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ORIGINAL ARTICLE

Identification of host miRNAs that may limit human rhinovirus replication

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Abstract

AIM: To test whether the replication of human rhinovirus (HRV) is regulated by microRNAs in human bronchial epithelial cells.

METHODS: For the present study, the human cell line BEAS-2B (derived from normal human bronchial epithelial cells) was adopted. DICER knock-down, by siRNA transfection in BEAS-2B cells, was performed in order to inhibit microRNA maturation globally. Alternatively, antisense oligonucleotides (anti-miRs) were transfected to inhibit the activity of specific microRNAs. Cells were infected with HRV-1B. Viral replication was assessed by measuring the genomic viral RNA by reverse transcription quantitative polymerase chain reaction (RT-qPCR). Association between microRNA-induced-silencing-complex and viral RNA was detected by Ago2 co-immunoprecipitation followed by RT-qPCR. Targetscan v.6 was used to predict microRNA target sites on several HRV strains.

RESULTS: Here, we show that microRNAs affect replication of HRV-1B. DICER knock-down significantly reduced the expression of mature microRNAs in a bronchial epithelial cell line (BEAS-2B) and in turn, increased the synthesis of HRV-1B RNA. Additionally, HRV-1B RNA co-immunoprecipitated with argonaute 2 protein, an important effector for microRNA activity suggesting that microRNAs bind to viral RNA during infection. In order to identify specific microRNAs involved in this interaction, we employed bioinformatics analysis, and selected a group of microRNAs that have been reported to be under-expressed in asthmatic bronchial epithelial cells and were predicted to target different strains of rhinoviruses (HRV-1B, -16, -14, -27). Our results suggest that, out of this group of microRNAs, miR-128 and miR-155 contribute to the innate defense against HRV-1B: transfection of specific anti-miRs increased viral replication, as anticipated *in-silico*.

CONCLUSION: Taken together, our results suggest that pathological changes in microRNA expression, as already reported for asthma or chronic obstructive pulmonary disease have the potential to affect Rhinovirus replication and therefore may play a role in virus-induced exacerbations.

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Key words: Human Rhinovirus; MicroRNAs; MiR-155; SiRNA; Lentiviral transduction; Antiviral innate immunity

Core tip: Our results show for the first time that: (1) DICER knock-down increases HRV-1B replication in hu-

man bronchial epithelial cells; (2) the genomic RNA of human rhinovirus (HRV)-1B interacts directly with the miRISC during infection; and (3) inhibition of two microRNAs predicted to target HRV-1B, *i.e.*, miR-128 and miR-155, favors viral replication. This supports a role for cellular microRNAs in the antiviral response to HRV-1B mounted by bronchial epithelial cells, and suggests that pathological microRNA dysregulation may contribute to the poor antiviral immunity in diseases such as asthma and chronic obstructive pulmonary disease.

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INTRODUCTION

MicroRNAs (or miRs) are small, non-coding RNAs that regulate gene expression at the post-transcriptional level. MicroRNAs are synthesized in the nucleus as long primary transcripts. These undergo a complex maturation process that includes cleavage by DICER in the cytoplasm, yielding the single-stranded mature form[1]. Mature microRNAs then associate with Argonaute (AGO) proteins[2]. These constitute the core of the effector complex often referred to as microRNA-induced silencing complex[3]. MicroRNAs modulate the rate of translation and the stability of target mRNAs by binding to partially complementary sites, within the 3' untranslated region (UTR) of their targets. Not requiring a perfect match, microRNAs of a given sequence can interact with many different mRNAs and therefore, the expression of several genes can be modulated by a single microRNA[4]. It is not straightforward to predict which genes can be regulated by a specific microRNA thus complex algorithms such as Targetscan[5] have been developed for this purpose.

Being part of the mechanisms that cells use to regulate and fine tune gene expression, it is not surprising that microRNAs play a role in many different biological processes such as development, cellular differentiation, maturation or apoptosis, highlighting their importance for normal cell function. As a consequence, their deregulation perturbs gene expression and can have pathological consequences, as evidenced by their involvement in cancer[6]. DICER is essential for mouse embryonic development[7] and later on for correct lung epithelium morphogenesis[8]. MicroRNA expression is highly regulated during lung development[9] in both mouse and human, suggesting an important role during this process[10]. Also in culture, the microRNA profile changes considerably during human bronchial epithelial cell differentiation, controlling the expression of genes involved in this process[11]. Several are the examples of microRNAs involved in the regulation of the immune response. For instance, miR-155 deficient mice showed impaired immune responses in addition to lungs with evident airway remodelling[12]. Importantly, the expression of microRNAs is not regulated only during development but also by a variety of extracellular stimuli such as cytokines, pathogen components and infection. For example, bacterial lipopolysaccharide (LPS) causes up-regulation of miR-155[13] but down-regulation of miR-125b in murine macrophages[14]. As expected by their opposite regulation, the two microRNAs have opposite effects on the expression of tumor necrosis factor α (TNF-α), which is secreted after LPS treatment. Often microRNAs are used by cells to fine tune or reinforce their responses, by targeting signalling molecules. For instance, in monocytes and macrophages, interleukin 1 β (IL-1β), TNF-α and LPS have been shown to up-regulate miR-146a/b. Perhaps in order to prevent excessive inflammation, the microRNA inhibits the expression of IL-1 receptor-associated kinase 1 and 2 and TNF receptor-associated factor 6 which are important signalling proteins of innate immunity pathways[15]. Also vesicular stomatitis virus (VSV) or Influenza A infection up-regulates miR-146a/b with the effect of promoting replication of the virus[16,17]. Respiratory syncytial virus can alter cellular microRNA expression[18,19]. In particular, in human bronchial epithelial cells the down-regulation of miR-221 favours viral replication by counteracting the induction of apoptosis in infected cells[20].

In addition to acting on host mRNAs, microRNAs can directly bind and regulate viral RNA and therefore hamper viral replication. Interestingly, such direct antiviral function has been conserved from plants to mammals[21]. In humans, antiviral microRNAs have been shown to act both independently and as important effectors of the interferon (IFN) system, which is paramount in innate antiviral immunity. For instance, Hepatits C Virus (HCV) exemplifies how microRNAs can influence viral tissue tropism and work either in favour or against viruses. In fact, miR-122, a liver-specific microRNA is able to facilitate HCV replication[22]. Conversely, IFN-β down-regulates miR-122 while up-regulating the expression of other microRNAs with sequence-specific antiviral activity[23]. Recently, IFN-β treatment of HeLa cells was shown to induce the expression of miR-23b[24] which in turn was able to limit HRV-1B replication, by reducing the levels of the very low-density lipoprotein receptor. DICER-deficient murine macrophages were also more permissive to VSV than wild type cells, with no difference in type I IFN production[25]. DICER and several microRNAs interfere also with the replication of Influenza A virus[26-28] and retroviruses such as Human Immune Deficiency Virus-1[29] and Primate Foamy Virus-1[30].

Considering the many examples of antiviral microRNAs reported, we hypothesized that Human Rhinoviruses (HRV) are targeted by microRNAs. While responsible for most of the common colds in healthy subjects, HRVs are a major trigger of chronic obstructive pulmonary disease (COPD) and asthma exacerbations[31-33] which represent a significant problem for disease management. HRVs predominantly infect epithelial cells of the upper and lower airways[34-37]. They are non-enveloped viruses of the

Picornaviridae family, and are subdivided in three species (A, B and C) based on sequence homology. HRV-A and -B comprise approximately 100 different strains which are also divided into two groups depending on receptor usage. Rhinoviruses of the major subgroup, such as HRV-16, use the intercellular adhesion molecule 1 for entry[38] while the low-density lipoprotein receptor family[39] is used by members of the minor subgroup, such as HRV-1B. Shortly after entry, the single stranded genomic RNA functions as mRNA, driving the expression of a single open reading frame that encodes all viral proteins. The double-stranded RNA, that forms during viral replication, is detected by cellular receptors such as Toll-like Receptor 3 and the RNA helicases *RIG-I* (retinoic acid inducible gene) and melanoma differentiation associated gene-5[40] leading to the induction of IFNs (and downstream IFN-regulated genes) and pro-inflammatory cytokines[41].

A bioinformatics study suggested that human microRNAs may effectively bind to the genome of Rhinoviruses[42] and artificial siRNAs have been shown to inhibit HRV-16 replication[43]. Here we show that microRNAs in general may be involved in the defence against Rhinovirus in human bronchial epithelial cells and that the inhibition of two microRNAs predicted to target HRV-1B, miR-128 and miR-155, increased the accumulation of intracellular viral RNA. To our knowledge, this is the first experimental study to provide evidence suggesting that constitutively expressed cellular microRNAs regulate HRV replication.

MATERIALS AND METHODS

Cell culture

BEAS-2B and THP-1 cells were grown in RPMI medium supplemented with Glutamax I (GIBCO) and 10% heat inactivated foetal bovine serum (FBS, GIBCO). All cells were cultured at 37 ℃ in a humidified 5% CO2 incubator. For the infection experiments, RPMI with Glutamax I supplemented with 2% FBS was used.

Propagation of HRV-1B and infection experiments

HRV-1B was a gift from Professor Sebastian L. Johnston (Imperial College, London). HRV-1B was grown on Ohio HeLa cells (obtained from the American Type Culture Collection) grown in Dulbecco's Modified Eagles Medium (DMEM, Gibco) supplemented with 10% FBS, 50 IU/mL penicillin, 50 mg/mL streptomycin, 2 mmol/L L-glutamine. Briefly, HeLa cells were grown up to around 70%-80% confluency. Cells were washed once with Hank's Balanced Salt Solution (GIBCO) and then HRV was added. The cell culture vessel was then kept at room temperature on a plate rocker for 1 h. Subsequently, more infection medium was added and finally the cell culture vessel was incubated at 33 ℃ (in a humidified incubator, in air with 5% CO_2) for 16-24 h. After performing three cycles of freeze-thaw at -80 ℃, the supernatant (containing the virus) was recovered, cell debris were removed by centrifugation at 800 × g at 4 ℃ and used to inoculate more HeLa cells. Finally, the virus-containing medium was centrifuged at 800 × g at 4 ℃ and then filtered through a 0.2 mm syringe filter. All the infection experiments presented here were performed with the same viral stock. The latter was titrated to 5.5 × 10^6 viral particles/mL by 50% tissue culture infective dose (TCID50) as previously described[44].

For infection experiments, BEAS-2B cells were grown until about 80% confluent, detached by trypsin treatment and seeded at 0.75 × 10^5 cells/well in 24-well plates. Twenty four hours later, growth medium was replaced with infection medium for about 16 h before infection with HRV-1B. In each experiment, before infection, the cells present in two wells were counted using an improved Neubauer 0.1 mm haemocytometer. Cells were detached by trypsin treatment and re-suspended in 1 mL/well. The cell count obtained was used to calculate the volume of viral stock necessary for the desired multiplicity of infection (MOI) according to the following formula: mL of viral stock = (MOI × number of cells)/TCID50. Cells to be infected were first washed with 1 mL/well of Phosphate buffered saline (PBS) and then 0.2 mL/well of the viral suspension were added. Cells were then incubated at room temperature on a plate rocker for one hour. At this point, residual virus was removed and cells were washed as above. Then, either 0.5 mL/well of infection medium was added, or cells were harvested (0 h post-infection) using 0.5 mL/well of TRI-Reagent (Ambion). Plates were placed in the incubator for the indicated time, before collection in TRI-Reagent.

Transfections

Negative control siRNA and an anti-DICER siRNA were purchased from Ambion (Silencer® Select Validated siRNA). Anti-DICER (Ambion siRNA ID: s23754) sense strand sequence (5'-3' sequence): GAUCCUAUGUUCAAUCUAAtt. Antisense strand sequence (5'-3' sequence): UUAGAUUGAACAUAGGAUCga. Negative control #2 (Ambion siRNA ID: 4390846) sequence not provided.

BEAS-2B cells were seeded at 0.75 × 10^5 cells/well in 24-well plates. Twenty four hours later cells were transfected as follows. Two hundred μL/well of growth medium; for each well to be transfected, 1.5 μL of Oligofectamine (Invitrogen) were diluted in 10 μL of plain RPMI and mixed by pipetting. In a separate microcentrifuge tube, 0.15 μL of 50 μmol/L siRNA were diluted in 40 μL of plain RPMI medium and mixed by pipetting. The tubes were left at room temperature. After 5 min, the contents of the two tubes were mixed together by pipetting and left at room temperature. Twenty minutes later, the mixture was mixed again by pipetting and dispensed on the cells (50 μL/well giving a final siRNA concentration of 30 nmol/L). The following day, the medium present in the wells was replaced with fresh infection medium. BEAS-2B cells were transfected every 36 h (3 d) following the protocol described. This required that 48 h post-transfection cells were detached by trypsin treatment and seeded at the density specified earlier. One day after the last transfection, the medium was replaced with infection medium. Cells were then infected with HRV-1B 24 h

later (48 h after the third transfection).

All the anti-miRs (Anti-miR™ miRNA Inhibitors) were purchased from Ambion: anti-miR negative control#1 cat.n: AM17010; anti-hsa-miR-18a, cat.n: AM12973; anti-hsa-miR-19b, cat.n: AM10629; anti-hsa-miR-106b, cat.n: AM10067; anti-hsa-miR-128, cat.n: AM11746; anti-hsa-miR-155, cat.n: AM12601.

Anti-miR transfections were performed using INTERFERin, as recommended by the manufacturer's protocol. BEAS-2B cells were seeded at 0.75×10^5 cells/well in 24-well plates. Twenty four hours later, the culture medium was replaced with 500 μL/well of growth medium. For each well to be transfected, 1.2 μL of 50 μmol/L anti-miR were diluted in 100 μL of plain RPMI and mixed by pipetting. Immediately after, 4 μL of INTERFERin were added and mixed by pipetting. After 20 min incubation at room temperature, 100 μL/well were used, giving a final anti-miR concentration of 100 nmol/L. The following day, cells were infected with HRV-1B.

Lentivirally transduced BEAS-2B cell lines

The lentivirally transduced BEAS-2B cell lines were generated as previously described[45]. The genomic region encompassing miR-155 was amplified using the following primers, forward: AAGCTTTATGCCTCATCCTCTGAGTGC; reverse: CTCGAGACGAAGGTTGAACATCCCAGTGACC. The insert was cloned into pSUPER plasmid between the sites HindⅢ and XhoI, excised with EcoRI and MluI and hence subcloned in pLVTHM, generating the construct pLVTHM_BIC.

HEK293T cells were co-transfected with 5 μg of pLVTHM_BIC (containing miR-155) or pLVTHM (used as negative control) 3.75 μg of pPAX2 and 1.5 μg of pMD2G using Superfect (Qiagen) according to the manufacturer's protocol. Supernatants were used to transduce BEAS-2B cells. Four days after transduction, selection of positively transduced cells (GFP+) was achieved by cell sorting, performed on a BD FACSAria Ⅲ cell sorter.

Reverse transcription-quantitative polymerase chain reaction analysis

Total cellular RNA was extracted using TRI-Reagent (Ambion) according to the manufacturer's instructions. RNA samples were quantified using the spectrophotometer Nanodrop 1000 (Thermo Scientific). Reverse transcription (RT) reactions were performed using the same amount of RNA for all samples, using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems part number: 4368813) with random hexamers as primers. MicroRNA reverse transcription quantitative polymerase chain reaction (RT-qPCR) was performed using Taqman microRNA assay kits from applied biosystems (part number: 4427975) that contain a specific RT primer and Taqman qPCR primers and probe necessary to quantify expression of a specific microRNA. In both cases, the cDNA obtained was used to perform qPCR using TaqMan® Universal PCR Master Mix, No AmpErase® UNG (Applied Biosystems part number: 4364341) on a 7900HT Real-Time PCR machine (Applied Biosystems) with the standard Taqman thermal cycling conditions for assays using "Taqman probes" (95 ℃ for 10 min; 95 ℃ for 15 s; 60 ℃ for 1 min - data collection) or modified as follows for assays using "Perfect probe" (95 ℃ for 10 min; 95 ℃ for 15 s; 50 ℃ for 30 s - data collection; 72 ℃ for 15 s). Fold differences in gene expression were calculated using the comparative Ct method for relative quantification[46]. Unless otherwise stated, gene expression was calculated using GAPDH as reference gene, and the average delta Ct value of the negative control as calibrator. Gene expression assays from Applied Biosystems (part n: 4331182): AGO2 (EIF2C2 - Assay ID: Hs01085579_m1); COL1A1 (Assay ID: Hs00164004_m1); DICER1 (Assay ID: Hs00229023_m1); GAPDH (part n: 4352934); SMAD2 (Assay ID: Hs00183425_m1). Gene expression assays from PrimerDesign Ltd (Southampton, United Kingdom): HRV-1B (Taqman probe); and IFNB1 (Perfect probe); IL13RA1 (Taqman probe); PU.1 (Taqman probe). MicroRNA expression assays (part n: 4427975): hsa-miR-18a (Assay ID: 002422); hsa-miR-19b (Assay ID: 000396); hsa-miR-106b (Assay ID: 000442); hsa-miR-128 (Assay ID: 002216); hsa-miR-155 (Assay ID: 002623).

Co-immunoprecipitation protocol

The antibodies used were: anti-AGO2, clone 11A9 (rat IgG2a) available from Ascenion GmbH, Munich, Germany[47]; normal rat IgG (cat.n: sc-2026, Santa Cruz biotechnology, inc). Protein G-coated Sepharose beads (Amersham) were prepared for IP reactions as follows. Beads were collected by centrifugation in a bench top centrifuge, at 3000 rpm (= 800 × g) for 1 min at 4 ℃. Beads were washed 3 times with ice-cold lysis buffer (PBS 1X, NP-40 0.5%, EDTA pH8 2 mmol/L, Glycerol 20%). Beads were then blocked in ice-cold lysis buffer with a final concentration of 1 mg/mL of BSA and 1 mg/mL of sonicated salmon sperm DNA (sspDNA) rotating end over end, for at least 1 h at 4 ℃. After this incubation, beads were collected by centrifugation and washed three times as above and resuspended in an equal volume of ice-cold lysis buffer.

THP-1 cells (human monocyte cell line, used as an additional control) were collected by centrifugation at 300 × g for 5 min at 4 ℃. BEAS-2B cells were first trypsinised to detach them from the plastic. Trypsin was inhibited by adding growth medium (containing 10% FBS) and cells were then collected by centrifugation as above. In both cases, cells were then washed twice in sterile ice-cold PBS (GIBCO) and finally the cell pellet was resuspended in 50 μL/10^6 cells of ice-cold complete lysis buffer (composed as specified above and containing in addition 100 U/mL RNAse inhibitor - Applied Biosystems; 1X complete cocktail protease inhibitor - Roche; DTT 0.5 mmol/L, all added just before use) incubated on ice for 10 min. Cell lysates were kept on ice during the entire procedure. They were cleared of the residual cell debris by centrifugation, at top speed in a bench top microcentrifuge, at 4 ℃ for 15 min. Pellets were discarded while supernatants were collected and diluted ten times, so that 1 mL of lysate would correspond to 2×10^6 cells. One mL aliquots were dispensed in pre-chilled eppendorfs, while 10 μL were

Table 1 Sequence details of miR-18a, -19b, -106b, -128, -155

miR name in text	miRBase accession number	mature miR sequence
miR-18a	MIMAT0000072	UAAGGUGCAUCUAGUGCAGAUAG
miR-19b	MIMAT0000074	UGUGCAAAUCCAUGCAAAACUGA
miR-106b	MIMAT0000680	UAAAGUGCUGACAGUGCAGAU
miR-128	MIMAT0000424	UCACAGUGAACCGGUCUCUUU
miR-155	MIMAT0000646	UUAAUGCUAAUCGUGAUAGGGGU

placed into 0.5 mL of TRI-Reagent and constitute the input sample. The same amount of anti-AGO2 specific antibody or the corresponding IgG negative control isotype antibody was then added to each 1mL-aliquot of cell lysate at this stage and finally, the samples were left rotating end over end at 4 ℃ overnight. 50 μL of protein G-coated sepharose beads, prepared as described above, were added to each co-IP reaction, and incubated rotating end over end at 4 ℃ for 2 h. Beads were then collected by centrifugation and then washed five times as already described, using 1mL of ice-cold washing buffer. The latter was different for THP-1 (PBS 1X, NaCl 160 mM, NP-40 0.05%, Glycerol 10%) or BEAS-2B cells (PBS 1X, NaCl 860 mmol/L, NP-40 0.05%, Glycerol 10%). Beads were then washed once more with ice-cold PBS (1x) and resuspended in 100 μL of RNAse-free water. 2.5 μL of proteinase K (20 mg/mL) was added to each tube. Beads were incubated for 15 min at 37 ℃ in a rotomixer at 1000 rpm. Finally, 500 μL of TRI-Reagent (Ambion) were added to each tube. Beads were then vortexed briefly and incubated at room temperature for about 10 min. Eppendorfs were then spun at top speed in a bench-top centrifuge for 1 min. Supernatants were transferred into new eppendorfs while the collected beads were discarded. RNA isolation was then performed as usual. The isolated RNA, was then either used straightaway for RT-qPCR reactions, or stored at -80 ℃ for later use.

AGO2 co-IP calculations

Relative RNA levels were calculated using the following formula: $2^{-[Ct(IP) - Ct(Input)]}/2^{-[Ct(GAPDH\ IgG) - Ct(Input)]}$. Inclusion of the Ct values from the input normalises for mRNA abundance in the cell lysate.

Bioinformatics analysis

The genomic sequences of different HRVs were obtained from the National Center for Biotechnology Information website. HRV-1B version number D00239.1; HRV-16 version number L24917.1; HRV-14 version number K02121.1; HRV-27 version number FJ445186.1.

The microRNA sequences used with Targetscan[48] were obtained from[49]. The microRNA sequences used with miRanda[50] and probability of interaction by target accessibility (PITA)[51] were obtained from miRBase 21[52]. Mature microRNA sequences for miR-18a, -19b, -106b, -128, -155, were identical across the two datasets and are reported in Table 1. MicroRNA target site predictions on viral genomes were obtained by running the appropriate program, locally on a Linux Ubuntu 10.04 LTS system. TargetScan software version 6, miRanda 3.3a and PITA were run with default parameters.

Results were then sorted by context+ score for Targetscan, Energy for miRanda and ddG for PITA. No cut-off value is recommended for Targetscan; Energy threshold of -7 (for miRanda) and ddG threshold of -10 (for PITA[53]) are recommended, besides the default parameters.

In Targetscan, the context+ score is calculated based on the seed class (6-mer, 7-mer-1a, 7-mer-m8 or 8-mer), the extent of miR 3'-binding, the miR:target stability, the AU composition of the target site, its distance from the closest end of the target RNA, and the number of sites on the same target RNA.

MiRanda first identifies regions of complementarity between miR and target RNA and afterwards calculates the free energy of the RNA duplex.

In PITA, after the identification of possible RNA duplexes, the algorithm calculates the net free energy (ddG). The latter is the difference between the stability of the miR:target (energy gained, dGduplex) minus the energy necessary to unfold the region surrounding the target site (dGopen) which is necessary for microRNA binding.

The schematics showing the bioinformatics predictions were generated using BioPerl[54].

Statistical analysis

Statistical significance was calculated using the tools integrated in the software GraphPad Prism v.6. Unless otherwise stated, the unpaired t-test was used for pairwise comparisons. If multiple conditions were compared altogether, one-way analysis of variance with Bonferroni post-test correction was used.

RESULTS

DICER knock-down favours HRV-1B replication

In order to test if microRNAs have a role during HRV infection of bronchial epithelial cells, we disrupted the biogenesis of microRNAs by knocking-down DICER in BEAS-2B cells. The latter is a virally immortalized cell line derived from healthy human bronchial epithelial cells[55] that has often been employed as a convenient model for HRV infection of this cell type, *e.g.*,[56]. To establish if DICER knock-down would compromise substantially the expression of mature microRNAs, we performed several rounds of transfection with either siRNA against DICER, or negative control siRNA. Total cellular RNA was extracted 48 h after each transfection (Figure 1A) and the siRNA efficacy was verified measuring

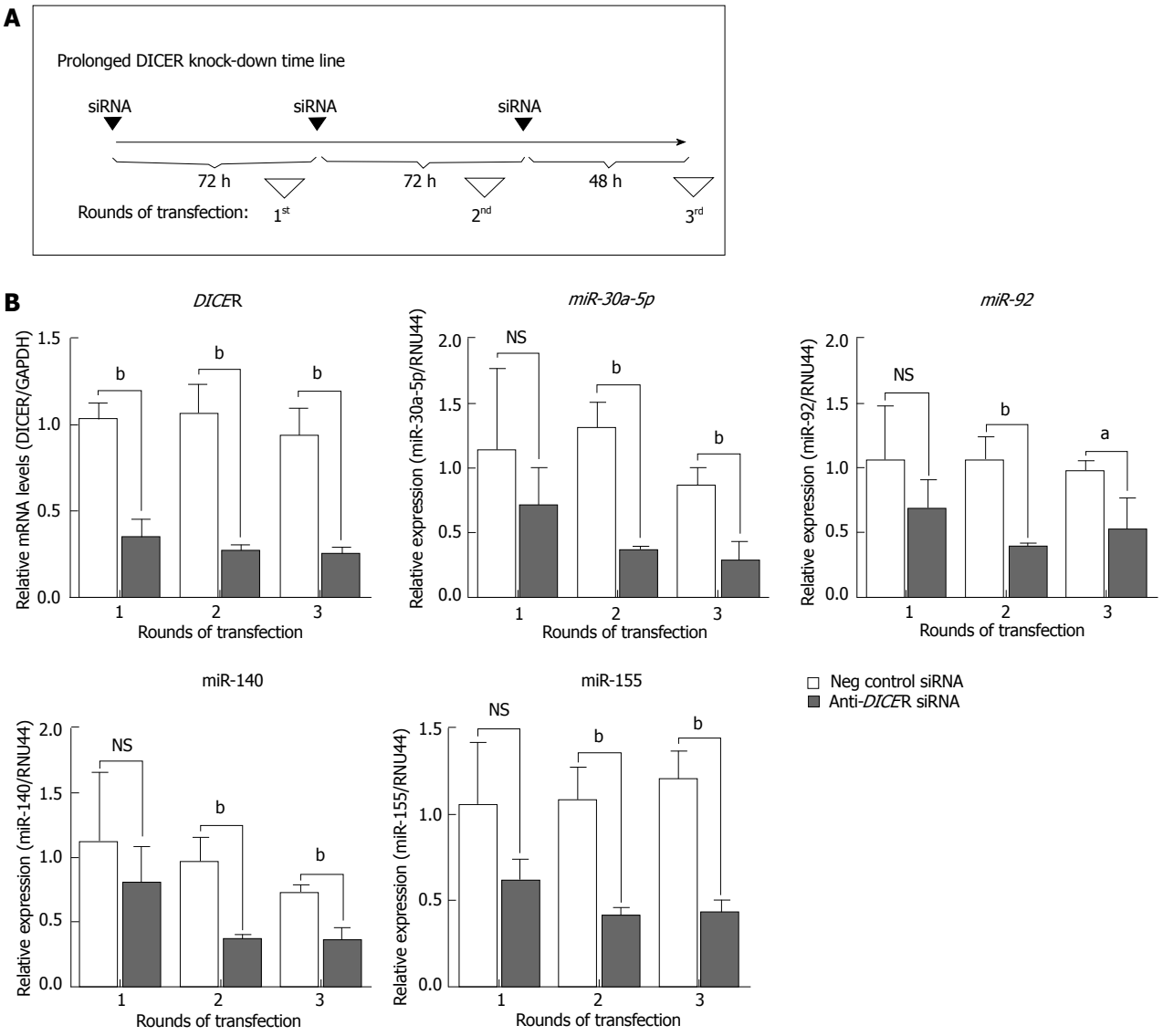

Figure 1 Prolonged knock-down of DICER is necessary in order to effectively lower the levels of mature microRNAs. Thirty nmol/L of either anti-DICER or negative control siRNA were used. A: Global timeline of the experiment; B: DICER mRNA or expression of the indicated microRNAs (miR-30a-5p, -92, -140, 155) was quantified by reverse transcription-quantitative polymerase chain reaction. The values plotted represent the mean ± SD, of three independent experiments. For all graphs, unpaired *t* test was used to calculate the *P* values for anti-DICER *vs* negative control siRNA samples. $^{a}P < 0.05$; $^{b}P < 0.01$. NS: Not significant.

DICER mRNA by RT-qPCR. In addition, we measured the expression of 4 miRs (miR-155, -140, -92, 30a-5p) to detect changes in the production of cellular microRNAs (Figure 1B). On the one hand, we found that DICER mRNA was significantly and consistently reduced by the anti-DICER siRNA since the first round of transfection. On the other hand, 48 h after the first transfection, the levels of mature microRNAs were only marginally affected compared to negative control. In fact, the microRNAs appeared to be reduced by less than 50% and such a difference was not statistically significant. After the second and third round of transfection instead, all four microRNAs were significantly reduced, with residual levels scoring below 50% of the respective negative controls. Therefore, we infected BEAS-2B cells with three different doses of HRV-1B, after three rounds of siRNA transfection (Figure 2). For each dose of virus used, DICER knock-down increased the intracellular levels of viral RNA, as measured 8 h post-infection. This experiment was repeated three more times using only the intermediate dose of virus (MOI = 0.01) confirming the previous results: cells lacking microRNAs (Figure 3A and B) had about 40% more viral RNA than negative control, both at 8 and 24 h post-infection (Figure 3C). Crucially, the levels of IFN-β mRNA were not different between cells transfected with either anti-DICER or negative control siRNA (Figure 3D). This suggested that DICER knock-down did not alter the expression of IFN-β neither before nor during viral infection. Therefore, higher levels of viral RNA were not due to altered IFN-β induction, suggesting that the disruption of microRNA maturation affected viral replication through a mechanism independent from the IFN-β pathway. Considering that there was no difference in the level of viral RNA that entered the cells (0 h post-infection) but differences were detected only at 8 and 24 h post-infection, the mechanism responsible for

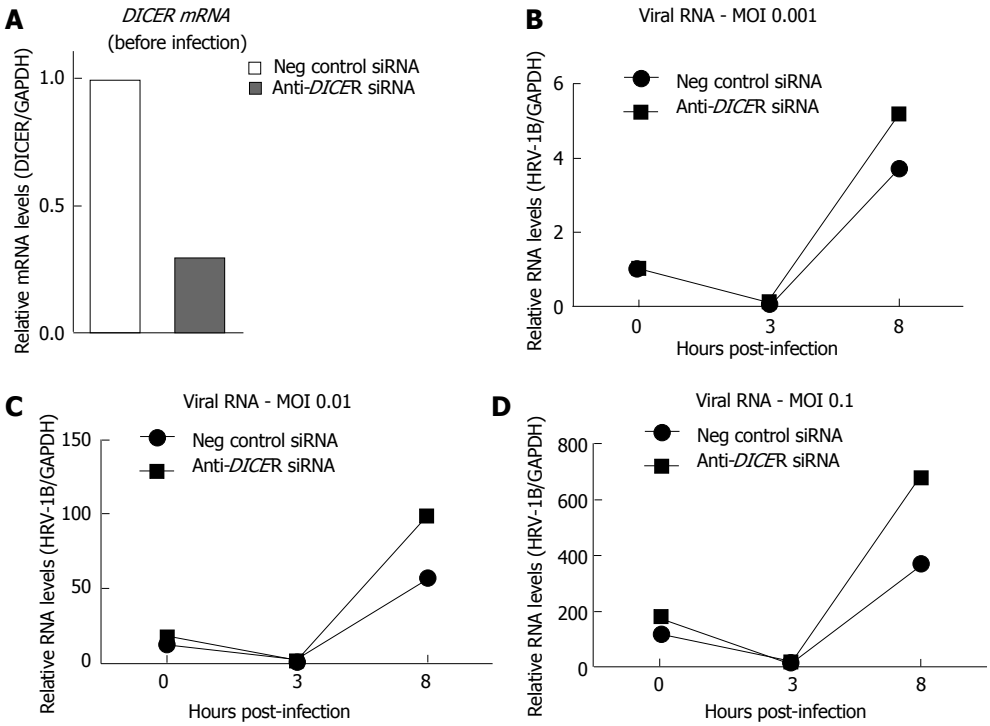

Figure 2 Prolonged DICER knock-down enhanced human rhinovirus-1B replication (preliminary experiments). BEAS-2B cells were transfected for three rounds with either a negative control siRNA or anti-DICER siRNA. Forty-eight hours after the third transfection, cells were infected with the indicated amount of HRV-1B, expressed as MOI (multiplicity of infection). Reverse transcription-quantitative polymerase chain reaction was used to quantify (A) DICER mRNA levels before infection or (B-D) HRV-1B RNA at 0, 3 or 8 h post-infection (HPI). The plotted values represent the average of qPCR duplicates from one experiment. The 0 HPI sample at MOI 0.001 was used as calibrator for all samples.

the higher accumulation of intracellular viral RNA was likely to involve intracellular events of viral replication. Given these considerations, we speculated that one possibility was that mature microRNAs interacted directly with the viral genome. Such interaction would hamper viral replication even if only mildly, as the results from our DICER knock-down experiment suggest.

AGO2 protein interacts with HRV-1B RNA

In order to test if microRNAs interacted directly with the RNA of HRV-1B, we performed RNA immunoprecipitations using an antibody specific for AGO2 (AGO2 co-IP thereafter). With this technique (Figure 4A), it is possible to identify the mRNA molecules that are bound by AGO2 protein and therefore, are targeted by microRNAs[47]. Although human cells express 4 Argonaute proteins (AGO1-4) several reports showed that they associate to largely overlapping pools of microRNAs and target mRNAs. Moreover, AGO2 co-IP has been shown to correctly identify direct targets of both endogenous and exogenously added microRNAs, *e.g.*,[57-61]. In addition, we validated the choice of AGO2 in our protocol by showing that microRNAs were strongly co-purified and that mRNAs, already known to be microRNA targets, could be readily detected in the immunoprecipitated fraction (Figure 5). BEAS-2B cells were infected with HRV-1B, and AGO2 co-IP was performed on cells harvested 6 h post-infection (Figure 4B). Under these conditions, AGO2 was as strongly associated with HRV-1B RNA as it was with SMAD2 mRNA. The latter constitutes a positive control, as it has already been shown to be a target of miR-155[62]. In contrast, GAPDH was not significantly co-precipitated with AGO2, as compared to when an irrelevant antibody was used. The interaction of AGO2 with the genomic RNA of HRV-1B suggested that the latter may be bound by microRNAs during infection of bronchial epithelial cells.

Antagonists of miR-155 or miR-128 enhance HRV-1B replication

To identify specific microRNAs that could directly target HRV-1B, we performed a bioinformatics analysis. We ran Targetscan[5] using a list of microRNAs that we found to be expressed in human primary bronchial epithelial cells (PBECs) as measured by Taqman low density microRNA arrays (Table 2). A number of those microRNAs were predicted to target HRV-1B with varying degrees of efficiency, as summarized by the "context+ score" where the lower the value the better. Among these, we focused on microRNAs that were also shown to be under-expressed in the asthmatic bronchial epithelium[63]. We selected one microRNA (miR-18a) predicted to be very efficient at targeting the virus, two microRNAs with average scores (miR-155 and miR-128) and two microRNAs predicted to be less likely to target the virus (miR-106b and miR-19b) (Table 3). In addition, the five microRNAs had putative sites also on other Rhinoviruses such as HRV-16, -14, -27. Interestingly, of the five microRNAs, miR-155 was predicted to target all the tested genomes in a very similar region at their 3' end (Table 3 and Figure 6).

In order to test experimentally the *in-silico* predictions,

Table 2 MicroRNAs expressed in healthy primary bronchial epithelial cells

Let-7a	miR-149	miR-218	miR-345	miR-501-5p	miR-93
Let-7b	miR-152	miR-221	miR-34a	miR-502-3p	miR-9
let-7c	miR-155	miR-222	miR-34c-5p	miR-502-5p	miR-95
let-7d	miR-15a	miR-223	miR-361-5p	miR-503	miR-96
let-7e	miR-15b	miR-22	miR-362-3p	miR-505	miR-98
let-7f	miR-16	miR-224	miR-362-5p	miR-519a	miR-99a
let-7g	miR-17	miR-23a	miR-365	miR-523	miR-99b
miR-100	miR-181a	miR-23b	miR-374a	miR-532-3p	
miR-101	miR-181c	miR-24	miR-374b	miR-532-5p	
miR-103	miR-182	miR-25	miR-375	miR-542-3p	
miR-106a	miR-183	miR-26a	miR-376a	miR-545	
miR-106b	miR-184	miR-26b	miR-376c	miR-548d-5p	
miR-107	miR-185	miR-27a	miR-379	miR-574-3p	
miR-10a	miR-186	miR-27b	miR-410	miR-576-3p	
miR-125a-3p	miR-18a	miR-28-3p	miR-411	miR-579	
miR-125a-5p	miR-18b	miR-28-5p	miR-422a	miR-582-3p	
miR-125b	miR-191	miR-296-5p	miR-423-5p	miR-582-5p	
miR-126	miR-192	miR-29a	miR-424	miR-589	
miR-127-3p	miR-193a-3p	miR-29b	miR-425	miR-590-5p	
miR-128	miR-193a-5p	miR-29c	miR-429	miR-597	
miR-129-3p	miR-193b	miR-301a	miR-449a	miR-598	
miR-130a	miR-194	miR-301b	miR-449b	miR-618	
miR-130b	miR-195	miR-30b	miR-450a	miR-625	
miR-132	miR-197	miR-30c	miR-450b-5p	miR-627	
miR-134	miR-198	miR-31	miR-452	miR-628-5p	
miR-135a	miR-19a	miR-320	miR-454	miR-629	
miR-135b	miR-19b	miR-323-3p	miR-455-3p	miR-636	
miR-136	miR-200a	miR-32	miR-455-5p	miR-642	
miR-138	miR-200b	miR-324-3p	miR-483-5p	miR-652	
miR-139-5p	miR-200c	miR-324-5p	miR-484	miR-655	
miR-140-3p	miR-202	miR-328	miR-485-3p	miR-660	
miR-140-5p	miR-203	miR-330-3p	miR-486-3p	miR-671-3p	
miR-141	miR-204	miR-331-3p	miR-486-5p	miR-708	
miR-142-3p	miR-205	miR-331-5p	miR-487a	miR-744	
miR-145	miR-20a	miR-335	miR-489	miR-758	
miR-146a	miR-20b	miR-339-3p	miR-491-5p	miR-885-5p	
miR-146b-5p	miR-210	miR-339-5p	miR-494	miR-886-3p	
miR-148a	miR-212	miR-340	miR-495	miR-886-5p	
miR-148b	miR-21	miR-342-3p	miR-500	miR-92a	

Here are reported only the miRs with Ct values lower than 40, in at least 2 (out of 4) samples of primary bronchial epithelial cells (PBECs) from healthy donors.

we transfected BEAS-2B cells with microRNA antagonists (anti-miRs). To test the efficiency of anti-miR transfection, we measured the expression of miR-18a, -106b, -128 and -155 by RT-qPCR, 24 h post-transfection (Figure 7) confirming reduction (from 70% to 90%) of the measured microRNAs. Twenty four hours after anti-miR transfection, BEAS-2B cells were infected with HRV-1B. Cells were collected 8 h post-infection and viral RNA was quantified by RT-qPCR (Figure 8). The results showed that, as expected given their positive "context + scores", miR-106b and miR-19b did not alter viral replication. The microRNA predicted to be the best match for HRV-1B, miR-18a, did not affect viral replication either. However, when miR-155 or miR-128 was silenced, we observed an increase in viral RNA of approximately 50% compared to a negative control anti-miR. Notably, the different constructs transfected did not alter the levels of GAPDH mRNA across all the samples of infected cells (Figure 9B). As an additional control we also noted that the total cellular RNA did not change among the samples transfected with specific anti-miRs (Figure 9A). In addition, the same analysis on independently transfected cells that were not subsequently infected with HRV-1B (Figure 9C and D) confirmed that these parameters were not affected by any of the anti-miRs used. These observations suggest that the measured increase of intracellular viral RNA was not influenced by the normalisation method adopted, and that in our experiments cell viability was not significantly altered by the different anti-miRs transfected.

Finally, we generated two lentivirally transduced BEAS-2B cell lines, in order to study the effect of miR-155 over-expression on the replication of HRV-1B (Figure 10). These experiments showed that from 40% to 50% less viral RNA accumulated in cells that over-expressed miR-155, compared to the relative negative control, thus reinforcing the results obtained for this microRNA in the anti-miR experiments.

DISCUSSION

Innate immunity plays a vital role in the antiviral response of human cells. In particular, the importance of the IFN

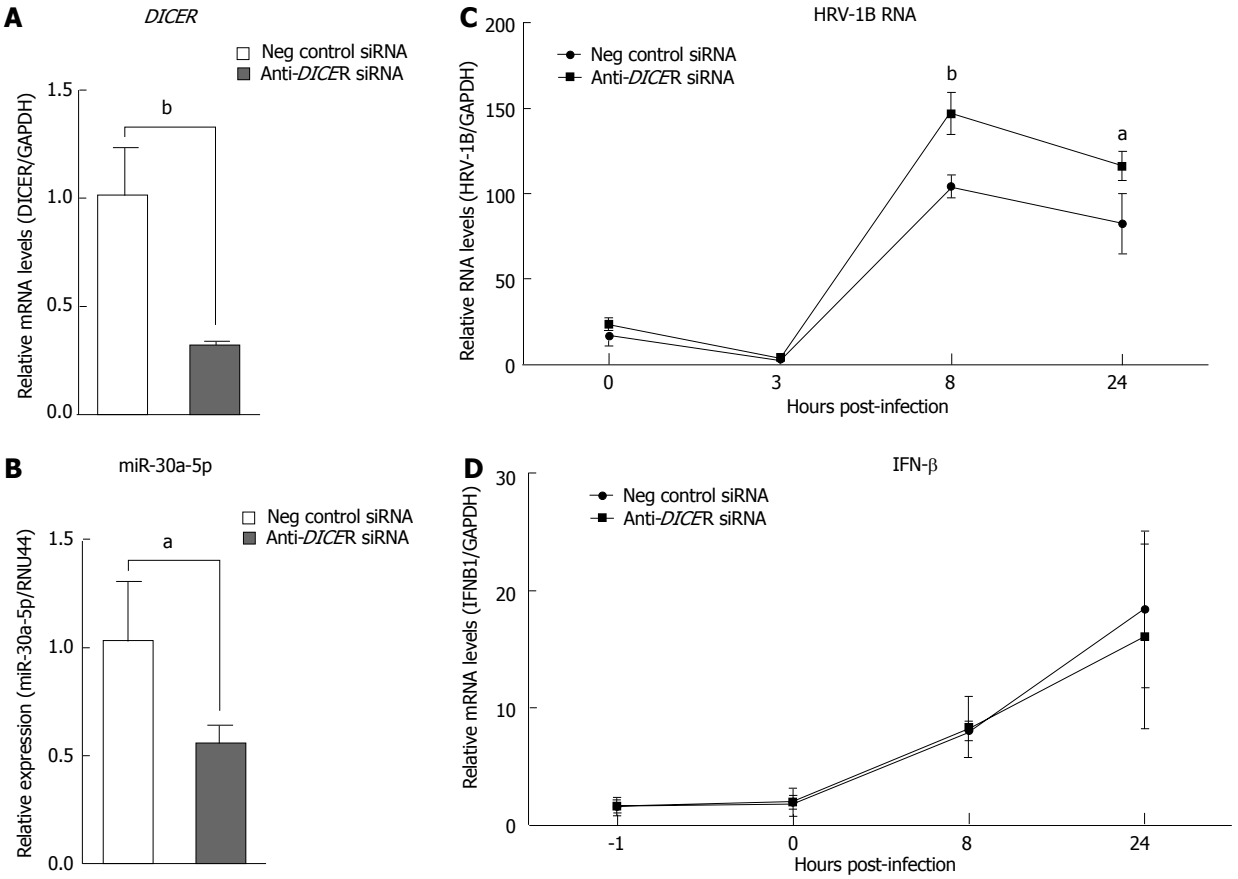

Figure 3 Prolonged DICER knock-down leads to higher levels of viral RNA. BEAS-2B cells were transfected for three rounds (using 30 nmol/L of siRNA) and then infected with human rhinovirus (HRV)-1B at MOI 0.01. Cells collected just before infection were used to measure DICER mRNA levels (A) and miR-30a-5p expression (B) by reverse transcription-quantitative polymerase chain reaction (RT-qPCR). HRV-1B RNA (C) and IFNB1 mRNA (D) levels in infected cells were measured by RT-qPCR at the indicated time points. In (D) the time point "-1" refers to uninfected cells, collected just before infection. In (C) all the samples were normalised as done for data in Figure 2 [0 h post-infection (HPI), MOI 0.001 sample as calibrator]. Plotted values represent the mean ± SD, of three independent experiments. For all panels, unpaired *t* test was used to calculate the *P* values for anti-DICER *vs* negative control siRNA samples.[a]*P* < 0.05; [b]*P* < 0.01. NS: Not significant; MOI: Multiplicity of infection.

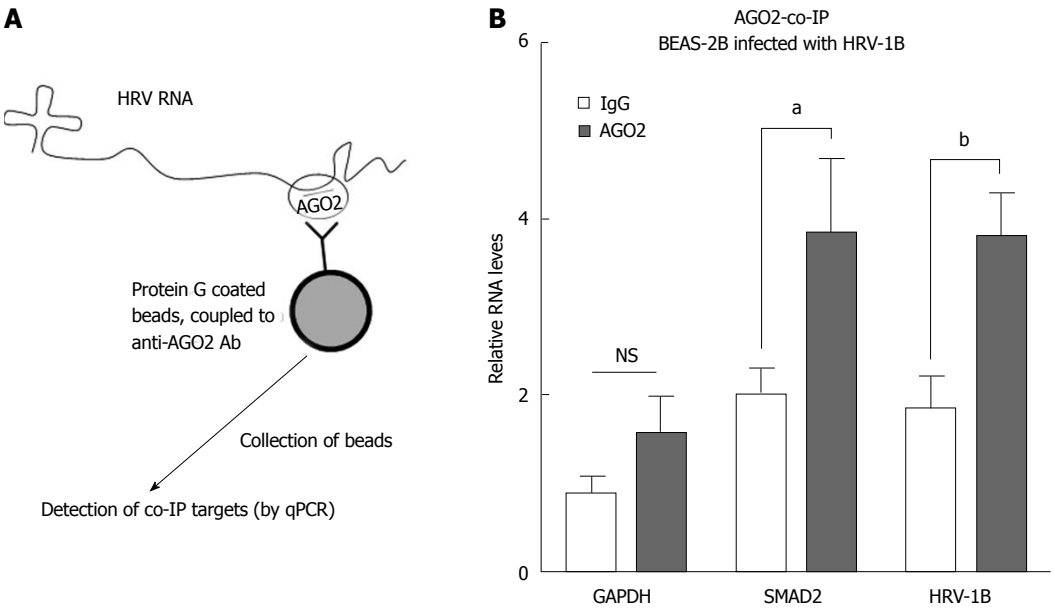

Figure 4 Human rhinovirus-1B RNA co-immunoprecipitates with AGO2 protein in BEAS-2B cells. A: Schematic representation of the rationale behind AGO2 co-IP experiments; B: BEAS-2B cells were infected with HRV-1B at MOI 0.01. Six hours post-infection cells were collected and AGO2 co-IP was performed. Plotted values represent the mean ± SD, of three independent experiments. The formula used takes into account the abundance of the RNAs before immunoprecipitation (see materials and methods). Unpaired *t* test was used to calculate the *P* values for AGO2 *vs* IgG control. NS: Not significant (*P* > 0.05); [a]*P* < 0.05; [b]*P* < 0.01. MOI: Multiplicity of infection; HRV: Human rhinovirus.

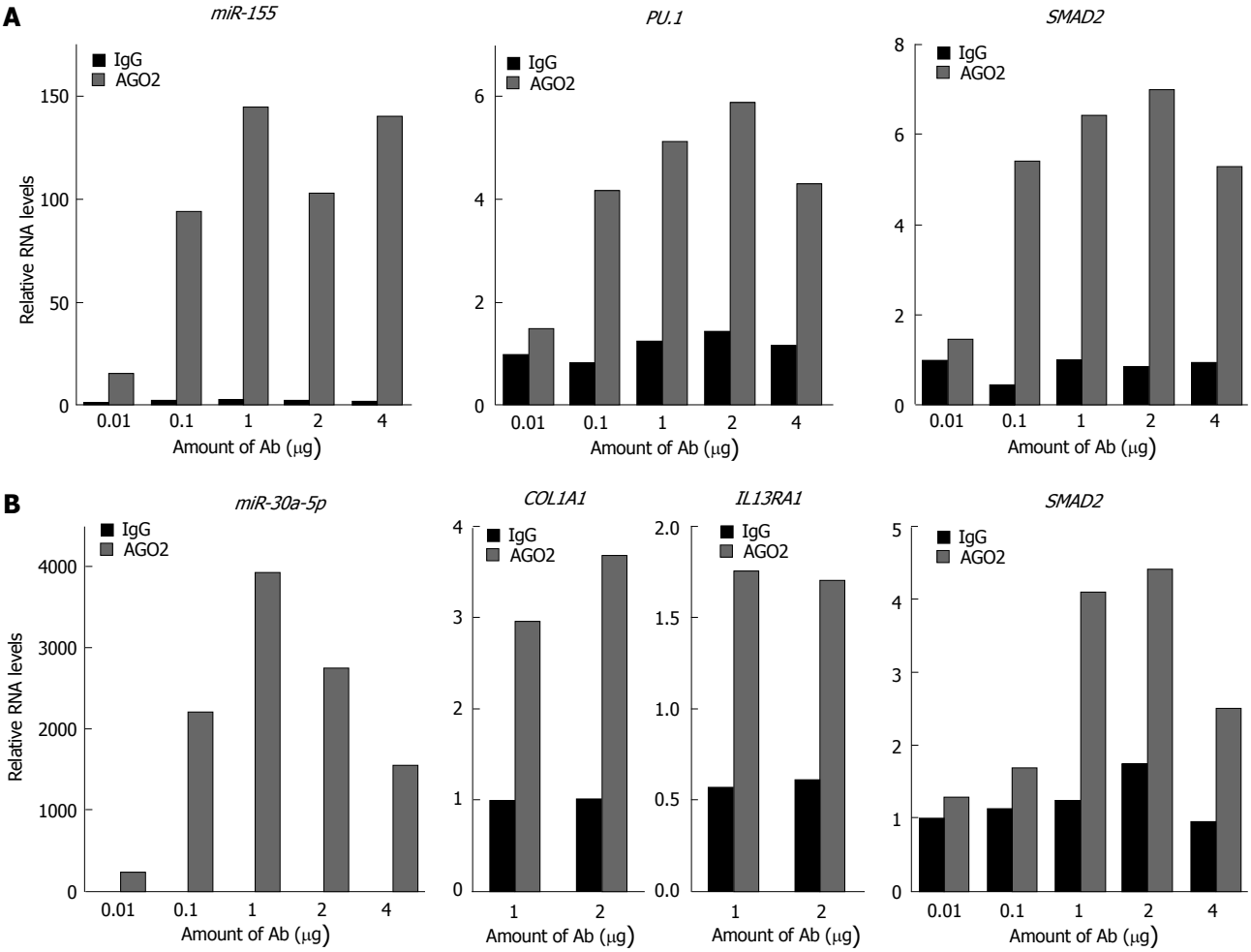

Figure 5 Antibody titration for AGO2 co-IP. These experiments were performed in order to determine the most convenient antibody concentration to use for (A) THP-1 (used as additional control) and (B) BEAS-2B cells: 1μg was chosen as it would give the highest enrichment of both microRNAs and target mRNAs. These results show that microRNAs are very tightly associated with AGO2 protein, and confirm that microRNA-regulated mRNAs are also considerably co-purified. Previous work from our group has shown that PU.1, SMAD2 and IL13RA1 are targeted by miR-155[45,62,89] while COL1A1 has been shown to be targeted by miR-29b elsewhere[90]. IgG refers to the negative control antibody, AGO2 refers to the anti-AGO2 antibody used. Plotted values represent the average of qPCR duplicates from one experiment.

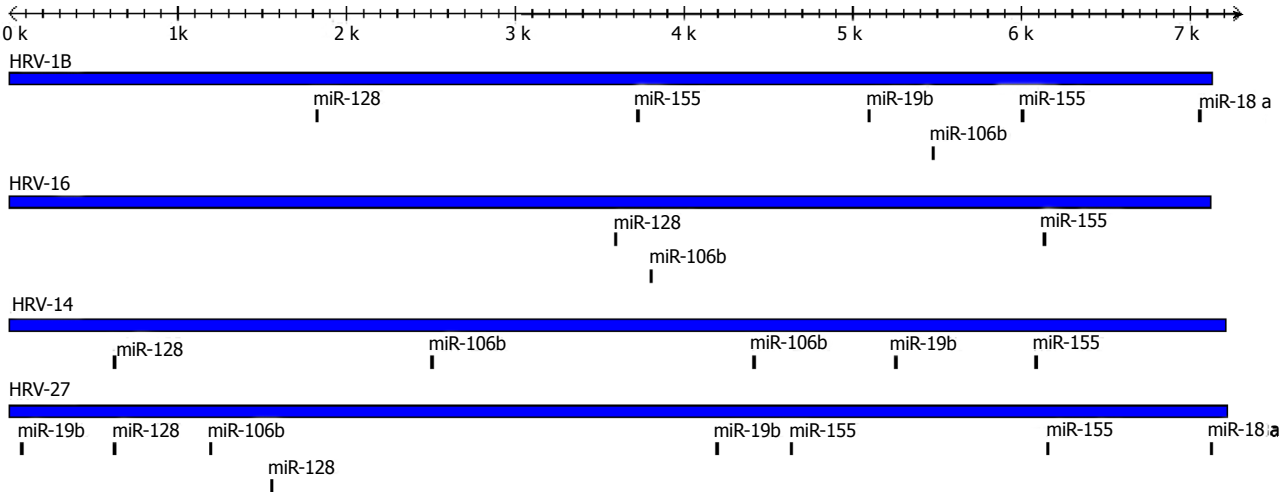

Figure 6 Schematic of targetscan predictions. Indicated are the target sites predicted for miR-18a, -19b, -106b, -128, -155 on two strains of HRV-A (HRV-1B and -16) and two strains of HRV-B (HRV-14 and -27). HRV: Human rhinovirus.

system in antiviral immunity has been long recognized and studied[64]. Early, innate immune responses are particularly well suited for protection from viruses that result in acute infections, such as Rhinoviruses. Although it is still

Table 3 Targetscan predictions on human rhinovirus -1B, -16, -14, -27

Virus	MiR	Site type	Site start	Site end	Context + score	Alignment
HRV-1B	18a	7mer-1a	7053	7059	0.163	site: GUGCACUGUAUAUCCCACCUUAU |||||| |||||| miR: GAUAGACGUGAUCUAC----- -GUGGAAU
HRV-1B	128	7mer-m8	1822	1828	-0.017	site: AAACAUUAGUAUGUACACUGUGC ||||||| miR: UUUCUCUGGCCAAGUGACACU
HRV-1B	155	7mer-m8	3716	3722	-0.014	site: AUAAAUCCAAUCAAU- - - - AGCAUUAG |||| ||||||| miR: UGGGGAUAGUGCUAAUCGUAAUU
HRV-1B	155	7mer-m8	5998	6004	-0.013	site: UGAUUCUAAACCAAUAGCAUUAG ||||||| miR: UGGGGAUAGUGCUAAUCGUAAUU
HRV-1B	19b	8mer-1a	5088	5095	0.021	Site: UAAUUUACAAAUUGUUUUGCACA ||||||| miR: AGUCAAAACGUACCUAAACGUGU
HRV-1B	106b	7mer-m8	5471	5477	0.025	Site: CCAGAAUGUAAUUUAGCACUUUC ||||||| miR: UAGACGUGACAGUCGUGAAAU
HRV-16	128	7mer-m8	3593	3599	-0.04	Site: GAUCUUAGACACUUUCACUGUGC |||| ||||||| miR: UUUCUCUGGCCAAGUGACACU
HRV-16	155	7mer-m8	6133	6139	-0.036	Site: UUCCAAACUUAAGCUAGCAUUAG ||||||| miR: UGGGGAUAGUGCUAAUCGUAAUU
HRV-16	106b	7mer-1a	3802	3808	0.107	Site: GCAAACAAUAAUUGCCACUUUAA |||||| miR: UAGACGUGACAGUCGUGAAAU
HRV-14	128	7mer-1a	620	626	-0.059	Site: CAUAUAUAUACAUAUACUGUGAU |||||| miR: UUUCUCUGGCCAAGUGACACU
HRV-14	155	7mer-m8	6082	6088	-0.056	Site: ACUUACACUAAAAGAAGCAUUAU ||||||| miR: UGGGGAUAGUGCUAAUCGUAAUU
HRV-14	19b	7mer-m8	5255	5261	-0.001	Site: GGACCAAACACAGAAUUUGCACU ||||||| miR: AGUCAAAACGUACCUAAACGUGU
HRV-14	106b	8mer-1a	2503	2510	0.047	Site: CAGAACUACCUACAUGCACUUUA ||||||| miR: UAGACGUGACAGUCGUGAAAU
HRV-14	106b	7mer-m8	4414	4420	0.057	Site: UCCACCAGAUCCCAAGCACUUUG ||||||| miR: UAGACGUGACAGUCGUGAAAU
HRV-27	18a	7mer-1a	7121	7127	-0.134	Site: AGGUUCUUGUGAUACCACCUUAC |||||| miR: GAUAGACGUGAUCUACGUGGAAU
HRV-27	19b	7mer-m8	66	72	-0.11	Site: CUGGUAUUUUGUACC- - - UUUGCACG ||||| ||||||| miR: AGUCAAAACGUACCUAAACGUGU
HRV-27	128	7mer-1a	619	625	-0.053	Site: AUAUAUAGCAUAUAUACUGUGAU |||||| miR: UUUCUCUGGCCAAGUGACACU
HRV-27	155	7mer-1a	6152	6158	0.018	Site: CUUAUGUUAGUUUGGGCAUUAAG |||||| miR: UGGGGAUAGUGCUAAUCGUAAUU
HRV-27	106b	8mer-1a	1193	1200	0.025	Site: CAAAAUUUCACAGUG--GCACUUUA ||| ||||||| miR: UAGACGUGACAGUCGUGAAAU
HRV-27	128	7mer-1a	1552	1558	0.049	Site: CCCAGUAUACCUGUU--ACUGUGAC ||| |||||| miR: UUUCUCUGGCCAAGUGACACU
HRV-27	19b	7mer-m8	4192	4198	0.058	Site: CAAAUGUCUCAGAAGUUUGCACC ||||||| miR: AGUCAAAACGUACCUAAACGUGU
HRV-27	155	7mer-m8	4632	4638	0.097	Site: UGUGUUGAACCCUGAAGCAUUAG ||||||| miR: UGGGGAUAGUGCUAAUCGUAAUU

HRV: Human rhinovirus.

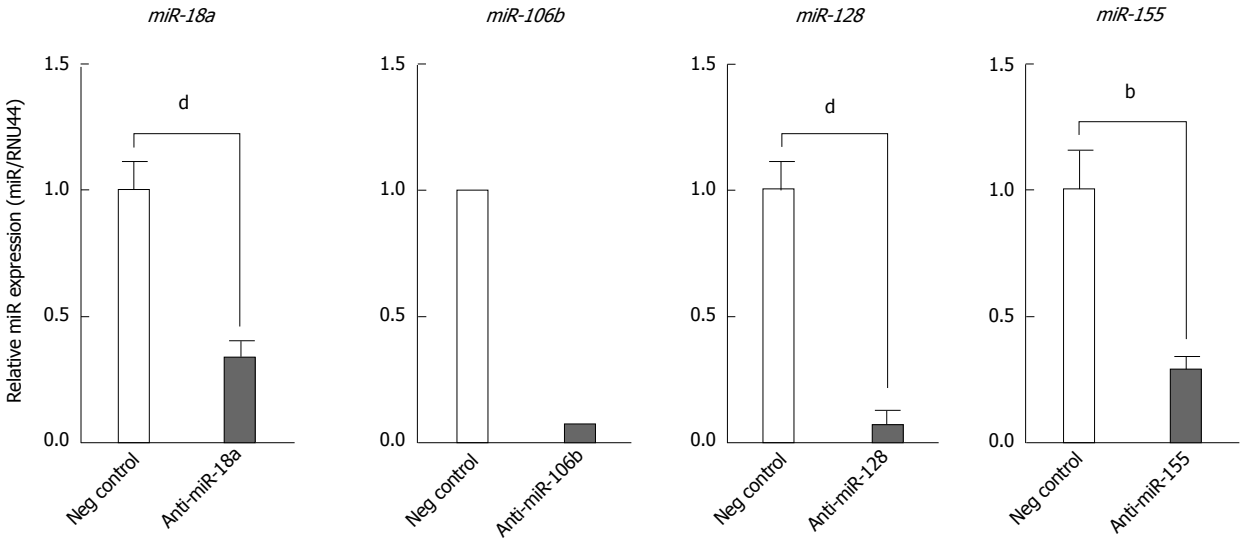

Figure 7 Effect of anti-miR transfection on microRNA expression in BEAS-2B cells. MicroRNA expression was quantified by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) 24 h post-transfection with either a negative control anti-miR or the indicated specific anti-miR. Plotted values for miR-18a, -128 and -155 represent the mean ± SD of three independent experiments. Unpaired *t* test was used to calculate *P* values for cells transfected with the specific anti-miR *vs* negative control anti-miR. $^{b}P < 0.01$; $^{d}P < 0.01$. The values plotted for miR-106b represent the average of qPCR duplicates from one experiment.

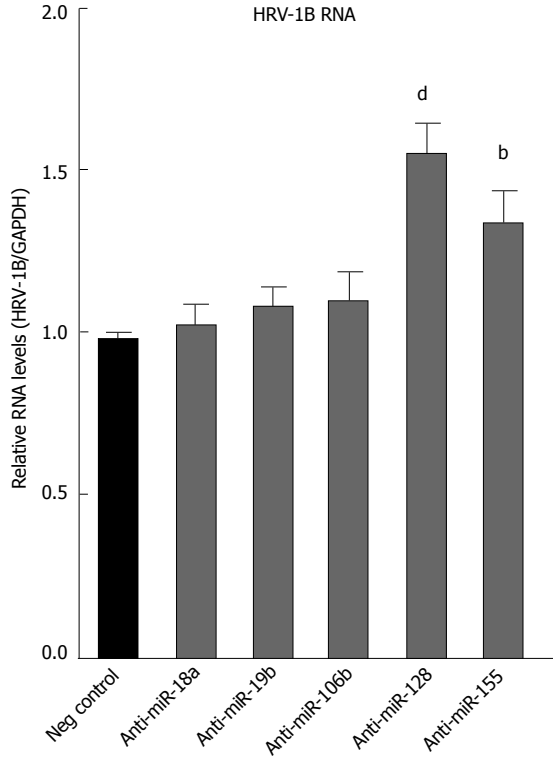

Figure 8 Antagonists of miR-155 and miR-128 enhance human rhinovirus-1B replication in BEAS-2B cells. BEAS-2B cells were transfected with 100 nmol/L of the indicated anti-miR. The following day, cells were infected with HRV-1B (multiplicity of infection of 0.01). HRV (human rhinovirus)-1B RNA was measured by RT-qPCR from samples collected at 8 h post-infection. Plotted values represent the mean ± SD, from 3 independent experiments. The *P* values were calculated for each specific anti-miR vs the negative control anti-miR, using one way ANOVA with Bonferroni correction. $^{b}P < 0.001$; $^{d}P < 0.0001$.NS: Not significant.

under debate whether virus-induced RNA interference (RNAi) is an antiviral mechanism actually conserved in human cells[65,66] it has become clear that microRNAs play an important role in human innate antiviral immunity.

We hypothesized that microRNAs inhibit the replication of human Rhinoviruses. This would be of interest in the fields of asthma and COPD, since Rhinoviruses constitute major exacerbation triggers of these conditions. In particular, it has been shown that Rhinoviruses replicate more in PBECs from asthmatics than in cells from healthy subjects and that the former express less IFN-β and IFN-gamma during infection[44,67]. Recently, it has been reported that the bronchial epithelium of asthmatics present a dysregulated microRNA profile[63] with prevalence of under-expressed microRNAs. Therefore, if microRNAs that are under-expressed in asthmatic PBECs were able to hold back Rhinovirus replication, it can be speculated that also their deficiency may contribute to the weaker antiviral immunity of asthmatic cells.

MicroRNA maturation involves the activity of two endonucleases that progressively shorten the initial transcript. DROSHA cleaves the primary microRNA (pri-miR) in the nucleus, while DICER is responsible for trimming the precursor microRNA (pre-miR) in the cytoplasm, and finally the mature microRNA assembles with AGO proteins in order to be functional[3,4]. Hence, impairing the activity of either DROSHA or DICER has been shown to lead to a reduction of mature microRNAs[68,69]. In order to test whether microRNAs affect the replication of Human Rhinovirus, we opted to target DICER in bronchial epithelial cells. A very specific protocol had to be followed to generate the desired knock down of microRNAs; transfecting BEAS-2B cells with anti-DICER siRNA for three consecutive times showed that microRNAs were reduced to about 40%-30% only after the second round of transfection (Figure 1) equivalent to five days of DICER silencing.

Our results are in agreement with other published work where, 48 h after transfection of anti-DICER siRNA, only a subset of microRNAs was affected, and in general their reduction was very rarely above 50%[68,70-72].

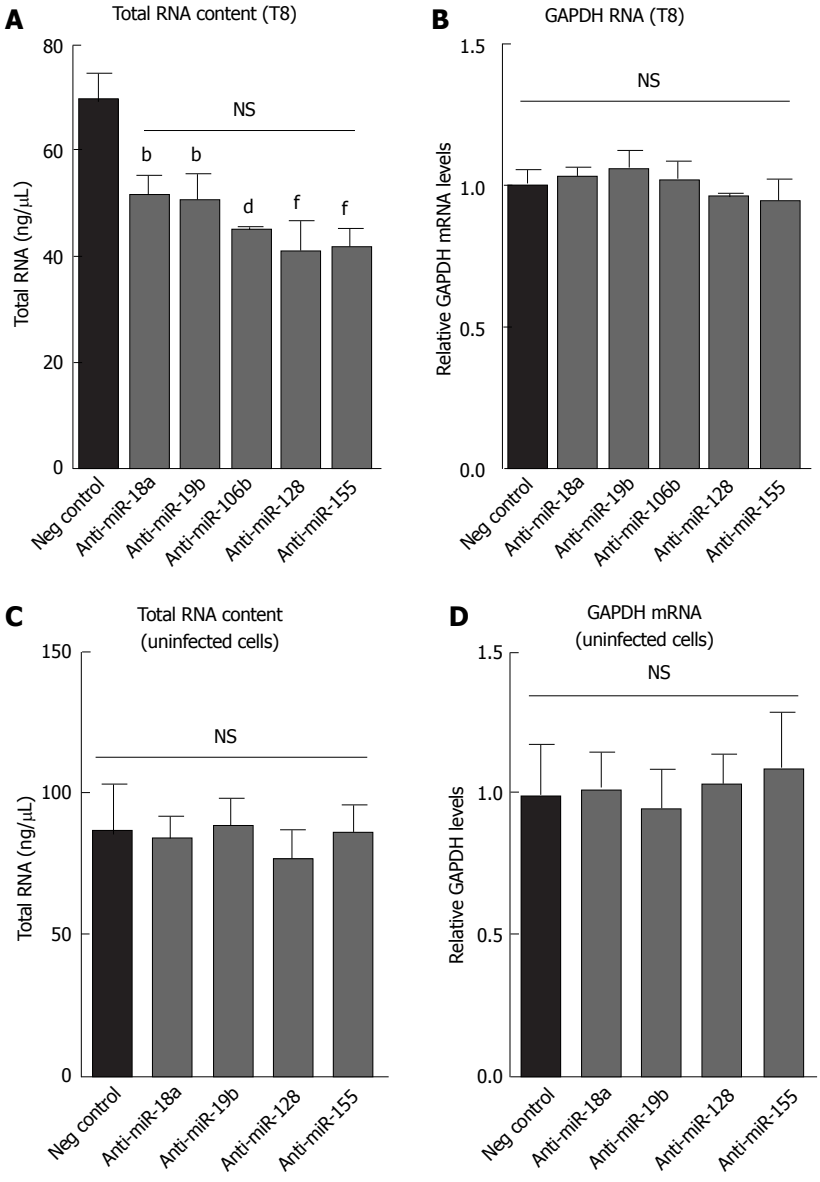

Figure 9 Effect of anti-miR transfection on total cell RNA and GAPDH expression in BEAS-2B cells. (A and B) Values obtained for the samples used for Figure 5 or (C and D) from cells transfected with the indicated anti-miRs but not infected with HRV-1B. The values plotted represent the mean ± SD of three independent experiments. The *P* values were calculated across all the anti-miRs, using one way ANOVA with Bonferroni correction. In A, each specific antimiR was significantly different *vs* negative control ($^{b}P < 0.01$; $^{d}P < 0.001$; $^{f}P < 0.0001$) but not significantly different to one another. NS: Not significant.

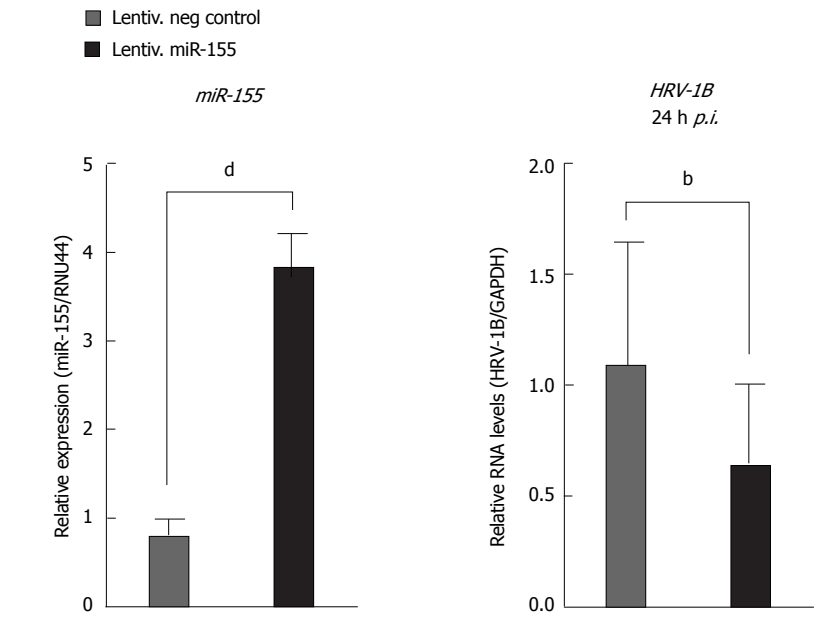

Figure 10 MiR-155 over-expression inhibits human rhinovirus-1B replication. Lentivirally transduced BEAS-2B cells were infected with human rhinovirus-1B (HRV-1B) at an MOI of 0.01. MiR-155 expression of uninfected cells or HRV-1B RNA (24 h post-infection) was measured by RT-qPCR from three independent experiments. *P* values for miR-155-overexpressing cells *vs* negative control cells were calculated using the unpaired *t* test for miR-155 expression, or the ratio paired *t* test for HRV-1B RNA; $^{b}P < 0.01$, $^{d}P < 0.001$.

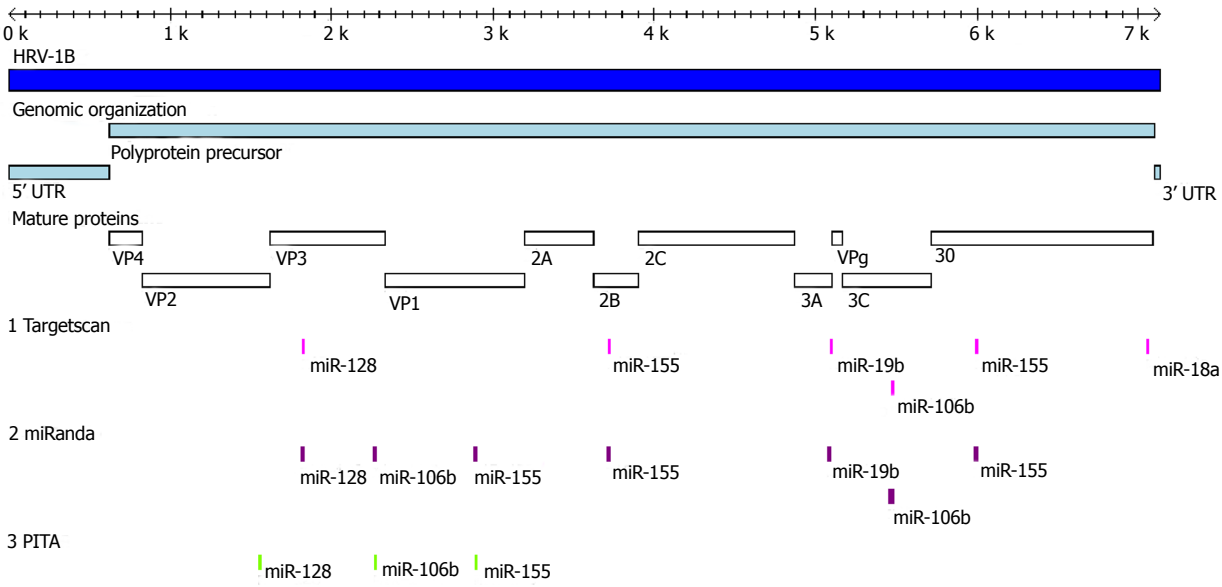

Figure 11 Schematic comparison of microRNA predictions on human rhinovirus-1B. Indicated are the target sites predicted for miR-18a, -19b, -106b, -128, -155 on HRV-1B by Targetscan, miRanda and PITA algorithms. In addition, the genomic organization and the boundaries of the viral mature proteins are shown.

Table 4 MiRanda predictions on human rhinovirus-1B

Virus	miR	Site start	Site end	Score	Energy (kcal/mol)		Alignment
HRV-1B	106b	2257	2276	141	-18.24	miR:	uaGACGUGACAGUCGUGAAAu
							||||| ||| || ||||
						site:	uuCUGCA-UGUAAGGACUUUu
HRV-1B	155	3704	3723	156	-17.59	miR:	ugGGGAUAGUGCUAAUCGUAAUu
							:|| |||| | ||||||||
						site:	aaUCCAAUCA-- --A-UAGCAUUAg
HRV-1B	106b	5459	5478	160	-14.6	miR:	uagACGUGACAGUCGUGAAAu
							||: | | |:||||||||
						site:	gaaUGUAAU--UUAGCACUUUc
HRV-1B	155	2878	2901	144	-14.2	miR:	ugGGGAUAGU--GCUAAUCGUAAUu
							|:|| || : ||||||||:
						site:	cuCUUUACCAUUUCUUAGCAUUGc
HRV-1B	155	5984	6005	159	-13.65	miR:	ugGGGAUAGUGCUAAUCGUAAUu
							:: ||| || ||||||||
						Site:	gaUUCUA--AACCAAUAGCAUUAg
HRV-1B	128	1809	1829	147	-13.12	miR:	uuucucuggcCAAGUGACACu
							|| ||||||
						Site:	acauuaguauGUACACUGUGc
HRV-1B	19b	5071	5095	153	-10.35	miR:	agucaAAACG- - - UACCUAAACGUGu
							||| | || | ||||||
						Site:	uguaaUUUACAAAUUGUUUUGCACa

HRV: Human rhinovirus.

Schmitter *et al*[69] showed that an anti-DICER shRNA had to be expressed for at least 3 to 7 d in HEK cells, in order to effectively abolish microRNA repression of a luciferase construct. In addition, Gantier *et al*[73] specifically studied microRNA stability, showing that different miRs have different turnover rates, and estimated that the average half-life in HEK cells is about 5 d.

DICER knock-down, and the subsequent reduction of microRNAs, allowed an increase of viral RNA following infection with HRV-1B (Figure 3) suggesting that the microRNA machinery affected the intracellular stages of Rhinovirus replication. In addition, considering that AGO proteins are responsible for the activity of microRNAs, our AGO2 co-IP results suggest that microRNAs bound the viral RNA during infection of bronchial epithelial cells (Figure 4). This encouraged us to use bioinformatics in order to identify possible antiviral microRNA candidates. From the candidates identified by Targetscan, we selected five microRNAs known to be deficient in asthmatic PBECs: miR-18a, -19b, -106b, -128 and -155 ([63] and our data, not shown). Using anti-miRs, we confirmed that, out of those microRNAs, miR-128 and miR-155 had antiviral activity against HRV-1B in BEAS-2B cells (Figure 8): inhibition of miR-128 or

Table 5 PITA predictions on human rhinovirus-1B

Virus	miR	Site start	Site end	dG duplex	ddG		Alignment
HRV-1B	155	2901	2893	-15.12	-11.87	Site:	UCUUUACCAUUUCUUAGCAUUGCG
							|| || |||||||:
						miR:	UGGGGAUAGU-GCUAAUCGUAAUU
HRV-1B	128	1562	1554	-16.4	-11.11	Site:	CUAACAUAAGACCAAUCACUGUAUCA
							|||||||
						miR:	UUUCUCUGGCCAAGUGACACU
HRV-1B	106b	2276	2268	-17.2	-10.9	Site:	GUUUCUGCAUGUAAGGACUUUUG
							|||||||||||
						miR:	UAGACGUGACAGUCGUGAAAU

The cut off was set at -10 (ddG). HRV: Human rhinovirus.

Table 6 Full target site list predicted by PITA on human rhinovirus-1B

Virus	microRNA	Start	End	dGduplex	dGopen	ddG
HRV-1B	miR-155	2901	2893	-15.12	-3.24	-11.87
HRV-1B	miR-128	1562	1554	-16.4	-5.28	-11.11
HRV-1B	miR-106b	2276	2268	-17.2	-6.29	-10.9
HRV-1B	miR-18a	2124	2116	-14.81	-4.9	-9.9
HRV-1B	miR-106b	4777	4769	-17.81	-7.96	-9.84
HRV-1B	miR-19b	6668	6660	-14.1	-5.07	-9.02
HRV-1B	miR-18a	7047	7039	-23.1	-15.01	-8.08
HRV-1B	miR-155	3186	3178	-14.3	-7.56	-6.73
HRV-1B[1]	miR-155	3723	3715	-14.9	-8.5	-6.39
HRV-1B	miR-19b	2016	2008	-12.11	-5.91	-6.19
HRV-1B	miR-18a	2570	2562	-16.8	-11.26	-5.53
HRV-1B	miR-18a	3108	3100	-13.5	-8.1	-5.39
HRV-1B	miR-128	3609	3601	-11.5	-6.32	-5.17
HRV-1B[1]	miR-19b	5095	5088	-12.53	-7.64	-4.88
HRV-1B[1]	miR-106b	5478	5470	-15.3	-10.46	-4.83
HRV-1B[1]	miR-155	6005	5997	-11.6	-7.01	-4.58
HRV-1B	miR-18a	4144	4138	-12.06	-7.91	-4.14
HRV-1B	miR-128	3513	3505	-14.3	-10.41	-3.88
HRV-1B	miR-19b	77	70	-10.42	-6.61	-3.8
HRV-1B	miR-106b	5542	5534	-10.6	-6.95	-3.64
HRV-1B	miR-155	5028	5020	-8.1	-4.94	-3.15
HRV-1B	miR-18a	4792	4784	-11.5	-8.37	-3.12
HRV-1B	miR-155	3552	3544	-12.6	-9.54	-3.05
HRV-1B	miR-106b	3585	3577	-12	-9.41	-2.58
HRV-1B	miR-18a	1686	1680	-14.19	-11.87	-2.31
HRV-1B	miR-106b	722	714	-4.36	-2.12	-2.23
HRV-1B	miR-128	2562	2554	-14.3	-12.1	-2.19
HRV-1B	miR-155	3975	3967	-13.8	-11.77	-2.02
HRV-1B	miR-128	442	434	-17.2	-15.21	-1.98
HRV-1B	miR-155	6621	6613	-6.5	-4.56	-1.93
HRV-1B	miR-155	4551	4543	-7.3	-5.68	-1.61
HRV-1B	miR-155	5978	5970	-7.9	-6.41	-1.48
HRV-1B	miR-18a	1431	1423	-6.6	-5.13	-1.46
HRV-1B	miR-19b	3248	3240	-8.71	-7.66	-1.04
HRV-1B	miR-106b	6598	6590	-11.8	-10.94	-0.85
HRV-1B[1]	miR-128	1829	1822	-12.9	-12.08	-0.81
HRV-1B	miR-155	6962	6954	-6	-5.38	-0.61
HRV-1B	miR-106b	569	563	-10.2	-9.77	-0.42
HRV-1B	miR-155	870	862	-9.57	-9.26	-0.3
HRV-1B	miR-18a	5478	5471	-10.7	-10.46	-0.23
HRV-1B	miR-155	3584	3576	-9.6	-9.41	-0.18
HRV-1B	miR-155	6258	6250	-9.17	-8.98	-0.18
HRV-1B	miR-19b	3550	3542	-10.4	-10.38	-0.019
HRV-1B	miR-106b	5269	5261	-10	-10	0.0096
HRV-1B	miR-106b	6533	6525	-11.4	-11.44	0.047
HRV-1B[1]	miR-18a	7059	7053	-18.7	-19.34	0.64
HRV-1B	miR-19b	2309	2301	-6.4	-7.08	0.68
HRV-1B	miR-19b	5076	5068	-9.2	-10.03	0.83
HRV-1B	miR-106b	177	169	-14.3	-15.51	1.21
HRV-1B	miR-155	624	616	-9.39	-11.23	1.84

HRV-1B	miR-106b	1105	1098	-9.2	-11.47	2.27
HRV-1B	miR-155	4265	4257	-6.91	-9.29	2.38
HRV-1B	miR-155	6641	6633	-5.8	-8.21	2.41
HRV-1B	miR-18a	4866	4858	-8.3	-11.02	2.72
HRV-1B	miR-18a	569	561	-7	-9.77	2.77
HRV-1B	miR-106b	6286	6278	-3.51	-6.53	3.02
HRV-1B	miR-128	6964	6956	-5.69	-8.77	3.08
HRV-1B	miR-155	2384	2376	-4.1	-7.44	3.34
HRV-1B	miR-155	234	226	-6.15	-9.76	3.61
HRV-1B	miR-19b	622	614	-7.4	-11.06	3.66
HRV-1B	miR-106b	441	435	-10.5	-15.27	4.77
HRV-1B	miR-155	6240	6232	-6	-12.02	6.02
HRV-1B	miR-106b	6184	6176	-7.9	-14.56	6.66
HRV-1B	miR-106b	549	541	-6.3	-15.77	9.47
HRV-1B	miR-106b	7007	6999	-7.61	-18.39	10.78

[1]Sites identified also by targetscan. HRV: Human rhinovirus.

miR-155 lead to a modest but significant increase of viral RNA, which was roughly similar to what obtained by the prolonged DICER silencing. Conversely, miR-155 over-expression inhibited viral replication (Figure 10).

MicroRNA-128 has been shown to play an important role in the control of apoptosis in glioblastoma[74-76]. MiR-128 has also been shown to regulate EGFR expression in non-small-cell lung cancer cell lines[77] and to be down-regulated in the airway epithelium of smokers[78]. It would certainly be of interest to investigate whether the lack of miR-128 in asthmatic PBECs contributes to their higher EGFR expression[79] or if this microRNA affects the virally induced apoptosis in bronchial epithelial cells[44]. However, considering the short time post-infection adopted (8 h), it seems unlikely that in our experiments the inhibition of miR-128 increased viral replication by affecting the induction of apoptosis.

MicroRNA-155 plays an important role in different processes[80] from physiological ones such as haematopoiesis, cellular differentiation and immune responses, to malignancies[13,81-85]. Importantly, miR-155 has been shown to be up-regulated by a series of microbial components such as LPS. Although the functions of miR-155 in non-hematopoietic cells have been much less studied, the antiviral activity we postulate for miR-155 in bronchial epithelial cells would fit with the large involvement of this microRNA in immunity.

It was surprising that the inhibition of miR-18a did not affect HRV-1B replication, despite the promising context+ score calculated by Targetscan. Therefore, we used two additional microRNA predictive programs on HRV-1B: miRanda and PITA (see Figure 11 and Tables 4 and 5). Despite large overlap between the sites identified by Targetscan and miRanda, the latter did not find a miR-18a target on HRV-1B. PITA identified favourable sites only for miR-155, -128 and -106b, in order of predicted efficiency. In combination with our experimental results (only miR-128 and -155 affected viral replication) this analysis highlights the importance to adopt multiple prediction algorithms. Moreover, the results from PITA suggest that the presence of secondary structures in the viral genome may prevent miR-18a from interacting with its target site. As a matter of fact, target site accessibility has been shown to be an important factor in determining microRNA efficacy[51]. Of the three algorithms, PITA is the only one to take into account this feature of microRNA biology (see materials and methods for more details). Notably, PITA could identify all the sites also predicted by Targetscan, but estimated their accessibility too low for the microRNA to have an effect on HRV-1B (Table 6).

The Rhinovirus genomic RNA is directly translated into a single polyprotein which further matures into separate proteins. Therefore, it is a single transcriptional unit *i.e.*, it is comparable to a single cellular mRNA. However, while miRs are often found to target the 3'UTR of cellular mRNAs, the putative miR sites for HRV-1B fall within the coding region (Figure 11). This is in agreement with what already shown for HCV[23] and Influenza virus[28] and represents a striking difference between microRNA targets on cellular mRNAs and those on viral RNA.

Further investigation is needed to understand the mechanistic details of what shown by our experiments. Our results cannot exclude the possibility that the observed effects were due to an indirect mechanism. It could be argued that the microRNA inhibitors could have affected the IFN-related antiviral machinery. For instance, miR-155 has been shown to target SOCS1, an inhibitor of type I IFN signalling[86]. So, it can be assumed that the inhibition of miR-155 would favour viral replication by increasing SOCS1. However, also miR-19b has been shown to target SOCS1[87] but, in our experiments, the inhibition of this microRNA did not have any effect on viral replication.

Despite these limitations, our data altogether suggest that HRV-1B may be directly targeted by microRNAs. In fact (1) prolonged DICER knock-down did not affect the basal levels of IFN-beta mRNA or its induction by infection; (2) AGO2 protein interacted with HRV-1B RNA during viral replication (6 h post-infection); (3) miR-155 and miR-128 have putative target sites on HRV-1B, as predicted by three different bioinformatics tools; and (4) inhibition of either miR led to a modest but significant increase of intracellular viral RNA, while over-expression of miR-155 had the opposite effect.

In conclusion, our study suggests that cellular microRNAs play a role in the innate immune response against

Rhinoviruses. We also showed that two microRNAs that are under-expressed in the asthmatic epithelium, can affect HRV-1B replication. Therefore, our work encourages speculating that the asthmatic microRNA deficiency recently uncovered[63,88] may contribute to the higher replication of Rhinoviruses in asthmatic PBECs.

COMMENTS

Background

Human Rhinovirus (HRV) is one of the major causes of asthma and chronic obstructive pulmonary disease (COPD) exacerbations, which can be life threatening and represent an unmet clinical need. MicroRNAs, non-coding RNA molecules that regulate gene expression, also participate in innate antiviral immunity.

Research frontiers

Bronchial epithelial cells from asthmatic patients have a defective innate immune response to HRV infection, notably by producing lower levels of interferons, compared to cells from healthy subjects. However, the role of microRNAs in such insufficient immune response has not been investigated, despite the recent finding that microRNA expression is dysregulated in asthmatic cells.

Innovations and breakthroughs

Several reports have highlighted the importance of microRNAs as part of the innate antiviral immunity. Often, microRNAs have been shown to inhibit viral replication by directly targeting viral RNA. While it has been demonstrated that miR-23b could limit HRV replication by an indirect mechanism, this is the first report showing evidence of direct interaction between the microRNA machinery and HRV RNA.

Applications

By uncovering a role for microRNAs in the antiviral response of bronchial epithelial cells to HRV, this study may inspire future strategies for therapeutic intervention in the management of HRV-induced exacerbations of asthma and COPD.

Terminology

MicroRNAs are short RNA molecules that generally inhibit gene expression by binding to partially complementary sequences in the 3' Un-Translated Region of cellular mRNAs. Such inhibition can involve both increased mRNA degradation and inhibition of translation.

Peer review

In the manuscript, some interesting results have been observed that both miR-128 and miR-155 target HRV-1B. Over-expression of miR-155 inhibits HRV-1B RNA accumulated in BEAS-2B cell line.

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