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An Integrative Study of Gene Expression of PMA Induced Differentiation in HL-60 Cells by DNA Microarray Analysis

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ABSTRACT

This study seeks to elucidate the molecular mechanisms by which the acute myeloid leukemia cell line, HL-60, is induced to differentiate into macrophage-like cells following exposure to phorbol 12-myristate 13-acetate (PMA). Changes in gene expression over the time course of PMA treatment were measured by DNA microarray analysis and select genes shown to be significantly up and down-regulated were validated by quantitative real-time PCR. Functional studies of transcription factor binding activities were performed by the electrophoretic mobility shift assay (EMSA). Novel findings include the rapid and sustained induction of mRNA levels for a group of CC chemokine genes (CCL2, CCL3, CCL3L1, CCL4, CCL4L1 and CCL4L2). These genes displayed 10 to 50-fold expression level increases that were observed within 3 hours of treatment and were maintained through 6 hours post-induction. Among the most highly induced early genes were others known to encode inflammatory response proteins (TNF, IL8, C3AR1). In addition, the transcription factor profile is dramatically altered early in the differentiation time course, with the predicted activation of AP-1 family members (JUNB, FOSL1) and the concomitant inhibition of NFKB (TNFAIP3, NFKBIA, NFKBID). c-Myc expression was significantly down regulated and remained low during the differentiation course. Early events in the genetic re-programming of HL-60 cells in response to the differentiation inducer PMA includes a massive up-regulation of chemokine genes and alteration of the transcription factor profile. The implication of these gene expression changes will be discussed in the context of differentiation therapies for acute myeloid leukemia.

INTRODUCTION

In 2009 there were approximately 9,000 deaths from Acute Myeloid Leukemia (AML) in the United States (American Cancer Society 2010). About 12,810 new cases were reported and the lifetime risk of developing AML is slightly higher for men than women, 1 in 250 and 1 in 300 respectively (American Cancer Society 2010). AML is a cancer of the blood and bone marrow cells, which progresses quickly and can be fatal if left untreated. Normally, the bone marrow produces stem cells that can mature into myeloid or lymphoid stem cells. In AML, the myeloid stem cells develop into myeloblasts, which do not become functional white blood cells and instead divide uncontrollably (National Cancer Institute 2009). Life expectancy has increased slightly for this disease, but the improvements are mostly due to supportive care, rather than drugs or new treatments (Ley *et al*. 2008). This research attempts to answer the question, "What is the difference between the leukemia cell genetic program and the "normal" myeloid cell genetic program?"

The human leukemia cell line, HL-60, was used as a model system for the study of genetic alterations associated with the development of myeloid leukemias. Dr R.C. Gallo's group at the National Institute of Health derived the HL-60 cell line from the bone marrow of a patient with AML about 30 years ago (Collins 1987, Tefferi *et al*. 2002). The HL-60 cells resemble blast cells because they are believed to be the cancerous derivatives of committed progenitors of granulocytes (Harris and Ralph 1985). Three key features contribute to this cell line's usefulness as a model system – proliferation, differentiation, and gene expression (Collins 1987). HL-60 cells continuously divide in culture with a doubling time of ~36 to 48 hours (Collins 1987). The ability to maintain HL-60 cells in culture is an unusual characteristic, which normal human myeloid leukemia cells do not typically possess. The normal human myeloid leukemia cells

typically undergo a limited number of cell divisions before growth arrest and cell death (Collins 1987). The histological origin of a cell line produces a specific gene expression pattern, which can then be used to compare gene expression patterns across various leukemia cell lines (Ross *et al*. 2000).

Differentiation occurs when cells commit to forming one specific type of tissue. The same set of genes is retained, but specific programs or gene networks are turned on or off depending on the cell type (Weinburg 2007). The HL-60 cells can be induced to differentiate *in vitro* into specialized blood cell types including granulocytes, monocytes, macrophage-like cells, and eosinophils (Collins 1987, Rovera *et al*. 1979). These differentiation pathways can be activated by a variety of inducing agents, which change the genetic program of the cells and cause them to exhibit a differentiated myeloid cell phenotype similar to normal myeloid stem cell differentiation *in vivo* (Collins 1987, Deeds *et al.* 2009) (Figure 1).

This project focuses on differentiating the HL-60 cells into the macrophage-like cells using the phorbol ester inducer 12-myristate 13-acetate (PMA). Several phenotypic characteristics clearly distinguish the HL-60 macrophage-like differentiated cell type from the HL-60 untreated cancer cell including adherence to surfaces, rapid loss of proliferative capacity, and eventually apoptosis (Deeds *et al.* 2009). Additionally, the expression of myelomonocytic enzymes such as myeloperoxidase, acid phosphatase, granulocyte and lysozyme, can also be observed in differentiated HL-60 cells (Harris and Ralph 1985). These phenotypic changes are due to changes in gene expression at the transcription level. HL-60 cells therefore serve as model system for the study of cell differentiation, oncogenesis and cancer "differentiation" therapies (Deeds *et al.* 2009).

There is considerable evidence that the receptor for PMA in HL-60 cells is protein kinase C because PMA can substitute for diacylglycerol to activate PKC irreversibly (Collins 1987, Seo *et al*. 2000). It is believed that activation of protein kinase C plays a role in the differentiation of HL-60 cells into the macrophage-like phenotype, but the specific genetic mechanisms by which PMA acts to differentiate cancer cells are still undefined (Collins 1987). This research applies a genome-wide analysis of PMA-induced differentiation, growth arrest, and apoptosis in HL-60 cells through cDNA microarray technology. DNA microarrays measure gene expression changes on a global level in order to identify key genes and pathways involved in the differentiation program in the HL-60 cells. DNA microarray gene expression studies between HL-60 cells and normal cells after differentiation have been previously performed (Seo *et al*. 2000, Zheng *et al*. 2002). These studies provide valuable groundwork for comparison, but did not ultimately characterize the most significant genes involved in the reprogramming of the HL-60 cell along the differentiation pathway.

Microarray technology involves extracting total RNA from target cells in both control and experimental conditions and making single-stranded cDNA copies. The data represents a snapshot of the genes actively expressed in the cell at the moment of isolation. The cDNA is then bound to a collection of spots containing synthetic oligonucleotides, representing a specific gene, attached to a small microscope slide in an ordered arrangement (Tefferi *et al*. 2002). The array measures expression levels of all the genes in a genome simultaneously. Red and green fluorochrome dyes are bound to the isolated cDNA, which was previously bound to the microarray slide (one dye to the control and one to the experimental condition). A microarray scanner can then be used to fluoresce the spots yielding quantitative measures of expression levels for ~ 37,000 transcribed sequences (Deeds *et al.* 2009) (Figure 2). A high or low red/green

ratio indicates different levels of expression for genes in the control and experimental conditions. Microarray studies are not just applicable to the study of cancer and are instead used in various fields including virology, botany, and evolutionary biology.

Microarrays study the gene expression levels of the entire cell, which provides the potential to identify previously unknown factors or genes involved in the development of AML. Before the advancement of this form of technology, researchers would have to be informed of which target gene they wanted to study in relation to the particular type of cancer. Microarrays allow researchers to essentially "throw out a net and see what comes back" without making a prior hypothesis as to which genes are essential. Additionally, these genome-wide studies can confirm and credit previous biochemical and genetic studies.

There are some practical limitations to microarray analysis, which are important in understanding the implications of the resulting data. Many proteins go through post-translational modifications, which can alter both the structure and function of the molecule. Since microarrays only investigate the gene expression levels (mRNA transcripts) the effect of modifications are not assessed and levels of the protein are not directly measured. However, there is a general correlation between the expression levels of a given gene and the levels of functional protein within the cell of that particular gene. This having been said, the data must be validated through follow-up assessments and functional assays, in order to ask questions for further research.

One method used to confirm the microarray data in this study is quantitative real time polymerase chain reactions (qRT-PCR). The goal of the qRT-PCR experiments is to directly compare the expression levels of a specific gene of interest between two separate populations. For this reason it makes sense to use the relative quantification (comparative C_T) method, which compares the expression between two populations. In this method, the amount of gene initially

present is determined by finding the cycle number at which the detected dye begins to elevate into log phase. The cycle number for this point is known as the threshold (C_T) (Figure 3).

When studying the amplification plot of two genes, a threshold at an earlier cycle determines which of the two samples is in higher quantity. It is important to establish that the two samples have the same amount of total cDNA loaded in each experiment so that the difference in expression are attributed solely to differences in the gene rather than the amount of cDNA in the sample. For this reason, it is necessary to run a reaction for an endogenous control gene of which equal expression across the two cell populations is expected. Comparing the thresholds for the two endogenous controls samples establishes whether the same amount of cDNA was loaded into each well. This method is referred to as the $\Delta\Delta C_T$ method because two ΔC_T values are calculated, the change between the two endogenous control samples and the change between the target gene in the two cell populations. These precise fold change values for each gene of interest in each sample can be directly compared to the fold changes obtained in the microarray experiment (Applied Biosystems 2010).

Electrophoretic Mobility Shift Assay (EMSA) is a functional assay involving a transcription factor binding to its target regulatory sequence. Nuclear protein extracts can be prepared to look for a particular transcriptional regulator of interest. Oligonucleotides containing the transcription binding site of interest are radio-labeled and incubated with the nuclear extracts. Complexes of specific transcription factors bound to their recognition sequences can be resolved using low ionic strength polyacrylamide gel electrophoresis (Chodish *et al*. 1986). In this way, quantitative measures of active (DNA bound) transcription factor activity can be made (Chodish *et al*. 1986) (Figure 4).

The large data sets produced by the microarray experiments require mathematical tools of data analysis to generate useful information. Hierarchical Cluster Analysis is one method used to organize large data sets for efficient retrieval of information. Ideally, individual cluster groupings display homogeneity while different clusters display separation (Forrester 2009). Since the DNA microarrays produce data on 40,000 genes in one experiment, cluster analysis is an ideal mathematical method for grouping similar and dissimilar genes in order to get the most information from the data collected. The first step in the cluster analysis is to conduct a distance measurement. Distance measurement allows you to take two objects and to produce a value relating the objects. There are several different types of metrics used including Euclidean, Manhattan, and Minkowski distances, which can be chosen depending on the data set (Forrester 2009). The next step is to cluster the objects either using agglomerative or divisive methods. The agglomerative method is most frequently used because it fuses the nearest points or groups together to form each cluster based on a linkage type such as single, complete, average, and ward linkages (Forrester 2009). A dendrogram is a graphical representation of the cluster procedure displayed as a tree diagram.

Using the above methods, this research project attempts to determine how the AML genetic program is converted into a normal macrophage-like, genetic program during differentiation of the human acute myeloid leukemia cell line HL-60 in the presence of PMA. It is hypothesized that signaling networks involving protein kinase C (PKC) are activated when the HL-60 cells are treated with PMA (Deeds *et al.* 2009). These networks then transduce signals via activation or deactivation of specific transcriptional regulators that direct changes in gene expression and affect cell cycle regulation and differentiation (Deeds *et al.* 2009).

In DNA microarray experiments, total RNA will be isolated from HL-60 cells treated with PMA and from untreated HL-60 cells to allow comparison of the gene expression levels at various time points. This will determine what genes are up and down-regulated in response to treatment with PMA. Quantitative RT-PCR will be used to validate the microarray data and to amplify and quantify a particular gene of interest. Total nuclear protein extracts from non-treated or PMA-treated HL-60 cells over the same PMA time course as the DNA microarrays will be isolated for EMSA experiments in order to quantify the presence of functional transcription factor binding.

MATERIAL AND METHODS

Cell Culture

HL-60 cells were cultured in media supplemented with 10% fetal bovine serum. Cultured cells were grown at 37°C in a humidified atmosphere of 5% CO_2 and were monitored to maintain a cell density under 2 x 10^6 cells/mL with a doubling time of ~36 hours. For the differentiation assay, HL-60 cells were seeded at a density of approximately 2 x 10^5 cells/mL, expect about 85% viable, and treated with 16 nM PMA for various time points up to 24 hours.

RNA Isolation

Cells treated with PMA at various time points were harvested by pouring the cell culture into 50 mL conical tubes, spinning for 10 minutes at 2,000 rpm, and aspirating the media. The Sigma Genelute total RNA isolation kit was used for the following procedure. Cell pellets were immediately immersed and resuspended in 500 mL Lysis Solution comprised of 10 μL 2-mercaptoethanol per 1 mL lysis buffer for each mL of cell culture. The cell lysate was transferred to a GenElute Filtration Column and centrifuged at 12,200 rpm for 2 minutes. The

filtered lysate was treated with 500 μL of 70% ethanol and a 500 μL aliquot of the lysate-ethanol mixture was transferred to a GenElute Binding Column and centrifuged at 12,200 rpm for 15 seconds. The flow-through was discarded and the remaining 500 μL of the lysate-ethanol mixture was applied to the column and centrifuged for an additional 15 seconds. The flow-through was discarded and 500 μL Wash Solution 1 was added to the column, and centrifuged at 12,000 rpm for 15 seconds. The column was transferred to a new collection tube and 500 μL of Wash Solution 2 was added, centrifuged at 12,000 rpm for 15 seconds. The flow-through was discarded and an additional 500 μL Wash Solution 2 was added and centrifuged for 2 minutes. This binding column was transferred to a new 2 mL collection tube and 50 μL of Elution Solution was added to the column and centrifuged at 12,200 rpm for 1 minute. An additional 50 μL Elution Solution was applied to the binding column, centrifuged, and the purified RNA was immediately stored on ice.

DNA Microarray Analysis of Expression Levels

Genisphere 3DNA 900 Kit protocol for microarray analysis was adapted for this experiment. This protocol along with the MAGICTool software are available online at the Genome Consortium for Active Teaching (GCAT) webpage (http://www.bio.davidson.edu/projects/GCAT/gcat.html). This protocol is designed for two microarray slides that allow for a dye reversal experiment that is completed on a separate microarray slide.

cDNA Synthesis Protocol

The isolated total RNA from HL-60 cells was converted to cDNA using the Genisphere 3DNA Array 900 kit. For each RNA sample, two populations of cDNA are synthesized, one

using the Cy3 RT Primer (green) and one using the Cy5 RT Primer (red). The four RNA-RT primer mixes are assembled in 0.5 mL PCR tubes as follows:

Tube 1: 2.5 μg RNA 0 hr, 1 μL Cy3
Tube 2: 2.5 μg RNA 0 hr, 1 μL Cy5
Tube 3: 2.5 μg RNA +PMA hr, 1 μL Cy3
Tube 4: 2.5 μg RNA +PMA hr, 1 μL Cy5

After mixing the solutions, the samples were heated to 80°C for 10 minutes and immediately transferred to ice for 3 minutes.

The next step is to prepare the Master Reaction Mix, which contains the remaining components from the Genisphere kit necessary for the reverse transcription reaction. The components of the master mix are as follows:

20 μL 5X Superscript (RT) reaction buffer
10 μL 0.1 M dithiothreitol (DTT)
5 μL Superase
5 μL dNTP mix
5 μL Superscript Enzyme (RT), 1000 units

The solution was mixed by pipetting, centrifuged, and stored on ice. A 9 μL aliquot of reaction master mix was added to each of the four, 11 μL RNA-RT primer mixes to produce a total reaction volume of 20 μL, the contents were mixed gently and incubated at 42°C for 2 hours. The polymerase chain reactions were stopped by adding 1.75 μL of 1.0 M NaOH/100mM EDTA to each reaction mixture in order to denature the DNA/RNA hybrids and degrade the RNA. The reaction was then neutralized by adding 2.5 μL of 2M Tris-HCl pH 7.5, mixed by pipetting, and stored at -20 °C until hybridization.

cDNA Hybridization to Microarray

Microarray chips were prepared by passing over a steam bath 3 times with the oligo side facing down (avoiding the formation of water droplets). The chip was then placed on a 100°C

heat block for 5 seconds with the oligo side up. The steaming process was then repeated, drying the chip for 1 minute on the heat block. The microarray chip was then washed as follows:

20 minutes in pre-warmed 58°C 2X SSC/0.2 SDS
5 minutes in room temperature 0.2X SSC
3 minutes at room temperature in deionized H_2O

The chips were then immediately centrifuged for 2 minutes at 1,000 rpm in uncapped 50 mL conical centrifuge tubes and then transferred to new, capped 50 mL tubes that were pre-warmed to hybridization temperature (58°C).

The cDNA hybridization mix was then prepared by first thawing and re-suspending the 2X Hybridization Buffer (Genisphere Kit Vial 5, 2X Enhanced cDNA Hybridization Buffer) by heating to 65–70°C for at least 10 minutes, vortexing until thoroughly mixed and re-suspended evenly, and microfuging for 1 minute at 10,000 rpm. For each array, the following cDNA Hybridization Mix for use with a 25x60 glass LifterSlip and 2X Enhanced Hybridization Buffer was prepared:

12.7 μL cDNA Synthesis #1
12.7 μL cDNA Synthesis #2
29 μL 2X Enhanced Hybridization Buffer (Vial 5)
1.6 μL Nuclease Free Water (Vial 10)
2 μL LNA dT Blocker (Vial 9)
1 μL Cot-1 DNA [Denatured at 100°C for 10 minutes prior to use]
59 μL total volume

There are typically two arrays done in one experiment to include the dye reversal in which one chip is hybridized with cDNA from Synthesis #1 with blue/cDNA and Synthesis #2 Red and the other with cDNA Synthesis #1 Red/cDNA and Synthesis #2 blue. The mixture was gently vortexed and briefly microfuged after the addition of all the components. The cDNA Hybridization Mix was first incubated at 78°C for 10 minutes and then at hybridization temperature (58°C) until loading the array. The microarrays were also pre-warmed to

hybridization temperature. The cDNA Hybridization Mix was added to the pre-warmed microarray by slowly pipetting under the glass LifterSlip for HEEBO chips, ensuring that air bubbles were avoided. The slides were then placed in tightly capped 50 mL tubes with a few drops of deionized H_2O and were incubated overnight in a dark humidified chamber at 58°C.

Post Hybridization Wash

Following the overnight hybridization, the arrays were placed into 50 mL conical vials filled with 2X SSC, 0.2%SDS wash buffer pre-warmed to 42°C. Once the LifterSlip fell off, the chips were placed in new conical vials filled 2X SSC, 0.2%SDS, pre-warmed to 42°C, followed by a 15 minute wash in 2X SSC at room temperature, and a final wash for 15 minutes in 0.2X SSC at room temperature. The array was then transferred to a dry 50mL centrifuge tube, orienting the chip so that the label is at the bottom of the tube and the oligo facing inward, and was immediately centrifuged with the cap off for 2 minutes at 1,000 rpm to dry the slide (delay in this step may result in high background).

3DNA Hybridization to Microarray

This process requires limited exposure of the dyes to light, which means the following steps were all preformed in the dark with a safe light. The first step was to prepare the 3DNA Capture Reagent (Vial 1) by thawing in the dark at room temperature for 20 minutes. The reagent was then vortexed, incubated at 50–55°C for 10 minutes, vortexed again and microfuged briefly to collect the contents. Then the 2X Hybridization Buffer (Vial 6) as thawed, re-suspended by heating to 70°C for at least 10 minutes and vortexed until all the components were evenly distributed. A 1 μL aliquot of Anti-Fade Reagent (Vial 8) was combined with 100 μL of the 2X Hybridiziation Buffer (Vial 6) to reduce fading of the fluorescent dyes during and after

the hybridization process. The 3DNA Hybridization Mix was prepared as follows for the two arrays:

5 μL 3DNA Capture Reagent #1 (Vial 1, red)
5 μL 3DNA Capture Reagent #1 (Vial 1, green)
40 μL Nuclease Free Water (Vial 10)
50 μL 2X Hybridiziation Buffer (Vial 6)
2 μL Cot-1 DNA [denatured at 95–100°C for 10 minutes prior to use]

The 3DNA Hybridization Mix was gently vortexed, briefly microfuged, incubated at 75–80°C for 10 minutes, and then stored at hybridization temperature (60°C) until loading the array. The 3DNA Hybridization Mix was then loaded onto the pre-warmed microarray slides (51μL total per chip), and the chips were incubated for 4 hours in the dark in a humidified chamber at 60°C.

Post 3DNA Hybridization Wash

These steps were also performed in the dark to avoid degradation of the fluorescent dyes. The chips were first washed and agitated in 2X SSC, 0.2% SDS wash buffer with 0.5 mM DTT pre-warmed to 65°C for 15 minutes, followed by a 15 minute wash-agitation in 2X SSC with 0.5 mM DTT at room temperature, and a wash-agitation for 15 minutes in 0.2X SSC at room temperature. The arrays were then immediately transferred to a dry 50 mL conical vial, orienting the slide so that the label is at the bottom and the oligo faces inward, and centrifuged without the cap for 2 minutes at 1,000 rpm to dry the slide. The microarrays were then packaged and shipped to Davidson College for scanning.

Microarray Data Analysis

There were several different types of methods employed in the data analysis of the microarray experiments. The signal ratios were generated from the GCAT program

MAGICTool, S-Plus software was used to perform the LOWESS standardization of the data, and Microsoft Excel was also used to sort data based on signal ratios.

MAGICTool Generation of Signal Ratios

The microarray scan produces two tiff files in grayscale. When the images are uploaded into the software, the user tells the program which file is the red signal and which is the green. The images on the second microarray are loaded backwards so that the "red image" was the green and the "green image" was the red to account for the dye reversal that was performed. The next step is to "grid" the microarray image to help the software locate the positions of the various spots on the array (Figure 6). This is an important step because the boxes formed around each spot specify the locations of each gene, which will match the gene list provided by HEEBO that designated each oligo complimentary sequence on the chip.

After gridding, the array must be segmented in order to identify which pixels are associated with signal (foreground) and which pixels can be considered background for each of the squares that were previously gridded around each spot (Figure 6). The segmentation method used was adaptive circle with a minimum radius of 5, a maximum radius of 6, and a threshold of 25. Once the software is able to distinguish between signal and background, the red/green ratios can be computed for each gene. The signal ratios were generated by using the total signal for all genes that did not have only black channels (this would indicate only background). These ratios represent the expression levels of control sample, untreated HL-60 cells (0 hours), to test sample, PMA treated HL-60 cells, to estimate the relative mRNA content in the two populations (Forrester 2009).

The raw signal ratios were then $\log_2$ transformed in order to generate a symmetric bell-shaped distribution of the ratios. This is important because the original graph of up and down-

regulated genes is positively skewed. For example, a gene up-regulated by a factor of 2 will have an expression ratio of 2, but a gene down-regulated by a factor of 2 will have and expression ratio of 0.5. This will result in a graph where the up-regulated genes have a much wider range than the down-regulated genes (Figure 7). When these ratios are $\log_2$ transformed, the up and down-regulated genes will be given equal weight. This is also helpful because it allows for repression to be represented by negative numbers and induction to be represented by positive numbers (Figure 7).

LOWESS Correction and Excel

One tool used to review a set of microarray data is the R-I plot. This method plots the log of the ratio (R) of the red/green signal at a spot against a measure of the intensity (I) of the two signals (red and green) at that spot (Forrester 2009) (Figures 8 and 9). The Ratio variable provides a measure of the induction or repression of the gene product while the Intensity variable provides a measure of the total signal strength in the two channels (Deeds *et al.* 2009). These types of diagnostic plots are useful in assessing the quality of the data and in suggesting appropriate normalization statistical manipulation (Deeds *et al.* 2009). There should be an approximate mean $\log_2(R/G)$ of zero because a large proportion of the genes in a genome are not expected to change their expression (Deeds *et al.* 2009, Forrester 2009).

Locally weighted scatter-plot smoothing (LOWESS) is a method used to estimate the dependence of the $\log_2(R/G)$ on a measure of total intensity (Forrester 2009) (Figure 9). The raw RI plot can be manipulated to correct for background effects during segmentation and to remove the systematic dependency of the plot on the total intensity (Deeds *et al.* 2009) (Appendix 1). Essentially, the LOWESS correction makes a window with 300 points (genes) and will move along the horizontal line computing the mean and standard deviation of the ratio and intensity

each gene (Figure 8 and 9). The computer will then retain the genes that fall within a 95% confidence interval and exclude the outliers in order to smooth out the graph and isolate significant genes. The $\log_2$ transformations and corrections based on LOWESS method were added to the excel spreadsheet. The dye reversals were swapped and an average fold ratio was determined for each gene. If the gene passed the confidence interval test it received a score of 1 for that experiment (Appendix 1). Once all of the data analysis for the microarrays was completed and lists were compiled of the most highly up-regulated genes it was necessary to validate and quantify the results by another method.

qRT-PCR Analysis of Up and Down-Regulated Genes

In order to validate the expression levels obtained from the microarray experiments, quantitative real-time PCR (qRT-PCR) experiments were run for approximately ten of the up and down-regulated genes as a preliminary screen. The isolated RNA used for the microarray experiments at various time points was reverse transcribed into cDNA using the High-Capacity cDNA Reverse Transcription Kit from Applied Biosystems (See TaqMan Gene Expression Assay Protocol. All of the PCR reactions were conducted using the gene specific primers (TaqMan probes) and 2x TaqMan Gene Expression Master Mix from Applied Biosystems. The PCR reaction started with 50°C, 2-minute and 95°C, 10-minute incubations and then 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. The reactions were run in an Applied Biosystems 7500 Fast System, using the comparative C_T method with a standard 2 hour run. The PCR reaction was set up in a clear 96-well Fast Reaction Plate with the 0 hr control and various time points run in triplicate for each of the target genes as well as for 18S rRNA, which was used as an endogenous control (Figure 10). Each well contained a 20 μL sample comprised of 1 μL

TaqMan probe (target gene or 18S), 9 μL cDNA or RNase-free water (negative controls), and 10 μL TaqMan Master Mix.

All data was analyzed using the 7500 Fast System software with automated C_T and baseline functions. 18S was set to be the endogenous control, the control samples were calibrator samples, and 95% confidence interval was set. Raw relative quantification scores were used setting the 0 hr control sample as a "1-fold" expression. Genes were determined to be significant if the error bars, calculated by evaluating the standard deviation of the triplicates, did not overlap when comparing any two histograms.

Cluster Analysis

Informatics data was collected on 80 genes found to be significantly up-regulated 3 hours after PMA treatment. The SABiosciences DECODE database was used to compare the number of binding sites of over 200 different transcription factors within the promoter region of each of the 80 genes. As a preliminary screen the Euclidean metric was used for hierarchical clustering followed by Ward's method for cluster fusion (Figure 22). The Euclidean metric is the most basic way of computing the distance between two points. Ward's linkage finds the sum of the squared distance from the combined centroid of groups and then chooses to fuse the smallest distance computed.

Eucidean distance equation:

$$d_{st}^2 = (x_s - x_t)(x_s - x_t)'$$ (MathWorks Inc. 2010)

Ward's linkage:

$$d^2(r,s) = n_r n_s \frac{\left\|\bar{x}_r - \bar{x}_s\right\|_2^2}{(n_r + n_s)}$$ (MathWorks Inc. 2010)

RESULTS

Microarray Analysis

DNA microarray experiments comparing untreated HL-60 cells to cells treated with PMA for 1, 3 and 6 hours were completed. Data analysis, including normalization, LOWESS, and highly stringent filtering criteria, have determined approximately 50 significantly up-regulated genes at 1 hour after exposure to PMA (Table 1), approximately 100 significantly up-regulated genes at 3 hours after exposure to PMA (Table 2) and approximately 115 significantly up-regulated genes at 6 hours after exposure to PMA (Table 3, Appendix 1). These numbers are generalizations based on each gene's confidence score from the LOWESS test (Appendix 1). There were fewer of the down-regulated genes that showed significant change within the 95% interval and this data has yet to be analyzed completely. A representative table of all genes showing fold induction or repression including $\log_2$ transformation and LOWESS correction is depicted in Appendix 1 for the 6 hour microarray. In general, these array results indicate novel, dramatic induction, 34 to 56-fold, of several chemokine ligand genes (Table 1-3, Figure 11-13). Additionally, several transcription factor and co-factor genes were identified to be up-regulated (Table 1-3, Figure 11-13).

A group of CC chemokine genes (CCL2, CCL3, CCL3L1, CCL4, CCL4L1 and CCL4L2) demonstrated rapid and sustained induction of mRNA levels starting 3 hours after exposure to PMA and continuing through 6 hours post-induction (Tables 2 and 3). Among the most highly

induced early genes were others known to encode inflammatory response proteins (TNF, IL8, C3AR1). Additionally, transcription factor gene expression was dramatically altered early in the differentiation time course, with the predicted activation of AP-1 family members (JUNB, FOSL1) and the concomitant inhibition of NFκB (TNFAIP3, NFκBIA, NFκBID). Another transcription factor found to be down-regulated was Myc.

qRT-PCR Validation of Expression Levels on Selected Targets

The comparative C_T qRT-PCR method was conducted on selected genes of interest and the expression ratios for each gene were computed. Based on the 1, 3, and 6 hour arrays the six selected genes of interest were four found to be generally up-regulated Jun-B (Figure 15), NFκBIA (Figure 17), TNF (Figure 18), and IL8 (Figure 19) and two found to be generally down-regulated Myc (Figure 14) and JDP2 (Figure 16). Graphical representations of select transcription regulators and chemokine genes from the 1, 3, and 6 hour arrays were organized to show the expression profiles of the genes and to easily compare the qRT-PCR data (Figures 20 and 21).

EMSA Validation of AP-1 Activity

Results from the Electrophoretic Mobility Shift Assay (EMSA) with AP-1 transcription factors was collected in order to validate that changes in gene expression in the microarrays was producing functional changes at the protein level **(**Figure 4). Figure 4, Lane NE, shows the free radio labeled specific AP-1 binding oligo loaded at the bottom with no protein extract present. The bands towards the top of Lanes 0, 1, 3, 6, 12, 16 and 24 show the presence of AP-1 protein-oligo complexes (Figure 4). The changing band intensity quantitatively indicates the amount of

AP-1 protein complex present at that time point and the location indicates variation in AP-1 subunit complexes (Figure 4). At this time, the specific components of each AP-1 complex are still uncharacterized, but it has been hypothesized that 0, 1, and 3 contain c-Jun and that JunB replaces c-Jun to form a heterodimer with Fos at the later time points (Figure 4). Microarray results indicate JDP inhibitor protein may be part of the complex at the earlier time points and that JDP leaves as JunB joins the complex (Figure 4, 15, 16). Both competition lanes were completed with the 24 hour nuclear extract. The AP-1 competition lane was comprised of 100x more unlabeled AP-1 specific oligo than radio-labeled AP-1 specific oligo. The absence of signal in the AP-1 competition lane demonstrates the selective binding of AP-1 complex to the unlabeled target oligo (Figure 4). The SP-1 competition lane was comprised of 100x more unlabeled SP-1 specific oligo than radio-labeled AP-1 specific oligo. The signal in the SP-1 competition lane demonstrates AP-1 specifically binding the AP-1 specifc radio-labeled oligo over the SP-1 specific unlabeled oligo (Figure 4).

Hierarchical Cluster Analysis of Transcription Factor Binding Sites

The hierarchical cluster analysis produced a dendogram with 7 unique clusters formed by Euclidean distance and Ward linkage (Figure 22). The following groupings seemed to be the primary similarities contributing to the formation of relevant clusters:

Cluster 1 (orange): mostly GR binding sites
Cluster 2 (green): No transcription regulator driving the cluster
Cluster 3 (blue): High numbers of AP-1 binding sites, some NFκB
Cluster 4 (yellow): No AP-1 binding sites, high numbers of NFκB and NFκB1
Cluster 5 (pink): some AP-1 binding sites, some CREB
Cluster 6 (purple): some AP-1 binding sites
Cluster 7 (red): some NFκB binding sites, high numbers of CREB binding sites

DISCUSSION

This research project used cDNA microarrays to perform a genome-wide analysis of PMA-induced differentiation, growth arrest, and apoptosis in the acute myeloid leukemia cell line, HL-60. The microarray data was analyzed using segmentation, generating expression ratios, log transformation, and LOWESS corrections. There were two main functional protein groups that predominantly arose from the DNA microarrays of up and down-regulated genes from the first three time points: proteins involved in inflammatory response (cytokines and interlukins) and transcription regulators.

Cytokines are growth factors that induce components of the hematopoietic system and also act as regulatory factors of the immune system including interferons and interleukins (Ugler *et al*. 2003). Cytokines that regulate differentiation pathways have been used to suppress malignancy in certain types of leukemia cells (Seo *et al*. 2000). Interlukins are growth and differentiation factors, which stimulate cellular components of the immune system (Weinburg 2007). Transcription regulators are proteins involved in regulating the transcription of a gene, often by associating with sequences in the promoter region of the gene (Weinburg 2007). The appearance of these functional groups identified to be highly up-regulated at these early time points (1, 3, and 6 hours) is consistent with the hypothesis that genetic re-programming of the HL-60 cells is an early event mediated by transcriptional regulators. The existence of genes coding for chemokines and interlukins also confirms the morphological and genetic changes observed to occur as the HL-60 cells differentiate into macrophage-like cells. Significant data on the down-regulated genes was not able unable to be produced with confidence.

Previous microarray studies have been completed on HL-60 cells with the addition of phorbol esters, either PMA or 12-O-tetradecanoylphorobol-13-acetate (TPA). Work published by

Zheng *et al*. in 2002 with TPA laid useful groundwork in studying a time-course of differentiation with HL-60 cells using DNA microarrays. A similar cell culture procedure was executed and the time course isolated total RNA at 0, 0.25, 0.5, 1, 2, 3, 6, 12, 24, and 48 hours after exposure to TPA (Zheng *et al*. 2002). The genome had fairly recently been sequenced and the microarrays used contained far fewer genes, approximately 4,600 including total genomic DNA, housekeeping genes and expressed sequence tags (Zheng *et al*. 2002). An interesting similarity was that JunD and FosB were found to be induced by TPA in the early time points and several cytokines were found to be up-regulated around 3 hours (Zheng *et al*. 2002).

A publication in 2000 by Seo *et al*. also studied the differentiation of HL-60 cells with PMA using cDNA microarrays. In this study, RNA was isolated 48 hours after exposure, which is interesting since we observed the HL-60 cells exhibiting apoptosis shortly after 24 hours of exposure. This microarray contained a total of 3,031 genes and up and down-regulated genes were determined based on fold increases between 0.5 and 2 (Seo *et al*. 2000). Overall, the data produced in the present study builds on the results from these previous publications by including the entire human genome, approximately 40,000 genes in each experiment, and by producing novel fold-inductions with stringent statistical analysis applied to each data point.

Up and down-regulated genes

A wide variety of protein functional groups including receptors, kinases, apoptosis regulators, and proteases were shown to be up and down-regulated from the DNA microarray data. Inflammatory response proteins, cytokines and interlukins, and transcription regulators were two functional groups of genes that conveyed the most interesting, relevant information on the differentiation program at these early time points. Cytokines are part of the inflammatory

response and regulate components of the hematopoietic system (Ugler *et al*. 2003). Cytokines that regulate differentiation pathways have been used to suppress malignancy in certain types of leukemia cells (Seo *et al*. 2000). mRNA transcript levels of CC chemokine genes including CCL2, CCL3, CCL3L1, CCL4, CCL4L1 and CCL4L2 were induced 10 to 50-fold within 3 hours of exposure to PMA and were sustained through to the 6 hour time point (Tables 2 and 3).

Monocytes are attracted to this CC family of chemokines, which are known to induce levels of monocyte chemoattractant proteins (MCP-1 an alias of CCL2) (Martin *et al.* 1997). CCL2 was found to be up-regulated 9-fold at 3 hours and 10-fold at 6 hours (Tables 2 and 3). MCP-1 is also known to be regulated by TNF, which was determined to be highly expressed in HL-60 cells exposed to PMA. MCP mRNA were found to be involved in pathological conditions of atherosclerosis and rheumatoid arthritis (Martin *et al*. 1997). Interestingly, the promoter region of MCP-1 contains binding sites for AP-1 and NFκB transcription factors, which were involved in the activation of many genes in this pathway (Martin *et al*. 1997).

IL8 and TNF are genes that code for inflammatory response and were among the most highly induced early genes. IL8 is one of the major mediators of inflammatory response and is secreted by several cell types. In the array experiments TNF was found to be up-regulated 6-fold at 1 hour and 29-fold at 3 hours (Tables 1 and 2). IL8 was found to be up-regulated 5-fold at 1 hour, 27-fold at 3 hours, and 45-fold at 6 hours (Table 1, 2, and 3). The initial induction of TNF was confirmed by qRT-PCR analysis; although the fold inductions are not identical, the trend of the expression profile was validated (Figure 18). The qRT-PCR experiment for IL8 produced a slightly different result in that the 6 hour time point showed a decreased expression level, while the array demonstrated further induction at 6 hours (Figure 19). IL8 was found to be up-regulated in HL-60 cells differentiated with TPA in a previous publication (Zheng *et al*. 2002).

Transcription regulators were another of the functional groups found to be highly up-regulated at the early time points in the microarray experiments. Specifically, proteins involved in forming the AP-1 complex such as JunB and Fos1. *c-jun* protooncogene codes for the major form of the transcription factor AP-1, which binds DNA and regulates the transcription of genes responsive to phorbol esters (Datta *et al*. 1991). AP-1 complex has been found have a dual role as an oncogenic protein and tumor suppressor as well as inducing apoptosis in specific cell types (Eferl and Wagner 2003). The components of the AP-1 complex also play an important role in determining the activation pathway of AP-1.

JunB was found to be up-regulated 5-fold at 1 hour, 6-fold at 3 hours, and 8-fold 6 hours after exposure to PMA (Table 1, 2, and 3). These findings support previous work done on the expression levels of JunB in response to phorbol esters and was confirmed by qRT-PCR (Datta *et al*. 1991) (Figure 15). c-Jun protein has been found to be present in proliferative tumors while JunB is selectively down-regulated (Eferl and Wagner 2003). It is hypothesized that JunB replaces c-Jun in HL-60 cells treated with PMA in order to repress the proliferation of the cells and to induce the differentiation and apoptosis pathways.

JDP2 was one transcription factor found to be down-regulated with confidence in the arrays. JDP2 is a negative regulator that represses JunB dimerization to Fos in the AP-1 complex. The array findings were validated by qRT-PCR analysis (Figure 16). It makes sense that the up-regulation of JunB and Fos1 coincide with the down-regulation of their inhibitor. If the AP-1 complex plays a critical role in the process of differentiating the HL-60 cells into using the macrophage-like program and initiating apoptosis, one of its inhibitors should be transcriptionally down-regulated.

Another transcription regulator that was found to be down-regulated was Myc. It is well established that the *c-myc* protooncogene is activated or amplified in a large number of naturally occurring malignancies (Salehi *et al*. 1987). This gene plays a key role in cellular proliferation and cellular differentiation, but PMA inhibits proliferation and the expression of *c-myc* during the differentiation of HL-60 cells to macrophage-like cells (Salehi *et al*. 1987). Preliminary findings from microarray experiments showed that expression of *c-myc* was down-regulated in HL-60 cells treated with PMA early in the time course and remained low, which was validated by qRT-PCR experiments (Figure 14). Myc expression is repressed as the PMA initiates differentiation and the HL-60 cells lose proliferative ability. A novel finding was the transient up-regulation of Myc at the 1 hour. Previous studies have indicated that c-Myc sensitizes cells to TNF-mediated apoptosis by inhibiting NFκB (You, *et al*. 2002). This could imply that the initial up-regulation of Myc at 1 hour plays a role in the down-stream programmed cell death experienced by the HL-60 cells.

The inhibition of NFκB was established by viewing the up-regulation of proteins such as TNFAIP3, NFκBIA, and NFκBID. In particular microarray analysis found NFκBIA to be up-regulated 12-fold at 1 hour and 9-fold 6 hours after exposure to PMA (Table 1, 2, and 3). The microarray results were validated by qRT-PCR (Figure 17). NFκB regulates genes involved in the immune response, apoptosis, and the cell cycle. NFκB is involved in the cell proliferation and has been associated with AML patients demonstrating increased binding activity of the transcription factor (Cilloni *et al*. 2007). The up-regulation of NFκBIA, an inhibitor of NFκB, could contribute to the cease of proliferative ability of the HL-60 cells after exposure to PMA.

Hierarchical clustering

Additional hierarchical clustering needs to be performed on transcription factor binding sites. Some of the clusters do provide valuable information about genes that contain similar promoter regions, but the green cluster 2 in particular generated a group that does not seem to have any true similarity (Figure 22). Although Euclidean distance provides a good preliminary screen of the data there is an inherent error in the clustering. When matching genes based on their similarity there will be some genes, which did not have the presence of a binding site in the promoter region for a certain transcription factor, which would receive a 0. The similarity measures unfortunately would group genes that have a zero-zero (co-absence) match even though this does not really contain useful information about the gene. Essentially, the fact that specific binding sites are not present in two genes is causing them to be grouped together and determined similar. Consider the usefulness of grouping a fish and a pig as similar animals because they do not have wings.

In order to avoid this inherent error it would be important to do another cluster using Jaccard's coefficient or another type of similarity measure in order to produce the most meaningful data on these transcription factor-binding sites. This data could ultimately be used as a predictive tool to anticipate which genes may be induced or repressed later in the time-course based on the presence of similar promoter regions. Software is available to do pathway predictions by searching for a particular gene in the database.

Future Experiments

The next step in this research would be to complete the time-course by collecting microarray data for HL-60 cells exposed to PMA for 12, 16, and 24 hours. Having a

comprehensive genetic profile for each of the genes involved in differentiating the leukemia cells into the macrophage-like cells is critical to completing this study. The next step would then be to perform hierarchical cluster analysis in order to group the genes over the time-course by their expression profiles and their function involvement in the differentiation program.

Looking forward, it would be useful to perform an RNA interference (RNAi) experiment. RNAi is a cellular pathway that can be used to shut down or "silence" genes allowing for a functional study to be performed in a gene-specific way (Martin and Caplen 2007). Double-stranded RNA (dsRNA) is first fragmented into short interfering RNA (siRNA), which then activate ribonuclease in order to specifically degrade the mRNA of a specific sequence (Cioca *et al*. 2003). It would be interesting to perform an RNAi experiment on one of the genes we found to be down-regulated in the HL-60 cells after exposure to PMA if we thought that gene was critical to initiating the differentiation program. Therapeutically, more work has been done in finding ways to shut down various proteins using small molecule drugs that render the protein inactive than to try and find a way to activate a previously inactive protein. Similarly, it has proven difficult to find an effective way to deliver genes to patients in order to activate that protein downstream. Because of this, an increased interest in shutting down over-expressed genes has emerged. RNAi could potentially be applied to oncogenes, genes that have had toxic gain of function mutations in cancer (Martin and Caplen 2007).

One such gene that was discussed in detail earlier is c-myc. Myc's central involvement in the proliferative capabilities of most cancers is one reason why this would be a good candidate gene for study. Another candidate gene would be NFκB because the up-regulation its inhibitor NFκBIA also seems to play a role in turning off proliferation. Specifically with HL-60 cells, an RNAi experiment was conducted looking at the c-Raf and bcl-2 genes because their protein

products are key factors involved in chemo-resistance in myeloid leukemia (Cioca *et al*. 2003). The study's results indicated that transfection with siRNA duplexes for the two genes significantly decreased the levels of Raf-1 and Bcl-2 proteins in the cell and ultimately contributed to the initiation of programmed cell death (Cioca *et al*. 2003). If the siRNA's are able to specifically target the leukemia cells, and to induce spontaneous apoptosis, the therapeutic application could be monumental.

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