

## Original Article

# Effects of down-regulation of clusterin by small interference RNA on human acute myeloid leukemia cells

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**Abstract:** Aims and background: Up-regulation of clusterin is associated with the survival and progression of various malignancies, and down-regulation of clusterin promotes apoptosis and inhibits invasion. The aim of this study was to explore the effect of clusterin small interference RNA (siRNA) on the proliferation, apoptosis and invasion of HL-60 acute myeloid leukemia (AML) cells. Methods: siRNA transfection was performed using Lipofectamine™2000 reagent. Relative protein expressions were quantified by Western blot. Trypan blue assay was performed to assess tumor cell proliferation after siRNA transfection. The cytotoxic effect of clusterin siRNA on leukemic cells was measured using MTT assay. Apoptosis was detected using fluorescence microscopy assay. Migration and invasion was detected after clusterin was silenced. Results: Clusterin siRNA clearly lowered clusterin protein levels in a time-dependent manner, leading to marked inhibition of cell survival, proliferation and invasion. Furthermore, clusterin down-regulation significantly enhanced the extent of HL-60 apoptotic cells. Conclusions: Our results suggest that the down-regulation of clusterin by siRNA can effectively trigger apoptosis and inhibit the proliferation and invasion of leukemic cells. Therefore, clusterin siRNA may be a potent adjuvant in AML therapy.

**Keywords:** Acute myeloid leukemia, apoptosis, invasion, clusterin, small interference RNA

## Introduction

Acute myeloid leukemia (AML) is a lethal disorder characterized by the accumulation of abnormal myeloid progenitor cells in the bone marrow, which results in hematopoietic failure. Despite various efforts in detection and treatment, many patients with AML continue to die of this cancer [1-3]. Therefore, the development of novel therapeutic options is imperative, particularly those employing strategies targeting genes involved in apoptosis and tumor progression.

Clusterin, also known as testosterone-repressed message-2, is overexpressed in the rat prostate during castration-induced programmed cell death [4]. Clusterin overexpression reaches a maximum 3-4 days post-castration and coincides with the onset of massive cell death [5]. The clusterin level also rises in various tissues during cell death responses [6, 7].

As clusterin is present during apoptosis, it was initially viewed as a cell death inducer, but other studies suggest that clusterin overproduction occurs in resistant cells [8, 9]. Thus, clusterin has been described as an anti-apoptotic factor, and it has also been implicated in prostate cancer progression to androgen independence.

Xie et al. has found that overexpression of clusterin was detected more frequently in metastatic lesions than that in their matched primary tumors, and overexpression of cytoplasmic clusterin in carcinomas was correlated inversely with the tumors' apoptotic index, demonstrating an antiapoptotic function of cytoplasmic clusterin in ovarian carcinomas [10]. In human hepatocellular carcinoma [11], renal cell carcinoma [12], pancreatic endocrine tumours [13], colorectal cancer [14], breast cancer [15], and cervical neoplasia [16], clusterin was also overexpressed in these cancer cells, and clusterin protein accumulation correlates with the

## Clusterin on acute myeloid leukemia cell

aggressiveness of these tumors. These features make clusterin an attractive target against which cancer therapeutics could be developed.

Niu et al. has found that sCLU silencing increased the chemosensitivity of A549<sup>DDP</sup> cells to DDP in vivo [17]. Zhang et al. has found that sCLU silencing promotes cisplatin antitumor activity in human non-small cell lung cancer xenografts in immunodeficient mice [18]. Other studies have found that clusterin silencing alone inhibited proliferation and reduces invasion in human laryngeal squamous carcinoma cells and breast cancer cells [19, 20]. Although clusterin has long been proposed to participate in cell survival and it has been extensively studied to inhibit apoptosis, no studies have been carried out to investigate the effect of clusterin silencing or overexpression on AML cells.

In this study, clusterin small interference RNA (siRNA) was transfected into AML cell line HL-60 in vitro, to investigate the impact of clusterin gene suppression on the human AML cell apoptosis, proliferation and invasion.

### Materials and methods

#### *Cell culture*

The HL-60 leukemic cells were maintained in RPMI-1640 culture medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Sigma-Aldrich), 1% antibiotics (100 IU/ml penicillin, 100 µg/mL streptomycin) (Sigma-Aldrich), 2 mM of glutamine, and 1% sodium pyruvate at 37°C in 95% humidified atmosphere containing 5% CO<sub>2</sub>. The cells were sub-cultured with an initial concentration of 5×10<sup>4</sup> cells/ml and used in the logarithmic growth phase in whole.

#### *Clusterin siRNA transfection*

Clusterin-shRNA and its control scrambled plasmid was kindly gifted by Dr Zhang [18]. Just before transfection, the cells were grown in RPMI-1640 medium free of antibiotics and FBS. shRNA transfection (at a final concentration of 50 nM in all experiments) was performed using Lipofectamine<sup>TM</sup>2000 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. In brief, siRNA lipofectamine (4 µL/mL of transfection medium) were separately diluted in Opti-MEM I Reduced

Serum Medium (Invitrogen) and incubated for 10 min at ambient temperature. The diluted solutions were then combined and incubated for 20 min at ambient temperature. Following on, the mixtures were added to each well containing medium and cells. Furthermore, the treated cells with only lipofectamine were considered as a siRNA blank control group. The cells were then incubated for 6 h at 37°C in a humidified CO<sub>2</sub> incubator. Subsequently, complete cell culture medium (with final FBS concentration of 15%) was added and the cells were incubated under the same mentioned conditions. To monitor the effect of siRNA on gene silencing, transfection (5×10<sup>5</sup> cells/well) were done in 6-well plates for 24-72 h. Down-regulation of clusterin expression was then measured by Western blot analysis.

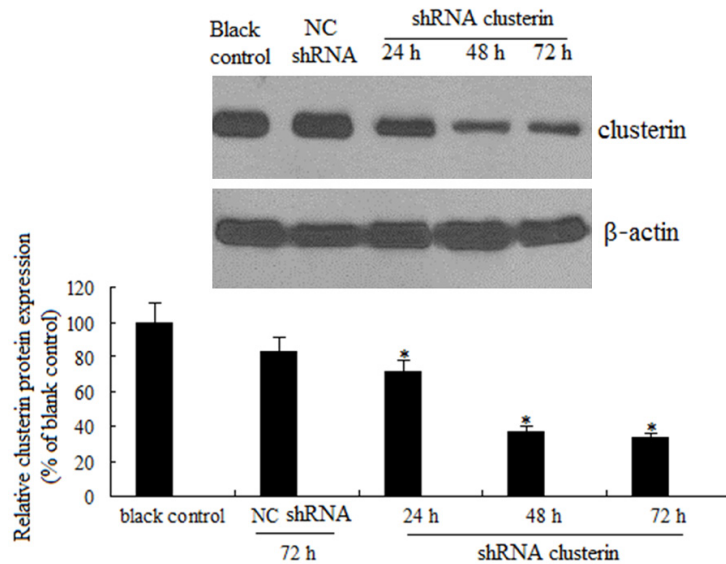
#### *Western blotting*

Following various transfection, cells were harvested by trypsinization (trypsin 0.25% w v<sup>-1</sup>, 1 mM ethylenediaminetetraacetic acid). The cells were lysed in a lysis buffer containing 150 mM NaCl, 1% Triton X-100 and 25 mM Tris (pH 7.5). Debris was sedimented by centrifugation for 5 min at 12 000 g, and the supernatants were heated for 5 min at 100°C in Laemmli's sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer containing 100 mM dithiothreitol. Protein concentrations of the lysates were determined with a protein quantitation kit (Bio-Rad Laboratories, Hercules, CA, USA), and 50 µg of each sample was separated on a 10% SDS-PAGE gel. Separated polypeptides were then electrophoretically transferred to 0.2-mm nitrocellulose membranes (Schleicher & Schuell, Keene, NH, USA). Membranes were blocked for 1 h in a Tris-buffered saline-Tween (25 mM Tris, pH 8.0, 150 mM NaCl, and 0.05% Tween-20) containing 5% (w v<sup>-1</sup>) nonfat dried milk. The blots were then probed overnight with primary antibodies and developed using species-specific secondary and tertiary antisera. Immunoreactive material was detected by the enhanced chemiluminescence technique (Amersham).

#### *Cytotoxicity assay*

The cytotoxic effect of clusterin shRNA on HL-60 was determined using 3-(4,5 Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide (MTT) assay. The experiment was subdivided

## Clusterin on acute myeloid leukemia cell



**Figure 1.** Down-regulation of clusterin expression by shRNA in HL-60 cells. The cells were transfected with negative control (NC) shRNA or clusterin shRNA for 24, 48 and 72 h. Representative western blot of  $\beta$ -actin and clusterin proteins from cells transfected with NC shRNA or clusterin shRNA. The expression level of each band was quantified using densitometry and normalized to the respective  $\beta$ -actin. The results are expressed as mean  $\pm$  SD (n = 3); \*P<0.05 versus blank control and NC shRNA.

into five groups: Clusterin shRNA, NC shRNA and blank control. Briefly, leukemic cells were seeded at a density of  $1.5 \times 10^4$  cells in 96-well cell culture plates. The cells were then transfected with shRNA as described previously. After 24 and 48 h of incubation, the cytotoxicities of the treatments were measured using the MTT assay kit (Roche Diagnostics GmbH, Mannheim, Germany) following the manufacturer's recommendations. The amounts of formazan dyes were quantified by measuring their absorbance (A) at 570 nm with a reference wavelength of 650 nm using an ELISA plate reader (Awareness Technology, Palm City, FL, USA). The cell survival rate (SR) was measured from the following formula:  $SR (\%) = (A_{\text{Experiment}}/A_{\text{Blank control}}) \times 100\%$ .

### Cell proliferation assay

The effect of clusterin shRNA was evaluated using trypan blue exclusion assay. The cells ( $5 \times 10^4$  cells/well) were transfected with clusterin shRNA and its control in 24-well cell culture plates and then incubated for 24-120 h. At different time points after transfections, the cells were collected and stained with equal volume of 0.4% trypan blue dye for 1 min. Following on, the number of viable cells (N, unstained cells) was quantified using a hemocytometer

and an inverted microscope (Nikon Instrument Inc., Melville, NY, USA). The percentage of viable cells was then determined from the equation as follows:  $\text{Cell viability } (\%) = (N_{\text{Experiment}}/N_{\text{Blank control}}) \times 100$ . The percentage of viable cells in each time was also considered as 100% for blank control group.

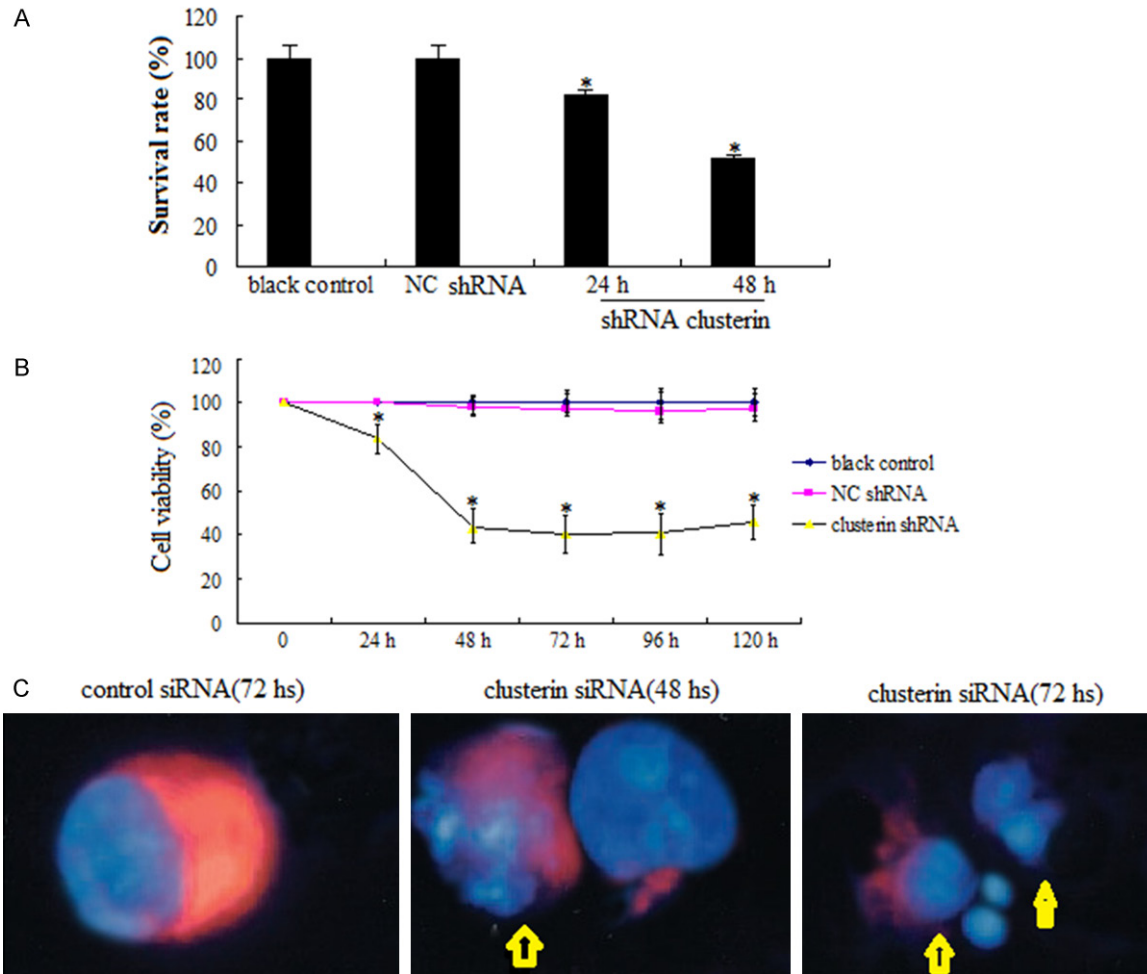
### Fluorescence microscopy

Clusterin shRNA and its control transfected cells (transfected for 48-72 hours) were trypsinized and loaded with 40 nM DiICl (5) and 10 mM Hoechst 33342 for 30 min at 37°C. They were then mounted on glass slides and imaged at 363 using an Olympus BX50 fluorescence microscope fitted with a Xillix MicroImager (Xillix, Vancouver, British Columbia, Canada). On excitation, DiICl (5) and Hoechst 33342 produced red and blue fluorescence, respectively. Apoptosis was determined by the identification of apoptotic nuclei with Hoechst 33342 and low DiICl (5) fluorescence.

### Cell invasion assay using matrigel

Cell invasion assays were performed using Transwell membrane filter inserts with 8-mm pore size (Corning Costar, Cambridge, MA). The upper surface of the Transwell membrane was coated with 250 mg/ml of growth factor-reduced Matrigel matrix (Becton Dickinson, Bedford, MA) overnight at 4°C, rehydrated once with 0.1% BSA in DMEM for 1 h at room temperature, and then placed in the upper compartment of six-well tissue culture plates. 48 hrs after transfection with clusterin shRNA and its control, HL-60 cells were removed from tissue culture flasks by a short exposure to 5 mM EDTA and washed once in PBS. Then  $2 \times 10^5$  cells in serum-free medium containing 0.1% BSA were added to each Transwell chamber and allowed to migrate toward the underside of the membrane for 18 h. After cells were fixed in 3.5% paraformaldehyde, cells on the upper surface of the membrane were removed by wiping with a cotton swab, and membranes were mounted onto glass slides. The relative number of invasion was determined by counting the

## Clusterin on acute myeloid leukemia cell



**Figure 2.** Effect of clusterin shRNA on the survival rate, proliferation and apoptosis of leukemia cells. A. HL-60 cells were transfected with NC shRNA or clusterin shRNA for 24 and 48 h and then the cytotoxicities were measured by MTT assay. The results are expressed as mean  $\pm$  SD (n = 4); \* $P$ <0.05 versus blank control and NC shRNA. B. Cell viability was determined by trypan blue assay over a period of 5 days. The results are expressed as mean  $\pm$  SD (n = 3); \* $P$ <0.05 versus blank control and NC siRNA. C. Fluorescence microscopic image. HL-60 cells transfected with clusterin shRNA for 48-72 hours were stained with DAPI (5) (red fluorescence) and Hoechst 33342 (blue fluorescence) to visualize nuclear integrity. Low clusterin expressed cells exhibit typical nuclear features of apoptosis (arrow).

number of invading EGFP-positive cells. The number of invading cells transfected with empty vector was assigned a value of 1.0 in each experiment. Twenty random fields/membrane were counted for each assay. Each determination represents the average of three separate experiments.

### Statistical analysis

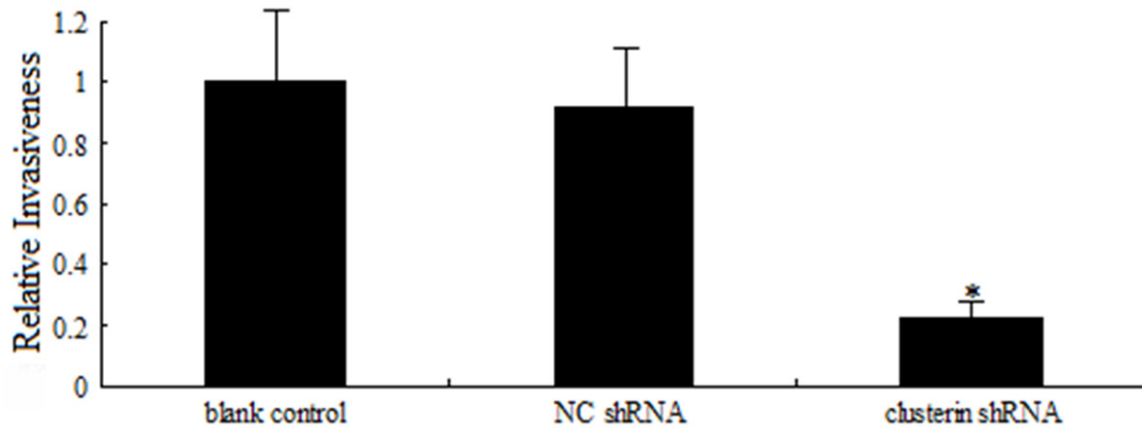
Data were expressed as mean  $\pm$  standard deviation (SD). Experiments were performed at least in triplicate. Comparisons were done with two tailed Student's  $t$  test. A value of  $P$ <0.05 was considered statistically significant.

## Results

### shRNA suppressed clusterin protein levels in leukemia cells

First, we explored the effect of shRNA on clusterin gene expression in HL-60 cells by Western blotting. Relative clusterin gene expression was calculated in relation to the blank control group, which was considered as 100%. As shown in **Figure 1**, clusterin shRNA led to a clear time-dependent reduction of clusterin protein levels ( $P$ <0.05; compared with the blank control and NC shRNA groups). At 24, 48 and 72 h post transfection, the relative clusterin protein

## Clusterin on acute myeloid leukemia cell



**Figure 3.** Decreased invasiveness potential of HL-60 cells transfected with clusterin shRNA to inhibit clusterin in a Matrigel invasion assay. The results are expressed as mean  $\pm$  SD (n = 3); \* $P$ <0.05 versus blank control and NC shRNA.

expression levels were 72.4%, 41.3% and 28.4%, respectively (**Figure 1**) ( $P$ <0.05). Notably, transfection with NC shRNA had an insignificant effect on clusterin expression compared to the blank control group.

### *Clusterin shRNA decreased the cell survival rate and inhibited the proliferation*

To assess the cytotoxic effect of clusterin down-regulation on HL-60 leukemia cells, the cells were treated with clusterin shRNA for 24 and 48 h and then analyzed in MTT assay. As shown in **Figure 2A**, the cytotoxicity significantly enhanced at 24 and 48 h in the clusterin shRNA transfected cells relative to the blank control and NC shRNA transfected cells. The results showed that clusterin shRNA significantly lowered the cell survival rate compared with the blank control group ( $P$ <0.05). In contrast, transfection with NC shRNA had a minimal cytotoxic effect on the leukemic cells relative to the blank control group ( $P$ >0.05; **Figure 2A**).

We next examined whether suppression of this protein could inhibit the proliferation of AML cells. The HL-60 cells were therefore transfected with clusterin shRNA and NC shRNA for 5 days and cell viability was measured every 24 h by trypan blue exclusion assay. Results showed that compared with the blank control or NC shRNA groups, clusterin shRNA significantly inhibited the proliferation of HL-60 cells over a period of 5 days ( $P$ <0.05; **Figure 3**). Meanwhile, no significant differences in cell proliferation

was observed between the NC shRNA group and the blank control groups ( $P$ >0.05; **Figure 2B**).

### *Effects of clusterin shRNA-induced apoptosis*

To analyze whether the observed cytotoxic effect of clusterin down-regulation was linked to the enhancement of apoptosis, the effect of clusterin shRNA on apoptosis were examined using fluorescence microscopy assay. Fluorescence microscopy with Hoechst 33342 and DiIc1 (5) dual labeling was used to examine the nuclei and energized mitochondria, respectively. Cells with low mitochondrial staining intensity (clusterin siRNA transfected cells) were observed to exhibit nuclear features of apoptosis such as nuclear fragmentation (**Figure 2C**). However, clusterin overexpressed cells did not show nuclear fragmentation (data not shown).

### *HL-60 cells transfected with clusterin shRNA decreased invasiveness*

We performed an *in vitro* cell invasion assay using Matrigel matrix to examine whether the invasiveness of HL-60 cells transfected with clusterin shRNA was decreased. Invading HL-60 cells transfected with control vector and clusterin shRNA. Invasive cells were assayed by counting EGFP positive cells using a fluorescence microscope. Invasiveness depicted as the average number of invading cells relative to controls in three individual experiments. As shown in **Figure 3**, suppression of the clusterin caused a >50% reduction ( $P$ <0.05) in the num-

ber of cells that traversed the membrane versus non-silencing control (by counting EGFP positive cells using a fluorescence microscope). These data suggest that the clusterin gene controls the motility and invasion of human HL-60 cells.

### Discussion

Molecular targeted therapy is a new emerging technology for treatment of cancer. Gene therapy is a potent kind of targeted therapy, in which the target is a specific gene overexpressed in tumor cells. The therapeutic agents, including ASO, ribozyme and siRNA are used to interfere with the expression of the target gene. As a result, the formation, growth and metastasis of tumors are inhibited [21-23].

Overexpression of clusterin is attributed to the tumor formation, development and metastasis [10-12]. On the contrary, different reports have shown that suppression of clusterin expression can induce apoptosis and inhibit the proliferation and invasion of tumor cells [17-20, 24]. Thus, we used a siRNA-based gene therapy strategy to target clusterin and evaluate its anti-leukemic effects.

Western blotting findings showed that transfection with clusterin shRNA led to steady decrease in the expression levels of clusterin protein over a 3-day period. These data revealed that clusterin shRNA effectively blocked the synthesis of the clusterin protein. The results of cytotoxicity assay exhibited that clusterin shRNA distinctly lowered the cell survival rate. Most notably, the results of the cell proliferation assay demonstrated that the suppression of clusterin expression significantly decreased the viability of HL-60 cells during a 5-day period, suggesting the critical role of clusterin in the proliferation of leukemic cells. In contrast, treatment with NC shRNA or lipofectamine displayed no significant changes in the gene expression and cellular events, demonstrating the specific impact of clusterin shRNA.

To further investigate the cellular role of clusterin in the development of leukemic cells, we examined the effect of clusterin suppression on induction of apoptosis. Results of fluorescence microscopy assay indicated that shRNA-mediated silencing of clusterin led to a remarkable spontaneous apoptosis. These results

illustrated the important biological role of clusterin in the survival and growth of leukemia cells. The above-mentioned results confirm that the presence of clusterin protein is required for the development and progression of HL-60 cells. Therefore, silencing of clusterin expression could induce spontaneous apoptosis and inhibit the proliferation of AML cells.

It has been reported that overexpression of clusterin prolongs cell survival under unfavorable conditions in the metastatic process, resulting in the enhanced metastatic potential of renal cell carcinoma [25]. In breast cancer in vivo and in vitro, over-expression of clusterin promoted invasion and metastasis of orthotopic primary MCF-7 tumors [26], and clusterin silencing could inhibit invasion and metastasis of MCF-7 tumors [20]. In this study, we show that clusterin suppression inhibited invasion in vitro in HL-60 cells. These data provide evidence that the clusterin gene may be associated with invasion and metastatic spread of human HL-60 cells.

In summary, we have demonstrated that clusterin has a critical role in the survival, growth and invasion of HL-60 cells. Specific knock-down of clusterin by shRNA induced apoptosis and inhibited the proliferation and invasion of leukemia cells in vitro. We therefore suggest that the shRNA-mediated silencing of clusterin may be considered as a novel treatment strategy for AML patients in the future. Future studies on animal models could further examine whether clusterin can be efficiently suppressed by shRNA expression.

### Disclosure of conflict of interest

None.

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## Clusterin on acute myeloid leukemia cell

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