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ORIGINAL ARTICLE

Modification of gene expression of the small airway epithelium in response to cigarette smoking

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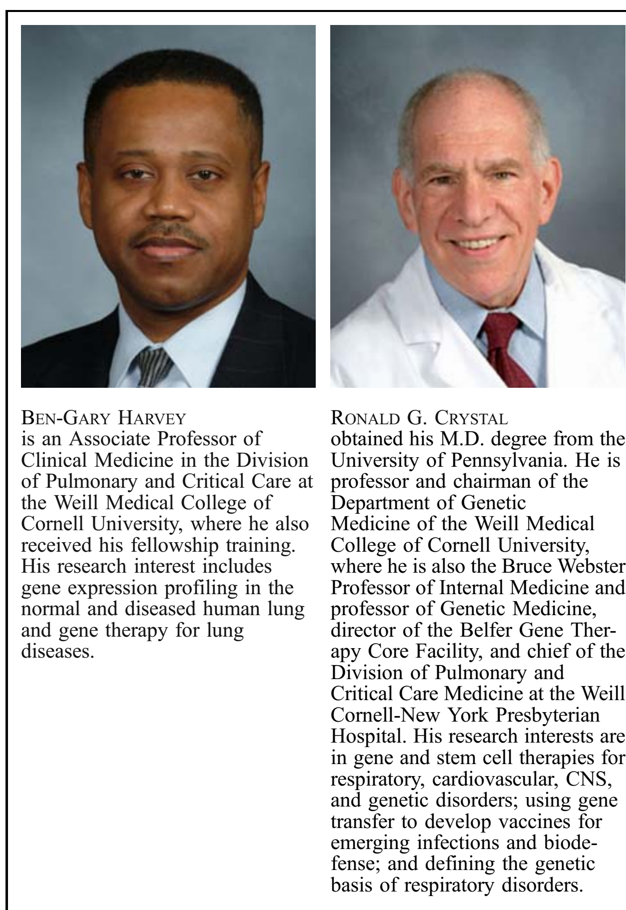
Abstract The earliest morphologic evidence of changes in the airways associated with chronic cigarette smoking is in the small airways. To help understand how smoking modifies small airway structure and function, we developed a strategy using fiberoptic bronchoscopy and brushing to sample the human small airway (10th–12th order) bronchial epithelium to assess gene expression (Affymetrix HG-U133A and HG-133 Plus 2.0 array) in phenotypically normal smokers (n=16, 25±7 pack-years) compared to matched nonsmokers (n=17). Compared to samples from large (second to third order) bronchi, the small airway samples had a higher proportion of ciliated cells, but less basal, undifferentiated, and secretory cells, and contained Clara cells. Even though the smokers were phenotypically normal, microarray analysis of gene expression of the small airway epithelium of the smokers compared to the non-smokers demonstrated up- and downregulation of genes in multiple categories relevant to the pathogenesis of chronic obstructive lung disease (COPD), including genes coding for cytokines/innate immunity, apoptosis, mucin, response to oxidants and xenobiotics, and general cellular processes. In the context that COPD starts in the small airways, these gene expression changes in the small airway epithelium in phenotypically normal smokers are candidates for the development of therapeutic strategies to prevent the onset of COPD.



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Introduction

Chronic obstructive pulmonary disease (COPD) associated with chronic cigarette smoking is characterized physiologically by limitation of expiratory airflow that, unlike asthma, is not reversed by pharmacologic intervention with bronchodilators [1–3]. The primary site of the airflow limitation is the small airways, defined as bronchi <2 mm in diameter [4–6]. While many affected individuals with COPD also have loss of elastic recoil and increased compliance secondary to destruction of central lobular alveoli, the initial site of the pathology in COPD is in the small airways [1–7]. Consistent with this concept, morphologic abnormalities are found in the small airways of cigarette smokers who are asymptomatic and have normal lung function [8–12]. Disease of the small airways is always a feature of COPD, independent of stage [13, 14], and the extent of small airway disease correlates with the extent of emphysema [13, 14].

Extensive data generated by many investigators supports the concept that the abnormalities of the small airways in COPD result from a combination of the toxic elements in cigarette smoke, a localized inflammatory host response, and biologic changes in the cells comprising the small airways, initially in the epithelium [1–4, 6, 7, 10, 14–17]. To help define the responses of the human airway epithelium to the stress of cigarette smoke, we along with others have employed microarray technology to assess the expression of the transcriptome of the large airways, using fiberoptic bronchoscopy and brushing to obtain pure populations of the epithelium of the second to third order bronchi [18–21]. While this approach has yielded valuable data regarding the responses of large airway epithelium to the stress of smoking, the large airways are not the initial site of airway injury in smokers [4–6]. This fact, and the knowledge that there are differences in the relative proportion of epithelial cell types in the small airways compared to the large airways (more ciliated cells, fewer goblet and basal cells) and the inclusion of a different cell type (Clara cells in the small, but not large, airways), leads to the question: What are the gene expression responses of the small airway epithelium to the stress of cigarette smoking?

To evaluate this question, we have developed methods utilizing fiberoptic bronchoscopy and brushing to sample the epithelium of small (10th to 12th order) airways of humans in high purity and in sufficient quantities to carry out microarray analysis. Assessment of the epithelial cell types recovered by small airway sampling demonstrated a cell composition consistent with small airways, including the presence of Clara cells, a cell type not present in the large airways. With this technology, we compared the expression of genes in the small airway epithelium of normal nonsmokers to that of phenotypically normal smokers with an average 25±7 pack-years of smoking, a smoking history that places these individuals at the cusp of risk for the development of smoking-induced lung disease [22–24]. Analysis of the microarray data demonstrated a large number of small airway epithelial genes that were significantly up- or downregulated in response to smoking. To place this in the context of the current concepts of pathogenesis of COPD, we have identified classes of genes previously implicated in the pathogenesis of COPD that our analysis demonstrated were significantly up- or downregulated in the small airway epithelium of smokers. While by no means complete, this subset of smoking-modulated genes provides a working list of potential targets for therapeutic intervention to prevent the development of COPD, and to assess the efficacy of therapies related to COPD.

Materials and methods

Study population

Normal nonsmokers and normal current cigarette smokers were recruited by posting ads in local newspapers. There were two groups: group A with n=11; 6 healthy smokers and 5 healthy nonsmokers, and group B with n=22; 10 healthy smokers and 12 healthy nonsmokers All 33 individuals were evaluated in the Weill Cornell NIH General Clinical Research Center under Institutional Review Board approved clinical protocols. All individuals were HIV-negative and determined to be phenotypically normal based on standard history, physical exam, complete blood count, coagulation studies, liver function tests, urine studies, chest X-ray, EKG, and pulmonary function tests (Supplemental Table 1). To verify smoking status, a complete smoking history was obtained and urine samples were evaluated for nicotine and cotinine, and venous blood was evaluated for carboxyhemoglobin. All chest X-rays and pulmonary function tests (spirometry, lung volumes, and diffusion capacity) were normal. The 16 normal smokers had a 25±7 pack-years smoking history, actively smoking 1.0±0.3 pack/day. There were no differences in age (p>0.2), sex (p>0.6), or race (p>0.7) among the smokers and nonsmokers.

Sampling the airway epithelium

Fiberoptic bronchoscopy was used to collect airway epithelial cells. After mild sedation was achieved with

Demerol and Versed, and routine anesthesia of the vocal cords and bronchial airways with topical lidocaine, the fiberoptic bronchoscope (Pentax, EB-1530T3) was positioned distal to the opening of the desired lobar bronchus. To obtain small airway epithelial cells, a 1.2-mm-diameter brush was advanced approximately 7 to 10 cm distally from the third order bronchial branching under fluoroscopic guidance (Supplemental Fig. 1). The distal end of the brush was wedged at about the 10th to12th generation branching of the right lower lobe, and small airway epithelial cells were obtained by gently gliding the brush back and forth on the epithelium five to ten times in ten different locations in the same general area. The cells were detached from the brush by flicking into 5 ml of ice-cold bronchial epithelial basal cell medium (BEBM, Clonetics, Walkersville, MD, USA). An aliquot of 0.5 ml was used for differential cell count and to develop slides for immunohistochemistry studies (typically 2×10^4 cells per slide). The remainder (4.5 ml) was processed immediately for RNA extraction. To compare cell types obtained from sampling the small airways to the cell types obtained from brushing the large airways, samples of the large airway epithelium were obtained in the same individuals using 2.0-mm disposable brushes to sample the epithelium of second and third order bronchi in the right lower lobe as previously described [18–20]. All individuals tolerated the fiberoptic bronchoscopy well; radiologic and fluoroscopic evaluations after fiberoptic bronchoscopy with sampling of large and small airways showed no evidence of pneumothorax.

Morphology of airway epithelial cells

The total number of cells recovered by bronchial brushing was determined by counting on a hemocytometer. To quantify the percentage of epithelial and inflammatory cells and the proportions of ciliated, basal, secretory, and undifferentiated epithelial cells, aliquots of 2×10^4 cells were prepared by centrifugation (Cytospin 11, Shandon Instruments, Pittsburgh, PA, USA) and stained with Diff-Quik (Dade Behring, Newark, NJ, USA). Aliquots were also assessed by immunohistochemistry with antibodies directed against surfactant protein A (SPA, Lab Vision, Fremont, CA, USA) and Clara cell protein 10 (CC10, BioVendor, Candler, NC, USA). Cytospin preparations were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) at pH 7.4 for 20 min at 23°C. Incubation with anti-SPA and anti-CC10 was carried out overnight at 4°C; subsequently, cytospins were washed with PBS, followed by incubation with a secondary peroxidase-coupled antibody for 30 min at 23°C. The final step included incubation with a 3,3′-diaminobenzidine chromogenic substrate detection system (Dako, Carpinteria, CA, USA), which rendered positive cells into brown. All cytospins were counterstained with hematoxylin. Species and subtype-matched antibodies were used as negative controls.

To assess the cell populations by transmission electron microscopy, the brushed airway epithelial cells were suspended in BEBM medium, pelleted, fixed, stained, cut, and viewed on a JSM 100 CX-II electron microscope (JEOL, Peabody, MA, USA) operated at 80 kV as previously described [25]. Images were recorded on Kodak 4489 Electron Image Film (Electron Microscopy Sciences) and then digitized on an Epson Expression 3200 Pro Scanner at 800 dpi (Epson America, Long Beach, CA, USA).

RNA and microarray processing

The HG-U133A and the HG-U133 Plus 2.0 arrays (Affymetrix, Santa Clara, CA, USA), including probes representing ~22,000 and ~39,000 full-length human genes, respectively, were used to evaluate gene expression. Total RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA, USA), yielding 2 to 4 μg from 10^6 cells. Quality control included an A_{260}/A_{280} ratio of 1.7 to 2.3. First and second strand cDNA were synthesized from 6 μg (HG-U133A chip) or 3 μg (HG-U133 2.0 Plus) of RNA, in vitro transcribed, and fragmented using the recommended Affymetrix reagents and kits. The quality of the RNA labeling was verified by hybridization to a test chip, and only test chips with a 3′ to 5′ ratio of <3 were deemed satisfactory. Samples passing the quality control criteria were then hybridized to the HG-U133A or the HG-U133 Plus 2.0 array, processed by the fluidics station to receive the appropriate reagents/washes, and then transferred to the scanner for duplicate scanning. The captured image data for HG-U133A arrays was processed using the Affymetrix Microarray Suite version 5 (MAS5) algorithm. Image data from the HG-U133 Plus 2.0 arrays was processed using MAS5 and also by the robust multi-array average (RMA) algorithm [26], using GeneSpring version 7.2 software (Agilent Technologies). MAS5 takes into account the perfect match and the mismatch values, while the RMA method utilizes only the perfect match values. MAS5-analyzed data were normalized using GeneSpring as follows: (1) per array, by dividing the raw data by the 50th percentile of all measurements; and (2) per gene, by dividing the raw data by the median of the expression level for the gene in all samples. RMA preprocessed data was normalized to the median measurement for the gene across all the arrays in the data set because the per array normalization step is included in this method.

Microarray data analysis

To determine the normal gene expression profile (the normal transcriptome) of the small airway epithelium in

healthy nonsmokers, RNA from the small airway epithelium of healthy nonsmokers was assessed for gene expression with the HG-U133 Plus 2.0 microarray. Expressed was defined as having an Affymetrix detection call of "Present" in >50% of the samples. The probe sets were grouped into functional categories, using the database from the Affymetrix NetAffx Analysis Center (http://www.affymetrix.com/analysis/index.affx) by the Gene Ontology (GO) Biological Processes classification.

Initial assessment of differentially expressed genes in small airway epithelium of smokers compared to nonsmokers was carried out in 11 healthy individuals (5 nonsmokers and 6 smokers, for convenience referred to as part A of the study; see below). To identify the categories of small airway epithelial genes up- and downregulated by smoking in these individuals, and to provide an overview of the relative fold changes of these genes by gene category relevant to the pathogenesis of COPD, microarray analysis was carried out using the Affymetrix HG-U133A microarray. Genes were considered significant if $p<0.05$ and the fold change (up- or down-regulation) was >2-fold between the two groups. The fold change was calculated by dividing the geometric mean expression value in all smoker samples by the geometric mean value in nonsmoker samples. The genes were categorized according to the GO annotations, in categories relevant to COPD pathogenesis, and additional general categories, such as signal transduction and transcription. Based on our assessment of patterns of gene expression in small airways of healthy smokers vs healthy nonsmokers, and from the data in the literature regarding molecular pathways in airway epithelium previously implicated in the pathogenesis of COPD, we generated a list of categories of genes expressed in the small airway epithelium relevant to the pathogenesis of COPD, including cytokines/innate immunity, apoptosis, profibrotic, mucin, response to oxidants, antiproteases, and general cellular processes. From the preliminary data comparing genes up- and downregulated in the small airway epithelium of smokers to nonsmokers in the first 11 individuals studied, a total of 152 genes with known function were identified and placed into various categories. From this catalog of genes, we chose examples representing the genes with (1) the highest fold differences in each group; and (2) literature suggesting that the pathway that includes the gene may be involved in the pathogenesis of COPD.

We independently assessed the small airway epithelial gene expression in an entirely new group of healthy individuals ($n=22$, 10 healthy smokers and 12 healthy nonsmokers, referred to as group B; see below) who shared similar phenotypic characteristics as the initial 11 individuals studied in group A. Assessment of the small airway epithelium gene expression of these new 10 healthy smokers vs 12 healthy nonsmokers (group B) was carried out with the newest generation Affymetrix chip, the HG-U133 Plus 2.0. Genes were considered significant if $p<0.05$ and the fold change (up- or downregulation) was >1.5-fold between the two groups in both the MAS5 and the RMA-generated datasets. To limit the number of false positives, we applied the Benjamini and Hochberg false discovery rate multiple test correction to both the MAS5 and the RMA-generated datasets [27]. Fold change was calculated by dividing the geometric mean expression value in all smoker samples by the geometric mean expression value in nonsmoker samples. Similar to the assessment of gene expression in group A, the genes differentially expressed in group B were classified according to categories relevant to COPD pathogenesis as described above. All data was deposited at the Gene Expression Omnibus site (http://www.ncbi.nlm.nih.gov/geo/), a high-throughput gene expression/molecular abundance data repository curated by the National Center for Bioinformatics site [28]. The accession number for the HG-133A data set is GSE3320, and for the HG-U133 Plus 2.0 dataset is GSE4498.

Cluster analysis

Unsupervised classification of samples was carried out by hierarchical cluster analysis, by gene and by individual sample, using the standard correlation, with the GeneSpring software (Agilent Technologies), using the expression levels of the expressed genes (called present in at least one array by the MAS5 algorithm) and by genes (up- and downregulated) modulated by smoking obtained by assessment of gene expression in group B. The goal of the cluster using the significant genes was to obtain a graphical representation of general variability within this population.

TaqMan RT-PCR

*Taq*Man real-time reverse transcriptase (RT) PCR was carried out for eight nonsmokers and eight smokers from group B, using the same RNA samples that had been used for the microarray analysis. First strand cDNA was synthesized from 2 μg of RNA in a 100-μl reaction volume, using the *Taq*Man Reverse Transcriptase Reaction Kit (Applied Biosystems), with random hexamers as primers. The cDNA was diluted 1:100 or 1:50, and each dilution was run in triplicate wells. Five microliters were used for each *Taq*Man PCR reaction in 25 μl of final reaction volume, using premade kits from Applied Biosystems. Relative expression levels were calculated using the ΔΔCt method (Applied Biosystems), using ribosomal RNA as the internal control (Human Ribosomal RNA Kit, Applied Biosystems), and the average value for nonsmokers, as the calibrator. The rRNA probe was labeled with VIC and the probes for the genes of interest with FAM. The PCR reactions were run in an Applied

Biosystems Sequence Detection System 7500. The relative quantity (ΔΔCt) was determined using the algorithm provided by Applied Biosystems. For comparison purposes, the data for each individual was normalized to the median across all nonsmokers and smoker samples, as was done with microarray data (see above).

Nonmicroarray-related statistical analyses

Comparison of the percentage cell types and demographic parameters in the nonsmokers and smokers was performed by two-tailed Student's *t* test. A two-way ANOVA with smoking status (smokers vs nonsmokers) and method (microarray vs *Taq*Man) as independent factors was carried out using StatView version 5.0 (SAS Institute) to demonstrate that smoking was significant but the methodology was not, therefore confirming the agreement between the two methodologies.

Results

Study population

The study individuals were divided into two groups (A and B). Airway epithelial samples from individuals in group A (n=11; 6 healthy smokers and 5 healthy nonsmokers) were used to establish the morphologic differences between large and small airway epithelium, to determine the presence of Clara cells in samples obtained from small airways, to demonstrate that airway epithelial cells from small airways but not the large airways expressed surfactant apoproteins-related genes, and to carry out preliminary assessment of the differences in gene expression among smokers compared to nonsmokers with the Affymetrix HG-U133A microarray chip. Gene expression in airway samples from individuals in group B (n=22; 10 healthy smokers and 12 nonsmokers) was assessed with the newest microarray chip, the Affymetrix HG-U133 Plus 2.0. Small airway epithelium RNA from individuals in group B was also used for *Taq*Man RT-PCR confirmation of a selected group of differentially expressed genes among smokers vs nonsmokers.

Sampling of the small airway epithelium

From a total of 5 to 10×10^6 epithelial cells, more than 95% of cells recovered from small and large airways from smokers and nonsmokers were epithelial (Table 1). The percentage of inflammatory cells in the large and small airways of smokers did not differ from that of the nonsmokers ($p>0.4$, both comparisons). Independent of smoking history, albeit low (≤5%, both large and small airways), the percentage of inflammatory cells in small airways evaluated in group A was higher than in large airways ($p<0.005$). Less than 1% of cells recovered from large and small airways were squamous cells in both smokers and nonsmokers ($p>0.7$). Assessment with Diff-Quik stain of the airway epithelial cells identified the four main epithelial cell types present in the human airways [ciliated, basal, undifferentiated, and secretory [29–33] in both the large and small airways (Table 1 and Fig. 1a)]. Evaluation by immunohistochemistry with SPA antibody [34] demonstrated the presence of Clara cells only in airway epithelial cells obtained from the small airways, not from large airways (Fig. 1b,c).

Assessment of the large and small airway epithelial cell populations by transmission electron microscopy demonstrated cells typical of Clara cells only in the small airway epithelial cell populations (Fig. 1d). These cells had 1 to 2 μm dense granules in the apical cytoplasm and contained microvilli, but not cilia, typical of ultrastructural descriptions of Clara cells in human small airways [35]. Because Clara cells are found only in airways <3 mm in diameter [35–37], the observation of Clara cells in the small airway samples confirms that the samples were, in fact, from the small airways.

Consistent with prior morphologic studies describing the composition of airway epithelial cells in the human lung, the small airways had a higher proportion of ciliated cells than large airways (nonsmokers $p<0.001$, smokers $p<0.001$). In contrast, the large airways had higher proportion of basal (nonsmokers $p<0.001$, smokers $p<0.001$), undifferentiated (nonsmokers $p<0.001$, smokers $p<0.001$), and secretory (nonsmokers $p<0.04$, smokers $p<0.04$) cells than small airways (Table 1).

As further evidence that the small airway epithelium was being sampled, independent of smoking status, gene expression of airway epithelium from small airways (evaluated in the individuals in group A) revealed the expression of surfactant apoprotein A2, surfactant apoprotein B, and surfactant apoprotein C genes (Fig. 2). Consistent with prior studies of surfactant gene expression in the small airway epithelium [34–36], the surfactant apoprotein genes were not expressed in the large airway epithelial samples.

Genes expressed in the small airway epithelium of normal nonsmokers

To determine the normal small airway epithelium transcriptome, RNA from small airway epithelial cells from the 12 healthy nonsmokers from group B was assessed with the HG-U133 Plus 2.0 microarray. In this analysis, 27,244 of the total 54,675 probe sets were "Present" or expressed according to the MAS5 algorithm in >50% of the samples. These genes were functionally grouped into 14 different

categories. Forty percent, representing 10,935 probe set IDs, were classified as unknown function and were not used to generate the data on the distribution of types of genes expressed. The remaining genes were classified in the general biological processes categories. The largest categories were transcription, transport, metabolism, signal transduction, followed by cell cycle, apoptosis, and cell adhesion; other categories included differentiation, immune response, proteolysis, electron transport, cell growth, and cell signaling related genes (Fig. 3).

Differentially expressed genes in the small airway epithelium of phenotypically normal smokers compared to normal nonsmokers

Relevant to the pathogenesis of COPD, assessment of gene expression in the small airway epithelium of smokers compared to nonsmokers showed a significant up- and downregulation of several genes in various functional categories (Table 2, Figs. 4 and 5). Initial assessment of gene expression in a small number of individuals (group A, n=11, 6 smokers vs 5 nonsmokers) demonstrated 152 genes differentially expressed, 103 genes upregulated, and 49 genes downregulated in several functional categories in the small airway epithelium of healthy smokers compared to nonsmokers (Supplemental Table 2). Of these 152 genes, 133 genes were of known function and were grouped into biologically relevant categories. Based on the assessment of the small airway gene expression and a review of the molecular pathways shown in the literature to be related to the pathogenesis of COPD, we chose the most relevant six of these categories, to generate a representative "small airway epithelial smoking-induced phenotype." These categories included cytokine/innate immunity, apoptosis, profibrotic, response to oxidants and xenobiotics, antiproteases, and general cellular processes (Supplemental Table 3).

After the initial assessment of differential gene expression in the first group of healthy individuals studied, we sought to verify these changes by studying a larger group of healthy individuals (group B, n=22, 10 smokers vs 12 nonsmokers). It is interesting to note that consistent with the initial assessment using the HG-U133A chip (group A), genes in similar categories were differentially expressed in small airway epithelium of healthy smokers compared to nonsmokers assessed with the HG-U133 Plus 2.0 chip (group B; Table 2). The group B assessment, which was subject to a more rigorous analysis (see "Materials and methods"), demonstrated a more restricted number of genes up- or downregulated [118 genes, 48 upregulated and 70 downregulated (Supplemental Table 4)] compared to the initial gene list of 152 observed in the initial analysis of group A (Supplemental Table 2). The 118 genes differentially

Table 1 Comparison of the cell types removed by brushing the airway epithelium

	Group A[a]				Group B[b]	
	Small airways		Large airways		Small airways	
	Nonsmokers	Smokers	Nonsmokers	Smokers	Nonsmokers	Smokers
Total number of cells recovered (×10^6)	10±6	7±4	7±2	9±3	5±2	7±2
Percentage of total cells						
Epithelial	96±4	96±4	98±5	98±1	99±1	97±1
Inflammatory	4±3	4±4	1±1	1±1	1±1	1±1
Squamous	0	0	1±1	1±1	0	0
Percentage of epithelial cells						
Ciliated	80±5	75±6	50±2	43±3	78±7	75±10
Secretory[c]	4±1	4±3	9±4	10±2	7±3	7±3
Basal	5±3	8±2	20±3	27±4	7±2	9±4
Undifferentiated	8±2	9±3	21±4	20±2	8±4	9±4
Clara[d]	2±1	4±1	0	0	ND	ND

ND Not determined

[a]Large airway epithelial cells were collected from second to third generation bronchi and small airway epithelial cells were collected from airways at the 10th to 12th generation under fluoroscopic guidance by advancing the brush 7 to 10 cm beyond the third generation bronchi. Cytospin preparations were stained with Diff-Quick to determine the percentage of epithelial vs inflammatory cells, and identification of ciliated, secretory, basal, and undifferentiated epithelial cells. The data is presented as mean±SD for each cell type in small and large airways for nonsmokers (n=5) and smokers (n=6).

[b]Small airway epithelial cells were collected from airways at the 10th to 12th generation under fluoroscopic guidance as described in table note a. The data is presented as mean±SD for each cell type in small airways for nonsmokers (n=12) and smokers (n=10).

[c]Secretory cells (percentage of total epithelial cells) include Clara cells and non-Clara cells.

[d]Immunostaining of large and small airway epithelial cells with SPA antibody with hematoxylin counterstaining was used to quantify Clara cells within the secretory subset (data as percentage of total epithelial cells); percentage of Clara cells were not determined for group B.

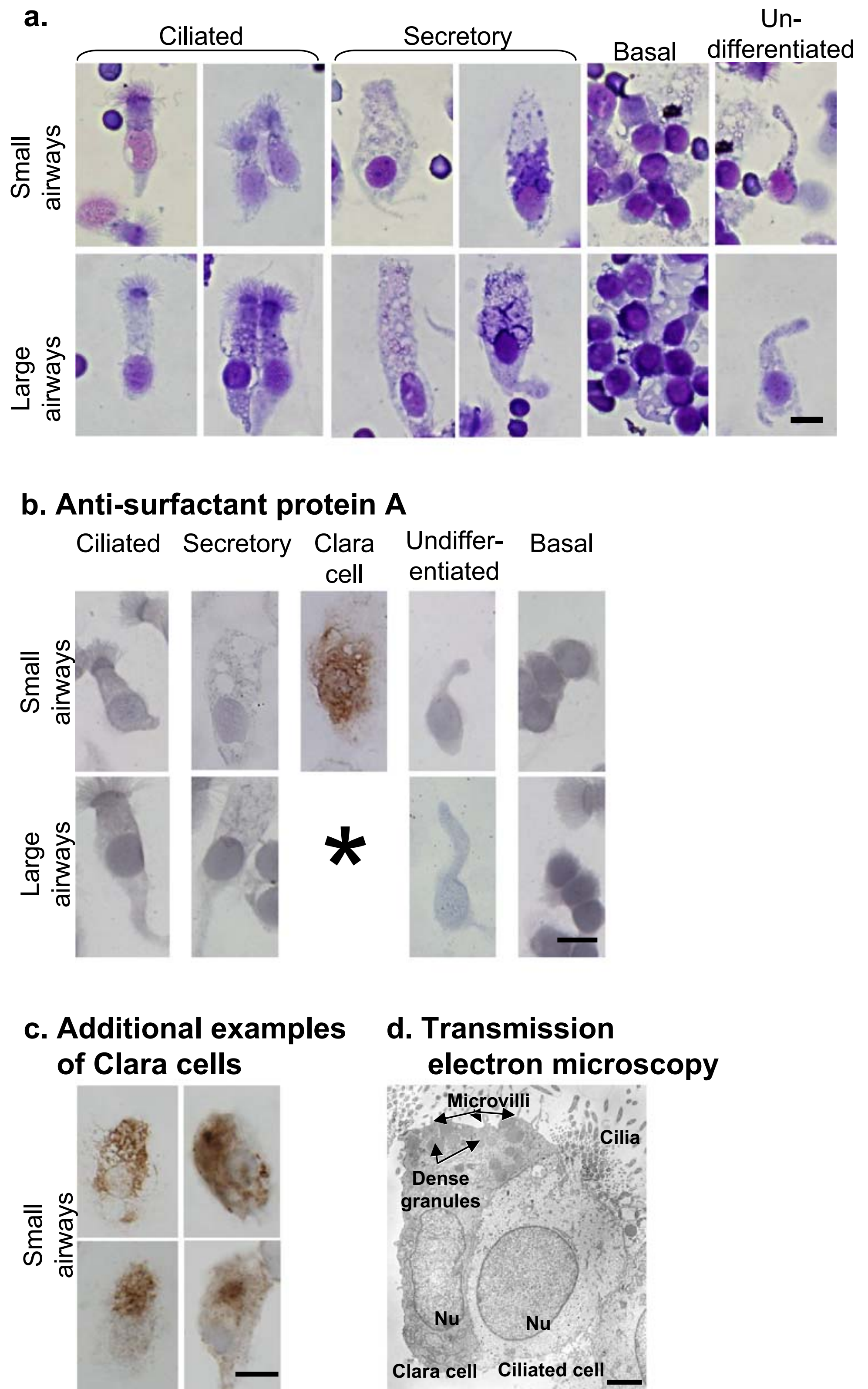

Fig. 1 Morphology of airway epithelial cells obtained by brushing small and large airways. **a** Epithelial cells from small and large airways stained with Diff-Quick. Shown are examples of ciliated cells, secretory cells, basal cells, and undifferentiated cells; bar=10 μm. **b** Clara cells detected in small airways by anti-SPA immunohistochemistry counterstained with hematoxylin. The SPA is the *brown* signal in the cytoplasm; nuclei are shown in *blue*. All cell types are negative except Clara cells; the *asterisk* indicates that no Clara cells were observed in the large airway samples; bar=10 μm. **c** Additional examples of Clara cells in small airway epithelial samples; bar=μm 10. **d** Transmission electron microscopy of cell pellets from small airway brushings. The Clara cell (*left*) contains apically positioned dense granules and microvilli. The adjacent cell (*right*) is a ciliated airway epithelial cell. The nuclei (*Nu*) of both cells are located toward the basolateral membrane; bar=5 μm. Transmission electron microscopy of the large airways showed no Clara cells (data not shown)

expressed in smokers vs nonsmokers in group B included genes in the categories cytokine/innate immunity, apoptosis, response to oxidants and xenobiotics, proteases/antiproteases, and general cellular processes (Supplemental Table 5). A comparison of the results of the HG-U133 2.0 Plus analysis with those from the HG-U133A microarray showed

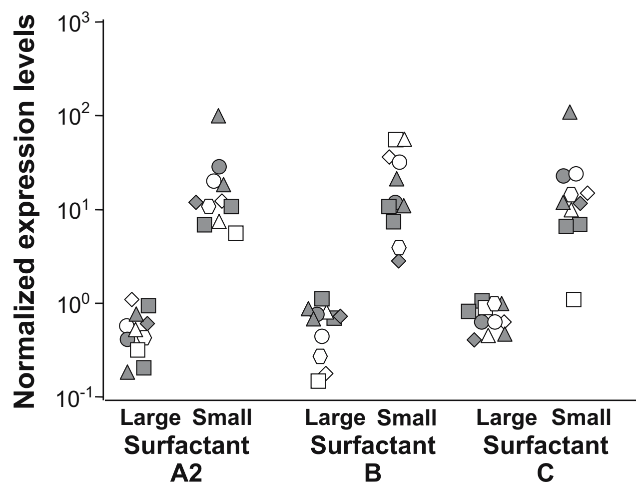

Fig. 2 Quantitative assessment of expression of surfactant apoprotein genes in small airway vs large airway epithelial cells of healthy nonsmokers and smokers. Five healthy individual nonsmokers and six healthy smokers (group A) were evaluated. Shown is the HG-133A-generated normalized expression level with a logarithmic scale for surfactant apoprotein A2, surfactant apoprotein B, and surfactant apoprotein C. Each *symbol* represents one individual. *Open symbols* represent nonsmokers; *closed symbols* represent smokers

that although there is a large degree of agreement between the two data sets, there are discrepancies (Supplemental Table 6). These differences can likely be explained by the fact that these are different microarrays with different number of probe sets and different hybridization conditions because of array density (group A consisted of n=6 smokers

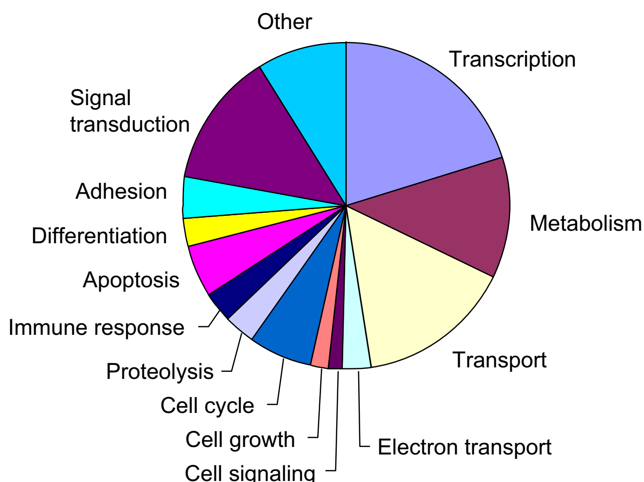

Fig. 3 Functional categories of genes expressed in the small airway epithelium of normal nonsmokers. The pie chart shows the different functional categories of the small airway epithelium transcriptome in 12 healthy nonsmokers; small airway epithelium gene expression was assessed with the Affymetrix HG-U133 Plus 2.0 microarray chip. The distribution data represents the gene expression in nonsmokers with probe sets representing genes expressed (Affymetrix detection call of present) in >50% of the small airway samples. Probe sets were categorized using the Affymetrix NetAffx Analysis Center by GO Biological Process. A total of 27,244 probe sets were grouped into functional categories; of these, 10,935 probe sets were classified as unknown function and were not used for the final analysis

and n=5 nonsmokers, while group B consisted of n=10 smokers and n=12 nonsmokers). Not only is the "n" of the groups different, but in group B we applied the Benjamin–Hochberg correction to reduce the number of false positives, which was not applied to group A because of the low n.

Assessment of gene expression levels for the 118 genes modulated by smoking in the small airway epithelium of the study individuals in group B, using an unsupervised assessment by hierarchical cluster analysis, showed, as expected, clustering of the samples according to smoking status. This suggests that taken as a group, similar changes are occurring among all healthy smokers. Likewise, as a group, healthy nonsmokers displayed a similar gene expression profile (Supplemental Fig. 2b), while cluster analysis using the complete list of expressed genes (30,963 probe sets, present in at least one array) did not segregate smokers from nonsmokers (Supplemental Fig. 2a), as noted previously for large airways [18].

After assessment of gene expression in small airway epithelium of healthy smokers vs nonsmokers in groups A and B, we generated a list showing examples of genes differentially expressed in a similar fashion in smokers vs nonsmokers in both groups A and B (Table 2). Assessment of the list of genes in the different pathways does not represent all the genes relevant for COPD; it nevertheless represents early gene expression responses in the small airway epithelium of individuals exposed to the insult of cigarette smoke, the main risk factor for the development of COPD.

In the context that the assessment of differential gene expression of healthy individuals in group B included a higher number of individuals (n=22, 10 smokers vs 12 nonsmokers), with assessment of gene expression with the Affymetrix HG-U133 Plus 2.0, and a more rigorous analysis of the gene expression data, which included independent assessment by RMA and MAS5 with Benjamini–Hochberg correction, the following description of differentially expressed genes in the different categories relevant to the pathogenesis of COPD will focus on the results obtained from small airway epithelial cells from these individuals (group B, Table 2, Figs. 4 and 5, and Supplemental Tables 4 and 5).

Expression of genes potentially relevant to the pathogenesis of COPD

Independent assessment of gene expression by RMA and MAS5 demonstrated that the small airway epithelium of smokers vs nonsmokers downregulated several immune-related genes. These genes included the interleukin-4 (IL4) receptor gene (p<0.002), the chemokine (C-X3-C motif) ligand 1 (p<0.02), also known as fractalkine, and the spondin 2 (p<0.04); these genes are involved in many inflammatory functions in human airways [38–41] (Table 2 and Fig. 5a).

Table 2 Examples of genes differentially expressed in the small airway epithelium in nonsmokers and smokers in functional categories that are relevant for the pathogenesis of COPD

Category	Gene	Gene symbol	References in literature that suggested pathways relevant for COPD pathogenesis[a]	Group A		Group B			
				S vs NS fold change[b]	p value S vs NS[c]	S vs NS fold change RMA[b]	p value S vs NS RMA[d]	S vs NS fold change MAS5[b]	p value S vs NS MAS5[e]
Cytokine/ innate immunity	Chemokine (C-X3-C motif) ligand 1	CX3CL1	[7, 16, 38–41]	−2.97	<0.040	−2.89	<0.016	−2.96	<0.004
Apoptosis	Pirin	PIR	[42–48, 56–61]	2.78	<0.001	2.64	<0.007	2.22	<0.028
	Growth arrest and DNA damage-inducible, beta	GADD45B	[42–48, 56–61]	−2.25	<0.043	−1.83	<0.024	−2.26	<0.014
Response to oxidants and xenobiotics	Cytochrome P450, family 1, subfamily B, polypeptide 1	CYP1B1	[7, 16, 21, 49, 62]	17.69	<0.001	20.73	<0.039	54.7	<0.004
	Aldo-keto reductase family 1, member B10	AKR1B10	[7, 16, 21, 49, 62]	11.73	<0.001	24.84	<0.003	20.76	<0.002
	Aldehyde dehydrogenase 3 family, memberA1	ALDH3A1	[7, 16, 21, 49, 62]	6.63	<0.001	75.6	<0.001	4.96	<0.001
	Alcohol dehydrogenase 7	ADH7	[7, 16, 21, 49, 62]	6.24	<0.001	7.21	<0.001	6.1	<0.001
	Glutathione peroxidase 2	GPX2	[7, 16, 21, 49, 62]	5.16	<0.001	2.69	<0.009	3.73	<0.001
	NAD(P)H dehydrogenase, quinone 1	NQO1	[7, 16, 21, 49, 62]	4.41	<0.001	3.37	<0.001	3.38	<0.001
	Aldo-keto reductase family 1, member C3	AKR1C3	[7, 16, 21, 49, 62]	3.09	<0.001	2.6	<0.01	2.32	<0.014
General cellular processes	Ubiquitin carboxyl-terminal esterase L1	UCHL1	[63, 64]	11.75	<0.001	15.85	<0.002	31.07	<0.001

Group A includes 11 healthy individuals (5 healthy nonsmokers and 6 healthy smokers) in whom small airway epithelial gene expression was assessed with the Affymetrix HG-U133A gene chip. Group B includes 22 healthy individuals (12 nonsmokers and 10 smokers) in whom small airway epithelial gene expression was assessed with the Affymetrix HG-U133 Plus 2.0 gene chip; for group B, expression values were independently generated using RMA and MAS5. Genes were considered expressed when they had Affymetrix present "P" calls in >50% of any given group of samples (nonsmokers or smokers) in both group A and group B study individuals.

[a] These references directly implicate the specific genes in some cases, or implicate pathways in which these genes are involved.

[b] Smokers (S) vs nonsmokers (NS) fold change was calculated by dividing the average expression value in the smokers by the average expression value in the nonsmokers.

[c] p values were calculated using the Welch t test (assuming unequal variances) using the Affymetrix HG-U133A gene chip; expression values were generated using MAS5.

[d] p values were calculated using the Welch t test (assuming unequal variances) using the Affymetrix HG-U133A Plus 2.0 gene chip; expression values were generated using RMA with Benjamini–Hochberg correction.

[e] Same as table note e except expression values were generated using MAS5.

We also observed differentially expressed apoptosis-related genes: consistent with prior studies demonstrating upregulation of pirin, a proapoptotic gene, in the large airways of smokers [20, 21, 42–45], we observed upregulation of pirin in the small airway epithelium of healthy smokers compared to nonsmokers ($p<0.03$; Table 2 and Fig. 5b). Similarly, the proapoptosis-related genes HIV-Tat interactive protein 2, 30 kDa gene also known as TIP30,

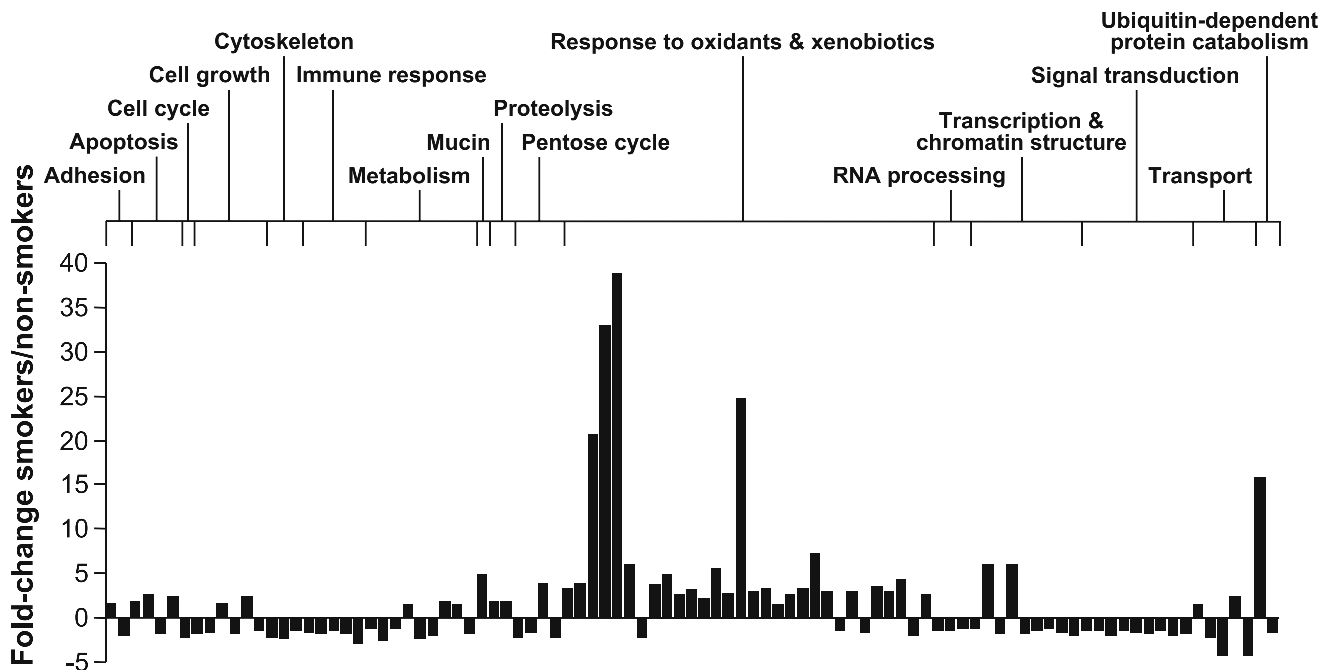

Fig. 4 Genes up- and downregulated in the small airway epithelium in response to smoking. The analyses are based on assessment of 12 nonsmokers and 10 smokers using the Affymetrix HG-U133 Plus 2.0 microarray chip. Fold changes in gene expression in small airway epithelium of healthy smokers compared to nonsmokers for the 95 significant genes with known function, out of the 118 significant genes represented. The fold change for genes upregulated by smoking was calculated by dividing the mean expression value for each gene in all smoker samples by the mean expression value in nonsmoker samples (positive numbers on the ordinate). For genes downregulated by smoking, the mean expression value for each gene in all nonsmoker samples was divided by the mean expression value in smoker samples, and identified with a *thin vertical line* (negative numbers on the ordinate). The genes were categorized according to the GO and Human Protein Reference Database annotations. The *abscissa* shows the individuals genes, randomly ordered by probe set ID within each category, as labeled

and the homeodomain interacting protein kinase genes [46, 47] were upregulated in smokers compared to nonsmokers ($p<0.03$). In contrast, the growth arrest and DNA damage-inducible, β-related gene, another proapoptotic gene [48], was downregulated in small airway epithelium of healthy smokers ($p<0.03$).

In agreement with prior gene expression studies in large airways of phenotypically normal smokers [18, 21], several oxidative stress and xenobiotic-related genes were differentially expressed in the small airway epithelium of smokers compared to nonsmokers (Table 2, Fig. 4, Fig. 5c). For example, the aldo-keto reductase family 1, member C1, and member C2 gene, the aldehyde dehydrogenase 3 family, member A1 gene, and the glutathione peroxidase 2 gene were upregulated in small airway epithelium of smokers compared to nonsmokers ($p<0.002$). Similarly, in the category of xenobiotics metabolism, the cytochrome P450, family 1, subfamily B, polypeptide 1 gene was upregulated in the small airway epithelium of healthy smokers compared to nonsmokers ($p<0.04$; for the entire list of genes in this category see Supplemental Tables 2 and 4).

Several genes involved in general cellular processes were differentially expressed in small airway epithelium of healthy smokers; for example, the ATPase H+ transporting, lysosomal V0 subunit a isoform 4, a gene involved in acidification of intracellular organelles for various intracellular processes such as protein sorting, receptor mediated endocytosis, and synaptic vesicle proton gradient generation, was upregulated in healthy smokers ($p<0.03$). In contrast, the coiled-coil alpha-helical rod protein 1 (CCHCR1) gene, which is involved in metabolism and cell differentiation, the forkhead box A2 (FOXA2) gene, important in cell differentiation, and the frizzled homolog 8 (Drosophila) FZD8 gene, involved in signal transduction, were downregulated ($p<0.03$; Fig. 5d and Table 2).

*Taq*Man RT-PCR

Independent analysis of differentially expressed genes in small airway epithelium of smokers vs nonsmokers in group B by real-time quantitative *Taq*Man RT-PCR confirmed the findings demonstrated by microarray assessment in a selected group of nine genes (Fig. 6). A two-way ANOVA with smoking status (smokers vs nonsmokers) and method (microarray vs *Taq*Man) as independent factors confirmed that expression levels of these nine genes were significantly affected by smoking status ($p<0.05$, all cases), and that method was not a significant factor ($p>0.2$, all cases).

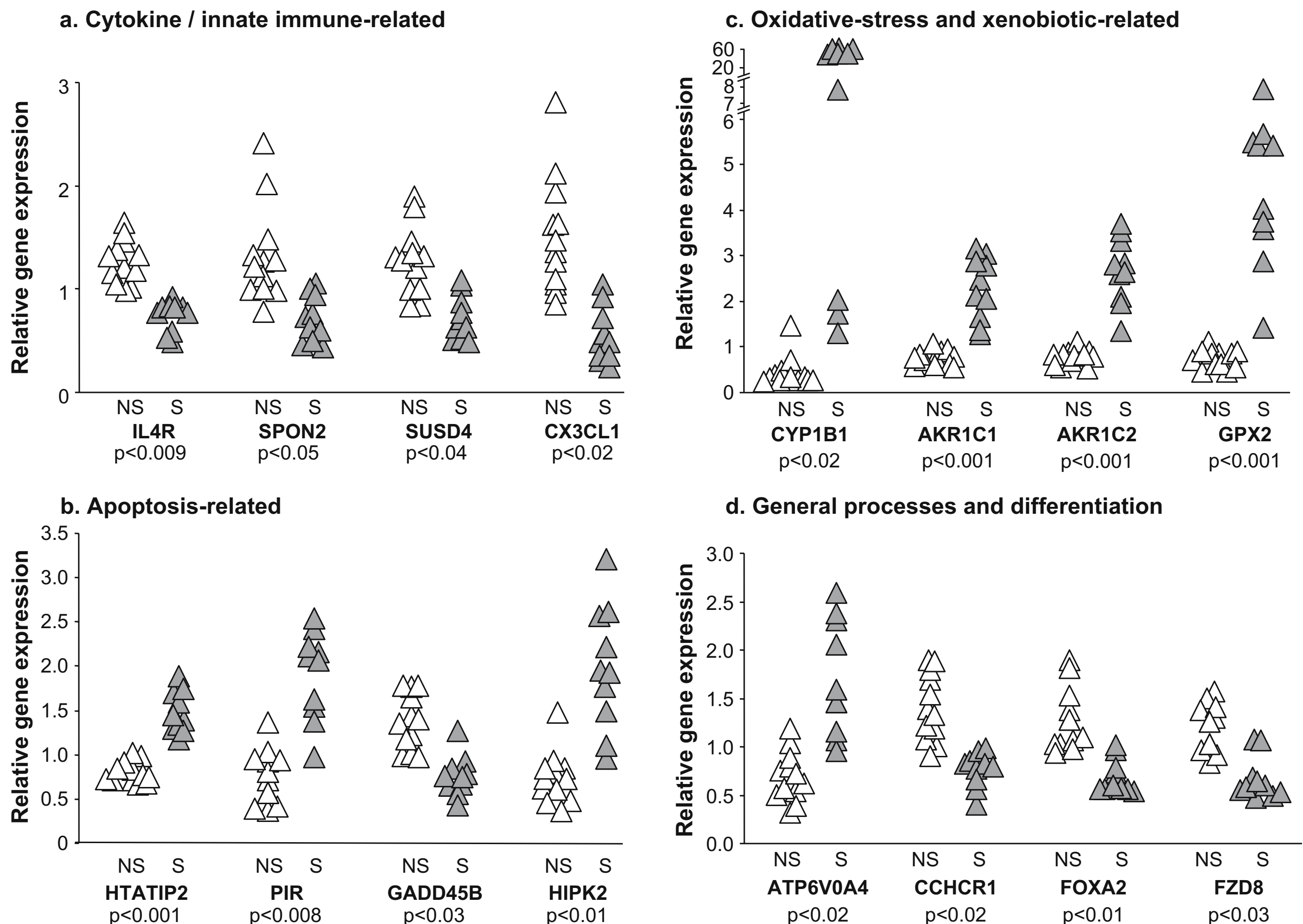

Fig. 5 Examples of genes up- and downregulated in the small airways of smokers compared to nonsmokers. The data is based on 10 healthy smokers compared to12 healthy nonsmokers assessed with the Affymetrix HG-U133 Plus 2.0 microarray chip, with Benjamini–Hochberg correction for multiple comparisons. The *abscissa* shows the specific genes; the *ordinate* shows the normalized gene expression levels. Each *symbol* represents an individual. *Open symbols* represent nonsmokers (*NS*); closed symbols represent smokers (*S*). *p* values are shown below each gene symbol. **a** Expression of cytokine/innate immune-related genes. The genes shown are the IL-4 receptor (*IL4R*) gene, the spondin 2 (*SPON2*) gene, the sushi domain containing four (*SUSD4*) genes, and the chemokine (C-X3-C motif) ligand 1 gene, also known as fractalkine (*CX3CL1*). **b** Expression of apoptosis-related genes. The genes shown are the HIV-Tat interactive protein 2, 30 kDa (*HTATIP2*) gene; the pirin (*PIR*) gene, the growth arrest and DNA damage-inducible, β-related (*GADD45B*) gene; and homeodomain interacting protein kinase 2 (*HIPK2*) gene. **c** Examples of expression of oxidative stress and xenobiotic-related genes. Shown are the cytochrome P450, family 1, subfamily B, polypeptide 1 (*CYP1B1*) gene; the aldo-keto reductase family 1, member C1 (*AKR1C1*) gene; the aldo-keto reductase family 1, member C2 (*AKR1C2*) gene; and the glutathione peroxidase 2 (*GPX2*) gene. **d** Examples of expression of general processes and differentiation genes. Shown are the ATPase H+ transporting, lysosomal V0 subunit a isoform 4 (*ATP6V0A4*) gene; the coiled-coil alpha-helical rod protein 1 (*CCHCR1*) gene, which is involved in metabolism and cell differentiation; the forkhead box A2 (*FOXA2*) gene, important in cell differentiation; and the frizzled homolog 8 (Drosophila) (*FZD8*) gene, involved in signal transduction

Discussion

While COPD associated with chronic cigarette smoking eventually involves all levels of the airways, the earliest smoking-induced changes are in the small airway epithelium [4–6, 13, 14]. To begin to understand the responses of the small airway epithelium to the stress of cigarette smoking, we developed a strategy with fiberoptic bronchoscopy and airway brushings to obtain highly pure epithelial cells from human small airways, and analyzed the epithelial cells gene expression with microarray technology. The small airway epithelial samples from healthy smokers and nonsmokers differed in composition from that of large airway samples, with the small airways having more ciliated cells, and less undifferentiated, basal, and secretory cells than the large airways. Consistent with the known composition of the secretory subset of airway epithelium in the small airways, epithelial cells of the small, but not large airways, demonstrated the presence of Clara cells and expression of surfactant apoprotein genes irrespective of smoking status.

Initial assessment of small airway epithelium gene expression in a small group (five nonsmokers and six smokers, group A) of healthy individuals with the HG-133A microchip array demonstrated 152 differentially

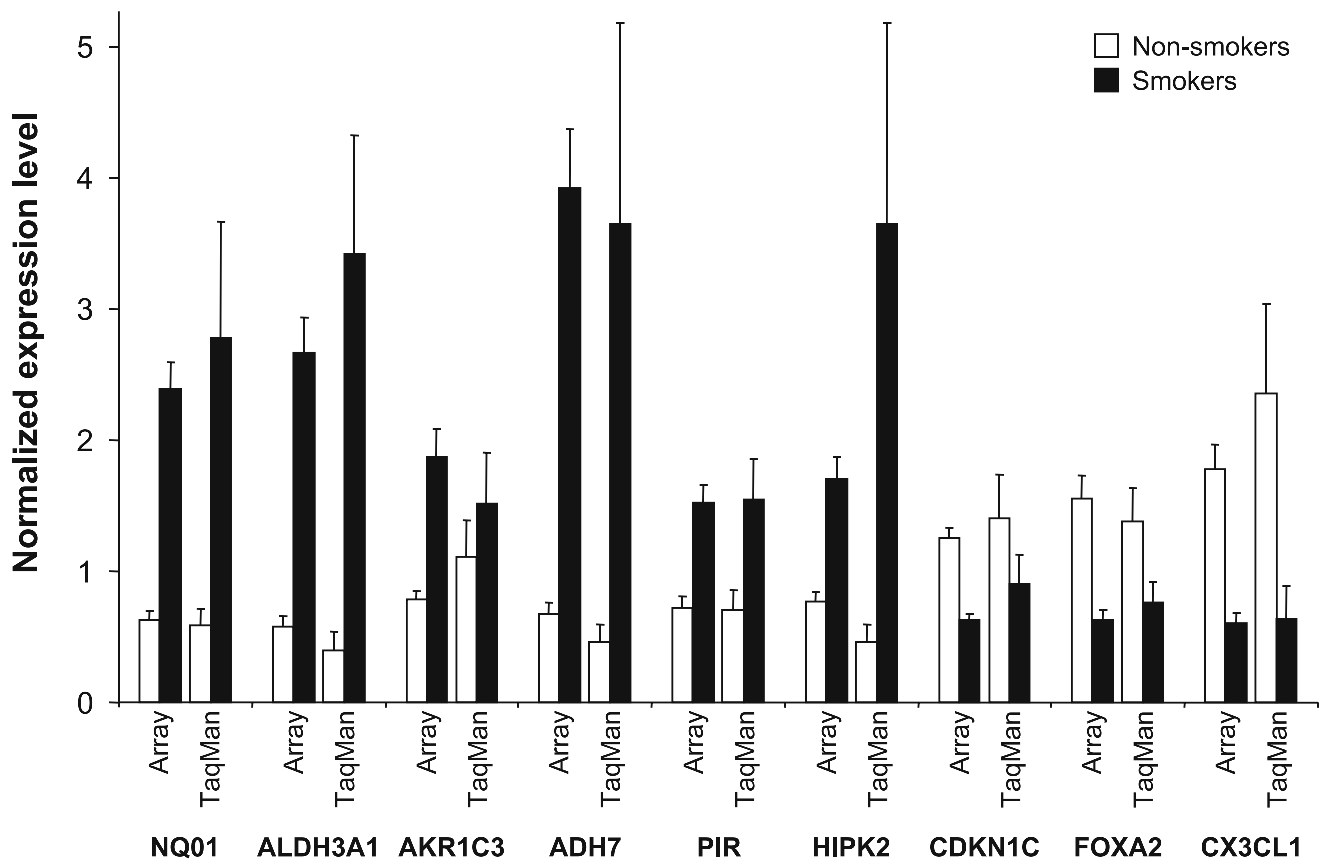

Fig. 6 Confirmation of microarray results with *Taq*Man real-time RT-PCR. Expression levels of six genes upregulated by smoking and three genes downregulated by smoking on initial assessment by microarray analysis (RMA-based) with the Affymetrix HG-U133 Plus 2.0 chip were confirmed with *Taq*Man real-time RT-PCR. To allow direct comparisons of values obtained using the two independent methods, *Taq*Man expression levels were normalized by dividing individual values by the median expression level of all nonsmokers and smokers for that method, as was done for microarray analysis. Relative expression levels (*ordinate*) are shown for six genes upregulated by smoking, as follows: four genes involved in the response to oxidative stress or xenobiotics, the NAD(P)H dehydrogenase, quinone 1 (*NQO1*) gene; the aldehyde dehydrogenase 3 family, member A1 (*ALDH3A1*) gene; the aldo-keto reductase family 1, member C3 (*AKR1C3*) gene; the alcohol dehydrogenase 7 (*ADH7*) gene; two genes involved in apoptosis, the pirin (*PIR*); and the homeodomain interacting protein kinase 2 (*HIPK2*) genes; and for three genes downregulated by smoking: the cyclin-dependent kinase inhibitor 1C (*CDKN1C*) gene, also known as p57 or Kip2, a cell cycle arrest protein; the transcription factor forkhead box A2 (*FOXA2*) gene, involved in transcription of the surfactant genes; and the chemokine (C-X3-C motif) ligand 1 (*CX3CL1*). A two-way ANOVA with smoking status (smokers vs nonsmokers) and method (microarray vs *Taq*Man) as independent factors confirmed that expression levels of these nine genes were significantly affected by smoking status ($p<0.05$, all cases), and that method was not a significant factor ($p>0.2$, all cases)

expressed genes (103 upregulated, 49 downregulated) in response to cigarette smoking; 133 of which are of known function and belong to several functional categories. After these initial assessments, we carried out an independent study in an entirely new and larger group of healthy smokers vs nonsmokers (12 nonsmokers vs 10 smokers, group B). This analysis, assessed with the HG-U133 Plus 2.0 microarray chip, with MAS5 and RMA (independently), and with Benjamini–Hochberg correction for false discovery rate, demonstrated 118 differentially expressed genes in healthy smokers vs nonsmokers.

Based on review of the literature on the different molecular pathways implicated in the pathogenesis of COPD involving the airway epithelium, and on the assessment of the differentially expressed genes in the small airways of smokers compared to nonsmokers, we developed a working list of genes divided into categories relevant to the pathogenesis of COPD. The categories of genes generated on the assessment of group A and group B included cytokine/innate immunity, apoptosis, response to oxidants and xenobiotics, proteolysis/antiproteases, and general cellular processes. These genes represent a snapshot of the early molecular changes in the small airway epithelium of healthy individuals who actively smoke, and are therefore at risk to develop COPD. These genes may represent COPD susceptibility genes and protective genes, and the risk for COPD may depend on an individual's specific pattern of combined expression for susceptibility

and protective genes. Furthermore, these genes may represent only a subset of the genes underlying the pathogenesis of COPD.

Small airways, smoking, and COPD

The small airways (<2 mm) represent the main site of airway obstruction in individuals with COPD [4–6, 13, 14]. Asymptomatic smokers display evidence of small airway inflammation; for example, Niewoehner et al. [8] studied the lungs of 19 young smokers and 20 nonsmokers, and demonstrated that the small airways of smokers had definitive pathologic abnormalities with denuded epithelium and increased number of mural inflammatory cells. These data are consistent with the concept that the small airways represent the earliest site of smoking-induced structural changes before the development of COPD [4–6, 8–14]. It is interesting to note that the extent of small airway disease correlates with the extent of alveolar destruction [13, 14].

Small airway epithelial smoking-induced phenotype

The airway epithelium plays an important role in controlling many airway functions and is capable of up- and downregulating genes in several categories as well as producing and secreting mediators important in several aspects of airway function [16–18, 21, 33, 49–54]. These genes and mediators, among others, include cytokines, chemokines, apoptosis-related, profibrotic-related, oxidative stress-related, proteolysis/antiproteases-related, mucin-related, and genes related to general processes [16–18, 21, 33, 49–54]. In this context, persistent activation of the small airway epithelium with the insult of cigarette smoke leads to an alteration of the "resting" state of the small airway epithelium with up- and downregulation of genes in different categories as observed in our study population.

The cytokine, innate, and immunomodulatory responses of the small airway epithelium to the insult of cigarette smoke play an important role, over time, in the eventual development of the small airway inflammatory component characteristic of individuals with established COPD. The small airways from healthy smokers demonstrate inflammation, as do symptomatic smokers, and individuals at different COPD stages [Global Initiative for Chronic Obstructive Lung Disease (GOLD) stages 1–4] [1, 2, 8–12]. It is interesting to note that although some degree of overlap in small airway inflammation is observed among asymptomatic smokers and individuals with COPD GOLD stages 0–3 [12], increased numbers of $CD8^+$ T lymphocytes are observed only in smokers who develop COPD [11]. This suggests that the cytokine/innate immune response seems to be nonspecific at earlier stages of smoking, but over time, for those individuals who develop COPD, the immune response undergoes a more specific change, which results in increased accumulation of $CD8^+$ T lymphocytes.

We found downregulation of the IL4 receptor, a mediator of several proinflammatory functions in human airways [38], and downregulation of chemokine (C-X3-C motif) ligand 1 (CX3CL1), which is involved in cell adhesion and recruitment of monocytes and T lymphocytes cells [39, 40]. It is interesting to note that microarray analysis of lung tissue from individuals with COPD demonstrated upregulation of the CX3CL1 gene in individuals with later stage COPD compared to individuals with early COPD [55]. Although lower mRNA levels do not necessarily reflect lower protein levels, it can be speculated that the downregulation of CX3CL1 in our study suggests that phenotypically healthy smokers attempt to maintain a balance in the inflammatory response in the small airways by suppressing signals that could potentially injure the epithelium.

The role of apoptosis in the pathogenesis of COPD is well recognized, and increased apoptosis of airway epithelial cells from individuals with COPD was documented even after cessation of smoking [56–61]. In our study, assessment of the small airway epithelium of healthy smokers showed smoking-related modulation of several proapoptotic genes [42–48], suggesting ongoing attempts of an "apoptosis balance" in the small airway epithelium of phenotypically healthy smokers.

It is well recognized that the oxidative stress of cigarette smoking plays an important role in the pathogenesis of COPD [49, 62]. We observed upregulation of several oxidative stress-related and xenobiotic genes in the small airway epithelium. This suggests that similar to studies of increased expression of oxidative stress-related genes in the large airway epithelium of healthy smokers [18, 21], the small airway epithelium responds to the insult of cigarette smoking by upregulating several oxidative stress-related and xenobiotic genes.

Several genes relevant to general cellular processes were upregulated, consistent with the increased energy expenditure observed in healthy smokers and in individuals with COPD [63, 64].

Implications for the understanding and treatment of COPD

Assessment of the molecular changes of the small airway epithelium in healthy smokers is relevant to establishing patterns of gene expression for comparison with the gene expression in small airways of individuals at various stages of COPD. The differential gene expression observed in the small airway epithelium of healthy smokers represents the initial deviations of gene expression observed in the main site of potential disease in individuals at risk for COPD.

This study may help in the identification of novel genes not related to already known mechanisms of COPD pathogenesis. Assessment of the expression of these genes in the small airway epithelium of individuals with COPD should be useful in identifying mechanisms relevant to the pathogenesis of COPD and potential new therapeutic targets for intervention.

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