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MicroRNAs affect GPCR and Ion channel genes needed for influenza replication

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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 for developing vaccine and therapeutic strategies. Identifying microRNAs (miRs) that affect host genes used by influenza virus for replication can support an antiviral strategy. In this study, G-protein coupled receptor (GPCR) and ion channel (IC) host genes in human alveolar epithelial (A549) cells used by influenza virus for replication (Orr-Burks *et al.*, 2021) were examined as miR target genes following A/CA/04/09- or B/Yamagata/16/1988 replication. Thirty-three miRs were predicted to target GPCR or IC genes and their miR mimics were evaluated for their ability to decrease influenza virus replication. Paired miR inhibitors were used as an ancillary measure to confirm or not the antiviral effects of a miR mimic. Fifteen miRs lowered influenza virus replication and four miRs were found to reduce replication irrespective of virus strain and type differences. These findings provide evidence for novel miR disease intervention strategies for influenza viruses.

INTRODUCTION

Influenza A viruses (IAV) and influenza B viruses (IBV) belong to the *Orthomyxoviridae* family and are composed of eight negative-sense, single-stranded viral RNA gene segments. IAV and IBV express ten primary viral proteins (PB2, PB1, PA, HA, NP, NA, M1, M2, NS1, NS2) and have different strain-dependent accessory proteins caused by frameshift and alternative splicing events [1–6]. IAV and IBV strains are responsible for seasonal epidemics and occasional pandemics resulting from genome reassortment [7, 8]. Vaccine failures most commonly occur as a result of antigenic drift in the HA surface protein, genome reassortment and strain mismatch [7, 8]. Influenza epidemics cause numerous hospitalizations and substantial deaths each year. The H1N1 2009 pandemic strain resulted in >60 million cases, >274000 hospitalizations, and >12400 deaths in the United States [9]. Seasonal viral burdens and subtypes vary each year. Vaccination is the most effective control measure, but influenza vaccines require annual reformulation, and the vaccine efficacy is decreased by strain mismatch [10].

Anti-influenza drugs may reduce infection, disease, or severity. There are several FDA-approved drugs for use against influenza. Specifically, peramivir, zanamivir, and oseltamivir are neuraminidase (NA) inhibitors [11]. Unfortunately, resistance among NA inhibitors has been observed. For example, the 2008–2009 seasonal H1N1 subtypes have 90% oseltamivir resistance due to point mutations within the NA [12–14]. Baloxavir marboxil targets and inhibits the cap-dependent endonuclease activity of the IAV and IBV polymerase inhibiting viral RNA synthesis [15, 16]. Unfortunately, the administration of baloxavir marboxil is at least three times more expensive compared to oseltamivir [16], and resistance to it is not well understood. Amantadine and rimantadine, both M2 ion channel inhibitors, are no longer recommended due to increased resistance and limited efficacy [17].

Influenza virus co-opts host genes for replication. Some of the pathways exploited include nuclear factor kappa B (NFκB), phosphatidylinositol-3-kinase (PI3K), mitogen-activated protein kinase (MAPK), protein kinase C (PKC), toll-like

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Abbreviations: GPCR, G protein-coupled receptor; HA, hemagglutinin; IAV, influenza A virus; IBV, influenza B virus; IC, ion channel; IPA, ingenuity pathway analysis; M1, matrix protein; M2, proton channel; MAPK, mitogen-activated protein kinase; miR, microRNA; mRNA, messanger RNA; NA, neuraminidase; NFkB, nuclear factor kappa B; NP, nucleoprotein; NS1, non-structural protein 1; NS2, nuclear export protein; NTC, non-targeting control; PA, polymerase acidic protein; PB1, RNA-directed RNA polymerase catalytic subunit; PB2, polymerase basic protein 2; PI3K, phosphoinositide 3-kinases; PKC, protein kinase C; qRT-PCR, quantitative real-time polymerase chain reaction; RNAi, RNA interference; TCID50, median tissue culture infectious dose; TLR, toll-like receptor; TPCK, L-(tosylamido-2-phenyl) ethyl chloromethyl ketone.
Four supplementary tables and three supplementary figures are available with the online version of this article.

receptor (TLR), and retinoic acid-inducible gene 1 (RIG-I) pathways [18–21]. Antiviral targeting of host factors needed for viral replication offers a recalcitrant approach to limit the development of drug resistance while providing broad-spectrum efficacy against 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 used to understand the virus-host interface and identify host genes used in influenza virus replication [22–27]. Understanding the host genes used by viruses for replication is advantageous for determining the miRs that regulate these genes and potentially affect virus replication. miRs are small (19–25 nt) noncoding RNAs fundamental in post-transcriptional gene regulation [28, 29]. The human genome encodes an estimated 2300 miRs, 1115 of which are annotated in the miRbase database as the number of validated human miRs continues to increase [30, 31]. miRs regulate host gene function by binding with host gene mRNA in a sequence-dependent manner via a short (~8 nt) seed region at the 5′ end of the mature miR, silencing its activity. Absolute complementarity of a miR with their target mRNA is not required to modify activity, thus miRs are promiscuous, i.e. having the ability to bind many targets with similar seed regions [31, 32]. miRs are predicted to regulate more than 50% of protein-coding genes [33]. Viral infection results in the temporal miR expression [34, 35]. For example, influenza virus infection results in strain-specific miR expression profiles [36, 37]. Evidence suggests miRs have an important role tempering the immune and inflammatory responses to infection [38–41], and may act as antiviral agents. For example, miR-134 inhibits poliovirus by modifying the host nuclear transport system by targeting the ras-related nuclear protein [42].

In this study, the findings from a previous RNAi screen that identified GPCR and IC host genes needed for influenza virus replication [43] were used to computationally shortlist miRs which target these host genes used for influenza virus replication A549 cells. We evaluated these miRs for their ability to decrease influenza replication using miR mimics and discovered several pan-antiviral miRs. These data show miR regulation of GPCR and IC host genes and are the basis for the development of novel antiviral miR therapeutic strategies to regulate influenza replication [42, 44, 45].

METHODS

Cells and viruses

Human alveolar 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 foetal bovine serum (HI-FBS) (Atlas Biologics Inc., Fort Collins, CO). All experiments were performed with log-phase A549 cells 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 [46]. B/Yamagata/16/1988 (BEI Resources) was grown in 9 day old embryonated chicken eggs as previously described to achieve acceptable titre for *in vitro* infection [47]. Viral titres (p.f.u. ml^{-1}) of stock viruses were determined by MDCK plaque assay and calculated using the Reed and Muench method [48–50].

Computational approaches for the identification of miR targets

GPCR and IC genes previously shown to be pro-influenza host genes [43] were examined using three miR target prediction programmes, i.e. IPA, TargetScan, and miRbase [51, 52]. 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 - 8mer complementary sequences and miR untranslated regions using miRanda and Ensembl [31]. 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 S1 (available in the online version of this article).

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 [24, 42]. Ninety-six-well plates were incubated with miRs in triplicate at 37 °C, 5% CO_2 for 48 h 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 1.5×10^4 cells were added to each well. Plates were incubated for 48 h at 37 °C, 5% CO_2. Following transfection, the media was discarded, the cells were washed twice with PBS, and infected for 48 h 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 1 ug ml^{-1} 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′-PAGU UGCUUCAAAUCUGCUCUU-3′, 5′-PAGAUGAAUUAG CUUUCUGGUU-3′) targets MAP2K previously shown to be required for influenza virus replication [53, 54]. 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 mRNA silencing was determined by qRT-PCR [24, 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 µl 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). Then 2 µg 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 µl) 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 S2) 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 [55].

Cell viability

Assay miR transfections were examined to determine if transfection mediated >20% loss in cell viability using Cell Titre 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 [24]. Following 48 h incubation, the transfected cell viability was determined according to the manufacturer's protocol. Briefly, 100 µl of media from each well was decanted and 20 µl of CTB reagent added to each well. Plates were mixed gently for 10 s and then incubated at 37 °C. 5% CO_2 for 2 h. Following incubation, the plates were gently rocked for 10 s 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 S3).

Plaque assay

Viral titres were determined by MDCK plaque assay [49, 56, 57]. Briefly, sample supernatants were diluted in MEM with 1 ug ml^{-1} TPCK-treated trypsin and serially diluted ten-fold and transferred to MDCK cell monolayers (90% confluent) in 12-well tissue culture plate format (Corning-Costar, MA). Following 1 h virus adsorption at 37 °C, 5% CO_2, 3 ml of overlay containing 1-part medium consisting of 10x MEM supplemented with 200 mM 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 twice 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 titres determined [56, 57].

$TCID_{50}$ assay

A $TCID_{50}$ assay was used to determine endpoint titres [43, 48, 58]. Briefly, sample supernatants were collected from influenza virus-infected A549 cells that were serially diluted ten-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 [48, 49]. 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 100 µl and a final concentration tRBC concentration of 0.5% in a round-bottom plate [58]. The $TCID_{50}$ titres were calculated using the Reed and Muench method [48].

Statistics

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

RESULTS

miRs affect GPCR and IC genes used for influenza virus replication

Nineteen GPCR and 13 IC genes were identified as needed for A/WSN/33 replication in A549 cells in a recent siRNA screen [43]. Using these previous results and computational approaches, we identified miR regulators of influenza virus replication that targeted the previously identified GPCR and IC host genes expression and confirmed that miRs were functional using miR mimic to knockdown the GPCR and IC target genes (Figs 1–4, Table S4). Transfection of miR mimics increases the cellular levels of the miR and mimics the endogenous function of naturally occurring miRs allowing for the evaluation of the miR on viral replication [59, 60]. Paired anti-sense miR inhibitors were also included in this study. miR inhibitor transfection reduces target cellular miR levels. A concentration of 25 nM was utilized in all experiments, as it was not within the scope of this study to determine the level of endogenous miR expression. miR inhibitor results were used as an ancillary measure to confirm or not the antiviral effects of a miR mimic [59–61]. miRs were considered functional if mimic transfection resulted in a fold-change reduction in virus titre (p.f.u. ml^{-1}), and transfection of the miR inhibitor had no change or an increase in fold-change in virus titre (p.f.u. ml^{-1}). A549 cells were transfected with 25 nM miR mimics or miR inhibitors, miR mimic non-targeting control, miR inhibitor non-targeting control, or siRNA targeting MAP2K (siMAP2K) in serum-free media for 48 h [24]. Mitogen-activated protein kinase one gene MAP2K

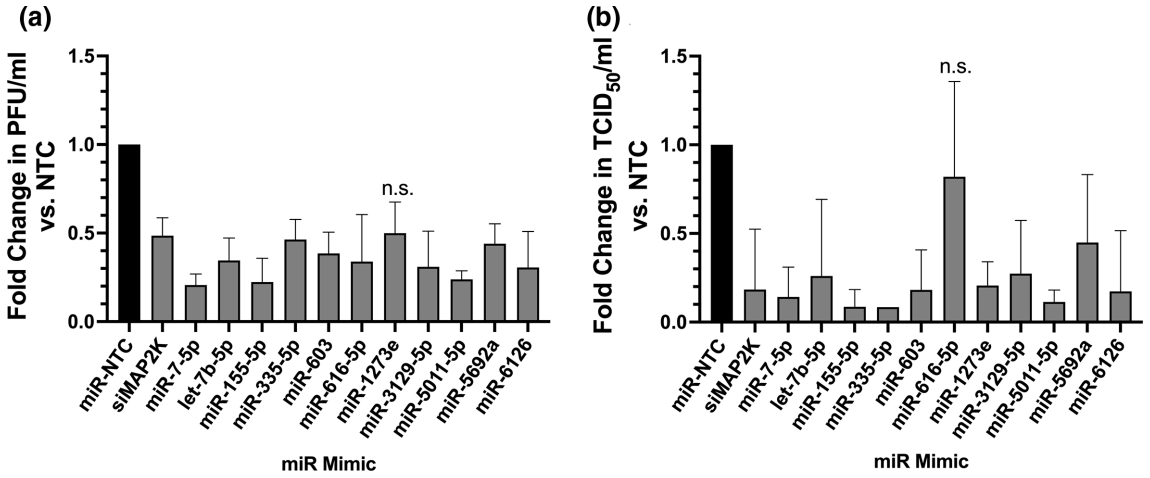

Fig. 1. miRs affecting A/WSN/33 replication in A549 cells. A549 cells were transfected (25 nM) with either miR mimic, its paired miR inhibitor, miR-NTC control, siMAP2K, or siTOX in triplicate and incubated for 48 h. Post-transfection, A549 cells were infected with A/WSN/33 (MOI=0.01), supernatants were collected 48 h pi, and MDCK plaque assays were performed to determine p.f.u. ml^{-1}. $TCID_{50}$ ml^{-1} titres were determined by sample titration on MDCK cells followed by HA assay. Plaque assay (a) and TCID, and $TCID_{50}$ assay (b) data is presented as fold-change in influenza virus, data is presented as fold-change in influenza virus titre (p.f.u. ml^{-1}) or $TCID_{50}$ ml^{-1} titre compared to miR-NTC and shown as mean fold-change ±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 p.f.u. ml^{-1} or $TCID_{50}$ ml^{-1} titre compared to control. A fold-change <1 equates to a decrease in p.f.u. ml^{-1} or $TCID_{50}$ ml^{-1} titre compared to control. A fold-change=1 equates to no change in p.f.u. ml^{-1} or $TCID_{50}$ ml^{-1} titre compared to control.

(Mitogen-activated protein kinase one gene) is required for influenza virus replication [53] and small interfering RNA MAP2K (siMAP2K) was used as a positive control for the reduction of influenza replication. All transfected A549 cells were examined for cell viability [62, 63] and no significant loss of viability was observed (Table S3). Following 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 titre determined by both plaque assay and $TCID_{50}$ assay [24]. Data are presented as fold-change comparing

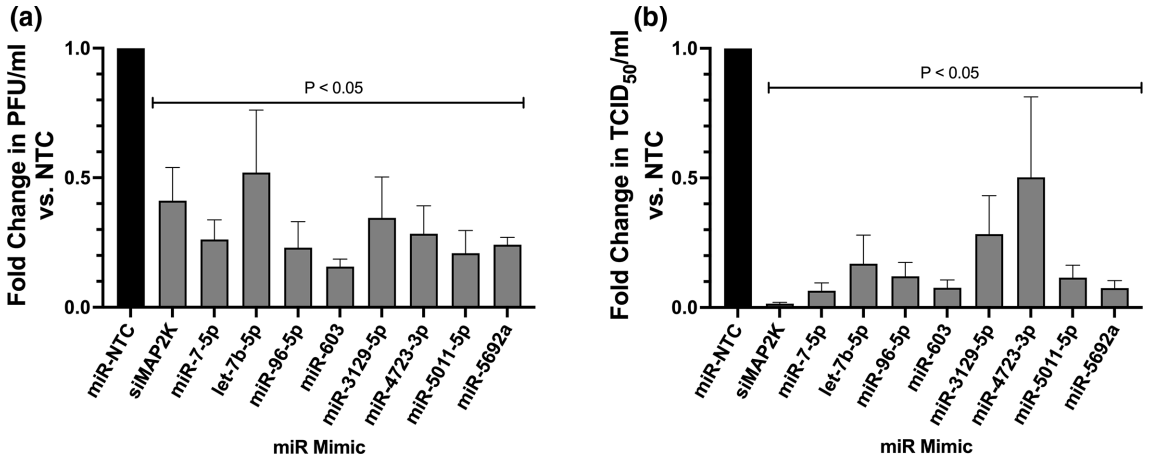

Fig. 2. miRs affecting A/CA/04/09 replication in A549 cells. A549 cells were transfected (25 nM) with either miR mimic, its paired miR inhibitor, miR-NTC control, siMAP2K, or siTOX in triplicate and incubated for 48 h. Post-transfection, A549 cells were infected with A/CA/04/09 (MOI=0.1), and supernatants were collected 48 h pi, and MDCK plaque assays were performed to determine p.f.u. ml^{-1}. $TCID_{50}$ ml^{-1} titres were determined by sample titration on MDCK cells followed by HA assay. Plaque assay (a) and TCID, and $TCID_{50}$ assay (b) data is presented as fold-change in influenza virus, data is presented as fold-change in influenza virus titre (p.f.u. ml^{-1}) or $TCID_{50}$ ml^{-1} titre compared to miR-NTC and shown as mean fold-change ±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 p.f.u. ml^{-1} or $TCID_{50}$ ml^{-1} titre compared to control. A fold-change <1 equates to a decrease in p.f.u. ml^{-1} or $TCID_{50}$ ml^{-1} titre compared to control. A fold-change=1 equates to no change in p.f.u. ml^{-1} or $TCID_{50}$ ml^{-1} titre compared to control.

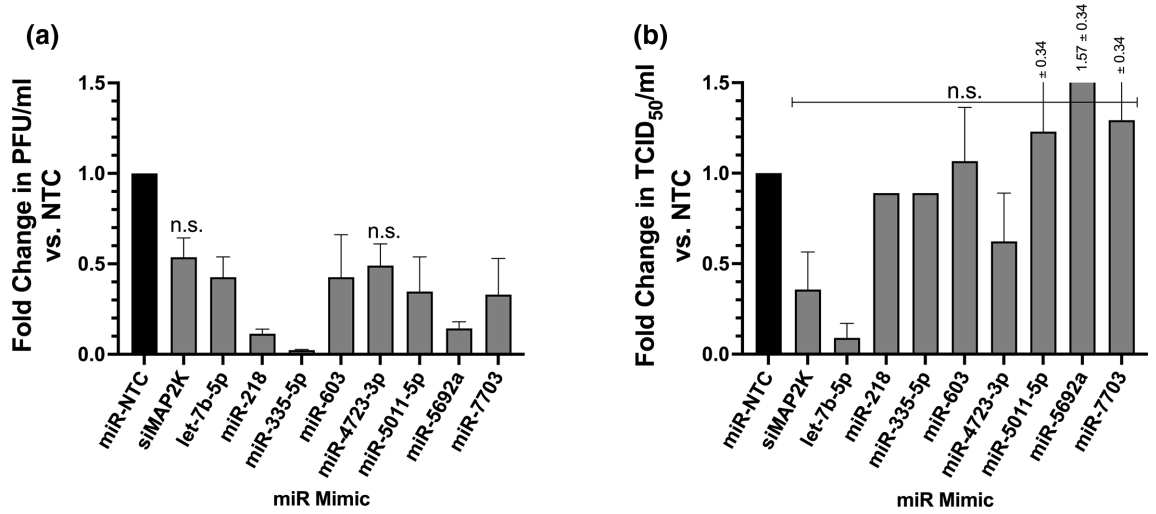

Fig. 3. miRs affecting B/Yamagata/16/1988 replication in A549 cells. A549 cells were transfected (25 nM) with either miR mimic, its paired miR inhibitor, miR-NTC control, siMAP2K, or siTOX in triplicate and incubated for 48 h. Post-transfection, A549 cells were infected with B/Yamagata/16/1988 (MOI=0.1), supernatants were collected 48 h pi, and MDCK plaque assays were performed to determine p.f.u. ml^{-1}. $TCID_{50}$ ml^{-1} titres were determined by sample titration on MDCK cells followed by HA assay. Plaque assay (a) and TCID, and $TCID_{50}$ assay (b) data is presented as fold-change in influenza virus, data is presented as fold-change in influenza virus titre (p.f.u. ml^{-1}) or $TCID_{50}$ ml^{-1} titre compared to miR-NTC and shown as mean fold-change ±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 p.f.u. ml^{-1} or $TCID_{50}$ ml^{-1} titre compared to control. A fold-change <1 equates to a decrease in p.f.u. ml^{-1} or $TCID_{50}$ ml^{-1} titre compared to control. A fold-change=1 equates to no change in p.f.u. ml^{-1} or $TCID_{50}$ ml^{-1} titre compared to control.

miR mimic or miR inhibitor to non-targeting control results. Fifteen miR mimics reduced influenza virus titres of which were four pan-anti-influenza miRs (Fig. 4).

Eleven miR mimics reduced A/WSN/33 plaque titres (p.f.u. ml^{-1}), specifically miR-7–5 p, let-7b-5p, miR-155–5 p, miR-335, miR-603, miR-616–5 p, miR-3129–5 p, miR-5011–5 p, miR-5692a and miR-6126 showed statistically significant (P<0.05) reduction in fold-change compared to non-targeting control (Table S1, Fig. 1a). Similar results were observed for the $TCID_{50}$ assay (Fig. 1b), and transfection of paired miR inhibitors resulted in either an increase or no change in fold-change of virus titre (Fig. S1a, b). Notably, miR-6126 inhibitor increased the $TCID_{50}$ ml^{-1} titre by 90-fold-change (Fig. S1b). The substantial increase in $TCID_{50}$ ml^{-1} fold-change compared to the plaque assay likely reflects the differences between the two assay endpoint readouts. Specifically, the plaque assay measures only the amount of infectious virus, whereas the $TCID_{50}$ assay detects both infectious and non-infectious virus that binds to red blood cells by HA. Nine miR mimics (miR-7–5 p, let-7b-5p, miR-155–5 p, miR-603, miR-616–5 p, miR-3129–5 p, miR-5011–5 p, miR-5692a and miR-6126) caused a statistically (P<0.05) significant fold-change reduction in A/WSN/33 plaque titres (p.f.u. ml^{-1}) beyond the siMAP2K control. These findings suggest these miRs are likely interacting with more than one mRNA (Fig. 1a, Table S4). Eight miR mimics (miR-7–5 p, let-7b-5p, miR-96–5 p, miR-603, miR-3129–5 p, miR-4723–3 p, miR-5011–5 p and miR-5692a) mediated a statistically significant fold change reduction in A/CA/04/09 plaque titre and $TCID_{50}$ titre (Fig. 2a, b, Table S1), with miR-603 mediating the greatest reduction in the fold-change of A/CA/04/09 titres (Fig. 2a, Table S1). These miR mimics, with exception of let-7b-5p, had a greater effect on reducing virus plaque titres compared to the siMAP2K control (Fig. 2a, Table S1). Transfection of paired miR inhibitors had either an increase or no fold-change in viral titre (Fig. S2a, b). miR mimics had a greater effect on reducing A/CA/04/09 replication compared to A/WSN/33 replication (Figs 1a and 2a, Table S1). Of note, miR-155–5 p, miR-335–5 p, miR-616–5 p, miR-1273e, and miR-6126 mimics reduced A/WSN/33 plaque formation but did not affect A/CA/04/09, whereas miR-96–5 p and miR-4723–3 p mimics reduced A/CA/04/09 plaque formation but not A/WSN/33 plaque formation (Figs 1a and 2a, Table S1) indicating strain differences. Eight miR mimics (let-7b-5p, miR-218, miR-335, miR-603, miR-4723–3 p, miR-5011–5 p, miR-5692a or miR-7703 miR) mediated a fold change reduction in B/Yamagata/16/1988 replication plaque titres (Fig. 3a, Table S1). Specifically, transfection of let-7b-5p, miR-218, miR-335, miR-603, miR-5011–5 p, miR-5692a or miR-7703 miR mimics resulted in a statistically significant reduction in fold change compared to non-targeting control (Fig. 3a, Table S1). miR-335 mimic transfection had the greatest reduction in B/Yamagata/16/1988 replication as determined by plaque numbers (Fig. 3a, Table S1). As predicted, transfection with miR inhibitors either increased or had no effect on plaque titres (Figs S3a, b). miR mimics let-7b-5p, miR-218, miR-335, miR-603, miR-4723–3 p, miR-5011–5 p, miR-5692a and miR-7703 reduced virus replication (p.f.u. ml^{-1}), however

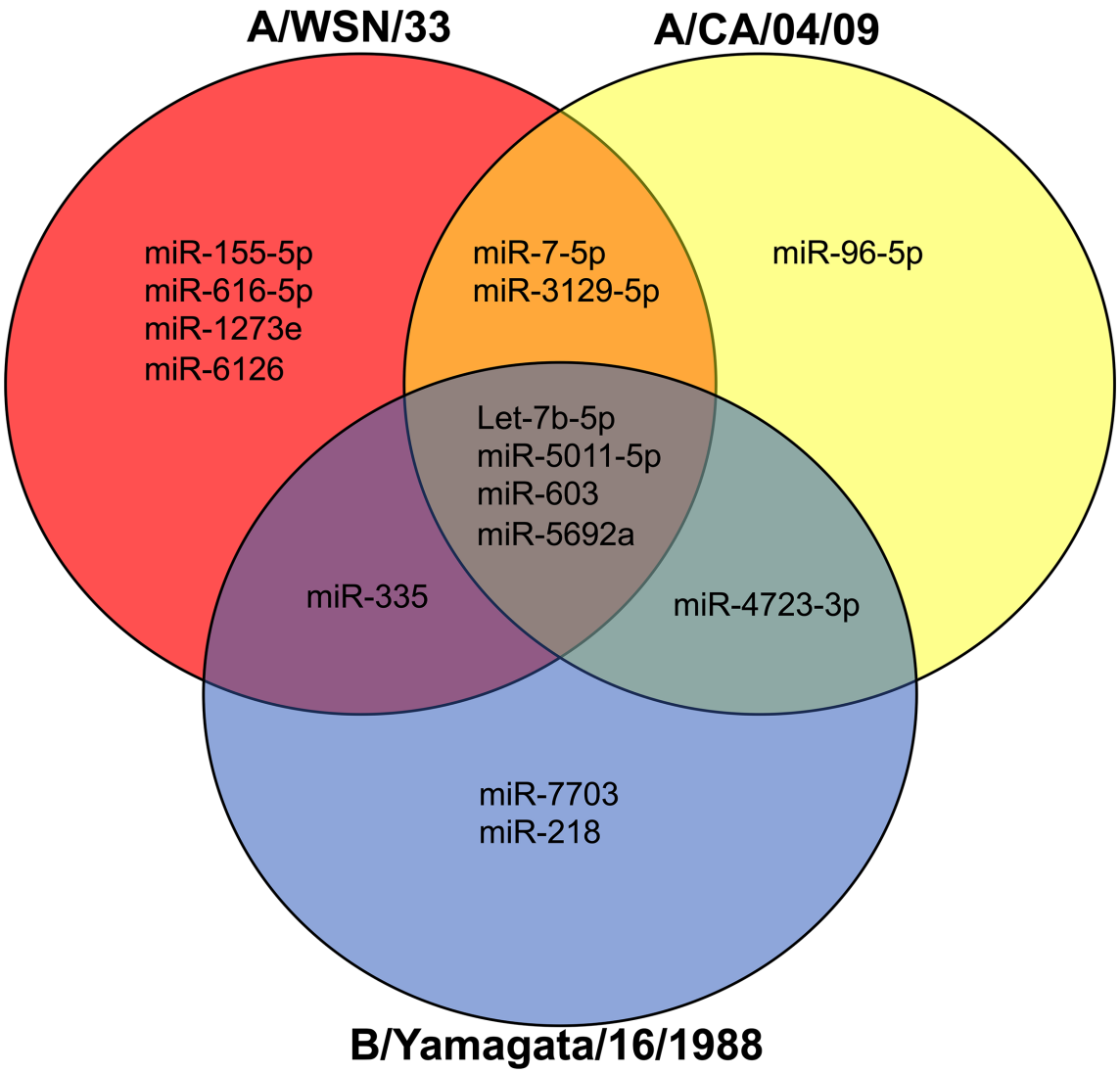

Fig. 4. Venn diagram of miR screening results. miR screening data clustered by the ability to reduce plaque titre with some clusters overlapping by strains and subtypes.

these miR mimics had nominal effects on $TCID_{50}$ titre (Fig. 3b). The variation in $TCID_{50}$ ml^{-1} results compared to plaque assay is likely due to the inherent differences in the two assays' endpoint readout methods (e.g. measurement of infectious virus vs HA). Of note, miR inhibitors (let-7b-5p, miR-603, miR-5022–5 p and miR-5692a) increased $TCID_{50}$ titres (Fig. S3b). Transfection of miR-335 mimic reduced both A/WSN/33 and B/Yamagata/16/1988 titres but did not affect A/CA/04/09 titres. Similarly, miR-4723–3 p mimic reduced both A/CA/04/09 and B/Yamagata/16/1988 titres but did not affect A/WSN/33 titres. Collectively, four pan-antiviral miR mimics (let-7b-5p, miR-603, miR-5011–5 p, miR-5692a) resulted in a fold-change reduction in virus titres of A/WSN/33, A/CA/04/09, and B/Yamagata/16/1988 titres (Fig. 4). These results show that miRs targeting specific GPCR or IC genes regulate influenza replication affecting the virus titres determined by plaque assays, or affect the production of defective particles linked to changes in $TCID_{50}$ [64].

miR validation

Previously, we determined 16 pro-influenza GPCR and five IC genes in A549 cells used by influenza A/WSN/33, A/CA/04/09 and B/Yamagata/16/1988 viruses for replication [43]. Here, we evaluate the predicted miRs targeting these host genes using highly potent synthetic miR mimics and inhibitors in gain- or loss-of-function studies. Synthetic miRNA targeting of host genes was validated by the manufacturer (Dharmacon) and others [65] confirming functional activity qPCR. To corroborate miR regulation, we evaluated the 16 GPCR genes we previously discovered (i.e. ADGRF1, ADORA1, ADRB2, AGTR1, C5AR2, CCKBR, FFAR1, HCAR3, HCRTR2, HRH2, HTR1B, LGR4, LPAR3, OXGR1, OXTR and P2RY12) and five

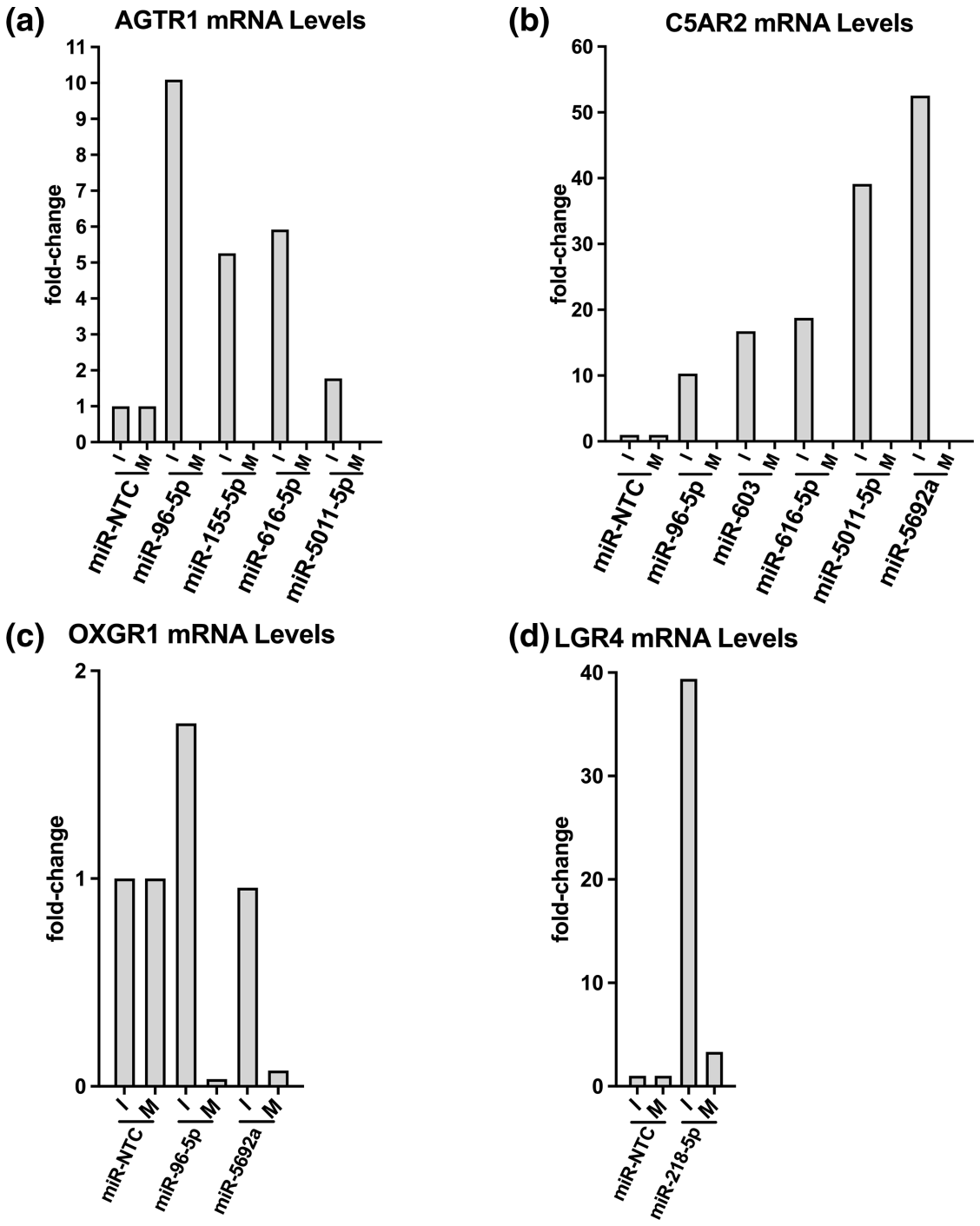

Fig. 5. qPCR of target gene mRNA following miR inhibitor or mimic (I/M) transfection. A549 cells were transfected (25 nM) with either miR mimic or its paired miR inhibitor, miR non-targeting inhibitor control (miR-NTC (I), miR non-targeting mimic control miR-NTC (), miR non-targeting mimic control miR-NTC (M), or siTOX transfection control for), or siTOX transfection control for 48 h. Cells were homogenized, and RNA isolated. Samples (*n*=3) were pooled and qPCR was performed to measure mRNA of predicted target genes AGTR1 (a), C5AR2 (), C5AR2 (b), OXGR1 (), OXGR1 (c), and LGR4 (), and LGR4 (d). Data were normalized to 18S rRNA and presented as fold-change of target mRNA in miR vs miR-NTC (I/M).

IC (ASIC1, GABRA3, GRID2, MCOLN2, and SCNN1D) to show that the predicted miRs regulated expression of these genes (Fig. 5). miR-5011–5 p, miR-603, and miR-5692a are pan anti-influenza virus miRs that decreased A/WSN/33, A/CA/04/09, and B/Yamagata/16/1988 replication. Transfection of miR-5011–5 p and miR-603 mimics reduced GPCR genes AGTR1 and C5AR2 mRNA expression while miR-5011–5 p and miR-603 inhibitors increased expression confirming their function (Fig. 5a, b). Similarly, transfection of miR-5692a mimic reduced expression of GPCR genes C5AR2 and OXGR1, while transfection of miR-5692a inhibitor increased expression as expected (Fig. 5b, c). MiR-155–5 p mimic reduced GPCR gene AGTR1 mRNA expression while miR-616–5 p reduced GPCR genes AGTR1 and C5AR2 mRNA expression compared to the control (Fig. 5a, b). As expected, transfection of their inhibitors resulted in increased mRNA expression (Fig. 5a, b). Transfection of the miR-96–5 p mimic reduced GPCR genes AGTR1, C5AR2, and OXGR1 mRNA levels (Fig. 5a–c) while the miR-96–5 p inhibitor increased the levels of all three genes (Fig. 5a–c). Transfection of miR-218–5 p mimic led to a small increase in GPCR gene LGR4, but inhibitor transfection led to a substantial increase in GPCR gene LGR4 compared to the control and mimic levels (Fig. 5d). These results show that miRs targeting select GPCR genes can modify influenza virus replication.

DISCUSSION

It is fundamental to understand how miRs affect the cadence of host gene expression, as well as important to determine miR regulation of influenza virus strains and types [66–68]. The identification of antiviral miRs may provide an avenue for the development of novel therapeutic strategies [42, 44, 45]. In this study, predicted miRs were examined and synthetic miR mimics and inhibitors were functionally assessed for their ability to reduce IAV and IBV replication (Fig. 4). Four miR mimics (let-7b-5p, miR-5011–5 p, miR-603, miR-5692a) were identified as pan-antiviral and shown to reduce in A/WSN/33, CA/04/09, and B/Yamagata/16/1988 replication in A549 cells, while other miRs were strain and type-specific or had shared effects on influenza strain and types. For example, four miRs (miR-155–5 p, miR-616–5 p, miR-1273e, miR-6126) were A/WSN/33-specific, while miR-96–5 p inhibited A/CA/04/09, and miR-218 and miR-7703 inhibited B/Yamagata/16/1988 replication in A549 cells (Fig. 4).

The miRs characterized in this study regulated pro-influenza host genes. For example, miR-218 mimic regulates the LGR4 (leucine-rich repeat-containing G protein-coupled receptor-4) gene which is an orphan GPCR receptor with no identified endogenous ligand [69]. The pan-anti-influenza miRs (miR-603, miR-5011–5 p, miR-5692a), the A/WSN/33-specific miR-616–5 p, and the A/CA/04/09-specific miR-96–5 p regulate the C5AR2 (complement component 5 a receptor 2) gene which is a non-classical GPCR [70, 71]. The AGTR1 gene is regulated by miR-5011–5 p, miR-155–5 p, miR-616–5 p, and miR-96–5 p. AGTR1 is a GPCR gene coupled to Gαq signalling [72]. The pan-anti-influenza miR-5692a and A/CA/04/09-specific miR-96–5 p regulates OXGR1 gene expression that is associated with Gαq signalling [73]. Of note, miR-7–5 p and miR-155–5 p mimics mediated the greatest reduction in A/WSN/33 titres (Fig. 1a) and miR-603 mimic had the greatest reduction in A/CA/04/09 titre (Fig. 2a), and miR-335–5 p mimic had the greatest reduction in A/CA/04/09 titre (Fig. 3a).

This study identified miRs affecting key pro-influenza virus GPCR and IC genes used for A/WSN/33, CA/04/09, and B/Yamagata/16/1988 replication in A549 cells. Of the 33 miRs evaluated, four pan-anti-influenza miRs were identified that reduced influenza virus titres of the three influenza viruses examined (Fig. 4).

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Conflicts of interest
The authors declare that there are no conflicts of interest.

References

1. Jagger BW, Wise HM, Kash JC, Walters K-. A, Wills NM. An overlapping protein-coding region in influenza A virus segment 3 modulates the host response. *Science* 2012;337:199–204.
2. Shi M, Jagger BW, Wise HM, Digard P, Holmes EC, *et al*. Evolutionary conservation of the PA-X open reading frame in segment 3 of influenza A virus. *J Virol* 2012;86:12411–12413.
3. Wise HM, Barbezange C, Jagger BW, Dalton RM, Gog JR, *et al*. Overlapping signals for translational regulation and packaging of influenza A virus segment 2. *Nucleic Acids Res* 2011;39:7775–7790.
4. Wise HM, Foeglein A, Sun J, Dalton RM, Patel S, *et al*. A complicated message: Identification of a novel PB1-related protein translated from influenza A virus segment 2 mRNA. *J Virol* 2009;83:8021–8031.
5. Yamayoshi S, Watanabe M, Goto H, Kawaoka Y. Identification of a novel viral protein expressed from the PB2 segment of influenza A virus. *J Virol* 2016;90:444–456.
6. Yang C-W, Chen M-F, Stambas J. Uncovering the potential pan proteomes encoded by genomic strand RNAs of influenza A viruses. *PLoS One* 2016;11:e0146936.
7. Sandbulte MR, Westgeest KB, Gao J, Xu X, Klimov AI, *et al*. Discordant antigenic drift of neuraminidase and hemagglutinin in H1N1 and H3N2 influenza viruses. *Proc Natl Acad Sci U S A* 2011;108:20748–20753.
8. Carrat F, Flahault A. fluenza vaccine: the challenge of antigenic drift. *Vaccine* 2007;25:6852–6862.
9. Shrestha SS, Swerdlow DL, Borse RH, Prabhu VS, Finelli L. Estimating the burden of 2009 pandemic influenza A (H1N1) in the United States (April 2009-April 2010). *Clin Infect Dis* 2010;52:S75–S82.
10. Zimmerman RK, Nowalk MP, Chung J, Jackson ML, Jackson LA, *et al*. 2014-2015 influenza vaccine effectiveness in the United States by vaccine type. *Clin Infect Dis* 2016;63:1564–1573.
11. McKimm-Breschkin JL. fluenza neuraminidase inhibitors: antiviral action and mechanisms of resistance. *Influenza Other Respir Viruses* 2013;7 Suppl 1:25–36.
12. Hussain M, Galvin HD, Haw TY, Nutsford AN, Husain M. Drug resistance in influenza A virus: the epidemiology and management. *Infect Drug Resist* 2017;10:121–134.
13. Samson M, Pizzorno A, Abed Y, Boivin G. fluenza virus resistance to neuraminidase inhibitors. *Antiviral Res* 2013;98::174–185.

14. Sheu TG, Deyde VM, Okomo-Adhiambo M, Garten RJ, Xu X, *et al*. Surveillance for neuraminidase inhibitor resistance among human influenza A and B viruses circulating worldwide from 2004 to 2008. *Antimicrob Agents Chemother* 2008;52:3284–3292.
15. Omoto S, Speranzini V, Hashimoto T, Noshi T, Yamaguchi H, *et al*. Characterization of influenza virus variants induced by treatment with the endonuclease inhibitor baloxavir marboxil. *Sci Rep* 2018;8:9633.
16. Yang T. Baloxavir marboxil: the first cap-dependent endonuclease inhibitor for the treatment of influenza. *Ann Pharmacother* 2019;53:754–759.
17. Deyde VM, Xu X, Bright RA, Shaw M, Smith CB, *et al*. Surveillance of resistance to adamantanes among influenza A(H3N2) and A(H1N1) viruses isolated worldwide. *J Infect Dis* 2007;196:249–257.
18. Shapira SD, Gat-Viks I, Shum BOV, Dricot A, de Grace MM, *et al*. A physical and regulatory map of host-influenza interactions reveals pathways in H1N1 infection. *Cell* 2009;139:1255–1267.
19. Fujioka Y, Tsuda M, Hattori T, Sasaki J, Sasaki T, *et al*. The Ras-PI3K signaling pathway is involved in clathrin-independent endocytosis and the internalization of influenza viruses. *PLoS One* 2011;6:e16324.
20. Ehrhardt C, Marjuki H, Wolff T, Nürnberg B, Planz O, *et al*. Bivalent role of the phosphatidylinositol-3-kinase (PI3K) during influenza virus infection and host cell defence. *Cell Microbiol* 2006;8:1336–1348.
21. Planz O. Development of cellular signaling pathway inhibitors as new antivirals against influenza. *Antiviral Res* 2013;98::457–468.
22. Bakre A, Andersen LE, Meliopoulos V, Coleman K, Yan X, *et al*. Identification of host kinase genes required for influenza virus replication and the regulatory role of MicroRNAs. *PLoS One* 2013;8:e66796.
23. Meliopoulos VA, Andersen LE, Birrer KF, Simpson KJ, Lowenthal JW, *et al*. Host gene targets for novel influenza therapies elucidated by high-throughput RNA interference screens. *FASEB J* 2012;26:1372–1386.
24. Meliopoulos VA, Andersen LE, Brooks P, Yan X, Bakre A, *et al*. MicroRNA regulation of human protease genes essential for influenza virus replication. *PLoS One* 2012;7:e37169.
25. Perwitasari O, Johnson S, Yan X, Howerth E, Shacham S, *et al*. Verdinexor, a novel selective inhibitor of nuclear export, reduces influenza a virus replication *in vitro* and *in vivo*. *J Virol* 2014;88:10228–10243.
26. Zhang W, Tripp RA. RNA interference inhibits respiratory syncytial virus replication and disease pathogenesis without inhibiting priming of the memory immune response. *J Virol* 2008;82:12221–12231.
27. Karlas A, Machuy N, Shin Y, Pleissner K-P, Artarini A. Genome-wide RNAi screen identifies human host factors crucial for influenza virus replication. *Nature* 2010;463:818–822.
28. Filipowicz W, Bhattacharyya SN, Sonenberg N. Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight? *Nat Rev Genet* 2008;9:102–114.
29. Lee Y, Kim M, Han J, Yeom K-H, Lee S, *et al*. MicroRNA genes are transcribed by RNA polymerase II. *EMBO J* 2004;23:4051–4060.
30. Alles J, Fehlmann T, Fischer U, Backes C, Galata V. An estimate of the total number of true human miRNAs. *Nucleic Acids Res* 2019;47:3353–3364.
31. Lewis BP, Burge CB, Bartel DP. Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. *Cell* 2005;120:15–20.
32. Kehl T, Backes C, Kern F, Fehlmann T, Ludwig N, *et al*. About miRNAs, miRNA seeds, target genes and target pathways. *Oncotarget* 2017;8:107167–107175.
33. Bartel DP. MicroRNAs: target recognition and regulatory functions. *Cell* 2009;136:215–233.
34. Loveday E-K, Svinti V, Diederich S, Pasick J, Jean F. Temporal- and strain-specific host microRNA molecular signatures associated with swine-origin H1N1 and avian-origin H7N7 influenza A virus infection. *J Virol* 2012;86:6109–6122.
35. Moheimani F, Koops J, Williams T, Reid AT, Hansbro PM, *et al*. fluenza A virus infection dysregulates the expression of microRNA-22 and its targets; CD147 and HDAC4, in epithelium of asthmatics. *Respir Res* 2018;19:145.
36. Makkoch J, Poomipak W, Saengchoowong S, Khongnomnan K, Praianantathavorn K, *et al*. Human microRNAs profiling in response to influenza A viruses (subtypes pH1N1, H3N2, and H5N1). *Exp Biol Med (Maywood)* 2015;241:409–420.
37. Bao Y, Gao Y, Jin Y, Cong W, Pan X, *et al*. MicroRNA expression profiles and networks in mouse lung infected with H1N1 influenza virus. *Mol Genet Genomics* 2015;290:1885–1897.
38. Skalsky RL, Cullen BR. Viruses, microRNAs, and host interactions. *Annu Rev Microbiol* 2010;64:123–141.
39. Umbach JL, Cullen BR. The role of RNAi and microRNAs in animal virus replication and antiviral immunity. *Genes Dev* 2009;23:1151–1164.
40. Nejad C, Stunden HJ, Gantier MP. A guide to miRNAs in inflammation and innate immune responses. *FEBS J* 2018;285:3695–3716.
41. Cullen BR. MicroRNAs as mediators of viral evasion of the immune system. *Nat Immunol* 2013;14:205–210.
42. Orr-Burks NL, Shim B-S, Wu W, Bakre AA, Karpilow J, *et al*. MicroRNA screening identifies miR-134 as a regulator of poliovirus and enterovirus 71 infection. *Sci Data* 2017;4:170023.
43. Orr-Burks N, Murray J, Todd KV, Bakre A, Tripp RA. G-Protein-coupled receptor and ion channel genes used by influenza virus for replication. *J Virol* 2021;95:e02410-20.
44. Christopher AF, Kaur RP, Kaur G, Kaur A, Gupta V, *et al*. MicroRNA therapeutics: Discovering novel targets and developing specific therapy. *Perspect Clin Res* 2016;7:68–74.
45. Rupaimoole R, Slack FJ. MicroRNA therapeutics: towards a new era for the management of cancer and other diseases. *Nat Rev Drug Discov* 2017;16:203–222.
46. Sun X, Tse LV, Ferguson AD, Whittaker GR. Modifications to the hemagglutinin cleavage site control the virulence of a neurotropic H1N1 influenza virus. *J Virol* 2010;84:8683–8690.
47. Woolcock PR. Avian influenza virus isolation and propagation in chicken eggs. Spackman E (eds). In: *Methods in Molecular Biology*, Vol. 436. Humana Press; 2008. pp. 35–46.
48. Reed LJ, Muench H. A simple method of estimating fifty per cent endpoints12. *Am J Hyg* 1938;27:493–497.
49. Klimov A, Balish A, Veguilla V, Sun H, Schiffer J, *et al*. Influenza virus titration, antigenic characterization, and serological methods for antibody detection. *Methods Mol Biol* 2012;865:25–51.
50. Appleyard G, Maber HB. Plaque formation by influenza viruses in the presence of trypsin. *J Gen Virol* 1974;25:351–357.
51. Griffiths-Jones S, Grocock RJ, van Dongen S, Bateman A, Enright AJ. miRBase: microRNA sequences, targets and gene nomenclature. *Nucleic Acids Res* 2006;34:D140-4.
52. Griffiths-Jones S, Saini HK, Dongen's VAN, Enright AJ. miRBase: tools for microRNA genomics. *Nucleic Acids Res* 2008;36:8.
53. Pleschka S, Wolff T, Ehrhardt C, Hobom G, Planz O, *et al*. fluenza virus propagation is impaired by inhibition of the Raf/MEK/ERK signalling cascade. *Nat Cell Biol* 2001;3:301–305.
54. Ludwig S, Wolff T, Ehrhardt C, Wurzer WJ, Reinhardt J, *et al*. MEK inhibition impairs influenza B virus propagation without emergence of resistant variants. *FEBS Lett* 2004;561:37–43.
55. Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, *et al*. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clin Chem* 2009;55:611–622.
56. Tobita K. Permanent canine kidney (MDCK) cells for isolation and plaque assay of influenza B viruses. *Med Microbiol Immunol* 1975;162:23–27.
57. Tobita K, Sugiura A, Enomote C, Furuyama M. Plaque assay and primary isolation of influenza a viruses in an established line of canine kidney cells (MDCK) in the presence of trypsin. *Med Microbiol Immunol* 1975;162:9–14.

58. Hirst GK. The quantitative determination of influenza virus and antibodies by means of red cell agglutination. *J Exp Med* 1942;75:49–64.
59. Thomson DW, Bracken CP, Szubert JM, Goodall GJ, Stoecklin G. On measuring miRNAs after transient transfection of mimics or antisense inhibitors. *PLoS One* 2013;8:e55214.
60. Wang Z. *The Guideline of the Design and Validation of MiRNA Mimics. MicroRNA and Cancer. Methods in Molecular Biology*. Totowa, NJ: Humana Press; 2011.
61. Robertson B, Dalby AB, Karpilow J, Khvorova A, Leake D, *et al*. Specificity and functionality of microRNA inhibitors. *Silence* 2010;1:1–9.
62. Wu W, Orr-Burks N, Karpilow J, Tripp RA. Development of improved vaccine cell lines against rotavirus. *Sci Data* 2017;4:170021.
63. Jorquera PA, Mathew C, Pickens J, Williams C, Luczo JM, *et al*. Verdinexor (KPT-335), a selective inhibitor of nuclear export, reduces respiratory syncytial virus replication *in vitro*. *J Virol* 2019;93:e01684-18.
64. Brooke CB. Biological activities of "Noninfectious" influenza A virus particles. *Future Virol* 2014;9:41–51.
65. Robertson B, Dalby AB, Karpilow J, Khvorova A, Leake D, *et al*. Specificity and functionality of microRNA inhibitors. *Silence* 2010;1:10.
66. Carthew RW, Sontheimer EJ. Origins and mechanisms of miRNAs and siRNAs. *Cell* 2009;136:642–655.
67. Djuranovic S, Nahvi A, Green R. A parsimonious model for gene regulation by miRNAs. *Science* 2011;331:550–553.
68. Davidson BL, McCray PB Jr. Current prospects for RNA interference-based therapies. *Nat Rev Genet* 2011;12:329–340.
69. Carmon KS, Gong X, Lin Q, Thomas A, Liu Q. R-spondins function as ligands of the orphan receptors LGR4 and LGR5 to regulate Wnt/beta-catenin signaling. *Proc Natl Acad Sci U S A* 2011;108:11452–11457.
70. Okinaga S, Slattery D, Humbles A, Zsengeller Z, Morteau O, *et al*. C5L2, a Nonsignaling C5A Binding Protein. *Biochemistry* 2003;42:9406–9415.
71. Van Lith LHC, Oosterom J, Van Elsas A, Zaman GJR. C5a-stimulated recruitment of beta-arrestin2 to the nonsignaling 7-transmembrane decoy receptor C5L2. *J Biomol Screen* 2009;14:1067–1075.
72. Gasparo DE, Catt K, Inagami T, Wright J, Unger T. International union of pharmacology. XXIII The Angiotensin II Receptors. *Pharmacological Reviews* 2000;52:415–472.
73. He W, Miao FJ-P, Lin DC-H, Schwandner RT, Wang Z, *et al*. Citric acid cycle intermediates as ligands for orphan G-protein-coupled receptors. *Nature* 2004;429:188–193.