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ORIGINAL RESEARCH

Effective-Component Compatibility of Bufei Yishen Formula (ECC-BYF) III Inhibits Mucus Hypersecretion by BEAS-2B Cells via miR-146a-5p-Mediated Regulation of the EGFR/MEK/ERK Pathway

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Purpose: To explore the role of the miR-146a-5p-mediated regulation of the EGFR/MEK/ERK pathway in the effect of effective-component compatibility of Bufei Yishen Formula III (ECC-BYF III) on ameliorating mucus hypersecretion by bronchial epithelial cells (BEAS-2B cells).

Methods: BEAS-2B cells exposed to cigarette smoke extract (CSE) were used to establish a mucus hypersecretion model of BEAS-2B cells. The optimal intervention concentration of ECC-BYF III was screened by CCK-8, qRT-PCR and ELISA, the effects of ECC-BYF III on MUC5AC, MUC5B, IL-4, IL-8, TNF-α, IL-1α, miR-146a-5p and EGFR/MEK/ERK pathway expression were assessed. Furthermore, dual luciferase reporter gene was used to verify the relationship between miR-146a-5p and EGFR/MEK/ERK, and to observe the effect of down-regulating miR-146a-5p on ECC-BYF III ameliorating mucus hypersecretion and EGFR/MEK/ERK pathway.

Results: ECC-BYF III reduced the expression of MUC5AC and MUC5B, decreased the mRNA expression of IL-1α, IL-8 and TNF-α, increased the mRNA expression of IL-4, and decreased the protein expression of TNF-α. Moreover, ECC-BYF III ameliorated CSE induced mucus hypersecretion in BEAS-2B cells through EGFR/MEK/ERK pathway. Finally, our results indicated that ECC-BYF III ameliorated the model by targeting miR-146a-5p and downregulating the EGFR/MEK/ERK pathway.

Conclusion: ECC-BYF III can ameliorate CSE induced mucus hypersecretion by BEAS-2B cells and reduce the inflammatory response. The underlying mechanism may be related to the regulation of miR-146a-5p and the EGFR/MEK/ERK pathway. ECC-BYF III can inhibit activation of the EGFR/MEK/ERK pathway by upregulating the expression of miR-146a-5p, thereby ameliorating mucus hypersecretion by BEAS-2B cells.

Keywords: mucus hypersecretion, COPD, effective-component compatibility of Bufei Yishen Formula III, traditional Chinese medicine, miR-146a-5p

Introduction

Chronic obstructive pulmonary disease (COPD) is a heterogeneous lung disease characterized by chronic respiratory symptoms, such as cough, phlegm, and dyspnea, and it is associated with airway abnormalities and/or alveoli (emphysema).[1] Chronic cough and sputum are the clinical manifestations of hypersecretion of airway mucus, an important pathophysiological characteristic of COPD. The mortality rate of COPD patients with mucus hypersecretion is 3.5 times greater than that of patients without mucus hypersecretion.[2] Hyperplasia of airway epithelial goblet cells and upregulated expression of MUC5AC are observed in patients with COPD.[3] Smoking or long-term exposure to biofuels can induce goblet

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cell metaplasia, hypertrophy of mucus glands, and a decrease in the number of cilia, resulting in an abnormal increase in mucus, which seriously affecting a patient's ability to work and quality of life.[4,5]

The mechanism underlying airway mucus hypersecretion is complex and is affected by multiple signal transduction pathways, among which EGFR is one of the most important pathways, and its activation plays a key role in the mechanism of airway mucus hypersecretion.[6] Activation of the EGFR pathway, which is stimulated by cigarette smoke and other factors, can trigger multiple signaling pathways, induce goblet cell differentiation, and thus increase mucin expression.[7–9] miRNAs are a class of small endogenous noncoding RNAs.[10] By regulating gene expression, miR-146a-5p plays an important role in cell homeostasis and disease pathogenesis. Studies have shown that miR-146a-5p is significantly correlated with COPD, that mR-146a-5p targets EGFR expression, and that EGFR can activate multiple downstream pathways, including the Ras/Raf/MEK/ERK pathway.[11–13] We explored this mechanism, as shown in Figure 1.

Expectorant treatment has been widely used to treat excessive mucus secretion, and this treatment approach can effectively relieve airway stenosis, prevent repeated infection, and delay lung function decline.[14,15] Drugs that have made progress in clinical practice include mucus modulators such as glucocorticoids and macrolide antibiotics, N-acetylcysteine and other mucolytic agents, Bronchodilators that improve mucus dynamics, Hypertonic saline and other expectorants.[16] The mechanism underlying mucus hypersecretion involves a variety of signaling pathways.[17] Important research directions for treating airway mucus hypersecretion include combining drugs with different pathways of action, restricting the synthesis and secretion of mucin, reducing the excessive production and secretion of mucus, and blocking signal transduction via multiple targets. In recent years, traditional chinese medicine has a broad prospect for the treatment of mucus hypersecretion in COPD due to its limited side effects, low toxicity, novel pharmacological mechanisms and diverse active ingredients.[18] Previous research from this group revealed that the effective component compatibility of Bufei Yishen Formula (ECC-BYF III, patent number: 201811115372.3) can effectively improve lung function and pathological injury in COPD model rats, alleviate the local inflammatory response, reduce the differentiation of goblet cells, and inhibit airway mucus differentiation. This formula is composed of five monomers: ginsenoside Rh1, astragaloside IV, icariin, nobiletin and paeonol. Ginsenoside Rh1 protects against

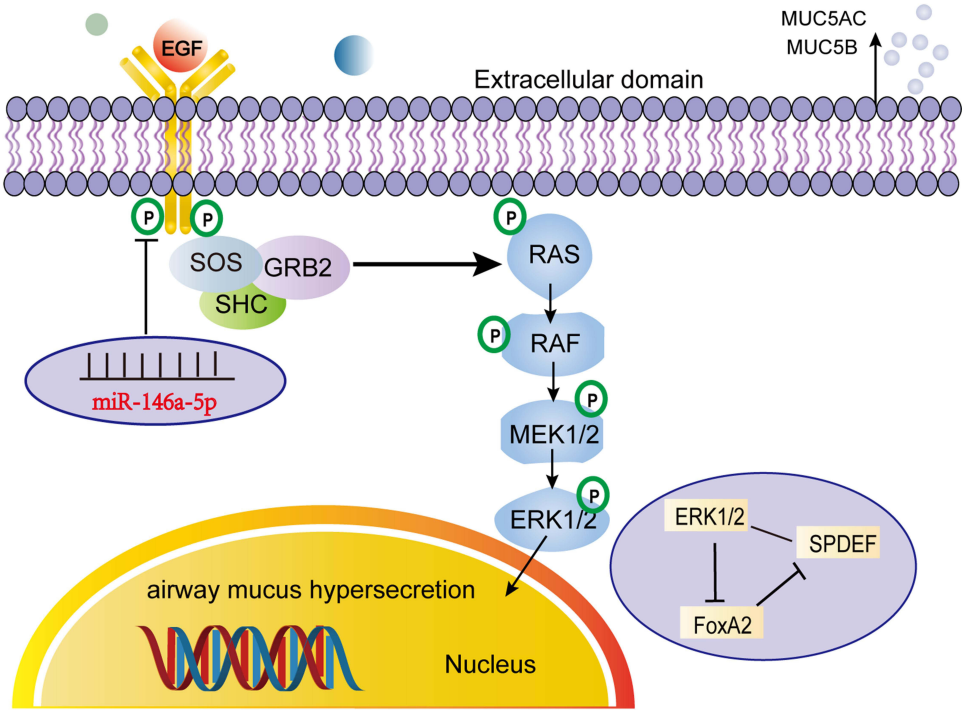

Figure 1 Schematic diagram of the miR-146a-5p/EGFR/MEK/ERK pathway.
Abbreviations: EGF, Epidermal Growth Factor; GRB2, growth factor receptor bound protein 2; SHC, Src homology and collagen; RAS, renin-angiotensin system; RAF, rapid acceleration of fibrosarcoma; MEK, mitogen-activated extracellular signal-regulated kinase; ERK, extracellular signal-regulated protein kinase; FOXA2, forkhead box A2; SPDEF, SAM pointed domain containing ETS transcription factor.

OVA/LPS-induced allergic asthma by suppressing immune cell infiltration by blocking the activation of MAPK, Akt, and NF-κB signaling pathways.[18] Astragaloside IV is a natural candidate molecule for the protection of pulmonary fibrosis, inhibiting the activity of RAS, interfering with the signal transduction of the RAS/RAF/FoxO pathway, and playing a key role both in decreasing the activation of fibroblasts and improving EMT.[19] Icariin can indirectly improve the lung function and pulmonary inflammatory infiltration of COPD rats by reducing airway inflammation.[20] The anti-inflammation role of nobiletin has been extensively demonstrated.[21] And many studies have determined that nobiletin is a potential candidate for COPD treatment. Paeonol can enhance the expression of antioxidants, reduce oxidative stress, and reduce ROS production in vitro.[22] It also has a protective effect on the lungs. The preliminary research foundation is solid, and the curative effect is precise.[23] We successfully established a rat model of COPD, and the results revealed that ECC-BYF III significantly suppressed the increase in airway goblet cells and airway mucin expression in COPD model rats, and a significant decrease in miR-146a-5p mRNA expression was observed in the COPD model group. ECC-BYF III can increase the expression of miR-146a-5p. The mechanism of action is further discussed in this study, as shown in Figure 2.

Materials and Methods

Cell Culture and Treatment

Human bronchial epithelial BEAS-2B cells were purchased from the National Collection of Authenticated Cell Cultures (catalog number: GNHu27). The cells were cultured in BEGM (CC-3170, Lonza, Switzerland) supplemented with 100 U/mL penicillin–streptomycin liquid (P1400, Solarbio, China). BEAS-2B cells were incubated at 37 °C and 5% CO_2.

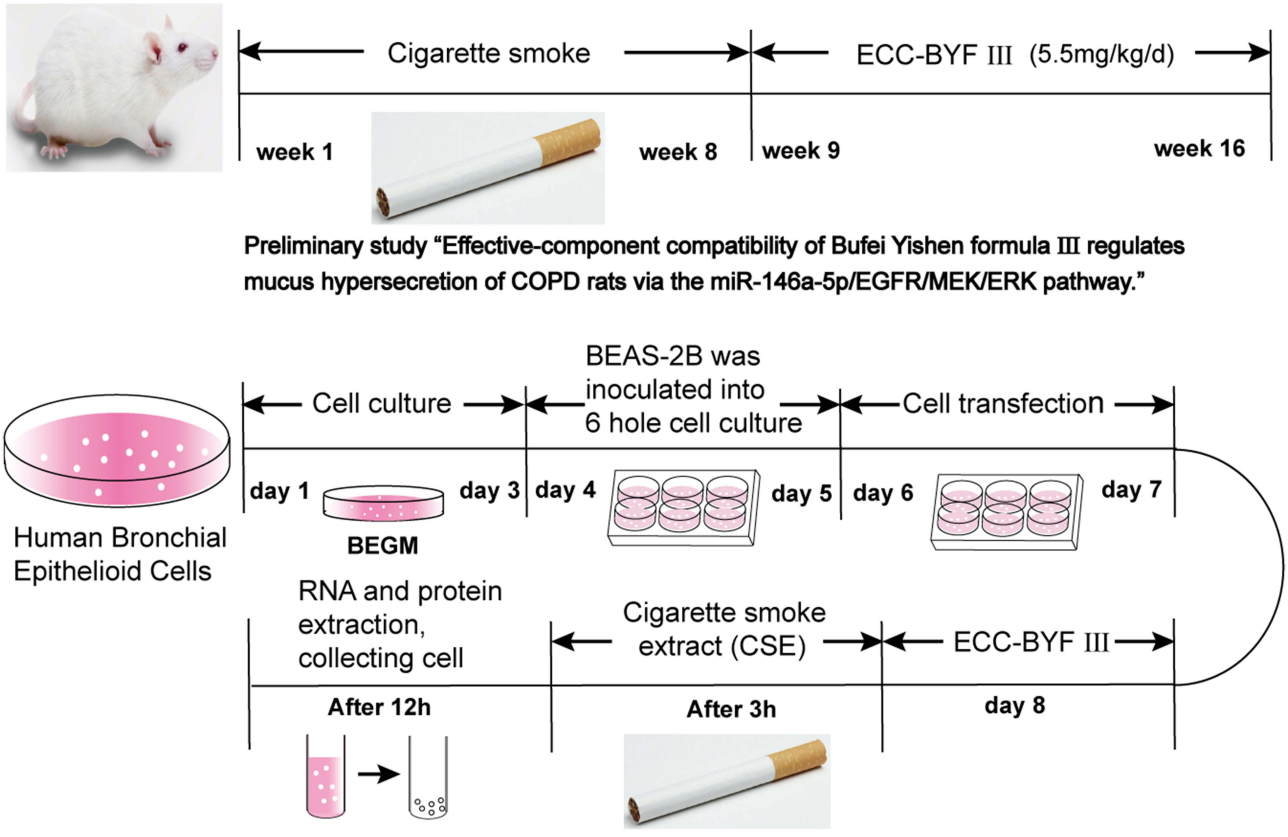

Figure 2 Flow chart of the preliminary study and BEAS-2B mucous hypersecretion model preparation and treatment.

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Drug Preparation

Effective-component compatibility of Bufei Yishen Formula III: Ginsenoside Rh1 (MUST-21121810), astragaloside IV (MUST-22031508), icariin (MUST-22081810), nobiletin (MUST-21100911), and paeonol (MUST-2041402) were purchased from Chengdu Must Technology Co., Ltd. Each monomer in ECC-BYF III was dissolved in dimethyl sulfoxide (DMSO) after compatibility in a certain proportion. After being packaged, ECC-BYF III was sealed and stored at −20 °C for later use.

Preparation of Cigarette Smoke Extract

To induce cellular mucus hypersecretion in vitro, a CSE solution was prepared via a custom-made smoking device, and a cigarette was burned. The cigarette was passed through 10 mL of DMEM (11965-092, Gibco, USA), and the smoke suspension was shaken to ensure full contact with the medium. The optical density (OD) value of the suspension was measured with an ultraviolet microspectrophotometer (wavelength parameter of 320 nm, optical path width of 10 nm), and the OD value of the suspension was adjusted with DMEM until the measurement result was in the range of 2.12 −2.16. BEAS-2B cells were cultured with CSE solution, which was filtered through a sterile 0.22μm filter and diluted to different concentrations in the culture medium.

Dual-Luciferase Reporter Gene

According to TargetScan V8 analysis, the targeting sequence of hsa-miR-146a-5p and the EGFR 3′UTR was GTTCTCT, and the binding site included residues 4151−4157 in EGFR 3′UTR. The restriction enzyme sites Nhe I/Sal II were used to ligate the EGFR 3′UTR sequence into pmir GLO (pmir GLO-EGFR 3′UTR-WT), and the 4151−4157 sequence was mutated from GTTCTCT to TGCTGAT to construct the mutant vector. The miR-146a-5p mimics and NC were cotransfected into BEAS-2B cells with pmirGLO-EGFR 3′UTR-WT and pmirGLO-EGFR 3′UTR-MUT, respectively. After transfection, the cells were cultured for 48 hours. A luciferase assay kit (E2920, Promega, USA) was used to read the results of the luciferase assay and analyze the regulatory effect of miR-146a-5p on EGFR expression.

Cell Viability Assay

CCK-8 reagent (GLPBIO, USA) was added after 6, 12, 24 and 48 hours of treatment, and the optical density was measured at 450 nm and 650 nm with a microplate reader for approximately 70 minutes. The inhibition rate (%) was calculated according to the OD value obtained.

Quantitative Real-Time PCR

Total RNA was isolated from BEAS-2B cells using TRIzol reagent (Thermo Fisher Scientific, USA), and the RNA concentration was determined with a Nanodrop 2000 spectrometer using a reverse transcription kit (Vazyme, China), which was used to obtain cDNA for quantitative real-time PCR (qRT-PCR). qRT-PCR (Applied Biosystems, USA) was performed on a QuantStudio6Flex system using SYBR Green Master Mix (RK21203, ABclonal, China). Information about the qRT-PCR primers is provided in Table 1.

Enzyme-Linked Immunosorbent Assay

The cells were cultured in 6-well plates and treated with CSE and ECC-BYF III. The cell supernatants were collected to measure TNF-α levels (E-EL-H2306, Elabscience, China), and the cells were collected to measure MUC5AC (ELK2098, ELK Biotechnology, China) and MUC5B (ELK2122, ELK Biotechnology, China) levels.

Western Blotting

Western blotting (WB) was used to measure EGFR (4267T, CST, USA), p-EGFR (3777T, CST, USA), MEK (8727T, CST, USA), p-MEK (9154T, CST, USA) and ERK (GTX134462, GeneTex, USA), p-ERK (GTX635617, CST, USA), FOXA2 (8186T, CST, USA), and SPDEF (A14114, ABclonal, China) expression. Denatured proteins were subjected to electrophoresis and transferred to polyvinylidene difluoride membranes. The membranes were blocked with 5% skim

Table 1 Primers Used in This Study

Gene		Primer Sequence	Length (bp)
MUC5AC	F	AGTGTCCCCCATGCACTGA	108
	R	CAGGGGCACAAGTTCCACTG	
MUC5B	F	GCCTACGAGGACTTCAACGTC	79
	R	CCTTGATGACAACACGGGTGA	
IL-4	F	CGGCAACTTTGTCCACGGA	111
	R	TCTGTTACGGTCAACTCGGTG	
IL-8	F	ACTGAGAGTGATTGAGAGTGGAC	112
	R	AACCCTCTGCACCCAGTTTTC	
IL-1α	F	AGATGCCTGAGATACCCAAAACC	147
	R	CCAAGCACACCCAGTAGTCT	
TNF-α	F	GAGGCCAAGCCCTGGTATG	91
	R	CGGGCCGATTGATCTCAGC	
GAPDH	F	GGAGCGAGATCCCTCCAAAAT	138
	R	GGCTGTTGTCATACTTCTCATGG	
β-actin	F	ATTGCCGACAGGATGCAGAA	150
	R	GCTGATCCACATCTGCTGGAA	
miR-146a-5p	F	TGAGAACTGAATTCCATGGGTT	150
U6	F	CTCGCTTCGGCAGCACA	94
	R	AACGCTTCACGAATTTGCGT	
miR-146a-5p mimics	S	UGAGAACUGAAUUCCAUGGGUU	
	AS	CCCAUGGAAUUCAGUUCUCAUU	
Negative control	S	5′-UUCUCCGAACGUGUCACGUTT-3	
	antisense	5′-ACGUGACACGUUCGGAGAATT-3′	
miR-146a-5p inhibitor	S	AACCCAUGGAAUUCAGUUCUCA	
MircoRNA inhibitor N.C	AS	CAGUACUUUUGUGUAGUACAA	

milk in 1× TBST. Next, they were incubated with primary antibody at 4 °C overnight and then with the corresponding secondary antibody. The protein bands were detected using enhanced chemiluminescence reagents.

Statistical Analysis

SPSS 23.0 was used to analyze the data, and one-way analysis of variance was used if the data were normally distributed. LSD was used for statistical analysis if the variance was equal. If the variance was uneven, Dunnett's T3 test was used for statistical analysis, and the results are expressed as the mean ± standard deviation ($\overline{x} \pm s$). GraphPad Prism 9 was used to analyze and process the bar graph. $P < 0.05$ indicated that the difference was statistically significant.

Results

Screening of Conditions to Establish a Model of Mucus Hypersecretion in CSE-Exposed BEAS-2B Cells

To establish a mucus hypersecretion model in BEAS-2B cells, BEAS-2B cells were exposed to CSE, which was prepared according to the relevant literature. The higher the concentration of CSE, the stronger the inhibitory effect on the BEAS-2B cells. Similarly, the inhibitory effect was more pronounced with longer durations of exposure. During the process of screening the exposure time and concentration of CSE, notable inhibitory effects of 20% CSE and 40% CSE were observed, so those conditions were eliminated (Figure 3A).

In the preliminary experiment, 6 h, 12 h, 24 h and 48 h were selected as the time points of CSE exposure, and 0%, 2.5%, 5%, 7.5%, 10% and 15% were selected as the CSE concentrations to evaluate activity. Because the 48 h treatment time was too long and the 15% concentration was too high, which strongly affected cell proliferation, these conditions were eliminated (Figure 3B and C). Next, we measured the mRNA expression of MUC5AC and MUC5B under different

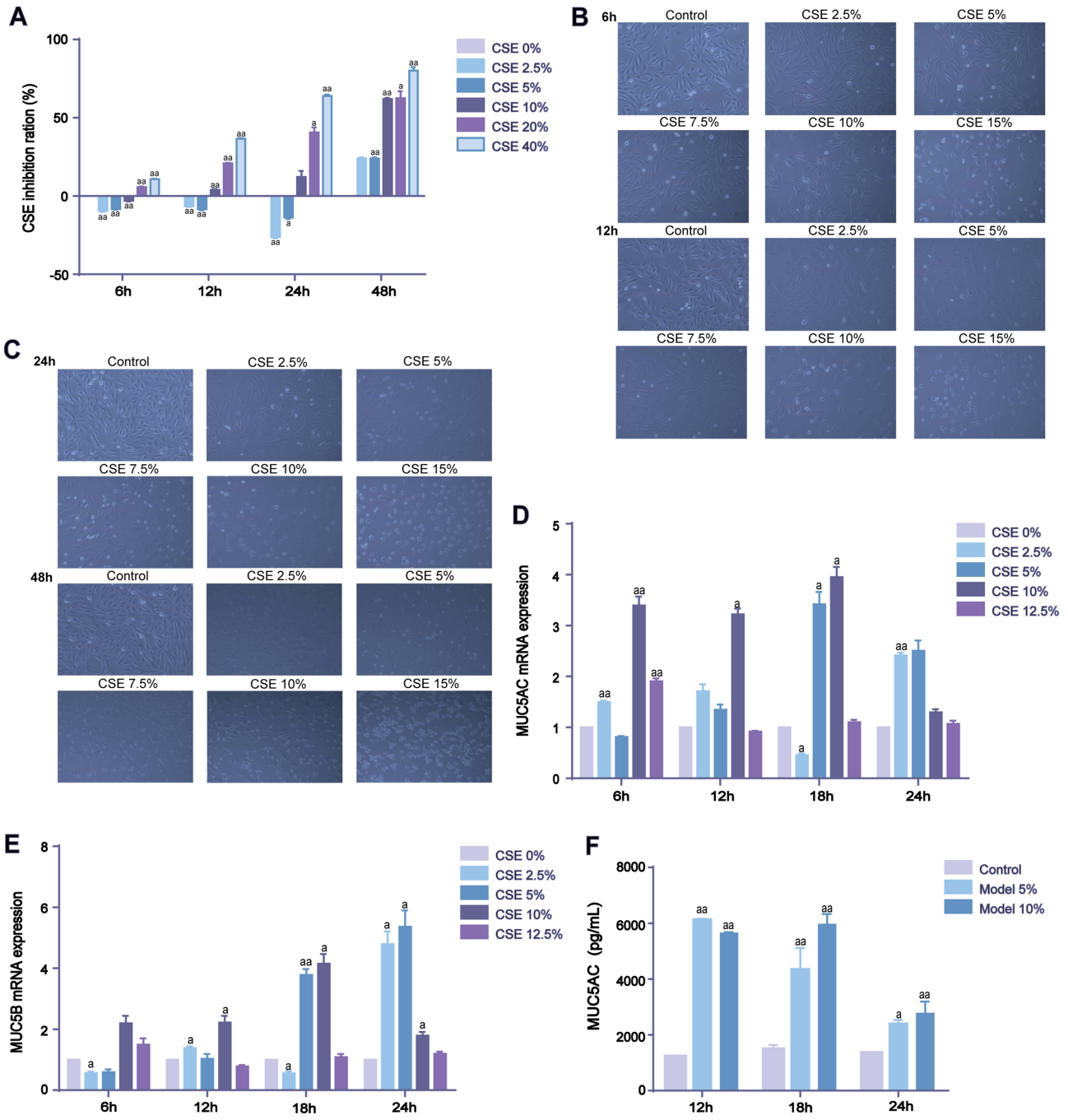

Figure 3 Screening of conditions to establish a model of mucus hypersecretion by BEAS-2B cells exposed to CSE. (**A**) Effects of different durations and concentrations of CSE on the proliferation of BEAS-2B cells. (**B** and **C**) Microscopic images of BEAS-2B cells treated with different concentrations of CSE (0%, 2.5%, 5%, 7.5%, 10%, 15%) for different times (6 h, 12 h, 24 h, 48 h) (×10). (**D** and **E**) Relative mRNA expression of MUC5AC and MUC5B in BEAS-2B cells after exposure to CSE for different durations and at different concentrations. (**F**) Relative protein expression of MUC5AC in BEAS-2B cells exposed to different concentrations of CSE for 12 h, 18 h and 24 h. All the data are presented as the means ± SDs (n = 3), $^{a}P < 0.05$, $^{aa}P < 0.01$ versus the control group.

induction times and concentrations of CSE (Figure 3D and E) and found that the expression of MUC5AC and MUC5B increased with increasing CSE treatment time and concentration, exhibiting a dynamic process of first increasing but then decreasing. We found that the expression was unstable under the 2.5% CSE and 12.5% CSE conditions and that the treatment effect was not obvious when the CSE treatment time was 6 h; thus, these conditions were excluded. In accordance with the conditions selected for qRT-PCR, 5% and 10% CSE were selected as the CSE concentrations, and MUC5AC protein expression was significantly increased after 12 h, 18 h and 24 h of CSE induction (Figure 3F).

Determining the Optimal Intervention Concentration of ECC-BYF III

Different concentrations of ECC-BYF III (0, 20, 40, 80, 160, and 320 μg/mL) and different exposure times (6 h, 12 h, 24 h, and 48 h) were selected to observe the effects of these concentrations of ECC-BYF III on the proliferation of BEAS-2B cells after different durations. The inhibition rate significantly increased with increasing concentrations of ECC-BYF III and longer durations, and the 160, 320 μg/mL and 48 h conditions were eliminated due to the relatively high inhibition rate of the cells (Figure 4A).

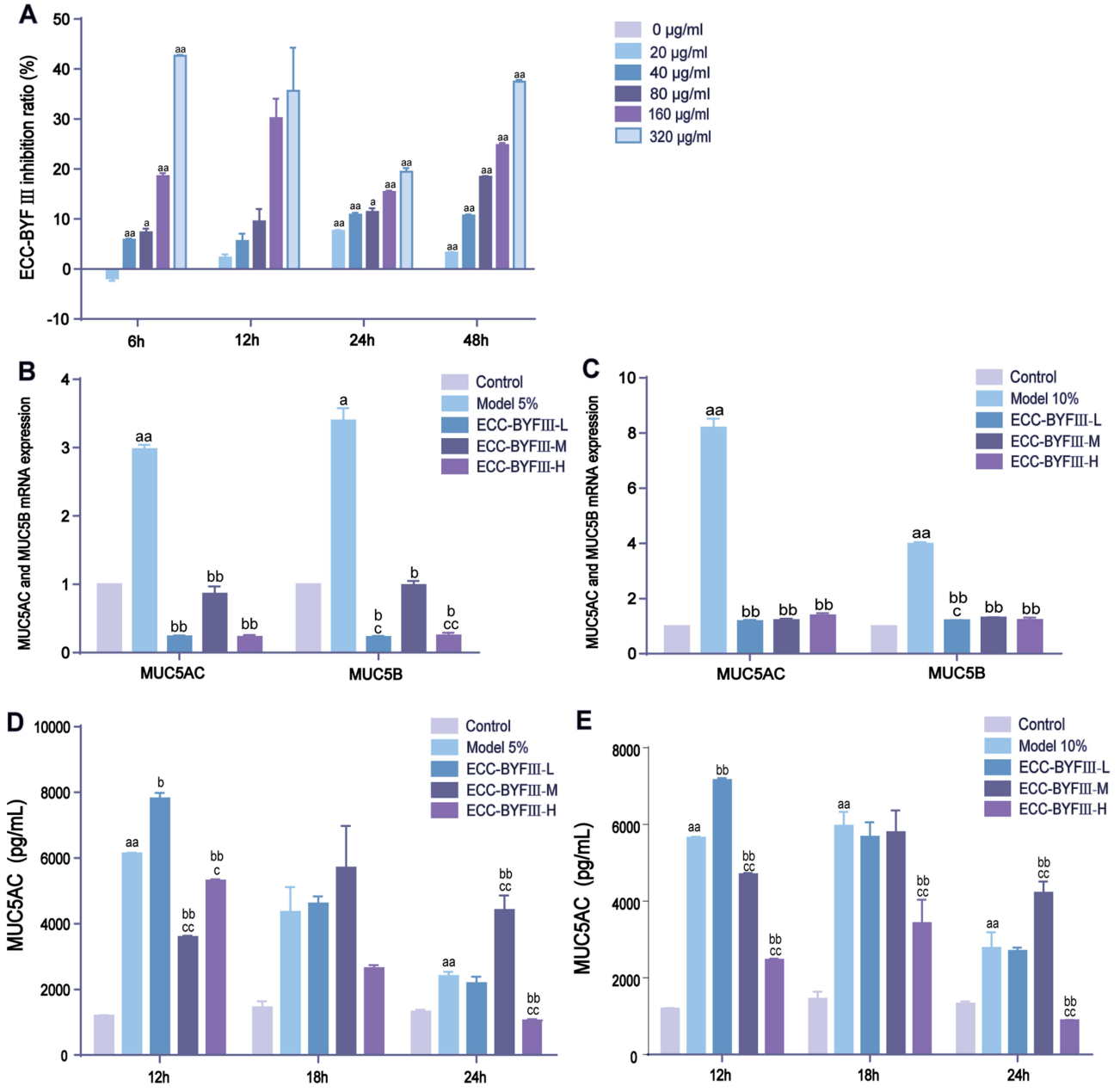

Figure 4 The optimal intervention concentration of ECC-BYF III was screened. (**A**) Effects of different durations and concentrations of ECC-BYF III on the proliferation of BEAS-2B cells. All the data are presented as the means ± SDs (n =3), $^{a}P < 0.05$, $^{aa}P < 0.01$ versus the control group. (**B** and **C**) Relative mRNA expression of MUC5AC and MUC5B in BEAS-2B cells exposed to 5% CSE or 10% CSE in the ECC-BYF III low-, medium- and high-dose groups. All the data are presented as the means ± SDs (n =3), $^{a}P < 0.05$, $^{aa}P < 0.01$ versus the control group. $^{b}P < 0.05$, $^{bb}P < 0.01$ versus the model group. $^{c}P < 0.05$, $^{cc}P < 0.01$ versus the ECC-BYF III -M group. (**D** and **E**) Relative protein expression of MUC5AC in BEAS-2B cells exposed to 5% CSE and 10% CSE in the ECC-BYF III low-, medium- and high-dose groups. All the data are presented as the means ± SDs (n =3), $^{a}P < 0.05$, $^{aa}P < 0.01$ versus the control group. $^{b}P < 0.05$, $^{bb}P < 0.01$ versus the model group. $^{c}P < 0.05$, $^{cc}P < 0.01$ versus the ECC-BYF III-L group.

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CSE was added to the cells for 12 h at concentrations of 0%, 5% and 10% (Control, Model 5% and Model 10%). The 20 μg/mL, 40 μg/mL, and 80 μg/mL concentrations were selected as the high-, medium-, and low-dose groups (ECC-BYF III-L, ECC-BYF III-M, and ECC-BYF III-H, respectively), and the mRNA expression levels of MUC5AC and MUC5B were observed. The mRNA expression of MUC5B in Model 10% was greater than that in Model 5%, but there was no significant difference in MUC5B mRNA expression. In terms of the drug dose, in Model 5%, the MUC5AC and MUC5B mRNA expression levels after treatment with ECC-BYF III-L and ECC-BYF III-H were lower than those after treatment with ECC-BYF III-M. But in Model 10%, compared with Model 10%, ECC-BYF-L, ECC-BYF-M and ECC-BYF-H were all significantly decreased. The groups treated with low-dose, medium-dose, and high-dose ECC-BYF III did not significantly differ (Figure 4B and C).

Combined with qRT-PCR results and relative protein expression results, it was found that compared with ECC-BYF-L, the protein expression of MUC5AC in ECC-BYF-H was decreased under the conditions of Model 5% and 12h. Compared with that in the ECC-BYF III-L group, MUC5AC protein expression was significantly decreased in the ECC-BYF III-H group of Model 5% after 24 hours ($P<0.01$). Compared with that in the ECC-BYF III-L group, MUC5AC protein expression was significantly decreased in the high-dose group at 12 h, 18 h, and 24 h after Model 10% treatment ($P<0.01$). In conclusion, high-dose ECC-BYF III was more effective than low- or medium-dose ECC-BYF III (Figure 4D and E).

Considering these experimental results, 10% CSE treatment for 12 h was selected to induce mucus hypersecretion by BEAS-2B cells (Model), and 80 μg/mL ECC-BYF III (ECC-BYF III) was selected as the drug concentration.

ECC-BYF III Can Ameliorate Mucus Hypersecretion and Affect the Levels of the Inflammatory Factors, miR-146a-5p and the EGFR/MEK/ERK Pathway

In terms of the effect of ECC-BYF III on mucus hypersecretion, protein expression of MUC5AC and MUC5B was increased in the model group ($P<0.01$). Additionally, protein expression of MUC5AC and MUC5B was decreased in the ECC-BYF III group ($P<0.01$) (Figure 5A and B).

Regarding the expression of genes involved in inflammation, we found decreased mRNA expression of IL-4 ($P<0.01$) in the model group, while the mRNA expression levels of IL-1α ($P<0.01$), IL-8 ($P<0.05$), TNF-α ($P<0.01$) was increased, and the protein expression of TNF-α was increased. In the ECC-BYF III group, mRNA expression of IL-4 was increased ($P<0.01$), mRNA expression of IL-1α, IL-8, and TNF-α was decreased ($P<0.01$), and protein expression of TNF-α was decreased ($P<0.05$) (Figure 5C and D).

The effects of ECC-BYF III on the mRNA expression of miR-146a-5p, MUC5AC, and MUC5B were then assessed. In the model group, the mRNA expression of miR-146a-5p was decreased ($P<0.05$), and mRNA expression of MUC5AC ($P<0.01$) and MUC5B ($P<0.05$) was increased. In the ECC-BYF III group, the mRNA expression of miR-146a-5p was increased ($P<0.05$), the mRNA expression of MUC5AC was decreased ($P<0.05$), and there was a trend toward decreased MUC5B mRNA expression, but the difference was not statistically significant (Figure 5E and F).

Effect of ECC-BYF III on the expression of EGFR/MEK/ERK pathway-related proteins: the protein expression of p-EGFR, p-MEK, p-ERK and SPDEF increased in the model group, and that of FOXA2 decreased. Protein expression of p-EGFR, p-MEK, p-ERK and SPDEF significantly decreased in the ECC-BYF III group, and that of FOXA2 significantly increased ($P<0.01$) (Figure 5G–K).

Dual-Luciferase Reporter Assay

To screen transfection conditions, different concentrations of Lipofectamine 3000 reagent and different transfection times were selected (Figure 6A). The dual-luciferase reporter gene of miR-146a-5p, which targets EGFR, was constructed, and the miR-146a-5p mimic or negative control was transfected into the cells. A luciferase detection system was used to observe the regulatory effect of miR-146a-5p on EGFR gene expression. The results showed that miR-146a-5p had an inhibitory effect on the EGFR gene, possibly by interacting with the EGFR 3′UTR sequence, leading to the down-regulation of luciferase expression of the reporter gene, further supporting the role of miR-146a-5p in EGFR regulation (Figure 6B–D).

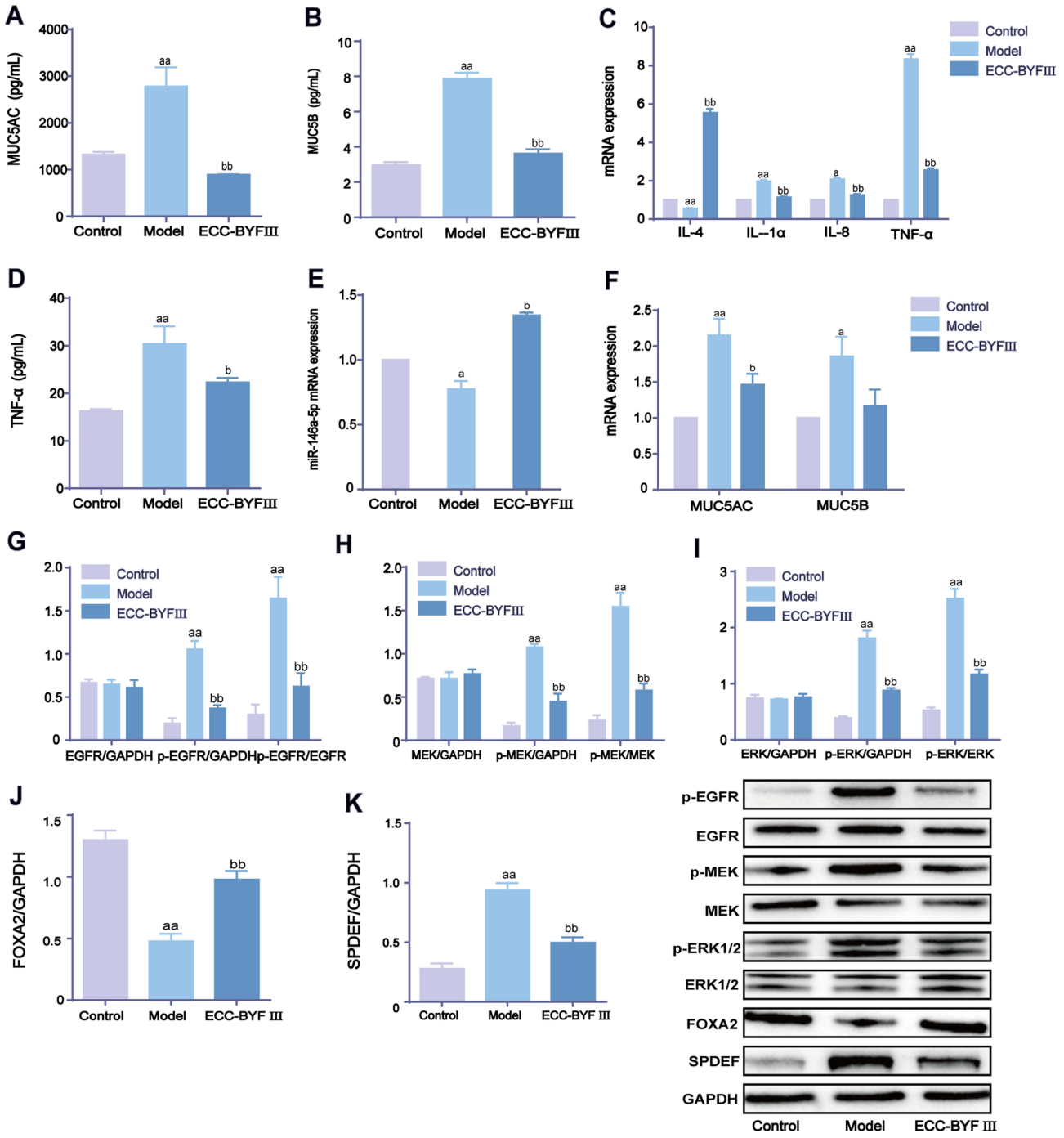

Figure 5 ECC-BYF III can ameliorate mucus hypersecretion and affect the levels of inflammatory factors, miR-146a-5p and EGFR/MEK/ERK pathway components. (**A** and **B**) Relative protein expression of MUC5AC and MUC5B in each group. (**C**) Relative mRNA expression of IL-4, IL-1α, IL-8 and TNF-α in each group. (**D**) Relative protein expression of TNF-α in each group. (**E** and **F**) Relative mRNA expression of miR-146a-5p, MUC5AC and MUC5B in each group. (**G**–**K**) Expression of EGFR/MEK/ERK pathway-related proteins affected by ECC-BYF III. All the data are presented as the mean ± SDs (n =3), $^{a}P < 0.05$, $^{aa}P < 0.01$ versus the control group. $^{b}P < 0.05$, $^{bb}P < 0.01$ versus the model group.

Effect of miR-146a-5p Overexpression on MUC5AC, MUC5B and the EGFR/MEK/ERK Pathway

Overexpression of miR-146a-5p affected the mRNA expression of MUC5AC and MUC5B: Compared with that in the NC control group, the mRNA expression of miR-146a-5p in NC Model group was significantly downregulated ($P<0.01$), and the mRNA expression of MUC5AC and MUC5B was significantly upregulated ($P<0.01$). The mRNA expression of

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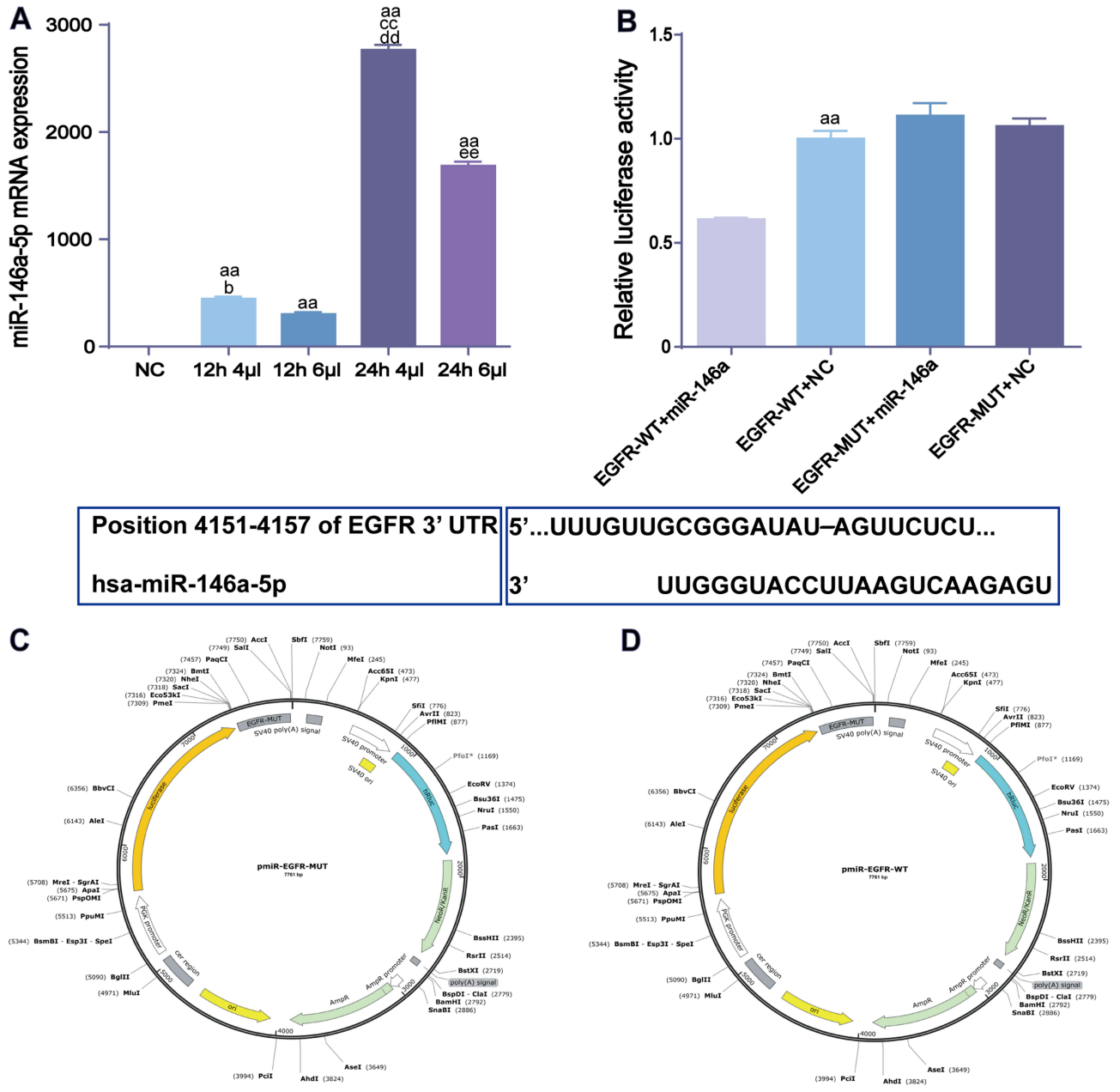

Figure 6 Transfection condition screening and dual luciferase reporter gene analysis. (**A**) Relative mRNA expression of miR-146a-5p under different transfection conditions. All the data are presented as the mean ± SDs (n =3), $^{aa}P < 0.01$ versus the NC group, $^{b}P < 0.05$ versus the 12 h and 6 μL group, $^{cc}P < 0.01$ versus the 24 h and 6 μL group, $^{dd}P < 0.01$ versus the 24 h and 4 μL group, $^{ee}P < 0.01$ versus the 12 h and 6 μL (**B**–**D**) Dual luciferase reporter assay and reporter vector. All the data are presented as the means ± SDs (n =3), $^{aa}P < 0.01$ versus the EGFR-WT+miR-146a group.

miR-146a-5p was significantly upregulated in the mimic Control group ($P<0.01$). Compared with that in the NC Model group, the mRNA expression of miR-146a-5p was upregulated ($P<0.01$), and that of MUC5AC ($P<0.01$) and MUC5B ($P<0.05$) was downregulated in the mimic Model group (Figure 7A and B). The trends in MUC5AC protein expression were generally consistent with those of the gene expression (Figure 7C).

Effect of miR-146a-5p overexpression on the EGFR pathway: Compared with those in the mimic Control group, the expression levels of p-EGFR ($P<0.05$), p-MEK ($P<0.05$) and p-ERK ($P<0.01$) were increased in the NC Control group, and the protein expression of FOXA2 was significantly decreased ($P<0.01$). Compared with those in the NC control group, the protein expression levels of p-EGFR ($P<0.05$), p-MEK ($P<0.05$) and p-ERK were increased in the NC Model group, but the differences were not statistically significant; the protein expression of FOXA2 was decreased ($P<0.01$).

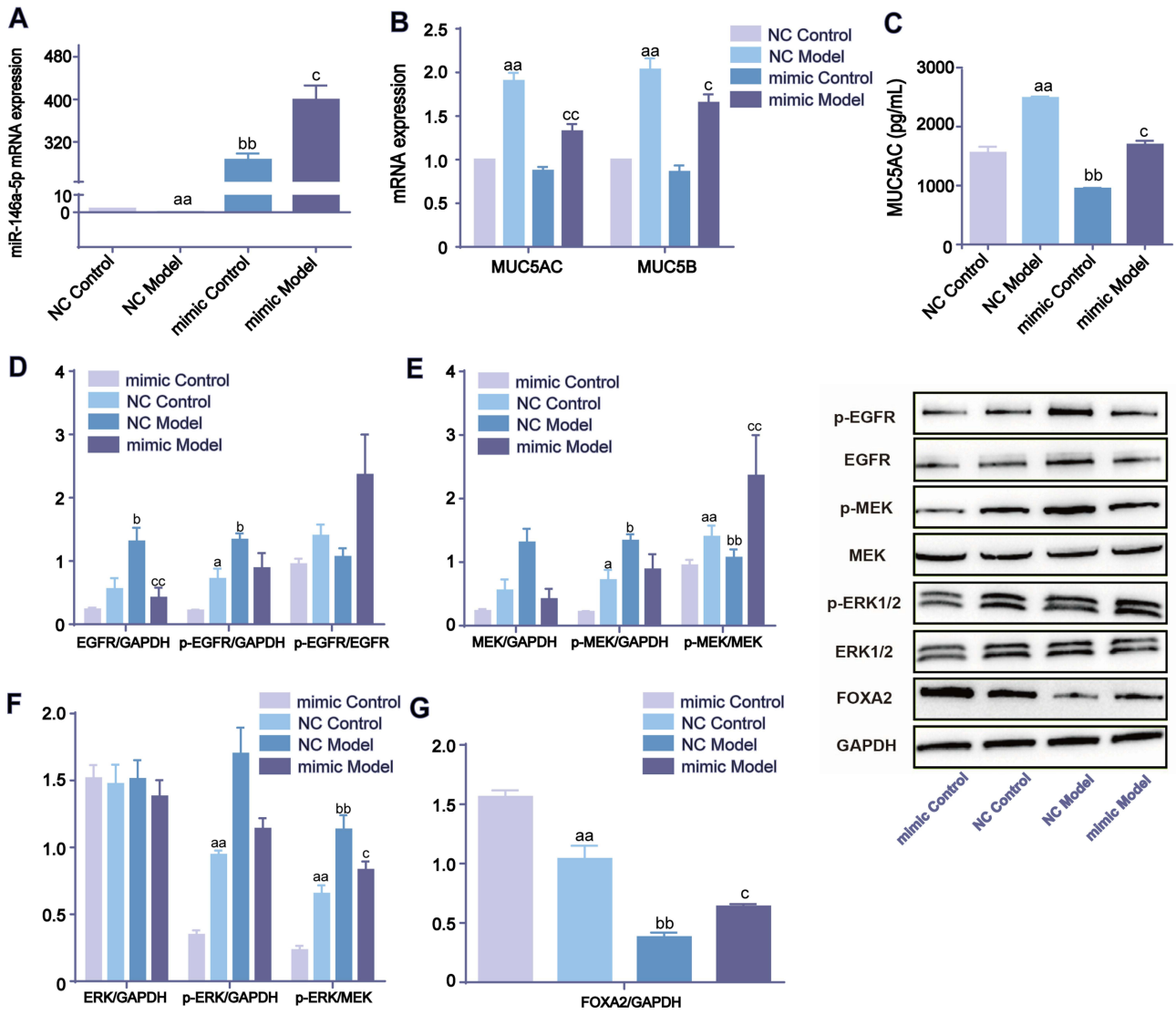

Figure 7 Effects of miR-146a-5p overexpression on MUC5AC, MUC5B and the EGFR/MEK/ERK pathway. (**A** and **B**) The relative mRNA expression levels of miR-146a-5p, MUC5AC and MUC5B in each group after transfection. (**C**) Protein expression of MUC5AC after transfection. All the data are presented as the means ± SDs (n = 3). NC Control group compared with NC Model group, $^{aa}P < 0.01$; NC Control group compared with mimic Control group, $^{bb}P < 0.01$; NC Model group compared with mimic Model group, $^{c}P < 0.05$, $^{cc}P < 0.01$. (**D**–**G**) Effect of miR-146a-5p overexpression on the EGFR/MEK/ERK pathway. All the data are presented as the means ± SDs (n =3). Mimic Control group compared with NC Control group, $^{a}P < 0.05$, $^{aa}P < 0.01$; NC Control group compared with NC Model group, $^{b}P < 0.05$, $^{bb}P < 0.01$; NC Model group compared with mimic Model group, $^{c}P < 0.05$, $^{cc}P < 0.01$.

Compared with those in the NC model group, the protein expression levels of p-EGFR, p-MEK and p-ERK were lower in the mimic Model group, and the protein expression of FOXA2 was increased ($P<0.05$) (Figure 7D–G).

Investigation of the Effect of miR-146a-5p Downregulation on ECC-BYF III-Mediated Regulation of Mucus Hypersecretion and the EGFR/MEK/ERK Pathway in BEAS-2B Cells

Effect of downregulating miR-146a-5p on ECC-BYF III-mediated regulation of mucus hypersecretion by BEAS-2B cells: Compared with that in the IN NC Control group, the expression of miR-146a-5p was significantly decreased in the IN NC Model group ($P<0.01$), and the mRNA expression of MUC5AC and MUC5B was significantly increased ($P<0.01$). Compared with that in the IN NC Model group, the expression of miR-146a-5p mRNA was increased in the IN NC ECC-BYF III group ($P<0.05$), and the mRNA expression of MUC5AC and MUC5B was significantly decreased ($P<0.01$). Compared with that in the IN NC ECC-BYF III group, the mRNA expression of miR-146a-5p was significantly downregulated in the IN ECC-BYF III group ($P<0.01$), and the mRNA expression of MUC5AC and

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MUC5B was significantly upregulated ($P<0.01$) (Figure 8A and B). The trends in MUC5AC protein expression were generally consistent with those in the gene expression analysis (Figure 8C).

Effect of downregulating miR-146a-5p on the EGFR pathway: Compared with that in the IN NC Control group, the protein expression of p-EGFR, p-MEK and p-ERK was increased in the IN NC Model group ($P<0.01$), whereas the protein expression of FOXA2 was decreased ($P<0.01$). Compared with those in the IN NC Model group, the protein expression levels of p-EGFR, p-MEK and p-ERK were decreased in the IN NC ECC-BYF III group ($P<0.01$). The protein expression of FOXA2 was increased ($P<0.01$). Compared with those in the IN NC ECC-BYF III group, the protein expression of p-EGFR and p-ERK was significantly increased in the IN ECC-BYF III group ($P<0.01$), but there was no significant difference in the protein expression of p-MEK. FOXA2 protein expression was decreased ($P<0.01$) (Figure 8D–G).

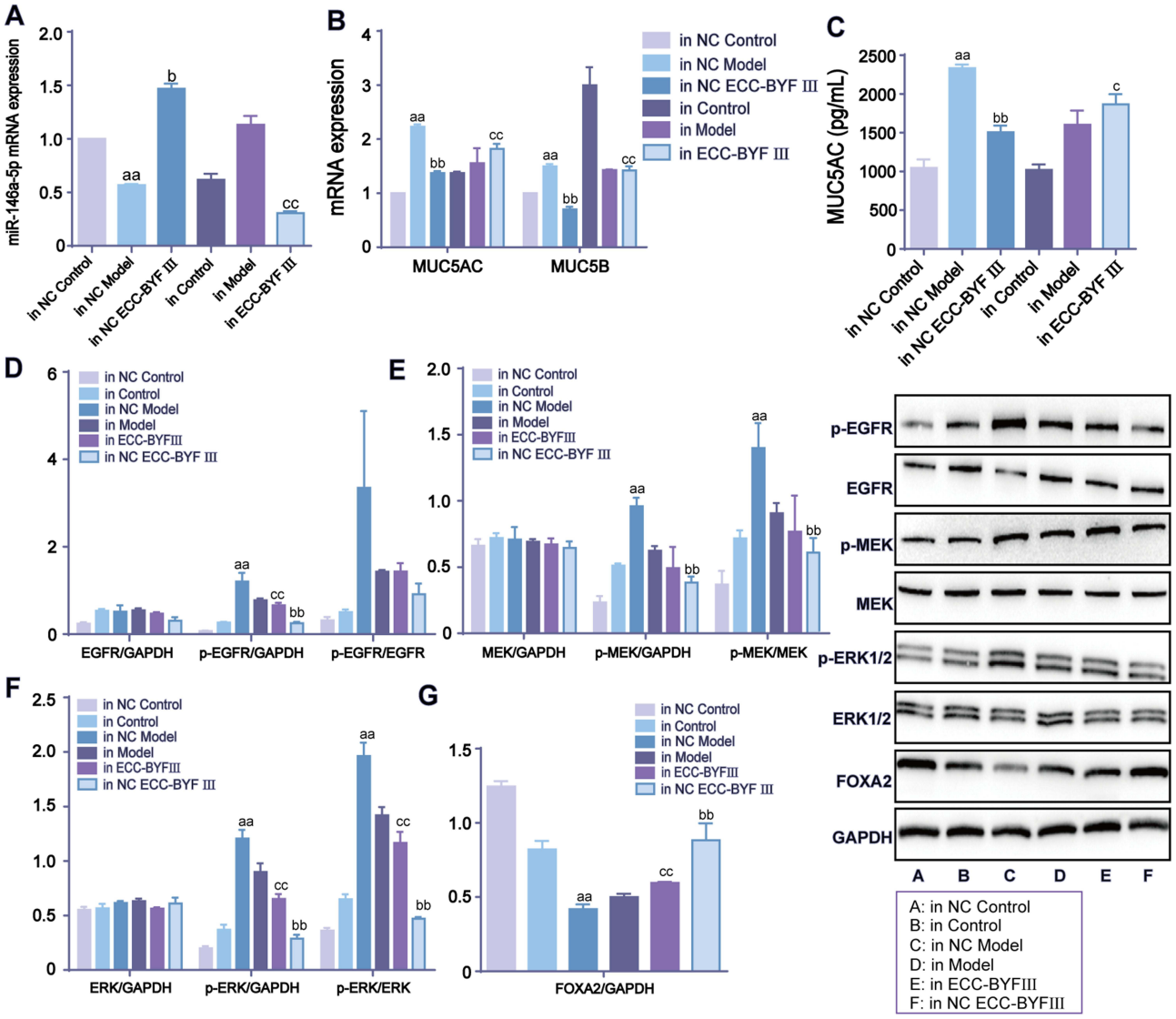

Figure 8 Effects of miR-146a-5p downregulation on the ability of ECC-BYF III to regulate mucus hypersecretion and the EGFR/MEK/ERK pathway in BEAS-2B cells. (**A** and **B**) Relative mRNA expression of miR-146a-5p, MUC5AC and MUC5B in each group after transfection. (**C**) Protein expression of MUC5AC after transfection. (**D**–**G**) Effect of downregulating miR-146a-5p on the EGFR/MRK/ERK pathway. All the data are presented as the means ± SDs (n =3). IN NC Control group compared with IN NC Model group, $^{aa}P < 0.01$; IN NC Model group compared with IN NC ECC-BYF III group, $^{b}P < 0.05$, $^{bb}P < 0.01$; IN NC ECC-BYF III group compared with IN ECC-BYF III group, $^{cc}P < 0.01$.

Discussion

COPD is a preventable and treatable chronic airway disease, which has become a major global public health challenge.[24] Its incidence is increasing annually and is expected to continue to over the next 40 years. By 2060, more than 5.4 million people may die from COPD and related diseases each year.[25] Mucus obstruction caused by mucus hypersecretion is common in patients with COPD and seriously affects the ability of patients to work as well as their quality of life.[26] Airway mucus hypersecretion is an important pathophysiological characteristic of COPD, and it is also an important risk factor for the onset and progression of COPD.[16] It is significantly related to a decrease in lung function, an increase in the number of acute exacerbations and an increase in the hospitalization rate of patients.

Traditional Chinese medicine has multicomponent, multitarget and multipathway characteristics and has broad prospects in the treatment of COPD airway mucus hypersecretion.[18] In our previous study, we found that ECC-BYF III had a good inhibitory effect on airway mucus hypersecretion in COPD rats caused by cigarette exposure combined with bacterial infection, and could alleviate local inflammatory response.[23] The mechanism may be related to miR-146a-5p/EGFR/MEK/ERK pathway. To further explore the role of miR-146a-5p in the EGFR/MEK/ERK pathway, we subsequently conducted cell experiments.

Neutrophil elastase (NE), Lipopolysaccharide (LPS), tumor necrosis factor (TNF)-α, PM2.5 and CSE are the most common factors that induce mucus hypersecretion in an vitro model of COPD.[27,28] Smoking is one of the main environmental factors of COPD, and it is also an important cause of the onset and progression of COPD, which is related to the mechanism of mucus hypersecretion. According to statistics, the relative risk of COPD in former smokers and current smokers was 2.9 times and 3.5 times higher, respectively, than that in people who had never smoked.[29] The 2024 Global initiative for Chronic Obstructive Disease (GOLD) guidelines propose that smoking cessation has the greatest impact on the natural history of COPD and can effectively reduce the frequency of acute exacerbation chronic obstructive pulmonary disease (AECOPD).[30] Studies have shown that ROS in tobacco can stimulate EGFR activation, thereby increasing MUC5AC.[31] Allyl alcohol can increase the expression of MUC5AC. Nicotine can hinder mucosal hydration, leading to increased viscosity. Nicotine can hinder mucosal hydration, leading to increased viscosity.[32] Studies have shown that cigarette smoke is one of the main causes of mucus hypersecretion, and it can induce the EGFR cascade, upregulate EGFR mRNA expression, induce phosphorylation, and promote the proliferation of goblet cells and mucin synthesis, thereby increasing the expression of MUC5AC.[33] Therefore, we selected CSE to induce cellular mucus hypersecretion.

Airway mucus is composed of a sol layer and a gel layer.[34] The gel layer is located above the sol layer and is composed of water, mucin, and lysozyme, which determine the mucus elasticity. Mucins are the main components of mucus gels and are divided into membrane-bound mucins and secreted mucins, of which MUC5AC and MUC5B are the most important mucins.[35–37] According to previous statistical studies, among the mucins that are expressed in COPD patients, the expression of MUC5AC and MUC5B is highest.[38] Therefore, we selected MUC5AC and MUC5B as indicators of successful establishment of mucus hypersecretion models.

During the process of establishing the model, we screened the effects of different concentrations and different durations on the proliferation of BEAS-2B cells, and it was found that higher concentrations of ECC-BYF III and longer durations resulted in a stronger inhibitory effect on BEAS-2B cells. The 20 μg/mL, 40 μg/mL and 80 μg/mL groups were selected as the low-, medium- and high-dose groups. In the Model 5% and Model 10% groups, the effect of low, medium and high doses of ECC-BYF III were observed. In the Model 5% group, the mRNA expression of MUC5AC and MUC5B was lower in the low-dose and the high-dose groups than that in the middle-dose group, but in the Model 10% group, there was no significant difference among the dose groups of ECC-BYF III. According to the results obtained for MUC5AC protein expression, different models and different durations, high-dose ECC-BYF III had a stronger effect than the other doses in terms of downregulating MUC5AC. Therefore, the high-dose of 80 μg/mL was selected as the drug concentration for subsequent experiments (ECC-BYF III group). Together with the previous experimental results, a concentration of 10% CSE for 12 h was selected as the Model group (Model group), which was the final mucus hypersecretion model of BEAS-2B cells.

Airway inflammation, a characteristic of COPD, is a key driver of COPD progression and deterioration.[39] It has been reported that airway mucus hypersecretion can lead to a variety of chronic airway inflammatory diseases, which are

closely related to patient prognosis.[40] It is a key factor for airway obstruction and decreases in lung function. Airway inflammation is usually caused by pathogens or exposure to irritants, allergens, and pollutants, among others.[41] The inflammatory mechanism recognizes the molecular patterns expressed by invading pathogens by recognizing receptors and activating inflammatory cells to produce growth factors, chemokines, and proinflammatory cytokines. The activated inflammatory cells are subsequently cleared, and damaged tissues are examined.[42,43] The harmful chemicals in cigarettes can activate alveolar macrophages, T lymphocytes and other inflammatory cells to release a variety of inflammatory mediators, resulting in chronic inflammatory responses in the airway and lung parenchyma.[44] On the basis of the model results, we investigated the effects of ECC-BYF III on IL-4, IL-8, IL-1α and TNF-α and found that ECC-BYF III ameliorated inflammation in BEAS-2B cells stimulated with CSE.

The expression of EGFR in COPD patients is greater than that in smokers with normal lung function.[45] COPD is indeed associated with the overexpression of EGFR. EGFR expression and activation are positively correlated with the proliferation of goblet cells, and the increase in the number of goblet cells is related to the total amount of mucin synthesis, which plays a key role in the mechanism of mucus hypersecretion.[46,47] After EGFR binds to ligands such as epithelial growth factor (EGF), transforming growth factor (TGF)-α and vascular endothelial growth factor (VEGF), its surface homodimers or heterodimers are activated, and intracellular tyrosine kinase domains are phosphorylated, leading to the activation of downstream signal transduction pathways.[48] One of the EGFR pathways involves Ras/Raf/MEK/ERK; in this pathway, growth factor receptor-bound protein 2 (Grb2) binds to tyrosine residues phosphorylated by activated EGFR and activates Ras with GMP exchange factor (GEF) via the guanosine diphosphate (GDP) exchange guanosine triphosphate (GTP) mechanism, which further activates Raf and MEK1/2 via phosphorylation.[13,49] This pathway further phosphorylates and activates ERK1/2. After stimulation by multiple factors, activation of the EGFR pathway induces the differentiation of goblet cells; this leads to increased expression of mucin, which plays a central role in the overexpression of MUC5AC.[50] By measuring the levels of p-EGFR, p-MEK, p-ERK, SPDEF, FOXA2 and other indicators, we found that the EGFR/MEK/ERK signaling pathway might be one of the pathways by which ECC-BYF III ameliorates mucus hypersecretion induced by CSE in BEAS-2B cells.

miRNAs are a class of small endogenous non-coding RNAs that play an important role in cell homeostasis and disease pathogenesis by regulating gene expression.[10] miRNAs are recognized as key regulators of chronic airway diseases.[51,52] miR-146a plays an important role in many inflammatory diseases and is a key factor in the pathogenesis of COPD.[53] Studies have shown that miR-146a can predict the risk of AECOPD and can be used as a biomarker between stable COPD patients and healthy individuals with AECOPD.[54] miR-134-5p and miR-146a-5p can be used as biomarkers of COPD and may provide treatment opportunities for patients with mucus hypersecretion.[55] miR-146a may be a target for the treatment of COPD, and the delivery of polymeric nanoparticles of miR-146a to treat COPD has been studied.[56]

Studies have confirmed that EGFR is a direct target of miR-146a-5p, and that miR-146a-5p targets and inhibits EGFR.[12] In the early stage of the experiment using the COPD rat model, the expression of miR-146a-5p was significantly decreased in the model group, and the levels of phosphorylated of EGFR, MEK and ERK1/2 were increased. ECC-BYF III effectively upregulated the expression of miR-146a-5p and inhibited activation of the EGFR/MEK/ERK pathway. To further verify the effect of ECC-BYF III on miR-146a-5p-mediated regulation of the EGFR/MEK/ERK pathway, based on the mucus hypersecretion model in BEAS-2B cells, we first measured the expression of MUC5AC and MUC5B mRNA in BEAS-2B cells without miR-146a-5p transfection. Subsequently, a dual luciferase reporter gene was used to verify and confirm that EGFR was the target gene of miR-146a-5p, and miR-146a-5p and EGFR had a negative feedback effect.

Using a model of mucus hypersecretion by BEAS-2B cells, we investigated the effects of miR-146a-5p overexpression on MUC5AC, MUC5B and the EGFR/MEK/ERK pathways. The effect of downregulating miR-146a-5p on ECC-BYF III-mediated amelioration of mucus hypersecretion and EGFR/MEK/ERK pathway activation was assessed. Downregulation of MUC5AC and MUC5B mRNA expression through inhibition of the EGFR/MEK/ERK pathway via miR-146a-5p upregulation may be an important pathway that regulates mucus hypersecretion. The expression of miR-146a-5p was downregulated by the transfection of miR-146a-5p inhibitors, indicating that ECC-BYF III could ameliorate BEAS-2B cell mucus hypersecretion by targeting miR-146a-5p and downregulating the EGFR/MEK/ERK pathway. Other mechanisms remain to be discussed.

Conclusion

The study did not consider the long-term implications of ECC-BYF III treatment on mucus hypersecretion and inflammation. We will further explore and verify it in clinical practice. ECC-BYF III can ameliorate mucus hypersecretion and reduce inflammation in BEAS-2B cells exposed to CSE. It can upregulate the expression of miR-146a-5p, thereby inhibiting activation of the EGFR/MEK/ERK pathway and ameliorating mucus hypersecretion by BEAS-2B cells. miR-146a-5p regulates EGFR/MEK/ERK, which is important but not the only mechanism by which ECC-BYF III ameliorates mucus hypersecretion by BEAS-2B cells.

Abbreviations

AECOPD, acute exacerbations of COPD; COPD, chronic obstructive pulmonary disease; CSE, cigarette smoke extract; DMSO, dimethyl sulfoxide; TCM, traditional Chinese medicine; ECC-BYF III, effective-component compatibility of Bufei Yishen formula III; ECC-BYF-H, Effective-Component Compatibility of Bufei Yishen Formula III high concentration; ECC-BYF-L, Effective-Component Compatibility of Bufei Yishen Formula III low concentration; ECC-BYF-M, Effective-Component Compatibility of Bufei Yishen Formula III middle concentration; EGFR, epidermal growth factor receptor; IL-1α, interleukin-1α; IL-4, interleukin-4; IL-8, interleukin-8; LPS, Lipopolysaccharide; miR-146a-5p inhibitors, has-microRNA-146a-5p inhibitors; miR-146a-5p mimics, mimics has-microRNA-146a-5p mimics; NC, Negative Control; NE, Neutrophil elastase; OD, optical density; TNF-α, tumor necrosis factor-α.

Data Sharing Statement

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors report no conflicts of interest in this work.

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