Microarray Profiling of Monocytic Differentiation Reveals miRNA–mRNA Intrinsic Correlation

Jing Wang,[1,2] Guangxing Xiang,[3] Keith Mitchelson,[1,3] and Yuxiang Zhou[1,2,3,4]*

[1]Medical Systems Biology Research Center, Tsinghua University, Beijing 100084, China

[2]Department of Biological Sciences and Biotechnology, Tsinghua University, Beijing 100084, China

[3]National Engineering Research Center for Beijing Biochip Technology, 18 Life Science Parkway, Beijing 102206, China

[4]The State Key Laboratory of Biomembrane and Membrane Biotechnology, Tsinghua University, Beijing 100084, China

ABSTRACT

MiRNAs (microRNAs) are small non-coding RNAs involved in mammalian gene expression of cellular processes including differentiation, apoptosis and cancer development. Both specific miRNAs and mRNAs have been identified during monocytic differentiation, but their interactions have not been fully characterized. Here we report that by genome-wide microarray analysis for U937 monocytic differentiation induced by TPA, a large number of miRNAs and mRNAs were differentially expressed, and by bioinformatics analysis could demonstrate that their functional pathway patterns overlap strongly. While expected negative correlation between the expression levels of miRNAs and their target mRNAs was seen, several positive correlations between miRNAs and host mRNAs were also observed, such as *C13orf25*/miR17, *MCM7*/miR93, and *MGC14376*/miR22. These microarray data were verified by quantitative RT-PCR, and the TPA-induced differentiation of U937 cells was confirmed by flow cytometric analysis. Our study suggests an intrinsic correlation between miRNAs and mRNAs underlying their interactions which would provide new insights for defining the mechanisms occurring during monocytic differentiation. J. Cell. Biochem. 112: 2443–2453, 2011.   © 2011 Wiley-Liss, Inc.

KEY WORDS: INTRINSIC CORRELATION; MICROARRAY PROFILING; microRNA; mRNAs; MONOCYTIC DIFFERENTIATION

Some noncoding RNA molecules (∼22nt long), called micro-RNA (miRNAs), were first discovered in *Caenorhabditis elegans* by two research groups in the early 1990s [Lee et al., 1993; Wightman et al., 1993]. MiRNAs play an important role in the regulation of gene expression by binding complementary sequences in target mRNAs and thus causing their selective degradation, or selectively inhibiting the translation of target mRNAs [He and Hannon, 2004]. Many studies reported that miRNAs interact in a combinational fashion with genes in complex biological processes, such as cell proliferation and cell differentiation [Cui et al., 2006]. The biogenesis of miRNA is not fully clarified, and in the recent years, it has been estimated that miRNAs that reside in intronic or exonic regions of other genes may be the dominating class, called "intragenic miRNAs." It is likely that intragenic miRNAs are processed from the same primary transcript as host genes and thus, their expression levels are supposed to be coordinated with the host gene mRNA [Hinske et al., 2010]. Given these properties of miRNAs

in gene expression, further investigating on the correlation between expression level of miRNAs and mRNAs could reveal a tight post-transcriptional regulatory network at both mRNA and protein level.

Bone marrow progenitors developed into monocytes proceeding through monoblast and promonocyte stages, and monocytes undergo further differentiation to become mature macrophages, which play a crucial role in host defense to pathogens, wound healing, and various types of chronic inflammation [Gordon and Taylor, 2005]. The U937 cell line originated from a human histolytic lymphoma, possessing immature promonocytic characteristics, is believed to retain the capacity to differentiate into monocyte/macrophage by various inducers such as TPA (12-O-tetradecanoyl-phorbol-13-acetate), VD3 (1, 25-dihydroxyvitamin D3), retinoic acids, DMSO and IFN-γ. Since the TPA-induced differentiation of U937 cells arrests cell growth in the G0/G1 phase of the cell cycle and presents functional differentiation markers as well as specific cell morphology, these cells are well accepted as an experimental

model for exploring the mechanisms of monocytic differentiation [Harris and Ralph, 1985; Kang et al., 2002].

In the present study, we use microarray-based approach to profile the mRNA transcriptional changes, as well as changes in the miRNA expression patterns during TPA-induced differentiation of U937 cells. Integrating microarray data and bioinformatics database, the correlations between expression of miRNAs and target mRNAs, as well as intronic miRNAs and host mRNAs, were characterized in the context of monocytic differentiation.

MATERIALS AND METHODS

CELL CULTURE AND ASSESSMENT OF MONOCYTIC DIFFERENTIATION

U937 cells were maintained in RPMI1640 with 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM glutamine. Differentiation of U937 cells was induced by adding TPA (Sigma-Aldrich, St. Louis, MO) to a final concentration of 30 nM to the culture media and incubating the cells for 48 h [Kang et al., 2002]. Untreated U937 cells were used as control. Cell morphologies were examined by light microscopy after Wright-Giemsa staining [Yazdanparast et al., 2006].

The differentiation marker CD11b was assessed as previously described [Yamamoto et al., 2009]. Briefly, approximately 5×10^5 cells were collected by centrifugation, and re-suspended in 20 μl CD11b-FITC working solution (eBioscience, San Diego). Following incubation for 30 min in the dark, the cells were washed twice with ice-cold PBS and analyzed by flow cytometry. For each sample, isotype antibody control was used to determine non-specific staining.

miRNA MICROARRAY ANALYSIS

For miRNA microarray analysis, the total RNA was extracted from cells using the Trizol reagent (Invitrogen, Carlsbad, CA). Microarray analysis was performed using CapitalBio Mammalian miRNA array V3.0 (consisting of 509 probes in triplicate), and the experimental procedures were performed as previously described [Guo et al., 2008; Li et al., 2009]. Briefly, the low-molecular-weight RNA was isolated by precipitation with 13% (v/v) PEG6000 and 1.6 M NaCl. 4 μg of low-molecular-weight RNA was labeled with 500 ng Cy3 (or Cy5) (Dharmacon, Lafayette, CO) with 2 units of T4 RNA ligase (NEB, Ipswich, MA). The labeling reaction was performed at 4°C for 2 h. Labeled RNA was precipitated with 0.3 M sodium acetate, 2.5 volumes ethanol, and re-suspended in 15 μl hybridization solution containing 3 × SSC, 0.2% SDS, and 15% formamide. Hybridization was performed at 42°C overnight. After washing and spin-drying, slides were scanned with LuxScan 10K-A laser confocal scanner (CapitalBio, Beijing, China) and the analog signal was transformed to a digital signal using LuxScan 3.0 software (CapitalBio, Beijing, China). Raw data were normalized, and the differentially expressed miRNAs were identified by Significance Analysis of Microarrays (SAM, Stanford University, CA) software [Tusher et al., 2001]. Three independent experiments were performed, and for each test and control sample, two hybridizations were performed by using a reversal fluorescent strategy. The original data of miRNA microarray are available from the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo), and the accession number is GSE26376.

mRNA MICROARRAY ANALYSIS

The same total RNA samples used for miRNA microarray analysis were also used for mRNA microarray analysis. A human oligonucleotide microarray (CapitalBio, Beijing, China) containing 21, 3295-amino-modified 70-mer probes was used for analysis. DNase-treated total RNA (5 μg) was prepared to produce fluorescent dye (Cy5 or Cy3) labeled cDNAs with Eberwine's linear RNA amplification method. Subsequently, the labeled cDNAs were purified and re-suspended in 80 μl hybridization solution containing 3 × SSC, 0.2% SDS, 25% formamide and 5 × Denhart's. DNA was denatured at 95°C for 3 min before loading on the microarray, and then the microarray was hybridized at 42°C overnight. Finally, microarray was washed and scanned as mentioned above. The operation of microarray analysis followed the CapitalBio standard procedures, and the details were described in the previously published protocols [Yu et al., 2008]. The original data of mRNA microarray are available from the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo), and the accession number is GSE26377.

QUANTITATIVE RT–PCR ANALYSIS OF miRNA

The primer design (Supplementary Table SI) and reverse transcriptase reactions were performed according to the method of Chen et al. [2005]. Briefly, reverse transcriptase reactions contained 100 ng total RNA, 50 nM stem-loop RT primer, 5 × RT buffer (Promega, Wisconsin), 0.25 mM each of dNTPs (Takara, Japan), 200 U/μl M-MLV (Promega, Wisconsin) and 40 U/μl RNase inhibitor (Promega, Wisconsin). Total 20 μl reactions were incubated for 30 min at 16°C, 30 min at 37°C, 10 min at 70°C, and maintained at 4°C.

PCR reaction was performed employing SuperGreen quantitative PCR Kit‖ (CapitalBio, Beijing, China), and the 20 μl PCR system contained 1 μl RT product, 1 × SuperGreen PCR Mix, 0.2 μM forward primer and universal reverse primer. U6 was used as an internal control. At least three independent reactions were performed for each miRNAs. The relative quantities of each miRNA were calculated from the Ct (cycle threshold) values scaled to internal control and corrected for efficiency of amplification according to the formula Q.rel. $= 2^{-\Delta Ct}$, where $^{\Delta}Ct =$ average Ct test gene − average Ct internal control.

QUANTITATIVE RT–PCR ANALYSIS OF mRNA

For the reverse transcriptase reactions of mRNAs, 10 μg total RNA was incubated with 10 U RNase-free DNase I (Promega, Wisconsin) at 37°C for 30 min, and then purified with NucleoSpin RNA Clean-up Kit (MACHEREY-NAGEL, Germany). Purified total RNA (2 μg) was reverse transcribed with 200 U/μl M-MLV (Promega, Wisconsin). Specific primers for quantification of mRNA were listed in Supplementary Table SI. Quantitative RT-PCR reactions were performed as described above, and *ACTB* was used as internal control. Three independent reactions were done for each gene.

KEGG PATHWAY ANALYSIS BASED ON MICROARRAY ANALYSIS

Potential targets of differentially expressed miRNAs were determined using the combination of two algorithms: TargetScan [Lewis et al., 2003] and miRanda [John et al., 2004]. Genes previously reported as targets for the differentially expressed miRNAs were obtained from Argonaute database [Shahi et al., 2006]. MiRNA targets from each database as well as differentially expressed mRNAs were subjected to pathway exploration using Molecule Annotation System (MAS) software (http://bioinfo.capitalbio.com/mas/) [Sun et al., 2008] and KEGG databases [Kanehisa and Goto, 2000].

STATISTIC ANALYSIS

SAM identified genes with statistically changes in expression by assimilating a set of gene-specific *t*-tests with a 5% false discovery rate (FDR) threshold. Quantitative RT-PCR data were expressed as means ± standard error (SE), and the differences with $P < 0.05$ were considered statistically significant using a two-sided unpaired Student's *t*-test.

RESULTS

MONOCYTIC DIFFERENTIATION OF U937 CELLS INDUCED BY TPA

After treatment of TPA, most cells adhered to the plate, and exhibited star-like or spindle-like morphologic changes under phase contrast microscopy, while control cells still remained in suspension as discrete cells. Cells were harvested and stained by Wright-Giemsa, observation by light microscope showed the obvious morphological feature changes of mature monocytes/macrophages including cell spreading, nuclear convolution, and disappearance of the nucleolus (Fig. 1A, B).

CD11b is a widely used marker for identification of mature monocytes/macrophages [Yamamoto et al., 2009]. Flow cytometric analysis showed that CD11b expression greatly increased in differentiated cells compared to control cells (Fig. 1C). These results
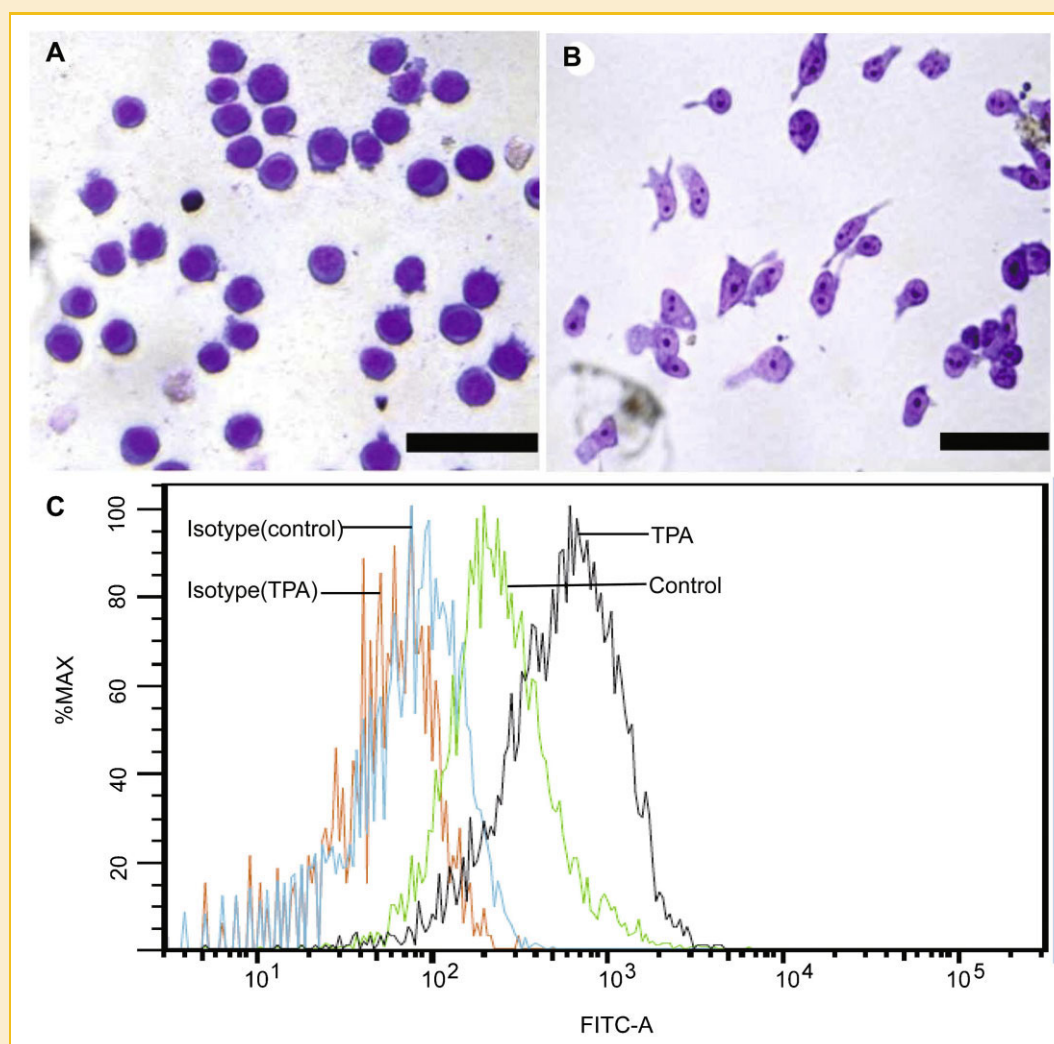

Fig. 1. Identification of TPA–induced monocytic differentiation of U937 cells. After Wright–Giemsa staining, the stained cells were observed under light microscopy (scale bar 100 μm): (A) U937 cells without TPA treatment; (B) U937 cells treated with TPA for 48 h. (C) Expression of CD11b in U937 cells with or without TPA was measured by FACS and analyzed by FlowJo software. Results in Figure 1 were repeated for at least three times, and (A), (B), and (C) are results of a typical experiment.

demonstrated that TPA induced U937 cells to differentiate to cells with mature monocytic characteristics.

DIFFERENTIAL EXPRESSION OF miRNAs IN MONOCYTIC DIFFERENTIATION

In order to investigate the changes in miRNA profiles during the monocytic differentiation, an oligonucleotide microarray-based miRNA detection platform was conducted. Differentially expressed miRNA in differentiated cells were identified by SAM statistics, and miRNAs filled with three criteria (fold change ≥ 1.5 or ≤ 0.65, q-value $\leq 5\%$, and SAM score > 2 or < -2) were considered as significantly regulated [Tusher et al., 2001]. In our study, SAM analysis generated a list of 44 miRNA differentially expressed during monocytic differentiation, with 32 up-regulated and 12 down-regulated. Based on these differentially expressed miRNAs, supervised hierarchical cluster generated a tree showing a high degree of agreement among the triplicate experiments, indicating the specificity of changes in miRNA expression and the reliability of microarray analysis (Fig. 2A).

To further validate the microarray data, quantitative RT-PCR was performed on 15 miRNAs using independent samples, and detailed analysis of fold changes for these miRNAs showed a good concordance ($R^2 \approx 0.92$) between the microarray and quantitative RT-PCR data (Fig. 2B). Taken together, using microarray and quantitative RT-PCR, a number of miRNAs were identified to be significantly regulated during monocytic differentiation.

DIFFERENTIAL EXPRESSION OF mRNAs IN MONOCYTIC DIFFERENTIATION

The mRNA expression changes in monocytic differentiation were simultaneously analyzed by human oligonucleotide microarray including approximately 22,000 gene transcripts, which allows a survey of the genome for gene expression. To reduce the false positive ratio in genome-wide oligonucleotide microarray platform, a more stringent criterion of at least 2-fold changes was adopt to select differentially expressed genes [Xiang et al., 2007]. Some 2522 genes (~10% of total genes examined) were thus found to be significantly regulated, in which 1296 genes were up-regulated and 1226 genes were down-regulated (Supplementary File 1).

Microarray profiling of differentiated U937 cells revealed a variety of regulated genes previously reported to involve in macrophages, inflammation and innate immunity including chemokines *IL-1B* and *TNF*, differentiation marker *ITGAM*, and matrix metalloproteinase protein *MMP7* and *MMP9*. A summary of these genes was listed in Table I. Interestingly, comparing our microarray data with other studies showed genes related to macrophage function such as *CCL24, IL6, MMP9,* and *CD9*, were constantly up-regulated in different differentiation conditions, while transcription factors like *FOS*, *HES1*, *BCL6*, and *NFKB1* were highly up-regulated only in the differentiation induced by TPA or LPS activation (Table I). In addition, in the same differentiation models of TPA-induced differentiation of U937, about 80% of checked genes showed consistent expression patterns between our microarray data and other microarray data from previously published study [Baek et al., 2009], which indicated the specificity of genetic regulation and the reliability of microarray data.

INTRINSIC CORRELATION BETWEEN EXPRESSION LEVELS OF miRNAs AND TARGET mRNAs REVEALED BY BIOINFORMATICS INTERPRETATION

Using TargetsScan and miRanda database, 583 differentially expressed mRNAs were predicted as 44 miRNA targets in monocytic differentiation (Fig. 3). To reduce the number of false positives, only targets predicted by both programs were accepted. Meanwhile, a list of the differentially expressed miRNAs and mRNAs was compared to the known miRNA-target interactions reported in the Argonaute database, a total of 183 differentially expressed mRNAs were listed as targets of 31 miRNAs regulated in monocytic differentiation (Fig. 3).

In most cases, the miRNAs function as negative regulators in gene expression, thus negative correlations between miRNAs and target mRNAs expression levels were scrutinized for these interactions (Table II, and see Supplementary data Table SII for complete list), eight negative correlations in which were confirmed by quantitative RT-PCR, and shown in Figure 4A.

Further functional implications of the negative correlations between miRNA and mRNA expression were also sought by application of the MAS software and its accompanying interaction database, from which canonical pathways associated with differentially expressed genes can be determined. Fisher's exact test was used to identify significant enriched pathways based on the incidence of identified differentially expressed genes.

This analysis indicated 183 reported targets of differentially expressed miRNAs involving in 96 pathways, among which 35 were significantly enriched pathways (P-value < 0.001), including MAPK [Matsumoto et al., 2006], TGF-β [Shi and Simon, 2006], Toll-like receptor signaling pathway [Krutzik et al., 2005], JAK/STAT [Tanuma et al., 2000], and Wnt signaling pathways [Tickenbrock et al., 2006], which have been previously reported to be closely related with the function of cell differentiation (Table SIII). According to KEGG terminology, 35 significant enriched pathways were categorized into 11 functional categories (Fig. 5A). Likewise, 583 predicted targets of miRNAs differentially expressed in monocytic differentiation participated in regulating 139 pathways (Table SIV), in which 40 significantly enriched pathways (P-value < 0.001) were found and grouped into 16 functional categories (Fig. 5B). During monocytic differentiation, 804 differentially expressed mRNA involved in 163 pathways (Table SV), Fisher's exact test showed 71 of them were significantly enriched (P-value < 0.001). Furthermore, these 71 significantly enriched pathways were categorized into 24 functional categories (Fig. 5C). As shown in Figure 5, a similarity can be observed between the functional patterns of predicted miRNA targets and differentially expressed mRNA.

INTRINSIC CORRELATION BETWEEN EXPRESSION LEVELS OF miRNAs AND HOST mRNAs IN MONOCYTIC DIFFERENTIATION

According to their genomic location, the miRNAs can be divided into intergenic miRNAs and intragenic (intronic/extronic) miRNAs [Hinske et al., 2010]. Using the National Center for Biotechnology Information (NCBI) mRNA reference sequences and miRBase release 16 (September 2010), of 44 differentially
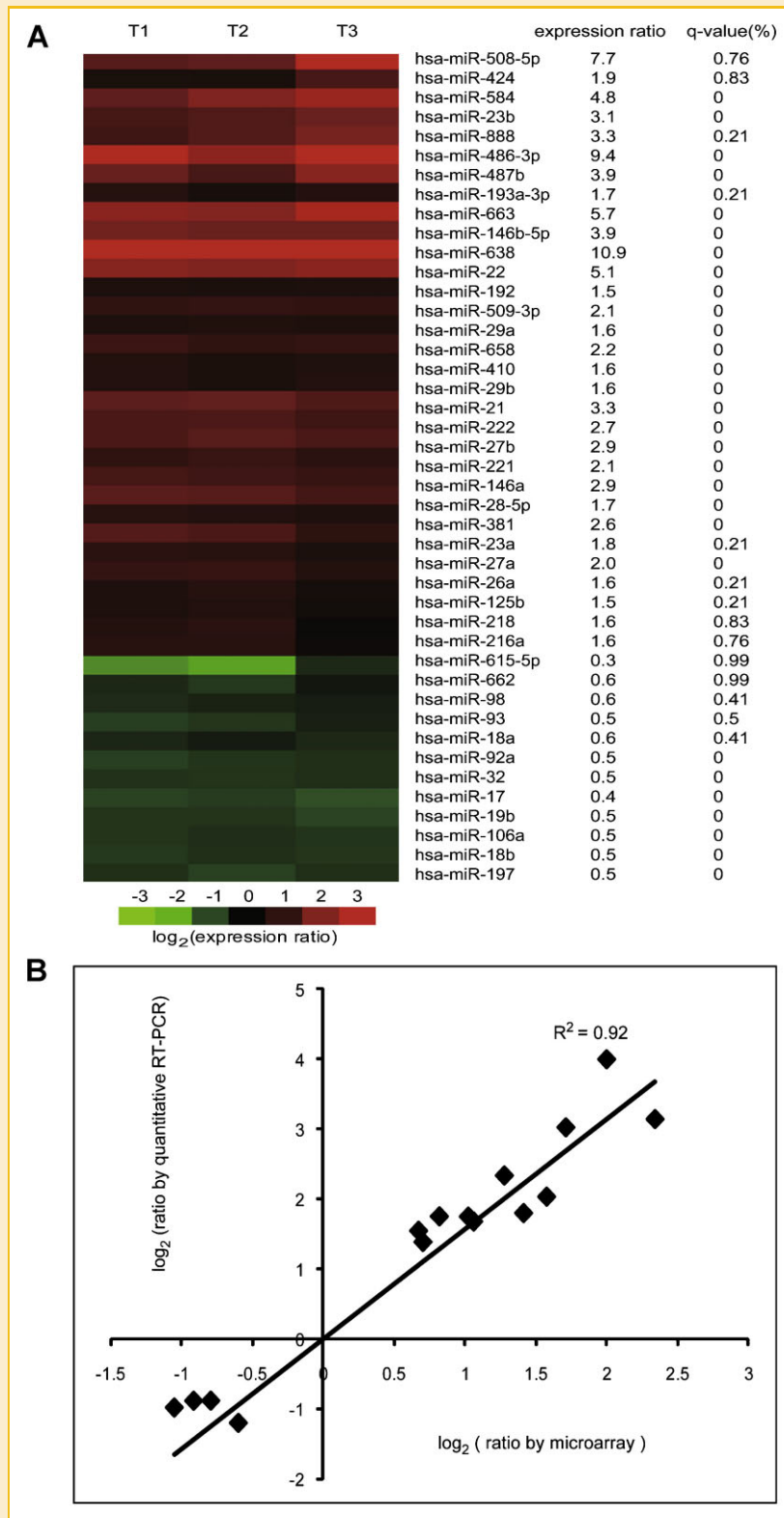
Fig. 2. Identification of differentially expressed miRNAs during monocytic differentiation. A: A tree generated by Cluster 3.0 software on the basis of differentially expressed miRNAs detected by microarray analysis, and red indicated expression upregulation and green indicated expression downregulation; column showed miRNAs list, expression ratio indicated the fold changes of expression of miRNAs in differentiated cells compared to the control cells, and q–value indicated the lowest positive False Discovery Rate at which the gene was called significant; row showed three independent biological replications, labeled as T1, T2, and T3. B: Pearson correlation scattered plots of comparisons of ratios measured by microarray and quantitative RT–PCR in fifteen miRNAs; R, the Pearson linear correlation coefficient value.

TABLE I. List of Representative Regulated Genes (Sorted by TPA Treatment).

Gene names	Gene symbols	Gene modulation		
		TPA[a]	LPS[b]	MΦ[c]
Cytokines and Chemokines				
Tumor necrosis factor (TNF superfamily, member 2)	TNF	13.5 (40.2)	↑	↑
Interleukin 1, alpha	IL1A	25.2	↑	↑
Interleukin 1, beta	IL1B	27.8 (13.3)	↑	↑
Chemokine (C-C motif) ligand 20	CCL20	38.3	↑	—
Interleukin 6 (interferon, beta 2)	IL6	12.6	↑	↑
Chemokine (C-C motif) ligand 1	CCL1	33.4	↑	↑
Chemokine (C-X-C motif) ligand 2	CXCL2	6.6	↑	↑
Chemokine (C-C motif) ligand 24	CCL24	40.0	↑	↑
Chemokine (C-C motif) ligand 5	CCL5	3.8	↑	—
Surface molecules				
CD83 molecule	CD83	9.8	↑	—
CD53 molecule	CD53	2.9	↑	↑
Integrin, alpha M	ITGAM	2.5	↑	↑
CD58 molecule	CD84	3.0	↑	↑
CD9 molecule	CD9	2.0 (4.5)	↑	↑
Signaling and effector molecules				
Endothelin 1	EDN1	24.7	↑	—
Matrix metallopeptidase 7 (matrilysin, uterine)	MMP7	102.0 (40.2)	↑	↑
Serum/glucocorticoid regulated kinase 1	SGK	12.1	↑	—
Cyclin-dependent kinase inhibitor 1A (p21, Cip1)	CDKN1A	11.5 (13.5)	↑	↓
Matrix metallopeptidase 9	MMP9	9.2	↑	↑
Myeloid differentiation primary response gene (88)	MYD88	2.1	↑	—
Complement component	C3	5.6	↑	↑
Cyclin D1	CCND1	5.8 (2.0)	↑	↑
Transforming growth factor, beta-induced, 68 kDa	TGFBI	5.8	↑	—
Cyclin A1	CCNA1	0.1	—	—
Coiled-coil domain containing 6	CCDC6	0.3 (0.2)	—	↓
Cyclin A2	CCNA2	0.1 (0.1)	↓	—
Cell division cycle 25 homolog A (S. pombe)	CDC25A	0.3 (0.1)	↓	—
Cyclin-dependent kinase	CDK4	0.2	↓	↑
Tumor necrosis factor (ligand) superfamily, member 13b	TNFSF13B	0.1	↓	↓
Transcription factors/Histone modifiers				
Early growth response 2 (Krox-20 homolog, Drosophila)	EGR2	31.5	↑	↑
B-cell CLL/lymphoma 6	BCL6	8.3	↑	—
Myocyte enhancer factor 2D	MEF2D	2.2	↑	↓
Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1	NFKB1	2.3 (14.4)	↑	—
Myocyte enhancer factor 2A	MEF2A	5.5	↑	↑
Sprouty homolog 2 (Drosophila)	SPRY2	5.7	↑	↑
v-fos FBJ murine osteosarcoma viral oncogene homolog	FOS	3.1	↑	↓
Hairy and enhancer of split 1, (Drosophila)	HES1	2.1	↑	↑
MAX dimerization protein 1	MAD	3.6	↑	↑
Activating transcription factor 5	ATF5	4.4	↑	↑
Myocyte enhancer factor 2C	MEF2C	2.7	↑	↑
Kruppel-like factor 13	KLF13	2.2	↑	—
Nuclear receptor subfamily 4, group A, member 3	NR4A3	41.1	↑	↓
Histone deacetylase 7	HDAC7A	2.3	—	—
BRCA1 interacting protein C-terminal helicase 1	BACH1	2.8	—	↓
Activating transcription factor 3	ATF3	3.2	—	↑
CCAAT/enhancer binding protein (C/EBP), beta	CEBPB	5.5	—	↑
Cytochrome b-245, beta polypeptide	CYBB	0.3	↓	↑
Hematopoietically expressed homeobox	HHEX	0.4	↓	↓
Forkhead box M1	FOXM1	0.2	↓	—
Histone deacetylase 2	HDAC2	0.4 (0.4)	↓	↑
Homeobox A5	HOXA5	0.4	↓	↓
v-myb myeloblastosis viral oncogene homolog (avian)	MYB	0.05	↓	—

[a]This column showed mean fold changes of regulated genes in differentiation of U937 induced by TPA; bracketed numbers indicated mean fold changes obtained by quantitative RT-PCR ($P < 0.05$).
[b]Results shown were for subsequent activation of TPA differentiated U937cells by LPS, obtained from the previous published studies [Baek et al., 2009]; the arrows indicated the direction of gene expression changes, and short strings indicated no data or no significant changes of gene expression.
[c]Results shown were for adherence-induced differentiation of primary human monocytes, which were obtained from GEO database (GES8286) [Liu et al., 2008].

expressed miRNAs identified in our study, 25 miRNAs can be located within corresponding host mRNA transcripts. Among them, 13 intragenic miRNAs have the same orientation with their host mRNAs, and 12 intragenic miRNAs reside in antisense orientation to their corresponding host mRNAs (Supplementary Table SVI). Previous studies indicated intragenic miRNAs might be transcribed in the sense orientation as part of the pre-mRNA gene structure and further released and processed to mature miRNA molecules following splicing events, while miRNAs in antisense orientation to their annotated host mRNAs might possess their own independent transcription units inside the host mRNAs [Hinske et al., 2010; Li et al., 2007].

Examining correlations of the expression between 25 intragenic miRNAs and their corresponding host genes, eight miRNAs were found to be positively correlated with their host genes in expression level (Table III). In addition, few negative correlations between
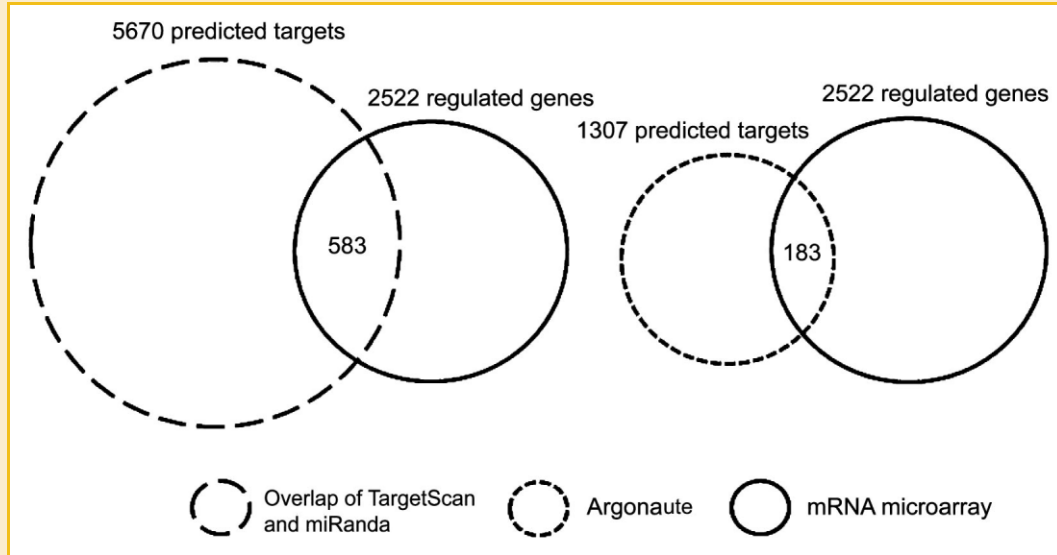
Fig. 3. The number of target genes used for analysis of correlations between miRNA and mRNA. Three data sources of miRNA targets were used, including TargetScan, miRanda, and Argonaute. The intersection of target database and microarray data was the number of genes used for further analysis.

expression of intragenic miRNAs and host genes were also observed (Table III). The expression levels of several host gene/intragenic miRNA pairs (*MGC14376*/miR22, *C13orf25*/miR17 cluster, and *MCM7*/miR93) were further validated by quantitative RT-PCR, supporting the positive correlation between them during monocytic differentiation (Fig. 4B).

DISCUSSION

This study included: (1) profiling the specific expression patterns of both miRNAs and mRNAs during monocytic differentiation simultaneously; (2) scrutinizing the negative correlations between the expression levels of miRNAs and targets mRNAs and revealing

TABLE II. Selected Negatively Correlated miRNAs and mRNAs

| miRNAs	Target symbols	Pathways	Identified by	Target modulation
Upregulated miRNAs				
has-miR-638	*GAMT*	Glycine, serine and threonine metabolism	Targetscan and miRanda	0.3
has-miR-508	*INHBB*	Cytokine-cytokine receptor interaction	Targetscan and miRanda	0.4
has-miR-663	*PPP5C*	MAPK signaling pathway	Targetscan and miRanda	0.3
hsa-miR-22	*GEMIN5*		Argonaute	0.2
has-miR-584	*DDB2*	Pyrimidine metabolism	Targetscan and miRanda	0.4
hsa-miR-146a	*CCDC6*	Thyroid cancer	Argonaute	0.3
hsa-miR-146b	*BRCA1*	Ubiquitin mediated proteolysis	Targetscan and miRanda	0.3
hsa-miR-487b	*CDK4*	Cell cycle	Argonaute	0.2
has-miR-888	*RBL1*	Cell cycle	Targetscan and miRanda	0.3
hsa-miR-21	*PARP1*	Base excision repair	Argonaute	0.3
hsa-miR-23a/b	*MYC*	MAPK signaling pathway	Argonaute	0.2
hsa-miR-27a/b	*PCNA*	DNA polymerase;	Argonaute	0.3
has-miR-221	*STMN1*	MAPK signaling pathway	Argonaute	0.3
has-miR-509	*PGM1*	Glycolysis/Gluconeogenesis	Targetscan and miRanda	0.3
has-miR-424	*CHEK1*	Cell cycle	Targetscan and miRanda	0.3
has-miR-28	*MAD2L1*	Cell cycle	Targetscan and miRanda	0.3
has-miR-193	*P2RX5*	Calcium signaling pathway	Targetscan and miRanda	0.4
hsa-miR-29a/b	*HMGB1*	Base excision repair	Argonaute	0.4
hsa-miR-24	*E2F2*	Cell cycle	Argonaute	0.1
has-miR-381	*CCNA2*	Cell cycle	Targetscan and miRanda	0.2
hsa-miR-218	*MYC*	MAPK signaling pathway	Argonaute	0.2
has-miR-410	*CTNNBIP1*	Wnt signaling pathway	Targetscan and miRanda	0.4
hsa-miR-26a	*EZH2*		Argonaute	0.5
hsa-miR-192	*SIP1*		Argonaute	0.3
hsa-miR-125b	*EIF4EBP1*	Wnt signaling pathway	Argonaute	0.2
Downregulated miRNAs				
hsa-miR-17/18a/92a-1	*CCND1*	Cell cycle	Argonaute	5.8
hsa-miR-106a/18b/19b/92a-2	*LIF*	Cytokine-cytokine receptor interaction	Argonaute	8.8
has-miR-662	*DUSP5*	MAPK signaling pathway	Targetscan and miRanda	11.9
hsa-miR-98	*TNF*	MAPK signaling pathway	Argonaute	13.5
hsa-miR-93	*CDKN1A*	ErbB signaling pathway	Argonaute	11.6
hsa-miR-32	*CDKN2A*	Cell cycle	Argonaute	2.8
has-miR-197	*ACVR1*	Cytokine-cytokine receptor interaction	Targetscan and miRanda	4.8
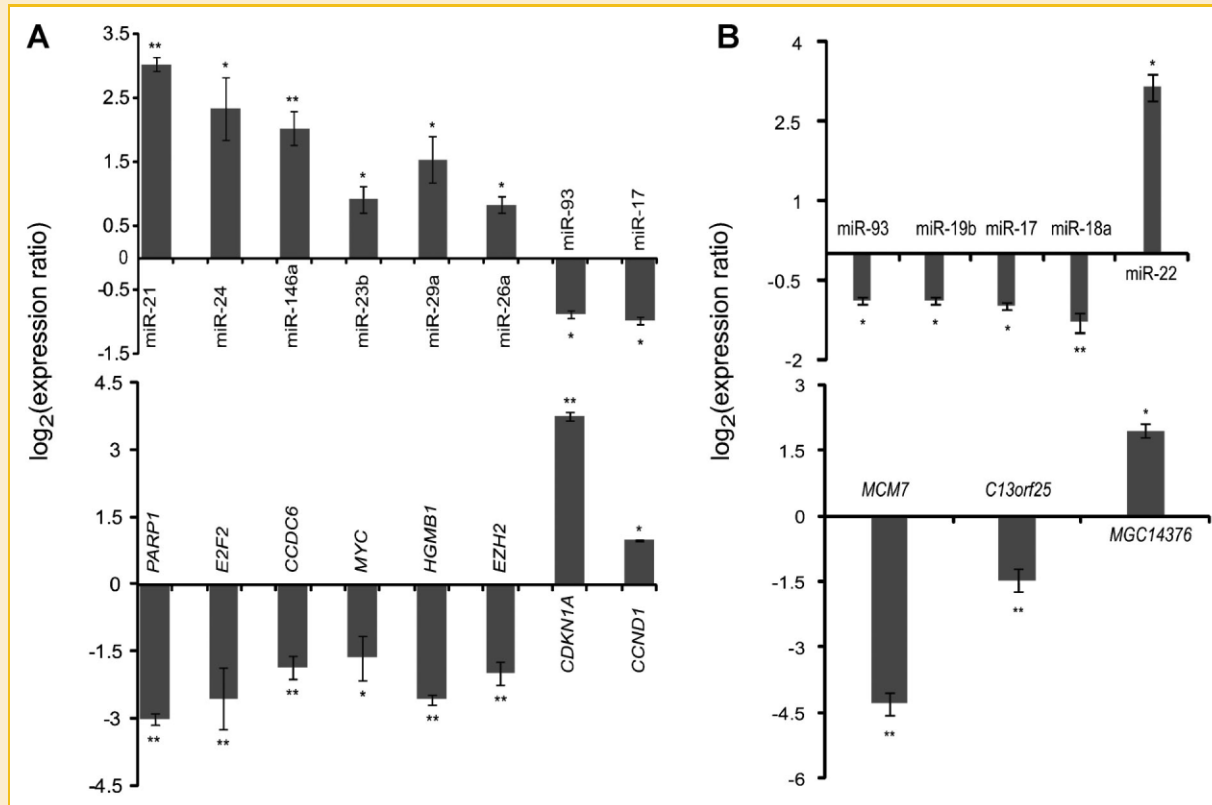
Fig. 4. Representative correlations between miRNA and mRNA revealed by quantitative RT–PCR. A: Quantitative RT–PCR validation with eight miRNAs and their targets predicted by TargetScans or Argonaute. Results shown were the fold changes of expression of these transcripts in differentiated cells compared to the control cells. The expression of *ACTB* was used as a baseline to calculate fold changes. B: Quantitative RT–PCR confirmation of five positive correlations between intragenic miRNAs and their host genes uncovered by microarray data, and relative quantities were calibrated to U6. Error bars represented SE of the expression ratios from triplicate biological replicates; $^*P < 0.05$, $^{**}P < 0.01$.

the functional interactions underlying negative correlations; (3) examining intrinsic correlations between the expression levels of intragenic miRNAs and host gene mRNAs in the context of monocytic differentiation.

The roles of miRNAs in gene expression received more and more attention, with the extensive applications of expression profiling analysis on miRNAs, systematic investigation on the correlations between miRNAs and target mRNAs using expression data could give us more information on the miRNA regulation [Huang et al., 2007]. For example, joint genome-wide profiling of miRNA and mRNA expression revealed pivotal functions of miRNAs in brain disease [Nunez-Iglesias et al., 2010]. MiRNAs-mRNAs interactions in NCI-60, a panel of 60 human cancer lines from several distinct tissues, were analyzed by computing the paired correlations based on the expression profiles of miRNA and mRNA [Wang and Li, 2009]. These studies focused on the negative correlations between expression of miRNA and mRNA, yet, positive correlations between host mRNAs and intragenic miRNAs have also been revealed by microarray analysis [Baskerville and Bartel, 2005]. Since the expression profile of miRNAs is specific in different tissues or under different physiological conditions, the correlations between miRNAs and mRNAs could vary under different biological circumstances. This is a study that also used expression profiles of miRNA and mRNA during monocytic differentiation to explore

the correlations between miRNAs and mRNA expression, either negative or positive.

During monocytic differentiation, the expression levels of numerous miRNAs were regulated (Fig. 2), including miR-21, miR-146a, miR-146b, miR-221, miR-222, and miR-424 as previously reported [Forrest et al., 2010; Kasashima et al., 2004; Taganov et al., 2006]. Moreover, some miRNAs not reported previously to be correlated with monocytic differentiation were also identified, and these newly identified miRNAs may participate in specific miRNA-target interactions which could be informative for potential new functions involved in monocytic differentiation.

Strategies for high-throughput miRNA target validation are still limited; therefore, target prediction algorithms are employed to assess large-scale miRNA-target interactions [Huang et al., 2007]. In this study, the data obtained from two popular algorithms (TargetScan and miRanda) were used to overcome individual biases caused by one specific method, because different target prediction algorithms are calculated from different types of information about miRNA targets and make different predictions. Furthermore, with the exponential growth of experimental knowledge about miRNA targets, the Argonaute database is also informative for exploring miRNA-target interactions underling complex biological conditions. Using Argonaute database, a lot of previously known miRNA-target interactions were also found during monocytic
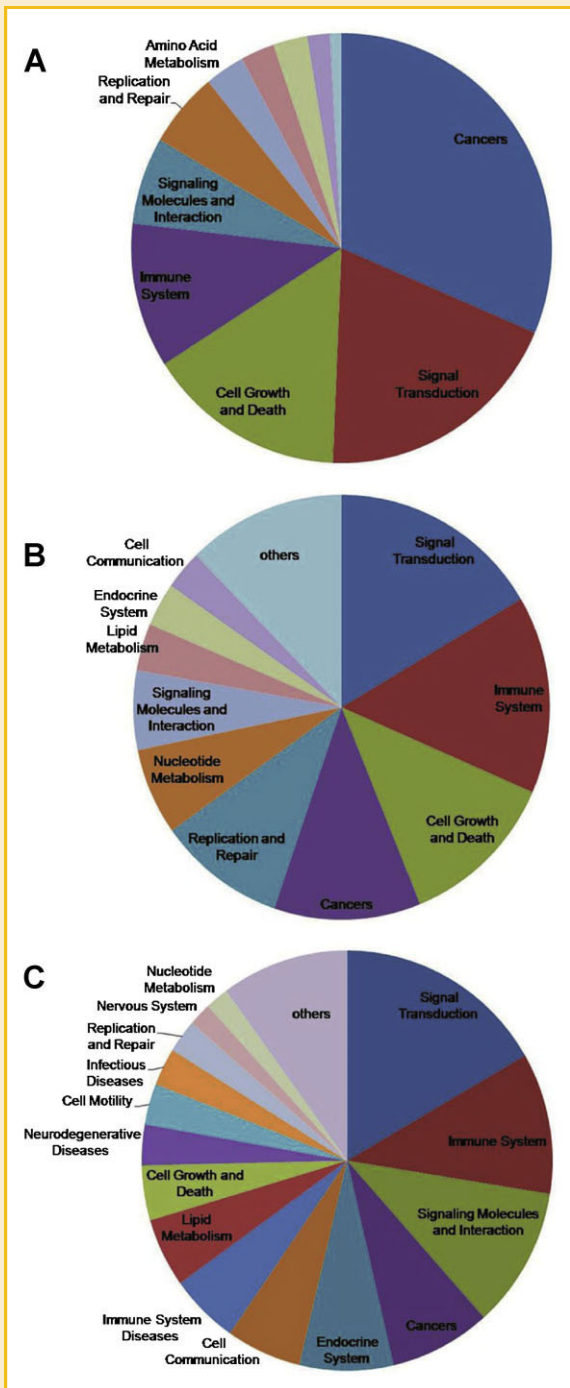
Fig. 5. Functional pattern analysis of mRNAs and miRNAs regulated in monocytic differentiation. The pie-charts shown were functional categories associated with validated miRNA targets obtained from Argonaute (A), predicted miRNA targets obtained from the combination of TargetScan and miRanda (B), and differentially expressed mRNAs identified by microarray (C); each pie indicated the proportion of the corresponding functional category.

differentiation (Table II and Table SII), for example, *CCDC6* and *CCNA2* as targets of miR-146a [Hsieh et al., 2010; Jazdzewski et al., 2008], *CDC25A*, *E2F2*, and *PARP1* as targets of miR-21 [Bhat-Nakshatri et al., 2009; Seike et al., 2009; Wang et al., 2009], and

EZH2 as target of miR-26a [Wong and Tellam, 2008]. It's worth noting that most miRNA targets identified were cell cycle control genes, epigenetic modifiers, and DNA replication components, which are overexpressed in pro-monocyte progenitors and involve in retaining proliferation of pro-monocyte progenitors [Asada et al., 1999; Iglesias-Ara et al., 2010]. As the expressions of these genes were inhibited by miRNAs, the proliferative potential could be repressed, as well as growth arrest involving in the differentiation could be induced. Thus, negative regulation of miRNAs is pivotal in controlling monocytic differentiation.

Importantly, negative correlations between expression levels of miRNAs and target mRNAs could shape functional interactions, for a given miRNA expression upregleted while corresponding to decreased expression of its targets related to certain pathways downregulated [Pandey et al., 2008]. In the present study, targets of regulated miRNAs were found to associate with specific functional pathways, which could be categorized into several functional categories; likewise, the pathways related with differential expressed mRNAs were also grouped into functional categories. Comparison of these functional categories from regulated miRNA and regulated mRNA emphasized our initial hypothesis that miRNA display targeting patterns appearing not only to influence targets' expression but also their functional environment, because their functional categories were found to be mainly overlapping in our study (Fig. 5). Among these functional categories, signal transduction, immune system, cell growth and death, and cancer took the dominant proportion of total categories; these results could help us better understand the mechanisms of monocytic differentiation.

Research on the correlation between intragenic miRNA and host mRNAs revealed different relations between miRNA and mRNA. In the past few years, a few studies reported the significant correlation between miRNA and host genes expression by comparing the expression profiles of miRNAs and their host genes, and indicated that intronic miRNAs and their host genes were regulated dependently [Baskerville and Bartel, 2005; Lionetti et al., 2009]. In the present study, several positive correlations between intragenic miRNA and host genes expression were observed during monocytic differentiation (Table III and Fig. 4B). Nevertheless, in seventeen out of twenty-five intragenic miRNAs indentified here were not coordinated with their corresponding host mRNAs in expression levels; besides, half of miRNAs residing in the same orientation with their host mRNA were still not co-regulated with their host mRNAs. Given this, a hypothesis was raised that positive correlation between intragenic miRNAs and host mRNAs expression may not be widespread phenomenon in cells.

In addition, our data also implied that even miRNAs in an antisense orientation to its host genes (e.g., miR-22 and miR-93) could be co-regulated with its host genes, while miRNAs in a sense orientation to its host genes might be regulated independently. Combing with previously observations, our findings support the idea that multiple regulation events regulate miRNA levels other than co-transcription from the host mRNA promoter. Even if intragenic miRNAs could be co-transcribed with the host mRNA, the cleavage of post-transcriptional processing of primary miRNAs to mature miRNAs may lead to diverse miRNA expression, compared to the host mRNA expression [Sikand et al., 2009]. Moreover, a recent

TABLE III. Intragenic miRNA Expression Correlates With Their Host Gene Expression

Intragenic miRNAs	Host genes	Genomic location	Strand direction[a]	Fold changes	
				miRNA	mRNA
hsa-miR-638[b]	DNM2	chr.19 intronic	+	10.9	2.1
hsa-miR-22[b]	MGC14376	chr.17 exonic	−	5.1	3.5
hsa-miR-28-5p[b]	LPP-019	chr.3 intronic	+	1.7	3.4
hsa-miR-26a[c,d]	CTDSPL	chr.3 intronic	+	1.6	0.6
hsa-miR-615-5p[c]	HOXC4	chr.12 intronic	+	0.3	3.7
hsa-miR-93[b,d]	MCM7	chr.7 intronic	−	0.5	0.1
has-miR-18a[b]	C13orf25	chr.13 intronic	+	0.6	0.3
hsa-miR-17[b]	C13orf25	chr.13 intronic	+	0.4	0.3
hsa-miR-92a[b]	C13orf25	chr.13 intronic	+	0.5	0.3
hsa-miR-19b[b]	C13orf25	chr.13 intronic	+	0.5	0.3

[a]+, miRNAs locate in the sense orientation of the pre-mRNA gene; −, miRNAs locate in the antisense orientation of pre-mRNA gene.
[b]These intragenic miRNAs expressions positively correlate with their host genes expressions.
[c]These intragenic miRNAs expressions negatively correlate with their host genes expressions.
[d]These intragenic miRNAs were also predicted to target their host genes.

study indicated transcription of roughly 30% of intragenic miRNAs may be initiated independently, and approximately 20% of intragenic miRNAs were also predicted to target their host mRNA transcripts [Hinske et al., 2010]. In this case, potential negative feedback loops between intragenic miRNA, host genes and miRNA targets may exist during cellular processes.

Most of host genes are protein coding genes, and have specific functions in cellular processes (Table SVI). In this study, miR-93 was found to be coexpressed with host gene MCM7, which has been indicated to involve in regulating cell proliferation [Blow and Hodgson, 2002], furthermore miR-93 has also been found to contribute to cell proliferation by regulating cell cycle controlling genes like E2F1, CDKN1. So the coexpression of intragenic miRNAs and host genes in certain physiological condition may imply the closely related functions between them. In addition, a few host genes have no definite functions, which are non-coding genes or even novel processed transcripts, yet their specific expression patterns with respect to intragenic miRNAs may add a new way to identify biomarkers, because miRNAs expression has been reported to be associated with tissue specific [Wienholds et al., 2005]. During monocytic differentiation the significant expression changes of C13orf25 and MGC14376 coordinated with corresponding intragenic miRNAs, indicating these genes could be a potential biomarker in maturation of monocytes. Until now, the funcional aspects of intragenic miRNAs are still largely unknown, the correlation between expressions of intragenic miRNA and host mRNA studied here is a starting point for an in-depth analysis of intragenic miRNA under monocytic differentiation.

In conclusion, our results suggested that microarray expression profiles could be used to assist the identification of intrinsic correlation between miRNAs and mRNAs. Examining correlations between miRNA and mRNA expression levels provided a multi-tiered approach for studying the gene regulations underlying complex biological process.

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