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The c-Myc-regulated miR-17-92 cluster mediates ATRA-induced APL cell differentiation

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Abstract

Background: Despite advances in the treatment of acute promyelocytic leukemia (APL) with all-*trans*-retinoic acid (ATRA), its underlying mechanism has not been fully elucidated. The oncogenic microRNA cluster miR-17-92 modulates multiple cellular processes, including survival, proliferation, and apoptosis. However, the role of miR-17-92 and its regulation has not yet been documented for APL.

Methods: We analyzed miR-17-92 expression in APL samples and cell lines by qRT-PCR. The expression of c-Myc was measured by western blot. Cell differentiation was assessed by measuring the surface CD11b antigen expression by flow cytometry analysis.

Results: We observed that miR-17-92 was upregulated in APL compared with healthy donors. Furthermore, we demonstrated that expressions of c-Myc and miR-17-92 are markedly suppressed during ATRA-induced NB4 cell differentiation. Importantly, we also demonstrated that miR-17-92 is directly regulated by c-Myc during the granulocytic differentiation of APL cells. Finally, the overexpression of miR-17-5p blocks ATRA-induced differentiation.

Conclusions: We report abnormal expression of the miR-17-92 cluster in APL cells, which is responsible for the differentiation block in blast cells in APL. In addition, we identified miR-17-92 as a target gene of c-Myc during ATRA-induced granulocytic differentiation.

KEYWORDS

acute myeloid leukemia, acute promyelocytic leukemia, all-*trans*-retinoic acid, differentiation, microRNA

1 | INTRODUCTION

Acute promyelocytic leukemia (APL) is a distinctive subtype of acute myeloid leukemia (AML), and is characterized by the chromosomal translocation t(15;17)-associated PML/RARα fusion, which leads to differentiation block at the promyelocytic stage.[1,2] The most common translocation, t(15;17), is the sole detectable genomic abnormality in most APLs. It is likely that APL shares various signal pathways downstream of APL oncoprotein, making them potential therapeutic targets.

APL is treated with all-*trans*-retinoic acid (ATRA), targeting the PML/RARα fusion protein. ATRA was found to differentiate leukemic blasts and cause disease regression specifically in t(15;17) APL.[3,4] Although important achievements in AML treatment have been made, ATRA-based treatments have not been effective and therapeutic failure remains high in non-APL AML patients. Understanding the molecular mechanisms involved in t(15;17) APL pathogenesis and their responsiveness to ATRA treatment will allow us to identify novel targets and extend the efficacy of ATRA-based treatments for patients with non-APL AML.

MicroRNAs (miRNAs) are endogenous small noncoding transcripts that regulate target gene expression mainly by binding to mRNAs, which leads to mRNA cleavage/degradation or translational suppression.[5,6] A number of studies have indicated that miRNAs play crucial roles in fundamental processes, including differentiation,[7] development,[8,9] and apoptosis.[10] Dysregulation of miRNAs is strongly associated with various cancers.[11] Our previous work demonstrated that miRNAs play a significant role in leukemia cell differentiation, proliferation, and apoptosis.[12–17] However, for many miRNAs, their

mechanism of regulation is not fully characterized. Furthermore, it has been suggested that miRNAs might be important for AML pathogenesis by interfering with signaling molecule essential for myeloid differentiation. The miR-17-92 polycistron encodes six mature miRNAs (miR-17, miR-18a, miR-19a, miR-20a, miR-19b-1, and miR-92a).[18] Previous studies have demonstrated crucial roles for miR-17-92 during various cancer development. MiR-17-92 is highly expressed in malignant lymphoma and acute lymphoid and myeloid leukemia.[19,20] More recently, the miR-17-92 cluster has been shown to be aberrantly expressed in mixed lineage leukemia (MLL)-rearranged AML.[21] However, the role of miR-17-92 and its regulation has not yet been documented for APL.

In this study, we found that the oncogenic miR-17-92 is aberrantly upregulated in APL cells, and treating these cells with ATRA induces differentiation and represses c-Myc and miR-17-92 expression. We identified miR-17-92 as a c-Myc target gene during the ATRA-induced terminal differentiation of APL cells. Of note, enforced expression of miR-17-5p is capable of inhibiting APL cell differentiation. Our results demonstrate that c-Myc directly affects miR-17-92 expression levels, and repression of miR-17-92 expression is critical for inducing the differentiation of APL cells.

2 | MATERIALS AND METHODS

2.1 | Patients and samples

A total of 113 peripheral blood (PB) samples, including 41 APL, 6 APL-complete remission (CR), 30 non-APL AML, 7 AML-CR, and 2 AML-non-remission (NR) samples at diagnosis plus 27 healthy donors (including 20 granulocyte samples from PB and 7 $CD34^+$ cells sorted from cord blood samples by CD34 microBeads) from the First Affiliated Hospital of Jinan University, were enrolled in this study. This study was approved by the Ethic committee of First Affiliated Hospital, Medical School of Jinan University. Studies were conducted in accordance with the Declaration of Helsinki.

2.2 | Cell lines and cell culture

The human APL cell line NB4, its ATRA-resistant clone NB4-R2, and the AML cell line HL60, KG1 were cultured in RPMI 1640 containing 10% fetal bovine serum at 37°C in a 5% CO_2 incubator. ATRA was purchased from Sigma-Aldrich and used at a final concentration of 1 μM.

2.3 | Quantitative real-time PCR analysis

Total RNA was isolated with the TRIzol reagent according to the manufacturer's instructions (Invitrogen). cDNA was reverse transcribed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Quantitative RT-PCR was performed with SYBR Green (TIANGEN) according to the manufacturer's instructions. The PCR primers are listed in Table S1. Mature miRNAs from the miR-17-92 cluster were detected using the miScript SYBR® Green PCR kit (QIAGEN), and *U6* served as an internal control.

2.4 | Transfection

Transfections were performed as described previously.[22] The siRNA sequences used to target c-Myc were as follows:

si-c-Myc-1: 5′-CGAUGUUGUUUCUGUGGAA-3′,
si-c-Myc-2: 5′-GCUUGUACCUGCAGGAUCU-3′, and
si-c-Myc-3: 5′-CGUCCAAGCAGAGGAGCAA-3′.

miR-17-5p agomir and miRNA agomir controls were purchased from RiboBio.

2.5 | Western blotting

NB4 cells were lysed in RIPA buffer with protease inhibitor. Protein extracts were separated by SDS-PAGE and then transferred to a PVDF membrane. The blots were incubated with anti-c-Myc (9402S, CST) and anti-β-actin (MA5-15739, Thermo Fisher Scientific) antibodies.

2.6 | Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) assays were performed following the instruction manual for EZ-ChIP™ #17-371 (Millipore). c-Myc (9402S, CST) or IgG control antibodies were used in the ChIP assay. ChIP-qPCR/PCR primers were designed as previously described[21,23] and are listed in Table S1. For the ChIP-qPCR assays, the ChIP efficiency for certain binding sites was normalized to control IgG, and negative control (NC) primers targeting non-c-Myc binding regions served as primer NC.

2.7 | Flow cytometry analysis

Cell differentiation was assessed as previously described,[14] in brief, surface ITGAM/CD11b antigen expression was measured by flow cytometry analysis. ITGAM/CD11b expression was determined using the APC anti-human CD11b/Mac-1 antibody (550019, BD Biosciences).

2.8 | Statistical analysis

Data are expressed as the mean ± SD of three independent experiments. All statistical analyses were performed using GraphPad Prism 5.0 software (GraphPad Software, Inc). The significance of the differences between two groups was determined by a two-tailed Student's *t* test. *P*-value < .05 was considered significant. $^*P < .05$, $^{**}P < .01$, $^{***}P < .001$.

3 | RESULTS

3.1 | pri-miR-17-92 is upregulated in APL

It has reported that the miR-17-92 cluster is overexpressed in MLL-rearranged acute leukemia.[21] To evaluate the role of the miR-17-92 cluster in APL, we first compared the expression of the miR-17-92

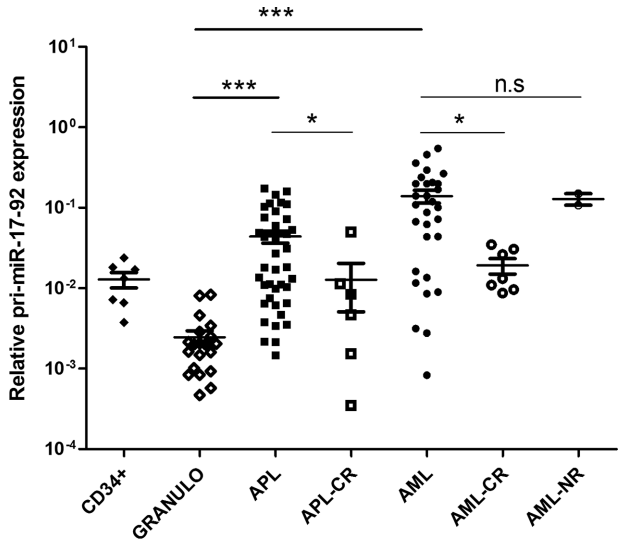

FIGURE 1 pri-miR-17-92 is significantly upregulated in samples from APL patients

Comparison of pri-miR-17-92 expression in healthy donors (normal, $n = 20$), primary APL patients ($n = 41$), APL-CR patients ($n = 6$), primary non-APL AML patients ($n = 30$), non-APL AML-CR patients ($n = 7$), non-APL AML-NR patients ($n = 2$), and CD34+ cord blood samples ($n = 7$). Pri-miR-17-92 expression was detected by qRT-PCR and normalized to the *ACTB* gene. *P* values between samples were obtained by performing a *t* test

cluster primary transcript (pri-miR-17-92) in primary t(15;17) APL patient samples with that of healthy donors. As shown in Figure 1, the pri-miR-17-92 expression level was markedly elevated in primary APL and non-APL AML patient samples compared with healthy donors (granulocyte and CD34-positive core blood cells). In addition, pri-miR-17-92 was highly expressed in primary APL and non-APL AML patient samples in comparison with the CR patients. This result suggests that pri-miR-17-92 upregulation may be associated with APL/AML pathogenesis.

3.2 | c-Myc and pri-miR-17-92 are suppressed during ATRA-induced cell differentiation

Because ATRA treatment leads to leukemia cell differentiation, we next investigated c-Myc and pri-miR-17-92 expression in the APL cell line NB4 treated with ATRA. As assessed by qRT-PCR, the c-Myc and pri-miR-17-92 expression levels in APL cells were downregulated upon ATRA treatment (Figure 2A and B). The c-Myc protein level was also downregulated upon treatment with ATRA as demonstrated by western blotting (Figure 2C). The expression of the mature miRNAs in the miR-17-92 had the same trend as the primary transcript expression (Figure 2D). There was no ATRA-mediated pri-miR-17-92 downregulation in the NB4-R2 (ATRA-resistant cell) (Figure S1A). In addition, arsenic trioxide and paclitaxel could also inhibit the expression of c-Myc/miR-17-92 in AML cells (Figure S1B). These results suggest that c-Myc may have a regulatory effect on miR-17-92 expression.

3.3 | c-Myc promotes pri-miR-17-92 expression by binding to the miR-17-92 promoter

We further investigated the effects of c-Myc on pri-miR-17-92 expression. As shown in Figure 3A-C, the depletion of c-Myc in NB4 cells led to pri-miR-17-92 downregulation. These results suggest that miR-17-92 may be regulated by c-Myc. Next, motif analysis revealed three conserved c-Myc-binding motifs, E-box CACATG (-2924, upstream of miR-17-5p), CATGTG (-1483, upstream of miR-17-5p), and CACGTG (-915, upstream of miR-17-5p), in the promoter of the miR-17-92.[23] To determine whether c-Myc directly binds to the miR-17-92 cluster promoter in APL cells, we performed a ChIP assay and found c-Myc binding. Together, these data demonstrate that miR-17-92 is a direct target gene of c-Myc in APL cells (Figure 3D and E).

3.4 | Overexpression of miR-17-5p blocks ATRA-induced APL cell differentiation

The above data demonstrate that c-Myc promotes miR-17-92 expression, and ATRA represses the expression of c-Myc and miR-17-92, suggesting that miR-17-92 may be involved in myeloid differentiation. miR-17-5p is a key component of the miR-17-92 cluster,[24,25] thus, we next explored the function of miR-17-5p in terminal differentiation of APL cells. miR-17-5p agomir was transfected into NB4 cells and treated with ATRA, and membrane antigen ITGAM/CD11b, which is the marker for granulocytic cell differentiation, was measured by flow cytometry analysis. As expected, ITGAM/CD11b levels were increased in cells treated with ATRA but repressed in NB4 cells overexpressing miR-17-5p (Figure 4A). In addition, the expression levels of C/EBP-β were also repressed by miR-17-5p (Figure 4B). These data confirm the involvement of miR-17-92 in myeloid differentiation.

4 | DISCUSSION

In this study, we demonstrate that miR-17-92 expression is upregulated in APL cells, and ATRA treatment represses c-Myc and miR-17-92 expression in APL cells. We also provide evidence that c-Myc directly affects miR-17-92 expression and demonstrate that repression of c-Myc/miR-17-92 expression is critical for inducing the terminal differentiation of APL cells.

Aberrant overexpression of the miR-17-92 cluster is detected in many cancers. However, results of analysis of miR-17-92 in AML have not been consistent. Li et al.[20] demonstrated that expression of the miR-17-92 cluster is high in MLL-rearranged AML and observed downregulation of miR-17-5p and miR-20a in t(15;17) AML samples compared with mononuclear cells using miRNA expression profiling. In this study, we also observed that pri-miR-17-92 is expressed at a lower level in APL compared with non-APL AML cases, but it was still significantly overexpressed in large APL patient cohorts compared with granulocytes from healthy donors. The expression of miR-17-92 is higher in NB4 APL cells although it may be associated with amplification of the miR-17-92 locus at 13q31.[21] We found that si-c-Myc

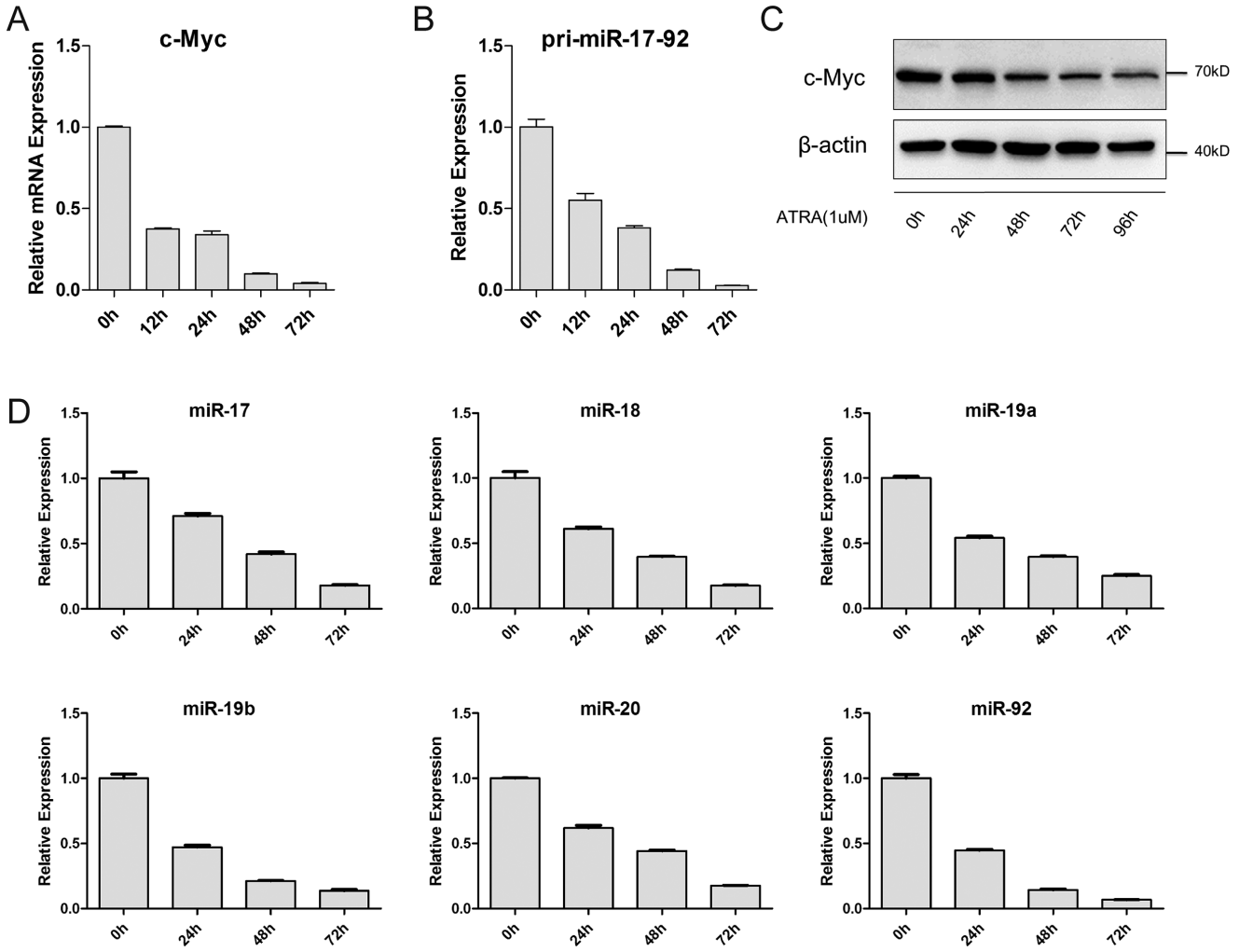

FIGURE 2 **c-Myc and the miR-17-92 cluster are significantly repressed in NB4 cells treated with ATRA**
NB4 cells were treated with 1 μM ATRA at the indicated time points. The (A) c-Myc and (B) pri-miR-17-92 mRNA levels were measured by qRT-PCR and normalized to *ACTB*. C, Western blot analysis of the c-Myc protein level after treatment. D, Expression of the mature miRNAs from the miR-17-92 cluster was analyzed by qRT-PCR and normalized to *U6*. Each panel shows the mean ± SD of a representative experiment performed in triplicate

or ATRA treatment significantly reduces the expression of miR-17-92 in NB4 cells. These data suggest that the aberrant expression of the miR-17-92 in APL is associated with c-Myc activity.

Previous studies have shown that the expression of miR-17-92 gradually downregulated during myeloid differentiation.[26] Consistent with these results, we show that the miR-17-92 cluster decreases with ATRA-mediated terminal differentiation. The downregulated expression of miR-17-92 suggests its critical role in the terminal differentiation of APL cells. Indeed, the overexpression of miR-17-5p inhibits granulocytic differentiation. The ATRA-induced derepression of miR-17-92 targets is likely required for mediating the differentiation effects. This finding is consistent with previously published data demonstrating that miR-17-92 is critical for myeloid differentiation of cord blood hematopoietic progenitors. Several transcription factors, including c-Myc, MYB, and p53, were reported to regulate the expression of miR-17-92 cluster at transcriptional level. Increased c-Myc oncogene is associated with poor prognosis and plays multiple roles in leukemogenesis.[27] Our study suggests that the miR-17-92 cluster is regulated by c-Myc in APL. It is notable that a previous report has suggested that increased expression of the miR-17-92 is controlled by C/EBP-β in HL60 cells.[28] However, we used three different siRNA sequences directed against c-Myc to ensure their effects are on target; all of the c-Myc siRNAs significantly reduced the expression of miR-17-92 (Figure 3A, 3C, and Figure S2). Importantly, our results demonstrate that c-Myc can act directly at the miR-17-92 promoter in NB4 cells. It is also of note that a previous report has shown that c-Myc inhibits the expression of C/EBP-β induced by ATRA.[29] Thus, in addition to c-Myc directly regulating miR-17-92 expression during APL cell granulocytic differentiation, it may also partially work through C/EBP-β.

In conclusion, we report abnormal expression of the miR-17-92 cluster in APL cells, which is involved in myeloid differentiation block in APL blast cells. In addition, we identified miR-17-92 as a target gene of c-Myc during ATRA-induced granulocytic differentiation. This study reveals a contribution of c-Myc/miR-17-92 in the development of APL.

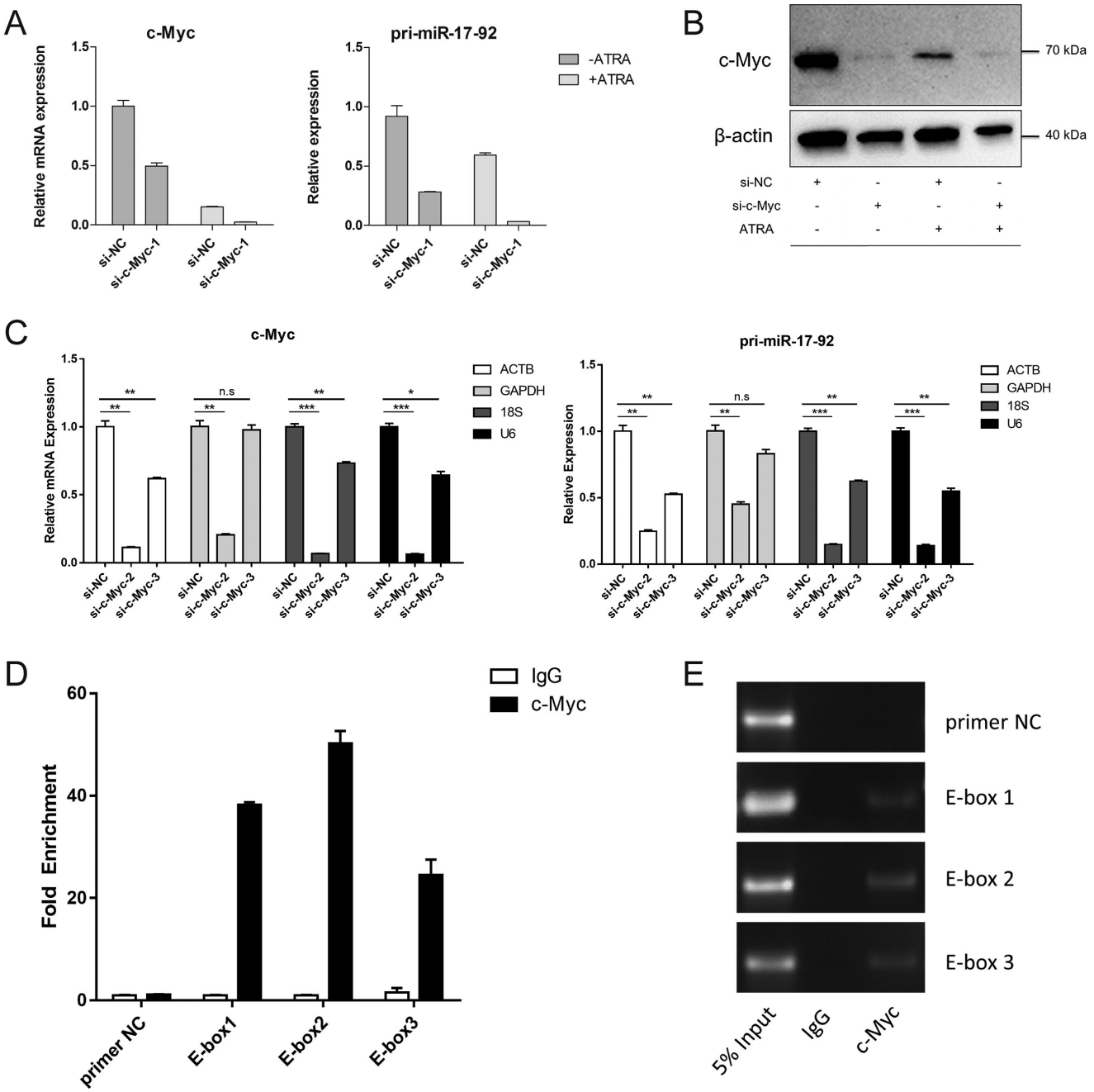

FIGURE 3 **c-Myc regulates the expression of the miR-17-92 cluster by binding to E-box elements in the miR-17-92 promoter**
A, NB4 cells transfected with siRNA specifically targeting *c-Myc* (si-c-Myc) or siRNA negative control (si-NC). Cells were treated with or without ATRA (1 μM) for 48 h. The expression of c-Myc and pri-miR-17-92 was evaluated by qRT-PCR. The values were normalized to *ACTB* expression. B, Western blot analyses of c-Myc after transfection with si-c-Myc followed by the treatment with or without ATRA (1 μM for 48 h). C, NB4 cells were transfected with si-c-Myc-2, si-c-Myc-3, or si-NC. The expression of c-Myc (left panel) and pri-miR-17-92 (right panel) was evaluated by qRT-PCR. The values were normalized to different housekeeping genes (*ACTB*, *GAPDH*, *18S*, *U6*). D and E, ChIP-qPCR/PCR analysis of NB4 cells demonstrated that immunoprecipitation (IP) with an anti-c-Myc antibody results in enrichment of the three putative binding sites compared with IP with an immunoglobulin control. Primers that amplify non-c-Myc binding regions served as negative controls (Primer NC)

DATA AVAILABILITY STATEMENT

The authenticity of this article has been validated by uploading the key raw data onto the Research Data Deposit public platform (www.researchdata.org.cn), with the approval RDD number as RDDB2019000573.

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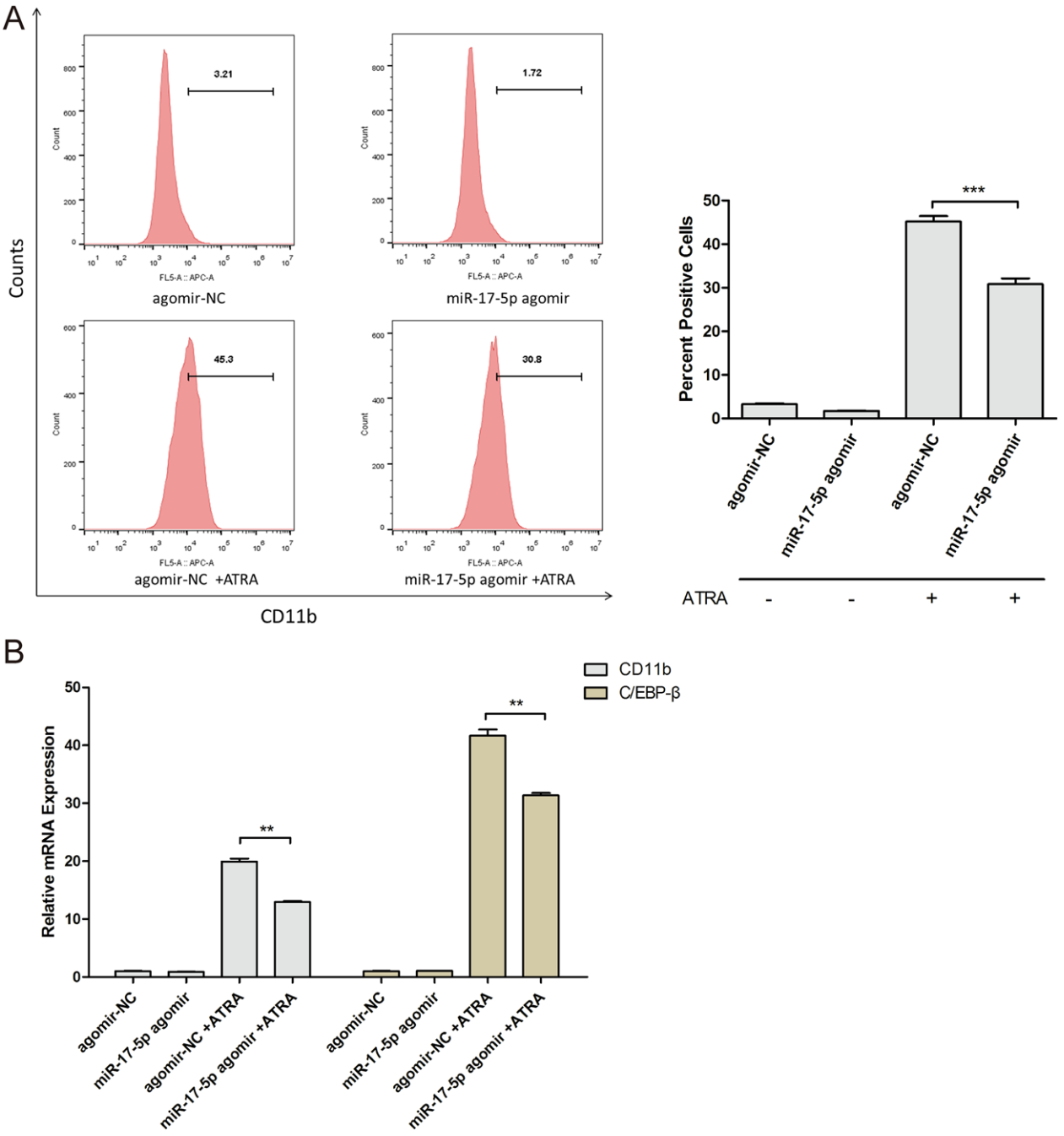

FIGURE 4 **miR-17-5p inhibits ATRA-induced differentiation**

A, Flow cytometry analysis of CD11b surface expression on miRNA agomir control and miR-17-5p agomir cells after 48 h of ATRA treatment (1 μM). CD11b expression was measured by flow cytometry, and values were normalized to untreated miRNA agomir control cells (left panel). The percentages of positive cells are represented in bar diagrams from three independent experiments (right panel). B, qRT-PCR analysis of the CD11b and C/EBP-β expression in cells transfected with the miRNA agomir control or the miR-17-5p agomir followed by 48 h ATRA treatment (1 μM) [Colour figure can be viewed at wileyonlinelibrary.com]

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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SUPPORTING INFORMATION

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

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