

MicroRNA-223 overexpression suppresses protein kinase C ϵ expression in human leukemia stem cell-like KG-1a cells

MALLIKA OSIRIPHAN^{1,2}, CHARAPAT INSUKHIN¹, SONGYOT ANUCHAPREEDA^{1,3,4},
PINYAPHAT KHAMPHIKHAM^{1,4} and SUWIT DUANGMANO^{1,3,4}

¹Division of Clinical Microscopy, Department of Medical Technology, Faculty of Associated Medical Sciences, Chiang Mai University, Mueang Chiang Mai, Chiang Mai 50200, Thailand; ²Master's Degree Program in Medical Technology (under the Chiang Mai University Presidential Scholarship), Faculty of Associated Medical Sciences, Chiang Mai University, Mueang Chiang Mai, Chiang Mai 50200, Thailand; ³Cancer Research Unit of Associated Medical Sciences, Department of Medical Technology, Faculty of Associated Medical Sciences, Chiang Mai University, Mueang Chiang Mai, Chiang Mai 50200, Thailand; ⁴Hematology and Health Technology Research Center, Department of Medical Technology, Faculty of Associated Medical Sciences, Chiang Mai University, Mueang Chiang Mai, Chiang Mai 50200, Thailand

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Abstract. MicroRNA-223 (miR-223) is dysregulated in various cancer types, including acute myeloid leukemia (AML). Despite this, there has been a lack of studies exploring the role of miR-223 in leukemic stem cells, particularly those involved in drug resistance, a major cause of chemotherapy failure in AML. The present study aimed to elucidate the impact of miR-223 on drug resistance in the leukemic stem-cell line, KG-1a. Two AML cell lines, KG-1 and KG-1a, differing in the proportion of CD34⁺CD38⁺ cells, were assessed for doxorubicin (DOX) sensitivity using the Cell Counting Kit-8 assay. The expression levels of miR-223 and protein kinase C ϵ (PKC ϵ) were evaluated via reverse transcription-quantitative PCR and western blot analysis. The association between miR-223 and its target, PKC ϵ , was confirmed by luciferase activity assay. The effects of miR-223 overexpression and PKC ϵ inhibition were also evaluated in KG-1a cells using miR-223 mimic and small interfering (si)RNA transfection, respectively. Daunorubicin was then used to assess drug sensitivity in the siRNA-transfected KG-1a cells. Compared with KG-1 cells, KG-1a cells displayed greater resistance to DOX, and had increased PKC ϵ levels and decreased miR-223 expression. Overexpression of miR-223 led to PKC ϵ protein downregulation in KG-1a cells, which was further confirmed

by a luciferase assay demonstrating miR-223 targeting of PKC ϵ . However, despite these effects, miR-223 overexpression and PKC ϵ inhibition did not change drug sensitivity in KG-1a cells compared with negative control cells. In summary, the present study demonstrated that miR-223 could target and silence PKC ϵ expression in KG-1a cells; however, the chemoresistance of KG-1a cells to anthracycline drugs may not be directly associated with the low expression of miR-223.

Introduction

Acute myeloid leukemia (AML) is one of the most prevalent hematological malignancies globally, constituting one-third of all adult leukemias (1-4). Despite advances in treatments leading to complete remission for most patients with AML, drug resistance remains a major factor in therapy failure and contributes to the short-term survival of these patients (4). Recently, accumulating evidence has implicated leukemic stem cells (LSCs), a dormant subset of leukemic cells, as pivotal in drug resistance and cancer relapse (5). These cells express the same markers as normal adult hematopoietic stem cells (HSCs) (CD34⁺ and CD38⁺) and exhibit stemness characteristics, such as self-renewal, proliferation and differentiation (5-8). In the past few years, several microRNAs (miRNAs/miRs), which represent small non-coding RNAs that act as post-transcriptional regulators, have been identified as participants in drug resistance mechanisms and LSC regulation, such as miR-22 and miR-126 (8,9). Therefore, investigations into miRNA-associated cancer pathogenesis are crucial for overcoming drug resistance events and regulating LSCs in AML.

Dysregulation of miR-223 has been documented in connection with various cancer types, including hepatocellular carcinoma, breast cancer and leukemia. Furthermore, numerous targets of miR-223, including insulin-like growth factor-1 receptor, monocytic enhancer factor 2C, microtubule destabilizer stathmin 1 and forkhead box protein

Correspondence to: Dr Suwit Duangmano, Division of Clinical Microscopy, Department of Medical Technology, Faculty of Associated Medical Sciences, Chiang Mai University, 110 Intawaroros Road, Sripoom, Mueang Chiang Mai, Chiang Mai 50200, Thailand
E-mail: suwit.du@cmu.ac.th

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O1A, have been associated with cancer-related traits, such as cell proliferation, carcinogenesis and metastasis (10). However, few studies have explored the role of miR-223 in AML. Previous studies demonstrated low miR-223 expression in patients with AML, especially in those with a poor prognosis (11,12). Moreover, a marked increase in miR-223 expression was reported in patients with AML after treatment, regardless of whether complete remission was achieved or not (12). Another study demonstrated that miR-223 suppressed cell proliferation and enhanced cell apoptosis in HL-60 and K-562 cell lines by specifically targeting the expression of F-box/WD repeat-containing protein 7 (*FBXW7*) (13).

Protein kinase C ϵ (PKC ϵ), an isoform of PKC, is encoded by the *PRKCE* gene. It phosphorylates a variety of protein targets and is known to participate in diverse cellular signaling pathways, including MAPK, ERK and PI3K/AKT pathways, which regulate various biological functions, such as proliferation, differentiation and apoptosis (14,15). PKC ϵ has been implicated in drug resistance in several cancer types, including gallbladder cancer, lung cancer, renal cell carcinoma and prostate cancer (14,16-19). Additionally, a recent study identified high levels of *PRKCE* mRNA in gallbladder cancer stem cells (19). Another recent study demonstrated that PKC ϵ overexpression has been shown to selectively confer resistance to daunorubicin (DNR) in the AML U937 and HEL cell lines. Furthermore, patients with high levels of PKC ϵ protein exhibit a lower rate of complete remission compared with those with lower PKC ϵ protein levels. Additionally, elevated PKC ϵ expression has been associated with decreased disease-free survival, indicating a consistent pattern of treatment resistance (20).

The present study aimed to elucidate the roles of miR-223 and PKC ϵ in the regulation of drug resistance mechanisms in LSCs to bridge current gaps in knowledge and offer valuable insights for the advancement of targeted therapies in AML.

Materials and methods

Cell culture. The human acute myeloblastic leukemia KG-1 cell line (cat. no. CCL-246) and its quiescent variant KG-1a (cat. no. CCL-246.1), obtained from the American Type Culture Collection, served as LSC models in the present study. KG-1a cells display less mature morphological, cytochemical and functional characteristics compared with KG-1 cells (21). Furthermore, the unresponsiveness of KG-1a cells to colony-stimulating factor and the lack of expression of human leukocyte antigen contributes to their increased resistance to differentiation-inducing drugs (22). Both cell lines were cultured in Iscove's modified Dulbecco's medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 20% FBS (Capricorn Scientific), 1 mM L-glutamine (Cytiva), 100 U/ml penicillin and 100 μ g/ml streptomycin (Gibco; Thermo Fisher Scientific, Inc.). 293T cells, used for the luciferase activity assay, were kindly provided by Dr Pinyaphat Khamphikham (an author of the present study). The 293T cells were cultured in DMEM (Nacalai Tesque, Inc.) containing 10% FBS, 1 mM L-glutamine, 100 U/ml penicillin and 100 μ g/ml streptomycin. All cell lines were cultured at 37°C in a humidified atmosphere with 5% CO₂ and were passaged every 2-3 days or upon reaching 70-80% confluence.

Drug sensitivity assay. KG-1 and KG-1a cells were seeded in 96-well plates at a density of 1.5×10^4 cells per well and were exposed to doxorubicin (DOX) (Fresenius Kabi Oncology, Ltd.) at concentrations of 0.03125, 0.0625, 0.125, 0.25, 0.5 and 1.0 μ g/ml. For the DNR sensitivity test, small interfering (si)RNA-transfected KG-1a cells were seeded at the same density and treated with DNR (APeXBIO Technology LLC) at concentrations of 0.03125, 0.0625, 0.125 and 0.25 μ g/ml. Blank wells containing only growth medium were utilized to subtract background signals. Following 48 h of incubation at 37°C with 5% CO₂, the Cell Counting Kit (CCK)-8 assay (Abbkine Scientific, Co., Ltd.) was conducted following the manufacturer's protocol. After incubation with the CCK-8 reagent for 2 h at 37°C, the absorbance at 450 nm was determined using a microplate reader (Metertech, Inc.). The experiment was repeated three times, and cell survival was determined using the following formula: % Cell viability = [mean optical density (OD) of the treated group-blank/mean OD of the untreated control group-blank] \times 100. The half-maximal inhibitory concentration (IC₅₀) value was established by plotting the cell viability rate (y-axis) against DOX concentration (x-axis) to generate a linear equation: $y = ax + b$. The IC₅₀ value was calculated as follows: $IC_{50} = (50 - b)/a$.

RNA isolation and reverse transcription-quantitative (RT-q)PCR. Total RNA was extracted from KG-1 and KG-1a cells using TRI Reagent® (Molecular Research Center, Inc.). The concentration of isolated total RNA was measured using a Qubit 4 fluorometer (Invitrogen; Thermo Fisher Scientific, Inc.) with the Qubit RNA High Sensitivity Assay kit (Invitrogen; Thermo Fisher Scientific, Inc.). To reverse transcribe mature miR-223, the RNA samples underwent conversion into U6 and miR-223-specific cDNA using SuperScript III Reverse Transcriptase (Invitrogen; Thermo Fisher Scientific, Inc.) with RT primers, according to the protocol of Varkonyi-Gasic and Hellens (23). For the conversion of mRNA into cDNA, total RNA (1 μ g) was reverse transcribed into cDNA using ReverTra Ace qPCR RT Master Mix with gDNA Remover (Toyobo Life Science) following the manufacturer's protocol. The RT reactions were performed on an MJ Mini thermocycler (Bio-rad Laboratories, Inc.). qPCR was performed using SensiFAST SYBR No-ROX reagent kit (Meridian Bioscience, Inc.). The primers used in the present study are listed in Table I. The qPCR was performed on a CFX Opus 96 Real-time PCR system (Bio-Rad Laboratories, Inc.) with the following cycling conditions: 95°C for 2 min, followed by 40 cycles at 95°C for 5 sec and 60°C for 15 sec. The samples were independently analyzed three times, and relative expression was assessed using the 2^{- $\Delta\Delta C_q$} method (24), with U6 and *GAPDH* as reference genes for miR-223 and *PRKCE*, respectively. As each replicated experiment was conducted individually at different time points, the expression levels of miR-223 or *PRKCE* of the control groups were standardized to 1, and the relative expression of the other groups were normalized to the expression of the control groups within each replicate.

Western blot analysis. Cells were lysed, and proteins were extracted using RIPA buffer composed of 50 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100, 0.5 mM EDTA, 0.1% SDS and a protease inhibitor cocktail (HiMedia Laboratories, LLC). The

Table I. Primer sequences used for RT-quantitative PCR.

Gene	Direction	Sequence (5'-3')
miR-223 RTp ^a	N/A	GTCGTATCCAGTGCAGGGTCCGAGGTATTTCGCACTGGATACGACTGGGGTA
U6 RTp ^a	N/A	CTCAACTGGTGTCTGTGGAGTCGGCAATTCAGTTGAGAAAAATATG
miR-223 ^a	Forward	AACACGCTGTCAAGTTTGTCAA
	Reverse	GTCGTATCCAGTGCAGGGT
U6 ^a	Forward	CTCGCTTCGGCAGCACA
	Reverse	AACGCTTCACGAATTTGCGT
GAPDH ^b	Forward	AACGGGAAGCTTGTCAATGGA
	Reverse	GCATCAGCAGAGGGGGCAGAG
PRKCE ^a	Forward	AGCCTCGTTCACGGTTCT
	Reverse	TGTCCAGCCATCATCTCG

^aSynthesized by Macrogen, Inc.; ^bsynthesized by Bio Basic, Inc. RTp, reverse transcription primer; N/A, not applicable; miR, microRNA; PRKCE, protein kinase C ε.

Table II. miRNA mimics and siRNAs.

Molecule	Strand	Sequence (5'-3')
mimic-NC	Sense	UUCUCCGAACGUGUCACGUTT
	Antisense	ACGUGACACGUUCGGAGAATT
miR-223 mimic	Sense	UGUCAGUUUGUCAAUACCCCA
	Antisense	GGGUAUUUGACAAACUGACAUU
si-NC	Sense	GGCAAGACCAGCUGCGACAUU
	Antisense	AAUGUCGCAGCUGGUCUUGCC
si-PRKCE#1	Sense	CCAGUCUGAAUACAGGUAGAUATA
	Antisense	UAAUAUCUACCUGUAUUCAGACUGGAA
si-PRKCE#2	Sense	GUCAUAAUUUUGAGCAAGACUUTA
	Antisense	UAAAGUCUUGGUCAAAAUUUAUUGACGU
si-PRKCE#3	Sense	UGAAAGCUUUAUGACGAAGAAUCC
	Antisense	GGAUUCUUCGUCAUGAAAGCUUUAAG

NC, negative control; miRNA/miR, microRNA; si, siRNA; PRKCE, protein kinase C ε.

quantification of the extracted proteins was determined using Qubit 4 fluorometer and a Qubit protein assay kit (Invitrogen; Thermo Fisher Scientific, Inc.). Subsequently, protein samples (50 µg per lane) were combined with loading buffer, boiled at 95°C for 5 min, separated on 7.5% SDS-PAGE gels, and then transferred to PVDF membranes. The membranes were blocked with 5% skimmed milk in PBS at room temperature for 3 h, followed by overnight incubation at 4°C with primary rabbit antibody targeting PKCε (1:1,000 dilution; cat. no. 2683; Cell Signaling Technology, Inc.) or for 1 h at room temperature with primary rabbit antibody targeting GAPDH (1:16,000 dilution; cat. no. ABS16; MilliporeSigma). The membranes were rinsed six times (5 min each time) with 0.1% Tween-PBS solution and subsequently incubated with HRP-conjugated anti-rabbit IgG secondary antibody (1:20,000 dilution; cat. no. W401B; Promega Corporation) for 1 h at room temperature. Immobilon Forte Western HRP substrate (MilliporeSigma) was used for signal development, and the density of protein bands was analyzed using Quantity One software version 4.6.8 (Bio-Rad

Laboratories, Inc.). Each replicated experiment was performed separately at a different time point. The levels of the control groups were standardized to 100%, and the relative expression of the other groups was normalized against the expression of the control group within each replicate.

Cell transfection. Mimic-negative control (NC) was obtained from Shanghai GenePharma Co., Ltd., while miR-223 mimic was purchased from Ambion (Thermo Fisher Scientific, Inc.). For the knockdown of PKCε, a TriFECTa RNAi kit, comprising siRNAs si-PRKCE#1, si-PRKCE#2 and si-PRKCE#3, was purchased from Integrated DNA Technologies, Inc. si-NC was purchased from Ambion; Thermo Fisher Scientific, Inc. The sequences of miRNA mimics and siRNAs utilized in the present study are listed in Table II. The transfection of miRNA mimics or siRNAs into the cells was performed using Lipofectamine® 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.) in accordance with the manufacturer's protocol.

For miRNA mimic transfection, KG-1a cells were transfected with either 25 nM miR-223 mimic or mimic-NC for 24 h at 37°C. Regarding siRNA transfection, KG-1a cells were transfected with 25 nM of either si-NC, si-*PRKCE*#1, si-*PRKCE*#2 or si-*PRKCE*#3 for 48 h at 37°C. Subsequent experiments were conducted instantly following transfection.

Dual-luciferase reporter assay. To investigate whether PKC ϵ serves as a direct target of miR-223, TargetScan software (https://www.targetscan.org/vert_80/) was used to predict the binding targets of miR-223, one of which was identified to be *PRKCE* mRNA. The pmiRGLO plasmid (Promega Corporation) was used to generate constructs with the 3' untranslated region (UTR) sequence of *PRKCE*, encompassing the miR-223 binding site. These constructs included pmiRGLO-*PRKCE*-3'UTR-wild-type (WT) and pmiRGLO-*PRKCE*-3'UTR-mutant (Mut). Co-transfection of these constructed plasmids (200 ng each) with miR-223 mimic or mimic-NC (10 pmol each) was performed in 293T cells using Lipofectamine 2000 reagent in accordance with the manufacturer's protocol. After 48 h of transfection at 37°C, firefly and *Renilla* luciferase activities were promptly quantified using the Dual-Glo Luciferase Assay System kit (Promega Corporation) and a CLARIOstar Plus microplate reader (BMG Labtech GmbH). The firefly luciferase activity was normalized to the *Renilla* luciferase activity of each sample. This experiment was independently repeated three times.

Statistical analysis. Data are presented as the mean \pm standard deviation. Data analysis was conducted using SPSS version 20 (IBM Corp.). An unpaired Student's t-test was used to make comparisons between two groups. Differences among multiple groups were analyzed using one-way ANOVA followed by Dunnett's post hoc test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

miR-223 is downregulated and PKC ϵ is upregulated in the drug-resistant KG-1a cell line. The assessment of drug resistance in LSC cell lines involved treating both KG-1 and KG-1a cells with varying concentrations of DOX. The findings indicated a significantly higher IC₅₀ in KG-1a cells (0.650 ± 0.004 μ g/ml) compared with that in KG-1 cells (0.435 ± 0.02 μ g/ml) ($P < 0.001$), revealing that KG-1a cells exhibited higher resistance to DOX (Fig. 1A).

Using the RT-qPCR method, miR-223 levels were examined in KG-1 and KG-1a cells. There was a significantly higher expression level of miR-223 in KG-1 cells compared with that in KG-1a cells ($P < 0.001$), indicating that miR-223 was expressed at a lower level in the KG-1a cell line (Fig. 1B). To further examine PKC ϵ expression in KG-1 and KG-1a cells, both RT-qPCR and western blot analysis were employed. The results demonstrated that PKC ϵ expression was significantly higher in KG-1a cells at both the mRNA ($P < 0.001$) and protein levels ($P < 0.01$), compared with that in the KG-1 cell line (Fig. 1C and D).

miR-223 targets and inhibits the expression of PKC ϵ . To investigate the association between miR-223 and PKC ϵ , KG-1a

cells were transfected with miR-223 mimic. After 24 h of transfection, RT-qPCR analysis revealed a significant increase in miR-223 expression in the miR-223 mimic group compared with that in the NC group ($P < 0.01$; Fig. 2A), indicating successful transfection.

Subsequent evaluation of PKC ϵ expression at both the mRNA and protein levels post-transfection showed no significant difference in *PRKCE* mRNA levels between the miR-223 mimic group and mimic-NC group ($P = 0.455$; Fig. 2B). However, there was a significant decrease in PKC ϵ protein level in the miR-223 mimic group compared with that in the mimic-NC group ($P < 0.01$; Fig. 2C), suggesting that the miR-223 mimic inhibited the protein expression of PKC ϵ .

Considering the predicted binding site of miR-223 on *PRKCE* mRNA, as obtained through the bioinformatics software TargetScan, a dual-luciferase reporter assay was conducted to validate this interaction. There was a significant reduction in luciferase activity in cells co-transfected with *PRKCE*-3'UTR-WT and miR-223 mimic ($P < 0.05$), while no changes were observed in the luciferase activity of cells co-transfected with *PRKCE*-3'UTR-Mut and miR-223 mimic, compared with that in the corresponding mimic-NC group ($P = 0.132$) (Fig. 2D). These findings indicate that miR-223 can target and suppress PKC ϵ expression.

Overexpression of miR-223 and inhibition of PKC ϵ are not associated with the drug sensitivity of KG-1a cells. To explore the impact of miR-223 overexpression on drug sensitivity in KG-1a cells, miR-223 mimic-transfected cells were subjected to varying concentrations of DOX. The results showed no significant difference in DOX sensitivity between the miR-223 mimic group and the mimic-NC group, with IC₅₀ values of 0.614 ± 0.15 and 0.558 ± 0.08 μ g/ml, respectively ($P = 0.598$; Fig. 3A). This suggests that the overexpression of miR-223 did not affect the DOX sensitivity of the KG-1a cell line.

To investigate the role of PKC ϵ in the drug sensitivity of KG-1a cells, the cells were transfected with si-NC, si-*PRKCE*#1, si-*PRKCE*#2 and si-*PRKCE*#3 to assess the effects of PKC ϵ knockdown. The results indicated that si-*PRKCE*#3 was the most effective siRNA for suppressing PKC ϵ expression, resulting in significantly lower *PRKCE* mRNA expression compared with the si-NC group ($P < 0.05$). Conversely, the si-*PRKCE*#1 ($P = 0.917$) and si-*PRKCE*#2 ($P = 0.291$) groups exhibited no significant differences in *PRKCE* mRNA expression compared with the si-NC group (Fig. 3B). PKC ϵ protein levels were evaluated in the si-*PRKCE*#3 group, confirming a reduction in PKC ϵ levels in si-*PRKCE*#3-transfected cells compared with those in the si-NC group ($P < 0.05$; Fig. 3C). Consequently, KG-1a cells transfected with si-*PRKCE*#3 were treated with various concentrations of DNR, as previous findings have suggested that PKC ϵ overexpression confers selective resistance to DNR in AML (20). However, the results demonstrated that DNR sensitivity was not changed after PKC ϵ knockdown compared with the si-NC group, with IC₅₀ values of 0.157 ± 0.01 and 0.172 ± 0.01 μ g/ml, respectively ($P = 0.076$; Fig. 3D). These findings suggest that neither miR-223 overexpression nor PKC ϵ knockdown appears to be directly associated with drug sensitivity in KG-1a cells.

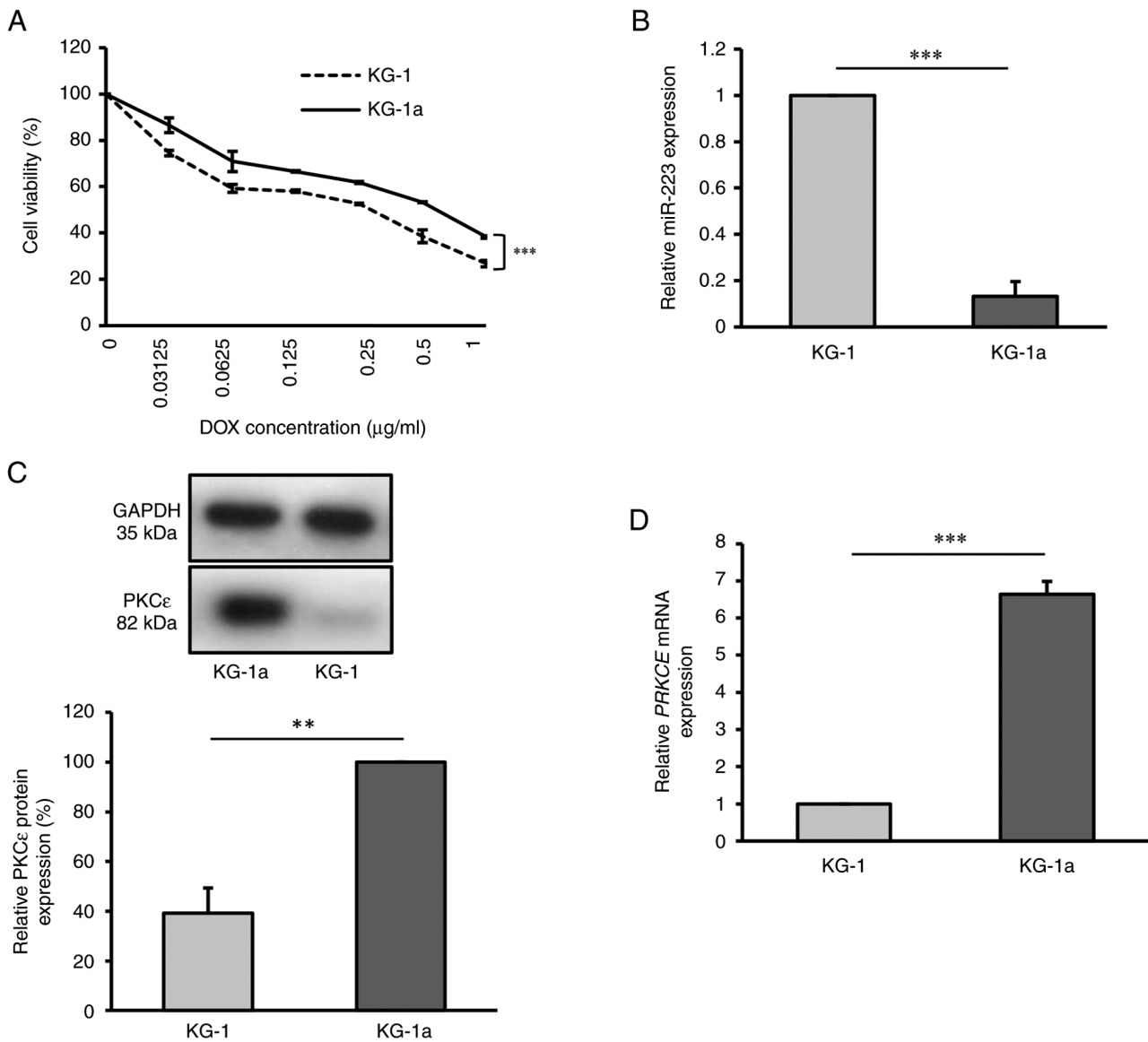


Figure 1. Drug resistant KG-1a cell line exhibits downregulation of miR-223 and upregulation of PKCε. (A) Cell viability of KG-1 and KG-1a cell lines after DOX treatment for 48 h, as detected by Cell Counting Kit-8 assay. (B) Relative miR-223 expression in KG-1 and KG-1a cells, as detected by RT-qPCR. (C) Relative PKCε protein level in KG-1 and KG-1a cells, as detected by western blot analysis. (D) Relative *PRKCE* mRNA expression in KG-1 and KG-1a cells, as detected by RT-qPCR. The values of KG-1 group in (B), (C) and (D) were normalized to 1 or 100% without accounting for standard error, due to the use of different batches for each replicate. ***P*<0.01; ****P*<0.001 (*n*=3 replicates/group). The data were analyzed using unpaired Student's t-test. DOX, doxorubicin; miR, microRNA; PKCε/*PRKCE*, protein kinase C ε; RT-qPCR, reverse transcription-quantitative PCR.

Discussion

AML is a prevalent global hematological malignancy (1-4), with significant advancements in molecular targeted therapy leading to complete remission in a number of patients. However, affordability remains a considerable challenge in developing countries, driven by high treatment expenses, limited accessibility, inadequate insurance coverage, significant out-of-pocket costs and income disparities. Furthermore, the lack of treatment options in certain countries poses a challenge, considering that targeted therapies may not be provided according to the standard of care or may not be available for specific cancer types (25-29). Additionally, drug resistance remains the primary cause of chemotherapy failure, impacting patient survival rates (4). Therefore, studies into traditional chemotherapy remain crucial to provide effective treatment

options. Given the association of miR-223 with various cancer types (10) and bioinformatics predictions identifying the *PRKCE* gene as one of the targets of miR-223, the present study aimed to elucidate the roles of miR-223 and PKCε in regulating drug resistance mechanisms in LSCs.

The current study showed that KG-1a cells exhibit a higher IC₅₀ for DOX compared with KG-1 cells, which is consistent with the findings of a previous study that reported that KG-1 cells were more responsive to DOX than KG-1a cells (30). The difference in the percentage of CD34⁺ CD38⁺ LSCs between the two cell lines (75.95% in KG-1 and 92.82% in KG-1a), as revealed in our previous study (31), indicates that the number of CD34⁺ CD38⁺ LSCs may impact the chemosensitivity of these cell lines. It is well-established that LSCs, characterized by dynamic origins, derive from various cell types within leukemia, demonstrating their complex adaptability in

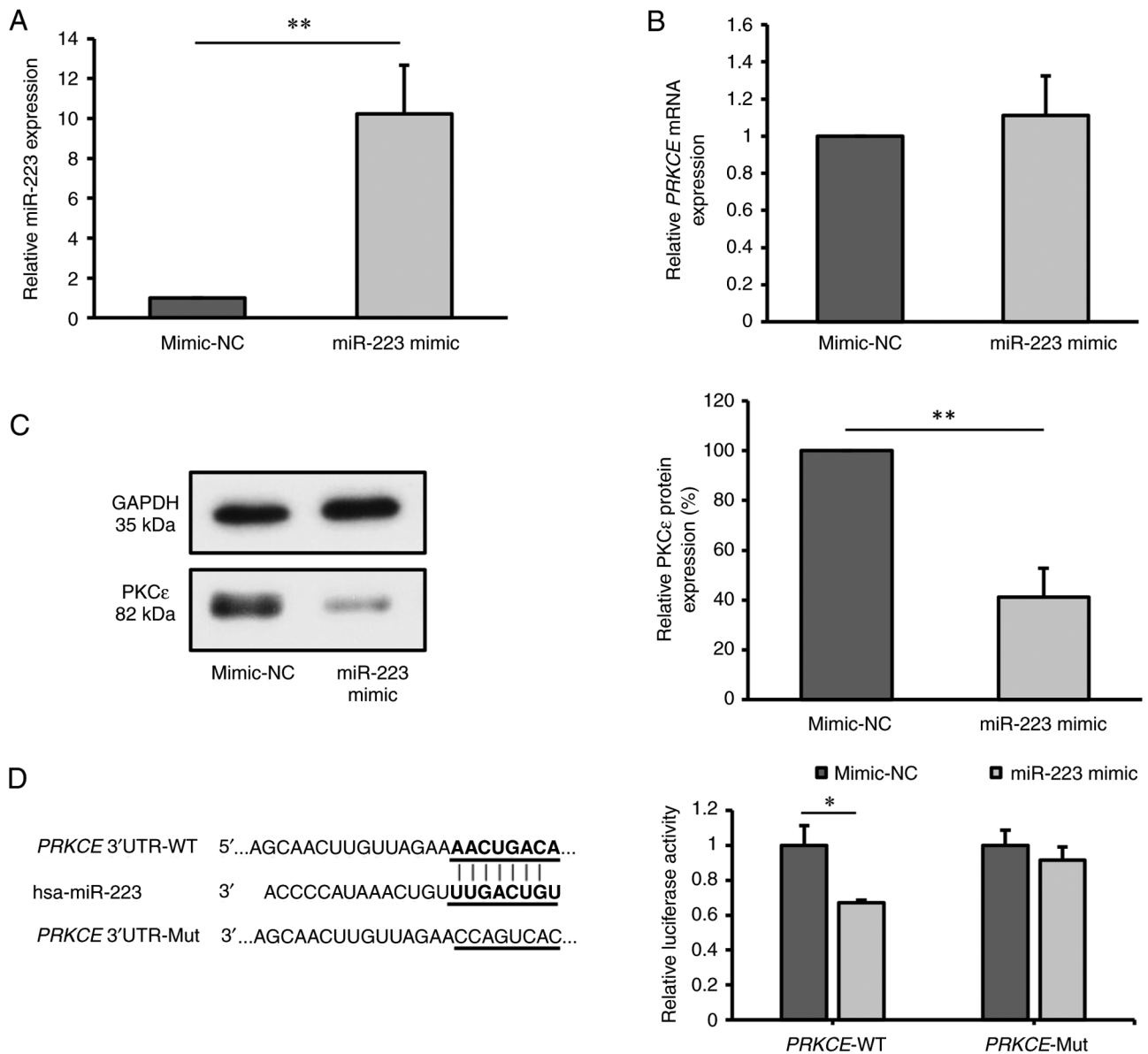


Figure 2. miR-223 targets and inhibits the expression of PKC ϵ . (A) Relative miR-223 expression in KG-1a cells after transfection with miR-223 mimic, detected by RT-qPCR; mimic-NC values were normalized to 1. (B) Relative *PRKCE* mRNA expression in KG-1a cells after transfection with miR-223 mimic, as detected by RT-qPCR. (C) Relative PKC ϵ protein level in KG-1a cells after transfection with miR-223 mimic, as detected by western blot analysis. (D) Binding sites between miR-223 and *PRKCE* mRNA predicted by TargetScan, and relative PKC ϵ luciferase activity measured by dual-luciferase assay. The values of mimic-NC group in (B) and (C) were normalized to 1 or 100% without considering standard error, as each replicate was conducted using different batches. * $P < 0.05$; ** $P < 0.01$ ($n = 3$ replicates/group). The data were analyzed using unpaired Student's t-test. miR, microRNA; NC, negative control; PKC ϵ /*PRKCE*, protein kinase C ϵ ; WT, wild-type; Mut, mutant; UTR, untranslated region; RT-qPCR, reverse transcription-quantitative PCR.

disease progression and treatment. These cells harbor various elusive resistance mechanisms, such as inherent dormancy, overexpression of ATP-binding cassette transporters, defects in apoptotic signals, resistance to apoptosis and senescent signals, metabolic reprogramming and epigenetic alterations, which contribute to differences in chemosensitivity (32).

In the present study, lower miR-223 expression was observed in KG-1a cells compared with KG-1 cells. Similarly, a previous study reported lower expression of miR-223 in blast cells from AML patients within fractions containing CD34⁺CD38⁻ LSCs compared with fractions of more committed leukemic cells lacking the CD34 marker (11). Downregulation of miR-223 has been observed in patients with AML, particularly those with intermediate and unfavorable prognoses (11-13). It is

therefore likely that miR-223 functions as a tumor-suppressing miRNA in AML.

In contrast to miR-223, PKC ϵ was observed to be upregulated in KG-1a cells at both the mRNA and protein levels, compared with the KG-1 cell line. The bioinformatics prediction software TargetScan, along with a luciferase assay, confirmed that miR-223 binds to the 3'UTR of *PRKCE* mRNA, resulting in the inhibition of the luciferase activity. Additionally, overexpression of miR-223 suppressed PKC ϵ protein expression in KG-1a cells, although there was no notable impact on *PRKCE* mRNA levels. A previous study similarly reported that, in non-small cell lung cancer cell lines, miR-143 mimic transfection resulted in the reduction of PKC ϵ protein levels, while mRNA levels remained unchanged (33). Typically, miRNAs function by

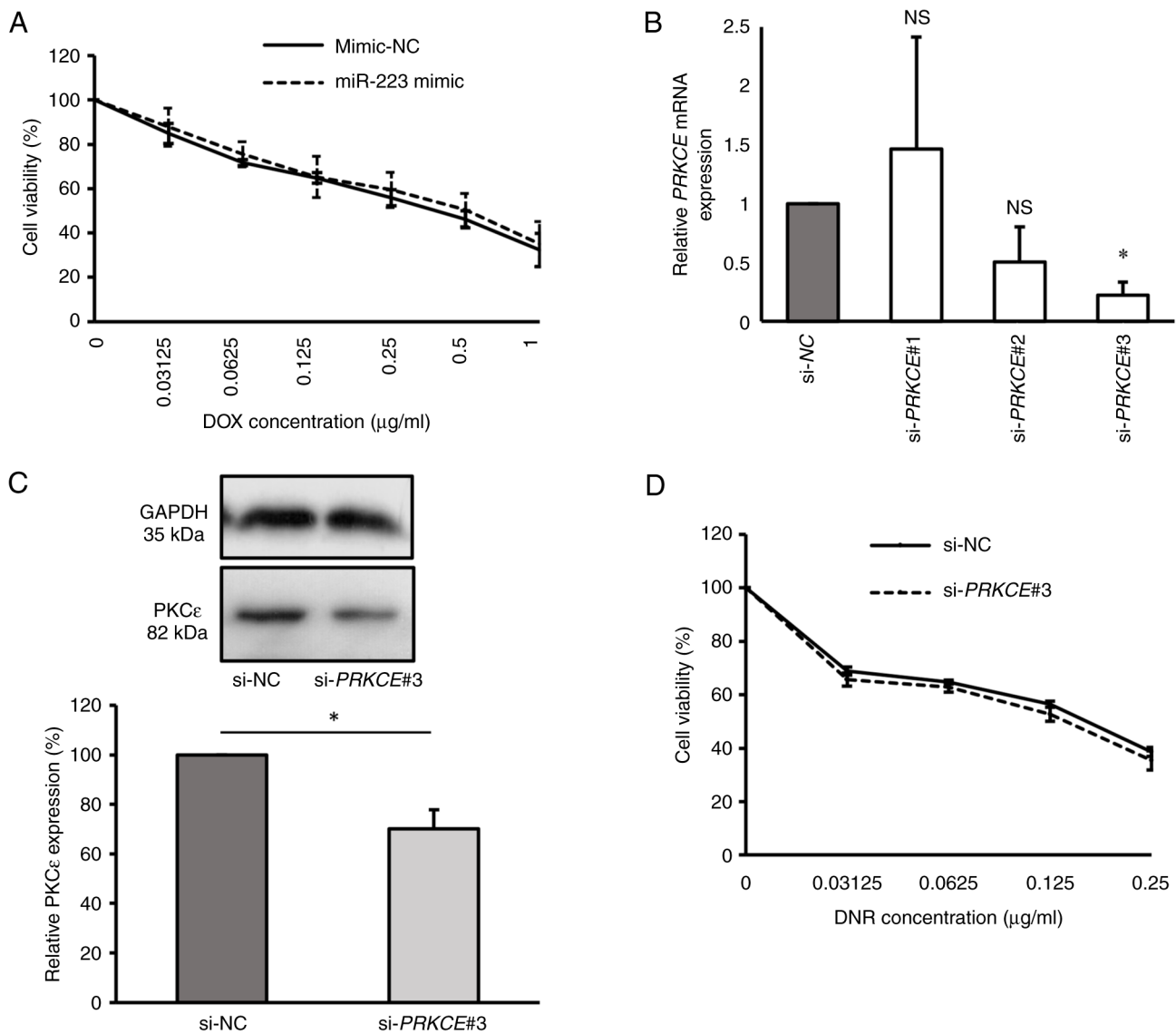


Figure 3. Overexpression of miR-223 and inhibition of PKCε are not associated with the drug sensitivity of KG-1a cells. (A) Cell viability of transfected KG-1a cells after DOX treatment for 48 h, as detected by CCK-8 assay. (B) Relative *PRKCE* mRNA expression in KG-1a cells after transfection with *PRKCE* siRNAs, as detected by reverse transcription-quantitative PCR. (C) Relative PKCε protein level in KG-1a cells after transfection with si-*PRKCE*#3, as detected by western blot analysis. (D) Cell viability of PKCε-knocked down KG-1a cells after DNR treatment for 48 h, as detected by CCK-8 assay. The values of si-NC group in (B) and (C) were normalized to 1 or 100% without considering standard error, as each replicate was conducted using different batches. * $P < 0.05$ vs. si-NC ($n = 3$ replicates/group). The data were analyzed using unpaired Student's t-test or one-way ANOVA followed by Dunnett's post hoc test. DOX, doxorubicin; miR, microRNA; NC, negative control; si, siRNA; PKCε/*PRKCE*; protein kinase C epsilon; DNR, daunorubicin; CCK-8, Cell Counting Kit-8; NS, non-significant.

inhibiting translation when they only partially match the 3'UTR of target genes. However, if there is a perfect match, miRNAs induce mRNA cleavage of the target genes (34,35). In animal cells, most interactions between miRNAs and their binding sequences are not completely complementary, and mismatches usually occur, especially in the central region of the target sequence (35). Based on the findings of the present study, miR-223 likely inhibits *PRKCE* mRNA translation. However, a previous study found that when miRNAs are temporarily overexpressed using mimics, the mRNA levels of target genes decrease 30 min after transfection, but start to recover after 12 h, while protein levels begin to recover after 24 h (36). In the present study, changes in *PRKCE* mRNA and protein levels were examined 24 h after transfection. It cannot therefore be definitively determined whether miR-223 functions as a

translation suppressor for *PRKCE* mRNA or if the observed effect is due to the recovery of *PRKCE* mRNA following transfection. Previous studies have also demonstrated the contrasting expression of miR-223 and PKCε. Reduced miR-223 expression and the accompanying elevation of PKCε levels were associated with the formation of Gottron's papules in dermatomyositis (37). Conversely, in ovarian cancer, increased miR-223 expression was observed alongside decreased levels of PKCε (38). Together with the results of the present study, this indicates that miR-223 can target and suppress PKCε protein expression.

Despite the aforementioned findings, the overexpression of miR-223 did not affect DOX sensitivity in the KG-1a cell line. As miRNAs have the capacity to regulate numerous genes by binding to the 3'UTR of target mRNAs, it is plausible that miR-223 may target multiple genes apart from PKCε.

For example, a previous study in colorectal cancer cells demonstrated that miR-223 promoted DOX resistance by regulating epithelial-mesenchymal transition via targeting of *FBXW7* (39). Therefore, it is possible that miR-223 may regulate other genes that have a more significant impact on drug resistance than PKC ϵ in the KG-1a LSC line.

It was also observed that downregulation of PKC ϵ using siRNA did not improve the sensitivity to DNR in KG-1a cells. Despite a recent study by Nicholson *et al* (20), which demonstrated that inducing PKC ϵ overexpression in AML cell lines resulted in specific resistance to DNR through an increase in P-glycoprotein levels, it was also observed that PKC ϵ knockdown in the AML U937 and MV4-11 cell lines did not impact chemosensitivity. This indicates that PKC ϵ inhibition alone may be insufficient to restore drug resistance in AML cells, possibly due to the redundancy among PKC isoforms. Drug resistance in LSCs can arise from various mechanisms beyond drug efflux transporters. LSCs employ crucial signaling pathways, such as Wnt/ β -catenin, Hedgehog, NOTCH and PI3K/AKT, to manage their stemness traits. This regulation induces a state of dormancy (G_0 state), which protects them from cell cycle-specific factors that target actively proliferating cells. Dysfunctions of apoptotic signals and senescence mechanisms in LSCs also contribute to chemotherapy failure. Metabolic reprogramming enables LSCs to adapt to energy level fluctuations, while epigenetic modifications and reprogramming further enhance their stemness properties (32). Some studies have discussed the role of PKC ϵ in the regulation of stemness features, such as differentiation and self-renewal. For example, downregulation of PKC ϵ was indicated to preferentially promote the differentiation of colorectal cancer cells (40). The phosphorylation of ERK-1/2 and AKT, which typically promotes differentiation, was reduced through short hairpin RNA-mediated knockdown of PKC ϵ in human pluripotent stem cells, resulting in a metastable undifferentiated state (15). In another study, PKC ϵ exhibited no notable influence on the efficiency of colony formation, but eliminated the formation of cobblestone area-forming cells, indicating that PKC ϵ selectively promotes the quiescence of HSCs (41). Based on this evidence, PKC ϵ may preferably function in the regulation of differentiation or self-renewal signaling pathways rather than by directly influencing drug sensitivity in AML.

The present study is constrained by certain limitations. Firstly, the experiments were conducted *in vitro*, specifically focusing on a single cell line, KG-1a cells, which may not fully represent the diversity of LSCs. The second limitation is the absence of clinical studies to confirm the results of the *in vitro* experiments, particularly the evaluation of miRNA-223 and PKC ϵ levels in newly diagnosed patients with AML before and after treatment, as well as the therapeutic response of these patients. Therefore, addressing these limitations should be a priority for future research endeavors.

Overall, the present study unveiled the downregulation of miR-223 in human LSC-like KG-1a cells. The biological function of miRNAs is to regulate target genes. In the present study, a luciferase reporter assay system and bioinformatics analysis were utilized to validate PKC ϵ as one of the target genes of miR-223 in KG-1a LSCs. However, both the overexpression of miR-223 and PKC ϵ knockdown failed to improve the chemosensitivity of KG-1a cells, suggesting that the miR-223/PKC ϵ

axis may not be associated with the sensitivity of KG-1a cells to anthracycline drugs, such as DOX and DNR. Consequently, further investigations are warranted to elucidate the roles of miR-223 and PKC ϵ in the drug resistance of LSCs. This understanding is crucial for unraveling drug resistance mechanisms and achieving objectives related to using miRNAs as biomarkers for diagnosis and prognosis, as well as developing targeted therapies in AML.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

MO performed the experiments, and contributed to data acquisition, statistical analysis and the preparation of the manuscript. SD contributed to the study conception and design and the manuscript review. CI performed the experiments regarding the PKC ϵ knockdown. SA advised on the design of the experiments regarding cell culture. PK provided the 293T cell line and advised on the use of molecular techniques during the experiments. SD and MO confirmed 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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