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### Periostin

Burgess, Janette K; Jonker, Marnix R; Berg, Marijn; Ten Hacken, Nick T H; Meyer, Kerstin B; van den Berge, Maarten; Nawijn, Martijn C; Heijink, Irene H

*Published in:*  
European Respiratory Journal

*DOI:*  
[10.1183/13993003.01286-2020](https://doi.org/10.1183/13993003.01286-2020)

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*Document Version*  
Publisher's PDF, also known as Version of record

*Publication date:*  
2021

[Link to publication in University of Groningen/UMCG research database](#)

*Citation for published version (APA):*

Burgess, J. K., Jonker, M. R., Berg, M., Ten Hacken, N. T. H., Meyer, K. B., van den Berge, M., Nawijn, M. C., & Heijink, I. H. (2021). Periostin: contributor to abnormal airway epithelial function in asthma? *European Respiratory Journal*, 57(2), [2001286]. <https://doi.org/10.1183/13993003.01286-2020>

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# Periostin: contributor to abnormal airway epithelial function in asthma?

Janette K. Burgess<sup>1,2</sup>, Marnix R. Jonker<sup>1,2</sup>, Marijn Berg<sup>1</sup>, Nick T.H. ten Hacken<sup>3</sup>, Kerstin B. Meyer<sup>4</sup>, Maarten van den Berge<sup>2,3</sup>, Martijn C. Nawijn<sup>1,2</sup> and Irene H. Heijink<sup>1,2,3</sup>

**Affiliations:** <sup>1</sup>Dept of Pathology and Medical Biology, Experimental Pulmonology and Inflammation Research, University of Groningen, University Medical Centre Groningen, Groningen, The Netherlands. <sup>2</sup>GRIAC Research Institute, University of Groningen, University Medical Centre Groningen, Groningen, The Netherlands. <sup>3</sup>Dept of Pulmonology, University of Groningen, University Medical Centre Groningen, Groningen, The Netherlands. <sup>4</sup>Wellcome Sanger Institute, Wellcome Genome Campus, Cambridge, UK.

**Correspondence:** Irene H. Heijink, University Medical Centre Groningen, Hanzeplein 1, NL-9713 GZ, Groningen, The Netherlands. E-mail: h.i.heijink@umcg.nl



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**High levels of IL-13 in Type-2 driven asthma play a crucial role in the development of the abnormal epithelial phenotype by induction of periostin (POSTN) secretion. POSTN induces mucin production in airway epithelial cells.** <https://bit.ly/3hg7ETu>

**Cite this article as:** Burgess JK, Jonker MR, Berg M, *et al.* Periostin: contributor to abnormal airway epithelial function in asthma? *Eur Respir J* 2021; 57: 2001286 [<https://doi.org/10.1183/13993003.01286-2020>].

**ABSTRACT** Periostin (POSTN) may serve as a biomarker for Type-2 mediated eosinophilic airway inflammation in asthma. We hypothesised that a Type-2 cytokine, interleukin (IL)-13, induces airway epithelial expression of *POSTN*, which in turn contributes to epithelial changes observed in asthma.

We studied the effect of IL-13 on *POSTN* expression in BEAS-2B and air-liquid interface differentiated primary bronchial epithelial cells (PBECs). Additionally, the effects of recombinant human POSTN on epithelial-to-mesenchymal transition (EMT) markers and mucin genes were assessed. *POSTN* single cell gene expression and protein levels were analysed in bronchial biopsies and induced sputum from asthma patients and healthy controls.

IL-13 increased *POSTN* expression in both cell types and this was accompanied by EMT-related features in BEAS-2B. In air-liquid interface differentiated PBECs, IL-13 increased *POSTN* basolateral and apical release. Apical administration of *POSTN* increased the expression of *MMP-9*, *MUC5B* and *MUC5AC*. In bronchial biopsies, *POSTN* expression was mainly confined to basal epithelial cells, ionocytes, endothelial cells and fibroblasts, showing higher expression in basal epithelial cells from asthma patients *versus* those from controls. A higher level of *POSTN* protein expression in epithelial and subepithelial layers was confirmed in bronchial biopsies from asthma patients when compared to healthy controls. Although sputum *POSTN* levels were not higher in asthma, levels correlated with eosinophil numbers and with the coughing-up of mucus.

*POSTN* expression is increased by IL-13 in bronchial epithelial cells and is higher in bronchial biopsies from asthma patients. This may have important consequences, as administration of *POSTN* increases epithelial expression of mucin genes, supporting the relationship of *POSTN* with Type-2 mediated asthma and mucus secretion.

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This article has supplementary material available from [erj.ersjournals.com](http://erj.ersjournals.com)

Received: 20 April 2020 | Accepted: 17 Aug 2020

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## Introduction

Many susceptibility genes for the development of asthma are expressed in the airway epithelium [1–3]. Airway epithelial barrier dysfunction is thought to play a central role in allergic sensitisation and the development of asthma [4, 5]. There are abnormalities in the epithelial barrier in asthma, including goblet cell metaplasia and loss of epithelial cell–cell adhesion molecule E-cadherin (CDH1) [5]. Loss of epithelial barrier function may have important consequences, not only increasing the access of allergens to the submucosa, but also propagating airway immunity, remodelling and hyper-responsiveness [4, 5]. The exact mechanisms through which aberrant expression of epithelial genes is translated into functionally altered responses to aero-allergens in asthma are still unknown.

Airway epithelial cells (AECs) from asthma patients display an intrinsic inefficiency in forming a functional barrier [6, 7]. It has been observed that loss of epithelial barrier function in asthma is accompanied by increased production of the Type-2 driving factors thymic stromal lymphopoietin (TSLP) and interleukin (IL)-33 *in vitro* [7], and increased expression of periostin (POSTN) in the airways *in vivo* [8]. Importantly, POSTN is a proposed systemic biomarker of IL-13 driven airway eosinophilia in asthma [9]. However, reduced *POSTN* expression upon treatment with the monoclonal IL-13 antibody lebrikizumab is not accompanied by reduced eosinophil numbers, limiting its use as a biomarker [10]. POSTN is an extracellular matrix-derived bioactive peptide (a matrikine) expressed by various lung resident cells and secreted by the airway epithelium in response to the Type-2 cytokines IL-4 and IL-13, as well as the profibrotic transforming growth factor- $\beta$  (TGF- $\beta$ ). Induction of *POSTN* expression is associated with epithelial-to-mesenchymal transition (EMT), a process of aberrant epithelial repair that may be involved in loss of epithelial barrier function and airway remodelling in asthma [5]. Both IL-4 and IL-13 downregulate *CDH1* expression in lung epithelial cells [11] disrupting epithelial barrier function [11, 12]. IL-13 has been shown to induce expression of CDH1 repressor and EMT marker, zinc finger protein (*SNAI1*), in intestinal epithelial cells [13]. Additionally, the asthma epithelium is reported to be more prone to undergoing EMT in response to TGF- $\beta$  compared to the healthy epithelium [14]. Our previous data indicates that during TGF- $\beta$  induced EMT, increased *POSTN* expression is accompanied by increased fibronectin (*FN*) and matrix metalloproteinase-2 (*MMP-2*) and *MMP-9* expression in the bronchial epithelial cell line BEAS-2B [15]. Overexpression of *POSTN* in BEAS-2B cells promotes epithelial expression of *MMP-2*, *MMP-9* and alpha-1 Type-I collagen (*COL1A1*) in a TGF- $\beta$  dependent manner [16]. Furthermore, it has been shown to mediate the effects of TGF- $\beta$  in fibroblasts [17], indicating that POSTN is not merely an EMT marker but may also play an active role in EMT. We hypothesise that IL-13 induced production of POSTN plays a crucial role in the abnormal phenotype of the asthma epithelium. We have assessed the expression of *POSTN* following exposure to IL-13 and have studied the effect of POSTN on expression of EMT markers, as well as mucins, in AECs. Additionally, we have assessed *POSTN* expression in bronchial biopsies (single-cell RNA-sequencing (scRNA-seq) and protein levels) and sputum from well-characterised asthma patients and healthy controls.

## Methods

### Subjects

We included bronchial biopsies and sputum samples from patients with asthma and from healthy controls with normal lung function, defined as forced expiratory volume in 1 s ( $FEV_1$ )/forced vital capacity (FVC) >lower limit of normal (LLN),  $FEV_1$  >80% predicted, an absence of bronchial hyper-responsiveness (BHR) to methacholine (provocative concentration causing a 20% fall in  $FEV_1$  ( $PC_{20}$ ) methacholine >16 mg·mL<sup>-1</sup>) and no allergies (see table 1 for subject characteristics). The medical ethics committee of University Medical Center Groningen (UMCG) approved the study and all subjects gave written informed consent.

Primary AECs were obtained by protease digestion of tracheal bronchial tissue from normal donor lungs received at the UMCG. The study protocol was consistent with the research code of the UMCG ([www.umcg.nl/EN/Research/Researchers/General/ResearchCode/Paginas/default.aspx](http://www.umcg.nl/EN/Research/Researchers/General/ResearchCode/Paginas/default.aspx)) and national ethical and professional guidelines (the Code of Conduct of the Dutch Federation of Biomedical Scientific Societies; [www.federa.org](http://www.federa.org)). Bronchial biopsies were subjected to immunohistochemical analysis for POSTN as described in the supplementary material.

### Sputum induction

Sputum was induced by inhalation of nebulised hypertonic saline (5%) for three consecutive periods of 5 min. Whole sputum samples were processed as described previously [19].

### Culture of human bronchial epithelial cells

Bronchial epithelial BEAS-2B cells and AECs were cultured and treated. Quantitative PCR and enzyme-linked immunosorbent assay (ELISA) were performed as described in the supplementary material.

TABLE 1 Subject characteristics

Subject	All		IHC		scRNA-seq	
	Asthma (n=87)	Control (n=73)	Asthma (n=26)	Control (n=22)	Asthma (n=6)	Control (n=6)
Age years	48 [21–71]	43 [20–70]	49 [25–67]	51 [35–70]	57 [51–65]	57 [50–64]
Gender						
Male	38	45	9	9	5	5
Female	49	28	17	13	1	1
Smoking pack-years	9 [0–47]	0 [0–51]	0.3 [0–44]	0 [0–51]	0 [0–0]	0 [0–5]
FEV <sub>1</sub> % predicted	91 [42–129]	106 [83–139]	90 [53–107]	111 [83–133]	87 [59–98]	118 [103–148]
Induced sputum						
Macrophage %	28 [3–85]	51 [1–95]	21 [9–82]	49 [1–76]	12.7 [7–51]	48 [35–66]
Neutrophil %	53 [15–97]	48 [4–99]	58 [10–87]	51 [24–99]	73 [35–78]	48 [22–58]
Eosinophil %	1.8 [0–73]	0.2 [0–3]	1.7 [0–3]	0.2 [0–5]	8 [1.7–16]	1 [0–3]
Lymphocyte %	0.3 [0–6.1]	0.2 [0–4.2]	0.3 [0–2.8]	0.7 [0–1.3]	4.5 [1–6]	4 [3–4.8]
Basophil %	0 [0–0.05]	0 [0–0]	0 [0–0.2]	0 [0–0]	0 [0–0]	0 [0–0]

Data are presented as n or median (range). Subjects with asthma were free of other lung diseases and were included on the basis of the presence of allergy (skin test or Phadiatop), documented BHR (defined as PC<sub>20</sub> AMP >320 mg·mL<sup>-1</sup>) and the presence of asthma symptoms (wheeze or asthma attacks) during the last 3 years. The presence of CMH was defined according to self-reporting CCQs [18]. Subjects were free of other lung diseases and had not used ICS, LABAs or long-acting anticholinergics for at least 4 weeks preceding the study. IHC: immunohistochemistry (subset of subjects with biopsies for POSTN staining); scRNA-seq: single-cell RNA-sequencing (subset of subjects with biopsies for scRNA-seq); FEV<sub>1</sub>: forced expiratory volume in 1 s; BHR: bronchial hyper-responsiveness; PC<sub>20</sub>: provocative concentration causing a 20% fall in FEV<sub>1</sub>; AMP: adenosine monophosphate; CMH: chronic mucous hypersecretion; CCQ: clinical COPD questionnaire; ICS: inhaled corticosteroids; LABA: long-acting β-agonist; POSTN: periostin.

### Single-cell RNA-sequencing

Bronchial biopsies were collected through bronchoscopy and processed for scRNA-seq with the Chromium platform (10x Genomics, Pleasanton, CA, USA) using the 3' protocol (version 2) according to the manufacturer's instructions. The details of this study have been described elsewhere [20]. Briefly, the data underwent quality control by use of SOUP-X correction, with removal of doublets as well as low quality cells [20]. We used Seurat, version 3 for analysis of *POSTN* gene expression in the dataset, using cluster annotation as previously described [20]. We combined the epithelial and non-epithelial datasets to perform principal component analysis (PCA), using the resulting principal components to calculate the uniform manifold approximation and projection (UMAP).

### Statistical analysis

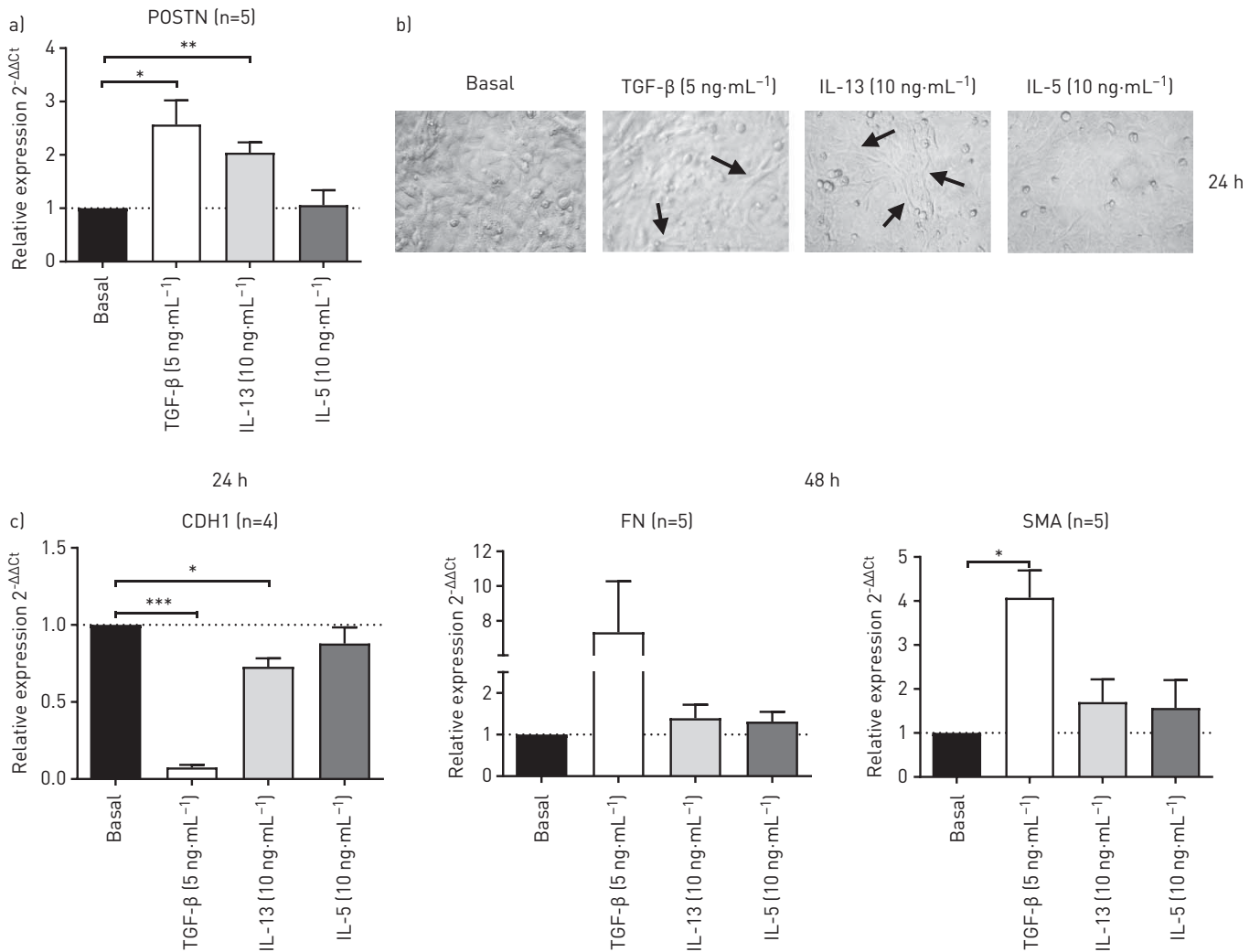
Data were analysed using the nonparametric, two-tailed Wilcoxon-signed rank test for paired observations in primary cells and the t-test for paired observations in the cell line, unless otherwise stated. Correlations were determined using a linear regression analysis.

## Results

### *IL-13 induces airway epithelial POSTN expression and induction of EMT markers in BEAS-2B cells*

We first assessed whether IL-13 induced *POSTN* expression and if this was accompanied by downregulation of *CDH1* and induction of EMT markers. We used Type-2 cytokine IL-5 as a negative control and TGF-β as a positive control. Treatment of BEAS-2B cells with both TGF-β and IL-13 for 48 h significantly upregulated *POSTN* gene expression (figure 1a). Furthermore, both TGF-β and IL-13 (but not IL-5) induced a change to a spindle-shaped morphology in BEAS-2B cells at 24 h (figure 1b) and downregulation of *CDH1* gene expression at 24 h (figure 1c). In contrast to our previous findings, the TGF-β induced increase in mRNA expression of EMT-marker *FN* did not reach significance [15], although TGF-β significantly increased mRNA expression of α-smooth muscle actin (*SMA*). *FN* and *SMA* were not induced by IL-13 or IL-5 (figure 1c). *MMP-9* expression was below the detection limit of the assay in BEAS-2B cells.

Next, we studied the effects of TGF-β, IL-13 and IL-5 on *POSTN* expression in air-liquid interface differentiated AECs. Interestingly, 48-h treatment with either TGF-β or IL-13 (but not IL-5) significantly increased *POSTN* expression in these cells, with the strongest increase, approximately 20-fold, observed after IL-13 treatment (figure 2a). *POSTN* protein levels were measured in cell-free supernatants at baseline and upon TGF-β and IL-13 stimulation. *POSTN* was detected with a higher concentration at baseline in



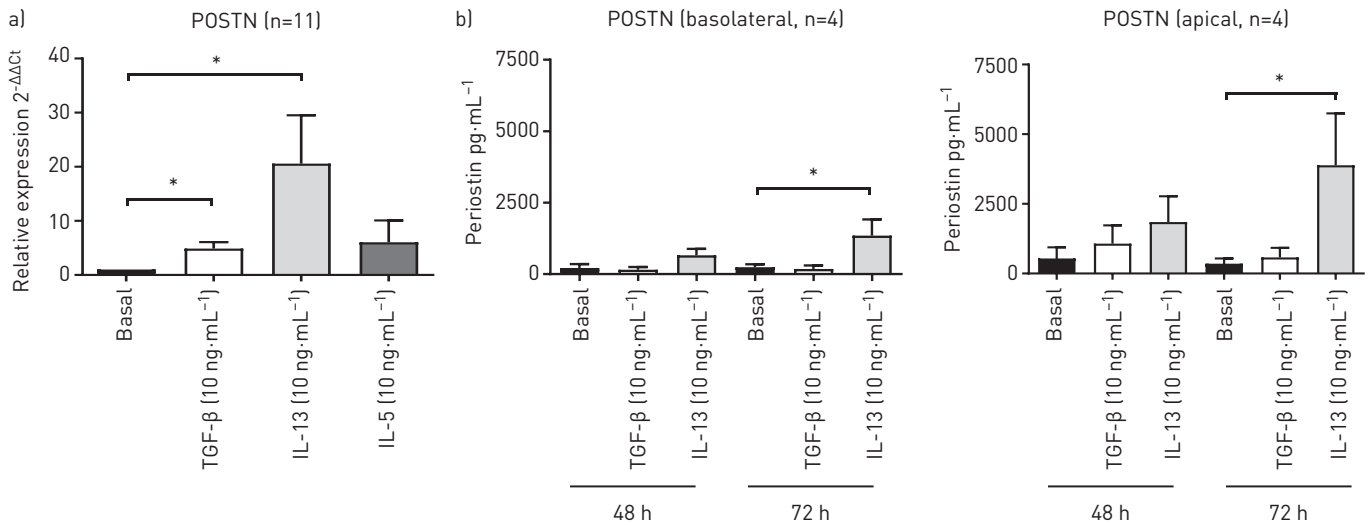
**FIGURE 1** Interleukin (IL)-13 induces changes in human bronchial epithelial cell morphology and epithelial plasticity with an increase in epithelial-to-mesenchymal transition (EMT) markers. BEAS-2B cells were seeded in 24-well plates in duplicate, grown to confluence in RPMI/10% fetal calf serum (FCS) and serum-deprived overnight. Subsequently, cells were exposed to vehicle control (medium), IL-13 (10 ng·mL $^{-1}$ ), transforming growth factor- $\beta$  (TGF- $\beta$ ) (5 ng·mL $^{-1}$ ) as a positive control, or IL-5 (10 ng·mL $^{-1}$ ) as a negative control. Techniques were then as follows: a) cells were harvested after 48 h for RNA isolation and periostin (*POSTN*) mRNA expression was measured by quantitative PCR. Expression of *POSTN* was related to the expression of the  $\beta_2$ -microglobulin (*B2M*) and peptidylprolyl isomerase A (*PPIA*) housekeeping genes, and expressed as  $2^{-\Delta\Delta Ct}$  compared to baseline; b) morphology was studied by phase-contrast microscopy at 24 h; c) cells were harvested for RNA isolation after 24 h or 48 h and expression of E-cadherin (*CDH1*), fibronectin (*FN*) and  $\alpha$ -smooth muscle actin (*SMA*) was related to the expression of the *B2M* and *PPIA* housekeeping genes, and expressed as  $2^{-\Delta\Delta Ct}$  compared to baseline. Data are presented as mean $\pm$ SEM (n=4–5). Ct: cycle threshold; SEM: standard error of the mean. \*: p<0.05; \*\*: p<0.01; \*\*\*: p<0.001 (between the indicated value and baseline, as analysed by t-test for paired observations).

the apical compartment. *POSTN* levels in both compartments were increased by IL-13 treatment, while no significant effect was observed for TGF- $\beta$  (figure 2b).

***POSTN induces expression of MMP-9, MUC5B and MUC5AC in differentiated primary epithelial cells***

To examine whether the IL-13 induced release of *POSTN* could be responsible for changes in the epithelial phenotype observed in asthma, we studied the effect of recombinant human *POSTN* on the expression of *CDH1* and EMT markers *SNAI1*, *MMP-9*, *FN*, *COL1A1* and *SMA* in air-liquid interface differentiated AECs at 48 h. TGF- $\beta$  (10 ng·mL $^{-1}$ ) was again used as a positive control based on previous findings on the induction of EMT by TGF- $\beta$  in human bronchial epithelial cells [14].

*POSTN* was applied from the apical side as IL-13 induced the strongest release of *POSTN* into the apical compartment (figure 2b). Apical stimulation with TGF- $\beta$  was used as a positive control. Similar to TGF- $\beta$ , *POSTN* significantly increased *MMP-9* expression, with a trend towards increased *FN* expression (figure 3a).



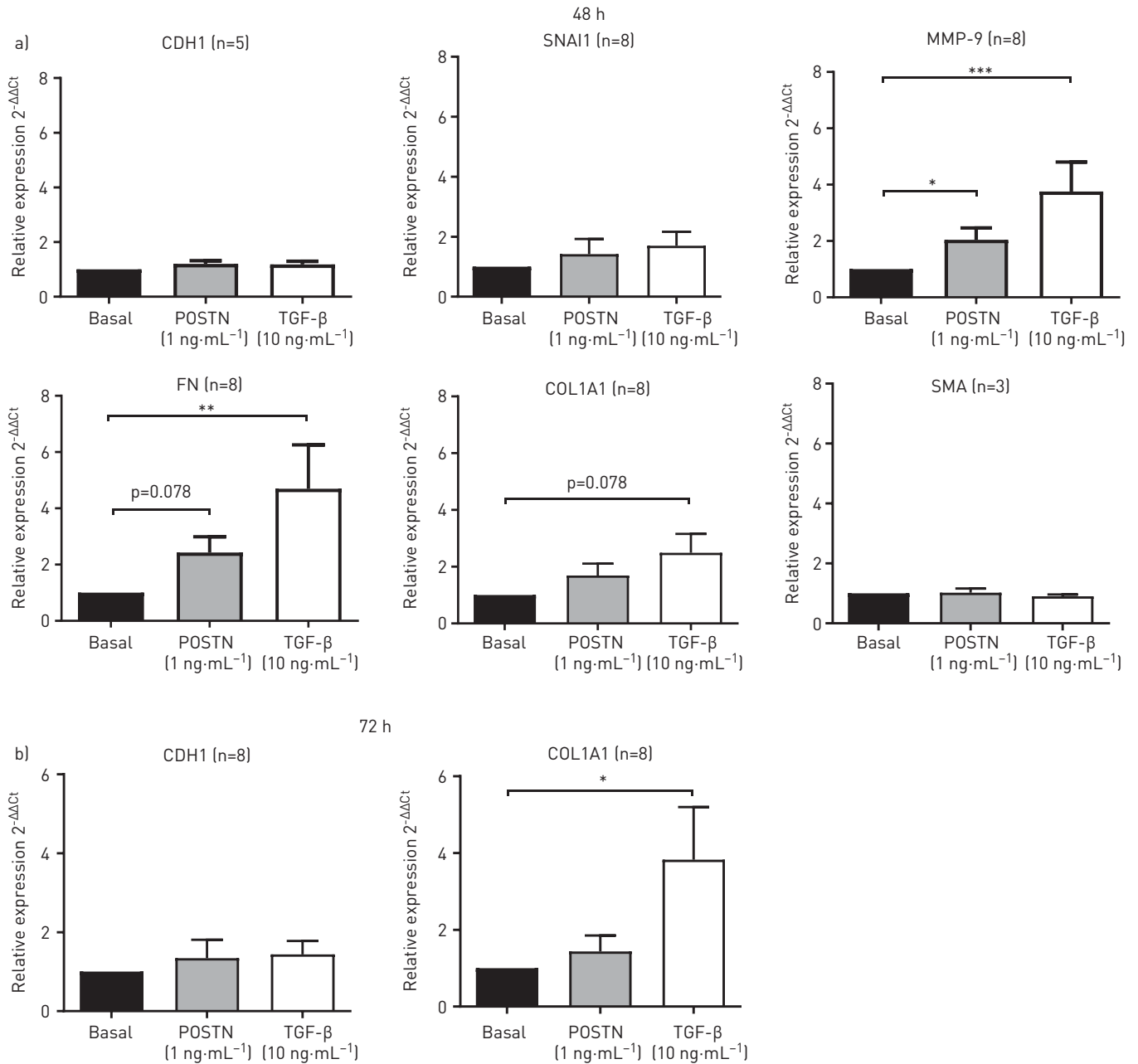
**FIGURE 2** Interleukin (IL)-13 induces periostin (*POSTN*) mRNA expression and release in human primary bronchial epithelial cells (PBECs). PBECs were differentiated at an air–liquid interface for 4 weeks, exposed to hormonally-deprived medium for 24 h (basolateral and apical side) and treated with or without transforming growth factor- $\beta$  (TGF- $\beta$ ) (10 ng·mL $^{-1}$ ) or IL-13 (10 ng·mL $^{-1}$ ) from the apical side for 48 h or 72 h. Techniques were then as follows: a) cells were harvested after 48 h for RNA isolation and *POSTN* mRNA expression was measured by quantitative PCR. Expression of *POSTN* was related to the expression of the  $\beta$ 2-microglobulin (*B2M*) and peptidylprolyl isomerase A (*PPIA*) housekeeping genes, and expressed as  $2^{-\Delta\Delta Ct}$  compared to baseline (n=11); b) at 48 h and 72 h, basolateral and apical supernatants were collected and *POSTN* was measured in cell-free supernatant (n=4). Data are presented as mean $\pm$ SEM. Ct: cycle threshold; SEM: standard error of the mean. \*: p<0.05 (between the indicated values as analysed by a) the Wilcoxon signed rank test or b) one-way ANOVA for repeated measurements with Bonferroni's multiple comparison test).

TGF- $\beta$  (but not *POSTN*) tended to increase *COL1A1* expression, while neither TGF- $\beta$  nor *POSTN* significantly affected the expression of *CDH1*, *SNAI1*, *COL1A1* (figure 3a) or *SMA* (data not shown). TGF- $\beta$  has previously been reported to reduce *CDH1* protein expression in air–liquid interface differentiated AECs after 72 h [14]. However, no significant difference was observed in *CDH1* expression, while TGF- $\beta$  (but not *POSTN*) significantly increased *COL1A1* expression at this time point (figure 3b). With MMP-9 being the only marker significantly affected by *POSTN*, we assessed MMP-9 protein levels in culture supernatants and observed a trend towards increased MMP-9 secretion with both TGF- $\beta$  (basolaterally and apically) and *POSTN* (apically) at 72 h (supplementary figure S1).

Since both IL-13 and MMP-9 have been implicated in mucus production and goblet cell hyperplasia, while reduced allergen-induced goblet cell metaplasia has been observed in *POSTN*-deficient mice [21], we determined the effect of apical *POSTN* exposure on mucins *MUC5B* and *MUC5AC* in air–liquid interface differentiated cells. We observed a significant increase in both *MUC5B* and *MUC5AC* mRNA expression (figure 4a). We did not detect *MUC5AC* protein in the apical compartment after 72 h of medium exposure of the air–liquid interface cultured cells, while *MUC5B* protein levels were significantly higher in the presence of *POSTN* despite the high variation between donors (figure 4b).

#### **Single cell analysis of *POSTN* expression in bronchial biopsies reveals expression in suprabasal epithelial cells and increased expression in asthma**

We recently generated a scRNA-seq dataset from airway wall biopsies of healthy individuals and asthma patients [20]. In this dataset, we observed the presence of increased numbers of goblet cells and mucous ciliated cells (ciliated cells expressing *MUC5AC* and other goblet cell genes) in the airway epithelium of asthma patients [20]. Using this dataset to explore *POSTN* expression in the different subsets of epithelial cells, we observed that club and ciliated cells hardly express *POSTN*, while substantial *POSTN* expression is observed in basal, activated basal (also referred to as suprabasal [22]), mucous ciliated and goblet cells, as well as in ionocytes (a rare bronchial epithelial cell type that has recently been described) (figures 5a–5c) [23]. In addition to epithelial cells, expression was also observed in endothelial cells and fibroblasts. When comparing expression levels between asthma patients and healthy subjects, *POSTN* gene expression in healthy controls was observed to be confined to the basal cell cluster, representing the most undifferentiated, resting basal cells. However, in asthma patients, *POSTN* expression was maintained and even increased in suprabasal, activated basal and cycling basal cells (figure 5b). *POSTN* expression in goblet cells and mucous ciliated cells was also only observed in asthma patients (figure 5b). Since this dataset consists of only six healthy donors and six asthma donors, we do not have the power to statistically



**FIGURE 3** Effects of periostin (POSTN) on epithelial-to-mesenchymal transition (EMT) markers and barrier function in human primary bronchial epithelial cells (PBECs). PBECs were differentiated at an air-liquid interface for 4 weeks, exposed to hormonally-deprived medium for 24 h and treated with or without POSTN (1 ng·mL<sup>-1</sup>) or transforming growth factor-β (TGF-β) (10 ng·mL<sup>-1</sup>) as a positive control, both from the apical side. Techniques were then as follows: a) cells were harvested for RNA isolation after 48 h and expression of E-cadherin (*CDH1*), zinc finger protein (*SNAI1*), matrix metalloproteinase-9 (*MMP-9*), fibronectin (*FN*), alpha-1 Type-I collagen (*COL1A1*) and α-smooth muscle actin (*SMA*) was related to the expression of the β2-microglobulin (*B2M*) and peptidylprolyl isomerase A (*PPIA*) housekeeping genes, and expressed as 2<sup>-ΔΔCt</sup> compared to baseline; b) cells were harvested for RNA isolation after 72 h and expression of *CDH1* and *COL1A1* was related to the expression of the *B2M* and *PPIA* housekeeping genes, and expressed as 2<sup>-ΔΔCt</sup> compared to baseline. Data are presented as mean±SEM (n=8). Ct: cycle threshold; SEM: standard error of the mean. \*: p<0.05; \*\*: p<0.01; \*\*\*: p<0.001 (between the indicated value and baseline, as analysed by the Wilcoxon signed rank test).

test whether the *POSTN* expression level is different between the two disease states for each epithelial cell subset. However, we have included the per-donor expression levels of *POSTN* for the different epithelial cell subsets in supplementary figure S2.

We have previously reported the presence of pathogenic, IL-13 expressing Type-2 T-helper (Th2) cells in airway wall biopsies from these asthma patients [20] and have observed increased expression of an IL-4/IL-13 induced gene signature (based on *in vitro* studies using primary AECs [24]) in the bronchial epithelial

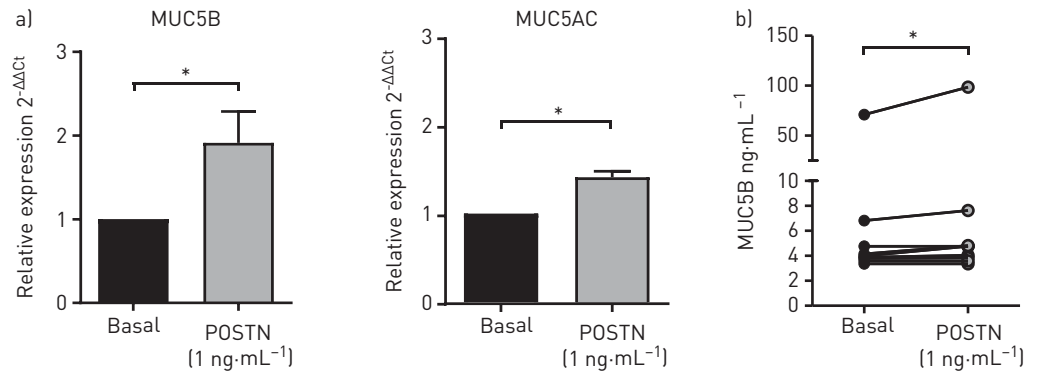


FIGURE 4 Effect of periostin (POSTN) on *MUC5B* and *MUC5AC* expression in human primary bronchial epithelial cells (PBECs). PBECs were differentiated at an air-liquid interface for 4 weeks, exposed to hormonally-deprived medium for 24 h and treated with or without POSTN (1 ng·mL<sup>-1</sup>) from the apical side. Techniques were then as follows: a) cells were harvested for RNA isolation after 72 h and expression of *MUC5B* (n=11) and *MUC5AC* (n=6) was related to the expression of the  $\beta$ 2-microglobulin (*B2M*) and peptidylprolyl isomerase A (*PPIA*) housekeeping genes, and expressed as 2<sup>-ΔΔCt</sup> compared to the control (basal); b) cell-free supernatants were harvested after 72 h and *MUC5B* levels were determined by enzyme-linked immunosorbent assay (ELISA) (n=9). Data are presented as mean±SEM. Ct: cycle threshold; SEM: standard error of the mean. \*: p<0.05 [between the indicated values, as analysed by the Wilcoxon signed rank test].

cells of asthma patients [20]. Therefore, we next asked whether the IL-13 induced gene signature [24] in the asthmatic airway epithelium [20] overlapped with *POSTN* gene expression patterns. The IL-13 induced gene signature was indeed increased in epithelial cells in asthma patients (figure 5d), with consistently increased expression in basal cell subsets as well as goblet and mucous ciliated cells (figure 5d).

**Increased expression of *POSTN* in bronchial biopsies of asthma patients and association of sputum levels of *POSTN* with sputum eosinophils and symptoms of mucus hypersecretion**

Given the small number of asthma patients and healthy controls in the scRNA-seq dataset, we next asked whether the increased expression of *POSTN* was also evident at the protein level in bronchial biopsies from a larger cohort of asthma patients and controls. In accordance with the scRNA-seq data, we observed that the intensity of *POSTN* in the biopsies from asthma patients was significantly stronger than in those from healthy controls (figures 6a and 6b).

Finally, we observed that *POSTN* was detectable in sputum of asthma patients and healthy control subjects. While we did not observe a significant difference in *POSTN* levels between the two groups (figure 6c), sputum *POSTN* levels in asthma patients positively correlated to sputum eosinophil numbers (figure 6d). Moreover, we observed that sputum *POSTN* levels in asthma patients were significantly correlated with coughing-up mucus (figure 6d).

**Discussion**

We hypothesised that high levels of IL-13 in Type-2 driven asthma may result in increased airway *POSTN* levels. Here, we show that IL-13 induces airway epithelial expression of *POSTN* and its release from both the apical and basolateral side. In bronchial biopsies, scRNA-seq analysis revealed that *POSTN* is expressed in basal epithelial cells, ionocytes, endothelial cells and fibroblasts, and is maintained in differentiated epithelial cells from asthma patients compared to healthy controls. Consistent with a role for IL-13 in epithelial *POSTN* expression, the IL-13 induced gene signature observed in the asthmatic AECs [20] overlapped with the *POSTN* gene expression patterns. At the protein level, *POSTN* was expressed in both airway epithelial and subepithelial layers, with higher expression in asthma patients. Exaggerated *POSTN* release may have important consequences for asthma pathophysiology, as administration of *POSTN* increased the expression of *MUC5B* and *MUC5AC* in differentiated AECs. Although *POSTN* levels in sputum of asthma patients were not significantly higher compared to those in healthy controls, the levels correlated with eosinophil numbers in asthma, supporting the relationship of *POSTN* with Type-2 mediated asthma and mucus hypersecretion.

We observed that IL-13 induces a strong increase in *POSTN* release by differentiated AECs, particularly into the apical compartment. This is in contrast to previous observations in undifferentiated primary epithelial cells, where *POSTN* could not be detected in apical washes, with or without 4-day IL-13 treatment [16]. Thus, *POSTN* may not be released by the bronchial epithelial cells under culture conditions that maintain them in a basal phenotype. Accordingly, SUZAKI *et al.* [25] demonstrated that



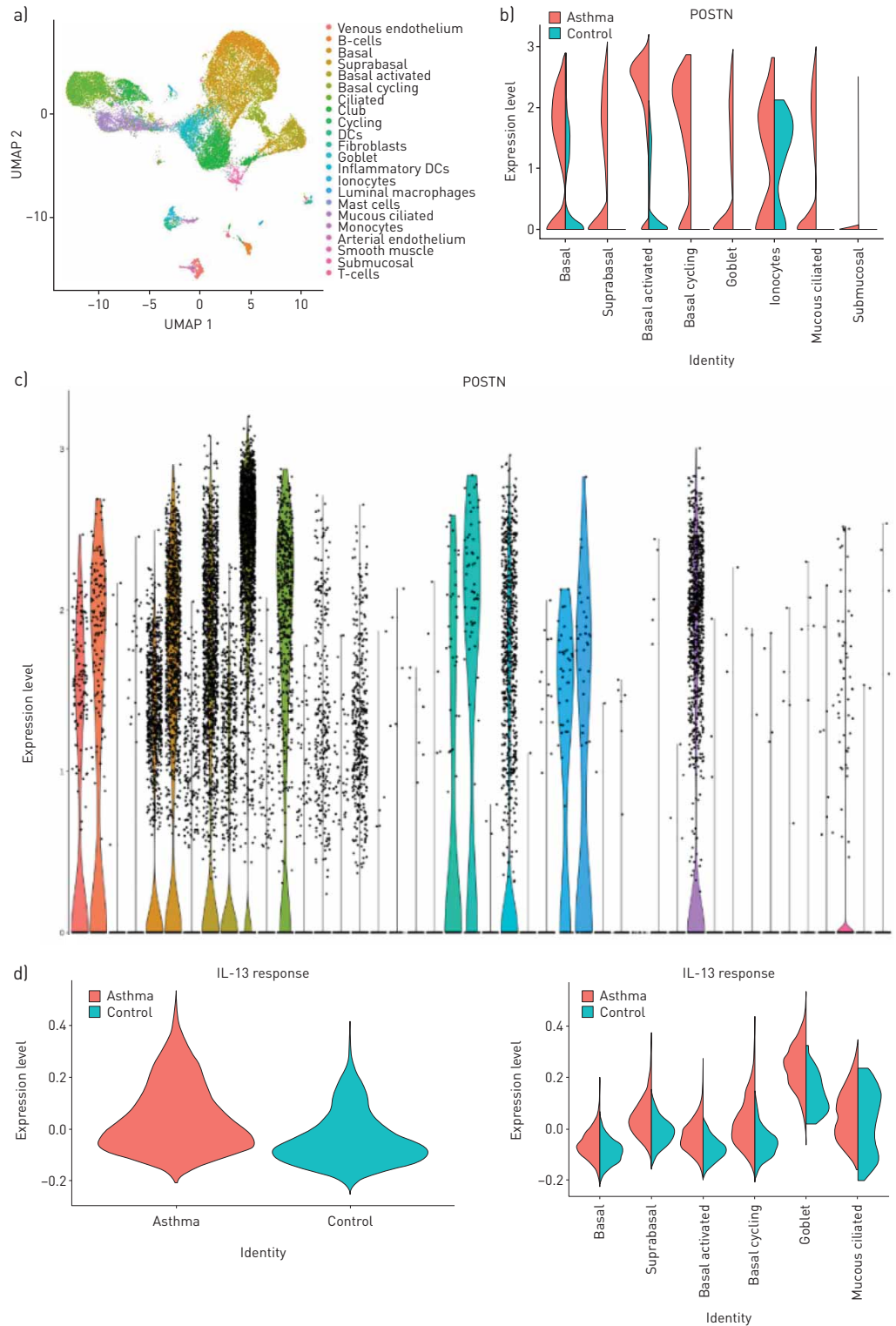


FIGURE 5 Single cell RNA expression of periostin (*POSTN*) and the interleukin (IL)-13 response gene expression signature in healthy controls and asthma patients. Bronchial biopsies were obtained from six healthy controls and six asthmatic subjects. Single-cell RNA-sequencing (scRNA-seq) was performed and the information obtained is displayed as follows: a) uniform manifold approximation and projection (UMAP) plot of the identified clusters of cells and their annotations; b) distribution of the normalised expression of *POSTN* split between asthmatic and control subjects in the expressing clusters; c) normalised expression of *POSTN* split between control subjects and asthmatic subjects (first and second plots, respectively) in the expressing cell types; d) expression of IL-13 response genes split between asthmatic and control subjects in all combined clusters (left) and the difference in the expression of IL-13 response genes between asthmatic and control subjects in different basal cell types/states (right). DC: dendritic cell.

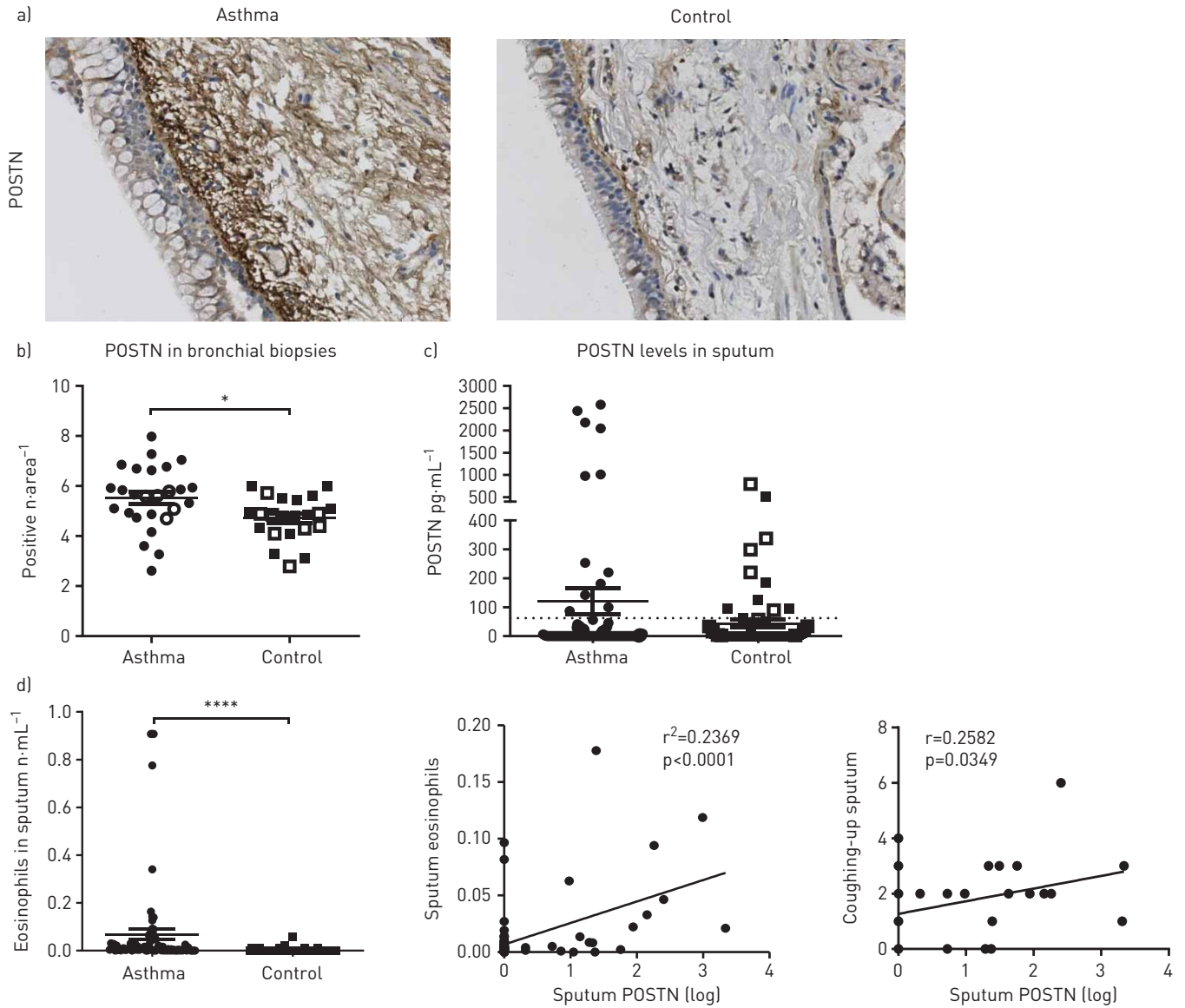


FIGURE 6 Levels of periostin (POSTN) protein in biopsies and sputum from asthma patients and healthy controls. Panels are as follows: a) exemplar immunohistochemistry results for POSTN in bronchial biopsies (26 asthma patients and 22 healthy control subjects); b) staining intensity for POSTN in bronchial biopsies [positive pixels per total tissue area (whole biopsy)] quantified using computer-assisted imaging; c) levels of POSTN (pg·mL<sup>-1</sup>) in induced sputum (73 asthma patients and 87 healthy control subjects) measured by enzyme-linked immunosorbent assay (ELISA). Mean levels±SEM are indicated; d) eosinophil counts in sputum, correlation between absolute eosinophil counts and log-transformed levels of POSTN in sputum, correlation between the score for coughing-up sputum (by questionnaire) and log-transformed levels of POSTN in sputum. \*: p<0.05; \*\*\*\*: p<0.0001 (between the indicated values, as analysed by the Mann–Whitney U-test). Correlations were analysed by linear regression and Pearson’s correlation for normally distributed data (eosinophils) or Spearman’s correlation for nonparametric data (coughing-up sputum). A p-value of less than 0.05 was considered statistically significant. Open symbols indicate smokers (some asthma group (n=13) and control group (n=15) smokers had POSTN levels in sputum below the detection limit and are thus not indicated).

14-day differentiation of AECs into goblet cells using IL-13 strongly increased POSTN secretion from both the basolateral and apical side. This suggests that more differentiated cells, such as the suprabasal, club, ciliated or goblet cells, were the main source of POSTN in this model. Whether goblet cells in particular were responsible for the apical release of POSTN in our study needs further investigation. Our scRNA-seq analysis revealed that *POSTN* was expressed mainly by basal epithelial cells in dissociated airway biopsies, with maintenance of expression in suprabasal, basal activated and cycling epithelial cells from asthma patients. Furthermore, *POSTN* was expressed in goblet and mucous ciliated cells exclusively in asthma patients. The overlapping expression patterns between the IL-13 induced gene signature and *POSTN* in epithelial cells from asthma patients further supports the notion that *POSTN* might well be an IL-13 induced gene *in vivo*, although a role for IL-4 cannot be excluded given the strong overlap between IL-13 and IL-4 induced gene expression patterns in primary human AECs [24].

It is tempting to speculate that the increased *POSTN* expression in asthma may have consequences, leading to higher basolateral as well as apical *POSTN* secretion and potentially also higher mucus production in the airways of asthmatic patients. As epithelial barrier integrity is compromised in asthma, *POSTN* may be less restricted to the basolateral side and our results indicate that apically secreted *POSTN* especially acts to induce transcriptional upregulation of mucin genes. We observed that administration of recombinant human *POSTN* from the apical side increases the expression of the mucin genes *MUC5B* and *MUC5AC* and also increases *MUC5B* protein levels. In line with a role for apical *POSTN* release in inducing mucus hypersecretion, we observed that sputum levels of *POSTN* correlate with symptoms of mucus hypersecretion in asthma patients. Mucus plugs in asthma patients were reported to contain both *MUC5AC* and *MUC5B*-containing domains [26]. *In vitro*, IL-13 induced the formation of heterogeneous mucus gels and impaired mucociliary transport. This was related to the tethering of *MUC5AC*-containing mucus gel domains to mucus-producing cells in the epithelium [26]. In our study, we did not observe an increase in mucin gene expression when cells were stimulated with either IL-13 or TGF- $\beta$ . A limitation of our study, however, is that we only administered these cytokines from the apical side to be able to compare them to the apical addition of *POSTN*.

Our findings that *POSTN* increased mucin expression correspond with previous data from BENTLEY *et al.* [21], who reported that *POSTN*-deficiency in a mouse model of allergic airway disease resulted in reduced house dust mite (HDM) induced goblet cell hyperplasia and reduced airway inflammation and hyper-responsiveness. However, these findings are in contrast with those of SEHRA *et al.* [27], which suggest an opposing role for *POSTN* in ovalbumin (OVA)-induced allergic airways disease, with increased *MUC5AC* expression upon allergen challenge in *POSTN*-deficient mice. Discrepancies between these two studies may be due to the different models that were used and the intranasal HDM-sensitisation model used by BENTLEY *et al.* [21] may be more relevant for human disease.

In addition to mucin genes, we observed that *POSTN* increases *MMP-9* mRNA in differentiated AECs. Increased *MMP-9* expression could be involved in the observed *POSTN*-induced increase in mucin expression and epithelial remodelling, as *MMP-9* has been implicated in epidermal growth factor receptor (EGFR)-induced *MUC5AC* production and goblet cell metaplasia [28, 29], thus affecting innate immune defense of the epithelial barrier. *MMP-9* is a marker of EMT but we did not find evidence for *POSTN* regulation of epithelial plasticity/EMT. Nevertheless, it will be of interest to focus future studies on the potential role of *POSTN* in airway hyper-responsiveness, since airway smooth muscle cell contractile responses may be regulated by *MMP-9* [30]. Furthermore, *MMP-9* may induce secretion of chemokines involved in the attraction of eosinophils, leading to peribronchial fibrosis and peribronchial numbers of eosinophils [31]. The mechanisms underlying the observed *POSTN*-induced upregulation of mucin genes and *MMP-9* need further investigation. Of note, *POSTN* acts on  $\alpha_M\beta_2/CD11b$  [32] and may thus directly induce eosinophil migration, which may subsequently act to further increase mucus secretion as eosinophilic cationic protein is a well-known mediator of mucus production. It is also of interest to mention that high levels of *POSTN* were detected in epithelial columnar cell-high sputum of asthma patients with eosinophilia [33]. This may reflect an eosinophil and/or Type-2 immune signalling function of epithelial cells that secrete high levels of *POSTN*.

Our observation that *POSTN* levels in sputum were associated with eosinophil levels in asthmatic patients is in line with a previous study on a cohort of poorly-controlled asthma patients [34], showing that sputum *POSTN* levels were significantly higher in eosinophilic asthma (defined as sputum eosinophils  $\geq 3\%$ ) compared to noneosinophilic asthma. Similarly, BOBOLEA *et al.* [35] observed that sputum levels of *POSTN* were higher in asthma patients with fixed airflow limitation compared to variable airflow limitation and in patients with eosinophilic (>2%) compared to mixed granulocytic asthma. The lack of a significant difference in sputum levels of *POSTN* between asthma patients and healthy controls in our study, with *POSTN* being undetectable in many of the asthma patients, can be explained by the fact that our patients had relatively mild asthma and were able to refrain from medication for at least 1 month prior to sample collection.

In addition to promoting mucus production, *POSTN* is known to have a structural role interacting with other extracellular matrix proteins to promote matrix production and subepithelial fibrosis, especially when secreted in the basolateral direction [16]. Our data do not support a role for *POSTN* in epithelial collagen synthesis, as reported previously for stable overexpression of *POSTN* [16], although we did not investigate the effects of basolateral secretion of *POSTN*, which is more likely to play a role in matrix remodelling.

All together, our data support the notion that IL-13 induced secretion of *POSTN* by AECs mediates mucus hypersecretion and acts to perpetuate eosinophilic inflammation, aggravating the symptoms of asthma. As such, targeting *POSTN* may potentially be a part of a novel, multipronged therapeutic approach in asthma.

Conflict of interest: J.K. Burgess has nothing to disclose. M.R. Jonker has nothing to disclose. M. Berg has nothing to disclose. N.T.H. ten Hacken has nothing to disclose. K.B. Meyer has nothing to disclose. M. van den Berge reports research grants paid to their institution from AstraZeneca, Novartis and Genentech, outside the submitted work. M.C. Nawijn reports grants from Lung Foundation Netherlands (grant numbers 5.1.14.020 and 4.1.18.226), GSK Ltd, the European Commission (grant number 874656, discovAIR) and the Chan Zuckerberg Initiative, during the conduct of the study. I.H. Heijink reports grants from Roche, during the conduct of the study; and grants from the Netherlands Lung Foundation and Boehringer Ingelheim, outside the submitted work.

Support statement: This study was supported by research grants from Roche (HLR-14) to I.H. Heijink and from the Netherlands Lung Foundation to M.C. Nawijn (LF5.1.14.020 and LF4.1.18.226). Funding information for this article has been deposited with the Crossref Funder Registry.

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