Methods Collection

Cell culture

Primary human bronchial epithelial cells (HBEpC) and small airway epithelial cells (SAEpCs) were purchased from PromoCell GmbH (Heidelberg, Germany) and sub-cultured in media supplemented with growth factors. Madin Darby Canine Kidney (MDCK) cells were cultured in Eagle's Minimum Essential Medium (ATCC, Manassas, VA) with 10% fetal bovine serum, and appropriate antibiotics. MDCK cells were used for the propagation of influenza virus H1N1 (A/WSN/33), H9N1 (1P10), and H9N1 (1WF10). Influenza virus H1N1 (A/WSN/33) was a kind gift from Prof. Robert A. Lamb (Northwestern University, Chicago, IL, USA), and H9N1 strains were from Daniel Perez (Georgia University, GA).

Viral infections

All infections of HBEpCs and SAEpCs were performed in 6-well plates (Corning, NY) at different multiplicity of infection (MOI). All experiments were done in duplicates and the experiments were repeated at 3 different days. Control cells were mock infected. Prior to infection, the cells were rinsed with PBS, and then virus diluted in Modified Hanks Buffer Saline Solution was added to each well. After a 45-minute incubation, excess virus was rinsed off using cold PBS. Fresh F12 media containing 1 µg/ml of TPCK (L-(tosylamido-2-phenyl) ethyl chloromethyl ketone) modified-trypsin (Sigma-Aldrich, St Louis, MO) was added to H9N1 strains and medium lacking TPCK was added to the H1N1strain. Both plates were incubated at 37 ℃ and 5% CO_2. Cells were harvested at different time intervals as described in methods.

Next generation sequencing (NGS)

NGS analysis was conducted on total RNA isolated from SAEpCs infected with influenza virus H1N1 and both H9N1 strains 1WF10 and 1P10 to identify the differentially expressed host miRNAs, both known in miRbase release 20 and unknown miRNAs (putative miRNA). SAEpCs were infected with influenza virus for 3 hours. Total RNA including miRNA was isolated from infected and uninfected cells using the miRNeasy kit (Qiagen, Rockville, MD) and was sent to Exiqon for NGS analysis. Quality of RNA was assessed by Agilent 2100 Bioanalyzer and Agilent RNA 6000 Nano Reagents (Santa Clara, CA).

HBEpC were transfected with mimic of put-miR-31, 34, and 35 for 24 h and the isolated RNA was used for NGS analysis. In addition, the cells transfected with the mimic of put-miR-22, 23, 31, and 34 miRNAs were subjected to AGO immunoprecipitation, and the resulting RNA was used for NGS analysis. The data used for miRNA sequence confirmation and the pathways targeted by these putative miRNAs using the Gene Ontology program.

For confirmation of putative miRNA expression in cells infected with influenza virus, we used the RT-PCR analysis, HBEpCs were infected with different strains of influenza virus for 3 h and RNA was extracted as described earlier. Analysis was contracted out to Exiqon. Briefly, 10 ng RNA was reverse transcribed in 10 µl reactions using the miRCURY LNA™ Universal RT microRNA PCR, Polyadenylation and cDNA synthesis kit (Exiqon, Aarhus, Denmark). cDNA was diluted 100 x and assayed in 10 µl PCR reactions according to the protocol for miRCURY LNA™ Universal RT microRNA PCR; each microRNA was assayed once by qPCR on the microRNA Ready-to-Use PCR, Custom Pick and Mix Panel using ExiLENT SYBR® Green master mix. Negative controls excluding template from the reverse transcription reaction were performed and profiled like the samples. Amplification was performed in a LightCycler® 480 Real-Time PCR System (Roche) in 384 well plates,

and amplification curves were analyzed using the Roche LC software, both for determination of Cq (by the 2nd derivative method) and for melting curve analysis.

NGS Data analysis

The amplification efficiency was calculated using algorithms similar to the LinRegPCR software. All assays were inspected for distinct melting curves and the Tm was checked to be within known specifications for the assay. Cq was calculated as the 2nd derivative. Using NormFinder the best normalizer was found to be the average of assays detected in all samples. All data were normalized to the average of assays detected in all samples (average - assay Cq). When comparing the two groups, a few miRNAs were found to be differentially expressed when using a p-value cutoff of 0.05. Two of these pass the Benjamini- Hochberg correction for multiple testing at a significance level of 0.05.

MicroRNA analysis

A time course study on putative miRNA expression in cells exposed to influenza virus was carried out by collecting samples at different time intervals post infection of influenza virus. All these experiments were conducted in duplicates and at 3 different days. Total RNA, including miRNA, was isolated from HBEpCs infected with influenza virus H1N1, H9N1(1P10) and H9N1 (1WF10) or mock infected cells using miRNeasy kit (Qiagen, Hilden, Germany) following the protocol of the supplier. The quality of the total RNA was verified by an Agilent 2100 Bioanalyzer profile following the procedure as suggested by the manufacturer. RT-PCRs were performed to determine the expression of selected miRNAs (put-miR-31, 34, 35) on cDNA synthesized from total RNAs, including miRNAs, isolated from HBEpCs or vehicle exposure. Specific TaqMan primers were synthesized (Thermo Fisher Scientific) and used with the TaqMan assay. Fold changes of miRNA were calculated using ddCt method, where Ct is the threshold cycle to detect fluorescence. The data were normalized to control, and fold change of miRNAs was calculated against control miRNA (U6 snRNA).

Transfection

HBEpC were transfected with put-miR-34 inhibitor oligonucleotide or a put-miR-34 mimic oligonucleotide (Thermo Fisher Scientific, Carlsbad, CA) using the lipid-based Lipofectamine 2000 reagent diluted in Opti-MEM-I reduced serum medium (Life Technologies, Carlsbad, CA). HBEpC were grown to 80% confluence in 6-well plates (Corning) and the transfection complexes were directly applied to the cells. As a negative control, cells were transfected with the same concentrations of scrambled oligonucleotides (Thermo Fisher Scientific). These cells were then exposed to 1.0 MOIs of virus, by following standard infection protocols for influenza A. Following the incubation, cells were harvested and used for RNA extraction using the RNeasy kit (Qiagen). For the transfection factor PCR array (Themofisher Scientific) the plates were run in duplicates, and hence no statistical analysis was reported.

Argonaute-immunoprecipitation

Co-immunoprecipitation of AGO proteins with associated RNAs is a powerful tool to validate miRNA targets. Briefly, cells were grown to confluency in a 6-well plates (Corning). After 24h of plating, cells were transfected with the mimics of put-miRNA-31, 34, 35 or a scrambled mimic miRNA (control) using lipofectamine 2000. After 24-36 h of transfection, cells were scrapped off by cold PBS and cells were lysed using the lysis buffer provided by the supplier (IP kit, Active motif, Carlsbad, CA) followed the manufactures instruction to complete the IP protocol to isolate the RNA and cDNA synthesis. The cDNA was used as template for the fold enrichment and identification of ago-associated miRNAs by RT-PCR,

using specific Taqman primers for put-miR-31, 34, and 35 (Thermo Fisher Scientific). The data were normalized to control, and fold enrichment of miRNAs was calculated as described in supplier's protocol.

Western immunoblotting

HBEpC cells were transfected with put miR-34 mimic or inhibitor for 36h and then mock infected or infected with H1N1 of MOI of 1.0. After 18 h post infection (p.i.), cells were lysed in 100 µl of radioimmunoprecipitation assay (RIPA) buffer (Thermo Scientific) containing protease inhibitor cocktail mixture (Thermo Fisher Scientific). Thirty micrograms of protein were solubilized in protein sample buffer and subjected to electrophoresis on 10% SDS-polyacrylamide gels (SDS-PAGE). Separated proteins were transferred to a nitrocellulose membrane (Bio-Rad). Membranes were blocked using Odyssey Blocking Buffer (LI-COR Biosciences, Lincoln, NE) for 1 h at room temperature and subjected to Western analysis with rabbit polyclonal anti-STAT3, anti-phospho-STAT3 (Abcam, MA), and mouse monoclonal anti-GAPDH (Abcam, MA) antibodies. Appropriate mouse and rabbit IRDye 680 or 800 secondary antibodies (LI-COR Biosciences) were used. Near-infrared fluorescence detection was performed on the Odyssey Imaging System (LI-COR Biosciences, Lincoln, NE), and the fluorescent signal intensities of the individual bands were determined and normalized to GAPDH. All quantitation was done using the manufacturer's software.