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MicroRNA-196b promotes cell proliferation and suppress cell differentiation *in vitro*

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ABSTRACT

MicroRNA-196b (miR-196b) is frequently amplified and aberrantly overexpressed in acute leukemias. To investigate the role of miR-196b in acute leukemias, it has been observed that forced expression of this miRNA increases proliferation and inhibits apoptosis in human cell lines. More importantly, we show that this miRNA can significantly increase the colony-forming capacity of mouse normal bone marrow progenitor cells alone, as well as partially blocking the cells from differentiation. Taken together, our studies suggest that miRNA-196b may play an essential role in the development of *MLL*-associated leukemias through inhibiting cell differentiation and apoptosis, while promoting cell proliferation.

1. Introduction

MicroRNAs (miRNAs, miRs) are endogenous ~22 nucleotides (nt) non-coding RNAs that play important regulatory roles in animals and plants by binding the 3′UTRs of messenger RNAs (mRNAs) of target genes, leading to mRNA cleavage/degradation or translational repression [1–4]. Rapidly accumulating evidence has revealed that miRNAs are strongly associated with cancer [3,5–7]. Mir-196b gene is located in a highly evolutionarily conserved region between *HOXA9* and *HOXA10* genes, at chromosome band 7p15.2 in human and 6qB3 in mouse. Recent studies show that it may function as an oncogene in haematopoiesis [8–11]. Chen's group reported previously that miR-196b was aberrantly overexpressed in *MLL*-rearranged AML, and its knockdown by antagomir abrogated *MLL*-fusion-mediated cell immortalization [12]. However, the involvement of this miRNA in the development of acute leukemia remains unclear.

In this report, we further analyzed the function of MicroRNA-196b (miR-196b) in cell proliferation, apoptosis, and its effects on differentiation. Our results suggested that miRNA-196b regulates leukemias through inhibiting cell differentiation and apoptosis, while promoting cell proliferation.

2. Materials and methods

2.1. Cell culture and transfections

HEK293T cells and Hela cells were grown in Dulbecco modified Eagle's medium (DMEM) supplemented with 10% FBS and 1% penicillin–streptomycin (Invitrogen). For transfection, HEK293T cells and Hela cells were plated in 96-well plates at a concentration of 10,000 cells per well or 5000 cells per well, respectively, and transfected with Effectene Transfection Reagent (Qiagen). THP-1 cells were grown in ATCC-formulated RPMI medium 1640 (ATCC) containing 1% penicillin–streptomycin, 0.05 mM 2-mercaptoethanol, and 10% FBS (Invitrogen). THP-1 were transfected with Cell Line Nucleofector kit V following program V01, using a nucleofector device (Amaxa Biosystems).

2.2. Cloning mir-196b expression vector

A 200-bp DNA fragment surrounding mir-196b was amplified using the primers: forward 5′_GAAGATCTTTCCTTGGCGGCGACA 3′ and reverse 5′CCCAAGCTTGATGGCCCGCCTA 3′. Gel-purified PCR product and vector were both digested with *Xho*I and *Eco*RI and ligated into pMSCVPIG or pMSCVneo retroviral vector (Addgene or Clontech).

2.3. Retrovirus production and colony forming assay

In vitro colony-forming (i.e., immortalization) assays were performed as described previously [8] with some modifications [9].

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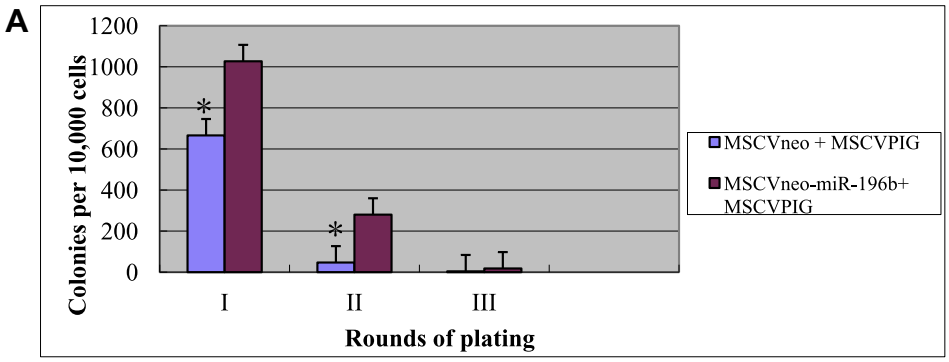

*, $P < 0.05$ (paired t test)

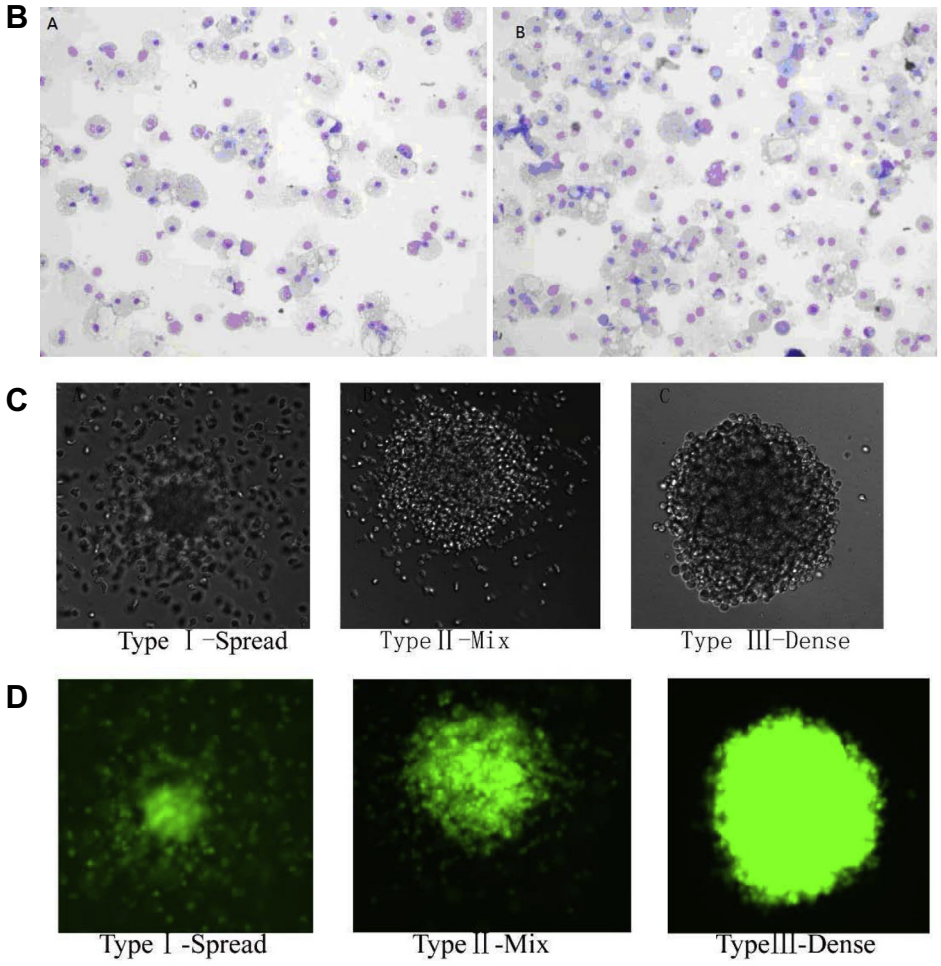

Fig. 1. *In vitro* colony-forming/replating assays. (A) Numbers of colonies per dish ($\geqslant$50 cells/colony; 1×10^4 input cells) of replating are shown (mean ± SD). (B) Morphology of cells of secondary or tertiary colonies (i.e., colonies at the second or third round of plating). Cells of secondary colonies with empty vector (Plot A); cells of secondary colonies with miR-196b (Plot B); cells were stained with Wright–Giemsa. (C) Morphological types of colonies. According to Lavau et al. [13], there were three morphological types of secondary or tertiary colonies generated by MSCV-miR-196b-infected hematopoietic cells. Similarly, we have also found three types of such tertiary colonies: (I) diffused colonies of migrating cells; (II) colonies with a dense center surrounded by a halo of migrating cells; and (III) extremely compact and resembled colonies. Lavau et al. [13] found that colonies of types III and II consisted of immature cells whereas the colonies of types I also included more differentiated cells. (D) Morphological types of colonies under fluorescence microscope. It showed that infected rate by MSCV-miR-196b was very high.

Briefly, retrovirus for each construct was produced in 293T cells by co-transfecting the retroviral construct and pCL-Eco packaging vector (IMGENEX, San Diego, CA). Rat1a cells were used to determine the viral titer. Then, hematopoietic progenitor cells obtained from a cohort of 4- to 6-week-old C57BL/6 mice 5 days after 5-FU treatment were transduced with the retroviruses of MSCVpuro or MSCVneo empty vector (both as negative controls; their colony-forming capacities are very similar), MSCVpuro-miR-196b, through "spinoculation" [8]. An aliquot of 1×10^4 of the transfected cells was plated into 35 mm Nunc Petri dishes in 1.1 ml of Methocult M3230 methylcellulose medium (Stem Cell Technologies Inc., Vancouver, BC, Canada) in duplicate containing 10 ng/ml each of murine recombinant IL-3, IL-6, and GM-CSF (R&D Systems, Minneapolis, MN) and 100 ng/ml of murine recombinant stem cell

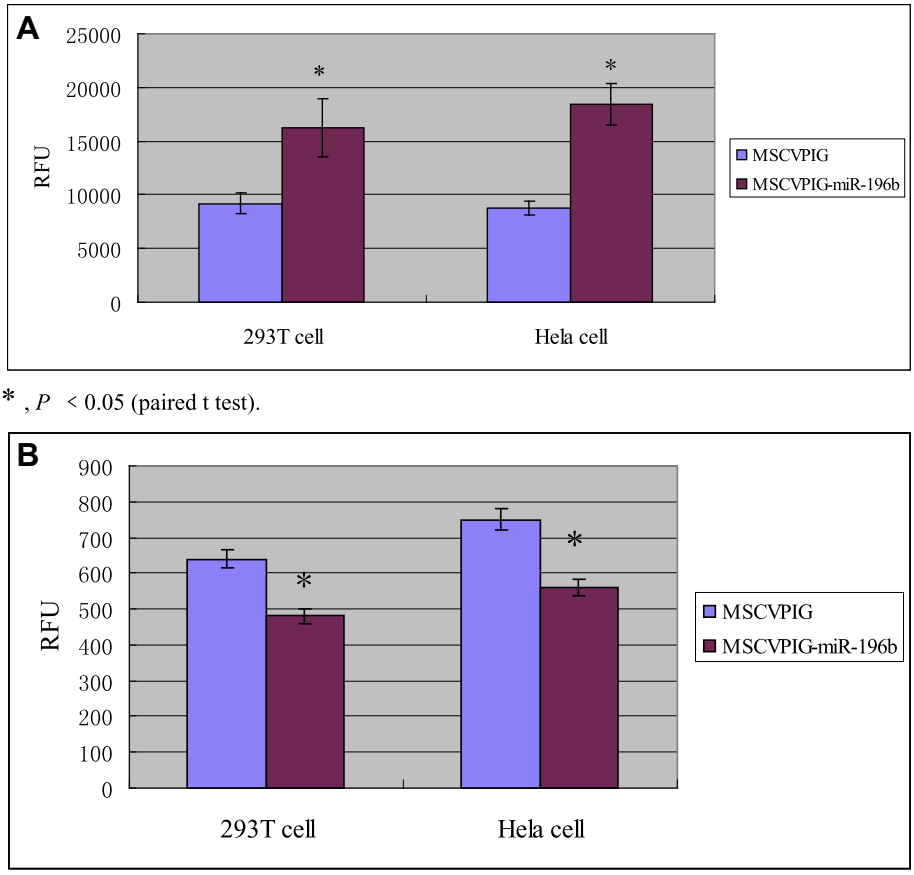

Fig. 2. The functional role of miR-196b. (A) Forced expression of miR-196b by transduced $MSCV_{PIG}$-miR-196b plasmid significantly increased proliferation in 293T and Hela cells. (B) Forced expression of miR-126 significantly decreased cell viability in 293T and Hela cells. Normalized mean values of three independent experiments and standard error (mean ± SE) are shown. $^{*}P < 0.05$ (paired *t* test). Relative Fluorescence Units (RFU) was measured.

factor (Sandoz, Holzkirchen, Germany), along with 1.0 mg/ml of G418 (Gibco BRL, Gaithersburg, MD) and/or 2.5 μg/ml of puromycin (Sigma, St. Louis, MO) accordingly. Cultures were incubated at 37 °C in a humidified atmosphere of 5% CO_2 in air. The colonies were replated every 7 days under the same conditions.

2.4. Cytospin

For cytospin preparation, 40,000 cells were washed twice and were diluted in 200 μl of cold PBS. Each sample was loaded into the appropriate wells of the cytospin, then spun at maximum speed for 1 min. The slides were dried in a desiccation chamber for 20 min and were stained with Wright–Giemsa.

2.5. Cell apoptosis and viability assays

Hela and 293T cells were plated at a concentration of 2500 or 5000 cells per well in triplicate in a 96-well plate. MSCVpuro-miR-196b or the control plasmid MSCVpuro was transfected into the cells with Effectene® Transfection Reagent (QIAGEN, Valencia, CA). Forty-eight hours after transfection, cell apoptosis was assessed through analyzing caspase-3 and caspase-7 activation using ApoONE Homogenous Caspase 3/7 Assay (Promega, Madison, WI); cell viability was assessed through analyzing metabolic activity of the cells using CellTiter-Blue Reagent (Promega, Madison, WI) following the manufacturer's manuals.

2.6. THP1 differentiation and miRNA-196b detection

Human THP-1 leukemia cells were differentiated along the monocytic lineage following the exposure to phorbol-12-myristate-13-acetate (PMA) as described previously [10] with minor changes. Briefly, to differentiate THP1 cells, PMA (Sigma) was added to a final concentration of 100 nM and cultured for 6 h, 12 h, 24 h, 48 h, 72 h and 96 h. Adherence was observed under ordinary light microscope, Wright–Giemsa staining was performed to observe the morphological changes of THP1 cells and expressions of CD 11b and CD14 were analyzed by flow cytometry. Based on cell adherence, morphology and expression of CD11b and CD14, the THP1 differentiation at different time point was analyzed.

Expression of miR-196b was detected by qRT-PCR. The TaqMan qPCR method [11] was used to validate the differential expression patterns of miRNAs by using kits from Applied Biosystems. U6 RNA was used as endogenous controls for qPCR of miRNA. PCRs were performed in an Applied Biosystems 7900HT system according to the manufacturer's recommendation, and each sample was analyzed in triplicate. Fold difference calculated using the $2^{-\triangle\triangle Ct}$ method. Primer sequences are available on request.

2.7. Transduction of mir-196b into THP1 cells and observation of differentiation phenotype change

Transduction of mir-196b into THP1 cells were performed using amaxa cell line Nucleofector® Kit V for THP1 cells (Lonza Cologne GmbH) according to the manufacturer's protocol. After adding

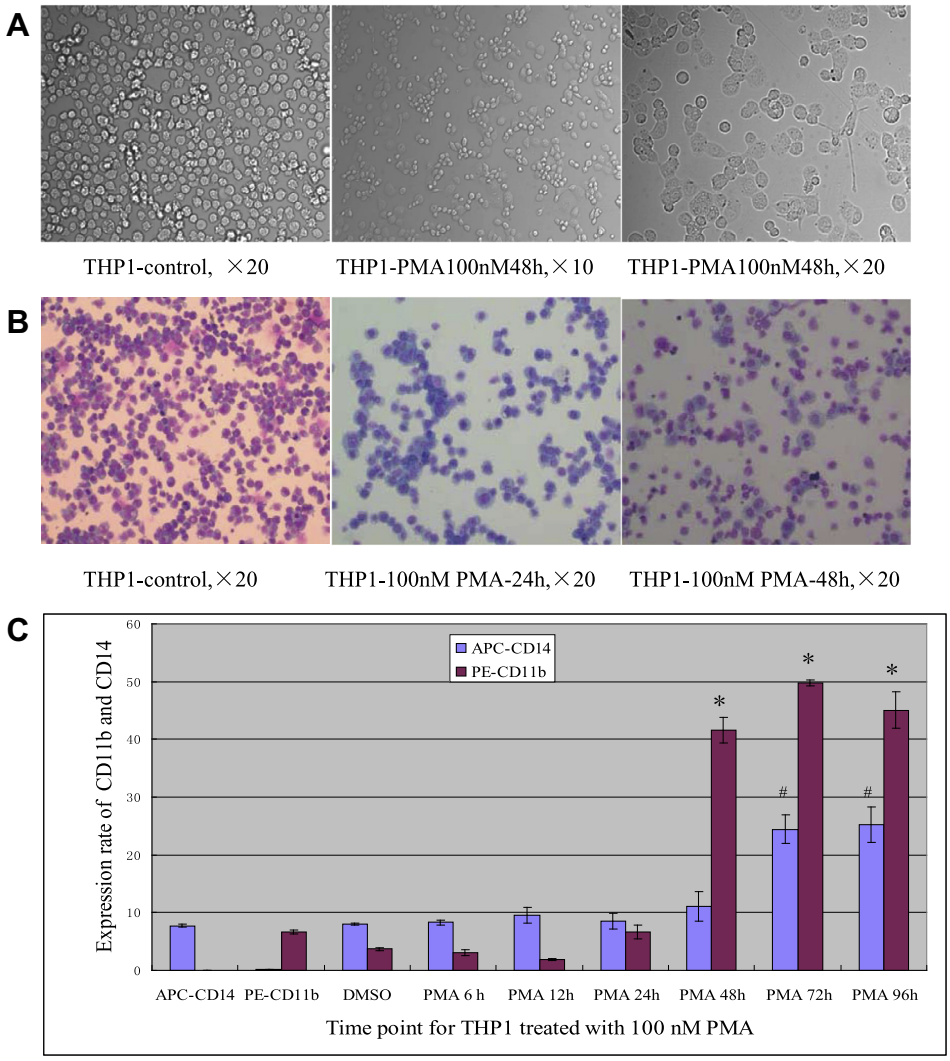

Fig. 3. Establishment of THP1 differentiation induced by 100 nM PMA cell model. (A) Morphological change of THP1 cell induced by 100 nM PMA observed under light microscopy. (B) Morphological change of THP1 cell induced by 100 nM PMA. (C) Expression rate change of CD11b, CD14 on THP1 cell induced by 100 nM PMA by FACS. $^{*,\#}$, *vs* THP1 (no PMA) or THP1 (+DMSO) group, $P < 0.05$.

100 nM PMA to induce differentiation of THP1 cells, the cells were monitored by changes in morphology, adherence, the expression of surface markers (CD11b, CD14) [12].

2.8. Statistical analysis

The results were presented as mean ± SD. Analyses were conducted with SPSS 18.0 software, using the unpaired Student's *t*-test for comparisons between two groups or one-way ANOVA for multiple comparisons. A *P* value of less than 0.05 was considered statistically significant.

3. Results

3.1. miR-196b enhances the colony forming capacity of mouse normal bone marrow progenitor cells and partially blocks differentiation

To evaluate the role of the miR-196b in leukemogenesis, we conducted colony-forming/replating assays. Mouse bone marrow progenitor cells were retrovirally transduced with (i) MSCVpuro or MSCVneo empty vector (i.e., control), (ii) MSCVpuro-miR-196b, respectively (see Section 2). As shown in Fig. 1A, forced expression of miR-196b alone could result in a significant increase in the number (>100) of colonies. The forced expression of miR-196b was confirmed by qPCR (data not shown). As shown in Fig. 1B, cells of the secondary colonies with empty vector (i.e., control; plot A) were mostly differentiated; cells of the secondary and tertiary colonies with miR-196b (plot B) were partially differentiated with some granulocytes. Similarly, in terms of morphology, the majority (~70%) of miR-196b secondary colonies were diffused colonies of migrating cells (i.e., Type I), and the minority (30%) of that were compact and dense colonies (i.e., Types III and II), while the secondary colonies of control (~95%) were diffuse colonies of migrating cells, compact and dense colonies were very low [13] (see Fig. 1C). The tertiary colonies of both groups were all diffused colonies of migrating cells. Taken together, our data indicate that miR-196b can enhance cell proliferation/colony forming capacity, and partially block cell differentiation. However, different from MLL fusion or other oncogenes, miR-196b does not be able to establish immortalized cells.

3.2. miR-196b inhibits apoptosis and increases viability of human cells lines

To examine the functional role of the miR-196b in human cells, gain- and loss- of function experiments were performed. We observed that forced expression of the miR-196b led to significantly decreased apoptosis (Fig. 2A) and increased viability

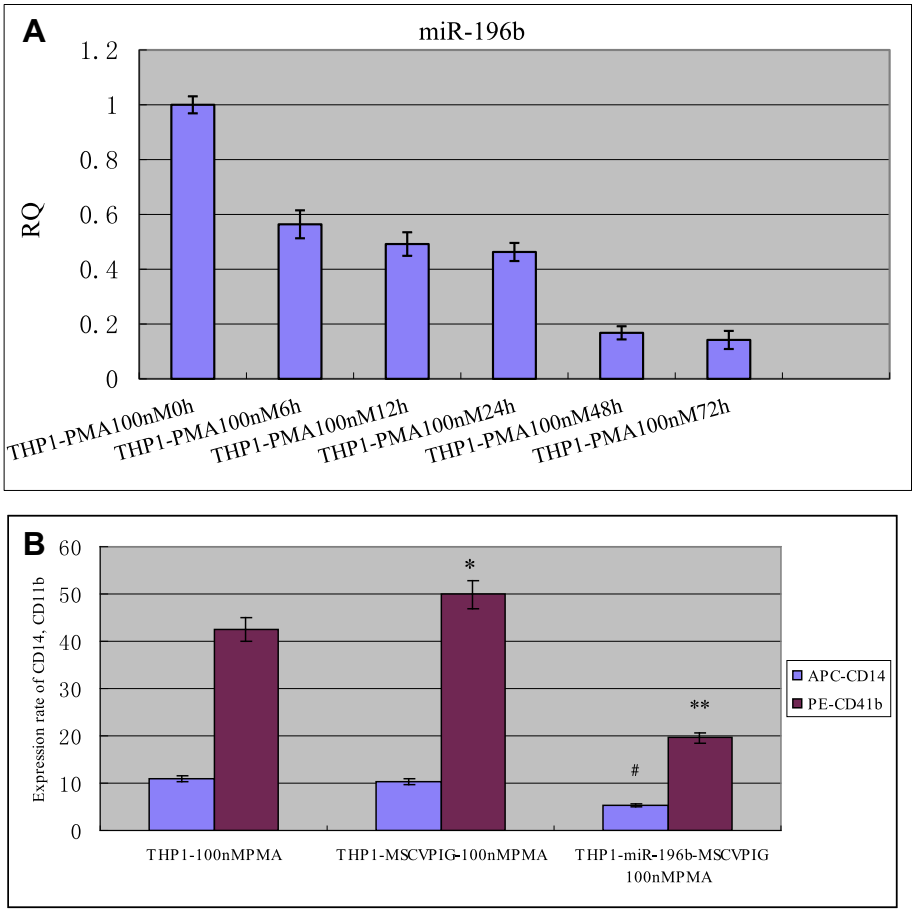

Fig. 4. Expression of miR-196b is inversely correlated with differentiation of THP1 cells. (A) Relative expression of mir-196b in 100 nM PMA-treated THP1 cell at different time point. (B) Expression rate of CD14 and CD11b on THP1 cell after forced expression of mir-196b for 48 h. *, *vs* THP1-100 nM PMA group, $P > 0.05$; #, *vs* THP1-100 nM PMA or THP1-MSCVPIG-100 nM PMA group, $P < 0.05$; **, *vs* THP1-100 nM PMA or THP1-MSCVPIG-100 nM PMA group, $P < 0.05$.

(Fig. 2B) in Hela (a human cervical cancer cell line) and 293T cells (a variant of human embryonic kidney 293 cell line). Forced expression of the miRNA after transfection was confirmed by qPCR (data not shown).

3.3. Expression of miR-196b is inversely correlated with differentiation of THP1 cells

To examine the relationship between expression of miR-196b and cell differentiation, THP1 cell differentiation was trigged using phorbol 12-myristate 13-acetate (PMA). As shown in Fig. 3A, under microscope, treatment with 100 nM phorbol 12-myristate 13-acetate (PMA) stimulated the differentiation of THP1 cells and enabled the cells to attach to the bottom of the culture dishes. Cell protrusions were also observed and some cells were fusiform. Wright–Giemsa staining revealed that the cells became smaller, nuclear-cytoplasmic ratio decreased, and nuclear became even smaller, concave and warped, most of which showed a rod-shaped or lobulated nucleus (Fig. 3B). The expression of specific molecular markers, such as CD11b or CD14, is indicative of the maturation of monocytes into macrophages. THP1 cells treated with 100 nM PMA revealed that average expression rates of CD11b and CD14 at different time point (6 h, 12 h, 24 h, 48 h, 72 h, 96 h) increased with the time ($F = 144.045$, $P = 0.000$; $F = 10.084$, $P = 0.003$, respectively). The expression rates of CD11b and CD14 at different time point (6 h, 12 h, 24 h, 48 h, 72 h, 96 h) were significantly higher than that of negative control (Fig. 3C), all of which are indicative of the differentiation of human myeloid THP1 monocytes into macrophages. Based on qRT-PCR assays, we observed relative expression level (RQ) of miR-196b decreased with time ($F = 1556.098$, $P = 0.000$) in this differentiation cell model, which was lowest at 72 h time point (Fig. 4A).

We then studied whether forced expression of miR-196b can block the differentiation of THP1 induced by PMA. We observed that forced expression of the miR-196b could decrease the expression of CD11b and CD14 significantly (Fig. 4B). Wright–Giemsa staining revealed that cells in the groups of THP1 + PMA and THP1 + MSCVPIG + PMA adhere to the bottom of the culture dishes and mostly differentiated. However, there are still some cells (monocytes) undifferentiated. Taken together, our data shows that expression of miR-196b is inversely correlated with differentiation of THP1 cells.

4. Discussion

Although large-scale, global miRNA expression profiling assays have reported the signatures of many miRNAs with cytogenetic and molecular subtypes of acute leukemia, as well as patient response to treatment [9,14–17], the mechanisms underlying the deregulation and the function of individual miRNAs in acute leukemia including *MLL*-rearranged AML are largely unknown. In the present study, We have showed that forced expression of miR-196b could significantly enhance viability and inhibit apoptosis of human Hela and 293T cells. More importantly, miR-196b could significantly increase proliferation and partially inhibit differentiation of mouse normal bone marrow progenitor cells. MicroRNAs

play a crucial role in the establishment, maintenance, and function of hematopoietic lineages [18,19]. For example, *miR-17-5p-20a-106a* controls monopoiesis [20]; miR-21 and miR-196b, regulated by Gfi1, are involved in myelopoiesis [21]; *miR-181* directs lymphoid progenitors toward B-lymphoid development; while *miR-146* and *m*iR-223 appear to favor T lymphopoiesis [18]. The advantages of RNA sequencing-based gene expression profiling (GEP) and its current and potential implications in AML are future directions [22]. Our data here suggest that aberrant overexpression of miR-196b in MLL-rearranged acute leukemia can promote cell proliferation and inhibit normal hematopoiesis, and thereby may play an important role in the development of *MLL*-rearranged leukemias.

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