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PU.1 can regulate the *ZNF300* promoter in APL-derived promyelocytes HL-60

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ABSTRACT

ZNF300, which plays the role in human embryonic development and some diseases, is a typical KRAB/C2H2 zinc finger gene expressed only in higher mammalians. Our data showed that expression of ZNF300 changed significantly in various leukemia blasts in the bone marrow aspirates of newly diagnosed leukemia patients. To investigate the potential relationship between expression of ZNF300 and the progression of leukemia development and hematopoietic differentiation, we cloned and characterized the putative human *ZNF300* gene promoter and identified its transcription start sites (TSSs). Deletion and mutagenesis analysis demonstrated that a myeloid-specific transcription factor PU.1 binding site was responsible for myeloid-specific regulation of *ZNF300* promoter activity. Furthermore, electrophoretic mobility shift and chromatin immunoprecipitation assays revealed that PU.1 bound to the PU.1 binding site within *ZNF300* promoter region *in vitro* and *in vivo*. Overexpression of PU.1 elevated *ZNF300* promoter activity, whereas silencing of PU.1 expression significantly reduced the activity in myeloid-derived HL-60 cell but not in T-cell Jurkat. *In vitro* induced HL-60 cells into CD11b expressing cells by DMSO demonstrated that ZNF300 was upregulated along with upregulation of PU.1 expression. These results demonstrated that *ZNF300* was activated by PU.1 and suggested that the regulation may be involved in the progression of leukemia development and hematopoietic differentiation.

1. Introduction

Zinc finger proteins are potential transcription factors and Krüppel-associated-box zinc finger proteins (KRAB-ZFPs), a novel subfamily of zinc finger proteins were regulated significantly during *in vitro* differentiation of human myeloid cells. Typically, KRAB-ZFPs, acting as transcriptional repressors [1], play important roles in diverse developmental and pathological processes [2,3].

ZNF300 gene was isolated from human embryos on the basis of the enrichment of C2H2-specific mRNAs [4]. ZNF300 belongs to KRAB-ZFP family and is a nuclear protein expressed in higher mammalian tissues but not in lower animals [4]. Recently, we found that ZNF300 could effectively activate IL-2Rβ promoter activity [5]. Moreover, it is reported that ZNF300 was downregulated during induced embryonic stem cell differentiation *in vitro* [6], and was found to be associated with 5q- syndrome, a distinct subtype of primary myelodysplastic syndrome (MDS), which is defined by interstitial deletion of chromosome 5q13–q33 [7,8]. Recently our data indicated that ZNF300 is markedly upregulated during myeloid differentiation and is highly expressed in colon carcinoma (data not shown). These findings suggested that ZNF300 was likely to play a role in the differentiation of blood cells and the pathogenesis of cancer. Therefore, identification of the signal networks and interactional processes regulating the expression of *ZNF300* gene during myeloid differentiation and cancerous pathogenesis is of vital importance.

PU.1, a member of the *ets* gene family, was originally identified as the product of the *Spi-1* oncogene in Friend virus-induced erythroleukemias. PU.1 binds to a purine-rich target sequence containing the 5′-AGGAA-3′ core sequence motif, which is crucial for the activity of numerous myeloid-specific promoters, including CD11b, G-CSF receptor, M-CSF receptor, and GM-CSF receptor expression. Gene targeting of PU.1 blocked myeloid hematopoiesis. Similarly, PU.1 targeting in ES cells or CD34+ cells further demonstrated that PU.1 was essential for myelopoiesis [9–12].

This work was aimed to elucidate that PU.1 regulates directly *ZNF300* gene promoter and the regulation may be involved in the progression of leukemia development and hematopoietic differentiation. Our results show that expression of ZNF300 changed gradually in various leukemia blasts of bone marrow aspirates from newly diagnosed and untreated leukemia patients. And we present the molecular cloning of *ZNF300* gene promoter, identi-

Abbreviations: ZNF300, zinc finger 300; APL, acute promyelocytic leukemia; CML, chronic myelogenous leukemia; DMSO, dimethyl sulfoxide; PU.1, purine-rich box 1; ChIP, chromatin immunoprecipitation; EMSA, electrophoretic mobility shift assays.

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Table 1
Patient and donor characteristics.

	AML patients ($n = 23$)	CML patients ($n = 11$)	Donors ($n = 5$)
Age (years)[a]	56 (21–76)	61 (24–78)	51 (19–67)
Sex (males/females)	15/8	7/4	3/2
FAB distribution[b]	5M1, 9M2, 5M3, 4M5	-	-

[a] Median (range).
[b] French–American–British classification for AML.

fication of the transcriptional start sites, and characterization of the ZNF300 promoter as a PU.1-dependent promoter in myeloid cell lines. We also exhibit that ZNF300 expression was upregulated along with PU.1 expression when HL-60 cells are induced to differentiate through the granulocytic pathway by DMSO. These data demonstrated that PU.1 could effectively activate *ZNF300* gene and it was required for myeloid-specific activation of gene expression from the *ZNF300* gene promoter by PU.1 binding to the putative PU.1 binding site.

2. Materials and methods

2.1. Bone marrow specimens and histological evaluation

Peripheral blood (PB) samples were obtained from 5 healthy donors, and bone marrow (BM) aspirates were obtained from 34 newly diagnosed and untreated patients (Table 1) with acute myelogenous leukemia (AML) and chronic myelogenous leukemia (CML). All these patients were from Wuhan Tongji Hospital, Huazhong University of Science and Technology and diagnosed based on French–American–British (FAB) criteria [13]. Leukemia blasts were defined as cells with high nuclear/cytoplasmic ratio, dispersed nuclear chromatin, and scant cytoplasm. The investigation was performed according to the revised Declaration of Helsinki [14] and all patients gave their informed consent.

2.2. In situ immunohistochemistry

In situ immunohistochemistry of ZNF300 expression assay was performed as reported [15,16]. To evaluate exactly the ZNF300 expression level of the bone marrow samples, we first estimated the cells' staining intensity and the number of positive cells. Specific evaluation methods are as the following. After *in situ* immunohistochemical staining with anti-ZNF300 antibody, a semi-quantitative method is used to estimate the ZNF300-positive leukemia cells: "1" represents 5–10% of positive cells, "2" represents 11–50% positive cells, "3" represents 51–80% positive cells, "4" represents more than 80% positive cells. Moreover, ZNF300 expression level is determined experientially by using a rating system, where "0" represents no reaction, "1" represents a weaker reaction, "2" represents moderate reaction, "3" represents strong reaction. The final results are the product of these two indicators, namely, arbitrary units (AU) [17,18] (Table 2). Data are expressed as mean ± SD from at least three experiments and analyzed by one-way ANOVA followed by a Student–Neuman–Keuls test with significance defined as $p < 0.05$.

2.3. Cell culture and in vitro induced differentiation

HL-60, K562 and Jurkat cells (CCTCC, Wuhan, China) were maintained as previously reported [5,19]. DMSO (Sigma) induced *in vitro* differentiation was performed as reported [20].

2.4. RNA extraction and RT-PCR analysis

Total RNA was extracted as previously reported [19]. Yeast RNA was obtained as a component of the RPA III Kit (Ambion). Reverse-transcription and PCR reaction were carried out as previously reported [19]. Various primers (sense: PTSS6, PTSS5, PTSS4, PTSS3 and PTSS2; antisense: PCSA2, see Table 3) were used for PCR amplification.

Table 2
ZNF300 staining scores [17,18].

	Number	ZNF300 staining scores (AU)[a]
Healthy donors	5	0
AML-M1 patients	5	0.9 (0.4–1.6)
AML-M2 patients	9	4.4 (2.3–5.9)
AML-M3 patients	5	4.0 (3.5–5.2)
AML-M5 patients	4	3.4 (2.5–4.8)
CML patients	11	3.2 (1.9–5.2)

[a] Values represent medians and interquartile ranges of arbitrary units (AU). Analysis of significance for overall differences between AML subtypes and CML was performed by the Student–Neuman–Keuls test with significance defined as $p < 0.05$.

2.5. Cloning of the 5′-flanking region of the ZNF300 gene

Human genomic DNA was extracted as previously reported to serve as the template for amplification of the *ZNF300* gene 5′-flanking region [19]. A 3980 bp fragment was amplified by primer set Pro300upstr5s/Pro300upstr5a (Table 3) and then inserted into the pGEM-T Easy vector (Promega) to generate pGEM-T-3980 and verified by DNA sequencing.

To map the *ZNF300* gene promoter region, different fragments of the 5′-flanking regions were inserted into the firefly luciferase reporter vector, pGL3-Basic (Promega), as the following (the numbers indicate the nucleotide positions relative to the TSS).

(1) The 895 bp PCR fragments (primer pair: luc−800/luc+95A, Table 3), 719 bp (luc−624/luc+95A), 592 bp (luc−497/luc+95A), 493 bp (luc−398/luc+95A), and 428 bp (luc−333/luc+95A) were generated by PCR amplification using pGEM-T-3980 as template and then inserted into pGL3-Basic to generate pGL3(−800/+95), pGL3(−624/+95), pGL3(−497/+95), pGL3(−398/+95), and pGL3(−333/+95).
(2) pGL3(−800/+95)-PU.1-m(−602 to −596), pGL3(−800/+95)-Myb-m(−475 to −469), pGL3(−800/+95)-C/EBPα-m(−772 to −767) and pGL3(−800/+95)-AML1-m(−415 to −409) were constructed by overlapping-extension-PCR method with template plasmid pGL3(−800/+95) as previously reported [5,19] and confirmed by DNA sequencing.

2.6. Primer extension assays

Primer extension assay was performed as described previously [19]. Total RNA isolated from HL-60 and K562 cells was used for primer extension assays with a 23 bp [γ-^{32}P]-labeled antisense oligonucleotide complementary ELA2 to the region +32 to +10 (Table 3). The resulting cDNA products were analyzed on polyacrylamide gels together with DNA sequencing reactions on genomic DNA performed with the labeled primer.

2.7. Transient transfection and dual luciferase assay

Transient transfection and dual luciferase assay were performed as described previously [5,19]. In brief, cells were transiently transfected with the indicated promoter constructs based on pGL3-Basic (which expresses firefly luciferase from the putative ZNF300 promoters) along with control construct pRL-TK (which expresses Renilla luciferase) and harvested 48 h after transfection for dual luciferase assay (Promega). Relative luciferase activity (F/R) was calculated by normalizing the firefly luciferase activity (F) from the promoter of interest against Renilla luciferase activity (R) from the co-transfected internal control pRL-TK construct. At least three independent experiments were conducted for each promoter construct, and the data are shown as mean values with standard errors. Student's t-test was used to statistically analyze the data.

2.8. Electrophoretic mobility shift assays (EMSA) and supershift assays

EMSA was performed as previously reported [5,19]. The binding buffer was prepared as reported [21] and anti-PU.1 antibody (sc-352) was purchased from Santa Cruz Biotechnology (Santa Cruz, USA). To achieve adequate separation, the gel was electrophoresed sufficiently long so that unbound (free) probe was run off the bottom [22].

2.9. Chromatin immunoprecipitation (ChIP) assays

ChIP assays were performed as described previously [5,19]. Briefly, HL-60 and K562 cells were crosslinked with 1% formaldehyde at 37 °C for 15 min. Chromatin extracts were prepared. 1.5 μl of each ChIP-precipitated DNA was amplified by PCR for 35 cycles with the primers PU.1-chip-s/PU.1-chip-a (Table 3) for the promoter region and ChIP-control-s/ChIP-control-a (Table 3) for an upstream region as a negative control.