Original Research

Enhancing the yield of seasonal influenza viruses through manipulation of microRNAs in Madin–Darby canine kidney cells

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Impact Statement

This study revealed that *cfa*-miR-340, *cfa*-miR-146b, *cfa*-miR-197, and *cfa*-miR-215 were the most frequently upregulated microRNAs after Madin–Darby canine kidney (MDCK) cells infecting with seasonal influenza viruses. Of these microRNAs, *cfa*-miR-146b, *cfa*-miR-215, and *cfa*-miR-197 could directly target and silence the polymerase genes of influenza viruses. The inhibition of candidate microRNAs can trigger the overexpression of the polymerase genes and enhance the replication of influenza viruses. Therefore, the utility of microRNA inhibitors might be useful for enhancing the influenza vaccine production based on the MDCK cell.

Abstract

Annual influenza vaccine is recommended to reduce the occurrence of seasonal influenza and its complications. Thus far, Madin–Darby canine kidney (MDCK) cell line has been used to manufacture cell-based influenza vaccines. Even though host microRNAs may facilitate viral replication, the interaction between MDCK cells-derived microRNAs and seasonal influenza viruses has been less frequently investigated. Therefore, this study highlighted microRNA profiles of MDCK cells to increase the yield of seasonal influenza virus production by manipulating cellular microRNAs. MDCK cells were infected with influenza A or B virus at a multiplicity of infection (MOI) of 0.01, and microRNA collections were then subjected to MiSeq (Illumina) Sequencing. The validated profiles revealed that *cfa*-miR-340, *cfa*-miR-146b, *cfa*-miR-197, and *cfa*-miR-215 were the most frequently upregulated microRNAs. The effect of candidate microRNA inhibition and overexpression on viral replication was determined using reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and enzyme-linked immunosorbent assay (ELISA). The hybridization pattern between candidate miRNAs and viral genes was performed using miRBase and RNAhybrid web-based programs. Moreover, the predicted microRNA-binding sites were validated by a 3'-UTR reporter assay. The results indicated that *cfa*-miR-146b could directly target the PB1 gene of A/pH1N1 and the PA gene of B/Yamagata. Furthermore, *cfa*-miR-215 could silence the PB1 gene of A/pH1N1 and the PB1 gene of B/Victoria. However, the PB2 gene of the A/H3N2 virus was silenced by *cfa*-miR-197. In addition, the HA and NA sequences of influenza viruses harvested from the cell cultures treated with microRNA inhibitors were analyzed. The sequencing results revealed no difference in the antigenic HA and NA sequences between viruses isolated from the cells treated with microRNA inhibitors and the parental viruses. In conclusion, these findings suggested that MDCK cell-derived microRNAs target viral genes in a strain-specific manner for suppressing viral replication. Conversely, the use of such microRNA inhibitors may facilitate the production of influenza viruses.

Keywords: Seasonal influenza viruses, microRNA, Madin–Darby canine kidney, enhancing the yield

***Experimental Biology and Medicine* 2022; 247: 1335–1349. DOI: 10.1177/15353702221098340**

Introduction

Seasonal influenza is a contagious respiratory disease caused by Influenza A viruses (IAVs) and B viruses (IBVs).[1] Currently, H1N1 and H3N2 subtypes of IAVs, along with Victoria and Yamagata lineages of IBVs, are circulating seasonally in humans.[2] Annual vaccination is an effective intervention to reduce complications, control seasonal influenza

transmission, and reduce its negative impacts on society and the economy.[3] At present, most influenza vaccines are produced using embryonated chicken eggs. While this manufacturing process is well-established, it is far from flexible or scalable. Due to capacity and supply constraints during pandemics, the traditional production of egg-based vaccines has been unable to meet growing demand promptly.[4,5] To address this shortcoming, a cell culture-based manufacturing system is currently being established.[6] In recent years, both the US Food and Drug Administration (FDA) and the European Medicines Agency (EMA) have approved the use of Madin–Darby canine kidney (MDCK) in the production of influenza vaccines.[7,8]

With the cultivation approach, it is necessary to understand virus–host interactions in order to optimize cell culture-based vaccine production. Recently, evidence has accumulated that microRNAs are shown to exert post-transcriptional control of gene expression by inhibiting translation or degrading messenger RNA.[9,10] In the case of viral infections, microRNAs may have a direct antiviral effect via sequence-specific binding to viral RNA, resulting in viral gene silencing. Recently, it was discovered that host microRNAs directly target the genomes of influenza viruses. For example, Song *et al.*[11] demonstrated that miRNA-323, miRNA-491, and miRNA-654 inhibited H1N1 IAV replication by binding to the *PB1* gene in H1N1-infected MDCK cell lines. It has also been shown that *let*-7c regulates IAV replication in H1N1-infected human A549 cell lines by degrading the viral *M1* gene (+) cRNA.[12] Our group recently demonstrated that human miR-3145 induced silencing IAV (pH1N1, H3N2, H5N1) viral *PB1* genes, resulting in the inhibition of influenza viral replication.[13] In another report, Terrier *et al.*[14] performed global microRNA profiling in H1N1 and H3N2-infected human lung epithelial A549 cells, discovering that a specific inhibitor of miR-146a could significantly enhance viral propagation.

While IAVs have been intensively investigated using human cell lines as a model, the studies of microRNAs in MDCK cells infected with influenza viruses remain limited. Thus, microRNA profiles in response to seasonal IAV and IBV infection were examined using next-generation sequencing (NGS) in this study. Apart from investigating the interactions between influenza virus genomes and host microRNAs, the ultimate objective is to enhance virus production for seasonal influenza vaccines via host microRNA regulation.

Materials and methods

Cell culture and virus inoculations

All the experiments in this study were performed in a biosafety level 2 (BSL-2) laboratory according to WHO recommendations. MDCK cells (5×10^4 cells per well) were cultured in Dulbecco's modified eagle medium (DMEM; GE Healthcare Life Sciences, Logan, UT, USA) supplemented with 10% fetal bovine serum (Gibco, Logan, UT, USA) in 24-well plates under 5% CO_2 at 37°C for overnight incubation. After reaching 80% confluence, the media was discarded and filled with IAV subtypes pH1N1 (A/Thailand/104/2009), H3N2 (A/Thailand/CU-H1817/2010), IBV Victoria lineage (B/Thailand/CU-B5522/2011), Yamagata lineage (B/Massachusetts/2/2012), or mock at a multiplicity of infection (MOI) of 0.01. Each viral suspension in overlay medium (DMEM supplemented with 0.2 μg/mL TPCK-treat trypsin [Sigma-Aldrich, St. Louis, MO, USA]) was incubated for 1 h at 37°C with periodic shaking under 5% CO_2. Then, the cells were washed and cultured in a fresh infection medium (DMEM supplemented with 0.2% [w/v] bovine serum albumin [Sigma-Aldrich] and 0.2 μg/mL TPCK-treat trypsin) and incubated at 37°C for 48 h under 5% CO_2.

MicroRNA isolation

The cells were harvested at 6-, 12-, and 24-hours postinfection (hpi). Briefly, the samples were washed twice with PBS and dissociated using 0.05% trypsin/EDTA (Gibco, Grand Island, NY, USA). According to the manufacturer's instructions, a MicroRNA purification kit (Geneaid, New Taipei City, Taiwan) was used to extract microRNA from the cell pellets. MicroRNA concentrations were determined using a Qubit fluorometer (Invitrogen, Singapore) and a Qubit™ microRNA assay kit (Invitrogen, Eugene, OR, USA).

Library preparation and high-throughput sequencing

Purified microRNAs were pooled from cells infected with the same viral strains and at the same time point. A 100 ng of the microRNAs from each group were used to construct libraries with different indexes using an NEBNext® Multiplex Small RNA Library Prep Set for Illumina® (New England BioLabs, Ipswich, MA, USA). KAPA Library Quantification Kits for Illumina® Platform were used to determine the concentrations of DNA libraries. The DNA libraries were pooled with equal concentration and sequenced single-end (50 cycles) on a MiSeq Desktop Sequencer (Illumina). To analyze the microRNA profile, the primary analysis of sequencing data was performed using the MiSeq reporter software version 2.4. Along with excluding low-quality reads (Q-score ≤ 30), the software trimmed low-quality regions of sequences. Passing filtered reads with a Q-score of 30 were aligned to canine genomic DNA (CanFam 3.1), mature and precursor canine miRNAs (from miRBase), and contaminant RNA (tRNA, rRNA, and mRNA). Sequencing reads that matched canine genomic DNA or contaminant RNA were discarded, whereas reads that matched the miRNA database were considered microRNAs. The microRNAs were identified and counted using the number of reads that matched the miRBase database (www.miRbase.org/). The differential expression analysis was performed using fold changes.[15]

Validation of microRNA expression

To determine the expression levels of candidate microRNAs, 100 ng of the microRNAs was polyuridylated using poly(U) polymerase (New England BioLabs). For cDNA generation, the microRNAs with poly(U) were reverse-transcribed by RevertAid™ reverse transcriptase (Thermo Scientific, Waltham, MA, USA) with stem-loop (SL) poly A primers (5′-GTCGTATCCAGTGCAGGGTCCGAG

GTATTCGCACTGGATACGAC-3′).[16] As an internal control for microRNAs, the canine U6 small nuclear RNA 2 (RNU6-2) expression was determined. Following the manufacturer's instructions, Maxima SYBR Green/ROX qPCR Master Mix (Thermo Scientific) was used to quantify the microRNAs and internal control. The real-time quantitative polymerase chain reaction (PCR) conditions and microRNA primers comprised an initial denaturation stage (95°C, 5 min), a cycling stage (Supplementary Table 1), and the melt curve stage. Amplifications using real-time PCR were performed on Step One Plus™ Real-time PCR Systems (Applied Biosystems, Waltham, MA, USA), and StepOne™ Software v.2.2 was used to analyze the results in triplicate. The comparative $\Delta\Delta C_t$ method was used to determine the relative quantitation.

Quantification of viral RNAs

One-hundred fifty microliters of each supernatant were collected for the isolation of viral RNAs using the GenUP™ viral RNA extraction kit (Biotechrabbit, Berlin, Germany). The concentration of RNAs was quantified with the NanoPhotometer™ (Implen, Westlake Village, CA, USA). To detect the expression levels of viral genes, extracted viral RNAs were reverse transcribed with random hexamers using RevertAid Reverse Transcriptase (Thermo Scientific), according to the manufacturer's protocol. Meanwhile, the RBC T&A cloning vector (Real-Biotech) carrying influenza A or influenza B gene was constructed for absolute quantification. The concentration of the plasmids was measured using the Implen NanoPhotometer, and the corresponding copy number was calculated.[17] The absolute expression was quantified using the Maxima SYBR Green/ROX qPCR Master Mix (Thermo Scientific). The real-time PCR conditions comprised an initial denaturation stage (95°C, 2 min), cycling stage (Supplementary Table 1), and the melt curve stage. Real-time PCR amplification was conducted on Step One Plus™ Real-time PCR Systems (Applied Biosystems). The results were analyzed in triplicate using StepOne™ Software v.2.2 analysis, and comparative fold changes between each group were reported.[18]

ELISA

Following the manufacturer's instructions, Influenza A H1N1 (Swine Flu 2009) HA ELISA Pair Set (Sino Biological; #SEK001), Influenza A H3N2 HA ELISA Pair Set (Sino Biological; #SEK11056), and Influenza B HA ELISA Pair Set (Sino Biological; #SEK11053) were used for quantification of viral proteins.

In silico prediction of microRNA target sites

The influenza viruses A/Thailand/104/2009, A/Thailand/CU-H1817/2010, B/Thailand/CU-B5522/2011, and B/Massachusetts/02/2012 genomes were retrieved from the NCBI and GISAID EpiFlu databases. Two web-based programs, miRBase[19] and RNAhybrid,[20] were used to predict the target sites. The criteria for selecting microRNA targets included effective hybridization patterns, particularly in the seeding region, and minimum free energy (MFE) for a base pairing of less than –15.0 kcal/mol. Only viral genomes with an effective hybridization pattern were chosen as candidate microRNA targets.

Plasmid construction

The vector backbones pSilencer 3.0-H1 (Ambion, Austin, TA, USA) and pmirGLO (Promega, Madison, WI, USA) were used to generate microRNA expression (Supplementary Table 2) and reporter vectors (Supplementary Table 3), respectively. Each 10 µL of top- and bottom-strand oligonucleotides (10 nM) was added to 5 µL of 5× rapid ligation buffer (Thermo Scientific), denatured for 5 min at 90°C, and then annealed for 1 h at 25°C. Meanwhile, 1 µg of pSilencer 3.0-H1 was cut with the restriction enzymes *Bam*HI and *Hind*III (New England BioLabs) and incubated for 4 h at 37°C. Meanwhile, pmirGLO was digested with *Nhe*I and *Xho*I (New England BioLabs) and then incubated for 4 h at 37°C. For pmirGLO, the plasmids were treated with 1 µL of Antarctic phosphatase (New England BioLabs). The annealed fragment was then ligated into linearized pSilencer 3.0-H1 or pmirGLO using T4 DNA ligase (Thermo Scientific). The plasmids were transformed into competent *Escherichia coli* strain JM109 cells (RBC Bioscience, Taiwan) by the heat shock method. Ampicillin-resistant colonies were selected and propagated, followed by plasmid extraction using the HiYield™ Plasmid Mini Kit (RBC Bioscience). NanoPhotometer™ (Implen, Westlake Village, CA, USA) was used to determine the concentration of each plasmid. The nucleotide inserts were investigated to verify the recombinant vectors by Sanger sequencing.

Overexpression and inhibition of microRNAs

MDCK cells were seeded into 24-well plates at 5×10^4 cells per well overnight. For overexpression, the transfection of the pSilencer silencing vectors was performed with Turbofect (Thermo Scientific), according to the manufacturers' recommendation. However, microRNA inhibitors (Ambion) and negative control inhibitors (Dharmacon, Lafayette, CO, USA) were transfected into the cells with Lipofectamine® 2000 (Thermo Scientific) for microRNA inhibition. After being transfected, the cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2 for 48 h. Following the incubation, microRNAs were quantified by RT-qPCR.

Dual-luciferase assay

MDCK cells were seeded at 10^4 cells/ well in media without antibiotic/antimycotic into 96-well plates and incubated for 24 h. For transfection into each well, pmirGLO and pSilencer were diluted with Opti-MEM (Gibco) and then co-transfected into the MDCK cells using Turbofect (Thermo Scientific), following the manufacturer's instruction. The transfected cells were incubated under 5% CO_2 at 37°C for 48 h and then harvested. The dual-luciferase assay was conducted using the Dual-Luciferase® Reporter Assay System (Promega) according to the manufacturer's protocol. The luciferase signals were determined in triplicate using Varioskan Flash Multimode (Thermo Scientific). The relative luciferase activity was calculated using signal intensities of firefly luciferase divided by *Renilla* luciferase from a reporter vector.

Sequencing of HA and NA genes

To generate cDNA, 12.3 µL of viral RNAs were added with 0.2 µL of either 10 µM MBT_Uni12 (5′-ACGCGTGATCAGC AAAAGCAGG-3′) for IAVs or 10 µM UniFlu_cDNA

(5′-IAGCARAAGC-3′) for influenza B viruses. The mixture was then incubated at 65°C for 5 min and chilled on ice for 2 min. The reverse transcription was performed at 42°C for IAVs or 37°C for influenza B viruses for 1.5 h, followed by heat inactivation at 70°C for 10 min. The PCR amplification was conducted on the Mastercycler Nexus GSX1 (Eppendorf, Hamburg, Germany). The PCR conditions and primers included an initial denaturation stage (94°C, 2 min), a cycling stage (Supplementary Table 1), and a final extension (68°C, 10 min). The PCR products of HA (~1800 bp) and NA (~1400 bp) were purified by 1% agarose gel electrophoresis. The gel slice containing HA and NA was cut and extracted using the HiYield™ Gel/PCR Fragments Extraction kit (RBC Bioscience), according to the manufacturer's protocol. The purified PCR products were verified by sequencing, and the results were illustrated using BioEdit version 7.2.

Statistical analysis

GraphPad Prism version 8.1 was used to conduct statistical analysis and visualization of the data. The mean value and SD (standard deviation) of triplicates are presented. To determine differences between each group, the Student's unpaired *t*-test and the Dunnett's multiple comparisons test were used for gene expression, ELISA, and luciferase activity. Statistical significance was defined as *P* values less than 0.05 ($P < 0.05$).

Results

Profiles of canine MicroRNAs upon seasonal influenza infection

MDCK cells were mock-infected or infected with one of four seasonal influenza viruses, IAV pH1N1, IAV H3N2, IBV Victoria lineage, or IBV Yamagata lineage, to determine microRNA profiles. After obtaining small RNA samples at 6, 12, and 24 hpi, they were subjected to library preparation for massively parallel sequencing on the Illumina MiSeq Platform. Small RNA libraries from the mock-infected, seasonal IAV-infected, and IBV-infected contained over 10^4 reads encoding microRNAs (Supplementary Table 4). Further analysis of these sequence tags was performed to determine whether specific microRNA expression was altered during IAV and IBV infection compared to the uninfected state. Since CanFam3.1 identified 453 mature canine microRNAs, a range of 147–178 microRNAs were identified in the library of IAV-infected groups (Supplementary Table 4). Meanwhile, a library of IBV-infected individuals revealed 139–174 microRNAs (Supplementary Table 4).

As shown in Table 1, three microRNAs – including *cfa*-miR-543, *cfa*-miR-340, and *cfa*-miR-125b – were overexpressed at 6, 12, and 24 h after pH1N1 infection, respectively. However, it was found that pH1N1 infection decreased the expression of 22 microRNAs. Among the downregulated microRNAs, *cfa*-miR-1249 expression was decreased at 12 and 24 hpi. Meanwhile, the expression of 19 microRNAs increased following H3N2 infection (Table 1). Interestingly, some of the upregulated microRNAs were detected at various time points. Specifically, overexpression of *cfa*-miR-1249 was observed at 6 and 24 hpi, whereas overexpression of *cfa*-miR-146b was observed at 6 and 12 hpi. In addition, at 12 and 24 hpi, the expression level of *cfa*-miR-215 increased. In comparison, 14 microRNAs were downregulated in the presence of H3N2. Among them, *cfa*-miR-18a expression decreased at 6 and 24 hpi. Twenty-seven microRNAs were upregulated and 14 microRNAs were downregulated in response to infection with IBV Victoria lineage (Table 2). Among these dysregulated microRNAs, *cfa*-miR-181a expression increased at 12 and 24 hpi, whereas *cfa*-miR-181c expression decreased at 6 and 24 hpi. High-throughput sequencing revealed an increase in the expression of 14 microRNAs and a decrease in the expression of five microRNAs during IBV Yamagata lineage infection (Table 2).

Interestingly, NGS demonstrated that several microRNAs were upregulated following infection with various influenza viruses (Tables 1 and 2 and Figure 1). For example, *cfa*-miR-340 expression was increased at 12 h after infection with two different IAV subtypes – pH1N1 and H3N2. However, overexpression of *cfa*-miR-361, *cfa*-miR-1841, *cfa*-miR-1842, and *cfa*-miR-330 was observed following infection with two distinct IBV lineages – Yamagata and Victoria. In addition, two microRNAs, *cfa*-miR-129 and *cfa*-miR-1249, were upregulated in response to A/H3N2 or B/Victoria influenza virus infection. Moreover, *cfa*-miR-197, *cfa*-miR-215, and *cfa*-miR-339-1 were upregulated when the cells were infected with A/H3N2, B/Victoria, or B/Yamagata influenza viruses. *cfa*-miR-146b was found to be upregulated in H3N2 or B/Yamagata-infected cells. However, *cfa*-miR-146b expression was increased by more than 1.5-fold when pH1N1-infected cells were compared to mock-infected cells.

Validation of microRNA profiles

The altered expression patterns of the NGS-identified microRNAs were further validated by RT-qPCR (Figure 2). *cfa*-miR-543, *cfa*-miR-340, and *cfa*-miR-125b were all highly expressed in response to pH1N1 virus infection. Notably, most tested microRNAs, except *cfa*-miR-125b, were upregulated, indicating a strong correlation between microRNA expression levels detected by NGS and RT-qPCR analysis. In H3N2-infected groups, some of the upregulated microRNAs identified by NGS were verified. The results indicated that validated microRNAs were mostly upregulated, except *cfa*-miR-339-1 at 12 hpi and *cfa*-miR-1249 at 24 hpi. *cfa*-miR-340 was found to have the highest level of expression, followed by *cfa*-miR-1249 (6 hpi), *cfa*-miR-122, *cfa*-miR-146b (6 hpi), and *cfa*-miR-132. In addition, two microRNAs that were upregulated at multiple time points were validated. *cfa*-miR-146b was overexpressed at 6 and 12 hpi, whereas *cfa*-miR-215 was upregulated at 12 and 24 hpi. These findings indicate that data from library sequencing analyses accurately reflect how microRNAs responded to pH1N1 and H3N2 virus infection.

For IBV Victoria infection, nine microRNAs identified through NGS screening were validated using RT-qPCR. The results indicated that *cfa*-miR-197, *cfa*-miR-215, *cfa*-miR-320, *cfa*-miR-500, *cfa*-miR-1307, and *cfa*-miR-1842 were overexpressed following infection of the Victoria lineage. In addition, most validated microRNAs increased in B/Yamagata-infected groups, except for *cfa*-miR-361 and *cfa*-miR-1842. The three most upregulated genes in B/Yamagata

Table 1. MicroRNA profiles of MDCK cells in response to influenza A virus infection at 6-, 12-, and 24-hours postinfection (hpi).

Subtype	6 hpi		12 hpi		24 hpi	
	miRNA	Fold change	miRNA	Fold change	miRNA	Fold change
pH1N1	miR-543	2.02	miR-340	2.42	miR-125b	7.03
	miR-106b	–2.07	miR-15b	–2.16	miR-1185	–2.32
	miR-26b	–2.25	miR-18a	–2.16	miR-494	–2.32
	miR-30e	–2.44	miR-151	–2.18	miR-543	–2.32
			miR-130a	–2.32	miR-374a	–2.91
			miR-194	–2.49	miR-8884	–2.91
			miR-503	–2.49	miR-1249	–5.57
			miR-1249	–2.64		
			miR-106a	–2.90		
			miR-205	–2.90		
			miR-29b	–2.90		
			miR-889	–2.90		
			miR-181c	–3.07		
			miR-152	–3.49		
			miR-590	–3.49		
H3N2	miR-1249	3.28	miR-215	3.49	miR-215	3.10
	miR-146b	2.77	miR-17	3.23	miR-129	2.61
	miR-1840	2.69	miR-1185	2.49	miR-1249	2.29
	miR-122	2.28	miR-132	2.49	miR-141	–2.03
	miR-8865	2.11	miR-146a	2.49	miR-181b	–2.03
	miR-18a	–2.21	miR-194	2.49	miR-22	–2.03
	miR-106b	–2.40	miR-340	2.49	miR-30e	–2.29
			miR-874	2.37	miR-374a	–2.29
			miR-147	2.32	miR-374b	–2.29
			miR-339-1	2.32	miR-210	–2.37
			miR-221	2.30	miR-151	–3.00
			miR-197	2.23	miR-18a	–3.17
			miR-375	2.23	miR-107	–3.62
			miR-146b	2.06		
			miR-8859b	–2.00		
			miR-181c	–2.26		

groups were *cfa*-miR-146b, *cfa*-miR-215, and *cfa*-miR-197. As a result, these data demonstrated that microRNA profiles obtained via NGS were consistent with those validated via RT-qPCR.

Some microRNAs of interest were reliant on the most common overexpression observed during infection with various subtypes. Unfortunately, no universal microRNAs were overexpressed in all four seasonal influenza virus strains. However, the results indicated that four microRNAs – including *cfa*-miR-146b, *cfa*-miR-197, *cfa*-miR-215, and *cfa*-miR-340 – were most frequently upregulated during seasonal influenza infections. Different IAV infections increased the expression of *cfa*-miR-340 at 12 hpi. In addition, *cfa*-miR-197 and *cfa*-miR-215 were overexpressed when the cells were infected with H3N2, Victoria, or Yamagata. Moreover, the cells infected with H3N2 or B/Yamagata showed increased expression of *cfa*-miR-146b.

Effect of candidate microRNA expression on viral propagation yield

The MDCK cells transfected with either microRNA inhibitors or microRNA overexpressing plasmids were infected with each strain of the influenza virus. The yield of each virus was determined using RT-qPCR and an ELISA assay. As illustrated in Figure 3, the effect of inhibiting and overexpressing candidate microRNAs on IAV pH1N1 replication was determined. The results indicated that inhibition of *cfa*-miR-146b and *cfa*-miR-215 increased the copy number of the pH1N1 *M* gene by approximately 3.3-fold and 1.7-fold, respectively (Figure 3(a) and (e)). In addition, the ELISA result showed a trend consistent with the RT-qPCR, implying that *cfa*-miR-146b and *cfa*-miR-215 inhibitors increased pH1N1 yield (Figure 3(b) and (f)). In contrast, when cells were treated with vectors overexpressing *cfa*-miR-146b and *cfa*-miR-215, the amount of the pH1N1 viral gene was significantly decreased (Figure 3(a) and (e)). Moreover, overexpression of *cfa*-miR-146b and *cfa*-miR-215 resulted in a decrease in viral titer (Figure 3(b) and (f)). Although *cfa*-miR-197 and *cfa*-miR-340 suppressed pH1N1 yields, the enhancing effect was not observed in cells treated with inhibitors of *cfa*-miR-197 and *cfa*-miR-340 (Figure 3(c) and (d) and (g) and (h), respectively). As a result, the role of these two microRNAs in the propagation of pH1N1 was uncertain. These findings indicated that *cfa*-miR-146b and *cfa*-miR-215 may inhibit pH1N1 replication and that the suppressive effect may be resolved using microRNA inhibitors.

As shown in Figure 3, the efficiency of H3N2 virus propagation in modified MDCK cells was also determined using RT-qPCR and an ELISA assay. The H3N2 viral gene in the

Table 2. MicroRNA profiles of MDCK cells in response to influenza B virus infection at 6-, 12-, and 24-hours postinfection (hpi).

Lineage	6 hpi		12 hpi		24 hpi	
	miRNA	Fold change	miRNA	Fold change	miRNA	Fold change
Victoria	miR-361	1.96	miR-184	7.64	miR-1842	3.62
	miR-874	–2.04	miR-191	6.90	miR-193a	3.32
	miR-500	–2.04	miR-183	6.32	miR-215	3.21
	miR-204	–2.04	miR-141	5.15	miR-1841	3.21
	miR-130b	–2.04	miR-186	4.95	miR-197	2.94
	miR-181c	–4.74	miR-185	4.35	miR-339-1	2.76
			miR-196a	3.34	miR-320	2.75
			miR-181a	2.87	miR-2483	2.62
			miR-18a	2.57	miR-133c	2.62
			miR-411	2.11	miR-1307	2.62
			miR-205	2.11	miR-1249	2.62
			miR-486	–2.22	miR-181a	2.31
			miR-15b	–3.22	miR-330	2.30
			miR-181d	–3.31	miR-500	2.04
			miR-181c	–3.44	miR-129	2.04
			miR-193a	–4.09	miR-423a	2.02
			miR-138a	–4.29		
			miR-1842	–4.48		
			miR-197	–6.79		
			miR-1839	–7.59		
			miR-1843	–8.32		
Yamagata	miR-361	3.33	miR-33b	2.58	miR-374a	4.21
	miR-339-1	3.07	miR-8859b	–2.06	miR-215	2.55
	miR-330	2.33	miR-130b	–2.32	miR-197	1.96
	miR-29c	2.33			miR-1841	1.96
	miR-1842	2.33			miR-146b	1.96
	miR-8859b	2.07			miR-486	–2.04
	miR-1839	2.07				
	miR-149	2.07				
	let-7f	–2.13				
	miR-16	–2.25				

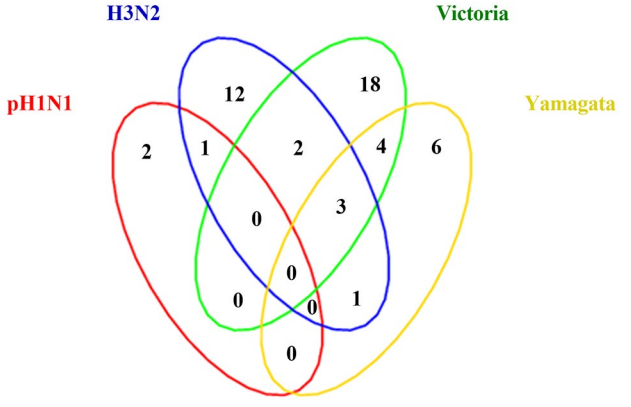

Figure 1. Venn diagram shows upregulated microRNAs of MDCK cells upon different strains of seasonal influenza infection. (A color version of this figure is available in the online journal.)

group treated with *cfa*-miR-197 inhibitor was increased by roughly 2.5-fold (Figure 3(c)). In addition, there was an approximately 1.5-fold increase in the viral protein following treatment with a *cfa*-miR-197 inhibitor (Figure 3(d)). However, overexpression of *cfa*-miR-197 has been shown to significantly reduce the number of H3N2 genes by approximately 70% compared to a scramble control group (Figure 3(c)). Furthermore, the ELISA result revealed a decreasing trend in cells treated with *cfa*-miR-197 (Figure 3(d)). Taken together, the results indicated that inhibiting *cfa*-miR-197 may increase H3N2 yield. While inhibition of *cfa*-miR-215 increased the viral titer, the inhibitory effect was not observed in the cells treated with a vector overexpressing *cfa*-miR-215 (Figure 3(e) and (f)). As a result, the role of *cfa*-miR-215 in H3N2 propagation was unclear.

Along with IAV propagation, the effect of microRNA on the production of IBV was investigated. As illustrated in Figure 3(e), B/Victoria viral RNA increased 1.5-fold following treatment with the *cfa*-miR-215 inhibitor. ELISA analysis revealed that the *cfa*-miR-215 inhibitor could significantly increase the viral protein compared to the negative control inhibitor (Figure 3(f)). In cells overexpressing *cfa*-miR-215, however, the viral gene copy number was decreased (Figure 3(e)). When cells were overexpressed with *cfa*-miR-215, a significant decrease in the viral protein was observed (Figure 3(f)). As a result of the antagonistic effect of the *cfa*-miR-215 inhibitor, the viral yield of the B/Victoria lineage was increased. The effect of candidate microRNAs on IBV Yamagata replication indicated that overexpression of *cfa*-miR-146b significantly decreased the viral protein compared to the scramble control group (Figure 3(b)). Moreover, a slightly increased in viral RNA and protein was observed in

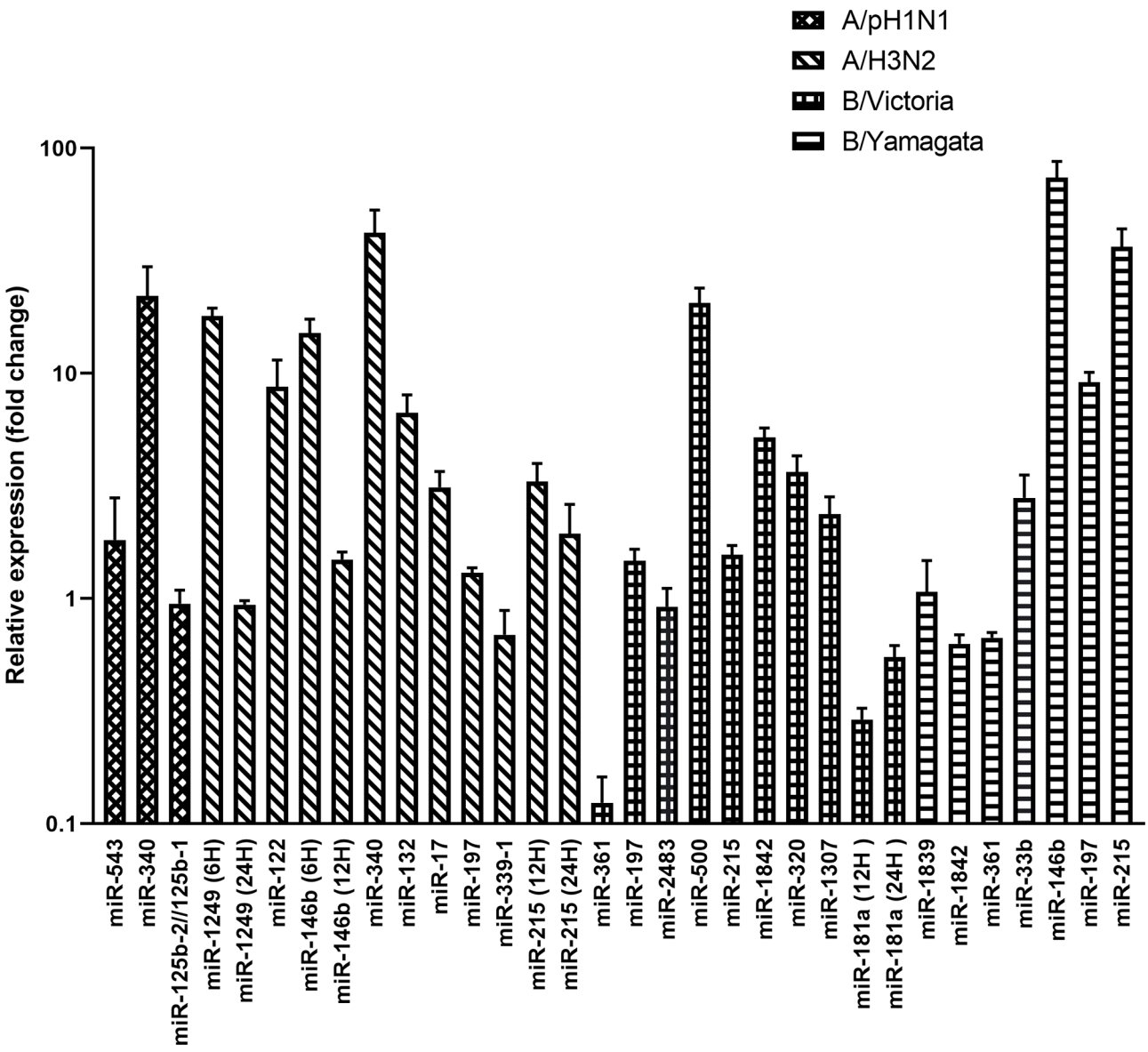

Figure 2. Validation of microRNA expression infection upon seasonal influenza infection.

the group treated with the *cfa*-miR-146b inhibitor compared to the negative control inhibitor (Figure 3(a) and (b)). Thus, the findings indicated that inhibiting *cfa*-miR-146b increases the yield of IBV Yamagata.

It is suggested that *cfa*-miR-146b is a candidate target of microRNA inhibitors for increasing the production of pH1N1 and B/Yamagata viruses. *cfa*-miR-215 may also be a viable candidate for increasing the production of pH1N1 and B/Victoria viruses. However, the microRNA inhibitor targeting *cfa*-miR-197 would benefit H3N2 virus propagation. According to our findings, microRNAs tended to target viral genes in a strain-specific manner, and the candidate microRNAs could target viral genomes, thereby suppressing viral replication.

In silico prediction of viral genomes targeted by canine MicroRNAs

As determined by RT-qPCR and ELISA, it is suggested that these microRNA inhibitors may antagonize the inhibitory effect of microRNAs on viral propagation. MicroRNA-binding sites on viral genomes remain to be investigated. Two web-based programs, miRBase and RNAhybrid, were used to predict target sites based on the hybridization patterns between microRNAs and viral genomes. As illustrated in Table 3, *cfa*-miR-146b specifically targeted three IAV pH1N1 genes: *PB2* (the position 1979), *PB1* (the position 2191), and *NA* (the position 693). In addition, *cfa*-miR-146b inhibited IBV Yamagata replication by targeting a single site on the *PA* gene (the position 534) and two sites on the *NP* gene (the positions 973 and 1290). However, the computational analysis revealed that two positions on IAV pH1N1 were direct targets of *cfa*-miR-215, including the *PB2* gene (the position 350) and the *PB1* gene (the position 2155). In addition, *cfa*-miR-215 may bind to three sites of IBV Victoria, including *PB1* (the position 2101), *HA* (the position 98), and *NP* (the position 617). *In silico* target prediction for IAV subtype H3N2 indicated that *cfa*-miR-197 could target two positions on the *PB2* gene, including 865 and 1447.

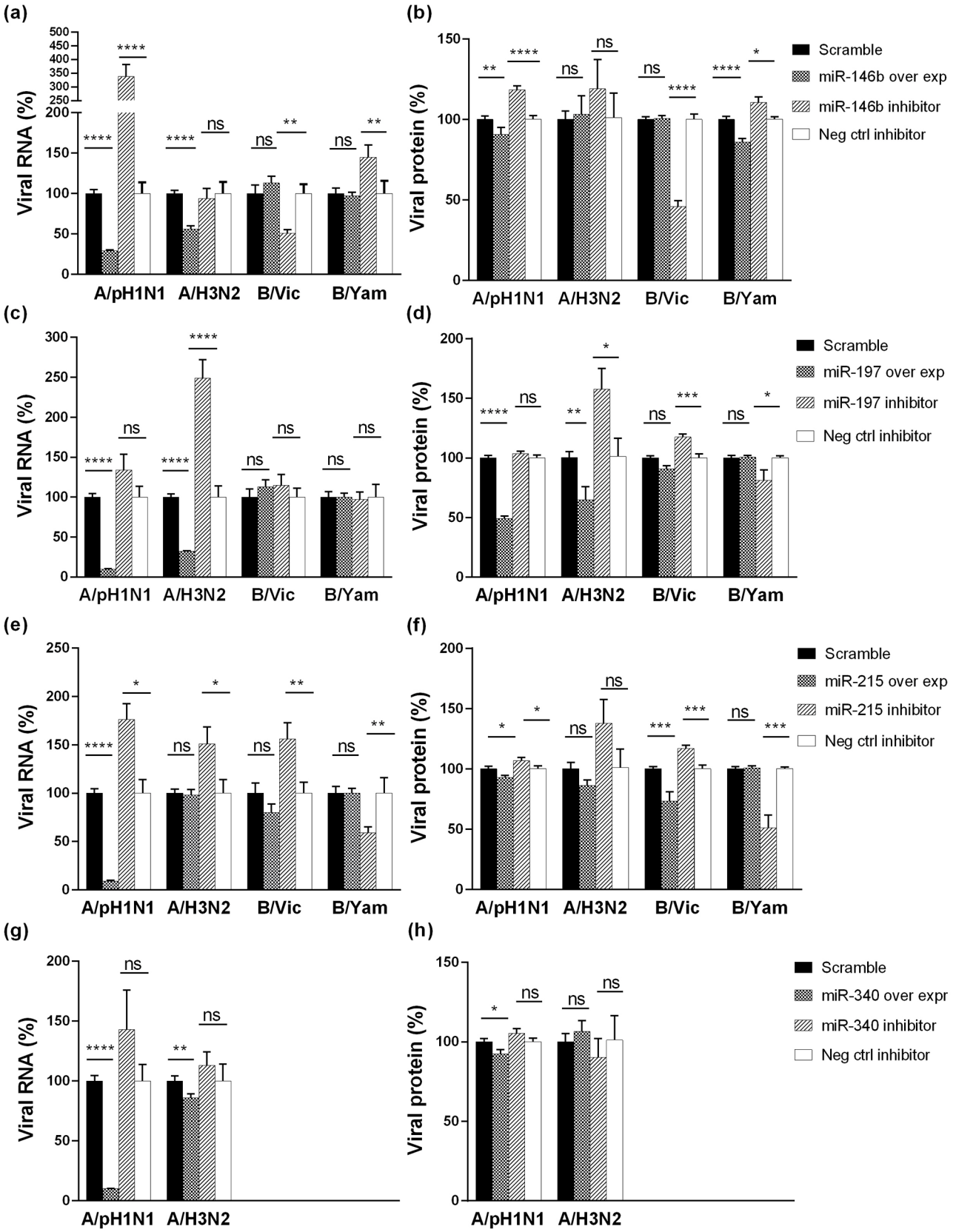

Figure 3. Effect of candidate microRNAs on the yield of seasonal influenza viruses. MDCK cells were transfected with microRNA overexpressing plasmids or microRNA inhibitors: *cfa*-miR-146b (a and b); *cfa*-miR-197 (c and d); *cfa*-miR-215 (e and f); *cfa*-miR-340 (g and h). Scramble plasmid or negative control inhibitors were used as a baseline to evaluate the effect of the controls and silencing plasmids or microRNA inhibitors on target gene expression, respectively. Following the infection for 48 h, viral RNA load and viral protein concentration were determined by RT-qPCR and ELISA assay, respectively.
$P \leqslant 0.05$ is designated as *; $P \leqslant 0.01$ is designated as **; $P \leqslant 0.001$ is designated as ***; $P \leqslant 0.0001$ is designated as ****.

Table 3. *In silico* analysis of microRNA target prediction.

Viruses	miRNAs	Target genes (position)	Hybridization pattern between miRNA (bottom strand) and target gene (top strand)	MFE (kcal/mol)
A/pH1N1	miR-146b	PB2 (1979)	5'…AGGCAACCAAACGACUUACAGUUCUUG…3' 3' UCGGAUAC---CUUAA-GUCAAGAGU 5'	–17.4
		PB1 (2191)	5'…GAGUCUGGACGGAUAUCAAGAAAGAAGAGUUCUCU…3' 3' UCGGAUA--CCUUAAG----------UCAAGAGU 5'	–21.4
		NA (693)	5'…UGCAUGUGUAAAU-GGUUCUUG…3' 3' UCGGAUACCUUAAGUCAAGAGU 5'	–16.3
	miR-215	PB2 (350)	5'…-----CUUAUUUCGAAAAGGUCGA…3' 3' ACAGAUAGUUAAGCA--UCCAGUA 5'	–16.7
		PB1 (2155)	5'…UGUCUAGGGCCCGGAUUGAUGCCAGGGUCGA…3' 3' ACAGAU----------AGUUAAGCAUCCAGUA 5'	–18.8
A/H3N2	miR-197	PB2 (865)	5'…AUUGGC-GGAACAAGGAUGGUGGAC…3' 3' CGACCCACCUC-UUCC-ACCACUU 5'	–24.3
		PB2 (1447)	5'…AUGGGUGUGGAUGAAUACUCCAGUACAGAGAGGGUGGUGGU…3' 3'CGACCCAC-----CU--------------CUUCCACCACUU 5'	–29.8
B/Victoria	miR-215	PB1 (2101)	5'…AGUGCAUCAUACAGGAAGCCAGUGGGUCAA…3' 3' ACAGAUAGUUAAG--------CAUCCAGUA 5'	–19.8
		HA (98)	5'…CUG-CUACUCAAGGGG-AGGUCAA…3' 3' ACAGAU-AGUUAAGCAUCCAGUA 5'	–19.2
		NP (617)	5'…AUGUCUGUU---UCCAAAGGUCAA…3' 3' ACAGAUAGUUAAGCA-UCCAGUA 5'	–22.4
B/Yamagata	miR-146b	PA (534)	5'…-ACCUAUGGCA---AGUUCUCAU …3' 3' UCGGAUACCUUAAGUCAAGAGU 5'	–26.2
		NP (973)	5'…AGGCCCUCUGUGGCGAGCAAAGUGGUUCUUC…3' 3' UCGG---AUACC-UUAA----GUCAAGAGU 5'	–21.0
		NP (1290)	5'…-UCUUCUGGAACUCGGUUUUCU …3' 3' UCGGAUACCUUAAGUCAAGAGU 5'	–23.0

Validation of MicroRNA target sites

To determine whether the predicted sites on influenza viral genomes were putative targets of candidate microRNAs in MDCK cells, luciferase reporter assays were performed 48 h after co-transfection of pmirGLO encoding viral sequences and pSilencer encoding microRNA mimic. For silencing control, the pSilencer siLuc2 was constructed to inhibit the expression of the *Luc2* gene, which serves as the reporter gene in pmirGLO. The pSilencer Scramble, however, was used as a non-targeting control. MicroRNA *cfa*-miR-146b had three target sites on IAV pH1N1 according to *in silico* analysis of microRNA targets (Figure 4(a) to (c)). In addition, pH1N1 contained two positions that *cfa*-miR-215 could target (Figure 4(d) and (e)). As shown in Figure 4(b), relative luciferase activity was significantly decreased ($P \leqslant 0.05$) when the pmirGLO containing *PB1* gene was co-transfected with silencing vectors encoding for *cfa*-miR-146b. As a result, the IAV pH1N1 *PB1* gene was identified as a putative target of *cfa*-miR-146b. In addition, *cfa*-miR-215 targeted the *PB1* gene of IAV pH1N1 (Figure 4(d)), but not the *NP* gene (Figure 4(e)), as evidenced by a significant decrease in luciferase activity ($P \leqslant 0.01$).

For IAV subtype H3N2, the computational analysis revealed that *cfa*-miR-197 could target two binding sites on the *PB2* gene. The results indicated that relative luciferase activity was significantly decreased ($P \leqslant 0.01$) when silencing vectors encoding for *cfa*-miR-197 were co-transfected

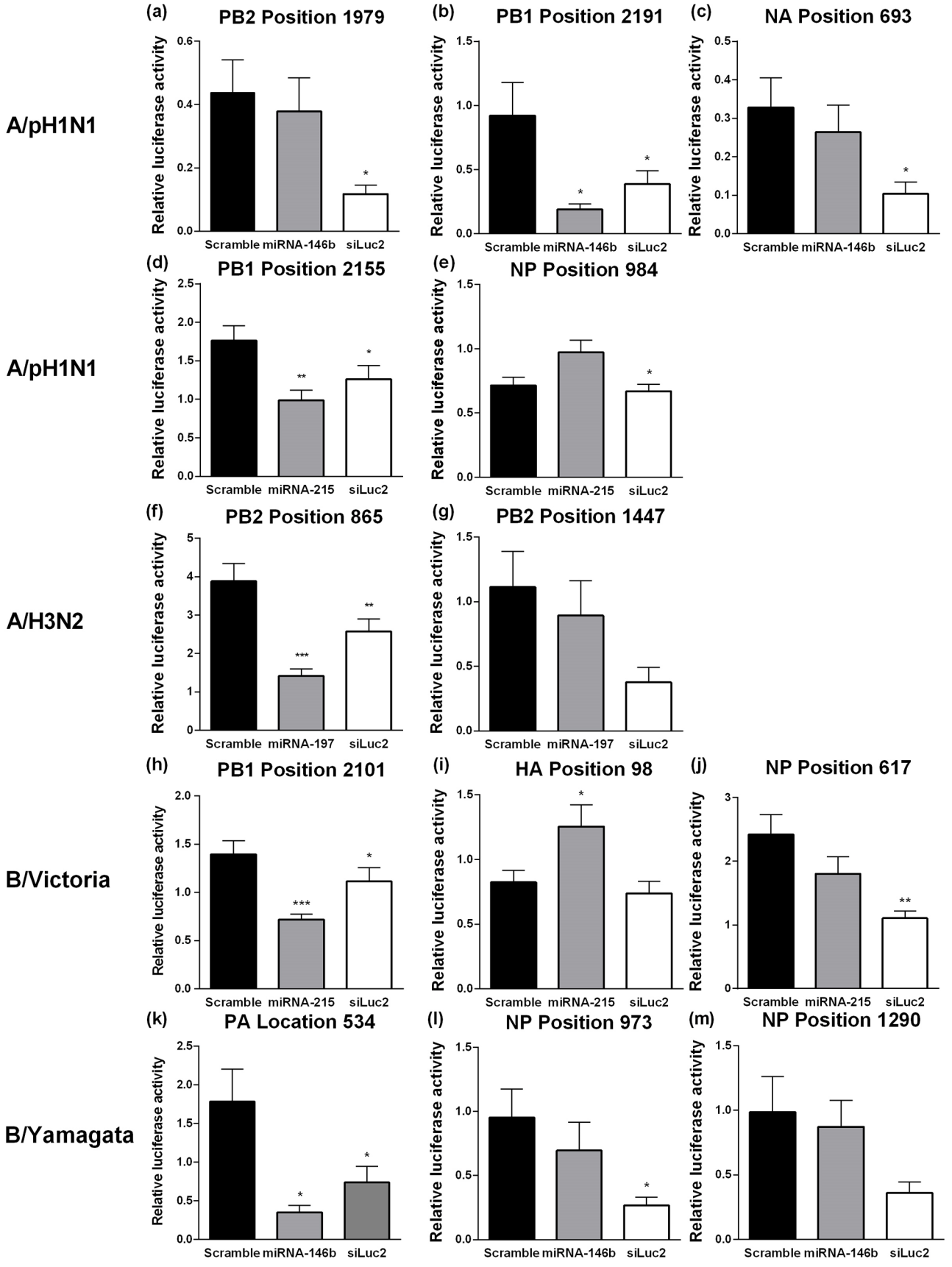

Figure 4. Luciferase assays were assessed for microRNA targets on influenza viruses after plasmid transfection for 48 h. IAV pH1N1 (a to e), IAV H3N2 (f and g), IBV Victoria lineage (h to j), or IBV Yamagata lineage (k to m).
$P \leqslant 0.05$ is designated as *; $P \leqslant 0.01$ is designated as **; $P \leqslant 0.001$ is designated as ***; $P \leqslant 0.0001$ is designated as ****.

Table 4. MicroRNA-binding sites of the experimental strains (top sequences) and other seed viral strains (lower sequences) used for influenza vaccine manufacturing during 2011–2019. (A color version of this table is available in the online journal.)

Gene segments (influenza strains)	MicroRNA-binding sites
PB1 (A/pH1N1)	5'…GAGUCUGGACGGAUAUCAAGAAAGAAGAGUUCUCU…3'
***Cfa*-miR-146b**	3' UCGGAUA--CCUUAAG----------UCAAGAGU 5'
PB1 A/Thailand/104/2009	GCATGGTGGAGGCCATGGTGTCTAGGGCCCGGATTGATGCCAGGGTCGACTTCGAGTCTGGACGGATCAAGAAAGAAGAGTTCTCTGAGAT
PB1 A/Brisbane/02/2018	..........A.....T.....
PB1 A/Michigan/45/2015	..........A.....T.....
PB1 A/California/07/2009	.....
PB1 (A/pH1N1)	5'…UGUCUAGGGCCCGGAUUGAUGCCAGGGUCGA…3'
***Cfa*-miR-215**	3' ACAGAU----------AGUUAAGCAUCCAGUA 5'
PB1 A/Thailand/104/2009	CCTAGCAGTTCATATAGGAGACCGGTTGGAATTTCTAGCATGGTGGAGGCCATGGTGTCTAGGGCCCGGATTGATGCCAGGGTCGACTTCG
PB1 A/Brisbane/02/2018	.....A.....T.
PB1 A/Michigan/45/2015	.....A.....T.
PB1 A/California/07/2009	.....
PB2 (A/H3N2)	5'…AUUGGC-GGAACAAGGAUGGUGGAC…3'
***Cfa*-miR-197**	3' CGACCCACCUC-UUCC-ACCACUU 5'
PB2 A/Thailand/CU-H1817/2010	GAAGAGCCGCAGTATCAGCAGATCCACTAGCATCTTTATTGGAGATGTGCCACAGCACACAAATTGGCGGAACAAGGATGGTGGACATTCT
PB2 A/Kansas/14/2017	.....G.....G.....
PB2 A/Switzerland/8060/2017	.....G.....G.....
PB2 A/Singapore/INFIMH-16-0019/2016	.....G.....G.....
PB2 A/Hong Kong/4801/2014	.....T.....G.....
PB2 A/Switzerland/9715293/2013	.....G.....G.....
PB2 A/Texas/50/2012	.....G.....
PB2 A/Victoria/361/2011	.....G.....
PB2 A/Perth/16/2009	.....
PB1 (B/Victoria)	5'…AGUGCAUCAUACAGGAAGCCAGUGGGUCAA…3'
***Cfa*-miR-215**	3' ACAGAUAGUUAAG--------CAUCCAGUA 5'
PB1 B/Thailand/CU-B5522/2011	TTCTTGAGGAACAATGCTACGCTAAATGTTGCAACCTATTTGAGGCCTGTTTTAATAGTGCATCATACAGGAAGCCAGTGGGTCAACATAG
PB1 B/Colorado/06/2017	.....G.....C.....
PB1 B/Brisbane/60/2008	.....C.....
PA (B/Yamagata)	5'…-ACCUAUGGCA---AGUUCUCAU …3'
***Cfa*-miR-146b**	3' UCGGAUACCUUAAGUCAAGAGU 5'
PA B/Massachusetts/02/2012	GAAGGGAAAGGGAGAGTGCTAAGCAGACTCACAGAACTTCAGGCTGAATTAAGTCTGAAAAACCTATGGCAAGTTCTCATAGGAG
PA B/Phuket/3073/2013	.....G.....A.....T.....

with the pmirGLO containing *PB2* at position 865 (Figure 4(f)) but not at position 1447 (Figure 4(g)). In addition, it was predicted that *cfa*-miR-215 and *cfa*-miR-146b would target three distinct positions on the IBV Victoria (Figure 4(h) to (j)) and Yamagata (Figure 4(k) to (m)) lineages, respectively. Specifically, silencing vectors encoding for *cfa*-miR-215 significantly decreased luciferase activity ($P \leqslant 0.001$) when co-transfected with pmirGLO containing the *PB1* gene from the IBV Victoria lineage (Figure 4(h)). However, luciferase activities associated with other predicted sites such as *HA* (Figure 4(i)) and *NP* (Figure 4(j)) did not decline significantly. In addition, a significant decrease in luciferase activity was observed ($P \leqslant 0.05$) when pmirGLO containing the *PA* gene from the IBV Yamagata lineage was co-transfected with *cfa*-miR-146b (Figure 4(k)). In contrast, no significant decrease in luciferase activity was observed when pmirGLO containing the *NP* gene was used (Figure 4(l) and (m)). As a result, *cfa*-miR-146b may directly target the *PA* gene of IBV Yamagata.

Effect of MicroRNA inhibitor treatments on microRNA-binding sites and antigenic sequences

Because of the unavailability of seed vaccine strains, the strains used in this experiment, except for Yamagata lineage, were not the same strains used in vaccine production. However, the sequences of viral seed strains obtained from NCBI or GISAID were compared to the experimental strains' sequences. The luciferase results indicate that *cfa*-miR-146b and *cfa*-miR-215 may target the *PB1* gene of IAV pH1N1. As illustrated in Table 4, the seed region of *cfa*-miR-146b has the potential to interact with the *PB1* of other pH1N1 seed strains. However, a nucleotide change from G to A was detected in the seed strains A/Michigan/45/2015 and A/Brisbane/02/2018. As a result, this mutation disrupted the binding of the *cfa*-miR-215 seed sequence to the PB1 of the pH1N1 seed strains. In addition, IAV H3N2 and IBV Victoria microRNA-binding sites were identified. The results indicated that *cfa*-miR-197 could bind to the *PB2* gene of other H3N2 seed strains, whereas *cfa*-miR-215 was capable of binding to the *PB1* gene of Victoria seed viral strains. A nucleotide substitution from C to T was observed in the *PA* gene of the seed strain B/Phuket/3073/2013 for the microRNA-binding site of the IBV Yamagata lineage. Although this mutation was located within the microRNA-binding site, it was outside the binding sites for the seed sequence of *cfa*-miR-146b. As a result, this mutation may not affect the pairing of *cfa*-miR-146b's seed region and the *PA* of the Yamagata seed strain. As a result, manipulation of microRNA inhibitors may be affected when viral mutations occur in microRNA-binding sites, particularly in the seed region of microRNAs. Antigenic variations play a critical role in vaccine effectiveness, apart from microRNA-binding sites. To assess the effect of microRNA inhibitors on antigenic alterations, the nucleotides of *HA* and *NA* genes were sequenced

compared to parental strains. The findings showed no nucleotide changes in the *HA* or *NA* genes were detected in IAV pH1N1 viruses treated with *cfa*-miR-146b or *cfa*-miR-215 inhibitors (Supplementary Figure 1). In addition, no antigenic changes were observed in IBV Victoria viruses grown in cells treated with *cfa*-miR-215 inhibitor (Supplementary Figure 2). As illustrated in Supplementary Figure 3, no *HA* or *NA* sequence changes were detected in IBV Yamagata propagated in cells treated with *cfa*-miR-146b inhibitor.

Discussion

Host factors required for viral replication might be a target for disease intervention. Among the host components, virus infection has been shown to alter cellular microRNA expression, thereby regulating various biological processes within an infected cell. As a result, microRNAs may be used to develop therapeutic interventions against infectious diseases,[21,22] biomarkers,[23–25] and vaccine development.[26,27] So far, influenza viruses, particularly IAVs, have been studied using human cell lines. Nonetheless, microRNA studies in MDCK cells infected with seasonal IAVs and IBVs remain limited. This study aimed to determine the microRNA profiles of MDCK cells following seasonal influenza virus infection. To date, 453 mature microRNAs have been predicted in the canine genome.[28] According to our microRNA profile data, dysregulated microRNAs were defined as those with greater than a twofold change in microRNA expression compared to mock-infected groups. In this study, three microRNAs were upregulated and 22 microRNAs were downregulated in MDCK cells infected with A/pH1N1. In addition, 19 microRNAs were upregulated and 13 microRNAs were downregulated following A/H3N2 infection. However, 27 microRNAs were upregulated and 14 microRNAs were downregulated in B/Victoria-infected cells. Finally, 14 microRNAs were found to be overexpressed and 5 microRNAs were downregulated in B/Yamagata-infected cells. Following validation of the profiles using RT-qPCR, some microRNAs of interest depended on common upregulation during infection with distinct subtypes. Even though no microRNAs were universally overexpressed across four strains, the four most common microRNAs, including *cfa*-miR-146b, *cfa*-miR-197, *cfa*-miR-215, and *cfa*-miR-340, were overexpressed in this study. *cfa*-miR-146b was overexpressed in canine lung and tracheal cells infected with canine influenza H3N2 virus, consistent with our findings.[29]

Besides the four microRNAs commonly overexpressed by different strains of seasonal influenza viruses, other microRNA expressions obtained from the present investigation were compared to previous experiments conducted in various subtypes of influenza viruses and cellular models. For instance, the current finding of *cfa*-miR-17 concurs well with the recent works utilizing pH1N1-infected human lung epithelial cells[15] and serum from H7N9-infected patients.[30] Nonetheless, some studies found that *hsa*-miR-17-3p was downregulated in H1N1 (PR8)-infected lung epithelial cells.[31] Furthermore, *cfa*-miR-122 was overexpressed, which corresponds to the investigation of avian influenza infection in broiler chicken lungs.[32] This study also discovered upregulation of *cfa*-miR-132, which is consistent with previous findings in human lung and bronchial epithelial cells infected with H1N1 (PR8)[33] and H3N2.[15] In addition, overexpression of *cfa*-miR-320 has been discovered in influenza B/Victoria-infected cells, which is consistent with a previous finding in H7N9-infected serum.[34] In contrast, *cfa*-miR-30e, *cfa*-miR-18a, and *cfa*-miR-374a were downregulated in pH1N1- and H3N2-infected cells in this study. It is controversial whether IAV infection causes *hsa*-miR-30e to be downregulated[35] or upregulated.[15] Previous research has shown that *hsa*-miR-18a[14,36] and *hsa*-miR-374a[14,35] were downregulated. Furthermore, this study demonstrated downregulated expression of *cfa*-miR-15b in cells infected with A/pH1N1 or B/Victoria virus. This result supports a recent investigation of IAV-infected human cells.[14]

In some previous investigations, host microRNAs could indirectly affect viral replications through silencing host genes. For example, Zhou *et al*.[37] demonstrated that overexpression of *cfa*-miR-143 decreased viral replication while *cfa*-miR-143 promoted apoptotic pathways in MDCK cells infected with canine influenza H3N2 virus. Our recent study found that *cfa*-miR-197 had significant down-expressions of KPNA6, thus decreasing viral loads of IBV Victoria lineage.[38] Intriguingly, direct binding between host microRNAs and viral RNAs leads to alterations in the pathogenesis or the translation and replication processes of the viruses. Recent findings showed that microRNAs could directly bind to many viruses, including hepatitis C virus,[39] enterovirus 71 (EV71),[40,41] and human T cell leukemia virus type I.[42] MicroRNAs have recently been demonstrated to interact with IAVs directly. For example, *hsa*-miR-584-5p and *hsa*-miR-1249 drastically inhibited replication of H5N1 and pH1N1 (A/Beijing/501/2009) IAVs in A549 cells by matching with the PB2-binding sequence.[43] In recent work, *hsa*-miR-3145 could inhibit the replication of IAVs (pH1N1, H3N2, H5N1) in human lung epithelial cell line A549 by silencing viral *PB1* genes.[13] *hsa*-miR-324-5p and *hsa*-miR-485 blocked viral replication in human cells upon infection with highly pathogenic avian influenza H5N1.[44,45] Besides viral polymerases *PB2* and *PB1*, *hsa*-let-7c binding to the 3′ UTR of the H1N1 M1 gene resulted in the control of viral replication in A549 cell lines.[12] However, these recent investigations have shown the effect of human microRNAs infected with IAVs, while only a few studies have reported canine microRNAs targeting viral genomes in response to IAVs so far. According to Song *et al*.,[11] *cfa*-miRNA-323, *cfa*-miRNA-491, and *cfa*-miRNA-654 blocked H1N1 (A/WSN/33) virus replication in MDCK cells by binding to the viral *PB1* gene. In addition, a recent investigation demonstrated that *cfa*-miR-26a inhibited H1N1 virus replication, while *cfa*-miR-939 facilitated the viral replication in MDCK cells.[46]

Unlike previous studies,[11,46] the current investigation showed canine microRNA profiles in MDCK cells infected with seasonal IAVs. This study demonstrated that *cfa*-miR-146b and *cfa*-miR-215 directly bind to the pH1N1 *PB1* gene, whereas *cfa*-miR-197 could interact with H3N2. Furthermore, this work studied canine microRNAs targeting IBVs, which have not been reported previously. The investigation discovered that *cfa*-miR-215 could target the *PB1* of the B/Victoria virus, whereas *cfa*-miR-146b could attach to the *PA* of the B/Yamagata virus. In addition, our microRNA profile results

revealed that *cfa*-miR-1249 was down-expressed upon infection with the influenza A/pH1N1 virus, which is compatible with previous findings.[43] Wang *et al.*[43] discovered that *hsa*-miR-1249-3p, which is bound to the PB2-binding sequence, significantly reduced the replication of influenza H5N1 and pH1N1 viruses in A549 cells. Although the present investigation found that *cfa*-miR-1307 was overexpressed and *cfa*-miR-486 was down-expressed in B/Victoria-infected cells, recent studies revealed both microRNAs targeting viral genomes in human cells. Specifically, *hsa*-miR-1307 could silence the *NS1* gene of pH1N1,[47] while *hsa*-miR-486-5p bound to multiple segments of H1N1 (PR8) or H3N2.[48]

Cellular microRNAs have been shown to attach to the 3′-UTR,[49] 5′-UTR,[50] and coding regions of viral proteins.[11,41] In general, the interactions between microRNA and viral genome result in translational inhibition of the viral genome, preventing viral replication. However, direct binding can stabilize viral RNA, and thus improve replication in some cases.[50–52] Interestingly, the synergistic effort between two or more miRNA-binding sites inside a gene enhanced mRNA translation repression.[53] As a result, the number and position of miRNA-binding sites within a viral genome may affect the function of microRNAs. Even though the current study used individual microRNA mimics/inhibitors, the findings showed that *cfa*-miR-146b and *cfa*-miR-215 could bind to the same *PB1* gene of the pH1N1 virus at positions 2191 and 2155, respectively. Furthermore, several studies have found that the combined efforts of several microRNAs targeting various gene segments had a greater inhibitory impact on some IAV strains than that of individual microRNA treatment.[48] However, the synergistic activity of microRNA mixtures is yet to be experimentally validated. Therefore, it is now feasible to state that canine microRNAs may inhibit the replication of seasonal influenza viruses by binding directly to viral RNAs. Treatment with microRNA inhibitors, however, was able to counteract the suppressive activity of microRNAs, resulting in increased viral propagation yields.

The following points about microRNAs that target viral RNAs deserve particular consideration. More specifically, the maintenance of microRNA-binding sites within the viral genome could pose a challenging issue. It is well-established that influenza viral RNA polymerase does not have a proofreading feature. As a result, incorrect nucleotides are usually incorporated during viral replication.[54–56] Since virus seed strains were unavailable, most of the strains in this experiment were derived from clinical specimens. Nonetheless, in comparison to the sequences of the experimental strains, the microRNA-binding sites of many viral seed strains obtained from NCBI or GISAID were observed. Unfortunately, some microRNA-binding sites of vaccine strains, especially the PB1 gene of pH1N1 viruses A/Michigan/45/2015 and A/Brisbane/02/2018, could not be targeted by *cfa*-miR-215. This finding aligns with a recent study by Bavagnoli *et al.*,[47] who found that a mutation in the *NS1* gene of A/pH1N1 strains found in Italy in 2010–2011 enabled the virus to evade the suppressive effect of *hsa*-miR-1307-3p. Another example is the expeditious loss of RISC-binding sites observed in the *in vitro* treatment of human immunodeficiency virus with siRNAs, which act similarly to microRNAs when they bind to RNAs in a complementary manner.[57,58] Although this study shows that host microRNAs negatively regulate influenza virus replication, the capacity of RNA viruses to evolve away from repression by particular microRNAs should be a concern. In addition, the impact of such microRNA inhibitor treatments on antigenic sequence changes should be taken into account.

To our understanding, acquired mutations in the influenza virus HA and NA surface glycoproteins cause viruses to evade defensive neutralizing antibody responses.[59] Furthermore, after serial passaging in cell culture, influenza viruses could develop mutations in the HA and NA proteins, reducing vaccine effectiveness.[60] As a result, the HA and NA sequences gained from the microRNA inhibitor-treated groups were investigated compared with the parental populations used in this study. Fortunately, the findings revealed no mutations in either *HA* or *NA* were present, although the viruses were propagated in the cells treated with microRNA inhibitors. Finally, the cost of production could be a concern of this approach. MicroRNA inhibitors could be an impractical method for mass production because of their transient function. This gene-editing tool has been shown to knockout or knockdown microRNAs *in vitro*[61–63] and *in vivo*.[64] Furthermore, this notion has been supported by a recent study by Waring *et al.*,[65] identifying that microRNA-21 targets various regions of the viral H1N1 (PR8) genome. The microRNA-21 deficient MDCK cells have the potential to be used as a vaccine platform to propagate viruses targeted by microRNA-21, potentially replacing egg-based vaccine production.[65]

In summary, the present investigation demonstrates the feasibility of manipulating host microRNA to improve viral propagation in MDCK cell-based manufacturing. It is the first time to report canine microRNA profiles in response to human seasonal influenza viruses. According to the findings, microRNAs tend to target viral genes in a strain-specific manner. Consequently, the candidate microRNAs could silence viral genes, causing viral replication to be suppressed. MicroRNA inhibitors, however, can counteract the effect of candidate microRNAs, resulting in enhanced viral yields.

AUTHORS' CONTRIBUTIONS

S.S. and S.P. contributed to the conception and design. S.S., W.P., and S.P. performed the data curation. S.S. and S.P. performed the formal analysis and visualization. SP acquired funding for the studies. S.S., P.N., K.K., W.P., and K.P. performed the investigations. S.S., Y.P., Q.Z., and S.P. performed the methodology. S.S., P.N., and K.K. validated the experiments. S.S. prepared the draft manuscript. Y.P. provided the clinical samples. S.S., S.R., Q.Z., and S.P. performed the final reviews and edits.

DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

ETHICAL APPROVAL

The study protocols were reviewed and approved by the Institutional Review Board (IRB No. 152/59) from the Faculty of Medicine, Chulalongkorn University.

FUNDING

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This study was supported by the National Science and Technology Development Agency (NSTDA) (P-17-51377); the Chulalongkorn Academic Advancement into its 2nd Century Project; the Royal Golden Jubilee (RGJ) PhD Program scholarship (PHD/0150/2558); the National Research Council of Thailand (NRCT; 2564NRCT321520); the Thailand Science Research and Innovation fund (TSRI; CU_FRB640001_01_30_4); the Graduate School, Chulalongkorn University (the 100th Anniversary Chulalongkorn University Fund for Doctoral Scholarship and the Overseas Research Experience Scholarship for Graduate Students of Chulalongkorn University).

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SUPPLEMENTAL MATERIAL

Supplemental material for this article is available online.

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(Received January 16, 2022, Accepted April 15, 2022)