Down-regulation of the Notch Pathway in Human Airway Epithelium in Association with Smoking and Chronic Obstructive Pulmonary Disease

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Rationale: **The airway epithelium of smokers is subject to a variety of mechanisms of injury with consequent modulation of epithelial regeneration and disordered differentiation. Several signaling pathways, including the Notch pathway, control epithelial differentiation in lung morphogenesis, but little is known about the role of these pathways in adults.**

Objectives: **We tested the hypotheses that Notch-related genes are expressed in the normal nonsmoker small airway epithelium of human adults, and that Notch-related gene expression is down-regulated in healthy smokers and smokers with chronic obstructive pulmonary disease (COPD).**

Methods: **We used microarray technology to evaluate the expression of 55 Notch-related genes in the small airway epithelium of nonsmokers. We used TaqMan quantitative polymerase chain reaction (PCR) to confirm the expression of key genes and we used immunohistochemistry to assess the expression of Notch-related proteins in the airway epithelium. Changes in expression of Notch genes in healthy smokers and smokers with COPD compared with nonsmokers were evaluated by PCR.**

Measurements and Main Results: **Microarray analysis demonstrated that 45 of 55 Notch-related genes are expressed in the small airway epithelium of adults. TaqMan PCR confirmed the expression of key genes with highest expression of the ligand DLL1, the receptor NOTCH2, and the downstream effector HES1. Immunohistochemistry demonstrated the expression of Jag1, Notch2, Hes1, and Hes5 in airway epithelium. Several Notch ligands, receptors, and downstream effector genes were down-regulated in smokers, with more genes down-regulated in smokers with COPD than in healthy smokers.**

Conclusions: **These observations are consistent with the hypothesis that the Notch pathway likely plays a role in the human adult airway epithelium, with down-regulation of Notch pathway gene expression in association with smoking and COPD.**

Keywords: gene expression; microarray analysis; delta-like ligand; basic helix-loop-helix transcription factors; Notch receptors

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Cigarette smoking places the airway epithelium under the stress of a variety of mechanisms of injury, with consequent need for epithelial regeneration and repair. The Notch pathway plays a critical role in lung development.

What This Study Adds to the Field

Genes encoding key Notch ligands, receptors, and downstream effectors are down-regulated in the airway epithelium of healthy smokers and smokers with chronic obstructive pulmonary disease, implying this pathway may be important in repair of smoking-induced injury.

The airway epithelium plays a major role in lung health and function by providing a barrier to the environment, maintaining the balance of water and small and large molecules between the basolateral and apical surfaces, mediating the mucociliary clearance of the epithelial surface, and expressing a variety of gene products that help maintain normal airway structure and function and contribute to host defenses (1–3). In normal individuals, as airway epithelial cells are injured or reach senescence, there is ongoing cell regeneration with basal cell proliferation and subsequent differentiation to maintain the normal pseudostratified epithelial barrier that lines the airways (4, 5). In the context of constant cell renewal and differentiation of the airway epithelium, signaling pathways that control cellular differentiation must play a role in the adult epithelium, with expression of genes involved in these pathways (6). However, although there has been considerable attention to the pathways that modulate epithelial differentiation in embryonic lung development, little is known about whether similar pathways are functional in the airway epithelium of adults (7–11).

One such differentiation control system is the Notch signaling pathway. Originally discovered in Drosophila, there is evidence in experimental animals that the Notch pathway plays an important role in the determination of cell fate in multiple organ systems (7, 13, 15, 16) including the lung (7, 12–14). The classical role of Notch signaling is the prevention of cell differentiation and the maintenance of an undifferentiated state in stem cells in various organs (7, 15, 37). When Notch is "on," differentiation is suppressed, whereas when Notch is "off," cells are allowed to proceed to a specific differentiation fate (13, 15, 16). Notch typically acts locally to define boundaries between different cell types with divergent fates; for example, it can act following division of a stem cell to induce differentiation in one daughter cell and keep the other as a stem cell (13, 16).

Notch signaling is simple in concept, but multiple regulatory components at every stage of the pathway add a level of complexity (13, 15–17). Notch signaling in humans is initiated by interaction of one of the five Notch ligands, Delta-like (DLL1, 3, or 4) or Jagged (JAG1 or 2) with one of the four receptors (NOTCH1, 2, 3, or 4). Both ligand and receptor are single-pass membrane proteins, and therefore Notch signaling typically involves cell-to-cell contact. Ligand-receptor binding is followed

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by cleavage of the Notch receptor and release of the Notch intracellular domain (NICD). NICD is vulnerable to proteolysis by modulators such as Numb and Itch; if not proteolyzed, NICD translocates to the nucleus and displaces CBF1, a transcription factor, from an inhibitory complex. CBF1 then associates with a stimulatory complex including the Mastermind-like (MAML) protein. The stimulatory complex binds to a consensus sequence (RTGGGAA), resulting in transcription of a group of inhibitory transcription factors known as basic helix-loop-helix (bHLH) genes, including HES1, 2, and 5, and HEY1, 2, and L. In turn, the bHLH genes suppress the expression of downstream differentiation factors including ASCL1, UBE2A and MYOD. Therefore, when Notch signaling is "on," bHLH genes are transcribed and suppress transcription of differentiation factors, maintaining "stemness." Conversely, when Notch signaling is "off," bHLH genes are not transcribed, permitting transcription of differentiation factors and differentiation to a specific cell fate.

With this background, we asked two questions. First, are Notch-related genes being expressed in the adult human airway epithelial transcriptome, and if so, what are the relative expression levels of the Notch pathway ligands (Delta-like ligands, DLL1, 3, and 4; Jagged, JAG1 and 2), receptors (NOTCH1, 2, 3, and 4), and downstream effectors (HES1, 2, and 5; HEY1, 2, and L)? Second, based on the knowledge that cigarette smoking and its associated disorder chronic obstructive pulmonary disease (COPD) are characterized by abnormal patterns of differentiation of the airway epithelium, is the expression of genes in the Notch pathway modulated in normal smokers and smokers with COPD? To assess these questions, we first compiled a list of 55 genes related to the Notch pathway and used microarray technology to assess the expression of these genes in the epithelium of small airways of healthy nonsmokers. Notch proteins were also assessed by performing immunohistochemical analysis on large airway biopsy samples from a subset of the subjects and on brushed small airway epithelial cells from an independent set of subjects. Following this screen of expression of the Notch pathway, we used TaqMan quantitative polymerase chain reaction (PCR) to assess relative expression levels of the key Notch genes in the small airway epithelium of normal nonsmokers, normal smokers, and smokers with COPD. The data demonstrate that most of the genes in the Notch pathway are expressed in the adult human small airway epithelium, and that the expression levels of key genes in the pathway are affected by smoking, both in phenotypic normal smokers and smokers who have developed COPD, observations that are consistent with this pathway playing a role in repair and differentiation in the adult lung.

Some of the results of these studies have been previously reported in the form of an abstract (18, 19).

METHODS

Study Population

All individuals were evaluated at the Weill Cornell NIH General Clinical Research Center (GCRC) and Department of Genetic Medicine Clinical Research Facility, using Institutional Review Board–approved clinical protocols. Normal nonsmokers and normal current cigarette smokers were recruited by posting advertisements in local newspapers. Individuals with COPD were recruited from the outpatient clinics of the Division of Pulmonary and Critical Care Medicine and from the population responding to advertisements that were documented to meet criteria for COPD. Individuals were determined to be phenotypically normal or to have COPD based on standard history, physical exam, complete blood count, coagulation studies, liver function tests, urine studies, chest X-ray (and, where relevant, chest CT scan), EKG, and pulmonary function tests. To verify smoking status, a complete smoking history was obtained, urine samples were evaluated for nicotine and cotinine, and venous blood was evaluated for carboxyhemoglobin.

Normal nonsmokers were defined as never-smokers with a normal history and physical exam, and normal lung function. Normal smokers were individuals currently smoking with a normal history and physical exam and normal lung function. Smokers with established COPD included current smokers who met the Global Initiative for Chronic Obstructive Lung Disease (GOLD) criteria (20). Individuals who demonstrated significant reversibility of airflow limitation, defined as an increase in FEV_1 or FVC of at least 200 cc and 12% in response to bronchodilators, were excluded from the study.

The gene expression arrays from a subset of the nonsmoker, healthy smoker, and COPD smoker groups used in this report have been used in previous reports (21, 22), and the raw data from the arrays has been made available to the public (Gene Expression Omnibus [GEO] accession numbers GSE4498 and GSE5058). Neither of these studies reported on an expression of genes related to the Notch signaling pathway.

Sampling Airway Epithelium

Fiberoptic bronchoscopy was used to collect small airway epithelial cells by brushing the epithelium as previously described (22). After mild sedation was achieved with meperidine and midazolam and routine anesthesia of the vocal cords and bronchial airways with topical lidocaine, a fiberoptic bronchoscope (Pentax, EB-1530T3) was positioned proximal to the opening of a desired lobar bronchus. A 2-mm diameter brush was advanced approximately 7 to 10 cm distally from the third-order bronchial branching under fluoroscopic guidance. The distal end of the brush was wedged at about the 10th to 12th 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 5 to 10 times in 10 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). An aliquot of 0.5 ml was used for differential cell count (typically 2×10^4 cells per slide). Each aliquot was prepared by centrifugation (Cytospin 11, Shandon Instruments, Pittsburgh, PA) and stained with Diff-Quik (Dade Behring, Newark, NJ). Differential cell counts were performed by experienced observers using light microscopy; these differential cell counts are highly reproducible with no significant interobserver variation (23). The remainder of cell medium (4.5 ml) was processed immediately for RNA extraction.

A subset of individuals from all three study groups also underwent endobronchial biopsies to be used for immunohistochemical analysis. These samples were collected by inserting a biopsy forceps via the working channel of the bronchoscope and taking several biopsies of the subcarinas of the right or left lower lobe under direct visualization.

RNA and Microarray Processing

The HG-U133 Plus 2.0 array (Affymetrix, Santa Clara, CA), which includes probes representing approximately 47,000 full-length human genes, was used to evaluate gene expression. Total RNA was extracted using a modified version of the TRIzol method (InVitrogen, Carlsbad, CA), in which RNA is purified directly from the aqueous phase by Rneasy purification (Rneasy MinElute RNA purification kit, Qiagen, Valencia, CA). RNA samples were stored in RNA Secure (Ambion, Austin, TX) at −80°C. RNA integrity was determined by running an aliquot of each RNA sample on an Agilent Bioanalyzer (Agilent Technologies, Palo Alto, CA). The concentration was determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). RNA samples accepted for further processing met three quality control criteria: (*1*) A_{260}/A_{280} ratio between 1.7 and 2.3; (*2*) concentration between 0.2 and 6 μg/ml; and (*3*) Agilent electropherogram displaying two distinct peaks corresponding to the 28S and 18S ribosomal RNA bands at a ratio of 0.5 or greater with minimal or no degradation. Double-stranded cDNA was synthesized from 3 μg total RNA using the GeneChip One-Cycle cDNA Synthesis Kit, followed by cleanup with GeneChip Sample Cleanup Module, *in vitro* transcription (IVT) reaction using the GeneChip IVT Labeling Kit, and cleanup and quantification of the biotin-labeled cDNA yield by spectrophotometry. All kits were from Affymetrix (Santa Clara, CA). Hybridizations to test chips and to the HG-U133 Plus 2.0 microarray were performed according to Affymetrix protocols, processed by the Affymetrix fluidics station, and scanned with an Affymetrix Gene Array Scanner 2500. Overall microarray quality was determined by taking into consideration the

percentage of "present" genes, the 3′/5′ signal ratio for control genes, and the average background signal, as recommended by Affymetrix.

Notch-related Gene Expression in the Small Airway Epithelium in Normal Nonsmokers

A list of 55 genes implicated in the Notch signaling pathway was compiled from the literature and genes were assigned to functional categories including ligands, receptors, proteases, transcription factors, modulators, and downstream effector genes (all of which belong to the basic helix-loop-helix family of transcription factors) (13, 16, 17, 24–26). As a screen, to determine whether these genes were expressed in the normal adult human airway epithelium, RNA from the small airway epithelium of normal nonsmokers was assessed with the HG-U133 Plus 2.0 microarray. A gene was categorized as "expressed" if the Affymetrix Detection Call was present in 50% or more of the normal nonsmokers small airway samples.

TaqMan Real-Time Reverse Transcriptase–PCR

TaqMan real-time reverse transcriptase (RT)-PCR for confirmation of gene expression levels and of changes in gene expression across groups was used with relative quantification using rRNA as the reference gene. Premade TaqMan Gene Expression Assays were obtained from Applied Biosystems (Foster City, CA). All samples were assessed in triplicate at two different cDNA concentrations to ensure that amplification efficiency for the reference gene and gene of interest was the same. All PCR reactions were run in an Applied Biosystems Sequence Detection System 7500 as previously described (21, 22).

Gene expression was assessed by TaqMan quantitative real-time RT-PCR using the ddCt method. dCt values were calculated using ribosomal RNA as a reference sample, and then ddCt values were calculated relative to the least-expressed gene. In this way, assessments of relative mRNA abundance were made for each gene of interest. In accordance with the recommendations of Applied Biosystems, the amplification efficiency of each Gene Expression Assay was assumed to be equivalent.

Immunohistochemistry

To determine which airway epithelial cells express Notch pathway proteins, bronchial biopsies were obtained from the large airway epithelium and brushed small airway epithelial cells were obtained from nonsmokers, normal smokers, and COPD smokers. Biopsies were fixed in 4% paraformaldehyde, embedded in paraffin, sectioned at a thickness of 5 microns, cleared with xylene, and rehydrated through a graded series of ethanols. Antigen recovery was performed using Dako Cytomation target retrieval solution (Carpenteria, CA) at 100°C for 15 minutes. Endogenous peroxidase activity was blocked for 30 minutes in 0.3% H_2O_2 in PBS. Tissues were blocked with either 5% goat serum or 5% donkey serum matched to the secondary antibody for 30 minutes. Tissues were incubated with the primary antibodies and controls at 4°C overnight in a humid chamber. For Jag1 the primary antibody was goat anti-Jag1 (C-terminus, #SC6011, Santa Cruz Biotechnology, Santa Cruz, CA) at 8 μg/ml and the control was the same antibody in the presence of a 10-fold excess (by protein concentration) of the Jag1 peptide immunogen (#SC6011P, Santa Cruz Biotechnology). For Notch2 the primary antibody was rabbit anti-Notch2 (C-terminus, #QC9031, Aviva Systems Biology, San Diego, CA) at 2 μg/ml, and the control was the same antibody in the presence of a 10-fold excess (by protein concentration) of the Notch2 peptide immunogen (#P21613, Aviva Systems Biology). For Hes1 the primary antibody was rabbit anti-Hes1 (N-terminus, #QC1851, Aviva Systems Biology) at 2 μg/ml and the control was the same antibody in the presence of a 10-fold excess (by protein concentration) of the Hes1 blocking peptide (#P03361, Aviva Systems Biology). For Hes5 the primary antibody was an affinity-purified rabbit anti-Hes5 (#H9288, Sigma Aldrich, St Louis, MO) applied at 2 μg/ml and the control was an equal concentration of rabbit IgG (Jackson Immunoresearch, West Grove, PA). Secondary antibodies were goat anti-rabbit antibodies conjugated to horseradish peroxidase (HRP) (Dako Cytomation) or donkey anti-goat antibodies conjugated to HRP (Jackson Immunoresearch). Aminoethylcarbazole (AEC) chromogenic substrate (Dako Cytomation) was used for visualizing the HRP.

Cytopreps were prepared as described above and were fixed in 4% paraformaldehyde. Endogenous peroxidase activity was blocked for 5 minutes in 0.3% H_2O_2. Antigen recovery was performed using 1× citrate buffer (Thermo Fisher Scientific, Fremont, CA) at 100°C for 20 minutes. Tissues were blocked with 5% goat serum for 30 minutes. Tissues were incubated with the primary antibodies and controls at 4°C overnight in a humid chamber. For Hes1 the primary antibody was rabbit anti-Hes1 (N-terminus, #QC1851, Aviva Systems Biology) at 2 μg/ml and the control was the same antibody in the presence of a 10-fold excess (by protein concentration) of the Hes1 blocking peptide (#P03361, Aviva Systems Biology). For Hes5 the primary antibody was an affinity-purified rabbit anti-Hes5 (#H9288, Sigma Aldrich, St. Louis, MO) applied at 2 μg/ml and the control was an equal concentration of rabbit IgG (Jackson Immunoresearch, West Grove, PA). Secondary antibodies were goat anti-rabbit antibodies conjugated to HRP (Dako Cytomation). AEC chromogenic substrate (Dako Cytomation) was used for visualizing the HRP.

Tissues were counterstained with Mayer's hematoxylin and mounted using GVA mounting medium (Zymed, San Francisco, CA). Brightfield microscopy was performed using a Nikon Microphot microscope equipped with a Plan 40× N.A. 0.70 objective lens. Images were captured with an Olympus DP70 CCD camera.

Statistical Analyses

To assess relative abundance of mRNA for genes in a particular category, the mean value among individuals was calculated for each gene in the group of nonsmokers after removing outlier values by the interquartile range method. Analysis of variance was used to assess for differences in mean expression level among genes in each functional category: ligands, receptors, and downstream effectors.

To evaluate for changes in gene expression across the study groups of nonsmokers, normal smokers, and smokers with COPD, means were

TABLE 1. STUDY POPULATION

Parameter	Normal nonsmokers	Normal smokers	Smokers with established COPD*	*P* Value
n	20	15	13	
Sex (male/female), n	16/4	12/3	12/1	>0.5
Age, yr	43 ± 8	46 ± 6	52 ± 6	<0.02†
Race B/W/H/A/O, n	9/7/3/0/1	10/5/0/0/0	4/8/0/1/0	>0.1
Smoking history, pack-yr	0	33 ± 18	42 ± 27	>0.3
Pulmonary function parameters‡				
FVC	106 ± 10	104 ± 13	97 ± 18	>0.1
FEV1	106 ± 11	106 ± 16	77 ± 24	<0.0001†
FEV1/FVC	82 ± 6	81 ± 3	64 ± 12	<0.0001†
TLC	98 ± 10	96 ± 12	107 ± 20	>0.09
DL_{CO}	95 ± 10	95 ± 10	75 ± 17	<0.0001†
Gold stage 0/I/II/III, n*	—	—	2/4/6/1	
Epithelial cells				
Number recovered × 10	5.5 ± 1.9	6.2 ± 3.3	5.3 ± 1.4	>0.8§
Epithelial cells, %	100 ± 1	99 ± 1	97 ± 1	<0.0001†
Inflammatory cells, %	0 ± 0	1 ± 1	3 ± 1	<0.0001†
Differential cell count, %				
Ciliated	73 ± 6	71 ± 6	66 ± 8	>0.2
Secretory	7 ± 4	10 ± 3	18 ± 6	<0.002†
Basal	14 ± 5	12 ± 3	7 ± 3	<0.002§
Undifferentiated	5 ± 2	8 ± 5	9 ± 1	>0.1
Medication use, n				
Beta-agonist			3	
Anticholinergic			2	
Inhaled steroid			1	

Definition of abbreviations: A = Asian; B = black; COPD = chronic obstructive pulmonary disease; DL_{CO} = carbon monoxide diffusing capacity; H = Hispanic; O = other; W = white.

Values are mean ± SD unless otherwise indicated.

* Smokers with established COPD were defined using GOLD criteria (20).

† *P* value significant for COPD versus nonsmoker and COPD versus smoker, but not for smoker versus nonsmoker.

‡ Pulmonary function testing parameters are given as percentage of predicted value with the exception of FEV1/FVC, which is reported as percentage observed. For individuals with COPD, FVC, FEV1, and FEV1/FVC are post-bronchodilator values.

§ *P* value significant for COPD versus nonsmoker only.

calculated for each gene in each group of study subjects. Analysis of covariance was used with age as a covariate to ensure that there was no effect of age on expression differences. Age was not a covariate for any gene. Because not all of the data met assumptions for normality, Kruskal-Wallis was then used to assess for differences among groups. For genes for which Kruskal-Wallis showed significant differences, post-hoc pairwise comparisons were done with Mann-Whitney U test to identify specific differences among each pair of study groups. Expression was considered to be significantly different if the magnitude of the fold-change between groups was 1.5 or greater (either up-regulated or down-regulated) and the P value was < 0.05.

Web Deposition of Data

All gene expression data has been deposited at the GEO site (http://www.ncbi.nlm.nih.gov/geo/), a high-throughput gene expression/molecular abundance data repository curated by the National Center for Bioinformatics (NCBI) site. The accession number for this data set is GSE7832.

RESULTS

Study Population and Biologic Samples

The overall study population consisted of 48 individuals, including 20 nonsmokers, 15 healthy smokers, and 13 smokers with COPD (Table 1). Microarray analysis was performed on the 20 nonsmokers. TaqMan analysis was performed on a subset of the nonsmokers (n = 12), all of the 15 healthy smokers, and 13 smokers with COPD. Large airway biopsies were taken from a subset of these subjects, and brushed small airway epithelial cells for immunohistochemistry were obtained from an independent set of 10 nonsmokers, 11 healthy smokers, and 10 COPD smokers. The three groups were of similar distribution of sex (predominately male, $P > 0.5$, all comparisons) and race (mostly of African or European descent, $P > 0.1$, all comparisons). The COPD group was older ($P < 0.02$) than the two other groups. The lung functions of the normal nonsmokers and normal smokers were similar ($P > 0.4$, all comparisons). On average, the smokers with COPD had a reduced FEV1, FEV1%, and DLCO compared with the normal nonsmokers and smokers ($P < 0.0001$, all comparisons). Among the COPD group, GOLD stages I and II dominated. Of the 13 individuals with COPD, 10 were using no medication for COPD and 3 were using combination therapies. The number of cells recovered in all groups was similar ($P > 0.8$). The proportion of epithelial cells recovered was, on average, 96% or greater in all groups, although the percentage of inflammatory cells was higher in the COPD group compared with the other groups ($P < 0.0001$ for both comparisons). The COPD group had a higher proportion of secretory cells compared with the other two groups, which was statistically significant ($P < 0.002$ for both comparisons), and a lower proportion of basal cells, which was statistically significant only when compared with nonsmokers ($P < 0.002$). Otherwise, the proportions of epithelial cell types were similar among the groups ($P > 0.05$, all other comparisons). Among all samples, the average RNA yield was 25.3 ± 9.0 μg per subject.

Expression of Notch-related Genes in Adult Nonsmoker Airway Epithelium

Based on a review of the literature, 55 genes involved in the Notch signaling pathway were identified, all of which were represented by at least one probe set on the Affymetrix HG-U133 Plus 2.0 microarray chip (Figure 1). Assessment of the airway epithelium from the healthy nonsmokers revealed that 45 (82%) of the Notch pathway-related genes were expressed in the human airway epithelium of normal nonsmokers (Table 2). The expressed genes represented all functional categories of the Notch pathway, including ligands, receptors, proteases, modulators, transcription factors, and downstream effectors.

TaqMan real-time quantitative RT-PCR was used to verify expression of Notch pathway ligands, receptors, and downstream effectors in the airway epithelium of nonsmokers (Figure 2). The 3 ligands expressed by microarray, DLL1, JAG1 and JAG2, were all confirmed to be expressed by TaqMan. DLL1 was the most

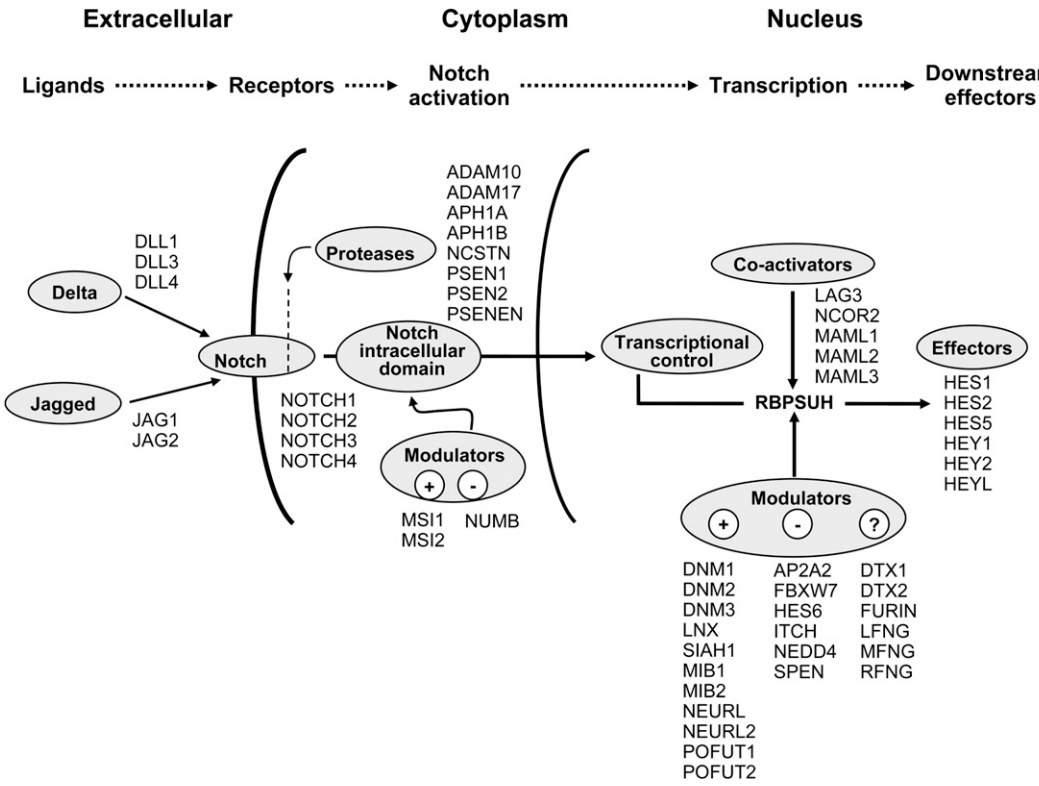

Figure 1. The Notch pathway in mammals. The pathway is triggered by the Delta-like or Jagged ligands interacting with one or more of the Notch receptors. The pathway is activated by intracellular cleavage of a Notch receptor, releasing the Notch intracellular domain, which translocates to the nucleus where it activates CBF1 (gene symbol, RBPSUH), the primary transcription factor to activate downstream Notch effector pathways (Hes1, 2, 5; Hey1, 2 and L). A variety of transcriptional coactivators and cytoplasmic and nuclear modulators tune the pathway, providing multiple points of control. When Notch signaling is active, differentiation is suppressed. When Notch signaling is turned "off," differentiation to specific cell fates is allowed to proceed.

TABLE 2. NOTCH PATHWAY GENES EXPRESSED IN THE SMALL AIRWAY EPITHELIUM OF NONSMOKERS*

Category	Gene title	Gene symbol	Nonsmokers small airways,† (*n = 20*)	Expressed in human airway epithelium‡
Ligands	Delta-like ligand 1	DLL1	100	yes
	Delta-like ligand 3	DLL3	5	no
	Delta-like ligand 4	DLL4	0	no
	Jagged 1	JAG1	100	yes
	Jagged 2	JAG2	100	yes
Receptors	Notch 1	NOTCH1	100	yes
	Notch 2	NOTCH2	100	yes
	Notch 3	NOTCH3	100	yes
	Notch 4	NOTCH4	90	yes
Proteases	Disintegrin and metalloprotease 10	ADAM10	100	yes
	Disintegrin and metalloprotease 17	ADAM17	100	yes
	Presenilin 1	PSEN1	100	yes
	Presenilin 2	PSEN2	100	yes
	Anterior pharynx defective 1 homolog A	APH1A	100	yes
	Anterior pharynx defective 1 homolog B	APH1B	100	yes
	Presenilin enhancer 2 homolog	PSENEN	100	yes
	Nicastrin	NCSTN	100	yes
	CBF1; CSL; recombining binding protein suppressor of hairless	RBPSUH	100	yes
Transcription factors	Mastermind-like 1	MAML1	100	yes
	Mastermind-like 2	MAML2	100	yes
	Mastermind-like 3	MAML3	100	yes
	Lymphocyte-activation gene 3	LAG3	5	no
	Nuclear receptor co-repressor 2	NCOR2	100	yes
Downstream effectors	Hairy & enhancer of split 1	HES1	100	yes
	Hairy & enhancer of split 2	HES2	90	yes
	Hairy & enhancer of split 5	HES5	50	yes
	Hairy/enhancer-of-split related with YRPW motif 1	HEY1	100	yes
	Hairy/enhancer-of-split related with YRPW motif 2	HEY2	100	yes
	Hairy/enhancer-of-split related with YRPW motif-like	HEYL	90	yes
Modulators, positive	Neuralized-like	NEURL	45	no
	Neuralized-like 2	NEURL2	0	no
	Mindbomb homolog 1	MIB1	100	yes
	Mindbomb homolog 2	MIB2	100	yes
	Protein O-fucosyltransferase 1	POFUT1	40	no
	Protein O-fucosyltransferase 2	POFUT2	100	yes
	Ligand of numb-protein X	LNX	100	yes
	Seven in absentia homolog 1	SIAH1	100	yes
	Musashi homolog 1	MSI1	0	no
	Musashi homolog 2	MSI2	100	yes
	Dynamin 1	DNM1	15	no
	Dynamin 2	DNM2	95	yes
	Dynamin 3	DNM3	35	no
Modulators, negative	Hairy & enhancer of split 6	HES6	95	yes
	ITCHY homolog	ITCH	100	yes
	Neural precursor cell expressed, developmentally down-regulated 4	NEDD4	90	yes
	NUMB homolog	NUMB	100	yes
	Adaptor-related protein complex 2, a2 subunit [or α2]	AP2A2	100	yes
	F-box & WD-40 protein 7	FBXW7	100	yes
	SPEN homolog	SPEN	100	yes
Modulators, mixed/unknown	Radical fringe	RFNG	90	yes
	Lunatic fringe	LFNG	60	yes
	Manic fringe	MFNG	75	yes
	Deltex homolog 1	DTX1	0	no
	Deltex homolog 2	DTX2	100	yes
	Furin	FURIN	75	yes

* Literature searches and Ingenuity Pathways software were used to identify components of the Notch signaling pathway, which were assigned to functional groups. Microarray data on small airway epithelial samples from the HG-U133 Plus 2.0 (n = 20 nonsmokers) were examined for the selected genes.

† Expressed as P call percentage (%), which is defined as the percentage of normal nonsmoker samples for which the Affymetrix algorithm determined that the gene was expressed or "Present" (P).

‡ Genes were categorized as expressed in human airway epithelium if the P call percentage was at least 50% in normal nonsmokers; for genes with more than one probe set, the percentage of P call listed is the highest value among the probe sets.

highly expressed Notch ligand in the normal adult nonsmoker human small airway epithelium, with average expression approximately 1.6-fold greater than JAG1, and 3.0-fold greater than JAG2 (both $P < 0.002$).

Expression of all 4 Notch receptors was also verified by TaqMan. The most highly expressed receptor was NOTCH2, which was expressed 1.3-fold more highly than NOTCH3 ($P < 0.05$) and approximately 3.3-fold more highly than NOTCH1

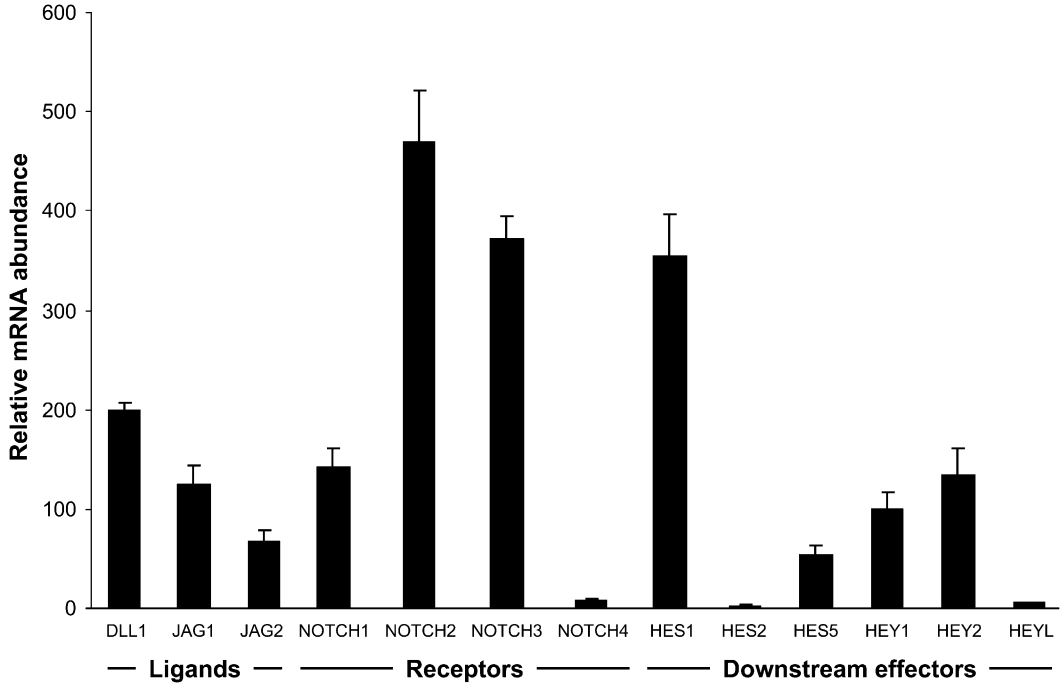

Figure 2. Relative expression of Notch ligands, receptors, and downstream effectors in the small airway epithelium of normal nonsmokers (n = 12). TaqMan quantitative real-time reverse transcriptase polymerase chain reaction was used to generate relative expression data for Notch-related ligands, receptors, and downstream effector genes. Each *bar* represents the mean expression level ± standard error for the gene in nonsmokers small airway epithelium.

($P < 0.001$) in the small airway epithelium. NOTCH4 mRNA, although detected, was present at levels approximately 50-fold less than NOTCH2 ($P < 0.001$).

TaqMan assessment also confirmed expression of six downstream genes: HES1, 2 and 5, and HEY1, 2 and L. HES1 was the downstream gene most highly expressed in the small airway samples, 6.6-fold more highly expressed than HES5, 3.6-fold more highly expressed than HEY1, and 2.6-fold more highly expressed than HEY2 ($P < 0.001$ for all comparisons). HES2 mRNA and HEYL mRNA were detected at very low levels, approximately 130-fold and 60-fold less than HES1, respectively (both $P < 0.001$).

Immunohistochemical Analysis of the Notch Pathway

To confirm expression of Notch pathway proteins in airway epithelium, we obtained large airway endobronchial biopsies from nonsmokers, normal smokers, and individuals with COPD and performed immunohistochemistry for Jag1, Notch2, Hes1 and Hes5. Staining for the Jag1 ligand was observed and was restricted to basal cells (Figure 3A and 3B). Conversely, staining for Notch2 appeared to be localized to the cytoplasm of differentiated cells present at the apical surface of the epithelium (Figure 3C and 3D). Hes1 and Hes5 were seen in both basal and differentiated cells (Figures 3E–3H).

To gain greater insight into exactly which epithelial cell types exhibit Notch signaling, small airway epithelial cells were obtained by bronchoscopic brushing in nonsmokers, healthy smokers and COPD smokers and immunohistochemistry was performed for the key downstream effector proteins, Hes1 and Hes5. Nuclear staining for Hes1 was observed in ciliated, secretory, basal and undifferentiated cells in all 3 phenotypic groups (Figure 4), as expected for a transcription factor. Similarly, nuclear staining for Hes5 was observed in all four epithelial cell populations (Figure 5).

In regard to variability, no significant difference was observed in the variability of numbers of cells staining positive for Hes1 and Hes5 among the three phenotypic groups. For Hes1, nonsmokers demonstrated a standard deviation of 16%, normal smokers 18%, and smokers with COPD 27%. For Hes5, nonsmokers demonstrated a standard deviation of 21%, normal smokers 15% and smokers with COPD 18%.

Modulation of the Notch Pathway by Smoking and COPD

Modulation of the Notch pathway in the small airway epithelium of normal smokers (n = 15) and smokers with COPD (n = 13), compared with nonsmokers (n = 12), was then assessed using TaqMan analysis for selected central genes in the pathway. With regard to ligands, this analysis demonstrated a decreased expression of DLL1 in smokers with COPD compared with normal smokers and normal nonsmokers ($P < 0.03$ and $P < 0.02$, respectively), although expression of DLL1 was similar among nonsmokers and normal smokers ($P > 0.7$) (Figure 6). JAG1 expression was similar in smokers with COPD compared with both normal smokers and normal nonsmokers ($P > 0.6$). Expression was next evaluated for the most highly expressed receptors, NOTCH2 and NOTCH3. No differences were seen in expression of NOTCH2 among the three groups ($P > 0.2$). In contrast, the expression of NOTCH3 was down-regulated in smokers with COPD and normal smokers compared with normal nonsmokers ($P < 0.02$ and $P < 0.002$, respectively), with no significant difference between smokers with COPD and normal smokers ($P > 0.3$) (Figure 7). Finally, for the downstream effectors, complex changes were also observed in association with smoking. No changes were noted for HES1, but TaqMan analysis revealed that for HES5 and HEY1, expression in smokers with COPD and normal smokers was down-regulated compared with normal nonsmokers (all significant to $P < 0.03$, except HEY1 for normal nonsmokers compared with normal smokers $P > 0.06$) (Figure 8). HEY2 was also down-regulated in smokers with COPD compared with nonsmokers ($P < 0.01$). Further down-regulation was observed for smokers with COPD compared with normal smokers for HEY2 ($P < 0.04$) and HES5 ($P < 0.002$), with a trend toward down-regulation of HEY1 (1.7-fold; $P > 0.2$).

To exclude a significant effect of medication use in three subjects from the COPD group, we compared mean expression values for each gene in the "COPD/medication" group compared with the mean expression in the "COPD/no medication" group using a *t* test. There were no significant differences (not shown).

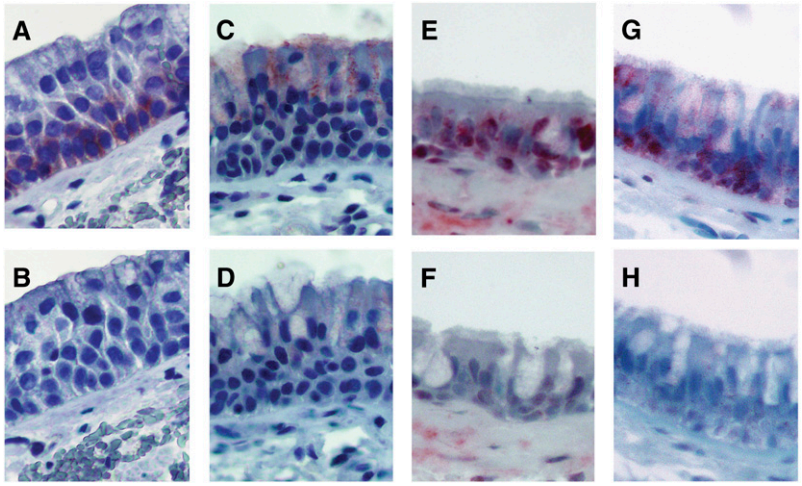

Figure 3. Immunostaining for key Notch proteins in airway epithelial biopsies. (*A*) Chronic obstructive pulmonary disease (COPD) smoker, goat anti-human Jag1 antibody. (*B*) COPD smoker, goat anti-human Jag1 antibody plus Jag1 peptide. (*C*) COPD smoker, rabbit anti-human Notch2 antibody. (*D*) COPD smoker, rabbit anti-human Notch2 antibody plus Notch2 peptide. (*E*) Normal nonsmoker, rabbit anti-human Hes1 antibody. (*F*) Normal nonsmoker, rabbit anti-human Hes1 antibody plus Hes1 peptide. (*G*) Normal smoker, rabbit anti-human Hes5 antibody. (*H*) Normal smoker, rabbit IgG control.

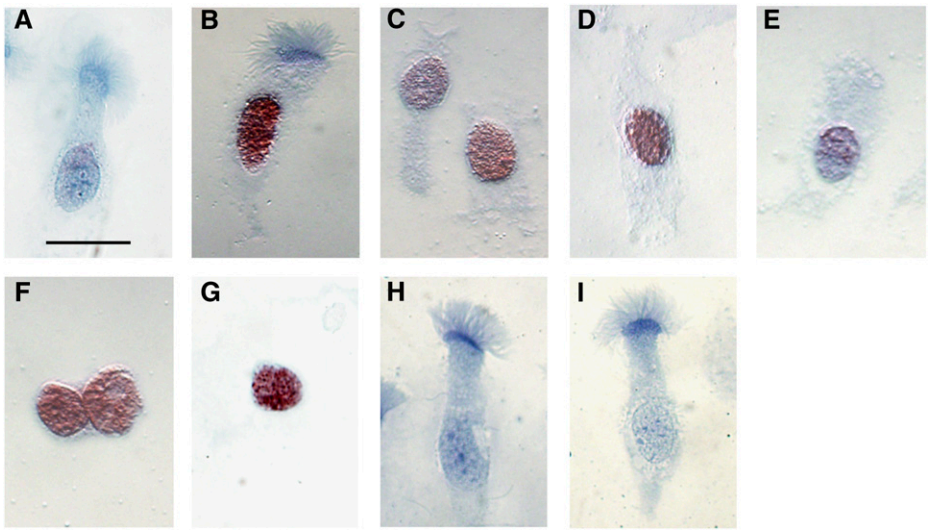

Figure 4. Immunohistochemistry assessment of Hes1 protein expression in brushed small airway epithelial cells. (*A*) Ciliated cell, nonsmoker, rabbit anti-Hes1 antibody. (*B*) Ciliated cell, nonsmoker, rabbit anti-Hes1 antibody. Note the variability in Hes1 abundance in 2 cells from the same individual. (*C*) Undifferentiated cell (*left*) and secretory cell (*right*), nonsmoker, rabbit anti-Hes1 antibody. (*D*) Secretory cell, nonsmoker, rabbit anti-Hes1 antibody. (*E*) Secretory cell, nonsmoker, rabbit anti-Hes1 antibody. (*F*) Basal cells, nonsmoker, rabbit anti-Hes1 antibody. (*G*) Basal cell, nonsmoker, rabbit anti-Hes1 antibody. (*H*) Ciliated cell, nonsmoker, rabbit anti-Hes1 antibody plus Hes1 peptide control. (*I*) Ciliated cell, nonsmoker, rabbit anti-Hes1 antibody plus Hes1 peptide control. *Bar*, 20 μm.

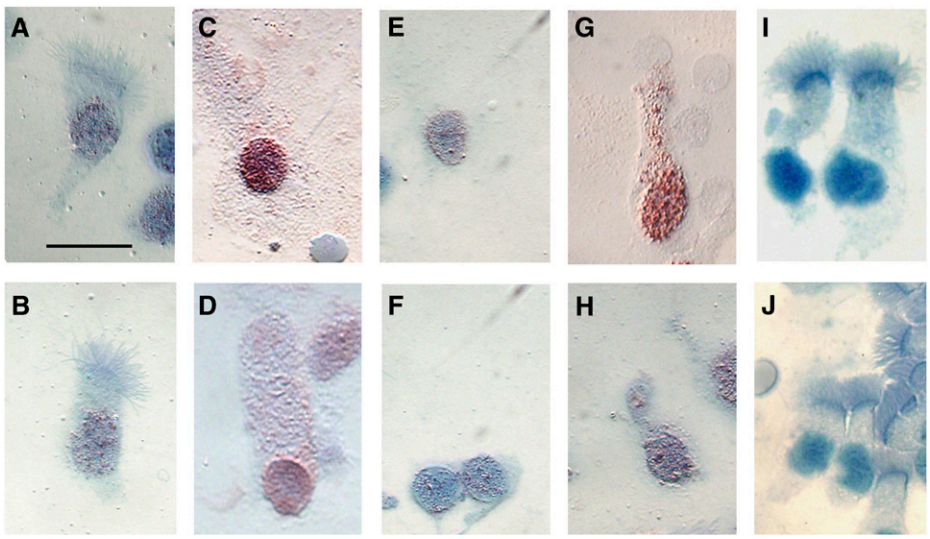

Figure 5. Immunohistochemistry assessment of Hes5 protein expression in brushed small airway epithelial cells. (*A*) Ciliated cell, nonsmoker, rabbit anti-Hes5 antibody. (*B*) Ciliated cell, nonsmoker, rabbit anti-Hes5 antibody. (*C*) Secretory cell, COPD smoker, rabbit anti-Hes5 antibody. (*D*) Secretory cell, COPD smoker, rabbit anti-Hes5 antibody. (*E*) Basal cell, nonsmoker, rabbit anti-Hes5 antibody. (*F*) Basal cells, nonsmoker, rabbit anti-Hes5 antibody. (*G*) Undifferentiated cell, COPD smoker, rabbit anti-Hes5 antibody. (*H*) Undifferentiated cell, nonsmoker, rabbit anti-Hes5 antibody. (*I*) Ciliated cells, nonsmoker rabbit IgG control. (*J*) Ciliated cells, nonsmoker rabbit IgG control. *Bar*, 20 μm.

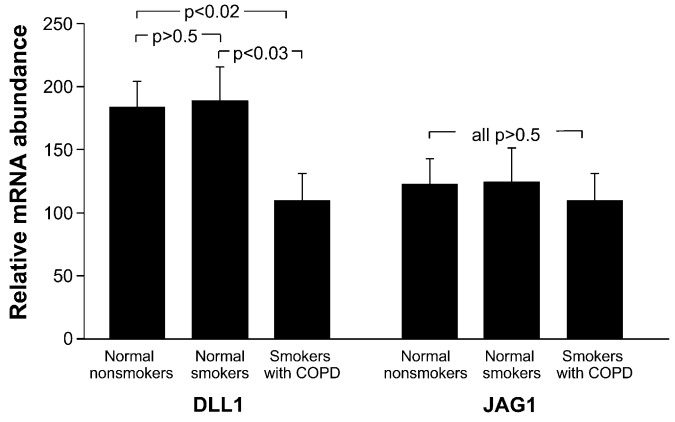

Figure 6. Comparison of the relative expression of selected ligands of the Notch pathway in nonsmokers (n = 12), normal smokers (n = 15), and smokers with chronic obstructive pulmonary disease (COPD) (n = 13). TaqMan polymerase chain reaction was used to generate the data. Each *bar* represents mean expression with standard error; *P* values are represented in *brackets* above the *bars*.

DISCUSSION

Smoking is the major risk factor for the development of COPD, which is estimated to become the third leading cause of death in the world by the year 2020 (27). By virtue of its anatomy, the airway epithelium takes the brunt of the burden of the toxic components of cigarette smoke, particularly the small airway epithelium, the site of the initial development of COPD (28–30). The morphologic changes in the small airway epithelium as a result of chronic smoking include basal cell hyperplasia, ciliated cells with absent cilia, and secretory cell hyperplasia with increased goblet cell hyperplasia (31–33). As COPD develops, the airway epithelial changes include increased numbers of basal and secretory cells, decreased numbers of ciliated cells, partial shedding of the epithelium, and squamous cell metaplasia (34–36). The airway epithelium of smokers and individuals with COPD expresses increased amounts of proliferating cell nuclear antigen (35, 36), and there is an increased rate of both cell division and apoptosis (35–38).

In the normal lung, in nonsmokers, there is ongoing epithelial cell regeneration in response to injury or to cell death (1–5). Basal cells, thought to include the stem-cell population of the airway epithelium, have the capacity to proliferate, dedifferentiate into undifferentiated cells, and then redifferentiate into the specialized cells of the normal airway epithelium (4, 39). In the setting of chronic cigarette smoke exposure, this redifferentiation/regeneration program does not lead to a normal epithelium but to the morphologically abnormal epithelium observed in smokers.

To begin to understand the molecular mechanisms responsible for this abnormal differentiation of the airways in association with smoking, we hypothesized that signaling pathways known to control epithelial differentiation likely play a role. As an initial strategy to assess this hypothesis we focused on the Notch pathway, which functions as a "gatekeeper" against epithelial differentiation (7, 15, 40), and looked for evidence that this pathway, of known importance in lung development during embryogenesis (7, 13, 15, 16), also plays a role in the adult lung. We hypothesized that key genes involved in the Notch pathway would be expressed in the adult human airway epithelium and that the expression of these genes might be altered in association with smoking, both in normal smokers and smokers with COPD. The data demonstrate that genes representing all the key functional categories in the Notch signaling pathway are expressed in the small airways of normal nonsmokers. Using TaqMan to obtain relative quantification data, we observed that DLL1 is the most highly expressed Notch ligand, NOTCH2 the most highly expressed receptor, and HES1 the most highly expressed downstream gene. At the protein level, expression was demonstrated for the ligand Jag1, the receptor Notch2, and the downstream effectors Hes1 and Hes5 in large airway biopsies, and Hes1 and Hes5 in all 4 major epithelial cell types in nonsmokers, healthy smokers and COPD smokers in brushed small airway cells.

Assessment for smoking-induced changes was done by performing TaqMan PCR for selected genes in nonsmokers, healthy smokers, and smokers with COPD. The data demonstrate down-regulation of key genes in the Notch pathway in healthy smokers and smokers with COPD, compared with nonsmokers. NOTCH3 and HES5 were down-regulated in healthy smokers and smokers with COPD, compared with nonsmokers. DLL1, HEY1, and HEY2 were also down-regulated in smokers with COPD compared with nonsmokers, and DLL1, HES5, and HEY2 were down-regulated in smokers with COPD compared with healthy smokers, with HEY1 showing a similar trend toward down-regulation.

Role of Notch in Experimental Animals

A number of animal studies have demonstrated the importance of Notch signaling in embryogenesis and specifically in epithelial

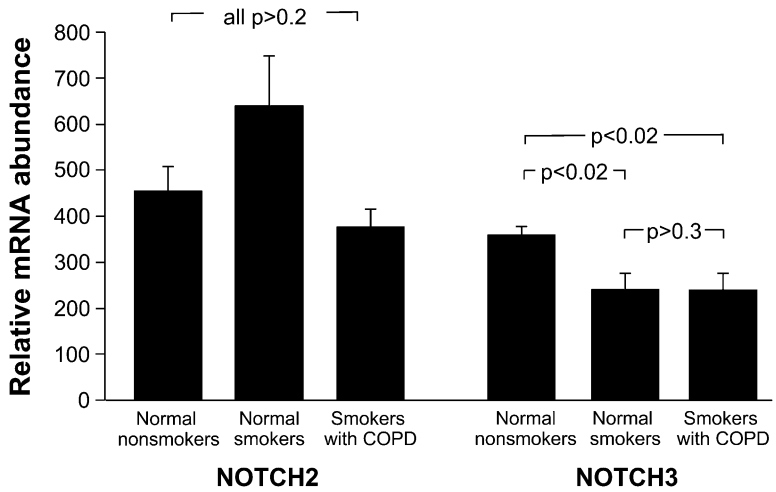

Figure 7. Comparison of the relative expression of selected receptors of the Notch pathway in nonsmokers (n = 12), normal smokers (n = 15), and smokers with COPD (n = 13). TaqMan PCR was used to generate the data. Each *bar* represents mean expression with standard error; *P* values are represented in *brackets* above the *bars*.

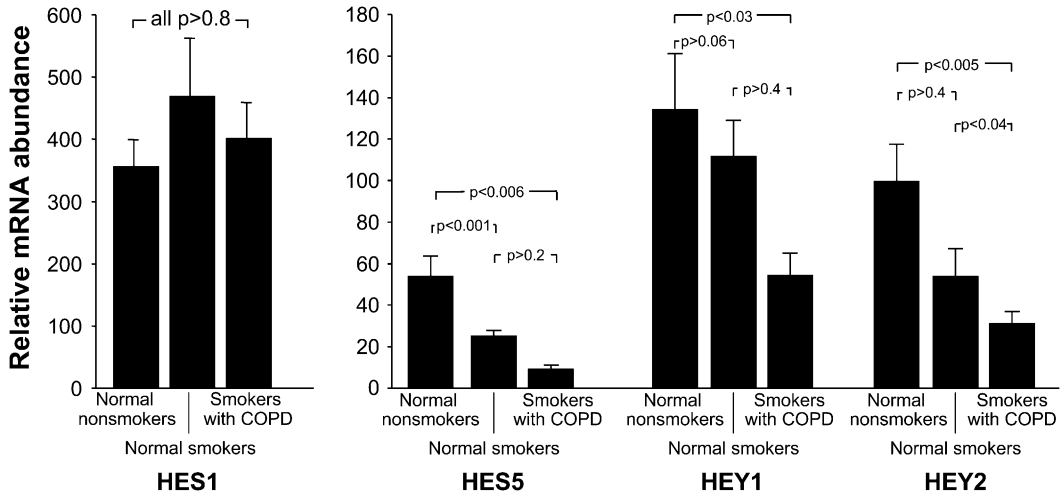

Figure 8. Comparison of the relative expression of selected downstream effectors of the Notch pathway in nonsmokers (n = 12), normal smokers (n = 15), and smokers with chronic obstructive pulmonary disease (COPD) (n = 13). TaqMan polymerase chain reaction was used to generate the data. Each *bar* represents mean expression with standard error; *P* values are represented in *brackets* above the *bars*. Note that the scale is different for Hes1 compared with Hes5, Hey1, and Hey2, reflecting the higher relative level of expression of Hes1 compared with the other downstream effectors.

differentiation in the developing lung. Knockout mice for the components of the Notch pathway have various phenotypes from early embryonic or perinatal lethal (for example Dll1 and 3, Notch1) to a lesser effect in embryogenesis (e.g., Notch3 and 4, Hey1) (7, 41). Jagged2 knockouts have a perinatal lethal phenotype with abnormal breathing (7). Hes1 knockouts have lung hypoplasia with increased neuroendocrine cells and reduced levels of Clara cells (42). Constitutive expression of Notch3 in the peripheral epithelium in the developing lung resulted in altered lung morphology and delayed development, leaving the majority of the epithelial cells undifferentiated (43).

Role of Notch in the Adult Human Airway Epithelium

A recent study by Ross and colleagues (6) examined gene expression in human airway epithelial cells as they were allowed to differentiate in culture at air-liquid interface. We examined levels of Notch gene expression in their data set. Interestingly, the key Notch downstream genes HES5, HEY1, and HEY2, which we found to be down-regulated in association with smoking and COPD, were expressed at low levels in the undifferentiated cells at the early time points in this study. Levels of these genes peaked at the late time points, when the cells were observed to be differentiated. This is consistent with the concept that Notch signaling activity is suppressed when cells are differentiating, and active when cells no longer need to differentiate.

To our knowledge, ours is the first study to assess the role of Notch signaling in the human airway epithelium of normal adults (7, 44). Using microarray technology, we demonstrated that the majority of known Notch-related genes are expressed in human small airway epithelium in normal adult nonsmokers. The expressed genes include representatives of every functional category within the Notch pathway, including ligands, receptors, proteases, the CBF1 transcriptional complex, downstream bHLH genes, and modulators of the pathway with positive, negative, and mixed or unknown effects.

We have used TaqMan quantitative real-time RT-PCR to quantify relative expression of genes in the key functional categories of ligand, receptor, and downstream effector. The data suggest that in the airway epithelium of normal adult humanss, Notch signaling may be predominantly mediated by the interaction of the DLL1 ligand with the NOTCH2 receptor, leading to transcription of HES1, which were the most abundant gene transcripts in these categories. Analysis by immunohistochemistry confirmed expression of Jag1, Notch2, Hes1, and Hes5 in the large airway epithelium in all three study groups and localized expression of Jag1 to basal cells and Notch2 to differentiated cells. Immunohistochemistry analysis for Hes1 and Hes5 in brushed small airway epithelial cells confirmed expression in the nuclei of all cell types in the differentiated airway epithelium in all three phenotypic groups. We used TaqMan PCR to assess for changes in expression of Notch ligands, receptors, and downstream effectors in healthy smokers and smokers with COPD compared with normal nonsmokers. The data demonstrate that the receptor NOTCH3 and the downstream gene HES5 are down-regulated in both normal smokers and smokers with COPD. Additionally, smokers with COPD were shown to down-regulate the ligand DLL1, as well as the downstream genes HEY1 and HEY2. Significant differences between normal smokers and smokers with COPD were decreased expression of DLL1, HES5 and HEY2 in the COPD group, with HEY1 showing a similar trend.

Issues for Future Studies

We have used freshly obtained, primary human airway epithelial cells to obtain data on the *in vivo* status of the Notch pathway in the adult human airway. Working with human samples poses many unique challenges and as such there are limitations to our study. First, it was not possible to perfectly match the groups of volunteers, and the COPD group was older than the other two groups. Second, the individuals with COPD may represent a somewhat heterogeneous group, as they included individuals ranging from the former classification of GOLD stage 0, to GOLD stage III. It is not possible to analyze what effect, if any, this may have had on gene expression, given the "n" of the study.

Working with primary human epithelial cells limits the ability to perform functional assays of the Notch pathway, thus a caveat of the study is that we cannot be certain that decreased expression of Notch pathway genes translates into decreased Notch signaling. There are a number of possible reasons for the observed changes in gene expression. In the setting of cigarette smoke–induced epithelial damage, progenitor cells with different patterns of gene expression may be recruited. The airways of smokers with and without COPD are known to have a different epithelial differentiation status, and this may also explain some of the observed changes. Confirming the observed changes at the protein level is limited by the relative insensitivity of immunohistochemistry to detect small changes in abundance and the low expression levels of the genes.

The observed changes in gene expression would be consistent with decreased Notch signaling in smokers, with a greater level of down-regulation of the pathway in those with evidence of COPD. Based on the knowledge that Notch signaling acts to maintain "stemness" and prevent differentiation of epithelial cells, we can hypothesize that, in the setting of the ongoing epithelial injury of cigarette smoking, Notch signaling is down-regulated to permit differentiation and repair of the airway epithelium. The present study suggests that the Notch pathway, previously thought to be important in the lung only during embryogenesis or in the context of cancer, may play a role in differentiation in the adult human airway epithelium.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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