

# Respiratory Syncytial Virus Infection Induces Airway Hyperresponsiveness Through miR-34b/c-5p/CXCL10 Axis

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## Research Article

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# Abstract

**Background:** There is growing evidence on miRNAs with pivotal role in viral infection-induced airway diseases such as asthma and chronic obstructive pulmonary disease (COPD). However, the related mechanism of RSV infection affecting airway inflammation and airway hyperresponsiveness (AHR) is largely unknown.

**Methods:** We obtained the miRNA and mRNA databases of patients with RSV infection, as well as the miRNA databases of asthma and COPD patients from the GEO database. Through integrated analysis we screened differentially expressed miRNAs (DEmiRs) and genes (DEGs). Further analysis was carried out to obtain the hub gene through the biological pathways and enrichment pathways of DEGs targeted by DEmiRs and construction of a protein-protein interaction (PPI) network. Finally, the expression of DEmiRs and hub gene were verified *in vivo* and *in vitro* experiments, and the regulatory relationship between DEmiRs and hub gene was further explored.

**Results:** The five differential molecules (miR-34b/c-5p, CD14, CXCL10 and RHOH) screened through animal experiments have the same expression trend in the acute and chronic phases of RSV infection. Following the infecting of BEAS-2B cells with RSV, we confirmed that RSV infection can down-regulate miR-34b/c-5p, and the expression levels of CXCL10 and CD14 were significantly increased in the infected group. Furthermore, the results of the dual-luciferase reporter assay showed that CXCL10 is the target of miR-34c-5p.

**Conclusion:** We confirmed that the increase of CXCL10 after RSV infection is regulated by miR-34b/c-5p. This study shed some lights on the mechanism of RSV infection affecting airway inflammation and AHR. In addition, the study provides diagnostic indicators and therapeutic targets for AHR caused by persistent RSV infection.

## Background

Airway hyperresponsive diseases (AHD) such as asthma and chronic obstructive pulmonary disease (COPD) are a serious public health burden worldwide [1]. Studies have shown that respiratory syncytial virus (RSV) infection with an important role in the acute attack of adult asthma, COPD and other diseases. RSV is an enveloped RNA virus with a single-stranded negative-chain genome and a common pathogen that causes respiratory tract infections in infants and young children [2]. RSV aggravates the inflammatory response and airway hyperresponsiveness (AHR) by inducing the release of cytokines, thereby exacerbating the symptoms of asthma and COPD [3,4]. Children who experienced severe RSV infection during infancy leading to bronchiolitis have an increased probability of recurrent wheezing or asthma at the age of 13[5]. Moreover, in some patients with COPD, persistent and low-grade RSV infection may induce lung immune disorders and even COPD[6]. At present, although there studies designed to study the mechanism of respiratory dysfunction caused by RSV infection, the mechanism of airway inflammation and AHR induced by RSV infection still remains unclear.

In animal models, RSV infection can significantly increase the infiltration of neutrophils, eosinophils and lymphocytes into the airways. Also it induces the releases of high levels of pro-inflammatory cytokines, including IL-1, IL-6, IL-12, IL-13, IFN- $\gamma$ , TNF- $\alpha$  and chemokines such as CCL5, CXCL10, CXCL11 and TNF-related apoptosis-inducing ligand (TRAIL)[7]. These contribute to airway inflammation, excessive mucus secretion and AHR. During the process of RSV infection, chemokines are the key drivers of the antiviral responses. They can interact with chemokine receptors to promote the recruitment and activation of peripheral immune cells to resist viral infections[8]. However, excessive inflammatory reaction leads to enlarged lung injury, causing related respiratory diseases. A variety of chemokines (such as CXCL9, CXCL10, CXCL11, CCL2, CCL5, CCL17 and CCL22) exhibit higher expression levels in the patients with airway diseases (such as asthma, COPD) than those in the control group[9,10]. There exist development of various small molecule antagonists of their receptors (such as CXCR3, CCR1, CCR4 and CCR5) [11].

MicroRNAs (miRNAs) are small non-coding single-stranded RNAs, usually composed of 18-25 nucleotides. They can induce degradation of messenger RNA (mRNA) to inhibit the translation process of target genes, and negatively regulate gene expression at the post-transcriptional level. In recent years, miRNAs have become a hot research topic because of their participation in many biological activities. To date, miRNAs are potential therapeutic targets via either inhibiting viral genome or promoting host antiviral gene signaling. This is not only for RSV, but also for the other respiratory viral infections[12]. The abnormal expression of miRNA caused by RSV infection do not only participates in the acute phase of inflammation, but also involves in the differentiation of immune cells, and the expression of immune tolerance-related genes associated with AHR after viral infection[13]. Therefore, targeting specific miRNAs may be a potential prophylactic or therapeutic strategy for infant RSV induced asthma.

In this study, we analyzed the differentially expressed miRNAs (DEmiRs) in RSV-infected patients, asthma patients, as well as COPD patients by screening miRNA profiling from public datasets. Then the integrated analysis was performed with the mRNA datasets obtained from the RSV-infected patients. We found that miR-34b/c-5p was downregulated and CXCL10 was the hub gene. The *in vivo* and *in vitro* experiments were conducted to verify the expression of miR-34b/c-5p and hub genes, which may explain the mechanism and predict the occurrence and development of RSV-induced AHR.

## Materials And Methods

### Microarray data

The four microarray expression profile datasets including GSE62306, GSE33336, GSE142237 and GSE117827 were obtained from the Gene Expression Omnibus (GEO) database (<https://www.ncbi.nlm.nih.gov/geo/>). The dataset GSE62306, GSE33336 and GSE142237 are miRNA expression profiles. GSE62306 included nasal mucosa samples from 13 healthy children and 14 severely RSV-infected children. GSE33336 contained 11 patients with mild COPD and 20 patients with moderate COPD from lung tissue. GSE142237 had a total of 10 samples derived from bronchial epithelial cells, including 3 healthy controls and 7 asthmatics. GSE117827 was an mRNA expression profile containing 6

control healthy samples and 5 RSV-infected samples, derived from nasal mucosa samples of children after excluding other irrelevant samples.

### **Data preprocessing and differential miRNAs (DEmiRs) and gene (DEGs) screening**

R software was used (version 4.0.3; <https://www.r-project.org/>) and bioconductor (<http://www.bioconductor.org/>) to process raw data. Sequencing platform files were used to convert probes and genes. When the genes had multiple probes, only the maximum probe corresponding data was taken. After the dataset was calibrated and normalized, the limma package was used for screening.

### **Prediction of target genes of miR-34b/c-5p and verification of target genes from DEGs**

The online tool miRDB (<http://mirdb.org/>) was used to obtain the potential target genes of miR-34b/c-5p. The predicted target genes of the two miRNAs and the up-regulated mRNAs in the GSE117827 dataset were subjected to Venn analysis, and the significant up-regulated target genes were obtained after the intersection.

### **GO and KEGG pathway analysis**

Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses (<https://amp.pharm.mssm.edu/Enrichr/>) were used to show functions and roles of the target genes.

### **Construction of PPI network and identification of the hub genes**

The differentially expressed target genes were uploaded to the online tool, STRING database (<https://string-db.org/>). Then the Cytoscape software (version 3.7.1) was used to analysis the PPI networks basing on the STRING results. In order to find the modules of the whole network, the Molecular Complex Detection (MCODE) plug-in of the Cytoscape software was applied. The hub genes were identified by using the plug-in cytoHubba of the Cytoscape software, including Density of Maximum Neighborhood Component (DMNC) and Maximal Clique Centrality (MCC). The top ten genes with the highest connectivity were identified as key genes.

### **Cell culture and viral preparation**

Human lung epithelial BEAS-2B cells and Hela cells were cultured in DMEM plus 10% fetal bovine serum (FBS) at 37°C and 5% CO<sub>2</sub>. RSV (Long strain/A2 type) was stored by the Department of Medical Microbiology, Central South University. RSV was propagated in Hela cells with DMEM containing 3% FBS and the viral titer was determined by plaque assay.

### **Transfection of miRNA mimics and RSV infection**

Synthetic miR-34b/c-5p mimics (50 nmol/L) or negative control (RiboBIO, Guangzhou,China) were transfected into BEAS-2B cells following riboFECT CP Transfection Kit (RiboBIO, C10511-05, Guangzhou,

China) for 24h. Subsequently, the cells were infected with RSV at MOI of 1 for 48h.

### **Dual-luciferase reporter assay**

Simply, the 3'-UTR fragments of CXCL10 containing miR-34b/c binding site were amplified by Nanjing Genscript Biological Technology Co., Ltd. and cloned into the pmirGLO vector (Promega, USA). Then, the wild type of CXCL10 or mutant CXCL10 3'-UTR (CXCL10-WT or CXCL10-MUT) was constructed. Next, the constructed plasmids were co-transfected with miR-34b/c-5p mimics or mimic-NC into 293T cells by using Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer's instructions. After 48 hours of transfection, luciferase activities were measured by the Dual-Luciferase Reporter System (Promega, USA).

### **Animal Models**

6-8 weeks BALB/c female mice weighing 16–20 g (purchased from Hunan Tianqin Biological Technology Co., Ltd) were kept in a pathogen-free environment. The mice were randomly divided into the control group (n = 12) and the RSV group (n = 12). After anaesthetizing the mice with isoflurane,  $5 \times 10^6$  pfu of RSV in 100  $\mu$ l, was intranasally inoculated. For mock infections, mice were administered an equivalent volume of sterile PBS. The airway resistances of mice were tested on day 7 and 28 post-infection (6 mice from each group). Then the mice were sacrificed and their lungs were taken for follow-up studies. RSV infection was verified by indirect immunofluorescence (IFA) with RSV major surface glycoprotein G monoclonal antibody (Bioss,bs-1264R, Beijing, China) as a primary antibody and CY3-conjugated antibody (Boster, BA1032, Wuhan, China) as secondary antibody.

### **Real-time RT-PCR**

Total RNA was extracted from lung tissue or cells using TRIzol reagent (Takara, Japan). Primers were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China), and the sequences were shown in Table 1. Each sample was reverse transcribed into cDNA using RR036A PrimeScript RT Master Mix (Perfect Real Time) (Takara, Japan). For miRNA cDNA synthesis, using RR037A PrimeScript RT Master Mix (Perfect Real Time) (Takara, Japan) with miRNA-specific stem-loop RT Primers. Then the cDNA was synthesized by reverse transcription and amplified using 2 $\times$  SYBR Green qPCR Master Mix (Bimake, USA) according to the manufacturer's instructions. qPCR was performed at 95°C for 3 min, and 40 cycles of 95°C for 15 s, 55°C for 30 s, and 72°C for 30 s. U6 was the internal reference for miR-34b/c, and GAPDH was the internal reference for the other target genes. The relative expression levels of mRNA were calculated using the  $2^{-\Delta\Delta CT}$  method.

### **Measurement of airway responsiveness to methacholine**

The airway resistances of mice were measured on the 7th and 28th day of RSV infection with by Buxco pulmonary function testing system (Buxco, Sharon, Connecticut, CT, USA). After anesthetizing the mice with sodium pentobarbital (60 mg/kg), the airway was separated and the tracheal intubation was

performed, and then the mice were mechanically ventilated with a small animal ventilator at a tidal volume of 10 ml/kg, and a frequency of 120 breaths/min. Then the mice were inhaled 10 $\mu$ l of methacholine (0.00, 6.25, 12.5, 25, and 50 mg/mL) and the values of airway resistances (Rn,cm H<sub>2</sub>O s / mL) were obtained by measuring airway flow and pressure.

### **Hematoxylin-eosin staining**

The left lung tissues taken from each animal were fixed in 10% formaldehyde solution for 24 h, and then embedded in paraffin and cut into 5  $\mu$ m sections. Hematoxylin-eosin staining was performed according to routine experimental procedures, and then the histomorphological changes were observed under an optical microscope.

### **Immunofluorescence**

The Lung tissue sections were fixed by 95% ethanol and 0.1% Triton-X100. The samples were then blocked with normal goat serum for 20 min, and incubated overnight with RSV major surface glycoprotein G monoclonal antibody (Bioss, bs-1264R, Beijing, China) rabbit primary antibody at 4 °C. Then the samples were incubated with a CY3-conjugated goat anti-rabbit secondary antibody (Boster, BA1032, Wuhan, China) at room temperature for 1 h. After counterstained with DAPI for 10 min, the samples were observed under a light microscope at high magnification (200 $\times$ ) (Leica, German).

### **ELISA**

The levels of CXCL10 in the culture supernatants of BEAS-2B cells were measured using ELISA kits (Jianglaibio, Shanghai, China), according to the manufacturer's protocol.

### **Statistical Analysis**

GraphPad Prism software version 7.0 was used for statistical analysis, and the data were expressed as mean  $\pm$  standard deviation. T-test was used for comparison between two groups; two-way ANOVA was used for comparison among multiple groups, and LSD was used for post hoc test.  $P < 0.05$  was considered significant.

## **Results**

### **The aberrantly expressed miRNA molecules in RSV infection, asthma or COPD**

The raw data of each dataset were normalized following data pretreatment. A total of 40 DE miRs (26 up-regulated and 14 down-regulated) were identified in GSE62306, whereas 10 DE miRs were identified in GSE33336, of which 4 were up-regulated and 6 were down-regulated. A total of 109 DE miRs were identified in GSE142237, of which 38 were up-regulated and 71 down-regulated. The heatmap and volcano plots (Fig. 1) exhibited the aberrantly expressed miRNA molecules. A total of 2 up-regulated miRNAs including miR-34b/c-5p (0 down-regulated miRNA) were finally screened in the 3 miRNA

microarray datasets following Venn analysis (Fig. 2A). Fig. 2B respectively demonstrated the expression of miR-34b/c-5p in RSV infected patients, asthmatics and patients with COPD.

### **DEGs expression profiles in RSV-infected patients and screening of DEGs targeted by miR-34b/c-5p**

For microarray data GSE117827, we constructed a heatmap of clustering analysis in Figure 3A. The left side of the samples was control group, while the right half was the RSV-infected group. Besides, DEGs were reflected in the volcano plot (Figure 3B). A total of 3,379 DEGs (497 up-regulated and 2,882 down-regulated) were identified in RSV-infected patients. Principal component analysis (PCA) showed that the evaluation of these DEGs expression was effective at separating two groups (Figure 3C). In order to further explore the DEGs related to the mechanism of RSV-induced airway inflammation and AHR, we obtained 1231 potential target genes of miR-34b/c-5p by the online tool miRDB. Then the predicted target genes were compared with the DEGs in microarray data GSE117827 and 23 up-regulated intersect genes were screened (Figure 3D).

### **Functional enrichment analysis, protein-protein interaction and hub gene identification**

The GO enrichment analysis displayed that the DEGs targeted by miR-34b/c-5p were mainly enriched in biological processes including cytokine-mediated signaling pathway, cellular response to bacterial molecules. Moreover, they enriched in molecular functions consisting of histone threonine kinase activity. In addition, the DEGs targeted by miR-34b/c-5p were mostly located at recycling endosome and trans-Golgi network transport vesicle. The KEGG enrichment analysis illuminated that the DEGs targeted by miR-34b/c-5p were predominantly enriched in inflammatory pathways such as the NF-kappa B signaling pathway and chemokine signaling pathway. The PPI network was constructed from (search tool for recurring instances of neighboring genes) online SRING tool (minimum required interaction score: 0.9) and the result was visualized with Cytoscape software[14]. About 23 intersect genes were uploaded and analyzed by SRING and Cytoscape software. Then, 10 hub genes including LCP1, CCR1, CXCL10, CD14, ZEB1, PRKCB, PZRY14, IRAK2, RHOH and LILRA1 were selected from the result analyzed by cytoHubba.

### **qRT-PCR verified the expression of miR-34b/c-5p and hub genes in the RSV-induced AHR mice.**

RSV (Long strain/A2 type) was propagated in Hela cells and the viral titer was determined by plaque assay. RSV-infected Hela cells showed obvious pathological changes containing syncytia (Fig. 5A) and the mice model of AHR induced by RSV infection was constructed. The RSV infection in the lungs of mice was verified using IFA and the RSV group showed significant red fluorescence compared to the control group (Fig. 5B). To confirm the promoting effect of RSV infection on airway inflammation, pathological examination was analyzed by H&E staining. As compared with control mice, peribronchial and perivascular infiltrating inflammatory cells were increased in RSV-infected mice (Fig. 5C). The  $R_n$  values after aerosolizing different concentrations of methacholine were significantly increased in RSV-infected group compared to those in the control group (Fig. 5D). During the RSV acute infection period (7 d), the AHR and airway inflammation of RSV-infected mice was the most severe. Over time, these symptoms were gradually alleviated during the chronic phase of infection (28 d). miR-34b/c-5p and hub genes were

detected by qRT-PCR. The results showed that miR-34b/c-5p were downregulated in the lung tissues of RSV-infected mice (7d and 28d) compared to those in the control mice (Fig. 5E). Furthermore, we found that the levels of CXCL10, CD14, ZEB1 and RHOH in lung tissues were significantly increased on day 7 of RSV infection, as compared to those in the control group (LILRA1 is not expressed in mice) (Fig. 5F). However, in the lung tissues of the chronic infection period, only the expressions of CXCL10, CD14 and RHOH were detected higher than those of control mice (Fig. 5G).

### **Down-regulated miR-34b/c-5p induced CXCL10 expression in RSV-infected BEAS-2B cells**

To further validate the results of animal experiments, we infected BEAS-2B cells with RSV for 48 h, and confirmed the infection using IFA. Compared with the uninfected cells, red fluorescence was seen in the BEAS-2B cells (Fig. 6A). Then, we tested the mRNA levels of miR-34b/c-5p and hub genes (CXCL10, CD14 and RHOH) using qRT-PCR. In RSV-infected BEAS-2B cells, the expression levels of miR-34b/c-5p were down-regulated (Fig. 6B), CXCL10 and CD14 levels were up-regulated (Fig. 6C), which were consistent with the tendency of animal experiments. However, RHOH showed no significant difference in two groups (Fig. 6C). To further estimate the function of miR-34b/c-5p in the expression of CXCL10 and CD14 after RSV infection, miR-34b/c-5p mimics were transfected into BEAS-2B cells before RSV infection (Fig. 6D). As shown in Fig. 6E, the mRNA levels of CXCL10 was decreased when transaction with miR-34b/c-5p mimics before RSV infection, while CD14 levels showed no difference. Moreover, after interfering with miR-34b/c-5p mimics prior to RSV infection for 48 h, the protein expression levels of CXCL10 were significantly reduced compared to the RSV+ miR-NC group (Fig. 6F). Then, we predicted that CXCL10 can bind to miR-34b/c-5p using bioinformatics analysis, and found that CXCL10 is a potential target gene of miR-34c-5p, but not miR-34b-5p (Fig. 6G). The luciferase assays were carried out to detect the relationship between CXCL10 and miR-34c-5p. As illustrated in Fig. 6G, over-expression of miR-34c-5p strikingly suppressed the luciferase activities of the CXCL10-WT reporter vector but not CXCL10-MUT reporter vector. Taken together, our findings deemed that miR-34c-5p can inhibit the expression of CXCL10 by directly targeting it.

## **Discussion**

Respiratory syncytial virus (RSV) can cause severe lower respiratory tract infections, especially in infants, immunocompromised individuals and among the elderly group [3]. RSV and other common respiratory pathogens play an important role not only in the initiation, but also in the exacerbation of asthma [15]. In addition, 64% of chronic obstructive pulmonary disease (COPD) exacerbations are caused by respiratory infections such as RV, influenza and RSV affecting the bronchial epithelium [1]. Therefore, the inflammatory mechanism caused by RSV infection may also be the basis for asthma and COPD.

In this study, the joint analysis of miRNA-mRNA expression profiles of three disease models was carried out to analyze the signal pathways involved in DEGs. This design was set to understand the mechanism of RSV infection affecting the occurrence and development of AHR at the system level. We constructed a RSV infection-induced AHR mice model and found that the expression of miR-34b/c-5p in the lungs of

RSV-infected mice was down-regulated, but the selected hub genes, which were only CXCL10, CD14 and RHOH, are highly expressed in RSV group.

The miR-34 family (miR-34s) is a group of highly conserved miRNAs. Human miR-34s include miR-34a, miR-34b and miR-34c. Hermeking et al. found that the expression of miR-34a is highest in the brain, while in the lung, miR-34b and miR-34c are mainly expressed[16]. The miR-34 family is very important in regulating the interaction between the virus and the host by participating in the immune response and virus replication. They can be used as biomarkers for viral infection [17]. Some studies have shown that miR-34 is closely related to the occurrence and development of lung diseases such as lung cancer and asthma[18-21]. Solberg et al. found that the expression of miR-34s (miR-34c, miR-34b) in the bronchial epithelial cells of asthma patients was significantly down-regulated. In addition, it was found that IL-13 can inhibit the expression of miR-34 in asthma patients, and speculated that miR-34 may play a role in asthma by regulating the proliferation and differentiation of epithelial cells [18]. Yin et al. reported that miR-34/449 can promote airway inflammation and fibrosis by modulating IGFBP-3 mediated autophagy activation [19]. However, the role of miR-34b/c during RSV infection has not yet been clarified.

CD14 is expressed in monocytes and macrophages, and it mediates the natural immune process of bacterial or viral infections. In patients with bronchiolitis infected with RSV, the concentration of serum CD14 is related to the subsequent progression of the disease, and its degree is affected by genetic polymorphisms [22]. The -159 C/T mutation in CD14 plays an important role in regulating CD14 expression [23]. Martin et al. reported that CD14 levels are higher in the plasma of patients with acute asthma [24]. Macrophages CD14 can initiate the recruitment of neutrophils into the airway, but CD14 may also need to interact with other cell types to develop AHR and produce cytokines.

CXCL10 is a Th1 chemokine involved in innate and adaptive immunity. It can be secreted by cells under the stimulation of type I and type II IFN and lipopolysaccharide. Studies have shown that CXCL10 is involved in virus-induced airway inflammation. For example, the concentration of CXCL10 is significantly increased in the plasma of bronchiolitis patients with acute RSV infection [25]. In rhinovirus (RV)-induced asthma, the concentration of serum CXCL10 increases, and the expression level of CXCL10 is correlated with the severity of clinical disease during RV-induced acute attacks [26]. The above results indicate that CXCL10 may play a role in the pathogenesis of virus-induced asthma exacerbation, and it can be used as a new marker of virus-induced acute asthma. However, its regulatory role in virus-induced AHR is still elusive.

Ras homologous gene family H (RHOH) is an atypical small GTPase without GTPase activity. RHOH plays a key role in immune cell function by acting as a membrane-bound adaptor for various proximal protein kinases. RHOH participates in anti-viral infections, RHOH deficiency can make T cells defective, which can lead to persistent viral infections [27]. As an adaptor of Syk, RHOH mediates the  $\gamma\delta$ TCR/Syk signaling axis required for the development of  $\gamma\delta$ T17, and then Syk induces  $\gamma\delta$ T17 by activating the PI3K/Akt pathway [28]. In addition, the PI3K/Akt pathway can also guide the differentiation of Th17 cells

and the production of IL-17[29], and the expression level of IL-17 is significantly related to respiratory diseases such as asthma and COPD.

In our study, the five differential molecules (miR-34b/c-5p, CD14, CXCL10 and RHOH) screened through animal experiments have the same expression trend in the acute and chronic phases of RSV infection. Differentially expressed molecules during the persistent infection period may affect the development of AHR in the later period, which serve as an indicator for the clinical diagnosis of RSV-induced AHR. Following the infecting of BEAS-2B cells with RSV, we confirmed that RSV infection can also down-regulate miR-34b/c-5p at the cellular level, and the expression levels of CXCL10 and CD14 were significantly increased in the infected group. We also found that the RSV-induced up-regulation of CXCL10 levels was affected by miR-34b/c-5p, but CD14 was not regulated.

CXCL10 secreted by epithelial cells may participate in airway inflammation and AHR through activation and recruitment of immune cells. CXCL10 can interact with CXCR3, which is expressed on activated CD4<sup>+</sup>, CD8<sup>+</sup> and NKT cells, mast cells and eosinophils, and stimulates the migration of these cells[30]. The presence of mast cells in the airways is characteristic of asthma. Viral infection may lead to the release of CXCL10 in the bronchial epithelium, which activates mast cells and allows them to migrate to airway smooth muscles to exacerbate airway inflammation and bronchoconstriction in asthma [31]. Takaku et al. reported that CXCL10 induces the adhesion of eosinophils to ICAM-1, which may play an important role in the activation and infiltration of eosinophils during virus-induced asthma exacerbation[32]. CD14 is a positive marker of macrophages, and chemokines are the main mediator of macrophage homing. We speculate that the secretion of CXCL10 is related to macrophages in this study. Xuan et al. have found that CXCL10 can induce the chemotaxis of M1 macrophages [33]. It is reported that CXCL10 secreted by activated fibroblasts can promote the expression of iNOS and CD86 on alveolar macrophages. It can be seen that CXCL10 is crucial in the M1 phenotype polarization of alveolar macrophages[34]. M1 macrophages have strong anti-microbial and anti-tumor activities. It can induce the expression of Th1 cell chemokines (CXCL9 and CXCL10), and can also release IL-1 $\beta$ , IL-6, IL-23 and other pro-inflammatory factors to promote the proliferation and differentiation of Th17 cells, increase the secretion of IL-17 to expand the inflammatory response.

Although this study found that RSV infection down-regulated the expression of miR-34b/c-5p, thereby promoting the secretion of CXCL10, the role of CXCL10 in airway inflammation and AHR was still unclear. In the later period, we hope to conduct in-depth research on the effects of miR-34b/c-5p/CXCL10 on the occurrence and development of AHR and their predictive and therapeutic values in AHR.

## Conclusion

This study used bioinformatics to jointly analyze the expression profiles of three disease models including RSV infection, COPD and asthma. We screened out five common differential molecules (miR-34b/c-5p, CD14, CXCL10 and RHOH), provided effective targets for clinical diagnosis and treatment of AHR caused by RSV infection. Through *in vitro* experiments, we confirmed that RSV infection down-

regulated miR-34b/c-5p, and the decreased expression of miR-34b/c-5p induced the secretion of CXCL10. In addition, miR-34c-5p can directly bind to CXCL10. The study provides new insights into the molecular mechanism of CXCL10 secretion in airway inflammation and AHR after RSV infection.

## Abbreviations

COPD: chronic obstructive pulmonary disease; AHR: airway hyperresponsiveness; DEmiRs: differentially expressed miRNAs; DEGs: differentially expressed genes; PPI: protein-protein interaction; miR-34b/c-5p: miR-34b-5p and miR-34c-5p; AHD: airway hyperresponsive diseases; RSV: respiratory syncytial virus; TRAIL: TNF-related apoptosis-inducing ligand; mRNA: messenger RNA; GEO: Gene Expression Omnibus; GO: Gene Ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes; MCODE: Molecular Complex Detection; DMNC: Density of Maximum Neighborhood Component; MCC: Maximal Clique Centrality; FBS: fetal bovine serum; RHOH: Ras homologous gene family H.

## Declarations

**Ethics approval and consent to participate:** The study was approved by the Institutional Ethic Committee of Xiangya School of Medicine, Central South University.

**Consent for publication:** Not applicable.

**Availability of data and material:** The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

**Competing interests:** The authors declare that they have no conflict of interest.

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**Authors' Contributions:** DL, ZT and OB carried out the study. GW performed the statistical analysis. LQ and YT participated in the design of the study. All authors read and approved the submission.

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## Tables

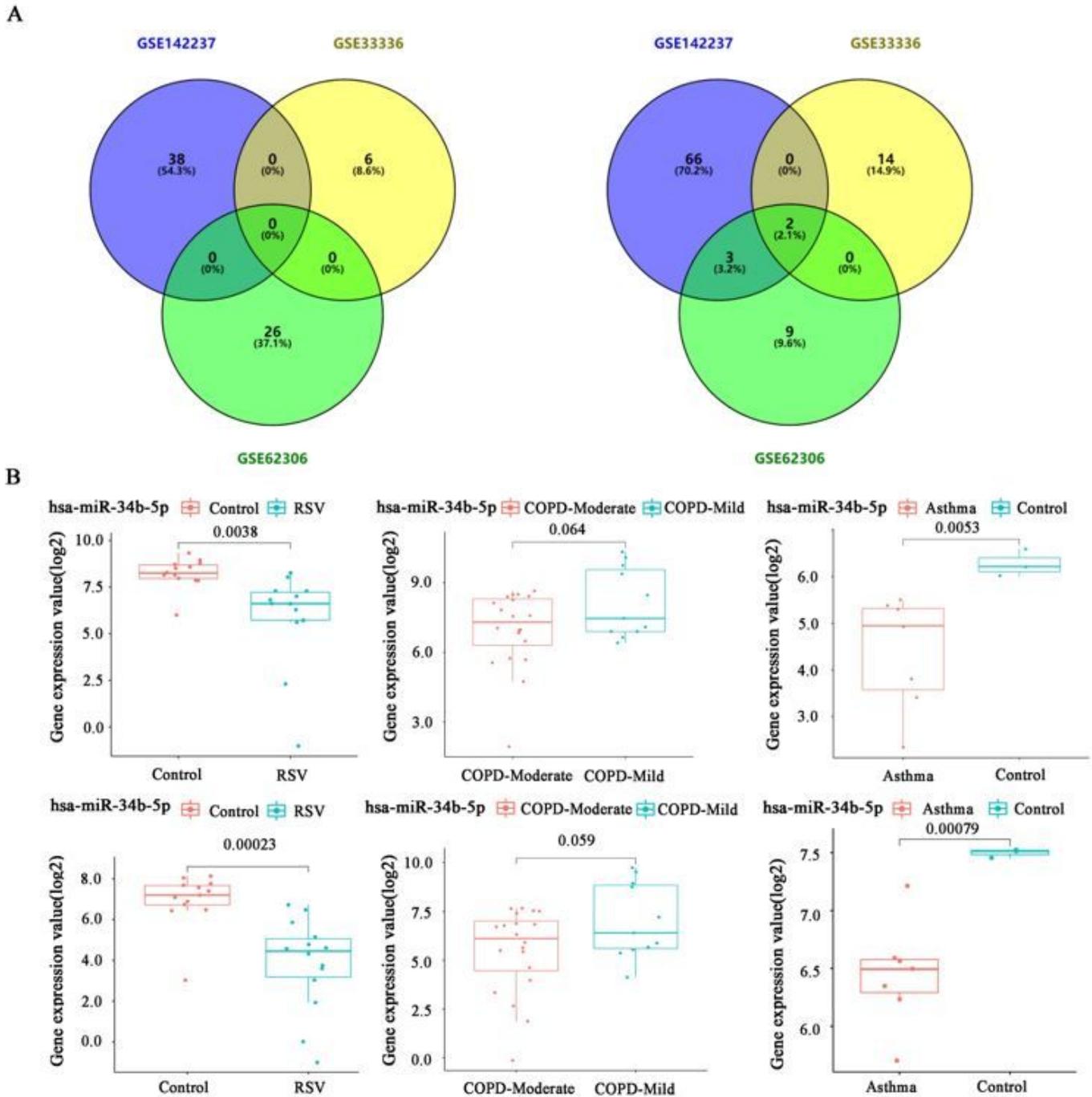
### Table1. Primers for quantitative polymerase chain reaction

Gene	Sequence	Length (bp)
LCP1 (Mouse)	TCTGTGCCAGACACGATTGACG	22
	GAGGCAGAGTTCAGAGCCAAGT	22
CCR1 (Mouse)	GCCAAAAGACTGCTGTAAGAGCC	23
	GCTTTGAAGCCTCCTATGCTGC	22
CXCL10 (Mouse)	ATCATCCCTGCGAGCCTATCCT	23
	GACCTTTTTTTGGCTAAACGCTTTC	24
CD14 (Mouse)	TTGAACCTCCGCAACGTGTCGT	22
	CGCAGGAAAAGTTGAGCGAGTG	22
ZEB1 (Mouse)	ATTCAGCTACTGTGAGCCCTGC	22
	CATTCTGGTCCTCCACAGTGGA	22
PRKCB (Mouse)	CCAAGATGACGATGTGGAGTGC	22
	CTCCATCACAAAGTACAGGCGG	22
PZRY14 (Mouse)	ACCTCCGTCAAGAGGAAGTCCA	22
	GCTGTAGTGACCTTCCGTCTGA	22
IRAK2 (Mouse)	GGACAGATTCCTACAGGCAGAG	22
	GGTAGATGAGGCTGTGGAAGTGC	22
RHOH (Mouse)	TGGATGGCATCCAGATCAGCCT	22
	GTAGCACATCAGTACCACGTCTG	23
GAPDH (Mouse)	CATCACTGCCACCCAGAAGACTG	23
	ATGCCAGTGAGCTTCCCGTTCAG	23
CXCL10 (Human)	GGTGAGAAGAGATGTCTGAATCC	24
	GTCCATCCTTGGAAGCACTGCA	22
CD14 (Human)	CTGGAACAGGTGCCTAAAGGAC	18
	GTCCAGTGTCAGGTTATCCACC	18
RHOH (Human)	TGGATGGCATCCAGATCAGCCT	22
	GCCACAGAGTAGCACATCAGCA	22
GAPDH (Human)	GTCTCCTCTGACTTCAACAGCG	22
	ACCACCCTGTTGCTGTAGCCAA	22

miR-34b-5p	CGCGTAGGCAGTGTCATTAGC	21
	AGTGCAGGGTCCGAGGTATT	20
miR-34b-5p (RT Primer)	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCAATCA	50
miR-34c-5p	CGCGAGGCAGTGTAGTTAGCT	21
	AGTGCAGGGTCCGAGGTATT	20
miR-34c-5p (RT Primer)	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGCAATC	50
U6	CTCGCTTCGGCAGCACA	17
	AACGCTTCACGAATTTGCGT	20

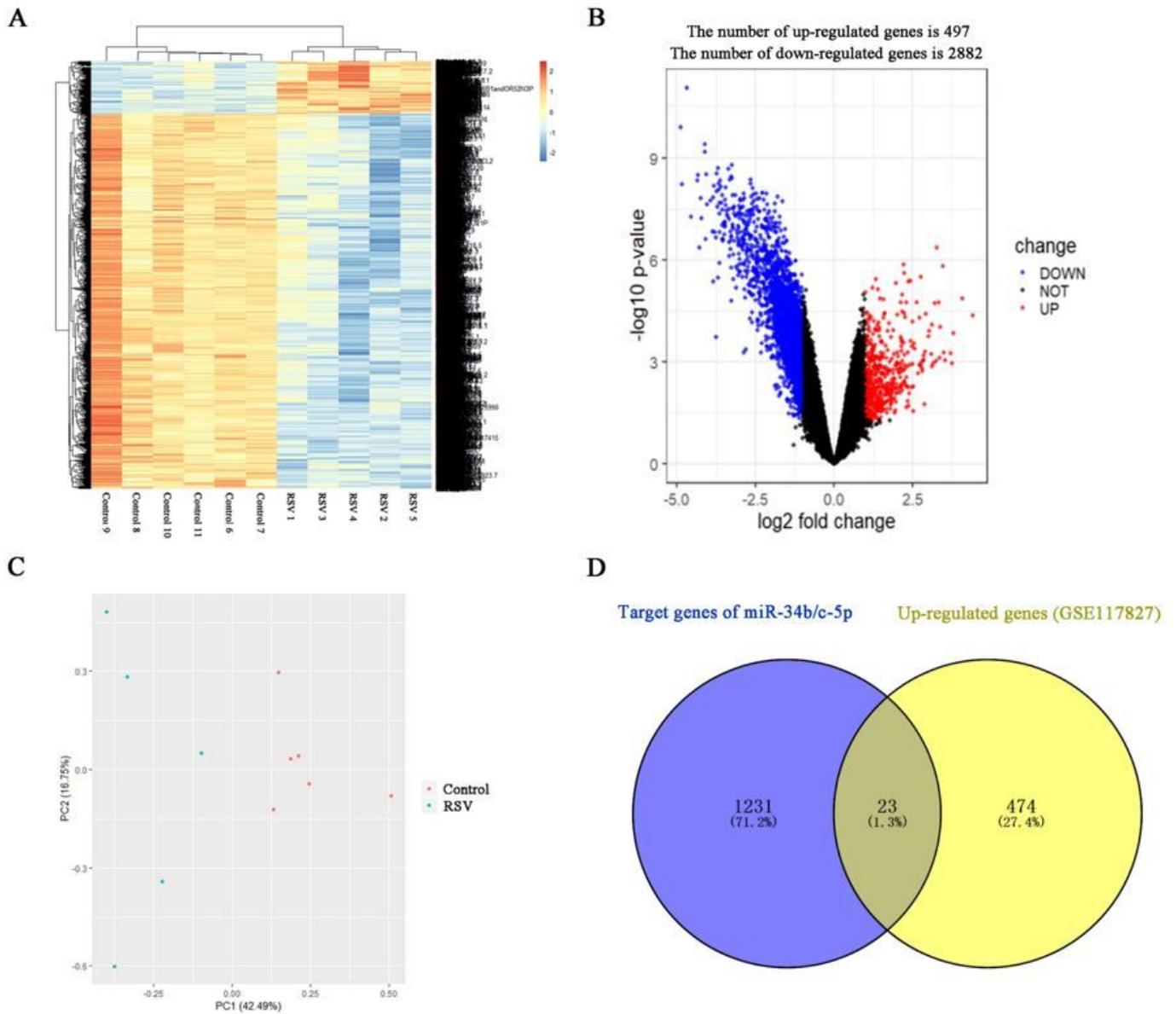
## Figures





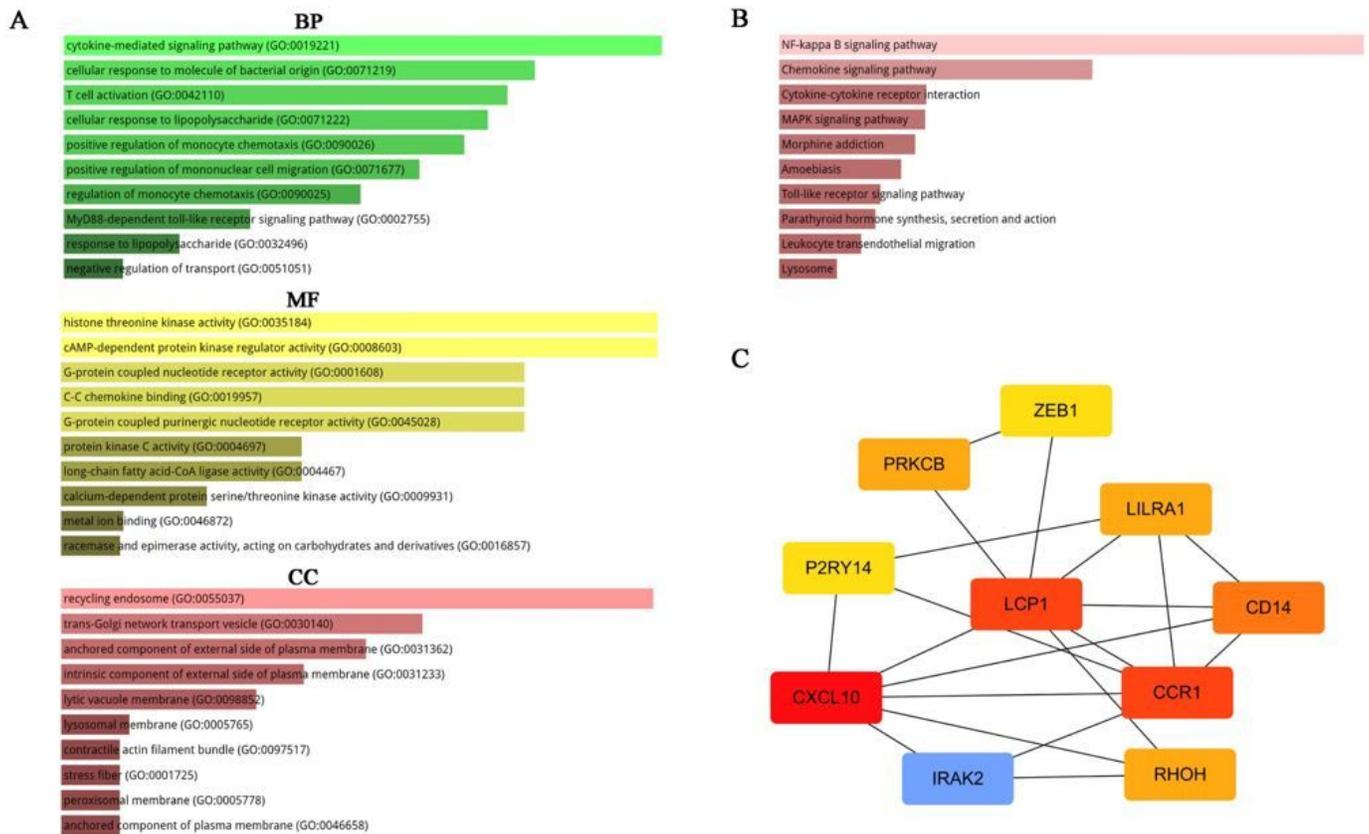
**Figure 2**

The intersecting expression of miR-34b/c-5p in RSV infected patients, asthma and COPD. A, Venn plots revealed 2 intersect miRNAs which include 2 up-regulated miRNAs in patients with RSV infection, asthma and moderate COPD and no down-regulated miRNA in these 3 miRNA microarray datasets. B, Expression levels of miR-34b/c-5p in GSE62306, GSE33336 and GSE142237. The values were expressed as log2 fold change.



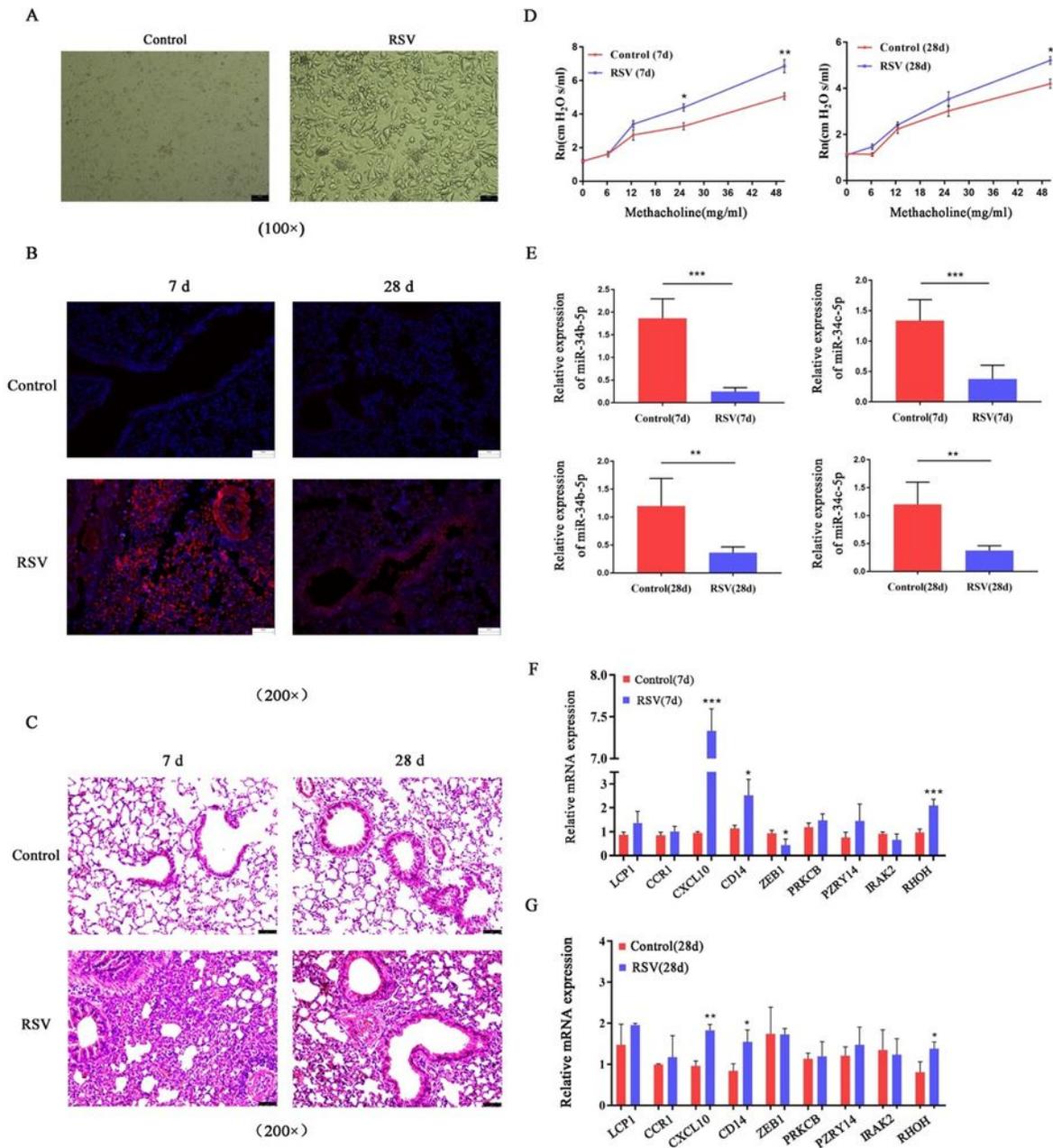
**Figure 3**

DEGs expression profiles in RSV-infected patients and screening of differentially expressed target genes. A, Heatmap of mRNA expression profile in uninfected and RSV infected patients. B, Volcano map for 3379 different expression mRNA. Dots in red and blue indicate high and low expression of mRNA in patients with RSV infection. C, PCA plot composed of nasal mucosa samples from healthy subjects and patients with severe RSV infection. D, Venn plot displayed the distribution of the predicted target genes and the upregulated in the DEGs.



**Figure 4**

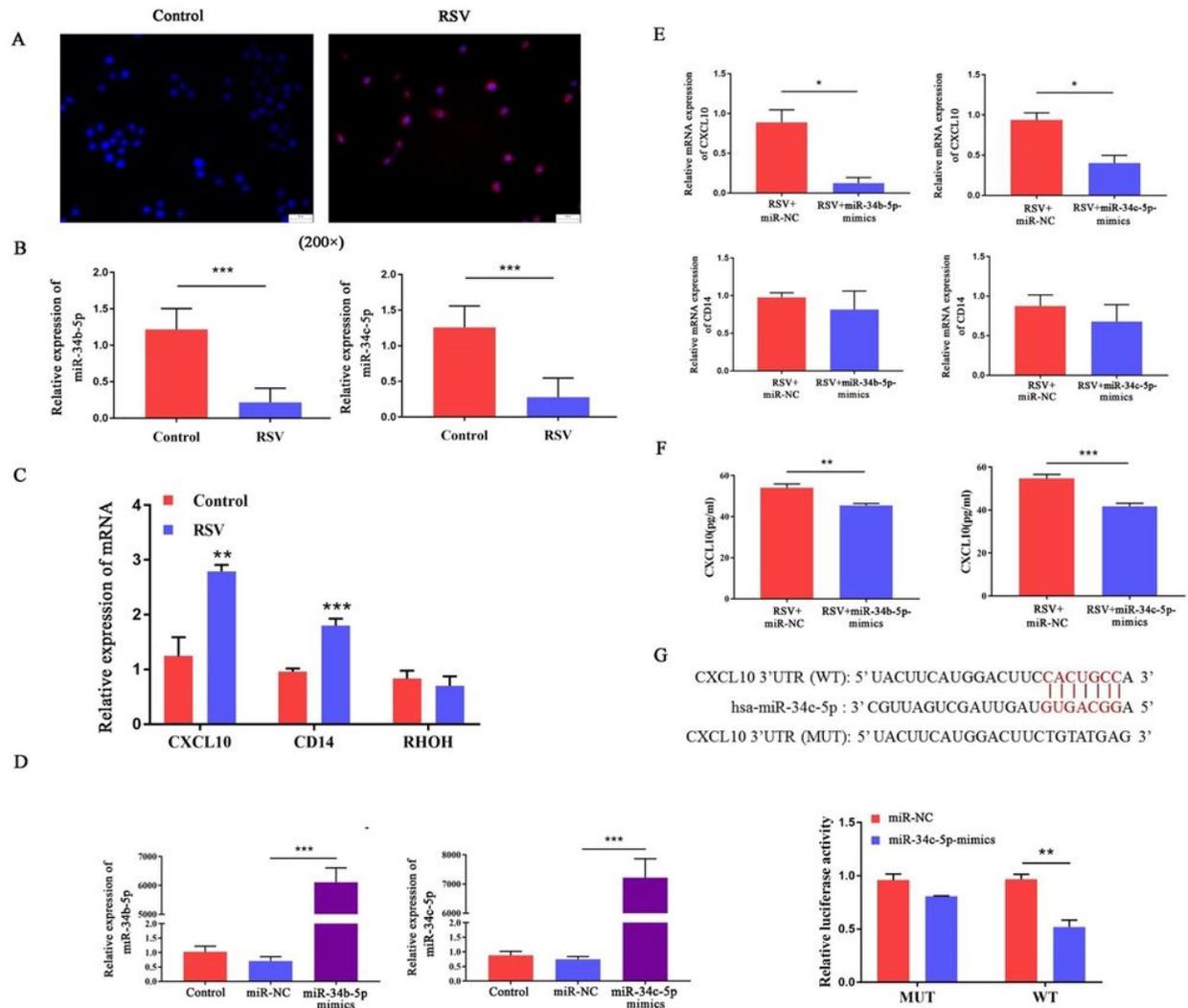
GO and KEGG pathway analysis, construction of PPI and hub gene identification. A, Results of BP, CC and MF in GO analysis revealed the relationship between DEGs targeted by DE miRNAs and functional pathways. B, KEGG pathway of DEGs targeted by DE miRNAs. C, The PPI network data from STRING was further analyzed by Cytoscape and hub genes identification was performed by cytoHubba. GO, gene ontology; BP, biological processes; CC, cellular components; MF, molecular functions; KEGG, Kyoto Encyclopedia of Genes and Genomes. PPI, protein-protein interaction.



**Figure 5**

The expression of miR-34b/c and hub genes in the lung tissues of RSV-induced AHR. A, The replication of RSV in Hela cells (magnification 100×). B, RSV infection in lung tissue was assayed using IFA (magnification 200×). C, Pathological examination was analyzed by H&E staining (magnification 200×). D, AHR to methacholine was evaluated by a Buxco pulmonary function testing system. E-G, detection of

mRNA levels of miR-34b/c-5p and hub genes in lung tissues using qRT-PCR (n = 6) (\*p < 0.05, \*\* p < 0.01 and\*\*\* p < 0.001 vs. Control).



**Figure 6**

Down-regulated miR-34b/c-5p induced CXCL10 expression in RSV-infected BEAS-2B cells. A, RSV infection in BEAS-2B cells was verified by indirect IFA with RSV G protein monoclonal antibody (magnification 200×). B-C, the expression levels of miR-34b/c-5p and hub genes (CXCL10, CD14 and RHOH) in BEAS-2B cells were assayed using qRT-PCR. D, BEAS-2B cells treated with miR-34b/c-5p mimics (mimics-miR-34b/c) or negative control mimics (miR-NC), qRT-PCR were used to detect the transfection effect. E, miR-34b/c-5p mimics or negative control mimic were transfected into BEAS-2B cells prior to 48 h of RSV infection, using qRT-PCR to detect the mRNA levels of CXCL10 and CD14. F, ELISA was used to detect the protein level of CXCL10. G, Putative binding sites of miR-34c within the CXCL10 predicted by

bioinformatics tools. Luciferase reporter assay was performed to validate relationship between CXCL10 and miR-34b/c-5p.