Gene expression networks underlying retinoic acid–induced differentiation of acute promyelocytic leukemia cells

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To elucidate the molecular mechanism of all-trans-retinoic acid (ATRA)–induced differentiation of acute promyelocytic leukemia (APL) cells, the gene expression patterns in the APL cell line NB4 before and after ATRA treatment were analyzed using complementary DNA array, suppression-subtractive hybridization, and differential-display–polymerase chain reaction. A total of 169 genes, including 8 novel ones, were modulated by ATRA. The ATRA-induced gene expression profiles were in high accord with the differentiation and proliferation status of the NB4 cells. The time courses of their modulation were interesting. Among the 100 up-regulated genes, the induction of expression occurred most frequently 12-48 hours after ATRA treatment, while 59 of 69 down-regulated genes found their expression suppressed within 8 hours. The transcriptional regulation of 8 induced and 24 repressed genes was not blocked by cycloheximide, which suggests that these genes may be direct targets of the ATRA signaling pathway. A balanced functional network seemed to emerge, and it formed the foundation of decreased cellular proliferation, maintenance of cell viability, increased protein modulation, and promotion of granulocytic maturation. Several cytosolic signaling pathways, including JAKs/STAT and MAPK, may also be implicated in the symphony of differentiation.

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Introduction

Acute promyelocytic leukemia (APL) is an interesting model in the study of human cancer because it is the first human malignancy that can be effectively treated with a cell differentiation inducer, all-trans-retinoic acid (ATRA). During the last decade, it was uncovered that the promyelocytic leukemia–retinoic acid response-α (PML/RARA) fusion gene, which was generated as a result of the APL-specific chromosome translocation t(15;17)(q22; q21) (translocation involving breaks at chromosome 15, long arm, band 22, and chromosome 17, long arm, band 21), plays a central role in leukemogenesis.1 On one hand, the creation of PML/RARA disrupts the normal location of the PML nuclear body or oncogenic domains (PODs)2,3 and interferes with the function of PML for growth inhibition and apoptosis regulation.4,5 On the other hand, the RARA/RXR pathway, which is indispensable to the granulocytic differentiation, was also damaged due to the sequestration of RXR by heterodimerization with PML/RARA.6 Moreover, both PML/RARA and RARA can interact with a large ubiquitous nuclear corepressor (N-CoR) complex (N-CoR/mSin3A/HDAC) by forming PML/RARA-CoR or RARA-CoR complexes.

Unlike the RARA-CoR complex, the PML/RARA-CoR complex is more stable and resistant to physiological ATRA treatment. PML/RARA competes with RARA for binding to the retinoic acid response elements (RAREs) of target genes and mediating the transcriptional repression through histone deacetylation by the CoR complex.7-13 Pharmacological doses of ATRA could trigger the degradation of PML/RARA and the reassembly of PODs.14 More recently, ATRA has been shown to cause a distinct conformational change of PML/RARA. This results in release of the CoR complex and subsequent recruitment of a coactivator (CoA) complex (CBP/P300, P/CAF, NcoA-1/SRC-1, P/CIP, and ACTR) and converts PML/RARA from transcription repressor to transcription activator. In contrast, the association of CoR with PLZF/RARA, a fusion receptor associated with an APL phenotype resistant to ATRA, could not be modulated by the ligand, even at very high concentrations.7-13 These findings further strengthen the concept that ATRA-induced differentiation is a novel cancer therapy based on transcriptional regulation.

Although the interaction between ATRA and the aberrant and wild type RAR-CoR/CoA complexes was largely elucidated, the molecular events downstream of the RAR complexes were still unclear. Therefore, using the ATRA-sensitive APL cell line NB1,15 as an in vitro model, we undertook a study based on methods allowing relatively large-scale transcriptional expression analysis to identify gene expression patterns in APL cells upon treatment with ATRA. A total of 169 genes, including transcriptional factors, signal transduction modulators, cell cycle promoters and inhibitors,

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and protein modulators, were confirmed to be ATRA-modulated and to form a harmonious network in the course of the ATRA-induced NB4 cell differentiation.

Materials and methods

Cell culture

Retinoic acid (RA)-sensitive NB4 cells were cultured under conditions previously described.\(^\text{16}\) The in vitro culture contained 1 μmol/L ATRA. For the cycloheximide inhibition test, NB4 cells were treated with 10 μg/mL cycloheximide for 30 minutes before ATRA addition and throughout the ATRA treatment. Total RNAs were extracted at indicated time-points using the Trizol reagent (Gibco BRL Life Technologies, Grand Island, NY). PolyA+ RNAs were purified using the Oligoex mRNA (messenger RNA) Kit (Qiagen, Hilden, Germany). Cell differentiation was evaluated for cell morphology changes using Wright’s staining, CD11b expression, and the nitroblue tetrazolium (NBT, Sigma Chemical Co, St Louis, MO) reduction test.\(^\text{2,18}\) For the detection of cell cycle, the cells were stained with propidium iodide (PI) (Sigma) and analyzed by a fluorescence-activated cell sorter (FACS).\(^\text{19}\) PML/RARA degradation was studied by indirect immunofluorescence with rabbit antihuman PML polyclonal antibodies.\(^\text{14}\)

Differential display-PCR

Differential display (DD)-PCR was performed with the Delta TM Differential Display Kit (Clontech Laboratories, Palo Alto, CA) following the instructions of the manufacturer. Reverse transcription reaction was performed with 2 μg total RNA, 1 μL complementary DNA (cDNA) synthesis primer (concentration, 1 μmol/L), and ribonuclease (RNase) free water to a total volume of 5 μL. After incubation for 3 minutes at 70°C, 2 μL 5 × first-strand buffer, 2 μL dNTP (deoxy nucleoside 5'-triphosphate) mix (total concentration, 5 mmol/L), and 1 μL MMLV-RT (reverse transcriptase) (concentration, 200 U/mL) were added and incubated for 1 hour at 42°C in an air incubator. The reactions were terminated by incubating for 10 minutes at 75°C before storage at −80°C. PCR amplification of a given reverse transcription reaction was carried out in duplicate. A 1-μL RT product was used for each subsequent PCR in a mixture (total volume, 20 μL) containing 1 × KlenTaq PCR reaction buffer, 50 μmol/L dNTP mix, 5 mmol/L Mg\(^{2+}\), 0.4 μL Advantage KlenTaq Polymerase with a different combination of 19 primers. The following PCR reactions were performed: 1 cycle of 5 minutes each at 94°C, 40°C, and 68°C; 1 cycle of 30 seconds at 94°C, 30 seconds at 40°C, and 5 minutes at 68°C; 23 cycles of 20 seconds at 94°C, 30 seconds at 60°C, and 2 minutes at 68°C; and an additional 7-minute cycle at 68°C. The amplified cDNAs were separated on 6% polyacrylamide sequencing gels. A total of 30 differentially expressed bands were obtained and subjected to further analysis.

Construction of cDNA libraries using suppression-subtractive hybridization

Suppression-subtractive hybridization (SSH) was performed according to the user’s manual (Clontech). The FORWARD library for isolating up-regulated genes was obtained by using cDNA of the NB4 cells treated with ATRA for 48 hours as a “test” and cDNA of un-treated NB4 cells was used as a “driver.” The REVERSE library for identifying down-regulated genes was constructed by replacing the cDNA of untreated cells as a “test,” while cDNA of NB4 cells treated with ATRA for 48 hours was used as the “driver.”

Differential screening of subtractive library

As indicated by the manufacturer’s protocol, 4 × 96 (384) clones from the FORWARD subtracted library were dotted in duplicate on nylon membranes and hybridized with α-\(^32\)P-labeled unsubtracted cDNA probes (tester and driver) as well as subtracted cDNA probes (FORWARD and REVERSE) to minimize the false-positive clones before embarking further confirmation. Genes showing equal hybridization signals were ruled out, while genes with different signals or without any signal were further investigated.

cDNA array

Membranes with 588 known genes (Clontech) were used, and hybridization was performed according to the manufacturer’s recommendations using the RNA from 3 independent sets of ATRA-stimulated NB4 cells at an indicated time-point. The signals were analyzed on Altabiassion software (Clontech) and normalized by the signal intensities of housekeeping genes. Only those genes with signal differences of more than 2-fold were considered as modulated.

Sequencing and bioinformatics analysis

Sequencing reactions were performed on a 9600 Thermal Cycler using the Dye Primer and Dye Terminator Cycle Sequencing Kit (Perkin-Elmer, Foster City, CA). All sequences were searched against GenBank and dbEST databases (version 108) for homology comparison by using the Genetics Computer Group (GCG) program package. The sequences were considered as part of the known genes if they shared 95% or more homology over at least a 100-bp (base pair) DNA sequence on the BLAST search. For those sequences representing previously unknown genes, GenBank and SWISSPROT searches were performed by using BLAST and FASTA. For those ESTs without significant homology, the motif search was carried out using the Blocks database, version 9.0 (http://www.blocks.fhcrc.org).

Northern blot analysis, semiquantitative RT-PCR, and real-time quantitative RT-PCR

Northern blot analysis was performed as previously described.\(^\text{20}\) For semiquantitative RT-PCR, G3PDH was used as an internal control with the following primer sets: sense, 5'-TGA AGG TCG GAG TCA ACG GAT TTG GT-3', and antisense, 5'-CAT GTG GCC CAT GAG GTC CAC CAC-3'. G3PDH and gene-specific primers were placed in a single tube PCR was performed in 20-μL volumes using the Perkin-Elmer Cyclase 480 (Perkin-Elmer) at the following conditions: 1 cycle for 5 minutes at 94°C; 20-30 cycles for 45 seconds at 94°C, 45 seconds at 56°C, and 1.5-2 minutes at 72°C. All RT-PCR reactions were repeated at least 3 times at different numbers of the extension cycle to avoid the false results of the PCR. The signals were normalized against 983-bp G3PDH using a densitometer. Triplicate real-time RT-PCR was performed using the ABI PRISM 7700 Sequence Detection System (Perkin-Elmer) with the same RNA templates of semiquantitative RT-PCR. β-Actin was coamplified as an endogenous control to standardize the amount of the sample RNA added to the reaction. The results of the indicated time-points after the ATRA treatments were plotted relative to the level at time zero.

Full-length cDNA cloning and sequencing

Two methods were used for full-length cDNA cloning: “in silico” cloning and the rapid amplification of the cDNA end (RACE). The in silico cloning started from novel cDNA sequences obtained in the current work, and overlapping dbEST sequences were assembled into contigs to get the open reading frames (ORFs), which were subject to further check by RT-PCR and subsequent sequencing. In RACE, self-made marathon-ready cDNAs were used as templates, and the touchdown PCR reaction was carried out according to the manufacturer’s protocol. Candidate bands were cut and cloned into pGEM-T (Promega Company, Madison, WI) or pT-Adv vector (Clontech). The sequences were analyzed for possible ORFs using the Autoassembler and Strider 1.2 program. If necessary, further RACE walking was performed until a complete ORF was obtained.

Results

Identification of the genes modulated by ATRA in NB4 cells

Using 3 techniques, a number of genes and cDNA clones were identified as being regulated in NB4 cells after treatment with
ATRA for approximately 8-72 hours. Triplicated cDNA array analysis revealed a total of 99 of 588 (16.8%) “hot spot” known genes to be modulated with more than a 2-fold difference of expression levels between ATRA-treated NB4 cells and untreated cells (Figure 1A). Among these 99 genes, 37 presented up-regulated signals, and 62 were down-regulated. The expression of 8 up-regulated genes and 2 down-regulated genes was confirmed by RT-PCR with coherent patterns (data not show). Using DD-PCR, 13 of 30 bands were identified and subsequently confirmed to represent genes with induced expression, while probes prepared from 7 of 30 bands gave signals of equal intensity before and after ATRA treatment, as detected by Northern blot analysis. Using Northern blotting, there were no signals in 10 of 30 bands.

SSH gave rise to a total of 703 clones (660 from FORWARD and 43 from REVERSE). Sequence analysis of these clones led to an assembly of 316 contigs (278 from FORWARD and 38 from REVERSE), of which 159 were known genes; 25 were matched mitochondria DNA, ribosomal RNA, and Alu repeats; and 132 were novel sequence fragments. The 159 known genes were subject to further analysis using differential screening and semiquan-


titative RT-PCR. Of these 159 genes, 39.6% were confirmed to be modulated (55 induced and 8 repressed), whereas the remainder showed either equal expression patterns or no obvious signals. Among these regulated genes, 10 were further confirmed by Northern blotting, which showed a pattern similar to RT-PCR. All novel sequence fragments were also further examined with differential screening and RT-PCR, and 49 of 132 showed an ATRA-modulated expression.

The expression patterns of 3 novel genes were further studied by real-time quantitative RT-PCR assay, and they turned out to be highly similar to those from the semiquantitative RT-PCR assay (Figure 1D). Moreover, 2 methods, in silico cloning (dbEST version 108.0) and RACE, were carried out to acquire full-length cDNA from novel gene sequences. The sequences were confirmed by RT-PCR and/or Northern blot analysis as being modulated by ATRA. Thus, we obtained 8 cDNA containing putative ORFs according to bioinformatic analysis. Of the 8 cDNA, 5 showed significant homology to known genes with potential structural and/or functional importance, whereas 3 exhibited only limited homology to known genes. Table 1 summarizes the major structural features of the 8 novel genes in the present work, temporarily

![Figure 1](https://www.bloodjournal.org)

Figure 1. The examples of 4 methods used to confirm ATRA-modulated genes in the differentiation of NB4 cells. (A) A partial view of a cDNA array result by comparing the transcriptional expression pattern in untreated NB4 cells (left) with that in ATRA-treated NB4 cells (right). The signals of housekeeping genes appeared in the bottom as an internal control. (B) The results of semi quantitative RT-PCR. The up-regulation of G3PDH and the down-regulation of myeloperoxidase seem to be protein synthesis-independent, while the up-regulation of transglutaminase and myeloid progenitor inhibitory factor was completely suppressed by the protein synthesis inhibitor cycloheximide. In the figure, A denotes ATRA, C, cycloheximide; and Ac, a combination of ATRA and cycloheximide. The number denotes hours after treatment. (C) The results of Northern blotting, with the lower panel showing the loading control (28s rRNA). (D) Relative mRNA levels of 3 novel genes (RIG-K, RIG-C, and RIG-I) were measured by semi quantitative RT-PCR and the Taq-man real-time quantitative PCR assay using the same samples. Data from the Taq-man analysis were normalized to mRNA concentrations according to the concentration of internal control actin and plotted relative to the level at time zero. The expression profiles of these genes, as measured by these 2 independent methods, were quite similar.
under the name of RIG (retinoic acid–induced gene), and their nucleic acid sequences, as well as deduced amino acid sequences, are now available in GenBank.

The overall results obtained using these 3 approaches seemed to be quite cohesive and complimentary. For example, RIG-E, RIG-G, and dioxin-inducible cytochrome P450 genes initially discovered by DD-PCR were also found to be up-regulated by SSH, whereas the induction of 16 genes, such as the monocyte chemoattractant protein (MCP), interleukin-8 (IL-8), the Src-like adaptor protein, and Bfl-1, were revealed by both cDNA array and SSH. The advantage of combining the 3 different methods was based on the concept that each of them might reflect a gene expression profile from a particular angle. The cDNA array used in this work only covers 588 known genes. DD-PCR generates a partial view of gene expression, while SSH may allow a more global approach for identification of both known and novel genes, although the efficiency of the method (in this work, 38.5%) needs to be further improved. After integration of the results by different techniques, a total of 169 genes were identified as ATRA-responsive, including 100 up-regulated and 69 down-regulated genes (Figure 2A-J).

Table 1. Details of 8 novel ATRA–up-regulated genes

<table>
<thead>
<tr>
<th>Gene name, perm</th>
<th>Length, bp</th>
<th>ORF, bp</th>
<th>Peptides, aa</th>
<th>Homology</th>
<th>Accession No.</th>
</tr>
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<tr>
<td>hUCE (RIG-A)</td>
<td>1223</td>
<td>48-506</td>
<td>153</td>
<td>mouse UBCM4</td>
<td>AF061736</td>
</tr>
<tr>
<td>hLAP (RIG-B)</td>
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<td>187-1743</td>
<td>519</td>
<td>leucine amino peptidase</td>
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<tr>
<td>hWSB-1 (RIG-C)</td>
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<td>266-1528</td>
<td>421</td>
<td>mouse WSB-1</td>
<td>AF069313</td>
</tr>
<tr>
<td>hPTP (RIG-D)</td>
<td>3610</td>
<td>73-2493</td>
<td>807</td>
<td>mouse hematopoietic cell protein tyrosine phosphatase</td>
<td>AF077031</td>
</tr>
<tr>
<td>hRD (RIG-H)</td>
<td>1390</td>
<td>8-976</td>
<td>323</td>
<td>rat retinol dehydrogenase type III</td>
<td>AF067174</td>
</tr>
<tr>
<td>SH001 (RIG-J)</td>
<td>1613</td>
<td>245-802</td>
<td>186</td>
<td>mouse FMLC1I (28.7% identities in 115 aa)</td>
<td>AF061739</td>
</tr>
<tr>
<td>SH002 (RIG-K)</td>
<td>4347</td>
<td>0-2319</td>
<td>773</td>
<td>human tilin (31.2% identities in 415 aa)</td>
<td>AF077041</td>
</tr>
<tr>
<td>SIH003 (RIG-L)</td>
<td>2107</td>
<td>381-1331</td>
<td>317</td>
<td>human ubiquitin C-terminal hydrolase (42.1% identities in 133 aa)</td>
<td>AF077040</td>
</tr>
</tbody>
</table>

The major structural features of the 8 novel genes in the present work, including their temporary name of RIG (retinoic acid–induced gene), nucleic acid sequences, open reading frames (ORFs), and deduced amino acid sequences, are now available in GenBank.

Figure 2. Functional classification of genes modulated by ATRA during differentiation of NB4 cells. A total of 169 ATRA-modulated genes (100 up-regulated and 69 down-regulated) are clustered into 10 groups (groups A-J) according to their functions. The expression pattern of each gene is displayed here as a horizontal strip, with the down-regulated genes on the left-hand side of the graph and the up-regulated ones on the right-hand side. For each gene, the ratio of mRNA levels in NB4 cells at the indicated time after ATRA treatment to its level in untreated (time zero) NB4 cells is represented by a color, according to the color scale at the bottom. Genes whose expression was resistant to cycloheximide are highlighted by using the yellow background in the column for gene names. Regarding genes also existing in Golub’s system with Affymetrix chips, different colors were used to label the column of their accession numbers. The genes with the same expression pattern in both systems are painted in rose; those modulated in our system but not in Golub’s are painted in sky-blue; while those with opposite patterns are painted in purple.
Functional categories of genes regulated by ATRA

The 100 up-regulated and 69 down-regulated genes by ATRA were classified into 10 categories according to their structures and/or functions (Figure 2A-J). Some transcription factors, such as c-JUN, ETR, ID-2, HOX-A1, and CEBPβ, were found to be induced. Interestingly, one component of CoA, ACTR, was also included in this group as a relatively early induced gene (12 hours). Genes participating in several important signal transduction pathways, including JAKs/STAT, cAMP/PKA, PKC, and calmodulin, were modulated. Other notable categories of genes included those responsible for protein modulation, such as UAE1 and SUMO-1, for apoptosis resistance, DAD-1 and Bcl-2, and GADD153, and p21WAF1/CIP1 for cell cycle exit. A number of genes reported to possess the function of proliferation suppression, including BTG1, Src-like adaptor protein, FGR, and LIMK, were also positively modified. The induced expression of some neutrophil function-related genes (eg, MCP-1, defensin, and X-CGD) may reflect biological activities needed in terminal granulocytic differentiation.

On the other hand, 69 down-regulated genes could be of functional importance in terms of cell growth regulation. Transcription factors known to be capable of promoting cell proliferation, including c-MYC, NFκB, and GATA2, were down-regulated shortly upon effect of ATRA. A cluster of genes responsible for DNA synthesis, repair, and recombination were also down-regulated. With regard to cell cycle checkpoint transition, the expression of both cyclin A and B1 was repressed. Of note, 3 families of mitogen-activated protein kinases, namely p38, JNK2, and ERK3, were all found with decreased levels of expression, suggesting that the MAPK/SAPK pathways could be transcriptionally modulated in favor of growth arrest. Some apoptosis agonists, such as the ICH-1L and FAST kinases, showed decreased levels of expression. In addition, myeloperoxidase, the cytotoxic marker of APL cells, was also down-regulated. It may be interesting to note that the expression of transcription factor AP-1 was biphasic because c-FOS was suppressed within 8 hours. This was followed by an increased expression at 24 hours, which coincided with the induction of c-Jun after 24 hours of ATRA treatment.

Identification of possible direct target genes of ATRA

We tried to find the genes whose transcriptional regulation was protein synthesis–independent and thus most likely represented target genes of RA receptors. By using the cycloheximide inhibition test, the response of 8 up-regulated genes to ATRA was not abolished by protein synthesis antagonist. That all these genes were up-regulated at an early stage (within 12 hours) was also in support of their direct induction by RA receptors. Among these 8 cycloheximide-resistant up-regulated genes, one of them is CEBPβ, which has been confirmed by other groups to be very important for the initiation of granulocytic differentiation. Cell cycle inhibitor p21WAF1/CIP1, apoptosis antagonists Bcl-2-related (Bcl-1) and GADD153 (CHOP), and some receptors and membrane proteins

Figure 2 (Cont’d)
(RIG-E, ICAM-1, and CD52) were also confirmed to be in this group. Surprisingly, the transcriptional modulation in 24 of 69 down-regulated genes appeared not to be inhibited by cycloheximide, and the onset of their down-regulation all occurred within 8 hours after treatment with ATRA (Figure 2A-J).

**Gene expression waves in concert with APL cell differentiation**

To receive insight into the gene expression network modulated by ATRA, it is important not only to obtain the gene catalog in the network but also to clarify the time course of the transcriptional regulation in each of the modulated genes. To this end, a better definition of the phenotypic changes of NB4 cells upon ATRA, with regard to time course, was necessary. At the early time-points (8 and 12 hours) of the ATRA treatment, there were no obvious morphological changes in the NB4 cells (Figure 3). The percentages of CD11b+ cells were still at the same level as that of time zero. PML/RARA degradation was not yet distinct. However, the increase of the G0/G1-phase cells and decrease of the S-phase cells were concomitant with the depression of cell proliferation that was started prior to 8 hours of ATRA treatment. After 24 hours of ATRA treatment, the differentiation markers of the NB4 cells began to be observable. CD11b+ and NBT+ cells gradually increased from 24-72 hours after treatment. The ratio of cytoplasm to nucleus was increased, and the chromatin condensation occurred gradually. There was no evident apoptosis observed during the whole course of the treatment. Meanwhile, PML/RARA degradation could be readily observed. Therefore, the time course of differentiation could be roughly divided into 2 phases: before 12 hours and after 12 hours of treatment.

In agreement with the above observation, the expression patterns of the 169 genes were studied using the cDNA array, semiquantitative RT-PCR, and/or Northern blot analysis on NB4 cells prior to ATRA treatment and at 8, 12, 24, 48, and 72 hours after treatment (Figure 1B,C). (For some genes, Northern blot analysis was performed as early as 4 hours.) Among the 100 genes with up-regulated expression, 53 genes (53%) were induced within 12 hours, 46 genes (46%) were induced between 24 and 48 hours, and only 1 gene was induced after 72 hours. In contrast, the modulation patterns of the 69 down-regulated genes were quite different because the expression level in 59 genes rapidly declined 8 hours after ATRA treatment, and the expression was suppressed in only 10 genes after 12 hours. Of note, 9 genes suppressed early by ATRA treatment showed increased expression or were restored to basal expression at a late stage (Figure 2A-I).

The time course of the regulated gene expression patterns was highly associated with the differentiation status of NB4 cells. Genes modulated before 12 hours (such as CEBPe and p21WAF/CIP) were thought to be more important for the initiation of differentiation. Those with modulated expression after 12 hours (such as ICAM1 and neutrophil cytosol factor–1) might be more tightly associated with the progression of differentiation and/or functional adjustment of the maturing cells. The modulation of cell cycle regulators (the up-regulation of p21WAF/CIP and the down-regulation of cyclin A, cyclin B, etc), DNA synthesis and/or repair, and recombination proteins (eg, double-stranded RNA binding proteins, ERCC3 and XRCC1) at quite an early time course of ATRA treatment corresponded to the cell cycle arrest and proliferation inhibition. Most of the protein modulators were up-regulated after 12 hours of ATRA treatment, and this was highly related to the degradation of PML/RARA and the restoration of PODs. The apoptosis-related genes were regulated at an early stage (8 hours) and lasted up to 72
Discussion

During the last few years, we and several other groups have isolated, in a piecemeal way, RA-regulated genes during ATRA-triggered granulocytic differentiation.\(^1\) However, there was no systematic survey of gene expression regulation upon the effect of ATRA, and few genes were known to be direct targets of RA in APL cells. Only recently have innovative tools, such as cDNA microarray, allowed a more global approach of the analysis of transcriptional regulation including regulation in APL cells.\(^40\) In the present work, which applied several new techniques, 100 genes, including 8 novel ones, were found to be up-regulated by ATRA, while 69 genes were down-regulated. A total of 169 genes were regulated by ATRA, and they may represent approximately 0.8%-1.7% of all genes expressed in APL cells (if the estimation that about 10 000-20 000 genes are expressed in a given cell type holds).\(^51\) There are some overlaps between our results and those of Tamayo et al.,\(^40\) whose group used Affymetrix chips to profile the transcriptional response of NB4 cells to ATRA.

A total of 48 up-regulated and 39 down-regulated genes in our system were also included in the system described by Tamayo et al.\(^40\) In addition, 42 of 48 up-regulated genes and 20 of 39 down-regulated genes were found to have similar expression patterns in both systems, which suggests a relatively good overall agreement (73.6%) between the results of these 2 groups with different technical approaches. However, 4 of 48 up-regulated genes and 15 of 39 down-regulated genes picked up in our system were not found to be modulated in the system of Tamayo et al. Moreover, the modulation patterns of 6 genes were contradictory between the 2 systems. It is worth pointing out that for the 2 up-regulated genes with a discrepancy (CD11A and LIMK1), our results of cDNA array were confirmed by RT-PCR. There could be several reasons to explain these differences. Because all genes with disparate results were those with only 2-fold to 5-fold modulation, we deduce that the difference could be caused by distinct sensitivities of the analysis systems employed. Another possibility is that NB4 cells in different laboratories could be subject to slightly distinct culture conditions, which could affect expression of some of the genes.

When RA-modulated genes were analyzed for their functions, a picture of well-coordinated choreography emerged, and this may reflect an elegant and intricate cellular program for the commitment to differentiation. For example, many studies suggested that the initiation of differentiation required the transcriptional activation of specific genes leading to proliferation arrest and cell cycle exit. Supporting this notion, indeed, a number of genes known to be able to suppress proliferation and cell cycle progress, such as c-MYC, c-MYB, GATA2, XRCCl, P55CDC, cyclin A, and cyclin B1, were down-regulated at 8 hours and 24 hours, respectively, after ATRA treatment. The cycloheximide inhibition test suggested that the dissection of different gene expression “waves” during cell differentiation. The cycloheximide inhibition test suggested that the dissection of different gene expression “waves” during cell differentiation.

\[\text{RA} \rightarrow \text{ΔN} \rightarrow \text{p21 WAF1/CIP1} \rightarrow \text{G1 arrest} \]

\[\text{RA} \rightarrow \text{ΔN} \rightarrow \text{p21 WAF1/CIP1} \rightarrow \text{G2 arrest} \]

\[\text{RA} \rightarrow \text{ΔN} \rightarrow \text{p21 WAF1/CIP1} \rightarrow \text{G2 arrest} \]

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models of differentiation, the induction of $p21^{WAF1/CIP1}$ appeared to be essentially required for the G1-S-phase arrest. One could therefore imagine that $p21^{WAF1/CIP1}$ may act in the upstream of the differentiation course (Figure 4). The puzzle that 24 genes seem to be down-regulated with no requirement for protein synthesis represents a challenge to the current model, which has no explanation for the ligand-dependent transcriptional repression. Several working hypotheses could, nevertheless, be proposed. First, there could be a general competition mechanism for the binding of transcription factors to CoA or CoR molecules. Second, the release of wild type PML and PLZF, which were both found to be sequestered by PML/RARA, could restore their function as growth suppressors. Of note, PLZF has been shown to repress the expression of the cyclin A gene. Third, there could be an opposition between the effects of RA signaling and AP-1.

The significance of cross-talk or coupling between nuclear and cytosolic signalings in APL cell differentiation has recently drawn much attention. In this work, we found that a number of genes encoding molecules involved in important cytosolic signal transduction pathways were modulated by ATRA. These include cytosolic kinase signal transducers such as cytokine receptors STAT5/ISGs, cAMP/PKA, and MAPK/P38/JNK. The functions of the 2 novel genes, RIG-C and RIG-D, should be further studied because they share, respectively, 92.6% and 79% amino acid identities with mouse WSB-1 and hematopoietic intracellular protein tyrosine phosphatase, both being important regulators in signal transduction.

In conclusion, by applying large-scale screening of the differentially expressed genes during ATRA-induced APL cell differentiation, we identified 169 modulated genes, which could be divided into 10 functional groups. A model of gene regulatory network during this process was thus obtained and provided previous datasets for further work to ultimately clarify the mechanism of APL leukemogenesis and ATRA-induced differentiation.

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