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Gene Expression Profiling during All-*trans* Retinoic Acid-Induced Cell Differentiation of Acute Promyelocytic Leukemia Cells

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Using cDNA microarrays we determined the gene expression patterns in the human acute promyelocytic leukemia (APL) cell line NB4 during all-trans retinoic acid (ATRA)-induced differentiation. We analyzed the expression of 12,288 genes in the NB4 cells after 12 hours, 24 hours, 48 hours, 72 hours, and 96 hours of ATRA exposure. During this time course, we found 168 up-regulated and more than 179 down-regulated genes, most of which have not been reported before. Many of the altered genes encode products that participate in signaling pathways, cell differentiation, programmed cell death, transcription regulation, and production of cytokines and chemokines. Of interest, the CD52 and protein kinase A regulatory subunit α (PKA-Rlα) genes, whose products are being used as therapeutic targets for certain human neoplasias in currently ongoing clinical trials, were among the genes observed to be markedly up-regulated after ATRA treatment. The present study provides valuable data to further understand the mechanism of ATRA-induced APL cell differentiation and suggests potential therapeutic alternatives for this leukemia. ***(J Mol Diagn 2003, 5:212–221)***

Acute promyelocytic leukemia (APL) is a subtype of acute myeloid leukemia characterized by the accumulation of cells arrested at the promyelocytic stage of myeloid differentiation. This leukemia exhibits a specific chromosomal translocation t(15;17) involving the promyelocytic leukemia (PML) gene locus on chromosome 15, and the retinoid acid receptor α (RARα) locus on chromosome 17. This translocation generates a chimeric fusion gene PML-RARα, which encodes a protein that functions as an aberrant nuclear receptor considered to be the cause of APL.[1,2]

APL cells are extremely sensitive to all-*trans* retinoic acid (ATRA), which induces APL cell differentiation into mature granulocytes and results in cell apoptosis.[3] Treatment of APL patients with ATRA in addition to chemotherapy yields a high rate of complete remission and long-term survival [4–6]. Therefore, ATRA had the distinction of being the first "differentiation therapy" drug for cancer.

Almost all APLs are associated with a chromosome translocation involving the rearrangement of the RARα gene. Five different chromosome translocations fusing RARα to distinct partner genes (PML, PLZF, NuMA, NPM, and STAT5b) have been described in APL.[1] However, the impairment of the myeloid differentiation is not a direct result of the translocation. Instead, the disruption of RARα function may alter the expression of a subset of ATRA-inducible genes critical for myeloid differentiation from the promyelocytic stage to the mature granulocytes. In the past decade great efforts have been devoted to the identification of novel gene(s) affected by the t(15;17) chromosome translocation resulting in blocked differentiation at the promyelocytic stage. Several candidates have been proposed, including ATRA-induced genes, RARα target genes, signaling pathway proteins, and transcription factors.[7–10]

It is generally believed that ATRA-induced differentiation of APL cells to a mature state is mediated, at least in part, through the regulation of gene transcription. Therefore, to gain insights into the molecular events associated with cell differentiation, we used the APL cell line NB4 and cDNA microarray technology to elucidate the transcription events occurring in the ATRA-induced cell differentiation process. DNA microarray technology allows the simultaneous analysis of multiple gene expression patterns,[11] an approach that should help elucidate the molecular mechanism of ATRA-induced NB4 cells differentiation. Due to the limitation of gene numbers and types in commercially available DNA arrays, we prepared cDNA chips with selected cDNA clones. The DNA chips used in our microarray analysis were composed of

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12,288 genes encoding proteins that are involved in the major signal transduction pathways (ie, cAMP-dependent protein kinase A, protein kinase C, and mitogen activated protein kinase), production of cytokines and chemokines, transcriptional regulation, cell growth and apoptosis, and the interferon system. We also included numerous expressed sequence tags (EST) unknown genes, to identify novel genes that may play important roles in APL cell differentiation. Our studies revealed that a large number of genes were modulated by ATRA, the majority of which have not been observed before. This approach provided a large amount of data that may help improve our understanding of the pathogenesis of APL.

Materials and Methods

Chemicals

All-*trans* retinoic acid (ATRA), nitroblue tetrazolium (NBT), and phorbol 12-myristate 13-acetate (PMA) were purchased from Sigma (St. Louis, MO).

Cell Culture

NB4 cells were cultured in RPMI 1640 medium (Life Technologies Inc., Grand Island, NY) plus 10% fetal calf serum, 50 units/ml penicillin G, and 50 μg/ml streptomycin. Cells (5×10^6/ml) were incubated at 37°C in the dark with or without 1 μmol/L ATRA for 12, 24, 48, 72, and 96 hours, respectively. At each time point, the cells with or without ATRA treatment were collected and washed with phosphate-buffered saline (PBS) three times and then stored at −70°C for RNA preparation and cDNA microarray analysis. Only ATRA-induced or modulated gene expression were highlighted by either increase or decrease in folds by comparison of treated sample to the corresponding control sample at the same time point. This type of design eliminated non-specific gene expression that occurred spontaneously in the cultured cells without ATRA treatment. Pretreatment cells were not collected because the purpose of the experimental design was to detect differential expression of ATRA-regulated genes.

cDNA Microarray

Total RNA was extracted from NB4 cells using RNeasy kit (Qiagen Inc., Valencia, CA). cDNA was synthesized from 15 μg of each RNA sample and labeled with Cy3 or Cy5 (Amersham Pharmacia Biotech, Uppsala, Sweden). The RNA extracted from untreated NB4 cells was labeled with Cy3 and the RNA from ATRA treated samples with Cy5. Once the reference and experimental probes were created, equal amounts of the two probes were combined and hybridized to our cDNA microarray slides. The microarray slide, which contains 12,288 sequence-verified clones obtained from Research Genetics (Huntsville, AL), were prepared using a MicroGrid TAS II microarrayer (Biorobotics Inc., Cambridge, UK). The hybridization was performed under a coverslip in a specially designed hybridization chamber (Array-It, Inc., Sunnyvale, CA). The hybridization of the probes to the array was allowed to proceed for 16 hours at 60°C. Following hybridization, the slides were washed and spun-dried. For each sample, the hybridization was repeated three times to confirm the results. Detection of probes hybridized to the array was accomplished using the GMS 418 Scanner (Affymetrix, Santa Clara, CA; Genetic Microsystems, Inc). The image data were captured by scanning the slide twice, the first time at 532 nm (Cy3 labeled) and second time at 635 nm (Cy5 labeled). This process generates two 16-bit TIFF image files, one for each wavelength. The two computer images produced from the scanner were combined and the numerical data for each spot was extracted using AnalyzerDG (Molecularware Inc., Cambridge, MA). The intensity data along with background and error measurements were stored into a text file. To determine whether the data quality for each spot was sufficiently good to warrant subsequent analyses, and to eliminate unreliable elements with expression statistically too close to the background, a statistical program was used that identifies (flags) spots with low intensity/background ratio. Reverse labeling of some housekeeping genes was performed to correct any potential artifactual differences in expression levels, which may result from differences in labeling efficiencies. This systemic variation was corrected during the data normalization. Normalization calculation was based on those spots with signal greater than 6 SD of the background distribution in both channels. Genes showing differences in expression between two groups that were statistically significant were clustered with cluster software.[12] Genes with more than twofold increase (>2.0) or decrease (<0.5) in signal intensity ratio were considered as ATRA-modulated genes.

RT-PCR

Total RNA was isolated from NB4 cells treated and untreated with ATRA (1μmol/L). The first-stranded cDNA synthesis was performed in a 20 μl reaction mixture containing 5 μg RNA, 5 mmol/L dNTP, 100 ng of random primers and 200 U of Superscript II reverse transcriptase (Life Technologies, Inc.). The mixture was incubated at 42°C for 50 minutes and the cDNA was amplified for 30 cycles with denaturation at 94°C for 30 seconds, annealing at 56°C for 30 seconds and extension at 72°C for 1 minute. The primers used were MAP4K2 (451 bp), forward: 5′-GAGTGGGAGCCTGCTGCAGTC-3′, reverse 5′-CAGGTTGAGTGTGTAGATGCC-3′. PKA-RIα (788 bp), forward 5′-CGCAGCCTTCGAGAATGTGAG-3′, reverse 5′-CACTGGTTCCAATGCATCAGC-3′. PKC-δ (461 bp), forward: 5′- TGGAAGTCGACGTTCGATGCC, reverse 5′-CATCTGCCGATGATCTTGTCG-3′. Tissue factor (311 bp), forward: 5′-CGCCAACTGGTAGACATGGAG-3′, reverse 5′-ACATCCTTCACAATCTCGTCG-3′; C/EBP-α (344 bp), forward 5′-AAGGCCAAGAAGTCGGTGGA-3′, reverse 5′-CAAGCCTCGAGATCCGGCGA-3′. The PCR products were separated by electrophoresis in a 2.0% agarose gel.

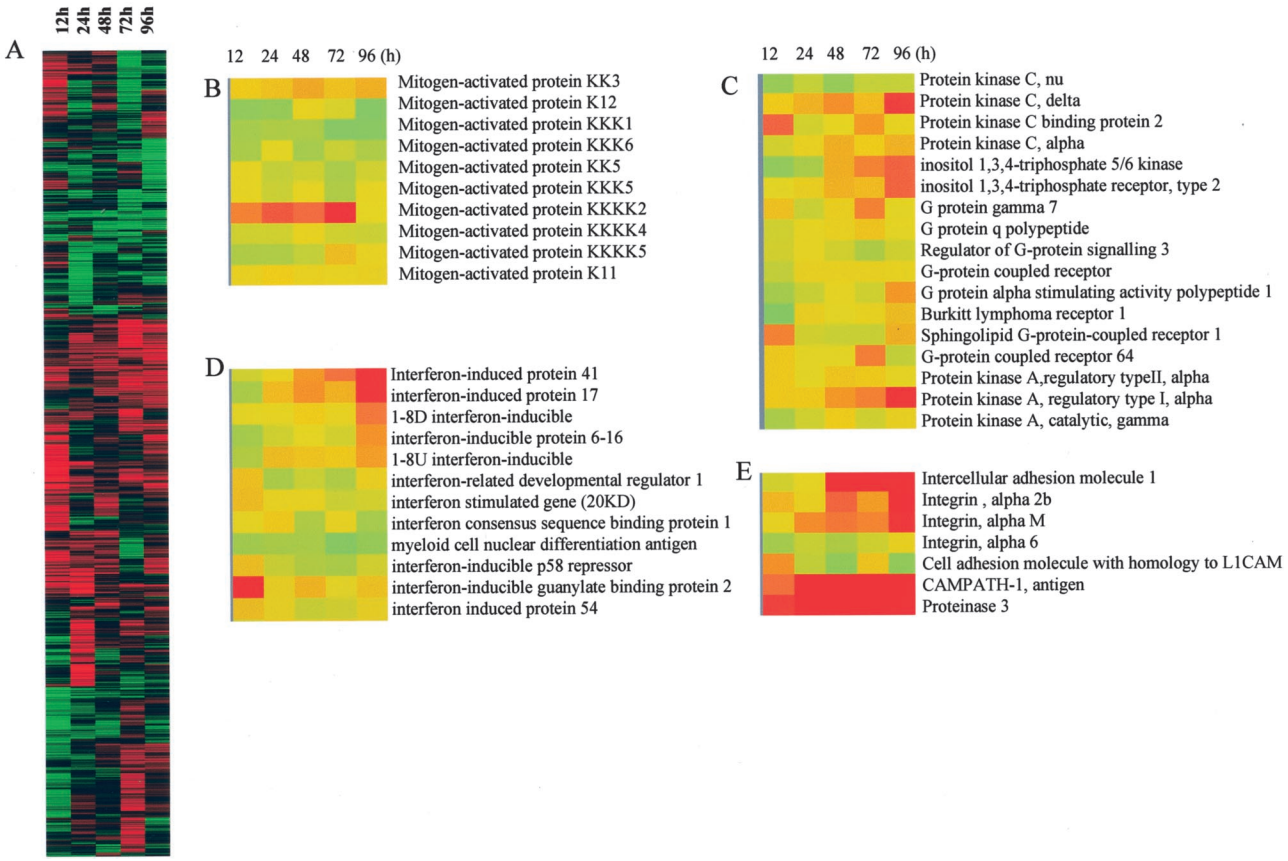

Figure 1. Expression profiles of 12,288 genes in NB4 cells with or without ATRA (1 μmol/L) treatment. **A:** Hierarchical clustering of 12,288 genes based on their expression profiles in NB4 cells treated with ATRA at 12 hours, 24 hours, 48 hours, 72 hours, and 96 hours. Each row represents a single gene on the microarray, and each column a separated sample at a different time point. The expression profile of functionally related genes: mitogen activated protein kinase (**B**), protein kinase C and protein kinase A-related genes (**C**), IFN-induced genes (**D**), and neutrophil marker protein genes (**E**).

NBT Reaction

Cells (2×10^5) were added to 200 μl of 1 mg/ml NBT solution contained 3×10^{-7} M PMA in PBS buffer, and incubated at 37°C for 40 minutes. Cytospin preparations were made from 100 μl of the cell suspension and allowed to air dry. The NBT-positive cells were scored by counting at least 200 cells under microscopic examination.[13]

Flow Cytometry Analysis of Cell Cycle and CD11b Expression

10^6 NB4 cells were incubated with an anti-CD11b-phycoerythrin (PE)-conjugated antibody (Serotec Inc., Raleigh, NC) in PBS buffer on ice for 30 minutes. PE-labeled isotype-matched immunoglobulin was used as negative control. The cells were washed three times with PBS and resuspended in 300 μl of PBS. PE fluorescence was measured using a FACScan flow cytometer (Becton Dickinson Biosciences, San Jose, CA) and 10^4 cells were analyzed per sample as previously described.[14] For cell cycle analysis, the cells were stained with propidium iodide (PI) and analyzed by flow cytometry, as described.[15]

Results and Discussion

Gene Expression Profiling of ATRA-Induced NB4 Cell Maturation

We compared the gene expression profiles of ATRA-treated cells to those of untreated cells to generate the fluorescence intensity ratio of treated *versus* control signals, as indicated in methods. The data generated from microarray experiments identified that approximately 347 genes, 2.8% of the total 12,288 cDNA elements on the array, were differentially expressed (> twofold up-regulated or down-regulated) in the ATRA treated cells compared to the control cells. It is worth noting that the up-regulated genes at each time point were not always the same ones (Figure 1A). Table 1 and 2 list the up-regulated and down-regulated known genes induced by ATRA at the indicated time points.

Confirmation of Altered Gene Expression by RT-PCR

We confirmed with RT-PCR the microarray data for ATRA-induced selective gene expression profiles at various

Table 1. List of Genes Up-Regulated in NB4 Cells by ATRA (Fold of Change)

Name		12 hour	24 hour	48 hour	72 hour	96 hour
DEFA4	defensin, alpha 4, corticostatin	1.5	7.25	38.06	41.44	152.62
HP-1	corticostatin/defensin HP-1 protein	1.94	3.86	15.09	24.7	58.55
ICAM1	intercellular adhesion molecule 1	1.06	3.06	3.12	7.2	12.38
K6HF	cytokeratin type II	1.21	1.32	4.54	5.27	10.01
PRTN3	proteinase 3	2.08	4.61	7.43	6.53	9.56
PDI2	peptidyl arginine deiminase, type II	1.98	1.98	3.56	1.85	7.26
PENK	proenkephalin	0.88	1.39	4.27	3.67	7.25
MYBL2	v-myb avian myeloblastosis viral oncogene homolog-like 2	1.2	0.94	2.17	1.45	6.74
CDW52	CAMPATH-1 antigen	1.77	3.66	5.38	3.51	6.49
AAD27764	tyrosine phosphatase homolog	1.61	1.75	2.5	2.35	5.22
CYP26A1	cytochrome P450, subfamily XXVIA, polypeptide 1	1.67	1.22	2.29	1.9	5.31
CAPN4	calpain, small polypeptide	0.87	1.35	1.56	2.4	4.87
CADPS	Ca2+-dependent activator protein for secretion	0.99	1.47	2.84	2.54	4.64
BM040	bone marrow protein	2.0	2.37	3.81	2.53	3.62
BC002447	cationic amino acid transporter	0.74	1.29	2.05	2.41	3.6
PSME2	proteasome activator subunit 2	1.56	2.65	4.26	2.37	3.6
DUSP6	dual specificity phosphatase 6	1.05	1.54	1.81	2.1	3.3
TCIRG1	T-cell, immune regulator 1	1.1	1.5	2.21	2.1	3.29
HOXB7	homeo box B7	1.62	1.4	1.92	1.37	3.27
ITGA2B	integrin, alpha 2b	0.95	1.16	2.17	1.69	3.23
OAS1	2′,5′-oligoadenylate synthetase 1	0.92	1.12	1.51	2.01	3.18
SH3BGRL	SH3-binding domain glutamic acid-rich protein like	0.98	1.38	1.99	2.29	3.1
XP03817	hypothetical protein	1.02	1.53	1.9	2.16	3.0
CREG	cellular repressor of E1A-stimulated genes	1.38	1.34	1.74	1.93	2.91
FYB	FYN-binding protein (FYB-120/130)	1.77	1.86	2.31	2.55	2.91
MAP4K2	mitogen-activated protein kinase kinase kinase kinase 2	0.99	2.31	2.13	2.88	1.04
ACAA1	acetyl-coenzyme A acyltransferase 1	0.94	1.17	1.5	1.56	2.9
HLA-G	HLA-G histocompatibility antigen, class I, G	1.57	1.51	2.01	1.79	2.87
IL5RA	interleukin 5 receptor, alpha	2.17	1.32	1.77	2.81	1.3
KCNC1	potassium voltage-gated channel, member 1	1.29	1.05	2.04	1.66	2.84
IFI41	interferon-induced protein 41, 30kD	1.47	1.11	1.71	2.05	2.76
PRKAR1A	protein kinase A regulatory subunit 1 alpha	1.26	1.29	1.71	1.92	2.75
ITGAM	integrin, alpha M	1.47	1.81	2.01	1.86	2.74
PHTF	putative hemeodomain transcription factor	1.51	1.78	1.2	1.64	3.58
IFI17	interferon-induced protein 17	0.91	1.42	1.76	1.5	2.72
BPI	bactericidal/permeability-increasing protein	0.88	0.88	1.51	2.52	2.67
FADSD6	delta-6 fatty acid desaturase	2.18	2.11	2.37	1.41	2.6
SAT	spermidine/spermine N1-acetyltransferase	0.85	1.34	1.95	1.62	2.57
PTPNS1	protein tyrosine phosphatase, non-receptor type substrate 1	0.88	1.1	1.64	1.71	2.57
SCYA20	small inducible cytokine superfamily A member 20	1.41	0.87	0.98	1.38	2.0
SCYA14	small inducible cytokine superfamily A member 14	0.77	1.17	1.17	2.52	1.37
PRKCD	protein kinase C, delta	1.28	1.53	1.81	1.43	2.56
PTPN22	protein tyrosine phosphatase, non-receptor type 22	0.93	1.42	1.49	1.8	2.44
DBI	diazepam binding inhibitor	1.34	1.38	2.02	1.36	2.39
AK3	adenylate kinase 3	1.02	1.27	1.87	1.65	2.27
MAFG	v-maf musculoaponeurotic fibrosarcoma, protein G	1.51	1.89	2.45	1.35	2.24
ADD3	adducin 3 (gamma)	1.41	1.26	1.62	1.88	2.24
MYL4	myosin, light polypeptide 4	1	1.55	1.91	1.54	2.23
ITPK1	inositol 1,3,4-triphosphate 5/6 kinase	0.8	0.72	1.58	1.9	2.22
ITPR2	inositol 1,4,5-triphosphate receptor, type 2	1.02	1.21	1.52	1.44	2.21
PPIF F	peptidylprolyl isomerase	0.83	1.38	2.03	1.19	2.2
SUPT3H	suppressor of Ty (S. cerevisiae) 3 homolog	1.24	0.94	1.15	3.05	2.17
CD68	CD68 antigen	1.21	1.34	1.94	1.25	2.16
MTMR2	myotubularin related protein 2	0.84	1.14	1.43	0.98	2.07
PTMA	prothymosin, alpha (gene sequence 28)	1.22	1.55	2.44	1.42	2.07
CYCL	cytochrome c-like antigen	0.97	1.09	1.47	1.58	2.05
SCD	stearoyl-CoA desaturase	1.23	1.58	2.6	1.44	2.05
TCF2	transcription factor 2	0.91	1.07	1.35	1.74	2.05
NIPSNAP1	4-nitrophenylphosphatase	0.83	1.25	1.44	1.11	2.04
1-8D	interferon-inducible	0.71	1.09	1.22	1.12	2.03
BIK	BCL-2 iteracting killer	1.01	1.35	1.33	1.04	2.02
PLSCR1	phospholipid scramblase 1	1.03	0.95	1.71	1.78	2.02

time points. The RT-PCR products were analyzed and normalized by using actin as an internal control. As shown in Figure 2, the expression patterns of three up-regulated genes (*PKA-RIα, MAP4K2*, and *PKC-δ*) and two down-regulated genes (*TF* and *C/EBP-α)* were consistent with the data obtained from microarray analysis. Our results were entirely consistent with previous findings of ATRA-induced protein(s) reported in the literature by other methods such as differential display, microarray analysis, and Northern blotting.[7,16] To verify the results of

Table 2. List of Genes Down-Regulated in NB4 Cells by ATRA (Fold of Change)

Name		12hr	24hr	48hr	72hr	96hr
NYP	neuropeptide Y	0.9	0.72	0.49	0.72	0.19
CFL1	cofilin 1 (non-muscle)	1.21	1.15	0.71	0.2	0.32
TPS1	tryptase, alpha	0.96	0.4	0.75	0.44	0.41
CDH1	cadherin 1, E-cadherin (epithelial)	0.41	0.68	0.38	0.16	0.34
CEP3	Cdc42 effector protein 3	0.66	0.58	0.35	0.18	0.09
GTF2H4	general transcription factor IIH	1	0.59	0.41	0.56	0.52
ASS	argininosuccinate synthetase	0.34	0.33	0.21	0.23	0.24
TP53TG3	TP53TG3 protein	0.9	0.1	0.08	0.23	0.25
P-B	salivary proline-rich protein	0.80	0.79	0.22	0.25	0.4
PCYT1B	phosphate cytidylyltransferase 1	0.8	0.9	0.33	0.29	0.04
IGFBP2	insulin-like growth factor binding protein 2	0.36	0.51	0.42	0.29	0.4
MNDA	myeloid cell nuclear differentiation antigen	1.08	0.74	0.72	0.29	0.21
MAP3K1	mitogen-activated protein kinase kinase kinase 1	0.65	0.76	0.79	0.31	0.38
EBBP	estrogen-responsive B box protein	0.71	0.71	0.73	0.32	0.44
KIAA0266	KIAA0266 gene product	0.71	0.43	0.75	0.34	0.25
SEMG2	semenogelin II	0.88	0.65	0.24	0.36	0.59
PTD010	PTD010 protein	0.48	1.28	0.68	0.37	0.46
CEBPA	CCAAT/EBP, alpha	1.19	0.62	0.8	0.38	0.29
RAD53	protein kinase Chk2	1.33	0.75	0.87	0.61	0.19
20D7-FC4	hypothetical protein	0.59	0.5	0.59	0.38	0.42
POLH	polymerase eta	1	0.38	0.03	0.71	0.19
SECTM1	secreted and transmembrane 1	1.08	1.15	1.33	0.38	0.7
BAI1	brain-specific angiogenesis inhibitor 1	1.05	0.68	0.33	0.87	0.32
GABRB3	gamma-aminobutyric acid A receptor beta 3	0.83	0.33	0.2	0.41	0.37
BC10	bladder cancer related protein	1.02	1.08	1.04	0.78	0.38
SPS2	selenophosphate synthetase 2	1.03	0.71	0.39	0.43	0.08
MSF	megakaryocyte stimulating factor	0.82	0.12	0.24	0.43	0.45
BCL2	B-cell CLL/lymphoma 2	0.65	0.57	0.49	0.55	0.75
TNF	tumor necrosis factor	1.23	1.21	0.69	0.43	0.52
MYO1E	myosin IE	1.45	0.43	0.04	0.43	0.59
KPNB1	karyopherin (importin) beta 1	0.58	0.82	0.71	0.43	0.68
UST	uronyl 2-sulfotransferase	0.49	0.67	0.32	0.44	0.52
PCYT2	phosphate cytidylyltransferase 2, ethanolamine	0.78	0.52	0.6	0.44	0.66
SOX10	SRY (sex-determining region Y)-box 10	0.5	0.28	0.46	0.45	0.44
FPGT	fucose-1-phosphate guanylyltransferase	1.37	0.96	1.07	0.45	0.61
PCDH2	protocadherin 2 (cadherin-like 2)	0.79	0.77	0.82	0.46	0.7
LY9	lymphocyte antigen	1.05	0.32	0.35	0.75	0.39
INPP4A	inositol polyphosphate-4-phosphatase, type 1, 107kD	0.75	0.4	0.3	0.5	0.47
WSX-1	class I cytokine receptor	0.99	0.74	0.99	0.5	0.61
PTPN3	protein tyrosine phosphatase, non-receptor type 3	0.58	0.72	0.48	0.5	0.69
TF (F3)	tissue factor	1.03	0.75	0.69	0.52	0.46
PLU-1	putative DNA/chromatin binding motif	0.67	0.53	0.52	0.51	0.5
HFL-EDDG1	erythroid differentiation and denucleation factor 1	1.07	0.91	0.78	0.52	0.55
ACVR1	actin A receptor type 1	1.18	0.7	0.99	0.79	0.58

microarray data, we also used neutrophil marker protein cDNAs as internal controls in our array chips, including defensins, proteinase 3, *CD11b, ICAM1, CCRL2*, and proenkephalin. The array data showed that the levels of gene expression of all of the above-mentioned genes paralleled ATRA-induced NB4 cell maturation (Figure 1E), supporting the notion that the microarray data reflected changes in gene transcriptional levels during the ATRA-induced myeloid differentiation process.

Activation of Multi-Signaling Pathways by ATRA

Multiple signaling pathways have been implicated in the ATRA-induced NB4 cells differentiation, including cAMP-dependent protein kinase A (*PKA*),[17] protein kinase C (*PKC*),[18] and mitogen-activated protein kinase (*MAPK*).[19] However, there is relatively little information regarding the effect of ATRA on the transcription level of genes involved in these signaling pathways. We analyzed the transcription level of 10 members of the MAP kinase family, six isoforms of protein kinase C, and two isoforms of cAMP-dependent protein kinase A. Our data revealed that only *MAP4K2*, one of 10 members of MAP kinases, was up-regulated threefold after treatment with ATRA (Figure 1B). *MAP4K2*, also called GC kinase, is a serine/threonine protein kinase, which has been identified in human lymphoid tissue and is activated during the stress response.[20] Recent studies confirmed that *MAP4K2* is an upstream activator of the MAP kinase cascade.[21] Activation of *MAP4K2* resulted in the activation of Jun N-terminal kinase (JNK) and p38 MAP kinase, but not the extracellular signal-regulated kinase (ERK). It was known that treatment of NB4 cells with ATRA leads to activation of p38 MAP kinase and inactivation of JNK.[22,23] We observed that ATRA treatment did not affect the gene expression of p38 kinase and JNK, indicating that the modulation of the activity of p38 kinase and JNK by ATRA is mediated by activation of the upstream activator MAP4K2

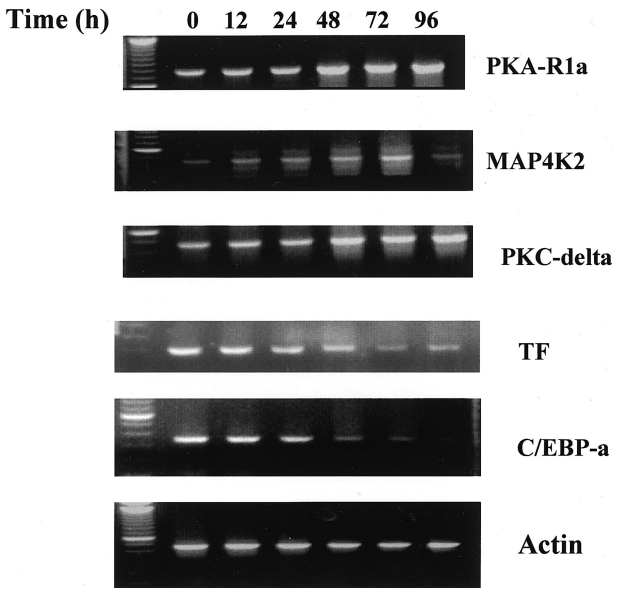

Figure 2. RT-PCR analysis of genes in NB4 cells treated with ATRA. cDNA was synthesized from equal amounts of RNA isolated from NB4 cells treated with ATRA at 0, 12, 24, 48, 72, and 96 hours separately. The data are representative examples of three independent experiments that gave similar results. Actin serves as internal control.

instead of a change in their gene expression. Our studies were also consistent with the previous observation that ATRA suppressed the transcription of c-jun and c-fos genes.[9]

Protein kinase C-δ is the major protein kinase in leukocyte extract that phosphorylates the cytoplasmic domain of CD18 subunit of CD11/CD18 intergrin during cell activation[24] and it also plays an important role in neutrophil activation and apoptosis.[25] Our array results demonstrated that the expression of protein kinase C δ and α, to a lesser extent, increased with ATRA treatment (Figure 1C), consistent with our previous observation that CD18 expressed on the cell surface in a time-dependent manner following ATRA treatment.[14] These findings also support the previous observations that only the α and δ isoforms of protein kinase C participate in myeloid differentiation[25] and neutrophil apoptosis.[26]

cAMP-dependent protein kinase A (PKA) includes two isoforms, *PKA-1* and *PKA-2*. They both share a common catalytic subunit but have distinct regulatory(R) subunits, R1 and R2, respectively. Our results revealed that ATRA only stimulated R1α gene expression, but not the other PKA subunits (Figure 1C). Expression of R1α of *PKA* is increased in various human cancers including those of breast,[27] ovary,[28] lung,[29] and colon.[30] Furthermore, the pathogenesis of malignancy and poor prognosis in cancer patients has been reported to correlate with overexpression of RIα of the PKA gene.[31] Previous experimental evidence has implicated different roles of regulatory subunits of PKA in the regulation of leukemic cell proliferation and differentiation. For instance, treatment of the leukemia cell line HL-60 with antisense oligonucleotide targeted against the mRNA of PKA-R1α caused cell growth inhibition and resulted in cell differentiation,[32] suggesting that PKA-R1α exerted inhibitory effects on HL-60 monocytic cell differentiation. In contrast, the present study demonstrates that ATRA-induced up-regulation of PKA-R1α expression resulted in NB4 cells undergoing granulocytic differentiation, suggesting that PKA-R1α exhibited a stimulatory influence on NB4 cell differentiation. Our results regarding PKA-R1α up-regulation by ATRA have been confirmed independently by a recent similar study using NB4 cells.[33] These conflicting results might be explained by the effects of PKA-R1α being cell-type dependent, due to activation of distinct signal pathways by binding to different regulatory subunits of PKA-R1α. Previous studies have shown that cAMP alone exhibited no differentiation effect on NB4 cells, but it substantially enhanced the maturation effect of ATRA on NB4 cells.[17] It was believed that ATRA "triggers" the cells to become responsive to cAMP and complete the maturation process. Our data have uncovered the molecular mechanism of the phenomenon that stimulation of the expression of R1α gene by ATRA results in NB4 cells being sensitive to cAMP.

Protein tyrosine phosphatase acts in conjunction with protein kinase to regulate the events of tyrosine phosphorylation that have been known to control cell activation and cell differentiation. Of this superfamily, lymphoid-specific protein tyrosine phosphatase non-receptor type 22 (*PTPN22*) and their substrate type 1 (*PTPNS1*) were up-regulated by ATRA. The response of protein tyrosine phosphatase to ATRA has not been observed before and the function of this enzyme remains unclear.[34]

Up-Regulation of Interferon (IFN) and IFN-Related Genes by ATRA

Previous studies have shown that ATRA is a strong inducer of interferon and interferon-mediated signaling pathways in ATRA-induced NB4 cells differentiation.[35–37] Our array data showed that among 11 interferon-induced genes contained in our chip, ATRA induced the expression of most IFN-induced genes, such as *OSA1, IFI41, IFI17, IFI6–16, 1–8U*, and *1–8D*, in a time-dependent manner. Three interferon-induced genes, ie, guanylate binding protein 2 (*GBP2*), interferon-stimulated gene (*ISG20*), and protein 54 (*IFIT2*), responded to ATRA immediately and were expressed in NB4 cells within 12 hours of ATRA treatment (Figure 1D). These early response proteins may be involved in the IFN-induced signaling pathway.

Regulation of the Expression of Cytokines and Chemokines by ATRA

Myeloid cell differentiation and maturation were also accompanied by different gene expression patterns of cytokines and chemokines.[38] The transcription regulation of cytokines and chemokines in ATRA-induced NB4 cells has not been fully explored. Therefore, we also added cDNAs of chemokines and cytokines in our array chips. We found that two chemokines, *HCC-1* (*SCYA14*) and *CCL20* (*SCYA20*) mRNA were increased after ATRA

treatment. *HCC-1* has been recently described as a human chemokine that is constitutively expressed in various tissues and is present at high concentrations in plasma.[39] However, the major source of circulating *HCC-1* is unknown. High expression of *HCC-1* in ATRA stimulated NB4 cells suggests that like *CCL20*,[40] the circulating *HCC-1* might be produced by neutrophils. In addition, interleukin-5 receptor α gene (*IL-5Rα)* was increased up to threefold over untreated cells. Interleukin-5 plays an important role in proliferation and differentiation of eosinophils and basophils. Recent observations by Denburg et al[41] indicated that ATRA could selectively inhibit membrane-bound IL-5 receptor and up-regulate soluble *IL-5* receptor transcription in $CD34^+$ cells. The up-regulation of *IL-5Rα* transcription by ATRA in NB-4 cells suggests that *IL-5* might also be involved in granulocytic cell differentiation. An alternative explanation is that the ATRA-induced terminal differentiated NB4 cells do not represent "pure" neutrophils and might exhibit some other kind of granulocytic features. This scenario was supported by our observations that ATRA also induced the expression of *CD52* antigen on differentiated NB4 cells (Figure 1E) expression and failed to induce CD16, a mature neutrophil marker expression (data not shown). It has been reported that *CD52* is present on the cell membrane of eosinophils, and is not present on neutrophils.[42,43]

Growth Inhibition and Apoptosis Induced by ATRA

We confirmed that ATRA inhibited NB4 cell growth as evidenced by the continuously decrease in cell S-phase and increase in cell apoptosis (data not shown). Our array data showed that ATRA treatment selectively down-regulated the expression of the anti-apoptosis *BCL-2* and *API3* genes and cell cycle related genes, and up-regulated the pro-apoptosis genes *BIK* and *Daxx*. Thus, induction of apoptosis may contribute to the therapeutic value of ATRA.

ATRA-Induced Effects on Transcriptional Factors

Various transcription factors have been associated with hematopoietic cell proliferation and myeloid differentiation. Critical to terminal granulocytic differentiation are the transcription factors *PU.1*, CCAAT/enhancer binding protein(C/EBP), core-binding factors (CBFs), and homeobox proteins.[44,45] We closely examined several transcriptional factors affected by ATRA treatment. After cells were exposed to ATRA, there was no change in the expression of *CBF-β* and *C/EBP-γ*. The expression of *C/EBP-α* was down-regulated. This result is consistent with previous studies showing that *C/EBP-α* plays a role in early myeloid progenitors but its expression is decreased with myeloid differentiation.[46]

Human *HoxB* cluster gene is located in chromosome 17q21.3[47] and the translocation t(15;17) in NB4 cells may affect the *HoxB* cluster gene expression. The results from our array showed that ATRA stimulated *HoxB7* but had no effect on *HoxB3, HoxA9* or, *HoxA10* gene expression. The putative homeodomain transcriptional factor (*PHTF*) was originally isolated from human erythroleukemic cells.[48] It is highly homologous with the human homeobox gene family. The biological function of *PHTF* is still unknown. Our data from the array studies showed that after exposure of cells to ATRA, *PHTF* gene transcription increased rapidly, suggesting that *PHTF* may play a role in myeloid cell differentiation.

Relationship between Gene Transcription and Cell Differentiation

We have also monitored ATRA-mediated NB4 cells differentiation using four classic assays for the study of myeloid cell maturation. They are cell morphology, *CD11b/CD18* expression, nitroblue tetrazolium (NBT) reduction test (a measure of granulocytic maturity), and cell cycle analysis at 0, 12, 24, 48, 72, and 96 hours to correlate the biological features with the gene transcription patterns. Using these assays, we observed no obvious morphological change in the NB4 cells during the first 24 hours of ATRA treatment. However, after 24 hours the differentiation markers began to be observed. The ratio of cytoplasm to nucleus increased and the chromatin condensed gradually. $CD11b^+$ expression and NBT-positive cells gradually increased from 24 hours to 96 hours of treatment (Figure 3). Maximal differentiation (ie, more than 85%) was observed at 96 hours after ATRA treatment. Furthermore, the decrease of the fraction of cells in S-phase paralleled the progression of cell differentiation and programmed cell death. These results indicate that the changes in gene expression observed in the microarray correlate with the degree of differentiation of NB4 cells. It must be emphasized that ATRA-induced granulocytic differentiation of NB4 cells is different from normal myeloid differentiation. NB4 cells strongly express myeloid markers, but they also express some T-cell marker[49] and lack some neutrophil-associated markers. For instance, both *CD16* and cathepsin G are neutrophil markers, but they were not expressed in NB4 cells before or after ATRA treatment in our study. The differentiated NB4 cells also expressed some proteins that are not expressed in normal neutrophils, such as *CD52* and interleukin 5 receptor. Therefore, the gene expression patterns reported here do not completely represent normal granulocytic differentiation.

Tissue factor (TF) is known to be an important factor in initiating coagulation cascade. As a result of increased TF expression in APL blast cells, patients with APL are often associated with disseminated intravascular coagulation. We have demonstrated that treatment of APL cells with ATRA significantly reduced TF expression (see Figure 2, and Table 2) from a ratio of 1.02 at 12 hours ATRA treatment to 0.46 at 96 hours ATRA treatment when compared to the untreated cells. This result is consistent with previous study[50] the clinical observation that ATRA treatment improves the APL-associated coagulopathy in patients with APL.[51]

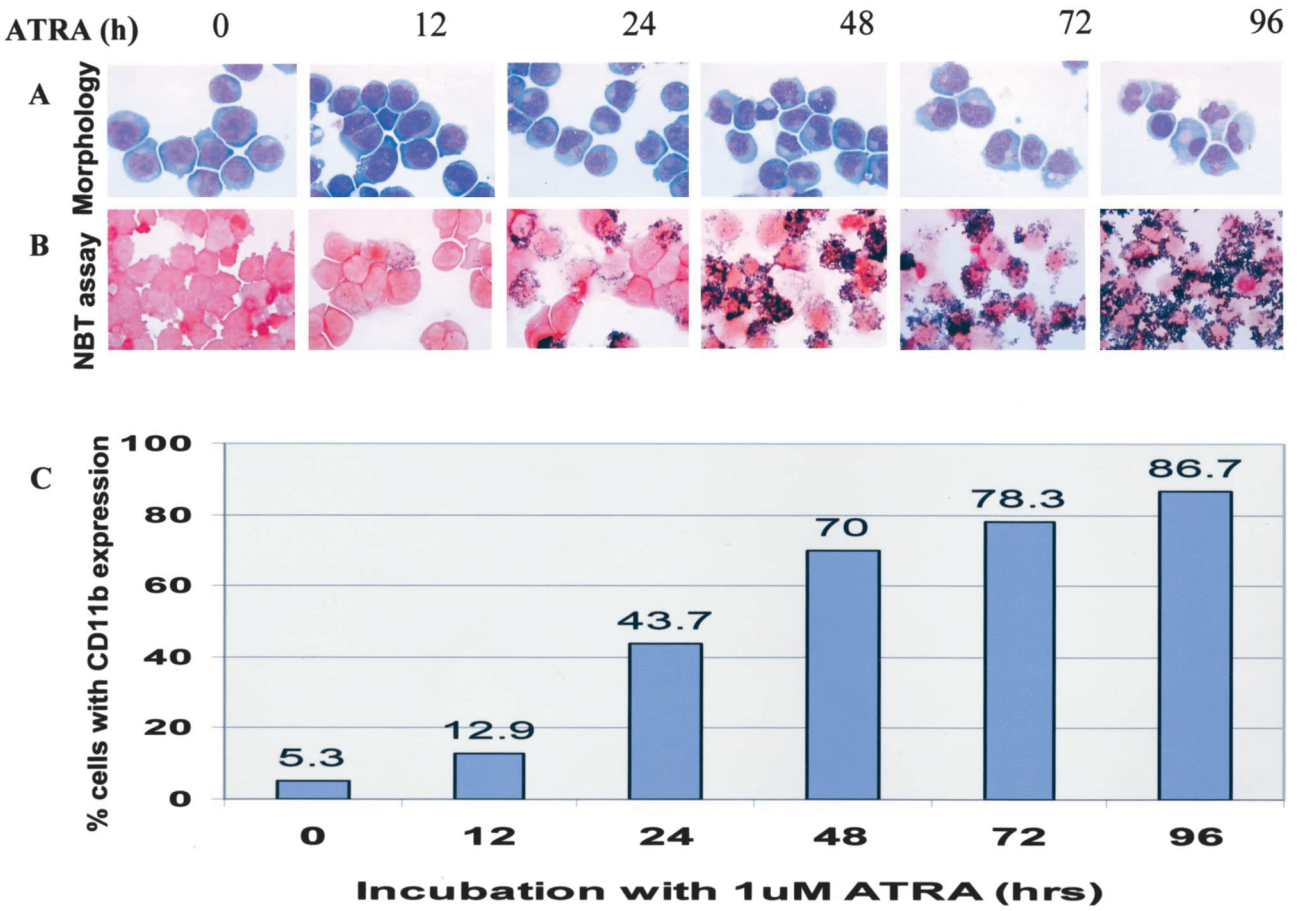

Figure 3. Differentiation of ATRA-induced NB4 cells. **A:** Morphological features of NB4 cells in response to ATRA treatment at different times. **B:** Nitroblue tetrazolium enzymatic reduction in ATRA-treated NB4 cells. **C:** CD11b intergrin expression following ATRA treatment of NB4 cells at each time point as assessed by flow cytometric analysis.

Conclusion

In summary, our results demonstrate that ATRA alters the expression of a large number of different genes, most of which have not been reported before. The ATRA-affected genes can be classified into several groups including genes involved in the regulation of signaling pathways, transcription factors, cell differentiation, programmed cell death, and the production of cytokines and chemokines.

Although incorporating ATRA into the treatment of APL has resulted in long-term survival and potential cure in nearly 70% of patients, the disease may relapse and resistance to further ATRA treatment and the retinoic acid syndrome occurred in almost all cases. Therefore, there is need to explore new drugs and new regimens for APL therapy. In this study, we showed that two potential target proteins, R1α subunit of PKA and *CD52* antigen, were highly expressed in the ATRA-treated APL cells. Recently, Sandrini et al[52] reported that PKA-R1α acted as a tumor-suppressor gene for sporadic thyroid cancer. The administration of ATRA has been used to treat patients with APL, but ATRA alone is not necessarily sufficient to maintain patients in stable remission. The finding of up-regulation of PKA-1α expression by ATRA support the strategy that combination of ATRA with cyclic AMP analogues synergistically induces APL cell differentiation and possibly reduces both the ATRA-induced side-effects and development of resistance to ATRA treatment. Another important finding in our study is that ATRA can induce *CD52* expression on the APL cell surface. *CD52* antigen is normally expressed on T and B lymphocytes, natural killer (NK) cells, monocytes, eosinophils and sperm cells, but not on leukemic myeloblasts and neutrophils.[42] *CD52* targeted therapy has been used in chronic lymphocytic leukemia and non-Hodgkin's lymphoma with promising results.[53] Thus, the induced up-regulation of *CD52* antigen expression in APL cells could provide a new target for an effective regimen of combination of ATRA and anti-*CD52* antibodies for the treatment of this disease.

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