Cellular MicroRNAs Inhibit Replication of the H1N1 Influenza A Virus in Infected Cells

Running title: MicroRNAs inhibit replication of influenza virus

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Abstract

MicroRNAs (miRNAs) are a class of non-coding RNAs of lengths ranging from 18-23 nucleotides that play critical roles in a wide variety of biological processes. There is a growing amount of evidence that miRNAs play critical roles in intricate host-pathogen interaction networks, but the involvement of miRNAs during influenza viral infection is unknown. To determine whether the cellular miRNAs play an important role in H1N1 influenza A viral infections, 3’UTR reporter analysis was used to identify putative miRNA targets in the influenza virus genome, and virus proliferation analysis was used to detect the effect of the screened miRNAs on the replication of H1N1 influenza A virus (A/WSN/33) in MDCK cells. The results showed that miR-323, miR-491, and miR-654 inhibit replication of the H1N1 influenza A virus through binding to pb1. Moreover mutational analysis of the predicted miRNA binding sites showed that the three miRNAs bind to the same conserved region of the pb1 gene. Intriguingly, despite the fact that the miRNAs and pb1 mRNA binding sequences are not a perfect match, the miRNAs downregulate pb1 expression through mRNA degradation instead of translation repression. This is the first demonstration that cellular miRNAs regulate influenza viral replication by degradation of the viral gene. Our findings support the notion that any miRNA has antiviral potential, independent of its cellular function, and that the cellular miRNAs play an important role in the host, defending against virus infection.
Introduction

MicroRNAs (miRNAs) are small RNA molecules with lengths of 21-23nt (21, 41). They have been detected in many plant and animal species, and even in some animal viral RNA genomes (3, 27, 39). MiRNAs regulate many cellular processes including cellular proliferation, apoptosis, homeostasis, and tumor formation by binding to the target mRNAs causing target cleavage or translational block (6, 36). Currently, it is believed that the choice of post-transcriptional mechanisms is determined by the extent to which the miRNAs and their target transcripts are complementary to one another (13, 20, 47). Perfect or near perfect matches, as is common in plant microRNAs and in a small class of animal microRNAs, cause target cleavage and degradation analogous to the action of siRNAs (42). However, in most animal cells, miRNA-mRNA base pairing is imperfect, and the mRNA is not cleaved. Instead, the translational efficiency of the mRNA is reduced (27, 34). In general, the 5` portion of the miRNA (2-8nt, termed the 5`seed region) is perfectly complementary to 3` untranslated region (3`UTR) elements in the mRNA and is thought to be important in mediating post-transcriptional repression (23, 24).

An increasing number of studies suggest that viral miRNAs are key in controlling viral infection in mammalian hosts via several distinct mechanisms (9, 18, 31, 43). Simian virus 40, a member of polyomavirus family, encodes miRNAs that target the gene encoding a major viral protein, the T antigen. The T antigen is a dominant target of the cytotoxic T lymphocyte (CTL) response, and downregulation of its expression decreases CTL-mediated lysis of infected cells (38). Another DNA virus, Herpes simplex virus-1 (HSV-1), is an example of a viral miRNA that targets a cellular gene. A remarkable feature of HSV-1 is the fact that it can establish latent infections and can remain undetected in cells for years. The viral latency associated transcript (LAT)
plays a critical role in this phenomenon by inhibiting apoptosis of infected cells. A miRNA produced from LAT, miR-LAT, targets the cellular mRNAs encoding two components of the TGF-β pathway (TGF-β and the transcription factor SMAD3) that regulate cell proliferation and programmed cell death (15). Therefore, it is clear that viral miRNAs can control expression of viral or cellular genes in order to interfere with the antiviral host defense.

In addition to the role of viral RNAs in the host-pathogen interaction, some reports suggest that cellular miRNAs can also regulate viral infections. For example, miR-32 has been shown to target a sequence in the genome of the primate foamy virus type 1 (PFV-1) (25). Two other cellular miRNAs, miR-24 and miR-93, target the viral large protein (L protein) and phosphoprotein (P protein) genes, and decreased miR-24 and miR-93 expression has been shown to lead to increased vesicular stomatitis virus (VSV) replication (32). Furthermore, Zhang reported that cellular miRNAs potently inhibit HIV-1 production in resting primary CD4\(^+\) T cells (19). They found that the 3`ends of HIV-1 mRNAs are targeted by a cluster of cellular miRNAs that include miR-28, miR-125b, miR-150, miR-223 and miR-382, which are enriched in resting CD4\(^+\) T cells compared to activated CD4\(^+\) T cells. Their data indicate that cellular miRNAs are pivotal to HIV-1 latency and suggest that manipulation of cellular miRNAs could represent a novel approach for purging the HIV-1 reservoir. Another miRNA, miR-122, is specifically expressed and is highly abundant in the human liver. Sequestration of miR-122 in liver cells results in a marked loss of autonomously replicating hepatitis C viral RNAs. Therefore, miR-122 likely facilitates replication of the viral RNA, suggesting that miR-122 may represent a potential therapeutic target for antiviral intervention (10, 22).

The H1N1 Influenza A viruses (IAV) continue to pose serious threats to public health,
as exemplified by the ongoing 2009 H1N1 influenza pandemic (14, 30). Katze report that cellular miRNAs expression can be altered during influenza virus infection, irrespective of the lethality of the virus (26). But the involvement of miRNAs during IAV infection or replication is still unclear. Since there is strong evidence that cellular miRNAs can be used by host cells to resist the viral infection we hypothesized that one or more cellular miRNAs could be involved in the replication of H1N1 IAV. In this report, we screened miR-323, miR-491 and miR-654 which inhibit the replication of the H1N1 IAV in MDCK cells through binding to the conserved region of the pb1 gene. To determine the action model of these miRNAs, we used real-time PCR to detect the pb1 mRNA level in infected MDCK cells overexpressing miR-323, miR-491, and miR-654. The results showed that despite the fact that the miRNAs and pb1 mRNA binding sequences are not a perfect match, the miRNAs downregulate pb1 expression through mRNA degradation. In addition, real-time PCR analysis showed that the endogenous miR-323, miR-491 and miR-654 are expressed in different cell lines and the expression of them can be altered during influenza virus infection. Our results suggest that cellular miRNAs can inhibit the replication of the H1N1 IAV through downregulating the viral gene expression in infected MDCK cells. Our results are consistent with the emerging notion that miRNAs might be broadly implicated in viral infection of mammalian cells, with either positive or negative effect on replication.
Materials and Methods

Cell culture

293T and MDCK cells (purchased from ATCC) were cultured in Earle’s Modified Eagle’s Medium containing 10% fetal bovine serum (HyClone), 2 mM L-glutamine, 100 units/ml penicillin, and 100 g/ml streptomycin at 37°C under 5% CO\textsubscript{2} with 95% air atmosphere.

Plasmid construction

The luciferase expression vector pRL-TK (Promega) was used as the parent vector for 3’UTR reporter analysis experiments. The eight segments of the H1N1 IAV (ha, na, np, ns, pa, pb1, pb2, m) and subfragments of pb1 (pb1-1, pb1-2, pb1-3, pb1-4, pb1-5) were amplified by PCR from the 12 plasmid IAV reverse-genetics system (H1N1, A/WSN/33) and were directionally cloned into the 3’UTR region of the luciferase gene in the pRL-TK vector as shown in Fig.1a. These constructed vectors were named pRL-TK-HA, pRL-TK-NA, pRL-TK-NP, pRL-TK-NS, pRL-TK-PA, pRL-TK-PB1, pRL-TK-PB2, pRL-TK-M, pRL-TK-PB1-1, pRL-TK-PB1-2, pRL-TK-PB1-3, pRL-TK-PB1-4, and pRL-TK-PB1-5. In order to facilitate cloning, an XbaI restriction site was added to the 5’primer and the 3’primer. For Western blot assays, the luciferase and luciferase-pb1-5 fragment were amplified by PCR using the pRL-TK-PB1-5 vector as a template. The amplified fragment was then cloned into the EcoRI and XhoI sites of the pcDNA3.0-FLAG vector (Invitrogen), generating the Flag-TK and Flag-TK-PB1-5 vector. The pb1 fragment was amplified by PCR using the PB1 vector in the 12 plasmid IAV reverse-genetics system as a template. The amplified fragment was then cloned into the Hind III and XhoI sites of the pcDNA3.0-FLAG vector (Invitrogen), generating the Flag-PB1 vector. All inserts were sequenced in their entirety in order to verify polymerase fidelity. The primer
sequences are available from the corresponding author upon request.

**Construction of mutant plasmids**

In order to identify the miRNA binding sites in the pb1 gene, the nucleotide sequences of putative binding sites in pb1 were mutated in pRL-TK-PB1-5, Flag-PB1, and PB1 in the 12 plasmid IAV reverse-genetics system using the Muta-direct™ kit (SBS) according to the manufacturer’s protocol. The primers were designed according to the instructions provided in kit (primer sequences are available from the corresponding author upon request). Due to the low efficiency of recovery of mutants when four nucleotides are changed at once, the mutant vectors were obtained through two successive rounds of PCR. Mutant plasmids were generated by PCR using 20ng of the parent vector as template under the following conditions: 95°C for 5min, followed by 25 cycles of 95°C for 30sec, 58°C for 1min and 72°C for 8min. The resulting mixture was digested with 1µl of Dpn-1 for 30min at 37°C in order to remove the parental DNA. The remaining DNA was used to transform DH5α (TaKaRa), and a reasonable number of colonies were obtained. Mutant plasmids were confirmed by sequencing.

The mutated pRL-TK-PB1-5, Flag-PB1 and PB1 vectors were named ΔpRL-TK-PB1-5, ΔFlag-PB1 and ΔPB1, respectively. In order to construct expression vectors for the mutant miRNAs, the mutant sequences were synthesized directly. The mutant miRNA vectors were named ΔmiR-323, ΔmiR-491 and ΔmiR-654.

**Prediction of miRNA-binding sites**

MiRNAs binding sites were predicted according to the principles of miRNA target recognition (5, 7, 33, 44). In brief, target sites for miRNAs were predicted using the MicroInspector algorithm at http://bioinfo.uni-plovdiv.bg/microinspector/. The cutoff values for hybridization temperature and free energy were set to 37°C and -17kcal/mol, respectively. Identified miRNA-target gene pairs were confirmed using
RNAHybrid at http://bibiserv.techfak.uni-bielefeld.de/ and rna22 miRNA target predictor at http://cbcsrv.watson.ibm.com/rna22.html. Finally, cross-species sequence comparison was used to detect whether the target sequence had been evolutionarily conserved between related species.

**MiRNA inhibitors and expression vectors**

MiRNA inhibitors were purchased from Ambion (AM17000). The psiSTRIKE™ vectors (Promega) were used to construct miRNA expression vectors according to the manufacturer’s protocol. Gene sequences for miRNAs were acquired from the Sanger miRNA Registry at http://microrna.sanger.ac.uk/sequences/. In short, the primers were annealed to form a double-stranded DNA and were then inserted into the psiSTRIKE™ vectors. Bacteria were transformed with the resulting constructs, and a reasonable number of colonies were obtained. The resulting miRNA expression vectors were confirmed by digestion with the restriction enzyme PstI according to the manufacturer’s protocol. In addition, we constructed the expression vector of *C. elegans* miR-239b and siRNA-pb1 as the negative control and the positive control, respectively. Primer sequences are available from the corresponding author upon request.

**Transfection**

The plasmids and miRNA inhibitors were transfected into cells using TransFast™ transfection reagent (Promega).

To determine whether miRNAs play a direct role in repression of luciferase expression from the pRL-TK vector containing the viral gene, 293T cells were plated in 24-well plates. When the cells reached 60-70% confluence, they were co-transfected with the pRL-TK vector containing the appropriate viral gene (0.5µg) and the pGL3-Control Vector (0.01µg). The empty pRL-TK vector and pGL3-Control
Vector were used as a negative control. After 30 hours, the cells were harvested for relative luciferase assay analysis.

To determine the effect of miRNAs on the luciferase expression from pRL-TK-PB1-5, 293T cells were plated in 24-well plates. When the cells reached 50-60% confluence, they were transfected with the indicated miRNA expression vectors (0.5µg). After 24 hours, the cells were co-transfected with the pGL3-Control Vector (0.01µg) and the pRL-TK-PB1-5 vector (0.5µg). The cells were harvested at 30 hours post-transfection and were analyzed for relative luciferase activity.

To determine whether miR-323, miR-491, and miR-654 could downregulate the luciferase expression in Flag-TK-PB1-5, 293T cells were plated in 6-well plates. When the cells reached a confluence of 50-60%, they were either transfected with the appropriate miRNA expression vector alone (2.5µg), or were cotransfected with the appropriate miRNA expression vector (1.5µg) and the appropriate miRNA inhibitor (150pmol). At 24 hours post-transfection, cells were transfected with the Flag-TK-PB1-5 vector or Flag-TK (2.5µg). After 48 hour, the cells were collected for Western blot analysis.

To investigate the miRNA binding sites in pb1, 293T cells were plated in 24-well plates. When the cells reached 50-60% confluence, they were transfected with wildtype or mutant miRNA expression vectors (0.5µg). After 24 hours, the cells were cotransfected with the pGL3-Control Vector (0.01µg) and the pRL-TK-PB1-5 vector or ΔpRL-TK-PB1-5 vector (0.5µg). The cells were harvested at 30 hours post-transfection and were analyzed for relative luciferase activity.

To determine the effect of miR-323, miR-491, and miR-654 on the pb1 expression from Flag-PB1, MDCK cells were plated in 6-wells plates. When the cells reached a confluence of 50-60%, they were transfected with the miRNA expression vector
(2.5µg). At 24 hours post-transfection, cells were transfected with the Flag-PB1 or ΔFlag-PB1 vector (2.5µg). After 48 hour, the cells were collected and analyzed with Western blot assay and real-time PCR.

In order to determine the effects of miRNAs on the replication of H1N1 IAV, MDCK cells were plated in 6-well plates. When the cells reached a confluence of 70-80%, the miRNA expression vectors (0.5µg) or specific miRNA inhibitors were transfected into cells. After 24 hours, the cells were infected with H1N1 IAV or with mutant H1N1 IAV at a hemagglutination (HA) value of 4. The virus replication was measured by HA assay, fluorescence and real-time PCR.

**Luciferase assay**

Luciferase assays were performed using the Dual-Luciferase reporter assay system kit (Promega) according to the manufacturer’s protocol. Collected cells were washed once with cold phosphate-buffered saline (PBS). Passive lysis buffer (100µl) was then added to the cells. After 10min, the supernatants were collected by centrifugation at 12000g for 30 sec and the relative luciferase expression values were analyzed using the Modulus™ Single Tube Multimode Reader (Promega).

The relative luciferase expression = the expression of Renilla luciferase (pRL-TK)/the expression of Firefly luciferase (pGL3-Control vector)

**Western blot**

After transfection, the cells were collected and washed with cold PBS three times. The PBS was decanted, and the cell pellet was resuspended in 100µl of lysis buffer (Promega) for 10min on ice. The resulting solution was then combined with 25µl 5×SDS sample buffer and boiled for 10 min. The samples were resolved on a 12% SDS-PAGE gel and transferred to a PVDF membrane (Amersham Bioscience). The membranes were blocked using 5% nonfat dry milk for 1h. Membranes were then
incubated overnight at 4°C in a purified primary mouse anti-flag antibody and an
anti-β-actin antibody (BD Biosciences), each at a 1:200 dilution in 5% nonfat milk.
After three washes with Tris-Buffered Saline containing 0.05% Triton-X100 (TBST),
the membranes were incubated for 1h at room temperature with the appropriate
horseradish peroxidase-conjugated secondary antibody (Santa Cruz) at a 1:5000
dilution in TBST. Protein bands were visualized using the Super ECL-plus system
(Applygen Technologies). β-actin was used as a loading control.

Real-time reverse transcription PCR analysis
In order to determine the level of expression of pb1 mRNA or ∆pb1 mRNA, total
RNA was prepared, and 2µg of total RNA was reverse transcribed into cDNA using
the QuantiTect® Reverse Transcription kit (Qiagen) according to the manufacturer’s
protocol. The level of β-actin mRNA was measured as control. PCR amplification of
β-actin and pb1 or ∆pb1 cDNAs was carried out.
In order to detect the cellular expression level of miRNAs, total RNA was prepared,
and 2µg of the total RNA was reverse transcribed into cDNA using the QuantiMir
Complete kit (SBI) according to the manufacturer’s protocol. PCR amplification of
the miRNA cDNAs was carried out. The expression of U6 was measured as a control
using the primers provided in the kit.
Real-time PCR was conducted using 2µl of cDNA diluted 1:50 and SYBR® Premix
Ex Taq™ II (TaKaRa). Cycling conditions for real-time PCR were as follows: 95°C
for 1min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. Real-time
PCR was conducted using the ABI PRISM 7300 sequence detection system, and the
data was analyzed with ABI PRISM 7300 SDS software (Applied Biosystems). The
primer sequences used in real-time PCR are available from the corresponding author
upon request.
**Virus infection**

H1N1 IAV (A/WSN/33) or mutant H1N1 IAV was used to infect the MDCK cells. The cells were washed with PBS three times, followed by infection with the virus in infection medium (culture medium lacking FCS and containing 2.5µg/ml trypsin). The HA value of the viruses was 4. After incubation for 1h, the cells were washed three times with PBS, and then infection medium was added to the cells. The infected cells were cultured at 35°C in 5% CO₂.

**HA assay**

The HA assay was carried out in V-bottom 96-well plates. Serial 2-fold dilutions of virus samples were mixed with an equal volume of a 0.5% suspension (vol/vol) of chicken erythrocytes and incubated for 20 min. Wells containing an adherent, homogeneous layer of erythrocytes were scored as positive.

**Fluorescence microscopy**

Infected MDCK cells were grown on coverslips. The cells were fixed by incubation in cold acetone for 10 min. The coverslips were washed three times with PBS. The cells were then incubated overnight at 4°C with an anti-HA mouse monoclonal antibody at 1:50 (Santa Cruz). The coverslips were washed three times with PBS. The cells were then incubated in the secondary antibody, goat anti-mouse-FITC (Santa Cruz) at 1:200, for 1h at room temperature. Following this incubation, the cells were washed three times with PBS and mounted onto slides in 90% glycerin and sealed with nail polish. Stained cells were observed by fluorescence microscopy (Olympus, MT6000 Fluorescent).

**Generation of mutant infectious influenza particles**

The 12 plasmid IAV reverse-genetics system was kindly provided by George F GAO (Institute of Microbiology, Chinese Academy of Sciences, Beijing). Using this system,
the pb1 nucleotide sequence was mutated without changing the acid amino sequence.

To generate the mutant IAV, 293T cells were plated in a 100 mm dish. When the cells reached 80-90% confluence, the 12 plasmid IAV system containing ΔPB1 (2µg/vector, totaling 24µg of vectors in 6ml of culture medium) was transfected into 293T cells using TransFast™ transfection reagent (Promega). After 72 hours, the influenza viruses were harvested and propagated in MDCK cells. The mutations in ΔPB1 were confirmed by sequencing.
Results

*Pb1* gene of the H1N1 IAV may harbor potential miRNA binding sites

Recently, an increasing number of reports suggest that cellular miRNAs play an important role in viral replication. Therefore, we utilized a 3’UTR reporter assay to determine whether cellular miRNAs have a direct role in repression of H1N1 IAV gene expression in cells. Eight fragments of the H1N1 IAV were subcloned into the luciferase reporter plasmid (pRL-TK) between the luciferase open reading frame and the polyadenylation signal (Fig. 1a). As shown in Fig. 1b, the relative luciferase activity in 293T cells cotransfected with pRL-TK-PB1 and pGL3-Control vector (bar 1) was approximately 30% less than that of cells cotransfected with pRL-TK and pGL3-Control vector (bar 9). We speculated that the decreased expression of the reporter gene may be attributed to miRNA mediated repression. In order to better characterize the location of this element, five smaller fragments of *pb1* (Fig.1c) were individually inserted into the 3’UTR of the luciferase gene in the pRL-TK vector (Fig. 1a). As shown in Fig. 1d, the relative luciferase activity in cells cotransfected with pRL-TK-PB1-5 and pGL3-Control vector (bar 5) was substantially decreased in comparison to the control (bar 6). These results suggest that *pb1*-5 might harbor potential binding sites for miRNAs that inhibit expression of the luciferase gene.

miR-323, miR-491, and miR-654 effectively inhibit luciferase expression in Flag-TK-PB1-5

To determine whether miRNAs are functional inhibitors of luciferase expression in pRL-TK-PB1-5, we searched for putative miRNA-binding sites in the *pb1*-5 fragment using the MicroInspector program. The putative binding sites were further verified using the RNA22 and RNAHybrid programs. It was determined that *pb1*-5 harbors putative binding sites for miR-323, miR-491, miR-654, miR-639, miR-591, miR-608, miR-608,
miR-601, miR-26a, miR-509, miR-378, miR-541, and miR-939 through these analyses. In order to determine whether these miRNAs inhibit expression of luciferase in pRL-TK-PB1-5, 293T cells were transfected with the miRNA expression vectors which could express the miRNAs effectively (data not shown), followed by cotransfection with pRL-TK-PB1-5 and pGL3-Control vector. The relative luciferase activity was determined using the Dual-Luciferase reporter assay system kit. As shown in Fig. 2a, the relative luciferase activity was downregulated in the cells with overexpressing miR-323, miR-654, miR-639, miR-591, miR-608, miR-601, miR-26a, miR-509, miR-939 or miR-491 in comparison to the control (p<0.01). The miRNAs which downregulate luciferase expressions in pRL-TK-PB1-5 by approximately 50% or less, compared to the control sample, were selected for further research. According to this criterion, miR-323, miR-654, miR-608, miR-601 and miR-491 were selected. The complementary sequences of 5’ seed region of miRNAs in the target gene were thought to be the putative binding sites of miRNAs, so we blasted the putative binding sites in NCBI database. The results showed that the putative binding site of miR-323, miR-491 and miR-654 are highly conserved across a variety of influenza viral strains (Table 1). So miR-323, miR-491 and miR-654 were thought to more likely to target and regulate the expression of pb1.

To further investigate whether miR-323, miR-491, and miR-654 could downregulate luciferase expression in pRL-TK-PB1-5, two additional experiments were carried out. As shown in Fig. 2b, luciferase expression from Flag-TK in 293T cells with overexpressing miR-323, miR-491, or miR-654 was similar to that of the control while the luciferase expression from Flag-TK-PB1-5 was less than that of the control. The results indicated that miRNAs downregulate the luciferase expression due to pb1-5 fragment. The second experiment utilized miRNA inhibitors purchased from Ambion.
to further verify the function of the miRNAs in inhibition of luciferase expression in Flag-TK-PB1-5. Consistent with the role of miRNA inhibitors in blocking miRNA function, expression of luciferase in 293T cells cotransfected with both the miRNA expression vector and its specific inhibitor was higher than that of cells cotransfected with miRNA expression vector and the negative control miRNA inhibitor (Fig. 2c).

These results demonstrate that miR-323, miR-491, and miR-654 downregulate the luciferase expression when the pb1-5 fragment was inserted into the 3′ UTR of luciferase. Therefore the pb1-5 gene fragment likely harbors binding sites of miR-323, miR-491, and miR-654.

**miR-323, miR-491, and miR-654 bind to the same conserved region in the pb1 gene**

The results imply miR-323, miR-491, and miR-654 downregulate the expression of *luciferase-pb1-5* through binding to a sequence in *pb1-5* fragment; however, the precise binding sites of the miRNAs are unknown. Sequence analysis indicates that the 5′ seed sequence of these miRNAs all contain the same nucleotide sequence 5′GUGG3′, which perfectly matches a 5′CCAC3′ sequence found in the *pb1* gene (Fig. 3a). The modes of hybridization between the miRNAs and the pb1 mRNA were predicted using RNAHybrid software as shown in Fig. 3b. Based on the similarity between the three 5′ seed sequences, the three miRNAs were thought to bind to the same region of the pb1 gene. To investigate the miRNA binding sites in pb1, the 5′CACC3′ predicted miRNA binding site in pb1 were mutated to 5′GGAA3′ in the pRL-TK-PB1-5 vector as shown in Fig. 3c, generating the ΔpRL-TK-PB1-5 vector. In order to analyze the effects of this mutation, 293T cells were transfected with miRNA expression vectors, followed by co-transfection with pGL3-Control vector and either ΔpRL-TK-PB1-5 or pRL-TK-PB1-5. The results from this experiment showed that
miRNA expression does not result in repression of the relative luciferase activity in cells transfected with ΔpRL-TK-PB1-5 (Fig. 3e). To further confirm these results, the 5′ seed sequences of the miRNAs were mutated in their respective expression vectors in a manner in that complements the mutations in ΔpRL-TK-PB1-5 as shown in Fig. 3d. The mutant miRNAs were named ΔmiR-323, ΔmiR-491, and ΔmiR-654. In order to test whether the mutant miRNAs could inhibit luciferase expression from ΔpRL-TK-PB1-5, 293T cells were transfected with vectors expressing the ΔmiRNAs, followed by co-transfection with ΔpRL-TK-PB1-5 and pGL3-Control vector. The results showed that the ΔmiRNAs inhibit the relative luciferase activity from ΔpRL-TK-PB1-5 (Fig. 3e). These results demonstrate that all three miRNAs bind to the same sequence 5′CCACC3′ in the pb1 gene. And the binding sites are critical for miRNA-mediated repression of the relative luciferase activity from pRL-TK-PB1-5 and pGL3-control vector. In addition, the binding sites of the miRNAs are located in the 3′ coding region of the pb1 gene and are highly conserved across a variety of influenza viral strains through alignment of strain genomes from the NCBI database (Table 1).

miR-323, miR-491, and miR-654 downregulate pb1 expression by degradating mRNA in MDCK cells

Since miR-323, miR-491, and miR-654 can downregulate the luciferase expression by binding to the pb1-5 fragment, we investigated whether these miRNAs directly regulate pb1 expression through the binding sites. As shown in Fig. 4a, the binding sites of miRNAs in the pb1 gene were mutated and the mutant vector was termed ΔFlag-PB1. The results showed that the pb1 expression in MDCK cells overexpressing miR-323, miR-491, or miR-654 was less than that of the control. When the binding sites of miRNAs in pb1 were mutated, three miRNAs could not...
inhbit pb1 expression (Fig. 4b). The results indicate that miR-323, miR-491, and miR-654 can inhibit the pb1 expression and that the specific binding sites on the pb1 gene are very important for inhibition.

In general, miRNAs downregulate the target genes through mRNA degradation or translation inhibition. In order to characterize the mechanism of inhibition in the present study, we utilized real-time PCR to detect the level of pb1 mRNA in MDCK cells overexpressing miRNAs. The results showed that the expression of pb1 in MDCK cells with overexpressing siRNA-pb1, miR-323, miR-491, or miR-654 was reduced (p<0.01) compared to the negative control (Fig. 4c). These observations indicate that the miRNAs downregulate the expression of pb1 through mRNA degradation. Additional, real-time PCR analysis was utilized to further assess the importance of the miRNA binding sites in the pb1 gene in suppression of gene expression. The results showed that none of the three miRNAs inhibited expression of the mutated pb1 mRNA while siRNA-pb1 still downregulated the expression level of the mutated pb1 mRNA (Fig. 4c). The results further confirmed that the miRNA binding sites 5'CCACC3' in pb1 gene are critical for miRNA function.

miR-323, miR-491, and miR-654 inhibit replication of the H1N1 IAV through binding to the pb1 gene.

To determine whether miR-323, miR-491, and miR-654 regulate replication of the H1N1 IAV in MDCK cells, the three miRNAs expression vectors were transfected individually and collectively as a mixture into MDCK cells. Twenty-four hours after transfection, the cells were infected with the H1N1 IAV, and the HA value was measured at different time points. The HA measurements increased dramatically between 0 and 24h, and continued to increase till 48h, but the value began to fall after 60h of infection. All of the miRNAs inhibited viral replication individually, and the
miRNA mixture had no greater antiviral capability than the individual miRNAs (Fig. 5a). These results were verified by fluorescence microscopy (Fig. 5b).

To further confirm that these miRNAs inhibit replication of the H1N1 IAV, specific miRNA inhibitors were cotransfected into MDCK cells with the miRNA expression vectors, and the transfected cells were infected by H1N1 IAV. After 24h, the infected cells were collected to analysis the expression of pb1 by real-time PCR. The results of real-time PCR showed that the relative expressions of pb1 in infected MDCK cells with overexpressing the miRNAs and negative miRNA inhibitors (bar 2, 4, and 6) were lower than that of in the infected cells with overexpressing miRNAs and specific miRNA inhibitor (bar 3, 5 and 7) (Fig. 5c).

In order to investigate whether the miRNA binding sites in the pb1 gene are important for inhibition of replication of the H1N1 IAV, we mutated the pb1 gene in the 12 plasmid influenza virus system as shown in Fig. 4a. The mutant viruses were generated by transfecting the 12 plasmid system containing the mutant pb1 gene into 293T cells. To analyze the effects of miRNAs on replication of the mutant virus, MDCK cells were transfected with vectors expressing C.elegans miR-239b, miR-323, miR-491, or miR-654. At 24h post-transfection, the cells were infected with the mutant IAV. The HA values were measured at 24h, 36h, 48h and 60h after infection. The results showed that the miRNAs did not inhibit replication of the mutant influenza virus as effectively as the wildtype virus (Fig.5d). These observations indicate that the specific miRNA binding sites in the pb1 gene are critical for inhibition of replication of the influenza virus.

The endogenous miR-323, miR-491 and miR-654 inhibit the replication of H1N1 IAV in MDCK cells

Since exogenous miR-323, miR-491 and miR-654 can inhibit the replication of H1N1
IAV in MDCK cells, whether endogenous miR-323, miR-491 and miR-654 are involved in the virus replication. To evaluate the possibility of endogenous miRNAs involvement in the replication of IAV, the endogenous expression level of miR-323, miR-491 and miR-654 were detected using real-time PCR in different cell lines. As shown in Fig.6, the endogenous miR-323, miR-491 and miR-654 were expressed in A549, HLF, QSG, MCF-7, 293T, MDCK, HeLa, 16HBE and WISN. Expression of miR-491 was not detected in HLF cells and miR-654 was particularly highly expressed in MDCK cells. The results suggest that endogenous miR-323, miR-491 and miR-654 could be utilized to regulate the replication of H1N1 IAV in MDCK cells.

We then investigated whether endogenous miR-323, miR-491 and miR-654 were involved in virus replication. The MDCK cells transfected with the specific miRNAs inhibitors were infected by H1N1 IAV and the HA values were determined 24 hours after infection. The results showed that blocking the function of these three endogenous miRNAs, by their specific inhibitors, resulted in increased IAV production from MDCK cells (Fig. 7). The results suggest that the endogenous three miRNAs have antiviral potential independent of their cellular function.
Discussion

Studies exploring the interaction between cellular miRNAs and the IAV are critical for providing insight into IAV resistance. In this report, we screened three cellular miRNAs which can inhibit the replication of the H1N1 IAV through the degradation of pb1 mRNA in MDCK cells.

It is well known that IAV has a major impact on both human health and on the global economy. The worst known case was the Spanish Flu of 1918 which caused the deaths of 30 to 50 million people (35). Few influenza viruses are sufficiently virulent to directly cause death in humans, instead, most deaths are due to an increased physiologic load in an already compromised host, or due to the combined effects of the viral disease and a secondary bacterial infection (29). Segment 2 of the influenza virus gene has two open reading frames (ORFs). One of them encodes PB1, which is involved in both transcription and replication of the RNA genome (40). The second ORF encodes the PB1-F2 protein which is proinflammatory, can contribute to virulence and facilitates secondary bacterial infections (12, 28). In this study, we have identified three cellular miRNAs which bind to and degrade pb1 mRNA. The low expression of the PB1 protein has been shown to inhibit replication of the influenza virus, and downregulate PB1-F2, reducing viral virulence and decreasing the host’s susceptibility to secondary bacterial infections (45, 46). Therefore, the three miRNAs identified not only inhibit the replication of H1N1 IAV but also have the potential to reduce the pathogenicity of IAV in humans.

The prevailing wisdom is that miRNAs regulate post-transcriptional processes by binding to the 3′UTR of target genes. However, Sarnow reported that miR-122 regulates viral replication by binding to the 5′-noncoding region (5′NCR) of the viral genome (22). In the present study, mutational analysis showed that miR-323, miR-491
and miR-654 bind to the same 3’ coding region rather than the 3’UTR in pb1 gene. The length of the influenza viral genome is limited, and the virus has to use every nucleotide efficiently. Therefore, it is unlikely that the pb1 gene could contain a 3’UTR sequence long enough to contain miRNA binding sites. The miRNAs have to bind to the coding region of the viral genes in order to perform their functions. Our finding raises the question of whether the coding regions in other viral or host cell mRNAs can also be targeted by miRNAs.

The extent to which sequences of the miRNA and the target mRNA complement each other is thought to play an important role in the effects caused by miRNAs. In general, if the sequences of the miRNA and the mRNA are perfectly matched, the target mRNA is degraded. Repression of translation is the most common effect if the sequence of miRNA and the mRNA do not match one another perfectly. However, perplexing observations contrary to this model have come from studies of plant miRNAs. For example, miR-172 appears to regulate APETALA2 via translational repression despite near-perfect match between the miRNA and its single complementary site in the APETALA2 ORF (2, 11). In this study, we used real-time PCR to detect pb1 mRNA expression levels in the presence of miR-323, miR-491, and miR-654 overexpression. The results showed that despite the fact that the miRNA sequences and the pb1 mRNA do not complement one another very well (Fig.3b), the miRNAs downregulate expression of the PB1 protein through mRNA degradation. These results indicate that other mechanisms may play a role in directing the action of miRNAs.

An intrinsic property of all viruses is their need to either subvert and/or subdue the host immune response in order to establish a productive replication cycle. The ability to subvert and disable host immunity is directly correlated with increased viral
pathogenicity. IAV has many mechanisms to avoid both innate and adaptive human immune responses (12). NS1, an unstructured IAV protein, is responsible for inactivation of the hosts innate immune response by preventing activation of PKR from INF-α/β signaling, thus allowing replication and viral protein synthesis to proceed unabated in the host cell (17). Additionally, IAV has the ability to escape the host’s humoral immunity through a phenomenon known as antigenic drift. Because of this new flu vaccines must be designed every year (37). Since IAV is an eight-segmented minus-sense RNA virus, its segmented nature allows for the swapping and exchange of gene segments between different strains. Specifically, this process occurs through human influenza viruses swapping the HA glycoprotein, NA glycoprotein, or polymerase (PB1, PB2, PA) segments with those of avian and pig IAV. As a result, a rearrangement of gene segments has occurred creating an entirely novel IAV strain capable of infecting humans (35). While growing evidence has shown that cellular miRNAs could be used by host cells to resist the viral infection, viruses have been found to possess mechanisms to subvert the antiviral function of miRNAs. Several mammalian viruses have been shown to encode viral factors that exhibit RNA silencing suppressor (RSSs) activity in animal cells. These factors include the IAV NS1, vaccinia virus E3L, hepatitis C virus Core, primate foamy virus type 1 (PFV-1) Tas, and the HIV-1 Tat proteins, as well as the adenovirus virus-associated RNAs I and II (VAI and VAIi) (1, 4, 8, 16, 25).

In our study, we found that miR-323, miR-491 and miR-654 can inhibit the replication of the virus in MDCK cells and the three of miRNAs are expressed in different cell lines. In addition, the expression levels of the three miRNAs can be altered during H1N1 IAV infection in MDCK cells (Fig. 8). The upregulation of miRNAs expression in MDCK cells reflect the mechanism of the host cells defense against viral infection.
The virus has to downregulate the expression of antiviral factors in order to replicate in host cells. The changing model of the three endogenous miRNAs after infection may reflect the resistant procession between the host cells and influenza virus. Our results suggest that cellular miRNAs are important factors in the host’s resistance to viral infection and provide a deeper understanding of the mechanisms underlying the defense system of host cells. The existence of such different defensive tools against new acquisitions to the viral genome underscore the importance of this phenomenon for viral and host interactions.

ACKNOWLEDGMENTS

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### Reference

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<td>12</td>
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<td>2007</td>
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123:368-71.


36:305-32.


Figure legends

Fig. 1 The pb1 gene may harbor potential binding sites for miRNAs
(a) Circular map of the pRL-TK vector. The IAV genes were inserted into the Xba I sites. (b) The relative luciferase activity from the pRL-TK reporter constructs carrying one of eight fragments of the H1N1 IAV and pGL3-Control vector. (c) Schematic map of the pb1 genome. Numbers in parentheses denote nucleotide positions spanned by fragments of the pb1 gene that were inserted into the 3′ UTR of the luciferase gene in pRL-TK. (d) The relative luciferase activity from pRL-TK reporter constructs carrying fragments from the dissection of pb1 gene and pGL3-Control vector.

Fig. 2. miR-323, miR-491, and miR-654 inhibit expression of the luciferase through binding to the pb1-5 fragment
(a) The relative luciferase activity from pRL-TK-PB1-5 and pGL3-Control vector in 293T cells with overexpressing miRNAs. The control is the expression vector of C. elegans miR-239b. (b) Western blotting was used to detect the effects of miR-323, miR-491, and miR-654 on luciferase expression in 293T cells transfected with Flag-TK-PB1-5 or Flag-TK. (c) Western blotting was used to further detect the effects of miRNA inhibitors on luciferase expression in Flag-TK-PB1-5. The expressions of β-actin were analyzed as a control in Western blot.

Fig. 3 miR-323, miR-491, and miR-654 bind to the same region in the pb1 gene
(a) The sequences of miR-323, miR-491, miR-654, and pb1-5 mRNA. The same nucleotide sequence in miRNAs 5′GUGG3′ perfectly matches 5′CCAC3′ sequence in the pb1 mRNA. (b) The model of hybridization between the miRNAs and pb1-5 mRNA were predicted using RNAHybrid software. (c) The mutant nucleotide
sequence of pRL-TK-PB1-5 and sequencing analysis. (d) The mutant nucleotide sequence of the miRNAs. And the mutant sequences 5’UUC3’ in ΔmiRNAs perfectly match 5’GGAA3’ sequence in ΔpRL-TK-PB1-5 mRNA. (e) Luciferase assay to analyze the importance of binding site of miRNAs in pb1 gene for miRNAs to inhibit the relative luciferase activity from pRL-TK-PB1-5 and pGL3-Control vector.

**Fig.4 miR-323, miR-491, and miR-654 downregulate the pb1 expression through mRNA degradation in MDCK cells**

(a) The mutant pb1 gene. The nucleotide sequence of pb1 was altered, but not the amino acid sequence. (b) Western blotting was used to detect the effects of miR-323, miR-491, and miR-654 on pb1 expression or mutant pb1 expression in MDCK cells. 1 to 5 represents the samples in MDCK cells and MDCK cells overexpressing *C. elegans* miR-239b, miR-323, miR-491, and miR-654 respectively. (c) Real-time PCR was used to detect the expression level of the pb1 mRNA and mutant pb1 mRNA in MDCK cells and MDCK cells overexpressing *C. elegans* miR-239b, siRNA-pb1, miR-323, miR-491, and miR-654 respectively. The expression vector of *C. elegans* miR-239b and siRNA-pb1 were used as a miRNA negative control or positive control respectively.

* The results were different significant, p<0.01.

**Fig.5 The miRNAs inhibit the replication of H1N1 IAV through binding to the pb1 gene in MDCK cells**

(a) The HA values were used to assess the effects of miRNAs on replication of H1N1 IAV in MDCK cells, the HA values were determined in triplicate at different time points. (b) Fluorescence microscopy was used to verify the effect of miRNAs on replication of H1N1 IAV in MDCK cells. (c) Real-time PCR was used to detect the
effects of miRNA expression vectors and miRNA inhibitors on the relative
expressions of pb1 in virus infected MDCK cells. (d) The HA values were used to
determine the effect miRNAs on replication of mutant H1N1 IAV in MDCK cells, the
HA values were determined in triplicate at different time points.

The expression vector of *C. elegans* miR-239b was used as negative control.

**Fig.6** The relative expression levels of miR-323, miR-491 and miR-654 in
different cell lines.

The relative expression of miR-323$=10^\times 2^{(Ct1-Ct2)}$

The relative expression of miR-491$=100^\times 2^{(Ct1-Ct2)}$

The relative expression of miR-654$=1000^\times 2^{(Ct1-Ct2)}$

Ct1: the Ct value of U6; Ct2: the Ct value of miRNAs

**Fig.7** The endogenous miR-323, miR-491 and miR-654 inhibit the replication of
HIN1 IAV in MDCK cells

* The results were different significant, $p<0.01$

**Fig.8** The relative expression of miR-323, miR-491 and miR-654 in MDCK cells
with or without HIN1 IAV infection

X-axis represents the different infected time point.

**Table 1** The conserved binding site of miR-323, miR-491, and miR-654 in the pb1
gene compared across a selection of influenza viral strains (collected from NCBI,
[22/01/2010]).
Table 1

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miR-323 5’AGGUGGUCCGUGCGGUCGC3’
miR-491 5’AGUGGGAGCCCUUCCAUGAGG3’
miR-654 5’UGGGUGCCUGCGGAAGAUCUAGC3’
pb1-5 mRNA 3’UACCACCUGUCUAGAAGUACUAG5’

miR-323 5’AGGUGGUCCGUGCGGUCGC3’
ΔmiR-323 5’AUUUCCGUGCGGUCGCUGC3’
Δpb1-5 mRNA 3’AAGGCUUGUCUAGAAG5’

miR-491 5’AGUGGGAGCCCUUCCAUGAGG3’
ΔmiR-491 5’UCCGGAGCCCUUCCAUGAGG3’
Δpb1-5 mRNA 3’AAGGCUUGUCUAGAAG5’

miR-654 5’UGGGUGCCUGCGGAAGAUCUAGC3’
ΔmiR-654 5’UUUCGCGCCUGCGGAAGAUCUAGC3’
Δpb1-5 mRNA 3’AAGGCUUGUCUAGAAG5’

pRL-TK-PB1-5 5’TTCACCCATTGAGAGCTCAGATC3’
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Relative Luciferase Activity

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a) The relative expression of miR-323

b) The relative expression of miR-491

c) The relative expression of miR-654
The relative expression of miR-323

The relative expression of miR-491

The relative expression of miR-654