

**Role of aberrant WNT signaling in the airway epithelial response to cigarette smoke in  
COPD**

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**Online data supplement**

## Methods

### *Epithelial cell culture*

The human alveolar type-II carcinoma cell line A549 was cultured on uncoated flasks in RPMI medium/10% FCS.

### *Realtime RT-PCR*

RNA was isolated from cells or lung tissue and cDNA was synthesized as described previously<sup>1</sup>. Samples were treated with RNase Free DNase and subsequently cleaned with RNeasy Mini Kit (Qiagen). cDNA was synthesized with the iScript cDNA Synthesis Kit (BioRad). Gene expression was analyzed by real-time PCR using the Taqman® according to the manufacturer's guidelines (Applied Biosystems, Foster City, CA). Validated probes for hemoxygenase-1 (HO-1), IL-8, MMP-2, MMP-9, fibronectin, FZD1, FZD2, FZD6, FZD8, WNT-4, WNT-5A, WNT-5B, WNT-7B, WNT-10B, WNT-16, WNT-inhibitory factor (WIF), WNT-induced secreted protein (WISP)1, Secreted-Frizzled Related Protein (SFRP)2,  $\beta$ -catenin, Dickkopf (DKK)1, axin-2, p21<sup>Waf1/CIP1</sup> and the housekeeping genes  $\beta_2$ -microglobulin ( $\beta_2\mu$ G) and Peptidylprolyl isomerase A (PPIA). The TaqMan Master Mix were purchased from Applied Biosystems.

### *Measurement of cytokines in supernatant*

VEGF and IL-8 protein levels were measured in cell-free supernatants using ELISA kits according to the manufacturer's guidelines (R&D systems Europe Ltd., Abingdon, UK). IL-1 $\alpha$ , IL-1 $\beta$ , IL-6, IL-8, CCL2, CCL3, CCL4 and CCL5 were measured by the use the Luminex-100

system as described before (Luminex Corporation, Austin, Tex., USA)<sup>2</sup>. using a Multiplex Panel (Biosource International, Inc., Camarillo, Calif., USA) according to the manufacturers' protocols.

*Immunodetection by western blotting*

Total cell lysates were obtained by resuspension of the cells in Laemmli buffer and immunodetection was performed as described<sup>3</sup>, using anti-WNT-4 (MAB4751, R&D Systems), anti-phospho-p38, anti-phospho-GSK-3 $\beta$  and anti-phospho-MLC (Cell Signaling Technology, Hitchin, Herts, UK) and anti-GAPDH (Abcam, Cambridge, UK) as loading control.

## Figure legends

**Figure E1.** Effects of CSE on the expression of WNT genes and target genes in bronchial epithelial 16HBE cells, PBECs and alveolar epithelial A549 cells. Cells were serum/growth factor-deprived overnight, incubated with or without 5 or 10% CSE for 6 hrs and RNA was isolated. **A)** HO-1 gene expression was related to the expression of the house keeping genes  $\beta 2\mu\text{G}$  and PPIA. mRNA levels are expressed as fold change compared to the unstimulated control value ( $2^{-\Delta\Delta\text{Ct}}$ ,  $n=4-6$ ) and medians are indicated. **B, C)** WNT gene (WNT-4, -5B, -7B, -10B and FZD2,-6,-8) and target gene (MMP-2,-9) expression was related to the expression of the house keeping genes  $\beta 2\mu\text{G}$  and PPIA. mRNA levels are expressed as fold change compared to the unstimulated control value ( $2^{-\Delta\Delta\text{Ct}}$ ,  $n=6-9$ ) and medians are indicated. WNT gene (WNT-4, -5B, -7B, -10B and FZD2,-6,-8) and target gene (MMP-2,-9) expression was related to the expression of the house keeping genes  $\beta 2\mu\text{G}$  and PPIA. mRNA levels are expressed as fold change compared to the unstimulated control value ( $2^{-\Delta\Delta\text{Ct}}$ ,  $n=6-9$ ) and medians are indicated.  $^* = p < 0.05$  versus control.

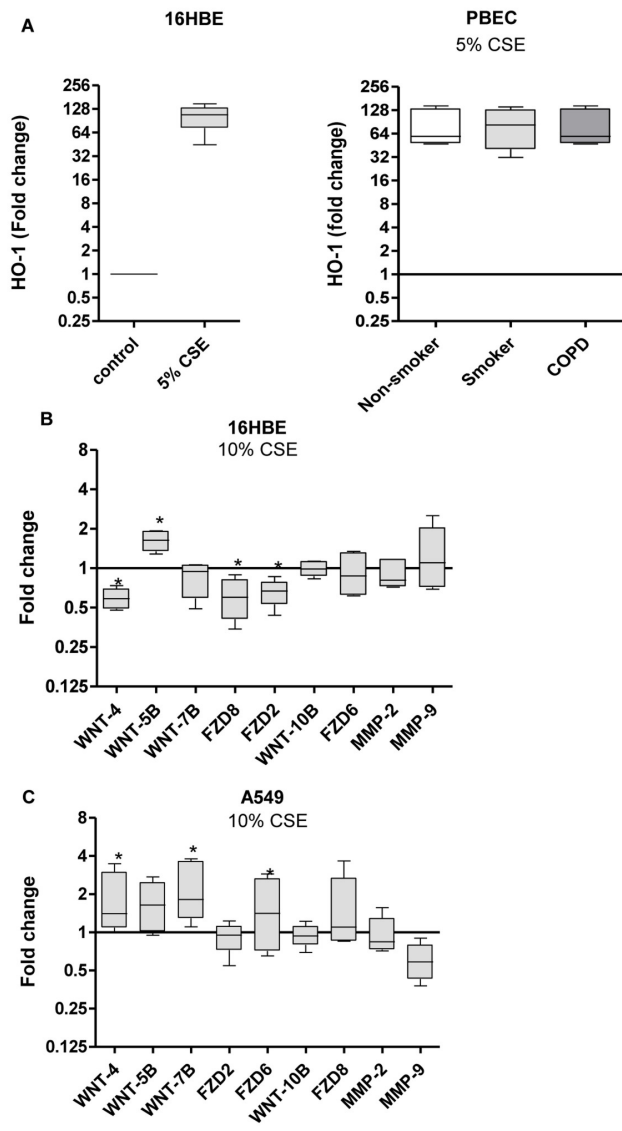


Figure E1

**Figure E2.** mRNA expression of WNT-4 is not different between PBECs from COPD current and ex-smokers or between brushed and Lonza PBECs derived from non-smoking and smoking donors. Cells were growth-factor deprived overnight, cultured in fresh growth factor-free medium for 2 hrs and mRNA was isolated. **A)** WNT-4 mRNA expression was assessed in PBECs from stage II-IV COPD patients (ex-smokers  $n=8$ , current smokers  $n=5$ ) at baseline. mRNA levels are expressed as fold change compared to one of the COPD ex-smokers ( $2^{-\Delta\Delta C_t}$ ) and medians are indicated. **B)** WNT-4 mRNA expression was assessed in brushed and Lonza PBECs from non-smokers and smokers at baseline. mRNA levels are expressed as fold change to of the non-smoking brushed cultures ( $2^{-\Delta\Delta C_t}$ ) and medians are indicated.

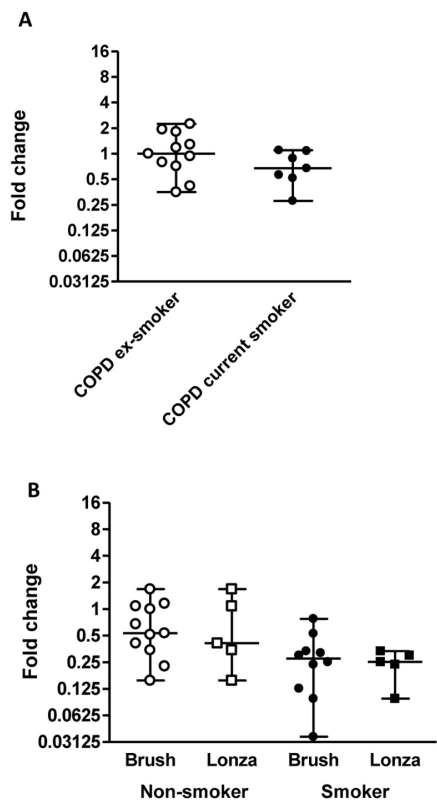


Figure E2

**Figure E3.** WNT-4 does not induce activity of the  $\beta$ -catenin-driven reporter TopFLASH in 16HBE cells. Cells were seeded in duplicates in 24-well plates, grown overnight and transfected with TopFLASH (0.5 $\mu$ g/ml) and the thymidine kinase-driven Renilla luciferase vector pRL-TK (0.05 $\mu$ g) as internal control, and stimulated with/without GSK-3 $\beta$  inhibitor LiCl as positive control WNT-4 for 24 hrs. Luciferase activity was determined, values were normalized (TopFLASH/Renilla ratio) and expressed as means  $\pm$  SEM ( $n=3$ ).



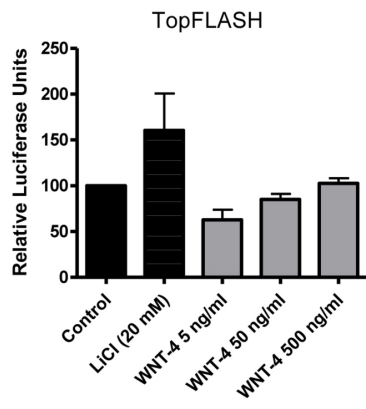


Figure E3

**Figure E4.** Recombinant human WNT-4 increases the release of IL-8 in A549 cells. A549 cells were treated with 500 ng/ml rhWNT-4. IL-8 levels were assessed upon 24 hrs of WNT-4 exposure in cell-free supernatants by ELISA. Absolute cytokine levels are shown (mean±SEM,  $n=3$ ).  $*=p<0.05$ .

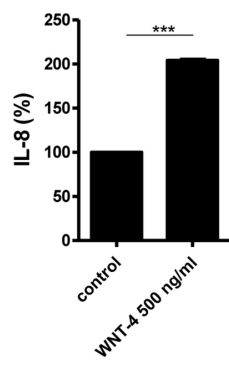


Figure E4

**Figure E5.** WNT-4 does not induce phosphorylation of MLC and GSK-3 $\beta$  in 16HBE cells. 16HBE cells were serum-deprived overnight and treated for with or without 50 ng/ml rhWNT-4. Total cell lysates were prepared upon 5-240 min. phospho-MLC and phospho-GSK-3 $\beta$  were detected by western blotting. GAPDH was used as loading control. A representative of 3 independent experiments.

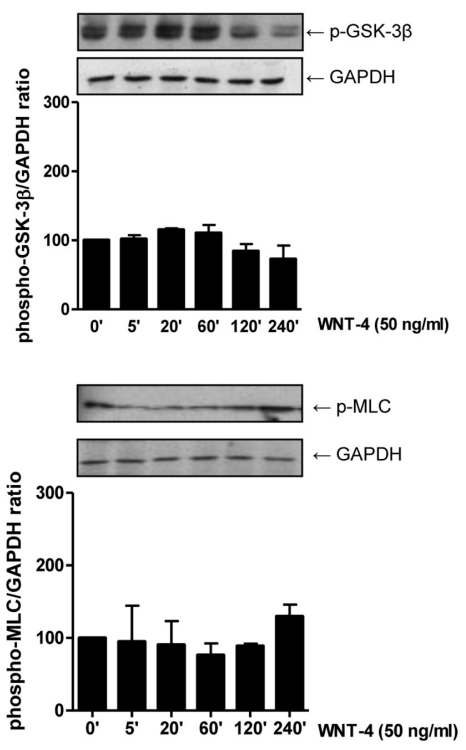


Figure E5

## Reference List

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