberger, 1998). The HA1 contains a receptor-binding domain, that specifically recognizes either α2,3-linked sialic acids or α2,6-linked sialic acids. Human influenza viruses preferentially recognize α2,6-linked sialic acids located on the surface of the upper and lower respiratory tract whereas avian influenza viruses preferentially recognize α2,3-linked sialic acids (Skehel and Wiley, 2000). This difference in receptor-binding specificity is determined by the amino acid located at position 226 of HA. An HA with a glutamine residue at position 226 binds to α2,6-linked sialic acids whereas a HA with a leucine residue at position 226 binds to α2,3-linked sialic acids (Fields et al., 2007). Binding of the HA to the sialic acid receptor stimulates entry into the cell through clatherin-mediated endocytosis (Matlin et al., 1981).

*Fusion and Uncoating*

The acidic environment within the late- and early-endosomes (pH 7.5-6) causes the HA to undergo an initial, reversible conformational change, and then a final, irreversible conformational change (pH < 6.0) that exposes the HA2 fusion peptide (Huang et al., 2003). In addition to inducing fusion of the viral and host membranes, the acid environment (pH < 6.0) within the endo-lysosome activates the M2 ion channel by protonating the histidine residue 37 (Wang et al., 1995). The activated M2 ion channel delivers protons into the viral core, which causes the pH to decrease and the dissociation of M1 proteins from the viral vRNP. The combination of fusion and acidification of the viral core leads to the release of the vRNP into the host cytoplasm (Li et al., 2014). Influenza BM2 and CM2 have a similar ion channel activity as the influenza A M2 and mediate viral uncoating (Hongo et al., 2004; Mould et al., 2003).

*Nuclear Import*

Once in the cytoplasm, the vRNA must be delivered into the nucleus where transcription and replication can occur. The proteins associated with the vRNA that form the vRNP (NP, PA, PB1, and PB2) contain nuclear localization signals (NLSs) to mediate delivery of the vRNP into the nucleus (Mukaigawa and Nayak, 1991; Nath and Nayak, 1990; Nieto et al., 1994; O'Neill et al., 1995; Wu et al., 2007). Transportation of the vRNP and other viral proteins into the nucleus through nuclear pore complexes requires recognition of NLSs on the viral proteins by importin α which forms a ternary complex with importin β that facilitates nuclear translocation (Goldfarb et al., 2004). The M1 protein also contains a NLS and is delivered into the nucleus by the same proteins (Ye et al., 1995). Localization of the NS2/NEP into the nucleus, which is required for export of the vRNP from the nucleus, does not seem to need a

NLS. Instead, due to its small size of 14 kDa, it can enter the nucleus by passive diffusion (Gao et al., 2014).

*Transcription and replication*

Negative-sense vRNA is transcribed in the nucleus by the viral RdRP. The conserved promoter, formed by complementary binding of the 5' and 3' noncoding regions of each RNA segment, is specifically recognized by the RdRP. Transcription is initiated when the 5' end of the vRNA is bound by PB1. This interaction induces a conformational change in the polymerase that allows the PB2 protein to bind to the 5' 7-methylguanosine cap of the host mRNAs (Fechter and Brownlee, 2005; Li et al., 1998). The conformational change also promotes binding of the 3' end of the vRNA by PB1, which activates the endonuclease activity PA (Fodor et al., 2002; Hagen et al., 1994). PA then cleaves the 5' 7-methylguanosine cap from the host mRNA which serves as a primer for transcription (Robertson et al., 1980). Transcription is mediated by PB1 (Kobayashi et al., 1996).  At the end of transcription, the PB1 reads a string of 5 to 7 uridine residues and then proceeds directly into the double-stranded conserved promoter. Through steric hindrance, the double-stranded conserved promoter causes the polymerase to stay bound and generate a poly-adenylated tail by a stuttering mechanism (Poon et al., 1999; Robertson et al., 1981). Splicing of the matrix and nonstructural transcripts involves cellular splicing machinery (Lamb et al., 1980; Lamb et al., 1981). The mRNA is transported into the cytoplasm where it can be translated into protein.

Replication of vRNA requires two rounds of transcription. The first round of transcription by the RdRP generates cRNA from the negative-sense vRNA template (Samji, 2009). During the second round

of transcription, the RdRP uses the cRNA as a template to generate more negative-sense vRNA. The influenza RdRP has a high mutation rate of about one mutation per genome replication, and therefore the vRNA generated isn't always an exact match of the parent vRNA (Drake, 1993). Accumulation of mutations causes the antigenicity of the viral proteins to change over time, a process known as antigenic drift. Both the cRNA and vRNA contain the 3' and 5' noncoding regions, whereas mRNA only contains the protein coding sequence (Cheung and Poon, 2007). The signals that cause the virus to switch from transcription to replication are not well understood. However, it has been proposed that the availability of NP in the nucleus is involved initiation of replication, perhaps by directly stabilizing the RdRP (Vreede et al., 2004). In addition, PB1 contains two domains that bind to either PB2 through PB1s C-terminal end or PA through PB1s N-terminal end (Gonzalez et al., 1996). A study by Honda et al. (2002) has suggested that transcription occurs while PB1 is bound to PB2, whereas replication occurs while PB1 is bound to BA.

*Nuclear Export*

After replication of the vRNA, the vRNPs are assembled and then transported out of the nucleus (Fields et al., 2007). The chromosome region maintenance 1 (CRM1) protein recognizes the two nuclear export signals (NES1 and NES2) of NS2/NEP and mediates the export of the vRNA (in addition to NEP and M1) by the CRM1-mediated pathway (Huang et al., 2013). The M1 protein is also involved in nuclear export of the vRNP by acting as a mediator between the vRNP and NEP. The C-terminal domain of M1 binds to the vRNP, whereas the N-terminal domain binds to NEP. This chain of proteins and vRNA can then be transported out of the nucleus through the nuclear pores after recognition of the NEP NESs by CRM1 (Samji, 2009). A similar process occurs for influenza B and C viruses (Fields et al., 2007).

*Packaging, budding, and release*

HA, NA, and M2, once translated by the ribosomes in the endoplasmic reticulum, are folded, post-translationally modified, and then transported to the Golgi apparatus (Fields et al., 2007). The apical sorting signals on the transmembrane domain of NA and HA recruit these proteins to the apical plasma membrane, along with M2 (Barman et al., 2001). How all of the viral proteins and vRNA assemble together to make a complete virion is still unknown. A study by Goto et al. (2013) identified packaging signals within the noncoding and coding regions of each viral RNA. It appears that the packaging signals within the noncoding region of influenza vRNA are essential for incorporation of each vRNA into the virion. After assembly of all of the viral components, M1 seems to be involved in budding of the virus from the apical plasma membrane, as deletion of the protein prevents viral budding, while expression of M1 alone can result in the production of virus-like particles (Gomez-Puertas et al., 2000). Finally, the neuraminidase located on the surface of the host's plasma membrane removes sialic acid residues from the cell surface in order to prevent re-binding to the same cell and production of viral aggregates (Palese et al., 1974).

## 2.7 Treatment and prevention

*Antiviral therapies*

There are two classes of antiviral therapies that are approved for use against influenza infections: matrix 2 inhibitors and neuraminidase inhibitors. Matrix 2 inhibitors, or adamantanes, are involved in preventing the release of the viral ribonucleoprotein into the cytoplasm (Dolin et al., 1982). Neuraminidase inhibitors specifically prevent the release of progeny virus from the host cell, thus

preventing the spread of infection (Davies, 2010; Hayden et al., 1999). Despite FDA approval, use of these antiviral therapies against influenza infection is controversial, as clinical studies evaluating their efficacy and ability to significantly reduce symptom burden and duration are limited.

*Matrix 2 inhibitors*

Adamantane drugs, such as amantadine and rimantadine, are a class of antiviral drugs known as matrix 2 inhibitors that block the translocation of ions through the M2. Adamantanes (derived from the greek work *adamantines,* meaning steel or diamond) consist of four interconnected cyclohexane rings that can exist in a stable boat-shaped or armchair-like structure. Adamantane drugs contain an organic backbone of adamantane ($C_{10}H_{16}$) and a functional group bound to one of four methynes (Senning, 2006). For amantadine, the extra functional group is an amine, while for rimantadine it is an ethylamine.

By performing nuclear diffraction nuclear magnetic resonance (NMR) spectroscopy analyses, amantadine was shown to bind to the outer region of M2 (Duff et al., 1994; Schnell and Chou, 2008). Prior to these studies, hydrophobic adamantanes were predicted to interact with hydrophobic residues lining the channel pore, thus actively blocking translocation. This theory was originally supported due to the observation that mutations of pore-lining, hydrophobic amino acid residues 26, 27, 30, and 34 are associated with influenza viruses with dual resistance to amantadine and rimantadine (Bright et al., 2005; Hay et al., 1985). In addition, Wang et al. (1993) showed that amantadines inhibit M2 activity with a 1:1 stoichiometry, therefore supporting the pore-blocking theory. Schnell and Chou (2008) proposed an alternative method in which adamantanes inhibit the translocation of ions through the M2 channel by an allosteric inhibition mechanism. They suggest that external binding of adamantanes cause the

channel to be more resistant to opening, but that mutations leading to the accumulation of hydrophilic amino acids within the pore allows the pore to open more readily in the presence of the adamantane, consequently allowing ion translocation. Interestingly, Schnell and Chou (2008) demonstrated through NMR spectroscopy that four molecules of amantadine can bind to influenza A M2, which opposes the findings of Wang et al. (1993).

Regardless of which mechanism is true, adamantanes are no longer recommended for influenza treatment due to the emergence of influenza viruses that have dual resistance to amantadine and rimantadine (CDC, 2006). During the 2005-2006 influenza season, 92% of influenza H3N2 and 25% of influenza H1N1 viruses contained an amino acid change at position 31 which confers dual-resistance to adamantanes (Bright et al., 2006).

Influenza A matrix protein 2 (M2) and influenza B matrix protein 2 (BM2) function as transmembrane ion channels, but the only homology between the two proteins is a Histidine-X-X-X-Tryptophan domain that facilitates ion translocation (Paterson et al., 2003). Structural mapping of the influenza A and B proteins indicate that the pore-lining residues within the influenza BM2 protein are hydrophilic, polar serines (Davies et al., 1964; Pinto and Lamb, 2006). Since influenza B viruses are not susceptible to adamantane inhibition, the presence of hydrophilic residues supports the allosteric inhibition mechanism.

A double-blind, placebo-controlled, randomized study of 450 volunteers was performed to test the prophylactic effects of amantandine and rimantadine during an influenza A outbreak. Treatment with 100 mg of amantadine or rimantadine twice a day for six weeks resulted in a 91% and 85% efficacy rate compared to placebo-controlled patients (Dolin et al., 1982). Treatment of influenza infection within 24 hours of symptom onset was associated with an average of one-day shorter fever duration after

treatment with amantadine 200mg/day (Wingfield et al., 1969). Similar results were obtained when patients with influenza A H3N2 infection were treated with rimantadine (Hayden and Monto, 1986).

Adamantanes have been used for the treatment of Parkinson's disease because they are weak antagonists to the N-methyl-D-aspartate receptor (NMDAR). Increased activity of NMDARs are associated with dyskinesia, or involuntary movement, in Parkinson's disease, and treatment with adamantanes can reduce these symptoms (Hallett and Standaert, 2004). Unfortunately, treatment with adamantanes during influenza infection has been associated with neurological side effects such as insomnia, hallucinations, and agitation due to its affinity for the neurological receptor NMDA (Jefferson et al., 2009).

*Neuraminidase inhibitors*

Oseltamivir, zanamivir, and peramivir are antiviral therapies that inhibit neuraminidase activity. Neuraminidases are surface-expressed viral glycoproteins that are involved in the release of newly formed viruses from the host cell (Gottschalk, 1957). The sialidase activity of neuraminidases mediates hydrolysis of the glycosidic linkage between a sialic acid and galactose. When sialic acids aren't actively removed from the surface of the host cell, influenza infection is attenuated because 1) the release of progeny virus from the host cell is limited, thus reducing the spread of infection, 2) the progeny virus re-enters the same cell from which it was derived, preventing the spread of infection, and/or 3) progeny viruses that escape from the host cell have residual sialic acid receptors on its surface and bind to other viruses via their hemagglutinin proteins, forming noninfectious aggregates (Palese et al., 1974). Neuraminidase inhibitors bind to the catalytic site of the neuraminidase, preventing the hydrolysis of

the glycosidic linkage (De Clercq, 2006). Therefore, sialic acids and the neuraminidase inhibitors compete for the neuraminidase catalytic site. The neuraminidase subtype 2 catalytic site consists of eight charged amino acid residues, R118, D151, R152, R224, E276, R292, R371, and Y406, that interact with the sialic acid receptor (Colman et al., 1983). These residues are also involved in hydrolysis of the glycosidic link, while surrounding residues E119, R156, W178, S179, D/N198, I222, E227, H274, E277, N294, and E425 are involved in supporting the structure of the catalytic domain (Colman, 1994; Colman et al., 1983). Neuraminidase inhibitors are designed to fit into the neuraminidase catalytic site and other domains; the charged amino acids on the neuraminidase typically form multiple hydrogen bonds with functional groups found on the neuraminidase inhibitors, thus preventing entry of the native sialic acids.

Oseltamivir and zanamivir are sialic acid analogs or mimics that actively bind to the catalytic site of the neuraminidase and prevent sialic acid cleavage. Of the two molecules, zanamivir most closely resembles the sialic acid molecule, Neu5Ac. In 1999, zanamivir was the first neuraminidase inhibitor to be approved by the FDA. The molecule consists of a neuraminidase backbone with C-4 hydroxyl group with a guanidinyl functional group (4-deoxy-4-guanidino-Neu5Acen). The functional group included in zanamivir improves affinity for the neuraminidase by forming an electrostatic interaction glutamic acid at residue 119 (Varghese et al., 1995). Zanamivir was found to have a low bioavailability when delivered by the oral route, average 2%, however delivery by inhalation resulted in 10-20% absorption (Cass et al., 1999).

In order to establish a delivery method that is conducive to compliant administration and improve bioavailability, oseltamivir was developed. Oseltamivir, ethyl (3R,4R,5S)-5-amino-4-acetamido-3-(pentan-3-yloxy)-cyclohex-1-ene-1-carboxylate is a pro-drug that uses the addition of an ethyl-ester group to improve lipophilicity, resulting in increased bioavailability of about 80% (Davies, 2010; von

Itzstein, 2007). The ethyl-ester is removed *in vivo* by esterases, resulting in an activated neuraminidase inhibitor.

Clinical trials evaluating the effects of early oseltamivir administration revealed that treatment within 12 hours of symptom onset can reduce the duration of symptoms an average of three days (Aoki et al., 2003), whereas treatment within 36-48 hours of symptom onset reduces the duration of symptoms by one day (Nicholson et al., 2000; Treanor et al., 2000). However, a systematic review by (Hsu et al., 2015) determined that a majority of the clinical studies associated with oseltamivir efficacy had very low quality of evidence due to factors such as imprecision and risk of bias.

A systematic review indicated that treatment with at least 10 mg of zanamivir per day resulted in about a 14-hour reduction in duration of symptoms compared to placebo treatment (Heneghan et al., 2014). Both adults and children included in these studies received similar benefits from zanamivir treatment. A clinical trial by Lalezari et al. (2001) determined that treatment of influenza A or B positive patients with zanamivir within 48 hours of symptom onset reduced the time to alleviate symptoms by 2.5 days. The incidence of complications that required antibiotic intervention was reduced by 43% compared to patients who received placebo treatment.

Peramivir was approved by the FDA in 2014 for intravenous use in influenza infected patients 18 years or older (FDA, 2014). It is structurally distinct from other neuraminidase inhibitors (Alame et al., 2016). Peramivir is a cyclopentane containing a C4-guanidino substitution and a hydrophobic side chain, which each interact with a hydrophilic pocket within the neuraminidase protein (Kim et al., 1997). The functional groups selected for the formation of peramivir provides additional hydrogen bonds that facilitate a tight interaction with the neuraminidase active site, compared to oseltamivir and zanamivir. Bantia et al. (2006) showed that oseltamivir and zanamivir have about a 16-fold lower dissociation rate

than peramivir. A study by Kohno et al. (2010) revealed that IV treatment with peramivir (300 or 600 mg) 48 hours after symptom onset resulted in a significant reduction in viral shedding and symptom duration.

There have been very few incidences of resistance toward neuraminidase inhibitors during treatment against influenza infection compared to treatment with adamantanes. During the 2015-2016 flu season, 0.8% of 2009 pandemic H1N1 influenza viruses that were circulating were resistant to oseltamivir and peramivir (CDC, 2016d). 100% of the 2009 pandemic H1N1 influenza A viruses were susceptible to zanamivir. In addition, all of the influenza A H3N2 and influenza B viruses that were tested were 100% susceptible to oseltamivir, peramivir, and zanamivir (CDC, 2016d). Adverse effects associated with neuraminidases are similar for oseltamivir, zanamivir, and peramivir and include nausea, vomiting and diarrhea (Kohno et al., 2011).  Mutations within the catalytic domain or the supporting structure is often associated with neuraminidase inhibitor resistance (Abed et al., 2006; Weinstock et al., 2003). One study examining influenza A (H3N2) isolates from 50 children treated with oseltamivir found that viruses containing mutations at amino acids 119, 292 and 294, which are found within the catalytic domain or are supporting amino acids, were resistant to oseltamivir treatment (Kiso et al., 2004). A R292K mutation was the most common and was found in 18% of the children tested; resistant strains were isolated as early as four days post-treatment.


*Vaccines*

Vaccines (derived from the Latin word *vaccinus*, or *vacca*, meaning cow) are substances that stimulates the host's immune response to produce antibodies or activated immune cells that can specifically target and clear influenza viruses and infected cells (Fields et al., 2007). Currently there are

seven types of flu vaccines available in the United States for the prevent of influenza A and B infection, including both trivalent and quadrivalent vaccines: inactivated quadrivalent vaccine (egg propagated or cell culture-based), inactivated trivalent vaccine, adjuvanted trivalent influenza vaccine, high dose inactivated trivalent vaccine, recombinant trivalent vaccine, and the live attenuated quadrivalent vaccine (CDC, 2016e). The type of vaccine recommended for use in a particular demographic is dependent on their immune composition. For instance, high dose inactivated trivalent vaccines and vaccines containing adjuvants, substances that enhances the host's immune response to an antigen, are recommended for use in patients 65 years or older in order to stimulate a stronger immune response against influenza viruses. The Fluzone High-Dose vaccine, which contains four-times more antigen than the standard dose, was found to be 24.2% more effective against protecting adult patients against influenza infection compared to the standard-dose Fluzone vaccine (DiazGranados et al., 2014). A larger number of patients were found to have a 4-fold increase in influenza-specific antibody titer or more than 128 hemagglutinin inhibitory ability after receiving the adjuvant-containing influenza vaccine, MF59, than patients receiving the flu vaccine without the adjuvant (Sindoni et al., 2009). The live attenuated vaccine is only recommended for those between the age of 2 and 49; however, the vaccine was not found to be effective in children less than 2 years of age, the elderly, and pregnant women and could cause complications in immunocompromised patients (CDC, 2013).

*Trivalent vs. Quadrivalent vaccines*

A monovalent, bivalent, trivalent, or quadrivalent vaccine is designed to target one, two, three, or four antigens or microorganisms, respectively. Current influenza viruses are manufactured as either trivalent or quadrivalent. Trivalent vaccines contain three different influenza viruses, two influenza A

viruses and one influenza B virus. Four influenza virus variants are included in quadrivalent vaccines, two influenza A viruses and two influenza B viruses. Quadrivalent flu vaccines give a broader range of protection, by stimulating an immune response against two influenza B lineages. One clinical study showed that there was no difference in the rate of seroconversion of B/Malaysia-specific antibodies when vaccinated with a trivalent or quadrivalent vaccine, both containing the influenza B/Malaysia lineage. However, the quadrivalent vaccine (containing the influenza B/Jiangsu lineage) was necessary to generate neutralizing antibodies against influenza B/Jiangsu (Beran et al., 2013). This information suggests that the quadrivalent vaccine gives broad protection against influenza viruses and that vaccination against one influenza B lineage does not provide sufficient protection against another influenza B lineage. The immune response provided by the vaccine is dependent on which antigens are included in the vaccine. Influenza vaccines are manufactured as whole virus, split virus, subunit, or recombinant vaccines (WHO, 2014). Whole viruses and split virus vaccines can stimulate immune responses against all influenza proteins, whereas subunit and recombination vaccines contain purified hemagglutinin and neuraminidase proteins or only hemagglutinin proteins, respectively (Beyer et al., 1998). The recombinant influenza vaccine (see below) is a trivalent vaccine that simulates an immune response against three hemagglutinins, whereas the quadrivalent live-attenuated vaccine (see below) stimulates an immune response against all influenza virus proteins.

*Inactivated vs. live-attenuated vaccines*

Current influenza vaccines contain either inactivated or live-attenuated influenza viruses, with the exception of the recombinant influenza vaccine which only contains purified hemagglutinin (Cox et al., 2008; Sridhar et al., 2015). Inactivated influenza vaccines consist of replicative-deficient whole or

portions (subunit or split-virion) of the influenza viruses selected for the vaccine. Whole inactivated influenza viruses are inactivated by chemical treatment (i.e. formalin or β-propiolactone) or heat (Wong and Webby, 2013). Split-virion inactivated vaccines undergo the same inactivation technique, but the lipid membrane of the virus is also disrupted by detergent treatment, thus exposing all viral proteins. Subunit vaccines are developed by specifically purifying the hemagglutinin and neuraminidase proteins from the rest of the virus structure. Live-attenuated vaccines contain viruses that are able to directly infect respiratory epithelial cells and replicate. The virus is attenuated, or weakened, in order to prevent extensive replication and disease (Fischer et al., 2015). The cold-adapted viruses within the live-attenuated influenza vaccine replicates well in the nasopharynx but poorly in the warm environment of the lower respiratory tract (Murphy and Coelingh, 2002). Attenuation is performed by serial passaging the virus through tissue culture or eggs until the virus accumulates enough mutations that weakens its ability to replicate efficiently (Maassab and DeBorde, 1985).

Live-attenuated vaccines are associated with a multifaceted immune response which includes the development of neutralizing antibodies specific for hemagglutinin and neuraminidase (Lee et al., 2004) (Iba et al., 2014; Margine et al., 2013), a localized immune response with the production of influenza specific IgA (Ambrose et al., 2012), and activation of CD4+ and CD8+ T cells (La Gruta and Turner, 2014; Whitmire et al., 2005). By vaccinating with an infectious virus, the viral proteins can enter the host epithelial cells and become presented by major histocompatibility complex I (MHC I) which stimulates the activation of influenza-specific cytotoxic (CD8+) T cells. Influenza-specific CD8+ T cells are important antiviral mediators that kill influenza-infected cells, thus preventing the spread of infection. In contrast, inactivated influenza vaccines are unable to infect epithelial cells and do not activate CD8+ T cells; instead, they only activate CD4+ T cells through major histocompatibility complex II (MHC II) on

antigen-presenting cells. Influenza-specific CD4+ T cells play an important role in secreting cytokines, such as interferon gamma and interleukin-2, that are involved in the activation of CD8+ T cells (Blachere et al., 2006; Whitmire et al., 2005) and isotype switching (Brown et al., 2004). Both inactivated and live-attenuated vaccines activate CD4+ T cells and promote the secretion of influenza-specific antibodies and activation of B cells. Although live-attenuated influenza vaccines produce a more complex immune response to influenza infection, many studies have identified inactivated influenza vaccines to be more effective in protecting against influenza infection (Monto et al., 2009). One clinical study of 6819 participants, 2703 of whom were vaccinated showed that there was no difference in vaccine efficacy between the inactivated vaccine and live-attenuated vaccine when targeting the A/H3N2 or influenza B viruses. However, efficacy was lower for the live-attenuated vaccine when protecting against the 2009 influenza A/H1N1 pandemic strain (Chung et al., 2016). Recently, the CDC's Advisory Committee on Immunization Practices voted that the live-attenuated vaccine nasal spray no longer be recommended for use during the 2016-2017 flu season, due to the lack of protection provided by the vaccine within patients between 2 years to 17 years of age (CDC, 2016a). The inactivated influenza vaccine was found to be more effective in this population, with an estimated 63% efficacy versus a 3% efficacy after vaccination with the live-attenuated vaccine.

*Manufacturing techniques*

A majority of the viruses included in influenza vaccines, inactivated or live-attenuated, are propagated in eggs in order to provide a cost-effective method that generates a large yield of influenza viruses. Influenza viruses readily replicate within the cells of the chorioallantoic membrane and progeny viruses accumulate within the allantoic fluid (Brauer and Chen, 2015).  A study by Osborne et al. (2011)

tested 2768 infants in Australia to determine the incidence of egg allergies. They found that about 9% of the infants tested were positive for egg-specific IgE. Because flu vaccines may contain residual egg-associated antigens, vaccination of patients with egg allergies are not recommended. Instead, people with who are immunocompromised or have severe egg allergies typically rely on herd immunity for protection against influenza infection (Plans-Rubió, 2012). CDC encourages those with minor allergies to eggs to still obtain a flu vaccine, as the amount of the ovalbumin allergen is minimal, and the rate of anaphylaxis after vaccination is 1.31 per one million vaccine doses given (CDC, 2016c).

An alternative approach to propagate influenza vaccine strains was approved by the FDA in 2012 (FDA, 2012). Madin-Darby canine kidney (MDCK) epithelial cells are used to culture the virus and have been found to provide a higher viral yield compared to egg propagation (Milián and Kamen, 2015). This method still uses egg-propagated viral strains to initiate propagation by cell culture, but the amount of egg antigen within the cell culture-based propagation would be significantly diluted compared to egg propagation (Katz and Webster, 1989). Currently, only one vaccine using the cell-based propagation technology, Flucelvax, is available in the United States (Moro et al., 2015).

Flublok is a trivalent vaccine that uses recombinant technology (Cox et al., 2008). Protein Science Corporation developed baculovirus expression vectors encoding for three hemagglutinin proteins. Once delivered to ovarian cells from the Fall Army worm, *Spodoptera frugiperda*, the recombinant hemagglutinin proteins that are synthesized can be purified and incorporated into the Flublock vaccine. The recombinant vaccine contains comparable amounts of total influenza protein to Fluzone; the difference is that Flublok contains three times more hemagglutinin (45 µg) than the trivalent inactive vaccine. Flublok acts similarly to inactivated flu vaccines, as the components of the vaccine do not replication within the host. However, the antigens provided are limited to the hemagglutinin. The Flublok

vaccine was shown to induce higher antibody titers to H3N2 influenza subtype than the trivalent inactivated vaccine (Treanor et al., 2006).

*Process of selection for seasonal vaccines*

Antigenic drift and shift are processes that occurs in influenza viruses when the viruses accumulates mutations or undergoes genomic rearrangement, thus changing their antigenic properties. Because of this, new vaccines must be produced yearly in order stimulate a strain-specific immune response. In 1952, the WHO Global Influenza Surveillance and Response System was developed to monitor changes in the virus' antigenicity and to aid in the selection of strains for the annual influenza vaccine (WHO, 2012). Throughout the year, the WHO and collaborators (CDC, the Francis Crick Institute, VIDRL, NIID, IVDC) collect epidemiological data through surveillance (identification of circulating strains, mutations, illnesses), laboratory testing (identifying antigenic testing and antiviral resistance) and clinical studies (CDC, 2016b, f, g). Twice a year, these institutions gather together to review this information and determine which influenza virus strains should be included in the upcoming seasonal flu vaccine; in February recommendations are made for the Northern Hemisphere's vaccine and in September, recommendations are made for the Southern Hemisphere's vaccine ((CDC, 2016g). The projected strains of influenza included in the annual influenza vaccine don't always match the circulating strains of the new flu season, and thus the vaccine doesn't always promote efficient immunity against those strains. The vaccine efficiency can range from 30% to 80% (Gupta et al., 2006).

A study during 47 flu seasons showed that both matching and mismatched flu vaccines offered significant protection against influenza infection. However, seven studies evaluating the efficacy of live-attenuated influenza vaccines containing strains that match the circulating strains of the season found an 83% vaccine efficacy, compared to 54% efficacy when the vaccine and circulating strains don't match

(Tricco et al., 2013). Trivalent inactivated vaccines had a similar ability to protect against mismatched strains, but they had a 65% vaccine efficacy against matched strains. The systematic review and meta-analysis provided by Tricco et al. (2013) demonstrated that vaccines that don't match circulating strains are less effective than when the vaccine and circulating strains match. This information, along with the fact that strains have emerged that are resistant to two classes of anti-influenza therapies suggests that new vaccines and antiviral therapies should be developed in order to account for the changing antigenicity and structure of influenza viruses.

## 2.8 Research for improved antiviral therapies and vaccines

In order to generate broad range protection against various antigenically district influenza viruses, scientists have focused on the development of improved antiviral therapies and universal influenza vaccines. The sections below provide a few examples of the many efforts that are involved in the development of these improved methods for anti-influenza therapeutics and the development of vaccines with a broad range of protection.

*Antiviral therapy research*

Laninamivir

Laninamivir, (2R,3R,4S)-3-acetamido-2-[(1R,2R)-2,3-dihydroxy- 1-methoxypropyl]-4-guanidino-3,4-dihydro-2H- pyran-6-carboxylic acid (or R-125489), is a new neuraminidase inhibitor that is currently in phase 2 clinical trials (Yamashita, 2010; Yamashita et al., 2009). It is a derivative of zanamivir, with a

7-methoxy chain. Treatment with laninamivir against oseltamivir-resistant viruses such as H274Y mutant 2009 pH1N1, H1N1 N294S and H3N2 E119V mutants has been successful (McKimm-Breschkin and Barrett, 2015; Nguyen et al., 2010). Zanamivir-related mutations in influenza B (D197E) and influenza A H1N9 (E119G) viruses are less susceptible to laninamivir treatment, which may be due to a rapid dissociation rate (McKimm-Breschkin and Barrett, 2015). Esterification of laninamivir resulted in the development of a pro-drug, 3-(O)-octanoyl laninamivir (or CS-8958), that is metabolized in the lungs and prolongs the life of laninamivir (McKimm-Breschkin and Barrett, 2015; Yamashita et al., 2009). The concentration at which neuraminidase activity was inhibited by 50% ($IC_{50}$) was 10-fold lower for CD-8958 than laninamivir when treating influenza A H3N2 and H2N2 viruses (Yamashita et al., 2009). Treatment of influenza A/PR/8/34 infected mice with a single intranasal dose (0.5µmol/kg) of CS-8958 one day prior to infection resulted in 90% survival of mice, whereas zanamivir treatment led to a 30% survival (Yamashita et al., 2009). A phase II trial performed in Japan during the 2008-2009 flu season found that CS-8958 treatment reduced the median time to alleviation of influenza A H3N2 symptoms by 6 hours compared to oseltamivir treatment, whereas no difference was seen when comparing influenza A H3N2 or influenza B symptom alleviation (Sugaya and Ohashi, 2010). Other studies and a phase III clinical trial in adults showed that a single dose of CS-8958 was as effective at relieving influenza-associated symptoms as treatment with a twice-daily dose of oseltamivir (Kashiwagi et al., 2016; Watanabe et al., 2010). Although treatment with CS-8958 may not be more effective than treatment with oseltamivir, the ability to deliver treatment with CS-8958 via a single dose might improve compliance.

Favipiravir (T-705)

An RNA-dependent RNA polymerase (RdRP) inhibitor called favipiravir (T-705) has been developed for the treatment of various RNA viruses, such as influenza, Rift Valley fever virus (Caroline et

al., 2014), and yellow fever virus (Julander et al., 2009). The chemical structure of favipiravir, 6-fluoro-3-hydroxy-2-pyrazinecarboxamide, is a prodrug that becomes phosphoribosylated to become favipiravir-RTP (Smee et al., 2009). Favipiravir-RTP is incorporated into the viral RNA during replication or mRNA during transcription by the RdRP. The addition of favipiravir-RTP then prevents elongation of the nascent RNA, thus preventing RdRP activity (Sangawa et al., 2013). Sequence analysis of influenza viruses derived from T-705 treated MDCK cells showed that the viruses acquired lethal mutations that resulted in the virus being defective or non-viable (Baranovich et al., 2013). Favipiravir specifically inhibits RdRP activity, as treatment with 1,000 μM of favipiravir was unable to inhibit the activity of human DNA polymerases (Kiso et al., 2010), and a high concentration, 905 μM, is required to inhibit the human RNA polymerase promoter (Takahashi et al., 2011).

Small interfering RNA (siRNA)

Since the discovery of RNA interference (RNAi), the use of small regulatory RNA to inhibit viral replication has been a focus of research (Ge et al., 2004; Henke et al., 2008; Janssen et al., 2013). However, siRNA therapeutics targeting influenza viruses are limited by the ability of the virus to mutate and undergo antigenic variation. Systematic in silico analyses have been performed in order to determine which nucleotide sequences within influenza A viruses are most conserved, thus potentially providing a broad-spectrum area to target via RNAi (ElHefnawi et al., 2011). Analysis of 22,000 complete segment sequence of influenza A viruses isolated between 1918 and 2007, identified 87 highly conserved (greater than 90% conservation) regions belonging to the PB2, PB1, PA, NP, M, and NS segments. The longest conserved motif was 17 nucleotides, located on the matrix segment, which is slightly shorter than the size of most miRNA or siRNA. Stoppani et al. (2015) identified a conserved sequence spanning nucleotides 1425-1494 of the nucleoprotein segment that could be targeted via eight putative 19-

nucleotide siRNA targeting sites. Small-hairpin RNA (shRNA) expression vectors were developed to test the efficacy of the two most potent putative siRNAs, NP6 an NP7, to inhibit viral replication. NP6 and NP7 expression vectors were able to reduce nucleoprotein mRNA 54,000-fold and 25,000-fold, respectively, and attenuated viral replication of various subtypes of influenza A viruses (H1N1, H1N2, H3N2, and H1N1). Nucleoprotein mRNA containing a single nucleotide mismatch to NP7 siRNA was significantly reduced as well (1,200-fold reduction). Although NP6 and NP7 were able to significantly reduce nucleoprotein mRNA expression, these siRNAs only target an estimated 19.72% and 9.11% of swine influenza with full complementarity, respectively, and 4.94% and 1.06% of human influenza viruses with full complementarity. Although the NP4 siRNA didn't reduce nucleoprotein mRNA to the same extent as NP6 and NP7, this siRNA should be further characterized as it can target an estimated 97.86% of swine influenza, 97.19% of avian influenza and 86.70% of human influenza with 100% complementary. This study shows the promise of the field of siRNA-mediated antiviral therapeutics to inhibit the replication of a broad range of influenza A viruses.

*Vaccine research*

Research efforts are currently attempting to develop a universal vaccine that would eliminate the need to develop a new vaccine each year. In theory, a universal vaccine would prime an immune response against all influenza strains, regardless of the virus' ever-changing surface proteins. Current efforts to create such a vaccine are aimed at producing immunity against conserved influenza antigens located on the surface of influenza viruses, such as the hemagglutinin and extracellular domain of matrix 2 (M2e).

Hemagglutinin targeting vaccines

Hemagglutinin (HA) proteins are surface-expressed glycoproteins that are involved in binding and fusion of the virus to the host cell membrane (Fields et al., 2007). The HA is a prime candidate for the development of a vaccine against influenza viruses because it is expressed abundantly on the surface of the virus. For this reason, the most prominent neutralizing antibodies generated during influenza infection recognize the HA antigen (Laursen and Wilson, 2013).

HA is expressed as a precursor molecule (HA0) that is later cleaved by specific proteases (i.e. human airway trypsin-like proteases or transmembrane protease serine S1 member 2) in the host (Böttcher-Friebertshäuser et al., 2010). The two new proteins, HA1 and HA2, continue to localize on the surface of the virus. HA1 constitutes the protein head, whereas the amino- and carboxyl-terminal ends of HA1 and the ectodomain of HA2 constitute the HA stalk (Wilson et al., 1981). Cleavage exposes the hemagglutinin fusion domain on the HA2 which is involved in fusing the viral and host membranes, thus mediating viral entry (Smrt et al., 2015). Because cleavage is necessary for the viral entry into the host cell and is conserved among influenza A and B viruses (Kawaoka and Webster, 1988; Macken et al., 2001; Nobusawa et al., 1991), the cleavage site has been studied for the development of universal vaccine. A study by Bianchi et al. (2005) developed a universal influenza B vaccine that includes 8 amino acids upstream, and 11 amino acids downstream of the HA cleavage site. Vaccination of BALB/c mice induced HA0-specific antibody responses in mice and protected mice from lethal viral challenge compared to mice vaccinated with the outer membrane protein complex of *Neisseria meningitides*. Passive transfer of serum from immunized mice protected naïve mice from influenza infection, indicating that protection was caused by influenza-specific antibodies. Cross-lineage protection between influenza B viruses was

also demonstrated. The same study showed that vaccination with an influenza A cleavage domain protects mice (60% survival) against lethal challenge with influenza B.

Most of the current vaccines develop antibodies targeting the highly variable, exposed globular head of hemagglutinin (Marozin et al., 2002). In order to develop a universal vaccine with broad protection against the globular head, antibodies targeting conserved regions of the HA head should be developed. Broadly-neutralizing human antibodies have been identified: one example is F005-126, which has been shown to bind to conserved regions found on influenza A H3N2 viruses, residues 91, 92, 171, 173, 239, 240 270, 273, 284, and 285 (Iba et al., 2014). Two other neutralizing antibody isolates, CH65 and C05, interact with the receptor binding site of HA (Ekiert et al., 2012; Whittle et al., 2011). CH65 was able to neutralize 83.3% of the viral H1N1 strains tested (total 36 viruses) and was shown, through crystal structure analysis, to mimic sialic acids in their ability to bind to the receptor binding pocket of HA1 (Whittle et al., 2011). C5 antibodies were able to protect mice from lethal challenge with influenza A/Memphis/3/2008 (H1N1; 100% survival) and influenza A/Aichi/2/X-31/1968 (H3N2; 80-100% survival) (Ekiert et al., 2012). Post-vaccination exposure to H1N1 or H3N2 resulted in 40-100% survival and 100% survival, respectively. Understanding which HA residues these naturally occurring neutralizing antibodies bind can aid in the development of antigens for use in universal vaccines.

The HA stalk is only exposed for a short period of time between initial cleavage of the HA0 and fusion of the virus with the host membrane. Therefore, immunological selection for a mutant stalk is limited, which allows for antigenic conservation (Mallajosyula et al., 2015). Broadly neutralizing human antibodies targeting the HA stalk have been developed after infection with H3N2 (Margine et al., 2013). In addition, a study by Dunand et al. (2015) determined that 3 out of 83 H3-reactive antibodies developed in previously vaccinated patients were able to neutralize influenza H7N9 strain by binding to

the HA stalk. Passive transfer of the neutralizing antibodies to BALB/c mice conferred 100% protection against lethal challenge with influenza A/Shanghai/1/2013 (H7N9) (Dunand et al., 2015). Headless HAs have been produced in order to expose the host's immune response to the stalk (Impagliazzo et al., 2015). Krammer et al. (2014) developed a conserved stalk domain-based vaccine, after identifying the antigen recognized by the cross-reactive 6F12 antibody. Delivery of an adenovirus 5 vector expressing the HA stalk (cH6/1) into ferrets resulted in the production of cross-reactive IgG antibodies that recognize H1, H2, and even H17 hemagglutinins.

Matrix targeting vaccines

Another protein that is of interest for the development of a universal vaccine is the extracellular domain of the M2 protein (M2e). It is localized to the viral surface and is conserved among all influenza A viruses that circulated between 1918 and 2008 (Deng et al., 2015; Neirynck et al., 1999). For this reason, it can serve as an antigen targeting influenza A viruses in a universal vaccine. Unfortunately, M2e is small in size (23 amino acid residues) and has low immunogenicity, and therefore it must be conjugated to carrier molecules in order for it to be recognized by the immune system (De Filette et al., 2008; Fan et al., 2004; Kang et al., 2011). To overcome the low antigenicity of the small M2e peptide, a hepatitis B virus core (HBc) fusion protein containing the M2e domain (M2HBc) was generated (De Filette et al., 2008). Purified M2HBc proteins were obtained from *Escherichia coli* cells transformed with an expression vector encoding for the M2HBc protein, under the control of the left-ward promoter ($P_L$) of phage λ. Eleven out of 12 mice vaccinated with the purified protein were protected from influenza A/Victoria/3/75 X-47 (H3N2) challenge; a similar response was seen when mice received passive immunization with serum from immunized mice. Vaccination also protected mice against lethal

challenge with A/PR/8/34 H1N1; thus, the antibody response generated by the vaccine produced broad-range protection against two subtypes of influenza viruses. A modified vaccine was produced, which included three consecutive M2e domains fused to HBc (De Filette et al., 2005). The modified vaccine proved higher IgG1 and IgG2a titer and slightly improved survival rates in mice challenged with a lethal dose of influenza A/Victoria/3/75 X-47 (H3N2) compared to mice vaccinated with unmodified M2HBc. ACAM-FLU-A vaccine delivers the same antigen and has shown good safety profiles in a phase I clinical trial.

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# Chapter 3 - Inhibition of influenza A virus matrix and nonstructural gene expression using RNA interference

# Inhibition of influenza A virus matrix and nonstructural gene expression using RNA interference.

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## 3.1 Abstract

Influenza antiviral drugs that use protein inhibitors can lose their efficacy as resistant strains emerge. As an alternative strategy, we investigated the use of small interfering RNA molecules (siRNAs) by characterizing three siRNAs (M747, M776 and M832) targeting the influenza matrix 2 gene and three (NS570, NS595 and NS615) targeting the nonstructural protein 1 and 2 genes. We also re-examined two previously reported siRNAs, M331 and M950, which target the matrix 1 and 2 genes. Treatment with M331-, M776-, M832-, and M950-siRNAs attenuated influenza titer. M776-siRNA treated cells had 29.8% less infectious virus than cells treated with the previously characterized siRNA, M950. NS570-, NS595- and NS615-siRNAs reduced nonstructural protein 1 and 2 expression and enhanced type I interferon expression by 50%. Combination siRNA treatment attenuated 20.9% more infectious virus than single siRNA treatment. Our results suggest a potential use for these siRNAs as an effective anti-influenza virus therapy.

## 3.2 Introduction

Influenza viruses cause respiratory infections that account for 3-5 million cases of severe illnesses and 250,000-500,000 deaths worldwide each year (Thompson et al., 2010; WHO, 2014). Classic symptoms of influenza virus infection include fever, malaise, headache, cough, and sore throat and are typically cleared within three to five days (Hayden F.G., 2009; Monto et al., 2000). However, those with weakened immune systems, such as children, pregnant mothers and fetuses, and the elderly, have an increased risk for severe complications or death (Haberg et al., 2013; Jamieson et al., 2009; Thompson et al., 2004). Consequently, vaccines and antiviral therapies have been developed to protect individuals from influenza infection.

Neuraminidase inhibitors (i.e. zanamivir and oseltamivir) and matrix 2 inhibitors (i.e. adamantine and rimantadine) are two common types of antiviral therapies approved by the United States Food and Drug Administration (FDA) that target influenza viruses (Gubareva et al., 2000; Wang et al., 1993). Neuraminidase inhibitors target both influenza A and B viruses, while matrix 2 inhibitors only target influenza A viruses, which limits their range of protection. These antiviral therapies have been reported to have significant side effects and minimal prophylactic or treatment effect (Dolin et al., 1982; Hanshaoworakul et al., 2009; Hsu et al., 2015; Millet et al., 1982). Furthermore, influenza virus genomes undergo a high rate of mutation (~1 mutation per genome replication;Drake, 1993), and cases of resistance against adamantine and oseltamivir have been reported. Sheu et al. (2008) identified the emergence of dual resistance to adamantine and oseltamivir among 28 influenza A (H1N1) viruses isolated during the 2008 influenza season. Consequently, there is an urgent need to develop alternative methods of antiviral treatment in order to address emerging resistance, adverse health effects, and the lack of efficacy associated with current antiviral therapies.

RNA interference (RNAi) is a post-transcriptional genetic mechanism in which gene expression is suppressed by RNA molecules such as microRNA (miRNA), small interfering RNA (siRNA), or anti-sense RNA oligonucleotides. RNAi involves RNA induced silencing complex (RISC) mediated transcript degradation or translational repression by miRNA or siRNA (Fire et al., 1998; Zamore et al., 2000). Alternatively, gene silencing via miRNA can be silenced using miRNA inhibitors. The study of gene silencing by RNAi has led to the development of a novel treatment for hepatitis C virus infection, Miravirsen, an anti-sense RNA molecule that silences the activity of the miRNA, miR122. In a phase 2a clinical trial, Miravirsen treatment showed sustained dose-dependent elimination or reduction in hepatitis C virus RNA levels, compared to placebo treated patients, without the emergence of viral resistance (Janssen et al., 2013). RNAi has several potential advantages as a means of therapeutic treatment compared to commonly used small-molecule drugs (i.e. antibodies, allosteric inhibitors, etc.). Effective siRNA can be designed easily by using various algorithms and can be quickly manufactured and altered as mutant strains emerge. Furthermore, siRNA can reduce the expression of targeted mRNA which can subsequently decrease the expression of both intracellular and extracellular proteins, and they can be effective at low concentrations (Smith et al., 2008; Tafer, 2014; Yamada and Morishita, 2005).

Thus far, only a handful of siRNAs have been characterized that effectively attenuate influenza infection (Ge et al., 2003; Hui et al., 2004; Sui et al., 2009; Zhou et al., 2007). Consequently, in the present study, we have designed six siRNAs targeting the matrix or nonstructural protein transcripts and compared the effect of treatment with a single siRNA to treatment with a combination of siRNAs against influenza A (H1N1) infection *in vitro* with the goal of characterizing new siRNAs that can be used as an effective anti-influenza therapy.

## 3.3 Materials and methods

*Cell lines and Viruses*

Madin-Darby Canine Kidney (MDCK) Epithelial cells were provided by Dr. Daniel Perez (University of Maryland, MD). MDCK cells were propagated and maintained in T75 cm$^2$ Corning CellBind Surface flasks (Corning, Inc.) using a complete growth media consisting of Eagle's Minimum Essential Medium (EMEM; American Type Culture Collection (ATCC)), 10% (v/v) fetal bovine serum (Hyclone Laboratories, Inc.), 200 units/mL penicillin, and 200 µg/mL streptomycin (Gibco, ThermoFisher Scientific). Cells were incubated at 35°C in a humidified 5% $CO_2$ incubator until 90% confluent.

Influenza strain A/WS/33 (H1N1) (VR-1520, ATCC) was maintained as described previously (Blachere et al., 2011). Total viral titer was determined by quantitative polymerase chain reaction (qPCR) and infectious viral titer was quantified by viral plaque assay (VPA).

*Small interfering RNAs (siRNAs)*

siRNAs against the matrix 1 and matrix 2 transcripts were designed by the BLOCK-iT™ RNAi Designer (ThermoFisher Scientific). Purified Stealth siRNAs M747, M776 and M832 were synthesized by Life Technologies (ThermoFisher Scientific), suspended in nuclease-free water and stored at -80°C (M747 sense: 5' ACGAUUCAAGUGAUCCUCUCGUCAU 3', M747 anti-sense: 5' AUGACGAGAGGAUCACUU GAAUCGU 3', M776 sense: 5'CAGCAAAUAUCAUUGGAAUCUUGCA 3', M776 anti-sense: 5' UGCA AGAUUCCAAUGAUAUUUGCU G 3', M832 sense: 5' CAAAUGCAUUUAUCGUCGCUUUAAA 3', M832 anti-sense: 5' UUUAAAGCGACGAUAAAUGCA UUUG 3').

siRNAs against the nonstructural protein 1 and 2 transcripts were aloso designed by the BLOCK-iT™ RNAi Designer (ThermoFisher Scientific). Purified Stealth siRNA NS570, NS595 and NS615 were

synthesized by Life Technologies, suspended in nuclease-free water and stored at -80℃ (NS570 sense: 5' CACAGUUCGAGUCUCUGAAACUCUA 3', NS570 anti-sense: 5' UAGAGUUUCAGAGAC UCGAACUGUG 3', NS595 sense: 5' CAGAGAUUCGCUUGGAGAAGCAGUA 3', NS595 anti-sense: 5' UACUGCUUCUCC AAGCGAAUCUCUG 3', NS615 sense: 5' CAGUAAUGAGAAUGGGAGACCUCCA 3', NS615 anti-sense: 5' UGGAGGU CUCCCAUUCUCAUUACUG 3'). Stealth RNAi siRNA Negative Control, Medium GC #2 (ThermoFisher Scientific) was used as a negative control siRNA. Stealth RNAi siRNA duplexes use proprietary next-generation chemistry in order to reduce off-target effects and activation of the protein kinase R/interferon response pathways (ThermoFisher Scientific).

M950-siRNA and M331-siRNA were synthesized by Integrated DNA Technologies, suspended in nuclease-free water and stored at -80°C (M950 sense: 5' ACAGCAGAAUGCUGUGGAUUU 3', M950 anti-sense: 5' AUCCACAGCAUUCUGCUGUUU 3', M331 sense: 5' GCTTAAGAGGGAGATAACATT 3', and M331 anti-sense: 5' AATGTTATCTCCCTCTTAAGC 3'). M950-siRNA was used as positive control for siRNA efficiency experiments.

The matrix-targeting siRNAs (M747, M776, and M832) and nonstructural-targeting siRNAs (NS570, NS595, and NS615) were characterized at a concentration of 100 nM. This concentration was selected after performing preliminary standardization studies that determined the most effective RNAi-mediating concentration (data not shown). The concentration of 50 nM used for further analysis of the M950-siRNA, was selected based on experiments performed by Sui et al. (2009).

*siRNA knockdown studies*

To test the efficiency of various siRNAs knocking down influenza virus transcripts *in vitro*, we used a protocol similar to what has been used in previous studies, with a few modifications (Ge et al., 2003;

Sui et al., 2009). Briefly, MDCK cells ($1 \times 10^6$ cells/well) were plated on a 6-well plate (Corning, Inc.) in complete growth media and incubated at 35°C in a humidified 5% $CO_2$ incubator. Twenty four hours later, cells were washed twice with phosphate-buffered saline (PBS) and 1.5 mL of OPTI-MEM I Reduced Serum Medium (Gibco) was added per well. Transfection was performed following the Lipofectamine 2000 Reagent protocol (ThermoFisher Scientific). Briefly, Lipofectamine reagent was prepared to a concentration of 32 µg/mL in 250 µL of OPTI-MEM I Reduced Serum Medium, mixed gently, and then incubated at room temperature for 5 min. Concurrently, corresponding siRNA was prepared to a concentration of 400 pmol/mL (50 nM) or 800 pmol/mL (100 nM) in 250 µL of OPTI-MEM I Reduced Serum Medium. The Lipofectamine and siRNA solutions were combined, mixed gently, and incubated for 20 min. Lipofectamine/siRNA solution (500 µL) was added to each well (50 nM or 100 nM siRNA final concentration). A negative control contained Lipofectamine solution (500 µL) without siRNA. Eight hours after transfection, the cells were washed twice with PBS and infected with influenza A virus at a multiplicity of infection (MOI) of 0.05 or 0.005 for 45 min. The viral solution was then removed, the cells were washed once with PBS and overlaid with 2 mL of Dulbecco's modified Eagle's medium (DMEM)/F12 (Gibco) supplemented with 100 U/mL penicillin G, 100 mg/ml streptomycin, 2 mM L-glutamine, 0.2% (v/v) bovine serum albumin (BSA), 10 mM HEPES (Gibco), 0.22% (v/v) sodium bicarbonate (Gibco), 0.01% (w/v) DEAE-dextran (MP BioMedicals LLC), and 2 mg/mL N-p-tosyl-L-phenylalanine chloromethyl ketone (TPCK) (Sigma-Aldrich). The culture supernatant and cell lysates were collected for analysis 24 h after infection. An MOI of 0.05 or 0.005 was selected to ensure reduced cytopathic effects after 24 h of infection (data not shown). Each treatment was performed in duplicate. The culture supernatant and cell lysates were stored at -80°C until processing.

*RNA isolation and cDNA transcription*

Total RNA was isolated from MDCK cells using the MagMax™-96 Total RNA Isolation Kit (Ambion). Briefly, upon thawing of the cellular lysate containing the Lysis/Binding Solution Concentrate, 500 μL of 100% isopropanol (Sigma-Aldrich) was added to each sample to complete the Lysis/Binding Solution preparation and samples were processed following the manufacturer's instructions. The final eluted total RNA volume was 60 μL. Total RNA was immediately transcribed into cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) in accordance with the manufacturer's instructions. Viral RNA was isolated from the culture supernatant using the MagMax™-96 Viral RNA Isolation Kit (Ambion) following the manufacturer's instructions.

*Analysis of RNA levels by real-time quantitative PCR (qPCR)*

qPCR analyses were performed using the following primers and probes: matrix 1 (Spackman et al., 2002), forward 5' AGATGAGTCTTCTAACCGAGGTCG3', reverse 5' TGCAAAAACATCTTCAAGTCTCTG 3', and probe 5' TCAGGCCCCCTCAAAGCCGA 3'; matrix 2, forward 5' GCACTTGATATTGTGGATTC 3', reverse 5' CAGCTCTATGTTGACAAAATG 3' and probe 5' AAGAATATCGAAAGGAACAGC 3'; nonstructural 1, forward 5' AATTGTTGGCGAAATCTCACC 3', reverse 5' TCCAAGCGAATCTCTGTAGA 3', and probe 5' AGGATGTCAAAAATG 3'; nonstructural 2, forward 5' TCGGAGGACTTGAATGGAATAATAA 3', reverse 5' GCTTCTCCAAGCGAATCTCTGT 3', and probe 5' ACAGTTCGAGTCTCTG 3'; and interferon-α, forward 5' TCTCTGTGGTCCACGTGATGA 3', reverse 5' GACGTGTCCGGGCAGAAG 3', and probe 5' CCAGAAGGTCTTCCACC 3'.

The matrix 1 primers and probe were used to detect total influenza virus in the culture supernatant (i.e. infectious and noninfectious virus) by detecting segment 7 of the influenza viral RNA.

The matrix 2, nonstructural 1, nonstructural 2 and canine specific interferon-α primers and probes were designed using the Primer Express 3.0 software (Applied Biosystems). The matrix 2 and nonstructural 2 primers and probes were used to detect all influenza-associated RNA (viral RNA, complementary RNA, and messenger RNA specific for that protein) in the culture lysates. The nonstructural 1 primers and probes were used to detect nonstructural viral RNA in the culture supernatant. Canine specific IFNβ1 Taqman Gene Expression Assay primers and probe (Cf03644503_s1, ThermoFisher Scientific) were used to detect interferon-β mRNA in culture lysates. The probes were designed with a 5' fluorescent dye, 6FAM, and 3' quencher, MGBNFQ. All primers and probes were synthesized by Applied Biosystems and used at a final concentration of 0.8 μM and 0.2 μM, respectively. Reactions were performed and analyzed using the Applied Biosystems 7500 Fast Real-Time PCR System under the following cycling conditions: 95°C for 20 seconds, followed by 40 cycles at 95°C for 3 seconds, and 60°C for 30 seconds. A negative control without template was included in all real-time PCR runs. All samples were run in duplicate. Relative gene expression was determined by the $\Delta\Delta C_T$ method and normalized to GAPDH (Hs03929097_g1, ThermoFisher Scientific). The PCR primers were tested on MDCK cell lysates and analyzed by gel electrophoresis to confirm specificity of GAPDH primers to canine GAPDH (data not shown).

*Analysis of infectious viral titers by viral plaque assay (VPA)*

MDCK cells were detached with 0.5% Trypsin-EDTA (Invitrogen), washed with PBS, re-suspended in complete growth media and $2.2 \times 10^6$ cells were seeded into each well of a 6-well CoStar tissue culture plate (Corning, Inc.). After 24 h, confluent cell monolayers were washed twice with PBS and inoculated with 800 μL of 1:10 serial dilutions of supernatant samples from transfected and influenza-infected cells or controls diluted in HBSS supplemented with 5% (v/v) penicillin/streptomycin and 10% (v/v) BSA

(Gibco). After 45 min, inoculated cells were washed once with PBS and overlaid with DMEM/F12 (Gibco)

supplemented with 100 U/mL penicillin G, 100 mg/ml streptomycin, 2 mM L-glutamine, 0.2% (v/v) BSA,

10 mM HEPES (ThermoFisher Scientific), 0.22% (vol/vol) sodium bicarbonate (ThermoFisher Scientific),

0.01% (w/v) DEAE-dextran (MP BioMedicals, LLC), 2 mg/mL N-p-tosyl-L-phenylalanine chloromethyl

ketone (Sigma-Aldrich) and 0.6% (w/v) agarose (Oxoid Ltd.). Post 48 h incubation, cells were fixed with

10% (v/v) formalin for 30 min and the agarose overlay was removed by washing with deionized water.

The MDCK cells were stained with 1% (w/v) crystal violet/ 0.19% (v/v) methanol for 30 min, rinsed with

deionized water, dried, counted, and the plaque forming units (PFU) were calculated.

*Analysis of protein levels by Western blot*

Cells were lysed with radioimmunoprecipitation assay (RIPA) buffer containing protease inhibitor

cocktail and EDTA (ThermoFisher Scientific). Protein concentration was determined with the BCA Protein

Assay Kit (Pierce) performed in triplicate. Thirty to fifty μg of sample protein were diluted 1:1 with 2x

Laemmli loading dye (Bio-Rad), denatured by boiling for 10 min, and immediately cooled on ice before

loading on to a 4% stacking/12% SDS-PAGE mini-gel. The electrophoresed protein was electroblotted

onto nitrocellulose membranes (0.2 μm) overnight at a constant 16 V. The blots were blocked in Odyssey

Blocking Buffer (LI-COR Biosciences) for 1 h at room temperature and then incubated overnight with

primary antibodies GAPDH (sc-25778, Santa Cruz), M1 (ab22396, AbCam), M2 (sc-32238, Santa Cruz),

NS1 (PA5-32243, Pierce), or NS2 (GTZ125952, GenTex) diluted in Odyssey Blocking Buffer. After four

washes with TBS-tween (0.1% (v/v) Tween-20), the blots were incubated with corresponding infrared

(680 or 800 nm) conjugated secondary antibodies (LI-COR Biosciences) for 1 h at room temperature.

Blots were washed four times in TBS-tween and then analyzed on the Odyssey Imaging System (LI-COR

Biosciences). Primary antibodies directed against GAPDH were used to verify equal sample loading and for normalization.

*Statistical analyses*

The analyses were generated using SAS/STAT software, Version 9.2 of the SAS system for Windows (SAS Institute). Data were transformed by calculating the natural log of each value prior to analysis to meet the assumptions of the statistical tests (homogeneity of variance). A two-way factorial mixed model analysis of variance (ANOVA) was performed on all variables. All pairwise comparisons were considered significant at $p < 0.05$. Asterisks above graphs refer to: * = $p<0.05$. All graphs are representative of three independent experiments (N=3) with two replicates per treatment group per experiment.

## 3.4 Results and discussion

To expand the number of effective influenza-targeting siRNA available for laboratory or therapeutic use, we designed three siRNAs that target the matrix 2 gene (M747, M776, M832), three siRNAs that target the nonstructural protein 1 and 2 genes (NS570, NS595, NS615), and re-examined two previously reported siRNAs: M331 and M950 (Hui et al., 2004; Sui et al., 2009), that target the matrix 1 and 2 genes, respectively, and tested their effect against influenza A virus infection *in vitro.* Our studies focused on targeting four influenza A virus proteins, the matrix proteins 1 and 2 and the nonstructural proteins 1 and 2, because the coding sequences for these proteins are more evolutionarily conserved than other influenza proteins (McSwiggen and Seth, 2008) and thus these proteins can be used to design influenza-specific siRNA that can target various strains of influenza viruses.

*M747-, M776-, and M832-siRNAs reduce viral RNA and protein expression in culture lysates and inhibit influenza A virus replication*

Segment seven of the influenza virus genome encodes for two proteins, the matrix protein 1 and matrix protein 2, both of which are have structural and nonstructural roles during influenza infection. The transcript containing the coding sequence for the matrix protein 1 is generated by reverse transcription of the seventh viral RNA (vRNA) segment into a primary transcript, whereas the coding sequence for the matrix protein 2 is generated after the primary transcript is post-transcriptionally spliced into a secondary transcript (Figure 3.1). The matrix protein 1 forms the viral capsid which serves as a boundary between the viral ribonucleoprotein (vRNP) and membrane (Ruigrok et al., 1989). In addition to its structural role, the matrix protein 1 has been shown to be involved in vRNP nuclear transport (Sakaguchi et al., 2003). The matrix protein 2 is an integral membrane protein that is embedded in the viral membrane (Lamb et al., 1985). The proton pump activity of the matrix protein 2 causes acidification of the viral capsid, allowing a structural change in the viral capsid and the release of the vRNA into the cytoplasm (Ciampor et al., 1992). Inhibiting the activity of the matrix protein 2 attenuates viral replication as seen by treatment with the matrix 2 antiviral therapies, amantadine and rimantadine (Schmidt, 2004; Wang et al., 1993), and RNAi treatment with matrix protein 2 targeting siRNAs (Hui et al., 2004; Sui et al., 2009; Zhou et al., 2007). For the present work, we designed three siRNAs that target the primary and secondary transcripts encoding for the matrix proteins 1 and 2, M747-, M776-, and M832-siRNA, and compared them to the previously published siRNA, M950 (Sui et al., 2009), which also targets both transcripts encoding for the matrix proteins (Figure 3.1). We hypothesize that a single siRNA that targets two transcripts can be used to knock down the expression of two proteins.

To test whether M747-, M776-, and M832-siRNA can attenuate influenza virus replication, MDCK cells were treated with individual matrix targeting siRNA, M950-siRNA, or negative control siRNA and then infected with influenza A/WS/33 (H1N1) at an MOI of 0.05 or 0.005.  In culture lysates infected at an MOI of 0.005, the siRNA treatments resulted in a 54.7% reduction in matrix RNA (viral RNA, complementary RNA, and messenger RNA) with M950, 30.6% with M747, and 48.9% with M832, compared to negative control siRNA treated cells (Figure 3.2A). Although treatment with the siRNAs reduced total matrix RNA in the culture lysates, reduction in RNA doesn't necessarily lead to a reduction in protein expression. Furthermore, analysis of total matrix RNA does not distinguish between reductions in primary or secondary matrix messenger RNA therefore culture lysates were analyzed by Western blot to determine whether matrix targeting siRNA treatment inhibited matrix protein 1 and 2 expression (Figure 3.2B). All four matrix targeting siRNAs significantly reduced matrix protein 2 expression in the culture lysates at an MOI of 0.005 compared to the negative control siRNA treated cells (M950, 80.2% reduction; M747, 75.4%; M776, 56.6%; and M832, 63.2%). At an MOI of 0.05, M747-siRNA reduced matrix protein 2 expression by 66.7%, and M832-siRNA reduced it by 42.9%, which shows a similar potency to M950-siRNA treatment (73.5% reduction; Figure 3.2C). On the other hand, M776-siRNA treatment was unable to knock down matrix protein 2 expression at higher MOI. None of the matrix targeting siRNAs, even the previously published M950-siRNA, all of which target the matrix protein 2 coding sequence, significantly reduced total matrix protein 1 in the culture lysates (Figure 3.2B & C). This may appear paradoxical as these siRNAs reduced the expression of the total matrix RNA, yet only the matrix 2 protein was reduced. However, since our assay of total matrix RNA can't differentiate between matrix 1 and matrix 2 RNA, one possible explanation is that, because the matrix siRNAs target the coding sequence of the matrix 2 protein, the matrix 1 coding sequence may be left largely intact as ribosomes bind and begin translation of the matrix 1 protein.

After determining that the matrix targeting siRNA treatment led to a reduction in total matrix RNA and matrix 2 protein expression in the culture lysates, we then assessed whether total influenza virus (which includes non-infectious and infectious virus) or infectious viral titer was attenuated in the culture supernatant of siRNA treated MDCK cells. Only M776-siRNA treatment significantly reduced total influenza virus (p=0.0042) in the culture supernatant, with a 40.5% reduction compared to negative control siRNA treated cells infected at an MOI of 0.005 (Figure 3.3A), but M950-, M776-, and M832-siRNAs all significantly reduced infectious influenza virus in the culture supernatant at both MOIs tested (Figure 3.3B). M832-siRNA reduced infectious virus by 24.2% at an MOI of 0.05 and 39.0% at an MOI of 0.005. M776-siRNA showed similar potency as M950-siRNA at an MOI of 0.05 (30.8% vs. 30.3%) (Figure 3.3B), whereas M776-siRNA was more effective at attenuating infectious virus than M950-siRNA at an MOI of 0.005 (54.1% vs. 34.6%) (Figure 3.3B). Although M747-siRNA was able to reduce matrix RNA and protein in the culture supernatant (Figure 3.2A-C), it was unable to attenuate infectious influenza virus in the culture supernatant (Figure 3.3B).

These results validate the use of siRNA targeting the matrix genes to attenuate influenza virus infection. The matrix targeting siRNAs significantly reduced viral RNA and protein, thus reducing the infectious viral titer. The matrix 1 and 2 proteins are structural proteins that are essential for the generation of progeny virus (Lamb et al., 1985; Ruigrok et al., 1989). Knocking down the expression of the matrix protein 2 was detrimental to viral replication and/or packaging into infectious virions, suggesting that defective virus is produced that are nonetheless detectable by PCR analysis of the matrix transcript. Although the matrix targeting siRNAs attenuated infectious viral titer, they failed to reduce the total amount of influenza virus (infectious and noninfectious) in the culture supernatant, with the exception of M776-siRNA treatment at an MOI of 0.005. The use of the matrix targeting siRNAs may be

beneficial for therapeutic strategies as the yield of total influenza virus can be used to stimulate influenza immunity, while still reducing the amount of infectious virus.

*M331-siRNA reduces matrix protein 1 and 2 expression and influenza A virus replication*

M950-, M747-, M776-, M832-siRNA treatment reduced the expression of matrix protein 2, but they were unable to reduce matrix protein 1 expression. We hypothesized that this result occurred because, although these siRNAs target both the primary and secondary transcripts, they only bind directly to the matrix 2 coding sequence. For this reason, we decided to further characterize a previously reported siRNA, M331, which targets the matrix protein 1 coding sequence in order to determine whether it could mediate RNAi and inhibit matrix protein expression (Hui et al., 2004).

Influenza A infected MDCK cells that were treated with M331-siRNA significantly reduced matrix RNA in culture supernatant by 81% at an MOI of 0.005, which is comparable to the 92% reduction seen with M950-siRNA treatment (Figure 3.4A). However, M331-siRNA had no effect at an MOI of 0.05. M331-siRNA treatment inhibited both matrix protein 1 and 2 expression (Figure 3.4B & C) whereas M950-, M747-, M776-, M832-siRNAs only inhibited matrix protein 2 expression (Figure 3.2B & C). Despite this, M331-siRNA treated cells yielded similar total influenza viral loads (Figure 3.4D) and infectious viral loads (Figure 3.4E) in the culture supernatant to MDCK cells treated with M950-siRNA. M331-siRNA reduced infectious virus in the culture supernatant by 80.5% while M950-siRNA attenuated infectious virus by 70.2%. These results indicate that siRNAs must target the primary transcript upstream of the target gene's stop codons in order to inhibit the expression of both proteins. However simply knocking down matrix protein 2 expression was sufficient to attenuate the infectious viral titer.

*NS570-, NS595-, and NS615-siRNA reduce viral RNA and both nonstructural protein 1 and 2 expression in culture lysates, but do not attenuate viral replication*

The eighth negative-sense RNA segment of influenza viruses encodes for two proteins, the nonstructural protein 1 and the nonstructural protein 2. Similar to the matrix transcripts, the transcript containing the coding sequence for the nonstructural protein 1 is generated by reverse transcription of the eighth viral RNA (vRNA) segment into a primary transcript, whereas the coding sequence for the nonstructural protein 2 is generated after the primary transcript is post-transcriptionally spliced into a secondary transcript (Figure 3.1). The nonstructural protein 1 promotes viral infection by inhibiting the intracellular sensor retinoic acid-inducible gene I (RIG-I) and subsequently preventing type I interferon expression (Pichlmair et al., 2006). The nonstructural protein 2 (also known as the nuclear export protein) is involved in the translocation of the vRNP out of the nucleus where the vRNP can be packaged into newly formed virus (O'Neill et al., 1998). In the previous experiments we saw that, treating MDCK cells with matrix targeting siRNAs that only target a single protein coding sequence resulted in reduced expression of only one of the matrix proteins. Consequently we designed three new siRNAs, NS570, NS595, and NS615 that target both the nonstructural protein 1 and 2 coding sequences (Figure 3.1) in order to knock down the expression of two proteins with a single siRNA, thus enhancing their utility.

NS570-, NS595-, and NS615-siRNAs were tested to determine whether they can mediate RNAi and attenuate influenza virus replication. As with the matrix targeting siRNA studies, MDCK cells were treated with individual nonstructural targeting siRNA then challenged with influenza A/WS/33 (H1N1) at an MOI of 0.05 or 0.005 for 24 hours. Nonstructural RNA (viral RNA, complementary RNA, and messenger RNA) was significantly reduced by NS570- (71.0%), NS595- (65.0%), and NS615-siRNA (45.0%) in culture lysates at an MOI of 0.05 (Figure 3.5A). With less virus (MOI 0.005), only NS570- and NS595-siRNA

significantly reduced nonstructural RNA (NS570, 71.5% reduction; NS595, 45.0%; Figure 3.5A). Positive control M950-siRNA treatment resulted in a significant reduction in matrix RNA in the culture lysates, but did not reduce nonstructural RNA, emphasizing the specificity of each siRNA to its corresponding target RNA (Figure 3.5A). Western blot and densitometry data confirmed that all three nonstructural targeting siRNAs, which were designed to target both the nonstructural protein 1 and 2 genes, were able to inhibit the expression of both nonstructural proteins in culture lysates (Figure 3.5B-D). The most notable reduction in nonstructural protein 1 expression occurred after infection at an MOI of 0.05 following treatment with NS570- (63.7% reduction), NS595- (57.1%) and NS615-siRNA (73.4% reduction; Figure 3.5C). A similar reduction in nonstructural protein 2 expression was detected as well (NS570-, 55.1%; NS595-, 65.4%; NS615-, 86.2%; Figure 3.5D). This suggests that designing a single siRNA targeting more than one protein coding sequence that is upstream of the stop codon on the primary transcript can knock down the expression of both proteins, as opposed to M747-, M776-, M832-, and M950-siRNAs which target one protein coding sequence and only had the capacity to reduce the expression of one protein.

The nonstructural targeting siRNAs were able to mediate RNAi and knock down nonstructural protein expression. For this reason, we assessed whether nonstructural targeting siRNA treatment could attenuate viral replication. Treatment with nonstructural targeting siRNAs resulted in a significant increase in both matrix RNA (NS570, 80.1% increase; NS595, 142.4%; NS615, 152.9%) and neuraminidase RNA (NS570, 263.2% increase; NS595, 142.2%; NS615, 128.8%) in the culture supernatant at an MOI of 0.05 (Figure 3.6A). In contrast, NS570-siRNA treated MDCK cells had significantly more nonstructural RNA (113.1% increase) in the culture supernatant at an MOI of 0.05, whereas NS595- and NS615-siRNA treatment retained a comparable level of nonstructural RNA compared to negative control treated cells

(Figure 3.6A). Nonstructural targeting siRNA treated cells infected at an MOI of 0.005 produced similar effects. These results indicate that NS595- and NS615-siRNA treatment promotes the replication of defective influenza virus that are void of the nonstructural RNA transcript, but does not promote the production of infectious virus (Figure 3.6B). On the other hand, NS570-siRNA treatment resulted in a significant increase in all three viral RNA (matrix, nonstructural, and neuraminidase) in the culture supernatant and results in a comparable 115.0% increase in infectious virus (p=0.0434; Figure 3.6B).

*Nonstructural targeting siRNAs increases type I interferon expression in culture supernatant*

The nonstructural protein 1 is known to act as a type I interferon antagonist (Pichlmair et al., 2006). Type I interferons are important cytokines that are produced following viral infection. They elicit direct antiviral effects and activate the host's immune cells, which can lead to attenuation of the viral titer (Garcia-Sastre, 2011; Staeheli et al., 1984). We hypothesized that NS-specific siRNA treatment could enhance type I interferon expression (interferon-α and interferon-β) due to reduced nonstructural protein 1 expression.

MDCK cells treated with the nonstructural-specific siRNAs displayed a significant increase in interferon-β mRNA expression (Figure 3.7A) at an MOI of 0.05 and 0.005. Interferon-β protein expression also increased in cells treated with NS570- (65.5%) and NS615-siRNA (76.7%) and infected at an MOI of 0.05, and with NS570-siRNA at an MOI of 0.005 (55.7% increase; Figure 3.7B &C). Relative interferon-α mRNA expression showed a trend towards increased expression in cells treated with NS-specific siRNAs, with NS595-, NS615- and M950-siRNA treated cells expressing significantly more interferon-α mRNA (Figure 3.7D). Although the M950-siRNA does not target the nonstructural genes, cells treated with M950-siRNA promoted the expression of interferon-β mRNA and protein as well as interferon-α mRNA (Figure 3.7A-D). These data suggest that nonstructural targeting siRNAs can boost the host's immune

response to viral infection by promoting the expression of type-I interferons that are typically repressed during influenza infection by the nonstructural protein 1, thus demonstrating its potential use to boost the host's immune response to viral infection. Nonstructural targeting siRNA treatment led to an increase in total influenza virus yield in the culture supernatant without changing the infectious viral titer, which suggests that these siRNAs could be used to generate additional virus that can act as a stimulus to enhance influenza specific immunity *in vivo*. Although an increase in viral antigen could stimulate a stronger influenza specific immune response, it is possible that an over stimulated immune response or increased viral load could result in an altered disease state *in vivo*. Future studies examining these effects should be considered in order to better characterize the suitability of these siRNAs for therapeutic intervention.

*Combination siRNA therapy is more effective than single siRNA treatment*

Treating cells with a combination of siRNAs targeting the same or different genes may enhance the efficacy of the treatment compared to a single siRNA treatment. For this reason, a combination of the siRNAs that showed the most promise in the previous studies (M950-, M776-, and NS595-siRNAs) were tested to determine whether the combination treatment results in further attenuation of influenza viral titer. MDCK cells treated with the combination therapy and then infected with influenza virus at an MOI of 0.005 demonstrated a 64.0% reduction in matrix RNA (viral RNA, complementary RNA, messenger RNA) in the culture lysates, whereas single siRNA treatment with M950 resulted in only a 40.0% reduction in matrix RNA (Figure 3.8A). Matrix protein 2 expression was similarly reduced in both M950-siRNA only treated cells (97.0%) and combination siRNA treated cells (98.9%), compared to the negative control siRNA treated cells infected with an MOI of 0.005 (Figure 3.8B & C). Only the combination treatment, containing the NS595-siRNA, showed a reduction (55.3%) in nonstructural

protein 1 expression at an MOI of 0.005 (Figure 3.8B & C). Combination siRNA and single siRNA treatment with M950-siRNA generated a similar reduction in total influenza virus in the culture supernatant (73.1% vs. 68.7%; Figure 3.8D). However, combination siRNA treatment resulted in a 62.4% reduction in infectious virus, whereas M950 siRNA treatment only reduced infectious virus by 41.4%, with combination siRNA treatment trending toward a significant difference in infectious virus compared to cells only treated with M950-siRNA ($p = 0.0650$; Figure 3.8E). MDCK cells treated with the combination siRNA had 35.7% less infectious virus than M950-siRNA treated cells.

Using a combination of siRNAs to treat influenza infection would have an advantage over matrix 2 inhibitors which can only target influenza A viruses (Schmidt, 2004; Wang et al., 1993), as the siRNAs used in the combination treatment can be designed to target both the influenza A and B proteins. A limited number of studies have examined the effect of combination siRNA treatment after influenza infection *in vitro* (Li et al., 2005) and *in vivo* (Tompkins et al., 2004). Our findings have significant implications for the use of these siRNAs, particularly M776- and NS595-siRNAs for therapeutic use against influenza infection. Future studies should be performed to determine whether using a combination of siRNAs targeting the same protein, as seen with our study, would be more effective than using a combination of siRNAs targeting different structural viral proteins.

## 3.5 Conclusions

The high burden of morbidity and mortality associated with influenza infection and the lack of effective broad-range antiviral therapies drives the need to design improved therapies that treat influenza infections. RNAi is a practical strategy that can be an effective alternative to current therapies

that employ a proteomic approach to treating disease due to its ease of design and manufacturing and its ability to target a broad range of proteins. By employing an RNAi approach through the use of siRNAs that target the influenza A virus matrix or nonstructural proteins, we have characterized siRNAs that can effectively attenuate influenza infection or boost cellular antiviral responses to influenza infection.

The matrix protein gene targeting siRNAs (M331, M747, M776, M832, and M950) mediated RNAi and knocked down matrix protein expression. M331-siRNA, which targets the primary transcript on the matrix protein 1 coding sequence, knocked down both matrix protein 1 and 2 expression, whereas M747-, M776-, M832-, and M950-siRNA, which directly target the matrix protein 2 coding sequence, knocked down the matrix protein 2 expression. M331-, M776-, M832- and M950-siRNA significantly attenuated the infectious influenza titer in the culture supernatant. The most effective treatment was with M776-siRNA which produced a 54.1% reduction in infectious viral titer, resulting in 29.8% less infectious virus than treatment with the previously published M950-siRNA. The nonstructural targeting siRNAs (NS570, NS595, and NS615) mediated RNAi in the influenza virus infected MDCK cells. These siRNAs target the nonstructural protein 1 and 2 coding sequences, and therefore treatment with these siRNAs resulted in a significant reduction in both proteins. This finding emphasizes the utility of using a single siRNA to knock down the expression of two proteins. Treatment with nonstructural protein gene targeting siRNAs did not attenuate the infectious influenza viral titer (with the exception of NS570-siRNA), but did result in a significant increase in defective influenza virus in the culture supernatant. These siRNAs also promoted the expression of type I interferon mRNAs (interferon-α and interferon-β) or enhanced interferon-β protein expression in the cell lysates. Studies further exploring the mechanism in which nonstructural-targeting siRNA treatment leads to an increase in noninfectious viral load should

be performed in the future. Furthermore, we show that using a combination of siRNAs can lead to a more potent viral attenuation than single siRNA treatment.

Overall, we have characterized three siRNAs targeting the nonstructural protein that can effectively boost the host's immune response to viral infection and target the expression of two influenza proteins. We also characterized two additional matrix protein targeting siRNAs that can be used to attenuate infectious viral titer, with M776-siRNA being more effective than the previously characterized M950-siRNA. Two proteins were able to be knocked down by a single siRNA that was designed to target upstream of both protein coding sequence stop codons (NS570-, NS595-, and NS615-siRNA), thus enhancing their utility. Finally, we have shown the practical application of a combination siRNA treatment to elicit a more potent attenuation of influenza infection compared to single siRNA treatment *in vitro*.

## 3.6 Acknowledgments:

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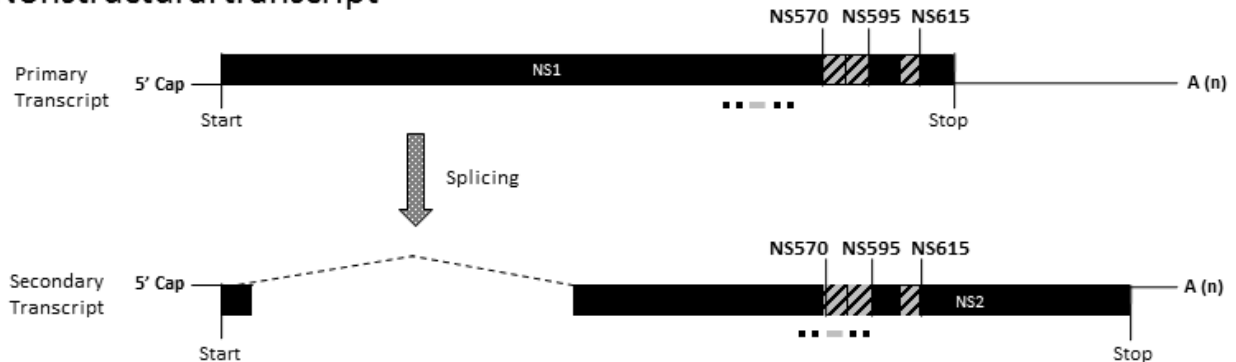
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## 3.8 Figures and figure legends
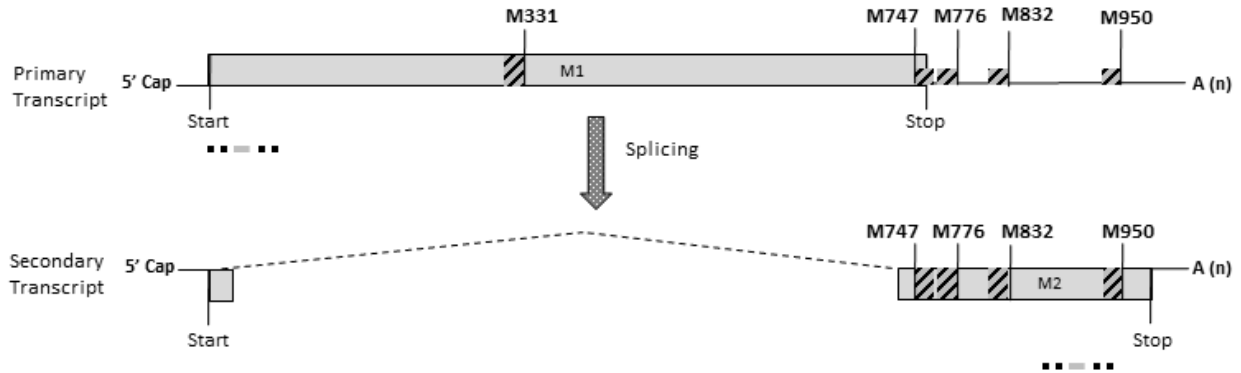


**Figure 3.1: Diagram of target sequences for matrix and nonstructural targeting siRNA.** M331- M747-, M776-, M832-, and M950-siRNAs bind to their complementary locations on the primary and secondary transcripts of the matrix proteins (binding location indicated by grey and black hashed blocks). M331-siRNA only binds to the matrix protein 1 (M1) coding sequence (grey solid block), whereas M747-, M776-, M832-, and M950-siRNAs bind to the matrix protein 2 (M2) coding sequence (grey solid block). The primary transcript encodes for the matrix protein 1 whereas the secondary transcript, generated after post-transcriptional splicing (grey arrow with white dots), encodes for the matrix protein 2. NS570-, NS595-, NS615-siRNAs bind to the primary and secondary transcripts of the nonstructural proteins,

including the nonstructural protein 1 (NS1) (black solid block) and nonstructural protein 2 (NS2) (black solid block) coding sequences. The primary transcript encodes for the nonstructural protein 1 whereas the secondary transcript, generated after post-transcriptional splicing (grey arrow with white dots), encodes for the nonstructural protein 2. Start and stop codons are indicated by "start" and "stop" labels, respectively. Representative qPCR primer (black hashed line) and probe (grey solid line) binding regions are shown under each transcript (matrix 1 (M1), matrix 2 (M2), nonstructural 1 (NS1), nonstructural 2 (NS2)).
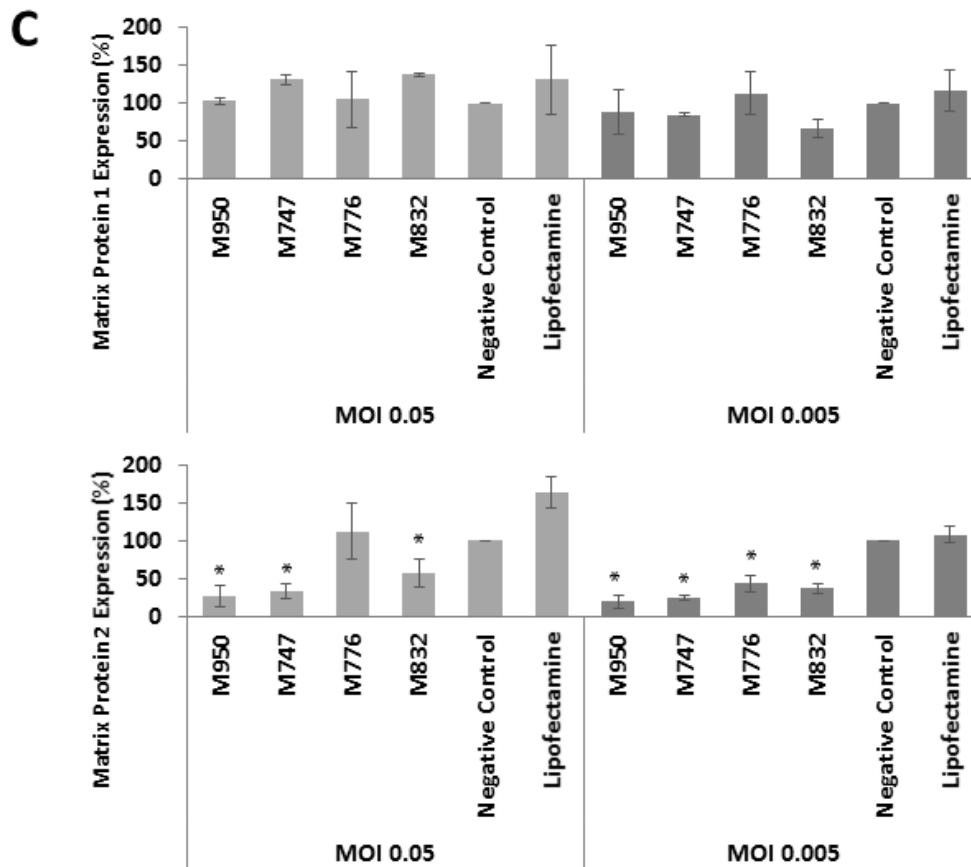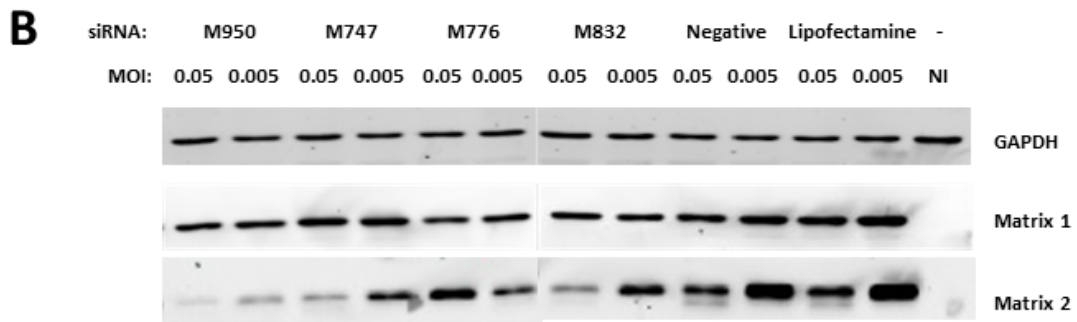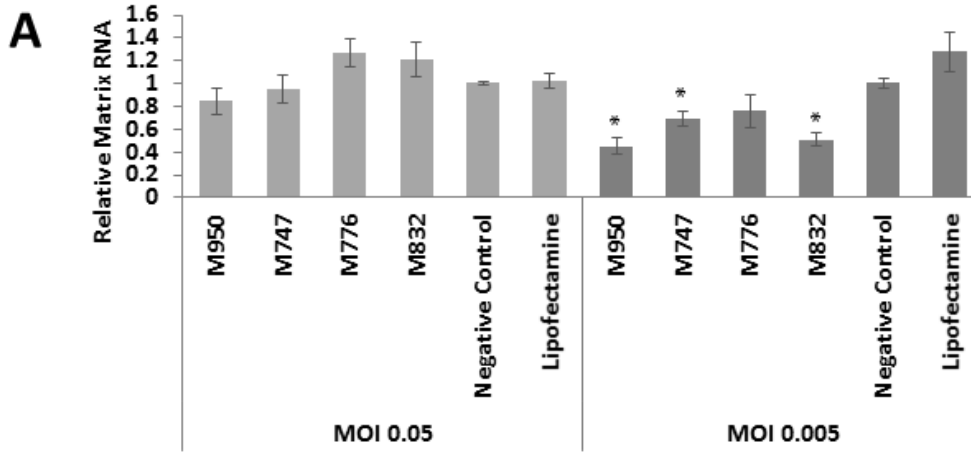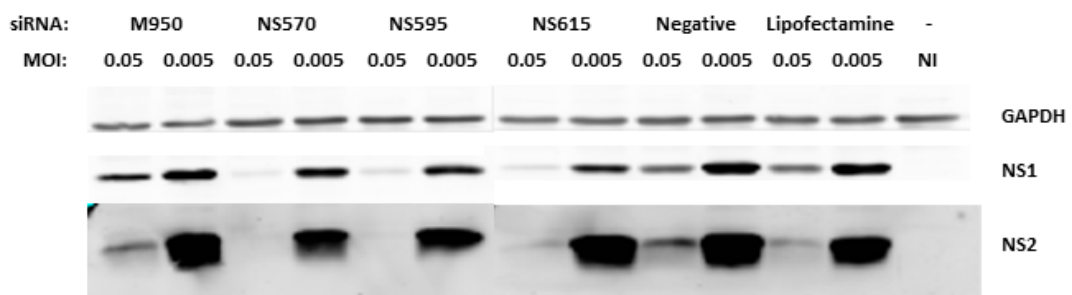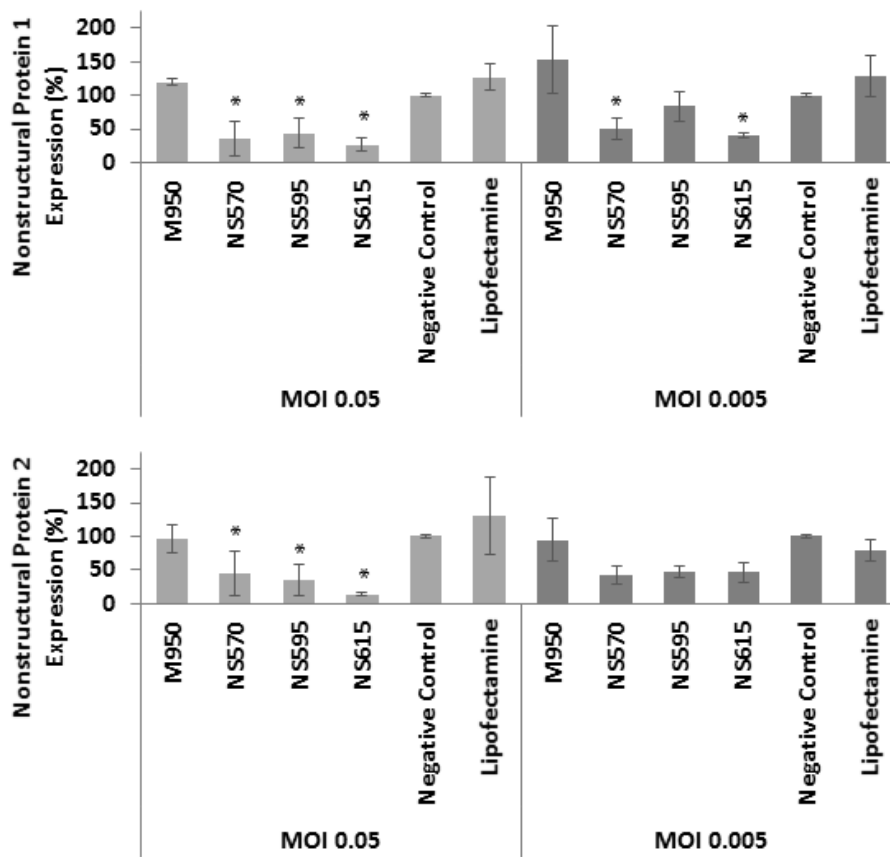
**Figure 3.2: M747-, M776-, & M832-siRNA reduce viral RNA and matrix protein 2 expression in culture lysates.** MDCK cells were transfected with siRNA [50 or 100 nM] for 8 h then infected with influenza A/WS/33 (H1N1) (MOI 0.05, 0.005) for 24 h. A) Relative influenza A/WS/33 matrix RNA expression was determined by qPCR analysis of culture lysates and normalized to GAPDH. Protein expression was determined by B) Western blot of culture lysates, and C) percent matrix protein 1 and 2 expression was determined by densitometry. Normalized to GAPDH. NI refers to no infection. (N=3)
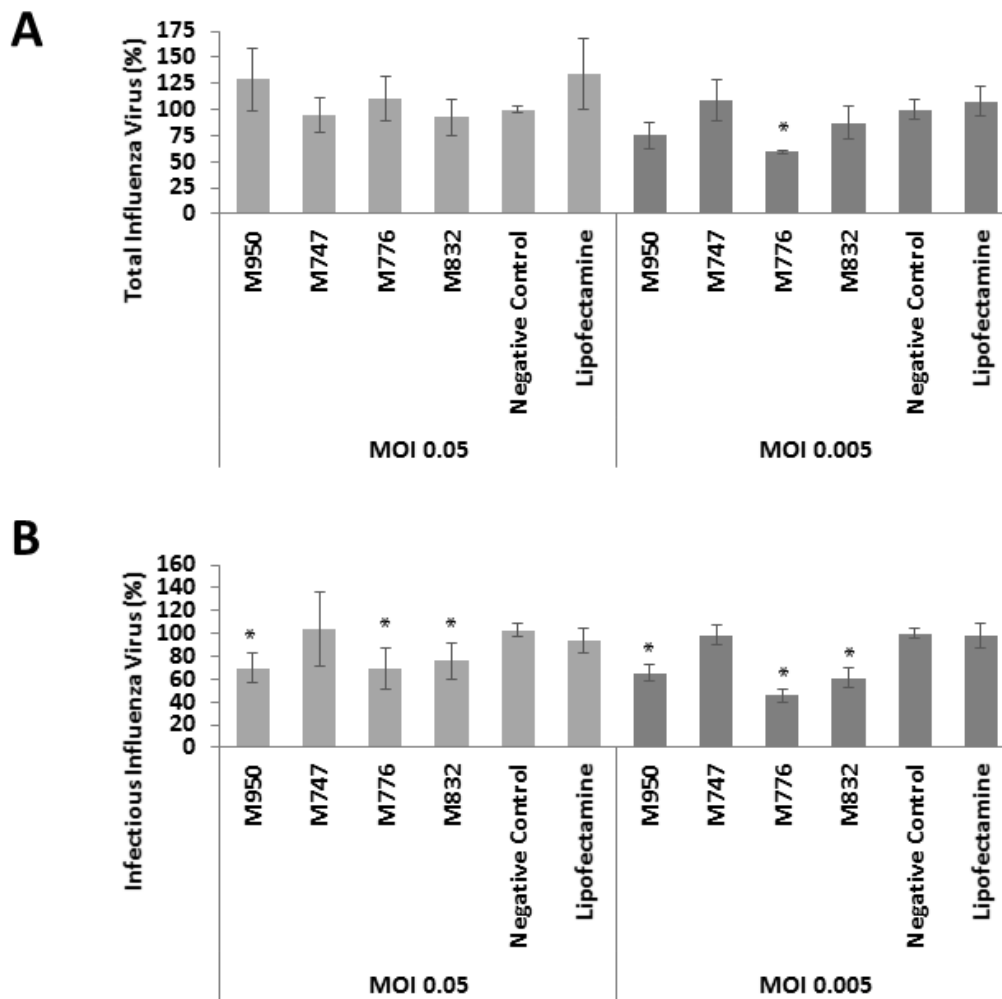
**Figure 3.3: M747-, M776-, M832-siRNA inhibits influenza A virus replication in culture supernatant.**
MDCK cells were transfected with matrix siRNA or control [50 or 100 nM] for 8 h then infected with influenza A/WS/33 (H1N1) (MOI 0.05, 0.005) for 24 h. A) Percent total influenza virus in culture supernatant was detected by matrix 1 specific qPCR. B) Percent infectious influenza virus was determined by viral plaque assay from culture supernatant. Percent calculations were normalized to negative control siRNA treated MDCK cells. (N=3)
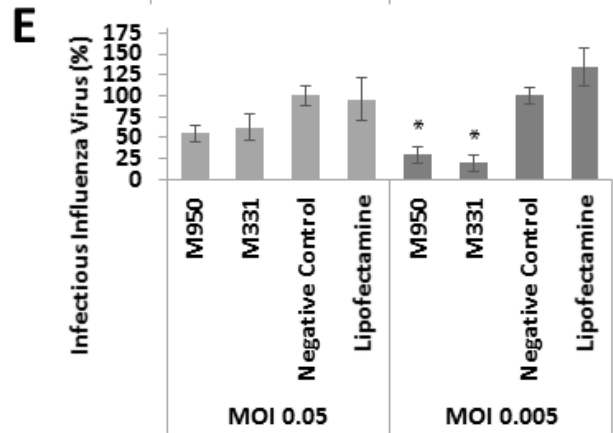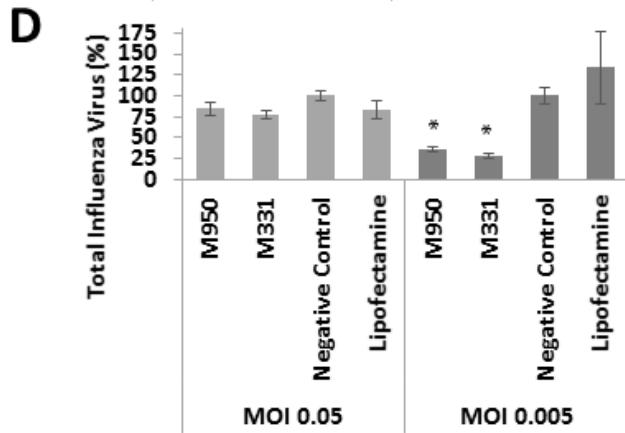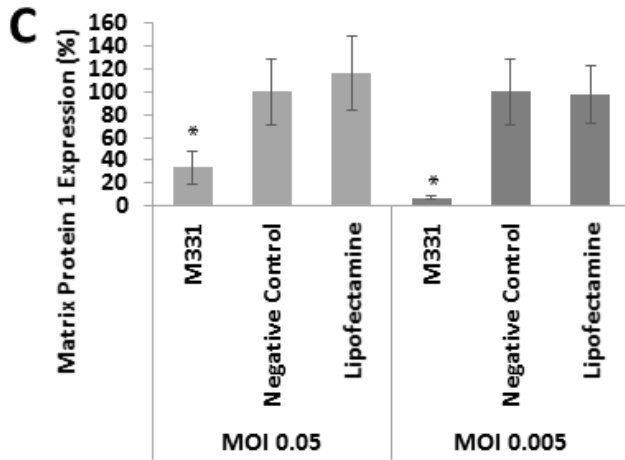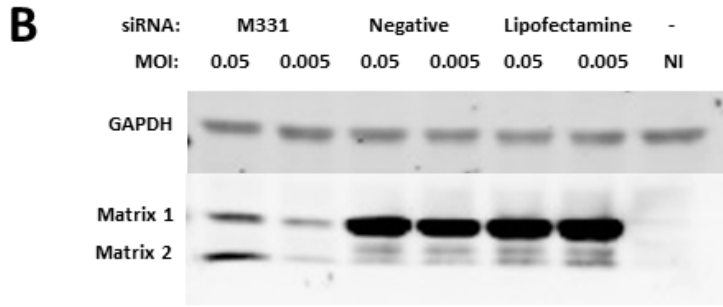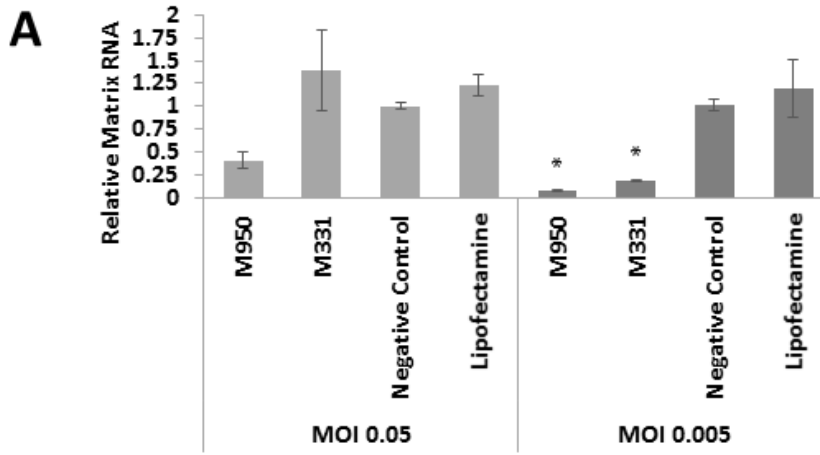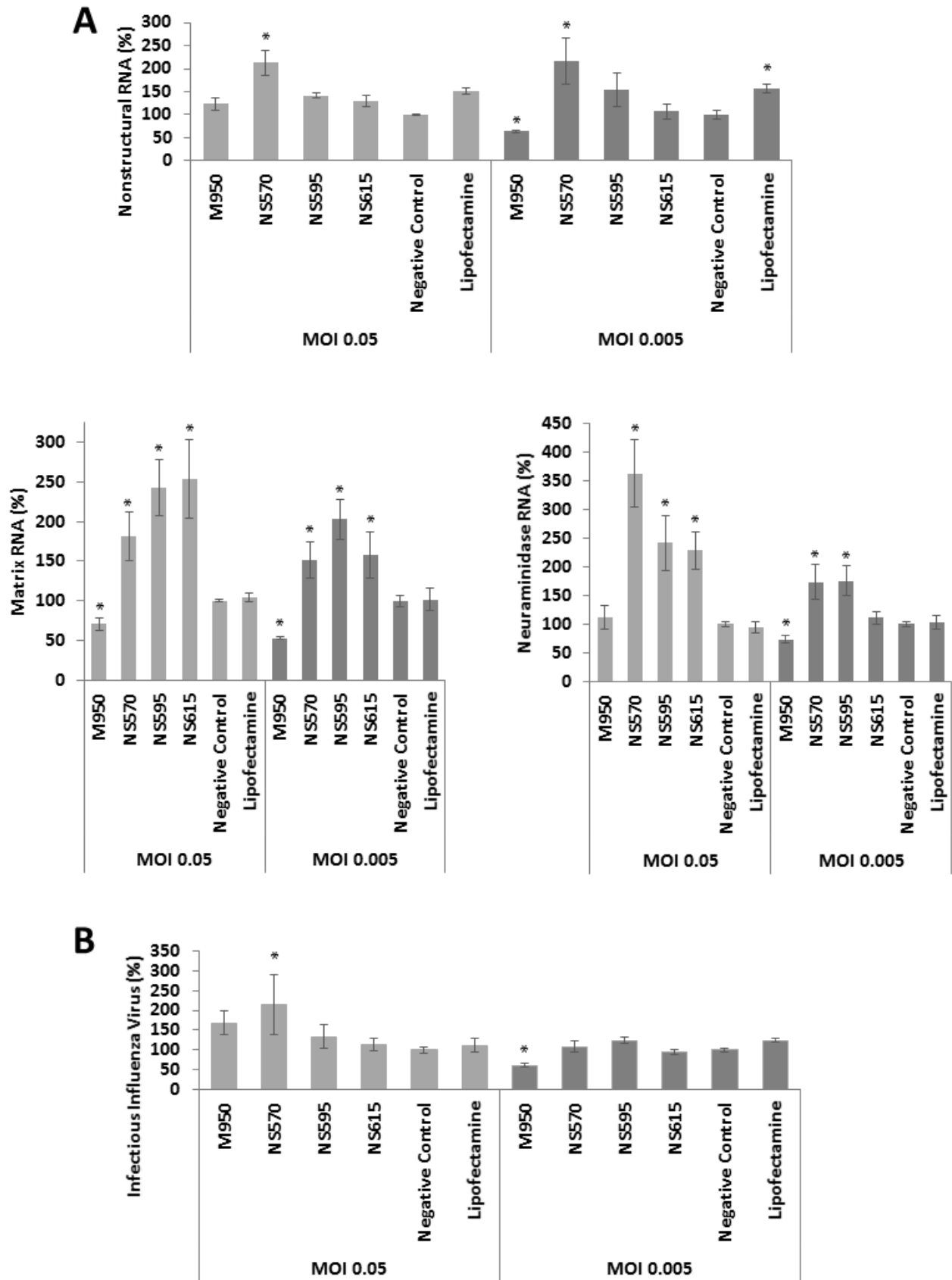
**Figure 3.4: M331-siRNA mediates RNAi and inhibits viral replication.** MDCK cells were transfected with siRNA [50 nM] for 8 h then infected with influenza A/WS/33 (H1N1) (MOI 0.05, 0.005) for 24 h. A) Relative influenza A/WS/33 matrix RNA expression was determined by qPCR analysis of culture lysates and normalized to GAPDH. Protein expression was determined by B) Western blot of culture lysates and C) percent matrix protein 1 or 2 expression was calculated by densitometry. Results were normalized to GAPDH. D) Percent total influenza virus in culture supernatant was detected by matrix 1 specific qPCR. C) Percent infectious influenza virus was determined by viral plaque assay from culture supernatant. Percent calculations were normalized to negative control siRNA treated MDCK cells. NI refers to no infection. (N=3)

**A**



**B**

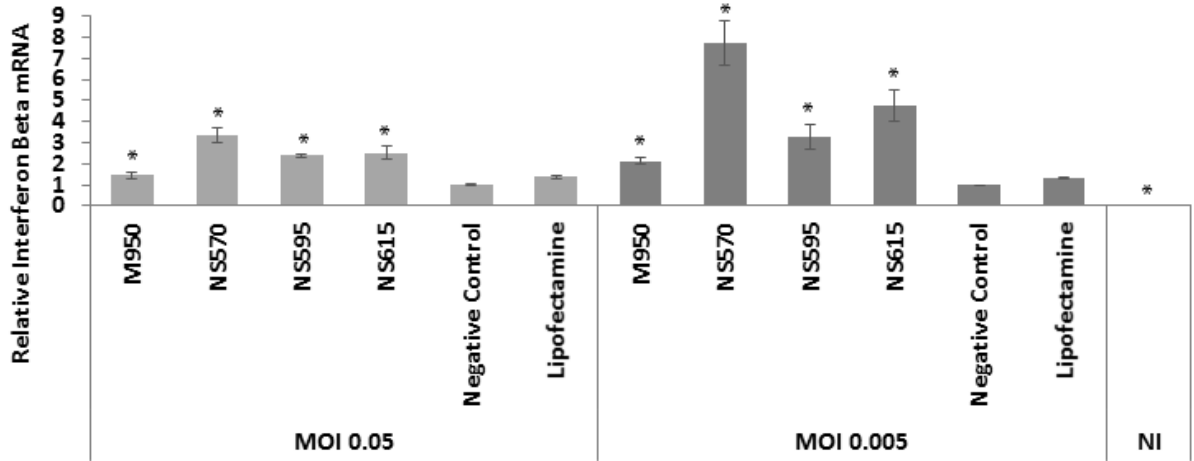| siRNA: | M950 | | NS570 | | NS595 | | NS615 | | Negative | | Lipofectamine | | - |
| MOI: | 0.05 | 0.005 | 0.05 | 0.005 | 0.05 | 0.005 | 0.05 | 0.005 | 0.05 | 0.005 | 0.05 | 0.005 | NI |

GAPDH

NS1

NS2
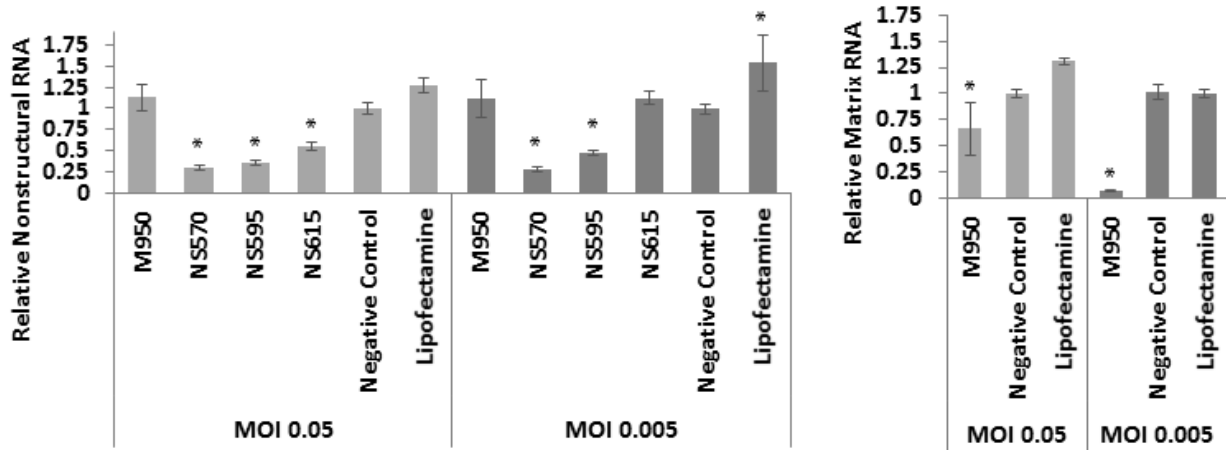
**C**

**Figure 3.5: NS570-, NS595-, NS615-siRNA reduce viral RNA and nonstructural protein 1 and 2 expression in culture lysates.** MDCK cells were transfected with siRNA [50 or 100 nM] for 8 h then infected with influenza A/WS/33 (H1N1) (MOI 0.05, 0.005) for 24 h. A) Relative influenza A/WS/33 nonstructural RNA expression was determined by qPCR analysis of culture lysates and normalized to GAPDH. To confirm transfection efficiency, relative matrix RNA expression was detected by qPCR and normalized to GAPDH for MDCK cells treated with positive control M950-siRNA. Protein expression was determined by B) Western blot of culture lysates and C) percent nonstructural protein 1 and 2 expression was determined by densitometry. Normalized to GAPDH. NI refers to no infection. (N=3)
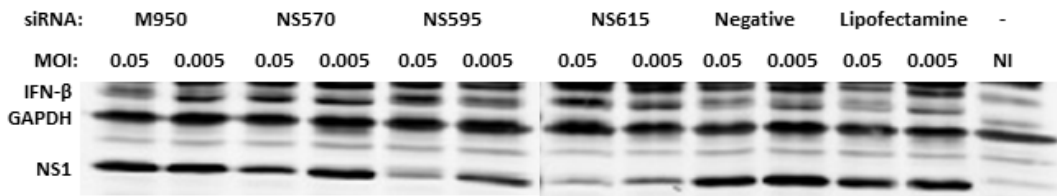
A





B

**Figure 3.6: NS570-, NS595-, NS615-siRNA do not attenuate influenza virus replication.** MDCK cells were transfected with matrix- or nonstructural-targeting siRNA or control siRNA [50 or 100 nM] for 8 h then infected with influenza A/WS/33 (H1N1) (MOI 0.05, 0.005) for 24 h. A) Percent total influenza virus in culture supernatant was detected by matrix 1, nonstructural 1, or neuraminidase specific qPCR. B) Percent infectious influenza virus was determined by viral plaque assay from culture supernatant. Percent calculations were normalized to negative control siRNA treated MDCK cells. (N=3)
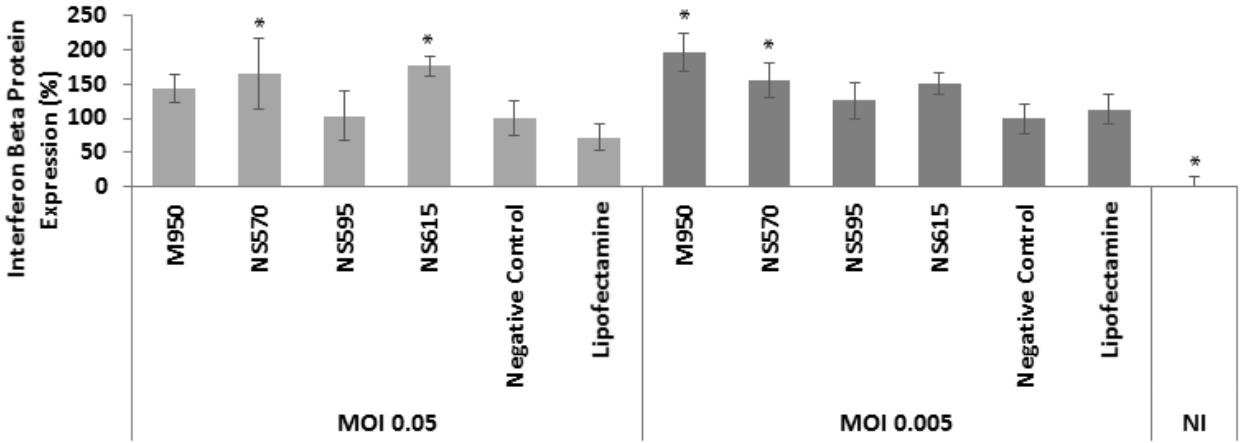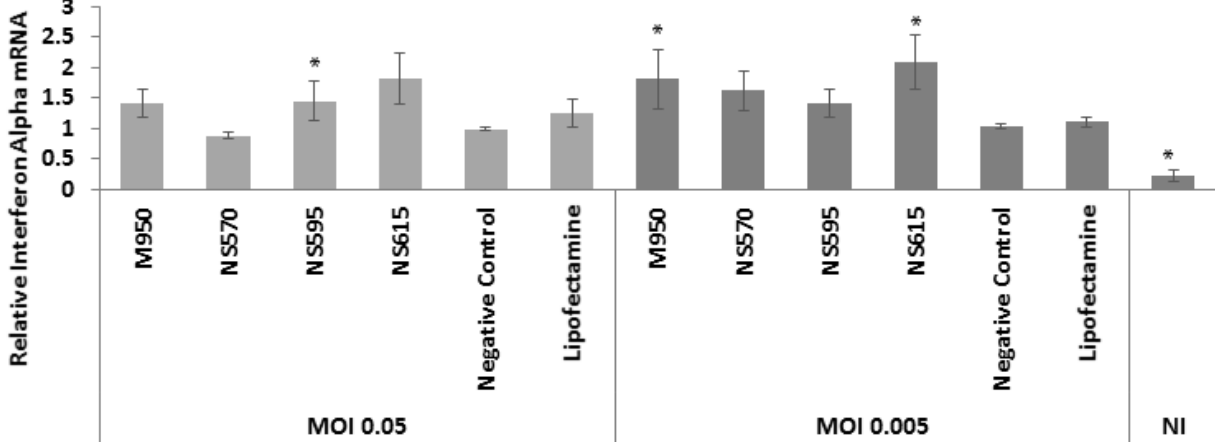
**A**



**B**

| siRNA: | M950 | | NS570 | | NS595 | | NS615 | | Negative | | Lipofectamine | | - |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| MOI: | 0.05 | 0.005 | 0.05 | 0.005 | 0.05 | 0.005 | 0.05 | 0.005 | 0.05 | 0.005 | 0.05 | 0.005 | NI |

IFN-β
GAPDH

NS1

**C**



**D**



116

**Figure 3.7: Nonstructural siRNA increases type-I interferon mRNA in culture supernatant.** MDCK cells were transfected with nonstructural-targetomg siRNA or control [50 or 100 nM] for 8 h then infected with influenza A/WS/33 (H1N1) (MOI 0.05, 0.005) for 24 h. A) Relative interferon-β (IFN-β) mRNA expression was determined by qPCR of culture lysates. IFN-β protein expression was determined by B) Western blot of culture lysates and C) percent IFN-β protein expression was calculated by densitometry. Percent calculations were normalized to negative control siRNA treated MDCK cells. D) Relative interferon-α (IFN-α) mRNA expression was determined by qPCR of culture lysates. Normalized to GAPDH. NI refers to no infection. (N=3)
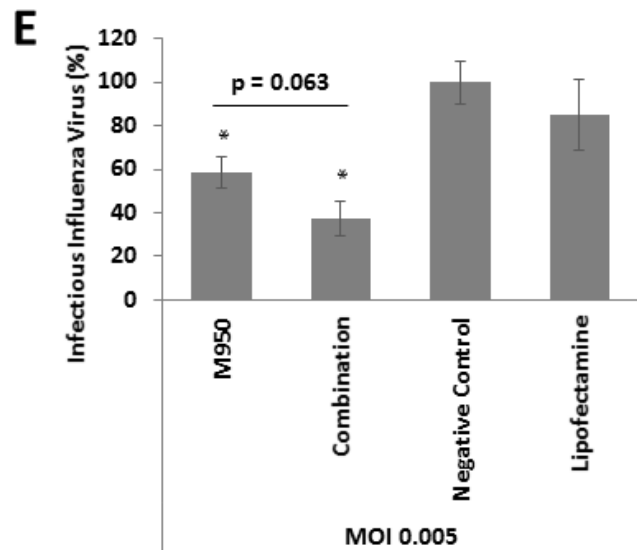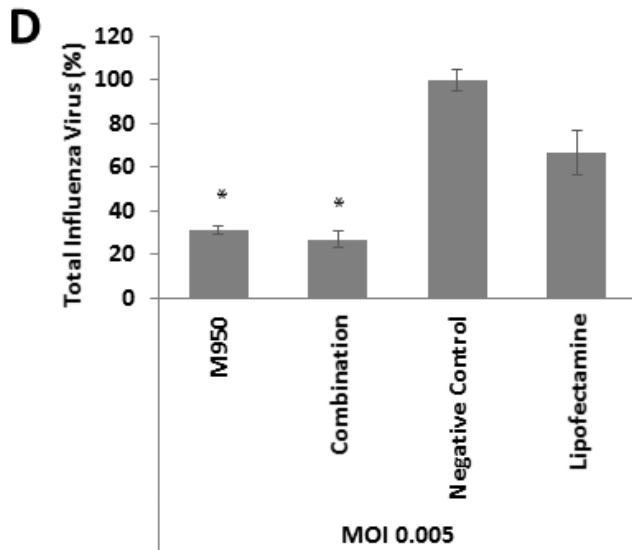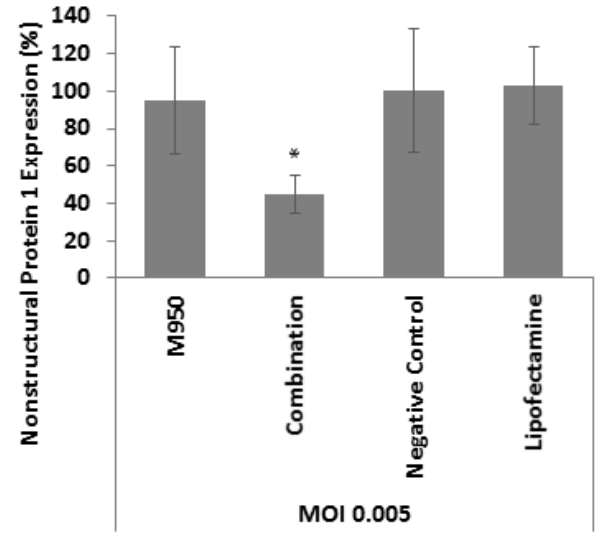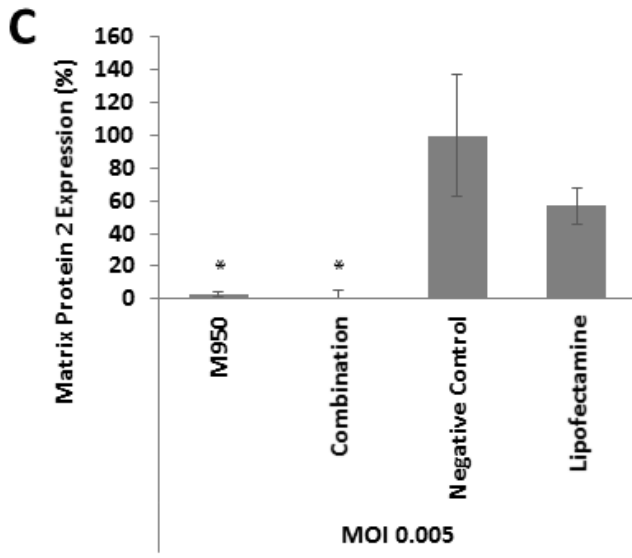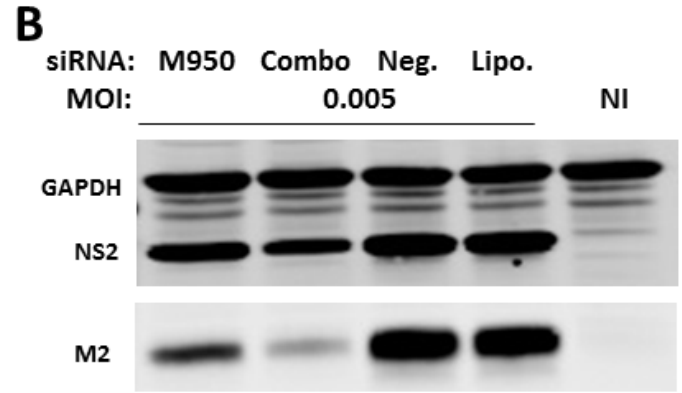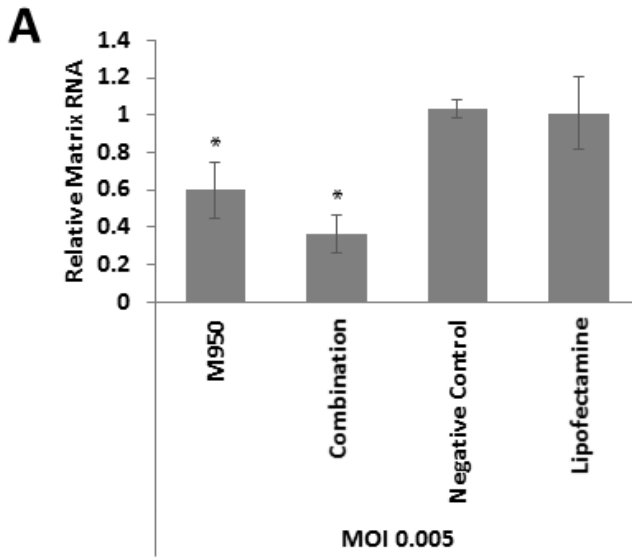
**Figure 3.8: Combination siRNA therapy is a more potent treatment than single siRNA therapy.** MDCK cells were transfected with M950 siRNA [50 nM], combination siRNA (M950 [50 nM], M776 [100 nM], NS595 [100 nM]), or negative control siRNA [100 nM] for 8 h then infected with influenza A/WS/33 (H1N1) (MOI 0.005) for 24 h. A) Relative matrix RNA expression was determined by qPCR analysis of culture lysates and normalized to GAPDH. Protein expression was determined by B) Western blot of culture lysates and C) percent matrix protein 2 and nonstructural protein 1 expression was calculated by densitometry and normalized to GAPDH. D) Percent total influenza virus in culture supernatant was detected by matrix 1 specific qPCR and E) percent infectious influenza virus was determined by viral plaque assay from culture supernatant. Percent calculations were normalized to negative control siRNA treated MDCK cells. Neg. refers to negative control siRNA, lipo. refers to lipofection, and NI refers to no infection. (N=3)

# Chapter 4 - Virus Induced Anti-sense RNA Expression Vector for the Development of an Inducible Anti-Influenza Therapy

**4.1 Abstract**

To overcome limitations with the clinical application of RNAi, we developed an inducible anti-sense RNA (asRNA) expression vector that utilizes the influenza virus conserved promoter to trigger expression of asRNAs upon recognition by the influenza virus RNA-dependent RNA polymerase (RdRP). We incorporated the sequence encoding for the asRNA strand of three previously characterized siRNA, M950, M776, and NS595, into the inducible asRNA expression vector and examined their ability to facilitate RNAi. Co-transfection of MDCK cells with an inducible asRNA expression vector and vectors expressing the viral RdRP resulted in the expression of asRNAs that were unable to knockdown plasmid-associated matrix 2 or nonstructural 2 transcripts. Upon further examination, the asRNA expression was 84- to 343-fold below the concentration needed to reduce influenza virus infection by RNAi. MDCK cells infected with influenza A/WS/33 (H1N1) at an MOI of 0.05 had up to 1.4-fold higher asRNA expression compared to control cells, yet the asRNAs were unable to reduce viral titer. Although a *Renilla* Luciferase reporter assay revealed that clinical influenza viral strains had a higher affinity for the influenza conserved promoter than cell culture adapted strains, asRNA expression still was not detected after infection with clinical isolates and did not alter viral titer after asRNA therapy treatment. The addition of enhancer elements or mutations to the influenza conserved promoter may improve the expression of asRNA. In addition, clinical isolates should be utilized for future antiviral research as adapted strains have altered affinity for the influenza promoter.

**4.2 Introduction**

RNA interference (RNAi) is a naturally occurring and conserved process that utilizes small RNA molecules, such as microRNAs (miRNA), short-hairpin RNAs (shRNA), small interfering RNAs (siRNA), or anti-sense RNA (asRNA), to facilitate posttranscriptional gene silencing by complementary messenger RNA (mRNA) degradation (Filipowicz, 2005; Fire et al., 1998; Zamore et al., 2000). The process of RNAi takes place when mature, endogenously expressed miRNA is introduced into the RNA-induced silencing complex (RISC) where the Arogonaute 2 (AGO2) protein degrades the passenger strand of the miRNA. The RISC uses the remaining guide strand to search for complementary mRNA that is then degraded by AGO2, thus silencing gene expression (Hammond et al., 2000). One hundred percent complementarity between the guide strand and the transcript results in transcript degradation, whereas imperfect complementarity results in translational repression. Gene silencing can occur through a similar mechanism by using exogenously synthesized or plasmid expressed siRNA or shRNA (Chen et al., 2005; Hui et al., 2004; Wacheck et al., 2003).  Alternatively, asRNA target complementary mRNA and silence gene expression via RNase-mediated degradation or inhibit protein translation by a steric blocking mechanism (Bertrand et al., 2002; Dias and Stein, 2002). RNAi by means of the RISC promotes repeated degradation of mRNA because one RNA molecule associated with the RISC can degrade multiple mRNAs whereas asRNAs can only be used once to degrade one mRNA because both the asRNA and its target are degraded by RNases (Crowley et al., 1985; Walder and Walder, 1988). A study by Martinez et al. (2002) showed that the single-stranded asRNA can also slip into the RISC and facilitate RNAi mirroring miRNA-mediated gene silencing.

Advances in the field of hepatitis C virus (HCV) treatment has emerged after the development of the anti-sense RNA molecule, Miravirsen. Miravirsen prevents RNAi by silencing the activity of the liver-

specific miRNA, miR122, that is known to support HCV protein translation (Henke et al., 2008). During a phase 2a clinical trial comprising of 36 patients with chronic HCV genotype 1 infection, Janssen et al. (2013) demonstrated that treatment with Miravirsen led to a continued dose-dependent reduction in HCV viral RNA levels and did not lead to the emergence of Miravirsen-resistant virus. Although RNAi can be an effective means to treat disease, its use as a therapeutic intervention is limited. For instance, unmodified siRNA and asRNA are highly susceptible to nuclease cleavage (Layzer et al., 2004), siRNA are readily cleared from the blood through the kidneys (van de Water et al., 2006), and siRNA or asRNA treatment can cause off-target gene silencing (Jackson et al., 2003) thus making it difficult to design, deliver and safely utilize RNAi molecules as a therapeutic.

To overcome the limitations associated with RNAi, we have developed influenza-induced expression vectors that will ultimately express RNAi-mediating asRNAs after influenza infection. By incorporating the highly conserved influenza promoter (Desselberger et al., 1980; Robertson, 1979) into a silencing RNA expression vector, asRNAs will only be expressed after the influenza-specific RNA-dependent RNA polymerase (RdRP) recognizes the influenza conserved promoter, thus limiting expression of the asRNA to influenza infected cells (Babar and Zaidi, 2015; Biswas and Nayak, 1994; Chu et al., 2012; Kawakami et al., 1981; Kukol and Hughes, 2014).

## 4.3 Materials and methods

*Cell lines and viruses*

Madin Darby Canine Kidney (MDCK) Epithelial cells were provided by Dr. Daniel Perez (University of Maryland, MD) and were maintained as described in McMillen et al. (2016).

Influenza strains A/WS/33 (H1N1) (ATCC, VR-1520), A/WSN/33 (H1N1), A/Swine/1976/31 (H1N1) (ATCC, VR-1682), A/Swine/Iowa/15/30 (H1N1) (ATCC, VR-1683), and A/Aichi/2/68 (H3N2) (ATCC, VR-1680) were maintained as described (Blachere et al., 2011). A/WSN/33 (H1N1) was provided by Dr. Robert A. Lamb (Northwestern University, IL). Clinical influenza virus isolates were maintained as follows: a Corning T-75 flask was seeded with 1 x 10$^7$ MDCK cells in complete growth media consisting of Eagle's Minimum Essential Medium (EMEM; American Type Culture Collection (ATCC)), 10% (v/v) fetal bovine serum (Hyclone Laboratories, Inc.), 200 units/mL penicillin, and 200 µg/mL streptomycin (Gibco, ThermoFisher Scientific; Lindsley et al., 2016). The cells were incubated overnight at 37° C in 5% $CO_2$ prior to inoculation. After 24 h, the media was removed from the T-75 flask and the cell monolayer was washed twice with phosphate-buffered saline (PBS), then once with using OPTI-MEM I Reduced Serum Medium (Gibco). Nasopharyngeal swab samples (Lindsley et al., 2016) were diluted 1:2 using OPTI-MEM I Reduced Serum Medium (Gibco) supplemented with 3 µg/mL N-p-tosyl-L-phenylalanine chloromethyl ketone (Sigma-Aldrich), applied to the cell monolayer, and incubated at 37° C for a 45 min adsorption period. Following the adsorption period, OPTI-MEM I Reduced Serium Medium (Gibco) supplemented with 3 µg/mL N-p-tosyl-L-phenylalanine chloromethyl ketone (Sigma-Aldrich) was applied to the inoculum and the MDCK cells were incubated at 37° C in 5% $CO_2$ for 48 h. Total viral titer and infectious viral titer were determined by quantitative polymerase chain reaction (qPCR) and viral plaque assay (VPA), respectively.

*Small interfering RNA (siRNA)*

Previously characterized siRNA targeting the matrix 1 and matrix 2 transcripts or nonstructural 1 and 2 transcripts were designed by the BLOCK-iT™ RNAi Designer (ThermoFisher Scientific). Purified Stealth siRNAs M776 and NS595 were synthesized by Life Technologies (ThermoFisher Scientific),

suspended in nuclease-free water and stored at -80° C (M776 sense: 5'CAGCAAAUAUCAUUGGAAUC UUGCA 3', M776 anti-sense: 5' UGCAAGAUUCCAAUGAUAUUUGCU G 3', NS595 sense: 5' CAGAGAUUCGCUUGGAGAAGCAGUA 3', NS595 anti-sense: 5' UACUGCUUCUCC AAGCGAAUCUCUG 3').

M950-siRNA was synthesized by Integrated DNA Technologies, suspended in nuclease-free water and stored at -80° C (M950 sense: 5' ACAGCAGAAUGCUGUGGAUUU 3', M950 anti-sense: 5' AUCCACAGCAUUCUGCUGUUU 3') (McMillen et al., 2016; Sui et al., 2009).

*Anti-influenza therapy constructs*

The pLucDel negative control vector was constructed by removing the *Renilla* luciferase gene in the canine RNA polymerase I (POL-I) promoter reporter plasmid, pk9POLI-RLuc vector, obtained from Dr. Daniel Perez (University of Georgia College of Veterinary Medicine, Athens, GA) (Hossain et al., 2010). The *Renilla* luciferase gene was deleted by site-directed mutagenesis using the QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies). Site directed mutagenesis was performed following the manufacturers protocol. To create pK9POLI-M950, -M776, and -NS595 inducible anti-influenza vectors (pM960, pM776, and pNS595, respectively), the anti-sense sequence for the corresponding siRNA was inserted between the 3' and 5' noncoding regions of the influenza nucleoprotein segment of influenza virus A/WSN/33 (Luytjes et al., 1989; Neumann and Hobom, 1995) by site-directed mutagenesis of the pLucDel negative control vector. Site-directed mutagenesis primers were designed by the web-based QuikChange Primer Design Program (Agilent Technologies) and were synthesized by Integrated DNA Technologies.

*Matrix protein 2 and nonstructural protein 2 expression vectors*

pUC57-SV40-M2 was generated by synthesizing DNA containing the following sequential sequences: SV40 promoter (Genbank: AY738229.1), influenza A/WS/33 (H1N1) matrix protein 2 coding

sequence (GenBank: CY009605.1), followed by the SV40 late poly (A) region (GenBank: AY738229.1) and inserting this sequence into the pUC57-Kan vector (GenBank: JF826242.2; Genewiz, Inc). pUC57-SV40-NS2 was generated by synthesizing DNA containing the SV40 promoter, influenza A/WS/33 (H1N1) nonstructural protein 2 (GenBank: CY009608.1), followed by the SV40 late poly (A) region and inserting this sequence into the pUC57-Kan vector (Genewiz, Inc.). DNA was synthesized and cloned into the pUC57-Kan vector by Genewiz, Inc. Optimal pUC57-SV40-M2 or –NS2 concentrations were determined by titered transfection of the expression vectors into MDCK cells for 24 h. The cell lysates were analyzed for expression by Western blot and the appropriate concentration was selected for further analysis (data not shown).

*RdRP induced asRNA via anti-influenza therapy*

To examine the ability of the influenza virus polymerase to induce the expression of asRNA, MDCK cells (5 x $10^5$ cells/well) were seeded on a 24-well plate (Corning) in complete growth media. Twenty-four hours later, transfection was performed following the Lipofectamine 2000 Reagent protocol (ThermoFisher Scientific). The MDCK cells were washed twice with PBS, then overlaid with 0.5 mL of OPTI-MEM I Reduced Serum Medium (Gibco, ThermoFisher Scientific) per well. Lipofectamine reagent was prepared to a concentration of 8 µg/mL in 50 µL of OPTI-MEM I Reduced Serum Medium, mixed gently, and then incubated at room temperature for 5 min. Concurrently, 0.16 µg of the corresponding anti-influenza therapy vectors were added to 50 µL of OPTI-MEM I Reduced Serum Medium. Cells were either transfected with each individual anti-influenza therapy alone or co-transfected with four vectors (0.16 µg each) that express the influenza RdRP (pCI-A-Brisbane-10-2007 (H3N2) NP, pCI-A-Brisbane-10-2007 (H3N2) PA, pCI-A-Brisbane-10-2007 (H3N2) PB1, pCI-A-Brisbane-10-2007 (H3N2) PB2; Hossain et al., 2010). The lipofectamine and antiviral therapy solutions were combined, mixed gently, and incubated

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for 20 min. Lipofectamine/antiviral therapy solution (100 µL) was added to each corresponding well. Lipofectamine solution (100 µL) without siRNA was used as a control. Four hours after transfection, the cells were washed twice with PBS to remove the lipofectamine reagent and overlaid with complete growth media. Twenty-four hours after transfection, the MDCK cells were lysed using Lysis/Binding Solution Concentration (Ambion) supplemented with 100% isopropanol (Sigma-Aldrich). The lysates were stored at -80° C until further processing for analysis by qPCR. Three independent experiments with two replicates per treatment were performed.

*Assessment of RdRP-induced asRNA facilitated RNAi of matrix protein 2 and nonstructural protein 2 expression vectors*

To evaluate whether the M776-asRNAs expressed by the influenza RdRP can knock down the expression of the influenza matrix protein 2, MDCK cells (1 x $10^6$ cells/well) were seeded on a 6-well plate in complete growth media. Twenty-four hours later, the MDCK cells were transfected with 0.8 µg of pUC57-SV40-M2, 0.4 µg of each of the four RdRP expression vectors, and 0.8µg of pM776 or pLucDel inducible asRNA expression vectors (3.2 µg total plasmid DNA) using 12.5 µL of Lipofectamine 2000 reagent (ThermoFisher Scientific) per well. The lipofectamine reagent was removed from the MDCK cells 8 h later by washing twice with PBS and overlaying the cells with complete growth media. Twenty-four hours after transfection, the complete growth media was removed from the cells and the cell were lysed with 100 µL of radioimmunoprecipitation assay (RIPA) buffer containing a protease inhibitor cocktail and ethylenediaminetetraacetic acid (EDTA; ThermoFisher Scientific) for analysis by Western blot. For qPCR analysis, the complete growth media was removed from the cells 24 h post transfection and suspended in Lysis/Binding Solution Concentration supplemented with 100% isopropanol (Sigma-Aldrich). The cell lysates were stored at -80° C until further use. For a control, MDCK cells transfected with pUC57-SV40-

M2 and pM776 (or pLucDel control), pCI-A-Brisbane-10-2007 (H3N2) NP, and pCI-A-Brisbane-10-2007 (H3N2) PB1 (not pCI-A-Brisbane-10-2007 (H3N2) PA or pCI-A-Brisbane-10-2007 (H3N2) PB2) with 0.8 µg pCBG-99 Basic Vector (Promega) to ensure a final amount of 3.2 µg of plasmid DNA transfected per well.

To evaluate whether the NS595-asRNAs expressed by the influenza RdRP can knock down the expression of the influenza nonstructural protein 2, MDCK cells were treated in a similar fashion as stated in the paragraph above, except that the pUC57-SV40-M2 expression vector was replaced with the pUC57-SV40-NS2 expression vector and the inducible asRNA expression vector, pNS595, was evaluated instead of pM776. Cells transfected with pLucDel instead of pNS595 were used as a control. Three independent experiments with one replicate per treatment were performed.

*Comparison of asRNA expressed by asRNA antiviral therapy and siRNA transfected into MDCK cells*

To evaluate the amount of asRNA expressed by the inducible asRNA antiviral therapy compared to the concentration of transfected siRNA needed to mediate RNAi, MDCK cells ($1 \times 10^6$ cells/well) were seeded on a 6-well plate in complete growth media. Twenty-four hours later, the MDCK cells were transfected with either 1) 200 pmol M950-, M776-, or NS595-siRNA alone, 2) 0.8 µg of pM950, pM776 or pNS595 and 0.6 µg of each of the four RdRP expression vectors or 3) 0.8 µg of pM950, pM776 or pNS595 and 0.6 µg of pCI-A-Brisbane-10-2007 (H3N2) NP and pCI-A-Brisbane-10-2007 (H3N2) PB1 with 1.2 µg pCBG-99 Basic Vector (Promega) using 12.5 µL of Lipofectamine 2000 reagent (ThermoFisher Scientific) per well. Transfection was performed following the Lipofectamine 2000 Reagent protocol (ThermoFisher Scientific). The lipofectamine reagent was removed from the MDCK cells 8 h later by washing twice with PBS and overlaying the cells with complete growth media. Twenty-four hours after transfection, the complete growth media was removed from the cells and the cell were lysed in 350 µL

Lysis Solution (Exiqon) and stored at -80° C until further processing by the miRCURY RNA Isolation Kit – Cell Plant (Exiqon).

As a control prior to transfection, 200 pmol of the corresponding siRNAs were directly suspended in lysis/binding concentration and stored at -80°C until further processing. Two independent experiments with two replicates per treatment were performed.

*A/WS/33 (H1N1) induction of asRNA via anti-influenza therapy*

To test the ability of infectious influenza virus to induce the expression of asRNA and examine the knockdown efficiency of the various asRNA, MDCK cells (1 x $10^6$ cells/well) were seeded on 6-well plate (Corning) in complete growth media. Twenty-four hours later, the MDCK cells were washed twice with PBS and overlaid with 1.5 mL of OPTI-MEM I Reduced Serum Medium (Gibco). Transfection was performed following the Lipofectamine 2000 Reagent protocol (ThermoFisher Scientific). 1.6 µg of corresponding asRNA anti-influenza therapy, pLucDel, or empty vector control, pAcGFP1-1 (Clontech), was added to the lipofectamine solution containing 8 µL lipofectamine per treatment. Eight hours later the cells were washed twice with PBS and then overlaid with complete growth media. Twenty-four hours after transfection, the MDCK cells were washed twice with PBS then inoculated with influenza virus at a multiplicity of infection (MOI) of 1.0, 0.05, or 0.005. After a 45 min adsorption period, the cells were washed with PBS and overlaid with 2 mL of Dulbecco's modified Eagle's medium (DMEM)/F12 (ATCC) supplemented with 100 U/mL penicillin G, 100 mg/ml streptomycin, 2 mM L-glutamine, 0.2% 9 (v/v) BSA, 10 mM HEPES (ThermoFisher Scientific), 0.22% (v/v) sodium bicarbonate (ThermoFisher Scientific), 0.01% (w/v) DEAE-dextran (MP BioMedicals, LLC), and 2 mg/mL N-p-tosyl-L-phenylalanine chloromethyl ketone (Sigma-Aldrich). The culture supernatant and cell lysates were collected for analysis 24 h after infection. Three independent experiments were performed with one replicate per experiment.

*Influenza virus affinity for influenza conserved promoter by luciferase reporter assay*

To test the affinity of adapted influenza viruses and clinical influenza virus isolates for the influenza conserved promoter, MDCK cells (5 x $10^5$ cells/well) were seeded on a 24-well plate (Corning) in complete growth media. Twenty-four hours later, the MDCK cells were washed twice with PBS and overlaid with 500 μL OPTI-MEM I Reduced Serum Medium (Gibco). Lipofectamine reagent was prepared to a concentration of 8 μg/mL in 50 μL of OPTI-MEM I Reduced Serum Medium, mixed gently, and then incubated at room temperature for 5 min. Concurrently, 0.8 μg of the canine RNA polymerase I (POL-I) promoter reporter plasmid, pk9POLI-RLuc vector, was added to 50 μL of OPTI-MEM I Reduced Serum Medium. The lipofectamine and antiviral therapy solutions were combined, mixed gently, and incubated for 20 min. Lipofectamine/antiviral therapy solution (100 μL) was added to each corresponding well. Lipofectamine solution (100 μL) without siRNA was used as a control. Four hours after transfection, the cells were washed twice with PBS to remove the lipofectamine reagent and overlaid with complete growth media. Twenty-four hours after transfection, the MDCK cells were washed twice with PBS then inoculated with adapted influenza viruses or clinical influenza virus isolates at an MOI of 1.0, 0.01, or 0.001. After a 45 min adsorption period, the cells were washed with PBS and overlaid with 2 mL of Dulbecco's modified Eagle's medium (DMEM)/F12 (ATCC) supplemented with 100 U/mL penicillin G, 100 mg/ml streptomycin, 2 mM L-glutamine, 0.2% 9 (v/v) BSA, 10 mM HEPES (ThermoFisher Scientific), 0.22% (v/v) sodium bicarbonate (ThermoFisher Scientific), 0.01% (w/v) DEAE-dextran (MP BioMedicals, LLC), and 2 mg/mL N-p-tosyl-L-phenylalanine chloromethyl ketone (Sigma-Aldrich). Culture supernatant and cell lysates were collected and centrifuged at 2,000 rpm for two minutes to collect intact cells. The supernatant was removed and the cell pellet was lysed in 100 μL PBL (Promega). Luciferase expression was determined using the Dual-Luciferase Reporter Assay Kit (Promega) following the manufacturer's

instructions. Mock infected cells treated only with supplemented DMEM/F12 were used as a control. Three independent experiments were performed with three replicates per experiment.

*Clinical influenza virus isolates' induction of asRNA via anti-influenza therapy*

To test the ability of infectious influenza virus to induce the expression of asRNA and examine the knockdown efficiency of the various asRNA, MDCK cells ($5 \times 10^5$ cells/well) were seeded on 24-well plate (Corning) in complete growth media. Twenty-four hours later, the MDCK cells were washed twice with PBS and overlaid with 500 µL of OPTI-MEM I Reduced Serum Medium (Gibco). Transfection was performed following the Lipofectamine 2000 Reagent protocol (ThermoFisher Scientific). 0.8 µg each of pM950, pM776, pNS595, and NA105 (referred to as combination treatment; for NA105 data, refer to Chapter 5) or, 3.2 µg of pLuc Del, or 3.2 µg of empty vector control, pCBG99 Basic Vector (Promega), was added to the lipofectamine solution containing 2 µL lipofectamine per treatment. Eight hours later the cells were washed twice with PBS then overlaid with complete growth media. Twenty-four hours after transfection, the MDCK cells were washed twice with PBS then inoculated with influenza virus at an MOI of 0.1 or 0.01. After a 45 min adsorption period, the cells were washed with PBS and overlaid with 1 mL of Dulbecco's modified Eagle's medium (DMEM)/F12 (ATCC) supplemented with 100 U/mL penicillin G, 100 mg/ml streptomycin, 2 mM L-glutamine, 0.2% 9 (v/v) BSA, 10 mM HEPES (ThermoFisher Scientific), 0.22% (v/v) sodium bicarbonate (ThermoFisher Scientific), 0.01% (w/v) DEAE-dextran (MP BioMedicals, LLC), and 2 mg/mL N-p-tosyl-L-phenylalanine chloromethyl ketone (Sigma-Aldrich). The culture supernatant and cell lysates were collected for analysis 24 h after infection. Three independent experiments were performed with three replicates per experiment.

*Total RNA isolation and cDNA transcription*

Total RNA was isolated from MDCK cells using the MagMax™-96 Total RNA Isolation Kit (Ambion). MDCK cells were lysed with Lysis/Binding Solution Concentration (Ambion) supplemented with 100% isopropanol (Sigma-Aldrich) and then processed following the manufacturer's instructions. Total RNA was immediately transcribed into cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) in accordance with the manufacturer's instructions.

For northern blot analysis and asRNA detection, total RNA was isolated from MDCK cells using the miRCURY™ RNA Isolation Kit (Exiqon) following the manufacturer's protocol. For northern blot analysis, total RNA was immediately stored at -80° C until further use. For asRNA detection, M950-, M776-, or NS595- asRNA was immediately reverse-transcribed into cDNA using the Taqman Small RNA Assays asRNA-specific primers (Applied Biosystems, following the manufacturer's instructions. Reverse transcription was performed using the Applied Biosystems 7500 Fast Real-Time PCR System under the following thermal cycling conditions: 16°C for 30 minutes, 42°C for 30 minutes and 85°C for 5 minutes. Total RNA isolated from the miRCURY™ RNA Isolation Kit (Exiqon) was immediately transcribed into cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) in accordance with the manufacturer's instructions.

Viral RNA was isolated from culture supernatant using the MagMax™-96 Total RNA Isolation Kit (Ambion). The culture supernatant was suspended in Lysis/Binding Solution Concentrate (Ambion) supplemented with 100% isopropanol (Sigma-Aldrich) and then processed following the manufacturer's instructions. Viral RNA was immediate reverse transcribed into cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) following the manufacturer's instructions.

For specific detection of mRNA, total RNA underwent a second round of isolation using oligo d(T)$_{20}$ magnetic beads (New England Biolabs), following the manufacturer's protocol. mRNA was reverse transcribed into mRNA-specific cDNA using the ThermoScript Reverse Transcriptase Kit from Invitrogen (ThermoFisher Scientific). Reverse transcription was performed following the manufacturer's protocol and using 50 µM of Oligo d(T)$_{20}$ Primer per reaction (Invitrogen, ThermoFisher Scientific).

*Analysis of asRNA and mRNA levels by real-time quantitative PCR (qPCR)*

qPCR analyses to detect asRNA were performed using the Taqman Small RNA Assays (Applied Biosystems). Primers for asRNA-specific reverse transcription and qPCR primers and probes specific for the asRNA were designed and synthesized by Applied Biosystems. Reactions were performed and analyzed using the Applied Biosystems 7500 Fast Real-Time PCR System under the following thermal cycling conditions: 50°C for 2 minutes, 95°C for 10 minutes, followed by 40 cycles at 95°C for 15 seconds and 60°C for 60 seconds. A negative control without template was included in all real-time PCRs.

qPCR analyses to detect influenza-specific mRNA were performed using the following primers and probes: Matrix 2, forward 5' GCACTTGATATTGTGGATTC 3', reverse 5' CAGCTCTATGTTGACAAAATG 3' and probe 5' AAGAATATCGAAAGGAACAGC 3'; Nonstructural 2, forward 5' TCGGAGGACTTGAATGGAATAATAA 3', reverse 5' GCTTCTCCAAGCGAATCTCTGT 3', and probe 5' ACAGTTCGAGTCTCTG 3'. The matrix 2 and nonstructural 2 primers and probes were designed in lab using the Primer Express 3.0 software by Applied Biosystems. The probes were designed with a 5' fluorescent dye, 6FAM, and 3' quencher, MGCNFQ. All primers and probes were synthesized by Applied Biosystems and used at a final concentration of 0.8 M and 0.2 M, respectively. Reactions were performed and analyzed using the Applied Biosystems 7500 Fast Real-Time PCR System under the following thermal

cycling conditions: 95°C for 20 seconds, followed by 40 cycles at 95°C for 3 seconds, and 60°C for 30 seconds. A negative control without template was included in all real-time PCRs.

All samples were run in duplicate. Relative gene expression was determined by ΔΔCT and normalized to GAPDH (Hs03929097_g1, ThermoFisher Scientific). PCR was performed on MDCK cell lysates and analyzed by gel electrophoresis to confirm specificity of GAPDH primers to canine GAPDH (data not shown).

*Analysis of infectious virus induced expression of asRNA by northern blot*

To detect asRNA expression from an inducible asRNA expression vector, MDCK cells (1 x $10^6$ cells/well) were seeded on 6-well plate (Corning) in complete growth media. Twenty-four hours later, the MDCK cells were washed twice with PBS and overlaid with 1.5 mL of OPTI-MEM I Reduced Serum Medium (Gibco). Transfection was performed following the Lipofectamine 2000 Reagent protocol (ThermoFisher Scientific). 1.0 µg of pM776 or pLuc Del was added to the lipofectamine solution containing 8 µL lipofectamine per treatment. Eight hours later the cells were washed twice with PBS then overlaid with complete growth media. Twenty-four hours after transfection, the MDCK cells were washed twice with PBS then inoculated with influenza virus at an MOI of 0.1 or 0.01. After a 45 min adsorption period, the cells were washed with PBS and overlaid with 2 mL of Dulbecco's modified Eagle's medium (DMEM)/F12 (ATCC) supplemented with 100 U/mL penicillin G, 100 mg/ml streptomycin, 2 mM L-glutamine, 0.2% 9 (v/v) BSA, 10 mM HEPES (ThermoFisher Scientific), 0.22% (v/v) sodium bicarbonate (ThermoFisher Scientific), 0.01% (w/v) DEAE-dextran (MP BioMedicals, LLC), and 2 mg/mL N-p-tosyl-L-phenylalanine chloromethyl ketone (Sigma-Aldrich). The culture supernatant and cell lysates were collected for analysis 24 h after infection. One independent experiment with one replicate per treatment group was performed.

Total RNA isolated following the miRCURY™ RNA Isolation Kit (Exiqon) protocol was analyzed by northern blot using the NorthernMax (Applied Biosystems) protocol, with a few modifications. 50 μg of total RNA was run by 17% polyacrylamide gel electrophoresis (PAGE). RNA was transferred to a nylon membrane by the capillary blotting method (Brown et al., 2004). RNA was linked to nylon membrane using the UV Stratalinker 1800 (Stratagene), pre-hybridized in hybridization buffer for 1 h, then hybridized overnight with 10 pmol of corresponding DIG-labeled probe (Exiqon). The membrane was washed twice under low stringency conditions, then washed once under high stringency conditions at 37°C. RNA was detected following the DIG nucleic acid detection kit protocol (Roche).

Double DIG-labeled locked nucleic acid (LNA) northern probes were synthesized by Exiqon to detect asRNAs (M776, 5' DIG-CAGCAAATATCATTGGAATCTTGCA-DIG 3'). For a loading control. A double DIG-labeled LNA northern probe was synthesized by Exiqon to detect the 5S rRNA of C*anis lupus familiaris* (5S rRNA probe, 5' DIG-TCTCCCATCCAAGTACTAACC-DIG 3').

*Analysis of protein levels by Western blot*

Cells were lysed with RIPA Buffer containing a protease inhibitor cocktail and EDTA (ThermoFisher Scientific). Protein concentration was determined with the BCA Protein Assay Kit (Pierce) performed in triplicate. Thirty to fifty μg of sample protein was diluted 1:1 with 2x Laemmli loading dye (Bio-Rad), denatured by boiling for 10 min, and immediately cooled on ice before loading on to a 4% stacking/12% sodium dodecyl sulfate (SDS)-PAGE mini-gel. The electrophoresed protein was electroblotted to nylon membranes overnight at a constant 16 V. The blots were blocked in Odyssey Blocking Buffer (LI-COR Biosciences) for 1 h at room temperature and then incubated overnight with primary antibodies GAPDH (sc-25778, Santa Cruz), M2 (sc-32238, Santa Cruz), or NS2 (GTZ125952, GenTex) diluted in Odyssey Blocking Buffer. After four washes with TBS-tween (0.1% tween), the blots

were incubated with corresponding infrared (680 or 800 nm) conjugated secondary antibodies (LI-COR Biosciences) for 1 h at room temperature. Blots were washed four times in TBS-tween and then analyzed on the Odyssey Imaging System (LI-COR Biosciences). Primary antibodies directed against GAPDH were used to verify equal sample loading and for normalization.

*Analysis of infectious viral titers by viral plaque assay (VPA)*

Viral plaque assays were performed as described in McMillen et al. (2016).

*Statistical analyses*

A two-way factorial mixed model analysis of variance (ANOVA) was performed on all variables. All pairwise comparisons were considered significant at $p < 0.05$. Asterisks above graphs (*) indicate $p<0.05$. All graphs are representative of three or one independent experiment with two replicates per treatment group per experiment.

## 4.4 Results and discussion

*Diagram of target sequences for matrix and nonstructural targeting asRNAs & model of the inducible-asRNA expression vector*

Influenza viruses cause a high rate of mortality and morbidity worldwide despite the development of antiviral therapies (Thompson et al., 2010; Thompson et al., 2004; WHO, 2014). Antiviral therapies that inhibit the activity of the matrix 2 protein have limited use because they are only effective against influenza A viruses  (Schmidt, 2004; Wang et al., 1993), whereas strains of influenza have emerged that are resistant to neuraminidase inhibitors (Sheu et al., 2008). As a result, there is a need to

develop alternative methods of antiviral therapies for influenza virus infection. We have designed three asRNA inducible vectors that express the asRNA strand of the siRNAs, M950, M776, or NS595. M950- and M776-siRNAs were previously characterized to knock down the expression of the matrix protein 2 and attenuate influenza replication (Sui et al., 2009), whereas NS595-siRNA was shown to knock down the expression of the nonstructural proteins 1 and 2 and enhance type I interferon expression *in vitro* (McMillen et al., 2016) (Figure 4.1A).

Each vector encoding for a particular asRNAs contain the following characteristics (Figure 4.1B): the anti-sense RNA coding region of M950-, M776-, or NS595-siRNA is flanked by the noncoding regions (NCR) (indicated by horizontal black stripes with white background) from the nucleoprotein gene of A/WSN/33 (Hossain et al., 2010). The sequences of the canine polymerase-I promoter (k9POL-I) and the canine polymerase-I termination (k9TI) sequences were included upstream to the 5' NCR or downstream to the 3' NCR, respectively. These sequences were placed under the pCAT3-basic vector background as previously stated in Hossain et al. (2010) (Figure 4.1B). The k9POL-I promoter allows for the expression of an RNA intermediate that mimics the genetic material of influenza viruses, as it is a negative-sense single-stranded RNA that contains the conserved promoter, however instead of the RNA intermediate containing an influenza-specific gene, it contains the coding region for a particular asRNA. Only after that same cell containing the RNA intermediate is infected with influenza will the influenza RdRPs recognize the conserved influenza promoter and transcribe the asRNAs. By limiting the expression of the asRNAs to cells that are infected with influenza, it will protect the surrounding healthy cells from unnecessary exposure to the asRNAs.

*Viral RNA-dependent RNA polymerase induces the expression of asRNAs*

To examine the ability of the influenza RdRP to induce the expression of asRNAs, MDCK cells were co-transfected with one of the three inducible asRNA expression vectors, pM950, pM776, or pNS595 and each of four individual expression vectors that encode for the four proteins that make up the influenza A/Brisbane/10/2007 (H3N2) RdRP. MDCK cells co-transfected with the pM776 or pNS595 inducible asRNA expression vectors and the RdRP expression vectors displayed a significant level of asRNA expression; cells containing pM776 had a 4,609-fold more M776-asRNA expression and pNS595 had a 28.4-fold more NS595-asRNA expression compared to cells transfected with the corresponding inducible asRNA expression vector alone (Figure 4.2). MDCK cells co-transfected with pM950 and the RdRP expression vectors had 3.4-fold more M950-asRNA expressed than cells containing the pM950 inducible asRNA expression vector alone (Figure 4.2). These results show that the influenza RdRP can prompt the expression asRNAs via the inducible asRNA expression vectors, whereas if the RdRP is not present, low to background levels of asRNAs are detected indicating that this is a RdRP-specific response.

*asRNAs expressed by RDRP induced asRNA expression vectors do not knockdown the expression of influenza proteins*

Next we examined the functional ability of the asRNAs expressed after exposure to the influenza RdRP to knock down the expression of influenza-specific genes. To study this, we designed and synthesized a matrix protein 2 (influenza A/WS/33 (H1N1)) expression vector, pUC57-SV40-M2. MDCK cells were co-transfected with pM776 and pUC57-SV40-M2 with or without the presence of the complete RdRP for 24 hours. There was no difference in the level of matrix 2 RNA in the culture lysates of cells treated with pM776 and the RdRP compared to pLucDel treated cells. Similarly, pM776 or pLucDel treated cells without the RdRP had similar levels of matrix 2 RNA (Figure 4.3A). To prevent the

detection residual pSV40-M2 expression vector by matrix 2-specific qPCR, mRNA was re-isolated from total RNA and evaluated by qPCR. pM776 treatment with the RdRP did not reduce matrix 2 transcript level in the culture lysates (Figure 4.3B).

To test the ability of the pNS595 induced asRNAs to facilitate RNAi we synthesized a nonstructural protein 2 (influenza A/WS/33 (H1N1)) expression vector. pNS595 was co-transfected with pUC57-SV40-NS2 and in the presence or absence of complete set of RdRP expression vectors into MDCK cells for 24 hours. Like the pM776 treatment, MDCK cells treated with the pNS595 in the presence of RDRP did not have reduced nonstructural 2 protein transcript when analyzed by cDNA generated from total RNA (Figure 4.3A) or mRNA (Figure 4.3B).

Western blot analysis also confirmed that treatment with pM776 or pNS595 in the presence of RdRP did not reduce matrix 2 or nonstructural 2 protein expression, respectively, when compared to MDCK cells treated with the pLucDel control vector (Figure 4.3C). These results indicated that although asRNA is expressed by the RdRP, the asRNA that is generated is unable to mediate RNAi and knockdown the expression of the corresponding transcript or protein.

*asRNAs expressed by RDRP induced asRNA expression vectors is much lower than what is needed to mediate RNAi*

To better understand why the asRNA that was expressed by the RdRP was incapable of causing RNAi, the level of asRNA induced by the RdRP was compared to the amount of siRNA that enters MDCK cells after transfection with 100 nM of M950-, M776-, or NS595-siRNA. McMillen et al. (2016) and (Sui et al., 2009) demonstrated that treatment of MDCK cells with 100 nM of siRNA can result in reduced protein expression or attenuated viral titer. asRNA generated by pM950-, pM776-, or pNS595- were 245.8-, 84.6- and 343.5-fold less than the amount of siRNA that is needed reduce matrix or nonstructural

protein expression *in vitro* (Figure 4.4). These results indicate that the inducible asRNA anti-influenza therapies do not generate enough asRNAs to mediate RNA interference. In the interest of increasing the likelihood of asRNA expression, manipulation of the canine polymerase I transcriptional activity or alterations to the conserved influenza promoter should be considered.

Enhancers are regulatory DNA regions within a promoter that act as binding sites for transcription factors (Shlyueva et al., 2014). The enhancers, which can be located upstream or downstream of the transcription start site, function to bring the sequence-specific transcription factors close to the transcription start site and expedite the transcription process (Pennacchio et al., 2013). A study trying to identify the complete canine polymerase I promoter observed that the addition of 457 nucleotides upstream of the transcriptional start site into a luciferase reporter assay resulted in 2-fold increased luciferase activity compared to the reporter assay containing 250 nucleotides (Murakami et al., 2008). Another study by Wang and Duke (2007) saw similar results where the addition of 469 nucleotides upstream of the of the transcriptional start site into a chloramphenicol acetyltransferase (CAT) reporter assay, had higher levels of CAT expression compared to the addition of only 230 nucleotides. The polymerase I promoter region included in the asRNA therapies only contains 346 nucleotides upstream of the transcriptional start site therefore, altering the asRNA antiviral therapy to contain 469 nucleotides upstream of the RNA polymerase I gene transcriptional start site may enhance its transcriptional activity. Alternatively a T7 RNA polymerase promoter has been utilized for the production of a reverse-genetics system for influenza A virus in MDCK cells (de Wit et al., 2007). The differential asRNA expression levels of asRNA therapies containing the T7 RNA polymerase promoter versus the canine polymerase I promoter should be tested in MDCK cells. Hence, the addition of regulatory elements, such as enhancer regions, to the inducible asRNA anti-influenza therapy could improve the expression of asRNA by increasing the number of RNA intermediates available to the viral RdRP.

Studies by Neumann and Hobom (1995) and Li and Palese (1992) performed mutational analysis of the influenza conserved promoter activity and discovered that certain point mutations resulted in increased promoter activity. For instance, mutating an adenine to a cytosine at position three of the 3' noncoding region of influenza resulted in a 3.5-fold increase in promoter activity compared to the wild-type promoter (Li and Palese, 1992). The addition of extra enhancer elements and mutations to the influenza conserved promoter could have a synergistic effect on asRNA transcription and should be evaluated further in future studies.

*A/WS/33 (H1N1) infection induces low levels of asRNA that are not able to reduce influenza replication and clinical influenza isolates have a higher affinity for the influenza conserved promoter than adapted influenza strains*

Delivery of the viral RdRP into cells occurs naturally during influenza infection, therefore we evaluated the ability of the RdRP from infectious virus to induce asRNA expression. Influenza virus infection resulted in a modest expression of asRNA in MDCK cells treated with pM950, pM776, and pNS595. MDCK cells treated with pM950 and then infected with influenza A/WS/33 (H1N1) at an MOI of 0.05 had 1.4-fold higher asRNA expression compared to pLucDel treated cells (Figure 4.5A). pM776 treated cells had a 1.4-fold higher asRNA expression after infection with influenza virus at an MOI of 0.05 and 0.005 whereas there was only a 0.7-fold increase in NS595 asRNA after MDCK cells treated with pNS595 were infected with influenza A/WS/33 (Figure 4.5A). Total viral titer was not affected after treatment with pM950, pM776 or pNS595, indicating that the asRNA expressed after influenza infection may not be sufficient to mediate RNAi (Figure 4.5B).

Because the influenza promoter is conserved for all strains and subtypes of influenza viruses and is specifically recognized by the influenza RdRP as a result, we hypothesized that various strains and

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subtypes of influenza viruses can induce the expression of the asRNAs. To have a better understanding of the affinity of the viral RdRP of various strains and subtypes of influenza viruses to the influenza conserved promoter, whether adapted or isolated from clinical samples, we utilized a luciferase reporter plasmid (Figure 4.5C) generated by Hossain et al. (2010). MDCK cells were transfected with pk9POLI-RLuc reporter plasmid for 24 hours and subsequently infected with various strains and subtypes of influenza A viruses for 24 hours. MDCK cells containing the reporter plasmid were infected with influenza A virus at an MOI of 0.1, 0.01 or 0.001 and compared to mock infected cells. We observed that regardless of strain and subtype, all influenza viruses were able to induce the expression of the *Renilla* luciferase protein compared to mock infected cells. Cells infected with the cell culture adapted influenza strains generated modest amount of *Renilla* luciferase protein, as determined by luciferase assay, compared to influenza virus collected from clinical isolates (Lindsley et al., 2016). Cells infected with the clinical isolate Clin 113NS (pH1N1) had 1012-fold and clinical isolate Clin 162NS (H3N2) had 2265-fold higher luciferase activity compared to the commonly studied influenza A/WS/1933 (H1N1) strain at an MOI of 0.1 (Figure 4.5C). A similar trend was observed for cells infected at an MOI of 0.01 and 0.001.

*Combination anti-influenza therapy does not reduce influenza virus in culture supernatant*

Two particular clinical isolates, Clin 113NS (pH1N1) and Clin 162NS (H3N2) had particular high luciferase protein expression. Therefore, we studied the ability of these strains to induce asRNA expression and the ability of inducible asRNA anti-influenza virus treatment to attenuate viral titer. MDCK cells were transfected with a combination treatment (pM950, pM776, pNS595, and NA105) and then infected with influenza A/WS/33 (H1N1), Clin 113NS, or Clin 162NS at an MOI of 0.1 or 0.01 for 24 hours. Treatment of MDCK cells with the combination therapy had no effect on total influenza virus in the culture supernatant compared to pLucDel or pCBG-99 treated control, regardless of the strain or

subtype tested (Figure 4.6A). In order to account for the potential for non-specific amplification of viral RNA when performing qPCR specific for the asRNA, a northern blot analysis was performed to detect the expression of M776-asRNA after influenza A/WS/33 (H1N1), Clin 113NS (pH1N1) or Clin 162NS (H3N2) infection. M776-asRNA was unable to be detected by northern blot compared to the positive control M776 siRNA [20 pmol], demonstrating that, like the vector expressed RdRP facilitated asRNA expression, only modest levels of asRNA are being expressed which are significantly lower than the amount of siRNA needed to cause RNAi *in vitro* (Figure 4.6B). We have shown that clinical isolates that have not been serially passaged through cell culture have a higher affinity for the conserved influenza promoter generated by the luciferase reporter plasmid. Although the clinical isolates are able to facilitate a higher expression level of luciferase protein, the amount of asRNA they generate is unable attenuate viral titer, further emphasizing the need to improve the promoter activity of the asRNA anti-influenza therapy.

In an effort to design a molecular-based approach that can overcome the limitations and adverse effects associated with RNAi, we designed a novel inducible system where influenza-specific RNA polymerases induce the expression of asRNAs. The influenza 5' and 3' untranslated regions are complementary regions found on all eight influenza genomic RNA that form the influenza promoter. This influenza promoter is highly conserved for all strains and subtypes of influenza viruses A, B and C and are specifically recognized by the influenza RdRP (Chow and Simpson, 1971; Desselberger et al., 1980; Robertson, 1979). We reasoned that by utilizing the conserved influenza promoter to induce the expression of asRNA by the influenza virus RdRPs, we could create a system that forces the virus to produce a molecule that inhibits viral protein expression and ultimately attenuate viral replication. Likewise, because the influenza promoter is recognized by RdRPs from all strains and subtypes of influenza, this antiviral therapy has the potential to be used as a universal therapy. As a proof of concept, the asRNA expression was able to be induced by vector expressed RdRP from influenza

A/Brisbane/10/2007 (H3N2) or by infection with A/WS/33 (H1N1), to a limited degree. Additionally, the asRNA inserted into the inducible asRNA expression vector can be easily manipulated to keep up with the ever changing influenza virus genome (Drake, 1993).

The use of the inducible anti-influenza therapy is limited by the anti-sense RNA designated to be incorporated into the therapy, as the asRNA, whether in combination or as a single asRNA, should effectively target a region of the influenza genome that is conserved among all strains and subtypes of influenzas A, B and C. More importantly, the asRNA should not target the PB1, PB2, PA, or NP genes as these proteins form the RdRP and are essential for the induction of asRNA expression.

There is a large concern for the emergence of a pandemic strain of influenza due to the ability of the virus to undergo antigenic drift and shift. Antigenic shift is a process in which subtypes of influenza virus from a human and avian undergo genetic reassortment within an intermediate mammalian reservoir, such as swine (Castrucci et al., 1993; Hinshaw et al., 1981; Kida et al., 1994; Scholtissek et al., 1985). As a result of antigenic shift an antigenically novel subtype of influenza emerges that can cause a pandemic. This molecular-based therapy can be utilized to develop genetically modified swine that can inherently express asRNA after influenza infection which will attenuate influenza titers, thus protecting swine from influenza infection and preventing the emergence of a pandemic strain of influenza. Recently, Whitworth et al. (2016) demonstrated the feasibility of generating gene-edited pigs that are no longer susceptible to porcine reproductive and respiratory syndrome (PRRS) due to the depletion of the PRRS virus receptor, CD163. Prevention of human diseases isn't limited to the production of genetically modified swine, as a genetically engineered dairy cow was created to express 96% less β-lactoglobulin in milk in order to accommodate the lactose-intolerant population (Jabed et al., 2012).

In conclusion, we have generated an inducible anti-influenza therapy that is induced by various strains and subtypes of influenza viruses. Alterations to the promoter regions of the anti-influenza therapy should be employed in order to generate more potent expression of asRNAs that can cause RNAi. Further studies using the enhanced versions of the inducible anti-influenza therapy *in vitro* and *in vivo* should be performed in order to further characterize the function and applicability of this therapy. We also recommend that influenza viruses collected from clinical isolates should be used for the screening of new antiviral therapies instead of adapted strains such as influenza A/WS/33 (H1N1), due to altered transcriptional activities and the possibility of false positive results. Although the current version of the inducible anti-influenza therapy needs to be improved, the process of utilizing the conserved promoter of a virus in order to generate an inducible vector that limits anti-sense RNA expression to infected cells should continue to be investigated for the treatment of influenza viruses and other negative-sense RNA viruses.

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**4.6 Figures and figure legends**
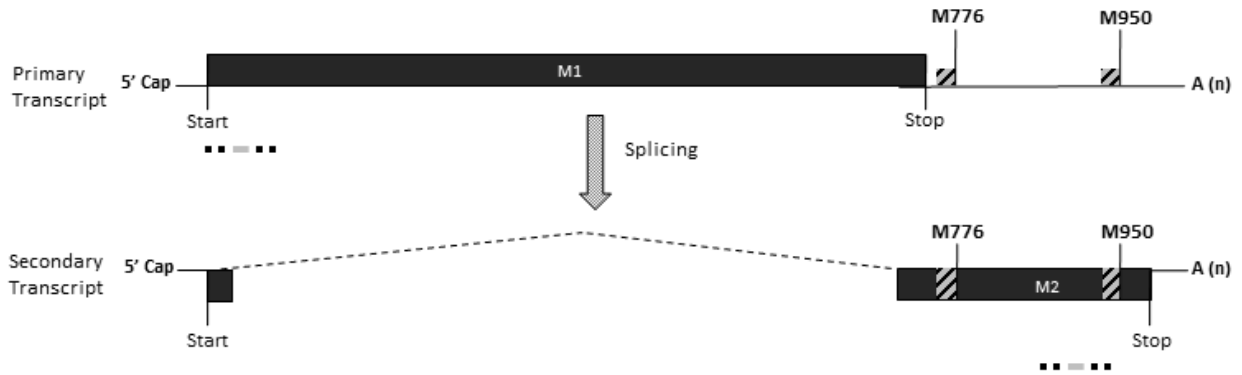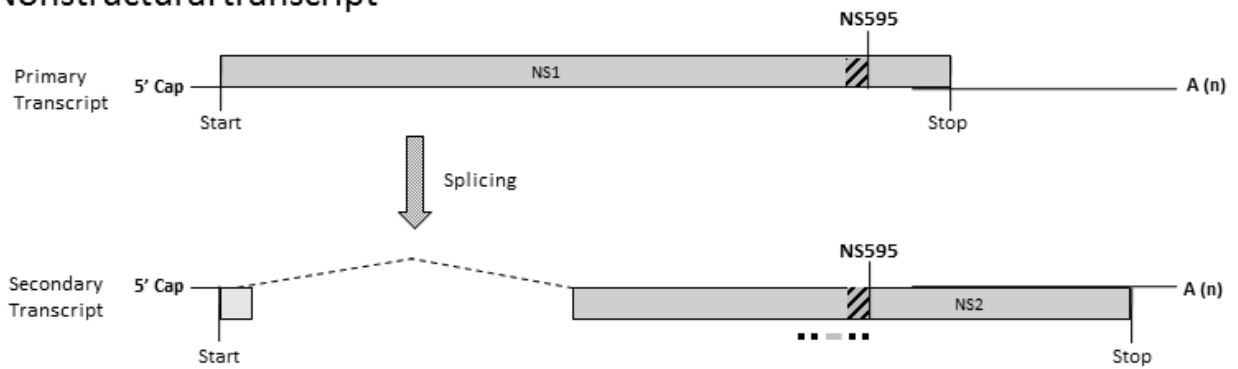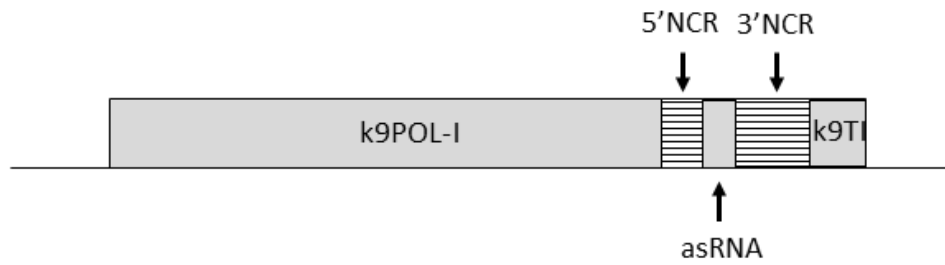
**Figure 4.1: Diagram of target sequences for matrix and nonstructural targeting asRNAs & model of the inducible-asRNA expression vector.** A) M776- and M950-asRNAs bind to their complementary locations on the primary and secondary transcripts of the matrix proteins (binding location indicated by grey and black hashed blocks). M776- and M950-asRNAs bind to the matrix protein 2 (M2) coding sequence (dark grey solid block). The primary transcript encodes for the matrix protein 1 whereas the secondary transcript, generated after post-transcriptional splicing (grey and white hashed arrow), encodes for the matrix protein 2. NS595-asRNAs bind to the primary and secondary transcripts of the nonstructural proteins, including the nonstructural protein 1 (NS1) (light grey solid block) and nonstructural protein 2 (NS2) (light grey solid block) coding sequences. The primary transcript encodes for the nonstructural protein 1 whereas the secondary transcript, generated after post-transcriptional splicing (grey and white hashed arrow), encodes for the nonstructural protein 2. Start and stop codons are indicated by "start" and "stop" labels, respectively. Representative qPCR primer (black hashed line) and probe (grey solid line) binding regions are shown under each transcript (matrix 1 (M1), matrix 2 (M2), nonstructural 2 (NS2)).  B) Each inducible asRNA anti-influenza therapy encoding for the various asRNA (M950-, M776, or NS595-asRNA) contain the following characteristics: the asRNA coding sequence is flanked by the 5' and 3' noncoding regions (NCR; indicated by horizontal black stripes with white background) from the nucleoprotein gene of influenza A/WSN/33 (H1N1; influenza conserved promoter), and the sequences of the canine polymerase-I promoter (k9POL-I) and the canine polymerase-I termination (k9TI) sequences were included upstream of the 5' NCR or downstream of the 3' NCR, respectively. The inducible vector consists of the pCAT3-basic vector (Promega) backbone.

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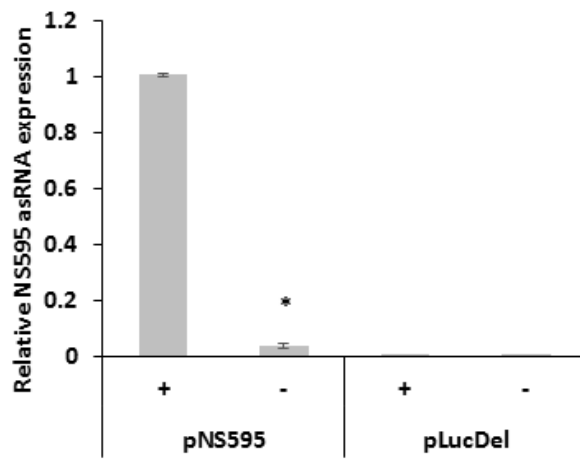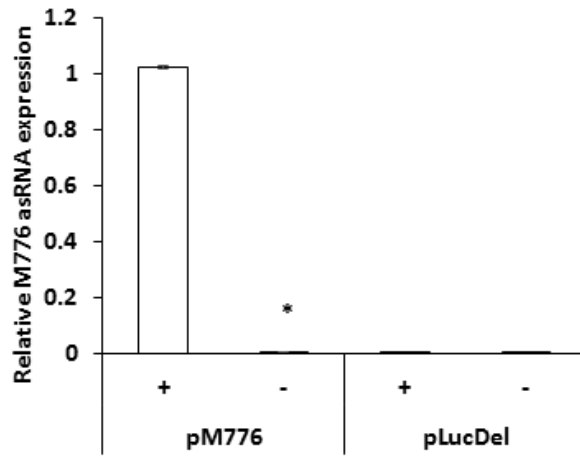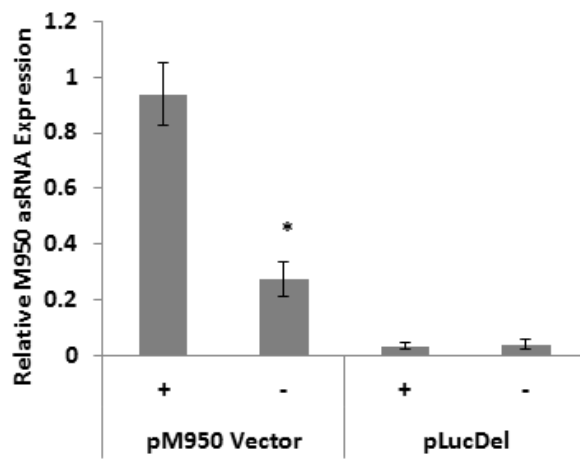
**Figure 4.2: Viral RNA-dependent RNA polymerase induces the expression of asRNAs.** MDCK cells were transfected with 1.6 μg of M950-, M776- or NS595-asRNA expressing vector or control vector, pLucDel, and co-transfected with the four vectors that express the proteins that form the viral RNA-dependent RNA polymerase (RDRP) for 24 h. asRNA expression, relative to cells treated with an asRNA expression vector and co-transfected with the RdRP, was determined by qPCR from culture lysates and normalized to GAPDH. (N=3)
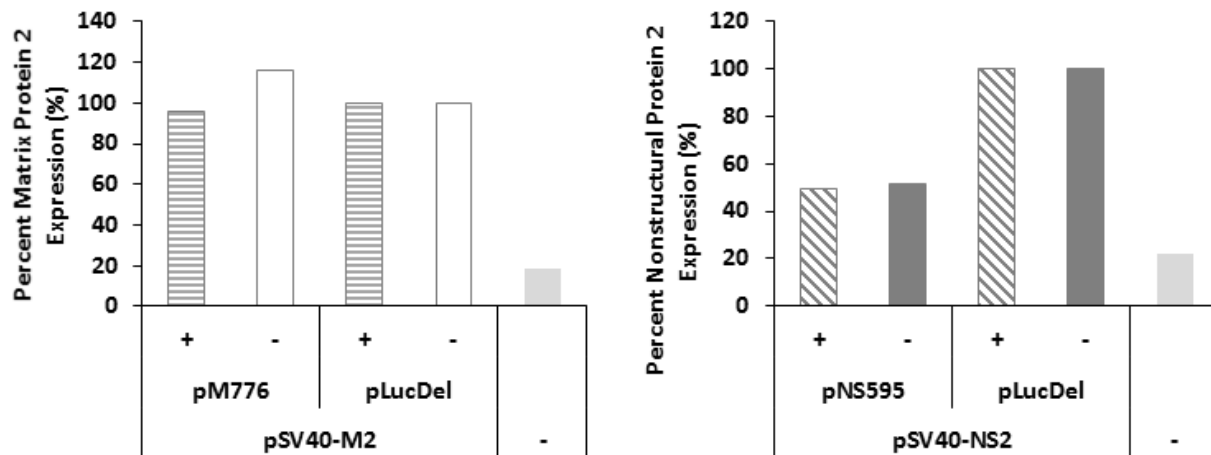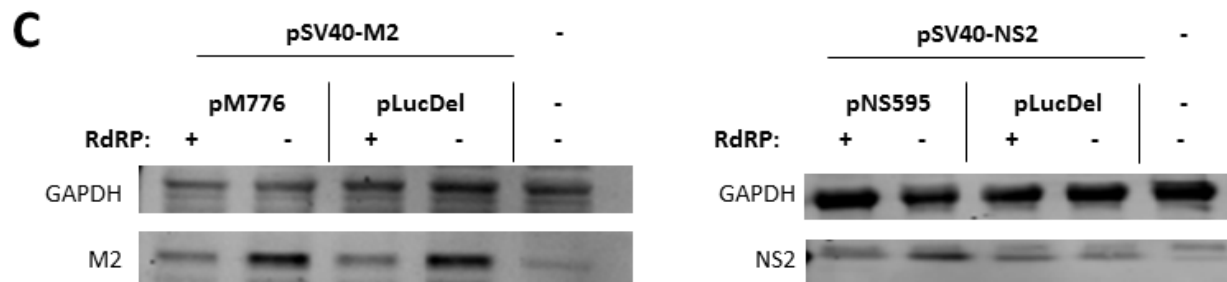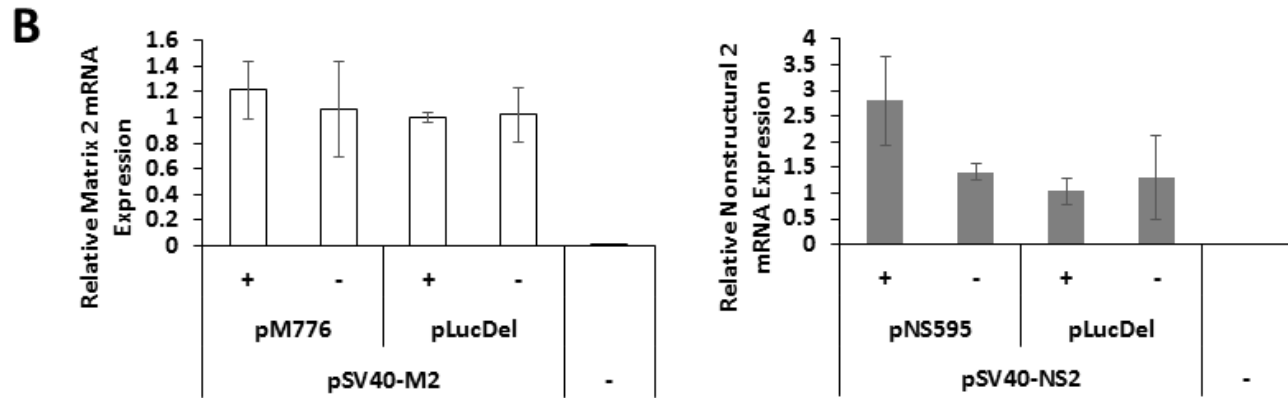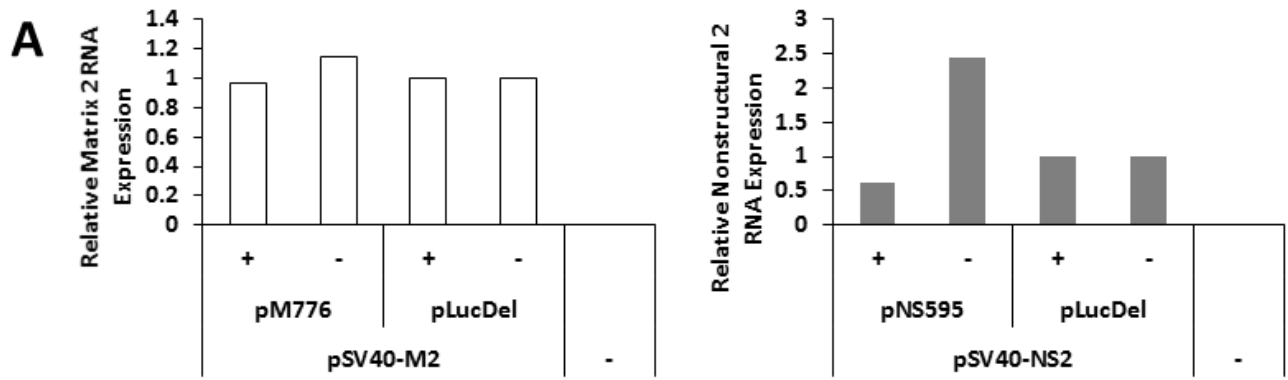
**Figure 4.3: asRNAs expressed by RDRP induced asRNA expression vectors do not knockdown the expression of influenza proteins.** MDCK cells were co-treated with pM776 or pNS595 and pSV40-M2 or pSV40-NS2, respectively, in the presence (+) or absence (-) of RdRP expression vectors for 24 h. Cell lysates were analyzed to detect A) total matrix 2 or nonstructural 2 RNA or B) mRNA by qPCR. Relative expression was normalized to GAPDH and compared to pLucDel treated cells. Protein expression was determined by C) Western blot of culture lysates and percent matrix protein 2 or nonstructural protein expression was determined by densitometry. Normalized to GAPDH. (N=1)
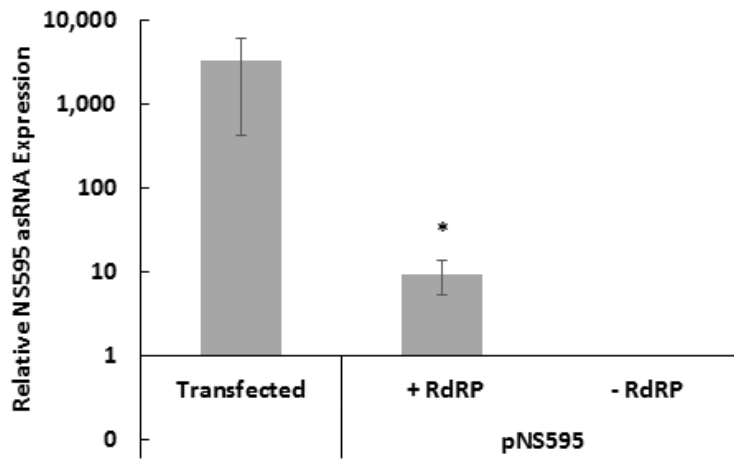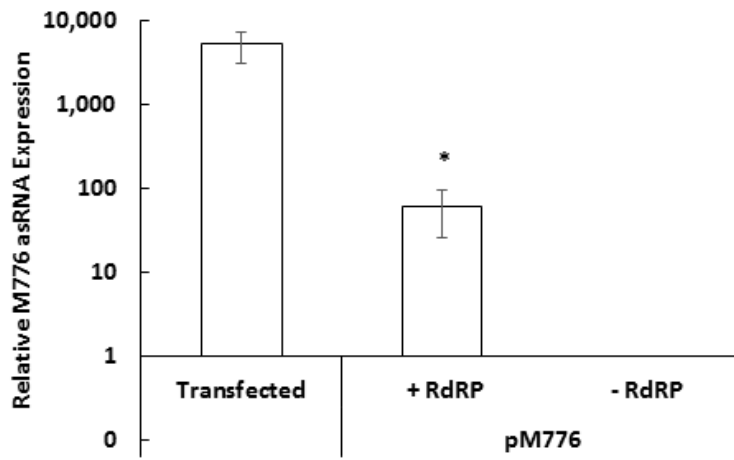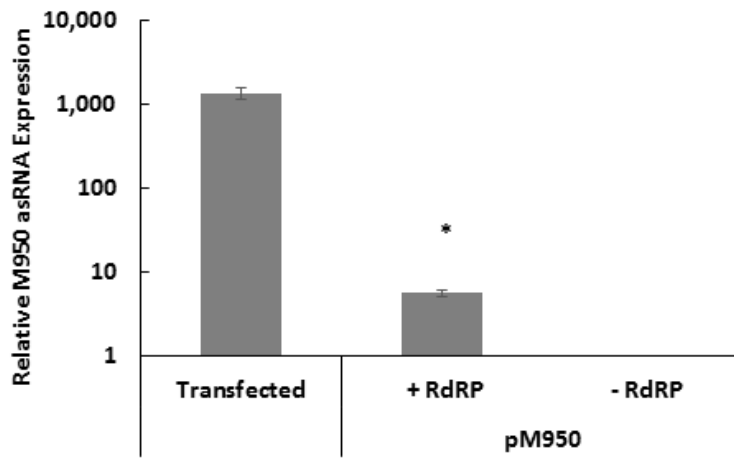
**Figure 4.4: asRNAs expressed by RDRP induced asRNA expression vectors is much lower than what is needed to mediate RNAi.** MDCK cells were transfected with 200pmol M950-, M776-, or NS595-siRNA or co-transfected with pM950, pM776 or pNS595, respectively, in the presence (+) or absence (-) of RdRP expression vectors for 24 h. Cell lysates were analyzed to detect asRNA expression. Relative expression was normalized to GAPDH. M950-, M776-, or NS595-siRNA [200 pmol] not transfected into MDCK cells were analyzed as a positive control. (N=2)
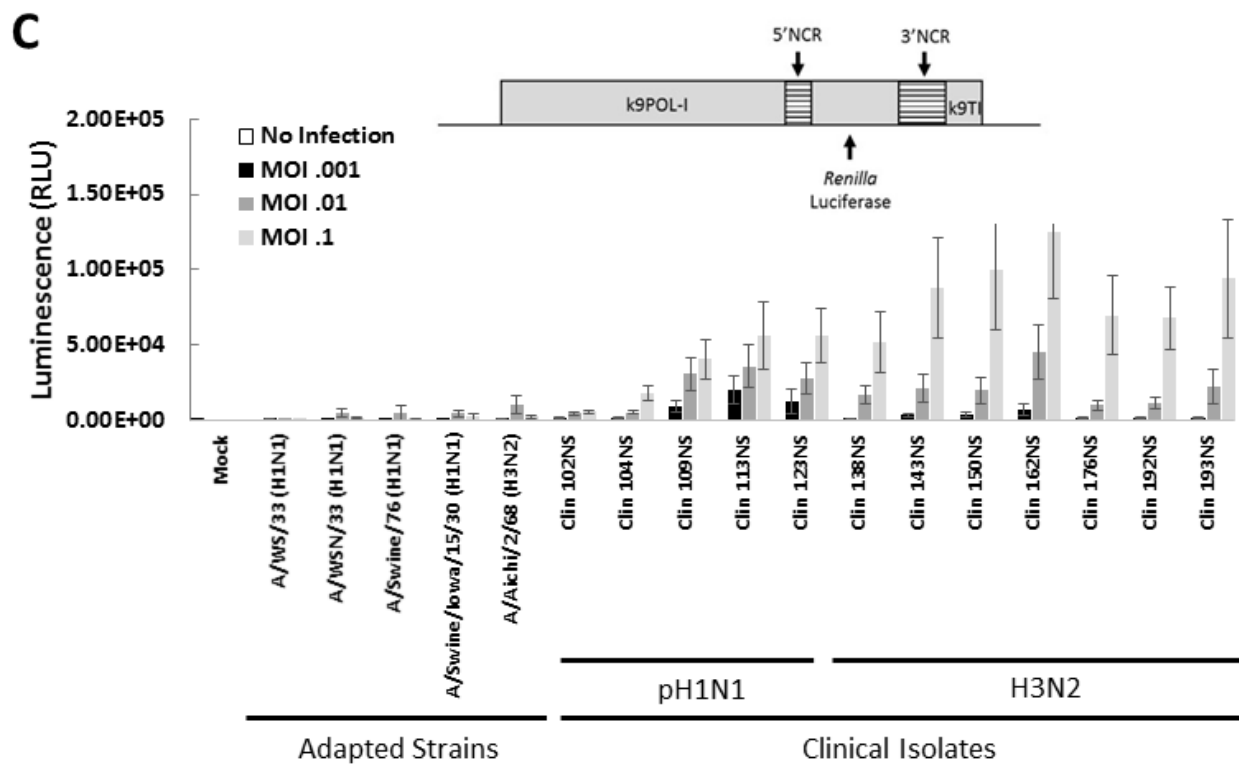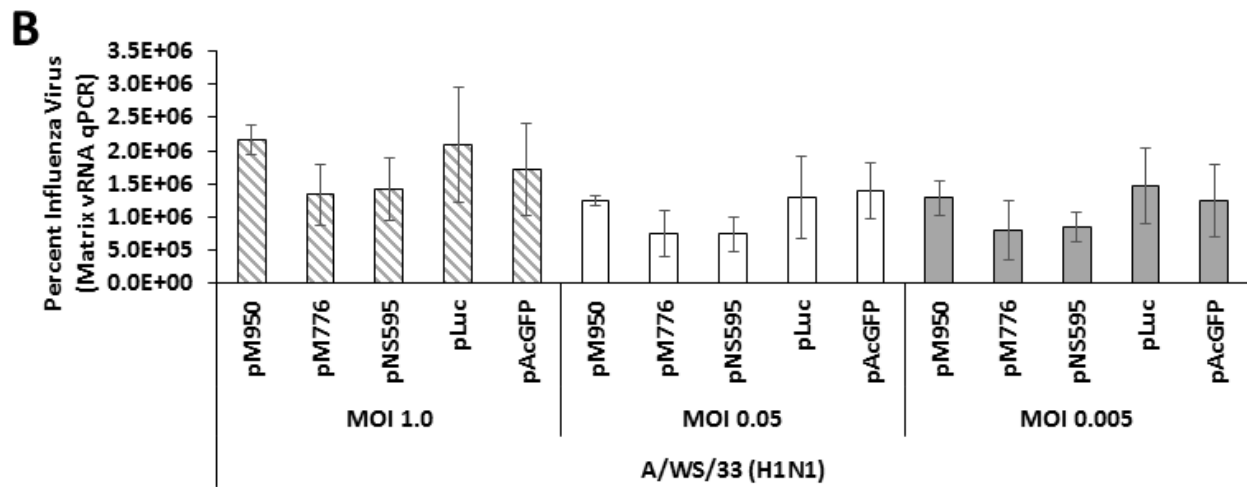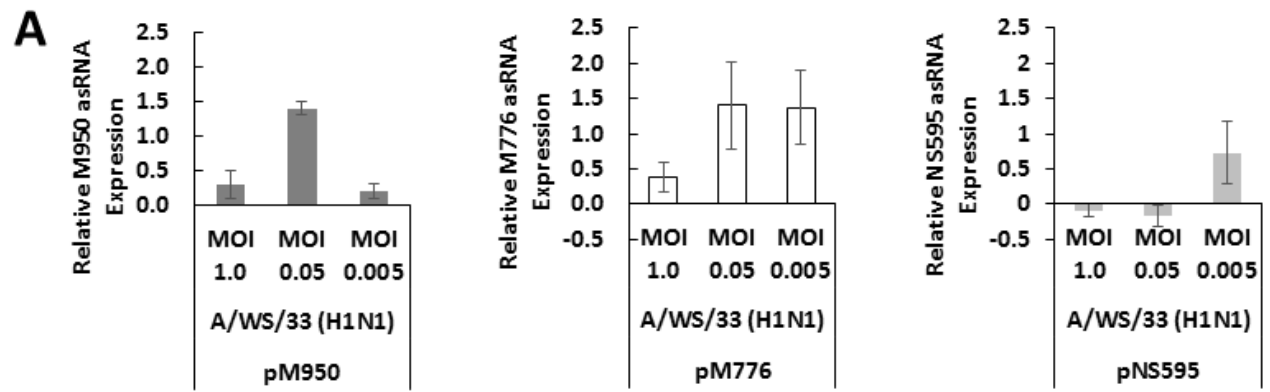
**Figure 4.5: A/WS/33 (H1N1) infection induces low levels of asRNA that are not able to reduce influenza replication and clinical influenza isolates have a higher affinity for the influenza conserved promoter than adapted influenza strains.** MDCK cells were treated with pM960, pM776, pNS595, or p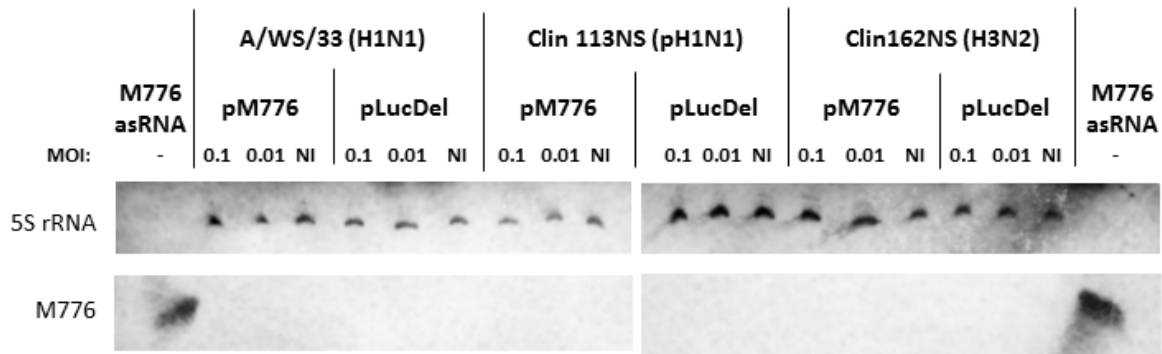LucDel control for 24 h then infected with influenza A/WS/33 (H1N1) for 24 h at an MOI of 1.0, 0.05, or 0.005. asRNA expression, relative to cells treated pLucDel at the corresponding MOI, was determined by qPCR from culture lysates and normalized to GAPDH. B) Total matrix vRNA in the culture supernatant was determined by matrix 1 qPCR. A Renilla luciferase reporter assay was used to study the affinity of adapted influenza viruses or clinical influenza isolates to the influenza conserved promoter. The pk9POLI-Rluc vector contains the following characteristics: the renilla luciferase coding sequence is flanked by the 5' and 3' noncoding regions (NCR; indicated by horizontal black stripes with white background) from the nucleoprotein gene of influenza A/WSN/33 (H1N1; influenza conserved promoter), and the sequences of the canine polymerase-I promoter (k9POL-I) and the canine polymerase-I termination (k9TI) sequences were included upstream of the 5' NCR or downstream of the 3' NCR, respectively. The luciferase reporter vector consists of the pCAT3-basic vector (Promega) backbone. MDCK cells were transfected with the canine RNA polymerase I (POL-I) promoter reporter plasmid, pk9POLI-RLuc vector, for 24 h then infected with adapted or clinical influenza virus for 24 h. C) Cell culture lysates were evaluated for relative luminescence units (RLU) by dual-luciferase assay. Mock infected cells were evaluated for a negative control. (N=3)
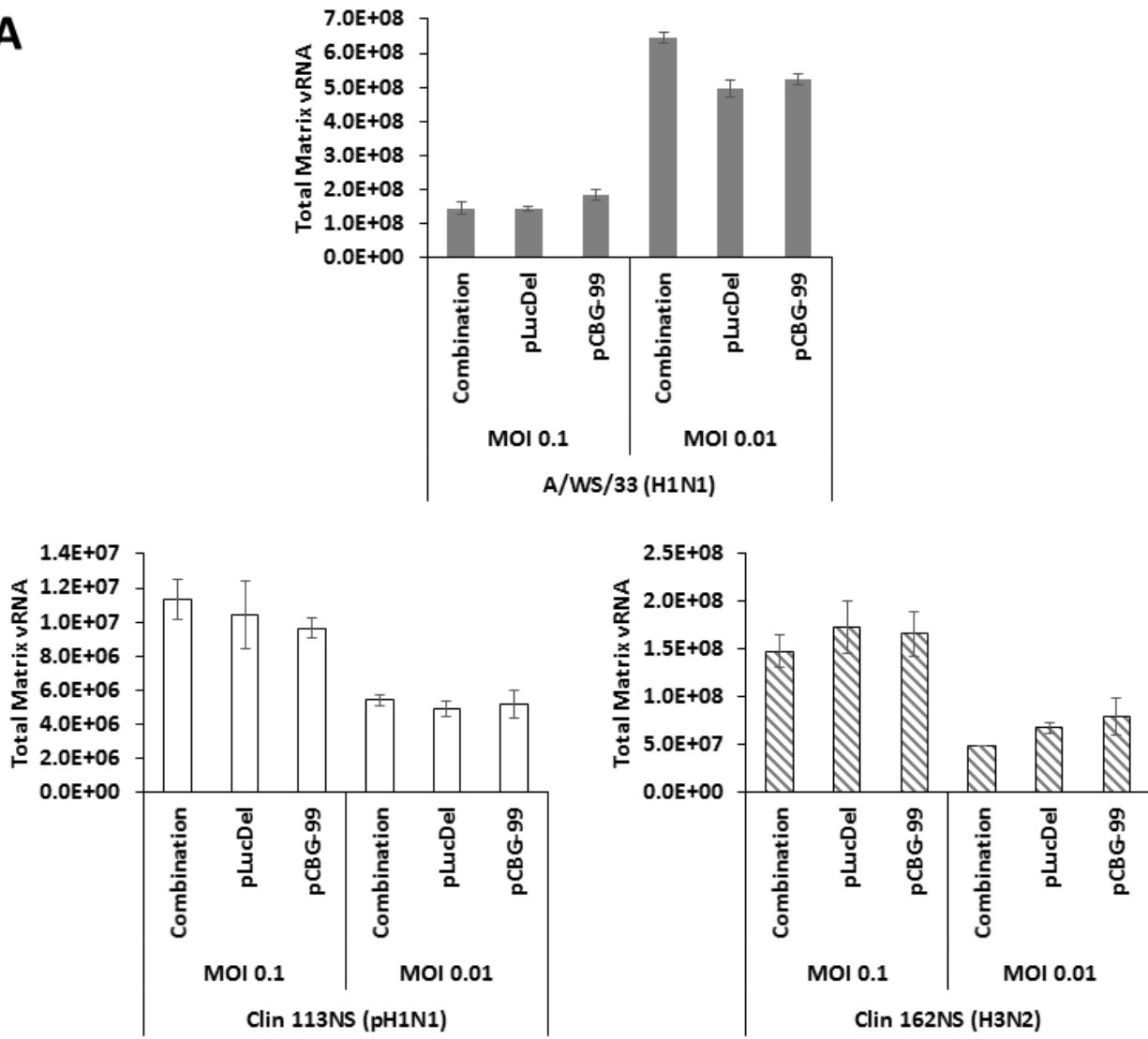
**Figure 4.6: Combination anti-influenza therapy does not reduce influenza virus in culture supernatant.**

MDCK cells were transfected with a combination of pM950, pM776, pNS595, or NA105, or controls pLucDel or pCBG-99 basic vector for 24 h then infected with influenza virus for 24 h at an MOI of 0.1 or 0.01. A) Total influenza virus in the culture supernatant was determined by matrix 1 qPCR. MDCK cells were transfected with pM776 or pLucDel for 24 h then infected with influenza virus for 24 h at an MOI of 0.1, 0.01. B) M776 asRNA expression was evaluated by northern blot. Cells not infected (NI) with influenza virus served as a negative control. Canine 5S rRNA was detected as a loading control. M776-asRNA [20 pmol] served as a positive control. (N=1)

# Chapter 5 – Inhibition of influenza A virus neuraminidase gene expression using RNAi

## 5.1 Abstract

As the emergence of pandemic strains of influenza continues to be a threat and strains resistant to multiple classes of antiviral therapeutics arise, alternative methods of therapeutic intervention should be evaluated. We have studied the use of RNA interference (RNAi), a biological pathway that regulates gene expression, as an alternative approach for the development of an anti-influenza therapeutic. A new siRNA, NA105, which targets the neuraminidase gene, was evaluated for its ability to attenuate influenza A/WS/33 (H1N1) infection by RNAi. RNAi by direct degradation of neuraminidase RNA did not occur after treatment of influenza-infected MDCK cells with NA105-siRNA or negative control siRNA. However, RNAi by translational repression occurred after NA105-siRNA treatment; neuraminidase protein expression was reduced by 62% when infected at an MOI of 0.05 and 95.5% when infected at an MOI of 0.005 compared to negative control siRNA treated MDCK cells. The total influenza virus in the culture supernatant was unaltered, but treatment with NA105-siRNA resulted in the attenuation of infectious influenza virus. NA105-siRNA treated cells had 33.0% less infectious virus at an MOI of 0.05 and 56.2% less at an MOI of 0.005. In order to limit anti-sense RNA (asRNA) expression to cells infected with influenza viruses, we developed a NA105-asRNA anti-influenza therapy, pNA105, which expresses NA105-asRNA after recognition of the influenza conserved promoter by influenza RNA-dependent RNA polymerases. These results demonstrate that a siRNA targeting neuraminidase protein expression can effectively attenuate influenza infection and suggests a potential use for this siRNA or asRNA as an anti-influenza therapeutic.

## 5.2 Introduction

Each year, particularly during the winter season, influenza epidemics account for a high rate of morbidity and mortality (WHO, 2014). Vaccines against influenza viruses are vital for the prevention and eradiation of disease. However, antiviral therapies can also play a considerable role in easing the burden of disease.

Neuraminidase inhibitors (oseltamivir and zanamivir) inhibit viral replication by preventing the release of progeny viruses. By binding to the active site of neuraminidases, neuraminidase inhibitors prevent the protein from removing sialic acid residues from sialic acid receptors (Gottschalk, 1957). When removal of the sialic acid residues from the host cell surface or the surface of progeny viruses is blocked, the viruses are unable to be released from the cell, or they form large aggregates due to the binding of the hemagglutinin glycoprotein to sialic acid receptors on neighboring influenza viruses (Palese et al., 1974). Early administration of oseltamivir, within 12 hours of symptom onset, can result in average reduced duration of symptoms of three days (Aoki et al., 2003), and treatment within 36-48 hours of symptom onset reduces the duration of symptoms by one day (Nicholson et al., 2000; Treanor et al., 2000). Treatment of influenza A or B positive patients with zanamivir within 48 hours of symptom onset reduced the time to alleviate symptoms by 2.5 days and reduced the incidence of complications that required antibiotic intervention by 43% compared to patients who received placebo treatment (Lalezari et al., 2001).

Adamantanes (amantadine and rimantadine) are a class of influenza antiviral therapy that are matrix 2 (M2) ion channel inhibitors. They work by entering the ion channels of the matrix 2 protein and blocking the translocation of hydrogen ions into the virions. By blocking hydrogen translocation, the interior of the virions doesn't become acidic and the virus is unable to release the ribonucleoprotein into

the cytoplasm for subsequent replication (Wang et al., 1993). Adamantanes are restricted to targeting only influenza A viruses, as the matrix 2 protein of influenza B viruses has a different structure that can't be blocked by adamantanes (Nicholson and Wiselka, 1991). Clinical trials testing the efficacy of amantadine treatment (200 mg/day) within 24 hours of symptom onset showed that treatment reduced the duration of a fever by one day (Wingfield et al., 1969). Treatment of influenza A H3N2 patients with rimantadine obtained similar results (Hayden and Monto, 1986). Unfortunately, adamantanes are no longer recommended for treatment of influenza infection due to the emergence of resistant influenza strains (Deyde et al., 2007).

Until the discovery of a universal influenza vaccine, the development of new and improved antiviral therapies should continue to be pursued. These new antiviral therapies need to overcome the limitations associated with current therapies, such as the inability of adamantanes to target influenza B viruses, the emerging resistance to both classes of antiviral therapies, and the inability to reduce symptom duration by more than two days. Here we evaluate the use of RNAi for antiviral intervention. RNAi is a biological process in which small RNA molecules silence gene expression (Fire et al., 1998). Thus far, only a handful of siRNAs have been characterized that effectively attenuate influenza infection (Ge et al., 2003; Hui et al., 2004; McMillen et al., 2016; Sui et al., 2009; Zhou et al., 2007). For the present study we have designed a siRNA molecule that targets the neuraminidase transcript and evaluated its ability to knockdown protein expression and attenuate influenza infection. To further expand the practical use of RNAi for an anti-viral therapy, an inducible asRNA anti-influenza therapy has been designed to express asRNA only after influenza infection.

## 5.3 Materials and methods

*Cell lines and viruses*

MDCK cells and influenza A/WS/33 (H1N1) was maintained as described in McMillen et al. (2016).

*Small interfering RNAs (siRNAs)*

A siRNA against the neuraminidase transcript was designed by the BLOCK-iT™ RNAi Designer (ThermoFisher Scientific). Purified Stealth NA105 siRNAs were synthesized by Life Technologies (ThermoFisher Scientific), suspended in nuclease-free water and stored at -80ºC (NA105 sense: 5' CCATTCAATTCAAACCGGAAATCAA 3', NA105 anti-sense: 5' TTGATTTCCGGTTTGAATTGAATGG 3'). Stealth RNAi siRNA Negative Control, Medium GC #2 (ThermoFisher Scientific) was used as a negative control siRNA. Stealth RNAi siRNA duplexes use proprietary modifications in order to reduce off-target effects and activation of the protein kinase R/interferon response pathways (ThermoFisher Scientific). The NA105-siRNA was characterized at a concentration of 100 nM.

M950-siRNA was synthesized by Integrated DNA Technologies, suspended in nuclease-free water and stored at -80°C (M950 sense: 5' ACAGCAGAAUGCUGUGGAUUU 3', M950 anti-sense: 5' AUCCACAGCAUUCUGCUGUUU 3'). M950-siRNA [50 nM] was used as positive control for siRNA efficiency experiments. This concentration was selected based on experiments performed by Sui et al. (2009).

*siRNA knockdown studies*

To test the efficiency of various siRNAs knocking down influenza virus transcripts *in vitro*, we used the protocol explained in McMillen et al. (2016) (refer to Chapter 3)

*Anti-influenza therapy constructs*

The pLucDel negative control vector was constructed by removing the *Renilla* luciferase gene in the canine RNA polymerase I (POL-I) promoter reporter plasmid, pk9POLI-RLuc vector, obtained from Dr. Ruben Donis (Influenza Division/NCIRD, Centers for Disease Control and Prevention, Atlanta, GA) (Hossain et al., 2010). The *Renilla* luciferase gene was deleted by site-directed mutagenesis using the QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies). Site directed mutagenesis was performed following the manufacturers protocol. To create the pK9POLI-NA105 (pNA105) inducible anti-influenza vector, the anti-sense sequence for the corresponding siRNA was inserted between the 3' and 5' noncoding regions of the influenza nucleoprotein segment of influenza virus A/WSN/33 (Luytjes et al., 1989; Neumann and Hobom, 1995) by site-directed mutagenesis of the pLucDel negative control vector. The QuikChange Primer Design Program (Agilent Technologies) was used to designed site-directed mutagenesis primers. Site-directed mutagenesis primers were synthesized by Integrated DNA Technologies.

*NA105-siRNA knockdown studies*

To examine the ability of NA105-siRNA to knock down the expression of influenza neuraminidase transcripts *in vitro*, we used a protocol similar to that used in previous studies, with a few modifications (Ge et al., 2003; Sui et al., 2009). Briefly, MDCK cells (1 x 10$^6$ cells/well) were plated on a 6-well plate (Corning, Inc.) in complete growth media and incubated at 35°C in a humidified 5% $CO_2$ incubator. Twenty-four hours later, cells were washed twice with phosphate-buffered saline (PBS) and 1.5 mL of OPTI-MEM I Reduced Serum Medium (Gibco) was added per well. Transfection was performed following the Lipofectamine 2000 Reagent protocol (ThermoFisher Scientific) as described in McMillen et al. (2016) and in Chapter 3. The culture supernatant and cell lysates were collected for analysis 24 h after infection.

An MOI of 0.05 or 0.005 was selected to ensure reduced cytopathic effects after 24 h of infection (data not shown). Each treatment was performed in duplicate. The culture supernatant and cell lysates were stored at -80°C until processing.

*RdRP induced NA105-asRNA expression via anti-influenza therapy*

To examine the ability of the influenza virus polymerase to induce the expression of NA105-asRNA, MDCK cells (5 x 10$^5$ cells/well) were seeded on a 24-well plate (Corning) in complete growth media. Twenty-four hours later, transfection was performed following the Lipofectamine 2000 Reagent protocol (ThermoFisher Scientific). The MDCK cells were washed twice with PBS, and then overlaid with 0.5 mL of OPTI-MEM I Reduced Serum Medium (Gibco, ThermoFisher Scientific) per well. Lipofectamine reagent was prepared to a concentration of 8 µg/mL in 50 µL of OPTI-MEM I Reduced Serum Medium, mixed gently, and then incubated at room temperature for 5 min. Simultaneously, 0.16 µg of the pNA105 or pLucDel negative control was added to 50 µL of OPTI-MEM I Reduced Serum Medium. Cells were either transfected with pNA105 or pLucDel alone or co-transfected with four plasmids (0.16 µg each) that express the influenza RdRP (pCI-A-Brisbane-10-2007 (H3N2) NP, pCI-A-Brisbane-10-2007 (H3N2) PA, pCI-A-Brisbane-10-2007 (H3N2) PB1, pCI-A-Brisbane-10-2007 (H3N2) PB2; Hossain et al., 2010). The lipofectamine and antiviral therapy solutions were combined, mixed gently, and incubated for 20 min. Lipofectamine/antiviral therapy solution (100 µL) was added to each corresponding well. Lipofectamine solution (100 µL) without siRNA was used as a control. Four hours after transfection, the cells were washed twice with PBS to remove the lipofectamine reagent and overlaid with complete growth media. Twenty-four hours after transfection, the MDCK cells were lysed using lysis solution and stored at -80° C until further processing for analysis by qPCR. Three independent experiments with two replicates per treatment were performed.

*RNA isolation and cDNA transcription*

Total RNA was isolated from MDCK cells using the MagMax™-96 Total RNA Isolation Kit (Ambion). Upon thawing of the cellular lysate containing the Lysis/Binding Solution Concentrate, 500 µL of 100% isopropanol (Sigma-Aldrich) was added to each sample to complete the Lysis/Binding Solution preparation and samples were processed following the manufacturer's instructions. Total RNA was immediately transcribed into cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) in accordance with the manufacturer's instructions. Viral RNA was isolated from the culture supernatant using the MagMax™-96 Viral RNA Isolation Kit (Ambion) following the manufacturer's instructions.

For asRNA detection, total RNA was isolated from MDCK cells using the miRCURY™ RNA Isolation Kit (Exiqon) following the manufacturer's protocol. NA105-asRNA was immediately reverse-transcribed into cDNA using the Taqman Small RNA Assays asRNA-specific primers (Applied Biosystems, following the manufacturer's instructions. Reverse transcription was performed using the Applied Biosystems 7500 Fast Real-Time PCR System under the following thermal cycling conditions: 16°C for 30 minutes, 42°C for 30 minutes and 85°C for 5 minutes. Total RNA isolated from the miRCURY™ RNA Isolation Kit (Exiqon) was immediately transcribed into cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) in accordance with the manufacturer's instructions.

*Analysis of RNA levels by real-time quantitative PCR (qPCR)*

qPCR analyses were performed using the following primers and probes: matrix 1 (Spackman et al., 2002), forward 5' AGATGAGTCTTCTAACCGAGGTCG3', reverse 5' TGCAAAAACATCTTCAAGTCTCTG 3', and probe 5' TCAGGCCCCCTCAAAGCCGA 3'; neuraminidase (N1), forward 5' AACCCGCGTCCCAAA GAT 3', reverse 5' TTTACTCCGTTTGCTCCATCAG 3', and probe 5' AACAGGCAGCTGTGGC 3'.

The matrix 1 primers and probe were used to detect total influenza virus in the culture supernatant (i.e. infectious and noninfectious virus) by detecting segment 7 of the influenza viral RNA. The neuraminidase (N1) primers and probes were designed using the Primer Express 3.0 software (Applied Biosystems). The probes were designed with a 5' fluorescent dye, 6FAM, and 3' quencher, MGBNFQ. All primers and probes were synthesized by Applied Biosystems and used at a final concentration of 0.8 µM and 0.2 µM, respectively. Reactions were performed and analyzed using the Applied Biosystems 7500 Fast Real-Time PCR System under the following cycling conditions: 95°C for 20 seconds, followed by 40 cycles at 95°C for 3 seconds, and 60°C for 30 seconds. A negative control without template was included in all real-time PCR runs. All samples were run in duplicate. Relative gene expression was determined by the $\Delta\Delta C_T$ method and normalized to GAPDH (Hs03929097_g1, ThermoFisher Scientific). The PCR primers were tested on MDCK cell lysates and analyzed by gel electrophoresis to confirm specificity of GAPDH primers to canine GAPDH (data not shown).

qPCR analyses to detect asRNA were performed using the Taqman Small RNA Assays (Applied Biosystems). Primers for NA105-asRNA-specific reverse transcription and qPCR primers and probes specific for the NA105-asRNA were designed and synthesized by Applied Biosystems. Reactions were performed and analyzed using the Applied Biosystems 7500 Fast Real-Time PCR System under the following thermal cycling conditions: 50°C for 2 minutes, 95°C for 10 minutes, followed by 40 cycles at 95°C for 15 seconds and 60°C for 60 seconds. A negative control without template was included in all real-time PCRs.

*Analysis of infectious viral titers by viral plaque assay (VPA)*

Viral plaque assays were performed following the methods explained in McMillen et al. (2016).

*Analysis of protein levels by Western blot*

Cells were lysed with radioimmunoprecipitation assay (RIPA) buffer containing a protease inhibitor cocktail and EDTA (ThermoFisher Scientific). Protein concentration was determined with the BCA Protein Assay Kit (Pierce) performed in triplicate. Fifty μg of total protein was diluted 1:1 with 2x Laemmli loading dye (Bio-Rad), denatured by boiling for 10 min, and immediately cooled on ice before loading on to a 4% stacking/12% SDS-PAGE mini-gel. The electrophoresed protein was electroblotted onto nitrocellulose membranes (0.2 μm) overnight at a constant 16 V. The blots were blocked in Odyssey Blocking Buffer (LI-COR Biosciences) for 1 h at room temperature and then incubated overnight with primary antibodies for GAPDH (sc-25778, Santa Cruz), M2 (sc-32238, Santa Cruz), and neuraminidase (N2) (40017-T60, Sino Biological, Inc.) diluted in Odyssey Blocking Buffer. After four washes with TBS-tween (0.1% (v/v) Tween-20), the blots were incubated with corresponding infrared (680 or 800 nm) conjugated secondary antibodies (LI-COR Biosciences) for 1 h at room temperature. Blots were washed four times in TBS-tween and then analyzed on the Odyssey Imaging System (LI-COR Biosciences). Primary antibodies directed against GAPDH were used to verify equal sample loading and for normalization.

*Statistical analyses*

A two-way factorial mixed model analysis of variance (ANOVA) was performed on all variables. All pairwise comparisons were considered significant at $p < 0.05$. Asterisks above graphs (*) indicate $p<0.05$. All graphs are representative of three independent experiment (N=3) with two replicates per treatment group per experiment.

## 5.4 Results and discussion

Research studies have only identified a few siRNAs that successfully attenuate influenza infection (Ge et al., 2003; Hui et al., 2004; McMillen et al., 2016; Sui et al., 2009; Zhou et al., 2007). In order to expand the number of siRNA that can be used for therapeutic intervention or in the laboratory setting, we have designed a new siRNA, NA105, that targets the neuraminidase transcript near the 3' end of the protein coding sequence (Figure 5.1A).

The neuraminidase of influenza viruses is a surface-expressed glycoprotein with enzymatic activity that cleaves the α-ketosidic linkage between a terminal sialic acid and a protein bound D-galactose or D-galactosamine residue found on the host cells or influenza viruses (Gottschalk, 1957). This protein is involved in the release of progeny virus from the host cell and prevents the aggregation of influenza viruses by preventing a hemagglutinin-sialic acid complex between viruses (Lentz et al., 1987; Palese et al., 1974). Other studies have implicated a role for neuraminidase in facilitating viral entry into the host cell (Matrosovich et al., 2004) and neuraminidase activity in the late endosome/lysosome has been shown to lead to enhanced viral replication (Suzuki et al., 2005). Although proteins located in the interior of the influenza virion are typically more conserved and are preferred targets for universal anti-influenza therapies (Babar and Zaidi, 2015; Biswas and Nayak, 1994), the fact that the neuraminidase protein is essential for the production of progeny virus and possibly enhances viral replication makes it a worthy candidate for an antiviral therapy target. Therefore, knocking down the expression of NA with NA105-siRNA is hypothesized to attenuate viral replication.

*NA105 siRNA does not reduce cellular neuraminidase transcript levels but does reduce neuraminidase protein expression*

To test whether NA105-siRNA can knockdown the expression of neuraminidase by RNA interference, MDCK cells were treated with NA105-siRNA, negative control siRNA, or M950 positive control siRNA, and then infected with influenza A/WS/33 (H1N1) at an MOI of 0.05 or 0.005 for 24 hrs. Although the M950-siRNA treated cells had a significant reduction in M1 RNA expression for both MOIs tested, cells treated with M950-siRNA did not affect the relative expression of influenza neuraminidase protein (Figure 5.2A).

RNA interference can inhibit the expression of proteins by either mRNA degradation or translational repression. mRNA degradation occurs when an RNA-induced silencing complex (RISC) containing a 100% complementary strand of RNA specifically recognizes a mRNA and the endonuclease argonaute cleaves the mRNA in two (Meister et al., 2004; Wang et al., 2008). Once the mRNA is cleaved by the endonuclease, the mRNA is susceptible to exonuclease degradation, thus preventing translation and protein expression (Orban and Izaurralde, 2005; Souret et al., 2004). The mechanism in which translation is repression by RNAi is less understood. Translational repression typically occurs when a RISC containing a strand of RNA that has imperfect complementary to the mRNA binds to the mRNA and, without directly degrading the mRNA, prevents the initiation of protein translation. The exact mechanisms in which translational repression occur isn't completely understood yet. It has been speculated that the RISC interferes with translational initiation altering activity of the cap-binding complex, eukaryotic initiation factor 4F (eIF4F) (Mathonnet et al., 2007) or the RISC inhibits actively translating polyribosomes, thus preventing polypeptide elongation (Nottrott et al., 2006). qPCR data suggested that RNAi did not occur by argonaute-mediated cleavage of the neuraminidase mRNA.

Therefore, in order to identify whether NA105-siRNA can cause RNAi by translational repression, a western blot was performed on the culture lysates. NA105-siRNA expression significantly reduced neuraminidase expression by 62.0% (p = 0.033) when infected with an MOI 0.05 and 95.5% (p < 0.0001) when infected at an MOI of 0.005 when compared to negative control treated MDCK cells (Figure 5.2B & C). These results indicate that NA105-siRNA can facilitate RNAi by translational repression, not mRNA degradation. The NA105-siRNA was designed by using the nucleoside sequence of the A/WS/33 (H1N1) neuraminidase gene provided by the National Center for Biotechnology Information (NCBI; GenBank: CY009606.1). Although the NA105-siRNA was designed with 100% complementary to A/WS/33 (H1N1) it is possible that through serial propagation in MDCK cells, that the A/WS/33 (H1N1) strain used in our laboratory has obtained mutations in the neuraminidase gene. A mismatch between the NA105-siRNA and the neuraminidase gene might explain why translational repression occurred and not direct mRNA degradation.

*NA105 siRNA attenuates virus infection*

Because the neuraminidase protein is essential for the release of progeny virus from the infected cell and the spread of the virus to surrounding cells (Lentz et al., 1987; Palese et al., 1974), we examined whether NA105-siRNA treatment can attenuate viral titer. The supernatant from siRNA treated cells infected with A/WS/33 (H1N1) and at MOI of 0.05 or 0.005 was collected and examined by qPCR and VPA for total and infectious virus, respectively. NA105-siRNA treatment of cells infected with an MOI of 0.05 did not alter the amount of total influenza virus (infectious and non-infectious virus) present in the culture supernatant compared to cells treated with the negative control siRNA (Figure 5.3A). Similarly, total influenza virus was unchanged in MDCK cells infected at an MOI of 0.005 (Figure 5.3A). Nevertheless, similar to previous studies (McMillen et al., 2016) M950-siRNA treatment resulted in a

significant decrease in total influenza virus at an MOI of 0.005 (Figure 5.3A). Although NA105-siRNA treatment did not affect the amount of total influenza virus in the culture supernatant, it did result in a significant decrease in total and percent infectious virus in the culture supernatant, compared to negative control siRNA treated cells (Figure 5.3B). NA105-siRNA treated cells had 33.0% less infectious virus at an MOI of 0.05 and 56.2% (p = 0.016) less infectious virus at an MOI of 0.005. Therefore, NA105-siRNA can facilitate RNAi and attenuate infectious viral titer.

*Viral RNA-dependent RNA polymerase induces the expression of NA105-asRNA*

The use of small RNA molecules to mediate RNAi can be an effective tool in the laboratory setting. However their use in the clinical setting is limited and can result in adverse effects. For instance, a study by Jackson et al. (2003) revealed that the seed region of siRNAs, a contiguous sequence of up to 11 nucleotides, can cause nonspecific gene silencing even if the rest of the siRNA is not specific for that particular transcript. Systemic delivery of siRNA would be an easy route to administer a therapeutic, but this form of delivery makes the siRNA or asRNA susceptible to nuclease cleavage (Layzer et al., 2004) or premature clearance by the kidney (van de Water et al., 2006). RNAi in dividing mammalian cells typically only last for 5-7 days (Chiu and Rana, 2002), whereas the effects of siRNA in non-dividing cells can last up to 3 weeks *in vivo* and *in vitro* (Bartlett and Davis, 2007). In order to effectively utilize siRNA for therapeutic intervention, the siRNA must be delivered by a system that can continuously express influenza-targeting asRNAs at the site of infection. By doing so, this form of delivery may prevent the clearance of the siRNA before it reaches the target organ. Moreover, the adverse effects associated with off-target gene silencing will be limited to cells that are already distressed.

To begin testing this hypothesis, we generated an inducible asRNA anti-influenza therapies that restricts asRNA expression to cells infected with influenza viruses. The pNA105 asRNA expression vector

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contains the following characteristics (Figure 5.1B): the anti-sense RNA coding region of NA105-siRNA (indicated by the black block) is flanked by the noncoding regions (NCR) (indicated by the white blocks with the black horizontal stripes) from the nucleoprotein gene of A/WSN/33 (Hossain et al., 2010). The canine polymerase-I promoter (k9POL-I) and the canine polymerase-I termination (k9TI) sequences (indicated by the grey blocks) were fused upstream of the 5' NCR or downstream of the 3' NCR, respectively. Expression of an RNA intermediate that mimics influenza viruses genetic RNA (vRNA) is under control of the k9POL-I promoter. The RNA intermediate mimics the vRNA because it contains the conserved promoter. However, instead of the RNA intermediate containing the coding sequence for an influenza protein, it contains the coding region for the NA105-asRNA. The RNA intermediate is transcribed by the canine polymerase I (Murakami et al., 2008), whereas the asRNA is only transcribed after the same cell containing the RNA intermediate is infected with influenza. Only then can the RdRP recognize the conserved influenza promoter and initiate asRNA transcription.

To assess whether the pNA105 asRNA anti-influenza therapy can be induced by influenza RdRPs, MDCK cells were co-transfected with pNA105 asRNA expression vectors and each of four individual expression vectors that encode for the four proteins that form the RdRP (influenza A/Brisbane/10/2007 (H3N2)). MDCK cells co-transfected with pNA105 and the RdRP expression vectors had 204-fold more NA105-asRNA expressed than cells containing the pNA105 inducible asRNA expression vector alone (Figure 5.4), whereas MDCK cells treated with the pLucDel control, with or without the RdRP, had background levels of NA105-asRNA expression. These results indicate that the inducible asRNA anti-influenza vector can be induced by the influenza RdRP.

In conclusion, we characterized a new siRNA, NA105, which can effectively mediate RNAi and attenuate infectious influenza virus titer. Future studies should be performed in order to quantitate how

much asRNA is being expressed by the asRNA anti-influenza therapy after influenza infection. Understanding how much asRNA is being expressed and comparing it to how much siRNA is typically needed to mediate effective RNAi will allow us to understand whether the amount of asRNA expressed by the anti-influenza therapy is sufficient to mediate RNAi. Experiments performed in Chapter 4 of this dissertation indicate that the inducible asRNA anti-influenza therapies should be modified in order to promote higher expression of asRNAs. Verification that the asRNAs are able to facilitate RNAi should be examined once the asRNA anti-influenza therapy has been modified to enhance asRNA that can reach the threshold needed for efficient RNAi. A study by Martinez et al. (2002) showed that asRNA is able to slip into the RISC without going through the same processing that miRNA and siRNA undergo. Identifying the presence of asRNA within the argonaute by immunoprecipitation and asRNA-specific qPCR can further verify the functional capability of these vector expressed asRNAs. These studies will help determine the practical use of this siRNA and the anti-influenza therapy in a clinical setting.

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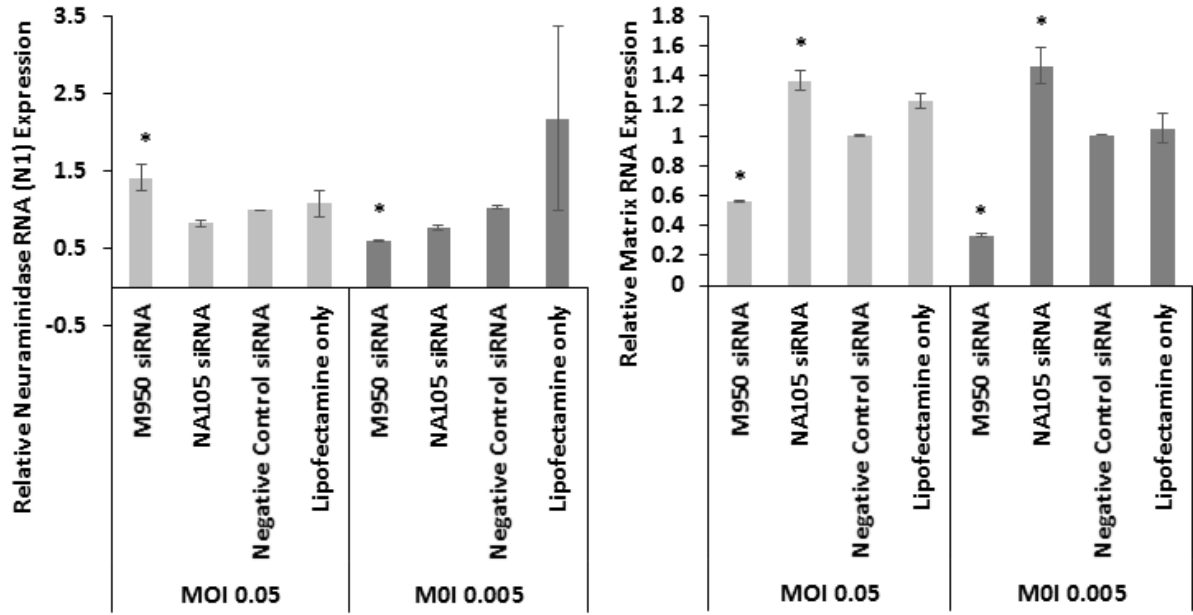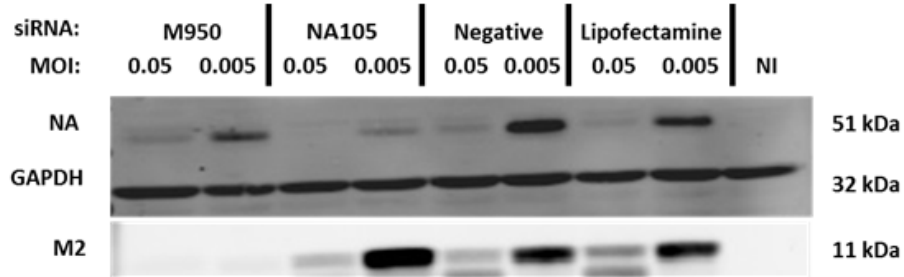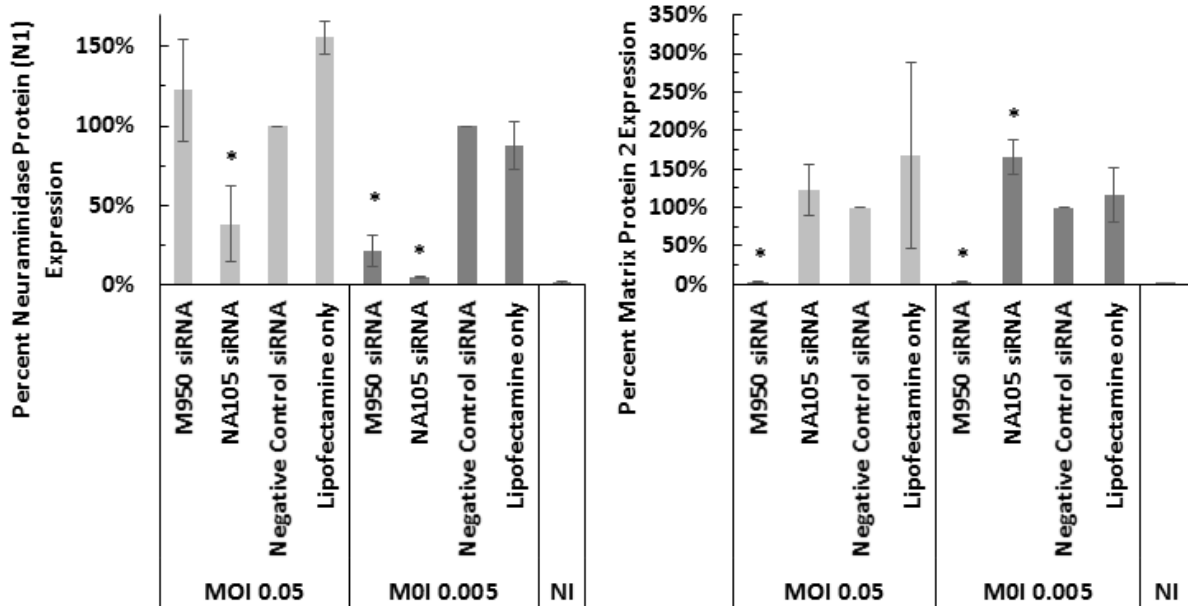
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**5.6 Figures and figure legends**
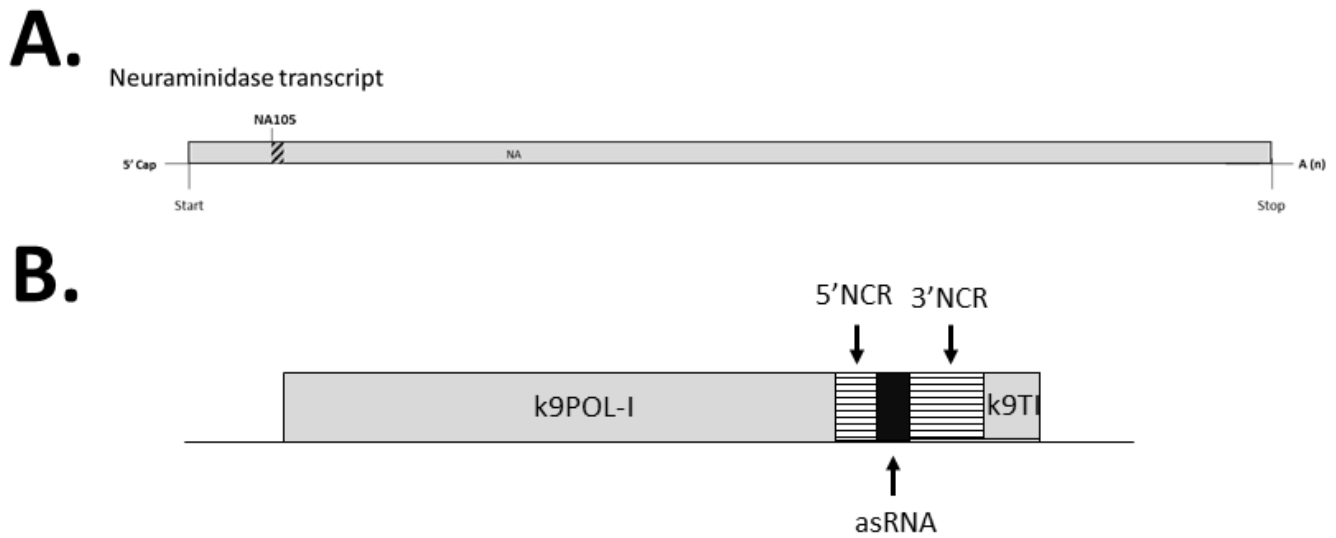


**Figure 5.1: Diagram of target sequence for NA105-siRNA & model of the inducible-asRNA expression vector.** A) NA105-siRNAs or asRNAs bind to their complementary locations (binding location indicated by grey and black hashed blocks) on the primary transcript of the neuraminidase protein (light grey solid block). B) The inducible asRNA anti-influenza therapy encoding for the NA105-asRNA contains the following characteristics: the asRNA coding sequence is flanked by the 5' and 3' noncoding regions (NCR; indicated by horizontal black stripes with white background) from the nucleoprotein gene of influenza A/WSN/33 (H1N1; influenza conserved promoter), and the sequences of the canine polymerase-I promoter (k9POL-I) and the canine polymerase-I termination (k9TI) sequences were included upstream of the 5' NCR or downstream of the 3' NCR, respectively. The inducible vector consists of the pCAT3-basic vector (Promega) backbone.

**A.**

Left chart: Relative Neuraminidase RNA (N1) Expression

MOI 0.05: M950 siRNA (*), NA105 siRNA, Negative Control siRNA, Lipofectamine only
MOI 0.005: M950 siRNA (*), NA105 siRNA, Negative Control siRNA, Lipofectamine only

Right chart: Relative Matrix RNA Expression

MOI 0.05: M950 siRNA (*), NA105 siRNA (*), Negative Control siRNA, Lipofectamine only
MOI 0.005: M950 siRNA (*), NA105 siRNA (*), Negative Control siRNA, Lipofectamine only

**B.**

siRNA: M950 | NA105 | Negative | Lipofectamine | NI
MOI: 0.05  0.005 | 0.05  0.005 | 0.05  0.005 | 0.05  0.005 |

NA — 51 kDa
GAPDH — 32 kDa
M2 — 11 kDa

**C.**

Left chart: Percent Neuraminidase Protein (N1) Expression

MOI 0.05: M950 siRNA, NA105 siRNA (*), Negative Control siRNA, Lipofectamine only
MOI 0.005: M950 siRNA (*), NA105 siRNA (*), Negative Control siRNA, Lipofectamine only
NI

Right chart: Percent Matrix Protein 2 Expression

MOI 0.05: M950 siRNA (*), NA105 siRNA, Negative Control siRNA, Lipofectamine only
MOI 0.005: M950 siRNA (*), NA105 siRNA (*), Negative Control siRNA, Lipofectamine only
NI

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**Figure 5.2: NA105 siRNA does not reduce cellular neuraminidase transcript levels but does reduce neuraminidase protein expression.** MDCK cells were transfected with siRNA [50 or 100 nM] for 8 h and then infected with influenza A/WS/33 (H1N1) (MOI 0.05, 0.005) for 24 h. A) Relative influenza A/WS/33 neuraminidase or matrix 1 RNA expression was determined by qPCR analysis of culture lysates and normalized to GAPDH. Protein expression was determined by B) Western blot of culture lysates, and C) percent neuraminidase and matrix protein 2 expression was determined by densitometry. Normalized to GAPDH. NI refers to no infection. (N=3)
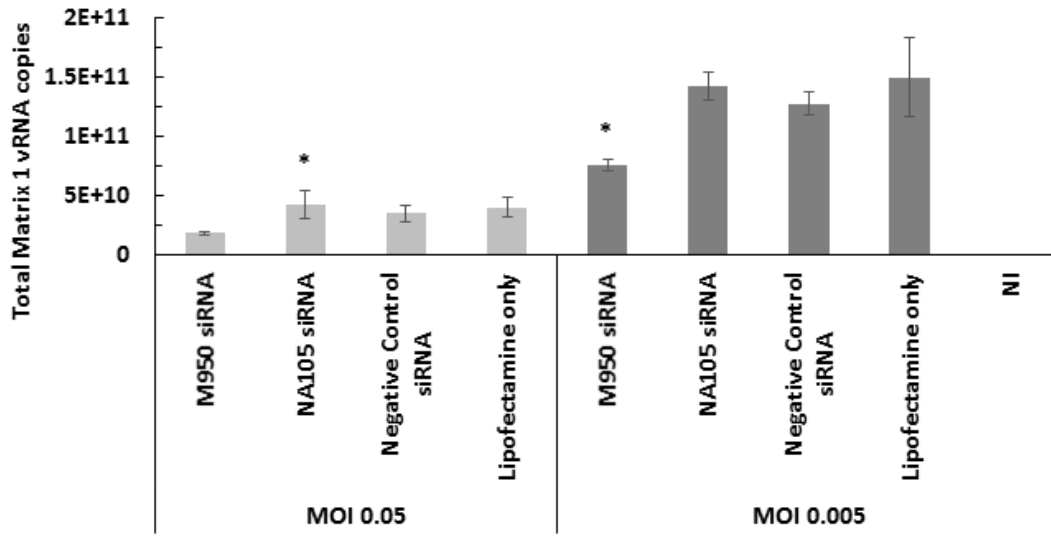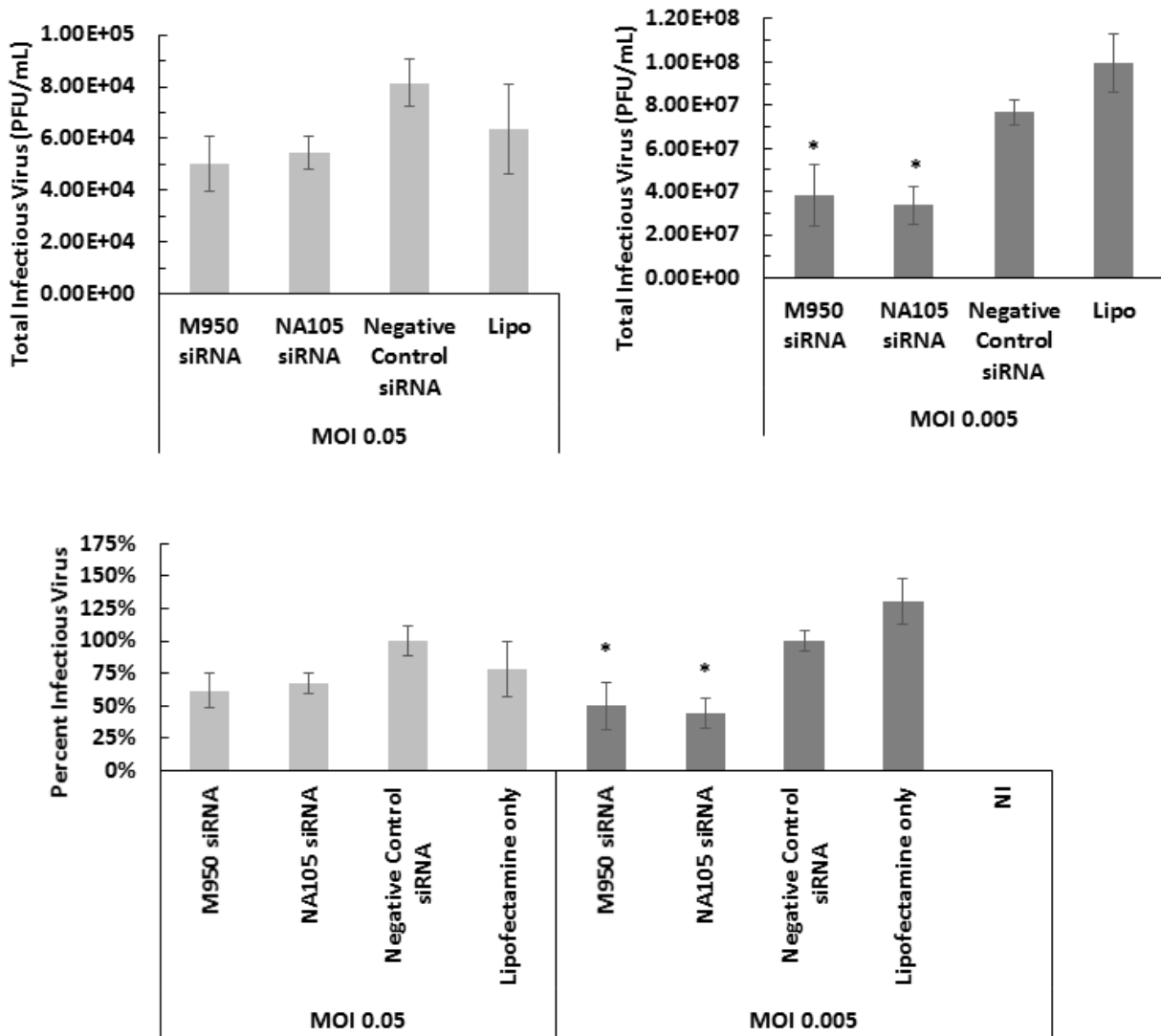
**Figure 5.3: NA105 siRNA attenuates virus infection.** MDCK cells were transfected with siRNA [50 or 100 nM] for 8 h and then infected with influenza A/WS/33 (H1N1) (MOI 0.05, 0.005) for 24 h. A) Total influenza virus in culture supernatant was determined by matrix 1 RNA expression 1 specific qPCR to determine matrix vRNA copy number. B) Total (PFU/mL) and percent infectious influenza virus was determined by viral plaque assay from culture supernatant. Percent calculations were normalized to negative control siRNA treated MDCK cells. NI refers to no infection control. (N=3)
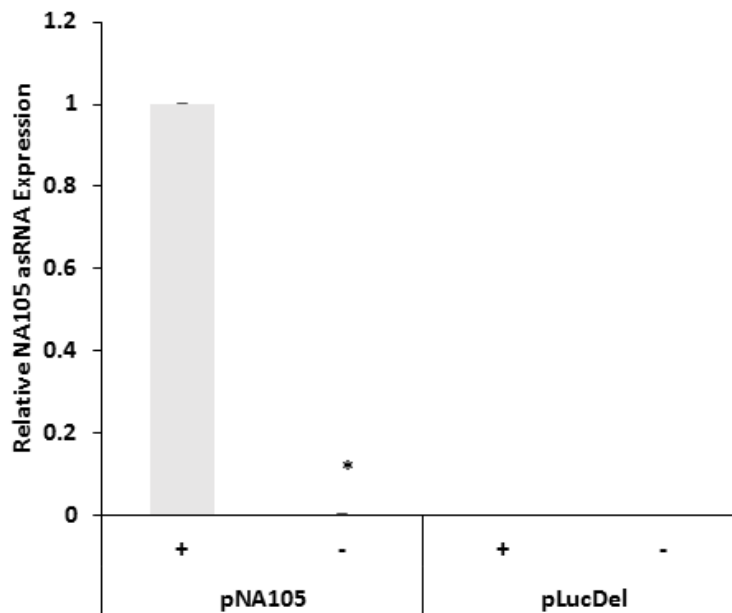


**Figure 5.4: Viral RNA-dependent RNA polymerase induces the expression of NA105-asRNA.** MDCK cells were transfected with 1.6 μg of NA105-asRNA expressing vector (pNA105) or control vector, pLucDel, and co-transfected with the four plasmids that express the proteins that form the viral RNA-dependent RNA polymerase (RDRP) for 24 h. asRNA expression, relative to cells treated with an asRNA expression vector and co-transfected with the RdRP, was determined by qPCR of culture lysates and normalized to GAPDH. (N=3)

# Chapter 6 - The Delivery of a Conserved Neuraminidase Antigen as a Novel Inducible Antiviral Therapy

## 6.1 Abstract

Antigenic drift and shift lead to the emergence of novel strains and subtypes of influenza viruses that can be resistant to current antiviral therapies. In an attempt to reverse antigenic variation and attenuate influenza titer, we have developed an inducible anti-influenza therapy that forces the virus to express a particular neuraminidase protein via the conserved influenza promoter. Recognition of the conserved promoter by the influenza RNA-dependent RNA polymerase (RdRP) drives expression of a nonfunctional neuraminidase (N2; pUC57 NF-NA) or functional neuraminidase (pUC57 F-NA) subtype 2. Vector performance was confirmed *in vitro* by co-transfection of MDCK cells with pUC57 NF-NA or F-NA and expression vectors encoding the RdRP. pUC57 NF-NA or F-NA transfected cells produced an RNA-intermediate regardless of the presence of the RdRP, whereas the polymerase was required for NF-NA mRNA and protein expression. The RdRP appeared to revert NF-NA protein back to a functional sialidase. Transfection of the inducible vectors into MDCK cells and subsequent infection with various influenza strains induced neuraminidase (N2) mRNA and protein expression. The clinical isolates of influenza virus induced up to 27.6-fold higher N2 RNA than adapted strains. Reinfection of MDCK cells with the supernatant from pUC57 NF-NA or F-NA treated and influenza (N1 subtype) infected cells revealed that the naïve MDCK cells generated N2 subtype viruses. The neuraminidase viral RNA could be incorporated into progeny viruses from a different influenza subtype, indicating that this multifaceted anti-influenza therapy can force viruses to become a particular subtype and be targeted by current antiviral therapies and vaccines.

## 6.2 Introduction

Each year, influenza epidemics occur that infect up to 5-15% of the world's population (WHO, 2014). The total economic burden in the United States during the 2003 influenza epidemic was estimated to be $87.1 billion, which accounts for direct costs, such as hospitalization and treatment, and indirect costs, such as absence from work and/or death (Mao et al., 2012). Antiviral therapies and vaccines that target influenza viruses have been developed in order to lessen the physical and economic burden associated with influenza virus infection.

One class of antiviral therapy that are currently used to target influenza A and B viruses are neuraminidase inhibitors (i.e. zanamivir and oseltamivir). Neuraminidase inhibitors are sialic acid mimics containing chemical modifications. The neuraminidase inhibitors bind to the active site of the neuraminidase, and through competitive inhibition, prevent the neuraminidases from removing the sialic acid residues from glycosylated residues and modulating the release of progeny virus (Palese and Compans, 1976). Although treatment with the neuraminidase inhibitor within 48 hours of symptom onset can decrease symptom duration by one day (Nicholson et al., 2000; Treanor et al., 2000), Sheu et al. (2008) reported that influenza virus resistant to neuraminidase inhibitors were isolated during the 2008 influenza season and are still in circulation today (CDC, 2016).

In the United States, inactivated or live-attenuated vaccines that can be delivered by intradermal, intramuscular, or intranasal routes are available for protection against influenza infection (Grohskopf, 2016). Trivalent vaccines, which include two strains of influenza A virus and one strain of influenza B virus, have been used in the United States since 1978 (Hannoun, 2013). During the winter of 2012, the United States Food and Drug Administration (FDA) approved the first quadrivalent vaccine, which include two strains of influenza A virus and two strains of influenza B virus, in order to supply

broader protection against the two lineages of influenza B viruses (Biere et al., 2010; FDA, 2012a). Each year the World Health Organization (WHO) Global Influenza Surveillance and Response System (GISRS) recommends which strains should be included in the influenza vaccine (Barr et al., 2014; Klimov et al., 2012). In addition, in November 2012 the FDA approved the use of cell-based vaccines. These vaccines use animal cells for virus production, instead of propagating the virus in eggs, thus allowing patients with egg allergies to become vaccinated against influenza viruses (FDA, 2012b).

Influenza viruses are negative-sense single-stranded RNA viruses that contain eight segmented RNAs encoding for various proteins (Desselberger et al., 1980). The viruses carry within their capsid RNA-dependent RNA polymerases (RdRP) that mediate replication and mRNA transcription. The RdRP lacks proof reading capabilities, which causes the influenza genome to undergo a high rate of mutation (Drake, 1993). On average, with each round of replication, the virus accumulates one new mutation. As the virus continues to replicate and expands its lineage, mutations accumulate over time, changing the viral antigenicity, and allowing mutated viruses to evade the host's immune response (van de Sandt et al., 2012). This phenomenon, called antigenic drift, causes influenza viruses to change from one season to the next and is partially responsible for the yearly recurrence of influenza epidemics and the emergence of antiviral-resistant strains of influenza (Deyde et al., 2007; Dolin, 1976). For this reason, the WHO GISRS was established to continually monitor which strains are present in a given year and predict which strains that should be included in the next seasonal vaccine (Barr et al., 2014; Klimov et al., 2012).

Another form of antigenic variation is called antigenic shift. Because influenza viruses contain segmented genomes, the segments from two different viruses that have infected the same cell are able to mix and match their genomes to create a new subtype of influenza (Dowdle and Schild, 1976; Webster et al., 1977). Due to the lack of protection against an antigenically novel influenza subtype, antigenic

shifts typically produce pandemic viruses (Kawaoka et al., 1989; Scholtissek, 1994; Scholtissek et al., 1978); the 1957 pandemic flu and the 1968 pandemic flu both emerged from genetic reassortment. Interestingly, the 1968 pandemic flu had a low mortality rate. Scientists speculate that because the virus still retained the neuraminidase (N2) antigen, this allowed for immunological memory against this antigen in subjects previously infected with the 1957 pandemic virus, thus resulting in a low case fatality rate (Gill et al., 1971; Kilbourne, 1997; Raoult and Drancourt, 2008).

Antigenic variation, either through antigenic drift or shift, makes it difficult to design effective vaccines because the strains included in the vaccine one year might not protect against the new strain present in the following year. In addition, antigenic variation can cause strains of influenza viruses to emerge that are resistant to antiviral therapies (Bright et al., 2006; Carr et al., 2011; de Jong et al., 2005). We have generated an inducible anti-influenza therapy that expresses non-functional neuraminidases after influenza infection. In an attempt to reverse antigenic variation and attenuate influenza virus titer, we have developed an inducible neuraminidase expression vector that forces the virus to express a particular neuraminidase protein through the use of the conserved influenza promoter. By doing so, we hope this inducible neuraminidase expression vector can force influenza viruses to converge into one subtype. Such a vector could be used as a therapy to enhance the efficacy of vaccines and antiviral therapies.

To further expand the utility of such a vector, we examined the ability of nonfunctional neuraminidases expressed by the inducible vector to attenuate influenza infection. The sialidase activity of neuraminidases are essential for the release of progeny viruses from the infected host cell. Without the neuraminidase-mediated cleavage of sialic acid residues from the surface of host cells and progeny viruses, influenza viral titer is reduced or attenuated due to the formation of viral aggregates (Hossain

et al., 2010; Shinya et al., 2004). Therefore, we hypothesize that overexpression of non-functional neuraminidases during influenza infection can attenuate influenza infection.

## 6.3 Materials and methods

*Cells and Virus*

Madin Darby Canine Kidney (MDCK) epithelial cells were provided by Dr. Daniel Perez (University of Maryland, MD). MDCK cells were propagated and maintained as described in McMillen et al. (2016).

Influenza strains A/WS/33 (H1N1) (ATCC, VR-1520), A/Swine/Iowa/15/30 (H1N1) (ATCC, VR-1683) were maintained as described in Blachere et al. (2011). Clinical influenza virus isolates, Clin 102NS (pH1N1) and Clin 123NS (pH1N1), were maintained as described in Chapter 4. Total viral titer and infectious viral titer were determined by quantitate polymerase chain reaction (qPCR) specific for the matrix 1 gene and viral plaque assay (VPA), respectively (Blachere et al., 2011).

*Anti-influenza therapy constructs*

The anti-influenza therapy construct that expresses a non-functional neuraminidase (NF-NA) mutant protein, pUC57 NF-NA, or the functional neuraminidase (F-NA) protein, pUC57 F-NA, was synthesized and cloned by Genewiz, Inc. The following DNA was synthesized to GeneWiz, Inc. and cloned into the EcoRV restriction site within the multiple cloning site of the pUC57-Kan plasmid: the coding sequence for influenza A/Tokyo/3/67 (H2N2) neuraminidase gene (GenBank: AY209929; pUC57 F-NA) or nonfunctional mutant with single nucleotide polymorphisms at nucleotides 826 and 828 (pUC57 NF-NA) is flanked by the noncoding regions (NCR) from the nucleoprotein gene of A/WSN/33 (Hossain et al., 2010; Luytjes et al., 1989; Neumann and Hobom, 1995) and the sequences of the canine polymerase-I

promoter (k9POL-I) and the canine polymerase-I termination (k9TI) sequences were included upstream to the 5' NCR or downstream to the 3' NCR, respectively. pUC57-Kan was used as an empty vector control for each study.

For confirmation that the mutations to the functional neuraminidase gene resulted in a non-functional protein, expression vectors containing the WT functional neuraminidase gene (pCI F-NA), the non-functional neuraminidase gene (pCI NF-NA) were generated. The corresponding genes were cloned into the pCI-neo vector (Promega). The pCI-neo vector was used as a negative control.

*Induced expression of neuraminidase (N2) by influenza RdRP*

To evaluate whether the influenza virus polymerase can induce the expression of neuraminidase transcripts, MDCK cells ($5 \times 10^5$ cells/well) were seeded on a 24-well plate (Corning) in complete growth media. Twenty-four hours later, transfection was performed following the protocol explained in the methods of Chapter 4 (Section: Assessment of RdRP-induced asRNA facilitated RNAi of matrix protein 2 and nonstructural protein 2 expression vectors). 0.16 μg of the corresponding anti-influenza therapy vectors (pUC57 NF-NA, pUC57 F-NA, or pUC57 empty vector) were added to 50 μL of OPTI-MEM I Reduced Serum Medium. Cells were either transfected with each individual anti-influenza therapy alone or co-transfected with four plasmids (0.16 μg each) that express the influenza RdRP (pCI-A-Brisbane-10-2007 (H3N2) NP, pCI-A-Brisbane-10-2007 (H3N2) PA, pCI-A-Brisbane-10-2007 (H3N2) PB1, pCI-A-Brisbane-10-2007 (H3N2) PB2; Hossain et al., 2010). Twenty-four hours after transfection, the MDCK cells were lysed using Lysis/Binding Solution Concentration (Ambion) supplemented with 100% isopropanol (Sigma-Aldrich). The lysates were stored at -80° C until further processing for analysis by qPCR. For protein analysis, the MDCK cells were lysed with radioimmunoprecipitation assay (RIPA) buffer containing protease inhibitor cocktail and EDTA (ThermoFisher Scientific) and stored at -80° C until

analysis by Western blot. Three independent experiments with two replicates per treatment were performed.

*Induced expression of neuraminidase (N2) by infection with influenza N1 subtype*

To determine whether infectious influenza virus can induce the expression of the neuraminidase (N2) protein, MDCK cells (1 x 10$^6$ cells/well) were seeded on a 6-well tissue culture plate (Corning) in complete growth media. Twenty-four hours later the cells were transfected with pUC57 NF-NA, pUC57 F-NA, or pUC57 empty vector control using the Lipofectamine 2000 Reagent (ThermoFisher Scientific) following the protocol explained in McMillen et al. (2016). The cells were infected with influenza A/WS/33 (H1N1), A/Swine/Iowa/15/30 (H1N1), Clin 102NS (pH1N1), or Clin 123 (pH1N1) 24 h later at a multiplicity of infection (MOI) of 0.1 or 0.001. After a 45 min adsorption period, the viral solution was removed, the cells were washed once with phosphate-buffered saline (PBS) and overlaid with 2 mL of Dulbecco's modified Eagle's medium (DMEM)/F12 (Gibco) supplemented with 100 U/mL penicillin G, 100 mg/ml streptomycin, 2 mM L-glutamine, 0.2% (v/v) bovine serum albumin (BSA), 10 mM HEPES (Gibco), 0.22% (v/v) sodium bicarbonate (Gibco), 0.01% (w/v) DEAE-dextran (MP BioMedicals LLC), and 2 mg/mL N-p-tosyl-L-phenylalanine chloromethyl ketone (TPCK) (Sigma-Aldrich). The culture supernatant and cell lysates were collected for analysis 24 h after infection. The MDCK cells were lysed with Lysis/Binding Solution Concentration (Ambion) supplemented with 100% isopropanol (Sigma-Aldrich) and stored at -80° C until further processing for analysis by qPCR. One independent experiment with two replicates per treatment was performed. For protein analysis, the MDCK cells were lysed with radioimmunoprecipitation assay (RIPA) buffer containing protease inhibitor cocktail and EDTA (ThermoFisher Scientific) and stored at -80° C until analysis by Western blot. One independent

experiment with one replicate per treatment was performed. The culture supernatant was stored at -80° C until further use.

To determine whether the neuraminidase (N2) gene was incorporated into progeny viruses, MDCK cells ($1 \times 10^6$ cells/well) were seeded on a 6-well tissue culture plate (Corning) in complete growth media. Twenty-four hours later the cells were inoculated with 100 µL of the culture supernatant from the experiment listed above. After a 45 min adsorption period, the viral solution was removed, the cells were washed once with PBS, and were then overlaid with 2 mL supplemented DMEM/F12 media. The culture supernatant was collected and the virus was lysed with Lysis/Binding Solution Concentration (Ambion) supplemented with 100% isopropanol (Sigma-Aldrich). The supernatant lysates were stored at -80° C until further processing for analysis by qPCR. One independent experiment with three replicates per treatment was performed.

*Total RNA isolation and cDNA transcription*

Total RNA was isolated from MDCK cells using the MagMax™-96 Total RNA Isolation Kit (Ambion) as explained in McMillen et al. (2016).

Neuraminidase mRNA-specific cDNA synthesis was performed using the ThermoScript Reverse Transcriptase Kit from Invitrogen (ThermoFisher Scientific). Reverse transcription was performed following the manufacturer's protocol and using Oligo $(dT)_{20}$ primer synthesized by Integrated DNA Technologies.

Neuraminidase viral RNA (vRNA)-specific cDNA synthesis was also performed using the ThermoScript Reverse Transcriptase Kit from Invitrogen (ThermoFisher Scientific). Reverse transcription was performed following the manufacturer's protocol using primers specific for the neuraminidase vRNA

3' noncoding region (5' TTTCTTGTGGACGAAAACGA 3'). Neuraminidase vRNA reverse transcription primers were synthesized by Integrated DNA Technologies.

*Viral RNA isolation and cDNA transcription*

Viral RNA was isolated from culture supernatant using the MagMax™-96 Total RNA Isolation Kit (Ambion) as described in McMillen et al. (2016).

*Analysis of mRNA and vRNA levels by real-time quantitative PCR (qPCR)*

qPCR analyses to detect influenza-specific mRNA or vRNA were performed using the following primers and probe: neuraminidase 2 (N2), forward 5' GAGAGAGGGACTCAAGGACTCAAAG 3', reverse 5' TTCTTCCCATCCACAAGTCATTT 3', probe 5' TGGGCCTTTGACAATG 3'. The neuraminidase 2 (N2) primers and probes were designed in lab using the Primer Express 3.0 software by Applied Biosystems. The probes were designed with a 5' fluorescent dye, 6FAM, and 3' quencher, MGCNFQ. All primers and probes targeting the coding sequence for influenza A/Tokyo/3/67 (H2N2) neuraminidase gene (GenBank: AY209929) were synthesized by Applied Biosystems and used at a final concentration of 0.8 M and 0.2 M, respectively. Reactions were performed and analyzed using the Applied Biosystems 7500 Fast Real-Time PCR System under the following thermal cycling conditions: 95°C for 20 seconds, followed by 40 cycles at 95°C for 3 seconds, and 60°C for 30 seconds. A negative control without template was included in all real-time PCRs.

All samples were run in duplicate. Relative gene expression was determined by $\Delta\Delta C_T$ and normalized to GAPDH (Hs03929097_g1, ThermoFisher Scientific). PCR was performed on MDCK cell lysates and analyzed by gel electrophoresis to confirm specificity of GAPDH primers to canine GAPDH (data not shown).

*Analysis of protein levels by Western blot*

Protein expression was analyzed by Western blot following the methods described in McMillen et al. (2016). Primary antibodies directed against GAPDH (sc-25778, Santa Cruz), neuraminidase 2 (N2; 40017-V07H, Sino Biological, Inc.) and nucleoprotein (ab66191, Abcam) were used for protein detection. Primary antibodies for GAPDH were used to verify equal sample loading and for normalization.

*Neuraminidase Assay*

To determine the sialidase activity of the expressed neuraminidase proteins, a neuraminidase assay was performed using the NA-Fluor™ Influenza Neuraminidase Assay Kit (Applied Biosystems, Life Technology), following the manufacturer's protocol. MDCK ($1 \times 10^6$) cells were seeded on a 6-well tissue culture plate (Corning) in complete growth media. Twenty-four hours later, the cells were transfected with 1.6 μg of pCI NF-NA, pCI F-NA, or pCI-neo empty vector for 24 h using Lipofectamine 2000 reagent (ThermoFisher Scientific) as described in McMillen et al. (2016). To lift the adherent MDCK cells off of the tissue culture plates, the cells were treated with 2 mL of 0.5% trypsin-EDTA (Gibco) and incubated in a for 10 min in a 35°C, 5% $CO_2$ incubator. The detached cells were centrifuged at 2,000 rpm for 3 min and the trypsin was removed from the cell pellet. The cell pellet was washed with 2 mL of PBS, briefly vortexed and centrifuged two times to remove residual trypsin. The cell pellet was resuspended in 200 μL of OPTI-MEM I Reduced Serum Medium (Gibco). 50 μL of each sample was used for detection via the neuraminidase assay. 0.1, 3.12 or 6.25 μM of 4-methylumbelliferone sodium salt (4-MU(SS)) was used for positive control.

To determine the sialidase activity of the neuraminidase proteins that were expressed by the influenza RdRP, a similar experiment as above was performed, with the exception that the MDCK cells instead were transfected with 1.6 μg pUC57 NF-NA, pUC57 F-NA, or pUC57 empty control in the presence

or absence of the four vectors that express the influenza RdRP. The neuraminidase activity of 1.2 x $10^7$ (2.5 x $10^5$ PFU) influenza A/WS/33 (H1N1) viruses was used as a positive control.

*Statistical analyses*

A two-way factorial mixed model analysis of variance (ANOVA) was performed on all variables. All pairwise comparisons were considered significant at $p < 0.05$. Asterisks above graphs (*) indicate $p<0.05$. All graphs are representative of three or one independent experiment with two replicates per treatment group per experiment.

## 6.4 Results and discussion

We have developed an inducible vector that expresses a particular subtype of neuraminidase upon recognition by the influenza RdRP. The inducible neuraminidase vector contains the following characteristics: the neuraminidase (N2; functional or non-functional) coding sequence is flanked by the noncoding regions (NCR) (indicated by horizontal black strips with white background) from the nucleoprotein gene of A/WSN/33 (H1N1), and the sequences of the canine polymerase-I promoter (k9POL-I) and the canine polymerase-I termination (k9TI) sequences were included upstream to the 5' NCR or downstream to the 3' NCR, respectively. These sequences were cloned into the pUC57-Kan expression vector (Figure 6.2A). Once the host cell, in this case MDCK cells, is supplied with the inducible neuraminidase vector, the host RNA polymerase I recognizes the k9POL-I and transcribes an RNA intermediate that mimics the genetic material of influenza viruses. The negative-sense single-stranded RNA intermediate contains the conserved influenza promoter in addition to the coding sequence for a functional (F-NA) or nonfunctional (NF-NA) neuraminidase protein. After the same cell, which has

expressed multiple copies of the RNA intermediate, is infected with an influenza virus, the influenza RdRPs recognizes the conserved promoter and generate neuraminidase transcripts which are then transcribed into protein. The neuraminidase proteins encoded by influenza viruses are foreign antigens that can stimulate an immune response within the host (Chow et al., 1979). By limiting the expression of the neuraminidase protein to cells already infected with the influenza virus it will prevent unnecessary inflammation and tissue damage (Iwasaki and Pillai, 2014).

We have hypothesized that expression of a non-functional neuraminidase in cells infected with influenza virus can attenuate influenza infection, due to neuraminidase's role in releasing progeny virus from the host cell (Lentz et al., 1987; Palese et al., 1974). In order to begin testing this hypothesis, we generated two inducible vectors that express either a functional neuraminidase (F-NA) or nonfunctional neuraminidase (NF-NA). A study by Lentz et al. (1987) showed that mutating the influenza A/Tokyo/3/67 (H2N2) at amino acid site 276 from a glutamate to a glutamine resulted in abolished sialidase activity. Consequently, we designed the pUC57 F-NA inducible vector to contain the coding sequence for the wild-type (functional) influenza A/Tokyo/3/67 (H2N2) gene, while the pUC57 NF-NA inducible vector contains the mutated coding sequence (Figure 6.1).

In order to distinguish between the neuraminidase expressed from the wild-type infectious virus and the neuraminidase that is expressed by the inducible vector we have utilized two different neuraminidase subtypes for our studies. We have incorporated the coding sequence for a neuraminidase from subtype 2 (N2) into the inducible vector, whereas the influenza viruses that are used to induce vector protein expression will contain neuraminidases from subtype 1 (N1). qPCR analyses have been designed to specifically recognizes neuraminidase subtype 1 or 2, without cross-reactivity of primers and probes.

*The influenza RNA-dependent RNA polymerase causes neuraminidase expression via the inducible neuraminidase vector*

The inducible neuraminidase vector contains the influenza conserved promoter and can be activated by the influenza RdRP. To examine whether the influenza RdRP can induce the expression of N2 proteins, MDCK cells were transfected with pUC57 NF-NA, pUC57 F-NA, or pUC57 empty vector in the presence or absence of the viral RdRP. Cells co-transfected with a neuraminidase expression vector and the RdRP had 6.9-fold (pUC57 NF-NA) or 6.0-fold (pUC57 F-NA) more neuraminidase mRNA than cells not containing the influenza RdRP (Figure 6.2B). As expected, cells containing the neuraminidase expression vector expressed up to about 187,000-fold more N2 viral RNA (vRNA) in the presence of the RdRP and up to about 257,000-fold more N2 vRNA in the absence of the RdRP (Figure 6.2B). These results indicate that the influenza RdRP is essential for the expression of the N2 mRNA, not vRNA. Instead, vRNA expression is under the control of the k9POL-I promoter. To further confirm that N2 expression is dependent on the presence of the influenza RdRP, a Western blot was performed to examine N2 protein expression after treatment of MDCK cells with a neuraminidase expression vector in the presence or absence of the influenza RdRP. Neuraminidase protein was present only in cells containing the pUC57 NF-NA or pUC57 F-NA inducible vector and the viral RdRP (Figure 6.2C). Cells transfected with the pUC57 empty vector control and not co-transfected with the plasmids expressing the viral RdRP did not express the N2 protein (Figure 6.2C).

*NF-NA expression under the cytomegalovirus (CMV) promoter results in the production of inactive protein, whereas NF-NA expression under the influenza conserved promoter reverts back to an active state*

A neuraminidase assay that measures sialidase activity by detecting the fluorogenic end product, 4-methylumbelliferone (4-MU) released after enzymatic cleavage of the substrate 2'-(4-methylumbelliferyl)-α-D-N-acetylneuraminic acid (MUNANA) was used to confirm that the non-functional neuraminidase does not have sialidase activity. MDCK cells treated with an expression vector that encodes for the NF-NA (pCI NF-NA) or F-NA (pCI F-NA) under the control of the CMV promoter were examined for neuraminidase sialidase activity. Only pCI F-NA treated cells displayed sialidase activity as indicated by the presence of 4-MU, whereas MDCK cells treated with pCI NF-NA or pCI-neo empty vector control only had baseline levels of fluorescence (Figure 6.3A).

Similarly, the sialidase activity of the N2 proteins expressed by cells treated with the inducible neuraminidase expression vectors in the presence or absence of the influenza RdRP was evaluated. The neuraminidase expressed from the pUC57 NF-NA and pUC57 F-NA inducible vectors had sialidase activity, however the neuraminidases expressed by the pUC57 NF-NA treated cells stimulated 55.4% less 4-MU release than pUC57 F-NA cells. Because the neuraminidases that were mutated to be nonfunctional had neuraminidase activity, it is possible that the RdRP that transcribes the vRNA into mRNA generated neuraminidase proteins that reverted back to their original active state. The influenza RdRP has an error rate of about 1 mutation per genome replication (Drake, 1993), thus it is possible that the RdRP generated vRNA or mRNA that contains the wild-type (functional) neuraminidase sequence. Sequence analyses of "non-functional" neuraminidase mRNA generated by the RdRP should be assessed to conclude that reversion of the mutant neuraminidase back to wild-type occurred. Because it appears

that the RdRP can cause the non-functional neuraminidase to revert back to its active state, the delivery of a non-functional neuraminidase via an inducible expression vector did not attenuate the influenza viral titer as originally predicted (data not shown).

*Influenza infection induces the expression of neuraminidase (N2) mRNA and protein*

To examine whether infectious influenza can induce the expression of N2 transcripts and protein, MDCK cells transfected with pUC57 NF-NA, pUC57 F-NA or pUC57 empty vector control were infected with various strains of influenza virus. Cells infected with adapted strains of influenza (A/WS/33 (H1N1) or A/Swine/Iowa/15/30 (H1N1)) expressed minimal amounts of neuraminidase RNA (Figure 6.4A). pUC57 NF-NA treated cells infected with clinical influenza isolates Clin 102NS or Clin 123NS (pH1N1) had 27.6- and 22.0-fold more N2 RNA, respectively, than cells infected with A/WS/33 (H1N1) at an MOI of 0.1 (Figure 6.4A). Similar results were observed when comparing inducible vector treated MDCK cells infected with clinical isolates to A/Swine/Iowa/15/30 (H1N1) infected cells (Figure 6.4A). A higher neuraminidase protein yield was also observed in pUC57 NF-NA or pUC57 F-NA treated cells that were infected with the clinical influenza isolates compared to the adapted strains (Figure 6.4B). Clin 102NS (pH1N1) infection induced the highest N2 expression via the pUC57-NF-NA expression vector. Clin 123NS (pH1N1) infection induced a similar amount of N2 (89.2% compared to Clin 102NS infected cells) whereas influenza A/Swine/Iowa (H1N1) induced a low level of N2 expression, 20.6%. A/WS/33 (H1N1) infection induced trace amounts of N2. Similar results were observed in cells treated with pUC57-NA expression vector. These studies imply that the clinical influenza isolates have a higher affinity for the antiviral therapy conserved promoter than the adapted strains, which allows these viruses to express a higher level of the N2 protein.

*Treatment of MDCK cells with culture supernatant from influenza A H1N1 infected cells treated with pNF-NA or pF-NA results in the production of H1N2 virus*

The canine polymerase I promoter generated multiple copies of a neuraminidase encoding RNA intermediate that mimics the influenza vRNA. To determine whether the progeny virus created after pUC57 NF-NA or pUC57 F-NA treated cells infected with H1N1 viruses obtained vRNA encoding for the N2 protein, MDCK cells were inoculated with the supernatant from experiment 3 for 24 h. The experiments using supernatant from the newly infected cells revealed that treatment of MDCK cells with a neuraminidase inducible vector and subsequent infection with an H1N1 influenza strain generated progeny virus that contains the N2 gene (Figure 6.5). Therefore, we have shown that the inducible anti-influenza therapy expressing a neuraminidase antigen can force naturally occurring H1N1 influenza viruses to become H1N2 viruses. It is uncertain whether the N2 strain of influenza itself is infectious, or whether co-infection with the N1 strain acts as a helper virus (Fodor et al., 1999) to complete the replication cycle.

Although the exact mechanism by which influenza viruses incorporate the eight unique vRNA into each virion is unknown, a study by Goto et al. (2013) showed that the packaging signals within the noncoding region of the influenza vRNA are needed for incorporation of each vRNA into the virion. They examined the nucleoprotein packaging signals and found that the packaging signals within the coding region of a particular vRNA are needed for efficient production of infectious virions, while the vRNA without the packaging signal is still incorporated in the virus. It is unclear whether the unique combination of noncoding and coding packaging signals must be present on the same vRNA in order to generate infectious virus, or if the noncoding packaging signal from one vRNA can be replaced with the packaging signal from another vRNA and still generate infectious virus. We propose that, because the

inducible neuraminidase expression vector contains the noncoding region from the nucleoprotein gene of A/WSN/33 (H1N1) and packaging signal from the coding region of the N2, one of three scenarios could have occurred: 1) the N2 is inserted into an incomplete virion because the noncoding and coding packaging signals don't match; 2) the N2 is inserted into a virion and takes the place of the WT NP vRNA, because it contains the noncoding packaging signal and relies on a helper virus for replication; or 3) the N2 is inserted into a virion and takes the place of the N1 vRNA, because it contains the coding packaging signal, and still generates infectious viruses. Future studies using reverse genetics should be performed in order to identify whether the N2 viruses are infectious and can replicate without a helper virus. As a means of simplification, generating an inducible neuraminidase expression vector that contains the noncoding region for the neuraminidase vRNA should also be examined to determine whether the inducible neuraminidase expression vector can generate infectious N2 viruses.

Many studies have been performed using reverse genetics systems that have either manipulated the virus to express fluorescent proteins (Watanabe et al., 2003) or other foreign proteins (Luytjes et al., 1989), studied the signals needed for viral packaging (Goto et al., 2013), or generated minimal viral RNA decoys that can inhibit viral replication (Luo et al., 1997). The inducible neuraminidase expression vector described here has important implications for the development of an anti-influenza therapy that primes the immune system to recognize a particular antigen by forcing the virus to express a certain neuraminidase subtype. To our knowledge, this is the first time the idea of forcing the influenza virus to express a particular neuraminidase has been considered. Because antigenic drift and shift have limited the efficacy of current antiviral therapies (Carr et al., 2011; Sheu et al., 2008) and vaccines (Carrat and Flahault, 2007; Donnelly et al., 1995), it is possible that this inducible neuraminidase expression vector can reverse antigenic drift and shift by continuously reintroducing a conserved antigen back into circulation.

A similar methodology could be used to force the virus to express a particular hemagglutinin subtype A dual inducible expression system, encoding for neuraminidase and hemagglutinin. Such a methodology should be considered in order to force the virus to express two important antigens involved in the generation of an influenza-specific immune response (Chow et al., 1979).

In conclusion, we have designed an inducible neuraminidase expression vector that forces progeny virus to incorporate a neuraminidase subtype 2 vRNA into its genome. This inducible therapy has the potential to force influenza viruses to converge into one subtype, thus reversing antigenic variation (Figure 6.6). In theory, a therapy of this type would then make it possible to generate effective antiviral therapies and vaccines that target the induced antigens present on the surface of the viruses and the vaccines will not lose efficacy over time, as all of the viruses that underwent antigenic shift will be susceptible to the vaccine primed immune system.

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**6.6 Figures and figure legends**



**A**

**Influenza A Virus (A/Tokyo/3/67 (H2N2)) neuraminidase gene**
**GenBank: AY209929**

|  |  |  |  |
|---|---|---|---|
| Functional (WT): | 811 nt | agtgctcagc atgta**GAG**ga gtgttcctgt | 840nt |
| Non-functional mutant: | 811 nt | agtgctcagc atgta**CAA**ga gtgttcctgt | 840nt |

|  |  |  |  |
|---|---|---|---|
| Functional (WT): | 271 aa | SAQHV**E**ECSC | 280 aa |
| Non-functional mutant: | 271 aa | SAQHV**Q**ECSC | 280 aa |

**Figure 6.1: Mutation of influenza A/Tokyo/3/67 (H2N2) neuraminidase gene from functional to non-functional.** The neuraminidase gene was mutated by making single nucleotide polymorphisms at nucleotides 826 and 828 which changes the amino acid at site 276 from a glutamate to a glutamine. This mutation changes the neuraminidase activity from an active (functional) state to a non-active (non-functional) state as determined by Lentz et al. (1987). GenBank: AY209929.

**A**
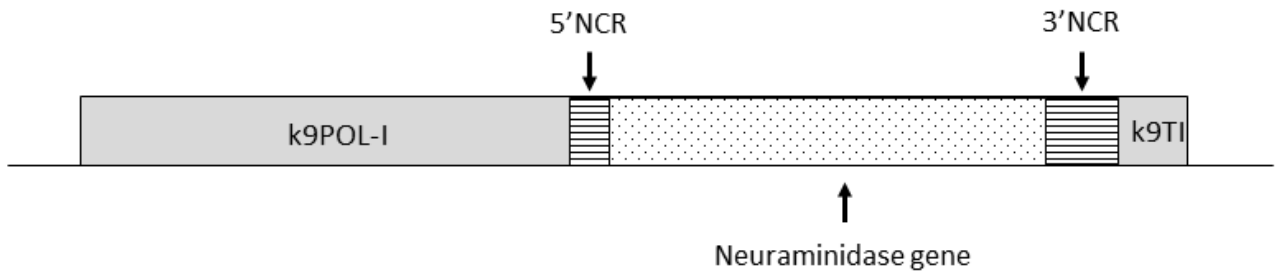
**B**
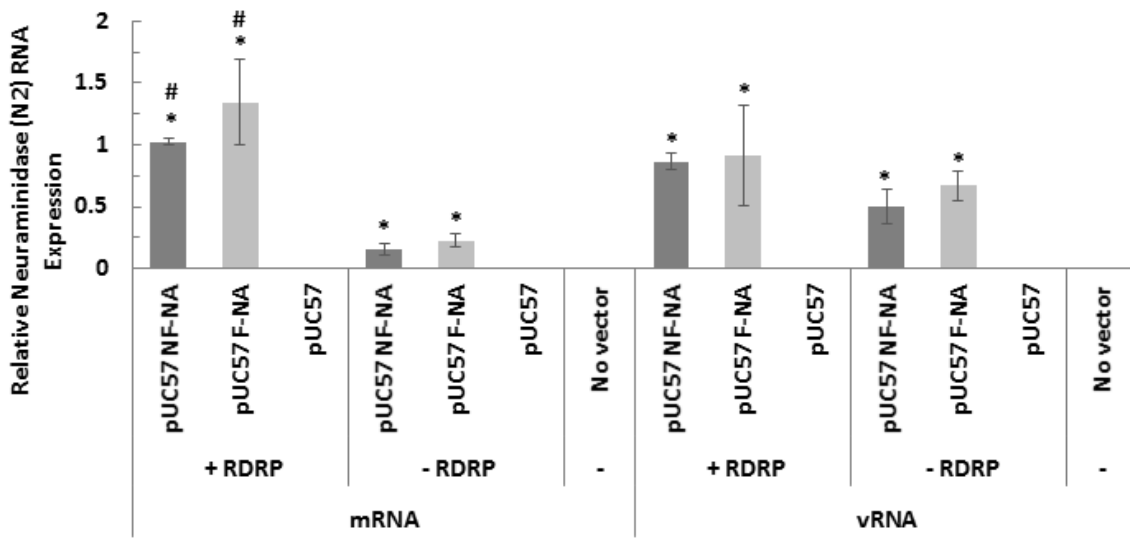
**C**
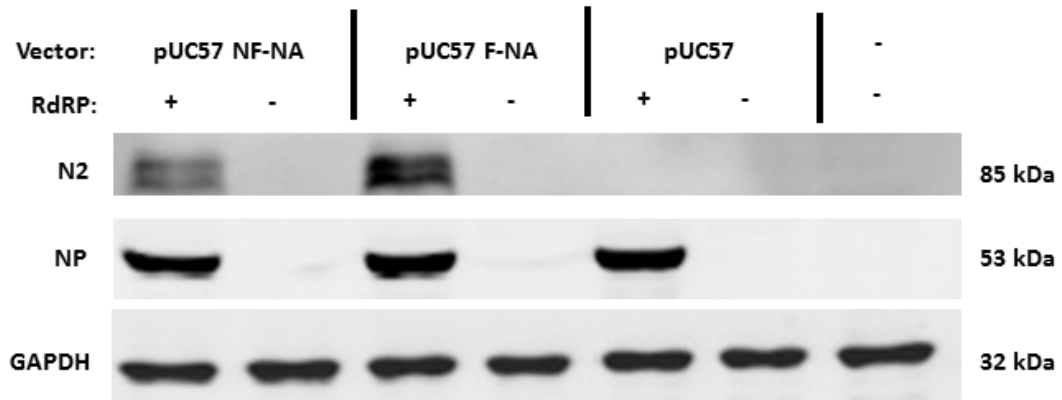
**Figure 6.2:** *The influenza RNA-dependent RNA polymerase causes neuraminidase expression via the inducible neuraminidase vector.* A) The inducible neuraminidase vector contains the following characteristics: the neuraminidase (functional or non-functional) coding sequence is flanked by the noncoding regions (NCR) (indicated by horizontal black strips with white background) from the nucleoprotein gene of A/WSN/33, and the sequences of the canine polymerase-I promoter (k9POL-I) and the canine polymerase-I termination (k9TI) sequences were included upstream to the 5' NCR or downstream to the 3' NCR, respectively. B & C) MDCK cells were co-transfected with 0.16 µg NF-NA, pF-NA, or empty vector and the four plasmids that express the four proteins that make up the influenza RNA-dependent polymerase for 24 hrs. B) RNA (mRNA or vRNA) specific for the influenza A/Tokyo/67 (H2N2) neuraminidase was measured by qPCR and normalized to GAPDH (N=3) and C) Neuraminidase (N2) protein levels were determined by Western blot. GAPDH served as a loading control. * indicates significance compared to pUC57 Empty vector, # indicates significance compared to treatment without RdRP.
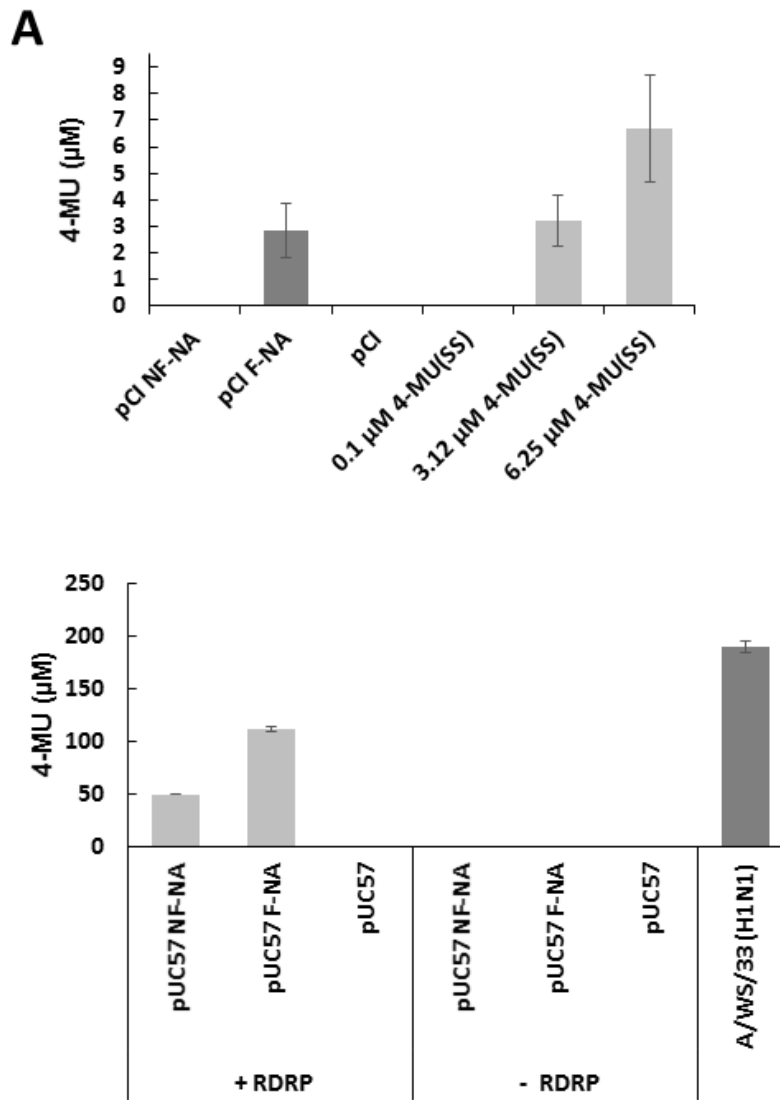
**Figure 6.3: NF-NA expression under the CMV promoter results in the production of inactive protein, whereas NF-NA expression under the influenza conserved promoter reverts back to an active state.** A) MDCK cells were transfected with 1.6 µg of pCI NF-NA, pCI F-NA, or pCI-neo empty vector for 24 h. Whole cells were collected and analyzed for the presence of active neuraminidases by neuraminidase assy. 0.1, 3.12 or 6.25 µM of 4-methylumbelliferone sodium salt (4-MU(SS)) was used for positive control and standard. B) MDCK cells were transfected with 1.6 µg of pUC57 NF-NA, pUC57 F-NA, or pUC57 empty vector in the presence or absence of the RdRP expression vector. The neuraminidase activity of $1.2 \times 10^7$ influenza A/WS/33 (H1N1; $2.5 \times 10^5$ PFU) was used as a positive control.
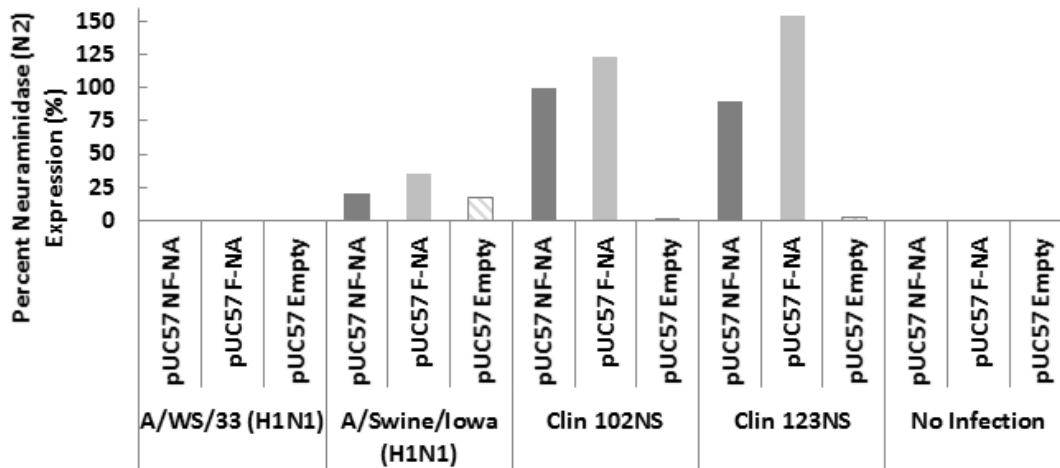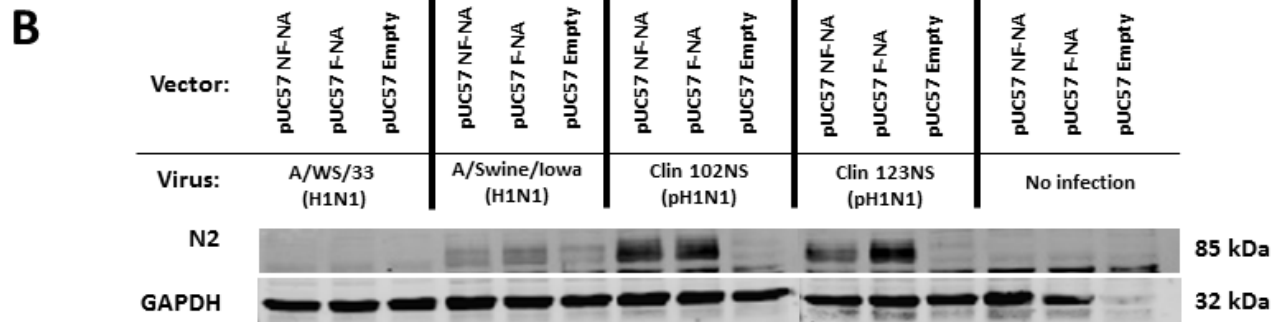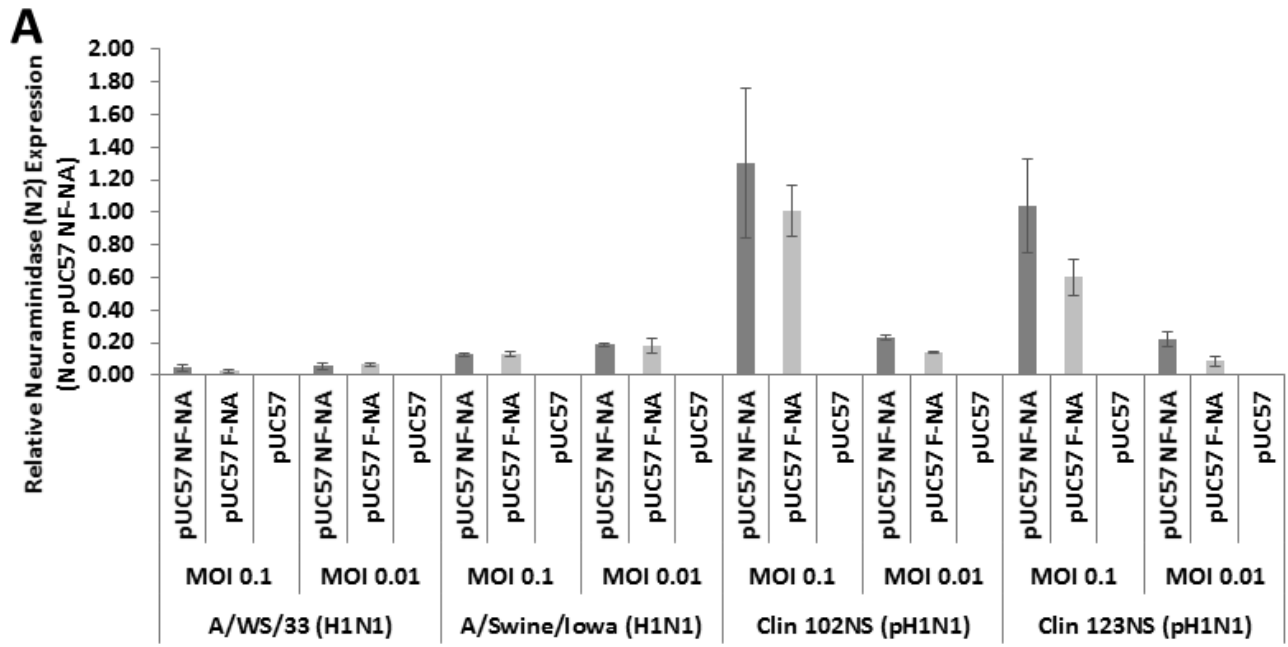
**Figure 6.4: Influenza infection induces the expression of neuraminidase (N2) mRNA and protein.** MDCK cells were transfected with 1.6 µg of pUC57 NF-NA, pUC57 F-NA, or pUC57 empty vector for 24 h then infected with influenza A/WS/33 (H1N1), A/Swine/Iowa (H1N1), Clin 102NS (pH1N1) or Clin 123NS (pH1N1) at an MOI of 0.1 or 0.01 for 24 h. A) Relative influenza A/Tokyo/67 (H2N2) neuraminidase expression was determined by qPCR and normalized to GAPDH. Value are relative to pUC57-NFNA Clin 123NS (pH1N1), MOI 0.1. B) Neuraminidase (N2) protein expression was determined by Western blot and normalized to GAPDH loading control. Densitometry was performed to percent protein expression compared to pUC57 NF-NA treated and Clin 102NS infected cells. (N=1)
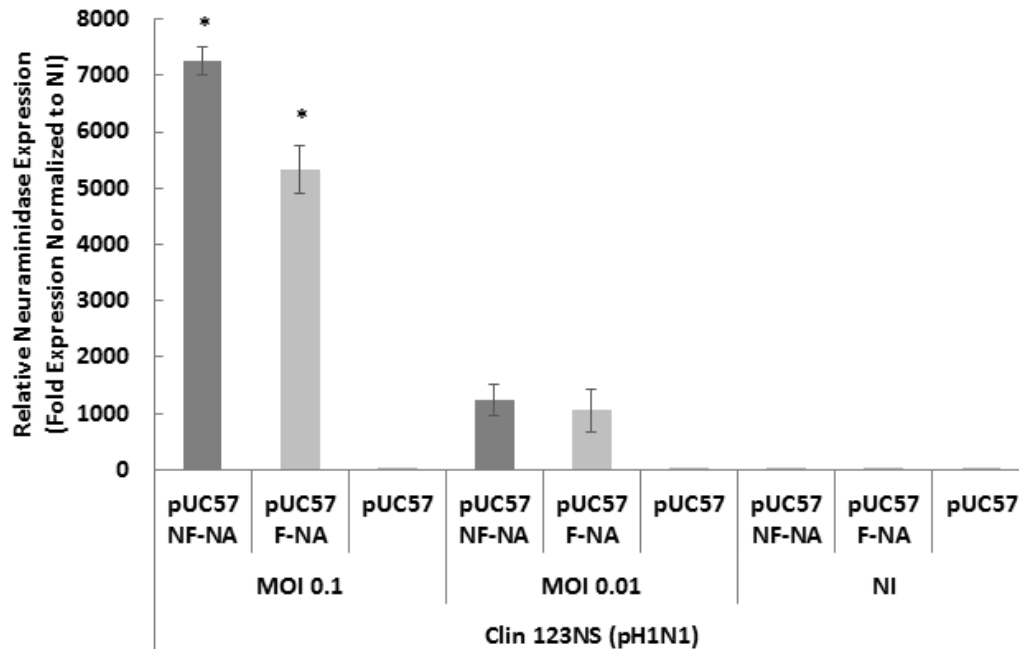
**Figure 6.5: Treatment of MDCK cells with culture supernatant from influenza A (H1N1) infected cells treated with pNF-NA or pF-NA results in the production of H1N2 virus.** MDCK cells were inoculated with 100 μL of supernatant from experiment 3 for 24 h. Relative neuraminidase (N2) viral RNA (vRNA) from the culture supernatant was quantified by qPCR and normalized to GAPDH. Values are relative to pUC57 NF-NA treated cells inoculated with the culture supernatant from uninfected cells. NI indicates no infection. (N=1)
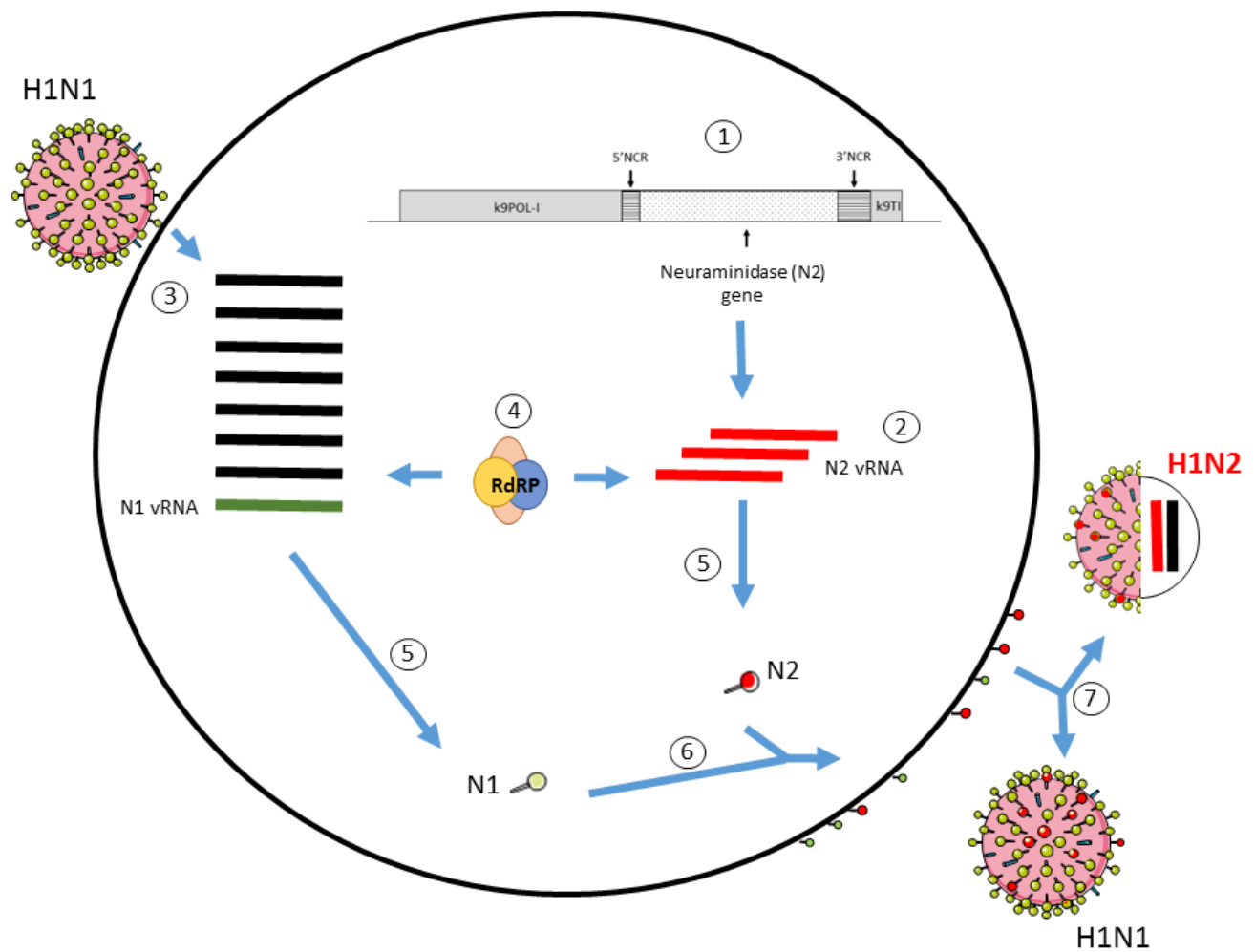
**Figure 6.6: Schematic of the theoretical mechanism of action for the inducible anti-influenza therapy expressing a decoy neuraminidase (N2).** 1) The molecular component of the inducible anti-influenza therapy is delivered to the target cell. The therapy contains the following sequences: the neuraminidase protein coding sequence is flanked by the noncoding regions (NCR; also referred to as the influenza conserved promoter) from the nucleoprotein gene of influenza A/WSN/33 (H1N1), and the sequences of the canine polymerase-I promoter (k9POL-I) and the canine polymerase-I termination (k9TI) sequences were included upstream to the 5' NCR or downstream to the 3' NCR, respectively. 2) The canine polymerase I transcribes multiple copies of an RNA intermediate that mimics influenza virus RNA (vRNA). The vRNAs encode for the neuraminidase subtype 2 (N2) protein (red). 3) The same cell is

infected with an influenza virus of the (H1N1) subtype. The virus brings 8 vRNA into the host cell, one of which encodes for the neuraminidase subtype 1 (N1) protein (green). 4) Only after the host cell, which contains the therapy, is infected with the virus that brings in the viral RNA-dependent RNA polymerase (RdRP) will the RNA-intermediate containing the influenza conserved promoter be recognized by the RdRP, which reverse transcribes the vRNA into messenger RNA (mRNA). At the same time, the 8 vRNA that comes in with the influenza (H1N1) virus is reverse transcribed into mRNA by the viral RdRP. 5) The mRNA that is generated from the inducible therapy will be translated into N2 protein (red), whereas the mRNA that is generated from the N1 vRNA will be translated into N1 protein (green). 6) Both neuraminidase proteins (N1 and N2) will be transported to the surface of the host cells. 7) When progeny virus is released from the host cell, they will contain both N1 and N2 surface expressed glycoproteins which can be targeted by the host's immune system, although the virus is genetically a H1N1 influenza virus. In addition to generating progeny virus with N1 and N2 expressed on the surface, the RNA intermediate generated by the inducible therapy can be incorporated into progeny virus, thus making an H1N2 influenza virus. This therapy has the ability to cause an influenza virus with a different neuraminidase subtype to undergo antigenic shift and become a neuraminidase subtype 2 virus.

# Chapter 7 - Discussion

## 7.1 Overview

In this dissertation, seven new small interfering RNA (siRNA; M747, M776, M832, NS570, NS595, NS615, and NA105) were characterized and two previously reported siRNAs (M950 and M331) were reinvestigated. Treatment of MDCK epithelial cells with M331-siRNA knocked down the expression of matrix proteins 1 and 2, which resulted in a significant attenuation of infectious virus. Treatment of MDCK epithelial cells with siRNA targeting the matrix transcripts (M747, M776, and M832) were able to attenuate infectious virus by knocking down the expression of matrix 2 protein expression. M776-siRNA treatment was the most effective at attenuating infectious influenza virus, with a reduction of 54.1% compared to negative treated cells. M776-siRNA treatment reduced infectious virus 19.5% more than the previously reported M950-siRNA. These results indicated that knocking down the expression of the matrix 2 protein alone was sufficient to attenuate infectious influenza titer.

Although nonstructural targeting siRNA (NS570, NS595, and NS615) were able to knock down nonstructural protein 1 and 2 expression and their corresponding transcripts, RNAi wasn't enough to attenuate infectious influenza virus. Instead, nonstructural-targeting siRNA treatment appeared to promote the production of defective interfering RNA, with the exception of NS570-siRNA treatment, which resulted in a slight increase in infectious virus. Nonstructural-targeting siRNA did, however, promote the production of type I interferons (interferon-α and interferon β) at the transcript and/or protein level. This could be attributed to the reduction in nonstructural protein 1 expression which is involved in antagonizing the expression of type I interferons during influenza infection (Pichlmair et al., 2006). Type I interferons are important immunomodulatory molecules that are involved in various antiviral responses such as translational repression and increased expression of RNases (Bergmann et al., 2000; Ronni et al., 1997). Accordingly, harnessing the ability to promote type I interferon expression

after nonstructural-targeting siRNA treatment could perform as an adjuvant to promote natural host-mediated anti-influenza responses.

As neuraminidases are essential for influenza infection and the release of progeny viruses from the host cell (Palese and Compans, 1976), I examined the ability of a new siRNA, NA105, to attenuate influenza virus infection. Treatment with NA105-siRNA did not result in neuraminidase-specific RNA degradation, but translational repression did occur. Knocking down the expression of the neuraminidase proteins with NA105-siRNA treatment attenuated influenza A/WS/33 (H1N1) infection by 56.2% compared to negative control treated cells.

Treatment of MDCK cells with a combination of siRNA prior to influenza infection (MOI 0.005) was evaluated in order to determine whether combination therapy elicited a more potent anti-influenza response than single siRNA treatment. Combination siRNA treatment resulted in a 62.4% reduction in infectious influenza virus, whereas treatment with a single siRNA (M950) resulted in a 41.4% reduction in infectious virus, compared to negative control siRNA treated MDCK cells. Treatment with a combination of siRNA may be a more efficient method of attenuating infectious virus, as combination treatment resulted in 35.7% less infectious virus than M950-siRNA treated cells. *In vivo* studies examining the use of different siRNA combinations and concentrations should be performed in order to determine the most effective RNAi-mediated anti-influenza treatment. Additional studies including M331- or NA105-siRNA treatment in the combination therapy may result in further attenuation of influenza viral titer.

In addition to revealing the utility of the new siRNA for therapeutic intervention or laboratory use, these studies also showed that a single siRNA can be used to knock down the expression of two proteins if it is designed to target two protein coding sequences. Treatment with M331-, NS570-, NS595-

, and NS615-siRNA, which targeted both matrix 1 and 2 or nonstructural 1 and 2 coding sequences, resulted in decreased expression of matrix proteins 1 and 2 (M331) or nonstructural proteins 1 and 2 (NS570, NS595, NS615). In contrast, M950-, M747-, M776-, and M832-siRNA, which just target a single protein coding sequence, only knocked down the expression of a single protein, matrix protein 2. Treatment with a single siRNA that can knock down the expression of two proteins might be a more potent means of RNAi and require lower concentrations of siRNA for therapeutic intervention.

Although siRNA treatments have been shown to be effective *in vitro* and *in vivo* (Hui et al., 2004; Li et al., 2010; Sui et al., 2009; Tompkins et al., 2004), off-target effects and other limitations are often associated with small regulatory molecule treatment (Jackson et al., 2003; Layzer et al., 2004). To circumvent these problems, an inducible anti-influenza therapy that expresses antisense RNA (asRNA) molecules under the control of the influenza promoter was developed. Co-transfection of MDCK cells with the inducible asRNA expression vector and vectors encoding for the RNA-dependent RNA polymerases (RdRP) resulted in expression of asRNAs (M950, M776, NS595, and NA105). These results indicate that expression is specifically induced by the influenza polymerase, as asRNA expression did not occur in cells treated with the inducible asRNA expression vector alone (no RdRP). Unfortunately, co-transfection of cells with the inducible asRNA expression vector and RdRP expression vectors did not produce enough asRNA to knock down vector expressed matrix protein 2 (pM776 treatment) or nonstructural protein 2 (pNS595 treatment).

I further examined the idea of using the conserved promoter to restrict anti-influenza targeting molecules to cells infected with influenza virus by creating another inducible vector that expresses neuraminidases. It was originally hypothesized that expression of a nonfunctional neuraminidase (NF-NA) could attenuate influenza infection by preventing the cleavage of sialic acid residues from the

surface of the host cell and progeny viruses, thus promoting viral aggregation and limiting the release of progeny viruses (Gottschalk, 1957; Palese et al., 1974). However, studies comparing the sialidase activity of nonfunctional and functional neuraminidases expressed by influenza RdRPs revealed that the RdRP appeared to revert the NF-NA to an active sialidase. Because the NF-NAs did not retain their inactive state, the effect of over-saturated expression of NF-NAs on viral attenuation could not be studied. RNA sequencing (RNA-seq) analyses can identify whether the RdRP did cause mutations that lead the nonfunctional neuraminidase to become a functional protein. Lentz et al. (1987) demonstrated that different amino acid substitutions can result in the production of NF-NA. For instance, in this study I evaluated whether point mutations of the influenza A/Tokyo/3/67 (H2N2) neuraminidase gene at nucleotides 826 and 828 resulting in replacing glutamate 276 amino acid with glutamine generated a neuraminidase with undetectable sialidase activity. Similar results were seen when amino acids 146, 152, 178, 277, and 406 were mutated. Therefore, in an effort to prevent the NF-NA from reverting back to a functional protein, studies evaluating the sialidase activity and protein structure of a neuraminidase containing multiple amino acid substitutions could be performed.

Upon further observation of the inducible neuraminidase therapy, I discovered that vector expressed RdRPs and infectious influenza RdRPs could promote the expression of neuraminidase viral RNA (vRNA) and proteins (subtype 2). Subsequent infection of naïve MDCK cells with the supernatant collected from inducible neuraminidase expression vector-treated and influenza pH1N1 infected MDCK cells gave rise to progeny influenza viruses containing neuraminidase subtype 2 encoding vRNA. This observation led us to propose that, although the inducible NF-NA expression vector is unable to directly attenuate influenza infection, the therapy can still be applied toward forcing one subtype of influenza viruses to undergo antigenic shift and become a different influenza subtype. By reversing antigenic variation and forcing circulating influenza viruses to converge to a particular subtype, current antiviral

therapies and recently developed vaccines would retain their effectiveness. For instance, the inducible neuraminidase expression vector can cause influenza viruses to converge and express a neuraminidase that is susceptible to oseltamivir or zanamivir. By expressing a neuraminidase that has recently been used in a seasonal influenza vaccination, such as the neuraminidase from A/Texas/50/2012 (H3N2), the population that has already been exposed to this virus through vaccination can clear infection quickly due to immunological memory (CDC, 2016). Alternatively, those that contract the virus and hadn't been vaccinated with A/Texas/50/2012 (H3N2) will produce an N2 subtype of influenza that would be susceptible to the previous vaccine.

The RdRP can cause a high rate of mutations due to its inability to proofread during replications and transcript (Kunkel and Mosbaugh, 1989), which could result in different strains of a particular neuraminidase subtype emerging from the inducible neuraminidase expression vector. The sequence encoding for the neuraminidase within the inducible anti-influenza therapy will remain static, meaning the anti-influenza therapy that is delivered can be screened to ensure different neuraminidase variants aren't delivered to the patient. Therefore, although the RdRP may generate mutant strains, the mutations won't accumulate over time causing more divergent and antigenically dissimilar strains (Lindstrom et al., 1998). Instead, with each new infection the therapy will begin with the wild-type N2 and only subsequent infections with the new N2 virus will gradually mutate into a different variant. Of course, future studies examining the rate of RdRP induced mutations should be examined in order to ensure that the RdRPs don't cause antigenically divergent neuraminidases that can evade the immune response of a vaccinated population or be resistant to antiviral therapies.

Chapter 4 showed that the inducible asRNA expression vector was able to generate asRNA molecules upon recognition of the influenza conserved promoter by the influenza RdRP. However, the

amount of asRNAs expressed by the vector was decreased up to 3 to 4-log less than the number of siRNAs needed to knockdown protein expression (refer to Chapter 3 & 4). Therefore, in order to reach the threshold of asRNAs needed to knockdown influenza protein expression, the promoters that drive the expression of the asRNAs should be modified. As discussed in Chapter 4, enhancer elements that are included upstream or downstream of the transcriptional start site can accelerate transcription initiation by gathering transcription factors to the promoter (Pennacchio et al., 2013; Shlyueva et al., 2014). Future studies examining the addition of either additional regulatory elements found upstream of the canine polymerase I promoter or the incorporation of virus-specific enhancers should be performed (Powell et al., 2015; Wang and Duke, 2007). For instance, incorporation of the CMV enhancer upstream of a species-specific promoter can yield up to a 50-fold higher gene expression, than an enhancer-less promoter (Liu et al., 2004a; Liu et al., 2004b; Muller et al., 2006). Increased expression of asRNAs can lead to a more efficient knock down influenza proteins and decreased viral titer.

The current structure of the inducible anti-influenza therapies is limited by their dependence on the canine RNA polymerase I promoter, which restricts the use of the therapy to canine cells. In order for the inducible anti-viral therapies to be evaluated across different species *in vitro*, the therapies must be modified to include a species-specific RNA polymerase I promoter. Developing anti-influenza therapies for different species can be a burdensome process. An alternative approach is to design the anti-influenza therapies with the T7 RNA polymerase promoter, a bacteriophage specific promoter, with a second expression cassette encoding for the T7 RNA polymerase. Because the T7 RNA polymerase promoter is specifically recognized by the T7 RNA polymerase, the polymerase must be supplemented into mammalian cells, as they do not naturally express the T7 promoter (de Wit et al., 2007). de Wit et al. (2007) used this principle to develop a reverse-genetics system for influenza A viruses and discovered that delivery of the T7 RNA polymerase with the reverse-genetics system was able to recover influenza

A viruses in human, canine, and avian cell lines. Transcription by the T7 RNA polymerase has been shown to be enhanced with the addition of guanosine residues at the start of transcription (de Wit et al., 2007; Pattnaik et al., 1992); incorporating two guanosine residues enhances gene expression by over 2-fold. Although this method could be effective *in vitro*, expression of the foreign bacteriophage T7 polymerase *in vivo* could induce an autoimmune-like response. The T7 promoter approach could possibly enable enhanced expression of the anti-influenza molecules, so comparative experiments examining the expression levels of asRNAs from vectors under the control of the canine RNA polymerase I promoter versus the T7 promoter and the various modifications should also be evaluated.

Another approach to increase the expression of asRNA molecules is to include multiple copies of anti-influenza molecule (neuraminidase or asRNA) coding sequences and promoters within the same plasmid. Instead of relying upon multiple plasmids to enter the same cell for enhanced expression, the delivery of one plasmid containing multiple expression cassettes can boost the expression of the anti-influenza molecules (Neumann et al., 2005). The size of the plasmid or deoxyribonucleic acid (DNA) should be considered as the use of large molecules can restrict efficient delivery into host cells (Yin et al., 2005) or packaging into viral vectors (Dong et al., 1996; Kumar et al., 2001).

While examining the amount of asRNAs or neuraminidases (N2) that are expressed after influenza infection, I discovered that clinical influenza isolates appeared to have a higher affinity for the influenza conserved promoter than cell culture-adapted strains. This was observed both by utilizing a luciferase reporter assay that contains the influenza conserved promoter (Chapter 4) and by examining the relative N2 expression levels after infection with adapted or clinical influenza isolates (Chapter 6). Clinical isolates Clin102 and Clin123 (pH1N1) had significantly more luciferase and neuraminidase expression compared to cell culture-adapted strains A/WS/33 (H1N1) or A/Swine/Iowa/15/30 (H1N1).

These observations highlighted the importance of using the proper infection model when testing the efficacy of a particular anti-viral therapy, as cell culture-adapted strains lose their wild-type phenotype over time and might not provide a reliable or realistic therapeutic outcome (Frensing et al., 2013).

Taken together, the studies reported in this dissertation have provided a number of new siRNA and alternative methods for anti-influenza therapeutics that should continue to be explored in order to create an effective anti-influenza therapy for clinical use.

## 7.2 Applications

Gene therapy is a treatment or preventative measure that involves the transplantation of functional genes in order to correct the activity of defective genes (Belmont and Caskey, 1986). Gene therapy can also be used to insert new genes into a host in order to cure or prevent a disease. The anti-influenza therapies described in this dissertation could be used as a gene therapy in order to prevent or treat influenza infection. The inducible neuraminidase expression vector could also be used to prevent the emergence of pandemic influenza viruses. Delivery of a new gene or genes into a human population is controversial due to safety (Fox, 1999; Yi et al., 2005) and ethical concerns (Berger and Gert, 1991). However, the development of genetically modified swine and poultry may be a realistic strategy.

Pandemic strains of influenza A viruses emerge by genetic reassortment between avian and human influenza viruses (i.e. 1957 and 1968 pandemic strains; Scholtissek et al., 1978). Swine have long been associated with being "mixing vessels" that allow for genetic reassortment to occur (Scholtissek, 1990). Epithelial cells within the swine upper respiratory tract, particularly the trachea, contain both $\alpha$-2,3-linked and $\alpha$-2,6-linked sialic acid receptors (Ito et al., 1998). Because human influenza viruses preferentially bind to $\alpha$-2,6-linked sialic acid receptors and avian influenza viruses preferentially bind to

α-2,3-linked sialic acid receptors (Connor et al., 1994), the mixing vessel hypothesis assumes that a swine cell, which contains both receptors, can be infected with both viruses at once. Studies have confirmed that avian and human influenza viruses are able to infect swine (Kida et al., 1994). When influenza viruses from avian and human reservoirs infect the same swine cell, the avian vRNA can be packaged into human influenza virus progeny, thus creating an antigenically novel and potentially pandemic influenza. Preventing genetic reassortment within swine could be a means to avoid the emergence of pandemic strains. Engineering genetically modified (GM) swine containing the inducible neuraminidase expression vector could force avian and human influenza viruses that infect swine to converge into a single subtype. If GM swine were vaccinated against pH1N1 and subsequently became infected with an avian strain of influenza that induces the expression of a conserved neuraminidase protein (pandemic H1N1/09 virus (pH1N1)), the immunological memory obtained from vaccination can clear the infection.

Transmission of avian influenza viruses directly to humans is associated with severe respiratory symptoms such as pneumonia that progress to multi-organ failure and pulmonary hemorrhage and result in a high mortality rate (Sandrock and Kelly, 2007). Between 2003 and 2016, there have been 854 human cases of avian influenza A virus infections and 450 deaths (Sandrock and Kelly, 2007; WHO, 2016). A majority of the infections were caused by human contact with infected poultry. The development of genetically modified poultry that express asRNAs, neuraminidase, or both, should be considered in order attenuate influenza infection in poultry, thus preventing transmission to humans and mortality.

Using a system that relies on the use of the influenza conserved promoter and polymerase is potentially beneficial because it limits protein expression to cells infected with the virus. However, there are also potential risks of using a system that generates intracellular RNA transcripts that can be integrated into the viral genome. Because RdRP has a high error rate, it could possibly add mutations to

the neuraminidase gene as it undergoes replication and transcription. Certain mutations in the neuraminidase gene might lead to pathogenic antigenic shift after integration of mutated RNA transcripts into the viral genome, leading to the emergence of highly pathogenic strains of influenza, an important potential concern for use of this inducible system.

## 7.3 Future directions

*In vivo* studies must be performed in order to better characterize the inducible anti-influenza therapies in a multicellular organism. The anti-influenza therapies used in this study are designed for expression in canine cells and must be modified to contain the RNA polymerase promoter specific to small animal models for influenza such as mice or ferrets (van der Laan et al., 2008). Alternatively, inducible anti-influenza therapies containing the T7 promoter and T7 RNA polymerase expression cassette could be utilized for studies in various species. Following successful modification of the anti-influenza therapies for enhanced and/or species-specific expression, studies confirming that the neuraminidase protein is delivered to the cell surface should be performed. Although it is assumed that the neuraminidase expressed by the anti-influenza therapy was delivered to the host surface because of the observed neuraminidase activity (refer to Chapter 4), flow cytometric or immunofluorescent microscopy can also be used to detect surface expressed neuraminidases. Once the presence of the neuraminidase on the host surface is confirmed, additional studies that evaluate the host's immune response to the neuraminidase (N2) protein should be evaluated. The neuraminidase protein will be present on the surface of progeny viruses and/or the host cell, and thus these proteins can succumb to immune recognition.  A primed immune response with immunological memory may be formed, thus clearing successive infected cells more rapidly and neutralizing progeny viruses that express the N2. Consequently, experiments evaluating neuraminidase (N2)-specific antibody production and activation

of neuraminidase (N2)-specific CD8+ and CD4+ T cells would be important indicators to determine whether this anti-influenza therapy can induce an influenza specific response.

After the inducible asRNA anti-influenza therapy has been improved for the *in vitro* studies, further work can be performed to confirm the functional ability of the asRNA to mediate RNAi. Rivas et al. (2005) demonstrated that asRNA can slip directly into Argonaute of the RISC. Therefore, further confirmation of the asRNAs' role in RNAi can be obtained by immunoprecipitation of the Argonaute and subsequent detection of the influenza-specific asRNA by qPCR.

The production of altered hemagglutinin and neuraminidase proteins as a result of antigenic shift is associated with the emergence of pandemic strains of influenza viruses (Scholtissek et al., 1978; Taubenberger, 2006). Thus, exchanging the neuraminidase gene for a hemagglutinin gene in the inducible neuraminidase expression vector should also be considered as an anti-influenza strategy. The development of an inducible anti-influenza therapy containing two expression cassettes, one encoding for a conserved neuraminidase and another encoding for a conserved hemagglutinin, could further promote the convergence of influenza viruses into a single influenza strain.

The Bunyaviridae and Arenaviridae family of segmented negative-strand RNA viruses have conserved and complementary 3' and 5' noncoding regions that form the viral promoter and is specifically recognized by their RdRP. The concept of using the viral conserved promoter to produce an inducible antiviral therapy could potentially be applied to these viral families (Walpita and Flick, 2005).

**7.4 Conclusions**

This dissertation explored a proof-of-concept study in which the influenza conserved promoter was used to induce precise expression of anti-influenza molecules during the time of influenza infection.

For instance, I have produced asRNA expression vectors that actively target influenza viruses only after the viral RdRP has entered the cell. The infection-induced expression of influenza-targeting molecules can ensure that the molecules are only delivered to the site at which they are needed and prevent adverse effects or unnecessary delivery of influenza-targeting molecules to healthy, uninfected cells.

Although the inducible nonfunctional neuraminidase expression vector was not able to attenuate infectious influenza virus by preventing the release of progeny virus, the therapy can still be used to promote the convergence of circulating influenza viruses into a particular subtype. The inducible neuraminidase expression vectors were shown to express vRNA under the control of the canine polymerase I promoter and neuraminidase mRNA after influenza infection. The vector expressed vRNA could be incorporated in infection-competent H1N1 influenza viruses, thus creating a H1N2 subtype.

The effective use of the anti-influenza therapies in the current form is constrained by limited expression levels and the use of canine specific promoters that are only active in canine cells. Yet, upon future modifications that allow for non-specific delivery of the inducible therapies and increased expression levels, the anti-influenza therapies could perform with high efficiency. More experimentation in vitro and in vivo should be performed in order to appreciate the utility of this unique system.

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# Chapter 8 - Appendix

# 8.1 Detection of Infectious Influenza Virus in Cough Aerosols Generated in a Simulated Patient Examination Room

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Keywords: influenza, transmission, aerosol, respirators, surgical masks

# Detection of Infectious Influenza Virus in Cough Aerosols Generated in a Simulated Patient Examination Room

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*Background.* The potential for aerosol transmission of infectious influenza virus (ie, in healthcare facilities) is controversial. We constructed a simulated patient examination room that contained coughing and breathing manikins to determine whether coughed influenza was infectious and assessed the effectiveness of an N95 respirator and surgical mask in blocking transmission.

*Methods.* National Institute for Occupational Safety and Health aerosol samplers collected size-fractionated aerosols for 60 minutes at the mouth of the breathing manikin, beside the mouth, and at 3 other locations in the room. Total recovered virus was quantitated by quantitative polymerase chain reaction and infectivity was determined by the viral plaque assay and an enhanced infectivity assay.

*Results.* Infectious influenza was recovered in all aerosol fractions (5.0% in >4 μm aerodynamic diameter, 75.5% in 1–4 μm, and 19.5% in <1 μm; n = 5). Tightly sealing a mask to the face blocked entry of 94.5% of total virus and 94.8% of infectious virus (n = 3). A tightly sealed respirator blocked 99.8% of total virus and 99.6% of infectious virus (n = 3). A poorly fitted respirator blocked 64.5% of total virus and 66.5% of infectious virus (n = 3). A mask documented to be loosely fitting by a PortaCount fit tester, to simulate how masks are worn by healthcare workers, blocked entry of 68.5% of total virus and 56.6% of infectious virus (n = 2).

*Conclusions.* These results support a role for aerosol transmission and represent the first reported laboratory study of the efficacy of masks and respirators in blocking inhalation of influenza in aerosols. The results indicate that a poorly fitted respirator performs no better than a loosely fitting mask.

Current evidence indicates that influenza can be transmitted by direct and indirect contact, droplet spray, and aerosol particles in the inhalable size range (≤10 μm) [1]. Transmission via respirable particles (≤4 μm), which can remain airborne for long periods and be inhaled into the lung alveoli, has been particularly controversial [2–5]. As early as 1941, aerosol transmission was demonstrated between ferrets that were

separated by up to 2.75 m [6]. More recent studies in ferrets [7–9] and guinea pigs [10–14] support airborne transmission (ie, by aerosol and/or large droplets and droplet nuclei) over considerably shorter distances (5–107 cm), although this transmission was strain dependent [9, 10, 14, 15]. Findings of studies in which influenza was administered experimentally by aerosol or intranasal inoculation provide indirect evidence that transmission of influenza in communities can occur by the aerosol route [16–18].

Transmission of influenza on respirable particles potentially generated during coughing, sneezing, and breathing is a concern in healthcare facilities because these particles may remain airborne for prolonged periods. Several studies have detected influenza RNA in the exhaled breath and coughs of patients with influenza

[19–23]. In 1 study, patients shed about 33 viral copies/min in aerosol particles ≥5 μm and 187 viral copies/min in particles <5 μm, and infectious virus was detected in the breath from 2 patients [22]. In another study, cough aerosols from 81% of the influenza-positive patients contained influenza RNA and 65% of the viral RNA was contained in particles <4 μm [23]. Two clinical studies showed that the highest concentrations of influenza RNA were detected in locations where the number of patients with influenza was highest and that 42%–53% of the viral RNA was contained in particles ≤4 μm [24, 25].

If it were known that infectious influenza virus is present on these small particles, the risk of infection could be properly assessed, and appropriate guidelines for prevention could then be established. To address this issue, a patient examination room containing a coughing manikin that "coughs" influenza virus into the room to simulate a patient with influenza and a breathing manikin to simulate a healthcare worker was constructed. National Institute for Occupational Safety and Health (NIOSH) aerosol samplers positioned within the breathing manikin and at various locations throughout the room were used to collect and size-fractionate the airborne particles. In this study, we show that infectious virus is present on a range of collected particles and we examine the effectiveness of surgical masks and N95 respirators in blocking virus inhalation.

## MATERIALS AND METHODS

### Cells and Virus
Madin-Darby canine kidney (MDCK) cells (American Type Culture Collection [ATCC] CCL-34) and influenza strain A/WS/33 (H1N1, ATCC VR-825, lot 58023547 at $1.58 \times 10^8$ 50% chicken embryo infectious dose [$CEID_{50}$]/mL, and lot 58772128 at $2.8 \times 10^6$ $CEID_{50}$/mL) were purchased from the ATCC and maintained as described elsewhere [26].

### Bioaerosol Samplers
NIOSH samplers, which collect and size-fractionate aerosols into 3 fractions (>4-, 1–4-, and <1-μm aerodynamic diameters), were used to collect influenza-containing aerosols [24, 27].

### Extraction of Virus From Surgical Gloves, Masks, and Respirators
Virus was eluted by overnight incubation at 4°C in 1 mL of supplemented [26] Hank's balanced salt solution.

### Real-Time Quantitative Polymerase Chain Reaction
Matrix gene copies were detected by real-time quantitative polymerase chain reaction analysis, as described elsewhere [26].

### Viral Plaque Assay
For viral plaque analysis, aerosol samples containing infectious influenza were inoculated onto a confluent lawn of MDCK cells and plaque-forming units were calculated, as described elsewhere [26].

### Viral Replication Assay
To enhance the ability to detect infectious virus, the copy number of infectious virus was amplified before detection by a modified 50% tissue culture infectious dose ($TCID_{50}$) assay, that is, the viral replication assay (VRA), as described elsewhere [26].
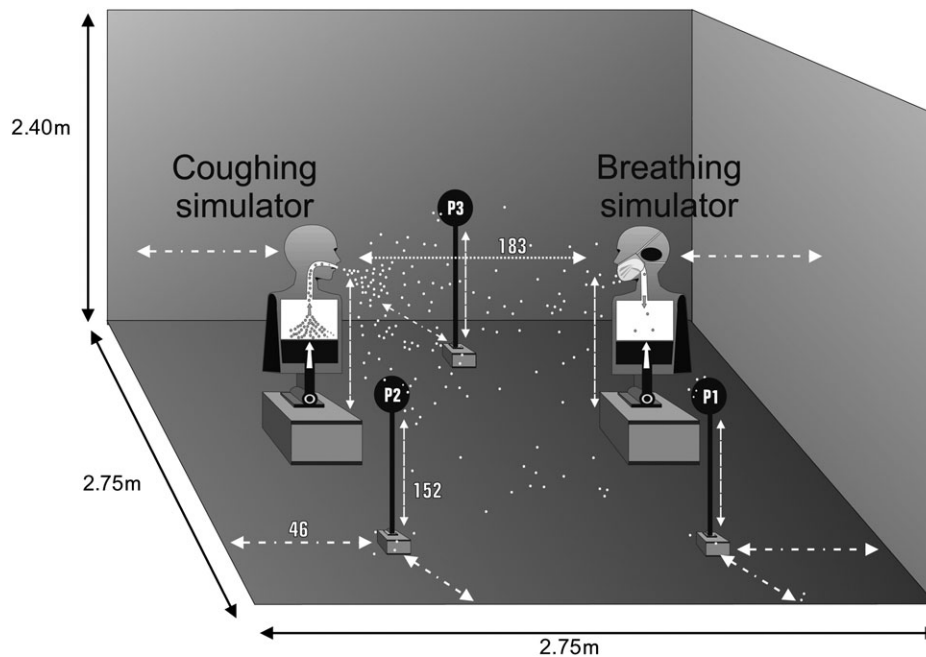
### Aerosol Exposure Simulation Chamber
The simulated examination room was 2.75 m × 2.75 m × 2.40 m and included a high-efficiency particulate air filter and a UV lamp [28] to disinfect the room. Influenza was aerosolized with an Aeroneb 2.5–4-μm micropump nebulizer (Aerogen), as described elsewhere [26], and loaded into the cough simulator remotely for a total of 5 coughs at approximately 2-minute intervals, also as described elsewhere [28]. The coughing simulator uses a metal bellows driven by a computer-controlled linear motor (Model STA2506; Copley Controls) to reproduce the flow and aerosol pattern of a human cough. The cough had a 4.2-L volume with a peak flow of 16.9 L/s and a mean flow of 5.28 L/s. The digital breathing simulator (Warwick Technologies) was equipped with a standard medium-sized head form (Sheffield model 189003; ISI). The breathing waveform was sinusoidal with a flow rate of 32 L/min (ISO standard for an adult 1.88 m tall with a mass of 85 kg engaged in moderate work) [29]. The coughing and breathing simulators were synchronized so that each cough was initiated at the start of an inhalation.

A surgical mask (Kimberly Clark 47625) or N95 respirator (3MM1860) was either tightly sealed over the mouth of the breathing simulator using silicone sealant or attached using the tie straps or elastic headbands of the mask or respirator. The fit factor of each mask or respirator was measured using a standard respirator fit-testing device (Model 8038 PortaCount Pro Plus; TSI). The fit factor is defined as 1/fraction of particles that pass through the mask.

## RESULTS

### Detection of Infectious Influenza on Aerosolized Particles
To determine whether infectious influenza could be recovered from airborne particles, influenza expelled by the coughing simulator was collected for 60 minutes by 5 NIOSH samplers. The samplers drew aerosol samples from a port located approximately 1 mm above the mouth (through mouth) of the breathing simulator, 10 cm to the right of the mouth (beside mouth), and at 3 other positions (P1, P2, P3) within the simulation chamber (Figure 1). Approximately $3.49 \times 10^6$ total virus was coughed into the simulation chamber (202 virus per liter of chamber air). The average total recovered virus per liter of collected air from each of the 5 samplers in 5 independent

**Figure 1.** Three-dimensional view of the aerosol exposure chamber. National Institute for Occupational Safety and Health samplers collected aerosols through the mouth (depicted as black oval in breathing mannequin's head), 10 cm beside the mouth of the breathing simulator, and in 3 other positions (P1, P2, P3), as shown. The mouths of the coughing and breathing simulators and sampler inlets at P1, P2, and P3 were located 152 cm above the floor (approximate height of a sitting patient and healthcare worker). For 3 experiments, fingertips from medical gloves were also placed on the manikin's forehead and alongside 3 of the aerosol samplers. All dimensions adjacent to white arrows within chamber are in centimeters.

experiments was $1.35 \times 10^4$ (standard error, $1.74 \times 10^3$) (Figure 2A). Most of the virus was recovered in the 1–4-μm aerosol fraction (75.5%) and <1-μm fraction (19.5%); the remainder was detected in the >4-μm fraction (5.0%) (Figure 2A). Infectious influenza, assessed by the viral plaque analysis, was recovered in all 3 fractions and from all NIOSH samplers regardless of their position within the simulation chamber (Figure 2B). The presence of infectious influenza was confirmed using an enhanced infectivity assay, the VRA (Figure 2C). There was no statistically significant difference in the percentages of virus that remained infectious in the 3 fractions or the 5 samplers.

### Tightly Fitted (Sealed) Surgical Masks and N95 Respirators and Exposure to Airborne Infectious Influenza

To examine the extent to which personal protective equipment (PPE) can effectively protect against aerosol exposure to influenza, surgical masks and N95 respirators were sealed to the breathing manikin's face to prevent aerosols from circumventing the PPE. Fit factors were determined to be 135 for the surgical masks and 200+ for the N95 respirators (for the sealed PPE, the fit factor measurement reflects the penetration of particles through the PPE, because face seal leakage was prevented). The total virus collected through the manikin's mouth by a NIOSH sampler compared with that collected beside the mouth revealed that 99.8% was blocked from entering the mouth by a tightly fitted respirator (Figure 3A). Furthermore, ≥99.5% of viral entry was blocked for all aerosol fractions. Similarly, 99.6% of the infectious virus was blocked from entering the mouth, with ≥99.4% of virus from each aerosol fraction blocked from entry (Figure 3B). The VRA confirmed these results and showed that 99.8% of the total infectious virus was blocked by the sealed respirator (Figure 3C).

A tightly fitted mask blocked 94.5% of the total virus and ≥91.8% was blocked regardless of which aerosol fraction was tested (Figure 4A). Similarly, 94.8% of the total infectious virus was blocked, with ≥92.7% being blocked regardless of which aerosol fraction was tested (Figure 4B). The VRA showed that 92.9% of the total infectious virus was blocked by the sealed mask (Figure 4C).

### Loosely Fitting (Unsealed) Surgical Masks and Poorly Fitting (Unsealed) N95 Respirators and Exposure to Airborne Infectious Influenza

Surgical masks typically have low fit factors owing to gaps and leaks between the mask and face. N95 respirators that are poorly fitted or improperly worn can also have a dramatically reduced fit factor [30–32]. To simulate low fit factors, masks and respirators were attached to the face using the tie strings or elastic headbands but without using sealant. Fit factors
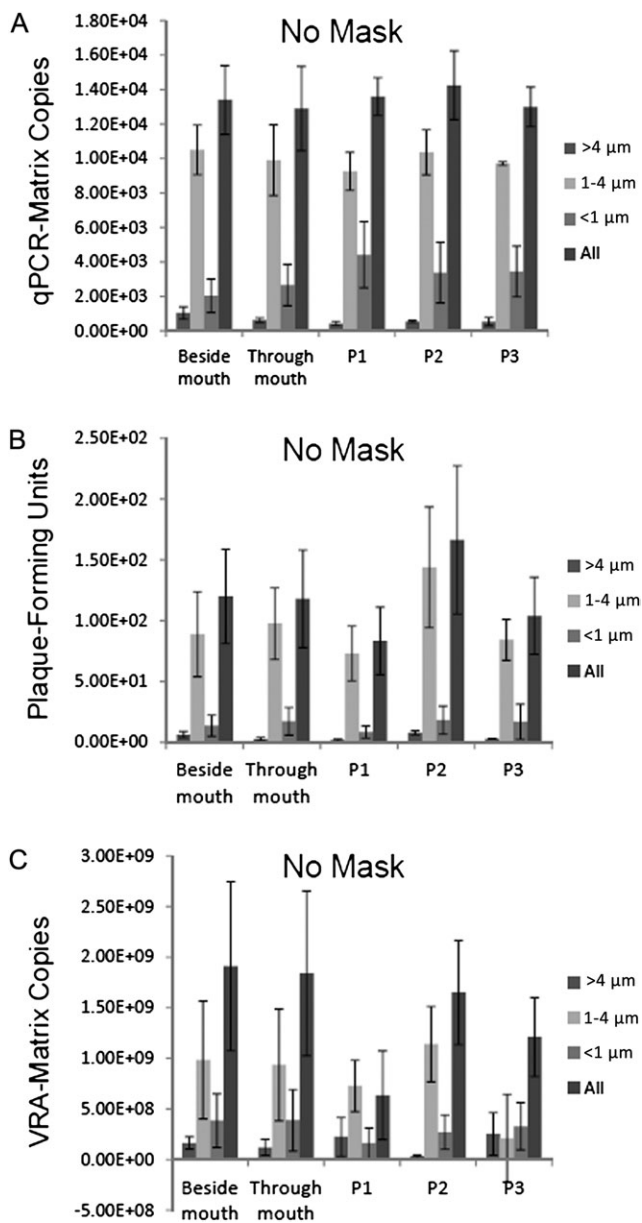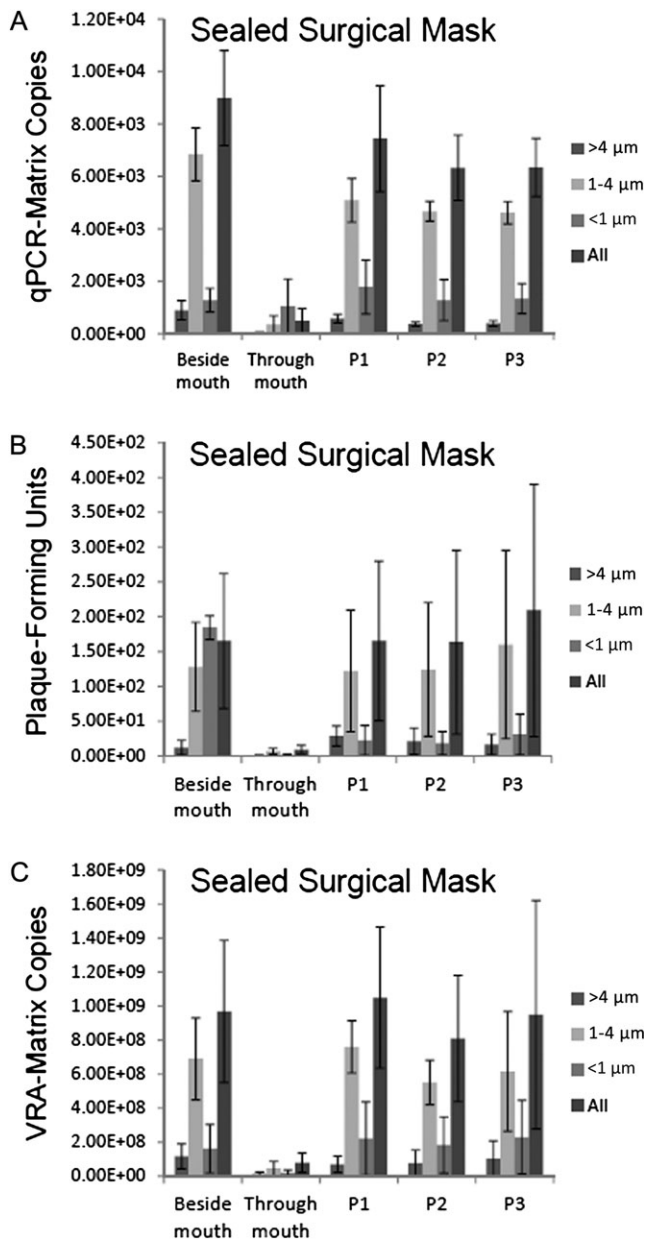
**Figure 2.** Detection of infectious influenza on aerosolized particles. National Institute for Occupational Safety and Health (NIOSH) samplers drew aerosol samples from a port located ∼1 mm above the mouth (through mouth) of the breathing simulator, 10 cm to the right of the mouth (beside mouth), and at 3 other positions (P1, P2, P3) within the environmental chamber. The amount of influenza virus detected in each fraction (>4, 1–4, and <1 μm) collected by the NIOSH sampler per liter of air collected is shown. *A, B,* Amounts of total virus (infectious and noninfectious) collected in each fraction was determined by quantification of the matrix gene by quantitative polymerase chain reaction (qPCR) (*A*) and by the plaque-forming unit assay (*B*). *C,* Viral replication assay (VRA) demonstrated the amount of infectious virus collected after amplification in Madin-Darby canine kidney cells to increase the sensitivity of detection. Data are means ± standard errors (n = 5).
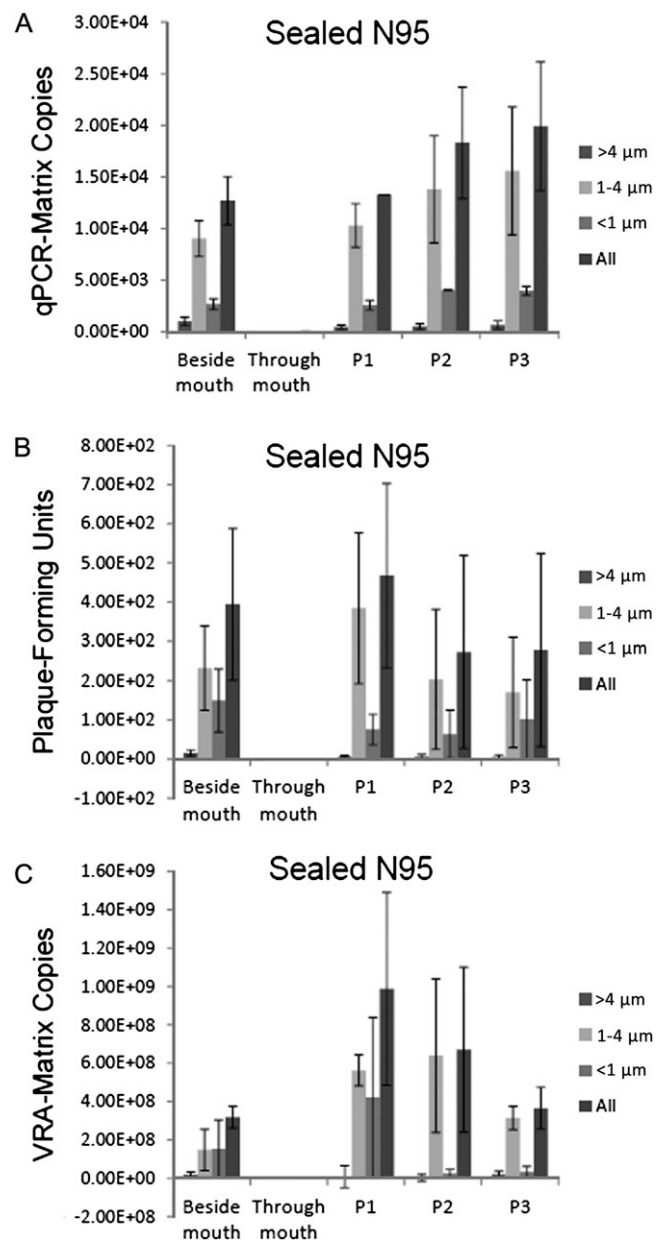
**Figure 3.** Tightly fitting (sealed) N95 respirators efficiently block exposure to airborne infectious influenza. An N95 respirator was sealed over the mouth of the breathing mannequin with silicone caulk. Amounts of infectious and noninfectious virus collected are as described for Figure 2. Data are means ± standard errors (n = 3); qPCR, quantitative polymerase chain reaction; VRA, viral replication assay.

ranged from 2.3 to 4.6 (100 is considered passing). The total virus collected through the manikin's mouth compared with that collected by a sampler beside the mouth showed that 69.9% was blocked from entering the mouth by a poorly fitting respirator (Figure 5A). Furthermore, ≥64.5% of virus from all 3 aerosol fractions was blocked from entering. Approximately 66.5% of the total infectious virus was blocked by the unsealed,

**Figure 4.** Tightly fitting (sealed) surgical masks efficiently block exposure to airborne infectious influenza. A surgical mask was sealed over the mouth of the breathing manikin with silicone caulk. The amount of infectious and noninfectious virus collected is as described for Figure 2. Data are means ± standard errors (n = 3); qPCR, quantitative polymerase chain reaction; VRA, viral replication assay.



**Figure 5.** Poorly fitting (unsealed) N95 respirators are less efficient at blocking exposure to airborne infectious influenza. An N95 respirator was fitted over the mouth of the breathing manikin with the mask's tie straps. The amount of infectious and noninfectious virus collected is as described for Figure 2. Data are means ± standard errors (n = 3); qPCR, quantitative polymerase chain reaction; VRA, viral replication assay.

poorly fitting respirator ($\geq$59.2% of the blocked infectious virus was in the $\leq$4 $\mu$m fractions; Figure 5B). Similarly, the VRA showed that 66.5% of the total infectious virus was blocked from entry (Figure 5C).

Similarly, a poorly fitting mask blocked 68.9% of the total virus (Figure 6A), and entry of 56.6% of the total infectious virus was blocked ($\geq$51.2% of the blocked infectious virus was in

the $\leq$4-$\mu$m fractions) (Figure 6B). In contrast, the VRA indicated that only 11.6% of the total infectious virus was blocked (Figure 6C).

### Recovery of Infectious Influenza Virus From PPE

Significant amounts of influenza were recovered from a 25-mm–diameter coupon punched out from the center of masks and
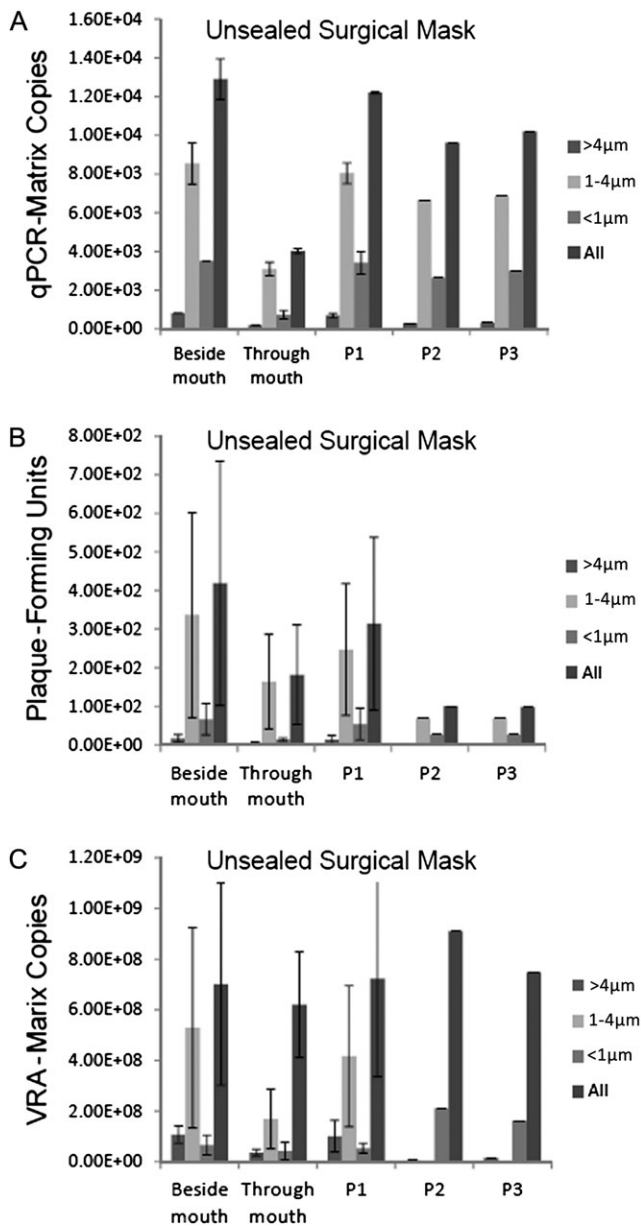
**Figure 6.** Loosely fitting (unsealed) surgical masks are less efficient at blocking exposure to airborne infectious influenza. A surgical mask was fitted over the mouth of the breathing manikin with the mask's tie straps. The amount of infectious and noninfectious virus collected is as described for Figure 2. Data are means ± standard errors (n = 2); qPCR, quantitative polymerase chain reaction; VRA, viral replication assay.

respirators worn by the breathing simulator (Table 1). The amount of virus recovered on the mask and respirator coupons were 5.6%–5.8% and 8.2%–11.0%, respectively, of the total amount recovered by NIOSH samplers positioned 10 cm beside the manikin's mouth. Infectious influenza was present on all mask and respirator coupons, regardless of whether or not they were sealed to the manikin's head, and infectivity of the

recovered virus was reduced approximately 4–8 fold from that of the viral preparation before aerosolization.

The location of virus within the coupons was also assessed. Coupons of 19-mm diameter were punched out from the center and side sections of a sealed mask and respirator, and the 3 layers of each coupon (outer water-repellent cover, middle filtering layer, and inner hydrophilic lining) were then separately processed. Most of the virus was located in the middle and outer layers of each coupon (Table 2). The inner layers of the coupons from the center and side sections of the mask contained only 2.3% and 0.8%, respectively, of the total virus recovered. The inner layers of the coupons from the center and side sections of the respirator contained only 0.4% and 0.2%, respectively, of the total virus recovered.

The tips (∼20 mm) of surgical gloves were attached to the manikin's forehead and to NIOSH samplers located at positions P1, P2, and P3 during 3 simulated examinations (Figure 1). Total influenza was recovered from the glove tips placed at all positions, and infectious virus was recovered from glove tips located on the manikin's forehead and at P1 (Table 3).

## DISCUSSION

To maintain the availability of healthcare workers during an influenza pandemic, it is imperative to assess the nature of the risk of transmission in healthcare settings, such as during patient examinations, and to develop appropriate mitigation measures. To address this, Lindsley et al [28] constructed a simulated examination room and showed that coughed aerosol particles of potassium chloride disperse within minutes throughout the room. The present study with influenza supports that finding and suggests that anyone present in a room with a patient who has influenza might be at risk of exposure.

Before aerosolization of the virus in the 16 simulated exposure experiments, an average 6.3% was infectious (the stock presumably contained >93% defective [noninfectious] virus) and 2.2% of the virus remained infectious after collection by the NIOSH samplers. However, the final infectivity varied considerably among the individual experiments. As reported by Cao et al [33], some of the losses were probably due to the use of the NIOSH sampler, which fractionates aerosolized virus on the dry walls of a collection tube and Teflon filter. In addition, humidity may have influenced the survival of infectious virus; 2 studies reported that maximal stability of influenza occurs at 20%–40% relative humidity, and minimal stability at 50% relative humidity [11, 34]. In our study, the relative humidity in the simulation chamber was 44%–63%.

In a real-world examination room, the actual number of aerosolized viral particles that a healthcare worker could potentially inhale would be dependent on the number of viral copies shed by infected individuals and the airflow in the room.

**Table 1. Recovery of Infectious Virus From Surgical Masks and N95 Respirators**

| | | | | Infectious Virus, % | |
|---|---|---|---|---|---|
| Mask Type | Total Matrix Copies Collected Beside Mouth | Total Matrix Copies on Mask Coupon | Total PFUs on Mask | Before Aerosolization | On Mask Coupon |
| Unsealed SM | $2.70 \times 10^6 \pm 2.20 \times 10^5$ | $1.52 \times 10^5 \pm 1.28 \times 10^5$ | $3.56 \times 10^3 \pm 2.81 \times 10^3$ | $9.8 \pm 2.7$ | $2.6 \pm 0.4$ |
| Unsealed N95 | $1.33 \times 10^6 \pm 7.10 \times 10^5$ | $1.46 \times 10^5 \pm 4.26 \times 10^4$ | $2.28 \times 10^4 \pm 2.01 \times 10^4$ | $5.6 \pm 1.9$ | $1.1 \pm 0.7$ |
| Sealed SM | $1.89 \times 10^6 \pm 3.78 \times 10^5$ | $1.1 \times 10^5 \pm 9.8 \times 10^4$ | $1.7 \times 10^3 \pm 1.7 \times 10^3$ | $7.1 \pm 2.1$ | $0.9 \pm 0.8$ |
| Sealed N95 | $3.11 \times 10^6 \pm 3.95 \times 10^5$ | $2.54 \times 10^5 \pm 1.12 \times 10^5$ | $2.70 \times 10^3 \pm 2.53 \times 10^3$ | $5.8 \pm 4.1$ | $0.8 \pm 0.7$ |

A 25-mm diameter coupon was punched out from the center of the mask and respirator (mouth area) and assayed for the presence of influenza. Data are the means ± standard errors of 2 experiments each for studies with unsealed surgical masks, sealed surgical masks, and sealed N95 respirators, or 3 experiments for studies with unsealed N95 respirators.

Abbreviations: N95, N95 respirator; PFUs, plaque-forming units; SM, surgical mask.

In 1 study, naturally infected participants shed 33 copies/min in aerosol particles $\geq 5$ μm and 187 viral copies/min in particles $< 5$ μm [22]. Assuming an examination room is occupied by $\geq 1$ infected patients for 60 minutes, up to $1.12 \times 10^4$ viral particles $< 5$ μm in size may be shed, and $1.23 \times 10^3$ viral particles could potentially be inhaled by a healthcare worker. Teunis et al [35] developed a dose-response model for infectivity and pathogenicity of influenza A using 3 clinical studies in which influenza was administered via aerosol and 12 studies in which it was administered through intranasal droplet inoculation. They concluded that the probabilities of infection by either aerosol or droplet transmission are approximately equal and that the probability of infection is significant ($P_{inf} = .2-.4$) at low doses ($10^{1-2}$ TCID$_{50}$ infectious units). They also noted that most of the freshly shed viruses are potentially infectious and that environmental conditions may rapidly decrease the fraction of infectious viruses.

Two systematic reviews on the use of surgical masks and N95 respirators came to different conclusions. One review was based primarily on data from severe acute respiratory syndrome outbreaks, and these authors concluded that interventions, including the use of masks or respirators, could reduce the spread of respiratory infections [36]. The other review found few data showing that masks are effective against influenza [37]. Similarly, a Canadian prospective randomized controlled trial of respirator and mask use by nurses found that use of a mask resulted in similar rates of laboratory-confirmed influenza, mostly documented by serological changes [38]. A recent large cluster randomized clinical trial conducted in China, where mask acceptance is high, compared the effectiveness of masks and respirators (fit tested and non–fit tested) in protecting healthcare workers from respiratory infection [39]. Their conclusion was that a benefit of respirators was suggested but would need to be confirmed by a larger trial

**Table 2. Penetration of Influenza Into Layers of Mask and Respirator**

| | Center Coupon | | | Side Coupon | | |
|---|---|---|---|---|---|---|
| Mask Layer | Total Matrix Copies | PFUs | Infectious Virus on Mask, % | Total Matrix Copies | PFUs | Infectious Virus on Mask, % |
| Sealed SM | | | | | | |
| Outer | $1.66 \times 10^3$ | $1.25 \times 10^1$ | 0.8 | $3.42 \times 10^3$ | $1.25 \times 10^1$ | 0.4 |
| Middle | $4.61 \times 10^3$ | 0.0 | 0.0 | $1.55 \times 10^4$ | $5.00 \times 10^1$ | 0.3 |
| Inner | $1.47 \times 10^2$ | 0.0 | 0.0 | $1.56 \times 10^2$ | 0.0 | 0.0 |
| Total | $6.42 \times 10^3$ | $1.25 \times 10^1$ | | $1.91 \times 10^4$ | $6.25 \times 10^1$ | |
| Sealed N95 | | | | | | |
| Outer | $1.46 \times 10^4$ | $3.13 \times 10^2$ | 2.1 | $4.14 \times 10^4$ | $6.25 \times 10^2$ | 1.5 |
| Middle | $3.45 \times 10^4$ | $5.38 \times 10^2$ | 1.6 | $1.50 \times 10^5$ | $1.24 \times 10^3$ | 0.8 |
| Inner | $2.04 \times 10^2$ | 0.0 | 0.0 | $3.13 \times 10^2$ | 0.0 | 0.0 |
| Total | $4.93 \times 10^4$ | $8.51 \times 10^2$ | | $1.92 \times 10^5$ | | |

A 19-mm-diameter coupon was punched out from the center and side of each mask and respirator. The 3 layers of each coupon were separated and assayed for the presence of influenza virus.

Abbreviations: N95, N95 respirator; PFUs, plaque-forming unit; SM, surgical mask.

**Table 3. Recovery of Infectious Virus From Surgical Gloves**

| Glove Location Within Environmental Chamber | Virus on Glove Tip | | Infectious Virus, % | |
| --- | --- | --- | --- | --- |
| | Total Matrix Copies | Total PFUs | Before Aerosolization | On Glove |
| On forehead | $2.93 \times 10^3 \pm 2.27 \times 10^3$ | $2.5 \pm 2.0$ | $9.2 \pm 2.1$ | $0.1 \pm 0.1$ |
| P1 | $6.58 \times 10^2 \pm 1.54 \times 10^2$ | $6.3 \pm 5.1$ | $9.2 \pm 2.1$ | $2.7 \pm 1.1$ |
| P2 | $3.13 \times 10^3 \pm 2.00 \times 10^3$ | $0$ | $9.2 \pm 2.1$ | $0$ |
| P3 | $3.08 \times 10^3 \pm 2.16 \times 10^3$ | $0$ | $9.2 \pm 2.1$ | $0$ |

The ~20-mm tip of the index finger of a surgical glove was assayed for the presence of influenza virus. Data are means ± standard errors of 3 experiments. A total of $1.18 \times 10^4 \pm 1.73 \times 10^3$ matrix copies per liter of air were collected from a National Institute for Occupational Safety and Health sampler positioned beside the mouth.

Abbreviations: P1, P2, P3, position 1, position 2, position 3; PFUs, plaque-forming units.

because the study was underpowered. Rates of fit test failure were very low, perhaps accounting for the study's finding that fit testing did not improve the efficacy of respirators. Thus, data from clinical settings has thus far failed to resolve uncertainty about the relative importance of aerosol transmission and the necessity for use of N95 respirators to prevent it.

In our study, we evaluated the effectiveness of surgical masks and N95 respirators when the masks and respirators were sealed to a manikin's face or unsealed to a manikin's face and documented to fit poorly (approximating how masks normally perform and how poorly fitting respirators might perform in the field). Sealed masks were not as effective as sealed respirators at blocking total influenza (94.5% vs 99.8% blocked) or at blocking infectious virus (95.8% vs 99.6% blocked). Rengasamy et al [40] examined the filtration efficiency of 5 models of masks using a standard filter tester and found penetration values ranging from <0.2% to 63% at 30 L/min. Our results were comparable to those for the mid-range masks in their study. Because filtration efficiencies of masks vary considerably, protection afforded by even a sealed mask would be further reduced. Unsealed masks and unsealed, poorly fitting respirators were not effective at blocking total influenza virus (68.5% vs 64.5% blocked) or infectious virus (56.6% vs 66.5%).

This result shows that gaps between the wearer's face and the PPE can have a tremendous impact on the protection offered. This is especially applicable for masks, which are not designed to seal to the wearer's face. Typical fit factors from volunteers wearing these types of masks have been reported to range from 2.5 to 9.6 [29, 30]. In contrast, respirators are required to have fit factors ≥100, and measurements from volunteers wearing properly fitted respirators have shown much higher factors than from those wearing masks [30–32]. In our study, the fit factors for sealed respirators and masks were 200+ and 135, respectively, whereas unsealed masks and respirators had fit factors of 2.3 and 4.6. Thus, the sealed respirators obtained fit factors similar to well-fitting respirators, and the unsealed masks obtained fit factors comparable

to those on human subjects during realistic use conditions. Therefore, our results support the use of properly fitted N95 respirators for maximal protection against infectious airborne influenza.

Finally, 2 important notes about our results should be made. First, the high fit factors seen with the sealed surgical masks in our study should not be interpreted to mean that surgical masks can be depended upon to provide respiratory protection. The filtration capacity of surgical masks varies tremendously from model to model, and large face seal leaks, which admit substantial amounts of aerosol particles, are normal even when surgical masks are tied tightly to the face. Second, the fit factor of respiratory PPE represents the protection offered by the PPE under ideal test conditions. In industrial hygiene, a distinction is made between this and the "assigned protection factor," which is the amount of protection that would be expected from the PPE during real-world usage and can be considerably lower. For PPE to provide the needed protection to workers, they must be part of a respiratory protection program that includes training and fit testing of workers for the PPE they will use.

## Notes

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# 8.2 High Humidity Leads to Loss of Infectious

# Influenza Virus from Simulated Coughs

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**Abstract**

**Background.** The role of relative humidity in the aerosol transmission of influenza was examined in a simulated examination room containing coughing and breathing manikins.

**Methods.** Nebulized influenza was coughed into the examination room and Bioaerosol samplers collected size-fractionated aerosols (<1 µm, 1-4 µm, and >4 µm aerodynamic diameters) adjacent to the breathing manikin's mouth and also at other locations within the room. At constant temperature, the RH was varied from 7-73% and infectivity was assessed by the viral plaque assay.

**Results.** Total virus collected for 60 minutes retained 70.6-77.3% infectivity at relative humidity ≤23% but only 14.6-22.2% at relative humidity ≥43%.  Analysis of the individual aerosol fractions showed a similar loss in infectivity among the fractions. Time interval analysis showed that most of the loss in infectivity within each aerosol fraction occurred 0-15 minutes after coughing. Thereafter, losses in infectivity continued up to 5 hours after coughing, however, the rate of decline at 45% relative humidity was not statistically different than that at 20% regardless of the aerosol fraction analyzed.

**Conclusion.** At low relative humidity, influenza retains maximal infectivity and inactivation of the virus at higher relative humidity occurs rapidly after coughing. Although virus carried on aerosol particles <4 µm have the potential for remaining suspended in air currents longer and traveling further distances than those on larger particles, their rapid inactivation at high humidity tempers this concern. Maintaining indoor relative humidity >40% will significantly reduce the infectivity of aerosolized virus.

**Introduction**

Winter influenza outbreaks occur with seasonal regularity in temperate climates and it has been suggested that humidity may affect transmission (Shaman et al., 2011; Shaman et al., 2010). Previous studies using influenza aerosols in small settling chambers generally concluded that aerosolized virus was inactivated at high relative humidity (RH) but survived much better at low RH (Harper, 1961; Hemmes et al., 1960; Hood, 1963). Other studies (Schaffer et al., 1976; Shechmeister, 1950) revealed that survival was optimum at low RH, moderate at high RH and minimum at middle RH. The aerodynamic diameters of the aerosolized particles were not determined in any of these studies; therefore, the influence of particle size on inactivation of virus has not been reported. Lowen et al. (2007) used a guinea pig model to directly test whether humidity affected aerosol transmission of influenza from infected animals to uninfected animals, housed in adjacent but separate cages in an environmental chamber with five RHs ranging from 20-80% at 20 $^{\circ}$C. In their study, transmission rates were 75-100% at 20%, 35%, and 65% RH, but only 25% at 50% RH and 0% at 80% RH. However, air samples were not collected to confirm that guinea pigs housed at different RHs shed similar amounts of aerosolized virus.

During the winter, people spend the majority of their time indoors and the risk of aerosol transmission of influenza by coughing, sneezing and breathing is a concern because respirable particles carrying influenza may remain airborne for prolonged periods. Influenza RNA has been detected in the exhaled breath and coughs of patients with influenza (Fabian et al., 2008; Huynh et al., 2008; Stelzer-Braid et al., 2009) and clinical studies during influenza seasons indicated that influenza was detected in aerosol particles ≤4 μm (Blachere et al., 2009; Lindsley et al., 2010a). A recent study of indoor locations where jet travelers are likely to interact with locals determined that RH is one of the primary factors associated with aerosol transmission of influenza (Hanley and Borup, 2010).

Healthcare workers treating influenza patients are particularly prone to infection as they can be exposed to multiple patients in closed examination rooms over the course of a day. A novel approach to assess risk factors is the use of manikins in a controlled environment. This approach has been used to study the flow of human respired air in a room (Bjorn and Nielsen, 2002), the effects of ventilation on respired air (Pantelic et al., 2009; Qian and Li, 2010; Qian et al., 2006), and the efficacy of surgical masks and respirators for protection of healthcare workers exposed to coughed influenza aerosols (Lindsley et al., 2012; Noti et al., 2012).

To address whether humidity contributes to the risk of aerosol transmission of influenza, a simulated examination room equipped with environmental controls was constructed that contained a coughing and breathing manikin to simulate a healthcare worker's exposure (Lindsley et al., 2012; Noti et al., 2012). In this study, the virus collected at the breathing manikin was separated into 3 size fractions according to their aerodynamic diameters (>4 µm, 1-4 µm, and <1 µm). We show that at low RH there is little loss in infectivity of virus from any particle fraction within the first hour but at moderate RH, 60-80% of the virus is inactivated and is dependent on viral particle size. The fastest rate of inactivation was seen in the >4 µm particle size where 78% of infectivity was reduced within 16-30 minutes of a cough.

**Materials and Methods**

*Cells and Virus*

Madin-Darby canine kidney (MDCK) cells (ATCC CCL-34) and Influenza strain A/WS/33 (H1N1, ATCC VR-825) were purchased from the American Type Culture Collection (ATCC, Manassas, VA) and maintained as described (Blachere et al., 2011).

*Bioaerosol Samplers*

National Institute for Occupational Safety and Health (NIOSH) bioaerosol samplers, which collect and size-fractionate aerosols into three fractions (> 4 μm, 1-4 μm, and < 1 μm aerodynamic diameters), were used to collect influenza-containing aerosols (Blachere et al., 2009; Lindsley et al., 2006).

*Real-time qPCR*

The amount of total virus (infectious and non-infectious) in an aerosol sample was determined by real-time qPCR analysis to assess the number of Matrix1 gene copies as described (Blachere et al., 2011).

*Viral Plaque Assay (VPA)*

The number of infectious virus within an aerosol sample was determined by the VPA. Aerosols containing infectious influenza were inoculated onto a confluent lawn of MDCK cells and plaque forming units (PFU) were calculated as described (Blachere et al., 2011).

*Aerosol Exposure Simulation Chamber*

The simulated examination room (aerosol exposure simulation chamber) is 3.16 m x 3.16 m x 2.27 m high and includes a HEPA filter and an ultraviolet lamp (Lindsley et al., 2012; Noti et al., 2012) to disinfect the chamber. The virus solution was aerosolized with an Aeroneb 2.5-4 μm micropump nebulizer (Aerogen, Galway, Ireland) and loaded into the cough simulator remotely for a total of 5 coughs at approximately 1 minute intervals as described (Blachere et al., 2011; Lindsley et al., 2012; Noti et al., 2012). The coughing simulator uses a metal bellows driven by a computer-controlled linear motor (Model STA2506, Copley Controls, Canton, MA) to reproduce the flow and aerosol pattern of a human cough. The cough had a 4.2 liter volume with a peak flow of 16.9 liters/second and a mean flow of 5.28

liters/second. The digital breathing simulator (Warwick Technologies Ltd., Warwick, UK) was equipped with a standard medium-sized head form (Sheffield model 189003, ISI Lawrenceville, GA). The breathing waveform was sinusoidal with a flow rate of 32 liters/minute (ISO standard for an adult 1.88 m tall with a mass of 85 kg engaged in moderate work) (ISO, 2007). The coughing and breathing simulators were synchronized so that each cough was initiated at the start of an inhalation. NIOSH aerosol samplers collected aerosols 1 mm above the manikin's mouth (through the mouth), 10 cm to the right and left of the mouth, and at two locations (P1 and P3) inside the room. For time course analysis, exam room air samples were collected from 3 samplers positioned outside the room (P2) to enable immediate processing of the collected samples. Aerosol particle concentrations in the exposure chamber were continuously monitored using an optical particle counter (OPC; Model 1.108, Grimm Technologies, Inc., Douglasville, GA) located 55 cm below the mouth of the coughing manikin The cough aerosol output from the cough simulator was measured using a Spraytec aerosol analyzer (Malvern Instruments, Worcestershire, UK).The aerosol exposure simulation chamber (Enviroline walk-in chamber, Norlake, Hudson, WI) maintained the selected temperature and humidity using a desiccant-based industrial dehumidifier (IAT-150-E, Innovative Air Technologies, Covington, GA), a centrifugal atomizer (Norlake), a remote heating/refrigeration system (NAWE150RL-3, Norlake) and a programmable temperature/humidity controller (CP8L, Norlake). After the chamber equilibrated at the desired temperature and humidity, the environmental control system was shut off and dampers within the system prevent aerosol particle losses in the dehumidifier and the heating/cooling air circulation system. The wall and floor seams of the chamber are sealed tightly with silicone caulk to prevent aerosol particles from leaking. The entrance door has manual locks that push the door tightly against seals that further prevent aerosol leakage during the equilibration and collection periods.

*Statistical Methods*

The analysis of the number of PFUs induced by viral particles collected from the samplers was generated using SAS/STAT software, Version 9.2 of the SAS system for Windows (SAS Institute, Cary, NC). Data were transformed by calculating the natural log of PFUs prior to analysis to meet the assumptions of the statistical tests (homogeneity of variance). For samples collected for 60 minutes under 7 different RHs, a two-way factorial mixed-model analysis of variance (ANOVA) was performed on RH and fraction. This was done using RH as a numeric independent variable to calculate slopes, as well as a categorical variable to allow comparisons between mean levels of PFUs in each fraction at each RH. A significant interaction in a model with humidity as a numeric variable indicates that the slopes of the lines which plot PFUs as a function of RH are not equal across fractions. The second experiment, which sampled for 15 minute intervals for 60 minutes at 2 different RHs was analyzed with a three-way factorial mixed model ANOVA on RH, time and fraction, each being utilized as class variables. The final experiment which sampled for 60 minutes between hours 4 and 5 following aerosol generation was analyzed using a two-way mixed model ANOVA on RH and fraction. In all analyses, trial was included as a random variable in 'Proc Mixed' to account for the lack of independence between fractions in a given trial. Interactions were analyzed by examining simple main effects using the 'slice' option. All pairwise comparisons were considered significant at p<0.05.

**Results**

*High Humidity Reduces the Infectivity of Influenza*

To assess the effect of humidity on infectivity, influenza virus was coughed into a simulated examination room where the RH was adjusted from 7-73%. The exam room contained coughing and breathing manikins facing each other and positioned 200 cm (~6.56 ft) apart (Fig. 1). Approximately 1.0 X $10^8$ total virus was coughed into the exam room which equilibrated to 4.5 X $10^3$ total virus/per liter of room air (assessed by qPCR Matrix gene copies).  A particle counter positioned just below the coughing manikin's mouth showed that the coughed particles optical diameters were largely within the respirable size range (Fig. 2). Most of the virus was recovered in the 1-4 µm aerosol fraction (74.6% ± standard error 1.99%) and <1 µm fraction (18.5% ± standard error 2.17%); the remainder was detected in the >4 µm fraction (7.5% ± standard error 0.70%). The total amount of virus captured by each sampler was approximately the same regardless of their position within the room (data not shown). Approximately 4.6% of the 4.5 X $10^3$ total virus /per liter of room air loaded into the exam room was infectious prior to coughing (assessed by VPA). The percentage of virus that retained infectivity (number of PFUs/number of qPCR Matrix copies in an aerosol sample) relative to that prior to coughing was determined to be highest (70.6-77.2%) at 7-23% RH with a dramatic drop to the lowest (14.6%) at 43% RH (Fig. 3A). Increasing the RH to 57% resulted in a modest increase in the retention of infectivity (22.2%). A similar pattern of infectivity in response to humidity was observed among the three aerosol fractions when examined after 60 minutes of collection (Fig. 3B-D). Specifically, in each of the 3 fractions there was a significant decline in infectivity as humidity levels increased. However this percentage decrease in infectivity as a function of humidity occurs to similar extent across the 3 fractions as the 3 slopes are not significantly different from one another.

*Loss of Infectivity at Moderate Humidity Occurs Rapidly After Coughing*

To determine how quickly aerosolized influenza is inactivated at increased RH, aerosol samples were collected at 5 intervals (0-15 min, 16-30 min, 31-45 min, 46-60 min and 4-5 h) after coughing and compared at 20% and 45% RH. The total amount of virus (assessed by qPCR of the matrix gene) collected during the initial 60 minutes after coughing was $1.8 \times 10^6$ at 20% RH and $1.4 \times 10^6$ at 45% RH (Fig. 4A). During this time, the total virus concentration within the exam room remained approximately the same throughout the 15 minute collection periods regardless of RH (Fig. 4A). Within the 0-15 min collection interval, 52% of the total infectious virus lost infectivity at 45% RH as compared to that found at 20% RH (Fig. 4B). Continued loss of viral infectivity occurred at each 15 min collection interval and at the later 4-5h interval, however, loses were similar at both 20% RH and 45% RH (Fig. 4B).

*Aerosol Particle Size Does Not Confer Increased Stability of Influenza at Low RH*

The amount of infectious virus present in the 3 aerosol fractions was then assessed to determine whether any one aerosol fraction carrying influenza virus retained infectivity longer at low RH. The amount of virus collected in the >4 μm aerosol fraction within the first 60 minutes of collection was approximately the same at 20% RH ($1.3 \times 10^5$ virus) and 45% RH ($9.9 \times 10^4$ virus) (Fig. 4C). Within the 0-15 min collection interval, >90% of the infectious virus in this fraction lost infectivity at 45% RH as compared to that found at 20% RH (Fig. 4D). Continued loss of viral infectivity occurred at each 15 min collection interval and at the later 4-5 h interval, however, loses were similar at both 20% RH and 45% RH (Fig. 4D).

The amount of virus collected in the 1-4 μm aerosol fraction within the first 60 minutes of collection was also approximately the same at 20% RH ($1.1 \times 10^6$ virus) and 45% RH ($1.2 \times 10^6$ virus) (Fig. 4E). Within 0-15 min after coughing, the loss in infectivity at 45% RH compared with that at 20% RH was

not as high as that in the >4 µm fraction (29% loss vs >90% loss). However, as seen in the >4 µm fraction, there were continued losses in viral infectivity at each 15 min collection interval and at the later 4-5 h interval that were approximately the same at either 20% RH and 45% RH (Fig. 4F).

The amount of virus collected in the <1 µm aerosol fraction within the first 60 minutes of collection was more variable at 20% RH (5.8 X $10^5$ virus) then at 45% RH (2.7 X $10^5$ virus) (Fig. 4G). However, this 2-3 fold variability was consistent throughout the 15 minute collection intervals. Within 0-15 min after coughing, 94% of the virus within this fraction lost infectivity at 45% RH as compared to that at 20% RH (Fig. 4H). Continued loss of viral infectivity occurred at each 15 min collection interval and at the later 4-5 h interval, however, rates of loss were similar at both 20% RH and 45% RH (Fig. 4H).

Statistical analysis of the first 60 minutes showed there are significant main effects for humidity, fraction and time on virus infectivity and a significant humidity by fraction interaction. Specifically, with respect to humidity in general, infectious virus are reduced in the higher 45% humidity relative to low 20% humidity (p<0.0001). With respect to fraction, the number of infectious virus is highest in the 1-4 µm fraction and is significantly reduced in the <1 µm fraction and further reduced in the >4 µm fraction (p<0.0001). There was also a significant main effect of time (p<0.0068) with the first and last 15 minute collection intervals significantly lower than the two middle time points. The humidity by fraction interaction simply reflects that the size of the difference between the two humidity conditions varies as a function of fraction. Specifically, the smallest difference (while still statistically different) was in the 1-4 µm fraction while the largest difference in the number of infectious virus was in the <1 µm fraction. However, there was no statistical difference in the rate of decay of infectious virus at 20% RH versus that at 45% RH in any of the 3 aerosol fractions once the initial loss in infectivity occurred within 0-15 min after coughing.

**Discussion**

The potential to transmit influenza by respirable aerosol particles (≤4 μm) is of particular concern as these particles can remain airborne for long periods and can be inhaled deeply into the lung to cause more severe infection (Brankston et al., 2007; Tellier, 2009; Weber and Stilianakis, 2008; Wein and Atkinson, 2009). Healthcare workers are at particular risk as they are directly exposed to the breaths and coughs of influenza patients which have been shown to contain virus (Lindsley et al., 2010b; Milton et al., 2010) and aerosolized virus has also been detected throughout clinic environments during flu seasons (Blachere et al., 2009; Lindsley et al., 2010a). The present study allowed us to assess viral infectivity under various levels of relative humidity and showed that one hour after coughing, ~5 times more virus remains infectious at 7-23% RH than at ≥43% RH.

Yang and Marr (2011) modeled the survival, size distributions, and dynamics of influenza emitted from a cough in an indoor environment and considered the roles of gravitational settling, ventilation, and virus inactivation at RHs of 10-90%. They concluded that settling can remove over 80% of airborne influenza 10 minutes after a cough and that RH increases the removal efficiency only slightly from 87% to 92% over the range of RHs.  Applying a similar model to the cough aerosol particle distribution shown in Fig. 2, we estimated the change in the concentration of airborne particles in our chamber over time due to gravitational settling and filtration by the breathing simulator and aerosol samplers.  We then predicted the amount of virus that should be collected in each stage of the aerosol sampler during the first hour (0 to 60 minutes) and the fifth hour (240 to 300 minutes) after the start of the series of coughs. Our results indicated that the amount of virus in the largest aerosol fraction (> 4 μm) collected during the fifth hour would be reduced to 6% of that seen during the first hour; the second fraction (1-4 μm) would be reduced to 30%; and the smallest fraction (< 1 μm) would be 58%.  These model results

compare very well to the actual viral particle collection results seen in Fig. 4B-D, where the amount of virus collected in each aerosol fraction during the fifth hour fell to 13%, 28% and 50% of the amounts detected during the first hour. The concentration of larger airborne particles decreases faster than smaller particles because larger particles settle much more quickly than smaller ones; in contrast, ventilation and filtration are not affected by particle size. Thus, settling accounts for much of the loss of particles >4 µm, whereas little settling occurred in the <1µm fraction.

Although most of the >4 µm particles were removed from the exam room at 4-5 h, a further decline in infectivity at 45% RH as compared to that at 20% RH nearly eliminates the potential for infection associated with particles of this size. Similarly, the potential for infection from influenza carried on the smaller particles was also further reduced at 45% RH, but the longer retention time of these particles in the air emphasizes the concern these sized particles still pose. The actual number of aerosolized viral particles that a healthcare worker could potentially inhale during a patient examination is largely dependent on the shedding rate of virus by the patient. Infected patients can shed 33 virus/min in aerosol particles ≥5 µm and 187 virus/min in particles <5 µm (Milton et al., 2010). Therefore, in 30 minutes a single patient in a room the size of our simulated exam room can shed up to $5.6 \times 10^3$ viral particles <5 µm in size and a healthcare worker could potentially inhale up to 237 viruses. A dose-response model developed by Teunis et al. (2010) shows that the probability of infection by influenza is significant ($P_{inf}$ = 0.2-0.4) at low doses ($10^{1-2}$ $TCID_{50}$ infectious units).

The effect of increasing humidity on viral survival differed among several reported studies as Hemmes et al. (1960), Hood (1963) and Harper (1961) concluded that survival was maximum at 10-25% RH and minimal at high >50% RH whereas, Shechmeister (1950) and Schaffer et al. (1976) found survival was maximal at 20-25% RH, minimal at 50% RH, and moderate at 70-80% RH. High salt concentrations

are deleterious to influenza (Brown et al., 2009) and protein concentrations in the viral preparation of less than 0.1 mg/ml adversely affect stability of influenza when aerosolized at high and mid-range RH (Schaffer et al., 1976). Yang and Marr (2011) suggest that, although Shechmeister (1950) and Schaffer et al. (1976) used significantly lower concentrations of protein in some of their viral preparations, the trends they obtained were the result of increasing salt concentrations followed by crystallization of the virus at the point of efflorescence (45-48% RH). In our study, 0.2% BSA was included to maintain stability of the virus, and our results support those obtained by Hemmes et al. (1960), Hood (1963), Harper (1961) and closely align with the Yang and Marr model.

Extrapolation of Harper (1961) data of influenza aerosolized into a settling chamber over a range of RHs by Yang and Marr (2011) revealed that infectivity of the total viral population is decreased faster at higher RHs and is evident 5 minutes after aerosolization. Our results indicate that the greatest effect of increased relative humidity occurs within 0-15 minutes after coughing and thereafter, the rates of inactivation of the virus within each aerosol fraction occurs at significantly slower rates regardless of humidity. Analysis of the aerosol fractions further indicates that the most rapid drop in infectivity within 0-15 min occurs in the >4 µM fraction (>90 %) and that virus in the 1-4 µM fraction losses only 29% of infectivity during this time. Moreover, after correction for the lowered amount of virus detected by qPCR in the <1 µM fraction at 45% RH over that detected at 20% RH, the loss in infectivity during 0-15 min after coughing is ~32%. Therefore, virus carried on smaller aerosol particles loose infectivity considerably slower. Yang and Marr (2011) found that droplets shrink to one-half of their original diameter at 90% RH but to only two-fifths at 10% RH but whether droplet shrinkage accounts for these losses is unclear.

Hanley and Borup (2010) examined aerosol transmission of influenza for indoor locations frequented by jet travelers and developed risk contours for temperature and humidity that were based

on studies reported in the literature. They concluded that, in addition to intervention strategies including the use of masks and gloves, climate control of indoor locations should be considered by public health planners in making recommendations to interrupt the spread of influenza. The environmental controls in health care facilities are primarily designed to satisfy human comfort criteria established under ASRAE and ISO standards (ASHRAE, 2010; ISO, 2005) with the exception of special cases where higher humidity is specified to reduce static charge in medical test equipment and/or computer areas. Raising the humidity levels in existing facilities may not be practical given design limitations built into the facilities under the existing standards. However, if functional areas of health care facilities were identified as high risk for flu transmission due to low humidity conditions, consideration could be given during the design and construction phase of these facilities to accommodate maintaining appropriate recommended humidity levels.

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**Figure 1. Three-dimensional view of the simulated examination room.** National Institute of Occupational Safety and Health (NIOSH) samplers collected aerosols through the mouth, 10 cm on either side of the manikin's mouth, and at 3 other positions (P1, P2, P3) as shown. The mouths of the coughing and breathing simulators and sampler inlets at P1, P2, and P3 were located 152 cm above the floor (approximate mouth height of a patient sitting on an examination table and a standing healthcare worker). All dimensions adjacent to white arrows within the room are in centimeters.

**Figure 2. Cough aerosol particle optical size distribution.** A particle counter was positioned just below the coughing manikin's mouth. Each bar represents the total volume of the aerosol particles in that size range expelled during a single cough. The amount of virus in the particles is proportional to the aerosol volume. The plot shows the mean and standard deviation of 30 coughs (six sets of five coughs each performed as described in the Methods).
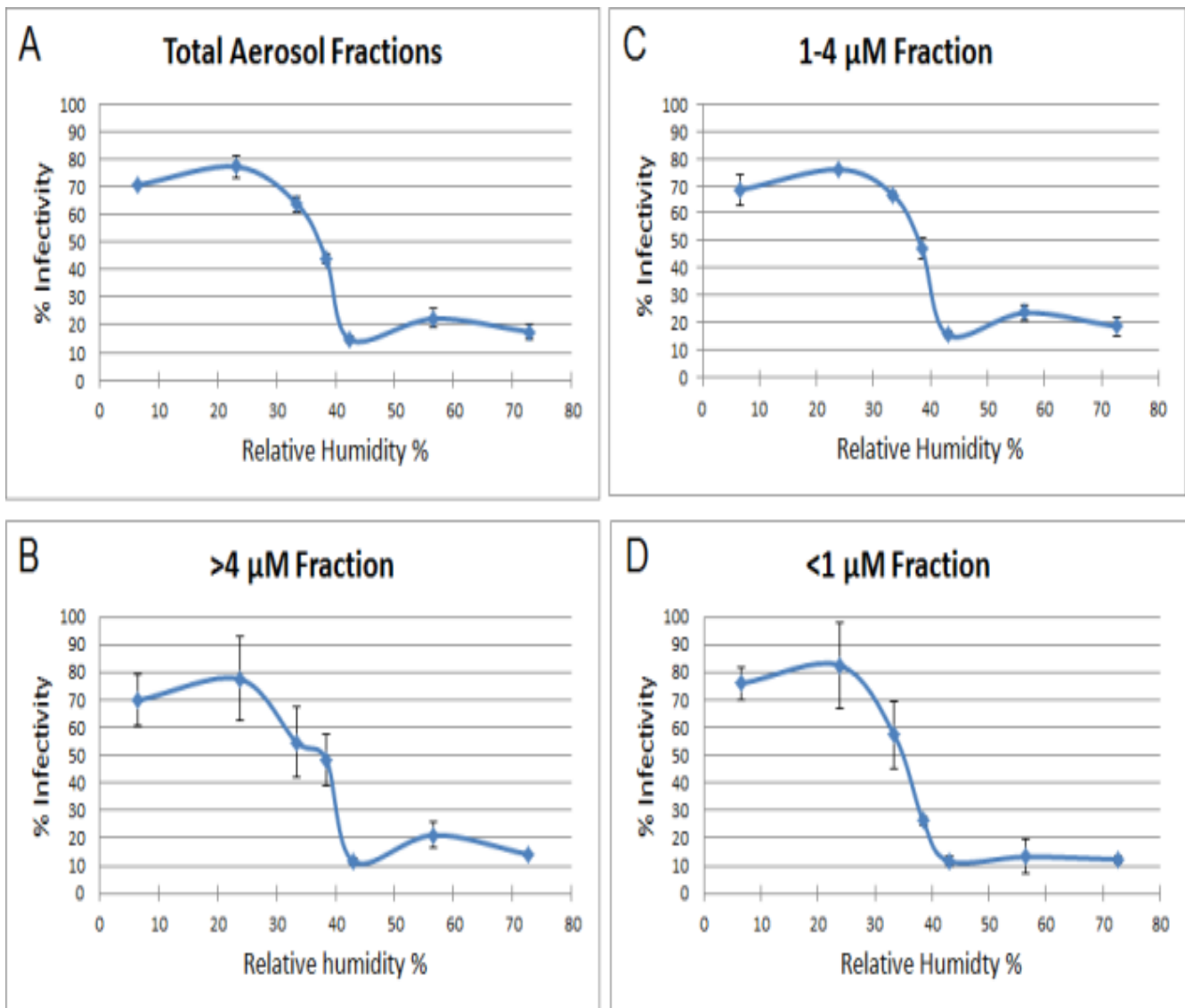
**Figure 3. High humidity reduces the infectivity of influenza.** Influenza virus was coughed into the examination room and NIOSH samplers collected aerosol samples for 60 minutes from the manikin's mouth, 10 cm to the right and left of the mouth, and at positions P1 and P2 within the room. At constant temperature (20°C), the RH was varied over 7-73%.The percentage of virus that retained infectivity relative to that prior to coughing is shown. *A,* The percentage of infectious virus from all fractions (>4 μm, 1-4 μm, and <1 μm) was determined by the viral plaque assay (VPA) and is shown. *B-D,* The percentage of infectious virus within each aerosol fraction is shown. Data are means ± standard error (n = 5).
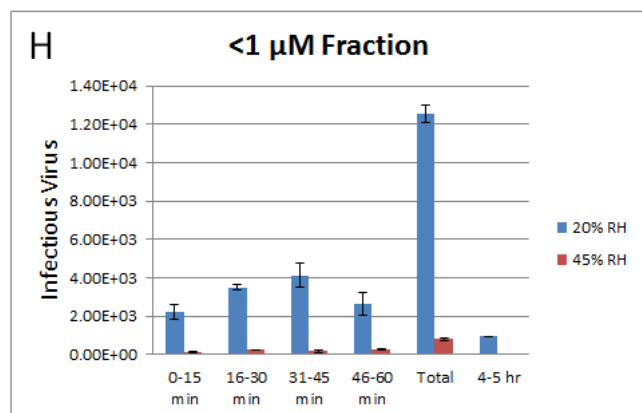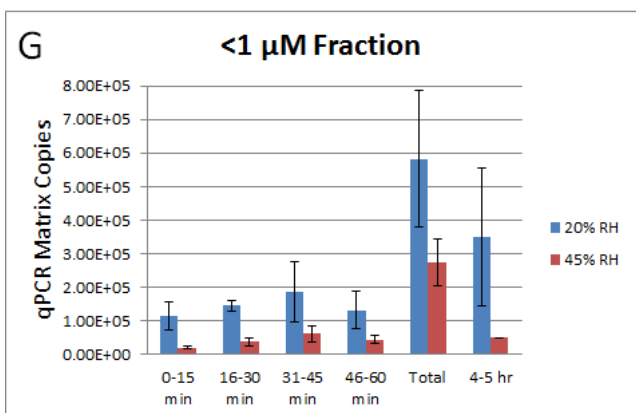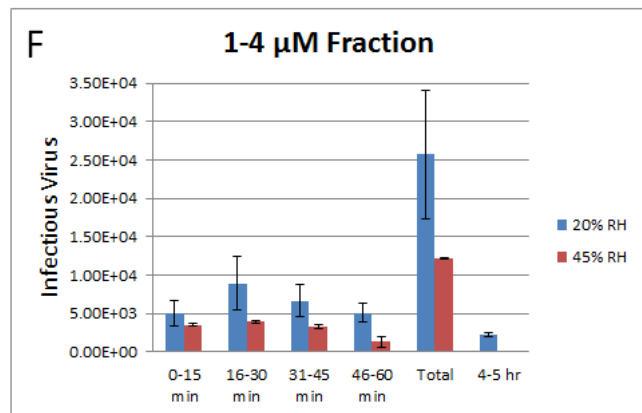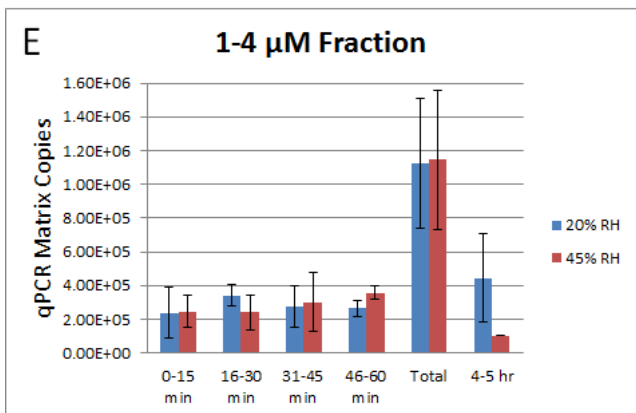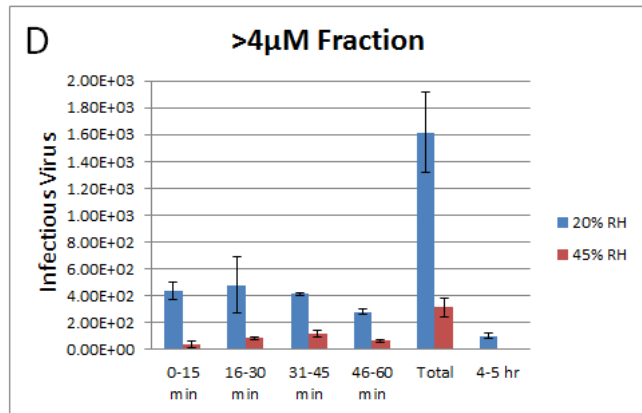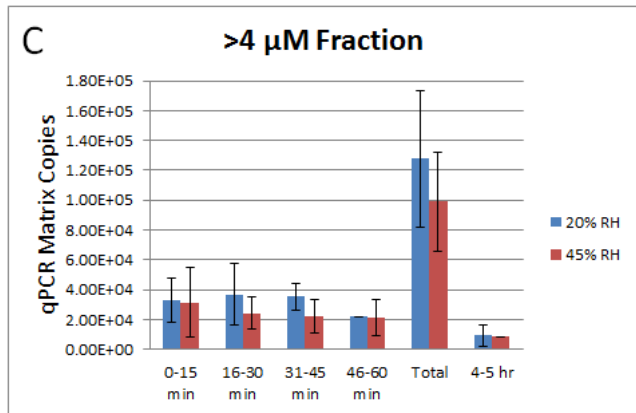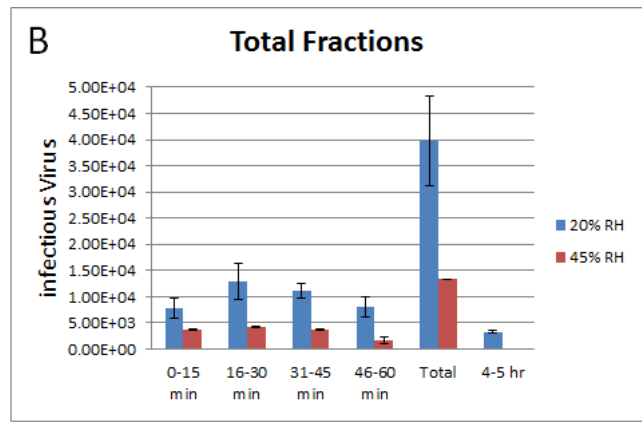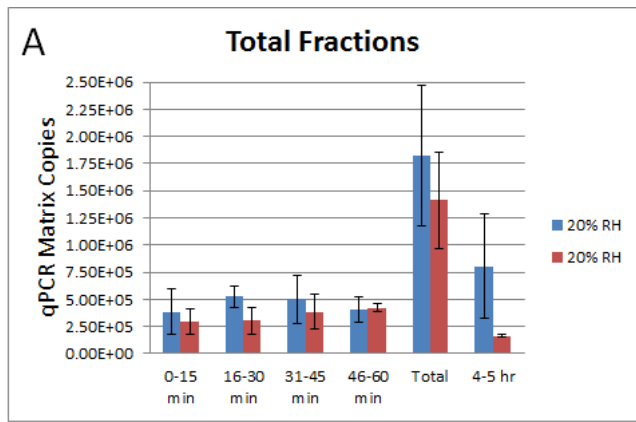
**Figure 4. Loss of infectivity at moderate humidity occurs rapidly after coughing.** Influenza virus was coughed into the examination room and NIOSH samplers collected aerosol samplers positioned on the outside wall of the examination room (P3) to enable immediate processing of the collected samples. Aerosol samples were collected at 16-30 min, 31-45 min, 46-60 min, and 4-5 h after coughing at 20% RH and 45% RH. The temperature of the examination room was maintained at 20$^O$C throughout the collection periods. *A,C,E,G,* Amounts of total virus (infectious and noninfectious) from all aerosol fractions (>4 μm, 1-4 μm, and <1 μm) collected at each time interval was determined by quantitative polymerase chain reaction (qPCR). *B,D,F,H,* The number of infectious virus collected at each time point from all aerosol fractions was determined by viral plaque assay. The amount of virus collected at each 15 minute interval during the initial 60 minutes was totaled and shown as the "Total" on the X-axis of each graph. Data are means ± standard errors (n = 3 for each aerosol fraction assayed).

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# 8.3 Curriculum Vitae

# Cynthia M. McMillen, PhD

## OFFICE ADDRESS

The University of Pittsburgh
Center for Vaccine Research
Regional Biocontainment Laboratory
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Pittsburgh, Pennsylvania 15261
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## RESIDENTIAL ADDRESS

1441 Summers School Road
Morgantown, WV 26508
Tel: (304) 376-1159
Email: cmcmill4@mix.wvu.edu
Email: cynthia.mcmillen@gmail.com

## EDUCATION

| | |
|---|---|
| Fall 2016 | **Doctor of Philosophy, Immunology and Microbial Pathogenesis**<br>Biomedical Sciences, Department of Microbiology, Immunology, and Cell Biology<br>Principle Investigator: Dr. John Noti<br>West Virginia University School of Medicine<br>Morgantown, West Virginia<br>**Thesis:** The Development of Inducible Anti-influenza Therapies |
| May 2010 | **Bachelor of Arts, Biology**<br>Eberly College of Arts and Sciences<br>Department of Biology<br>GPA: 3.58/4.00 (Cum Laude)<br>West Virginia University<br>Morgantown, WV |

## FELLOWSHIPS

| | |
|---|---|
| 2009 | University of Cincinnati, College of Medicine<br>Research and Graduate Education<br>Summer Undergraduate Research Fellowship<br>Principle Investigator: Dr. Kasper Hoebe<br>Research topic: Analysis of small hairpin RNA toll-like receptor activity |

## PROFESSIONAL EXPERIENCE

| | |
|---|---|
| 2017 - present | University of Pittsburgh<br>Center for Vaccine Research<br>Regional Biocontainment Laboratory<br>Pittsburgh, PA<br>Post-doctoral Fellow |
| 2011 - 2016 | Centers for Disease Control and Prevention/ National Institute for Occupational Safety & Health (CDC/NIOSH)<br>Health Effects Laboratory Division<br>Allergy & Clinical Immunology Branch<br>Infectious Diseases Transmission Program<br>Morgantown, WV<br>Regular Fellow |
| 2009 - 2010 | West Virginia University<br>Eberly College of Arts and Sciences<br>Department of Biology<br>Principle Investigator: Dr. Rita Rio<br>Undergraduate Research Assistant |
| 2007 - 2010 | West Virginia University Center for Health Ethics and Law<br>Morgantown, WV<br>Student Intern |

## TECHNICAL SKILLS
*Proficient in the following:*

**Virology**
- influenza virus propagation
- viral plaque assay
- hemagglutination assay
- neuraminidase assay

**Cell biology**
- cell culture
- DNA and RNA transfection
- RNA interference
- stable cell line generation

**Molecular biology**
- gene and recombinant DNA cloning
- quantitative RT-PCR
- site-directed mutagenesis
- gel electrophoresis
- genomic PCR
- DNA/RNA isolation

**Protein biology**
- western blot
- ELISA
- BCA assay
- dual-luciferase assay

**Aerosol biology**

- generation of aerosols containing influenza virus via AeronebLab Micropump nebulizer (Aerogen©)
- collection and processing of aerosol samples from NIOSH and/or SKC sampler

**Microbiology**
- bacterial staining
- bacterial quantitation
- microbial enzymes

- fermentation
- bacterial selection
- bacterial differentiation

**Microscopy**
- confocal
- light

- fluorescent microscopy

*Experience in the following:*

**Molecular biology**
- southern blot
- northern blot

- non-isotopic labeling of DNA probe

**Animal techniques**
*Tsetse fly colony*
- feeding blood-meals
- sex determination

- mating

*Mouse colony*
- necropsy
- tail-vein injection

- orbital bleeding
- cervical dislocation


## <u>TEACHING EXPERIENCE/MENTORING</u>

| | |
|---|---|
| March 2016 | MICB 801: Medical Microbiology for Medical Students, West Virginia University School of Medicine. Self-Directed Learning "Vaccines", Facilitator |
| 2015 | BMS 715: Molecular Genetics, West Virginia University Office of Research and Graduate Education. Tutor |
| 2015 | BMS 730: Cancer Cell Biology, West Virginia University Office of Research and Graduate Education. Tutor |
| November 2015 | Graduate School Panel Discussion, Allegheny Branch of the American Society for Microbiology Annual Meeting. West Virginia University Panel Representative |

| | |
|---|---|
| September 2015 | MICB 702: Medical Microbiology for Dental Students, West Virginia University. Tetanus Immunity Patient-Oriented Problem Solving Class, Facilitator |
| March 2015 | 62nd Annual West Virginia State Science and Engineering Fair - High School Students, Biochemistry and Microbiology Judge |
| March 2014 | BMS 736: Immunology & Microbial Pathogenesis, West Virginia University. "Microbes and Cancer Diagnosis/Therapy" Guest Lecturer |
| 2014 | BMS 783a/b: Functions of Contemporary Biomedical Research 1&2, West Virginia University, Tutor |
| 2012, 2013 | MICB 323: Medical Microbiology Laboratory, West Virginia University. Laboratory Assistant |
| 2011 | Immunology, WVU School of Dentistry. Private Tutor |

## LEADERSHIP/SERVICE POSITIONS

| | |
|---|---|
| 2013 - 2014 | Student Representative, Biomedical Sciences PhD Admission Committee |
| 2013 - 2014 | President Emeritus, Health Sciences Center Graduate Student Organization, West Virginia University School of Medicine |
| 2014 | Coordinator, Non-Academic Careers in Biomedical Sciences. West Virginia University Health Sciences Center Graduate Student Organization Symposium<br><br>Keynote Speaker: Dr. Emily Morey-Holton (Ames Research Center, NASA) |
| 2012 - 2014 | Academic Committee, Health Sciences Center Graduate Student Organization, West Virginia University School of Medicine |
| 2012 - 2013 | President, Health Sciences Center Graduate Student Organization. West Virginia University School of Medicine |
| 2011 - 2012 | Treasurer, Health Sciences Center Graduate Student Organization. West Virginia University School of Medicine |

## HONORS/AWARDS

| | |
|---|---|
| 2015 | First Place Graduate Student Poster Presentation Award, Allegheny Branch American Society for Microbiology (ABASM) |
| 2014 | Young Investigator, 2014 NIOSH Intramural Science Meeting, CDC/NIOSH, Morgantown, WV. *Title of work:* Inducible anti-influenza therapy utilizing RNA interference. |
| 2014 | CDC Nominee for the Charles C. Shepard Science Award for Outstanding Scientific Paper in Laboratory Science |
| 2014 | First Place Poster Presentation Award, E.J. Van Liere Memorial Convocation and HSC Research Day, West Virginia University |
| 2014 | Travel Award for Graduate Students, West Virginia University Office of Research and Graduate Education |
| 2014 | Travel Award, West Virginia University Microbiology, Immunology, and Cell Biology Department |
| 2013 | CDC Nominee for the Charles C. Shepard Science Award for Outstanding Scientific Paper in Prevention and Control |
| 2012 | NIOSH Alice Hamilton Honorable Mention Award in Exposure and Risk Assessment Category |
| 2010 | Undergraduate Academic Enrichment Recipient, West Virginia University Eberly College of Arts and Sciences |
| Spring 2010 | President's List, West Virginia University Eberly College of Arts and Sciences |
| Fall 2009 | President's List, West Virginia University Eberly College of Arts and Sciences |
| Spring 2009 | Dean's List, West Virginia University Eberly College of Arts and Sciences |
| Fall 2008 | Dean's List, West Virginia University Eberly College of Arts and Sciences |

## PRESENTATIONS

**Inducible anti-influenza therapy utilizing RNA interference.** Infectious Diseases World Summit, 4th Influenza Research & Development Conference, Boston, MA, July 2015.

**Involuntary suicide: an update on the development of an inducible anti-influenza therapy.** Microbiology, Immunology, and Cell Biology Department Seminar Series. West Virginia University. Morgantown, WV. January 2015.

**Inducible anti-influenza therapy utilizing RNA interference.** 2014 NIOSH Intramural Science Meeting. CDC/NIOSH. Morgantown, WV. July 2014.

**RNAi: inhibiting virus replication.** Microbiology, Immunology, and Cell Biology Department Seminar Series. West Virginia University. Morgantown, WV. January 2014.

**Inducible influenza anti-viral therapy that utilizes the viral RNA-dependent-RNA polymerase.** Microbiology, Immunology, and Cell Biology Department Seminar Series. West Virginia University. Morgantown, WV. May 2013.

**Detection of influenza virus in aerosols: understanding transmission, environmental factors influencing infectivity & protection.** Microbiology, Immunology, and Cell Biology Department Seminar Series. West Virginia University. Morgantown, WV. February 2012.

*Poster Presentations*

**McMillen CM,** Noti J, Blachere F, Othumpangat S, Beezhold D. Inducible Anti-influenza Therapy Utilizing RNA Interference. Allegheny Branch American Society for Microbiology Annual Meeting. Greensburg, PA. November 2015.

**McMillen CM,** Beezhold D, Noti J. The Delivery of a Conserved Decoy Antigen as a Novel Inducible Influenza Vaccine. Infectious Diseases World Summit, 13th Vaccines Research & Development Conference, Boston, MA, July 2015.

**McMillen CM,** Noti J, Blachere F, Othumpangat S, Beezhold D. Inducible Anti-influenza Therapy Utilizing RNA Interference. Infectious Diseases World Summit, 4th Influenza Research & Development Conference, Boston, MA, July 2015.

**McMillen CM,** Beezhold D, Noti JD. Inducible Influenza Vaccine Delivering a Non-functional Neuraminidase Antigen. 2015 Annual Conference on Vaccine Research, Bethesda, MD, April 2015.

**McMillen CM,** Noti J, Beezhold D. Inducible anti-influenza therapy utilizing RNA interference. American Society for Microbiology General Meeting. Boston, MA. May 2014.

**McMillen CM,** Noti JD, Blachere FM, Beezhold DH. Inducible anti-influenza therapy utilizing RNA interference. 2014 E.J. Van Liere Convocation and HSC Research Day, West Virginia University. Morgantown, WV. February 2014.

**McMillen CM**, Noti JD, Blachere FM, Lindsley WG, Kashon ML, Slaughter DR, Beezhold DH. Catching the flu: the effect of relative humidity on aerosolized influenza virus infectivity. E.J. Van Liere Convocation and HSC Research Day, West Virginia University. Morgantown, WV. February 2013.

**McMillen CM**, Harris N, Hoebe K. Analysis of small hairpin RNA toll-like receptor ligand activity. University of Cincinnati, College of Medicine, Research and Graduate Education 2009 Summer Undergraduate Research Fellowship Capstone Poster Symposium. Cincinnati, OH. August 2009.


## PUBLICATIONS

*Manuscripts*

**McMillen CM,** Noti JD, Blachere FM, Othumpangat S, Kashon ML, Beezhold DH. Characterization of an inducible anti-influenza therapy utilizing RNA interference. Virology 2016;494:171-84.

Lindsley WG, Blachere FM, Beezhold DH, Thewlis RE, Noorbakhsh B, Othumpangat S, Goldsmith WT, **McMillen CM**, Andrew ME, Burrell CN, Noti JD. Viable Influenza A Virus in Airborne Particles Expelled during Coughs vs. Exhalations. Influenza and Other Respiratory Viruses. 2016; DOI: 10.1111/irv.12390.

Othumpangat S, Noti JD, **McMillen CM**, Beezhold DH. ICAM-1 regulates the survival of influenza virus in lung epithelial cells during the early stages of infection. Virology 2016;487:85-94.

Noti JD, Blachere FM, **McMillen CM,** Lindsley WG, Kashon ML, Slaughter DR, Beezhold DH. High humidity leads to loss of infectious influenza virus from simulated coughs. PLoS One 2013;8(2):e57485.
  2012 NIOSH Alice Hamilton Award Honorable Mention in Exposure and Risk Assessment Category
  2013 CDC Nominee for the Charles C. Shepard Science Award for Outstanding Scientific Paper in Prevention and Control

Noti JD, Lindsley WG, Blachere FM, Cao G, Kashon ML, Thewlis RE, **McMillen CM,** King KP, Szalajda JC, Beezhold DH. Detection of infectious influenza virus in cough aerosols generated in a simulated patient examination room. Clinical Infectious Diseases 2012;11:1569-77.
  2014 CDC Nominee for the Charles C. Shepard Science Award for Outstanding Scientific Paper in Laboratory Science

Snyder AK, **McMillen CM,** Wallenhorst P, Rio RVM. The phylogeny of sodalis-like symbionts as reconstructed using surface encoding loci. FEMS Microbiology Letters 2014;317(2):143-51.

**McMillen CM,** Noti JD, Blachere FM, Othumpangat S, Beezhold D. Involuntary suicide: an update on the development of an inducible anti-influenza therapy. Microbiology, Immunology, and Cell Biology Department Seminar Series. West Virginia University. Morgantown, WV. January 2015.

**McMillen CM,** Noti J, Beezhold D. Inducible anti-influenza therapy utilizing RNA interference. The 114th Annual American Society for Microbiology General Meeting. Boston, MA. May 2014.

**McMillen CM,** Noti J, Blachere FM, Beezhold D. Inducible anti-influenza therapy utilizing RNA interference. 2014 NIOSH Intramural Science Meeting. CDC/NIOSH. Morgantown, WV. July 2014.

**McMillen CM,** Noti JD, Blachere FM, Beezhold DH. Inducible anti-influenza therapy utilizing RNA interference. 2014 E.J. Van Liere Convocation and HSC Research Day, West Virginia University. Morgantown, WV. February 2014.

**McMillen CM,** Noti JD, Blachere FM, Beezhold DH. RNAi: inhibiting virus replication. Microbiology, Immunology, and Cell Biology Department Seminar Series. West Virginia University. Morgantown, WV. January 2014.

**McMillen CM**, Noti JD, Blachere FM, Beezhold DH. Inducible influenza anti-viral therapy that utilizes the viral RNA-dependent-RNA polymerase. Microbiology, Immunology, and Cell Biology Department Seminar Series. May 2013.

**McMillen CM,** Noti JD, Blachere FM, Lindsley WG, Kashon ML, Slaughter DR, Beezhold DH. Catching the flu: the effect of relative humidity on influenza infectivity. Microbiology, Immunology, and Cell Biology Department Seminar Series. March 2013.

**McMillen CM**, Noti JD, Blachere FM, Lindsley WG, Kashon ML, Slaughter DR, Beezhold DH. Catching the flu: the effect of relative humidity on aerosolized influenza virus infectivity. E.J. Van Liere Convocation and HSC Research Day, West Virginia University. Morgantown, WV. February 2013.

Lindsley WG, Ahrenholz SH, Beezhold D, Blachere F, Brueck SE, Cummings KJ, De Perio MA, Fisher E, King WP, Martin SB, **McMillen CM,** Noti JD, Othumpangat S, Reynolds JS, Shaffer R, Szalajda JV, Thewlis RE. Airborne transmission of influenza: a research update. Cambridge, OH. August 2012.

Lindsley WG, Noti JD, King WP, Blachere F, **McMillen CM,** Thewlis RE, Reynolds JS, Szalajda JV, Beezhold D. Respiratory protection and influenza-laden cough aerosols in a simulated medical examination room. NIOSH Personal Protective Technology Program Stakeholders Meeting. Pittsburgh, PA. March 2012.

**McMillen CM,** Noti JD, Blachere FM, Lindsley WG, Kashon ML, Slaughter DR, Beezhold DH. Detection of influenza virus in aerosols: understanding transmission, environmental factors influencing infectivity & protection. Microbiology, Immunology, and Cell Biology Department Seminar Series. February 2012.

Noti JD, Lindsley WG, Blachere FM, Cao G, Kashon ML, Thewlis RE, **McMillen CM,** King WP, Szalajda JV, Beezhold DH. Detection of infectious influenza virus in cough aerosols generated in a simulated patient examination room. Pandemic Influenza Scientific Agenda (PISA) conference, Centers for Disease Control and Prevention, Atlanta Georgia. 2011.

Beezhold DH, Blachere FM, Cao G, Lindsley WG, **McMillen CM**, Thewlis CM, Noti JD. Detection of influenza virus in aerosols. Aerobiology Mini-Symposium-Mechanics of Respiratory Transmission. Pittsburgh, PA. November 2011.

Snyder AK, **McMillen CM,** Rio RVM. Phylogeny of recently diverged insect symbionts based on hypervariable genome regions. 110th Annual American Society for Microbiology General Meeting. San Diego, CA. May 2010.