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Differential global gene expression in cystic fibrosis nasal and bronchial epithelium

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ABSTRACT

Respiratory epithelium is the target of therapies, such as gene therapy, for cystic fibrosis (CF) lung disease. To determine the usefulness of the nasal epithelium as a pre-screen for lung-directed therapies, we profiled gene expression in CF and non-CF nasal and bronchial epithelium samples using Illumina HumanRef-8 Expression BeadChips. 863 genes were differentially expressed between CF and non-CF bronchial epithelium but only 15 were differentially expressed between CF and non-CF nasal epithelium ($\geq$1.5-fold, $P \leq 0.05$). The most enriched pathway in CF bronchial epithelium was inflammatory response, whereas in CF nasal epithelium it was amino acid metabolism. We also compared nasal and bronchial epithelium in each group and identified differential expression of cellular movement genes in CF patients and cellular growth genes in non-CF subjects. We conclude that CF and non-CF nasal and bronchial epithelium are transcriptionally distinct and CF nasal epithelium is not a good surrogate for the lung respiratory epithelium.

1. Introduction

Mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene cause abnormal fluid and ion transport across the apical membrane of epithelial cells and give rise to lung disease, the leading cause of morbidity in cystic fibrosis (CF) [1–3]. Nasal respiratory epithelium displays the CF ion transport abnormality but little, if any, CF-related pathology [4]. Due to the accessibility of nasal epithelial tissue much effort has been focussed on using it as a pre-screen for therapies, such as gene therapy, that target the lungs [5]. Consequently, it is of relevance to determine in more detail and more globally differences between CF and non-CF nasal and bronchial epithelium that might support or contradict the utility of the nasal epithelium as surrogate for the bronchial epithelium in the assessment of new therapies.

Microarrays enable the analysis of global patterns of gene expression and are being used to uncover the genomic response to diseases, such as CF [6]. Microarray analysis of lung tissue from CF knock-out and control mice revealed differential expression of genes involved in transcription, inflammation, signal transduction, ion transport and protein trafficking [7]. Two studies profiled gene expression in human airway epithelium. One compared primary cultures of airway epithelial cells from CF and non-CF donor lungs and identified a limited number of differentially expressed genes [8]. A second study compared nasal epithelium from CF patients with epithelium from healthy subjects and reported differences in the expression of genes involved in a wide range of cellular processes, including protein and lipid turnover, mitochondrial function and airway defence [9].

Comparison of paired human CF and non-CF native nasal and bronchial epithelium samples may help to determine whether nasal epithelium is a suitable surrogate for bronchial epithelium in gene directed intervention studies. To this end, we undertook an analysis of global patterns of gene expression in paired freshly brushed nasal and bronchial epithelium comparing CF patients with healthy controls. Differentially expressed genes identified may improve our understanding of the *in vivo* transcriptional consequences of CFTR mutations in the respiratory epithelium of the nose and lungs.

2. Results

2.1. Sample cellularity and CFTR expression

A total of 60 airway samples (CF and non-CF nasal and bronchial epithelium) were used for microarray analysis (Supplemental Table S1). A proportion of these were suitable for cytological analysis and

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immunocytochemistry using CFTR antibodies to assess cell content. Epithelial cell content ranged from a mean of 45% to 79% across the four sample types with a 5-fold higher percentage of neutrophils in CF bronchial epithelium samples being the most notable difference (Table 1). Epithelial cells comprised ciliated or non-ciliated tall columnar cells but also included goblet cells and basal cells (Supplemental Fig. S1). Few CF bronchial epithelium samples were of sufficient quality for immunocytochemistry or cytological analysis and even suitable samples were difficult to decipher due to the presence of debris, mucus and inflammatory cells (Supplemental Table S2A). The percentage of epithelial cells and tall columnar epithelial cells expressing CFTR at the apical surface was 3-fold higher in non-CF subjects than in CF subjects (Supplemental Table S2B).

2.2. Global gene expression analysis reveals many differentially expressed genes in CF bronchial epithelium but few in CF nasal epithelium

We profiled gene expression in 8 CF bronchial epithelium samples, 16 non-CF bronchial epithelium samples, 20 CF nasal epithelium samples and 16 non-CF nasal epithelium samples (Supplemental Table S1). Using a cut-off of $\geq$1.5 fold-change and $P \leq 0.05$ we found that 863 genes were differentially expressed between CF and non-CF bronchial epithelium but only 15 genes were differentially expressed between CF and non-CF nasal epithelium (Figs. 1A and B). This represents a 58-fold difference in the proportion of differentially expressed genes between CF nasal and bronchial epithelium. None of the differentially expressed genes in CF nasal epithelium reached a 2-fold cut-off whereas 227 genes were differentially expressed by $\geq$ 2-fold in CF bronchial epithelium ($P \leq 0.01$).

2.3. Differential gene expression in CF nasal and bronchial epithelium

Since no statistically significantly differences were found between CF and non-CF nasal epithelium samples using a stringent cut-off (2-fold-change, $P \leq 0.01$) we focussed on the genes that passed the lower cut-off (1.5-fold, $P \leq 0.05$) in both CF nasal and bronchial epithelium samples. Differentially expressed genes encoded proteins with roles in a wide range of cellular processes. Of the 863 differentially expressed genes in CF bronchial epithelium, 438 were up-regulated and 425 were down-regulated (Supplemental Table S3). The most highly up-regulated of these included the putative lymphocyte G0/G1 switch gene (G0S2), plasminogen activator, urokinase (PLAU), chemokine receptor 4 (CXCR4), prokineticin 2 (PROK2) and interleukin 8 (IL8) (Table 2A and Supplemental Table S4). G0S2 encodes a mitochondrial protein and pro-apoptotic factor that is also an all-*trans*-retinoic acid target [10]. Plasminogen activator, urokinase (PLAU) has a critical role in regulating the activity of TGFB1, involved in tissue repair and fibrosis [11]. CXCR4 is a growth factor receptor that in response to infection interacts with toll-like receptor 2 (TLR2) in lipid rafts on the apical surface of epithelial cells [12]. PROK2 encodes a secreted protein that is involved in neurogenesis [13]. IL8 is a potent pro-inflammatory cytokine that is also a hallmark of CF lung inflammation [14]. The expression of IL8 is regulated by the transcription factor, NFKB1, which has a crucial role in regulating innate immune defences. NFKB1 and several other downstream targets were up-regulated in CF bronchial epithelium, including TNF, IL1B, PLAU, PLAUR and the IL2 receptor. Upstream mediators of NFKB1 activation, such as TLR2, RAC1, phosphatidylinisotol-3 kinase (PIK3CA) and protein kinase B (PTK2B), were also up-regulated. Genes encoding hypothetical proteins with homology to 3 Rho GTPases and a Ras protein activator, as well as 3 transmembrane proteins, were also up-regulated in CF (Supplemental Table S5). In addition, RNAs encoding multiple members of the same protein family, such as those encoding proteins involved in inflammation, cell-to-cell signalling and cellular trafficking, were more abundant in CF bronchial epithelium (Supplemental Table S6).

Of the most highly down-regulated genes in CF bronchial epithelium two encoded secretoglobin, secretoglobin family 3A member 1 (SCGB3A1) and secretoglobin family 1A member 1 (SCGB1A1) (Table 2B and Supplemental Table S4). These genes encode secreted anti-inflammatory cytokines, one of which (SCGB1A1) is known to be expressed at reduced levels in the induced sputum of CF patients [15]. The other (SCGB3A1) is highly expressed in the airways and has been shown to be up-regulated during retinoic acid-induced differentiation of bronchial epithelial cells [16]. Glutathione S-transferase A2 (GSTA2) is one of four down-regulated glutathione *S*-transferase enzymes which, along with a further four down-regulated genes encoding cytochrome P450s (CYPs), perform essential roles in drug metabolism and detoxification (Supplemental Table S6). The calcium-activated chloride channel, CLCA2, is expressed mainly in basal cells of stratified epithelia and has the dual function of mediating chloride conductance and cell-cell adhesion, and has recently been shown to be a stress-inducible gene and candidate tumour suppressor [17,18]. Also down-regulated in CF were RNAs encoding hypothetical proteins with homology to 11 zinc finger proteins, two dynein proteins and a sodium/potassium ion transporter (Supplemental Table S5). A further 11 transcripts encoding zinc finger proteins were less abundant in CF bronchial epithelium (Supplemental Table S6).

Genes differentially expressed in CF nasal epithelium included a sodium-coupled borate co-transporter (SLC4A11); tubulin alpha 6 (TUBA1C), a major component of microtubules ; claudin 7 (CLDN7), a tight junction protein that has a role in paracellular ion transport [19]; and beta defensin 1 (DEFB1), an antimicrobial peptide that has an important role in immune defence and inflammation [20] (Table 3).

2.4. Differentially expressed genes common to both CF epithelial tissues

Comparing the differentially expressed genes in CF nasal (n = 15 genes) and bronchial epithelium (n = 863 genes) we identified five genes in common (Table 3). These were transgelin 2 (TAGLN2), aldolase A fructose bisphophate transcript variant 2 (ALDOA), cathepsin D (CTSD), NTS and integral membrane protein 2A (ITM2A). TAGLN2 is known to be expressed in neoplastic lung epithelial cells [21]. ALDOA is a glycolytic enzyme bound to the cytoskeleton which has a role in glucose metabolism and in inducing lamellipodia formation during keratinocyte migration [22]. NTS is a neuropeptide which has important physiological roles such as in increasing pain thresholds and in regulating blood pressure and gut function [23]. It has also been shown to mediate the production of IL8 upon stimulation by curcumin [24]. CTSD functions as a protease and

Table 1
Cellular content.

	% epithelial[a] (range)	% macrophage (range)	% neutrophil (range)	% denuded nuclei (range)
CF BBr (n = 3)	45 (38–52)	2 (1–6)	33 (27–44)	20 (10–35)
non-CF BBr (n = 15)	74 (48–85)	2 (0–8)	6 (0–27)	18 (11–26)
CF NBr (n = 13)	68 (26–84)	4 (0–10)	4 (0–40)	24 (10–49)
non-CF NBr (n = 15)	79 (50–89)	1 (0–4)	2 (0–11)	18 (4–39)

Definition of abbreviations: BBr = bronchial brushings; NBr = nasal brushings; % = percentage of total number of nucleated cells counted.
[a] Epithelial = ciliated or non-ciliated tall columnar epithelial cells, goblet cells or basal cells.

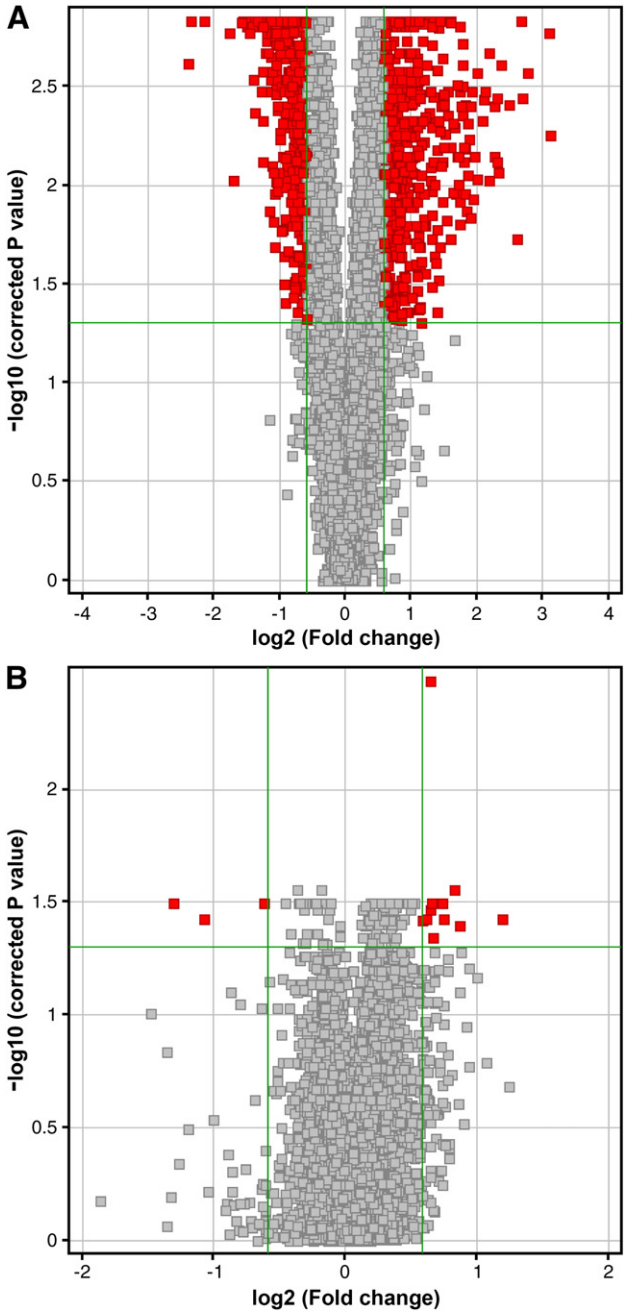


Fig. 1. Global gene expression profiles of CF and non-CF nasal and bronchial epithelium. RNA from eight CF bronchial epithelial samples, 16 non-CF bronchial epithelial samples, 20 CF nasal epithelial samples and 16 non-CF nasal epithelial samples were hybridised to Illumina HumanRef-8 microarrays displaying probes for 24, 354 genes. Microarray data were log-transformed and normalised using quantile. After filtering to remove unreliable genes, 11,080 genes were statistically analysed to identify genes with significant differential expression. Each square represents a different gene. Volcano plots of (A) CF versus non-CF bronchial epithelium and (B) CF versus non-CF nasal epithelium (fold-change ≥ 1.5 and $P<0.05$). Genes are distributed according to statistical significance (y-axis) and magnitude of change (log ratio CF:non-CF) (x-axis). Red squares represent genes that pass the statistical and fold-change cut-offs (P values were calculated using Mann–Whitney U test unpaired and were corrected for multiple testing using Benjamini Hochberg False Discovery Rate).

plays an active role in cytokine-induced programmed cell death [25]. ITM2A is an integral membrane protein found in large cytoplasmic vehicles and the Golgi apparatus, as well as on the cell surface [26]. These genes were not differentially expressed between CF or non-CF nasal and bronchial epithelium.

2.5. Differential pathways activated in CF versus non-CF nasal and bronchial epithelium

To identify pathways enriched in the CF tissues we used Ingenuity Pathways Analysis software to group genes by molecular and cellular function. Inflammatory response was the most significant pathway in CF bronchial epithelium samples, followed by cellular movement and cell-to-cell signalling and interaction (Table 4). In contrast, amino acid metabolism, cellular morphology and cellular compromise were the most significant in CF nasal epithelium samples, but these were less significant (Table 4). Together, these pathways indicate that CF bronchial epithelium has an activated inflammatory response and an increased capacity or need for sensing and mediating cellular movement compared with non-CF bronchial tissue, whereas CF nasal epithelium has higher metabolic requirements than non-CF nasal tissue.

2.6. Differential gene expression in the nasal and bronchial epithelium of CF patients and healthy subjects

We also compared the gene expression profiles of nasal and bronchial epithelium in each group to identify tissue differences. We found that the proportion of genes differentially expressed between paired CF nasal and bronchial epithelium was similar to that of paired non-CF nasal and bronchial epithelium with 306 and 308 genes identified, respectively (Supplemental Tables S7A and S7B). 17% (89 out of 525) of genes differentially expressed between the nasal and bronchial epithelium of CF patients or healthy subjects overlapped. Pathway analysis showed that these 89 genes are involved in small molecular biochemistry, molecular transport and drug and lipid metabolism. Of the 306 genes differentially expressed between CF nasal and bronchial epithelium, 174 were up-regulated and 132 were down-regulated in bronchial epithelial tissue. Between non-CF nasal and bronchial epithelium 237 out of 308 differentially expressed genes were up-regulated and 71 were down-regulated in bronchial epithelial tissue. To determine the maximum, feasible amount of overlap in differentially expressed genes between nasal and bronchial epithelium in CF patients and healthy controls we used a fold-change based gene selection method [27] and identified a 30% overlap (data not shown). In CF nasal versus bronchial tissue, the inflammatory response pathway was not enriched but pathways involved in cellular movement, cell-to-cell signalling and interaction and cell signalling were enriched (Table 5). In contrast, in non-CF nasal versus bronchial tissue, cellular growth and proliferation, cell cycle and DNA replication pathways were most enriched, but these were less significant (Table 5).

2.7. QPCR for selected genes confirms microarray data

We selected 10 genes differentially expressed in at least one of the four comparisons to analyse by QPCR; these were CYP26A1, DUSP5, FOS, IFITM3, LTF, MMP10, NTS, SCGB1A1, TSC22D3 and TUBGCP4. Two genes (LTF and MMP10) were selected because they were differentially expressed between nasal and bronchial epithelium in both CF patients and healthy subjects. Five were differentially expressed between CF and non-CF bronchial epithelium samples, including SCGB1A1, one of the top 5 differentially expressed genes in CF bronchial epithelium. FOS was selected because it was differentially expressed in three out of the four comparisons. NTS was one of the five genes that overlapped between CF and non-CF nasal and bronchial epithelium samples. CYP26A1 was selected because it was differentially expressed between the nasal and bronchial epithelium of CF patients only. We confirmed differences in the appropriate tissues for 9 out of 10 genes, the exception was TUBGCP4. Statistically significant changes were confirmed for 7 out of 17 comparisons made. These included SCGB1A1 (6.3 fold down-regulated in CF versus non-CF

Table 2A
Top 50 genes up-regulated in CF versus non-CF bronchial epithelium.

Gene	Symbol	Fold change	*P* value[a]	Accession no.
Putative lymphocyte G0/G1 switch gene	G0S2	8.71	0.006	NM_015714.2
Plasminogen activator, urokinase	PLAU	8.61	0.002	NM_002658.1
Prokineticin 2	PROK2	6.85	0.003	NM_021935.2
Chemokine (C-X-C motif) receptor 4	CXCR4	6.50	0.004	NM_003467.1
Interleukin 8	IL8	6.35	0.001	NM_000584.2
Chemokine (C-C motif) receptor-like 2	CCRL2	5.62	0.004	NM_003965.3
Serum amyloid A1	SAA1	5.15	0.002	NM_000331.2
Superoxide dismutase 2	SOD2	5.04	0.009	NM_000636.1
S100 calcium binding protein A12 (calgranulin C)	S100A12	4.98	0.008	NM_005621.1
Pleckstrin	PLEK	4.96	0.004	NM_002664.1
Interleukin 1	IL1B	4.84	0.004	NM_000576.2
Colony stimulating factor 3 receptor (granulocyte)	CSF3R	4.82	0.007	NM_172313.1
Tryptase beta 1	TPSAB1	4.56	0.009	NM_003294.2
Interferon	ISG15	4.55	0.002	NM_005101.1
Putative chemokine receptor	HM74	4.38	0.003	NM_006018.1
Integrin alpha polypeptide	ITGAX	4.29	0.004	NM_000887.3
S100 calcium binding protein A9 (calgranulin B)	S100A9	4.24	0.005	NM_002965.2
Tumor necrosis factor receptor superfamily	TNFRSF1B	4.03	0.009	NM_001066.2
Plasminogen activator, urokinase receptor	PLAUR	4.02	0.003	NM_002659.1
Lymphocyte cytosolic protein 1 (L-plastin)	LCP1	3.93	0.007	NM_002298.2
Normal mucosa of esophagus specific 1	C15orf48	3.80	0.003	NM_197955.1
Leukocyte immunoglobulin-like receptor member 3	LILRB3	3.75	0.004	NM_006864.1
Chemokine (C-C motif) ligand 3-like 1	CCL3L1	3.69	0.006	NM_021006.3
Interleukin 1 receptor transcript variant 2	IL1R2	3.65	0.005	NM_173343.1
BCL2-related protein A1	BCL2A1	3.63	0.009	NM_004049.2
Interferon-induced transmembrane protein 1 (9–27)	IFITM1	3.56	0.004	NM_003641.2
CD22 antigen	CD22	3.52	0.007	NM_001771.1
Leukocyte Ig-like receptor 9	LILRA5	3.51	0.008	NM_021250.2
Pre-B-cell colony enhancing factor 1	NAMPT	3.49	0.006	NM_005746.1
Triggering receptor expressed on myeloid cells 1	TREM1	3.44	0.003	NM_018643.2
Interleukin 1 receptor antagonist	IL1RN	3.44	0.002	NM_173842.1
Serum amyloid A2	SAA2	3.44	0.002	NM_030754.2
Matrix metalloproteinase 25	MMP25	3.42	0.004	NM_022468.3
Gardner–Rasheed feline sarcoma viral (v-fgr) oncogene homolog	FGR	3.39	0.004	NM_005248.1
Serum amyloid A4	SAA4	3.36	0.003	NM_006512.1
Interferon-induced transmembrane protein 3 (1-8U)	IFITM3	3.35	0.002	NM_021034.1
Neutrophil cytosolic factor 1 (47 kDa autosomal 1)	NCF1	3.30	0.007	NM_000265.1
Matrix metalloproteinase 9	MMP9	3.30	0.010	NM_004994.1
Prostaglandin-endoperoxide synthase 2	PTGS2	3.26	0.003	NM_000963.1
Hypothetical protein	GLT1D1	3.23	0.007	NM_144669.1
Protease inhibitor 3	PI3	3.18	0.005	NM_002638.2
Hypothetical protein	LPCAT1	3.12	0.005	NM_024830.3
AXIN1 up-regulated 1	CSRNP1	3.05	0.001	NM_033027.2
Regulator of G-protein signalling 2	RGS2	3.05	0.006	NM_002923.1
Peptidylprolyl isomerase F	PPIF	3.05	0.001	NM_005729.3
CD83 antigen	CD83	2.96	0.004	NM_004233.2
Cytidine deaminase	CDA	2.95	0.006	NM_001785.1
Tumor necrosis factor (ligand) superfamily	TNFSF13B	2.93	0.003	NM_006573.3
Interferon-induced protein with tetratricopeptide repeats 4	IFIT3	2.88	0.005	NM_001549.2
Pleckstrin homology binding protein	CYTIP	2.84	0.004	NM_004288.2

Definition of abbreviations: Fold-change, mean ratio (CF:non-CF); *P*-value ≤ 0.01 (corrected for multiple testing using Benjamini Hochberg False Discovery Rate).
[a] The full list of genes most highly up-regulated in CF versus non-CF bronchial epithelium can be found in Supplemental Table S4 (≥2-fold, $P \leq 0.01$).

bronchial epithelium, $P = 0.0007$; 4.4-fold up-regulated in non-CF bronchial versus nasal epithelium, $P = 0.0004$); CYP26A1 (3.6 fold down-regulated in CF bronchial versus nasal epithelium, $P = 0.0008$); and NTS (2.1-fold down-regulated in CF versus non-CF bronchial epithelium but was of borderline statistical significance, $P = 0.056$ and 2.7 fold down-regulated in CF versus non-CF nasal epithelium, $P = 0.0073$) (Fig. 2).

2.8. Microarrays can distinguish CF patients and healthy subjects by their bronchial epithelial tissue but not by their nasal epithelial tissue

A heatmap generated by unsupervised hierarchical clustering of the 24 bronchial epithelium samples and the 227 most differentially expressed genes demonstrated that CF patients and healthy subjects could be distinguished by the gene expression profiles of their bronchial epithelium (Fig. 3). In comparison, clustering of the 36 nasal samples and the 15 differentially expressed genes that passed moderately stringent filters was inadequate, resulting in two groups that consisted of 16 CF patients (80%) and 4 healthy subjects, and 11 healthy subjects (69%) and 2 CF patients (Supplemental Fig. S2). There was no obvious correlation with age, gender or recent infection; however, a group of nine CF nasal samples clustered by the batch in which they were processed. Clustering the bronchial epithelium samples by the expression patterns of the same 15 genes was also ineffective in distinguishing CF patients and healthy subjects (data not shown).

3. Discussion

In this study of global patterns of gene expression in samples from the nasal and bronchial epithelium of CF patients and healthy controls, we provide evidence that suggests caution in the use of nasal epithelium as a pre-screen for lung-directed therapies, such as gene therapy or novel CF therapeutics.

Table 2B
Top 50 genes down-regulated in CF versus non-CF bronchial epithelium.

Gene	Symbol	Fold-change	*P* value[a]	Accession no.
Secretoglobin, family 3A, member 1	SCGB3A1	5.22	0.002	NM_052863.1
Secretoglobin, family 1A, member 1	SCGB1A1	5.11	0.001	NM_003357.3
Glutathione S-transferase A2	GSTA2	4.41	0.001	NM_000846.3
Chloride channel family member 2	CLCA2	3.41	0.002	NM_006536.3
Neurotensin	NTS	3.25	0.009	NM_006183.3
BTB (POZ) domain containing 5	KLHL28	2.98	0.001	NM_017658.1
ATPase lysosomal 56/58 kDa	ATP6V1B1	2.93	0.001	NM_001692.2
Cytochrome P450 subfamily J	CYP2J2	2.82	0.001	NM_000775.2
DMC1 dosage suppressor of mck1 homolog	DMC1	2.81	0.002	NM_007068.2
Zinc finger protein 14 (KOX 6)	ZNF14	2.81	0.001	NM_021030.2
Hypothetical protein	FLJ34047	2.75	0.001	NM_173669.1
Activator of S phase kinase	DBF4	2.74	0.002	NM_006716.3
Gamma tubulin ring complex protein	TUBGCP4	2.72	0.001	NM_014444.2
Transcriptional adaptor 3 (NGG1 homolog transcript variant 2)	TADA3	2.71	0.001	NM_133480.1
Hypothetical protein	FLJ34278	2.70	0.001	NM_173602.1
Claudin 8	CLDN8	2.67	0.002	NM_012132.3
Glutathione S-transferase A5	GSTA5	2.62	0.002	NM_153699.1
Protein S (alpha)	PROS1	2.62	0.003	NM_000313.1
Alcohol dehydrogenase 1A (class I)	ADH1A	2.61	0.004	NM_000667.2
ATP-binding cassette member 13	ABCC13	2.61	0.002	NM_172024.1
Hypothetical zinc finger protein	ZNF667	2.60	0.001	NM_022103.2
SLIT and NTRK-like family	SLITRK6	2.59	0.001	NM_032229.2
Hypothetical protein	C19orf39	2.59	0.001	NM_175871.2
Interleukin 18 (interferon-gamma-inducing factor)	IL18	2.59	0.001	NM_001562.2
Hypothetical protein	FLJ20700	2.54	0.001	NM_017932.1
Small nuclear RNA activating complex 43 kDa	SNAPC1	2.52	0.001	NM_003082.2
Golgin-67	GOLGA8A	2.51	0.002	NM_181077.1
FLJ35093 protein	FAM73A	2.50	0.001	NM_198549.1
Erythrocyte transmembrane protein	LOC51145	2.47	0.001	NM_016158.1
Hypothetical protein	LOC286177	2.47	0.001	XM_379582.1
Similar to alpha tubulin	LOC375049	2.43	0.002	XM_351316.1
Aldehyde dehydrogenase 1 family	ALDH1A1	2.42	0.003	NM_000689.3
Tripartite motif-containing 50C	TRIM74	2.41	0.001	NM_198853.1
Keratin 15	KRT15	2.40	0.008	NM_002275.2
Alcohol dehydrogenase 1C (class I)	ADH1C	2.40	0.005	NM_000669.2
Chromosome 14 open reading frame 127 (C14orf127)	NUBPL	2.39	0.001	NM_025152.1
NADH dehydrogenase (ubiquinone) 12	NDUFC2	2.38	0.001	NM_004549.3
FK506 binding protein 14	FKBP14	2.38	0.002	NM_017946.1
CD164 antigen	CD164	2.37	0.003	NM_006016.3
Hypothetical protein	FAM55B	2.36	0.001	NM_182495.3
Zinc finger protein 339	OVOL2	2.35	0.001	NM_021220.1
Hypothetical	LOC91170	2.35	0.001	XM_036612.8
Similar to CG9117-PA	MBLAC1	2.35	0.001	XM_171171.2
Hydroxysteroid (17-beta) dehydrogenase 7	HSD17B7	2.33	0.001	NM_016371.1
Hypothetical protein FLJ21657	C5orf28	2.32	0.002	NM_022483.2
Folate receptor 1 (adult)	FOLR1	2.29	0.003	NM_016731.2
Protocadherin beta 9	PCDHB9	2.29	0.002	NM_019119.3
Nuclear factor of activated T-cells	NFATC3	2.28	0.001	NM_173164.1
Basic leucine zipper nuclear factor 1 (JEM-1)	BLZF1	2.28	0.002	NM_003666.2
Flavoprotein oxidoreductase	MICAL3	2.28	0.001	XM_032997.4

Definition of abbreviations: Fold-change, mean ratio (CF:non-CF); *P*-value ≤ 0.01 (corrected for multiple testing using Benjamini Hochberg False Discovery Rate).
[a] The full list of genes most highly down-regulated in CF versus non-CF bronchial epithelium can be found in Supplemental Table S4 (≥2-fold, *P* ≤ 0.01).

Given the similar ion transport phenotype of CF nasal and bronchial respiratory epithelium [28,29], we hypothesized that they would display similar global patterns of gene expression. However, we show that they have different patterns of differential gene expression, with little overlap between the two tissues. One reason for this difference might be that there is little, if any, CF-related pathology in CF nasal epithelium whereas there is significant pathology in CF bronchial epithelium e.g. chronic infection and neutrophil-dominated inflammation [30]. Also, mucociliary clearance in the nose has been shown to be normal unless significant sinonasal disease is present [31]. Enrichment in CF versus non-CF bronchial epithelium, but not in CF versus non-CF nasal epithelium, of pathways involved in the inflammatory response and immune cell trafficking support this at the molecular level. A second possible reason for the difference is that CF bronchial epithelium samples contain a higher proportion of neutrophils (33%) than non-CF bronchial epithelium samples (6%) or CF nasal samples (4%) such that non-epithelial cells might contribute to the overall signal obtained in CF bronchial epithelium samples. However, a survey of the literature on global analysis of gene expression in neutrophils revealed that only 22 of the 863 genes (2.5%) differentially expressed in CF bronchial epithelium were reported to be differentially expressed in either human CF neutrophils or stimulated neutrophils; including, beta actin (ACTB), pre-B cell enhancing factor CXCL10, FOS, heat shock protein 70 (HSP70), IL8, IL1B and CTSD [32,33]. A third possible reason might be that there is a greater diversity of epithelial subtypes in the bronchial versus nasal epithelium samples. Fourthly, the chronic effects of fibrosis and airway remodelling, downstream of infection and inflammation, in the lung may account for the differences.

With respect to the proportion of differentially expressed genes identified in CF nasal and bronchial epithelium, our work contrasts with that of others who have profiled gene expression in human CF airway epithelial tissue. In a study of primary cultures of airway epithelial cells from CF and non-CF donor lung tissue 24 differentially expressed genes (0.1% of genes analysed) were identified [8]. This contrasts with the 863 genes (3.5% of genes analysed) that we found

Table 3
Differentially expressed genes in CF versus non-CF nasal epithelium.

Gene	Symbol	Fold-change	*P* value[a]	Accession no.
NADH dehydrogenase 1	MT-ND1	2.29	0.037	NM_173708.1
Calcyphosine transcript variant 1	CAPS	1.82	0.040	NM_004058.2
Transgelin 2	*TAGLN2*	1.78	0.028	NM_003564.1
Aldolase A fructose bisphosphate transcript variant 2	*ALDOA*	1.68	0.037	NM_184041.1
Claudin 7	CLDN7	1.67	0.032	NM_001307.3
Solute carrier family 4 member 11	SLC4A11	1.65	0.032	NM_032034.1
Insulin-like growth factor binding protein 2 (36kD)	IGFBP2	1.58	0.046	NM_000597.1
Cathepsin D (lysosomal aspartyl protease)	*CTSD*	1.57	0.032	NM_001909.3
Aldo-keto reductase family 1	AKR1B1	1.56	0.003	NM_001628.2
β defensin-1	DEFB1	1.56	0.034	NM_005218.2
Tubulin alpha 6	TUBA1C	1.54	0.037	NM_032704.2
Junction plakoglobin mRNA	JUP	1.51	0.038	NM_021991.1
Calcineurin homologous protein	LOC63928	0.41	0.032	NM_022097.1
Neurotensin	*NTS*	0.47	0.037	NM_006183.3
Integral membrane protein 2A	*ITM2A*	0.65	0.032	NM_004867.2

Definition of abbreviations: Fold-change, mean ratio (CF:non-CF); *P*-value ≤ 0.05 (corrected for multiple testing using Benjamini Hochberg False Discovery Rate). Italics, genes differentially expressed between CF and non-CF bronchial epithelium.

to be differentially expressed between CF and non-CF bronchial epithelium. Even the 227 differentially expressed genes that passed stringent filtering represent a 9-fold larger proportion of differentially expressed genes than those reported by Zabner *et al.* [8]. As the authors analysed resting cultures of differentiated airway epithelial cells grown at air-liquid interface the *in vitro* culture conditions used in this study may explain why they reported minimal CF and non-CF differences. Our results in which we identify many differences between freshly brushed CF and non-CF bronchial epithelium samples likely reflect the complex patterns of airway epithelial cell gene expression in physiologically relevant lung milieu.

In a second microarray study, 0.15%–1.27% of genes analysed were reported to be differentially expressed [9]. This contrasts with the 15 genes (0.06% of genes analysed) we found to be differentially expressed in CF nasal epithelium. One reason for the difference may be that 7 out of the 8 patients included in our study were undergoing antibiotic treatment at the time of brushing. More likely, however, are the differences in study design, the patient cohort and the stringency of cut-offs applied.

There is some overlap in the differentially expressed genes reported in our study and those reported in the studies cited above. Although none of the differentially expressed genes reported by Zabner *et al.* [8] were identical to those in CF bronchial epithelium, there were eight genes out of 11 known genes with functions in common. Analogous genes in CF bronchial epithelium encoded proteins involved in ion transport, lipid and amino acid metabolism, signal transduction, cell-to-cell signalling and inflammation (the exceptions were Copine VII (CPNE7), transferrin receptor (TF) and topoisomerase I (TOP1)). In terms of CF nasal epithelium, integral membrane protein 2A (ITM2A), a gene encoding a T lymphocyte-inducible integral membrane protein, has also been reported to be down-regulated in CF nasal epithelium from patients with low lung function (Wright *et al.* [9]). ITM2A and five further genes (ACTB, CD74, interferon-induced transmembrane protein 1 (IFITM1), SP110 nuclear body protein and epithelial stromal interaction protein 1 (EPSTI1)) were also differentially expressed in CF bronchial epithelium. Furthermore, as for CF bronchial epithelium, Wright *et al.* [9] reported differential expression of genes involved in a wide range of cellular processes, including protein and lipid turnover, mitochondrial function and airway defence. It is noteworthy that 10 genes differentially expressed in CF bronchial epithelium were differentially expressed in CF mouse knock-out lung tissue [7]. Additionally, the authors reported differential expression of genes involved in a wide range of cellular processes, including transcription, inflammation, signal transduction, ion transport and protein trafficking. Overall, the overlap between our transcriptome data and that of the studies cited above supports the general view that data from different platforms can give rise to concordant differentially expressed genes and pathways [27].

To identify tissue differences between nasal and bronchial epithelium we compared the expression profiles of these tissues in CF patients and in healthy subjects. We showed that a similar

Table 4
Pathways enriched in CF versus non-CF airway epithelium.

Molecular and cellular pathways	
CF bronchial epithelium	*P* value
Inflammatory response	1.18E−36
Cellular movement	1.66E−29
Cell-to-cell signalling and interaction	3.38E−26
Immune cell trafficking	5.58E−24
Cellular growth and proliferation	2.98E−23
Hematological system development and function	6.71E−23
Cell death	3.58E−21
Tissue development	5.28E−20
Cellular development	2.91E−19
Connective tissue disorders	3.38E−19
CF nasal epithelium	
Amino acid metabolism	1.15E−02
Cell morphology	3.07E−02
Cellular compromise	3.93E−02
Drug metabolism	3.85E−02
Molecular transport	4.87E−02

Definition of abbreviations: P value, gene network generated by IPA and ranked according to the presence of differentially expressed genes within a curated gene network. The five most significant gene networks in each category are shown ($P \leq 0.05$).

Table 5
Pathways enriched in bronchial versus nasal epithelium of CF patients and healthy subjects.

Molecular and cellular pathways	
CF patients	*P* value
Cellular movement	1.37E−24
Cell-to-cell signalling and interaction	1.48E−16
Cell signaling	5.94E−16
Molecular transport	5.94E−16
Vitamin and mineral metabolism	5.94E−16
Healthy subjects	
Cellular growth and proliferation	7.44E−04
Cell cycle	1.27E−03
DNA replication, recombination and repair	1.27E−03
Antigen presentation	6.05E−03
Cell morphology	6.05E−03

Definition of abbreviations: P value, gene network generated by IPA and ranked according to the presence of differentially expressed genes within a curated gene network. The five most significant gene networks in each category are shown ($P \leq 0.05$).

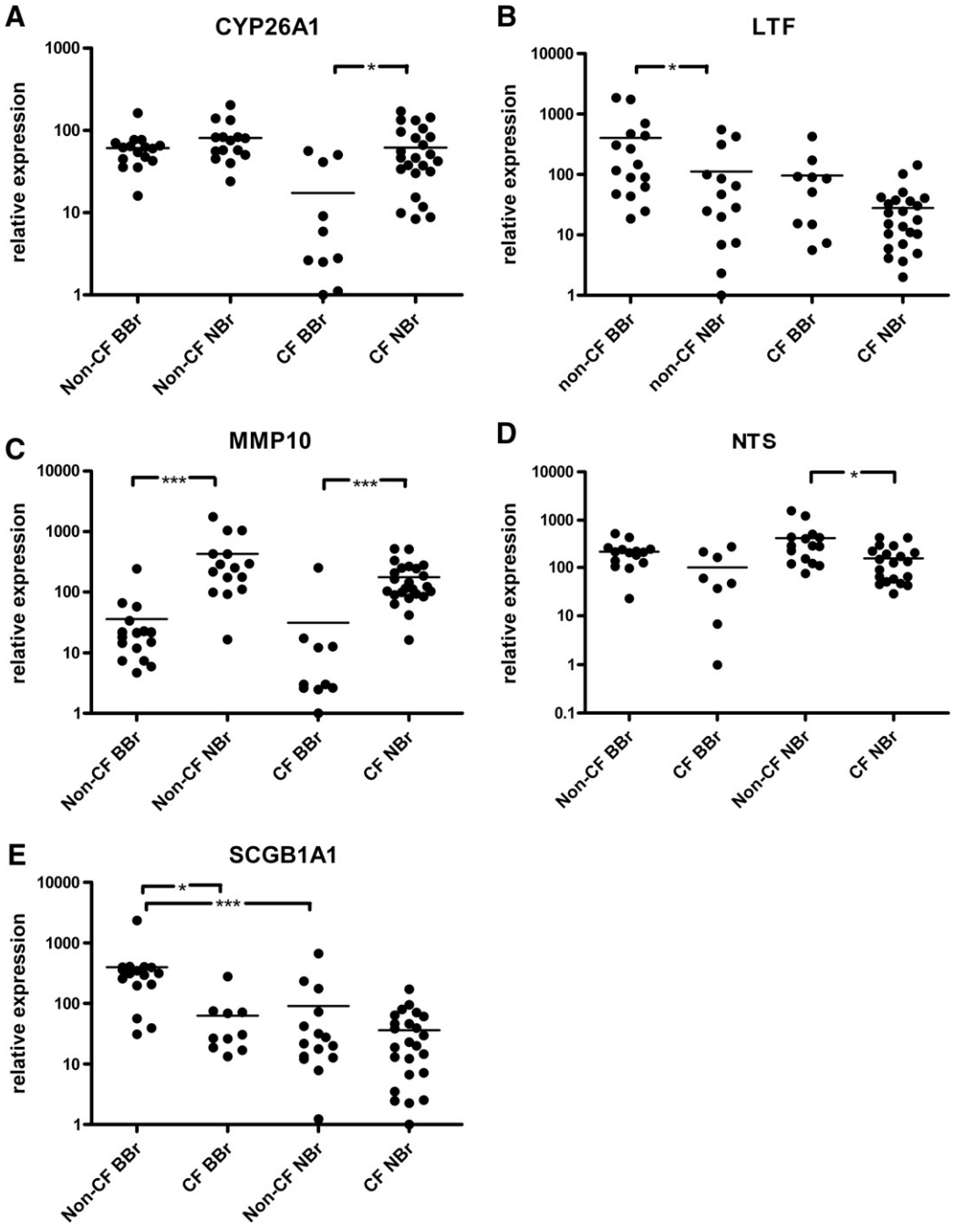
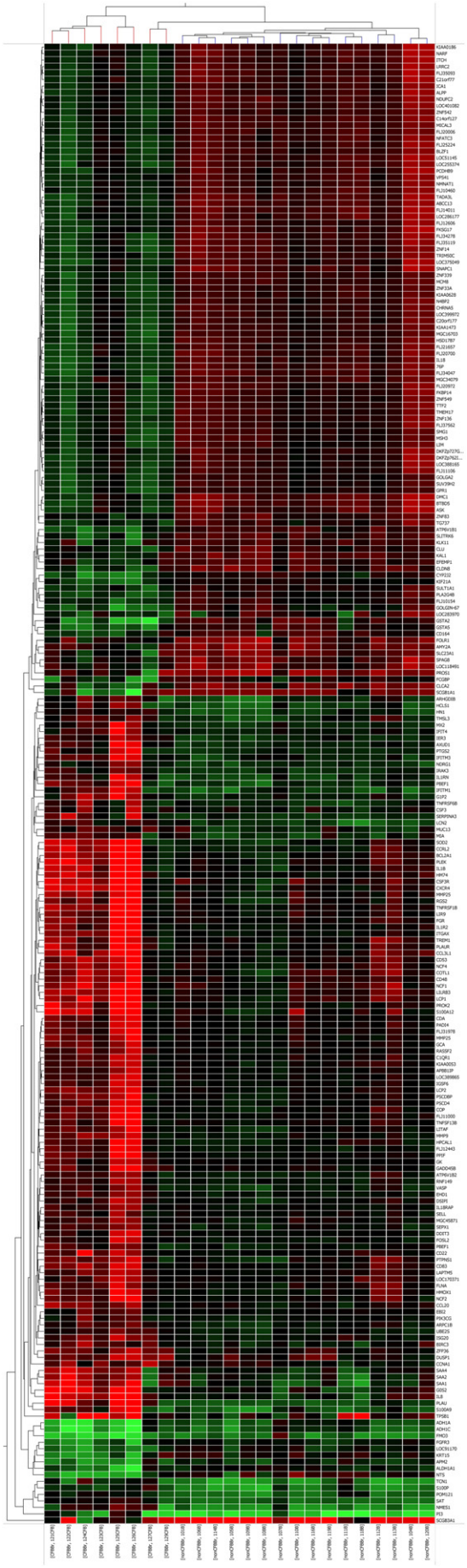


Fig. 2. Selected genes analysed by quantitative real-time PCR (QPCR). QPCR analysis of gene expression in CF and non-CF nasal and bronchial epithelium was performed using TaqMan assays (Applied Biosystems) for (A) cytochrome P450 (CYP26A1), (B) lactoferrin (LTF), (C) matrix metalloproteinase 10 (MMP10), (D) neurotensin (NTS) and (E) secretoglobin (SCGB1A1). RNA levels are normalised to large ribosomal protein 0 (RPLP0). The sample with the lowest relative expression value in the four conditions was used as the calibrator (1×) sample. Data are plotted on a log scale. Statistical analysis was performed using Prism 4.0. software (GraphPad, San Diego, USA). $P \leq 0.05$ was considered statistically significant (Mann–Whitney unpaired test). *, $P \leq 0.05$, ** $P < 0.01$, *** $P < 0.001$.

proportion of genes were differentially expressed between the CF tissues and between the non-CF tissues but that the majority (83%) of genes did not overlap. Pathways involved in tissue repair, such as cellular movement and cell-to-cell signalling, were differentially expressed between CF nasal and bronchial epithelium samples but were not differentially expressed between non-CF nasal and bronchial epithelium samples where pathways involved in tissue maintenance, such as cellular growth and proliferation, were enriched. These results indicate that CF and healthy nasal and bronchial epithelium have differential functions *in vivo*. Furthermore, some of the differentially expressed genes and perturbed pathways in patient airways suggest that the bronchial epithelium is adapting to its environment by initiating cellular changes that may eventually lead to epithelial metaplasia. For example, the gene SCGB1A1 is known as a Clara cell marker (although it is also expressed by other cell types) and is down-regulated in CF bronchial epithelium compared with non-CF bronchial epithelium but is expressed at similar levels to both CF and non-CF nasal epithelium (Fig. 2). This could suggest that CF bronchial epithelium has a drastically reduced Clara cell content since the nasal epithelium lacks Clara cells. Changes in the epithelial phenotype of CF airways, such as changes in the proportion, morphology or gene expression patterns of Clara cells or goblet cells, have been proposed to contribute to the development of chronic lung disease in CF but studies of goblet cells have yielded conflicting results [34–36]. Our



transcriptome data revealed discordant patterns of expression for goblet cell markers, MUC1 and MUC5B, in CF nasal versus bronchial epithelium (Supplemental Table S7A). Cellular changes may explain some of the differences in global patterns of gene expression between CF and non-CF nasal and bronchial epithelium. Strikingly, the inflammatory response pathway was only enriched when CF and non-CF bronchial epithelium were compared. This suggests that only bronchial tissue will be useful in predicting the inflammatory response of the lung to new treatments, such as gene therapy. Notably, the nose has not proved useful as a surrogate for the lung with regards to toxicity in a recent gene therapy study [37]. As children's lungs may be more amenable to treatment than adults' lungs it might be expected that changes in the inflammatory profile of bronchial epithelium may be more readily detected in children than in adults after treatment.

Given the heterogeneity of CF lung disease, we were surprised to find highly consistent patterns of gene expression in the bronchial epithelium, but not the nasal epithelium, of individuals of the same group. These results suggest that microarrays can detect altered global gene expression in CF bronchial epithelium samples but may be less informative in CF nasal epithelium samples due to the limited number of genes with detectable changes in this tissue. QPCR may be better suited to the analysis of gene expression in CF nasal epithelium in the study of new CF therapies, such as gene therapy, because it is highly sensitive and enables detailed analysis of a small number of genes. Difficulties in identifying a consistently expressed reference gene for nasal epithelial tissue will need to be overcome if this approach is taken. However, our results do not negate the utility of nasal epithelium as a model tissue for the direct assessment of CFTR gene transfer.

It was important to identify differences between CF and non-CF nasal and bronchial epithelial tissue because successful CF therapies might be expected to induce changes in the expression levels of the genes we have identified towards non-CF levels. By establishing CF and non-CF differences in nasal and bronchial epithelial tissue we may be able to understand the biological significance of changes in global patterns of gene expression in these tissues after treatment. Our results lay the foundation for the development of a focussed, hypothesis-driven approach to microarray analysis of bronchial epithelium in the study of lung-directed therapies, such as gene therapy. Microarrays are being used for a similar purpose in cancer treatment studies [38]. It is noteworthy that the vast majority of genes did not show statistically significant differential expression in CF nasal or bronchial epithelium (99.9% and 96.5% of genes analysed, respectively) indicating that the detectable *in vivo* transcriptional consequences of CFTR mutations in respiratory epithelium are minimal. We were unable to gather sufficient differential data from CF versus non-CF bronchial epithelium samples using immunocytochemical CFTR antibodies to allow us to draw any robust conclusions. This was due to the limited number of CF bronchial epithelium samples of sufficient quality for detailed cellular analysis.

A potential limitation of our study is that CF bronchial epithelium samples were obtained under general anaesthetic whereas non-CF bronchial samples, and nasal samples, were not. There was also a significant age difference between CF patients and healthy subjects. Therefore, some of the CF and non-CF differences identified may be

Fig. 3. Heatmap of bronchial epithelium samples. A heatmap was generated by unsupervised hierarchical clustering of 277 genes (fold-change ≥ 2.0, $P \leq 0.01$) and bronchial samples (n = 8 CF patients, n = 16 non-CF subjects) using Euclidean distance and average linkage rule. Branch length indicates the degree of similarity between samples (top) and genes (right side) where short branches indicate high similarity. After clustering, samples were decoded to identify CF patients (red branches) and non-CF healthy people (blue branches). Red bars indicate up-regulation in CF. Green bars indicate downregulation in CF. Black indicates no differential expression (fold-change < 2.0, $P > 0.01$).

age-related or due to anaesthetic but the effects of these factors are likely to be low compared with the effects of the disease itself. Use of paired nasal and bronchial epithelium samples from CF patients and healthy subjects was more pertinent to the hypothesis being tested in this study.

Our exploratory results highlight the need to consider alternative methods of sampling the bronchial epithelium from patients undergoing bronchoscopy. Given the cost and effort involved in generating transcriptome data from CF patients it is important that optimal approaches for sample collection and handling are agreed on for future studies. Our findings have important implications not only for microarray analysis of gene expression but also for new rapidly evolving profiling technologies, such as next generation sequencing.

In conclusion, our results do not support the use of the nasal epithelium as a pre-screen for lung-directed therapies due to differences in global patterns of gene expression between CF and non-CF nasal and bronchial epithelium samples. However, the CF nasal epithelium may have some use in predicting the molecular response to new treatments, such as gene therapy or novel CF therapeutics e.g. in identifying genes most proximal to the primary genetic defect. Our results suggest that bronchial epithelium samples should be directly used to assess the biological effects of new treatments on inflammation in the lung.

4. Materials and methods

4.1. Nasal and bronchial brushings

CF had been diagnosed on standard criteria [39] and 23 out of 28 were homozygous for the ΔF508 mutation; 2 were compound heterozygotes for the ΔF508 mutation; and 3 were sweat test-positive but had not been genotyped. Healthy volunteers had been screened for CFTR mutations, were free of any respiratory tract symptoms, were non-smokers and had no allergies. Protocols had been approved by the local Research Ethics Committee and informed written consent (and where appropriate, assent for children) had been obtained from the subjects. Additional detail on the subjects can be found in Supplemental Tables S8 and S9. Bronchial brushings were obtained by bronchoscopy from five airways of approximately 5-6th generation using a cytology brush (Olympus BD-202D-5010). Nasal brushings were obtained from the mucosal surface of the inferior turbinate using a 2.7 mm interdental brush (Dent-O-Care, London, UK Ltd.). Immunocytochemistry was used to determine epithelial cell content, as previously described [40]. Brushings were double-stained using cytokeratin and CFTR (G449; [41]) antibodies. Cellular content was determined by cytological analysis.

4.2. RNA preparation and microarray hybridisation

Total RNA was extracted using RNeasy mini columns (Qiagen Ltd., Crawley, UK) and treated with DNase I (DNA-free kit, Applied Biosystems™, Warrington, UK). RNA quality and quantity was determined using a 2100 Bioanalyser, according to manufacturers' instructions (Agilent Technologies UK Ltd., Wokingham, UK). An RNA Integrity Number (RIN) ranging from a mean of 6.6 ± 2.6 to 8.8 ± 1.0 indicated quality (Supplemental Table S10). Biotin-labelled, antisense copy RNA (cRNA) was synthesised from 100 ng of total RNA (Illumina® TotalPrep RNA Amplification kit, Cat. No. IL1791, Ambion, UK) as described by the manufacturer. 850 ng of cRNA was hybridised to a Sentrix HumanRef-8 v1 Expression BeadChip microarray according to manufacturer's instructions (Illumina® San Diego, USA). Microarrays were scanned using a BeadArray Reader (Illumina®) and data were processed using BeadStudio software v2 (Illumina®). Additional detail on the methods is provided in supplementary data.

4.3. Microarray data analysis

Data were analysed using GeneSpring GX10 software (Agilent Technologies), and results confirmed by limma [42] and SAM [43] analyses using R [44] and Bioconductor [45] (Supplemental Fig. S3). Data were normalised by Quantile and filtered. Significant changes were calculated by the Mann–Whitney *U* test unpaired and *P* values were corrected for multiple testing using Benjamini–Hochburg false discovery rate. Additional detail on the method is provided in supplementary data. Ingenuity pathway analysis software (Ingenuity Systems Inc., www.ingenuity.com) was used to identify significant pathways. The Basic Local Alignment Search Tool (BLAST) was used to identify homologues ($\geq$90% identity) for genes encoding hypothetical proteins.

4.4. Quantitative real-time polymerase chain reaction (QPCR)

100 ng of cRNA was reverse transcribed using random hexamer primers and a first-strand cDNA synthesis kit for RT-PCR (Roche, West Sussex, UK). cDNA was quantified by PicoGreen (PicoGreen dsDNA assay kit, Molecular Probes®, Paisley, UK). QPCR was performed using TaqMan® Universal PCR Mastermix and gene-specific assays on an ABI 7900HT Fast Real-Time PCR system (Applied Biosystems™, Warrington, UK). Additional detail on the method is provided in supplementary data.

Supplementary materials related to this article can be found online at doi:10.1016/j.ygeno.2011.06.008.

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