Influenza A Virus Infection of Human Respiratory Cells Induces Primary MicroRNA Expression*

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Background: The mammalian antiviral response is regulated by virus-induced changes in cellular gene expression.

Results: Cellular miRNAs are induced by influenza virus to regulate antiviral response genes including IRAK1 and MAPK3.

Conclusion: Influenza virus infection alters the miRNA profile of human cells to regulate innate immune signaling.

Significance: The miRNA response to influenza virus identifies new pathways of cell regulation that modulate the mammalian innate antiviral response.

The cellular response to virus infection is initiated by recognition of the invading pathogen and subsequent changes in gene expression mediated by both transcriptional and translational mechanisms. In addition to well established means of regulating antiviral gene expression, it has been demonstrated that RNA interference (RNAi) can play an important role in antiviral responses. Virus-derived small interfering RNA (siRNA) is a primary antiviral response exploited by plants and invertebrate animals, and host-encoded microRNA (miRNA) species have been clearly implicated in the regulation of innate and adaptive immune responses in mammals and other vertebrates. Examination of miRNA abundance in human lung cell lines revealed endogenous miRNAs, including miR-7, miR-132, miR-146a, miR-187, miR-200c, and miR-1275, to specifically accumulate in response to infection with two influenza A virus strains, A/Udorn/72 and A/WSN/33. Known antiviral response pathways, including Toll-like receptor, RIG-I-like receptor, and direct interferon or cytokine stimulation did not alter the abundance of the tested miRNAs to the extent of influenza A virus infection, which initiates primary miRNA transcription via a secondary response pathway. Gene expression profiling identified 26 cellular miRNAs targeted by these miRNAs, including IRAK1, MAPK3, and other components of innate immune signaling systems.

The cellular innate immune response is an important early line of defense against virus infection. Components of virions and virus replication intermediates are detected by several receptors, including Toll-like receptors (TLRs)3 and RIG-I-like receptors (RLRs), which signal through several intermediates to stimulate transcription factor activation and expression of an antiviral gene program (1). The protein products of these genes produce a broadly effective cellular resistance to virus infection referred to as the antiviral state. In addition to direct antiviral mediators, such as type I interferon (IFN), many virus infections also activate the secretion of proinflammatory cytokines, chemokines, and other immune stimulators that contribute to innate and adaptive defenses and comprise well established paradigms that control gene expression in response to virus infections.

It has become clearly established that small RNAs contribute to antiviral responses in plants and invertebrate animals via RNA interference involving virus-derived small interfering RNAs (siRNAs) (2). However, there is little evidence to support a similar role for siRNAs in mediating general or specific antiviral responses in mammalian hosts. Mounting evidence does suggest a role for miRNA modulated responses during infection (3).

Mature miRNAs are small, endogenously produced 17–24-nucleotide RNAs that regulate gene expression either by inhibiting mRNA translation through seed-region annealing to the 3’ untranslated regions (UTRs) of mRNAs or by inducing mRNA degradation. MicroRNAs are generated from primary miRNA transcripts (pri-miRNAs) that are produced in the nucleus by RNA polymerase II as independent transcriptional units or within intronic regions of mRNAs. Short hairpin RNAs, known as precursor miRNAs (pre-miRNAs), are excised from the pri-miRNA by the ribonuclease, DROSHA. The pre-miRNAs are exported to the cytoplasm and further processed by cleavage with DICER, resulting in a mature miRNA duplex that is incorporated into the RNA-induced silencing complex primed for translational inhibition (4–7). Translational inhibition can occur either by miRNA-induced inhibition of translation initiation, or by miRNA-induced mRNA degradation, or a combination of the two mechanisms. MicroRNAs, once associated with RNA-induced silencing complex, are able to bind their target and recruits deadenylation factors to remove the poly(A) tail of mRNA transcripts (8, 9). Once deadenylation occurs, decapping enzymes remove the m7G cap on the 5’ end of mRNA transcripts and promotes 5’ to 3’ mRNA decay (10). Mounting evidence indicates that target degradation is the predominant form of miRNA-mediated translational repression (11, 12).
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Although miRNAs have been implicated as potentially important regulators of mammalian antiviral responses, their precise roles in general or specific antiviral systems have not yet been extensively reported (13–16). Recent studies have shown that miRNAs play subtle roles in the antiviral response of mammals. The abundance of several miRNAs is modulated in response to virus infections, and in some cases the regulated miRNAs have been linked to important innate immune signaling factors. For example, miR-146a has been described as a negative regulator of signaling from TLR and RLR pathways (4, 15). Viruses such as vesicular stomatitis virus (VSV) and Epstein-Barr virus stimulate expression of miR-146a and down-regulate signaling from these innate immune signaling pathways (4, 17, 18). Other cellular miRNAs have been reported to interact with viral genomes or mRNAs, resulting in both positive and negative effects on virus fitness (19–21).

Recent studies have suggested miRNA involvement in influenza A virus infection and replication (16, 22, 23). Influenza A virus is the causative agent of a contagious respiratory infection, which is well known to modulate the host antiviral defense systems (24, 25). In the present study, we identify cellular miRNAs that are induced in human lung cells after influenza A virus infection and demonstrate that miRNA accumulation is due to an increase in primary miRNA transcription in the infected cell. Analysis of mRNA and protein targets indicates a role for the induced miRNAs in the regulation of innate immune signaling systems.

EXPERIMENTAL PROCEDURES

Cell Culture, Viruses, Treatments, and Transfection—A549 cells (ATCC) were maintained in Ham’s F-12 media with Kaighn’s modification (F12K, Invitrogen) with 10% cosmic calf serum (Hyclone) and 500 units/ml of penicillin and 500 μg/ml of streptomycin. BEAS-2B cells (ATCC) were maintained in BEGM media (Lonza, Walkersville, MD). Primary differentiated human airway epithelial (HAE) cells were purchased from Mattek Corporation (Ashland, MA) and maintained according to the manufacturer’s instructions.

The A/Udorn/72 and A/WSN/33 strains of influenza virus (gift of R. A. Lamb, Northwestern University) were propagated and titered on Madin-Darby canine kidney cells. Encephalomyocarditis virus (EMCV) and parainfluenza virus 5 (PIV5) were propagated and titered on Vero cells. VSV was propagated and titered on CV1 cells. Sendai virus (Cantell strain) was propagated in embryonated chicken eggs and titered on Vero cells. Virus infections were performed in serum-free media (SFM) supplemented with 1% BSA. At 2 h post-infection, the inoculation media was replaced with medium containing 2% cosmic calf serum and cells were washed with serum-free media prior to RNA purification.

Cytokine treatments of A549 cells were as follows: 1000 units/ml of IFNa (Hoffman LaRoche), 500 ng/ml of IFNy (PBL Interferon Source), 400 ng/ml of IL-6, and 500 ng/ml of soluble IL-6 receptor (Peprotech), or 10 ng/ml of TNFa (R&D Systems). Cells were incubated with cytokines for 10 h before RNA purification. For poly(I:C) transfections, synthetic dsRNA was transfected into A549 cells (5 μg/ml media) poly(I:C) (Ambion). Cells were transiently transfected with 800 ng of 4xM67 pTATA TK LUC plasmid and 20 ng of constitutively active Renilla luciferase (pRenilla null) plasmid. Twenty-four hours post-transfection, cells were left untreated or treated with IL-6 and soluble IL-6 receptor for 10 h harvested, and assayed for luciferase activity with the dual luciferase assay system (Promega, Madison, WI). For miRNA mimic transfections, A549 cells were transfected with miR-7, miR-132, miR-146a, miR-187, miR-200c, miR-1275, and negative control miRNA mimics (Ambion pre-miR®) at 50 nM total concentration using Lipofectamine 2000 (Invitrogen) in F12K media lacking penicillin and streptomycin.

MicroRNA Microarray Analysis—Total RNA was isolated using the miRNeasy kit (Qiagen) and fractioned using flashPAGE (Ambion) to isolate RNAs smaller than 40 nucleotides in length. MicroRNA microarrays were performed at LC Sciences (Houston, TX) using the complete miRBase 11.0. MicroRNA microarrays were performed as a single biological replicate with at least 3 independent verifications by qRT-PCR using TaqMan microRNA assays.

Gene Expression Profiling and Pathway Analysis—Total RNA was purified from A549 cells that were mock infected, infected with A/WSN/33 at multiplicity of infection of 5 pfu/cell or A549 cells expressing miRNA mimics and infected with A/WSN/33 at a multiplicity of infection of 5 pfu/cell. Total RNA from three biological replicates was purified 10 h post-infection and hybridized to Illumina Bead Array whole genome expression microarrays (26, 27). Determination of potential miRNA targets was performed with TargetScan and MiR-Walk algorithms (28, 29). Pathway analysis to identify functions of miRNA target genes was performed using the Innate DB database (30). Interactome analysis was generated by literature search for interacting partners and visualized within Cytoscape with the Agilent Literature Search Software (Agilent Technologies, Santa Clara, CA) (31).

RNA Purification, Reverse Transcription, Real-time PCR, and miRNA Analysis—For mRNA analysis, total RNA was purified from 1 x 10⁶ A549 or BEAS-2B cells using the miRNeasy RNA isolation kit (Qiagen), reverse transcribed using oligo(dT) primers and Superscript III reverse transcriptase (Invitrogen). Real-time PCR was performed using SYBR Green detection and primers specific for: IFNB, forward, 5'-CATTACCTGAAGGCACCAAGGA-3'; reverse, 5'-CAATTGTCCAGTCCCAGAGG-3'; IL-28a, forward, 5'-GGAGGTTCAGACACAGAGGT-3'; reverse, 5'-GAGGCCTCTTGACCTTTCAA-3'; ISG15, forward, 5'-AATGGCAGCAAACTCTGTAAC-3'; reverse, 5'-GAAAGGTCCGAGCAACAGGT-3'; CC15, forward, 5'-CGCTGTCATCTCCTATTGCTA-3'; reverse, 5'-GGACCTTGGCAGTTGTAAGA-3'; PB1, forward, 5'-AATGGTCATTTAGGGCAAGG-3'; reverse, 5'-CGAATTTTTTGTGCCTGTG-3'; GAPDH, forward, 5'-ACAGTACGGCGCATCTTCT-3'; reverse, 5'-ACGACAAATCCGGTTGACTC-3'.

Relative mRNA abundance was determined by normalizing the mRNA of interest to GAPDH. All real-time PCR data are presented as 2^-ΔΔCt (32). For analysis of mature miRNAs, TaqMan miRNA assays (Applied Biosystems) were used. Total RNA was reverse transcribed using a miRNA-specific primer and Multiscribe reverse transcriptase (Applied Biosystems).
Real-time PCR was performed with a miRNA-specific probe according to the manufacturer’s instructions (Applied Biosystems). Relative miRNA abundance was determined by normalizing the miRNA of interest to U6 small nuclear RNA. The following TaqMan miRNA assays were used: dme-miR-7, hsa-miR-16, hsa-miR-132, hsa-miR-187, hsa-miR-200c, and hsa-miR-1275.

For detection of the primary miRNA transcripts, total RNA was reverse transcribed using random primers (Invitrogen) and the Superscript III first strand kit (Invitrogen). Real-time PCR was performed as above with TaqMan probes specific for hsa-pri-miR-132, hsa-pri-miR-146a, hsa-pri-miR-187, and hsa-pri-miR-200c (Applied Biosystems). Relative pri-miRNA abundance was determined by normalizing the pri-miRNA of interest to U6 small nuclear RNA. All real-time PCR was performed using MX3005p real-time PCR machines (Agilent Technologies, Santa Clara, CA).

**Indirect Immunofluorescence**—Cells were fixed in 3.7% formaldehyde, permeabilized in ice-cold methanol, and stained with antibodies specific for the influenza A virus nucleoprotein, NP (Abcam), and a fluorescein-conjugated secondary antibody. Stained cells were visualized using a Nikon Eclipse Ti-U inverted fluorescence microscope.

**Immunoblotting**—A549 cells were washed in ice-cold phosphate-buffered saline and then lysed in whole cell extract buffer containing 50 mM Tris, pH 8.0, 280 mM NaCl, 0.5% IGEPAL, 0.2

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![Figure 1](image_url)

**FIGURE 1. Cellular response to influenza A virus infection.** A549 and BEAS-2B cells were mock infected or infected with 1 pfu/cell A/Udorn/72 or A/WSN/33 for 8, 12, or 24 h. The relative abundance of antiviral mRNAs IFNβ, ISG15, CCL5, and the influenza virus mRNA PB1 was measured. p.i., postinfection.
mM EDTA, 2 mM EGTA, 10% glycerol, 1 mM DTT, 0.1 mM sodium vanadate, and protease inhibitor mixture. Forty μg of total protein was separated by SDS-PAGE. Protein was transferred to a nitrocellulose membrane, blocked in 5% nonfat milk in TBST, and detected by specific antibodies to influenza NP (Abcam), MAPK1 + MAPK3 (Abcam), IRAK1 (Santa Cruz Biotechnologies), and GAPDH (Santa Cruz Biotechnologies). Antibody detection was visualized by chemiluminescence (PerkinElmer Life Sciences).

RESULTS

MicroRNA Abundance Changes during Influenza A Virus Infection of Human Cells—To determine whether the abundance of cellular miRNAs was altered by infection, two com-
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To determine whether induction of miRNAs by influenza A virus in these cell lines is also observed under more physiological conditions, similar miRNA analysis was conducted in differentiated primary HAE tissue cultures. These three-dimensional ciliated airway tissues are readily infected with influenza A/WSN/33, although not as uniformly as homogenous monolayer cells such as A549 (Fig. 3C). The level of antiviral miRNAs for IFNβ, ISG15, IL-28a, and CCL5 and the influenza virus PB1 mRNA were examined at 8 and 24 hpi, and all genes were significantly induced (Fig. 3A). In parallel, mature miRNA levels were examined and found to increase in abundance in the HAE cells at 8 hpi, and most of the miRNA increase was resolved to near baseline levels by 24 hpi (Fig. 3B). The magnitude of miRNA induction in HAE cells ranged from 3- to 6-fold and miRNA accumulation preceded mRNA accumulation (Fig. 3, A and B). Importantly, the level of a control miRNA, miR-21, was unchanged in HAE cells after infection with A/WSN/33 (Fig. 3B).

Several of these miRNA changes were consistent across the three cell systems tested (miR-7, miR-132, miR-146a, miR-187, miR-1275) and others exhibited cell type differences (miR-16 and miR-200c). Together, these data support the conclusion that selected miRNA levels change as a result of influenza A virus infection, and that this is a property of both primary human airway cells and human respiratory-derived cell lines.

**Influenza Virus Activates miRNA Induction**—To determine whether the effect of influenza A virus infection on miRNA abundance is a general feature of RNA virus infection or is specific to influenza A virus, A549 cells were mock infected or infected with EMCV, Sendai virus (SeV), PIV5, or VSV in parallel with A/Udorn/72 and A/WSN/33. After 10 h, total RNA was prepared and the relative abundance of the miRNAs was evaluated by real-time PCR. As a control to ensure that all virus infections were generating an antiviral response, the transcriptional induction of CCL5 mRNA was measured by RT-PCR (Fig. 4). Although several of the miRNAs were found to accumulate in response to infection with other viruses (Fig. 4), the magnitude of the increase was greatest in response to influenza A virus infection, with the notable exception of PIV5, which also caused increased expression of miR-132. The abundance of miR-146a, miR-187, miR-200c, and miR-1275 was dramatically increased in the influenza A virus-infected cells when compared with cells infected with the other viruses tested. All viruses tested increased the abundance of miR-146a between 3- and 7-fold (Fig. 4, inset), and other minor fluctuations in miRNA abundance were observed, but influenza virus infection was consistently the most robust miRNA activator. These results suggest that miRNA induction is caused by a signal that is more strongly induced during influenza virus infection than during infection with the other viruses tested.

### TABLE 1

| MicroRNA | A/Udorn/72 | A/WSN/33 | BEAS-2B | A/Udorn/72 | A/WSN/33 |
|----------|------------|----------|---------|------------|----------|
| miR-7    | 1.37       | 1.66     | 2.58    | 1.85       | 2.05     | 1.90     | 1.33     | 1.41     | 1.41     | 1.76     | 2.47     | 3.78     |
| miR-132  | 1.75       | 1.45     | 1.79    | 1.25       | 1.28     | 1.33     | 1.33     | 1.41     | 1.41     | 1.76     | 2.47     | 3.78     |
| miR-146a | 2.21       | 3.25     | 3.00    | 4.46       | 6.62     | 11.23    | 3.34     | 4.77     | 4.77     | 3.82     | 6.03     | 6.52     |
| miR-187  | 2.13       | 3.20     | 1.86    | 2.41       | 3.39     | 2.62     | 1.65     | 1.98     | 1.98     | 1.76     | 2.59     | 2.37     |
| miR-200c | 4.10       | 4.91     | 6.62    | 3.87       | 4.60     | 4.70     | 0.80     | 0.61     | 0.61     | 0.74     | 1.33     | 0.05     |
| miR-1275 | 6.42       | 7.35     | 7.04    | 4.02       | 5.06     | 3.85     | 1.60     | 2.10     | 2.10     | 1.92     | 2.77     | 2.86     |
| miR-16   | 1.00       | 0.92     | 0.61    | 0.94       | 1.05     | 0.83     | 0.85     | 0.91     | 0.91     | 0.98     | 0.98     | 0.73     |

* MicroRNAs displaying significant increases (p value < 0.05) in abundance throughout the time course of infection. Numbers indicate fold-change of miRNA abundance of infected sample compared to mock infected sample.

* A549 or BEAS-2B cells were infected with A/Udorn/72 or A/WSN/33 strains of influenza virus (1 pfu/cell).

* Hours postinfection.
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Influenza A Virus Infection Induces Primary miRNA Transcription—Alterations in miRNA abundance have been reported in many systems, and changes in mature miRNA levels may occur via enhancement of primary miRNA transcription, or through post-transcriptional mechanisms that regulate the processing of a pre-existing pool of pri-miRNAs or pre-miRNA (33–35). To determine whether the increase in miRNA abundance was due to increased pri-miRNA transcription or enhanced miRNA processing, miR-132, miR-146a, miR-200c, miR-1275, and miR-21 was measured. C, immunofluorescence of HAE cells that were mock infected or infected as above for 24 h. Cells were immunostained for the influenza nucleoprotein (NP). p.i., postinfection.

### Table: mRNA and miRNA Abundance Changes during Influenza A Virus Infection in Primary Airway Epithelial Cells

| mRNA          | miRNA        | miRNA          |
|---------------|--------------|----------------|
| IFNβ          | miR-16       | miR-187        |
| ISG15         | miR-7        |                |
| IL-28a        | miR-132      |                |
| CCL5          | miR-146a     |                |
| PB1           | miR-200c     |                |

A, relative abundance of antiviral mRNAs IFNβ, ISG15, IL-28a, and the influenza virus PB1 mRNA were measured. B, relative abundance of miR-16, miR-7, miR-132, miR-146a, miR-200c, miR-1275, and miR-21 was measured. C, immunofluorescence of HAE cells that were mock infected or infected as above for 24 h. Cells were immunostained for the influenza nucleoprotein (NP). p.i., postinfection.

**FIGURE 3.** mRNA and miRNA abundance changes during influenza A virus infection in primary airway epithelial cells. HAE cells were mock infected or infected with 10⁶ pfu/cell culture chamber of A/Udorn/72 for 8 or 24 h. A, relative abundance of antiviral mRNAs IFNβ, ISG15, IL-28a, and the influenza virus PB1 mRNA were measured. B, relative abundance of miR-16, miR-7, miR-132, miR-146a, miR-200c, miR-1275, and miR-21 was measured. C, immunofluorescence of HAE cells that were mock infected or infected as above for 24 h. Cells were immunostained for the influenza nucleoprotein (NP). p.i., postinfection.
the pri-miRNA transcripts were found to increase detectably between 2 and 4 h after infection, and continued to increase throughout the time course. In all cases, the appearance of the pri-miRNA preceded the mature miRNA accumulation, suggesting that the observed induction of miRNAs is regulated at the level of pri-miRNA accumulation rather than enhanced processing of a pre-existing pool of pri-miRNAs or pre-miRNAs (Fig. 5).

**New Protein Synthesis Is Required for Influenza Virus-induced Pri-miRNA Increase**—To investigate whether primary response pathways or secondary mechanisms lead to pri-miRNA transcription, the role of newly synthesized protein in pri-miRNA levels was examined. A549 cells were infected with A/Udorn/72 or mock infected in the presence or absence of cycloheximide (CHX, 100 μg/ml), and pri-miRNA levels were measured. As a control, the mRNA levels of two known primary responders in the innate immune response, IL-28α and CCL5, were analyzed. The accumulation of these mRNAs was not altered by CHX treatment. However, CHX did inhibit the production of a secondary immune responder mRNA, ISG15, and the viral mRNA encoding the polymerase protein, PB1 (Fig. 5B). Similar to the pattern observed for the secondary response genes, the infection-induced increase of both pri-miRNAs and mature miRNAs was abrogated by treatment with CHX (Fig. 5, A and C). Primary miRNAs continued to accumulate until 8 h postinfection, then declined by 24 h, concomitant with increases in mature miRNA levels, consistent with a precursor-product relationship (Fig. 5D). These data suggest that the influenza virus induced an increase in pri-miRNA abundance, and thereby the accumulation of mature miRNAs, observed following infection with influenza virus, and involves newly synthesized proteins. Examination of miRNA induction by expression of influenza virus mRNAs, either individually or in combination, was insufficient to induce the expression of the miRNAs (not shown), implying that a secondary cellular response pathway is responsible.

**Antiviral Mediators Are Insufficient for miRNA Induction**—The production and secretion of cytokines, specifically interferons (IFN), interleukins (IL), and tumor necrosis factor α (TNFα) are important in antiviral responses, and have been implicated in miRNA regulation (15, 36). To investigate if signaling by these cytokines contributes to influenza virus induction of miRNAs, A549 cells were mock infected or infected with A/Udorn/72 or A/WSN/33 or treated with a panel of inducers: IFNα (1000 units/ml), IFNγ (500 ng/ml), IL-6 (400 plus 500 ng/ml of soluble IL-6 receptor), or TNFα (10 ng/ml) for 10 h. As
a control, the abundance of ISG15 mRNA, IP10 mRNA, and CCL5 mRNA was measured to ensure cells were responding to IFN-α, IFN-γ, and TNF-α, respectively. Additionally, a parallel sample was used to ensure IL-6 activation, using a STAT3-responsive m67-GAS luciferase reporter gene assay (Fig. 6C).

The abundance of the influenza virus-induced miRNAs was determined by real-time PCR. Although most of the tested miRNAs remained unchanged by these cytokine treatments (Fig. 6A), the abundance of miR-146a was increased 14.3-fold after treatment with TNF-α, in agreement with prior literature (15). However, this level of miR-146a induction was dramatically lower than that observed in influenza A virus-infected cells, which increased miR-146a by 150-fold (Fig. 6A). These results suggest that signaling by IFNα, IL-6, and TNFα are, by themselves, unable to account for the vast majority of influenza-induced miRNA accumulation.

Recognition of influenza virus by the innate immune system can occur through several pattern recognition pathways (37–39). The RLRs are the main pattern recognition receptors for viruses in A549 cells because these cells lack TLR7 and TLR8 expression (40). To determine whether the synthetic double-stranded RNA, poly(I:C), induced changes in miRNA expression, A549 cells were transfected with poly(I:C) prior to RNA isolation 12 h later (41). As a control, the level of IFNβ and
CCL5 mRNA was measured to verify the efficacy of the poly(I:C) transfection in inducing RLR signaling (Fig. 6C). Under these conditions, miR-146a was induced 2.6-fold, but miR-7, miR-132, miR-187, miR-200c, and miR-1275 remained constant or below a 2-fold increase threshold (Fig. 6B). These results indicate that signaling induced by poly(I:C) may contribute to miR-146a induction, but is insufficient to account for other miRNAs that are increased in response to influenza A virus infection.

Influenza Virus-induced miRNAs Target Cell Signaling Pathways—As miRNAs are able to repress translation by inducing mRNA degradation, gene expression profiling was performed to identify miRNA-targeted mRNA transcripts that decrease in abundance during infection and contain miRNA seed matches within their 3′ UTR. Genomewide mRNA abundance was assessed in A549 cells that were mock infected or infected with A/WSN/33 in the presence or absence of miR-7, miR-132, miR-146a, miR-187, miR-200c, and miR-1275 mimics. Total RNA was purified from cells 10 h postinfection and mRNA was measured using an Illumina whole genome expression microarray.

In the absence of miRNA mimics, 1970 genes were differentially regulated by infection greater than 1.5-fold, with 1177 genes displaying decreased expression and 793 genes displaying increased expression. There are many known mechanisms that might account for the decreased mRNA expression during influenza virus infection including cap-snatching, inhibition of pre-mRNA formation, and degradation of RNA PolII, in addition to antiviral gene products such as RNase L that can randomly target cellular and viral mRNAs (42, 43). However, it is notable that 448 of the 1177 down-regulated genes (38%) contained seed matches for the tested miRNAs, as determined by 3 separate algorithms using the MiR-Walk software (29).

In the presence of miRNA mimics, most mRNA levels were identical to the control samples. However, data analysis identified 36 genes that were differentially expressed during infection.
in the presence of miRNA mimics: decreased expression was observed for 26 genes, whereas 10 genes increased expression (greater than 1.5-fold threshold, see Table 2). Of the 26 repressed genes, all but one contained at least one seed match to the specifically expressed miRNAs. At the top of this list is IRAK1, a previously identified target of miR-146a, affirming the efficacy of this mRNA expression profiling approach for identification of miRNA targets.

To identify functional pathways correlated with the gene expression changes observed due to miRNA expression, the Innate DB database was used for a systems biology pathway analysis (30). The identified miRNA-targeted genes were connected to 22 cellular innate response pathways including TLR, JAK-STAT, TNFα, insulin, IL-1, and IL-7 signal transduction systems (Table 3). To further analyze these targets, an interactome network was constructed based on literature search terms connected with the gene targets (Fig. 7A). Twelve of the target genes were clustered by this analysis, with a set of central interconnected nodes surrounding MAPK3, IRAK1, and STMNI (Fig. 7B). Two of these targets, IRAK1 and MAPK3, connect diverse and interrelated pathways and signaling proteins known to be important for the regulation of cellular responses to infections, and these nodes are deeply rooted in TLR and cytokine signaling pathways (44, 45).

To verify the mRNA microarray data at the protein level, MAPK3 and IRAK1 protein abundance was measured by immunoblotting before and after infection with influenza A/WSN/33, both in the absence and presence of specific miRNA mimics. For MAPK3, miR-132- and miR-1275-specific mimics were used, and for IRAK1, miR-146a and miR-1275 mimics were used as these miRNAs were identified to bind the 3’ UTR of respective mRNA (Table 2). At 10 h postinfection, whole cell extracts were generated, separated by SDS-PAGE, transferred to nitrocellulose membranes, and probed with antibodies that recognize MAPK1 + MAPK3, IRAK1, influenza NP, and GAPDH. Results indicate that both MAPK3 and IRAK1 protein levels decrease during influenza virus infection, and that this decrease is more pronounced in the presence of specific mimics, but not control mimics (Fig. 8). These conditions did not alter the levels of control proteins, MAPK1, GAPDH, or influenza NP. These data confirm the microarray findings and demonstrate that miR-132, miR-146a, and miR-1275 work in conjunction to negatively regulate MAPK3 and IRAK1 at both RNA and protein levels during influenza A virus infection.

**DISCUSSION**

Most of the cell-autonomous responses to RNA virus infections are mediated at the level of regulated gene expression. In addition to the alteration in mRNA expression profiles, protein expression is also modulated in response to infection through mechanisms that regulate mRNA translation, such as the action of the antiviral protein kinase, PKR, or pathways that control translation initiation downstream of IFN signaling (46). The present study adds a further dimension to the cellular response to virus infection, through the analysis of endogenous human miRNAs that are induced by infection with influenza A viruses. Specifically, experiments indicate that miR-7, miR-132, miR-146a, miR-187, miR-200c, and miR-1275 increase in abundance during influenza virus infection of human respiratory cells,
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causing down-regulation of antiviral proteins such as IRAK1 and MAPK3.

In addition to the influenza virus-induced human miRNAs identified in the present study, infection of chicken and mouse lungs with influenza A viruses also has been reported to change the levels of several miRNAs (22, 47). Despite the conceptual similarities among these studies, comparing the results reveals little or no commonality among the miRNAs reported to be induced by influenza A viruses in these diverse systems. For example, miR-21 was found to be elevated in infected mouse lungs with influenza A viruses and chicken lungs with influenza A virus infection (47). In human cells, the chicken miR-146a level was strongly reduced in response to avian influenza virus infection (47). There are several potential explanations for the apparent discrepancies among these studies, including the use of different host species and virus strains for each experiment: for example, miR-21 is one of the most abundant miRNAs present in A549 cells at steady state, but this is not the case for other cells tested, and both strain-specific and temporal regulation of miRNA abundance was observed during infection of A549 cells with swine and avian origin influenza viruses (16). A further complication may arise from the analysis of heterogeneous endogenous tissue samples, in which only a small number of infected cells is likely to be present and therefore only partially contributing to differential miRNA expression. Not only would the contribution of infected cells to the total miRNA pool be diluted in tissue RNA preparations, but also non-cell autonomous effects such as cytokine or apoptotic signaling could cause differential miRNA expression in uninfected diverse cell types that are present in complex tissue samples.

Identification of influenza A virus-regulated miRNAs raises a question of their potential endogenous host miRNA targets. As most miRNA target prediction algorithms are based on 6–8-bp imperfect seed region matches, hundreds of potential targets can be computationally identified for each miRNA analyzed. None of the miRNAs that were characterized had the potential ability, as determined by seed sequence match, to target the influenza virus genome or miRNAs. Such a specific targeting mechanism has been demonstrated to be theoretically possible, as computationally-predicted miRNAs (miR-323, miR-491, and miR-654) with seed matches to influenza virus genomes were shown to control influenza virus replication when overexpressed in Madin-Darby canine kidney cells (23). However, this type of regulation by miRNAs is evolutionarily unfavorable, as variant viruses with disruptions to the seed sequences will be selected.

Considering that many miRNA-regulated targets are also subject to combinatorial recognition by several miRNAs, deciphering the exact target(s) for individual miRNAs represents a formidable challenge, especially in the context of changes induced during acute virus infections (48). Both known and novel miRNA targets were observed during infection in the absence or presence of miRNA mimics. The 3’ UTR of the 26 identified target miRNAs were analyzed to find target sites. The data indicate that two genes involved in several innate immune processes, MAPK3 and IRAK1, are targeted for miRNA degradation and protein translation inhibition by the combinatorial effect of miR-132 and miR-1275, and miR-146a and miR-1275, respectively. This suggests that at least three of the six influenza virus-induced miRNAs tested have the ability to regulate innate immune signaling pathways. Interestingly, miR-132 has been shown to be regulated transcriptionally by a MAPK3 (ERK1) pathway and this study suggests that MAPK3 may use miR-132 to regulate its activity in a negative feedback loop (49, 50). The extent of pathways targeted by just a small number of influenza virus-induced miRNAs suggests that miRNA regulation of cellular responses is an ongoing process in the infected cell that can fine-tune the cellular response. Considering the potential for activation of even more miRNAs in the infected cell, we predict they will have a significant impact in regulating cellular mRNA and protein levels.

Several of the miRNAs identified in this study have been previously linked to biological responses related to cellular responses to virus infection. MicroRNA-132 has been shown to be induced in cells infected with Kaposi’s sarcoma-associated herpesvirus, and was proposed to target the histone acetyltransferase protein, p300, which is required for the production of IFNβ (51). Our study indicates that influenza A virus is also capable of inducing miR-132 accumulation, although not to the same degree as reported for the Kaposi’s sarcoma-associated herpesvirus study. Again, this discrepancy might be explained...
by variations in both the cell types and the viruses under investigation. Notably, within our gene expression analysis for targets of miR-132, CBP/p300 was not identified as differentially regulated at the mRNA level. Another well characterized miRNA, miR-146a, already shown to be induced by LPS, IL-1β, VSV, and LMP-1 of Epstein-Barr virus, is dramatically induced by influenza A virus infection (14, 15, 52). This miRNA has been shown to be capable of regulating the activation of NF-κB by...
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model is consistent with our finding that miR-132, miR-146a, and miR-1275 may work in conjunction to negatively regulate MAPK3 and IRAK1 at both RNA and protein levels during influenza A virus infection. This regulatory event is achieved by one or more secondary response pathway(s) that can regulate miRNA accumulation during the time course of influenza virus infection. As this study demonstrates alteration of the abundance of a select group of miRNAs can dramatically influence mRNA targeting, it will be of great interest to expand the measurement of miRNA changes genome-wide using next-generation sequencing technology.

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FIGURE 8. MAPK3 and IRAK1 protein levels are reduced during influenza virus infection. A549 cells were mock infected (M) or infected with A/WSN/33 (m.o.i. of 5 pfu/cell; 10 h) in the absence or presence of control (CTRL) or specific miRNA mimics for miR-132, miR-146a, or miR-1275 as indicated. 40 μg of total protein was separated by SDS-PAGE and probed with antisera for MAPK1 + MAPK3, IRAK1, IRAK2, and TRAF6.

targeting the expression of the upstream signaling molecules, IRAK1, IRAK2, and TRAF6 (14, 15). Our analysis indicates that miR-146a can target IRAK1, and that miR-1275 may also contribute to this effect. The present study is the first to link miR-7, miR-132, miR-200c, or miR-1275 with virus infections or cellular antiviral responses.

An interesting feature of the miRNAs described here is their unique regulation in response to influenza virus infection. Other RNA viruses tested, EMCV, Sendai virus, PIV5, and VSV, are among the most commonly used viruses for studying innate antiviral responses, yet they are unable to increase the abundance of these miRNAs to the same extent as influenza virus. Treatment of cells with several antiviral mediators, including IFNα, IFNγ, IL-6, and TNFα, or transfection with poly(I:C), did not induce changes in any miRNA to the degree observed with influenza virus infection. It is possible that additional untested mediators are fully responsible for some or all of the miRNA induction observed, or that combinations of these individual treatments might better imitate the milieu of influenza virus infection. TNFα was found to induce miR-146a accumulation, in agreement with prior studies, but influenza A virus infection was a much more effective inducer (15).

This apparent specificity and potency of activation might be explained by cumulative effects of multiple cellular pathways and signaling systems activated by influenza virus infection that could then converge synergistically on miRNA regulation.
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