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Identification of target genes of transcription factor CEBPB in acute promyelocytic leukemia cells induced by all-trans retinoic acid

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ABSTRACT

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Objective: To identify target genes of transcription factor CCAAT enhancer-binding protein β (CEBPB) in acute promyelocytic leukemia cells induced by all-trans retinoic acid. **Methods:** A new strategy for high-throughput identification of direct target genes was established by combining chromatin immunoprecipitation (ChIP) with *in vitro* selection. Then, 106 potential CEBPB binding fragments from the genome of the all-trans retinoic acid (ATRA)-treated NB4 cells were identified. **Results:** Of them, 82 were mapped in proximity to known or previously predicted genes; 7 were randomly picked up for further confirmation by ChIP-PCR and 3 genes (GALM, ITPR2 and ORM2) were found to be specifically up-regulated in the ATRA-treated NB4 cells, indicating that they might be the down-stream target genes of ATRA. **Conclusions:** Our results provided new insight into the mechanisms of ATRA-induced granulocytic differentiation.

1. Introduction

Acute promyelocytic leukemia (APL) is a form of acute myeloid leukemia, which harbors the translocation t (15;17) and results in the formation of promyelocytic leukemia-retinoic acid receptor α (PML-RARA) fusion gene[1-6]. The aberrant PML-RARA chimeric protein encoded by the fusion gene disrupts the retinoic acid signaling pathway and arrests the blast at the promyelocytic stage[7-9]. The PML-RARA-bearing blasts are acutely sensitive to the therapeutic doses of all-trans retinoic acid (ATRA) which is used effectively for the treatment of t (15;17) patients[10-14]. Evidences showed that PML-RARA protein not only participates in the block of granulocyte differentiation but also mediates the sensitivity of APL cells to ATRA[15,16].

As a major PML-RARA responsive gene, transcription

factor CEBPB plays a key role in ATRA-induced differentiation of APL cells[15,16]. The protein expression and DNA binding activity of CEBPB in APL cells increase markedly and quickly following ATRA treatment[15,16]. Moreover the forced expression of CEBPB in multipotent leukemia cells is enough for differentiation toward granulocytes and inhibition of CEBPB expression dramatically reduces APL cells response to ATRA[15,16]. Recently it has been reported that CEBPB is essential for granulopoiesis during emergency responses to acute infections[4,17]. Therefore, identifying target genes of CEBPB will yield a more complete understanding of this transcription factor regulating the differentiation of APL cells and emergency granulopoiesis.

Chromatin immunoprecipitation (ChIP) is a powerful tool to identify target genes[18]. Several groups have used a combination of ChIP with subsequent cloning to identify the target genes of transcription factors[19-22]. But such ChIP cloning is difficult due to the much excess of non-specifically precipitated DNA fragments. To enhance the enrichment for targets, we added an *in vitro* selection to

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ligation-mediated PCR amplification of ChIP products. We got 106 potential CEBPB binding sequences after cloning and sequencing and seven of sixteen binding sites were confirmed *in vivo* and *in vitro*. Three CEBPB target genes (ORM2, ITPR2 and GALM) involved in acute phase response, Ca²⁺ signaling pathway and galactose metabolism respectively were induced after ATRA treatment.

2. Materials and methods

2.1. Cell culture and differentiation

APL cell lines NB4 and HL60 were maintained in RPMI medium 1640 supplemented with 10% fetal calf serum (GIBCO) and antibiotics in a humidified atmosphere with 5% CO₂ at 37 °C. ATRA (Sigma) was used at final concentration of 1 μM. Differentiated cells were examined by morphology with Wright's staining and NBT staining, and subsequently quantified by flow cytometry analysis. Immunofluorescence was used to detect the differentiation-related surface markers with phycoerythrin conjugated mouse anti-human CD11b, a surface marker of granulocytes and monocytes; and fluorescein isothiocyanate conjugated mouse anti-human CD14 (BD Bioscience), a surface marker of monocytes.

2.2. Expression and purification of CEBPB

CEBPB was expressed in *Escherichia coli* by the glutathione S-transferase fusion protein system. Since it is an intronless gene, CEBPB was directly amplified from human genomic DNA and cloned into a pGEX-4T-2 expression vector using primers with FLAG-tag: sense, 5'-gcGGATCCatgcaacgcctggtggcctg-3'; antisense, 5'-aaCTC GAGctactgtcgtcatcgtctttagtgcagtgccggaggag-3'. The recombinant plasmid was transformed into *Escherichia coli* BL21 strain and confirmed by sequencing. The CEBPB with GST and FLAG tag was purified with glutathione Sepharose 4B (Pharmacia) and confirmed by anti-CEBPB, GST and FLAG antibodies.

2.3. Electrophoretic mobility shift assay

Nuclear extracts were prepared from 48-hour ATRA-induced NB4 cells. Synthetic double-stranded oligonucleotide 5'-TGCAGATTGCGCAATCTGCA (C/EBPcon) including a C/EBP binding site was labeled with biotin. The sequences of unlabeled oligonucleotides used as competitive probes were listed in Figure 1A. Electrophoretic mobility shift assays was carried out using the LightShift Chemiluminescent EMSA Kit (Pierce) as previously described. For each gel shift reaction (10 μL), a total of 20 fmol biotin-labeled probe was combined with 6 μg nuclear extract or 0.1 μg purified GST-CEBPB, 1 μg poly(dI-dC) in binding buffer (10 mM HEPES pH 7.9, 50 mM KCl,

2.5 mM MgCl₂, 1 mM DTT, 10% glycerol, and 1 μg bovine serum albumin). For competition analysis, a 100-fold molar excess of unlabeled oligo-nucleotides was pre-incubated for 10 min at room temperature with nuclear extracts before the addition of the labeled probe. The reaction mixture was resolved on a non-denaturing 5% acrylamide gel in 0.5× TBE buffer and the electrophoresed binding reactions were then transferred to nylon membrane and cross-link was performed for 10 min with a hand-held UV lamp. The biotin-labeled DNA in membrane was detected by using the stabilized Streptavidin-horseradish peroxidase conjugate.

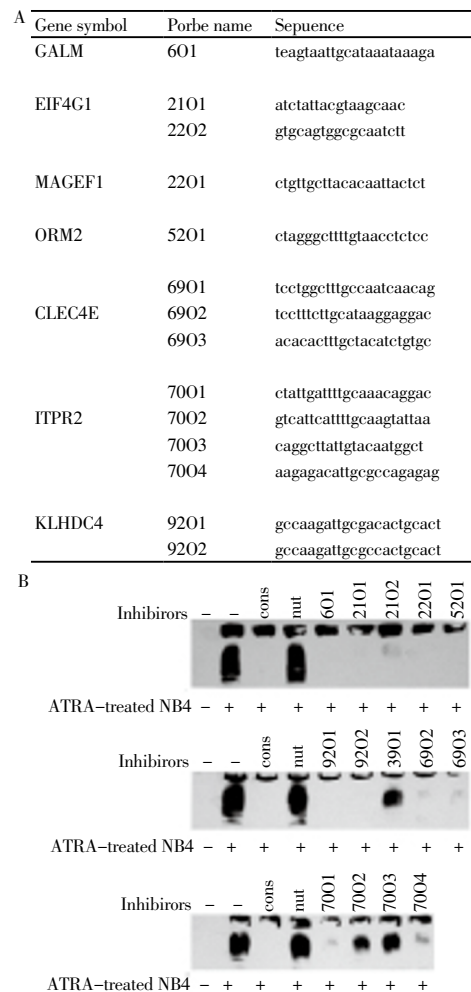


Figure 1. CEBP binding sites in the targets were confirmed by EMSA. (A) The sequences of probes for EMSA; (B) EMSA was carried out using biotin-labeled CEBP consensus probe (5'-TGCAGATTGCGCAATCTGCA) and nuclear extract from 48-hour ATRA-induced NB4 cells. 100-fold molar excess unlabeled probes were added as the competitive inhibitors. The unlabeled CEBP consensus probe and mutant probe (TGCAGAGACTAGTCTCTGCA) were used as positive and negative control. Typical results for experiments were performed in duplicate.

2.4. Chromatin immunoprecipitation

NB4 cells untreated and ATRA-treated were cross-linked with 1% formaldehyde for 15 min in room temperature.

After washing with ice-cold phosphate-buffered saline, cells were lysed in 200 μ L of a solution containing 1% SDS, 10 mM EDTA, 50 mM Tris-HCl (pH 8.1) and protease inhibitor cocktail (Roche). Chromatin samples were sonicated to 200–1 000 bp fragments and were diluted to 2 mL with 0.01% SDS, 1.1% Triton \times -100, 1.2 mM EDTA, 167 mM NaCl, and 16.7 mM Tris-HCl (pH 8.1). And 20 μ L of this solution was removed for later PCR analysis (input). After preclearing with salmon sperm DNA/ protein A agarose for 1 h at 4 $^{\circ}$ C, antibody (C/EBPb, sc-150, Santa Cruz Biotechnology, CA, USA) were added and the sonicated lysates were incubated overnight at 4 $^{\circ}$ C in a rocking platform. Then 60 μ L Protein A/G agarose was added and incubated 2 h. After washing agarose with low salt wash buffer one time, high salt wash buffer one time, LiCl wash buffer one time, TE buffer two times, the complexes were eluted from beads two times using 100 μ L fresh elution buffer (0.1 M NaHCO₃, 1%SDS). The eluates were combined and add 8 μ L 5 M NaCl to reverse crosslinks of protein/DNA complex at 65 $^{\circ}$ C for 4–5 h. Samples were then treated with Proteinase K and the DNA was collected by using the Qiagen QIAquick PCR purification Kits according to the manufacturer's instruction. Five percent of purified DNA was analysed by PCR with the primers.

2.5. *In vitro* selection

The chromatin samples were prepared as above. ChIP DNA fragments were digested with 5 U of MboI (NEB) for 4 h at 37 $^{\circ}$ C. After purified the DNA fragments were ligated with linkers (5'-GCACTAGTGGCCTATGCGGCC ATGGTACCTTCGTTGCCG-3' and 5'-GATCCGGCAACG AAGGTACCATGGCCGCATAGGCCACTAGTGC-3') using 8U T4 ligase (NEB). Following overnight incubation at 16 $^{\circ}$ C the DNA was purified using Qiaquick columns (Qiagen). Ten microliters of the ligation product was used as template in a 100- μ L PCR reaction with 0.1 M of primer (5'-GCACTAGTGGCCTATGCGG-3'), 0.2 mM dNTP, 1.5 mM MgCl₂, 2 units of HotStar Taq in 1 \times buffer (Qiagen, Chatsworth, CA). PCR program used the following parameters: 94 $^{\circ}$ C, 1 min; 60 $^{\circ}$ C, 1 min; 72 $^{\circ}$ C, 2 min; 30 cycles. The purified PCR product was incubated with 5 μ g recombinant CEBPB protein, 1 μ g anti-Flag antibody, 0.5 μ g poly-dIdC in 20 μ L binding buffer (20 mM Tris pH 7.6, 50 mM NaCl, 1 mM MgCl₂, 0.2 mM EDTA, 5% glycerol, 0.5 mM DTT, 1 \times protease inhibitor cocktail) on ice for 20 min. Then 120 μ L of protein A/G plus agarose was added and incubated for an additional 20 min. The agarose beads were washed once in TN buffer (10 mM Tris-HCl pH 7.5, 150 mM NaCl). The DNA was recovered in 120 μ L dissociation buffer (500 mM Tris-HCl pH 9.0, 20 mM EDTA, 10 mM NaCl, 0.2% SDS). The 10% recovered DNA was used as templates for amplification. The selections were repeated four rounds and the forth-round PCR product was sub-cloned into T vector for sequencing. The sequencing was performed at Chinese

National Human Genome Center at Beijing.

2.6. *Quantitative real-time PCR*

NB4 cells and HL60 cells were treated with 1 μ M ATRA and harvested at 0, 4, 24, 48 and 72 h. The total RNA was prepared by using TRIzol (Invitrogen) and reverse-transcribed into cDNA primed with oligo (dT). PCR reactions were performed by using Platinum[®] SYBR Green qPCR SuperMix (Invitrogen) with ABI7500 Real-Time PCR System according to manufacturer's instruction. All reactions were performed with three biological replicates and three technical replicates with reference dye normalization. Results are given as fold induction of the target genes/GAPDH ratio compared with the value of NB4 and HL60 cells before treatment (0 h). mRNA of bone marrow samples were available from pretreatment and ATRA-induced complete remission of a patient with APL from Rui Jin Hospital, China. Informed consent was obtained from the patient according to procedures approved by the respective institutional review boards.

2.7. *Transient transfection and luciferase assay*

Suspension cell line 2×10^5 NB4 cells were transiently transfected with 1 μ g reporter plasmid 20 ng pRL-CMV vector using Effectene[®] transfection reagent (Qiagen). Twenty four hours after transfection cells were treated with 1 μ M ATRA for thirty hours and lysed for luciferase assay. HEK293T cells were seeded in 24-well plate and transfected after 16 h using lipofectine 2000 (Invitrogen) with 0.6 μ g reporter plasmid, 0.2 μ g expression vector, and 2 ng pRL-CMV vector. Dual luciferase assays were carried out 24 h after transfection according to the manufacturer's protocol. Promoter activity was calculated for each of the constructs as a ratio of luciferase activity to pGL3 basic vector.

3. Results

NB4 cells were harvested at 0, 4, 24, 48 and 72 hours after 1 μ M ATRA treatment and the differentiation was assessed by visualizing morphological changes with Wright's staining (data not shown). The expression of CD11b and CD14, two markers of myelomonocytic differentiation. CD11b is a surface marker of granulocytes and monocytes, while CD14 expresses on monocytes. The expression levels of CD11b on NB4 cells increased markedly after ATRA treatment whereas those of CD14 remained at measurable levels (Figure 2A). We also detected the protein expression of CEBPA and CEBPB in NB4 cells after ATRA treatment (Figure 2B). The CEBPB expression in NB4 cells also increased quickly after ATRA treatment, which is similar to the results reported [16]. Then we monitored CEBPB binding to the promoter of transcription factor PU.1, a known target of CEBPB in APL cells after

ATRA treatment. We amplified PU.1 promoter in CEBPA and CEBPB ChIP products using quantitative real-time PCR. The binding activity of CEBPB to PU.1 promoter was induced to 5-fold after ATRA treatment while that of CEBPA did not change significantly (Figure 2C). These results indicated that DNA specifically bound by CEBPB in APL cells after ATRA treatment can be successfully immunoprecipitated.

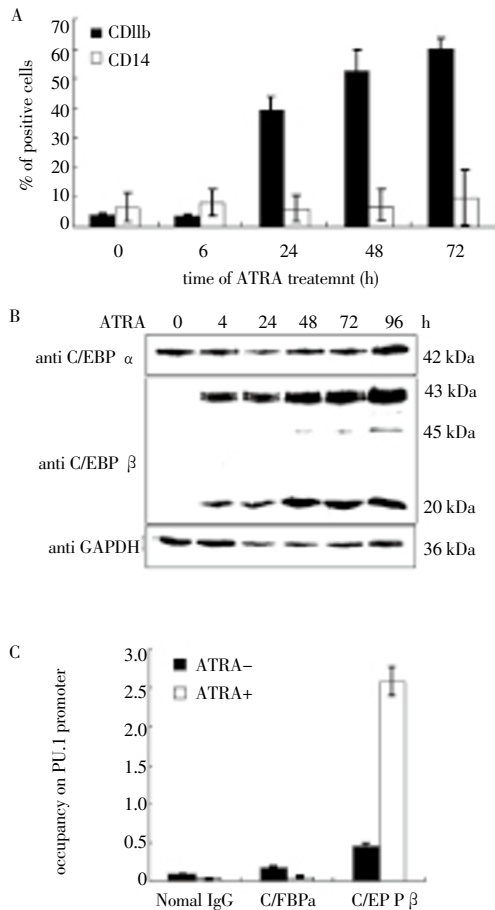


Figure 2. All-trans retinoic acid induced differentiation of NB4 cells. (A) NB4 cells were treated with 1 μ M ATRA 0, 4, 24, 48, 72 h and CD11b⁺ or CD14⁺ cells were analyzed by FACS; Error bars represent standard deviation and indicate the average values of three culture replicates. (B) CEBPA and CEBPB expression in NB4 cells treated with ATRA were detected by western blotting with anti-CEBPA and anti-CEBPB antibody, GAPDH was shown as loading control. (C) Chromatin immunoprecipitation was performed in untreated or ATRA-treated 48 hours NB4 cells with anti-CEBPA, anti-CEBPB, or nonimmune IgG as indicated and quantitative real-time PCR analysis of ChIP was used to amplify PU.1 promoter.

Then ChIP and subsequent *in vitro* selection was exploited to clone novel CEBPB target sites in ATRA-induced APL cells. We performed ChIP to get the DNA fragments bound by CEBPB in NB4 cells after 48-hour ATRA treatment and the recovered DNA was amplified by ligation-mediated PCR. Then the purified PCR products were used for *in vitro* selection with the CEBPB protein expressed in *Escherichia coli*. After four rounds of selection and amplification, the DNA fragments were cloned into vector for sequencing. We

got 106 unique targets in human genome after mapping the location of the sequences using the UCSC Human BLAT. The fragments which were located in human genome were analyzed for the presences of C/EBP consensus binding sites using AliBaba2.1, a program for predicting transcription factor binding sites. Of the fragments located in human genome, 98% contained putative C/EBP binding sites. Among these 82 were mapped in 30-kb distance of annotated genes in human genome (Table 1). Moreover, twenty one fragments (20%) were located less than 5-kb distance of transcription start site, suggesting that they are associated with gene promoter. Two target sequences (located near the genes *EIF4G1* and *SAMD8*) were cloned twice, which indicates that they may be the CEBPB specific binding targets. We noticed a target sequence is located in 5' upstream of *CLEC4E* (also named Mincle) gene encoding a C-type lectin protein which is one of CEBPB target genes in macrophages^[23].

Table 1

Distribution of CEBPB binding sites relative to genes.

Genomic location	Number of ChIP fragments
Intronic	41 (7 TIS ^a < 5 kb)
<30 kb upstream	31 (13 TIS ^a < 5 kb)
<30 kb downstream	10 (1 TIS ^a < 5 kb)
>30 kb from gene	24 ^a
Total	106

^aTIS, transcription initiation start.

We used conventional ChIP-PCR to confirm the *in vivo* specificity of CEBPB binding to the sixteen targets cloned from ATRA-induced APL cells which selected according the binding sites location in the genes and the function of the candidate genes. The target sequences were amplified from the DNA samples immunoprecipitated with anti-CEBPB, anti-RNAPolII and pre-immune serum in ATRA-treated or untreated NB4 cells. We amplified the GAPDH promoter as a negative control, which was enriched in ChIP products of anti RNAPolII but not in anti-CEBPB and pre-immune serum (Figure 3A). Seven of sixteen potential targets (43%) were specifically enriched in the CEBPB ChIP of ATRA-treated NB4 cells (Figure 3A and Table 2). We noticed that *EIF4G1* which was cloned twice is in the list of ChIP-PCR confirmed target genes. The most of targets weren't enriched in untreated NB4 cells in which the CEBPB expression and DNA binding activity is very low (Figure 3A). Then we quantified the amount of PCR product amplified from ChIP products in ATRA treated or not NB4 cells and in another APL cell line HL60 cells with using quantitative real-time PCR with SYBR green I dye (Figure 3B). The binding activities of CEBPB to the target sequences after ATRA treatment were induced from 3-fold to 8-fold respectively (Figure 3B). These data showed that the binding of CEBPB to targets increased after ATRA treatment, indicating that the expression of these genes may be regulated by CEBPB

in ATRA induced differentiation of acute promyelocytic leukemia.

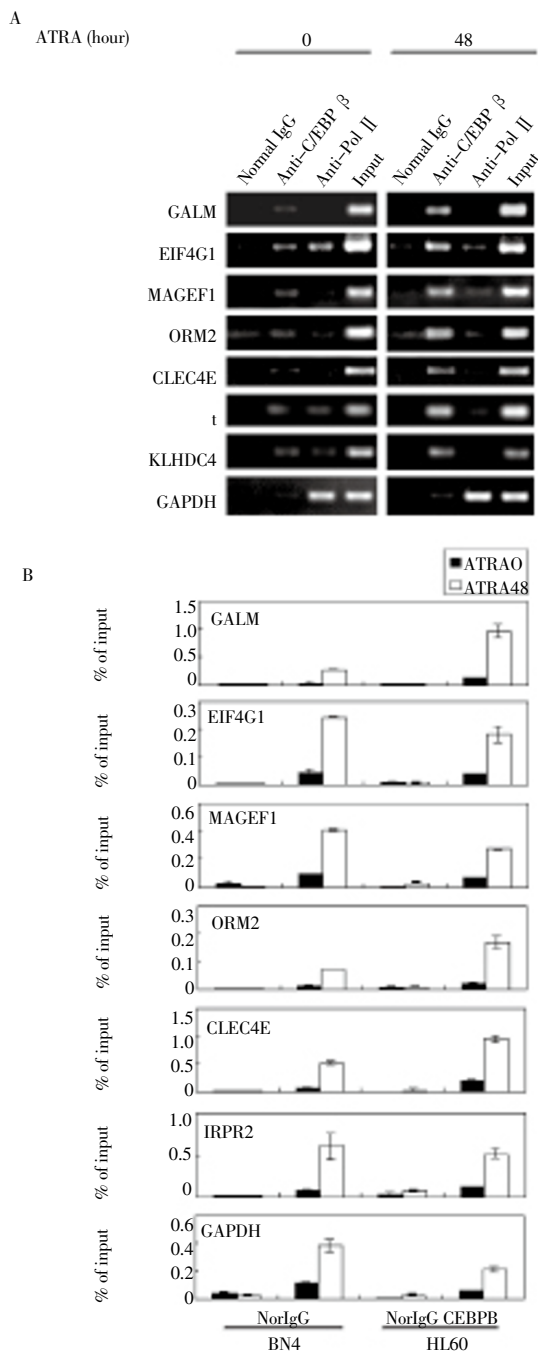


Figure 3. Confirmation of CEBPB targets by using ChIP-PCR.

(A) Chromatin was prepared from NB4 cells untreated or ATRA treated 48 h and immunoprecipitated with anti-Pol II, anti-CEBPB and nonimmune IgG. The input and ChIP products were subjected to PCR by using the specific primers for the identified targets and GAPDH as negative control. (B) Quantitative PCR was performed in ChIP products with anti-CEBPB antibody and non immune serum from NB4 or HL60 cells which were untreated (ATRA0) or treated with ATRA 48 h (ATRA48). The samples were normalized by input.

Table 2

Candidate target genes and the location of CEBPB binding sites.

Gene symbol	GenBank accession no.	Description	Location	Distance from TIS ^a (kb)
GALM	NM_138801	galactose mutarotase	Intron 1	<1
MAGEF1	NM_022149	melanoma antigen family F, 1	5'	4.7
ORM2	NM_000608	orosomuroid 2	5'	<1
EIF4G1	NM_198244	eukaryotic translation initiation factor 4	5'	2.4
KLHDC4	NM_017566	Kelch domain containing4	5'	10
CLEC4E	NM_014358	C-type lectin, superfamily member 9	5'	26
ITPR2	NM_002223	inositol 1,4,5-trisphosphate receptor, type 2	5'	20

^aTIS, transcription initiation start.

The members of C/EBP family have similar DNA binding sequence preference and the consensus sequence is RTTGCGYAAAY (R is A or G and Y is C or T)[24]. We analyzed sequences of the seven targets and got fourteen potential CEBPB binding elements for gel shift assay (Figure 1A). The probes harboring each of these fourteen elements were used to competitively inhibit the binding of CEBPB protein to biotin-labeled CEBP consensus binding probe (5'-TGCAGATTGCCAATCTGCA). The shift band generated by biotin-labeled CEBP consensus probe and ATRA-treated NB4 cell nuclear extracts was abolished by both unlabeled CEBP consensus probe and eleven probes harboring potential CEBPB binding elements but not by CEBP mutant probe and three putative elements (Figure 1B). Moreover each CEBPB targets at least has one CEBP binding element. We got the same results by using CEBPB expressed in *Escherichia coli* to substitute nuclear extracts from ATRA-treated NB4 cells (data not shown). These data showed that the targets have the C/EBP binding elements which can be bound by CEBPB protein *in vitro*.

After confirmation of targets bound by CEBPB protein *in vivo* and *in vitro*, we detected whether the expressions of these CEBPB target genes were modulated in ATRA-treated NB4 cells and HL60 cells. The mRNA expression levels of the target genes were quantified using real-time quantitative reverse transcription PCR. The expression of ORM2, ITPR2 and GALM were induced in both NB4 and HL60 cells after ATRA treatment and CLEC4E were suppressed only in NB4 cells.

We observed a steady increase of ORM2 mRNA to more than 10-fold after ATRA treatment, reaching a plateau in

both NB4 and HL60 cells 48 h after ATRA treatment. Human orosomucoid (ORM), also known as α_1 -acid glycoprotein (AGP), is a major acute phase plasma protein predominantly produced in liver^[25]. In plasma ORM proteins are mixture of ORM1 and ORM2, which are encoded by two closely linked loci, ORM1 and ORM2^[25]. ORM1 protein is also synthesized and stored in secondary granules during granulocytic differentiation and released by activated neutrophils to mediate immuno-modulating effects at the sites of infection or injury^[26]. We also observed significant increase of ITPR2 and GALM mRNA 48 hours after ATRA treatment. ITPR2 encodes one of inositol 1,4,5 trisphosphate receptors involved in Ca^{2+} signaling pathway^[27] and ITPR2 protein may mediate the phagocytic activity of neutrophils^[28]. Galactose mutarotase encoded by GALM gene catalyzes the first step in normal galactose metabolism by catalyzing the conversion of β -D-galactose to α -D-galactose^[29]. The increased expression of GALM reflects the increased α -D-galactose requirement of neutrophils due to a great deal glycoprotein synthesis. Although it was reported that CLEC4E is induced by CEBPB in monocytes^[30], its expression decreased in ATRA-treated HL60 cells, suggesting the difference between monocytes and neutrophils. The expressions of other genes bound by CEBPB have no significant alteration, illustrating the redundancy present in regulatory networks.

It has been reported that three C/EBP members CEBPA, CEBPB and CEBPE are involved in the differentiation of APL cells after ATRA-treatment. Then we analyzed the potential of the three C/EBP members to activate the three ORM2 promoter constructs in adherent HEK 293T cells by transiently transfection. In absence of C/EBP members, the -1 158 bp and -203 bp ORM2 promoter constructs have 5–10 folds activities compared with mock vector while -95 ORM2 promoter construct only has a weak activity.

4. Discussion

Identifying target genes of transcription factors is important for constructing transcriptional networks and understanding cellular processes. Interfering expression of the transcription factor in question and analyzing of the resulting changes of gene expression by using microarray technology allow high-throughput identification of downstream genes^[30–33]. However, it may be biased the special genomic regions if the detection is only dependent on microarray types. A lot of false positives limited its application. Several approaches have been developed to improve the efficiency of ChIP-cloning, such as double ChIP, combined with differential display technology (ChIP display) or with one yeast-hybrid assay^[34–36]. Here we demonstrate a new method, ChIP with

in vitro selection to identify the target genes of transcription factors with enhancing the specificity. However, in present study seven of sixteen (44%) potential target fragments were confirmed by ChIP-PCR and this is likely to be underestimated because some of the negative genes may be bound by CEBPB at a location too far from the ChIP-PCR amplicon to be detected by this method.

CEBPB is a transcription factor essential for numerous biological processes, including differentiation, metabolic homeostasis, proliferation, tumorigenesis, inflammation, and apoptosis^[37]. To date researchers have identified more than one hundred genes directly regulated by CEBPB, including cytochrome P450 genes, coagulation factor VIII, serum amyloid A2, lactoferrin, G-CSF receptor, *etc*^[38]. Friedman *et al* performed orthogonal analysis through combining expression profiling and ChIP-mouse promoter micro-array to identify the direct CEBPB target genes in mouse liver after partial hepatectomy^[39,40]. Compared to the Friedman's results, the CEBPB target genes identified in ATRA-induced APL cells in our experiments did not indicated the overlapped CEBPB targets. There are two possible explanations to this: first although it plays a key role both in liver regeneration and in APL differentiation, CEBPB regulates the different target genes between liver cells and ATRA-induced APL cells. Second it may be due to the difference of technology. There are three of seven confirmed target genes the expressions of which are significantly altered in both APL cell lines after ATRA treatment. The genes identified in this study are involved in different cellular processes, including acute phase response, calcium signaling pathway and metabolism. CEBPB plays a pivotal role in acute phase response by regulating expression of acute phase proteins such as serum amyloid A, C-reactive protein, haptoglobin, *etc*^[38]. As acute phase reactants, both ORM1 and ORM2 are produced by hepatocytes and secreted into plasma in response to infection/injury. Extensive studies have showed that the induction of ORM1 expression is mediated by CEBPB via binding the acute phase response element (APRE) in its promoter^[25–38]. Recent study showed that ORM1 is still synthesized in neutrophils and its expression is induced by CEBPE^[26]. We noticed that both CEBPA and CEBPB can enhance the ORM2 promoter activities. The expression of ORM2 regulated by both CEBPA and CEBPB suggested that it may function in steady-state and emergency granulopoiesis.

ITPR2 is one of inositol 1,4,5-trisphosphate receptors which are located on endoplasmic reticulum and mediate Ca^{2+} mobilization from ER to the cytoplasm in response to the binding of second messenger, inositol 1,4,5-trisphosphate (IP3)^[27]. ITPR2 and ITPR3 double knockout mice present with a lack of Ca^{2+} release capability from ER and thus are unable to induce secretion of saliva and pancreatic

juice^[41]. ITPR2 is expressed in various tissues and cell lines including hematopoietic cell lineages. Several reports plus this study showed that ITPR2 is induced in APL cells after ATRA treatment^[1,28].

The expressions of the other genes including the CEBPB-binding sites don't response to the ATRA stimulation in APL cells. This non-functional CEBPB-binding illustrates the redundancy in CEBPB regulation network, which also was found in a study of the CEBPB target gene identification in liver regeneration^[39]. This study has identified the CEBPB target genes and further research is needed to investigate the role that these target genes play in the ATRA-induced differentiation of APL cells and granulopoiesis. Knockdown with specific siRNA and over-expression experiments will be helpful.

In summary, we provided a new choice of combining *in vivo* chromatin immunoprecipitation with *in vitro* selection to identify the direct target genes of transcription factors. Using the approaches, we identified eighty-two potential target genes of transcription factor CEBPB in APL cells after ATRA treatment. Seven of sixteen were confirmed as CEBPB binding sites by ChIP-PCR and EMSA, and the expression of three genes were altered in the granulocytic differentiation of ATRA-treated APL cells. These results also helped to understand the regulatory network of CEBPB in granulopoiesis.

Conflict of interest statement

We declare that we have no conflict of interest.

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