miR-let-7c-3p targeting on Egr-1 contributes to the committed differentiation of leukemia cells into monocyte/macrophages

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Abstract. In preliminary experiments, it was found that the expression of early growth response-1 (Egr-1) was upregulated during the committed differentiation of leukemia cells into monocytes/macrophages. The cross-analysis of gene chip detection and database prediction indicated that Egr-1 was associated with upstream microRNA (miR)-let-7c-3p, thus the present study focused on the role of the miR-let-7c-3p/Egr-1 signaling axis in the committed differentiation of leukemia cells into monocytes/macrophages. Phorbol 12-myristate 13-acetate (PMA) was used to induce the directed differentiation of human K562 leukemia cells into monocytes/macrophages and the differentiation of K562 leukemia cells was determined by cell morphology observation and expression of differentiation antigens CD11b and CD14 by flow cytometry. The expression levels of Egr-1 and miR-let-7c-3p were detected by reverse transcription-quantitative PCR and the protein expression of Egr-1 was detected by western blotting. The effect of Egr-1 on the differentiation of K562 cells was detected by short interfering (si)RNA interference assay. A dual-luciferase reporter assay was used to detect target binding of miR-let-7c-3p on the 3'UTR of Egr-1. Cell transfection of miR-let-7c-3p mimics and inhibitors was used to modulate the expression of miR-let-7c-3p, as indicated by RT-qPCR assays. Western blotting was also used to examine the effect of miR-let-7c-3p on Egr-1 expression. The PMA-induced differentiation of K562 cells was transfected with miR-let-7c-3p and the expression of differentiation antigen was detected by flow cytometry. A differentiation model of K562 leukemia cells into monocytes/macrophages was induced by PMA, which was indicated by morphological observations and upregulation of CD11b and CD14 antigens. The gene or protein expression of Egr-1 was significantly higher compared with that of the control group, while the expression of miR-let-7c-3p was significantly lower compared with that of the control group. siRNA interference experiments showed that the expression of cell differentiation antigen CD14 in the 100 μg/ml PMA + si-Egr-1 group was significantly lower compared with that in the 100 μg/ml PMA + si-ctrl group. The dual luciferase reporter gene results showed that the luciferase activity of the co-transfected mimic and Egr-1 WT groups was significantly lower than that of the NC control group, while the luciferase activity of the co-transfected mimic and Egr-1 MUT groups was comparable to that of the NC control group. Therefore, the dual-luciferase reporter gene assay confirmed that miR-let-7c-3p can target Egr-1. Western blotting showed that the expression of Egr-1 following transfection with miR-let-7c-3p inhibitor was significantly higher compared with that of the negative control and the expression of Egr-1 after transfection with miR-let-7c-3p mimic was significantly lower than that of the negative control. Following exposure to PMA, the expressions of CD11b and CD14 in the miR-let-7c-3p inhibitor group were significantly higher than those in the miR-let-7c-3p NC group, as indicated by CD11b and CD14 respectively. In conclusion, miR-let-7c-3p could bind to the 3'UTR of Egr-1 and negatively regulated Egr-1 expression. The miR-let-7c-3p/Egr-1 signaling axis was closely associated with the committed differentiation of K562 cells from leukemia cells to monocytes/macrophages.

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Introduction

Myeloid leukemia is a type of hematopoietic stem cell malignant tumor, with differentiation disorder, uncontrolled proliferation, or the inability of terminal differentiation of primitive cells to retain malignant proliferation ability and accounting for ~15% of new cases of adult leukemia (1,2). It has been confirmed that the occurrence of myeloid leukemia is associated with certain gene mutations, abnormal gene expression, epigenetic disorders or abnormal expression of non-coding RNA (3-6). Compared with traditional chemotherapy, induced differentiation therapy has become an ideal method for the treatment of leukemia due to its non-toxic side effects (7,8). However, so far, only patients with acute promyelocytic leukemia could get the complete remission induced by differentiation-inducing drugs such as all-trans retinoic acid; other types of leukemia have not benefited from them (9-11). Therefore, it is necessary to actively explore new intervention targets and corresponding targeted drugs on the basis of in-depth exploration of the key mechanisms of leukemia differentiation disorders. It is the superiority of the aforementioned induced differentiation therapy that has made differentiation induction a research hotspot in recent years (12-15).

Expression of early growth response-1 (Egr-1) is a member of the early growth response protein family, which has been considered to be of great significance in a variety of physiological processes and has been extensively studied (16,17), especially in cell proliferation, angiogenesis, invasion and immune response of tumors (18,19). Egr-1 can act as a transcriptional regulator by combining the C2H2 type zinc finger with the DNA motif of the 5'-GCG(T/G)GGGCG-3' sequence. Regardless of the methylation status of cytosine, it can bind to double-stranded target DNA and the target DNA that does not bind to cytosine is oxidized to 5-formylcytosine or 5-carboxycytosine (20,21). As it is an important part of certain signal pathways in the process of cell signal transduction, it can mediate the coupling of intracellular signal cascades and regulate the transcription and transcription of a number of downstream long-term response genes that determine cell karyotype changes (16,17). To a certain extent, the role of Egr-1 in cell proliferation and differentiation is heterogeneous, especially in normal somatic cells and malignant tumor cells. For example, in normal somatic cells, Egr-1 is in a dormant state and the expression level is very low or even not expressed in the normal state. However, when the cell is stimulated by some physical and chemical factors, the rapid activation of Egr-1 allows cells to enter the proliferation phase from the resting phase, which in turn leads to cell proliferation (22,23). In tumor cells, the role of Egr-1 is more complex and can be expressed as an oncogene or tumor suppressor gene in different types of tumor, for example, Egr-1 promotes the malignant behaviors of LC cells (24), circCSPP1-miR-520h-Egr-1 activation axis lead to the progression of prostate tumor (25), and Egr-1 as a potential oncogene that promotes cell proliferation and defines Egr-1 as a new molecular target in DLBCL non-Hodgkin lymphomar (26). As far as proliferation and differentiation of leukemia cells are concerned, although there are some studies associating it with the inhibition of proliferation and induction of differentiation of leukemia cells (27,28), its role in the committed differentiation of leukemia cells into monocyte/macrophages is rarely reported.

micro (mi)RNAs are non-coding small RNAs with post-transcriptional regulation. They are endogenous small RNAs with a length of 18-24 nucleotides. Usually, they can base pair with the 3' untranslated region (UTR) of target mRNAs and silence genes at the post-transcriptional level by inhibiting mRNA translation or directly causing mRNA degradation and abnormal expression often appears in the occurrence and development of tumors (29). Among which the miRNA (MiR)-let-7 family is downregulated in various types of tumor tissues and has been widely studied as a tumor suppressor gene (30,31). Increasing evidence shows that Let-7 also has the same properties as other miRNAs, which not only participate in the occurrence and development of leukemia, but also serve as a potential biomarker for the diagnosis and prognosis of leukemia (32). However, whether the miRNAs Let-7 is involved in the directional monocyte-macrophage maturation and differentiation of leukemia cells remains to be elucidated.

In the authors' previous work (data not published), distinct changes in miR-let-7c-3p and Egr-1 expression were detected in a PMA-induced differentiation model of K562 cells. The present study focused on the role of the miR-let-7c-3p/Egr-1 signaling axis in the committed differentiation of K562 leukemia cells into more mature monocytes/macrophages. The results demonstrated that the miR-let-7c-3p/Egr-1 axis was closely associated with the differentiation of K562 cells from leukemia cells into more matured monocytes/macrophages induced by PMA.

Materials and methods

Materials. Human chronic myeloid leukemia cell line K562 was purchased from Shanghai Cell Bank, Chinese Academy of Sciences. PMA was purchased from American Sigma Company (cat. no. P1585-1MG). Fetal bovine serum (FBS), BCA protein assay kit and SDS-PAGE gel rapid preparation kit were purchased from Shanghai Biyuntian Biotechnology Co., Ltd. RPMI 1640 medium was purchased from HyClone (Cytiva). Swiss-Giemsa staining solution, double antibody, RIPA protein lysis solution and 5X protein loading buffer were purchased from Beijing Solarbio Science & Technology Co., Ltd. Standard protein marker and Lipofectamine® 2000 transfection kit were purchased from Thermo Fisher Scientific, Inc. The ECL luminescence kit was purchased from Shandong Sparkjade Scientific Instruments Co., Ltd. Egr-1 (cat. no. 22008-1-AP), GAPDH (cat. no. 10494-1-AP) and β-actin (cat. no. 20536-1-AP) primary antibodies were purchased from ProteinTech Group, Inc. HRP-labeled rabbit secondary antibody (cat. no. ZB-2301) was purchased from OriGene Technologies, Inc. Primer design was provided by Sangon Biotech Co., Ltd. Reverse transcription kit PrimeScript RT reagent kit with gDNA Eraser (Perfect Real Time), chimeric fluorescence detection kit and TB Green Premix Ex Taq™ (Tli RNaseH Plus) kit was purchased from Takara Biotechnology Co., Ltd. The cycle kit was purchased from Jiangsu KGI Biotechnology Co., Ltd. PE-CD11b (cat. no. 301306) and FITC-CD14 (cat. no. 301804) fluorescent conjugated antibodies were purchased from BioLegend, Inc. TRIzol® reagent was purchased from Thermo Fisher Scientific. Inc.

Establishment of differentiation model of K562 leukemia cells. K562 cells were grown in culture flasks containing 10% FBS in RPMI-1640 complete medium, cultured at 37°C, 5% CO_2. In the logarithmic growth phase, an appropriate amount of K562 cell suspension and PMA solution were added to 96-well plates, so that the cell concentration in each well was $1x10^5$/ml and the corresponding dose of PMA solution was added. A total of three duplicate wells were set up in each group and one zero-adjusting well was set up in each plate with only an equal volume of RPMI1640 culture medium (100 μl) added and then cultured at 37°C, 5% CO_2 with saturated humidity. At 24, 48 and 72 h of culture 10 μl of CCK8 solution was added to each well, except the blank well and incubated at 37°C for 2 h and detected at 450 nm. According to the IC_{50} experimental results of 48 h of culture, the control group (PMA 0 ng/ml) and the experimental group (the final concentration of PMA 100 ng/ml) were selected.

Observation of cell morphology by Swiss-Giemsa staining. The control group with 0 ng/ml PMA and the experiment group with 100 ng/ml PMA of K562 cells induced for 48 h without staining were observed directly under an inverted optical microscope. Cells of the above-mentioned control and experiment group were collected at 48 h, washed twice with cold PBS, resuspended with 100 μl PBS and mixed by gently pipetting to make a cell suspension. After centrifugation at 200 x g for 3 min at 4°C, the centrifuged smears were dried and stained with Wright-Giemsa Stain Solution for 5 min at room temperature. The changes of cell morphology were observed under different magnifications of the optical microscope and the resulting images were captured.

Expression of differentiation antigen CD11b and CD14 by flow cytometry. K562 cells were collected into flow tubes, resuspended in PBS, washed twice by centrifugation at 200 x g for 3 min at 4°C, and adjusted to a cell concentration of $1x10^6$ cells/ml. PBS (100 μl) was added to each tube, followed by 5 μl PE-labeled mouse anti-human CD11b fluorescent antibody and FITC-labeled mouse anti-human CD14 fluorescent antibody respectively and incubated at 4°C for 30 min in the dark. The cells were centrifuged at 200 x g for 4 min at 4°C and washed twice with PBS to remove excess monoclonal antibody. The cells were resuspended in 200 μl PBS and then fixed in 1% paraformaldehyde. The expressions of CD11b and CD14 in different treatment groups were analyzed on FACSVerse (BD Biosciences Pharmingen) Flow cytometer. Isotypic rat IgG was also used to check for nonspecific binding. The experiment was repeated three times.

Protein expression by western blotting. The cells of the control group and the experimental group were collected, washed twice with pre-cooled PBS, cells were lysed with Protein Extraction reagent (Beijing Solarbio Science & Technology Co., Ltd.), the total cell protein was extracted, the protein concentration was determined by BCA method and 5x protein loading buffer was added and boiled for denaturation at 95°C for 10 min. Protein (20 μg) was loaded for SDS-PAGE (10%) electrophoresis, the separated protein was transferred to PVDF membrane, blocked with 5% skimmed milk for 90 min at room temperature and then incubated with the corresponding primary antibody;

Egr-1 (1:800), GAPDH (1:8,000) and β-actin (1:2,000) at 4°C overnight, then washed with 1X TBST for 30 min, then incubated with goat anti-rabbit IgG secondary antibody (1:20,000) at room temperature for 90 min and finally washed for 30 min, and proteins were detected using an ECL kit (Sparkjade ECL super, ED0015-B, Shandong Sparkjade Scientific Instruments Co., Ltd.). ImageJ v1.51j8 was used for densitometry (National Institutes of Health). The experiment was repeated three times.

Relationship Analysis between miR-let-7c-3p and Egr-1. TargetScan, PITA and microRNAorg databases were used to predict target genes of possible upstream miRNAs of Egr-1 and intersected the predicted target miRNAs by crosstalk of the three databases. The common target miRNAs in the three databases were obtained, and the top 10 miRNAs were selected according to the P-value and literature research. In addition, GeneChip miRNA 4.0 (Affymetrix Co., Ltd.) was used to detect the different miRNA profiles between control and PMA-induced K562 cells, and 10 miRNAs that were reduced after induced-differentiation were screened according to the P-value.

Gene expression by reverse transcription-quantitative (RT-q) PCR. When the K562 cells were cultured to the logarithmic growth phase, the cells ($1x10^6$) were collected and the total RNA was extracted by TRIzol® (Thermo Fisher Scientific, Inc.) method and the total RNA concentration was measured by an ultra-trace nucleic acid and protein analyzer. cDNA was synthesized according to the instructions of the PrimeScript RT reagent kit with gDNA Eraser (Perfect Real Time) reverse transcription kit. For reverse transcription, samples were incubated in an Eppendorf PCR system at 42°C for 30 min, then at 90°C for 5 min and at 5°C for 5 min. cDNA was used as a template for PCR amplification. The sense primer of miR-let-7c-3p was 5'-GCGCGCTGTACAACC TTCTAG-3', the antisense primer was 5'-AGTGCAGGGTCC GAGGTATT-3'; the U6 sense primer was 5'-AGAGAAGAT TAGCATGGCCCCTG-3', Antisense is 5'-AGTGCAGGG TCCGAGGTATT-3'; Egr-1 sense primer was 5'-AGCAGC AGCAGCACCTTCAAC-3', antisense was 5'-CCACCAGCA CCTTCTCGTTGTTC-3'; GAPDH sense primer is 5'-CAA CTTTGGTATCGTGGAAGG-3', antisense was 5'-GCCATC ACGCCAGAGTTTC-3'. The real-time fluorescence quantitative amplification reaction was performed according to the instructions of the TB Green® Premix Ex Taq (Tli RNaseH Plus) kit, PCR was conducted with the following conditions: 10 sec at 95°C; 40 cycles of 5 sec at 60°C and 10 sec at 72°C; 34 sec at 60°C, and the relative quantitative analysis was performed using the $2^{-\Delta\Delta Cq}$ method (33). The experiment was repeated three times.

Dual-luciferase reporter gene analysis between miR-let-7c-3p and Egr-1. Using the bioinformatics prediction website (http://www.targetscan.org) to predict the binding fragments of Egr-1 and miR-let-7c-3p, pmirGLO-Egr-1-wt wild plasmid vector and pmirGLO-Egr-1-mut plasmid vector were constructed (Jinan Boshng Biotechnology Co., Ltd.) respectively, and cells co-transfected by transfection reagent kit (jetPRIME; Polyplus-transfection SA) with the above Egr-1 wild plasmid, Egr-1 mutant plasmid and miR-let-7c-3p mimics

(sequence: CUGUACAACCUUCUAGCUUUCC) or mimics NC (sequence: UUCUCCGAACGUGUCACGUTT). The luciferase activity was measured by Dual-Luciferase Reporter Assay System (Envision; PerkinElmer, Inc.) 48 h following culture and *Renilla* luciferase was used as an internal control

siRNA interference. An appropriate amount of short inter-fering RNA (siRNA) and its corresponding negative control were mixed with the transfection reagent to form a transfec-tion complex, which was added to the 6-well plate that had been seeded with cells and cultured at 37˚C for 48 h for subsequent experiments. Egr-1 siRNA-1: 5'-CCAUGGACA ACUACCCUAATT-3', Egr-1 siRNA-2: 5'-GCCUAGUGA GCAUGACCAATT-3' and Egr-1 siRNA-3: 5'-GCUGUCACC AACUCCUUCATT-3' synthesized by Shanghai BioSune Co., Ltd. were selected. According to the preliminary experimental results, the Egr-1 siRNA-1 with the most obvious interference effect (5'-CCAUGGACAACUACCCUAATT-3') was selected as the target siRNA (hereinafter referred to as siEgr-1). As to the si-RNA interference experiment, there were four groups. In addition to the above 100 μg/l PMA experimental group and 0 μg/l PMA control group, the 100 μg/l PMA experi-mental group was transfected with si-ctrl at the same time as the PMA + si-Ctrl group. The 100 μg/l PMA experimental group was transfected with siEgr-1 at the same time as PMA + si-Egr-1 group.

Expression regulation of Egr-1 by miR-let-7c-3p mimic and inhibitor. miR-let-7c-3p mimic and inhibitor were synthesized by Shanghai GenePharma Co., Ltd. and their sequences were 5'-CUGUACAACCUUCUAGCUUUCC-3', 5'-GGAAAGCUAGAAGGUUGUACAG-3', corresponding NCs were: 5'-UUCUCCGAACGUGUCACGUTT-3' and 5'-GGAAAGCUAGAAGGUUGUACAG-3', respectively. The conventional transfection method of Lipofectamine® 2000 (Thermo Fisher Scientific, Inc.) was used. Following transfection, RT-qPCR and western blotting were used to detect the expression changes of miR-let-7c-3p and down-stream Egr-1 protein expression, respectively. iii) The effect of miR-let-7c-3p on the expression of differentiation antigens in K562 cells: In the above experiments of transfection of miR-let-7c-3p inhibitor, the expression changes of K562 cell differentiation antigens CD11b and CD14 were detected at the same time and let-7c-3p inhibitor NC was used as a control. The expression levels of differentiation antigens in the 100 μg/l PMA experimental group and the 0 μg/l PMA control group were also observed.

Statistical analysis. GraphPad Prism 8 software (GraphPad Software, Inc.) was used for data processing and Shapiro-Wilk (S-W) normal distribution was used for quantitative data. Each experiment was repeated three times and the measurement data were expressed as mean ± standard deviation. Comparisons between two groups were performed using independent samples t-test and ANOVA was used for multiple-group comparisons. The Bonferroni test was used as the post-hoc test for the one-way ANOVA test. Pearson analysis was used for the correlation between miR-let-7c-3p and Egr-1. All data were analyzed by two-tailed test. P<0.05 was considered to indicate a statistically significant difference.

Results

Changes in cell morphology and level of proliferation. The *in vitro* growth characteristics of the K562 cell line were directly observed under an inverted microscope. The results showed that, compared with the control group, after exposure to 100 ng/ml of PMA for 48 h, the K562 cells density was significantly reduced and the cells grew from a suspension state to an adherent state gradually (Fig. 1A and B). The CCK8 experiment confirmed that there was no significant difference between the PMA group and the control group before differentiation induction. After 48 h of differentiation induction, the proliferation ability of the PMA-induced differ-entiation group was significantly decreased compared with the control group (0.85±0.03 vs. 0.46±0.03; t=16.05; P<0.0001; Fig. 1C). Swiss-Giemsa staining showed that the cell volume after PMA-induced differentiation for 48 h increased signifi-cantly compared with the control group and the cytoplasmic volume increased, the nuclear-cytoplasmic ratio decreased and the nuclei became smaller. There was a trend towards monocyte-macrophage differentiation (Fig. 1D and E) and the number of more matured monocyte-macrophages increased (5.34±2.12 vs. 45.21±3.18; t=18.07; P<0.0001; Fig. 1F). The results indicated that the model of leukemia cell line differ-entiation into monocytes/macrophages was successfully established.

CD11b and CD14 differentiation antigens before and after exposure to PMA. To further validate the committed differ-entiation of leukemia cells into monocytes/macrophages, the present study examined the expression of monocyte/macro-phage-specific surface markers CD11b and CD14 in K562 cells treated with PMA for 48 h. The results of flow cytometry showed that the expression of CD11b in the PMA-induced group was significantly higher compared with that in the control group (49.47±3.48 vs. 3.54±0.54; t=24.070; P=0.002) and CD14 in the PMA-induced group was significantly higher compared with that in the control group (59.84±5.26 vs. 6.79±0.66; t=16.670; P=0.004; Fig. 2). The results showed that PMA could induce K562 cells to differentiate into mono-cytes/macrophages.

Expression of Egr-1 in K562 cells before and after exposure to PMA. The expression level of Egr-1 in normal human periph-eral blood mononuclear cells and K562 cells was detected by western blotting and the results showed that the expression of Egr-1 protein in K562 cells was significantly lower compared with that in normal controls (Fig. 3A and B). The gene expres-sion of Egr-1 gene was detected at the indicated different time points (Fig. 3C) and it was also found that PMA induced an increase in the expression of Egr-1 gene in K562 cells and the protein expression of Egr-1 was also significantly increased (Fig. 3D and E), which contributed to the induced differentia-tion from leukemia cells into monocytes/macrophages *in vitro*.

Effect of siRNA-Egr-1 on the differentiation of K562 cells induced by PMA. The expression changes of Egr-1 in K562 cells before and after PMA-induced differentiation were detected. The results confirmed that compared with the control group, the expression of Egr-1 was significantly increased after
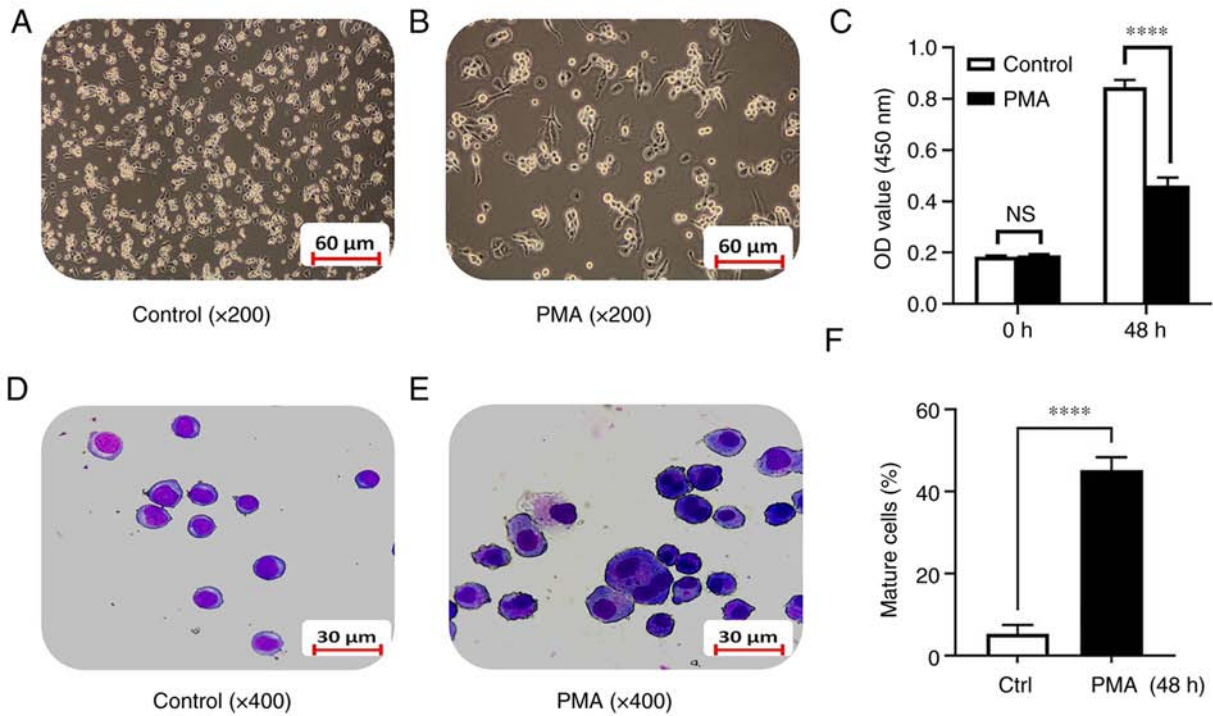
Figure 1. Changes in morphology and proliferation of K562 cells after 48 h of PMA treatment. (A) Cell growth density before induction (magnification, x200). (B) Cell growth density at 48 h after induction (magnification, x200). (C) CCK8 detection of cell proliferation prior to and following induction. (D) Swiss-Giemsa staining prior to induction (magnification, x400). (E) Swiss-Giemsa staining of cells following induction (magnification, x400). (F) Changes in percentage of mature cells prior to and following induction (****P<0.0001; NS, no significance). PMA, phorbol 12-myristate 13-acetate; Ctrl, control.
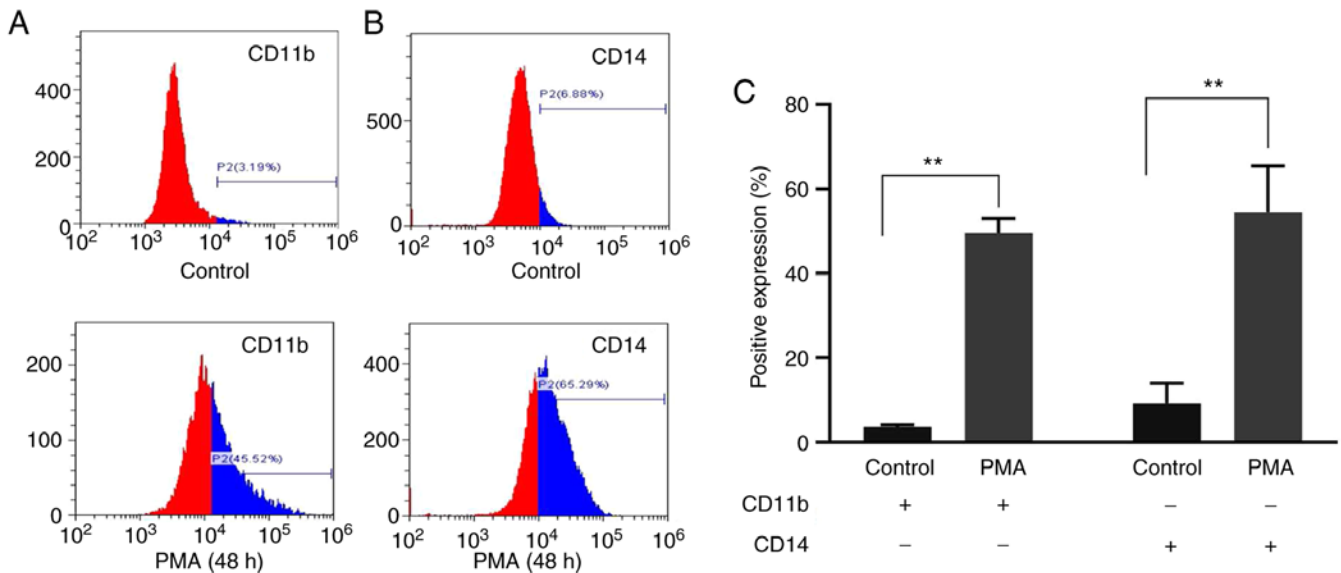
Figure 2. Effect of PMA on the expression of CD11b and CD14 on the surface molecule of K562 cells. (A) CD11b expression group prior to and following induction (B) CD14 expression group prior to and following induction. (C) Average expression levels of CD11b and CD14 (**P<0.01). PMA, phorbol 12-myristate 13-acetate.

PMA induction (0.19±0.02 vs. 0.85±0.03; t=24.800; P<0.001; Fig. 4A and B). It was found that this elevated expression of Egr-1 protein was accompanied by an elevated expression level of K562 cell differentiation antigen CD14 (4.30±1.01 vs. 36.67±4.31; t=12.66; P=0.0002; Fig. 4C and D). Compared with the PMA alone group, the Egr-1 protein expression in the PMA + siEgr-1 co-action group was decreased (0.22±0.03 vs. 0.48±0.03; t=11.380; P<0.001; Fig. 4E and F) and the expression

of the differentiation antigen CD14 was significantly decreased (7.03±1.45 vs. 24.40±4.70; t=6.113; P=0.004; Fig. 4G and H).

Target relationship between miR-let-7c-3p and Egr-1. In the preliminary experiments, TargetScan, PITA and microR-NAorg databases were used to predict target genes of possible upstream miRNAs of Egr-1 and intersected the predicted target miRNAs by crosstalk of the three databases. According
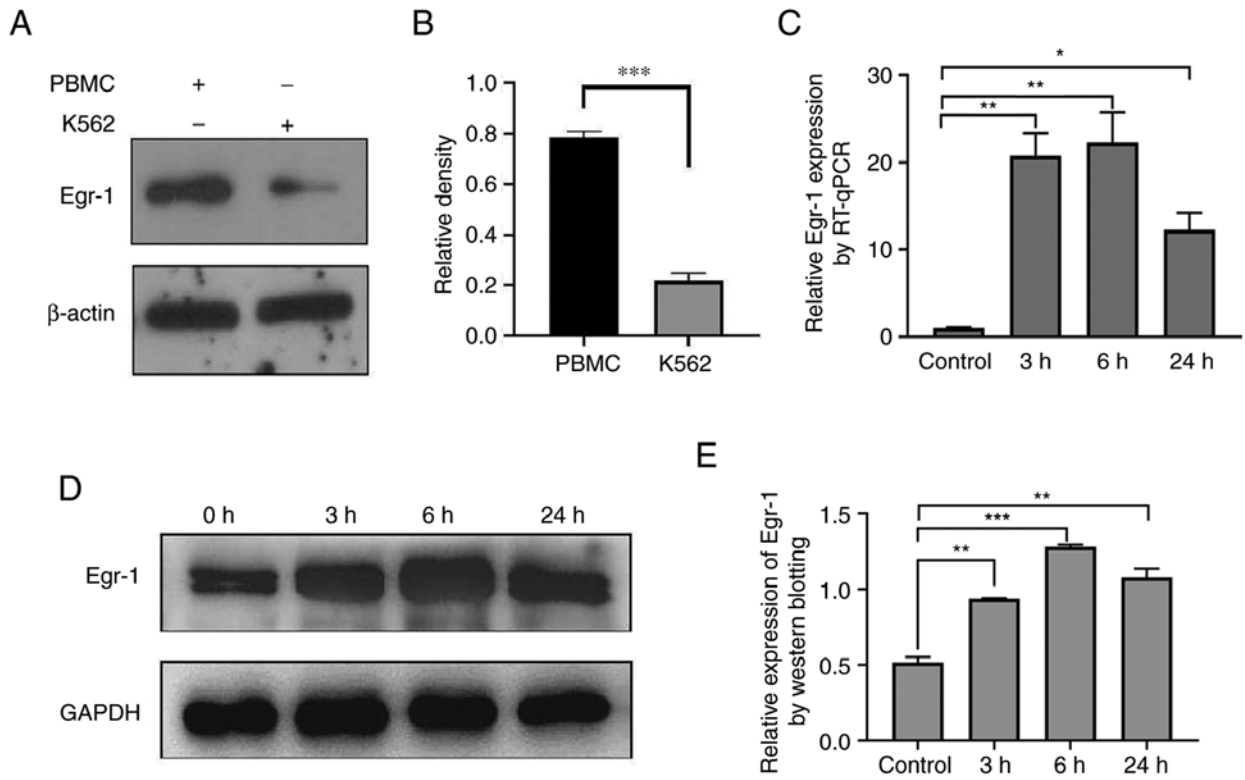
Figure 3. Differences in expression of Egr-1 between K562 cell control group and induced group. (A and B) Egr-1 expression levels in normal human PBMC and K562 cells. (C) Egr-1 mRNA relative expression statistics prior to and following induction. (D and E) Egr-1 expression prior to and following K562 cell differentiation variety (*$P<0.05$, **$P<0.01$, ***$P<0.001$). Egr-1, early growth response-1; PBMC, peripheral blood mononuclear cells.
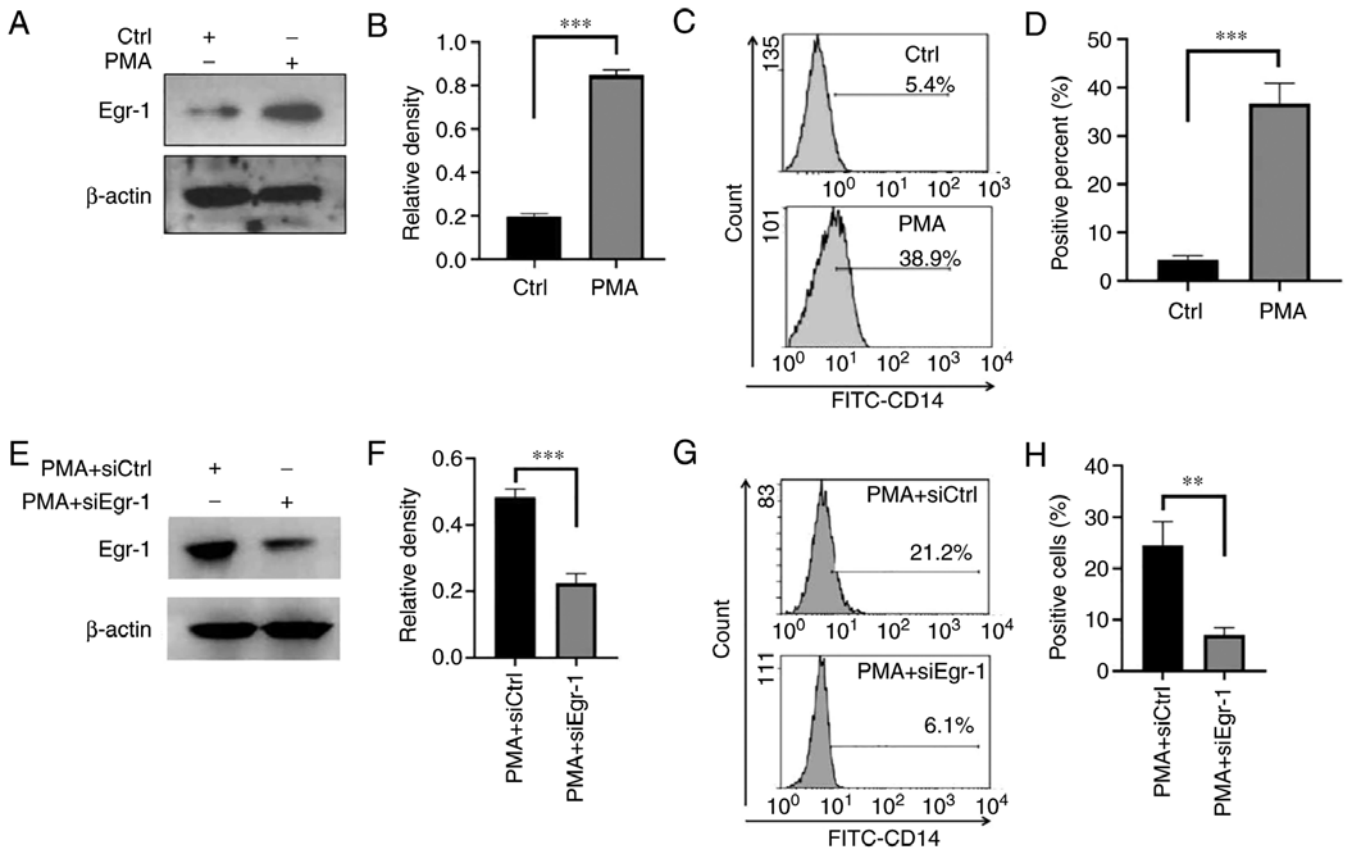

Figure 4. The effect of siRNA-Egr-1 on differentiation of K562 cells induced by PMA. (A and B) Changes of Egr-1 expression following PMA induction. (C and D) Changes of expression levels of differentiation antigen CD14 following PMA induction. (E and F) Egr-1 protein expression in PMA + siEgr-1 co-action group. (G and H) Changes in the expression of CD14 differentiating antigen in the PMA + siEgr-1 group (**$P<0.01$, ***$P<0.001$). si, short interfering; Egr-1, early growth response-1; PMA, phorbol 12-myristate 13-acetate.
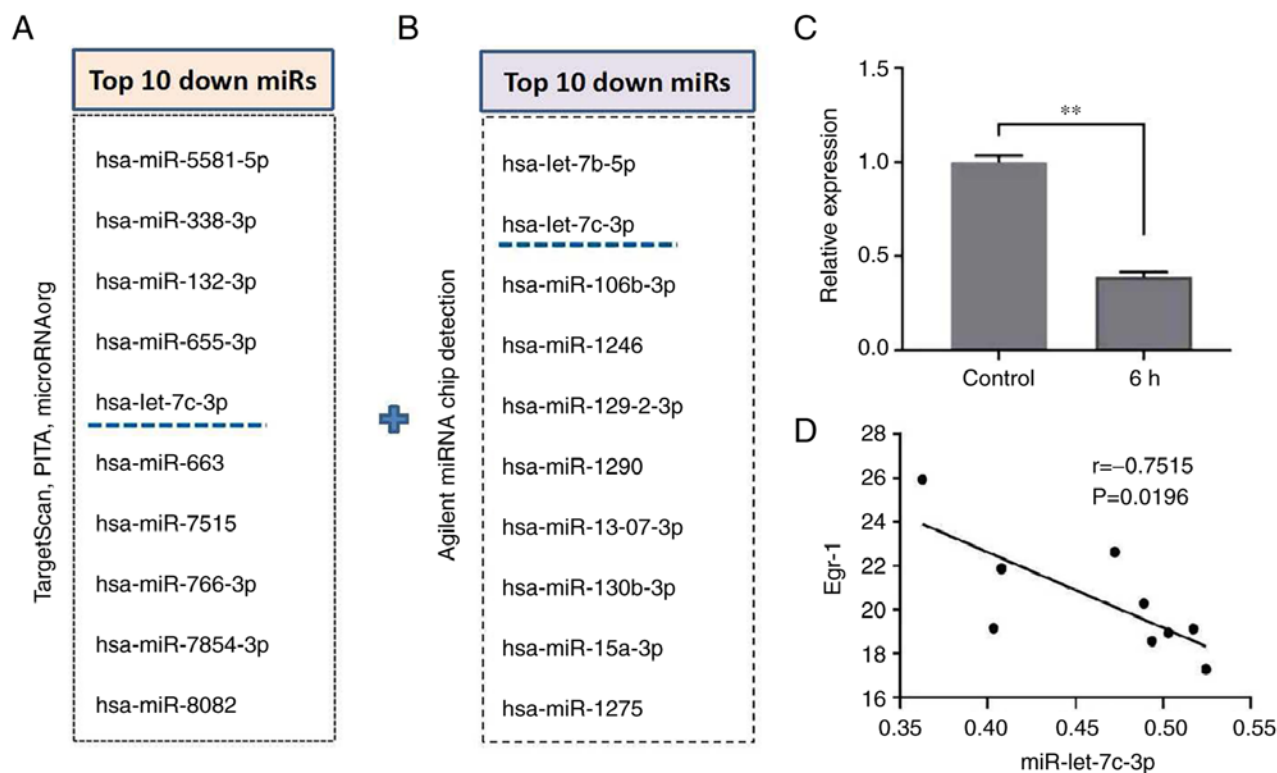
Figure 5. Relationship between miR-let-7c-3p and Egr-1. (A) TargetScan, PITA and microRNAorg databases predicted the top ten miRNAs. (B) Agilent miRNA Chip detected 10 reduced miRNAs. (C) Reverse transcription-quantitative PCR detected the expression level of miR-let-7c-3p following PMA induction. (D) Correlation of changes in miR-let-7c-3p and Egr-1 ($^{**}P<0.01$). miR, microRNA; Egr-1, early growth response-1; PMA, phorbol 12-myristate 13-acetate.

to the results of target gene prediction, the common target miRNAs in the three databases were obtained. After sorting according to the P-value and literature research, the top 10 miRNAs were selected (Fig. 5A). In addition, following Agilent miRNA Chip detection, 10 miRNAs that were reduced after induced-differentiation were screened according to the P-value (Fig. 5B). Then the cross-analysis of the database analysis and the actual detection results of the chip was performed and it was found that miR-let-7c-3p was the only candidate miRNA. RT-qPCR results showed that the expression level of miR-let-7c-3p in the PMA-induced group was significantly lower than that in the control group (1.00 ± 0.04 vs. 0.39 ± 0.03; $t=20.040$; $P=0.003$; Fig. 5C). This indicated that in the process of PMA-induced differentiation of K562 cells, the expression level of miR-let-7c-3p was decreased. The three different time points at which K562 cells were induced to differentiate were randomly selected and three replicate samples were observed. Following Pearson correlation analysis, it was found that the changes of miR-let-7c-3p and Egr-1 were negatively correlated (Fig. 5D).

The expression of Egr-1 by miR-let-7c-3p mimic and inhibitor. The regulatory effect of miR-let-7c-3p mimic and inhibitor on the expression of miR-let-7c-3p were first verified and the results confirmed that the expression level of miR-let-7c-3p in the miR-let-7c-3p inhibitor group was significantly lower compared with that in its negative control group (0.44 ± 0.42 vs. 0.96 ± 0.05; $t=12.870$; $P=0.006$). The expression level of miR-let-7c-3p in the miR-let-7c-3p mimic group was significantly higher compared with that in the negative

control group (418.80 ± 17.33 vs. 1.01 ± 0.02; $t=41.760$; $P<0.001$; Fig. 6A and B). On the regulation of Egr-1 expression by miR-let-7c-3p, the results of western blotting showed that the expression of Egr-1 was significantly increased following transfection of miR-let-7c-3p inhibitor as compared with the control (0.83 ± 0.12 vs. 0.39 ± 0.00; $t=6.315$; $P=0.024$; Fig. 6C and D), while the expression of Egr-1 was significantly decreased following transfection of miR-let-7c-3p mimic compared with the control group (0.18 ± 0.01 vs. 0.48 ± 0.06; $t=7.809$; $P=0.016$; Fig. 6E and F).

Targeted binding and regulation of Egr-1 by miR-let-7c-3p. Further analysis of the StarBase database revealed a binding site between miR-let-7c-3p and Egr-1, while the TargetScan database predicted a pairing site between miR-let-7c-3p and Egr-1 (Fig. 7A). The results of dual luciferase reporter gene showed that the luciferase activity in the co-transfected mimic and Egr-1 WT groups was significantly lower than that in the co-transfected mimic and Egr-1 MUT groups (0.77 ± 0.01 vs. 1.00 ± 0.01; $t=27.582$; $P<0.001$; Fig. 7B). The dual-luciferase reporter gene assay confirmed that there was a targeted binding activity regulatory relationship between miR-let-7c-3p and Egr-1, indicating that miR-let-7c-3p can target Egr-1.

The effect of miR-let-7c-3p on PMA-induced differentiation of K562 cells. To explore the effect of miR-let-7c-3p on PMA-induced differentiation of K562 cells, K562 cells were transfected with miR-let-7c-3p inhibitor and its negative control, treated with PMA (100 ng/ml) for 48 h to induce differentiation and the expression of cell surface markers
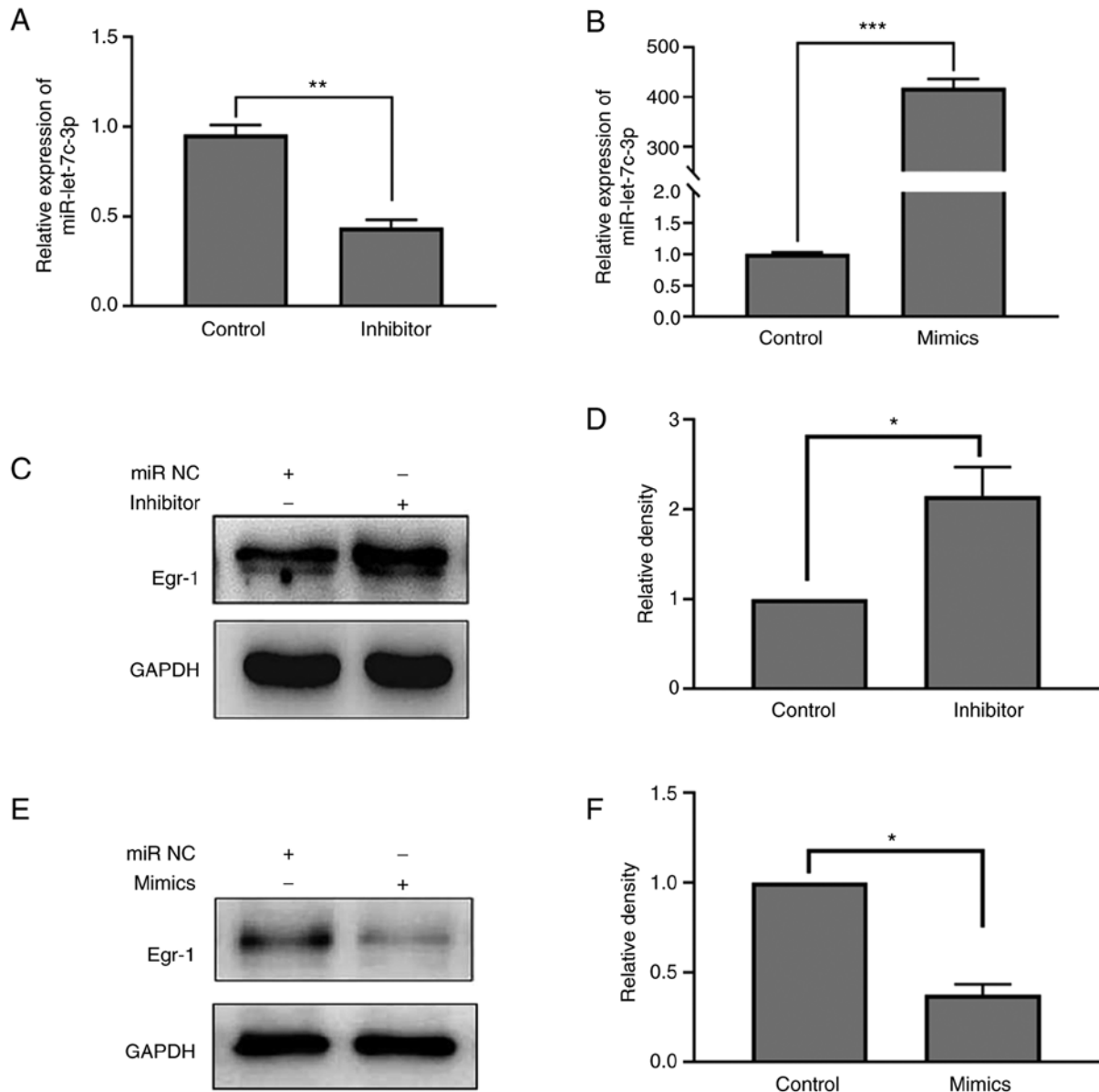
Figure 6. Effect of upregulation or downregulation of miR-let-7c-3p on the expression of Egr-1 in K562 cells. (A) Verification of the regulation of miR-let-7c-3p inhibitor on miR-let-7c-3p. (B) Verification of the regulation of miR-let-7c-3p mimic on miR-let-7c-3p. (C) The effect of miR-let-7c-3p inhibitor on Egr-1 protein expression. (D) The effect of miR-let-7c-3p inhibitor on the average level of Egr-1 protein expression. (E) The effect of miR-let-7c-3p mimic on Egr-1 protein expression. (F) The effect of miR-let-7c-3p mimic on the average level of Egr-1 protein expression ($^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$). miR, microRNA; Egr-1, early growth response-1; NC, negative control.
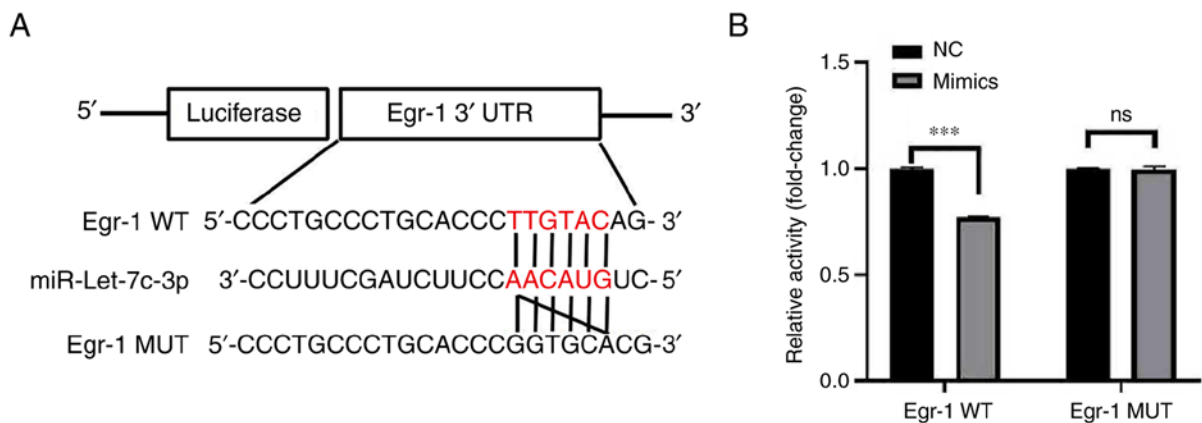

Figure 7. Expression of miR-let-7c-3p and targeting on Egr1. (A) 3' UTR binding region of miR-let-7c-3p and Egr-1. (B) Luciferase activity detection (ns, no significance; $^{***}P<0.001$). miR, microRNA; Egr-1, early growth response-1; WT, wild-type; MUT, mutant.
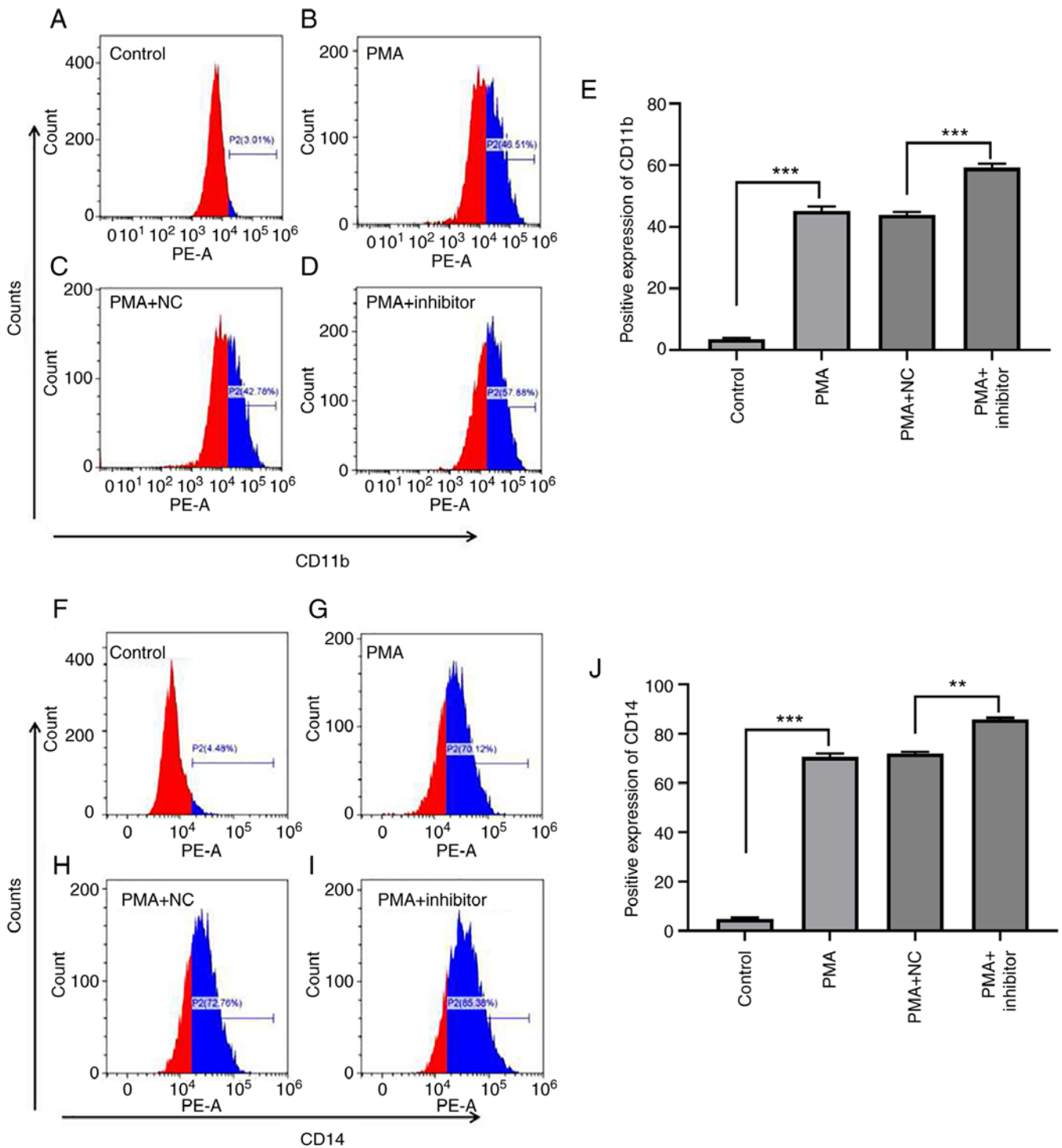
Figure 8. Effect of miR-let-7c-3p on PMA-induced differentiation of K562 cells. (A) CD11b expression in control group. (B) CD11b expression in K562 cells induced by PMA. (C) CD11b expression in PMA + NC group. (D) CD11b expression in PMA + miR-let-7c-3p inhibitor group. (E) Average level of CD11b in each group. (F) CD14 expression in control group. (G) CD14 expression in K562 cells induced by PMA. (H) CD14 expression in PMA + NC group. (I) CD14 expression in PMA + miR-let-7c-3p inhibitor group. (J) Average level of CD14 in each group (**P<0.01, ***P<0.001). miR, microRNA; PMA, phorbol 12-myristate 13-acetate; NC, negative control.

CD11b and CD14 in each group was detected by flow cytometry. The results, as shown in Fig. 8, demonstrated that PMA clearly induced the committed differentiation of K562 cells, which was manifested as increased CD11b expression; for example, the expression levels of CD11b in the control group, PMA group, PMA + NC group and PMA + inhibitor group were 3.44±0.43%, 45.14±1.40%, 43.91±1.00% and 59.22±1.28%, respectively (Fig. 8A-E). The difference in ANOVA analysis for CD11b between groups was statistically significant (F=1460.318, P<0.001), and multiple Bonferroni test analysis showed that, except for PMA and PMA+NC with P=0.238, the remaining P-values were all <0.001, which was in line with the expected results. The difference was statistically significant in ANOVA analysis for CD14 between groups

(F=5163.346, P<0.001), multiple Bonferroni test analysis found that except for PMA and PMA+NC were 0.544, the remaining P-values were all <0.001, which was in line with the expected results. Among them, the expression level of CD11b in the PMA group was significantly higher than that in the control group (t=39.570; P<0.001), the expression level of CD11b in the PMA + inhibitor group was significantly higher than that in the PMA + NC group (t=37.350; P<0.001; Fig. 8E), The expression level of CD11b in PMA + inhibitor group was significantly higher than that in PMA group (t=9.147; P=0.012).

PMA also significantly increased the expression of CD14 in K562 cells. The expression levels of CD14 in the control group, PMA group, PMA + NC group and PMA + inhibitor group were 4.91±0.42%, 70.54±1.44%, 71.91±0.74% and 85.98±0.52%, respectively (Fig. 8F-J), the difference was statistically significant following ANOVA analysis between groups (F=5163.346; P<0.001), multiple LSD analysis found that except for PMA and PMA + NC (P=0.91), the remaining P-values were all <0.001, which was in line with the expected results. The expression level of CD14 in the PMA group was significantly higher compared with the control group (t=94.720; P<0.001), the expression level of CD14 in the PMA + inhibitor group was significantly higher compared with the PMA + NC group (t=19.310; P=0.003) and the expression of CD14 in the PMA + inhibitor group was significantly higher compared with the PMA group (t=18.660; P=0.003; Fig. 8J). The results indicated that reduction of the expression of miR-let-7c-3p could promote the directed differentiation of PMA-induced leukemia.

Discussion

Myeloid leukemia is a type of hematopoietic stem cell malignant tumor with the characteristics of differentiation disorder and uncontrolled proliferation (1,2). At present, the treatment of leukemia is mainly based on the combination of chemotherapy and targeted therapy, but there are obvious side effects and a high recurrence rate. The differentiation induction therapy represented by all-trans retinoic acid is one of the best methods for the treatment of leukemia (9-11); in that, the leukemia cells are induced to differentiate into mature promyelocytes by all trans retinoic acid (ATRA) and their malignant proliferation is inhibited. It is precisely because of this selective effect on leukemia cells that it does not affect normal hematopoietic and immune functions, making it a research hotspot (12-15). However, elucidating the molecular mechanism of development and occurrence of patients with leukemia and how to find new differentiation-inducing drugs has become a challenge in the field of differentiation induction. At present, in addition to a number of studies on the induction of leukemia cells into myeloid cells (8,34-36), there are also some reports on the induction of leukemia cells into monocytes/macrophages. For example, Chou and Hsu (37) show that PMA can induce the differentiation of chronic myeloid leukemia cell line K562 into monocyte-macrophages. On this basis, the present study first used multiple methods to verify that PMA induced K562 to differentiate into monocytes/macrophages; it was found that the cells gradually changed from the suspension growth to the adherent state, the cell volume increased, the antennae were prominent, the cytoplasmic folds increased,

the nucleocytoplasmic ratio was significantly reduced and multinucleation appeared, showing the development trend of mature monocytes/macrophages following PMA induction. At the same time, the monocyte/macrophage surface molecules CD11b and CD14 were significantly increased (38). CCK8 experiments also showed that PMA could effectively inhibit the proliferation of K562 cells. All of the above results indicated that PMA possessed a strong ability to induce K562 to differentiate into monocytes/macrophages. Therefore, this differentiation induction model provides a model for studying the molecular mechanism of leukemia cells to differentiate into monocytes/macrophages.

The Egr-1 gene, as a member of the zinc finger structure transcription factor family, is located on human chromosome 5q31 and encodes a DNA-binding transcription factor of ~80 kDa (39). It has been demonstrated that Egr-1 serves an important role in cell growth, differentiation and apoptosis (16,40-44). At the same time, Egr-1 is closely associated with the occurrence and development of certain diseases, for example tutor and leukemia, however the specific signaling pathway of each of them remains to be elucidated. It is also found so far that Egr-1 is an important multifunctional transcriptional regulator, and various factors can affect the expression of Egr-1. Usually, in unstimulated cells, Egr-1 expression was difficult to detect or only detectable at very low levels. However, Egr-1 can be upregulated in a rapid and transient manner under the activation by different extracellular stimuli (45-48), such as some cytokines or differentiation inducers (39,49,50). Egr-1 includes transactivation and repression regions, as well as three DNA-binding zinc finger regions that recognize GC-rich fragments of the promoter regions of target genes (51). Egr-1 has a wide range of functions, involving the control of synaptic plasticity, wound repair, inflammation, blood coagulation, pulmonary vascular permeability, growth and apoptosis of a number of cells (50,52-56). Some findings suggest that Egr-1 can reverse the disease progression of acute myeloid leukemia by regulating changes in the downstream target genes c-myc and E2F1 (57). In chronic myeloid leukemia, a decrease in Egr-1 leads to an increase in the number of leukemia stem cells in the blood, accelerating disease progression, whereas Egr-1 serves an important role in normal hematopoietic stem cell differentiation, quiescence and terminal differentiation of monocyte/macrophage cells (58). As early as the last century, some researchers identified Egr-1 as an important differentiation response gene during monocyte/macrophage development (59), it is greatly upregulated in HL-60 and U-937 leukemia cells following exposure to PMA. In the present study, the expression of Egr-1 in K562 cells also showed a trend of upregulation, which was induced by PMA. Combined with the Egr-1 interference experiment, the PMA-induced upregulation of CD14 expression was reduced by the si-Egr-1 group, as compared with that of PMA + si-ctrl group, indicating that Egr-1 was involved in the differentiation of leukemia K562 cells into monocyte/macrophage.

It has been demonstrated that some important transcriptional regulatory proteins are often regulated by epigenetic factors, such as non-coding RNAs, at the same time that some important transcriptional regulatory proteins are involved in transcriptional regulation. Usually, one important pathway for microRNAs (miRNAs) to result in gene silencing or translational

repression was mainly contributed to binding to 3'UTR of target mRNAs (60-62). The regulatory factors of miRNA not only participate in the occurrence and development of various human tumors (63), but also show great potential in disease diagnosis, prognosis judgment and targeted therapy (64). Among them, it has been found that miRNAs are involved in differentiation, proliferation, apoptosis and other processes of leukemia cells, and its relatively specific mutational and deregulated expression profiles also have potential as diagnostic or prognostic biomarkers (65). In addition, non-coding RNAs also serve an important role in the chemoresistance of tumors or leukemias. Different miRNA species serve different roles in the formation or reversal of drug resistance. It can be used both as a biomarker of drug resistance and as one of the targets for drug resistance intervention (66,67). For example, miRNA expression profiling of drug-resistant melanoma patients and their cell lines reveals that miRNA-181a and miRNA-181b are significantly downregulated in drug-resistant melanoma patients and drug-resistant cell lines. Reconstruction of miR-181a/b expression reversed the resistance of melanoma cells to the BRAF inhibitor dabrafenib. Clinical observations show that melanoma patients with high expression of miRNA-181a and miRNA-181b have longer progression-free survival time (68).

miR-let-7, as the earliest discovered human miRNA, is one of the most widely studied miRNAs. Its family members are abnormally expressed in various malignant tumors and become a new target for tumor prevention and treatment (32,69). For example, in cervical cancer, nanocarriers can target miR-let-7c-5p to inhibit tumor cells and exhibit reduced cytotoxicity (70). The miR-let-7 family has been shown to be downregulated in various types of tumor tissues and has been extensively studied as a tumor suppressor gene (30,31,71). Accumulating evidence suggests that Let-7 also has the same properties as other miRNAs and is not only involved in the occurrence and development of leukemia, but also a potential biomarker for leukemia diagnosis and prognosis (32). However, it has not been elucidated whether miRNA Let-7 is involved in leukemia cell-directed monocyte-macrophage maturation and differentiation.

In the preliminary experiments of the present study, it was found that some miRNAs changed significantly during the process of differentiation of leukemia cells into mature monocytes/macrophages induced by PMA. Further analysis of the StarBase database revealed a binding site between miR-let-7c-3p and Egr-1, while the TargetScan database predicted a pairing site between miR-let-7c-3p and Egr-1. In addition, following Agilent miRNA Chip assay, miR-let-7c-3p also reduced after induced-differentiation by PMA with the upregulation of Egr-1 mRNA. Furthermore, it was found that the expressions of miR-let-7c-3p and Egr-1 showed an inverse relationship by Pearson analysis in the differentiation process induced by PMA and that they had a nucleic acid sequence basis for targeted binding. In addition, Egr-1 is widely associated with miRNAs and can be regulated by miRNAs in a variety of tumors (72,73). Therefore, it was hypothesized that the miR-let-7c-3p signal axis may serve a role in the committed differentiation of leukemia cells into monocytes/macrophages. First, to detect the possible effect of miR-let-7c-3p on binding 3'UTR and modulating activity of Egr-1 transcription, luciferase assay was performed and the results indicated that the

miR-let-7c-3p could bind 3'UTR of Egr-1 and modulate its activity. The luciferase activity of the co-transfected mimic and Egr-1 WT group was significantly lower than that of the co-transfected mimic and Egr-1 MUT group. Furthermore, the miR's endogenous expression in K562 cells was also demonstrated by miR-let-7c-3p mimics transfection; the expression level of Egr-1 in miR-let-7c-3p mimic group was significantly lower than that in the negative control group. The expression of miR-let-7c-3p in K562 cells before and after treatment with PMA was detected by RT-qPCR assay and the results showed that the expression level of miR-let-7c-3p in the induction group was significantly lower than that in the control group, as in agreement with the effect of miR-let-7c-3p inhibitor and the expression level of Egr-1 in the miR-let-7c-3p inhibitor group was significantly lower compared with the negative control group.

Some other studies (39,74,75) report that the role of Egr-1 can be modulated by some miRNAs, including miR-424, miR-146a, miR181a and miR-337. In the present study, the expression of Egr-1 is regulated in K562 cells transfected with miR-let-7c-3p mimics and inhibitor. Western blotting showed that compared with the control group, the expression of Egr-1 in the miR-let-7c-3p mimics transfected group was decreased and the expression of Egr-1 in the miR-let-7c-3p inhibitor transfected group was increased. Thus, miR-let-7c-3p can be targeted to bind to Egr-1 and has a negative regulatory relationship. In brief, the expression of Egr-1 and miR-let-7c-3p was upregulated or downregulated after exposure to PMA *in vitro* and miR-let-7c-3p could directly bind to the 3'UTR of Egr-1 and modulated its promoter activity. The miR-let-7c-3p/Egr-1 signaling axis contributed to the differentiation from K562 leukemia cells into more matured monocytes/macrophage cells.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

GSJ, XDW, FQ, XPW and CZW made substantial contributions to the conception and design and also critically reviewed the study. FQ, XPW, CZW, RJS and HW performed

the experiments. FQ, SZZ, PCD and JW analyzed the data and wrote the manuscript. GSJ and XDW confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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