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Oxidative stress modulates the expression of genes involved in cell survival in ΔF508 cystic fibrosis airway epithelial cells

Grégory Voisin,[1]* Guillaume F. Bouvet,[2]* Pierre Legendre,[3] André Dagenais,[2] Chantal Massé,[2] and Yves Berthiaume[2,4]

[1]*Centre de recherche, Centre hospitalier de l'Université de Montréal - Hôtel Dieu, Montréal, Quebec, Canada;* [2]*Institut de recherches cliniques de Montréal, Montréal, Quebec, Canada;* [3]*Département de sciences biologiques, Université de Montréal, Succursale Centre-ville, Montréal, Quebec, Canada; and* [4]*Département de médecine, Faculté de médecine, Université de Montréal, Montréal, Quebec, Canada*

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Voisin G, Bouvet GF, Legendre P, Dagenais A, Massé C, Berthiaume Y. Oxidative stress modulates the expression of genes involved in cell survival in ΔF508 cystic fibrosis airway epithelial cells. *Physiol Genomics* 46: 634–646, 2014. First published June 3, 2014; doi:10.1152/physiolgenomics.00003.2014.—Although cystic fibrosis (CF) pathophysiology is explained by a defect in CF transmembrane conductance regulator (CFTR) protein, the broad spectrum of disease severity is the consequence of environmental and genetic factors. Among them, oxidative stress has been demonstrated to play an important role in the evolution of this disease, with susceptibility to oxidative damage, decline of pulmonary function, and impaired lung antioxidant defense. Although oxidative stress has been implicated in the regulation of inflammation, its molecular outcomes in CF cells remain to be evaluated. To address the question, we compared the gene expression profile in *NuLi-1* cells with wild-type CFTR and *CuFi-1* cells homozygous for ΔF508 mutation cultured at air-liquid interface. We analyzed the transcriptomic response of these cell lines with microarray technology, under basal culture conditions and after 24 h oxidative stress induced by 15 μM 2,3-dimethoxy-1,4-naphtoquinone. In the absence of oxidative conditions, *CuFi-1* gene profiling showed typical dysregulated inflammatory responses compared with *NuLi-1*. In the presence of oxidative conditions, the transcriptome of *CuFi-1* cells reflected apoptotic transcript modulation. These results were confirmed in the *CFBE41o-* and *corrCFBE41o-* cell lines as well as in primary culture of human CF airway epithelial cells. Altogether, our data point to the influence of oxidative stress on cell survival functions in CF and identify several genes that could be implicated in the inflammation response observed in CF patients.

ΔF508; cystic fibrosis; oxidative stress; apoptosis; microarray; two-way permutational multivariate analysis of variance

CYSTIC FIBROSIS (CF) IS THE most frequent autosomal recessive genetic disorder in Caucasians (40). Respiratory manifestations predominate, with chronic airway infection and inflammation beginning in early childhood and leading to progressive loss of lung functions (40). Although the pathophysiology of CF lung disease is not fully understood, it is relatively well established that lung epithelial cells are central to this process. The key role of the epithelium has been emphasized by experimental data showing that CF airway ion transport is abnormal, including reduced transepithelial chloride conductance and increased basal sodium absorption (46). Multiple and controversial hypotheses have been proposed to explain CF pathophysiology, such as the modulation of cAMP-dependent chloride transport driven by CFTR (14), pH dysregulation of airway surface liquid (10), or dysregulation of other ion channels and of intracellular membrane transport processes (49). A number of studies also support the concept that there is an excessive intrinsic inflammatory response in CF lungs. Although the mechanisms underlying this response are still under investigation, the epithelial cells are thought to be a central player in this response (38). Several hypotheses have been proposed to explain the proinflammatory conditions prevalent in CF environment, including increased nuclear factor-κB signaling (26, 33), annexin A1 depletion (5), and aggresome formation in the cells (32). However, the implication of CFTR in regulating the inflammatory response in epithelial cells (20, 33) is still controversial.

Gene expression profiling is an interesting unbiased approach to explore the exaggerated inflammatory response in CF. Several investigations of CF mouse models have reported overexpression of inflammatory genes in CF lungs (19, 23, 53) and intestinal tissue (36). However, these observations could not be consistently reproduced in CF bronchial epithelial cell lines or primary culture of CF epithelial cells (20). Increased modulation of inflammatory genes [interleukin-1 (IL-1), IL-6, and IL-8] has been encountered in CFT-2 cells, an immortalized human tracheal fetal (ΔF508) cell line (50), but has not been found in similar experiments on primary airway epithelial cell culture (56) or in cells obtained by nasal brushing (52). The most striking feature of gene profiling of CF epithelial cells is the lack of consistency in terms of pathways and genes reported to be regulated in CF. It stimulated Hampton and Stanton (21) to reanalyze, via a recently developed statistical approach, the original data from four studies that evaluated the effect of ΔF508-cystic fibrosis transmembrane conductance regulator (CFTR) mutation on gene expression in human airway epithelial (HAE) cells. Their work suggested that CF genotype may impair immune functions in airway epithelial cells but is not associated with overexpression of genes involved in the inflammatory response. However, it might be difficult to identify a common gene expression pattern among all experiments conducted on different cells, tissues, or culture conditions (air-liquid interface or liquid-liquid interface).

Although gene array studies have been performed to ascertain gene expression differences between CF and non-CF cells, few experiments have examined the gene profiling response in CF cells under stress. We choose to explore the transcription response to oxidative stress since CF patients exhibit elevated markers of oxidative stress compared with non-CF healthy

* G. Voisin and G. F. Bouvet contributed equally to this work.
Address for reprint requests and other correspondence: Y. Berthiaume, Inst. de recherches cliniques de Montréal (IRCM), 110 av. des Pins Ouest, Montréal, QC, Canada H2W 1R7 (e-mail: yves.berthiaume@umontreal.ca).

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controls (9, 57). Furthermore, CFTR dysfunction drives mitochondrial defects, alterations in oxidative phosphorylation, calcium homeostasis, oxidative stress, apoptosis, and innate immune response (48). We choose to work with 2,3-dimethoxy-1,4-naphtoquinone (DMNQ) to enhance oxidative stress in CF cells without inducing major perturbations of epithelial functions. Investigations were conducted in two human immortalized bronchial epithelial cell lines, *NuLi-1* cells, isolated from a non-CF patient expressing wild-type (wt) CFTR, and *CuFi-1* cells, a CF cell line homozygous for ΔF508 mutation. Our results demonstrated that *CuFi-1* cells, under oxidative conditions, expressed more genes involved in cell survival than *NuLi-1* cells. To validate these results, the expression of these genes was also tested in ΔF508 CF (*CFBE41o-*) and non-CF CFTR corrected (*corrCFBE41o-*) cell lines. We found that they were modulated similarly as in *CuFi-1/NuLi-1*, which lends a more comprehensive dimension to our bioinformatic analysis. To complete our study, the internucleosomal degradation of genomic DNA was tested to determine if apoptosis was driven similarly by a DMNQ stress in CF and non-CF cell lines. Taken together, these experiments indicate that under oxidative stress, CF epithelial cells are resistant to apoptosis compared with non-CF epithelial cells.

EXPERIMENTAL PROCEDURES

Cell Lines, Primary Bronchial Cells, Experimental Conditions, and RNA Extraction

Two CF and non-CF matched cell lines were studied: first, *CuFi-1* (homozygous ΔF508/ΔF508) derived from CF bronchial epithelium and immortalized E6/E7 and hTERT expression and *NuLi-1* (non-CF), normal HAE cells, were a gift from Dr. J. Zabner, University of Iowa (55). Second, a ΔF508-CFTR (homozygous ΔF508/ΔF508) named *CFBE41o-* is derived from bronchial epithelial cells isolated from CF bronchus at the first bifurcation and immortalized with pSVori-plasmid (18) and a corrected version of *CFBE41o-* transduced with wt-CFTR, named *corrCFBE41o-* (for corrected *CFBE41o-*), were kindly provided by Dr. J. Hanrahan, McGill University. Finally, primary human bronchial cells were isolated from CF homozygous ΔF508/ΔF508 lung transplants (median age 30 yr, mean forced expiratory volume in 1 s: 22.6 ± 1.9%) and non-CF donors. In brief, after lung dissection, bronchi were digested overnight at 4°C under gentle agitation in MEM (Invitrogen) supplemented with 7.5% $NaHCO_3$ (Sigma-Aldrich), 2 mM L-glutamine, 10 mM HEPES (Fisher, Ottawa, Canada), 0.05 mg/ml gentamycin, 25 U/ml penicillin-streptomycin, 0.25 μg/ml Fongizone (Invitrogen), 0.1% pronase E (Sigma-Aldrich), 10 μg/ml DNAse (Sigma-Aldrich), and antibiotic specific from each patient. After pronase-DNase neutralization, the cells were gently scraped off the remaining tissue. The cell suspension was washed and seeded into flasks coated with Purecol (Cedarlane) and cultured in complete SAGM (LHC basal medium supplemented with the SAGM kit, Clonetics) and 25 ng/ml EGF, 100 U/ml of penicillin-streptomycin, 0.07 μg/ml phosphorylethanolamine (Sigma-Aldrich), 1.86 ng/ml ethanolamine (Sigma-Aldrich), 0.05 nM retinoic acid (Sigma-Aldrich), and stock 11, stock 4, and trace elements (kindly provided by Dr. Scott Randell, University of North Carolina). Cultured under air-liquid conditions, the cells were coated on collagen plastic dishes with serum-free bronchial epithelial cell growth medium and supplements (Lonza, Allendale, NJ). The passage number of each type was recorded, and the cells were analyzed at passage 14. After differentiation by cell culture at the air-liquid interface, the cellular monolayer was treated on the apical side with 15 μM DMNQ for 24 h. These conditions were optimal for generating oxidative stress without affecting cellular integrity. At the beginning, 75,000 cells were cultured per filter. The proliferation rate is ~10-fold after 1 wk. The final cell number, although difficult to estimate on filter, should be ~7.5 million. Total RNAs were isolated from each cell line after or no DMNQ treatment with Trizol (Invitrogen), and RNA was purified with RNeasy Mini Kit (Qiagen, Valencia, CA). The quality of total RNA was evaluated by Agilent 2100 Bioanalyzer (Agilent, Palo Alto, CA). All 28s/18S ratios gave values between 1.9 and 2.1 and RNA integrity number ranging from 9.8 to 9.9, where 10 indicates the highest possible RNA quality. We obtained an average of 700 ng/μl eluted in 35 μl, so a final concentration of 24.5 μg of total RNA. Only 4 μg of total ARN were used for cDNA and hybridization.

Glutathione Level

Glutathione (GSH) and glutathione disulfide (GSSG) in bronchial epithelial cells were quantified by a previously described technique (2). In brief, samples were mixed with an equal amount of 10 mM 5,5′-dithiobis(2-nitrobenzoic acid)/0.1 M potassium phosphate, pH 7.5/17.5 mM EDTA, and then centrifuged at 2,000 *g* for 10 min. A 50 μl sample of supernatant was added in a cuvette containing 0.5 unit of glutathione reductase in 0.1 M potassium phosphate, pH 7.5. After 1 min incubation at 25°C, we started the assay reaction by adding 200 nM NADPH/0.1 M potassium phosphate, pH 7.5/5 mM EDTA in a final volume of 1 ml. The rate of 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) reduction was recorded spectrophotometrically at a wavelength of 412 nm using a Envision multiplate reader (Perkin Elmer, Waltham, MA). GSH/GSSG levels were quantified by standard curves generated from known GSSG concentrations (0.125 μM) in PBS (pH 7.4).

To quantify GSSG, a sample of bronchial epithelial cells was mixed immediately after recovery with an equal volume of 10 mM N-ethylmaleimide/0.1 M potassium phosphate, pH 6.5/17.5 mM EDTA and centrifuged at 2,000 *g* for 10 min. Subsequently, 250 μl of supernatant was passed through a Sep-Pak cartridge previously washed with 3 ml of methanol, followed by 3 ml of distilled water. GSSG was eluted from the column with 1 ml of 0.1 M potassium phosphate, pH 7.5/5 mM EDTA/800 μM DTNB/glutathione reductase at 2 units/ml/1 mM NADPH, and the rate of DTNB reduction was measured by spectrometry at 412 nm. Standard curves were derived from dilutions of known GSSG concentrations (0125–4 μM) mixed with 10 mM N-ethylmaleimide and chromatographed in a Sep-Pak C18 column. We quantified GSH by subtracting GSSG from GSH/GSSG levels.

Electrophysiology

Short-circuit currents (I_{sc}) were measured at 37°C in cell monolayers grown at the air-liquid interface on polycarbonate membrane inserts (24 mm diameter; Corning Transwell, Tewksbury, MA) placed horizontally in a modified Ussing chamber. For I_{sc} measurement, the monolayers were bathed in physiological buffer (140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM glucose, 10 mM TES, pH 7.4 at 315 mOsm/kg). Voltage (calomel) and current (Ag/AgCl) electrode pairs were in contact with the apical and basolateral bathing solutions and connected via 2 M KCl/5% agarose bridges. I_{sc} were generated by VCC MC2 voltage clamp amplifier (Physiological Instruments, San Diego, CA) linked to a 4sp PowerLab data acquisition and analysis system (ADInstruments, Grand Junction, CO). The currents were recorded and analyzed with PowerLabs Chart 5 software. We calculated transepithelial resistance (R_{te}) according to Ohm's law by recording the current jump obtained after applying a 1 mV square pulse for 1 s every 10 s.

Microarray Experiment

This experiment was designed to study two factors (ΔF508 mutation and oxidative stress) at two levels (absence and presence) in triplicate. Microarray hybridization was performed with human ex-

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pression HG-U133 Plus 2.0 Genechip arrays (Affymetrix, Santa Clara, CA) following the manufacturer's recommendations. In brief, for each experimental condition, 4 μg of total RNA were reverse-transcribed using T7-$(dT)_{24}$ oligonucleotide as primer. The probes were labeled with biotin and fragmented by Affymetrix's reagents according to a standard protocol. After being washed and stained with streptavidin-phycoerythrin (Invitrogen, Carlsbad, CA), 10 μg of resulting cRNA were loaded on each chip and scanned in a Genechip Scanner 3000 workstation. Raw and processed microarray data were deposited in the publicly accessible Gene Expression Omnibus database (3): accession number GSE39843. We used 12 Agilent chips in these experiments: 2 cell lines (*CuFi-1* and *NuLi-1*) × 2 treatments (1, no treatment; 2, DMNQ treatment 15 μM for 24 h) × 3 replicates per cell line and conditions.

Microarray Data Processing

For each hybridized chip, probe intensity levels were extracted by Affymetrix Scanner 3000 7G, and the data analyzed by Microarray Analysis Suite (version 5.0) algorithm. Quality of the microarray experiments was evaluated according to Affymetrix quality control criteria through internal controls in the Affymetrix platform. First, the absence of hybridization bias was controlled for each chip. Second, several features, including background correction, reduction factor, present percentage, 3′/5′ ratio, and internal controls, were evaluated as per Affymetrix recommendations. In the absence of bias for all these parameters, data from the different chips comprising our experiment were concluded to be of good quality and suitable for further analysis. The expression data had to be transformed by a normalization step to compare the chips to one another. The Robust Multichip Average method (25) was used for this purpose: *1*) quantile transformation was applied, *2*) data from the 1,352,569 probes were summarized to 54,675 probe sets, and *3*) $\log_2$ transformation was carried out. We used 12 Agilent chips: 2 cell lines (CuFi-1 and NuLi-1) × two treatments (1, no treatment; 2, DMNQ treatment 15 μM for 24 h) × 3 replicates per cell line and conditions.

Differential Analysis and Ontology Assessment

Quality control, normalization, and univariate and multivariate differential analysis were assessed by routines from Bioconductor version 2.7 (http://www.bioconductor.org). Differential expression between cell lines in the absence of DMNQ treatment was detected with linear models from the microarray data package (42). A list of genes expressed with significant statistical differences ($P < 0.05$) was generated for *CuFi-1* compared with *NuLi-1*. To study the effect of DMNQ on the gene expression profile of both cell lines, we developed methodology to isolate transcripts that were modulated and provided relevant biological information. Because biological data rarely follow normal distribution, interquartile range (IQR), a nonparametric measure, identified the most relevant expression data. This value, more robust than variance, indicates dispersion around the median and includes 50% of expression level data. The *rda()* function of the vegan package in R statistical language, for canonical redundancy analysis (37), served as a form of two-way multivariate analysis of variance (ANOVA) with which to evaluate the quantitative effect induced by mutation and oxidative stress (30, 31). This approach estimates fractions of variation in gene expression attributable to the experimental factors mutation and oxidative stress. With this method, tests of significance do not require normality because they use the permutation method. Ontological analysis was performed with function hyperGTest of the GOstats package (15). Only significantly overrepresented biological functions ($P \leq 0.05$), composed of a minimum of 20 genes, were taken into account.

mRNA Quantification

mRNA expression levels were quantified by assays designed with the Universal Probe Library from Roche (http://www.universalprobelibrary.com). The high melting temperature characteristic of longer probes is retained by Locked Nucleic Acid nucleotide chemistry in these shorter probes. The reverse transcription-quantitative polymerase chain reaction (RT-qPCR) of selected genes and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and hypoxanthine phosphoribosyltransferase 1 (HPRT1) was performed as described below. Reverse transcription was undertaken with Quantiteck (Qiagen, Toronto, ON, Canada) following the manufacturer's instructions and using either poly-TT or random primers. The PCR amplification was carried out with SYBR Green Master Mix (Applied Biosystems, Grand Island, NY) according to the manufacturer's instructions on Rotor-Gene Q (Qiagen). Reactions contained: 2× Universal PCR Master Mix, 2 μl of template cDNA, 200 nM of primers (all primers deployed in these studies are listed in Table 1) in a final volume of 15 μl. Relative transcript quantity was measured by the comparative threshold (CT) cycle method and normalized with GAPDH or HPRT1 as internal control (Applied Biosystems) by Rotor-Gene 6000 software. cDNA ratio was calculated on the basis of CT values standardized by the amount of GAPDH/HPRT1 housekeeping gene expression. Each PCR reaction was performed in triplicate.

Apoptosis Measurements

Quantitative estimation of oligonucleosomes after DMNQ-induced stress. Photometric enzyme immunoassay (Cell death detection ELISA^{plus}; Roche Applied Science, Grand Island, NY) quantified cytoplasmic histone-associated DNA fragments in vitro after oxidative stress induced by DMNQ. The following protocol modification was suggested by the company for sample preparation: apical supernatants of *CuFi-1/NuLi-1* or *CFBE41o-/corrCFBE41o-* were aspirated and discarded. The monolayers were then rinsed twice with warm PBS before trypsin (0.25%) was added for 7–8 min at 37°C. Following trypsin treatment, cells from the monolayer are collected and resuspended in 2 or 5 ml size in specific medium (DMEM-F-12 + USG + antibiotics), depending on pellet size. About 20,000 cells were counted and resuspended in 100 μl of culture medium (DMEM-F-12) and then recentrifuged (10 min at 3,500 rpm at 4°C). After supernatant removal, the cell pellets were resuspended in 200 μl of lysis buffer for 30 min at room temperature. Finally, 20 μl of supernatant, which represent the cytosolic fraction, were subjected to ELISA according to a standard procedure. For immunofluorescence analysis, the absor-

Table 1.

Gene Symbol	Forward Primer (5′ → 3′)	Reverse Primer (5′ → 3′)
ALOX5	gcctaagaagctcccagtgac	actggtgtgtacaggggtcag
BCL2A1	catcaagaaacttctacgacagc	ttacaaagccattttcccagc
C1S	cttccgctacatcaccac	ttgtcctcacagacatggt
CCL5	cttccgctacatcaccac	ttgtcctcacagacatggt
CXCL11	atgagtgtgaagggcatggc	tcactgcttttaccccaggg
CXLC10	ccagaatcgaaggccatcaa	catttccttgctaactgctttcag
FOS	ctggcgttgtgaagaccat	ccttttctcttcttcttctggaga
HLA-DQB1	gcgatgctgagctcccta	gcccttaaactggaacacga
HLA-DRB1	ccgggctgttcatctacttc	gtggtcatctgcatttcagc
HPRT1	catcaagaaacttctacgacagc	cactaatcacgacgccagggct
IL1A	ggttgagtttaagccaatcca	tgctgacctaggcttgatga
IL1B	ctgtcctgcgtgttgaaaga	ttgggtaatttttgggatctaca
IL1RN	aagacaatgctgactcaaaggag	ctgaaggcttgcatcttgct
IL6	ttcaatgaggagacttgcctg	acaacacaatctgaggtgcc
IL8	ttcaatgaggagacttgcctg	acaacacaatctgaggtgcc
MAK5P3	aatctttagctcaggggggct	gaagtccagctccagtttca
S100A12	agacaccgaagctactctccttca	atccctactctttgtgggtgtggt
S100A8	caagtccgtgggcatcat	gacgtcgatgatagagttcaagg
S100A9	gtgcgaaaagatctgcaaaa	tcagctgcttgtctgcattt
STAT1	tccatatggcttgaacgagcagc	cttagcttctgcgtcggtgagc
THBS1	gccacagttcctgatggag	ccatggagaccagccatc
TNFAIP6	tccatatggcttgaacgagcagc	cttagcttctgcgtcggtgagc
VEGFA	caccatgcagattatgcgga	gctttcgtttttgccccttt

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bance was measured at 405 nm and subtracted from absorbance measured at 490 nm after background correction. Two-way ANOVA was used to analyze the data.

Caspase 3/7 activity. Synergy HT Multi-Mode Microplate Reader (BioTek) in combination with Promega's Caspase-Glo Assay kit (Promega) provides a convenient procedure for measuring caspase-3 or -7 activities. Caspase 3/7 activity was measured in duplicate on CuFi-1/NuLi-1 cell lines using 96-well lumitrac 200 (Biolynx) according to the standard protocol excepted for incubation time, which was optimized for 90 min at room temperature.

RESULTS

Expression Profiling and Gene Ontology Term Analysis of CF (ΔF508) Airway Epithelial vs. Non-CF Cell Lines

The gene expression profile of *CuFi-1* cells, an HAE cell line homozygous for the ΔF508 mutation of CFTR (the most common mutation present in CF), was compared with that of non-CF *NuLi-1* cells by HG-U133 Plus 2.0 Affymetrix microarray. The platform is composed of 54,675 probe sets, representing ~38,500 genes (estimated by UniGene identifiers). Between *CuFi-1* and *NuLi-1* cells, 9,755 transcripts were expressed differentially as monitored by moderated *t*-test ($P < 0.05$, Fig. 1). From these selected transcripts, 4,438 were upregulated and 5,317 downregulated in *CuFi-1* compared with *NuLi-1* cells. To define the biological processes modulated in *CuFi-1* cells, Gene Ontology (GO) term analysis of all significantly altered transcripts was undertaken. As reported in Fig. 2*A*, GO term analysis highlighted three preponderant groups of processes: cellular survival, ribonucleotide metabolism, and regulation of signaling pathways. In a second step, we chose transcripts classified as upregulated (Fig. 2*B*) and downregulated (Fig. 2*C*), to identify biological processes specifically overrepresented in these selections. Inflammation, regulation of signal transduction, and cellular survival were also overrepresented in upregulated transcripts, and ribonucleotide metabolism, chromatin modification, and translational elongation were significantly overrepresented in downregulated transcripts. To summarize, the transcriptomic response of *CuFi-1* cells can be divided into four groups of processes: cellular survival, ribonucleotide metabolism, signal transduction, and inflammation.

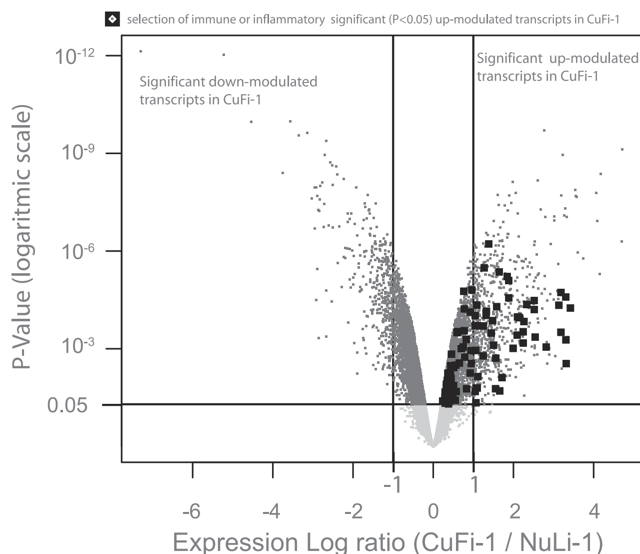

Fig. 1. Volcano plot showing distribution of the expression ratio in *CuFi-1* compared with *NuLi-1* cells. Upregulated, downregulated are illustrated in gray, and immune or inflammatory transcripts are illustrated in black squares. $P < 0.05$ values are considered significant, and the vertical lines indicate a 2-fold difference between the 2 cell lines.

Modulation of Inflammatory and Immune Response Transcripts in CuFi-1 Compared With NuLi-1 Cells in Culture

After GO term analysis, we identified the overrepresentation of several processes associated with immune and inflammatory responses represented by 123 upregulated transcripts. From these 123 transcripts, 19 genes described in the literature as being involved in CF were validated by RT-qPCR to confirm the modulation observed in the microarray experiment. As shown in Fig. 3, the two techniques gave similar *CuFi-1/NuLi-1* expression ratios, except for HLADRB1 and HLADQB1. Several transcripts already reported to be upregulated in CF pathophysiology (IL-6, IL-8, IL-1B and IL-1A) were found to be significantly modulated in *CuFi-1* cells, and these changes were confirmed by RT-qPCR (Fig. 3). Genes indirectly linked to inflammatory responses, such as signal transducer and activator of transcription 1 (STAT1, modulation confirmed by RT-qPCR) and FBJ murine osteosarcoma viral oncogene homolog (cFOS, modulation not confirmed by RT-qPCR), were also upregulated. Also related to inflammatory processes, genes of the alarmin family were shown to be upregulated in CF cells (in the microarray experiment), such as IL-33 (22), lactotransferrin (11), heat shock protein 70, defensin beta 4, or calcium-chelating S100 family proteins (7) (also called calgranulins S100A8-A9-A12, confirmed by RT-qPCR). An alarmin-like protein, chitinase-like 1 protein (29), was also upregulated.

Gene Modulation Induced by Oxidative Stress in CF Cell Lines

Several lines of evidence indicate that oxidative stress is increased in CF patients (9, 28, 57), impacting several biological functions involved in CF pathophysiology (9). Nevertheless, its involvement in transcriptomic modulation has not yet been investigated. To evaluate this aspect, we exposed *CuFi-1* and *NuLi-1* cells for 24 h to DMNQ, an oxidative stress inducer. The cellular level of total GSH (pmol/μg of protein) was measured to assess the oxidative effect of DMNQ. DMNQ treatment for 24 h elicited a significant increase of total GSH in *CuFi-1* and *NuLi-1* cells at all concentrations tested (Fig. 4*A*). In addition to its oxidative action, DMNQ affected the electrophysiological properties of *CuFi-1* cells in a dose-dependent manner. At high concentration (20 μM), DMNQ significantly decreased the total I_{sc} of *CuFi-1* (Fig. 4*B*), but R_{te} remained relatively stable (Fig. 4*C*). No significant modification of I_{sc} or R_{te} was evident in *NuLi-1* cells. After obtaining these preliminary results, we treated *CuFi-1* and *NuLi-1* cells with 15 μM DMNQ in transcriptomic experiments to evoke significant oxidative stress without affecting cellular integrity and bioelectrical properties.

To investigate the effects of oxidative stress in these cell lines, we studied gene expression as previously explained. Because we needed to consider two conditions, CF mutation

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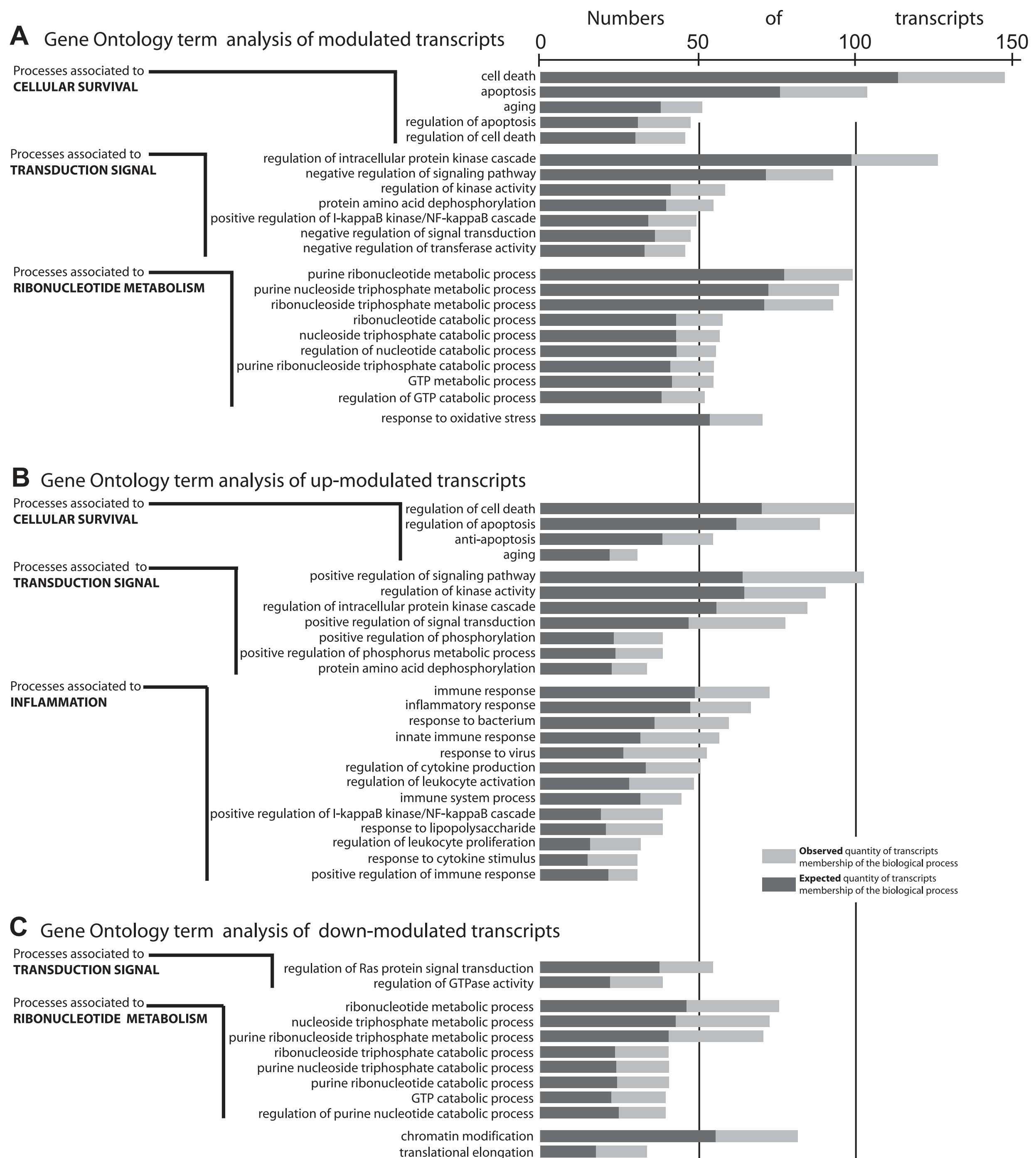

Fig. 2. Gene Ontology (GO) term analysis of genes expressed differentially in *CuFi-1* and *NuLi-1* cells. These bar plots summarize the results of GO term analysis for 3 selections of transcripts: modulated transcripts (*A*), upmodulated transcripts (*B*), downmodulated transcripts (*C*). In this representation, biological functions fitting 4 criteria are shown: significantly overrepresented ($P < 0.05$), composed of at least >30 transcripts (for a minimum of 50 transcripts in *A* and for a minimum of 30 transcripts in *B* and *C*), odds-ratio <1.5, associated with 1 of 4 biological processes: cellular survival, inflammation, ribonucleotide metabolism, transduction signal. The observed (in light gray) and expected (dark gray) number of transcripts for each GO term are illustrated in the bar plot. The GO terms that were overrepresented in the CuFi but not associated with the 4 selected biological processes are not included in the figure.

and oxidative stress, to select modulated transcripts, we proceeded in two steps: first, we retained those genes, with an IQR of 0.5, where the expression was modulated significantly by mutation and/or oxidative stress (see *Differential Analysis and Ontology Assessment* in EXPERIMENTAL PROCEDURES). Second, the selected transcripts underwent Rao's redundancy analysis (RDA), to quantify the percentage of the variance of the gene expression levels that can be attributed to each factor (mutation and oxidative stress). Using an IQR threshold of at least 0.5 and a percentage of explained variance of at least 20% for each experimental condition, we isolated 687 interesting transcripts (Fig. 5) (see *Differential Analysis and Ontology*

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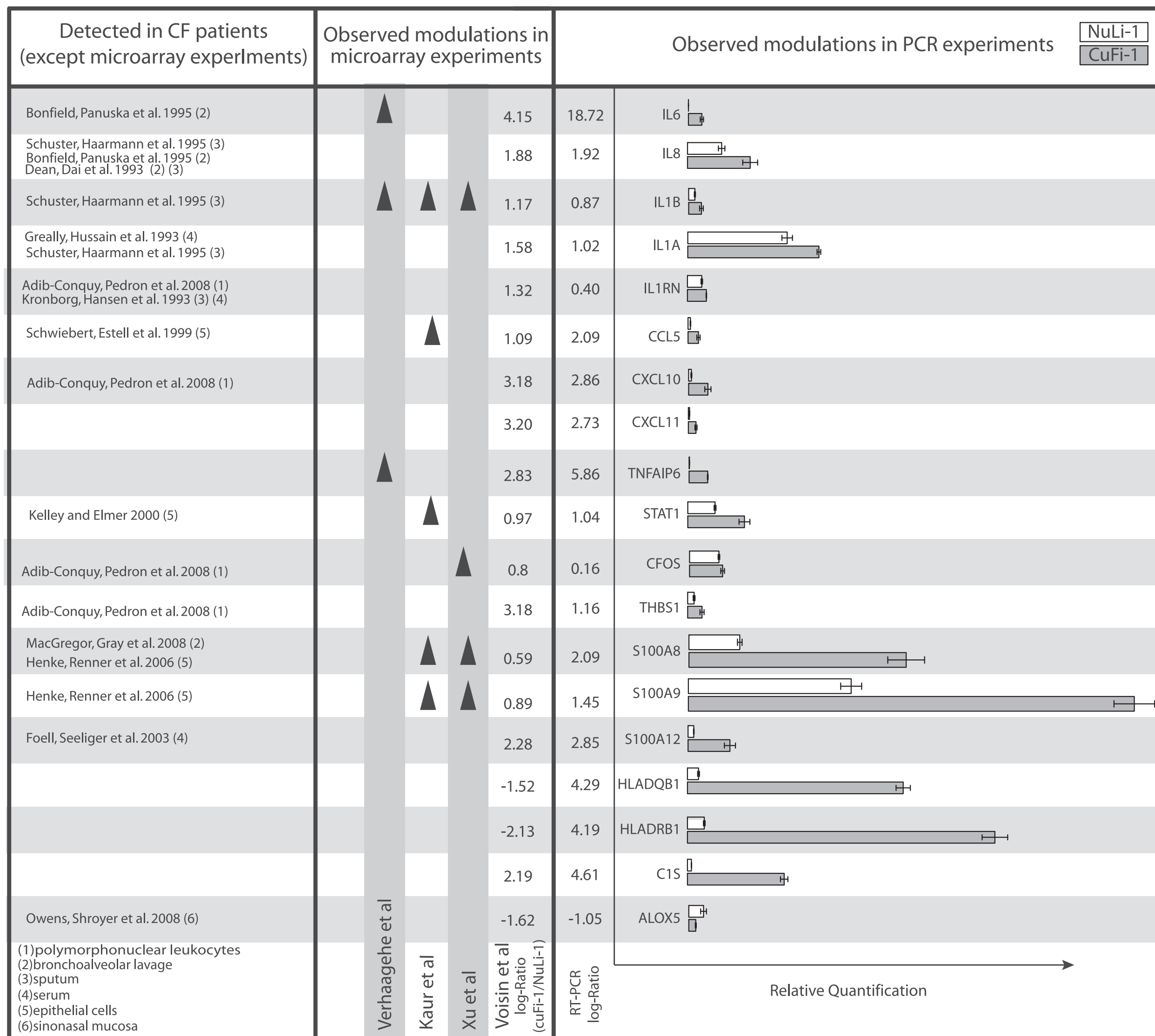

Fig. 3. Comparison of expression log-ratios between microarray and RT-qPCR experiments. The expression log-ratio microarray results of 19 transcripts were compared with their expression log-ratio by RT-qPCR. We also report the observed modulation or detection of these transcripts in other experiments. All the expression data were done in triplicate, starting with 3 distinct cultures of each cell lines (with or without treatment). For each gene, error bars on the histogram for the relative expression are indicated.

Assessment in EXPERIMENTAL PROCEDURES). We then applied a GO term analysis to determine the biological processes that were overrepresented processes in theses transcripts. Applying IQR filter, RDA, and GO term analyses, we identified three significant biological processes: oxidation/reduction, cell death, and apoptosis (Fig. 5). Supported by the GO database, 59 transcripts were found to participate in the cell death process (GO: 0008219); they included 29 transcripts associated with apoptosis (GO: 0006915). For this reason, we only assessed the expression of these 59 transcripts in the next analysis, to examine the impact of mutation and oxidative stress on the processes implicated in cell death.

To investigate the expression profiles of the 59 transcripts in *CuFi-1* and *NuLi-1* cells and highlight the modulation induced by DMNQ, we applied supervised classification based on the Mfuzz c-means algorithm (16). Among the 59 transcripts selected, only 44 showed DMNQ-dependent expression profiles, which were segregated into two tendencies: downregulation of 25 transcripts (Fig. 6*A*) and upregulation of 19 transcripts (Fig. 6*B*). Based on the RDA, we were able to rank these transcripts as a function of the ratio variance explained by experimental conditions (ΔF508 mutation and oxidative stress)/ total explained variance. Other genes indirectly involved in the modulation of survival were also present. The vascular endothelial growth factor A (VEGFA) is implicated in activation of apoptosis and associated with hyperinflammation in CF. In addition, stratifin is involved in VEGFA signaling and in regulation of the mitogen-activated protein kinase (MAPK) cascade. Finally, many RDA-selected transcripts are involved in MAPK cascade regulation, such as the MAP kinases MAP3K5 and MAPK1 and some indirect regulators (ERBB3, CASP1, CD14, TNFRSF10A). Finally, we noted the presence of BCL2A1, an element that is important in the regulation of apoptosis.

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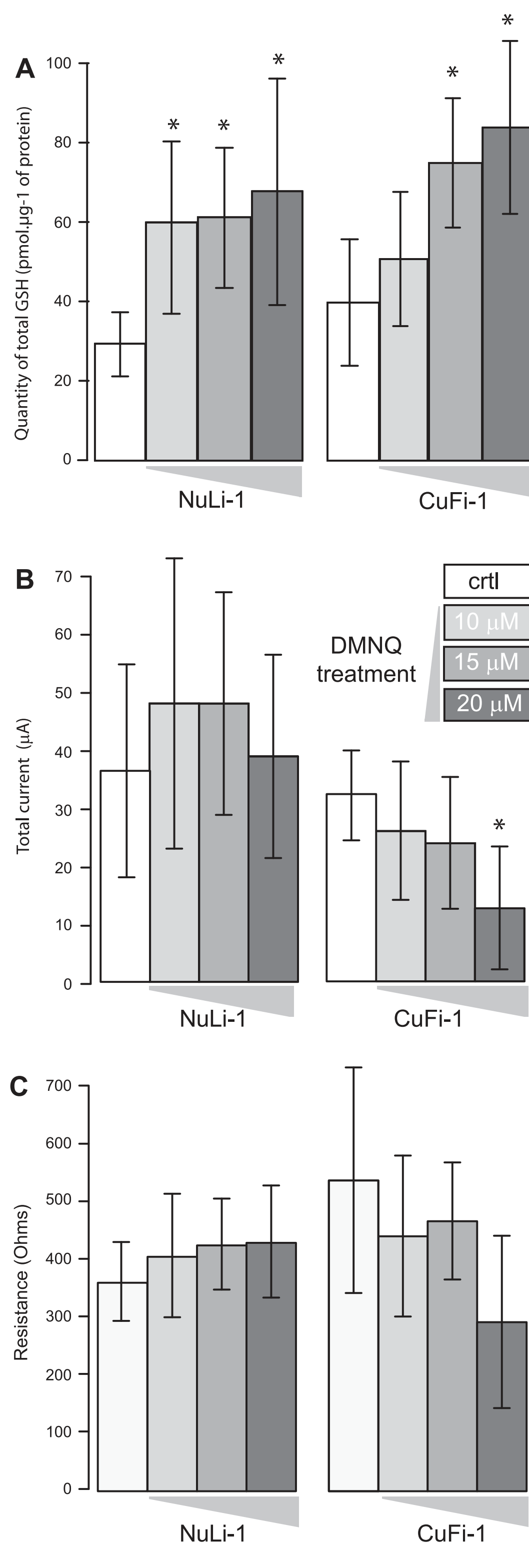

Fig. 4. Dose-response study evaluating the effect of 2,3-dimethoxy-1,4-naphtoquinone (DMNQ) on *CuFi-1* and *NuLi-1* cell lines. *A*: total glutathione (GSH) was measured to evaluate the oxidative stress generated by different DMNQ concentrations (0, 10, 15, 20 μM) on *CuFi-1* and *NuLi-1* cell lines. *B*: the impact of DMNQ on total current (μA) was also recorded. *C*: the physiological impact of DMNQ was also estimated by measuring the transepithelial resistance (Ohms). The data are shown as means ± SD. Significant differences were revealed by parametric 2-way ANOVA (*$P \leq 0.05$ of difference from the controls). The gene expression study was done with a concentration of 15 μM.

Validation of Targeted Genes After Oxidative Stress in CFBE Cell Lines

Based on the above results, we decided to validate the expression of MAP3K5, VEGFA, and BLC2A1 in another cell model, CFBE (homozygous ΔF508) and *corrCFBE41o-*, a corrected version of *CFBE41o-* transduced with wt-CFTR (see EXPERIMENTAL PROCEDURES). As illustrated in Fig. 7*A*, MAP3K5 expression was significantly decreased after DMNQ treatment in the *CFBE41o-* and the *corrCFBE41o-*, whereas VEGFA and BCL2A1 were increased. These results are comparable to those obtained in *CuFi-1/NuLi-1* with microarray and RDA selection. The results were also validated in CF and non-CF primary bronchial cells. Results demonstrated an upregulation of BCL2A1 and VEFGA as shown in cell lines. However, we could not show any significant modulation of expression of MAK3P5 in primary bronchial CF cells (Fig. 7*B*).

Absence of Apoptosis Induction in CF Cell Lines After Oxidative Stress

The internucleosomal degradation of genomic DNA to oligonucleosomal fragments generated by Ca^{2+}/Mg^{2+}-dependent endonucleases is well accepted as a biochemical marker of apoptosis. Based on our transcriptional results, we hypothesized that apoptosis would be modulated in CF cell line. Therefore, we quantified oligonucleosome fractions in CF (*CuFi-1/CFBE41o-*) and non-CF (*NuLi-1/corrCFBE41o-*) cell lines before and after DMNQ stress (Fig. 8). In the absence of DMNQ in *CuFi-1* cells, we did not observe a significant increase in the release of oligonucleosomal fragments compared with the *NuLi-1* cells. However, after DMNQ-induced stress, there is a significant increase in the release of oligonucleosomes in the *NuLi-1* cells compared with the *CuFi-1* cells. *CFBE41o-* and *corrCFBE41o-* cell lines were tested to confirm defective apoptosis in another CF cell type background. Cells were treated with 10 μM DMNQ, and the results were similar to those obtained with oligonucleosomes in *CuFi-1/NuLi-1*: in both cell types, DMNQ induced an increase of oligonucleosome release. However, the response of *corrCFBE41o-* was stronger compared with *CFBE41o-* cells, again indicating a tendency of CF cell lines to resist apoptosis.

To confirm these observations, we measured Caspase 3/7 activity in *CuFi-1/NuLi-1* in the presence or absence of DMNQ. Results show no increase in Caspase 3/7 activity, confirming a decreased in apoptotic activity in CF cells as observed with the oligonucleosomes assay (Fig. 9).

DISCUSSION

On the basis of the complexity of CF pathophysiology, influenced by environmental conditions as well as genetic background, we adopted a whole ORFeome microarray approach to study the transcriptional adaptation of CF cells exposed to oxidative stress. First, we demonstrate that there is an overrepresentation of several processes associated with immune and inflammatory responses in the CF cell line studied (*CuFi-1*). Second, using a novel approach based on canonical analysis, which estimates the contribution of two experimental conditions to the variance of each gene, we show that oxidative stress induced by DMNQ treatment elicited upregulation of genes involved in inflammatory responses and apoptosis. Our

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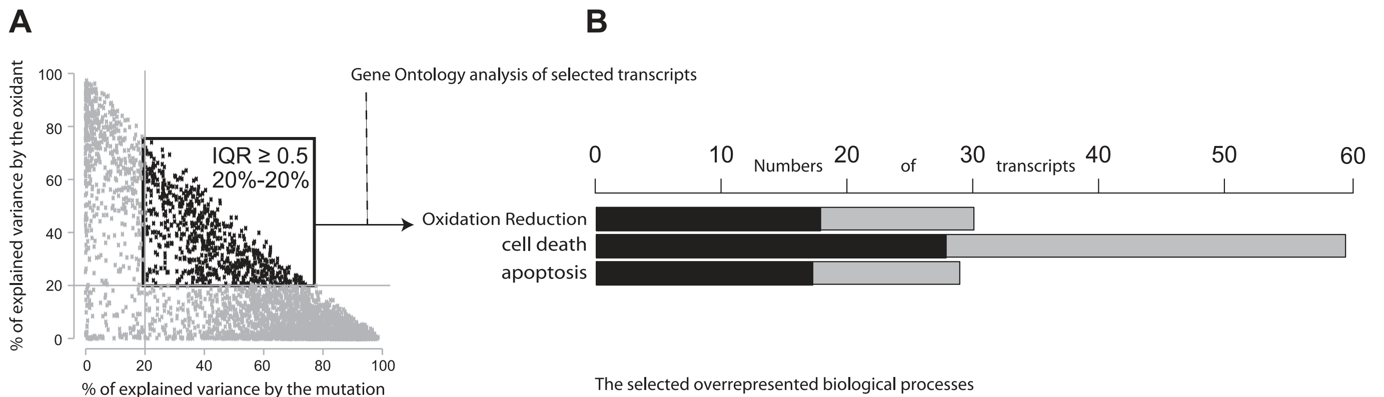

Fig. 5. Comparative GO term analysis. Enrichment of GO term following the selection of transcripts based on interquartile range (IQR) and taking into account the % of explained variance. *A*: based on the IQR filters, selection of 687 transcripts having an IQR value ≥ 0.5 that have % of explained variance >40% (decomposed in at least 20% explained by the mutation and at least 20% explained by the oxidant). *B*: a GO analysis was performed on the selected gene to test the overrepresentation of biological processes for this selection. Among the most represented pathways, oxidation/reduction, cell death and apoptosis were significantly overrepresented.

results point to the involvement of important molecules, such as BCL2A1 and VEGFA, in the mechanisms of apoptotic modulation of CF cell lines under oxidative stress.

Proinflammatory Profile of CuFi-1/NuLi-1 Cell Lines

In the present study, the CF model tested was derived from HAE cells carrying the ΔF508 mutation (*CuFi-1*) (55). This cell line constitutes a polarized monolayer with expected trans-epithelial activity in air-liquid interface conditions that mimic the behavior of airway epithelial cells in vivo. The CuFi-1/NuLi-1 model has been investigated in many CF studies to explore the CF inflammatory response (6, 12) and the CF epithelial repair processes (47) and to compare the transcriptomic response in CF epithelia (17). Our transcriptome analysis of *CuFi-1* and *NuLi-1* cells demonstrated overrepresentation of genes associated with the inflammatory response, such as of

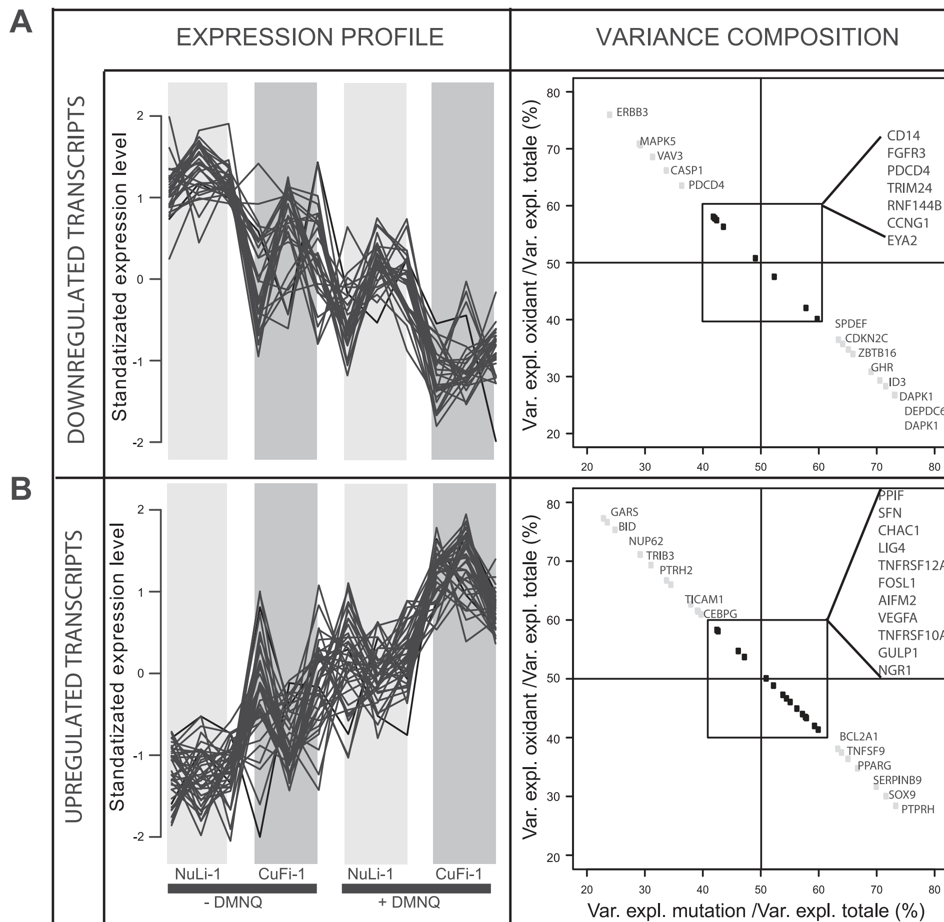

Fig. 6. Downmodulated and upmodulated trends of gene expression for *CuFi-1* vs *NuLi-1* cells in the absence/presence of DMNQ. Expression profiles were clustered according to the Mfuzz algorithm from standardized expression data on 96 probes (corresponding to 59 distinct transcripts) with IQR >0.5 associated with cell death and apoptotic processes. Expression profiles of upregulated transcripts dependent on DMNQ and ΔF508 mutation (*B*) and downregulated transcripts dependent on DMNQ and ΔF508 (*A*) for *CuFi-1* vs *NuLi-1*. To the *right* of each cluster, we illustrate for each transcript the repartition of the total variance between the factors mutation and oxidant. The central black square highlights those transcripts where the total variance is explained by at least 40% but not more than 60% for 1 factor.

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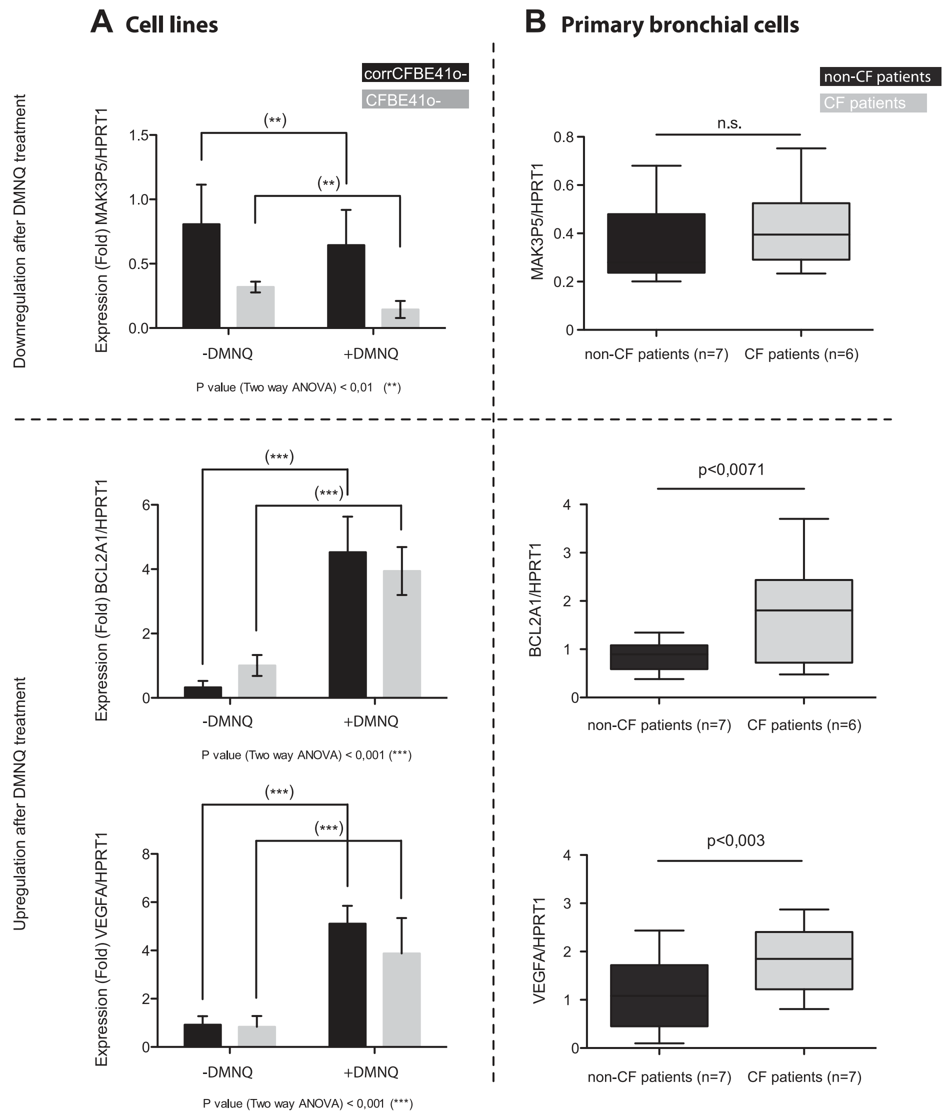

Fig. 7. Evaluation of MAP3K5, VEGFA, and BCL2A1 mRNA expression by RT-qPCR on CF and non-CF cell lines after oxidative stress and in CF and non-CF primary bronchial cells. *A*: the HPRT1 gene was used to normalize MAP3K5, VEGFA, and BCL2A1 expression. VEGFA and BCL2A1 expression in CF and non-CF cells was significantly upregulated (***$P < 0.001$) after DMNQ treatment, as described previously in *CuFi*/*NuLi* cell lines. MAP3K5 was significantly decreased (**$P < 0.01$) in CF and non-CF cell lines after DMNQ treatment. *B*: the HPRT1 gene was used to normalize MAP3K5, VEGFA, and BCL2A1 expression. VEGFA and BCL2A1 expression was significantly upregulated in bronchial CF cells. There was no significant increase of the MAP3K5 gene in the bronchial CF cells. The data are reported as fold expression obtained by standard curve analysis.

interleukins (IL-1A, IL-1B, IL-6, and IL-8) and chemokines (CXCL1, CXCL10, CXCL11, CXCL2, CXCL3, CXCL5, and CXCL6) in *CuFi* cells. We were also able to confirm the modulation of selected genes (Fig. 3) by RT-qPCR. Furthermore, many of the upregulated genes identified in our study, such as IL-6, IL-8, IL-1B, and IL-1A, have also been identified in other CF tissues (19, 23, 36, 53). However, in recent experiments on CFBEs, the authors report no compelling evidence that mutations in CFTR induce a hyperinflammatory response in CF epithelial cells (20). This discrepancy in the expression of inflammatory genes could be explained by the diversity of experimental models of CF tissues or CF epithelial cells to study this question (1, 4, 20, 33). Second, the difference between our data and theirs could argue in favor of other regulatory mechanisms involved in the regulation of inflammation in CF cell lines. One of these mechanisms could be epigenetic regulation. Indeed, DNA methylation or histone tail modification profiles, which are well known to influence gene expression, could be cell line specific, independent of ΔF508 mutation or dependent on cell culture conditions. We have recently demonstrated different DNA methylation profiles of *CFBE* and *CuFi-1/NuLi-1* cell lines (8). Further studies are necessary to understand the role of these epigenetic modifications in CF cells.

RDA Analysis Highlights Pathways Modulated by Oxidative Stress in CF Cell Lines

To understand the impact of oxidative stress on the transcriptome of CF cells, we included two factors (experimental conditions: cell lines and oxidative stress) in the experimental design, which allowed us to draw relevant biological conclusions. The challenge was to perform single two-way ANOVA of multivariate response data. Some approaches have been developed with multifactorial design, such as comparison of Student's test results by Venn diagram (42) or two-way ANOVA (38), but are debatable because their robustness is very dependent on biological replication included in the study and present the problem of correction for multiple testing. To resolve this issue, we adopted a robust method of two-way ANOVA for multivariate response data, as proposed by Legendre and Anderson (30), which uses RDA for variance parti-

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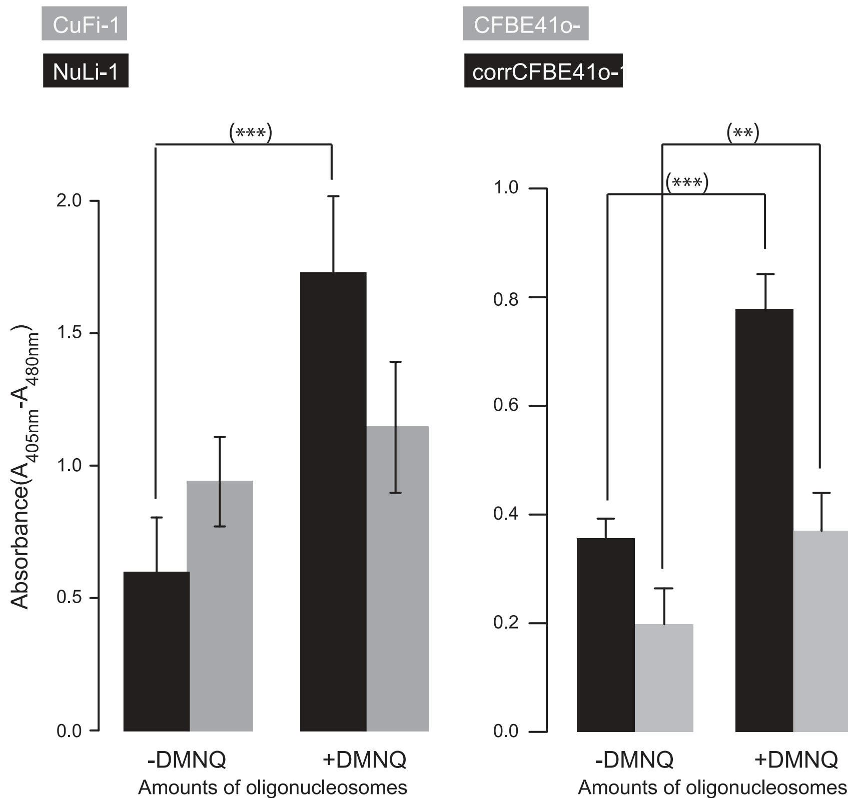

Fig. 8. Oligonucleosomes release after DMNQ treatment in CF and non-CF cell lines. As a specific marker of apoptosis induction, histone-associated DNA fragments (mono- and oligonucleosomes) were quantified by photometric enzyme immunoassay in CF and non-CF cell lines. We performed 2-way ANOVA on background-normalized absorbance. There is a significant increase in the oligonucleosome release in the cytosol of the *NuLi-1* cells compared with the *CuFi-1* cells in presence of DMNQ. In the *corrCFBE41o-* (non-CF) and *CFBE41o-* (CF) cells, in presence of DMNQ, There is an increase of oligonucleosomes in the CF and non CF cells, but the increase was more important in *corrCFBE41o-* (non-CF) compared with *CFBE41o-* (CF) cells.

tioning. The method is largely applied in ecological research (31). Its robustness derives from permutation testing for the effects of main factors and the interaction between them; permutation tests do not require the normality of residuals.

This approach combines multiple regression and principal component analysis to establish relationships between response variables (expression level) and explicative variables (experimental conditions). The method highlights trends in the scatter of data points that are maximally and linearly related to a set of constraining (explanatory) variables. In other words, RDA determines the proportion of expression level variance that is accounted for by experimental conditions. Hence, for a specific probe, the expression levels measured in several experimental conditions could be summarized by the proportion of explained variance of each condition. For example, the RDA of an experimental design with two factors (A and B) at two levels (absence, presence), in triplicate conditions, allows the representation of a list of 12 expression level values associated with a specific probe (genes) by only two values, each being the proportion of the explained variance of each factor. This representation of data is realized without loss of generality. This approach, based on the ordination method, proposes a different analytical view for multifactorial design, compared with classical multivariate approaches, which could be powerless for the analysis of design with few biological replicates. Its advantage is that it quantifies the effects associated with each experimental condition and establishes a threshold for the selection of candidate transcripts that is more realistic than the classical statistical threshold (i.e., the percentage of variance explained by an experimental condition is more interpretable of a *P* value in biological problems). Nevertheless, RDA assumes that the relationships between explanatory and response variables are linear and may underestimate the percentage of variance that could be explained by nonlinear models (true for any form of linear ANOVA).

In the present study, based on such representation, we selected probes that had at least 20% of variance explained by each experimental condition to investigate transcripts modulated by ΔF508 mutation and oxidative conditions. Using this analytical technique we were able to demonstrate that pathways involved in cell death and apoptosis were overrepresented in the biological processes modulated by oxidative stress in CF cells (Fig. 5). The overrepresentation of these biological processes adds to the body of knowledge regarding the link between CFTR defects, inflammation, and apoptosis (43).

Antiapoptotic Profile of CF Cell Lines Under Oxidative Stress

To study the transcriptomic response of CF cells in the presence of oxidative stress, we decided to work with DMNQ, which elicited intracellular oxidative stress and oxidative DNA damage. With this oxidative stress, we were able to evoke a chronic stress response, as demonstrated by increased GSH after 24 h exposure (Fig. 4*A*), without significant biological perturbation of resistance integrity and cell function (transepithelial transport; Fig. 4, *B* and *C*). The DMNQ concentration

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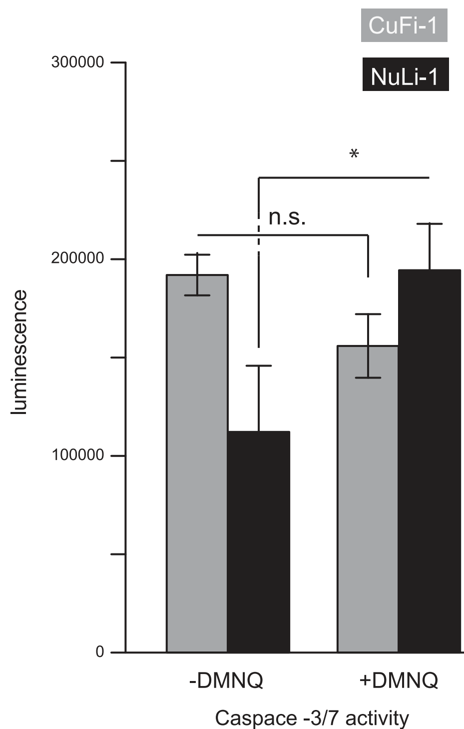

Fig. 9. Caspase 3/7 after DMNQ treatment in CF and non-CF cell lines. As a specific marker of apoptosis induction, Caspase 3/7 activity was quantified by liminometric assay in CF (CuFi-1) and non-CF (NuLi-1) cell lines. Following DMNQ treatment, there is a significant increase (* $P < 0.05$) in the caspase activity in the *NuLi* cells but not in the *CuFi* cells.

used in our protocol induced intracellular stress that mimicked the oxidative stress present in bronchial epithelial cells of CF patients. DMNQ was also chosen because it produces apoptosis via mitochondrial release of cytochrome c and caspase-9 and -3 activation in C17.2 cells (45), and intracellular oxidative stress is associated with enhanced apoptosis in pancreatic (CFPAC1) and tracheal CF (CFT-2) cells (41).

In our study, after chronic oxidative stress induction, the amount of oligonucleosomes (Fig. 8) released by the *CuFi-1* cell line after exposure to DMNQ was lower in *CuFi-1* cells compared with their controls (*NuLi-1*), suggesting resistance of the CF cell lines to apoptosis under oxidative stress. This intriguing result was confirmed by the same technique in the *CFBE41o-* cell line. Furthermore, we saw no activation of the Caspase 3/7 in *CuFi-1* cells compared with their controls (*NuLi-1*) following DMNQ exposure (Fig. 9).

These results add to the controversy regarding the susceptibility of CFTR-deficient epithelial cells to apoptosis (43). Rottner et al. (41) has shown that CF cells are more sensitive to apoptosis induced by actinomycin D or staurosporine. However, Yalcin et al. (54) have reported that increased apoptosis in the epithelial cells may be related to the presence of chronic infections rather than CFTR dysfunction. Antiapoptotic profiles have also been described in $CFTR^{(-/-)}$ mice (13). Although these results highlight the potential role of CFTR in cell survival, the impact on apoptosis might be cell specific and depend on the environmental condition (intrinsic or extrinsic stimulus) surrounding the CFTR-deficient cells.

To identify potential molecular pathways that could be involved in the modulation of cell survival by CFTR, we performed supervised classification based on the Mfuzz c-means algorithm (Fig. 6). This analysis demonstrates that there are transcripts that are upregulated in presence of CFTR defect and further upregulated in presence of DMNQ. There are also transcripts that are downregulated in presence of CFTR defect and further dowregulated in presence of DMNQ. Furthermore, the level of expression of each of these transcripts is dependent, in our experimental model, on at least two factors (oxidative stress and CFTR mutation). The variance of some of the transcripts is mainly dependent on the presence of oxidative stress, whereas other transcripts depend more on the CFTR mutation (Fig. 6). From these results, we can hypothesize that depending on the type and level of stress applied to CF cells that the molecular survival or death pathways activated might be different.

To identify potential pathways that could be implicated in the resistance of CF cells to oxidative stress induced by DMNQ we have confirmed by RT-qPCR the expression level of three molecular targets well studied in cell survival in another cell line (*CFBE41o-/corrCFBE41o-*), as well as in a primary culture of bronchial epithelial cells. We demonstrated that oxidative stress was associated with increased VEGF in both cellular models (Fig. 7). This observation is particularly interesting since it has been shown that VEGF protein level is elevated in CF patients (34, 35). VEGF's protective activity in the oxidative condition is dependent on BCL2A1 (24), the other molecular pathway potentially involved in resistance to apoptosis and potentially involved in CF resistance to apoptosis (27). BCL2A1 protein also reduces the release of proapoptotic cytochrome c from mitochondria and blocks caspase activation. It is the only member of its family to be associated with the inflammatory response (51). Our microarray and RT-qPCR analyses revealed upregulation of BCL2A1 gene after DMNQ-induced stress (Fig. 7), and this upregulation in CF cells indicates its potential participation in antiapoptosis action. According to our results, VEGFA and BCL2A1 are upregulated in both cell lines but also in primary bronchial cells of CF patients, arguing in favor of an increase antiapoptotic activity in CF.

Finally, we also obtained potential evidence of MAPK signaling pathway participation in this process. MAPK genes comprise a large family of protein kinases that enable cells to respond to exogenous and endogenous stimuli (39). They build up an intracellular network that translates and integrates signals into cytoplasmic and nuclear processes. The phosphorylation states of MAPK signaling pathways modulate multiple cellular functions, such as proliferation, differentiation, and apoptosis (44). We have observed modulation of mRNA expression of several molecules in the MAPK pathway. MAP3K5 (also named ASK1) is downregulated in *CuFi-1* and *CFBE41o-* exposed to DMNQ. However, we could not identify a significant decrease of MAP3K5 in the primary culture of epithelial cells. This lack of decrease in the primary culture could be secondary to a lack of power of our sample size or to the complexity and variability of the signaling pathways involved in apoptotic regulation. Taken together, these elements suggest that deficient apoptotic signaling in CF cell lines, either *CuFi-1* or *CFBE41o-*, under oxidative conditions, could derive from a complex regulatory system implicating VEGFA, BCL2A1 and the MAPK signaling pathway.

In conclusion, the new, permutational, multivariate, two-way ANOVA used in our study identified interesting tran-

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scripts by computing the proportion of their variance explained by experimental conditions. The approach could be relevant in biomedical problems by integrating more than one experimental factor. Based on this analysis, we demonstrated the modulation of biological processes associated with survival functions in CF cell lines under oxidative stress. Modulation could potentially be explained by dysregulation of the MAPK signaling pathway and the potential role of BCL2A1 and VEGFA, pro- and antiapoptotic genes, respectively, in the apoptotic response.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: G.V., G.F.B., and Y.B. conception and design of research; G.V., G.F.B., C.M., and Y.B. performed experiments; G.V., G.F.B., P.L., C.M., and Y.B. analyzed data; G.V., G.F.B., P.L., A.D., and Y.B. interpreted results of experiments; G.V., G.F.B., and Y.B. prepared figures; G.V., G.F.B., and Y.B. drafted manuscript; G.V., G.F.B., P.L., A.D., and Y.B. edited and revised manuscript; G.V., G.F.B., and Y.B. approved final version of manuscript.

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