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 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. 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.
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'-GGCG(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.

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 Biotecnology Co., Ltd. RPMI 1640 medium was purchased from HyClone (Cytiva). Swiss-Giemsia 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 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 Biotecnology Co., Ltd. The cycle kit was purchased from Jiangsu KGI Biotecnology 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% CO2. 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 1x10^3/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% CO2 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 IC50 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 4 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 FACSVersa (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 (1:2,000) 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 crossstalk 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'-GGCGCGCTGATACACCTCCTTTAG-3', the antisense primer was 5'-AGTCGACGGTTCCAGGTAAT-3'; the U6 sense primer was 5'-AGAGAAGATAGTCATGGCCTTG-3', antisense is 5'-AGATGCAGGGTCCGAGGTAAT-3'; Egr-1 sense primer was 5'-AGCACGCAGGCACACCTTCACAC-3', antisense is 5'-CCACCAGCACCCTTCTCTGTTTCCT-3'; GAPDH sense primer is 5'-CAAAGTTGGATACGTGAAGG-3', antisense is 5'-GCCATAGCCCAAGATTTCCCGTAAC-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 2ΔΔCq 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.
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'-CUGUACACCUUAGCUUCC-3', 5'-GGAAAGCUAGGAAGGGUACAG-3', corresponding NCs were: 5'-UUCUCAGCGUUCACGUTT-3' and 5'-GGAAAGCUAGGAAGGGUACAG-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 downstream 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/ml PMA experimental group and the 0 µg/ml 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 differentiation 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 significantly 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 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 differentiation into monocytes/macrophages was successfully established.

Expression of Egr-1 in K562 cells before and after exposure to PMA. To further validate the committed differentiation of leukemia cells into monocytes/macrophages, the present study examined the expression of monocyte/macrophage-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 monocytes/macrophages.

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
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 microRNA.org 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
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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.
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 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.
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.
However, elucidating the molecular mechanism of development and immune functions, making it a research hotspot (12-15). It is precisely because of this selective effect on trans retinoic acid (ATRA) and their malignant proliferation are induced to differentiate into mature promyelocytes by all the induction of leukemia cells into monocytes/macrophages. At present, in addition new differentiation-inducing drugs has become a challenge in the field of differentiation induction. At present, in addition new differentiation-inducing drugs has become a challenge in the field of differentiation induction. At present, in addition new differentiation-inducing drugs has become a challenge in the field of differentiation induction. At present, in addition new differentiation-inducing drugs has become a challenge in the field of differentiation induction. At present, in addition new differentiation-inducing drugs has become a challenge in the field of differentiation induction. 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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 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 Egr-1 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 immature monocytes/macrophage cells.

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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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