PML-RARα interferes with erythropoiesis by repressing LMO2 in acute promyelocytic leukaemia

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Abstract
The PML-RARα fusion gene, generated by the t(15;17) chromosome translocation, is regarded as the initiating factor of acute promyelocytic leukaemia (APL). In addition to the well-known effects on blocking myeloid differentiation at the promyelocytic stage, promyelocytic leukaemia-retinoic acid receptor α (PML-RARα) has also been reported to interfere with multiple differentiation processes, including erythroid differentiation. However, the detailed molecular mechanism by which PML-RARα impairs erythropoiesis has not yet been fully addressed. By chromatin immunoprecipitation-PCR assay, we found that PML-RARα bound to the distal promoter region of LMO2 (LIM-only protein 2), a critical erythroid-specific transcription factor. Luciferase reporter assays and qRT-PCR results demonstrated that PML-RARα down-regulated the expression of the LMO2 distal transcript through transrepressing its promoter activity. Analysis of gene expression profiling data from large cohorts of acute myeloid leukaemia (AML) patients confirmed that LMO2 expressed at a markedly lower level in APL patients in comparison to non-APL AML patients. Further flow cytometry analysis demonstrated that PML-RARα inhibited erythropoietin-induced erythroid differentiation by down-regulating LMO2 expression. Our findings reveal a previously unidentified mechanism, by which PML-RARα interferes with erythropoiesis through directly targeting and transrepressing LMO2 expression in the development of APL.

KEYWORDS
acute promyelocytic leukaemia, erythropoiesis, LMO2, PML-RARα

1 INTRODUCTION

Acute promyelocytic leukaemia (APL), a subtype of acute myeloid leukaemia (AML), is characterized by the t(15;17)(q22;q21) chromosomal translocation. The resultant fusion protein promyelocytic leukaemia-retinoic acid receptor α (PML-RARα) is well-known to be responsible for a differentiation block at the promyelocytic stage,1,2 resulting in the aberrant accumulation of immature promyelocytes in bone marrow and peripheral blood. Leukaemia initiating cells (LICs) in APL have been reported from the different models. Some studies regard that APL LICs are myeloid committed cells, based on the transgenic mouse models in which PML-RARα expression is under control of more differentiated myeloid specific promoters.3–5 Interestingly, other studies have also indicated that PML-RARα is

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expressed at the early stage of hematopoietic hierarchy such as multistep progenitors rather than committed myeloid progenitors and promyelocytes only, indicating that the influence of PML-RARα may not be limited to myeloid cells but other lineages of blood cells as well. Furthermore, PML is consistent with the previous finding in early hematopoiesis and erythropoiesis, suggesting that the disrupted expression pattern of PML by PML-RARα may affect normal erythropoiesis. Indeed, it has been reported that PML-RARα can interfere with hemin-induced erythroid differentiation in K562 cells, further supporting the idea that PML-RARα may impair erythropoiesis. However, the molecular mechanism by which PML-RARα influences erythroid differentiation is not yet clear.

LMO2 (LIM-only protein 2, also known as RBNT2), is an important regulator of hematopoietic stem cell development and erythropoiesis, as mice deficient in Lmo2 show a complete lack of blood cells and defects in the formation of foetal erythrocytes. LMO2 has been demonstrated to function as a bridge molecule and assist in the assembly of multimeric transcription factor complexes. LMO2 is capable of inducing erythroid differentiation through the interaction with transcription factors, including SCL, E2A, LDB1 and GATA-1. Such a transcriptional complex regulates the expression of erythroid-specific genes, such as the α-globin genes. EKLF and glycoporphin A (GPA). Knockdown of LMO2 results in the disassembly of this transcriptional complex and thereby attenuates the chromatin occupancy of GATA-1 and LDB1, ultimately leading to the dysregulated expression of erythroid-specific genes. Moreover, forced expression of LMO2 is able to rescue the defective erythroid differentiation caused by c-myb silencing in CD34 positive cells. The above findings indicate the important role of LMO2 in erythropoiesis.

In the present work, we found that PML-RARα bound to the distal promoter of LMO2 and thereby down-regulated the expression of LMO2 through decreasing the promoter activity. We showed that LMO2 expression was significantly lower in APL patients than that in non-APL AML patients. Functionally, LMO2 expression was up-regulated in umbilical cord blood (UCB)-derived CD34 positive cells upon erythropoietin (EPO)-induction of erythropoiesis. Forced expression of PML-RARα into the CD34 positive cells arrested EPO-induced erythropoiesis by repressing LMO2 expression. Taken together, our results demonstrated that PML-RARα interfered with erythroid differentiation through directly targeting the LMO2 distal transcript and repressing LMO2 expression in the pathogenesis of APL.

2 MATERIALS AND METHODS

2.1 Cell lines culture

U937-PR9 was a gift from Dr. PG Pellicci (Milan, Italy). NB4 was a gift from Dr. M Lanotte (Hospital St Louis, Paris, France). 293T was obtained from the Cell Bank at the Chinese Academy of Sciences (Shanghai, China). U937-PR9 and NB4 cells were maintained in RPMI 1640 (Gibco, Carlsbad, CA, USA) each containing 10% foetal bovine serum (FBS) (Gibco). 293T cells were cultured in DMEM (Gibco) containing 10% FBS. Cells were cultured in an incubator at 37°C with 5% CO2. ZnSO4 (Sigma, St. Louis, MO, USA) was used to induce the expression of PML-RARα in U937-PR9 cells at the final concentration of 100 μM. Both all-trans retinoic acid (ATRA) (Sigma) and arsenic trioxide (ATO) (Sigma) were dissolved in absolute ethanol and MQ water respectively. ATRA and ATO are used at the final concentration of 1 μM.

2.2 Human UCB specimens

The study was approved by the Ethics Committee of Ruijin Hospital affiliated to Shanghai Jiaotong University School of Medicine and was adherent to the regulation of the declaration of Helsinki. The approval number is ChiCTR-OPC-15006492. Fresh human UCB specimens were obtained from volunteer donors attending obstetrics department at Ruijin Hospital. Informed consent was obtained according to institutional guidelines.

2.3 Isolation of UCB-derived CD34 positive cell stimulation of erythroid differentiation in vitro

CD34 positive cells were isolated by Ficoll (Axis-Shield, Oslo, Norway) density centrifugation for mononuclear cells and subsequent magnetic cell sorting for cells stained with anti-CD34 antibody (Becton Dickinson, Franklin Lakes, NJ, USA). Freshly isolated CD34 positive cells were cultured in the Serum-Free expansion Medium (StemCell Technologies, Vancouver, BC, Canada) containing 40 ng/mL of granulocyte-macrophage colony-stimulating factor (Baote Biology Co., Ltd, China), 20 ng/mL of interleukin-3 (Sigma) and 100 ng/mL of stem cell factor (Sigma). EPO (Sanfang Pharmaceutical Co., Ltd, Shenyang, China) was added to stimulate cell differentiation along with the erythroid lineage at the final concentration of 5 IU/mL. The cells were collected at a series of time-points after treatment. These cells were immunostained with CD235a antibody (Becton Dickinson) and subsequently analysed by flow cytometry (Becton Dickinson).

2.4 RNA extraction and RT-PCR

Total RNA of leukaemic cell lines and UCB-derived CD34 positive cells with/without manipulation were extracted using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and RNeasy Micro Kit (Qia-gen, Santa Clarita, CA, USA) respectively. cDNA was converted using the SuperScript II Reverse Transcriptase (Invitrogen) with random hexamer primers according to the manufacturer’s protocol. RT-PCR was performed to measure the mRNA levels of PML-RARα and LMO2. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control. The information of primer sequences is as follows, LMO2-F: 5′-CAAAAGCGCCATATATGCC-3′; LMO2-R: 5′-CCCTCCACCTACTGTGCTG-3′; PML-RARα-F: 5′-GAAGGTAGGTCCTCTGGCCCA-3′; PML-RARα-R: 5′-GGCTTGCGACTTCTTTCAGA-3′; GAPDH-F: 5′-GAAGGTGAAGGTCGGAGTC-3′; GAPDH-R: 5′-GAAGATGATGATTTCAGTGGATTTCC-3′; each experiment was performed in triplicate.
2.5 Plasmid construction, transient transfection and luciferase assays

The LMO2 distal promoter regions including both the full length (approximately 2.3 kb upstream of the LMO2 transcription start site) and truncated form were cloned into PGL3-basic vector (Promega, Madison, WI, USA) respectively. Plasmids were transfected into 293T cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. UCB-derived CD34 positive cells were transfected using Amaza Human CD34+ cell Nucleofector Kit (Amaza, Cologne, Germany). Luciferase assays were performed with Dual-Luciferase Reporter Assay (Promega) 48 hours after transfection. Briefly, the transfected 293T cells were lysed with passive lysis buffer (Promega) and 10 μL of cell lysate was aspirated for measurement. Luciferase activities were normalized by cotransfecting a plasmid expressing Renilla luciferase. The Primers for luciferase constructs are as follows, LMO2-full length-F: 5′-cgcctcagcTGACATAACCCCTCAAG-3′; LMO2-full length-R: 5′-ccttcaagtt-GATGCTCTGTGCCGGAATC-3′; LMO2-truncated-F: 5′-cgcctcagcCTCTGCTTCAGGTGAAG-3′; LMO2-truncated R is the same with LMO2-full length R; LMO2-RARE (1st mutation) R: 5′-GCTTGTTGAGCGATGATCCACTCCCTCtcaaagTGGTAAGACTGAGTCTCTTCGATCCTC-3′; LMO2-RARE (2nd mutation) R: 5′-TCTCAATGTAAAATTGctaaagCAATTTCACATTGAGAAGGCTGGACACGTCTTTACACTTACAAGG-3′; LMO2-RARE (2nd mutation) R: 5′-TCTATGCTTCAATGTAAAATTGctaaagCAATTTCACATTGAGAAGGCTGGACACGTCTTTACACTTACAAGG-3′; LMO2-RARE (2nd mutation) R: 5′-TCTCTCGATCCTCTAAATTCCTCtcaaagTGATGAACTCCTCTG-3′.

2.6 Chromatin immunoprecipitation analysis

Chromatin immunoprecipitation (Chip) assays were performed according to the Affymetrix protocol as described, with the following antibodies: anti-RARα (C-20 X; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-PML (H238 X; Santa Cruz Biotechnology) and the rabbit immunoglobulin G (ab46540; Abcam, Cambridge, UK). PCR was performed to detect the enrichment. Each experiment was performed in triplicate and equivalent results were observed. Promoter primers used for ChIP-PCR are as follows: LMO2-DP-F: 5′-GCACCTTATAACTGTCAGGACC-3′; LMO2-DP-R: 5′-CCATGCTATGACACACAC-3′; LMO2-N-F: 5′-GTTGAGTGATGCTCCTAAACC-3′; LMO2-N-R: 5′-ACTGAGATATCTGGGAAGAGGA-3′.

2.7 Gene expression analysis

Three transcriptome data sets of AML patients, including TCGA, GSE103582 and GSE115922 were used to compare the expression of LMO2 between APL and non-APL patient samples. To perform interarray comparison, the CEL files were analysed by Affymetrix MAS 5.0 software (Affymetrix, Santa Clara, CA, USA). Two-tailed t-tests were used to validate the significance of the observed differences, which were considered statistically significant when P < 0.05.

2.8 Gene ontology analysis

ChiP-Seq data set GSM55223723 by using Lmo2 antibody in mouse hematopoietic progenitor cell line (HPC-7) was retrieved to investigate the downstream target genes of Lmo2. To compare the expression of the Lmo2 targets genes between human AML samples, conversion of the genomic co-ordinates from mouse to human orthology was performed based on the Mouse Genomic Informatics database. Gene Ontology (GO) analysis was performed on differentially expressed LMO2 target genes by using the ClueGO of the Cytoscape software including the following databases: Kyoto Encyclopedia of Genes and Genomes (KEGG), GO Molecular Function, GO Cellular Component and GO biological Process. The P-values denote the significance of GO terms enrichment. The P-value <0.05 is considered statistically significant.

3 RESULTS

3.1 PML-RARα binds to the distal promoter of LMO2

To identify the potential genes that might be involved in the inhibition of erythroid differentiation in the pathogenesis of APL, we screened the PML-RARα targets that we previously discovered from genome-wide studies. Interestingly, we found that PML-RARα was significantly enriched in the distal promoter region of LMO2 (Figure 1A). Three alternative transcripts of LMO2 have been identified so far, among which the distal promoter is regarded as an erythroid-specific promoter due to the direct regulation by GATA-1. To verify the PML-RARα binding on the distal promoter of LMO2, we performed ChiP-PCR assays in ZnSO4-treated PR9 cells and APL patient-derived NB4 cells using anti-PML and anti-RARα antibodies. As illustrated in Figure 1B, the positive bands were only amplified in the ChiPed region in ZnSO4-treated PR9 cells and NB4 cells but not in untreated PR9 cells. These results indicate that PML-RARα rather than wild-type RARα binds to the distal promoter of LMO2 in APL cells.

3.2 PML-RARα down-regulates the expression of LMO2 through transcriptional repression of the LMO2 distal transcript

The next question we asked was whether such binding affected the transcription of LMO2. To answer this question, we first scanned the enriched motifs within the LMO2 distal promoter. As shown in Figure 2A, we found two half sites of retinoic acid responsive elements (RAREs) with 300 bps of each other within the PML-RARα binding peak. To determine if PML-RARα represses LMO2 transcriptional activity, we conducted promoter reporter assays using the distal promoter of LMO2 in 293T cells, a non-hematopoietic cell line. As illustrated in Figure 2B, after cotransferring the PML-RARα expression construct, we observed that the distal promoter activity of LMO2 was transrepressed by PML-
RARα. Interestingly, wild-type RARα had no impact on LMO2 transcriptional activity, which was in line with the ChIP result that wild-type RARα did not bind the LMO2 distal promoter. Furthermore, PML-RARα transrepressed LMO2 distal promoter activity in a dose-dependent manner (Figure 2C), demonstrating that LMO2 was a transcriptional target of PML-RARα. To further investigate if these two RARE half sites are involved in PML-RARα-mediated repression of LMO2, we generated three truncated or mutated LMO2 distal promoters, one lacking these two RARE half sites and the other two with each mutated RAREh site, and then compared the luciferase activity upon PML-RARα expression between the full length and truncated/mutated constructs. As shown in Figure 2D, PML-RARα failed to repress the transcriptional activity of all three truncated/mutated constructs. The above observations suggested that PML-RARα transrepressed the transcriptional activity of LMO2 distal promoter through binding these two RARE half sites and both RAREh sites were required in this repression.

Next, to investigate if the expression of the LMO2 distal transcript was subjected to the repressed transcriptional activity of the LMO2 distal promoter, we performed qRT-PCR in PR9 cells treated with ZnSO4 in a time series. As shown in Figure 2E, the expression level of the LMO2 distal transcript was gradually decreased upon the PML-RARα induction, indicating that the repression of the LMO2 distal promoter by PML-RARα resulted in the reduction in LMO2 expression.

### 3.3 LMO2 is expressed at a lower level in APL than in non-APL AML subtypes

The above observations demonstrated that PML-RARα repressed the LMO2 expression via targeting the LMO2 distal promoter, which indicated a negative correlation between PML-RARα and LMO2 in APL. To further verify the correlation between PML-RARα and LMO2 in a large population, we retrieved three data sets (TCGA, GSE10358 and GSE1159) on the expression profiling of 743 AML patients,20–22 including 76 APL patients and 667 patients with other AML subtypes. Using these data sets, we compared the LMO2 expression values between APL patients and non-APL AML patients. As shown in Figure 3, the large-scale gene expression revealed that LMO2 was expressed at a lower level in APL patients as compared with non-APL AML patients, further confirming that LMO2 expression was specifically down-regulated with the expression of PML-RARα in APL.

### 3.4 PML-RARα interferes with erythroid differentiation through repressing LMO2 in APL

Since LMO2 plays a pivotal role in erythropoiesis, we postulated that the decreased LMO2 expression caused by PML-RARα might lead to the defective erythroid differentiation in APL. To test this hypothesis, we first treated UCB-derived CD34 positive cells with EPO and then measured the expression of CD235 on the cell surface, which

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**FIGURE 1** PML-RARα binds to the distal promoter of LMO2. (A) Schematic diagram showing the binding of PML-RARα to the distal promoter regions of LMO2. ChIP assays were performed in the PML-RARα-inducible PR9 cells using anti-RARα and anti-PML antibodies. The peaks represent the PML-RARα-enriched ChIP regions. (B) PML-RARα bound to the distal promoter of LMO2 in PML-RARα-inducible PR9 cells and APL patient-derived NB4 cells. ChIP was performed with anti-RARα, anti-PML or normal immunoglobulin G (IgG) antibodies. ChIP-PCR was performed with primers specific for the distal promoter region of LMO2 (LMO2-AP) or a non-relevant region far from the LMO2 locus (LMO2-N). Total DNA or chromatin DNA immunoprecipitated with different antibodies was used for PCR amplification.
is a cell surface marker only expressing in mature erythroid cells. As shown in Figure 4A, the expression of CD235 was continuously up-regulated after EPO treatment and the increase was maintained up to 72 hours, indicating that EPO was capable of stimulating the CD34 positive cells to differentiate into mature erythroid cells. Considering the efficiency of nucleofection, we selected the 24-hour time-point for the subsequent experiments. We further overexpressed the PML-RARα expressing plasmid in CD34 positive cells to

**FIGURE 2** PML-RARα down-regulates the expression of LMO2 through transcriptional repression of the LMO2 distal transcript. (A) Schematic representation of the LMO2 distal promoter. The half sites of retinoic acid responsive elements (RAREs) are defined using TRANSFAC with the core and matrix similarity. (B) PML-RARα rather than wild-type RARα repressed the transcriptional activity of the LMO2 distal promoter. Luciferase reporter assays were performed in 293T cells. (-) absence and (+) presence of the indicated plasmid. (C) The distal promoter activity of LMO2 was repressed by PML-RARα via a dose-dependent manner. The LMO2 distal promoter was transfected into 293T cells along with increasing amounts of the PML-RARα expression construct. (D) Both RAREh sites were required for PML-RARα-mediated LMO2 repression. Schematic representation of the LMO2 distal promoter luciferase constructs including wild-type, truncated construct and mutants (left panel). PML-RARα failed to repress the luciferase activities of the truncated construct and mutants of the LMO2 promoter. (E) LMO2 expression was decreased after PML-RARα induction in ZnSO₄-treated PR9 cells at a series of time-points. RT-PCR was performed to detect the expression of PML-RARα, the LMO2 distal transcript and GAPDH respectively. Data represent the mean of three replicates ± SD, **P < 0.001; ***P < 0.0001.
detect whether PML-RARα would affect the EPO-induced erythropoiesis. As illustrated in Figure 4B, we observed that EPO failed to induce the expression of CD235 on the surface of PML-RARα expressing cells, suggesting that PML-RARα might interfere with erythroid differentiation. Furthermore, we evaluated the mRNA levels of PML-RARα and the LMO2 distal transcript to compare the LMO2 expression before and after EPO treatment. We found that in control cells, EPO was able to up-regulate LMO2 expression. In contrast, in PML-RARα expressing cells, LMO2 expression had almost no change after EPO treatment (illustrated in Figure 4C), indicating that PML-RARα inhibited EPO-induced increase in LMO2 expression. Taken together, our results suggest that PML-RARα interferes with erythroid differentiation through inhibiting the expression of the LMO2 distal transcript.

In the light of the observations that PML-RARα interfered with erythropoiesis via LMO2 suppression, we therefore assumed that PML-RARα deregulated the LMO2-dependent erythroid differentiation programme. To test this assumption, we retrieved ChIP-Seq data using Lmo2 antibody in mouse haematopoietic progenitor HPC-7 cell line and identified 4660 genes targeted by Lmo2. Among these target genes, 293 genes were differentially expressed between APL and non-APL AML patients, which suggests that PML-RARα deregulated the expression of these target genes through LMO2 suppression in APL. GO and KEGG pathway analysis showed that the differentially expressed genes downstream of LMO2 were enriched for pathways associated with haematopoietic development and haematopoietic progenitors differentiation as well as several erythropoiesis related signalling pathways (Figure 4D), such as Ras, PI3-kinase and hypoxic inducible factor 1 (HIF-1) signalling pathways. Our results suggest that PML-RARα disrupts erythroid differentiation programme through repression of LMO2, and thereby leads to the inhibition of erythropoiesis in APL.

4 | DISCUSSION

Haematopoiesis is a tightly regulated process by which various lineage differentiation and commitment are controlled in a highly co-ordinated manner. Leukaemia-associated fusion proteins can disrupt this tightly controlled process through the aberrant transcriptional programmes, which results in a global differentiation block. The oncogenic PML-RARα fusion protein dysregulates key regulators of normal haematopoesis, such as PU.1, RUNX1 and many others, as well as different pathways such as RAR signalling, thus resulting in the repression of critical myeloid gene expression and thereby contributing to the block at the promyelocytic stage. We show here that PML-RARα also interfered with erythroid differentiation by directly targeting and repressing the expression of LMO2 in the pathogenesis of APL.

Previous studies have described APL LICs from the different cell models. On the one hand, some studies suggest that APL LICs are myeloid committed progenitors. Interestingly, these studies are all based on the transgenic mouse models in which PML-RARα expression is under the control of more committed myeloid specific promoters, such as CTSG, MRP8 and CD11b. It is therefore not surprising that the influence of PML-RARα action in these models is restricted to the myeloid/granulocytic compartment. On the other hand, some studies performed on normal CD34+ Lin- cells suggest that PML-RARα expression induces an APL phenotype possibly through three major sequential events, that is, differentiation commitment, rapid differentiation and promyeloid arrest. Furthermore, several studies have reported that the translocation of PML-RARα occurs in pluripotent stem cells in APL patients. These observations raise the possibility that PML-RARα-mediated cell transformation may be involved in different cell origins, although the true origin of leukaemia is still unknown because of the complexity of the disease origin and the limitations of current research methods.

Erythroid differentiation blocked by the expression of PML-RARα has been demonstrated in several cell models. For example, expression of PML-RARα in CD34+Lin- cells enables normal haematopoietic progenitor/stem cells to reach the promyelocytic level of differentiation but not to go further along the erythroid or the thrombocytic lineage, even if cells are cultivated in an adequate cytokine cocktail. Disrupted erythroid differentiation by the oncogenic fusion proteins is also associated with the pathogenesis of t (8;21) AML. AML1-ETO is able to result in a gross inhibition of erythroid colony formation and thus inhibit early erythroid development. These observations strongly suggest a global differentiation...
block induced by the fusion proteins, which functions—at least for the erythroid lineage—already at a very early level, whereas the granulocytic precursors are blocked at a late stage of differentiation, such as at the promyelocytic level by PML-RARα. We indeed provided the experimental evidence that PML-RARα inhibited EPO-induced erythropoiesis of human CD34 positive cells, which suggests a direct link between PML-RARα and disruption of erythroid differentiation.

Erythropoiesis is orchestrated by a series of erythropoietic transcriptional factors. Many studies have demonstrated that these transcriptional factors promote erythroid development by forming the complex through the protein-protein interaction.\textsuperscript{12,14,15} LMO2,
similar to the well-known erythropoietic transcriptional factor GATA-1, is also regarded as the central factor in this transcriptional complex because it mediates the interaction between this complex and chromatin. LMO2 is required to maintain at a relatively high expression level across erythroid development from haematopoietic stem cells to erythroblast. Down-regulation of LMO2 leads to inhibition of erythropoiesis. We found that PML-RARα directly bound to the regulatory regions of LMO2 and further repressed its expression, thus contributing to the disrupted erythroid differentiation in APL. Interestingly, in addition to LMO2, other factors in the transcriptional complex, including GATA-1, LDB1, TCF3 and TAL1, showed no change or even higher expression in U937-PR9 cells after PML-RARα induction (Figure S2), suggesting the indispensable role of LMO2 in erythropoiesis. Of note, we cannot exclude the possibilities that PML-RARα can interfere with erythropoiesis at the protein-protein interaction level. Indeed, PML-RARα is able to interact with several haematopoietic specific transcription factors, such as AP-1, GATA2, and PU.1. Mass spectrometry-based screening can be applied to search for novel proteins that interact with PML-RARα and are also involved in the regulation of erythropoiesis. The disruption of erythroid differentiation is also observed in other subtypes of leukaemia through impaired expression or activity of erythroid transcription factors by fusion proteins. For example, the AML1-ETO fusion protein generated by t(8;21) has the capability to repress the expression of GATA-1. Our findings emphasize the importance of LMO2 in erythropoiesis and reveal a previously unidentified mechanism of defective erythropoiesis in APL, by which PML-RARα specifically transrepressed LMO2, and thereby interfered with erythroid differentiation.

As part of our studies to determine how the expression of LMO2 is disrupted by PML-RARα in APL, we carried out an extensive analysis of the LMO2 distal promoter region. Although three alternative promoters have been identified in the LMO2 gene, our studies focused on the distal promoter since this region was specifically targeted and repressed by PML-RARα. Indeed, it has been demonstrated that of the three promoters of LMO2, the distal promoter displays a hematopoietic restricted pattern, directing the hematopoietic-specific expression of LMO2. Our luciferase assays and RT-PCR results provide the experimental evidence that PML-RARα transrepressed the expression of the LMO2 distal transcript. Furthermore, we also demonstrated the requirement of two RAREh sites within the LMO2 promoter in PML-RARα-mediated repression of LMO2. Our previous findings have shown that RAREh is significantly enriched in PML-RARα binding sites and the RAREh sites are arranged in different orientations and with widely variable spacing in between. The two RAREh sites within the LMO2 promoter were around 300 bp apart, raising the possibility that the two RAREh sites could be spatially close due to the high-order structure of chromatin.

In addition to LMO2 per se, we also looked at LMO2 target genes and focused on the genes with differential expression between APL and non-APL patients. Interestingly, we found that these genes were enriched in several signalling pathways critical for erythropoiesis. For instance, activation of PI3-kinase is crucial for cell proliferation of erythroid progenitors. Moreover, PI3-kinase/AKT signalling pathway is regarded as a mediator in EPO-induced erythropoiesis through favoring GATA-1 transcription. Ras signalling pathway negatively regulates erythroid maturation by observing that overexpression of RAS blocks the differentiation of erythroid progenitor cells. HIF signalling is capable of promoting erythropoiesis at multiple levels, including regulation of EPO synthesis, enhancement of iron uptake and utilization as well as adjustment of bone marrow microenvironment for erythroid progenitor differentiation and maturation. Dysregulation of such erythropoiesis associated signalling pathways by repression of LMO2 may have multifaceted effects on inhibition of erythropoiesis, further emphasizing the importance of repression of LMO2 in erythroid deficiency in APL patients.

ATRA and ATO are two commonly and clinically used treatments applied for APL therapy. The therapeutic mechanisms of ATRA and ATO are different. ATRA mainly induces granulocytic terminal differentiation through transcriptional activation of the differentiation-associated programme. ATO can rapidly degrade PML-RARα fusion protein and induce the apoptosis of APL cells, thereby relieving the repression of genes targeted by PML-RARα. In our study, we observed different changes in LMO2 expression upon ATRA or ATO treatment in NB4 cells (Figure S1A and B). A similar observation has been found in our previous findings, in which PSMB8, PSMB9 and PSMB10 show response to ATRA but not ATO. LMO2 expression showed no change or even further down-regulation in ATRA-treated NB4 cells (Figure S1B). The result observed upon ATRA treatment was reasonable, since miR-223 is reported to repress LMO2 expression, which is up-regulated during the ATRA-induced differentiation process from promyelocytes to neutrophils. In contrast, ATO treatment could restore LMO2 expression in NB4 cells (Figure S1A). Our data likely indicate that ATO but not ATRA has the ability to reactivate LMO2 expression in APL cells.

Collectively, our findings identify LMO2, as a downstream target of PML-RARα, whose dysregulated expression is associated with the failure of erythropoiesis in APL. Our data not only reveal a molecular mechanism of PML-RARα-mediated erythropoiesis inhibition but also provides evidence that PML-RARα has broad impacts on multiple lineages of blood cells rather than myeloid lineage only.

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**CONFLICT OF INTEREST**

The authors declare no conflict of interest.
AUTHOR CONTRIBUTION

XWW designed the study, performed experiments and wrote the manuscripts; YT, PW, HZ, MZ and XJZ performed experiments; KKW designed the study, interpreted the results and wrote the manuscript. All authors read and approved the final manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.