Pathways related to PMA-differentiated THP1 human monocytic leukemia cells revealed by RNA-Seq

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Previous analyses have reported that the human monocytic cell line THP1 can be differentiated into cells with macrophage-like characteristics by phorbol 12-myristate 13-acetate (PMA). However, little is known about the mechanism responsible for regulating this differentiation process. Here, we performed high-throughput RNA-Seq analysis to investigate the genes differently expressed in THP1 cells treated with and without PMA and examined those that may be responsible for the PMA-induced differentiation of monocytes into macrophages. We found 3,000 genes to be differentially expressed after PMA treatment. Gene ontology analysis revealed that genes related to cellular processes and regulation of biological processes were significantly enriched. KEGG analysis also demonstrated that the differentially expressed genes (DEGs) were significantly enriched in the PI3K/AKT signaling pathway and phagosome pathway. Importantly, we reveal an important role of the PI3K/AKT pathway in PMA-induced THP1 cell differentiation. The identified DEGs and pathways may facilitate further study of the detailed molecular mechanisms of THP1 differentiation. Thus, our results provide numerous potential therapeutic targets for modulation of the differentiation of this disease.

acute myeloid leukemia, differentiation, macrophage, PI3K/AKT pathway, RNA sequencing

Acute myeloid leukemia (AML) is a hematologic disease characterized by abnormal proliferation, apoptosis repression and differentiation blockage in hematopoietic stem/progenitor cells [1]. One approach for the treatment of leukemia is chemical-induced differentiation. Because of the relationship between the differentiation of leukemia blasts and disease regression, this treatment is clinically referred to as differentiation therapy [2]. Retinoic acid (RA)

treatment for acute promyelocytic leukemia has been considered a clinical success for differentiation therapy. Although several transcription factors have been identified to play important roles in leukemogenesis [3,4], the aberrant expression of oncogenes and disruption of tumor suppressor genes are most likely involved in processes responsible for blocking leukemia cell differentiation [5–11].

Treatment of human monocytic leukemia cells with PMA can overcome blocks in terminal differentiation, such as that in myeloid lineage THP1 cells, and result in growth arrest and differentiation into an adherent monocytic phenotype.

Understanding the dynamics of the transcriptome during differentiation will allow us to identify novel therapeutic targets. Transcriptional profiling has proven to be useful for determining general patterns of differential gene expression among samples [12]. Recent reports have shown that RNA-Seq is highly sensitive and can quantitatively measure gene expression in a large dynamic range of gene abundance [5,13–15].

The aim of this study was to gain an understanding of the molecular mechanisms associated with the PMA-induced differentiation of monocytes into macrophages and establish a sound foundation for future molecular studies.

1 Materials and methods

1.1 Cell culture and reagents

The human monocytic leukemia cell line THP1 was cultured in RPMI 1640 (HyClone, SH30027, USA) containing 10% fetal bovine serum (HyClone, SV30160). The cells were maintained in a humidified atmosphere containing 5% CO_2 at 37°C. PMA was purchased from Sigma (USA) and dissolved in dimethyl sulfoxide (DMSO). Cell differentiation was assessed by measuring surface marker integrin alpha M (ITGAM/CD11b) and CD14 molecule (CD14) expression (eBioscience, 11-0113, USA) by flow cytometry analysis.

1.2 Quantitative real-time PCR analysis

RNA was reverse-transcribed into cDNA using M-MLV reverse transcriptase (Promega, M1705, USA). PCR of the cDNA was performed using specific primers for the genes of interest. The oligonucleotide sequences are available in Table S1. *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) was used as an internal control.

1.3 Cell counting kit-8 (CCK-8) assay

THP1 cells were plated at a density of 5×10^3 cells/well in 96 well plates, and cell viability was assessed using the CCK-8 assay according to the manufacturer's instructions. After treatment with LY294002 (PI3K/Akt inhibitor) and PMA for 24, 48, and 72 h, the CCK-8 (Dojingdo, Japan) reagent (10 μL) was added to each well followed by a 2-hour incubation.

1.4 RNA isolation and RNA-Seq (quantification)

To obtain an overview of the macrophage differentiation transcriptome, cDNA libraries generated from THP1 cells stimulated with PMA at several time points were used. The cDNA libraries were prepared as previously described [16]. The library products were used for sequencing by the Illu-mina HiSeq™ 2000 platform (USA). Gene expression levels were calculated by assessing the number of uniquely mapped reads per kilobase of exon region per million mappable reads (RPKM) using Chen's method [17]. RPKM values were used to compare differences in gene expression among samples. We identified differentially expressed genes (DEGs) between samples using the following criteria: FDR (false discovery rate) ≤0.001 and $\log_2$ fold-change ratio≥1.

2 Results

2.1 Differentially expressed genes in monocytes and macrophages

Treatment of THP1 cells with PMA is a well-established model for monocyte/macrophage differentiation. THP1 cells were treated with PMA (50 ng mL^{-1}) for 24, 48 and 72 h. A marked increase in cell size and adhesion was observed for PMA-treated THP1 cells, which had a typical macrophage shape and signs of macrophage differentiation (Figure S1). Next, we performed RNA sequencing (RNA-Seq) to identify the genes involved in macrophage differentiation. The gene expression profiles derived from RNA-Seq were calculated using the RPKM method. There were approximately 20,000 genes detected on average in the individual libraries, and greater than 3,000 genes were differentially expressed after PMA treatment. Using hierarchical clustering analysis, we found that a large number of genes were robustly induced during the process of macrophage differentiation (Figure 1), including *p21*$^{WAF1/Cip1}$, *MCL1* (myeloid cell leukemia 1) and *BCL2L1* (BCL2-like 1), which were previously reported to be induced during differentiation [18–20]. The DEGs are shown in Table S2.

2.2 qRT-PCR for data validation

To validate the DEGs identified by RNA-Seq, we assessed nine of the DEGs (i.e., *ITGAM/CD11b*, *CD14*, *SPIB* (Spi-B transcription factor), *VEGF* (vascular endothelial growth factor), *MMP9* (matrix metallopeptidase 9), *HOXA9* (homeobox A9), *ATG7* (autophagy related 7), *CXCR4* (chemokine (C-X-C motif) receptor 4), and *EGR1* (early growth response 1)) that exhibited highly significant differences in expression or were involved in important signaling pathways by quantitative RT-PCR. The results of the qRT-PCR analysis were generally in good agreement with the RNA-Seq data (Figure 2). Importantly, RT-PCR detection demonstrated that the mRNA level of some genes, including the macrophage lineage surface markers ITGAM/CD11b and CD14, increased in response to PMA, which is consistent with previous studies [1,18]. These results confirmed that RNA-Seq could provide reliable data for differential RNA expression analysis.
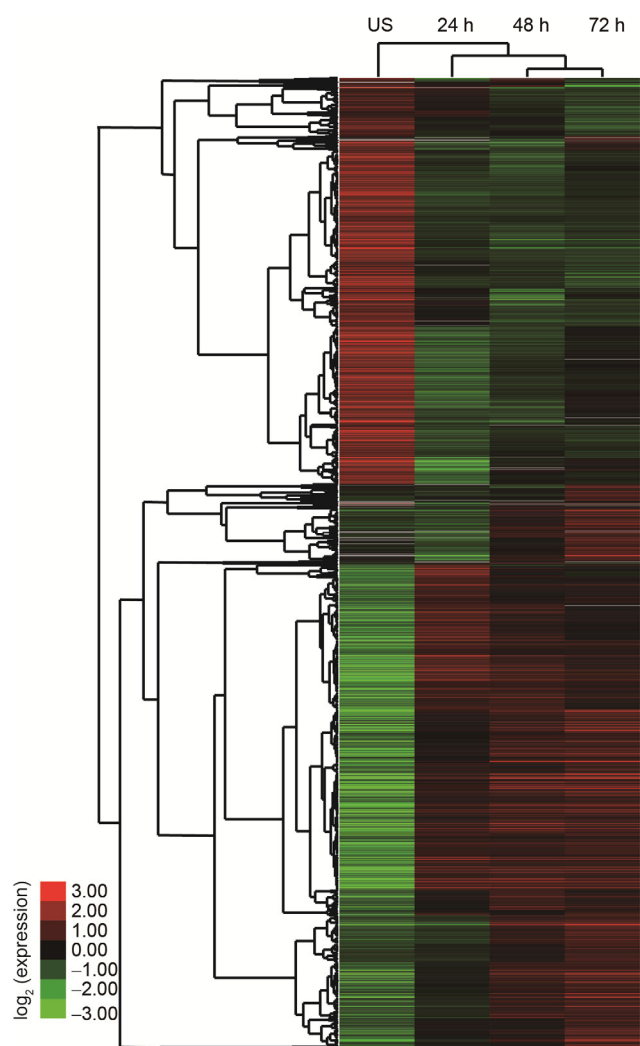
Figure 1 Hierarchical clustering analysis of differentially expressed genes. The heatmap shows hierarchical clustering of the genes expressed during the differentiation of monocytes into macrophages. The color intensity represents the degree of expression. A red-green color scale was used to reflect standardized gene expression with red representing high expression and green representing low expression (scale shown in the lower left). Cutoff values of FDR$\leqslant$0.001 and $\log_2$ fold-change ratio$\geqslant$1 relative to unstimulated cells were selected. The identities of these genes are listed in Supplemental table 2. US: unstimulated.

2.3 Gene ontology (GO) and KEGG pathway enrichment analysis of DEGs

We performed GO and KEGG analysis to classify the functions of the DEGs. To avoid experimental variation, we only used DEGs that had a $\log_2$ fold-change ratio$\geqslant$2. We compared the three time points of the PMA-treated and untreated cells. Analysis of the DEGs based on the biological process GO categories revealed that genes related to cellular processes and the regulation of biological processes were significantly enriched. The most significant biological process terms are listed in Table S3.

Pathway-based analysis helps to further understand the

biological functions of DEGs. The KeggChart tool in the DAVID program was used to detect enriched pathways in the KEGG database [21]. Several pathways were found to be significantly enriched for in the DEGs (Table S4). Pathways most commonly reported to be related to proliferation and differentiation, such as the PI3K/AKT, Notch, MAPK and NF-κB signaling pathway, were identified [19,22–24]. Importantly, the PI3K/AKT signaling pathway was found to be significantly enriched for in the DEGs. These findings are particularly interesting because the PI3K/AKT signaling pathway has been closely linked with PMA-induced p21$^{\text{WAF1/Cip1}}$ expression in human leukemia cells [20].

2.4 The PI3K/AKT pathway in PMA-induced THP1 cell differentiation and growth inhibition

Studies of human leukemia cell lines have indicated that PMA inhibits THP1 cell proliferation before the initiation of differentiation occurs [25]. Interestingly, previous reports have also shown that the PI3K/AKT pathway plays an important role in regulation of p$^{21\text{WAF1/Cip1}}$ expression during prostate cancer cell proliferation [26]. We therefore asked whether PMA could induce differentiation via PI3K/AKT pathway-dependent proliferation inhibition. We examined the effects of LY294002 (PI3K/AKT inhibitor) on cell proliferation and differentiation in PMA-stimulated THP1 cells by measuring CD11b and CD14 surface antigen expression. As shown in Figure 3A, we found that LY294002 treatment inhibits PMA-induced THP1 cell differentiation but has no effect on THP1 cell growth (Figure 3B). Collectively, these results further support the data of our RNA-Seq analysis and suggest that the effects of the PI3K/AKT pathway on THP1 cell differentiation may not depend on growth inhibition.

3 Discussion

In this study, we examined the molecular mechanisms associated with the PMA-induced differentiation of monocytes into macrophages. For this purpose, THP1 macrophages generated by stimulation with PMA at several time points were used. We obtained the comprehensive transcriptome of the process of monocyte-to-macrophage differentiation using RNA-Seq. Additionally, genes differentially expressed during the different stages of the PMA-induced differentiation of monocytes into macrophages were identified and functionally annotated using the GO and KEGG databases.

To elucidate the fundamental roles of these DEGs, we analyzed putative genes using GO "biological process" categories. This analysis revealed that a set of DEGs related to cellular processes and regulation of biological processes may be associated with macrophage function. The enriched KEGG pathways associated with the DEGs clustered into
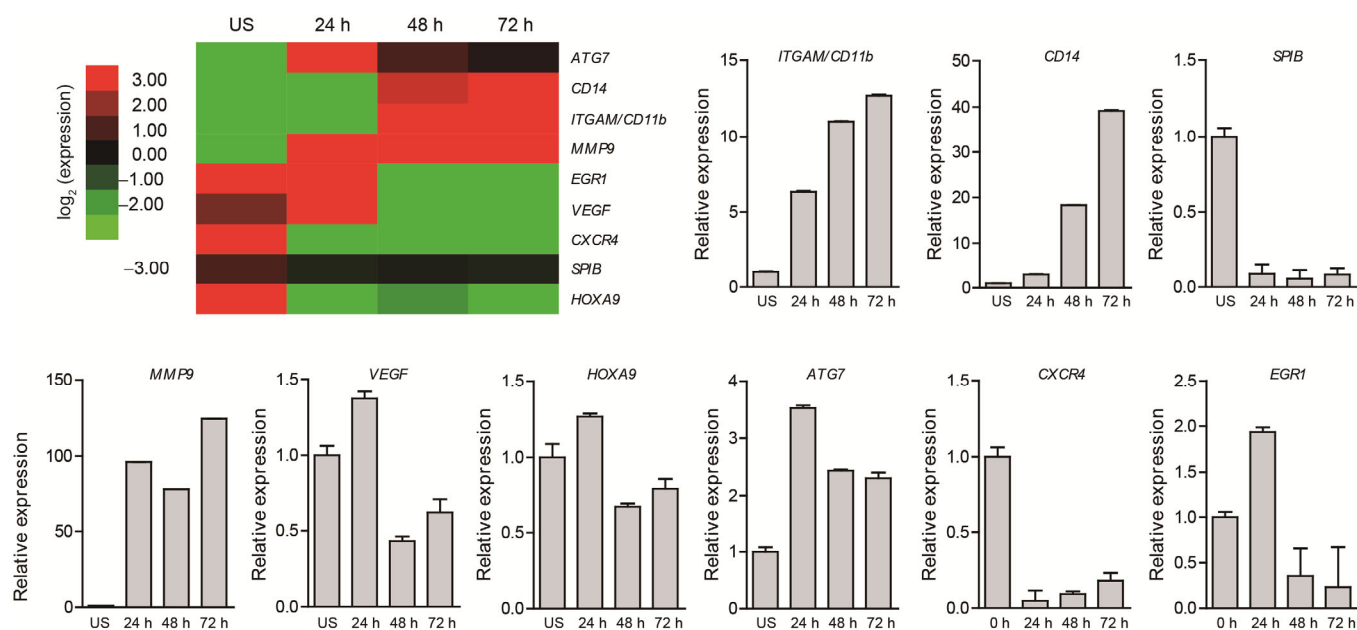
Figure 2 Quantitative real-time PCR validation of RNA-Seq data. The color panels indicate the log$_2$-transformed mean expression values obtained by RNA-Seq (see reference color bar). mRNA levels were measured by qRT-PCR and are shown as n-fold changes compared with untreated cells normalized to the housekeeping gene *GAPDH*. The panels show the $\bar{x}$ ±SD of a representative experiment performed in triplicate.
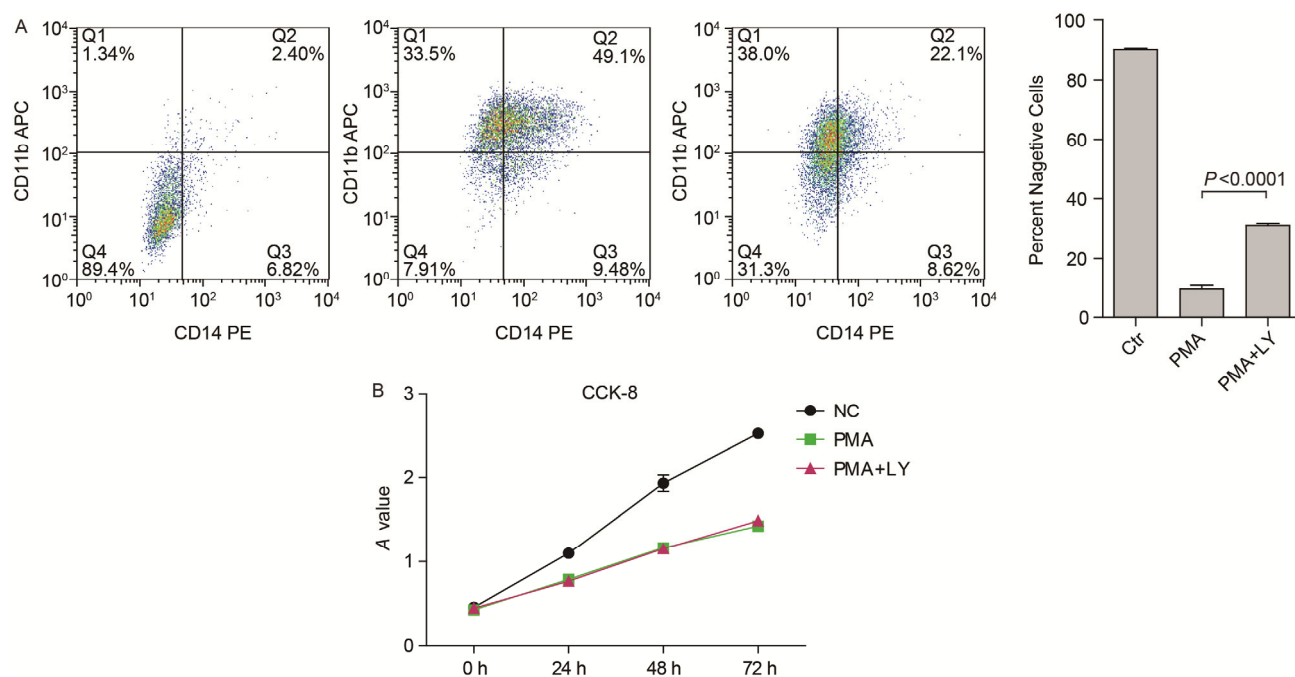

Figure 3 Effects of LY294002 on PMA-induced THP1 cell differentiation and growth. A, THP1 cells were seeded in 6-well plates in RPMI containing 10% fetal bovine serum, and they were treated with PMA (50 ng mL^{-1}) alone or in combination with LY294002 (10 μmol L^{-1}) for 48 h. Following differentiation, the cells were evaluated for cell-surface markers (CD11b and CD14) by flow cytometry. The values shown are derived from *n*=3 independent experiments, and data are reported as $\bar{x}$ ±SD. B, THP1 cells were co-treated with LY294002 and PMA (50 ng mL^{-1}) or PMA alone for the indicated times. Cell viability was analyzed using the CCK-8 assay.

several categories. We demonstrated that the most widely reported pathways related to proliferation and differentiation, such as the PI3K/AKT and NF-κB signaling pathways, were also included. Of these pathways, we were particularly interested in the PI3K/AKT pathway, which was significantly activated. A recent study has reported that PI3K/AKT regulates survival during the differentiation of human macrophages [19]. Interestingly, by measuring CD11b and

CD14 surface antigen expression, we found that inhibition of PI3K activity by LY294002 inhibits PMA-induced THP1 cell differentiation. Inhibition of the PI3K/ AKT pathway suppressed the PMA-induced morphological changes, suggesting that the PI3K/AKT pathway is required for PMA-induced THP1 differentiation. It is understood that before initiating cell differentiation, PMA treatment must first inhibit cell growth [25]. Regulation of p21$^{WAF1/CIP1}$ gene expression has been extensively studied in macrophage differentiation [20]. *p21$^{WAF1/CIP1}$* gene expression was upregulated in our RNA-Seq dataset, which is similar to other reports. Previous reports have shown that the PI3K/AKT pathway plays an important role in regulation of *p21$^{WAF1/Cip1}$* expression during proliferation [26]. We therefore tested the effects of LY294002 on cell proliferation during PMA-stimulated differentiation. However, we found that LY294002 and PMA co-treatment had no effect on THP1 cell growth during macrophage differentiation. Previously reported results in differentiated macrophages demonstrated that a signaling pathway consisting of PI3K/Akt and NF-κB regulates cell survival during the differentiation process [19]. Indeed, we found significant changes in BCL2 family genes in our data (Table S5). Thus, the functions of the PI3K/AKT pathway in the differentiation of PMA-treated THP1 cells may not depend on growth inhibition.

In conclusion, in this study, by using RNA-Seq to evaluate the effects of PMA at different time points, we identified a number of genes associated with the PMA-induced differentiation of monocytes into macrophages. Functional and pathway analyses of the genes further demonstrated the importance of the PI3K/AKT signaling pathway in regulating the differentiation process. These data will provide potential molecular targets for functional studies.

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Supporting Information

Figure S1   PMA induces cell differentiation in THP1. Cell morphology was observed under a phase-contrast microscope and photographed by a digital camera.

Table S1   Real-time PCR primers

Table S2   Gene expression level in THP1 cells with or without PMA treatment

Table S3   GO-Biological Process analysis of DEGs in THP1 cells with or without PMA treatment

Table S4   KEGG pathway analysis of DEGs in THP1 cells with or without PMA treatment

Table S5   Gene expression level of BCL2 family in THP1 cells with or without PMA treatment

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