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MiR-15b regulates cell differentiation and survival by targeting CCNE1 in APL cell lines

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ABSTRACT

MicroRNAs have been shown to be involved in various cell processes, including proliferation, apoptosis and differentiation. However, little is known about their function in granulopoiesis. In the present study, overexpression and knockdown experiments revealed that miR-15b was required to block the proliferation of NB4 and HL60 cells and induce them differentiated to granulocyte lineage. Moreover, we identified CCNE1 as a direct target of miR-15b, and demonstrated that CCNE1 was involved in cell differentiation and proliferation in acute promyelocytic leukemia cells. In addition, we demonstrated a novel pathway in which miR-15b regulated cells arrested in the G0/G1 phase and promoted terminal differentiation of cells by targeting CCNE1, which could modulate the cell cycle effort pRb in APL cells. These events blocked cell proliferation and promoted granulocyte differentiation. In conclusion, our data highlighted, for the first time, the important role of miR-15b in myeloid differentiation and suggested the potential role of miR-15b in cancer therapy.

1. Introduction

Acute promyelocytic leukemia, a subtype of acute myeloid leukemia, is driven by a chromosomal translocation in which the N-terminal promyelocytic leukemia protein (PML) on chromosome 15 fuses with the C-terminal retinoic receptor a (RARa) on chromosome 17 [1,2]. This chromosomal translocation results in a novel fusion protein known as promyelocytic leukemia-retinoic acid receptor a (PML/RARa), which is capable of blocking cell differentiation and neoplastic transformation by acting as a transcriptional factor that represses RARa [3] and non-RARa [4] target genes, interfering with the normal assembly of PML protein, and disrupting the function of PML nuclear bodies (PML-NBs) involved in growth inhibition and apoptosis [5]. Accruing reports indicate that pharmacological doses of all-trans-retinoic acid (ATRA) reverses the transcriptional repression and induces the differentiation of acute promyelocytic leukemia (APL) blast cell by disrupting PML-RARa fusion protein [6,7].

MicroRNAs (miRNAs)—small, non-protein-coding RNAs—play a critical role in the regulation of gene expression by post-transcriptional mRNA silencing [8–10]. Dysregulated miRNA can modulate a variety of biological pathways, which play a crucial role in the development of malignancies [11–13]. Several studies have focused on miRNAs for their clinical significance in tumor diagnosis and prognosis. For example, Liu et al. [14] reported that miR-182 acts as an early diagnostic biomarker in colorectal cancer because of its enhanced expression in tissue and serum samples. Decreased expression of miR-185 can serve as an independent prognostic factor for gastric cancer [15]. Furthermore, in acute myeloid leukemia (AML), miR-29a and miR-142-3p are potential candidates for diagnosis and therapeutic strategy [16].

MiR-15b, located on chromosome 3q25, is associated with cell differentiation, proliferation, and apoptosis in a wide variety of cancers [17,18]. Loss of miR-15b/16-2 results in B-cell malignancy [19]. Numerous studies have reported that miR-15b could target RECK [20], BMI1 [21], and IGF1R [22] in prostate cancer, tongue cancer, and glioblastoma respectively. Ectopic expression of miR-15b has been reported in patients with APL and APL cell lines during ATRA treatment [23,24]. However, the precise functional role of miR-15b in APL remains unclear and needs to be investigated.

In this study, we found that the expression of miR-15b was significantly increased in APL cell lines with the treatment of ATRA. In

Abbreviations: AML, Acute myeloid leukemia; APL, Acute promyelocytic leukemia; CCK8, Cell Counting Kit-8; NBT, Nitroblue tetrazolium; qRT-PCR, Quantitative real-time PCR; PVDF, Polyvinylidene difluoride; siRNA, small interfering RNA; CCNE1, cyclin E1; ATRA, all-trans retinoic acid; miRNA, microRNA; miR-15b, microRNA-15b; Rb, retinoblastoma; SDS, Sodium dodecyl sulfate.

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addition, gain- and loss-of-function assays elucidated that miR-15b induced APL cells differentiation and suppressed cell proliferation by inhibiting cyclinE1 (CCNE1), which in turn led to a decrease in the level of phosphorylated retinoblastoma protein (pRb). In a word, our data reveals that miR-15b induces cellular differentiation and suppresses cell proliferation by targeting CCNE1.

2. Materials and methods

2.1. Microarray analysis

ATRA-induced miRNA Microarray dataset GSE11379 was downloaded from Gene Expression Omnibus (GEO) datasets (https://www.ncbi.nlm.nih.gov/gds/). The dataset GSE11379 based on the platform as GPL4717. Means of ratio from ATRA-treated/control were calculated for each cell line.

2.2. Cell line and culture

Human leukemia cells NB4, HL60 were chosen for this study. All cell lines were stored at our laboratory. All cell lines were cultured in RPMI 1640 medium (Gibco, MD, USA), supplemented with 10% fetal bovine serum (FBS; Gibco, Melbourne, Australia) and maintained in 5% carbon dioxide at 37 °C.

2.3. Western blot analysis

After the specified treatment, cells were harvested and washed twice with cold PBS. Whole cell lysates were extracted by RIPA lysis Buffer containing a protease inhibitor cocktail (Beyotime Biotechnology, Shanghai, China). Total protein concentration of supernatants was measured using the BCA protein assay (Beyotime, Shanghai, China); equal amounts of proteins were loaded on 10% SDS-PAGE and subsequently transferred to PVDF membrane (Millipore, MA, USA). Primary antibodies used were CCNE1, Rb, p-Rb (Abcam, Cambrige, UK) and β-actin (Beijing Zhongshan Golden Bridge Biotechnology, Beijing, China). Horseradish peroxidase-conjugated secondary antibodies (Biosharp, China) were also used. Signals were detected using an ECL kit (Millipore, USA). The results of immunoblots were quantified by densitometric analysis using the ImageJ software.

2.4. RT-PCR assay

Total RNA from cells in each group was extracted by TRIzol reagent (TAKARA, Kyoto, Japan) following the manufacturer's instructions, and reverse transcribed into cDNA using the PrimeScript™ RT reagent Kit (TAKARA, Kyoto, Japan).

Quantitative real-time PCR (qRT-PCR) was performed using the SYBR® Premix Ex Taq™ II (Takara, Japan) kit on a CFX Connect™ real-time PCR operating system (Bio-Rad, USA). The following primers were used for real-time amplification: miR-15b (Forward 5′-CGCGCGTAGC AGCACATCATGG-3′, Reverse 5′-ATCCAGTGCAGGGTCCGAGG-3′ and RT 5′-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGAC TGTAAA-3′); U6 (Forward 5′-CTCGCTTCGGCAGCACA-3′, Reverse 5′-AACGCTTCACGAATTTGCGT-3′ and RT 5′-CGCTTCACGAATTTG CGT-3′); CD11b (Forward 5′-CAGAGCGTGGTCCAGCTTCA-3′ and Reverse 5′-CCTTCATCCGCCGAAAGTCA-3′); CSF3R (Forward 5′-TCAA GTTGGTGCTATGGCAAGG-3′ and Reverse 5′-GCTCCCAGTCTCCACAG AATC-3′); β-actin (Forward 5′-TAGTTGCGTTACACCCTTTCTTG-3′ and Reverse 5′-TGCTGTCACCTTCACCGTTC-3′). The relative expression of miRNA and mRNA were measured by $2^{-\Delta\Delta Ct}$ analysis method, where U6 and β-actin were used as control, respectively.

2.5. Cell viability assay

Cell viability was determined using the Cell Counting Kit-8 (CCK8, Abcam, Cambrige, UK). Cells in a 96-well plate were transfected with miR-15b mimic or scramble, and incubated in RPMI-1640 containing 10% fetal bovine serum. The CCK8 assay was conducted at indicated time points, and the results were determined by measuring absorbance at 450 nm using the microplate reader (Eon, BioTeck, CA, USA).

2.6. Wright-Giemsa staining

To assess morphologic differentiation, NB4 and HL60 cells were treated for 72 h as indicated. Cells were fixed on glass slides and stained with Wright-Giemsa. The slide was washed with deionized water and observed under a light microscope (YS100; Nikon, Japan). The percentage of segmented neutrophils was determined by counting 200 cells on the slide.

2.7. Nitroblue tetrazolium (NBT) reduction assay

NB4 and HL60 cells, untreated or treated with ATRA, were collected after 72 h and resuspended in RPMI1640 medium containing 10% fetal bovine serum, and 3×10^5 cells per well were incubated with 0.2% nitro blue tetrazolium (NBT, Beyotime, Shanghai, China) and 240 μg/mL 12-O-tetradecanoylphorbol-13-acetate (TPA, Beyotime, Shanghai, China) in 96-well plates for 1 h. Finally, 10 μL CCK8 was added to each well and the absorbance was measured at 570 nm.

2.8. Flow cytometry

Following the indicated protocol, cells were harvested, washed twice with cold phosphate-buffered saline (PBS), resuspended in 50 μL PBS, and incubated with phycoerythrin (PE)-conjugated anti-CD11b (BD Biosciences, CA, USA) in dark for 30 min. Cells were washed twice with PBS and resuspended in 200 μL PBS. The percentage of CD11b-positive cells was measured using an Accuri C6 flow cytometer (BD Biosciences, CA, USA).

2.9. Luciferase reporter assay

Target genes of miR-15b were obtained from TargetScan (www.genes.mit.edu/targetscan), MiRanda (www.MicroRNA.org/Miranda), PicTar (www.pictar.bio.nyu.edu) and miRDB (http://mirdb.org/miRDB/). Among the total number of targets predicted, CCNE1 was selected for this study. The CCNE1-3′UTR-wild-type and mutant segments were amplified by PCR and cloned into the pGL3 luciferase reporter control vector. Either wild-type or mutant vector was co-transfected along with miR-15b mimic or scramble into HEK293T cells using Lipofectamine 2000 (Invitrogen, CA, USA). HEK293T cells were collected 48 h after transfection,Luciferase activity was measured using the Dual-Luciferase Reporter Assay System kit (Promega, WI, USA). Firefly luciferase activity was normalized by Renilla luciferase.

2.10. Transfection

The miR-15b mimic, inhibitor, and the controls were synthesized by Gene Pharma (Shanghai, China), and transfected into NB4 and HL60 cells using Lipofectamine 2000 (Invitrogen, USA) following the manufacturer's instructions. For small interfering RNA (siRNA) experiments, siRNAs against CCNE1 were also synthesized by Gene Pharma (Shanghai, China), and transfected into NB4 and HL60 cells using Lipofectamine 2000 (Invitrogen, CA, USA) following the manufacturer's instructions, while using non-targeting human siRNA as control.

2.11. Statistical analysis

All experimental data were obtained from three independent experiments and the results were summarized as mean ± s.d. Students' *t*-test was used for comparison between two groups, and One-way

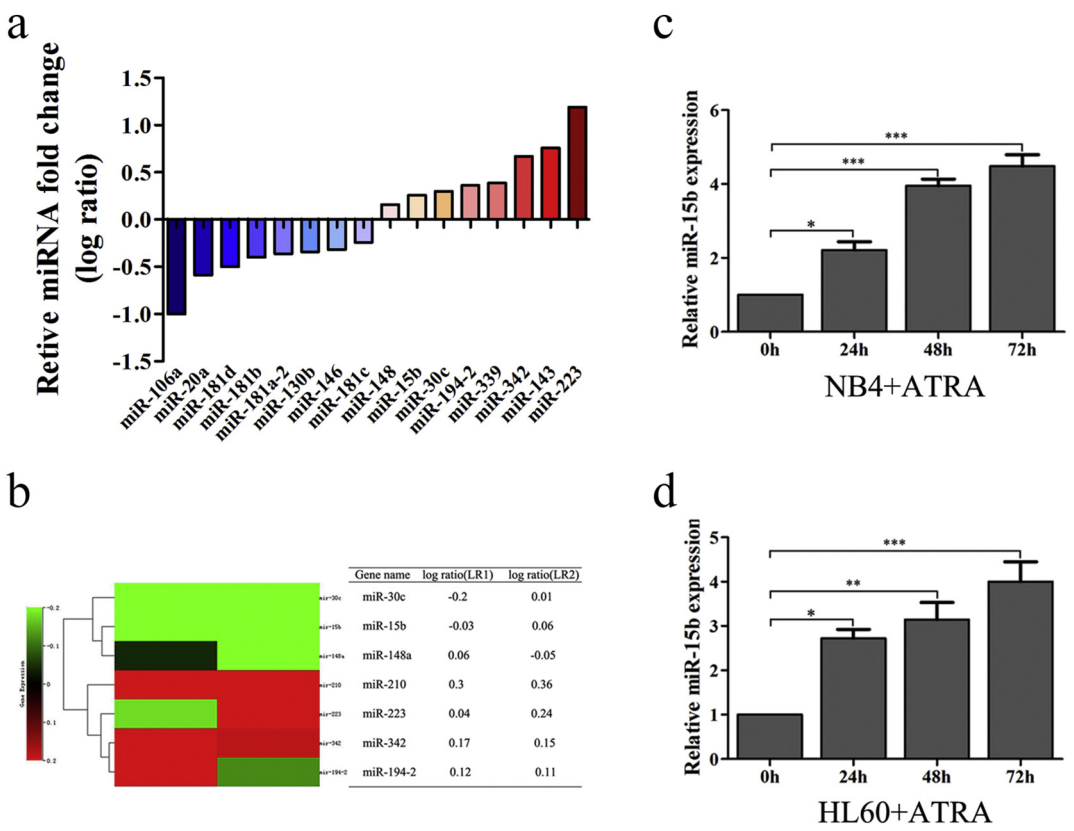

Fig. 1. The expression of miR-15b during ATRA treatment. **a** 8 up-regulated and down-regulated miRNAs with treatment of ATRA. **b** MicroRNAs differentially expressed during ATRA treatment of NB4-LR1 and NB4-LR2 cells. **c** A time-dependent upregulation of miR-15b in NB4 **d** and HL60 cells was observed following exposure to ATRA. Three independent experiments were performed. *$P < .05$, **$P < .01$, ***$P < .001$.

ANOVA followed by Tukey comparison test was applied for comparison between at least three groups. All statistical analyses were performed using GraphPad (Prism 5.0) software.

3. Result

3.1. MiR-15b expression is significantly enhanced in APL cells upon ATRA stimulation

To explore miRNA expression profiles of APL cells treated with ATRA, we first searched online database Gene Expression Omnibus (GEO) for dysregulated miRNAs with the treatment of ATRA. Fig. 1a showed 8 up-regulated and down-regulated miRNAs based on microarray data (GSE11379) [25]. Among these miRNAs, the miR-181 family had been shown to be down-regulated by ATRA in APL cells [26], and the miR-223 [27], and the miR-342 [28], 2 miRNAs previously found up-regulated in similar settings. Notably, heat map revealed that ATRA could not induce miR-15b level in ATRA-resistant APL cells, NB4-LR1 and NB4-LR2 (Fig. 1b), which indicated the function of miR-15b in myeloid mature. Moreover, qRT-PCR analysis revealed that the expression of miR-15b gradually increased in NB4 (Fig. 1c) and HL60 (Fig. 1d) cells in a time dependent manner. Together, these results strongly implied the relationship of miR-15b and myeloid cell's differentiation.

3.2. Upregulation of miR-15b promotes granulocytic differentiation and inhibits cell survival

To uncover the role of miR-15b in hematopoietic cell differentiation, we transiently transfected NB4 and HL60 cells with either miR-15b mimic or anti-15b. Transfection efficiency was verified by qRT-PCR (Fig. 2a). Flow cytometry data showed a higher percentage of mature cells was observed among NB4 and HL60 cells overexpressing miR-15b, after the treatment with ATRA, compared to the scramble cells (Fig. 2b). The acceleration of maturation resulting from miR-15b transfection was also confirmed by nitroblue tetrazolium staining (NBT, Fig. 2c). Furthermore, we quantified the mRNA level of differentiation markers by qRT-PCR in NB4 cells (Fig. 2d) and HL60 cells (Fig. 2e). Finally, compared to control-transfected cells, miR-15b mimic transfection demonstrated more features of mature cell morphology (decreased cell size and nucleus:cytoplasm ratio; indented-shape; and convoluted nuclei) (Fig. 2f). Moreover, to further obtain evidence of the potential function of miR-15b during granulocytic differentiation in NB4 and HL60 cells, Transfection efficiency of anti-15b was verified by qRT-PCR (Fig. 2g). And we found that cell differentiation was significantly decreased by anti-15b (Fig. 2h, i). These results demonstrated that miR-15b was a crucial factor during ATRA-induced granulocytic differentiation.

In addition, we validated the effects of miR-15b on cell proliferation in NB4 and HL60 cells. The proliferation assay showed that overexpression of miR-15b decreased the rate of proliferation in NB4 and HL60 cells (Fig. 2j).

3.3. CCNE1 was identified as a target of miR-15b

The elucidation of miRNA targets was a major issue in functional investigations of miRNAs, several prediction programs including TargetScan, PicTar, miRBase and miRDB were used to search potential targets of miR-15b. Based on its biological function, the selected list of miR-15b targets were grouped in Fig. 3a. We focused our attention on CCNE1, which had been investigated previously and were involved in leukemia formation [29]. To demonstrate the interaction between miR-15b and CCNE1, the wild-type and mutated 3′UTR of CCNE1 was cloned into pGL3-Promoter, and were transfected into 293 T cells that

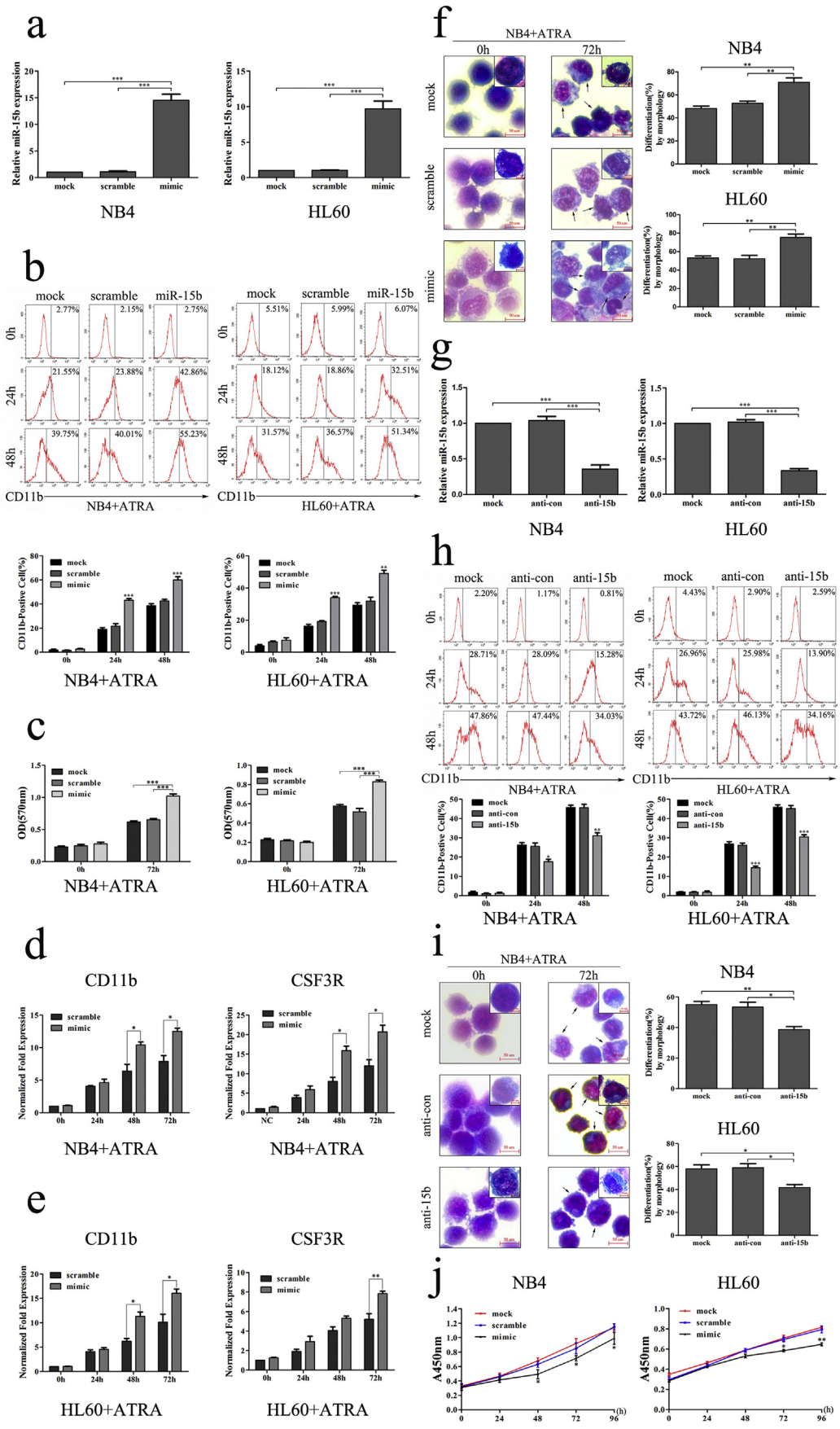

Fig. 2. Role of miR-15b in granulocytic differentiation and cell proliferation. **a** qRT-PCR of miR-15b in NB4 and HL60 cells transfected with miR-15b mimic or scramble. **b** CD11b positive cells were measured by flow cytometry in NB4 and HL60 cells transfected with scramble or miR-15b mimic under ATRA treatment. **c** NBT assay in NB4 and HL60 cells transfected with miR-15b mimic or scramble. **d** Expression levels of CD11b and CSF3R mRNA were analyzed by qRT-PCR in NB4 cells. **e** and HL60 cells. **f** Wright-Giemsa staining image of transfected cells treated with ATRA in NB4 and HL60 cells. **g** qRT-PCR of miR-15b in NB4 and HL60 cells transfected with anti-15b or anti-con. **h** Flow cytometry analysis of CD11b positive cells in NB4 and HL60 cells transfected with anti-con or anti-15b under ATRA treatment. **i** Morphological analysis of NB4 and HL60 cells transfected with anti-con or anti-15b with the treatment of ATRA. **j** Cell proliferation assay of NB4 and HL60 cells transfected with scramble or miR-15b mimic using CCK8 assay. Three independent experiments were performed. *P < .05, **P < .01, ***P < .001.

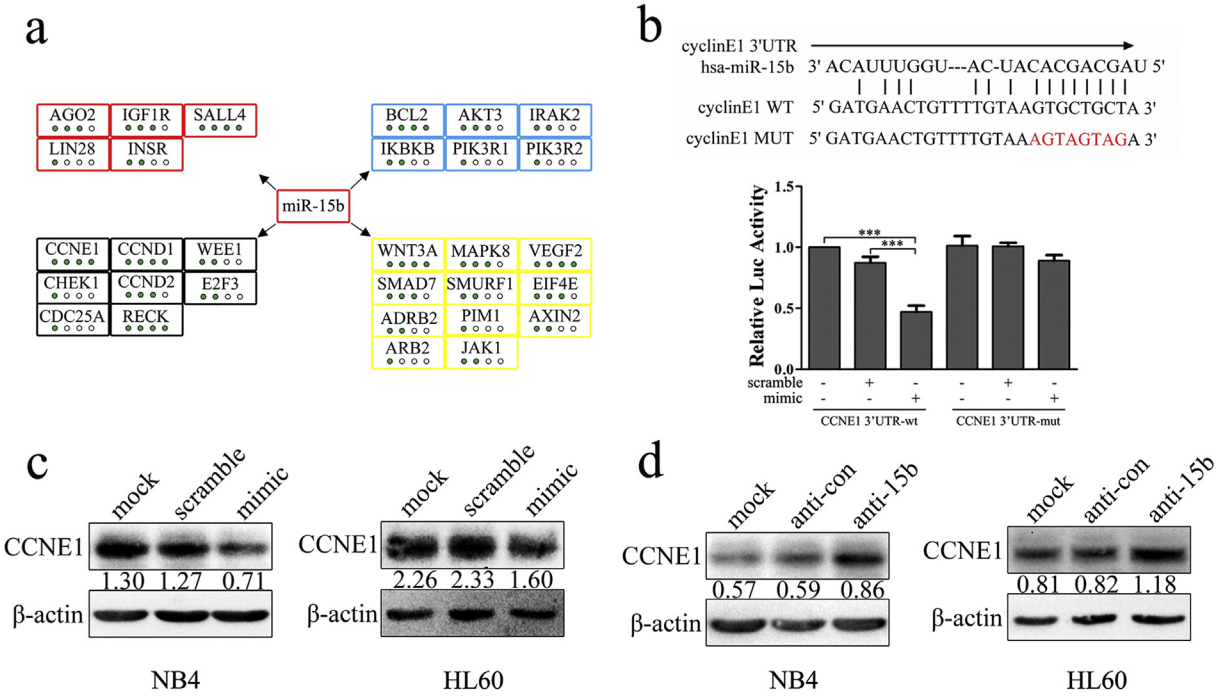

Fig. 3. CCNE1 is a target gene of miR-15b. **a** Scheme of target genes of miR-15b screened by miRanda, TargetScan, Pictar and miRDB algorithms. Filled spots represented the number of algorithms able to predict the putative target gene of miR-15b. **b** Luciferase activity in 293 T cells cotransfected with either the wild-type or mutant, and miR-15b mimic or scramble. **c** The protein expression levels of CCNE1 were detected by western blot in NB4 and HL60 cells after they were transfected with miR-15b mimic. **d** or anti-15b. Three independent experiments were performed. ***P < .001.

were treated with the miR-15b mimic or scramble. Luciferase reporter activity showed that the wild-type 3′UTR of CCNE1 was significantly repressed by miR-15b compared to the mutant (Fig. 3b).

To confirm that the CCNE1 protein was suppressed by miR-15b, we performed both miR-15b gain- and loss-experiments in NB4 and HL60 cells and detected expression levels of CCNE1 protein by western blot. As shown in Fig. 3c, a significant reduction of CCNE1 protein levels in NB4 and HL60 cells were detected after transfecting with miR-15b mimic, but it was increased in cells that were transfected with anti-15b (Fig. 3d). These results indicated that miR-15b fulfilled its function by targeting CCNE1.

3.4. Effects of CCNE1 on granulocytic differentiation and cell proliferation

To corroborate whether CCNE1 was involved in the differentiation of APL cells, we first detected the expression of CCNE1 in NB4 and HL60 cells. A significant decline in CCNE1 protein levels were determined in respond to the treatment of ATRA in a time-dependent manner (Fig. 4a), and this phenomenon correlated negatively with miR-15b expression. Wright-Giemsa staining showed morphologic changes in ATRA treated cell populations of NB4 and HL60 cells transfected with siRNA (Fig. 4b). Moreover, we found that the marker of granulocytic differentiation was significantly upregulated compared to the cells transfected with siControl in the presence of ATRA (Fig. 4c), which highlighted that CCNE1 knockdown led to a higher percentage of mature granulocytic cells. These results demonstrated that CCNE1 inhibited cell differentiation. We further demonstrated the role of CCNE1 in cell proliferation using CCK8 assay. NB4 and HL60 cells proliferation gradually decreased in a time-depended manner following transfection with siCCNE1 (Fig. 4d). These results were similar to the effects of miR-15b, which indicated that CCNE1 had a role in cell growth.

Previous studies had indicated that CCNE1 could influence the phosphorylation status of Rb. Thus, we asked whether CCNE1 had a role in the phosphorylated form of Rb protein in NB4 and HL60 cells. To reveal this issue, we transfected siCCNE1 into NB4 and HL60 cells and detected the expression of pRb decreased with the downregulation of CCNE1 by western blot (Fig. 4e), while the amount of total Rb protein remained the same in the treatment.

3.5. MiR-15b regulated cell differentiation and growth in APL cells by targeting CCNE1, which in turn modulated pRb expression

As we demonstrated that miR-15b could induce cell differentiation and survival in APL cells by repressing the expression of CCNE1, which had a crucial role in cellular differentiation and proliferation in APL cells though phosphorylating Rb. Thus, we hypothesized that miR-15b might play its function in APL via the CCNE1-pRb pathway. To reveal this hypothesis, we tested the expression of CCNE1 and pRb protein level after transfecting miR-15b mimic (Fig. 5a) and anti-15b (Fig. 5b) into NB4 and HL60 cells. These results support the hypothesis that miR-15b regulates the CCNE1-pRb pathway at the level of translation.

Given that Rb was a master cell cycle regulator, we also examined the cell cycle by FACS to identify S activation via the overexpression of miR-15b. As shown in Fig. 5c, a significantly increased percentage of cells in G1 was detected among these cells transfected with miR-15b mimic.

4. Discussion

Hematopoietic precursor differentiation is mediated by different miRNAs [30–32]. Emerging studies have used miRNA microarray platforms to reveal a number of regulated miRNAs during myeloid differentiation [33,34]. However, the molecular mechanisms underlying the role of miRNAs acting in APL therapy have been largely neglected. In this study, our data revealed that miR-15b promoted cell differentiation toward granulocyte lineages, and highlighted the role of miR-15b in APL therapeutic strategy.

It has been documented that miR-15/16 family played a vital role in tumorigenesis and progression [35,36]. As an important member of miR-15/16 family, miR-15b has been reported as a tumor suppressor in

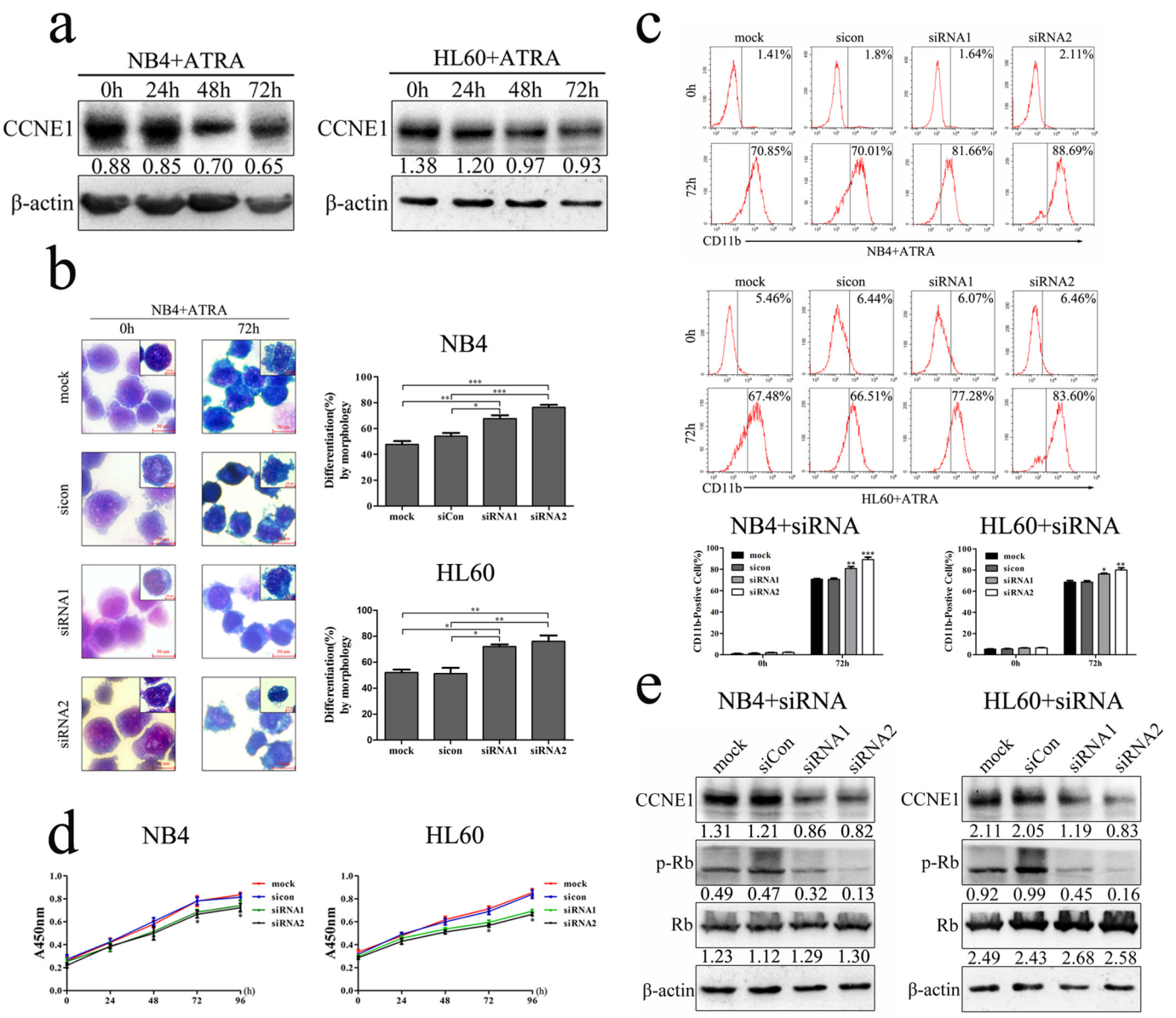

Fig. 4. Roles of CCNE1 in cell differentiation and proliferation. **a** Western blot showed CCNE1 protein levels in NB4 and HL60 cells with ATRA treatment. **b** The differentiated cells was measured by Wright-Giemsa staining. **c** or flow cytometry. **d** Cell proliferation assay of NB4 and HL60 cells transfected with siCCNE1 or siControl. **e** Western blot analysis showed the expression of CCNE1, pRb and Rb in CCNE1 knockdown cells. Three independent experiments were performed. *P < .05, **P < .01, ***P < .001.

hepatocellular carcinoma [37], colorectal cancer [38] and B lymphocytes [39]. However, little is known of its physiological function in myeloid differentiation and survival. Herein, we first used GEO database to evaluate the expression of miRNAs with the treatment of ATRA, and miR-15b demonstrated a expression alteration. Next, we analyzed expression level of miR-15b in NB4 and HL60 cells with the treatment of ATRA. These results revealed that ATRA could induce miR-15b expression. In order to understand the functions and effects of miR-15b in APL cells, a synthetic miR-15b mimic and anti-15b were designed to perform gain- and loss-of-function experiments in NB4 and HL60 cells. We detected that overexpression of miR-15b facilitated the differentiation and inhibited proliferation of NB4 and HL60 cells. Conversely, knockdown of miR-15b provided the opposite results. These results implied that an increase in the level of miR-15b might be an important step during granulocytic differentiation and cell survival. In a future study, the functional role of miR-15b in APL mouse models needs to be further investigated.

Mechanistically, the potential target genes of miR-15b were screened by in-silico analysis. Base on bioinformatics analysis and dual-luciferase reporter assays, we confirmed that CCNE1 was the target of miR-15b. CCNE1 belongs to cyclins gene family and functions as an oncogene, and its overexpression is associated with poor clinical prognosis of ovarian cancer, bladder cancer and breast cancer [40-42]. In detail, CCNE1 physically interacts with CDK2, and subsequently phosphorylates Rb to promote cell proliferation and accelerate G1-S progression [43,44]. In the present study, western blot assays demonstrated that miR-15b could regulate CCNE1, which in turn regulated pRb expression in NB4 and HL60 cells. Similar to our results, previous research reported that miR-15b inhibited the osteoblastic proliferation through targeting CCNE1 [45], indicating that miR-15b-inhibited CCNE1 expression might be universal in different tumors. Finally, we observed that knockdown of CCNE1 by RNA interference significantly promoted cell differentiation with the treatment of ATRA, consistent with the results in miR-15b overexpressing cells.

Taken together, our data reveals the importance of miR-15b in promoting APL cell differentiation and arresting cell proliferation. We

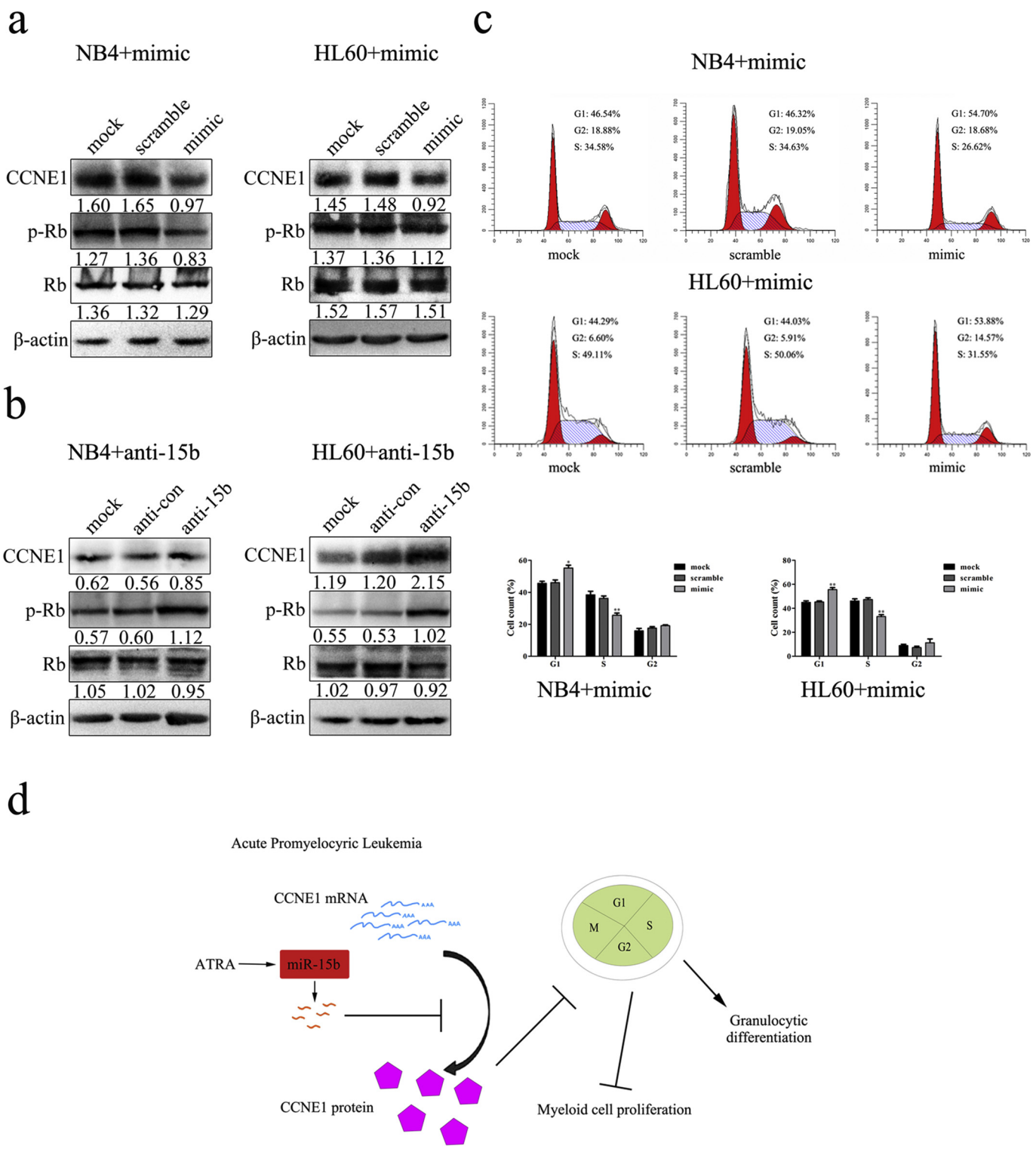

Fig. 5. MiR-15b regulated APL cells differentiation and proliferation by modulating CCNE1-pRb pathway. **a** The protein expression levels of CCNE1, p-Rb and Rb were detected by western blot in NB4 and HL60 cells after they were transfected with miR-15b mimic. **b** or anti-15b. **c** Cell cycle phase data in NB4 and HL60 cells transfected with scramble or miR-15b mimic. **d** Schematic diagram describing the function of miR-15b in APL cells.

also provide evidence for a direct link between miR-15b and CCNE1 in APL cells. Moreover, increasing endogenous expression or ectopic implantation of miR-15b may be a potential strategy for APL treatment.

5. Conclusion

In summary, our data demonstrate that the induced effect of ATRA exerted on APL cell differentiation was mediated by miR-15b, and suggest CCNE1 was the potential target of this process. This may provide a novel therapeutic strategy for APL in the future.

Competing interests

None.

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