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Respiratory syncytial virus persistent infection causes acquired CFTR dysfunction in human bronchial epithelial cells

LONG Chunjiao[1, 2], QI Mingming[3], WANG Jinmei[1], LUO Jinhua[1], QIN Xiaoqun[1], GAO Ge[4, 5], XIANG Yang[1]

(1. Department of Physiology, School of Basic Medical Science, Central South University, Changsha 410013; 2. Department of Nephrology Medicine, Third Xiangya Hospital, Central South University, Changsha 410013; 3. Department of Obstetrics, Zhuzhou Central Hospital/Zhuzhou Hospital Affiliated to Xiangya School of Medicine, Central South University, Zhuzhou Hunan 412007; 4. Department of Laboratory Medicine, Third Xiangya Hospital, Central South University, Changsha 410013; 5. Department of Medical Laboratory Science, Xiangya School of Medicine, Central South University, Changsha 410013, China)

ABSTRACT

Objective: Many studies have shown that respiratory syncytial virus persistent infection may be the main cause of chronic respiratory pathology. However, the mechanism is unclear. Cystic fibrosis transmembrane conduction regulator (CFTR) is an apical membrane chloride channel, which is very important for the regulation of epithelial fluid, chloride ion, and bicarbonate transport. CFTR dysfunction will lead to changes in bronchial secretions and impair mucus clearance, which is related to airway inflammation. In our previous study, we observed the down-regulation of CFTR in airway epithelial cells in respiratory syncytial virus (RSV) infected mouse model. In this study, we further investigated the expression and function of CFTR by constructing an airway epithelial cell model of RSV persistent infection.

Methods: 16HBE14o- cells were infected with RSV at 0.01 multiplicity of infection (MOI). The expression of CFTR was detected by real-time RT-PCR, immunofluorescence, and Western blotting. The intracellular chloride concentration was measured by N-(ethoxycarbonylmethyl)-6-methoxyquinolium bromide (MQAE) and the chloride current was measured by whole-cell patch clamp recording.

Results: 16HBE14o- cells infected with RSV were survived to successive passages of the third generation (G3), while the expression and function of CFTR was progressively decreased upon RSV infection from the first generation (G1) to G3. Exposure of 16HBE14o- cells to RSV led to the gradual increase of TGF-β1 as well as phosphorylation of Smad2

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First author: LONG Chunjiao, Email: longchunjiao2511@sina.com, ORCID: 0000-0001-8337-0370

Corresponding author: XIANG Yang, Email: xiangyang@csu.edu.cn, ORCID: 0000-0002-7865-2565; GAO Ge, Email: gaoge96@csu.edu.cn, ORCID: 0000-0002-1224-1413

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following progressive RSV infection. Disruption of TGF-β1 signaling by SB431542 prevented Smad2 phosphorylation and rescued the expression of CFTR.

Conclusion: RSV infection can lead to defective CFTR function in airway epithelial cells, which may be mediated via activation of TGF-β1 signaling pathway.

KEY WORDS respiratory syncytial virus; cystic fibrosis transmembrane conductance regulator; human bronchial epithelial cells; transforming growth factor-β1

呼吸道合胞病毒持续感染导致人支气管上皮细胞囊性纤维化穿膜传导调节蛋白功能障碍

龙春姣[1,2]，柒铭铭[3]，王金美[1]，罗金华[1]，秦晓群[1]，高戈[4,5]，向阳[1]

（1. 中南大学基础医学院生理学系，长沙 410013；2. 中南大学湘雅三医院肾内科，长沙 410013；3. 株洲市中心医院/中南大学湘雅医学院附属株洲医院产科，湖南 株洲 412007；4. 中南大学湘雅三医院医学实验中心，长沙 410013；5. 中南大学湘雅医学院医学检验系，长沙 410013）

[摘 要] 目的：呼吸道合胞病毒(respiratory syncytial virus，RSV)持续感染可能是导致气道慢性疾病的病理原因，然而，其作用机制尚不清楚。囊性纤维化穿膜传导调节蛋白(cystic fibrosis transmembrane conductance regulator，CFTR)是一种顶端膜氯离子(Cl^-)通道，对上皮液体、Cl^-和碳酸氢盐运输的调节至关重要。CFTR功能障碍将导致支气管分泌物改变和黏液清除受损，与气道炎症有关。我们前期在RSV感染的小鼠模型中观察到气道上皮细胞CFTR表达下调。本研究通过构建RSV持续感染的气道上皮细胞模型，进一步研究其对CFTR表达和功能的影响。**方法：**以病毒感染复数(multiplicity of infection，MOI)为0.01的RSV感染16HBE14o-细胞后继续传代。以real-time RT-PCR、免疫荧光及蛋白质印迹法检测CFTR的表达，以新型氯离子荧光探针MQAE测量细胞内Cl^-浓度和全细胞膜片钳记录Cl^-电流。**结果：**16HBE14o-细胞感染RSV病毒后存活至第3代(G3)。CFTR的表达在RSV感染后从G1到G3逐渐降低，同时伴随着CFTR功能的降低。16HBE14o-细胞暴露于RSV可导致TGF-β1表达进行性升高及其下游信号分子Smad2磷酸化。用SB431542阻断TGF-β1信号通路可阻止Smad2磷酸化，增加CFTR的表达。**结论：**RSV感染可导致气道上皮细胞CFTR功能缺陷，其作用可能是通过激活TGF-β1信号通路介导的。

[关键词] 呼吸道合胞病毒；囊性纤维化穿膜传导调节蛋白；人支气管上皮细胞；转化生长因子β1

Respiratory syncytial virus (RSV) often causes lower airway infection in infants. In addition to acute infection, the persistence of RSV is considered as a potential cause of long-term complications[1]. It is associated with an increased risk of airway hyperresponsiveness and asthma for years[2-3]. Although the mechanism remains unclear, it is speculated that persistent low-level RSV infection might alter the mechanism of airway epithelial stress response, leading to impaired lung function[4].

Airway epithelium is the first barrier between the external environment and the lung, which is indispensable in host defense[5]. Cystic fibrosis transmembrane conductance regulatory (CFTR) is a kind of chloride channel located in the apical membrane of cells and participates in the transport of liquids, chloridion (Cl^-), and bicarbonates in epithelial cells[6]. The dysfunction of CFTR leads to an alteration in the bronchial secretions, with impaired mucus clearance, which is associated with airway inflammation and obstruction[7].

Emerging evidence demonstrates that the absence of CFTR expression leaves human airway cells vulnerable to epithelial injury by RSV[8]. CFTR gene-deficient mice are more susceptible to RSV[9]. However, the effect of RSV infection on CFTR function has not been investigated. In a preliminary experiment, we observed the decreased expression of CFTR in bronchial epithelium of an RSV infected mouse model. In this study, we are interested in whether the persistent RSV

infection of airway epithelial cells affects the expression and function of CFTR, which may lead to infection, inflammation, and progressive decreased lung function.

1 Materials and methods

1.1 RSV infected mouse model

BALB/c mice (6−8 weeks, male, weighing 15−20 g) were held under specific pathogen-free conditions in the Laboratory Animal Centre of Central South University. Mice were infected intranasally (50 μL/animal) with 1.0×10^5 plaque forming unit (PFU) of RSV A2. Analysis of the viral response was performed on the 7th day after infection. Animal experiments were approved by the Ethics Committee of Xiangya Hospital of Central South University (201803246) and carried out in accordance with the National Institute of Health (NIH, USA) guide for the care and use of laboratory animals.

1.2 16HBE14o- cell culture and RSV infection

Persistent RSV infected human bronchial epithelial (16HBE14o-) cells were established as described previously[1]. Briefly, 16HBE14o- cells[10] were cultured in DMEM∶F12 containing 10% FBS at 37 ℃. Cells were infected with RSV at a multiplicity of infection (MOI) of 0.01 in 70%−80% confluent. After incubated for 2 h at 37 ℃ in serum-free DMEM, non-absorbed virus was removed. Cells were cultured in fresh medium and passaged twice a week. During passages, the expression of non-structural (NS) protein NS1 was detected by RT-PCR to verify RSV persistence. Cells were successively passaged from the first generation (G1) to G3.

1.3 Histology, immunochemistry, and immuno-fluorescence

Mice were sacrificed with an intra-peritoneally injection of sodium pentobarbitone (100 mg/kg), and lung tissues were obtained. The lower part of the right lung was embedded in paraffin. Tissue structure and inflammatory reaction were observed by hematoxylin-eosin staining. Immunohistochemistry (IHC) analysis was performed by employing anti-CFTR (SAB40734, Sabbiotech, China).

For immunofluorescence analysis, cells with or without RSV infection were incubated with primary antibodies to CFTR (sc-376683, Santa Cruz Biotech, American) at 4 ℃ overnight, followed by incubation with FITC-labeled secondary antibody at room temperature for 1 h. Images were taken on an Olympus IX170 inverted fluorescence microscope at 400× magnification.

1.4 Detection of chloride concentration in 16HBE14o- cells

A Cl^- fluorescence probe, N-(ethoxycarbonylmethyl)-6-methoxyquinolium bromide (MQAE; ab145418, Abcam, England), was used to detected intracellular chloride concentration ($[Cl^-]_i$). It detects ion levels through a diffusion-limited collision quenching method. When $[Cl^-]_i$ increases, its fluorescence intensity decreases proportionally with the increase of $[Cl^-]_i$[11]. Briefly, 16HBE14o- cells were incubated with 5 mmol/L MQAE. After 1 h of incubation in the dark at 37 ℃, unbound probe was removed. Fluorescence images were taken by a laser scanning confocal microscope (Zeiss LSM710, Germany) at an excitation wavelength of 350 nm.

1.5 Electrophysiological experiments on Cl^- channel regulated by CFTR in RSV infected 16HBE14o- cells

Whole cell recordings with a List EPC-10 Patch-Clamp Amplifier were performed according to procedures previously described[12]. Forskolin (5 μmol/L) was used to stimulated CFTR Cl^- current. RSV infected G1 cells and control cells were clamped at −40 mV and pulsed from −80 to +80 mV by 20 mV increments. The maximum current value under each voltage state was taken to draw the current-voltage (*I-V*) relation curve.

1.6 ELISA

The contents of transforming growth factor-β1 (TGF-β1) in the culture supernatants of RSV infected 16HBE14o- cells were quantified by ELISA (Sigma, St. Louis, MO, USA) according to the manufacturers' instructions.

1.7 Real-time RT-PCR

Total RNA was extracted from lung tissues or 16HBE14o- cells. Total RNA (1.0 μg) was used to synthesize cDNA employing the PrimeScript™ RT master mix kit (Takara, Japan). Real-time RT-PCR analyses were conducted using SYBR® Premix Ex Taq™ II system (Takara, Japan) on a deep well Real-Time PCR Detection System (CFX96 Touch™; BioRad, CA, USA). Primer sequences used in this study are shown in Table 1.

Table 1 Primers used for real-time PCR analysis

Gene	Sequence of primers
RSV NS1	Forward 5'-TAGCCAAAGCAGCAATAC-3'
	Reverse 5'-CAAGCCCAAGTAAATCAG-3'
mouse HPRT	Forward 5'-AGGCCAGACTTTGTTGGATTTGAA-3'
	Reverse 5'-CAACTTGCGCTCATCTTAGGCTTT-3'
mouse TLR4	Forward 5'-AGCTTCTCCAATTTTTCAGAACTTC-3'
	Reverse 5'-TGAGAGGTGGTGTAAGCCATGC-3'
mouse IL-6	Forward 5'-GACTGGGGATGTCTGTAGCTC-3'
	Reverse 5'-TGAAGTAGGGAAGGCCGTG-3'
mouse TGF-β1	Forward 5'-TCAGACATTCGGGAAGCAGT-3'
	Reverse 5'-TCGAAAGCCCTGTATTCCGT-3'
mouse CFTR	Forward 5'-TCCCCACTGCCGTACAGATA-3'
	Reverse 5'-CTGTTTGGGTTTGCTCCACG-3'
human TLR4	Forward 5'-CCCTGAGGCATTTAGGCAGCTA-3'
	Reverse 5'-AGGTAGAGAGGTGGCTTAGGCT-3'
human CFTR	Forward 5'-GGAGTAGCCGACACTTTGCT-3'
	Reverse 5'-GCTGGAGTTTACAGCCCACT-3'
human GAPDH	Forward 5'-GAAGGTGAAGGTCGGAGTC-3'
	Reverse 5'-GAAGATGGTGATGGGATTTC-3'

1.8 Western blotting

Total protein in 16HBE14o- cells was extracted by RIPA containing protease inhibitor. Proteins was fractionized by 10% SDS-PAGE gels and transferred to a PVDF membrane. The membrane was blocked with 5% skim milk for 2 h at room temperature. Then, primary antibody was added and incubated at 4 ℃ overnight. The following day, membrane was incubated with secondary antibody at room temperature for 1 h. The expressions of CFTR (SAB40734, Sabbiotech, China), Smad2 (ab228765, Abcam, England), and phosphorylated Smad2 (ab188334, Abcam, England) were determined with corresponding antibodies. To block TGF-β1, 16HBE14o- cells were cultured with SB431542 (10 μmol/L) (Selleck Chemicals, USA) for 24 h.

1.9 Statistics analysis

Data were expressed as mean±standard deviation ($\bar{x}\pm s$). All experiments were independently repeated at least 3 times. Student's *t*-test was used for comparison between the 2 groups, and the analysis of variance (ANOVA) was used for the comparison between multiple groups. Statistically significant was considered when $P<0.05$.

2 Results

2.1 CFTR expression in airway epithelium decreased after RSV infection in vivo

To confirm the changes in CFTR protein expression induced by RSV infection, we constructed a RSV infected mouse model, which was verified by increased viral load (Figure 1A), increased infiltration of inflammatory cells in lungs (Figure 1B), and increased mRNA expression of TLR4, IL-6, and TGF-β1 (Figure 1C). We also observed the decreased expression of CFTR in RSV infected mouse (Figure 1C and 1D), especially on airway epithelial cells.

2.2 Dynamic changes of CFTR expression in 16HBE14o- cells with progressive RSV infection

RT-PCR result showed that RSV NS1 protein was progressively increased in 16HBE14o- cells during successive passages from G1 to G3 (Figure 2A). Correspondingly, the mRNA expression of TLR4 also increased from G1 to G3 in RSV-infected cells (Figure 2B). By using immunofluorescence and Western blotting, we revealed that after infection with RSV, the expression of CFTR protein gradually decreased from G1 to G3 (Figure 2C and 2D).

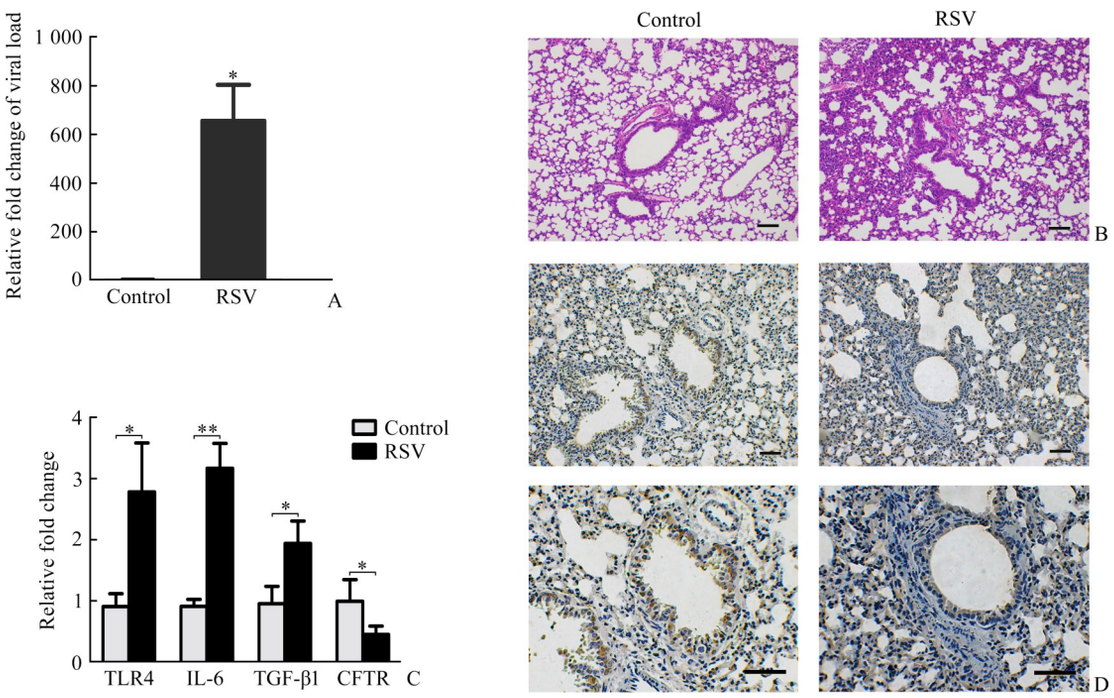

Figure 1 CFTR expression in airway epithelium decreased after RSV infection in vivo

A: RT-PCR validation of RSV NS1 protein in mouse lung, **P*<0.05 vs control; B: Representative images of HE staining of lung sections (scale bar=50 μm); C: RT-PCR validation of the expression of TLR4, IL-6, TGF-β1, and CFTR mRNA in mouse lung after RSV infection, **P*<0.05, ***P*<0.01 vs control; D: Representative images for immunohistochemical staining of CFTR in lung sections from mice exposed to RSV (scale bar=50 μm).

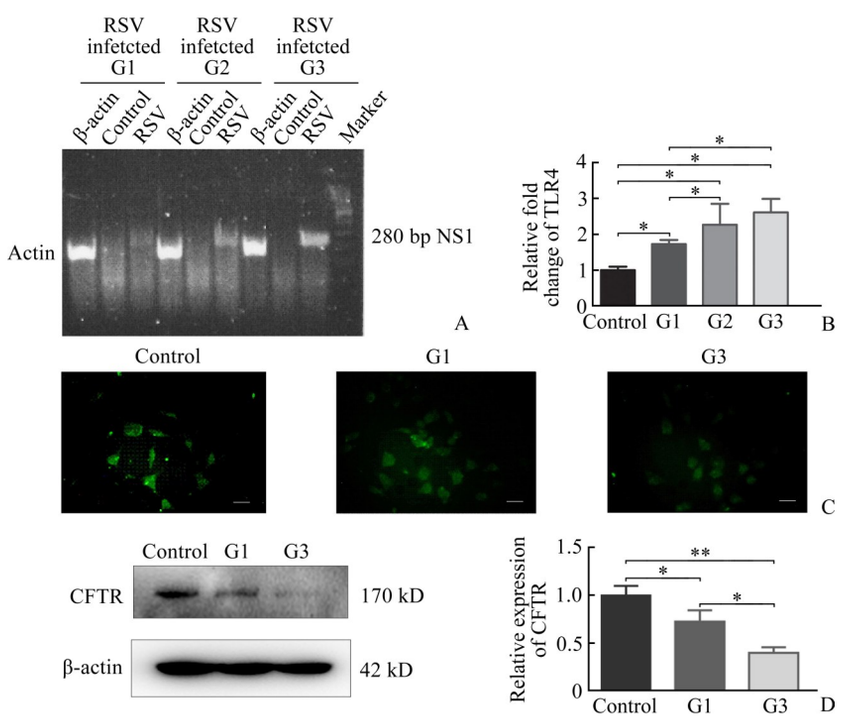

Figure 2 Dynamic changes of CFTR expression in 16HBE14o- cells with progressive RSV infection

A: RT-PCR amplification of RSV NS1 mRNA (280 bp) in different generations of 16HBE14o- cells infected by RSV; B: RT-PCR validation of the expression of TLR4 mRNA in different generations of 16HBE14o- cells infected by RSV (*n*=5, **P*<0.05); C: Immunofluorescence of CFTR in 16HBE14o- cells infected by RSV (scale bar=20 μm); D: Western blotting validation of the expression of CFTR protein in 16HBE14o- cells infected by RSV (*n*=3, **P*<0.05, ***P*<0.01).

2.3 $[Cl^-]_i$ in 16HBE14o- cells increased with progressive RSV infection

To clarify the functional significance of the decrease expression of CFTR, we detected intracellular $[Cl^-]_i$ in 16HBE14o- cells with MQAE fluorescence. The fluorescence intensity decreased proportionally with the increase of $[Cl^-]_i$. As a positive control, forskolin induced a significant decrease in $[Cl^-]_i$ (high intensity fluorescence), indicating a dramatically chloride efflux stimulated by forskolin. After infection with RSV, fluorescence intensity gradually decreased in G1 to G3 cells, indicating the increasing $[Cl^-]_i$ induced by progressive RSV infection (Figure 3A and 3B). These results suggested that the Cl^- channel function of CFTR is suppressed due to RSV infection.

2.4 Inhibition of CFTR currents by RSV infection

Cl^- currents in 16HBE14o- cells were completed by whole cell recordings in the presence or absence of RSV infection. Typical whole cell currents and associated *I-V* plots are presented in Figure 3C and 3D. Forskolin was used to stimulated CFTR Cl^- currents in 16HBE14o- cells. RSV infection caused a significant decrease of Cl^- currents. The Cl^- currents were inhibited by glibenclamide (CFTR Cl^- blockers), but not by 4,4'-diisothiocyanatostilbene-2,2'-disulphonic acid (DIDS) (non CFTR dependent Cl^- blocker), indicating they are functional CFTR currents.

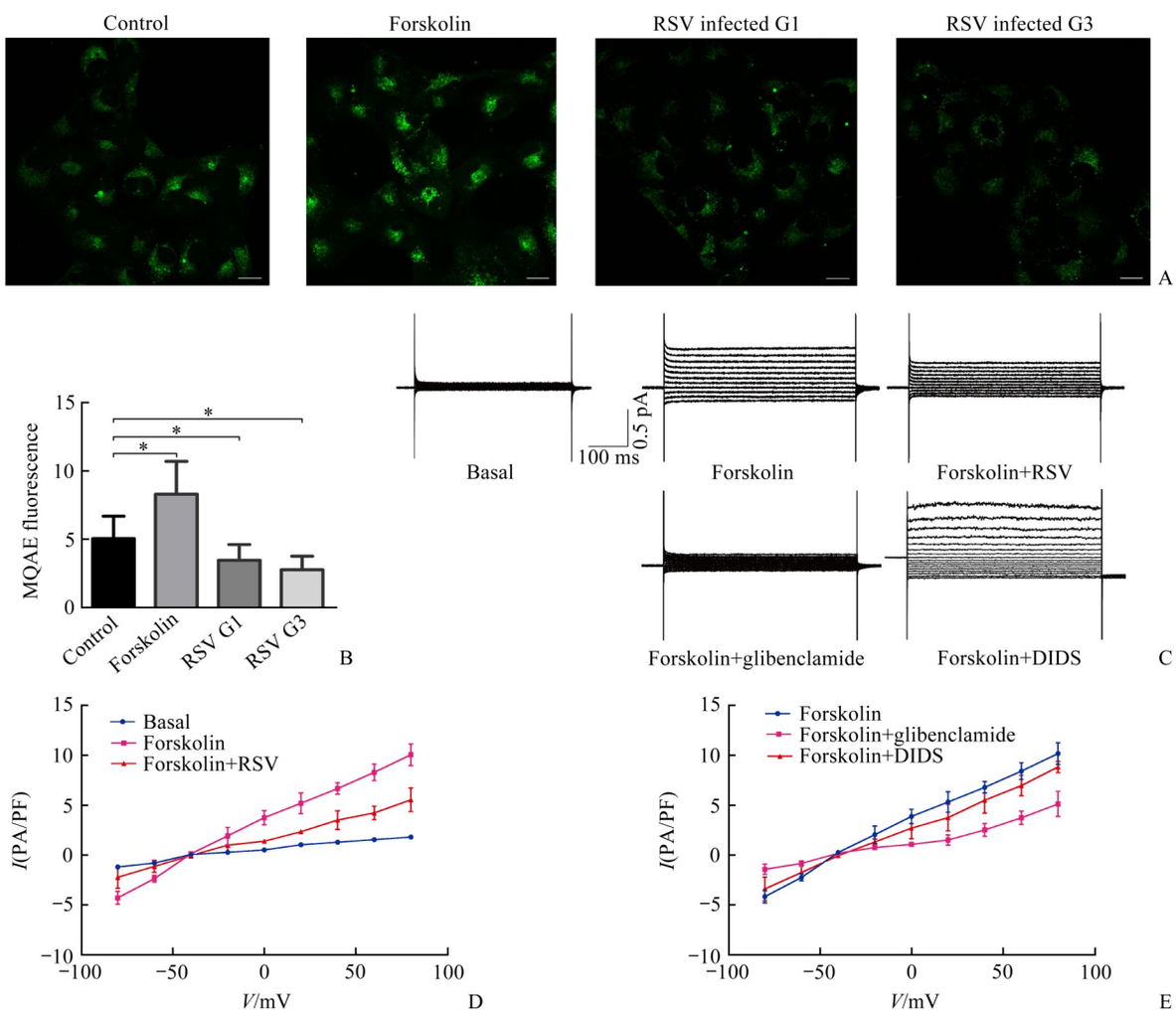

Figure 3 RSV infection decreased the function of CFTR in 16HBE14o- cells

A: Confocal fluorescent images of living 16HBE14o- cells stained with MQAE (scale bar=20 μm); B: Fluorescence intensity of MQAE in different groups of 16HBE14o- cells (n=100, *P<0.05); C: Current traces for CFTR in representative 16HBE14o- cells; D−E: *I-V* curves for CFTR current. Cells were held at −40 mV and cells pulsed from −80 to +80 mV by 20 mV increments (n=5).

2.5 RSV infection decreased CFTR expression through activation of TGF-β1 signaling

TGF-β1 mRNA expression in lung tissues of RSV-infected mice was significantly elevated (Figure 1C), RSV infected 16HBE14o- cells produced TGF-β1 (Figure 4A). Phosphorylation of Smad2 was also observed following progressive RSV infection (Figure 4B). Disruption of TGF-β1 signaling by SB431542 prevented Smad2 phosphorylation (Figure 4B) and rescued the expression of CFTR (Figure 4C and 4D).

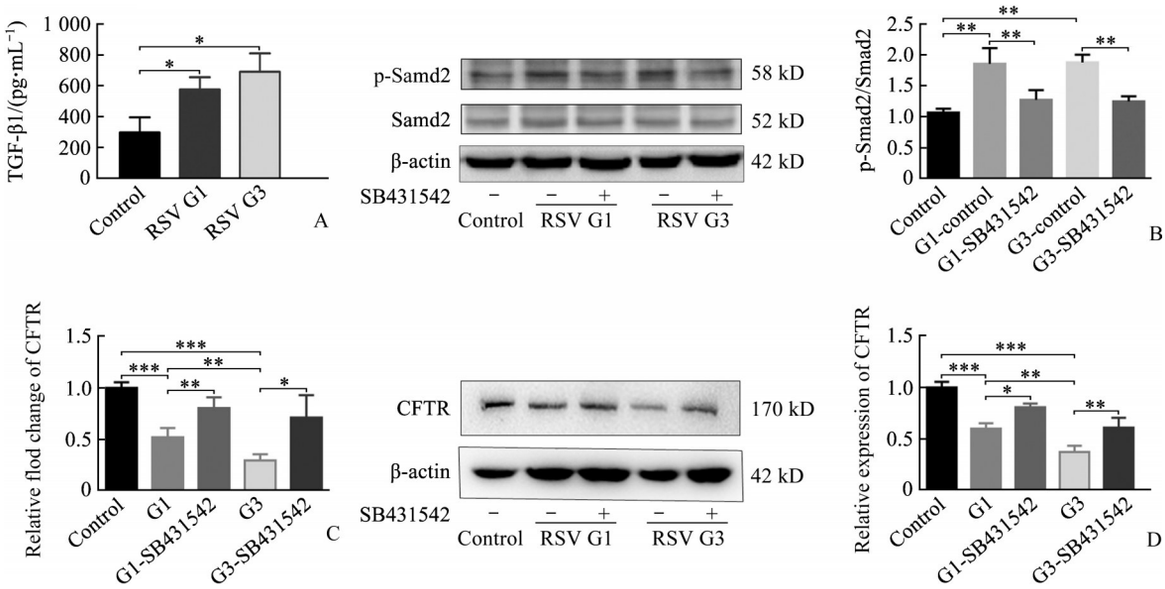

Figure 4 RSV infection decreased CFTR expression through activation of TGF-β1 signaling

A: ELISA validation of the contents of TGF-β1 in the supernatants of RSV infected 16HBE14o- cells (n=3, *P<0.05). B: Smad2 and p-Smad2 expression in RSV-infected 16HBE14o- cells by Western blotting with or without SB431542 (n=3, **P<0.01). C: RT-PCR validation of the expression of CFTR mRNA in different generation of 16HBE14o- cells infected by RSV with or without SB431542 treatment (n=3, *P<0.05, **P<0.01, ***P<0.001). D: CFTR expression in RSV-infected 16HBE14o- cells by Western blotting with or without SB431542 (n=3, *P<0.05, **P<0.01, ***P<0.001).

3 Discussion

Many prospective studies have shown that lower respiratory tract infection caused by RSV in early life is related to the development of asthma[13-15]. However, the mechanisms of the effect of RSV infection on respiratory physiology remain unclear. A number of studies indicated that RSV can potentially persist in airway epithelial cells. The persistent infection of RSV is closely associated with chronic respiratory disease[1, 16-17]. Transport of Cl^- by CFTR accounts for the fluid secretion and is crucial for airway surface liquid homeostasis[6]. Emerging evidence supports the roles for CFTR in epithelial barrier integrity, wound repair, mucus secretion, and clearance, which are critical in airway host defense[18-19]. CFTR dysfunction is associated with the pathogenesis of chronic obstructive pulmonary disease (COPD) and hyperresponsiveness of asthma.

In this study, we observed a down-regulation of CFTR expression in airway epithelial cells of RSV infected mice. By using a progressive RSV infected 16HBE14o- cell model[1], we found that the expression of CFTR progressively decreased upon RSV infection from passages of G1 to G3. Decreased protein expression was accompanied by the decrease of CFTR function, which is characterized by the determination of intracellular $[Cl^-]_i$ by MQAE and the measurement of $[Cl^-]_i$ by whole-cell patch clamp recordings. RSV infection-induced CFTR dysfunction may occur by activating the signaling pathway of TGF-β1.

Airway dysfunction caused by RSV infection may

be a direct damage caused by virus replication in airway epithelial cells, but it may also be promoted by airway inflammation[20]. In the process of RSV infection, TLR4 plays a key regulatory role in both innate and acquired immunity[21-22].

This receptor activates nuclear factor-κB, and thus, inflammatory cytokine such as IL-1 and IL-6 production[23]. RSV can inhibit cell innate immunity through a variety of different mechanisms to maintain its growth in the host. Two RSV NS proteins, NS1 and NS2, are involved in anti-host defense by IFN suppression[24].

In this study, 16HBE14o- cells were infected with RSV at a MOI of 0.01. TLR4 and NS1 were progressively increased in 16HBE14o- cells during passages of G1 to G3 and cells survived to G3. The increased expression of NS1 gene may contribute to the success of RSV in establishing sustained infections in 16HBE14o- cells. Our findings demonstrated that progressive RSV infection may cause acquired CFTR dysfunction, including decreased CFTR expression, reduced Cl^- secretion and channel gating in 16HBE14o- cell. Previous studies have also implicated cigarette-smoke exposure[25] and oxidant stress[26] to CFTR dysfunction, which will potentially contribute to more severe pulmonary manifestations.

Next, we demonstrated that the TGF-β1 signaling pathway is involved in the regulation of CFTR expression in airway epithelial cells infected by RSV. TGF-β1 is a pleiotropic cytokine that acts as an important immunomodulatory factor in airway. Studies demonstrated that children with severe asthma have elevated levels of TGF-β1, and are susceptible to exacerbation of asthma when infected with RSV[27]. TGF-βl produced by RSV infected macrophages inhibits the type I interferon response and impairs mitochondrial function[28]. Consistent with these studies, in our study, RSV infected 16HBE14o- cells showed a progressively increases of TGF-β1, which in turn activates TGF-β1 dependent signaling pathway of Smad2. Disruption of TGF-β1 signaling by SB431542 prevented Smad2 phosphorylation and rescued the expression of CFTR.

In summary, we validated the decreased expression of CFTR and the suppression of CFTR function in response to the progressive RSV infection. Given the reduced ciliated cells and excessive mucus production as common pathophysiological mechanisms for many airways diseases such as asthma and COPD, the decrease of CFTR function will impose marked effects on mucus clearance and airway homeostasis. Treatments aimed at increasing CFTR function may contribute to reduce the inflammation and airway obstruction associated with RSV infection, and may help to alleviate its long-term effects.

Conflict of interest: The authors declare that they have no conflicts of interest to disclose.

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