DISSERTATION

RNA INTERFERENCE AS AN ALTERNATIVE PREVENTIVE MEASURE FOR AVIAN INFLUENZA IN POULTRY

Submitted by

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ABSTRACT

RNA INTERFERENCE AS AN ALTERNATIVE PREVENTIVE MEASURE FOR AVIAN INFLUENZA IN POULTRY

Avian influenza virus (AIV) is a viral pathogen that causes a wide range of disease in poultry, from subclinical to severe clinical illness and can often result in death. In 1878, AIV was first described as a disease affecting poultry. Nearly 80 years later this disease-causing agent was identified as influenza A virus and a member of the family *Orthomyxoviridae*. AIV was not considered a significant human pathogen until 1997, when high pathogenic AIV H5N1 emerged from the wildfowl reservoir and was directly transmitted from domestic poultry to humans. Despite a long history of outbreaks in animals, this incident propelled AIV into a globally recognized disease associated with socioeconomic and animal health consequences. Each AIV outbreak highlights ways to improve upon current control strategies and stimulates new ideas for developing novel approaches and technologies to better mitigate AIV outbreaks worldwide.

AIV is a dynamic pathogen to study. Host range and adaptation, pathogenicity, pathology, molecular composition, and the epidemiology of AIV all make this virus particularly challenging to control in poultry. Vaccines against AIV are available but the protection they provide for poultry is limited, especially when administered at the onset or in the midst of an outbreak.

The most efficacious vaccines must be administered subcutaneously or intramuscularly, an impediment to successfully immunizing large numbers of poultry in a short window of time. Frequently, improper storage and handling leads to vaccine failure. To elicit efficient protection the vaccine must be HA-subtype specific to the outbreak virus. Often stockpiles of vaccines become obsolete and new vaccines must be generated. This is a time-consuming process and

can take months to secure and additional time to disseminate and administer. In the naïve animal, protective antibody production takes two to three weeks to acquire following vaccination. Even if the decision to vaccinate during an outbreak is rapid and an appropriate vaccine is available for immediate use in poultry, vaccination alone would do little to protect against the threat of infection and break the chain of transmission, especially in areas lacking appropriate biosecurity measures. These limitations convey a genuine need to develop a prophylactic that would offer universal protection against any subtype or strain of AIV and would provide rapid protection in the face of an outbreak.

Using RNA interference (RNAi) methodologies, this dissertation focuses on developing an innovative antiviral prophylactic that works rapidly to protect poultry against AIV shedding and transmission. The innovation behind this prophylactic technology lies in combining RNAi with the transkingdom RNAi (tkRNAi) delivery platform. This anti-AIV technology specifically targets conserved viral gene segments using small interfering RNA (siRNA) generated and delivered to chicken mucosal respiratory tissues using the tkRNAi system. The work presented in this dissertation details the steps taken to show proof of concept for using this technology to prevent AIV replication and shedding *in vitro* using an avian cell model and *in vivo* using commercial chickens.

The overarching vision for this anti-AIV technology is to provide a cost effective means to protect commercial and backyard flocks against AIV outbreaks. The long-term goal is to promote this prophylactic as a complement to vaccination with the intention of developing a more effective and robust control plan against AIV in poultry. If this technology is successful, it could be applied in the face of an outbreak to reduce the shedding and transmission of virus within poultry, between farms, and across borders, thereby improving animal health and reducing the economic impact of outbreaks worldwide. Additionally, this work could provide the framework and valuable evidence for developing a similar anti-influenza prophylactic for humans.

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TABLE OF CONTENTS

DEFINITION OF ABREVIATIONS

Abbreviation	Meaning
AAF	Amnioallantoic fluid
AGID	Agar gel immune-diffusion
AIV	Avian influenza virus
APC	Antigen presenting cell
APEC	avian pathogenic *Escherichia coli*
ATCC	American Type Culture Collection
BHI	Brain Heart Infusion agar
BID_{50}	50% bird infectious dose
BLAST	Basic Local Alignment Search Tool
bp	Base pair
cDNA	Complementary DNA
CEQ221	competent cells transformed with pMBV43 plasmid
CFU	Colony forming units
CI	Confidence interval
Cocktail-siRNA	siRNA cocktail for NP and PA genes
CPE	Cytopathic effect
CSREES	Cooperative State Research, Education, and Extension Service
Dap	2,3-Diaminopropionic Acid
DAPI	4',6-diamidino-2-phenylindole
dNTP	Deoxyribonucleotide
Dpi	Days post infection
dsRNA	Double-stranded RNA
E. coli	*Escherichia coli*

EID_{50}/mL	50% egg infectious titer
EID_{50} eq/mL	EID_{50} equivalents per mL
FBS	Fetal bovine serum
HA	Hemagglutinin protein
HPAI	High pathogenic avian influenza
hpi	Hours post infection
H6N2	A/Chicken/Texas/473-2/10
H8N4	A/turkey/Colorado/1/05
IACUC	Institutional Animal Care and Use Committee
IM	Inoculation medium
IN	Intranasal
inv	Invasin gene
IPTG	Isopropyl β-D-1-thiogalactopyranoside
Kan	Kanamycin
kb	Kilo base
LB	Luria Broth
LCL	Lower 95% confidence level
LLO	Listerolysin O
LMH	Chicken hepatocellular carcinoma epithelial cells
LPAI	Low pathogenic avian influenza
LPS	Lipopolysaccharide
LRT	Lower respiratory tissues
MDCK	Madin-Darby canine kidney cells
MEM	Minimum essential medium
MEM/EBSS	Minimum essential medium with Earle's balanced salts
MLR	Multivariable linear regression

MOI	Multiplicity of infection
mRNA	Messenger RNA
M1	Matrix protein
M2	Matrix 2 ion channel protein
NA	Neuraminidase protein
NASS	National Agricultural Statistics Service
NCBI	National Center for Biotechnology Information database
NEAA	Non-essential amino acids
NEP	Nuclear export protein
NP	Nucleoprotein
NP-siRNA	siRNA for NP gene
NSP1	Non-structural protein 1
NSP2	Non-structural protein 2
nt	Nucleotide
NVSL	National Veterinary Services Laboratory
OIE	Office of International Education
OP	Oropharyngeal
OR	Odds ratio
PA	Polymerase acidic protein
PAMP	Pathogen-associated molecular pattern
PA-siRNA	siRNA for PA gene
PBS	Phosphate buffered saline
PB1	Polymerase basic protein 1
PB1-F2	Polymerase basic protein 1-F2
PB2	Polymerase basic protein 2
PC	Positive control

pMBV43	tkRNAi shRNA plasmid
POC	Proof of concept
R_0	Basic reproductive rate
RFP	Red fluorescent protein
RISC	RNA-induced silencing complex
RNAi	RNA interference
RT-qPCR	Real time reverse transcriptase PCR
SA	Sialic acid
SD	Standard deviation
shRNA	Short hairpin RNA
siRNA	Small interfering RNA
SPF	Specific pathogen-free
$TCID_{50}$	50% tissue culture infectious dose
tkRNAi	Transkingdom RNAi
TLR	Toll-like receptor
TPCK	L-1-Tosylamide-2-phenylethyl chloromethyl ketone
UCL	Upper 95% confidence level
URT	Upper respiratory tissues
USDA	United States Department of Agriculture
VDL	Veterinary Diagnostic Laboratory
vRNP	Viral ribonucleoprotein
WHO	World Health Organization

CHAPTER 1

INTRODUCTION AND REVIEW OF AVIAN INFLUENZA VIRUS

1. Introduction

AIV is a viral pathogen that infects specific tissues in many avian species, including the respiratory, digestive and nervous system. Due to its contagious nature in poultry often resulting in death, outbreaks can be devastating, resulting in severe economic consequences to the commercial industry and developing countries. High pathogenic avian influenza (HPAI) often causes fatal disease in domestic poultry, with a case-fatality rate approaching 100% (Horimoto and Kawaoka 2001). Over 250 million poultry have died or been destroyed internationally due to HPAI outbreaks (Monke and Corn 2007). The economic impact of low pathogenic avian influenza (LPAI) lies in loss of production rather than mortality. LPAI outbreaks are still significant threats to the industry and regions around the world where poultry are a source of livelihood. In the past, LPAI outbreaks have been handled with traditional stamping-out practices. This was the case in 2002, when Virginia responded to the H7N2 LPAI outbreak with a massive culling effort. The federal eradication cost per farm was estimated at $461,000 (Swayne and Halvorson 2008). The international response to AIV outbreaks from 2002 to 2008 alone was estimated to be ≥ $2 billion (McLeod 2008). In addition to bearing large economic effects, lack of effective control measures is problematic because influenza A viruses originating from animals, including poultry, represent a major public health threat due to their potential to reassort and cause the next human pandemic. This disease has attracted the attention of veterinarians, virologists, the poultry industry, public health professionals, and researchers worldwide.

2. Ecology of AIV in avian species

2.1 Etiology

In 1878, HPAI was first described as a disease of poultry (Perroncito, 1878). However, in 1955 it was determined that the causative agent was influenza A virus (Schäfer, 1955). AIV is a type A influenza virus and a member of the family *Orthomyxoviridae*. The viral genome is comprised of 8 segments of single-stranded, negative sense RNA that encode a total of 12 structural and non-structural proteins (Jagger et al 2012, He et al 2009). These include the hemagglutinin (HA), neuraminidase (NA), matrix (M1), matrix 2 ion channel protein (M2), nucleoprotein (NP), non-structural protein 1 (NSP1), non-structural protein 2 (NSP2); also known as nuclear export protein, (NEP), polymerase acidic protein (PA), polymerase basic protein 1 (PB1), polymerase basic protein 2 (PB2) and polymerase basic protein 1-F2 (PB1-F2) (Fields et al 2007). The viral ribonucleoproteins (vRNPs) consist of a single negative sense RNA segment, which is encapsidated by NP molecules and a RNA-dependent RNA polymerase, consisting of one copy of PB1, PB2, and PA each (Elton et al 2001). These vRNP structures are packaged within a shell of the viral M1 protein underlying the lipid bilayer that contains the three integral envelope glycoproteins HA, NA, and M2 (Elton et al 2001).

The AIV replication cycle can be divided into the following stages: entry into the host cell, entry of the vRNP complexes into the nucleus, transcription and replication of the viral genome, splicing of viral messenger RNA (mRNA) using the host splicing machinery, nuclear export and viral mRNA translation, export of the vRNPs from the nucleus, post-translational processing and assembly and budding of the progeny virion at the host cell plasma membrane.

After membrane fusion and release of the vRNPs into the cell's cytoplasm, the vRNPs are actively transported into the host cell nucleus. While inside the cell's nucleus, the influenza virus transcribes and replicates its RNA genome. This requires a functional vRNP complex and the presence of NP, PA, PB1, and PB2 (Nagata et al 2008, Deng et al 2006, Elton et al 2001). As an obligate intracellular pathogen, AIV transcription and replication depends on the vRNP

proteins and the exploitation of specific host factors. These host factors include those with pivotal roles in influenza virus replication. For example, nuclear export and import factors (Hao et al 2008, Nagata et al 2008, Wang et al 2008, Deng et al 2006) vacuolar ATPases (Karlas et al 2010, Hao et al 2008, Mayer et al 2007), splicing factors (Jorba et al 2008), transcription factors (Karlas et al 2010, Nagata et al 2008), and those with chaperone-like activity for viral protein transportation (Nagata et al 2008).

AIV is classified by the antigenicity of its two major envelope surface glycoproteins, HA and NA. To date, 18 HA (H1-H17) and 11 NA subtypes (N1-N9) have been identified (Tong et al 2012). AIVs are characterized based on pathogenicity: high pathogenicity avian influenza or low pathogenicity avian influenza. Those classified as LPAI can include any combination of HA (H1-16) and NA (N1-9), while HPAI is restricted to subtypes H5 and H7. While many factors play a role in determining or predicting pathogenicity of a particular virus, pathogenicity of AIV correlates with the ability of trypsin to cleave the HA molecule into two subunits, allowing the virus to enter the host cell. HPAI viruses have HA proteins with multibasic cleavage sites that allow these HA proteins to be cleaved by ubiquitous cellular proteases (Stieneke-Gröber et al 1992). Phenotypically, this is one molecular characteristic that allows HPAI to infect systemically and cause high mortality and morbidity in poultry (Bertram et al 2010).

2.2 Host range

With the exception of subtypes H17 and H18, recently isolated from bats (Guo et al 2013, Tong et al 2012), histories of serological results have confirmed all types of HA have been isolated from aquatic wild birds and all classified as LPAI viruses. This validates that wild avian species serve as the reservoir for maintaining AIV (Alexander, 2007; Olsen et al 2006; Hinshaw et al 1979). Although AIV is usually asymptomatic in wild birds, it is considered endemic in wild bird populations. With the exception of H5N1, AIVs have actually reached an evolutionary stasis in aquatic birds. This adaptive optimum allows the virus to select for mutations that

reduce mortality and other deleterious effects to the host (Webster et al 1992). The resulting symbiotic relationship allows the virus to replicate efficiently and infect large populations of wild birds, particularly waterfowl, without causing disease to the host. The characteristics of this relationship between wild birds and AIV allow the virus to be persistent in the environment and in a state of continuous circulation throughout the year (Webster et al 1992).

An unusual characteristic of AIV is their ability to infect a wide range of hosts, including but not limited to chickens, turkeys, swine, horses, dogs and humans. However, the ability of the virus to infect a new host and be maintained depends on necessary adaptations affecting the interplay between the host and virus. Influenza A viruses are believed to have partial host-range restriction, meaning that while viruses can transmit between species fairly frequently, these viruses are rarely maintained within the new species. Two mechanisms contribute to the virus' ability to establish infections in new host species. First, the virus has an error prone RNA polymerase. This equates to a high rate of mutation. Second, the virus has the ability to reassort gene segments with other co-circulating viruses (Manrubia et al 2005). Both of these mechanisms allow the virus to quickly change and adapt when infecting a new host species. When a virus circulates long enough in a new host, it becomes adapted to that new species and results in a unique phylogenetic lineage (Gorman et al 1990).

The first step in AIV infection depends on attachment of the HA protein to the host cell sialic acid (SA) receptor. These SA molecules are classified based on the manner in which they are linked to the underlying sugars by the α-2 carbon. The most common linkages are the α-2,3 and α-2,6 linkage (Suzuki 2005). It is these two SA linkages that affect the conformation of the host's receptor and the ability of the HA viral surface protein to bind these SA receptors.

Levels of expression of α-2,3 and α-2,6 differ by species as well as the type of tissue within a particular species. These expression differences, both in terms of sensitivity (array, availability, and abundance) and specificity (receptor type), help dictate the binding affinity between the viral HA proteins and the host's SA receptors (Thompson et al 2006, Gagneux et al

2003). Because influenza viruses have a strong preference for either α-2,3 or α-2,6 sialic acid, this relationship affects host specificity and results in different levels of species barriers for AIV infection and replication (Lin et al 2012, Gagneux et al 2003). Epithelial tissues in avian species commonly express α-2,3 SA, and avian influenza viruses bind more efficiently to α-2,3 SA receptors (Lin et al 2012, Rogers and Paulson 1983). Alternatively, human influenza viruses preferentially bind to α-2,6 SA receptors which are found more abundantly in human epithelial tissues (Rogers and Paulson 1983). However, as outlined above, the type of SA receptors expressed (specificity) varies depending on the location and type of tissue in a single host. This means not all avian and human viruses exclusively infect avian and human species, respectively. Nevertheless, SA receptor tissue tropism, meaning the propensity for a particular SA receptor to be expressed and the particular location of its expression, does influence a virus' potential to infect, replicate, and cause disease. As an example, humans do express both α-2,3 and α-2,6 SA receptors. However, α-2,6 is expressed more abundantly in the upper respiratory tissues (URT), whereas α-2,3 is almost exclusively expressed in the lower respiratory tissues (LRT) in humans (Thompson et al 2006, van Riel et al 2006, Matrosovich et al 2004). These expression patterns have specific implications on the potential for AIV to successfully infect and replicate in a human host. To efficiently attach to α-2,3 SA receptors, virion particles must be to be deposited in the LRT.

During infection of a host, efficient delivery to tissues expressing appropriate SA receptors partially explains this host-range restriction phenomenon. Following attachment, fusion, and entry into the host cell, the virus must efficiently replicate within this cell. However, just as each virus preferentially binds a type of SA receptor, the efficiency of viral replication depends on several factors, one of which is the temperature within the tissue (Schrauwen et al 2014, Scull et al 2009). Not all viruses replicate proficiently under the same temperature conditions and not all tissues maintain the same temperature. AIVs tend to prefer higher replication temperatures, whereas human influenza viruses prefer lower replication

temperatures (Massin et al 2001). The PB2 proteins of AIV favor higher temperatures, enabling better replication in the LRT and intestinal tract where temperatures are generally higher compared to proximal tissues (Scull et al 2009). Interestingly, research has shown that a simple amino acid switch at position 626 in the virus's PB2 protein allows for efficient replication at lower temperatures (Steel et al 2009). However, to maintain optimal fitness in an avian host, the amino acids comprising the AIV PB2 proteins are adapted to support efficient replication.

AIV could infect the LTR in a human host, but just as infection might not result in efficient replication, infection and replication might not support sustained transmission. The few documented cases of non-sustained human-to-human transmission have occurred in blood-related family members and probably due to close, unprotected contact with a severely ill relative (World Health Organization (WHO) 2006, Ungchusak et al 2005). These reports suggest efficient AIV transmission between human hosts is related to exposure dose and is dependent on the quantity of biologically viable virions generated and delivered to the LRT of another susceptible human. Transmission might be further restricted by inefficient NA cleavage related to temperature restrictions (Scull et al 2009). Given these barriers and unique viral characteristics, AIV host range is a multifactorial equation. There is no simple way to determine host restriction, making it difficult to predict the ability of an existing or even novel reassortant virus to infect, replicate and maintain transmission in a particular species.

3. Epidemiology of AIV in poultry

AIV is rarely fatal in wild birds and this avirulent nature can be attributed to viral adaptation over many centuries (Webster et al 1992). However, AIV is highly contagious and can be associated with morbidity and mortality in domestic poultry (Swayne and Halvorson 2008). Transmission of AIV occurs through the migration of infected wild birds, direct contact between wild birds and domestic poultry, movement of infected poultry, and through fomites carried by humans and equipment. AIV outbreaks have been reported by 69 countries

between 1996 and 2013 (2014: Office of International Education (OIE) World Organization for Animal Health).

LPAI transmission from wild bird populations to domestic poultry is most efficient through contamination of water into a common environment (Keeler et al 2012, Swayne 2008, Webster et al 1992). This occurs when infected wild migratory birds shed large quantities of the virus into the common environment via respiratory and fecal secretions (Keeler et al 2012, Swayne 2008, Hinshaw et al 1979). When lakes or ponds are used as sources of drinking water for domestic poultry, AIV can be introduced. Live bird markets provide an ideal environment to introduce AIV to poultry (Halvorson 2008, Kung et al 2003, Suarez et al 1999). This occurs when domestic waterfowl are raised in outdoor ponds and become infected. When these birds are transported to a live bird market, they become a source of infection and AIV spread to poultry, which eventually supports AIV transmission to commercial poultry. Poor biosecurity and outdoor rearing (backyard flocks) also favor introduction to domestic poultry due to the close proximity to wild birds (Cardona 2008, Swayne 2008).

Exposure does not always lead to infection, which is partially dependent on infectious dose, host immunity, and the degree of viral adaptation to the new host species. However, once LPAI infects domestic poultry and continues to circulate, it becomes well adapted to this new host species. Increased replication leads to higher shedding titers, which ultimately increases the risk for transmission within and between flocks. The incubation period for AIV may last as long as 10 days and the majority of infected poultry shed virus for 7-10 days, allowing the virus to circulate among and between poultry flocks for a long period of time (Easterday et al 1997). This potentially long shedding period also increases the transmission risk to poultry, especially within larger populations (Easterday et al 1997). When uncontrolled circulation of LPAI occurs in poultry populations, this increases the risk for H5 and H7 mutation into HPAI (Swayne 2008, Perdue and Suarez 2000).

4. Current AIV control strategies in poultry

The strategies developed to control AIV in poultry depend on many factors. These include geographical location or country, poultry species, pathogenicity of the virus, financial resources, public perception, veterinary infrastructure, and the desired outcome. Three possible goals exist with any control strategy. These include prevention, management, and eradication (Swayne 2004). Each one of these goals is accomplished using a combination of different strategies.

Education is a component often overlooked, but is a critical aspect to controlling AIV, especially in developing countries. One of the primary ways to spread AIV is through contaminated fomites transported by humans (Swayne 2008). Properly educating, training, and communicating with individuals and the public on how these events occur and how they can be prevented, will limit risky behavior and activities associated with viral transmission. Detecting the virus using proper diagnostics and demonstrating the presence or absence of disease using surveillance are both important tools to control and manage outbreaks. Mass depopulation and the proper disposal of infected poultry play a significant role in preventing environmental contamination and the dissemination of virus (Krushinskie et al 2008).

To date, biosecurity practices are perhaps one of the most important preventative measures to help control influenza outbreaks in poultry (Cardona 2008). Biosecurity practices are directed at preventing contact between wild migratory birds and poultry, preventing virus transmission via fomites, and limiting human contact with flocks. Among the major egg producing operations in the United States, flocks of 100,000 are common and can reach 1 million or more birds (National Agricultural Statistics Service 2013). These large farms invest more in biosecurity measures than smaller farms due to the increased risk associated with higher bird densities (Thomas et al 2005). The recent increase in small-scale producers raising antibiotic-free and free-range poultry has also added concerns about AIV emergence in such small poultry productions (Jacob et al 2008). Biosecurity also includes filtering drinking water

used by poultry farms, especially when water sources are coming from ecosystems that harbor aquatic wildfowl (Keeler et al 2012, Swayne and Suarez 2005). Practicing sound biosecurity requires proper education and training. Once again, education is often lacking in developing countries where AIV is endemic and this creates a barrier to understanding the importance of maintaining biosecurity measures to protect against AIV (Paudel et al 2013, Neupane et al 2012, Maton et al 2007).

The final component to preventing, managing, and eradicating AIV in poultry is increasing host resistance. In the United States, state veterinarians regulate the use of LPAI vaccines (Swayne and Suarez 2005). However, the poultry industry does not commonly adopt preventative vaccination as a viable control option against AIV outbreaks (Swayne and Suarez 2005). The exception is commercial turkey operations because LPAI is often endemic in turkeys and turkey breeders are very susceptible to swine influenza (Halvorson 2008, Swayne and Suarez 2005, Swayne 2001). Although most state veterinarians approve the use of LPAI vaccines, several reasons have created an adverse stance on preventative vaccination in the United States. First, the most potent vaccines must be administered subcutaneously (Halvorson 2008). On a large poultry operation, this makes administration slow and cumbersome. Second, the vaccine must be HA-subtype specific for efficient protection (Swayne 2014, Swayne and Kapczynski 2008). Frequently, a new vaccine must be generated to achieve subtype specificity and this is a time-consuming process. It can take 2 months or more just to acquire the vaccine and additional time to disseminate and administer. In the end, vaccination is not viewed as a cost effective strategy to LPAI (Swayne 2014, Swayne and Kapczynski 2008). The United States Department of Agriculture (USDA) regulates vaccination for HPAI (H5 or H7) at the federal level (Swayne and Suarez 2005). HPAI vaccination has never been granted, as the United States has yet to deal with a sizable outbreak. When HPAI virus is detected, as has been the case in several New York and New Jersey live bird markets (Bulaga et al 2003), a stamping out strategy is used to depopulate.

The largest demand for an AIV vaccine worldwide has stemmed from the four H5N1 HPAI enzootic countries, China, Egypt, Indonesia, and Vietnam, where vaccination programs are directed to all poultry (Swayne and Spackman 2013). Cost benefits and risk analyses all contribute to selecting appropriate populations of birds to vaccinate and determining appropriate vaccination zones (Halvorson 2008, Swayne 2006). Vaccination of birds in a high-risk (outbreak zone) is most important, followed by vaccination of rare captive birds, valuable genetic stocks, long-lived poultry (commercial layers or hatchers), and lastly meat poultry (Swayne 2006). An important consideration when deciding to vaccinate is cost. The average cost, including administration, is $0.10 - $0.15 per bird (Halvorson 2008). Often the economic consequences that could ensue after failure to contain an AIV outbreak play the greatest role in the decision to vaccinate (McLeod 2008).

The ultimate goal or ideal outcome with any disease control plan is complete eradication. With respect to AIV, complete eradication is nearly an impossible feat, given that the virus is maintained in wild migratory bird populations. However, preventing and managing outbreaks to reduce economic losses is a very realistic goal. Success depends on adopting a comprehensive strategy, as no single approach is adequate.

5. RNA interference

RNAi is canonically known as a conserved post-transcriptional gene silencing mechanism that occurs in many eukaryotic organisms. RNAi was first described in the late 1990s in *Caenorhabditis elegans* when Andrew Fire and Craig Mello attempted to enhance gonad genes but instead blocked genes using exogenous double-stranded RNA (dsRNA) (Fire et al 1998). The mechanism is initiated when long dsRNA is cleaved by an enzyme known as Dicer to yield 20-25 short nucleotide pieces of dsRNA or siRNA duplexes (Elbashir et al 2001). The antisense strand of the resulting siRNA is incorporated into the RNA-induced silencing complex (RISC) and acts as a guide strand to mediate the degradation of target mRNAs. When

the siRNA binds to target RNA with perfect sequence complementarity, this catalyzes the slicing and degradation of the complementary mRNA sequence by Argonaute, the catalytic component of the RISC complex (Elbashir et al 2001, Tuschl et al 1999, Fire et al 1998).

While the canonical RNAi pathway has been characterized most extensively in *Drosophila melanogaster*, researchers have demonstrated that RNAi acts as a natural antiviral defense in plants, insects, nematodes, and fungi (Guo et al 2012, Han et al 2011, Segers et al 2007, Diaz-Pendon et al 2007). Lending to the notion that RNAi is a natural antiviral pathway, a more recent study has indicated RNAi might function as an antiviral mechanism in mammals (Li et al 2013).

RNAi can be used as a valuable tool to study gene expression, cellular processes, and to develop new antiviral technologies. Just as cellular mRNAs are susceptible to RNAi silencing, viral mRNAs can become targets of synthetic siRNAs. When these synthetic siRNAs are designed to target specific viral mRNA sequences and are transfected into recipient cells, the introduced siRNAs incorporate into the cellular RNAi machinery and target their complementary viral RNA for degradation (Jin et al 2014, Rothe et al 2011). This has raised the possibility of using synthetic siRNA as promising antivirals (Thakur et al 2012, O'Neill 2007) and provided the foundation for the research presented in this dissertation.

6. Project justification and long-term goal

Experimental data for both LPAI and HPAI show that vaccination protects against morbidity and mortality, reduces virus shedding, slows virus transmission and increases resistance to infection (van der Goot et al 2007, Capua et al 2004). However, there are limitations when relying on a vaccine to adequately control AIV spread. Vaccination against AIV does not confer sterilizing immunity (i.e. with 100% flock immunity) as evidenced by the ability of the virus to replicate in vaccinated birds (Capua et al 2004). Further limitations of vaccination as a control measure are attributable to a reliance on a healthy immune system and antigenic

evolution of the virus, which could render even the most current vaccines ineffective (Arzt et al 2010, Zhou et al 2008, Bennink et al 2004, Ge et al 2004). The limited practicality in administrating the vaccine by injection is another significant hurdle. A low cost method for mass immunization or treatment of poultry would translate into an economic incentive to vaccinate, especially in areas where vaccination is needed to control AIV. The speed and timeliness in which vaccination can be implemented following an outbreak should not be overlooked. Protective antibody production in chickens takes two to three weeks to acquire following vaccination (Swayne et al 1997). As such, vaccination during an outbreak does little to protect against the threat of infection, thereby failing to break the chain of AIV transmission among and between flocks.

These findings indicate it is important to consider new prophylactic strategies that can provide universal protection against any strain or subtype of AIV, including HPAI. Furthermore, because the vaccine relies on protective antibody production, even when preventative vaccination is applied at the onset of an outbreak, the risk of spreading virus is high due to a lack of protection. Developing a prophylactic technology that can be easily and readily administered during an outbreak to provide rapid and universal protection against AIV could have a significant impact on preventing viral transmission and would contribute to building a more comprehensive control strategy.

Using RNAi methodologies, this dissertation project is focused on developing an innovative anti-influenza prophylactic that provides rapid protection against AIV in an avian model. I envision this technology could be used by the commercial poultry industry and by developing countries as a cost effective means to protect commercial and backyard flocks against AIV outbreaks. The long-term goal is to promote this novel anti-AIV technology as a complement to vaccination with the intention of developing a more effective and robust control plan against AIV in poultry. If successful, this technology could be used in the face of an outbreak to reduce the shedding and transmission of virus within poultry, between farms, and

across borders, thereby improving animal health and reducing the economic impact of outbreaks worldwide. This approach provides a new point of view that could positively contribute to improving mainstream perspectives on preventing influenza in poultry and reducing the risk of transmission to humans. Finally, this work could provide the framework and valuable proof-of-concept to the scientific and medical community for developing a similar anti-influenza prophylactic for humans.

7. Dissertation objective and specific aims

Identifying viral genes required for efficient AIV replication and silencing these genes using RNAi could provide the basis for developing a new anti-AIV platform for poultry. However, the delivery of these RNAi-mediating agents has historically been an obstacle to validating its clinical relevance for future translation into a viable technology. TkRNAi is a delivery platform using bacteria to generate and deliver siRNA to target mucosal tissues (Xiang et al 2006). These tkRNAi vectors can be engineered to generate and deliver siRNA targeting viral genes necessary for efficient AIV replication, and could represent a technology ideal for preventing avian influenza via administration to the upper airways in chickens.

The overall objective of this dissertation was to provide proof of concept for inhibiting AIV shedding in chickens using tkRNAi vectors (termed anti-AIV vectors) engineered to generate and deliver viral siRNAs to avian respiratory tissues. The central hypothesis of this dissertation was administration of anti-AIV vectors expressing specific viral siRNAs would significantly inhibit AIV replication in an avian model. Applying the tkRNAi delivery approach in an avian model is innovative and represents the first instance of such a delivery mechanism to inhibit influenza replication. The main objective of this dissertation was achieved by pursing the following three aims:

1. ***Demonstrate in vitro proof of concept for using viral specific siRNAs to inhibit avian influenza in an avian cell model***. An avian cell model was established after optimizing

transfection, infection, and viral quantitation techniques. Viral siRNAs targeting specific AIV genes were constructed and assessed for their ability to inhibit viral replication and shedding in chicken epithelial cells, independent of the tkRNAi system.

2. Design the anti-AIV vectors and demonstrate in vitro proof of concept for using vectors to inhibit avian influenza in the established avian cell model. Using the siRNA sequences tested and validated in aim 1, the tkRNAi vectors were constructed and tested *in vitro*. Vector uptake into chicken epithelial cells and associated cell toxicity were both evaluated, independent of viral siRNA delivery. Vectors were subsequently assessed for their ability to inhibit AIV replication and shedding in chicken epithelial cells.

3. Demonstrate in vivo proof of concept for using the anti-AIV vectors to inhibit avian influenza in chickens. The vectors designed and tested in aim 2 were delivered intranasally into commercial chickens. Vector uptake into chicken respiratory tissues and associated gross and microscopic pathology were evaluated. Anti-AIV vector protection against AIV was subsequently assessed in experimentally challenged chickens.

A concluding summary of the strengths and limitations of this work is presented in the final chapter of this dissertation. Also discussed is the potential to further evaluate the anti-AIV vector technology using randomized field trials, and methods in which the technology could be improved to elicit broad-spectrum protection against avian influenza virus infection in poultry. This final chapter addresses the poultry industry's needs and specific ways these vectors could effectively meet these requirements. Finally, the feasibility and value of translating this technology into a usable product is considered.

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CHAPTER 2

PREVENTING AVIAN INFLUENZA VIRUS REPLICATION IN A CHICKEN CELL MODEL USING VIRAL SiRNAs

Summary

AIV represents one of the most significant economic threats to poultry worldwide. Vaccines for AIV are limited, highlighting the need to consider new prophylactic strategies that can protect poultry against outbreaks. An appropriate avian tissue transfection and AIV infection model was developed. This avian model was used to demonstrate the antiviral potential of siRNAs targeting two key AIV genes required for viral replication, NP and PA. Chicken LMH cells were transfected with siRNAs targeting NP and PA mRNA and cells were infected with two different LPAI subtypes, H8N4 and H6N2. Multivariable linear regression analysis, controlling for day, revealed significant differences in adjusted mean shedding titers between samples treated with siRNA and those untreated ($p<0.05$). Individual siRNAs and tested siRNA cocktails led to a decrease of up to 100 fold in shedding titers in chicken cell supernatants ($p < 0.05$). This work demonstrates *in vitro* proof of concept for using viral specific siRNAs to inhibit avian influenza replication in the established avian cell model.

1. Introduction

While asymptomatic and rarely fatal in wild birds, AIV is highly contagious and infection can result in mild to severe clinical illness when transmitted to domestic poultry (Swayne and Halvorson 2008, Capua and Marangon 2006, Horimoto and Kawaoka 2001). HPAI viruses rapidly infect poultry and are often fatal (Horimoto and Kawaoka 2001). In contrast, LPAI viruses typically cause mild disease that can go undetected. However, the economic effect of LPAI lies

in loss of production, the risk for both LPAI and HPAI outbreaks are threats to the poultry industry, domestic and internationally.

Current prophylactic methods for AIV in poultry are limited. Available vaccines do not confer complete immunity, as evidenced by the ability of the virus to replicate in vaccinated birds (Capua et al 2004). Vaccines rely on a healthy immune system and could be rendered ineffective due to viral antigenic evolution (Arzt et al 2010, Escorcia et al 2008, Zhou et al 2008, Bennink et al 2004, Ge et al 2004). Results from previous studies showed that AIV field isolates from poultry exhibited constant drifts in their genetic information and the persistence of the virus in the field was likely aided by antigenic differences attributed to the vaccine strain (Escorcia et al 2008, Lee et al 2004). Vaccine protection can take more than one week to acquire (Kim et al 2009); therefore, vaccination during an outbreak offers little protection. Vaccination is most effective via intramuscular injection (Halvorson 2008), which is not practical for large-scale vaccine administration. Finally, improper storage and handling often leads to vaccine failure (Swayne and Kapczynski 2008). There are also growing concerns that current avian H5N1 viruses are becoming resistant to amantadine, rimantadine and oseltamivir (Cheung et al 2006, Le et al 2005, de Jong et al 2005). Reports have linked treatment with these common drugs to the shedding of drug resistant viruses in both humans and poultry (WHO Global Influenza Program Surveillance Network). As an example, in a desperate attempt to protect their poultry farms, China was reported to have administered amantadine during the 2005 H5N1 outbreaks. This misuse has led to drug resistant strains that are circulating in China and southeastern Asia (Cheung et al 2006). The lack of robust prophylactics and the endemic nature of AIV underline the urgency to develop more effective control measures in poultry, as a means of controlling AIV transmission and reducing the impact outbreaks have on poultry operations. It is specifically valuable to consider new prophylactic strategies that can protect poultry against any subtype or strain of AIV.

Because the NP and PA proteins both play a critical role in AIV transcription and replication, it is possible to inhibit virus replication by targeting these genes with siRNA (Zhou et al 2007, Li et al 2005, Tompkins et al 2004, Ge et al 2003). Designing siRNAs that can remain potent despite antigenic drift and shift requires targeting regions within a gene that are conserved among different subtypes and strains of AIV. Possibly, due to their role in viral transcription and replication and as a potential way of maintaining viral fitness, NP and PA each contain a stretch of conserved nucleotides found in most type A influenza viruses. The specific roles these short sequences play in viral replication has yet to be elucidated. However, these short conserved sequences are found in chicken, human, canine, equine, and swine influenza genomes (the influenza sequence database at www.flu.lanl.gov, Heiny et al 2007, Bennink et al 2004, Ge et al 2004, Ge et al 2003). Unlike these two genes, the HA and NA segments contain no stretch of 21 conserved nucleotides for siRNA design (Ge et al 2004). These conserved regions render these short sequences in the NP and PA genes prime targets for siRNA design.

Other researchers have investigated the use of siRNAs targeting the viral genome, specifically NP and PA as a way of preventing viral replication in mammalian cell culture and in mice (Khantasup et al 2014, Jiao et al 2013, Zhou et al 2008, Zhou et al 2007, Ge et al 2004, Tompkins et al 2004). However, areas of research aimed at using small inhibitory RNAs as antivirals for AIV are needed. Research has shown that these viral siRNAs are effective singly, but it is practical to determine if their combined potency is multiplicative or at best, additive. Most research has been assessed in mammalian cells (Khantasup et al 2014, Jiao et al 2013, Li et al 2005, Zhou et al 2008, Zhou et al 2007, Ge et al 2003) and few studies using avian models are available in the literature. AIV is a disease of avian species. As such, it is imperative to use an avian cell model to determine if RNAi is a valid approach to preventing AIV replication in poultry. The viruses typically selected for previous RNAi transfection and viral infection studies have been lab adapted strains (Zhou et al 2007, Ge et al 2004, Ge et al 2003), primarily isolated from mammals. If we seek to determine if RNAi is a valid approach to preventing AIV replication

in poultry, it is critical to conduct these studies using naturally occurring avian influenza viruses commonly isolated from poultry.

The objective of this research was to develop an avian cell model and determine if siRNA mediated knockdown targeting these two key genes required for viral replication, NP and PA, inhibits AIV replication *in vitro*, as a model for use in poultry.

2. Materials and methods

2.1 Cell cultures

Chicken hepatocellular carcinoma epithelial cells (LMH cells, CRL-2117, American Type Culture Collection (ATCC), Manassas, VA) were chosen as the model avian cell line. These cells are commercially available, specifically intended for transfection studies, and as epithelial hepatocytes, they represent an appropriate avian tissue for type A influenza infection (Swayne and Pantin-Jackwood 2006, Shinya et al 1995). LMH cells were grown in Waymouth's MB 752/1 medium (Life Technologies, Grand Island, NY), with 10% fetal bovine serum (FBS) (Life Technologies), 2 millimolar (mM) L-Glutamine (Life Technologies), 100 Units/mL penicillin and 1mg/mL streptomycin (Life Technologies) at 37°C in the presence of 7% CO_2. All culture vessels used to propagate the LMH cells were precoated with 0.1% gelatin (EMD Millipore Corporation, Billerica, MA). Madin-Darby canine kidney (MDCK) cells were used to determine viral titers and to quantify infectious viral particles following all transfection and infection assays conduced in LMH cells. MDCK cells were grown in minimum essential medium with Earle's balanced salts (MEM/EBSS), 2 mM L-Glutamine, 10% FBS, 0.5% sodium pyruvate (100 mM solution, Life Technologies), 0.5% MEM non-essential amino acids (NEAA) (100x solution, Life Technologies), and 100 Units/mL penicillin and 1mg/ml streptomycin (Life Technologies) at 37°C in the presence of 7% CO_2.

2.2 Viruses

The following LPAI avian influenza virus strains were propagated in LMH cells: A/Chicken/Texas/473-2/10 (H6N2) and A/turkey/Colorado/1/05 (H8N4) (subtype was previously determined by hemagglutination-inhibition and neuraminidase-inhibition panels). Virus titers were measured by 50% tissue culture infectious dose ($TCID_{50}$) assay in MDCK cells. H6N2 and H8N4 LPAI strains were chosen for the study because they replicated to high titers in LMH cells with visual damage to the cells during virus invasion indicative of a cytopathic effect (CPE) at 48 hours post infection (hpi). The H8N4 virus was passaged 3 times and the H6N2 virus was passaged once in LMH cells. Viral stocks were generated in LMH cells and supernatants were collected, centrifuged to remove cell debris, and stored at -80°C. A working stock titer was measured by $TCID_{50}$ and optimal multiplicity of infection (MOI) was empirically derived.

2.3 siRNAs

RNA oligonucleotides were synthesized, desalted, and duplexed prior to shipment (Dharmacon, Thermo Scientific, Lafayette, CO). Sense and antisense RNA sequences for NP (NP-siRNA) and PA (PA-siRNA) were previously published (Ge et al 2003). The mRNA target sequence for each siRNA are as follows: NP: 5'- GGA TCT TAT TTC TTC GGA G - 3' and PA: 5' -GCA ATT GAG GAG TGC CTG A - 3'. BLAST was used to verify each siRNA sense strand sequence was 100% complementary to the NP and PA viral gene sequences associated with H6N2 and H8N4. The National Veterinary Services Laboratory (NVSL) previously sequenced the NP and PA genes from the H6N2 virus. Because the sequences for the current H8N4 virus were not previously annotated, the full length NP and PA sequences corresponding with twenty different H8N4 viruses available on the National Center for Biotechnology Information (NCBI) database were used to screen for complementarity. The H8N4 viruses included in this screen had been isolated in 2005 +/- 2 years and from domestic turkey (n=6) and from other domestic poultry and wild birds (n=14). Additionally, all siRNA anti-sense seed region sequences were

screened for high homology to chicken sequences to decrease the possibility of off-target silencing.

2.4 Transfection efficiency

Several transfection reagents were tested for visual signs of CPE independent of siRNA. These include Lipofectamine® 2000 (Life Technologies), Lipofectamine® RNAiMax (Life Technologies), FuGene® 6 (Promega Corporation, Madison, WI) and XtremeGene (Roche Diagnostics, Indianapolis, IN). Transfection efficiencies were determined in LMH cells using BLOCK-iT™ Alexa Fluor® Red Fluorescent dsRNA Control (Life Technologies). Alexa Fluor®-positive cells were counted in eight random ocular fields at 40X using the LSM 510 Zeiss confocal fluorescent microscope. Fluorescent microscopy was used to select the final transfection reagent, determine the optimal concentration range of siRNA and transfection reagent, and the optimal siRNA incubation period. The transfection efficiency ($\alpha = 0.05$) was determined after 24 hours incubation.

2.5 Transfection of siRNA into chicken cells

LMH cells were seeded one day prior to transfection in 24-well plates to allow cell monolayers to reach 80% confluency. Cells were transfected with NP-siRNA, PA-siRNA and both NP/PA (cocktail-siRNA) (32nM individually or 64nM total in a cocktail format) using Lipofectamine® 2000 according to the manufacturer's instruction. Briefly, Lipofectamine® 2000 was diluted 1:50 in Opti-MEM medium (Life Technologies). Each test siRNA was subsequently diluted in the Lipofectamine® 2000 – Opti-MEM mixture and allowed to incubate at room temperature for 15 minutes. The LMH cells were prepared for transfection by removing the complete growth medium and washing each well with Opti-MEM medium only. The wash was replaced with 687 µL/well transfection medium (Opti-MEM, 10% FBS, 2 mM L-glutamine) and 63 µL of siRNA-Lipofectamine® 2000 complex or the Lipofectamine® 2000 mixture without

siRNA (positive control mock-transfection wells) was added. All control or treated cells were transfected in triplicate wells.

2.6 Virus infection

Twenty four hours post transfection the LMH cells were prepared for infection by removing the transfection medium and washing each well with inoculation medium (IM) containing 0.25 µg/mL TPCK treated trypsin (Sigma-Aldrich, St Louis, MO), 3% bovine serum albumin (BSA) (Gemini Bio-Products, Sacramento, CA), 2 mM L-glutamine, 100 Units/mL penicillin and 1mg/mL streptomycin. The wash was removed and a total of 250 µL of IM containing either H8N4 or H6N2 virus at an MOI of 0.01 or 0.001 was added to each appropriate well. Plates were incubated on a rocking platform at 37°C in the presence of 7% CO_2 for 1 hour before removing the virus and replacing with 750 µL/well of fresh IM. Forty-eight hpi cell culture supernatants were harvested, centrifuged to remove cell debris, and frozen at -80°C. Each experiment included a positive control (mock-transfection and LPAI infected) and a negative control (untreated LMH cells) tested in triplicate wells. An initial experiment compared virus titers between infected only controls (LMH cells infected with LPAI virus) and the positive control (PC) samples.

2.7 Evaluation of infectious virus titer

LMH cell culture supernatants were quantitated by titration on MDCK cells using CPE as the indicator of viral presence, and titer was expressed as the $TCID_{50}$/mL. Briefly, MDCK cells were seeded into 96 well plates. For each culture supernatant, ten-fold serial dilutions from 1:10 to $1:10^8$ were made in MEM/EBSS, 2 mM L-Glutamine, 3% BSA, 0.5% sodium pyruvate, 0.5% MEM-NEAA, 100 U/mL penicillin and 1 mg/mL streptomycin, containing 1 µg/mL L-1-Tosylamide-2-phenylethyl chloromethyl ketone (TPCK) treated trypsin (Worthington Biochemical Corporation, Lakewood, NJ). When cell monolayers were 80-90% confluent, growth medium

was removed and the cells were inoculated with 100 µL/well of virus suspension in triplicate. At 48 hpi cells were fixed and stained with crystal violet (0.1% crystal violet) in phosphate buffered saline (PBS) and wells with CPE were scored as positive for virus growth. $TCID_{50}$/mL was calculated by the Reed and Muench mathematical technique (Reed and Muench 1938).

2.8 Statistical analysis

All experiments were repeated three times and on different days. Using a statistical significance level of 0.05, the Wilcoxon rank-sum test was performed to compare median viral titers with 95% confidence intervals (CI) between siRNA transfected and PC samples performed on the same day. To determine if adjusted mean $LogTCID_{50}$/mL values between siRNA transfected and PC samples were different ($p < 0.05$), data was analyzed using multivariable linear regression (MLR), controlling for day. Statistical analyses were performed using the data analysis and statistical software STATA 10 IC (StataCorp, 2009, Stata Statistical Software: Release 10. College Station, TX).

3. Results

3.1 Design of effective siRNAs

The wide range of genetic variation among different strains and subtypes of AIV is largely responsible for viral evasion when the vaccine administered is different from the AIV subtype in circulation. Likewise, this genetic variability makes it difficult to design siRNAs that can remain effective against multiple AIVs. As little as one nucleotide difference between the mRNA target site and the siRNA antisense seed region can completely abolish any RNAi antiviral activity. Therefore, it is important to design siRNAs targeting highly conserved sequences observed across many different strains, including HPAI viruses. Basic Local Alignment Search Tool (BLAST) (www.ncbi.nlm.nih.gov/BLAST) was used to search for sequence homology in type A influenza viruses as well as off-target matches between the NP

and PA siRNA sequences and chicken sequences. This screening revealed neither siRNA had high homology to any known chicken sequences, specifically between the siRNA seed region and host mRNA. The NP and PA siRNA sequences both aligned against all twenty full length NP and PA H8N4 viral genes used for screening and the H6N2 NP and PA sequences with 100% homology (Table 3.1). Using the nucleotide collection database (nr/nt) for both 19 nucleotide (nt) siRNA sequences, the BLAST search reveals 100% query cover in over 10,000 type A influenza viruses, including those isolated from swine, wild birds, poultry, equine, canine, and humans. These query matches include pathogenic H5, H9, and H7 subtypes isolated as recently as 2013 from national and international outbreaks.

3.2 siRNA antiviral activity in chicken cells

Lipofectamine® RNAiMax, FuGene® 6 and XtremeGene all resulted in observable CPE in LMH cells. Therefore, transfection efficiencies were not further assessed using these three reagents. The transfection procedure was optimized to achieve up to 81% transfection rate in LMH cells using Lipofectamine® 2000 and siRNA concentration between 32nM and 64nM. This transfection reagent, optimized procedure, and range of siRNA concentration allowed for adequate transfection, without inducing CPE. Prior to infection with H6N2 or H8N4 virus at MOI 0.01 and 0.001, NP and PA-siRNA were transfected into LMH cells and culture supernatants were collected for $TCID_{50}$ analysis. Lack of significant differences in virus titer between infected only controls and PC samples indicate the transfection procedure itself did not affect virus production (data not shown).

The antiviral activity of each siRNA transfection varies by day, virus, and MOI used for infection (Figure 3.1). However, significant antiviral activity is observed in NP, PA, or cocktail-siRNA samples from both H6N2 and H8N4 infections. On each experimental day, at least one siRNA transfection significantly reduced median virus titers in both viruses and at both MOIs, compared to the corresponding PC samples. On at least one experimental day, the cocktail

siRNA significantly reduced median virus titers in both viruses and at both MOIs, except H8N4 at MOI 0.001. Despite a lack of significant differences likely associated with a small sample size, 70%, 50%, and 100% of all NP-siRNA, PA-siRNA, and cocktail-siRNA transfections resulted in lower median viral titers compared to their appropriate untreated PCs, respectively.

To compare siRNA transfected to untreated PC samples across all experimental days, MLR analysis was used, controlling for day. MLR results reveal significant differences in adjusted mean viral titers between siRNA transfected and untreated PC samples (Table 3.2). Significant differences were observed in both NP-siRNA and cocktail-siRNA samples from H8N4 MOI 0.01 and H6N2 MOI 0.001 infected cells. These mean titers ($LogTCID_{50}$/mL) are as follows: NP-siRNA (H8N4 MOI 0.01) (4.1; 95% CI= 3.4, 4.8) compared to PC (5.0; 95% CI= 4.3, 5.7); cocktail-siRNA (H8N4 MOI 0.01) (3.7; 95% CI = 3.1, 4.3) compared to PC (5.0; 95% CI= 4.4, 5.6); NP-siRNA (H6N2 MOI 0.001) (8.4; 95% CI= 7.2, 9.7) compared to PC (10.4; 95% CI= 9.5, 11.4); and cocktail-siRNA (H6N2 MOI 0.001) (8.7; 95% CI = 7.4, 10.0) compared to PC (10.4; 95% CI= 9.4, 11.5). The log_{10} reduction in infectious virus between siRNA transfected and untreated samples was calculated. With the exception of PA-siRNA transfection from H8N4 MOI 0.001 and H6N2 MOI 0.01 samples, all siRNA transfections resulted in at least 0.3 log_{10} reduction (2.1 fold reduction). Most notable were H8N4/MOI 0.01 titers after cocktail-siRNA transfection, H8N4/MOI 0.001 titers after NP-siRNA transfection, and H6N2/MOI 0.001 titers following cocktail and NP-siRNA transfections, resulting in a 1.3 (20-fold), 1.0 (10-fold), and 1.7 (50-fold), 2.0 log_{10} reduction (100-fold), respectively.

4. Discussion

This work demonstrates *in vitro* proof of concept for using viral specific siRNAs to inhibit avian influenza virus in an avian cell model. Chicken LMH cell transfection with siRNAs targeting NP and PA mRNA significantly reduced infectious titers following infection with two different AIV subtypes, H8N4 and H6N2.

Even when a significant difference between siRNA transfection and untreated PC samples was not observed, $\log_{10}$ reductions in infectious viral titers were observed in all siRNA samples, except two PA-siRNA transfection sets. The most potent inhibition of infectious viral titers occurred after LMH cells were transfected with either NP-siRNA or cocktail-siRNA, resulting in $\log_{10}$ reductions ranging from 0.5 - 2.0 (3.2 - 100 fold reduction). Several research groups have used different RNAi approaches to demonstrate the antiviral activity of siRNAs against human and avian influenza virus and report various degrees of viral inhibition in multiple subtypes tested (Khantasup et al 2014, Jiao et al 2013, Zhou et al 2008, Zhou et al 2007, Li et al 2005, Ge et al 2004, Tompkins et al 2004, Ge et al 2003). However, the majority of these studies demonstrate inhibition of human influenza virus in a mammalian model or the inhibition of AIV in a non-avian model. In one of the first studies, siRNA targeting NP and PA mRNA associated with the lab-adapted PR8 virus (A/PR/8/34 (H1N1) MOI of 0.001) resulted in 4.5 $\log_{10}$ (30,000-fold) reduction in MDCK cells (Ge et al 2003). In mice experiments, NP and PA siRNAs and short hairpin RNAs (shRNAs) resulted in inhibition ranging from 9-56 fold from multiple influenza subtypes, including H5N1, mouse-adapted H1N1, H7N7 and H9N2 (Zhou et al 2008, Ge et al 2004, Tompkins et al 2004). These are all notable results. However, either these reported reductions represent those associated in mice and non-avian cell models or the virus used for challenge was a lab/mouse adapted virus. To our knowledge, the avian cell model and AIVs utilized in the work presented in this chapter have not been reported in the literature. Both of the LPAI viruses utilized in this current work were minimally adapted through serial passage in LMH cells. Both were isolated from naturally infected animals from a past outbreak in poultry that occurred in the United States. These viruses are field isolates and required minimal passage in LMH cells. Therefore, they were suitable for testing this siRNA antiviral approach.

While these previous studies have demonstrated the antiviral potential of using siRNAs against influenza, these studies have similarly reported variations in the level of viral inhibition

observed when the same siRNA construct is used against different type A influenza viruses. It is challenging to suggest reasons for this variation, given these siRNAs are targeting highly conserved mRNA regions. Despite the obvious conservation of these 21 nucleotide sequences across all type A influenza viruses, it is possible that in different viruses these sequences correspond with slightly different functions. Complicated viral-host interactions are associated with optimal viral replication and these targeted mRNA sequences might interact differently with host factors or interact with entirely different host factors, all resulting in differences in siRNA potency. As such, targeting the NP sequence of H6N2 might have a more detrimental effect on viral replication compared to targeting the same NP sequence associated with H8N4 virus. However, evaluating the specific replication and host-interaction function of these viral mRNA sequences was beyond the scope of this work. Despite 81% transfection efficiency, some siRNA preparations may have been less efficient at knocking down NP and PA mRNAs. This could be due to moderate mRNA inactivation, inefficient siRNA loading with the transfection reagent, or inefficient siRNA delivery into the cell cytoplasm. Measuring NP and PA mRNA knockdown efficiencies or their corresponding protein levels might have helped explain the variation in antiviral activity. It is also possible that despite efficient mRNA knockdown, gene level inhibition due to siRNA silencing does not correlate with lower NP and PA protein levels. The outcome of interest was the ability of these viral siRNA to inhibit the overall quantity of virus released into the supernatants by measuring viral titer. Therefore, measuring reductions in NP and PA mRNA or protein was deemed unnecessary for this proof of concept work.

The sustainability of an AIV outbreak in poultry is characterized by sufficient shedding of infectious virus into the environment to infect the next host, consequently sustaining transmission between birds and thereby between farms. This raises the question, could the viral titer reductions observed in this current work represent biological indicators for the efficacy of siRNA transfection against AIV challenge in chickens? A previous study showed that a 10 to 100-fold (1 to 2 $\log_{10}$) reduction in HPAI H5N1 and 2009 pandemic H1N1 titers following

vaccination in mice fully protected from death and reduced pathology in the lungs (Easterbrook et al 2012). Another study showed that a 2 to 4 $\log_{10}$ reduction in oral shedding of infectious virus following vaccination in birds established efficacy of an H5 vaccine against HPAI H5N1 (Bublot et al 2007). While it is not possible to confirm if the reductions observed in the work presented in this chapter could translate into a clinically relevant reduction observed in chicken experiments, this work may provide a basis for defining measures eliciting sufficient siRNA protection that prevents virus shedding and subsequent disease transmission during an AIV outbreak in poultry. Additionally, influenza viruses mutate quickly, often rendering vaccines ineffective. Designing RNAi constructs targeting these conserved mRNA sites could allow for more efficient RNAi silencing, but moreover, targeting multiple conserved sites using these siRNA cocktails could further prevent RNAi escape by mutation.

RNAi is a tool that can help researchers achieve what has not yet been possible and provide an opportunity for a new anti-influenza development strategy. The work presented in this chapter was successful at developing an *in vitro* avian cell model to validate these siRNAs used with this RNAi anti-influenza approach. This work showed that siRNA mediated knockdown targeting both the viral NP and PA genes, in a cocktail format, inhibits AIV replication *in vitro*. However, despite the potential these siRNA have to inhibit infectious virus shedding in this avian model, these RNAi-mediating agents require a better mechanism of delivery to be deemed clinically applicable. The efficiency of delivery associated with using a synthetic transfection vehicle, such as Lipofectamine® 2000, is limited for several reasons. First, low delivery efficiencies require higher doses, which are not only cost prohibitive, but often toxic (Ge et al 2004). Second, these transfection reagents only achieve systemic delivery, not targeted delivery. This again requires higher doses to sufficiently transfect a specific tissue. Developing an RNAi antiviral technology that would allow for intranasal delivery would be uniquely promising for the prevention of AIV. Consequently, subsequent work presented in this

dissertation is focused on designing a better delivery mechanism for this RNAi based antiviral, specifically for the prevention of AIV in poultry.

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5. Tables and Figures

Table 3.1

Sequences of top strand siRNAs and AIV genes for NP and PA.

AIV gene (bp region)	Sense strand sequence (5' - 3')
NP-siRNA	GGA TCT TAT TTC TTC GGA G-dTdT
H8N4 NP (1486 - 1504)	GGA TCT TAT TTC TTC GGA G
H6N2 NP (1453 - 1471)	GGA TCT TAT TTC TTC GGA G
PA-siRNA	GCA ATT GAG GAG TGC CTG A-dTdT
H8N4 PA (2089 - 2107)	GCA ATT GAG GAG TGC CTG A
H6N2 PA (2065 - 2083)	GCA ATT GAG GAG TGC CTG A

Table 3.2

SiRNA protection in chicken epithelial cells as measured by log and fold-reductions in shedding titers. Results are from multivariable linear regression, controlling for day. [a] Expressed as adjusted mean $LogTCID_{50}$/mL. [b] Comparing adjusted mean $LogTCID_{50}$/mL from treated to untreated PC samples ($p<0.05$). [c] Log reduction in mean infectious titer compared to untreated control. [d] Fold reduction in geometric mean compared to untreated control.

Sample	*n*	Adjusted Mean [a]	95% CI	Adjusted Mean (untreated)	95% CI (untreated)	*P*-value [b]	Log reduction [c]	fold reduction [d]
H8N4 LPAI virus MOI 0.01								
NP-siRNA	10	4.1	(3.4, 4.8)	5.0	(4.3, 5.7)	0.010	0.9	7.9
PA-siRNA	10	5.5	(4.5, 6.5)	5.8	(4.8, 6.8)	0.547	0.3	2.0
cocktail-siRNA	10	3.7	(3.1, 4.3)	5.0	(4.4, 5.6)	< 0.001	1.3	20.0
H8N4 LPAI virus MOI 0.001								
NP-siRNA	10	7.5	(6.4, 8.7)	8.5	(7.6, 9.4)	0.088	1.0	10.0
PA-siRNA	10	8.6	(7.3, 10.0)	8.5	(7.5, 9.6)	0.892	0.0	0.8
cocktail-siRNA	10	8.3	(7.1, 9.4)	9.0	(8.1, 9.9)	0.190	0.7	5.0
H6N2 LPAI virus MOI 0.01								
NP-siRNA	10	6.3	(5.5, 7.2)	6.6	(5.7, 7.5)	0.443	0.3	2.1
PA-siRNA	10	7.5	(6.7, 8.2)	6.9	(6.1, 7.7)	0.109	0.0	0.3
cocktail-siRNA	10	5.8	(4.9, 6.6)	6.3	(5.5, 7.1)	0.188	0.5	3.2
H6N2 LPAI virus MOI 0.001								
NP-siRNA	10	8.4	(7.2, 9.7)	10.4	(9.5, 11.4)	0.004	2.0	100.0
PA-siRNA	10	10.0	(8.4, 11.7)	10.4	(9.2, 11.7)	0.615	0.4	2.5
cocktail-siRNA	10	8.7	(7.4, 10.0)	10.4	(9.4, 11.5)	0.012	1.7	50.1

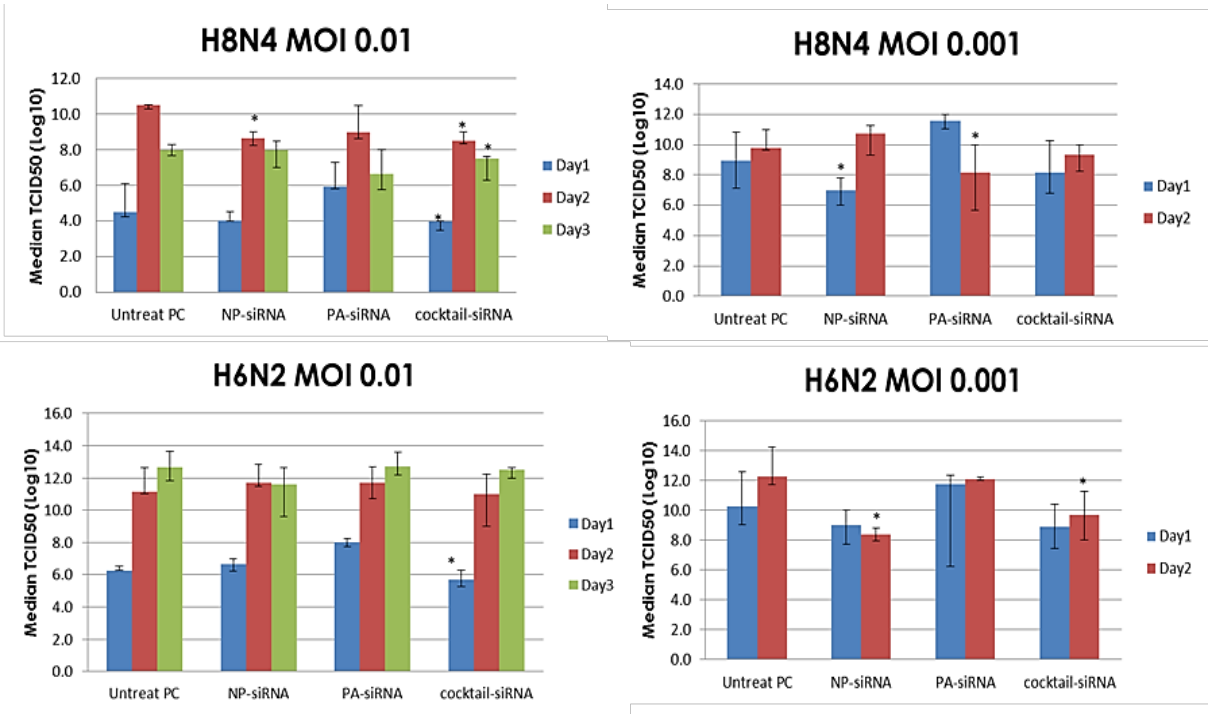

Figure 3.1

SiRNA protection as measured by reduction in median titers by day, MOI, and virus.

*Statistically significant difference with corresponding untreated sample using Wilcoxon rank-sum test ($p < 0.05$).

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CHAPTER 3

INHIBITING AVIAN INFLUENZA VIRUS REPLICATION IN A CHICKEN CELL MODEL USING A UNIQUE RNAi DELIVERY TECHNOLOGY

Summary

Economic incentives to vaccinate poultry against AIV are low and often owed to several limitations of the vaccine. These limitations and lack of incentive pose significant hurdles for effectively controlling AIV outbreaks in poultry. Developing a new anti-influenza technology is a critical step towards effectively managing and controlling the spread of this disease in poultry, minimizing financial losses, and reducing the risk for transmission to other animals, including humans. Applying RNAi methodologies to develop an alternative antiviral against AIV is one possibility. However, the delivery of RNAi-mediating agents is an obstacle to harnessing its clinical application. TkRNAi uses nonpathogenic bacteria to generate and deliver siRNAs to target tissues, and could be the key to attaining clinical application of an RNAi approach. TkRNAi vectors (anti-AIV vectors) were constructed to generate siRNA targeting the viral genes, NP and PA, and the protective efficacy of these vectors was evaluated in the established avian cell model. Vector uptake and invasion was first verified in chicken LMH cells treated with vectors tagged with a red fluorescent protein. Next, cells were treated with the anti-AIV vectors and infected with two different LPAI subtypes, H8N4 and H6N2. Multivariable linear regression analysis, controlling for day, revealed significant differences in adjusted mean shedding titers between samples treated with the anti-AIV vectors and those untreated. Vector cocktails targeting both NP and PA genes provided up to a 10,960-fold reduction in shedding titers in chicken LMH cell supernatants. This work demonstrates *in vitro* proof of concept for using these novel anti-AIV vectors to inhibit AIV in the established avian cell model. This work

presented in this dissertation represents the first such instance for using the tkRNAi system in an avian model and to inhibit influenza virus replication.

1. Introduction

Historically, AIV outbreaks in poultry are devastating and estimates of potential economic loss are enormous. This is specifically true when AIV epidemics hit areas that have a higher density of poultry farms. These areas become high-risk locations for outbreaks and often face considerable challenges to control AIV transmission, despite strict biosecurity measures and depopulation efforts. Once transmitted via respiratory secretions and feces, the incubation period for AIV may last as long as 10 days and the majority of infected poultry shed virus for 7 to 10 days, allowing the virus to circulate in a flock for a long period of time (Easterday et al 1997). This potentially long shedding period increases the transmission risk to poultry, especially within larger populations (Easterday et al 1997). Developing an anti-influenza technology is a critical step to effectively manage and control the spread of this disease in poultry to minimize financial losses and risks for transmission to other susceptible species, including humans.

Current vaccination strategies for birds are limited, as they do not confer complete immunity, are reliant on a healthy immune system, are susceptible to viral antigenic evolution, require individual handling of every bird in a large scale commercial poultry operation, have a limited shelf-life, and antibody protection following vaccination takes several weeks to acquire. Because of these challenges, AIV vaccination within the US is rarely favored for prophylactic use in poultry. If an emergency vaccination program were adopted, it would offer little protection if administered during an outbreak. Furthermore, growing evidence for the emergence of drug resistance AIV variants poses a risk for the use of drug therapies, such as amantadine, rimantadine and oseltamivir. These limitations indicate a real need to develop a prophylactic that can not only protect against different subtypes and drug resistant strains of virus, but would

provide rapid protection should an outbreak occur and vaccination is not feasible or effective in a short period.

Using RNAi to develop alternative antivirals has created a wealth of research focused on controlling diseases in humans and livestock species using siRNAs (Lyall et al 2011, Long et al 2010, Chen et al 2009). The delivery of RNAi-mediating agents, however, has historically been an obstacle to harnessing its capabilities and its clinical application (Li and Shen 2009). Since siRNAs are unable to cross cell membranes independently, they require a delivery vehicle such as genetically engineered viruses and synthetic carriers (Aigner 2009, Li et al 2006, Ge et al 2004). These viral and synthetic siRNA vehicles pose serious limitations and concerns for clinical efficacy (Ge et al 2004). Viral vectors can elicit strong immune responses causing cell death (Davidson and McCray 2011, Ge et el 2004). Controlling the dose of RNAi agents with viral vectors is difficult and can result in saturation of the RNAi/microRNA systems resulting in hepatotoxicity (Beer et al 2010, Hacein-Bey-Abina et al 2008, Grimm et al 2006). Viral vectors can integrate into the host's genome and lead to tumorigenesis (Davidson and McCray 2011, Beer et al 2010, Hacein-Bey-Abina et al 2008, Ge et al 2004). In fact, recent studies warn of off-target effects from shRNA viral vectors resulting in cell death and organ problems in transgenic animals (Grimm et al 2006). Finally, synthetic RNA vehicles have low delivery efficiencies and require higher doses, which is not only cost prohibitive, but often toxic (Ge et al 2004). Consequently, it is imperative to focus on better delivery mechanisms for RNAi based antivirals, specifically against AIV in poultry. A safe and effective siRNA delivery vehicle that would specifically target the lung and respiratory tissues, the main sites of AIV infection and replication, would represent a particularly promising approach. This type of system would improve the specificity of delivery for a more effective anti-influenza prophylactic, while minimizing siRNA losses due to systemic administration and reducing siRNA related toxicity.

Chapter 2 of this dissertation aimed at developing an appropriate *in vitro* avian model to validate an RNAi anti-influenza approach. Using a synthetic transfection carrier, this work

showed that siRNA mediated knockdown targeting both the viral NP and PA genes inhibits AIV replication in chicken epithelial cells. Despite the potential these siRNA have to inhibit the shedding of infectious virus in this avian model, these RNAi-mediating agents require a better mechanism of delivery to be deemed clinically applicable. The objective of chapter 3 of this dissertation was to investigate the inhibition of AIV in chicken epithelial cells using a unique RNAi delivery platform, tkRNAi. TkRNAi uses nonpathogenic bacteria to generate and deliver silencing RNAs to mucosal epithelial tissues, and could be an ideal delivery approach for preventing influenza via administration to the upper airways in chickens. These bacteria are genetically engineered to produce shRNA specific for an mRNA target, invade mucosal epithelial cells, and release their shRNA payload into the host cells' cytoplasm. Once released into the cytoplasm these shRNA are processed into siRNA and subsequently silence complementary mRNA targets, thereby triggering RNAi (Buttaro and Fruehauf 2010, Xiang et al 2006). The tkRNAi system utilized in this work has been previously described (Xiang et al 2006) and became available for this work after establishing a collaboration with the small private biotech company, Cambridge Biolabs, and the co-founder of this technology, Dr. Johannes Fruehauf. The tkRNAi system is comprised of *Escherichia coli* (*E. coli)* bacteria that have been transformed with a specific shRNA generating plasmid (pMBV43). These pMBV43 plasmids and carrier *E. coli* bacteria are characterized by several necessary components. These include a shRNA expression cassette under the control of the T7 RNA polymerase promoter, and terminator for successful release and generation of siRNAs in the cytoplasm of influenza susceptible respiratory epithelial cells. The introduction of an invasin gene (*inv*) from *Yersinia pseudotuberculosis* is necessary for expression of invasin protein on the *E. coli* surface (Xiang et al 2006). Invasin interacts with β1 integrin receptors present on the surface of mucosal epithelial cells leading to uptake of the carrier bacteria into endosomes of the target epithelial cells (Isberg and Barnes 2001, Conte et al 1994, Isberg and Leong 1990). Once the *E.coli* are taken up into the host cell, they need to escape the host vacuole for release in the cytosol. The

bacteria are encoded to express Listerolysin O (LLO) from the *hlyA* gene, a pore-forming toxin from *Listeria monocytogenes* (Xiang et al 2006, Mathew et al 2003, Radford et al 2002, Grillot-Courvalin et al 1998). Due to the low pH environment of the host lysosome and a lack of nutrients, the bacteria are lysed inside the endosome, subsequently releasing this bacterial toxin, which leads to the rupture of the endosomal membrane (Nguyen and Fruehauf 2009). Now in the cytoplasm, the released shRNA are processed into siRNA by Dicer, incorporated into RISC, and trigger the RNAi pathway to silence the genes being targeted for knockdown. Figure 3.1 provides a diagrammatic picture of the tkRNAi pathway. Using tkRNAi, we developed a novel RNAi antiviral capable of generating and delivering siRNAs targeting the NP and PA AIV genes. We applied this novel approach to the previously developed avian cell model with the aim of demonstrating *in vitro* proof of concept for using anti-AIV vectors to inhibit AIV shedding.

2. Materials and methods

2.1 Cell cultures

Based on previous work, chicken LMH cells and MDCK cells were used for all invasion and infection, and viral titer assays, respectively. Cells were maintained in appropriate growth medium, as previously described in chapter 2 of this dissertation, with slight modification. To minimize host complement inactivation of the anti-AIV vectors during the invasion step, LMH cells were continuously grown in medium containing heat inactivated FBS (Life Technologies). This was to avoid possible activation of the alternative complement pathway in response to the presence of bacteria, leading to inactivation of the bacterial vectors prior to intracellular uptake.

2.2 Viruses

The following LPAI avian influenza virus strains were chosen for use in all invasion and infection studies: A/Chicken/Texas/473-2/10 (H6N2) and A/turkey/Colorado/1/05 (H8N4). Viral titers for each LPAI were measured by $TCID_{50}$ assay. Both viruses replicate to high titers in LMH

cells with CPE at 48 hpi. As previously described in chapter 2 of this dissertation, stocks were generated and optimal MOIs were chosen.

2.3 Beta-1 integrin validation in chicken cells

Because avian tissues are a novel target for tkRNAi, the presence of β(1) integrin receptors on the surface of LMH cells under normal conditions (uninfected) and post AIV infection was important to validate. Total RNA was extracted (E.Z.N.A.® Total RNA Kit I, Omega Bio-Tek, Norcross, GA) from LMH cell cultures that were uninfected (normal growth conditions) and from cultures at 6 and 24 hours post infection with H8N4 virus. First strand complementary DNA (cDNA) was synthesized from 0.75 µg of total RNA using Oligo(dT) Primer (Promega Corporation) and the cDNA synthesis was completed using 4mM deoxyribonucleotide (dNTPs) and the AffinityScript Multiple Temperature Reverse Transcriptase kit (Stratagene, La Jolla, CA), according to the manufacture's recommendations. The housekeeping gene, β-actin, was used as an internal control for β(1) integrin expression in LMH cells. Primers for β-actin and β(1) integrin were previously published (Caprile et al 2009) using GenBank chicken sequences. Conventional PCR amplification was completed using a 25 µL reaction containing: 2.5 µL of cDNA, 0.8 mM dNTPs, 1.6 mM $MgCl_2$, 2.5 µL 10X Amplitaq Gold Buffer® II, 0.8 U Taq DNA polymerase (Life Technologies), and 0.4 µM forward and reverse primers. Each reaction was overlaid with 30 µL Chill Out® wax (Bio-Rad, Hercules, CA) to prevent evaporation and placed into an MJ Research 60 place thermal cycler (Bio-Rad). The PCR reaction mixture was incubated at 95°C for 10 minutes and 35 cycles of 95°C for 30 seconds, 57°C for 60 seconds, and 72°C for 20 seconds. PCR product was analyzed by agarose gel electrophoresis using the FlashGel® DNA System (Lonza Group Ltd, Basel, Switzerland). Amplified products were visualized by UV light transillumination and 100 base pair (bp) molecular weight ladder (Lonza Group Ltd) was concordantly run on the gels to aid in the calculation of the size of the amplified

DNA fragments. The expected band size for β-actin and β(1) integrin was 282 and 308 bp, respectively.

2.4 Construction and generation of tkRNAi shRNA vectors

The shRNA expression vector, pmbv43, has been previously described (Buttaro and Fruehauf 2010). Pmbv43 contains an expression cassette driven by T7 promoter with cloning sites containing BamHI and SalI restriction enzyme sequences. Upon receiving parent_pmbv43 plasmid from Cambridge Biolabs (Cambridge, MA), the plasmid was digested 2 hours at 37°C in a 50 µL reaction containing: 2 µg pmbv43, 2.0 µL BamHI (New England Biolabs, Ipswich, MA), 2.0 µL SalI (New England Biolabs), 5.0 µL Buffer 3 (New England Biolabs), 0.5 µL 100X bovine serum albumin (New England Biolabs), and 24.2 µL water. Digested parent_pmbv43 was subsequently treated with Alkaline Phosphatase, Calf intestinal (New England Biolabs) and phenol/chloroform and ethanol precipitated. The resulting 8.4 kilo base (kb) linear parent_pmbv43 plasmid was gel extracted from a 1% agarose gel following gel electrophoresis and isolated using dialysis.

The RNAi targeting sequence of NP was 5' - GGA TCT TAT TTC TTC GGA G - 3' and PA was 5' - GCA ATT GAG GAG TGC CTG A - 3'. The DNA template encoding the shRNA specific for NP and PA was: BamHI site - sense sequence-hairpin loop (5' - TTC AAG AGA - 3') - antisense sequence - TTTTTTTTTT - SalI site. Integrated DNA Technologies synthesized the top and bottom strands for the PA DNA oligonucleotide sequence. Each PA oligonucleotide was resuspended to 300 µM and 120 µM of each strand were annealed together in a 20 µL reaction. After annealing, the duplex was phosphorylated and ligated into linear pmbv43 for 24 hours at 4°C in a 10 µL reaction containing: 1 µL T4 DNA ligase buffer (New England Biolabs), 1 µL of annealed and phosphorylated PA oligonucleotide, 2 µL pmbv43, 1 µL T4 DNA ligase (New England Biolabs), and 5 µL water. The resulting ligation mixture was transformed into DH5α™ competent cells (Life Technologies) and the resulting transformed cells were plated onto Luria

Broth (LB) plates containing 10 µg/mL kanamycin (Kan) and incubated at 37°C overnight. Resulting colonies were screened by PCR using PA_shRNA specific primers that amplified a 209 bp product according to the following thermal profile: 94°C for 4 minutes, 30 cycles of 94°C for 30 seconds, 45°C for 30 seconds, 72°C for 30 seconds, and a final elongation at 72°C for 10 minutes. A single PCR positive PA_pmbv43 plasmid clone was purified using the PureLink® HiPure Plasmid Maxiprep Kit (Life Technologies) and sequenced to verify proper PA_shRNA insertion. Using standard cloning and plasmid purification methods, the NP_pmbv43 plasmid was commercially synthesized, generated using DH10-beta cells, and purified by the commercial company DNA2.0, Incorporated (Menlo Park, CA). Upon receiving NP_pmbv43, the plasmid was resuspended in molecular water to a final concentration of 100 ng/µL. NP_pmbv43, PA_pmbv43, and parent_pmbv43 were transformed into CEQ221 competent cells (NP_pmbv43/CEQ, PA_pmbv43/CEQ, and parent_pmbv43/CEQ vectors) and plated onto Brain Heart Infusion (BHI) agar containing 25 µg/mL Kan and 50 µg/ml 2,3-Diaminopropionic Acid (BHI/Kan/Dap). Plates were incubated overnight at 37°C and resulting colonies were screened by PCR using sets of NP, PA, and parent_shRNA specific primers. A single PCR positive clone representing NP_pmbv43/CEQ, PA_pmbv43/CEQ, and parent_pmbv43/CEQ was sequenced. Following sequence validation, each clone was subsequently propagated in BHI/Kan/Dap broth. Stocks were generated at mid-log (OD_{600} = 0.4-0.6) and at stationary phase (OD_{600} = 1.0), and frozen back at -80°C in 20% glycerol. A single frozen aliquot from each vector stock representing OD_{600} = 1.0 was thawed for plate enumeration. Briefly, each 1 mL aliquot was thawed, centrifuged for 5 minutes at 5,000 x g, and resuspended in 1 mL of PBS containing 100 µg/mL Dap (PBS/Dap). The resulting vectors were diluted in PBS/Dap 1:10, 1:100, 1:1,000, 1:10,000, 1:60,000, 1:90,000, 1:135,000, 1:270,000. A total of 50 µl from each theoretical dilution representing 1:60,000 - 1:270,000 were plated in duplicate on BHI/Kan/Dap agar and incubated overnight at 37°C. Colony counts at each dilution were averaged and used to calculate overall colony forming units (CFU) per mL (CFU/mL). These enumeration values

represented an appropriate viable CFU/mL concentration for NP_pmbv43/CEQ (anti-AIV_NP), PA_pmbv43/CEQ (anti-AIV_PA), and parent_pmbv43/CEQ (anti-AIV_scramble) vector stocks. This system allowed aliquots to be thawed and used directly in all future *in vitro* invasion assays.

2.5 Intracellular uptake of tkRNAi vectors

To verify bacterial uptake and intracellular invasion into LMH cells, anti-AIV_scramble vector was tagged with a red fluorescent protein (RFP). Briefly, anti-AIV_scramble vector was co-transformed with the RFP prokaryotic expression vector, pE2-Crimson (Clontech, Mountain View, CA), using standard transformation methods. This RFP was chosen because it is a far-red fluorescent protein with fast maturation, high photostability, reduced cytotoxicity, and is expressed from the *lac* promoter to allow for Isopropyl β-D-1-thiogalactopyranoside (IPTG) activation of RFP expression (Bevis and Glick 2002). After growing the anti-AIV_scramble_RFP vectors (RFP-vector), stocks were generated and enumerated, as previously described. LMH cells were seeded in 8-well chamber slides one day prior to invasion to allow cell monolayers to reach 60% confluency. On the day of vector invasion, a 1 ml aliquot of RFP-vector was thawed, centrifuged at 5,000 x g for 5 minutes, and resuspended in PBS/Dap. The RFP vector was then placed on ice and serially diluted 1:4 in Waymouth's MB 752/1 medium containing 2 mM L-glutamine and 50 µg/mL of Dap starting with 5e7 CFU/mL and ending at 7.8e5 CFU/mL. To prepare LMH cells for invasion and remove antibiotics from the growth medium, medium was aspirated and each well was washed twice with fresh Waymouth's MB 752/1 medium containing 2 mM L-glutamine and 50 µg/ml of Dap (invasion medium). Rinse medium was aspirated and 1 mL of RFP-vector at each dilution was added to an appropriate well and allowed to incubate for 2 hours at 37°C in the presence of 7% CO_2. One untreated well (Waymouth's MB 752/1 complete medium), one mock-invasion control well (invasion medium only) and one non-RFP invasion control well (anti-AIV_scramble vector at 3.1e6 CFU/mL) was included with each

chamber slide experiment. After 2 hours incubation, invasion medium was aspirated and wells were washed twice with Waymouth's MB 752/1 complete medium, before fresh Waymouth's MB 752/1 complete medium was replaced and cells were allowed to continue incubating for an additional 2-24 hours. To fix cells, growth medium was aspirated and wells were rinsed twice with calcium/magnesium free PBS. The chamber was removed from the slide and 10 µL of ProLong® Gold Antifade Reagent with 4',6-diamidino-2-phenylindole (DAPI) (Life Technologies) was added to each well grid and the slide was mounted with a cover slip. The resulting slide was incubated at room temperature in the absence of UV light for 24 hours before images were captured using the Eclipse Ti inverted fluorescent microscope (Nikon Instruments Inc., Melville, NY) at 40X. Filter cubes for DAPI and CY5 were used to visualize the DAPI and RFP fluorophores using 461 and 670 emission, respectively.

2.6 Optimal tkRNAi vector concentration

To determine the optimal vector concentration allowable, without concomitant induction of CPE, LMH cells were seeded in 24-well plates one day prior to invasion to allow cell monolayers to reach 80% confluency. Anti-AIV_scramble vector was prepared, as previously described, and diluted in invasion medium to 5e7, 1.25e7, 3.1e6 and 7.8e5 CFU/mL. LMH cells were prepared for invasion, as described above. Rinse medium was aspirated and 1 mL of anti-AIV_scramble vector at each dilution was added to an appropriate well, in triplicate, and allowed to incubate for 2 hours at 37°C in the presence of 7% CO_2. Each plate included an untreated well (Waymouth's MB 752/1 complete medium) and a mock-invasion well (invasion medium only). After 2 hours incubation, invasion medium was aspirated and wells were washed twice with Waymouth's MB 752/1 complete medium before fresh Waymouth's MB 752/1 complete medium was replaced and cells were allowed to continue incubating for an additional 24-48 hours. At multiple time points post invasion, wells were observed for visual signs of CPE, compared to the untreated control wells. The maximum anti-AIV_scramble vector concentration

allowable, without inducing CPE, was selected for all subsequent anti-AIV_NP and anti-AIV_PA invasion assays in LMH cells.

2.7 Chicken cell invasion with tkRNAi shRNA vectors

LMH cells were seeded in 24-well plates one day prior to invasion to allow cell monolayers to reach 80% confluency. On the day of vector invasion, a 1 mL aliquot of anti-AIV_NP, anti-AIV_PA, and anti-AIV_scramble vector was thawed, centrifuged at 5,000 x g for 5 minutes, and resuspended in PBS/Dap. Each vector was then placed on ice and diluted appropriately for the invasion assay. LMH cells were prepared for invasion, as previously described. Wells were tested in triplicate and included anti-AIV_NP, anti-AIV_PA, anti-AIV_scramble, and a cocktail of anti-AIV_NP + anti-AIV_PA vector (anti-AIV_cocktail). Control wells were also included in triplicate (mock-invasion and untreated). After 2 hours incubation, LMH cells were washed (previously described) and fresh Waymouth's MB 752/1 complete medium was replaced.

2.8 Virus infection

Twenty four hours post invasion the LMH cells were prepared for infection by removing the growth medium and washing each well with IM containing 0.25 µg/mL TPCK, as previously described. The wash was removed and a total of 250 µL of IM containing either H8N4 or H6N2 virus at an MOI of 0.01 or 0.001 was added to each appropriate well. Plates were incubated on a rocking platform at 37°C in the presence of 7% CO_2 for 1 hour before removing the virus and replacing with 750 µL/well of fresh IM. Cell culture supernatants were harvested 48 hpi, centrifuged, and frozen at -80°C. Each experiment included a positive control (AIV infected), negative control (untreated), and mock-invasion control (mock-invasion + AIV infected) each tested in triplicate wells.

2.9 Evaluation of infectious virus titer

LMH cell culture supernatants were quantitated by end-point titration on MDCK cells as previously described using $TCID_{50}$/mL calculations. Briefly, the harvested supernatants were thawed, diluted ten-fold, and 100 µL/well of virus suspension was overlaid in triplicate into 96 well plates seeded with MDCKs. Cells were stained with crystal violet 48 hpi and wells with CPE were scored as positive for virus growth. $TCID_{50}$/mL was calculated by the Reed and Muench mathematical technique (Reed and Muench 1938).

2.10 Statistical analysis

All experiments were repeated three times and on different days for a total of 9 or 10 independent samples. Using a statistical significance level of 0.05, the Wilcoxon rank-sum test was performed to compare median viral titers between vector treated and untreated PC samples performed on the same day. To determine if adjusted mean $LogTCID_{50}$/mL values between vector treated and PC samples were statistically different ($p < 0.05$), data was analyzed using multivariable linear regression, controlling for day. Statistical analyses were performed using the data analysis and statistical software STATA 10 IC (StataCorp 2009, Stata Statistical Software: Release 10).

3. Results

3.1 Verifying intracellular uptake of tkRNAi vectors in chicken cells

Uptake of the tkRNAi vectors by epithelial cells works by receptor mediated endocytosis when the bacterial surface protein, invasin, interacts with the host receptor, β(1) integrin. It was therefore important to verify the presence and stable expression of β(1) integrin in chicken LMH cells during normal growth conditions and during infection with AIV. Following RNA extraction and cDNA synthesis, the expression of β(1) integrin in LMH cells grown using normal growth conditions, as well as in cells infected with H8N4 virus (MOI 0.01) at 24 hours post infection

(Figure 3.2) was observed. Assuming that β(1) integrin mRNA was efficiently translated, these results indicated the tkRNAi vector should appropriately attach to LMH cells, even when the cells are showing signs of CPE and diseased post infection. Tagging the anti-AIV_scramble vector with an RFP verified intracellular expression inside LMH cells at 2 and 24 hours post invasion (Figure 3.3). The maximum RFP-vector concentration allowable, without inducing CPE, was 7.8e5 CFU/mL. Therefore, all subsequent experiments adopted this vector concentration. Together, these results indicate the tkRNAi delivery platform is appropriate for chicken epithelial cells.

3.2 Antiviral activity of tkRNAi shRNA vectors in chicken cells

It was previously shown in chapter 2 of this dissertation that the NP and PA siRNA sequences align against the H8N4 and H6N2 NP and PA sequences with 100% homology and are devoid of any off-target matching to any known chicken sequence. Prior to infection with H6N2 or H8N4 virus, LMH cells were first treated with vector and following infection culture supernatants were collected for $TCID_{50}$ analysis.

In Figure 3.4, median titers ($LogTCID_{50}$/mL) in treated compared to PC samples are shown by individual day and for each virus and corresponding MOI used for infection. The graphs indicate median titers from each sample set vary by day and by virus and MOI used for infection. However, significant antiviral activity was observed in anti-AIV_NP, anti-AIV_PA, and anti-AIV_cocktail samples from both H6N2 and H8N4 infections. On at least one experimental test day, anti-AIV_NP, anti-AIV_PA, and the anti-AIV_cocktail significantly reduced median virus titers in both viruses at an MOI 0.01, compared to the corresponding PC samples. The vectors appeared to have a slightly less profound antiviral effect at a viral MOI 0.001. Despite a lack of significant differences likely associated with a small sample size, 100%, 89%, and 100% of all NP, PA, and anti-AIV_cocktail vector treatments resulted in lower median viral titers compared to their appropriate untreated PCs, respectively. It should be noted that the anti-AIV_scramble

vector also resulted in lower median viral titers, some of which were significantly different from the PC samples (Figure 3.4).

By controlling for day, MLR analysis was used to compare vector treated LMH cells to untreated PC cells across all experimental days. Significant differences in adjusted mean viral titers between vector treated and untreated PC samples are shown in Table 3.1. MLR analysis indicates significant differences between all vector treatments (excluding anti-AIV_scramble) and their corresponding PC samples after infection with both H6N2 and H8N4 at MOI 0.01. With the exception of infection with H8N4 MOI 0.001, in all experimental sets the cocktail vector resulted in significantly lower viral titers compared to the corresponding PC titers ($p<0.05$). The log10 reduction in infectious virus between vector treated and untreated samples were calculated. All vector treated samples resulted in at least 0.8 log10 reduction (6 fold reduction). These sample sets and the corresponding reductions include H8N4 infection at MOI 0.01 after NP (3.8 log or 6,918 fold reduction), PA (2.5 log or 331 fold reduction), and anti-AIV_cocktail vector treatment (4 log or 10,965 fold reduction) as well as H8N4 at MOI 0.001 after anti-AIV_PA vector treatment (3.0 log or 1,000 fold reduction). The scramble vector also showed antiviral activity in all experimental sets, except for infection with H6N2 at MOI 0.001 (Table 3.1)

4. Discussion

The objective of this study was not to prevent infection, but to reduce the amount of infectious virus shed into the culture supernatants after treating LMH cells with the anti-AIV vectors. *In vitro* treatment with these antiviral vectors significantly reduced infectious titers following infection with two different AIV subtypes isolated in poultry, H8N4 and H6N2. In all but one sample set (H8N4/MOI 0.001), the cocktail vector significantly reduced viral titers. However, even when a significant difference between vector treated and untreated PC samples was not observed, a notable $\log_{10}$ reduction in infectious titer was observed from all vector treated sample sets. The most potent inhibition of infectious virus shedding occurred after LMH cells

were treated with anti-AIV_cocktail and infected with H8N4 or H6N2, resulting in significant log_{10} reductions ranging from 1.7 - 4.0 (50 - 10,965 fold reduction).

As was noted above, treatment with the scramble vector also reduced infectious titers in all but the H6N2/MOI 0.001 infected sample set. There is a possible explanation for this observation. The innate immune response is part of the host's early defense mechanism. Toll-like receptors (TLRs) play a key role in the innate response by recognizing and binding bacterial components, which are markers of infection to host cells. These bacterial components, also referred to as pathogen-associated molecular patterns (PAMPs), include endotoxins like lipopolysaccharide (LPS) which act as immune enhancers (Bessler et al 1990). These anti-AIV vectors are delivered by non-pathogenic *E. coli* bacteria, therefore it is not surprising that these chicken epithelial cells would detect the extracellular presence of these bacterial endotoxins, likely via TLR-4 recognition (Schoen et al 2004). This binding event would lead to the expression of the inflammatory transcription factor, nuclear factor kappa(β), and ultimately the release of downstream proinflammatory cytokines and chemokines as part of the innate response. In this case, these bacterial components could be acting much like a vaccine adjuvant, thereby stimulating the innate response prior to AIV infection and essentially mounting an additional level of protection against viral infection.

While this is the first time tkRNAi has been assessed as a delivery vehicle in an avian cell model and as a vector to deliver siRNA targeting influenza virus, several other studies have successfully targeted and silenced other disease associated genes in mammalian models using this delivery approach (Xiang et al 2006, Kruhn et al 2009, Zhao et al 2005). This concept is being verified *in vivo* (mice and non-human primates) including several studies that are entering the early stages of clinical testing (Buttaro and Fruehauf, 2010). Marina Biotech (Bothell, WA) recently developed the first human therapeutic based on the tkRNAi platform (CEQ508), which is currently in clinical testing for the treatment of Familial Adenomatous Polyposis.

This work provides initial proof of concept and demonstrates both successful tkRNAi delivery and the antiviral potential of these novel anti-AIV vectors in an avian cell model. However, do these data suggest an anti-AIV vector cocktail targeting NP and PA could significantly reduce AIV shedding in chickens? Could this approach translate into an antiviral technology that limits AIV outbreaks and transmission in poultry? Even more, could this represent a transformative approach for controlling influenza in other species? This work represents the first steps towards answering these questions. The overarching long-term goal of this research is to develop this prophylactic into a novel complement to the traditional AIV vaccine for poultry. The future framework behind this goal relies on testing this approach *in vivo*. As such, the findings from this work have been translated into efforts aimed at assessing the efficacy of these antiviral vectors in experimentally challenged chickens. The objective of this future work is to develop an innovative anti-influenza prophylactic that would represent of a more effective and robust control method for AIV in poultry.

Acknowledgments

I would like to thank Cambridge Biolabs for their valuable collaboration on this work and for kindly donating the pmbv43 plasmid and CEQ221 bacteria. Additional thanks to Laura Holberger (Cambridge Biolabs) for her technical expertise and input on constructing these anti-AIV vectors. I am grateful to the entire Wilusz^2 Laboratory, including John Anderson for his guidance and invaluable training related to cloning and transformation, and for the generous use of their space and equipment. Thanks to Jan Pederson at NVSL in Ames, IA for her help in selecting the LPAI H6N2 for this and ongoing work.

5. Tables and figures

Table 3.1

Anti-AIV vector protection in chicken LMH cells as measured by log and fold-reductions in shedding titers. Results are from multivariable linear regression, controlling for day. [a] Expressed as adjusted mean LogTCID50/mL. [b] Comparing adjusted mean LogTCID50/mL from treated to untreated PC samples ($p<0.05$). [c] Log reduction in mean infectious titer compared to untreated control. [d] Fold reduction in geometric mean compared to untreated control.

Sample	*n*	Adjusted Mean [a]	95% CI	Adjusted Mean (untreated)	95% CI (untreated)	*P*-value [b]	Log reduction [c]	fold reduction [d]
				H8N4 LPAI virus MOI 0.01				
Anti-AIV_NP	9	1.1	(0.0, 2.6)	4.9	(3.7, 6.2)	< 0.001	3.8	6918
Anti-AIV_PA	9	3.0	(2.1, 3.8)	5.5	(4.6, 6.4)	< 0.001	2.5	331
Anti-AIV cocktail	9	0.9	(0.0, 2.1)	4.9	(3.9, 6.0)	< 0.001	4.0	10965
Anti-AIV_scramble	10	2.8	(2.0, 3.5)	4.9	(4.1, 5.8)	< 0.001	2.1	138
				H8N4 LPAI virus MOI 0.001				
Anti-AIV_NP	9	8.1	(6.3, 9.8)	8.9	(7.9, 10.0)	0.309	0.8	6
Anti-AIV_PA	9	5.8	(4.9, 6.8)	8.8	(8.0, 9.5)	< 0.001	3.0	1000
Anti-AIV cocktail	9	7.9	(6.3, 9.5)	8.9	(7.9, 9.9)	0.213	1.0	10
Anti-AIV_scramble	10	7.3	(6.0, 8.6)	8.5	(7.5, 9.5)	0.064	1.2	16
				H6N2 LPAI virus MOI 0.01				
Anti-AIV_NP	9	4.7	(3.1, 6.2)	6.3	(4.8, 8.0)	0.043	1.6	43
Anti-AIV_PA	9	4.9	(3.6, 6.2)	6.3	(4.8, 7.9)	0.031	1.4	27
Anti-AIV cocktail	9	4.4	(3.0, 5.9)	6.3	(4.9, 7.8)	0.013	1.9	85
Anti-AIV_scramble	9	5.5	(4.6, 6.3)	6.3	(5.3, 7.4)	0.053	0.8	7
				H6N2 LPAI virus MOI 0.001				
Anti-AIV_NP	9	9.2	(7.5, 9.3)	10.1	(9.1, 11.1)	0.270	0.9	8
Anti-AIV_PA	9	9.5	(8.1, 10.9)	10.5	(9.4, 11.7)	0.149	1.0	10
Anti-AIV cocktail	9	8.4	(7.1, 9.8)	10.1	(9.2, 10.9)	0.023	1.7	50
Anti-AIV_scramble	9	11.3	(9.8, 12.8)	10.5	(9.3, 11.6)	0.269	0.0	0

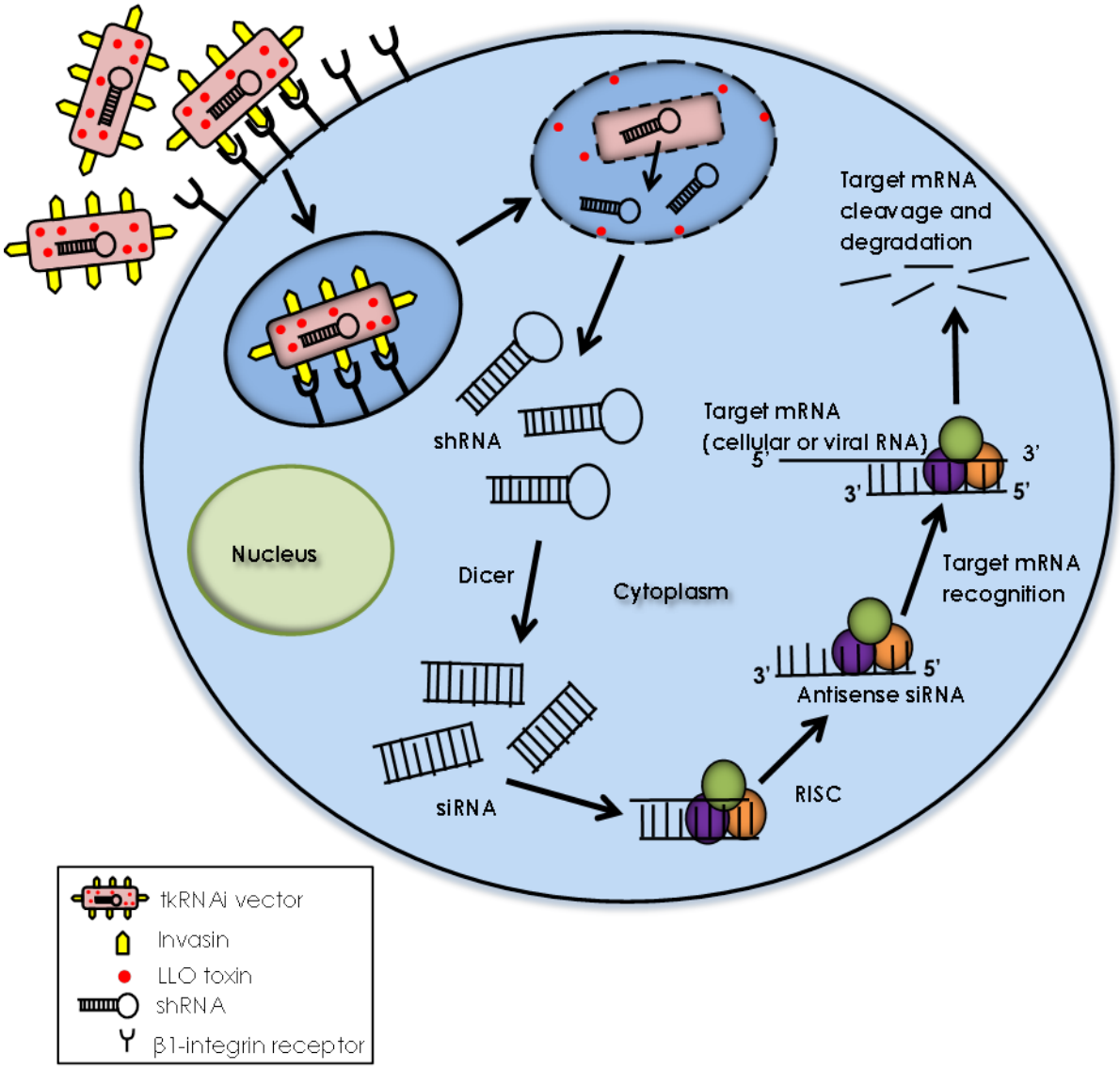

Figure 3.1

Diagrammatic picture of the tkRNAi pathway.

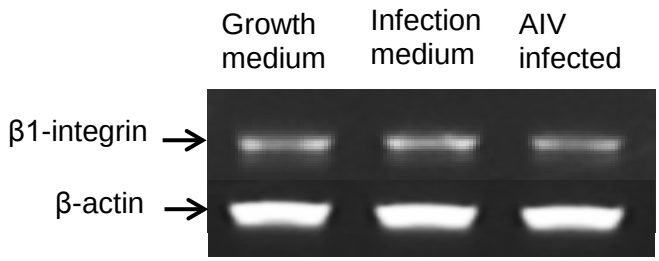

Figure 3.2

mRNA expression of β-actin and β1-integrin in LMH cells cultured in growth normal medium, infection medium without virus, or infection medium 24 hpi with H8N4 virus (AIV infected).

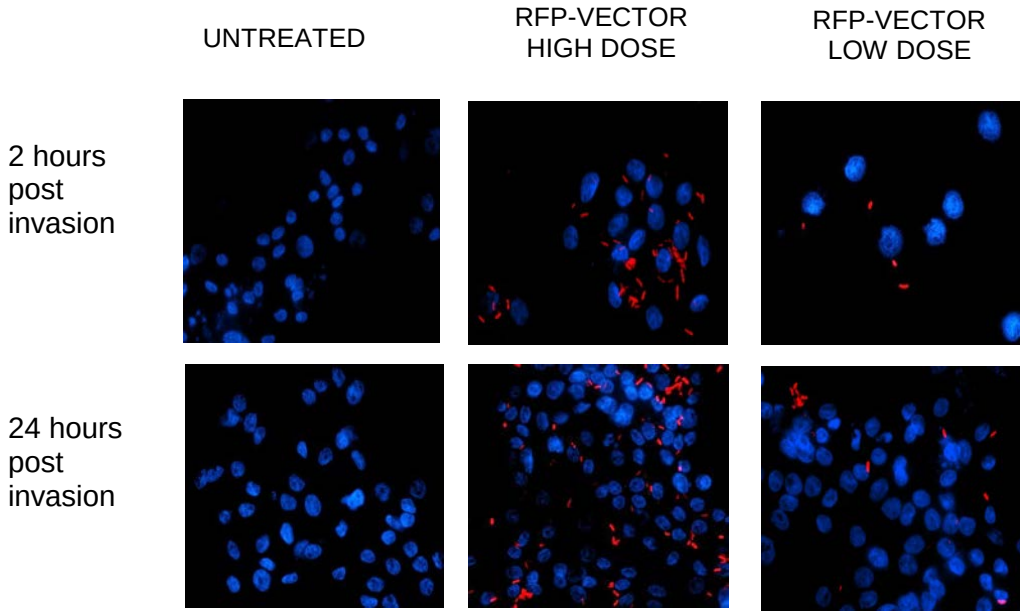

Figure 3.3

Anti-AIV_scramble vector tagged with RFP. Chicken LMH cells given RFP-vector at two doses (high=7.8e5 CFU/mL and low=1.95e5 CFU/mL). Vector uptake assessed at 2 and 24 hours post invasion.

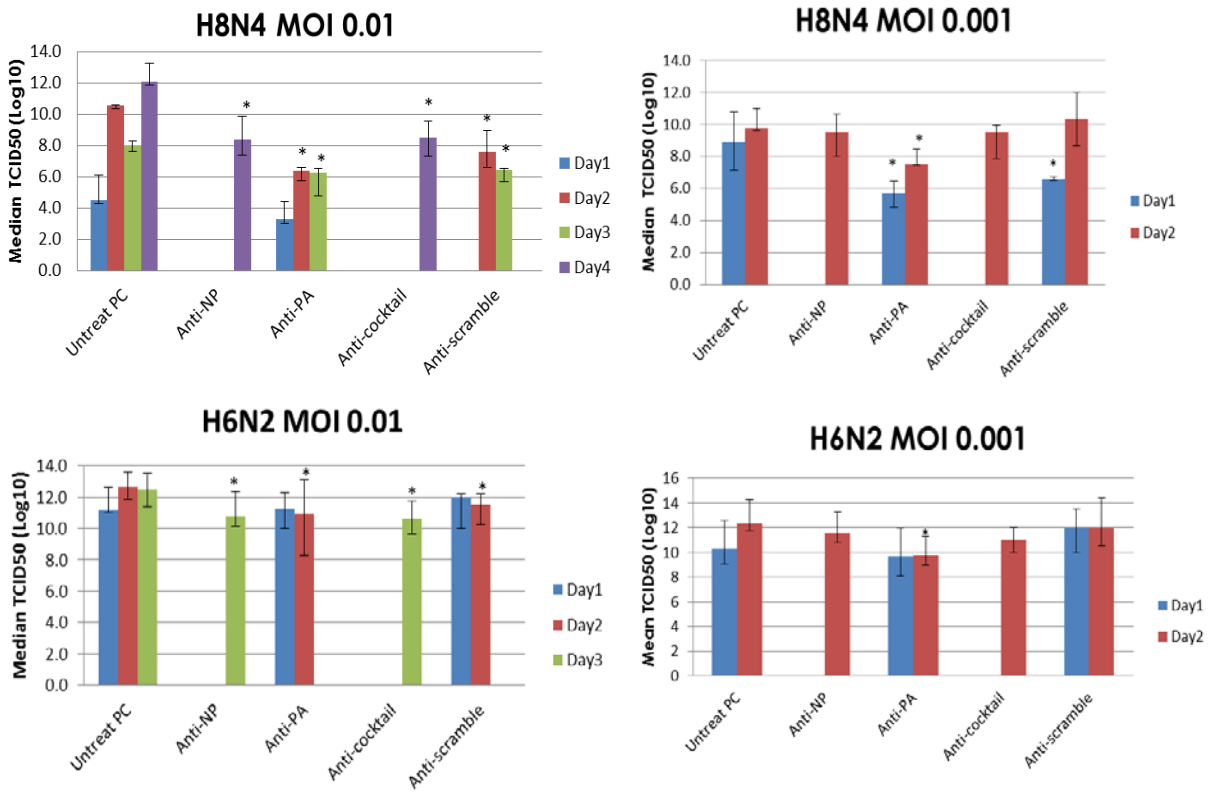

Figure 3.4

Anti-AIV vector protection as measured by reduction in median titers by day, MOI, and virus. *Statistically significant difference with corresponding untreated sample using Wilcoxon rank-sum test ($p < 0.05$).

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CHAPTER 4

A NOVEL AVIAN INFLUENZA RNAi ANTIVIRAL TECHNOLOGY FOR POULTRY: PROOF OF CONCEPT IN EXPERIMENTALLY CHALLENGED CHICKENS

Summary

In the face of an AIV outbreak, several factors are critical to effectively controlling the spread of virus within poultry populations and across farms. These include the speed at which a control method or vaccine is applied, how rapidly a prophylactic works to protect, and the ability to protect against any subtype or strain of AIV. Developing an anti-influenza technology for poultry is a critical step to effectively manage and control the spread of this disease worldwide. Chapter 3 of this dissertation demonstrated the value of using novel anti-AIV vectors targeting the viral NP and PA genes to reduce viral shedding titers *in vitro* but had yet to be tested *in vivo* using experimentally challenged chickens. Vector uptake into chicken respiratory tissues was first assessed using vectors tagged with fluorescent red protein for visualization. Once vector uptake and a lack of vector associated pathogenicity was demonstrated in chickens, groups of commercial chickens were treated with the cocktail vectors (n=10), a scramble vector (n=10), or a placebo (n=10). Twenty-four hours later, all chickens were challenged with H6N2 virus. Chickens treated prophylactically with the anti-AIV cocktail vectors shed significantly less virus compared to untreated chickens ($p<0.05$). Likewise, the proportion of chickens shedding virus was significantly less in the anti-AIV cocktail treated chickens compared to the untreated chickens ($p<0.05$). The anti-AIV cocktails also prevented AIV transmission to sentinel chickens. This work demonstrates *in vivo* proof of concept for using this novel RNAi antiviral technology to protect chickens against AIV replication, viral shedding, and transmission.

1. Introduction

AIV is recognized as a National Institute of Allergy and Infectious Diseases bio-defense category C priority pathogen, an economically important disease of poultry, and a major public health threat because viruses originating from animals have the potential to cause the next human pandemic (Carver and Krushinskie 2006, Webster 1997). AIV affects many species of food production birds, including chickens, turkeys, ducks, quails, and guinea fowl. Throughout the literature, there are countless descriptions of significant global outbreaks of LPAI and HPAI in domestic poultry. These outbreaks are of concern, not only because of the degree of virulence observed in poultry resulting in severe economic consequences, but due to the ability to transmit to mammalian species. Highly pathogenic H5N1 avian strains are currently circulating in multiple countries. HPAI outbreaks in poultry have led to the culling of millions of animals and the net loss of billions of dollars. Since 2003, H5N1 strains have resulted in 648 human cases, of which 384 were fatal (WHO Influenza at the human animal interface 2013). Human infection with the LPAI (H7N9) virus has now been reported, with approximately 150 cases of which nearly 33% have resulted in death (Centers for Disease Control and Prevention 2014).

A major risk factor for AIV transmission to humans is direct contact and handling of domestic birds (Vong et al 2009, Areechokchai et al 2006, Dinh et al 2006, Wang et al 2006, Bridges el al 2002, Mounts et al 1999), so the majority of human cases have occurred when close proximity between humans and livestock leads to transmission. As the number of circulating viruses increases in domestic poultry, so does the risk for transmission to humans and the potential to reassort into a form that is more transmissible among humans (Gatherer 2010). Therefore, with respect to reducing the risk to humans, AIV prevention in domestic poultry must be a major focus. The emergence of a novel, transmissible AIV in a population that has little or no immunity, would cause a global pandemic for which no vaccine is available. These strains are of particular concern because they are resistant to the most cost-effective

antiviral drugs, amantadine and rimantadine (He et al 2008, Lee et al 2008, Pinto and Lamb 1995, Belshe et al 1988).

Just as it is critical to have early detection protocols and warning systems in place as means to control and manage AIV in poultry, effective prevention measures must be available to prepare for potential outbreaks. Vaccinating domestic poultry may help to control HPAI viruses and reduce economic losses and zoonotic transmission, however vaccination is not a common practice on poultry farms because it is not seen as a viable option to prevent LPAI. If vaccination were adopted in an emergency, it would likely occur after an outbreak has already been identified. Vaccine protection takes two to three weeks to acquire (Kim et al 2009), and often requires repeated doses to induce full protection (Poetri et al 2011). Therefore, vaccination during an outbreak might do little to control the chain of transmission, especially in densely populated poultry areas. Developing a powerful anti-influenza technology for poultry is a critical step to effectively manage and control the spread of this disease worldwide.

The work previously described in chapter 3 of this dissertation investigated the inhibition of AIV in chicken epithelial cells using the RNAi delivery platform, tkRNAi. This tkRNAi system uses engineered *E. coli* to produce short hairpin RNA against the NP and PA viral genes and successfully delivered the shRNA intracellularly. Within twenty-four hours of administration, these vectors lead to significant reductions in AIV replication *in vitro*. These proceedings demonstrated the rapid antiviral potential of these novel anti-AIV vectors in our avian cell model. In continuing pursuit of our long-term goal to develop this anti-influenza technology into a prophylactic that would contribute to the development of a more effective and robust control method for AIV in poultry, the objective of the present study was to evaluate the intranasal administration and the protective efficacy of these antiviral vectors in experimentally challenged chickens.

Vaccine efficacy is evaluated based on clinical protection (reduced morbidity and mortality) and through the detection of virus shedding after challenge. An additional goal of AIV

vaccination in poultry is to stimulate an immune response and prevent the individual animal from being infected. The prophylactic vector in this current work is not engineered to prevent infection in the individual animal. This technology's mode of action is inhibiting viral replication, thereby reducing the shedding of infectious virus following infection. Therefore, to provide initial efficacy for these anti-AIV vectors, the prophylactic ability of these vectors to reduce viral shedding in experimentally challenged chickens was evaluated. If this prophylactic can reduce shedding in an individual bird and/or reduce the proportion of birds shedding in a flock, this will reduce the risk for transmission within and between populations of birds, respectively. In addition to monitoring shedding, the potential for transmission was tested using sentinel birds housed among AIV challenged birds that received the anti-AIV vectors. This work was conducted using 3 pilot studies and one proof of concept (POC) study.

2. Materials and methods

2.1 Animals

Four week old commercial leghorn chickens were donated by a commercial production farm in Colorado and housed in a biosafety level 2 facility. Upon arrival, birds were randomly allocated to a treatment group by blindly selecting colored coded leg bands placed in a box. On the day of arrival, blood was collected from the brachial vein and an oropharyngeal (OP) swab was taken to verify AIV free status using serology testing and real time reverse transcriptase PCR (RT-qPCR). Groups of birds were housed in a room containing separate 12 square-foot suites, in accordance with the Guide for the Care and Use of Laboratory Animals. Negative airflow was maintained to prevent cross contamination between suites, whereby the air was positive to the central corridor of the room during the entire course of the study. Groups of chickens were allowed to roam freely within each suite and feed and water were provided with *ad libitum* access. Room temperature was maintained at 70°C and lights were controlled daily to allow adequate time for daytime activity and resting. Animal care and each experiment was

conducted under the approval of the Institutional Animal Care and Use Committee (IACUC) committee at Colorado State University. To give chickens time to acclimate to their new surroundings, all of the experimental studies commenced at least 4 days after arrival.

2.2 Virus

The LPAI A/Chicken/Texas/473-2/10 (H6N2) virus was chosen due to its history of naturally infecting chickens and ability to be isolated from chicken respiratory swabs collected during an outbreak in 2010 occurring in Texas. The virus was propagated and titrated in the allantoic cavity of 10-day old embryonated specific pathogen-free (SPF) chicken eggs for 2 days at 37°C. Briefly, after growing the virus, the amnioallantoic fluid (AAF) was harvested and pooled to represent a single virus stock. To titrate the virus, an aliquot of the stock was diluted ten-fold from 1:10 to $1:10^{10}$ in BHI containing 1X antibiotic cocktail (2000 U/mL penicillin G, 4 mg/mL streptomycin, 16 µg/mL gentamycin, 100 U/mL nyastatin, 650 µg/mL kanamycin) and 0.1 mL was used to inoculate 3 SPF eggs at each dilution. AAF was harvested and the 50% egg infectious (EID_{50}/mL) titer calculated according to the Reed and Muench mathematical formula.

2.3 Vector administration

Vector stocks were generated and frozen back at appropriate CFU/ml concentrations (as previously described) so that additional growth and enumerations were not necessary. This essentially allowed vector aliquots to be used directly as the treatment dose appropriate for direct administration to a bird. Briefly, an appropriate volume of vector stock was thawed, centrifuged at 5,000 x g for 5 minutes, and resuspended in PBS containing 50 µg/mL Dap. The cocktail vector represented an equal concentration (CFU/mL) in and equal volume (based on total dose volume) of the NP (NP_pmbv43/CEQ) and PA (PA_pmbv43/CEQ) vectors. The vectors were transported on ice to the facility housing the chickens. Vector was administered by the intranasal route (0.3 - 0.5 mL per bird). Each chicken was gently restrained during dosing

and an equal volume was administered into each nostril. The bird was restrained for one additional minute to prohibit sneezing and allow the vector to settle into the nasal tissues for efficient invasion.

2.4 Virus challenge

To emulate the natural route of infection for AIV in an experimental setting, chickens were inoculated at 5 weeks (± 4 days) of age with 10^6 EID_{50}/0.1 mL solution of H6N2 virus. Prior to inoculation, virus was diluted in sterile PBS to the desired dose and stored cold until ready for challenge. During challenge, each animal was gently restrained while trained personnel inoculated 50 µL of virus into each nostril. After challenge, each bird was restrained for an additional 1 minute to prohibit sneezing and allow the virus to settle into the nasal tissues for efficient infection. Chickens were evaluated daily for clinical illness

2.5 Data and sample collection

Post vector and/or viral inoculation, chickens were inspected daily using the clinical illness (0-4) scoring system: 1 = Mild lethargy evidence by lack of interest in feed, slow movement, and low reaction to external environment; 2 = Mild respiratory disease (open mouth breathing, snicking, sneezing), accompanied by mild lethargy; 3 = Moderate respiratory disease (snicking, sneezing, mild cough, raspy breathing), accompanied by moderate lethargy; and 4 = Severe respiratory disease (coughing, sneezing, labored breathing), accompanied by lack of feeding with limited movement or reaction to the external environment. After challenge with H6N2 virus, OP swabs were collected from infected birds at 2, 3, 4, 5, 6, 7, and 10 days post infection (dpi). Swabs were immediately submerged and rinsed in a falcon tube containing 1 mL BHI broth storage medium and placed on ice. Personnel collecting the swabs changed gloves and booties between groups to avoid potential cross contamination. Samples were stored at −70°C until subsequent RT-qPCR. At appropriate times, birds were euthanized by CO_2 and

tissues collected at necropsy to assess RFP-vector uptake, vector associated pathogenesis, or viral infection.

2.6 Detection of viral shedding by RT-qPCR

All OP swab samples collected over the infection period were processed for RT-qPCR to determine H6N2 viral titers. According to the manufacturer's recommendations, total RNA was extracted from 50 µL of OP swab sample using Trizol LS (Life Technologies, Grand Island, NY) and the MagMAX 96 AI/ND Viral RNA isolation kit (Life Technologies) with the KingFisher magnetic particle processor (Thermo Scientific, Waltham, MA). RT-qPCR was performed using the ABI 7500 platform (Life Technologies) and with primers and probe specific for conserved sequences in avian influenza matrix gene as previously described (Spackman et al 2002) and in accordance with the NVSL protocol AVSOP1521.01. Primer and probe sequences were as follows: Forward primer (5′-AGA TGA GTC TTC TAA CCG AGG TCG-3′), Reverse primer (5′-TGC AAA AAC ATC TTC AAG TCT CTG-3′), and 5′ reporter dye (5'- 6-carboxyfluorescein [FAM]) (5′- FAM-TCA GGC CCC CTC AAA GCC GA-TAMRA-3′) 3' reporter dye (6-carboxytetramethylrhodamine [TAMRA]) labeled probe. The RT step conditions were 30 min at 50°C and 15 min at 94°C, followed by 45 cycles of 94°C for 0 seconds and 60°C for 20 seconds. The standard curve for virus quantification was generated in triplicate using a series of 10-fold dilutions from 1:10 to $1:10^{10}$ of the H6N2 stock virus from which EID_{50} equivalents per mL (EID_{50} eq/mL) sample medium of each RNA sample could be calculated. The limit of detection was determined to be 10^1 EID_{50}/ml (1 $\log_{10}$ EID_{50}/ml) per reaction.

2.7 Serology

Upon arrival at the housing facility, serum collected from all birds was tested by the agar gel immune-diffusion (AGID) test and shown to be negative for antibodies to any influenza type

A viral antigen. Infected chickens were bled on day 10 post infection and sera were tested again using the AGID test.

2.8 *Experimental design*

This exploratory work included three initial pilot studies, each requiring the comparative examination of birds, in order to verify anti-AIV vector uptake into chicken respiratory tissues, determine proper anti-AIV vector dose, and verify H6N2 virus is appropriate for challenging the chickens. The findings from the following three pilot experiments were an essential part of planning the fourth part of the described *in vivo* work, the final POC study. The findings from the initial pilot studies were not used to extrapolate to chicken populations due to the limited sample size and lack of power.

2.8.1 Pilot 1

Two groups of two chickens were housed in separate suites and were administered the RFP-vector (parent_pmbv43/CEQ_RFP, previously described) at 1.6e8 CFU in a single 500 µL dose. Each group of two chickens were euthanized by CO_2 inhalation at 15 and 27 hours post RFP-vector treatment. Respiratory tissues were collected at necropsy, including sinus, trachea, pharynx and lungs, and the proventriculus of each animal was similarly collected. Within three hours, these tissues were prepared for fluorescent microscopy and subsequent observation of RFP expression indicating vector adherence and intracellular uptake. Briefly, the tissues were cryogenically frozen and sectioned using a cryostat machine. Tissue sections were mounted on glass coverslips using ProLong® Gold Antifade Reagent with DAPI and covered with a coverslip. After 24 hours incubation at room temperature, slides were maintained at 4°C until images were captured using the Eclipse Ti inverted fluorescent microscope using 461 and 670 emission. The objective of this pilot study was to verify that these *E. coli* bacteria are appropriate vehicles to specifically target and deliver the anti-AIV vectors to the chicken

respiratory tissues. These were qualitative observations, not aimed at detecting a difference in the measured effects between the two groups but rather interested in determining if these vectors are optimal delivery vehicles to chicken respiratory tissues.

2.8.2 Pilot 2

Two groups of five chickens were housed in separate suites and were administered the scramble vector (parent_pmbv43/CEQ, previously described) with one of two doses, 1.36e7 or 3.6e8 CFU per 500 µL dose. Pilot 2 required one group of five chickens to serve as the untreated control group. These chickens were administered 500 µL of PBS/Dap and served as the baseline for comparison to clinical illness scores and histology. Chickens were monitored daily for clinical illness and vector treated chickens euthanized at day 3 (n=2), day 7 (n=2), and at day 14 (n=1). The untreated control birds were euthanized at day 3 (n=1), day 7 (n=2), and at day 14 (n=2). During necropsy, chickens were assessed for gross signs of pathogenesis and/or inflammation indicative of bacterial infection and sinus, trachea, pharynx, lungs, and the proventriculus tissues were collected. Tissue were fixed in 10% neutral buffered formalin solution, sectioned, and stained with hematoxylin and eosin. The objective of this pilot study was to verify these vectors are well tolerated at the two administered doses, without a concomitant induction of gross or microscopic signs of epithelial damage at day 3, 7 and 14 post treatment, compared to the untreated tissues. These were qualitative observations, aimed at determining the highest vector dose tolerable, based on lowest average clinical illness and least observable gross or microscopic signs of epithelial damage. Pilot 2 was necessary to determine the optimal anti-AIV vector dose for the final POC experiment.

2.8.3 Pilot 3

One group of ten chickens were challenged with LPAI H6N2 virus. Chickens were evaluated daily for clinical illness and OP swabs collected to detect viral shedding using RT-

qPCR. At 10 dpi, chickens were euthanized. The purpose of pilot 3 was to verify chicken susceptibility to infection following H6N2 challenge by way of clinical illness scores and/or detection with RT-qPCR.

2.8.4 Proof of concept

This work required three groups of animals (n=10 for each group) to statistically determine differences in viral shedding between chickens treated with the anti-AIV vectors and untreated positive control chickens. Estimated sample sizes were calculated to achieve at least 80% power with a level of significance of 95% using the statistical analyses previously indicated (Wilcoxon rank-sum test, Fisher's exact test, multivariable linear regression, and logistic regression). The most important quantitative measurement is viral titer (EID_{50}/mL), a value that represents viral shedding. Therefore, power calculation was based on expected EID_{50}/mL measurements. It was estimated that the mean viral titer in the untreated chickens would be 5.5 log EID_{50}/mL with standard deviation (SD) ± 0.5 log EID_{50}/mL (Swayne and Beck, 2005). For this work, at least 5-fold reduction (0.7 log EID_{50}/mL difference) in viral titers would be deemed clinically relevant. Therefore, the estimated mean viral titer for the treated chickens would be 4.8 log EID_{50}/mL with SD ± 0.5 log EID_{50}/mL. The calculated effect size was 1.4 ((largest mean - smallest mean)/SD = (5.5-4.8)/0.5). Using the Stata code, fpower, the output table indicated a sample size between 9 and 10 was required to achieve a power of 0.8 with $\alpha = 0.05$. A sample size of ten was sufficient to demonstrate a ≥ 5-fold reduction in viral shedding associated with chickens treated with the cocktail vector expressing viral specific shRNAs compared to untreated PC chickens or scramble vector treated chickens. Three groups of ten chickens, housed in three separate suites, were administered 3.6e8 CFU of vector or a placebo in a 300 µL dose. These groups included group 1 = anti-AIV cocktail vector, group 2 = scramble vector, and group 3 = positive control (PBS/Dap solution). All birds were challenged via intranasal (IN) route with H6N2 virus at 20 hours post treatment (± 2 hours). Chickens were evaluated daily for

clinical illness and OP swabs were collected. At 10 dpi, chickens were euthanized and necropsied for signs of disease and collection of sinus, trachea, pharynx, lungs, liver, and spleen. One-half of the tissue section was fixed in 10% neutral buffered formalin solution, while the remaining half of fresh tissue was stored at -80°C.

2.8.5 Sentinels

Two additional 4 week old commercial chickens were added to the shipment of thirty animals for the final POC study. In lieu of euthanizing these extra animals, they were housed among the ten H6N2 challenged chickens that had additionally received the anti-AIV cocktail vector. These sentinels were in direct contact with these infected animals and shared the same food and water. The purpose for including these two sentinel chickens was to monitor possible transmission.

2.9 *Statistical analysis*

Descriptive statistics included median viral titers with 95% CI, proportions of chickens shedding, and fold-reductions in shedding titers. Differences in the proportion of chickens shedding virus were analyzed using Fisher's exact test ($p<0.05$). Differences in the median viral titers between treated and untreated chickens were analyzed using the Wilcoxon rank-sum test ($p<0.05$). MLR, using ranks and controlling for day, was used to determine if adjusted median Log EID_{50} eq/mL values from OP samples collected from vector treated and untreated PC chickens were statistically different ($p < 0.05$). Logistic regression analysis was used to determine the odds ratio (OR) of shedding in treated chickens compared to untreated PC chickens ($p<0.05$), by individual day as well as across all days. Statistical analyses were performed using the statistical computing software STATA 10 IC (StataCorp 2009, Stata Statistical Software: Release 10).

3. Results

3.1 RFP-vector localization in chicken respiratory tissues

In pilot 1, animals were euthanized and tissues were collected 15 and 27 hours after chickens were treated with the RFP-vector. These tissues were prepared for fluorescent microscopy and images of treated and untreated tissues were captured. This RFP is generated by the prokaryotic expression vector, pE2-Crimson. This fluorescent protein is very bright, photostable, and noncytotoxic due to its solubility in the cytosol of host cells (Strack et al 2011). It was also chosen because of its far-red excitation and emission properties, allowing differentiation between this red fluorescent signal and possible background due to auto-fluorescence. Shown in Figure 4.1, the RFP expressing bacteria appear to localize to the epithelium of the sinus tissues, trachea, and lungs at both time points. This is in contrast to a lack of red expression visible in those tissues collected from PBS/Dap treated chickens.

3.2 Optimal vector dose and associated pathogenicity

In pilot 2, chickens were administered one of two doses of scramble-vector and were observed for 14 days. Between days 1-14 post treatment, birds in all groups did not show signs of any distress or clinical illness. At both vector doses, groups of chickens were euthanized at day 3 (n=2), 7 (n=2) and 14 (n=1) post treatment and at necropsy all tissues appeared normal compared to tissues from birds treated with the placebo (PBS/Dap). No obvious pathogenesis was observed in the proventriculus tissues. Histological assessment indicated vector treatment was not associated with respiratory tissue pathogenesis when compared to PBS/Dap treated tissues. Histology did indicate minor underlying disease present in some treated and untreated tissues, which may have been present in these commercial chickens prior to the start of the study. Therefore, it was difficult to determine if disease was associated with the vector, the treatment volume (500 µL), or if the animals came from a population previously diseased.

For this reason, it was decided to repeat this work using birds given only the higher vector dose (3.6e8 CFU per 500 µL dose) compared to birds treated with 500 µL PBS/Dap, birds that were untreated, and birds arriving fresh from the commercial operation. The last group of birds was included to determine if disease found in the untreated birds was present prior to arrival or if disease developed after arrival to the housing facility. Briefly, two birds were randomly allocated to each of the four groups. With the exception of the fresh arrival birds, which were euthanized and necropsied on arrival, birds in the remaining three groups were treated with vector, PBS/Dap, or nothing and observed for 14 days. On day 14, all birds were euthanized and necropsied. At necropsy, none of the birds in any of the groups displayed any signs of disease or abnormalities and the pathologist, Dr. Sushan Han, analyzing the histological slides was blinded to remove any potential bias. Histology revealed very little pathology or macrophage infiltration in the lungs, trachea, and air sacs of any bird that would indicate an adverse reaction to the vector or dose volume, especially in the in the airways and interstitium. There was mild perivascular inflammation noted in some of the tissues, but the presentation was minimal and random among all groups. Furthermore this presentation is not unusual given these birds are commercial and not SPF.

3.3 Susceptibility to H6N2 challenge

In pilot 3, ten chickens were challenged with H6N2 virus. OP swabs were collected and clinical illness noted. One chicken was found dead at 7 dpi. At 8 dpi, 6/9 remaining chickens were AIV seropositive after AGID testing and chickens were euthanized at 10 dpi. The purpose of this pilot was to verify H6N2 infection and shedding after experimental challenge. Therefore, OP swabs collected on 3, 4 and 5 dpi were the first and only group of samples tested using RT-qPCR. Results revealed shedding in 7/10, 10/10, and 8/10 chickens at 3, 4, and 5 dpi, respectively. These data were sufficient to verify chicken susceptibility to H6N2 challenge and no further RT-qPCR testing was conducted on remaining samples.

3.4 Vector antiviral activity in experimentally challenged chickens

In the final POC study, groups of birds were prophylactically treated with the cocktail vector, scramble vector, or given a placebo (PBS/Dap) prior to infection with H6N2 virus. OP swabs were collected and clinical illness was noted daily. At 8 dpi, one chicken in the PC group died. This was the only mortality in all groups. The remaining chickens were euthanized at 10 dpi. Blood collected prior to euthanasia revealed 4/10, 6/10, and 6/9, cocktail, scramble and PC chickens were AIV seropositive, respectively. H6N2 challenge produced subclinical illness, as none of the chickens presented any notable signs of disease. As such, the clinical illness scoring system was not used and scores were not analyzed.

Vector protection as measured by a cumulative reduction in median virus titer and the proportion of positive OP swabs out of the total swabs collected from vector treated compared to PC chickens is shown in Table 4.1. This data represents crude differences across the entire study, without adjusting for day. A significant difference in median titer (log EID_{50} eq/mL) between the cocktail treated (1.5; 95% CI = 0, 3.0) and the PC chickens (4.1; 95% CI = 2.8, 4.8) (Wilcoxon rank sum, $p<0.0001$) is shown. Likewise, there is a significant difference in the median titer between scramble treated (3.2; 95% CI = 0, 3.9) and PC chickens ($p=0.003$). There is a significant difference in the proportion of positive OP swabs collected from the cocktail group, 36/70 (51%), compared with 58/70 (83%) from the PC group (Fisher's exact test, $p<0.0001$). This significant difference was also observed between scramble treated (42/70, 60%) and PC birds ($p=0.002$). Although there is no significant difference in the cumulative median titer or proportion of positive swabs between the cocktail and scramble groups, in both measurements, the antiviral effect in the cocktail group is more profound. Table 4.1 indicates vector protection, but does not reveal which day during infection these significant differences are occurring.

Table 4.2 portrays vector protection on each day. For each treatment group, median titer and shedding proportions on each day post infection are shown. The Wilcoxon rank-sum test for

a difference in median titer indicates significant differences between cocktail treated and PC chickens at days 3 and 4 post infection. No significant difference in median titers on any individual day was observed between the scramble treated and PC chickens. There was a significant difference in the proportion of birds shedding in the cocktail group (5/10) compared to the PC group (10/10) at 3 dpi ($p=0.016$). However, the differences in shedding proportions are insignificant between the scramble treated and PC group on any individual day.

Median H6N2 virus titers and the proportion of chickens shedding virus at each daily interval are shown in Figure 4.2. Looking at the plot of median titer by day representing all chickens in each group (including those not shedding), there a distinct trend in the load of virus shed between the cocktail chickens and those in the scramble and PC group (Figure 4.2 A). Between day 2 and 7 post infection, median titers in both the scramble and PC groups appear to rise, while the median titer in the cocktail group drops and continues to decline until day 5 post infection. The titer in the cocktail group does briefly rise at 6 and 7 dpi, but the load of virus shed is still much lower than the virus load shed from the scramble and PC chickens. A less profound trend is observed when the median titer by day representing those chickens shedding in each group is plotted (Figure 4.2 B). Here each median titer represents the daily proportion of chickens shedding from Figure 4.2 C. With the exception of day 3 and 10 post infection, the daily median load of virus shed is less in the cocktail compared to the PC shedding birds. Despite this apparent switch in observed virus titer between the cocktail and PC group on these two days, it is important to recognize that still a greater number of birds are shedding (Figure 4.2 C) on day 3 and 10 in the PC group (10/10 and 5/9) compared to the cocktail group (5/10 and 4/10).

Median shedding titers from birds in each group varies by day. Significant differences between crude median titers are observed (Table 4.1), however to determine significant differences across all days post infection, it is necessary to control for day in the analysis. Vector protection as measured by a reduction in median titer was analyzed using MLR,

controlling for day (data not shown). Regardless of the day post infection, there was a significant difference in median titer in the PC chickens compared to the cocktail ($p<0.0001$) and compared to the scramble ($p=0.002$) chickens. In the majority of data presented, the cocktail vector provided greater protection compared to the scramble vector. However using MLR and controlling for day, this difference is not statistically significant ($p=0.251$).

Table 4.3 presents ORs, 95% CIs, and level of significance for each individual day and after controlling for day (across all days). With the exception of 3 dpi, the odds of shedding on an individual day in the PC chickens was not significantly different compared to the cocktail or scramble treated chickens. On day 3 post infection 10/10 (100%) PC chickens were shedding virus. When controlling for day, the odds of shedding H6N2 among PC chickens was 4.83 (95% CI = 2.17, 10.72) greater compared to cocktail treated chickens ($p<0.0001$). Compared to the scramble vector treated chickens, the odds of shedding detectable virus across all days in the PC chickens was slightly less (3.48; 95% CI = 1.57, 7.86), but still significant ($p=0.003$). Albeit not statistically significant, across all days the odds of shedding virus in the scramble group was 1.42 greater compared to the odds of shedding in the cocktail group ($p=0.303$).

Vector protection as measured by log reduction and fold reduction in replication and shedding titers is shown in Table 4.4. Differences in median titer (log EID_{50} eq/mL) between vector treated and PC chickens was calculated as the log reduction. Using the geometric median values, fold reductions in virus titers were calculated. Both reduction measurements were computed across all days (using the unadjusted median titer) and for each individual day. Across all days, the cocktail treatment was associated with a 2.6 median log reduction (398-fold reduction) in virus titer shed compared to the PC chickens. At each individual day post infection, the cocktail treatment resulted in a measureable median log reduction. Most notable was at days 3, 4, 5, 6, and 7 post infection, in which viral shedding was between 2.5 and 3.8 median logs lower (288 to 6309-fold lower) compared to the PC chickens. Log reductions in viral shedding are much less pronounced in the scramble treated chickens. However, overall still

resulted in a 0.9 median log reduction (7.9-fold reduction) and at each individual day at least a 0.3 median log reduction (1.8-fold reduction). Most notable for the scramble vector treatment was at day 10 post infection (1.4 median log reduction), which represents the least potent reduction observed in the cocktail group, also at day 10 post infection.

3.5 Sentinels

Two naïve chickens were housed with the cocktail treated birds from the time of vector administration to 10 days post H6N2 challenge. From both of these birds, only one OP positive swab was detected at day 7 post introduction (1.5 log EID50 eq/mL). None of the other swabs collected between day 2 and 10 post infection from these sentinel birds were positive for the AIV M gene. Overall, 13/14 (92.8%) of OP swabs collected from these contact birds were negative for the AIV M gene.

4. Discussion

This study is the first to show efficient delivery and bacteria-mediated invasion into avian sinus, trachea, and lung tissues using the tkRNAi delivery platform. RFP-vector uptake was assessed at two time points, 15 and 27 hours post treatment. These time points were selected to demonstrate vector delivery and uptake occurred within 24 hours, as a way of verifying vector delivery preceded AIV challenge and infection. After delivery and invasion to the chicken respiratory tissues, it is unknown how long these vectors would actively supply shRNA to these recipient non-phagocytic epithelial cells. To show initial efficacy, it was decided to assess and verify rapid vector uptake to simulate viral protection within 24 hours. Images captured in Figure 4.1 showing RFP-vector localization to the respiratory tissues at both time points was sufficient to conclude that vector delivery and theoretically, the unloading of shRNA would precede viral challenge.

Intranasal administration of the scramble vector was not associated with any observed pathogenesis within 14 days post treatment as compared to placebo (PBS/Dap) and untreated birds. Lack of any observed clinical illness and favorable histopathology results suggests these vectors were well tolerated at the dose administered. There are limitations to this work. Pilot 2 did not test if tissue specific pathogenesis would be present if sequential vector doses were administered across several days. Also not evaluated in this work are alternative ways of quantifiably measuring vector treatment response, including a drop in egg production for layer birds and lack of weight gain in broilers. In future work, this prophylactic technology would need to be assessed beyond 14 days and after multiple doses. However, these vectors are Dap auxotrophic and once administered, are unable to survive or proliferate outside of a supplemented medium. It is doubtful a chronic inflammatory response would incur beyond 14 days due to a lack of any gross or microscopic tissue damage at 2 weeks post treatment. These initial pilot results appear promising and indicate this tkRNAi system is appropriate for avian respiratory tissue delivery and does not trigger an inflammatory response that the avian host is unable to overcome within 14 days.

Pilot 3 verified that H6N2 virus at the challenge dose was infectious in 5 week old commercial layer chickens. This dose (10^6 EID_{50}/0.1 mL) is common for LPAI vaccine efficacy trials and other experimental challenge studies in chickens (Claes et al 2013, Pantin-Jackwood et al 2012, Abbas et al 2011). Together pilot 1, 2, and 3 verified vector uptake within 24 hours, identified an appropriate vector dose, and an optimal LPAI challenge virus. These parameters were incorporated into the final POC study.

The crude data presented in Table 4.1 indicates significant differences in shedding titers and the proportion of positive swabs collected across all days post infection when comparing the cocktail or scramble treated birds to the PC birds. These results are further supported when MLR analysis is conducted, controlling for day. It is possible to expand these results by day and identify where and allude to why these differences occurred between treatment groups.

Following H6N2 challenge, it appears that the proportion of chickens shedding on 2 dpi was relatively similar; meaning the success of experimental infection was relatively equal in all groups. Yet, of birds shedding on day 2, shedding titers were lower in the cocktail compared to both the scramble and PC birds. Additionally, individual bird shedding (data not presented) indicates one cocktail treated bird was shedding at 2 dpi, but stopped shedding at a detectable level on subsequent days. This intuitively makes sense because the vector's mode of action is aimed at preventing replication, not infection.

At day three, while shedding proportions in the scramble (7/10) and PC (10/10) groups appear to spike, the cocktail group significantly drops to 5/10 and plateaus through days 3, 4, and 5. In support of this observation, shedding titers are significantly different at days 3 and 4 post infection between cocktail treated and PC chickens. Again, looking at shedding trends (Figure 4.2 A), at day 3 through 5 post infection, median titers drop in the cocktail group, but continue to increase out to day 7 post infection in both the scramble and PC groups. These observations might be explained by the NP and PA shRNA antiviral activity, interfering with efficient viral replication during the infectious period. There is an alternative way to interpret these observations. In the cocktail treated group, more chickens were shedding virus at 2 days post infection compared to days 3-5. This might indicate vector protection would be more effective if administered > 24 hours prior to challenge, allowing greater time for shRNA unloading and processing.

Shedding titers and proportions by day support a proposed model of vector protection. Following viral entry and infection, the vector interferes with viral replication, inhibiting the proliferation of infectious virions that go on to infect neighboring cells and tissues. This reduces the infectious viral load within the respiratory tissues, thereby decreasing viral shedding in each chicken. Because AIV is primarily transmitted through direct contact in chickens, this prevents subsequent infection of susceptible birds in that group. Together these findings imply cocktail vector treatment disrupts efficient viral replication and shedding.

Shedding duration directly influences the infectious period or the time from when virus is first detected to when the virus is no longer detected. It is provides another way to assess vector protection. However, it can be argued that shedding duration plays a less significant role in transmission compared to shedding titer. An animal might be shedding over a longer duration, but shedding titers too low to support successful transmission. Additionally, bird density might not support transmission, despite longer shedding periods. For that reason, shedding duration is not typically used as a measurement for evaluating vaccine efficacy. This study was terminated at 10 dpi. Had the study continued beyond 10 days, it would have been possible to measure the vector's effect on shedding duration. The cocktail vectors reduced oropharyngeal viral shedding titers, but do not appear to limit the duration of shedding. Perhaps this indicates the vectors appropriately provided rapid and transient protection early on in the infection cycle, but also might indicate a need to administer a subsequent vector dose, much like a booster vaccine. This might further protect susceptible birds from those infected, thereby blocking the transmission chain.

In addition to assessing shedding duration and differences in shedding titers and shedding proportions, the odds of shedding virus in the PC chickens compared to vector treated chickens provides an alternative way to measure vector protection. Controlling across all days post infection, the PC chickens were 4.83 times more likely to shed H6N2 virus, compared to the cocktail treated chickens ($p<0.0001$). PC chickens were also more likely to shed virus compared to the scramble treated chickens. While these odds ratios provide greater support for vector protection, they are not a standardized method of evaluating vaccine efficacy in the industry.

The quantitative reduction in virus replication in the respiratory tract is a critical measurement used to evaluate a prophylactic's capacity to limit virus transmission and control disease (Swayne and Kapczynski 2008). To be deemed clinically relevant, an AIV vaccine must demonstrate a minimum reduction in replication and shedding titers of 10^2 EID_{50} (2 log or 100

fold) less virus from respiratory tracts in vaccinated compared to nonvaccinated birds (Suarez et al 2006), and/or the difference should be statistically significant (Swayne et al 1997). While the scramble vector did not demonstrate this minimum required reduction, it did demonstrate a statistically significant difference in shedding titers across all days. With the exception of day 2 and 10 post infection, shedding titers from the cocktail vector demonstrated a minimum of 2.5 log (288-fold) and a maximum of 3.8 log (6,309-fold) less virus compared to the PC birds. Across all days, the anti-AIV cocktail group was shedding 398-fold less infectious virus compared to the PC group. These measurements were all statistically significant. Based on both of these measurable criteria, the cocktail vector treatment was well within the parameters to be regarded as providing clinically relevant protection against AIV.

Several other measurements are used to assess vaccine protection. Vaccinated birds are often tested for quantifiable resistance after challenge with 10^2 to 10^5 EID_{50} greater dose compared with unvaccinated birds (Swayne and Kapczynski 2008). In addition, clinical illness as defined by a drop in egg production in layer birds can quantifiably measure protection. Egg production does not begin until 18-20 weeks of age. To mimic the age at which the industry typically applies vaccine regimens, the birds utilized in this study were 4-6 weeks of age. Therefore measuring egg production would not have been possible. Future studies utilizing this vector system would use both of these assessments to quantifiably measure vector protection. The prevention of mortality is another direct measurement of vaccine protection. LPAI is by definition low pathogenic and not often associated with mortality. However, this H6N2 virus is well adapted to chickens, contributing to 10% mortality in the PC chickens and chickens challenged in pilot 3. In contrast, the cocktail and scramble vectors both appeared to prevent mortality, as none of the birds receiving either vector died following H6N2 challenge.

Finally, prevention of contact transmission is a direct method to demonstrate a vaccine's protective efficacy and propensity to limit field transmission. Therefore, clinically relevant reductions in shedding can be demonstrated by showing reduced spread to contact birds, a

practice often requested by national veterinary biologic regulatory agencies. This is difficult to assess in experimental settings because factors that play a role in transmission are not standardized. These factors include bird density, ventilation, humidity, temperature, challenge virus and dose, route of administration, age at challenge, virus-host adaptation, and room sanitation. The intranasal 50% bird infectious dose (BID_{50}) has been suggested as a way to quantifiably assess the potential for AIV to initiate infection and support transmission in chickens challenged in a laboratory setting (Tumpey et al, 2004). In this current study, it can be said that the birds were challenged with an appropriate BID_{50} to initiate infection and support transmission because greater than 50% of the PC chickens were shedding virus. Two naïve sentinel chickens, housed with the cocktail treated birds, served as a way to monitor viral spread within this group at a density of 1.0 square-feet per bird. With the exception of one positive swab at 7 days post introduction, no other evidence of bird-to-bird transmission was detected in the two sentinels. The titer detected from this one swab was just above the limit of detection of the RT-qPCR assay. It is possible that this positive sample was truly due to H6N2 transmission. However, it is also possible this sporadic positive swab was a result of cross contamination from gloves during collection or viral RNA during the extraction procedure. To minimize the chance for cross contamination between birds in different treatment groups, clean sets of gloves were used to collect swabs between groups. This would not eliminate the chance for cross contamination between swabs collected in the same group, had a glove been in contact with a shedding animal and become contaminated with virus prior to collecting the swab from the sentinel animal. Precaution was taken to avoid this scenario, but the sampling procedure would not have eliminated this potential risk. During the viral RNA extraction procedure, samples were processed in an open 96 well plate. One negative extraction control (water only) per 96 well plate was included, however this practice only indicates and does not verify the lack of cross-contamination between all samples.

In a previous laboratory based transmission study, researchers experimentally challenged 3 week old SPF chickens (n=10) with LPAI A/Ck/HN/1/98 (H9N2) (Guan et al 2013). When housed at a density of 0.5 square-feet per bird, mean OP viral titers of 2.1 log 10 EID_{50} eq/mL at 2 dpi was sufficient for transmission to naïve chickens (n=2). A second study intranasally challenged 46 week old SPF leghorn chickens (n=10) with 0.1 mL containing 10^6 EID_{50} of A/chicken/CA/1255/02 (H6N2) virus and at 3 dpi two sentinel birds were added to monitor contact transmission (Pantin-Jackwood et al 2012). In the challenged chickens, mean OP titers ± SD (log10 EID_{50} eq/mL) at 2 and 4 dpi were 4.8 ±0.5 and 5.3 ±0.5, respectively. At 4 days post introduction, both sentinels were shedding virus (3.7 and 3.1 log10 EID_{50} eq/mL titers). While bird density is not presented in this referenced study, shedding titers in the challenged birds are similar to those titers detected in our current PC chickens at day 2 and 4 post infection. Vector protection as measured by reduced transmission potential would have been better illustrated had sentinel birds also been housed with the PC chickens. Unfortunately, this was not possible as only two extra birds were provided by the commercial farm. In addition to using an appropriate BID_{50}, these previous studies indicate shedding titers would have been adequate to support transmission if sentinels had been housed among PC chickens. Regardless, it appears that the cocktail vector successfully inhibited transmission to these contact birds. To make any valid inferences about capacity of these anti-AIV vectors to break the transmission chain, a standardized laboratory contact-transmission model is required to more accurately reproduce a field or on farm environment.

Mucosal surfaces of the poultry respiratory tract are known gateways of entry for AIV (Zarzaur and Kudsk 2001). Because AIV invades the mucosal surfaces, targeted delivery of these vectors to the mucosal respiratory tracts in chickens is an added benefit to targeting viral replication. In this way, these vectors could provide strong protection at the first line of defense for the host. However, concerns over immune activation are important to consider, as there are pros and cons to activating an immune response in the respiratory tissues. Unlike the natural

microflora present in poultry gastrointestinal tracts, it is unlikely respiratory tissues have developed immune tolerance against bacterial components. A moderate immune response could be beneficial, especially against influenza infection. This might help explain the antiviral activity associated with scramble vector treatment.

However, it is important to avoid stimulating a strong mucosal immune response. This could interfere with the efficacy of this intranasally applied vector, especially if multiple doses are necessary for better protection against AIV. After concluding the POC work, it was learned that these 4 week old pullets had been vaccinated at 7 days of age with the Poulvac® *E. coli* modified-live vaccine (Zoetis, Florham Park, NJ). This newly approved vaccine has been studied to determine its broad-spectrum protection mechanism against avian pathogenic *Escherichia coli* (APEC). When administered by spray or drinking water, it appears the vaccine stimulates the production of APEC specific immunoglobulin A in the mucosa tissues and proliferation of CD8 memory cells, both of which indicate class I MHC activation leading to a cellular, rather than a humoral response (Filho et al 2013). Activation of a cellular immune response would help prevent future tissue invasion by APEC, especially in the respiratory tract, as this is the primary route of APEC infection (Sadeyen et al 2014). Vaccination may have resulted in mucosal inflammation at 4 weeks of age. Perhaps Poulvac® *E. coli* vaccination explains the underlying disease observed in pilot 2 (first round) and resulted in observed inflammation and lymphoid aggregates in the respiratory tissues of both control and vector treated birds.

Besides concerns over Poulvac® *E. coli* vaccine induced mucosal inflammation, an obvious caveat to this *E. coli* vaccine is if it affected vector efficacy by preventing vector invasion of the respiratory epithelium via priming a cell-mediated response. While this concern is valid, it can be addressed in several ways. The bacteria used in the anti-AIV vector system are the non-mobile *E. coli* strain CEQ221. These bacteria are a K-12 derivative, phenotypically rough, and while their LPS has a complete core structure, they lack O antigen (Liu and Reeves

1994). This means these vectors are defective in LPS O-antigen biosynthesis and may lack efficient LPS expression resulting in lower levels of LPS on their outer membranes. It is therefore unlikely CEQ221 would induce a robust immune response. CEQ221 are non-pathogenic, invasive intracellular bacteria and have been genetically engineered to target mucosal epithelial cells. These invasive *E. coli* require (β)1 integrin expression on the surface of mucosal epithelial cells. Entry is dependent on this invasin-(β)1 integrin receptor interaction allowing for bacterial uptake via receptor mediated endocytosis (Xiang et al 2006, Isberg and Barnes 2001, Conte et al 1994, Isberg and Leong 1990). If not for the genetically engineered bacterial expression of the invasin protein, these CEQ221 bacteria would otherwise be extracellular and recognized by professional antigen presenting cells (APCs). Epithelial cells are not typically characterized as professional APCs. Because of this specific mucosal epithelium targeting system, dendritic cells and other APCs are less likely to interact or respond to these vectors. CEQ221 is a Dap auxotroph and undergoes rapid lysis after invasion into host cells. Even if CEQ221 were invasive to APCs and other immune cells, the expression of LLO after bacterial lysis in the phagosome would disrupt the fusion between the cell's phagosome and lysosome. This disruption would block class I MHC presentation and prevent activation of a cell-mediated immune response via CD8 memory cell proliferation (Grillot-Courvalin et al 1998). Therefore, these CEQ221 vectors are not well suited to develop a cell-mediated or even a humoral immune response. These characteristics, or lack thereof, might explain this vector's ability to evade the cellular response generated by the Poulvac® *E. coli* vaccine in these commercial chickens.

Had the animals utilized in this study been SPF birds, vector protection might have been more directly measured. However, to best mimic the efficacy of this vector system in the field, an argument can be made for using these commercial birds. Ultimately, it is difficult to decipher the effects this Poulvac® *E. coli* vaccine may have played on the anti-AIV vectors in this

preliminary pilot work. However, if the *E. coli* vaccine had any deleterious effects this would suggest the results from this work underestimate vector protection against AIV in chickens.

While results suggest the cocktail vector provided greater protection, it is not unusual that the scramble vector alone has antiviral capabilities. These bacterial vectors are characteristically LPS rough, however even very low levels of LPS can act as immune enhancers (Bessler et al 1990). LPS is commonly recognized by host tissues and stimulates an innate immune response. LPS recognition can lead to downstream signaling pathways that stimulate type I IFN production. This scenario is probable given some past research related to CEQ508 *E. coli* bacteria, a derivative of the CEQ221 strain that serves as anti-AIV vector delivery vehicle. In previous work (Steinbach et al 2010), oral dosing with CEQ508 did not elicit a significant increase in circulating pro-inflammatory cytokines in mice as compared to LPS injections. However, these bacterial vectors did stimulate very low levels of these cytokines, including TNF-α, IL-6, MCP-1, and IL-12. These are all key cytokines involved in the innate immune response, a well-known cellular defense mechanism against viral infection, specifically influenza (Garcia-Sastre and Biron 2006). When these bacterial vectors were administered via the intranasal route to the chickens, it is quite possible that these vectors triggered an innate response, resulting in an added protection against H6N2 shedding. The production of pro-inflammatory cytokines during this innate activation would increase the ability of any uninfected host cells to resist new infection by newly generated virus.

As has been presented and discussed, there are several ways to measure and describe vector protection from this POC work. Whether looking at differences in shedding proportions, shedding titers, or odds ratios both by day and across all days, a similar pattern is observed. Compared to the positive controls, chickens treated prophylactically with the anti-AIV cocktail vector were protected following experimental challenge with H6N2. Although results were not statistically significant on any individual day, overall (across all days) protection was linked to scramble vector treatment. Albeit, protection from the scramble vector was less profound,

suggesting the cocktail vector has greater antiviral potential. This added protection is likely due to the dual antiviral action of NP and PA shRNA.

A fundamental question arising from this work is if a reduced transmission potential can be estimated from the viral shedding data. When considering the severity of an AIV outbreak in poultry, how critical is the proportion of chickens shedding and the quantity being shed during the infectious period to sustaining virus transmission? Moreover, what is the minimum reduction in the proportion of chickens shedding virus necessary to break the chain of transmission? On average between days 3-7 post infection, 48% of the cocktail vector treated chickens were not shedding. In contrast, only 10% of the chickens in the PC group were not shedding. Results analyzing reductions in shedding titer showed that the cocktail vector provided clinically relevant protection. As was observed in this POC work, if 38% less birds were shedding virus on a farm, would this reduction along with the clinically relevant reduction observed in shedding titers be sufficient to inhibit the spread of this infectious disease within a flock and between farms?

A quantitative standardized model would need to be developed to assess the anti-AIV vector's ability to reduce transmission. Along with incorporating cocktail vector protection as measured by the quantitative reductions identified in this current work, the model would have to incorporate other relevant factors. These include route of infection, infectious dose, housing parameters, climate, animal age, virus strain and pathogenicity, contact rate, vector protective dose, the basic reproductive rate (R_0), and other factors specific to the target population. This type of standardized model could be used as an epidemiological tool used to predict outbreak spread with and without anti-AIV vector protection. It would identify the proportion of a population that would need vector administration to prevent sustained spread of infection. It would help assess the impact this anti-AIV vector technology would have on reducing transmission and ultimately preventing major outbreaks in chickens.

As stated previously in chapter 1 of this dissertation, the long-term goal of this dissertation work is to develop this anti-influenza technology into a prophylactic that would

contribute to the development of a more effective and robust control method for AIV in poultry. Successfully developing this technology would not only reduce the economic burden outbreaks have on the industry and developing countries, it would directly reduce the transmission risk to humans and provide proof-of-concept to the scientific and medical community for developing a similar anti-influenza prophylactic for humans. As with developing any new technology, additional studies are needed and should not be underestimated. This work demonstrates the protective efficacy of these anti-AIV vectors against avian influenza in chickens and provides a strong argument for the continued evaluation of this technology.

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5. Tables and figures

Table 4.1

Vector protection as measured by reduction in virus titer and number of positive OP swabs from vector treated chickens. [a] Represents log EID50 eq/mL from all OP swabs collected over infection period. [b] Number of positive/total OP swabs tested by RT-qPCR. [†] Wilcoxon rank-sum test for difference in median titer values ($p<0.05$). [‡] Fisher's exact test for difference in proportion of positive swabs ($p < 0.05$).

Group	Median Titer (95% CI)[a]	Positive OP Swabs[b]
Cocktail	1.5 (0, 3.0)	36/70 (51%)
Scramble	3.2 (0, 3.9)	42/70 (60%)
PC	4.1 (2.8, 4.8)	58/70 (83%)
	P-value†	P-value‡
Cocktail PC	<0.0001	<0.0001
Scramble PC	0.003	0.002
Cocktail Scramble	0.247	0.198

Table 4.2

Vector protection as measured by reduction in virus titer and number of vector treated chickens shedding H6N2 by day. [a] Titers represent Log EID50 eq/mL values. [†]Wilcoxon rank-sum test for difference in median titer compared to positive control group ($p<0.05$). [‡] One-sided Fisher's exact test for difference in proportion of chickens shedding in vector treated compared to positive control group ($p<0.05$). * One bird died between 7 and 10 dpi.

Group (n=10)	Days post infection with H6N2 virus							
	0	**2**	**3**	**4**	**5**	**6**	**7**	**10**
COCKTAIL								
Median titer (95% CI)[a]	0	2.2 (0, 3.3)	1.2 (0, 4.4)	1.2 (0, 4.6)	0.8 (0, 4.8)	2.2 (0, 4.9)	1.6 (0, 5.3)	0 (0, 4.4)
P-value†	N/A	0.105	0.05	0.042	0.089	0.147	0.18	1.0
Shedding proportion	0/10	6/10	5/10	5/10	5/10	6/10	5/10	4/10
P-value‡	N/A	0.500	0.016	0.070	0.175	0.152	0.070	0.328
SCRAMBLE								
Median titer (95% CI)	0	3.0 (0, 4.4)	3.2 (0, 4.8)	3.9 (0, 5.2)	3.5 (0, 4.8)	4.4 (0, 4.8)	3.8 (0, 4.7)	0 (0, 3.3)
P-value	N/A	0.279	0.289	0.322	0.145	0.223	0.102	0.658
Shedding proportion	0/10	6/10	7/10	6/10	6/10	6/10	7/10	4/10
P-value	N/A	0.500	0.105	0.152	0.314	0.152	0.291	0.328
POSITIVE CONTROL								
Median titer (95% CI)	0	3.9 (0, 5.3)	4.1 (2.1, 5.2)	4.8 (2.0, 5.3)	4.6 (0.9, 5.5)	4.7 (2.3, 5.4)	4.8 (2.0, 5.6)	1.4 (0, 2.9)
Shedding proportion	0/10	7/10	10/10	9/10	8/10	9/10	9/10	5/9 *

Table 4.3

Vector protection as measured by the odds of shedding virus in untreated positive control chickens. [a] Odds ratio calculated using logistic regression analysis. [b] Lower 95% confidence level (LCL). [c] Upper 95% confidence level (UCL). [d] Calculated using simple and multiple logistic regression analysis ($p<0.05$). [e] Controlling across all days. * 10/10 chickens shedding in untreated PC group; OR is not applicable.

		Untreated Positive Control				Scramble Vector			
Reference Group	Dpi	OR[a]	LCL[b]	UCL[c]	P-value[d]	OR	LCL	UCL	P-value
Cocktail Vector	Across all days	4.83	2.17	10.72	<0.0001	1.42	0.73	2.8	0.303
	2	1.55	0.24	9.91	0.640	1.0	0.17	5.98	1.0
	3	-- *	--	--	--	2.33	0.37	14.6	0.365
	4	9.0	0.81	100.14	0.074	1.5	0.25	8.82	0.654
	5	4.0	0.55	29.1	0.171	1.5	0.25	8.82	0.654
	6	6.0	0.53	67.65	0.147	1.0	0.17	5.98	1.0
	7	9.0	0.81	100.14	0.074	2.33	0.37	14.6	0.365
	10	2.25	0.38	13.47	0.374	1.0	0.17	5.98	1.0
Scramble Vector	Across all days	3.48	1.54	7.86	0.003				
	2	1.55	0.24	9.91	0.640				
	3	-- *	--	--	--				
	4	6.0	0.53	67.65	0.147				
	5	2.67	0.36	19.71	0.337				
	6	6.0	0.53	67.65	0.147				
	7	3.86	0.33	45.57	0.284				
	10	2.25	0.38	13.47	0.374				

Table 4.4

Vector protection as measured by log and fold-reduction in shedding titers. [a] Reduction in median log EID_{50} eq/mL titer compared to positive control. [b] Reduction in geometric median compared to positive control. [c] Based on crude median log EID_{50} eq/mL not adjusted for day.

Treatment Group	Dpi	Log reduction [a]	Fold reduction [b]
Cocktail	Across all days [c]	2.6	398.1
	2	1.7	51.3
	3	2.9	794.3
	4	3.6	3715.4
	5	3.8	6309.6
	6	2.5	288.4
	7	3.2	1659.6
	10	1.4	24.0
Scramble	Across all days	0.9	7.9
	2	0.9	8.5
	3	0.8	6.8
	4	0.9	7.1
	5	1.1	11.2
	6	0.3	1.8
	7	1.0	9.8
	10	1.4	24.0

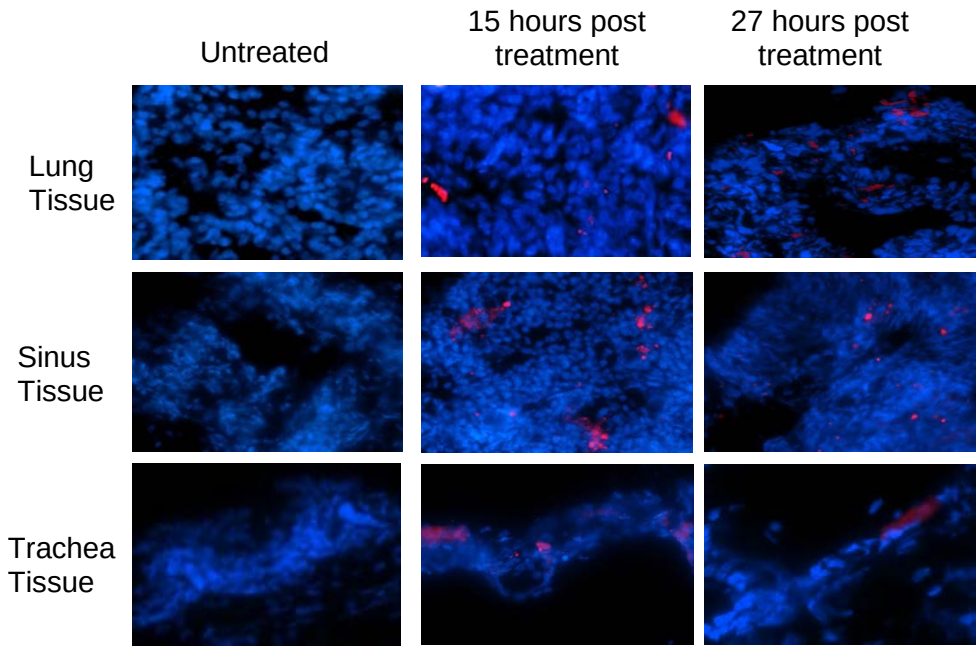

Figure 4.1

Anti-AIV_scramble vector tagged with RFP. Chickens were treated intranasally with RFP vector and uptake assessed at 15 and 27 hours post treatment.

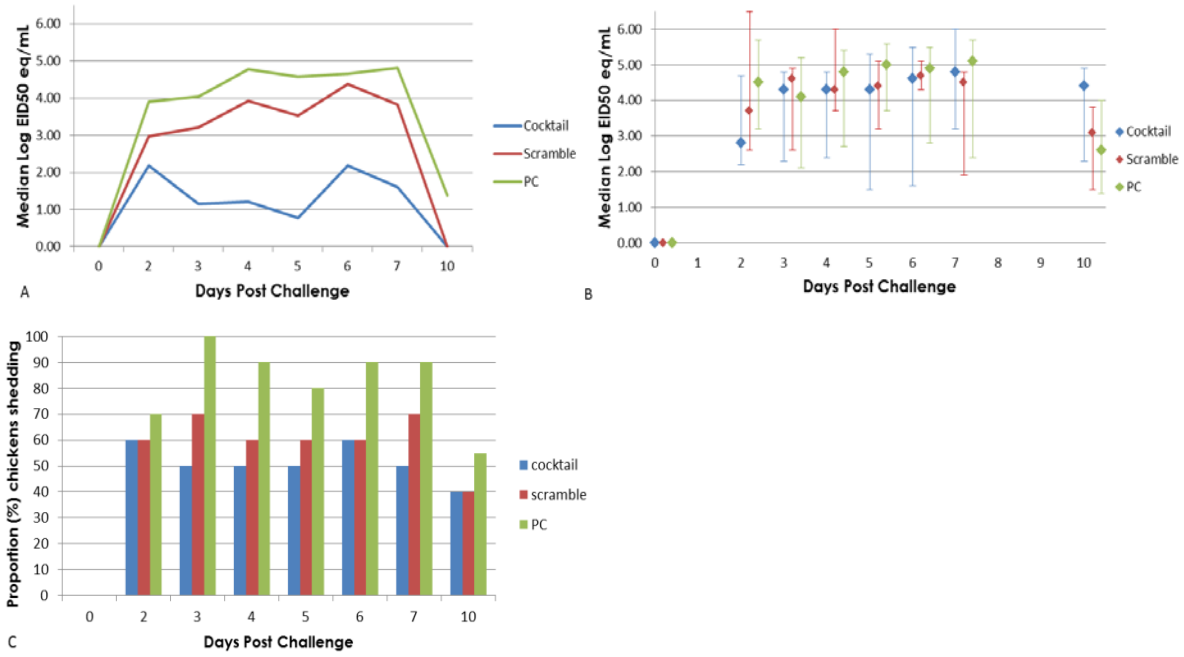

Figure 4.2.

Daily H6N2 virus titer and proportion of chickens shedding virus over the study period. A) Median virus titer (Log EID50 eq/mL) representing all chickens (shedding and not shedding) plotted by day. B) Median virus titer representing chickens shedding and plotted by day. C) Proportion (%) of chickens shedding H6N2 virus by day, out of n=10 total chickens in each group; however day 10 represents n=9 observations in the PC group due to one mortality. Median titer by day (B) represents the proportion of chickens shedding H6N2 virus at that day (C).

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CHAPTER 5

SUMMARY, DISCUSSION, AND FUTURE DIRECTION

Concluding summary

The research presented in this dissertation demonstrates the antiviral capabilities of these anti-AIV vectors, both *in vitro* and *in vivo*. This work allows several conclusions to be drawn on the strengths and limitations of this technology and has advanced our understanding surrounding the potential these vectors have to inhibit AIV shedding in chickens.

Chapter 2 of this dissertation introduced two viral specific siRNAs and demonstrated *in vitro* proof of concept for using these siRNAs to reduce AIV shedding in an avian cell model. Developing this cell model and highlighting the value of using these siRNAs allowed us to translate these siRNA sequences and design the anti-AIV vectors. Chapter 3 of this dissertation provided evidence that these vectors are capable of inhibiting AIV shedding in the established avian cell model.

These siRNAs and corresponding anti-AIV vectors reduced viral shedding titers when tested against two different LPAI viruses. In theory, an advantage for using these viral siRNAs is their potential to target a wide range of AIVs, including HPAI H5 and H7 subtypes. While the findings presented in this dissertation are adequate to illustrate *in vitro* proof of concept for using these vectors against AIV, empirically it cannot be determined if these vectors would have similar antiviral capabilities against HPAI subtypes or even alternative LPAI viruses. Moreover, the avian cell model utilized in this work was comprised of chicken epithelial cells derived from liver tissues. While the advantages to working with these cells were described in chapter 2 of this dissertation, there are limitations to using these cells. This cell model does lack external validity, as these LMH cells have been immortalized and LPAI does not typically infect and replicate in chicken liver tissues during a natural infection.

Chapter 4 of this dissertation provided supportive evidence that these vectors can be administered intranasally for targeted delivery to the respiratory tissues in chickens. Clinical observations, necropsy assessments, and histology further indicate that after these vectors are administered to chickens, they are well tolerated and do not elicit undesirable side effects to the host. The data presented does not provide supportive evidence that these vectors are safe for long term use, beyond 14 days.

The final work presented in chapter 4 of this dissertation demonstrated *in vivo* proof of concept for using the anti-AIV vectors to inhibit AIV shedding in experimentally challenged chickens. As measured by AIV vaccine efficacy standards, the cocktail vector provides clinically relevant reductions in shedding titers compared to untreated chickens. However, testing the protective efficacy against one LPAI virus does not provide supportive evidence that these vectors would offer universal AIV protection. Using commercial chickens as an alternative to SPF chickens does not allow us to establish causality between anti-AIV vector treatment and the reduction of AIV shedding. However, the use of commercial chickens does lend to the external validity of these experiments. The inclusion of only two sentinel chickens in the cocktail vector group and the lack of sentinels in the positive control group limits our ability to draw conclusions regarding the vector's capacity to inhibit viral transmission.

1. Assessing the value of the anti-AIV vector using randomized field trials

Before planning a randomized field trial, it is necessary to complete several additional experimental challenge studies using standardized parameters. Just as the current study appropriately highlighted gold standard publication checklist items for standardizing animal studies (Hooijmans et al 2010), future studies would indicate experimental groups and controls, temperature, humidity, bedding, cage size, group size, cage enrichment, nutrition/water, handling practices, intervention, blinding, and randomization. However, future challenge studies would differ in several ways to better assess vector protection.

Prior to completing any follow-up experimental challenge studies, a safety design test would be conducted on a greater number of SPF layer chickens to appropriately determine the safety of the anti-AIV vectors, independent of AIV challenge. This would include assessing different vector doses, frequencies of dosing, and routes of administering the vector. Measurements to assess vector safety would be more comprehensive to include clinical illness, weight gain, egg production, and gross and histopathology at different times post treatment to monitor any transient or chronic (beyond 1 month) disease. Potential consumer risk would also need to be determined. This would require testing for the presence of the bacterial vector (viable and nonviable) in the tissues including meat and in chicken eggs. This might also include testing eggs after spraying with the vector to ensure vectors are unable to penetrate the porous surface and survive. Assessing the risk to the environmental would also be performed by monitoring shedding of the anti-AIV bacteria via fecal and respiratory secretions as well as into the common environment (bedding, feed, water, etc.). This would indicate any potential to spread to contact animals and persist in the environment, taking into account human health as well.

Subsequent challenge studies would utilize SPF chickens to better isolate the protective effect of vector treatment, thereby eliminating possible confounding factors such as prior disease or vaccinations. Older birds (20+ weeks) would be used to measure vector protection by inhibiting drops in egg production. Transmissibility via direct and indirect contact would be assessed using sentinel animals in all treatment groups. Vector protection against different avian influenza viruses, both LPAI as well as HPAI, would be necessary to demonstrate the universality of this technology. The potency of vector protection would be quantified by treating chickens with alternate doses, at different frequencies, and using different administration routes pre and post AIV challenge to determine combinations resulting in optimal protection as compared to untreated controls.

Not to be overlooked is the equipment that would need to be developed to appropriately administer these anti-AIV vectors. This would require proper engineering and input from poultry owners and farmers to generate a new method or expand on an existing method. The latter would represent a more realistic and feasible approach. There are several ways to apply vaccinations and drugs to poultry in production housing environments. Many productions contain high numbers of birds, so it has become necessary to evolve efficient methods for mass vaccination. Pressured spray apparatus, such as backpack sprayers, are one mechanism to administer live respiratory vaccines (Cobb-Vantress 2013, Jenkins et al 2013). Agricultural sprayers can also be modified to accomplish this spraying technique (Cobb-Vantress 2013). Spray cabinets that administer a defined amount of vaccine to a box containing birds are also often employed (Cobb-Vantress 2013, La Ragione et al 2013, Vermeulen et al 2002). Drinking water is commonly utilized as a route of administration for live respiratory virus vaccines (Vermeulen et al 2002, Cargill 1999). Drinking water administration is accomplished using water pumps or specified water tanks that are mixed with vaccine and drive the vaccine into the drinking water lines (Cobb-Vantress 2013). For both methods, dyes are commonly added to the vaccine cocktail as visible indicators for successful administration (Cobb-Vantress 2013).

After conducting these experimental challenge studies and refining the method of delivery, the next step towards developing and further assessing the value of this anti-AIV technology would include a clinical trial in the form of a randomized field trial. It would be valuable to confirm the safety and evaluate the efficacy of this technology on both layer chicken farms and turkey farms, with broiler farms being of lesser importance simply due to the short lifespan of these production animals. In addition, a field trial could be conducted on free-range backyard flocks, live bird markets, or in areas where poultry are highly domesticated and in close contact with humans. Asia in particular is one of the largest poultry-producing regions in the world. The high density of poultry and their undeveloped practices make it challenging to control an infectious disease like avian influenza. Conducting a field trial in an Asian country like

China or Indonesia could highlight the value of this technology and ultimately lead to significant improvements in controlling the spread of this disease among poultry and reducing the risk for transmission to humans.

After identifying the target population to conduct the study, a field trial would be designed. An optimal target population would be small-scale commercial or backyard layer chickens that characterize the majority of poultry production in a given geographical location, like Indonesia. The location of these farms would need to represent a high-risk area where AIV is considered endemic in poultry. However, because this technology is intended to be used at the onset or during an AIV outbreak, implementation could be a delicate situation. A population of birds that had previously received AIV vaccine would not be excluded from this field trial, as the technology is anticipated to be used in conjunction with the vaccine, not as a replacement. Enrollment into the study would have to include monetary supplementation, should losses incur.

Ideally, a population of high-risk commercial or backyard layer chickens from one farm would be divided into two subgroups. To avoid introducing selection bias into the design, an individual other than the farmer or owner of the flocks would randomly divide these animals into each group. Division would not necessary mean physically dividing these two groups to prevent contact. This detail would be decided depending on the outcome of interest. The farmer and any individuals providing care for these animals would be blinded to the treatment groups. These animals would be of similar age and breed and would be cared for using identical husbandry techniques. An appropriate dose of anti-AIV vector would be administered to one group, while the second group would receive nothing. Any abnormalities and all production measurements (feed/water intake, egg production) would be recorded by a blinded individual. These measurements would be compared to those taken prior to intervention with the vector. Blood would be collected prior to vector administration for serological testing to indicate AIV infection status. Oropharyngeal and cloacal swabs and blood would be collected from all animals in each group at set times (i.e. weekly). Collected samples would need to be properly

stored to subsequently measure shedding titers using RT-qPCR. This field trial would assess anti-AIV vector efficacy by way of reducing the incidence, intensity, and spread of AIV within a flock, but would not assess transmissibility between farms.

To best capture the effect this vector technology could have on preventing AIV transmissibility between farms, an addition field trial would need to be organized. This field trial would need to enroll two different commercial flocks in separate locations, but both representing poultry production in that given geographical location. Like before, chickens on these two farms would be of similar age and breed and would be cared for using similar husbandry techniques. An appropriate dose of anti-AIV vector would be administered to one farm, while the second farm would receive nothing. Oropharyngeal and cloacal swabs and blood would be collected to represent shedding titers and AIV infection status, respectively. This type of field trial would aim to assess whether outbreaks in the area near the vector treated flock differed substantially from those within the control flock area. If this were the case, we would attempt to infer the extent to which this difference was attributable to vector protection.

Unlike vaccine field studies that can use serology to assess protective antibody titers, it would be much more difficult to measure vector protection in the absence of an AIV infection during a known outbreak. Depending on the duration of vector protection and optimal route of administration determined in previous experimental studies, the vector dose would need to be re-administered at set time points (i.e. weekly). Evaluating anti-AIV vector efficacy would require long field trials designed over several months and possibly across seasons to assess whether vector administration feasibly reduces the incidence, intensity, and transmissibility of AIV outbreaks within this population.

2. Addressing the needs of the poultry industry

Beyond demonstrating proof-of-concept for this unique antiviral technology both in the work presented in this dissertation and through future experimental and randomized field trials,

it is equally important to consider the value this technology could provide to the industry and developing countries worldwide. Unlike showing efficacy based on quantifiable measurements, this is a more subjective assessment that can only be truly evaluated while using this technology during an outbreak situation. As is the case with vaccination, the value imparted by this technology might differ depending on the severity of an outbreak, pathogenicity of the virus, target population, and geographic location. However, based on the vector's mechanism of action, the mode of protection would certainly differ from what is available from current vaccines and other methods of control. Theoretically, these novel anti-AIV vectors would offer several advantages over current poultry AIV vaccines and would overcome known hurdles associated with RNAi antiviral techniques *in vivo*. Moreover, from the industry's perspective, several criteria need to be met, all of which I anticipate these proposed anti-AIV vectors could fulfill. The main objective of poultry vaccines is to improve overall production for the producers, because in the end the cost-benefit resulting from vaccination or treatment is the bottom line for this industry. For developing countries, the main objective of vaccination is to maintain the health of the animal to sustain livelihoods through the consumption of poultry and reduce the risk for transmission to humans.

2.1 Universality

LPAI vaccines are based on economic needs. However, available vaccines often lack antigenic similarity to the field strain. A universal vaccine, effective against any AIV subtype or strain would be a great benefit to the industry. These vectors have been engineered to express siRNA specific for highly conserved regions of the vRNP complex genes, NP and PA. The NP and PA siRNA each target short sequences within these two genes with extreme sequence stability observed in all type A influenza viruses (Li et al 2005, Bennink et al 2004, Ge et al 2004, Tompkins et al 2004, Ge et al 2003). Targeting these conserved regions within these genes gives this technology an advantage over traditional vaccines, as it could be used to

prevent all avian influenza viruses, including HPAI and LPAI subtypes. In this way, these vectors would provide universal protection against AIV instead of specific HA and NA subtypes as traditionally applied with the vaccine. The influenza viruses' rapid rate of mutation makes them famous at evading vaccines. If viral mutation reduced the efficacy of the vector, the viral targeting shRNA sequence generated and delivered by the anti-AIV vector could be rapidly changed to counter the mutation, unlike traditional vaccines.

2.2 Surveillance and international trade compatibility

Poultry treated with the anti-AIV vectors would not develop antibodies against AIV. This is typically a limitation associated with vaccination practices because the vaccine stimulates the production of antibodies against the virus. This creates problems related to trade barriers, making it difficult to distinguish antibody reactivity due to vaccination verses natural AIV infection. If an effective conventional vaccine is available, it often cannot be used. A country cannot risk losing AIV disease-free status, which is often the case when positive serological tests due to vaccination interfere with disease surveillance. Unlike vaccinated poultry, those treated with the anti-AIV vectors would not test positive for AIV antibodies, and would not require further testing using the Differentiate Infected from Vaccinated Assay (DIVA). In this way, trade barriers associated with the reactivity of vaccinated animals and additional screening tests for AIV would be avoided.

2.3 Cost effective

The poultry industry will not adopt a commercial vaccine or any other prophylactic without demonstrating that its use is a cost-effective countermeasure to outbreaks of AIV. This is especially critical in low-density poultry areas where the risk for an outbreak is perceived as negligible. Factors that affect the cost of vaccination include shelf life or sustainability of the vaccine, method of administration, ease at which it can be generated, and accessibility.

Generally, the benefit must be higher than the cost of vaccination or treatment to be viewed as a good investment.

One of the limitations associated with other RNAi therapeutic approaches is the cost associated with the commercial manufacturing of the silencing RNA. An obvious advantage of the proposed technology is the ability of the bacteria themselves to produce the shRNA for cleavage into the subsequent siRNA sequences. This characteristic eliminates expensive siRNA manufacturing and high production costs (Xiang et al 2009, Keates et al 2008, Xiang et al 2006, Gardlik et al, 2005). In areas where vaccines are considered necessary, countries will stockpile millions of doses of vaccine each year for emergency preparedness. Many times these vaccines become obsolete when they are found to be distant to the field virus. It is simple and fast to generate large quantities of bacteria. Therefore, unlike a vaccine that can take months to formulate and even longer to acquire in sufficient quantities, these vectors could be readily generated in massive quantities from a small stock. If properly stored, these bacterial vectors could have an indefinite shelf life. This is yet another major advantage over current vaccines.

Vaccination and other RNAi approaches have their share of hurdles related to administration and targeted delivery. Most AIV vaccines must be administered subcutaneously making them cumbersome and inefficient to administer. These anti-AIV vectors allow for targeted respiratory delivery and specific invasion into mucosal epithelial cells expressing β1-integrins. Unlike other tissues and organs, respiratory tissues including the lungs are incredibly accessible, making this anti-AIV technology ideal for preventing respiratory diseases like influenza. Chickens have a gland located medial to the eye called the Harderian gland, and this gland is thought to be critical for developing a local immune response in the upper respiratory tract (Wight et al 1971). For this reason, eye drop vaccinations are believed to stimulate a mucosal immune reaction via the Harderian gland and the same can be said if these vectors were administered into the eye. The anti-AIV vectors could be easily amendable for

administering intranasally, orally, via eye drop, or as a sprayed aerosol suitable for inhalation or ocular delivery.

These vectors would represent a cost effective, sustainable product readily available for large-scale application on a commercial poultry operation. The versatility of delivery and the ease at which it could be amendable for large-scale application gives this technology an advantage over current vaccines and equates to a system that provides efficient delivery, and localization to lung tissues. These qualities make this technology especially advantageous during an outbreak situation to reduce shedding and transmission of virus between poultry populations.

2.4 Efficacy and proven safety

Efficacy is an essential characteristic of an ideal AIV vaccine or alternative prophylactic for poultry. The term efficacious is used to determine if a vaccine or alternative prophylactic is protective in standardized experimental challenge studies, as measured by preventing mortality, morbidity, shedding, and transmission of AIV among poultry. The industry standards for vaccine efficacy with HPAI are reduced morbidity and mortality. Very little morbidity and mortality are observed in LPAI experimentally challenged SPF chickens, making it difficult to determine vaccine efficacy for LPAI isolates. Quantifying oropharyngeal titers from chickens is routinely how efficacy is determined for vaccines against LPAI isolates. Because vaccines currently available for poultry do not provide sterilizing immunity, an effective control tool should be reducing virus shedding to the greatest extent possible (Sylte and Suarez 2012). The results from these initial pilot studies indicate that anti-AIV vector treatment reduces virus replication and shedding titers to levels considered clinically relevant. However, additional studies need to be conducted to further demonstrate the protective nature of this technology.

Vaccination for AIV in poultry is more difficult than vaccination used against most other disease causing viruses. This is owed to large antigenic variations that require prior knowledge

of circulating subtypes, reducing vaccine efficacy. Low vaccine efficacy and high cost to benefit ratios usually offset any proposed vaccination policies. When AIV is detected in poultry, generally a 'stamping out' or culling policy is used in the efforts to eradicate the disease. Assuming efficacy, potency, and environmental safety is demonstrated in a future challenge studies and randomized field trials, I envision this prophylactic technology could be a solution to preemptive culling of poultry in high-risk areas. However, even when vaccination is selected as an emergency tool during outbreaks, it is often in conjunction with other control methods, especially if rapid culling is difficult. Previous experiences have indicated that in order to be successful at controlling and ultimately in eradicating AIV, vaccination programs must be part of a wider control strategy (Capua and Marangon 2006). This includes strict biosecurity measures, surveillance, culling, and possibly implementing the use of this prophylactic technology.

Another issue related to efficacy is the time interval necessary to obtain protective immunity. It requires a minimum of 7 to 10 days to stimulate an initial immune response following vaccination, and another 2 weeks to generate protective antibody levels. This implies that even if the decision-making process is fast-tracked and an appropriate vaccine is available for immediate use, vaccination might not be efficacious if applied during an active outbreak in poultry. A key benefit this anti-AIV vector technology could have over the vaccine is its ability to provide rapid protection. This characteristic would be incredibly advantageous during an outbreak situation.

Proven safety, as measured by low toxicity, absence of unwanted side effects, and limited environmental impact and risk to the consumer, are also essential aspects of an ideal AIV vaccine or alternative prophylactic for poultry. A lack of clinical illness and gross or microscopic pathology associated with delivering these anti-AIV vectors to the respiratory tissues of chickens indicates this technology is not associated with any toxicity and can be safely administered without inducing side effects to the host. As was earlier discussed in Chapter 4, these vectors are not likely to elicit a significant host immune response. Because

these vectors do not integrate into host DNA, they should not pose a tumorigenic threat or pose concerns related to regulatory or animal welfare concerns. Of course, additional studies are needed to further validate these observations and assumptions. Risk to the environment, humans, and other animals consuming the eggs or meat from chickens treated with these vectors would need to be assessed. That said, I would hypothesize these vectors would be of low risk for several reasons. These bacterial vectors are non-pathogenic and unable to survive outside of a supplemented environment. This is due to the inactivation of the Dap gene on these bacterial vectors, suggesting that transmission via shedding into the environment or between animals will not occur. Even if transmission did occur, the Dap amino acid is not present in sufficient amounts in mammals, birds, or in the environment to support bacterial survival (Buttaro and Fruehauf 2010, McCoy et al 2006, Burns-Keliher et al 1998, Harb et al 1998). These bacteria are non-conjugative and have a confirmed safety record for clinical purposes (Grillot-Courvalin et al 2011, Buttaro and Fruehauf 2010, Li et al 2006, Xiang et al 2006, Isberg and Leong 1990). Furthermore, the tkRNAi system is currently being tested in clinical trials for prevention or treatment of human disease (Nguyen et al 2008, Xiang et al 2006), including clinical testing for the treatment of Familial Adenomatous Polyposis.

3. Targeting host-viral interactions for broad spectrum protection

Recent studies and scientific groups have exposed the potential that RNAi holds for developing influenza-resistant transgenic animals and alternative techniques to preventing infection. Besides limitations due to high cost, poor delivery, and unwanted side-effects to the host, an area of research in this anti-influenza field of RNAi is lacking. Previous studies report reduction in influenza infection and replication via siRNA mediated knockdown or from transgenic alterations that target viral genes (Lyall et al 2011, Zhou et al 2007, Li et al 2005, Tompkins et al 2004, Ge et al 2003). We have yet to consider the power of targeting host genes. The influenza virus transcribes and replicates its RNA genome within the nucleus of the

host cell and requires a functional vRNP complex comprised NP, PA, PB1, and PB2 (Nagata et al 2008, Deng et al 2006, Elton et al 2001). However, the influenza virus also hijacks host genes necessary for virus replication has developed sophisticated ways to utilize the host cell's machinery to transcribe and replicate its RNA. Inevitably, complex interplay between viral and cellular components occurs. These host factors include those with pivotal roles in influenza virus replication, for example, nuclear export and import factors, vacuolar ATPases, transcription factors and those with chaperone-like activity for the viral proteins.

Using genome-wide RNAi screens for host cellular factors, researchers have compiled lists of genes that are required for influenza virus infection (König et al 2010, Hao et al 2008, Nagata et al 2008, Wang et al 2008, Mayer et al 2007, Kash et al 2006). Many of the host genes required for both human and avian influenza infection in mammals have been identified, but have yet to be evaluated for their application in inhibiting influenza infection with RNAi. Lack of such knowledge presents a barrier to improving control methods for AIV in poultry and humans. Understanding the roles these host factors play in influenza virus infection would provide new insight into key host-viral interactions and new opportunities for the development of host factor directed antiviral therapies, including integration into the anti-AIV vector technology.

Developing an intervention that targets host genes offers an advantage over viral genes because it limits the virus's ability to escape silencing by mutation. This might seem unlikely due to the high level of sequence homology observed in these short siRNA target regions among type A influenza viruses, indicating these sequences are 'safe' targets. Using the current approach and targeting only viral genes, I must recognize the possibility that these viral siRNAs could become ineffective over time due to viral mutations at these siRNA recognition sites. Targeting host genes offers an advantage over existing RNAi approaches because it limits the generation of viral escape mutants due to selective pressure. To greatly reduce the possibility of viral escape by gene mutation, future work should aim to improve the current anti-AIV vector technology by developing an approach that targets both host and viral factors. These anti-AIV

vector cocktails would provide universal as well as broad-spectrum coverage for maximum protection against avian influenza virus.

4. Future direction and final remarks

Since 2012, more than 20 new veterinary vaccine products have been introduced worldwide. A growing global market for animal vaccines means there is huge potential for growth in this arena. Compared to human medicine, vaccines developed for veterinary use typically go through less stringent regulatory and preclinical trial requirements (Meeusen et al 2007). This means experimental infections, safety trials, and dose-response studies can be completed faster in relevant animal models, rather than less appropriate rodent studies. Successful translation into a usable product is reliant on the ability to execute good basic science, incorporate relevant industry or local requirements, appreciate global perspectives and the impact of a specific disease in a target population, and the knowhow to address a need. However, one of the most important factors that determine the longevity of an experimental vaccine for animals is successful commercialization. Successful commercialization is contingent on cost, quality, uniformity, and performance in the field (Meeusen et al 2007).

Commercialization is driven by a need. Where to commercialize a vaccine as part of an AIV control strategy in poultry depends largely on knowledge related to the characteristics of the particular virus and its epidemiology. Representing one of four H5N1 HPAI enzootic countries and the world's largest AIV vaccine user, China will play a significant role in the commercialization of new vaccine technologies. Good technologies that have the lowest cost to manufacture will dominate the market. Fortunately, recent advances in biotechnology have made possible the development and commercialization of new biologicals, especially category II and III products. These include products containing live modified microorganisms carrying plasmid DNA and live vectors carrying foreign genes (OIE Terrestrial Manual 2012). These products, like the anti-AIV vectors, represent a new and innovative approach to vaccine and

antiviral development. There are caveats, as guidelines for the development, production, characterization, and control of these novel products are still preliminary and fluid. Evolving data and new knowledge may present additional unforeseen hurdles.

Preventative vaccination or use of a prophylactic antiviral is based on economic need and the severity of economic consequences that could result from an AIV outbreak. Industrial poultry operations are usually at lower risk for outbreaks due to higher biosecurity practices. In contrast, backyard poultry, especially in low-resource rural settings, are at much higher risk for outbreaks. Avian influenza in developing countries is one of the biggest threats to rural poultry and sustaining human livelihoods, especially when compensation due to loss is not possible. A technology that works rapidly and is cheap and convenient for poor farmers to transport, store, and use holds the key to enabling much of the world's poor to protect their village poultry against AIV. Developing the anti-AIV vectors into a practical and valuable technology could represent a high-impact, low-cost solution in areas where poultry are a lifeline.

In my opinion, translating this technology for use in remote rural settings should be a priority. This scenario might not be the most lucrative, but could have the biggest impact on the success and acceptance of this 'out of the box' approach. Remember, the single most important factor that will determine the longevity of an experimental vaccine or drug is successful commercialization, and successful commercialization is dependent on field performance. If this anti-AIV vector technology proved to be a viable solution, effective at curtailing AIV outbreaks in high-risk areas and in low-income countries, it certainly would increase the likelihood of licensure to commercial poultry industries in developed countries.

I believe that reducing the spread and circulation of AIV will also decrease the zoonotic impact of these viruses on human and other animal populations. The future intent for this technology is to be used in the face of an outbreak to reduce the shedding and transmission of virus within and between poultry flocks. This would not only help reduce the burden of this economically important disease of poultry worldwide, but would reduce the risk for spread to

humans due to direct bird handling practices. Ultimately, this reduces the risk for a human pandemic. In this way, this preventive technology could directly address and improve poultry health worldwide, and have a significant impact on the public health programs in the US and around the world. The economic benefit of preventing or at minimum reducing the annual incidence of human influenza is so enormous it is superfluous to argue the case. In the future, I envision this technology could represent a transformative approach to preventing and controlling human influenza with great potential to have a sustained and significant impact on human disease.

In David Swayne's book *Avian Influenza,* the final chapter is dedicated to a global strategy to control HPAI (Swayne 2008). A section on 'applied research' is part of the proposed implementation strategy. Under this section, the authors outline several main aspects necessary to control AIV, one of which is to develop novel technologies to enhance vaccine efficacy and delivery. One could expand upon this point and conclude that developing novel technologies means changing mainstream perspectives on preventative medicine. Could this unconventional anti-AIV vector technology represent a viable alternative? In my opinion, the outlook appears promising. I would like to close with a quote by the famous science philosopher, Thomas Kuhn, who wrote in his book, *The Structure of Scientific Revolutions*, 1962:

> "The scientific enterprise as a whole does from time to time prove useful, open up new territory, display order, and test long-accepted belief. Nevertheless, the individual engaged on a normal research problem is almost never doing any one of these things. Once engaged, [her] motivation is of a rather different sort. What then challenges [her] is the conviction that, if only [she] is skillful enough, [she] will succeed in solving a puzzle that no one before has solved or solved so well."
>
> *T.S. Kuhn (1962:38)*

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