Original Article
PRAME induces apoptosis and inhibits proliferation of leukemic cells in vitro and in vivo

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Abstract: PRAME is a germinal tissue-specific gene that is expressed at high levels in haematological malignancies, but the physiological functions of PRAME in leukemia cells are unknown. It has reported that PRAME was found to be predominantly expressed in acute leukemias and high PRAME expression is correlated with a favorable prognosis in childhood acute leukemias, which suggested that PRAME could be involved in the regulation of cell death or apoptosis. In the present study, we tested a hypothesis that the PRAME gene plays a role in the regulation of apoptosis and proliferation of leukemia cells. We observed that KG-1 cells transient overexpressing the PRAME gene (when transfected with pcDNA3.1-PRAME plasmid) significantly induces apoptosis and decreases proliferation in vitro, and repression of PRAME expression by a short interfering RNA exhibited a increased proliferation in K562 cells in vitro and increases tumorigenicity of K562 leukemic cells in nude mice. Our results suggest that the leukemias expressing high levels of PRAME has favorable prognosis. PRAME may be as an attractive target for potential immunotherapy for acute leukemic.

Keywords: Acute leukemias, PRAME, apoptosis, proliferation

Introduction

PRAME (preferentially expressed antigen of melanoma) was originally identified as a tumor antigen recognized by HLA-A24- and HLA-A2-restricted cytotoxic T cells against a melanoma surface antigen [1, 2]. It is considered a melanocyte differentiation antigen which is overexpressed in both solid and hematologic tumors. Hematologic malignancies reported to overexpress PRAME include acute lymphoblastic and myelogenous leukemias (ALL and AML) [3-5], chronic myelogenous leukemia (CML) [6], chronic lymphocytic leukemia (CLL) and mantle cell lymphoma (MCL) [7, 8].

Overexpression of PRAME in human cancer cells confers growth or survival advantages and promotes malignant differentiation of stem cells [9]. After PRAME siRNA transfection, proliferation was suppressed and cell cycle analysis showed G(0)/G(1) arrest, followed by apoptosis. PRAME siRNA-treated cells also showed changes in the genes affecting erythroid differentiation [10]. However, a high expression of PRAME seems to be predominantly found in acute leukemias carrying a favorable prognosis [3, 11]. In addition, overexpression of PRAME seems to be associated with significantly higher rates of overall and disease-free survival and lower relapse rate, compared with patients with no or low PRAME expression [5]. The function of PRAME and its effect on gene expression in leukemic cells remains controversial due to conflicting observations in the literature.

In the present study, we aim at evaluating the contribution of PRAME in the regulation of cell proliferation, apoptosis, and tumorigenicity in vitro and in vivo.

Materials and methods

Cell line

KG-1 and K562 cells were purchased from the Shanghai Cell Bank of Chinese Academy of Sciences. KG-1 and K562 cells were main-

tained in RPMI l640 containing 10% fetal bovine serum (FBS) at 37°C in an environment with 5% CO_2.

PRAME plasmid construct and transfection

To construct a plasmid expressing PRAME, the full-length human PRAME gene cDNA clone (Tzrd.co, Beijing, China) was digested with Sall and BamHI, then cloned into pCDNA3 to generate pCDNA3-PRAME (pPRAME). All the constructions were verified by sequence analysis. For transfection studies, KG-1 cells were plated at a density of 1×10^6 cells per well in six-well plates and incubated for 24 h in complete medium. The cells were then

Transfected with 2 μg of the pPRAME by using an Lipofectamine 2000 Reagent (Invitrogen, Carlsbad, CA) for 48 h following the instructions of the manufacturer. For controls, the same amount of empty vector pcDNA3.1 was also transfected. For selection of stably transfected KG-1 cells, G418 (Life Technologies) was added to the culture medium 48 hours after transfection at a concentration of 600 ug/mL. After 4 weeks of selection by G418, these stably transfected cells were screened by Western blot assay.

PRAME short interfering RNA transfection

PRAME-short interfering RNA [PRAME siRNA (h)] was purchased by Invivogen (Guangzhou, China). It was cloned in a psiRNA-h7SKblasti expression vector (Invivogen). As control, we used a siRNA targeted to green fluorescent protein (GFP-siRNA). Control and PRAME-siRNA-encoding plasmids were transfected into K562 cells. Cells were selected byG418 (400 ug/Ml) during at least 2 weeks. PRAME silencing was confirmed by real-time PCR and Western blot.

RT-PCR

Total RNA was isolated from the transfected cells using the Qiagen RNeasy kit (Qiagen, Inc., Valencia, CA) according to the manufacturer's protocol, and OneStep RT-PCR kit (Qiagen) was used for detecting mRNA expression of PRAME. First-strand cDNA was prepared using Omniscript and Sensiscript reverse transcriptases at 50°C for 30 min. PCR amplification was then carried out under the following conditions:

95°C for 15 min, followed by 35 cycles at 94°C for 1 min and GAPDH as an internal control) for 1 min, and at 72°C for 1 min. The final extension was completed at 72°C for 10 min. The primers used are as below. 5'-CTCTATGTGGAC-TCTTTATTTTTCCTTAGA-3', 5'-CGAAAGCCGGC-AGTTAGTTATT-3'.

Western blot analysis

Western blot analysis was performed with the standard method with antibodies to PRAME and GAPDH (Abcam, Shanghai, China). Cells were lysed by sonication and the debris cleared in a microcentrifuge. A total of 40 μg of lysate were loaded into each well of 6% or 10% Tris-glycine polyacrylamide minigels (Invitrogen) for SDS-PAGE analysis. Proteins were transferred to PVDF membranes (Invitrogen), blocked for 1 h in TBS-T plus 5% (w/v) powdered blotting grade milk (Bio-Rad Laboratories, Hercules, CA, USA), and then probed overnight at 40°C with primary antibodies at a 1:2000 dilution in blocking solution. Blots were developed using enhanced chemiluminescence (ECL Plus) reagents from Amersham Biosciences (GE Healthcare, Buckinghamshire, UK).

Cell proliferation assay

Cells transfected with the pPRAME plasmid or siRNA plasmid or with a control plasmid were seeded at a density of 1×10^3/well in 96-well culture dishes. After 24 h, the cells were incubated with MTT (0.5 mg/ml; Sigma) at 37°C for 4 h and then with DMSO at room temperature for 1 h. At 72 h, the spectrophotometric absorbance of the samples was determined by using Ultra Multifunctional Microplate Reader at 495 nm.

Colony formation assay

Cells transfected with the pPRAME plasmid or siRNA plasmid or with a control plasmid were mixed with RPMI medium 1640 containing 10% FCS and 0.5% agar (bottom layer). Cells (5,000 cells per well) in 20% FCS/0.7% agarose (top layer) were plated and incubated at 37°C for 14 days. After incubation, plates were stained with 0.005% crystal violet for more than 2 h, and colonies were counted in two colony grids per well by using a microscope.
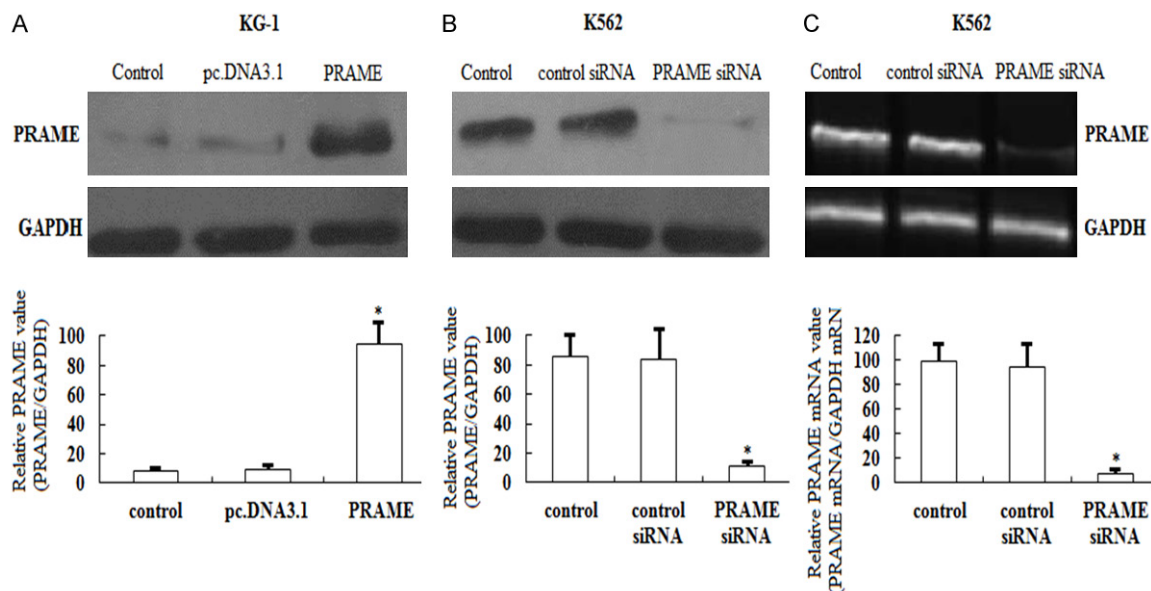
Figure 1. PRAME detection in PRAME-silenced K562 and PRAME-overexpressing KG-1 leukemic cells. A. Cell extracts were analyzed for expression of the PRAME proteins using specific antibodies in PRAME -overexpressing KG-1 leukemic cells. B. Cell extracts were analyzed for expression of the PRAME proteins using specific antibodies in PRAME-silenced K562 leukemic cells. C. PRAME mRNA were analyzed in PRAME-silenced K562 leukemic cells. The experiment was repeated twice with similar results. Levels are reported as percent of control ± SD. Vs pc.DNA3.1 or control siRNA, *P<0.01.

Terminal dUTP nick-end labeling (TUNEL) assay

Transfected cells were cultured on chamber slides and allowed to adhere overnight. Apoptosis of the cells was evaluated on the basis of the TUNEL assay using the Dead End Fluorometric TUNEL System (Promega, Madison, WI) according to the manufacturer's instructions. All assays were performed in quadruplicate.

Flow cytometry

Cells (1×10^6) were harvested and the pellets were washed twice with PBS. Cells were then fixed in cold 70% ethanol added dropwise while vortexing gently. Fixed cells were kept overnight at 4°C. Cells were centrifuged and pellets were resuspended in 1 mL propidium iodide/RNase staining buffer (BD Biosciences). Reactions were incubated for 20 min at 4°C and protected from the light. Samples were analyzed by flow cytometry using a FACSCalibur flow cytometer (BD Biosciences).

Caspase-3 activity assay

Lysates were assayed for caspase-3 activity using the BD ApoAlert fluorometric Caspase

Assay Plate (BD Biosciences Clontech, Palo Alto, CA) in accordance with the manufacturer's instructions. Plates were read (excitation, 360 nm; emission, 480 nm) using a CytoFluor 4000 multiwell fluorescence plate reader (Applied Biosystems, Foster City, CA). A minimum of three determinations was done for each sample.

Tumor formation in nude mice

For in vivo experiment, stable PRAME-siRNA-expressing K562 cells or control cells (1×10^6) in 200 µL serum-free DMEM (Life Technologies) were injected s.c. into the left flank of 4 week-old nude mice (6 mice). Tumor-bearing mice were sacrificed after 3 weeks and tumor masses were excised and weighed. All experiments were done in accordance with institutional standard guidelines of People's hospital of Linyi for animal experiments.

Statistics

Student's t test was used to determine whether the difference between control and experimental samples was significant (P<0.05).
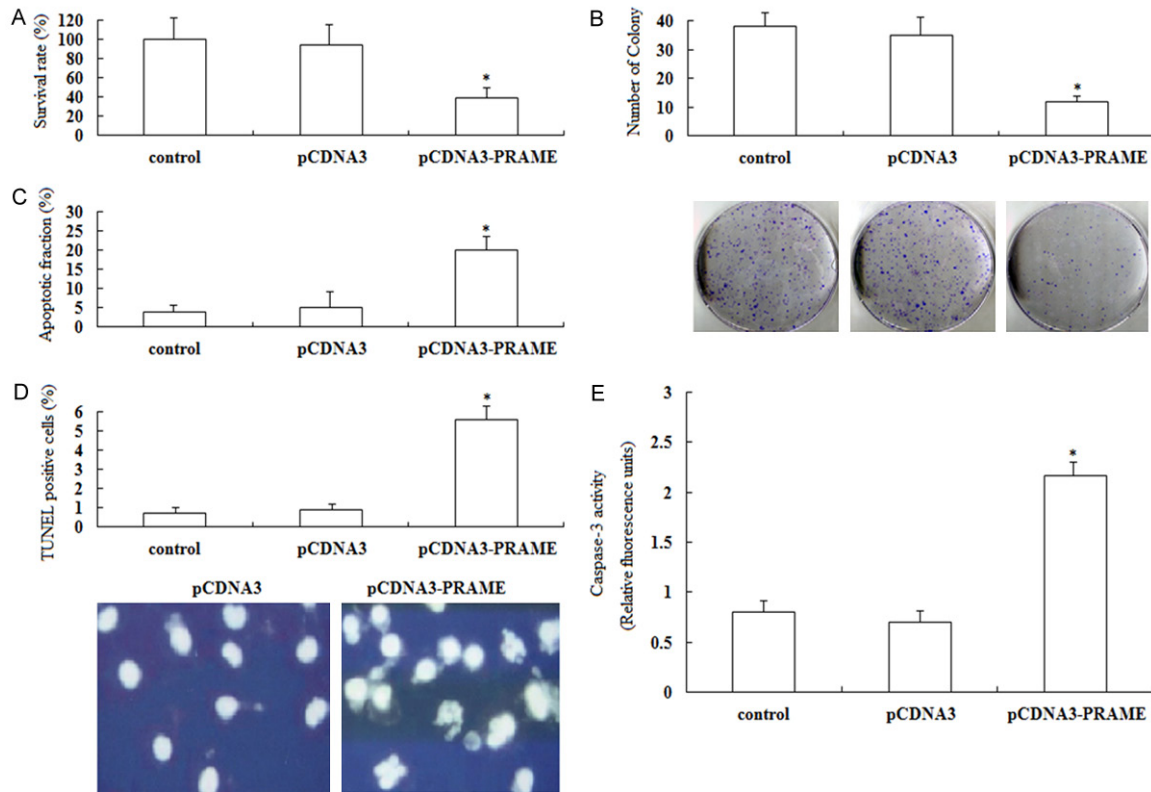
Figure 2. Effect of transfection on cell proliferation and apoptosis in pCDNA3-PRAME transfected KG-1 cells. A. Effect of pCDNA3-PRAME transfection on cell proliferation by the MTT assay. B. Representative of colony formation in soft agar. Percentage of colonies formed was summarized. Values were the mean ± SD of at least three independent experiments. C. Apoptotic fraction of cells was determined by flow cytometric analysis of at least 10,000 cells. D. Terminal deoxynucleotidyl transferase biotin-dUTP nick-end labeling apoptosis assay was done for DNA fragmentation/fluorescence staining. E. Cellular caspase 3 activity in pCDNA3-PRAME transfected KG-1 cells. Vs pc.DNA3.1, *P<0.01.

Results

Effect of pCDNA3-PRAME transfection on PRAME expression in KG-1 cell

KG-1 cells display very low PRAME expression in the KG-1 cells by western blot assay. KG-1 cells transfected with pc.DNA3.1-PRAME plasmid for 48 h displayed a significant increase in the expression levels of PRAME as compared with vector control (**Figure 1A**). Because PRAME expression was observed to be very high 48 h after transfection, we selected this time point for further studies.

Effect of PRAME-siRNA transfection on PRAME expression in K562 cells

High PRAME expression was found in the K562 cells by western blot (**Figure 1B**) and RT-PCR (**Figure 1C**) assay. In stable PRAME-siRNA

transfected K562 cells, PRAME expression was significantly suppressed by western blot (**Figure 1B**) and RT-PCR (**Figure 1C**) assay. Nonsilencing siRNA did not exhibit any effect on protein levels (**Figure 1B**) and gene levels (**Figure 1C**) of PRAME. These data confirmed the suppression effect of siRNA transfection.

PRAME overexpression inhibits KG-1 cell proliferation and colony formation

KG-1 cells were transiently transfected with pCDNA3-PRAME for 72 h, and the cell viability was determined by MTT assay. The KG-1 cells transfected with pCDNA3-PRAME resulted in a significant inhibition of cell proliferation compared to the control (**Figure 2A**). To investigate the effect of PRAME overexpression on the growth of KG-1 cells, we performed a soft agar colony-formation assay. As shown in **Figure 2B**, pCDNA3-PRAME -transfected KG-1 cells formed
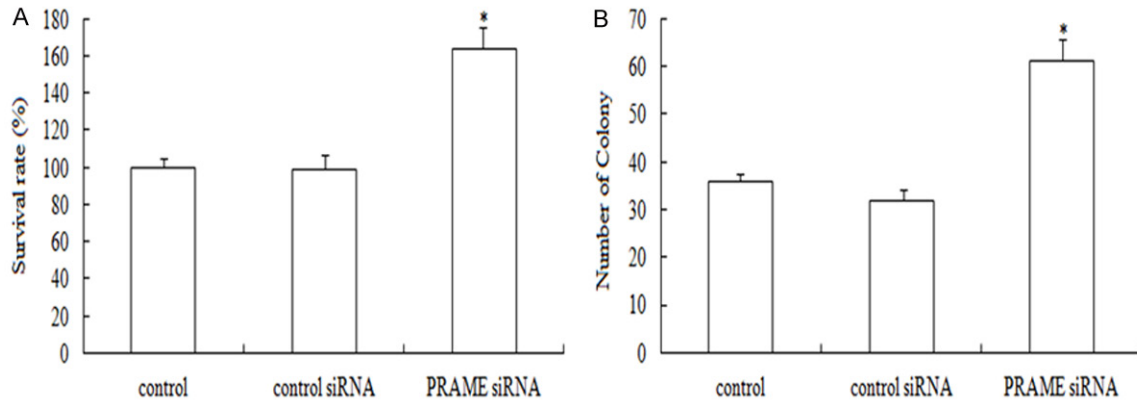
Figure 3. Effect of transfection on cell proliferation s in PRAME siRNA transfected K562 cells. A. Effect of PRAME siRNA transfection on cell proliferation by the MTT assay. B. Percentage of colonies formed was summarized. Values were the mean ± SD of at least three independent experiments. Vs control siRNA, *P<0.01.
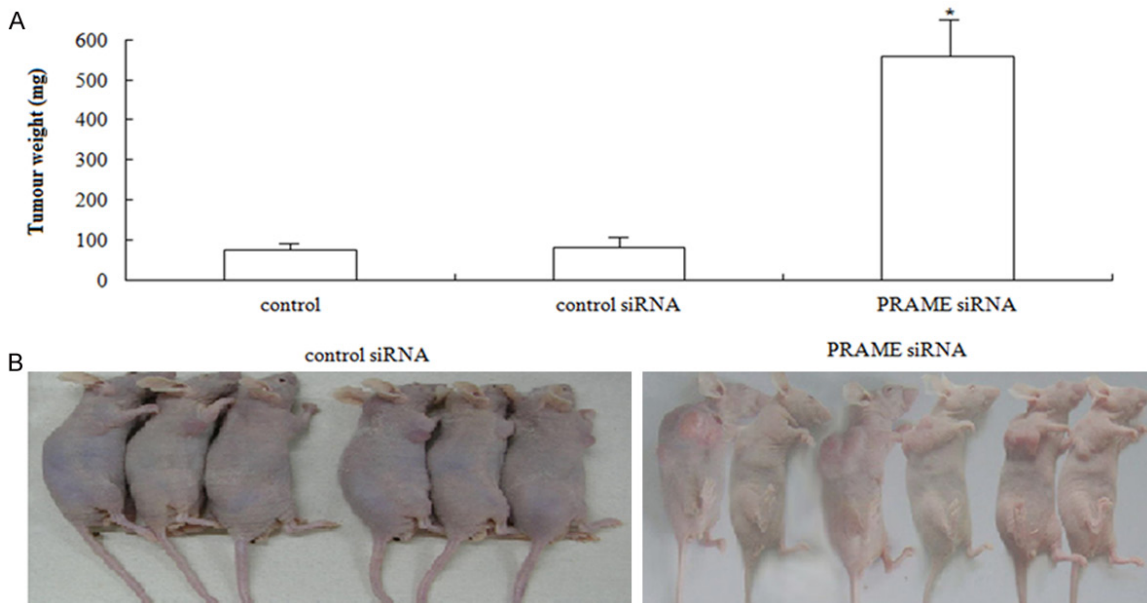

Figure 4. Tumorigenicity of PRAME knockdown leukemic cells in nude mice. A. Tumor weights are quantified. B. Nude mice injected with control siRNA transfected cells or PRAME-siRNA transfected cells. Vs control siRNA, *P<0.01.

a significantly reduced (P<0.01) percentage of colonies compared with control transfected cells, demonstrating the growth-inhibiting effects of PRAME on KG-1 cells.

PRAME overexpression promotes apoptosis in KG-1 cells

KG-1 cells were transiently transfected with pCDNA3-PRAME for 72 h, cells were fixed and stained with propidium iodide for flow cytometric analysis. In PRAME overexpressed KG-1 cell, apoptosis was markedly increased at 72 h time point (P<0.01), compared to the control pCDNA3 transfected KG-1 cells (**Figure 2C**).

TUNEL assay has the same results as flow cytometric (**Figure 2D**).

Caspase 3 activities of cell lysates, obtained following pCDNA3-PRAME transfection for 72 h in KG-1 cells were quantified by fluorometric assay. KG-1/pPRAME transfectants exhibited a 48% higher level of caspase-3 activation than control pCDNA3 transfectants (**Figure 2E**).

Knockdown of PRAME promotes proliferation and colony formation in K562 cells

K562 cells transiently transfected with PRAME siRNA for 72 h resulted in a significant increase

of cell proliferation compared to the control determined by MTT assay (**Figure 3A**). Soft agar colony-formation assay showed that PRAME siRNA-transfected K562 cells formed a significantly increased (P<0.01) percentage of colonies compared with control transfected cells (**Figure 3B**), demonstrating the growth-promoting effects of PRAME inhibition on K562 cells.

Knockdown of PRAME promotes tumorigenicity of leukemic cells in nude mice

Having shown that knockdown of PRAME promotes proliferation and colony formation in K562 cells in vitro, we tested the growth of the tumors accomplished by PRAME siRNA transfected K562 cells using an s.c. K562 cells model. As shown in **Figure 4**, the PRAME siRNA-transfected tumor cells showed a significant increased weigh as compared with control siRNA transfected groups.

Discussion

Though PRAME is absent or expressed at very low levels in most normal tissues tested, high levels of PRAME mRNAs are encountered in malignant cells, including the vast majority of primary and metastatic melanomas (88% and 95% respectively) [12]. Microarray and PCR studies have shown that PRAME is absent in normal haematopoietic tissues including bone marrow, CD34+sorted bone marrow cells, unsorted peripheral blood cells and sorted B and T lymphocytes [5, 6, 13, 14]. However, numerous studies have reported highly elevated levels of PRAME in both acute and chronic leukaemias [3-8]. PRAME up-regulation was observed in most AML cases with t(8; 21) karyotype and 45% of AML cases with t(15;17). In CML, PRAME expression was found to correlate with disease progression, showing increased expression in blast crisis as compared with chronic phase disease [6, 14].

Although the role of PRAME in acute leukaemia is complex, it has promise both as a cancer biomarker and as a therapeutic target. In AML, PRAME is usually associated with a favourable response to chemotherapy and favourable progress [13, 15]. In contrast, over-expression of PRAME mRNA is associated with poor prognosis in solid organ malignancies [16-18]. This raises the possibility that PRAME may have different roles in oncogenesis or tumour suppression dependent on the tumour type. This raises the possibility that PRAME may have different

roles in oncogenesis or tumour suppression dependent on the tumour type. Therefore, its usefulness in predicting clinical outcome in solid tumours remains unclear. However PRAME remains relevant in acute leukaemias for risk stratification, to monitor residual disease and as a potential target for immunotherapies. In the present study, we investigate whether PRAME may be as a potential target for eukaemias treatment, and tested a hypothesis that the PRAME gene plays a role in the regulation of apoptosis and proliferation of leukemia cells.

In this study, we show that overexpression of the PRAME induced apoptosis and inhibited proliferation and colony-formation in KG-1 cells in vitro. We also show that suppression of PRAME gene by siRNA promotes the growth and proliferative potential of human K562 cells.

On the basis of our results in vitro, we propose a model to describe the role of suppression of PRAME gene by siRNA. In this model, we found PRAME-siRNA-transfected leukemic K562 cells were injected into nude mice for 20 days. We observed that PRAME-siRNA-transfected cells proliferated more rapidly in vivo than control-siRNA-transfected cells. The tumor weight was significantly higher in mice injected with PRAME-siRNA than in mice injected with control-siRNA-transfected cells. These results show that PRAME down-regulation promotes proliferation of leukemic cells in vivo.

In conclusion, we shows that overexpression of PRAME in cultured cells induces cell apoptosis and decreases proliferation, and inhibition of PRAME in cultured cells promotes proliferation and tumor formation in nude mice.

We suggest that these observations could explain the goodprognosis of PRAME-expressing childhood acute leukemias. PRAME may be as a potential target for eukaemias treatment.

Disclosure of conflict of interest

None.

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