



# Exaggerated BMP4 signalling alters human airway basal progenitor cell differentiation to cigarette smoking-related phenotypes

Wu-Lin Zuo, Jing Yang, Yael Strulovici-Barel, Jacqueline Salit, Mahboubeh Rostami, Jason G. Mezey, Sarah L. O'Beirne, Robert J. Kaner and Ronald G. Crystal

**Affiliation:** Dept of Genetic Medicine, Weill Cornell Medical College, New York, NY, USA.

**Correspondence:** Ronald G. Crystal, Dept of Genetic Medicine, Weill Cornell Medical College, 1300 York Avenue, Box 164, New York, NY 10065, USA. E-mail: geneticmedicine@med.cornell.edu



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**COPD pathogenesis is mediated by BMP4 reprogrammed stem/progenitor cells in human airway**  
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**ABSTRACT** Airway remodelling in chronic obstructive pulmonary disease (COPD) originates, in part, from smoking-induced changes in airway basal stem/progenitor cells (BCs). Based on the knowledge that bone morphogenetic protein 4 (BMP4) influences epithelial progenitor function in the developing and adult mouse lung, we hypothesised that BMP4 signalling may regulate the biology of adult human airway BCs relevant to COPD.

BMP4 signalling components in human airway epithelium were analysed at the mRNA and protein levels, and the differentiation of BCs was assessed using the BC expansion and air-liquid interface models in the absence/presence of BMP4, BMP receptor inhibitor and/or small interfering RNAs against BMP receptors and downstream signalling.

The data demonstrate that in cigarette smokers, BMP4 is upregulated in ciliated and intermediate undifferentiated cells, and expression of the BMP4 receptor BMPRI1A is enriched in BCs. BMP4 induced BCs to acquire a smoking-related abnormal phenotype *in vitro* mediated by BMPRI1A/Smad signalling, characterised by decreased capacity to differentiate into normal mucociliary epithelium, while generating squamous metaplasia.

Exaggerated BMP4 signalling promotes cigarette smoking-relevant airway epithelial remodelling by inducing abnormal phenotypes in human airway BCs. Targeting of BMP4 signalling in airway BCs may represent a novel target to prevent/treat COPD-associated airway disease.

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## Introduction

The human airway epithelium is a pseudo-stratified columnar epithelium, composed of basal stem/progenitor cells (BCs), intermediate undifferentiated cells, ciliated cells and secretory cells [1–3]. BCs, small cuboidal cells residing on the airway basement membrane, serve as the stem/progenitor cells of human airway epithelium, capable of differentiating into the ciliated and secretory cells that are central to the physical barrier, host defence and mucociliary clearance [1, 2]. The intermediate cells are BC-derived precursors committed to differentiate into ciliated and secretory cells [4, 5].

Cigarette smoking, the major cause of chronic obstructive pulmonary disease (COPD), is associated with marked changes to the morphology of airway epithelium, including BC and intermediate cell hyperplasia, mucous cell hyperplasia, squamous cell metaplasia, loss of ciliated cells, and shortened cilia [5–13]. Increasing evidence supports the concept that reprogramming of airway BCs is central to the early pathogenesis of cigarette smoking-induced derangement of airway epithelium [14, 15]. BCs isolated from smokers have a significant transcriptomic difference compared with BCs from nonsmokers, including reprogramming of genes associated with the genetic risk for COPD [16]. Smoking skews the normal function of BCs by suppression of airway epithelial differentiation and tight junction formation [12, 17], in part through the cross-talk between BCs and other cell types [5, 18]. For example, activation of epidermal growth factor (EGF) receptor (EGFR) in BCs alters the differentiation programme of BCs towards the squamous and epithelial–mesenchymal transition (EMT)-like phenotypes characteristic of smoking-induced changes to airway epithelium *in vivo* [5].

In the assessment of human airway epithelium in healthy nonsmokers, asymptomatic smokers and COPD smokers, we observed that bone morphogenetic protein 4 (BMP4) expression is upregulated in airway epithelium of asymptomatic smokers and COPD smokers. Based on the knowledge that BMP4, a secreted transforming growth factor (TGF)- $\beta$  family member, controls epithelial progenitor function in the developing and adult mouse lung by regulating early mesoderm formation [19], airway branching morphogenesis [20] and promoting proximal airway differentiation [21], and induces EMT-like changes in a human airway epithelial cell line [22], we hypothesised that excessive BMP4 signalling may play a functional role in dysregulating the biology of adult human airway BCs relevant to smoking and COPD. The data demonstrate that in cigarette smokers, BMP4 expression is upregulated in ciliated and intermediate undifferentiated cells, and BMP4 receptor BMPRI1A expression is enriched in BCs. BMP4-treated normal BCs acquire a COPD-like “fatigue” phenotype, characterised by decreased capacity to differentiate into normal mucociliary epithelium, while generating squamous metaplasia. These effects are mediated by BMPRI1A/Smad signalling and inhibition of BMPRI1A restores the stem/progenitor cell potential of airway BCs, providing a possible target for therapeutic intervention in smoking-induced lung disorders.

## Methods

Human airway epithelium and BCs were obtained from nonsmokers, asymptomatic smokers and COPD smokers by bronchoscopy and *in vitro* primary BC culture. *In vitro* primary BC and air–liquid interface (ALI) cultures were carried out to study the effects of BMP4 on BC differentiation. Gene expression of airway epithelium and BCs *in vivo* and *in vitro* was assessed by RNA sequencing/microarray and TaqMan real-time (quantitative) PCR (Applied Biosystems, Foster City, CA, USA). Airway epithelial morphology and localisation of proteins were accessed by immunohistochemistry/immunofluorescence staining. Phosphorylation of BMP4 downstream signalling was assessed by Western blot.

Full details are provided in the supplementary material.

## Results

### ***BMP4 is upregulated in airway epithelium of asymptomatic and COPD smokers***

To determine whether cigarette smoking was associated with abnormal BMP signalling in airway epithelium, gene expression of BMPs and BMP antagonists in the large airway epithelium and BCs from nonsmokers, asymptomatic smokers and COPD smokers was quantified by RNA sequencing. Expression of *BMP5*, *BMP6* and BMP antagonists *NOG*, *CHRD* and *CHRD2* was very low in both airway epithelium and airway BCs (average fragments per kilobase of transcript per million mapped reads <0.2). *BMP2* and BMP antagonists *FST*, *FSTL3* and *TWSG1* were highly expressed in airway BCs compared with expression in complete airway epithelium, and expression did not change in asymptomatic smokers and COPD smokers (supplementary figure S1a and b, and supplementary table SI). *BMP7* was expressed in nonsmokers, and mildly increased in asymptomatic smokers and COPD smokers (supplementary figure S1a and supplementary table SI). *BMP4* expression was relatively low in nonsmokers, but was significantly upregulated in asymptomatic smokers and COPD smokers in airway epithelium. There was no significant difference between asymptomatic smokers and COPD smokers (figure 1a, supplementary figure S2a and

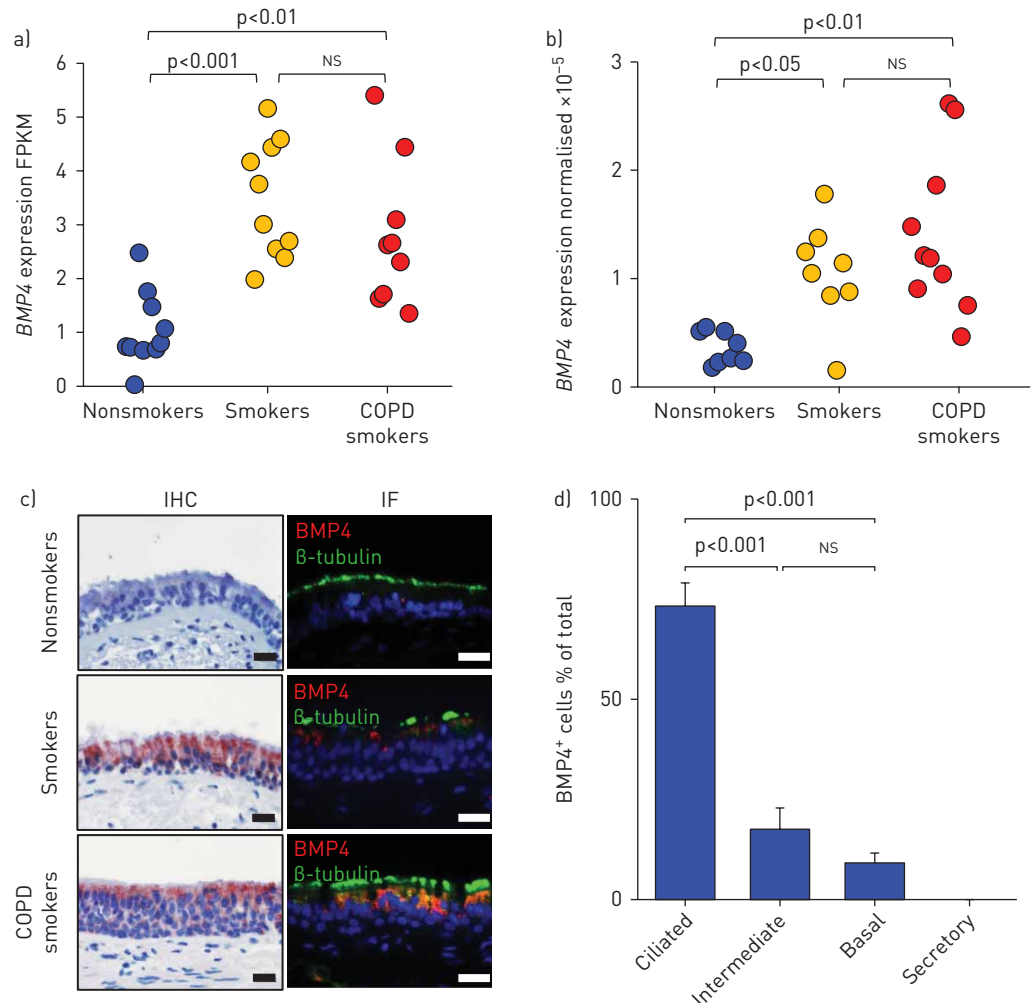


FIGURE 1 Upregulation of bone morphogenetic protein 4 (BMP4) in human airway epithelium of asymptomatic smokers and chronic obstructive pulmonary disease (COPD) smokers. FPKM: fragments per kilobase of transcript per million mapped reads; IHC: immunohistochemistry; IF: immunofluorescence. a) RNA sequencing assessment of *BMP4* expression in human airway epithelium of nonsmokers (n=10), asymptomatic smokers (n=10) and COPD smokers (n=9). b) TaqMan quantitative PCR normalised *BMP4* gene expression in human airway epithelium of nonsmokers (n=8), asymptomatic smokers (n=8) and COPD smokers (n=10). c) *BMP4* protein expression in human airway epithelium: IHC staining and IF colocalisation of *BMP4* (red) and ciliated cell marker  $\beta$ -tubulin (green) in human airway epithelium of nonsmokers, asymptomatic smokers and COPD smokers. Scale bar: 20  $\mu$ m. Nuclei are stained with 4',6-diamidino-2-phenylindole (blue). d) Distribution (percentage of total airway epithelial cells) of *BMP4*<sup>+</sup> cells in ciliated, intermediate, basal and secretory cells based on IHC staining on asymptomatic smokers (n=6) and COPD smokers (n=6) [based on assessment of a total of 444 cells from 12 different samples]. No secretory cells expressed *BMP4*. NS: nonsignificant.

supplementary table S1). *BMP4* upregulation in airway epithelium of asymptomatic smokers and COPD smokers was further confirmed by TaqMan quantitative PCR (figure 1b). Although there was no significant difference in *BMP4* expression of airway BCs from nonsmokers, asymptomatic smokers and COPD smokers, a subgroup of asymptomatic smokers and COPD smokers had higher *BMP4* expression than any of the nonsmokers (supplementary figure S2a). Comparing airway epithelium and airway BCs from asymptomatic smokers, *BMP4* was highly expressed in the total airway epithelium (supplementary figure S2a), suggesting that upregulated *BMP4* is mainly expressed in differentiated airway epithelial cells. The data are in agreement with the *in vitro* TaqMan data that *BMP4* expression increased during BC differentiation in the ALI model (supplementary figure S2b).

Using a unique microarray dataset from human small airway epithelium at multiple time-points (months 0, 3, 6 and 12), we observed that *BMP4* was upregulated at all time-points in COPD smokers when compared with nonsmokers (supplementary figure S3). When COPD smokers quit smoking after the initial bronchoscopy at month 0, their *BMP4* expression in small airway epithelium dramatically decreased

at month 3, and approached the normal level in nonsmokers at months 6 and 12. However, when COPD smokers stopped quitting smoking and re-started smoking after month 3, their *BMP4* expression in small airway epithelium increased again (supplementary figure S3). In summary, in addition to large airway epithelium RNA sequencing data, the microarray data confirm that *BMP4* expression in human small airway epithelium is also upregulated in COPD smokers and cigarette smoking is a “trigger” for *BMP4* expression in airway epithelium.

To determine the expression and localisation of BMP4 protein in human airway epithelium, immunohistochemistry and immunofluorescence staining were carried out on human airway epithelial biopsies. BMP4 protein was barely detected in normal airway epithelium of nonsmokers, but was markedly increased in  $\beta$ -tubulin<sup>+</sup> ciliated cells of normal airway epithelium from asymptomatic smokers and COPD smokers (figure 1c and supplementary figure S4). In abnormal airway epithelium with BC hyperplasia, mucous cell hyperplasia and squamous metaplasia from asymptomatic smokers and COPD smokers, BMP4 protein was upregulated in ciliated cells as well (figure 3a and supplementary figure S4). In addition to expression in ciliated cells, BMP4 was also upregulated in a subset of BCs and intermediate cells from asymptomatic smokers and COPD smokers, but was not expressed in secretory cells (figure 1d and supplementary figure S4), which was consistent with the RNA sequencing data in airway BCs. The cellular distribution of BMP4<sup>+</sup> cells from asymptomatic smokers and COPD smokers, which included both normal and abnormal morphologies of airway epithelium, showed that BMP4 was predominately induced in ciliated cells, as well a few intermediate cells and BCs (figure 1d). However, BMP4 protein was not detected in the mesenchyme underneath the epithelial cells (figures 1c and 3a, and supplementary figure S4).

#### ***BMP4 suppresses normal BC proliferation and differentiation***

BCs are the stem/progenitor cells in human airways, and are able to self-renew and differentiate into normal ciliated and secretory cells. To determine the effect of BMP4 on BC self-renewal and proliferation, we applied BMP4 to cultured normal nonsmoker BCs and identified that BMP4 significantly decreased BC number and suppressed BC proliferation (supplementary figure S5).

To study the effects of upregulated BMP4 on normal BC differentiation, we used ALI culture to assess human airway BC differentiation. The data demonstrated that BMP4 had a broad inhibitory effect on normal BC differentiation into a mucociliary epithelium. Compared with control, airway epithelium generated from BMP4-treated BCs had significantly lower expression of ciliated cell-related genes, including dynein *DNAI1*, transcription factor *FOXJ1*, and the transcription factors *MCIDAS* and *RFX2* that control early ciliogenesis (figure 2a and supplementary figure S6a). Consistent with the gene expression data, immunofluorescence staining of DNAI1 protein showed that BMP4 decreased the proportion of DNAI1<sup>+</sup> ciliated cells that were derived from BCs in ALI culture (figure 2b, and supplementary figure S6b and c). Moreover, BMP4 significantly suppressed the cilia beating of ciliated cells derived from healthy nonsmoker BCs (supplementary video S1a and d).

Similarly, BMP4 had an inhibitory effect on secretory cell differentiation. The secretory cell-related genes *SCGB1A1*, *SPDEF*, *MUC5B* and *TFF3* were significantly downregulated by BMP4 stimulation (figure 2a and supplementary figure S6a). Stimulation of BCs with different levels of BMP4 at various time-points in ALI culture showed that the suppression effects of ciliated and secretory cell differentiation by BMP4 were sustained during differentiation in ALI culture in a concentration-dependent manner (supplementary figure S6a). *SCGB1A1* protein was also suppressed by BMP4 stimulation (figure 2b). TaqMan analysis also showed that expression of early differentiation markers for ciliated cells (*MCIDAS*, *DNAH5* and *IFT172*) and secretory cells (*SPDEF* and *TFF3*) was suppressed (supplementary figure S6d) in cultured BCs by BMP4 stimulation. These data suggest that BMP4 begins to modulate the ciliated and secretory cell differentiation programme in BCs at a very early stage.

#### ***BMP4 promotes squamous cell differentiation***

As described earlier, BMP4 upregulation in asymptomatic smokers and COPD smokers was associated with abnormal morphology of airway epithelium, including squamous metaplasia (figure 3a and supplementary figure S4). To further confirm this observation, immunofluorescence co-staining of BMP4 with intermediate/squamous cell marker KRT6 was assessed in airway epithelium from nonsmokers, asymptomatic smokers and COPD smokers. In the nonsmoker samples with normal morphology, BMP4 and KRT6 expression levels were very low. However, in the samples from asymptomatic smokers and COPD smokers with normal and squamous metaplasia morphology, expression levels of both BMP4 and KRT6 were markedly increased, and a small subset of BMP4<sup>+</sup> cells expressed KRT6 (figure 3a). Based on the staining data that BMP4 upregulation appeared to be related to squamous metaplasia, we hypothesised that BMP4 shifted the normal BC differentiation to abnormal squamous cells. To assess this hypothesis, we stimulated human airway BCs with BMP4 in both ALI differentiation culture and BC culture models.

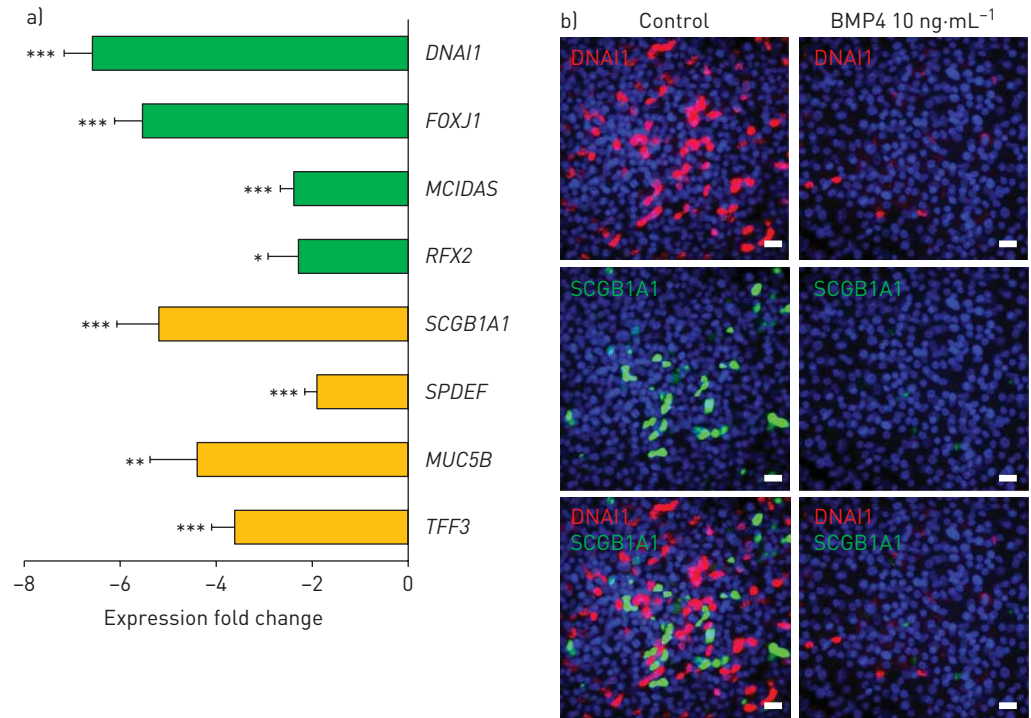


FIGURE 2 Bone morphogenetic protein 4 (BMP4) suppression of normal airway basal stem/progenitor cell (BC) differentiation. ALI: air-liquid interface. To mimic the effects of upregulated BMP4 observed in airway epithelium in asymptomatic smokers and chronic obstructive pulmonary disease smokers, BMP4 (10 ng·mL<sup>-1</sup>) was added to the basolateral side when the ALI cultures were established, and expression levels of airway epithelial differentiation-related genes and protein were assessed. a) TaqMan assessment of fold change (log<sub>2</sub>, BMP4 versus control) in mRNA expression of ciliated cell differentiation-related genes (*FOXJ1*, *DNAI1*, *MCIDAS* and *RFX2*; green bars) and secretory cell differentiation-related genes (*SCGB1A1*, *SPDEF*, *MUC5B* and *TFF3*; yellow bars) on airway epithelium after 14 days in ALI culture with BMP4 treatment versus untreated control. n=3 or 4. \*: p<0.05; \*\*: p<0.01; \*\*\*: p<0.001. b) Immunofluorescence top staining of ciliated cell marker DNAI1 (red) and secretory cell marker SCGB1A1 (green) on airway epithelium after 28 days in ALI culture with BMP4 (10 ng·mL<sup>-1</sup>) stimulation from the basolateral side versus untreated control. Nuclei are stained with 4',6-diamidino-2-phenylindole (blue). Scale bar: 20 μm.

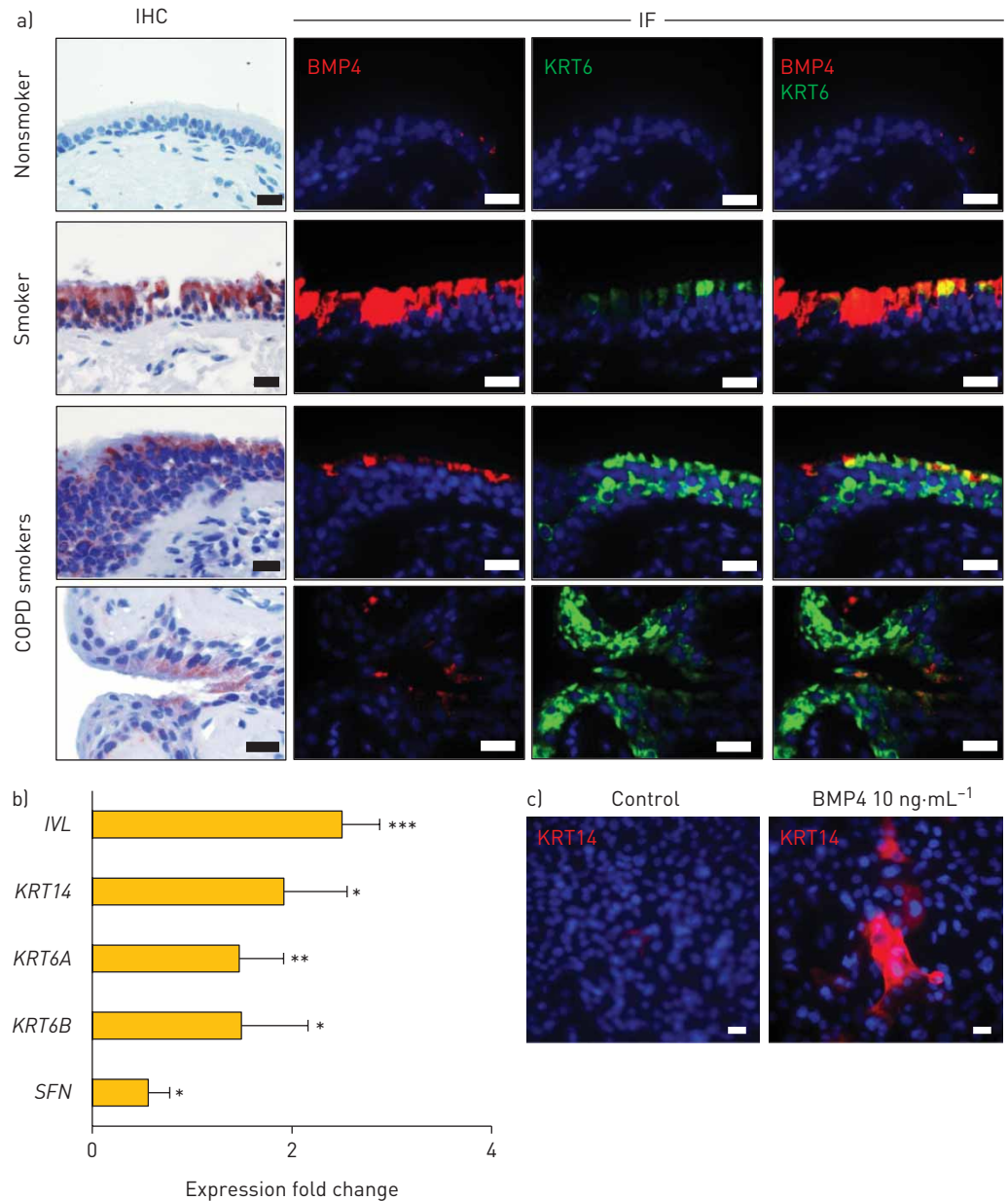
The TaqMan data showed that BMP4 could induce upregulation of a series of squamous cell markers, including *IVL*, *KRT14*, *KRT6A* and *KRT6B*, in both models (figure 3b, and supplementary figure S7a and e). BMP4 induced upregulation of *SFN* in ALI culture, but not in BC culture (figure 3b and supplementary figure S7e). The TaqMan data were strengthened by the immunofluorescence staining of squamous cell markers on airway epithelium derived from BCs with or without BMP4 stimulation. In airway epithelium derived from BMP4-treated BCs, more cells were positive for squamous cell markers KRT14 and IVL, while in the control groups, the percentages of IVL<sup>+</sup> and KRT14<sup>+</sup> cells were significantly lower (figure 3c and supplementary figure S7b–d). BMP4 not only suppressed ciliated and secretory cell differentiation, but also induced squamous cell differentiation in a concentration-dependent manner (supplementary figure S7a).

#### Effects of BMP4 on fully differentiated airway epithelium

In addition, stimulation of fully differentiated airway epithelium with BMP4 from the basolateral side also suppressed expression of ciliated cell (*FOXJ1* and *MCIDAS*) and secretory cell (*SCGB1A1*, *SPDEF*, *MUC5B* and *TFF3*) markers, and induced the upregulation of squamous cell markers (*IVL*, *KRT14* and *KRT6A*) (supplementary figure S8). However, apical application of BMP4 had no effects on the expression of ciliated cell (*FOXJ1*), secretory cell (*SCGB1A1*, *MUC5B* and *TFF3*) and squamous cell (*IVL* and *KRT14*) markers, and mildly suppressed the expression of ciliated cell marker *DNAI1* (supplementary figure S9).

#### BMP type I receptor enriched in airway BCs mediates BMP4-induced epithelial remodelling

BMP4 signals through two receptor families: BMP receptors (BMPRI1, BMPRI1B and BMPRI2) and activin receptors (ACVR1, ACVR2A and ACVR2B) [23]. Using the RNA sequencing data, we examined expression of BMP4 receptors in total human airway epithelium and BC samples. The data demonstrated that BMPRI1 expression slightly decreased and BMPRI1B expression increased in airway epithelium from



**FIGURE 3** Bone morphogenetic protein 4 [BMP4] induction of differentiation towards squamous cells. IHC: immunohistochemistry; IF: immunofluorescence; COPD: chronic obstructive pulmonary disease; ALI: air-liquid interface. a) From left to right: representative BMP4 IHC staining [positive cells are shown in red], IF staining of BMP4 (red), IF staining of squamous cell marker KRT6 (green), and overlap of BMP4 (red) and KRT6 (green) IF staining. These stains were performed on human airway epithelium of a nonsmoker with normal morphology, an asymptomatic smoker and COPD smokers with abnormal morphology. Nuclei are stained with 4',6-diamidino-2-phenylindole (blue). Scale bar: 20  $\mu$ m. The biopsy images with IHC and IF staining in each row were from the same sample. b) TaqMan assessment of fold change ( $\log_2$ , normalised to control) in the expression of squamous cell-related genes [IVL, KRT14, KRT6A, KRT6B and SFN] from airway epithelium after 14 days in ALI culture with BMP4 (10 ng·mL<sup>-1</sup>) stimulation from the basolateral side *versus* untreated control. n=3 or 4. \*: p<0.05; \*\*: p<0.01; \*\*\*: p<0.001. c) IF top staining of the squamous cell marker KRT14 (red) on airway epithelium after 14 days in ALI culture with BMP4 (10 ng·mL<sup>-1</sup>) stimulation from the basolateral side *versus* untreated control. Nuclei are stained with 4',6-diamidino-2-phenylindole (blue). Scale bar: 20  $\mu$ m.

asymptomatic smokers and COPD smokers, but not in airway BCs. Expression levels of *BMPR1B*, *ACVR2A* and *ACVR2B* were higher in airway epithelium compared with expression in BCs (supplementary figure S1c and supplementary table SII). In contrast, *BMPR1A*, *BMPR2* and *ACVR1* expression levels were higher in BCs than in complete airway epithelium, suggesting that BMP4 may regulate BC function through these receptors enriched in BCs. The same pattern of BMP4 receptor enrichment in BCs was observed in nonsmokers, asymptomatic smokers and COPD smokers (figure 4a,

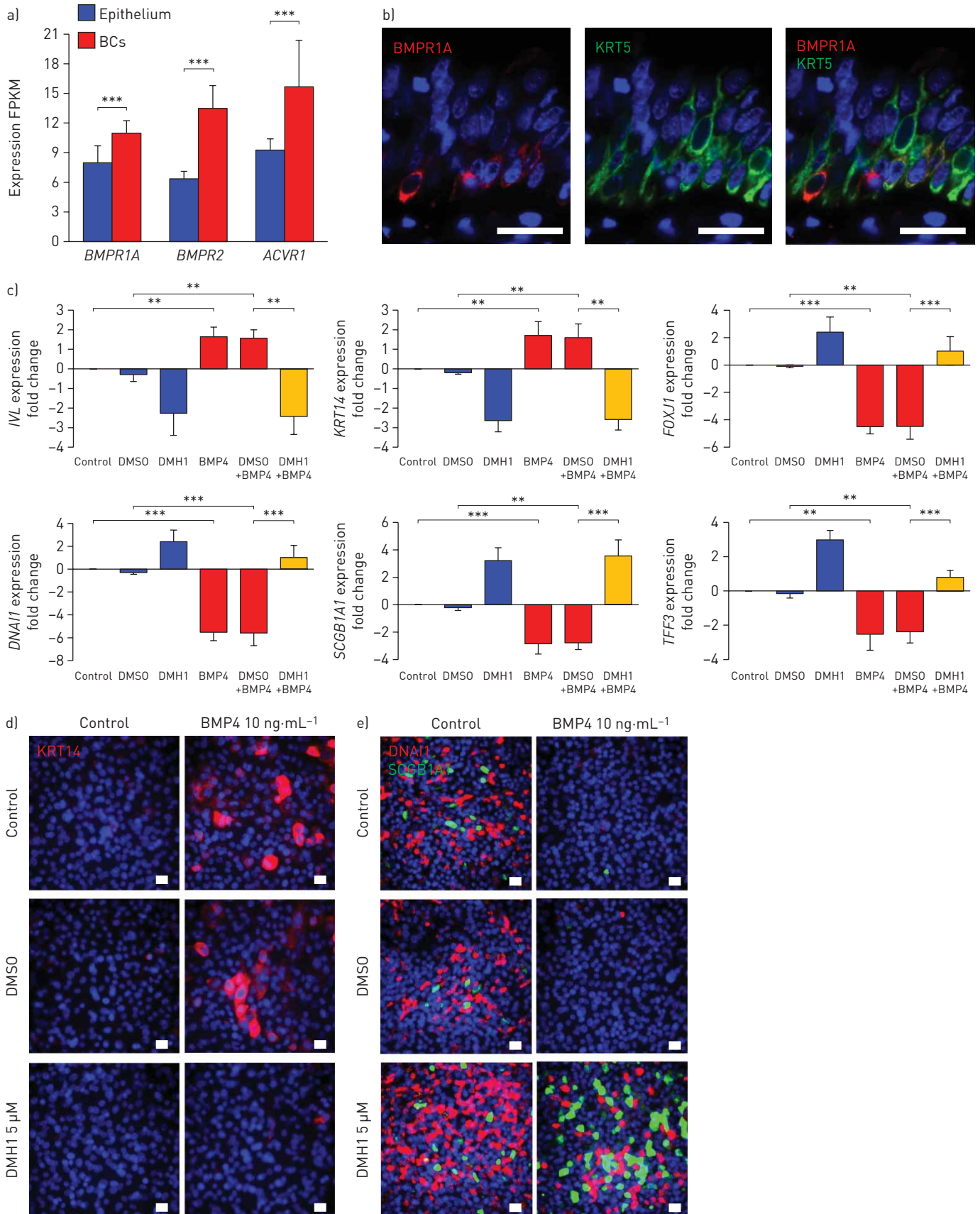
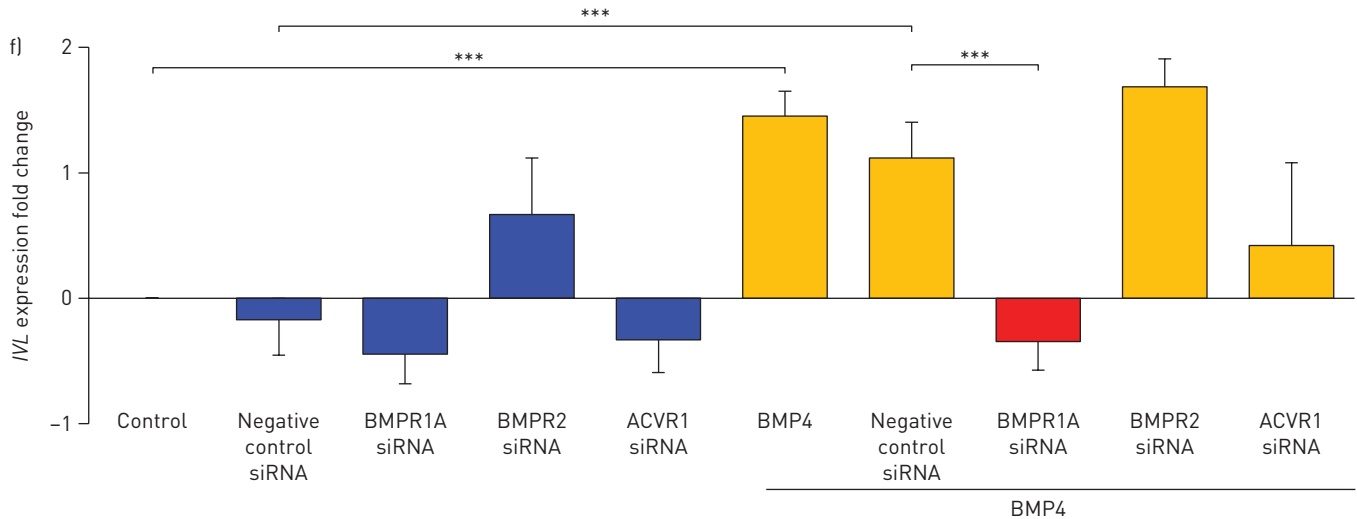


FIGURE 4 Continued.



**FIGURE 4** Bone morphogenetic protein (BMP) receptor-mediated BMP4-induced airway epithelial remodelling. FPKM: fragments per kilobase of transcript per million mapped reads; BC: basal stem/progenitor cell; DMSO: dimethylsulfoxide; ALI: air-liquid interface. **a)** RNA sequencing assessment of BMP4 receptors (*BMPR1A*, *BMPR2* and *ACVR1*) in human airway epithelium (n=29) and airway BCs (n=42). Detailed expression data of BMP4 receptors for individuals with different phenotypes are shown in supplementary figure S1c and supplementary table SII. **b)** Representative immunofluorescence co-localisation of BMPR1A (red) and BC marker KRT5 (green) in normal human airway epithelium. Scale bar: 20  $\mu$ m. Nuclei are stained with 4',6-diamidino-2-phenylindole (blue). **c)** TaqMan analysis of fold change ( $\log_2$ , normalised to control) in the expression of various genes related to airway BC differentiation (*IVL* and *KRT14*: squamous cells; *FOXJ1* and *DNAI1*: ciliated cells; *SCGB1A1* and *TF3*: secretory cells). Data were generated from airway epithelium in ALI culture (day 14) with BMP4 (10 ng·mL<sup>-1</sup>) stimulation from the basolateral side *versus* untreated control in the absence or presence of BMP type I receptor inhibitor DMH1 (5  $\mu$ M). DMSO was used as the negative control. n=3. **d)** Representative immunofluorescence top staining of squamous cell marker KRT14 (red) on airway epithelium after 14 days in ALI culture. **e)** Representative immunofluorescence top staining of ciliated cell marker DNAI1 (red) and secretory cell marker SCGB1A1 (green) on airway epithelium after 28 days in ALI culture. Nuclei are stained with 4',6-diamidino-2-phenylindole (blue). Scale bar: 20  $\mu$ m. **f)** TaqMan assessment of expression of the squamous cell marker *IVL* in BCs with BMP4 (10 ng·mL<sup>-1</sup>) stimulation *versus* untreated control for 48 h in the presence of small interfering RNA silencing of BMPR1A, BMPR2 or ACVR1. The data were normalised to the untreated control ( $\log_2$ ). n=3 or 4. \*\*: p<0.01; \*\*\*, p<0.001.

supplementary figure S1c and supplementary table SII). Immunofluorescence staining further confirmed that BMPR1A protein was mainly expressed in a subset of KRT5<sup>+</sup> BCs (figure 4b).

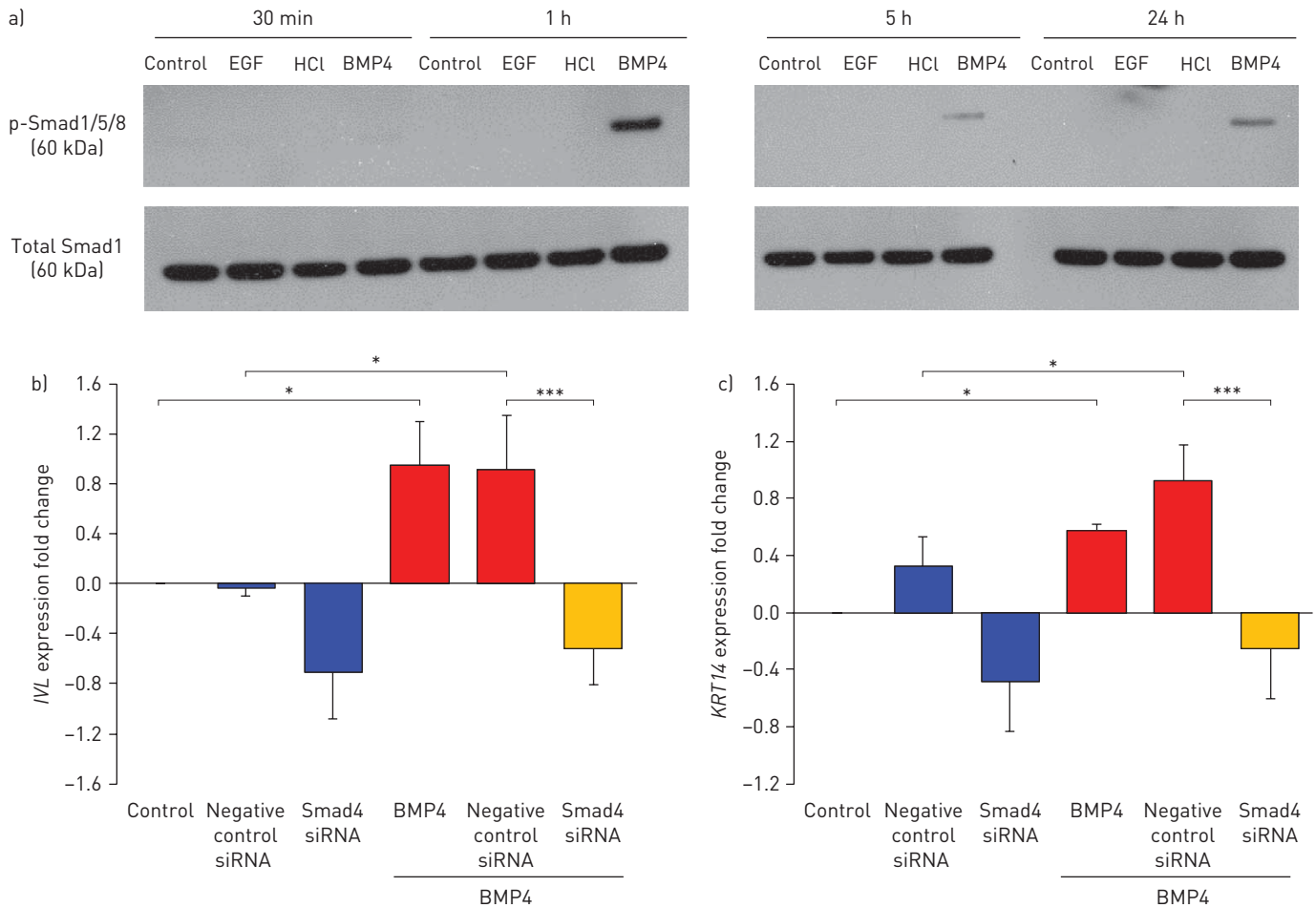
To assess whether BMP receptors mediate BMP4-induced airway epithelial remodelling, DMH1, a selective inhibitor of BMP type I receptors, was applied to the ALI culture while stimulating BCs with BMP4. The data showed that DMH1 reversed BMP4-induced upregulation of squamous cell markers (*IVL* and *KRT14*), and downregulation of ciliated (*FOXJ1* and *DNAI1*) and secretory cell (*SCGB1A1* and *TF3*) markers (figure 4c). Immunofluorescence staining of airway epithelium with squamous cell marker KRT14, ciliated cell marker DNAI1 and secretory cell marker SCGB1A1 validated that DMH1 could block BMP4-induced airway epithelial remodelling (figure 4d and e). Furthermore, DMH1 also reversed BMP4-suppressed cilia cell beating (supplementary video S1).

To further evaluate the specific receptor that mediates BMP4-induced abnormal BC differentiation, BMPR1A, BMPR2 and ACVR1 small interfering RNAs (siRNAs) were applied to cultured BCs with BMP4 treatment *versus* control. BMPR2 siRNA had no effect on cultured BCs, but BMPR1A siRNA significantly decreased BMP4-induced upregulation of squamous cell marker *IVL* (figure 4f). Although not significant, ACVR1 siRNA tended to suppress BMP4-induced upregulation of *IVL*. Additional evidence in ALI differentiation culture confirmed that silencing of BMPR1A could reverse BMP4-induced squamous metaplasia (supplementary figure S10a) and suppression of ciliated cell differentiation (supplementary figure S10b). Efficiency of all the siRNAs was validated by TaqMan analysis of receptor expression in cultured BCs and airway epithelium in ALI culture (supplementary figure S11).

#### **Smad signalling mediates BMP4-induced squamous metaplasia**

To assess whether BMP4 downstream Smad signalling was activated by BMP4 on human airway BCs, Western blot analysis of phosphorylated (p)-Smad1/5/8 was performed on BCs in ALI culture. BMP4 activated Smad1/5/8 phosphorylation at 1 h, and the activation continued but with weaker signalling at 5 and 24 h (figure 5a). To evaluate whether Smad signalling mediates BMP4-induced squamous metaplasia, Smad4, which translocates p-Smad1/5/8 to the nucleus, was silenced by siRNA in cultured BCs (supplementary figure S12). TaqMan analysis showed that BMP4-induced expression of squamous cell





**FIGURE 5** Smad signalling mediated bone morphogenetic protein 4 (BMP4)-induced squamous cell differentiation. p-Smad: phosphorylated Smad; EGF: epidermal growth factor; siRNA: small interfering RNA; BC: basal stem/progenitor cell. **a)** Western blot analysis shows p-Smad1/5/8 and total Smad1 protein expression of airway BCs in air-liquid interface culture (day 0) with or without BMP4 ( $10 \text{ ng}\cdot\text{mL}^{-1}$ ) stimulation for 30 min, 1, 5 and 24 h. BMP4 solvent HCl and EGF ( $10 \text{ ng}\cdot\text{mL}^{-1}$ ; EGF does not activate BMP4 downstream Smad signalling [23]) were used as negative controls. **b, c)** TaqMan assessment of expression of the squamous cell markers **b)** *IVL* and **c)** *KRT14* in BCs with BMP4 ( $10 \text{ ng}\cdot\text{mL}^{-1}$ ) stimulation versus untreated control for 48 h while silencing Smad4 by siRNA. Negative control siRNA was used as the negative control and the data were normalised to the untreated control ( $\log_2$ ).  $n=4$ . \*:  $p<0.05$ ; \*\*\*:  $p<0.001$ .

markers (*IVL* and *KRT14*) was decreased (figure 5b and c), suggesting that Smad signalling mediated BMP4-induced human airway squamous metaplasia.

#### ***Inhibition of BMP4 signalling reverses cigarette smoke extract- and EGF-induced abnormal phenotypes***

Cigarette smoke extract (CSE) is known to alter BC differentiation to suppress the generation of mucociliary epithelium and induce squamous cell differentiation (figure 6 and supplementary figure S13) [12]. To assess whether BMP4 signalling was involved in CSE-induced airway epithelial remodelling, BMP type I receptor inhibitor DMH1 was applied to BC differentiation culture in ALI culture when treated by CSE. The gene expression data showed that DMH1 significantly reversed CSE-induced upregulation of the squamous cell markers *IVL* (figure 6a) and *KRT14* (figure 6b), and downregulation of ciliated cell markers (*FOXJ1* and *DNAI1*) (supplementary figure S13a) and secretory cell markers (*SCGB1A1* and *TFF3*) (supplementary figure S13b).

As with CSE, we previously identified that EGF signalling plays a role in reprogramming BC differentiation to the smoking-relevant phenotypes [5, 18]. Here, we found that inhibition of BMP4 signalling by DMH1 could restore EGF-induced abnormal phenotypes in the BC differentiation process, including downregulation of ciliated (*FOXJ1* and *DNAI1*) and secretory cell (*SCGB1A1*) markers, and upregulation of squamous cell markers (*IVL* and *KRT14*) (supplementary figure S14a). However, BMP4 had no effect on the expression of amphiregulin (*AREG*; an EGFR ligand) (supplementary figure S14b), the upregulation of which is associated with BC and mucous cell hyperplasia [18].

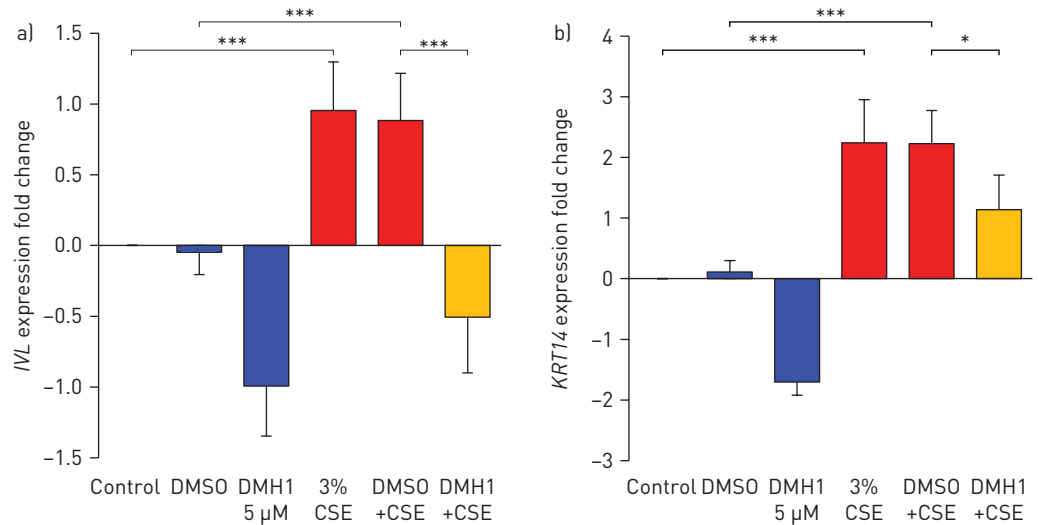


FIGURE 6 DMH1 suppressed cigarette smoke extract (CSE)-induced squamous cell differentiation. DMSO: dimethylsulfoxide; BC: basal stem/progenitor cell. a, b) TaqMan assessment of fold change ( $\log_2$ , normalised to control) in gene expression of the squamous cell markers a) *IVL* and b) *KRT14* in airway epithelium derived from BCs after 14 days in air-liquid interface culture. In CSE-treated groups, BCs were exposed to 3% CSE stimulation from the basolateral side *versus* untreated control in the absence or presence of bone morphogenetic protein 4 type I receptor inhibitor DMH1 (5  $\mu$ M). DMSO was used as negative control. n=4. \*: p<0.05; \*\*\*: p<0.001.

## Discussion

COPD is a cigarette smoking-related chronic lung disease characterised by long-term reduced airflow with chronic cough, increased sputum production and shortness of breath [24]. The early pathogenesis of COPD is associated with dramatic architectural changes of airway epithelium, including BC and intermediate cell hyperplasia, decreased number of ciliated cells, shortening cilia length, mucus overproduction, goblet cell hyperplasia, and squamous metaplasia [5–13]. The present study identifies a novel mechanism that regulates airway epithelial remodelling in asymptomatic smokers and COPD smokers based on the observation that expression of BMP4 is upregulated in differentiated airway epithelium of asymptomatic smokers and COPD smokers. The upregulated BMP4 interacts with BCs, the stem/progenitor cells in the human airway, to suppress normal mucociliary differentiation and induce squamous metaplasia, *i.e.* processes directly relevant to the pathogenesis of COPD (supplementary figure S17).

### *BMP4 expression in airway epithelium*

BMP4, a ligand classified with the TGF- $\beta$  superfamily, is expressed in many tissues, and plays a critical role in embryogenesis, embryonic development and homeostasis of adult organs [23]. In the developing embryonic mouse lung, BMP4 is expressed in the distal tips of the epithelium [25], and dynamics of BMP4 expression in endoderm during lung development determine lung bud morphogenesis [20] and epithelial proximal–distal patterning [21]. In the adult mouse airway, BMP4 is highly expressed in fibroblast-like cells in the mesenchyme and weakly expressed in some luminal epithelial cells in the steady state. During injury, BMP4 expression decreases in the mesenchyme and disappears in epithelial cells [26]. In addition, BMP4 has been reported to be expressed in mouse lung endothelial cells, suppressed by naphthalene injury and induced by bleomycin injury [27].

There is limited information available on BMP4 expression in human airway epithelium. Consistent with our data, BMP4 mRNA is detected in primary cultured human bronchial epithelial cells and the expression of BMP4 is increased in ALI culture [28], suggesting BMP4 may be involved in mucociliary differentiation. In the present study, taking advantage of RNA sequencing of bronchoscopy brushing epithelial cells and immunostaining of human airway epithelial biopsies from nonsmokers, asymptomatic smokers and COPD smokers, we found that BMP4 expression is very low in normal airway epithelium. In contrast, in asymptomatic smokers and COPD smokers, BMP4 expression was significantly upregulated, mostly in ciliated and intermediate cells, as well as in a subset of BCs. Unlike BMP4 expression in the mouse airway, BMP4 expression in asymptomatic healthy smokers and COPD smokers was not detected in mesenchyme and epithelial secretory cells in the human airway. Importantly, BMP4<sup>+</sup> cells were not only observed in normal airway epithelium of smokers, but were markedly increased in abnormal airway

epithelium in asymptomatic smokers and COPD smokers that also had abundant KRT6 expression. The expression correlation between BMP4 and KRT6, which has been related to squamous differentiation [5, 18, 29], suggests that BMP4 is relevant to the generation of remodelled airway epithelium. In contrast to the upregulation of BMP4, the expression of other BMPs (BMP2, BMP5 and BMP6), BMP antagonists (NOG, FST, FSTL3, CHRDL1, CHRDL2 and TWIG2) and BMP4 receptors (BMPRI1, BMPRI2 and ACVR2A) did not change in airway epithelium obtained from asymptomatic smokers and COPD smokers. BMP7 and BMPRI1B expression levels were increased, and BMPRI1A expression levels were decreased, in asymptomatic smokers and COPD smokers, respectively, but with less dramatic change. Taken together, these data suggest that BMP4 is the main component in the BMP pathway that induces abnormal airway epithelium in asymptomatic smokers and COPD smokers. However, the mechanism of how cigarette smoking enhances BMP4 expression is unknown. It is reported that Hedgehog signalling is evoked by chronic cigarette smoke exposure in human airway epithelial cells [30] and Hedgehog signalling induces expression of BMP4 [31]. If so, the exaggerated BMP4 in smokers may be the result of activation of Hedgehog signalling in epithelial cells. BCs are stem/progenitor cells capable of differentiating into mucociliary epithelium and BCs from COPD smokers are quite different from BCs from nonsmokers [32, 33]. These observations suggest another hypothesis, *i.e.* that the enhanced BMP4 in differentiated airway epithelium is due to altered differentiation of BCs in COPD smokers.

#### ***Exaggerated BMP4 in airway epithelium induces cigarette smoking-relevant phenotypes***

In the mouse, conditional knockout of BMP4 in the anterior foregut causes loss of the trachea [34]. During morphogenesis of the mouse lung, BMP4 in endoderm cooperates with fibroblast growth factor 10 in the distal mesenchyme to regulate branching morphogenesis [20]. BMP4 also controls endodermal proximal–distal patterning during lung development [21]. In resting adult mouse airway epithelium, BMP4 inhibits BC proliferation, while following an injury, BMP4 signalling is suppressed, allowing airway epithelial proliferation and repair [26].

In humans, BMP4 has been shown to activate the EMT response in airway epithelial cells [22, 35]. BMP4 is also crucial for human pluripotent stem cell differentiation to lung cells [36, 37]. However, the effects of BMP4 on human BCs are still unknown. BCs are stem/progenitor cells in human airway epithelium and BCs from COPD smokers fail to generate normal airway epithelium [33], suggesting human airway BCs play a central role in the pathogenesis of smoking-related lung diseases. Based on 1) cigarette smoking reduces the junctional barrier integrity of airway epithelium, partially mediated by EGF–EGFR signalling [5, 17, 18], to allow the particles/proteins to cross the barrier junctions of airway epithelium, and 2) our observation that BMP4 is expressed abundantly in airway epithelium of smokers, we hypothesised that upregulated BMP4 passes through the damaged barrier junctions of airway epithelium, and interacts with BCs to switch the normal BC self-renewal and differentiation towards smoking-relevant abnormal airway epithelium. From our data, BCs exposed to BMP4 have a decreased proliferation rate, and were not able to differentiate into normal mucociliary epithelium and were reprogrammed to generate squamous cells. Interestingly, the expression of SPDEF, a transcription factor that regulates goblet and club cell differentiation [38, 39], was also suppressed by BMP4. These data are in agreement with the reduction of club cells in COPD smokers [40], while opposite to the expected phenotypes of goblet cell hyperplasia in smokers [7, 10], suggesting a complicated mechanism that regulates the smoking-related phenotypes in human airway epithelium.

#### ***BMP signalling pathway reprogramming of human airway epithelial differentiation***

BMP4 signals through canonical Smad-dependent and other noncanonical signalling pathways [23]. In the canonical signalling pathway, BMP4 binds to receptors, including type I and II receptors, to initiate the transduction cascade. Activation of the receptor by BMP4 phosphorylates intracellular Smad1/5/8 [23]. With the help of Smad4, the phosphorylated Smad1/5/8 translocates to the nucleus to regulate gene expression. Noncanonical pathways, like those of mitogen-activated protein kinase (MAPK), phosphoinositide 3-kinase and p38, can also be activated by BMP4 [41–43]. BMPRI1A, one of the BMP type I receptors, is critical for mouse lung development [44]. Deletion of BMPRI1A decreases epithelial proliferation in the distal embryonic lung, and leads to apoptosis and abnormal morphogenesis in the mouse lung [44]. We identified that BMPRI1A serves as the major receptor to mediate BMP4-induced smoking-relevant abnormal phenotypes and its downstream Smad signalling mediates squamous cell differentiation as well. As BMPRI1A is only expressed in a subset of BCs *in vivo*, we consider the BMP4-induced smoking-relevant phenotypes (decreased mucociliary epithelium and increased squamous metaplasia) would reside in a “local” area of airway epithelium. The “local changes” of airway epithelium mediated by BMP4 may represent an early pathogenesis of smoking-related diseases, including COPD.

We previously reported that CSE and EGF/amphiregulin–EGFR signalling altered the BC differentiation programme towards smoking-related lesions [5, 12, 18], which is similar to the phenotype induced by

BMP4. Interestingly, our data showed that blocking the BMP receptor reversed CSE/EGF-induced smoking-related lesions, suggesting that CSE/EGF and BMP4 might share some common signalling pathways in BCs. MAPK signalling might be a candidate, as it is the downstream signalling pathway for EGF and noncanonical downstream signalling for BMP4 [45]. However, BMP4 did not change the expression of amphiregulin, suggesting that BMP4 signalling is not entirely overlapping with EGF/amphiregulin signalling.

In summary, our study identified a novel BMP4-dependent mechanism related to abnormal airway epithelial reprogramming associated with smoking and COPD. Modulation of BMP4 or the downstream receptors and signalling cascades in human airway BCs may be a potential therapeutic approach to cure or prevent the pathogenesis of smoking-induced airway disease.

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