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Epigenetic regulation of the metallothionein-1A promoter by PU.1 during differentiation of THP-1 cells

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ABSTRACT

We recently demonstrated that metallothionein (MT)-1 A is a direct target gene negatively regulated by PU.1. In this study, we revealed that the expression of PU.1 was increased and accompanied by downregulation of MT-1A expression during TPA-induced THP-1 monocyte differentiation. Chromatin immunoprecipitation (ChIP) analysis demonstrated that PU.1 and the methyl CpG binding protein (MeCP) 2 bound to the same −887 to −602 region in the MT-1A promoter, and the binding of these proteins to this promoter was enhanced during differentiation. Consistently, bisulfite sequencing analyses around this region revealed that the proportion of methylated CpG sites was obivously increased during differentiation. In addition, ChIP analysis demonstrated that acetylated histone H4 around this region tended to be reduced and this may also play a role in the reduction of MT-1A expression during differentiation. Taken together, these findings suggest that MT-1A is epigenetically regulated by PU.1 during monocytic differentiation.

1. Introduction

PU.1 is a member of the Ezb transformation-specific sequence family of transcription factors, and is expressed in granulocytic, monocytic and B-lymphoid cells [1]. PU.1-deficient mice exhibit defects in the development of neutrophils, macrophages and B cells [2]. Thus, PU.1 is indispensable for myelomonocytic and B cell differentiation during normal hematopoiesis. In addition, mice carrying hypomorphic PU.1 alleles that reduce PU.1 expression to 20% of its normal levels have been reported to develop acute myeloid leukemia (AML) [3]. Moreover, downregulation of PU.1 has been reported to play a role in the pathogenesis of multiple myeloma [4], and is related to poor prognoses of myelodysplastic syndrome [5]. Therefore, clarifying the function of the PU.1 transcription factor during hematopoietic cell differentiation is important to understand the molecular biology of hematopoiesis and oncogenesis.

In dual microarray analyses of PU.1-knockdown K562 (K562PU.1KD) cells stably expressing PU.1 siRNA and PU.1-overexpressing K562 (K562PU.1OE) cells, we found that the expression of MT-1A is markedly induced in K562PU.1KD cells and suppressed in K562PU.1OE cells [6]. We revealed that PU.1 directly binds to the MT-1A promoter for epigenetic suppression though histone deacetylase (HDAC) and DNA methyltransferase (Dnmt) activities. Reduction of PU.1 expression leads to aberrant induction of MT-1A through suppression of HDAC and Dnmt enzyme activities [6].

The human monocytic leukemia cell line THP-1 differentiates into mature cells with the functions of macrophages by stimulation with 12-*O*-tetradecanoylphorbol-13-acetate (TPA) [7]. In this study, we examined whether the expression of PU.1 and MT-1A is indeed correlated to each other, and whether MT-1A expression is regulated by PU.1 during TPA-induced THP-1 monocyte differentiation.

2. Materials and methods

2.1. Cell culture and reagents

THP-1 cells were grown in RPMI medium (GIBCO BRL, Rockville, MD) containing 10% heat-inactivated fetal bovine serum (Biowest, Miami, FL), 100 U/ml penicillin and 100 μg/ml streptomycin (GIBCO BRL) as described previously [8]. TPA was obtained from Sigma (St. Louis, MO), and a stock solution was prepared in dimethyl

Abbreviations: MT, metallothionein; ChIP, chromatin immunoprecipitation; AML, acute myeloid leukemia; K562PU.1KD, PU.1-knockdown K562; K562PU.1OE, PU.1-overexpressing K562; HDAC, histone deacetylase; Dnmt, DNA methyltransferase; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; DMSO, dimethyl sulfoxide; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HSCs, hematopoietic stem cells; ROS, reactive oxygen species.

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sulfoxide (DMSO) and stored at −20 °C. For THP-1 cell differentiation, TPA was added to the medium at a final concentration of 10 ng/ml, and the same concentration of DMSO was added for the control. For morphological examination, cells were stained by the Wright-Giemsa staining method.

2.2. mRNA expression analyses

For RNA preparation and real-time PCR analyses, THP-1 cells and their controls were seeded at a density of 1×10^5 cells/ml and treated with 10 ng/ml TPA or an equal volume of DMSO, and then harvested at the indicated times. cDNA was prepared from cells using a Transcriptor first strand cDNA synthesis kit (Roche, Indianapolis, IN). Quantitative PCR was performed using Quantitect SYBR Green PCR Reagent (Qiagen, Miami, FL) according to the manufacturer's protocol and an Opticon Mini Real-time PCR instrument (Bio-Rad, Hercules, CA) as described previously [9]. The primer sequences were as follows. PU.1: forward, 5′-GTGCCCTATGACAACGGATCT-3′, reverse, 5′-GAAGCTCTCGAAC TCGCTGT-3′; MT-1A: forward, 5′-CTCGAAATGGACCCCAACT-3′, reverse, 5′-ATATCTTCGAGCAGGGCTGTC-3′, MT-1G: forward, 5′-CTTCTCGCTTGGGAACTCTA-3′, reverse, 5′-AGGGGTCAAGATTG-TAGCAAA-3′ CD11c: forward, 5′-ACCACAAGCAGTAGCTCCTTC-3′, reverse, 5′-AAGTAGGAGCCGATCTGAGTC-3′; CD14: forward, 5′-GTCTGCAACTTCTCCGAACC-3′, reverse, 5′-CCAGTAGCTGAGCAG-GAACC-3′; glyceraldehyde-3-phosphate dehydrogenase (GAPDH): forward, 5′-GAAGGTGAAGGTCGGAGT-3′, reverse, 5′-GAAGATGGT-GATGGGATTTC-3′. The thermal cycling conditions for PU.1 were: 95 °C for 15 min, and then 35 cycles of 95 °C for 15 s and 60 °C for 1 min. PCR conditions for the other genes were: 95 °C for 15 min, and then 35 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s. The copy number of each sample was calculated as described previously [10].

2.3. Bisulfite DNA sequencing analysis

Genomic DNA was prepared from differentiated or undifferentiated THP-1 cells using a Blood & Cell Culture DNA Mini Kit (Qiagen) according to the manufacturer's protocol. The isolated genomic DNA was subjected to bisulfite DNA treatment using an EZ DNA Methylation-Gold Kit (Zymo Research, Orange, CA) according to the manufacturer's protocol. The resulting DNA was subjected to

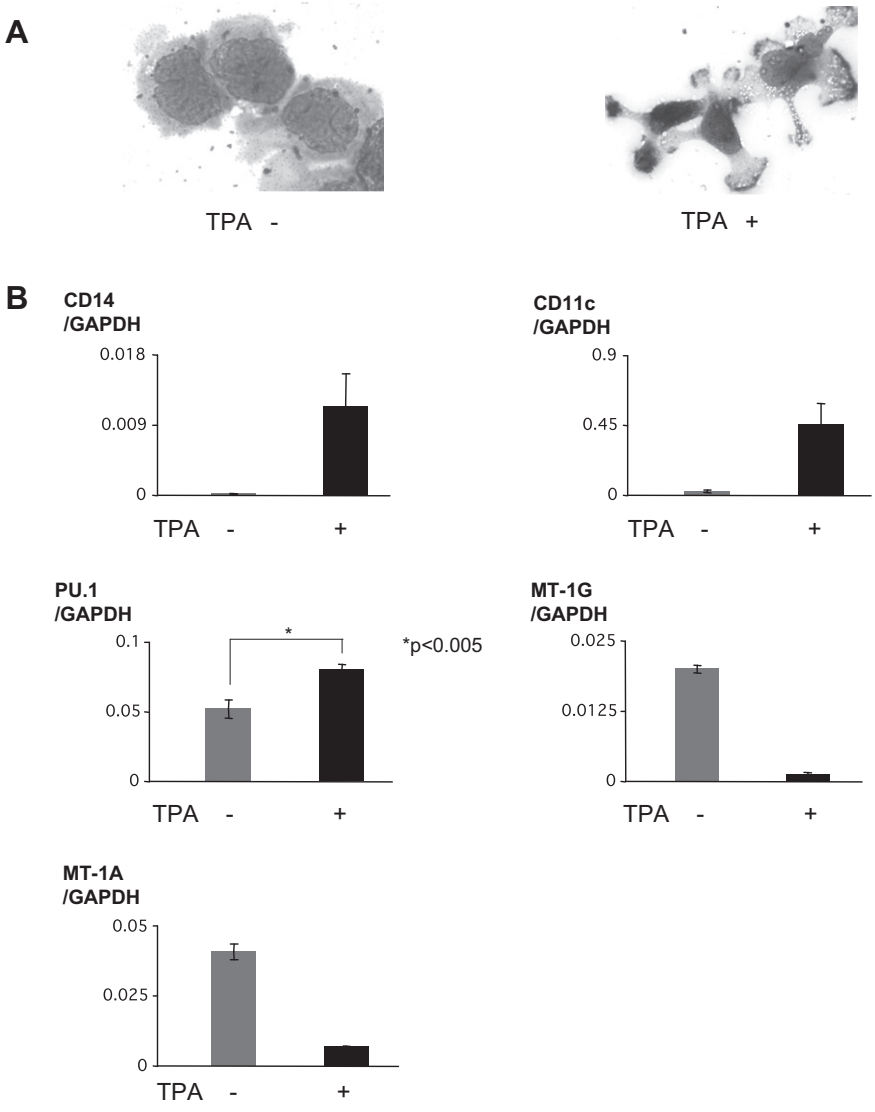

Fig. 1. TPA induces the PU.1 expression, while reducing MT-1A expression in THP-1 cells. (A) Morphology of THP-1 cells untreated (left panel) and treated (right panel) with 10 ng/ml TPA for 5 days. Original magnification, 1000×. (B) Expression of *CD14*, *CD11c*, *PU.1*, *MT-1G* and *MT-1A* was examined by real-time PCR. The *p*-value from the Student's *t*-test is shown. The data presented were obtained from three replicates and reproducibility was confirmed by two independent preparations of cDNA.

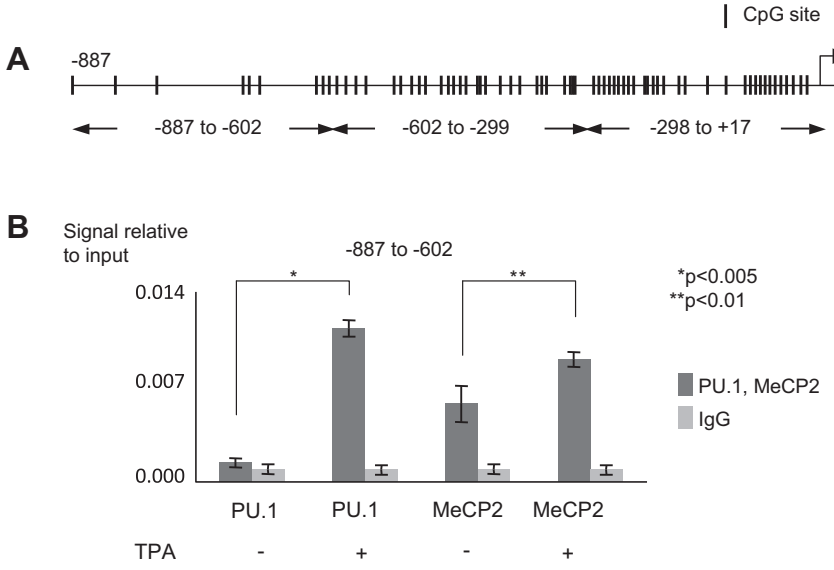

Fig. 2. PU.1 and MeCP2 bind to the same region in the MT-1A promoter, and the binding activities of these proteins are increased in TPA-induced THP-1 cells. (A) Structure of the MT-1A promoter. Indicated regions were examined by ChIP analysis. (B) ChIP assays were performed with crosslinked chromatin from 1×10^7 THP-1 cells cultured in the presence or absence of TPA. Either the anti-PU.1 or anti-MeCP2 antibody, or same amount of rabbit IgG were employed using a ChIP IT express kit as described in Section 2. The amount of immunoprecipitated DNA in each sample is represented as the signal relative to the total amount of input chromatin, which is equivalent to one, shown in the *y*-axis. Data are representative of two independent experiments and similar results were obtained. *P*-values from the Student's *t*-test are shown. Binding of PU.1 and MeCP2 to the MT-1A promoter was not observed at −602 to −299 or −298 to +17 (data not shown).

PCR amplification using the following primers. MT-1A: forward, 5′-GGAGGATTTGGATAAATGTG-3′, reverse, 5′-CTAAATACAACCA-CAACCTC-3′. The amplified fragment was purified by 2% agarose gel electrophoresis, recovered using a Wizard SV Gel and PCR Clean-up System (Promega, Madison, WI), and then cloned into a pGEM-T Easy vector (Promega). The M13R primer was used for sequencing with a Big Dye Sequencing Kit (Applied Biosystems, Foster City, CA). The combination of bisulfite treatment and PCR amplification resulted in the conversion of unmethylated cytosine (C) residues to thymine (T) residues, while methylated C residues remained unconverted. The products were analyzed by an ABI Prism DNA Sequencer 3130 (Applied Biosystems) to detect changes from C to T.

2.4. Chromatin immunoprecipitation (ChIP) assay

ChIP assays were performed using a ChIP-IT Express kit (Active Motif, Carlsbad, CA) following manufacturer's protocol. Briefly, cells were fixed, thawed, lysed, and then transferred to an ice-cold Dounce homogenizer. After nuclear release, cells were pelleted and resuspended in shearing buffer. The lysates were subjected to ultrasonic sonication, and then centrifuged to remove cellular debris. For immunoprecipitation, protein G magnetic beads were combined with 20 μg sheared chromatin and the following antibodies. Rabbit polyclonal anti-PU.1 (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit polyclonal anti-MeCP2 (Santa Cruz Biotechnology), rabbit polyclonal anti-acetyl-histone H3 (Upstate Biotechnology, Waltham, MA), rabbit antiserum anti-acetyl-histone H4 (Upstate Biotechnology), or rabbit IgG (R&D Systems, Minneapolis, MN) as the isotype control. To examine PU.1 and MeCP2 binding to the MT-1A promoter, 8 μg of antibodies and the same amount of control IgG were employed. To examine histone modification, 2 μg of antibodies and the same amount of control IgG were used. After overnight incubation at 4 °C, the magnetic beads were washed, eluted, reverse crosslinked and treated with proteinase K as instructed. Then, the samples were subjected to 40 cycles of real-time PCR.

2.5. Statistical analysis

Statistical differences were assessed using the Student's *t*-test for paired data.

3. Results

3.1. Expression of PU.1 and its target gene, MT-A, is inversely correlated during THP-1 cell differentiation

We recently found that the expression of PU.1 and its target gene, MT-1A and MT-1G, is inversely correlated by analyzing PU.1 transgenic cells and AML patient samples [6]. In this study, we first examined whether these correlations are also observed during monocyte cell differentiation. After examined the morphological changes (Fig. 1A), we found that the expression of monocyte markers CD14 and CD11c was potently increased in the presence of 10 ng/ml TPA for 120 h (Fig. 1B). Next, we examined the expression of PU.1 and its target genes. As shown in Fig. 1B, the expression of PU.1 was significantly induced by the addition of TPA. In contrast, the expression of MT isoforms, MT-1A and MT-1G, was dramatically suppressed during THP-1 cell differentiation induced by TPA.

3.2. PU.1 binds to the MT-1A promoter in THP-1 cells

Because we previously demonstrated direct regulation of MT-1A by the hematopoietic transcription factor PU.1 [6], we next examined the mechanism through which PU.1 regulates MT-1A in TPA-induced THP-1 cell differentiation. The ChIP assay demonstrated that PU.1 bound modestly to the MT-1A promoter at −887 to −602 in uninduced THP-1 cells. Of note is that the binding activity of PU.1 to this promoter was potently induced in TPA-induced THP-1 cells (Fig. 2B). PU.1 forms a complex with methyl CpG binding protein (MeCP)2, which recruits mSin3A-HDAC and Dnmt [11]. MeCP2 is known to induce transcriptional repression of methylated CpG promoters by recruitment of Dnmts and HDACs [12], and we previously observed overlap with PU.1 occupancy and

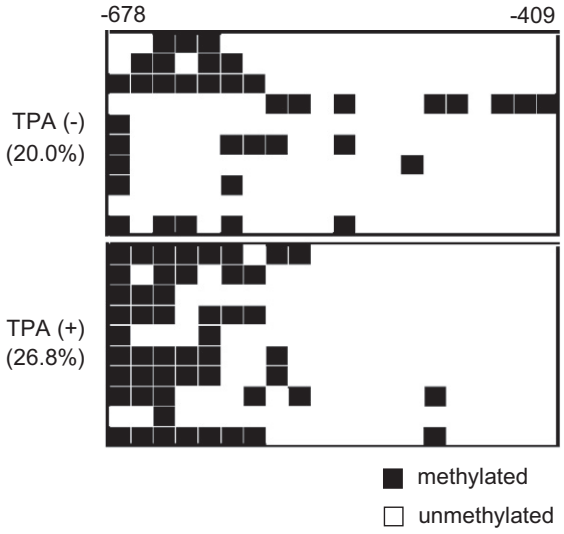

Fig. 3. TPA-induced differentiation of THP-1 cells is related to an increased proportion of methylated CpG sites in the MT-1A promoter. Results of bisulfite DNA sequencing analyses of the MT-1A promoter are shown (−678 to −409, 20 CpG sites). Genomic DNA extracts from the indicated cells were treated with sodium bisulfite and amplified. The PCR products were cloned, and individual clones were randomly selected for DNA sequencing. Filled squares represent methylated CpG sites and open squares represent unmethylated CpG sites. The percentage of methylated CpG sites in this region is shown in parenthesis.

MeCP2 in the MT-1A promoter of K562 cells [6]. Here, we found enhanced binding of MeCP2 to the MT-1A promoter in TPA-induced, but not uninduced THP-1 cells, which was the same region for binding of PU.1 (−887 to −602) (Fig. 2B). However, binding of PU.1 or MeCP2 was not observed in another region of the MT-1A promoter (−602 to +17) (data not shown).

3.3. Methylation of CpG sites in the MT-1A promoter is highly correlated with PU.1 expression levels during THP-1 cell differentiation

We next determined the methylation status of the distal CpG rich region (−678 to −409) of the MT-1A promoter by bisulfite DNA sequencing analyses. As shown in Fig. 3, the proportion of methylated CpG islands in the MT-1A promoter was obviously increased during TPA-induced THP-1 differentiation. These findings suggest that MT-1A is regulated by promoter methylation associated with PU.1 expression.

3.4. Histone modifications in the MT-1A promoter during TPA-induced differentiation of THP-1 cells

To examine whether histone modifications in the MT-1A promoter are correlated with changes in PU.1 expression, quantitative ChIP assays were performed using polyclonal antibodies against acetylated histones H3 and H4. As a result, in −887 to −602 of the MT-1A promoter, although the levels of acetylated histone H3 of the PU.1-bound region were unchanged during differentiation, acetylated H4 tended to be slightly but reproducibly decreased during differentiation (Fig. 4A). However, this change was not observed in another region of the MT-1A promoter (−602 to +17) (Fig. 4B and C). These data suggest that histone H4 modification of the MT-1A promoter in the PU.1- and MeCP2-binding region may also play a role in the reduction of MT-1A expression during THP-1 cell differentiation.

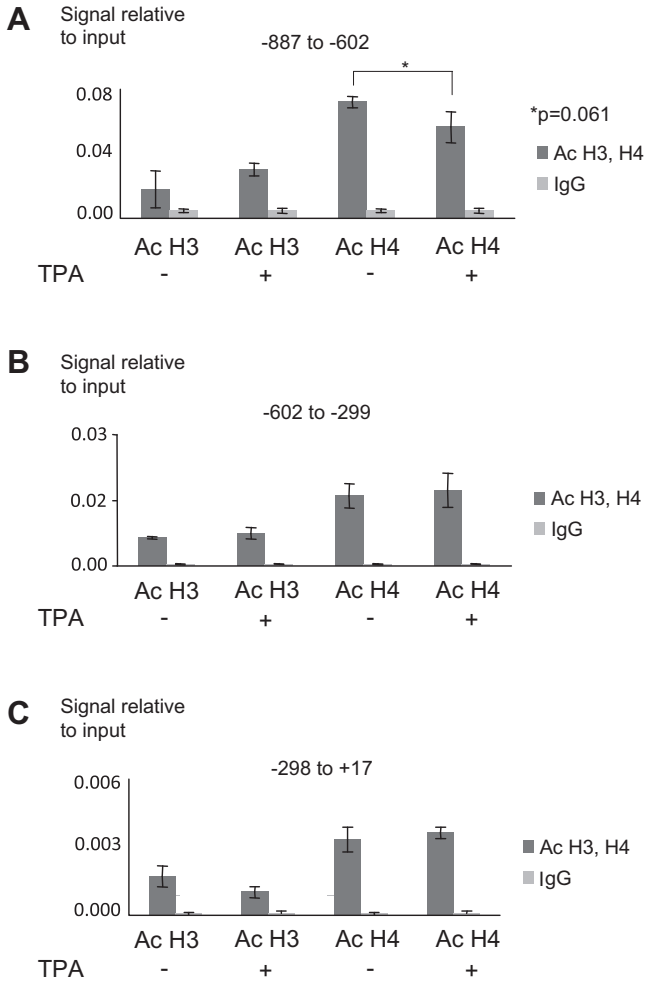

Fig. 4. Analyses of acetylated histones by ChIP assays. Chromatin modifications at −887 to −602 (A), −602 to −299 (B) and −298 to +17 (C) in the MT-1A promoter were analyzed. Antibodies against acetylated histones H3 (Ac-H3) or H4 (Ac-H4) were used to assess chromatin modification. ChIP products were quantified by PCR. The amount of immunoprecipitated DNA in each sample is represented as the signal relative to the total amount of input chromatin, which is equivalent to one, shown in the *y*-axis. The *p*-value from the Student's *t*-test is shown. Data are representative of two independent experiments with very similar results.

4. Discussion

This is the first report describing epigenetic activity of PU.1 to control target gene expression during myeloid cell differentiation. Stiedl et al. [13] revealed that ~21,500 of 45,000 transcripts are expressed in PU.1-knockdown hematopoietic stem cells (HSCs), among which 225 transcripts are downregulated and 97 are upregulated [13]. These findings indicate that PU.1 not only activates but also represses a substantial number of genes in HSCs. Several PU.1 suppressive targets have been identified and reported to date [14]. PU.1 represses the promoters of several genes (i.e., *c-myc*, *c-fos*, *SV-40*, *β-actin*, *PCNA*, and *Flt3*) independently of active PU.1-binding sites [9,15]. Consistent with this notion, in the current study and our previous report [6], we found that PU.1 binds to the methylated CpG-rich region with MeCP2 in THP-1 cells without consensus PU.1-binding sites. In contrast to its suppressive target genes, the majority of PU.1-dependent myeloid promoters (i.e., *G-CSFR*, *M-CSFR*, *GM-CSFR*, *CD11b*, and *neutrophil elastase*), which are positively regulated by PU.1, share several structural features including a PU.1-binding site close to the site(s) of transcriptional initiation, no TATA box, and a cluster of binding sites for Sp1 and members of

the CCAAT/enhancer binding protein or core-binding factor families [16,17]. Therefore, the structure of the target gene promoter may be important for the versatility of PU.1.

MT proteins comprise a group of low molecular weight cysteine-rich intracellular proteins encoded by a family of genes with several isoforms in humans [18]. Two major isoforms of MT, designated MT-1 and MT-2, have been identified in mammals, and are found in all types of tissues [18]. Two other members, designated MT-3 and MT-4, are expressed in limited tissues as minor isoforms [18]. MT-1A is one of the major isoforms of MT, and its mRNA is abundantly expressed in various cell types [18]. Human MT genes are located on chromosome 16 in a cluster, and can be activated by a variety of stimuli including metal ions, cytokines, growth factors and oxidative stress [19]. MT is a potential negative regulator of apoptosis, and a report has suggested that MT may play roles in carcinogenesis and drug resistance in at least a proportion of cancer cells [19]. Compared with analyses of solid tumors, few studies have analyzed the roles of MT in hematopoietic tissues [19]. In the myeloid lineage, the roles of MT in differentiation have not yet been analyzed. However, because MT possesses potent antioxidant functions [20], and the generation of reactive oxygen species (ROS) is important for the antibacterial activities of monocytes and macrophages [21], down-regulation of MT-1A during THP-1 differentiation may be necessary for monocytic differentiation to modulate the activity of ROS.

In conclusion, we revealed that PU.1 expression is increased during TPA-induced THP-1 cell differentiation, which is associated with downregulation of MT-1A expression. Downregulation of MT-1A is accompanied by increased binding of PU.1 and MeCP2 to the distal region of the MT-1A promoter, which increases the proportion of methylated CpG sites in this region. Taken together, not only proper expression of myeloid-specific genes (i.e., *G-CSFR, M-CSFR, GM-CSFR, CD11b,* and *neutrophil elastase*) by upregulation of PU.1, but also proper gene suppression (i.e., *MT-1A*) by the epigenetic activities of PU.1 may be necessary for myelomonocytic cell differentiation.

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