

A pro-inflammatory role for the Frizzled-8 receptor in chronic bronchitis

Supplementary File

Authors

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Extended Methods section

Ethics statement

For the genetics study on chronic mucus hypersecretion (CMH) approval by the local medical ethics committee and written informed consent from all patients was obtained. At Laval, lung specimens were collected from patients undergoing lung cancer surgery and stored at the “Institut universitaire de cardiologie et de pneumologie de Québec” (IUCPQ) site of the Respiratory Health Network Tissue Bank of the “Fonds de recherche du Québec – Santé” (www.tissuebank.ca). Written informed consent was obtained from all subjects and the study was approved by the IUCPQ ethics committee. At Groningen, lung specimens were provided by the local tissue bank of the Department of Pathology and the study protocol was consistent with the Research Code of the University Medical Center Groningen and Dutch national ethical and professional guidelines (“Code of conduct; Dutch federation of biomedical scientific societies”; <http://www.federa.org>). At Vancouver, the lung specimens were provided by the James Hogg Research Center Biobank at St. Paul's Hospital and subjects provided written informed consent. The study was approved by the ethics committees at the UBC-Providence Health Care Research Institute Ethics Board.

Animal studies

Heterozygous, inbred, specified-pathogen-free breeding colonies FZD8^{+/-} mice (C57BL/6;129P2-FZD8^{tm1Dgen}/J), showing no obvious phenotype, were obtained from the Jackson Laboratory (USA). After breeding, homozygous FZD8^{-/-} mice and wild-type^{+/+} (WT) littermates were used for experiments. Animals were housed under a 12 hour light-dark cycle and received food and water *ad libitum*. Male FZD8^{-/-} and WT mice were subjected for four

successive days to fresh air or cigarette smoke from Kentucky 3R4F research cigarettes (Tobacco Research Institute, University of Kentucky, Lexington, KY, USA) by whole body exposure, as described previously.[1] In brief, cigarette smoke was directly circulated into a 6-liter Perspex box. After removing the filter, each cigarette was smoked in five minutes at a rate of 5L/hour in a ratio of 60L/hour air using a peristaltic pump (45 rpm, Watson Marlow 323 E/D, Rotterdam, the Netherlands). On the first day, mice were exposed to one cigarette in the morning and three cigarettes in the afternoon. On the second to fourth day, mice were exposed to five cigarettes in the morning and five cigarettes in the afternoon. Control animals were exposed at the same time intervals to fresh air. Eight mice were included in each group. Sixteen hours after the last cigarette smoke exposure, mice were euthanized by subcutaneous injection with a mixture of medetomidine (0.5 mg/kg Dormitor[®], Orion Pharma, Mechelen, Belgium) and ketamine (40.0 mg/kg, Alfasan, Woerden, the Netherlands) followed by exsanguination. The lungs were lavaged five times with 1 mL PBS. The bronchial alveolar lavage fluid (BALF) fractions were pooled. From these, cytopins were prepared to determine total and inflammatory cell numbers. Cytopins were stained with May-Grünwald and Giemsa (Sigma, St. Louis, MO, USA). Differential cell count was performed by counting 400 cells in duplicate in a blinded manner. Preceding immunohistochemistry, the upper right lung lobe was taken up in formalin and paraffin-embedded. Preceding mRNA isolation, the post caval lobe was snap frozen, mechanically crushed under liquid nitrogen and taken up in lysis buffer.

Fibroblast cell culture

Human airway and parenchymal lung fibroblasts from ex-smoking GOLD stage IV COPD patients with and without CMH were isolated from transplanted lungs as has been described previously.[2] Presence of CMH was defined by patient records. Patient characteristics are shown in table 1. MRC-5 human lung fibroblasts[3] were obtained from Sigma (St. Louis,

MO, USA). Primary human airway and parenchymal lung fibroblasts and primary MRC-5 human lung fibroblasts were cultured in Ham's F12 medium supplemented with 10% (v/v) fetal bovine serum (FBS), 2 mM L-glutamine, 50 µg/ml streptomycin, 50 U/ml penicillin and 1.5 µg/ml amphotericin B. Prior to the experiment, cells were grown to confluence in 6-well or 24-well cluster plates and placed in Ham's F12 medium with 0.5% (v/v) FBS for 24 hours. Subsequently, cells were stimulated with either 2 ng/ml recombinant human transforming growth factor (TGF)- β_1 , 1 ng/ml recombinant human interleukin (IL)-1 β , 10 ng/ml recombinant human tumor necrosis factor (TNF)- α , 10 ng/ml recombinant human epidermal growth factor (EGF) or 5% cigarette smoke extract (CSE) for several time points. To prepare CSE, two cigarettes were smoked sequentially using a peristaltic pump (323 E/D; Watson Marlow, Rotterdam, the Netherlands) at 45 rpm through 25 ml of Ham's F12 medium supplemented with 0.5% (v/v) FBS, referred to as 100% CSE. 100% CSE was diluted to a working concentration of 5% CSE. CSE was freshly prepared before each experiment.

Air liquid interface (ALI) cell culture

Human airway epithelial cells were isolated after incubating tracheal tissue from donor lungs for 2 hours at 37°C in Protease IX (Sigma, St. Louis, MO, USA) and plated on coated culture dishes. Coating consisted of 10 µg/ml bovine serum albumin, 10 µg/ml fibronectin (both from Sigma, St. Louis, MO, USA) and 30 µg/ml collagen (PureCol[®], Advanced Biomatrix, San Diego, CA, USA) in phosphate buffered saline (PBS). Human airway epithelial cells were grown to approximately 70% confluence in keratinocyte serum-free medium (KSFM) supplemented with 25 µg/ml streptomycin, 25 U/ml penicillin, 1 µM isoproterenol, 0.2 ng/ml EGF, 25 µg/ml bovine pituitary extract. For the first week after isolation, KSFM medium was additionally supplemented with 1.5 µg/ml amphotericin B and 5 µg/ml ciproxin. Cells were plated on a 0.4 µm polyester membrane 12 mm inserts (Transwell[®] Permeable Supports,

Corning, NY, USA) coated well in 1:1 Bronchial Epithelial Cell Growth Medium (Lonza, Walkersville, MD, USA)/Dulbecco's Modified Eagle's Medium (Gibco, Grand Island, NY, USA) (BEGM/DMEM) medium supplemented with 25 µg/ml streptomycin, 25 U/ml penicillin, 5.5 mg/ml sodium pyruvate (all from Gibco, Grand Island, NY, USA) 0.4% (w/v) bovine pituitary extract, 0.5 ng/ml EGF, 5 µg/ml insulin, 10 µg/ml transferrin, 1 µM hydrocortisone, 6.5 ng/ml T3, 0.5 µg/ml epinephrine (all from Lonza, Walkersville, MD, USA), 15 ng/ml retinoic acid and 1.5 µg/ml bovine serum albumin (Sigma, St. Louis, MO, USA). When confluence was reached, cells were air-exposed and allowed to differentiate into mucociliary epithelium for 14 days. Subsequently, cells were serum deprived for 16 hours in BEGM + ITS (5 µg/ml insulin, 5 µg/ml transferrin, and 5 ng/ml selenium) and stimulated basolaterally for 24 hours with 10 ng/ml recombinant human IL-6 or 10 ng/ml recombinant human chemokine ligand (CXCL)8 to measure MUC5AC gene expression and to perform immunohistochemistry for goblet cells.

Immunohistochemistry

Inserts of ALI cell culture were taken up in formalin and paraffin-embedded according to the protocol 'Preparation of Costar[®] Transwell[®] Inserts for Histology'. Transverse cross-sections of 5 µm thick were used for morphometric analyses. Paraffin-embedded sections were stained for goblet cells with Periodic Schiff's (PAS, Sigma-Aldrich, Zwijndrecht, the Netherlands). PAS-positive cells were counted and expressed per mm basement membrane.

After sacrificing the mice, the upper right lung lobe was taken up in formalin and paraffin-embedded. Transverse cross-sections of 5 µm thick were used for morphometric analyses. Paraffin-embedded sections were stained for α-smooth muscle(sm)-actin using rabbit anti-α-sm-actin antibody (Abcam, Cambridge, UK) and visualized using a goat anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibody (Santa Cruz

Biotechnology, CA, USA) and diaminobenzidine (Sigma-Aldrich, Zwijndrecht, the Netherlands). The presence of sm- α -actin around the airway was quantified using ImageJ.[4] The surface of positively stained tissue was expressed as mm² per mm² basement membrane. MUC5AC positive cells were stained in paraffin-embedded sections using a MUC5AC antibody staining (Neomarkers, Fremont, CA, USA).

Small interfering (si)RNA transfection

Human fibroblasts were grown to 90% confluence and transfected with specific siRNA against the FZD8 transcript to knockdown FZD8. Cells were transfected in serum-free Ham's F12 medium without supplements using 100 pmol FZD8-targeted siRNA or non-targeting control siRNA and Lipofectamine[®] 2000 Transfection Reagent. After 6 hours, the medium was changed to medium supplemented with 10% (v/v) FBS for 18 hours and subsequently to medium supplemented with 0.5% (v/v) FBS for 24 hours. Cells were stimulated with either recombinant human IL-1 β or EGF in increasing concentrations (0.03-3 ng/ml IL-1 β and 0.1-10 ng/ml EGF) for 24 hours. Knockdown was considered successful when FZD8 gene expression was reduced by 60%.

Cytokine release from fibroblasts

24 hours after stimulation, culture medium was collected for the determination of cytokines using enzyme-linked immunosorbent assay (ELISA) and Milliplex[®]. We used Milliplex[®] (MILLIPLEX MAP Human Cytokine/Chemokine - Premixed 26 Plex, Millipore, Billerica, MA, USA) to screen for primary MRC-5 human lung fibroblast cytokine release. We specifically measured concentrations of secreted IL-6 and CXCL8 in primary MRC-5 human

lung fibroblasts by ELISA according to the manufacturer's instructions (#M1916 (IL-6), #M1918 (CXCL8); Sanquin, Amsterdam, the Netherlands).

mRNA isolation and real-time PCR analysis

Total mRNA from primary MRC-5 human lung fibroblasts and from mice lung tissue was extracted using the NucleoSpin[®] RNA II kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany). Total mRNA from primary human airway and parenchymal lung fibroblasts was extracted using the miRNeasy Micro Kit (Qiagen, Venlo, the Netherlands). Total mRNA from differentiated primary human airway epithelial cells was extracted using Tri Reagent[®] Solution (Applied Biosystems, Life Technologies Europe BV, Bleiswijk, the Netherlands). The eluted mRNA was quantified using spectrophotometry (Nanodrop, Thermo Scientific[™], Wilmington, DE, USA). Equal amounts of mRNA (1 µg) were then reverse transcribed using the Reverse Transcription System (Promega Benelux b.v., Leiden, the Netherlands) and the cDNA was stored at -20°C till further use. mRNA expression was determined using real-time PCR, which was performed with the Illumina Eco Personal QPCR System (Westburg, Leusden, the Netherlands). Primer sets are listed in table S1-2.

Materials and reagents

Recombinant human TGF- β_1 , was obtained from R&D Systems (Minneapolis, MN, USA), recombinant human IL-1 β , recombinant human TNF- α and recombinant human EGF were obtained from Sigma (St. Louis, MO, USA). Recombinant human IL-6 and recombinant human CXCL8 were obtained from ImmunoTools (Friesoythe, Germany). For CSE, Kentucky 3R4F research cigarettes (Tobacco Research Institute, University of Kentucky, Lexington, KY, USA) were used. FZD8 targeted siRNA was obtained from Santa Cruz

Biotechnology Inc. (Heidelberg, Germany), non-targeting control siRNA from Qiagen (Venlo, the Netherlands). Lipofectamine[®] 2000 Transfection Reagent was obtained from Invitrogen (Paisley, UK). All other chemicals were of analytical grade.

References

- 1 Kistemaker LE, Bos IS, Hylkema MN, *et al.* Muscarinic receptor subtype-specific effects on cigarette smoke-induced inflammation in mice. *Eur Respir J* 2013;42(6):1677-1688.
- 2 Brandsma CA, Timens W, Jonker MR, *et al.* Differential effects of fluticasone on extracellular matrix production by airway and parenchymal fibroblasts in severe COPD. *Am J Physiol Lung Cell Mol Physiol* 2013;305(8):L582-9.
- 3 Jacobs JP, Jones CM, Baille JP. Characteristics of a human diploid cell designated MRC-5. *Nature* 1970;227(5254):168-170.
- 4 Schindelin J, Arganda-Carreras I, Frise E, *et al.* Fiji: an open-source platform for biological-image analysis. *Nat Methods* 2012;9(7):676-682.

Supplementary Figure legends

Figure S1: characterization of FZD8^{-/-} mice.

WT and FZD8^{-/-} mice were exposed to PBS and paraffin-embedded sections were stained for α -sm-actin. (A) Quantification of the area positive for α -sm-actin around the airways in WT and FZD8^{-/-} mice. (B) Quantification of the area positive for α -sm-actin around the vessels in WT and FZD8^{-/-} mice. Data represent mean \pm s.e.m. of 10 mice per group. (C) Representative staining for α -sm-actin in WT and FZD8^{-/-} mice. WT and FZD8^{-/-} mice were exposed to cigarette smoke for 4 days. mRNA expression was determined in whole lung homogenates. (D) FZD8 gene expression in whole lung homogenates of WT mice. (E) WNT-5A gene expression in whole lung homogenates of WT and FZD8^{-/-} mice. (F) WNT-5B gene expression in whole lung homogenates of WT and FZD8^{-/-} mice. (G) MUC5AC gene expression in whole lung homogenates of WT and FZD8^{-/-} mice. Data represent mean \pm s.e.m. of 8 mice per group. * $p < 0.05$ compared to air exposed WT mice (two-way ANOVA with Student-Newman-Keuls multiple comparisons test).

Figure S2: WNT/FZD gene expression in primary human parenchymal lung fibroblasts.

Primary parenchymal lung fibroblasts of GOLD stage IV COPD patients with and without CMH were stimulated for 4 hours with 5% CSE, IL-1 β (1 ng/ml), TNF- α (10 ng/ml), TGF- β (2 ng/ml) or EGF (10 ng/ml) to study WNT-5A, WNT-5B, WNT-16, FZD2, FZD6 and FZD8 gene expression. (A) IL-1 β -induced gene expression. Basal delta Cq-values did not differ between groups: parenchymal fibroblasts GOLD stage IV COPD: 16.29 \pm 0.61, airway fibroblasts GOLD stage IV COPD: 16.28 \pm 0.28, parenchymal fibroblasts GOLD stage IV COPD with CMH: 15.96 \pm 0.38, airway fibroblasts GOLD stage IV COPD with CMH: 15.99 \pm 0.51. (B) EGF-induced gene expression. (C) CSE, IL-1 β , TNF- α , TGF- β and EGF effects on FZD8 gene expression. Data represent mean \pm s.e.m. of induction of FZD8 gene

expression of 8 patients per group. *** $p < 0.001$ compared to control (one-way ANOVA with Student-Newman-Keuls multiple comparisons test).

Figure S3: IL-1 β - and EGF-induced increase of FZD8 gene expression in parenchymal lung fibroblasts of GOLD stage IV COPD patients: correlation to SNP rs663700.

Correlation of the fold induction in FZD8 gene expression in parenchymal lung fibroblasts of GOLD stage IV COPD patients after stimulation with IL-1 β or EGF to the genotype of the patients, concerning SNP rs663700. (A) Correlation between the fold induction in FZD8 gene expression after stimulation of parenchymal lung fibroblasts with IL-1 β and patient genotype. (B) Correlation between the fold induction in FZD8 gene expression after stimulation of parenchymal lung fibroblasts with EGF and patient genotype. Data represent 5 patients with CC and 6 patients with CT; (one-tailed Mann-Whitney test).

Figure S4: EGF effects on IL-6 secretion by MRC-5 human lung fibroblasts. Primary MRC-5 human lung fibroblasts were transfected with specific FZD8 siRNA or non-targeting siRNA and stimulated for 24 hours with increasing concentrations of EGF (0.1-10 ng/ml). (A) EGF-induced IL-6 secretion by primary MRC-5 human lung fibroblasts. Data represent mean \pm s.e.m. of 4 independent experiments. (B) FZD8 knockdown for the experiments shown in figures 6A-B. Data represent mean \pm s.e.m. of 5 independent experiments. (C) FZD8 knockdown for the experiments shown in figure 6C and S4A. Data represent mean \pm s.e.m. of 4 independent experiments. * $p < 0.05$ compared to control siRNA # $p < 0.05$ ## $p < 0.01$ compared to stimulated control siRNA (two-way ANOVA with Student-Newman-Keuls multiple comparisons test).

Supplementary Tables

Table S1. Human primers used for the determination of specific genes of interest.

		Primer sequence			
FZD2	Forward	5'	CCC GACT TCAC GGT CTA CAT		3'
	Reverse	5'	CTG TTG GTG AGG CGA GTG TA		3'
FZD6	Forward	5'	TTG TTG GCA TCT CTG CTG TC		3'
	Reverse	5'	CCA TGG ATT TGG AAA TGA CC		3'
FZD8	Forward	5'	GAC ACT TGA TGG GCT GAG GT		3'
	Reverse	5'	CAA ATC TCG GGT TCT GGA AA		3'
WNT-5A	Forward	5'	GGG TGG GAA CCA AGA AAA AT		3'
	Reverse	5'	TGG AAC CTA CCC ATC CCA TA		3'
WNT-5B	Forward	5'	ACG CTG GAG ATC TCT GAG GA		3'
	Reverse	5'	CGA GGT TGA AGC TGA GTT CC		3'
WNT-16	Forward	5'	GCT CCT GTG CTG TGA AAA CA		3'
	Reverse	5'	ACC CTC TGA TGT ACG GTT GC		3'
MUC5AC	Forward	5'	ATT TTT TCC CCA CTC CTG ATG		3'
	Reverse	5'	AAG ACA ACC CAC TCC CAA CC		3'
18S rRNA	Forward	5'	CGC CGC TAG AGG TGA AAT TC		3'
	Reverse	5'	TTG GCA AAT GCT TTC GCT C		3'

Table S2. Mouse primers used for the determination of specific genes of interest.

		Primer sequence			
CXCL2	Forward	5'	AAG TTT GCC TTG ACC CTG AA		3'
	Reverse	5'	AGG CAC ATC AGG TAC GAT CC		3'
CXCL5	Forward	5'	GAA AGC TAA GCG GAA TGC AC		3'
	Reverse	5'	GGG ACA ATG GTT TCC CTT TT		3'
KC	Forward	5'	GCT GGG ATT CAC CTC AAG AA		3'
	Reverse	5'	AGG TGC CAT CAG AGC AGT CT		3'
FZD8	Forward	5'	TCC GTT CAG TCA TCA AGC AG		3'
	Reverse	5'	CGG TTG TGC TGC TCA TAG AA		3'
WNT-5A	Forward	5'	CAA ATA GGC AGC CGA GAG AC		3'
	Reverse	5'	CTC TAG CGT CCA CGA ACT CC		3'
WNT-5B	Forward	5'	GGT TCC ACT GGT GTT GCT TT		3'
	Reverse	5'	AGA CTT TTG TGA GGC GGA GA		3'
MUC5AC	Forward	5'	GAG ATG GAG GAT CTG GGT CA		3'
	Reverse	5'	GCA GAA GCA GGG AGT GGT AG		3'
18S rRNA	Forward	5'	AAA CGG CTA CCA CAT CCA AG		3'
	Reverse	5'	CCT CCA ATG GAT CCT CGT TA		3'

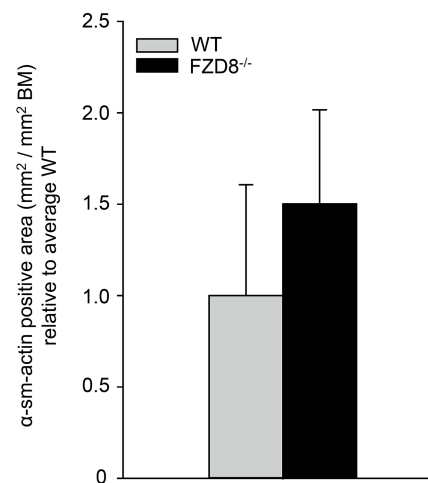
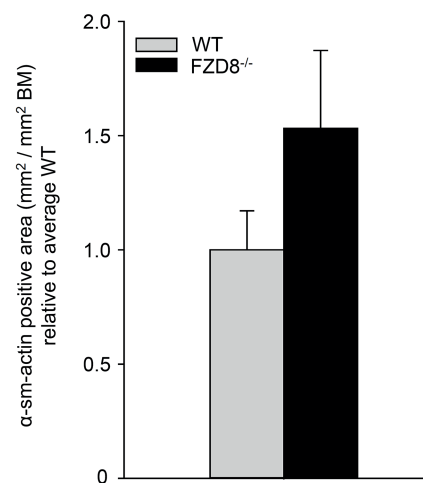
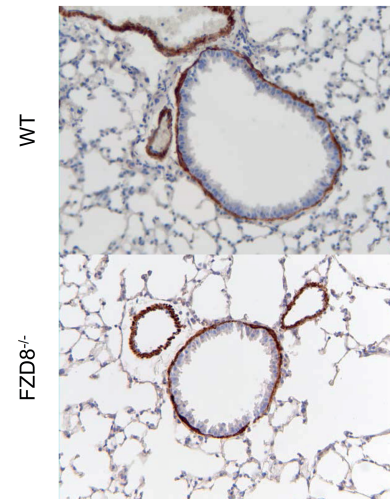
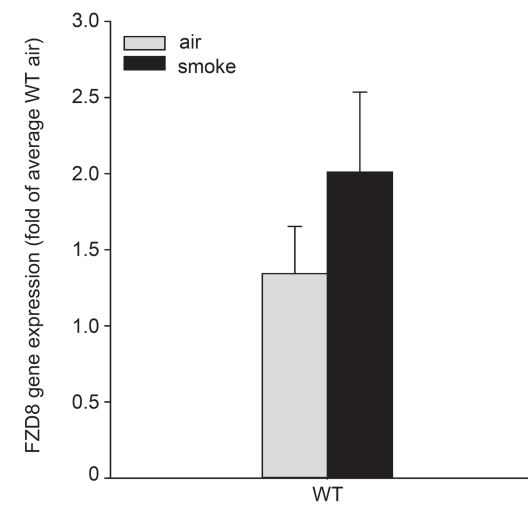
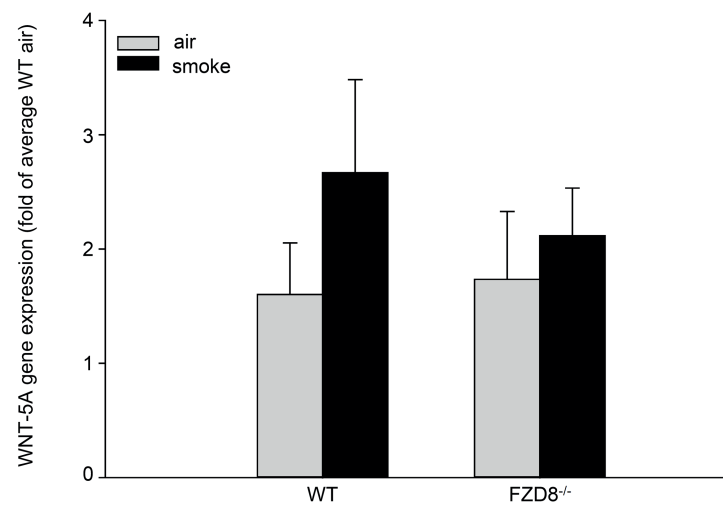
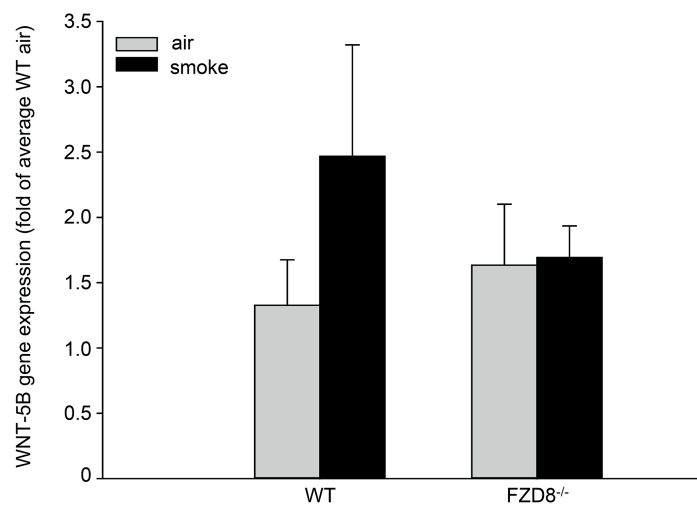
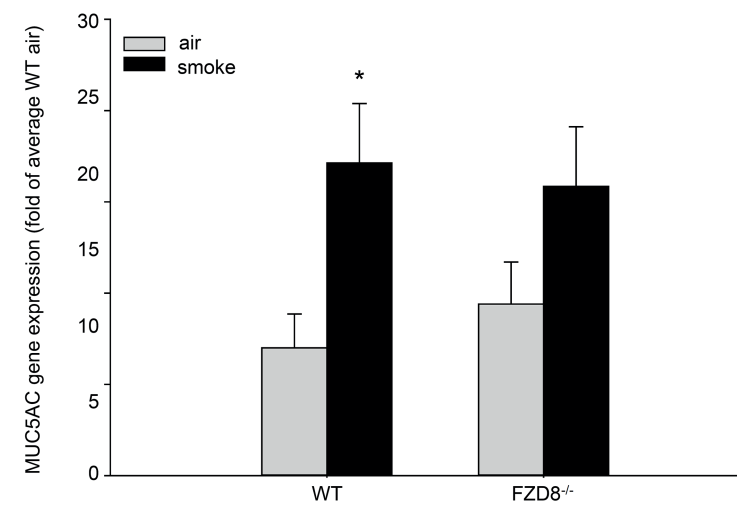
Table S3. OR for CMH for the 6 tested SNPs in the *FZD8* region.

SNP (CHR * 10)	base pair position	OR	SE	L95 [†]	U95 [‡]	p-value	left gene	right gene
rs640827	35941721	0.8499	0.07413	0.735	0.9828	$2.82 * 10^{-02}$	GJD4	FZD8
rs10827519	35952397	0.7996	0.08744	0.6737	0.9491	$1.05 * 10^{-02}$	GJD4	FZD8
rs663700	35956378	0.8095	0.08367	0.684	0.9495	$9.89 * 10^{-03}$	GJD4	FZD8
rs596642	35976759	0.8652	0.07958	0.7403	1.011	$6.89 * 10^{-02}$	FZD8	LOC439954
rs11010252	35987557	1.143	0.1164	0.9102	1.437	$2.49 * 10^{-01}$	FZD8	LOC439954
rs618443	35988716	1.128	0.1019	0.9238	1.377	$2.37 * 10^{-01}$	FZD8	LOC43995

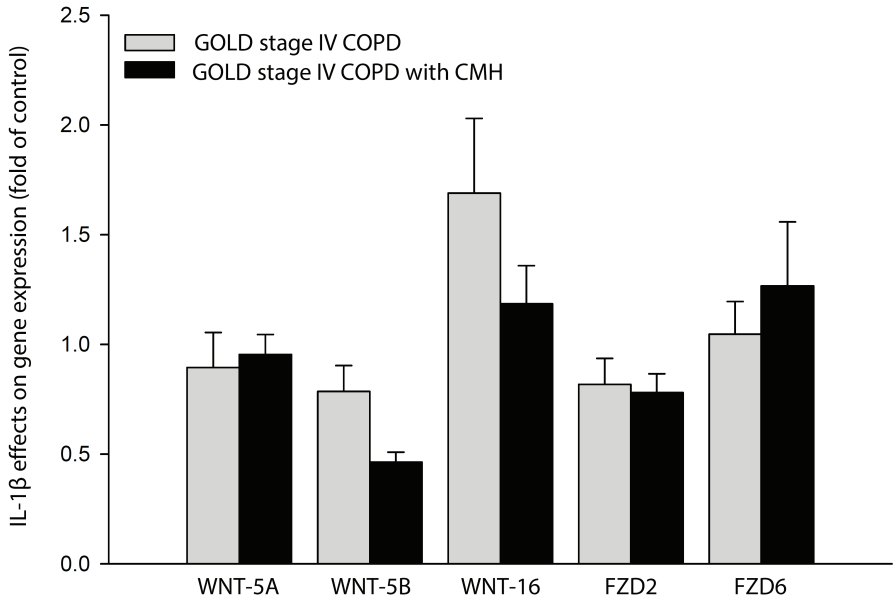
*CHR = chromosome

[†] L95 = lower limit

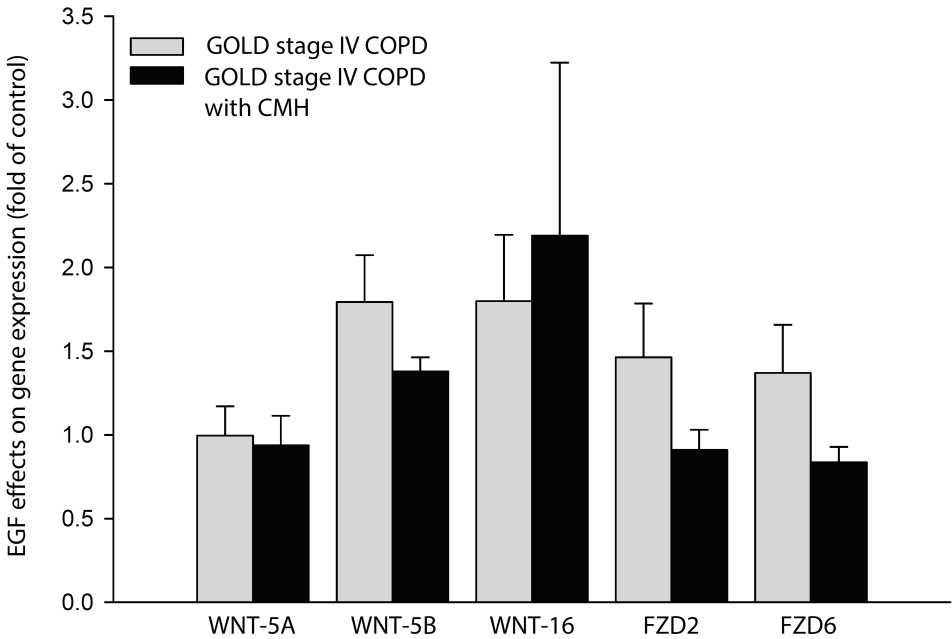
[‡] U95 = upper limit

A α -sm-actin around the airways**B** α -sm-actin around the vessels**C** PBS**D****E****F****G**

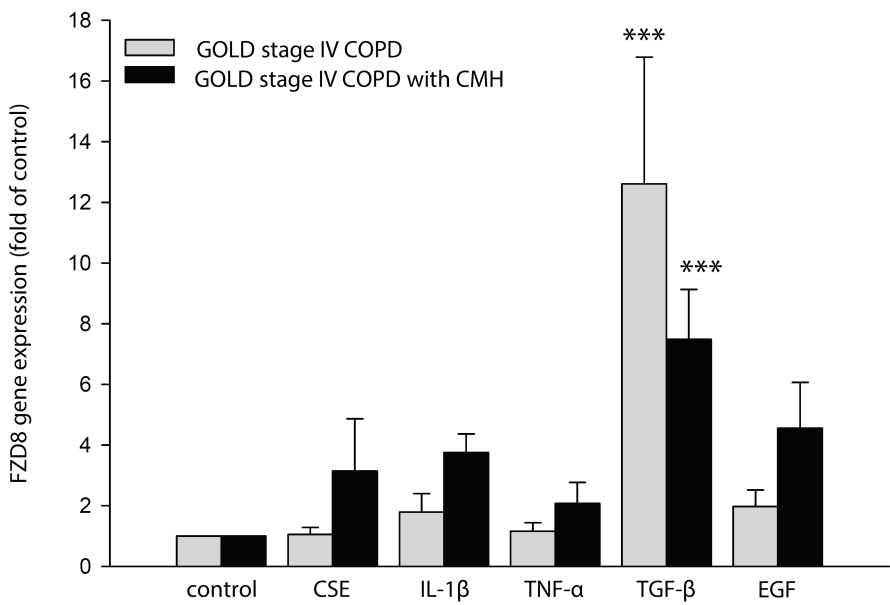
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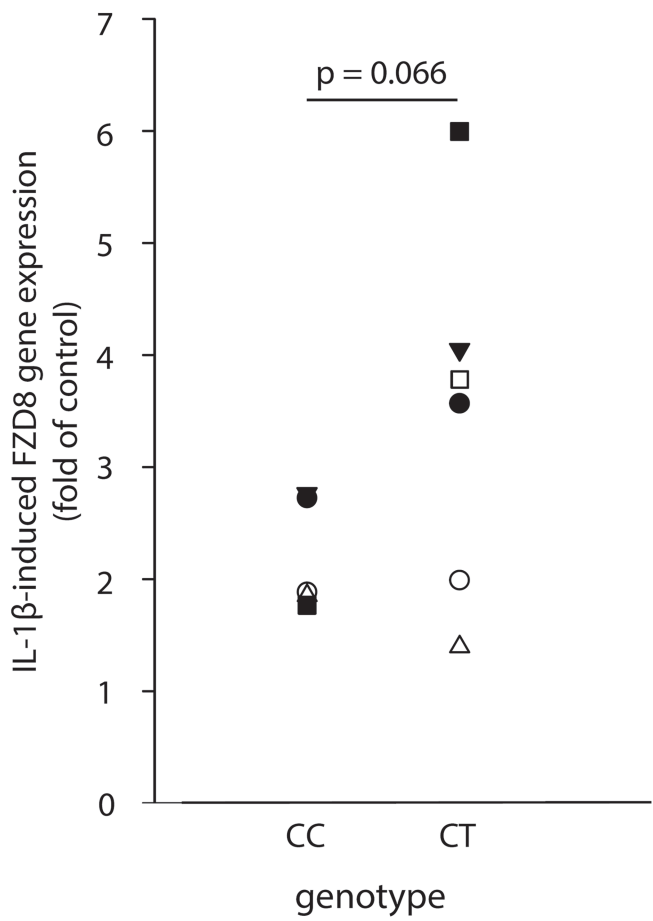
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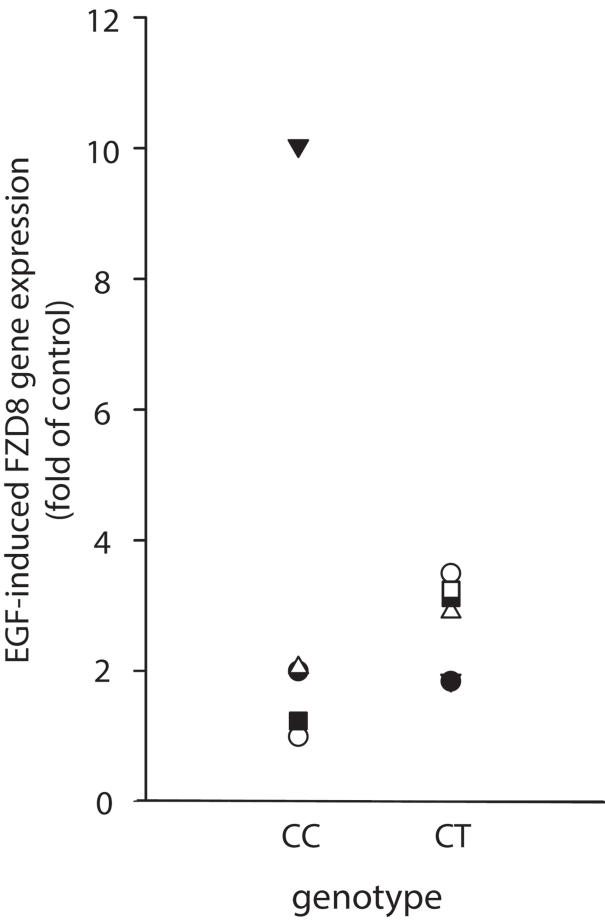
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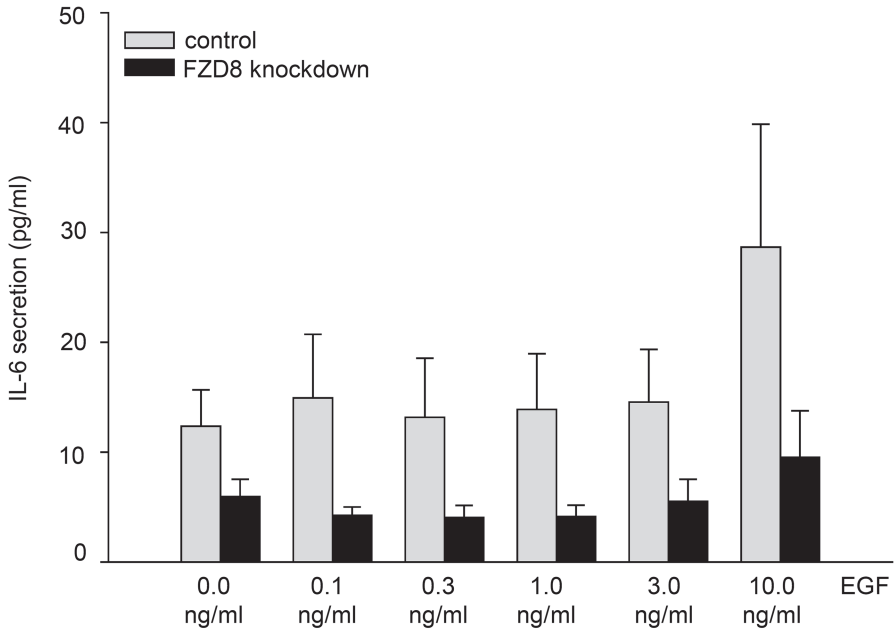
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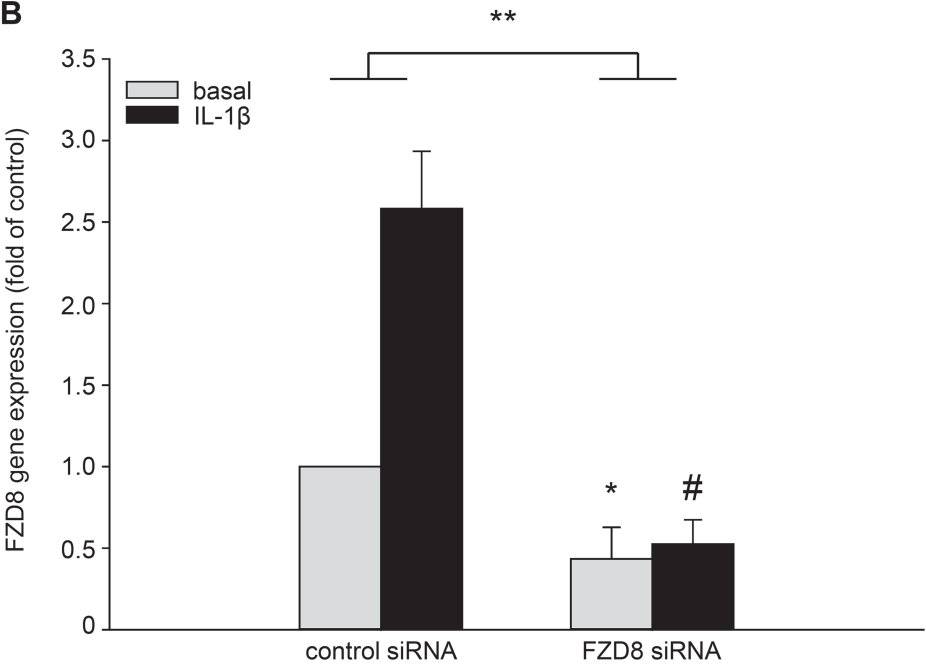
B



A



B



C

