HOST GENES AS ANTIVIRAL TARGETS FOR INFLUENZA VIRUS

by

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ABSTRACT

Influenza virus causes mild to severe respiratory tract infections and is a substantial health concern causing seasonal epidemics and sporadic pandemics resulting in morbidity, mortality, and economic losses worldwide. Several anti-influenza drugs are available, but these agents target viral components and are susceptible to drug resistance. Influenza viruses require host genes to replicate. RNA interference (RNAi) screens can identify specific host genes coopted by influenza for replication. Targeting these pro-influenza genes can provide therapeutic strategies to reduce virus replication. Identifying microRNAs (miRs) that regulate these host genes can support an antiviral strategy while repurposing of clinically approved drugs that target cellular machinery necessary for influenza virus replication can provide a therapeutic approach for inhibiting influenza virus replication. Herein, we used RNA interference screening to identify key host cell genes required for influenza replication using human lung (A549) cells and identified 19 pro-influenza GPCR and 13 pro-influenza ion channel genes using small inferring RNAs (siRNA). These pro-influenza genes were authenticated by infecting siRNA transfected cells with A/WSN/33, A/CA/04/09, and B/Yamagata/16/1988 resulting in validation of 16 pro-influenza GPCR and 5 pro-influenza ion channel genes. These findings showed that

several GPCR and ion channel genes are needed for production of infectious influenza virus and provide potential targets for the development of host-directed therapeutic strategies to impede the influenza productive cycle to limit infection. Thirty-three miRs predicted to target these pro-influenza host GPCR or ion channel genes were screened using A/WSN/33 A/CA/04/09, or B/Yamagata/16/1988 infected A549 cells using miR mimics and their paired inhibitors and evaluated for their effect on influenza replication. Several miRs prevented influenza virus replication through GPCR or ion channel gene regulation and 4 pan-anti-influenza miRs were identified. There is a need for new antiviral drug strategies that include repurposing of clinically approved drugs. Herein, we identified the FDA-approved drugs Clopidogrel and Triamterene and evaluated these drugs' ability to inhibit A/WSN/33 and A/CA/04/09 influenza A strains, and Yamagata/16/1988 influenza B replication *in vitro*. Both Clopidogrel and Triamterene reduced influenza replication and spread across multiple strains and subtypes providing a potential druggable approach to influenza treatment.

INDEX WORDS: influenza virus, GPCR, ion channel, drug repurposing, microRNA, host-directed therapeutics, antiviral

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DEDICATION

This work is dedicated to my parents who through their many sacrifices and unyielding support have taught me to persevere against the odds and who have been a consistent source of love and inspiration. To Me-Me, who at an early age inspired creativity and independence. Thank you all for teaching me that anything is achievable if you are willing to work for it. To my husband Brad, who has always supported my dreams as his own and inspires me to keep dreaming. And finally, our son Luke, without whom this would be meaningless. Luke, I hope you are as proud of Mommy as I am of you every day.

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CHAPTER 1

INTRODUCTION

Influenza A viruses (IAV) and influenza B viruses (IBV) are members of the Orthomyxoviridae family. IAVs and IBVs contain 8 negative-sense, single-stranded viral RNA gene segments which encode for 10 primary viral proteins: hemagglutinin (HA), neuraminidase (NA), matrix 1 (M1), matrix 2 (M2), nucleoprotein (NP), non-structural protein 1 (NSP1), non-structural protein 2 also known as nuclear export protein (NS2 or NEP), polymerase acidic protein (PA), polymerase basic protein 1 (PB1), polymerase basic protein 2 (PB2) and polymerase basic protein 1-F2 (PB1-F2), as well as several strain-dependent accessory proteins via frame shifts and alternative splicing event (1-6).

The RNA dependent RNA polymerase drives the viral replication cycle of influenza but has no proof reading ability making it highly error prone ($>7.3x10^{-5}$ mutation rate per replication per base pair) (7). Accumulation of point mutations within the HA globular head region which contains hypervariable antigenic sites or at key antigenic sites of the NA can lead to alterations of the glycoproteins' antibody-binding sites resulting in antigenic drift. Drift variants emerge that are no longer inhibited by antibodies to previous strains and are responsible for annual seasonal epidemics (8). Antigenic shift, or genomic reassortment events, results in an abrupt antigenic change and typically occurs when a human lineage virus reassorts with an avian or swine lineage virus to produce a novel subtype virus that is antigenically distinct from circulating influenza viruses (8). These viruses may result in IAV pandemics associate with significant morbidity, mortality,

morbidity, and economic losses (9, 10). Globally, approximately 10% of adults and 20% - 30% of children are affected by seasonal influenza epidemics resulting in substantial illness, some hospitalizations, and 290,000 - 650,000 deaths annually (11, 12). Annual vaccination remains the most effective control measure against influenza infection. Since the first attempted inactivated influenza vaccine in 1942, much progress has been made, but vaccine efficacy is still variable and has ranged from 60% to as low as 19% (2009-2019) when mismatch or pandemic strains emerge due to waning vaccine-induced antibody responses, antigenic drift and occasional antigenic shift, despite advancements of vaccine technology (13, 14). Supplementary counter measures include the use of anti-influenza chemotherapeutics (antivirals).

There are four FDA-approved anti-influenza drugs recommended by the CDC for use against circulating influenza strains: peramivir, zanamivir, oseltamivir and baloxavir marboxil. Peramivir, zanamivir and oseltamivir belong to the neuraminidase inhibitor (NAI) class of antivirals and reduce influenza by reduction of viral budding from infected cells. Although cross-resistance among NAIs has not yet been observed, point mutations within the neuraminidase glycoprotein of circulating strains in 2007-2008 and 2008-2009 influenza season resulted in up to 90% of circulating strains conferring oseltamivir resistance suggesting overuse of NAIs provides the pressure needed to induce resistance (15, 16). Baloxavir marboxil, licensed for use in the USA and Japan in 2018, targets and inhibits the cap-dependent endonuclease activity of the IAV and IBV polymerase acidic protein (PA) inhibiting viral RNA synthesis (17). Unfortunately, administration of Baloxavir marboxil is ~3x more expensive compared to oseltamivir (18). Due to its relatively new existence, resistance to baloxavir marboxil in not well defined, but it is likely

as evidenced by a pediatric study in Japan that resulted in ~20% of viruses isolated from treated children to develop mutations (19). As of the 2004-2005 influenza season, the adamantine derivatives amantadine and ramantadine, both M2 ion channel inhibitors are no longer recommended due to increased resistance and limited efficacy (20, 21). Collectively, decreased sensitivity to anti-influenza antivirals generates inconsistency among therapies and foreshadows inevitable shortcomings for influenza disease intervention strategies (22). The targeting of host cell factors required for influenza replication offers a refractory approach to limit or prevent drug resistance while also providing a broader spectrum of efficacy across multiple viruses.

Influenza hijacks and exploits host proteins and affects associated cellular pathways at various stages of viral entry, replication and egress. Some of the most studied pathways exploited by influenza include nuclear factor kappa B (NFκB), phosphatidylinositol-3-kinase (PI3K), mitogen-activated protein kinase (MAPK), protein kinase C/protein kinase R (PKC/PKR), toll-like receptor (TLR) and retinoic acid-inducible gene 1 (RIG-I) pathways (23-26). Currently, approved anti-influenza chemotherapeutics rely on targeting viral proteins. Unfortunately, due to influenza's high rate of mutation and ability to recombine its genome segments during assembly, escape mutants emerge resulting in decreased effectiveness, thus demonstrating a need for novel strategies for intervention. Targeting host factors offers a refractory approach to limit or prevent drug resistance while also providing a broader spectrum of efficacy across multiple viruses in a genus.

RNA interference (RNAi) is an evolutionary conserved mechanism of post-transcriptional gene-specific regulation which can be exploited to probe the virus-host interface and identify novel host genes and the associated cellular processes amenable to

influenza replication (27-32). RNAi screening probes the virus-host interface and provides data for the identification of novel host genes. These data can be applied to develop novel host targeted antiviral strategies against influenza (30, 33, 34). siRNAs mediate post-transcriptional gene silencing via sequence-specific nucleolytic cleavage or translational inhibition upon interaction with their target mRNA (35). Similar to siRNAs, miRNAs are noncoding RNAs central in gene regulation. siRNAs and miRNAs share many similarities however siRNAs are rationally designed to be specific for one mRNA target, whereas miRNAs can bind to multiple targets and regulate RNAi pathways (36).

GPCRs are a large superfamily of ubiquitous seven-transmembrane cell-surface receptor proteins which facilitate intracellular communication via activation of signal transduction pathways upon binding of external ligand (36). Pathways controlled by GPCRs are numerous and often hijacked by viruses at various nodes to facilitate entry, replication and egress. For example, HIV tropism is associated with CXCR4/CCR5 co-receptor and GPCR15 (37-39). In addition, blocking select GPCRs with drug antagonists obstructs Marburg virus and Ebola virus cell entry and replication (40). The overarching influence of GPCRs on cellular processes makes strategies that target GPCRs amenable to disease intervention.

ICs transverse cellular membranes and allow ion exchange between the extracellular and intracellular space as well as the cytoplasmic space and compartments of the cell (41). ICs facilitate the influx/efflux of Na^{+}, K^{+}, Cl^{-}, or Ca^{+2} ions which regulate effector pathways. For example, inhibition of K^{+} channels at the early stages of Bunyamwera virus infection hinders virus replication post-entry (42). In addition, Cl^{-} channels are important for herpes simplex virus 1 entry and viral-host fusion (43). Further, the Na^{+} channel opener,

SDZ-201106, can inhibit IAV replication via the PKC pathway inhibition (44), and modulation of Cl^- or Na^+ secretion/absorption in the respiratory tract contributes to regulation of respiratory disease (45).

The overall objective of this project is to use RNAi to identify GPCR and IC genes that are co-opted for influenza replication. siRNA silencing of these pro-influenza host genes can provide leads for host-target strategies for intervention and add to our understanding of the cellular factors and networks required for influenza replication. These data can be used to identify potential anti-influenza miRs and provide new host factors enabling rational development, repurposing and rescuing of existing FDA-approved drugs to be used to combat influenza. In this way, this study will contribute to our overall knowledge of the host-virus interface, identify negative regulators of that interface via miR validation and identify potential therapeutics providing a platform for novel intervention strategies.

Central Hypothesis: Silencing pro-influenza IC or GPCR genes using RNAi will reduce influenza virus replication *in vitro*. These data will further our understanding of host factors and processes required for influenza replication, identify miR which negatively regulate influenza replication and identify novel targets for drug repurposing.

Specific Aim 1. Determine GPCR and IC genes that when silenced inhibits influenza replication following A/WSN/33, A/CA/04/09, and B/Yamagata/16/1988 infection of A549 cells.

Specific Aim 2. Identify miRNAs that regulate pro-influenza host genes and determine their ability to modify A/WSN33, A/CA/04/09, and B/Yamagata/16/1988 replication.

Specific Aim 3. Identify FDA-approved drugs that can be repurposed to inhibit GPCRs and IC genes co-opted by A/WSN33, A/CA/04/09, and B/Yamagata/16/1988 for replication.

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CHAPTER 2

LITERATURE REVIEW

Overview of influenza A and influenza B viruses

Influenza viruses are members of the Orthomyxoviridae family classified into types A, B, C or D based on genetic divergence and protein expression. IAV and IBV contain 8 genome segments while ICV contain 7. This is made possible by the expressing of a single major surface glycoprotein, hemagglutinin-esterase-fusion protein (HEF), which replaces the IAV and IBV HA and NA, and a single minor envelope protein CM2 (1). Influenza D (IDV), first identified in swine, has a genome structure nearly identical to ICV with unique features. For example, IDV has an adenine at position 5 at it 3' end of its segments which is in contrast to ICV which has a cysteine at the same position (2). IAV, IBV and ICV are known to infect humans. IBV and ICV typically infect humans while IAV has the ability to infect mammalian and avian species (3). IAV is responsible for seasonal epidemics and occasional pandemics. IAV and IBV causes some morbidity and mortality, and ICV is not responsible for any significant disease in humans (4). IAV and IBV viruses contain 8 negative-sense, single-stranded viral RNA genome segments which encode for 10 viral proteins: HA, NA, M1, M2, NP, NSP1, NS2 (aka NEP), PA, PB1, PB2, and PB1-F2 as well as several strain-dependent accessory proteins via frame shifts and alternative splicing event (5-10). A-type viruses are further subtyped based on HA and NA. Eighteen HA (H1-H18) and 11 NA (NA1-NA11) proteins have been identified. Furthermore, type-A viruses are divided into different strains within the subtype based on variations of the surface

proteins which result in HA/NA isoforms. B-type and C-type viruses are not subtyped. B-type viruses are instead divided into lineages, B/Yamagata and B/Victoria, and strains. Influenza viruses are named using a WHO/CDC naming convention which incorporates the host origin if other than human, antigenic type (e.g. A, B, C, or D), geographical origin of isolation, strain number and year of isolation (e.g. B/Yamagata/16/1988). For IAV, the HA and NA antigenic description is also noted, (e.g. A/Sydney/05/97(H3N2). Furthermore, pandemic viruses are assigned a distinct name designated with the pdm and year (e.g. A/H1N1/pdm09) and variant viruses which normally circulate in swine but have been isolated from humans are denoted with 'v' (e.g. A/H3N2/v) (11).

Influenza viruses are formed from host-cell derived lipid envelopes which form roughly spherical with a diameter of ~100nm or filaments reaching up to 20μm in length (12). The viral envelope surface is decorated with the viral membrane proteins, in descending concentration, HA, NA and the M2 ion channels (13). The cell derived envelope may also contain host-derived membrane proteins which can differ between cell types (14-16). M2 transverses the envelope through a supportive M1 layer beneath and into the virions core (17). Within the viral core is a segmented RNA genome. IAV and IBV contain 8 single-stranded negative-sense vRNAs. Each vRNA is bound to NP and associated with a single copy of heterotrimeric viral polymerase (PB1, PB2 and PA) at the helical hairpin to form individual viral ribonucleoprotein complexes (vRNPs) (18, 19). These vRNPs code for 10 primary viral proteins (5-10). Influenza A segments 1, 2 and 3 encode the components of the viral RNA-dependent RNA polymerase, segments 2 and 3 also encode for strain dependent non-essential accessory proteins such as PB1-N40 and

PA-X. Segment 4, 5 and 6 encode the HA, NP and NA proteins, respectively. Segment 7 encodes the M1 and M2 protein by alternative splicing. Segment 8 encodes NSP1 and NS2 also known as NEP via alternative splicing (6, 7, 9). Largely similar to IAV, IBV differs in its accessory proteins, notably segment 6 which encode NA which also encodes the accessory protein NB. Segment 7 of IBV encodes M1 and M2, but M2 is translated via termination–reinitiation rather than alternative splicing (20).

Influenza replication

Influenza viruses initiate infection by host receptor binding of HA to surface glycoproteins that contain sialic acid residues, and enters the host cell by receptor-mediated endocytosis triggered by the interaction between the HA1 subunit of the viral membrane protein HA and the host cell surface sialic acid receptors (3). Endocytosis of the virion occurs either by a clathrin-dependent mechanism involving dynamin and adapter protein Espsin-1 or by micropinocytosis (21, 22). Preferential binding of HA depends on the type of sialic acid receptor linkage. Human influenza viruses preferentially bind α(2,6) while avian preferentially bind α(2,3). Swine influenza viruses have demonstrated preferential binding for both types lending to their importance as mixing vessels for both avian and human viruses (17). Following endocytosis, the virion is enclosed within an endosome inside the cytoplasm. The environment within the endosome has relatively low pH (5-6) compared to the extracellar space. This low pH triggers M2 activation and induces a conformational change in HA0 to expose to expose the fusion peptide of the HA2 subunit. Activated M2 allows acidification of the viral particle causing disassociation of the vRNPs from M1 preparing the genome segments for transfer to the cytoplasm. Concurrently, the

fusion peptide inserts into the endosomal membrane bringing viral and endosomal membranes in close contact with each other to promote the formation of a hairpin. Following collapse of the hairpin the two membranes are pulled closer together until fusion of the two membranes occurs. The vRNPs are released and are trafficked to the nucleus by host cell machinery (23). At the nucleus, translocation is facilitated via nuclear localization signals (NLS) located on the vRNPs (17, 24, 25).

A viral RNA dependent RNA polymerase complex (RdRp) is associated with the 5' and 3' ends of each of the negative sense vRNA segments and is necessary for initial transcription due to the polarity. Following nuclear translocation, cap snatching directed by the PB2 subunit binding to the 5' cap of host mRNA, leads to initiation of viral mRNA transcription using the 5'cap as a primer (26). Transcription of viral mRNAs is terminated by a stuttering process which occurs upon the vRdRp encountering consecutive uracil bases at the 5' end of the vRNA producing a polyadenylated 3' end on the newly formed viral mRNA (26). Newly synthesized viral mRNAs are either exported to cytoplasm as synthesized or undergo alternative splicing. Viral mRNAs within the cytoplasm are translated into viral proteins by the host ribosomes. Newly synthesized PA, PB1 and PB2 (viral polymerase subunits) and NP proteins are trafficked back to the nucleus to assemble vRNPs for packaging. Composed vRNPs are exported to the cytoplasm via M1 binding to viral RNA segments and its association with NEP, which masks the NLS used during vRNP import facilitating the exit of vRNPs from the nucleus (27).

HA, NA and M2 proteins are translated at the rough endoplasmic reticulum via membrane-bound polyribosomes. These proteins are further modified at the Golgi

apparatus where they subsequently loaded into transport vesicles which bud from the trans-face (3, 27). Vesicles are transported to the cell membrane via sorting signals on the proteins. The process by which M1 and vRNPs are transported to the apical surface of the cell is unclear. It is thought that M1 and NP proteins are translated via non-membrane-bound polyribosomes and transported absent the exocytic pathway (61). Viral RNPs are most likely transported via interaction with HA and NA to cell membrane. How the genome segments are sorted and subsequently packaged into virions is not completely understood, but there is strong evidence to support a model which suggests sorting via internal signals located at the 3' and 5' ends of the vRNPs (28). The transportation of early (HA, NA and M2) and late (M1) as well as the vRNPs to the site of assembly at the apical surface of polarized cells triggers the budding and subsequent formation of the virion. The M1 protein facilitates the complete association of the membrane to form the closed envelope. The virion is release from the membrane following neuraminidase cleavage of sialic acid residues (27).

Influenza and disease

IAV is responsible for seasonal epidemics and periodical pandemics resulting in morbidity and mortality around the world. Antigenic drift in the HA gene can lead to changes in viral surface proteins resulting in variation of circulating strains and the unpredictable and often inconsistent length and severity of the flu season (29, 30). The number of influenza virus-associated illness and deaths varies due to strain differences and the length and severity of the influenza season. Globally, seasonal influenza epidemics results in numerous hospitalizations and 290,000 - 650,000 deaths per year (4, 31). The

most recent pandemic influenza strain (i.e. H1N1 2009) resulted in >60 million cases, >274,000 hospitalizations and >12,400 deaths in the United States (32). IAV vaccines require annual reformulation to prevent vaccine failure (33). The 2014-2015 influenza vaccine composed of A/Texas/50/2012 (H3N2), A/California/7/2009 (H1N1) and B/Massachusetts/2/2012-like strains had low vaccine efficacy against the IAV H3N2 strains largely due to drift events which most likely occurred post-selection (34).

There are four known routes of transmission between hosts: direct contact, indirect contact, droplet. and airborne. Direct contact occurs following the transfer of virus as a result of physical contact between and infected host and a susceptible host. Indirect transmission occurs passively when a susceptible host interacts with contaminated objects. The ability of the virus to survive on a surface is relative to the microenvironment and porosity of the surface. Droplet transmission occurs through direct or indirect contact with surfaces contaminated by coughing and/or sneezing which propels droplets (≥5μm) containing virions contaminating the immediate vicinity (1m radius) (35). Infection results as a consequence of these droplets coming in contact with nasal, oral or ocular mucosal surfaces. Airborne transmission occurs following aerosolization of influenza virus (<5μm droplets) and/or contact with contaminated debris suspended in the air (35). Influenza virus can remain suspended in the air in low humidity for up to 24 h or up to 1 h in high humidity at relatively low levels depending on the host origin and strain. Direct, indirect and droplet transmission can be avoided with proper hygiene and the reduction of contact between the hands and mucosal surfaces. Airborne transmission is more difficult to control because it requires the use of ventilation systems (35).

Immune response to influenza virus

The interwoven nature of the innate and adaptive immune responses are important for the control and clearance of influenza virus infection. The innate immune response is the initial line of defense against infection. In the human host, influenza virus primarily infects the respiratory epithelial cells but also has the ability to infect alveolar macrophages and dendritic cells (DCs) (36). These cells assist in viral clearance by limiting viral propagation as part of the innate immunity and priming of the adaptive immune response. Macrophages and dendritic cells have ability to respond rapidly to influenza infection upon stimulation triggered by the recognition of viral nucleic acids by the toll-like receptor family of pattern recognition receptors, specifically TLRs 3, 7, 8 and 9 (37). TLR activation induces the expression of proinflammatory cytokines as well as interferons. These immune modulators non-specifically inhibit viral replication and spread by initiating the antiviral response while also modulating tissue inflammation and the activation of the adaptive immunity via the recruitment and subsequent activation of leukocytes.

Type I and III IFNs have an important role during influenza infection. These two classes of interferons are expressed in response to viral components and their expression is upregulated by ISGs within the airway epithelia (38). Silencing studies have shown that RIG-I is a primary mediator of the IFN response during influenza infection in the airway epithelium and that this pathway can yield type I or type III interferons (39). It is debatable whether type I or III IFNs have a greater role in controlling influenza infection. While type I IFNs have been shown to promote systemic immunity linked to DC and alveolar macrophage responses, type III IFNs promote a more localized response as the IFN

receptors are restricted within the airway epithelium during infection (40). It has been proposed that type III IFNs are increased in the lung compared to type I IFNs in an effort to restrict the pro-inflammatory response thus reducing lung tissue damage (41).

Type I IFN receptors consist of IFNAR1 and IFNAR2. Binding induces signal-transduction pathways which result in the transcription of IFN-inducible genes or IFN-stimulated genes (ISGs). These ISGs activate the antiviral state which includes but is not limited to rendering cells more recalcitrant to infection, activating effectors cells and promoting the development of the acquired immune response (42). Type II IFN include IFN-γ which is produced by NK cells and T cells in response to viral infection. Type III IFNs include λ1 (IL-29), λ2 (IL-28A) and λ3 (IL-28B). Type III IFN are induced directly by viral infection utilizing a similar pathway to that of types I IFNs. These IFNs bind the heterodimeric IL-28 receptor composed of IL-10R and IFNLR1 to elicit similar immune responses by STAT phosphorylation (43, 44). Differences in the regulation of these two classes of IFNs have been demonstrated. It was shown in mice that acute viral infections were controlled better by type III IFNs, while systemic infections were controlled by type I IFNs (45). In other examples, IFN responses and the ability to continue to response to IFNs depends on the cell types/tissue types involved as well as the IFN type involved during the response (46). These data suggest that type III IFN responses are largely consigned to the epithelium. Type III IFN signaling relies on the activation of on the mitogen activated protein kinase signaling pathway during promotion of the antiviral state in contrast to type I IFN which does not. This divergence may provide the explanation for the functional differences in these two classes of IFNs (45).

There are many pathways by which viral infection can activate IFN expression including recognition of extracellular dsRNA, dsRNA within endosomes, ssRNA within endosomes, DNA within endosomes, intracellular viral RNA, cytoplasmic viral DNA, and viral proteins. Recognition of ssRNA at the cell surface or within the endosome is dominated by TLR7. In pDC activation of TLR7 plays a major role in the recognition of influenza infection during virion entry. TLR7 recognizes ssRNA in the absence of sequence specificity requiring only the recognition of a string of uridine activation (47, 48). TLR7 activation induces the production of type I IFN through recruitment of MyD88 and either the IRF-7 or NFkB pathways (49-51). These cascades while utilizing various adaptor proteins predominately signal through MyD88 and to a lesser extent NFkB (49). TLR 3 and 8 also sense viral components, but their response is secondary to TLR 7. TLR 3 in macrophages and DC senses dsRNA at the cell surface or within the endosome to trigger IFN-B and proinflammatory cytokines production through TRIF and NFkB. TLR-8 in human monocytes and macrophages sense ssRNA trigger the production of IL-12. Recognition of intracellular viral RNA and the subsequent induction of IFN is independent of TLR activation and is instead dominated by RNA helicase molecules RIG-I and MDA-5. Both RIG-I and MDA-5 can be activated by dsRNA, but RIG-I can also be activated by RNA with 5’ triphosphates. Both molecules recruit Cardif/VISA/MAVS/IPS-1 via their N-terminal CARD domains and elicit IFN-B promotor transcription through IF-7, IF-3 or NFkB pathways (42). Robust interferon induction requires transcription, indicated by the correlation between the accumulation of viral RNA and increased IFN production, but replication is not required for activation (42). This is further confirmed as the major IAV PAMPs require the synthesis and nuclear export of an RNA product or products from

infecting genomes and the synthesis of these RNAs is uninhibited by the impairment of cRNPs and vRNPs accumulation. Furthermore, distinct PAMPs are made at different junctures of the viral replication cycle making the incoming influenza A virus genomes a minor contributor to the initial activation of IFN at early time points post infection (42).

Recognition of influenza virus by TLRs leads to the transcription of IFN promotors and the production and secretion of IFNs. Following secretion, IFN α/β bind the type I IFN receptor composed of IFNAR1 and IFNAR2 to induce oligomerization. Binding of IFN induces a confirmation change in the receptor promoting the phosphorylation of the receptor associated tyrosine kinase 2 and JAK1 and subsequent recruitment and phosphorylation of STAT2 and STAT1, respectively. These phosphorylated proteins dimerize to form a stable heterodimer which is translocated to the nucleus. Inside the nucleus it interacts with IRF-9 to form ISGF3. ISGF3 binds to the IFN-stimulated response element (ISRE) within the promoters of ISGs and enhances their transcription. Example ISGs include: IFITMs, TRIM, Mx proteins, zinc finger antiviral protein (ZAP), oligoadenylate synthase ribonuclease L (OAS-RNAseL), protein kinase R (PKR), ISG15, Viperin and Tetherin (52). Type III interferons follow a similar pathway (42).

The production of IFNs enhance the expression of hundreds of ISGs to promote the antiviral state and limit viral replication. PKR is produced in the inactive form within the cell and can undergo dimerization and activation following IFN signaling. PKR controls viral replication via many mechanisms such as induction of apoptosis and cell-cycle arrest effective halting viral replication due to lack of functional use of cellular machinery. PKR can also directly bind dsRNA and suppress viral replication (52). OAS is activated by IFN to oligomerize ATP triggering it binding and subsequent activation of RNase L. This

complex binds and degrades cellular and viral RNA. This process may also further induces IFN secretion (53). ZAP inhibit PB2 and PA expression by reducing viral mRNA expression and translation (54). Viperin restricts microdomain formation within lipid rafts at budding sites reducing influenza virus budding (55). Tetherin limits release of viral progeny at the cell surface by degrading newly budding viruses (56). The Mx family of genes encodes a large family of GTPases with poorly defined antiviral activity. In short, MxA/B and Mx1/2 in humans and mice, respectively, bind viral proteins like nucleocapsid protein and inhibits its translocation to the nucleus (57). ISGs may reduce virus replication by stabilizing cellular proteins. For example, ISG15 covalently binds cellular proteins and acts like a cytokine in IFN-treated cells (58). It is thought that ISG15 reduces viral replication by stabilizing antiviral immune system proteins and downregulating cap-dependent translations and by interaction and direct inhibition or viral proteins (59). ISG15 has been shown to bind influenza B virus NS1 protein and prevents its action (60). Upregulation of IFITMs restricts viral entry indirectly by alteration of cell adhesion, fluidity and topography (42). The tripartite motif (TRIM) family ISGs play various roles to inhibit influenza. For example, TRIM25, an E3 ubiquitin ligase regulates the relocalization of RIG-I to the mitochondria while TRIM22 blocks IAV interacts with NP resulting in polyubiquination and degradation in a proteasome-dependent manner (61, 62). Additionally, TRIM32 directly binds PB1 or the RNA polymerase and restricts its activity (63).

Plasmacytoid DCs are activated by signaling through TLR7. RIG-I is also affected by the recognition of viral RNAs within the cytoplasm which induces similar responses in other cells. IFNs are important for the initiation of an antiviral response and activation of

the JAK/STAT pathway that affects ISGs and DCs antigen presentation to T cells, and activation of the NLRP3 containing inflammasome (64). Induction of IFNs also has a role in the upregulation of MHC I molecules and other APC components following infection. This upregulation often counters the downregulation elicited by viruses during infection. This upregulation helps to shield these infected cells from possible killing by NK cells due to low levels of MHC I. IFN also upregulates the production of perforin and granzymes to activate NK cells during infection (65). IFN α/β promotes the maturation of DC and helps to sustain the proliferation of antigen-presenting CD8+ T cells (66). IFNs interact with APCs to induce the secretion of IL-15 to promote the division of memory CTLs and sustain NK cell populations (67, 68). Alveolar macrophages are important innate immune cells which bridge the gap between the innate and adaptive immune responses having a key role in pathogen clearance and antigen presentation to T cells (36). Activation also induces the production of nitric oxide synthase 2 (NOS2) and tumor necrosis factor (TNFα) that contribute to influenza associated lung pathology. DCs are located between the epithelial and basal layers of the epithelium. Following internalization of a pathogen, DCs may home to the draining lymph node where they present pathogen epitopes to T cells thereby activating them. Virus-derived peptides are presented to CD8+ T cells and CD4+ T helper (Th) via the class I and II major histocompatibility complexes (MHC), respectively. NK cells are involved in pathogen defense. These cells induce lysis of infected cells via antibody dependent cell cytotoxicity (ADCC). The ADCC process is facilitated by the recognition of antibody-bound influenza infected cells. Collectively, the innate immunity is the first line of defense against influenza infection, providing the cytokines and chemokines to induce non-specific defense and the recruitment of T cells. In this way, the

innate immunity is linked to the second arm of the immune response, the adaptive immunity. The adaptive response, response is activated in part following the stimulation of these cells to release proinflammatory cytokines and chemokines, confers the maturation of virus-specific T cells and antibodies via the cellular and humoral arms of the adaptive response, respectively. Internalization of the virus by macrophages and dendritic cells allows for the presentation of influenza epitopes to T cells, priming an antigen-specific T cell response as previously discussed (64). As part of cell-mediated immunity, activated Th2 cells produce IL-4 and IL-13, which promote B cell proliferation and maturation. Activated Th1 cells produce IFN-γ and IL-2 promoting a robust cellular immune response. Th17 and Treg cells also contribute to cellular responses. Virus-specific cytotoxic T cells (CTLs) prevent the production and release of progeny virus via destruction of virally infected cells. CTLs recognition of infected cells induces the production of pro-inflammatory cytokines, perforin and granzymes. Perforin destroys the cell membrane allowing the granzyme to enter the cells inducing apoptosis. Virus replication is also limited by proteolytic cleavage of viral and host proteins required for influenza replication. Apoptosis can also be induced through the Fas/FasL system (64).

Host Cellular Pathways and Influenza Replication

Influenza virus uses host cellular machinery to facilitate transcription of its genome, translation of it viral proteins and assembly of these products to produce progeny virions. Interaction between viral and cellular proteins enables the manipulation of specific cellular pathways. The most studied pathways utilized during influenza infection include NF-κB signaling, the PI3K/Akt pathway, the MAPK pathways, the PKC/PKR, TLR, and RIG-I

pathways (69). The NF-κB pathways are vital to many cellular functions including regulation of the immune response, inflammation, host cell proliferation and apoptosis. Influenza HA, NP and M1 proteins activate NF-κB following infection, inducing an immune response, which is subverted by the viral protein NS1 and IFN antagonists. NF-κB is linked to viral RNA synthesis and its inhibition of produces influenza resistant cells (70, 71). The PI3K/Akt pathway is involved in cell survival, proliferation, differentiation, apoptosis, etc. and has been shown to be important during influenza replication and propagation. Influenza NS1 protein indirectly activated PI3K inhibiting cell death while regulating antiviral functions (72). This process is not fully understood. Ribosomal protein S6 kinase beta-1 (p70S6K) acts downstream of PIP3 and PDK1 in the PI3K pathways and is an alternative substrate for mTOR as part of the autophagy pathway during the cellular stress response. Influenza NS1 and M2 proteins have been reported to bind adaptor proteins to extend autophagy at moderate levels to limit apoptosis allowing prolonged viral replication (73, 74). Blocking p70S6K or associated mTORC2 activity decreases production of IAV (75). Of note, greater extension of autophagy is associated H9N2/G1 infection and the induction of CXCL10 and IFN-α responses as compared to H1N1 and novel pandemic swine origin H1N1 viruses. Inhibition of a critical component of the autophagy pathway resulted in 50% decrease in CXCL10 and IFN-α markers of increased pathogeneses and disease (76). The MAPK family of kinases is crucial for the regulation of the inflammatory response to influenza. These kinases (as participants of more complex host cell pathways), promote trafficking of viral ribonucleoprotein and virus production. There are four subsets of MAPKs, all of which are activated during influenza infection. These cascades include: the extracellular signal-regulated kinases (ERK), c-jun N-terminal

or stress activated protein kinases (JNK/SAPK), EKR5/ big MAP kinase 1 (BMK1) and p38 MAPK (69, 77). The PKC/PKR signaling cascade is an important modulator of the antiviral response. Following activation, PKR phosphorylates the eukaryotic initiation factor 2 (eIF2α) arresting translation and inducing apoptosis. PKC, an upstream modulator of Raf/MEK/ERK pathway, has been shown to phosphorylate viral M1 aiding in viral replication. This pathway is also important for the entry of influenza and other enveloped viruses (69). As previously discussed, TLR and RIG-I activation by influenza induce the antiviral response. NS1 diminishes this response by sequestering RNA generated during viral replication and by interacting with tripartite motif-containing protein 25 (TRIM25) a RIG-I activator thus inhibiting its action. PKA has been implicated in late stages of influenza infection, specifically virus budding (78). Targeted inhibition of PKA results in reduced budding of influenza virus (79, 80). Subtype specific infection, e.g. H1N1 but not H3N2, leads to activation of phospholipase γ1 (PLC-γ1) (81). Activation of PLC leads to hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP2) and the release of diacylglycerol (DAG) and 1,4,5-inositol trisphosphate (IP3). DAG in turn activates protein kinase C (PKC) while IP3 bind IP3 receptor ligand gated calcium channels on the surface of ER leading to increase concentrations of Ca^{+2} into the cytosol. This increase in Ca^{+2} may lead to the opening of cell surface calcium channel and the increased intracellular concentration of Ca^{+2} (82). Intracellular Ca^{+2} has a central regulatory role in the clathrin-mediated and clathrin-independent endocytosis pathways utilized by influenza to enter the host cell. Prevention of Ca^{+2} concentrations induced by IAV attenuates virion internalization and replication (83). Rho-associated protein kinase (ROCK) is a kinase belonging to the PKA/PKG/PKC family of serine-threonine kinases and is involved

regulating cell movement acting on the cytoskeleton (83). ROCK functions as a downstream regulator of virus-induced Ca^{+2} and inducer of Ca^{+2} signaling creating a circuit of regulation for regulating both pathways of endocytosis (83).

Anti-influenza agents

There are four FDA-approved anti-influenza drugs currently recommended to treatment IAV and IBV in the United States. These include the NAIs oseltamivir phosphate, zanamivir and peramivir as well as the cap-dependent endonuclease inhibitor baloxavir marboxil. The M2 inhibitors, amantadine and ramantadine, are no longer recommended due to reduced increased drug resistance resulting from point mutations within the M2 of circulating strains. Oseltamivir phosphate is an oral drug approved for the treatment of acute uncomplicated influenza infection in patients 2 weeks and older as well as prevention of infections in patients one year and older. Zanamivir a powder for oral inhalation approved for the prevention and treatment of uncomplicated influenza infection in patients 7 years and older. Peramivir is an injectable approved for the treatment but not prevention of acute uncomplicated influenza infection in patients 2 years or older. Baloxavir marboxil is the most recently approved anti-influenza antiviral as an oral tablet for the treatment of acute uncomplicated influenza infection in patients 12 years and older or for the prevention of infection following exposure. It is not currently recommended for patients with immune systems complicated by pregnancy, progressive illness, etc. Unfortunately, increased drug resistance is occurring hat is correlated with point mutations found within the NA protein. Resistance to oseltamivir increased by 95% between the 2006/2007 season and the 2008/2009 season (84). Furthermore, all current therapeutics

target viral proteins which have the propensity to mutate when placed under pressure due to the error prone viral RdRp. Collectively, increased resistance to NAIs leads to inconsistency with therapies (85). Consequently, there is an urgency for the development of antivirals with novel mechanisms of action to reduce the development of widespread resistance.

Drug repurposing and novel antivirals

Conventionally, drug development has three general approaches: 1) structure-based virtual screening (SBVS), 2) target-based virtual screening (TBVS) and 3) large-scale chemical library screens (LSCLS). SBVS and TBVS aim to predict the most favorable interaction between ligands and the molecular target to form a complex using the resolved 3D-structure of the target molecule. LSCLS require vast compound libraries and can be accomplished *in silico* or direct phenotype screening. Both methods can be cumbersome, lengthy, and costly and yield minimal results for considerable amounts of work. This is the bottleneck which restricts drug discovery and development presenting a need for alternative methods for target discovery and drug identification. On average, the costs associated with research, development and approval per new drug is on average US $4 billion and takes 10-15 years from preclinical testing to approval (86). The FDA estimates roughly 0.1% of compounds screened progress to development by virtue of an investigational new drug application (IND) and institutional review board approval. Compounds which receive an approved IND have a 40% conditional probability of success and will move on to clinical testing phases I, II, III safety and efficacy trials. This stage comprises the most costly and time-consuming portion of the drug development process

(FDA.gov/drugs). Alternatively, drug repurposing or repositioning of licensed drugs or compounds which have not been licensed for reasons other than safety, elevates the majority of the aforementioned impediments. In the majority of examples, the expense and time required towards approval are significantly reduced as result of the drugs previous demonstration of safety in humans.

Conventional routes for the identification of targets and development of subsequent therapies for influenza virus intervention suffer many bottlenecks during development as previously mentioned. One such alternative method for target discovery identifies host factors required for the efficient entry, replication and egress, but which are temporally dispensable to the cell. This method exploits the parasitic nature of influenza and its reliance on the host cells endogenous network of cellular pathways to replicate. Cellular host factors and their respective pathways can be essential or inhibitory to influenza virus replication. Understanding these host factors provides a pool of potential pharmacological targets while also adding to our knowledge of the molecular mechanisms involved during virus replication. While influenza viral proteins are prone to mutation, host proteins are typically conserved. Accordingly, host protein structures are roughly consistent throughout different individuals allowing for accurate targeting relative to viral proteins. Targeting host factors may provide a platform for intervention which is recalcitrant to the emergence of viral resistance as there is less environmental pressure placed directly upon the virus itself increasing the likelihood of prolonged efficacy of the drug. There is considerable overlap amongst viruses regarding the cellular pathways which are required for replication. In this way, a single drug and/or therapy has the potential to prove efficacious in treating

multiple infections owing to the conservation of host proteins and pathways throughout cell types and tissues including various subtypes of influenza virus. This approach also affords the opportunity to develop combination therapies, which may have a synergistic or additive effect when combined with current antivirals (87).

Functional analysis of host factors during infection historically employed the characterization of mutant phenotypes a lengthy cumbersome process which requires the alteration of cellular genetics. Alternatively, methods which involve loss-of-function are of particular interest due to their ability to transiently, specifically and within a relatively short period of time modulate endogenous host cellular processes. Of these methods, RNA interference provides a multifaceted, relatively quick, cost effective methodology to characterize gene function in various contexts.

Overview of RNA interference mechanisms

RNA interference (RNAi) is an evolutionary conserved process that affords post-transcriptional gene-specific regulation were gene silencing is facilitated by the production and processing of small RNA molecules. These RNA constructs mediate gene silencing via binding of small RNA molecules to target mRNA leading to mRNA degradation and halted protein expression by impeding translation. RNAi regulation is typically facilitated by the endogenous production (or exogenous introduction) of small RNA molecules to a host cell. RNAi has been used as a means to probe the virus-host interface to identify the host gene expression required for virus replication (88, 89). Of particular interest are the endogenously expressed miRNAs. miRs are small non-coding RNAs expressed from non-

coding cellular genes comprising an estimated 0.5-1% of predicted genes. Initially expressed as hairpin structures within the nucleus they are exported to the cytoplasm and processed to form single-stranded 19-25 nt RNA oligonucleotides (90). In the nucleus, RNA polymerase II produces primary miRNAs (pri-miR) ~2 kb in length (91). Pri-miR are processed within the nucleus by the RNase III enzyme Drosha and the double-stranded-RNA-binding protein Pasha/DGCR8 and folded into stem loop structures (92). Pri-miRs are then exported to the cytoplasm by karyopherin exportin 5 (Exp5) and Ran-GTP complex (93). In the cytoplasm the pri-miRNAs are further processed into miRs (~22 nt) by the RNAse III enzyme Dicer. Concurrently, Dicer initiates the formation of the RNA-induced silencing complex (RISC) (94). The miR:RISC complex is responsible for initiating mRNA contact and silencing. While miRNAs are genetically encoded, siRNAs are a result of the processing of exogenous synthetic double-stranded RNA oligonucleotides duplexes (exo-RNAi) or from the production of siRNAs from endogenous transcripts (endo-RNAi) (95-97). Unlike miRNAs, siRNAs are 20-24 nt with a two nt 3' overhang and require minimal processing by cellular kinases to enter the RISC complex. Following loading of the dsRNA into the RISC complex, regardless of miR or siRNA origin, Ago2 cleaves on strand (the passenger strand) and allows the other strand (the guide strand) to remain within the complex. Site directed cleavage or translational inhibition of target mRNAs and subsequent gene silencing is facilitated by the sequence dependent recognition of seed region complements within the target mRNA. siRNAs share complete complementarity to their target mRNA while miRNA seed site pairing (2-7nt) is partially complimentary yielding the possibility for multiple mRNA targets. Sequence-specific nucleolytic cleavage or translational inhibition upon interaction with their target mRNA is

directed by the Ago2 catalytic domain or inhibition of ribosome association (98). This process is utilized as an *in vitro* and *in vivo* tool to characterize host machinery and survey the virus-host interface providing a platform for the target discovery and successive identification of novel anti-influenza drugs (99).

miRs are key regulators of gene expression and thereby protein expression. Given that influenza virus requires host proteins to replicate, it is very likely that miRNA expression has a role in the host's response to viral infection (100). Several viruses are known to express miRNAs. These viruses like Epstein-Barr virus, herpes simplex viruses and cytomegaloviruses, usurp the host RNAi pathway. Influenza A expresses small viral leader RNAs, but these RNAs do not function like classical miR (101). As of yet, there is no evidence that influenza A codes for miRs, but the lack of evidence does not mean that influenza viruses are incapable of expressing miRs (102). miR expression profiles are dynamic and depend on the needs of the host. Modulation of specific miRs via replacement or inhibition can manipulate the host response to influenza infection and thus is a viable strategy to enhance treatment outcome. Host miRs have been documented to target viral mRNA. For example, miR-323, miR-491 and miR-654 directly target viral mRNA, specifically PB1 gene transcripts thereby limiting production of PB1 diminishing viral replication (103). It was demonstrated that demonstrated that host let-7c directly inhibits M1 synthesis and that hsa-miR-145 and -92a target influenza HA (104, 105). During influenza infection, miRs profiles change. Identification and evaluation of varying levels of aberrantly expressed miRs following influenza infection can be used to gain a better understanding of the pathogenicity of influenza as well as regulatory pathways, which

influence influenza infection. These data can be used as non-invasive molecular diagnostic tools (biomarkers) for monitoring treatments and patient stratifications following infection.

GPCR genes and virus replication

GPCRs are a large superfamily of ubiquitous seven-transmembrane cell-surface receptor proteins which facilitate intracellular communication via activation of signal transduction pathways upon binding of external ligand. GPCR signaling is composed of complex assemblage of networks with each GPCR complex having several isoforms and splice variants that creates hundreds of combinations of G proteins (106). The specific composition of the G-protein affects not only which transmembrane receptor it can bind to, but also which downstream target is affected conditioning its effector function (106-108). The G-protein consists of α and βγ subunits where theβγ subunit is bound to GDP when associated with its receptor. Activation is triggered by the binding of peptide or non-peptide ligands including but not limited to viral proteins, chemokine, nucleotides and chemokines ligand binding to the extracellular domain of the receptor protein. Upon ligand binding, a conformational change in the seven transmembrane α-helices and presents the intracellular guanosine binding site of the α-subunit to the cytoplasm making it available for guanosine binding causes the G-proteins to act as a guanine nucleotide exchange factor (GEF) exchanging GDP for GTP. Association of GTP initiates disassociation of the subunits from their membrane bound receptor and each other to produce Gα-GTP and Gβγ. Gα-GTP and Gβγ act as second messengers regulating the activity of enzymatic effectors to promote or stunt downstream signaling cascades (107). G-protein deactivation is a result of interaction with regulator of G-protein signaling proteins (RGS), accelerating the Gα

GTPase activity and thus hydrolyzing GTP to GDP. Deactivation leads to the re-association of the G-protein to the cell surface receptor (109). In the absence of external signal, the GPCR remains inactive and GTP unbound. Mammalian GPCR Gα subunits are grouped into four families (Gαs, Gαi, Gαq/11, Gα12/13) based on sequence homology consisting of ~20 distinct Gα subunit proteins due to splice variants (110). Gαi signaling preeminently inhibits adenyl cyclase which decreases intracellular cAMP inhibiting the activity of cAMP-dependent protein kinases such as protein kinase A (PKA), while Gαs signaling principally stimulates adenyl cyclase prompting the opposing effect and activating PKA and other host factors such as exchange protein directly activated by cAMP. The modulation of cAMP levels regulates the duration and intensity of cAMP signaling via feedback mechanisms by regulating specific phosphodiesterases (111). Gαq/11 signaling activates multiple downstream pathways following GPCR activation, but the most well characterized are those associated with phospholipase Cβ (PLC) activation and PI3K. Activation of PLC leads to hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP2) and the release of diacylglycerol (DAG) and 1,4,5-inositol trisphosphate (IP3). DAG in turn activates PKC while IP3 bind IP3 receptor ligand gated calcium channels on the surface of ER leading to increase concentrations of Ca^{+2} into the cytosol. This increase in Ca^{+2} may lead to the opening of cell surface calcium channel and the increased intracellular concentration of Ca^{+2} (82). Intracellular Ca^{+2} has a central regulatory role in the clathrin-mediated and clathrin-independent endocytosis pathways utilized by influenza to enter the host cell. Gα12/13 principally activates RhoA GTPase. ROCK is a kinase belonging to the PKA/ PKG/PKC family of serine-threonine kinases and is involved regulating cell movement acting on the cytoskeleton (83). ROCK functions as a downstream regulator of

virus-induced Ca^{+2} and inducer of Ca^{+2} signaling creating a circuit of regulation for regulating both pathways of endocytosis (83). Although, the α subunits provide effector target specificity the pathways effected by GPCR signaling often overlap and/or merge at various nodes within pathways either directly or indirectly via expression or inhibition of second messengers such as Ca^{+2} (108).

Host cell GPCRs influence virus tropism, attachment, entry, and replicative ability. Pathways controlled by GPCRs are abundant and are adopted by viruses to facilitate entry, replication and egress. For influenza virus, the most studied pathways include the NF-κB signaling pathway, PI3K/Akt pathway, the MAPK and PKC/PKR pathway, the TLR, and RIG-I pathways which have been associated with influenza virus infection and replication (72, 112-114). These pathways are among the pathways associated with GPCR modulation. Other virus specific examples include HIVs tropism, which is in part defined by interaction of the viral envelope with its choice of co-receptor CXCR4 or CCR5 GPCRs in most cases. Alternative GPCRs GPR15/Bob, CXCR6 and CCR3 have also been identified as co-receptors (115-117). Individuals that possess a homozygous mutation within the CCR5 allele are resistant to infection, while those who possess, and heterozygous mutation show slowed progression towards AIDS revealing further its importance for infection and spread of HIV/AIDS (118-120). It has been shown that blocking certain host GPCRs with drug antagonists effectively blocks entry of Marburg virus and Ebola viruses into cells and inhibited their replication (121). The overarching nature of GPCRs and their scope of influence on host cells makes drug targeting GPCRs pathways amenable to restrict influenza replication for disease intervention.

Host IC genes and virus replication

ICs form a transmembrane passageway allowing transmembrane passage of ions between the extracellular environment and the intracellular components of the cell (122). The flow of ions (Na^+, K^+, Cl^-, Ca^{+2}) into and out of the cell and cellular compartments and can function as effectors and/or generate second messengers. Binding of a ligand or excitation via provokes a conformational change opening or closing of these channels. These channels are selectively permeable and only transport ions of a particular size and/or charge based on concentration gradient or presence of ATP for active transport against the gradient. Specificity is dictated by the amino acids lining the barrel-like channel and the physical diameter of the channel. In general, the barrel-like structure is composed of 4 or 5 helices with repeat domains. The topology and sequences amongst ion channels vary (122). Examples include acid-sensing, voltage-dependent L-type calcium, NMDA receptor coupled, cholinergic receptor couple and GAB) receptor coupled ion channels. IC can facilitate the regulation and/or activation of effector pathways. It has been previously shown that inhibition of K^+ ion channels at the early stages of BUNV infection hinders BUNV replication processes which occur post-entry, but prior to viral RNA synthesis (123). Cl^- channels have been shown to be important for HSV-1 entry, specifically, modulation of Cl^- channels was able to inhibit HSV-1 viral-host fusion (124). The Na^+ channel opener SDZ-201106 was shown to significantly inhibit IAV replication via PKC pathway inhibition (125). Furthermore, modulation of secretion and absorption of Cl^- or Na^+ within the respiratory environment contributes to the regulation of many components

which may affect viral respiratory disease (126). To date, these gene families have been underrepresented in the context of IAV infection.

Ca^{2+} signaling is essential to IAV infection (83). Crystallization of the influenza neuraminidase 1 protein and subsequent proton induced X-ray emission analysis has predicted a high affinity calcium binding site near the active site of this enzyme with the specific purpose of this site unknown (127). Data suggests calcium ion binding at this site is important for enzymatic activity and thermostability (128, 129). Withholding calcium from group 2 neuraminidases results in destabilization at the active site residue R292 near this predicted calcium-binding site and thus it's most probable that calcium binding contributes to the integrity of the active site as well as the biding free energies and thus impacts substrate binding (130). Calcium concentrations are also important for the activation and deactivation of the pathways important for influenza replication, previously mentioned above. Influenza A enters the cell via clathrin-mediated and clathrin-independent endocytosis. Both processes are mediated by activation of RhoA, Rho-kinase, phosphatidylinositol 4-phosphate 5-kinase (PIP5K) and PLC, all of which are regulated by intracellular Ca^{2+}. These clathrin-dependent and independent pathways are part of a positive feedback loop controlled by intracellular Ca^{2+} (83). As previously discussed, protons are essential to the release of the virus from the endosome. Briefly, the viral M2 ion channel allows the influx of protons acidifying the viral core disassociating the vRNPs (NP, PA, PB1 and PB2) from M1 allowing the vRNPs to enter the cytoplasm. The M2 ion channel facilitate the efflux of sodium and potassium cation ions to maintain the electrical

neutrality. In doing so, the proton gradient remains intact allowing the further influx of protons into the viral core (131).

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CHAPTER 3

GPCR AND IC GENES USED BY INFLUENZA VIRUS FOR REPLICATION[1]

[1] Nichole Orr-Burks, Jackelyn Murray, Kyle V. Todd, Abhijeet Bakre, and Ralph A. Tripp. 2021. *The Journal of Virology.* 10.1128/JVI.02410-20. Reprinted here with permission of publisher.

Abstract

Influenza virus causes epidemics and sporadic pandemics resulting in morbidity, mortality and economic losses. Influenza viruses require host genes to replicate. RNA interference (RNAi) screens can identify host genes coopted by influenza for replication. Targeting these pro-influenza genes can provide therapeutic strategies to reduce virus replication. Using human lung (A549) cells, 19 pro-influenza GPCR and 13 pro-influenza ion channel genes were identified using small inferring RNAs (siRNA). These pro-influenza genes were authenticated by testing A/WSN/33, A/CA/04/09, and B/Yamagata/16/1988-infected A549 cells resulting in 16 pro-influenza GPCR and 5 pro-influenza ion channel genes being validated. These findings showed that several GPCR and ion channel genes are needed for production of infectious influenza virus. These data provide potential targets for the development of host-directed therapeutic strategies to impede the influenza productive cycle to limit infection.

Introduction

Influenza A viruses (IAV) and influenza B viruses (IBV) are members of the *Orthomyxoviridae* family. IAVs and IBVs contain 8 negative-sense, single-stranded viral RNA gene segments which encode for 10 primary viral proteins, e.g. PB2, PB1, PA, HA, NP, NA, M1, M2, NS1, NS2, as well as strain-dependent accessory proteins mediated by frame shifts and alternative splicing events (1-6). Antigenic drift in the hemagglutinin (HA) gene can lead to changes in viral surface proteins and are responsible for seasonal epidemics, whereas genomic reassortment events may result in pandemics (7, 8). The number of influenza-associated illnesses and deaths varies by strains and the length and

severity of the influenza season. Globally, influenza epidemics result in numerous hospitalizations and 290,000 - 650,000 deaths per year (9, 10). The most recent pandemic influenza strain, i.e. H1N1 2009 resulted in >60 million cases, >274,000 hospitalizations and >12,400 deaths in the United States (11). IAV vaccines require annual reformulation to prevent vaccine failure (12). The 2014-2015 influenza vaccine composed of A/Texas/50/2012 (H3N2), A/California/7/2009 (H1N1) and B/Massachusetts/2/2012-like strains had low vaccine efficacy against the IAV H3N2 strains largely due to drift events which most likely occurred post-selection (13).

Viruses exploit host genes and their pathways to support entry, replication and egress. Some of the most studied pathways exploited by influenza virus include nuclear factor kappa B (NFκB), phosphatidylinositol-3-kinase (PI3K), mitogen-activated protein kinase (MAPK), protein kinase C/protein kinase R (PKC/PKR), toll-like receptor (TLR) and retinoic acid-inducible gene 1 (RIG-I) pathways (14-17). Anti-influenza drugs typically target viral proteins, but often these drugs can have reduced efficacy due to drug resistance acquired through antigenic shift and drift (18). For example, amantadine is no longer recommended for treatment of influenza virus infection due to increased drug resistance, and the reduced efficacy of oseltamivir observed is linked to NA mutations (19) creating inconsistencies among therapies (20). In contrast, therapeutics targeting host genes necessary for virus replication could offer a refractory approach toward drug resistance while providing broader spectrum drug efficacy.

RNA interference (RNAi) is a conserved mechanism of post-transcriptional gene-specific regulation (21). RNAi can probe the virus-host interface to identify host genes necessary for virus replication (22-26). Genome-wide RNAi screening has uncovered key

virus-host interactions, helped identify drug targets for influenza viruses (27), and has been used to validate host genes important for virus replication (28-32). siRNAs mediate post-transcriptional gene silencing via sequence-specific nucleolytic cleavage or translational inhibition upon interaction with their target mRNA (29). siRNAs are rationally designed to be specific for one mRNA target (33).

G-protein coupled receptors (GPCRs) are a family of seven-transmembrane cell-surface receptor proteins that facilitate intracellular communication via activation of signal transduction pathways (34). Viruses use GPCRs to facilitate attachment, entry, replication and egress. For example, HIV tropism is associated with the CXCR4/CCR5 co-receptor and GPCR15 (35-37). In addition, blocking select GPCRs with drug antagonists obstructs Marburg virus and Ebola virus cell entry and replication (38). The overarching influence of GPCRs on the cell makes drugs that target GPCRs amenable to disease intervention. Similarly, ion channels (ICs) are assemblages of integral protein domains that allow transmembrane passage between the extracellular and the intracellular components of the cell (39). ICs enable the influx/efflux of Na^+, K^+, Cl^-, or Ca^{+2} ions which regulate effector pathways. For example, inhibition of K^+ channels at the early stages of Bunyamwera virus infection hinders virus replication post-entry (40). In addition, Cl^- channels are important for herpes simplex virus 1 entry and viral-host fusion (41). Further, the Na^+ channel opener, SDZ-201106, can inhibit IAV replication via the PKC pathway inhibition (42), and modulation of Cl^- or Na^+ secretion/absorption in the respiratory tract contributes to regulation of respiratory disease (43).

In this study, we used RNAi as a tool to survey the virus-host interface connected to GPCR and IC genes needed for influenza virus replication. Using siRNA pools to mediate

RNAi, we examined GPCR and IC genes for their effect on influenza virus replication in A549 cells based on: (1) z-score, (2) Ingenuity Pathway Analysis (IPA; i.e. searching public databases and published texts), (3) availability of small molecule inhibitors and antagonists, and (4) targeting by miRs. The gene hits from the RNAi screen of A/WSN/33-infected A549 cells were validated following deconvolution using A/WSN/33. Confirmed hits were re-examined using A/CA/04/09 or B/Yamagata/16/1988 infected A549 cells. The findings from this study provide a better understanding of the virus-host interface and host genes needed for influenza virus replication, and provide drug target information for the development of new drugs, or for repurposing existing FDA-approved drugs to combat influenza.

Materials and Methods

Cells and Viruses

Type II human lung epithelial (A549) cells (ATCC CCL-185) were propagated in Dulbecco's modified Eagle's Medium (DMEM; HyClone, Logan, UT) supplemented with 5% heat-inactivated fetal bovine serum (HI-FBS; Atlas Biologics Inc., Fort Collins, CO). Madin-Darby Canine Kidney (MDCK) cells (ATCC CCL-34) were propagated in DMEM supplemented with 5% HI-FBS. All experiments were performed using log-phase A549 or MDCK cells.

A/WSN/33 (H1N1; ATCC VR-825), is lab-adapted and trypsin-independent (44), A/CA/04/2009 (H1N1, BEI Resources), and B/Yamagata/16/1988 (BEI Resources) were grown in 9-day old embryonated chicken eggs as previously described (45). The A/WSN/33 and A/CA/04/2009 used in siRNA validation and miR studies was propagated

in MDCK cells. Viral titer was determined by plaque assay and calculated using the Reed and Muench method (46-48).

siGenome Screen

siGENOME plates received from Dharmacon/Horizon Discovery were preloaded with 0.5nmol of pooled, lyophilized siRNAs targeting 390 GPCR or 349 IC genes. siRNAs were designed to ensure ≥85% silencing of target gene expression (49), and optimal antisense strand RISC complex loading is guaranteed (50, 51). siRNA pools were resuspended in siRNA resuspension buffer to a concentration of 1 µM, aliquoted and stored at -80°C until use. For the screen, A549 cells were reverse transfected with siRNA SMARTpools or siRNA controls (50 nM) and incubated at 37°C, 5% CO_2 for 48h to allow for silencing of the targeted gene prior to virus infection as previously described (24, 30). Briefly, transfections were performed in a 96-well plate format in triplicate. The siRNA SMARTpools were diluted in Hank's balanced salt solution (HBSS; GIBCO) added to the plate, and incubated at RT for 5 min. Following incubation, 0.4ul of DharmaFECT 1 transfection reagent (Horizon Discovery) and 9.6 ul of HBSS was per well and incubated for 20 min at RT. Lastly, 80ul containing 1.5 x 10^4 A549 cells in DMEM supplemented with 5% HI-FBS was added to each well and incubated at 37°C, 5% CO_2 for 48h. Post-transfection the cells were washed 2x with PBS, and infected with A/WSN/33 at a MOI = 0.001 to reduce defective interfering particles and incubated at 37°C, 5% CO_2 for 48h. Post-infection supernatant was collected and analyzed by $TCID_{50}$ for virus replication by HA titer as previously described (24). HA titer results were normalized to siNTC. A primary screen was performed twice in two independent experiments. Results were pooled

and analyzed. All RNA interference (RNAi) experiments were completed according to the Minimum Information for an RNAi Experiments (MIARE) guidelines (52).

Host genes having a z-score $\leq$ -1.0 were considered pro-influenza because siRNA silencing reduced virus replication compared to non-targeting controls. Of the GPCR and IC genes, 185 GPCR and 173 IC genes were pro-influenza genes. These genes were evaluated by Ingenuity Pathway Analysis (IPA; Ingenuity Systems, Inc. Redwood City, CA) and gene ontology (GO) analysis. Comprehensive gene interaction networks were determined by combining IPA and GO analysis to identify relationships, functions, mechanism, and pathways. Following IPA and GO analysis, the GPCR and IC host genes identified were evaluated for their ability to be targeted by miRs. These data were used to select 19 pro-influenza GPCR and 13 pro-influenza ion channel candidates for further examination.

siRNA Pool Deconvolution and Validation

The four siRNAs per SMARTpool were individually examined in a deconvolution assay to eliminate false-positives and determine the most effective siRNA reducing influenza virus replication. Plates containing 0.5 nmol of individual lyophilized ON-TARGETplus (OTP) siRNAs (Horizon Discovery) against a single host gene target were tested. OTP-modified siRNAs contained a modification within seed-regions to reduce off-target effects, increase selectivity, and effectiveness. siRNAs were suspended in siRNA buffer according to manufacturer recommendations to a concentration of 1 μM, aliquoted, and stored at -80°C until use.

A549 cells were reverse transfected with one of four OTP siRNAs as describe (25, 53). Briefly, siRNAs targeting a given GPCR or IC gene (Table 1), siRNA control (siNTC, non-targeting), siMAP2K (mitogen-activated protein kinase 1 gene), or siTOX were used at a final concentration of 50 nM and incubated at 37°C, 5% CO2 for 48h to allow for gene silencing prior to infection. Transfections were performed in a 96-well plate in triplicate. Briefly, siRNA reverse transfection was done using 0.4% DharmaFECT 1 transfection reagent where siRNA was pre-incubated with DharmaFECT 1 in serum-free DMEM at RT for 20 min. A549 cells were suspended in DMEM supplemented with 5% HI-FBS, and 1.5 x 10^4 cells were added to each well. Transfection plates were incubated at 37°C, 5% CO_2 for 48h. Post-transfection, the media was decanted, and the cells were washed 2x with PBS then infected with A/WSN/33 (MOI = 0.001) diluted in infection media (MEM+0.3% BSA + 1ug/ml TPCK-Trypsin Worthington, Columbus, OH). Infections were incubated for 48h at 37°C 5% CO_2 and included siNTC, and a siTOX siRNA control. siRNA NTC (5'-UAGCGACUAAACACAUCAA-3') targets no known sequence, siMAP2K (5'-PAGAACCUCCAUCCAUGUGCUU-3', 5'-PUCAAAUCUGCUCUCUCUGCUU-3', 5'-PAGUUGCUUCAAAUCUGCUCUU-3', 5'-PAGAUGAAUUAGCUUUCUGGUU-3') targets MAP2K required for influenza virus replication was used a positive control, i.e. host targeted decrease of influenza replication (54), and siTOX was used to confirm siRNA transfection under transfection conditions. Following incubation, supernatants were collected and stored at -80°C until tested by plaque assay. For the selected gene targets, the two siRNAs that gave the greatest reduction in virus titer were used for all remaining studies.

Validated Hits

A549 cells were transfected with individual OTP siRNAs (2 siRNAs/gene target) from the deconvolution screen or with a control siRNA (siNTC, siMAP2K, siTOX) at a final concentration of 50 nM in triplicate. Following transfection, the cells were infected with A/WSN/33 (MOI = 0.01) or A/CA/04/2009 (MOI = 0.1) or B/Yamagata/12/1988 (MOI = 0.1). The MOIs mediated low or no CPE. Following incubation, supernatants were removed and stored at -80°C until tested by plaque assay and $TCID_{50}$. Two independent experiments were performed.

Cytotoxicity Assay

Any cytotoxic effects associated with siRNA silencing was determined using a Toxilight kit (Lonza, Rockland, ME). Results were normalized to siTOX transfection control that results in complete cell death 48h post-transfection. SMARTpools were considered toxic if transfection resulted in luminescence ≥20% of siTOX control.

Plaque Assay

Infectious virus titers were determined by plaque assay as described (25, 47, 55). Briefly, supernatants were serially diluted 10-fold in MEM with 1 ug/ml TPCK-trypsin and inoculated onto 90% confluent MDCK cell monolayers in 12-well tissue culture plates (Corning-Costar, Cambridge, MA). The virus was adsorbed for 1h at 37°C, 5% CO_2 before adding 3 ml of overlay. Overlay media contained 1-part liquid medium containing: 10x MEM supplemented with 200mm L-glutamine (Gibco), HEPES solution (Gibco), 7.5% $NaCHO_3$ (Gibco), Pen/Strep/Amp B solution (Gibco), and 1-part 2.4% Avicel (FMC

BioPolymer, Philadelphia, PA) in water, or 1-part 1% agarose in water. Samples from A/WSN/33 or A/CA/0409 wells were incubated at 37°C, 5% CO_2 for 3 days. B/Yamagata/16/1988 was incubated at 37°C, 5% CO_2 for 5 days to allow for better plaque formation. The overlays were removed, the plates were washed 2x with PBS, and the cell monolayers fixed with acetone/methanol (80:20) for 20 min at RT. Following fixation, the plates were stained with crystal violet as previously described, and the viral titers determined (25, 47, 55).

$TCID_{50}$ Assay

Endpoint titers were determined by $TCID_{50}$ as previously described (46, 47). Briefly, supernatants collected from influenza virus infected A549 cells were serially diluted 10-fold in triplicate on MDCK cells in 96-well plates. Influenza virus infected MDCK plates were incubated 5 days using cell culture conditions as described (25, 56). Following incubation, an HA test was performed using 50ul of supernatant from infected MDCKs and 50 ul of 1% turkey red blood cells for a final concentration of 0.5% in a round-bottom plate (46, 47, 57). The TCID50 titers were calculated using the Reed and Muench method (46).

Hemagglutination Assay

Hemagglutination was used for viral diagnosis of influenza viruses (46, 57). Briefly, two-fold serial dilutions of virus in PBS were dispensed into individual wells of a 96-well round-bottom microtiter plate (Corning-Costar, Cambridge, MA) then aliquots of

turkey RBC are added to each well to 0.5% of final volume. The highest dilution at which clumping is observed is regarded as the HA titer of the sample.

Statistics

HA assay results were normalized to siNTC transfected controls. The non-targeting control was set to an arbitrary value of 1. Genes were specified a z-score where: $z = (x-\mu)/(s / \sqrt{n})$ where x is equal to the average HA value of each gene, μ is equal to the population mean of the HA, s is equal to the standard deviation of each gene across the two independent experiments, and n is equal to the number of genes within the populations (22). Genes in the primary screen which were <1.5 standard deviation from the plate mean in both duplicates was considered primary hits.

Results

RNAi Screen Identify GPCR Genes

GPCR genes permit intracellular communication via signal transduction following activation (34), and GPCR genes are involved in virus replication (38, 58-60). We performed a genome-wide RNAi screen of GPCR genes required for influenza virus replication in A549 cells. Briefly, A549 cells were reverse transfected with siRNA SMARTpools, and 48h post-transfection the cells were infected (MOI = 0.001) with A/WSN/33. The levels of virus replication were determined, and a z-score applied that showed the number standard deviations the gene silencing event was from the mean. A negative z-score ($\leq$ -1.0) indicated decreased virus replication, while a positive z-score ($\geq$ 1.0) indicated increased viral replication. Our study focused on gene silencing events that

decreased influenza virus titer as the goal was to determine strategies for host cell targeted antiviral therapeutics.

We identified 185 GPCR genes that when silencing resulted in z-scores $\leq$ -1.0. Further evaluation of these genes with IPA and GO analyses as well as implementing selection criterion identified 19 critical GPCR genes, i.e. ADGRF1, ADORA1, ADRB2, AGTR1, C5AR2, CCKBR, FFAR1, HCAR3, HCRTR2, HRH2, HTR1B, LGR4, LPAR3, MTNR1B, NMUR2, OXGR1, OXTR, P2RY12 AND PRLHR (Table 3.2). GPCRs are grouped into 6 classes (A-F) based on sequence homology and functional similarity (34). There were 16 of 19 GPCR genes identified as class A with ADGRF1 being class B, C5AR2 being a non-classical GPCR, and LGR4 an orphan receptor. To limit off-target results, the 19 GPCR genes identified by SMARTpool screens were re-examined by deconvolution of the siRNA pools (24, 61). Here, A549 cells were transfected with individual ON TARGETplus (OTP)-modified siRNAs from the SMARTpool. OTP-siRNAs have improved gene targeting due to a dual strand modification that provides increased interaction with the RISC complex decreasing off-target effects by antisense strands (62).

OTP-siRNA transfected A549 cells were infected (MOI = 0.01) with A/WSN/33 and after 48h the levels of infectious virus production determined by plaque assay. GPCR genes knocked down by OTP-siRNAs having a decrease in virus plaque titer for two or more individual OTP-siRNAs were further evaluated. For example, silencing the MTNR1B gene markedly reduced influenza virus titer when transfected with siRNA 4 from the SMARTpool, but transfection of siRNAs-1, -2 or -3 had a modest effect (Figure 3.1A), thus the MTNR1B gene was not considered further. Additionally, silencing of

NMUR2 or PRLHR genes had no substantial effect on viral titer (Figure 3.1A). In contrast, OTP-siRNA silencing of ADGRF1, ADORA1, ADRB2, AGTR1, C5AR2, CCKBR, FFAR1, HCAR3, HCRTR2, HRH2, HTR1B, LGR4, LPAR3, OXGR1, OXTR or P2RY12 genes resulted in decreased virus titers ($\leq$ -1.0) for 2 or more siRNAs (Figure 3.1A and 3.1B), and silencing of C5AR2, CCKBR, OXTR, or P2RY12 genes gave the greatest reduction in virus titer for two or more siRNAs (Figure 3.1B). Knocking down of ADGRF1, ADRB2, C5AR2, CCKBR, HCRTR2, LPAR3, OXTR or P2RY12 genes yielded a greater reduction in infectious viral titer than knocking down of MAP2K (-9.54 fold reduction) known to limit the replication of influenza virus and thus reduce infectious viral titer (Figure 3.1A and 3.1B) (54, 63). Thus, 16 GPCR genes (ADGRF1, ADORA1, ADRB2, AGTR1, C5AR2, CCKBR, FFAR1, HCAR3, HCRTR2, HRH2, HTR1B, LGR4, LPAR3, OXGR1, OXTR and P2RY12) were further evaluated.

RNAi Screen Identifies Ion Channel (IC) Genes

ICs are membrane-spanning proteins that allow for ion flux across cellular membranes (64) which effects signaling cascades and effector functions as well as the activity and stability of viral proteins (65). Thus, ion channels affect influenza virus replication (42, 43) as influenza viruses attach to the cell membranes during infection and incorporate the membrane into an acidified endosome triggering conformational changes in HA (66, 67). We screened 352 IC genes for their importance in influenza virus replication and found z-scores $\leq$ -1.0 yielded 173 IC genes. These pro-viral genes were analyzed by IPA and GO analyses yielding 13 IC genes (ASIC1, CACNA1C, CHRNA1, GABRA3, GRID2, GRIN3A, KCNA7, KCNE2, KCNIP2, KCNMB2, MCOLN2, SCN7A and SCNN1D) (Table 3.3). OTP-siRNAs SMARTpools were deconvoluted (1 siRNA pool per treatment/

4 siRNAs per target), reverse transfected into A549 cells, and then the cells were infected (MOI = 0.01) with A/WSN/33 after 48h. Levels of infectious influenza virus were determined by plaque assay. IC genes showing decreased plaque titers for two or more individual OTP-siRNAs were further evaluated. Silencing CACNA1C, CHRNA1, GRIN3A, KCNA7, KCNE2, KCNIP2, KCNMB2 or SCN7A genes did not detectably affect virus titer compared to siNTC controls (Figure 3.1C), however silencing ASIC1, GABRA3, GRID2, MCOLN2, or SCNN1D genes resulted in a < -1.0 fold-change. Silencing ASIC1 led to a greater reduction in influenza virus titer compared to silencing MAP2K (-4.29 fold reduction). Silencing SCNN1D resulted in a small decrease in viral titer, however as SCNN1D is targeted by the ion channel inhibitor triamterene, this gene was further evaluated as a potential repurposed drug was available (68-70), and 5 ion channel genes, i.e. ASIC1, GABRA3, GRID2, MCOLN2, and SCNN1D were further evaluated.

Distinctive GPCR and IC Genes are Utilized for Replication of Influenza Virus Strains and Subtypes

To better understand GPCR and IC genes that have influenza virus strain and type differences the GPCR and IC genes were evaluated following A/CA/04/09 or B/Yamagata/16/1988 in A549 cells using plaque assay and $TCID_{50}$ assays. Our initial RNAi screen investigated A/WSN/33 infection of A549 cells at a lower MOI = 0.001. To corroborate earlier data, gene hits were confirmed using individual OTP-siRNAs and a higher MOI = 0.01 of A/WSN/33. The higher MOI = 0.01 was repeated for RNAi silencing of GPCR and IC genes in A549 cells infected with A/CA/04/09 or B/Yamagata/16/1988. Briefly, A549 cells were transfected with OTP-siRNAs (2 siRNAs per target transfected

individually) targeting a GPCR or ion channel gene selected from the A/WSN/33 deconvolution screen. Following reverse transfection for 48h the A549 cells were infected with either A/WSN/33 (MOI = 0.01), A/CA/04/2009 (MOI = 0.1), or B/Yamagata/12/1988 (MOI = 0.1). Forty-eight hours post-infection, the titer and 50% tissue culture infective dose was determined by plaque assay and $TCID_{50}$ HA assay. The results showed that silencing 16 GPCR and 5 IC pro-influenza genes had a >2-fold decrease in influenza plaque formation for A/WSN/33 (Figure 3.2A and 3.2B), A/CA/04/2009 (Figure 3.3A and 3.3B), or B/Yamagata/12/1988 (Figure 3.4A and 3.4B) infected A549 cells. Notably, there was a >100-fold decrease in $TCID_{50}$ for A/WSN/33 (Figure 3.2C and 3.2D), a >10-fold decrease by TCID50 for CA/04/09 (Figure 3.3C and 3.3D), and a >10-fold decrease by $TCID_{50}$ for B/Yamagata/16/1988 (Figure 3.4C and 3.4D). These differences in the fold-change are likely related to the virus replication dynamics and growth kinetics. A/WSN/33 and CA/04/09 strains replicate at a higher tempo and to higher titers compared to B/Yamagata/16/1988 (71, 72). As shown in Figure 3.2, siRNA silencing of LGR4, LPAR3, OXGR1, ASIC1, GABRA3 or MCOLN2 genes markedly reduced A/WSN/33 virus titer compared to siNTC while also showing a reduction in virus titer compared to siMAP2K (4.4-fold change decrease) (Figure 3.2A and 3.2B). The effect of individually silencing the 16 GPCR and 5 IC genes on A/CA/04/2009 replication was also determined (Figure 3.3). The results show that siRNAs targeting AGTR1, HCRTR2, P2RY12 or GRID2 genes substantially reduced A/CA/04/2009 replication (Figure 3.3A and 3.3B). Silencing P2RY12 also had considerable reduction in virus titer compared to siMAP2K (6.84 fold change reduction) (Figure 3.3A). The result of individually silencing 16 GPCR genes and 5 IC genes on B/Yamagata/16/1988 replication was also determined (Figure

3.4). Importantly, silencing HRH2 or GRID2 genes substantially reduced B/Yamagata/16/1988 titer and targeting HRH2 resulted in a reduction in virus titer greater than siMAP2K (30-fold change reduction) gene silencing (Figure 3.4A and 3.4B). These results confirm earlier results from the A/WSN/33 screen and show that several GPCR and IC genes affect A/CA/04/09 and B/Yamagata/16/1988 replication.

Discussion

RNAi screens have aided in the discovery of essential features of the host-virus interface specifically the host pathways used to facilitate virus replication (23, 73), and have provided information used to develop disease intervention strategies (28, 29). GPCR and IC are implicated in the replication mechanisms of several RNA viruses including SARS-CoV-2, Marburg virus, Ebola virus and HIV, but have not been well described for influenza virus (38, 40, 41, 60, 74). In this study, we identified GPCR and IC genes used by influenza virus for replication and determined influenza strain and type differences. We screened 390 GPCR and 349 IC genes of which 19 GPCR and 13 IC genes selected for validation studies. Secondary validation by siRNA pool deconvolution yielded in 16 confirmed GPCR genes, namely ADGRF1, ADORA1, ADRB2, AGTR1, C5AR2, CCKBR, FFAR1, HCAR3, HCRTR2, HRH2, HTR1B, LGR4, LPAR3, OXGR1, OXTR, P2RY12, as well as 5 IC genes namely ASIC1, GABRA3, GRID2, MCOLN2 and SCNN1D (Figure 3.1). Validation of the genes from the RNAi screen was done using two individual OTP-siRNAs and testing the effect on A/WSN/33 replication using a higher MOI=0.01 to ensure robust infection. These studies used two endpoints to evaluate effects of silencing on influenza replication, i.e. plaque assays to quantitate infectious virus titers

(pfu/ml) and $TCID_{50}$ assays to measure the amount of virus required to infect 50% of cells as determined by HA assay (Figure 3.2). siRNA silencing of GPCR genes LGR4, LPAR3, and OXGR1, and silencing of IC genes ASIC1, GABRA3 and MCOLN2 in A549 cells yielded substantial decreases in A/WSN/33 titer showing these genes are needed for A/WSN/33 replication. Of note, the decreases in virus plaque numbers were greater than the control, i.e. siMAP2K (4-fold change decrease) which targets mitogen activated protein kinase shown to be required for influenza virus replication (54, 63).

To examine influenza virus strain differences, siRNA transfected A549 cells were infected with A/CA/04/09, a representative circulating strain of human influenza A strain, and levels of virus replication were determined by quantification of infectious virus (plaque assay) and $TCID_{50}$ to determine 50% infectious dose following transfection (Figure 3.3). Silencing of GPCR and IC genes gave similar results as for A/WSN/33-infected A549 cells where influenza virus titers linked to GPCR genes AGTR1, HCRTR2 and P2RY12 and the IC gene GRID2 were considerably reduced. Of note, silencing P2RY12 reduced virus titer, i.e. 6-fold reduction compared to the siMAP2K control. We also examined the potential for influenza virus type differences linked to GPCR and IC genes in A549 cells by evaluating B/Yamagata/16/1988 replication after siRNA transfection (Figure 3.4). siRNA silencing of GPCR and IC genes also yielded reduced B/Yamagata/16/1988 replication but was only statistically ($p<0.01$) significant for IC genes HRH2 and GRID2 and targeting HRH2 yielded a reduction in virus titer greater than siMAP2K (30-fold change).

The results suggest that influenza virus strains and types coopt similar GPCR and IC genes as part of the replication process in A549 cells but have the ability to utilize different genes in similar pathways (73, 75). It has been reported that the tempo of signal

transduction and host gene expression is associated with viral replication and virus production dynamics (75). It is possible that different host genes may be used for influenza virus replication in other cell types particularly as transformed cell lines can have distinct gene expression (76). This is a caveat of A549 cells as some host genes identified as important may not translate to primary cell cultures. Additionally, the findings in this study were limited to 48h pi due to the high-throughput screening procedure, and the later phases of the virus replication were not evaluated. Additionally, GPCR signaling is a complex network as each GPCR complex may have a number of isoforms and splice variants that creates hundreds of combinations of G proteins. Thus, differences in cell signaling associated with the kinetics of infection, and/or GPCR isoforms/splice variants can go unnoticed (34). In addition, the configuration of the G-protein affects not only which transmembrane receptor it can bind to, but also which downstream target is affected (34, 77, 78). GPCR Gα subunits are grouped into four families ($G\alpha_s$, $G\alpha_i$, $G\alpha_q$, $G\alpha_{12/13}$) based on sequence homology consisting of approximately 20 distinct Gα subunit proteins due to splice variants (79). The host genes ADORA1, AGTR1, HTR1B and P2RY12 are coupled to Gαi (Figure 3.5), while ADRB2, HCAR3 and HRH2 genes are coupled to Gαs (Figure 3.6). Gαi signaling inhibits adenyl cyclase which decreases intracellular cAMP, while Gαs signaling stimulates adenyl cyclase prompting the opposing effect. Modulation of cAMP levels regulates the duration and intensity of cAMP signaling via feedback mechanisms (80). G proteins have been implicated in late stages of influenza infection specifically virus budding (81-83). The host genes AGTR1, CCKBR, FFAR1, HCRTR2, OXGR1 and OXTR were associated with Gαq signaling by IPA (Figure 3.7). Gαq signaling is associated with multiple downstream pathways, but the most well characterized are those

associated with phospholipase Cβ (PLC) activation and phosphoinositide 3-kinase (PI3K) (84). Alteration of this pathway has been shown to play a regulatory role in the clathrin-mediated and clathrin-independent endocytosis pathways utilized by influenza at entry (85). Host genes ADGRF1 and LGR4 are orphan receptors with no identified endogenous ligand (86-88). C5AR2 is a non-classical GPCR, and although it is a seven transmembrane receptor, it does not couple to a G-protein and instead binds β-arrestins (89, 90). In this study, we show that siRNA silencing of GPCR genes AGTR1, CCKBR, FFAR1, HCRTR2, OXGR1 and OXTR inhibits A/WSN/33, A/CA/04/2009 and B/Yamagata/16/1988 replication in A549 cells.

IPA of the validated IC genes determined in this study suggested that several genes affected influenza replication. ASIC1 is an acid-sensing sodium channel gene whose regulation is controlled by activation of the PKC pathway (91), however it remains unclear how ASIC1 is necessary for viral replication. Similarly, GRID2 (or GluRδ2) is an orphan glutamate receptor gene whose function is poorly understood (92). SCNN1D (or δENaC) is one of four subunits that compose the epithelial sodium channel located on the apical surface of polarized tissues, e.g. the lung. It is involved in Na+ transport across the transepithelial surface during reabsorption of Na+ (70, 93). In this study silencing of SCNN1D reduced virus replication suggesting a novel role for this subunit compared to its αβγ counterparts (94). GABRA3 has been shown to be expressed in the lung (95), and its activation is linked to autophagy (95) which is a strategy that influenza virus uses to promote replication (96). We show that GABRA3 silencing reduces viral replication. It has been shown that MCOLN2 is associated with improved influenza, dengue virus, yellow fever virus, and equine arteritis virus infectivity (97) possibly by promoting virus

trafficking between the early and late endosomes and releasing virus into the cytosol independent of IFN signaling (97). Our findings concur and showing that siRNA silencing of MCOLN2 decreases influenza virus replication and show that MCOLN2 is an important host factor for influenza A, but also B virus replication which was not previously known.

Understanding the host factors used by influenza virus during entry, replication, and egress can help identify targets for drug repurposing or for the development of novel antiviral drugs. Targeting host factors is refractory to the development of drug resistance generated by viral mutations (18). Here, we identify several GPCR and ion channel genes that can be targeted by FDA-approved drug antagonists and/or inhibitors (Table 3.4). For example, P2RY12 (a GPCR gene) can be targeted by the drug Plavix (clopidogrel bisulfate) which is currently approved to inhibit platelet aggregation and treat patients with acute coronary syndrome (98-100). Interestingly, the AGTR1 gene (a GPCR gene) has been shown to be associated with coronavirus infection pathway having a possible link between ACE2 and lung injury. AGTR1 can be targeted by angiotensin-receptor blockers (ARBs) including Candesartan that has been suggested as a treatment for COVID-19 (101). ARBs have shown efficacy in decreasing lung injury in animal models of ARDS, but not without potential side effects (102). Further studies are needed to determine the importance of this association and COVID-19 disease. The ion channels, ASIC1 and SCNN1D, can be inhibited by amiloride which has been shown to suppress Coxsackievirus B3 (CVB3) replication and foot-and-mouth disease virus (FMDV) replication (103, 104). These examples show the therapeutic potential of drug repurposing to target host factors needed for virus replication.

Taken together, this study identified and evaluated GPCR and IC genes coopted by influenza (A/WSN/33, CA/04/2009, B/Yamagata/16/1988) for replication and identified strain and type differences. Collectively, the identification of these GPCR and IC genes provides the opportunity to develop host-directed virus control strategies to limit influenza replication and disease using drug repurposing or the development of novel antivirals.

Tables

siRNA #	Gene Symbol	Gene ID	Gene Accession	Target Sequence
si1	ADGRF1	266977	NM_025048	CACAUGGGCUAAUUAGAAU
si2	ADGRF1	266977	NM_025048	CUAUAGAGAUUCCAAGGAG
si3	ADGRF1	266977	NM_025048	GUGAAUGUCAUCUCAACAA
si4	ADGRF1	266977	NM_025048	GGAGUGCUGUGGCUCAUUU
si1	ADORA1	134	NM_000674	AGAGAGGCCUGAUGACUAG
si2	ADORA1	134	NM_000674	GGAACAAUCUGAGUGCGGU
si3	ADORA1	134	NM_000674	CCACAGACCUACUUCCACA
si4	ADORA1	134	NM_000674	CAAGAUCCCUCUCCGGUAC
si1	ADRB2	154	NM_000024	UGAUCAUGGUCUUCGUCUA
si2	ADRB2	154	NM_000024	GGGCAUGGACUCCGCAGAU
si3	ADRB2	154	NM_000024	CGUCCUGGCCAUCGUGUUU
si4	ADRB2	154	NM_000024	UUGCCAAGUUCGAGCGUCU
si1	AGTR1	185	NM_032049	UGGAAGGCAUAAUUACAUA
si2	AGTR1	185	NM_032049	CCUGUACGCUAGUGUGUUU
si3	AGTR1	185	NM_032049	GAAUACCGCUGGCCCUUUG
si4	AGTR1	185	NM_032049	AUACGUGACUGUAGAAUUG
si1	C5AR2	27202	NM_018485	GGAACGAUUCUGUCAGCUA
si2	C5AR2	27202	NM_018485	ACGAAAGUGUGGACAGCAA
si3	C5AR2	27202	NM_018485	UGCAGUGUGUGGUGGACUA
si4	C5AR2	27202	NM_018485	GACCAUGUAUGCCAGCGUC
si1	CCKBR	887	NM_176875	GUGAGUGUGUCCACGCUAA
si2	CCKBR	887	NM_176875	GAAUGUUGCUGGUGAUCGU
si3	CCKBR	887	NM_176875	GAAUCACUCUUUACGCAGU
si4	CCKBR	887	NM_176875	GAUGAGCGUUGGAGGAAAU
si1	FFAR1	2864	NM_005303	CGCUCAACGUCCUGGCCAU
si2	FFAR1	2864	NM_005303	CCUACAACGCCUCCAACGU
si3	FFAR1	2864	NM_005303	GUGACCGGUUACUUGGGAA

si4	FFAR1	2864	NM_005303	UUCCGGAGGCCGUGCUAUU
si1	HCAR3	8843	NM_006018	UCAAAUAACCAUUCCAAGA
si2	HCAR3	8843	NM_006018	AGAAGUUGCUGAUCCAGAA
si3	HCAR3	8843	NM_006018	CGUUCGUGAUGGACUACUA
si4	HCAR3	8843	NM_006018	CGCCAGGGCAGCAUCAUAU
si1	HCRTR2	3062	NM_001526	GGUGUUGGCUUAUCUGCAA
si2	HCRTR2	3062	NM_001526	CUGCGAAUCCAAUUAUUUA
si3	HCRTR2	3062	NM_001526	GGAGCUGAAUGAAACUCAA
si4	HCRTR2	3062	NM_001526	UGUCACCCUUUGAUGUUUA
si1	HRH2	3274	NM_022304	CCAAGAGGAUCAAUCACAU
si2	HRH2	3274	NM_022304	GCAAUGUGGUCGUCUGUCU
si3	HRH2	3274	NM_022304	GUGCAAAGUCCAGGUCAAU
si4	HRH2	3274	NM_022304	UCAAUGAGGUGUUAGAAGC
si1	HTR1B	3351	NM_000863	GGAAAGUACUGCUGGUUAU
si2	HTR1B	3351	NM_000863	GAAUCCGGAUCUCCUGUGU
si3	HTR1B	3351	NM_000863	UCUAUUAACUCGCGGGUUC
si4	HTR1B	3351	NM_000863	GAGCCCAGCUGAUAACCGA
si1	LGR4	55366	NM_018490	AGGAUUCACUGUAACGUUA
si2	LGR4	55366	NM_018490	UUACUGAAGCGACGUGUUA
si3	LGR4	55366	NM_018490	UAACAACAUUUGCAUCUUG
si4	LGR4	55366	NM_018490	GCCAAUAACUAACCUAGAU
si1	LPAR3	23566	NM_012152	GGACACCCAUGAAGCUAAU
si2	LPAR3	23566	NM_012152	UCUACUACCUGUUGGCUAA
si3	LPAR3	23566	NM_012152	CAACACUGAUACUGUCGAU
si4	LPAR3	23566	NM_012152	UCAUCAUGGUUGUGGUGUA
si1	MTNR1B	4544	NM_005959	GCUACUUACUGGCUUAUUU
si2	MTNR1B	4544	NM_005959	GUACGACCCACGCAUCUAU
si3	MTNR1B	4544	NM_005959	GGUAAUUUGUUCUUGGUGA
si4	MTNR1B	4544	NM_005959	GAGAACGGCUCCUUCGCCA
si1	NMUR2	56923	NM_020167	CCAUGUGGAUCUACAAUUU
si2	NMUR2	56923	NM_020167	GGUGUCAGGUGUCUUCUUC
si3	NMUR2	56923	NM_020167	UGAAGGGAAUGCAAAUAUU
si4	NMUR2	56923	NM_020167	GGAGCUGACCGAAGAUAUA
si1	OXGR1	27199	NM_080818	CGGAUGAACUCAAUACUAU
si2	OXGR1	27199	NM_080818	CAUCGUUUCUAGACCAUUA
si3	OXGR1	27199	NM_080818	CCGAUGACCUUCUUGAUCA
si4	OXGR1	27199	NM_080818	CCACUAGACUAUUUAGCAA
si1	OXTR	5021	NM_000916	GGAUCACGCUAGCUGUCUA
si2	OXTR	5021	NM_000916	UGGCAGAACUUGCGGCUCA
si3	OXTR	5021	NM_000916	GCGUCAAGCUCAUCUCCAA
si4	OXTR	5021	NM_000916	GAGCAACUCGUCCUCCUUU
si1	P2RY12	64805	NM_176876	GGUCUAGUCUGGCAUGAAA
si2	P2RY12	64805	NM_176876	GUACCGGUCAUACGUAAGA
si3	P2RY12	64805	NM_176876	CAAGUUACCUCCGUCAUAU

si4	P2RY12	64805	NM_176876	CAAGUCAUUUUCUGGAUUA
si1	PRLHR	2834	NM_004248	CAUCGACCCUUACGCCUUU
si2	PRLHR	2834	NM_004248	GGUCACAACUCCCGCCAAC
si3	PRLHR	2834	NM_004248	CAGGGUUUCUGACUUAUUU
si4	PRLHR	2834	NM_004248	GCAAACUGUUGGUCGCUUG
si1	ASIC1	41	NM_001095	GGAAAGUGCUACACGUUCA
si2	ASIC1	41	NM_001095	CUUCGAAGCAGGCAUCAAA
si3	ASIC1	41	NM_001095	CAACAACAGGUAUGAGAUA
si4	ASIC1	41	NM_001095	UCAACAAAUCUGAGCAAUA
si1	CACNA1C	775	NM_000719	GGAGGAGCACAUUCGAUAA
si2	CACNA1C	775	NM_000719	GGAUGUUAGUCUGUAUUUA
si3	CACNA1C	775	NM_000719	GGGUAGCAUUGUUGAUAUA
si4	CACNA1C	775	NM_000719	GAAGAUGACUGCUUAUGGG
si1	CHRNA1	1134	NM_000079	GCCCAGACCUUGUUCUCUA
si2	CHRNA1	1134	NM_000079	UAACUGGCCUGGUAUUCUA
si3	CHRNA1	1134	NM_000079	GACCAGGAGUCUAACAAUG
si4	CHRNA1	1134	NM_000079	UAAAUCAGAUCGUGACAAC
si1	GABRA3	2556	NM_000808	GAGAUAAUCCGGUCUAGUA
si2	GABRA3	2556	NM_000808	ACAAUGAGGUUAACAAUUC
si3	GABRA3	2556	NM_000808	CGACUGAGACCAAGACCUA
si4	GABRA3	2556	NM_000808	ACAAGUCACUGUUACAUGA
si1	GRID2	2895	NM_001510	GAGCGAUCCUUGUUAUGAA
si2	GRID2	2895	NM_001510	GGUAGGAGAACUUGUCUUU
si3	GRID2	2895	NM_001510	GGACUCACCCGGAGCAACA
si4	GRID2	2895	NM_001510	UCCUAGACUCUGCGGUAUA
si1	GRIN3A	116443	NM_133445	CGACGGAAAUACAUCUUUA
si2	GRIN3A	116443	NM_133445	CAGCUUACCGUAUGGAAUA
si3	GRIN3A	116443	NM_133445	CAACAUAUCCGAGCUAAUC
si4	GRIN3A	116443	NM_133445	GAAGAGUCCAUUUGGUUUG
si1	KCNA7	3743	NM_031886	GCGAAGAGGCUGGGAUGUU
si2	KCNA7	3743	NM_031886	GAGACGCUGUGUAUUUGUU
si3	KCNA7	3743	NM_031886	GGAAACACCUGGUCACCGA
si4	KCNA7	3743	NM_031886	CACUGUGGGUGGCAAGAUA
si1	KCNE2	9992	NM_172201	GAACUUCUACUAUGUCAUC
si2	KCNE2	9992	NM_172201	GACGGGAACACUCCAAUGA
si3	KCNE2	9992	NM_172201	CGAAGGCCACCAUCCAUGA
si4	KCNE2	9992	NM_172201	ACACAACAGCUGAGCAAGA
si1	KCNIP2	30819	NM_173197	GAAUGUCCCAGCGGAAUUG
si2	KCNIP2	30819	NM_173197	CAGCGUGGACGAUGAAUUU
si3	KCNIP2	30819	NM_173197	AAACCAAAUUCACGCGCAA
si4	KCNIP2	30819	NM_173197	GGAAUUCAUUGAGUCUUGU
si1	KCNMB2	10242	NM_005832	CCAACGUGCUGUUCCAUUC
si2	KCNMB2	10242	NM_005832	UCCAACGGAUCAAUAGAUA
si3	KCNMB2	10242	NM_005832	UCACACUCCUGCGCUCAUA

si4	KCNMB2	10242	NM_005832	GUACCUCUCCCUACUAUGU
si1	MCOLN2	255231	NM_153259	GCUCUAAGGUUACGGAAGA
si2	MCOLN2	255231	NM_153259	GACCAUACCAUGACAAGUU
si3	MCOLN2	255231	NM_153259	UCAGAUACCUGGGUUAUUU
si4	MCOLN2	255231	NM_153259	UCAGUCGUCUGUAUUUAUA
si1	SCNN1D	6339	NM_002978	GCAUCAGGGUCAUGGUUCA
si2	SCNN1D	6339	NM_002978	GCUACUACCUCCACCCUCU
si3	SCNN1D	6339	NM_002978	GAGAAUGGAAGCAGCCACA
si4	SCNN1D	6339	NM_002978	CUACACAACACCUCCUACA

Table 3.1. Summary of siRNA information for deconvolution experiments. A genome-wide RNAi screen was performed with siRNA SMARTpools to determine GPCR and IC gene hits for A/WSN/33-infected A549 cells. Hits were validated by deconvolution of the SMARTpools by testing each siRNA individually at 50nM final concentration. The table includes 4 siRNAs from each pool and relative gene sequence and target information. Gene hits were considered validated when two or more siRNAs yielded reduced viral replication when transfected individually.

	Symbol	Gene Name	z-Score
GPCR Targets	*ADGRF1*	G protein-coupled receptor 110	-2.0
	ADORA1	adenosine A1 receptor	-2.1
	ADRB2	adrenoceptor beta 2, surface	-1.8
	AGTR1	angiotensin II receptor, type 1	-1.6
	C5AR2	complement component 5a receptor 2	-1.9
	CCKBR	cholecystokinin B receptor	-2.8
	FFAR1	free fatty acid receptor 1	-2.1
	HCAR3	hydroxycarboxylic acid receptor 3	-1.8
	HCRTR2	hypocretin (orexin) receptor 2	-1.9
	HRH2	histamine receptor H2	-2.3
	HTR1B	5-hydroxytryptamine (serotonin) receptor 1B, G protein-coupled	-1.5
	LGR4	leucine-rich repeat containing G protein-coupled receptor 4	-1.6
	LPAR3	lysophosphatidic acid receptor 3	-1.6
	MTNR1B	melatonin receptor 1B	-1.7
	NMUR2	neuromedin U receptor 2	-1.7
	OXGR1	oxoglutarate (alpha-ketoglutarate) receptor 1	-1.3
	OXTR	oxytocin receptor	-1.4
	P2RY12	purinergic receptor P2Y, G-protein coupled, 12	-1.5
	PRLHR	prolactin releasing hormone receptor	-1.9

Table 3.2. GPCR genes from a genome-wide RNAi screen. A negative z-score indicates a gene is pro-influenza.

	Symbol	Gene Name	z-Score
IC Targets	*ASIC1*	acid-sensing (proton-gated) ion channel 1	-1.8
	CACNA1C	calcium channel, voltage-dependent, L type, alpha 1C subunit	-2.2
	CHRNA1	cholinergic receptor, nicotinic, alpha 1 (muscle)	-1.5
	GABRA3	gamma-aminobutyric acid (GABA) A receptor, alpha 3	-1.5
	GRID2	glutamate receptor, ionotropic, delta 2	-1.8
	GRIN3A	glutamate receptor, ionotropic, N-methyl-D-aspartate 3A	-1.5
	KCNA7	potassium voltage-gated channel, shaker-related subfamily, member 7	-1.5
	KCNAB2	potassium voltage-gated channel, shaker-related subfamily, beta member 2	-1.7
	KCNE2	potassium voltage-gated channel, Isk-related family, member 2	-1.4
	KCNIP2	Kv channel interacting protein 2	-1.9
	MCOLN2	mucolipin 2	-1.9
	SCN7A	sodium channel, non-voltage-gated 1, delta subunit	-1.5
	SCNN1D	sodium channel, voltage-gated, type VII, alpha subunit	-2.0

Table 3.3. IC genes from a genome-wide RNAi screen. A negative z-score indicates a gene is pro-influenza.

Target	Drug Name	PubChemID	CAS #	Action	Chemical Formula	Reference
ADORA1	**Aminophylline**	9433	317-34-0	antagonist	C16H24N10O4	(105)
	Dyphylline	3182	479-18-5	antagonist	C10H14N4O4	(105)
	Istradefylline	5311037	155270-99-8	antagonist	C20H24N4O4	(105)
	Pentoxifylline	4740	6493-05-6	Unknown	C13H18N4O3	(106, 107)
	Theophylline	2153	58-55-9	antagonist	C7H8N4O2	(105)
AGTR1	**Azilsartan**	135415867	147403-03-0	antagonist	C25H20N4O5	(108, 109)
	Candesartan	2541	139481-59-7	antagonist	C24H20N6O3	(101, 109, 110)
	Eprosartan	5281037	133040-01-4	antagonist	C23H24N2O4S	(109)
	Irbesartan	3749	138402-11-6	antagonist	C25H28N6O	(109, 111, 112)
	Losartan	3961	114798-26-4	antagonist	C22H23ClN6O	(109)
	Valsartan	60846	137862-53-4	antagonist	C24H29N5O3	(109, 113)
HTR1B	**Asenapine**	3036780	65576-45-6	antagonist	C17H16ClNO	(114)
P2RY12	**Cangrelor**	9854012	163706-06-7	inhibitor	C17H25Cl2F3N5O12P3S2	(115)
	Clopidogrel	60606	113665-84-2	antagonist	C16H16ClNO2S	(98-100, 109, 116-118)
	Prasugrel	6918456	150322-43-3	antagonist	C20H20FNO3S	(109, 116, 119)
	Ticagrelor	9871419	274693-27-5	inhibitor	C23H28F2N6O4S	(120)

	Ticlopidine	5472	55142-85-3	antagonist	C14H14ClNS	(100, 109, 116, 117)
ADRB2	**Carteolol HCL**	40127	51781-21-6	antagonist	C16H25ClN2O3	(121)
	Labetalol	3869	36894-69-6	antagonist	C19H24N2O3	(109)
	Levobunolol	39468	47141-42-4	antagonist	C17H25NO3	(109, 122)
	Metipranolol	31477	22664-55-7	antagonist	C17H27NO4	(68, 123)
	Sotalol	5253	3930-20-9	antagonist	C12H20N2O3S	(124)
	Timolol	33624	26839-75-8	antagonist	C13H24N4O3S	(125)
HRH2	**Asenapine**	3036780	65576-45-6	antagonist	C17H16ClNO	(114)
	Famotidine	5702160	76824-35-6	antagonist	C8H15N7O2S3	(126)
	Lafutidine	5282136	118288-08-7	antagonist	C22H29N3O4S	(127)
ASIC1	**Amiloride**	16231	2609-46-3	inhibitor	C6H8ClN7O	(104)
	diclofenac	3033	15307-86-5	inhibitor	C14H11Cl2NO2	(128)
GABRA3	**Bicuculline**	10237	485-49-4	antagonist	C20H17NO6	(129)
OXTR	**Atosiban**	5311010	90779-69-4	antagonist	C43H67N11O12S2	(130, 131)
SCNN1D	**Amiloride**	16231	2609-46-3	inhibitor	C6H8ClN7O	(132)
	Triamterene	5546	396-01-0	inhibitor	C12H11N7	(68, 123, 133)

Table 3.4. Summary of FDA Approve Drugs which Target Pro-Influenza GPCR and ion channels.

Figures

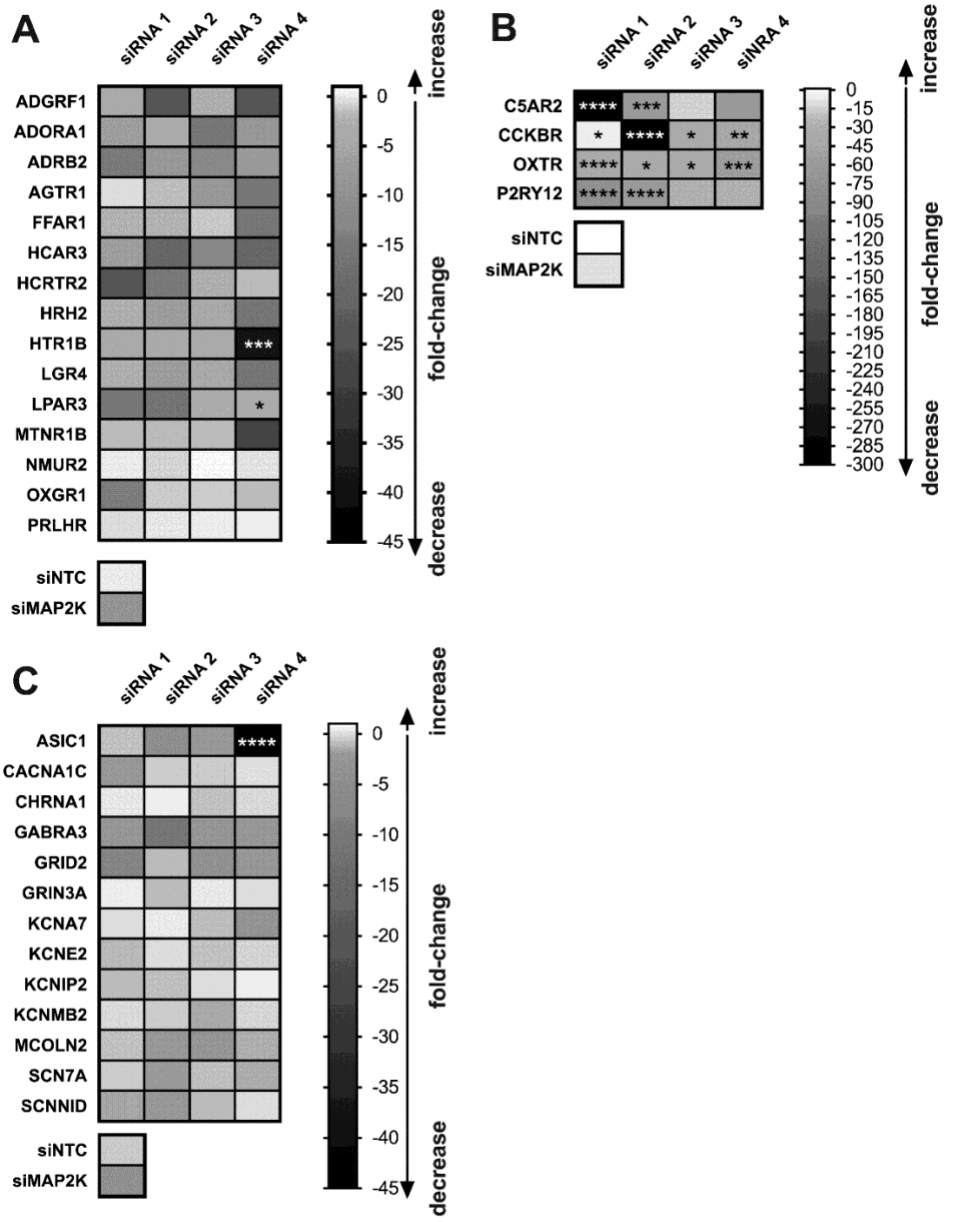

Figure 3.1. Deconvolution of siRNA pools. siRNA pools targeting GPCR (A, B) and IC (C) genes were deconvoluted and reverse transfected at a final concentration of 50nM in A549 cells. At 48h post-siRNA transfection, the A549 cells were infected (MOI = 0.001) with A/WSN/33 and supernatants were collected and virus titers determined by MDCK plaque assay. Experiments were performed in triplicate and assayed in duplicate. Results are presented as heat maps depicting fold-change in influenza virus titer (PFU/ml) compared to non-targeting control siRNA (siNTC). Positive fold-change equates to an increase in PFU/ml compared to control

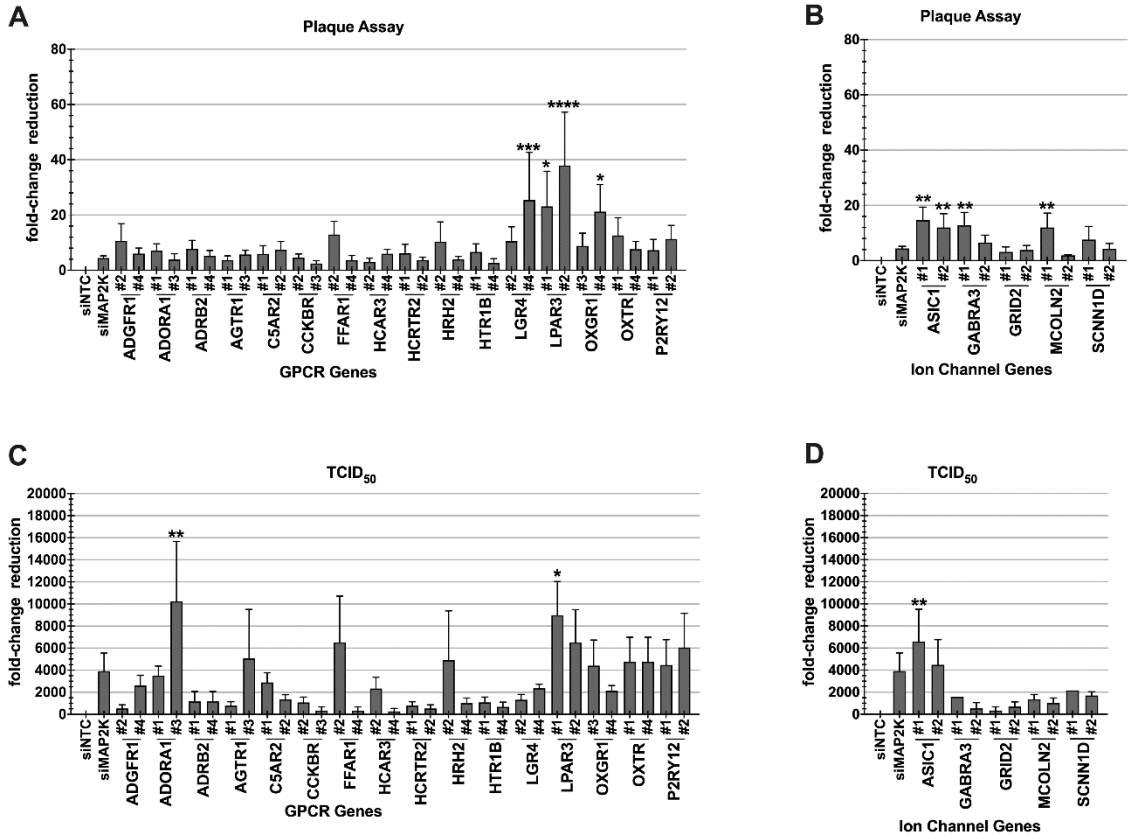

Figure 3.2. Validation of host gene targets for A/WSN/33 infected A549 cells. A549 cells were reverse transfected (50 nM) with OTP-modified siRNAs (2 siRNAs per gene target) from the deconvolution siRNA screen in triplicate and incubated for 48h. The A549 cells were infected (MOI = 0.01) with A/WSN/33. Supernatants were collected 48h post-infection. Infectious viral titer (PFU/ml) and $TCID_{50}$ titers were determined by MDCK plaque assay and sample titration on MDCK cells followed by HA assay, respectively. Plaque assay data for GPCR (A) and ion channel (B) genes and TCID50 data for GPCR (C) and ion channel genes (D) is presented as the inverse of the fold-change decrease compared to non-targeting control siRNA (siNTC) of three independent experiments performed in triplicate. A positive increase in fold-change equates to a decrease in PFU/ml or $TCID_{50}$/ml compared to siNTC control. Data shows mean ± SEM of 3 independent experiments performed in triplicate. Ordinary one-way ANOVA with Dunnett's Multiple Comparisons Post-Test *$p<0.05$ **$p<0.001$ ***$p<0.0001$ and ****$p<0.00001$ compared to control. siNTC results are corrected to zero to reflect baseline change in replication is 0. Results are normalized to siNTC control. The siRNA targeting a particular gene and # refers to the siRNA used from the SMARTpool where # refers to the individual siRNA transfected.

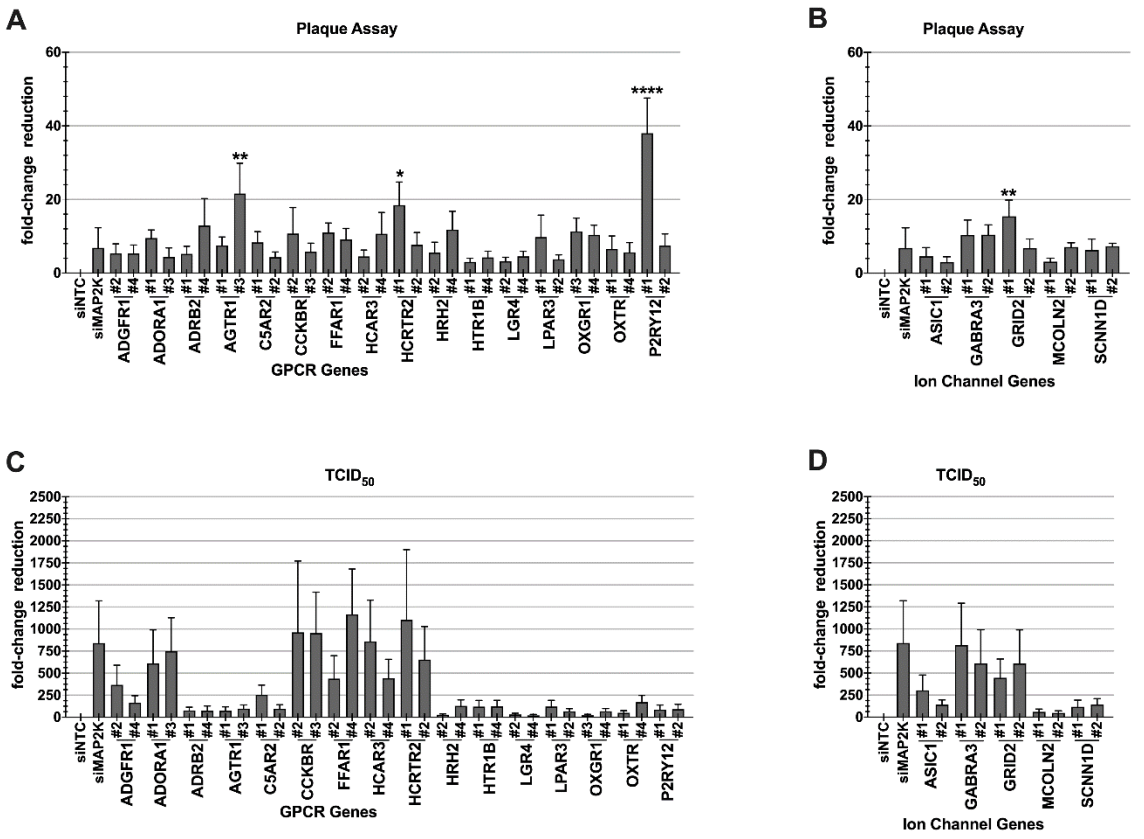

Figure 3.3. Validation of host gene targets for A/CA/04/09 infected A549 cells. A549 cells were reverse transfected (50 nM) with OTP-modified siRNAs (2 siRNAs per gene target) from the deconvolution siRNA screen in triplicate and incubated for 48h. The A549 cells were infected (MOI = 0.01) with A/CA/04/09. Supernatants were collected 48h post-infection. Infectious viral titer (PFU/ml) and $TCID_{50}$ titers were determined by MDCK plaque assay and sample titration on MDCK cells followed by HA assay, respectively. Plaque assay data for GPCR (A) and ion channel (B) genes and TCID50 data for GPCR (C) and ion channel genes (D) is presented as the inverse of the fold-change decrease compared to non-targeting control siRNA (siNTC) of three independent experiments performed in triplicate. A positive increase in fold-change equates to a decrease in PFU/ml or $TCID_{50}$/ml compared to siNTC control. Data shows mean ± SEM of 3 independent experiments performed in triplicate. Ordinary one-way ANOVA with Dunnett's Multiple Comparisons Post-Test *p<0.05 **p<0.001 ***p<0.0001 and ****p<0.00001 compared to control. siNTC results are corrected to zero to reflect baseline change in replication is 0. Results are normalized to siNTC control. The siRNA targeting a particular gene and # refers to the siRNA used from the SMARTpool where # refers to the individual siRNA transfected.

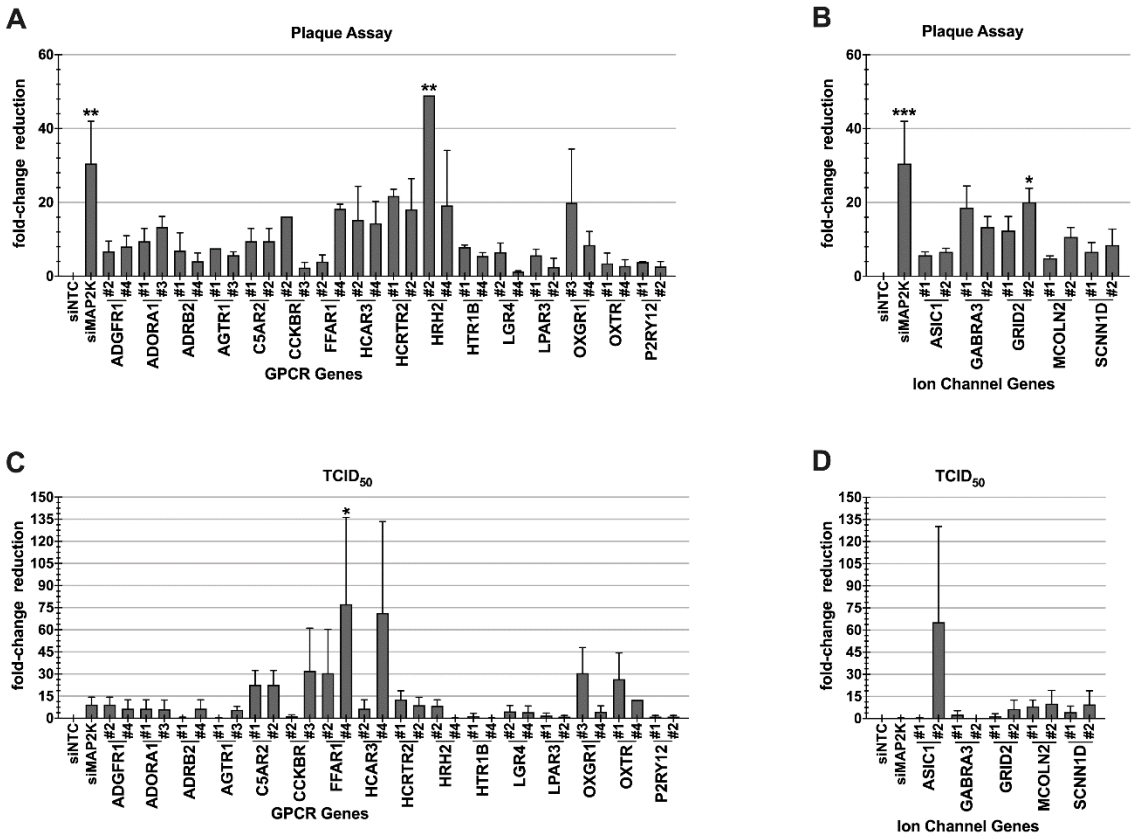

Figure 3.4. Validation of host gene targets for B/Yamagata/16/1988 infected A549 cells. A549 cells were reverse transfected (50 nM) with OTP-modified siRNAs (2 siRNAs per gene target) from the deconvolution siRNA screen in triplicate and incubated for 48h. The A549 cells were infected (MOI = 0.01) with B/Yamagata/16/1988. Supernatants were collected 48h post-infection. Infectious viral titer (PFU/ml) and $TCID_{50}$ titers were determined by MDCK plaque assay and sample titration on MDCK cells followed by HA assay, respectively. Plaque assay data for GPCR (A) and ion channel (B) genes and TCID50 data for GPCR (C) and ion channel genes (D) is presented as the inverse of the fold-change decrease compared to non-targeting control siRNA (siNTC) of three independent experiments performed in triplicate. A positive increase in fold-change equates to a decrease in PFU/ml or $TCID_{50}$/ml compared to siNTC control. Data shows mean ± SEM of 3 independent experiments performed in triplicate. Ordinary one-way ANOVA with Dunnett's Multiple Comparisons Post-Test *p<0.05 **p<0.001 ***p<0.0001 and ****p<0.00001 compared to control. siNTC results are corrected to zero to reflect baseline change in replication is 0. Results are normalized to siNTC control. The siRNA targeting a particular gene and # refers to the siRNA used from the SMARTpool where # refers to the individual siRNA transfected.

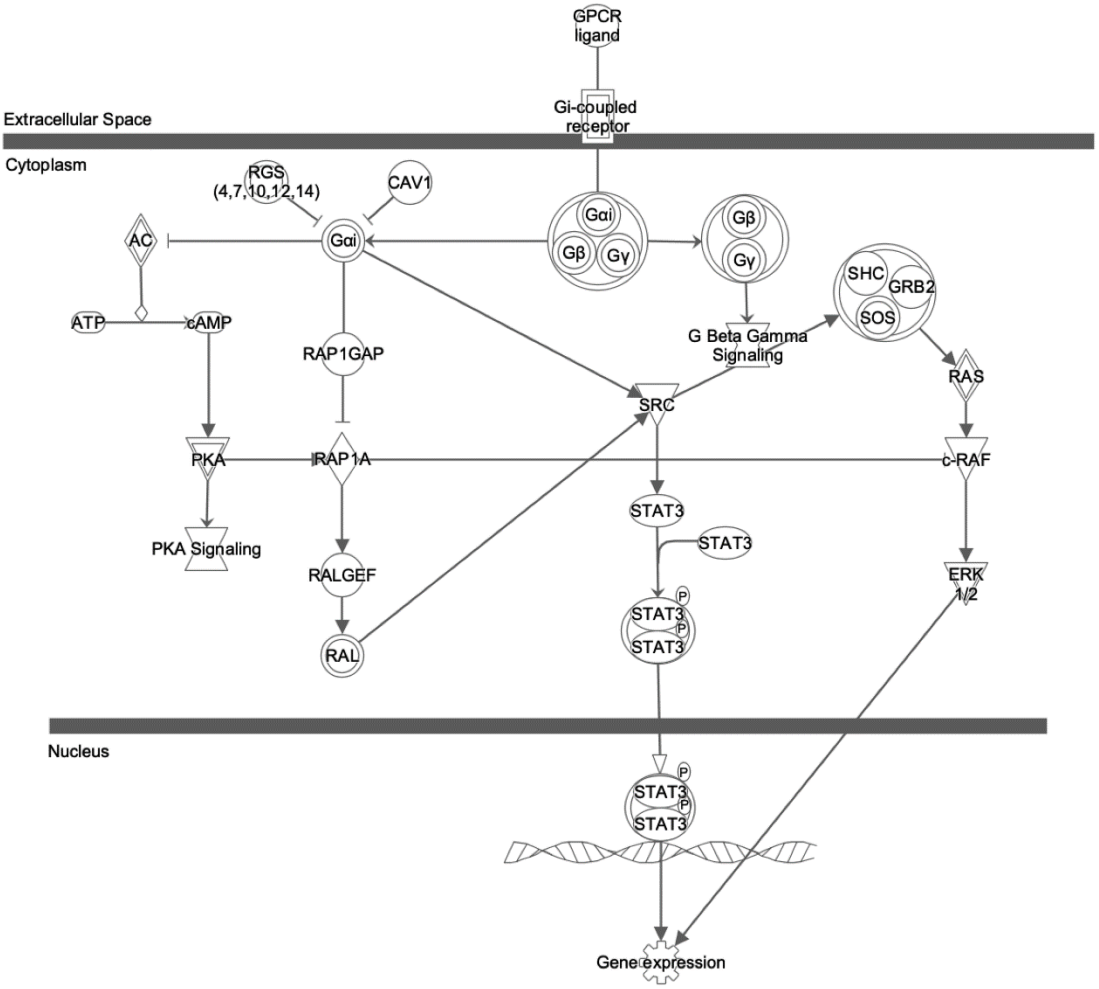

Figure 3.5. Gαi signaling pathway generated by IPA. ADORA1, AGTR1, HTR1B and PYR12 genes were associated with Gαi signaling as part of IPA. AC = adenylyl cyclase; ATP = adenosine triphosphate; cAMP = cyclic AMP; PKA = protein kinase A; RGS = regulators of G protein signaling; CAV1 = Caveolin-1; RAP1GAP = RAP1 GTPase activating protein; RAP1A = Ras-related protein Rap-1A; RALGEF = Ras-like small GTPase; RAL = Ras-like protein; SRC = Src protein kinase; STAT3 = signal transducer and activator of transcription 3; GRB2 = growth factor receptor-bound protein 2; SHC = adaptor protein; SOS = guanine nucleotide exchange protein; c-RAF = RAF proto-oncogene serine/threonine-protein kinase; ERK 1/2 = extracellular signal-regulated kinase

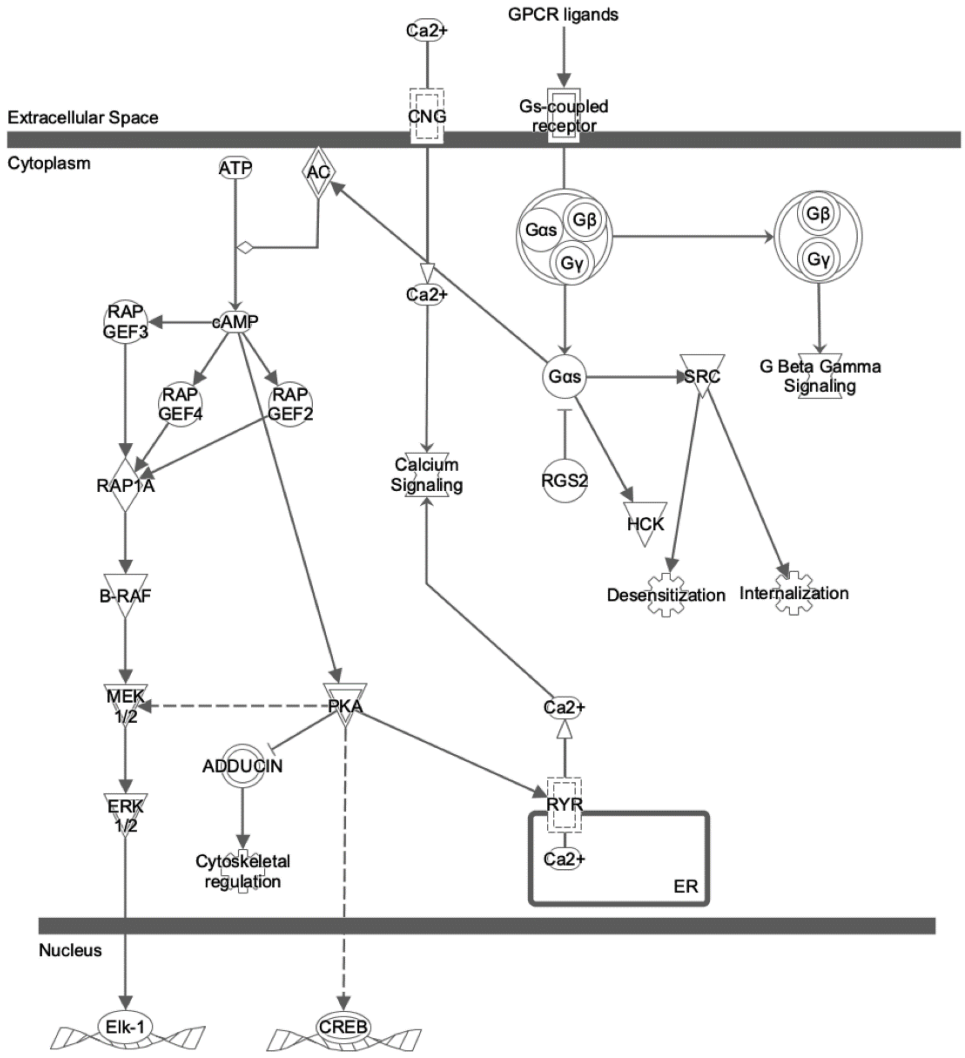

Figure 3.6. Gαs signaling pathway generated by IPA. ADRB2, HCAR3 and HRH2 genes were associated with Gαs signaling as part of IPA. AC = adenylyl cyclase; ATP = adenosine triphosphate; cAMP = cyclic AMP; PKA = protein kinase A; RGS2 = regulators of G protein signaling; RAP1A = Ras-related protein Rap-1A; RAPGEF 2, 3, 4 = rap guanine nucleotide exchange factor 2, 3, 4; SRC = Src protein kinase; B-RAF = RAF proto-oncogene serine/threonine-protein kinase; MEK1/2 = mitogen-activated kinase 1 and 2; ERK 1/2 = extracellular signal-regulated kinase 1 and 2; CNG = cyclic-nucleotide-gated ion channel; HCK = tyrosine protein kinase; RYR = ryanodine receptor; ER = endoplasmic reticulum; CREB = cAMP response element-binding protein; Elk-1 = ETS like-1 protein

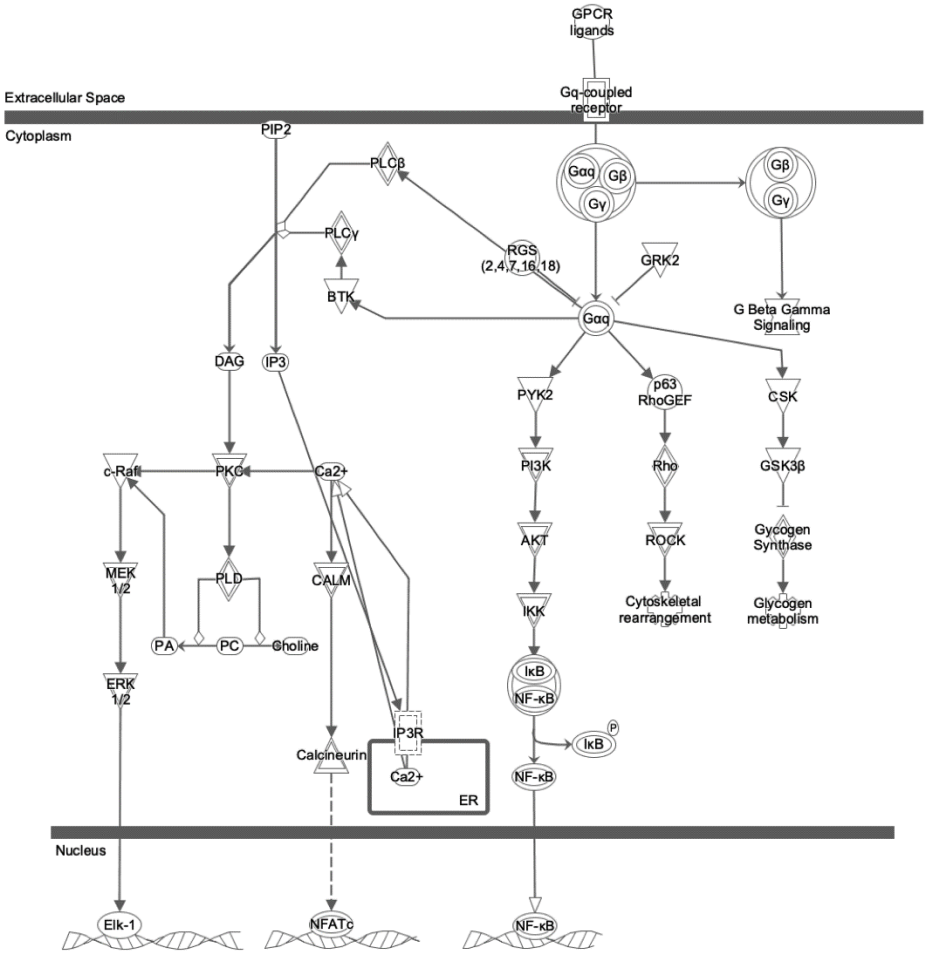

Figure 3.7. Gαq signaling pathway generated by IPA. AGTR1, CCKBR, FFAR1, HCRTR2, OXGR1 and OXTR genes were associated with Gαq signaling. RGS = regulators of G protein signaling; c-RAF = RAF proto-oncogene serine/threonine-protein kinase; MEK1/2 = mitogen-activated kinase 1 and 2; ERK 1/2 = extracellular signal-regulated kinase; PIP2 = phosphatidylinositol biphosphate; IP3 = inositol triphosphate; DAG = diacylglycerol; PKC = protein kinase C; PLD = phospholipase D; PA = phosphatidic acidic; PC = phosphatidylcholine; CALM = clathrin assembly lymphoid myeloid leukemia protein; NFATc = nuclear factor activated T-cells, cytoplasmic; PYK2 = Tau tyrosine kinase; PI3K = phosphoinositide 3-kinase; AKT = protein kinase B; IKK = IκB kinase; NFκB = nuclear factor kappa-light-chain-enhancer of activated B cells; RhoGEF = Rho guanine nucleotide exchange factor; ROCK = Rho associate protein kinase; CSK = tyrosine protein kinase; GSK3β = glycogen synthase kinase-3 beta; PLCβ = phospholipase C beta; BTK = Bruton tyrosine kinase

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CHAPTER 4

MICRORNA REGULATION OF GPCR AND IC GENES THAT AFFECT INFLUENZA REPLICATION[2]

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Abstract

Influenza virus causes seasonal epidemics and sporadic pandemics resulting in morbidity, mortality, and economic losses worldwide. Understanding how to regulate influenza virus replication is important in vaccine and therapeutic employment. Identifying microRNAs (miRs) that regulate host genes used by influenza virus for replication can support an antiviral strategy. In this study, thirty-three miRs predicted to target pro-influenza host GPCR or ion channel genes were screened using A/WSN/33 A/CA/04/09, or B/Yamagata/16/1988 infected A549 cells. miR mimics and their paired inhibitors were evaluated for their effect on influenza replication. The findings show that several miRs reduce influenza virus replication through GPCR or ion channel gene regulation. These findings also identified several pan-anti-influenza miRs.

Introduction

Influenza A viruses (IAV) and influenza B viruses (IBV) belong to the *Orthomyxoviridae* family and are composed of 8 negative-sense, single-stranded viral RNA gene segments. IAV and IBV express 10 primary viral proteins (PB2, PB1, PA, HA, NP, NA, M1, M2, NS1, NS2) and different strain-dependent accessory proteins caused a frameshift and alternative splicing events (1-6). Antigenic drift in HA can lead to epidemics, and reassortment events may lead to pandemics and vaccine failure (7, 8). Influenza epidemics cause numerous hospitalizations and substantial deaths each year. The H1N1 2009 pandemic strain resulted in >60 million cases, >274,000 hospitalizations, and >12,400 deaths in the United States (9). Vaccination is the most effective control measure, but influenza vaccines require annual reformulation to be effective and their efficacy is

decreased by strain mismatch. For example, the 2014-2015 influenza vaccine containing A/Texas/50/2012 (H3N2), A/California/7/2009 (H1N1) and B/Massachusetts/2/2012-like strains had reduced efficacy against IAV H3N2 strains largely due to post-selection drift events (10).

Antiviral drugs are used to limit infection, disease duration, or severity. There are several FDA-approved drugs for use against influenza. Specifically, peramivir, zanamivir, and oseltamivir that act as neuraminidase (NA) inhibitors (11). Unfortunately, resistance among NA inhibitors has been observed. Evaluation of the 2008-2009 seasonal H1N1 circulating subtypes revealed 90% oseltamivir resistance worldwide due to point mutations within the NA (12-14). Baloxavir marboxil is licensed for use in the USA and Japan. It targets and inhibits the cap-dependent endonuclease activity of the IAV and IBV polymerase acidic protein (PA) inhibiting viral RNA synthesis (15, 16). Unfortunately, the administration of baloxavir marboxil is at least three times more expensive compared to oseltamivir (18). Resistance to baloxavir marboxil is not well understood, but A/H1N1 2009 pandemic and A/H3N2 having a I38T mutation in the acidic polymerase from patients before and after baloxavir marboxil treatment are less sensitive (17). Amantadine and rimantadine, both M2 ion channel inhibitors, are no longer recommended due to increased resistance and limited efficacy (18).

Influenza virus hijacks host genes to aid viral replication. Some of the most studied pathways exploited by the influenza virus include nuclear factor kappa B (NFκB), phosphatidylinositol-3-kinase (PI3K), mitogen-activated protein kinase (MAPK), protein kinase C/protein kinase R (PKC/PKR), toll-like receptor (TLR), and retinoic acid-inducible gene 1 (RIG-I) pathways (19-22). Antiviral targeting of host factors typically offers a

recalcitrant approach to limit drug resistance while providing a broad spectrum of efficacy across multiple viruses that may use the same genes or host pathways to replicate. RNA interference (RNAi) is an evolutionarily conserved mechanism of post-transcriptional gene-specific regulation that can be exploited to probe the virus-host interface and identify novel host genes and associated pathways used in influenza virus replication (23-28). MicroRNAs (miRs) are small (19-25 nt) noncoding RNAs central in post-transcriptional gene regulation including the RNAi pathways (29, 30). The human genome encodes an estimated 2,300 miRs, 1115 of which are annotated in the miRbase database as the number of validated human miRs continues to increase (31, 32). miRs bind mRNA as their target in a sequence-dependent manner, but do not require complete complementarity, and thus are promiscuous (32). miRs are predicted to regulate more than 50% of protein-coding genes (33). Viral infection results in the temporal expression of viral proteins that alter cellular miR expression (34, 35). Influenza virus infection results in strain-specific host-miR expression profiles (36, 37). Evidence suggests miRs have a critical role at the host-virus interface during infection tempering the immune and inflammatory responses to infection (38-41). Furthermore, miRs have the potential to act as host-targeting antiviral agents. For example, miR-134 inhibits poliovirus by modifying the host nuclear transport system by targeting the ras-related nuclear protein (RAN) (42). Likewise, miR-555 has a profound antiviral effect against three poliovirus vaccine strains most likely through targeting of heterogeneous nuclear ribonucleoprotein C1/C2 (hnRNP C) (43). Also, Japanese Encephalitis virus (JEV) infection decreases expression of miR-33a-5p *in vitro*, but removal by transfection of miR-33a-5p mimics decreases JEV replication through targeting eukaryotic translation elongation factor 1A1 (44). Further, pulmonary delivery of

an adenovirus vector expressing miR-206 resulted in decreased influenza virus loads, increased type I IFN responses, and increased survival compared to controls.

In this study, miR screening was used to explore the virus-host interface. We previously screened host G-protein coupled receptor (GPCR) and ion channel (IC) genes using siRNAs in influenza A/WSN/33, A/CA/04/09 and B/Yamagata/16/1988 infected A549 cells. We examined virus replication following gene silencing and identified multiple pro-influenza GPCR and ion channel genes (45). These findings allowed us to short-list potential antiviral miRs based on computational target prediction (46, 47). Herein, we examined the miRs' ability to inhibit influenza virus, and elucidated potential strain and type differences amongst the miR inhibition profiles. In addition, it allowed for the recognition of potential pan-antiviral miRs targeting the critical pro-influenza GPCR and ion channel genes, thus providing the foundation for the development of novel antiviral miR therapeutic strategies to impede influenza replication (42, 48, 49).

Materials and Methods

Cells and Viruses

Human lung epithelial (A549) cells (ATCC CCL-185) and Madin-Darby canine kidney (MDCK) cells (ATCC CCL-34) were cultured in Dulbecco's modified Eagle's Medium (DMEM; HyClone, Logan, UT) supplemented with 5% heat-inactivated fetal bovine serum (HI-FBS) (Atlas Biologics Inc., Fort Collins, CO). All experiments were performed with log-phase A549 or MDCK cells.

A/WSN/33 (H1N1; ATCC VR-825) is lab-adapted and trypsin-independent, and A/CA/04/2009 (H1N1, BEI Resources) viruses were propagated in MDCK cells with the

minimal passage (50). B/Yamagata/16/1988 (BEI Resources) was grown in 9-day old embryonated chicken eggs as previously described to achieve acceptable titer for *in vitro* infection (51). Viral titers (PFU/ml) of stock viruses were determined by MDCK plaque assay and calculated using the Reed and Muench method (52-54).

Computational Approaches for the Identification of miR Targets

GPCR and IC genes previously validated as pro-influenza host genes (45) were examine using three miR target prediction programs, i.e. IPA, TargetScan, and miRbase (46, 47). Briefly, IPA (Qiagen, CA) was used to identify potential miR regulators of validated GPCR and IC genes, while TargetScan (Whitehead Institute for Biomedical Research) was used to predict miR-mRNA seed region match sites on conserved 6 - 8 mer complementary sequences and miR untranslated regions using miRanda and Ensembl (32). miR results were categorized into broadly conserved, conserved, or poorly conserved where broadly conserved was defined throughout vertebrates, conserved defined across mammals, and poorly conserved defining as all other miRs. Only results that were assigned as broadly conserved or conserved were considered as miR regulators. Results were limited to experimentally supported data, and only human results were included. This workflow resulted in 33 potential anti-influenza miRNAs. A detailed summary of miRs reducing influenza replication is in Table 4.S3.

miR Screen

To determine the miRs affecting influenza replication, A549 cells were transfected with 25 nM concentrations of miR mimic or miR inhibitor (Horizon Discovery) and subsequently

infected with influenza as described (25, 42). 96-well plates were incubated with miRs in triplicate at 37°C, 5% CO_2 for 48h to allow for miR activity before infection. Briefly, miRs were mixed with DharmaFECT-1 in SF-DMEM at room temperature (RT) for 20 min. A549 cells were suspended in DMEM supplemented with 5% HI-FBS and 1.5 x 10^4 cells were added to each well. Plates were incubated for 48h at 37°C, 5% CO_2. Following transfection, the media was discarded, the cells were washed twice with PBS, and infected for 48h with A/WSN/33 (MOI = 0.01) or A/CA/04/2009 (MOI = 0.1) or B/Yamagata/12/1988 (MOI = 0.1) diluted in MEM supplemented with 0.3% BSA and 1ug/ml TPCK-Trypsin. All experiments included a non-targeting control miR inhibitor and non-targeting control miR mimic, i.e. siMAP2K, and a siTOX control, respectively. The non-targeting miR controls are designed based on miR sequences from *C. elegans* miR and target no known human sequence, while the MAP2K positive control (5'-PAGAACCUCCAUCCAUGUGCUU-3', 5'-PUCAAAUCUGCUCUCUCUGCUU-3', 5'-PAGUUGCUUCAAAUCUGCUCUU-3', 5'-PAGAUGAAUUAGCUUUCUGGUU-3') targets MAP2K previously shown to be required for influenza virus replication (55, 56). Following incubation, supernatants were removed and stored at -80°C until tested by $TCID_{50}$ assay and plaque assay.

Quantitative Real-time PCR of miR-Mediated Silencing of Host Genes

A549 cells were transfected and gene-specific mRNA silencing was determined by qRT-PCR (25, 42). Briefly, cells were removed from the plate for RNA isolation using RNAzol RT reagent (Sigma). Replicates were pooled and RNA was extracted following the manufacturer protocol. RNA pellets were resuspended in 10 ul nuclease-free water and

stored at -20°C until testing. The quantity of total RNA was determined using an Epoch microplate spectrophotometer (BioTek; Winooski, VT). 2 ug RNA was treated with DNase I (Thermofisher) to remove DNA contamination before cDNA synthesis. Total RNA from DNase-treated samples was determined and equal amounts of RNA (50 ng or 100 ng) were reverse transcribed to cDNA using LunaScript RT SuperMix Kit (NEB; MA). Equal volumes of cDNA (2 ul) were used to perform qPCR using Luna Universal qPCR Master Mix (NEB) and predesigned primer assays (Integrated DNA Technologies; Iowa) specific for target genes AGTR1, C5AR2, OXGR1, and LGR4 which were previously validated as pro-influenza host genes and predicted targets of lead miRs during the miR identification process (Table 4.S1) per the manufacturer's protocol. All samples were normalized to 18S RNA and compared to matched I or M control. Methodology and data analysis for qPCR experiments was performed following the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines (57).

Cell Viability Assay

miR transfections were examined to determine if transfection mediated >20% loss in cell viability using Cell Titer Blue (CTB; Promega, WI) for any miR mimic or inhibitor pair. Briefly, A549 cells were transfected with miR mimic, miR inhibitor, siTOX, or mock-transfected (25). Following 48h incubation, the transfected cell viability was determined according to the manufacturer's protocol. Briefly, 100 ul of media from each well was decanted and 20 ul of CTB reagent added to each well. Plates were mixed gently for 10 sec and then incubated at 37ºC. 5% CO_2 for 2h. Following incubation, the plates were gently rocked for 10 sec before reading absorbance with Tecan plate reader at 570 nm with

reference at 600 nm. Percent viability was calculated by comparing mock-transfected to miR-transfected (Table S4.2).

Plaque Assay

Viral titers were determined by MDCK plaque assay (53, 58, 59). Briefly, sample supernatants were diluted in MEM with 1 ug/ml TPCK-treated trypsin and serially diluted 10-fold and transferred to MDCK cell monolayers (90% confluent) in 12-well tissue culture plate format (Corning-Costar, MA). Following 1h virus adsorption at 37°C, 5% CO_2, 3 ml of overlay containing 1-part medium consisting of 10x MEM supplemented with 200mm L-glutamine (Gibco), HEPES solution (Gibco), 7.5% NaCHO3 (Gibco), Pen/Strep/Amp B solution (Gibco), and 1-part 2.4% Avicel (FMC BioPolymer, PA) in water, or 1-part 1% agarose in water was added/well. A/WSN/33 or A/CA/0409 samples were assayed for 3 days at 37°C, 5% CO_2. B/Yamagata/16/1988 samples were assayed for 5 days at 37°C, 5% CO_2 to allow for better plaque formation. Following incubation, overlays were removed, the plates were washed 2x with PBS, and monolayers fixed with acetone/methanol (80:20) for 20 min at RT. Plaques were visualized with crystal violet staining, counted and the viral titers determined (58, 59).

$TCID_{50}$ Assay

A $TCID_{50}$ assay was used to determine endpoint titers (45, 52, 60). Briefly, sample supernatants were collected from influenza virus-infected A549 cells that were serially diluted 10-fold in triplicate on MDCK cells in 96-well plates. Plates were incubated for 5 days under cell culture conditions 37°C, 5% CO_2 (52, 53). The presence of HA was

determined by HA assay post-incubation. Briefly, supernatants were diluted 1:1 with 1% turkey red blood cells (tRBC) to a final volume of 100ul and a final concentration tRBC concentration of 0.5% in a round-bottom plate (60). The $TCID_{50}$ titers were calculated using the Reed and Muench method (52).

Statistics

Statistical analyses for cross-strain/cross-type miR validation were performed using GraphPad Prism software using a two-way ANOVA with Dunnett post-test comparing values to miR-NTC inhibitor or miR-NTC mimic control.

Results

miRs Regulate Influenza Replication

Computational approaches were used to identify antiviral miR regulators of influenza. miRs were evaluated for their potential to target pro-influenza GPCR or IC genes previously identified (45). miRs were short-listed and examined using miR mimics to determine a miRs ability to inhibit influenza replication and a paired anti-sense miR inhibitor was also included (Figures 4.1-4; Table 4.S4). Transfection of miR mimics increases cellular levels of a miR compared to biological levels allowing the evaluation of its effect on viral replication (61, 62). Transfection of miR inhibitors aims to reduce cellular miR levels and is used here as a control where null change or increase in viral replication is expected for antiviral due to endogenous expression of target miR affecting miR inhibitors effect and/or cross-reactivity of miR inhibitor within a miR family (61-63). 33

miRs were predicted to interact with GPCR and IC mRNA based on IPA, TargetScan, and miRbase using high-stringency analysis (Table 4.S3).

The 33 miRs were evaluated against A/WSN/33, A/CA/04/09, and B/Yamagata/16/1988 viruses for their ability to reduce infectious viral titer and $TCID_{50}$ titer using miR mimics. miR inhibitors were used as controls to validate miR mimic results. miR were considered to have an antiviral effect if mimic transfection resulted in a reduction in infectious viral titer and miR inhibitor transfection resulted in either null change or increase in infectious viral titer. All 33 miRs were screened against each virus and the antiviral miR are reported. Briefly, A549 cells were transfected for 48h with 25 nM miR mimics or miR inhibitors, or miR non-targeting control or miR inhibitor non-targeting control or siRNA targeting MAP2K in serum-free media (25). Mitogen-activated protein kinase 1 gene (MAP2K) is required for influenza replication (35, 36) and was targeted here by siMAP2K as a positive control for the reduction of influenza replication. Transfected A549 cells were examined for cell viability (64), and as expected, no loss was detectable (data are not shown). Post-transfection, A549 cells were infected with A/WSN/33 (MOI = 0.01), A/CA/04/09 (MOI = 0.1), or B/Yamagata/16/1988 (MOI = 0.1) and the virus titer determined by plaque assay and TCID50 assay (25). Collectively, 15 miR mimics reduced influenza plaque formation. Eleven miR mimics reduced A/WSN/33 plaque titer with miR-7-5p, let-7b-5p, miR-155-5p, miR-603, miR-616-5p, miR-3129-5p, miR-5011-5p and miR-5692a resulting in substantial decreases compared to non-targeting control (Table 4.S4; Figure 4.1A). This phenotype was observed in the $TCID_{50}$ assay where miR-7-5p, let-7b-5p, miR-155-5p, miR-335-5p, miR-603, miR-1273e, miR-3129-5p, miR-5011-5p and miR-5692a resulted in substantially reduced $TCID_{50}$ titers (Figure 4.1C). Alternatively,

transfection of paired miR inhibitors resulted in either an increase or no change in virus titer (Figure 4.1B, 4.1D). Of interest, miR-6126 inhibitor increased $TCID_{50}$ titer 90-fold, the largest increase in $TCID_{50}$ of any miRs tested (Figure 4.1D). Nine miR mimics miR-7-5p, let-7b-5p, miR-155-5p, miR-603, miR-616-5p, miR-3129-5p, miR-5011-5p, miR-5692a and miR-6126 had a greater effect on plaque titers compared to siMAP2K (Figure 4.1A; Table 4.S4).

Eight miR mimics (miR-7-5p, let-7b-5p, miR-96-5p, miR-603, miR-3129-5p, miR-4723-3p, miR-5011-5p and miR-5692a) reduced A/CA/04/09 titer and $TCID_{50}$ titer (Figure 4.2A, 4.2C; Table 4.S4). miR-603 mimic resulted in the largest reduction in the mean fold-change of A/CA/04/09 titer reduction (Figure 4.2A; Table 4.S4). These miR mimics with exception of let-7b-5p had a greater effect on plaque titers compared to siMAP2K (Figure 4.2A; Table 4.S4). Alternatively, transfection of paired miR inhibitors resulted in either an increase or no change in viral titer (Figure 4.2B, 4.2D). Of note, miR-5692a inhibitor had the greatest effect on $TCID_{50}$ titer showing a substantial increase compared to miR-NTC (Figure 4.2D). Of the miR mimics that reduced viral titers, 6 reduced both influenza A virus titers, but in most cases, miR mimics had a greater effect on reducing A/CA/04/09 replication compared to A/WSN/33 except for let-7b-5p (Figure 4.1A, 4.2A; Table 4.S4). Of note, miR-155-5p, miR-335-5p, miR-616-5p, miR-1273e, and miR-6126 reduced A/WSN/33 plaque formation but did not affect A/CA/04/09, whereas miR-96-5p and miR-4723-3p reduced A/CA/04/09 plaque formation but not A/WSN/33 plaque formation (Figure 4.1A, 4.2A; Table 4.S4).

Individual transfection of 8 miRs (let-7b-5p, miR-218, miR-335, miR-603, miR-4723-3p, miR-5011-5p, miR-5692a and miR-7703) considerably reduced

B/Yamagata/16/1988 replication (Figure 4.3A; Table 4.S4). miR-335 mimic had the greatest reduction in B/Yamagata/16/1988 titer (Figure 4.3A) and transfection of let-7b-5p mimic had the greatest reduction in $TCID_{50}$ titers, although not statistically significant (Figure 4.3C). Transfection of miR inhibitors resulted in an increased or no effect on plaque numbers (Figure 4.3B). Intriguingly, transfection of these miR mimics reduced virus replication as revealed by plaque numbers, however, miR-218, miR-335, miR-603, miR-5011, miR-5692a, and miR-7703 mimics had nominal effects on $TCID_{50}$ titer (Figure 4.3C). Of note, transfection of let-7b-5p, miR-603, miR-5022-5p and miR-5692a inhibitors resulted in significant increases in $TCID_{50}$ (Figure 4.3D). These results suggest these miRs may be affecting production of viral particles, possibly allowing for the production of viral proteins like HA while disrupting the production of progeny resulting in the production of defective particles (65).

Transfection of miR-335 mimic reduced both A/WSN/33 and B/Yamagata/16/1988 titers did not affect A/CA/04/09 titers. Similarly, miR-4723-3p mimic reduced both A/CA/04/09 and B/Yamagata/16/1988 titers did not affect A/WSN/33 titers. Collectively, 4 pan-antiviral miR mimics, i.e. let-7b-5p, miR-603, miR-5011-5p, miR-5692a were identified that reduced viral titers (PFU/ml) of all three influenza viruses evaluated (Figure 4.4).

Anti-influenza miRs regulate host gene expression

Recently, 16 pro-influenza GPCR and 5 pro-influenza ion channel genes were identified using RNAi, and the genes were validated by testing A/WSN/33, A/CA/04/09, and B/Yamagata/16/1988 replication in A549 cells (45). To confirm miR-mediated

regulation of host gene expression, qPCR of target host-gene mRNA was determined. miR-5011-5p, miR-603, and miR-5692a, which are pan anti-influenza miRs as they reduce A/WSN/33, A/CA/04/09, and B/Yamagata/16/1988 replication were selected for further evaluation (Figure 4.4). Also examined were miRs that targeted A/WSN/33 (miR-155-5p, miR-616-5p), A/CA/04/09 (miR-96-5p) and B/Yamagata/16/1988 (miR-218) (Figure 4.4). Transfection of miR-5011-5p and miR-603 mimics reduced AGTR1 and C5AR2 mRNA compared to miR-NTC mimic control, while miR-5011-5p and miR-603 inhibitors increased expression (Figure 4.5A and 4.5B). Similarly, transfection of miR-5692a mimics reduced expression of C5AR2 and OXGR1 compared to the control, while transfection of miR-5692a inhibitor increased expression (Figure 4.5B and 4.5C). Transfection of miR-155-5p and miR-616-5p reduced A/WSN/33 replication (Figure 4.1; Figure 4.4; Table 4.S4) and miR-155-5p mimic reduced AGTR1 mRNA expression while miR-616-5p reduced AGTR1 and C5AR2 mRNA expression compared to the control. As expected, transfection of their inhibitors resulted in increased mRNA expression (Figure 4.5A and 4.5B). Transfection of miR-96-5p reduced A/CA/04/09 replication (Figure 4.2; Figure 4.4; Table 4.S4) and miR-96-5p mimic transfection resulted in reduced AGTR1, C5AR2, and OXGR1 mRNA levels compared to control. Transfection of miR-96-5p inhibitor resulted in increased levels of all three genes (Figure 4.5A-C). miR-218-5p mimic transfection reduced B/Yamagata/16/1988 replication (Figure 4.3; Figure 4.4; Table 4.S4). Transfection of miR-218-5p mimic led to a small increase in LGR4, but inhibitor transfection led to a substantial increase in LGR4 compared to the control and mimic levels (Figure 4.5D). These results show these miRs may regulate pro-influenza host gene expression, however because of miR promiscuity, these genes represent a very small

portion of the targets for each miR and the possibility of off-target events contributing to the reduction in viral replication are possible.

Discussion

It is central to understand how miRs may affect host gene regulation and cadence of expression, as well as to determine miRs regulation of influenza virus strains and types (66-68). The identification and validation of antiviral miRs can provide an avenue for the development of novel therapeutic strategies (42, 48, 49). In this study, the miRs examined 15 reduced influenza virus replication (Figure 4.4). Four miR mimics (let-7b-5p, miR-5011-5p, miR-603, miR-5692a) were identified as pan-antiviral and reduced replication of A/WSN/33, CA/04/09, and B/Yamagata/16/1988 in A549 cells, while other miRs were strain and type-specific or had shared effects on influenza strain and types. For example, 4 miRs (miR-155-5p, miR-616-5p, miR-1273e, miR-6126) were A/WSN/33-specific, while miR-96-5p inhibited A/CA/04/09, and miR-218 and miR-7703 inhibited B/Yamagata/16/1988 replication in A549 cells (Figure 4.4).

The miRs identified may regulate pro-influenza host genes (45). The miR-218 mimic regulates LGR4 (leucine-rich repeat-containing G protein-coupled receptor 4) gene expression. LGR4 is an orphan GPCR receptor with no identified endogenous ligand (45-47). The pan-anti-influenza miRs (miR-603, miR-5011-5p, miR-5692a), the A/WSN/33-specific miR-616-5p, and the A/CA/04/09-specific miR-96-5p reduced mRNA expression by C5AR2 (complement component 5a receptor 2) which is a non-classical GPCR comprised of a seven-transmembrane receptor that lacks G-protein coupling but instead binds β-arrestin (69, 70). AGTR1 mRNA is targeted by the pan-anti-influenza miR-5011-

5p, the A/WSN/33-specific miR-155-5p and miR-616-5p, and the A/CA/04/09-specific miR-96-5p. AGTR1 is a GPCR gene coupled to Gαq signaling (71). The pan-anti-influenza miR-5692a and A/CA/04/09-specific miR-96-5p regulate OXGR1 gene expression. OXGR1 is also a GPCR gene that is associated with Gαq signaling (72) and is targeted by pan-anti-influenza miR-5692a and A/CA/04/09-specific miR-96-5p. Gαq signaling is relevant to phospholipase Cβ (PLC) activation and phosphoinositide 3-kinase (PI3K) (73). Alteration of this pathway has been shown to play a regulatory role in the clathrin-mediated and clathrin-independent endocytosis pathways utilized by influenza at entry (74). Of note, the miR-155-5p mimic mediated in the greatest reduction in A/WSN/33 titer (Figure 4.1B), and the miR-96-5p mimic had the greatest reduction in A/CA/04/09 titer (Figure 4.2A).

This study identified miRs with antiviral effects on A/WSN/33, CA/04/2009, and B/Yamagata/16/1988 replication in A549 cells revealing strain and type-specific differences. Of the 33 miRs evaluated, four pan-anti-influenza miRs were identified that reduced influenza viral titer of all three influenza viruses examined (Figure 4.4). miRs were examined by qPCR to confirm their ability to regulate pro-influenza host genes. Collectively, this study can be used to develop disease intervention strategies and therapeutic modulation of host genes to control influenza replication and disease.

Figures

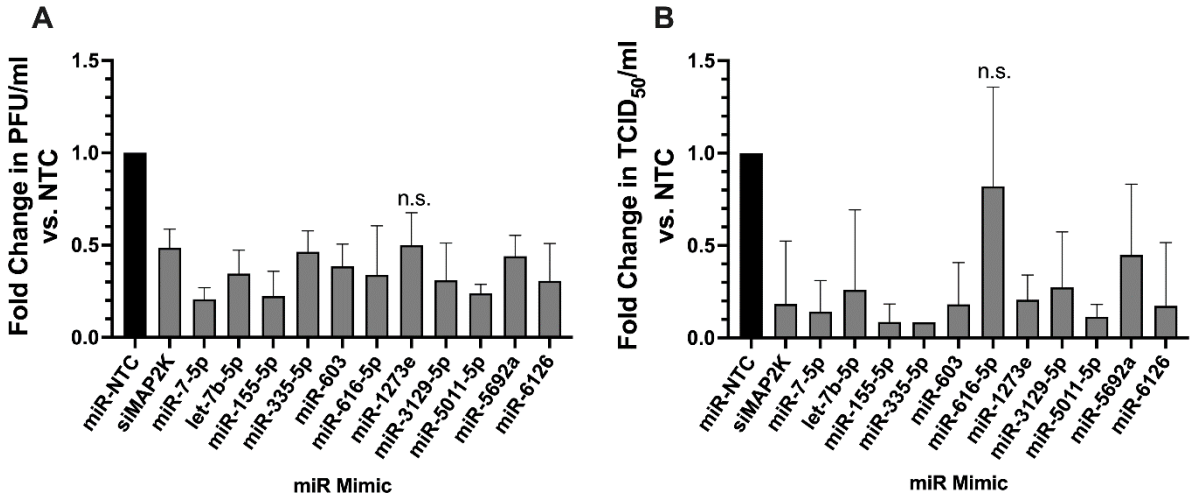

Figure 4.1. miRs affecting A/WSN/33 replication in A549 cells. A549 cells were transfected (25 nM) with either miR mimic, its paired miR inhibitor, miR-NTC control, siMAP2K, or siTOX in triplicate and incubated for 48h. Post-transfection, A549 cells were infected with A/WSN/33 (MOI = 0.01), supernatants were collected 48h pi, and MDCK plaque assays were performed to determine PFU/ml. $TCID_{50}$/ml titers were determined by sample titration on MDCK cells followed by HA assay. Plaque assay (A, B) and $TCID_{50}$ assay (C, D) data is presented as fold-change in influenza virus titer or $TCID_{50}$/ml titer compared to miR-NTC and shown as mean ± SEM of two independent experiments performed in triplicate. Ordinary one-way ANOVA with Dunnett's Multiple Comparisons Post-Test *p<0.05 **p<0.001 ***p<0.0001 and ****p<0.00001 compared to NTC control. A fold-change >1 equates to an increase in PFU or $TCID_{50}$ titer compared to control. A fold-change <1 equates to a decrease in PFU or $TCID_{50}$ titer compared to control. A fold-change = 1 equates to no change in PFU or $TCID_{50}$ titer compared to control.

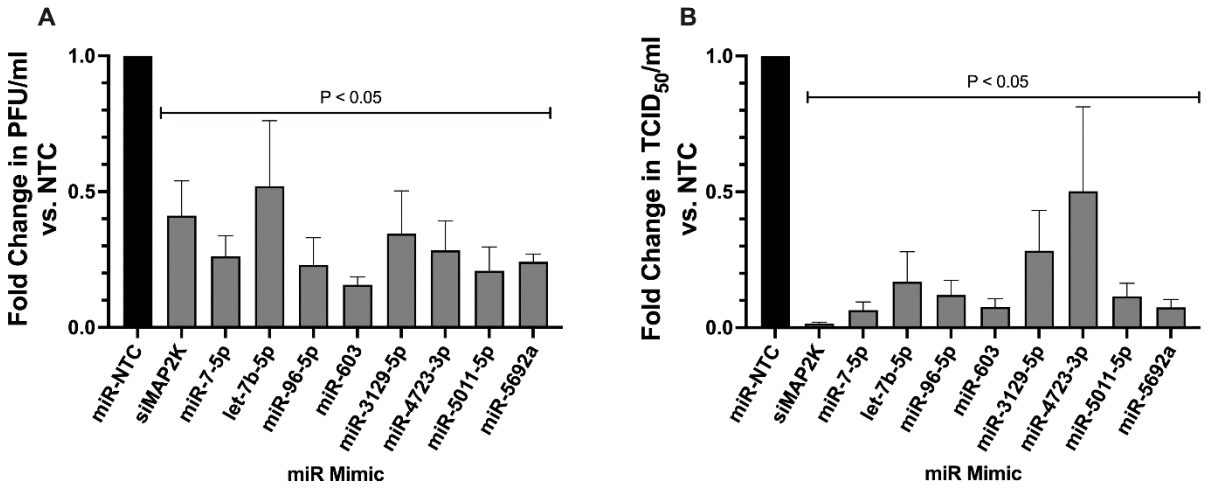

Figure 4.2. miRs affecting A/CA/04/09 replication in A549 cells. A549 cells were transfected (25 nM) with either miR mimic, its paired miR inhibitor, miR-NTC control, siMAP2K, or siTOX in triplicate, and incubated for 48h. Post-transfection, A549 cells were infected with A/CA/04/09 (MOI = 0.1), and supernatants were collected 48h pi, and MDCK plaque assays were performed to determine PFU/ml. $TCID_{50}$/ml titers were determined by sample titration on MDCK cells followed by HA assay. Plaque assay (A, B) and $TCID_{50}$ assay (C, D) data is presented as fold-change in influenza virus titer or $TCID_{50}$ titer compared to miR-NTC and shown as mean ± SEM of two independent experiments performed in triplicate. Ordinary one-way ANOVA with Dunnett's Multiple Comparisons Post-Test *p<0.05 **p<0.001 ***p<0.0001 and ****p<0.00001 compared to NTC control. A fold-change >1 equates to an increase in PFU or $TCID_{50}$l titer compared to control. A fold-change <1 equates to a decrease in PFU or $TCID_{50}$l titer compared to control. A fold-change = 1 equates to no change in PFU or $TCID_{50}$ titer compared to control.

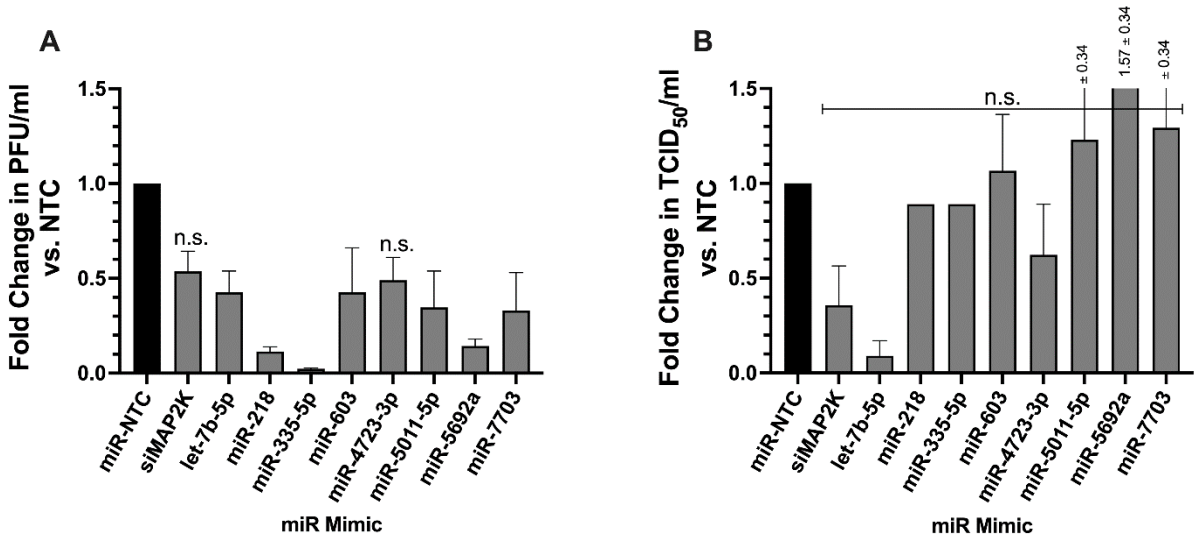

Figure 4.3. miRs affecting B/Yamagata/16/1988 replication in A549 cells. A549 cells were transfected (25 nM) with either miR mimic, its paired miR inhibitor, miR-NTC control, siMAP2K, or siTOX in triplicate and incubated for 48h. Post-transfection, A549 cells were infected with B/Yamagata/16/1988 (MOI = 0.1), supernatants were collected 48h pi, and MDCK plaque assays were performed to determine PFU/ml. $TCID_{50}$/ml titers were determined by sample titration on MDCK cells followed by HA assay. Plaque assay (A, B) and $TCID_{50}$ assay (C, D) data is presented as fold-change in influenza virus titer or TCID50 titer compared to miR-NTC and shown as mean ± SEM of two independent experiments performed in triplicate. Ordinary one-way ANOVA with Dunnett's Multiple Comparisons Post-Test *p<0.05 **p<0.001 ***p<0.0001 and ****p<0.00001 compared to NTC control. A fold-change >1 equates to an increase in PFU or $TCID_{50}$ titer compared to control. A fold-change <1 equates to a decrease in PFU or $TCID_{50}$ titer compared to control. A fold-change = 1 equates to no change in PFU or $TCID_{50}$ titer compared to control.

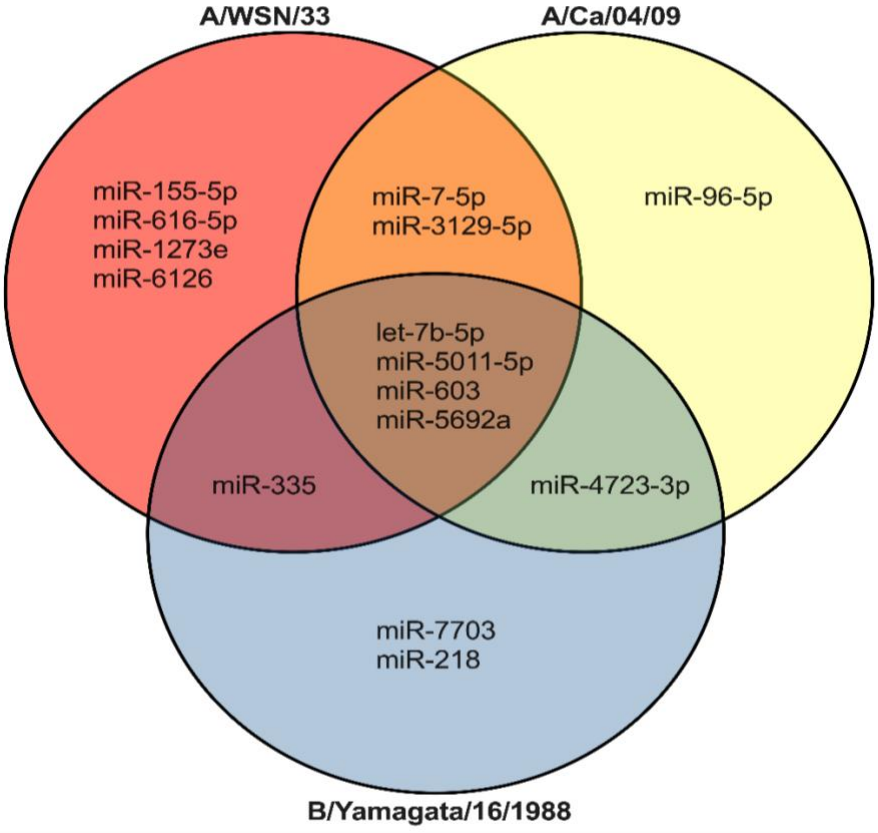

Figure 4.4. Venn diagram of miR screening results. miR screening data clustered by the ability to reduce plaque titer with some clusters overlapping by strains and subtypes.

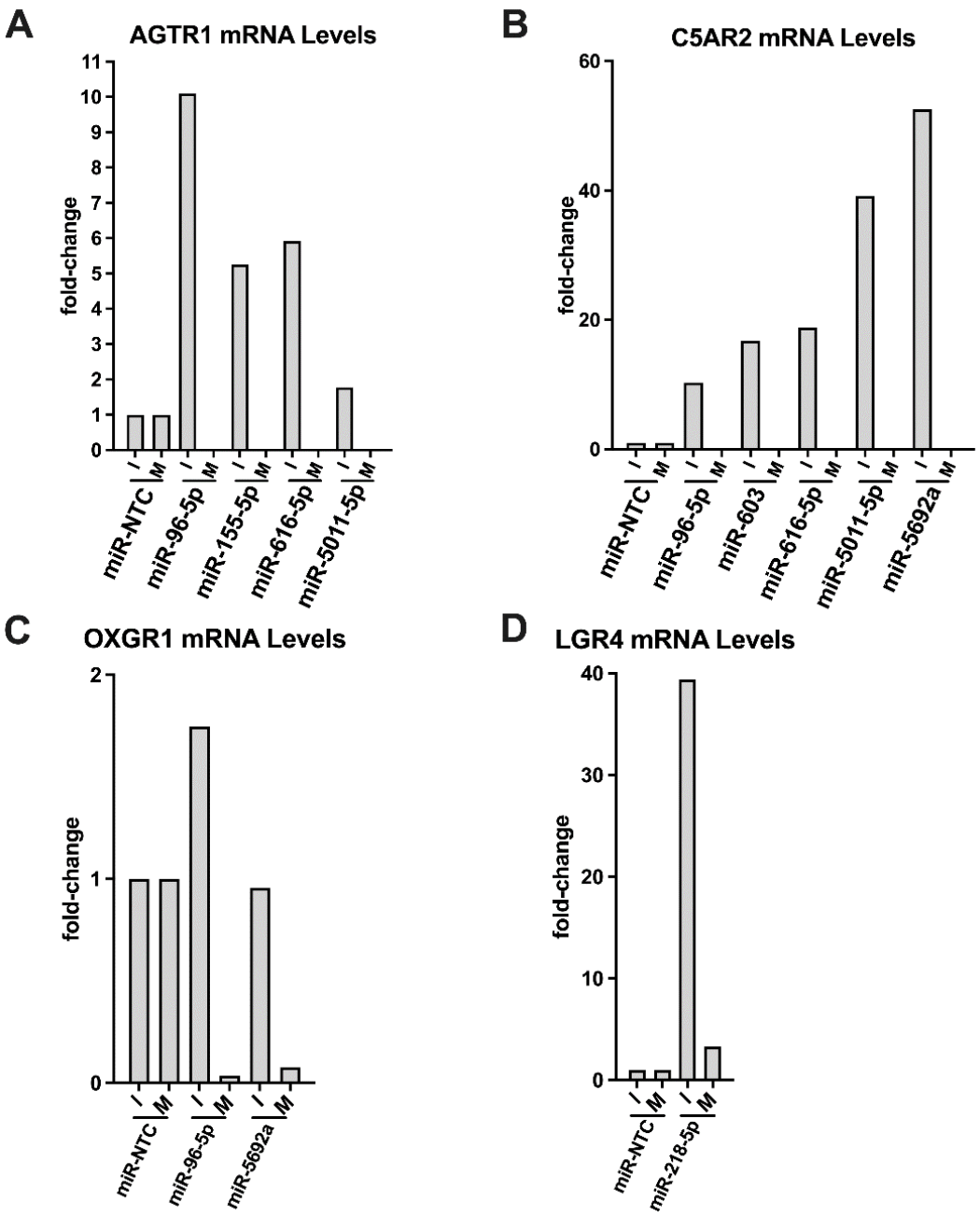

Figure 4.5. qPCR of target gene mRNA following miR (I/M) transfection. A549 cells were transfected (25 nM) with either miR mimic or its paired miR inhibitor, miR non-targeting inhibitor control (miR-NTC (I), miR non-targeting mimic control miR-NTC (M), or siTOX transfection control for 48h. Cells were homogenized, and RNA isolated. Samples were pooled and qPCR was performed to measure mRNA of predicted target genes AGTR1 (A), C5AR2 (B), OXGR1 (C), and LGR4 (D). Data were normalized to 18s rRNA and presented as fold-change of target mRNA in miR vs miR-NTC (I/M).

Supplementary Tables and Figures

Gene	RefSeq Number	Exon Location	Assay Name
LGR4	NM_018490	9-11	Hs.PT.58.40488761
AGTR1	NM_004835	0 - 0	Hs.PT.56a.39468776.g
C5AR1	NM_001271749	4 - 4	Hs.PT.58.26353905.g
OXGR1	NM_080818	1 - 3	Hs.PT.58.19515878

Table 4.S1. qPCR primer assay information. Predesigned primer assays (Integrated DNA Technologies; Iowa) specific for target genes AGTR1, C5AR2, OXGR1, and LGR4 which were previously validated as pro-influenza host genes and predicted targets of lead miRs during the miR identification process were purchased and resuspended per manufacturer recommendations.

Treatment	Percentage of Mock Transfected (Mean ± SEM)
mock transfected	-
siMAP2K	98.61 ± 9.0
siTOX	29.44 ± 2.1
NTC miRNA IH	110.1 ± 2.8
NTC miRNA M	104 ± 2.3
IH_miR-7-5p	107.5 ± 2.6
M_miR-7-5p	105.6 ± 5.2
IH_let-7b-5p	118.7 ± 2.2
M_let-7b-5p	117.7 ± 5.3
IH_miR-26b-5p	120.8 ± 10.5
M_miR-26b-5p	99.64 ± 22.6
IH_miR-32-5p	104.1 ± 1.6
M_miR-32-5p	108.6 ± 0.4
IH_miR-92a-3p	97.9 ± 2.0
M_miR-92a-3p	97.95 ± 5.9
IH_miR-96-5p	109.8 ± 1.4
M_miR-96-5p	109.1 ± 1.317
IH_miR-98-5p	92.14 ± 4.6
M_miR-98-5p	104.2 ± 2.4

IH_miR-100-3p	101.5 ± 2.7
M_miR-100-3p	106.3 ± 2.9
IH_miR-132-3p	106.3 ± 1.1
M_miR-132-3p	110.6 ± 1.7
IH_miR-142-3p	105 ± 2.4
M_miR-142-3p	111.4 ± 0.9
IH_miR-152-3p	126.1 ± 2.1
M_miR-152-3p	117.7 ± 0.3
IH_miR-155-5p	130.8 ± 2.7
M_miR-155-5p	126.4 ± 0.9
IH_miR-190a-3p	110.8 ± 0.5
M_miR-190a-3p	116.3 ± 2.7
IH_miR-218-5p	102.9 ± 4.3
M_miR-218-5p	105.8 ± 2.8
IH_miR-328-5p	103.6 ± 4.1
M_miR-328-5p	97.52 ± 8.2
IH_miR-335-5p	102.6 ± 6.7
M_miR-335-5p	101.9 ± 10.7
IH_miR-365a-3p	93.36 ± 7.9
M_miR-365a-3p	103.5 ± 1.1
IH_miR-374c-3p	95.64 ± 5.4
M_miR-374c-3p	102.2 ± 7.6
IH_miR-448	96.12 ± 9.8
M_miR448	102.6 ± 6.0
IH_miR-603	102.8 ± 3.1
M_miR-603	98.58 ± 9.3
IH_miR-616-5p	102.5 ± 7.9
M_miR-616-5p	119.1 ± 1.6
IH_miR-642a-5p	112.1 ± 2.5
M_miR-642a-5p	115.2 ± 1.8
IH_miR-665	110.2 ± 5.1
M_miR-665	108.8 ± 4.3
IH_miR-762	111.4 ± 3.2
M_hsa-miR-762	113.6 ± 0.3
IH_miR-1273e	104.7 ± 7.0
M_miR-1273e	118.9 ± 3.2
IH_miR-3129-5p	102.9 ± 2.9
M_miR-3129-5p	102.9 ± 1.2
IH_miR-4685-5p	121 ± 3.5
M_miR-4685-5p	120.1 ± 2.1
IH_miR-4723-3p	116.1 ± 2.8
M_miR-4723-3p	117.1 ± 2.8
IH_miR-5011-5p	113.7 ± 0.9
M_miR-5011-5p	111.1 ± 2.3
IH_miR-5692a	95.67 ± 14.8

M_miR-5692a	112.8 ± 5.2
IH_miR-6126	101.1 ± 1.3
M_miR-6126	104.8 ± 0.9
IH_miR-6803-5p	118.6 ± 4.1
M_miR-6803-5p	120.7 ± 1.7
IH_hsa-miR-7703	111.3 ± 9.8
M_hsa-miR-7703	101.4 ± 16.6

Table 4.S2. miR transfection does not reduce cell viability. CellTiter Blue non-destructive cell viability assay was used to examine miR transfections and determine if transfection mediated toxicity via loss in cell viability for any miR mimic or inhibitor pair. Data is presented as mean of percentage of mock transfected control ± standard error (Mean ± SEM). Toxicity is defined as ≥20% loss of viability compared to mock control.

miRNA Inhibitor/Mimic	Mature Accession	Inhibitor Target/Mature miRNA Sequence	Precursor Accession
IH_hsa-miR-7-5p	MIMAT0000252	UGGAAGACUAGUGAUUUUGUUGU	MI0000264
M_hsa-miR-7-5p	MIMAT0000252	UGGAAGACUAGUGAUUUUGUUGU	MI0000264
IH_hsa-let-7b-5p	MIMAT0000063	UGAGGUAGUAGGUUGUGUGGUU	MI0000063
M_hsa-let-7b-5p	MIMAT0000063	UGAGGUAGUAGGUUGUGUGGUU	MI0000063
IH_hsa-miR-26b-5p	MIMAT0000083	UUCAAGUAAUUCAGGAUAGGU	MI0000084
M_hsa-miR-26b-5p	MIMAT0000083	UUCAAGUAAUUCAGGAUAGGU	MI0000084
IH_hsa-miR-32-5p	MIMAT0000090	UAUUGCACAUUACUAAGUUGCA	MI0000090
M_hsa-miR-32-5p	MIMAT0000090	UAUUGCACAUUACUAAGUUGCA	MI0000090
IH_hsa-miR-92a-3p	MIMAT0000092	UAUUGCACUUGUCCCGGCCUGU	MI0000093
M_hsa-miR-92a-3p	MIMAT0000092	UAUUGCACUUGUCCCGGCCUGU	MI0000093
IH_hsa-miR-96-5p	MIMAT0000095	UUUGGCACUAGCACAUUUUUGCU	MI0000098
M_hsa-miR-96-5p	MIMAT0000095	UUUGGCACUAGCACAUUUUUGCU	MI0000098
IH_hsa-miR-98-5p	MIMAT0000096	UGAGGUAGUAAGUUGUAUUGUU	MI0000100
M_hsa-miR-98-5p	MIMAT0000096	UGAGGUAGUAAGUUGUAUUGUU	MI0000100
IH_hsa-miR-100-3p	MIMAT0004512	CAAGCUUGUAUCUAUAGGUAUG	MI0000102
M_hsa-miR-100-3p	MIMAT0004512	CAAGCUUGUAUCUAUAGGUAUG	MI0000102
IH_hsa-miR-132-3p	MIMAT0000426	UAACAGUCUACAGCCAUGGUCG	MI0000449
M_hsa-miR-132-3p	MIMAT0000426	UAACAGUCUACAGCCAUGGUCG	MI0000449
IH_hsa-miR-142-3p	MIMAT0000434	UGUAGUGUUUCCUACUUUAUGGA	MI0000458
M_hsa-miR-142-3p	MIMAT0000434	UGUAGUGUUUCCUACUUUAUGGA	MI0000458
IH_hsa-miR-152-3p	MIMAT0000438	UCAGUGCAUGACAGAACUUGG	MI0000462
M_hsa-miR-152-3p	MIMAT0000438	UCAGUGCAUGACAGAACUUGG	MI0000462
IH_hsa-miR-155-5p	MIMAT0000646	UUAAUGCUAAUCGUGAUAGGGGU	MI0000681
M_hsa-miR-155-5p	MIMAT0000646	UUAAUGCUAAUCGUGAUAGGGGU	MI0000681
IH_hsa-miR-190a-3p	MIMAT0026482	CUAUAUAUCAAACAUAUUCCU	MI0000486
M_hsa-miR-190a-3p	MIMAT0026482	CUAUAUAUCAAACAUAUUCCU	MI0000486
IH_hsa-miR-218-5p	MIMAT0000275	UUGUGCUUGAUCUAACCAUGU	MI0000295
M_hsa-miR-218-5p	MIMAT0000275	UUGUGCUUGAUCUAACCAUGU	MI0000295

IH_hsa-miR-328-5p	MIMAT0026486	GGGGGGGCAGGAGGGGCUCAGGG	MI0000804
M_hsa-miR-328-5p	MIMAT0026486	GGGGGGGCAGGAGGGGCUCAGGG	MI0000804
IH_hsa-miR-335-5p	MIMAT0000765	UCAAGAGCAAUAACGAAAAAUGU	MI0000816
M_hsa-miR-335-5p	MIMAT0000765	UCAAGAGCAAUAACGAAAAAUGU	MI0000816
IH_hsa-miR-365a-3p	MIMAT0000710	UAAUGCCCCUAAAAAUCCUUAU	MI0000767
M_hsa-miR-365a-3p	MIMAT0000710	UAAUGCCCCUAAAAAUCCUUAU	MI0000767
IH_hsa-miR-374c-3p	MIMAT0022735	CACUUAGCAGGUUGUAUUAUAU	MI0016684
M_hsa-miR-374c-3p	MIMAT0022735	CACUUAGCAGGUUGUAUUAUAU	MI0016684
IH_hsa-miR-448	MIMAT0001532	UUGCAUAUGUAGGAUGUCCCAU	MI0001637
M_hsa-miR-448	MIMAT0001532	UUGCAUAUGUAGGAUGUCCCAU	MI0001637
IH_hsa-miR-603	MIMAT0003271	CACACACUGCAAUUACUUUUGC	MI0003616
M_hsa-miR-603	MIMAT0003271	CACACACUGCAAUUACUUUUGC	MI0003616
IH_hsa-miR-616-5p	MIMAT0003284	ACUCAAAACCCUUCAGUGACUU	MI0003629
M_hsa-miR-616-5p	MIMAT0003284	ACUCAAAACCCUUCAGUGACUU	MI0003629
IH_hsa-miR-642a-5p	MIMAT0003312	GUCCCUCUCCAAAUGUGUCUUG	MI0003657
M_hsa-miR-642a-5p	MIMAT0003312	GUCCCUCUCCAAAUGUGUCUUG	MI0003657
IH_hsa-miR-665	MIMAT0004952	ACCAGGAGGCUGAGGCCCCU	MI0005563
M_hsa-miR-665	MIMAT0004952	ACCAGGAGGCUGAGGCCCCU	MI0005563
IH_hsa-miR-762	MIMAT0010313	GGGGCUGGGGCCGGGGCCGAGC	MI0003892
M_hsa-miR-762	MIMAT0010313	GGGGCUGGGGCCGGGGCCGAGC	MI0003892
IH_hsa-miR-1273e	MIMAT0018079	UUGCUUGAACCCAGGAAGUGGA	MI0016059
M_hsa-miR-1273e	MIMAT0018079	UUGCUUGAACCCAGGAAGUGGA	MI0016059
IH_hsa-miR-3129-5p	MIMAT0014992	GCAGUAGUGUAGAGAUUGGUUU	MI0014146
M_hsa-miR-3129-5p	MIMAT0014992	GCAGUAGUGUAGAGAUUGGUUU	MI0014146
IH_hsa-miR-4685-5p	MIMAT0019771	CCCAGGGCUUGGAGUGGGGCAAGGUU	MI0017317
M_hsa-miR-4685-5p	MIMAT0019771	CCCAGGGCUUGGAGUGGGGCAAGGUU	MI0017317
IH_hsa-miR-4723-3p	MIMAT0019839	CCCUCUCUGGCUCCUCCCCAAA	MI0017359
M_hsa-miR-4723-3p	MIMAT0019839	CCCUCUCUGGCUCCUCCCCAAA	MI0017359
IH_hsa-miR-5011-5p	MIMAT0021045	UAUAUAUACAGCCAUGCACUC	MI0017879
M_hsa-miR-5011-5p	MIMAT0021045	UAUAUAUACAGCCAUGCACUC	MI0017879
IH_hsa-miR-5692a	MIMAT0022484	CAAAUAAUACCACAGUGGGUGU	MI0019297
M_hsa-miR-5692a	MIMAT0022484	CAAAUAAUACCACAGUGGGUGU	MI0019297
IH_hsa-miR-6126	MIMAT0024599	GUGAAGGCCCGGCGGAGA	MI0021260
M_hsa-miR-6126	MIMAT0024599	GUGAAGGCCCGGCGGAGA	MI0021260
IH_hsa-miR-6803-5p	MIMAT0027506	CUGGGGGUGGGGGGCUGGGCGU	MI0022648
M_hsa-miR-6803-5p	MIMAT0027506	CUGGGGGUGGGGGGCUGGGCGU	MI0022648
IH_hsa-miR-7703	MIMAT0030018	UUGCACUCUGGCCUUCUCCCAGG	MI0025239
M_hsa-miR-7703	MIMAT0030018	UUGCACUCUGGCCUUCUCCCAGG	MI0025239

Table 4.S3. miRs determined by computational search of GPCR and ion channel genes used for A/WSN/33, A/CA/04/09, or B/Yamagata/16/1988 replication. A549 cells were transfected with miR mimic and inhibitor pairs for 48h (25, 42). Following transfection the cells were infected with A/WSN/33 (MOI=0.01), A/CA/04/09 (MOI=0.1), or B/Yamagata/16/1988 (MOI=0.1) for 48h. Post-infection supernatants were collected and tested for virus titer by plaque assay and HA $TCID_{50}$ assay. miRs which reduced viral replication as determined by MDCK plaque assay were considered anti-viral and evaluated by $TCID_{50}$ HA assay

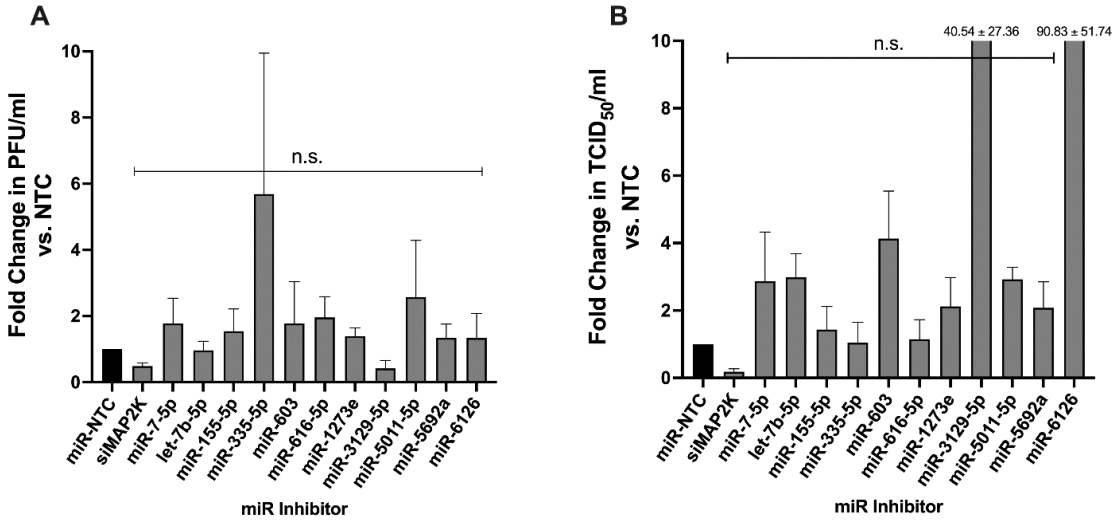

Figure 4.S1. Paired miR inhibitors for miRs affecting A/WSN/33 replication in A549 cells. A549 cells were transfected (25 nM) with either miR inhibitor, miR-NTC control, siMAP2K, or siTOX in triplicate and incubated for 48h. Post-transfection, A549 cells were infected with A/WSN/33 (MOI = 0.01), supernatants were collected 48h pi, and MDCK plaque assays were performed to determine PFU/ml. $TCID_{50}$/ml titers were determined by sample titration on MDCK cells followed by HA assay. Plaque assay (A) and $TCID_{50}$ assay (B) data is presented as fold-change in influenza virus titer or $TCID_{50}$/ml titer compared to miR-NTC and shown as mean ± SEM of two independent experiments performed in triplicate. Ordinary one-way ANOVA with Dunnett's Multiple Comparisons Post-Test ($p < 0.05$) compared to NTC control. A fold-change >1 equates to an increase in PFU or $TCID_{50}$ titer compared to control. A fold-change <1 equates to a decrease in PFU or $TCID_{50}$ titer compared to control. A fold-change = 1 equates to no change in PFU or $TCID_{50}$ titer compared to control.

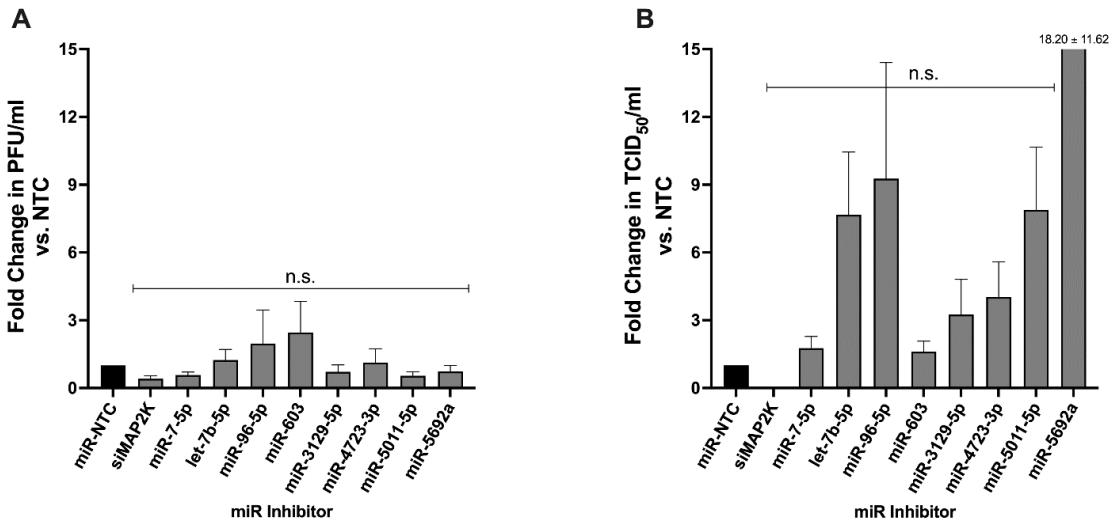

Figure 4.S2. Paired miR inhibitors for miRs affecting A/CA/04/09 replication in A549 cells. A549 cells were transfected (25 nM) with either miR inhibitor, miR-NTC control, siMAP2K, or siTOX in triplicate, and incubated for 48h. Post-transfection, A549 cells were infected with A/CA/04/09 (MOI = 0.1), and supernatants were collected 48h pi, and MDCK plaque assays were performed to determine PFU/ml. $TCID_{50}$/ml titers were determined by sample titration on MDCK cells followed by HA assay. Plaque assay (A) and $TCID_{50}$ assay (B) data is presented as fold-change in influenza virus titer or $TCID_{50}$ titer compared to miR-NTC and shown as mean ± SEM of two independent experiments performed in triplicate. Ordinary one-way ANOVA with Dunnett's Multiple Comparisons Post-Test ($p< 0.05$) compared to NTC control. A fold-change >1 equates to an increase in PFU or $TCID_{50}$l titer compared to control. A fold-change <1 equates to a decrease in PFU or $TCID_{50}$l titer compared to control. A fold-change = 1 equates to no change in PFU or $TCID_{50}$ titer compared to control.

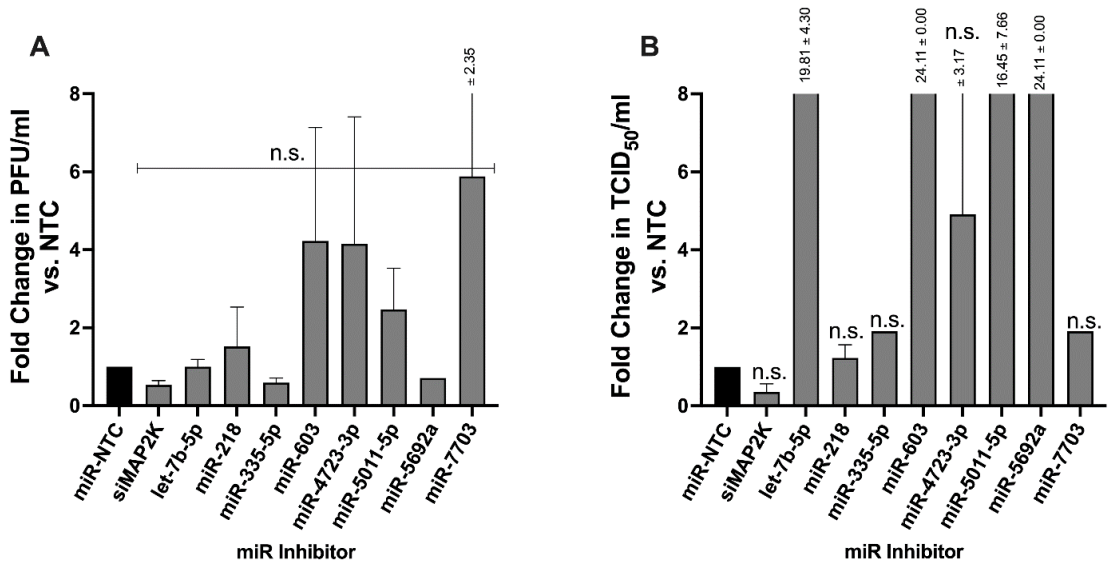

Figure 4.S3. Paired miR inhibitors for miRs affecting B/Yamagata/16/1988 replication in A549 cells. A549 cells were transfected (25 nM) with either miR inhibitor, miR-NTC control, siMAP2K, or siTOX in triplicate and incubated for 48h. Post-transfection, A549 cells were infected with B/Yamagata/16/1988 (MOI = 0.1), supernatants were collected 48h pi, and MDCK plaque assays were performed to determine PFU/ml. $TCID_{50}$/ml titers were determined by sample titration on MDCK cells followed by HA assay. Plaque assay (A) and $TCID_{50}$ assay (B) data is presented as fold-change in influenza virus titer or TCID50 titer compared to miR-NTC and shown as mean ± SEM of two independent experiments performed in triplicate. Ordinary one-way ANOVA with Dunnett's Multiple Comparisons Post-Test ($p < 0.05$) compared to NTC control. A fold-change >1 equates to an increase in PFU or $TCID_{50}$ titer compared to control. A fold-change <1 equates to a decrease in PFU or $TCID_{50}$ titer compared to control. A fold-change = 1 equates to no change in PFU or $TCID_{50}$ titer compared to control.

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CHAPTER 5

REPURPOSING CLOPIDOGREL OR TRIAMTERENE AS AN ANTI-INFLUENZA AGENT[3]

[3] Nichole Orr-Burks, Jackelyn Murray, Kyle V. Todd, Abhijeet Bakre, and Ralph A. Tripp. Submitted to *The Journal of General Virology*, 7/12/21.

Abstract

Influenza viruses cause respiratory tract infections and substantial health concerns. Infection may result in mild to severe respiratory disease associated with morbidity and some mortality. Several anti-influenza drugs are available, but these agents target viral components and are susceptible to drug resistance. There is a need for new antiviral drug strategies that include repurposing of clinically approved drugs. Drugs that target cellular machinery necessary for influenza virus replication can provide a means for inhibiting influenza virus replication. We used RNA interference screening to identify key host cell genes required for influenza replication, and then FDA-approved drugs that could be repurposed for targeting host genes. We focused on Clopidogrel and Triamterene to inhibit A/WSN/33 and A/CA/04/09 influenza A strains, and Yamagata/16/1988 influenza B replication. Clopidogrel and Triamterene provide a druggable approach to influenza treatment across multiple strains and subtypes.

Introduction

There are four types of influenza viruses (types A, B, C, and D) but only influenza A and B infect humans and cause seasonal epidemics (1). Influenza A virus (IAV) is divided into subtypes based on the hemagglutinin (HA) and neuraminidase (NA) genes. There are 18 different HA subtypes and 11 different NA subtypes. Influenza A subtypes are further divided into genetic clades. Influenza B viruses (IBV) are not divided into subtypes but are classified into two lineages: B/Yamagata and B/Victoria (1). IAV and IBV are enveloped and contain eight negative-sense single-stranded RNA genome segments encoding 10 primary viral proteins (PB2, PB1, PA, HA, NP, NA, M1, M2, NS1, NS2) and

various strain-dependent accessory proteins resulting from frameshift and alternative splicing events (2-7). Seasonal epidemics arise from antigenic drift in the HA or NA surface proteins whereas pandemics are the result of viral genome reassortment events leading to vaccine failures (8, 9). Young children, older individuals, and those immunocompromised are at greater risk of more serious influenza illness (10, 11). Vaccines are the most effective available method of control against influenza disease; however, vaccine efficacy is variable within the population and may diminish in the event of strain mismatch (12, 13). The CDC recommends four FDA-approved drugs (peramivir, zanamivir, oseltamivir, and baloxavir marboxil (14) for use against circulating influenza strains. Peramivir, zanamivir, and oseltamivir are neuraminidase (NA) inhibitors that impede influenza replication by inhibiting the budding of progeny virus from infected cells (15, 16). Unfortunately, the accumulation of point mutations within the NA gene during the 2007-2009 influenza season resulted in ~90% of circulating strains having oseltamivir resistance suggesting NAI overuse leads to drug resistance (17, 18). Baloxavir marboxil inhibits the endonuclease activity of the polymerase acidic (PA) protein impeding viral RNA synthesis (19). Unfortunately, baloxavir marboxil administration is nearly three times more expensive compared to oseltamivir (20), and as evidenced by a pediatric study in Japan, approximately 20% of the influenza viruses isolated from treated children developed mutations (21). As of the 2004-2005 influenza season, amantadine and rimantadine, both M2 ion channel inhibitors, are no longer recommended due to increased resistance and limited efficacy (22, 23). Taken together, this demonstrates a need for new disease intervention strategies and that target genes refractory to mutation such a cellular genes and pathways (24, 25). As influenza virus is susceptible to drug-induced changes leading

to resistance, targeting cell genes is recalcitrant to drug-induced changes or resistance. This approach also affords one the opportunity to develop combination therapies that may have a synergistic or additive effect when combined with current antivirals (26).

Traditional drug discovery involves structure-based screening or target-based screening approaches. Both methods can be cumbersome, lengthy, and costly. Approximately 0.1% of all compounds screened progress to an investigational new drug (IND) application, and less than half of these will move to phase III testing. The costs associated with research, development, and approval per IND are on average USD 4 billion and takes 10-15 years from preclinical testing to approval (27). This bottleneck can be overcome using drug repurposing. In this study, we build upon previous RNA interference (RNAi) screening which identified host GPCR and ion channel targets required for influenza replication (28). GPCRs are a superfamily of cell-surface receptor proteins that facilitate activation of intracellular signaling and downstream transcriptional events upon ligand binding (29). Viruses usurp GPCRs to facilitate entry, replication, or egress (30-34). Similarly, ion channels that facilitate Na^{+}, K^{+}, Cl^{-}, or Ca^{+2} ion influx/efflux between the extracellular and intracellular compartments are used to modulate effector pathways and influenza replication (35, 36). Based on the findings from GPCR and ion channel RNAi screens (28), we studied a panel of druggable host genes and focused on Clopidogrel and Triamterene as two repurposed drugs that inhibit influenza A (A/WSN/33 and A/CA/04/09), and influenza B (Yamagata/16/1988) replication. Host genes are potential pharmacological targets involved in virus replication.

Materials and Methods

Cells and Viruses

Type II human lung epithelial (A549) cells (ATCC CCL-185) and Madin-Darby Canine Kidney (MDCK) cells (ATCC CCL-34) were maintained in Dulbecco's modified Eagle's Medium (DMEM; HyClone, Logan, UT) supplemented with 5% heat-inactivated fetal bovine serum (HI-FBS; Atlas Biologics Inc., Fort Collins, CO). Calu-3 lung epithelium cells (ATCC HTB-55) were propagated in DMEM containing 10% HI-FBS, 1% nonessential amino acid solution, 1% L-glutamine solution, and 1% HEPES solution (all from GIBCO). Cells were propagated as described (28).

A/WSN/33 (H1N1; ATCC VR-825) is a lab-adapted, trypsin-independent influenza A strain. A/CA/04/2009 (H1N1, BEI Resources) is a circulating influenza A strain. Both viruses were propagated in MDCK cells (37, 38). B/Yamagata/16/1988 (BEI Resources), an influenza B strain, was propagated in 9-day old embryonated chicken eggs to achieve appropriate working titers as previously described (37). Viral titer (PFU/ml) was determined by plaque assay using MDCK cells, and the titer calculated using the Reed and Muench method (39-41).

Identification of Drugs

GPCR and ion channel genes potentially targeted by repurposed drugs were identified using Ingenuity Pathway Analysis (IPA, Qiagen, CA), Drugbank 3.0, the Drug Gene Interaction Database (DGIdg), and PubChem literature searches. Briefly, IPA, Drugbank, and DGIdg were used to identify potential reverse agonists/inhibitors and antagonist drugs

targeting validated pro-influenza GPCR and ion channel genes based on data extracted from public databases (42-44). Stringency filters were limited to drugs with direct reverse agonist/inhibitor or antagonistic action, human specificity, experimental evaluation, and current FDA approval. Commercially available drugs were purchased from SelleckChem (Houston, TX) or Tocris Bioscience (Bristol, UK). Drug stocks were prepared in DMSO to a stock concentration of 10 mM, aliquoted into working volumes, and stored at -20°C until needed. Drug stocks were discarded following freeze-thaw.

CellTiter Blue viability assay

The selected drugs were evaluated for cytotoxicity using A549 cells and Calu-3 cells using a CellTiter blue viability assay (Promega, WI). Drugs that did not cause cytotoxicity when compared to the DMSO control (<20%) were further evaluated. Briefly, 1.5 x 10^4 A549 cells or Calu-3 cells were seeded into 96-well flat-bottom plates (Costar) and incubated at 37ºC/5% CO_2. Subsequently, the cells were gently washed 1x with PBS (GIBCO), and minimum essential media (MEM; HyClone, Logan, UT) supplemented with 0.3% (v/v) bovine serum albumin (BSA; Gibco, Waltham, Massachusetts) which was added to the plates at 37ºC/5% CO_2. Drug stocks were prepared in filter-sterilized DMSO (Sigma) to a stock concentration of 10 mM. Drugs were dispensed into 96-well plates using a D300 BioPrinter digital drug dispenser (HP, Palo Alto, CA) in MEM supplemented with 0.3% BSA to final concentrations of 500, 200, 150 and 100 for A549 cells and 250, 200, 150, 100, 50, 30, 20, 10, 5, 2.5, 2 and 1 uM for Calu3 cells. Dilutions were transferred to A549 or Calu-3 plates for final drug concentrations of 250, 100, 75, and 50 uM and 125, 100, 75, 50, 25, 15, 10, 5, 2.5, 1.25, 1, and 0.5 uM, respectively. All wells were normalized

to 1% DMSO for A549 cell evaluation, and 0.833% DMSO for Calu-3 cell evaluation. Cells were incubated for the relevant timepoints at 37°C/5% CO_2. Following incubation, CellTiter blue was added to each well and incubated at 37°C/5% CO_2 for 2 h. The absorbance of the plates was determined using a spectrophotometer plate reader (Tecan Trading; AG, Switzerland) at 570 nm with reference at 600 nm. Percent viability was determined by comparing the DMSO control to drug-treated cells.

Drug Screening

Time of addition

96-well flat-bottom plates (Costar) were seeded with 1.5 x 10^4 A549 cells/well and incubated overnight at 37ºC/5%CO_2. Clopidogrel and Triamterene were prepared in DMSO to a stock concentration of 10 mM. Drugs plates were prepared using the D300 BioPrinter digital drug dispenser (HP, Palo Alto, CA) and dispensed into 96-well plates containing virus infection media containing MEM supplemented with 0.3% BSA and TPCK-treated trypsin (1 ug/ml) (Worthington, Lakewood, New Jersey) to the final concentrations 500, 200, 150, or 100 uM. A/WSN/33 virus (MOI = 0.01) was prepared in infection media. A549 cell plates were washed 2x with PBS. Equal volumes of infection and drug dilutions were transferred to the A549 cells (excluding control wells) to infect cells (MOI = 0.01) and achieve final concentrations of 250, 100, 75, or 50 uM of the drug. The wells were normalized to 1% DMSO. The plates were incubated for 12h or 24h at 37ºC/5% CO_2. Following incubation, the supernatants were removed, the plates were washed 2x with PBS, and the cells fixed with methanol: acetone (80:20; Sigma). Leptomycin B (LMB) (Sigma, St. Louis, MO) was used as the positive control for

inhibition of influenza replication and was administer on cells 2h prior to infection (45, 46).

Prophylactic treatment with Clopidogrel and Triamterene

Calu-3 cells were plated in 96-well plates at 1.5 x 10^4 cells per well and incubated overnight at 37ºC/5% CO_2. Clopidogrel and Triamterene were prepared in DMSO to a stock concentration of 10mM. Drugs plates were prepared by the D300 BioPrinter digital drug dispenser (HP, Palo Alto, CA) and dispensed into 96-well plates containing 200 ul DMEM (HyClone) plus with 4% BSA (GIBCO) to the final concentrations of 125, 100, 50, 25, 15, 10, 5, 2.5, 1.25, 1 and 0.5 uM. All wells were normalized to 0.833% DMSO. Plates were incubated with the drug for 24h at 37ºC/5% CO_2. Following incubation, a new 96-well plate was prepared where the drugs in infection media plus TPCK-treated trypsin were added at the final concentrations of 250, 200, 100, 50, 30, 20, 10, 5, 2.5, 2, and 1 uM. Either A/WSN/33 (MOI = 0.01), A/CA/04/09 (MOI = 0.1), or B/Yamagata/16/1988 (MOI = 0.1) were diluted in infection media and added to the wells of the new drug plate excluding the control wells bringing the final concentrations of drugs to 125, 100, 50, 25, 15, 10, 5, 2.5, 1.25, 1, and 0.5 uM, and incubated for 24h at 37ºC/5% CO_2. Following incubation, supernatants were collected and stored at -80ºC until tested by plaque assay and $TCID_{50}$ assay. A/WSN/33 and A/CA/04/09 infected cells were fixed in methanol: acetone (80:20) and B/Yamagata/16/1988 infected cells were fixed with 4% formalin in PBS and permeabilized with 0.5% Triton X-100 prior to staining for Cellomics analysis. Leptomycin B (LMB) (Sigma, St. Louis, MO) was used as the positive control for inhibition of influenza replication and was administered to cells 2h prior to infection (45, 46).

Cellomics

A549 cells and Calu3 cells infected with either A/WSN/33 or A/CA/04/09 were fixed in methanol: acetone (80:20) for 10 minutes and stained with murine anti-NP IgG (National Cell Culture Center, Minneapolis, MN) and DAPI (Invitrogen, Carlsbad, CA). Calu3 cells infected with B/Yamagata/16/1988 were fixed in 4% formalin in PBS for 20 min then permeabilized with 0.5% Triton X-100 for 10 minutes prior to staining with Ferret Sera raised against B/Yamagata/16/1988 and DAPI. The percentage of NP+ cells was quantified using an Arrayscan VTI HCS Reader and Cellomics software (Thermo Fisher, Waltham, MA). Briefly, for NP staining the fixed cells were blocked with Blotto (4% BSA fraction + 4% dry milk in KPL buffer) for 1h at room temperature. The blocking solution was decanted, replaced with murine anti-NP IgG in Blotto, and incubated at room temperature for 1h. Following the incubation, the antibody was removed, and plates washed 3x with KPL buffer, and then a secondary antibody goat anti-mouse IgG AlexaFluor488 (Invitrogen, Carlsbad, CA) in Blotto was added to the wells and incubated at room temperature for 1h. Briefly, for B/Yamagata staining fixed cells were blocked with Blotto (3 fixed cells were blocked with Blotto (3% BSA (Cohn fraction) in KPL buffer) for 1h at 37°C. The blocking solution was decanted, replaced with ferret B/Yamagata/16/1988 anti-sera in Blotto, and incubated at 37°C for 1h. Following the incubation, the antibody was removed, and plates washed 3x with KPL buffer, and then a secondary antibody goat anti-ferret IgG FITC (Abcam, Cambridge, UK) in Blotto was added to the wells and incubated at 37°C for 1h. In all cases following incubation, the antibody was removed, and plates were washed 3x with KPL buffer. The plates were

stained with DAPI in PBS for 20 min and subsequently washed 2x with PBS. Following washing, PBS was added to each well and the percentage of NP+ cells was quantified using the Cellomics software, i.e. green (AlexaFluor488) and blue (DAPI). Background fluorescence was determined, and the baselines set using antibody-treated uninfected control wells. Cells were positive for infection if their average intensity for AlexaFluor488 or FITC within the cytoplasmic mask was higher than that of the control.

Influenza plaque assay

MDCK cells were used in plaque assays to determine viral titers (28, 40, 47, 48). Briefly, supernatants were serially diluted 10-fold in MEM with 1 ug/ml TPCK-trypsin and inoculated onto 90% confluent MDCK cell monolayers in 12-well tissue culture plates (Costar). The virus was adsorbed for 1h at 37°C/5% CO_2 before adding 3 ml of an overlay. Overlay media contained 1-part liquid medium containing: 10x MEM supplemented with 200 mm L-glutamine, HEPES solution, 7.5% $NaCHO_3$, Pen/Strep/Amp B solution (all from Gibco, Waltham, MA), and 1% agarose (Sigma) in water. Samples from A/WSN/33 or A/CA/0409 wells were incubated at 37°C/5% CO_2 for 3 days. B/Yamagata/16/1988 infected cells were incubated at 37°C/5% CO_2 for 5 days to allow for improved plaque formation. Following incubation, the plates were washed 2x with PBS, and the cell monolayers were fixed with methanol: acetone (80:20) for 20 min at room temperature. Following fixation, the plates were stained with 0.2% crystal violet (Fisher Scientific, Waltham, MA) as described to determine the virus titers (28, 40, 47, 48).

$TCID_{50}$ HA assay

Endpoint titers were determined by $TCID_{50}$ (28, 39, 49). Briefly, supernatants that were collected from influenza virus-infected cells were serially diluted 10-fold in triplicate on MDCK cells in 96-well plates (Costar). Influenza virus-infected MDCK plates were incubated for 5 days using cell culture conditions as described (39, 40). Following incubation, an HA test was performed using the supernatants from influenza virus-infected MDCKs and 0.5% turkey red blood cells in round-bottom plates (Costar) (49). The $TCID_{50}$ titers were calculated using the Reed and Muench method (39).

CellTiter Blue Viability Assay

The selected drugs were evaluated for cytotoxicity using A549 cells and Calu-3 cells using a CellTiter blue cell viability assay (Promega, WI). Drugs which did not cause cytotoxicity (<20%) when compared to the DMSO control were further evaluated. Briefly, 1.5 x 104 A549 or Calu-3 cells were seeded into 96-well flat-bottom plates (Costar) and incubated for the relevant timepoints at 37oC/5% CO2. Subsequently, the cells were gently washed 1x with PBS (GIBCO), and minimum essential media (MEM; HyClone, Logan, UT) supplemented with 0.3% (v/v) bovine serum albumin (BSA; Gibco, Waltham, Massachusetts) was added to the plates and plates placed at 37oC/5% CO2. Drug stocks were prepared in filter-sterilized DMSO (Sigma) to a stock concentration of 10 mM. Drugs were dispensed into 96-well plates using a D300 BioPrinter digital drug dispenser (HP, Palo Alto, CA) in MEM supplemented with 0.3% BSA to final concentrations of 500, 200, 150 and 100 for A549 cells and 250, 200, 150, 100, 50, 30, 20, 10, 5, 2.5, 2 and 1 uM for Calu3 cells. Dilutions were transferred to A549 or Calu-3 plates for final drug

concentrations of 250, 100, 75, and 50 uM and 125, 100, 75, 50, 25, 15, 10, 5, 2.5, 1.25, 1, and 0.5 uM, respectively. All wells were normalized to 1% DMSO for A549 cell evaluation, and 0.833% DMSO for Calu-3 cell evaluation. Cells were incubated for the relevant timepoints at 37°C/5% CO2. Following incubation, CellTiter blue reagent was added to each well and incubated at 37°C/5% CO2 for 2 h. The absorbance of the plates was determined using a spectrophotometer plate reader (Tecan Trading; AG, Switzerland) at 570 nm with reference at 600 nm. Percent viability was determined by comparing the DMSO control to drug- treated cells.

Results

Clopidogrel and Triamterene

A dataset of 16 GPCR and 5 ion channel genes were previously identified and validated for replication of A/WSN/33, A/CA/04/09, and B/Yamagata/16/1988 influenza viruses (28). We evaluated commercially available FDA-approved drugs that targeted either the GPCR or IC genes and short-listed this to 21 drugs or compounds. The drugs were purchased from SelleckChem or Tocris Bioscience and resuspended to 10 mM in DMSO. The short-listed drugs were screened against A/WSN/33 (MOI = 0.01) in a time of addition assay where A549 cells were infected for 1h before drug treatment. The percentage of influenza-infected cells was determined at 12h and 24h post-treatment using influenza nucleoprotein as a marker for infection. Two drugs, Clopidogrel bisulfate (Figure 5.1C) and Triamterene (Figure 5.1F) reduced the percentage of influenza-infected A549 cells following treatment at 12h and 24h (Figure 5.1). Clopidogrel significantly ($p<0.05$) reduced the percentage of influenza-infected cells at 12hp for cells treated with 250 uM

and 100 uM, and at 24hpt at 250 uM ($p < 0.05$) with a dose-dependent increase in infected cells (Figure 5.1A and 5.1B). Triamterene significantly ($p<0.05$) reduced the percentage of influenza-infected cells at 12h when cells were treated with 250 uM and marked reduction was observed when cells were treated with 100 uM and 75 uM (Figure 5.1D). Triamterene treatment (250 uM) significantly ($p<0.05$) reduced infection at 24h (Figure 5.1E). The results were not due to a loss of cell viability as determined by a CellTiter Blue assay (Figure 5.S1).

Clopidogrel pretreatment inhibits A/WSN/33 replication in Calu-3 cells.

Calu-3 human bronchial epithelium cells are widely used for the evaluation of influenza and other respiratory viruses (50-52). Calu-3 cells recapitulate the lung compared to A549 cells and may provide more translatable results (53). Clopidogrel was not cytotoxic to Calu-3 cells as determined by a CellTiter Blue assay (Figure 5.S2). We tested Clopidogrel for its ability to reduce A/WSN/33 replication in Calu-3 cells. Leptomycin B (LMB) was used as the positive control as it has been previously shown to inhibit transport of vRNP from the nucleus reducing influenza virus replication and spread (26, 54). LMB had no detectable effect on cell viability (Figure 5.S2 and 5.S3). Calu-3 cells were pretreated with Clopidogrel for 24h before infection with A/WSN/33 (MOI = 0.01). Calu-3 cells were pretreated for 2h with 10 nM LMB prior to A/WSN/33 (MOI= 0.01) infection. At the time of infection, the media was removed and replaced with A/WSN/33, infection media, and Clopidogrel and incubated for 24h. Following infection, the supernatants were collected and evaluated by plaque assay and $TCID_{50}$ HA assay. Calu-3 cells were fixed with acetone/methanol, immunostained for influenza nucleoprotein (NP), and DAPI stained for

Cellomics analysis (Figure 5.2). Influenza virus titers were determined by titration of sample supernatants on MDCK cell monolayers as described (28, 39, 48). Compared to DMSO-treated controls, Clopidogrel pretreatment significantly ($p<0.05$) reduced influenza titers using 125 - 50 uM concentrations (Figure 5.2A). The fold-change in $TCID_{50}$ HA titer was also significantly ($p<0.05$) reduced using 125 - 25 uM concentrations compared to the control (Figure 5.2B). The LMB control had a significant ($p<0.05$) decrease in fold-change compared to the DMSO control for influenza titer (PFU) and $TCID_{50}$ HA titer (Figure 5.2A and 5.2B). The percent influenza NP positive cells was determined using an Arrayscan comparing nuclei (DAPI) to NP, i.e. AlexaFluor488 stained cells. Cells were determined to be NP positive if their average intensity within the cytoplasm was higher than the control threshold. The findings showed that Clopidogrel treatment significantly ($p<0.05$) reduced influenza-infected cells using 125 - 15 uM concentrations (Figure 5.2C and 5.2D). Of note, at higher concentrations, Clopidogrel staining localized with DAPI staining suggesting nuclear retention of vRNPs (Figure 5.2D). These results show that Clopidogrel pretreatment effectively inhibits A/WSN/33 replication in Calu-3 cells.

Triamterene Pretreatment inhibits A/WSN/33 replication in Calu-3 cells.

Using Calu-3 cells, we evaluated Triamterene for its antiviral activity. Triamterene was not cytotoxic for Calu-3 cells in a CellTiter Blue assay (Figure 5.S3). Calu-3 cells were pretreated with Triamterene for 24h before A/WSN/33 (MOI= 0.01) infection. As a positive control, Calu-3 cells were pretreated for 2h with 10 nM LMB before A/WSN/33 (MOI= 0.01) infection. At the time of infection, the media was removed and replaced with A/WSN/33 and Triamterene. The virus infection was incubated for 24h and supernatants

were collected and evaluated by plaque assay and $TCID_{50}$ HA assay. Calu-3 cells were fixed with acetone/methanol, immunostained for influenza NP, and stained with DAPI for Cellomics assays (Figure 5.3). Triamterene pretreatment significantly ($p< 0.05$) reduced influenza titers and the $TCID_{50}$ HA titer using 125 - 100 uM concentrations (Figure 5.3A, 5.3C, and 5.3D). The influenza titers for the LMB control were also significantly ($p<0.05$) reduced (Figure 5.3A and 5.3B). These results show that Triamterene pretreatment effectively inhibits A/WSN/33 replication in Calu-3 cells.

The antiviral activity of Clopidogrel is not influenza virus strain or type-specific.

Clopidogrel is a small molecule inhibitor of P2RY12, a GPCR gene (55-57). We have previously shown that RNAi silencing of P2RY12 reduces influenza replication (28). Associated with P2RY12 is $G\alpha_i$ signaling and activation of the Raf/MEK/Erk pathway (58). Inhibition of the Raf/MEK/Erk cascade during influenza infection leads to reduced influenza virus production and retention of vRNP within the nucleus (59, 60). We sought to determine if the antiviral activities of Clopidogrel were influenza strain or type-specific. Calu-3 cells were pretreated with Clopidogrel for 24h or with 10 nM LMB for 2h before infection with A/CA/04/2009 (MOI = 0.1). At the time of infection, the media was removed and A/CA/04/2009 was added and Clopidogrel replenishment. The infection was incubated for 24h, the cell supernatants were collected at 24hpt and evaluated by plaque assay and $TCID_{50}$ HA assay. Calu-3 cells were fixed with acetone/methanol, immunostained for influenza NP, and stained with DAPI for Cellomics assays (Figure 5.4A-D). Clopidogrel pretreatment significantly ($p<0.05$) reduced A/CA/04/09 titer using 125 - 50 uM concentrations (Figure 5.4A). $TCID_{50}$ HA titers were significantly ($p<0.05$) reduced using

125 - 50uM concentrations (Figure 5.4B). As expected, the LMB control significantly ($p<0.05$) reduced for both influenza titer and $TCID_{50}$ HA titer (Figure 5.4A and 5.4B). The percentage of influenza-infected cells was significantly ($p<0.05$) reduced using 125 - 50 uM concentrations, and markedly reduced using 25 - 2.5 uM treatment (Figure 5.4C and 5.4D). As observed earlier, NP staining localized with DAPI at higher Clopidogrel treatment concentrations suggesting nuclear retention of vRNPs (Figure 5.4D).

To determine if the antiviral activity of Clopidogrel was influenza type-specific it was evaluated against B/Yamagata/16/1988 for antiviral efficacy. Calu-3 cells were pretreated with Clopidogrel for 24h, or LMB 2h, before infection with B/Yamagata/16/1988 (MOI = 0.1), incubated for 24h and evaluated by plaque assay and $TCID_{50}$ assay. Clopidogrel pretreatment reduced B/Yamagata/16/1988 titers using 125 - 1.25 uM concentrations (Figure 5.4E). Pretreatment significantly ($p<0.05$) reduced the HA titer using 125 - 10uM concentrations and at 125 - 50 concentrations there was a greater reduction of $TCID_{50}$ titer compared to LMB (Figure 5.4F). The LMB control reduced influenza titer and $TCID_{50}$ HA titer (Figure 5.4E and 5.4F). The percentage of influenza-infected cells was consistently reduced compared to untreated control with all concentrations and significantly ($p<0.05$) reduced with nearly all concentrations of drug (Figure 5.4G and 5.4H). These data show broad antiviral effects of Clopidogrel treatment against host genes used by influenza to aid viral replication of influenza A and B strains.

The antiviral activity of Triamterene is not influenza virus strain or type-specific.

Triamterene affects the expression of SCNN1 (or δENaC), a sodium channel host gene located on lung cells that are involved in sodium ion transport and reabsorption (61, 62).

We have previously shown that RNAi silencing of the SCNN1D reduces influenza replication (28). We examined Triamterene for its ability to inhibit influenza replication across strains and types (Figure 5.5). Calu-3 cells were pretreated with Triamterene for 24h, or LMB 2h, before infection with A/CA/04/2009 (MOI = 0.1), incubated for 24h, and assayed evaluated by plaque assay and $TCID_{50}$ assay. Triamterene pretreatment significantly ($p<0.05$) reduced A/CA/04/2009 titer and $TCID_{50}$ using 125 - 100 uM concentrations (Figure 5.5A and 5.5B). The LMB control significantly ($p<0.05$) as expected reduced influenza titer (PFU/ml) and $TCID_{50}$ HA titer (Figure 5.5A and 5.5B). The percentage of influenza-infected cells was significantly ($p<0.05$) reduced using 125 - 50 uM concentrations (Figure 5.5C and 5.5D).

Triamterene efficacy was evaluated against B/Yamagata/16/1988 infection. Calu-3 cells were pretreated with Triamterene 24h, or LMB 2h before infection with B/Yamagata/16/1988 (MOI = 0.1), incubated for 24h, and assayed evaluated by plaque assay and $TCID_{50}$ assay. Triamterene pretreatment significantly ($p<0.05$) reduced B/Yamagata/16/1988 titers at all concentrations tested (Figure 5.5E). Pretreatment of Calu-3 cells with Triamterene for 24h led to a significant ($p<0.05$) dose-dependent reduction in $TCID_{50}$ HA titer using 125 - 10 uM concentrations compared to the LMB control. (Figure 5.5E and 5.5F). The percentage of influenza-infected cells was significantly ($p<0.05$) reduced at all concentrations (Figure 5.5G and 5.5H). These data show that pretreatment with Triamterene for 24h can reduce A/CA/04/09 and B/Yamagata/16/1988 replication affirming the pan anti-influenza effects of Triamterene.

Discussion

FDA-approved influenza antiviral drugs may reduce virus replication but this strategy may lead to virus resistance (24, 25).Thus, it is important to consider new strategies to combat influenza such as targeting host genes needed for replication (26, 54, 63, 64). Host genes used for virus replication are recalcitrant to resistance and may provide the potential for pan-antiviral therapies as often viruses usurp the same or similar host pathways for replication (28). In addition, drug discovery is time-consuming, costly, and fraught with failure. In contrast, drug repurposing can mitigate bottlenecks associated with drug discovery and generally reduces the time and cost for implementation (65). We previously utilized RNAi host gene screens to identify GPCR and ion channel genes required for influenza A and B virus replication (28, 42-44), and to identify drugs for repurposing. Our host gene pathway analysis led us to investigate two FDA-approved drugs, i.e. Clopidogrel and Triamterene, which when tested prophylactically reduced influenza A and B virus replication in human respiratory epithelial cell lines, i.e. A549 and Calu-3 cell lines.

GPCRs represent the largest drug target family, and ion channels are the second largest of approved drugs (66, 67). The genes facilitate the activation and modulation of host pathways often hijacked by viruses to facilitate entry, replication, and egress (33, 34). For example, replication of the Marburg virus and Ebola virus replication is linked to GPCR usage (30-32, 68), while modulation of specific ion channels reduces Bunyamvera virus, herpes simplex virus-1, and influenza A viruses (35, 69-71). Furthermore, ion channels affect the efficient viral replication of influenza viruses (71, 72). We short-listed 21 commercially available drugs targeting 16 GPCR and 5 ion channel genes required for

A/WSN/33, A/CA/04/09, or B/Yamagata/16/1988 replication in A549 cells (28). We used Ingenuity Pathway Analysis, Drugbank 3.0, the Drug Gene Interaction Database (DGIdg), and PubChem information to identify potential agonists or inhibitors drugs that directly affected the gene targets (28, 42-44). Drug candidates were evaluated for their therapeutic potential to inhibit A/WSN/33 replication in A549 cells infected. The percent A/WSN/33 infected cells was determined at 12h and 24h post-treatment by Cellomics. Pretreatment with Clopidogrel or Triamterene reduced the percentage of influenza-infected cells at multiple time points (Figure 5.1). Inhibition was only significant at higher drug concentrations, but neither drug affected cell viability (Figure 5.S1). These findings prompted the further evaluation of Clopidogrel and Triamterene.

Clopidogrel is a small molecule thienopyridine antagonist which specifically and irreversibly binds to the purinergic receptor P2RY12 blocking adenosine 5'-diphosphate (ADP) binding although its exact mechanism is not fully understood (73). Clopidogrel received FDA approval in late 1997 to reduce the risk of vascular and cerebrovascular disease (74). We evaluated Clopidogrel pretreatment of Calu-3 cells for its ability to inhibit influenza replication. Calu-3 cells were pretreated with Clopidogrel for 24h, or with LMB control for 2h before A/WSN/33 (MOI = 0.01) infection, then plaque assays, $TCID_{50}$ assays, and the percentage of influenza-infected cells were determined. Based on these endpoint assays, Clopidogrel pretreatment consistently significantly, ($p<0.05$) reduced A/WSN/33 virus production using 125 - 50uM concentrations (Figure 5.2A and 5.2B). Although pretreatment did not completely inhibit virus replication, the results suggest that Clopidogrel treatment affected virus spread as shown by Cellomics image analysis (Figure 5.2C). Interestingly, NP staining of Clopidogrel treated cells showed that like LMB

treatment NP staining was localized to DAPI-stained nuclei (Figure 5.2D). We further showed that host-directed antiviral therapy provides an antiviral strategy across strains and types of influenza viruses.

We show that Clopidogrel pretreatment is effective against circulating A/CA/04/09 influenza A virus and B/Yamagata/16/1988 strains (Figure 5.4). Clopidogrel pretreatment recapitulated previous results and significantly ($p<0.05$) inhibited A/CA/04/09 replication using 125 - 50 uM concentrations (Figure 5.4A and 5.4B). Importantly, the percent of A/CA/04/09 infected cells was significantly ($p<0.05$) reduced when treated using 125 - 50 uM concentrations (Figure 5.4C). Similarly, Clopidogrel pretreatment significantly ($p<0.05$) reduced B/Yamagata/16/1988 titers using 125 - 10 uM concentrations (Figure 5.4E and 5.4F) and reduced the percentage of infected cells at all concentrations and significantly ($p<0.05$) reduced with nearly all concentrations of drug (Figure 5.4G). These data show the antiviral potential of repurposing drugs targeting host genes.

The ability of Clopidogrel to inhibit A/WSN/33, A/CA/04/09, or B/Yamagata/16/1988 replication is likely linked to inhibition of signaling pathways associated with P2RY12. RNAi silencing of P2RY12 inhibits A/CA/04/09 and B/Yamagata/16/1988 replication (28). This GPCR purinergic receptor modulates the activation of the Raf/MEK/Erk pathway (58). Inhibition of the Raf/MEK/Erk cascade during influenza replication leads to reduced influenza virus production and retention of vRNP within the nucleus (59, 60). We confirmed that pretreatment with Clopidogrel localizes NP staining to the nucleus suggesting retention of NP-coated vRNPs in the nucleus as a mechanism inhibiting viral replication (75). The observation that higher Clopidogrel concentrations are needed to achieve the NP staining phenotype and greater reduction in virus replication is likely

because Clopidogrel is a pro-drug. It is believed that Clopidogrel undergoes sequential oxidative steps facilitated by cytochrome P450 enzymes to form an active metabolite (73, 76). The requirement for activation is not clear as studies report that Clopidogrel is active *in vitro* without bioactivation (73, 77, 78). Of note, it has been shown in animal studies that higher doses of Clopidogrel are needed for activity compared to the lower dosing requirements used in humans suggesting differences in metabolism (73).

Triamterene is a diuretic that inhibits epithelial sodium channels (ENaC) (79) and was FDA-approved in 1964 for the treatment of edema (80). The sodium channel gene SCNN1D was previously shown to be a pro-influenza host gene (28, 67, 81). SCNN1D is not expressed in rodents limiting its evaluation (82). We evaluated Triamterene pretreatment of Calu-3 cells for its ability to inhibit influenza replication. Calu-3 cells were pretreated with Triamterene for 24h, or with 10 nM LMB control for 2h before A/WSN/33 (MOI=0.01) infection, then plaque assays, $TCID_{50}$ assays, and the percentage of influenza-infected cells were determined. Based on these endpoint assays, pretreatment with Triamterene significantly ($p<0.05$) reduced A/WSN/33 replication using 125 - 100 uM concentrations (Figure 5.3A and 5.3B). The percent influenza infected Calu-3 cells was significantly ($p<0.05$) reduced following treatment using 125 - 0.5 uM concentrations (Figure 5.3C and 5.3D). We examined Triamterene pretreatment of Calu-3 cells on A/CA/04/09 and B/Yamagata/16/1988 replication (Figure 5.5). Triamterene pretreatment recapitulated previous results showing significant ($p<0.05$) inhibition of A/CA/04/09 replication using 125 - 100 uM concentrations (Figure 5.5A and 5.5B). The percent A/CA/04/09 infected cells was also significantly ($p<0.05$) reduced following pretreatment using 125 - 50 uM concentrations (Figure 5.5C). Triamterene pretreatment was effective

in inhibiting B/Yamagata/16/1988 replication (Figure 5.5E), significantly ($p<0.05$) reduced HA titer using 125 -10 uM concentrations (Figure 5.5F) and significantly ($p<0.05$) reduced the percentage of influenza-infected cells at all concentrations (Figure 5.5G). These results show the broad antiviral potential of repurposing Triamterene.

Triamterene targets epithelial sodium channels (EaNC), which are composed of 4 subunits (α, β, γ and δ), thus it is difficult to attribute the inhibitory effects to a specific subunit or action (83). Based on our previous RNAi screen, it is likely that the δ subunit gene SCNN1D is contributing to limiting replication (28). These results, linked to Triamterene-induced changes in ion gradients and subsequent signaling cascades, affect the membrane transport of proteins required for influenza replication. For example, influenza virus entry into the cell is regulated by a Ca^{+2} dependent signaling cycle which directly regulates clathrin-mediated or clathrin-independent endocytosis (84, 85). As Clopidogrel and Triamterene are available by prescription, their use as repurposed drugs may be useful as an adjunct treatment for antiviral drug-resistant influenza strains.

Figures

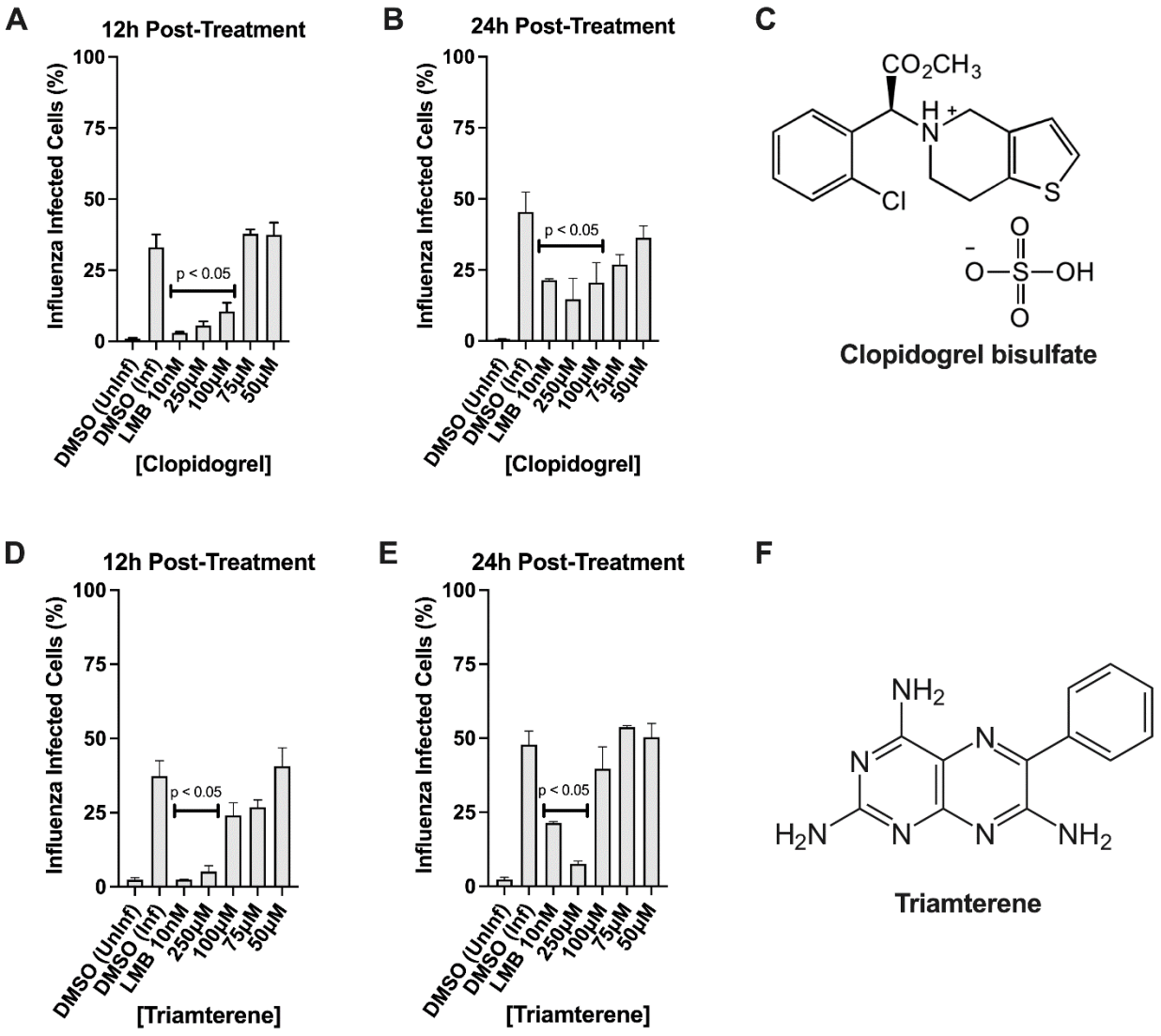
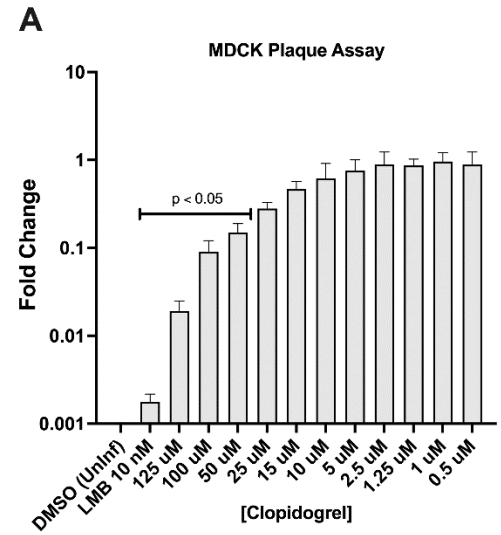


Figure 5.1. Clopidogrel and Triamterene have anti-influenza activity. A549 cells were infected for 1h before treatment with Clopidogrel for 12h (B) and 24h (C) or treated with Triamterene for 12h (E) and 24h (F) or LMB for 2h before infection after which the percent influenza-infected 293 cells was determined using an ArrayScan VTI HCS Reader and Cellomics Software. Presented are the structural formulas for compound SR-25990C, or Clopidogrel bisulfate (C) and compound SKF8542, or Triamterene (F). Shown is the mean ± standard errors for two independent experiments performed in triplicate. Asterisks indicate significant differences from DMSO-treated controls a determined by one-way analysis of variance with Dunnett's multiple-comparison test ($p<0.05$).


A
MDCK Plaque Assay
Fold Change
10
1
0.1
0.01
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p < 0.05
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1 uM
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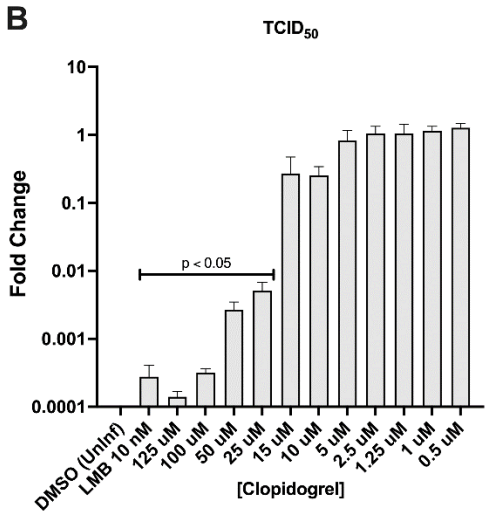
[Clopidogrel]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LMB 10 nM
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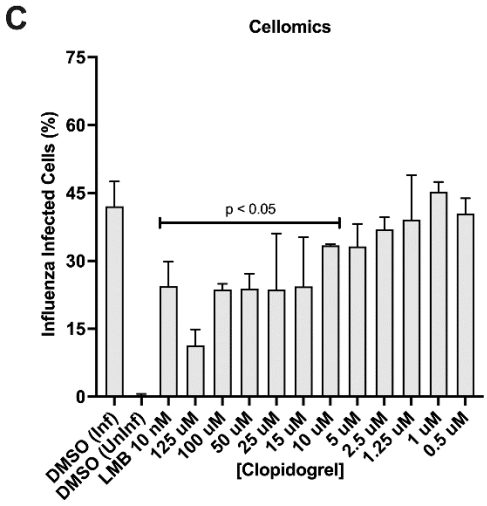
100 uM
50 uM
25 uM
15 uM
10 uM
5 uM
2.5 uM
1.25 uM
1 uM
0.5 uM
[Clopidogrel]


C
Cellomics
Influenza Infected Cells (%)
75
60
45
30
15
0
p < 0.05
DMSO (Inf)
DMSO (UnInf)
LMB 10 nM
125 uM
100 uM
50 uM
25 uM
15 uM
10 uM
5 uM
2.5 uM
1.25 uM
1 uM
0.5 uM
[Clopidogrel]

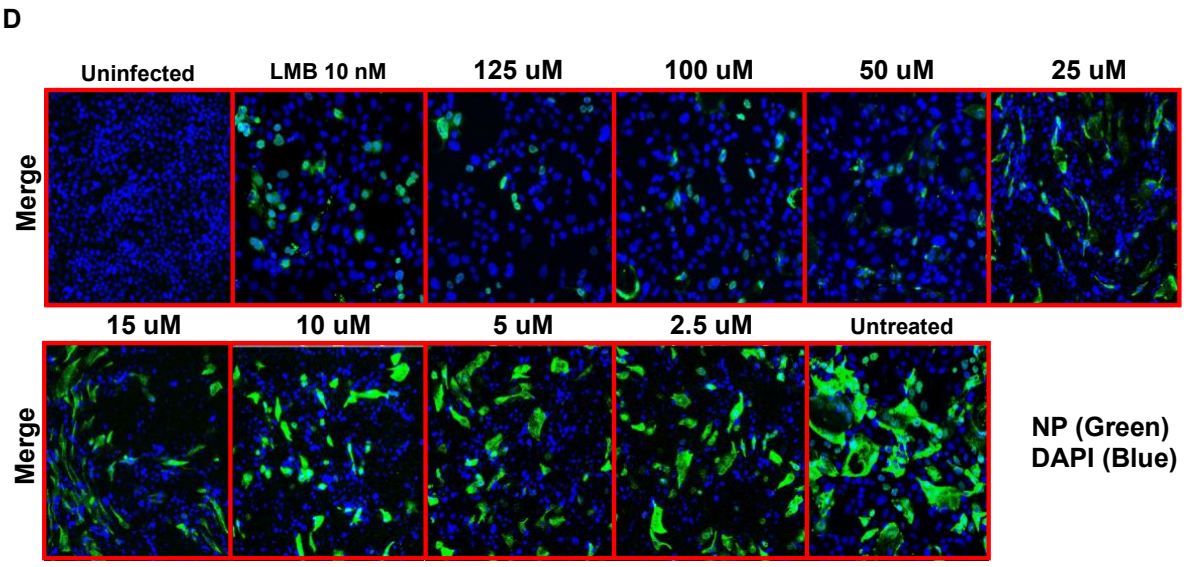


Figure 5.2. Pretreatment with Clopidogrel reduces A/WSN/33 replication. Calu-3 cells were treated with Clopidogrel for 24h or LMB for 2h before infection with A/WSN/33 (MOI=0.01). At the time of infection, the media was removed and the virus was added with drug replenishment. The infection was incubated for 24h. Post-infection, supernatants were collected and evaluated by plaque assay (A) and $TCID_{50}$ HA endpoint assay (B). Fixed Calu-3 cells were immunostained for NP and DAPI for Cellomics analysis (C) and imaging (D). Data show means ± standard errors of the means for two independent experiments performed in triplicate. Asterisks indicate significant differences from the DMSO-treated control by one-way analysis of variance with Dunnett's multiple-comparison test ($p < 0.05$). Influenza NP is green; nuclei are blue.

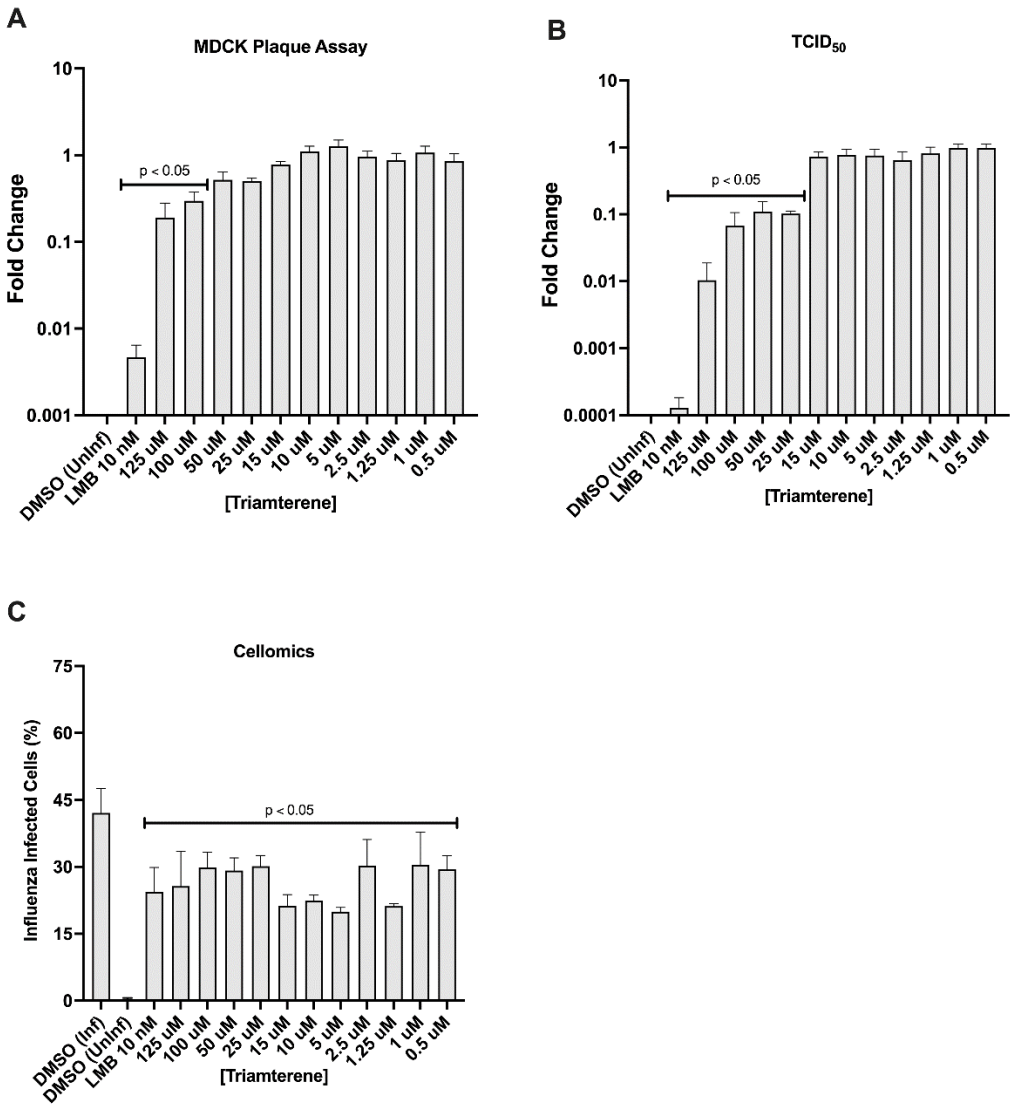
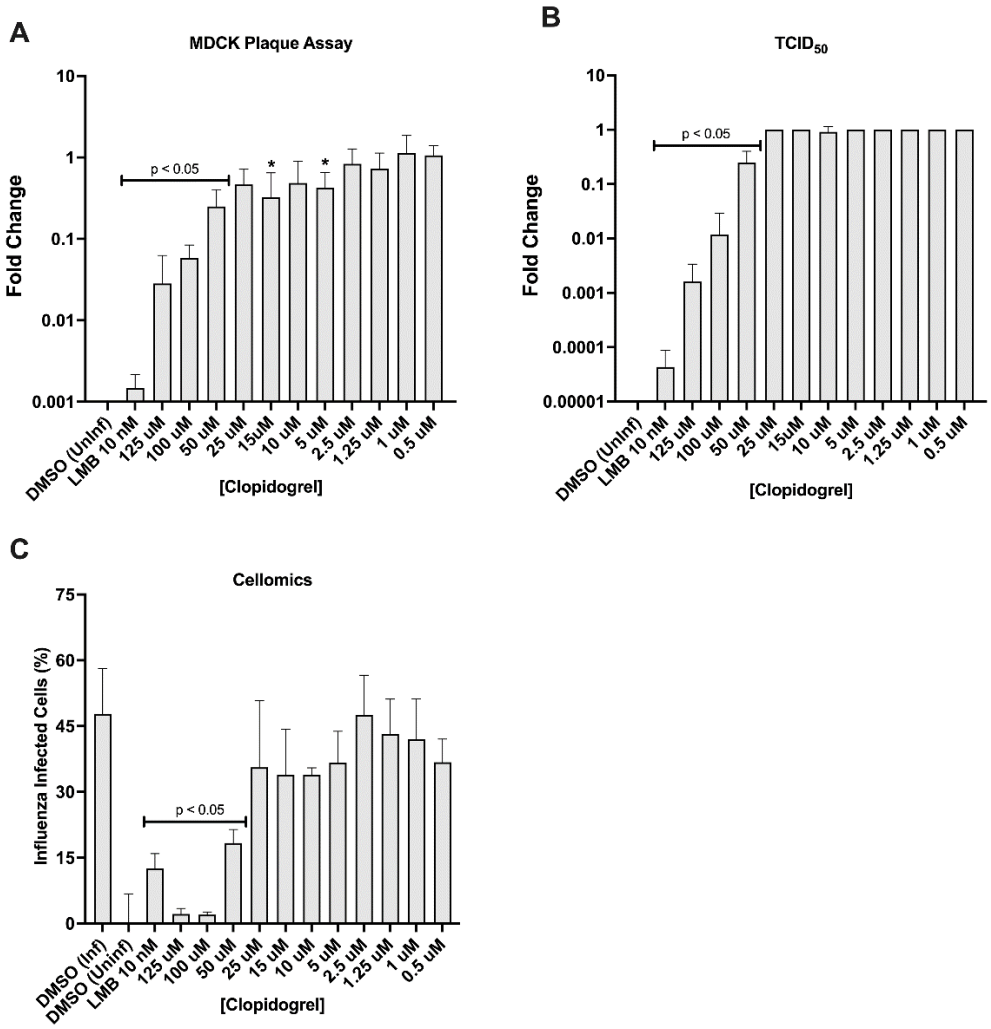
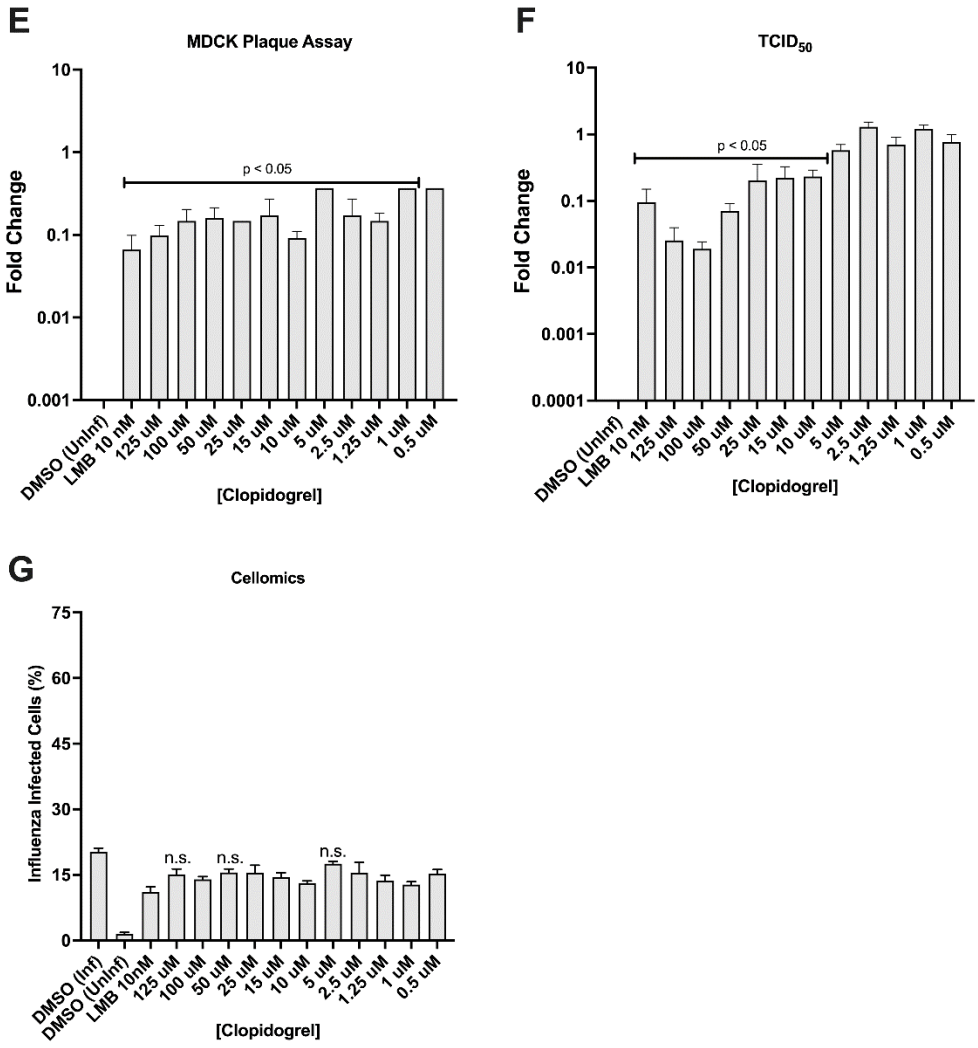

A
MDCK Plaque Assay
Fold Change
p < 0.05
DMSO (UnInf)
LMB 10 nM
125 uM
100 uM
50 uM
25 uM
15 uM
10 uM
5 uM
2.5 uM
1.25 uM
1 uM
0.5 uM
[Triamterene]
B
TCID50
Fold Change
p < 0.05
[Triamterene]
C
Cellomics
Influenza Infected Cells (%)
p < 0.05
DMSO (Inf)
[Triamterene]
D
Uninfected
LMB 10 nM
125 uM
100 uM
50 uM
25 uM
Merge
15 uM
10 uM
5 uM
2.5 uM
Untreated
NP (Green)
DAPI (Blue)

Figure 5.3. Pretreatment with Triamterene reduces A/WSN/33 replication. Calu-3 cells were pretreated with Triamterene for 24h, or LMB for 2h before infection with A/WSN/33 (MOI=0.01). At the time of infection, the media was removed and the virus was added with drug replenishment. The infection was incubated for 24h. Post-infection, supernatants were collected and evaluated by plaque assay (A) and $TCID_{50}$ assay with HA endpoint (B). Fixed Calu-3 cells were immunostained for NP and with DAPI for Cellomics analysis (C) and imaging (D). The results were determined as means ± standard errors of the means for two independent experiments performed in triplicate. Asterisks indicate significant differences from the DMSO-treated control by one-way analysis of variance with Dunnett's multiple-comparison test ($p<0.05$). Influenza NP is green; nuclei are blue.


A
MDCK Plaque Assay
Fold Change
p < 0.05
[Clopidogrel]
B
TCID50
Fold Change
p < 0.05
[Clopidogrel]
C
Cellomics
Influenza Infected Cells (%)
p < 0.05
[Clopidogrel]
D
Uninfected
LMB 10 nM
125 uM
100 uM
50 uM
25 uM
Merge
15 uM
10 uM
5 uM
2.5 uM
Untreated
Merge
NP (Green)
DAPI (Blue)


E
MDCK Plaque Assay
Fold Change
p < 0.05
[Clopidogrel]
F
TCID50
Fold Change
p < 0.05
[Clopidogrel]
G
Cellomics
Influenza Infected Cells (%)
n.s.
[Clopidogrel]

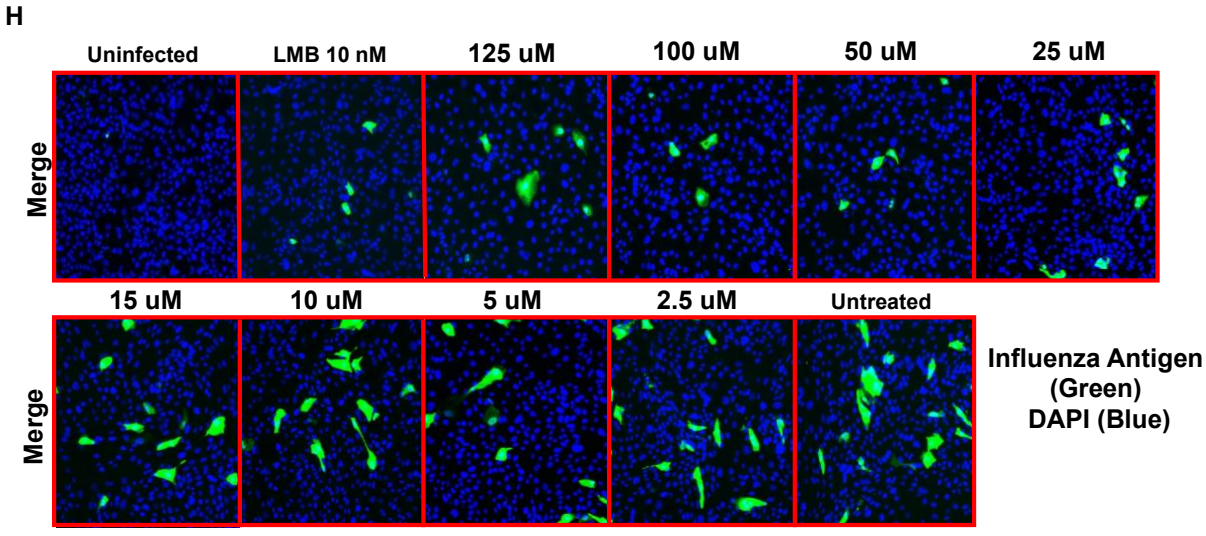
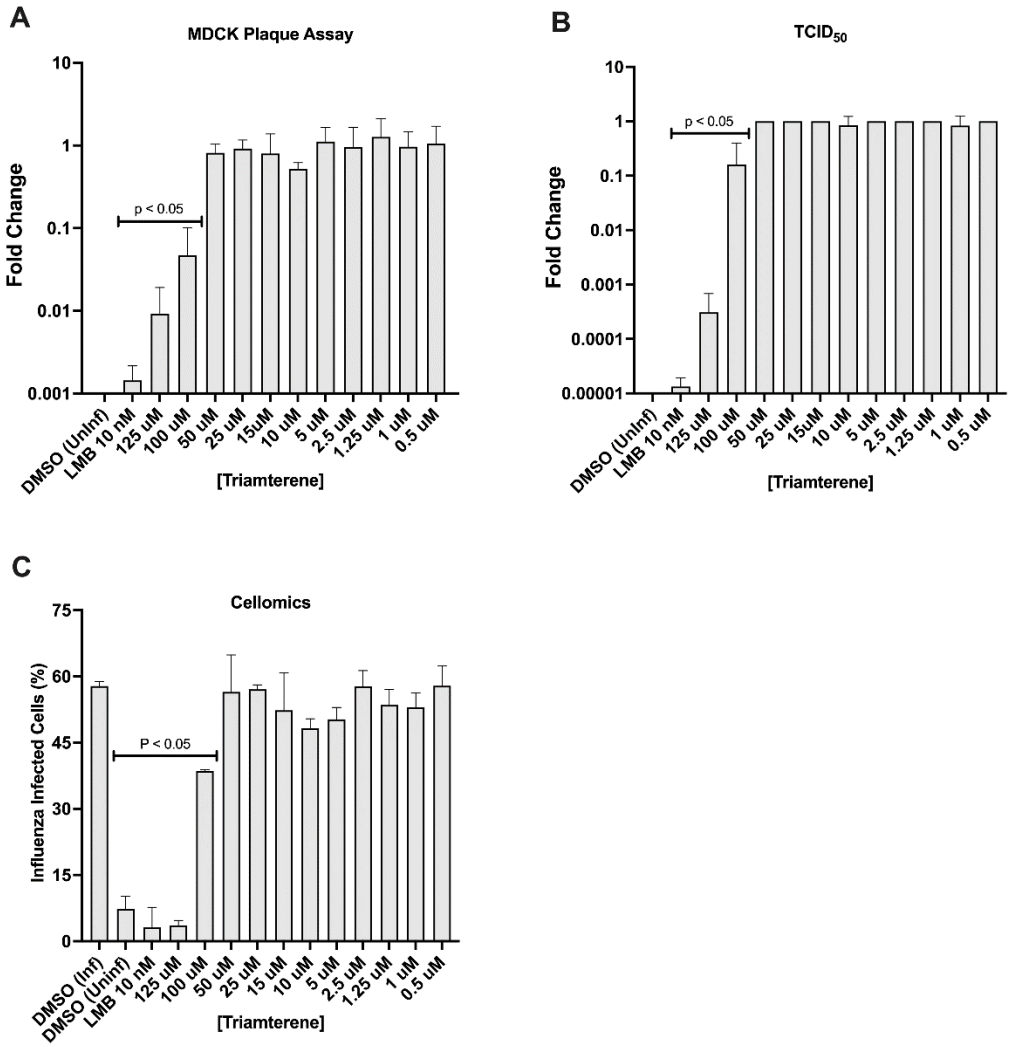

H
Uninfected
LMB 10 nM
125 uM
100 uM
50 uM
25 uM
Merge
15 uM
10 uM
5 uM
2.5 uM
Untreated
Merge
Influenza Antigen (Green)
DAPI (Blue)

Figure 5.4. Pretreatment with Clopidogrel reduces A/CA/04/09 and B/Yamagata/16/1988 replication. Calu-3 cells were treated with Clopidogrel for 24h, or with LMB for 2h before infection with either A/CA/04/09 (MOI=0.1) (A-D) or B/Yamagata/16/1988 (MOI=0.1) (E-H). At the time of infection, the media was removed, and the virus was added with drug replenishment. The infection was incubated for 24h. Post-infection, supernatants were collected and evaluated by plaque assay (A, E) and $TCID_{50}$ assay with HA endpoint (B, F). Fixed Calu-3 cells were immunostained for NP (C, D) or B/Yamagata/16/1988 antigen (G, H) and with DAPI for Cellomics analysis and imaging. Data show means ± standard errors of the means for two independent experiments performed in triplicate. Asterisks indicate significant differences from the DMSO treated control by one-way analysis of variance with Dunnett's multiple-comparison test ($p<0.05$). Influenza NP is green; nuclei are blue.


A
MDCK Plaque Assay
Fold Change
p < 0.05
[Triamterene]
B
TCID50
Fold Change
p < 0.05
[Triamterene]
C
Cellomics
Influenza Infected Cells (%)
P < 0.05
[Triamterene]
D
Uninfected
LMB 10 nM
125 uM
100 uM
50 uM
25 uM
Merge
15 uM
10 uM
5 uM
2.5 uM
Untreated
Merge
NP (Green)
DAPI (Blue)

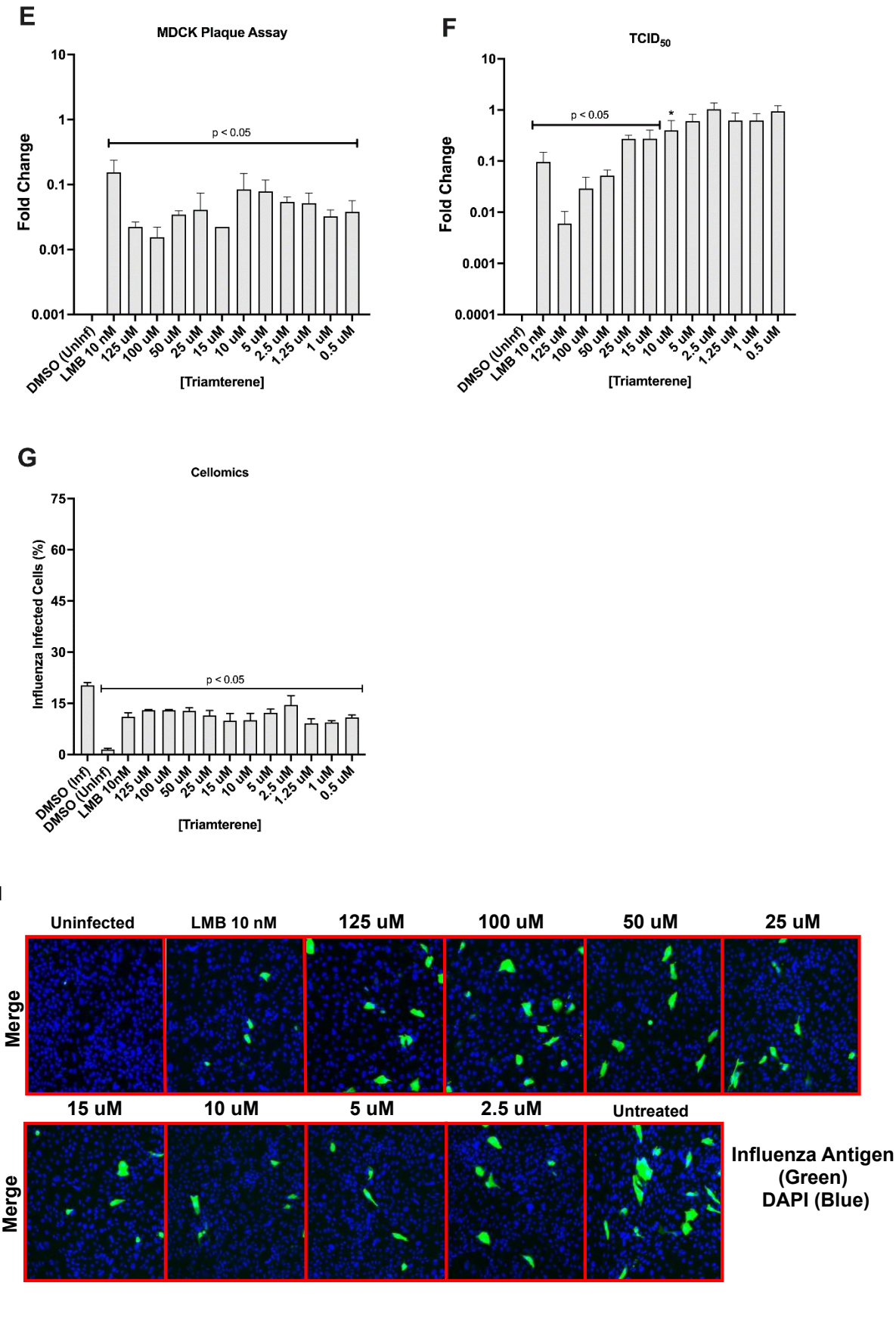
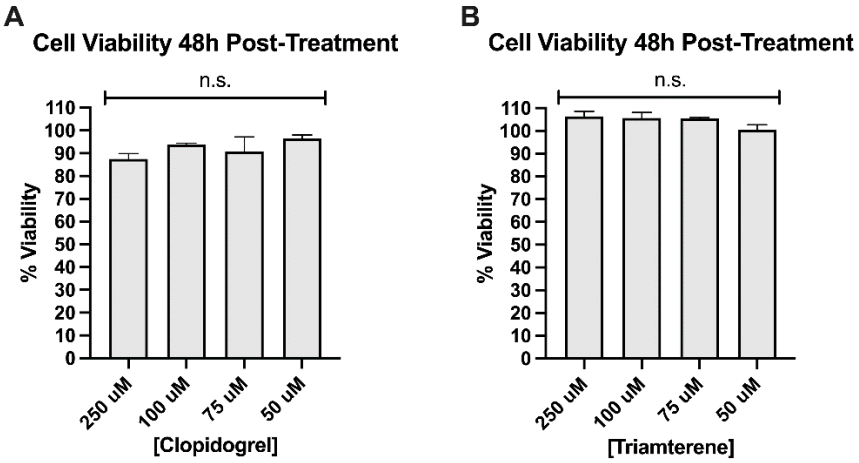
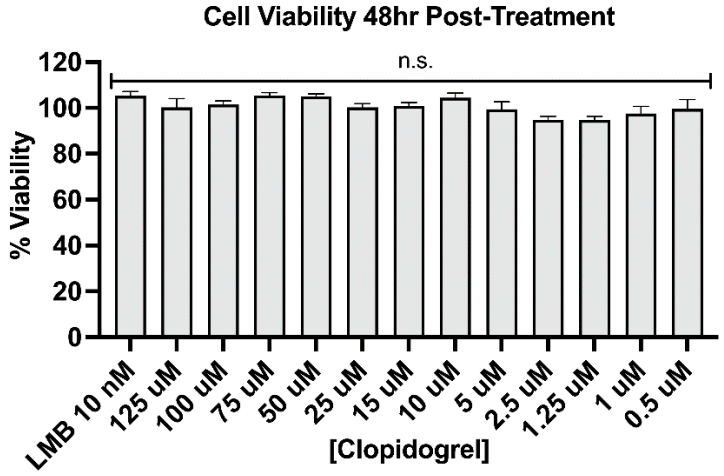

E
MDCK Plaque Assay
Fold Change
p < 0.05
[Triamterene]
F
TCID50
Fold Change
p < 0.05
*
[Triamterene]
G
Cellomics
Influenza Infected Cells (%)
p < 0.05
[Triamterene]
H
Uninfected
LMB 10 nM
125 uM
100 uM
50 uM
25 uM
Merge
15 uM
10 uM
5 uM
2.5 uM
Untreated
Merge
Influenza Antigen (Green)
DAPI (Blue)

Figure 5.5. Pretreatment with Triamterene reduces A/CA/04/09 and B/Yamagata/16/1988 replication. Calu-3 cells were pretreated with Triamterene for 24h, or LMB for 2h before infection with either A/CA/04/09 (MOI=0.1) (A-D) or B/Yamagata/16/1988 (MOI=0.1) (E-H). At the time of infection, the media was removed and the virus was added with drug replenishment. The infection was incubated for 24h. Post-infection, supernatants were collected and evaluated by plaque assay (A, E) and $TCID_{50}$ assay with HA endpoint (B, F). Fixed Calu-3 cells were immunostained for NP (C, D) or B/Yamagata/16/1988 antigen (G, H) and with DAPI for Cellomics analysis and imaging. Data show means ± standard errors of the means for two independent experiments performed in triplicate. Asterisks indicate significant differences from the DMSO treated control by one-way analysis of variance with Dunnett's multiple-comparison test ($p<0.05$). Influenza NP is green; nuclei are blue.

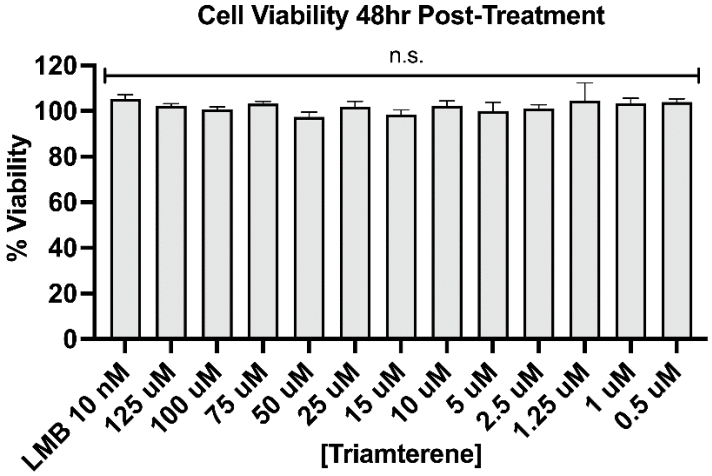
Supplementary Figures



Supplemental Figure 5.S1. Clopidogrel and Triamterene do not reduce A549 viability. A CellTiter Blue assay was used to evaluate changes in A549 cell viability following 48h treatment with Clopidogrel or Triamterene. Results are shown as the mean percent of the DMSO-treated control ± standard error. Toxicity is defined as ≥20% loss of viability compared to mock control. Asterisks indicate significant differences from the DMSO treated control by one-way analysis of variance with Dunnett's multiple-comparison test ($P < 0.05$).



Supplemental Figure 5.S2. Clopidogrel pretreatment does not reduce Calu-3 viability. A CellTiter Blue assay was used to evaluate Calu-3 cell viability. Following 48h treatment with Clopidogrel where the drug and media were replaced at 24h the mean percent of DMSO-treated control ± standard error was determined. Toxicity was defined as ≥20% loss of viability compared to the mock control. Asterisks indicate significant differences from the DMSO treated control by one-way analysis of variance with Dunnett's multiple-comparison test ($P < 0.05$).



Supplemental Figure 5.S3. Triamterene does not reduce CALU-3 viability. CellTiter Blue non-destructive assay was used to evaluate changes in cell viability of CALU-3 cells following 48h treatment with Triamterene following a replenishment protocol where drug and media were replaced at 24h. Data is presented as mean of percentage of DMSO treated control ± standard error (Mean ± SEM). Toxicity is defined as >20% loss of viability compared to mock control. Asterisks indicate significant differences from the DMSO treated control by ordinary one-way analysis of variance with Dunnett's multiple-comparison test ($P < 0.05$).

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CHAPTER 6

CONCLUSIONS

Influenza A and B viruses continue to be a major global health concern. Seasonal influenza epidemics results in numerous hospitalizations and 290,000 - 650,000 deaths per year. Vaccines are currently the most effective preventive measure available against influenza infection, but IAV vaccines require annual reformulation as mismatch of vaccine strains can result in vaccine failure. Safe and effective drugs are needed to combat infection when vaccine efforts fail. Currently available drugs target viral proteins, allowing for reduced efficacy due to subtype bias and reduced drug sensitivity and drug resistance acquired through antigenic shift and drift. Herein we address the *hypothesis* that silencing pro-influenza ion channel or G-protein coupled receptor genes using RNAi will lead to reduced virus replication in vitro and that these data will further our understanding of host factors and processes required for influenza replication, identify miR which negatively regulate influenza replication and identify novel targets for drug repurposing. We used siRNA screening to identify pro-influenza GPCR and ion channel host genes required for multiple strains and types of influenza viruses. As effective strategies are needed to combat influenza infection, we utilized these data to examined miRNAs (miRs) targeting these pro-influenza GPCR and ion channel host genes for their ability inhibit influenza virus replication. These studies led to the discovery of several pan-antiviral miRs. We continued to utilize these data to repurpose drugs against influenza. Herein we identified FDA

approved drugs Clopidogrel and Triamterene which target host factors identified herein as pro-influenza host factors needed offering an antiviral strategy refractory drug resistance while providing broader spectrum drug efficacy. These studies provide the foundation for the development of novel broad-spectrum antiviral miR therapeutic strategies and drug repurposing of clopidogrel and triamterene to limit influenza replication. The *specific aims* addressed were:

Specific Aim 1: Determine GPCR and ion channel (IC) genes that when silenced inhibits influenza replication and validate these genes against A/WSN/33 A/WSN33, A/CA/04/09 and influenza B strain B/Yamagata/16/1988. The data presented in Chapter 3 identified 16 GPCR genes (ADGRF1, ADORA1, ADRB2, AGTR1, C5AR2, CCKBR, FFAR1, HCAR3, HCRTR2, HRH2, HTR1B, LGR4, LPAR3, OXGR1, OXTR, P2RY12) and 5 ion channels genes ASIC1, GABRA3, GRID2, MCOLN2 and SCNN1D) as pro-influenza host genes by deconvolution of siRNA pools and subsequent validation against influenza A strains A/WSN/33 and A/CA/04/09 as well as influenza B strain B/Yamagata/16/1988. siRNA silencing of these genes reduced viral titer in all strains and thus are required for efficient replication suggesting that influenza viruses coopt GPCR and IC genes as part of the replication cycle.

Specific Aim 2. Identify microRNAs which regulate \pro-influenza host genes and determine their ability to modify influenza A A/WSN33, A/CA/04/09 and influenza B strain B/Yamagata/16/1988 replication. The data in chapter 4 identifies multiple strain specific antiviral miRs and highlights the strain variation associated with miR regulation.

These data also identified four pan-antiviral miRs (let-7b-5p, miR-5011-5p, miR-603 and miR-5692a) which reduced influenza viral titer and demonstrates that these miRs, at least in part, are regulating the expression of the pro-influenza GPCR genes identified in aim 1.

Specific Aim 3. Identify FDA-approved drugs which can be repurposed to inhibit key GPCRs and ion channels genes used by A/WSN33, A/CA/04/09 and influenza B strain B/Yamagata/16/1988 to replicate. The data presented in chapter 5 identifies the FDA approved drugs clopidogrel and triamterene as candidates for repurposing as antiviral agents against influenza virus. The data shows clopidogrel and triamterene pretreatment reduces influenza replication and spread for H1N1 and type B Yamagata. Specifically, clopidogrel at higher concentrations led to viral NP (likely vRNPs) sequestering within the nucleus – an effect limiting viral replication and spread. This phenotype is likely a result of clopidogrel targeting P2RY12, a GPCR identified in aim 1, and its association with signaling pathways (e.g. Raf/MEK/Erk) required for influenza replication. Whereas, triamterene's affects are likely a result of perturbations in the ion gradient and subsequent secondary effects on signal cascades required for replication.

Collectively, the identification of these pro-influenza GPCR and IC genes further our basic understanding of the virus-host interface and provides the basis to develop host-directed virus control strategies to limit influenza replication and disease. The identification of pan-antiviral miR and FDA approved drugs targeting these genes strengthens these findings. These data showing reduction of replication across strain and type differences solidifies the importance of investigating host directed strategies moving

forward. In conclusion, this data can be used to develop disease intervention strategies which employ therapeutic modulation of host genes to control influenza replication and disease as well as repurpose drugs which may be useful as an adjunct treatment for antiviral drug-resistant influenza strains.