doi:10.4149/neo_2009_02_141

Combination of mRNA and protein microarray analysis in complex cell profiling

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Received June 5, 2008

This study combines mRNA and protein analysis using cDNA and antibody microarray techniques, respectively. These create a novel, integrated perspective into cellular molecular profiles. The aims of this study were to establish a reliable way of integrating these two approaches in order to obtain complex molecular profiles of the cell and to find suitable methods to normalize the data obtained using these approaches.

Antibody microarray and cDNA microarray techniques were used to study expression alterations in HL-60 cells that were differentiated into granulocytes using all-*trans* retinoic acid (ATRA). We selected this model to evaluate this combined profiling technique because the expression levels of most of the mRNA and protein species in these cells are not altered; therefore it is easier to track and define those species that are changed. The proteins whose levels were altered included c-myc, c-jun, Pyk2, FAK, PKC, TRF1, NF-kappaB and certain caspase types. These proteins are involved in apoptosis and hematopoietic differentiation pathways, and some have also been reported to have oncogenic potential. We compared the results obtained using the two methods, verified them by immunoblotting analysis, and devised normalization approaches.

This is one of the first demonstrations that a combination of antibody microarray and cDNA microarray techniques is required for complex molecular profiling of cells based on multiple parameters. This approach allows a more detailed molecular phenotype of the given sample to be obtained. The results obtained using a combination of the two profiling methods are consistent with those from previous studies that used more traditional methods.

Keywords: microarray, cell profiling, protein expression, mRNA expression, HL-60

Protein and cDNA microarrays are based on the same principles and aim to simultaneously detect a large number of molecules in a biological sample. The main difference between cDNA and protein microarrays lies in the structures of the target and probe molecules. Protein microarrays can be manufactured and read using DNA microarray methods, however, only a subset of the available methods can be used for protein arrays. Data analysis and modeling tools are also similar for both techniques [1]. The antibody microarray is a subset of protein microarrays, which provides an excellent format for protein expression profiling. The use of cDNA microarrays for profiling cellular mRNA expression in a massively parallel fashion has provided valuable insights into many biological areas. However, these results are only correlative because proteins are the major orchestrators of cellular functions; therefore, proteins and their interactions with one another should be assessed in order to profile the actual cellular proteome. A direct correlation between mRNA levels and protein expression has not been proven [2], and mRNA expression profiling does not allow the extent of post-translational modification to be determined. For example, antibody-based microarrays can detect protein phosphorylation in order to assess the function of a given signaling pathway [3]. Phosphorylation plays a key role in regulation of many aspects of cell proliferation and differentiation [4]. Therefore, the combination of cDNA and antibody microarrays in a single experiment represents an advance in complex cell expression profiling, and allows the complete expression profile to be evaluated.

The examination of gene expression and protein profiles in cancer is an important area for the application of microarrays. Profiling cancer-associated changes requires simultaneous measurement of many mRNA transcripts and proteins in a single sample. Identifying these changes may lead to the discovery of new biomarkers. Precise phenotyping

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of the disease origin is especially required in the case of leukemia. [5]

We chose to evaluate the human myeloid leukemia cell line HL-60 in this study because these cells are widely used as a model of leukemic myeloid cell differentiation, and this line has been demonstrated to be a representative model system for studying human granulopoiesis *in vitro*. All-*trans* retinoic acid (ATRA) treatment of HL-60 cells *in vitro* induces granulocytic differentiation, which involves the reorganization of the nucleus and cytoplasm. Retinoic acid treatment over a period of 6-8 days resulted in a progressive increase in the proportion of cells with mature neutrophil morphologies and an increase in the proportion of cells that exhibited the morphological characteristics of apoptosis [6, 7]. ATRA is currently being used for granulocytic differentiation of various cell types [8]. In addition, ATRA has been widely used as a differentiation therapy for acute promyelocytic leukemia [9] and for chronic myeloid leukemia treatment [10].

Materials and methods

Cells **.**HL-60 cells were cultured in Roswell Park Memorial Institute (RPMI)-1640 medium supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 U/ml streptomycin (PAN-Biotech, Aidenbach, Germany) in an atmosphere supplemented with 5% CO_2 at 37°C and 100% humidity. Granulocytic differentiation was induced with 1 μM ATRA. All cells were incubated for 4 days and ATRA treated cells were cultivated in fresh medium with retinoic acid for an additional 3 days after apoptotic cells were isolated. Cell numbers were assessed using a Bürker counting chamber.

Cell isolation, extraction and sample preparation. Cells were washed two times in phosphate buffered saline (PBS). A MACS® Dead Cell Removal Kit, along with a magnetic MiniMACS™ cell separator and a MACS® MS Column (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) were used to eliminate apoptotic cells from ATRA-treated cell culture. Total cellular protein extracts were prepared using an extraction buffer from the Panorama™ Ab Microarray - Cell Signaling Kit (Sigma-Aldrich, St. Louis, USA) for antibody microarray assays and immunoblot analysis. RNA was isolated using TRI REAGENT (Sigma-Aldrich, St. Louis, USA), and then purified using an Rneasy Kit (Qiagen, Inc., Valencia, CA, USA), according to the manufacturer's protocol. RNA samples were reverse transcribed into cDNA. RNA was labeled by incorporating either Cy3- or Cy5- dCTP (Amersham Biosciences, Buckinghamshire, UK) using Oligo (dT) Primers and Superscript II (Invitrogen, Carlsbad, CA, USA) and the reverse transcriptase-polymerase chain reaction.

3D nuclear staining Cell differentiation was analyzed by 3D nuclear staining. Cells in PBS were attached to poly-L-lysine coated microscopic slides for 15 min, and the cells were then fixed with 3.7% paraformaldehyde in 250 mM HEPEM (12 min) [11] and washed three times in 1×PBS. After washing, the cells were permeabilized in 0.5% Triton X-100 in 1×PBS (10 min), 0.2% saponin in 1×PBS (10 min) and washed three times in 1×PBS. DNA was stained using 0.2 μl/ml DAPI in Vectashield (Vector, Burlingame, CA, USA).

cDNA microarrays. The microarray slides used in this study (SS-H19k6 QuantArray, Clinical Genomic Centre, Toronto, Canada) contained 19,200 human cDNAs, positive controls and negative controls. The microarray was used to compare mRNA from HL-60 cells with mRNA from differentiated granulocytes. Reverse transcribed cDNA was precipitated using isopropanol. The microarray assay was carried out according to the manufacturer's instructions (protocols from the OCI Microarray Centre, Ontario, Canada, 2000). We made two replicates for each cell type, resulting in a total of four slides.

The signals were scanned using a ProScanArray, 4-Laser scanner (PerkinElmer, IL, USA).

Antibody microarrays. The Panorama™ Ab Microarray - Cell Signaling Kit, CSAA1 (Sigma-Aldrich, St. Louis, USA) used in this study contains 224 different antibodies, each of which are spotted in two copies onto nitrocellulose-coated glass slides. A complete list of the antibodies on the microarray can be found at www.sigmaaldrich.com/antibodyarray. All antibodies (monoclonals and polyclonals) used to construct the microarray were also from Sigma-Aldrich, as were the chemicals and solvents in the kit. The Cy3 and Cy5 fluorescent dyes were purchased from Amersham Biosciences (Buckinghamshire, UK).

The assays were carried out according to the manufacturer's instructions. The microarrays were used to compare the protein expression profiles of two samples, the test (Granulocytes) samples versus the reference samples (HL-60). We used independent microarrays for each sample, and labeled each sample with both Cy3 and Cy5, allowing us to obtain reliable internal controls by first comparing the two channels for the same sample. The expression profile of each sample (Cy3 and Cy5) was individually recorded and normalized, and mean values of each slide were compared. We made two replicates for each cell type, resulting in a total of four slides. The signals were scanned using a ProScanArray, 4-Laser scanner (PerkinElmer, IL, USA). Antibody microarray slides can be preserved and rescanned multiple times using a laser scanner over a period of days or weeks [12].

*Microarray data analy**sis.*** The scanned images were analyzed using JABS-I software [13]. This software produced a set of datasets containing foreground and local background spot intensities for each slide. In order to visualize the data, we used the MeV (MultiExperiment Viewer), v4.0 software [14].

The normalization of spot intensities is a mandatory step in data analysis. Spot intensities among the copies of a single spot may vary due to the spatial distribution of the signal. The intensities may also vary among the different slides used in analysis. Many normalization approaches have previously been described [15]. The intra-slide normalization process consists of a running method [16], which is called a "Two-parameter signal normalization approach in sliding windows".

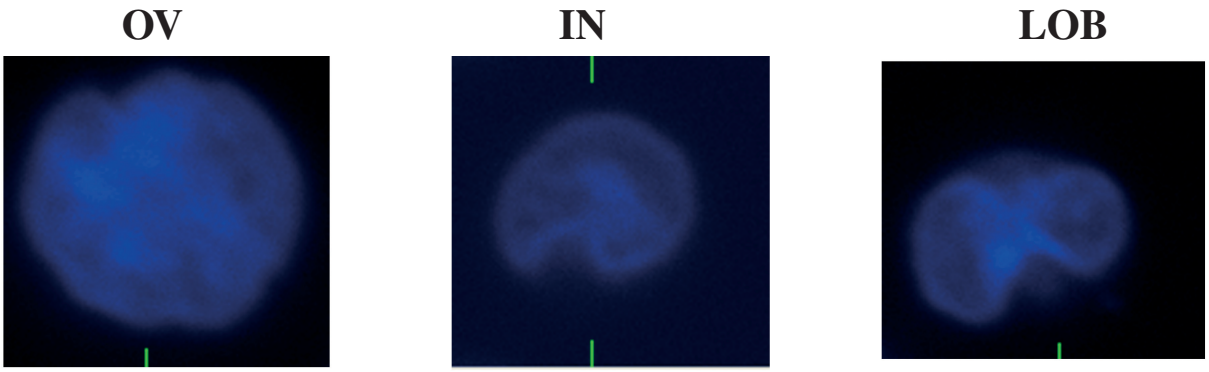

Figure 1. Confocal microscope images of 3D nuclear staining of HL-60 cells. OV, undifferentiated cell with ovoid nuclear shape; IN, differentiated metamyelocyte with a horseshoe-shaped (intended) nucleus; LOB, fully differentiated granulocyte with a lobular nucleus.

For inter-slide normalization of antibody microarray data we used housekeeping proteins. Because we used independent microarrays for each sample, we normalized the fluorescence intensities between slides according to the average of several housekeeping proteins uniformly distributed on the slide. After these transformations had been performed, all of the intensities could be directly compared with one another.

We chose four isotypes of beta tubulin as our set of housekeeping proteins (beta tubulin I, beta tubulin II, beta tubulin III and beta tubulin IV). However, the expression levels of proteins that are commonly used as internal controls in classical expression studies may not always be constant [17]. In order to show that the housekeeping proteins we selected were suitable for normalization purposes, we analyzed the levels of beta tubulin via immunoblotting with a monoclonal anti-beta tubulin antibody (clone 2-28-33, Sigma, USA). In order to obtain more reliable results, we decided to normalize the antibody microarrays with an appropriate average of the levels of several isotypes of the reference protein, beta tubulin.

There were 224 distinct antibodies spotted on the microarray in 448 spots. The query sample (Gra) was compared with the reference sample (HL). The ratio (T) of the ith protein (where i is an index that runs over all of the arrayed antibodies from 1 to 448) can be expressed as

$T_i = Gra_i/HL_i$

For visualization of the data using the MultiExperiment Viewer, the expression ratio is transformed using the logarithm base 2, which produces a continuous spectrum of values, and treats up- and down-regulated genes in a similar manner [18].

We used the LOWESS method [19] for intra-slide normalization of cDNA microarray data, which resulted in two normalized channels per slide.

We compared the two different formats of microarrays, cDNA and antibody. In order to find an appropriate RNA transcript for each protein, we created an intersection of the 19,200 RNA transcripts and 224 antibodies.

Immunoblotting. The cellular protein extract was added to SDS Sample Loading Buffer and boiled for 5 min. Eight micrograms of protein were analyzed on an SDS polycrylamide gel, and electrotransferred to a PVDF membrane using the Mini-PROTEAN® 3 system (Bio-Rad Laboratories, Hercules, CA, USA). We used 10% polyacrylamide precast gels (Precise™ Protein Gels; Pierce Biotechnology, Rockford, IL, USA). The proteins were electrophoresed in Tris-HEPES-SDS buffer at 150V for 45 minutes. The transfer buffer was composed of Tris Base, glycine and methanol. The membranes were blocked for 1 h using a solution of 5% non-fat milk in Tris Buffered Saline (TBS) containing 0.1% Tween-20. They were then incubated overnight at 4°C with the following specific mouse monoclonal antibodies: anti-beta tubulin and anti-c-myc purchased from Sigma-Aldrich, USA. The working dilutions of the primary antibodies were 1:1000 for anti-beta tubulin and 1:7000 for anti-c-myc. The antibodies were diluted in TBS with Tween-20. The secondary antibody was conjugated with horseradish peroxidase and was used at a dilution of 1:15000 (anti-mouse IgG - whole molecule; Sigma-Aldrich, USA). The majority of the chemicals were obtained from Sigma-Aldrich, USA. The molecular marker was purchased from Fermentas GmbH (Ontario, Canada). Antibody complexes were detected by chemiluminiscence on photographic film (Agfa-Gevaert Group, Mortsel, Belgium).

Results

Nuclear shape changes were quantified in DAPI stained ATRA-treated HL-60 cells. When the cells were exposed to 1 μM ATRA, the vast majority exhibited nuclear properties associated with differentiated cells (~27% indented and ~55% lobulated nuclei) (Figure 1). However, we still observed a small number of undifferentiated cells with ovoid nuclei (~18%). Antibody microarray and cDNA microarray analyses were used to compare the levels of mRNA and proteins in undifferentiated human HL-60 cells and in HL-60 cells after ATRA-induced differentiation.

We found that certain proteins were strongly up- or down-regulated; differences were observed in the levels of 27

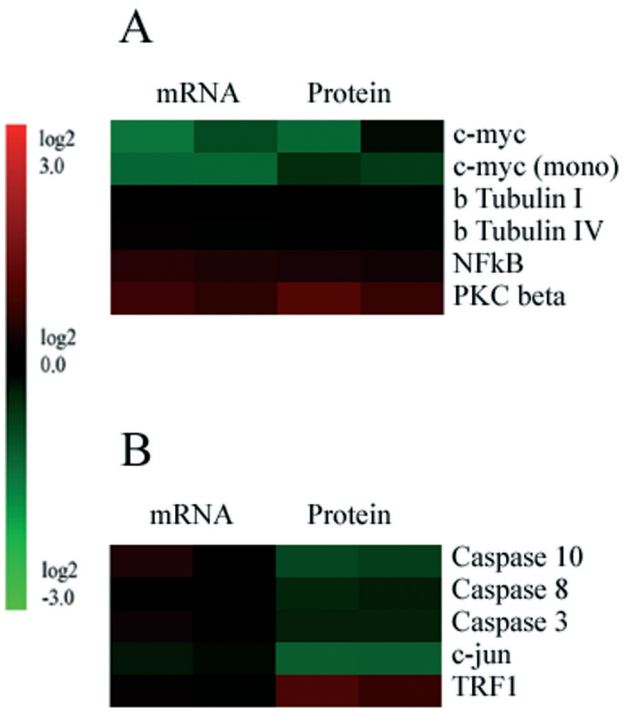

Figure 2. MultiExperiment Viewer (v4.0) visualization of the data with a high degree of correlation between the levels of mRNA and protein (A) and data with a low degree of correlation between the levels of mRNA and protein (B). Down-regulation (green), up-regulation (red) and no change (black) in mRNA and protein level.

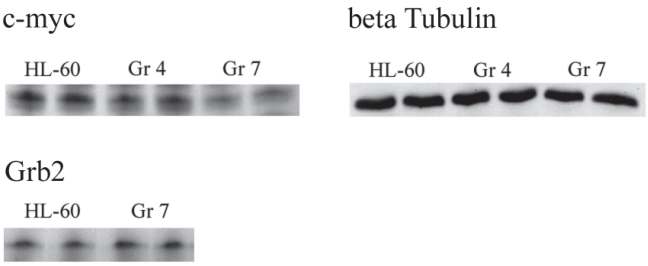

Figure 3. **Confirmation of microarray results by immunoblotting. Eight micrograms of total cell extract from treated or non-treated cells were electrophoresed on SDS polyacrylamide gels and electroblotted. For c-myc there is an obvious decreasing trend: the level of c-myc was slightly lower in cells treated for 4 days by ATRA (Gr 4), but decreased strongly after 7 days of ATRA treatment (Gr 7) when compared with untreated cells (HL-60). The same amount of beta tubulin was found in undifferentiated cells (HL-60) after 4 days (Gr 4) and 7 days (Gr 7) of ATRA treatment. Grb2 protein does not change its expression during 7 days of ATRA treatment. Equal loading in each lane was assessed by the Bradford assay.**

proteins in total (Table 1). For some genes, among them c-myc, beta tubulin, PKC beta, or NF-kappaB, the changes in protein levels correlated with the changes in mRNA levels (Figure 2A). On the other hand, the levels of mRNA did not correlate for caspase 3, caspase 8, caspase 10, TRF1 or c-jun (Figure 2B). The mRNA levels of the transcription factor c-myc decreased as HL-60 cells were differentiated with ATRA, and this correlated with a significant decrease in the expression of this protein in the differentiated cells. The spot intensity for the c-myc mRNA was over 2-fold lower in the differentiated cells, and 1.5-fold lower for the c-myc protein. We observed a 2-fold decrease in protein spot intensity for another transcription factor, c-jun. In contrast, certain proteins were induced during granulocytic differentiation. We observed enhanced levels (between 2- and 2.5-fold increases in spot intensity for certain specific phosphorylated forms) of phosphorylated proteins of the FAK family of protein tyrosine kinases. However, levels of the unphosphorylated forms of PTK2 (also known as FAK) and Pyk2 remained unchanged upon differentiation. The beta form of protein kinase C (PKC) was also enhanced (1.82-fold on the antibody microarray and

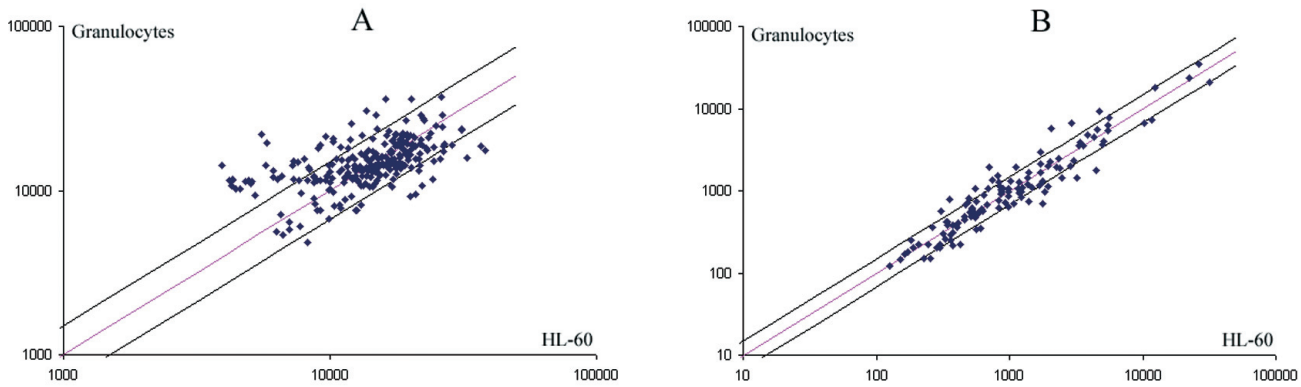

Figure 4. Scatter plots of the merged normalized data from four slides of antibody microarrays (A) and four slides of cDNA microarrays (B); HL-60 plotted against granulocyte values. The central line is the midpoint with the lines above and below at 1.5 fold. Each point represents a single antibody

Table 1. Differentially expressed proteins within the antibody microarray with a fold change (T_i ratio) lower than 0.667 and higher than 1.5.

Gene name	Gene function	Fold change PROTEIN	Fold change mRNA
Lower in granulocytes			
Smad4	Transcriptional activation	0.47	1.12
cJUN	Transcription factor, involved in tumor metastasis, inflammation, and response to stress factors	0.48	0.88
Cyclin A (poly)	Regulation of cell cycle progression	0.54	0.96
c-Myc	Proto-oncogene, effect on proliferation and differentiation	0.60	0.48
Caspase 10	Intracellular protease, effector of apoptosis	0.61	1.22
Cofilin	Actin-binding protein	0.62	0.68
Higher in granulocytes			
p57	Cell cycle inhibitor	1.51	0.75
S-100	Cell-growth regulator	1.59	0.96
Spectrin	Cytoskeletal protein, associated with the plasma membrane	1.59	1.06
Cdc7 Kinase	Cell cycle control	1.65	1.00
DOPA Decarboxylase	Decarboxylase, conversion of dopa to dopamine in central nervous system	1.66	1.05
FAK-pSer910	Myeloid cells differentiation, adhesion, migration and cell cycle control	1.72	
TRF1	Binds to human telomers, has a negative effect on the length of the telomer	1.75	1.06
cytokeratin 7	Cytoskeletal protein	1.83	0.64
PKC b	Signal transduction, cell growth, differentiation and apoptosis	1.83	1.61
HSP70		1.87	1.09
PKC g	Signal transduction, cell growth, differentiation and apoptosis	1.90	1.33
Cyclin D1	Cell cycle control	1.98	1.07
RAF-pSer621	Member of the MEK kinase family, involved in the cell cycle, apoptosis and differentiation	1.98	
Tau-pSer199/202	Promotes tubulin polymerization and stabilizes microtubules	2.26	
FAK-pTyr397	Myeloid cells differentiation, adhesion, migration and cell cycle control	2.28	
Pyk2 - pTyr579	Myeloid cells differentiation, adhesion, migration and cell cycle control	2.30	
Pyk2 - pTyr580	Myeloid cells differentiation, adhesion, migration and cell cycle control	2.36	
FAK-pTyr577	Myeloid cells differentiation, adhesion, migration and cell cycle control	2.51	
Pyk2 - pTyr881	Myeloid cells differentiation, adhesion, migration and cell cycle control	2.58	
Nerve growth factor receptor	Binds Nerve Growth Factor	2.62	1.53
PKB-pThr 308	Control of cell cycle, proliferation, differentiation and apoptosis	2.82	

1.6-fold on the cDNA microarray) upon differentiation. NF-kappaB showed a 1.4-fold up-regulation in its mRNA levels, and there was a slight up-regulation of the NF-kappaB p65 subunit protein. mRNA level screening showed no difference in the level of TRF1 or caspases 3, 8, and 10, but changes in proteins abundance were observed. The TRF1 protein level increased in granulocytes 1.75-fold. In contrast, the caspase 10 protein level decreased 1.6-fold and protein levels of caspases 3 and 8 decreased 1.25-fold. The antibody microarray data were obtained using independent microarrays for each sample that had been Cy3 and Cy5 labeled.

Our immunoblotting analyses confirmed the microarray results for c-myc, Grb2 and beta tubulin (Figure 3). We showed that there was a decrease in the level of the c-myc protein in HL-60 cells treated with ATRA for 7 days with specific bands seen at the predicted molecular weight of 49 kDa. A slight decrease in the level of this protein was observed after only 4 days of treatment. The expression level of beta tubulin (MW 50 kDa) remained unchanged during granulocytic differentiation of HL-60 cells, which also correlated with the microarray data. Probing with the Grb2 antibody produced the predicted molecular weight band of 24kDa, with no clear difference between the HL-60 and granulocytic cell sample.

Discussion

Studies at the protein level have the potential to provide more direct information than studies at the genetic level, since most genes function through their protein products. However, mRNA expression profiles are also extremely valuable, since they provide other crucial points of view of cellular changes. The combination of both cDNA and antibody microarrays into a single experiment provides a huge source of data, which is invaluable from a global point of view, and greatly saves time and money.

Researchers have tried to find correlations between mRNA levels and limited protein expression data in order to predict the protein profile of the cell using mRNA experiments, which are more abundant and technically easier to perform than protein analyses. However, limited correlations between mRNA and protein expression data have been reported, and these correlations have been reported mainly in human cancers. [20–22]. Recent advances in high throughput protein analyses allow more robust analyses of the relationship between mRNA expression and protein abundance to be performed. The quantities of both molecules may be used as independent sources of information. There are two main

reasons for the low correlation between the quantities of these two molecules. First, there are many complicated post-transcriptional mechanisms involved the conversion of mRNA into proteins, and these have not yet been completely defined. The genes that show a large degree of variation in their expression throughout the cell cycle are controlled at the transcriptional level, and a high degree of correlation has been shown between the levels of mRNA and protein for these genes. In contrast, those genes that show minimal variation in their mRNA expression are more likely to show little or no correlation with the final protein levels, as the cell would likely control these proteins at the post-translational level [23]. Similarly, highly expressed genes tend to show a greater correlation between protein and mRNA levels than genes that are expressed at low levels. A low correlation between protein and mRNA levels has also been described for a group of less abundant proteins [24]. Second, proteins may differ substantially regarding their half lives *in vivo*. The cell can control the rates of degradation or synthesis for a given protein, and there is heterogeneity even among proteins that have similar functions [25].

Antibody microarrays have been widely used to profile proteins that define specific cancer types [26–28], and to detect post-translational modifications in malignant processes [29, 30]. The approach of combining antibody microarrays and cDNA microarrays for complex molecular cell profiling was used previously to look for differences in the expression patterns of muscle cells from a spinal muscular atrophy patient [31]. Differences were shown in the protein levels of 11 proteins. Nine of them were down-regulated and those were proven to be disease-specific. Although the down-regulation was relatively moderate, from 1.4- to 1.8-fold, these changes were confirmed for some of these proteins via western blot analysis. mRNA microarray analysis revealed down-regulation of the mRNA transcripts for most of proteins. For screening cellular pathways of interest, the specialized antibody microarrays may also be used. These only contain antibodies against a subset of proteins involved in a desired process (e.g., apoptosis, cell cycle or p53 pathway). There are further options for large-scale proteomic analysis other than protein microarrays. 2D-gels have been used widely to separate and visualize a few thousand proteins in a single experiment. Proteins can be identified subsequently by mass spectrometry (MS). MS is becoming a very popular protein profiling method, especially when coupled with matrix-assisted laser desorption/ionization (MALDI) or surface-enhanced laser desorption/ionization (SELDI) [32]. These approaches are very labor intensive.

The aims of our study were to use both cDNA and antibody microarray analyses to identify changes in mRNA and protein levels in HL-60 cells after ATRA treatment, and to determine how changes in mRNA levels correlate with changes in protein levels. We obtained a unique and complex molecular profile of the HL-60 human leukemic cell differentiation model. The HL-60 cells are predominantly promyelocytes but can spontaneously differentiate into morphologically mature cells, including metamyelocytes and neutrophils [33]. Addition of specific inducing agents such as ATRA significantly increases the levels of spontaneous differentiation, and most of the treated cells acquire the characteristics of mature granulocytes [34]. The HL-60 cell line is therefore a convenient model for functional and morphological hematopoietic differentiation *in vitro*.

Different tissues show increased levels of gene expression in certain chromosomal domains, and peaks are found in corresponding chromosomal regions. Therefore, it is predicted that the expression levels of the majority of RNA transcripts do not change during granulocytic differentiation [35, 36]. Our results indicate that the mRNA and protein levels of the majority of genes remain unchanged upon differentiation. However, there was modulation of the expression of a group of mRNA molecules. Many changes in mRNA transcription levels corresponded with changes in protein levels. The expression levels of these proteins were thus altered as a consequence of changes in mRNA levels, rather than by post-transcriptional effects. However, we also observed alterations in protein levels that were independent of mRNA levels.

If different slides are used to analyze different samples, the data must be normalized, because fluorescence units are arbitrary, not absolute. Therefore comparing the data from two different slides may not provide accurate results. To normalize the 19,200 spots on the cDNA microarray we used the LOWESS method, which is suitable for the large number of spots. However, the number of spots (448) on the antibody microarray is too small for reliable normalization by the LOWESS method. Therefore, we chose to normalize the antibody microarrays according to levels of proteins against house-keeping proteins on the microarray, even though normalizing the microarray data by summing the fluorescence intensities over all elements of the microarray had a similar result. Technically, the antibody microarray worked well with a high degree of similarity between the two data sets. The dynamic range of signal intensity on the antibody microarrays was over 30,000, showing that proteins expressed at a wide range of levels can be assessed. The microarray data was analyzed initially with JABS-I and then within Excel and MeV. The scatter plot of HL-60 signal intensity compared with that of the granulocyte sample showed the majority of antibody microarray data had a T_i ratio close to 1 (78% of the proteins and 84% of the mRNAs have T_i ratio between 0.667 and 1.5) (Figure 4A, B).

Using the antibody microarrays, we found that there were groups of proteins that were up- or down-regulated in the differentiated cells. The c-myc, c-jun, PKC beta, NF-kappaB, TRF1 and phosphorylated forms of Pyk2 and FAK are the most altered proteins in the differentiated cells (Table 1). Down-regulation of c-myc at the mRNA level correlates with the antibody microarray results. It has already been reported that there is a lower level of c-myc mRNA in ATRA-treated

HL-60 cells [37, 38]. The decreased c-myc expression in differentiating HL-60 cells appears to mimic the expression patterns in normal hematopoiesis, since a similar decrease in c-myc mRNA levels has been observed in normal bone marrow progenitor cells undergoing terminal differentiation in liquid suspension culture [39]. The c-myc gene is known to have oncogenic potential, and high levels of c-myc expression are associated with proliferation and block differentiation [40]. Some proteins were up-regulated during granulocytic differentiation. Increased levels of the phosphorylated forms of the FAK family members Pyk2 and PTK2 (FAK) were observed, however, the levels of the non-phosphorylated forms remained unchanged. These protein tyrosine kinases (PTKs) are involved in the differentiation and function of myeloid cells. Pyk2 was the second member of the FAK family of PTKs isolated. Pyk2 is known to be highly expressed in the cells of hematopoietic lineages [41]. Increased amounts of Pyk2 have been observed previously during granulocytic differentiation of HL-60 cells with dimethyl sulfoxide (DMSO) [42]. We observed increased levels of protein kinase C beta. Insufficient levels of this enzyme have been shown previously to be responsible for HL525 cell resistance to apoptosis, thus implicating this enzyme in the signaling pathway that leads to apoptosis in the HL-60 cell system [43]. NF-kappaB (p65) was activated by ATRA in HL-60 cells. Induction of differentiation, but not of proliferation, selectively activates diverse members of the NF-kappaB family of proteins. In differentiation-induced cells, NF-kappaB (p65) was translocated from the cytoplasm to the nucleus [44]. NF-kappaB has been reported to activate the tumor suppressor protein PTEN in leukemia cells stimulated by DMSO [45]. Our data suggest ATRA has an effect similar to DMSO upon NF-kappaB activation, which could be investigated further to improve the effects of ATRA against cancers such as various types of leukemia. Caspase types 3, 8 and 10 were shown to be down-regulated, although their mRNA levels remained unchanged, which would suggest protein degradation in the cell. Increased caspase expression was previously observed during granulocytic differentiation of HL-60 cells by ATRA, which resulted in spontaneous apoptosis [46]. Lower expression in our differentiated cell sample may be caused by removal of the apoptic cells out of the suspension using MACS® Dead Cell Removal Kit. Removal of the minority of apoptotic cells was reflected by the antibody microarray results with high sensitivity. Another gene product with a lack of correlation between mRNA and protein levels was TRF1. TRF1 is the major protein that binds to human telomeres and has a negative effect on the length of the telomere [47]. Hence, an enhanced level of TRF1 protein in differentiated cells may cause shortening of their telomeres and potential loss of their self-renewal capability.

The immunoblotting results suggest that antibody microarrays are relevant tools for large-scale protein analysis. The fact that the expression levels of beta tubulin were unchanged in western-blotting analysis indicates that this protein is suitable for microarray data normalization in this tissue.

The results obtained from cDNA microarrays and antibody microarrays clearly show the importance of integration of these progressive methods. This is demonstrated primarily by the existence of proteins for which expression levels do not correlate with the corresponding mRNA levels. On the other hand, a large number of proteins reflected their corresponding mRNA transcript levels. A normalization method based on housekeeping proteins allowed us to directly compare between independent antibody microarray slides. The number of genes on the cDNA chip is greater than the number of proteins available for microarray analysis. The automation and standardization regularly used with cDNA chips is not yet available for protein chips, however, important advances are expected in this field in the near future [48]. In conclusion, the findings of this study show that antibody microarrays in combination with cDNA microarrays are a very powerful tool. They may be used together to globally assess the molecular profile of cellular populations, especially in clinical studies.

This work was supported by MSM grant NPV II 2BO6052 and MSM 0021622430.

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