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, >274 000 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 (NFkB), phosphatidylinositol-3-kinase (PI3K), mitogen-activated protein kinase (MAPK), protein kinase C (PKC), toll-like...
receptor (TLR), and retinoic acid-inducible gene 1 (RIG-1) 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 its 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 as defined through vertebrates, conserved defined across mammals, and poorly conserved defined 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% CO₂ 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 1.5×10⁵ cells were added to each well. Plates were incubated for 48 h at 37°C, 5% CO₂. 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 μg 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 and target no known human sequence, while the MAP2K positive control (5‘-PAGAACCUCUCAUGUGCUU-3’, 5‘-PUCAAAUCGUCUCUCUGCUU-3’, 5‘-PAGUUGCUCUAAUCUGUCUU-3’, 5‘-PAGAUAAUAGCUUUCGGUU-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 TCID₅₀ 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 RNeazol RT reagent (Sigma). Replicates were pooled and RNA was extracted following the manufacturer protocol. RNA pellets were resuspended in 10µl 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 2µg 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 pre-designed 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% CO2 for 2h. 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 µg/ml 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% CO2, 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% CO2. B/Yamagata/16/1988 samples were assayed for 5 days at 37°C, 5% CO2 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].

TCID50 assay

A TCID50 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% CO2 [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 TCID50 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 anti-viral 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 preserves), and transfection of the miR inhibitor had no change or an increase in fold-change in virus titre (p.f.u. ml preserves). 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
(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\textsubscript{50} assay [24]. Data are presented as fold-change comparing
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-7–5p, let-7b-5p, miR-155–5p, miR-603, miR-616–5p, miR-3129–5p, miR-5011–5p, 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 TCID₅₀ 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 TCID₅₀ ml⁻¹ titre by 90-fold-change (Fig. S1b). The substantial increase in TCID₅₀ 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 TCID₅₀ assay detects both infectious and non-infectious virus that binds to red blood cells by HA. 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) caused a statistically significant fold-change reduction in A/CA/04/09 plaque titre and TCID₅₀ 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-155–5p, miR-335–5p, miR-616–5p, miR-1273e, and miR-6126 mimics reduced A/WSN/33 plaque formation but did not affect A/CA/04/09, whereas miR-96–5p and miR-4723–3p 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-4723–3p, miR-5011–5p, miR-5692a or miR-7703) reduced virus replication (p.f.u. ml⁻¹), however
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-5022–5p 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-4723–3p 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-5011–5p, 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

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**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.
Fig. 5. qPCR of target gene mRNA following miR inhibitor or mimic (I/M) transfection. A549 cells were transfected (25nM) with either miR mimic or its paired miR inhibitor, miR non-targeting inhibitor control (miR-NTC (I)), miR non-targeting mimic control miR-NTC (I), or siTOX transfection control, 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 (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).
IC (ASIC1, GABRA3, GRID2, MCOLN2, and SCNN1D) to show that the predicted miRs regulated expression of these genes (Fig. 5). miR-5011–5p, 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-5011–5p and miR-603 mimics reduced GPCR genes AGTR1 and C5AR2 mRNA expression while miR-5011–5p and miR-603 inhibitors increased expression confirming their function (Fig. 5a, b). Similarly, transfection of miR-5692a mimicked reduced expression of GPCR genes C5AR2 and OXGR1, while transfection of miR-5692a inhibitor increased expression as expected (Fig. 5b, c). MiR-155–5p mimic reduced GPCR gene AGTR1 mRNA expression while miR-616–5p 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-96–5p mimic reduced GPCR genes AGTR1, C5AR2, and OXGR1 mRNA levels (Fig. 5a–c) while the miR-96–5p inhibitor increased the levels of all three genes (Fig. 5a–c). Transfection of miR-218–5p 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-5011–5p, 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-155–5p, 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 (Fig. 4).

The miRs characterized in this study regulated pro-influenza host genes. For example, miR-218 mimic regulated 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-5011–5p, miR-5692a), the A/WSN/33-specific miR-616–5p, and the A/CA/04/09-specific miR-96–5p regulate the C5AR2 (complement component 5a receptor 2) gene which is a non-classical GPCR [70, 71]. The AGTR1 gene is regulated by miR-5011–5p, miR-155–5p, miR-616–5p, and miR-96–5p. AGTR1 is a GPCR gene coupled to Gαq signalling [72]. The pan anti-influenza miR-5692a and A/CA/04/09-specific miR-96–5p regulates OXGR1 gene expression that is associated with Gαq signalling [73]. Of note, miR-7–5p and miR-155–5p 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-335–5p 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.

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