

# Induction of differentiation-specific miRNAs in TPA-induced myeloid leukemia cells through MEK/ERK activation

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Received August 7, 2012; Accepted September 21, 2012

DOI: 10.3892/ijmm.2012.1191

**Abstract.** Cellular microRNAs (miRNAs) are pivotal regulators involved in various biological processes through the post-transcriptional regulation of gene expression. Signaling pathways are extensively activated during 12-O-Tetradecanoylphorbol-13-acetate (TPA)-induced differentiation of human leukemia cells, but the modulation of miRNA expression and processing in this context has yet to be fully explored. In this study, we comprehensively analyzed 10 miRNAs that are consistently upregulated during TPA-induced differentiation of various leukemia cell lines by employing microarray technology. The upregulation of these miRNAs was further verified by quantitative RT-PCR, and, markedly, a subset of the miRNAs was found to be induced via the MEK/ERK signaling pathway using TPA and specific pharmacological inhibitors. Moreover, immunoblotting and quantitative RT-PCR analysis demonstrated that the expression levels of key miRNA processing machineries (i.e., Drosha, Dicer, Ago1 and Ago2) were not induced in this context, but the transcription of the miRNA products was triggered by MEK/ERK activation. Therefore, we identified the unique miRNAs that respond to TPA treatment in leukemia cells and demonstrated the essential role of the MEK/ERK signaling pathway in the induction of these miRNA transcripts.

## Introduction

Dysregulation of hematopoietic cellular differentiation contributes to leukemogenesis (1). The use of differentiation agents can force malignant cells to undergo terminal differentiation, which is viewed as a promising and revolutionary approach for the therapy of leukemia diseases (2). The drug

12-O-Tetradecanoylphorbol-13-acetate (TPA) is a phase I clinical therapeutic agent for patients with relapsed/refractory myelocytic leukemia due to its capacity to induce differentiation or apoptosis in malignant cells (3). Further molecular and genetic analyses of the effects of TPA could promote a better understanding of its mechanisms contributing to differentiation and therapeutic response.

A new class of small non-coding RNA molecules known as microRNAs (miRNAs) has introduced a whole new layer of gene regulation in eukaryotes (4). The miRNAs are transcribed by RNA polymerase II as large primary non-coding transcripts or from regions of a known gene, and the transcribed primary miRNAs are processed through the concerted actions of biogenesis machineries, including their sequential cleavage, export, and incorporation into the RNA-induced silencing complex (RISC) to mediate the expression of target genes (5,6). miRNA expression profiling in TPA-treated leukemia cells has previously been performed in several studies (7-9). However, considering the number of miRNAs that exist in cells and the various types of myeloid leukemia, additional differentiation-related miRNAs need to be identified (10). Moreover, miRNA expression profiling should be conducted and compared among various leukemia cell lines to identify the specifically induced miRNAs that respond to TPA treatment.

The differentiation of leukemia cells induced by TPA is highly dependent on the involvement of multiple signaling pathways, including the mitogen-activated protein kinase (MAPK) pathways, involving MEK/ERK/MAP kinase and c-Jun NH2-terminal kinase, as well as MAPK-independent pathways such as the PI3K pathway (11). These signaling pathways are responsible for appropriately mediating gene transcription with respect to cellular behavior (12). To better understand the mechanism of TPA action, it is useful to investigate which pathways are activated and how they mediate the induction of miRNAs in response to TPA treatment in leukemia cells.

Here, we present the results of a microarray-based screen for miRNAs that respond to TPA treatment in various leukemia cell lines. We identified a series of specific differentiation-induced miRNAs and analyzed their responses to signal transduction by using pharmacological inhibitors, showing the essential role of MEK/ERK signaling in miRNA induction. Moreover, the

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**Key words:** myeloid leukemia, 12-O-Tetradecanoylphorbol-13-acetate, microRNA, microRNA biogenesis machinery, MEK/ERK signaling

regulation of both miRNA biogenesis machineries and primary transcripts was analyzed in the same context, revealing the major mechanisms for the induction of miRNA products.

## Materials and methods

**Cell culture and reagents.** The NB4, HL-60, K562 and U937 cell lines were obtained from the American Type Culture Collection (ATCC, Richmond, MD, USA) and cultured in RPMI-1640 medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (PAA Laboratories Pty Ltd, Morningside, Australia), 2 mM L-glutamine and antibiotics in a humidified incubator containing 5% CO<sub>2</sub> at 37°C. TPA, U0126 and LY294002 were purchased from Sigma (St. Louis, MO, USA) and dissolved in dimethylsulfoxide (DMSO) according to the supplier's instructions. To induce cell differentiation, TPA was added to the medium to a final concentration of 30 nM (13). To inhibit signal transduction, cells were pretreated with specific inhibitors for 30 min prior to TPA treatment (14,15). Antibodies against phospho-ERK1/2, ERK1/2, Dicer, Drosha, Ago1, and Ago2 were purchased from Cell Signaling Technology (Danvers, MA, USA). Antibodies against phospho-Akt, Akt and  $\beta$ -actin were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The horseradish peroxidase (HRP)-conjugated secondary antibodies were also obtained from Santa Cruz Biotechnology, Inc.

**Assessment of surface antigen expression.** Surface antigen expression was measured by flow cytometry according to our previously published protocol (13). Briefly, cells were harvested at the indicated times, washed twice with PBS, and then incubated with 20  $\mu$ l FITC-labeled anti-CD14 antibody working solution (eBioscience, San Diego, CA, USA) for 30 min at 4°C in the dark. For every sample, separate aliquots of cells were also incubated with isotype antibody controls (eBioscience) to determine non-specific staining. Following incubation, cells were washed twice with PBS and analyzed by flow cytometry with a 488 nm argon laser. For each sample, a total of 10,000 cells were analyzed.

**Assessment of cell cycle.** Cell cycle was profiled by flow cytometry as previously described (14). Briefly, cells were harvested and fixed in 70% ethanol at 4°C overnight. After washing with PBS, the fixed cells were re-suspended in PBS containing 100  $\mu$ g/ml RNase A and incubated for 30 min at 37°C. Finally, the cells were collected by centrifugation and incubated in PBS containing 50  $\mu$ g/ml of propidium iodide (PI) (Sigma) for 20 min in the dark, and then analyzed using flow cytometry with a 488 nm argon laser. A minimum of 20,000 cells was analyzed for each sample.

**miRNA microarray analysis.** For miRNA microarray analysis, total RNA was extracted using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. CapitalBio Mammalian miRNA Array V3.0 (CapitalBio, Beijing, China) microarrays were then probed, which include 924 probes for human, mouse and rat miRNAs. The microarray analyses were performed according to our previously published protocols (13,16). Significance Analysis of Microarrays (SAM; Stanford University, CA, USA) software was used to determine

the differentially expressed miRNAs, and the selection criteria included a fold change  $\geq 1.5$  or  $\leq 0.65$ , a q-value  $\leq 5\%$ , and a SAM score  $> 2$  or  $< -2$  (17). The entire dataset described here is available from the Gene Expression Omnibus (GEO, <http://www.ncbi.nlm.nih.gov/geo>) through accession number GSE33537.

**Quantitative real-time PCR analysis.** To quantify the mature miRNAs, stem-loop RT-PCR assays were performed according to the method of Chen *et al* (18); snRNA U6 was used as an internal standard. To quantify the pri-miRNAs, total RNA was subjected to DNase I treatment and reverse-transcribed into cDNA with M-MLV (Promega, Wisconsin, WI, USA) (19). Following reverse transcription, PCR reactions were performed using a SuperGreen quantitative PCR kit II (CapitalBio) according to the manufacturer's instructions. ACTB was used as an internal standard. Relative expression levels were calculated using the formula:  $Q_{rel} = 2^{-\Delta CT}$ , where  $\Delta CT$  = average CT test gene - average CT internal standard, and CT is the cycle threshold. All primers used for quantitative RT-PCR are listed in Table I.

**Immunoblotting analysis.** The cells were quickly lysed on ice using lysis buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1% Triton X-100, 10% glycerol, 2 mM EDTA, 1 mM Na<sub>3</sub>VO<sub>4</sub>, and protease inhibitors) and clarified by centrifugation at 14,000 rpm for 10 min; the supernatants represented the cellular protein extracts. Equal quantities of protein extracts were resolved by SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked in TBST (10 mM Tris pH 7.6, 150 mM NaCl, and 1% Tween-20) containing 5% low-fat milk for 1 h. Subsequently, the membranes were incubated with primary antibody dilutions at 4°C overnight, followed by three washes with TBST at room temperature. The membranes were then incubated with secondary antibody dilutions for 1 h at room temperature, followed by four washes with TBST. The enhanced chemiluminescence (ECL) system (Pierce, Rockford, IL, USA) was used to detect reactive proteins.

**Statistical analysis.** Experiments were performed in duplicate or triplicate and independently repeated at least three times. Data are presented as the means  $\pm$  standard deviation (SD). Statistical significance was determined using two-tailed t-tests, and  $P < 0.05$  was considered to indicate statistically significant differences.

## Results

**Analysis of the miRNA expression pattern of myeloid leukemia cell lines following TPA treatment.** NB4 (acute promyelocytic leukemia), HL-60 (acute myeloblastic leukemia), U937 (monoblastic leukemia), and K562 (chronic myelogenous leukemia) cell lines are the typical models for studies of human leukemia cell differentiation *in vitro*. The differentiation of these cells induced by TPA was determined by assessing cell morphology and the expression of a differentiation marker. After TPA treatment, the cells spread and adhered to the culture dishes, and some cells displayed pseudopod-like protrusions (Fig. 1A). A significant increase in CD14 expression was also observed in leukemia cells treated with TPA (Fig. 1B). These results suggest that cellular differentiation was induced.

Table I. PCR primers designed for the amplification of the investigated microRNAs and primary transcripts.

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
hsa-miR-21	GCCGCTAGCTTATCAGACTGATGT	GTGCAGGGTCCGAGGT
hsa-miR-22	CGAAGCTGCCAGTTGAAGAA	GTGCAGGGTCCGAGGT
hsa-miR-23a	GATATCACATTGCCAGGGATT	GTGCAGGGTCCGAGGT
hsa-miR-24	GGTGGCTCAGTTCAGCAGGA	GTGCAGGGTCCGAGGT
hsa-miR-23b	GCATCACATTGCCAGGGATT	GTGCAGGGTCCGAGGT
hsa-miR-29a	CGTAGCACCATCTGAAATCGG	GTGCAGGGTCCGAGGT
hsa-miR-29b	GCATAGCACCATTGAAATAGT	GTGCAGGGTCCGAGGT
hsa-miR-146b	CGGCTGAGAACTGAATTCCATAG	GTGCAGGGTCCGAGGT
hsa-miR-638	AGGGATCGCGGGCGG	GTGCAGGGTCCGAGGT
SnRNA U6	CTCGCTTCGGCAGCACA	AACGCTTCACGAATTTGCGT
Pri-miR-21	TTTTGTTTTGCTTGGGAGGA	AGCAGACAGTCAGGCAGGAT
Pri-miR-22	GCAGAAAGCCTTGGGTTG	CGAACAGCAGGGTGGATGA
Pri-23a-24-2	TCACCCCTGTGCCACG	CAAACCAACTGTGTTTCAGCT
Pri-miR-146b	GAGCAGCGTCCAGGCTG	CCGGGCACCAGAACTGAGT
ACTB	CATGTACGTTGCTATCCAGGC	CTCCTTAATGTACGCACGAT

Table II. Significantly upregulated miRNAs obtained by microarray analysis.

Cell lines	miRNAs (fold changes <sup>a</sup> $\geq 1.5$ -folds; q-value <sup>b</sup> $\leq 0.05$ )
HL-60	hsa-miR-21, hsa-miR-22, hsa-miR-146a, hsa-miR-27a, hsa-miR-222, hsa-miR-27b, hsa-miR-24, hsa-miR-146b-5p, hsa-miR-23b, hsa-miR-29a, hsa-miR-23a, hsa-miR-221, hsa-miR-509-3p, hsa-miR-17, hsa-miR-29b, PREDICTED_MIR191, hsa-miR-638
NB4	hsa-miR-146a, hsa-miR-222, hsa-miR-22, hsa-miR-23b, hsa-miR-146b-5p, hsa-miR-221, hsa-miR-638, hsa-miR-21, PREDICTED_MIR191, hsa-miR-23a, hsa-miR-24, hsa-miR-27b, hsa-miR-663, hsa-miR-509-3p, hsa-miR-155, hsa-miR-124, mmu-miR-193b, hsa-miR-193a-3p, hsa-miR-29b, hsa-let-7g, hsa-miR-29a
U937	hsa-miR-638, hsa-miR-486-3p, hsa-miR-508-5p, PREDICTED_MIR191, hsa-miR-663, hsa-miR-22, hsa-miR-584, hsa-miR-146b-5p, hsa-miR-487b, hsa-miR-888, hsa-miR-21, hsa-miR-23b, hsa-miR-146a, hsa-miR-27b, PREDICTED_MIR255, hsa-miR-222, hsa-miR-381, hsa-miR-24, hsa-miR-658, hsa-miR-221, hsa-miR-509-3p, hsa-miR-27a, hsa-miR-424, hsa-miR-23a, hsa-miR-28-5p, hsa-miR-193a-3p, hsa-miR-29b, hsa-miR-218, hsa-miR-410, hsa-miR-216a, hsa-miR-29a, hsa-miR-26a, PREDICTED_MIR160, hsa-miR-192, hsa-miR-125b
K562	PREDICTED_MIR191, hsa-miR-638, hsa-miR-663, mmu-miR-762, hsa-miR-22, hsa-miR-21, hsa-miR-146b-5p, hsa-miR-24, hsa-miR-29b, hsa-miR-23b, hsa-miR-27a, hsa-miR-29a, hsa-miR-23a, hsa-miR-17, hsa-miR-16

<sup>a</sup>Fold changes indicate the expression level of miRNAs in TPA-treated cells compared to the untreated cells; <sup>b</sup>q-value indicates the lowest positive false discovery rate (FDR) at which the gene was considered significant.

Using microarrays, the global changes in miRNA expression were analyzed after treatment of the myeloid leukemia cell lines with TPA. The microarray chips contained 924 probes, allowing a survey of 802 mature human, mouse and rat miRNAs after discarding redundant sequences, and a further 122 predicted miRNAs from published data. To reduce individual variability, replicate array analysis used independently treated samples, and a technical replicate was also performed for each sample.

Employing hierarchical cluster analysis, the global expression patterns of miRNAs in the four leukemia cell lines were

obtained (Fig. 1C). Through SAM statistics (17), significantly regulated miRNAs in differentiated cells were identified. TPA induction resulted in the upregulation of 21 miRNAs in the NB4 cell line, 17 miRNAs in the HL-60 cell line, 15 miRNAs in the K562 cell line, and 35 miRNAs in the U937 cell line (Table II). To identify specific differentiation-induced miRNAs, the overlaps of upregulated miRNAs were evaluated between these four myeloid leukemia lines (20). The cell lines each exhibited characteristic miRNA profiling due to their different origins. However, 10 unique miRNAs were consistently induced in all four leukemia cell lines after exposure

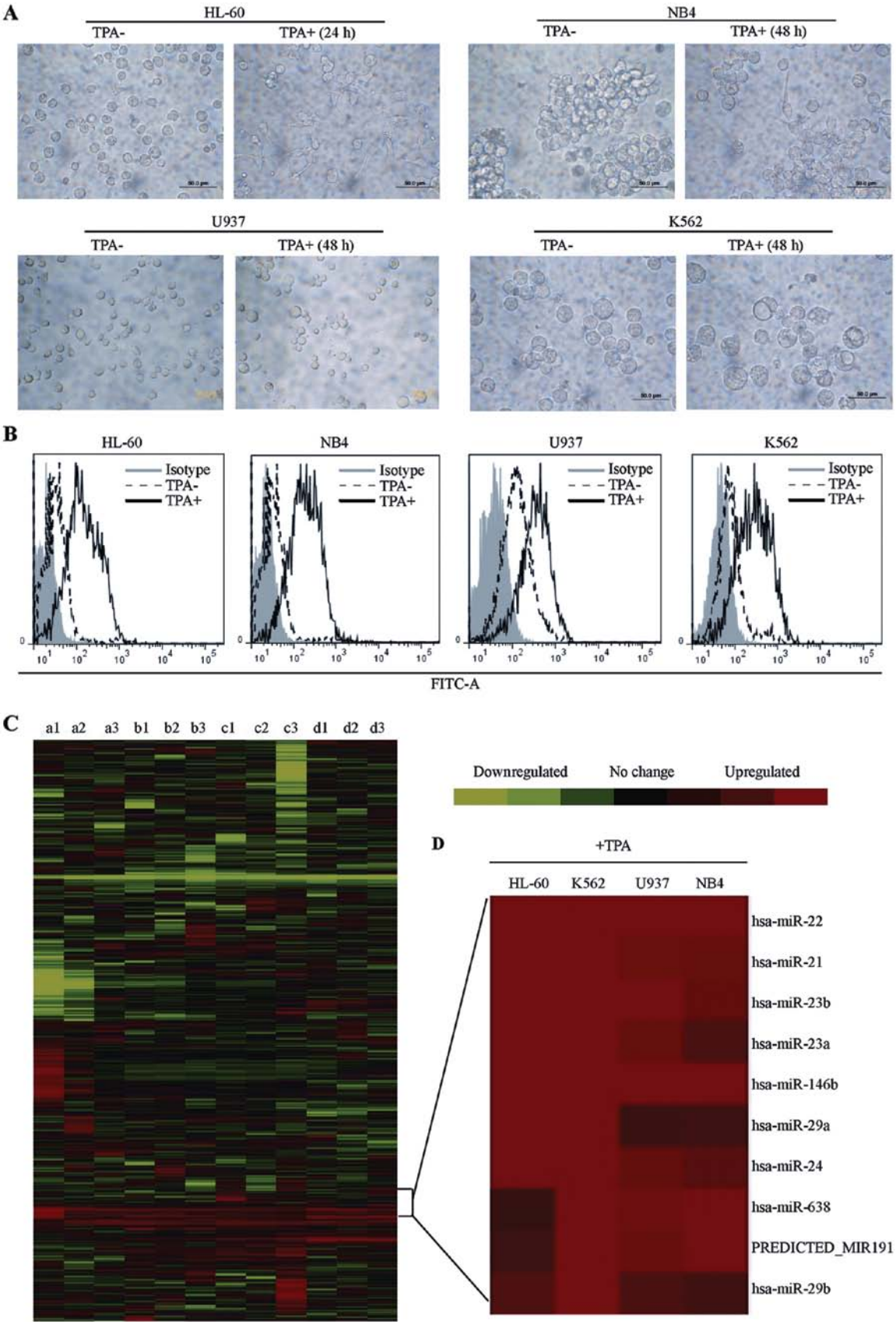


Figure 1. Microarray profiling of miRNAs in myeloid leukemia cell lines treated with TPA. (A) Morphological changes of leukemia cell lines exposed to 30 nM TPA (scale bar, 50  $\mu$ m). (B) Flow cytometric analysis of CD14 expression in leukemia cell lines treated with TPA. (C) Hierarchical clustering showing the expression patterns of 924 mature miRNAs in four myeloid leukemia cell lines treated with TPA. Samples are labeled a-d: a1-a3, HL-60 (TPA) vs. HL-60 (normal); b1-b3, K562 (TPA) vs. K562 (normal); c1-c3, NB4 (TPA) vs. NB4 (normal); and d1-d3, U937 (TPA) vs. U937 (normal). Biologically independent triplicates are indicated by 1-3. The color bar indicates the expression changes of miRNAs: red represents upregulation, green represents downregulation, and black represents no change in expression. (D) Cluster diagram of miRNAs consistently induced among the four myeloid leukemia cell lines treated with TPA (fold changes  $\geq 1.5$ ; q-value  $\leq 5\%$ ).

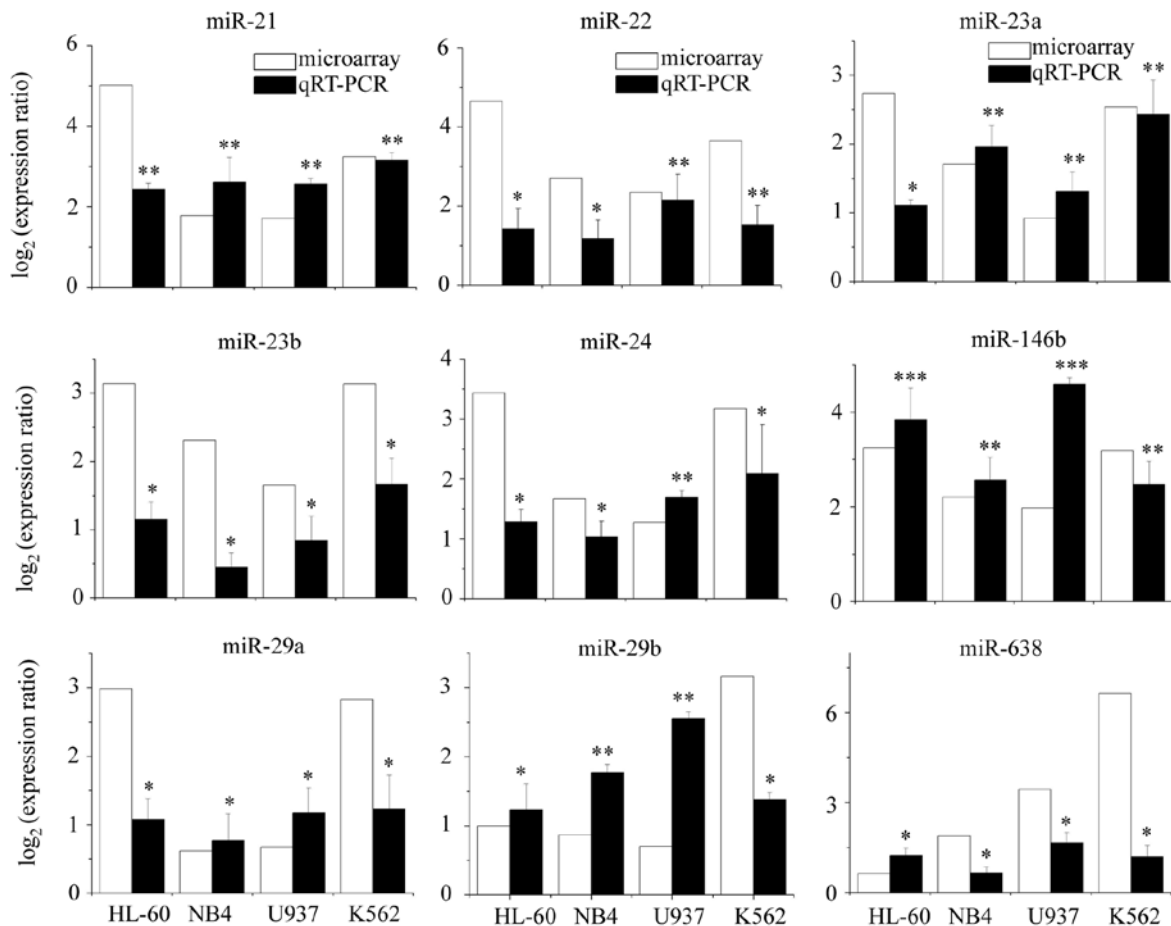


Figure 2. Validation of the microarray results by stem-loop RT-PCR. Stem-loop RT-PCR analysis of nine differentiation-specific miRNAs identified by microarray using independent samples. Data are shown as fold changes of miRNA levels in TPA-treated vs. untreated cells and represent the means  $\pm$  SD of three independent experiments. \* $P < 0.05$ , \*\* $P < 0.01$  and \*\*\* $P < 0.001$ .

to TPA (Fig. 1D). We support that the differentially regulated miRNAs may represent individual characteristics of each cell line, while the commonly regulated miRNAs may be 'key players' in the differentiation of leukemia cells.

**Validation of differentiation-specific miRNAs by quantitative RT-PCR.** To confirm the accuracy of our microarray analysis, stem-loop RT-PCR assays were performed on the identified differentiation-specific miRNAs (except predicted-miR191) using independent samples. The results of qRT-PCR were consistent with those of microarray analysis in all four cell lines (Fig. 2). These nine miRNAs were confirmed to be differentiation-specific in leukemia cells induced by TPA, of which a subset (miR-21, miR-22, miR-146b, miR-23a and miR-24) was selected for further investigation largely based on the magnitude of the detected induction.

**Pharmacological inhibition of MEK/ERK activation suppresses the upregulation of differentiation-specific miRNAs.** To ascertain how the expression of these differentiation-specific miRNAs was induced, cell signaling analysis was performed. Both the MEK/ERK and PI3K/Akt pathways are associated with the induction of differentiation by TPA in leukemia cells (11). To investigate the effects of these pathways on miRNA induction, MEK/ERK and PI3K/Akt signal trans-

duction was blocked using pharmacological inhibitors: U0126 (MEK1/2 inhibitor) and LY294002 (PI3K inhibitor). U0126 blocked the TPA-stimulated phosphorylation of ERK1/2 (Fig. 3A), and inhibited the induction of miR-21, miR-22, miR-146b, miR-23a and miR-24 (Fig. 3B). The changes in the expression of the differentiation marker, growth arrest, and cell morphology were also inhibited by pretreatment with U0126 (Figs. 3C and 4). By contrast, LY294002 failed to suppress the TPA-induced miRNA expression and cellular differentiation (Figs. 3B, C and 4). The reduction of Akt phosphorylation proved the inhibitory effect of LY294002 on PI3K/Akt signaling (Fig. 3A). Thus, MEK/ERK activation contributed to the induction of these differentiation-specific miRNAs.

**MEK/ERK activation triggers the transcription of differentiation-specific miRNAs.** To investigate the mechanism by which MEK/ERK activation caused the upregulation of differentiation-specific miRNAs, the expression of both miRNA biogenesis machineries and primary transcripts was examined. Using immunoblotting analysis, four proteins that are the key machineries of miRNA biogenesis pathways (Drosha, Dicer, Ago1 and Ago2) were investigated. Drosha and Dicer are involved in successively cleaving primary transcripts into mature miRNAs; Ago1 and Ago2 are the major components of RISC (5,6). The expression of Dicer, Ago1 and Ago2 was not significantly altered

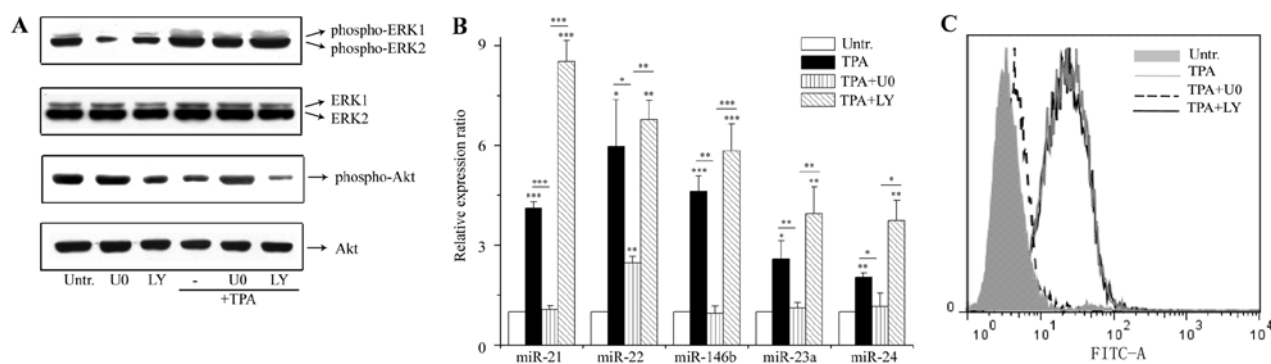


Figure 3. Effects of the MEK1/2 inhibitor U0126 and the PI3K inhibitor LY294002 on the induction of differentiation-specific miRNAs. NB4 cells were pretreated with 10  $\mu$ M U0126 (U0) or LY294002 (LY) for 30 min and then treated with 10 nM TPA for 24 h. (A) The levels of ERK, phospho-ERK, Akt and phospho-Akt in cells were analyzed by immunoblotting. The expression levels of total ERK and total Akt were regarded as controls. (B) The induction of miR-21, miR-22, miR-146b, miR-23a and miR-24 was analyzed by qRT-PCR. Data are shown as fold changes of miRNA levels in treated vs. untreated cells and represent the means  $\pm$  SD of three independent experiments. \* $P$ <0.05, \*\* $P$ <0.01 and \*\*\* $P$ <0.001 compared to the corresponding control. (C) Flow cytometric analysis of differentiation marker CD14 in cells after different treatments. Results were repeated at least three times, and a typical experimental result is shown.

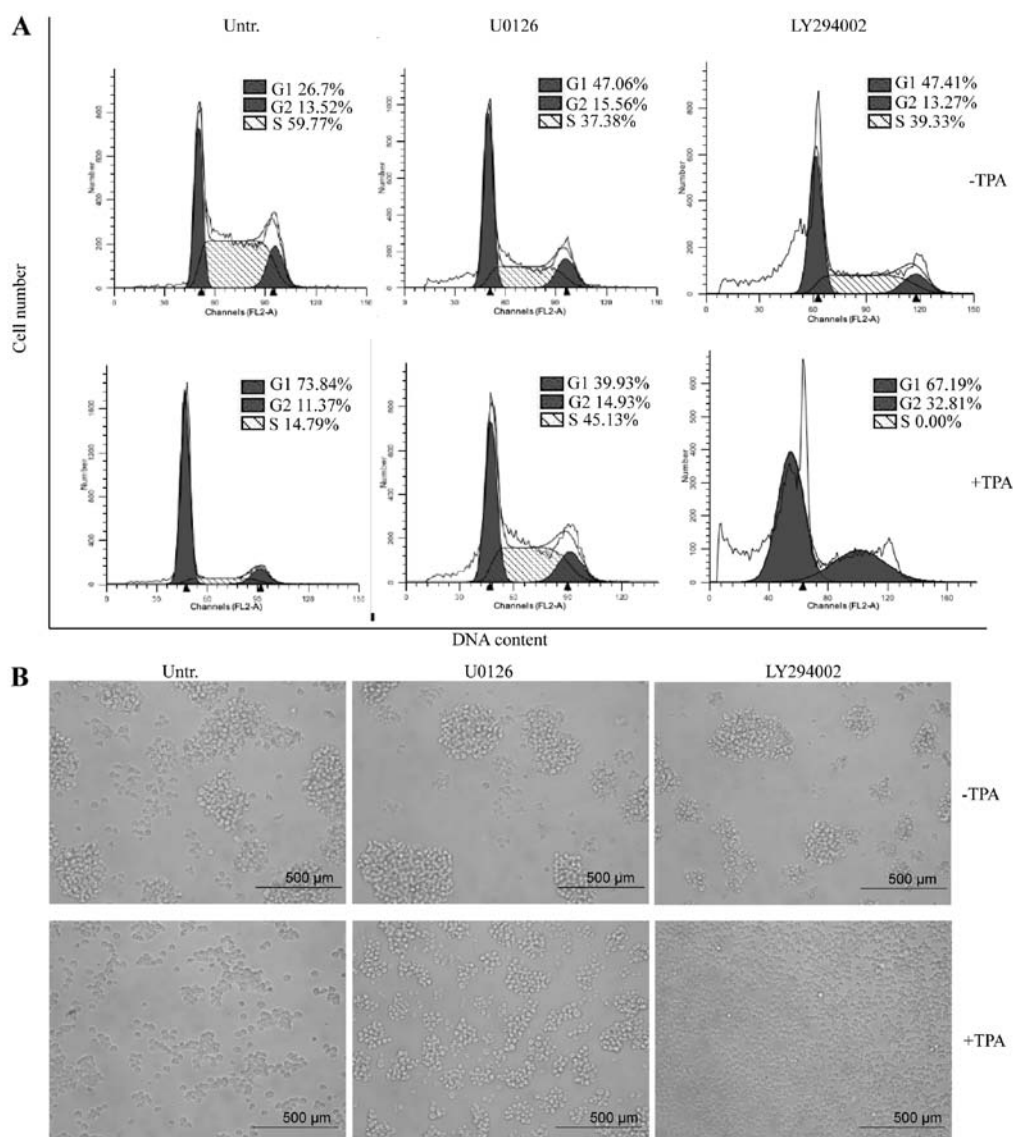


Figure 4. Effects of the MEK1/2 inhibitor U0126 and the PI3K inhibitor LY294002 on the differentiation of leukemia cells induced by TPA. NB4 cells were pretreated with 10  $\mu$ M U0126 or LY294002 for 30 min and then treated with 10 nM TPA for 24 h. (A) Flow cytometric analysis of cell cycle distributions. (B) The morphology of cells was observed under light microscopy (scale bar, 500  $\mu$ m). All experiments were repeated at least three times, and a typical experimental result is shown.

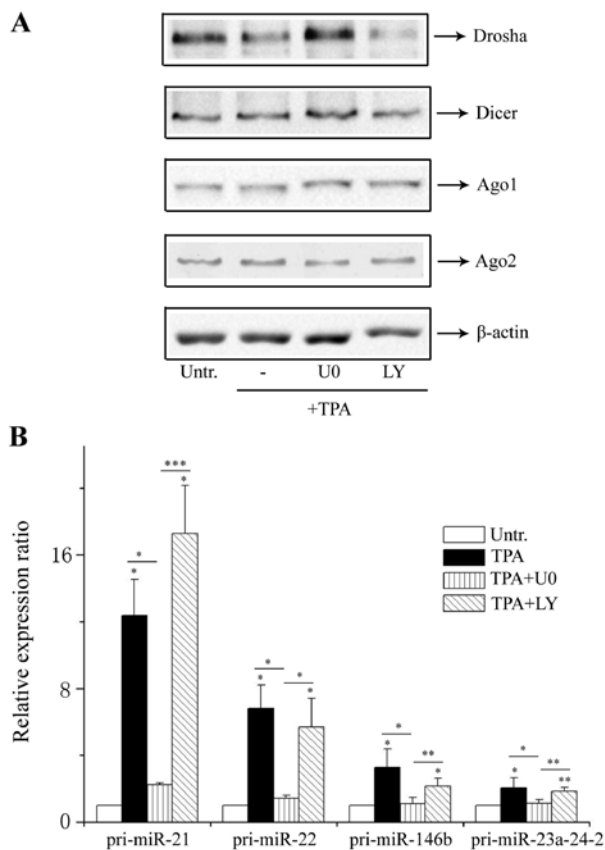


Figure 5. Effects of the MEK1/2 inhibitor U0126 and the PI3K inhibitor LY294002 on the miRNA biogenesis machineries and their transcription. NB4 cells were pretreated with 10  $\mu$ M U0126 (U0) or LY294002 (LY) for 30 min and then treated with 10 nM TPA for 24 h. (A) The levels of Drosha, Dicer, Ago1 and Ago2 were analyzed by immunoblotting in whole cell lysates. The results are representative of three independent experiments, and  $\beta$ -actin was used as a loading control. (B) The levels of pri-miR-21, pri-miR-22, pri-miR-146b, pri-miR-23a and pri-miR-146b were determined by qRT-PCR. Data are shown as fold changes of miRNA levels in treated vs. untreated cells and represent the means  $\pm$  SD of three independent experiments. \*P<0.05, \*\*P<0.01 and \*\*\*P<0.001 compared to the corresponding control.

in differentiated cells. Only Drosha was downregulated in cells after TPA treatment, and U0126 inhibited the downregulation of Drosha caused by TPA (Fig. 5A). These results suggest that the expression levels of the key miRNA biogenesis machineries in differentiated cells were not responsible for the increased expression of the aforementioned miRNAs induced by TPA.

Next, we assessed if the MEK/ERK activation induced the upregulation of the differentiation-specific miRNAs at the transcriptional level. qRT-PCR was performed to examine the primary transcripts of miR-21, miR-22, miR-146b, miR-23a and miR-24. Since there was a coordinated effect of processing at the miR-23a and miR-24 loci (21), a pair of specific primers was designed to quantify the expression levels of the pri-23a-24-2 transcript loci. The basal levels of these miRNA primary transcripts were increased after TPA induction, and the induction of these transcripts was significantly inhibited by U0126 but not by LY294002 (Fig. 5B). Therefore, the transcription of these differentiation-specific miRNAs was induced by MEK/ERK activation, suggesting a major mechanism for the upregulation of these miRNAs in the differentiation of leukemia cells induced by TPA.

## Discussion

This study included: i) miRNA expression (induced by TPA) profiling in four distinct leukemia cell lines; ii) confirmation of the differentiation-specific miRNAs in this process; and iii) investigation of the association of signaling pathways with the induction of differentiation-specific miRNAs, revealing the essential role of MEK/ERK signaling in regulating miRNA transcription in response to TPA treatment.

Since miRNAs are expressed in a tissue-specific and context-dependent manner, the large-scale profiling of miRNAs using microarrays has aided drug research and disease diagnosis (22). Previous studies on miRNA profiling of TPA-treated leukemia cells demonstrated heterogeneous results due to the differences in platform, drug dose, and cell types (7-9). In our study, miRNA expression profiling was analyzed in four distinct leukemia cells using the same experimental platform with the same concentration of drug, which is a better approach for further data mining. Through our comparison, 10 commonly upregulated miRNAs were identified in the four cell lines, representing the differentiation-specific miRNAs. Among them, miR-146b and miR-29a were previously reported to be upregulated in TPA-induced HL-60 cells, and they were also identified in K562 and NB4 cells by our analysis. In addition, miR-23b and miR-24 are induced in K562 cells treated with TPA, and these two miRNAs were also upregulated in TPA-induced U937, NB4 and HL-60 cells by our analysis. Such specifically induced genes that respond to drug treatment are largely regarded as potentially major targets of drug action (20). Therefore, the differentiation-specific miRNAs identified in this study provide significant insights into the thorough understanding of the mechanism of TPA action in leukemia cells.

Signal transduction is an important mechanism for gene regulation in cells (12), and a large number of miRNAs are under the control of various important signal pathways (23). Using specific signal transduction inhibitors, we demonstrated that MEK/ERK activation contributes to the induction of several differentiation-specific miRNAs (miR-21, miR-22, miR-23a, miR-146b and miR-24). These miRNAs target several genes related to differentiation, and most of them function as tumor suppressors (24-26). Their expression patterns correspond to cellular behaviors, i.e., induction in differentiated cells and inhibition in undifferentiated cells. Moreover, these MEK/ERK signaling-induced miRNAs (such as miR-21 and miR-24) can in turn regulate the MEK/ERK signaling pathway by targeting the components of this pathway or other related pathways, forming a complex regulatory network in TPA-induced leukemia differentiation (27,28). These differentiation-specific miRNAs are an important molecular link between MEK/ERK signal transduction and TPA-induced differentiation.

The common upregulation of differentiation-specific miRNAs via MEK/ERK activation may indicate a uniform regulatory program. To explore this possibility, we examined both miRNA biogenesis machineries and primary transcripts. The expression changes of miRNA biogenesis enzymes can affect the miRNA expression in some cases (29-31). We found that the expression levels of several key miRNA biogenesis machineries were not increased in TPA-induced NB4 cells, and the same trend was also observed in HL-60 and K562 cells (data not shown). Based on this, we hypothesized that MEK/



ERK activation may work on the transcriptional level for miRNA production. Indeed, we found that MEK/ERK activation contributed to the induction of the primary transcripts of the differentiation-specific miRNAs by qRT-PCR. Among these transcripts, the upregulation of miR-21 and miR-24 is also observed in other biological processes induced by MEK/ERK activation (32,33). In addition, the promoter regions of these induced miRNAs also contain potential binding sites for the transcription factors RUNX1, NF- $\kappa$ B and RREB-1 (34,35), all of which are downstream effectors of Raf/MEK/ERK signaling. These previous findings and our data indicate a causal effect of the MEK/ERK signaling pathway on the induction of the investigated miRNAs, and further studies will be conducted to confirm the direct targets of the MEK/ERK pathways that contribute to the induction of the differentiation-specific miRNAs.

In conclusion, elucidating the modulation of miRNA expression related to signal transduction advanced our understanding of an intracellular signaling network. Moreover, with increasing clinical administration of differentiation therapy in leukemia patients, the miRNA expression signature reported in this study may facilitate the development of differentiation therapeutic strategies and ultimately be predictive of response to therapy.

## Acknowledgements

We thank Yonggui Wang and Xiaoyu Zhang for their microarray technical assistance, Yu Liu for microarray data analysis, and Chao Wang for flow cytometric analysis. We thank Dr JunWei Guo for the helpful discussion and comments on the manuscript. We also thank Dr M. Bochman for checking the manuscript.

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