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GENE EXPRESSION OF PRIMARY HUMAN BRONCHIAL EPITHELIAL CELLS IN RESPONSE TO COAL DUSTS WITH DIFFERENT PREVALENCE OF COAL WORKERS' PNEUMOCONIOSIS

Wenwei Hu, Qi Zhang, Wei Cheng Su, Zhaohui Feng, William N. Rom, Lung Chi Chen, Moonshong Tang, Xi Huang

Department of Environmental Medicine, New York University School of Medicine, New York, USA

Striking regional differences in the prevalence of coal workers' pneumoconiosis (CWP) have been observed but not fully understood. This study investigated the early biological responses of primary lung cells to treatment with coal dusts from various seams. High-density oligoarray technology (GeneChip, Affymetrix, Santa Clara, CA) was used to compile gene expression profiles of primary human bronchial epithelial cells to low concentrations (2 $\mu g/cm^2$) of coals for 6 h or 24 h of treatment. Data showed that a total of 1050 out of 12,000 genes on the chip were altered by 2 coal dusts. The coal from the Pennsylvania (PA) coal-mine region with a high prevalence of CWP altered 908 genes, many more than the coal from Utah (UT) with a low prevalence of CWP, which affected 356 genes. Many genes decreased their expression levels in response to the PA coal at 6 h and/or 24 h of treatment. For example, transferrin receptor, a gene known to control cellular iron uptake, was downregulated in the cells treated with the iron-containing PA coal in order to protect cells from iron overload. The UT coal without bioavailable iron had no such effect. The downregulation patterns of genes were confirmed by reverse-transcription polymerase chain reaction (RT-PCR). This study is one of the first in profiling gene expressions of primary bronchial epithelial cells treated with coals from various seams, which may set stages for future studies on specific genes.

Epidemiological studies of the relationship between the prevalence of coal workers' pneumoconiosis (CWP) and environmental measurements have consistently revealed that the predominant adverse exposure factor is respirable mixed coal dusts (Attfield & Wagner, 1993). It has been shown in United States, Great Britain, France, and Germany that the prevalence and severity of CWP differed remarkably between different coal mines despite the comparable exposures to respirable dusts (Amoudru, 1987; Attfield & Castellan, 1992; Coggon et al., 1995; Hurley et al., 1982; Reisner & Robock, 1977). For example,

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Address correspondence to Xi Huang, Department of Environmental Medicine, New York University School of Medicine, 550 First Ave, PHL Rm 802, New York, NY 10016, USA. E-mail:xihuang@env.med.nyu.edu

the disease is more prevalent in Pennsylvania (PA) coal miners and least common in coal miners from Utah (UT) (Attfield & Castellan, 1992; Morgan et al., 1973; Rom et al., 1981). Chronic obstructive pulmonary diseases such as asthma and emphysema, indistinguishable from their nonoccupational analogues, also appear to be more prevalent in PA than UT coal workers.

In the first round of the National Study of CWP (NSCWP) conducted between 1969 and 1971, 31 coal seams from 10 states were selected, and over 9000 coal workers participated in the survey. After adjusting for the age of the miners, the average tenure in mining, the job categories, and the estimated dust concentration and exposure, the prevalence of CWP was highest in PA coal miners (26%), lower in West Virginia (WV) coal miners (10%), and lowest in Utah (UT) coal miners (4%) (Morgan et al., 1973). Follow-up studies at the same mines (in 1972–1975, 1977–1981, and 1985–1988) showed that the overall prevalence of CWP was decreased in coal miners (due to the lower mandatory exposure levels), but the regional difference persisted with greater risk to eastern (PA and WV) than western coal miners (UT and Colorado) (Attfield & Morring, 1992; Attfield & Seixas, 1995). Coal rank (Miller & Jacobsen, 1985), quartz content (Walton et al., 1977), carbon-centered free radicals (Dalal et al., 1989, 1990), and bioavailable iron (BAI) present in the coal dusts (Huang et al., 1998, 1999) have been suggested to play important roles in the development of CWP. Levels of oxidants, inflammatory cytokines, and growth factors were increased in the lung lavage fluids of coal workers diagnosed with pneumoconiosis, after years of coal dust exposure (Borm & Schins, 2001; Castranova & Vallyathan, 2000; Rom, 1991; Rom et al., 1987). Coal increases inducible nitric oxide synthase and nitric oxide–stimulated inflammation (Blackford et al., 1997). However, little is known about the early biological responses of primary lung cells to treatment with coal dusts from various seams.

Recent technological advances have made possible the simultaneous detection of thousands of gene transcripts using a small tissue sample (Alizadeh et al., 2001; Nadadur & Kodavanti, 2002; Wu, 2001). The Affymetrix GeneChip arrays have been used to rapidly compile extensive gene expression information (Huang et al., 2001). This technique measures the relative concentration of mRNA transcripts based on the hybridization of entire mRNA populations to high-density arrays of oligonucleotides representing thousands of genes (Alizadeh et al., 2001; Nadadur & Kodavanti, 2002; Wu, 2001). A clear benefit of applying GeneChip technology is the ability to look at thousands of gene expression profiles from the same sample without prior knowledge about the genes being tested. Since bronchial epithelial cells are the first type of cells that encounter the inhaled coal dusts, the acute gene expression profiles of these cells were investigated after exposure to low doses of two coal dusts with striking differences in the prevalence of CWP. The data presented here clearly show that coal from the PA coal-mine region with a high prevalence of CWP alters more genes than the coal from UT with a low prevalence of CWP. The information provided on the global gene changes induced by the dusts may set the stage

for numerous future studies on specific genes. As a whole, this study provides some insights into the mechanisms as well as the disease variation of lung injury induced by coal dusts from various regions.

MATERIALS AND METHODS

Selection of Coal Samples

Two bituminous coal samples, one from the PA coal-mine region (PSOC number 1198) with a 26% prevalence of CWP, the other from UT (PSCO number 459) with a 4% prevalence of CWP, were selected from the 28 coal samples pool that was previously studied in relation to coal's toxicity and fibrogenicity (Zhang et al., 2002). The selection was based on the published epidemiological data, which reported a different prevalence of CWP for specific coal seams in specific counties and states (Huang et al., 1998; Zhang et al., 2002). The coal samples were collected during the time period when the epidemiological studies were performed (Attfield & Castellan, 1992). Based on our experimental results, samples from PA and UT regions were quite homogeneous (Huang et al., 1998; Zhang et al., 2002). Therefore, these two coal samples are relatively representative of their respective coal-mine regions. The coal samples were purchased from the Penn State Coal Sample Bank (University Park, PA). Physicochemical characteristics of the coal that are thought to play important roles in CWP, such as coal rank (carbon content or molar ratio of carbon/hydrogen), quartz content, and BAI, are described elsewhere (Huang et al., 1998; Zhang et al., 2002). Coal samples were ground and size-classified using the Mercer Impactor (Intox, Albuquerque, NM). Coal particles less than 5 μm with similar size distribution were used for cell treatments.

Cell Culture and Treatment

Primary cultured normal human bronchial epithelial cells (Clonetics, Bio Whittaker Co, San Diego, CA) were isolated from normal lung tissue of a 9-year-old Caucasian male. Cells were grown in medium (BEGM BulletKit) provided by the vendor, which contains 52 μg/ml bovine pituitary extract, 0.5 μg/ml hydrocortisone, 0.5 ng/ml human recombinant epidermal growth factor, 0.5 μg/ml epinephrine, and 10 μg/ml transferrin. Cells were used before passage 10. For coal exposure experiments, cells were grown to 50–70% confluence. Twelve hours before treatment, cells were cultured in medium without transferrin, which was to ensure that BAI present in the coals would not be rendered inactive by the capture of transferrin added to culture media. Two coals, one from the PA coal-mine region, the other from UT, were added to the cells in triplicate per time point at a concentration of 2 $\mu g/cm^2$, and the cells were incubated at 37 °C for 6 h or 24 h, respectively. At these low concentrations and short time period treatment, the coal samples were not toxic to the primary cells (data not shown, MTT assay). After treatment with coal, the

medium was aspirated, and cells were washed with fresh Dulbecco's phosphate-buffered saline (pH 7.0) and harvested for RNA isolation.

RNA Extraction

RNA was isolated from each sample using the Qiagen RNeasy Mini kit (Qiagen, Inc., Valencia, CA) according to the manufacturer's instructions. Extracted total RNA was spectrophotometrically quantitated by ultraviolet (UV) and then examined by 1% agarose gel electrophoresis to determine its quality.

Microarrays, Probes, Hybridization, and Scanning

Human Genome U95A microarray was used for hybridization (Affymetrix, Santa Clara, CA). This single array represents ~12,000 sequences that have been previously characterized in terms of function or disease association. Sample preparation, hybridization, and the array wash procedure were performed as described in the standard protocol outlined in the GeneChip Expression Analysis Technical Manual (Affymetrix). Total RNA from triplicate treatments was first pooled and hybridized on one chip for expression analysis. In brief, 10 μg total RNA was used to synthesize double-strand cDNA that was then used as a template to generate biotinylated cRNA by an in vitro transcription reaction. The RNA yield was determined by UV spectrophotometer after performing a cleanup procedure, and an adjusted cRNA yield was calculated to reflect carryover of unlabeled total RNA. Fifteen micrograms adjusted cRNA from each sample was fragmented and added to the U95A probe. It was then hybridized to the oligonucleotide probes on the probe array during a 16-h incubation at 45 °C in the Affymetrix hybridization oven 640. After sample hybridization, microarrays were washed, stained with a steptavidin-conjugated fluorescent stain, and followed by antibody amplification on the Affymetrix Fluidics Station 400. Each microarray was scanned twice. The first scanning was performed after steptavidin-conjugated fluorescent staining and data were used for analyzing expression level of abundant RNAs. It was scanned again after the whole washing and staining procedure was complete. The scanner acquires an image of each of the probe cells and the computer workstation automatically averages the intensities of each probe cell for the greatest assay sensitivity. RNA quality was also checked by examination of the 3′ to 5′ ratios for human actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) oligonucleotides on Affymetrix Test 2 chips first. Each gene on the GeneChip is represented by 20 probes, 25-mer oligonucleotide. The expression levels of genes were measured by comparing the signal intensities of hybridization of these probe sets to the one-base, mismatched oligonucleotide probes by Affimetrix Microarray Suite 4.0 (Santa Clara, CA; average difference). Expression of the genes was scored as "present" when the perfect match signal exceeded its mismatched counterpart.

Data Analysis

Microarray primary data were also examined using GeneSpring version 4.1 (Silicon Genetics, Redwood City, CA), and S plus package (MathSoft, Cambridge,

MA). The intensities of genes without the "present" call were replaced by twofold background value. This was to eliminate the bias generated from non- or low-expressed levels when the normalization was performed. Data sets were then subjected to standardization using the median intensities of housekeeping genes among different chips, and correction factors were then calculated for each chip. To categorize the patterns of gene expression changes that occurred in the cells, principal-component analysis method was applied to profile genes, followed by extensive manual data mining and compiling gene profiles that have been related to certain pathways such as activator protein-1 (AP-1) activation.

RT-PCR

Expression status of five genes from the same batches of RNA as for the GeneChip were tested using reverse-transcription polymerase chain reaction (RT-PCR), except the sample treated with UT coal for 6 h, due to the insufficient amount of RNA left for the measurement. Primers used were: 5′-CAG CCC AGC AGA AGC ATT ATC-3′ and 5′-GGA AGT AGC ACG GAA GAA GTC-3′ for transferrin receptor (TfR), 5′-ATG GAT CCC AAC TGC TCC TGC G-3′ and 5′-AGG GCT GTC CCA ACA TCA GGC-3′ for metallothionein (MT), 5′-GTG GAA GTC GCG TTC TTT ACA-3′ and 5′-CCG ACT CTA GCC GCC TCT T-3′ for MT-2A, 5′-CGG AGT CAA CGG ATT TGG TCG TAT-3′ and 5′-AGC CTT CTC CAT GGT TGG TGA AGA C-3′ for GAPDH, and 5′-AAC CAC GTG ACT TTG TCA CAG C-3′ and 5′-CTG CTC ATA CAT CAA ACA TG-3′ for β_2-microglobulin (β_2-m) (Cao & Cousins, 2000; Mididoddi et al., 1996; Sposi et al., 2000). After determining the linear range of RT-PCR for each of the target genes, the RT-PCR bands were analyzed by densitometric scanning (Imaging Densitometer, model GS-700; Bio-Rad Laboratories) using the molecular analyst software. For semiquantitative analyses, mRNA of the housekeeping gene β_2-m was coamplified using the same RT-PCR conditions as for each experiment. Ratios of all experimental bands to β_2-m were used to measure changes in mRNA expression between different treatments groups.

RESULTS

Overview of Global Genomic Expression Induced by Coal Dusts from the PA and UT Coal-Mine Regions

In total, five gene chips were used to test the gene expression profiles induced by two coal dusts at two time points (6 h and 24 h) as compared to control. In order to correct for differences in intensity levels among samples, mainly due to saturation effects of a given mRNA in the hybridization process, or the nonlinear quenching effects of phosphorescent dyes (Wu, 2001), normalizations among samples were performed. A cluster of about 30 constitutively expressed "housekeeping" genes was entered into the database. This resulted in 13 genes, which were "present" in all five samples as determined by the

Affymetrix software. Since housekeeping genes are more likely to be expressed equally in two given samples (Wu, 2001), these genes were used to compute the median intensity of the housekeeping genes among samples. The correction factor for each chip was then calculated using the median intensity of the housekeeping gene on the chip divided by the median intensity of the housekeeping genes among the five chips. The average correction factors found were 0.93, 1.15, 0.89, 1.16, and 0.82 for control, UT coal at 6 and 24 h, and PA coal at 6 and 24 h chips, respectively. These analyses suggest that the microarray chips were well hybridized and equally handled during experimental processes. As a result, only slight correction factors were applied to each test sample for standardization. Among the 12,000 genes on the chip, approximately 9000 genes were "absent" in all 5 samples and were filtered out. Therefore, only about 3000 genes, which were 'present' in at least 1 sample, were used for further analyses. Using principal component analysis, nine groups of genes were altered as shown in Figure 1A. The majority of genes located in the center of the picture showed no changes in levels of expression. Figure 1B shows a total of 1050 genes altered by the two coals at two time points and the number of genes altered in each group. For example, 47 genes in Group 3 were upregulated by the PA coal and UT coal at 6 and 24 h treatments. Results of Figure 1B show that the genes altered by the PA coal with a high prevalence of CWP outnumbered those by the UT coal with a low prevalence of CWP. A lot of genes were downregulated by the PA coal at 6 h and/or 24 h of treatment. There were some genes downregulated by the UT coal at 6 h, but returned to normal at 24 h.

Genes Commonly Induced by the Coals (Group 3)

As shown in Figure 2, a total of 47 genes were commonly induced by the two coals (6 and 24 h treatment), indifferent from the coal mine regions. Figure 2 illustrates the average-fold changes of gene expressions over the control induced by each coal at each time point and also examples of a few genes, which were strongly induced by both coals. After researching the functions of each gene, it was found that TYRO3 protein tyrosine kinase, an enzyme possibly involved in cell adhesion processes, which may be related to coal dust–induced pneumoconiosis and fibrosis, is upregulated by both the PA and UT coals. Some dehydrogenases such as NADH dehydrogenase (role in mitochondrial respiratory chain) or phosphate synthases such as uridine monophosphates synthase (common to other phosphoribosyltransferases) or uridine phosphorylase were induced by the coals from the PA and UT coal-mine regions.

Transient Downregulation of Genes by the PA Coal at 6 h Only (Group 4)

In total, 406 genes were transiently downregulated by the PA coal at 6 h but returned to the control levels at 24 h. In contrast, the coal from UT had no such effects on those genes at 6 or 24 h of treatment. Figure 3 shows some of the

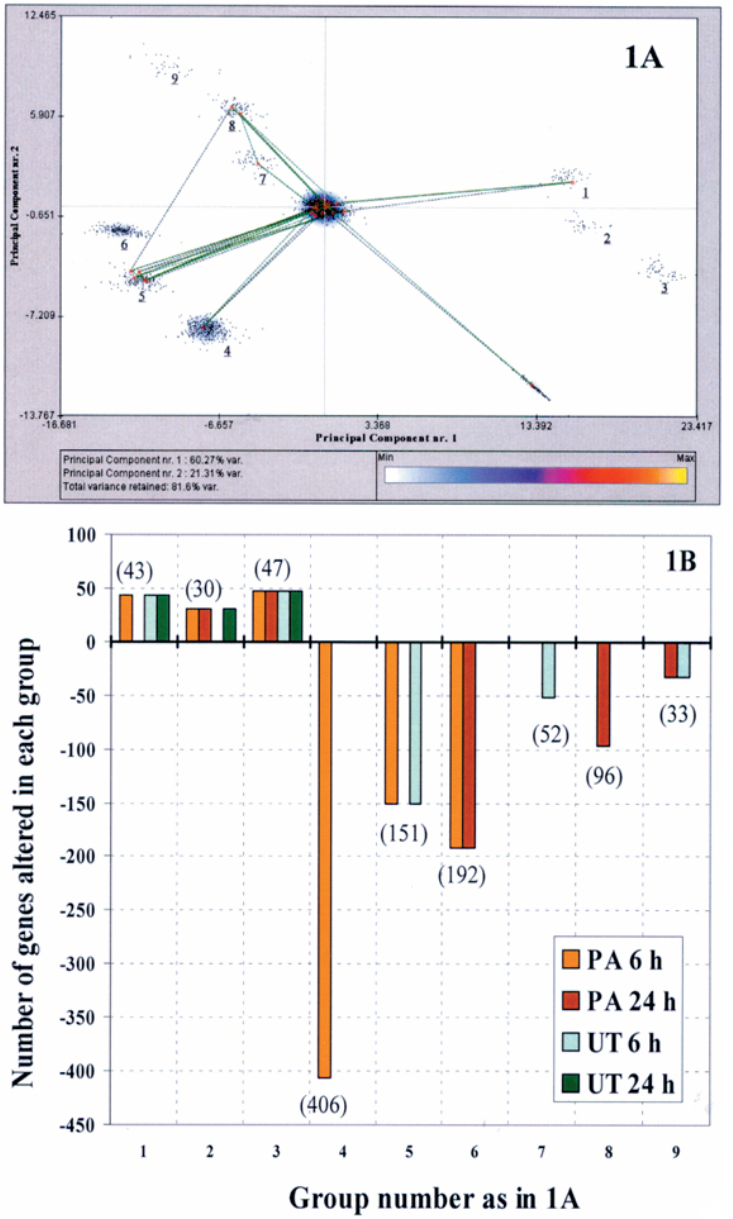
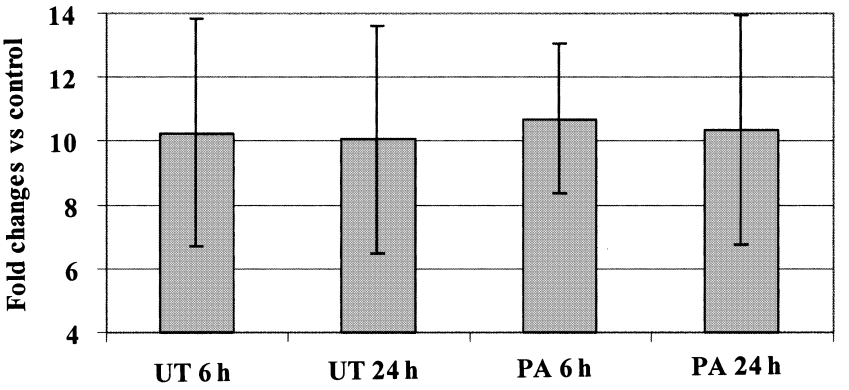
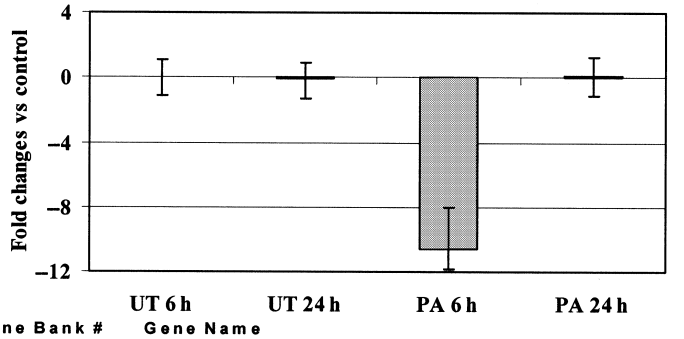


FIGURE 1. Principal-component analyses of gene expression profiles in primary human bronchial epithelial cells in response to coal dust treatment (A) and the number of genes altered in each group by two different coals (B). The two coal samples, one from the PA coal-mine region with a high prevalence of CWP (26%), the other from UT (4%), were used for cell treatments (6 and 24 h). Using Gene Spring software, gene expression profiles were automatically clustered for 9 groups (A) and were shown (B) according to their pattern of induction or repression by different coals at different time points.



Gene Bank #	Gene Name
U66711	lymphocyte antigen 6 complex, locus E
M16941	Human MHC class II HLA-DR7-associated glycoprotein
M19481	follistatin isoform FST344 precursor
J03626	uridine monophosphate synthetase
Z97074	p40
X59303	valyl-tRNA synthetase
AF026291	chaperonin containing TCP1, subunit 4 (delta)
AF025654	RNA guanylyltransferase and 5'-phosphatase
AF084260	thyroid receptor interacting protein 15
AF055006	sec6 homolog
D87461	BCL2-like 2
X90858	uridine phosphorylase
U50383	NN8-4AG
X78669	reticulocalbin 2, EF-hand calcium binding domain
AF050640	NADH dehydrogenase (ubiquinone) Fe-S protein 2
HG511	Ras Inhibitor Inf
L33801	glycogen synthase kinase 3 beta
X71874	proteasome (prosome, macropain) subunit, beta type, 10
X15331	phosphoribosyl pyrophosphate synthetase 1
M15400	retinoblastoma 1 (including osteosarcoma)
X16665	homeo box B2
X65550	antigen identified by monoclonal antibody Ki-67
L77886	protein tyrosine phosphatase, receptor type, K
AJ010842	ATP(GTP)-binding protein
U43944	cytosolic NADP(+)-dependent malic enzyme
M93425	protein tyrosine phosphatase, non-receptor type 12
D17517	TYRO3 protein tyrosine kinase
AF001903	3-hydroxyacyl-CoA dehydrogenase, isoform 2

FIGURE 2. Genes commonly induced by the PA and UT coals (Group 3). Changes over control were the average expression levels of all 47 induced genes over control ± standard deviation (SD). Some genes with known functions and possibly involved in coal dust–induced toxicity and pneumoconiosis are listed in increasing order of expression from top to bottom.



Gene Bank #	Gene Name
U68723	checkpoint suppressor 1
AF091433	cyclin E2
X77743	cyclin-dependent kinase 7
L04282	zinc finger protein 148 (pHZ-52)
M37197	CCAAT-box-binding transcription factor
Z46973	phosphoinositide-3-kinase, class 3
X61587	ras homolog gene family, member G (rho G)
Y13834	zinc metalloproteinase, STE24 (yeast, homolog)
X96717	transcription factor binding to IGHM enhancer 3
U78575	phosphatidylinositol-4-phosphate 5-kinase, type I, alpha
D88357	cell division cycle 2, G1 to S and G2 to M
X61498	nuclear factor of kappa light polypeptide gene enhancer
AF055479	lung cancer candidate
AF025304	protein-tyrosine kinase EPHB2v
Z36714	cyclin F
X66363	serine/threonine protein kinase
M63838	interferon, gamma-inducible protein 16
HG3521	Ras-Related Protein Rap1b
U47414	cyclin G2
AJ222700	transforming growth factor beta-stimulated protein TSC-22
X95384	translational inhibitor protein p14.5
Z22555	CD36 antigen (collagen type I receptor)-like 1
U08438	beta adrenergic receptor kinase 1
Y10659	interleukin 13 receptor, alpha 1
U25435	CCCTC-binding factor (zinc finger protein)
AF061034	tumor necrosis factor alpha-inducible cellular protein
AF047469	arsenite translocating ATPase
U43899	signal transducing adaptor molecule 1
AF041248	cyclin-dependent kinase inhibitor 2C
U57627	phosphatidylinositol polyphosphate 5-phosphotase
AF110377	transformation/transcription domain-associated protein
L29277	signal transducer and activator of transcription 3
Y07661	upstream transcription factor 2, c-fos interacting
AF069250	acid-inducible phosphoprotein
AF003938	thioredoxin-like, 32kD
X98654	phosphatidylinositol transfer protein, membrane-associated
Z56281	interferon regulatory factor 3
L33799	procollagen C-endopeptidase enhancer
X60188	protein serine/threonine kinase
AB014560	Ras-GTPase activating protein SH3 domain-binding protein 2
X79067	butyrate response factor 1 (EGF-response factor 1)
U65785	oxygen regulated protein
U09937	plasminogen activator, urokinase receptor
M37435	colony stimulating factor 1 (macrophage)
X64037	general transcription factor IIF, polypeptide 1
X01060	transferrin receptor

FIGURE 3. Genes transiently repressed by the PA coal at 6 h treatment (Group 4). Changes over control were average expression levels of all 406 repressed genes over control ± SD. Some genes with known functions and possibly involved in coal dust–induced toxicity and pneumoconiosis are listed in increasing order of repression from top to bottom.

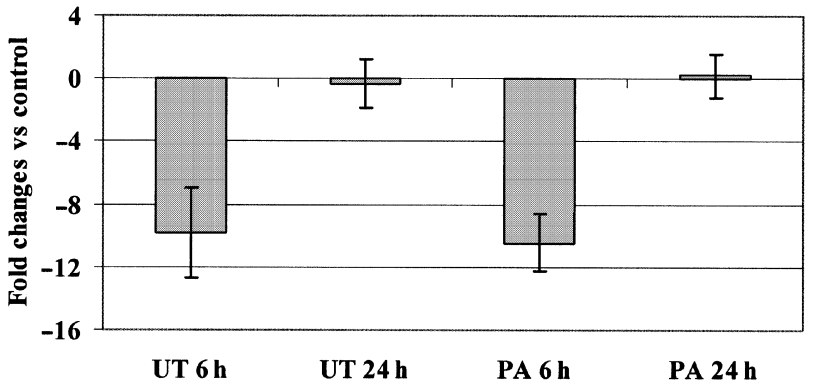
repressed genes that may be involved in coal dust–induced oxidative stress as well as pneumoconiosis and fibrosis. For example, in response to the PA coal-induced stress, serine/threonine protein kinase, phosphoinositide 3-kinase, as well as upstream transcription factor 2 (c-fos interacting) were transiently downregulated by the PA coal at 6 h. Acute-phase response factor, a transcription factor that binds to the interleukin-6 (IL-6)-responsive elements identified in the promoters of various acute-phase protein genes, was also downregulated by the PA coal. It is worth noting that transferrin receptor, zinc finger proteins 148 and 207, arsenite translocating ATPase, zinc metalloproteinase, an enzyme involved in proteolytic processing of farnesylated proteins, thioredoxinlike protein, oxygen-regulated protein, and quinone oxidoreductase homologue were also downregulated in response to the PA coal treatment at 6 h.

Transient Downregulation of Genes by the PA and UT Coals at 6 h (Group 5)

A total of 151 genes were transiently repressed by both PA and UT coals at 6 h (Figure 4). In response to the environmental stress, cells downregulate mitogen-activated protein kinase–activated protein kinases 2 and 3, mitogen-activated protein kinase (MAPK) 7, mitogen-activated protein kinase kinase kinase (MAPKKK) 7, and p50-NF-kappa B homolog. The coals from the PA and UT coal-mine regions are rich in bioavailable calcium (Zhang et al., 2002); putative Ca^{2+}-transporting ATPase was repressed by both coals. Various transferases were downregulated by the two coals, such as farnesyltransferase, glucosyltransferase, phenylethanolamine *N*-methyltransferase, thiopurine *S*-methyltransferase, serine hydroxymethyltransferase 1, acetylcoenzyme A, acetyltransferase 2, glutathione *S*-transferase theta 1, DNA (cytosine-5-)methyltransferase 1, phosphoribosylglycinamide formyltransferase, glyceronephosphate *O*-acyltransferase, and histone acetyltransferase 1. Two proinflammatory cytokine-related genes, interleukin-1-beta and interleukin enhancing binding factor 1, were also repressed by the two coals.

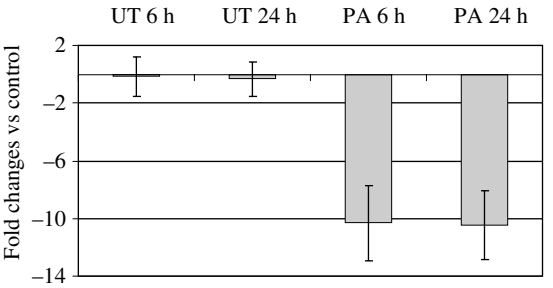
Downregulation of Genes by the PA Coal at 6 and 24 h (Group 6)

A sustained downregulation of genes may have greater adverse effects on cells. Figure 5 shows a few examples of a total of 192 genes repressed by the PA coal at 6 and 24 h. The downregulation of these genes appears to be specific to the PA coal. Since the coals from this region are highly fibrogenic compared to the coal from the UT coal-mine region, alteration of genes in this group may play very important roles in coal dust–induced pneumoconiosis. For example, tissue inhibitor of metalloproteinases-3, collagens type IV and VIII, insulinlike growth factor binding protein 3, tumor necrosis factor receptor-associated factor 4, and transforming growth factor-β–inducible early growth response were downregulated by the PA coal at 6 h and 24 h of treatment.



Gene Bank #	Gene Name
AB014519	Rho-associated, coiled-coil containing protein kinase 2
M97388	down-regulator of transcription 1
L12535	ras suppressor protein 1
M15330	interleukin 1, beta
AJ010953	putative Ca2+-transporting ATPase
AB009356	mitogen-activated protein kinase7
L11931	serine hydroxymethyltransferase 1 (soluble)
AF030424	histone acetyltransferase 1
X75346	mitogen-activated protein kinase-activated protein kinase 2
U29725	mitogen-activated protein kinase 7
L00634	farnesyltransferase, CAAX box, alpha
S70154	acetyl-Coenzyme A acetyltransferase
L26336	heat shock 70kD protein 2
M64673	heat shock transcription factor 1
X84908	phosphorylase kinase, beta
AB007851	phosphoribosyl pyrophosphate synthetase-associated protein
U58198	interleukin enhancer binding factor 1
U43784	mitogen-activated protein kinase-activated protein kinase 3
AJ002190	glyceronephosphate. O-acyltransferase
AB019517	protein kinase (cAMP-dependent, catalytic) inhibitor gamma
S76638	p50-NF-kappa B homolog
X62535	diacylglycerol kinase, alpha (80kD)
D63780	serine/threonine kinase 25 (Ste20, yeast homolog)
AJ224875	glucosyltransferase
AF019369	thiopurine S-methyltransferase
U09585	interferon-related developmental regulator 2
X54199	phosphoribosylglycinamide formyltransferase,
X74262	retinoblastoma-binding protein 4
Z84718	glutathione S-transferase theta 1
X63692	DNA (cytosine-5-)-methyltransferase 1
X52730	phenylethanolamine N-methyltransferase
HG3236	Neurofibromatosis 2 Tumor Suppressor

FIGURE 4. Genes transiently repressed by the PA and UT coals at 6 h of treatment (Group 5). Changes over control were expressed the same way as described in the legend of Figure 3.



Gene Bank #	Gene Name
U14394	tissue inhibitor of metalloproteinases-3
M34065	cell division cycle 25C
AF050110	TGFB inducible early growth response
M27968	fibroblast growth factor 2 (basic)
X57527	collagen, type VIII, alpha 1
D25248	actin filament associated protein
M35878	insulin-like growth factor binding protein 3
AF049891	tyrosylprotein sulfotransferase 2
M16750	pim-1 oncogene
M64673	heat shock transcription factor 1
U41745	PDGF associated protein
U77949	CDC6 (cell division cycle 6, S. cerevisiae) homolog
M92287	cyclin D3
AL035079	dJ53C18.1 (Catalase)
U09578	mitogen-activated protein kinase-activated protein kinase 3
D86550	dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 1A
U01062	inositol 1,4,5-triphosphate receptor, type 3
L76703	protein phosphatase 2, regulatory subunit B (B56)
L33930	CD24 antigen (small cell lung carcinoma cluster 4 antigen)
M24594	interferon-induced protein with tetratricopeptide repeats 1
U02570	Rho GTPase activating protein 1
AF046059	cytokine receptor-like molecule 9
U94836	calcium (ca2+) homeostasis endoplasmic reticulum protein
X67155	mitotic kinase-like protein-1
U10868	aldehyde dehydrogenase 7
U69609	transcription repressor
U79270	COX11 (yeast) homolog, cytochrome c oxidase assembly protein
AF046798	glycogen phosphorylase
X53390	upstream binding transcription factor, RNA polymerase I
M26576	collagen, type IV, alpha 1
U04209	microfibrillar-associated protein 1
X55503	metallothionein
M76125	tyrosine kinase receptor
X80200	TNF receptor-associated factor 4
U28014	caspase 4, apoptosis-related cysteine protease
U15131	suppression of tumorigenicity 5

FIGURE 5. Genes stably repressed by the PA coal (Group 6). Changes over control were expressed the same way as described in the legend of Figure 3.

Genes Induced or Repressed by Both PA and UT Coals at Various Time Points (Groups 1, 2, 7, 8, and 9)

Forty-three genes were induced by the PA coal at 6 h only and by the UT coal at 6 h and 24 h treatment (Group 1, see Figure 1 for reference). Examples of the induced genes are histone H2B, tumor necrosis factor receptor 2–associated protein, and G protein–coupled receptor kinase. On the other hand, 30 genes were induced by the PA coal at 6 h and 24 h of treatment but by the UT coal at 6 h only (Group 2), such as c-myc binding protein, tumor protein p53-binding protein 1, and calcium/calmodulin-dependent protein kinase (CaM kinase) II gamma. Fifty-two genes repressed by the UT coal at 6 h but returned to normal at 24 h (Group 7), including nuclear factor (NF)-kappa-B transcription factor subunit, NF-kappa-B DNA binding subunit, protein kinase (interferon-inducible, double-stranded RNA–dependent activator), glutathione synthase, and matrix metalloproteinase 1 (interstitial collagenase). Ninety-two genes repressed by the PA coal at 24 h of treatment only (Group 8) include glutathione transferases (Zeta 1 and M4), cyclin-dependent kinase 2, cyclin H, tumor protein 53, mitogen-activated protein kinase 3, surfactant (pulmonary-associated protein D), fibroblast growth factor receptor 1, tumor necrosis factor alpha–induced protein 1, RAS p2l protein activator 1 (GTPase activating protein), E2F dimerization partner 2, and plasminogen activator inhibitor-1 gene. Thirty-three genes (Group 9), which were commonly downregulated by the UT coal at 6 h and PA coal at 24 h, include activated p21cdc42Hs kinase, chimeric DNA binding factor, and zinc finger protein 261. In these three groups, many genes with unknown functions were downregulated.

Confirmation of Gene Alteration by RT-PCR

To validate the results of microarray analyses and to minimize the occurrence of "false positives," the expression levels of five genes were measured by using relative quantitative RT-PCR. Because bioavailable metals, particularly iron, were well characterized in these coal samples (Zhang et al., 2002), TfR and MT genes were used for RT-PCR confirmation. For example, TfR gene repressed by PA coal at 6 h and MT and MT-2A genes repressed by the same coal at 6 and 24 h on the chip were confirmed by RT-PCR (Figure 6). GAPDH, a glycolytic protein sensitive to oxidative stress (Dastoor & Dreyer 2001; Ito et al., 1996), was upregulated by the PA coal at 6 h and 24 h treatment, but to a much lesser extent, by the UT coal at 24 h of treatment. The results in Figure 6 showed that the gene expression patterns from two independent methods were similar.

DISCUSSION

In the present study, data showed that 1050 genes out of 12,000 genes on the chip were altered in the primary human bronchial epithelial cells treated with the 2 coal dusts at 2 time points in comparison to control cells. The coal

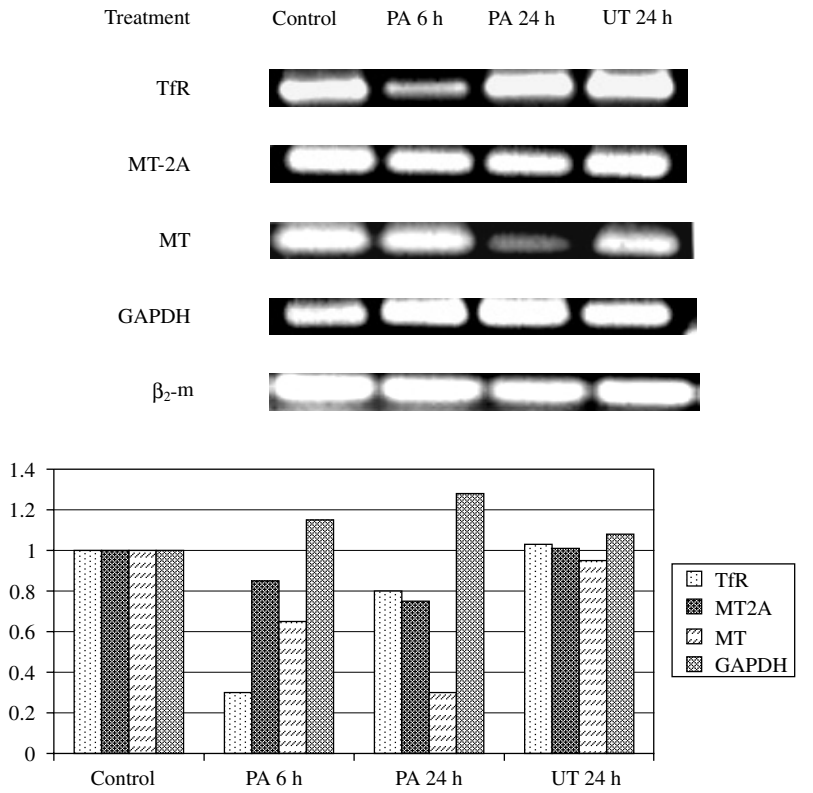


FIGURE 6. Confirmation by RT-PCR of gene downregulation or upregulation patterns Observed in the Genechip. The same RNA samples used for Genechip were used for RT-PCR except the sample treated with UT coal for 6 h (insufficient amounts of RNA for the assay).

from the PA coal-mine region with a high prevalence of CWP alters many more genes than coal from UT with a low prevalence. In the present study, a down-regulation of many genes, some of which were confirmed by RT-PCR using β_2-m as a housekeeping gene, was observed. GAPDH, which is usually used as a housekeeping gene, was upregulated by the PA coal. Previously, it was shown that the coals from PA coal-mine region contained large amounts of bioavailable iron (BAI) and were active in forming oxidants (Huang et al., 1998). The over-expression of GAPDH in the PA coal-treated cells is in agreement with data previously published by others, showing that oxidative stress increases mRNA levels of GAPDH and that the increase can be attenuated by the pretreatment of cells with iron chelator 1,10-phenanthroline (Ito et al., 1996). Our data also suggest that using one housekeeping gene, such as GAPDH, may not be sufficient for normalization of gene chip analyses. In the present study, the median intensities of 13 housekeeping genes were used for normalization, as mentioned in the Results section.

Examples of genes that were downregulated by the PA coal (6 h and 24 h of treatment) in the primary human bronchial epithelial cells include tissue inhibitor of metalloproteinase-3, zinc metalloproteinase, collagen, types VIII and IV, and insulinlike growth factor binding protein 3. It is known that the gene expression profile in the present study is the acute response of these primary cells under in vitro conditions to low concentrations of coal dust (2 μg/cm^2 for 6 or 24 h). Longer in vivo exposure may have different gene expression profile. However, it is still unclear why the highly fibrogenic PA coal downregulates some genes possibly involved in the development of CWP and progressive massive fibrosis (PMF). In fact, CWP and lung fibrosis is characterized by a substantial increase in the collagen content of the lung accompanied by major changes in lung architecture, after years of coal dust inhalation (Castranova & Vallyathan, 2000).

Bearing this in mind, genes that were related to iron metabolism were examined. Previously, it was shown that the coals from the PA coal-mine region with a high prevalence of CWP contain large amounts of BAI. The coals from UT with a low prevalence of CWP had low amounts of BAI (Huang et al., 1998; Zhang et al., 2002). The gene expression profile showed that TfR was repressed by PA coal but not by UT coal. It is known that cellular uptake of iron depends on the number of membrane TfRs. Increase in intracellular iron downregulates the TfR number, which is modulated by posttranscriptional regulation of TfR mRNA. In the setting of low cellular and cytosolic iron, the iron-responsive element-binding protein binds to a 3′ regulated untranslated region in TfR mRNA, increasing mRNA stability, and leads to increased transcription and a greater TfR number (Koeller et al., 1991). Conversely, when intracellular iron is elevated, which is the case in the cells treated with PA coal, iron interacts with the iron-binding protein and markedly reduces the protein affinity for TfR mRNA. Without bound iron-binding proteins, the stability of TfR mRNA is greatly decreased, leading to a reduction in TfR receptor number (Eisenstein, 2000; Eisenstein & Blemings, 1998). Therefore, as a protective measure of cells against the elevated iron concentration in the PA coal, TfR mRNA was downregulated. This observation was confirmed by RT-PCR (Figure 6). Interestingly, MT and MT-2a were also downregulated by the PA coal at 6 and 24 h treatment along with many other zinc-binding proteins. It has been shown that MT downregulation occurs when human cells become immortalized (Duncan & Reddel, 1999). No significant differences in levels of ferritin mRNA among samples were found, which was due to the high fluorescence readings of ferritin transcripts (saturation on the gene chips, data not shown). These results suggest that primary human bronchial epithelial cells, in the presence of iron-containing PA coal, may temporarily protect themselves from intracellular iron increase by downregulating TfR. For the same reason, it is postulated that early biological response of primary human bronchial epithelial cells to the high fibrogenicity of the PA coal may downregulate the fibrogenic genes.

In summary, the results of the present study show that microarray-based analysis of gene expression provides an effective tool for the identification of

gene targets that are involved in primary human bronchial epithelial cells in response to coal dust treatment. It was clearly shown that the coal from the PA coal-mine region with a high prevalence of CWP alter many more genes than coal from UT with a low prevalence of CWP, which may provide an explanation for the observed regional differences in the prevalence of CWP. Longer treatment times or higher concentrations might have changed the expression profile, particularly for gene induction. Macrophages may have given different etiologic results. Since our experiment was performed only one time on each chip (four conditions plus controls from triplicate treatments, which were pooled and hybridized on one chip), it is not possible to comment on reproducibility of the Genechip.

REFERENCES

Alizadeh, A. A., Ross, D. R., Perou, C. M., and van de Rijn, M. 2001. Towards a novel classification of human malignancies based on gene expression patterns. *J. Pathol.* 195:41–52.

Amoudru, C. 1987. Les pneumoconioses—Dimension actuelle des problemes. *Colloque INSERM* 155:3–40.

Attfield, M. D., and Castellan, R. M. 1992. Epidemiological data on US coal miners' pneumoconiosis, 1960 to 1988. *Am. J. Public Health* 82:964–970.

Attfield, M. D., and Morring, K. 1992. An investigation into the relationship between coal workers' pneumoconiosis and dust exposure in U.S. coal miners. *Am. Ind. Hyg. Assoc. J.* 53:486–492.

Attfield, M. D., and Seixas, N. S. 1995. Prevalence of pneumoconiosis and its relationship to dust exposure in a cohort of U.S. bituminous coal miners and ex-miners. *Am. J. Ind. Med.* 27:137–151.

Attfield, M., and Wagner, G. 1993. Respiratory disease in coal miners. In *Environmental and occupational medicine*, ed. W. N. Rom, 2nd ed., pp. 325–344. Boston: Little, Brown.

Blackford, J. A., Jr., Jones, W., Dey, R. D., and Castranova, V. 1997. Comparison of inducible nitric oxide synthase gene expression and lung inflammation following intratracheal instillation of silica, coal, carbonyl iron, or titanium dioxide in rats. *J. Toxicol. Environ. Health* 51:203–218.

Borm, P. J., and Schins, R. P. 2001. Genotype and phenotype in susceptibility to coal workers' pneumoconiosis. The use of cytokines in perspective. *Eur. Respir. J. Suppl.* 32:127s–133s.

Cao, J., and Cousins, R. J. 2002. Metallothionein mRNA in monocytes and peripheral blood mononuclear cells and in cells from dried blood spots increases after zinc supplementation of men. *J. Nutr.* 130:2180–2187.

Castranova, V., and Vallyathan, V. 2000. Silicosis and coal workers' pneumoconiosis. *Environ. Health Perspect.* 108 (suppl. 4):675–684.

Coggon, D., Inskip, H., Winter, P., and Pannet, B. 1995. Contrasting geographical distribution of mortality from pneumoconiosis and chronic bronchitis and emphysema in British coal miners. *Occup. Environ. Med.* 52:554–555.

Dalal, N. S., Shi, X. L., and Vallyathan, V. 1990. Role of free radicals in the mechanisms of hemolysis and lipid peroxidation by silica: Comparative ESR and cytotoxicity studies. *J. Toxicol. Environ. Health.* 29:307–316.

Dalal, N. S., Suryan, M. M., Vallyathan, V., Green, F. H., Jafari, B., and Wheeler, R. 1989. Detection of reactive free radicals in fresh coal mine dust and their implication for pulmonary injury. *Ann. Occupy. Hyg.* 33:79–84.

Dastoor, Z., and Dreyer, J. L. 2001. Potential role of nuclear translocation of glyceraldehyde-3-phosphate dehydrogenase in apoptosis and oxidative stress. *J. Cell. Sci.* 114:1643–1653.

Duncan, E. L., and Reddel, R. R. 1999. Downregulation of metallothionein-IIA expression occurs at immortalization. *Oncogene* 18:897–903.

Eisenstein, R. S. 2000. Iron regulatory proteins and the molecular control of mammalian iron metabolism. *Annu. Rev. Nutr.* 26:627–662.

Eisenstein, R. S., and Blemings, K. P. 1998. Iron regulatory proteins, iron responsive elements and iron homeostasis. *J. Nutr*. 128:2295–2298.

Huang, Q., Liu, D., Majewski, P., Schulte, L. C., Korn, J. M., Young, R. A., Lander, E. S., and Hacohen, N. 2001. The plasticity of dendritic cell responses to pathogens and their components. *Science* 294:870–875.

Huang, X., Fournier, J., Koenig, K., and Chen, L. C. 1998. Buffering capacity of coal and its acid-soluble Fe2 + content: Possible role in coal workers' pneumoconiosis. *Chem. Res. Toxicol*. 11:722–729.

Huang, X., Zahna, R., and Pezerat, H. 1999. Chemical reactivity of the carbon-centered free radicals and ferrous iron in coals: Role of bioavailable Fe^{2+} in coal workers pneumoconiosis. *Free Radical Res*. 30:439–451.

Hurley, J. F., Burns, J., Copland L., Dodgson, J., and Jacobsen, M. 1982. Coalworkers' simple pneumoconiosis and exposure to dust at 10 British coalmines. *Br. J. Ind. Med*. 39:120–127.

lto, Y., Pagano, P. J., Tornheim, K., Brecher, P., and Cohen, R. A. 1996. Oxidative stress increases glyceraldehyde-3-phosphate dehydrogenase mRNA levels in isolated rabbit aorta. *Am. J. Physiol*. 270:H81–H87.

Koeller, D. M., Horowitz, J. A., Casey, J. L., Klausner, R. D., and Harford, J. B. 1991. Translation and the stability of mRNAs encoding the transferrin receptor and c-fos. *Proc. Natl. Acad. Sci*. USA 88:7778–7782.

Mididoddi, S., McGuirt, J. P., Sens, M. A., Todd, J. H., and Sens, D. A. 1996. Isoform-specific expression of metallothionein mRNA in the developing and adult human kidney. *Toxicol. Lett*. 85:17–27.

Miller, B. G., and Jacobsen, M. 1985. Dust exposure, pneumoconiosis, and mortality of coalminers. *Br. J. Ind. Med*. 42:723–733.

Morgan, W. K. C., Burgess, D. B., Jacobson, G., O'Brien, R. J., Pendergrass, E., Reger, R. B., and Shoub, E. P. 1973. The prevalence of coal workers' pneumoconiosis in U.S. coal miners. *Arch. Environ. Health* 27:221–230.

Nadadur, S. S., and Kodavanti, U. P. 2002. Altered gene expression profiles of rat lung in response to an emission particulate and its metal constituents. *J. Toxicol. Environ. Health A* 65:1333–1350.

Reisner, M. T. R., and Robock, K. 1977. Results of epidemiological, mineralogical and cytotoxicological studies on the pathogenicity of coal mine dusts. In *Inhaled particles IV*, ed. W. H. Walton, pp. 703–715. Oxford, UK: Pergamon Press.

Rom, W. N. 1991. Relationship of inflammatory cell cytokines to disease severity in individuals with occupational inorganic dust exposure. *Am. J. Ind. Med*. 19:15–27.

Rom, W. N., Bitterman, P. B., Rennard, S. I., Cantin, A., and Crystal R. G. 1987. Characterization of the lower respiratory tract inflammation of nonsmoking individuals with interstitial lung disease associated with chronic inhalation of inorganic dusts. *Am. Rev. Respir. Dis*. 136:1429–1434.

Rom, W. N., Kanner, R. E., Renzetti, A. D., Jr., Shigeoka, J. W., Barkman H. W., Nichols, M., Turner, W. A., Coleman, M., and Wright, W. E. 1981. Respiratory disease in Utah coal miners. *Am. Rev. Respir. Dis*. 123:372–377.

Sposi, N. M., Cianetti, L., Tritarelli, E., Pelosi, E., Militi, S., Barberi, T., Gabbianelli, M., Saulle, E., Kuhn, L., Peschle, C., and Testa, U. 2000. Mechanisms of differential transferrin receptor expression in normal hematopoiesis. *Eur. J. Biochem*. 267:6762–6774.

Walton, W. H., Dodgson, J., Hadden, G. G., and Jacobsen, M. 1977. The effect of quartz and other non-coal dusts in coal workers' pneumoconiosis. In *Inhaled particles IV*, ed. W. H. Walton, pp. 669–689. Oxford: Pergamon Press.

Wu, T. D. 2001. Analysing gene expression data from DNA microarrays to identify candidate genes. *J. Pathol*. 195:53–65.

Zhang, Q., Dai, J., Ali, Chen, L., and Huang, X. 2002. Roles of bioavailable iron and calcium in coal dust-induced oxidative stress: Possible implications in coal workers' lung disease. *Free Radical Res*. 36:285–294.