

Vitamin D3 alleviates cigarette smoke extract-mediated epithelial-mesenchymal transition and fibrogenesis by upregulating CC16 expression in bronchial epithelial cells

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Abstract. Vitamin D3 supplementation has been previously reported to inhibit the occurrence and development of chronic obstructive pulmonary disease (COPD). However, the underlying mechanism remains unclear. Epithelial-mesenchymal transition (EMT) and fibrogenesis have been associated with the development of COPD. The aim of the present study was to investigate the potential effects and mechanism of vitamin D3 in an *in vitro* model of cigarette smoke (CS)-induced EMT and fibrosis, with specific focus on the role of club cell protein 16 (CC16). CS extract (CSE) at different concentrations (5, 10 and 20%) was used to treat 16-HBE cells to induce EMT and fibrogenesis following which they were treated with vitamin D3. Subsequently, the 20% CSE group was selected for further experiments, where 16-HBE cells were divided into the following five groups: The control group; the CSE group; the low-dose vitamin D3 group (250 nM); the medium-dose vitamin D3 group (500 nM); and the high-dose vitamin D3 group (1,000 nM). Western blot analysis was used to detect the protein expression levels of the EMT-related proteins E-cadherin, N-cadherin, Slug and α -SMA, fibrogenesis-related proteins collagen IV and fibronectin 1, proteins involved in the TGF- β 1/SMAD3 signaling pathway and CC16. Immunofluorescence was used to measure the protein expression levels of E-cadherin, N-cadherin and collagen IV. Specific CC16 knockdown was performed using short hairpin RNA transfection to investigate the role of CC16. The results of the present study found that vitamin D3 could increase the protein expression level of CC16 to inhibit the activation of the TGF- β 1/SMAD3 signaling pathway;

thereby reducing the 20% increase in CSE-induced EMT- and fibrogenesis-related protein expression levels. Following CC16 knockdown, the inhibitory effects of vitamin D3 on EMT- and fibrogenesis-related protein expression were partially reversed. To conclude, these results suggest that vitamin D3 can inhibit the protein expression levels of EMT- and fibrogenesis-related proteins induced by CSE, at least partially through the function of CC16. These findings are expected to provide novel theoretical foundations and ideas for the pathogenesis and treatment of COPD.

Introduction

Chronic obstructive pulmonary disease (COPD) is characterized by persistent airflow limitation to and from the lung, which has become a growing global health issue (1,2). In addition, air pollution and genetic factors are important risk factors for COPD (3-6). However, currently available therapeutic options for COPD, including methylxanthines, β 2-agonists, anticholinergics, corticosteroids and phosphodiesterase 4 inhibitors, were not able to completely halt the progression of COPD pathogenesis nor reduce the mortality rate (7-9). Therefore, there remains to be an urgent demand to explore novel treatment strategies to prevent the development of COPD.

Although the pathogenesis of COPD has not been fully elucidated, it has been proposed that epithelial-mesenchymal transition (EMT) and fibrogenesis may be involved (10,11). During EMT, epithelial cells gradually lose their epithelial phenotype and acquire typical mesenchymal characteristics (12-15). These are characterized by enhanced mitotic ability and extracellular matrix production, accompanied by the upregulation of N-cadherin, slug and α -SMA expression and the downregulation of E-cadherin expression (12-15). The main pathological changes that occur during fibrosis are increases in fibrous connective tissue generation in the organ tissue, decreases in parenchymal cells (16). Continuous EMT and fibrosis progression can lead to the destruction of organ structure and functional decline (17). This serves to be one of the main mechanism of small airway narrowing and is now considered to be the most important mechanism of COPD progression (18). Epithelial cell fibrogenesis

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process is frequently accompanied with upregulation of fibrogenesis-related protein expression, such as collagen IV and fibronectin 1 (FN1) (19). Previous studies have provided evidence that EMT is active in the airway of smokers, particularly in patients with COPD who smoke, suggesting that smoking-induced EMT can contribute to the pathogenesis of COPD (15,20).

Patients with COPD and acute obstructive pulmonary disease were frequently found to have vitamin D deficiency (21). Vitamin D (calcitonin) deficiency was reported to be positively associated with the severity of COPD (21). In addition, patients with COPD or more severe disease were found to have lower serum vitamin D levels (22). A previous study has suggested that treatment with vitamin D in patients with COPD and vitamin D deficiency can reduce the risk of moderate-to-severe disease (23). Furthermore, a recent study on patients with COPD caused by COVID-19 showed that although vitamin D deficiency is not a determinant of disease severity, supplementation does confer a positive role in alleviation (24). Cigarette smoke extract (CSE) can inhibit vitamin D-induced vitamin D receptor (VDR) translocation (25). Mathysen *et al* (26) previously documented that the use of vitamin D supplements may reduce CSE-mediated cellular inflammatory responses by upregulating cathelicidin expression in bronchial epithelial cells. However, the underlying molecular mechanism remain unknown (26). In another previous study, although vitamin D deficiency exacerbated pulmonary fibrosis and EMT (27), no significant associations between the two conditions could be found. Vitamin supplements have been found to delay the process of fibrosis (28). However, the role of vitamins in CSE-induced fibrogenesis in bronchial epithelial cells remains unclear.

Club cell protein 16 (CC16) is the most abundant protein in the bronchoalveolar lavage fluid (29,30). It is encoded by the *secretoglobin family 1A member 1* gene and serves an anti-inflammatory and antioxidant role *in vivo* (29,30). Previous studies have shown that CC16 mainly exerts anti-inflammatory effects in smoke-exposed lungs, such that COPD has been associated with CC16 deficiency (31,32). Smokers and patients with COPD were found to exhibit reduced airway CC16 immunostaining, which decreased further with increasing COPD severity (31-33). Exposing mice to CSE was found to reduce the airway expression of CC16 (34). Therefore, CC16 is becoming of interest as a target molecule for the treatment of COPD. CC16 has been associated with the occurrence and development of COPD, with the severity and prognosis of the disease (34). The dynamic changes in CC16 expression can be used to predict changes in the condition of patients with COPD and to evaluate the clinical outcome. Combined with the comprehensive analysis of other common clinical indicators, the length of the stay in hospital can be shortened as analyzed by linear correlation analysis and multiple linear regression analysis (33,35,36). Therefore, the present study hypothesized that CC16 is associated with lung fibrogenesis during COPD. However, the effects of vitamin D3 on the expression of CC16 after CSE exposure and its underlying molecular mechanism remain unclear.

In COPD, the bronchial epithelium is the first immune barrier triggered by cigarette smoke (37). The present study aimed to investigate the effect of vitamin D3 supplementation

on EMT and fibrogenesis in bronchial epithelial cells after CSE treatment. In addition, the role of CC16 in these processes and the expression of EMT and fibrogenesis-related markers were detected.

Materials and methods

Bioinformatics Methods. The interaction between CC16 and FN1 was analyzed using the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) database (version 11.5; <https://string-db.org/cgi/input.pl>).

Bronchial epithelial cell culture. The 16-HBE cells were purchased from the American Type Culture Collection and were cultured in DMEM (Sigma-Aldrich; Merck KGaA) supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin G sodium and 100 µg/ml streptomycin sulfate (Invitrogen; Thermo Fisher Scientific, Inc.). They were cultured at 37°C in a humidified incubator with 5% CO₂. The cells were cultured until they reached 70-80% confluence.

CC16 short hairpin (sh) RNA. The pLentiLox 3.7 lentiviral plasmid shRNAs targeting CC16 and scramble control shRNA were purchased from Hanbio Biotechnology Co., Ltd. The cells were seeded into a 96-well plate for 24 h at 37°C until 70-80% confluence, before the cells were transfected with the shRNAs (50 nM) using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocols. The efficiency of transfection was determined using reverse transcription-quantitative PCR (RT-qPCR) and western blot analyzes after 48 h of transfection. The sequences were as follows: ShRNA-CC16-1, sense 5'-CCAGAGAAAGCA TCATTA-3', antisense 5'-TTAATGATGCTTTCTCTGG-3'; shRNA-CC16-2, sense 5'-CCCAAAGCTCACTGTGTAA-3', antisense 5'-TTACACAGTGAGCTTTGGG-3'; shRNA-NC, sense 5'-GATCCCCTTCTCCGAACG-3', antisense 5'-AGC TAAAAATTCTCCGAAC-3'.

Preparation of CSE. The smoke from 15 lit cigarettes (Sichuan China Tobacco Industry Co., Ltd.) was slowly inhaled into a 50 ml syringe, and then injected it into DMEM pre-heated in a 37°C water bath (38). The pH of DMEM was adjusted to 7.4 and sterilized using a 0.22-µm filter (EMD Millipore) and stored at -80°C. Serum-free DMEM was used to dilute 100% CSE to the required CSE concentrations (5, 10 and 20%). Cells were treated with vitamin D3 (250, 500 or 1,000 nM; Sigma-Aldrich; Merck KGaA) or vehicle (0.1% ethanol) for 30 min prior to 24 h treatment at 37°C with CSE and vitamin D3 or vehicle, after shRNA transfection (when it was required) (26).

Cell Counting Kit (CCK)-8 assay. Cell viability was performed using CCK-8 assay. A total of 1x10⁴ 16-HBE cells/well were seeded into 96-well plates and pre-treated with CSE followed by vitamin D3. In total, 10 µl CCK-8 reagent (Abcam) was added into each well and the samples were incubated at 37°C for 1-4 h. Subsequently, the absorbance in each well was measured at 460 nm using a microplate spectrophotometer (Bio-Rad Laboratories, Inc.).

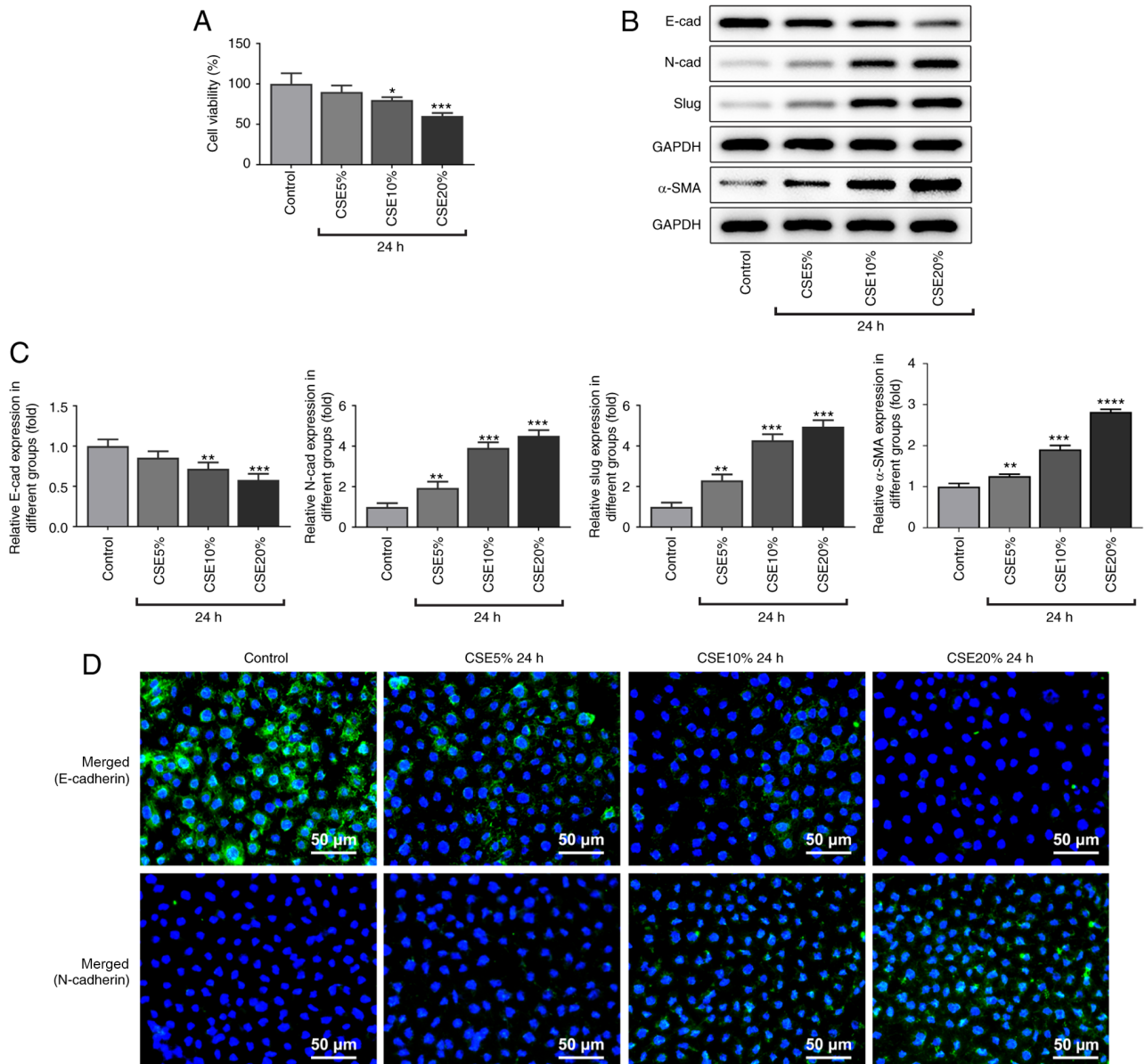


Figure 1. Protein expression levels of E-cadherin, N-cadherin, slug and α-SMA and the viability of 16-HBE cells following treatment with CSE. (A) Cell viability. (B) Protein expression levels of E-cadherin, N-cadherin, slug and α-SMA were measured by western blotting, (C) which were quantified. (D) Immunofluorescence staining of E-cadherin and N-cadherin. Scale bar, 50 μm. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001 vs. Control. CSE, cigarette smoke extract; α-SMA, α-smooth muscle actin.

RT-qPCR. Total RNA was extracted from the cells using TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc.). The concentration was assessed using a Nanodrop® 2000 spectrophotometer (Thermo Fisher Scientific, Inc.) before being reverse transcribed into cDNA. cDNA was synthesized using the PrimeScript™ RT kit (cat. no. RR037A; Takara Bio, Inc.) under the following conditions: 15 min at 37°C and 5 sec at 85°C. The reactions were performed using SYBR Green Taq Mix (cat. no. RR096A; Takara Bio, Inc.) in a StepOnePlus™ PCR system (Thermo Fisher Scientific, Inc.) under the following conditions: 45°C for 3 min, 95°C for 10 sec, 40 cycles at 95°C for 15 sec then 58°C for 1 min. The primer sequences used were as follows: CC16 forward, 5'-CAGAGACGGAAC CAGAGACG-3' and reverse, 5'-CAGATCTCTGCAGAA GCGGA-3' and GAPDH forward, 5'-GGAGCGAGATCCCTC CAAAAT-3' and reverse, 5'-GGCTGTTGTCATACTTCT

CATGG-3'. Gene expression was evaluated using the $2^{-\Delta\Delta C_q}$ method (39) using GAPDH as a reference gene.

Western blot analysis. The total protein was extracted from the cells using RIPA lysate buffer (Beyotime Institute of Biotechnology) containing protease inhibitors, phosphatase inhibitors and phenylmethylsulfonyl fluoride (all from Beyotime Institute of Biotechnology). The protein concentration was determined using a BCA protein determination kit (Beyotime Institute of Biotechnology). A total of 30 μg protein per lane extract was separated using 6-12% SDS-PAGE and transferred onto PVDF membranes. Subsequently, the PVDF membranes were blocked with TBS containing 5% BSA (Sigma-Aldrich; Merck KGaA) for 2 h at room temperature, before being incubated overnight at 4°C with the following primary antibodies: E-cadherin (1:1,000; cat. no. 14472;

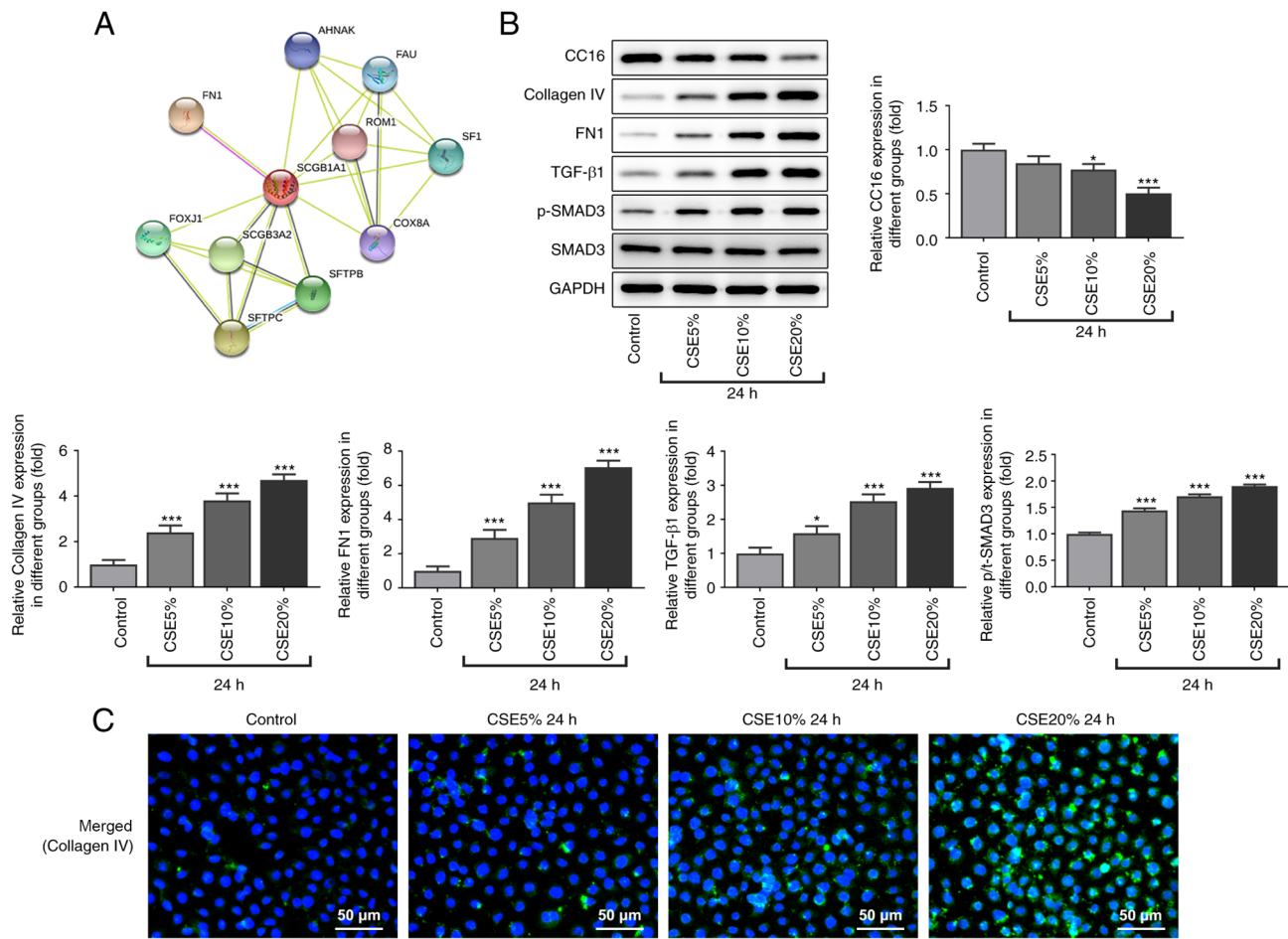


Figure 2. Effect of CSE on fibrogenesis-related protein expression in 16-HBE cells. (A) Interactions between CC16 and FN1 were predicted using the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) database. (B) Western blot analysis of CC16, collagen IV, FN1, TGF-β1, p-SMAD3 and SMAD3 protein expression levels. (C) 16-HBE cells were stained for collagen IV (green), whilst the cell nuclei were stained with DAPI (blue). Scale bar, 50 μm. * $P < 0.05$ and *** $P < 0.001$ vs. Control. CSE, cigarette smoke extract; p-, phosphorylated; CC16, club cell protein 16; FN1, fibronectin 1.

Cell Signaling Technology, Inc.), N-cadherin (1:1,000; cat. no. 13116; Cell Signaling Technology, Inc.), slug (1:1,000; cat. no. 9585; Cell Signaling Technology, Inc.), α-smooth muscle actin (1:2,000; α-SMA; cat. no. NBP2-78836; Novus Biologicals, LLC), collagen IV (1:1,000; cat. no. ab6586; Abcam), FN1 (1:1,000; cat. no. 26836; Cell Signaling Technology, Inc.), TGF-β1 (1:2,000; cat. no. sc-130348; Santa Cruz Biotechnology, Inc.), SMAD3 (1:1,000; cat. no. 9523; Cell Signaling Technology, Inc.), phosphorylated (p-) SMAD3 (1:1,000; cat. no. 9520; Cell Signaling Technology, Inc.) and CC16 (5 μg/ml; cat. no. RD181022220-01; BioVender). The membranes were then incubated with the goat anti-mouse IgG H&L HRP-conjugated secondary antibodies (1:20,000; cat. no. ab205719; Abcam) and goat anti-rabbit IgG H&L (1:50,000; cat. no. ab205718; Abcam) at room temperature for 2 h. Afterwards, they were washed for 25 min with TBS-0.1% Tween-20. After using an enhanced chemiluminescence reagent (Thermo Fisher Scientific, Inc.), the protein bands were detected using a Bio-Rad Imaging system (Bio-Rad Laboratories, Inc.) and analyzed using ImageJ software (version 1.43; National Institutes of Health).

Immunofluorescence staining. The cells were fixed in 4% paraformaldehyde (Beyotime Institute of Biotechnology)

for 1 h at room temperature, washed in PBS and blocked for 15 min in QuickBlock™ Blocking buffer for immunol staining (Beyotime Institute of Biotechnology) at room temperature. The samples were then incubated with primary antibodies overnight at 4°C. The following primary antibodies used targeted E-cadherin (1:200; cat. no. 14472; Cell Signaling Technology, Inc.), N-cadherin (1:200; cat. no. 13116; Cell Signaling Technology, Inc.), collagen IV (1:500; cat. no. ab6586; Abcam). Subsequently, the samples were incubated with corresponding Alexa Fluor® 488-conjugated secondary antibodies (Goat Anti-Rabbit IgG H&L; 1:1,000; cat. no. 150077; Abcam and Goat Anti-Mouse IgG H&L; 1:1,000; cat. no. 150113; Abcam) for 1 h at room temperature after washing with PBS. Then these samples were incubated for 5 min at room temperature with DAPI (1 μg/ml; cat. no. D1306; Invitrogen; Thermo Fisher Scientific, Inc.), and observations were performed using a fluorescence microscope (magnification, x100; Olympus Corporation) and were analyzed with ImageJ version 1.43 software.

Statistical analysis. Statistical analysis was performed using GraphPad 8.0 (GraphPad Software, Inc.). Data were expressed as means ± SEM of three independent experiments. Comparisons were performed using one-way analysis

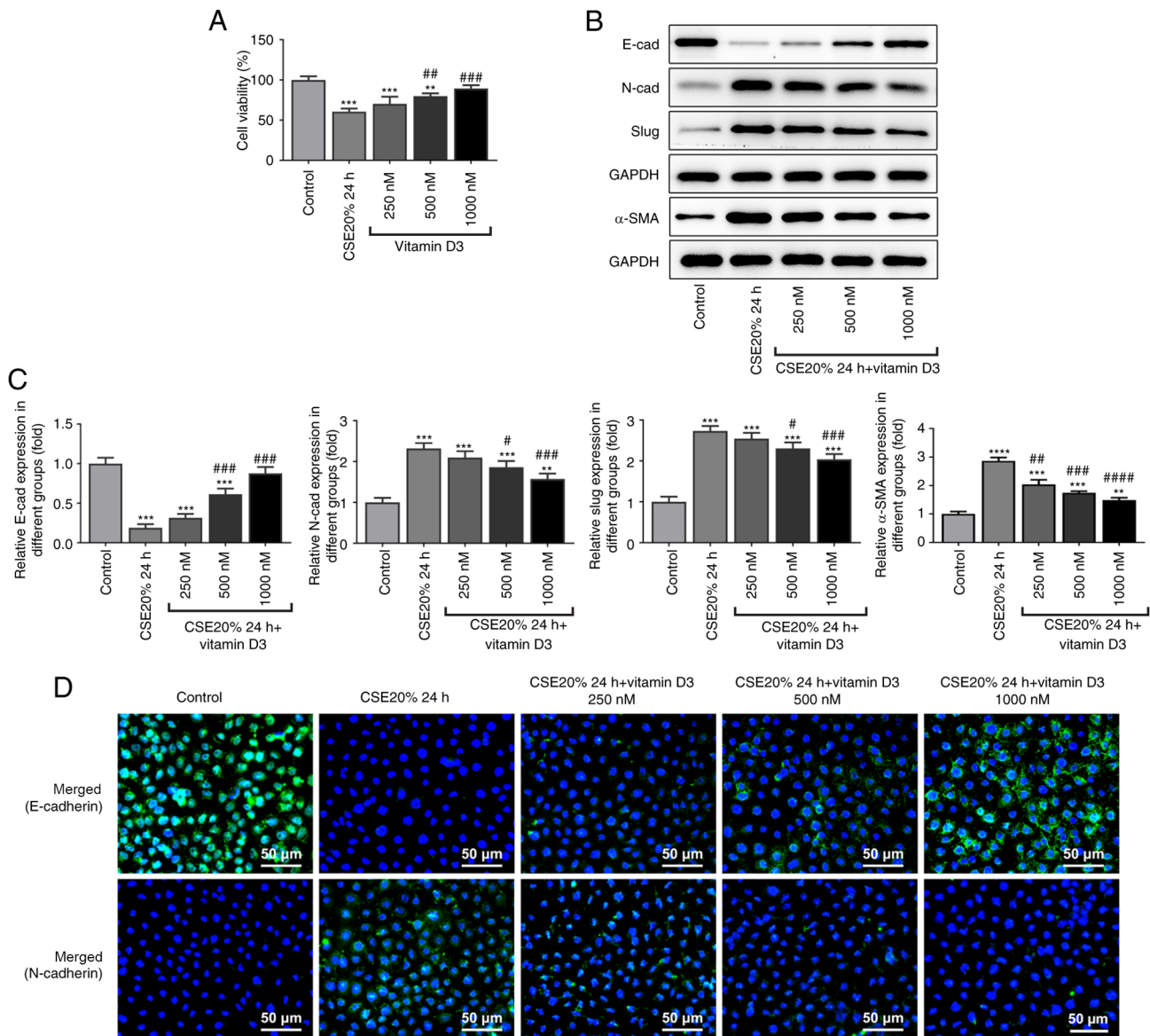


Figure 3. Effect of vitamin D3 on E-cadherin, N-cadherin, slug and α-SMA protein expression and viability in CSE-induced 16-HBE cells. (A) Cell viability. (B) Protein expression levels of E-cadherin, N-cadherin, slug and α-SMA were measured by western blotting, (C) which were quantified. (D) Immunofluorescence staining showing E-cadherin and N-cadherin expression. Scale bar, 50 μm. ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$ vs. Control. # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$ and #### $P < 0.0001$ vs. CSE 20% 24 h. CSE, cigarette smoke extract; α-SMA, α-smooth muscle actin.

of variance with Tukey-Kramer post hoc test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

CES reduces cell viability and induces EMT. After treating the 16-HBE cells with different concentrations of CSE (5, 10 and 20%) for 24 h, cell viability was decreased in a dose-dependent manner, comparing with that in the control group (Fig. 1A). CSE at a concentration of 20% was used for subsequent experiments. Western blot analysis revealed that the protein expression level of E-cadherin was decreased, whilst the protein expression levels of N-cadherin, slug and α-SMA were increased, in a dose-dependent manner following CSE treatment (Fig. 1B and C). The results of immunofluorescence were consistent with those in the western blot analysis results, revealing a decrease in E-cadherin protein expression and

an increase in N-cadherin protein expression compared with those in the control group (Fig. 1D). These results suggest that CSE promoted the transformation of these 16-HBE epithelial cells into mesenchymal-like cells.

CSE induces fibrogenesis in 16-HBE cells. It was first found that CC16 interacted with FN1, which is associated with fibrogenesis (40), from analysis using the STRING database (Fig. 2A). Therefore, western blot analysis was used to measure the protein expression level of fibrogenesis-related proteins. Compared with that in the control group, the protein expression level of CC16 in 16-HBE cells were decreased following CSE treatment. Furthermore, the protein expression levels of the fibrogenesis-related proteins collagen IV and FN1, in addition to those in the TGF-β1/SMAD3 signaling pathway, were increased in the CSE treatment groups in a dose-dependent manner compared with that in the control group (Fig. 2B). Immunofluorescence

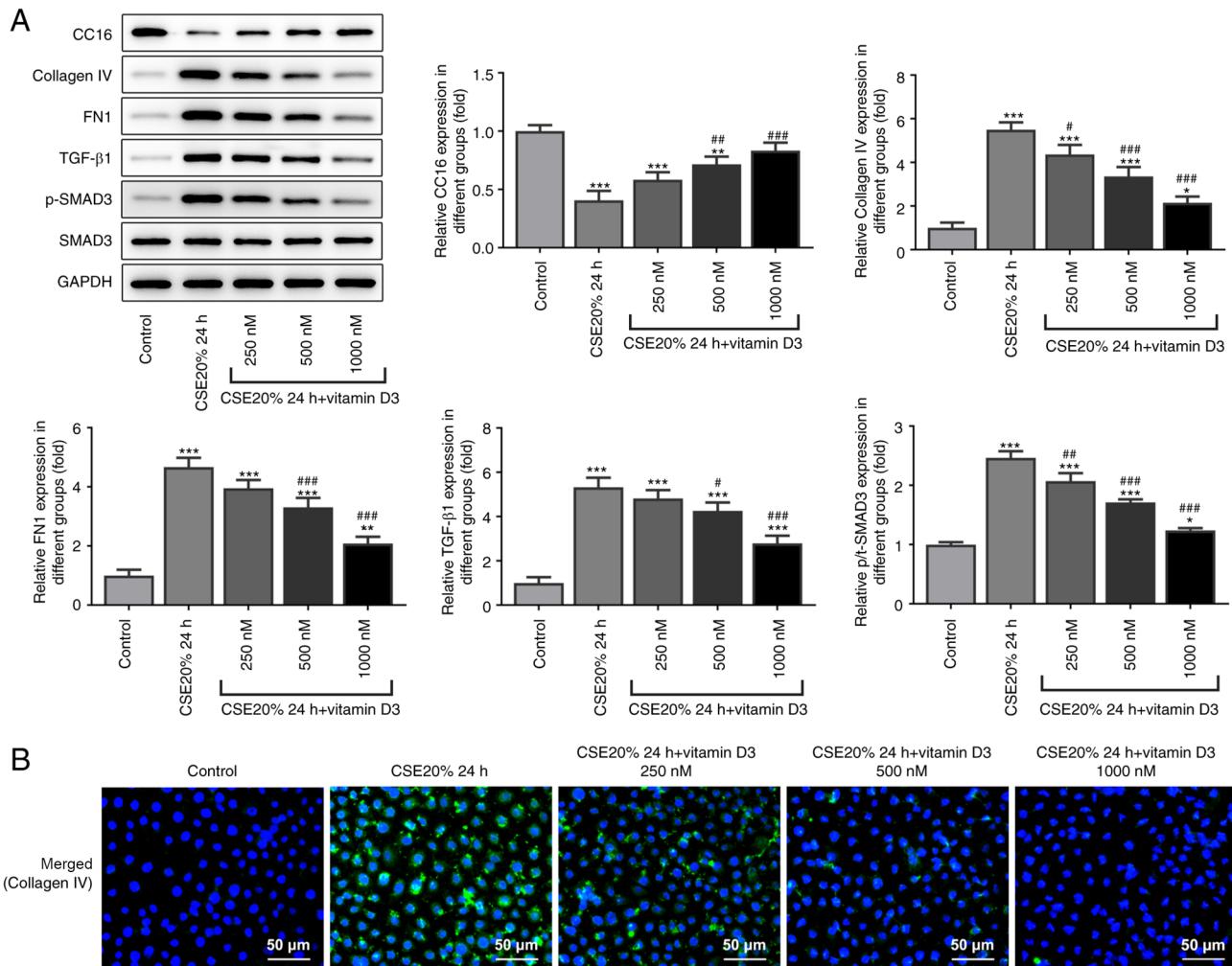


Figure 4. Effects of vitamin D3 on fibrogenesis-related protein expression levels in CSE-induced 16-HBE cells. (A) Western blot analysis of CC16, collagen IV, FN1, TGF-β1, p-SMAD3 and SMAD3 protein expression. (B) 16-HBE cells were stained for collagen IV (green) whereas all cell nuclei were stained with DAPI (blue). Scale bar, 50 μm. *P<0.05, **P<0.01 and ***P<0.001 vs. Control group. #P<0.05, ##P<0.01 and ###P<0.001 vs. CSE 20% 4 h. CSE, cigarette smoke extract; p-, phosphorylated; CC16, club cell protein 16; FN1, fibronectin 1.

results also showed that the expression level of collagen IV was also increased in the CSE treatment group compared with that in the control group (Fig. 2C). In summary, these results suggest that CSE induced fibrogenesis in the 16-HBE cell line.

Supplementation of vitamin D3 inhibits EMT caused by CSE in the 16-HBE cells. Vitamin D3 treatment did not appear to cause toxicity to 16-HBE cells treated with 20% CSE, instead increasing cell viability (Fig. 3A). Western blot analysis found that the protein expression level of E-cadherin was increased, whilst the protein expression levels of N-cadherin and slug were decreased following supplementation with vitamin D3, compared with those in the 20% CSE treatment alone group for 24 h (Fig. 3B and C). The results from immunofluorescence were consistent with those in western blot analysis (Fig. 3C), suggesting that vitamin D3 supplementation can reverse EMT induced by CSE.

Vitamin D3 supplementation inhibits fibrogenesis caused by CSE in the 16-HBE cell line. Compared with that in the CSE alone group, the protein expression level of CC16 was increased following supplementation with vitamin D3.

Western blot analysis was used to also measure the protein expression level of fibrogenesis-related proteins. The results showed that compared with that in the CSE alone group, the protein expression level of fibrogenesis-related proteins collagen IV, FN1, TGF-β1 and SMAD phosphorylation were decreased following administration with vitamin D3 (Fig. 4A). Immunofluorescence results showed that compared with that in the CSE alone group, the protein expression level of collagen IV was inhibited following supplementation with vitamin D3 (Fig. 4B). These results suggest that vitamin D3 can inhibit the CSE-induced fibrogenesis of bronchial epithelial cells (Fig. 4B).

Inhibitory effects of vitamin D3 on EMT and fibrogenesis is weakened following transfection with shRNAs targeting CC16 in the 16-HBE cells. To assess the role of CC16 in fibrogenesis and EMT, specific shRNAs were used to inhibit the expression level of CC16 in the 16-HBE cell line. Compared with that in the scrambled shRNA control group, both shRNA-CC16-1 and shRNA-CC16-2 significantly reduced CC16 expression in the 16-HBE cell line, with shRNA-CC16-1 being more potent (Fig. 5A). According to western blot analysis, CC16

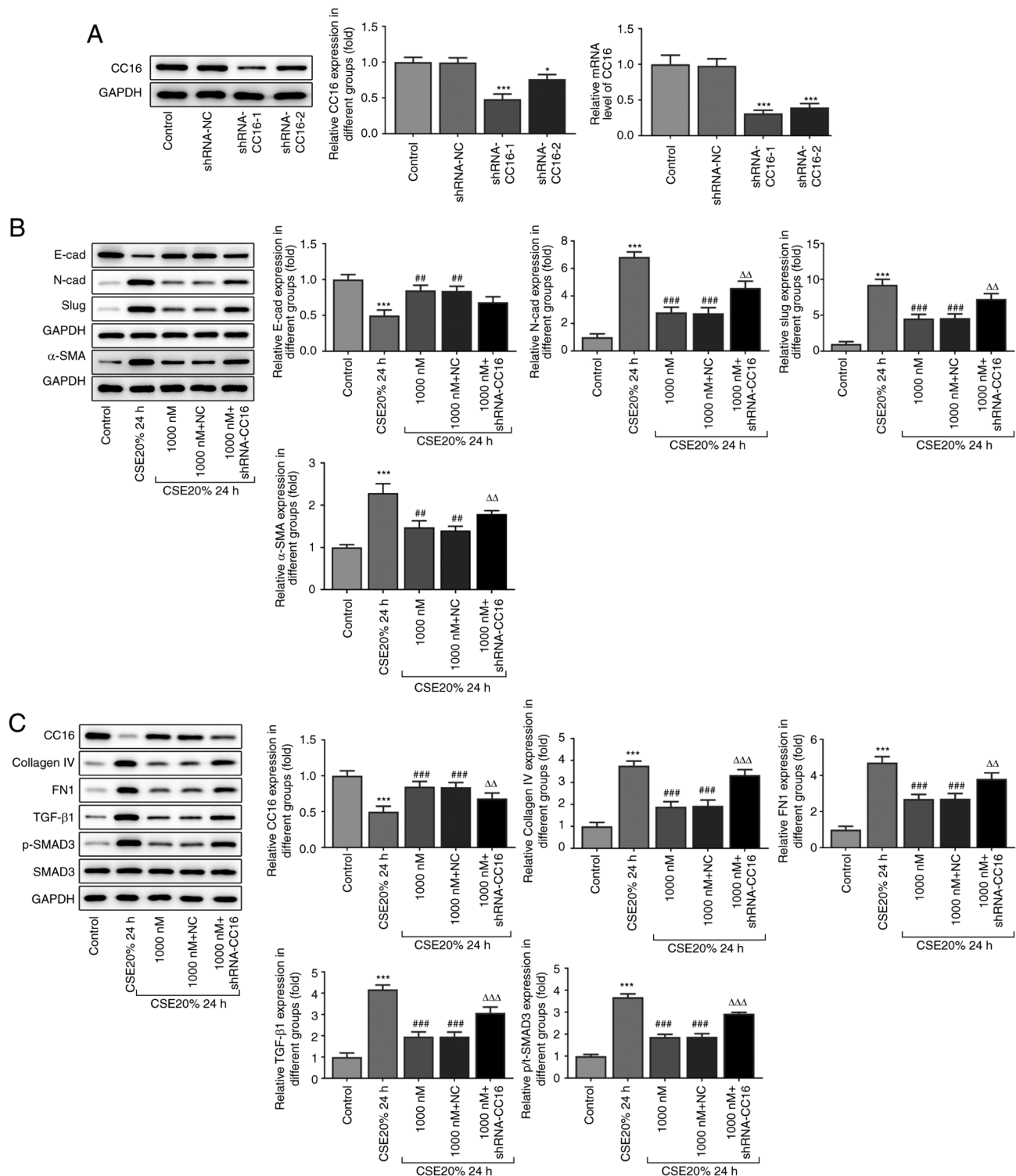


Figure 5. Effect of vitamin D3 on fibrogenesis- and epithelial-mesenchymal transition-related protein expression in CSE-induced 16-HBE cells after CC16 knockdown. (A) Western blotting and reverse transcription-quantitative PCR analysis of protein and mRNA expression levels of CC16 in 16-HBE cells following transfection with shRNA-CC16. The protein expression levels of (B) E-cadherin, N-cadherin, slug and α-SMA, in addition to (C) CC16, collagen IV, FN1, TGF-β1, p-SMAD3 and SMAD3 were measured using western blotting and quantified. * $P < 0.05$ and *** $P < 0.001$ vs. Control. ## $P < 0.01$ and ### $P < 0.001$ vs. CSE 20% 24 h. ΔΔ $P < 0.01$ and ΔΔΔ $P < 0.001$ vs. 1,000 nM + NC. shRNA, short hairpin RNA; NC, negative control; p-, phosphorylated; CC16, club cell protein 16; CSE, cigarette smoke extract; α-SMA, α-smooth muscle actin; FN1, fibronectin 1.

knockdown markedly reduced the protein expression level of E-cadherin whilst promoting the protein expression levels of N-cadherin, slug and α-SMA, compared with those in the vitamin D3 treatment group (Fig. 5B). Subsequent western blot analysis revealed that knocking down CC16 expression

led to significant increases in the protein expression levels of fibrogenesis-related proteins compared with those in the vitamin D3 treatment group (Fig. 5C). These results suggest that after transfection with shRNA-CC16-1, the inhibitory effects of vitamin D3 on EMT and fibrogenesis were reversed

in the 16-HBE cell line. In addition, these suppressive effects of vitamin D3 on CSE-induced EMT and fibrogenesis was at least partially mediated by CC16.

Discussion

COPD is a type of chronic bronchitis and/or emphysema with the pathological characteristics of airflow obstruction, which can further develop into common chronic diseases such as pulmonary heart disease and respiratory failure (41). COPD is associated with aberrant inflammatory reactions caused by harmful gases and harmful particles (42). Among these harmful factors, cigarette smoke considered to be one of the most common harmful particles that can cause COPD (43). Metal substances (such as Arsenic, cadmium and lead) and tobacco derivatives contained within cigarette smoke increases the risk of lung adenocarcinoma, such that ~6 million deaths are caused by tobacco use every year worldwide (44-46). Previous studies have shown that smoking contributes to lung remodeling prior to COPD, where CSE induces bronchial epithelial damage by increasing the susceptibility to respiratory infections (47-49). During this process, cells tend to undergo EMT and anchor-independent growth (50). In the present study, 16-HBE cells exposed to CSE exhibited EMT characteristics in a dose-dependent manner, with the highest N-cadherin, slug and α -SMA protein expression levels and the lowest E-cadherin protein expression levels being observed following 20% CSE exposure. Previous studies have found signs of peribronchiolar fibrosis in the small airways of patients with COPD, which EMT appearing to be involved in this process (47,51). The present study observed that the expression of fibrogenesis-related proteins collagen IV and FN1 were significantly upregulated in 16-HBE cells after being treated with 20% CSE for 24 h. These results suggest that CSE can induce EMT and fibrogenesis in bronchial epithelial cells.

According to analysis using the STRING database, CC16 is associated with the fibrosis-related protein FN1. CC16 is a major protein that is secreted by club cells in the bronchial epithelium and is eliminated by the kidneys (30). After observing this association between CC16 and fibrogenesis, the expression levels of CC16 was measured after CSE exposure. Previous study reported that CC16 can exert anti-inflammatory properties in smoke-exposed lungs of mice and proposed that COPD may be associated with CC16 deficiency (33). In the present study, downregulation of CC16 expression in CSE treated 16-HBE cells was also observed.

Over the past decade, a number of studies have shown that vitamins serve an important role in COPD (52-54). Vitamin A is essential for the preservation and integrity of the lung epithelium (55). Compared with that in the healthy group, the levels and intake of vitamin A in patients with COPD was significantly reduced whilst serum vitamin A levels were also found to be positively associated with the severity of COPD (56). In a previous clinical study that involved Korean patients with COPD, vitamin C was found to exert protective effects against COPD (57). In addition, another previous study hypothesized that the protective effects of vitamin C against COPD was due to its antioxidant effects (52). Vitamin B12, vitamin E and vitamin K were also found to serve key roles in inhibiting the occurrence and development of COPD (58-61). However, accumulating

evidence has found that vitamin D, which is a class of fat-soluble vitamins that includes vitamin D3 and D2, can exert a role in COPD (62,63). Its reported physiological effects include regulation of calcium and phosphorus metabolism, cell proliferation and differentiation, immune regulation and promotion of bone growth (64). Therefore, vitamin D has been of interest for the potential treatment and prevention of various diseases, such as chronic kidney disease and cystic fibrosis (65-67). In a previous *in vivo* study, vitamin D3 supplementation was found to alleviate lung injury in rats with COPD whilst also reducing cell apoptosis in the lung tissues (68). Clinical studies have also shown that vitamin D3 supplementation can alleviate moderate or severe COPD to reduce the incidence of upper respiratory tract infections (23-69). Furthermore, CSE has been reported to inhibit vitamin D-induced VDR translocation (25), but the vitamin D3/VDR axis can inhibit emphysema in patients with COPD (70). However, the specific mechanism of the protective effects of vitamin D3 against COPD remains unclear. Therefore, the present study investigated the effects of vitamin D3 on CSE-induced EMT and fibrogenesis in 16-HBE cells.

A previous study found that combining aerobic exercise with vitamin D3 supplementation can upregulate the levels of serum CC16 in patients with lung injury caused by smoking (71). However, the specific mechanism was not elucidated (71). Compared with the findings reported by Mathysen *et al* (26), this previous study found no clear association between vitamin D3 supplementation and the degree of EMT of bronchial epithelial cells (71). In the present study, EMT and fibrogenesis was induced in 16-HBE cells following exposure to CSE, which were alleviated by vitamin D3 in a dose-dependent manner. In addition, to verify the association between Vitamin D3 and CC16 in this process, CC16 shRNA was used to knock down its expression. The results showed that after CC16 expression was reduced, the protective effects of vitamin D3 on 16-HBE cells were significantly reversed, with EMT and fibrogenesis restored. This suggests that vitamin D3 can mediate protective effects by increasing the protein expression of CC16. This provides a novel mechanism to further the understanding of the protective role of vitamin D3 against COPD. However, further investigation is required.

During EMT, the TGF- β 1/SMAD signaling pathway serves an important role (72). A previous study has shown that smoking can increase TGF- β 1 production (73). The levels of EMT and TGF- β in the airway epithelial cells of patients with COPD were associated with the severity of peribronchial fibrosis and airway obstruction (74). In the present study, upregulation of TGF- β 1 expression was observed in CSE-treated 16-HBE cells. A previous study also suggested that the TGF- β 1/SMAD2/3 pathway can be a potential therapeutic target for EMT in malignant tumor cells derived from epithelial cells (75). Compared with that in healthy individuals, the expression level of TGF- β 1 and its downstream signal, SMAD2/3 was found to be significantly increased in the airways of patients with COPD (73). The present study showed that SMAD3 was activated in 16-HBE cells exposed to CSE. After treating the cells with vitamin D3, the expression of TGF- β 1 and the activation of SMAD3 was partially inhibited whilst the expression of CC16 was upregulated.

Bronchial epithelial cells were used for the present study. However, *in vivo* models must be applied for verification. In

addition, it is of importance to investigate further the exact mechanistic relationship between vitamin D3 and CC16.

In conclusion, vitamin D3 supplementation could inhibit CSE-mediated EMT and fibrogenesis by increasing the protein expression levels of CC16. After CC16 expression was knocked down using shRNA, the inhibitory effects of vitamin D3 on EMT and fibrogenesis was reversed, suggesting that vitamin D3 protected the bronchial epithelial cells by inhibiting EMT and fibrogenesis. To the best of our knowledge, the present study was the first to suggest that vitamin D3 can serve a inhibitory role in EMT and fibrogenesis by regulating CC16. This may furthering the understanding of the mechanism underlying the effects exerted by vitamin D3 supplementation in preventing the development of COPD.

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Availability of data and materials

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

Authors' contributions

HF guided the project, analyzed the data and wrote the manuscript. YM conceived the technical details and designed the experiments. HF and YM performed the experiments. YM and HF confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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