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

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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 metallothionein 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 hyperkeratoses and, in many cases, carcinogenesis (12,13). 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
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-ethyl-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 (NaAsO₂), cadmium chloride (CdCl₂), chromium oxide (CrO₃), chromium chloride (CrCl₃), lead acetate [(C₂H₃O₂)₂Pb·3H₂O], 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 × 10⁵ cells per 75-cm² 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 As³⁺, 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 LD₂⁵, LD₅₀, and LD₁₀⁰, 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 Cr³⁺ and Cr⁶⁺. 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 LD₅₀ (low mixture) and LD₁₀₀ (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 (LD₅₀ mixture); and Mix-High (LD₁₀₀ 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 × 10⁴ cells/mL were plated in 35-mm gridded dishes in triplicate at 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 × 10⁴/cm²) were plated in 25-cm² 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 × 10⁶) in 0.1 mL PBS were injected subcutaneously into the interscapular region of 4- to 8-week-old male Balb/c nude 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 RNeasy Mini 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-32P]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 × 10⁶ 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 0.5x SSC/0.01% SDS, 0.06x SSC/0.01% SDS, and 0.06x SSC, respectively, the glass slide was placed in a 50-µL polypropylene tube and centrifuged at 500×g for 5 min to remove excess liquid before scanning. The slide was scanned in a BioChip Image (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 MNNNG 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 MNNNG, 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 spindly, 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 MNNNG 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 MNNNG, 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 MNNNG (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 MNNNG. 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 most pronounced in the control and As treatment. In addition, these cultures had no piling or rounded cells, as was seen with the MNNNG-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 was 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 the same passage 25, MC cloning ability in OM3 was approximately 19%. OM2 did not at any time exhibit a significantly higher cloning efficiency than OM1. In contrast to previous findings (22), treatment of RHEK-1 with the lower concentration of MNNNG (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 MNNNG-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.

| Treatment group | Time in culture |
|-----------------|-----------------|
|                 | 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 MNNNG) | 0.03 ± 0.006 | 0.06 ± 0.02 | 3.2 ± 0.67 |
| OM3 (0.1 µg/mL MNNNG) | 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 ± 0.06 | 2.63 ± 0.13 | 2.67 ± 0.10 |
| Mix-Low (LD50) | 1.42 ± 0.04* | 1.49 ± 0.05* | 2.51 ± 0.08 |
| Mix-High (LD10) | 0.38 ± 0.03* | 0.98 ± 0.06* | 2.06 ± 0.04* |

*ND, not determined. *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, Cz, 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.

### Table 2. Saturation density of chemically treated RHEK-1 cultures.

| Treatment group | Saturation density (x 10⁶/cm²) |
|-----------------|--------------------------------|
| OM1 (DMSO control) | 5.7 ± 0.06 |
| OM2 (0.01 µg/mL MNNG) | 6.7 ± 0.08* |
| 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* |
| Mix control (water) | 4.0 ± 0.05 |
| Mix-Low (LD₃) | 3.1 ± 0.17* |
| Mix-High (LD₃) | 2.9 ± 0.08* |

*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.

| Treatment group | No. with tumors/no. inoculated at week 3 | Average tumor size of existing tumors at week 3 (mm)² |
|-----------------|----------------------------------------|-------------------------------------------------------|
| 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 Mix control (water) | 9/10 | 5.2 ± 0.6 |
| p25 Mix-Low (LD₃) | 9/10 | 5.0 ± 0.6 |
| p25 Mix-High (LD₃) | 2/10 | 2.2 ± 0.2* |

Abbreviations: p16, passage 16 cells; p25, passage 25 cells. *The tumorigenicity assay was performed 3 times. Data were pooled and are represented as mean number or size of all measurable tumors. **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 accession no. | Name                                                                 | Function                        | Array | Fold |
|-----------------------|----------------------------------------------------------------------|---------------------------------|-------|------|
| M74088                | Adenomatous polyposis coli protein (APC)                             | Tumor marker                    | 1     | 4    |
| S81317                | 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     | 3    |
| U72014                | Caspase-4 precursor                                                 | Apoptosis                       | 1     | 5    |
| AF011792              | Cell cycle progression 2 protein                                    | Cell cycle regulation           | 1     | 3    |
| X60364                | 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 A22; cell surface glycoprotein MUC 18   | Tumor marker                    | 1     | 12   |
| J02958                | Met proto-oncogene; hepatocyte growth factor receptor precursor      | Protein tyrosine kinase         | 1     | 3    |
| U25905                | Mito kinesin-responsive phosphoprotein DOC2                          | Tumor suppressor from ovarian carcinoma cells | 1     | 36   |
| Y10313                | Nerve growth factor (NGF)-inducible PC4 homolog                      | Growth factor                   | 1     | 6    |
| U48256                | PTPCAAX1 nuclear tyrosine phosphatase PRL-1                          | Nuclear phosphatase             | 1     | 4    |
| X95134                | RBG-3                                                                | RB protein–binding protein       | 1     | 36   |
| X83670                | TRAM protein                                                        | Extracellular protein involved 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    |
| S87538                | Actin depolymerizing factor                                          | Actin depolymerization          | 2     | 4    |
| U25944                | Breast carcinoma fatty acid synthase                                 | Involvement in breast carcinoma | 2     | 3    |
| M33011                | [Cone carcinoma 33–2–2] carcinoma-associated antigen GA733–2        | Cancer antigen from colorectal and pancreatic carcinoma | 2     | 2    |
| U66838                | Cyclin A                                                             | Cell cycle regulation           | 2     | 5    |
| L37385                | Homolog of mouse MAT-1 oncogene                                     | Expression in breast cancer cells | 2     | 3    |
| J02958                | Human 20-kDa myosin light chain (MLC) 2 mRNA                          | Smooth muscle and nonmuscle cell contractile activity | 2     | 5    |
| AF02705               | Kunutz-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 squamous epoxidase                                          | Metabolism                      | 2     | 3    |
| X56160                | mRNA for tenasin                                                     | Extracellular matrix protein     | 3     | 2    |
| X06959                | mRNA fragment for class II histocompatibility antigen β-chain (pII-β-3) | Histocompatibility antigen       | 3     | 2    |
| M13866                | Plasma protease inhibitor                                            | Protease inhibitor              | 2     | 2    |
| U41303                | Small nuclear ribonucleoprotein particle N                           | T-cell receptor protein alpha splicing | 2     | 5    |
| AF009615              | ADAM10                                                               | Prostate cancer suppressor ATP processing enzyme | 3     | 2    |
| M32325                | Adenocarcinoma-associated antigen (KSA)                              | Lung cell surface glycoprotein  | 3     | 3    |
| X60807                | 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     | 3    |
| X84229                | Dek mRNA                                                             | Putative oncogene; gene translation in acute myeloid leukemia | 3     | 3    |
| U70785                | Epithelial membrane protein                                         | Squamous cell–associated gene   | 3     | 3    |
| J04101                | Erythroblastosis virus oncogene homolog 1 (ets-1)                   | Proto-oncogene; TF              | 3     | 3    |
| S82582                | 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    |
| O04945                | 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    |
| D68374                | mRNA for HM1                                                        | Malignant transformation in gastrointestinal adenocarcinoma | 3     | 2    |
| X07876                | mRNA for rpr protein                                                | GF-like proto-oncogene          | 3     | 3    |
| Z36715                | mRNA for Net transcription factor                                   | A new ets TF that is activated by Ras | 3     | 10   |
| X05354                | mRNA for trk oncogene                                               | Tyrosine kinase; a transforming gene in a human colon carcinoma | 3     | 3    |
| M92424                | p63-associated mRNA                                                 | p53-associated gene in human sarcomas | 3     | 2    |
| K03199                | p63 cellular tumor antigen                                          | Tumor antigen from human vulva carcinoma cell line | 3     | 2    |
| L78132                | Prostate carcinoma tumor antigen                                    | Tumorigenesis and metastasis    | 3     | 2    |
| Y00705                | Pst1 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                | Tral1 mRNA for homolog of murine tumor rejection antigen gp96        | Cell surface glycoprotein       | 3     | 3    |
| M76125                | Tyrosine kinase receptor (axl)                                      | A transforming gene             | 3     | 4    |
| X18391                | 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 accession no. | Name | Function | Array | Fold |
|-----------------------|------|----------|-------|------|
| V00572                | mRNA encoding phosphoglycerate kinase | Kinase | 4    | 2    |
| A8001406              | mRNA for alkali phosphatase | Phosphatase | 4    | 3    |
| X04770                | mRNA for Araf-1 oncogene | Downstream signal molecule for Ras signal transduction | 4    | 2    |
| X7576                | mRNA for protein kinase C (PKC) | Kinase | 4    | 2    |
| X52192               | RNA for c-fos | Oncogene; protein tyrosine kinase | 4    | 4    |
| X59727               | 63-kDa protein kinase related to rat ERK3 | MAP kinase signaling | 4    | 9    |

Suppression (total 41)

| GenBank accession no. | Name | Function | Array | Fold |
|-----------------------|------|----------|-------|------|
| L22253               | Arginine/serine-rich splicing factor 7 | mRNA splicing | 1    | 3    |
| U78095               | Bikinin | Hepatocyte GF activator inhibitor 2 | 1    | 2    |
| L34860               | Cadherin B | Cell differentiation | 1 >100 | 1    |
| M36067               | DNA ligase I | DNA replication | 1    | 3    |
| X16707               | Fos-related antigen (FRA1) | AP1 | 1    | 5    |
| U34683               | Glutathione synthase | Cell protection | 1    | 2    |
| L07516               | Heterochromatin protein homolog 1 | Chromatin structure | 1    | 13   |
| X62534               | High mobility group protein 2 | Malignant transformation | 1    | 4    |
| X62654               | Histone H4 | Chromosome structure | 1    | 4    |
| L22155               | IgG receptor FC large subunit P51 precursor | Immunoglobulin structure | 1    | 3    |
| X05347               | 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    |
| U76704               | Microsomal glutathione S-transferase I | Cell protection | 1    | 4    |
| M15796               | Proliferating cell nuclear antigen (PCNA) | Cell cycle regulation | 1    | 3    |
| J20040               | 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    | 2    |
| S79986               | Idl (Idl-a) | Inhibit transcription by forming inactive heterodimer | 2    | 7    |
| U68018               | Mad protein homolog (MAD-2) | Downstream molecule in TGFβ receptor activation | 2    | 3    |
| X62570               | mRNA for IP53 | IFN-inducible protein | 2    | 9    |
| X62571               | mRNA for keratin-related protein | Cell differentiation | 2    | 3    |
| X71835               | mRNA for neuropeptide Y-like receptor | Cell differentiation | 2    | 3    |
| A2272700             | mRNA for TSS-22 protein | G protein-coupled receptor | 2    | 5    |
| U51478               | Sodium/potassium-transporting ATPase (P3 subunit) | TGFβ-stimulated clone 22; apoptosis | 2    | 4    |
| AF007165             | Suppression | A novel suppressor of cell cycle entry | 2    | 3    |
| U49406               | Translation initiation factor 5 | Gene transcription/translation | 2    | 5    |
| L26510               | Cyclin-dependent kinase inhibitor; p21CIP1 | 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 | 3    | 2    |
| U46691               | Pdutative chromatin structure regulator | Neuroblastoma tumor suppressor gene | 3    | 2    |
| AFO0704              | Putative tumor suppressor protein 101F6 | Transcriptional regulation | 3    | 2    |
| AFO060228            | Retinoic acid receptor responder 3 (RARE3) | Putative tumor suppressor | 3    | 2    |
| X75208               | HEK-2 mRNA for protein tyrosine kinase receptor | Protein tyrosine kinase receptor | 4    | 3    |
| A0064757             | mRNA for PCDH7 | Metastasis inhibitor | 4    | 5    |
| U48995               | MUC-18 | Kinase | 4    | 4    |
| M34668             | Protein tyrosine phosphatase (PTPase-α) mRNA | Kinase | 4    | 4    |
| U40317               | Protein tyrosine phosphatase (PTPase-α) | Phosphatase | 4    | 2    |
| X62055               | PTP1C mRNA for protein-tyrosine phosphatase 1C | Phosphatase | 4    | 2    |
| L08924               | Receptor-type protein tyrosine phosphatase γ | Phosphatase | 4    | 2    |
| AF989988             | Sto-26 related kinase SNAK | MAP kinase signaling | 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 necrosis factor. *Gene accession numbers are from the GenBank database (http://www.ncbi.nlm.nih.gov/genbank/genbankoverview.html). †Clontech Cancer 1.2, 2, NEN Human 2400, 3, NEN Oncogene/Tumor Suppressor; 4, NEN Kinase/Phosphatase array. ‡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 (HNT1), 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); 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 bikinin. 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 expression 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 receptor 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, MAPK6, GATA3, and the Mitogen-responsive phosphoprotein DDC2. Many oncogenes were suppressed, including int-1, Ret, Bcl-2, 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 accession no. | Name                                                                 | Function                                                                                     | Arraya | Foldb |
|-----------------------|----------------------------------------------------------------------|-----------------------------------------------------------------------------------------------|--------|-------|
| M63959                | α2-Macroglobulin receptor–associated protein precursor (α2-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 (CDK4)                           | Cell cycle regulation                                                                          | 1      | 2     |
| U04718                | DNA mismatch repair protein MLH1, COCA2                              | DNA repair                                                                                    | 1      | 4     |
| M36089                | DNA-repair protein XRC1                                               | DNA repair                                                                                    | 1      | 4     |
| X92541                | Early growth response protein 1 (EGR1)                               | Early response gene                                                                            | 1      | 6     |
| X79067                | EGF response factor 1                                                | DNA repair                                                                                    | 1      | 2     |
| U79178                | Endonuclease III homolog (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/germline multiple exostoses candidate gene | Putative tumor suppressor                                                                       |        |       |
|                     |                                                                     |                                                                                               | 2      | 3     |
| M36803                | General β-spectrin                                                  | Membrane skeleton protein                                                                       | 2      | 3     |
| U40992                | HIV 40 homolog                                                      | Cell protection                                                                                | 2      | 3     |
| X95425                | mRNA for EHK-1 receptor tyrosine kinase                              | Receptor tyrosine kinase; formation of neuronal pathway                                         | 2      | 4     |
| A8803220              | mRNA for semaphorin E                                               | Non-MDR drug resistance gene                                                                   | 2      | 5     |
| U14394                | Tissue inhibitor of metalloproteinas-3 (TIMP-3)                     | Metalloproteinase inhibitor                                                                    | 2      | 9     |
| M19154                | TGFβ2                                                               | TGFβ superfamily                                                                               | 2      | 3     |
| M85787                | 22-kDa smooth muscle protein                                         | Structural protein                                                                              | 2      | 3     |
| U42488                | Cyclophilin-related protein                                          | Function of natural killer cells                                                                | 3      | 3     |
| M98833                | ERGβ transcription factor; FLI-1 homolog                            | A new Ets TF                                                                                  | 3      | 6     |
| AF081936              | 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     |
| U92013                | Glutathione S-transferase homolog                                  | Cell protection                                                                                | 1      | 3     |
| AF019770              | MC1                                                                 | 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 cytoplasm intermediate filaments                             | 2      | 8     |
| M20681                | Glucose transporter-like protein-III (GLUT3)                        | Expression in fetal skeletal muscle                                                            | 2      | 3     |
| A8000712              | hCPE-R mRNA for Clostridium perfringens enterotoxin (CPE) receptor  | CPE receptor                                                                                  | 2      | 6     |
| X80872                | hGATA3 mRNA for trans-acting T-cell specific transcription factor  | TF                                                                                           | 2      | 6     |
| L42611                | Keratin 6 isofrom (K6a)                                            | Cell differentiation                                                                           | 2      | 3     |
| M26512                | Keratin 8 mRNA, S’ end                                             | Cell differentiation                                                                           | 2      | 4     |
| M21389                | Keratin type II (58 kDa)                                           | Cell differentiation                                                                           | 2      | 3     |
| X92426                | mRNA for cytokeratin 13                                            | Cell differentiation                                                                           | 2      | 5     |
| X10497                | mRNA for HLA-DR antigens associated invariant chain p33            | Transmembrane polarity                                                                        | 2      | 10    |
| X69990                | mRNA for intercellular adhesion molecule (ICAM)-1                  | Cell adhesion molecule                                                                         | 2      | 2     |
| X98549                | mRNA for rho GDP-dissociation inhibitor 2                          | Intron STP binding; disruption of actin cytoskeleton                                          | 2      | 3     |
| L41351                | Prostasin                                                          | Prostate-specific marker                                                                        | 2      | 5     |
| L33404                | Stratum corneum chymotrypsin enzyme                                 | Serine protease                                                                               | 2      | 15    |
| M73554                | Bcl-1                                                              | Anti-apoptosis                                                                                 | 3      | 3     |
| U66694                | Epithelium-restricted Ets protein ESX                              | Oncogene, TF                                                                                  | 3      | 4     |
| X51602                | Fli mRNA for receptor-related tyrosine kinase                      | Fms-related tyrosine kinase                                                                    | 3      | 6     |
| M32325                | KSA                                                                | Lung cell surface glycophotin                                                                  | 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     |
| M90857                | Tumor antigen L6                                                   | Tumor-associated cell surface antigen                                                          | 3      | 3     |

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

**MSH2, TSG, 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 TGFβ 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.

| GenBank accession no. | Name | Function | Array | Fold |
|-----------------------|------|----------|-------|------|
| **Induction (total 13)** | | | | |
| M63969 | 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 | FRα1 | Ap1 | 1 | 4 |
| D13385 | Growth inhibitory factor; metallothionein-III (MT-III) | Cell growth regulation | 1 | 3 |
| X53587 | Integrin π4 | Cell differentiation | 1 | 2 |
| M29039 | Jun-B | Ap1 | 1 | 4 |
| L7211 | MT51; CDK41; p16-INK4 | Cell cycle regulation | 1 | 3 |
| U96551 | Histone ZA-like protein | Nuclear protein | 2 | 3 |
| X76717 | MT-11 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 |
| X52222 | DNA excision repair protein ERCC2 | DNA repair | 1 | 11 |
| L20046 | DNA excision repair protein ERCC5 | DNA repair | 1 | 2 |
| U60405 | DNA mismatch repair protein MSH2 | DNA repair | 1 | 4 |
| U38657 | Dual-specificity mitogen-activated protein kinase | MAP kinase signaling | 1 | 23 |
| U51166 | G/T mismatch-specific thymine DNA glycosylase (TDG) | DNA repair | 1 | 2 |
| L31951 | Integrin β4 | Cell differentiation | 1 | 2 |
| AF047347 | Adaptor protein X11α | Suppresses cellular amyloid precursor protein processing and reduces Aβ40 and Aβ42 secretion | 2 | 4 |
| | | | | |
| | | | | |
| **Array** | | | | |
| 1, Clontech Cancer 1.2; 2, NEN Human 2400; 3, NEN Oncogene/Tumor Suppressor; 4, NEN Kinase/Phosphatase array.

*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. Gene accession numbers are from the GenBank database ([http://www.ncbi.nlm.nih.gov/genbank/genbankoverview.html](http://www.ncbi.nlm.nih.gov/genbank/genbankoverview.html)). 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 TGFβ 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 β4, 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 As/Mix/MNNG. To further elaborate on the picture, genes altered in 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 non-tumorigenic, 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...
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 time-wise 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.

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