Characterization of Gene Expression Changes Associated with MNNG, Arsenic, or Metal Mixture Treatment in Human Keratinocytes: Application of cDNA Microarray Technology

Dong-Soon Bae,[1] William H. Hanneman,[2] Raymond S.H. Yang,[1] and Julie A. Campain[1]

The identification of molecular markers related to critical biological processes during carcinogenesis may aid in the evaluation of carcinogenic potentials of chemicals and chemical mixtures. Work from our laboratory demonstrated that a single treatment with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) enhanced spontaneous malignant transformation of the human keratinocyte cell line RHEK-1. In contrast, chronic low-level exposure of cells to arsenic alone or in a mixture containing arsenic, cadmium, chromium, and lead inhibited malignant conversion. To identify changes in gene expression that influence these different outcomes, cDNA microarray technology was used. Analysis of multiple human arrays in MNNG-transformed RHEK-1 cells, designated OM3, and those treated with arsenic or the arsenic-containing metal mixture showed unique patterns of gene expression. Genes that were overexpressed in OM3 included oncogenes, cell cycle regulators, and those involved in signal transduction, whereas genes for DNA repair enzymes and inhibitors of transformation and metastasis were suppressed. In arsenic-treated cells, multiple DNA repair proteins were overexpressed. Mixture-treated cells showed increased expression of a variety of genes including metallothioneins and integrin β4. These cells showed decreased expression of oncogenes, DNA repair proteins, and genes involved in the mitogen-activated protein kinase pathway. For comparison we are currently analyzing gene expression changes in RHEK-1 cells transformed by other means. The goal of these studies is to identify common batteries of genes affected by chemical modulators of the carcinogenic process. Mechanistic studies may allow us to correlate alterations in their expression with sequential stages in the carcinogenic process and may aid in the risk assessment of other xenobiotics. *Key words:* **arsenic, cDNA microarray, cell transformation, chemical carcinogenesis, gene expression, human keratinocytes, metal mixture, molecular markers.** *Environ Health Perspect* **110(suppl 6):931–941 (2002).**
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Epidemiological evidence suggests that some, if not all, environmentally relevant metals, including arsenic (As), cadmium (Cd), chromium (Cr), and lead (Pb), are human carcinogens. Unfortunately, human exposures to such metals in both the occupational and environmental setting are common occurrences. In fact, because of high As (and other metal) concentrations in the drinking water supplies in many countries, chronic toxicity and development of neoplastic lesions have become health problems of global proportions (*1,2*). In the United States, As, Cd, Cr, and Pb are the top four metals in site frequency count by the Agency for Toxic Substances and Disease Registry (ATSDR) Completed Exposure Pathway Site Count Report (*3*); three of these, As, Pb, and Cd, are among the Superfund's top 10 priority hazardous substances (*4*). In addition, these metals most often occur together; they are present in 8 of 10 and 5 of 10 of the top 10 binary combinations of contaminants in soil and water, respectively (*5*).

The mechanisms mediating metal-induced cytotoxicity and carcinogenicity are currently unclear. Many laboratories, using a variety of experimental systems, have carried out detailed studies in attempts to address these issues. From this work, it has become apparent that metals affect multiple intracellular targets and exert a variety of diverse effects on cells *in vitro* (*6,7*). Studies suggest that different metals have unique primary mechanisms of action that are cell specific and/or tissue specific (*6,7*). Additionally, the activity of a metal in any given tissue is dependent on its speciation and metabolism (*6*). To further complicate the picture, metals have been shown to interact at multiple levels and, most likely, modify one another's cytotoxicity and/or carcinogenic potential (*8–11*). As a result, we are still a long way from a fundamental understanding of the actions of metals or metal mixtures at the cellular level, particularly as they relate to toxic end points. Accurate risk assessment of these highly relevant chemicals awaits our progress in this area.

The skin is one important target organ for As-mediated pathological effects and is a useful model system for mechanistic studies in this area. Chronic exposure to As leads to skin disorders such as hyperkeratosis and, in many cases, carcinogenesis (*12,13*). Both As and Cr, a well-known skin sensitizer, have substantial effects on epidermal keratinocytes *in vitro* and *in vivo*; these metals have been shown to alter expression of numerous growth regulatory factors, to stimulate cell proliferation at low concentrations, and to inhibit the normal process of differentiation (*11,14–19*). They have not, however, been shown to be directly transforming in this cell type. In transgenic Tg.AC mice, As acts as co-promoter during skin carcinogenesis in standard two-stage models (*20*). These studies have suggested that transforming growth factor-α (TGFα) and granulocyte/macrophage-colony stimulating factor may be useful biomarkers for As-associated carcinogenesis in keratinocytes; data from As-exposed human subjects support this hypothesis (*20*). It is likely, however, that the picture is much more complex than this and that the genes involved are more numerous.

New technologies in expression analysis at the RNA and protein levels have led to the development of the field of toxicogenomics, that is, the use of genetic information to address issues such as these that are crucial in toxicology. As an approach to defining the mechanism(s) behind selective chemical toxicity, one may analyze gene expression changes in cells after exposure to the chemical(s) of interest. Methodologies such as microarray analysis allow one to gain a comprehensive view of the cellular pathways affected by the chemical(s) under scrutiny; comparison may then be made between multiple chemicals having the same or differing toxicities. Characterization of the relationship among chemical exposure, gene expression alterations, and development of acute or chronic toxicity should help in delineating important molecular events that are mechanistically

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Address correspondence to J.A. Campain, Quantitative and Computational Toxicology Group, Center for Environmental Toxicology and Technology, Dept. of Environmental and Radiological Health Sciences, Colorado State University, Ft. Collins, CO 80523 USA. Telephone: (970) 491-8383. Fax: (970) 491-8304. E-mail: julie.campain@colostate.edu

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linked to the different toxic end points. In addition, once gene expression changes induced by individual chemicals are identified and linked to functional end points, interactions in chemical mixtures will be substantially easier to understand and predict.

We have used human keratinocytes as an experimental model to define molecular events that may mediate the cytotoxicity and/or carcinogenicity of As alone and in environmentally relevant metal mixtures. We describe here an evaluation of the transforming potential of As alone and together with Cd, Cr, and Pb in previously immortalized human epidermal keratinocytes compared with the potent carcinogen *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) and negative controls. Genetic alterations induced by the different chemical treatments and that may be involved in their selective toxicity and/or carcinogenicity were analyzed by cDNA microarray technology.

Materials and Methods

Chemicals. Sodium meta-arsenite ($NaAsO_2$), cadmium chloride ($CdCl_2$), chromium oxide (CrO_3), chromium chloride ($CrCl_3$), lead acetate [$(C_2H_3O_2)_2Pb{\cdot}3H_2O$], and dimethyl sulfoxide (DMSO) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). MNNG was obtained from Aldrich (Milwaukee, WI, USA).

Cell lines and culture reagents. The Ad12/SV40-immortalized human keratinocyte cell line (RHEK-1) was obtained from J. Rhim (Center for Prostate Disease Research, Rockville, Maryland, USA) (*21–23*). RHEK-1 was cultured in Dulbecco's modified Eagle's medium supplemented with 100 U/mL penicillin, 0.1 mg/mL streptomycin, 10 mM L-glutamine, and 10% fetal bovine serum (Summit Biotechnology, Ft. Collins, CO, USA). Methylcellulose (MC)-based medium for determination of anchorage-independent growth (AIG) was obtained as MethoCult from Stem Cell Technologies (Vancouver, Canada).

Establishment of keratinocyte cell lines after exposure to MNNG, As, or As-containing mixture. RHEK-1 cells were plated at 2.5×10^5 cells per 75-cm^2 flask. The conditions used for MNNG treatment were those described by Rhim et al. (*22*). Briefly, 24 hr after plating, cultures were fed with medium containing the positive control MNNG, at 0.01 or 0.1 μg/mL, or 0.5% DMSO vehicle control. After 24 hr of exposure (one treatment only), cells were washed with 1× phosphate-buffered saline (PBS) and then refed with culture medium. Cells were subsequently subcultured weekly. RHEK-1 cells were also exposed to low doses (9, 11, and 14 ppb) of As^{3+}, the metal mixture, or water vehicle controls, continuously for approximately 6 months, or 25 passages; that is, the test chemicals were added to the culture medium at each subculturing. The concentrations of As used corresponded to the $LD_{2.5}$, LD_5, and LD_{10}, as determined in our laboratory for this cell type (*11*). The low-mixture treatment group was composed of 1, 10, 62, and 33 ppb of As, Cr, Cd, and Pb, respectively. In efforts to more closely mimic the actual exposure scenario with Cr, these chronic studies were carried out with a mixture of 1:1 Cr^{3+} and Cr^{6+}. The high-mixture treatment group was exposed to 14, 104, 618, and 332 ppb of As, Cr, Cd, and Pb, respectively. The concentrations of the four-metal mixture used corresponded to the LD_1 (low mixture) and LD_{10} (high mixture) of each individual metal in RHEK-1 cells. The resulting cultures were designated as follows: OM1 (DMSO-treated control cells); OM2 (0.01 μg/mL MNNG); OM3 (0.1 μg/mL MNNG); water control; As-Low (9 ppb As); As-Med (11 ppb As); As-High (14 ppb As); Mix-Low (LD_1 mixture); and Mix-High (LD_{10} mixture).

MC cloning. MC cloning as an index for AIG was carried out every two or three passages for the cultures treated with As, metal mixture, or MNNG. For MC cloning, 1×10^4 cells/mL were plated in 35-mm gridded dishes in triplicate in 1.3% MC. The number of colonies was counted via manual inspection under phase-contrast microscopy after 2 weeks and is expressed as percentage cloning efficiency.

Analysis of saturation density. Saturation density was measured as the maximum number of cells obtained in cultures as a function of time. Cells (1×10^4 /cm^2) were plated in 25-cm^2 culture flasks in triplicate. Viable cells at 5, 8, 10, 12, 15, and 17 days after initial plating were counted by trypan blue exclusion on a hemocytometer. Culture medium was changed every 3 days. Unattached cells in the culture medium were pelleted by centrifugation and also counted.

Analysis of tumorigenicity in immunocompromised mice. The tumorigenicity assay was carried out using a modification of Rhim et al. (*22*). Briefly, cells from passage 16 and passage 25 (OM1 and OM3) or passage 25 (As-Low, As-High, Mix-Low, Mix-High, and the appropriate water controls) were collected by 0.05% trypsin treatment. Cells (2×10^6) in 0.1 mL PBS were injected subcutaneously into the interscapular region of 4- to 8-week-old male Balb/c *nu/nu* mice. The mice were observed weekly for 3 months for tumor development and growth. The tumors were measured by caliper, excised, and fixed in 10% formalin before sectioning and slide preparation. Tissue sections were stained with hematoxylin and eosin and characterized by histopathological analysis.

RNA preparation. Total RNA was isolated from cultures of control and chemically treated RHEK-1 cells (at ~70% confluence) at passage 25 using the RNAqueous kit (Ambion, Austin, TX, USA) and following the manufacturer's directions. RNA purity and concentration were assessed by determination of absorption at 260 and 280 nm.

cDNA synthesis and radioactive labeling for the Clontech Atlas Human Cancer 1.2 Array. Total RNA (2 μg per reaction) was reverse transcribed from each test sample with superscript in the presence of [α-^{32}P]deoxyadenosine 5′-triphosphate (Amersham, Piscataway, NJ, USA) using the Atlas Pure Total RNA Labeling System (Clontech, Palo Alto, CA, USA). Unincorporated isotope was removed by gel filtration in Chroma Spin-200 columns (Clontech). The Atlas Human Cancer 1.2 Array was supplied by the manufacturer on nylon membranes; 1,185 genes were analyzed using this array. These membranes were prehybridized for 30 min at 68°C in ExpressHyb (Clontech) containing 0.1 mg/mL sheared salmon sperm DNA. They were then incubated with 2×10^6 cpm of radiolabeled cDNA probe (control OM1 or test sample OM3; water control or test samples As-High and Mix-High) per milliliter of ExpressHyb buffer overnight at 68°C. After high-stringency washes in 2× standard saline citrate (SSC), 1% sodium dodecyl sulfate (SDS) at 68°C, the blots were exposed to storage phosphor screens (Molecular Dynamics, Sunnyvale, CA, USA). Signals were scanned and captured using a Storm 860 Phosphorimager and ImageQuant software (Molecular Dynamics). Gene expression images were quantified using AtlasImage 1.0 program (Clontech) by the Atlas Technology Center. Relative changes in gene expression were determined by normalizing the hybridization signals to the signals obtained from all the genes included in the array. Genes that demonstrated ≥ 2-fold changes in expression between control and treatment were reported.

cDNA synthesis and fluorescent labeling for New England Nuclear arrays. Two microarrays from New England Nuclear (NEN; Boston, MA, USA) were used to analyze gene expression changes in OM3 versus OM1 and As-High and Mix-High versus the appropriate water controls. These were the NEN Human 2400 (2,400 genes analyzed) and Oncogene/Tumor Suppressor (325 genes analyzed) arrays. In addition, gene expression in OM3 compared with OM1 was analyzed by the NEN Kinase/Phosphatase array (275 genes analyzed); this latter analysis was not carried out on the metal-treated cultures. We do not, therefore, know how the genes contained within this array are affected in the metal-treated cells. Synthesis and labeling of cDNA were carried using the MICROMAX Direct cDNA Microarray System (NEN) following the manufacturer's directions. Briefly, 100 μg or 40 μg of total RNA for the Human

2400 or Oncogene/Tumor Suppressor arrays, respectively, was reverse transcribed from each test sample with AMV Reverse Transcriptase in the presence of cyanine 3 (Cy3) (for OM1 and water controls) and cyanine 5 (Cy5) (for OM3, As-High, and Mix-High) using MICROMAX. For the NEN Kinase/Phosphatase array, 40 µg of RNA was used from OM1 and OM3. Labeled cDNA from control and treated samples was purified by isopropyl alcohol precipitation. MICROMAX microarray slides were used that contained the three different arrays. The entire reaction from the combined Cy3- and Cy5-labeled probes in hybridization buffer (NEN) was pipetted underneath slide coverslips. Overnight hybridization was performed in a microarray hybridization cassette from Corning (Corning, NY, USA) at 65°C. After three consecutive washes at room temperature in 0.5× SSC/0.01% SDS, 0.06× SSC/0.01% SDS, and 0.06× SSC, respectively, the glass slide was placed in a 50-mL polypropylene tube and centrifuged at 500×*g* for 5 min to remove excess liquid before scanning. The slide was scanned in a BioChip Imager (Packard, Meriden, CT, USA). Laser and photomultiplier tube voltages were adjusted manually to maximize the signal-to-noise ratio. Cy3 and Cy5 signal intensities were standardized relative to one another by comparing the total signal intensities of all spots in each channel. The scanner output images were quantified using ScanAlyze (software developed by M. Eisen, University of California at Berkeley).

Statistical analysis. One-way analysis of variance (ANOVA) followed by Dunnett's test (*24*) was used to analyze differences between control and chemical-treated samples in saturation density, MC cloning, and tumorigenicity studies. *p*-Values < 0.05 were considered statistically significant.

Results

As and an As-containing metal mixture inhibit and MNNG enhances malignant transformation in RHEK-1 human keratinocytes. To analyze the effects of As, both alone and in metal mixtures, on malignant transformation, we used the virally immortalized human epidermal keratinocyte cell line RHEK-1. To carry out this analysis, RHEK-1 cells were treated chronically with increasing concentrations of either As or a mixture of As, Cd, Cr, and Pb; this scenario was chosen to more closely reflect actual human exposures. For comparison, we also treated RHEK-1 with MNNG, a potent carcinogen that has previously been shown to malignantly transform this cell type.

With continued culture after chemical treatment, we were able to observe substantial changes in morphology in our RHEK-1 populations; however, these alterations were not the same in all cultures and were not consistently associated with malignant transformation. Solvent control RHEK-1 cells underwent substantial morphological changes with increasing time in culture, becoming very pleiomorphic with distinctive nests of cobblestone-like cells surrounded by spindlier, elongated layers of cells. It was noteworthy, however, that at approximately passage 13, RHEK-1 cells treated with both the low and high concentrations of MNNG began to develop foci of piled cells from which round cells were continually being released; these alterations were similar to those previously described by Rhim and colleagues (*21–23*) and were not present in the corresponding DMSO-treated OM1 cells. With continued subculturing, in populations treated with 0.1 µg/mL MNNG, these foci began to dominate the entire flask. By passage 16, these latter cultures consisted of substantially larger cells that were relatively homogeneous in size and shape; this line was named OM3. Cultures treated with 0.01 µg/mL MNNG (OM2) also began to pile up in foci; however, the cells in these cultures remained small, similar to the control cells. The situation with the As- and metal mixture–treated populations was very different from that of cells treated with MNNG. After undergoing chronic, long-term exposure to either As or the metal mixture at multiple concentrations, populations became increasingly uniform in both size and morphology compared with the water control cultures. The cells in the As- and mixture-treated cultures were flat and had a regular polygonal epithelial appearance. This effect was dose dependent for both As and the metal mixture. In addition, these cultures had no piling or rounded cells, as was seen with the MNNG-treated populations.

At passage 4 after treatment, all cultures were analyzed biweekly for their ability to grow in semisolid medium, that is, in an anchorage-independent manner. As early as passage 11, OM3 gained the AIG+ phenotype (Table 1). The cloning efficiency of OM3 at passage 11 averaged 0.34% compared with 0.02% in control OM1. While working with RHEK-1, we have observed that these cells spontaneously become less dependent on adherence for growth with increasing time in culture. After 25 passages we noted an increase in the AIG of OM1; these cells formed colonies with an efficiency of 2.1% in MC. However, at this same passage 25, MC cloning ability in OM3 was approximately 19%. OM2 did not at any time tested exhibit a significantly higher cloning efficiency than OM1. In contrast to previous findings (*22*), treatment of RHEK-1 with the lower concentration of MNNG (0.01 µg/mL) did not detectably affect the malignant behavior of the cells during the time course of our studies.

In contrast to what we observed with MNNG-treated cells, As-High and Mix-High cultures did not exhibit increased AIG compared with water controls at any passage or under any condition examined in our studies. However, spontaneous progression in water control RHEK-1 cells was quite rapid, even compared with the progression observed in OM1; by passage 11, the water controls exhibited AIG+ growth of 1.4–1.9%. By passage 16, these controls formed colonies in MC with efficiencies of 1.46 and 2.63%, respectively. Through passage 16, chronic treatment of RHEK-1 cells with As or the metal mixture acted in a dose-dependent manner to partially inhibit this spontaneous acquisition of AIG+ in RHEK-1. By passage 25, however, AIG+ in As-High and Mix-High were very similar to water control cultures. The results of this analysis are shown in Table 1.

Increased saturation density may be another characteristic of malignant transformation. Thus, we measured the saturation density of OM1, OM2, OM3, As-High, Mix-High, and the water controls (Table 2). Interestingly, and unexpectedly, when assayed at passage 16, OM3 showed decreased saturation density compared with OM1; the maximum cell densities reached in these cultures were 5.7 and 3.2×10^5 cells/cm^2 for OM1 and OM3, respectively. OM2 did exhibit significantly increased saturation density compared with both OM1 and OM3; this phenotypic change is likely related to the smaller size of OM2 cells

Table 1. Anchorage-independent growth of chemically treated human RHEK-1 keratinocytes.[a]

	Time in culture		
Treatment group	Passage 11	Passage 16	Passage 25
OM1 (DMSO control)	0.02 ± 0.006	0.05 ± 0.04	2.1 ± 0.21
OM2 (0.01 µg/mL MNNG)	0.03 ± 0.006	0.06 ± 0.02	3.2 ± 0.67
OM3 (0.1 µg/mL MNNG)	0.34 ± 0.04*	1.60 ± 0.22*	18.9 ± 0.66*
As control (water)	1.40 ± 0.05	1.46 ± 0.04	2.45 ± 0.03
As-Low (9 ppb)	1.41 ± 0.03	1.40 ± 0.05	2.11 ± 0.02*
As-Med (11 ppb)	0.73 ± 0.01*	1.17 ± 0.04*	ND
As-High (14 ppb)	0.95 ± 0.04*	0.77 ± 0.05*	2.39 ± 0.03
Mixture control (water)	1.86 ± 0.006	2.63 ± 0.13	2.67 ± 0.10
Mix-Low (LD_1)	1.42 ± 0.04*	1.49 ± 0.05*	2.51 ± 0.08
Mix-High (LD_{10})	0.38 ± 0.03*	0.98 ± 0.06*	2.06 ± 0.04*

ND, not determined. [a]MC cloning assay was carried out as described in "Materials and Methods." Values in MC colony formation study are expressed as mean ± SE (n = 3). *Significantly different from control using one-way ANOVA followed by Dunnett's test, $p < 0.05$.

(especially when compared with OM3) and not to malignant transformation. We also observed lower saturation density in RHEK-1 populations treated with the high concentrations of As and the metal mixture compared with controls; the ratios of the maximum cell density in these cultures were 4.2:6.2 and 2.9:4.0 for As-High and Mix-High versus the water controls, respectively.

To test the tumorigenic potential of the chemically treated RHEK-1 cells, Balb/c *nu/nu* mice were used. After subcutaneous injection of the control and treated cell populations into immunocompromised mice, several cultures rapidly (within 3 weeks) and consistently formed large dorsal tumors (Table 3). Passage 16 OM3 cells formed tumors in all injected mice in 3 weeks. Tumors formed in mice by passage 25 OM3 cells were significantly larger; the average sizes of the resulting tumors from these time points were 6.6 mm and 10.7 mm, respectively. At 3 weeks, in mice injected with passage 16 OM1 cells, no tumors were observed; however when OM1 cells were cultured through 25 passages before being tested for tumorigenicity, the results were somewhat different. These passage 25 cells had progressed to the point where they formed small tumors (2.4 mm average tumors in 3 of 10 mice) by 3 weeks after injection. With both passage 16 and passage 25 OM1 cells, by 3 months after injection tumors of approximately 6–7 mm were observed in recipient mice, again supporting the hypothesis that RHEK-1 spontaneously progresses to a low-level malignancy with continued time in culture. Observations from our studies on As and the metal mixture–treated cultures were not highly surprising, given the MC cloning results described above. Neither As-High nor Mix-High cells were tumorigenic under these conditions, even at passage 25, when they had acquired the ability to grow in an anchorage-independent manner. In contrast, by 3 weeks, both water control cell populations formed tumors in a portion of injected mice; cells treated with the lower concentrations of As and the metal mixture also were tumorigenic (Table 3). Histopathological exam of excised tumors from each culture demonstrated that they were all poorly differentiated squamous cell carcinomas. Chromosome painting and karyotypic analysis confirmed that these tumors arose from the parental RHEK-1 cells.

Analysis of changes in gene expression that arise during treatment of RHEK-1 cells with MNNG, As, or the four-metal mixture. We were interested in characterizing changes in gene expression that may be involved in mediating the different outcomes after treatment of RHEK-1 cells with MNNG, As, or the mixture of As-High, Cd or the mixture of, Cr, and Pb-High. To explore this issue, we have begun to use cDNA microarray technology to identify mRNA species that are over- or underexpressed in chemically treated versus control cells. Our first observation from this analysis was that the patterns of gene expression in the treated cultures were unique, being distinct both from their respective controls and from cells that were exposed to different chemicals (Tables 4–6). Among the three chemically treated cell populations, OM3 showed the most numerous alterations in gene expression (Table 4). Not only did we use an additional array for studying OM1 and OM3, but this finding may also be attributed in part to the fact that MNNG is a very effective DNA-damaging agent and mutagen and likely has genomewide effects. In all, 72 and 41 genes represented on the combined arrays were induced and suppressed, respectively, in OM3 compared with OM1 cells. In As-High a total of 52 genes were altered in their expression compared with water controls; of these, 23 showed increased and 29 showed decreased expression (Table 5). Last, 13 genes were induced in the Mix-High populations, and 51 were suppressed (Table 6). A comprehensive list of genes induced or suppressed under each exposure scenario, along with their assigned (putative) function, is shown in Tables 4–6.

Among the genes showing increased expression in OM3 compared with the OM1 control population were many that could potentially have impacts on cell proliferation, including *a*) cell cycle regulators (*RBQ-3*, *cyclins H* and *A1*, and *CDK5*); *b*) growth factors (*int-6*, *irp,* and *PDGF2*); and *c*) oncogenes, several of which are involved in the mitogen-activated protein pathway (MAP) kinase signaling pathways, including an *Erk-3*–related protein, *JNK-2, PKC*μ, *A-raf*-1, and *Net* (Table 4). Additionally, genes encoding proteins that modulate cell–cell or cell–matrix interactions were also induced; among these genes were macrophage inhibitory cytokine MIC1, nerve growth factor–inducible PC4 homolog, and several protease inhibitors. Increased expression of several tumor-associated markers, such as melanoma (A32)-associated and prostate carcinoma-associated antigens and the adenomatus polyposis coli (APC) protein, was consistent with the malignant phenotype of OM3. Last, several proteins involved in DNA damage response and/or apoptosis, including p53-associated protein and caspase 4 were expressed at higher levels in the transformed line.

There were substantially fewer genes with decreased expression in OM3 compared with OM1 (Table 4). Among these genes were representatives from several functional categories. Particularly striking were repressions in *a*) multiple protein tyrosine phosphatases, including PTP1C, PTPα, PTPσ, and receptor-type protein tyrosine phosphatase γ; *b*) cell-protective mechanisms such as the ultraviolet (UV) excision repair protein RAD23A, glutathione synthase, and glutathione-*S*-transferase (GST); and *c*) integrin β4, cadherin 8, and a keratin-related protein. Additionally, several putative inhibitors of transformation and metastasis such as RARRES3, suppressin, tumor suppressor protein 101F6, and PCDH7 exhibited decreased expression in OM3.

In our studies, several noteworthy genes/gene families were altered in their expression in As-treated cells (Table 5). In these populations, the most striking induction was seen in DNA

Table 2. Saturation density of chemically treated RHEK-1 cultures.[a]

Treatment group	Saturation density (x 10^5/cm^2)
OM1 (DMSO control)	5.7 ± 0.06
OM2 (0.01 μg/mL MNNG)	6.7 ± 0.06*
OM3 (0.1 μg/mL MNNG)	3.2 ± 0.09*
As control (water)	6.2 ± 0.09
As-Low (9 ppb)	4.5 ± 0.08*
As-Med (11 ppb)	4.6 ± 0.15*
As-High (14 ppb)	4.2 ± 0.07*
Mixture control (water)	4.0 ± 0.05
Mix-Low (LD_1)	3.1 ± 0.17*
Mix-High (LD_{10})	2.9 ± 0.06*

[a]Experiments were conducted with passage 16 cells. Saturation density analysis was carried out as described in "Materials and Methods." Values in saturation density are expressed as mean ± SE (n = 3). *Significantly different from control using one-way ANOVA followed by Dunnett's test, $p < 0.05$.

Table 3. Tumorigenicity of MNNG-, As-, and metal mixture–treated RHEK-1 cells.[a]

Treatment group	No. with tumors/no. inoculated at week 3	Average tumor size of existing tumors at week 3 (mm)[b]
p16 OM1 (DMSO control)	0/10	0
p16 OM3 (0.1 μg/mL MNNG)	10/10	6.6 ± 0.4*
p25 OM1 (DMSO control)	3/10	2.4 ± 0.3
p25 OM3 (0.1 μg/mL MNNG)	10/10	10.7 ± 0.4 *
p25 As control (water)	7/10	3.6 ± 0.4
p25 As-Low (9 ppb)	4/10	4.0 ± 0.4
p25 As-High (14 ppb)	1/10	1.3 ± 0.7
p25 Mixture control (water)	9/10	5.2 ± 0.6
p25 Mix-Low (LD_1)	9/10	5.0 ± 0.6
p25 Mix-High (LD_{10})	2/10	2.2 ± 0.2*

Abbreviations: p16, passage 16 cells; p25, passage 25 cells. [a]The tumorigenicity assay was performed 3 times. Data were pooled and are represented as mean number or size of all measurable tumors. [b]Tumor size was expressed as mean ± SE (n = 10). *Significantly different from corresponding control using one-way ANOVA followed by Dunnett's test, $p < 0.05$.

Table 4. Alterations in gene expression detected by microarray analysis of MNNG-treated RHEK-1 cells.

GenBank[a] accession no.	Name	Function	Array[b]	Fold[c]
		Induction (total 72)		
M74088	Adenomatous polyposis coli protein (APC protein)	Tumor marker	1	4
S83171	BCL-2 binding athanogene-1 (BAG-1); glucocorticoid receptor–associated protein RAP46	Steroid receptor	1	>100
U43746	BRCA2	Zinc finger domain	1	3
X66141	Cardiac ventricular myosin light chain 2	Filament	1	>100
U28014	Caspase-4 precursor	Apoptosis	1	5
AF011792	Cell cycle progression 2 protein	Cell cycle regulation	1	3
X66364	Cell division protein kinase 5	Cell cycle regulation	1	3
L31951	C-jun N-terminal kinase 2 (JNK2)	Transcription regulation	1	49
U43901	Colon carcinoma laminin-binding protein	Cancer marker	1	4
U11791	Cyclin H	Cell cycle regulation	1	5
J04164	Interferon-inducible protein 9–27	Cell growth regulation	1	61
AF019770	Macrophage inhibitory cytokine 1 (MIC1)	TGFβ superfamily	1	7
M28882	Melanoma-associated antigen A32; cell surface glycoprotein MUC 18	Tumor marker	1	12
J02958	Met proto-oncogene; hepatocyte growth factor receptor precursor	Protein tyrosine kinase	1	3
U39050	Mitogen-responsive phosphoprotein DOC2	Tumor suppressor from ovarian carcinoma cells	1	36
Y10313	Nerve growth factor (NGF)-inducible PC4 homolog	Growth factor	1	6
U48296	PTPCAAX1 nuclear tyrosine phosphatase PRL-1	Nuclear phosphatase	1	4
X85134	RBQ-3	RB protein–binding protein	1	36
X63679	TRAM protein	ER protein involved early in polypeptide translocation	1	4
X87852	Transmembrane protein sex precursor	Homology to cMET; a novel transmembrane protein	1	3
X56134	Vimentin	Intermediate filament	1	3
S65738	Actin depolymerizing factor	Actin depolymerization	2	4
U29344	Breast carcinoma fatty acid synthase	Involvement in breast carcinoma	2	3
M33011	(Clone GA733–2–2) carcinoma-associated antigen GA733–2	Cancer antigen from colorectal and pancreatic carcinoma	2	2
U66838	Cyclin A1	Cell cycle regulation	2	5
L37385	Homolog of mouse MAT-1 oncogene	Expression in breast cancer cells	2	3
J02854	Human 20-kDa myosin light chain (MLC) 2 mRNA	Smooth muscle and nonmuscle cell contractile activity	2	5
AF027205	Kunitz-type protease inhibitor	Protease inhibitor	2	3
X04741	mRNA for protein gene product 9.5	A novel cytoplasmic neuroendocrine marker protein	2	3
D78130	mRNA for squalene epoxidase	Metabolism	2	3
X56160	mRNA for tenascin	Extracellular matrix protein	2	3
X00699	mRNA fragment for class II histocompatibility antigen β-chain (pII-β-3)	Histocompatibility antigen	2	5
M13656	Plasma protease inhibitor	Protease inhibitor	2	2
U41303	Small nuclear ribonuleoprotein particle N	Pre-mRNA splicing	2	5
AF009615	ADAM10	Protumor necrosis factor α processing enzyme	3	2
M32325	Adenocarcinoma-associated antigen (KSA)	Lung cell surface glycoprotein	3	3
X66087	A-myb mRNA	Nuclear protein, TF	3	13
U74611	Apo-3	TNF receptor family	3	3
U14680	BRCA1	Zinc finger domain	3	3
X06182	C-kit proto-oncogene mRNA	A new cell surface receptor tyrosine kinase	3	6
M12783	C-sis/platelet-derived growth factor 2 (SIS/PDGF2)	Proto-oncogene	3	3
M14333	C-syn proto-oncogene	Protein tyrosine kinase family	3	2
X64229	Dek mRNA	Putative oncogene; gene translocation in acute myeloid leukemia	3	3
U77085	Epithelial membrane protein	Squamous cell–associated gene	3	3
J04101	Erythroblastosis virus oncogene homolog 1 (ets-1)	Proto-oncogene; TF	3	3
S82592	Evi-1, Evi-1 protein	TF; overexpression in myeloid leukemia	3	4
M64240	Helix-loop-helix zipper protein (max)	TF; complex with myc	3	3
O04045	hMSH2	Human mismatch repair gene	3	3
U37547	IAP homolog B	Apoptosis inhibitory protein	3	4
U62962	Int-6	GF-like proto-oncogene	3	3
AF042857	Lung cancer antigen NY-LU-12 variant A	Nuclear zinc finger protein	3	3
Y18046	mRNA for FOP	FGFR1 oncogene partner	3	3
D63874	mRNA for HMG-1	Malignant transformation in gastrointestinal adenocarcinoma	3	2
X07876	mRNA for irp protein	GF-like proto-oncogene	3	3
Z36715	mRNA for Net transcription factor	A new ets TF that is activated by Ras	3	10
X03541	mRNA of trk oncogene	Tyrosine kinase; a transforming gene in a human colon carcinoma	3	3
M92424	p53-associated mRNA	p53-associated gene in human sarcomas	3	2
K03199	p53 cellular tumor antigen	Tumor antigen from human vulva carcinoma cell line	3	2
L78132	Prostate carcinoma tumor antigen	Tumorigenesis and metastasis	3	2
Y00705	PstI mRNA for pancreatic secretory inhibitor (expressed in neoplastic tissue)	Trypsin inhibitor in cancer	3	5
L07868	Receptor tyrosine kinase (ERBB4) gene	EGF receptor family	3	3
U16296	T-lymphoma invasion and metastasis inducing TIAM1 protein	Found in virtually all analyzed tumor cell lines of human origin	3	3
X15187	Tra1 mRNA for homolog of murine tumor rejection antigen gp96	Cell surface glycoprotein	3	3
M76125	Tyrosine kinase receptor (axl)	A transforming gene	3	4
M18391	Tyrosine kinase receptor (eph)	Overexpression in several human carcinomas	3	3
M11730	Tyrosine kinase-type receptor (HER2)	Oncogene	3	3

(*Continued*)

Table 4. *(Continued)*

GenBank[a] accession no.	Name	Function	Array[b]	Fold[c]
		Induction (total 72)		
V00572	mRNA encoding phosphoglycerate kinase	Kinase	4	2
AB011406	mRNA for alkalin phosphatase	Phosphatase	4	3
X04790	mRNA for A-raf-1 oncogene	Downstream signal molecule for Ras signal transduction	4	2
X75756	mRNA for protein kinase C (PKC) μ	Kinase	4	2
X52192	RNA for c-fes	Oncogene; protein tyrosine kinase	4	4
X59727	63-kDa protein kinase related to rat ERK3	MAP kinase signaling	4	9
		Suppression (total 41)		
L22253	Arginine/serine-rich splicing factor 7	mRNA splicing	1	3
U78095	Bikunin	Hepatocyte GF activator inhibitor 2	1	2
L34060	Cadherin 8	Cell differentiation	1	>100
M36067	DNA ligase I	DNA replication	1	3
X16707	Fos-related antigen (FRA1)	AP1	1	5
U34683	Glutathione synthase	Cell protection	1	2
L07515	Heterochromatin protein homolog 1	Chromatin stucture	1	13
X62534	High mobility group protein 2	Malignant transformation	1	4
X67081	Histone H4	Chromosome structure	1	4
U12255	IgG receptor FC large subunit P51 precursor	Immunoglobulin structure	1	3
X53587	Integrin β4	Cell differentiation	1	6
D21063	MCM2 DNA replication licensing factor	Nuclear protein	1	3
X74794	MCM4 DNA replication licensing factor	Nuclear protein	1	3
U77604	Microsomal glutathione *S*-transferase II	Cell protection	1	4
M15796	Proliferating cell nuclear antigen (PCNA)	Cell cycle regulation	1	3
J03040	Secreted protein acidic and rich in cysteine precursor	Secretory protein	1	4
D21235	Ultraviolet excision repair protein RAD23A	DNA repair	1	3
L47647	Creatine kinase B	Kinase	2	7
S78986	Id1 (Id1-a)	Inhibit transcription by forming inactive heterodimer	2	7
U68018	Mad protein homolog (hMAD-2)	Downstream molecule in TGFβ receptor activation	2	3
X62570	mRNA for IFP53	IFN-inducible protein	2	9
X62571	mRNA for keratin-related protein	Cell differentiation	2	3
X71635	mRNA for neuropeptide Y-like receptor	G protein coupled receptor	2	5
AJ222700	mRNA for TSC-22 protein	TGFβ-stimulated clone 22; apoptosis	2	4
U51478	Sodium/potassium-transporting ATPase β3 subunit	Ion transporter	2	5
AF007165	Suppressin	A novel suppressor of cell cycle entry	2	3
U49436	Translation initiation factor 5	Gene transcription/ translation	2	5
L25610	Cyclin-dependent kinase inhibitor; p21ClP1	Cdk-interacting protein	3	6
AF027964	MAD-related protein Smad2	Downstream molecule in TGFβ receptor activation	3	4
D28124	mRNA for unknown product; putative	Putative tumor suppressor neuroblastoma tumor suppressor gene	3	2
U46691	Putative chromatin structure regulator	Transcriptional regulation	3	2
AF040704	Putative tumor suppressor protein 101F6	Putative tumor suppressor	3	2
AF060228	Retinoic acid receptor responder 3 (RARRES3)	Retinoid-induced class II tumor suppressor	3	7
X75208	HEK2 mRNA for protein tyrosine kinase receptor	Protein tyrosine kinase receptor	4	3
AB006757	mRNA for PCDH7	Metastasis inhibitor	4	5
U48959	MLC kinase	Kinase	4	4
M34668	Protein tyrosine phosphatase (PTPase-α) mRNA	Phosphatase	4	2
U40317	Protein tyrosine phosphatase (PTPase-σ)	Phosphatase	4	2
X62055	PTP1C mRNA for protein-tyrosine phosphatase 1C	Phosphatase	4	2
L09247	Receptor-type protein tyrosine phosphatase γ	Phosphatase	4	2
AF099989	Ste-20 related kinase SPAK	Kinase	4	2

Abbreviations: EGF, epidermal growth factor; ER, endoplasmic reticulum; FC, crystallizable fragment of an immunoglobin. FGFR, fibroblast growth factor receptor; GF, growth factor; IFN, interferon; RB, retinoblastoma; TF, transcription factor; TNF, tumor neurosis factor. [a]Gene accession numbers are from the GenBank database (*http://www.ncbi.nlm.nih.gov/genbank/genbankoverview.html*). [b]1, Clontech Cancer 1.2; 2, NEN Human 2400; 3, NEN Oncogene/Tumor Suppressor; 4, NEN Kinase/Phosphatase array. [c]Values are represented as mean from two experiments for arrays 2, 3, and 4 in chemically treated cell population.

damage response genes, including *XRCC1*, *RAD23A*, endonuclease III homolog 1 (*HNTH1*), DNA repair protein *MLH1*, and a *heat shock protein* (*HSP*) *40 homolog*. Among other representative inductions were genes involved in cell cycle regulation (*jun-B, FRA-1, MTS1/p16-INK4, PCNA, early growth response protein 1*); oncogenes (*EHK-1 receptor tyrosine kinase*); two putative tumor suppressor genes (*EXT1* and *RDA32*); and genes for proteins regulating invasion and/or cell–cell interactions, *BMP4* and *TIMP-3*. Genes suppressed in As-treated populations included those for cytoprotective molecules (cytosolic superoxide dismutase [SOD], *glutathione synthetase*, and *glutathione-S-transferase*), *ICAM-1*, *stratum corneum chymotryptic enzyme*, *MIC1*, and *bikunin*. In our studies, treatment with As also was observed to inhibit expression of a variety of cytokeratins, including 6E, 8, 13, 18, and an unidentified 58-kDa type II protein.

The metal mixture–treated populations had a somewhat different spectrum of gene alterations than did cells exposed to As alone (Table 6). Relatively few genes showed increased expression under this exposure scenario. Genes involved in cell cycle regulation that were induced included *jun-B, MTS1/p16-INK4, FRA1,* and *nuclear factor 1-X*. DNA damage response/cytoprotective/apoptosis mechanisms induced included multiple metallothioneins, caspase 10, and HNTH1. Also induced under these conditions were integrin β4 and BMP4. In contrast, many genes were repressed by metal mixture treatment compared with control. Cell cycle regulatory proteins and cytokines showing decreased expression included WAF1/CIP1, MAPKK6, GATA6, JNK2, TGFβ2, and mitogen-responsive phosphoprotein DOC2. Many oncogenes were suppressed, including *int-1*, *Ret*, *Blym-1*, *n-myc*, *DBL*, and the *EHK-1 receptor tyrosine kinase*. Among the DNA damage response/cytoprotective/apoptosis genes showing decreased expression were *ERCC2*, *ERCC5*,

Table 5. Alterations in gene expression detected by microarray analysis of As-treated RHEK-1 cells.

GenBank[a] accession no.	Name	Function	Array[b]	Fold[c]
		Induction (total 23)		
M63959	α_2-Macroglobulin receptor–associated protein precursor (α_2-MRAP)	Human homolog of a Heymann nephritis antigen	1	2
D30751	Bone morphogenetic protein 4 (BMP4)	Production of skeletal structure during development; TGFβ family	1	5
L27211	Cyclin-dependent kinase 4 inhibitor (CDK4I); multiple tumor suppressor 1 (MTS1); p16-INK4	Cell cycle regulation	1	2
U07418	DNA mismatch repair protein MLH1; COCA2	DNA repair	1	4
M36089	DNA-repair protein XRCC1	DNA repair	1	4
X52541	Early growth response protein 1 (hEGR1)	TF	1	2
X79067	EGF response factor 1	Early response gene	1	6
U79718	Endonuclease III homolog 1 (HNTH1)	DNA repair	1	2
X16707	FRA1	AP1	1	3
M29039	Jun-B	AP1	1	3
M15796	PCNA	Cell cycle regulation	1	3
D21235	UV excision repair protein RAD23A	DNA repair	1	6
S79639	EXT1, putative tumor suppressor/hereditary multiple exostoses candidate gene	Putative tumor suppressor	2	3
M96803	General β-spectrin	Membrane skeleton protein	2	3
U40992	HSP 40 homolog	Cell protection	2	3
X95425	mRNA for EHK-1 receptor tyrosine kinase	Receptor tyrosine kinase; formation of neuronal pathway	2	4
AB000220	mRNA for semaphorin E	Non-MDR drug resistance gene	2	5
U14394	Tissue inhibitor of metalloproteinases-3 (TIMP-3)	Metalloprotease inhibitor	2	9
M19154	TGFβ2	TGFβ superfamily	2	3
M95787	22-kDa smooth muscle protein	Structural protein	2	3
L04288	Cyclophilin-related protein	Function of natural killer cells	3	3
M98833	ERGB transcription factor; FLI-1 homolog	A new Ets TF	3	6
AF061836	Putative tumor suppressor protein RDA32	Putative tumor suppressor	3	6
		Suppression (total 29)		
U78095	Bikunin	HGF activator inhibitor	1	7
M34225	Cytokeratin 8 (K8)	Cell differentiation	1	3
M26326	Cytokeratin 18 (K18)	Cell differentiation	1	3
K00065	Cytosolic superoxide dismutase 1 (SOD1)	Cell protection	1	4
U34683	Glutathione synthase	Cell protection	1	7
U90313	Glutathione *S*-transferase homolog	Cell protection	1	3
AF019770	MIC1	TGFβ superfamily	1	35
L33930	CD24 signal transducer and 3´ region	A potential early tumor marker in human hepatocellular carcinoma	2	18
M77830	Desmoplakin I	Cell surface attachment site for cytoplasmic intermediate filaments	2	8
M20681	Glucose transporter-like protein-III (GLUT3)	Expression in fetal skeletal muscle	2	3
AB000712	hCPE-R mRNA for Clostridium perfringens enterotoxin (CPE) receptor	CPE receptor	2	6
X58072	hGATA3 mRNA for trans-acting T-cell specific transcription factor	TF	2	6
L42611	Keratin 6 isoform (K6e)	Cell differentiation	2	3
M26512	Keratin 8 mRNA, 5´ end	Cell differentiation	2	4
M21389	Keratin type II (58 kDa)	Cell differentiation	2	3
X52426	mRNA for cytokeratin 13	Cell differentiation	2	5
X00497	mRNA for HLA-DR antigens associated invariant chain p33	Transmembrane polarity	2	10
X06990	mRNA for intercellular adhesion molecule (ICAM)-1	Cell adhesion molecule	2	2
X69549	mRNA for rho GDP-dissociation inhibitor 2	Inhibit GTP binding; disruption of actin cytoskeleton	2	3
L41351	Prostasin	Prostate-specific marker	2	5
L33404	Stratum corneum chymotryptic enzyme	Serine protease	2	15
M73554	Bcl-1	Anti-apoptosis	3	3
U66894	Epithelium-restricted Ets protein ESX	Oncogene; TF	3	4
X51602	Flt mRNA for receptor-related tyrosine kinase	Fms-related tyrosine kinase	3	6
M32325	KSA	Lung cell surface glycoprotein	3	4
Z13009	mRNA for E-cadherin	Invasion suppressor; Ca-dependent cell adhesion molecule	3	6
AF060228	RARRES3	Retinoid-induced class II tumor suppressor	3	4
AF070675	TNF-inducible protein CG12–1	Vascular endothelial gene	3	4
M90657	Tumor antigen L6	Tumor-associated cell surface antigen	3	3

Abbreviations: GDP, guanosine diphosphate; HGF, hepatocyte growth factor; MDR, multiple drug resistance protein/gene. [a]Gene accession numbers are from the GenBank database (*http://www.ncbi.nlm.nih.gov/genbank/genbankoverview.html*). [b]1, Clontech Cancer 1.2; 2, NEN Human 2400; 3, NEN Oncogene/Tumor Suppressor; 4, NEN Kinase/Phosphatase array. [c]Values are represented as mean from two experiments for Arrays 2, 3, and 4 in chemically treated cell population.

MSH2, *TDG*, cytosolic SOD, and catalase. Many additional kinases/phosphatases were altered in their expression, including an *ERK3*-related protein kinase, HCK, several creatine kinases, HYL, and PRL-1.

Detailed analysis of data from OM3, As-High, and Mix-High demonstrated that, in addition to numerous chemical-specific gene changes, several genes were altered in a similar or opposite manner under the different exposure conditions (Table 7). There were no genes commonly induced by MNNG and As or the metal mixture, although the *RARRES3* gene (a retinoid-induced tumor suppressor) was suppressed under all three treatment conditions. As and metal mixture treatment did increase expression of a common group of genes, including *JunB, FRA1, MTS1,* and a member of the *TGF*β family, *BMP4* (*25,26*). These two exposures also commonly suppressed expression of tumor antigen L6,

Table 6. Alterations in gene expression detected by microarray analysis of mixture-treated RHEK-1 cells.[a]

GenBank[b] accession no.	Name	Function	Array[c]	Fold[d]
		Induction (total 13)		
M63959	α-2-MRAP	Human homolog of a Heymann nephritis antigen	1	3
D30751	BMP 4	TGFβ family	1	4
U60519	Caspase-10 precursor	Apoptosis	1	4
M34570	Collagen 6 α 2 subunit	Structural protein	1	5
U79718	HNTH1	DNA repair	1	2
X16707	FRA1	AP1	1	4
D13365	Growth inhibitory factor; metallothionein-III (MT-III)	Cell growth regulation	1	3
X53587	Integrin β4	Cell differentiation	1	2
M29039	Jun-B	AP1	1	2
L27211	MTS1; CDK4I; p16-INK4	Cell cycle regulation	1	3
U90551	Histone 2A-like protein	Nuclear protein	2	3
X76717	MT-1l mRNA	Cell protection	2	3
L31881	Nuclear factor I-X	Interference with transcriptional activation	2	3
		Suppression (total 51)		
S83171	BAG-1	Steroid receptor	1	8
U09579	Cyclin-dependent kinase inhibitor 1; WAF1/CIP1	Cell cycle regulation	1	6
X52221	DNA excision repair protein ERCC2	DNA repair	1	11
L20046	DNA excision repair protein ERCC5	DNA repair	1	2
U04045	DNA mismatch repair protein MSH2	DNA repair	1	4
U39657	Dual-specificity mitogen-activated protein kinase kinase 6 (MAPKK 6)	MAP kinase signaling	1	23
U51166	G/T mismatch-specific thymine DNA glycosylase (TDG)	DNA repair	1	2
L31951	JNK2	Transcription regulation	1	3
AF019770	MIC1	TGFβ superfamily	1	7
U53446	Mitogen-responsive phosphoprotein DOC2	Tumor suppressor from ovarian carcinoma cells	1	4
U48296	PTPCAAX1 nuclear tyrosine phosphatase PRL-1	Nuclear phosphatase	1	8
K00065	SOD1	Cell protection	1	3
X85960	Trk-T3 oncoprotein	Oncogene; tyrosine kinase	1	6
X56134	Vimentin	Intermediate filament	1	4
AF047347	Adaptor protein X11α	Slows cellular amyloid precursor protein processing and reduces Aβ40 and Aβ42 secretion	2	4
M22489	BMP-2A	TGFβ family	2	6
L47647	Creatine kinase B	Kinase	2	5
M14780	Creatine kinase M	Kinase	2	5
M81635	Erythrocyte membrane protein	Cation transporter inhibitor	2	4
M20681	GLUT3	Expression in fetal skeletal muscle	2	19
M97796	Helix-loop-helix protein Id-2	Nuclear protein; expression in early development	2	3
X77278	HYL tyrosine kinase mRNA	Nonreceptor protein tyrosine kinase	2	3
S78986	Id1; Id1-a	transcription regulator; helix-loop-helix protein	2	6
X04076	Kidney mRNA for catalase	Cell protection	2	4
X95425	mRNA for EHK-1 receptor tyrosine kinase	Formation of neuronal pathway	2	3
D87811	mRNA for GATA-6	TF	2	4
X71635	mRNA for neuropeptide Y-like receptor	G protein-coupled receptor	2	9
Y10032	mRNA for putative serine/threonine protein kinase	Kinase	2	4
X92494	mRNA for STM-7 protein	A novel phosphatidyl inosine-4-phosphate-5-kinase	2	5
S75725	Prostacyclin-stimulating factor	PGI2-stimulating factor from fibroblast cells	2	6
X59727	63 kDa protein kinase related to rat ERK3	MAP kinase signaling	2	4
U49857	Transcriptional activator	Nuclear protein	2	3
M19154	Transforming growth factor β2	TGFβ superfamily	2	4
M90657	Tumor antigen L6	Tumor-associated cell surface antigen	2	15
U57059	Apo-2 ligand	TNF receptor family	3	7
K01884	Blym-1 transforming gene	Transforming gene	3	6
M62397	Colorectal mutant cancer protein.	Cancer marker	3	5
J03639	DBL oncogene encoding a transforming protein	Transforming oncogene	3	28
M34309	Epidermal growth factor receptor	GF receptor	3	3
Y00664	Germ line n-myc gene	TF	3	8
M16591	Hemopoietic cell protein-tyrosine kinase gene (HCK), clone λ-a2/1a	Protein tyrosine kinase	3	5
X03072	Int-1 mammary oncogene	GF-like proto-oncogene	3	5
X07384	mRNA for GLI protein	Oncogene; zinc finger protein	3	5
X87241	mRNA for hFat protein	Cadherin superfamily	3	4
X07876	mRNA for irp protein	GF-like proto-oncogene	3	4
Y00705	PstI mRNA for pancreatic secretory inhibitor	Trypsin inhibitor in cancer	3	4
AF069072	Putative lung tumor suppressor DAL1	Putative tumor suppressor	3	10
AF060228	RARRES 3	Retinoid-induced class II tumor suppressor	3	6
M57464	Ret proto-oncogene	Oncogene; tyrosine kinase	3	7
AF016028	Sarcospan-2	25-kDa Transmembrane component of dystrophin glycoprotein complex	3	3
M55994	Tumor necrosis factor receptor II (TNFrII)	TNF receptor family	3	8

[a]Microarray analysis was carried out using the Clontech Cancer 1.2, NEN Human 2400, and NEN Oncogene/Tumor Suppressor arrays for all samples. NEN Kinase/Phosphatase array was analyzed for MNNG-treated cells only. [b]Gene accession numbers are from the GenBank database (*http://www.ncbi.nlm.nih.gov/genbank/genbankoverview.html*). [c]1, Clontech Cancer 1.2; 2, NEN Human 2400; 3, NEN Oncogene/Tumor Suppressor; 4, NEN Kinase/Phosphatase array.[d]Values are represented as mean from two experiments for arrays 2, 3, and 4 in chemically treated cell population.

SOD1, and MIC1, another member of the *TGF*β family. Interestingly, the largest group of genes was those oppositely regulated by MNNG and the metal mixture, among which were *JNK2, ERK3*, *nuclear phosphatase PRL-1,* an unidentified Ser/Thr protein kinase, *integrin* β*4*, and *vimentin*.

Discussion

To develop more efficient methodologies for evaluating carcinogenic potentials for environmentally relevant chemicals such as As and other metals, we have attempted to identify molecular markers involved in the process of carcinogenesis in keratinocytes. In our studies, the Ad12/SV40-immortalized human epidermal keratinocyte cell line RHEK-1 slowly and spontaneously progressed to a malignant phenotype with continued passage. Progression of RHEK-1 was enhanced greatly by treatment of the cells with the strong initiating agent MNNG. In contrast, treatment of RHEK-1 with DMSO, As alone, or As in the presence of Cd, Cr, or Pb acted to inhibit this progression. Microarray analysis allowed us to catalog widespread changes in gene expression in treated cells that may potentially correlate with these different toxicological end points.

Several investigators have taken advantage of the SV40 virus in development of immortalized and/or "transformed" cell lines from normal primary tissues (*27–29*). These studies have described a variety of phenotypic changes frequently associated with expression of viral T antigens in infected cultures, including increased cloning efficiency and proliferative potential, unlimited life span in culture, and anchorage- and/or growth factor–independent growth. In these studies, clones surviving "crisis" are highly variable in their growth properties initially and change fairly rapidly with increasing time in culture (*27,30*). Transformation by SV40 appears to progress over time, with acquisition of AIG+ and tumorigenicity occurring spontaneously in some cell lines. In our studies, RHEK-1 progressed to the AIG+ or tumorigenic phenotype at vastly different rates depending on the chemical treatment the cells received. Although this phenomenon may have been due to either genetic or epigenetic mechanisms, depending on the chemical, specific alterations in gene expression are, without doubt, involved.

In studies such as these, where large numbers of genes are identified and assignment of a mechanistic role to specific gene changes is the desired goal, it is the analysis and interpretation of data that become difficult. In our studies, we need to compare not only each chemically treated RHEK-1 line with its appropriate control, but also gene expression changes in cells treated with transformation-enhancing (MNNG) versus transformation-inhibitory (As and the metal mixture) chemicals. To further complicate the picture, genes altered in their expression after treatment of cells with potentially carcinogenic agents likely fall into at least two categories. The first would be genes directly involved in or mediating some aspect of malignant transformation, that is, genes whose function or lack thereof is necessary for neoplastic progression. The second group would be composed of genes that are altered as a result of cytotoxic stress on the cell and are not involved in the malignant phenotype at all. As a first approach, analysis of the known or putative functions of identified genes may yield some insight into their potential roles in the toxicological end point of interest, that is, transformation or toxicity.

From the alterations in gene expression that we observed in our studies, one could formulate several interesting hypotheses concerning transformation-specific effects on RHEK-1. More rapid conversion of this keratinocyte cell line to the tumorigenic phenotype by MNNG could potentially be mediated by constitutively increased expression of growth factors and/or oncogenes such as PDGF, members of the MAP kinase signaling pathway, and/or the cyclins or cyclin-dependent kinases. Activation of the MAP kinase pathway is the primary response to mitogenic stimuli in all cell types (*31*). Multiple genes involved in this pathway were selectively induced in MNNG-transformed cells compared with As- and metal-mixture–treated populations. In Mix-High populations, which were nontumorigenic, the *JNK2*, the *ERK3 homolog*, and *MAPKK6* genes demonstrated substantially decreased expression compared with water controls; these findings are consistent with a role for activation of the MAP kinase pathway in progression of RHEK-1. One can also speculate that altered expression of a host of protein phosphatases in a cell, such as was observed in OM3, would have profound impacts on its proliferative potential and facilitate its ultimate transformation. Protein phosphatases are crucial players in regulation of the mitogenic cascade, among other functions, and changes in their expression have been strongly linked to carcinogenesis in many studies (*32*). Because malignant transformation is a multistep, and very complex, process, it is likely that many of the alterations in gene expression that we detected (as well as others) are involved.

Rhim et al. (*21,22*) and Yang et al. (*23*) have been able to derive multiple malignant lines from RHEK-1 by treating the cells with chemicals such as MNNG, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), and 4-nitroquinoline-1-oxide (4NQO), exposing

Table 7. Genes altered in their expression by multiple chemical treatments.

	Treatment			
	As/Mix/MNNG	As/MNNG	Mix/MNNG	As/Mix
Genes commonly induced by treatment				*JunB* *FRA1* *MTS1* *Endonuclease III homolog 1 (HNTH1)* *BMP4* *α-2-MRAP*
Genes commonly suppressed by treatment	*RARRES3*	*GSH synthase B* *Bikunin*	*Creatine kinase* *Id1-a* mRNA for *neuropeptide Y-like receptor*	*Tumor antigen L6* *SOD1* *MIC1* *GLUT3*
Opposite regulation by treatment	*MIC1* (⇓⇓⇑) *FRA1* (⇑⇑⇓)	*KSA* (⇓⇑) *RAD23A* (⇑⇓) *PCNA* (⇑⇓)	*JNK2* (⇓⇑) *ERK3* (⇓⇑) *DOC2* (⇓⇑) *Nuclear tyrosine phosphatase* (*PRL-1*) (⇓⇑) mRNA for irp protein (⇓⇑) *BAG-1* (⇓⇑) *Vimentin* (⇓⇑) *Pancreatic secretory inhibitor* (⇓⇑) *Integrin β4* (⇑⇓)	*TGFβ2* (⇑⇓) mRNA for *EHK-1 receptor tyrosine kinase* (⇑⇓)

them to X ray, and transfecting them with oncogenic viruses. In collaborative studies with these investigators, we will compare gene expression patterns in these various lines with OM1 and OM3; the primary goal here would be to identify, if there is one, a common battery of genes altered during progressive transformation of RHEK-1 by multiple chemical and physical agents. Having several transformed lines with the same basic wild-type background of gene expression should greatly facilitate our identification of genes potentially involved in malignant progression of this cell type. Our results from these studies could then be tested with other cell types transformed by various means.

Many types of studies, both epidemiological and in the laboratory, have demonstrated that most, if not all, of the metals used in our work are human carcinogens. However, in our hands, both As and the As-containing metal mixture were inhibitory to malignant progression of RHEK-1. This is not the first demonstration of an "anticarcinogenic" effect of As; the metal has been shown to inhibit formation of GST-P–positive hepatic foci in chemically treated rats *in vivo* and is currently being used in chemotherapeutic regimens for acute promyelocytic leukemia (*33–36*). Although the mechanism of arsenic trioxide's clinical effects remain unclear, it has been shown to induce apoptosis in leukemic and lymphoid cell lines *in vitro* (*35,36*). The observed changes in gene expression after exposure to As alone were not inconsistent with an anticarcinogenic effect and indicated that the metal generally stimulated DNA-protective mechanisms in exposed cells. Particularly interesting was the strong induction of multiple DNA repair proteins, including XRCC1, HNTH1, RAD23A, and MLH1, in the As-High populations, which may be a function of the clastogenic and/or comutagenic effects of the metal (*37–44*). Induction by As of multiple regulators of the cell cycle (jun-B, c-fos/FRA-1, and EGR1) has also been seen in other studies where it is assumed that the metal is acting to promote carcinogenesis (*45,46*). Obviously, given the complexity of the cell, it is highly likely that the carcinogenic or anticarcinogenic effect of the metal in any one situation or cell type is dependent on batteries of genes working together and not any single gene change. Because the metal mixture also acted to inhibit transformation of RHEK-1 in our studies, common gene expression changes seen in both As-High and Mix-High cells may be important in the process and worth exploring in more detail.

Alterations in gene expression that differ depending on whether As is alone or mixed with other metals are also highly interesting and may potentially help us to understand the dose-dependent metal–metal interactions we have observed in these cells in other short-term cytotoxicity studies in the lab (*11,47*). For example, in contrast to the situation in As-High, only one of the same DNA repair genes, *HNTH1*, was induced in cells treated with the As-containing metal mixture, despite the fact that the concentration of As was the same in both cultures. In fact, we identified four DNA repair proteins in this latter population that were suppressed, likely by one of the other metals in the mixture. In addition, two metallothionein genes showed increased expression in Mix-High, certainly a result of the presence of Cd in the mix; cells treated with As alone did not exhibit increased expression of these important cytoprotective molecules and, in fact, showed decreased glutathione synthase and GST levels. Certainly, these findings have implications for the cytotoxicity of the metals alone and together in simple or complex mixtures.

In conclusion, we have used DNA microarray analysis to identify changes in gene expression in the human keratinocyte cell line RHEK-1 in response to treatment with chemicals that enhance or inhibit its spontaneous malignant transformation. Our studies have shown unique and intriguing gene expression patterns in cells treated with either As, an As-containing chemical mixture, or the potent mutagen MNNG. Meticulous analysis of gene expression patterns in a variety of cell types, as described above, and timewise comparison of defined changes with acquisition of transformation-associated characteristics such as AIG and tumorigenicity should allow us to identify potential players in each step of the process of malignant conversion. In future studies, these "transformation-associated" molecular markers will be used in biologically based dose–response models to predict the carcinogenic potentials of other xenobiotics. Additionally, once we have a clear mechanistic understanding of how single carcinogenic agents work and have been able to model the process using computational techniques, chemical mixtures will be much more amenable to study. Linkage of models through common metabolic pathways and/or mechanisms of cytotoxicity will allow a more comprehensive view of the potential health/carcinogenic effects of complex chemical mixtures.

References and Notes

1. Chen C-J, Chen CW, Wu M-M, Kuo T-L. Cancer potential in liver, lung, bladder, and kidney due to ingested inorganic arsenic in drinking water. Br J Cancer 66:888–892 (1992).
2. Tseng WP. Effects and dose–response relationships of skin cancer and blackfoot disease with arsenic. Environ Health Perspect 19:109–119 (1977).
3. ATSDR. 1997 CERCLA Priority List of Hazardous Substances That Will Be the Subjects of Toxicological Profiles & Support Document. Atlanta, GA:Agency for Toxic Substances and Disease Registry, 1997.
4. De Rosa CT, Johnson BL, Fay M, Hansen H, Mumtaz MM. Public health implications of hazardous waste sites: findings, assessment, and research. Food Chem Toxicol 34:1131–1138 (1996).
5. Fay M, Mumtaz MM. Development of a priority list of chemical mixtures occurring at 1188 hazardous waste sites using the HazDat database. Food Chem Toxicol 34:1163–1165 (1996).
6. Goyer RA. Toxic effects of metals. In: Casarett & Doull's Toxicology: The Basic Science of Poisons (Klaassen CD, ed). New York:McGraw-Hill, 1996;691–736.
7. Snow ET. Metal carcinogenesis: mechanistic implications. Pharmacol Ther 53:31–65 (1992).
8. Diaz-Barriga F, Llamas E, Mejia JJ, Carrizales L, Santoyo ME, Vega-Vega L, Yanez L. Arsenic-cadmium interaction in rats. Toxicology 64:191–203 (1990).
9. Elsenhans B, Schmolke K, Kolb K, Stokes J, Forth W. Metal-metal interactions among dietary toxic and essential trace elements in the rat. Ecotoxicol Environ Saf 14:275–287 (1987).
10. Nordberg GF, Anderson O. Metal interactions in carcinogenesis: enhancement, inhibition. Environ Health Perspect 40:65–81 (1981).
11. Bae DS, Gennings C, Carter WH Jr, Yang RSH, Campain JA. Toxicological interactions among arsenic, cadmium, chromium, and lead in human keratinocytes. Toxicol Sci 63:132–142 (2001).
12. Chen C-J, Chuang Y-C, Lin T-M, Wu H-Y. Malignant neoplasms among residents of a blackfoot disease-endemic area in Taiwan: high-arsenic artesian well water and cancer. Cancer Res 45:5895–5899 (1985).
13. Nriagu JO. Arsenic in the Environment. Part II: Human Health and Ecosystem Effects. New York:Wiley,1994.
14. Cohen MD, Kargacin CB, Klein CB, Costa M. Mechanisms of chromium carcinogenicity and toxicology. Crit Rev Toxicol 23:255–281 (1993).
15. Germolec DR, Spalding J, Boorman GA, Wilmer JL, Yoshida T, Simeonova PP, Bruccoleri A, Kayama F, Gaido K, Tennant R, et al. Arsenic can mediate skin neoplasia by chronic stimulation of keratinocyte-derived growth factors. Mutat Res 386:209–218 (1997).
16. Germolec DR, Yoshida T, Gaido K, Wilmer JL, Simeonova PP, Kayama F, Burleson F, Dong W, Lang RW, Luster MI. Arsenic induces overexpression of growth factors in human keratinocytes. Toxicol Appl Pharmacol 141:308–318 (1996).
17. Kachinskas DJ, Qin Q, Phillips MA, Rice RA. Arsenate suppression of human keratinocyte programming. Mutat Res 386:253–261 (1997).
18. Ye J, Zhang X, Young HA, Mao Y, Shi X. Chromium(VI)-induced nuclear factor-κB activation in intact cells via free radical reactions. Carcinogenesis 16:2401–2405 (1995).
19. Yen H-T, Chiang L-C, Wen K-H, Chang S-F, Tsai C-C, Yu C-L, Yu H-S. Arsenic induces interleukin-8 expression in cultured keratinocytes. Arch Dermatol Res 288:716–717 (1996).
20. Germolec DR, Spalding J, Yu H-S, Chen GS, Simeonova PP, Humble MC, Bruccoleri A, Boorman GA, Foley JF, Yoshida T, et al. Arsenic enhancement of skin neoplasia by chronic stimulation of growth factors. Am J Pathol 153:1775–1785 (1998).
21. Rhim JS, Jay G, Arnstein P, Price FM, Sanford KK, Aaronson SA. Neoplastic transformation of human epidermal keratinocytes by AD12-SV40 and Kirsten sarcoma viruses. Science 227:1250–1252 (1985).
22. Rhim JS, Fujita J, Arnstein P, Aaronson SA. Neoplastic conversion of human keratinocytes by adenovirus 12-SV40 virus and chemical carcinogens. Science 232:385–388 (1986).
23. Yang JH, Thraves P, Dritschilo A, Rhim JS. Neoplastic transformation of immortalized human keratinocytes by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. Cancer Res 52:3478–3482 (1992).
24. Tamhane AC, Dunlop DD, eds. Multiple comparisons of means. In: Statistics and Data Analysis from Elementary to Intermediate. Upper Saddle River, NJ:Prentiss-Hall, 2000;475–476.
25. Glozak MA, Rogers MB. Specific induction of apoptosis in P19 embryonal carcinoma cells by retinoic acid and BMP2 or BMP4. Dev Biol 179:458–470 (1996).

26. Wozney JM, Rosen V, Celeste AJ, Mitsock LM, Whitters MJ, Kriz RW, Hewick RM, Wang EA. Novel regulators of bone formation: molecular clones and activities. Science 242:1528–1534 (1988).
27. Sack GH Jr. Human cell transformation by simian virus 40. In Vitro 17:1–19 (1981).
28. Rhim JS. Development of human cell lines from multiple organs. Ann NY Acad Sci 919:16–25 (2000).
29. Rundell K, Parakati R. The role of the SV40 ST antigen in cell growth promotion and transformation. Semin Cancer Biol 11:5–13 (2001).
30. Steinberg ML, Defendi V. Transformation and immortalization of human keratinocytes by SV40. J Invest Dermatol 81:131s–136s (1983).
31. Wilkinson MG, Millar JBA. Control of the eukaryotic cell cycle by MAP kinase signaling pathways. FASEB J 14:2147–2157 (2000).
32. Parsons R. Phosphatases and tumorigenesis. Curr Opin Oncol 10:88–91 (1998).
33. Pott WA, Benjamin SA, Yang RS. Antagonistic interactions of an arsenic-containing mixture in a multiple organ carcinogenicity bioassay. Cancer Lett 133:185–190 (1998).
34. Pott WA, Benjamin SA, Yang RSH. Arsenic, alone and in chemical mixtures, antagonizes the development of glutathione *S*-transferase π (GST-P) positive foci in the rat liver. Toxicologist 54:135 (2000).
35. Soignet SL, Maslak P, Wang ZG, Jhanwar S, Calleja E, Dardashti LJ, Corso D, DeBlasio A, Gabrilove J, Scheinberg DA, et al. Complete remission after treatment of acute promyelocytic leukemia with arsenic trioxide [see comments]. N Engl J Med 339:1341–1348 (1998).
36. Zhang W, Ohnishi K, Shigeno K, Fujisawa S, Naito K, Nakamura S, Takeshita K, Takeshita A, Ohno R. The induction of apoptosis and cell cycle arrest by arsenic trioxide in lymphoid neoplasms. Leukemia 12:1383–1391 (1998).
37. Jan KY, Huang RY, Lee T-C. Different modes of action of sodium arsenite, 3-aminobenzamide and caffeine on the enhancement of ethyl methanesulfonate clastogenicity. Cytogenet Cell Genet 41:202–208 (1986).
38. Lee T-C, Huang RY, Jan KY. Sodium arsenite enhances the cytotoxicity, clastogenicity, and 6-thioguanine resistant mutagenicity of ultraviolet light in Chinese hamster ovary cells. Mutat Res 148:83–89 (1985).
39. Lee T-C, Wang-Wuu S, Huang RY, Lee KCC, Jan KY. Differential effects of pre- and posttreatment of sodium arsenite on the genotoxicity of methyl methanesulfonate in Chinese hamster ovary cells. Cancer Res 46:1854–1857 (1986).
40. Li JH, Rossman TG. Mechanism of comutagenesis of sodium arsenite with *N*-methyl-*N*-nitrosourea. Biol Trace Elem Res 21:381 (1989).
41. Li JH, Rossman TG. Comutagenesis of sodium arsenite with ultraviolet radiation in Chinese hamster V79 cells. Biol Metals 4:197–200 (1991).
42. Okui T, Fujiwara Y. Inhibition of human excision repair by inorganic arsenic and the co-mutagenic effect in V79 Chinese hamster cells. Mutat Res 172:69–76 (1986).
43. Wiencke JK, Yager JW. Specificity of arsenite in potentiating cytogenetic damage induced by the DNA crosslinking agent diepoxybutane. Environ Mol Mutagen 19:195–200 (1992).
44. Wiencke JK, Yager JW, Varkonyi A, Hultner M, Lutze LH. Study of arsenic mutagenesis using the plasmid shuttle vector pZ189 propagated in DNA repair proficient human cells. Mutat Res 38:335–344 (1993).
45. Simeonova PP, Wang S, Kashon ML, Kommineni C, Crecelius E, Luster MI. Quantitative relationship between arsenic exposure and AP-1 activity in mouse urinary bladder epithelium. Toxicol Sci 60:279–284 (2001).
46. Simeonova PP, Wang S, Toriuma W, Kommineni V, Matheson J, Unimye N, Kayama F, Harki D, Ding M, Vallyathan V, et al. Arsenic mediates cell proliferation and gene expression in the bladder epithelium: association with activating protein-1 transactivation. Cancer Res 60:3445–3453 (2000).
47. Gennings C, Carter WH Jr, Campain JA, Bae DS, Yang RSH. Statistical analysis of interactive cytotoxicity in human epidermal keratinocytes following exposure to a mixture of four metals. J Agric Biol Environ Stat 7:58–73 (2002).