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ORIGINAL ARTICLE

Cadmium-induced ER stress and inflammation are mediated through C/EBP–DDIT3 signaling in human bronchial epithelial cells

Jeeyoung Kim[1,2,5], Haengseok Song[3,5], Hye-Ryeon Heo[1,2,5], Jung Woon Kim[1,2], Hye-Ryun Kim[3], Yoonki Hong[1,2], Se-Ran Yang[4], Seon-Sook Han[1,2], Seung-Joon Lee[1,2], Woo Jin Kim[1,2] and Seok-Ho Hong[1,2]

Cadmium (Cd), a major component of cigarette smoke, disrupts the normal functions of airway cells and can lead to the development of various pulmonary diseases such as chronic obstructive pulmonary disease (COPD). However, the molecular mechanisms involved in Cd-induced pulmonary diseases are poorly understood. Here, we identified a cluster of genes that are altered in response to Cd exposure in human bronchial epithelial cells (BEAS-2B) and demonstrated that Cd-induced ER stress and inflammation are mediated via CCAAT-enhancer-binding proteins (C/EBP)-DNA-damaged-inducible transcript 3 (DDIT3) signaling in BEAS-2B cells. Cd treatment led to marked upregulation and downregulation of genes associated with the cell cycle, apoptosis, oxidative stress and inflammation as well as various signal transduction pathways. Gene set enrichment analysis revealed that Cd treatment stimulated the C/EBP signaling pathway and induced transcriptional activation of its downstream target genes, including DDIT3. Suppression of DDIT3 expression using specific small interfering RNA effectively alleviated Cd-induced ER stress and inflammatory responses in both BEAS-2B and normal primary normal human bronchial epithelial cells. Taken together, these data suggest that C/EBP signaling may have a pivotal role in the early induction of ER stress and inflammatory responses by Cd exposure and could be a molecular target for Cd-induced pulmonary disease.
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INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is a major cause of morbidity and mortality, the prevalence of which is increasing worldwide.[1,2] COPD is characterized by progressive airway obstruction with chronic and irreversible inflammation of the airways and lung tissues, as a result of prolonged exposure to inhaled irritants such as cigarette smoke.[1] Long-term cigarette smoking is the most significant and commonly encountered risk factor for COPD. Chronic inflammation caused by cigarette smoke in COPD patients likely has an important role in the pathogenesis of lung cancer.[3]

Cadmium (Cd), a major component of cigarette smoke, has a significant impact on lung function and might be associated with the development of COPD by disrupting homeostasis in the endoplasmic reticulum (so-called ER stress) and subsequent pro-apoptotic signaling.[4–6] Therefore, understanding the molecular alterations initiated by Cd exposure to lung tissue is essential to prevent and manage the development of COPD. The ER is the primary intracellular organelle for proper protein synthesis, folding and assembly. ER stress triggers an evolutionarily conserved intracellular response called the unfolded protein response (UPR), which is initiated by the activation of ER stress transducers including inositol-requiring enzyme 1, protein kinase RNA-like ER kinase and activating transcription factor (ATF) 6. Binding immunoglobulin protein (BiP) has a central role in ER stress signaling. Upon ER stress, the release of these transducers from BiP triggers the UPR, which regulates the balance between cell survival and apoptosis.[7,8] Cd induces cell death, apoptosis and DNA damage through ER stress-triggered UPR in several cell types. For example, Cd can induce DNA damage by activating the ER stress response in hepatocarcinoma cells.[9] Cd also induces neuronal cell death by the generation of reactive oxygen species (ROS) followed by disruption of ER homeostasis.[10] However,

[1]Department of Internal Medicine, School of Medicine, Kangwon National University, Chuncheon, South Korea; [2]Environmental Health Center, Kangwon National University Hospital, Chuncheon, South Korea; [3]Department of Biomedical Science, College of Life Science, CHA University, Seongnam, South Korea and [4]Department of Thoracic and Cardiovascular Surgery, School of Medicine, Kangwon National University, Chuncheon, South Korea
[5]These authors contributed equally to this work.
Correspondence: Dr S-H Hong, Department of Internal Medicine, School of Medicine, Kangwon National University, Kangwondaehakgil-1, Chuncheon, Gangwon-do 24341 South Korea.
E-mail: shhong@kangwon.ac.kr
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the molecular mechanisms involved in Cd-induced ER stress and apoptotic pathways in human bronchial epithelial cells have not been fully elucidated.

To investigate the genes and cellular pathways related to Cd-induced cytotoxicity in human bronchial epithelial cells (BEAS-2B), we compared the gene expression profiles of Cd (10 and 30 μM)-treated BEAS-2B cells to those of non-treated control cells. A number of genes associated with cell proliferation, apoptosis, oxidative stress and inflammation, as well as various signal transduction pathways, were affected by Cd exposure to BEAS-2B cells. Transcriptomic analysis using a functional enrichment assay revealed that Cd treatment stimulated the CCAAT-enhancer-binding protein (C/EBP) signaling pathway and induced the transcriptional activation of its downstream target genes, including DNA-damaged-inducible transcript 3 (DDIT3, also called CHOP). DDIT3 is known as an ER-mediated pro-apoptotic factor, which is activated by cytotoxic materials and leads to cell death.[11] However, the role of DDIT3 in relation to Cd toxicity in bronchial epithelial cells has not yet been clarified. Here, we show that the suppression of DDIT3 expression alleviates Cd-induced inflammatory and ER stress responses in BEAS-2B and primary normal human bronchial epithelial (NHBE) cells, suggesting that C/EBP–DDIT3 signaling could be a molecular target for COPD therapy.

MATERIALS AND METHODS

Cell culturing

The human bronchial epithelial cell line (BEAS-2B) was kindly provided by the Biomedical Research Institute at Seoul National University Hospital. BEAS-2B cells were maintained in defined keratinocyte serum-free medium containing epidermal growth factor, 100 U ml^{-1} penicillin, and 100 μg ml^{-1} streptomycin (Thermo Fisher Scientific, Waltham, MA, USA). Primary NHBE cells (CC-2540, Lonza Group, Allendale, NJ, USA) isolated from the epithelial lining of airways above the bifurcation of a normal human donor lung were cultured in Bronchial Epithelial Growth Medium (BEGM BulletKit medium, CC-3171, Lonza) and used before passage 3 in all experiments. Cell cultures were incubated at 37 °C in humidified atmosphere containing 5% CO_2.

Chemicals

Unless otherwise indicated, all heavy metals were obtained from Sigma-Aldrich (St Louis, MO, USA). All reagents were freshly dissolved in sterile water at a concentration of 100 mM and were diluted in medium to reach the indicated concentration.

Cell viability assay

BEAS-2B cells (10 000 cells per well) were plated in a 96-well plate and allowed to attach overnight. Cells were grown to 80% confluence and then were either sham-exposed or treated with different concentrations of heavy metals (0, 1, 5, 10, 30, 50 and 100 μM) to determine subtoxic doses *in vitro*. The cells were treated with the heavy metals for 24 h and replaced with fresh medium followed by further incubation for 3 h. Cell viability was measured by MTS assay (Abcam, Cambridge, UK) according to the manufacturer's protocol. Incubation with Cd at 30–100 μM markedly decreased cell viability at 24 h. Thus, we performed all experiments with the concentration of 10 and 30 μM of Cd, which has been employed in many studies to examine early genetic alteration and inflammatory responses.[10,12–15]

Western blotting analysis

Cell lysates were separated on an SDS-PAGE gel (10% or 15%), transferred onto polyvinyldiflouride (PVDF) membranes (Millipore, Billerica, MA, USA), and blocked with 3% skim milk. Membranes were probed with primary antibodies against anti-human IL-1β antibody (AF-201-NA, R&D Systems, Minneapolis, MN, USA), anti-IL-6 antibody (#12153S, CST, Danvers, MA, USA), anti-human COX2 antibody (SC-1745, Santa Cruz Biotechnology, Dallas, TX, USA), anti-human iNOS antibody (ab15323, Abcam), ER stress sampler kit antibodies (#9956S, CST), NF-κB sampler kit antibodies (#9936, CST), anti-PTEN antibody (#9952, CST), anti-AKT and phospho-$AKT^{Ser473/Thr308}$ antibodies (#33748, #11054 and #11055, Signalway Antibody, College Park, MD, USA) or anti-β-actin antibody (#4970P, CST) overnight at 4 °C. The membranes were further probed with HRP-conjugated secondary anti-sera (A9917, A6667, or A5420, Sigma-Aldrich, St Louis, MO, USA) and visualized with PierceFast western blot kit (Thermo Fisher Scientific) and a cooled CCD camera System (Bio-Rad Laboratories, Hercules, CA, USA).

ELISA

To quantitate secreted IL-6, culture supernatants from BEAS-2B cells were measured using the human IL-6 Quantikine ELISA Kit (R&D Systems). The ELISA plates were read using a microplate reader (Molecular Devices, Sunnyvale, CA, USA).

RNA extraction and real-time qPCR

BEAS-2B cells (2×10^6 cells per well) in 6-well culture plates were treated in the absence or presence of Cd (Sigma) for 24 h. Total RNA was extracted using Trizol (Life Technologies, Carlsbad, CA, USA) and reverse-transcribed to first-stand complementary DNA (cDNA) using a random primer (9-mer) and QuantiTect reverse transcriptase (Qiagen, Hilden, Germany) according to the manufacturer's protocols. Transcripts were quantitated using Power SYBR Green PCR (Applied Biosystems by Life Technologies, Warrington, UK) and the QuantStudio 6 Flex Real-Time PCR system (Applied Biosystems). Quantitation was normalized to 18 s rRNA (internal control). Quantitative RT-PCR was performed using the primer sequences in Supplementary Table 1.

Microarray and data analysis

Following Cd treatment (10 and 30 μM) for 24 h, BEAS-2B cells were collected as described previously, and the total RNA was extracted using Trizol. Affymetrix Primeview Arrays (Affymetrix) were hybridized with cRNA probes at the GenoCheck core facility (Ansan, Kyunggi, Korea). The expression value and detection calls were computed from the raw data and gene set enrichment analysis (GSEA) version 4.0 (Broad Institute, Cambridge, MA, USA) to interpret expression profiles from microarrays.[16,17] GSEA was originally developed to identify cohorts of genes whose functions are integrated into certain biological processes and/or specific signaling pathways. Pathways were ranked according to the significance of the enrichment, and the validation mode measure of significance was used to identify pathways of greatest enrichment. Significance was tested by comparing the observed enrichment with the enrichment seen in data sets in which sample labels were randomly permutated ($n = 1000$).

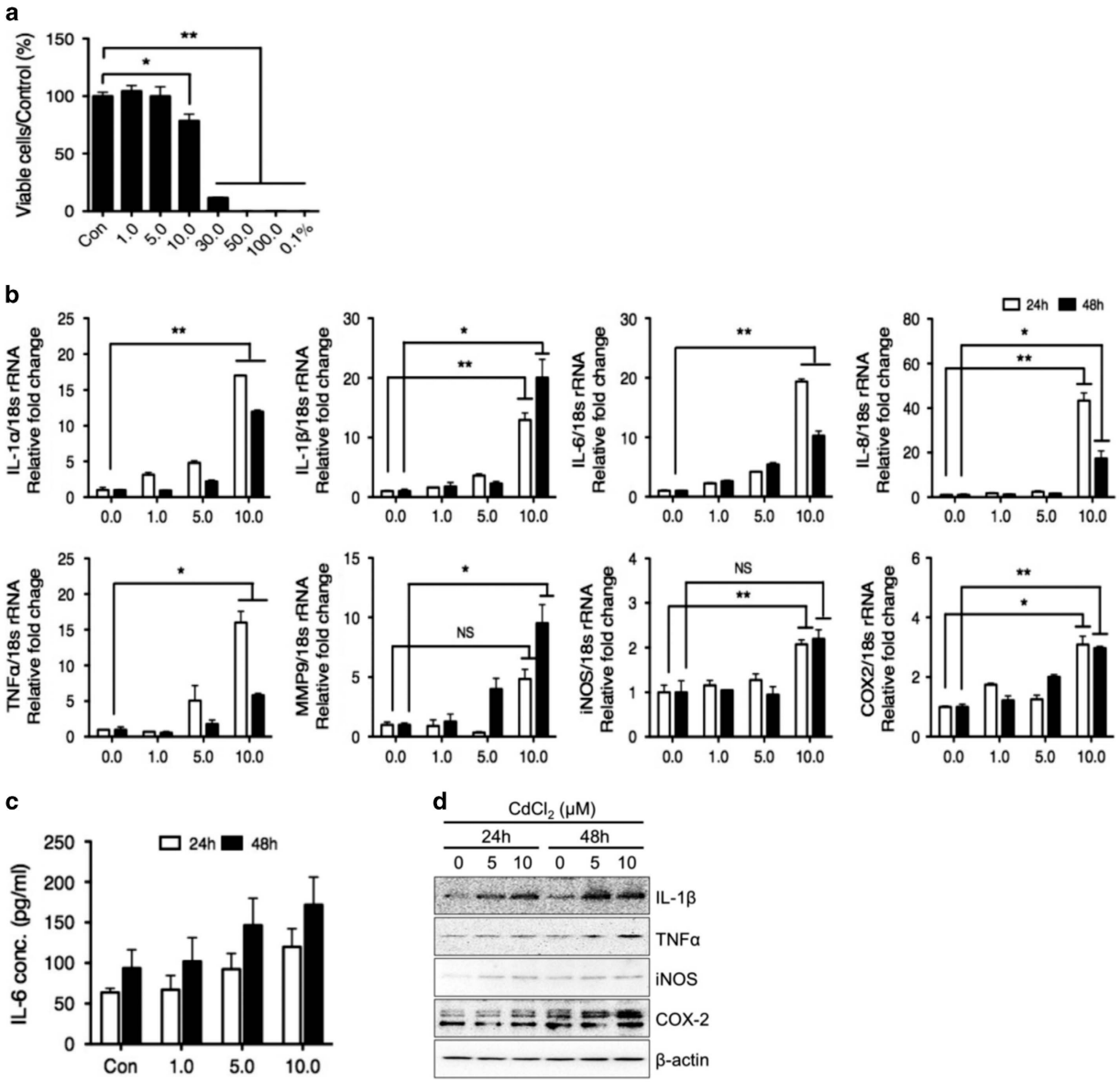

Figure 1 Effect of cadmium (Cd) on cell viability and pro-inflammatory cytokine expression. BEAS-2B cells were treated with Cd (1 to 100 μM) in medium containing antibiotics and incubated for 24 or 48 h. Cell viability was measured by the MTS assay. Triton X-100 (0.1%, Triton), as suggested by the manufacturer, leads to complete cell death, and so the Triton-treated group was set as 0% of the survival rate and non-treated group sets as 100% (**a**). Transcriptional levels of pro-inflammatory cytokines (IL-1α, IL-1β, IL-6, IL-8, TNFα, MMP9, COX2 and iNOS) were analyzed using SYBR green-based quantitative real-time PCR (**b**). Supernatant IL-6 levels were measured by enzyme-linked immunosorbent assay (ELISA) (**c**). Protein levels of pro-inflammatory cytokine (IL-1β, TNFα, IL-6, iNOS or COX2) were detected by immunoblotting (**d**). All data shown are representative of three biological replicates. $*P<0.05$; $**P<0.01$.

Gene sets consisting of <15 or more than 500 genes were filtered out by gene set size filters.

Knockdown of DDIT3 transcript using siRNA transfection

BEAS-2B cells were transfected with human DDIT3 siRNA (DDIT3 ON-TARGET plus SMART pool) or a negative control siRNA (ON-TARGET plus non-targeting pool) at a final concentration of 10 nM in the presence of DharmaFECT reagent (Dharmacon, Lafayette, CO, USA), as per the manufacturer's protocol. After transfection, the cells were collected at 24 or 48 h for qPCR analysis and western blotting performed. All experiments were performed in triplicate, and siRNA knockdown efficiency was confirmed by qPCR.

Statistical analyses

Statistical analyses were performed with one-way ANOVA for multiple groups using GraphPad Prism (GraphPad Software, San Diego, CA, USA). *P*-values are indicated in the figures.

RESULTS

Effects of Cd on the viability and inflammatory response of human BEAS-2B cells

To evaluate the toxic effects of Cd on bronchial epithelial cells, the viability of BEAS-2B cells exposed to various concentrations for 24 h was measured using the 3-(4,5-dimethylthiazol-2-yl)-

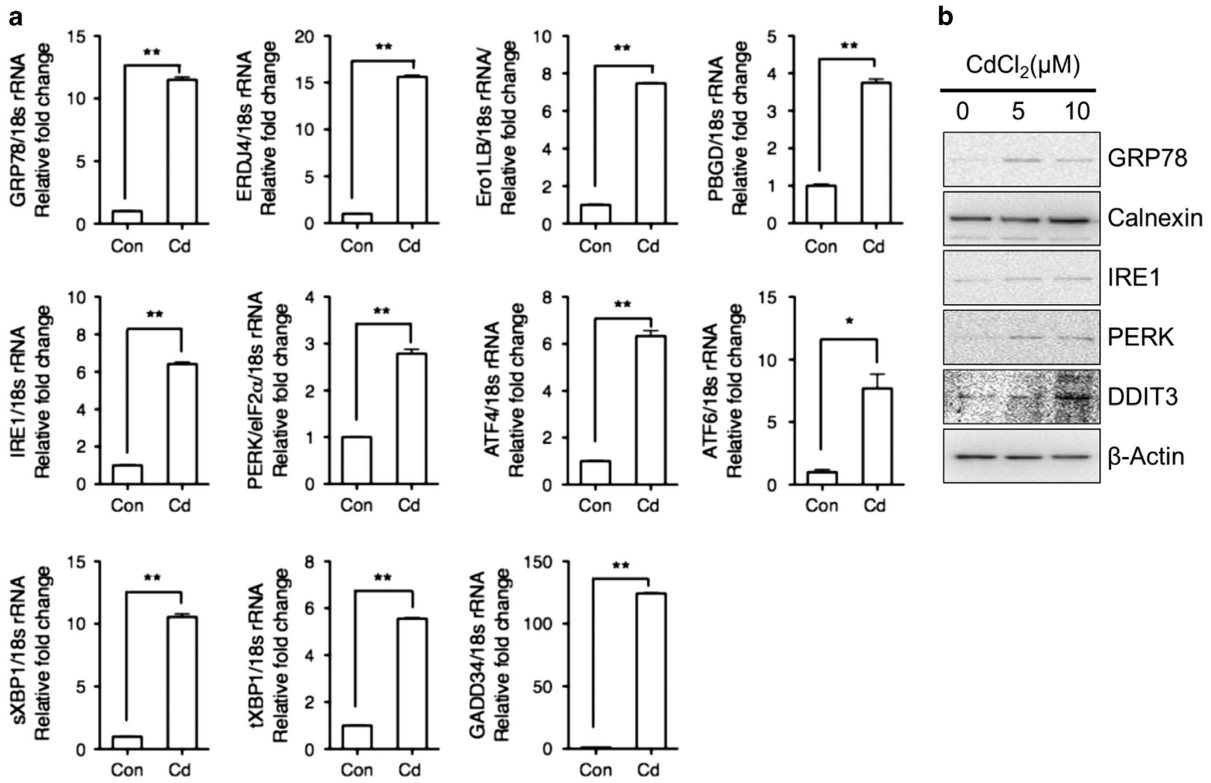

Figure 2 Effect of cadmium (Cd) on endoplasmic reticulum (ER) stress-related gene expression. BEAS-2B (2×10^5 cells per well for qPCR and immunoblotting) were treated with the indicated concentrations of Cd (1–10 μM). Levels of transcripts for GRP78, ERDJ4, Ero1LB, PBGD, IRE1, PERK/eIF2α, ATF4, ATF6, sXBP1, XBP1 and GADD34 were analyzed using SYBR green-based quantitative real-time PCR. Data represent the mean ± s.e.m for three biological replicates. $^*P<0.05$; $^{**}P<0.01$ (**a**). Effects of Cd on the levels of ER stress-related proteins were detected by western blotting (**b**). All data shown are representative of at least three biological replicates.

5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay. A significant toxic effect of Cd on viability was observed in cells treated with 10 μM, but not in cells treated with lower doses (1 and 5 μM) of Cd (Figure 1a). To further confirm the cytotoxicity of other heavy metals, BEAS-2B cells were treated with various concentrations of $PbCl_2$ (Pb), $Cr_2Na_2O_7H_2O$ (Cr), $NaAsO_2$ (As) and NiCl (Ni). Cr, Ni and As tended to have a higher cytotoxic effect than Cd, whereas Pb did not alter cell viability (Supplementary Figure S1). On the basis of the results of the MTS assay, we investigated whether Cd could induce the secretion of pro-inflammatory cytokines and mediators at the transcriptional and translational levels under our experimental conditions. We found that Cd upregulates the transcription of pro-inflammatory cytokines and mediators (such as interleukin [IL]-1α, IL-1β, IL-6, IL-8, tumor necrosis factor [TNF]α, matrix metalloproteinase [MMP]9, cyclooxygenase [COX]-2 and inducible nitric oxide synthase [iNOS]) and stimulates their secretion in a dose-dependent manner (Figure 1b–d). These findings suggest that inhalation exposure to Cd is related to the early development of lung diseases by promoting inflammatory responses.

Cd impairs ER homeostasis and induces ER stress in human BEAS-2B cells

It is known that toxic heavy metals can impair ER homeostasis and induce UPR via ER stress.[5,6,9,10] Therefore, we investigated whether Cd could induce ER stress in human bronchial epithelial cells. Real-time PCR results showed that transcript levels of GRP78, ERDJ4, Ero1LB, PBGD, IRE1, PERK/eIF2α, ATF4, ATF6, sXBP1, XBP1 and GADD34, which are canonical markers of UPR and ER stress, were significantly increased by treatment with 10 μM Cd (Figure 2a). We further found that protein levels of UPR markers (GRP78, Calnexin, IRE1, PERK and DDIT3) were also increased upon Cd treatment (Figure 2b). Collectively, these results suggest that Cd treatment promotes ER stress by disrupting ER homeostasis in human bronchial epithelial cells, which may impair normal metabolism and lead to bronchial epithelial cell death.

Cd alters genome-wide gene expression profiles in human BEAS-2B cells

To further understand Cd-induced alterations in bronchial epithelial cells at the molecular level, we asked whether Cd exposure alters global gene expression in human bronchial epithelial cells. The expression profiles of total RNA of BEAS-2B cells exposed to Cd (10 and 30 μM) for 24 h were analyzed using a cDNA microarray with 49 293 human cDNA probes, in an attempt to obtain a comprehensive view of the harmful effects of Cd. Unsupervised hierarchical clustering clearly showed that the global expression patterns of BEAS-2B cells were significantly altered by Cd treatment in a

dose-dependent manner (Figure 3a). A total of 1851 (1158 upregulated and 693 downregulated) and 5186 genes (2439 upregulated and 2747 downregulated) were significantly altered (>1.5-fold) upon treatment with 10 and 30 μM Cd, respectively. GSEA provided heat maps that represent lists of the top 50 genes showing the greatest increase and decrease in expression upon Cd treatment (Figure 3b). Interestingly, many subtypes of metallothionein (MT) including MT1A, MT1F,

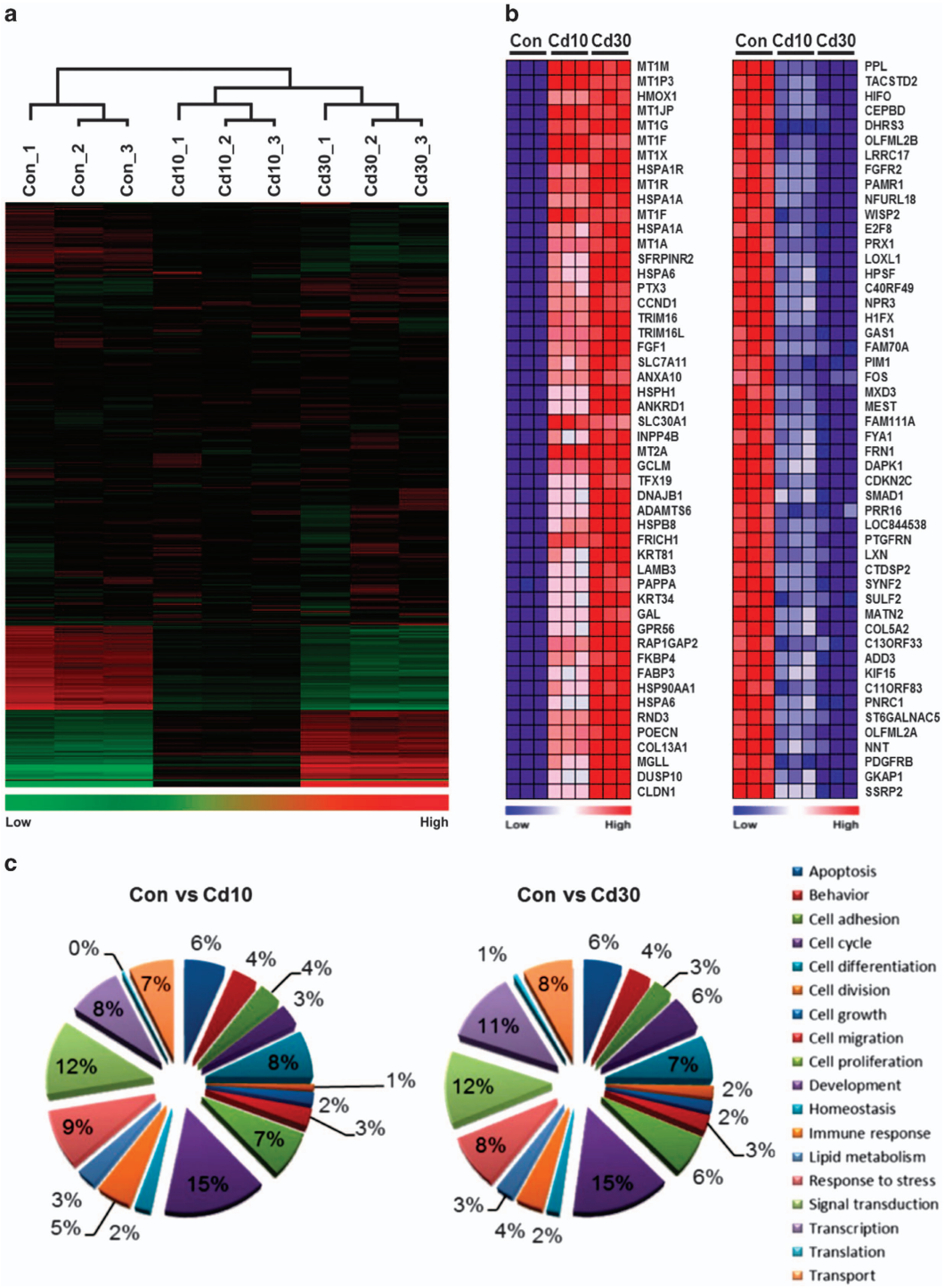

Figure 3 Dysregulated genome-wide expression profiles of cells exposed to cadmium (Cd). Unsupervised hierarchical clustering analysis of cDNA microarray data from Cd-treated cells. The color spectrum from green to red indicates low to high expression (**a**). Heat maps for the 50 genes with the greatest increase and decrease in expression in Cd-treated cells. The color spectrum from blue to red represents low to high expression (**b**). Gene ontology (GO) analyses for genes that are differentially regulated in cells treated with 10 and 30 μM Cd. Differentially expressed genes upon Cd exposure were filtered using the selection criteria of fold-change ⩾1.5 (**c**). Cd and Con indicate cells cultured with and without Cd, respectively.

Table 1 Upregulated gene sets in Cd-treated BEAS-2B cells

Name	*Size*	*Nominal* P	*FDR* P
HDAC1_AND_HDAC2_TARGETS_UP	157	0.000	0.000
CEBP_TARGETS	86	0.000	0.000
NRG1_SIGNALING_UP	143	0.000	0.000
RESPONSE-TO-SALIRASIB_UP	228	0.000	0.000
INFLAMMATORY_RESPONSE_LECTIN_VS_LPS_UP	418	0.000	0.000
EGF_RESPONSE_480_HELA	148	0.000	0.000
HRAS_ONCOGENIC_SIGNATURE	185	0.000	0.000
ANGIOIMMUNOBLASTIC_LYMPHOMA_DN	109	0.000	0.000
HYPOXIA_VIA_KDM3A	44	0.000	0.000
IL2_SIGNALING_UP	76	0.000	0.001
CYTOKINE_CYTOKINE_RECEPTOR_INTERACTION	86	0.002	0.058
MAPK_SIGNALING_PATHWAY	167	0.000	0.051
NEUROACTIVE_LIGAND_RECEPTOR_INTERACTION	57	0.013	0.083
AMINO_SUGAR_AND_NUCLEOTIDE-SUGAR_METABOLISM	38	0.036	0.145
NOD_LIKE_RECEPTOR_SIGNALING_PATHWAY	40	0.018	0.144

Table 2 Downregulated gene sets in Cd-treated BEAS-2B cells

Name	*Size*	*Nominal* P	*FDR* P
EARLY_T_LYMPHOCYTE_UP	86	0.000	0.000
RESPONSE_TO_SALIRASIB_DN	308	0.000	0.000
ADIPOGENESIS_3	93	0.000	0.000
REGULATED-BY-METHYLATION_DN	111	0.000	0.000
GAMMA_RADIATION_RESPONSE	36	0.000	0.000
BREAST_CANCER_CLUSTER_2	31	0.000	0.000
ERYTHROID_DIFFERENTIATION	71	0.000	0.000
LEUKEMIC_STEM_CELL_DN	141	0.000	0.001
EGFR_SIGNALING_24HR_DN	236	0.000	0.001
METASTASIS_UP	194	0.000	0.003
HDAC1_AND_HDAC2_TARGETS_DN	159	0.000	0.015
GLUTAMINE_DEPRIVATION_UP	31	0.007	0.023
RESPONSE_TO_ANDROGEN_DN	191	0.000	0.032
CELL_CYCLE	115	0.000	0.026
ECM_RECEPTOR_INTERACTION	46	0.014	0.227
TGF_BETA_SIGNALING_PATHWAY	59	0.021	0.227

MT1G and MT2A that provide protection against metal toxicity were collectively upregulated. To assess the biological relevance of these differentially expressed genes (DEGs), gene ontology (GO) analyses were performed using total expression data from BEAS-2B cells treated with Cd. The main GO categories that included DEGs were response to stress, immune response, apoptosis, cell differentiation and proliferation, development, transcription, transport and signal transduction (Figure 3c).

The C/EBP-regulated pathway is aberrantly stimulated by Cd

To understand in detail the underlying mechanisms by which Cd affects cell homeostasis, it is essential to identify aberrantly regulated signaling pathways and biological processes. GSEA, a supervised analysis, showed that various signaling pathways were either upregulated or downregulated in the Cd-treated cells (Tables 1 and 2). For example, the 'TGF-β_signaling_pathway' gene set was significantly downregulated and the 'NRG1_signaling_up' gene set was upregulated in Cd-treated cells (Supplementary Figure S2). Interestingly, gene sets consisting of target genes of histone deacetylase 1 (HDAC1) and HDAC2 were collectively either upregulated or downregulated by Cd, suggesting that Cd treatment mimics the action(s) of HDACs (Supplementary Figure S2). Furthermore, the 'C/EBP_targets' gene set is significantly upregulated in Cd-treated cells. As Cd-induced inflammatory responses in BEAS-2B cells are mediated, at least in part, by ER stress and C/EBP transcript factors are associated with ER stress, we further investigated the downstream target genes of C/EBP transcription factors in cells treated with Cd. The heat map and enrichment plot of genes in the 'C/EBP targets' gene set showed that most hits in ranking order were enriched in the area of Cd treatment (Figure 4a and b), suggesting that many genes associated with the C/EBP signaling pathways are upregulated in cells exposed to Cd. Real-time reverse transcription polymerase chain reaction (RT-PCR) validated that a set of genes regulated by C/EBPs, including GADD45b/g, regulator of G-protein signaling 2 (RGS2), BCL2-associated athanogene 3 (BAG3) and DDIT3, was systemically upregulated by Cd (Figure 4c). Among all members of the C/EBP family, C/EBPγ was the most significantly increased upon treatment with 10 and 30 μM Cd (~ twofold) (Figure 4d). In addition, Cd induced a moderate increase in C/EBPβ. These findings collectively suggest that Cd triggers ER stress-mediated inflammatory responses in human bronchial epithelial cells via the C/EBP signaling pathway.

Cd induces inflammatory cytokine secretion in human bronchial epithelial cells via the C/EBP–DDIT3 signaling pathway

Previous studies demonstrated that DDIT3 induces cell death and inflammation in several cell types.[10,11] To substantiate the

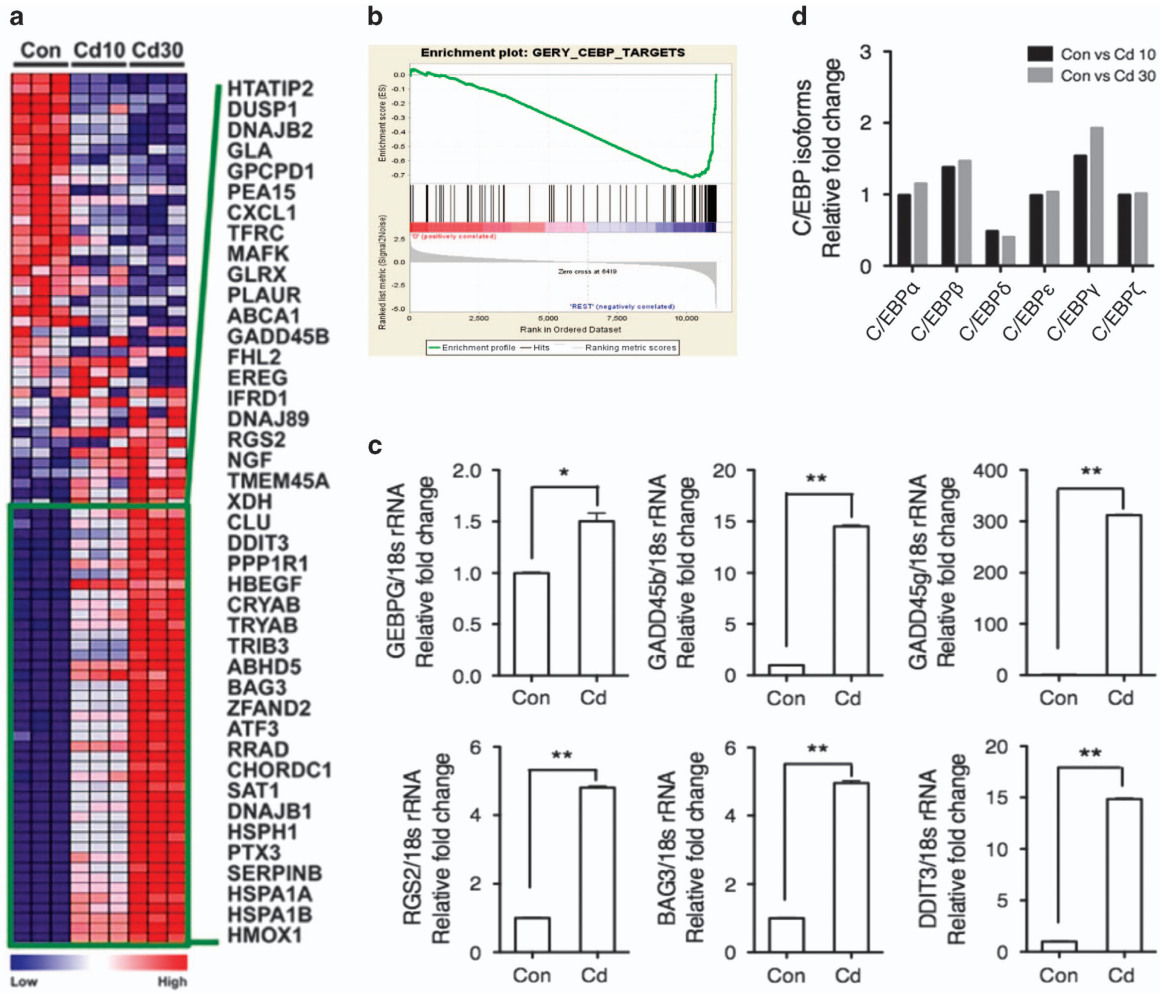

Figure 4 Collective upregulation of C/EBP target genes by cadmium (Cd) treatment. Heat map of genes in the 'C/EBP targets' gene set. The color spectrum from blue to red indicates low to high expression (**a**). Enrichment plot of the 'C/EBP targets' gene set. Genes within the green box in **a** are leading candidates for building enrichment scores in Cd-treated cells (Cd) (**b**). The results of real-time PCR for several genes included in the green box in **a** to measure the relative differences in their expression levels between Con and Cd. $*P<0.05$; $**P<0.01$ (**c**). Real-time PCR analyses to evaluate expression levels of C/EBP family members in cells treated with Cd (**d**). Cd and Con indicate cells cultured with and without Cd, respectively.

role of DDIT3 in the inflammatory response in human bronchial epithelial cells, BESA-2B cells were transfected with siRNA against the DDIT3 transcript. To test the efficiency of DDIT3 transcript suppression, BEAS-2B cells were transfected with 10 nM siRNA and cultured for 24 h. Then, the cells were processed for western blot analysis to determine the expression of DDIT3. As expected, transfection of BEAS-2B cells with 10 nM siRNA resulted in ~70% silencing of DDIT3 gene expression levels, compared with control (Supplementary Figure S3a). More importantly, the silencing of DDIT3 before the addition of Cd suppressed the inflammatory responses of BEAS-2B cells compared with Cd-treated control cells (Figure 5a–c). These results clearly showed that silencing of DDIT3 significantly protected against Cd-induced pro-inflammatory cytokine production in BEAS-2B cells. Next, we determined whether DDIT3 has an impact(s) on Cd-induced nuclear factor kappa B (NF-κB) activity. NF-κB activity is controlled by the inhibitor of kappa B (IκB) complex. Under control conditions, p65 is sequestered in the cytosol by the IκB complex. Upon cytokine treatment, IκB-α and β are degraded, allowing p65 translocation to the nucleus and the induction of NF-κB target genes. In an attempt to explain the effect of DDIT3 on NF-κB activity, we studied the impact of DDIT3 on p65 translocation and the stability of the IκB complex. Knockdown of DDIT3 attenuated p65 nuclear translocation induced by Cd toxicity and prevented IκB-α degradation (Figure 5d). PI3K-Akt signaling has an important role in regulating cell growth, proliferation, survival and motility.[18,19] Thus, we determined whether Cd toxicity is regulated through the Akt signaling pathway in a DDIT3-dependent manner. Knockdown of DDIT3 prevented the dephosphorylation of Akt (Ser473) induced by Cd treatment (Figure 5e). To reinforce the biological relevance of our findings in BEAS-2B cells exposed to Cd, we examined whether Cd also provokes inflammatory responses via activation C/EBP–DDIT3 signaling in primary cultured NHBE cells. As seen in BEAS-2B cells, Cd induced

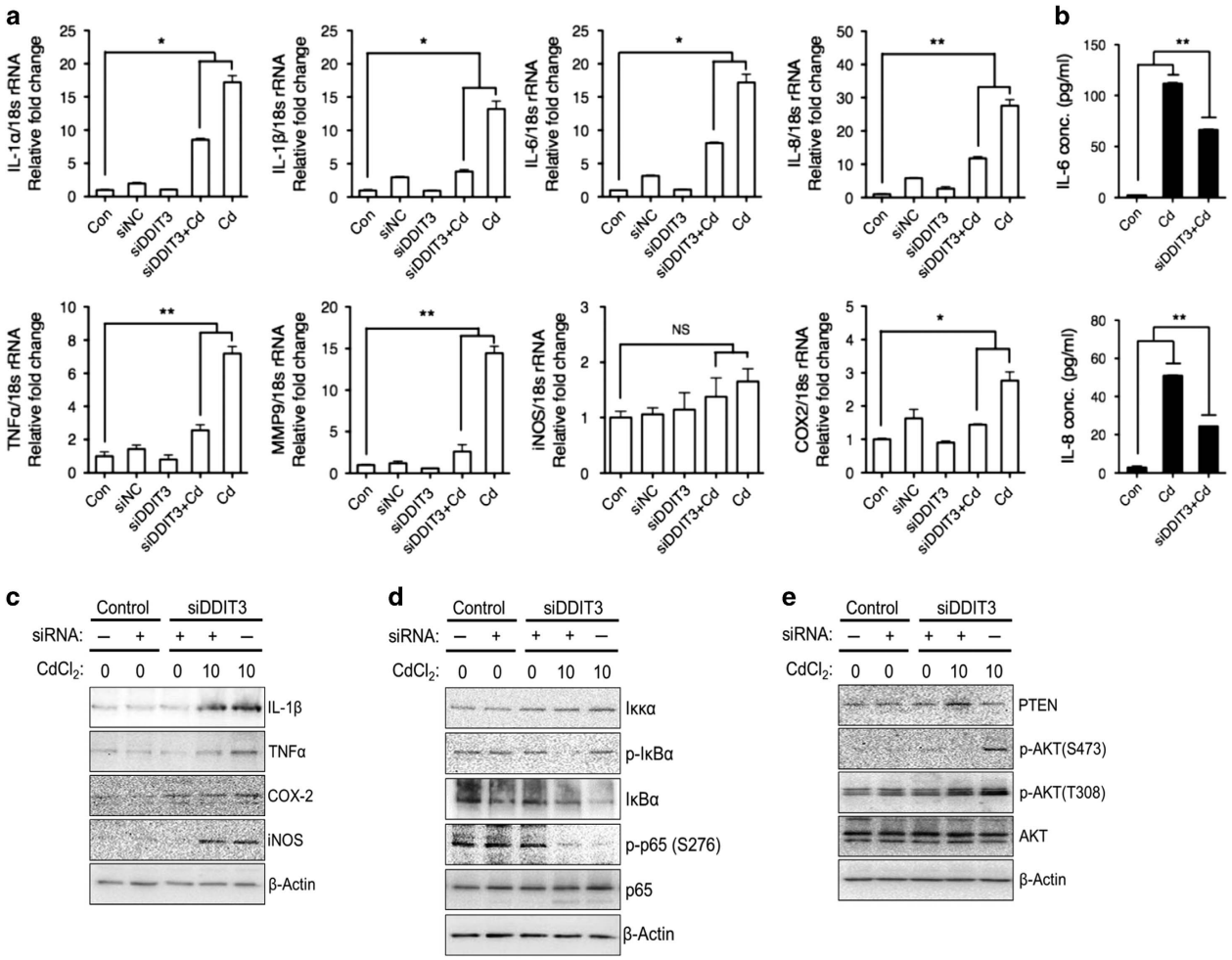

Figure 5 Effect of DDIT3 knockdown on the inflammatory response to cadmium (Cd) in BEAS-2B cells. BEAS-2B cells were transiently transfected with DDIT3 siRNA or a control, non-targeting siRNA (siNC). After incubation for 24 h, the cells were treated with Cd (10 μM) for 24 h. Levels of pro-inflammatory cytokine mRNAs were analyzed by quantitative real-time PCR (**a**). Cell supernatants were subjected to ELISA to measure IL-6 or IL-8 levels (**b**). Cell lysates were subjected to western blot analysis to determine the protein level of IL-1β, TNFα, iNOS or COX2 (**c**) and NF-κB activity (**d**). The PTEN/AKT pathway was examined by western blotting (**e**). All data shown are representative of at least three biological replicates. $*P<0.05$; $**P<0.01$.

upregulation of C/EBPs and their target genes, including GADD45b/g, RGS2, BAG3 and DDIT3 (Figure 6a and b). In addition, silencing of DDIT3 expression in NHBE cells suppressed inflammatory responses in a similar manner (Figure 6c; Supplementary Figure S3b). In conclusion, our study shows that Cd induces inflammatory cytokine secretion in human bronchial epithelial cells via the C/EBP–DDIT3 signaling pathway. DDIT3 directly contributes to Cd-induced cell apoptosis by promoting the activation of pro-apoptotic NF-κB-dependent pathways and Akt phosphorylation.

DISCUSSION

Previous studies have shown that Cd induces apoptotic cell death via the activation of ER stress-related signal transduction pathways in various cell types.[9,10,20–25] Some of these studies demonstrated the pro-apoptotic role of DDIT3 in cells exposed to Cd, which is mediated by ER stress and leads to cell death. For example, Cd-initiated apoptosis of human renal proximal tubular cells via the induction of DDIT3 is mediated by the activation of either ATF4 or ATF6.[21,22] Cd also induces the activation of DDIT3, ATF4 and ATF6 in rat cardiomyocytes and thus leads to cardiac cell death by disrupting glucose metabolism.[25] The exposure of neuroblastoma cells to Cd leads to an increase in intracellular ROS generation, which results in cell death by DDIT3 induction.[10] In addition, transcriptional activation of the DDIT3 promoter by Cd exposure in HepG2 hepatoma cells increases DNA damage and cell death.[9] These results are consistent with our finding that DDIT3 is upregulated in Cd-treated human bronchial epithelial cells and induces cell death by stimulating pro-apoptotic and pro-inflammatory responses. Interestingly, As and Cr also elevate the transcriptional and translational activation of DDIT3 and ER stress in liver cells via the upregulation of the ATF5 and ATF6 genes and lead to cell death.[24,26] These findings together indicate that DDIT3 could be a common cytotoxic marker induced by exposure to toxic heavy metals, as

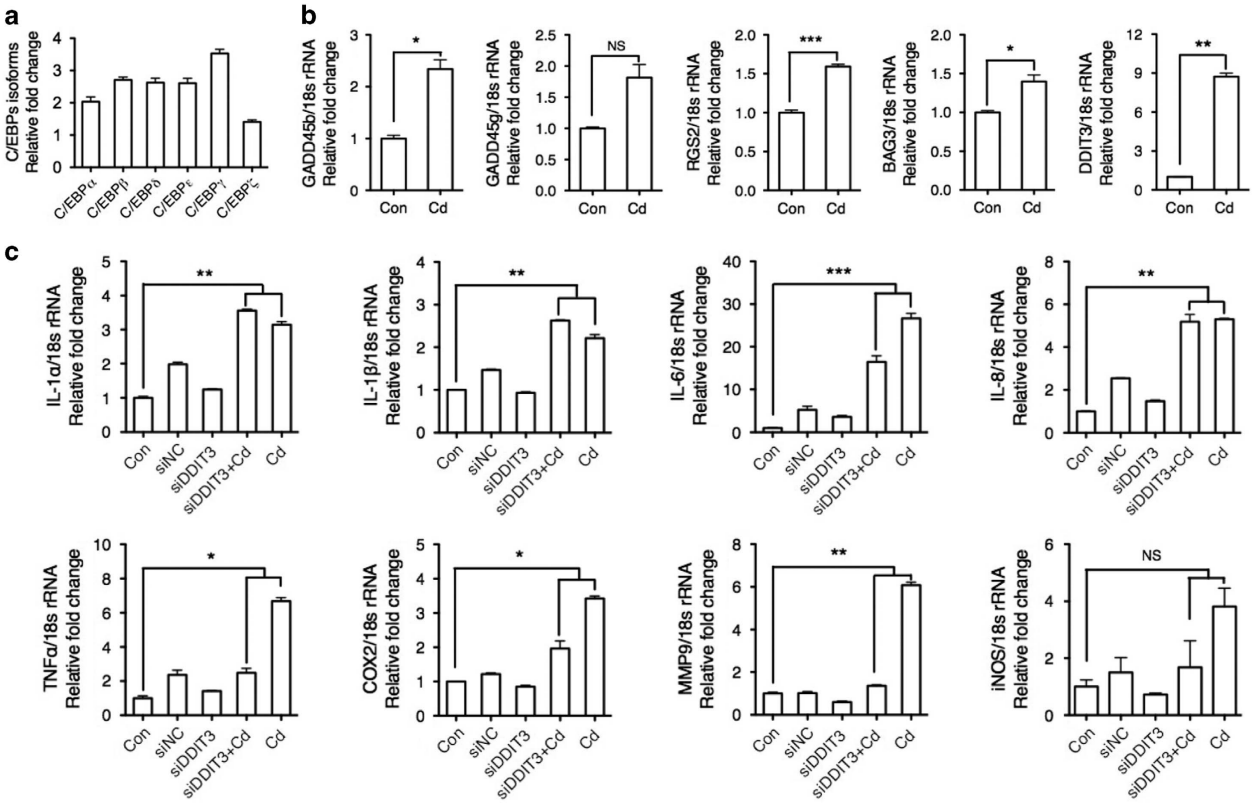

Figure 6 Effect of DDIT3 knockdown on the inflammatory response to Cd in primary normal human bronchial epithelial (NHBE) cells. Expression levels of C/EBP family members in cells treated with Cd (**a**). Expression levels of C/EBP target genes between Con and Cd. (**b**). Primary NHBE cells were transiently transfected with DDIT3 siRNA or non-targeting siRNA (siNC). After incubation for 24 h, the cells were treated with Cd (10 μM) for 24 h. Levels of pro-inflammatory cytokine mRNAs were analyzed by quantitative real-time PCR (**c**). All data shown are representative of at least three biological replicates. $*P<0.05$; $**P<0.01$, $***P<0.001$.

well as a molecular target for blocking apoptotic cell death and for the treatment of pulmonary diseases including COPD. ATFs are known as ER stress transducers that function to initiate the UPR and regulate DDIT3 expression in cells exposed to a toxic environment, and their functions may be cell type-specific.[27] In our current finding, ATF4 and ATF5 were strongly upregulated in Cd-treated BEAS-2B cells, suggesting that DDIT3 is a potential target for ATF4 or ATF5. We also found that knockdown of the DDIT3 transcript attenuated secretion of IL-8 and p65 nuclear translocation induced by Cd toxicity, suggesting that the NF-κB signaling pathway may be involved in the inflammatory response and cell death upon Cd exposure of human airway epithelial cells.[12] In contrast, NF-κB-independent secretion of IL-8 in human airway epithelial cells exposed to Cd has been reported.[14] Thus, further study will be needed to reveal the regulatory role of ATF4, ATF5 and NF-κB-signaling pathway in Cd-induced inflammatory responses, which may provide the exact molecular mechanisms underlying the initiation and development of pulmonary diseases upon Cd exposure.

Our GSEA revealed that the 'C/EBP_targets' gene set is significantly upregulated in Cd-treated BEAS-2B cells. DDIT3 is included in this gene set as a C/EBP downstream target. We further confirmed that the suppression of DDIT3 using siRNA alleviated the inflammatory and ER stress responses elevated by Cd exposure, suggesting that the C/EBP–DDIT3 signaling pathway could be a therapeutic target for the treatment of pulmonary diseases including COPD. The precise role of C/EBP genes in the initiation and development of COPD remains unclear. However, several studies indicate that C/EBP transcription factors are primarily responsible for ER stress and inflammation-related gene expression and might be implicated in COPD. For example, Mori *et al.*[28] reported elevated expression of C/EBPβ in advanced COPD patients compared with the asymptomatic smokers. Elevated expression of C/EBPβ induces the downregulation of elastin mRNA in lung alveoli because C/EBPβ acts as a negative regulator of elastin transcription.[29] Similar to these results, our study detected increased expression of C/EBPβ in BEAS-2B cells treated with Cd. These findings suggest that an increased C/EBPβ level may correlate with the destruction of normal lung function and structure in COPD patients. In contrast, Didon *et al.*[30] reported that the binding activity of C/EBPβ in the airway epithelium is decreased in COPD patients compared with healthy smokers. They further reported that C/EBPβ expression is significantly downregulated in the airway epithelial cells of smokers compared with those who had never been smokers and in primary human bronchial epithelial cell cultures treated with

cigarette smoke extract (CSE). Furthermore, CSE treatment caused compromised induction of pro-inflammatory cytokines and neutrophil chemoattractants in C/EBPβ-inactivated mouse lung epithelial cells.[31] These results suggest that decreased expression of C/EBPβ might render the lung epithelium resistant to efficient regeneration and more sensitive to irritant toxic materials. The identification of two different regulatory pathways for C/EBPβ translation may be the cause of this discrepancy between these studies.[32,33] Another possibility is that the activity of C/EBPβ can be regulated by other C/EBP family members, especially C/EBPγ. C/EBPγ is a truncated isoform that appears to lack the N-terminal activation domains present in most other C/EBP family members.[34,35] Under physiological and pathophysiological conditions, C/EBPγ serves as a regulator or reservoir against the transcription activity of C/EBP family members through heterodimerization.[36] Recent studies have provided evidence that C/EBPγ is an important regulator of airway epithelial proliferation, apoptosis and development.[36,37] Interestingly, in our study, among all members of the C/EBP family, C/EBPγ was the most significantly increased upon treatment with 10 and 30 μM Cd (~twofold). In fact, *in silico* analyses for the DDIT3 promoter suggest that putative-binding sites for both C/EBPβ and C/EBPγ are present in the proximal region of conserved DDIT3 promoters (data not shown). Although further studies are needed, it is conceivable that regulation of C/EBPγ expression may result in improvements for the treatment of COPD by controlling the activity of both the C/EBPα and C/EBPβ proteins.

In summary, our study extends and further integrates present knowledge regarding the molecular mechanisms linking Cd-induced ER stress and inflammatory responses to pulmonary diseases. Insights into the network regulating DDIT3-mediated apoptosis and the inflammatory response will potentially provide a basis for C/EBP–DDIT3 signaling-targeted therapeutic approaches to ER stress-associated pulmonary diseases.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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