Cigarette smoke-induced epithelial expression of WNT-5B: implications for COPD

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ABSTRACT Wingless/integrase-1 (WNT) signalling is associated with lung inflammation and repair, but its role in chronic obstructive pulmonary disease (COPD) pathogenesis is unclear. We investigated whether cigarette smoke-induced dysregulation of WNT-5B contributes to airway remodelling in COPD.

We analysed WNT-5B protein expression in the lung tissue of COPD patients and (non)smoking controls, and investigated the effects of cigarette smoke exposure on WNT-5B expression in COPD and control-derived primary bronchial epithelial cells (PBECs). Additionally, we studied downstream effects of WNT-5B on remodelling related genes fibronectin, matrix metalloproteinase (MMP)-2, MMP-9 and Snail in BEAS-2B and air–liquid interface (ALI)-cultured PBECs.

We observed that airway epithelial WNT-5B expression is significantly higher in lung tissue from COPD patients than controls. Cigarette smoke extract significantly increased mRNA expression of WNT-5B in COPD, but not control-derived PBECs. Exogenously added WNT-5B augmented the expression of remodelling related genes in BEAS-2B cells, which was mediated by transforming growth factor (TGF)-β/Smad3 signalling. In addition, WNT-5B upregulated the expression of these genes in ALI-cultured PBECs, particularly PBECs from COPD patients.

Together, our results provide evidence that exaggerated WNT-5B expression upon cigarette smoke exposure in the bronchial epithelium of COPD patients leads to TGF-β/Smad3-dependent expression of genes related to airway remodelling.

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Introduction
Chronic obstructive pulmonary disease (COPD) is a life-threatening disease with a worldwide increase in morbidity and mortality, it is characterised by chronic airway inflammation, irreversible airflow limitation and accelerated lung function decline [1]. Abnormal tissue repair upon cigarette smoking is an important pathophysiological feature of the disease, leading to increased extracellular matrix (ECM) deposition and small airway wall thickening. These structural changes cannot be cured and there is an urgent need for a better understanding of the underlying mechanisms.

The airway epithelium forms the first barrier to inhaled cigarette smoke, separating the underlying mesenchyme from the environment. Cigarette smoke causes epithelial damage, which is thought to play a pivotal role in airway remodelling. Especially when the epithelial layer is damaged, epithelial cells produce growth factors (e.g. transforming growth factor (TGF)-β) that act on underlying tissue to regulate repair and remodelling processes [2]. TGF-β is a key mediator of airway remodelling in COPD [2] and a well-known inducer of epithelial-to-mesenchymal transition (EMT), a process involved in tissue repair. During EMT, epithelial markers like E-cadherin are lost and mesenchymal markers, e.g. fibronectin, matrix metalloproteinases (MMPs) and Snail, are gained. Cigarette smoke has been shown to induce EMT in airway epithelium in a TGF-β-dependent manner [3].

Wingless/integrase-1 (WNT) signalling [4], a pathway critically involved in the regulation of lung development, is re-activated upon tissue damage and inflammation in the lungs [5]. The autocrine and paracrine effects of WNT ligands are mediated by frizzled receptors (FZD), leading to transcription of ECM proteins, MMPs and other remodelling genes [6]. Canonical WNT signalling results in β-catenin-mediated transcription, an important aspect of EMT. In addition, WNT ligands can induce gene transcription through noncanonical pathways, including p38, Ca2+/nuclear factor of activated T-cell (NFAT) and RhoA-dependent signalling [7, 8]. Of interest, WNT signalling has been implicated in lung epithelial injury and repair processes [5, 9, 10] and dysregulated expression of WNT genes has been observed in COPD lung tissue and airway epithelium [11–13].

The role of WNT signalling in the development and progression of airway remodelling in COPD is still largely unknown. In the current study, we hypothesised that aberrant epithelial expression of WNT ligands upon cigarette smoke exposure contributes to airway remodelling in COPD. We show that WNT-5B expression is increased upon cigarette smoke exposure in bronchial epithelial cells, particularly in cells from COPD patients, and that WNT-5B induces TGF-β/Smad3-dependent transcription of EMT/remodelling genes.

Methods
Subjects
Lung tissue was derived from lungs of seven smoking COPD patients and control lungs of seven smokers with normal lung function and eight nonsmokers (table 1). The tissue was derived from tumour resection surgery left-over lung tissue, taken far distant from the tumour and checked for abnormalities by experienced pathologists. Primary bronchial epithelial cells (PBECs) were obtained by protease digestion, as described previously [14], from tracheobronchial tissue of nine ex-smokers with severe COPD undergoing lung transplantation (table 1), and six non-COPD lung donors about whom no further information was available. The study protocol was consistent with the Research Code of the University Medical Center Groningen (www.umcg.nl/SiteCollectionDocuments/English/Researchcode/UMCG-Researchcode.%20basic%20principles%202013.pdf) and national ethical and professional guidelines (“Code of conduct; Dutch federation of biomedical scientific societies”; www.federa.org). In addition, PBECs were obtained by fibre-optic bronchial brushing according to standard guidelines in five smokers with COPD and six
nonsmoking controls in a study approved by the Medical Ethics Committee of our centre (table 2). All participants gave their written informed consent.

**Immunohistochemistry**

Lung tissue from seven smokers with COPD, seven control smokers and eight nonsmokers was embedded in paraffin, used for immunohistochemistry and quantified by automated computer-assisted image analysis as described in the online supplementary material.

**Cell culture**

BEAS-2B cells were cultured on collagen-coated flasks in RPMI medium (Biowhittaker, Verviers, Belgium)/10% fetal calf serum. PBECs were obtained as described earlier and cultured in hormonally supplemented bronchial epithelium growth medium (BEGM; Lonza, Walkersville, MD, USA) in collagen/fibronectin-coated flasks. PBECs were stored in liquid nitrogen before use in passage three. For experimentation, cells were grown in duplicates in 24-well plates until 80–90% confluence and serum/hormonally deprived overnight. For mucociliary-differentiated epithelium, PBECs were grown at the air–liquid interface (ALI) for 2 weeks, as described previously [15].

**Cell stimulation**

Prior to stimulation, cells were transfected with small interfering (si)RNA or reporter constructs or pretreated with/without specific inhibitors as described in the online supplementary material. Subsequently, cells were exposed to 5–10% cigarette smoke extract (CSE), which does not affect cell viability [16], 5 ng·mL$^{-1}$ TGF-$eta$1 and/or recombinant human WNT-5B (5–500 ng·mL$^{-1}$, R&D systems, Minneapolis, MN, USA) for 6 or 24 h. Cell-free supernatants were collected and cells were harvested in TRIreagent (MRC Inc, Cincinatti, OH, USA) for RNA isolation or in Laemmli buffer for cell lysate preparation. See the online supplementary material for methods on cell transfection, concentration of inhibitors, CSE preparation, RNA isolation, cDNA synthesis, real-time PCR, western blotting, MMP-2 measurement and dual luciferase assay.

**Statistical analysis**

Data were analysed using the nonparametric rank-sum Mann–Whitney U-test between subject groups, nonparametric Wilcoxon-signed rank test for paired observations within subject groups, and repeated measurements ANOVA with Bonferroni’s multiple comparison test or t-test for analysis of paired observations within the cell line, based on the assumption of a normal distribution.

**Results**

**Increased WNT-5B protein expression in lung tissue upon smoke exposure in vivo**

We previously reported that human bronchial epithelial cells express substantial levels of WNT-4, WNT-5A, WNT-5B and WNT-7B mRNA, and that cigarette smoke exposure specifically upregulates WNT-5B mRNA expression in vitro [11]. To study whether cigarette smoking increases airway epithelial WNT-5B expression in vivo, we compared the protein expression of WNT-5B in lung tissue from nonsmokers to control smokers and smokers with COPD using immunohistochemistry. Importantly, WNT-5B was strongly expressed in lung tissue from smokers with COPD, with significantly higher airway epithelial expression than nonsmokers as well as control smokers, without a significant difference between the latter two groups (figure 1). This suggests that higher WNT-5B expression is not smoking related but disease related, or that airway epithelium from COPD patients is especially susceptible to cigarette smoke-induced upregulation of WNT-5B.

**Cigarette smoke upregulates WNT-5B expression in airway epithelium**

To investigate this further, we studied the effect of cigarette smoke exposure on WNT-5B expression in primary airway epithelial cells from COPD patients and nonsmoking controls in vitro. We previously

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**TABLE 2 Characteristics of the subjects undergoing bronchial brushings**

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Control nonsmoker</th>
<th>COPD stage II $^9$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subjects n</td>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td>Female %</td>
<td>17</td>
<td>0</td>
</tr>
<tr>
<td>Age years</td>
<td>61 (49–73)</td>
<td>66 (64–69)</td>
</tr>
<tr>
<td>Smoking status</td>
<td>Nonsmoker</td>
<td>Current $^8$</td>
</tr>
<tr>
<td>FEV1 % predicted</td>
<td>113 (89–129)</td>
<td>65 (49–79)</td>
</tr>
</tbody>
</table>

Data are presented as median [range], unless otherwise stated. COPD: chronic obstructive pulmonary disease; FEV1: forced expiratory volume in 1 s. $^1$: COPD patients were included based on the Global Initiative for Chronic Obstructive Lung Disease standard [1]; $^8$: ⩾10 pack-years.
reported that PBECs from COPD patients express higher mRNA levels of WNT-4 compared with nonsmokers and control smokers at baseline, without differential expression of WNT-5B [11]. Again, we did not observe significant differences in baseline expression of WNT-5B mRNA in COPD- and control-derived PBECs (figure 2a). 6-h exposure to 5% CSE significantly enhanced WNT-5B expression in PBECs from five current smokers and three ex-smokers with COPD, whereas no significant CSE-induced increase was present in control-derived PBECs (figure 2a). At the protein level, we studied the effect of both 5% and 10% CSE. Although we did not observe significant differences, the lower concentration of CSE had a maximal effect in COPD-derived cells, while in control-derived cells the maximal effect was observed at the higher concentration (figure 2b). Together, these data suggest that COPD epithelium is more prone to upregulate WNT-5B expression upon cigarette smoke exposure. In contrast, 5% CSE induced an equally strong increase in expression of the redox-sensitive gene haem oxygenase in PBECs from COPD patients and controls (figure S1).

Because of the limited number of COPD-derived PBECs, we used a human bronchial cell line to further investigate the role of WNT-5B in airway epithelial remodelling mechanistically. We used BEAS-2B, since this cell line is susceptible to undergo CSE-induced EMT [17]. Here, 10% CSE significantly increased
WNT-5B mRNA expression, whereas 5% CSE did not (figure 2c). The upregulatory effect was confirmed at the protein level (figure 2d).

**WNT-5B regulates the expression of remodelling markers in BEAS-2B**

Next, we assessed the effects of exogenously added WNT-5B on markers of cigarette smoke-induced airway remodelling in COPD, i.e. fibronectin, MMP-2, MMP-9 and E-cadherin [18–21]. 6-h exposure to 500 ng·mL$^{-1}$, but not 5 or 50 ng·mL$^{-1}$, WNT-5B significantly increased fibronectin mRNA expression in BEAS-2B cells (figure 3a). WNT-5B (500 ng·mL$^{-1}$) also significantly increased the expression of MMP-2, MMP-9 and the E-cadherin repressor SnaiI, and reduced E-cadherin mRNA (figure 3b). These effects were confirmed at the protein level for fibronectin and E-cadherin (figure 3c). Furthermore, WNT-5B significantly increased the release of MMP-2 protein (figure 3d), while levels of MMP-9 were below the
detection limit of the assay. Part of the secreted MMP-2 was active, as indicated by the reduction in MMP-2 levels upon extraction of active MMPs with tumour necrosis factor protease inhibitor 2 (TAPI-2)-labelled beads (figure S2).

WNT-5B induces activation of Smad3 and p38 signalling in BEAS-2B

To elucidate the signalling pathways involved in these effects of WNT-5B, we used reporter constructs containing TCF/LEF-1 or NFAT and AP-1 binding sites for canonical WNT/β-catenin and non-canonical WNT signalling, respectively, in BEAS-2B cells. We also included a Smad-binding element (SBE) reporter, given the implications of TGF-β, a downstream molecule Smad3, for EMT [3, 22]. WNT-5B significantly increased SBE activity, while it had no effect on β-catenin (figure 4a, b), NFAT and AP-1 activation (figure S3). To assess whether Smad was indeed activated by WNT-5B, we studied Smad3 phosphorylation and observed a significant increase upon WNT-5B exposure (figure 4c, d). In addition, WNT-5B significantly increased phosphorylation of the non-canonical signalling molecule p38.

WNT-5B-induced expression of remodelling-related genes depends on TGF-β/Smad3 signalling in BEAS-2B

We next examined whether p38 and Smad3 mediate the effect of WNT-5B on gene transcription using selective inhibitors. The Smad3 inhibitor SIS3 significantly reduced the effect of WNT-5B on fibronectin...
mRNA expression, and a similar effect was observed for the p38 mitogen-activated protein kinase (MAPK) inhibitor SB203580, albeit to a smaller extent (figure 5a). Smad3 and p38 MAPK are both downstream targets of TGF-β signalling and are activated upon binding of TGF-β to its receptor (TβRII/Alk5) [23]. To assess involvement of TGF-β/TβRII signalling in the transcriptional effects of WNT-5B, we used the TβRII/Alk5 inhibitor SB431542, and this completely abrogated the effect of WNT-5B on fibronectin mRNA expression (figure 5b). Given the relatively short time that this took to occur, we anticipate that the effects of WNT-5B are exerted by activation of the TGF-β receptor and downstream signalling rather than by increasing de novo synthesis of TGF-β, although WNT-5B significantly increased TGF-β mRNA expression (figure S4). TGF-β is synthesised and secreted as a latent complex, in which it is incorporated into the ECM. Upon tissue damage, TGF-β can be converted from the latent into the active form to bind its receptor by several mechanisms, including changes in the ECM and proteolytic activation by plasmin and MMP-2/9 [24]. Of interest, WNT-5B-induced fibronectin mRNA expression was almost completely abrogated by the use of the broad-spectrum MMP inhibitor TAPI-2 (figure 5c). Thus, WNT-5B may induce TGF-β activation and downstream signalling by increasing active MMP levels. In further support of a role for TGF-β signalling in the effects of WNT-5B, the upregulatory effect of WNT-5B on fibronectin was blocked by addition of a neutralising TGF-β antibody (figure 5b). Furthermore, exogenously added TGF-β1 mimicked the effects of WNT-5B, with a

FIGURE 4 WNT-5B does not induce activity of the β-catenin-driven reporter TopFLASH, but activates a Smad binding element (SBE) driven reporter and increases phospho-Smad3 (p-Smad3) and phospho-p38 (p-p38) levels in BEAS-2B cells. a, b) Cells were grown overnight and transfected with a) the TopFLASH reporter (200 ng) with/without 200 ng constitutively active β-catenin (CA-β-catenin) construct (positive control for β-catenin signalling) or b) with the SBE reporter (200 ng) and the thymidine kinase-driven Renilla luciferase vector pRL-TK (20 ng) as the internal control, and stimulated with/without 500 ng·mL⁻¹ WNT-5B or 5 ng·mL⁻¹ transforming growth factor (TGF)-β (positive control for Smad signalling) for 24 h. Luciferase activity was determined, values were normalised (reporter/Renilla ratio) and expressed as means±SEM (n=5). c, d) Serum-deprived cells were incubated with 500 ng·mL⁻¹ WNT-5B for 0–240 min. Total cell lysates were prepared and c) p-Smad3 and d) p-p38 were detected by Western blotting. Actin was used as the loading control. Densitometry was performed and levels were related to actin levels. Representative blots and the means±SEM ratios are depicted (n=4). *: p<0.05; and **: p<0.01 between the indicated value and the unstimulated control tested using t-tests for paired observations (panels a and b) or repeated measures ANOVA (panels c and d).
downregulatory effect on E-cadherin mRNA expression and an upregulatory effect on fibronectin, MMP-2 and MMP-9 mRNA expression (figure 5d).

WNT-5B effects in PBECs from controls and COPD patients
To enhance the translational relevance of our findings in BEAS-2B cells, we assessed whether WNT-5B induces similar effects in PBECs. WNT-5B strongly and significantly increased the expression of fibronectin, MMP-2 and MMP-9 mRNA in PBECs derived from three severe COPD patients and three non-COPD controls (figure 6a), with equal effects of WNT-5B in cells from COPD patients and controls. Next, we used ALI-differentiated PBECs from six COPD patients and six non-COPD controls to more closely reflect the situation in vivo [25]. Baseline levels of WNT-5B (figure 5b), fibronectin, MMP-2, MMP-9 and Snail mRNA expression was related to the expression of the house keeping genes β2m and PPIA. mRNA levels are expressed as the mean±SEM fold change compared with the unstimulated control value (2^ΔΔCT). *: p<0.05; **: p<0.01; and ***: p<0.001 between the indicated values as tested by repeated measures ANOVA (panels a–c) or t-test for paired observations (panel d).

Together, our results suggest that WNT-5B may contribute to airway remodelling in COPD patients by promoting TGF-β signalling in a positive feedback loop.
Our data in human lung tissue and epithelial cells indicate an important role for WNT-5B in cigarette smoke-induced airway epithelial remodelling in COPD. Airway epithelial expression of the WNT-5B protein was significantly higher in lung tissue from smokers with COPD than in tissue from control smokers and nonsmokers, without a significant difference between the latter two. Accordingly, cigarette smoke exposure increased WNT-5B expression in bronchial epithelial cells, particularly in COPD-derived epithelium. This

Discussion
Our data in human lung tissue and epithelial cells indicate an important role for WNT-5B in cigarette smoke-induced airway epithelial remodelling in COPD. Airway epithelial expression of the WNT-5B protein was significantly higher in lung tissue from smokers with COPD than in tissue from control smokers and nonsmokers, without a significant difference between the latter two. Accordingly, cigarette smoke exposure increased WNT-5B expression in bronchial epithelial cells, particularly in COPD-derived epithelium. This
confirms our hypothesis that cigarette smoking leads to aberrant WNT-5B expression in COPD airways. In line with our hypothesis, WNT-5B upregulated epithelial expression of fibronectin, MMP-2, MMP-9 and Snail, potentially contributing to airway remodelling in COPD. Finally, our data show for the first time that these downstream effects of WNT-5B are mediated by TGF-β/Smad3-dependent signalling.

We provide evidence that airway epithelium from COPD patients is more prone to upregulate WNT-5B expression in response to cigarette smoking, as supported by the in vitro observation that CSE only induces a significant increase in WNT-5B expression in COPD-derived but not control-derived bronchial epithelial cells. In line with these findings, our group previously demonstrated that fibroblasts from COPD patients are more prone to upregulate WNT-5B expression upon TGF-β stimulation than fibroblasts from controls matched for smoking history [26]. This may contribute to the susceptibility to develop structural changes in the airways, as we have shown that WNT-5B acts in a positive feedback loop to facilitate TGF-β signalling, promoting the expression of the EMT markers fibronectin, MMP-2, MMP-9 and Snail. Recently, a role for EMT in COPD airway remodelling and peribronchial fibrosis has been proposed [4].

Interestingly, specific single-nucleotide polymorphisms in SMAD3 were reported to associate with COPD in a Chinese population [33, 34]. Smad3 null mice were resistant to TGF-β and bleomycin-induced fibrosis, indicating that Smad3 signalling may have detrimental effects in fibrotic disease [35]. By contrast, Smad3 null mice developed spontaneous age-related airspace enlargement, consistent with emphysema [36]. Similar to TGF-β/Smad3 signalling, we speculate that WNT signalling may have a dual role in COPD. We and others have shown increased WNT-4 expression in bronchial epithelial cells [36] and bronchial biopsies [11] of COPD patients compared with controls. In an elastase-induced emphysema mouse model, however, reduced expression of WNT (target) genes, e.g. WNT-10B, WNT-2, FZD1 and axin1/2, was observed [37]. Here, WNT/β-catenin activation attenuated airspace enlargement [12], pointing towards a protective role of the WNT pathway in peripheral lung tissue destruction, while our data suggest that WNT-5B signalling may exert detrimental effects in the airways of COPD patients, promoting airway remodelling.

Together, our data show that cigarette smoke increases WNT-5B expression in airway epithelial cells, especially those from COPD individuals, leading to increased TGF-β/Smad3 signalling with a downstream increase in remodelling markers. Thus, targeting WNT-5B may constitute a novel therapeutic approach in the treatment of airway remodelling in COPD patients.
References


