UNDERSTANDING HOST PROTEASE GENE INVOLVEMENT DURING INFLUENZA VIRUS REPLICATION AS A POTENTIAL DISEASE INTERVENTION STRATEGY

by

VICTORIA A. MELIOPOULOS

(Under the Direction of Ralph A. Tripp)

ABSTRACT

Influenza A virus causes seasonal epidemics and periodic pandemics threatening the health of millions of people each year. Vaccination is an effective strategy for reducing morbidity and mortality, and in the absence of drug resistance, the efficacy of chemoprophylaxis is comparable to that of vaccines. However, the rapid emergence of drug resistance has emphasized the need for new drug targets. Knowledge of the host cell components required for influenza replication has been an area targeted for disease intervention. We screened human protease genes for those involved in influenza virus replication, and validated five human protease genes using RNA interference approaches. Pathway analysis determined three global cellular pathways governing inflammation, cAMP/calcium signaling, and apoptosis, in which the five protease genes were involved. Analyses of host microRNAs predicted to govern expression of these genes showed that eight miRNAs regulated gene expression during virus replication. We also investigated the role of the protease gene, TMPRSS2, known to cleave and activate influenza

hemagglutinin in influenza cell-to-cell spread. Together, these findings identify unique host genes and microRNAs important for influenza replication providing potential new targets for disease intervention strategies.

INDEX WORDS: influenza, RNA interference, protease, TMPRSS2, siRNA screen, miRNA

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DEDICATION

This work is dedicated to my husband, Daniel Taylor, for his support and infinite patience during this endeavor.

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CHAPTER 1

INTRODUCTION

Influenza continues to be responsible for widespread respiratory disease and morbidity, mortality, and economic loss despite worldwide vaccination and eradication programs (22, 29, 32, 43). This is of particular concern in view of recent outbreaks of avian and swine influenza in humans, and in particular, the novel H1N1 “swine flu” pandemic that began in the summer of 2009. Seasonal vaccines are generally available for circulating influenza strains, however influenza is constantly undergoing antigenic drift and periodic shift (2, 3, 16) which can render vaccines less or ineffective (14, 16, 30). In addition to affecting vaccine efficacy, antigenic drift and shift has also resulted in increasing resistance to antiviral drugs such as adamantanes and neuraminidase inhibitors (2, 4, 11, 18, 26, 27, 31).

Influenza viruses are a member of the *Orthomyxoviridae* family, and possess a negative-sense, single-stranded, RNA genome of eight segments (34). These eight RNA segments encode individual specific viral proteins, and because of this simplicity, the virus must utilize many features of the host cell during the viral infection cycle (35, 37, 38). Therefore, adaptation to the host cell system is the most important issue for influenza infection and transmission (1, 20, 25). Although several factors which relate to host cell adaptation by influenza are known, e.g. the preference for sialic acid moieties (12, 25, 28)

and expression of endogenous host cell proteases (33, 36, 41), the elucidation of the host cell mechanisms co-opted by the virus for replication and transmission remains a challenging research area due to the sheer number of host cell genes and pathways that can be affected.

Recent advances in our understanding of RNA interference (RNAi) have provided a means to perform genome-wide screens to determine and validate host cell genes that may be required for replication and transmission of influenza (8, 17, 19, 23, 40). RNAi is an efficient mechanism for the sequence-specific inhibition of gene expression, and is mediated by small interfering RNAs (siRNA), generally processed from double-stranded RNA molecules by the nuclease Dicer, and incorporated into the RNA-induced silencing complex (RISC) where the antisense or guiding strand of the siRNA can direct degradation of messenger RNAs that contain homologous sequences (15, 21, 46). Synthetic siRNAs are now frequently used to target viral and host genes and have been successfully applied as therapeutic disease intervention strategies in animals and humans (13, 44).

Although there have been many studies showing successful treatment of influenza using siRNAs targeting viral genes (44, 48), targeting host genes affecting viral replication is an attractive option (23). Protease expression in the respiratory tract is a major determinant of influenza virulence and tissue tropism (33, 36, 41). Little is known about the host cell proteases that contribute to or are required for influenza replication and the contribution of these proteases in the human respiratory tract is still not well characterized. A better understanding of host cell proteases is required for understanding

the biology of influenza replication, to determine mechanisms of pathogenicity and tissue tropism, and for developing disease intervention strategies.

Post-transcriptional regulation of genes may affect influenza replication. A recently identified mechanism governing gene expression is endogenously expressed micro (mi)RNAs in the host cell, whose expression can be modified during viral infection (10, 47). miRNAs are synthesized in the host cell nucleus as small hairpin precursors, which are exported to the cytoplasm before entering the RISC pathway similar to siRNAs (21, 39). miRNAs may have multiple targets due a tendency to bind to their mRNA targets with imperfect sequence homology, and thus one miRNA may inhibit multiple gene expression at either the transcriptional or the translational level (9, 21). miRNAs are beginning to be explored as a novel therapeutic option (24, 42), so understanding host miRNA expression during influenza infection can be invaluable.

The central hypothesis of this study is that genome-wide small interfering RNA (siRNA) libraries that target individual genes in the host protease library can be used to identify gene knockdown events that increase or inhibit production of viral progeny. The study includes the following specific aims:

Specific aim 1. To identify human protease genes involved in influenza replication using a genome-wide siRNA library screen targeting the human protease library, and to determine whether silencing of these genes results in increase or decrease of influenza infection levels. The *working hypothesis* is that silencing of human protease genes by siRNA will either reduce/eliminate influenza replication in the case of genes essential for virus replication, or substantially increase influenza replication in the case of antiviral

genes. Since influenza must use host cell machinery to replicate within the cell, certain host genes, presumably including protease genes, are important to facilitate this process.

Specific aim 2. To determine the global cellular pathways involved in influenza replication as they relate to the genes identified in aim 1. The *working hypothesis* is that the protease genes identified in aim 1 are involved in global cellular pathways and silencing of the hit protease genes will have an inhibitory or enhancing effect on these signaling pathways. After a stringent validation process by a novel siRNA targeting a different seed site on the target gene to confirm the phenotype seen in the primary screen, the genes are subject to a universal pathway analysis to determine global host cell pathways in which the protease genes play a role. The benefits from this analysis are twofold: since influenza must co-opt cellular pathways to successfully infect and replicate, linking the hit protease genes to established pathways can further understanding of the viral replication process; in addition, global cellular pathways may already have established drug targets and a previously unused therapy may be harnessed to control influenza infection.

Specific aim 3. To understand post-transcriptional regulation of the protease genes by miRNAs that were predicted to interact with the genes and cellular pathways identified by aims 1 and 2. The *working hypothesis* is that inhibition of specific miRNAs predicted by the pathway analysis will have an effect not only on protease mRNA levels and gene expression, but also on influenza replication. Understanding modulation of host gene expression by miRNAs in the context of influenza infection may open a new avenue to miRNA-based therapeutics.

An additional goal of this research was to further characterize the mechanism of a human protease gene, TMPRSS2 (transmembrane protease, serine 2), known to affect influenza infection by its ability to cleave and activate the HA protein (5-7). TMPRSS2 is expressed on the surface of a variety of cells, including kidney, liver, prostate, and lung tissue (6, 28, 45). However, HA cleavage mediated by TMPRSS2 occurs only inside the cell (7), suggesting that incoming virions will not be cleaved and activated by TMPRSS2. Based on the decrease of viral titers in cells where TMPRSS2 was inhibited (7), the final specific aim was examined:

Specific aim 4. To determine if silencing of TMPRSS2 by RNAi affects cell-to-cell spread during influenza infection. The *working hypothesis* is that silencing of TMPRSS2 in alveolar epithelial cells will decrease the ability of the influenza virus to spread easily to adjacent cells during infection.

The evaluation of these specific aims will allow better understanding of the mechanism of influenza infection in the context of human protease gene expression. The identification of protease genes essential for influenza replication can provide novel drug targets, and the determination of global cellular pathways affected by those protease genes may lead to the application of previously established therapeutics targeting those pathways to the treatment of influenza. miRNAs regulating expression of host protease genes may also lead to the development of novel influenza therapeutics. Understanding the mechanism of inhibition of the host protease gene TMPRSS2 can also deepen understanding of virus biology. Taken together, this research may lead to supplementary therapies to influenza infection that are not as deeply affected by viral mutations as the current vaccine and antiviral drugs.

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CHAPTER 2

LITERATURE REVIEW

Overview of influenza virus

Many emerging infectious diseases (EIDs) in humans are of zoonotic origin, deriving from animals or animal products. EIDs are broadly defined to include 1) new agents, 2) existing yet previously undetected agents, 3) the re-emergence of known agents, and/or 4) expansion of a known agent into a new geographic range (72). Influenza viruses are well-documented examples of EIDs. In 2009, the H1N1 pandemic virus erupted from the swine population and quickly spread to over 200 countries overtaking pre-existing H1N1 and H3N2 viruses in the human population and became the dominant circulating strain (295). Similarly, outbreaks of highly pathogenic H5N1 influenza viruses continue to emerge and may show up to 60% mortality rates in humans (155). These outbreaks highlight the persistent and devastating nature of influenza infections, and increase the risk of new pandemics.

Despite scientific advancements over the last three decades, EIDs continue to cause substantial social and economic costs. For example, influenza is a leading cause of morbidity and mortality in the world (322) with seasonal viruses affecting up to 15% of the human population, causing severe illness in 3 – 5 million people and fatality of approximately 500,000 individuals per year (323). Secondary complications such as

bacterial pneumonia can further increase mortality rates (203, 296). Coupled with this is the economic burden that is associated with widespread influenza infection. In the United States alone, financial losses resulting from seasonal influenza infection are estimated to exceed 87 billion dollars annually (213).

The ability of influenza viruses to continuously mutate has made vaccine control strategies difficult. Simultaneously, resistance to antivirals is increasing. For these reasons, new strategies to defeat EIDs such as influenza are required and an expanded knowledge of host-virus interactions is a crucial first step. Despite the complexity of influenza biology, viruses of this class contain only 8 gene segments (219), and therefore lack the full complement of proteins required for the production of infectious virus. Influenza, like all viruses, must co-opt a wide array of host proteins, non-coding RNAs, and cellular processes (for example, vesicle transport) to generate infectious viral particles. Understanding the host contribution to viral replication and immune evasion is essential for discovering new therapeutic strategies. Genome screening technologies utilizing RNA interference (RNAi), together with bioinformatics provide the ability to rapidly identify the complement of essential host functions and pathways that are essential to the virus.

Influenza Reservoirs

Influenza virus exists in several natural reservoirs, namely various species of aquatic birds and in swine. It manifests in avian species primarily as a gastrointestinal disease and is spread through fecal-oral contact (45). Proteolytic cleavage of HA0 is indispensable for influenza virus infectivity and tropism (283). It is thought that

ubiquitous tissue expression of cellular proteases such as transmembrane serine proteases (TMPRSS2, TMPRSS4, TMPRSS13), human airway tryptase (HAT), and matrix metalloproteinases (MMPs) necessary to cleave HA0 impact viral tropism and pathogenicity (10, 25, 54, 238, 317). In addition, the binding preference of the HA molecules to surface sialic acid moieties is thought to explain host preference and host cell tropism. In general terms, influenza virus from humans has been shown to preferentially bind α(2,6) sialic acid linkages, whereas those from avian and equine origin prefer α(2,3) linkages (70, 162, 205), while those from swine bind to both (225). However, recent evidence indicates that this HA-sialic acid partitioning may not be obligatory (66, 132, 200, 227, 241).

Besides the species barrier, there are internal barriers to successful influenza infection within the host. First, the virus must successfully cross the respiratory epithelium, crossing a layer of mucus, avoiding alveolar macrophages, and penetrating the epithelial cell layer (163). Some mucins can even bind glycoproteins on the virus itself (239). The viral neuraminidase has been hypothesized to reduce mucus viscosity by cleaving sialic acids from the mucin and allowing the virus to reach the epithelium (51, 180). Once inside the cell, the virus must be equipped to harness cellular pathways to replicate. The virus must also avoid the immune response of the host, particularly type I interferons early in infection (11). Tissue restriction is also important, as a cell type that does not express viral receptors cannot be infected. For influenza virus to replicate systemically, it must leave the respiratory tissue and get into the blood, and be transported to tissue where it can perpetuate infection (282).

Global Impact

Influenza virus can undergo various mutations during viral replication. The concept of viral quasispecies is based on the high error rate of RNA viruses and subsequent high variability in their genomes (75). Influenza virus has an error-prone polymerase, and like other RNA viruses it exists very close to the error threshold (3, 17). As the influenza virus replicates in a cell, it is constantly exposed to selection pressures of the particular host cell it has infected, which results in the creation of progeny viruses that vary from the infecting population due to selection pressures. Examples of selection pressures include antibody and cellular immunity, whether or not the host has been vaccinated, host cell defense such as interferon, and cellular transcription and translation entities for modification or inhibition (21, 75). These variable sequences, however, cluster around what is called the consensus sequence, which is the 'average' of the known variable sequences (267). The advantage to an RNA virus such as influenza for existing close to a mutation error threshold is rapid adaptation to host conditions and an overall increase in viral fitness, and this is part of the reason why some viruses are so easily able to persist in host cells and tissues (3). Antigenic drift is the accumulation of single nucleotide mutations over time (1). Some of these mutations can be silent, or others can cause mutations of single amino acids. In this way the virus can evade the immune system and reinfect the population over time by the introduction of new viral epitopes to the immune system.

Influenza viruses can undergo antigenic shift, due to the rearrangement of viral gene segments in cells simultaneously infected with multiple influenza viruses.

Occasionally, a pandemic will arise due to viral reassortment, or antigenic shift. The 1918 Spanish influenza (H1N1) pandemic caused 20 – 50 million deaths worldwide (54). The 1957 H2N2 pandemic was caused by a reassortant virus containing the HA, NA, and PB1 from an avian strain in a background with human tropism, and the 1968 H3N2 pandemic was from reassortment of an avian HA and PB1 into a circulating human strain (145, 268). The highly pathogenic H5N1 currently circulating among the human population in Asia was caused by several reassortant events in avian strains (176). The recent 2009 H1N1 influenza pandemic, while not as severe as originally projected, was caused by reassortment of classical swine influenza with the North American triple reassortant (human/avian/swine) influenza strain (97).

Influenza Detection

Influenza was originally postulated to be caused by a bacterium, *Haemophilus influenzae*, however in 1933 Richard Shope successfully identified the influenza virus by infecting pigs with swine mucus samples that had been filtered to remove bacteria (310). Influenza virus was first grown in eggs in the laboratory setting (50), but can now be grown in tissue culture, most often in Madin-Darby canine kidney (MDCK) cells, which are the gold standard for influenza assays (104). Growing virus in eggs is best for generation of large quantities of virus, and the influenza vaccine is made this way (89).

There are a variety of established laboratory methods for quantification and culture of influenza virus. Viral replication in eggs is determined by the ability of the infected allantoic fluid to agglutinate red blood cells at the maximum dilution, called a hemagglutination assay (HA) (1, 328). The hemagglutination inhibition test (HAI) detects

subtype-specific antibodies against HA, prohibiting agglutination of red blood cells (289).

Plaque assays are based on the virus's ability to cause cytopathic effects. Based on the number of plaques formed over the course of an infection at a particular dilution, the virus can be quantified as plaque forming units per ml (199). Another assay to measure infectious virus is the 50% tissue culture infectious dose ($TCID_{50}$). This assay is also quantified based on viral dilutions, but calculated as the amount of virus that kills 50% of infected cells, normally MDCK cells (258). Both plaque assays and $TCID_{50}$ assays determine the amount of infectious virus produced, as in order for the assays to work the virus must spread from cell to cell (plaque assay) or successfully infect a cell monolayer ($TCID_{50}$). For most influenza viruses grown in cell-based assays, the addition of exogenous trypsin to cleave and activate the HA protein is required (283).

PCR can be used to detect the presence of virus in tissues. This involves targeting the viral genes with specific primers to determine whether gene copies by viral RNA are present in the sample. Although any influenza gene can be targeted, popular choices are HA which is useful for viral subtyping, and the M gene that is a conserved target and offers broad specificity (284). This method is useful both for determining presence of virus and quantifying genomic replication. Another useful technique to detect influenza virus is by immunofluorescent staining. An infected cell monolayer can be fixed and permeabilized, followed by incubation with influenza-specific antibodies conjugated to fluorescent tags. In this way, the virus localization within the cell can be visualized quite easily.

Influenza Virus Replication

The influenza virus life cycle can be divided into the following stages: 1) entry into the host cell; 2) entry of vRNPs into the nucleus; 3) transcription and replication of the viral genome; 4) export of the vRNPs from the nucleus; and 5) assembly and budding at the host cell plasma membrane. The HA protein is a homotrimer that forms spikes on the viral lipid membrane. HA binds to sialic acid moieties found on the surface of the host cell's membrane (1, 287). The HA precursor, HA0, is made up of two subunits: HA1, which contains the receptor binding domain, and HA2, which contains the fusion peptide (34, 35, 140).

The HA protein is the major antigenic protein of the virus, and it mediates attachment to the host cell membrane through sialic acid moieties on the host cell membrane and entry, although recent studies suggest NA may also play a role (235). The virus is then encapsulated in at least one of four ways: either by 1) clathrin-coated pits, 2) caveolae, 3) a non-clathrin or non-caveolae mediated pathway dependent on the low pH in the late endosome, or 4) macropinocytosis (1, 69, 235). The virus then fuses with the endosomal membrane and structural changes in HA occur due to the acidic pH of the endosome (166). HA is cleaved into HA1 and HA2, which remain associated by disulfide bonds, activating the protein (34, 35, 274). Only the cleaved form of HA is active, and if the HA0 precursor is not cleaved, the protein is nonfunctional and progeny virus cannot enter host cells to perpetuate infection (35, 148), since the fusion peptide located on HA is exposed by this cleavage (1). Thus, efforts are underway to identify host cell HA-processing proteases.

At the same time, M2, the ion channel protein, allows an influx of H^+ atoms from the endosome to the virus particle, essentially disrupting protein-protein interaction and allowing a pore to open and the viral RNP to be released into the host cell cytoplasm (1, 140). vRNP complexes are then actively transported to the nucleus by host cell nucleotrafficking machinery. This can be easily accomplished because all vRNP proteins contain a nuclear localization signal (NLS) that allows the RNP to be targeted for nuclear uptake. Cellular importin-α binds the RNP directly at the NLS, followed by importin-β binding which allows association of the vRNP with a nuclear pore (1). Inside the nucleus, the viral RNA (vRNA) is transcribed to complementary RNA (cRNA) and message RNA (mRNA) for protein synthesis and genome replication respectively. Transcription of viral RNA to mRNA occurs via a primer dependent mechanism. During transcription to mRNA, viral mRNA is capped and polyadenylated, and the mRNA is then used as a template (161). Two processes can occur using the template mRNA: translation of the mRNA into viral proteins to package and produce progeny virus, and replication of the mRNA where a full length positive-sense copy is made (cRNA) which is then used as a template to make more vRNA (219). Replication is controlled by influenza's polymerase complex, consisting of PA, PB1, and PB2, which can interact with the large subunit of RNA polymerase II (Pol II) of the host (39). Each unit of the viral polymerase has its own function. PB1 sequentially adds nucleotides to the viral RNA (161). It binds to the ends of vRNA and the cRNA complementary copy to initiate both transcription and replication (100, 101). It also contains endonuclease activity necessary for generation of a capped primer for mRNA synthesis. PB2 is important for 'cap-snatching': influenza mRNAs cannot be translated without a cap, and PB2 binds the cap on host mRNA and 'snatches'

it by forming an aromatic sandwich of amino acids around the cap (1). Each vRNA has a promoter of noncoding flanking sequences at both its 5' and 3' end. Viral mRNAs also require a polyA tail for translation, and this is done using the cellular Pol II (178). Two of the vRNAs, the M segment and the NS segment, must be regulated through cellular splicing machinery to produce two different viral proteins each. However, splicing of viral mRNA is not nearly as efficient as cellular mRNA, since proteins are expressed from both the spliced and unspliced mRNAs (1). cRNA does not require capping or polyadenylation for replication and can be transcribed to vRNA. However, cRNA must be encapsidated by NP to stabilize the cRNA, or the cRNA is degraded (1, 316). NP therefore has a role in control simply by its presence, whether the viral mRNA is fated to become genomic RNA or to be translated into protein, as do the structural modifications to the mRNA such as capping and the addition of the poly-A tail. NF-κB is also reported to be involved in regulation of vRNA synthesis, but the exact mechanism is not well understood (165).

The virus itself can exert both transcriptional and translational regulation (8, 172, 249). An example of transcriptional regulation is the C4-containing promoter in the polymerase segments. The C4 promoter is associated with down-regulation of transcription and upregulation of replication. Simply put, there is a 'C' instead of a 'U' located at the fourth position of the vRNA promoter from the 3' end. Translation control includes the degradation of host mRNAs caused by cap-snatching (since the presence of the cap serves to stabilize an mRNA), inhibition of host mRNA processes by monopolizing machinery such as Pol II for the virus's own purposes, and preferential translation of viral mRNAs over host mRNAs (24, 92, 134, 143, 144). The virus also

regulates translation of particular viral proteins temporally (1). For example, NP is produced early on in the process since NP is needed to stabilize the viral cRNA and is thus essential for replication. NS1 is also produced early in infection because it is required to modulate the host antiviral response (119), helps to inhibit host mRNA processing and translation (208), and suppresses host RNAi used as antiviral defense in an RNA-binding dependent mechanism (114, 201). HA, NA, and M1 proteins, since they are not involved in actual translation and replication, are not produced until later when viral packaging needs to occur.

After replication is completed the NEP/NS2 protein together with help from M1 exports the vRNPs from the cell nucleus. This occurs by association of the vRNP with M1 to form an RNP complex, and NEP/NS2 serves to bridge interaction between the cellular export machinery and M1 (224, 233). Late expression of M1 discussed above ensures that export will not occur until viral replication is complete. The newly translated viral proteins M2, HA, and NA enter the cell's endoplasmic reticulum (ER) for glycosylation and folding (76, 294, 307). The HA protein is then cleaved and activated, and the three proteins are then directed to the apical membrane for packaging and budding. HA, NA, and M2 associate with lipid rafts on the cell membrane, and that particular area pushes out until the inner core containing all genome material and the required viral proteins is enveloped (261). Finally, the viral particle buds from the host cell nucleus and is released by cleavage of sialic acid moieties by the NA protein, and the cycle begins anew (1).

Pathogenesis and Host Immune Response to Influenza Virus

Symptoms of influenza infection can range from asymptomatic to viral pneumonia leading to fatality. However, influenza infection most typically presents in adults as an upper respiratory infection including nasal obstruction, cough, body aches, fever, and sneezing (1). Children may suffer from high fevers as well that increase the risk for seizure (256). Children can also develop otitis media, croup, and pneumonia as secondary complications (243). If the infection is severe it can spread to the lower respiratory tract where it becomes more serious. This is common with young children, the elderly population, and the immune compromised, where primary viral pneumonia, combined viral-bacterial pneumonia, or secondary bacterial pneumonia can develop (1). Rarely, but in the most severe cases, cardiac involvement can lead to death by myocarditis (49) or CNS involvement can cause a variety of neurological symptoms (117).

Influenza. The influenza A virus is an enveloped virus with a negative sense, single-stranded, segmented RNA genome belonging to the family *Orthomyxoviridae*. The virus is pleomorphic and can exist either as a sphere or as a long filamentous structure (215). Although the M1 protein is mainly responsible for the determination of viral morphology (37, 83), recent evidence suggests that cellular trafficking factors such as Rab11 may also play a role (46). Influenza A viruses have eight segments that encode for either 10 or 11 viral genes: hemagglutinin (HA), neuraminidase (NA), matrix 1 (M1), matrix 2 (M2), nucleoprotein (NP), non-structural protein 1 (NSP1), non-structural protein 2 (NS2; also known as nuclear export protein, NEP), polymerase acidic protein (PA), polymerase basic protein 1 (PB1), polymerase basic protein 2 (PB2) and in some cases the polymerase basic protein 1-F2 (PB1-F2) (1, 56, 219). The viral envelope is

made up of a lipid bilayer that contains three of the viral transmembrane proteins: HA, NA, and M2. This lipid bilayer is derived from the host's plasma membrane and is known to contain both cholesterol-enriched lipid rafts and non-raft lipids (1, 16, 285). HA is the most abundant envelope protein, followed by NA, and M2 is a very minor component of the viral envelope. HA and NA are exclusively associated with the lipid rafts in the viral lipid membrane, whereas M2 is not (16). Underneath the viral lipid membrane is M1, which forms a matrix holding the viral ribonucleoproteins (vRNPs). These vRNPs are the core of the virus and are made up of the viral negative stranded RNAs, which are wrapped around NP and very small amounts of NEP. At one end of the vRNPs are the three polymerase proteins (PB1, PB2 and PA) that make up the viral RNA polymerase complex (219). Influenza A viruses have the highest impact on public health and are the focus of this research.

Influenza B. The influenza B strains can cause mild illness in humans and an influenza B strain is included in the trivalent influenza vaccine (204). The disease caused by influenza B is similar to influenza A, ranging from mild to serious (60, 99). Like influenza A, influenza B viruses include eight gene segments, however they have four proteins expressed on their lipid envelopes: HA, NA, NB, and BM2 (27, 43, 234). Influenza B infection is primarily restricted to humans (248).

Influenza C. The influenza C strain is substantially different from influenzas type A and B. It only has seven gene segments and a different surface structure: hexagonal netlike structures (7) and very long cordlike structures on the host cell surface (218, 229). The major glycoprotein, HEF (hemagglutinin-esterase-fusion), of influenza C is also slightly different in structure and function in that it serves as both an HA and an NA

protein (220, 221, 246). Although influenza C can infect both humans and swine (111), it rarely causes serious disease in humans (214). Most people have protective antibodies to influenza C by the time they are adults (232).

Immune response to influenza A. The host immune response to influenza A is multifaceted. Once the virus enters the host cell, the innate immune response is activated. Toll-like receptors (TLR) such as TLR3 and TLR7 are stimulated by double-stranded (ds)RNA (from a viral replication intermediate) and single-stranded (ss)RNA, respectively. TLR3 is expressed on respiratory epithelial cells and TLR7 on dendritic cells, some of the first responders of the immune system (1). Activation of TLR3 induces type I interferon production through the IRF3 activation (109), while TLR7 stimulates induction of proinflammatory cytokines IL-6 and IFN-α, corresponding with clinical symptoms, while IL-8 is induced later (13, 118, 192). The type I interferon response and induction of the antiviral state is very important during influenza infection, as the NS1 protein is known to be a type I interferon antagonist (95, 114). Mutant influenza viruses where the NS1 protein has been deleted are highly attenuated (96).

Humoral immunity is very important for protection against subsequent infection with influenza virus. Serum antibodies play an important role in this protection. During infection, antibodies are made to the HA, NA, NP, and M proteins. The presence of HA and NA serum antibodies is associated with resistance to and protection from infection (61). Antibodies to HA are neutralizing (313) while NA antibodies prevent the NA protein from cleaving sialic acid receptors and releasing progeny virus, in effect limiting the spread of infection (1). Antibodies can also aid in viral clearance (265) and may contribute to heterosubtypic immunity (226). However, antibodies to HA and NA do not

offer protection against antigenically different strains, so reinfection occurs as the circulating strains of influenza virus change (1). Additionally, neutralizing mucosal antibodies in the form of IgA are found in nasal secretions (90).

Finally, cell mediated immunity also plays a role in influenza infection and pathogenesis. $CD8^+$ T cells (cytotoxic T lymphocytes, CTLs) are known to mediate viral clearance by lysing infected cells (333), and CTLs recognize MHC I molecules expressing epitopes to HA, M, NP, and PB2 (87). Because of their ability to recognize epitopes of the more conserved internal viral antigens, CTLs often have cross-reactive specificity. $CD4^+$ T cells, however, can also aid in clearance, as shown by $CD8^+$ deficient mice that can still clear influenza virus (81). $CD4^+$ T cells can also augment the antibody response of B cells secreting anti-HA antibodies (1), thus increasing neutralizing antibody titer and therefore protection from influenza virus.

Virulence Factors and Cellular Networks Involved in the Influenza Virus Life Cycle

HA. Proteases expressed in lung tissue facilitate influenza infection and spread of the virus. For example, human influenza strains typically include a monobasic cleavage site and depend upon HA processing and activation by endogenous cellular trypsin-like proteases (238). Extracellular proteases that can cleave influenza HA protein include tryptase Clara, plasmin/plasminogen, human airway tryptase (HAT), type II transmembrane serine proteases, and some bacterial proteases (88). Highly pathogenic avian influenza strains, however, contain a polybasic HA cleavage site that can be cleaved by ubiquitously expressed proteases, such as the endoproteases furin and PC6, which allows infection to become systemic and therefore more dangerous (216, 286).

PB2. The PB2 protein has also been recognized as a virulence determinant. Studies have shown that avian influenza viruses are unable to replicate in mice due to amino acid 627 in the PB2 protein; however when this residue is mutated, the virus can replicate (11, 116). Generally, if residue 627 is a glutamic acid, the virus exhibits low pathogenicity, but if the glutamic acid is replaced by a lysine, the virus becomes highly pathogenic in both a mouse model and during human infection (116).

NA. Although the main function of the influenza neuraminidase protein is to cleave sialic acid residues and release budding progeny virus from the infected host cell, it has also been shown to play a role in pathogenicity (102). Similarly to HA, NA can show a preference toward certain sialic acid linkages, which may be determined by the amino acid 275 (154). Furthermore, the NA protein of the A/WSN/33 (H1N1) virus is critical for the neurovirulence phenotype unique to that influenza virus (269). The NA virus of certain avian strains can withstand a lower pH than human or swine-derived strains (292). Both of these attributes of the NA protein have been linked to the loss of a carbohydrate chain at position 146 (102, 177).

NS1. The influenza NS1 protein plays a variety of roles during viral replication, including modulation of the host innate immune response and apoptosis (114). NS1 is known to be a type I interferon antagonist, which can lessen the immune response mounted by the host against the virus. NS1 can limit IFN-β production itself, either pre-transcriptionally by preventing activation of transcription factors such as IRF-3, NF-κB, or c-Jun/ATF-2 (190, 293, 318), or after transcription by preventing post-transcriptional processing of RNA pol II transcripts (119, 120). The NS1 protein also has both pro- and anti-apoptotic functions, which serve to both facilitate viral genome replication by

preventing apoptosis, and release of progeny virus once replication is complete (114). Modulation of apoptosis is a common mechanism of viral pathogenesis (71). The exact mechanism for NS1 mediation of apoptosis is unknown but there is evidence to suggest it may occur through PI3K pathway activation (80, 336). Temporal modulation of apoptosis during infection is indispensable for efficient influenza virus replication (114).

cAMP response element. CRE/CREB (cyclic AMP response element binding) signaling is an important cellular process that serves a variety of functions. Most notably with respect to influenza infection, CRE/CREB signaling has been shown to activate protein kinase A (PKA) and thus have a role in protein synthesis (285). PKA is a cAMP-dependent protein kinase that activates a variety of signaling cascades in the cell. In an MDCK cell model, PKA was found to aid in transport of newly synthesized influenza HA protein from the trans-Golgi network to the apical surface in preparation for budding (252), but not to affect the act of viral budding itself (130).

Receptor tyrosine kinase (RTK) signaling. Within the host cell, a major mechanism that transmits extracellular signals to intracellular signaling is the engagement of receptor tyrosine kinases (RTKs). Among the family of RTKs are the group of epidermal growth factor receptors (EGFR), consisting of four members (EGF, ErbB2, ErbB3 and ErbB4 receptors) (255). ERBB expression has been associated with a multitude of cellular functions and responses, including proliferation, cell migration, differentiation and apoptosis (137, 139, 184, 320). Cellular endocytosis of influenza co-opts pathways used by EGFRs, resulting in protein ubiquitinylation and sorting into the vacuolar pathway (147). In addition, influenza virus particles are sorted into the same population of late endosomes as EGFRs (169). Specific inhibition of tyrosine kinases by

small molecule inhibitors as well as specific EGFR inhibition via RNAi reduces virus uptake and subsequent virus titers (82). Furthermore, attachment of influenza virus to the host cell causes clustering of plasma membrane lipids, similar to that seen following EGF stimulation. Therefore, upon influenza virus binding to host cell sialic acids, it is able to cluster and activate EGFR and other RTKs to form a lipid raft-based signaling platform (82). This leads to receptor-mediated signaling events, which enhance influenza virus uptake and subsequent viral replication. It is thought that this activation is not mediated by viral engagement of a particular receptor kinase but is a more general phenomenon that affects several RTKs (82). This is additionally supported by results of a recent siRNA screening study, which identified the involvement of fibroblast growth factor receptors FGFR 1, 2 and 4 as RTKs in the very early steps of viral infection (156). Therefore, influenza virus entry accompanied by down-regulation of signaling receptors promotes co-endocytosis of the virus into the host cell.

Protein kinase C (PKC) signaling. Protein kinase C (PKC) belongs to large family of serine/threonine kinases involved in a multitude of physiological processes (301). PKC plays an integral role in sodium ion transport, important for maintaining the low pH in the endosome (125, 167, 171, 277). PKC has also been shown to be critical for the entry of enveloped viruses via receptor-medicated endocytosis (64). Upon influenza virus infection, the hemagglutinin rapidly activates PKC (64, 167) and it has been shown that a specific inhibitor of PKC prevents influenza virus replication by inhibiting the entry of the virus. Similarly, influenza virus replication has also been reported in cells expressing a phosphorylation-deficient form of PKC (260).

Phosphatidylinositol 3-kinase signaling. The family of phosphatidylinositol 3-kinases (PI3Ks) regulates various cellular events, such as cell metabolism, proliferation and survival (223, 311). The major function of the PI3K is to phosphorylate membrane phospholipids. Upon PI3K activation phosphatidylinositol-3,4,5-triphosphate is generated by phosphorylation of phosphatidylinositol-4,5-bisphosphate which functions as a second messenger through interaction with pleckstrin homology domain-containing proteins such as Akt/PKB and phosphoinositide-dependent kinase (PDK)-1 (223). Cells treated with inhibitors of PI3K or PIP3 show significantly decreased influenza virus titers (79), suggesting that PI3K performs influenza-supportive functions. Furthermore, influenza A NS1 protein acts as a suppressor of PI3K signaling. The absence of PI3K activation in ΔNS1-infected cells led to the intriguing conclusion that NS1 itself may induce PI3K-mediated signaling (79) as it has been shown that the NS1 protein is required and sufficient for activation of the kinase (80, 113, 275, 336). It has been shown that NS1 can bind to the regulatory subunit p85 of PI3K, which could lead to activation of PI3K (80, 113, 275).

Raf/MEK/ERK signaling. Infection with influenza virus leads to activation of a variety of different MAP-kinase (MAPK) cascades (164, 188, 189, 254). They are activated by a variety of extracellular stimuli such as growths factors, cytokines, and environmental stress factors like osmotic stress or ultraviolet light. Downstream substrates for MAPKs are transcription factors (e.g., ATF2, ELK-1, or c-Jun) and other protein kinases such as the MAP kinase-activated protein kinases MK2 and MK3. MAPK pathways thereby regulate a variety of cellular responses such as gene expression, proliferation, differentiation, apoptosis, and immune responses (77, 245, 253).

Influenza ribonucleoprotein (RNP) formation and nuclear export are important steps in the life cycle of influenza virus and data indicate that Raf/MEK/ERK cascade is required for an efficient nuclear RNP export as indicated by several studies (191, 254). Inhibition of Raf signaling results in nuclear retention of viral RNP and the concomitant inhibition of virus production (254). Influenza virus HA membrane accumulation and its tight association with lipid-raft domain trigger the activation of MAPK cascades via PKC-α activation and RNP export (195). HA membrane accumulation is enhanced by the higher polymerase activity of influenza virus, resulting in up-regulation of the MAPK cascade and more efficient nuclear RNP-export, along with virus production (196). In addition, p38 MAPK and JNK have been shown to regulate the expression of pro-inflammatory cytokines in influenza virus infected cells (109, 141, 164, 190).

NF-κB signaling. An important influenza virus-induced signaling mediator is the transcription factor nuclear factor κB (NF-κB). This factor regulates induction of a variety of antiviral responses, including the type I interferon response cytokine interferon (IFN)-β, the initiator of a strong type I IFN defense program (242). Although NF-κB is generally regarded as a central factor in the innate immune defense (58), independent studies demonstrated that replication of influenza viruses was impaired rather than enhanced in cells where the pathway was blocked (165, 228, 330). For example, NF-κB acts to induce pro-apoptotic factors, such as TNF-related apoptosis-inducing ligand (TRAIL) or FasL (330), and subsequently activates caspases (331). This results in enhanced nuclear export of viral RNPs presumably by specific cleavage of nuclear pore proteins (84, 159). NF-κB-dependent antagonism of type I IFN induced gene (ISG) expression may occur by upregulation of the suppressor of cytokine signaling-3 (SOCS-

3) (244) and/or by direct suppression of ISG promoter regions (319). Additionally, it is known that NF-κB differentially regulates viral RNA synthesis (165). Furthermore, besides direct antiviral action, inhibition of NF-κB may also indirectly influence pathogenesis of influenza virus. In the case of highly pathogenic avian influenza, the exact mechanism for the high mortality and severe disease it causes in humans is unknown (327). A burst of cytokines and chemokines are induced early during infection with highly pathogenic influenza viruses that are regulated by NF-κB (59, 242). Infection is also associated with multi-organ damage, necrosis of lymphoid tissue, pulmonary damage, and intravascular coagulation (300). However, treatment with immune modulators such as corticosteroids has not been beneficial in highly pathogenic H5N1 infections (23).

Disease Management

Influenza vaccines are generally available as a prophylactic measure; however, the vaccine must be specifically tailored to the circulating strain of each season. There are two vaccine formulations currently in use: the trivalent inactivated subunit vaccine consisting of HA and NA proteins, and the live-attenuated influenza vaccine (FluMist) (303). Influenza viruses antigenically drift and can undergo rapid antigenic shift (114) reducing vaccine efficacy. Additionally, the vaccine may not demonstrate a high efficacy in the populations that most need protection, such as the elderly, the very young, and the immune compromised (107, 207). Furthermore, production is problematic in that the vaccine is grown in eggs, and is therefore unsuitable for a large portion of the population who suffer from egg allergies. The vaccine also requires six to nine months from

selection of the strains for inclusion to completion of the vaccine supply and the strains must be predicted before the influenza season begins (2).

A second strategy used for disease intervention is the administration of antiviral drugs. Currently, several antiviral drugs have shown efficacy in the treatment and prophylaxis of influenza A infections: namely, two M2 inhibitors (amantadine and rimantadine) and two neuraminidase inhibitors (zanamivir and oseltamivir) (209). Early treatment with these antiviral drugs reduces the duration of symptoms and the time to recovery. The M2 inhibitors, or adamantanes, are antiviral drugs that target the M2 ion channel of influenza (20, 26, 128, 185), however, the use of adamantanes has been associated with the rapid emergence of resistant viruses capable of transmission (26, 128), compromising their potential as a prophylactic, as well as a treatment. The neuraminidase inhibitors are effective at preventing release of the virus from the infected host cell (103, 128, 168, 266, 315). Despite the utility of antiviral drugs, they come with unwelcome side effects, particularly for at-risk groups, and could possibly increase vulnerability in a pandemic situation due to lack of seroconversion, as well as drive drug resistance among circulating influenza virus strains (63, 198). Existing influenza antivirals can also require complex dosing regimens for severe seasonal or pandemic influenza (22).

Despite the availability of both inactivated and live-attenuated vaccines and antiviral drugs against influenza, there is still a need to develop novel therapeutics. The vaccines are slow to make and problematic (302, 303), and the rapidly mutating influenza virus could render current antiviral drugs useless in the future (63, 140, 266). RNA interference is a promising new avenue for the development of novel antiviral drugs.

RNA Interference (RNAi)

Post-transcriptional gene silencing was first discovered in petunias. The introduction of a pigment-deepening gene under a strong promoter to petunias resulted not in the deep purple color expected but in flowers of variegated color (222). This phenomenon was named “cosuppression” due to the suppression of not only the newly introduced transgene but also the endogenous pigmentation gene (222). It was only later that the presence of small RNAs was noted in nematodes in 1993 (173), and injection of not only anti-sense RNA against the *par-1* gene in nematodes would silence *par-1* as expected, but that injection of sense RNA was having the same effect (110). This was studied more substantially where it was found that injection of nematodes with both the sense and anti-sense strands together (dsRNA) resulted in extremely efficient and potent gene silencing (86). Further characterization of the effects of dsRNA on gene knockout in nematodes (291, 297, 298) received the Nobel Prize. This phenomenon, named RNA interference, has since been studied in a variety of organisms, including plants, *Drosophila*, and mammals (91, 146, 314, 329). The discovery of RNA interference has been crucial as it has not only defined an entirely novel mechanism for post-transcriptional gene control, but has also become an indispensable tool in the laboratory.

RNAi is an efficient mechanism for the sequence-specific inhibition of gene expression (175, 304), and is mediated by small interfering RNAs (siRNA) incorporated in the RNA-induced silencing complex (RISC) where the antisense or guide strand of the siRNA can suppress protein expression or direct degradation of messenger RNAs that contain homologous sequences (112, 150, 326). Since then, it has been characterized as

an extensive class of short, non-coding, regulatory RNA molecules that can control gene expression at both the transcriptional and translational levels (53, 121). There are several classes of RNAi: 1) piwiRNA (piRNA), 2) small nuclear RNA (snRNA), and small nucleolar RNA (snoRNA), 3) small interfering RNA (siRNA), and 4) microRNA (miRNA), which differ slightly in size, targeting parameters, and biogenesis pathways (53, 150).

piRNA. piwiRNAs derive their name from their ability to bind to the Piwi clade of Argonaute proteins (98). piRNAs do not bind to Dicer proteins (either Dcr-1 or Dcr-2) and unlike mammalian miRNAs, have a 2-O'-methyl group at the 3'-terminus (247, 262, 309). Interestingly, this modification is similar to siRNA in *Drosophila* (127). piRNAs are thought to be important for germline development (67, 68). However, they have been characterized mostly in *Drosophila*, and their role in mammals is still not well understood (98).

snRNA and snoRNA. Small nuclear RNA and small nucleolar RNAs perform their function within the center of the cell. snRNAs are found in the nucleus of the eukaryotic cell (250). They are transcribed by RNA pol II or III (240) and play a role in RNA splicing (273) and regulation of transcription factors (250). Small nucleolar RNAs (snoRNA) reside in nucleoli where ribosomal (r)RNA synthesis occurs. They cleave rRNA into 18S and 28S subunits (174, 259), methylate the rRNA (153), and remodel certain uridine residues on the rRNA to pseudouridines (33). snoRNAs can even act upon snRNAs in some cases (94, 305). Both snRNA and snoRNAs are rapidly increasing in number and they are increasingly being found to have more and more diverse functions (reviewed in (250, 281)).

siRNA. Mammalian biogenesis of siRNA and miRNA is similar (Figure 2.1). In the case of siRNA, it begins in the cytoplasm as a short (18 – 22 nt) duplex of RNA that is processed by the nuclease Dicer. The antisense strand of the siRNA is then incorporated into the RNA-induced silencing complex (RISC), and can either suppress protein expression or direct degradation of messenger RNAs that contain homologous sequence(s) (48, 78, 112, 150, 326). siRNAs in particular have become a valuable research tool for gene knockdown and silencing studies, providing quick and efficient removal of the gene of interest without the need for the development of complex knockout systems (212). Furthermore, cell-based RNAi screens have the potential to allow the study of genes involved in the entire viral infection cycle (57), from early events such as attachment and entry, through genome replication and movement of the genetic material through the host cell, to assembly and budding.

miRNA. MicroRNAs are processed in a similar manner, however they derive from precursor hairpin structures occurring in the genome (150, 257). The primary (pri)-miRNA transcript is created by RNA polymerase II (Pol II), which are then processed by the RNase III enzyme Drosha to create stem-loop precursor (pre)-miRNAs. The pre-miRNAs are exported from the nucleus to the cytoplasm where they enter the RISC pathway after Dicer processing similar to siRNAs (150). The purpose of miRNA expression in the host cell is to fine-tune gene expression. To date, over a thousand miRNAs have been identified in the human genome, and there are surely more to be discovered (105, 106, 152). The vast majority of the human genome is non-coding (4), and we are only just beginning to unravel the miRNAs encoded in this portion of the genome.

Post-transcriptional gene regulation by miRNA. There are multiple mechanisms of regulation by miRNAs, and they are very complex. miRNAs can regulate gene expression at both the transcriptional and the translational level in a variety of ways, dependent on the degree of homology between the miRNA and its target strand. The position, number, and type of mismatches between the miRNA and the target can have different effects on gene regulation. The miRNA strand generally targets the 3'-UTR of the cellular mRNA. This targeting is due to Watson-Crick base pairing of the miRNA to the mRNA. However, these matches are not always 100% complementary (44). In this case, the miRNA will bind to the mRNA as much as its complementarity will allow, but the mRNA will not be targeted for degradation. Depending on the way the miRNA strand binds, transcriptional or translational inhibition can occur, and by a variety of different mechanisms. If binding is perfectly complementary, then inhibition occurs at the transcriptional level and the mRNA can be cleaved and degraded by the Argonaut (Ago) subunit of the RISC complex, much like a traditional siRNA mechanism (152). The miRNA can also compete for cap binding with the mRNA, so the mRNA is not as stable and is therefore degraded in an exonucleolytic manner (152). When the binding is imperfect, which is more often the case with miRNAs, translation is repressed via a variety of mechanisms. Several models exist for translational repression. The miRNA can compete for binding of the actual ribosome by interfering with the elF6/60S subunit preventing it from binding to the 40S ribosomal subunit in order for translation to begin. Alternatively, the miRNA can stimulate deadenylation of the poly-A tail, another feature of a cellular mRNA that stabilizes it. It can also promote degradation of the translational

protein complex itself, or cause the ribosome to drop off the mRNA entirely, so that the mRNA is not translated (53).

An miRNA is generated from an endogenous transcript generated from the genome. This primary transcript can be generated from an miRNA gene, or intronic miRNA (93). The primary transcript is generated by RNA polymerase II, known as the pri-miRNA, and is capped and polyadenylated (53, 152). These miRNA transcripts can have multiple isoforms, and one transcript can potentially be cleaved into several different miRNAs, or one miRNA and one protein (53, 152). The pri-miRNA contains extensions to the actual miRNA sequence on both the 5' and the 3' end. The pri-RNA is processed in the cell nucleus by an RNase III-type enzyme known as Drosha, into a hairpin structure known as the pre-miRNA. In animal systems, the pre-miRNA is exported from the cell nucleus by exportin 5 driven by RanGTP/GDP, still in hairpin form, and it encounters the enzyme Dicer in the cytoplasm (152). Dicer makes two alterations to the pre-miRNA. First, it removes the loop from the top of the hairpin, leaving a mature duplex of around 22 nucleotides in length. The second alteration is a cut at the 3' overhang at the other end of the duplex, leaving a double-stranded miRNA. This duplex is extremely short lived and quickly associates with an Ago protein (236), the catalytic component of the RNA-induced silencing complex (RISC). After association with Ago, the miRNA unwinds, a mechanism that is not well understood for miRNAs although it is better characterized for siRNAs (53). The passenger strand, also called the miRNA* strand, is generally lost, although it has been detected in the Ago complex in certain situations (237). The other strand, the actual single-stranded microRNA, is retained in the complex and can target and regulate gene expression (19).

RNAi for disease intervention. Synthetic siRNAs can be readily developed to target viral or host genes and have been successfully applied in disease intervention approaches. For example, siRNA targeting respiratory syncytial virus has shown efficacy for silencing virus replication (5, 14, 32, 73, 334, 335), a feature that has led to RNAi-based clinical trials as a new therapeutic option (73). In addition, there are promising results from targeting host genes, such as the use of siRNA silencing for the treatment of age-related macular degeneration (12), and in the case of influenza, inhibiting the host gene CAMK2B prevented vRNA transcription in vitro (156). Recently, several studies employed genome-wide RNAi screens to identify host genes required for influenza virus infection and replication (42, 115, 142, 156, 272), and genes have also been identified by random homozygous gene perturbation (290) and by a proteomic screen (40). From these studies, we hope to be able to identify novel gene targets and cellular pathways involved in influenza replication within the host cell. These new targets might be applied as RNAi-based antiviral therapies, or may lead to a new understanding of influenza infection.

The use of synthetic siRNAs to target viral and host genes has been successfully applied in animal models (6, 334) and this has led to the initiation of RNAi-based clinical trials as a new therapeutic option. For example, there are promising results from research studies and ongoing clinical trials for the treatment of cancer, age-related macular degeneration and respiratory syncytial virus (RSV) that suggest RNAi therapeutics targeting host or viral genes can be effective (15, 74, 230, 321). There is also evidence that viruses such as Epstein-Barr virus, herpes simplex virus, and cytomegalovirus encode their own miRNAs that regulate host gene expression and may have a substantial

effect on host-pathogen interactions (108, 123, 251, 299). Host miRNAs are known to interact with influenza virus (122) and microRNA expression profiles are altered during influenza infection in a mouse model (179). RNAi antiviral therapy that targets host genes offers a variety of advantages. There are potentially a wide variety of host genes that may be involved in virus replication as opposed to limited conserved viral target genes. The host targets are less likely to mutate, and it is likely that similar pathways will be co-opted by related viruses thereby offering a potential RNAi therapeutic with broad efficacy.

The identification of pro- and antiviral miRNAs (264) opens the possibility of using miRNAs as potential therapeutics. However, clinical application of miRNA is dependent on our understanding of the consequences of perturbations in miRNA expression (197, 271). As miRNAs are known to be involved in basic cellular pathways such as proliferation and apoptosis (325), which can be dysregulated during cancer or viral infection (52, 55, 186), miRNAs can be harnessed as a means to control gene expression during illness. miRNAs can also aid in activation of the innate immune response (29).

Simple characterization of host miRNA expression can lead to understanding of disease pathogenesis. Aberrant miRNA expression profiles are known to be indicative of disease, and the miRNAs let-7 and miR-155 have been shown to be dysregulated in non-small cell lung cancer (135). miRNA expression profiles can also be correlated with cancer relapse, survival rates, and treatment success (206). miRNAs have also been used as therapeutics themselves. Delivery of miR-26a suppressed tumorigenesis in a murine hepatocarcinoma model (158), and overexpression of miR-155 was shown to enhance

innate antiviral immunity and aid in hepatitis B virus clearance (288). Similarly, miR-122 was found to facilitate hepatitis C virus replication of the viral genome, implying miR-122 is a potential therapeutic target for HCV (138, 170), as well as miR-199a (217).

Host Factors Required for Influenza Virus Replication

RNAi high-throughput screens. siRNA libraries targeting each gene in the human genome are now available. siRNAs are fairly easy to synthesize and more cost-effective than other related reagents (129) and offer an alternative to proteomic screens (65). Most libraries consist of pools of several siRNAs, to ensure efficient silencing of the target gene(s) (42). These libraries have been especially useful in the study of virus-host interactions, used to screen both for resistance host genes and genes required by the virus for efficient replication for HIV (41, 157, 337), dengue virus (42, 270), West Nile virus (42, 160), and influenza virus (42, 115, 142, 156, 272).

Human immunodeficiency virus (HIV) is a tremendous public health burden and the need to understand HIV virus-host interactions is crucial for the development of new therapies, since the virus has the ability to mutate quickly, rendering current therapeutics useless (62). RNAi screens for host factors exploited by the retrovirus HIV have utilized different methodologies. Zhou et al employed an HIV LTR-driven β-galactosidase reporter assay as readout for viral replication at both 48 hours post infection (hpi) to assay factors associated with viral entry and 96 hpi to study the complete replication cycle (337). This study used a classic laboratory cell line (HeLa cells) altered to express CD4 to render the cells susceptible to HIV infection, and also expressing the β-gal reporter (337). This screen identified 311 hit genes that were expressed in human $CD4^+$

cells and macrophages of which 232 genes were validated by repeating the assay with a novel pool of siRNAs (337). In contrast, an independent HIV RNAi screen examined host genes involved in early events of virus replication using an HIV reporter virus expressing luciferase that undergoes only a single round of replication (157). This screen identified 213 host factors that were then used to conduct a meta-analysis to determine overall cellular factors and processes connected with HIV replication, some of which had not previously been identified (157).

RNAi screens have also been performed for flaviviruses that include dengue virus (DENV) and West Nile virus (WNV). Dengue fever is of particular concern as there is no vaccine available and previous exposure to one serotype can negatively impact a subsequent infection of another serotype (270). A DENV screen looked at host factors involved in DENV-2 infection and identified 116 associated cellular factors, some that had previously been associated with DENV pathogenesis such as v-ATPases and α-glucosidases, and others that were entirely novel. The screen was carried out in *Drosophila* cells, but 82 candidates had human homologs, and a secondary screen validated 42 of those genes in a human cell line (270). WNV, a causative agent of the neuroinvasive West Nile encephalitis, represents another serious global public health problem where RNAi screens for host factors may prove useful. Krishnan et al. used immunofluorescence of the viral envelope protein after HeLa cells were transfected with a pool of siRNAs to determine host genes involved in viral replication steps of entry through translation. A rescreen with a second siRNA to confirm phenotype served as validation to identify 305 associated host factors (160).

Meta-analysis of influenza siRNA screens. To date, five RNAi interference screens of the human genome have been published for influenza virus (Table 2.1). Each screen used varying methodologies and endpoint assays. Some screens included early replication events only (115, 156) while others included both late and early events (42, 142, 272). The subset of host genes examined from screen to screen varied as well, and the cell model varied from permissive cells (142, 156) to cells which the influenza virus cannot naturally infect (42, 115, 272). The five screens generated a list of hit genes that represented approximately 2% of the genes screened except for one study (272), which had a 35% success rate, but this was likely due to the gene availability constraints of the Y2H system. However, these screens had very little overlap of hit genes among each other. Only three genes overall were independently validated in four of the six screens, nine genes were validated in 2 of 6 screens, and 86 genes were validated across two of the screens. The lack of consistency is troubling at first glance, but the relationships of the hit genes to each other may be more helpful to interpret the data than the individual genes themselves. A meta-analysis of the hits identified in other influenza screens shows that while the individual hit genes differ, the cellular pathway nodes connected to the hits show repeatability from screen to screen.

The first RNAi screen for influenza was done in *Drosophila* cells using a recombinant A/WSN/33 virus where the hemagglutinin and neuraminidase segments had been replaced with VSV-G and a luciferase reporter, respectively (115). After transfection, cells were infected with the recombinant virus and infection was quantified by luciferase expression. However, this screen only allowed identification of host genes that affected viral entry, as the recombinant virus was unable to replicate. Furthermore,

Drosophila cells are not permissive for influenza infection, so host genes identified may not be translational despite identifying 121 genes involved with early infection events (115).

A recent screen in human U2OS cells (260 genes identified) was performed (42), but these cells were also not permissive for natural influenza infection and the methodology again only allowed for single round viral replication. Virus (A/PR8) was measured by staining for HA protein, which is an indirect method of assaying viral replication (42). Another screen (616 genes identified) was performed in HBECs that more closely mimic a natural infection and utilized a yeast two-hybrid (Y2H) system (272). However, the library available for the human ORFeome v3.1 used in the Y2H screen contains only 1200 genes so the selection of available host genes was limited. Additionally, the A/PR8 virus used was lacking the NS1 protein, which is vital in modulation of host expression of innate immune response genes as well as apoptosis-related genes (114). Another screen utilized A549 cells and both recombinant A/WSN/33 and SOIV with luciferase readout (156); however a recombinant virus may have different requirements than the wild-type virus. Of the 295 gene hits identified, 219 were confirmed in wild-type WSN virus while 76 were not (156). Finally, it was shown that 168 genes are involved in the A549 cell system which affect influenza virus (A/WSN/33) replication as measured by a 293T luciferase reporter cell line (142).

Each of the primary screens resulted in a large number of hit genes but not all genes identified may be a genuine hit. With any high-throughput screen, there is the potential for the generation of both false negatives and false positives (18). While the design of the screen methodology is important to take into consideration, the validation

process is crucial. Off-target effects of siRNAs are a major concern. After an siRNA screen it is important to determine whether the hits are valid; if the phenotype cannot be repeated with an siRNA with a novel nucleotide sequence from the sequences used in the screen, the phenotype observed in the initial screen was most likely due to off-target effects (278). An siRNA will target its mRNA with perfect complementarity (124, 278). However, since siRNAs enter the same biological pathway as an endogenously derived miRNA, there is the possibility of the short siRNA sequence acting as an miRNA and targeting multiple other mRNAs with imperfect complementarity instead of binding only to the target mRNA (30, 124, 278). Furthermore, siRNAs longer than ~23-25 base pairs strongly unregulated interferon stimulated genes (ISGs) in some cell lines (9, 149), and siRNAs can also activate a type I IFN response through TLR7 and TLR8 (126, 280). Elimination of these false positives from the pool of hit genes is accomplished by validating the screen hits.

Validation methods varied across each of the five influenza screens. One common method for validation is to deconvolute the pools of siRNA used to target each individual gene. For example, Brass et al utilized pools of four individual siRNAs for each host gene. A repeat of the assay using the four siRNAs separately to confirm that the same phenotype was repeated between each siRNA served as the validation step (42). Others used certain cutoffs of reduction of virus that were required of multiple unique siRNAs, for example more than 2 unique siRNAs targeting the candidate gene reduced virus by 35% (156) or five-fold (142, 156). Another screen validated phenotype by using the same phenotype but looking at multiple endpoint assays (272).

As well as false positives, false negatives are also an important consideration. The statistical methods employed to analyze a high-throughput screen may result in too strict of a cutoff resulting in missing some of the valid gene hits. These genes may have been missed in the screen due to the fact that an RNAi screen simply cannot be optimized for every siRNA/gene pair included in the study. Proteins with a long half-life may not be completely gone by the time of assay even though the siRNA has effectively silenced the message, some genes may have functional redundancy meaning other genes can serve its purpose even after the target gene has been silenced, and some genes are simply difficult to knockout by siRNA (278).

Other screens. In addition to screening with siRNA, it is now possible to perform high-throughput screens based on miRNA, which can subtly modulate both host and viral gene expression. For example, a library of miRNA mimics and inhibitors was used to investigate the miRNAs involved in β-herpesvirus (MCMV, HCMV, HSV-1) infection (264). The miRNA mimics increase the cellular concentration of miRNAs that can be loaded onto the RISC, while the miRNA inhibitors are designed to bind to mature miRNAs and prohibit cleavage (312). By evaluating the effect on phenotype from both the miRNA mimic and corresponding inhibitor, the authors were able to identify four antiviral miRNAs and three proviral miRNAs that acted across three separate β-herpesviruses. Since the screen was performed using GFP-reporter viruses, testing with wild-type virus validated the identified miRNAs. Further analysis implicated the miRNAs in a variety of host signaling networks, including ERK/MAPK and PI3K/AKT signaling, among others (264). Since these pathways are also implicated in influenza

infection (80, 253), this implies miRNAs can be used as potential therapeutics with broad application.

There are however limitations to miRNA screens. Careful consideration must be used when choosing the cell model, as all miRNAs are not expressed in similar amounts, if at all, across multiple cell types due to the tight regulatory control over cellular processes that they exert (325). Additionally, miRNA function in the host cell is subtle and multi-targeted; therefore results must be thoroughly validated to prohibit confusion from off-target effects (31). Finally, since one miRNA may potentially have hundreds of targets (91), one particular miRNA-mRNA interaction may not be sufficient to produce or explain the intended effect. Effective miRNA antivirals may require targeting multiple genes using different miRNAs (264).

Host Proteases and Influenza Infection

There are known HA-processing proteases in the human respiratory tract, including transmembrane serine proteases (such as TMPRSS2, TMPRSS4, and TMPRSS13) and human airway trypsin-like protease, (HAT) (34-36, 238). TMPRSS2 is expressed as a 70 kDa full-length protein with a 32 kDa auto-catalytically cleaved protease domain (47, 151, 324). The function of TMPRSS2 is not well characterized, but it is homologous to enterokinase, which specifically cleaves the acidic propeptide from trypsinogen to yield active trypsin (332). TMPRSS2 is thought to have an important role in physiological and pathological processes such as protein catabolism, blood coagulation, cell growth and migration, and inflammation (308). The functions of TMPRSS2 appear redundant as TMPRSS2-deficient mice have no discernable defects (151). TMPRSS2 is expressed by

various cell types, and particularly by cell types for which influenza virus has tropism, e.g. normal human bronchial epithelial cells (200) and type II human alveolar epithelial (A549) cells (35). Recently, MDCK cells expressing TMPRSS2 and HAT have been shown to allow human and avian strains of influenza to replicate independently of trypsin (34).

In addition to known HA-processing proteases, other proteases may play a role in influenza infection. Inhibition of serine proteases other than TMPRSS2 and HAT has been shown to decrease influenza replication in a mouse model (10), and HIV protease inhibition drugs can block influenza replication as well (85). Due to the limited number of viral genes, a virus must rely on host cellular pathways to facilitate viral replication. Host proteases can contribute to replication in a variety of ways. HA processing and activation can allow viral entry through the exposure of the HA fusion peptide, however once inside the cell proteases can be used for degradation of viral proteins for MHC presentation (202). This can be beneficial if the virus is using mimic peptides but detrimental if display of the viral peptides alerts the immune system (202). Some viruses can use host proteases for cap-snatching activities to remove the 5'-cap from a cellular mRNA and apply it to a viral mRNA (38). Additionally, many viruses modulate apoptosis in the infected cells to suit viral needs, and therefore control the proteases that activate the apoptotic signaling cascade (306).

Proteases occur throughout the human body and serve a variety of functions. ADAMTS7 (ADAM metallopeptidase with thrombospondin, type 1, motif 7) is a member of the ADAM metallopeptidase family, a group of proteins that share a common thrombospondin type I motif and similar zinc metalloproteinase and disintegrin-like

domains (131, 193). ADAMTS7 itself has been implicated as a major player in the pathogenesis of arthritis (181). The extracellular matrix of cartilage, specifically cartilage oligomeric matrix protein (COMP), has been shown to be a target of both ADAMTS7 and ADAMTS5, among other members of this family (182). Degradation of COMP by these peptidases can contribute to joint pain and inflammation. ADAMTS7 was not only shown to be highly expressed in musculoskeletal tissue and upregulated in patients with rheumatoid arthritis, but also to bind tightly to and cleave COMP both *in vitro* and *in vivo* (182, 187). In addition to its important role in the development and pathogenesis of arthritis, ADAMTS7 has also been implicated in retinal matrix turnover and in inflammatory eye disease, such as macular degeneration (28). In the context of influenza infection, ADAMTS7 may play a role in viral pathogenesis specifically because of its ability to destroy the extracellular matrix. In a highly pathogenic influenza infection, degradation of the extracellular matrix could lead to increased vascular permeability and allow the infection to become systemic (317). CPE (carboxypeptidase E) and MST1 [macrophage stimulating 1 (hepatocyte growth factor-like)] have also been implicated in tissue injury and inflammation along with ADAMTS7 (136, 187, 194). DPP3 (dipeptidyl-peptidase III) is a metallopeptidase that plays a role in the defense against oxidative stress (183) and is involved in the pain-modulatory system (263). DPP3 shows upregulated activity in malignant ovarian tumors (279) and cancerous cells (276). PRSS12, also known as neurotrypsin or motopsin, is a serine protease similar to the TMPRSS family of proteases whose function has mostly been characterized in nervous tissue and the brain (133, 210, 211, 231).

Conclusions

Influenza is the causative agent of a considerable public health burden. The threat of emerging pandemic influenza strains is constant, and recent events have highlighted the need for pandemic preparedness. As development of the influenza vaccine is time-consuming, and resistance to the current arsenal of antiviral drugs is steadily increasing, it becomes crucial to develop novel influenza therapeutics. Targeting host genes instead of viral genes is an attractive option to circumvent viral mutation. RNA interference offers the opportunity to screen a large amount of host genes quickly for those that are involved with virus replication, and to increase our knowledge of global cellular pathways co-opted by the influenza virus. Protease genes are critical in viral pathogenesis, replication, and the host immune response, and offer a unique perspective to elucidate the biology of influenza virus replication.

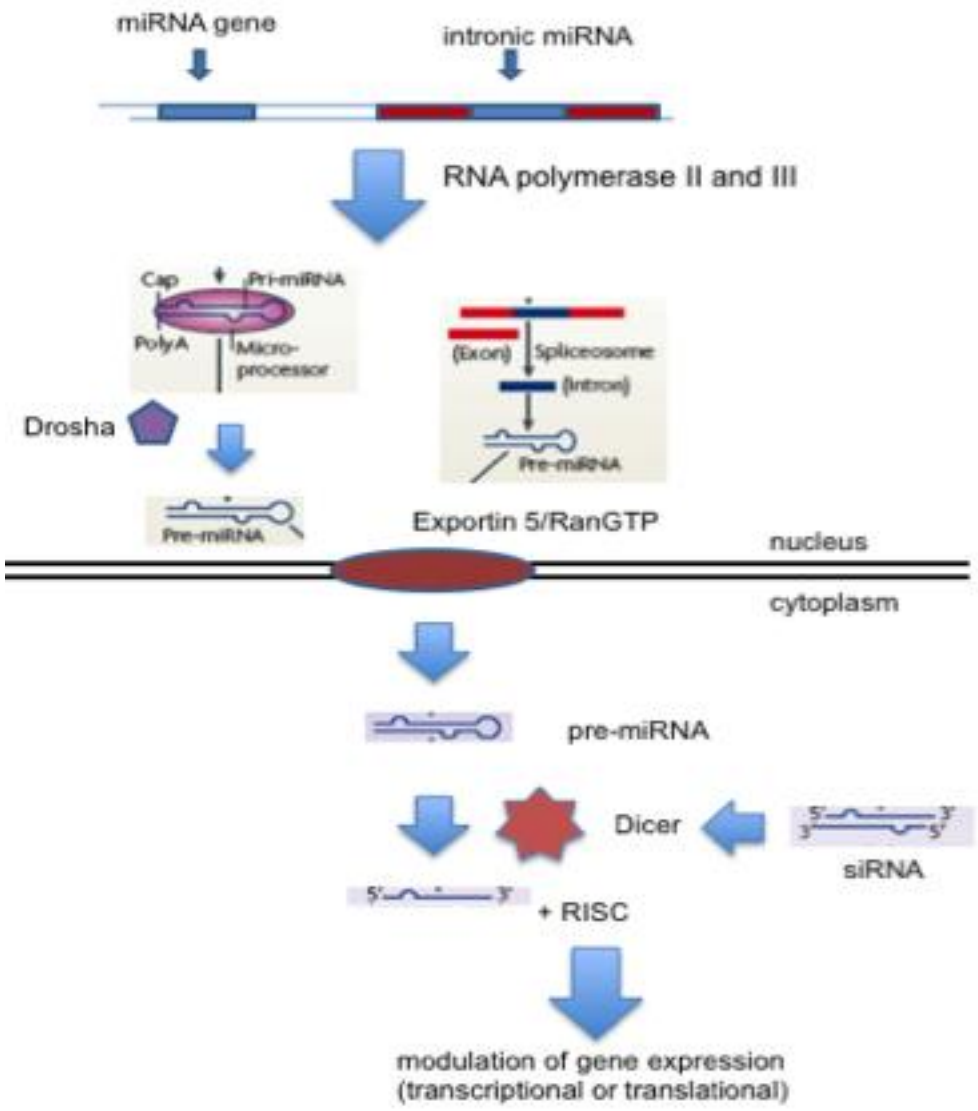

Figure 2.1: The RNAi biogenesis pathway. miRNAs are generated from endogenous transcripts in the nucleus. RNA Pol II (or Pol III) removes the primary miRNA (pri-miRNA) as a hairpin structure. The pri-miRNA is processed by the Drosha enzyme to the precursor miRNA (pre-miRNA), which is exported from the cell nucleus. The pre-miRNA is processed by Dicer to the mature miRNA, where it is directed to the RISC complex for targeting. A cytoplasmic siRNA is also processed by Dicer before associating with the RISC complex for mRNA targeting and degradation by the catalytic Ago component of RISC. Figure adapted from *Gangaraju et al. Nat Rev Mol Cell Bio 2009.*

Table 2.1 siRNA screens for host genes affecting influenza virus replication

Screen	Cell line	Influenza virus	Readout	Genes screened	Validated hits	Validation	Reference
siRNA screen (Ambion)	*Drosophila* D-Mel2	Recombinant A/WSN/33	Luciferase activity	13,071	121 (110 ↓[a] and 11 ↑[b])	Decreased luciferase expression in two replicates, inhibiting ≥ mean ± 2.5σ in at least one replicate, and phenotype consistent when targeted with an alternate dsRNA amplicon	Hao, L. *et al. Nature* 454, 890-893 (2008).
siRNA screen (Dharmacon)	Human U2OS	A/PR/8/34 H1N1	HA immunostain	17,877	260 (250 ↓[a] and 10 ↑[b])	Rescreen with individual siRNAs from pool	Brass, A.L. *et al. Cell* 139, 1243-1254 (2009).
virus-host direct interactions (Y2H), transcriptional responses (microarray), and pathway association (IPA)	Human HBEC	A/PR/8/34, ΔNS1[c], or vRNA	Infectious virus or IFNβ□production	1745	616	siRNA to candidate gene affected the phenotype in ≥ 1 of 3 functional assays	Shapira, S.D. *et al. Cell* 139, 1255-1267 (2009).
siRNA screen (Qiagen, Invitrogen, IDT)	Human A549	Recombinant A/WSN/33, SOIV A/NL/602/09	Luciferase activity	19,628	295 (295 WSN, 12 SOIV)	≥ 2 unique siRNAs to candidate gene reduced viral infection by ≥35%	Konig, R. *et al. Nature* 463, 813-817 (2010).

siRNA screen (Qiagen)	Human A549	A/WSN/33, SOIV A/Hamburg/04, HPAI A/VN/1203/04	Infectious virus quantified using a 293T cell reporter system and NP immunostain and viral replication was measured by titrating the treated A549 supernatant on MDCK cells.	22,843	168 (119 WSN, 121 SOIV, 6 HPAI)	≥ 2 unique siRNAs to candidate gene decreased virus replication by more than fivefold	Karlas, A. *et al.* *Nature* 463, 818-822 (2010).
siRNA screen (Dharmacon)	Human A549	A/WSN/33	Amount of infectious virus, NP expression, M gene levels	1201	28 (25 ↓[a] and 3 ↑[b])	Phenotype is emulated using a novel siRNA targeting a different seed region of the hit gene	Andersen, L.E. *et al.*[#], Meliopoulos, V.A. *et al.**

[a]↓, hits that decreased virus replication. [b]↑, hits that increased virus replication. [c]ΔNS1, PR8 virus lacking the non-structural gene.
[#]unpublished data, *submitted

HA, hemagglutinin; NA, neuraminidase; VSV-G, vesicular stomatitis virus glycoprotein G; eGFP, enhanced green fluorescence protein; HBEC, human bronchial epithelial cells; NP, influenza virus nucleoprotein; M, influenza virus matrix protein; Y2H, yeast two-hybrid; IPA, Ingenuity Pathway Analysis; vRNA, viral RNA; IFN, interferon; SOIV, swine-origin influenza virus; HPAI, highly pathogenic avian-origin influenza virus; MDCK, Madin-Darby canine kidney.

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CHAPTER 3

IDENTIFICATION OF HOST PROTEASE GENES INVOLVED IN INFLUENZA REPLICATION[1]

[1]Meliopoulos, V. A., L. A. Andersen, P. Brooks, X. Yan, J. K. Coleman, S. M. Tompkins, R. A. Tripp. Submitted to *PLoS One* September 14, 2011.

Abstract

Influenza is a considerable public health concern. Although vaccines and antivirals against the influenza virus are available, the need to develop new therapeutics is essential due to the ongoing mutation of the virus. Owing to the high mutation rate of influenza, therapeutic drugs targeting host genes required for virus replication may be better targets than the virus itself. An RNA interference (RNAi) screen of the human protease library (481 genes) was performed to identify host genes involved in influenza infection and replication. The primary screen identified 24 human protease genes that had an effect on viral titers when silenced in human lung epithelial (A549) cells. These genes were further evaluated for cytotoxicity and assayed for their effect on aspects of influenza replication, genomic replication and cellular localization. In total, this study identified 24 potential therapeutic targets for novel influenza disease intervention.

Introduction

Influenza virus is a major public health concern, causing seasonal disease and occasionally pandemics, resulting in increased morbidity and mortality in the population (24, 45). Vaccines are generally available, however due to mutation and antigenic drift, they must be tailored to the current circulating strain or risk losing efficacy (18). Antiviral drugs that include the M2 ion channel inhibitors (amantadine and rimantadine) and neuraminidase inhibitors (zamamivir and oseltamivir) are available but early treatment is required to reduce symptoms and transmission (4, 6, 21, 23, 33, 34). Furthermore, the use of antiviral drugs is complicated by the emergence of drug resistant viruses (5, 6, 14, 21, 31). Thus, it is critical to discover new targets for chemoprophylactics and treatment.

RNA interference (RNAi) is a gene silencing method that can be used as a platform enabling technology to perform genome-wide screens to identify host cell genes that may be required for influenza virus replication (20, 29). RNAi is used for the sequence-specific inhibition of gene expression (32, 46), where a small interfering RNA molecule (siRNA) can suppress protein expression or directly degrade messenger RNAs that contain homologous sequences (17, 27, 49). Synthetic siRNAs can be readily developed to target viral or host genes (22) and have been successfully applied in disease intervention approaches. For instance, siRNA targeting respiratory syncytial virus proteins has shown efficacy for silencing virus replication (1, 3, 7, 16, 51, 52), which has led to RNAi-based clinical trials as a novel therapeutic option (16). There are also promising results from targeting host genes in place of viral genes, such as the use of siRNA for treatment of age-related macular degeneration (2), and in the case of

influenza, inhibition of the host gene CAMK2B prevented vRNA transcription *in vitro* (28). Recently, several studies employed genome-wide RNAi screens to identify host genes required for influenza virus infection and replication (13, 19, 25, 28, 39), and genes have also been identified by random homozygous gene perturbation (43), and by a proteomic screen (12).

Of the host genes known to affect influenza virus, the proteases are important for infection and replication. Proteases may affect virus infection and replication in several ways including viral entry and hemagglutinin (HA) processing (8-10, 26), degradation of viral components for MHC presentation (35), cap-snatching (11), induction of apoptosis (47), and by increasing vascular permeability aiding in the development of systemic infection in cases of severe infection (48). However, it remains unclear which protease genes are essential in the biology of influenza virus replication.

To comprehensively determine the host proteases required by influenza virus for infection and replication, a siRNA screen of all known 481 protease genes was performed in human lung type II epithelial (A549) cells using the influenza virus A/WSN/33. Twenty-four primary gene hits were found in the siRNA screen, of which eight increased viral titers when silenced, implying a role in antiviral resistance, and 16 decreased viral titers, which may be genes essential for influenza virus replication. Of the 24 genes, only one was cytotoxic when silenced in A549 cells. The genes were further evaluated for their effect on NP localization and viral replication to further validate the phenotype seen from the primary screen.

Materials and Methods

Cells and virus stocks. A549 cells (ATCC CCL-185) were cultured in Dulbecco's modified Eagle's medium (DMEM) (HyClone, Logan, UT) containing 5% heat-inactivated FBS (HyClone, Logan, UT). Cells were frozen in 10% DMSO and 90% FBS to create one stock of a single cell passage that was used for the entire screen. A stock of MDCK cells (ATCC CCL-34) was also propagated and stored using the same conditions. A/WSN/33 (H1N1) influenza virus was used for the primary siRNA screen as this virus has the ability to replicate without the need for exogenous trypsin (40) and was propagated in 9-day-old embryonated chicken eggs as previously described (50). Virus was titrated in MDCK cells and titers calculated by the method developed by Reed and Muench (37).

Protease library screen. A primary screen using four pooled siRNAs (sequences in Table 3.1) to target each gene of the 481 genes in the human protease library (SMARTpool; Dharmacon ThermoFisher, Lafayette, CO) was performed using A/WSN/33 influenza virus and type II human alveolar pneumocytes (A549 cells) similar to a method previously described (28). siRNAs were resuspended in Dharamcon siRNA buffer to a concentration of 1 uM and stored at -80°C until use. An siRNA targeting the MEK gene (siMEK), a well-characterized human kinase gene important for influenza replication (4, 30), was used to control for the transfection efficiency and host gene silencing. A non-targeting siRNA control (siNEG) was also used in all assays. The siRNA SMARTpool library constituents, siMEK, and siNEG transfected A549 cells at >85% efficiency, and a few (<2%) siRNA pools targeting human protease genes induced

cytotoxicity as determined by adenylate kinase (AK) release in the cell culture supernatant (15, 36) and by visual inspection of cell morphology.

A549 cells were reverse transfected with the siRNA pool 48 hours before infection with influenza A/WSN/33 (MOI = 0.001). The amount of infectious virus was measured 48 hpi by titration of A549 cell supernatant on MDCK cells, and the results normalized to siNEG-treated cells. All assays were run in duplicate and the entire screen assay was repeated twice. Both plate-based controls and assay-wide controls, e.g. siMEK, siNEG, and siTOX, were included on each individual assay plate. The results were normalized to the plate median for the SMARTpools. The primary screen, performed as two independent studies, was analyzed using a scaling methodology that sets the non-targeting control siRNA (siNEG) at an arbitrary value based on the number of dilutions where virus was detectable by $TCID_{50}$, and the negative control siTOX at zero. siRNAs targeting host genes were assigned a score based on the distribution of these values. Z-scores were computed based on this data for each gene hit and those $\geq 1.5\sigma$ and $\leq -1.5\sigma$ were considered for validation. Of the 481 human protease genes targeted, twenty-four genes were identified as "positives" or "primary hits". Hits that caused greater than 20% cytotoxicity in A549 cells were considered cytotoxic.

Reverse transfection. Lyophilized siRNAs in 96-well plates were diluted with HBSS (HyClone, Logan, UT) and allowed to incubate for 5 minutes. Dharmafect-1 transfection reagent (Lafayette, CO) and HBSS were added such that each well received 0.004 ml of transfection reagent and 0.096 ml of HBSS. The siRNA/transfection reagent mix was allowed to incubate for 20 minutes at room temperature after which 0.08 ml of 1.5×10^4 A549 cells suspended in DMEM/5% FBS was added to each well, and the plate

incubated for 48 hours at 37°C in 5% CO_2. The final concentration of siRNA for all primary screen transfections was 50 nM.

Cytotoxicity and virus infection. To determine if siRNA gene silencing was cytotoxic, the cell supernatants from siRNA transfected A549 cells were analyzed for adenylate kinase (AK) release using a Toxilight kit (Lonza, Rockland, ME). Results were normalized to a siTOX control, a siRNA control (Dharmacon) causing complete cell death by 48 hours, as a maximum, and the siNEG control as a minimum. siRNA transfected cells with luminescence greater than or equal to 20% of the siTOX control were not considered for further evaluation. A549 cells were subsequently infected with A/WSN/33 at an MOI of 0.001 pfu/cell. Cells were incubated for 48 hours at 37°C/5% CO_2.

Endpoint assays. Virus titers in siRNA-treated A549 cells infected with A/WSN/33 were determined by modified $TCID_{50}$ or hemagglutination assay (HA). Briefly, virus infected A549 cell culture supernatants were serially diluted ten-fold, and added to MDCK cells. The MDCK cell plates were incubated for 72 h, followed by an HA using 0.5% chicken red blood cells as previously described (42). To further identify gene hits, the cells were screened for the magnitude and localization of nucleoprotein (NP) staining. For NP staining, the cells were fixed with 3.7% formaldehyde and stained with anti-NP monoclonal antibody (5 ug/ml; H16-L10-4R5) and the antibody staining detected using AlexaFluor 488 labeled goat anti-mouse IgG (1 ug/ml; Invitrogen, Carlsbad, CA). Cells were counterstained with DAPI (2 ug/ml) (Invitrogen, Carlsbad, CA) and visualized by immunofluorescent microscopy (20X, EVOS digital inverted fluorescent microscope, Advanced Microscopy Group, Bothell, WA). As an additional hit

identification endpoint, real-time qRT-PCR analysis was performed to quantify influenza M gene copy number in the cells as previously described (41). Briefly, RNA was purified using the RNeasy kit (Qiagen, Valencia, CA), and cDNA was synthesized using a SuperScript First Strand cDNA synthesis kit (Invitrogen, Carlsbad, CA) and appropriate primer/probe set (41) to detect the M gene. PCR was performed using the amplification cycle: 10 minutes at 95ºC followed by 40 cycles of 95ºC for 30 seconds, 60ºC for 1 minute, and 72ºC for 30 seconds. M gene copies were compared to infected cells treated with the siNEG control.

Results

Primary screen. The primary screen was performed as depicted in Figure 3.1. Additional endpoint assays of NP staining by immunofluorescent assay and M gene copy number by qPCR were performed on the gene hits identified by the primary screen. A549 cells were treated with a pool of four individual siRNAs, called the SMARTpool, targeting each gene at a different site in a reverse transfection format. The SMARTpool was used to ensure the maximal silencing possible (13). Three controls were included on each plate: siNEG, siMEK, and siTOX, so comparisons could be made across assay plates. The siNEG control consisted of a non-targeting siRNA that does not match any known human sequence and would therefore not specifically silence any gene in the host cells. An siRNA targeting MAP kinase kinase (MEK) was used as a positive control as inhibition of MEK is well-documented to inhibit influenza virus replication (4, 30). The siTOX control was used for cytotoxicity studies, and transfection with siTOX results in complete cell death 24 – 48 hours post transfection but only if taken up by the cell. The siRNA

transfection mixture was incubated with the cells for 48 hours to allow for maximum gene silencing. The cellular supernatant was then removed and tested for adenylate kinase (AK) release, which is indicative of toxicity (15, 36). The cells were then infected using A/WSN/33 (H1N1) at an MOI of 0.001 because of its unique ability to replicate in the absence of exogenous trypsin (40), a feature important for the cell model chosen, as A549 cells are sensitive to trypsin. At 48 hpi the cellular supernatant was harvested and virus titers determined by a modified $TCID_{50}$ assay. To determine primary hits from the screen, the $TCID_{50}$ results were assigned a score based on the furthest dilution where virus was detected. The scores were compared to the siNEG control and the siTOX control (score = 0). Z-scores were calculated from the $TCID_{50}$ score. The primary hits were considered $\geq 1.5\sigma$ and $\leq -1.5\sigma$ from the mean Z-score (Figure 3.2).

Most primary hits were not cytotoxic. Of the 481 human protease genes screened, 24 genes from the primary screen were identified as positives that when silenced, had the effect to up- or down-regulat influenza virus replication (Figure 3.2, Table 3.1). Most hit genes were not cytotoxic to the cell when silenced by RNAi. Genes were considered cytotoxic if AK levels were 20% or greater when compared with the siTOX control. Of the 24 primary hits, only siRNA targeting SENP1 (siSENP1) was cytotoxic to A549 cells, causing AK levels of 30% of the siTOX control (Figure 3.3). In fact, some genes were actually less toxic than the siNEG control, as indicated by genes whose percent cytotoxicity was below zero (Figure 3.3).

Effect of host gene silencing on infectious virus production. The modified $TCID_{50}$ assay (37) determined infectious virus production in A549 cells where the host protease gene of interest had been silenced. The $TCID_{50}$ assay was modified to allow for direct

transfer of supernatant in the same plate format as the primary screen plates, and such that all controls were included on each $TCID_{50}$ plate. Some genes resulted in an increase of infectious virus production relative to the siNEG control, while some resulted in a comparative decrease. The siMEK control consistently resulted in approximately 2 logs less infectious influenza virus. Eight human protease genes, including the cytotoxic SENPI, caused increased virus production when silenced, ranging from 1 – 2 logs more virus compared to the siNEG control (Figure 3.4). Sixteen of the hit genes resulted in a decrease of influenza virus, and silencing of 12 of the genes gave no detectable virus titers (Figure 3.4). The $TCID_{50}$ assay is indicative of two complete independent screens of the human protease library, and the 24 hit genes tested gave repeatable results as indicated by the calculated z-score (Figure 3.2). Viral titers are summarized in Table 3.1.

Effect of host gene silencing on influenza NP localization. To better characterize the effect of host gene silencing on influenza replication, influenza nucleoprotein (NP) localization 48 hours post infection (hpi) was examined in A549 cells that had been treated with 50 nM of the siRNA pool for the primary hit genes. Cells were counterstained with DAPI to indicate cell nuclei (Figure 3.5). Compared to the siNEG control, cells treated with siMEK showed little to no influenza NP at 48 hpi (Figure 3.5), although there was $10^{2.3}$ $TCID_{50}$/ml of infectious virus detected in siMEK-treated cells (Figure 3.4). Of the antiviral genes discovered (SERPIND1, MPN, CPZ, SENP1, KLK14, ELA1, CASP7, ADAM30), SERPIND1, CPZ, and SENP1 showed greatly increased NP staining compared to the siNEG control. Cells treated with siMPN showed increased staining, but the NP protein was not diffusely located throughout the well but instead isolated in large clumps of clustered, infected cells. Cells treated with siRNA against

KLK14, ELA1, CASP7, and ADAM30 showed reduced NP staining compared to the siNEG control. Genes that when silenced resulted in a decrease in viral titers as shown in Figure 3.4 correlated well with that data in the case of MMP14, CPE, GGTLA1, TFR2, PRSS12, PAPPA, MST1, MMP13, KLK12, DPP3, and ADAMTS7, with little to no NP detected, comparable to the siMEK control. However, siUSP52 and siCTSH-treated wells showed an increased amount of viral NP present in the cells, counter to the viral titers determined earlier. Cells treated with siGZMB also showed higher NP levels compared to cells treated with other downregulating siRNAs, but the overall NP detected was still less than the siNEG control. Cells treated with siADAMDEC1 had several large, strongly NP positive single cells throughout the well; however, the infection appeared not to spread to adjacent cells. Effect of gene silencing on the level of NP detected in transfected cells is summarized in Table 3.1.

Effect of host gene silencing on genomic replication. To determine the effect of host protease silencing on genomic replication, influenza M gene copies in the cell were quantified by qPCR. Briefly, cells were transfected per the standard protocol (Figure 3.1), and infected after 48 hours. Total RNA was isolated 48 hpi and tested for viral M gene levels. M gene levels did not correlate well with the phenotypes observed from the $TCID_{50}$ or NP assays. Cells treated with siNEG had $10^{7.6}$ M gene copies in the cell while siMEK-treated cells had a log less M gene copies. The 24 hit protease genes did not separate into two clear groups, less or more M gene, based on the phenotype observed in the other endpoint assays. Effect of gene silencing on the number of M gene copies in transfected cells is summarized in Table 3.1.

Discussion

The primary screen of the human protease library identified 24 positive genes (~5% of the human protease library) that when silenced had an effect on influenza replication. Of those genes, eight resulted in an increase of influenza viral titers when they were silenced, implying that these genes have a role in antiviral resistance, and silencing of the remaining 16 genes resulted in less or no viral titers, implying that these genes were somehow required for influenza replication and/or infection to occur. RNAi silencing of the genes that may be essential for influenza replication could provide new options for antivirals. However, to use a host gene drug target for a potential RNAi therapeutic, the gene must not be cytotoxic to the host cell when silenced. Of all the genes tested, only one gene hit was above the 20% cytotoxicity cutoff, SENP1 (Figure 3.3).

While most (63%) of the genes (SERPIND1, CPZ, SENP1, CPE, TFR2, PRSS12, PAPPA, MST1, MMP13, KLK12, GZMB, DPP3, CPA4, ADAMTS7, ADAMDEC1) showed a consistent phenotype across all three endpoint assays, several of the genes resulted in the opposite phenotype in one endpoint assay. For example, GGTLA1 showed decrease in both viral titers and the level of detectable NP when cells were treated with siGGTLA1, but M gene copy number was increased slightly above siNEG-treated cells. This could because the mechanism of inhibition when GGTLA1 is silenced is unknown, or it could be due to off-target effects, which are not repeatable across assays due to their nature. Similarly, siCTSH treatment resulted in decreased viral titers and M gene copies, but increased NP staining, and siELA1 showed increased titers and decreased NP staining and decreased M gene copies. This finding implies that silencing of some of the hit protease genes are likely off-target effects. For example, examination of the ELA1 gene

revealed that the ELA1 is evolutionarily silenced in human cells (38), indicating that a positive gene hit here is likely a off-target effect. Further work is needed to validate the 24 hit protease genes beyond the primary screen and ensure that the effect on phenotype seen is directly due to silencing of the target gene, and not to an off-target effect caused by the SMARTpool of siRNA itself.

The idea of targeting viral genes using RNAi as therapies is not new (1, 44, 52). Recently, targeting host genes instead of quickly mutating viral genes has shown promise for a variety of viral infections (28). In order to consider the hit protease genes for therapeutic action further validation of these genes is need. For example, SENP1 is not a good candidate for evaluation because silencing of SENP1 caused cytotoxicity beyond our designated cutoff. Furthermore, genes such as ELA1 and perhaps others simply may not be expressed ubiquitously in human respiratory tissue. Additionally, it will be important to validate whether these genes truly have an effect on influenza infection by using novel siRNAs targeting different portions of the mRNA, to ensure silencing of the target is the reason for the phenotype observed. Finally, elucidation of global cellular pathways in which these genes are involved may allow targeting of related, already established drug targets for other conditions, allowing us to quickly adapt therapies to other diseases and to allow us to more deeply understand the influenza virus.

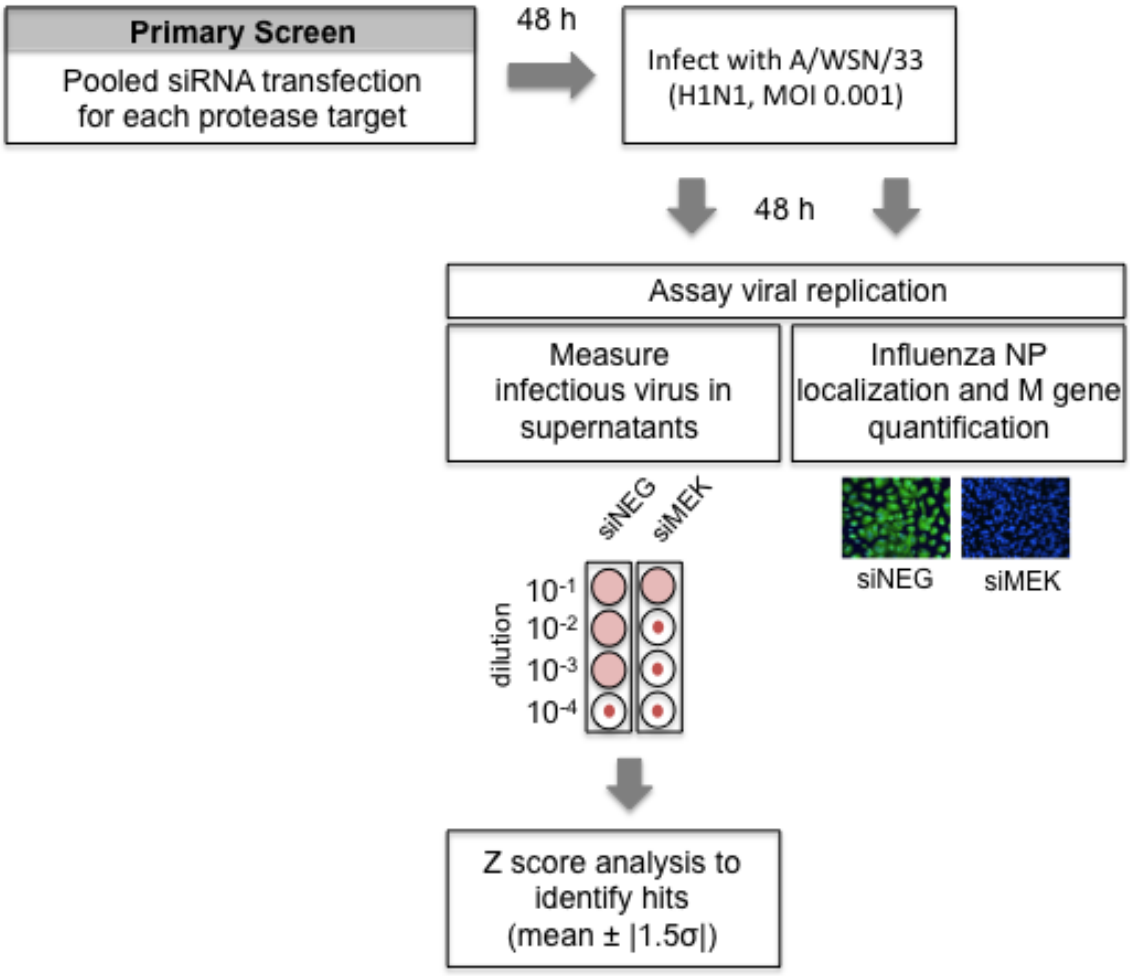

Figure 3.1: Methodology of the human protease screen. As the standard protocol for the primary RNAi screen, A549 cells were reverse transfected using a pool of siRNAs targeting each gene. After 48 hours, the cells were infected with A/WSN/33 (H1N1) at an MOI of 0.001. The infection was allowed to proceed 48 hours and infectious virus titers were determined by a modified $TCID_{50}$. Additional endpoints were determined by influenza NP localization by immunofluorescent staining and M gene quantification by qPCR. A gene was considered a hit if the z-score was > |1.5σ| from the mean z-score.

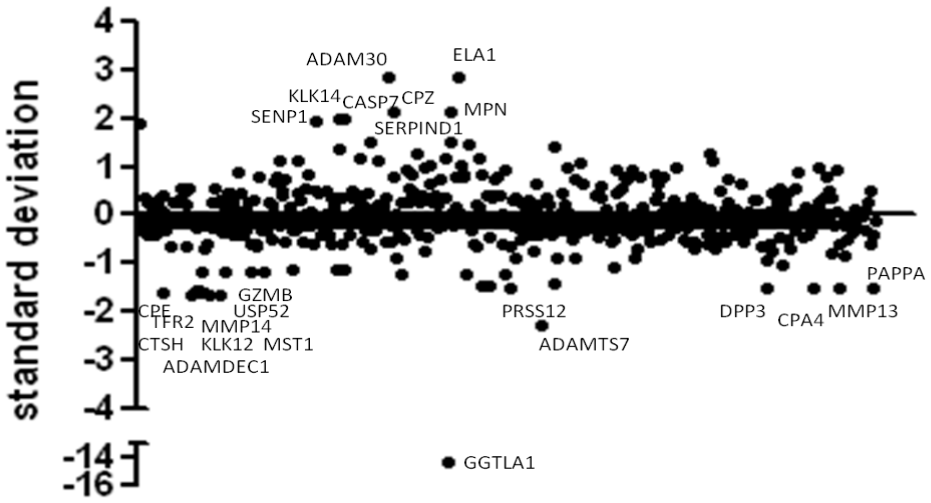

Figure 3.2: Z-scores of the primary screen. The y-axis indicates the standard deviation of the z-score. Labels indicate hit protease genes. The human protease library primary screen results were analyzed using a scaling methodology that sets the siNEG control at an arbitrary value based on the number of dilutions where virus was detectable by $TCID_{50}$, and the negative control siTOX at zero. siRNAs targeting host genes were assigned a score based on the distribution of these values. Z-scores were computed based on this data for each gene hit and those $\geq 1.5\sigma$ and $\leq -1.5\sigma$ were considered for validation. Data is indicative of two independent screens.

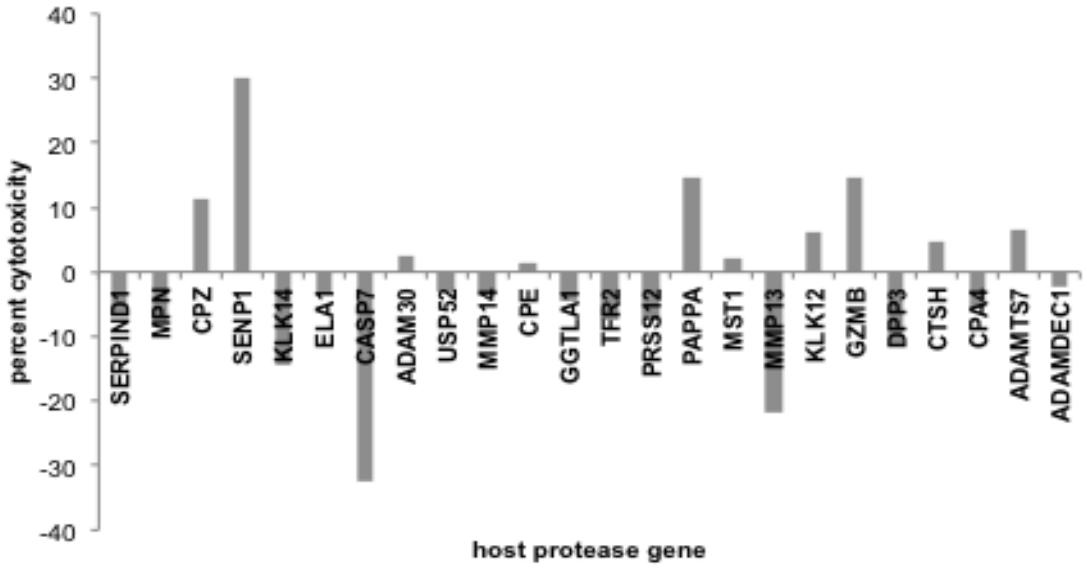

Figure 3.3: Cytotoxicity in A549 cells linked to protease gene silencing. Cytotoxicity was determined by adenylate kinase (AK) release in transfected cells. AK was normalized as a percentage to cells treated with the siTOX siRNA, which causes complete cell death, as a maximum, and to cells treated with siNEG as a minimum measure of cytotoxicity. Data is representative of two independent screens.

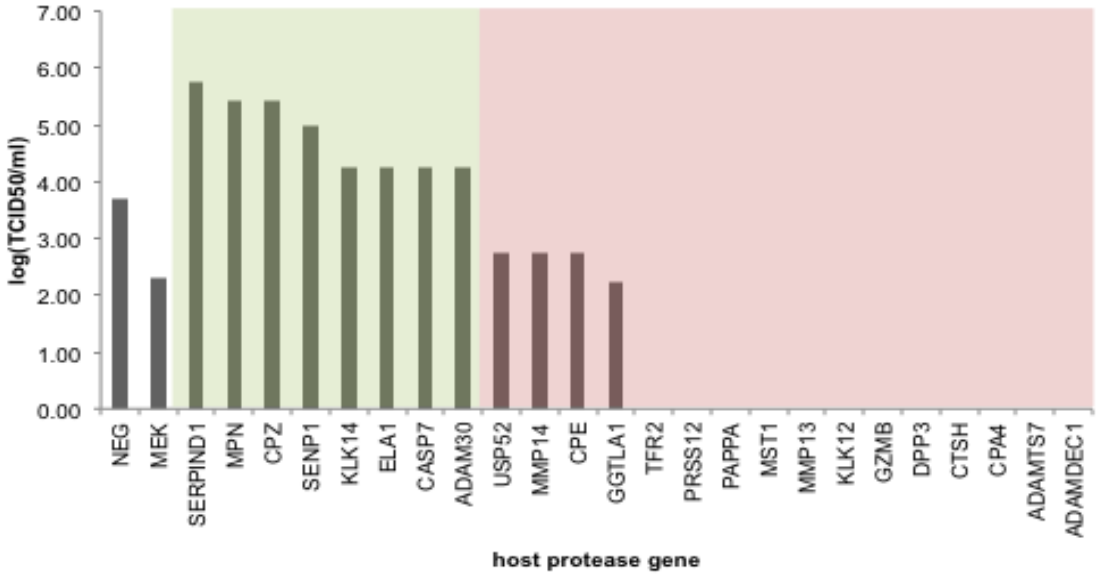

Figure 3.4: Infectious virus production after silencing of hit protease genes. For the primary screen, A549 cells were transfected with a pool of siRNA for the indicated gene. 48 hpi, virus titer was determined by modified $TCID_{50}$. Titers are expressed as $log(TCID_{50}/ml)$. The green box indicates genes that when silenced resulted in an increase of virus titers relative to the siNEG control, while the red box shows genes whose silencing resulted in a decrease of infectious virus. Data shown is from two independent screens.

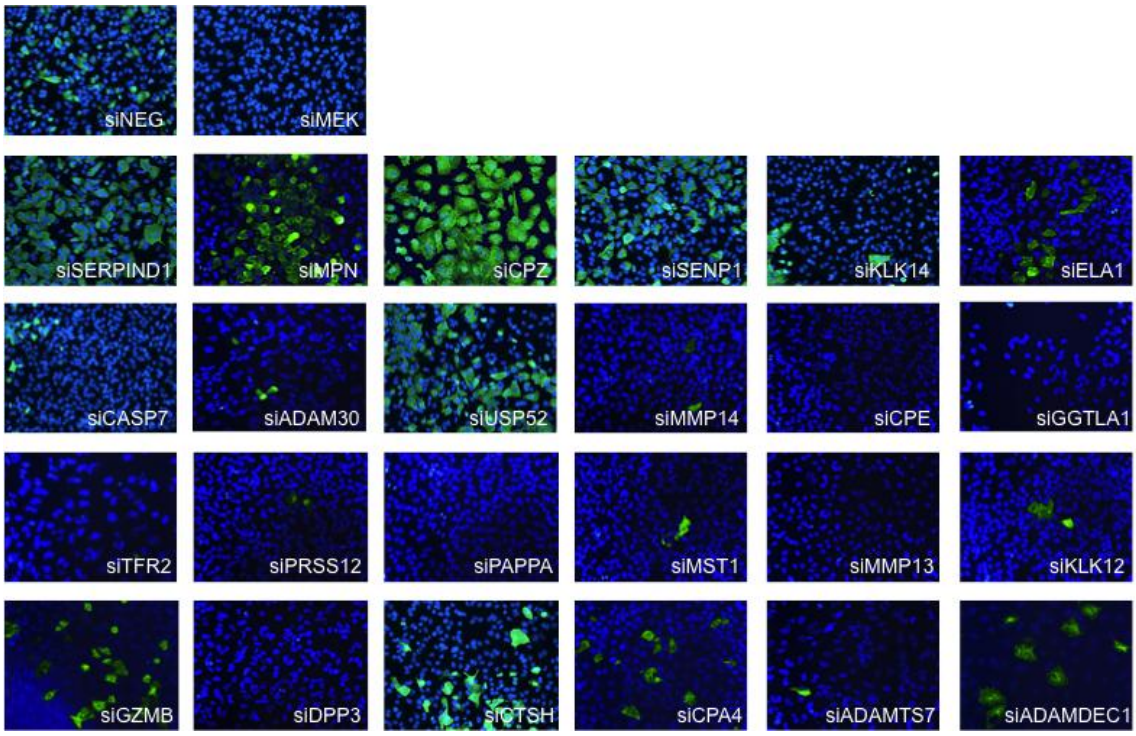

Figure 3.5: Effect of gene silencing on influenza NP localization. A549 cells were treated with the indicated siRNA and infected with influenza virus per the standard protocol. 48 hpi, cells were fixed and stained for NP protein (green). Nuclei were counterstained with DAPI (blue). Data is representative of two independent screens. Magnification is 20X.

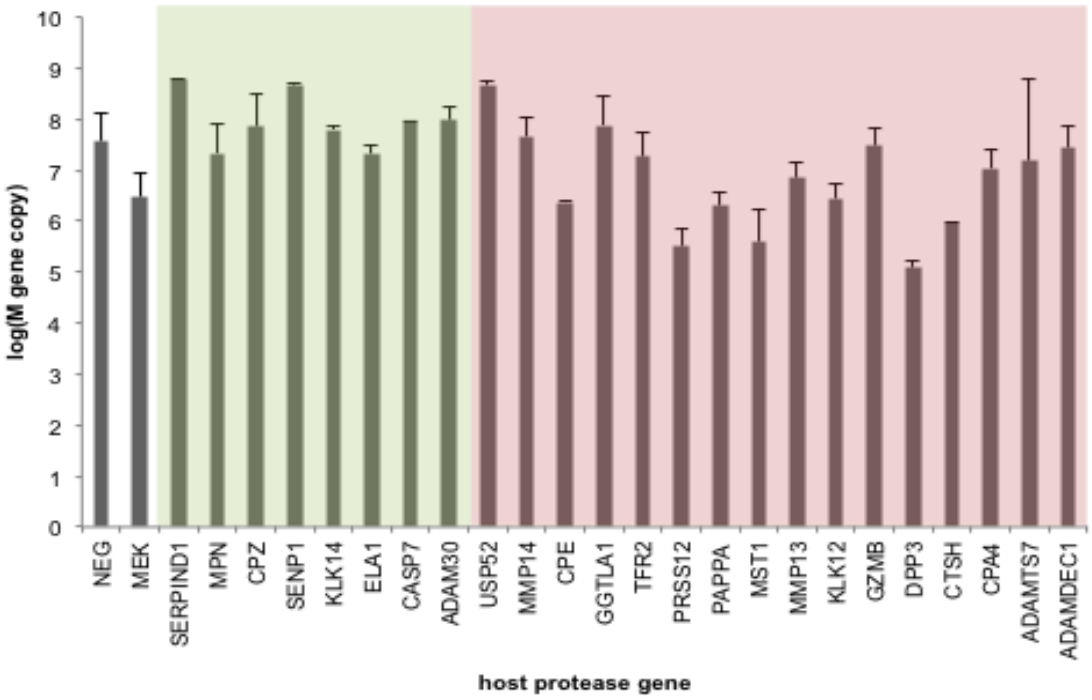

Figure 3.6: Effect of siRNA treatment on M gene levels. A549 cells were transfected with the siRNA pool for the indicated gene followed by influenza infection per the standard protocol. Cellular RNA was isolated 48 hpi and M gene levels were determined by qPCR. Data is expressed as log(M gene copy number). The green box indicates genes that when silenced resulted in an increase of virus titers relative to the siNEG control in the primary screen, while the red box shows genes whose silencing resulted in a decrease of infectious virus. M gene copy was normalized to an M plasmid control. Data is representative of two independent screens.

Table 3.1 Primary screen hits and effect on influenza replication.

	Gene name	Accession number	Effect when silenced in A549 cells			SMARTpool RNAi sequences
			log($TCID_{50}$/ml)[1]	NP localization[1]	log(M gene copy)[1]	
NEG	–	–	3.69	–	7.56	UAGCGACUAAACACAUCAA
MEK	mitogen-activated protein kinase kinase 1	NM_002755	↓(2.31)	↓	↓(6.47)	GCACAUGGAUGGAGGUUCU GCAGAGAGAGCAGAUUUGA GAGCAGAUUUGAAGCAACU CCAGAAAGCUAAUUCAUCU
SERPIND1	serpin peptidase inhibitor, clade D	NM_000185	↑(5.75)	↑	↑(8.77)	GAGAAGUGCUUCUGCCGAA CGAAAUUCAAGCUGGAGAA CGAAAUUCAAGCUGGAGAA GAACAAGUGCACUCGAUUU
MPN	(PRSS27) protease, serine 27 (marapsin)	NM_031948	↑(5.41)	↑	↓(7.33)	CCAGUGCCCUUCACCAAUU CCAGGAUGCUGAACCGAAU GCGCACUGCUUCCGCAACA CAGCAAAGACACCGAGUUU
CPZ	carboxypeptidase Z	NM_003652	↑(5.41)	↑	↑(7.85)	CAAAGGCAAUCAUGAAGUG UCAAAGGUGUGGUGACAGA GCUAUGACCCGCUGGAGAA GCUUUGAGAUCACGGUAGA
SENP1	SUMO1/sentrin specific peptidase 1	NM_014554	↑(4.99)	↑	↑(8.66)	GGAAAGAGUUUGACACCAA GCAAAUAUGCUGACUGUAU GAUCAUCAAUUUCUACAUG GCAUUUCGCCUGACCAUUA
KLK14	kallikrein-related peptidase 14	NM_022046	↑(4.25)	↓	↑(7.80)	CAGAAGGCCUAUCCUAGAA GAAACGAUGCGGGACAAAU GAUAAUUGGUGGCCAUACG GAAGCUGGAUUGAGGAAAC
ELA1	elatase 1, pancreatic	NM_001971	↑(4.25)	↓	↓(7.32)	GCUUACAUCUCCUGGAUAA UAACGUGGCUGCCGGCUAU GCGUUACCCUCAAUAGCUA CAAUAGGACUUGCGAUCAA
CASP7	caspase 7, apoptosis-related cysteine peptidase	NM_033340	↑(4.25)	↓	↑(7.95)	GGGCAAAUGCAUCAUAAUA GAACUCUACUUCAGUCAAU UACCGUCCCUCUUCAGUAA

						CCAGACCGGUCCUCGUUUG
ADAM30	ADAM metallopeptidase domain 30	NM_021794	↑(4.25)	↓	↑(7.98)	GGAGCAACAUGUCUAAAUA GCGAUUCCCUCGUCAAUUU GAACAACAAUCUUUCUCAA GAGCAUACGACUAUAAUUU
USP52	ubiquitin specific peptidase 52	NM_014871	↓(2.75)	↑	↑(8.67)	GCAUGAAGCUGUGCAGUUU GCCGAUAUCUUUCAUGUGA GAACAAGGAAUUUGCUUUG UAACAACUCUCAAGUCUAC
MMP14	matrix metallopeptidase 14	NM_004995	↓(2.75)	↓	↑(7.66)	GAACAAAUACUGGAAAUUC GGUCUCAAAUGGCAACAUA GCAAAUUCGUCUUCUUCAA UCAAAUGGCAACAUAAUGA
CPE	carboxypeptidase E	NM_001873	↓(2.75)	↓	↓(6.37)	GAAAGAAGGUGGUCCAAAU GCUUAUACCUGGAAACUAU GGAAUAGACCACGAUGUUA GGAUGCAAGACUUCAAUUA
GGTLA1	gamma-glutamyltransferase-like activity 1	NM_001099782	↓(2.75)	↓	↑(7.88)	GGACAGGCAUCAUCCUCAA GGUCGAAGCUAGUGAUUGG GGAGGCCGCAGGCUACUAA CUGAAGGGAGGGUGAACGU
TFR2	transferrin receptor 2	NM_003227	↓(n.v.)[2]	↓	↓(7.26)	ACACAAAGGAGGACACUUA CUCAAGGAGUGCUCAUAUA UCUGGAACAUUGAUAACAA AGGAGAGAGACGAGCGACU
PRSS12	protease, serine, 12 neurotypsin (motopsin)	NM_003619	↓(n.v.)	↓	↓(5.49)	GGACUGAGCUGAAUACAUA GAUGAUGGAUGGACUGAUA GAGCAUAACUGUGGCCAUA GGACGAGCCUAUUCAAGAA
PAPPA	pregnancy-associated plasma protein A, pappalysin 1	NM_002581	↓(n.v.)	↓	↓(6.30)	GGACAGACAUUGUGUGACA GAACCAAGGUGAUAGAUCU GGAGAAUCCUGGUGCAGUA AGCCCAAGGUGGUGCGCUA
MST1	macrophage stimulating 1 (hepatocyte growth factor-like)	NM_020998	↓(n.v.)	↓	↓(5.58)	GUACGGACCUGCAUCAUGA GGAAUGGCCUGGAAGAGAA GCUGUGACCUCUUCCAGAA CGACAACUAUUGCCGGAAU
MMP13	matrix	NM_002427	↓(n.v.)	↓	↓(6.85)	GAAGAUAAGUGCAGCUGUU

	metallopeptidase 13					GGAGAAACAAUGAUCUUUA GGAGAUAUGAUGAUACUAA CUACAAAUCUCGCGGGAAU
KLK12	kallikrein-related peptidase 12	NM_145895	↓(n.v.)	↓	↓(6.44)	GGGAGAAUCACGAGCAACA GCACUGAGUGUGGGCGUAA CUGGGAACUUCUUGGAACU GCAGCCACACCGAAGAUUU
GZMB	granzyme B (granzyme 2, cytotoxic T-lymphocyte-associated serine esterase 1)	NM_004131	↓(n.v.)	↓	↓(7.47)	CUACAUGGCUUAUCUUAUG CCACGAGCCUGCACCAAAG CGACAGUACCAUUGAGUUG GAAGAUCGAAAGUGCGAAU
DPP3	dipeptidyl peptidase-3	NM_005700	↓(n.v.)	↓	↓(5.09)	ACACGGUGCUGCUGCGUAA GAGGGAAUCACCACCUAUU GCAGCAAGAUCCGGUCUGU GCUCAGACGUGCAGCUUCU
CTSH	cathepsin H	NM_148979	↓(n.v.)	↑	↓(5.96)	GACAUGAGCUUUGCUGAAA GAUAAACGCCCACAACAAU GCCAGGCUUUCGAGUAUAU GCGCCCAGGACUUCAAUAA
CPA4	Carboxypeptidase A4	NM_016352	↓(n.v.)	↓	↓(7.03)	GAACGGAGCAGUAAUAACU GAGACGAGAUCAGCAAAUU GGAUAUGUGUAUACUCAAA UGACAACGGCAUCAAAUUU
ADAMTS7	ADAM metallopeptidase with thrombospondin type 1 motif, 7	NM_014272	↓(n.v.)	↓	↓(7.18)	GCGAGGACCCGGAGAAGUA GAACGUGGGCUGUGACUUC CAACGAGGACUACUUCAUU GCAGAUACUGUGUGGGUGA
ADAMDEC1	ADAM-like, decysin 1	NM_014479	↓(n.v.)	↓	↓(7.42)	GGAAACAUCCUAAAUGAAA GAGAGGAAAUUACCACGAA GCAAACACCUGAAUUAACG CACCAUAGAUGUUCAAGUG

[1]Relative to siNEG control, [2]n.v., no virus

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CHAPTER 4

PATHWAY ANALYSIS AND MicroRNA REGULATION OF HUMAN PROTEASE GENES ESSENTIAL FOR INFLUENZA VIRUS REPLICATION[1]

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Abstract

Influenza A virus causes seasonal epidemics and periodic pandemics threatening the health of millions of people each year. Vaccination is an effective strategy for reducing morbidity and mortality, and in the absence of drug resistance, the efficacy of chemoprophylaxis is comparable to that of vaccines. However, the rapid emergence of drug resistance has emphasized the need for new drug targets. Knowledge of the host cell components required for influenza replication has been an area targeted for disease intervention. In this study, the human protease genes required for influenza virus replication were determined and validated using RNA interference approaches. The genes validated as critical for influenza virus replication were ADAMTS7, CPE, DPP3, MST1, and PRSS12, and pathway analysis showed these genes were in global host cell pathways governing inflammation (NF-κB), cAMP/calcium signaling (CRE/CREB), and apoptosis. Analyses of host microRNAs predicted to govern expression of these genes showed that eight miRNAs regulated gene expression during virus replication. These findings identify unique host genes and microRNAs important for influenza replication providing potential new targets for disease intervention strategies.

Introduction

Influenza A viruses generally cause seasonal epidemics however they have the potential to cause pandemics associated with substantial morbidity and mortality (30, 60). Development of seasonal vaccines is required for influenza virus due to high viral mutation rates that lead to antigenic drift, and also because of periodic antigenic shift which can render vaccines less or ineffective (23). There are four antiviral drugs that have proven efficacy in the treatment of influenza infections: two M2 ion channel inhibitors (amantadine and rimantadine) and two neuraminidase inhibitors (zamamivir and oseltamivir) (4, 6, 27, 28, 42, 45). Early treatment with these antiviral drugs reduces the duration of symptoms and the time to recovery; however, the use of antiviral drugs is complicated by the emergence of drug resistant viruses (5, 6, 17, 27, 38). In addition, antiviral drug use may come with unwelcome effects that could include an increase in population vulnerability due to lack of seroconversion, as well as driving drug resistance among circulating strains (17). Thus, it is critical to discover new targets for chemoprophylactics and treatment.

Recent advances in our understanding of RNA interference (RNAi) have provided a means to perform genome-wide screens to determine and validate host cell genes that may be required for influenza virus replication (26, 35). RNAi is an efficient mechanism for the sequence-specific inhibition of gene expression (40, 61), and is mediated by small interfering RNAs (siRNA) incorporated in the RNA-induced silencing complex (RISC) where the antisense or guide strand of the siRNA can suppress protein expression or direct degradation of messenger RNAs that contain homologous sequences (22, 33, 65). Synthetic siRNAs can be readily developed to target viral or host genes and have been

successfully applied in disease intervention approaches. For example, siRNA targeting respiratory syncytial virus has shown efficacy for silencing virus replication (1, 3, 7, 19, 67, 68), a feature that has led to RNAi-based clinical trials as a new therapeutic option (19). In addition, there are promising results from targeting host genes, such as the use of siRNA silencing for the treatment of age-related macular degeneration (2), and in the case of influenza, inhibiting the host gene CAMK2B prevented vRNA transcription in vitro (34). Recently, several studies employed genome-wide RNAi screens to identify host genes required for influenza virus infection and replication (13, 24, 31, 34, 52), and genes have also been identified by random homozygous gene perturbation (59) and by a proteomic screen (12). Although there were few common genes detected among the studies, meta-analysis revealed that influenza virus was co-opting many of the same host cell pathways (13, 24, 31, 34, 52). Thus, the inability to find the same genes among the studies is not unexpected given that multiple genes may be affected in the same host cell pathway, that the tempo of gene expression may vary among the cell lines studied, and that differences can be attributed to variations in methodologies, viruses, and cell lines used among the studies (13, 24, 34).

Of the host genes known to affect influenza virus, the proteases are important for infection and replication. Proteases may affect virus infection and replication in several ways including viral entry and hemagglutinin (HA) processing (8-10, 32), degradation of viral components for MHC presentation (46), cap-snatching (11), induction of apoptosis (62), and by increasing vascular permeability aiding in the development of systemic infection in cases of severe infection (64). However, it remains unclear which protease genes are essential in the biology of influenza virus replication.

In addition to host gene involvement during viral infection, the tempo of host gene expression may be altered by a variety of factors, such as by microRNAs (miRNA). Host miRNAs are similar to siRNAs in their silencing mechanism, but miRNAs are generated in the nucleus from short hairpin precursors and must be processed and exported before entering the RISC pathway (16). miRNAs can be generated by the virus (21, 48) or the host and can silence genes by similar mechanisms to siRNAs; however, unlike siRNA silencing, miRNA sequences do not need to be homologous to the target mRNA (16). Little is known about the role of miRNAs during virus infection, however host-derived miRNAs have been shown to negatively affect influenza replication (55) and miRNAs have even been used as therapeutics (39, 50). We considered that host miRNAs might also have a role in host gene modulation during influenza infection.

To comprehensively determine the host proteases required by influenza virus for infection and replication, a siRNA screen of all known 481 protease genes was performed in human lung epithelial (A549) cells using the influenza virus, A/WSN/33. Twenty-four primary gene hits were found in the siRNA screen of which five (ADAMTS7, CPE, DPP3, MST1, and PRSS12) were validated as critical for replication. Pathway analysis revealed that these five genes were linked to cAMP responsive binding element (CREB), NF-κB, and apoptosis pathways, thus we examined and verified host gene regulation of these pathways. As the tempo of host gene expression is governed by miRNAs which have important roles in regulating host genes during viral infection and replication (14, 41, 48), an additional analysis was performed to identify miRNAs that might potentially regulate the five validated host genes required for influenza virus replication. A library of miRNA antagonists was used to confirm miRNA regulation, and several miRNAs were

identified to affect virus replication and host gene regulation, notably miR-1254, miR-106B, miR-106B*, miR-124-a, and miR-124*. This finding shows varied miRNA expression patterns associated with the regulation of various genes involved in global host cell pathways governing inflammation (NF-κB), cAMP/calcium signaling (CRE/CREB), and apoptosis, and adds to our understanding of how miRNAs may govern host gene regulatory networks involved in the response to influenza virus replication.

Materials and Methods

Cells and virus stocks. A549 cells (ATCC CCL-185) were cultured in Dulbecco's modified Eagle's medium (DMEM) (HyClone, Logan, UT) containing 5% heat-inactivated FBS (HyClone, Logan, UT). Cells were frozen in 10% DMSO and 90% FBS to create one stock of a single cell passage that was used for the entire screen. A stock of MDCK cells (ATCC CCL-34) was also propagated and stored using the same conditions. A/WSN/33 (H1N1) influenza virus was used for the primary siRNA screen as this virus has the ability to replicate without the need for exogenous trypsin (54). For the validation studies, A/New Caledonia/20/99 (H1N1) influenza virus was also used. All viruses were propagated in 9-day-old embryonated chicken eggs as previously described (66). Viruses were titrated in MDCK cells and titers calculated by the method developed by Reed and Muench (49).

Protease library screen. A primary screen using four pooled siRNAs to target each gene of the 481 genes in the human protease library (SMARTpool; Dharmacon ThermoFisher, Lafayette, CO) was performed using A/WSN/33 influenza virus and type II human alveolar pneumocytes (A549 cells) similar to a method previously described

(34). siRNAs were resuspended in Dharamcon siRNA buffer to a concentration of 1 uM and stored at -80°C until use. The screen was conducted in two steps, a primary screen targeting all 481 human protease genes, followed by a smaller-scale validation screen of the primary hits to confirm the genes identified were essential for influenza virus replication. In all studies, a siRNA targeting the MEK gene (siMEK), a well-characterized human kinase gene important for influenza replication (4, 36), was used to control for the transfection efficiency and host gene silencing. A non-targeting siRNA control (siNEG) was also used in all assays. The siRNA SMARTpool library constituents, siMEK, and siNEG transfected A549 cells at >85% efficiency, and a few (<2%) siRNA pools targeting HP genes induced cytotoxicity as determined by adenylate kinase (AK) release in the cell culture supernatant (18, 47) and by visual inspection of cell morphology.

A549 cells were reverse transfected with the siRNA 48 hours before infection with influenza A/WSN/33 (MOI = 0.001). The amount of infectious virus was measured 48 hpi by titration of A549 cell supernatant on MDCK cells, and the results normalized to siNEG-treated cells. All assays were run in duplicate and the entire screen assay was repeated twice. Both plate-based controls and assay-wide controls, e.g. siMEK, siNEG, and siTOX, were included on each individual assay plate. The results were normalized to the plate median for the SMARTpools. After excluding cytotoxic siRNAs based on detection of AK, primary screen data was subject to analysis. The primary screen, performed as two independent studies, was analyzed using a scaling methodology that sets the non-targeting control siRNA (siNEG) at an arbitrary value of 1.0, and the negative control siTOX at zero. siRNAs targeting host genes were assigned a score based

on the distribution of these values. Wells in the primary screen with a percent of differentiation $\geq|1.5|$ standard deviations from the plate mean in both duplicate assays were considered primary hits. Of the 481 HP genes targeted, twenty-four genes were identified as "primary hits". Hits that caused greater than 20% cytotoxicity in A549 cells were excluded (Figure 4.1A). Z-scores were computed based on this data for each gene hit and those $\geq 1.5\sigma$ and $\leq -1.5\sigma$ were considered for validation.

Reverse transfection. Lyophilized siRNAs in 96-well plates were diluted with HBSS (HyClone, Logan, UT) and allowed to incubate for 5 minutes. Dharmafect-1 transfection reagent (Lafayette, CO) and HBSS were added such that each well received 0.004 ml of transfection reagent and 0.096 ml of HBSS. The siRNA/transfection reagent mix was allowed to incubate for 20 minutes at room temperature after which 0.08 ml of 1.5×10^4 A549 cells suspended in DMEM/5% FBS was added to each well, and the plate incubated for 48 hours at 37°C in 5% CO_2. The final concentration of siRNA for all primary screen transfections was 50 nM.

Cytotoxicity and virus infection. To determine if siRNA gene silencing was cytotoxic, the cell supernatants from siRNA transfected A549 cells were analyzed for adenylate kinase (AK) using a Toxilight kit (Lonza, Rockland, ME). Results were normalized to a siTOX control, i.e. a siRNA control (Dharmacon) causing complete cell death by 48 hours. siRNA transfected cells with luminescence greater than or equal to 20% of the siTOX control were not considered for further evaluation. A549 cells were subsequently infected with A/WSN/33 at an MOI of 0.001 pfu/cell. Cells were incubated for 48 hours at 37°C/5% CO_2.

Endpoint assays. Virus titers in siRNA-treated A549 cells infected with A/WSN/33 were determined by modified $TCID_{50}$ or hemagglutination assay (HA). Briefly, virus infected A549 cell culture supernatants were serially diluted ten-fold, and added to MDCK cells. The MDCK cell plates were incubated for 72 h, followed by an HA using 0.5% chicken red blood cells as previously described (58). To further identify gene hits, the cells were screened for the magnitude and localization of nucleoprotein (NP) staining. For NP staining, the cells were fixed with 3.7% formaldehyde and stained with anti-NP monoclonal antibody (5 ug/ml; H16-L10-4R5) and the antibody staining detected using Alexa Fluor 488 labeled goat anti-mouse IgG (1 ug/ml; Invitrogen, Carlsbad, CA). Cells were counterstained with DAPI (2 ug/ml) (Invitrogen, Carlsbad, CA) and visualized by immunofluorescent microscopy (20X, EVOS digital inverted fluorescent microscope, Advanced Microscopy Group, Bothell, WA). As an additional hit identification endpoint, real-time qRT-PCR analysis was performed to quantify influenza M gene copy number in the cells as previously described (56). Briefly, RNA was purified using the RNeasy kit (Qiagen, Valencia, CA), and cDNA was synthesized using a SuperScript First Strand cDNA synthesis kit (Invitrogen, Carlsbad, CA) and appropriate primer/probe set (56) to detect the M gene. PCR was performed using the amplification cycle: 10 minutes at 95ºC followed by 40 cycles of 95ºC for 30 seconds, 60ºC for 1 minute, and 72ºC for 30 seconds. M gene copies were normalized to the siNEG control.

Validation of gene hits. Individual novel siRNAs (Dharmacon) were used to target a different seed site on the same gene for gene hits identified during the primary screen (Table 4.1). Gene silencing was confirmed by qPCR using SybrGreen (Qiagen, Valencia, CA) to detect the dsDNA product allowing for quantification of gene silencing relative to

cells treated with the siNEG control. Primers targeting each individual hit were compared to control GAPDH levels. For phenotype validation, A549 cells were transfected with 100 nM siRNA, incubated 48h at 37°C in 5% CO_2, infected with A/WSN/33 (MOI = 0.001), and the amount of infectious virus was measured 48 hpi by $TCID_{50}$ and NP staining assays to validate the screen phenotype previously observed. From the primary gene hits, five genes essential for influenza virus replication were validated using the novel siRNAs. The five genes were also tested using A/New Caledonia/20/99 at an MOI of 0.001 as described above; however infection was done in the presence of TPCK-trypsin (1 ug/ml).

Host cell pathway analysis. To survey the spectrum of host cell pathways that may be linked to the validated gene hits, pathway analysis was performed using Ingenuity Pathway Analysis software (Ingenuity Systems, http://www.ingenuity.com). The results provided network modeling of the validated genes and identified several functional groups (NF-κB, CRE/CREB signaling, apoptosis) linked to protease gene expression, host cell miRNA regulation, and influenza replication.

Reporter system assays. Reporter plasmids used to confirm pathway were obtained from SABiosciences/Qiagen as a dual luciferase Cignal Reporter Assay Kit. The reporters consisted of a transcription factor of interest linked to a firefly luciferase gene, a positive control plasmid (luciferase linked to a CMV promoter), and a negative control plasmid (luciferase with no promoter). A549 cells were co-transfected with 100 nM of siRNA and 100 ng of the appropriate plasmid (reporter, positive, or negative control plasmids) using either the SureFECT (SABiosciences) or Attractene (Qiagen) transfection reagents. As a transfection control, the plasmid kit also contains a Renilla

luciferase plasmid fused to a CMV promoter, and all data was normalized to Renilla luciferase expression. After 24 h of transfection, cells were allowed to rest for one day and then infected with A/WSN/33 (MOI = 0.001) for 24 h. Cell lysate was taken and luciferase expression was measured using a Safire2 Microplate reader (Tecan, Männedorf, Switzerland).

Apoptosis array. A human apoptosis array was obtained from SABiosciences/Qiagen. A549 cells were transfected with 50 nM of either siDPP3 or siNEG. After 48h, cells were infected with A/WSN/33 at an MOI of 0.001. RNA was isolated 18 hpi, a time point chosen because it is late enough for apoptosis modulation to become evident at the transcriptional level, but sufficiently early such that cells were not destroyed (51), and then treated with DNase I to remove any genomic DNA. cDNA was synthesized using an RT^2 First Strand Kit (SABiosciences) and PCR was performed using RT^2 SYBR Green Master Mix (SABiosciences) per the manufacturer protocol. Data was analyzed by calculating $2^{(-\Delta\Delta Ct)}$. Silencing of siDPP3 relative to siNEG-treated cells was confirmed for each independent experiment.

miRNA studies. A library of miRNA hairpin inhibitors synthesized as RNA oligonucleotides with novel secondary structure designed to inhibit the function of endogenous miRNA, and chemically enhanced to improve efficacy and longevity (miRIDIAN, Dharmacon, Lafayette, CO) were used to target host cell miRNAs identified as potential regulators of host genes validated as important for influenza virus replication. miRNA mimics were not used since treatment with mimics results in extremely high concentrations of miRNA relative to biological levels. In these studies, A549 cells (1.5×10^4) were transfected with 25 nM of an appropriate miRNA inhibitor using Dharmafect-1

per the manufacturer's protocol. After 48 h, the cells were infected with A/WSN/33 at an MOI of 0.001 for an additional 24h or 48h. Viral replication was assayed by $TCID_{50}$ and qPCR for M gene as described above. Gene specific primers were also used to quantify changes in host gene expression in cells receiving the miRNA inhibitors using SybrGreen as described above.

Statistics. The primary siRNA protease gene screen was performed independently and in duplicate. HA results for each gene were assigned values 0 – 8 based on the dilution of the HA readout. HA values were normalized to the average of the siNEG control readout per plate. Robust z-scores ($Z = xi - x/sx$) were used as the normalizing method and calculated for each gene to determine hits based upon the standard deviation where *xi* is the raw measurement on the *i*th siRNA, and *sx* are the mean and the standard deviation, respectively, of all measurements within the plate. Normalization of raw data removes systematic plate-to-plate variation, making measurements comparable across plates. Statistical analyses for the cross-strain validation studies, the reporter studies, and miRNA studies were performed using GraphPad Prism software using the Mann-Whitney U test.

Results

Human protease genes required for influenza replication. A primary RNAi screen of 481 host protease genes in a human type II respiratory epithelial cell line (A549) identified 24 genes important for A/WSN/33 influenza virus replication (Figure 3.1). The gene hits identified were $\geq 1.5\sigma$ and $\leq -1.5\sigma$ from the mean Z-score. Endpoint validation of the gene hits in the primary screen included influenza NP cell localization determined by

immunohistochemistry, determining the level of infectious virus by $TCID_{50}$ assay, as well as influenza M gene copy number determined by qPCR (Figure A.2). For validation of primary gene hits, a novel siRNA targeting the same gene but at a different seed site (Table 4.1) was required to produce the same phenotype as observed in the screen. After validation, five genes were identified that decreased virus replication when silenced: ADAMTS7, CPE, DPP3, MST1, and PRSS12 (Table 4.1). Silencing of these protease genes did not cause cytotoxicity in A549 cells compared to the siTOX control. siMEK and siNEG both displayed low level cytotoxicity possibly due to the importance of MEK in cellular signaling, and the induction of an inflammatory response to siNEG due to off-target effects (Figure 4.1A). Silencing of the five validated genes resulted in considerably decreased influenza NP staining compared to the negative (-) control, and was consistent with low NP staining in cells treated with the positive (+) siRNA control, siMEK (Figure 4.1B). The level of infectious virus from A549 cells treated with 100 nM of a single siRNA targeting each of the validated genes was greatly (siCPE, siMST1 and siPRSS12) and significantly ($p < 0.05$; siADAMTS7 and siDPP3) reduced (Figure 4.1C). Influenza virus M gene levels reflected the low level of infectious virus (Figure A.2) and NP staining observed for A549 cells treated with siRNAs targeting the five validated genes, as well.

Host genes validated with a clinical influenza virus isolate. Since A/WSN/33 was used in the primary and secondary screens because of its ability to grow in the absence of trypsin, a feature facilitating high throughput screening, cross-validation of ADAMTS7, CPE, DPP3, MST1, and PRSS12 genes was performed in A549 cells with A/New Caledonia/20/99. Using the same siRNAs previously used to validate the genes (Figure

4.1C), A549 cells treated with 100 nM of the siRNAs significantly ($p < 0.05$) reduced A/New Caledonia/20/99 virus replication (Figure 4.2). The differences in viral titer were at least 1.5 logs lower compared to siNEG-treated cells for all five genes examined. These results show that ADAMTS7, CPE, DPP3, MST1, and PRSS12 contribute to influenza A virus replication, and provide promising disease intervention targets.

Cell pathways associated with the validated host genes affect influenza replication. Expanding from the primary screen toward defining host cell pathways involved in influenza virus replication requires assessment of the signaling pathways, molecular networks, and biological processes that are linked to the five validated host genes. Dynamic pathway analysis identified three major pathways for the five validated genes, i.e. NF-κB pathway, CRE/CREB signaling pathway, and cellular apoptosis (Figure 4.3). To verify the host genes in these pathways, A549 cells were transfected with a luciferase assay reporter plasmid governed by the promoter of interest linked to a firefly luciferase gene. The level of pathway activation was determined by luciferase expression, which was normalized to a control plasmid expressing Renilla luciferase to account for transfection efficiency. Pathway analysis suggested that MST1 and PRSS12 genes participate in the CRE/CREB signaling pathway. RNAi of MST1 by siRNA targeting (siMST1) led to a significant ($p < 0.05$) increase in CRE/CREB signaling compared to the siNEG and CRE controls in the presence or absence of influenza virus infection (Figure 4.4A). RNAi of PRSS12 by siPRSS12 did not affect CRE/CREB activation relative to controls. Interestingly, RNAi of DPP3 using siDPP3 significantly ($p < 0.05$) increased CRE/CREB activation under mock-infected conditions despite DPP3 not being implicated in the CRE/CREB pathway, and silencing of ADAMTS7 significantly ($p <$

0.05) abrogated CRE/CREB activation during infection. Pathway analysis also indicated that ADAMTS7, CPE, and MST1 genes were potentially involved in the NF-κB activation pathway (Figure 4.3). siADAMTS7 significantly ($p < 0.01$) abrogated NF-κB activation regardless of infection or mock-treatment (Figure 4.4B). Interestingly, a similar finding was observed for DPP3, a gene not previously linked to the NF-κB signaling pathway by our analysis. siCPE did not affect NF-κB activation under mock conditions, but during infection NF-κB activation levels were significantly ($p < 0.05$) downregulated. siMST1 also had a negative effect on NF-κB activation regardless of infection. Elevated NF-κB levels were also seen in controls receiving the NF-κB plasmid alone but this was not significant compared of siNEG controls.

Dynamic pathway analysis predicted DPP3 to be involved in apoptotic pathways (Figure 4.3), and as a luciferase/Renilla pathway reporter system was unavailable, a human apoptosis array was performed to determine the level of mRNA expression of various pro- and anti-apoptotic host genes in relation to DPP3 expression (Figure 4.5). After confirming RNAi silencing (> 95%) of DPP3 by PCR (data not shown), gene expression was compared in cellular RNA extracted from infected A549 cells treated with either siDPP3 or siNEG (50 nM). While most genes tested did not vary in expression levels relative to siNEG-treated cells, four pro-apoptotic genes were found to be substantially increased when DPP3 was silenced: BCL2L10, TNFSF10, TNFSF25, and TNFSF8. Of these four genes, TNFSF10 was significantly ($p < 0.05$) increased 6-fold, and TNFSF8 was significantly ($p < 0.05$) increased 5-fold compared to siNEG control treated cells. TNFSF25 was substantially increased as well. These findings show that inhibition of DPP3 expression initiates higher expression of pro-apoptotic factors,

features that may negatively impact influenza virus replication and associated modulation of cellular apoptosis.

miRNAs govern host genes required for influenza replication. Pathway analysis of the five validated host genes revealed potential miRNA interaction (Figure 4.6) with eight miRNAs (miR-1254, miR-1272, miR-17-5p, miR-17-3p, miR-106B, miR-106B*, miR-124-a, and miR-124*). To determine the role of these miRNAs in regulating ADAMTS7, CPE, DPP3, MST1, and PRSS12, A549 cells were treated with miRNA hairpin inhibitors as previously described (63), or treated with a negative control from *C. elegans* that shares no homology with known human miRNA sequences, and gene expression levels determined by qPCR. As gene modulation by miRNAs is usually subtle and multi-targeted, the effect of the miRNA inhibitors on host gene mRNA levels was determined 24 hours post-treatment. All 8 miRNA inhibitors were tested for their effect on each of the 5 validated genes; however, only those genes whose expression was affected by miRNAs are shown (Figure 4.7). At 24h post-treatment, ADAMTS7 gene expression levels increased 20-fold when miR-106B was inhibited, and 40-fold when miR-124* was inhibited (Figure 4.7A). Treatment with siRNA targeting the gene examined was performed as a control. CPE expression was slightly decreased by miRNA inhibitors, as there was no response above 1.0 (Figure 4.7B). In contrast, inhibition of miR-106B and miR-124* resulted in a >20-fold and >40-fold increase of DPP3 gene expression, respectively (Figure 4.7C), while miR-1254, miR-1272, and miR-17-3p inhibition caused a decrease of DPP3 expression. MST1 expression was increased to similar levels as DPP3 with the same miRNA inhibitors, while the other miRNA inhibitors resulted in a slight (but significant for miR-17-3p) decrease in expression

(Figure 4.7D). Finally, PRSS12 expression levels were significantly ($p < 0.05$) increased in response to miR-106B inhibition, but a slight decrease was detected by inhibition of miR-1254 (Figure 4.7E). These results show the same miRNAs can regulate different genes both subtly and robustly.

Given the evidence that miR-1254, miR-1272, miR-17-5p, miR-17-3p, miR-106B, miR-106B*, miR-124-a, and miR-124* are involved in governing aspects of ADAMTS7, CPE, DPP3, MST1, and PRSS12 gene expression (Figure 4.7), the role of these miRNAs in the regulation of influenza virus replication was determined (Figure 4.8). A549 cells were treated with individual miRNA inhibitors and the cells infected with A/WSN/33 to determine the effect on virus replication (Figure 4.8). Of the 8 miRNA inhibitors tested, inhibition of miR-106B was associated with a decrease in influenza virus replication, while inhibition of miR-124 resulted in an increase in virus replication with respect to the negative control. Inhibition of the other eight miRNAs had more subtle effects with slight increases or decreases of influenza virus replication. The decrease of virus replication associated with inhibition of miR-106B is likely associated with a decrease of CPE gene expression as RNAi silencing of CPE was associated with low levels of virus replication (Figures 4.1 and 4.2). The level of virus replication was confirmed by qPCR M gene levels and was consistent with the findings observed in Figure 5 (Figure A.2). The results show that some of the miRNAs modulate host genes critical for influenza virus replication, thus it likely that the tempo of host gene expression is differentially regulated in response to influenza virus infection. Further, the results provide evidence that targeting miRNAs may offer an alternative disease intervention approach to control influenza virus replication.

Discussion

Some human proteases are known to have a direct function in the replication of influenza virus (10, 32), but the role of other proteases in the biology of virus replication and host cell pathways they affect are not fully elucidated. To better understand protease gene requirements for influenza virus replication, and discover novel disease intervention targets, we conducted an RNAi screen of 481 genes comprising the human protease genome and validated five genes, ADAMTS7, CPE, DPP3, MST1, and PRSS12 that are required for influenza replication. The primary and secondary RNAi screens were performed with A/WSN/33 influenza virus because of its ability to replicate in the absence of trypsin in a type II respiratory epithelial (A549) cell line. All five genes showed reduced A/WSN/33 titers and some showed a substantial decrease in NP staining to the level where NP was not detected using a NP-specific monoclonal antibody. This may have been due to an inability of the virus to reenter the host cells for subsequent infection as a consequence of protease gene silencing. To confirm the findings for WSN/33 infection, the five validated genes were assessed against a clinical influenza virus strain, i.e. A/New Caledonia/20/99. RNAi silencing of all five genes resulted in decreased levels of A/New Caledonia/20/99 replication. Although previous RNAi host factor screens for influenza virus replication did not identify any of the five protease genes in this study, the same host cell pathways were identified which included the genes validated in this study (13, 24, 31, 34, 52). Specifically, our pathway analysis for this study showed that ADAMTS7, CPE, DPP3, MST1, and PRSS12 were involved in three previously identified host cell pathways, specifically CRE/CREB signaling, NF-κB

activation, and apoptosis. It is likely that different pathway interaction is co-opted by the virus at different stages in the virus replication process, but further study is needed to determine the exact process.

CRE/CREB signaling is an important cellular process that serves a variety of functions. Most notably with respect to influenza infection, CRE/CREB signaling has been shown to activate protein kinase A (PKA) and thus have a role in protein synthesis (57). In this study, MST1 and PRSS12 were implicated in CRE/CREB signaling; however, in response to influenza infection, CRE signaling levels in cells treated with siPRSS12 were unchanged compared to both pathway reporter and siNEG controls. However, RNAi of the MST1 gene resulted in significantly higher CRE signaling levels regardless of infection. These findings may suggest that MST1 is involved in some aspect of CRE signaling or the cAMP response, and without MST1, the calcium response element is not activated.

ADAMTS7, CPE, and MST1 have been shown to have a role in tissue injury and inflammation (29, 43, 44), and pathway analysis performed in this study identifying their linkage to NF-κB pathway activation is consistent with these earlier findings. However, there was no significant effect of these genes on the NF-κB pathway relative to controls. However, A549 cells treated with siRNAs targeting ADAMTS7 and DPP3, a gene not predicted to be involved in the NF-κB pathway, resulted in complete abrogation of NF-κB regardless of influenza infection. This finding suggests that NF-κB signaling may involve ADAMTS7 and DPP3. As ADAMTS7 plays a role in maintenance of the extracellular matrix (43) and DPP3 is a metallopeptidase, it is possible that silencing

these genes is decreasing the inflammatory response because less damage is being done to basement tissue.

DPP3 is involved in apoptosis modulation and is over-expressed in cancerous cells (53). RNAi of the DPP3 gene was performed to evaluate the effect on apoptotic gene expression. The results showed induction of BCL2L10, TNFSF10, TNFSF25 and TNFSF8 pro-apoptotic genes. Since DPP3 silencing resulted in a sharp decrease of influenza replication as quantified by $TCID_{50}$ and NP staining, the findings suggest that reducing DPP3 gene expression causes influenza virus infected cells to initiate apoptosis without interference by the known anti-apoptosis activities of the influenza NS1 protein (23). It is possible that without the NS1-mediated apoptosis delay, the virus is not able to replicate and bud before the host cell is eliminated. Furthermore, as NF-κB appears to have a role in apoptosis modulation, the effect of DPP3 silencing on NF-κB activation observed in this study is predictable and consistent with earlier findings (37).

Host gene expression is governed by miRNAs (16, 20), thus it is important to understand potential miRNA regulation of host genes validated as required for influenza virus replication. The results from the miRNA hairpin inhibitor studies showed that targeting the predicted miRNAs had a considerable and significant outcome on most host gene expression. For example, inhibition of miR-106B and miR-124* resulted in 20-fold and 40-fold increased ADAMTS7 gene expression levels, respectively, while CPE gene expression was slightly reduced by miRNA inhibitors. As expected, some miRNA inhibitors had differential effects on host genes required for influenza virus replication. For example, inhibition of miR-106B had little effect on CPE gene expression, but dramatically increased DPP3 gene expression. These findings show that expression of

host genes required for influenza virus replication can be regulated by multiple miRNAs, and suggests that targeting miRNAs to regulate host gene expression may be a strategy to regulate influenza virus replication.

As predicted from the host gene expression studies, miRNA inhibitors also affected influenza virus replication. For example, inhibition of miR-106B, a miRNA known to cause cell cycle arrest when inhibited in a laryngeal cancer model (15), resulted in substantially decreased virus replication. Human papillomavirus oncoproteins E6 and E7 have also been reported to dysregulate miR-106B (69). Inhibition of miR-124-a resulted in an increase of influenza virus replication relative to the negative control. Although miR-124-a was not found to regulate any of the 5 host protease genes from our screen, it is reported to have a putative target in both swine influenza virus and the 2009 pandemic H1N1 strain (25). Inhibition of other miRNAs also subtly modulated infectious virus levels, but not to a level significantly different from the NEG control. In this study, inhibition of miR-106B increased ADAMTS7, DPP3, MST1 and PRSSS12 gene expression, but subtly reduced CPE gene expression, an effect that resulted in reduced influenza virus replication, implying the effect of miR-106B inhibition on CPE may be dominant. Additionally, inhibition of miR-1254 resulted in slightly decreased CPE, DPP3, MST1 and PRSS12 gene expression levels, and this translated to slightly decreased influenza virus replication. Since none of the 5 validated genes were increased by inhibition of miR-124-a, it is likely miR-124-a affects influenza replication through other genes yet identified. These findings indicate that while miRNAs do modulate host gene expression, they likely modulate influenza replication indirectly and perhaps at different points in the replication pathway. Further study is needed to validate miRNA

involvement and mechanism of virus inhibition or assistance. Taken together, these findings suggest novel targets and potentially new therapies for regulating influenza replication and the pathways co-opted during infection.

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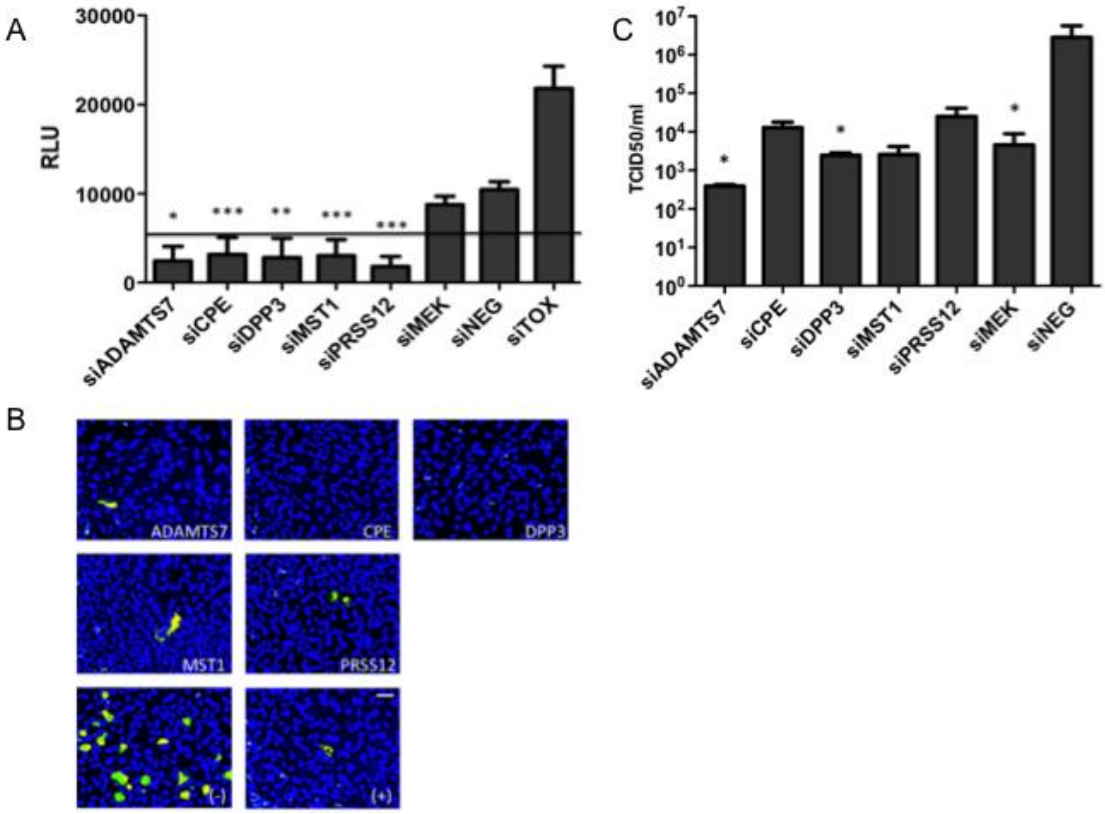

Figure 4.1: RNAi of five host protease genes down-regulates influenza virus replication. A: A549 cells were reverse transfected with 50 nM of siRNA (SMARTpool) specific for the indicated genes (ADAMTS7, CPE, DPP3, MST1, PRSS12). After 48 hours, cytotoxicity was determined by adenylate kinase (AK) release. Cells treated with the siTOX control were considered 100% cytotoxic and all values were normalized to siTOX. * $p < 0.05$ vs siNEG, ** $p < 0.01$ vs siNEG, ***$p < 0.005$ vs siNEG; siTOX vs all samples: $p < 0.001$ (not shown). Line shows 20% of siTOX control. B: A549 cells were reverse transfected with 50 nM of siRNA (SMARTpool) specific for the indicated genes (ADAMTS7, CPE, DPP3, MST1, PRSS12). After 48 hours, cells were infected with A/WSN/33 at an MOI of 0.001. 48 hours post-infection, cells were fixed in 4% formaldehyde and stained with an anti-NP (green) monoclonal antibody followed by counterstain with DAPI (blue.) Positive (+) control: siMEK, negative (-) control: siNEG. Magnification is 20X (bar is 100 microns). C: Cells were transfected with 100 nM of a novel siRNA targeting a different seed site from the SMARTpool used in the primary

screen and infected as in B. After 48 hours of infection, cellular supernatant was tested for infectious virus production by a modified $TCID_{50}$. Data is expressed as $TCID_{50}$/ml. Data is representative of two independent experiments. (*$p < 0.05$ vs siNEG)

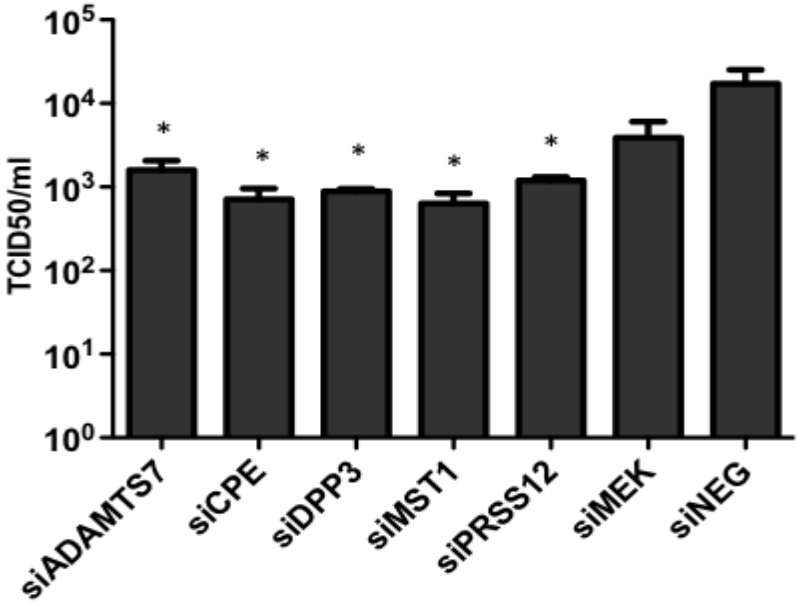

Figure 4.2: RNAi of individual host protease genes downregulates replication of a clinical influenza isolate. A549 cells were reverse transfected with 100 nM of the novel siRNA targeting siADAMTS7, siCPE, siDPP3, siMST1, and siPRSS12. After 48 hours, cells were infected with A/New Caledonia/20/99 at an MOI of 0.1 in the presence of 1 ug/ml TPCK-trypsin. After 48 hours of infection, cellular supernatant was tested for infectious virus production by a modified $TCID_{50}$. Data is expressed as $TCID_{50}$/ml. Data is representative of two independent experiments. (*$p < 0.05$ vs. siNEG)

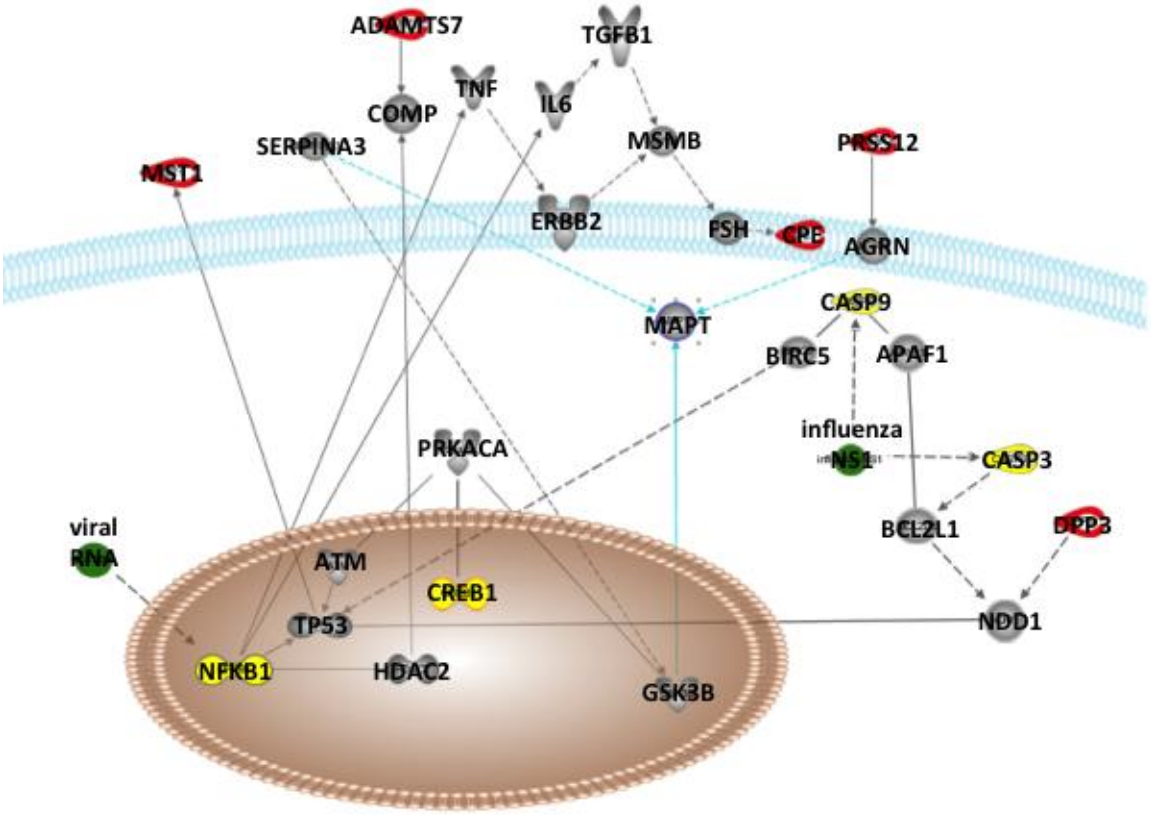

Figure 4.3: Pathway analysis identifying global cellular pathways related to hit protease genes. An Ingenuity pathway analysis linked global cellular pathways and the five host protease genes of interest. Hit protease genes (ADAMTS7, CPE, DPP3, MST1, PRSS12) are shown in red and relevant influenza proteins are shown in green. Nodes indicating global cellular pathways linked with the hit genes are shown in yellow (CREB, NF-κB, caspases).

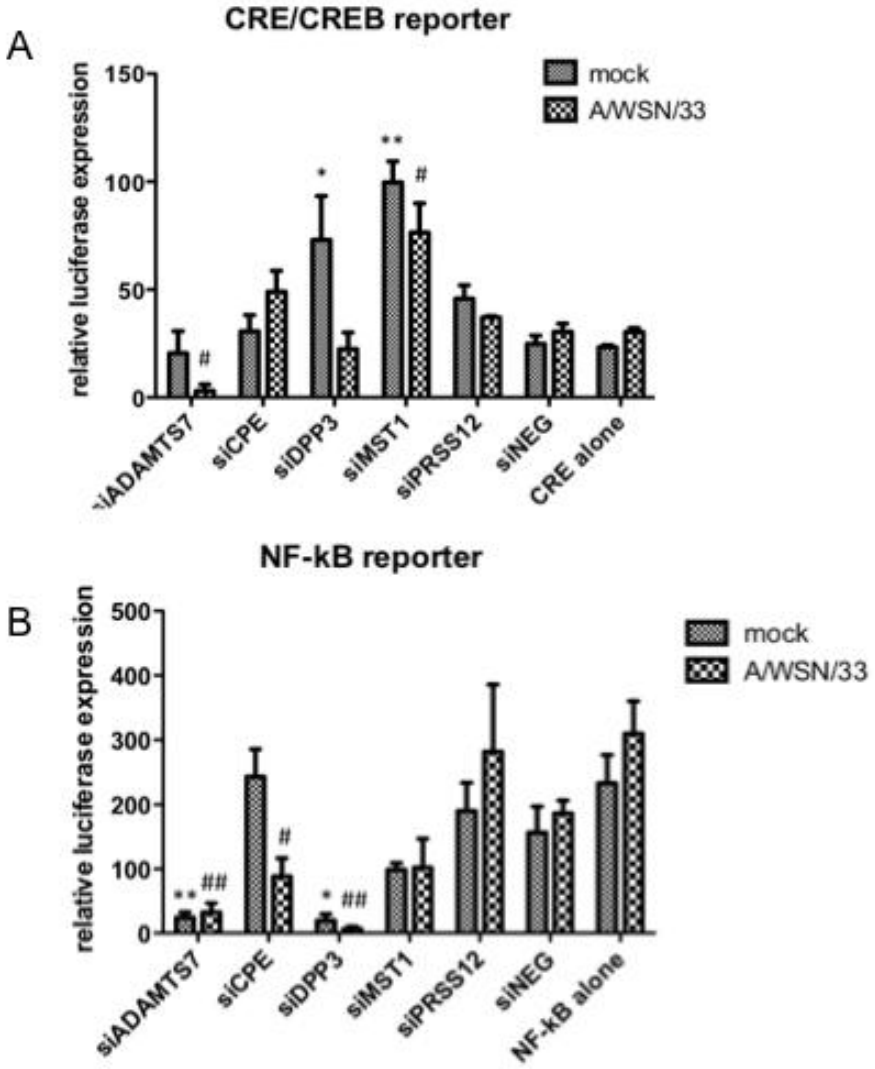

Figure 4.4: Analysis of host gene involvement in major cellular pathways. A: A549 cells were reverse cotransfected with CRE/CREB reporter plasmid or the appropriate control plasmid and 100 nM of the novel siRNA. After 24 h incubation, the transfection media was replaced with culture media. Cells were mock infected or infected with A/WSN/33 at an MOI of 0.001 the following day. After 24 h, culture supernatant was analyzed for luciferase expression. Luciferase units were normalized to Renilla expression. * $p < 0.05$, ** $p < 0.01$ compared to siNEG control (mock), # $p < 0.05$, ## $p < 0.01$ compared to siNEG control (A/WSN/33) B: A549 cells were reversed cotransfected with NF-κB reporter plasmid or the appropriate control plasmid and 100 nM of the novel

siRNA. After 24 h incubation, the transfection media was replaced with culture media. Cells were mock infected or infected with A/WSN/33 at an MOI of 0.001 the following day. After 24 h, culture supernatant was analyzed for luciferase expression. Luciferase units were normalized to Renilla expression. * $p < 0.05$, ** $p < 0.01$ compared to siNEG control (mock), # $p < 0.05$, ## $p < 0.01$ compared to siNEG control (A/WSN/33). Data is representative of three independent experiments.

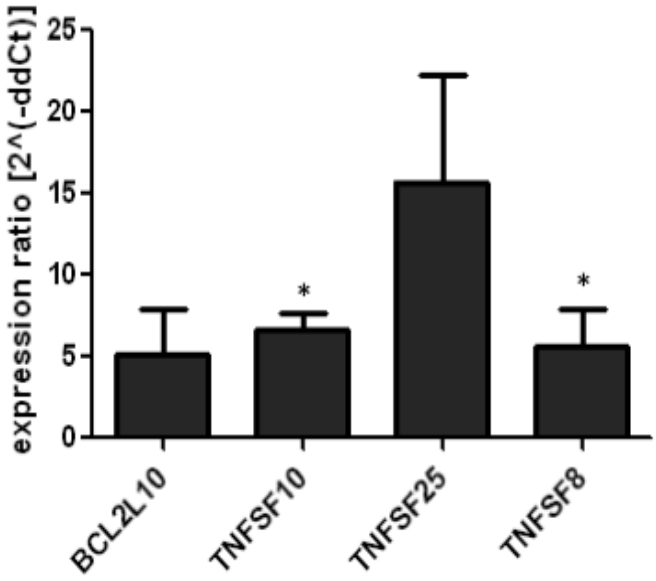

Figure 4.5: RNAi of DPP3 (siDPP3) inhibits influenza replication by modulation of apoptotic genes. A549 cells were reverse transfected with 50 nM of siDPP3 or siNEG. After 48 hours, cells were infected with A/WSN/33 at an MOI of 0.001. After 18 hours of infection, cellular RNA was isolated and apoptosis gene expression profiles were determined by array. Gene expression was normalized to GAPDH levels. Silencing DPP3 resulted in upregulated levels of the pro-apoptotic genes BCL2L10, TNFSF10, TNFSF25 and TNFSF8. Data is representative of three independent experiments. * $p < 0.05$

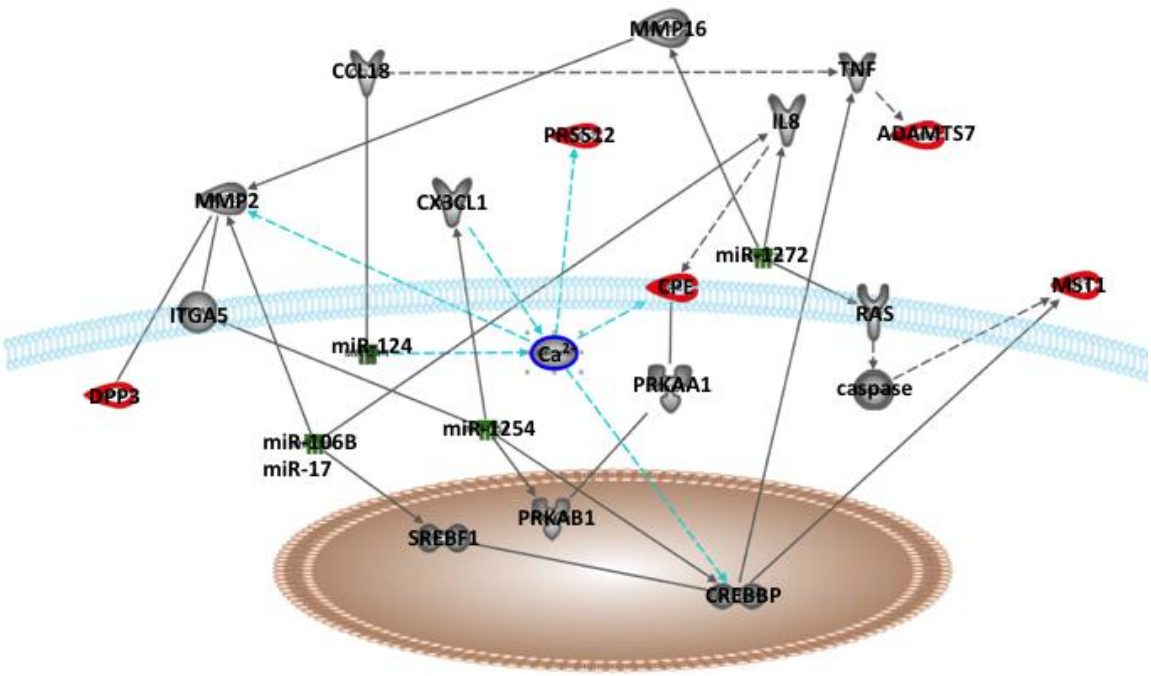

Figure 4.6: miRNAs interact with host protease genes. An Ingenuity pathway analysis implicated several miRNAs connected with host protease genes of interest. Hit protease genes are shown in red and miRNAs are shown in green.

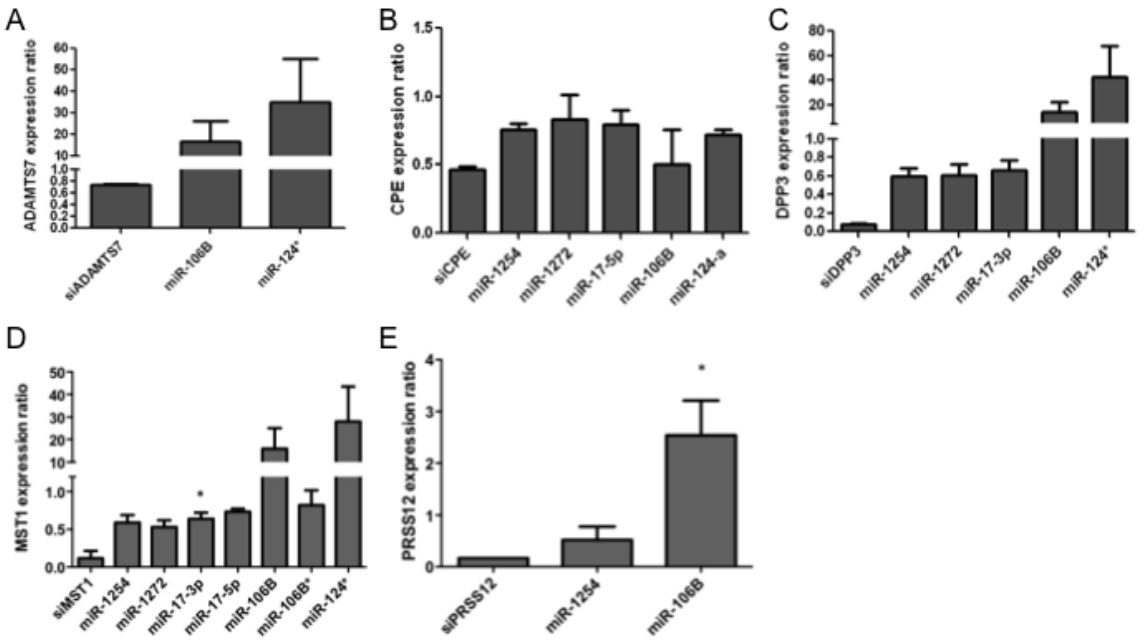

Figure 4.7: Effect of miRNA inhibition on host protease gene expression 24 h post miRNA inhibitor treatment. Host cell miRNAs of interest were evaluated for their effect on host gene hits by qPCR. A549 cells were treated with the appropriate miRNA inhibitor (25 nM) for 24 hours. Cellular RNA was isolated 24 hpi and evaluated by qPCR for host gene expression using a SYBRgreen assay with gene-specific primers. Gene expression was compared to cells transfected with siNEG (for siRNA) or NEG (non-targeting miRNA inhibitor) at the equivalent concentration. Data is normalized to GAPDH expression. miRNAs indicated on the x-axis refer to inhibition of those miRNAs. A: ADAMTS7 expression levels, B: CPE, C: DPP3, D: MST1, E: PRSS12. Data is representative of two independent experiments. (*$p < 0.05$ versus siRNA treatment.)

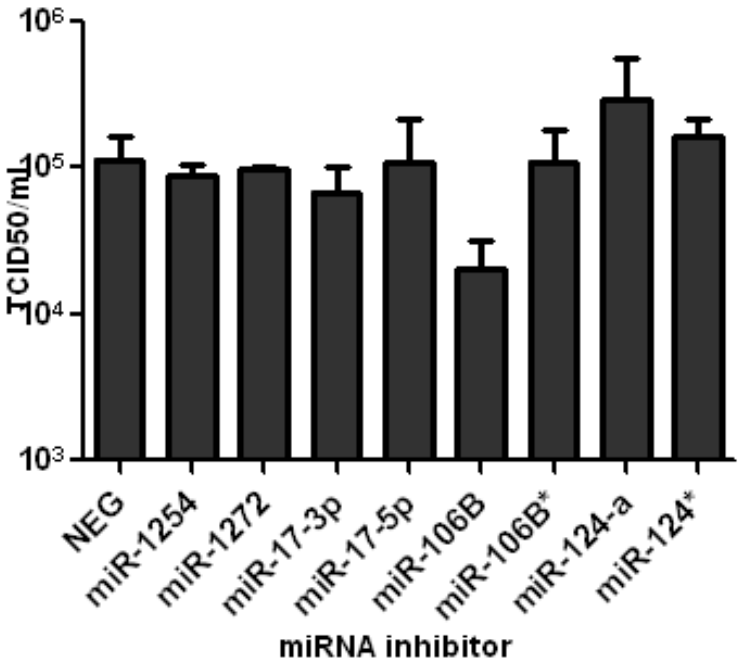

Figure 4.8: Effect of miRNA inhibition on influenza replication. A549 cells were treated with the appropriate miRNA inhibitor (25 nM) for 48 hours, followed by infection with A/WSN/33 (MOI = 0.001). Cellular supernatant was tested for infectious virus production by a modified $TCID_{50}$ 48 hpi. Data is expressed as $TCID_{50}$/ml and is representative of two independent experiments.

Table 4.1: Validated human protease gene hits

Gene	Name	Accession number	siRNA sequence[#]	Function
ADAMTS7	ADAM metallopeptidase with thrombospondin type 1, motif 7	NM_014272	CCAAGGACAUUAUCGACUU	Role in inflammation of extracellular matrix
CPE	Carboxypeptidase E	NM_001873	GAUGAGACGCGGAGUGGUA	Role in local opioid network in the lung
DPP3	Dipeptidyl-peptidase 3	NM_005700	GAUCCUUCUCUGAGCGUUU	Role in inflammation and apoptosis
MST1	Macrophage stimulating 1 (hepatocyte growth factor-like)	NM_020998	GACCAAAGGUACGGGUAAU	Role in inflammation and response to tissue injury
PRSS12	Neurotrypsin, motopsin	NM_003619	GAGCAAGAACCAUGGCUUA	Unknown

[#]Refers to the individual siRNA used for the validation step.

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CHAPTER 5

THE ROLE OF TRANSMEMBRANE SERINE PROTEASE 2 (TMPRSS2) DURING EARLY EVENTS IN INFLUENZA INFECTION[1]

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Abstract

Influenza may impact people of all ages causing substantial morbidity and some mortality. Vaccination is the most effective means of reducing disease, although antiviral drugs may improve disease outcome. Many circulating influenza strains have developed drug resistance creating the need for new antivirals. Host genes involved in viral replication offer an attractive target. For example, a transmembrane serine protease, TMPRSS2, has previously been shown to cleave and activate the HA protein of influenza. In this study, the role of TMPRSS2 in influenza infection of type II alveolar epithelial (A549) cells was determined. Despite efficient RNAi-mediated silencing of TMPRSS2, there was no substantial difference in viral titers 48 hours pi. However, at earlier times during infection, there was a noticeable lag in viral replication during TMPRSS2 silencing, confirmed by immunostaining of infected cells. Thus, TMPRSS2 contributes to cell-to-cell spread early during infection.

Introduction

Influenza virus threatens the health of millions of people each year with clinical outcomes ranging from mild upper respiratory tract illness to life threatening lower respiratory tract disease. Influenza vaccines are generally available, however influenza viruses antigenically drift and can undergo antigenic shift reducing vaccine efficacy (26). Approved antiviral drugs are used to treat influenza infections: two M2 channel inhibitors (amantadine and rimantadine) and neuraminidase inhibitors (zanamivir and oseltamivir) (5, 9, 27, 29, 36, 37). Early treatment with these antiviral drugs reduces the duration of symptoms and the time to recovery; however, the use of amantadines and neuraminidase inhibitors has been linked to the emergence of resistant viruses (6, 9, 19, 27, 35).

RNA interference (RNAi) is an evolutionarily conserved mechanism for the sequence-specific inhibition of gene expression. RNAi is mediated by small interfering RNAs (siRNA) which are incorporated into the RNA-induced silencing complex (RISC) where the antisense/guide strand can either suppress protein expression or direct degradation of messenger RNAs that contain homologous sequence(s) (25, 32, 48). In addition to siRNA, small hairpin (sh)RNAs inserted into the host genome via a lentiviral vector can also afford efficient silencing (48). The use of synthetic siRNAs to target viral genes has been successfully applied, particularly for respiratory syncytial virus (1, 3, 11, 21, 51, 52), and this has led to the initiation of RNAi-based clinical trials as a new therapeutic option (21). RNAi has also yielded promising results for silencing host genes, such as for the treatment of age-related macular degeneration, suggesting that RNAi therapeutics targeting host or viral genes can be effective (2). RNAi antiviral therapy targeting host genes offers several advantages. For example, several recent studies that

aimed to determine the host genes required for influenza virus replication have identified new genes in every step of the influenza virus life cycle (17, 30, 34, 40). As host gene targets are typically conserved and different viruses may require similar genes for replication, RNAi therapeutics targeting host genes may offer broad efficacy.

Type II transmembrane serine proteases (TTSPs) are expressed at the cell surface and generally function to regulate cell-cell and cell-matrix interactions (18). Recently three known TTSPs, i.e. transmembrane serine protease-2 (TMPRSS2), -4 (TMPRSS4) and human airway trypsin-like protease (HAT) (13, 14) have been implicated to have a role in influenza replication, specifically HA-processing. Mosaic serine protease large-form (MSPL) and its splice variant, TMPRSS13, have also been suggested to process the HA of highly pathogenic avian influenza virus (31). TMPRSS2 is detectable as a 55 kDa protein and is expressed in a variety of human tissues, such and the kidney, the prostate, and the lung (13, 45), although there is some discrepancy as some papers have reported TMPRSS2 as a 70 kDa protein with a cleaved 32 kDa serine protease domain (47). TMPRSS2 is not completely characterized, but it is considered to have a role in physiological and pathological processes (45), and it has similarity with enterokinase, which specifically cleaves the acidic propeptide from trypsinogen to yield active trypsin (50). Many functions of TMPRSS2 may be redundant as TMPRSS2-deficient mice have no discernable defects (33). TMPRSS2 is expressed by various cell types, and by cell types for which influenza virus has tropism, e.g. bronchial epithelial cells (38) and type II human alveolar epithelial cells (14). Recently, MDCK cells expressing TMPRSS2 have been shown to allow human and avian strains of influenza to replicate independently of trypsin (7, 13).

Previous studies shown that TMPRSS2 functions as an HA-processing protease (14), that engineered over-expression of TMPRSS2 allows for HA cleavage (7, 15), and that the over-expression of TMPRSS2 and TMPRSS4 in cell lines correlated with an ability to support influenza spread in the absence of trypsin (7, 13, 15). In addition, a recent study showed that RNAi of both TMPRSS2 and TMPRSS4 in human epithelial colorectal (Caco-2) cells reduced influenza virus spread demonstrating that these proteases were responsible for efficient proteolytic activation of HA in this cell line (7). Given this evidence, we evaluated the effect of RNAi-mediated silencing of TMPRSS2 in type II alveolar epithelial (A549) cells that naturally express TMPRSS2, and are a more relevant cell model for influenza than Caco-2. RNAi was found to efficiently silence TMPRSS2 mRNA in A549 cells, and although this silencing had no effect on virus replication 48 hours post infection (hpi), TMPRSS2 appeared to affect early events during infection.

Materials and Methods

Cells, virus stock, and reagents. A549 cells (ATCC CCL-185) were cultured in Dulbecco's modified Eagle's medium (DMEM) (HyClone, Logan, UT) containing 5% heat-inactivated FBS (Hyclone, Logan UT). MDCK cells (ATCC CCL-34) were cultured under the same conditions. A/New Caledonia/20/99 (H1N1) influenza virus was propagated in 9-day-old embryonated chicken eggs as previously described (49). Virus was titrated in MDCK cells and titers calculated by the method developed by Reed and Muench (39). For infections, MDCK cells were cultured in modified Eagle's medium (MEM) (HyClone, Logan, UT) and 1 ug/ml TPCK-trypsin (Worthington, Lakewood,

NJ). A549 cells were supplemented with 0.3% BSA (Invitrogen, Carlsbad, CA) and 2 ug/ml TPCK-trypsin to increase cellular adherence (53). Cells were infected with A/New Caledonia/20/99 (H1N1) at an MOI of 0.5. Cells were rinsed after the infection step to remove any unattached virus. Doxycycline (Fisher Bioreagents, Fair Lawn, NJ) was used to induce shTMPRSS2 expression in transduced A549 cells (shTMPRSS2-A549).

siRNA transfection. For all siRNA studies, cells were transfected using siTMPRSS2 or siNEG (a non-targeting control siRNA whose sequence targets nothing in the human genome). Cells were transfected using the Accell transfection reagent. (Dharmacon, Lafayette, CO). Accell siRNA is specially modified for use without a transfection reagent. Following siRNA transfection, the cells were rinsed and incubated at 37ºC/5% CO_2 for 72 hours with normal growth media. siRNA sequences for siTMPRSS2: GACUUAACCUUGAAAUGGA, CGUGCAUGAUUUACUCUUA, GUGUGCACCUCAAAGACUA, CUGUAAAGUUCAAUUGUGA; siNEG sequence: UAGCGACUAAACACAUCAA.

PCR. Cellular RNA was purified using the RNeasy kit (Qiagen, Valencia, CA), and cDNA was synthesized using the SuperScript First Strand cDNA synthesis kit (Invitrogen), or the Verso cDNA synthesis kit (Invitrogen). PCR was performed using the following primers: TMPRSS2_F: CAG GGT CAC CAC CAG CTA TT, TMPRSS2_R: CCG CTG TCA TCC ACT ATT CC, ACTB_F: GGC ATC CAC GAA ACT ACC TT, ACTB_R: AGC ACT GTG TTG GCG TAC AG. To assess the efficacy of siTMPRSS2 on influenza virus replication, qPCR was used to quantify copies of the influenza matrix protein (M) gene present in the cells (43). PCR was performed using the amplification

cycle: 10 minutes at 95ºC followed by 40 cycles of 95ºC for 30 seconds, 60ºC for 1 minute, and 72ºC for 30 seconds. Gene copies were normalized to a standard curve.

Protein detection. Cells were lysed in 1% TritonX-100 and stored at -80ºC until use. Total protein was quantified using the BCA Protein Assay kit (ThermoScientific Pierce, Rockford, IL). Proteins were separated on a 4 – 20% SDS-PAGE gel (125V for 2 hours) and transferred to a PVDF membrane (15 V for 1 hour). The membrane was blocked in 5% BSA/TBS/0.05% tween (wash buffer) following detection with goat pAb to TMPRSS2 (Abcam, Cambridge, MA) diluted in wash buffer and detection with an alkaline-phosphatase conjugated rabbit anti-goat secondary (Invitrogen) in wash buffer.

$TCID_{50}$ assay. After transfection, MDCK cells were inoculated with ten-fold dilutions of the A549 or shTMPRSS2-A549 supernatant, and the cells were incubated in MEM/0.3%BSA/2ug/ml TPCK-trypsin for 72 hours (24). After incubation, a hemagglutination assay was performed using the MDCK cell supernatant with 0.5% chicken red blood cells (cRBCs) (44). For kinetics study, shTMPRSS2-transduced A549 cells (shTMPRSS2-A549) were induced (4 ug/ml doxycycline) or cultured in normal growth media. Cells were infected with A/New Caledonia/20/99 at an MOI of 0.5. Supernatant was taken 0, 8, 12, 24, 36, and 48 hpi and assayed for virus titer by $TCID_{50}$. Statistical analyses were performed using GraphPad Prism software and the Mann-Whitney U test.

Lentivirus transduction. An shTMPRSS2 plasmid (ThermoScientific Open Biosystems, Huntsville, AL) was harvested from *E. coli.* The plasmid contained a shTMPRSS2 hairpin with a tetracycline response element and RFP expression. The plasmid was transduced into HEK293T cells (ATCC CRL-11268) along with a lentiviral

packaging mix and the Arrest-In transfection reagent (ThermoScientific Open Biosystems). Lentivirus containing the shTMPRSS2 plasmid was harvested and titrated according to the manufacturer's protocol. A549 cells were infected with the lentivirus at an MOI of 5. After infection, cells were placed under puromycin selection to eliminate cells not successfully infected with the lentiviral vector, cells expressing shTMPRSS2 were sorted based on RFP expression, and a stock of clones was generated from the single cells. Clones were assayed for TMPRSS2 silencing in response to doxycycline induction and a single stock with optimal silencing was chosen to use for experiments. The mature sequence of the shTMPRSS2 hairpin was CCGGCATGTCGATATCTA (sense), TAGATATCGACATTGCCGG (antisense).

Immunostaining. Cells (A549 and shTMPRSS2-A549) were induced with doxycycline (4 ug/ml) or cultured under normal conditions. Following induction, cells were infected as described. Cells were fixed with 3.7% formaldehyde at 8, 12, 24, 36, and 48 hpi, permeabilized in 0.5% TritonX-100 and stained with anti-NP monoclonal antibody (5 ug/ml; H16-L10-4R5) and the antibody staining detected using AlexaFluor 488 labeled goat anti-mouse IgG (1 ug/ml; Invitrogen, Carlsbad, CA). Cells were counterstained with DAPI (2 ug/ml) (Invitrogen, Carlsbad, CA) and visualized by immunofluorescent microscopy (EVOS digital inverted fluorescent microscope, Advanced Microscopy Group, Bothell, WA).

Results

RNAi silencing of TMPRSS2. To determine whether TMPRSS2 expression could be down-regulated by RNAi-mediated silencing, A549 cells were treated with siRNA

specific for TMPRSS2 (siTMPRSS2) or a non-targeting control (siNEG) for 72 hours. Cellular RNA was harvested and PCR was performed to determine TMPRSS2 expression (Figure 5.1A). In untreated A549 cells, TMPRSS2 mRNA was evident at 601 bp. In cells treated with siNEG, TMPRSS2 message was still detected. However, in cells treated with siTMPRSS2 at both a low concentration (0.1 uM) and a higher concentration (1.5 uM), no TMPRSS2 product was detected in cellular RNA. All samples expressed similar levels of mRNA per β-actin. To determine protein expression, cells were treated as previously indicated but total cell lysate was examined for TMPRSS2 protein expression (Figure 5.1B). Equivalent amounts of proteins from untreated A549 cells and A549 cells treated with a low (0.5 uM) and higher (1.5 uM) concentration of siNEG or siTMPRSS2 were compared. A faint band at 55 kDa indicating TMPRSS2 was observed in the untreated A549 cells. However, no TMPRSS2 band was detected in cells treated with either siNEG or siTMPRSS2, indicating that the siNEG control may be mediating translational repression.

TMPRSS2 silencing does not affect genomic replication or influenza virus titers. To determine the effect of TMPRSS2 silencing on genomic replication, A549 cells were transfected with siTMPRSS2 or siNEG control. After 72 hours, the cells were infected at an MOI of 0.5 for 48 hours. Influenza M gene copy numbers determined by qPCR were used to measure genomic replication (Figure 5.2A). There was no significant ($p < 0.05$) difference in M gene copy numbers between siTMPRSS2 and siNEG treated cells at any concentration tested. In addition to testing the effect of TMPRSS2 silencing on genomic replication, virus titers from cells transfected with siNEG or siTMPRSS2 were quantified (Figure 5.2B). A549 cells were transfected as previously described and infected at an

MOI of 0.5 for 48 hours. Supernatant was harvested and virus titers were determined as $TCID_{50}$. The results showed no significant ($p < 0.05$) difference between cells transfected with siTMPRSS2 or siNEG despite TMPRSS2 message being adequately silenced by RNAi. The average viral titer in cells treated with 1.5 uM siTMPRSS2 was a log (10X) lower than the corresponding siNEG-treated cells; however several values were outliers which may indicate an off-target effect from the high concentration of siTMPRSS2 used. However, to properly clarify the result, a different RNAi model was used to determine the effects of TMPRSS2 on influenza replication.

shTMPRSS2-A549 cells efficiently silence TMPRSS2 expression. RNAi is often limited by off-target effects, particularly when using siRNAs to mediate gene silencing (4, 10, 28). To overcome this propensity, an shTMPRSS2 stably transfected cell line was developed to study the effects of TMPRSS2 on influenza replication. Briefly, a lentiviral vector was used to deliver and insert a shTMPRSS2 hairpin under the control of a doxycycline promoter into the genome of A549 cells, referred to as shTMPRSS2-A549 cells. After sorting and clonal selection, a single stock was grown that demonstrated efficient TMPRSS2 silencing under induction conditions (Figure 5.3). Although 2ug/ml of doxycycline was able to induce TMPRSS2 mRNA silencing by 48 hours post induction, the studies using the shTMPRSS2-A549 cells were done at 4 ug/ml doxycycline to ensure maximal induction. After doxycycline was added to culture media, TMPRSS2 mRNA and protein levels were extremely low in cells given ≥2 ug/ml doxycycline.

TMPRSS2 has no effect on viral titers at 48 hpi. To confirm the phenotype seen in the siRNA-transfected cells, shTMPRSS2-A549 cells were induced with 4 ug/ml

doxycycline or untreated, and infected with A/New Caledonia/20/99 at an MOI of 0.5 (Figure 5.4). Untreated A549 cells were included as a control. At 48 hpi, no significant difference was detectable in viral titers whether the cells were induced, untreated, or as wild type.

TMPRSS2 does affect viral titers early after infection. Using the shTMPRSS2-A549 cells, virus replication and growth was measured over 48 hpi. Virus titers were compared between induced and non-induced shTMPRSS2-A549 cells, since TMPRSS2 expression was similar between A549 and non-induced shTMPRSS2-A549 cells (Figure 5.3). Cells were infected with A/New Caledonia/20/99 at an MOI of 0.5, supernatant was harvested at 0, 8, 12, 24, 36, and 48 hpi, and viral titers were determined by $TCID_{50}$ (Figure 5.5). At 8 hpi, viral titers were lower in cells treated with doxycycline (4 ug/ml) compared to non-induced cells. By 12 hpi, viral titers in induced cells were similar to the non-induced cells, and virus levels remained the same out to 48 hpi.

TMPRSS2 silencing affects cell-to-cell spread early during infection. To determine the mechanism contributing to the delay in viral titers early during infection, shTMPRSS2-A549 cells were either induced (4 ug/ml doxycycline) or cultured with normal growth media. At 8 and 12 hpi, cells were fixed in 3.7% formaldehyde, permeabilized, and stained for influenza NP protein to visualize virus accumulation in cells (Figure 5.6). Cells were counterstained with DAPI to visualize cell nuclei. At 8 hpi, the number of infected cells in induced shTMPRSS2-A549 cells was significantly lower ($p < 0.05$) than in the non-induced cells, with cells adjacent to infected cells showing no NP staining. By 12 hpi, the number of infected cells in induced cells had increased relative to 8 hpi and influenza NP localization was generally constrained to the

cytoplasm, but some adjacent cells were still not infected. In contrast, by 12 hpi the majority of non-induced cells were positive for influenza NP throughout the cytoplasm and nucleus (Figure 5.6A). At later points in infection, there was no discernable difference in NP localization between induced and non-induced cells (Figure 5.6B). Mock-infected cells were negative for influenza NP (data not shown).

Discussion

In this study, RNAi was used to silence TMPRSS2 expression in A549 cells, both at the transcriptional level as indicated by lack of TMPRSS2 mRNA, and at the protein level as confirmed by Western blot (Figures 5.1 and 5.3). Although siRNA-mediated TMPRSS2 silencing was efficient, transfection with higher siRNA concentrations seemed to induce off-target effects, possibly by activating the innate immune response due to its double-stranded nature (42), or by less than 100% complementary siRNA-mRNA pairing and mimicking of miRNA behavior (10, 28, 46). To circumvent this problem, an shTMPRSS2-A549 cell line was developed to further study the role of TMPRSS2 during influenza infection. The benefits of using a shRNA vector include reduced off-target activity, more efficient RNAi, and the ability to generate a stably transfected cell line (12, 48).

Silencing of TMPRSS2 by siRNA in A549 cells did not modify viral titers or genomic replication at 48 hpi (Figures 5.2 and 5.3). However, transfection with the siNEG control appeared to induce TMPRSS2 translational repression, possibly by acting as a miRNA by having less complementary base-pairing to the 3'-UTR of TMPRSS2 mRNA which is common during treatment with higher concentrations of siRNA (10).

Although no substantial difference was detected in influenza replication between siNEG- and siTMPRSS2-treated cells (Figure 5.2), there was concern that the lack of difference may be due to siNEG-mediated off-target silencing. The shTMPRSS2-A549 cell line had efficient and specific TMPRSS2-silencing when induced with doxycycline, although there was a slight decrease in TMPRSS2 protein in the non-induced shTMPRSS2-A549 cells compared to wild type A549. This is likely due to low levels of background activity and is to be expected with this type of construct (20). When the shTMPRSS2-A549 cell line was used, a similar phenotype to the siRNA-transfected cells was observed (Figure 5.4).

Numerous publications have shown that TMPRSS2 aids in influenza infection by cleaving and activating the HA protein (8, 13, 14, 16), and TMPRSS2 has also been shown to activate other viruses such as coronavirus and metapneumovirus (23, 41). Most models for these studies have been performed in cells that do not endogenously express TMPRSS2, and so have been transfected with an overexpression plasmid (13, 14). As TMPRSS2 levels are lower in lung tissue than in other tissues (45), the overexpression of TMPRSS2 may account for the large differences seen in viral titers. Additionally, many of the cell lines used such as Vero cells and MDCK cells do not express endogenous proteases, so removal of TMPRSS2 from the system severely inhibits influenza replication (13, 14, 41).

Although there was no difference in viral titers at 48 hpi, early during infection, i.e. 8 hpi there was a delay in virus replication in doxycycline-induced shTMPRSS2-A549 cells (Figure 5.5). A549 cells express multiple proteases including TMPRSS4 and HAT (16, 22). It is possible that TMPRSS2 is one of the first endogenous proteases

recruited during viral infection, and at later points during infection the virus can use other proteases. This may account for the correction of virus replication to normal levels. Interestingly, there was no difference in virus replication whether or not exogenous trypsin was added to the infection media (data not shown), implying that proteases expressed by A549 cells are sufficient for infection. When virus replication and localization within the infected cells was examined early during infection, fewer infected cells were observed in doxycycline-induced shTMPRSS2-A549 cells (Figure 5.6A). In particular, cells adjacent to infected cells were still not infected at 12 hpi, while in the non-induced cells the virus appeared to spread throughout the monolayer. In contrast, at later times (24 and 48 hpi) in infection (Figure 5.6B), there was no discernable difference in between induced and non-induced cells.

It is well established that TMPRSS2 cleaves HA (8, 13, 14, 16). Recent work has shown that the cleavage and activation of HA is occurring in a subcellular compartment, so presumably incoming HA virions are not being activated by TMPRSS2 (13). This may be a mechanism for the inhibition of cell-to-cell spread that is observed early in infection (Figure 5.6). TMPRSS2 is also known to be co-expressed with α(2,6)-sialic acid receptors, the preferred receptor to which HA binds so the virus can enter the cell (8). As this receptor is important for the first step in viral replication, this may explain why TMPRSS2 silencing appears to affect virus replication early. Although TMPRSS2 silencing appears to inhibit influenza infection early on, disease intervention strategies based on targeting host proteases may require a tandem approach.

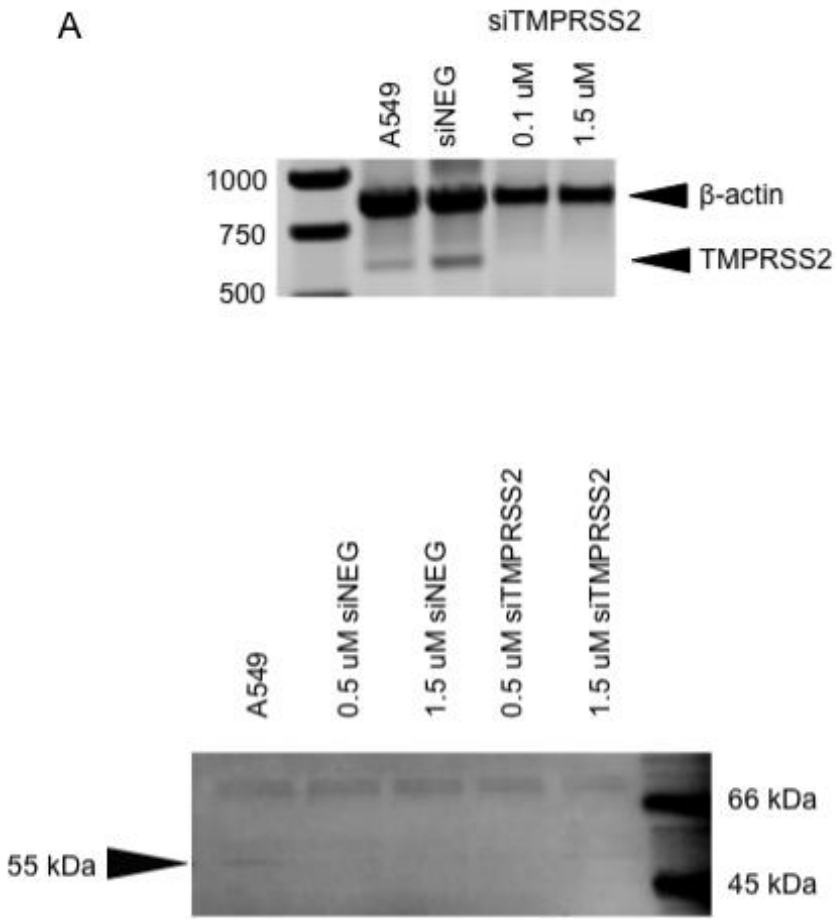

Figure 5.1: siRNA transfection silences TMPRSS2 expression in A549 cells. Cells were transfected with siRNA specific for TMPRSS2 (0.1 uM or 1.5 uM) for 72 hours. A: TMPRSS2 mRNA levels were determined by qPCR with product run on a 1% agarose gel. TMPRSS2 mRNA levels were compared to untreated A549 cells and cells transfected with the siNEG control. TMPRSS2 expression was compared to β-actin. B: Cells were lysed with 1% TritonX-100 and proteins were detected by immunoblotting with an anti-TMPRSS2 polyclonal antibody to determine TMPRSS2 protein expression.

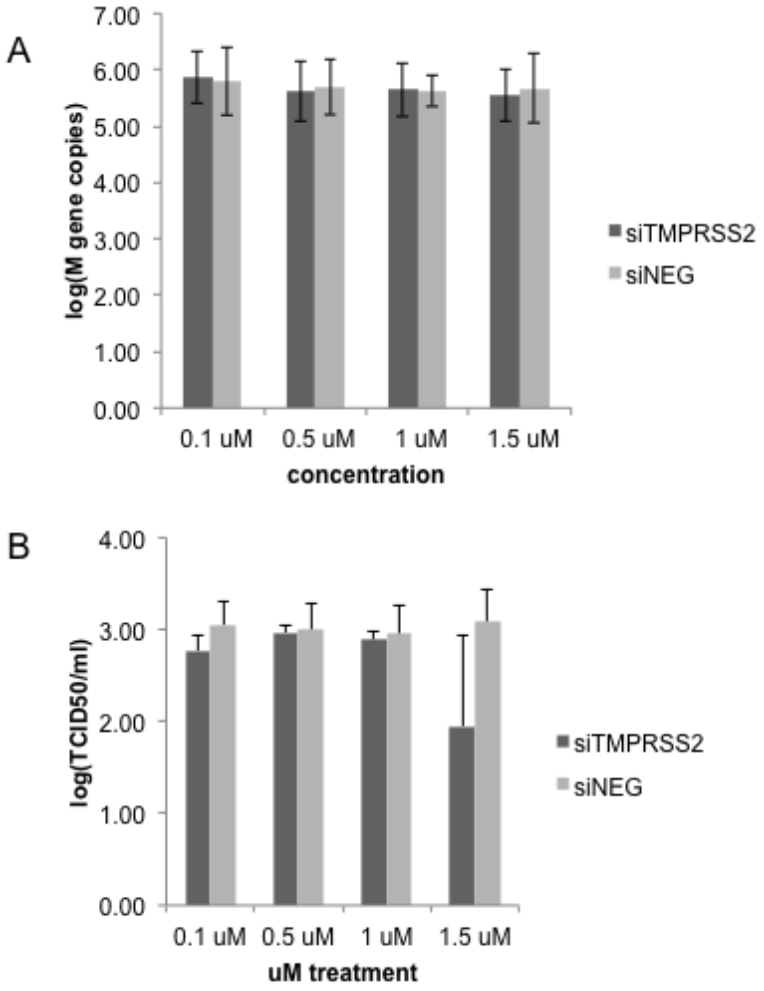

Figure 5.2: TMPRSS2-siRNA silencing has negligible effect on genomic viral replication and infectious virus production in A549 cells. A: Seventy-two hours post siTMPRSS2 transfection, A549 cells were infected with A/New Caledonia/20/99 (MOI of 0.5) and incubated for 48 hours. Influenza M gene copies were determined by qPCR in cellular RNA from siTMPRSS2 transfected cells and compared to siNEG controls. The results represent mean values from three independent assays. Error bars indicate SEM. B: Seventy-two hours post siTMPRSS2 transfection; A549 cells were infected with A/New Caledonia/20/99 (MOI of 0.5) and incubated for 48 hours. The level of infectious virus in

cell supernatants from A549 cells was determined by $TCID_{50}$ assay. The results represent mean values from four independent assays. Error bars indicate SEM.

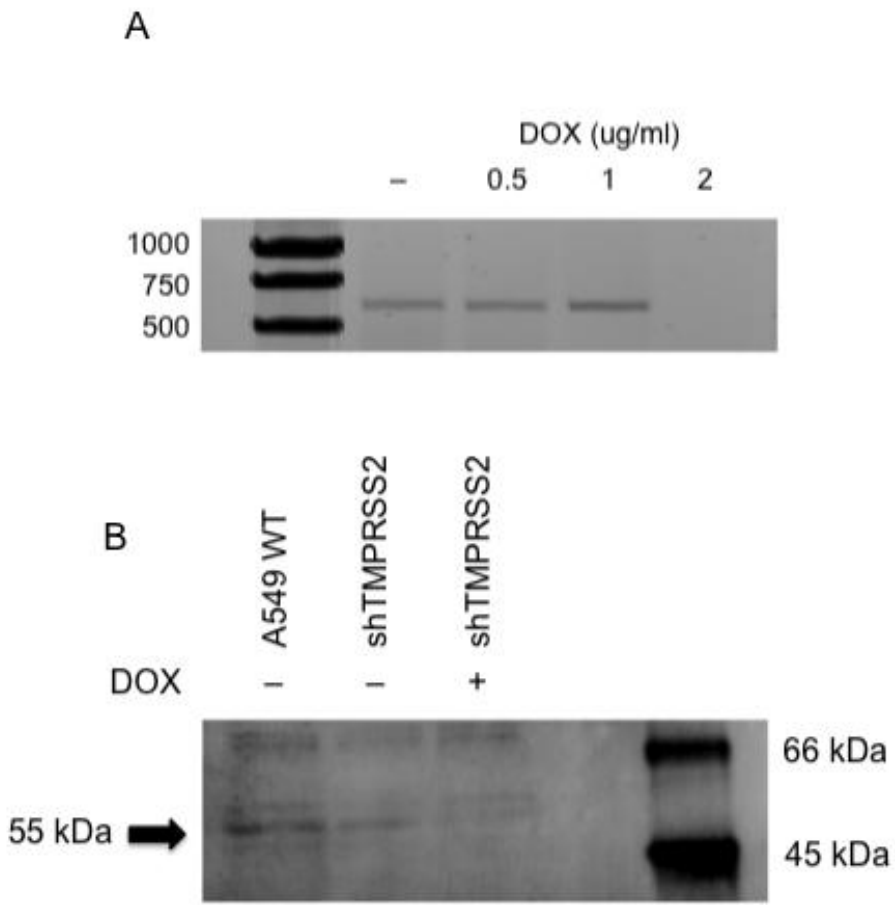

Figure 5.3: TMPRSS2 is silenced during doxycycline induction of shTMPRSS2-transduced A549 cells. A549 cells were transduced with a lentiviral vector carrying shTMPRSS2. Cells were induced with doxycycline or treated with normal growth media. TMPRSS2 expressed was compared to non-transduced, wild type A549 cells. A: RNA was harvested and TMPRSS2 expression was determined by PCR in cells. B: Cells were lysed with 1% TritonX-100 and proteins were detected by immunoblotting with an anti-TMPRSS2 polyclonal antibody.

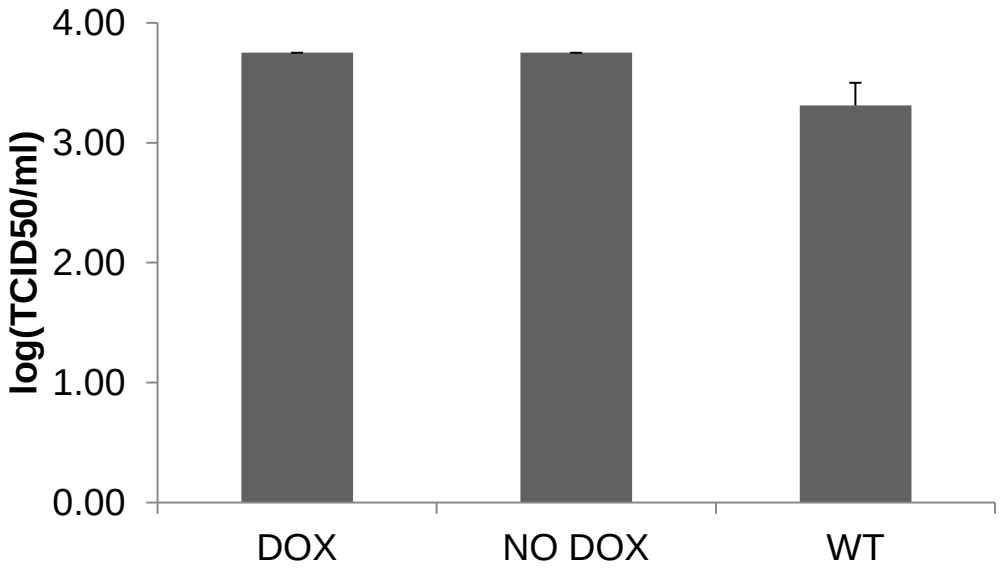

Figure 5.4: shTMPRSS2 mediated silencing has negligible effect on infectious virus replication in A549 cells. A549 cells transduced with the shTMPRSS2 vector were induced with doxycycline (4 ug/ml) or cultured in normal growth media. Cells were infected with A/New Caledonia/20/99 at an MOI of 0.5 in the presence of TPCK-trypsin (2 ug/ml) and incubated for 48 hours. The level of infectious virus in cell supernatants from A549 cells was determined by $TCID_{50}$ assay. The results represent mean values from two independent assays. Error bars indicate SEM.

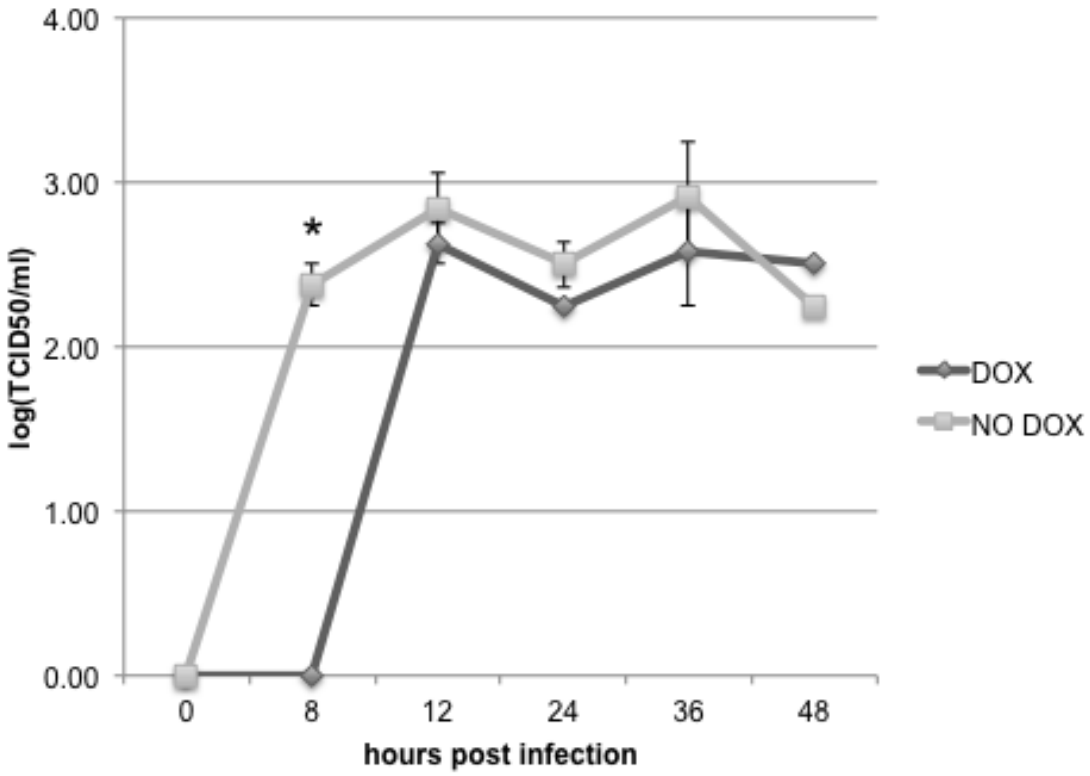

Figure 5.5: shTMPRSS2 silencing affects viral kinetics early during infection. A549 cells transduced with the shTMPRSS2 vector were induced with doxycycline (4 ug/ml) or cultured in normal growth media. Cells were infected with A/New Caledonia/20/99 at an MOI of 0.5 in the presence of TPCK-trypsin (2 ug/ml). Supernatant was harvested at 8, 12, 24, 36, and 48 hpi and tested for virus titers by $TCID_{50}$ assay. Error bars indicate SEM. * $p < 0.05$

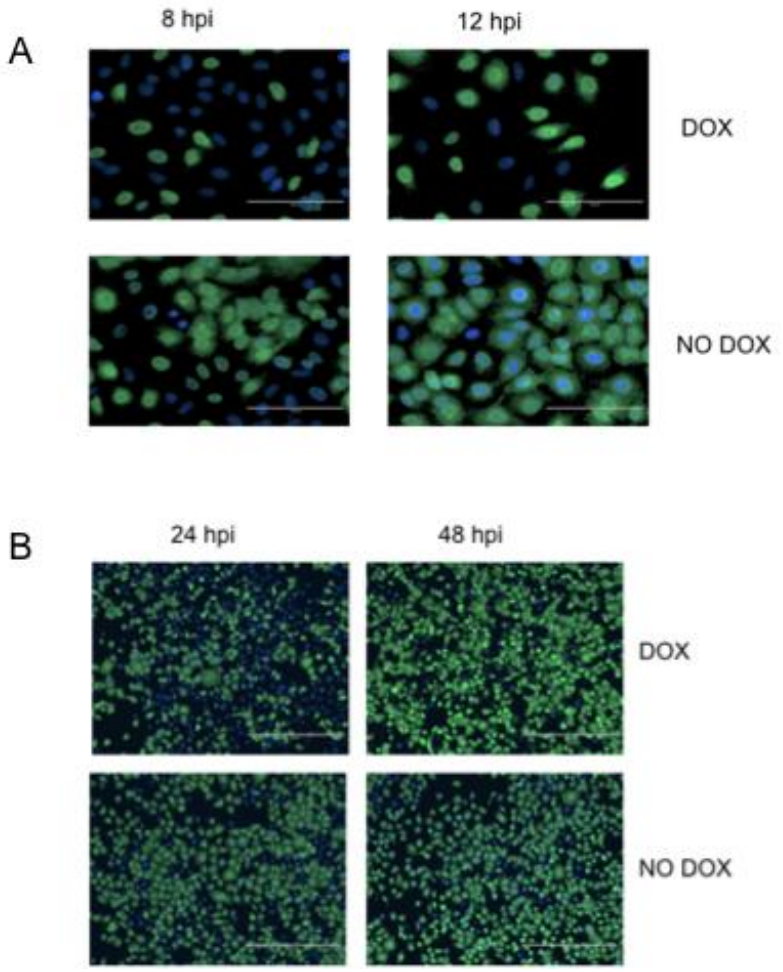

Figure 5.6: TMPRSS2 silencing affects cell-to-cell spread early in viral infection. shTMPRSS2-A549 cells were either cultured under normal growth conditions or induced with doxycycline (4 ug/ml). Cells were infected with A/New Caledonia/20/99 at an MOI of 0.5 in the presence of TPCK-trypsin (2 ug/ml). A: Early post-infection (8 and 12 hpi), cells were fixed and stained with anti-influenza NP antibody (green) and counterstained with DAPI (blue). Results reflect two independent experiments. Magnification is 40X. B: By 24 and 48 hpi NP localization was similar regardless of TMPRSS2 silencing. Magnification is 10X. Results reflect two independent experiments.

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CHAPTER 6

CONCLUSIONS

Influenza remains a public health problem with the threat of pandemic due to antigenic drift and shift. Although yearly influenza virus vaccines are available, vaccine development remains problematic due to the time taken to synthesize the vaccine, the need to tailor the vaccine to current circulating strains that must be predicted before the influenza season, and the reduced efficacy in young children and the elderly. In addition to issues linked with vaccine availability, the efficacy of existing antiviral drugs is complicated by the emergence of resistant viruses highlighting the need for the development of novel influenza therapeutics. Some influenza therapies target the virus, but due to the mutation high rate of influenza, an alternate approach targeting host genes required for virus replication is becoming attractive. This avenue for novel drug development is made possible by furthering our understanding of host pathways co-opted by influenza virus. The experiments completed in this study were designed to elucidate host protease genes involved during viral replication. The *hypothesis* addressed was that genome-wide small interfering RNA (siRNA) libraries that target individual genes in the host protease library can be used to identify gene knockdown events that increase or inhibit production of viral progeny. The *specific aims* addressed were:

Specific aim 1. Identify and validate human protease genes involved in influenza replication that when silenced by RNAi affect influenza replication. The *working hypothesis* is that siRNA silencing of human protease genes would allow for identification of genes important for influenza virus replication. The data presented in Chapter 3 and the Appendix show that from a primary screen of 481 human protease genes, 24 individual genes were identified as essential for virus replication or were antiviral genes affecting replication. The assay endpoints used to evaluate the phenotype or effect of gene silencing included evaluating levels of infectious virus production ($TCID_{50}$), genomic replication (M gene copy number), and/or NP localization (immunostaining) in cells. To confirm that the phenotype observed was due to silencing of the target gene and not to off-target effects of siRNA treatment, the 24 genes identified as important in the screen were validated as described in the Appendix. Of the 24 primary positive hits, five human protease genes (ADAMTS7, CPE, DPP3, MST1, and PRSS12) were validated as essential for influenza replication as RNAi silencing of these genes lowered viral replication for each of the endpoint assays tested.

Specific aim 2. Determine the cellular pathways involved in influenza replication. The *working hypothesis* is that the protease genes identified in *Specific Aim 1* are involved in cellular pathways co-opted by influenza virus and that RNAi silencing of protease genes along this pathway will modulate influenza virus replication. The pathway analysis described in Chapter 4 revealed that the five human protease genes were involved in three major cellular pathways, i.e. calcium response element binding (CREB) signaling, NF-κB pathways, and apoptosis. All three of these pathways have been previously linked to have an important role in influenza infection and replication. When

individually silenced by RNAi, each of the five human protease genes affected the activity of the pathways activated within the host cell during infection and replication. Specifically, siRNA silencing of ADAMTS7 lowered CRE/CREB signaling compared to basal expression levels following influenza infection. RNAi silencing of the MST1 gene resulted in increased CRE/CREB signaling regardless of whether the cells were infected with influenza virus. Silencing of siADAMTS7 and siDPP3 with siRNA resulted in abrogation of NF-κB activation, while siRNA silencing of the CPE gene lowered NF-κB activation following influenza virus infection. Notwithstanding, RNAi of the siDPP3 gene resulted in increased expression of pro-apoptotic genes. Although predicted by the pathway analysis, the PRSS12 gene was not validated to be involved in any of the three global cellular pathways examined, thus ADAMTS7, CPE, DPP3, MST1, and PRSS12 are important in the cellular pathways required for influenza replication.

Specific aim 3. Identify the role of miRNA in post-transcriptional regulation of validated protease genes. The *working hypothesis* is that miRNAs govern the expression of the human protease genes required for influenza virus, and that inhibition of specific miRNAs predicted to target these genes will affect influenza replication. As miRNAs subtly govern gene expression, miRNAs targeting the host genes identified from the pathway analysis were investigated to determine whether the protease genes were being post-transcriptionally regulated. In Chapter 4, inhibition of eight miRNAs (miR-1254, miR-1272, miR-17-3p, miR-17-5p, miR-106B, miR-106B*, miR-124-a, and miR-124*) was found to affect host gene transcripts. Inhibition of miR-106B resulted in a decrease of influenza virus titers, and inhibition of miR-124-a increased influenza virus titers.

An additional goal of this research was to characterize the role of a sixth protease gene, TMPRSS2, during influenza infection. TMPRSS2 is well established to aid in cleavage of the influenza HA protein, as well as other viruses. However, this cleavage occurs in subcellular compartments, suggesting that incoming virions are not affected by TMPRSS2. The purpose of this portion of the study was to further characterize the contribution of TMPRSS2 to influenza infection. This was addressed by the following specific aim:

Specific aim 4. Determine if RNAi-mediated silencing of TMPRSS2 affects influenza virus cell-to-cell spread during infection. The *working hypothesis* is that silencing of TMPRSS2 in alveolar epithelial cells will decrease the ability of the influenza virus to spread to adjacent cells during infection. The data shown in Chapter 5 indicates that TMPRSS2 has a role in cell-to-cell spread of the influenza virus. TMPRSS2 mRNA and protein was efficiently silenced using siRNA or shRNA mRNA and protein, an effect that resulted in decreased viral titers at early time post-infection (8 hpi) in a human respiratory epithelial cell line, and had an early inhibitory effect on infection spread to adjacent cells.

Taken together, this research identified five human protease genes that can serve as potential drug targets for the development of novel influenza therapies. The five genes identified: ADAMTS7, CPE, DPP3, MST1, and PRSS12, were also implicated in major cellular pathways that govern processes indispensable during influenza infection such as PKA signaling and protein synthesis (CRE/CREB), inflammation and the innate immune response (NF-κB), and cellular apoptosis. In addition to cellular pathways, post-transcriptional regulation of protease gene expression was regulated by miRNAs,

particularly miR-106B and miR-124-a, which decreased and increased influenza virus titers, respectively. Finally, a sixth protease gene, TMPRSS2, was shown to affect cell-to-cell spread early in infection and may represent an attractive drug target for treatment in conjunction with other endogenous host cell proteases.

APPENDIX

SUPPLEMENTARY DATA: VALIDATION SCREEN

Abstract

After performing an RNAi screen of the human protease library (481 genes), 24 genes were identified that when silenced caused an increase or decrease of influenza replication. In this study, the 24 primary hits were validated by silencing with a novel siRNA targeting a different seed sequence on the target gene and determining whether transfection with the novel siRNA affected influenza virus titers and M gene copy number, as well as determining by quantitative PCR whether the target gene was silenced. In addition, a global pathway analysis was conducted to determine how the primary hits participated in global cellular pathways. Of the 24 primary hits, five protease genes were validated where RNAi-mediated silencing of these genes was not cytotoxic and caused a decrease in influenza virus replication.

Introduction

When performing RNA interference (RNAi) screens, it is necessary to validate the gene hits generated from the screen. A major caveat of RNAi screening is the ability of siRNAs to mediate off-target effects (9). Although evaluation of multiple endpoints can help to reduce false positives (2), further validation is necessary to confirm that the effect, or phenotype, is due to direct silencing of the target gene. Off-target effects can be caused by several factors. For example, it is possible that the siRNA may act as a miRNA because of imperfect seed site complementarity and affect the expression of a different host gene (3, 4, 9). Treatment of cells with siRNA can also activate the type I interferon response through TLRs (5, 10) and activate ISGs (interferon stimulated genes) (1, 6).

For these reasons, the 24 primary hits generated from the human protease library siRNA screen were validated to ensure that the effect on influenza virus replication was due to silencing of the target gene. A robust primary screen was performed that included the evaluation of three assay endpoints: viral titers, localization of influenza NP protein within the cells, and M gene copy number. This primary screen was performed using a SMARTpool consisting of four individual siRNAs targeting different seed sites on each protease gene. To validate the primary hits, a novel siRNA was used that targeted each primary hit at a different seed sequence than any of the siRNAs included in the SMARTpool. Viral titers and M gene copy numbers were determined after silencing the target protease genes with the novel siRNA. Additionally, a subset of genes was tested to determine directly whether mRNA of the target protease gene was decreased by siRNA treatment. Concurrent to these validation studies, a global pathway analysis was performed on the 24 primary hits to determine to which global cellular pathways the

primary hits contributed. Taken together, the data showed five human protease genes essential for influenza replication, ADAMTS7, CPE, DPP3, MST1, and PRSS12, which were validated as decreasing virus replication when silenced in A549 cells. None of the antiviral primary hits from the human protease library were validated.

Materials and Methods

Cells and virus stocks. A549 cells (ATCC CCL-185) were cultured in Dulbecco's modified Eagle's medium (DMEM) (HyClone, Logan, UT) containing 5% heat-inactivated FBS (HyClone, Logan, UT). Cells were frozen in 10% DMSO and 90% FBS to create one stock of a single cell passage that was used for the entire study. A stock of MDCK cells (ATCC CCL-34) was also propagated and stored using the same conditions. A/WSN/33 (H1N1) influenza virus was used as this virus has the ability to replicate without the need for exogenous trypsin (11). Virus was propagated in 9-day-old embryonated chicken eggs as previously described (12). Viruses were titrated in MDCK cells and titers calculated by the method developed by Reed and Muench (7).

Reverse transfection. siRNAs were diluted with HBSS (HyClone, Logan, UT) and allowed to incubate for 5 minutes. Dharmafect-1 transfection reagent (Lafayette, CO) and HBSS were added such that each well received 0.004 ml of transfection reagent and 0.096 ml of HBSS. The siRNA/transfection reagent mix was allowed to incubate for 20 minutes at room temperature after which 0.08 ml of 1.5×10^4 A549 cells suspended in DMEM/5% FBS was added to each well, and the plate incubated for 48 hours at 37°C in 5% CO_2. The final concentration of siRNA was 100 nM.

Validation of gene hits. Individual novel siRNAs (Dharmacon) were used to target a different seed site on the same gene for gene hits identified during the primary screen (Table 4.1). Gene silencing was confirmed by qPCR using SybrGreen (Qiagen, Valencia, CA) to detect the dsDNA product allowing for quantification of gene silencing relative to cells treated with the siNEG control. Primers targeting each individual hit were compared to control GAPDH levels (Table A.1). For phenotype validation, A549 cells were transfected with 100 nM siRNA, incubated 48h at 37°C in 5% CO_2, infected with A/WSN/33 (MOI = 0.001), and the amount of infectious virus was measured 48 hpi by $TCID_{50}$ and M gene levels to validate the screen phenotype previously observed. From the primary gene hits, five genes essential for influenza virus replication were validated using the novel siRNAs.

Host cell pathway analysis. To survey the spectrum of host cell pathways that may be linked to the validated gene hits, pathway analysis was performed using Ingenuity Pathway Analysis software (Ingenuity Systems, http://www.ingenuity.com). The results provided network modeling of the validated genes and identified several functional groups linked to protease gene expression, host cell miRNA regulation, and influenza replication.

Results

Initial pathway analysis implicated 16 primary hits in global cellular pathways. Using Ingenuity Pathway Analysis (IPA) software, a detailed pathway analysis was performed on the 24 primary hit genes (Table A.2). Pathways were classified based on direct or indirect interaction of the hit protease genes with established nodes related to the global

cellular pathways. An indirect interaction is defined as one or more signaling intermediates between the cellular pathway node and the protease gene of interest. Pathways of note include cell death, cellular function and maintenance, connective tissue disorders (inflammation), and cell-to-cell signaling. Of the 24 primary hit genes, nine implicated in the pathway analysis. Genes that were not involved in the global pathways identified included MPN, CPZ, KLK14, CASP7, ADAM30, USP52, MMP14, TFR2, PAPPA, MMP13, KLK12, GZMB, CTSH, CPA4, and ADAMDEC1. Genes that did not come up in the pathway analysis were not pursued beyond the validation step.

Phenotype validation by novel siRNAs. Concurrent with the pathway analysis, phenotype using the individual and novel siRNAs was evaluated. After transfection with the novel siRNA (100 nM), viral titers and M gene copy numbers were evaluated to confirm the phenotype seen in the primary screen. In cells treated with siNEG, infection generated a viral titer of $10^{5.2}$ $TCID_{50}$/ml. Compared to siNEG-treated cells, cells where SERPIND1, MPN, CPZ, and GGTLA1 had been silenced by RNAi did not generated the same phenotype observed in the primary screen. However, cells treated with siRNA targeting CPE, PRSS12, PAPPA, MST1, MMP13, DPP3, and ADAMTS7 showed the same phenotype as in the primary screen, i.e. less infectious virus compared to siNEG-treated cells. As expected, the siMEK control resulted in a decrease of viral titers by three logs.

In addition to virus titers, the novel siRNAs were also tested for their ability to affect virus replication by the determination of M gene copy number within the infected cells (Figure A.2). Compared to the siNEG control, cells treated with siRNA targeting SERPIND1, CPE, PRSS12, PAPPA, MST1, MMP13, ADAMTS7, and DPP3 resulted in

a similar phenotype as the one observed in the primary screen, while cells where MPN and CPZ had been silencing did not correlate. However, in the primary screen the M gene levels (Figure 3.6) did not correlate well with the phenotypes seen in the $TCID_{50}$ and IFA endpoint assays (Figures 3.4 and 3.5).

Silencing of host protease genes validated by qPCR. As a final step in the validation process, a subset of gene hits was tested to ensure that gene-specific silencing was occurring (Figure A.3). The genes selected for testing were genes with a conflicting phenotype in the validation screen or genes that validated across both endpoint assays of the secondary validation screen, and thus silencing needed to be confirmed. The siMEK control was 95% silenced compared to siNEG-treated cells. The novel siRNA did not silence SERPIND1, MPN, or GGTLA1, and these genes were in fact up-regulated compared to controls. Although siCPZ resulted in 95% CPZ knockdown, treatment with siCPZ resulted in decreased viral titers relative to siNEG (Figure A.1), the opposite phenotype expected.

Discussion

Of the 24 primary screen hits, most were not cytotoxic when silenced in A549 cells, excluding SENP1 (Figure 3.3). Another gene, ELA1, was found to be evolutionarily silenced in human tissue (8), rendering it an ineffective potential therapeutic target. Therefore these genes were not considered for further validation. Other genes (MPN, CPZ, KLK14, CASP7, ADAM30, USP52, MMP14, TFR2, PAPPA, MMP13, KLK12, GZMB, CTSH, CPA4, ADAMDEC1) did not appear in the initial pathway analysis and therefore were not considered for further evaluation in this study, beyond the validation

screen. Although some of these genes were tested further (Figures A.1 – A.3), this was done only because the validation experiments were performed concurrently with the pathway analysis.

Detection of viral titers provided a straightforward method to determine whether silencing of the target protease gene by the novel siRNA was resulting in the same phenotype as the SMARTpool used in the primary screen. From the genes tested, only four did not show similar phenotype: SERPIND1, MPN, CPZ, which caused a substantial increase in virus in the primary screen, and GGTLA1, which substantially decreased viral titers. Because these results were not consistent with the primary screen data, these genes were not considered validated.

M gene data was not considered as a deciding factor but was done to provide further information. This decision was made because in the primary screen, the M gene results did not correlate well with the other endpoint assays. For instance, some genes with high M gene copy number detected in the cells produced extremely low titers. This may simply be a mechanism of inhibition issue of that particular target gene, or, in the case of genes that did not validate, the result of off-target effects. In the primary screen siADAMTS7 treatment resulted in, on average, a decrease in M gene copy number (Figure 3.6). However the error for this particular gene was very large, and compared with the decrease in NP protein and virus titers seen in the primary and secondary screen, together with the fact that ADAMTS7 mRNA was decreased by the novel siRNA, ADAMTS7 was considered validated.

Finally, a subset of genes was tested to determine whether treatment with the novel siRNA resulted in a decrease in cellular mRNA for the target gene. Of the subset of

genes tested, CPZ, CPE, PRSS12, MST1, DPP3, and ADAMTS7 showed decreased expression. Since CPZ had already been discounted due to its failure to appear in the pathway analysis and the difference in viral titers seen from the primary screen to the validation screen, only CPE, PRSS12, MST1, DPP3, and ADAMST7 were further considered.

Taken together, the secondary validation screen resulted in five human protease genes, ADAMTS7, CPE, DPP3, MST1, and PRSS12, that when silenced in A549 cells, resulted in a decrease in influenza virus replication. Of these protease genes, all five caused a decrease in viral titers, M gene levels (except ADAMTS7), and were adequately silenced by treatment with the novel siRNAs. Additionally, all five of the genes were identified as belonging to global cellular pathways governing pathways related to inflammation, cell signaling, and cell maintenance (Table A.2). This five human protease genes were selected to analyze further in specific aims 2 and 3, for more stringent pathway analysis, to study the effects of these genes on pathway activation and signaling, and to determine mechanisms of post-transcriptional regulation of these genes.

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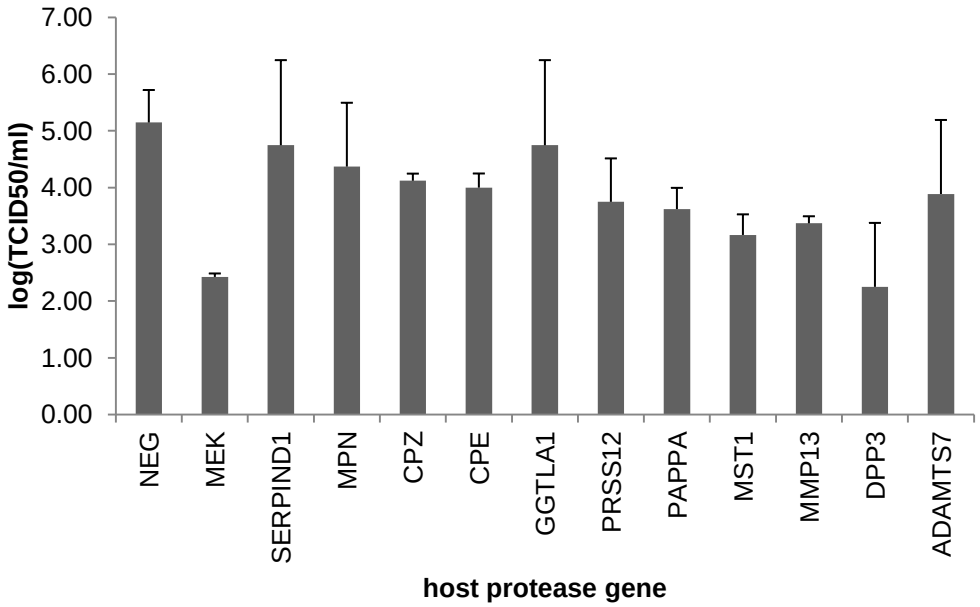

Figure A.1: Validation of infectious virus production using novel siRNAs. A549 cells were reverse transfected with 100 nM of an individual siRNA targeting the protease gene of interest at a different seed site from any of the siRNAs used in the primary screen, as described in the methods section. After 48 hours, cells were infected with A/WSN/33 at an MOI of 0.001 and incubated for an additional 48 hours. Cellular supernatant was evaluated for virus titer by $TCID_{50}$. Data is representative of two independent experiments.

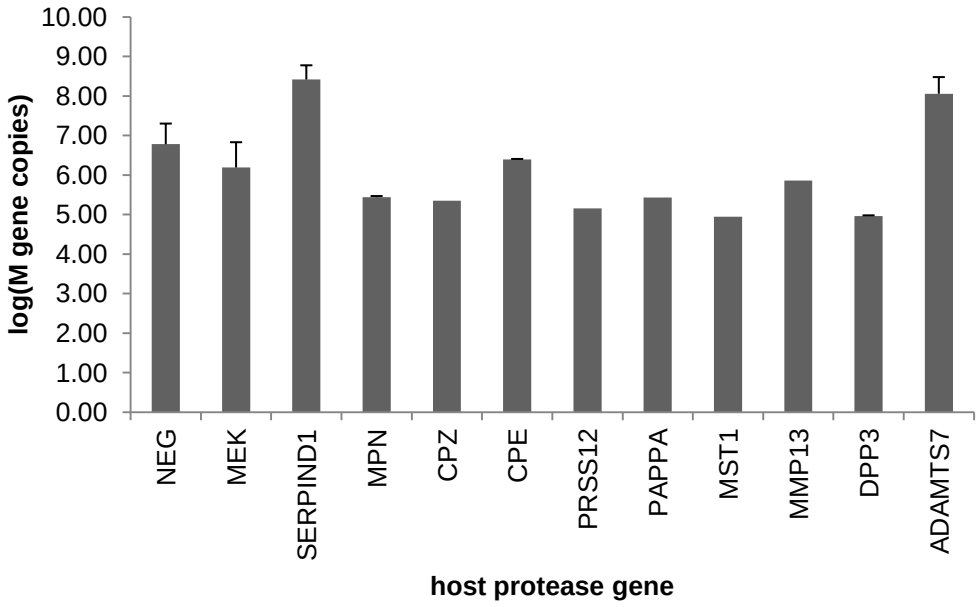

Figure A.2: Validation of genomic virus replication using novel siRNAs. A549 cells were reverse transfected with 100 nM of an individual siRNA targeting the protease gene of interest at a different seed site from any of the siRNAs used in the primary screen, as described in the methods section. After 48 hours, cells were infected with A/WSN/33 at an MOI of 0.001 and incubated for an additional 48 hours. Cellular RNA was harvested and influenza M gene copy was determined by qPCR. Data is representative of two independent experiments.

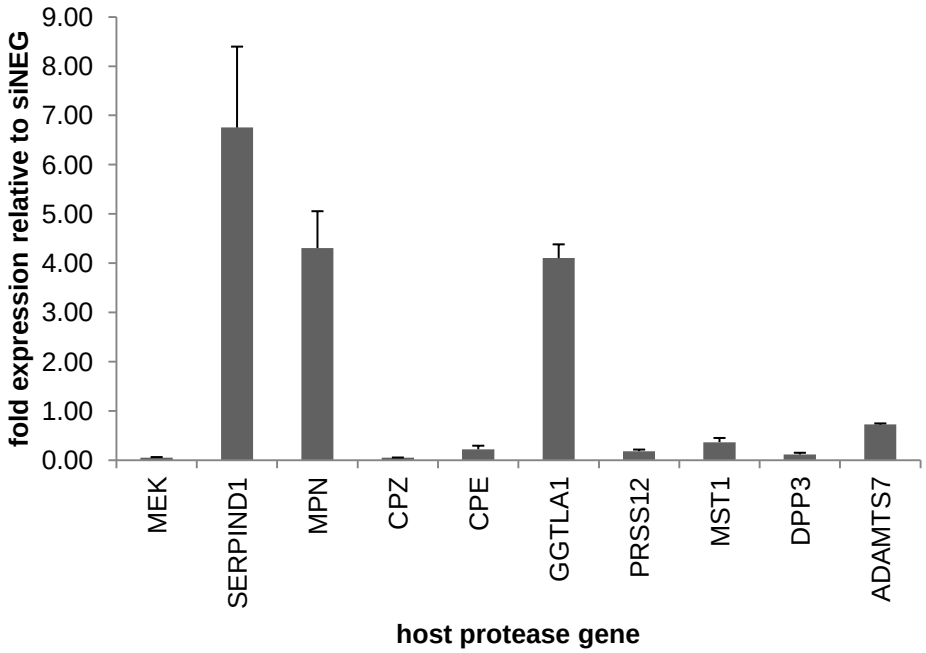

Figure A.3: Novel siRNAs were tested to determine targeted gene silencing by qPCR. A549 cells were reverse transfected with 100 nM of the novel siRNA as described in the methods section. After 48 hours, cellular RNA was harvested and mRNA expression of the target gene was determined by qPCR using gene specific primers. Values above 1 indicate increased expression relative to siNEG-treated controls, and values below 1 indicate silencing. Gene expression was normalized to GAPDH.

Table A.1: Gene specific primers from secondary validation screen.

Gene	Primer	Sequence (5'→3')
MEK	MEK_F	GCCCTCCAACATCCTAGTCA
	MEK_R	GACAGTCCCATGCTCCAGAT
SERPIND1	SERPIND1_F	CCCGCTGGACTATCACCTTA
	SERPIND1_R	CCTCGGGTTTTCTTCTAGGG
MPN	MPN_F	CTTTGAGACGGGCATGAACT
	MPN_R	TGCTGTAGAGCAGGTTGCAC
CPZ	CPZ_F	GATGTTCAAGCTGCTGTCCA
	CPZ_R	GTTGTGCTGCCAGAGTGTGT
CPE	CPE_F	TACCTGGAGGGATGCAAGAC
	CPE_R	TGTTTTGAGATCACCGTGGA
GGTLA1	GGTLA1_F	GCCGCAGGCTACTAAGACAC
	GGTLA1_R	GCCACCTCTTCCACTCTCAG
PRSS12	PRSS12_F	TGAGTTTGAAGGCACAGTGG
	PRSS12_R	CACCCTGCCAGATGTCTTTT
MST1	MST1_F	TTCACGTTTACCTCCGAACC
	MST1_R	TGCCACACTTCTCAAACTGC
DPP3	DPP3_F	TAGCAAGTTCAGCACCATCG
	DPP3_R	AAAGCCAAAGATCTCCAGCA
ADAMTS7	ADAMTS7_F	AGCAAAGAGAAGTGGGTGGA
	ADAMTS7_R	CCATGTTCATGATGGTCAGC
GAPDH	GAPDH_F	TGAACGGGAAGCTCACTGG
	GAPDH_R	TCCACCACCCTGTTGCTGTA

Table A.2: Direct and indirect network analysis of the human protease library.

Interaction	Cellular networks	Protease genes
Direct	Connective tissue disorders, genetic disorder, protein degradation	PRSS12, SERPIND1
Direct	Protein degradation, cancer, cell-to-cell signaling and interaction	ADAM30, ADAMTS7, CPE, ELA1, MST1
Direct	Cellular development, hepatic system development and function, cancer	SENP1
Direct	Cell death, embryonic development, gene expression	GGTLA1
Direct	Cellular assembly and organization, molecular transport, nucleic acid metabolism	DPP3
Indirect	Connective tissue disorders, genetic disorder, protein degradation	CPE, PRSS12
Indirect	Gene expression, cellular development, cellular function and maintenance	DPP3, SENP1
Indirect	Molecular transport, cancer, cellular function and maintenance	ADAMTS7, ELA1, SERPIND1

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