Gene Expression Differences in Primary Tracheobronchial Airway Epithelial Cells from Human Donors Diagnosed with Asthma or Chronic Obstructive Pulmonary Disorder (COPD)

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The respiratory disease therapeutic market has seen considerable growth over the past five years, however there are few marketed treatments that address the underlying disease pathology and prevent or reverse disease progression. According to the World Health Organization (WHO), 300 million people suffer from asthma, and it is the most common chronic disease among children.[1] The COPD Foundation estimates that over 210 million people worldwide have Chronic Obstructive Pulmonary Disease (COPD) and that the total worldwide deaths from the disease are expected to increase by more than 30% over the next decade without new treatments and interventions to cut risks.[2]

Current drug targets of both diseases include certain enzymes or receptors which are known to modulate key cells involved in regulating pathogenic immunity and inflammatory responses. Airway epithelial cells are also another new and more direct target as therapeutic developers look to medicines with a specific, targeted mechanism of action either through the development of new delivery systems, or by the identification of new drug targets. Advances in molecular testing to identify disease sub-types or categories are also assisting drug development to identify new biomarkers. A recent FDA approved treatment for asthma, bronchial thermoplasty[3] permanently treats a symptom of asthma, constricted airway lumen, but there is still a question of other pathologies seen with the disease including increased mucous production and longer term effects, such as airway remodeling. COPD has no known cure, only treatments for symptoms of the disease including bronchodilators, pulmonary rehabilitation, oxygen therapy, and in some cases, specialized surgery to remove pieces of effected lung tissue.[4]

Access to diseased primary cells from patients diagnosed with COPD or asthma provides a convenient, biologically relevant model to assess genetic and in some cases phenotypic changes from normal, healthy donor tissues. In this study, we grew human bronchial epithelial (HBE) cells from normal, COPD-, and asthma-diagnosed human donor lung tissues in an air-liquid interface (ALI) model to assess potential phenotypic changes *in vitro* as well as provide a differentiated layer of cells to harvest for gene expression studies using the StellARray™ Gene Expression System.

Methods and Materials

Cells and Cell Culture

HBE cells from normal, COPD and asthma donors were screened for cell performance for number of population doublings and morphology in BEGM™ Bronchial Epithelial Growth Medium in submerged cultures, and for differentiation in B-ALI™ Bronchial Air-liquid Interface Differentiation Media. For airlifted cultures, HBE lots (normal airway epithelial cells, NHBE, Cat. No. CC-2540, lot 0000139014; COPD airway epithelial cells, DHBE-COPD Cat. No. 00195275, lot 0F3313; asthma airway epithelial cells, DHBE-As, Cat. No. 00194911, lot 9F3134; donor characteristics reported in Table 1) were thawed and expanded for 3 days in B-ALI™

Description	Cat. No.	Lot No.	Donor History	PD[a]	TEER[b] (Ω*cm²)	ZO-1[c]	β-catenin[c]	Mucin[d]	Cilia[d]
NHBE - Normal Human Bronchial Epithelial Cells	CC-2540	0000139014	1Y0 Hispanic male, no history of heart disease, hypertension, non-smoking.	18	842 ± 47	+	+	+	+
DHBE-COPD - Diseased Human Bronchial Epithelial Cells (COPD)	00195275	0F3313	48Y0 Caucasian female, history of hypertension, smoked 2 - 3 P/D for 34 years, positive diagnosis for COPD and Emphysema.	19	864 ± 36	+	+	+	+
DBHE-Asthma - Diseased Human Bronchial Epithelial Cells (Asthma)	00194911	9F3134	52Y0 Caucasian male, no history of heart disease or hypertension, non-smoking, diagnosed with asthma as a child, taking Albuterol, Advair®, Singulair®, Proventil®, Prednisone, and Decadron® at time of death and 3 months prior to death.	11	933 ± 10	+	+	+	+

Table 1

HBE cell culture endpoints and donor information. [a] in BEGM™, traditional submerged culture; [b] measured by day 7 in ALI culture; [c] measured by day 35 in ALI culture; [d] measured by day 20 in ALI culture.

Growth Media (Lonza Cat. No. 00193514) then seeded in transparent, collagen-coated PET 0.4 µm filter inserts (Corning Cat. No. 3470) at a density of 60,000 live cells and maintained per Lonza manufacturer instructions. The trans epithelial electrical resistance (TEER, a measure of barrier function), was measured with EVOM equipped with STX2 electrodes. The presence of tight junction proteins was assessed through immunostaining with mouse anti-human ZO-1 (Life Technologies Cat. No. 33-9100) and mouse anti-human β-catenin (Zymed 13-8400), both detected with goat anti-mouse ALEXA Fluor® 488 (Life Technologies A11017). Secreted mucin was assessed through direct staining with Alcian Blue, and the cilia formation was assessed through direct immunostaining with mouse monoclonal anti-β-tubulin FITC conjugated (Sigma Cat. No. F-2043, clone TUB 2.1).

qPCR Experimental Design

To generate data with biologically relevant variance, four replicate samples were independently assayed from each cell type.

RNA Isolation and cDNA Synthesis

After airlift, media in all inserts were changed every second day until day 15 when cell surfaces were washed twice with PBS and lysed directly on the transwell membrane with 100 µl Qiagen RLT Buffer. Each sample consisted of the pooled lysates from three inserts. Sample lysates were frozen at -80°C and shipped to Bar Harbor BioTechnology (BHB) for further processing and analysis. At BHB, thawed lysates were clarified by centrifugation, and RNA was isolated with Qiagen's RNeasy® Mini Kit (Cat. No. 74104). 2 µL of SUPERase-In™ (Life Technologies, Inc. Cat. No. AM2694) were added to the bottom of the RNA elution receiver tube (just prior to elution) to improve RNA stability, and eluates were treated with DNA-*free*™ Kit (Life Technologies, Inc. Cat. No. AM1906) at 37°C for 1 hour. RNA quality was assessed with the RNA 6000 Nano Kit (Agilent Technologies, Inc. Cat. No. 5067-1511) and the 2100 Bioanalyzer microfluidics-based platform (2100 Bioanalyzer System, Cat. No. G2940CA, Agilent Technologies, Inc.). cDNA was synthesized using MessageSensor™ RT Kit (Life Technologies, Inc. Cat. No. AM1745) with the Two Step RT-PCR procedure. For primers, an equal mix of random decamers and oligo dT primers (Life Technologies, Inc. Cat. No. 5722G and 5730G) was used. Input RNA was normalized to 660 ng per sample. cDNA synthesis reactions were incubated with the following thermal program: 25°C for 10 minutes, 42°C for 90 minutes, 95°C for 10 minutes, hold at 4°C.

Real-time qPCR

For each sample 4224 µL SYBR® Green Master Mix was made. One half of the cDNA was included in this master mix as a template (the equivalent of 330 ng input RNA). The master mixes contained hot-start *Taq* Polymerase, AmpliTaq Gold® (Life Technologies/Applied Biosystems). 10 µL of master mix was loaded into each well of a 384-well custom StellARray™ qPCR Array Plate. Arrays were run on a 7900HT Sequence Detection System (Applied Biosystems, Inc.) using default cycling parameters for 40 cycles (one cycle of 50°C for 2 minutes, one cycle of 95°C for 10 minutes, and 40 cycles of 95°C for 15 seconds and 60°C for 1 minute). Fluorescence data was acquired during the 60°C anneal/extension plateau. Post-run data collection involved the setting of a common threshold (Ct) across all arrays within an experiment, exportation and collation of the Ct values, and analysis via GPR.

GPR Algorithm

Data input for GPR consists of a list of Ct values, derived directly from real-time PCR instruments, for each sample (control or experimental). After designating the control and experimental sets of Ct values, GPR filters expression data to separate genes into two groups – genes considered for analysis (G) and genes that can be used as potential normalizers (N). Genes that are not expressed in either sample are not considered further. After filtering the data into two sets, GPR performs a proprietary global normalization and statistical analysis by comparing each G to each N. The magnitude of change in expression ("fold change") for each gene is subsequently determined using the ten best N genes, as defined within each experiment.

Results and Discussion

The COPD and asthma bronchial epithelial cells used here reached >10 population doublings in standard culture conditions and appear to be morphologically indistinguishable from NHBEs. Each of the three cell types was differentiated in a 3D ALI culture using Lonza B-ALI™ Media

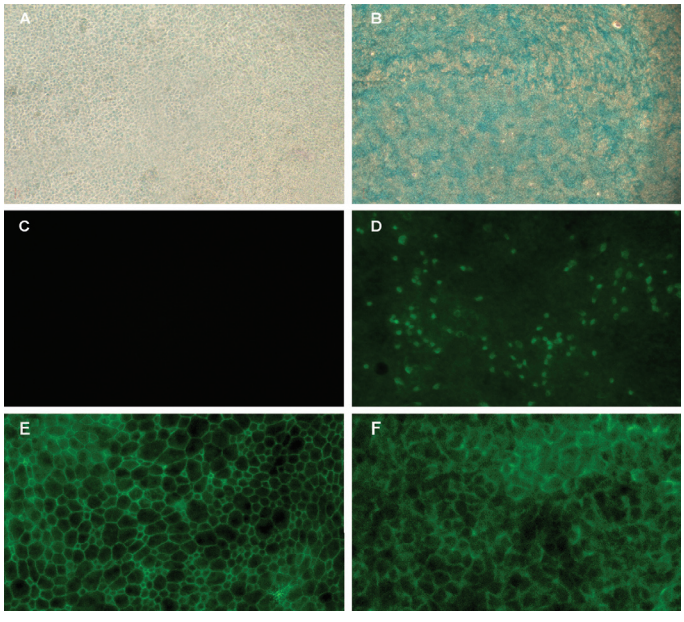

Figure 1
Typical differentiated characteristics of bronchial epithelial cells in B-ALI™ Air-liquid Interface Conditions. Each of the epithelial cell types used in this paper (normal, asthma and COPD) were positive for mucin secretion, cilia formation and tight junctions, with no significant differences observed between the normal and diseased cells. The data here are from the COPD samples. **A.** Alcian blue stain for mucin (negative control), **B.** Alcian blue stain for mucin, **C.** β-tubulin stain for cilia (negative control), **D.** β-tubulin stain for cilia, **E.** ZO-1 staining for tight junctions, **F.** β-catenin stain for tight junctions.

and Protocols. These cells show good barrier function (TEER ranges of 840 Ω*cm^2 to 930 Ω*cm^2; Table 1) as early as day 7 after airlift, and tight junctions were confirmed by ZO-1 and β-catenin expression. Mucin production was present in every COPD and asthma cell lot, but not clearly correlated with donor health history. Cilia formation frequency was donor dependent and observed by day 20 after airlift. Representative photos of mucin, β-tubulin, ZO-1 and β-catenin staining are shown in Figure 1. The three endpoints used to characterize COPD and asthma HBE cells relative to normal cells validate successful differentiation, but do not show obvious differences between normal and diseased tissues in the 21 day comparison study we conducted on the differentiated tissues. Therefore, the cells in ALI culture conditions were subjected to pathway-specific array analysis to determine if there were any differences in gene expression between normal cells and those from the diseased donors at these time points. A custom 384-well StellARray™ qPCR Array Plate was developed and contained genes that focused on inflammation, known airway and airway diseased genes, and those in related pathways. In each case, statistically relevant differences in gene expression were detected in the diseased cells compared to the normal HBE cells. Genes that were up- or down-regulated at least 1.9-fold, and whose P-values are 0.05 or better are reported (Figure 2, normal vs. asthma, and Figure 3 for normal vs. COPD). Out of the 380 unique genes on the array, the expression of 52 genes changed by at least 1.9-fold in the asthma sample compared to normal cells (Figure 2), and 48 genes had altered expression in the COPD samples compared to the normal HBE cells (Figure 3). When comparing the asthma to the COPD samples, there are 67 statistically significant gene expression changes (data not shown). While this article is not meant to be a comprehensive analysis of the genes and their functions in airway disease, we will point out several genes that have been previously determined to be involved in these diseases. For example, urokinase plasminogen activator (PLAU) which activates latent forms of TGF-β1 and MMPs, and is suspected of being involved in COPD,[5] is up-regulated in the asthma sample but down-regulated in the COPD sample. There was a 4.3-fold decrease in the mucin gene MUC5b expression in asthma, which correlates with past studies.[6] In the asthma sample, many inflammatory interleukin and ECM-related MMP genes are overexpressed, while in the COPD sample, MMP genes for the most part have decreased expression relative to the normal sample. Exhaled nitric oxide is a widely used asthma biomarker,[7] and we found that nitric oxide synthase 1 (NOS1) is elevated in both the asthma and COPD samples. Interestingly, in the COPD sample, many of the human leukocyte antigen (HLA) genes are up-regulated (HLA-DQB1, -DQB2, -DRB1, -DQA1 and -DRA). These genes are sometimes linked to autoimmunity, and more COPD samples need to be screened in order to determine if the HLA genes are a marker of COPD status.

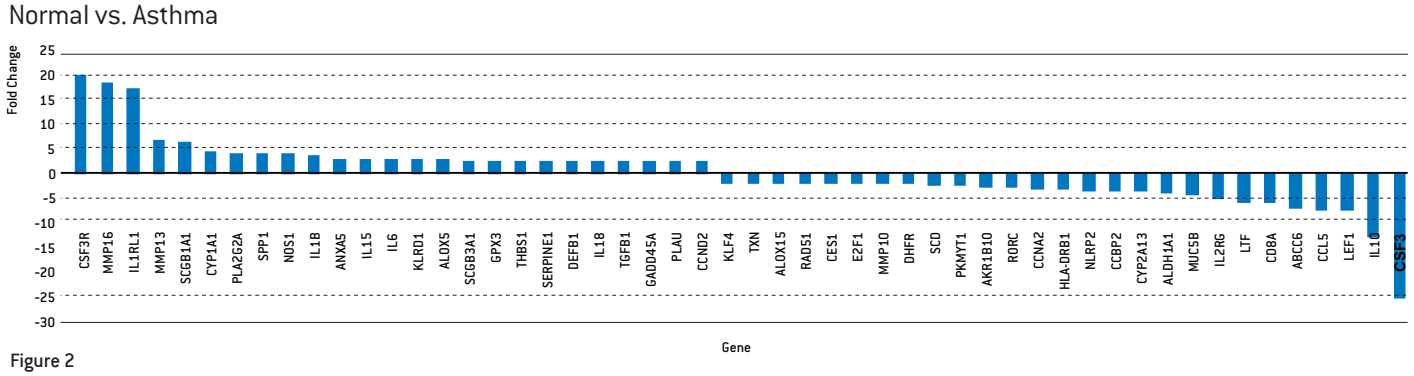

Figure 2

Genes up- or down-regulated in asthma sample compared to normal bronchial epithelial cells. P-values ranged from 0.04 to 0.0003 for the genes shown.

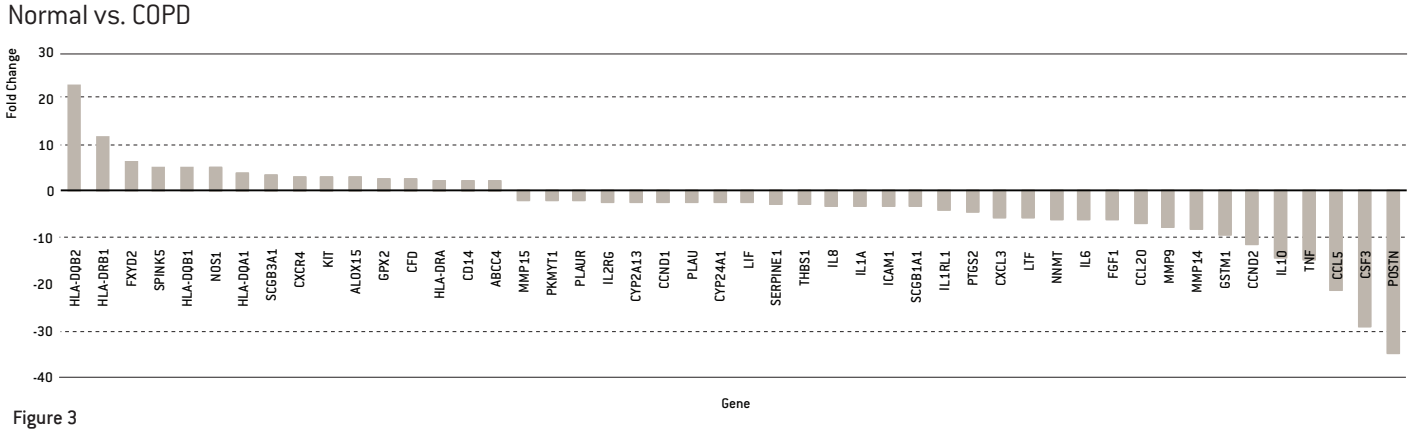

Figure 3

Genes up- or down-regulated in COPD sample compared to normal bronchial epithelial cells. P-values ranged from 0.05 to 0.0002 for the genes shown.

Summary

We have described a method in which normal and diseased bronchial epithelial cells were grown in ALI culture using the B-ALI™ Media Kit, and then gene expression analysis was examined using a custom 384-well StellARray™ qPCR Array focused on genes involved in inflammation and respiratory processes. Our data indicate that this methodology was successful and that differences in gene expression were revealed between these cell types. Several of the genes detected were previously implicated in asthma and/or COPD which suggests that, even though the data are from single donors, the cells and methods used here yielded valuable information which is consistent with other published data.

Lonza now offers a broad selection of cryopreserved primary human diseased airway cells including bronchial epithelial and smooth muscle cells, and lung fibroblasts from both Chronic Obstructive Pulmonary Disease (COPD) and asthmatic donors. Now, researchers can easily access ready-to-use airway cells from donors diagnosed with COPD or asthma. More in-depth patient donor information is accessible through Lonza's Scientific Support Team. The new cells are tested and guaranteed to perform with existing Clonetics™ Media Kits and Reagents making it easy to culture both normal and diseased cells using the same cell culture systems. With Lonza's new disease cell offering, researchers now have direct and easy access to these cells to advance and accelerate their studies in potentially uncovering the causes and developing new treatments for them.

www.lonza.com/airway-cells

References

1. Information from the World Health Organization website "Fact sheet N°307-Asthma" available at: http://www.who.int/mediacentre /factsheets/fs307/en/print.html. Nov. 2010.
2. Information from the COPD Foundation Organization website "About COPD Fact sheet" available at: http://www.copdfoundation.org/COPDersCaregivers/About COPD/tabid/81/language/en-US/Default.aspx. Nov. 2010.
3. Wall Street Journal, M. Beck. Health Journal, New Surgery to Treat Asthma, Nov. 2010.
4. Information from the NHLBI available at: http://www.nhlbi.nih.gov/health/dci/Diseases/Copd/Copd_Treatments.html. Nov. 2010.
5. Xiao, W, Hsu, YP, Ishizaka, A, Kirikae, T and Moss, RB. 2005, Sputum cathelicidin, urokinase palminogen activation system components, and cytokines discriminate cystic fibrosis, COPD and asthma inflammation, *Chest 128: 2316–2326.*
6. Woodruff, PG, Boushey, HA, Dolganov, GM *et al.* 2007, Genome-wide profiling identifies epithelial cell genes associated with asthma and with treatment response to corticosteroids, *Pro. Na. Aca. Sc. USA, 104: 15858–15863.*
7. Louhelainen, L, Myllärniemi, M, Rahman, I and Kinnula, VL (2008) Airway biomarkers of the oxidant burden in asthma and chronic obstructive pulmonary disease: Current and future perspectives, *Int J Chron Obstruct Pulmon Dis. 3: 585–603.*

Ordering Information

https://shop.lonza.com

Cat. No.	Description	Size
New Human Diseased Primary Cell Types		
00194911	DHBE-As, Asthmatic Human Bronchial Epithelial Cells	≥500,000 cells
00194912	DHLF-As, Asthmatic Human Lung Fibroblasts	≥500,000 cells
00194850	DBSMC-As, Asthmatic Human Bronchial Smooth Muscle Cells	≥500,000 cells
00195275	DHBE-COPD, Human Bronchial Epithelial Cells	≥500,000 cells
00195277	DHLF-COPD, Human Lung Fibroblasts	≥500,000 cells
00195274	DBSMC-COPD, Human Bronchial Smooth Muscle Cells	≥500,000 cells
Normal Human Primary Cell Types		
CC-2540	NHBE, Normal Human Bronchial Epithelial Cells	≥500,000 cells
CC-2512	NHLF, Normal Human Lung Fibroblasts	≥500,000 cells
CC-2576	BSMC, Normal Human Bronchial Smooth Muscle Cells	≥500,000 cells
Related Media		
00193514	B-ALI™ Bronchial Air-liquid Interface Media BulletKit™	Kit
CC-3170	BEGM™ Bronchial Epithelial Growth Media BulletKit™	Kit
CC-3132	FGM™-2 Fibroblast Growth Media BulletKit™	Kit
CC-3182	SmGM™-2 Smooth Muscle Growth Media BulletKit™	Kit

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AD-RNSpring2011 02/11 GM-NE008