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WNT/β-catenin signaling regulates cigarette smoke-induced airway inflammation via the PPARδ/p38 pathway

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The mechanisms of WNT/β-catenin signaling involved in airway inflammation of chronic obstructive pulmonary disease (COPD) remain unknown, although recent observations have suggested an important contribution of the pathway in pulmonary parenchymal tissue repair and airway epithelium differentiation. We investigated the role of WNT/β-catenin signaling in cigarette smoke (CS)-related airway inflammation using patient lung tissues, human bronchial epithelial cells *(16HBECs),* and mouse models. Reduced activity of WNT/β-catenin signaling was observed in the airway epithelium of smokers with or without COPD. The mRNA expression of WNT transcription factor TCF4 negatively correlated with the pack year. The mRNA levels of WNT receptor FZD4 negatively correlated with the mRNA levels of IL-1β. CS exposure decreased the activity of WNT/β-catenin signaling in both 16HBECs and mice. *In vitro* studies demonstrated the upregulation of inflammatory cytokines TNF-α and IL-1β secretion induced by CS extract (CSE) could be attenuated by β-catenin activator SB216763 and be exacerbated by β-catenin small-interfering RNA (siRNA), respectively. Furthermore, the decrease in the expression of peroxisome proliferator-activated receptor (PPARδ) induced by CSE stimulation could be rescued by SB216763. SB216763 also attenuated the upregulation of phosphorylated p38 mitogen-activated protein kinase (MAPK) stimulated by CSE. Both PPARδ agonist and p38 MAPK inhibitor could suppress the TNF-α and IL-1β release induced by CSE treatment. In addition, PPARδ activation could abolish β-catenin siRNA-mediated aggravation of phosphorylated p38 MAPK in response to CSE. Finally, SB216763 treatment significantly ameliorated peribronchial inflammatory cell infiltration, leukocyte influx, and the release of TNF-α and IL-1β in the bronchoalveolar lavage fluid of CS-exposed mice. Taken together, our findings indicate that the reduced activity of WNT/β-catenin signaling induced by CS may promote inflammatory cytokine production in airway epithelium and have an essential role in airway inflammation in COPD by PPARδ/p38 MAPK pathway.

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Chronic obstructive pulmonary disease (COPD), which is characterized by persistent chronic inflammation in the airway and lung parenchyma,[1] is a growing cause of morbidity and mortality.[2,3] Cigarette smoke (CS), the most important etiological factor in the development of COPD, induces airway inflammation, airway epithelium injury, and repair. The airway epithelium acts as the first line of defense against environmental stimuli via the secretion of inflammatory mediators and recruitment of inflammatory cells.[4] Epithelium dysfunction has long been recognized to contribute to the development of inflammatory disorders of the lung.[5]

The WNT/β-catenin signaling, a regulator of lung morphogenesis and lung diseases,[6–8] has been shown to contribute to allergic airway inflammation.[9] β-Catenin is an important component of the WNT/β-catenin signaling pathway and its accumulation contributes to pathway activation. Briefly, in the absence of WNT ligands, β-catenin is captured by a destruction complex that contains glycogen synthase kinase 3β (GSK3β), APC, and casein kinase 1 (CK1), resulting

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in proteosome-mediated degradation. In contrast, in the presence of WNT ligands, activated frizzled receptors (FZD) bind to co-receptors (LRP), followed by the dephosphorylation and stabilization of β-catenin in the cytoplasm and its translocation into the nucleus, where it combines with the TCF/LEF transcription factor family to regulate the expression of important downstream target genes.[10,11] Recently, WNT/β-catenin signaling has been implicated in tissue injury and the regeneration of COPD.[12–14] The activity of WNT/β-catenin signaling was decreased in the alveolar epithelial type II (ATII) cells of COPD patients and an emphysema mice model.[12] Wang *et al*[13] found that the expression profile of WNT/β-catenin signaling was decreased in the small airway epithelium of COPD patients and smokers, suggesting its important role in airway epithelium differentiation. In addition, TGF-β1-induced WNT/β-catenin signaling activation was enhanced in the lung fibroblasts of COPD subjects, indicating a role for WNT/β-catenin signaling in the fibroblast phenotype and extracellular matrix (ECM) production.[14] However, little is known about the involvement of WNT/β-catenin signaling in CS-induced airway inflammation in COPD.

Peroxisome proliferator-activated receptor (PPARδ), which has been reported to have a role in inflammatory response, was a target of β-catenin/TCF regulation elements.[15,16] PPARs are nuclear receptor/ligand-activated transcription factors, composed of three known isoforms: PPARα, PPARδ, and PPARγ.[17] PPARs regulate various cellular processes involving lipid metabolism, glucose homeostasis, and inflammation.[18] Evidences concerning the impact of PPARδ in inflammatory process have increased in the last few years. PPARδ activation effectively inhibited very low-density lipoprotein-induced IL-1β release in THP-1 macrophages,[19] lipopolysaccharide-induced TNFα production in cardiomyocytes,[20] and TNFα-induced expression of vascular cell adhesion molecule-1 in human umbilical vein endothelial cells.[21] However, the potential roles of PPARδ in inflammatory responses of lung remain unknown.

Herein, the aim of this study was to investigate the function and the molecular mechanism of WNT/β-catenin pathway involved in the development of CS-induced airway inflammation. We found that CS reduced the activity of WNT/β-catenin pathway in human bronchial epithelial cells (16HBECs), lung tissues of mice, and COPD patients. Moreover, decreased WNT/β-catenin pathway contributed to CS-related inflammation via PPARδ/p38 mitogen-activated protein kinase (MAPK) cascade.

MATERIALS AND METHODS

Study Subjects

The patients enrolled in this study underwent lung resection for peripheral lung cancers. The tissue samples were resected >5 cm away from the cancer tissue. All samples were tumor-free tissues by examining tissues under a microscope. The patients were classified into the following groups: (1) non-smoking patients without COPD (controls) ($n=7$); (2) smoking patients without COPD (smokers) ($n=7$); (3) smoking patients with COPD (COPD smokers) ($n=18$) (Table 1). COPD was defined according to the Global Initiative for Chronic Obstructive Lung Disease (GOLD) guidelines (http://www.goldcopd.com). Patients in controls and smokers groups had a forced expiratory volume in 1 s (FEV1)/forced vital capacity (FVC) ratio ≥70%, whereas patients in COPD smokers group had airflow limitation (FEV1/FVC <70%). Spirometry was performed after using bronchodilator. Subjects with other systemic diseases, lung diseases other than COPD and lung tumors, pulmonary infection and antibiotic treatment for 4 weeks before the operation, those who used inhaled or oral glucocorticoids for 3 months before the operation, those who received chemotherapy or radiotherapy at any time before the absence of tumor- or pneumonia-free lung tissue specimens, and those who used biomass fuel and had history of occupational exposure were excluded. This study was approved by the Clinical Trial and Ethics Committee of West China Hospital, Sichuan University (approval no. ChiCTR-OCC-12002143), and all participants provided written informed consent.

Table 1 Clinical characteristics of study subjects

Clinical characteristics	Controls ($n=7$)	Smokers ($n=7$)	COPD smokers		
			GOLD I ($n=9$)	GOLD II ($n=5$)	GOLD III ($n=1$)
Male/female	0/7	7/0*	15/1*		
Age	58.9 ± 7.2	59.1 ± 12.5	59.4 ± 7.9		
Pack year		31.5 (18, 40)	40 (20, 60)		
FEV1/FVC%	75.3 ± 5.28	78 ± 2.4	59.6 ± 7.9 *†		
FEV1% pred	92.1 ± 8.6	90.3 ± 8.7	83.4 ± 20.7		

COPD, chronic obstructive pulmonary disease; FEV1, forced expiratory volume in 1 s; FVC, forced volume capacity; GOLD, Global Initiative for Chronic Obstructive Lung Disease.
Values are mean ± s.d. for normally distributed data or median (75th, 25th percentile) for non-normally distributed data.
P-values were calculated by one-way ANOVA tests (for age, FEV1% pred, and FEV1/FVC%; normally distributed data), Kruskal-Wallis H tests (for pack year; non-normally distributed data) or chi-square test (for male/female). *$P<0.05$ *vs* controls; †$P<0.05$ *vs* smokers.

Preparation of CS Extract

CS extract (CSE) was derived from three cigarettes (Jiao Zi, Chengdu Cigarette Factory, Chengdu, China; 1.0 mg nicotine and 14 mg tar per cigarette) was drawn slowly into a 50-ml syringe and bubbled through 10 ml of Dulbecco's modified Eagle's medium (DMEM). One cigarette yielded five draws of 50 ml with the syringe, with each individual draw requiring approximately 10 s to complete. This preparation, considered to be 100% CSE, was titrated to pH 7.4 and sterilized with a

0.22-mm syringe filter. Serum-free cell culture medium was used to dilute 100% CSE to the required CSE concentrations.

Cell Culture

The human airway epithelial cell line (*16HBECs*) was obtained from the American Type Culture Collections (Manassas, VA, USA). The cells were cultured in DMEM supplemented with 10% fetal bovine serum, 50 U/ml penicillin G sodium and 50 μg/ml streptomycin sulfate (Invitrogen, Carlsbad, CA, USA) and maintained at 37 °C in a humidified atmosphere containing 5% CO_2. The confluent *16HBECs* cultured in dishes were serum-starved overnight and then exposed to CSE for 24 h with and without a 4-h pretreatment of 10 μM SB216763 (Sigma-Aldrich, St Louis, MO, USA), or 30-min pretreatment of 10 μM SB203580 (Beyotime, Shanghai, China) or 30-min pretreatment of 1 μM GW0742 (Selleck Chemicals, Houston, TX, USA). The control groups studied in parallel received DMSO.

Small-Interfering RNA Preparation and Transfection

16HBECs was cultured in 12-well plate overnight to 40–60% confluence in complete medium without antibiotics. Validated small-interfering RNA (siRNA) oligonucleotides specific to β-catenin and negative control siRNA were purchased from Sigma (St Louis, MO, USA). Transient transfections were performed by using HiPerFect Transfection Reagent (Qiagen, Valencia, CA, USA) following the manufacturer's protocols. To avoid stress-induced secretion of cytokines in response to transfection, the control groups and CSE groups studied in parallel received the same volume transfection reagents.

Cell Viability Assay

The cell viability was determined with a Cell Counting Kit-8 (CCK-8, Dojindo Laboratories, Tokyo, Japan) to count living cells.[20] Briefly, *16HBECs* were seeded into 96-well plates at an initial density of 5000 cells per well. After incubation with the indicated concentrations of CSE for 24 h, 10 μl of kit reagent was added and incubated for a further 3 h. The cell viability was obtained by scanning with a Model 680 microplate reader (Bio-Rad Laboratories, Hercules, CA, USA) at 450 nm.

Treatment of Animals

Specific pathogen-free, male C57BL/6 mice weighing 18–20 g were housed under controlled conditions in standard laboratory cages. Adult animals were exposed to whole-body mainstream CS at 200 mg TPM/m^3 for 4 h per day, 5 days per week for up to 4 weeks using a Baumgartner-Jaeger CSM2082i automated cigarette smoking machine (CH Technologies, West-Wood, NJ, USA). The smoke was generated from Jiao Zi cigarettes. The control mice were exposed to filtered air in an identical manner. SB216763 (Sigma-Aldrich) was first dissolved in DMSO to 10 mg/ml and then diluted 10-fold with phosphate-buffered saline (PBS). After 2 weeks of exposure, the mice were treated with a daily i.p. injection of 0.6 mg/kg SB216763 or DMSO/PBS mixture 1 h before CS exposure. All experimental protocols were approved by the Institutional Animal Care and Use Committee of Sichuan University (Chengdu, China).

Bronchoalveolar Lavage and Cell Count

After 4 weeks of CS exposure, the mice were intraperitoneally anesthetized with 50 mg/kg sodium pentobarbital, followed by drawing blood from the right ventricle to allow tissue sample collection. The left lung was lavaged twice with 0.8 ml of sterile saline, with a recovery rate of 80%, and the recovered saline was centrifuged at 400 *g* for 5 min. The supernatant was immediately frozen at − 80 °C to measure the cytokines. The pellet was resuspended in 200 μl of cold PBS. The total bronchoalveolar lavage fluid (BALF) cell numbers were detected with a hemocytometer, and the differential cell counts were determined in cytospin preparations stained with Wright–Giemsa.[21] An experienced investigator, who was blinded to the experimental conditions, performed all enumerations.

Isolation of RNA and Real-Time PCR

The total RNA was isolated from lung tissues and *16HBECs* using the RNApure High-Purity Total RNA Rapid Extraction Kit (Bioteke, Beijing, China) according to the manufacturer's protocol. cDNA was then synthesized using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories). Quantitative real-time RT-PCR analysis was performed in triplicate with the CFX96 real-time PCR detection system using SsoFast EvaGreen Supermix according to the manufacturer's specifications (Bio-Rad Laboratories). The primer sequences and PCR conditions were shown in Supplementary Table S1. All data were normalized to internal control gene expression, and relative expression levels were determined using the $2^{-\Delta\Delta Ct}$ method.

Western Blot

Western blot analyses were performed as previously described with a few modifications.[22] Briefly, 16HBECs and tissues were prepared in lysis buffer containing 50 mM Tris-HCl, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 2 mM NaF, 2 mM EDTA, 0.1% SDS, and a protease inhibitor cocktail tablet (Roche Applied Science, Indianapolis, IN, USA). The lysates were sonicated on ice and centrifuged at 12 000 *g* for 30 min. The protein concentration in the cell lysates was measured using the bicinchoninic acid method (Pierce, Rockford, IL, USA). The cell lysate proteins were diluted with 5 × concentrated sample buffer and heated at 98 °C for 10 min before loading. After SDS–PAGE, the proteins were transferred to PVDF membranes (Millipore, Bedford, MA, USA). The membranes were incubated with a 1:1000 dilution of rabbit antibody to phosphorylated or total p38 MAPK, phosphorylated or total extracellular regulating kinase (ERK) MAPK, phosphorylated or total GSK3β, β-catenin, β-actin (Cell Signaling Technology, MA, USA) and PPARδ (GeneTex,

Irvine, CA, USA). The signals were developed using Super-Signal West Pico chemiluminescent substrate (Pierce).

Enzyme-Linked Immunosorbent Assay (ELISA)

The TNF-α and IL-1β secretion in cell the culture supernatant and non-diluted BALF were measured following the manufacturer's instructions (Neobioscience Technology, Beijing, China).

Lung Histopathology and Immunohistochemistry

The lung tissue was fixed in 4% formaldehyde (pH 7.4), embedded in paraffin and cut into 4-mm-thick sections. The paraffin sections were then stained with hematoxylin and eosin (H&E) to evaluate the morphological changes and inflammation in the lungs. For the immunohistochemical detection of β-catenin, the sections were stained with a human monoclonal antibody to β-catenin (1:200, Cell Signaling Technology). Lung cancer tissues, known to widely express β-catenin,[6,23,24] were used as positive control. The immunostaining pattern in the epithelium of airways was blindly evaluated by two investigators. The scoring method was performed according to Chiappara *et al*[25] with slight modification. The expression of β-catenin was classified according to the following grading system: the staining intensities were categorized as no staining (0), weak staining (1), moderate staining (2), or strong staining (3). The staining areas were scored as 0 (0%), 1 (1–10%), 2 (11–50%), and 3 (51–100%). The staining intensity and staining area scores were multiplied to yield a composite IHC scores. High-power fields of bronchial tissues were randomly sampled, and calculations were performed on five images per section. The average score of five images from each tissue was used for statistical analysis.

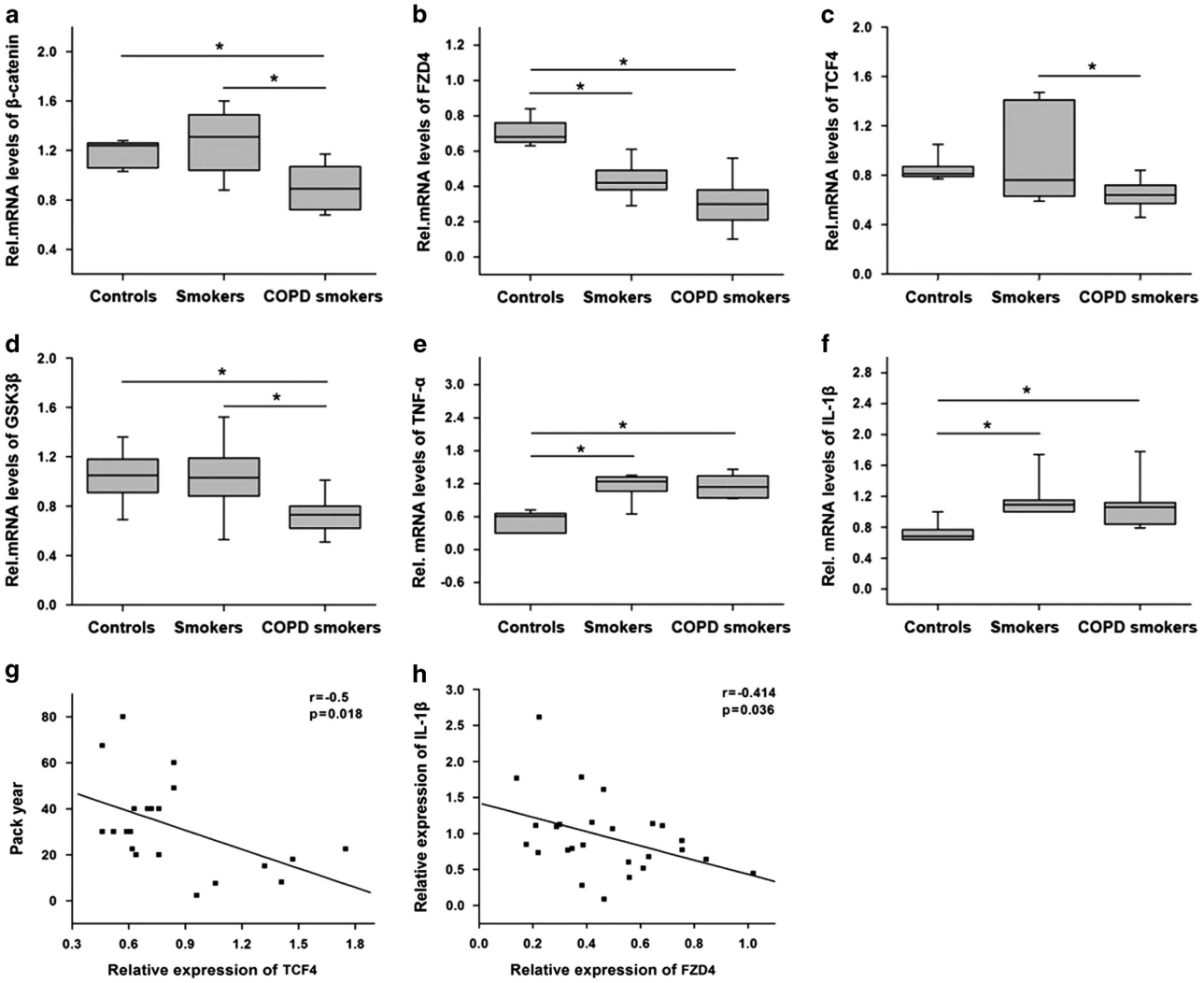

Figure 1 The mRNA expression of WNT/β-catenin signaling components and pro-inflammatory cytokines in human peripheral lung tissues. The mRNA expression of (**a**) β-catenin, (**b**) FZD4, (**c**) TCF4, (**d**) GSK3β, (**e**)TNF-α, and (**f**) IL-1β in lung tissue homogenates of the controls (no smoking patients), smokers (smoking patients without COPD), and COPD smokers (smoking patients with COPD) were assayed by real-time RT-PCR. The levels of mRNA were normalized to the β-actin values ($n = 7$–15). *$P < 0.05$. Correlation analysis was determined in **g** and **h**. COPD, chronic obstructive pulmonary disease.

Immunofluorescent Assay

The 16HBECs were plated in 24-well plate chambers. The cells were washed with 1× PBS and fixed with 4% paraformaldehyde for 15 min and then washed three times with 1× PBS, followed by blocking with normal goat serum (Boster, Wuhan, China) for 60 min at room temperature. The cells were incubated with primary antibodies against β-catenin (1:100) diluted in 1× PBS at 4 °C overnight in a humidified chamber. Subsequently, the cells were washed three times in 1× PBS and hybridized with Alexa Fluor-conjugated secondary antibody (Invitrogen) for 60 min at room temperature. DAPI (Sigma-Aldrich) was used to stain the nuclei. The images of cells were acquired using a Nikon fluorescence microscope.

Statistical Analysis

The data are expressed as the mean ± s.d. A statistical analysis was performed using a one-way analysis of variance (ANOVA) for studies with more than two groups. Correlations were determined with a Pearson correlation test. A significant difference was accepted at $P<0.05$.

RESULTS

The Activity of the WNT/β-Catenin Signaling Pathway Decreased in the Peripheral Lung of Smokers With or Without COPD

The baseline characteristics of study participants are shown in Table 1. The age, pack year, and FEV1% pred did not differ between groups, whereas the sex ratio and FEV1/FVC% did differ.

First, real-time PCR was used to investigate the mRNA levels of WNT/β-catenin signaling main components in the lung homogenates of controls, smokers, and COPD smokers. The results showed that the levels of β-catenin, FZD4, and GSK3β mRNA were decreased in the lung tissue specimens of COPD smokers compared with controls (Figures 1a, b, and d). The expression of FZD4 mRNA was lower in smokers than in controls (Figure 1b). Moreover, the mRNA expression of β-catenin, TCF4, and GSK3β was decreased in COPD smokers compared with smokers (Figures 1a, c, and d). The expression of WNT3A, LRP5/6, and LEF1 did not significantly differ between groups (data not shown). The levels of TCF4 mRNA negatively correlated with pack year (Figure 1g).

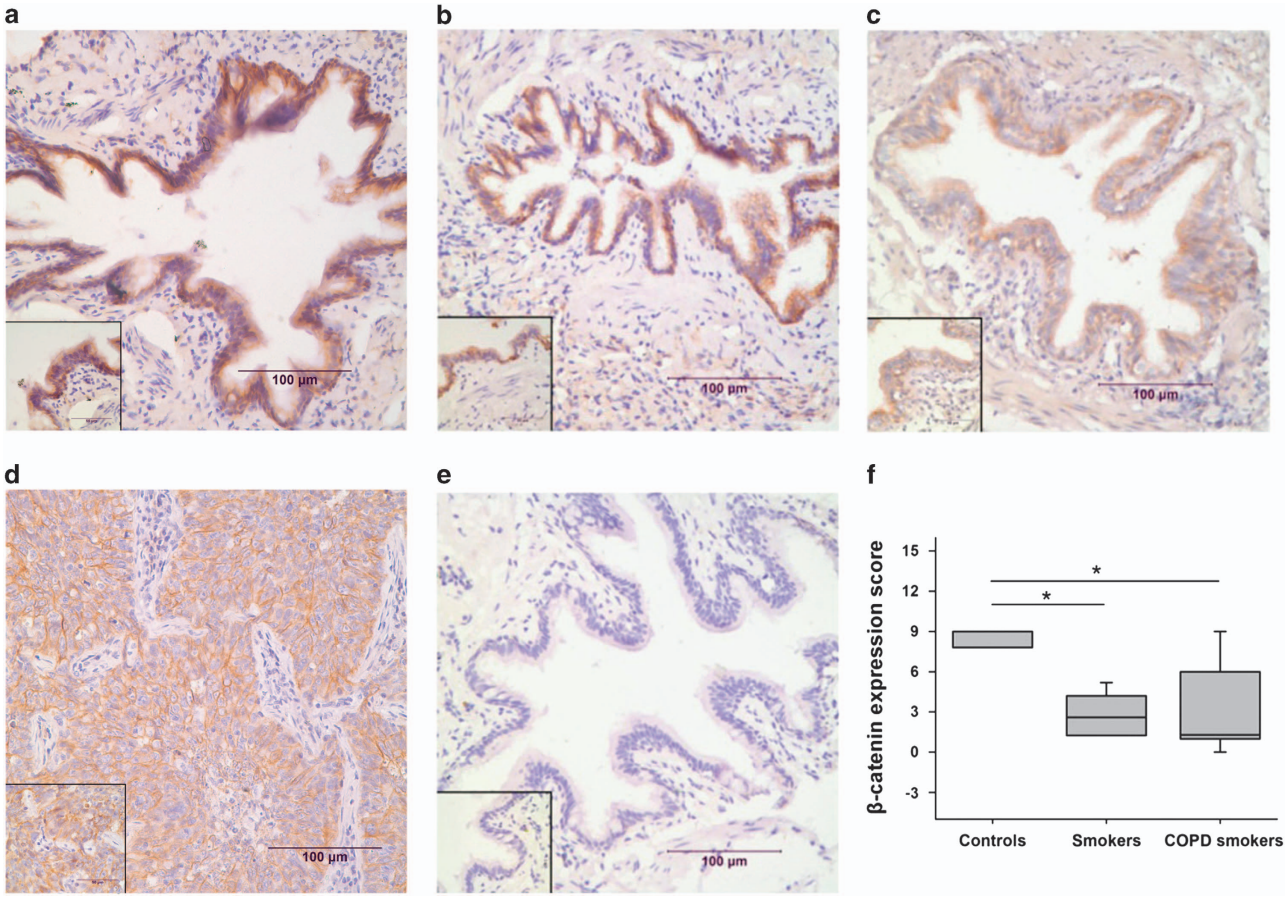

Figure 2 β-Catenin expression in airways. The location and level of β-catenin protein in airway epithelium of (**a**) controls, (**b**) smokers, and (**c**) COPD smokers were demonstrated by immunohistochemistry staining. (**d**) Lung cancer tissues stained with β-catenin antibody were designed to be a positive control. (**e**) Rabbit IgG staining was designed to be an isotype control. (**f**) Statistical analysis with a score of β-catenin level was performed for the airway epithelium of human lung tissues (see Materials and Methods for details). Original magnification is ×40. *$P<0.05$. COPD, chronic obstructive pulmonary disease.

The immunohistochemistry analysis showed that the staining of β-catenin was distributed in the airway epithelium and that the expression of β-catenin protein in the airway epithelium of smokers and COPD smokers was significantly lower than in controls (Figure 2). The results indicated that the WNT/β-catenin signaling activity was decreased in the airway epithelium of COPD patients, and smoking may be a key factor that decreases its activity.

Next, we measured the expression of TNF-α and IL-1β mRNA in the lung tissues of subjects. The results showed that the levels of TNF-α and IL-1β mRNA in smokers and COPD smokers were significantly higher than in controls (Figures 1e and f). The levels of FZD4 mRNA negatively correlated with the levels of IL-1β mRNA (Figure 1h), suggesting the important role of WNT/β-catenin signaling in airway inflammation of COPD.

CSE Treatment Decreased the Activity of WNT/β-Catenin Signaling in Human Bronchial Epithelial Cells

Next, we investigated the effect of CS on the activity of WNT/β-catenin signaling *in vitro*. First, the effect of CSE on the cell viability of 16HBECs was evaluated with a CCK-8 assay. As shown in Supplementary Figure S1, CSE exerted a dose-dependent cytotoxic effect on 16HBECs when the concentration exceeded 2%. Subsequently, we treated 16HBECs with 0.5–2% CSE for 24 h. Our results revealed that CSE decreased the expression of nuclear β-catenin in a dose-dependent manner (Figure 4a). Real-time RT-PCR was used to investigate the mRNA levels of WNT/β-catenin signaling components, including ligand WNT3A and WNT7B, receptor FZD1, FZD4, and LRP6, signal transducer β-catenin and GSK3β, transcriptional factors TCF4 and LEF1, target genes cyclin D1, MMP-2, MMP-9, and PPARδ in the 2% CSE-treated 16HBECs. The results showed that the mRNA levels of WNT3A, FZD1, FZD4, β-catenin, TCF4, cyclin D1, MMP-2, MMP-9, and PPARδ were decreased in the CSE-treated group compared with the control group (Figure 3). Furthermore, CSE treatment reduced both nuclear and total β-catenin protein in 16HBECs (Figures 4b and c). Similar results were obtained by immunofluorescent assay (Figure 4d). These results revealed that CSE reduced the activity of WNT/β-catenin signaling.

CSE-Induced Inflammatory Cytokine Release Dependent on WNT/β-Catenin Signaling in Human Bronchial Epithelial Cells

To functionally evaluate whether WNT/β-catenin signaling regulates CS-induced airway inflammation, we tested the effect of pharmacological activators or siRNA of β-catenin on the secretion of inflammatory cytokines. As a β-catenin activator, we used SB216763 that stabilizes β-catenin at the protein level.[26,27] Results showed that SB216763 significantly increased the protein levels of total and nuclear β-catenin (Figures 4a and c). Moreover, SB216763 also reversed CSE-induced downregulation of β-catenin protein

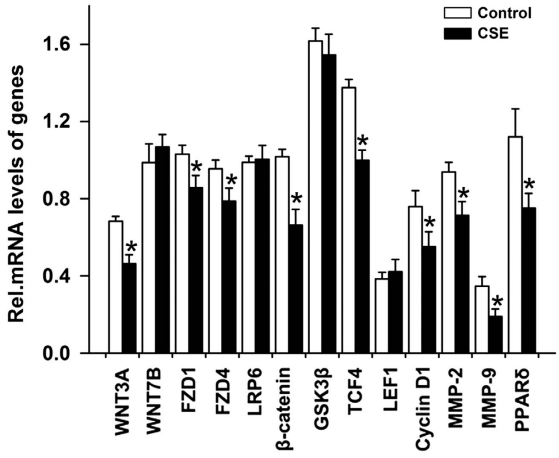

Figure 3 The mRNA expression of WNT/β-catenin signaling components in CSE-treated human bronchial epithelial cells. Human bronchial epithelial cells (16HBECs) were exposed to CSE for 24 h. The mRNA expression of WNT ligand WNT3A and WNT7B, receptor FZD1, FZD4, and LRP6, signal transducer β-catenin and GSK3β, transcriptional factors TCF4 and LEF1, target genes cyclin D1, MMP-2, MMP-9, and PPARδ were assayed by real-time RT-PCR. The levels of mRNA were normalized to the GAPDH values. The data were averaged from a duplicate of each sample and from three independent experiments. $^*P<0.05$. CSE, cigarette smoke extract; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

(Figures 4a and b). As shown in Figure 5c, all three β-catenin siRNAs (50, 100 nM) effectively inhibited the levels of β-catenin. Fifty nanomolar β-catenin siRNA3, which reduced the β-catenin expression by >90%, was used for the following experiments.

Then, the expression of inflammatory cytokine TNF-α and IL-1β were determined in CSE-treated 16HBECs. As shown in Figures 5a and b, CSE exposure significantly increased the secretion of both IL-1β and TNF-α, as well as the mRNA expression of IL-1β, but not TNF-α, which could be partially abrogated by SB216763 treatment. On the contrary, the downregulation of WNT/β-catenin signaling by β-catenin siRNA augmented the secretion of IL-1β and TNF-α stimulated by CSE treatment (Figure 5d). These results indicated that WNT/β-catenin signaling had an essential role in the CSE-induced inflammatory cytokine release in 16HBECs.

WNT/β-Catenin Signaling Pathway Regulated CS-Stimulated Airway Inflammation by a PPARδ/p38 MAPK–Dependent Manner

PPARδ, which has been reported to be involved in inflammation,[21,28] was a downstream target gene of WNT/β-catenin signaling.[16] Our PCR results in Figure 3 showed that the mRNA levels of PPARδ were decreased by CSE stimulation. To elucidate the molecular mechanism responsible for the WNT/β-catenin signaling in CS-induced airway inflammation, we first analyzed the effect of β-catenin signaling activation on the levels of PPARδ and p38 MAPK signaling in CSE-exposed 16HBECs. As demonstrated in Figures 6a and b, 24-h CSE exposure induced a decrease in

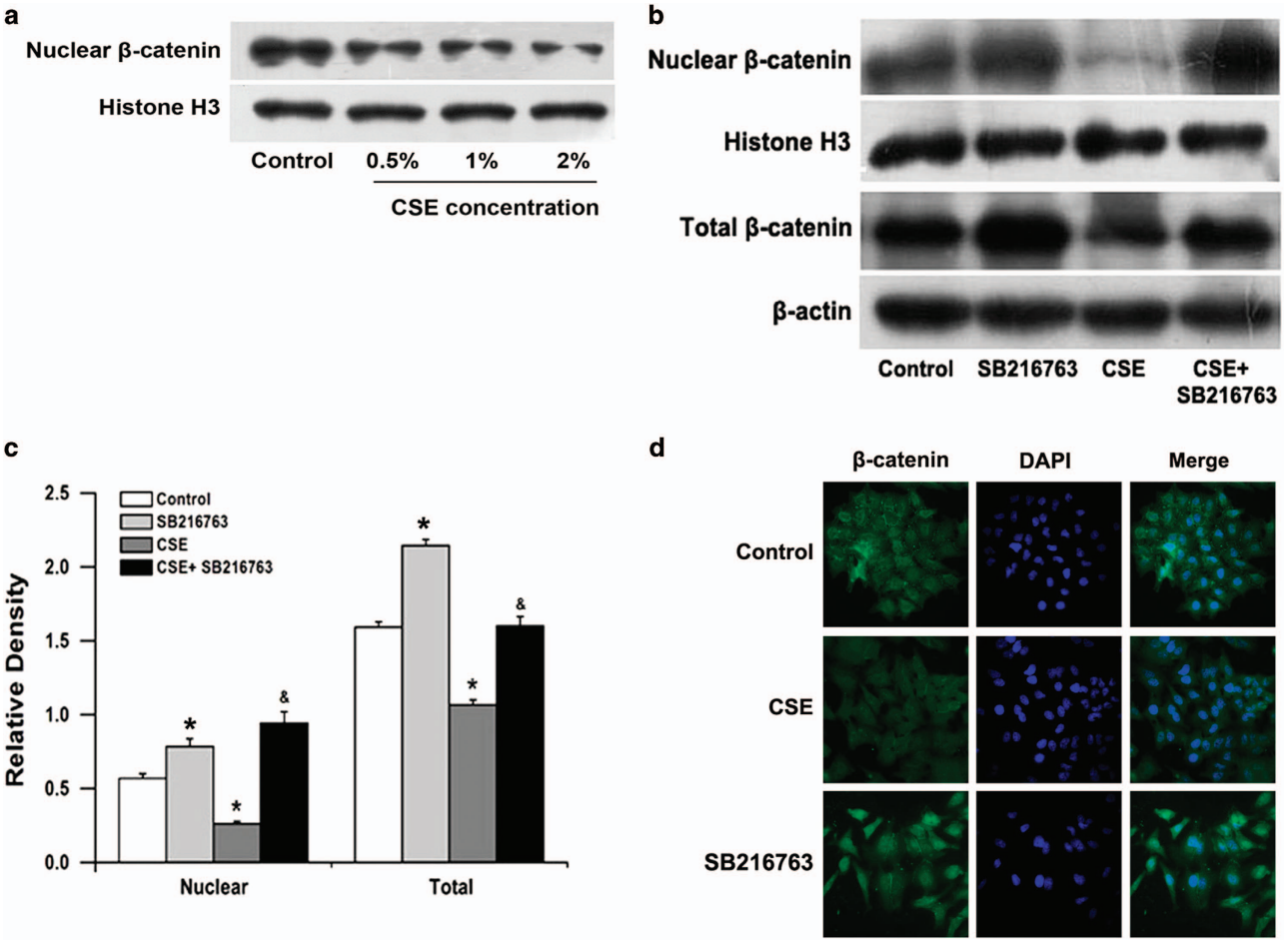

Figure 4 The activity of WNT/β-catenin signaling in CSE-treated 16HBECs. 16HBECs pretreated with or without β-catenin activator (SB216763, 10 μM, 4 h) were exposed to CSE for 24 h. (**a** and **b**) The levels of nuclear and total β-catenin protein in cells were measured by western blot. (**c**) Densitometry was performed, and the nuclear and total β-catenin protein levels were normalized to histone H3 and β-actin levels, respectively. Representative immunoblots of three independent experiments are shown. (**d**) The cells were processed for the immunofluorescent labeling of β-catenin (green). The nuclei were counterstained with DAPI (blue). All images show ×60 original magnifications. $^{*}P<0.05$ *vs* control group; $^{\&}P<0.05$ *vs* CSE group. CSE, cigarette smoke extract; DAPI, 4′,6-diamidino-2-phenylindole; 16HBEC, human bronchial epithelial cell.

the levels of PPARδ and an increase in the levels of phosphorylated p38 MAPK, which were inverted by SB216763 treatment. However, SB216763 has no effect on the expression of phosphorylated ERK MAPK stimulated by CSE (Figures 6a and b). The results suggested that PPARδ and p38 MAPK were the downstream target of β-catenin signaling in 16HBECs.

To determine whether PPARδ and p38 MAPK had an essential role in CSE-induced cytokine production in 16HBECs, PPARδ agonist GW0742 and p38 MAPK inhibitor SB203580 were prepared. First, western blot analysis showed that GW0742 significantly enhanced the expression of PPARδ (Figure 6c) and SB203580 obviously suppressed the levels of p-p38 (Figure 6d), respectively. Furthermore, both GW0742 and SB203580 effectively reversed the secretion of TNF-α and IL-1β induced by CSE treatment (Figures 6e and f), suggesting a requirement for PPARδ and p38 MAPK in CS-related airway inflammation. Then, our results showed that activated PPARδ by GW0742 pretreatment could effectively attenuate β-catenin siRNA-mediated aggravation of phosphorylated p38 MAPK in response to CSE (Figure 6g), which indicated WNT/β-catenin signaling may regulate CS-related airway inflammation dependent on PPARδ/p38 MAPK signaling.

Activation of WNT/β-Catenin Signaling Effectively Attenuated Experimental Airway Inflammation

Finally, we validated the role of WNT/β-catenin signaling in a mouse model of airway inflammation. The results showed that the levels of β-catenin and TCF4 mRNA (Figure 7a) and β-catenin protein (Figure 7b) in lung tissues of mice were decreased after CS exposure for 4 weeks, which could be reverted by SB216763 treatment. We then assessed whether WNT/β-catenin signaling activation could inhibit airway

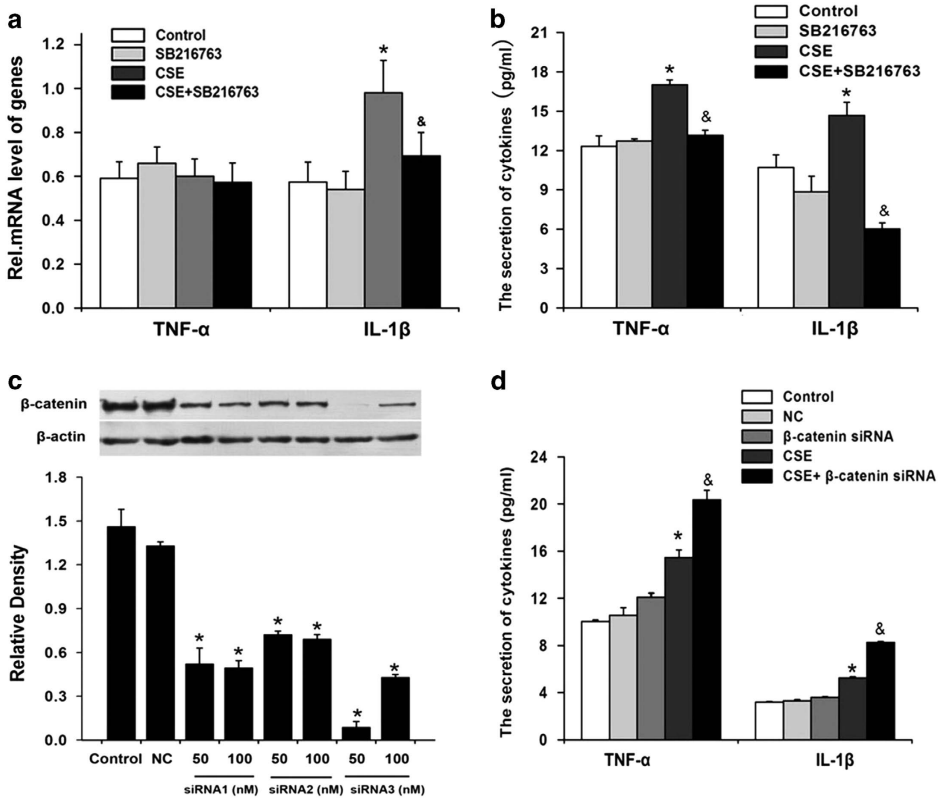

Figure 5 Effects of β-catenin activation or inhibition on the expression of inflammatory cytokines in response to CSE treatment in 16HBECs. 16HBECs pretreated with SB216763 (10 μM, 4 h) or transfected with negative control/β-catenin siRNA (50 nM, 24 h) were exposed to CSE for 24 h. The (**a**) mRNA and (**b** and **d**) protein levels of TNF-α and IL-1β were detected using real-time RT-PCR and ELISA, respectively. The data were averaged from a duplicate of each sample and from three independent experiments. (**c**) The total protein levels of β-catenin were detected by western blot. Densitometry was performed, and the levels were normalized to the β-actin levels. Representative immunoblots of three independent experiments are shown. $^{*}P<0.05$ *vs* control group; $^{\&}P<0.05$ *vs* CSE group. CSE, cigarette smoke extract; ELISA, enzyme-linked immunosorbent assay; 16HBEC, human bronchial epithelial cell.

inflammation in mice exposed to CS. As shown in Figure 8, SB216763 administration markedly attenuated the CS-induced peribronchial inflammatory cell infiltration, total leukocyte, and macrophage counts and secretion of TNF-α and IL-1β in BALF. A further investigation showed that the phosphorylated p38 MAPK levels markedly increased after CS exposure, which could be relieved by SB216763 treatment (Supplementary Figure S2). These findings indicated that the activation of WNT/β-catenin signaling may be a potential strategy to ameliorate CS-induced airway inflammation.

DISCUSSION

To better understand the development of COPD, the role of WNT/β-catenin signaling in CS-related airway inflammation was demonstrated. Studies in the lung tissues of patients showed that the activity of WNT/β-catenin signaling in lung specimens of smokers and COPD smokers was lower than in controls. A correlation analysis revealed that the pack year negatively correlated with the expression of TCF4 mRNA, and the mRNA levels of FZD4 negatively correlated with the mRNA levels of IL-1β. CS treatment significantly decreased the activity of WNT/β-catenin signaling in 16HBECs and in the mouse lung. Activation of WNT/β-catenin signaling effectively reduced CS-induced inflammatory responses and the secretion of pro-inflammatory cytokine TNF-α and IL-1β. We also found that the WNT/β-catenin signaling may have an essential role in CS-induced secretion of TNF-α and IL-1β via the PPARδ/p38 MAPK pathway. Taken together, these results showed, for the first time to the best of our knowledge, that aberrant WNT/β-catenin signaling was involved in the CS-related airway inflammation of COPD by a PPARδ/p38-dependent way.

WNT signaling pathway has been known to have a critical role in embryonic development and in adults.[29] The three established WNT signaling pathways are referred to as the WNT/β-catenin pathway (also known as canonical WNT pathway), the planar cell polarity pathway (also known as non-canonical WNT pathway), and the Wnt-Ca^{2+} pathway.[10] It has been reported that non-canonical WNT ligand WNT4 protein levels were higher in PBEC from COPD patients than both smokers and non-smokers, and treated cells with WNT4 increased the secretion of pro-inflammatory cytokines/chemokines and the activity of p38 MAPK.[30] Another study also found the upregulation of WNT4 in patients with COPD,

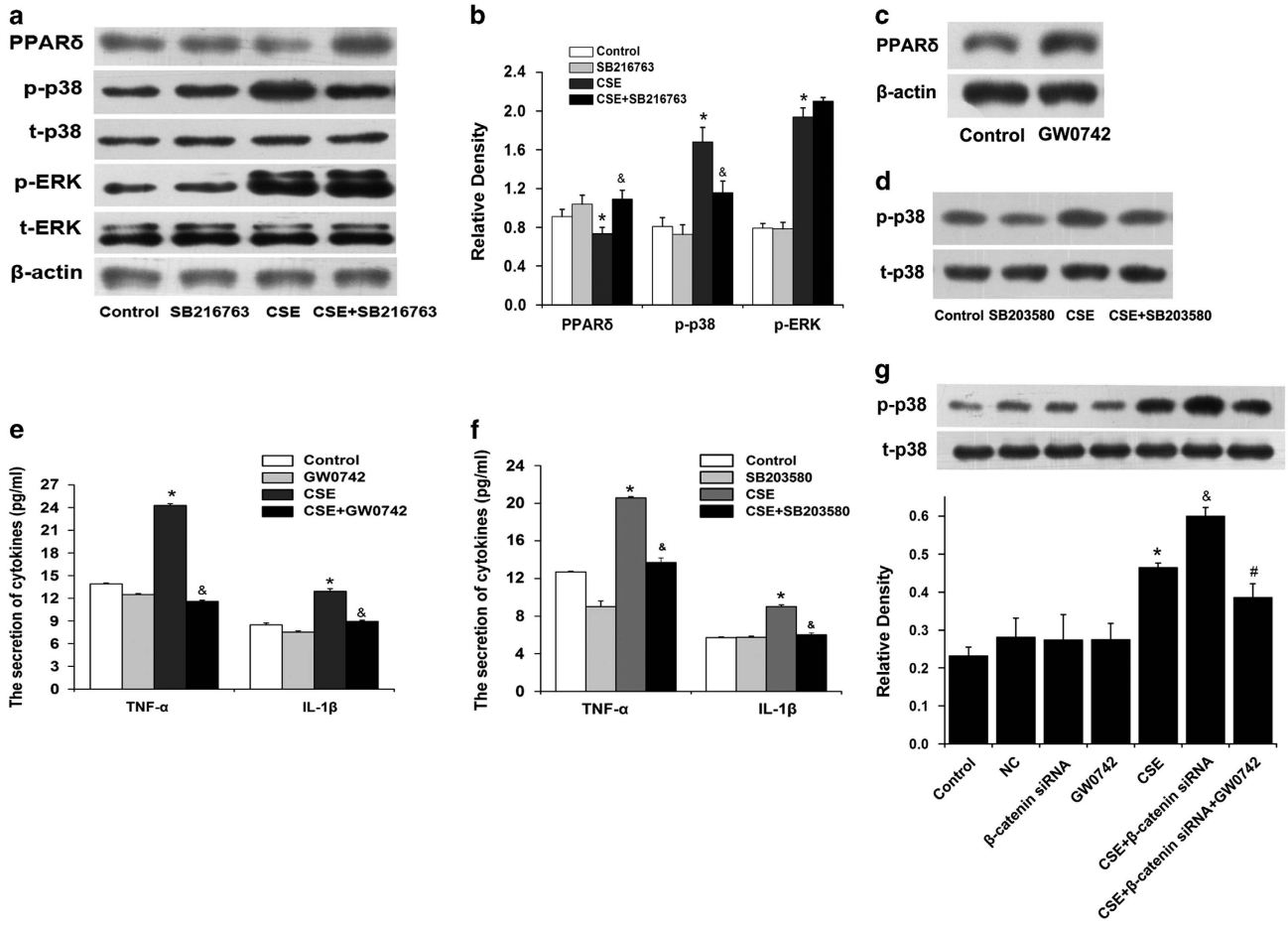

Figure 6 WNT/β-catenin signaling regulated CS-induced airway inflammation by a PPARδ/p38 MAPK-dependent pathway. 16HBECs pretreated with SB216763 (10 μM, 4 h), SB203580 (10 μM, 30 min), GW0742 (1 μM, 30 min), or pre-transfected with negative control/β-catenin siRNA (50 nM, 24 h) were exposed to CSE for 24 h. (**a**, **c**, **d** and **g**) The protein levels of PPARδ, phosphorylated and total p38 MAPK and phosphorylated and total ERK MAPK were detected by western blot. (**b**) Densitometry was performed, and the ratio of PPARδ/ β-actin, p-p38 MAPK/t-p38 MAPK, and p-ERK MAPK/t-ERK MAPK were calculated. Representative immunoblots of three independent experiments are shown. (**e** and **f**)The secretion of TNF-α and IL-1β in supernatants were detected by ELISA. The data were averaged from a duplicate of each sample and from three independent experiments. $^{*}P<0.05$ *vs* control group; $^{\&}P<0.05$ *vs* CSE group; $^{\#}P<0.05$ *vs* CSE +β-catenin siRNA group. CSE, cigarette smoke extract; ELISA, enzyme-linked immunosorbent assay; ERK, extracellular regulating kinase; 16HBEC, human bronchial epithelial cell; MAPK, mitogen-activated protein kinase; PPARδ, peroxisome proliferator-activated receptor.

and WNT4-induced airway epithelium proliferation and IL8 expression through the non-canonical pathway.[31] These two studies suggested that WNT4 promoted CS-independent airway inflammation in COPD via non-canonical WNT pathway. However, the effect of WNT pathway in CS-induced airway inflammation remains unknown. Although Heijink *et al* found that CSE decreased WNT4 and other WNT components in 16HBECs,[30] they did not study the potential role of the decreased WNT pathway in CS-related airway inflammation. Interestingly, it has been found that the mRNA levels of β-catenin were downregulated in the primary airway epithelium of smokers and smokers with COPD[13] and the number of β-catenin-positive staining alveolar epithelial cells were decreased in COPD patients compared with controls.[12] These studies suggested a crucial role of the WNT/β-catenin signaling in airway differentiation and alveolar tissue repair in COPD respectively. However, whether WNT/β-catenin signaling has an effect on CS-induced airway inflammation in COPD remains unclear. In this study, we found that β-catenin, which mainly distributed in the airway epithelium, was decreased in smokers and COPD smokers compared with controls. In addition, the pack year (smoking index) negatively related to the levels of TCF4 mRNA in the lung homogenates of patients. Moreover, CS stimulation reduced the activity of WNT/β-catenin signaling in 16HBECs and mice. These findings indicated that CS is a key factor reducing activity of WNT/β-catenin signaling in the airway epithelium. Subsequently, we observed that activation of β-catenin signaling by SB216763 attenuated the CS-induced inflammaion *in vivo* and *in vitro* study. In addition, suppression of β-catenin levels by siRNA aggravated the secretion of pro-inflammatory cytokines induced by CSE in 16HBECs. Our results pointed toward a protective role of the canonical WNT pathway in CS-induced airway inflammation.

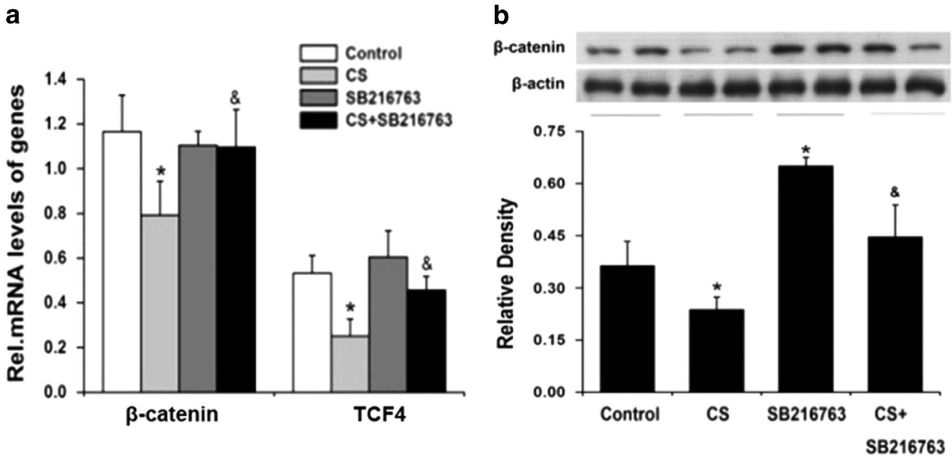

Figure 7 The activity of WNT/β-catenin signaling in lung tissues of CS-exposed mice. C57BL/6 mice were subjected to CS exposure (200 mg TPM/m^3, $n = 5–10$ per group) followed by SB216763 treatment (i.p., 0.6 mg/kg). (**a**) The mRNA expression of WNT/β-catenin signaling components were detected by real-time RT-PCR, and the levels of mRNA were normalized to the beta-2-microglobulin (B2M) values. (**b**) The levels of β-catenin protein were measured by western blot. Densitometry was performed, and the levels were normalized to the β-actin levels. Representative immunoblots of three independent experiments are shown. $^{*}P < 0.05$ *vs* control group; $^{\&}P < 0.05$ *vs* CS group. CS, cigarette smoke.

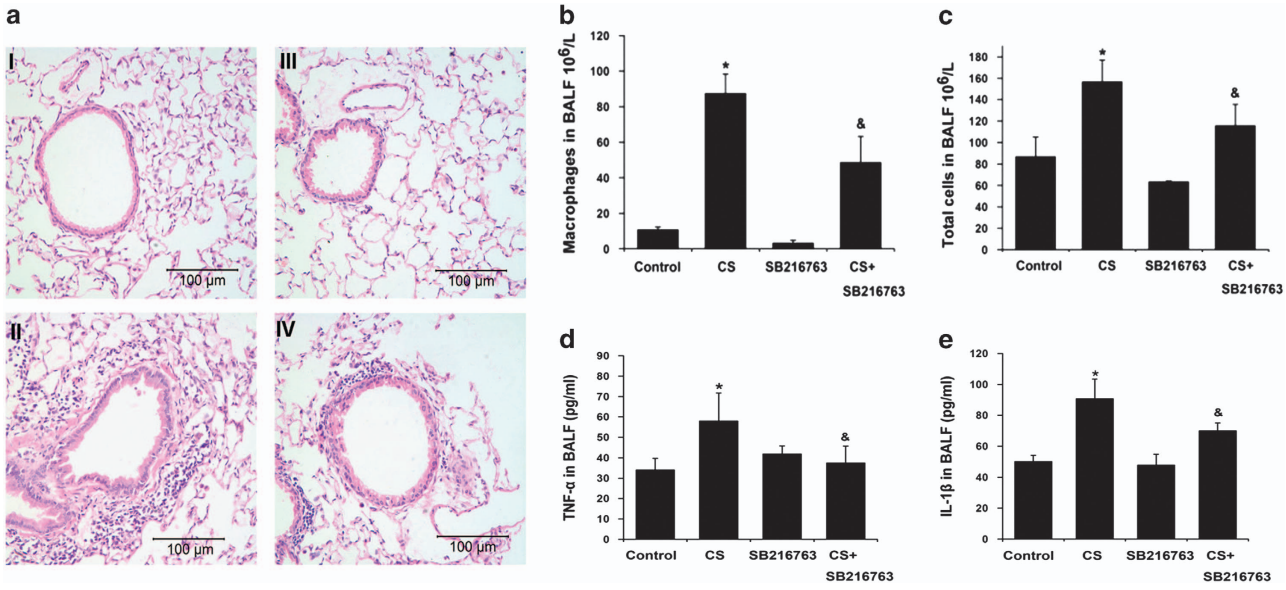

Figure 8 Pharmacological activation of WNT/β-catenin signaling attenuated the airway inflammation. C57BL/6 mice were subjected to CS exposure (200 mg TPM/m^3, $n = 5–10$ per group) followed by SB216763 treatment (i.p., 0.6 mg/kg). (**a**) Lung tissues were analyzed by hematoxylin and eosin staining (**a**I, control group; **a**II, CS group; **a**III, 0.6 mg/kg SB216763 group; **a**IV, CS+0.6 mg/kg SB216763 group). All images show ×40 original magnifications. (**b** and **c**) The numbers of macrophages and total cells in mice BALF were measured with a cell count. (**d** and **e**) The secretion of TNF-α and IL-1β in BALF were detected by ELISA. $^{*}P < 0.05$ *vs* control group; $^{\&}P < 0.05$ *vs* CS group. BALF, bronchoalveolar lavage fluid; CS, cigarette smoke; ELISA, enzyme-linked immunosorbent assay.

PPARδ, a widely expressed transcriptional factor, was reported to be a downstream target of WNT/β-catenin signaling.[15,16] He *et al*[16] found that transfected CRC cells with a dnTCF-4 expression vector significantly suppressed the activity of PPARδ promoter, which suggested that PPARδ was regulated by β-catenin/TCF pathway. We found that activation of WNT/β-catenin signaling by SB216763 reversed the reduction in the PPARδ protein induced by CSE stimulation, which suggested PPARδ may be a downstream target of β-catenin signaling (Figure 9). Whether TCF/LEF elements directly target the promoter of PPARδ or not in our study needs further investigation. Moreover, PPARδ was involved in inflammatory process,[21] while little is known in CS-related airway inflammation. We found that pretreated 16HBECs with PPARδ agonist GW0742 could attenuate CSE-induced upregulation of TNF-α and IL-1β, indicating an important

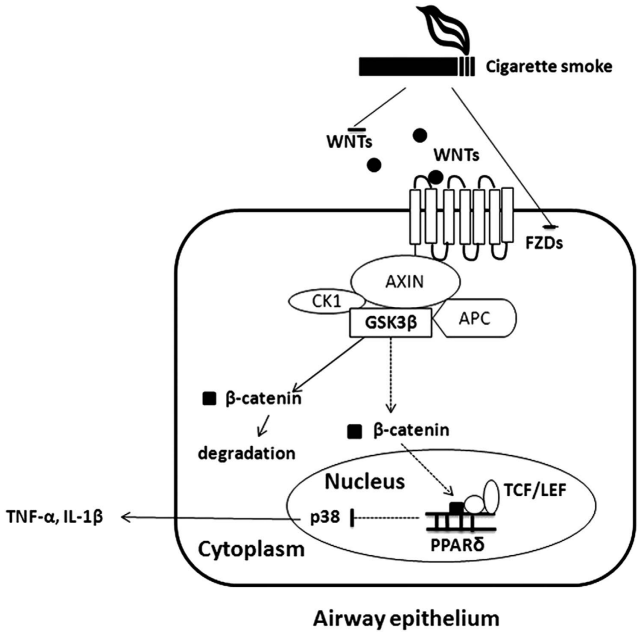

Figure 9 Proposed model of the mechanism of WNT/β-catenin signaling in CS-induced airway inflammation. When WNT ligands bind to WNT receptor FZDs, a destruction complex that includes APC, AXIN, GSK3β, and CK1 disassembles, followed by the stabilization of β-catenin in the cytoplasm and its translocation to the nucleus, where it combines with the TCF/LEF transcription factor family to promote the expression of downstream target gene PPARδ that can suppress the activity of p38 MAPK and the secretion of the pro-inflammatory cytokine TNF-α and IL-1β. In contrast, CS promotes the proteosome-mediated degradation of β-catenin, perhaps by inhibiting the expression of WNTs and FZDs. Thus, the TCF/LEF family cannot combine with PPARδ to promote its expression, leading to the upregulation of p38 MAPK activity, as well as TNF-α and IL-1β secretion. CS, cigarette smoke; MAPK, mitogen-activated protein kinase; PPARδ, peroxisome proliferator-activated receptor.

anti-inflammatory role of PPARδ in CS-related airway inflammation. It has been found that treatment with a synthetic PPARδ agonist inhibited activation of p38 MAP kinases in inflammatory circumstance,[19,28,32] which suggested a tight link between PPARδ and p38 MAPK.

The MAPK family includes three members: p38, ERK and c-Jun N-terminal kinase (JNK).[33,34] p38 MAPK has been observed in airway epithelial cells and macrophages responding to environmental stresses, and this pathway appeared to be important for the secretion of pro-inflammatory cytokines and chemokines.[35–38] Our findings observed that CS stimulated the activity of p38 MAPK and the release of TNF-α and IL-1β in airway epithelial cells and in a mouse model. In our previous study, we showed that the p38 MAPK inhibitor SB203580 effectively reduced acrolein (a component of CS)-induced TNF-α release in BALF and the lungs of mice.[39] In addition, SB203580 could also attenuate the CS-induced upregulation of TNF-α and IL-1β protein in mouse lung homogenates [40] and LPS-induced upregulation of IL-1β protein in monocytic cell lines.[41] Consistent with these studies, our present results revealed that SB203580 could alleviate the secretion of TNF-α and IL-1β induced by CSE in 16HBECs. Furthermore, we found that SB216763 and β-catenin siRNA decreased and increased CS-induced p38 MAPK phosphorylation, respectively, whereas it had no effect on ERK phosphorylation, suggesting that p38 MAPK may be a downstream target of β-catenin signaling in CS-induced airway inflammation (Figure 9). In addition, our study showed that PPARδ activation relieved the aggravation of CSE-induced p38 MAPK phosphorylation mediated by β-catenin siRNA. These results indicated that WNT/β-catenin signaling may have an anti-inflammatory role in CS-induced airway inflammation through PPARδ/p38 MAPK pathway (Figure 9), whereas the detail mechanism of PPARδ regulating the phosphorylation of p38 MAPK need further investigation.

We have found that the decreased WNT/β-catenin signaling was involved in CS-induced airway inflammation by regulating PPARδ/p38 MAPK pathway. However, how CS decreased WNT/β-catenin signaling remains unclear. Considerable evidences suggested that CS decreased the levels of antioxidants such as HO-1,[42] SOD,[43] and GSH,[44] and oxidant stress contributed to CS-induced pulmonary inflammation in COPD.[45] Moreover, oxidant stress could suppress the activity of WNT/β-catenin signaling,[46] and antioxidant HO-1 is the upstream of WNT/β-catenin signaling.[47,48] Therefore, we speculated that oxidant stress may have an important role in CS-induced airway inflammation by regulating antioxidants/β-catenin signaling in our study, and this needs our further investigation.

In summary, our data demonstrated that CS was a determinant of the WNT/β-catenin signaling activity in airway epithelium. The WNT/β-catenin signaling had a protective role in CS-induced airway inflammation via the PPARδ/p38 MAPK pathway. Understanding the specific molecular mechanism that underlies CS-induced airway inflammation may allow us to identify potential interesting target to modulate inflammatory responses.

Supplementary Information accompanies the paper on the Laboratory Investigation website (http://www.laboratoryinvestigation.org)

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DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

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