

# miR-107 promotes the erythroid differentiation of leukemia cells via the downregulation of *Cacna2d1*

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**Abstract.** microRNAs (miRNAs) have been reported to be involved in various human diseases. They may have uses in diagnosis and as therapeutic targets, thus the discovery of novel miRNAs has the potential to provide clinical tools or shed light on novel mechanisms. In the current study, miR-107 was revealed to be downregulated in chronic myeloid leukemia cells. Overexpression of miR-107 in K562 and KCL-22 chronic myeloid leukemia cells promotes erythroid differentiation, while having no effect on cell proliferation. Further bioinformatics predicted that one target of miR-107 may be *Cacna2d1*, a calcium channel protein. A luciferase reporter assay and quantitative polymerase chain reaction were utilized to confirm that *Cacna2d1* is a target molecule of miR-107. The effect of miR-107 on K562 and KCL-22 cells was mediated through the downregulation of *Cacna2d1*, as rescued expression of *Cacna2d1* reversed the effects of miR-107. In summary, the current study identified a novel miRNA that is involved in chronic myeloid leukemia cell erythroid differentiation and the associated mechanisms, making it a potential therapeutic target in the treatment of chronic myeloid leukemia.

## Introduction

Chronic myeloid leukemia (CML), is a clonal myeloproliferative disorder of the hematopoietic stem cells, which is defined by the overproduction of myeloid cells, leading to marked splenomegaly and leukocytosis. If ill-controlled, CML advances to the accelerated and blastic phases. The annual

incidence of CML in 2010 was reported to be one to two cases per 100,000 adults, accounting for 15% of newly diagnosed cases of leukemia in adults (1). Exposure to nuclear radiation increases the risk of CML, the peak incidence occurs 5-12 years post-exposure and the risk is dose-dependent (1,2). Due to an increase in exposure of the general population to radiation, either medically (cancer patients undergoing radiotherapy and radiologists) or incidentally (exposure as a result of a nuclear accident, for example in Fukushima), it is predicted that the prevalence of CML will increase gradually over time. Hence, the development of more effective therapeutic options for leukemia patients is indispensable.

In the human genome, protein-coding exons account for only 1.5% of the DNA (3) and the vast non protein-coding portions of the genome are of critical functional importance. One class of small non-coding RNAs, microRNAs, is of particular interest (4-6). miRNAs have been previously reported to be involved in a number of human diseases, including cancer, and it has been demonstrated that epigenetic and genetic defects in miRNAs and their processing machinery are common hallmarks of disease (3,7). Certain miRNAs (for example miR-15, miR-21 and miR-29) are considered to be therapeutic targets in the treatment of cardiovascular diseases (8), and it is almost certain that an increasing number of miRNAs will be investigated for their functions in disease diagnosis, development and therapeutics. In the present study, the involvement of miRNAs in chronic myeloid leukemia was investigated using various Bcr-Abl-expressing cell lines.

## Materials and methods

**Cell culture and erythroid differentiation.** K562 and KCL-22 human chronic myelogenous leukemia cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA). The cell lines were cultivated in RPMI-1640 medium with 10% (v/v; Invitrogen Life Technologies, Carlsbad, CA, USA) fetal calf serum (FCS; Invitrogen Life Technologies) and 1% (v/v) penicillin/streptomycin (Gibco-BRL, Carlsbad, CA, USA). To induce erythroid differentiation, cells were treated with 2 mM sodium butyrate.

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Table I. Primers for miR-107, miR-17, miR-34c, Cacna2d1. Primers for Cacna2d1-3'-UTR in the luciferase reporter assay.

	Forward	Reverse
miR-107	5'-AAAGAATTCCTGTTTCACTCGCC AAGC-3'	5'-AAAGGATCCAGCGAGTGAGGAGGG AGA-3'
miR-17	5'-GCCGCAAAGTGCTTACAGTG-3'	5'-TGCAGGGTCCGAGGTAT-3'
miR-34c	5'-GCGCTAGGCAGTGTAGTTAG-3'	5'-GTGCAGGGTCCGAGGT-3'
Cacna2d1	5'-GCTCATTCTGGTGGACGTGAG-3'	5'-TCACGGCATCTTTCAACACCT-3'
(for luciferase)	5'-TCTCCCATCGCCGTGGGCCAA AACATGAGCCCTCAGT-3'	5'-CTTCTAGATCACTTCGCCGTCT GCCTAACCC-3'
miR-107 mimics	5'-AGCAGCAUUGUACAGGGCUAUCA-3'	5'-AUAGCCCUGUACAAUGCUGCUUU-3'
miR-107 inhibitors	5'-UGAUAGCCCUGUACAAUGCUGCU-3'	5'-TCATTGGCATGTACCATGCAGCT-3'

**Quantitative polymerase chain reaction (qPCR).** Total RNA was extracted from cultured cells using TRIzol reagent (Invitrogen Life technologies) and cDNA was synthesized using the PrimeScript RT reagent kit (Takara Biotechnology Co. Ltd., Dalian, China). qPCR was performed using SYBR<sup>®</sup> Green master mix, Universal RT kit for the detection of microRNA (Exiqon, Vedbaek, Denmark) and the relative primers were all obtained from Exiqon. qPCR was carried out with primers for miR-107, miR-17, miR-34c and Cacna2d1 (Table I) with SYBR in the 7900 HT real-time PCR System (Qiagen, Valencia, CA, USA) using the following protocol: 95°C for 5 min, followed by 40 cycles of 95°C for 10 sec and 60°C for 30 sec. All experiments were performed in triplicate. U6 RNA was used as an endogenous control.

**Luciferase reporter assay.** The target gene predicted by scanning the database, TargetScan (www.targetscan.org), was Cacna2d1. A wild-type 3'-UTR segment of murine Cacna2d1 mRNA (1099 bp) containing the putative binding site for miR-107 was PCR-amplified and inserted into the pGL3 firefly luciferase reporter, which was based upon the pGL3-control (Promega, Madison, WI, USA) and created by Professor Shi-mei Zhuang (Sun Yat-sen University, Guangzhou, China). The primers used are presented in Table I.

The firefly luciferase construct was cotransfected with a control *Renilla* luciferase vector into cells in the presence of either miR-107 mimics or miRNA inhibitors. A dual luciferase assay (Promega) was performed 48 h following transfection. The experiments were performed independently and in triplicate.

**Oligonucleotide transfection.** miR-107 inhibitors and mimics were synthesized by Genepharma (Shanghai, China). Oligonucleotide transfection was performed with the Lipofectamine 2000 reagent (Invitrogen Life Technologies). miR-107 mimics were used at a concentration of 50 nM, while miR-107 inhibitors were transfected at a concentration of 100 nM.

**Western blot analysis.** Whole cell lysates were prepared using a radioimmunoprecipitation assay buffer (Sigma-Aldrich, St. Louis, MO, USA). Equal amounts of proteins

(10 mg/lane) were separated by 10% SDS-PAGE and transferred to polyvinylidene fluoride membranes. Rabbit anti-mouse Cacna2d1 polyclonal antibody was purchased from Santa Cruz Biotechnology (sc-133436; Santa Cruz, CA, USA). Secondary goat anti-rabbit horseradish peroxidase-conjugated antibody was obtained from Cell Signaling Technology (Beverly, MA, USA). Signals were detected using an ECL chemiluminescence kit (EMD Millipore, Billerica, MA, USA).

**MTT assay.** K562 and KCL-22 cells at the exponential growth phase were harvested and adjusted to a final concentration of  $5 \times 10^4$  cells per ml. The cells that were pretreated with miR-107 or miR-control using Lipofectamine 2000 were set as experiment groups, while untreated cells were used as the control group, and cells that were cultured with gabapentin adjusted to  $10^{-6}$  mol/l served as the positive control group. Cells ( $5 \times 10^3$ ) were seeded into 96-well plates, with each of the four groups assigned three columns of wells, and the plates were incubated at 37°C in 5% CO<sub>2</sub> for 5 days. Cell Counting Kit-8 solution (10 µl; Beyotime Institute of Biotechnology, Jiangsu, China) was added to each well and incubated for 2 h at 37°C, with 5% CO<sub>2</sub>. The absorbance was detected at a wavelength of 450 nm to measure the growth of the cells.

**Hemoglobin histochemical stain.** The cells were treated as previously described for the MTT assay and seeded into 24-well plates. Hemoglobin histochemical staining was performed to measure the erythroid differentiation level of the K562 and KCL-22 cells. Briefly, the cells were harvested on day 4,  $1 \times 10^6$  cells were incubated with 1 ml benzidine (2 mg/ml) and 10 µl 30% H<sub>2</sub>O<sub>2</sub> and subsequently seeded on to a 96-well plate with a total volume of 200 µl in each well. The absorbance was detected at a wavelength of 570 nm.

**Flow cytometric analysis.** Cells ( $1 \times 10^6$  from each group) were harvested and 1 µl OKT9 anti-human CD71-FITC antibody (eBioscience, San Diego, USA) was added. Following incubation at 4°C for 30 min, the cells were washed with phosphate buffered-saline (PBS) and resuspended in 100 µl PBS for further analysis with a Calibur flow cytometer equipped with Cellquest software (BD Biosciences, Franklin Lakes, NJ, USA).

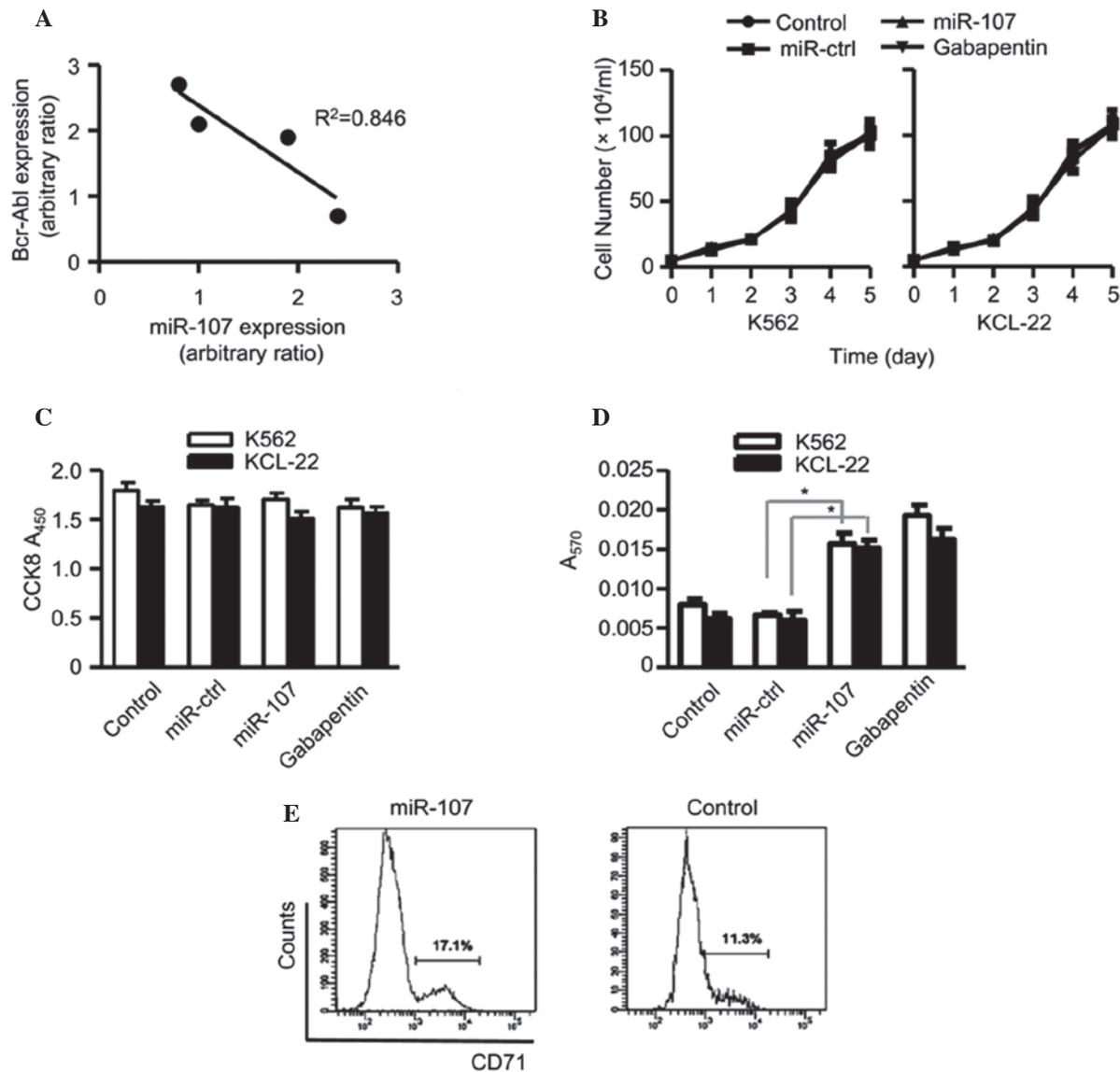


Figure 1. Overexpression of microRNA (miR)-107 may promote the erythroid differentiation of chronic myeloid leukemia cells. Cell lines were harvested at the exponential growth phase, adjusted to a final concentration of  $5 \times 10^4$ /ml and divided into four groups: Control, no treatment; miR-control, transfected with unrelated miRNAs; miR-107, transfected with 10 nM miR-107 and gabapentin-positive control, treated with 10 nM gabapentin. (A) The relative expression of Bcr-Abl and miR-107 were set as the Y-axis and the X-axis, respectively. (B) The cells were cultured in six-well plates for five days. Each day, the number of cells was counted. There was no difference in the growth rate between the four groups. (C) The cells were cultured in 96-well plates for four days. On day four, Cell Counting kit-8 solution was added to the medium and A450 was measured. There was no difference in the cellular proliferation between the four groups. (D) The cells were seeded in 24-well plates in conditional medium with sodium butyrate. Hemoglobin histochemical staining was performed to measure the erythroid differentiation level of K562 and KCL-22 cells. The absorbance was detected at 570 nm. In the miR-107 and gabapentin groups, the differentiation rate was higher than that in the control group. (E) The CD71 level was analyzed by flow cytometry. In the miR-107 group, the level was higher than that in control group. \* $P < 0.05$ .

**Statistical analysis.** All data are presented as the mean  $\pm$  standard deviation. Statistical analysis was performed using a two-way analysis of variance and a two-tailed paired Student's t-test where appropriate.  $P \leq 0.05$  was considered to indicate a statistically significant difference. All experiments were performed at least three times.

## Results

**miR-107 promotes erythroid differentiation of chronic myeloid leukemia cells.** Through microRNA screening, it has previously been demonstrated that miR-107 is downregulated in chronic myeloid leukemia cells (manuscript in preparation). The current study used qPCR to detect the expression of

Bcr-Abl, a marker for the chronic myeloid leukemia and miR-107. The results revealed a reciprocal association between miR-107 and Bcr-Abl (Fig. 1A). The aim of this study was to investigate the role of miR-107 in the biological behavior of leukemia cells using the K562 and KCL-22 chronic myeloid leukemia cell lines. The endogenous miR-107 expression of the cells was altered via transfection with miR-107 using Lipofectamine 2000. K562 and KCL-22 cells overexpressing miR-107 were cultured for 4 days. When compared with that of the miR-107-control or gabapentin-treated cells, the proliferation rate of the miR-107 cells displayed no significant differences in the K562 or KCL-22 cell lines (Fig. 1B and C).

Subsequently the differentiation status of the cells was analyzed. Since it has previously been reported that K562

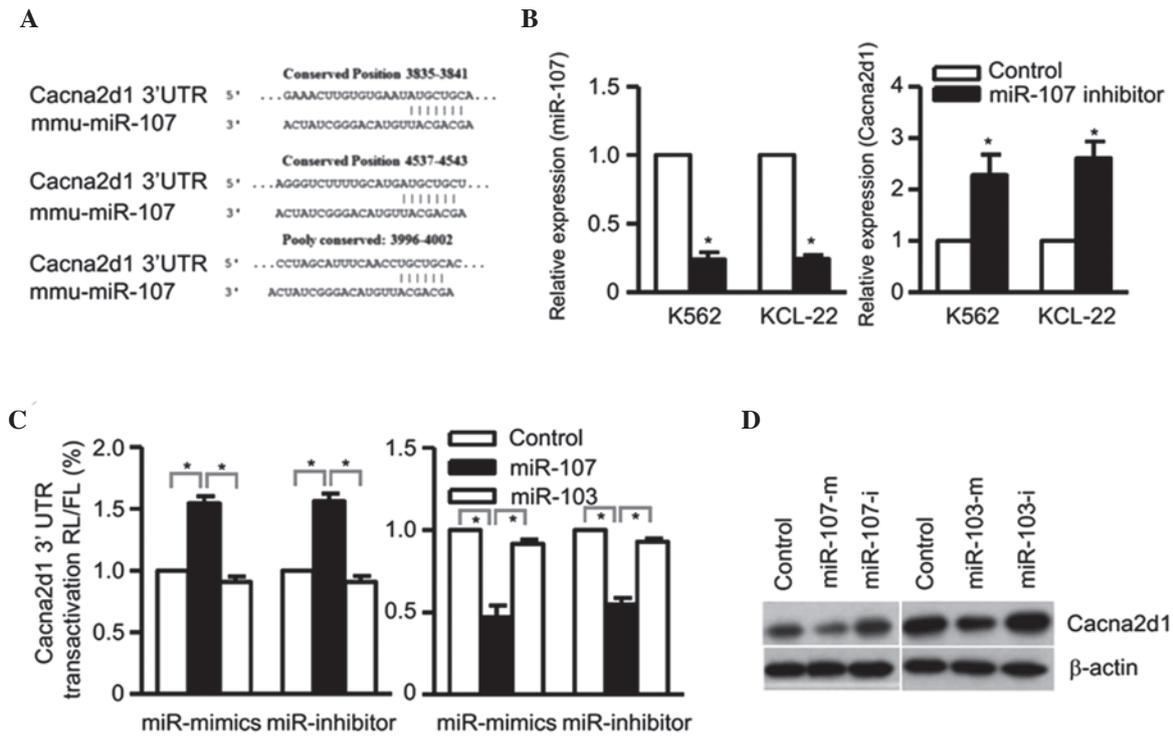


Figure 2. *Cacna2d1* is a target of miR-107. (A) The putative miR-107 binding site at the 3'-UTR of *Cacna2d1* at the position 3835-3841. (B) The miR-107 inhibitors were transfected into K562 and KCL-22 cells with Lipofectamine 2000, quantitative polymerase chain reaction revealed that miR-107 was downregulated and *Cacna2d1* was upregulated. (C) The firefly luciferase construct was co-transfected with a control *Renilla* luciferase vector into K562 cells in the presence of either miR-107/103 mimics or inhibitors. A dual luciferase assay showed that with miR-107/103 mimics, the expression of *Cacna2d1* was downregulated while with miR-107/103 inhibitors, the expression of *Cacna2d1* was upregulated. (D) Western blot analysis showed that the expression level of *Cacna2d1* was upregulated in K562 cells treated with miR-107/103 inhibitors and was downregulated in K562 cells treated with miR-107/103 mimics, compared with the controls. Data are presented as the mean ± standard deviation. \*P<0.05 and \*\*P<0.01, vs. the controls.

cells have the ability to undergo erythroid differentiation, the current study investigated the effect of miR-107 on this process. The cells were treated with sodium butyrate to induce erythroid differentiation. The results showed that the levels of hemoglobin and CD71 were considerably higher in miR-107 overexpressed cells than those in the controls (Fig. 1D and E). These results demonstrate that miR-107 promotes erythroid differentiation of chronic myeloid leukemia cells, while having no effect on cellular proliferation.

*Cacna2d1* is a target of miR-107. In order to further elucidate the underlying mechanism of miR-107 in K562 and KCL-22 erythroid differentiation, the target of miR-107 had to be determined. By searching the potential targets of miR-107 with TargetScan, Pictar (<http://pictar.mdc-berlin.de>), miRanda ([www.microrna.org](http://www.microrna.org)) and Microcosm (<http://www.ebi.ac.uk/enright-srv/microcosm>) software, a list was compiled of all the predicted genes for functional clustering analysis classified by the DAVID 6.7 (<http://david.abcc.ncifcrf.gov>), Kegg ([www.genome.jp/kegg](http://www.genome.jp/kegg)) and Panther ([www.pantherdb.org](http://www.pantherdb.org)) databases. *Cacna2d1* contains a putative target sequence, with two conserved and one poorly conserved positions, and was identified as the possible target of miR-107 (Fig. 2A). To validate this *in silico* prediction, qPCR was used to determine whether the expression of *Cacna2d1* could be repressed by miR-107. The results showed that with the miR-107 inhibitors in K562 and KCL-22 cells, the mRNA expression level of

*Cacna2d1* was significantly downregulated compared with that in the negative control (P<0.05) (Fig. 2B).

To determine whether *Cacna2d1* is a direct downstream target of miR-107, a fragment of the 3'-UTR of *Cacna2d1* (1099 bp) containing the potential miR-107 binding site was cloned into a vector with the firefly luciferase reporter gene. Luciferase activity in cells treated with miR-107 was reduced by ~20% compared with the control. Subsequently, K562 or KCL-22 cells were transfected with either miR-107 mimics or inhibitors. The results showed that miR-107 mimics downregulate the *Cacna2d1* expression levels while miR-107 inhibitors upregulate the *Cacna2d1* expression levels at the mRNA level, compared with those observed in the controls. It has been reported that miR-103 and miR-107 share numerous functions and are always co-expressed (10), hence, in the current study the expression of miR-103 was also analyzed, and the results indicated that miR-103 does not exhibit the same effects on *Cacna2d1*, implying this is a specific function of miR-107 (Fig. 2C). This ensures the specificity of the binding between miR-107 and the 3'-UTR of *Cacna2d1*. The *Cacna2d1* expression levels were also monitored in K562 cells subjected to both overexpression and knockdown of miRNAs, and the results demonstrated that miR-107 had strong effects on *Cacna2d1* expression, while miR-103 had marginal effects (Fig. 2D).

Taken together, these results indicated that the downregulation of miR-107 may cause the increased expression levels of *Cacna2d1*, which is the target gene of miR-107.

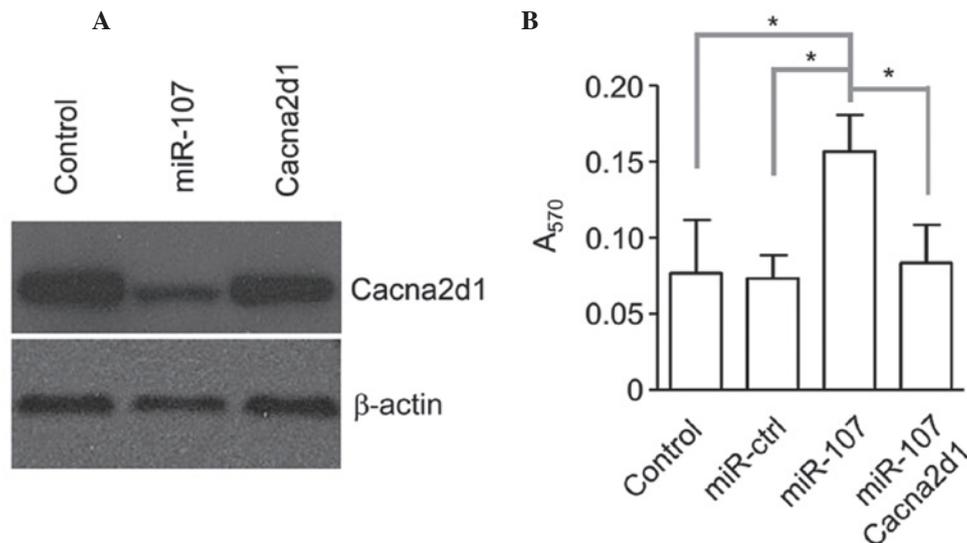


Figure 3. miR-107 promotes K562 erythroid differentiation via downregulation of *Cacna2d1*. (A) K562 cells transfected with or without indicated miRNA alone or combined with plasmid. After lysis, the cells were subjected to SDS-PAGE, and transferred to polyvinylidene fluoride membranes, followed by incubation with *Cacna2d1* antibody and the appropriate secondary antibody. (B) The cells were seeded in 24-well plates in conditional medium with sodium butyrate, and transfected with indicated plasmids. The hemoglobin histochemical stain was performed to measure the erythroid differentiation level of K562 cells. The absorbance at 570 nm was detected. In miR-107 and gabapentin groups, the differentiation rate was higher than that in the control group. Data are presented as the mean  $\pm$  standard deviation. \* $P < 0.05$ .

*miR-107 promotes erythroid differentiation of chronic myeloid leukemia cells via downregulation of Cacna2d1*. As shown in Fig. 2D, miR-107 overexpression contributes to the accelerated downregulation of *Cacna2d1*. To further confirm the function of miR-107 in K562 cells, a rescue experiment was performed via the transfection of a plasmid expressing *Cacna2d1* and the detection of erythroid differentiation. The rescue reversed the downregulation of miR-107, as observed using an immunoblotting assay (Fig. 3A). Meanwhile, the K562 cells with rescued *Cacna2d1* expression demonstrated better erythroid differentiation (Fig. 3B). Therefore, this study clearly showed that miR-107 promotes K562 erythroid differentiation via the downregulation of *Cacna2d1*.

## Discussion

miRNAs have been reported to have important roles in the development and progression of a number of diseases through the repression of downstream target genes (11,12). Since this initial discovery, abnormal miRNA expression levels have been detected in numerous types of hereditary diseases. However, little is known about the dysregulation of miRNAs in laminopathies. It has been reported that miR-107 has strong influences on health and disease in human biology. It can regulate the expression of genes involved in metabolism, angiogenesis, cell division and stress response in numerous vertebrate species. miR-107 has also been implicated in cardiovascular disease, a number of cancers and certain neurodegenerative diseases in humans (13-15). miR profiling experiments have revealed that miR-107 is expressed in numerous mammalian tissues in moderate to high levels, including heart, skeletal muscle, brain, lung, liver, kidney, spleen and placenta tissues (4,10).

The present study investigated the target gene that is regulated by miR-107. Through the luciferase reporter

assay combined with target prediction software, including Targetscan, Pictar, Miranda and Microcosm, a number of possible target genes were identified (data not shown). *Cacna2d1* was the most likely candidate. The official name of this gene is calcium channel, voltage-dependent,  $\alpha$ -2/ $\delta$  subunit 1. This gene encodes a member of the  $\alpha$ -2/ $\delta$  subunit family, which is a protein in the voltage-dependent calcium channel complex. Calcium channels mediate the influx of calcium ions into the cell upon membrane polarization, and they comprise a complex of  $\alpha$ -1,  $\alpha$ -2/ $\delta$ ,  $\beta$  and  $\gamma$  subunits in a 1:1:1:1 ratio. Studies on a similar protein in rabbits indicate that the protein described in this record is cleaved into  $\alpha$ -2 and  $\delta$  subunits. Alternative transcriptional splice variants of this gene have been observed, however, they have not been thoroughly characterized (16,17).

In the current study, miR-107 was revealed to promote the erythroid differentiation of K562 and KCL-22 chronic myeloid leukemia cells via the downregulation of *Cacna2d1*, while having no effect on the cell growth. Further investigation of the regulation mechanism is required to provide information on the association between *Cacna2d1* and other diseases, which may offer a novel insight into *Cacna2d1*.

In conclusion, the present study indicated that miR-107 promotes the erythroid differentiation of K562 and KCL-22 cells via the downregulation of *Cacna2d1*, providing potential therapeutic approaches for the treatment of chronic myeloid leukemia.

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