

LncRNA MEG3 ameliorates respiratory syncytial virus infection by suppressing TLR4 signaling

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Abstract. Maternally expressed gene 3 (MEG3), a long noncoding RNA (lncRNA) has been dysregulated in various tumors. However, the expression level and functional role of MEG3 in the progression of respiratory syncytial virus (RSV) infection remains to be elucidated. The present study quantified the expression level of MEG3 in the nasopharyngeal (NPA) samples of RSV-infected patients and in BEAS-2B cells infected with RSV. The findings of the present study demonstrated that the expression level of lncRNA MEG3 was reduced in the NPA samples of RSV-infected patients and in BEAS-2B cells infected with RSV. *In vitro* transfection revealed increased mRNA expression levels of toll-like receptor 4 (TLR4), tumor necrosis factor- α (TNF α) and interleukin (IL)-8 following RSV infection in BEAS-2B cells. Additionally, ectopic expression of MEG3 reduced the expression level of TLR4, subsequently suppressing the mRNA expression levels of TNF α and IL-8, indicating the protective role of MEG3 in the process of RSV infection. It is of note, that RSV infection-induced p38 mitogen activated protein kinase (MAPK) and nuclear factor- κ B (NF- κ B) activation was partly abolished by overexpression of MEG3. In conclusion, to the best of our knowledge, the present study provided the first evidence that lncRNA MEG3 expression level was reduced in the NPA samples of patients with RSV infection and RSV-infected cells. Additionally, it was demonstrated that MEG3 protected human airway epithelial cells from RSV infection, primarily by suppressing TLR4-dependent p38 MAPK and NF- κ B signaling.

Introduction

Respiratory syncytial virus (RSV) remains one of the most common pathogens in the world among young children (1,2). It has been previously reported that RSV is a serious threat to children with chronic lung disease, congenital heart disease or prematurity compared with healthy children (3,4). Due to the high incidence of RSV infection and its potential for negative outcomes, it is important to investigate, identify and prioritize children who are at high risk of developing RSV-associated acute lower respiratory infection.

Toll-like receptor 4 (TLR4) is a primary receptor for Gram-negative lipopolysaccharides (LPS) (5). The structure of the RSV fusion protein is different from LPS; however, it may also trigger TLR4 signaling similarly to LPS (6,7). It has been previously suggested that RSV infection is markedly reduced in mice with TLR4 mutations (8,9). Early lung nuclear factor- κ B (NF- κ B) response to RSV has been previously reported to be TLR4-dependent, indicating that enhanced susceptibility to RSV may induce an appropriate inflammatory response by regulating TLR4 (10). Therefore, elucidating the underlying mechanism of TLR4 regulation may aid in identifying the treatment of RSV infection in children.

Maternally expressed gene 3 (MEG3), a long non-coding RNA (lncRNA), has been extensively identified in various normal tissues (11). In various types of tumor, loss of MEG3 expression enhances the progression of tumors; therefore, ectopic expression of MEG3 may suppress cancer cell proliferation (12,13). Reduced MEG3 expression has been previously identified in non-small cell lung cancer (NSCLC) tissues and may be involved in p53 activation, subsequently prompting the progression of NSCLC (14). However, the expression level and functional role of MEG3 in the progression of RSV infection remains to be elucidated.

The present study demonstrated that the level of MEG3 was reduced in the nasopharyngeal (NPA) samples of patients infected with RSV. Furthermore, overexpression of MEG3 may upregulate the expression level of TLR4, subsequently suppressing RSV infection-induced inflammatory responses.

Materials and methods

NPA samples for mRNA analysis. NPA samples were collected from RSV bronchiolitis patients within 24 h after being

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admitted in the hospital (n=104) or from healthy controls (n=40) in Wuhan Children's Hospital (Wuhan, China) from December 2015 to May 2016. The exclusion criteria were as previously described (15), including corticosteroid use in the past 48 h, significant congenital heart or lung disease and immunodeficiency, or the presence of one of 15 different viral pathogens. The clinical characteristics are presented in Table I. NPAs were collected from the nostrils by deep nasal suctioning. RSV infection was detected using a rapid antigen test (BinaxNOW RSV Card; Alere, Inc., Waltham, MA, USA) and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) was performed in the hospital. The respiratory secretions were subsequently removed from the NPAs. Nasal epithelial cells were collected from each nostril by rotating a cytology brush (Medscand Medical Cytobrush Plus; CooperSurgical, Inc., Trumbull, CT, USA) over the anterior nasal mucosa. Immediately afterwards, the brushes were immersed in RNA stabilization reagent RNAlater (Sigma-Aldrich; Merck Millipore, Darmstadt, Germany) and epithelial cells were detached from the brushes and stored at -80°C in RNAlater for subsequent mRNA analysis.

Cell culture. BEAS-2B human bronchial epithelial cells were purchased from The American Type Culture Collection (Manassas, VA, USA) and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) horse serum (GE Healthcare, Logan, UT, USA), 100 U/ml penicillin (Invitrogen; Thermo Fisher Scientific, Inc.), and 0.1 mg/ml streptomycin (GE Healthcare) at 37°C in a humidified atmosphere with 5% CO₂.

RSV propagation and titer determination. Viral stock of human RSV A2-strain was purchased from the Viral Laboratory at Beijing Children's Hospital (Capital University of Medical Sciences, Beijing, China). RSV and ultraviolet light-inactivated RSV was prepared as previously described (16). Multiplicity of infection was 10 in the subsequent experiments.

RNA extraction. Whole blood samples (5 ml) were collected in tubes containing EDTA. The total RNA from the blood samples or the epithelial cells was isolated using RNeasy LS (Vigorous Biotechnology, Beijing, China) in accordance with the manufacturer's protocol. The concentration and the purity of the RNA samples were determined at 260-280 nm.

RT-qPCR. For synthesis of cDNA of the specific mRNA, 1 µg of the total RNA was reverse transcribed using the PrimeScript RT reagent Kit (Takara Biotechnology Co., Ltd., Dalian, China) with specific primers. To quantify the relative mRNA expression levels, a qPCR assay was performed using SYBR Green supermix (Bio-Rad Laboratories, Inc., Hercules, CA, USA) on a Bio-Rad iCycleriQ Real-Time PCR detection system. The PCR amplifications were performed in a 10 µl total reaction volume, containing 5 µl SYBR-Green supermix, 0.4 µl forward primer, 0.4 µl reverse primer, 2.2 µl ddH₂O and 2 µl template cDNA (1 µg). Initial denaturation occurred at 95°C for 10 min, followed by 40 cycles at 95°C for 15 sec and 60°C for 1 min. The relative expression level of mRNA was determined using the 2^{-ΔΔC_q} method (17). GAPDH was chosen as the internal control. The primers used in the

Table I. Clinical characteristics of control and RSV-positive patients included in the quantitative polymerase chain reaction analysis.

Characteristic	Control	RSV patients
Number of participants	40	104
Age (years), median (IQR)	6.5±4.1	6.1±3.8
Male gender, n (%)	22 (55%)	52 (50%)
Weight (g) mean (SD)	5,873 (1,647)	6,998 (2,350)
Duration of symptoms (days), median (IQR)	NA	4.6 (3-6)
Admission, n (%)	NA	84 (80.8%)
Length of stay, median (IQR)	NA	0 (0-0)
Length of stay >3 days, n (%)	NA	0 (0%)

RSV, respiratory syncytial virus; IQR, inter-quartile range; SD, standard deviation; NA, not applicable.

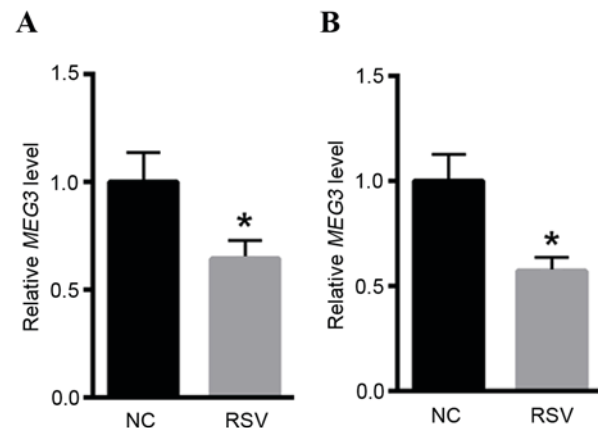


Figure 1. Decreased long noncoding RNA MEG3 level after RSV infection. (A) Expression level of MEG3 was markedly decreased in the nasal mucosal samples of RSV-infected patients compared with the healthy controls. (B) Expression level of lncRNA MEG3 was reduced in RSV-infected BEAS-2B cells. *P<0.05 vs. control. NC, healthy control; RSV, respiratory syncytial virus; MEG3, maternally expressed gene 3.

present study were listed as follows: MEG3 forward, 5'-CCA CCCCTCTTGCTTGTCTT-3' and reverse, 5'-CTTTGTGAG CGTGCTTCCAC-3'; TNF-α forward, 5'-CTGGGCAGGTCT ACTTTGGG-3' and reverse, 5'-CTGGAGGCCCCAGTTTGA AT-3'; IL-8 forward, 5'-GCCACCATCTTACCTCACAGT-3' and reverse, 5'-CACAGCACTACCAACACAGC-3'; TLR4 forward, ATCCCTCCCCTGTACCCTTC and reverse TCA AGGAGCATTGCCCAACA; GAPDH forward, TCCTCC GGGTGATGCTTTTC and reverse, TTCCCGTTCTCAGCC TTGAC.

Construction of adenovirus associated virus (AAV)-MEG3. The present study used AAV subtype 9. AAV-MEG3 was constructed by GeneChem Co., Ltd., (Shanghai, China) as previously described (18). The single-stranded AAV vectors were generated by cloning the green fluorescent protein or human MEG3 coding sequences under control of the ubiquitous CAG hybrid

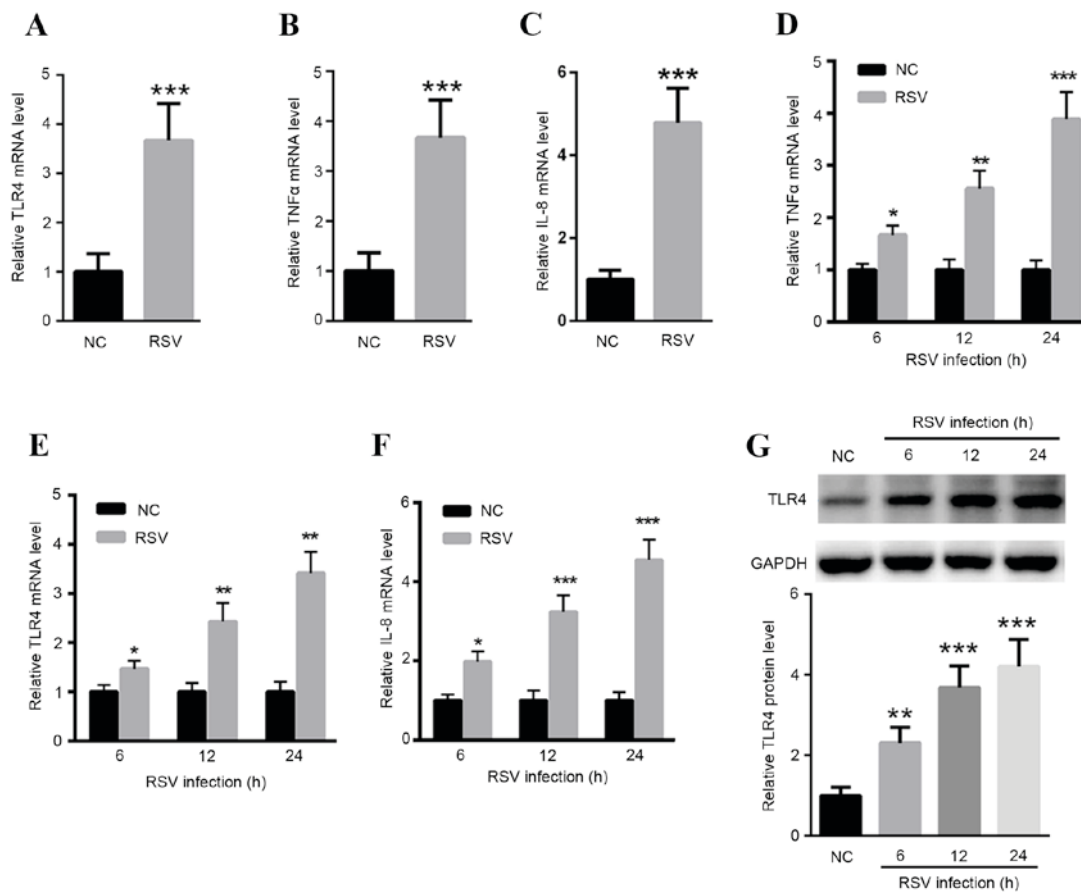


Figure 2. RSV infection increased mRNA expression levels of TLR4, TNF α and IL-8. The mRNA expression levels of (A) TLR4, (B) TNF- α and (C) IL-8 in RSV infected samples from patients were detected. RSV infection enhanced the mRNA levels of (D) TLR4, (E) TNF α and (F) IL-8 as assessed by reverse transcription-quantitative polymerase chain reaction analysis in BEAS-2B cells at different time points. (G) Western blot analysis indicated that the protein levels of TLR4 were significantly increased following RSV infection for 6, 12 and 24 h. *P<0.05, **P<0.01 and ***P<0.001 vs. control. NC, healthy control; RSV, respiratory syncytial virus; TLR4, toll-like receptor 4; TNF α , tumor necrosis factor- α ; IL-8, interleukin-8.

promoter (CMV enhancer, chicken β -actin promoter) into AAV backbone plasmids. AAV8 were produced by triple transfection of 293 cells and purified by an optimized CsCl-based gradient method that renders high purity vectors preps as previously described (19). Vector titers were verified using qPCR.

Transient transfection. Cells were seeded at a density of 6×10^5 cells/well in 6-well plates with 2 ml DMEM culture medium containing 10% (v/v) horse serum and antibiotics as aforementioned. A small interfering RNA (siRNA) targeting TLR4 (CCAGGUGCAUUUAAA-GAAATT) or a negative control (UUCUCCGAACGUGUCACGUTT) were mixed with HiperFect transfection reagent (Qiagen GmbH, Hilden, Germany) and incubated at room temperature for 10 min. Subsequently, each complex was transfected into BEAS-2B cells and the cells were collected for other experiments 48 h post-transfection.

Western blotting. Cell protein was extracted using radioimmunoprecipitation lysis buffer (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) and was collected following centrifugation at 12,000 \times g. A bicinchoninic protein assay kit (Pierce; Thermo Fisher Scientific, Inc.) was used to determine the protein concentration. A total of 15 μ g protein was loaded per lane, separated by 10% SDS-PAGE

and transferred to polyvinylidene difluoride membranes. The membranes were blocked with 8% non-fat dry milk at 4°C overnight. Following three washes with PBS with Tween 20 (5 min/wash), the membranes were incubated with the following primary antibodies at 4°C overnight: TLR4 (cat. no. 14358; 1:1,000; Cell Signaling Technology, Inc., Danvers, MA, USA), NF- κ B (cat. no. 8801; 1:1,000; Cell Signaling Technology, Inc.), phosphorylated (p)-NF- κ B (cat. no. 3033; 1:1,000; Cell Signaling Technology, Inc.), p38MAPK (cat. no. 8690; 1:1,000; Cell Signaling Technology, Inc.), p-p38MAPK (cat. no. 4511; 1:1,000; Cell Signaling Technology, Inc.), GAPDH (cat. no. 5174; 1:1,000; Cell Signaling Technology, Inc.) were used. Following several washes with TBST, blots were incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit and anti-mouse IgG secondary antibodies (cat no. TA130023 and TA140002; 1:5,000; OriGene Technologies, Inc., Beijing, China) for 2 h at room temperature. Blots were washed again and bands were subsequently visualized with enhanced chemiluminescent substrate (EMD Millipore, Billerica, MA, USA). GAPDH was used as an internal control. ImageJ software (version 1.43b; National Institutes of Health, Bethesda, MD, USA) was used for densitometry.

Statistical analysis. Data are presented as the mean \pm standard deviation. Two-tailed unpaired Student's t-test were used for

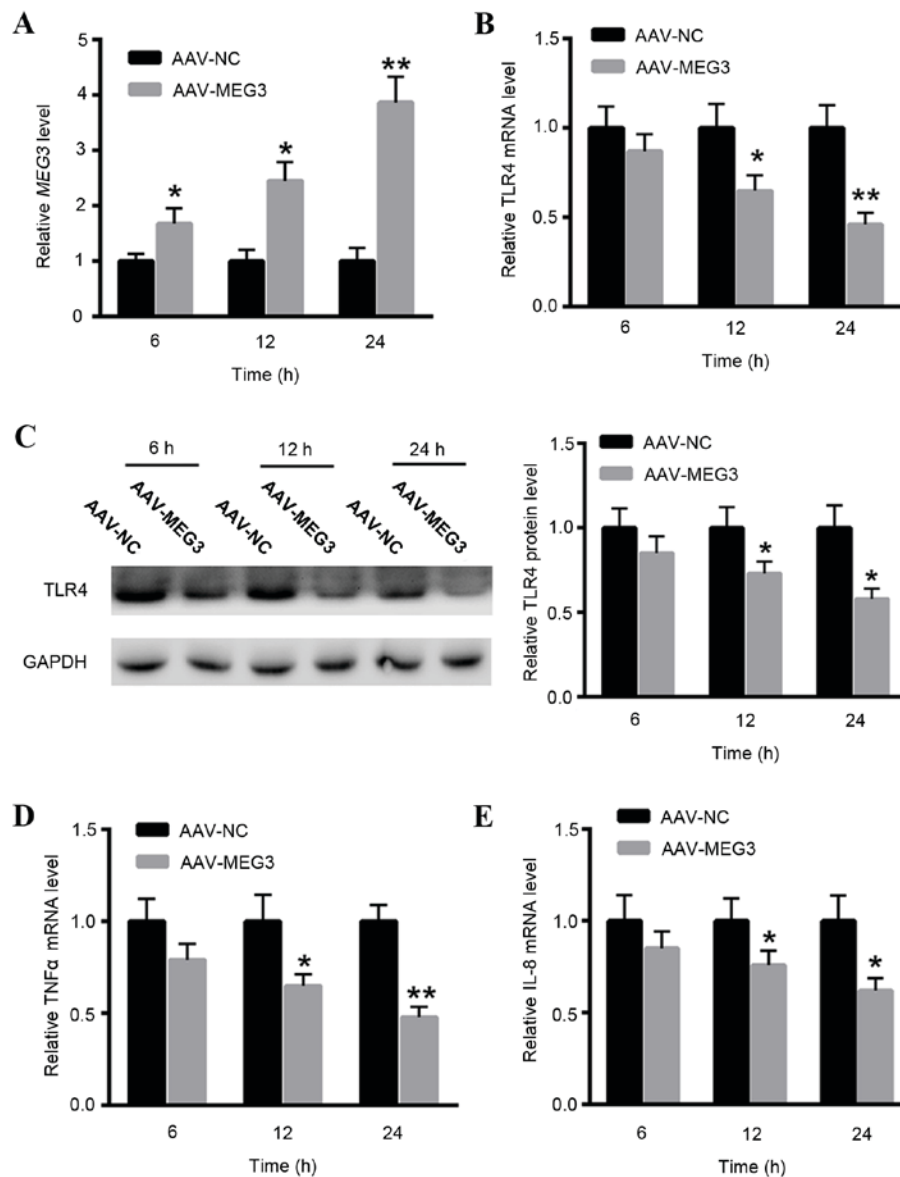


Figure 3. LncRNA MEG3 suppressed the mRNA expression level of TLR4, TNF α and IL-8. (A) qPCR analysis indicated that transfection with AAV-MEG3 significantly increased the level of lncRNA MEG3 at 6, 12, 24 h in BEAS-2B cells. Upregulation of lncRNA MEG3 was able to reduce RSV infection-induced TLR4 upregulation at 6, 12, 24 h, as indicated by (B) qPCR and (C) western blot analysis. RSV-induced upregulation of (D) TNF α and (E) IL-8 was also reduced by ectopic expression of lncRNA MEG3. *P<0.05, **P<0.01 vs. control. AAV, adenovirus-associated virus; NC, healthy control; RSV, respiratory syncytial virus; MEG3, maternally expressed gene 3; TLR4, toll-like receptor 4; TNF α , tumor necrosis factor- α ; IL-8, interleukin-8; lncRNA, long noncoding RNA; qPCR, quantitative polymerase chain reaction.

comparisons of two groups. Analysis of variance multiple comparisons test followed by Tukey's post-hoc test was used for comparisons of more than two groups. All statistical analysis was performed using SPSS version 13.0 (SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

lncRNA MEG3 expression level is reduced following RSV infection. The expression level of MEG3 was markedly reduced in the NPAs of RSV-infected patients (Fig. 1A) when compared with healthy controls. BEAS-2B cells were infected with RSV for 24 h. Subsequently, RNA was extracted from BEAS-2B cells, compared with normal controls, the expression level of

lncRNA MEG3 was reduced in RSV-infected BEAS-2B cells (Fig. 1B). These data revealed the involvement of lncRNA MEG3 in the progression of RSV infection-associated disease.

RSV infection enhances TLR4, TNF α and IL-8 mRNA expression levels. The mRNA expression levels of TLR4, TNF α and IL-8 in RSV infected samples from patients were quantified. As presented in Fig. 2A-C, the expression levels of TLR4, TNF α and IL-8 were significantly increased in patients with RSV compared with healthy controls. Subsequently the TLR4 mRNA expression level in BEAS-2B cells transfected with RSV was determined. RT-qPCR revealed that the expression level of TLR4 was increased by ~1.67, ~2.56 and ~3.89-fold in RSV-infected cells at 6, 12 and 24 h, respectively (Fig. 2D). The mRNA expression levels of inflammatory factors,

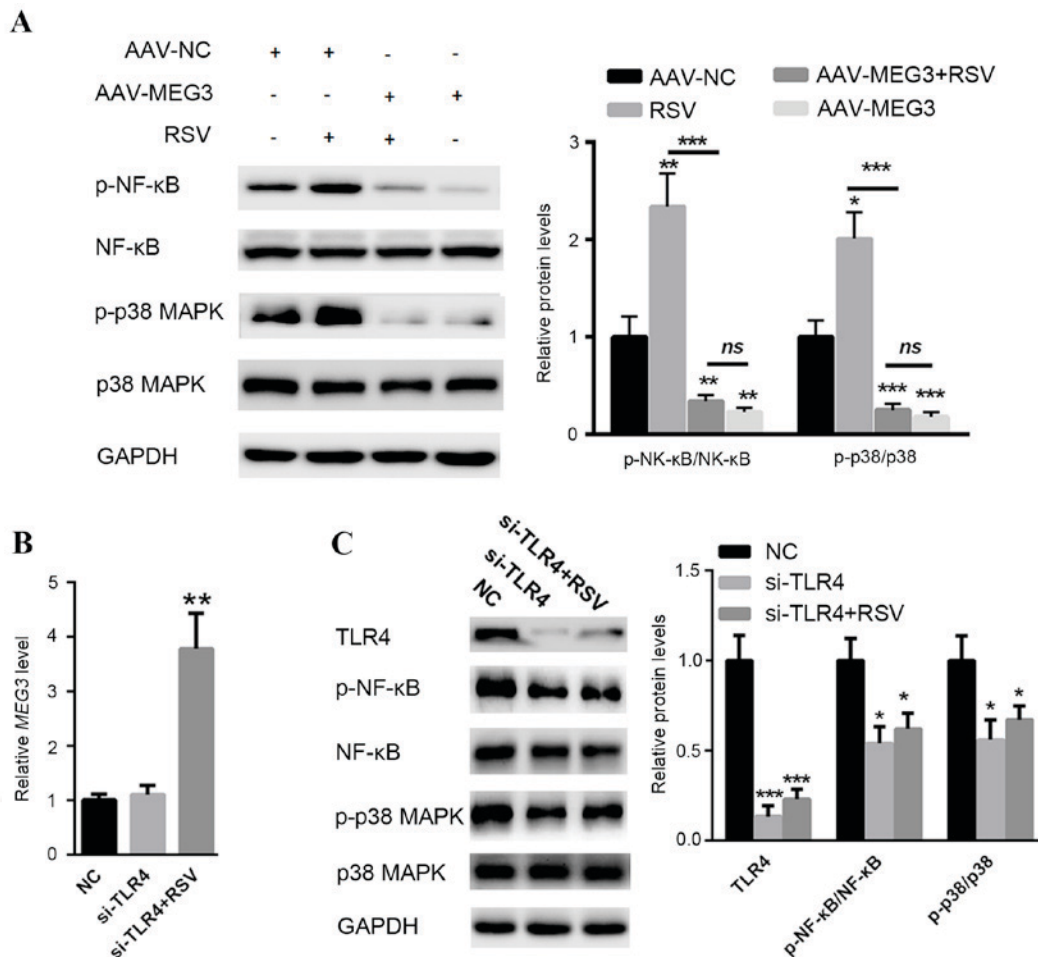


Figure 4. (A) Long noncoding RNA MEG3 reduced the activation of NF- κ B and p38 MAPK signaling. (B) Silencing of TLR4 did not affect the level of MEG3 in samples with or without RSV infection. (C) Western blot assay revealed that RSV infection-induced activation of p38 MAPK and NF- κ B signaling may be partially abolished by knockdown of TLR4. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ vs. control. AAV, adenovirus-associated virus; NC, healthy control; RSV, respiratory syncytial virus; MEG3, maternally expressed gene 3; p-, phosphorylated; NF- κ B, nuclear factor- κ B; p38 MAPK, p38 mitogen-activated protein kinase; TLR4, toll-like receptor 4; si, small interfering; lncRNA, long noncoding RNA.

including TNF α and IL-8 were quantified. As presented in Fig. 2E and F, infection with RSV significantly increased TNF α and IL-8 mRNA levels of compared with the control. Additionally, the present study also investigated the protein expression of TLR4 following infection of RSV. Western blot analysis indicated that the TLR4 protein levels were significantly increased following RSV infection for 6, 12 and 24 h (Fig. 2G).

LncRNA MEG3 suppresses TLR4, TNF α and IL-8 mRNA expression levels. In order to investigate the role of lncRNA MEG3 in RSV infection-induced inflammatory responses, the present study overexpressed lncRNA MEG3 in RSV-infected BEAS-2B cells. RT-qPCR analysis indicated that transfection with AAV-MEG3 significantly increased the level of lncRNA MEG3 at 6, 12 and 24 h in BEAS-2B cells (Fig. 3A). It is of note that an upregulation of lncRNA MEG3 was able to partially reduce RSV infection-induced TLR4 upregulation at 6, 12 and 24 h, as indicated by RT-qPCR and western blot analysis (Fig. 3B and C). Additionally, RSV-induced upregulation of TNF α and IL-8 may also be reduced by ectopic expression of lncRNA MEG3 (Fig. 3D and E), indicating the protective role of lncRNA MEG3 in RSV infection.

LncRNA MEG3 reduces the activation of NF- κ B and p38 mitogen-activated protein kinase (MAPK) signaling. Previous studies have indicated the key role of NF- κ B and p38 MAPK signaling in the activation of virus internalization (20,21); therefore, the present study investigated the effect of lncRNA MEG3 on the NF- κ B and p38 MAPK pathways. Western blot analysis revealed that RSV infection significantly increased the activation of NF- κ B and p38 MAPK signaling (Fig. 4A). Conversely, overexpression of lncRNA MEG3 may significantly suppress RSV infection-induced NF- κ B and p38 MAPK activation (Fig. 4A). In order to verify whether MEG3 protected human airway epithelial cells from RSV infection primarily by suppressing TLR4-dependent p38 MAPK and NF- κ B signaling, the present study silenced TLR4 in BEAS-2B cells. As presented in Fig. 4B, silencing of TLR4 did not affect the expression level of MEG3 in cells transfected with or without RSV. Western blotting revealed that knockdown of TLR4 suppressed p38 MAPK and NF- κ B signaling (Fig. 4C). However, RSV infection-induced activation of p38 MAPK and NF- κ B signaling may be partially reduced by knockdown of TLR4 (Fig. 4C). These findings confirmed that TLR4 has a key role in MEG3-mediated NF- κ B and p38 MAPK activation by RSV infection.

Discussion

RSV is frequently identified in the environment and it may lead to serious illness through its interaction with respiratory epithelial cells (22). There are 11 genes and encoded proteins involved in the infection process of RSV (23). The fusion protein is one of the most important viral attachment protein, primarily exerting its function through TLR4 signaling (23,24). It has been previously suggested that in the early RSV response, TLR4 signaling is an important component, enhancing the inflammatory responses in sequelae to RSV infection phenotype (25).

A previous study revealed that RSV infection enhances the airway epithelial cell receptors, which may enhance the binding of inhaled environmental agents (26). The current study revealed that the level of TLR4 was increased by ~1.67-, ~2.56- and ~3.89-fold in RSV infected cells at 6, 12 and 24 h, respectively. Additionally, the inflammatory factors, such as TNF α and IL-8, were markedly increased following RSV infection compared with the controls. These findings indicate that increased TLR4 expression may lead to the activation of inflammatory cytokine production in human airway epithelial cells.

Genome-wide surveys have previously demonstrated that lncRNAs are ubiquitously involved in diverse biological processes, including cell cycle control and cell differentiation through distinct mechanisms (27,28). Various lncRNAs have been previously identified in cancer biology (29,30). However, the involvement of lncRNAs in RSV infection remains to be determined. The lncRNA MEG3 expression has been reported to be significantly reduced in primary bladder tumors, which increased cancer cell metastasis (31). To highlight the impact of MEG3 in RSV infection, the present study investigated the expression level of lncRNA MEG3 in the NPA samples of RSV-positive patients. The expression level of MEG3 was markedly reduced in the NPAs of RSV-infected patients compared with the healthy controls. Furthermore, the expression level of lncRNA MEG3 was reduced in RSV-infected BEAS-2B cells. These findings suggest that reduced MEG3 expression level may promote RSV infection in BEAS-2B cells.

It is of note that the current findings revealed that lncRNA MEG3 suppressed the mRNA expression level of TLR4, TNF α and IL-8, indicating a protective role of lncRNA MEG3 in the progression of RSV-associated disease. TLR4, a signaling receptor for structurally diverse microbe-associated molecular patterns, has been previously revealed to be activated by the RSV fusion protein (32). Furthermore, TLR4-mediated activation of MAPK and NF- κ B signaling has been extensively reported in multiple previous studies (33,34). For example, Ugonin M, a *Helminthostachys zeylanica* constituent, has been found to prevent LPS-induced acute lung injury by suppressing the TLR4-mediated MAPK and NF- κ B signaling pathways (33). The p38 MAPK signaling activated by pattern recognition receptors has been previously reported as part of the antiviral immune response (35). It has been previously reported that RSV infection activates cellular MAPKs, which enhances host-cell targeting and virus-cell fusion (20). In addition, TLR4-mediated inflammatory response may be, in part, dependent on NF- κ B activation (25). Additionally, TLR4 signaling has been

previously identified to induce functional nerve growth factor receptor p75NTR in mouse dendritic cells through p38 MAPK and NF- κ B pathways (36). The present study validated that infection with RSV significantly increased the activation of p38 MAPK and NF- κ B signaling which was in agreement with the previous studies. By contrast, ectopic expression of MEG3 by transfection partially blocked p38 MAPK and NF- κ B activation. These findings suggest the involvement of MEG3 in p38 MAPK and NF- κ B signaling to suppress RSV infection.

In conclusion, to the best of our knowledge, the present study for the first time, identified reduced lncRNA MEG3 expression levels in patients with RSV infection. The current study also demonstrated that MEG3 protected human airway epithelial cells from RSV infection primarily by suppressing TLR4-dependent p38 MAPK and NF- κ B signaling.

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