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Effects of Selenomethionine on the Gene Expression Profile of Cloned Human Prostate Cancer Cells Representing a Phenotypic Continuum of Cancer Progression

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Abstract

We have previously characterized three cell clones that were derived by limiting dilution from a human prostate cancer cell line (LNCaP) representing a phenotypic continuum of cancer progression (1). The present study was undertaken to examine the effects of L-selenomethionine (SeM), a clinically approved cancer chemopreventive agent, on the gene expression profile of the cultured cell clones. Following a three day incubation period with SeM, total RNA was extracted and the gene expression profile was evaluated using Affymetrix human HG U133A microarrays and analyzed by ViaLogy's VMAxS® platform deploying quantum resonance interferometry (QRI) processing. The differentially expressed genes and corresponding biological processes were compared across the different treatments and cells types. Whereas SeM significantly affected RNA-DNA metabolism, and protein transport and metabolism in all of the cell types evaluated, other effects by SeM were observed only in certain cell clones.

Introduction

Prostate cancer is the most common male malignancy and the second leading cause of male cancer death in the United States (2). Current clinical cancer prevention trials focus on the use of L-selenomethionine (SeM) and vitamin E for the prevention of prostate cancer (3). Since prevention trials are largely anticipatory wherein ostensibly normal individuals are treated, it is prudent to gain insight about the basic mechanisms that underlie prostate cancer progression and the effects of clinically validated chemopreventive agents such as SeM on such processes. We propose that the establishment of a phenotypic continuum for cancer progression through clonal selection of parental tumor cell lines is likely to address aspects of drug response at various stages of cancer development. During a previous study, therefore, a series of LNCaP-derived cell clones were established and characterized in terms of cell population doubling time, hormonal dependency, morphology and/or the ability to form colonies in soft agar (1). Among the selected LNCaP cell clones, the most "normal" phenotype was observed in clone 21, which grew as a monolayer on tissue culture dishes, did not form colonies in soft agar, and was highly-hormone sensitive. The most malignant phenotype was observed in clone 6, which grew as densely packed multilayered patches on tissue culture dishes, readily formed colonies in soft agar and was less sensitive to hormone-deprivation. Clone 17 appeared intermediate, and mainly grew as densely packed multilayered patches on tissue culture dishes, formed colonies in soft agar with moderate efficiency and retained hormone-sensitivity. Here we determine the effects of SeM on gene expression patterns in the three LNCaP cell clones, as well as in the parental cell line. The

differences in gene expression profiles and response to SeM treatment among the three LNCaP cell clones and the parental cell line were also evaluated. Thus, the present study assesses the effects of SeM on gene expression as a function of tumor cell heterogeneity, using cell clones that represent the phenotypic continuum of prostate cancer progression. This approach, we feel, is likely to provide considerable insight and better strategies for the use of SeM in future clinical prevention trials.

Materials and Methods

Cells and cell treatment with SeM

Several LNCaP cell clones with different phenotypic characteristics were established using a limiting dilution approach, as described elsewhere (1). Parental LNCaP cells and three LNCaP cell clones (Clones 6, 17 and 21) were chosen for the present study based on the differences of these clones in anchorage-independent growth capability.

Cells of each clone and the parental line were plated in T-75 flasks. On day-1 of the experiments, the medium was changed and fresh plain medium (Control) or medium containing 5 μ M SeM was added to the cell cultures. The cells were cultured for an additional three days, and then harvested on day-4 for RNA isolation.

RNA extraction and cDNA synthesis

RNA was prepared using the Totally RNA™ kit (Ambion, Austin, TX). The precipitated RNA pellets were resuspended in DEPC (0.1% diethylpyrocarbonate)-treated water and stored frozen at -70 °C before use. The RNA samples were thawed on ice, vortexed and an aliquot was diluted in 10 mM Tris, pH 7.5 for quantitation on a GeneQuant™ spectrophotometer (Amersham BioSciences, Piscataway, NJ). The quality of RNA preparation was assessed by the ratio of absorbance readings at wavelengths of 260 and 280 nm followed by visualization of ribosomal 28S and 18S bands either on a 1% agarose gel with ethidium bromide staining or by using an Agilent 2100 BioAnalyzer™ (Agilent, Palo Alto, CA). RNA samples having a 260/280 ratio less than 1.8 were processed with RNAeasy™ columns (Qiagen, Valencia, CA) and an aliquot was re-assessed for quality prior to being used for cDNA synthesis reactions.

The target cDNA was prepared using 10 μ g of total RNA. First strand cDNA was synthesized using Superscript II™ Reverse Transcriptase (Life Technologies, Bethesda, MD) and a T7-(dT)24 primer (IDT, Coralville, IA) to incorporate the T7 priming site into the cDNA. Following RNA degradation with RNase H and the second strand cDNA synthesis with DNA polymerase I, the resulting double-stranded cDNA was extracted with phenol, chloroform and isolamyl alcohol (at a volume ratio of 25:24:1). For some samples, cDNA cleanup was performed using the Affymetrix Sample Cleanup Module.

cRNA labeling and hybridization

Approximately 1 μ g of cDNA was used as a template for each in vitro transcription assay reaction (Enzo Biochem, New York, NY), which incorporated biotin into the resulting cRNA. cRNA was purified using either Qiagen RNAeasy™ columns or the Affymetrix Sample Cleanup Module. The cRNA was fragmented to a size range of 35–200 bases by incubation at 94 °C for 35 minutes in fragmentation buffer (40 mM Tris, acetate, pH 8.1, 125 mM KOAc, 30 mM MgOAc) prior to use in hybridization. Fifteen μ g of fragmented probe was mixed with the Gene Microarray Eukaryotic Hybridization Controls, herring sperm DNA, and acetylated bovine serum albumin (BSA) in hybridization buffer (100 mM MES; 1 M [Na⁺]; 20 mM EDTA; 0.01% Tween 20). The hybridization mixture was heated at 99 °C for five minutes, incubated at 45 °C for five minutes and then centrifuged at 13,000

x g for 5 minutes. Test microarrays were prehybridized with 200 μ l of 1X hybridization buffer for 10 minutes at 45 °C and 60 RPM in the hybridization oven. Following the removal of prehybridization buffer, the microarrays were filled with 200 μ l of the hybridization mixture and incubated at 45 °C and 60 RPM for 16 hours.

For each cell clone–treatment combination, a total of three Affymetrix HG U133A microarrays were used. Two of these were technical replicates, in which the same target preparation was split between the two microarrays for hybridization. The third microarray was an independent biological experimental replicate, in which the RNA sample from a separate cell culture experiment was extracted, labeled and hybridized onto the microarray.

Washing, staining and scanning

After the hybridization reaction, the hybridization mixture was removed and saved, and the microarray was filled with 250 μ l of non-stringent wash buffer [6X SSPE (1X SSPE contains 0.18 M NaCl, 10 mM NaH₂PO₄, 1 mM EDTA at pH 7.7) and 0.01% Tween 20]. Further washing and staining of the microarrays was conducted on the fluidics station using sequentially the non-stringent washing buffer, stringent washing buffer (100 mM MES, 0.1 M [Na⁺] and 0.01% Tween 20), and stain buffer (100 mM MES, 1 M [Na⁺] and 0.05% Tween 20) containing 2 mg/ml acetylated BSA and 10 μ g /ml of streptavidin phycoerythrin (SAPE). The signal was amplified by additional treatment with stain buffer (100 mM MES; 1 M [Na⁺] and 0.05% Tween 20) containing acetylated BSA (2 mg/ml), goat IgG (0.1 mg/ml), biotinylated antibody (3 μ g/ml) and a second staining with SAPE.

Each HG U133A microarray was scanned twice at 570 nm using an Agilent confocal scanner. The output fluorescence was obtained using the Affymetrix Microarray Analysis Suite (MAS) 5.0 software and the average of the two scans was used to produce an image file for further data analysis.

Signal processing and Quality Control

The Affymetrix human HG U133A image (DAT) files were processed using the VMAxS® microarray analysis service from ViaLog, Altadena, CA. VMAxS processing entails determination of presence as well as an absolute expression value for each probe's feature cell on the HG U133A microarray using QRI active signal processing algorithm. The detected and quantitated individual feature-level expression values are then combined to quantitate a gene-level expression for each probe set on the HG U133A microarray. If a critical mass of features is not detected, the gene is called absent and an expression value of zero is generated. Quality Control was performed initially using global and quantile normalizations across all microarrays. Global normalizations were tested for scaling factors to ensure microarray-to-microarray comparability of overall signal strength. Quantile normalized data were used to generate M (log intensity ratios) versus A (average log intensities) plots to test for the presence of any significant technical problems or anomalies on any of the microarrays. In the case of the individual comparisons, quantile normalizations were performed only across the data being compared in individual small groups. The M versus A plot showed that the difference between the results of the third microarray, which represents an independent biological experimental replicate, and the first and second microarrays, which are technical hybridization replicates using the same target preparation for hybridization, are larger than the difference between the results of the first and second microarrays (data not shown). This is typical of microarray experiments on high density expression arrays due to variation in labeling and hybridization efficacy. Some of this variability can be attributed to variations in starting RNA pooled from independent runs of cell culture experiments.

Pre-processing

For comparison of SeM treatment with the Control treatment, quantile normalization was performed across the six microarrays (three for the SeM treatment and three for the Control treatment), and M versus A plots were generated to ensure even distribution of gene intensities. If a gene is determined by VMAxS® to be absent, it is assigned an expression value of '0'. As many post-analysis algorithms using fold change do not work well with values of zero, nor with the zero values modified to a finite low number, genes with one or more zero values across the compared arrays were analyzed using an alternative method, as described below.

Determination of differentially expressed genes

The data were analyzed using three algorithms: 1) SAM as described in Tusher et al (4), 2) Cyber-T as described in Long et al (5), and 3) J-score, an algorithm which ranks genes based on the following statistic:

$$j\text{-score} = \frac{\bar{r}_i \sqrt{n_i}}{s_i + 0.5}$$

This formula is loosely based on the SAM d-score. For each gene, the natural logarithm of the ratio of gene expression was computed for each of the three untreated samples versus each of the three treated samples. In the above expression, n_i is the number of log2 ratios computed for gene_i, where r_i is the absolute value of the mean of the log2 ratios, and s_i denotes the standard deviation of the n_i log2-ratios.

Further processing was conducted to minimize significance bias due to any false positives and false negatives. Results from the three above methods were compiled separately in three lists. Subsequently, the three independent scores were combined to enforce a more stringent significance control during the downstream analysis. The presumptive differentially expressed genes were compared across the three algorithms using a simple ranking scheme. The three individual rankings generated by each algorithm were averaged to generate a single rank, with one exception. When a gene was ranked above a thousand in any of the individual rankings (less significant), the ranking number used to calculate the overall ranking average was kept constant at a thousand. This was implemented to avoid unfairly punishing a gene that for some reason had a bad ranking in one system.

For genes that had one or more absent calls (i.e., they had the intensity value of zero), different rules were applied relating to the number of zero values in each set of replicates, in connection with the fold change observed for the non-zero values. This procedure generated a second list of potential differentially expressed genes.

The differentially expressed genes after SeM treatment, as determined by their final ranking values, were compared across the four cell lines to identify genes affected by the same treatment, and also to identify genes that were affected in a particular cell line, but not in the others.

Mapping to biological processes

Lists of differentially expressed genes were generated and analyzed for the preponderance of genes associated with certain biological processes or molecular functions. The lists, which included both up- and down-regulated genes, were examined using EASE, the Expression Analysis Systematic Explorer from the National Institute of Allergy and Infectious Diseases at the National Institute of Health (6). EASE Online is available at <http://>

apps1.niaid.nih.gov/david/upload.asp. Ranked lists according to EASE score were generated, and the resulting biological processes were compared across treatments and cell lines, as above, to determine both shared and differentially affected processes.

Analysis of impact of SeM treatment on gene expression

The impact of SeM treatment on gene expression was determined by regression analysis using the level of gene expression in the control cells as the independent variable and the level of gene expression in the SeM-treated cells as the dependent variable. A separate regression analysis was performed for each of the three LNCaP cell clones and the parental line. Absent genes (genes with absent calls) were not included in the regression analysis since the inclusion of a large number of data pairs with a numeric value of zero would have forced the regression line through zero, thereby skewing the regression line for genes that were expressed.

Comparison of gene expression and SeM response among three LNCaP cells clones and the parental line

The gene expression profile was compared between the three LNCaP cell clones and the parental lines by regression analysis using the level of gene expression in one LNCaP cell clone or the parental line as the independent variable and the level of gene expression in another LNCaP cell clone or the parental line as the dependent variable. A separate regression analysis was performed for each pair of LNCaP cell clones and the parental line. Absent genes were again excluded in the regression analysis to avoid skewing the regression line for genes that were expressed.

The SeM response for each gene was defined as the difference in the gene expression levels between the control cells and the SeM-treated cells. The SeM responses were also compared between the three LNCaP cell clones and the parental lines by regression analysis as described above, and a separate regression analysis was performed for each pair of LNCaP cell clones and the parental line.

Results

The results of these studies have been described in detail in a Vialogy Technical Report (7).

Similarly and differentially expressed genes in cells treated with SeM

To identify genes that have a similar response to the SeM treatment regardless of the cloned cell line, and to detect genes exhibiting differences among clones undergoing the same SeM treatment, the ranked gene lists were compared across the three different cloned cell lines and the parental cell line. The results showed that there were 170 genes that exhibited similar responses (up or down regulation) to the SeM treatment in all three LNCaP cell clones and the parental line (Table 1, the last row). In contrast, the number of genes which responded to SeM treatment in one clone or parental cell line, but not in other clones or the parental cell line, ranged from 555 to 848 (Table 1, the first 4 rows).

Among the three LNCaP cell clones compared, Clone 21 shared 215 and 144 genes with Clones 6 and 17, respectively, with common responses to the SeM treatment. In contrast, Clones 6 and 17 only shared 88 genes with common responses to the SeM treatment. These results indicate that Clones 6 and 17 were each more similar to Clone 21 than to each other in the response to SeM treatment.

Similarities and differences between biological processes involved in different cell clones subjected to the SeM treatment

In addition to analyzing individual gene expression levels, entire biological processes implicated in SeM treatment of the cloned cell lines were also investigated. For each combination of cell clone and treatment, the list of significantly regulated genes (up and down regulated genes combined) was used to deduce the biological processes affected by the treatment. This analysis was conducted using EASE (6), which determines whether there are biological processes with significantly more genes regulated than expected at random. As the EASE listing also includes several non-significant processes in its output, hits with an EASE score higher than 0.2 were discarded as being non-significant. The number of processes differentially affected in one, two or three LNCaP cell clones or the parental line (Table 2, the first 14 rows) was generally not more than the number of processes commonly affected in all three LNCaP cell clones and the parental line (Table 2, the last row). This is in contrast to the results at the gene level, as described above (Table 1), indicating that a particular process may not always be regulated by the same set of genes, but different genes can lead to the same effect.

SeM Treatment had highly significant effects on some processes in all of the different cells lines examined. Specifically these processes were RNA-DNA metabolism, and protein transport and metabolism, as indicated in Table 3. Other effects of SeM were observed only in certain cell lines. Treatment with SeM was shown to alter the expression of many genes that belong to the processes designated as Cell cycle, Growth and Differentiation in Clone 6 but not in the other two clones (Table 3). Since Clone 6 is the most malignant among the three LNCaP clones, the results imply that the treatment with SeM may selectively control growth and differentiation in more advanced cancer cells. Treatment with SeM also appeared to regulate programmed cell death in Clones 6 and 21 but not in Clone 17 (Table 3). The significant effect of SeM on apoptosis was registered in four sub-processes for Clone 6 but only in one subprocess for Clone 21 (Table 4).

Impact of SeM treatment on gene expression levels in the LNCaP cell clones and parental cells

To further evaluate the significance of SeM induced changes in gene expression, the gene expression levels were compared among the three LNCaP lines and the parental line by regression analysis. The results indicate that there were 1590 genes differentially expressed in Clone 6 with and without SeM treatment, and 426, 650 and 646 of them were also differentially expressed in Clone 17, Clone 21 and the parental line, respectively (Table 5). For these genes, the basal level of expression (in Control cells) was highly correlated with a nearly perfect linear fit ($r^2 = 0.97$). Similar results were also observed in all other pairs of LNCaP cell clones or the parental line compared. These results indicate that the expression of the genes differentially expressed in at least two LNCaP cell clones or the parental line with and without SeM treatment was highly conserved among the three LNCaP cell clones and the parental line.

The impact of SeM treatment on gene expression was evaluated by regression analysis of levels of gene expression in SeM-treated cells and the control cells in each of the three LNCaP cell clones and the parental line. For each cell clone and parental line, a separate regression analysis was performed for genes that were up-regulated by the SeM treatment and genes that were down-regulated by SeM treatment. The results demonstrate a highly linear correlation between the levels of gene expression in the SeM-treated cells and control cells in each LNCaP cell clone and the parental line (Figure 1). The slope value of the regression line, which represents the ratio of the expression levels of genes in the SeM-treated cells to the expression levels of corresponding genes in the control cells, was

compared among the three LNCaP cell clones and the parental line to determine the effect of SeM treatment in these cells. For genes that are up-regulated by the SeM treatment, the slope value of the regression line was 1.18, 1.09, 1.14, and 1.22 for Clones 6, 17, 21 and the parental line, respectively. These results indicate that SeM treatment up-regulated the expression of these genes by 18%, 9%, 14% and 22% in Clones 6, 17, 21 and the parental line (a slope value of 1.00 is expected if there is no change in the gene expression level after SeM treatment), and the differences among the three LNCaP cell clones and the parental line are statistically significant ($p < 0.01$ by Tukey-Kramer multiple comparison test).

For genes that are down-regulated by SeM treatment, the slope value of the regression line was 0.86, 0.92, 0.87, and 0.85 for Clones 6, 17, 21 and the parental line, respectively. These results indicate that SeM treatment down-regulated the expression of these genes by 14%, 8%, 13% and 15% in Clones 6, 17, 21 and the parental line. The magnitude of down-regulation is significantly smaller in Clone 17 than in the other two clones or the parental line ($p < 0.01$ by Tukey-Kramer multiple comparison test) whereas the differences among Clones 6, 21 and the parental line are not statistically significant ($p > 0.05$).

The SeM responses were also compared among the three LNCaP clones and the parental line by regression analysis (Table 5). The strongest correlation was observed between Clone 21 and Clone 6, Clone 17 or the parental line ($r^2 = 0.90$) and between Clone 6 and the parental line ($r^2 = 0.90$). Slightly less strong correlation was observed between Clone 6 and Clone 17 ($r^2 = 0.84$) and between Clone 17 and the parental line ($r^2 = 0.74$). In general, the SeM response was conserved to a lesser degree as compared to the basal gene expression levels among the three LNCaP cell clones and the parental line.

Differentially expressed genes in three LNCaP cell clones and parental LNCaP cells

To investigate whether the LNCaP cell clones and the parental cells have different gene expression patterns, the gene expression levels were compared among the three clones and the parental cells, which presumably represent a mixture of ancestors of the cloned cells as well as other cells not yet cloned and characterized. As Clone 21 most closely resembles normal prostate epithelium, for purposes of this study its gene expression levels were assumed to represent a gene expression baseline in the normal prostate epithelium.

Gene expression levels were compared in clones 6, 17 and the parental cell line to the gene expression levels in clone 21 to assess whether the different phenotypes observed in these cell lines would reflect the difference in gene expression. When compared to clone 21, clones 6, 17 and parental cells exhibited differential expression in 1600, 1654 and 1625 genes, respectively. These numbers represent about 7 % of the total number of genes present on the HG U133A microarray. Most of the fold changes were small but 649, 446 and 685 genes were different by 50 % or more for Clones 6, 17 and parental cells, respectively, as compared to Clone 21.

Among the 1600, 1654 and 1625 genes differentially expressed in Clone 6, 17 or the parental cells as compared to Clone 21, 119 genes were common to Clones 6, 17 and the parental cells. Since they may represent commonly regulated genes in malignant transformed LNCaP cells, these 119 genes were further analyzed using the Functional annotation tool by the Database for Annotation, Visualization and Integrated Discovery (DAVID, <http://niaid.abcc.ncifcrf.gov>). Three functional annotations, that are significantly represented in the list of these 119 genes, contain genes that are potentially responsible for the phenotypes observed in clones 6, 17 and the parental cell line: “Cell organization and biogenesis” is represented by 17 genes (Table 6), “Establishment of localization” is represented by 28 genes (Table 7) and “Transmembrane” is represented by 33 genes (Table 8). These results indicate that LNCaP parental cells and clones with phenotypes related to

malignant potential do exhibit significant differences in gene expression levels as compared to Clone 21, which resemble the “normal” phenotype of the prostate cells. These genes, which are common to LNCaP clones with phenotypes related to malignant potential and parental LNCaP cells, are a good resource for further exploration of causes and effects of malignant transformation.

Discussion

In the present study, gene expression profiling was used to evaluate human LNCaP prostate cancer cells and three clones treated with SeM. These cloned cell lines were chosen to mimic different stages of prostate carcinogenesis. It was proposed that studying the similarities and differences in gene expression profiles in these cloned LNCaP cells may lead to discovery of potential new markers and targets, as well as the mechanisms of SeM treatment effects in the cells at different stages of prostate carcinogenesis. The genes involved in RNA-DNA metabolism, and protein transport and metabolism were affected by SeM in all of the different cell lines evaluated. Other effects of SeM were observed only in certain cell lines. Genes involved in Cell cycle, Growth and Differentiation were found to be significantly affected by SeM in Clone 6 but not in the other two clones. This finding allows speculation that *in vivo* treatment with SeM may control the growth and differentiation of advanced cancer cells while not affecting the more normal cells. In addition, treatment with SeM seems to have profound effect on programmed cell death in Clone 6 but not in the other clones, giving evidence that SeM may selectively induce apoptosis in malignant cells while sparing the non-transformed or more normal cells. The fact that Clone 6 is the most likely candidate among all the cell lines to undergo apoptosis when treated with SeM was corroborated by EASE analysis.

The magnitude of the effects of SeM treatment on gene expression was evaluated in each of the three LNCaP cell clones and the parental line by linear regression analysis. When the genes in each LNCaP cell clone or the parental line were separated into two groups based on the direction of response to SeM treatment (increased or decreased gene expression in response to SeM treatment) and examined separately by linear regression analysis, the largest magnitude of gene expression change was found in the parental LNCaP cells, in which genes responded positively to the SeM treatment with an increased expression by an average of 22% (compared to the baseline expression level) whereas genes which responded negatively to the SeM treatment decreased expression by an average of 15%. Among the three LNCaP cell clones, the magnitude of the gene expression change in response to the SeM treatment was the largest in Clone 6 (18% increase and 14% decrease) and the smallest in Clone 17 (9% increase and 8% decrease).

To evaluate whether the baseline gene expression profile of each cloned cell line and parental cell line could explain the difference in the magnitude of response to SeM treatment, pairwise comparison of SeM regulated genes in LNCaP cell clones and the parental line was performed using linear regression analysis. The results indicate that for genes that were regulated by SeM in the LNCaP cell clones/the parental line compared in each pair, the baseline expression was highly conserved ($r^2 = 0.97$). It is to be noted that these genes only accounted for 25% ($426/1590 = 0.25$ for the comparison between Clones 6 and 17) to 41% ($650/1590 = 0.41$ for the comparison between Clones 6 and 21) of the genes that were differentially expressed in a particular LNCaP cell clone with and without SeM treatment. These same groups of genes, whose expression appeared to be conserved on the basal level, were further evaluated in SeM treated cell lines by pairwise comparison using linear regression analysis. The results indicate that the SeM response is less well conserved than the baseline gene expression among the three LNCaP cell clones as evidenced by the slightly lower correlation coefficients (r^2 were between 0.74 and 0.91). It is interesting to

note that SeM responses in Clones 6 and 17 were more similar to the SeM response in Clone 21 ($r^2 = 0.90$) than to each other ($r^2 = 0.84$). Given the observation that Clone 6 has the most malignant phenotype and Clone 21 had a relatively “normal” phenotype with Clone 17 in between (1), it is hypothesized that Clone 21 represents the relative normal phenotype of ancestor cells from which cells with more malignant potential, represented by Clones 6 and 17 were derived. An explanation to the dissimilar response between Clones 6 and 17 may be that these two clones do not represent cells at different stages of a linear carcinogenesis pathway but rather represent cells which diverged from common ancestor cells along different directions during carcinogenesis.

The three cloned cell lines, Clones 6, 17 and 21, as well as the parental cell line, previously evaluated in terms of morphology, proliferation characteristics, anchorage independent growth, hormone sensitivity and propensity to undergo apoptosis (1), exhibit different phenotypes. Parental cell line and clones 6 and 17 represent malignant forms of prostate epithelium while clone 21 resembles normal prostate epithelial cells. Microarray results of this study indicate that while all three cloned cell lines and the parental cell line share a number of common responses to SeM treatment, some biological processes appear to be regulated in response to SeM treatment only in some of the clones. Comparison of gene expression levels in untreated clones 6, 17 and parental line to the gene expression levels in untreated clone 21 showed differential expression of a large number of genes, 1600, 1654 and 1625 genes, respectively, indicating that the phenotypically different clones and the parental cell line are also different at the gene expression level. Further, some of these differences in basal gene expression could be causally related to the differential responses observed through the microarray analysis.

A group of 119 genes were found to be commonly expressed in the two malignant LNCaP cell clones and the parental line but differentially expressed in Clone 21, which represents a normal phenotype. To search for a unifying theme in malignantly transformed cells, these 119 genes were examined using a functional annotation tool. “Cell organization and biogenesis”, “Establishment of localization” and “Transmembrane” functional annotations were found to be significantly represented in the 119 gene list. Further research should reveal if genes listed in these three functional annotations could explain phenotypes described for these cloned cell lines, such as growth in multilayers, attachment independent growth or decreased doubling time.

The effects of SeM on biologic endpoints related to malignant potential reported previously (8) indicate that SeM had the strongest growth inhibitory effect on clone 6 cells compared to clone 17 and clone 21 cells, and for anchorage independent growth, SeM had a stronger inhibitory effect on clone 6 cells than it had on clone 17 cells (as clone 21 cells do not exhibit anchorage independent growth, the effect of SeM on clone 21 cells was not evaluated). The results for these biologic endpoints correlate well with the effects of SeM on gene expression reported in this study. In the previous study, SeM had the strongest effects on apoptosis in clone 21 cells, while not having significant effects in clone 17 or clone 6 cells. These results do not correlate well with the effects of SeM on gene expression related to apoptosis in the cell lines evaluated in this study. This discrepancy could be related to the significant increase in the expression of anti-apoptosis genes in Clone 6 cells (Table 3).

Analysis of gene expression using microarray technology has allowed the evaluation of three phenotypically different cloned cell lines and the parental LNCaP cell line on a molecular level. The data from this study indicated that the evaluated cell lines exhibit differences on the gene expression level, and that these differences could explain the different phenotypes of the cell lines. It is also possible that the observed differences in basal gene expression could be causally related to the observed differences in transcriptional

responses to SeM. The results from this study also reveal that SeM can trigger many biological processes; some of these biological processes are common for all cell lines tested (i.e., RNA-DNA metabolism and protein transport and metabolism), but some of the responses are specific and occur only in cells with a malignant phenotype (i.e., Cell cycle, Growth and Differentiation and Apoptosis). Taken together, this study has yielded a wealth of data, which will in the future serve as a basis for hypothesis derivation and further hypothesis-driven research.

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Figure 1.
Correlation of expression levels corresponding genes between control cells and SeM-treated cells.

Table 1

Quantitative analysis of genes regulated in the same fashion in different cell lines by SeM

Cell clone(s)/line with genes regulated in the same fashion by SeM treatment	Number of genes regulated significantly by SeM treatment
Parental line (not in Clones 6, 17 or 21)	687
Clone 21 (not in parental or clones 6 or 17)	680
Clone 17 (not in parental or clones 6 or 21)	848
Clone 6 (not in parental or in clones 17 or 21)	555
Clone 21 and parental line * (not in clones 6 or 17)	128
Clone 17 and parental line * (not in clones 6 or 21)	187
Clone 6 and parental line * (not in clones 17 or 21)	209
Clones 17 and 21 * (not in parental or clone 6)	144
Clones 6 and 21 * (not in parental or in clone 17)	215
Clones 6 and 17 * (not in parental or in clone 21)	88
Clones 17, 21 and parental line * (not in clone 6)	78
Clones 6, 21 and parental line * (not in clone 17)	192
Clones 6, 17 and parental line * (not in clone 21)	88
Clones 6, 17 and 21 * (not in parental line)	99
Clones 6, 17, 21 and parental line	170

* Combination of LNCaP cell clone(s) and the parental line compared for genes regulated in the same fashion (up or down regulation) by the SeM treatment.

As an example, 128 genes were found to be significantly regulated by SeM in the Parent cell line as well as cell line 21.

Table 2

Quantitative analysis of biological processes regulated in the same fashion in different cell lines by SeM

Cell clone(s)/line with biological processes regulated in the same fashion by SeM treatment	Number of biological processes regulated significantly by SeM treatment
Parental line (not in Clones 6, 17 or 21)	42
Clone 21 (not in parental or clones 6 or 17)	35
Clone 17 (not in parental or clones 6 or 21)	63
Clone 6 (not in parental or in clones 17 or 21)	55
Clone 21 and parental line [*] (not in clones 6 or 17)	6
Clone 17 and parental line [*] (not in clones 6 or 21)	23
Clone 6 and parental line [*] (not in clones 17 or 21)	15
Clones 17 and 21 [*] (not in parental or clone 6)	13
Clones 6 and 21 [*] (not in parental or in clone 17)	22
Clones 6 and 17 [*] (not in parental or in clone 21)	15
Clones 17, 21 and parental line [*] (not in clone 6)	7
Clones 6, 21 and parental line [*] (not in clone 17)	33
Clones 6, 17 and parental line [*] (not in clone 21)	22
Clones 6, 17 and 21 [*] (not in parental line)	17
Clones 6, 17, 21 and parental line	71

^{*} Combination of LNCaP cell clone(s) and the parental line compared for biological processes regulated in the same fashion by SeM.

As an example, 6 biological processes were found significantly regulated in the Parent cell line as well as cell line 21 when treated with SeM.

Table 3

Summary of biological processes involved in SeM treatment. Biological processes are grouped together into super processes. Six of these are summarized, based on their average ranking in the treatment and cell line in question.

Super Processes	Cell Line			
	Clone 6	Clone 17	Clone 21	Parental
Protein transport and metabolism	++++	++++	++++	++++
RNA-DNA metabolism	++++	+++	++++	++++
Cell Cycle/Growth/Differentiation	+++	–	–	–
Steroid metabolism	–	–	–	–
Protein kinase activity	–	–	–	–
Apoptosis	++	–	+	–

Table 4

Significance of apoptosis related processes as determined by EASE analysis of significantly regulated genes

Apoptosis related processes	EASE ranking of process significantly regulated by SeM treatment		
	Clone 6	Clone 17	Clone 21
apoptosis	69	124	58
negative regulation of apoptosis	77	295	427
apoptotic program	115	246	345
induction of apoptosis	394	160	493
positive regulation of apoptosis	390	446	526
anti-apoptosis	100	443	552
regulation of apoptosis	146	312	550
induction of apoptosis by extracellular signals	333	464	286

The EASE results were specifically searched for apoptosis related processes. The table is showing the rank results for each LNCaP cell clone after SeM treatment. Results with EASE scores better than $p < 0.05$ have been highlighted with ***bold italic*** font.

Table 5

Differences and similarities of gene expression and SeM treatment response among three clones and the parental LNCaP cells

Pair of cell clones or the parental line compared		Pairs of data	Control Cells	SeM Response
Clone 6 (1590 genes differentially expressed)	Clone 17	426	$y = 1.34 + 0.93x$ $r^2 = 0.97$	$y = 10.95 + 0.53x$ $r^2 = 0.84$
	Clone 21	650	$y = 23.34 + 0.96x$ $r^2 = 0.99$	$y = 13.02 + 0.90x$ $r^2 = 0.91$
	Parental Line	646	$y = 17.73 + 1.03x$ $r^2 = 0.99$	$y = 6.13 + 1.12x$ $r^2 = 0.90$
Clone 17 (1678 genes differentially expressed)	Clone 6	426	$y = 14.01 + 1.05x$ $r^2 = 0.97$	$y = -4.82 + 1.59x$ $r^2 = 0.84$
	Clone 21	471	$y = 25.07 + 1.04x$ $r^2 = 0.99$	$y = 7.79 + 1.43x$ $r^2 = 0.90$
	Parental Line	512	$y = 25.30 + 1.10x$ $r^2 = 0.97$	$y = 20.18 + 1.53x$ $r^2 = 0.74$
Clone 21 (1679 genes differentially expressed)	Clone 6	650	$y = -17.71 + 1.03x$ $r^2 = 0.99$	$y = -8.28 + 1.01x$ $r^2 = 0.91$
	Clone 17	471	$y = -17.03 + 0.95x$ $r^2 = 0.99$	$y = -0.14 + 0.63x$ $r^2 = 0.90$
	Parental Line	553	$y = -5.79 + 1.05x$ $r^2 = 0.99$	$y = 4.55 + 1.14x$ $r^2 = 0.90$
Parental Line (1725 genes differentially expressed)	Clone 6	646	$y = -9.00 + 0.96x$ $r^2 = 0.99$	$y = -0.14 + 0.81x$ $r^2 = 0.90$
	Clone 17	512	$y = -8.21 + 0.89x$ $r^2 = 0.97$	$y = 3.91 + 0.48x$ $r^2 = 0.74$
	Clone 21	553	$y = 11.72 + 0.94x$ $r^2 = 0.99$	$y = 2.92 + 0.79x$ $r^2 = 0.90$

Table 6

Gene list for functional annotation “Cell organization and biogenesis”.

AFFY_ID	Gene Name
216985_s_at	syntaxin 3a
217790_s_at	signal sequence receptor, gamma (translocon-associated protein gamma)
209806_at	histone 1, h2bk
209230_s_at	p8 protein (candidate of metastasis 1)
208490_x_at, 208527_x_at, 209911_x_at, 208523_x_at	histone 1, h2bd
216205_s_at	mitofusin 2
205142_x_at	ATP-binding cassette, sub-family d (ald), member 1
205436_s_at	H2A histone family, member X
201774_s_at	chromosome condensation-related smc-associated protein 1
210001_s_at	suppressor of cytokine signaling 1
206491_s_at	n-ethylmaleimide-sensitive factor attachment protein, alpha
205539_at	advillin
201005_at	cd9 antigen (p24)
209026_x_at, 211714_x_at	tubulin, beta
205423_at	adaptor-related protein complex 1, beta 1 subunit
201714_at	tubulin, gamma 1
211972_x_at	ribosomal protein, large, p0

Table 7

Gene list for functional annotation “Establishment of localization”.

AFFY_ID	Gene Name
216985_s_at	syntaxin 3a
203453_at	sodium channel, nonvoltage-gated 1 alpha
209453_at	solute carrier family 9 (sodium/hydrogen exchanger), member 1 (antiporter, Na ⁺ /H ⁺ , amiloride sensitive)
217790_s_at	signal sequence receptor, gamma (translocon-associated protein gamma)
202804_at	ATP-binding cassette, sub-family c (cfr/mrp), member 1
204981_at	imprinted polyspecific membrane transporter 1
205530_at	electron-transferring-flavoprotein dehydrogenase
200602_at, 214953_s_at	amyloid beta (a4) precursor protein (peptidase nexin-II, alzheimer disease)
216205_s_at	mitofusin 2
205142_x_at	ATP-binding cassette, sub-family d (ald), member 1
206491_s_at	n-ethylmaleimide-sensitive factor attachment protein, alpha
201005_at	cd9 antigen (p24)
205421_at	solute carrier family 22 (extraneuronal monoamine transporter), member 3
201714_at	tubulin, gamma 1
209099_x_at	jagged 1 (alagille syndrome)
201801_s_at	solute carrier family 29 (nucleoside transporters), member 1
210448_s_at	purinergic receptor p2x, ligand-gated ion channel, 5
215916_at	cholinergic receptor, nicotinic, epsilon
200030_s_at	solute carrier family 25 (mitochondrial carrier; phosphate carrier), member 3
222258_s_at	SH3-domain binding protein 4
201192_s_at	phosphatidylinositol transfer protein, alpha
209163_at	cytochrome b-561
205770_at	glutathione reductase
201556_s_at	vesicle-associated membrane protein 2 (synaptobrevin 2)
210519_s_at	NAD(P)H dehydrogenase, quinone 1
218309_at	calcium/calmodulin-dependent protein kinase II inhibitor 1
209026_x_at, 211714_x_at	tubulin, beta
205423_at	adaptor-related protein complex 1, beta 1 subunit

Table 8

Gene list for functional annotation “Transmembrane”.

AFFY_ID	Gene Name
203627_at, 203628_at	insulin-like growth factor 1 receptor
216985_s_at	syntaxin 3a
203453_at	sodium channel, nonvoltage-gated 1 alpha
204750_s_at	desmocollin 2
209453_at	solute carrier family 9 (sodium/hydrogen exchanger), member 1 (antiporter, Na ⁺ /H ⁺ , amiloride sensitive)
216687_x_at	UDP glucuronosyltransferase 2 family, polypeptide b15
217790_s_at	signal sequence receptor, gamma (translocon-associated protein gamma)
217483_at	folate hydrolase (prostate-specific membrane antigen) 1
202804_at	ATP-binding cassette, sub-family c (cftr/mrp), member 1
213094_at	g protein-coupled receptor 126
204981_at	imprinted polyspecific membrane transporter 1
202948_at	interleukin 1 receptor, type I
213716_s_at	secreted and transmembrane 1
205530_at	electron-transferring-flavoprotein dehydrogenase
200602_at, 214953_s_at	amyloid beta (a4) precursor protein (peptidase nexin-II, alzheimer disease)
205142_x_at	ATP-binding cassette, sub-family d (ald), member 1
216205_s_at	mitofusin 2
205421_at	solute carrier family 22 (extraneuronal monoamine transporter), member 3
201005_at	cd9 antigen (p24)
209099_x_at	jagged 1 (alagille syndrome)
207415_at	phospholipase a2 receptor 1, 180kDa
201801_s_at	solute carrier family 29 (nucleoside transporters), member 1
210448_s_at	purinergic receptor p2x, ligand-gated ion channel, 5
215916_at	cholinergic receptor, nicotinic, epsilon
200030_s_at	solute carrier family 25 (mitochondrial carrier; phosphate carrier), member 3
201248_s_at	sterol regulatory element binding transcription factor 2
211708_s_at	stearoyl-CoA desaturase (delta-9-desaturase)
209163_at	cytochrome b-561
221471_at	serine incorporator 3
201556_s_at	vesicle-associated membrane protein 2 (synaptobrevin 2)
202669_s_at	ephrin-b2
212070_at	g protein-coupled receptor 56
218140_x_at	signal recognition particle receptor, b subunit