MicroRNA Profiling of Sendai Virus Infected A549 Cells Identifies miR-203 as an Interferon-inducible Regulator of IFIT1/ISG56

William A. Buggele and Curt M. Horvath*

Department of Molecular Biosciences
Northwestern University
Evanston, IL 60208

Running Title: MiR-203 Regulation of IFIT1/ISG56

*Corresponding Author
Northwestern University
Pancoe Pavilion, Room 4401
2200 Campus Drive
Evanston, IL 60208, USA
Office (847) 491-5530
Lab (847) 491-5484
Fax (847) 494-1604
Email: horvath@northwestern.edu

Abstract Word Count: 166
Text Word Count: 4956
Abstract

The mammalian type I interferon (IFN) response is a primary barrier for virus infection and is essential for complete innate and adaptive immunity. Both IFN production and IFN-mediated antiviral signaling are the result of differential cellular gene expression, a process that is tightly controlled at transcriptional and translational levels. To determine the potential for microRNA (miRNA) mediated regulation of the antiviral response, small RNA profiling was used to analyze the miRNA content of human A549 cells at steady state and following infection with the Cantell strain of Sendai virus, a potent inducer of IFN and cellular antiviral responses. While the miRNA content of the cells was largely unaltered by infection, specific changes in miRNA abundance were identified during Sendai virus infection. One miRNA, miR-203, was found to accumulate in infected cells and in response to IFN treatment. Results indicate that miR-203 is an IFN-inducible miRNA that can negatively regulate a number of cellular mRNAs, including an IFN-stimulated gene target, IFIT1/ISG56, by destabilizing its mRNA transcript.
Introduction

Virus infection of mammalian cells induces immediate and robust changes in cellular gene expression. Detection of virus infection by cellular signaling machinery triggers the transcription of antiviral genes including primary antiviral cytokines in the type I interferon (IFN) family as well as diverse effectors of the antiviral state (1). These cytokines and antiviral genes also drive further gene expression to amplify and regulate a primary cellular antiviral response that not only serves as a barrier to virus replication, but also functions to educate the innate and adaptive immune systems. Inappropriate activation of these antiviral programs can lead to cytotoxicity and cell death; accordingly, diverse feedback inhibitors and other signal attenuators have evolved that serve to modulate the intensity and duration of IFN signaling and antiviral responses.

One of the primary mediators of antiviral gene expression is the IFN-JAK-STAT signaling system that directly links IFN production to target gene expression. IFN-receptor engagement induces the assembly of the heterotrimeric transcription factor interferon stimulated gene factor 3 (ISGF3), from pre-existing latent reservoirs of STAT1, STAT2, and IRF9 (2, 3). ISGF3 translocates to the nucleus, binds to target
sites in interferon stimulated gene (ISG) promoters, and activates the transcription of a large number of antiviral effector genes.

In addition to the virus-activated expression of protein coding mRNAs, it has become widely recognized that virus infections can also regulate the expression of non-coding RNAs, including miRNAs involved in RNA interference pathways (4-6). MicroRNAs are generated from primary RNA polymerase II transcripts that are processed in the nucleus to create precursor miRNA hairpins. The precursor hairpins are further processed in the cytoplasm to create a mature 17-24 bp miRNA duplex that is incorporated into the RNA-induced silencing complex. MicroRNAs function to regulate the level of protein production by base pairing with short seed regions typically within the 3'UTR of target mRNAs (7-11). Recognition of mRNA targets by miRNAs can reduce protein expression either by inhibiting target mRNA translation or by promoting target mRNA degradation. Mounting evidence indicates that mRNA destabilization is a predominant means of miRNA-mediated translational repression (12-17).

Antiviral responses mediated by RNA interference are well documented in organisms such as flies, worms, and plants (18). In these cases, double stranded viral genomic
RNA or RNA replication intermediates are used to generate siRNAs that directly target the viral genome or mRNAs for efficient degradation (19). While there is scant evidence for a similar mechanism occurring naturally during the mammalian antiviral response (20), intact RNA interference pathways are required for optimal murine antiviral responses during vesicular stomatitis virus infection (21). The requirement of RNA interference machinery for mounting an antiviral response suggests an evolutionarily conserved role for the RNA interference pathway during virus infections. Further support of this concept has derived from studies that describe individual miRNAs that are regulated by virus infections or that control the response to virus infection. For example, influenza virus, VSV, KSHV, and pathogenic bacteria induce miRNAs miR-132 and miR-146a, resulting in attenuation of cell signaling proteins MAPK3, p300, and IRAK1 to modulate antiviral signaling (6, 22-24). The well characterized hepatocyte miRNA, miR-122, is used by hepatitis C virus for efficient replication (25), but IFN signaling can decrease miR-122 abundance to limit virus replication (26). IFN has also been implicated in regulation of hepatocyte miRNAs that were reported to directly target the HCV genome in order to prevent its replication (27, 28). Diverse virus infections, including porcine reproductive and respiratory syndrome virus, respiratory syncytial virus, rabies virus, Epstein-Barr virus, influenza virus, human immunodeficiency virus 1,
primate foamy virus, and hepatitis C virus have been found to alter the cellular miRNA content during infection (29), and regulate the cellular antiviral response (6, 30, 31) or virus replication (28, 32-35, 27). These examples provide and reinforce strong correlative links between antiviral signaling and miRNA regulation.

To explore the importance of miRNA regulation during the cellular antiviral response, small RNA profiling by next generation sequencing was carried out to characterize the miRNA content of human cells at steady state and following Sendai virus infection. Differentially expressed miRNAs were identified that responded to virus infection, including miR-203, which is demonstrated to be an IFN inducible miRNA capable of targeting the IFN-stimulated antiviral mediator, IFIT1/ISG56. These findings provide evidence for diverse miRNA regulation by a negative strand RNA virus infection, and characterize an example of a complete miRNA regulatory circuit in the IFN antiviral response.
Materials and Methods

Cell Culture and Viruses

A549 cells (ATCC) were maintained in Ham’s F12 media with Kaign’s modification (F12K, Gibco) supplemented with 10% cosmic calf serum (CCS, Hyclone), 500 units/mL penicillin, and 500 ug/mL streptomycin. Vero cells were maintained in Dulbecco’s modified eagle medium (DMEM) supplemented with 10% CCS, 500 units/mL penicillin, and 500ug/mL streptomycin.

Sendai virus (Cantell strain) was grown in embryonated chicken eggs and titered on Vero cells. The A/Udorn/72 and A/WSN/33 strains of influenza virus (gift of R.A. Lamb, Northwestern University) were propagated and titered on MDCK cells. Virus infections were performed in serum-free media (SFM) supplemented with 1% BSA. At 2 hours post-infection, the inoculation media was replaced with media containing 2% CCS and cells were washed with SFM prior to RNA purification.

Cell Treatments and Transfection
Recombinant human IFNα (Hoffman LaRoche) was added to F12K media at a concentration of 1000 units/mL. Cells were treated for 10 hours prior to RNA purification.

MicroRNA non-targeting control mimic and inhibitor or miR-203 specific mimic and inhibitor (Dharmacon) was transfected into A549 cells at a concentration of 25nM using Lipofectamine 2000 (Invitrogen) according to the manufacturer's recommended RNAi transfection protocol.

For cycloheximide (CHX) treatments, A549 cells were treated with 100μg/mL CHX in F12K media for 1 hour prior to IFN treatment or Sendai virus infection. Cells were maintained in F12K media containing CHX for the duration of the experiment.

RNA Purification, Reverse Transcription, Real-time PCR, and miRNA Analysis

Total RNA was purified from cells using the miRNeasy RNA isolation kit (Qiagen) and size fractionated with the RNeasy MinElute Cleanup Kit (Qiagen). For mRNA analysis, high molecular weight RNA was reverse transcribed using oligo(dT) primers and
Superscript III reverse transcriptase (Invitrogen). PCR was performed using SYBR green detection and primers specific for:

- **IFNβ**: Forward: 5’-CATTACCTGAAGGCAAGGA-3’
  Reverse: 3’-CAATTGTCCAGTCCCCAGAGG-3’

- **ISG15**: Forward: 5’-AATGCACGAACCTCTGAAC-3’
  Reverse: 5’-GAAGGTCAGCCAGACAGGT-3’

- **IFIT1/ISG56**: Forward: 5’-GCAGCCAAGTTTTACCCGAAG-3’
  Reverse: 5’-AGCCCTATCTGGTGATGCAG-3’

- **Actin**: Forward: 5’-GCGATCCTCACCCTGAAGTA-3’
  Reverse: 5’-AGGTGTGGTGCCAGATTTTC-3’

- **GAPDH**: Forward: 5’-ACAGTCAGCCGCATCTTCTT-3’
  Reverse: 5’-ACGACAAATCCGTTGACTC-3’

Relative mRNA abundance was determined by normalizing the mRNA of interest to GAPDH. All real-time PCR data is presented as $2^{-\Delta\Delta Ct}$ (36).

For analysis of miRNAs, TaqMan miRNA assays (Applied Biosystems) were used. Low molecular weight RNA was reverse transcribed using a miRNA specific primer and Multiscribe reverse transcriptase (Applied Biosystems.) PCR was performed with a
Relative miRNA abundance was determined by normalizing the miRNA of interest to U6 small nuclear RNA using $2^{-\Delta\Delta Ct}$, as above. The following TaqMan miRNA assays were used: hsa-miR-16, hsa-miR-125b, hsa-miR-147b, mmu-miR-187, hsa-miR-203, hsa-miR-376, hsa-miR-449b, hsa-miR-483-3p, and hsa-miR-652.

### Library Preparation and Bioinformatics Analysis

To generate a library for SOLiD small RNA sequencing, A549 cells were either mock infected or infected with Sendai virus (5 p.f.u./cell). RNA was purified from cells 10 hours post infection, and size fractionated. One μg of low molecular weight RNA was used to generate the cDNA libraries using the SOLiD Small RNA Expression Kit (Applied Biosystems) according to manufacturer’s instructions. Emulsion PCR and SOLiD sequencing using the SOLiD4 platform (Applied Biosystems) was performed at the Center for Genetic Medicine at Northwestern University.

Sequence tags were analyzed using the BioScope Software package (Applied Biosystems). The sequence tags were aligned to the human genome with high stringency, allowing for zero mismatches between the sequence tag and the genome.
Sequence tags that mapped to the human genome were further characterized to determine their identity by BLAST. Sequence tags from the virus-infected library that were unable to be mapped to the human genome were then mapped to the Sendai virus genome. All sequence information can be found in the NCBI GEO database under accession number GSE43966.
**Immunoblotting**

A549 cells were washed in ice cold phosphate buffered saline before being lysed in whole cell extract buffer containing 50nM Tris, pH 8.0, 280nM NaCl, 0.5% IGEPAL, 0.2mM EDTA, 2mM EGTA, 10% glycerol, 1mM DTT, 0.1mM sodium vanadate, and protease inhibitor mixture. Five μg of total protein was separated by SDS-PAGE and protein was then transferred to nitrocellulose membrane, blocked in 5% non-fat milk in TBST, and detected by specific antibodies for IFIT1/ISG56 (Thermo Pierce), GAPDH (Santa Cruz Biotechnologies), and MDA5 specific antisera. Antibody detection was visualized by chemiluminescence (Perkin Elmer Life Sciences). Densitometry analysis was performed using Vision Works software (UVP, LLC., Upland, CA).

**Gene Expression Profiling**

Gene expression profiling and analysis was performed as in (6). A549 cells were left untreated, treated with 1000 units/mL IFN, infected with Sendai virus (5 pfu/cell), and treated or infected in the presence of 50nM miR-203 mimic. RNA was purified from cells 10 hours after treatment and hybridized to a whole genome microarray (Illumina bead array) (37, 38). MicroRNA seed matches were determined with the MiR-Walk
algorithm (39) and gene ontology analysis was performed utilizing InnateDB and DAVID (40-43). Microarray data can be found in the GEO database with accession numbers GSM1152886, GSM1152887, GSM1152888, GSM1152890, GSM1152891, GSM1152892, GSM1152894, GSM1152895, GSM1152896, GSM1152897, GSM1152898, GSM1152997, GSM1152998, GSM1152999, GSM1153000, GSM1153003, GSM1153004, GSM1153005, GSM1153006, and GSM1153007.
Results

MicroRNA profile of Sendai virus infected cells

A small RNA deep sequencing experiment was performed to profile cellular miRNAs and determine abundance changes induced by Sendai virus infection. A549 cells, a human alveolar epithelial cell line, were mock infected or infected with Sendai virus for 10 hours, a time point previously determined to be suitable for both inducible mRNA and miRNA expression (6). Total RNA was purified and size fractionated to yield low molecular weight RNA (<200 nucleotides). The low molecular weight RNA was used to construct a library for SOLiD sequencing (Applied Biosystems). Both infected and uninfected cell libraries yielded in excess of $10^8$ sequence tags. These sequence tags were mapped to the human genome with high stringency; no mismatches were allowed between the genome sequence and the sequence tags. This resulted in ~$3.5 \times 10^7$ unique mappable sequences per condition (Table 1). The mappable sequences were subjected to BLAST alignment (44) to determine their locus of origin, resulting in ~$1.5 \times 10^6$ miRNA sequence tags in each library. This procedure identified 778 miRNAs in the mock-infected library and 800 miRNAs in the Sendai virus-infected cell library (Supplemental Table 1). In addition to miRNAs, snRNA and snoRNA classes were
identified. Fragmented large RNA classes including rRNA and mRNA were also identified. Sequence tags from the virus-infected cell library that were unable to be mapped to the human genome could be mapped to the Sendai virus genome. These small Sendai virus-derived RNAs displayed a size distribution between 18-24 nucleotides, and genomic derived RNAs mapped uniquely to the 5’ end, similar to the reported RIG-I ligand generated during Sendai virus infection (45). Of the miRNAs identified, only 343 miRNAs in the mock library and 351 miRNAs in the Sendai virus library were identified by more than 100 sequence tags. To identify if any gross changes in miRNA abundance were the result of Sendai virus infection, the number of miRNAs identified in each library were compared. The mock library contained 19 exclusive miRNAs and the Sendai library contained 27 exclusive miRNAs, while both libraries contained a common 324 miRNAs (Fig 1A). This analysis indicated that the vast majority of miRNAs identified by more than 100 sequence tags were common between libraries, consistent with the interpretation that miRNA expression is overall largely similar before and after infection. In addition, the 25 most abundant miRNAs comprised greater than 87% of all miRNAs in either library. Comparing normalized miRNA sequence tag abundances (miRNA sequence tags per thousand mappable sequence tags) between the two conditions revealed that the vast majority of miRNAs in
A549 cells do not display greater than two-fold change during infection (Fig 1B).

Nonetheless, 52 miRNAs that increase in abundance by ≥ 1.5-fold and 33 miRNAs that decreased by ≥ 1.5-fold following infection were identified (Table S2). Together, these data suggest that infection does not cause a bulk change in the miRNAs present within the cell, however it does cause changes in abundance of specific miRNAs.

**Cellular miRNAs respond to Sendai virus infection**

To verify the sequencing data, independent RNA samples were generated from mock or Sendai virus-infected cells and subjected to individual miRNA analysis by RT-qPCR using TaqMan miRNA assays (Applied Biosystems). A group of miRNAs was selected for validation based on two criteria, initial abundance and differential expression induced by infection. These miRNAs are represented by more than 100 sequence tags in either library and also exhibit greater than 1.5 fold change in response to Sendai virus infection. Several miRNAs were analyzed that displayed either positive (miR-125b, miR-147b, miR-203, miR-376, miR-449b, and miR-483-3p) or negative (miR-187 and miR-652) changes in abundance (Fig 2). A high correlation between sequencing and independent RT-qPCR detection measurement was observed for all the tested miRNAs, confirming their reproducible differential expression. These findings verify that changes
in miRNA abundance identified by small RNA sequencing accurately reflect the miRNA content of uninfected and infected cells.

IFN-mediated accumulation of miR-203

A prior investigation of miRNAs induced by influenza virus infection revealed remarkable virus-specific responses (6). To determine if the Sendai virus inducible miRNAs, miR-203 and miR-449b, are sensitive to other RNA virus infections, A549 cells were either mock-infected or inoculated with 5 p.f.u./cell of Sendai virus, influenza A/Udorn/72, or influenza A/WSN/33 for 10 hrs prior to miRNA analysis. The level of a control miRNA, miR-16, did not change in response to either Sendai virus or influenza virus infection, and miR-449b, was induced by Sendai virus (~2.5-fold) and even more dramatically in response to either strain of influenza virus (~13.5-fold) (Fig. 3A). These results are consistent with our previously reported observation that influenza virus infection can be a more potent inducer of miRNA expression than other RNA viruses (6). However, a distinct behavior was observed for miR-203, which increased by >15-fold in response to Sendai virus infection, but only ~3-fold during influenza virus infections (Fig 3A).
One significant difference in the cellular response to influenza virus infection compared to Sendai virus infection is that Sendai virus is a more robust activator of the antiviral cytokine, IFNβ. The reason for this difference is known: the Cantell strain used for these studies is well documented to induce IFN production due to the presence of defective interfering genomes that activate antiviral responses (46). The IFNβ mRNA is highly induced by Sendai virus infection of A549 cells, but only weakly induced by the influenza viruses (Fig 3B). To determine the effect of IFN signaling on miR-203 expression a dose response experiment was performed. A549 cells were left untreated, treated with PBS, or treated with increasing concentration of IFNα (250, 500, 1000, or 5000 units/mL). RNA was purified ten hours after treatment and the abundance of miR-203 was determined by RT-qPCR. MicroRNA-203 abundance increased as the concentration of IFN increased to 1000 units/mL (Fig 3C). This concentration proved saturating for miR-203 induction when compared to treatment of 5000 units/mL. Higher IFN concentrations induce miR-203 to accumulate to higher abundance, similar to the enhanced accumulation observed during Sendai virus infection.
To compare miR-203 induction by Sendai virus infection with that of IFN treatment, A549 cells were treated with 1000 units/ml IFNα or infected with Sendai virus (5 p.f.u./cell). Ten hours later, total RNA was prepared, size fractionated, and the low molecular weight RNA was used to measure the abundance of miR-203. IFNα stimulation increased miR-203 levels by 3.4-fold, and infection with Sendai virus increased miR-203 by 8.3-fold (Fig 3D). These data indicate that miR-203 is inducible by direct IFNα stimulation but Sendai virus infection is a more potent miR-203 inducer than IFNα treatment alone, possibly indicating additional factors are generated during Sendai virus infection that further enhance miR-203 expression or stability.

To assess the role of RLR signaling in the activation of miR-203, A549 cells were untreated, treated with PBS, treated with 1000 units/mL IFNα, or transfected with the synthetic double-stranded RNA, Poly (I:C). RNA was purified ten hours after treatment and the abundance of miR-203 was determined by RT-qPCR. RLR signaling induced miR-203 to levels statistically identical to IFN treatment alone (Fig 3E). Together the data indicate that while IFN mediates induction, robust activation of miR-203 requires synergism of multiple antiviral pathways activated during Sendai virus infection but not influenza virus infection, IFN stimulation, or RLR signaling alone.
To determine if type I IFN is required for miR-203 induction during Sendai virus infection, an experiment was performed using Vero cells, a cell line that does not induce IFN in response to infection due to genetic defects (47, 48). Vero cells were left untreated, treated with PBS, treated with 1000 units/mL IFNα or infected with Sendai virus (5 pfu/cell). Similar to the A549 cells, miR-203 increased 3.3-fold following IFNα treatment. Unlike the A549 cells, miR-203 did not increase in response to Sendai virus-infection compared to a mock-infected sample (Fig 3F). This result further corroborates the IFNα inducibility of miR-203, and also is consistent with the notion that IFN production initiated by Sendai virus infection is required for miR-203 activation, although it remains possible that there may be additional factors produced in A549 cells that are not produced in Vero cells that could contribute to the differential miR-203 expression.

Activation of miR-203 by immediate and delayed signaling pathways

To further explore the kinetics of miR-203 induction, a time-course experiment was performed. A549 cells were infected with Sendai virus (5 p.f.u./cell), RNA was purified at 2, 4, 10, and 24 hours post infection, and the abundance of miR-203 was measured. Elevated miR-203 was detected as early as 2 hours post infection and continued to
increase during the 24 hours of infection despite loss of cells due to cytopathic effects. These results suggested that signaling pathway accumulation is required for continuous miR-203 expression throughout infection (Fig 4A).

To determine if the changes in miR-203 abundance result from a primary or secondary response to cell stimulation, a time course experiment was carried out in the presence of the protein synthesis inhibitor, cycloheximide (CHX). A549 cells were treated with IFNα (1000 units/ml) or infected with Sendai virus (5 p.f.u./cell) in the presence or absence of CHX. RNA samples were prepared 4, 10, and 24 hours after treatment and subjected to miRNA analysis (Fig 4B). Treatment with CHX did not inhibit miRNA production in general, as miR-449b remains virus-inducible regardless of CHX. After 4 hours of IFN treatment, the level of miR-203 increased two fold, and this increase was unaffected by CHX treatment. A similar increase in miR-203 was observed during the first 4 hours of Sendai virus-infection, but this increase was not observed in the presence of CHX. These data indicate that protein synthesis during Sendai virus-infection is required for miR-203 induction whereas a preexisting signaling complex downstream of IFN, possibly the IFN-activated JAK-STAT pathway, mediates the initial increase in miR-203.
During prolonged stimulation with IFNα or infection with Sendai virus (i.e., 10h and 24h), miR-203 continues to accumulate, but in all cases this accumulation is dramatically reduced in the presence of CHX. These data indicate that the sustained expression of miR-203 is mediated by factors that are generated during the antiviral response as a result of new protein synthesis.

**IFIT1/ISG56 is a target of miR-203**

To identify potential mRNA targets of miR-203, the TargetScan algorithm was used to locate miR-203 seed matches within 3' UTRs of cellular mRNAs (49). One potential target of miR-203 was identified as the IFN inducible gene IFIT1/ISG56, which contains two slightly overlapping seed matches for miR-203 (Fig 5A). To determine if miR-203 can target IFIT1/ISG56, A549 cells were transfected with miRNA mimics or antagonists in combination with IFN treatment, and the effect of these miR-203 agonists and antagonists on IFIT1/ISG56 mRNA levels were determined by RT-qPCR (Fig 5B). The IFIT1/ISG56 mRNA is strongly induced by IFN stimulation, and was unaffected by the non-targeting control miRNA mimic or antagonist. In contrast, the miR-203 mimic
suppressed IFIT1/ISG56 mRNA accumulation by 43% compared to the non-targeting control. In the complementary experiment, the miR-203 antagonist increased the level of IFIT1/ISG56 mRNA by 47% compared to the control. These results demonstrate that miR-203 can specifically regulate the level of IFIT1/ISG56 mRNA even as it is undergoing potent induction during IFN stimulation.

To verify the mRNA analysis, the effect of the miRNA agonists and antagonists on the level of IFIT1/ISG56 protein was determined by immunoblotting. A549 cells transfected with a miR-203 mimic had a 34% lower level of IFIT1/ISG56 protein, while miR-203 antagonist increased IFIT1/ISG56 protein level by 51% compared to the non-targeting control mimic and inhibitor (Fig 5C). The levels of control proteins MDA5 and GAPDH did not change due to miR-203 perturbation. These findings demonstrate that miR-203 regulates the expression of IFIT1/ISG56 at both RNA and protein levels.

Identification of additional miR-203 targets during IFN stimulation and Sendai virus infection

In order to determine additional targets of miR-203, a gene expression profiling experiment was performed. A549 cells were either left untreated, treated with IFN or
infected with Sendai virus, both in the presence or absence of exogenously expressed miR-203. RNA was purified 10 hours post treatment and used to probe a microarray to determine changes in gene expression due to miR-203. The analysis indicated that 21 genes are differentially regulated during IFN treatment and 12 genes during Sendai virus infection by greater than 1.5 fold as a consequence of elevated miR-203 (Table 2). The most differentially regulated gene during IFN treatment and the second most differentially regulated gene during Sendai virus infection is LAMP1, which contains 2 seed matches for miR-203 and has been previously identified as a miR-203 target gene (50, 51). The identification of a known mir-203 target validates the gene expression profiling method for identifying miRNA target genes. Additional gene targets that were identified by both IFN stimulation and Sendai virus infection include RAPGEF1, SEPN1, and DNAJB6. The mRNA levels for these genes were reduced by 1.93, 1.54, and 1.5 -fold due to miR-203 expression during IFN treatment and 1.54, 1.63, 1.7 -fold, respectively, due to miR-203 expression during Sendai virus infection. These three genes contain miR-203 target sites in their 3'UTR suggesting they are directly regulated by miR-203. Consistent with our direct analysis above, the level of IFIT1/ISG56 mRNA expression was affected by miR-203 during Sendai virus infection and IFN treatment; IFIT1/ISG56 expression was reduced by 22.3% and 9.6%, respectively. However, as
these differences represent the net change achieved by slow miRNA destabilization compared to the rapid transcriptional activation of IFIT1/ISG56, these levels did not meet our strict cutoff requirements for the differential screen due to the limited dynamic range of hybridization-based microarrays for observation of small changes in highly abundant RNAs. Underestimation of relative mRNA expression changes during microarray experiments has been reported (52), and suggests that small changes observed during the profiling experiment are indicative of larger changes within the cell, as we observe by direct analysis.

The observation that miR-203 partially reduced the level of an IFN-stimulated gene product raised the question of whether miR-203 by itself has the ability to demonstrably alter the course of Sendai virus infection under these conditions. In order to test the effect of miR-203 on Sendai virus infectivity and replication, experiments were performed to evaluate virus replication in A549 cells in the presence of miRNA mimics and antagonists. However, neither expression nor inhibition of miR-203 was found to significantly alter the Sendai virus replication when compared to infection alone or in the presence of control miRNAs (not shown). In the absence of detectable direct effects on virus replication, it is hypothesized that miR-203 may play a role related to more subtle
regulation of cellular response pathways. In order to evaluate potential effects of miR-203 on cellular gene expression, gene ontology analysis was performed on the identified miR-203-regulated genes. The miR-203 regulated genes were found to fall into three significant functional annotation clusters that include diverse cellular processes such as regulation of cytoskeleton, response to organic stimuli, regulation of cell death, and protein complex biogenesis (Table 3). This analysis indicates that miR-203 is capable of regulating diverse cellular processes during IFN stimulation and the cellular response to Sendai virus infection.
Discussion

The mammalian antiviral response is initiated by dramatic changes in gene expression that produce effective barriers to virus replication. Many transcriptional and post-transcriptional mechanisms underlying cellular antiviral effects have been characterized in detail, and endogenous miRNA regulation of cellular responses to virus infections are an area of intense investigation. Next-generation sequencing has enabled analysis of the small RNA profile of human A549 cells in the steady state or following Sendai virus infection. However, while the majority of cellular miRNAs maintain constant levels irrespective of infection, direct comparison has revealed specific examples of miRNAs that significantly accumulate in response to Sendai virus infection (Supplemental Table 1).

One of the Sendai virus-induced miRNAs, miR-203, was studied in detail and found to be potently induced by Sendai virus through the virus-induced IFN antiviral response. Biphasic expression of miR-203 through both direct and indirect IFN-mediated antiviral signaling pathways was found to contribute to the production of miR-203 continuously throughout Sendai virus infection. The absolute level of miRNA induction was found to vary slightly among experiments, possibly related to cell passage number, cell density,
or slight differences in infection efficiencies, but miR-203 was consistently induced by Sendai virus, generally in the range of 8-14-fold compared to mock infected cells. Pre-existing factors were found to drive the immediate expression of miR-203 following IFN stimulation, and newly synthesized downstream factors are required to mediate the continued expression of miR-203. It is noteworthy that multiple ISRE/IRF binding sites were identified within 2 kbp of the putative miR-203 precursor transcriptional start site, possibly implicating the IFN-activated JAK-STAT-ISGF3 pathway in the immediate regulation of miR-203 activation, and interferon regulatory factors or other ISG products as the newly synthesized factors driving late expression. This type of regulation is reminiscent of IFN-stimulated regulation of mRNA coding genes, such as GBP, which is regulated by both immediate-acting STAT transcription complexes and later by IFN regulatory factors such as IRF1 (53). Activation of mir-203 by Sendai virus infection is blocked by CHX treatment at all time points, consistent with the requirement for virus-induced IFN biosynthesis to drive miRNA expression.

Computer-aided target analysis identified the IFIT1/ISG56 mRNA as potential miR-203 target, and the ability of miR-203 to destabilize the mRNA was demonstrated using miRNA agonists and antagonists. Together, these results indicate that an IFN-inducible
miRNA can regulate the abundance of an IFN-inducible target gene. Although this arrangement of co-regulated antiviral activators and their inhibitors may seem counterintuitive, this pattern of regulation resembles a classic example of an incoherent feed-forward loop, in which a single signal (i.e., infection or IFN) activates two independent responders (IFIT1/ISG56 and miR-203) and one responder (miR-203) serves to negatively regulate the other (IFIT1/ISG56) (54). The results described here fit well to such a model for an antiviral regulatory pathway (Fig 6). After infection with Sendai virus, cells mount an antiviral response by producing type I IFN, which signals to activate the transcription of a variety of ISGs, including IFIT1/ISG56, miR-203, and other mediators such as IRF family proteins. MiR-203 is potentially positively regulated by IRFs for sustained expression in a positive feed forward loop during the antiviral response. The IFIT1/ISG56 mRNA is regulated by miR-203 in an incoherent feed-forward loop, thereby modulating the production of IFIT1/ISG56 protein (Fig 6). MiR-203 expression was found to be sensitive to CHX treatment at two points during Sendai virus infection. The production of type I IFN requires translation of nascent mRNAs during the early phase of infection, and the production of additional signaling molecules that participate in miR-203 regulation during late stages of infection, these are represented in the model as IFN regulatory factors.
Beyond regulation of IFIT1, several other mRNA targets of miR-203 were identified using a microarray screening approach. Despite these connections, miR-203 expression or antagonism failed to reveal dramatic effects on Sendai virus replication, kinetics, or cytopathicity. These findings are distinct from cases of virus-hijacked microRNA variants that have been linked to efficient virus and cellular regulatory phenomena (55), and may indicate that miR-203 serves to fine-tune cellular protein expression during the cellular response to virus infection without directly limiting Sendai virus replication. A role for miR-203 as a mediator of more subtle alterations to its protein target is consistent with the current understanding of miRNA action (56). By analogy, while it is well established that IFN is a potent antiviral cytokine, not all of the virus-inducible IFN target genes imitate this antiviral activity when expressed alone (57). Therefore, it is not surprising that the partial attenuation of IFIT1/ISG56 does not have a measurable impact on Sendai virus replication under these conditions. Both IFIT1/ISG56 and miR-203 are regulated with distinct kinetics and to varying degrees by both ISGF3 and other virus-induced transcription factors (58-60), supporting a model in which miR-203 functions as a regulator rather than an on-off switch to regulate rather than eliminate IFIT1/ISG56 expression. Additional miR-203 targets identified here, and
in other studies have implicated miR-203 in diverse processes related to virus replication and immune regulation including modulation of MyD88 (61), TNFα, IL-24 (62), suppressor of cytokine signaling (SOCS)1 and SOCS3 (63), regulation of papillomavirus infection in keratinocytes (64, 65), and regulation of miR-203 abundance during rabies virus (30), Epstein-Barr virus (66), and Cocksackie B3 virus infection (67). It is likely that IFN and virus induction of mir-203 plays both general and tissue specific roles in the regulation of antiviral responses.
Acknowledgements

The authors acknowledge the Northwestern University Genomics Core Facility for assistance with the deep sequencing experiment and associated bioinformatics, Dr. Robert A. Lamb for the generous gift of influenza virus strains and expertise in propagating Sendai virus, and members of the Horvath lab for critical and helpful discussions. William A. Buggele is supported by the Malkin Scholars Fund and the Cell and Molecular Basis of Disease Pre-doctoral training grant (T32 GM008061). This work was supported by the NIH Grant U01AI082984 from the NIAID IMVC program.


Figure 1. Small RNA Profile of uninfected and Sendai virus-infected A549 cells.

A549 cells were mock infected or infected with Sendai virus (Cantell strain; 5 p.f.u./cell). RNA was purified 10 h later, size fractionated to yield RNA <200 nt in length, and cDNA libraries were prepared for SOLiD sequencing. A.) Venn diagram illustrates the number of miRNAs identified (by greater than 100 sequence tags) that are either unique or common to Mock infected or Sendai virus infected libraries. B.) Scatter plot indicates the abundance of each identified miRNA in the mock infected or Sendai virus-infected libraries with greater than 100 sequence tags. Blue dots represent miRNAs with less than 1.5-fold change, and red dots represent miRNAs with greater than 1.5-fold change.

Figure 2. Validation of changes in miRNA abundance induced by Sendai virus infection.

For each indicated miRNA, normalized sequence tag abundance is plotted to the left of the dashed line. Individual miRNAs were also measured in freshly prepared RNA derived from A549 cells that were either mock infected or infected with Sendai virus (5 p.f.u./cell, 10 h) using specific TaqMan microRNA RT-qPCR assays are plotted to the
right of the dashed line. Statistical significance was determined by a two-tailed t-test
(p<0.05=*, p<0.01=**, p<0.001=***).

Figure 3. IFN mediates regulation of miR-203.

A.) A549 cells were mock infected or infected with either influenza virus A/Udorn/72
(Udorn), A/WSN/33 (WSN), or Sendai virus Cantell Strain (Sendai) (5 p.f.u./cell, 10h).

RNA was purified 10 hours post infection, size fractionated to yield RNA <200nt in
length, and the abundance of miR-203, miR-449b, and miR-16 was analyzed using
TaqMan miRNA RT-qPCR assays.

B.) High molecular weight RNA was used to measure the abundance of IFNβ mRNA
during Sendai virus and influenza virus infection by RT-qPCR as indicated.

C.) A549 cells were left untreated, or treated with PBS or increasing concentrations of
IFNα (250 units/mL, 500 units/mL, 1000 units/mL, or 5000 units/mL). RNA was purified
10 hours post treatment and the abundance of miR-203 was analyzed by TaqMan
miRNA assays.
D.) A549 cells were left untreated, or treated as indicated with PBS or IFNα (1000 units/mL), mock infected, or infected with Sendai virus (5 p.f.u./cell). RNA was purified 10 hours post treatment and the abundance of miR-203 was analyzed by TaqMan miRNA assays.

E.) A549 cells were left untreated, treated with PBS or IFNα (1000 units/mL) or transfected with Poly(I:C) (5μg/mL). RNA was purified 10 hours post treatment and the abundance of miR-203 was analyzed by TaqMan miRNA assays.

F.) Vero cells were left untreated, or treated as indicated with PBS or IFNα (1000 units/mL), mock infected, or infected with Sendai virus (5 p.f.u./cell). RNA was purified 10 hours post treatment and the abundance of miR-203 was analyzed by TaqMan miRNA assays.

Statistical significance was determined by a two-tailed t-test (p<0.05=*, p<0.01=**, p<0.001=***).
Figure 4. Activation of miR-203 by immediate and delayed signaling pathways.

A.) A549 cells were infected with Sendai virus (5 p.f.u./cell) and the abundance of miR-203 was measured at indicated times after Sendai virus infection by TaqMan miRNA RT-qPCR.

B.) A549 cells were stimulated with IFNα (1000 units/mL) or infected with Sendai virus (5 p.f.u./cell) for 4, 10, and 24 hours in the presence (+) or absence (-) of cycloheximide (CHX 100 ng/mL; 1h pre-treatment and continuous thereafter). Abundance of miR-203 and miR-449b was determined by TaqMan microRNA assays at indicated time points. Statistical significance was determined by a two-tailed t-test (p<0.05=*, p<0.01=**, p<0.001=***).

Figure 5. Identification and characterization of IFIT1/ISG56 as a miR-203 target.

A.) Illustration of the 3’UTR of IFIT1/ISG56, with boxes indicating the positions of two partially overlapping miR-203 seed matches.

B.) A549 cells were transfected with 25nM of non-targeting miRNA mimic (Control), or non-targeting control miRNA inhibitor (Inhib.), or miR-203-specific mimic, or miR-203
inhibitor as indicated. Cells were either untreated (-) or treated (+) with 1000 units/mL IFNα. RNA was purified from cells 10 h later and analyzed by RT-qPCR for the induction of IFIT1/ISG56 mRNAs. In parallel, levels of miR-203 were measured by TaqMan assay. Statistical significance was determined by two-tailed t-test (p<0.05=*, p<0.01=**, p<0.001=***).

C.) A549 were transfected with miRNA mimics and inhibitors, then treated with IFNα as in panel (B.) Whole cell lysates were prepared 10 h later and analyzed by immunoblotting with antisera specific for IFIT1/ISG56, MDA5, or GAPDH proteins.

Figure 6. Model of the regulation of miR-203 and IFIT1/ISG56 during Sendai virus infection.

Cells infected with Sendai virus generate an antiviral response by producing type I IFN which signals through the JAK-STAT-ISGF3 pathway to induce IFN stimulated gene products including IFIT1/ISG56, miR-203, and IFN regulatory factors (IRFs). Both IFN production and IRF induction are sensitive to treatment with CHX. MiR-203 can be induced by IFN initially by pre-existing proteins, but sustained miR-203 expression requires newly synthesized signaling proteins, potentially, but not limited to, IRF family
members (IRF?). MiR-203 accumulation functions to destabilize IFIT1/ISG56 mRNA and regulate its translation. See text for more details of the model.
Table 1. Sequencing Library Statistics

<table>
<thead>
<tr>
<th>RNA Sequence Tag Classification</th>
<th>Mock(^a)</th>
<th>Sendai virus(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Sequence Tags</td>
<td>97542060(^c)</td>
<td>108423000(^c)</td>
</tr>
<tr>
<td>Mappable Sequence Tags</td>
<td>32188879</td>
<td>35247578</td>
</tr>
<tr>
<td>microRNA Sequence Tags</td>
<td>18012238</td>
<td>11917137</td>
</tr>
<tr>
<td>miscRNA Sequence Tags</td>
<td>183009</td>
<td>411399</td>
</tr>
<tr>
<td>miRNA Sequence Tags</td>
<td>483546</td>
<td>389294</td>
</tr>
<tr>
<td>rRNA Sequence Tags</td>
<td>1297566</td>
<td>1898927</td>
</tr>
<tr>
<td>snoRNA Sequence Tags</td>
<td>1143405</td>
<td>626386</td>
</tr>
<tr>
<td>snRNA Sequence Tags</td>
<td>188817</td>
<td>127418</td>
</tr>
<tr>
<td>Other RNA Sequence Tags</td>
<td>11294960</td>
<td>19877017</td>
</tr>
</tbody>
</table>

\(^a\) RNA sequence tag classification identified through small RNA deep sequencing

\(^b\) A549 cells were either mock-infected or infected with Sendai virus (5 p.f.u./cell) for 10 hours

\(^c\) Number of sequence tags that correspond to particular RNA feature identified in library
Table 2. MicroRNA-203 Regulated Genes During IFNα Treatment and Sendai Virus Infection

<table>
<thead>
<tr>
<th>Gene IDa</th>
<th>Treatmentb</th>
<th>p-Value (IFN)c</th>
<th>Fold Change (IFN)d</th>
<th>p-Value (Sendai)e</th>
<th>Fold Change (Sendai)f</th>
<th>miR-203 Seed Matchesg</th>
</tr>
</thead>
<tbody>
<tr>
<td>LASP1</td>
<td>IFN, Sendai</td>
<td>9.70x10^{-13}</td>
<td>-1.94</td>
<td>3.89x10^{-10}</td>
<td>-1.83</td>
<td>2</td>
</tr>
<tr>
<td>RAPGEF1</td>
<td>IFN, Sendai</td>
<td>1.22x10^{-11}</td>
<td>-1.93</td>
<td>5.09x10^{-11}</td>
<td>-1.54</td>
<td>2</td>
</tr>
<tr>
<td>PRDX3</td>
<td>IFN, Sendai</td>
<td>5.89x10^{-12}</td>
<td>-1.82</td>
<td>2.01x10^{-10}</td>
<td>-2.53</td>
<td>N/A</td>
</tr>
<tr>
<td>COL4A1</td>
<td>IFN</td>
<td>7.62x10^{-11}</td>
<td>-1.71</td>
<td>N/A</td>
<td>N/A</td>
<td>1</td>
</tr>
<tr>
<td>TUBGCP2</td>
<td>IFN, Sendai</td>
<td>6.83x10^{-11}</td>
<td>-1.70</td>
<td>2.07x10^{-07}</td>
<td>-1.71</td>
<td>N/A</td>
</tr>
<tr>
<td>C12orf75</td>
<td>IFN, Sendai</td>
<td>5.80x10^{-11}</td>
<td>-1.69</td>
<td>5.78x10^{-07}</td>
<td>-1.62</td>
<td>N/A</td>
</tr>
<tr>
<td>SRC</td>
<td>IFN</td>
<td>8.92x10^{-11}</td>
<td>-1.61</td>
<td>N/A</td>
<td>N/A</td>
<td>2</td>
</tr>
<tr>
<td>SELT</td>
<td>IFN</td>
<td>3.93x10^{-08}</td>
<td>-1.58</td>
<td>N/A</td>
<td>N/A</td>
<td>1</td>
</tr>
<tr>
<td>BANF1</td>
<td>IFN, Sendai</td>
<td>6.23x10^{-09}</td>
<td>-1.55</td>
<td>2.37x10^{-09}</td>
<td>-1.67</td>
<td>N/A</td>
</tr>
<tr>
<td>SEPN1</td>
<td>IFN, Sendai</td>
<td>6.29x10^{-08}</td>
<td>-1.54</td>
<td>4.96x10^{-07}</td>
<td>-1.63</td>
<td>2</td>
</tr>
<tr>
<td>POLR1E</td>
<td>IFN</td>
<td>3.37x10^{-09}</td>
<td>-1.54</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>LAMP2</td>
<td>IFN</td>
<td>1.15x10^{-08}</td>
<td>-1.54</td>
<td>N/A</td>
<td>N/A</td>
<td>3</td>
</tr>
<tr>
<td>TCP1</td>
<td>IFN</td>
<td>1.92x10^{-08}</td>
<td>-1.53</td>
<td>N/A</td>
<td>N/A</td>
<td>2</td>
</tr>
<tr>
<td>CHMP3</td>
<td>IFN</td>
<td>7.71x10^{-07}</td>
<td>-1.52</td>
<td>N/A</td>
<td>N/A</td>
<td>2</td>
</tr>
<tr>
<td>SLC12A2</td>
<td>IFN</td>
<td>1.15x10^{-07}</td>
<td>-1.51</td>
<td>N/A</td>
<td>N/A</td>
<td>1</td>
</tr>
<tr>
<td>CHMP3</td>
<td>IFN</td>
<td>4.74x10^{-08}</td>
<td>-1.50</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>PHLD3</td>
<td>IFN, Sendai</td>
<td>1.03x10^{-08}</td>
<td>-1.50</td>
<td>2.66x10^{-08}</td>
<td>-1.54</td>
<td>N/A</td>
</tr>
<tr>
<td>DNAJB6</td>
<td>IFN, Sendai</td>
<td>2.23x10^{-07}</td>
<td>-1.50</td>
<td>1.40x10^{-07}</td>
<td>-1.70</td>
<td>1</td>
</tr>
<tr>
<td>PPAP2B</td>
<td>Sendai</td>
<td>N/A</td>
<td>N/A</td>
<td>1.38x10^{-06}</td>
<td>-1.72</td>
<td>N/A</td>
</tr>
<tr>
<td>PHLD3</td>
<td>Sendai</td>
<td>N/A</td>
<td>N/A</td>
<td>2.66x10^{-06}</td>
<td>-1.54</td>
<td>1</td>
</tr>
<tr>
<td>NCL</td>
<td>Sendai</td>
<td>N/A</td>
<td>N/A</td>
<td>6.96x10^{-07}</td>
<td>-1.54</td>
<td>N/A</td>
</tr>
<tr>
<td>SLC2A3</td>
<td>IFN</td>
<td>2.50x10^{-07}</td>
<td>1.51</td>
<td>N/A</td>
<td>N/A</td>
<td>1</td>
</tr>
<tr>
<td>IGFBP1</td>
<td>IFN</td>
<td>3.63x10^{-06}</td>
<td>1.57</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>ALDOC</td>
<td>IFN</td>
<td>5.08x10^{-06}</td>
<td>1.62</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
</tbody>
</table>

a. Gene identifiers that were differentially regulated by ≥1.5 fold during IFN treatment or Sendai virus infection during over-expression of miR-203
b. A549 cells were treated with 1000 units/mL IFNα, IFN, or infected with Sendai virus, Sendai (moi 5pfu/cell)
c. Statistical significance of gene expression due to the presence of exogenous miR-203
d. Fold change of gene due to exogenous miR-203
e. Number of miR-203 seed matches in the 3' UTR of indicated gene as determined by at least 2 predictive algorithms using MiR-Walk (39).
Table 3. Functional annotation clustering of miR-203 regulated genes.

<table>
<thead>
<tr>
<th>Functional Cluster</th>
<th>Enriched Biological Processes</th>
<th>MiR-203 regulated genes associated with cluster</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cluster 1</td>
<td>Response to Organic Substance</td>
<td>DANJ6, ALDOC, IGFBP1, PRDX3, SRC</td>
</tr>
<tr>
<td></td>
<td>Regulation of Apoptosis</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Regulation of programmed cell death</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Regulation of Cell Death</td>
<td></td>
</tr>
<tr>
<td>Cluster 2</td>
<td>Protein complex assembly</td>
<td>ALDOC, TCP1, TUBGCP2, SRC</td>
</tr>
<tr>
<td></td>
<td>Protein complex biogenesis</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Macromolecular complex subunit</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Macromolecular complex subunit organization</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cytoskeleton</td>
<td></td>
</tr>
<tr>
<td>Cluster 3</td>
<td>Transmembrane receptor protein tyrosine kinase signaling pathway</td>
<td>RAPGEF1, IGFBP1, SLC12A2, SRC</td>
</tr>
</tbody>
</table>

a. Statistically significant functional clusters of miR-203 regulated genes determined by the DAVID bioinformatics resource (42, 43)