Discrimination of Vanadium from Zinc Using Gene Profiling in Human Bronchial Epithelial Cells

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We hypothesized that gene expression profiling may discriminate vanadium from zinc in human bronchial epithelial cells (HBECS). RNA from HBECS exposed to vehicle, V (50 µM), or Zn (50 µM) for 4 hr (n = 4 paired experiments) was hybridized to Affymetrix Hu133A chips. Using one-class t-test with p < 0.01, we identified 140 and 76 genes with treatment:control ratios ≥ 2.0 or ≤ 0.5 for V and Zn, respectively. We then categorized these genes into functional pathways and compared the number of genes in each pathway between V and Zn using Fisher’s exact test. Three pathways regulating gene transcription, inflammatory response, and cell proliferation distinguished V from Zn. When genes in these three pathways were matched with the 163 genes flagged by the same statistical filtration for V:Zn ratios, 12 genes were identified. The hierarchical clustering analysis showed that these 12 genes discriminated V from Zn and consisted of two clusters. Cluster 1 genes (ZBTB1, PML, ZNF44, SIX1, BCL6, ZNF450) were down-regulated by V and involved in gene transcription, whereas cluster 2 genes (IL8, IL1A, PTGS2, DTR, TFBAP3, CXL3) were up-regulated and linked to inflammatory response and cell proliferation. Also, metallothionein 1 genes (MT1F, MT1G, MT1K) were up-regulated by Zn only. Thus, using microarray analysis, we identified a small set of genes that may be used as biomarkers for discriminating V from Zn. The novel genes and pathways identified by the microarray may help us understand the pathogenesis of health effects caused by environmental V and Zn exposure. Key words: cell proliferation, inflammation, interleukin-1, interleukin-8, metal, microarray, transcription.

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The advancement of microarray technology has allowed investigators to examine simultaneously changes in thousands of genes induced by environmental toxins. McDowell et al. (2000), using gene array with more than 8,000 cDNAs, found patterns of gene expression consistent with acute lung injury in nickel-treated mice. Sato et al. (1999) showed changes in genes related to cell growth and possibly carcinogenesis in rat lungs treated with diesel particles. More recently, Andrew et al. (2003) demonstrated distinct expression patterns in human lung cells exposed to low and high doses of arsenic. The capability of microarrays to provide a snapshot view of expression of a large number of genes may help us generate mechanistic hypotheses as well as identify biomarkers of exposure specific to environmental toxins. The availability of such specific genomic biomarkers may be important in determining the nature of environmental exposures.

Vanadium is present in several environmental settings, for example, during overheating of oil-fired boilers and burning of heavy fuel in power plants. Exposures to high levels of V-rich particles produce upper and lower respiratory symptoms (Levy et al. 1984; Woodin et al. 1999, 2000). Intratracheal administration of vanadyl sulfate (VOSO4) and a V-rich pollutant dust, residual oil fly ash (ROFA), increased pulmonary artery pressure acutely in buffer-perfused rabbit lungs (Huang et al. 2002) and constricted isolated rat aortic rings (Cadene et al. 1997). Particulate air V concentration correlated with increases in heart rate variability index in boilermakers (Magari et al. 2002). V or ROFA altered the expression of many genes and their protein products related to acute stress (Carter et al. 1997; Gavett et al. 1997, 1999; Nadadur et al. 2000; Samet et al. 1998) and cell survival and tissue growth in cultured cells (Chen et al. 2001; Huang et al. 2000; Zhang et al. 2001).

Zinc is ubiquitous in the natural environment, including ambient air (Walsh et al. 1994). Exposure to excessive Zn (via metal fumes) is a potential hazard for industrial workers who perform welding and smelting operations. Inhalation of high concentrations of zinc oxide or zinc chloride produce respiratory epithelial cell damage, inflammation, and acute injury (Doig and Challen 1964; Evans 1945; Kuchar et al. 1995; Matarrese and Matthews 1986; Nemery 1990; Pare and Sandler 1954). Treatment of lung epithelial cells in vitro with Zn compounds enhanced inflammatory signaling and produced cytotoxicity and cell death (Riley et al. 2003; Samet et al. 1998, 1999).

Although V and Zn belong to different elemental classes in the periodic table, they share many biologic properties. For example, both metals are potent enhancers for phosphorylation of signaling proteins, including mitogen-activated protein kinase (Samet et al. 1998) and epidermal growth factor receptors (Wu et al. 1999), and both increase Ras activity (Wu et al. 2002) and interleukin-8 (IL8) release (Samet et al. 1998). Many of these effects may be attributed to the capability of these metals to inhibit protein tyrosine phosphatase activity (Samet et al. 1999). Both V and Zn also inhibit metabolic activity of the cells (Riley et al. 2003). V and Zn may coexist in the ambient environment after being released from different emission sources (Nriagu and Pacyna 1988). The development of a biomarker that discriminates these metals thus may help define the sources and nature of exposures. In this study we hypothesized that gene profiling may be used to discriminate V from Zn in human bronchial epithelial cells (HBECS). We sought to identify a small group of genes that may serve as biomarkers of exposure.

Materials and Methods

Cell culture. Two bronchoscopists obtained bronchial epithelial cells from normal volunteers through bronchoscopic bronchial brushings following the same operational guidelines (Ghio et al. 2000; Huang et al. 2003). Subjects were informed of the procedures and potential risks, and each gave written informed consent. The protocol was approved by the University of North Carolina School of Medicine Committee on Protection of the Rights of Human Subjects and by the U.S. Environmental Protection Agency. A single experienced technician processed all brushings by following the...
established standard of procedures in our laboratory. The cells (passage 2 or 3) were maintained in bronchial epithelial growth medium (BEGM) (Clonetics, San Diego, CA), supplemented with bovine pituitary extract, insulin (5 µg/mL), hydrocortisone (0.5 µg/mL), gentamicin (50 µg/mL), retinoic acid (0.1 ng/mL), transferrin (10 µg/mL), triiodothyronine (6.5 µg/mL), epinephrine (0.5 µg/mL), and human epidermal growth factor (0.5 ng/mL). The fragmented RNA was diluted in hybridization buffer (Tris, magnesium (20 µg) was incubated at 94°C for 35 min, and then injected into a GeneChip cartridge. The GeneChip array was incubated at 42°C for at least 16 hr in a rotating oven at 60 rpm. GeneChips were washed with a series of non-stringent (25°C) and stringent (50°C) solutions containing variable amounts of 2-morpholinoethanesulfonic acid, Tween 20, and SSPE (3 M NaCl, 0.2 M, NaH₂PO₄, 0.02 M EDTA). The microarrays were then stained with streptavidin phycoerythrin, and the fluorescent signal was amplified using a biotinylated antibody solution. Fluorescent images were detected in a GeneChip Scanner 3000 (Affymetrix), and expression data were extracted using the default settings in the MicroArray Suite 5.0 software (Affymetrix). All GeneChips were scaled to a median intensity setting of 500. Four independent sets of experiments were performed on HBECs obtained from four different individuals. Each set consisted of control (vehicle), VOSO₄, and ZnSO₄.

**Quantitative polymerase chain reaction.** Quantitative polymerase chain reaction (Q-PCR) was performed for selected genes to validate microarray results. HBECs were lysed in guanidine isothiocyanate (GIC) buffer (4 M GIC (Boehringer Mannheim, Indianapolis, IN)), 25 mM sodium citrate (pH 7.0), 0.5% sarkosyl, and 0.1 M DTT, and RNA was pelleted at 80,000 rpm through a cesium chloride gradient for 2 hr at 15°C. cDNAs were synthesized from 0.4 µg of total RNA in 100 µL of a buffer containing 5 µM random hexaoligonucleotide primers (Pharmacia, Piscataway, NJ), 10 U/µL Moloney murine leukemia virus reverse transcriptase and cyclooxygenase)

| Gene accession no. | Fold change | Gene symbol | Gene name |
|-------------------|-------------|-------------|-----------|
| Hs.624            | 8.04        | IL8         | interleukin 8 |
| Hs.230873         | 6.67        | PPEF2       | protein phosphatase, EF hand calcium-binding domain 2 |
| Hs.519417         | 5.52        | STX6        | syntaxin 6 |
| Hs.233988         | 5.98        | CVL         | carboxypeptidase, vitellogenic-like |
| Hs.196384         | 4.67        | PTGS2       | prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase) |
| Hs.248189         | 4.46        | KRTHA6      | keratin, hair, acidic, 6 |
| Hs.211600         | 4.33        | TNFAP3      | tumor necrosis factor, alpha-induced protein 3 |
| Hs.477070         | 4.30        | CSNK1B      | casein kinase 1, delta |
| Hs.431460         | 4.24        | ICAM2       | intercellular adhesion molecule 2 |
| Hs.443854         | 4.24        | SBF1        | stoned B-like factor |
| Hs.799            | 4.21        | DTR         | diaphtheria toxin receptor (heparin-binding epidermal growth factor-like factor) |
| Hs.418167         | 4.16        | ALB         | albumin |
| Hs.246310         | 4.11        | ALM2        | junctional adhesion molecule 2 |
| Hs.406990         | 4.06        | ROC4DP      | phosphoserine 4D interacting protein (myomegalin) |
| Hs.992            | 4.04        | PL2G1B      | phospholipase A2, group IB (pancreas) |
| Hs.454822         | 3.97        | ANGPT1L     | angiopeptin-like 1 |
| Hs.65758          | 3.78        | ITPR3       | inositol 1,4,5-trisphosphate receptor, type 3 |
| Hs.65713          | 3.70        | DIPA        | hepatitis delta antigen-interacting protein A |
| Hs.519884         | 3.65        | GCNT2       | glucosaminyl (N-acetyl) transferase 2, l-branching enzyme |
| Hs.157259         | 3.64        | SIPA1L2     | signal-induced proliferation-associated 1-like 3 |
| Hs.436203         | 3.63        | PRDM1       | PR domain containing 1, with ZNF domain |
| Hs.303980         | 3.51        | CYP11A1     | cytochrome P450, family 11, subfamily A, polypeptide 1 |
| Hs.236646         | 3.49        | HOXD9       | homeo box D9 |
| Hs.171695         | 3.46        | DUSP1       | dual specificity phosphatase 1 |
| Hs.197683         | 3.44        | CACNG2      | calcium channel, voltage-dependent, gamma subunit 2 |
| Hs.485910         | 3.34        | RARSL       | arginyl-tRNA synthetase-like |
| Hs.211233         | 3.30        | IL1F9       | interleukin 1, family, member 9 |
| Hs.520319         | 3.30        | SLC22A16     | solute carrier family 22, organic cation transporter, member 16 |
| Hs.445555         | 3.22        | SERPIN2     | serine (or cysteine) proteinase inhibitor, clade D (neurosperin), member 2 |
| Hs.256667         | 3.20        | PKD2        | pyruvate dehydrogenase kinase, isozyme 2 |
| Hs.248122         | 3.10        | EPR24       | G-protein-coupled receptor 24 |
| Hs.511899         | 3.07        | EDN1        | endothelin 1 |
| Hs.525306         | 2.99        | WARS2       | tryptophanyl tRNA synthetase 2 (mitochondrial) |
| Hs.333175         | 2.86        | PL2G12B     | phospholipase A2, group XIB |
| Hs.410817         | 2.87        | RPL13       | ribosomal protein L13 |
| Hs.520942         | 2.77        | CLDN4       | claudin 4 |
| Hs.508230         | 2.74        | PDCD2       | programmed cell death 6 |
| Hs.550498         | 2.72        | RCE1        | RCE1 homolog, prenyl protein protease (S. cerevisiae) |
| Hs.511899         | 2.72        | PRDM1       | PR domain containing 1, with ZNF domain |
| Hs.241724         | 2.66        | CTSG        | cathepsin G |

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transcriptase (GIBCO BRL Life Technologies), 1 U/µL RNase inhibitor (RNasin; Promega, Madison, WI), 0.5 mM dNTP (Pharmacia), 50 mM KCl, 3 mM MgCl₂, and 10 mM Tris-HCl (pH 9.3). After 1 hr of incubation at 39°C, the reverse transcriptase was heat inactivated at 94°C for 4 min.

Q-PCR of specimen cDNA and standard cDNA was performed using TaqMan master mix (Perkin Elmer, Foster City, CA), 1.25 µM probe, 3 µM forward primer, and 3 µM reverse primer in a 50-µL volume. The probe, which contains both a fluorescence reporter dye at the 5’-end (6-carboxytetramethyl rhodamine, TAMRA: maximum emission wavelength = 518 nm) and a 5’-end (6-carboxyfluorescein, 6-FAM: maximum emission wavelength = 518 nm) and a quencher dye at the 3’-end (carboxy-X-rhodamine, AQ-2: maximum emission wavelength = 562 nm), is degraded by the 5’-3’ exonuclease activity of the Taq DNA polymerase, and the resulting fluorescence is detected by a laser in the sequence detector (TaqMan ABI Prism 7700 Sequence Detector System; PerkinElmer). The relative abundance of mRNA levels was determined from standard curves generated from a serially diluted standard pool of cDNA prepared from BEAS-2B cells. The relative abundance of glycoldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was used to normalize levels of the mRNAs of interest. Six additional sets of Q-PCR experiments consisting of control (vehicle), VOSO₄, and ZnSO₄ were performed using HBECs from six different individuals.

**Microarray data analysis.** The microarray data were deposited in the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo; accession number GSE2111). Gene expression values were background-corrected and normalized globally using the default setting of the Affymetrix Microarray Suite 5.0 software, and log₂-transformed according to the Affymetrix Statistical Algorithm Reference Guide (Affymetrix, Inc. 2004b). The log₂ ratios of treatment (V or Zn) over control and V over Zn for all probe sets were analyzed using the one-class t-test against the null hypothesis of 0 (ratio = 1) using the Multiexperiment Viewer (version 3.0; The Institute of Genomic Research, Rockville, MD). A p-value of < 0.01 was considered statistically significant. If more than one probe set for the same gene were flagged, their ratios were averaged.

**Functional classification of genes.** Biologic processes represented by the differentially expressed genes were compiled using the GOCharts in the Database for Annotation, Visualization and Integrated Discovery (DAVID) (http://apps1.nciaid.nih.gov/david/) with the coverage and specificity set at level 5 (high) and the hits threshold at 1; with the classification of the Gene Ontology Consortium (http://www.geneontology.org); and with the human gene resources from NCBI (http://www.ncbi.nlm.nih.gov). Comparison of the probe sets in the biologic processes between V and Zn was determined by the Fisher’s exact test (p < 0.05) (StatView 4.0; SAS Inc., Cary, NC).

**Results**

**Differentially expressed genes associated with V treatment.** Incubation of HBECs with VOSO₄ at 50 µM for 4 hr showed no cytotoxicity as supported by the lack of lactate dehydrogenase (LDH) release (data not shown). There were 140 differentially expressed genes with known protein products. Seventy-six genes were up-regulated with a treatment:control ratio ≥ 2.0 (Table 1), and 64 genes were down-regulated with a treatment:control ratio ≤ 0.5 (Table 2). The expression of five up-regulated genes (IL8, prostaglandin-endoperoxide synthase 2 (PTGS2), intercellular adhesion molecule 2 (ICAM2), diphtheria toxin receptor (heparin-binding epidermal growth factor-like growth factor) (DTR), and dual specificity phosphatase 1 (DUSP1) was confirmed by Q-PCR in additional experiments (Figure 1). The 140 genes could be further classified functionally into 28 biologic processes containing at least three gene hits.

**Differentially expressed genes associated with Zn treatment.** Incubation of HBECS with ZnSO₄ at 50 µM for 4 hr also showed no LDH release (data not shown). There were 76 differentially expressed genes with known protein products. Forty-three genes were up-regulated with a treatment:control ratio ≥ 2.0 (Table 3), and 33 genes were down-regulated with a treatment:control ratio ≤ 0.5 (Table 4). The up-regulation of metallothionein 1F (MT1F) and heme oxygenase 1 (HMOX1) was confirmed by Q-PCR (Figure 1). The 76 genes could be further

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**Table 1. Continued.**

| Gene accession no. | Fold change | Gene symbol | Gene name |
|--------------------|-------------|-------------|------------|
| Hs.2250            | 2.63        | LIF         | leukemia inhibitory factor (cholinergic differentiation factor) polymerase (RNA) III (DNA directed) (32 kDa) |
| Hs.262397          | 2.58        | RP52        | ras homolog gene family, member J |
| Hs.525898          | 2.56        | ARHI        | protein phosphatase 1, regulatory subunit 10 |
| Hs.106019          | 2.54        | PPP1R10     | solute carrier family 13 (sodium-dependent dicarboxylate transporter), member 3 |
| Hs.250281          | 2.52        | SLC13A3     | zinc finger protein 306 |
| Hs.2128            | 2.48        | DUSP5       | dual-specificity phosphatase 5 |
| Hs.89690           | 2.45        | CXCL3       | chemokine (C-X-C motif) ligand 3 |
| Hs.111690          | 2.45        | MIG-6       | mitogen-inducible gene 6 |
| Hs.788             | 2.41        | CXCL1       | chemokine (C-X-C motif) ligand 1 (melanoma growth-stimulating activity, alpha) |
| Hs.485004          | 2.37        | ZNF306      | zinc finger protein 306 |
| Hs.534478          | 2.36        | DUSP21      | dual-specificity phosphatase 21 |
| Hs.441972          | 2.34        | IFNT1       | interferon tau-1 |
| Hs.503598          | 2.33        | JMJD20      | jumonji domain containing 2D |
| Hs.546252          | 2.25        | EGC3        | endothelial differentiation, sphingolipid G-protein-coupled receptor, 3 |
| Hs.85862           | 2.23        | PDLIM3      | PDZ and LIM domain 3 |
| Hs.445489          | 2.22        | PLEKH1B     | pleckstrin homology domain containing, family B (e vecnins), member 1 |
| Hs.1722            | 2.21        | IL1A        | interleukin 1, alpha |
| Hs.466871          | 2.21        | PLAUR       | plasminogen activator, urkinase receptor |
| Hs.159291          | 2.20        | DNP2        | dystrophin-related protein 2 |
| Hs.303649          | 2.19        | CCL2        | chemokine (C-C motif) ligand 2 |
| Hs.111944          | 2.19        | CYP3A7      | cytochrome P450, family 3, subfamily A, polypeptide 7 |
| Hs.533683          | 2.19        | FGR2        | fibroblast growth factor receptor 2 |
| Hs.50550           | 2.19        | KBTBD10     | kelch repeat and BTB (POZ) domain containing 10 |
| Hs.78844           | 2.19        | RGS2        | regulator of G-protein signaling 2, 24 kDa |
| Hs.190783          | 2.17        | HAL         | histidine ammonia-lyase |
| Hs.463059          | 2.17        | STAT3       | signal transducer and activator of transcription 3 (acute-phase response factor) |
| Hs.25847           | 2.16        | FOS         | v-fos FBJ murine osteosarcoma viral oncogene homolog |
| Hs.127022          | 2.14        | PTPRE       | protein tyrosine phosphatase, receptor type, E |
| Hs.447899          | 2.13        | SIGLEC8     | sialic acid-binding Ig-like lectin 8 |
| Hs.344812          | 2.13        | TREC1       | three prime repair exonuclease 1 |
| Hs.528610          | 2.12        | MMP25       | matrix metallocproteinase 25 |
| Hs.514913          | 2.11        | SERPNB2     | serine (or cysteine) proteinase inhibitor, clade B (ovalbumin), member 2 |
| Hs.506381          | 2.07        | FGID6       | FYVE, RhoGEF and PH domain containing 6 |
| Hs.278658          | 2.06        | KRTHB6      | keratin, hair, basic, 6 (monilethrix) |
| Hs.439060          | 2.08        | CLDN1       | claudin 1 |
| Hs.507348          | 2.05        | HSSST1      | heparan sulfate (glucosamine) 3-O-sulfotransferase 1 |

*Only genes with known protein products are shown.
*Fold changes are the average of four individuals.
Hs.498292 –2.89 SDCCAG8 serologically defined colon cancer antigen 8
Hs.142167 –2.11 HSZFP36 ZFP-36 for a zinc finger protein
Hs.535499 –2.02 RARA retinoic acid receptor, alpha
Hs.131494 –2.00 ARNT aryl hydrocarbon receptor nuclear translocator
Hs.195710 –2.08 ZNF503 zinc finger protein 503
Hs.518438 –2.08 SOX2 SRY (sex determining region Y)-box 2
Hs.63335 –2.12 TERF2 telomeric repeat binding factor 2
Hs.105633 –2.12 WINS1 WIN51 protein with Drosophila Lin homologous domain
Hs.497868 –2.41 BCL6 B-cell CLL/lymphoma 6 (BCL6), IL1α (IL1A), IL8, PTGS2, DTR, chemokine (C-X-C motif) ligand 3 (CXCL3), promyelocytic leukemia (PML), sine oculis homeobox homolog 1 (Drosophila) (SIX1), tumor necrosis factor (TNF), α-induced protein 3 (TNFAIP3), Zn finger and BTB domain containing 1 (ZBTB1), Zn finger protein 44 (KOX 7) (ZNF44), and Zn finger protein 450 (ZNF450). The hierarchical cluster analysis showed that these 12 genes clearly discriminated the V group from the Zn group (Figure 2) and could be separated into two clusters (Figure 2). Cluster 1 contained ZBTB1, PML, ZNF44, SIX1, BCL6, and ZNF450 that were down-regulated by V and involved in gene transcription. Cluster 2 contained IL8, IL1α, PTGS2, DTR, TNFAIP3, and CXCL3 that were up-regulated and linked primarily to inflammatory response and cell proliferation.

Discussion
In the present study we first determined the differential gene expression patterns in HBECs exposed to 50 µM of V and Zn and found 147 and 76 genes altered by V and Zn, respectively, compared with control. These genes could be classified into 14 biologic processes containing at least three gene hits.

Identification of genes differentiating V from Zn.
To identify genes that would discriminate V from Zn, we first analyzed V:Zn ratios using the same statistical filtration method. A total of 163 genes were identified. The results of the hierarchical clustering analysis using these genes are shown in Figure 2. We next compared biologic processes associated with V with those associated with Zn. We found that four biologic processes, regulation of transcription (24 genes), DNA-dependent transcription (22 genes), inflammatory responses (11 genes), and regulation of cell proliferation (10 genes), contained a disproportionately greater number of V-induced genes. Because all genes involved in the DNA-dependent transcription pathway were also flagged in the regulation of transcription pathway, these two processes were combined into one, designated “gene transcription.” The number of probe sets in the three biologic pathways associated with V and Zn treatment was compared using the Fisher’s exact test. The p-values for these three pathways, gene transcription, inflammatory response, and regulation of cell proliferation, are 0.004, 0.037, and 0.013, respectively.

We next matched genes in these three pathways with the 163 genes and identified 12 candidate genes: B-cell CLL/lymphoma 6 (BCL6), IL1α (IL1A), IL8, PTGS2, DTR, chemokine (C-X-C motif) ligand 3 (CXCL3), promyelocytic leukemia (PML), sine oculis homeobox homolog 1 (Drosophila) (SIX1), tumor necrosis factor (TNF), α-induced protein 3 (TNFAIP3), Zn finger and BTB domain containing 1 (ZBTB1), Zn finger protein 44 (KOX 7) (ZNF44), and Zn finger protein 450 (ZNF450). The hierarchical cluster analysis showed that these 12 genes clearly discriminated the V group from the Zn group (Figure 2) and could be separated into two clusters (Figure 2). Cluster 1 contained ZBTB1, PML, ZNF44, SIX1, BCL6, and ZNF450 that were down-regulated by V and involved in gene transcription. Cluster 2 contained IL8, IL1α, PTGS2, DTR, TNFAIP3, and CXCL3 that were up-regulated and linked primarily to inflammatory response and cell proliferation.

Discussion
In the present study we first determined the differential gene expression patterns in HBECs exposed to 50 µM of V and Zn and found 147 and 76 genes altered by V and Zn, respectively, compared with control. These genes could be
classified into 28 and 14 biologic pathways, respectively, that each had at least three gene hits. Seven differentially expressed genes were validated prospectively in six additional experiments using HEBCs from six different individuals. When the numbers of genes in the pathways were compared between V and Zn, three biologic processes (gene transcription, inflammatory response, and regulation of cell proliferation) contained a disproportionately greater number of V-induced genes. We then matched the genes in these three pathways with the 163 genes that differentiated V from Zn, and identified 12 candidate genes.

These 12 genes clearly discriminated the V group from the Zn group based on the hierarchical clustering analysis and could be separated into two clusters. The first cluster consisted of 6 genes (ZBTB1, PML, ZNF44, SIX1, BCL6, ZNF436) that were down-regulated by V but mildly up-regulated by Zn. All 6 genes were involved in gene transcription, and BCL6 was also linked to inflammatory response and regulation of cell proliferation. The inhibitory effects of V on the expression of these genes have not been reported. Five of these genes encode Zn finger proteins (ZBTB1, ZNF44, BCL6, ZNF436) or proteins containing Zn-binding domains (PML) that play a role in DNA binding (Bray et al. 1991; Zhong et al. 2000). SIX1 encodes a protein characterized by a divergent DNA-binding homeodomain and an upstream SIX domain, which may be involved in determining DNA-binding specificity and protein–protein interactions. Mice lacking the SIX1 gene have impaired organogenesis of skeletal muscle and kidney during embryonic development (Laclef et al. 1996), but its function is unclear. Six biologic pathways were compared between V and Zn, with the 163 genes that differentiated V from Zn. The numbers of genes in the 163 genes that differentiated V from Zn and identified 12 candidate genes.

Gene profiling for vanadium and zinc. Only genes with known protein products are shown.

**Table 3. Genes up-regulated by ZnSO4**

| Gene accessiona | Fold changeb | Gene symbola | Gene namea |
|-----------------|--------------|--------------|------------|
| Hs.188518       | 81.01        | MT1K         | metallothionein 1K |
| Hs.433391       | 28.87        | MT1G         | metallothionein 1G |
| Hs.283678       | 8.40         | PCDHBI4      | protocadherin beta 14 |
| Hs.412106       | 8.09         | ESRBBL1      | estrogen-related receptor beta-like 1 |
| Hs.502182       | 5.46         | BDNF         | brain-derived neurotrophic factor |
| Hs.517581       | 4.78         | HMOX1        | heme oxygenase (decycling) 1 |
| Hs.165736       | 4.67         | SCAND2       | SCAND domain containing 2 |
| Hs.519469       | 4.05         | SLC30A1      | solute carrier family 30 (zinc transporter), member 1 |
| Hs.513626       | 4.58         | MT1F         | metallothionein 1F (functional) |
| Hs.154296       | 4.58         | TLR2         | toll-like 2 |
| Hs.303090       | 3.94         | PPR1R3C      | protein phosphatase 1, regulatory (inhibitor) subunit 3C |
| Hs.118354       | 3.66         | PRB3         | proline-rich polypeptide 3 |
| Hs.468691       | 3.55         | ZNF232       | zinc finger protein 233 |
| Hs.59889        | 3.47         | HMGCS2       | 3-hydroxy-3-methylglutaryl-coenzyme A synthase 2 (mitochondrial) |

**Figure 1.** Gene expression ratios measured by Q-PCR. The expression of a gene associated with V or Zn treatment, relative to the control; n = 6 independent experiments in cells from six different individuals for Q-PCR. Dashed line denotes an expression ratio of 1 (no change). Data are mean ± SE.

**Table 3. Genes up-regulated by ZnSO4**

- **Gene accession**
- **Fold change**
- **Gene symbol**
- **Gene name**

### Only genes with known protein products are shown.

*Gene annotations are from NCBI (http://www.ncbi.nlm.nih.gov).* 
*Fold changes are the average of four individuals.*
Table 4. Genes down-regulated by ZnSO₄.

| Gene accession no. | Fold change | Gene symbol | Gene name |
|--------------------|-------------|-------------|-----------|
| Hs.376873          | -6.25       | ZNF390      | zinc finger protein 390 |
| Hs.106513          | -6.09       | TLL1        | toll-like 1 |
| Hs.200309          | -5.87       | IL23R       | interleukin-23 receptor |
| Hs.268581          | -5.47       | LPIN2       | lip 2 |
| Hs.112218          | -5.36       | CAPN10      | calpain 10 |
| Hs.532082          | -5.23       | IL6ST       | interleukin 6 signal transducer (gp130, oncostatin M receptor) |
| Hs.463136          | -4.53       | COMMD10     | COMM domain containing 10 |
| Hs.141349          | -4.39       | MOS         | myeloid oligodendrocyte glycoprotein |
| Hs.7138            | -4.10       | CHRNA3      | cholinergic receptor, muscarinic 3 |
| Hs.126033          | -4.08       | SESN3       | stress 3 |
| Hs.512567          | -3.58       | MST1        | macrophage stimulating 1 (hepatocyte growth factor-like) |
| Hs.370510          | -3.23       | IGF2F       | immunoglobulin superfamily, member 4 |
| Hs.533040          | -3.21       | PDLM47      | PDZ and LIM domain 7 (enigma) |
| Hs.552578          | -3.03       | TCF1        | transcription factor 1, hepatic; LF-B1, hepatic nuclear factor (HNF1), albumin proximal factor |
| Hs.472558          | -2.92       | SDCBG84     | sertolocal immunoglobulin superfamily member 84 |
| Hs.506394          | -2.77       | ubiquitin specific protease 44 |
| Hs.438989          | -2.69       | ZNF544      | zinc finger protein 544 |
| Hs.32721           | -2.61       | SAG         | S-antigen, retina and pineal gland (arrestin) |
| Hs.74082           | -2.48       | KLRC3       | killer cell lectin-like receptor subfamily C, member 3 |
| Hs.382683          | -2.47       | PRG-3       | plasticity-related gene 3 |
| Hs.522291          | -2.42       | PRKWNK2     | protein kinase, lysine deficient 2 |
| Hs.493275          | -2.34       | TRIM31      | tripartite motif-containing 31 |
| Hs.129899          | -2.29       | TBX3        | T-box 3 (ulnar mammary syndrome) |
| Hs.546233          | -2.29       | KIR3DL2     | killer cell immunoglobulin-like receptor, three domains, long cytoplasmic tail, 2 |
| Hs.546354          | -2.21       | RRP4        | homolog of yeast RRP4 (ribosomal RNA processing 4), 3′-5′-exoribonuclease |
| Hs.19385           | -2.19       | ABHD5       | abhydrolase domain containing 5 |
| Hs.344400          | -2.19       | MPHOSPH6    | M-phase phosphoprotein 6 |
| Hs.411311          | -2.17       | IL24        | interleukin 24 |
| Hs.492236          | -2.17       | H2B29       | histone H2B |
| Hs.255423          | -2.06       | CIB3        | calcium and integrin binding family member 3 |
| Hs.476852          | -2.02       | SNRK        | SNF1-related kinase |
| Hs.432889          | -2.01       | MAP2K13     | mitogen-activated protein kinase kinase kinase 13 |

Only genes with known protein products are shown.

*Gene annotations are from NCBI (http://www.ncbi.nlm.nih.gov). Fold changes are the average of four individuals.

Figure 2. The hierarchical clustering analysis for the 163 genes that discriminated V from Zn (A) and the 12 genes from this list identified by additional filtration algorithms described in the text (B). Each row represents one single gene, and each column represents one experiment. Red areas are up-regulation, and green areas are down-regulation, relative to control. The 12 genes clearly discriminate the V group and the Zn group. The analysis also divided the genes into two clusters. Gene names are from NCBI (http://www.ncbi.nlm.nih.gov).
fume increased mRNA of MTs in rat lungs (Cosma et al. 1992). Systemic administration of Zn enhanced MT levels in the liver (Conrad et al. 1997). Mice lacking MTs were more sensitive to Zn toxicity compared with wild-type mice (Park et al. 2001). In our study, in addition to increases in MT1F (4.6-fold), MT1G (29-fold), and MT1K (81-fold), other MTs, although not identified by our statistical filtration, also had elevated ratios: 1.36 for metallothionein 1X (MT1X), 1.17 for metallothionein 1H (MT1H) and 1.21 for metallothionein 2A (MT2A). These results confirm that up-regulation of the MTs may represent that up-regulation of the MTs may represent

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