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Diverse Effects of Methylseleninic Acid on the Transcriptional Program of Human Prostate Cancer Cells

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Methylseleninic acid (MSA) has been shown to have potent anticancer activity and is an excellent compound for studying the anticancer effects of selenium in vitro. To gain insights into the effects of MSA in prostate cancer, we characterized the global transcriptional response of LNCaP, an androgen-sensitive human prostate cancer cell line, to MSA by using high-density cDNA microarrays. We identified 951 genes whose expression shows striking dose- and time-dependent changes in response to 3–30 μM MSA over the time course of 48 h. Transcript levels of many cell cycle-regulated genes change in response to MSA, suggesting that MSA inhibits proliferation. Consistent with these gene expression changes, cell proliferation, monitored by carboxyfluoroscein succinimidyl ester staining, was decreased after MSA treatment, and an accumulation of cells at G0/G1 phase was detected by flow cytometry. Surprisingly, MSA also modulated expression of many androgen-regulated genes, suppressed androgen receptor (AR) expression at both mRNA and protein level, and decreased levels of prostate specific antigen secreted into the medium. Low concentrations of MSA also induced significant increases in transcript levels of phase 2 detoxification enzymes and induced NADPH dehydrogenase, quinone 1 enzymatic activity, a surrogate marker of global phase 2 enzyme activity. Our results suggest that MSA may protect against prostate cancer by inhibiting cell proliferation, by modulating the expression of AR and AR-regulated genes and by inducing carcinogen defenses.

INTRODUCTION

Increasing evidence suggests that selenium compounds have promise as prostate cancer preventive agents. Several epidemiological studies have shown an inverse association between selenium levels in the serum or toenails and the subsequent risk of developing prostate cancer (Willett *et al*., 1983; Yoshizawa *et al*., 1998; Helzlsouer *et al*., 2000; Nomura *et al*., 2000; Brooks *et al*., 2001a). Animal and human intervention trials have shown that a daily supplementation with selenium-containing compounds reduces the risk of several malignancies, particularly human prostate cancer (Ip and White, 1987; el-Bayoumy, 1994; Reddy *et al*., 1994; Clark *et al*., 1996, 1998; Medina *et al*., 2001; Rao *et al*., 2001; Davis *et al*., 2002; Duffield-Lillico *et al*., 2002). The Nutritional Prevention of Cancer Trial, for instance, showed significantly lower incidence of prostate cancer diagnosis in subjects randomized to receive 200 μg of selenized yeast after 6.4 and 7.4 yr of follow-up, as well as reduced total cancer incidence (Clark *et al*., 1996; Duffield-Lillico *et al*., 2002). Although this study has been criticized for its use of secondary endpoints, it has provided compelling rationale for the recently initiated Selenium and Vitamin E Cancer Prevention Trial (SELECT), a 12-year prospective, randomized trial involving 32,000 men (Hoque *et al*., 2001; Klein *et al*., 2001).

The inverse relationship between selenium intake and prostate cancer risk has prompted a great deal of interest in understanding the mechanisms of selenium chemoprevention. Diverse forms of selenium have been shown to affect a variety of biological processes important in carcinogenesis (Ip, 1998; Combs, 2001; El-Bayoumy, 2001; Fleming *et al*., 2001; Ganther, 2001; Kim and Milner, 2001; Lu and Jiang, 2001; Youn *et al*., 2001). Selenium compounds have been shown to inhibit cell proliferation and induce apoptosis, and these are thought to be major mechanisms by which selenium prevents tumor initiation or progression (Ip *et al*., 2000a; Combs, 2001; Ganther, 2001; Lu, 2001). Selenium compounds also protect cells against oxidative stress and genetic damage, and block tumor angiogenesis (El-Bayoumy, 2001; Lu and Jiang, 2001). However, a comprehensive understanding of the mechanisms underlying selenium's anticancer effects is currently lacking.

Monomethylated forms of selenium are highly potent and efficacious chemopreventive agents. Methylselenocysteine (MSC) and methylseleninic acid (MSA) have been shown to be more active in cancer prevention than inorganic selenite, or selenomethionine, the form of selenium being used in SELECT (Ip *et al*., 1991; Ip, 1998; Combs, 2001; Hoque *et al*., 2001; Klein *et al*., 2001). It is believed that they are the direct precursors of methylselenol, possibly the key metabolite responsible for selenium's anticancer activity. Whereas MSC

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Abbreviations used: AR, androgen receptor; CFSE, carboxyfluoroscein succinimidyl ester; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MSA, methylseleninic acid; MSC, methylselenocysteine; NQO1, NADPH dehydrogenase, quinone 1; PSA, prostate-specific antigen.

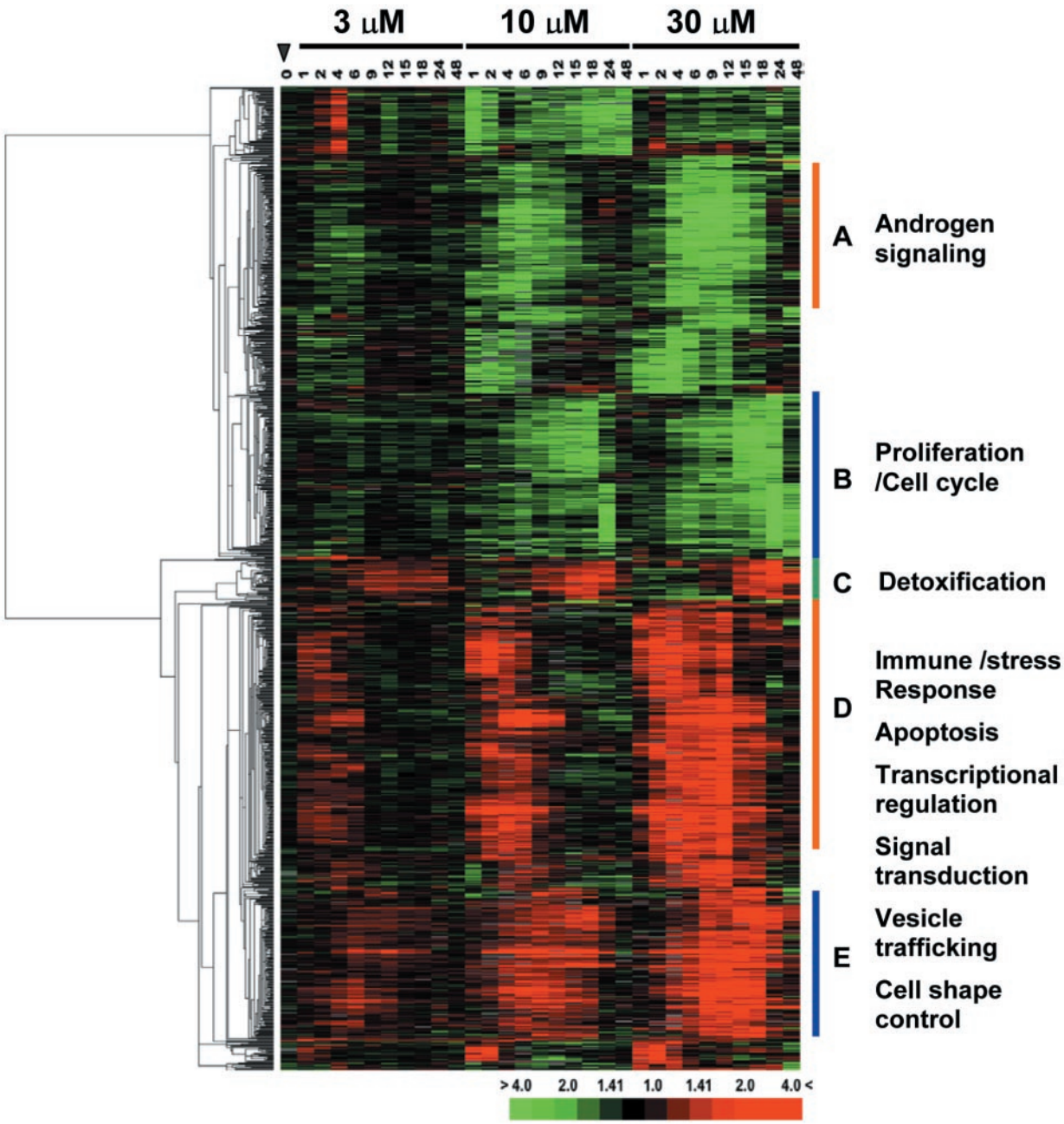


Figure 1. Hierarchical clustering analysis of MSA-responsive genes in LNCaP cells. Each column represents data from a single time point after treatment with MSA, and each row represents expression levels for a single gene across the time course. The 1128 transcripts were up-regulated (red) or down-regulated (green) after exposure to 3, 10, or 30 μM MSA as indicated at the top of the image. The degree of color saturation corresponds with the ratio of gene expression shown at the bottom of the image. For comparison, the gene expression pattern of untreated cells at time 0 is shown at the closed arrowhead. The data from each treatment condition were arranged in a time ascending order (1, 2, 4, 6, 9, 12, 15, 18, 24, and 48 h) as indicated on top of the image. The gene tree shown at the left of the image corresponds to the degree of similarity (Pearson correlation) of the pattern of expression for genes across the experiments. Genes in cluster A–E show different temporal response to MSA in a dose-dependent manner. Full transcript identities and raw data are available at http://www.Stanford.edu/~hongjuan/MSA.

requires the action of cysteine conjugate β-lyase or related lyases to be converted to methylselenol, MSA does not (Andreadou *et al*., 1996; Ganther and Lawrence, 1997; Ip, 1998; Ip *et al*., 2000b). It is 10 times more potent than MSC in affecting biological processes in vitro, probably because of limited β-lyase activity in cultured eukaryotic cells (Ip *et al*., 2000b). Therefore, MSA is an ideal compound for studying the anticancer effects of selenium in vitro.

DNA microarrays provide a genome-wide view of the biological processes affected by cellular perturbations and offer an opportunity to gain new insights into the mechanisms by which preventive agents exert their effects (Wil-

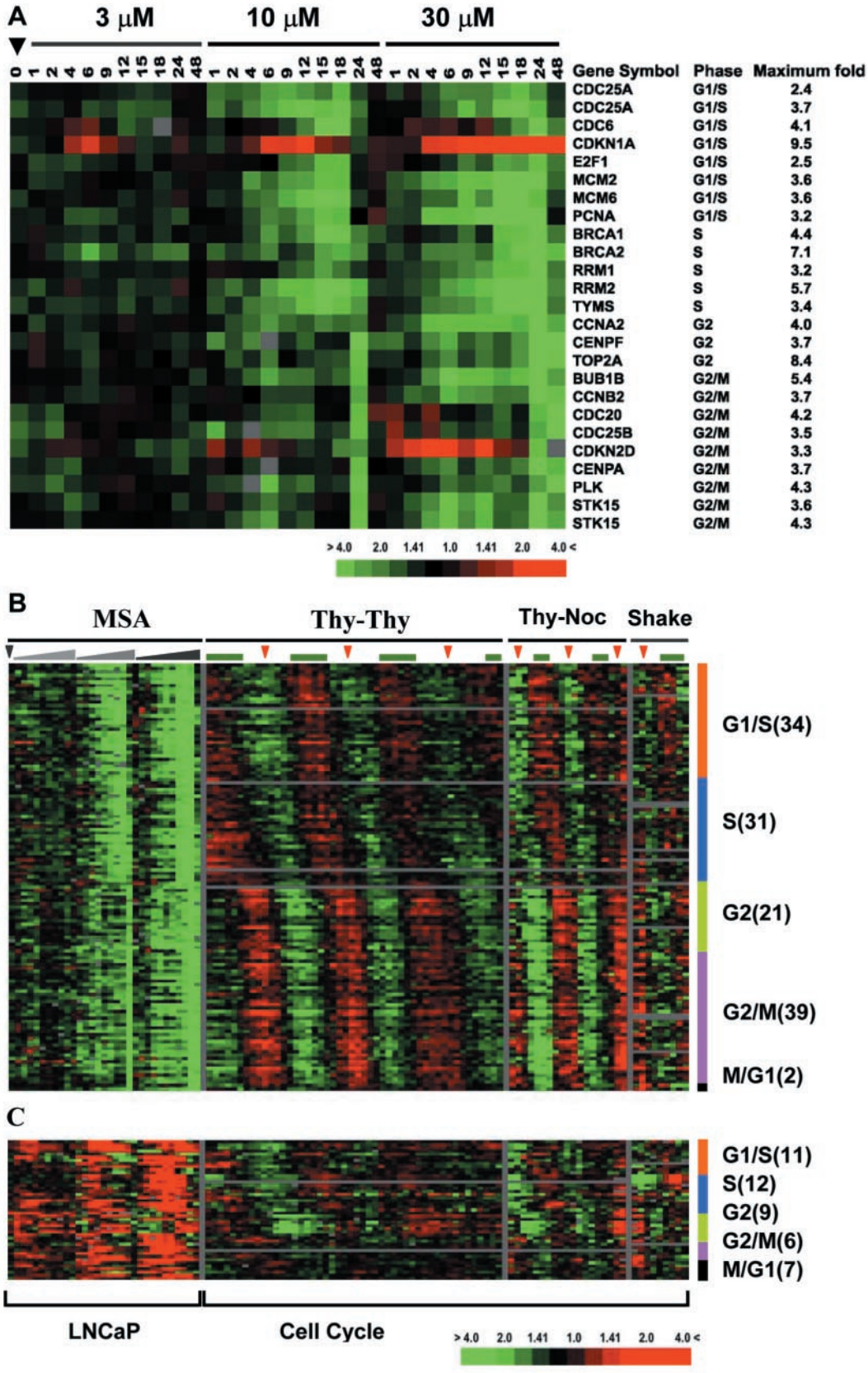


Figure 2. Cell cycle-regulated genes modulated by MSA. Genes that occur more than once are represented by multiple clones on arrays. (A) Transcripts representing previously characterized cell cycle-regulated genes. (B) Cell cycle-regulated transcripts identified by Whitfield *et al.* (2002) that are down-regulated by MSA. The number of transcripts belonging to different cell cycle phases is shown at the right of the image.

liams and Brooks, 2001). Herein, we have undertaken a systematic evaluation of the changes in gene expression that result from treatment of the androgen-sensitive prostate cancer cell line LNCaP with MSA. We identified 1128 clones representing 951 genes whose expression levels are affected by MSA in a time- and dose-dependent manner. The transcriptional profiles and confirmatory experiments suggest that MSA causes cell accumulation at G0/G1 modulates the expression of androgen receptor (AR) and its regulated genes, and induces enzymes that detoxify carcinogens.

MATERIALS AND METHODS

Cell Culture and Treatment

LNCaP cells were cultured in RPMI 1640 medium with 2 mM L-glutamine, 100 U/ml penicillin/100 μg/ml streptomycin (Invitrogen, Carlsbad, CA), and 5% defined fetal bovine serum that contributed 13 nM selenium to the medium (Hyclone Laboratories, Logan, UT). When cells reached ~40–60% confluence, the medium was changed, and 12–24 h later the cells were treated with 3, 10, or 30 μM MSA (pH adjusted to 7.0) (Selenium Technologies, Lubbock, TX). The doses used in this study were chosen based on previous studies using MSA in vitro and reported selenium levels in human serum (Ip *et al*., 2000b; Nomura *et al*., 2000; Brooks *et al*., 2001a; Jiang *et al*., 2001; Sinha *et al*., 2001; Dong *et al*., 2002; Zhu *et al*., 2002). At several time points after exposure, total RNA was harvested as described below. Untreated cells cultured in parallel were used as controls for each time point.

Total RNA Isolation

Medium was aspirated from each 150-mm cell culture plate, and 5 ml of TRIzol solution (Invitrogen) was added. After 5 min of gentle agitation, lysates were extracted with chloroform, and the organic and aqueous layers were separated using Phase Lock Gel (Eppendorf-5 Prime, Inc., Boulder, CO). Total RNA was precipitated with isopropanol and further purified with RNeasy mini kit (QIAGEN, Valenica, CA). The concentration of total RNA was determined using an MBA 2000 spectrometer (PerkinElmer Life Sciences, Boston, MA), and the integrity of total RNA was assessed using a 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA).

cDNA Microarray Hybridizations

Fluorescently labeled cDNA probes were prepared from 70 μg of total RNA isolated from MSA-treated cells (Cy5 labeled) and control cells (Cy3 labeled) by reverse transcription with an Oligo dT primer 5′-TTTTTTTTTTTTTTT-3′ (QIAGEN) as described previously (Zhao *et al*., 2002). Labeled probes from MSA-treated and control cells for each time point were mixed and hybridized overnight to spotted cDNA microarrays with 42,941 elements (Stanford Functional Genomics Facility). Microarray slides were then washed to remove unbound probe and analyzed as described previously (Zhao *et al*., 2002).

Data Processing and Analysis

Fluorescence intensities for each fluoroprobe were acquired using an Axon scanner 4000B and analyzed with GenePix Pro3.0 software (Axon Instruments, Union City, CA). Spots of poor quality were removed from further analysis by visual inspection. Data files containing fluorescence ratios were entered into the Stanford Microarray Database where biological data were associated with fluorescence ratios, and genes were selected for further analysis (Sherlock *et al*., 2001). Only spots with a signal intensity >150% above background in both Cy5 and Cy3 channels in at least 80% of the microarray experiments were used in the subsequent analysis. We arbitrarily selected transcripts whose expression level varied at least twofold after treatment compared with controls in at least three of the experiments examined. Prior work has shown that twofold variations in expression reliably reflect changes in expression levels measured by other methods (Blader *et al*., 2001; Jones and Arvin, 2003). The genes in the resulting data table were ordered by their patterns of gene expression by using hierarchical clustering analysis (Eisen *et al*., 1998) and visualized using Treeview software (http://rana.lbl.gov/EisenSoftware.htm). The data for all 1128 clones as well as the primary data are available at http://www.stanford.edu/~hongjuan/MSA.

Cell Proliferation and Cell Cycle Assay

Cell proliferation was determined using 5- or 6-(*N*-succinimidyloxycarbonyl)-3′,6′-*O*,*O*′-diacetylfluorescein (CFSE) (Dojindo Molecular Technologies, Gaithersburg, MD) staining (Lyons, 2000; Groszer *et al*., 2001) Untreated cells were stained with 1 μM CFSE in RPMI 1640 medium at 37°C for 10 min before being seeded in 60-mm plates with fresh media. After cells were cultured overnight, the media were again changed to eliminate residual CFSE that may have leaked from the cells. Half of the plates were treated with MSA for different lengths of time and harvested by trypsinization, and the remaining untreated plates cultured in parallel were used as controls. The absolute intensity of CFSE within each cell was measured by flow cytometry, and the average intensity of CFSE within the population calculated using Flow Jo software (http://www.flowjo.com/v4/html/overview.html).

Cell cycle distribution was determined by propidium iodide (PI) (Sigma-Aldrich, St. Louis, MO) staining. After aspirating the media, treated and control cells were collected by trypsinization and washed with 1× phosphate-buffered saline. Duplicate samples were collected for each growth condition. Cells were fixed with 70% ice-cold ethanol overnight and stained with PI (20 μg/ml) in presence of RNase A (300 μg/ml) at 37°C for 30 min. The DNA content of the cells was determined by flow cytometry, and cell cycle distribution was analyzed with Flow Jo software.

Western Blotting

Treated and control cells were lysed with 1 ml of radioimmunoprecipitation assay buffer (pH 7.4, 50 mM Tris-HCl, 1% NP-40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin). The cell lysate was passed through a 21-gauge needle to shear the cellular DNA. Protein concentration was determined using a BCA protein assay kit (Pierce Chemical, Rockford, IL). Ten to 15 μg of protein was separated using a 4–20% Tris-HCl precast gel (Bio-Rad, Hercules, CA), and transferred to a Hybond-P membrane (Amersham Life Sciences, Arlington Heights, IL). AR was detected with a rabbit polyclonal antibody against the amino terminus of human AR, sc-816 (Santa Cruz Biotechnology, Santa Cruz, CA) and visualized with an ECL Plus kit (Amersham Biosciences, Piscataway, NJ). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was detected with a monoclonal mouse anti-rabbit antibody, MoAb 6C5, which reacts with human GAPDH (Research Diagnostics, Flanders, NJ). AR and GAPDH signal intensities were quantified with a GS-700 densitometer (Bio-Rad).

Determination of Secreted Prostate-specific Antigen (PSA) Levels

Media from MSA-treated and control cells cultured on a 24-well plate was aspirated and stored at −80°C. PSA concentration in the thawed medium was measured using a human prostate specific antigen ELISA kit (Alpha Diagnostic International, San Antonio, TX) and was normalized to total protein of cells cultured in the same well where the medium was taken.

NADPH Dehydrogenase, Quinone 1 (NQO1) Enzymatic Activity Assay

After aspirating the media, treated and control cells cultured in a 96-well plate were lysed with 200 μl of 0.08% digitonin (Sigma-Aldrich)/2 mM EDTA (pH 8.0) at 37°C for 30 min. NQO1 enzymatic activity was assessed in triplicate by the menadione-coupled reduction of tetrazolium dye as described previously (Brooks *et al*., 2001b). Enzymatic activity for each sample was averaged across the triplicate and normalized to total cell protein in each sample.

RESULTS

MSA Affects Gene Expression in LNCaP Cells in a Dose- and Time-dependent Manner

To study systematically the effects of MSA in human prostate cancer cells in vitro, we characterized the temporal program of gene expression induced by treating LNCaP cells with three different concentrations of MSA. Thirty-one samples (10 samples/concentration over the course of 48 h plus one sample from untreated cells) were analyzed on microarrays containing ~42,941 features representing ~29,587 different human genes as inferred from UNIGENE clusters. The 1128 clones representing 951 genes displayed changes in expression levels of at least twofold after MSA treatment compared with controls in at least three samples.

Figure 2 (cont). The effect of MSA on expression of these genes is shown to the left organized in the same order as in A. The pattern of these genes across multiple cell cycles in HeLa cells is shown to the right. Thy-Thy indicates a double thymidine block to synchronize cells at S phase before release. Thy-Noc indicates a thymidine-nocodazole block to synchronize cells at mitosis before release. Shake indicates cells collected with an automated cell shaker that were used as synchronized in mitosis. The green bar above each column represents S phase, and the red arrowheads indicate mitosis as estimated by flow cytometry or bromodeoxyuridine labeling. (C) Cell cycle-regulated transcripts identified by Whitfield *et al.* (2002) that are up-regulated by MSA.

Many of the transcripts represent poorly characterized genes or expressed sequence tags. The data for the 1128 transcripts were ordered by their patterns of gene expression by hierarchical clustering (Eisen *et al.*, 1998) (Figure 1). The complete data set, including raw data, is available at http://www.stanford.edu/~hongjuan/MSA.

MSA produced discrete, reproducible, time- and dose-dependent changes in gene expression in LNCaP cells. Expression changes were largely similar among cells treated with 3, 10, and 30 μM MSA; however, with higher concentrations of MSA, changes in gene expression were larger in both the magnitude and duration. The number of transcripts whose expression increased or decreased was similar (541 and 587, respectively). Approximately one-half of the transcripts showed changes within 1–2 h after treatment with peak variation occurring within 8 h and returned to baseline expression levels by 24 h (Figure 1, clusters A and D). Many of the functionally characterized genes in cluster A are known to be involved in androgen signaling pathways. The remaining transcripts were delayed in their response, with expression changes that peaked between 12 and 24 h and that remained apparent at 48 h (Figure 1, clusters B, C, and E). These included genes involved in cell cycle regulation (cluster B) and phase 2 detoxification enzymes (cluster C). Known genes in clusters D and E are involved in diverse biological processes, including immune and stress responses (IGSF3, IGSF4, and NFIL3), apoptosis regulation (BIRC2, BIRC3, and TNFAIP3), transcriptional regulation (ATF3, ELF3, and MAD), signal transduction (JAK1, ARHB, and SH3BP5), tumor suppression (MEN1, ING1, and IRF1), vesicle trafficking (SEC24D, STX1A, and RAB31), and cell shape control (KLHL2, WASF1, and MAP1B).

MSA Changes Expression of Cell Cycle-regulated Genes

MSA has been shown to inhibit cell growth through its effects on the cell cycle in several model systems, although not in the LNCaP cell line. A subset of the 1128 transcripts (Figure 1, cluster B) modulated by MSA in LNCaP cells represent known cell cycle-regulated genes (Figure 2A). To gain insight into the effect of MSA on cell cycle-regulated genes, we compared these 1128 transcripts to a set of 1134 transcripts (representing >850 genes) that vary periodically as synchronized HeLa cells pass through the cell cycle (Whitfield *et al.*, 2002). In the latter data set, all 1134 transcripts were grouped according to the phase in the cell cycle where their expression peaked. Between the MSA and cell cycle data sets, 172 transcripts were found in common. The 127 transcripts that showed decreased expression were distributed throughout all phases of the cell cycle and included genes involved in DNA replication initiation (CDC6, MCM2, and MCM6), DNA repair (PCNA), and cell cycle control (CDC25A and E2F1) expressed in G1/S phase, DNA replication (RRM1, RRM2, and TYMS) expressed in S phase, chromosome condensation and organization (TOP2A and CENPA), mitotic spindle checkpoint (CDC20 and BUB1B), and centrosome duplication (PLK and STK15) expressed in G2 and M phase (Figure 2B). There were 45 clones in common between the data sets that were up-regulated by MSA that, again, were distributed throughout all phases of the cell cycle. These transcripts show periodic expression in HeLa cells with an expression pattern that was the inverse of the genes that are down-regulated by MSA. In this set of transcripts are known inhibitors of proliferation, most notably CDKN1A (p21), CDKN2D (p19), and CDKN1C (p57), all of which are potent negative regulators of G1 cyclin/cdk complexes (Sherr and Roberts, 1999; Gitig and Koff, 2000). This suggests that induction of this set of genes by MSA may modulate decreased proliferation in LNCaP cells.

The distribution of transcripts affected by MSA across all phases of the cell cycle suggested that MSA might cause LNCaP cells to exit the cell cycle, rather than induce an arrest at a specific cell cycle phase or slow cell cycle progression. In the HeLa cell cycle experiments, cell cycle arrest was associated with high expression of transcripts typically expressed during the phase of the cell cycle at which arrest occurs (see Thy-Thy, Thy-Noc, and Shake off in Figure 2B). In LNCaP treated with MSA, on the other hand, expression variations of cell cycle-regulated transcripts were not selectively associated with any particular phase of the cell cycle; cell cycle-regulated transcripts typically expressed in a particular phase of the cell cycle (i.e., G1, S, or G2/M) all showed decreased expression and the transcripts that displayed increased expression are known to inhibit cell proliferation. These expression changes, therefore, suggest that cells are exiting the cell cycle in response to MSA, rather than arresting at a particular phase in the cell cycle.

MSA Inhibits Cell Proliferation by Induction of Cell Accumulation at G0/G1

Based on the expression changes in the cell cycle-regulated genes, we assessed the effect of MSA on the proliferation of the LNCaP cells after pulse exposure to CFSE. CFSE diffuses freely into cells where it is converted to a fluorescently tagged membrane impermeable dye that is retained in the cytoplasm. With each round of cell division, the retained CFSE is partitioned equally to daughter cells and the relative intensity of the dye becomes decreased by half. At concentrations between 3 and 30 μM, MSA produced a dose-dependent inhibition of LNCaP cell growth, evident by the significantly higher mean intensity of CFSE in treated cells compared with controls (Figure 3). CFSE levels in MSA-treated cells remained high relative to control cells up to 48 h and then the inhibitory effect began to diminish (our unpublished data). Exchange of the medium at 72 h and retreatment with MSA produced growth inhibition out to 120 h similar in magnitude to that produced by the first treatment. Therefore, as predicted from gene expression profiling, MSA inhibits LNCaP cell growth and cells retain sensitivity to this inhibition with repeated treatments.

To evaluate whether the decreased proliferation we observed was most consistent with cell cycle arrest or exit from the cell cycle, we performed flow cytometry on MSA-treated and untreated LNCaP cells. The proportion of cells at G0/G1, S, and G2/M phase was determined after 24-h exposure to different concentrations of MSA. Cells treated with 3, 6, 10, and 30 μM MSA all showed an increase in the percentage of cells at G0/G1 phase with a corresponding depletion of cells in S and G2/M phase (Figure 4). The most pronounced effects were seen with 6 and 10 μM MSA, where the fraction of cells in S and G2/M phase decreased by 66 and 63%, respectively. We did not see evidence of apoptosis at any of the doses tested. These results are most consistent with MSA inducing either G1 arrest or causing cells to exit the cell cycle (G0).

MSA Modulates Transcript Levels of AR and Androgen-responsive Genes

To our surprise, we found that MSA modulated the expression of AR and a group of well-characterized androgen-regulated genes in a time- and dose-dependent manner. Two clones representing AR showed decreased

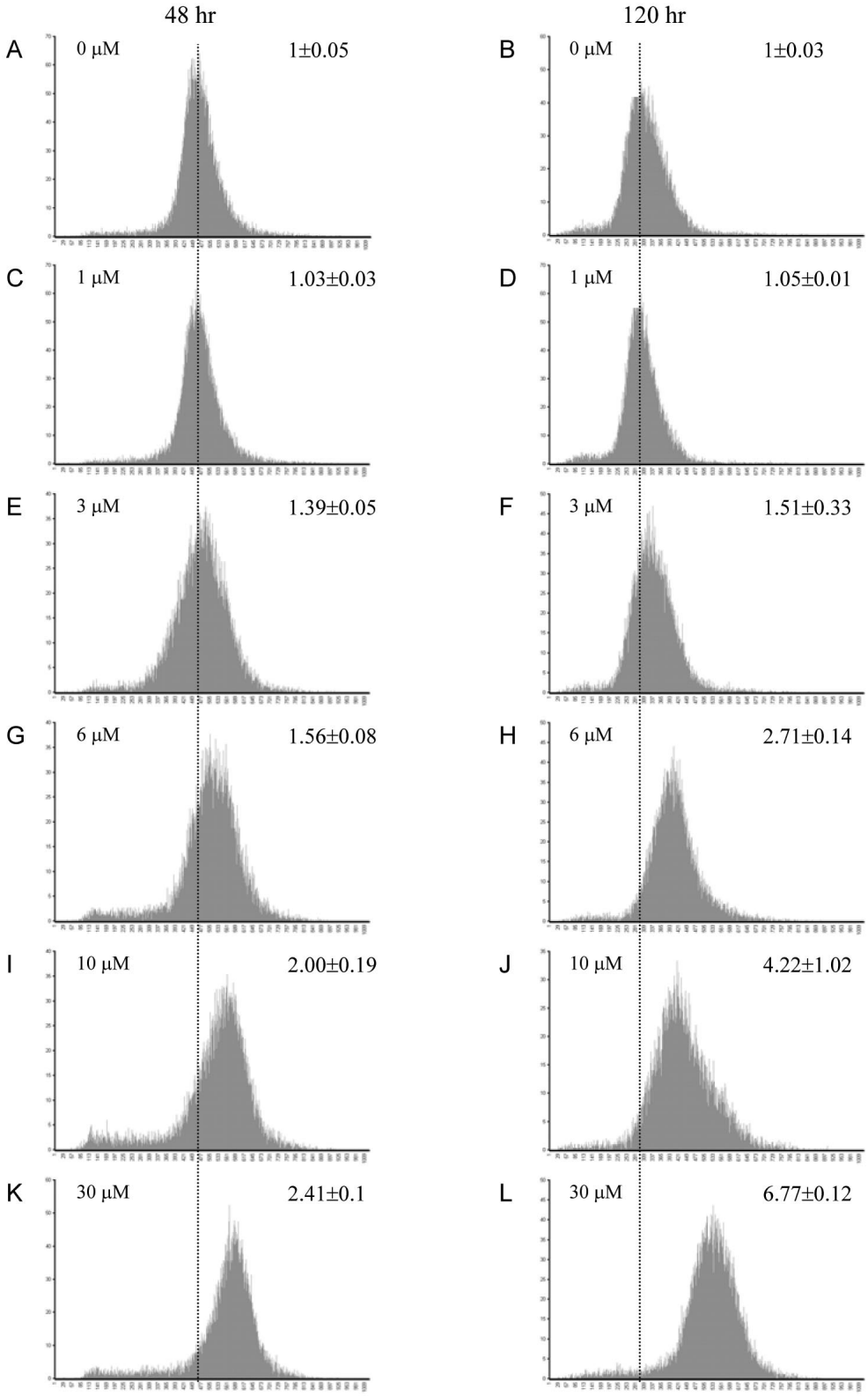


Figure 3. Cell proliferation monitored by CFSE staining and flow cytometry with and without MSA exposure. The y-axis represents the number of cells, and the x-axis represents the intensity of CFSE in the cells. Cells harvested 48 h after CESF staining (left) and 120 h (right). Media with fresh MSA were exchanged at 72 h after CFSE staining. The concentration of MSA used to treat the cells is shown at the top left corner of each graph. The mean average intensity of CFSE in treated cells was normalized against that of the control cells and is shown at the top right corner of each graph. Each graph represents data from triplicate samples.

transcript levels in response to MSA, and 19 known androgen-regulated genes showed altered transcript levels. MSA suppressed expression of 12 androgen-induced genes (KLK3, KLK2, ACPP, NKX3A, TMPRSS2, E2F1, ARSDR1, FKBP5, TUBA2, TUBB2, PPFIA1, and AIBZIP) and increased expression of six of seven genes normally suppressed by androgen (APOD, CLU, PEG3, UGD, NDRG1, and SERPINB5) (Figure 5A). Myc transcript levels, previously shown to be suppressed by androgen, showed a biphasic response to MSA.

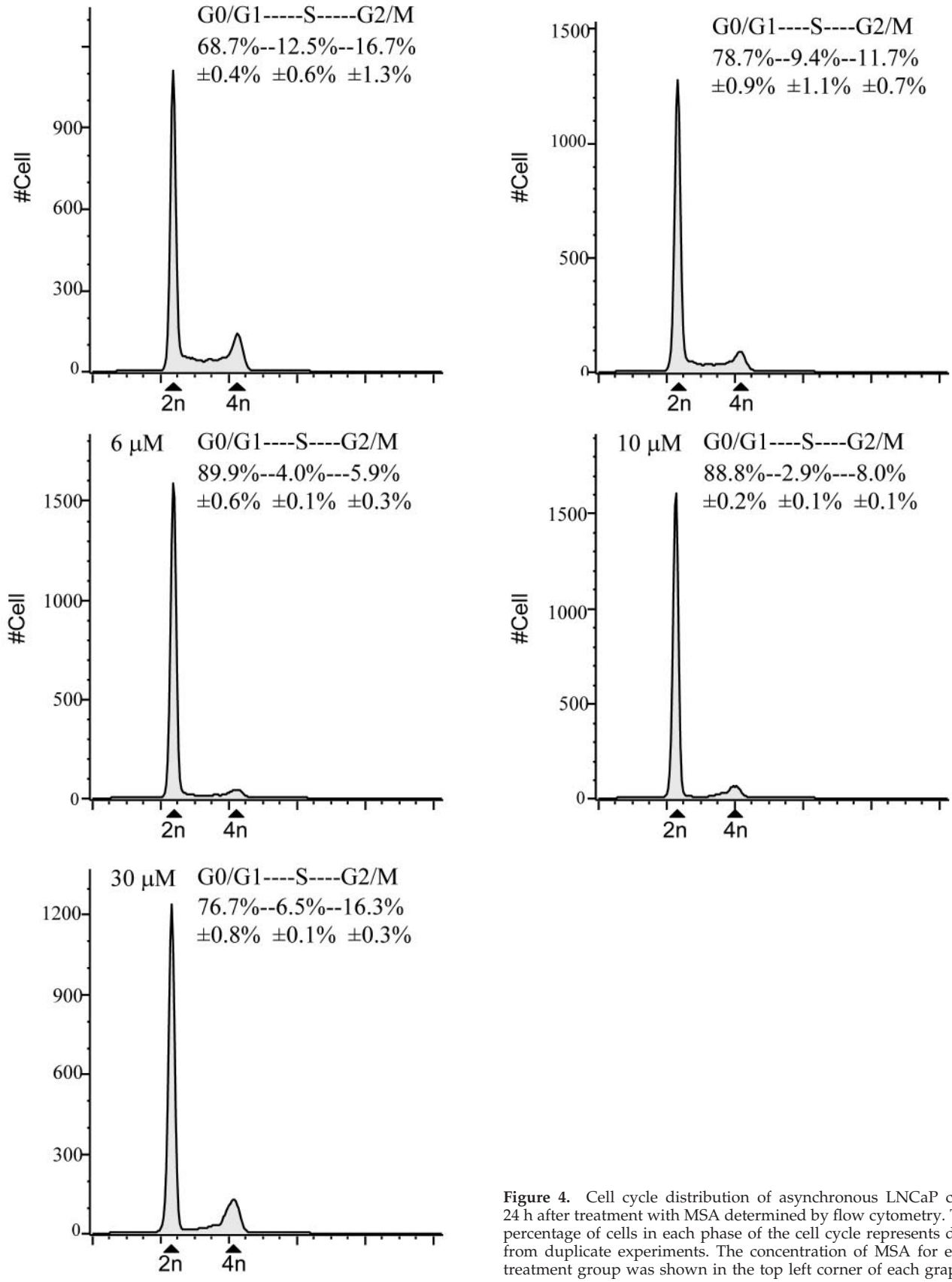


Figure 4. Cell cycle distribution of asynchronous LNCaP cells 24 h after treatment with MSA determined by flow cytometry. The percentage of cells in each phase of the cell cycle represents data from duplicate experiments. The concentration of MSA for each treatment group was shown in the top left corner of each graph.

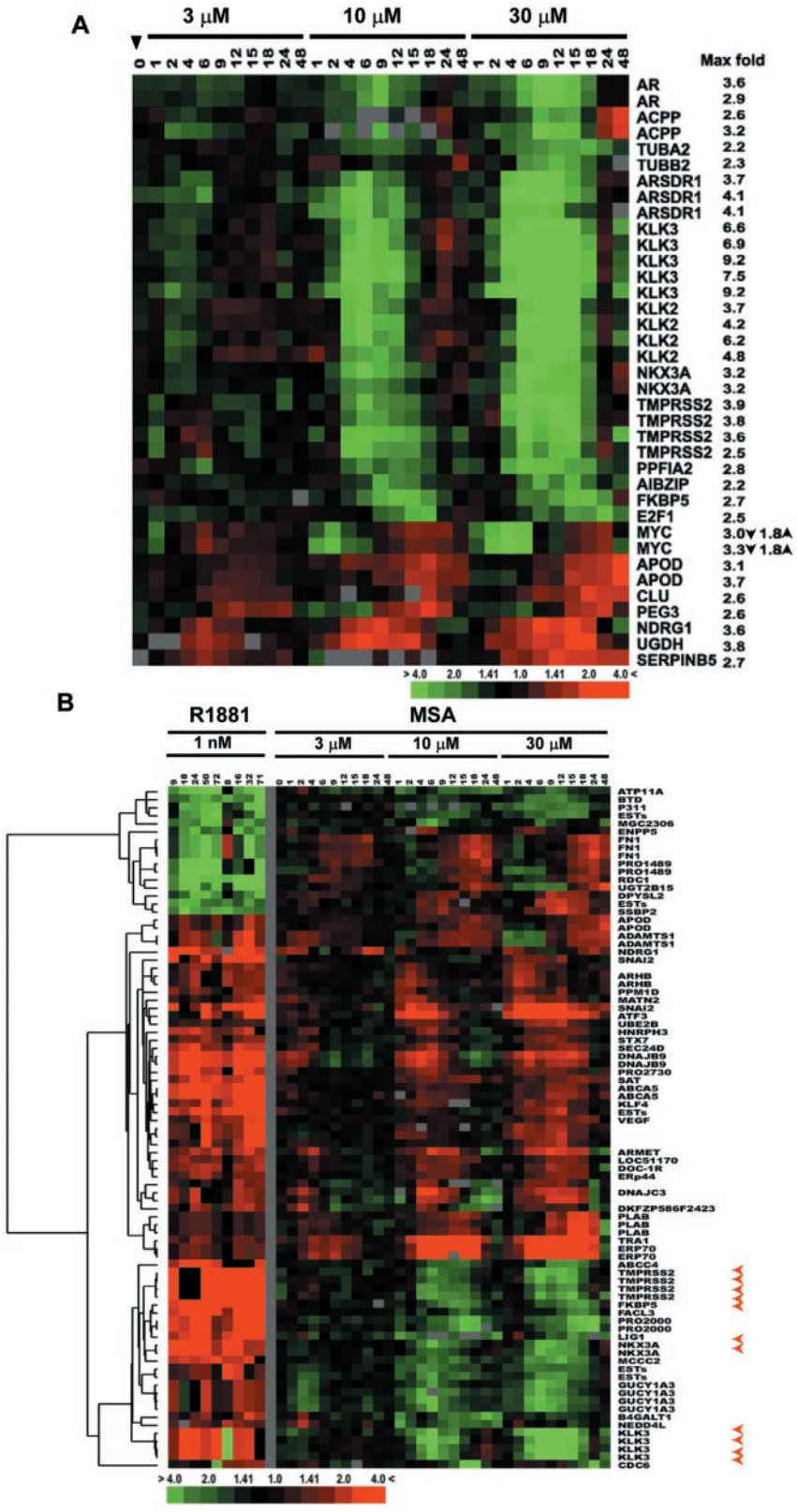


Figure 5. Androgen-responsive genes modulated by MSA. Genes that occur more than once are represented by multiple clones on arrays. (A) MSA-induced expression changes of known androgen-regulated genes. (B) MSA-affected transcripts that are present in a list of androgen-responsive transcripts identified by DePrimo *et al.* (2002). On the left are gene expression patterns from two separate time courses induced by treatment of LNCaP cells with the synthetic androgen R1881. On the right are expression patterns of this same set of genes after MSA treatment. The red arrowheads point to well-characterized androgen-regulated genes.

We compared our MSA-regulated data set to a recently reported set of 103 androgen-regulated genes (Nelson *et al.*, 2002) and found that 18 of 26 genes found in both data sets showed a reciprocal response to MSA (Table 1). Intriguingly, when compared with a set of 567 androgen-regulated transcripts we had identified previously (DePrimo *et al.*, 2002), 85 of the MSA-regulated transcripts representing 61 genes were found in common, and only one-half of the transcripts

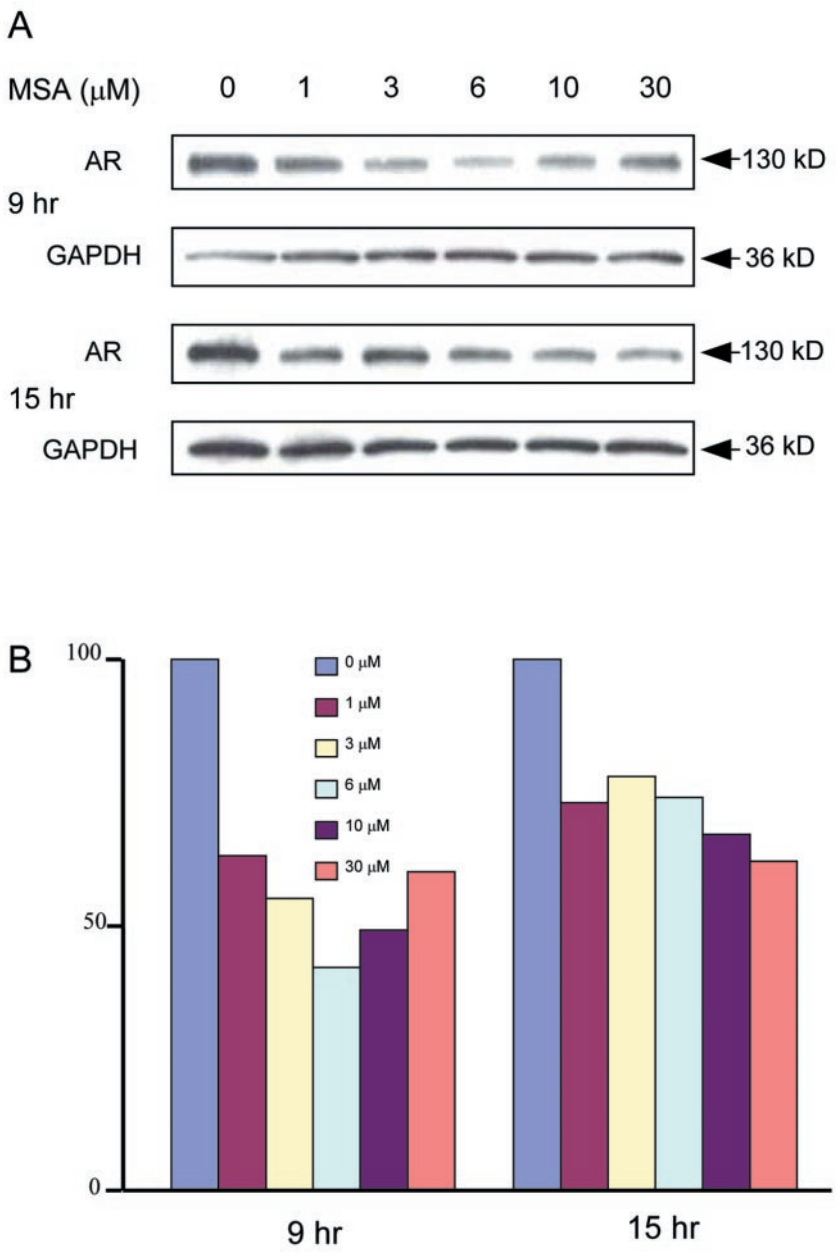


Figure 6. MSA decreases AR protein expression. (A) AR protein level after 9 and 15 h of exposure to different concentrations of MSA by western blotting analysis. GAPDH from each sample is shown as an internal control. (B) Quantitation of AR protein levels by using a densitometer. The signal intensity of AR was normalized to GAPDH in each same sample. AR intensity of treated cells was normalized against that of the untreated control cells.

were reciprocally regulated (Figure 5B). Therefore, comparison of the MSA expression data set to this larger androgen-regulated data set suggested that MSA has mixed effects on androgen-responsive genes.

MSA Represses AR Protein Expression and the Level of Secreted PSA

To characterize further the effects of MSA on the androgen axis, we performed Western blotting to compare AR protein levels from treated and untreated LNCaP cells (Figure 6A). The decreased AR transcript levels we observed on the microarrays were associated with decreased AR protein levels at 9 and 15 h after MSA exposure, even at relatively low doses (1 μM). AR protein levels decreased 40–60% after 9 h of MSA exposure, and 30–40% after 15-h exposure. There did not seem to be a significant difference in the degree of AR down-regulation for different MSA concentrations at 15 h; however, 6 μM MSA produced more striking suppression of AR protein levels at 9 h (Figure 6B).

To evaluate further the effects of MSA on androgen-regulated genes, we determined the level of secreted PSA in the cell culture media after exposure of cells to MSA (Figure 7). A dose-dependent decrease in secreted PSA level was detected within 12 h after MSA exposure and continued out to 48 h. Therefore, protein levels of PSA, a well-known androgen target, show modulation similar to that observed for transcript levels using microarray analysis.

MSA Up-Regulates Detoxification Enzymes

Phase 2 detoxification enzymes function in metabolizing and inactivating xenobiotics and toxins and thereby protect cells against carcinogens. We noted 12 transcripts representing seven genes encoding phase 2 enzymes were up-regulated by MSA (Figure 8A). The mRNA levels of NQO1, a surro-

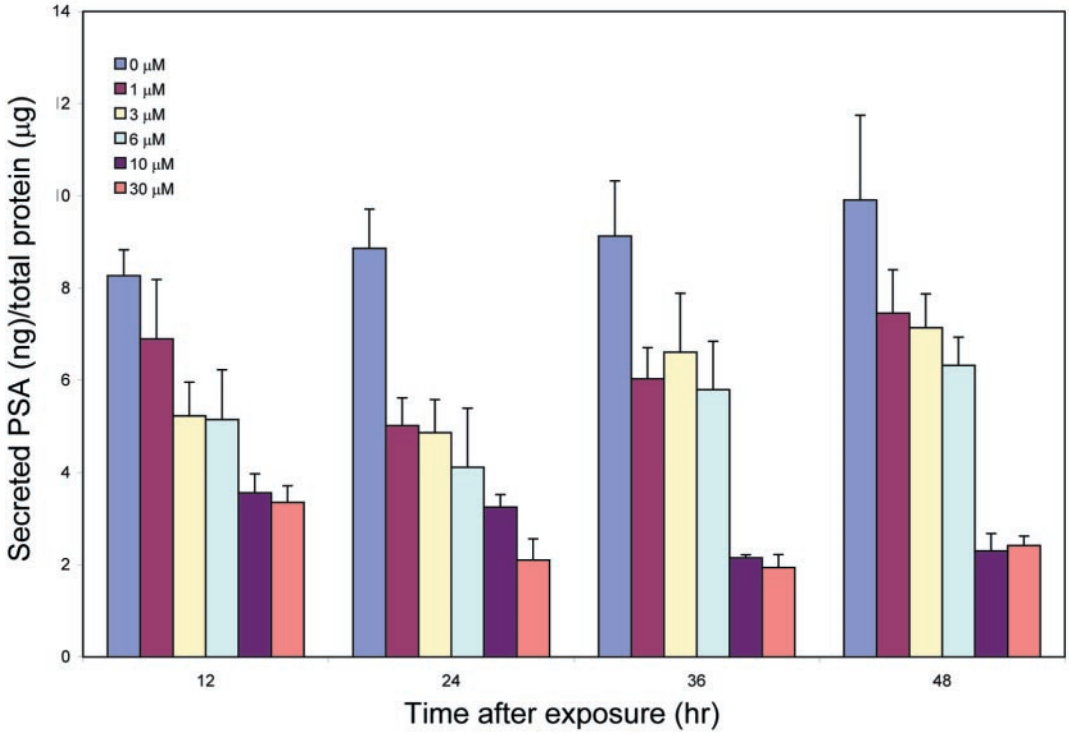


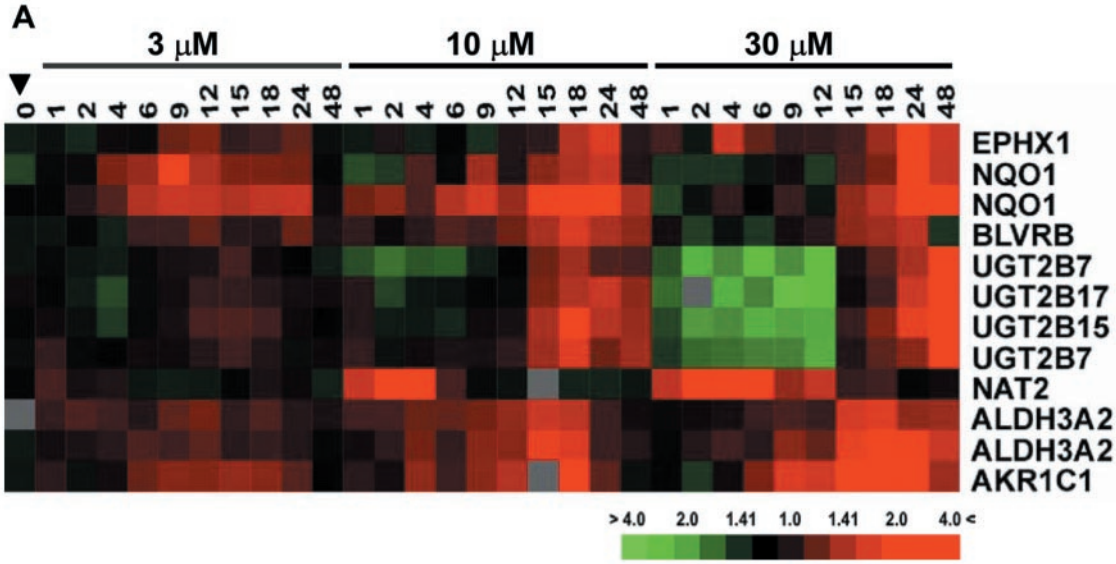
Figure 7. MSA decreases levels of PSA secreted into the media in LNCaP cells. PSA levels in the cell culture medium measured by ELISA and normalized against the total protein of the cultured cells. Each column represents data from experiments performed in triplicate.

Table 1. Comparison of gene expression changes induced by MSA and androgen reported by Nelson *et al.* (2002)

Gene symbol	Description	Expression change: Androgen 24 hr	Androgen 48 hr	MSA Max fold	Biological process
CDC14B	Cell division cycle 14 homolog B	3.0 ↑	3.1 ↑	2.9 ↓	Proliferation/differentiation/apoptosis
ID2[a]	Hes6 neuronal differentiation gene ortholog	1.6 ↑	3.8 ↑	2.7 ↑	Proliferation/differentiation/apoptosis
NDRG1[b]	N-myc downstream regulated	13.7 ↑	14.8 ↑	3.6 ↓	Proliferation/differentiation/apoptosis
KLK2	Kallikrein 2, prostatic	8.8 ↑	9.0 ↑	6.2 ↓	Protease/protease Inhibitor
KLK3[b]	Kallikrein 3, prostate specific antigen	7.9 ↑	10.2 ↑	9.2 ↓	Protease/protease Inhibitor
TMPRSS2[b]	Transmembrane protease, serine 2	15.5 ↑	18.3 ↑	3.9 ↓	Protease/protease Inhibitor
GUCY1A3[b]	Guanylate cyclase 1, soluble, alpha 3	2.9 ↑	3.3 ↑	4.3 ↓	Signal transduction
INPP4B	Inositol polyphosphate-4-phosphatase, type II	2.3 ↑	4.6 ↑	2.3 ↓	Signal transduction
PEG3	Paternally expressed 3	3.2 ↓	4 ↓	2.6 ↑	Signal transduction
FN1[b]	Fibronectin 1	2.5 ↓	4.4 ↓	4.1 ↑	Structure/motility/adhesion
H1F0	Histone family, member 0	2.9 ↑	3.2 ↑	7.4 ↓	Structure/motility/adhesion
B4GALT1[b]	BetaGlcNAc beta 1,4-galactosyltransferase	3.3 ↑	3.3 ↑	3.5 ↓	Metabolism
FACL3[b]	Fatty-acid-Coenzyme A ligase, long-chain 3	2.7 ↑	3.7 ↑	2.4 ↓	Metabolism
SAT[a]	Spermidine/spermine N1-acetyltransferase	3.7 ↑	7.3 ↑	2.3 ↑	Metabolism
SCD	Stearoyl-CoA desaturase	5.9 ↑	4.5 ↑	5.8 ↓	Metabolism
UGDH	UDP-glucose dehydrogenase	2.9 ↑	4.0 ↑	3.8 ↓	Metabolism
KLF4[a]	Kruppel-like factor 4	2.3 ↑	3.0 ↑	2.4 ↑	Transcription regulation
MYC[a]	V-myc myelocytomatosis viral oncogene homolog	2.7 ↓	2.8 ↓	3.3 ↓ 1.8 ↑	Transcription regulation
NKX3A[b]	NK3 transcription factor homolog A (Drosophila)	14.9 ↑	14.1 ↑	3.2 ↓	Transcription regulation
ABCC4[b]	ATP-binding cassette, sub-family C	5.5 ↑	7.8 ↑	2.5 ↓	Transport/trafficking
FKBP5[b]	FK506 binding protein 5	24.4 ↑	25.4 ↑	2.7 ↓	Transport/trafficking
SEC24D[a,b]	SEC24 related gene family, member D	3.0 ↑	2.6 ↑	2.9 ↑	Transport/trafficking
RDC1[b]	G protein-coupled receptor	7.8 ↓	4.5 ↓	2.4 ↑	Stress response
DNAJB9[a,b]	DnaJ (Hsp40)homolog, subfamily B	4.0 ↑	3.6 ↑	4.2 ↑	Stress response
SGK[a]	Serum/glucocorticoid regulated kinase	4.4 ↑	2.4 ↑	2.5 ↑	Stress response
ST7[a]	Suppression of tumorigenicity 7	2.7 ↓	4.2 ↓	2.5 ↓	Other functions

[a] Genes show similar expression changes under the influence of androgen and MSA.

[b] Genes that are also represented in dataset from DePrimo *et al.* (2002).



B

MSA concentration (μM)	Time after exposure (hr)		
	15	24	48
	Percentage increase in NQO1 activity		
1	52.5±10.0	49.9±13.9	32.4±7.8
3	59.1±10.2	68.6±7.2	67.3±11.7
6	79.0±15.3	72.4±8.7	82.6±11.3

Figure 8. MSA induces expression of several phase 2 enzymes. Genes that occur more than once are represented by multiple clones on arrays. (A) Transcript levels of phase 2 enzymes after treatment with 3, 10, and 30 μM MSA. (B) Percentage increase of NQO1 enzymatic activity after treatment with 1, 3, and 6 μM MSA compared with untreated cells. Results shown represent the average of triplicate experiments.

gate marker of global phase 2 enzyme activity, were induced by as little as 3 μM MSA. At higher concentrations, several other phase 2 enzymes were induced coordinately with NQO1. We tested whether MSA also increases the enzymatic activity of NQO1 in LNCaP cells by a colorimetric assay involving the mendione-coupled reduction of tetrazolium dye (Brooks *et al.*, 2001b). Treated and control LNCaP cells were harvested at 15, 24, or 48 h after exposed to 1, 3, or 6 μM MSA. The NQO1 activity in each sample was normalized to the total protein of that sample, and the percentage of increase of NQO1 activity compared with control is shown in Figure 8B. NQO1 activity was induced similarly by all three concentrations of MSA and increased over time. Therefore, the increases in NQO1 transcript levels observed in the microarray experiments correlated well with induction of NQO1 enzymatic activity.

DISCUSSION

MSA induces striking dose- and time-dependent changes in gene expression in LNCaP cells, suggesting that selenium acts by diverse mechanisms as a putative prostate cancer preventive agent. MSA decreases proliferation of LNCaP cells, possibly by causing cells to exit the cell cycle, alters the expression of many genes in the androgen axis, including AR and many androgen-responsive genes, and induces expression of phase 2 detoxification enzymes, an effect that could be particularly relevant to human prostate cancer chemoprevention. Our findings support the hypothesis that monomethylated selenium may be responsible, at least in part, for the potential anticancer activity of selenium supplements.

Several reports using a variety of model systems have shown that selenium inhibits cell proliferation, and this

inhibition is thought to underlie selenium chemoprevention (Ip *et al*., 2000a; Combs, 2001; Ganther, 2001; Lu, 2001). Decreased proliferation has been attributed to cell cycle arrest, although in prostate cancer cell lines no consistent pattern of arrest has been observed. After treatment with sodium selenite or selenomethionine, growth arrest has been reported in the G1 and G2/M phases of the cell cycle, depending on the prostate cancer cell line in which these compounds were tested (Redman *et al*., 1998; Menter *et al*., 2000; Venkateswaran *et al*., 2002; Bhamre *et al*., 2003). This lack of consistency may be due to innate differences between the cell lines or to differences in metabolism of the forms of selenium used in these studies. Based on compelling evidence that methylselenol is largely responsible for the chemopreventive activities of selenium compounds, we used MSA in our studies because it can be converted directly into methylselenol in vitro (Ip *et al*., 2000b). MSA produced a dose-dependent inhibition of cell growth of LNCaP with an accumulation of cells in G0/G1 phase. Similar inhibition of proliferation and accumulation of cells in G0/G1 has been observed in breast cancer and endothelial cells treated with MSA (Sinha *et al*., 2001; Wang *et al*., 2001; Dong *et al*., 2002).

We noted that a striking decrease in expression of many cell cycle-regulated genes from all phases of the cell cycle accompanied growth inhibition in LNCaP cells. Microarray analysis has been used in mammary cancer cells and PC-3 prostate cancer cells, and down-regulation of cell cycle-regulated genes has been observed along with increased expression of CDK inhibitors (Dong *et al*., 2002, 2003). In these reports, decreased proliferation had been attributed to cell cycle arrest due to modulation of key regulators of the cell cycle, many of which are seen in our data set. Comparison of our data set to genes whose expression varies periodically as HeLa cells pass through the cell cycle provides a broader view of the effects of MSA on the cell cycle. The coordinate, decreased expression of genes involved in all phases of the cell cycle coupled with the increased expression of CDK-inhibitors (CDKN1A, CDKN2D, and CDKN1C) suggest MSA causes LNCaP cells to exit the cell cycle, rather than inducing an arrest at a specific phase in the cell cycle. Whether this is the primary mechanism by which selenium compounds inhibit cell growth awaits further study. Certainly, assessment of the effects of other forms of selenium on the expression of cell cycle genes in prostate cells could provide additional information on the means by which selenium compounds inhibit prostate cancer growth. Ultimately, it will be necessary to evaluate the effects of selenium on prostate cancer growth in vivo, and the cell cycle-regulated genes identified in this and other studies could serve as biomarkers of response.

Perhaps the most striking observation from our microarray experiments is that MSA produced changes in transcript levels of AR and AR-regulated genes. Androgens are critical to prostate carcinogenesis, and androgen deprivation therapy is a mainstay of prostate cancer treatment. MSA suppresses the expression of AR at both mRNA and protein levels, decreases transcript levels of PSA, and decreases PSA protein excretion into the media. A small set of well-characterized androgen-regulated genes, including those with androgen response regulatory elements, show expression changes that are reciprocal to those induced by androgen. Comparison of the MSA data set with a large data set of genes modulated in response to androgens shows that many, but not all, androgen-regulated genes show expression changes opposite to what is seen after treatment with androgens. Some genes were regulated similarly in the two data sets, suggesting that MSA has mixed effects on the transcription of AR-regulated genes. It is possible that genes that are regulated similarly by MSA and androgens are not direct targets of androgen signaling pathways. For instance, androgen treatment of LNCaP cells is known to produce cellular stress by inducing an oxidative burst, and induction of stress response genes has been observed with expression profiling after androgen treatment (Xu *et al*., 2001; DePrimo *et al*., 2002). Therefore, the transcripts regulated similarly by androgens and MSA (DNAJB9, ATF3, and VEGF) might reflect cellular stress or other pathways that have been activated secondarily.

Effects of selenium on AR and AR-regulated genes in prostate cancer cell lines have not been observed with other selenium compounds; in fact, two reports have shown that selenomethionine does not have an effect on AR function or PSA secretion in LNCaP cells (Zhang *et al*., 2002; Bhamre *et al*., 2003). One possible explanation for the lack of effect of selenomethionine on androgen-regulated genes is its poor conversion to methylselenol in vitro. Intriguingly, men supplemented with selenized yeast do show small but significant decreases in their serum PSA levels compared with control subjects, suggesting the possibility that selenium compounds can affect AR-regulated genes in vivo where they can be metabolized to methylselenol (El-Bayoumy *et al*., 2002). In addition, effects of MSA on AR-regulated genes in PC-3 cells were not observed by Dong *et al*. (2002, 2003), suggesting that MSA may affect transcription of AR-regulated genes through AR.

It is tempting to speculate that MSA blocks proliferation in prostate cells through its effects on AR and AR-regulated genes. Consistent with our findings, Venkateswaran *et al.* (200) observed that selenomethionine did not affect the growth of wild-type (AR-null) PC-3 prostate cancer cell lines, but did inhibit growth of PC-3 cells stably expressing AR. However, three other groups have observed growth inhibition by selenium compounds in prostate cancer cell lines that do not express AR (Redman *et al*., 1998; Menter *et al*., 2000; Dong *et al*., 2003). Additional work will be necessary to understand the role of MSA on androgen signaling pathways and cell growth.

Our studies suggest that enhancement of detoxification is another mechanism that underlies the chemopreventive effects of MSA. MSA up-regulates mRNA levels of several phase 2 enzymes, including EPHX1, NQO1, NAT2, and members of the UGTB family, as well as the enzymatic activity of NQO1. We have observed similar induction of NQO1 enzymatic activity in LNCaP cells treated with sodium selenite and selenium dioxide (Brooks *et al*., 2002), demonstrating that several forms of selenium are capable of inducing phase 2 enzymatic activity in prostate cells. Induction of phase 2 enzymatic activity has been proposed as a promising avenue of prostate cancer prevention after the discovery that virtually all human prostate cancers and precursor lesions (PIN) lose expression of the phase 2 enzyme glutathione S-transferase π (GSTP1) (DePrimo *et al*., 2001; Nelson *et al*., 2001). Global induction of phase 2 enzymes by selenium compounds might compensate for the loss of GSTP1 expression that occurs early in prostate carcinogenesis thereby and protect vulnerable prostatic epithelial cells against genome damage.

In summary, we have characterized the global transcriptional response program of LNCaP to MSA. The expression changes we observed imply that MSA exerts its anticancer activity through diverse mechanisms, including inhibition of cell proliferation, modulation of the expression of AR and its regulated genes, and induction of enzymes involved in carcinogen detoxification. Therefore, this data set provides a potential resource for understanding the modes of action of

MSA and serves as a source for candidate biomarkers of selenium's effects that could be measured in vivo. Discovery of such markers could help in the design and interpretation of selenium intervention trials currently in progress.

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