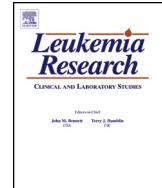




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Polymeric nanoparticle-mediated silencing of CD44 receptor in CD34⁺ acute myeloid leukemia cells

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ABSTRACT

The adhesion receptor CD44 plays an important role in the survival and retention of leukemic stem/progenitor cells (LSPC) within the bone marrow (BM) niche, as well as in the high relapse rates of acute myeloid leukemia (AML). Down-regulating CD44 could be clinically relevant not only for suppression of the deregulated function of LSPC but also in LSPC response to chemotherapeutic agents. Small interfering RNA (siRNA) delivery is a promising approach for AML treatment, and we recently reported effective siRNA delivery into difficult-to-transfect AML cell lines using lipid-substituted polyethylenimine/siRNA complexes (polymeric nanoparticles). In this study, we investigated polymeric nanoparticle-mediated silencing of CD44 in CD34⁺ LSPC cell models (leukemic KG-1 and KG-1a cell lines) as well as primary AML cells. Polymeric nanoparticle-mediated silencing decreased surface CD44 levels in KG-1, KG-1a and primary AML cells by up to 27%, 30% and 20% at day 3, respectively. Moreover, CD44 silencing resulted in induction of apoptosis in KG-1 cells, reduced adhesion of KG-1 and KG-1a cells to hyaluronic acid-coated cell culture plates and BM-MSC, and decreased adhesion of primary AML cells to BM-MSC. Our results suggest that polymeric nanoparticle-mediated silencing of CD44 might be a useful technique for inhibiting LSPC interactions with their microenvironment, thereby prohibiting leukemia progression or sensitizing LSPC to chemotherapy.

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1. Introduction

Acute myeloid leukemia (AML), a heterogeneous group of disorders, arises from critical genetic alterations in hematopoietic stem/progenitor cells (HSPC) that alter normal mechanisms of self-renewal, proliferation and differentiation [1]. The most effective way to eradicate AML is to use cytotoxic drugs, the current standard of clinical care. AML cells generally respond well to therapy at the onset of treatment, but even the most advanced drugs lose their effectiveness over the long term, and relapse rates remain quite high (more than 85%) [2,3]. The high relapse rate in AML

patients has been attributed to existence of a rare population of leukemia stem/progenitor cells (LSPC) [4,5]. LSPC, which are capable of propagating leukemia, are able to engraft, self-renew, and interact similar to normal HSPC with cells within the bone marrow (BM) niche (e.g., mesenchymal stromal cells (MSC) and osteoblasts) [6,7]. In fact, during the development of leukemia, the BM niche converts into an environment with dominant signals that favor LSPC survival and expansion and protect LSPC from chemotherapy [8–10]. Thus, the development of novel strategies to displace these cells from the BM niche is required for the inhibition of AML progression and elimination of drug resistance.

The adhesion receptor CD44, a ubiquitously expressed trans-membrane glycoprotein that mediates cell-cell and cell-extracellular matrix interactions through binding with its major ligand hyaluronic acid (HA) [11], has been shown to play a pivotal role in the trafficking of HSPC [11] and in the homing and maintenance of AML LSPC [12]. In fact, it has been reported that

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Table 1

Clinical characteristic and surface CD44 and CD34 levels of patients.

Pt #	Age/sex	Diagnosis (WHO classification)	Karyotype	WBC 10 ⁹ /L PB	% Blasts in PB	% Blasts in BM	CD44%	CD34%
1	22/M	AML without maturation	51~54, XY, +Y, +4,+8,+10,+13,+20,+21,+22(CP20)	166	87	94	98.9	60
2	58/M	AML without maturation	46, XY (20)	59.8	73	83	95	86
3	78/M	AML without maturation	46, XY (20)	101.4	81	88	98	84

WHO: World Health Organization; WBC: white blood cell count at diagnosis; PB: peripheral blood; BM: bone marrow.

the inhibition of CD44 by anti-CD44 monoclonal antibody (moAb) was able to block human AML LSPC reconstitution in NOD-SCID mice without affecting normal HSPC engraftment. The antibody bound to CD44 and selectively eliminated AML LSPC *in vivo* by blocking LSPC interactions with the niche [12]. In addition, CD44 was identified as a crucial surface receptor on BCR-ABL-positive LSPC of chronic myeloid leukemia (CML) involved in homing, migration and retention in the BM [13]. More importantly, clinical experience indicates that CD44 is associated with poor prognosis of AML, and high CD44 levels are associated with higher relapse rates [14,15]. In support of its crucial role in AML relapse, it has been recently reported that the high level expression of CD44 by AML cells was sufficient to generate leukemia by leukemia-initiating cells even after withdrawal of the HoxA10 gene overexpression event that initiated the leukemia [16]. Therefore, down-regulating CD44 might be a feasible approach to inhibit leukemia progression, sensitize LSPC to drug therapy, and prevent relapse by dislodging the LSPC from the protective BM niche. Although this could be achieved using anti-CD44-moAb-based approaches or small molecule inhibitors [17], strategic alternatives are continually being sought to overcome the low efficacy/safety ratio, toxicity and immunogenicity of moAbs and the toxicity and short half-life of inhibitors [18–21].

Non-viral lipid or polymer-based gene silencing with RNA interference (RNAi) is a promising therapeutic approach for treatment of various diseases including cancer [22–27]. Synthetic small interfering RNAs (siRNAs) promote mRNA degradation in the process of RNAi and prevent the translation of proteins. Li et al. and Subramaniam et al. showed that siRNA directed against CD44 could efficiently inhibit CD44 expression in ovarian and colon cancer cell lines [28,29]. More importantly, CD44 silencing led to inhibition of *in vitro* adhesion, invasion and resistance to apoptosis of ovarian cancer cells, and suppressed the growth of colon and ovarian tumors. Furthermore, Shah et al. and Van Phuc et al. showed that CD44-siRNA treatment enhanced the efficiency of chemotherapy in ovarian and breast cancer cells, respectively [30,31]. Although lipid-based approaches have been widely used for transfecting siRNAs into adherent cells, success in leukemic cells has been limited [32–34]. Use of RNAi in leukemic cells mainly relies on viral delivery [35,36], which is not amenable for clinical practice due to the undesirable consequences of the viral integration process and potential development of unwanted immune responses against vectors [37]. In fact, siRNA silencing in LSPC has been shown to be the most challenging [38], making siRNA-mediated anti-CD44 approaches a difficult therapeutic modality. To overcome this limitation, we recently developed functional lipid-substituted polymeric materials that could efficiently deliver siRNA in both adherent breast cancer cells and leukemic cells grown in suspension [25–27,34,39]. We pursued silencing of various model (e.g., GFP) and targets (e.g., CXCR4, BCR-ABL) in differentiated types of AML and CML cell lines [34,39]. Importantly, we observed that cellular delivery of siRNA was dependent on the nature of lipid substituent, the extent of lipid substitution, and varied among the different AML cell lines as well as CML cell lines. We capitalized on our initial findings on the carrier features responsible for effective siRNA delivery and therefore, here we used caprylic acid and linoleic acid to modify polyethyleneimine. In this study, we investigated the silencing

of CD44 receptor by lipid-substituted polymeric nanoparticles in immature CD34⁺ leukemic cell lines as well as primary AML cells.

2. Materials and methods

2.1. Cells and cultures

The AML cell lines KG-1 (CD34⁺/CD38⁺) and KG-1a (CD34⁺/CD38⁻) were purchased from American Type Culture Collection (Rockville, MD, USA). Cells were then maintained in Iscove's Modified Dulbecco's Medium (IMDM, GibcoBRL, Long Island, NY, USA) supplemented with 20% bovine growth serum (BGS; Hyclone, Logan, UT, USA).

Blood samples were obtained from patients diagnosed with AML at the University of Alberta Hospital (Edmonton, AB, Canada) with patients' informed consents and the approval of the Health Research Ethics Board. AML subtypes were classified according to the World Health Organization guidelines, and only patient samples that contained a high percentage of CD34⁺ cells were selected (60–86%, Table 1). Light density mononuclear cells (MNC) were isolated by Ficoll/Hyphaque density centrifugation as described previously and frozen until further use [40]. AML cells were thawed and maintained in IMDM supplemented with 20% BGS for at least 4 h prior to siRNA delivery studies. Cell viability was measured by the trypan blue exclusion assay.

Bone marrow-derived mesenchymal stromal cells (BM-MSC) were established from femoral reaming during total hip arthroplasty procedures with the patients' informed consent and the approval of the Health Research Ethics Board as described previously [41]. BM-MSC were cultured in IMDM supplemented with 10% fetal bovine serum (FBS; PAA Laboratories Inc., Etobicoke, ON, Canada).

2.2. Synthesis of lipid-substituted polymers

Lipid-modified polyethylenimines (PEIs) were synthesized using a modification of previously published procedures [34,39]. Caprylic acid (CA) and linoleic acid (LA) were used as the specific lipids for N-acylation of 2 kDa PEI (PEI2; purified by freeze-drying of manufacturer-supplied product). In a typical reaction, lipid chlorides individually dissolved in 5 mL of anhydrous dichloromethane were added drop-wise to 100 mg of PEI2 in 15 mL of dichloromethane (containing 50 μL of triethylamine). After 24 h at room temperature under argon, the solutions were concentrated by a rotary evaporator, polymers precipitated in ethyl ether and then washed with excess ethyl ether. After dissolving the precipitated polymers in Ultrapure water, the solutions were freeze-dried at room temperature to obtain polymer powders. The CA- and LA-substituted polymers were designated as PEI2-CA and PEI2-LA, respectively. The polymers were analyzed by ¹H NMR in D₂O using the characteristic proton shifts of lipids (δ ~0.8 ppm; –CH₃) and PEI (δ ~2.5–2.8 ppm; NH—CH₂—CH₂—NH—) to calculate the lipid substitution levels. The levels of substitution were 6.9 CA/PEI2 and 2.1 LA/PEI2.

2.3. Polymeric nanoparticle preparation and cell delivery

For the preparation of siRNA complexes with lipid-modified polymers (polymeric nanoparticles), an aliquot of scrambled siRNA (Ambion, Austin, TX, USA) and CD44 (standard isoform, CD44s) specific siRNA (HSC.RNAI.N000610.12.1; Integrated DNA Technologies (IDT), Coralville, IA, USA) stock solution (100 μM in RNase-free water) was first dissolved in a 150 mM NaCl solution in polypropylene sterile tubes. siRNA was then added to 150 mM NaCl for final siRNA concentrations of 25, 50 and 100 nM in cell suspension. The polymers (dissolved at 1 mg/mL in ddH₂O) were then added to the siRNA solution to give the desired polymer:siRNA weight ratios (4:1 and 8:1, designated as R4 and R8, respectively) bringing the final volume to 150 μL, followed by incubation for 30 min prior to adding cells.

50,000 cells were plated in Nunc® MicroWell™ 96-well polystyrene plates (Thermo Scientific, Lafayette, CO, USA) and maintained in 100 μL IMDM supplemented with 20% BGS and incubated for 3 h. The polymeric nanoparticle complexes (25–50 μL/well) were then added in triplicate to the cells in 96-well plates. Cells were collected 48 and 72 h after polymeric nanoparticle treatment and analyzed for CD44 silencing efficiency and viability.

2.4. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Total RNA was extracted using TRIzol reagent (GibcoBRL, Gaithersburg, MD, USA) according to the manufacturer's instructions. Two μg RNA was

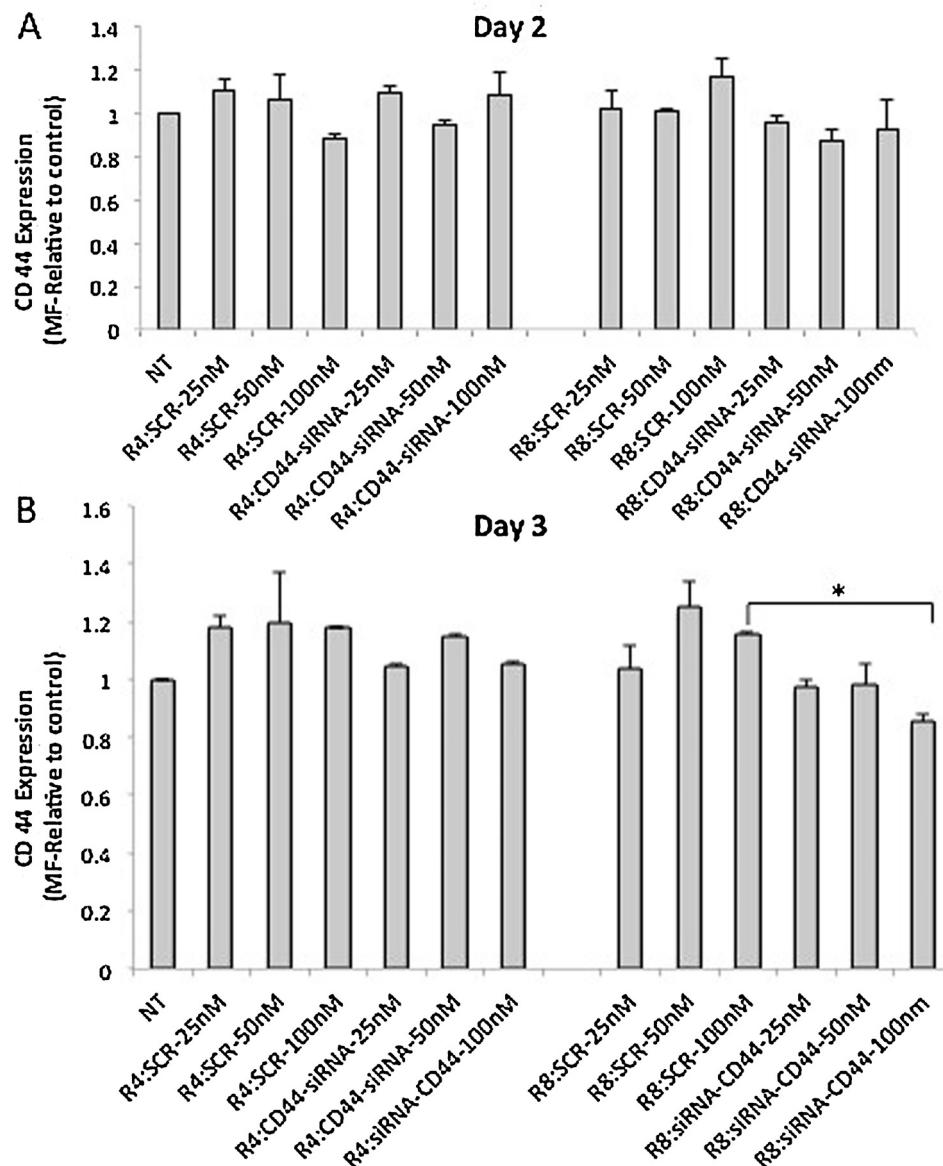


Fig. 1. PEI-LA-mediated CD44 silencing in KG-1 cells at day 2 (A) and day 3 (B) after siRNA treatment. KG-1 cells were exposed to three different concentrations of siRNA complexes (25, 50 and 100 nM) with 4:1 and 8:1 polymer:siRNA weight ratios. The data are presented as relative MF (mean fluorescence), which was normalized with the negative control (no-treatment control). Means and standard deviations have been calculated from 2 (for KG-1a cells) to 3 (for KG-1 cells) independent experiments; * $p \leq 0.05$ compared to SCR control.

reverse-transcribed into cDNA as previously described [39]. Human beta-actin RNA (forward primer: 5'-CCA CCC CAC TTC TCT CTA AGG A-3'; reverse primer: 5'-AAT TTA CAC GAA AGC AAT GCT ATC A-3', IDT) was used as an internal control to evaluate transcripts for CD44s (forward primer: 5'-GGC CTT GGC TTT GAT TCT TG-3'; reverse primer: 5'-CCA CTT GGC TTT CTG TCC T-3', IDT). Quantification was performed using SYBR green master mix with ROX (MAF Center, University of Alberta, Edmonton, AB, Canada) in a 96-well plate on the StepOne Real-time PCR system (Applied Biosystems, Foster City, CA, USA). The amplification cycle consists of inactivation at 95 °C for 5 min and amplification at 95 °C for 10 s and 60 °C for 30 s for 40 cycles, with each sample analyzed in triplicate. The level of target gene expression was determined using the comparative threshold cycle method ($\Delta\Delta C_T$) and presented as fold-change of the target primer relative to the beta-actin expression (StepOne Software v2.2.2, Applied Biosystems).

2.5. Flow cytometry

The cells were stained with PE-anti-CD44s (CD44 standard form), PE-anti-CD34, FITC-anti-CD38, FITC-anti-CD14, and PE-anti-CD15 monoclonal antibodies and PE-goat-anti-mouse IgG as an isotype control (BD Biosciences, Oakville, ON, Canada) for 45 min at 4 °C. After the final wash (PBS + 0.1% bovine serum albumin (BSA)), cells

were fixed in 1% paraformaldehyde prior to flow cytometric analysis (FACSCalibur, Becton-Dickinson, San Jose, CA, USA).

2.6. Cell viability

AML cell viability was evaluated by trypan blue exclusion assay, whereas the viability of both KG-1 and KG-1a cells was assessed by MTT assay at day 3 after siRNA delivery. The MTT solution (40 µL, 4 mg/mL in HBSS) was added to each well and the cells were incubated for 2 h. The medium was removed, and 200 µL of DMSO was added to each well to dissolve the MTT crystals. The optical density of the solutions was measured at 570 nm by an ELx800 Universal Microplate Reader (BioTek Instruments). The percentage viability was calculated as follows: 100% × (absorbance of polymeric nanoparticle treated cells/absorbance of untreated cells).

2.7. Apoptosis assay

Apoptosis was assessed by an annexin V-FITC apoptosis detection kit (BD Biosciences) as previously described [39]. Three days after siRNA delivery, cells were collected, washed twice with PBS, and re-suspended in 500 µL of staining solution containing FITC-conjugated annexin V antibody (5 µL). After

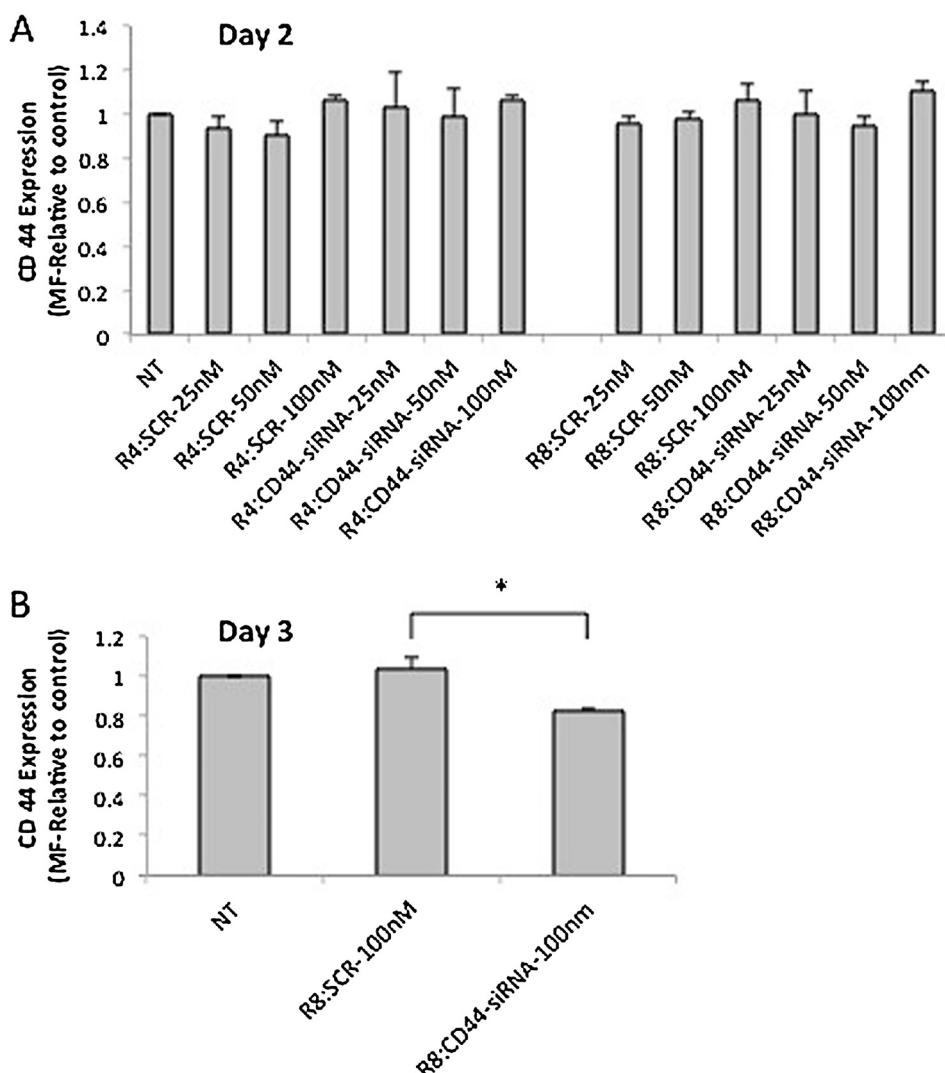


Fig. 2. CD44 silencing using PEI-CA/siRNA complexes in KG-1 cells at day 2 (A) and day 3 (B) after siRNA treatment. * $p \leq 0.05$, compared to SCR control.

incubation on ice for 30 min, cells were analyzed by flow cytometry. The percentage of cells undergoing apoptosis was determined by two independent experiments using four or five replicates each time for both KG-1 and KG-1a cells.

2.8. Hyaluronic acid (HA) adhesion assay

HA binding assay was performed as previously described [42] with minor modifications. Briefly, flat bottom 96-well tissue culture plates (Falcon, BD Biosciences) were coated with 3 mg/mL HA in PBS or 2% BSA (control) and incubated overnight at 4 °C, then blocked with 1% BSA. The wells were washed with PBS and seeded with cells (50,000 cells/well) previously incubated with 2.5 μM Dil (carbocyanine dye) in serum-free medium for 15 min. Adhesion proceeded at 37 °C for 1 h. The wells were then washed twice with PBS and plate was read ($\lambda_{\text{EX}} = 549$ nm, $\lambda_{\text{EM}} = 565$ nm) on an ELx800 Universal Microplate Reader (BioTek Instruments).

2.9. BM-MSC adhesion assay

BM-MSC adhesion assay was carried out using the method of Zepeda-Moreno et al. [43] with minor modifications. 5000–20,000 BM-MSC were plated in flat bottom 96-well tissue plate (Falcon; BD Biosciences) and maintained for 2 days to reach confluence. After removal of the medium, IMDM with 20% BGS and ~30,000 (AML patient cells) or 150,000 (KG-1 and KG-1a cells) Dil-labeled cells were added per well and incubated at 37 °C, 5% CO₂ for at least 1 h to let the cells make contact with the BM-MSC. After incubation the plate was turned upside down and further incubated at 37 °C, 5% CO₂ for 3 h. The lid was taken off and media and non-adherent or weakly adherent cells were gently removed. The plate was read on an ELx800 Universal Microplate Reader as above.

2.10. Statistical analysis

Arithmetic means and standard deviations were calculated. Statistical significance was defined as $p \leq 0.05$ using the Mann-Whitney U-test.

3. Results

3.1. Efficient CD44-silencing in KG-1 and KG-1a cells by polymeric nanoparticles

Previously, we performed extensive studies to determine effective polymeric materials to efficiently deliver siRNA (using reporter targets with no functional studies) and reported that cellular delivery of siRNA was dependent on the nature of the lipid substituent and degree of lipid substitution and that the delivery varied among different AML and CML cell lines, e.g. PEI2-LA led to successful delivery of siRNA into leukemic progenitor cell line KG-1, whereas PEI2-CA resulted in silencing of genes in the more differentiated monocytic AML cell line THP-1 [34,39]. On the other hand, palmitic acid (PA)-substituted 1.2 kDa PEI (PEI1.2-PA) gave a superior silencing efficiency in CML cell lines [39]. In this work, we therefore first investigated siRNA delivery with LA-substituted polymers to silence CD44 gene expression in the models of AML LSPC, namely KG-1 and KG-1a cell lines. It is known that AML LSPC can reside within CD34⁺/CD38⁻ or CD34⁺/CD38⁺ immunophenotypic

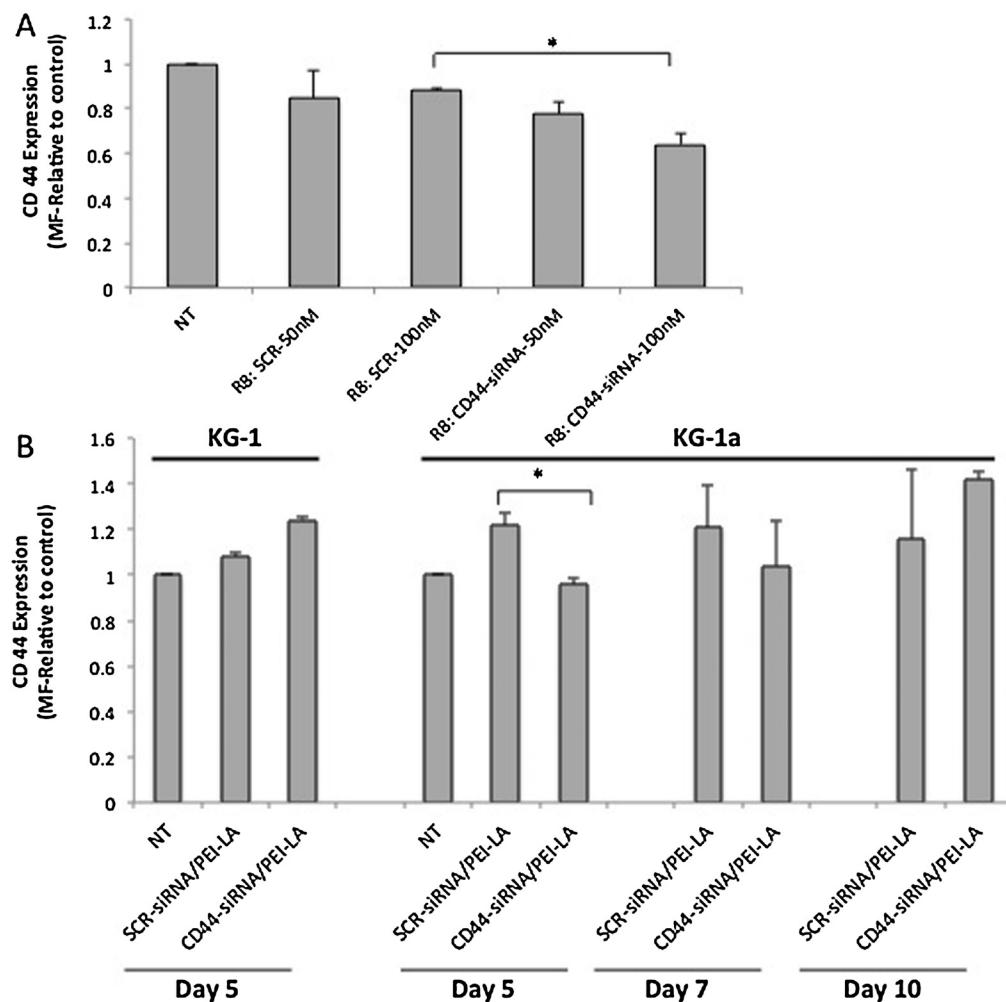


Fig. 3. CD44 silencing using PEI-LA/siRNA complexes in KG-1 cells at day 3 (A) and in KG-1 and KG-1a cells at day 5, 7 and 10 (B) after siRNA treatment. Results represent the mean \pm SD of two independent experiments using triplicates per condition; * $p \leq 0.05$, compared to SCR control.

compartments [44,45]. Therefore, these cell lines were chosen because they are both CD34⁺ with high CD44 surface expression (~100% positive cell population with fluorescence intensity (MF)=~260 for KG-1, ~245 for KG-1a cells; data not shown) [46]. KG-1a cells represent the more immature leukemic cell fraction (CD38⁻). Initial studies were performed in KG-1 cells using PEI2-LA/CD44-siRNA complexes. Three concentrations of siRNA complexes (25, 50 and 100 nM) with 4:1 and 8:1 polymer:siRNA weight ratios were used in this study, while scrambled siRNA (SCR) served as a control. We did not include high molecular weight PEI (PEI25, 25 kDa) or PEI2 in our study design as they were not effective delivery agents for the KG-1 cells [34]. The concentration of siRNA, the types of polymers, the polymer:siRNA weight ratios, and the period of incubation initially used were the optimal conditions found for KG-1 cells as described in our previous work [34].

In the current study we found that at day two 50 nM and 100 nM CD44-siRNA at 8:1 polymer:siRNA weight ratios led to ~12% CD44 silencing in KG-1 cells, whereas no effect was observed with lower concentration of CD44-siRNA or 4:1 polymer:siRNA weight ratios (Fig. 1). However, at day three, silencing was 27% using 100 nM of CD44-siRNA at 8:1 polymer:siRNA (Fig. 1). We also observed a slight increase of CD44 expression in cells exposed to PEI2-LA/SCR alone. This could be due to the background fluorescence generated by polymers (i.e., increased non-specific binding of antibodies to cell surfaces containing cationic polymers) as we observed this phenomenon with other cell types as well as with other non-viral

transfection reagents [34,47]. We also explored the silencing efficiency of CD44 using PEI2-CA polymer; no CD44 silencing was observed at day 2 but ~17% CD44 silencing was observed at day 3 (Fig. 2). These results indicated that PEI2-LA is more efficient than PEI2-CA for CD44 silencing in KG-1 cells. We next investigated CD44 silencing using PEI2-LA/CD44-siRNA complex in more primitive KG-1a cells at day three and obtained a similar CD44 silencing efficiency (~30%) (Fig. 3A).

We also evaluated the CD44 expression at day 5, 7 and 10 after siRNA delivery into both KG-1 and KG-1a cells. We found that CD44 silencing was evident in KG-1a cells, but not in KG-1 cells, 5 days after treatment (Fig. 3B). The slower growth rate of KG-1a cells in comparison to KG-1 cells might have been the reason for this prolonged silencing. Increased CD44 levels at day 5 for KG-1 cells and at day 10 for KG-1a cells were also observed (Fig. 3B).

3.2. Effect of polymeric nanoparticle-mediated CD44 silencing on cell viability and differentiation

To investigate the cytotoxicity of polymeric nanoparticle-mediated CD44 silencing, we evaluated the viability of KG-1 and KG-1a cells at day 3 by the MTT assay. We did not observe any growth inhibition of the polymeric nanoparticles in KG-1 or KG-1a cells using this assay (Fig. 4A). On the other hand, we observed increased (~20%) induction of apoptosis in CD44-silenced KG-1 cells at day 3 at 100 nM siRNA concentration

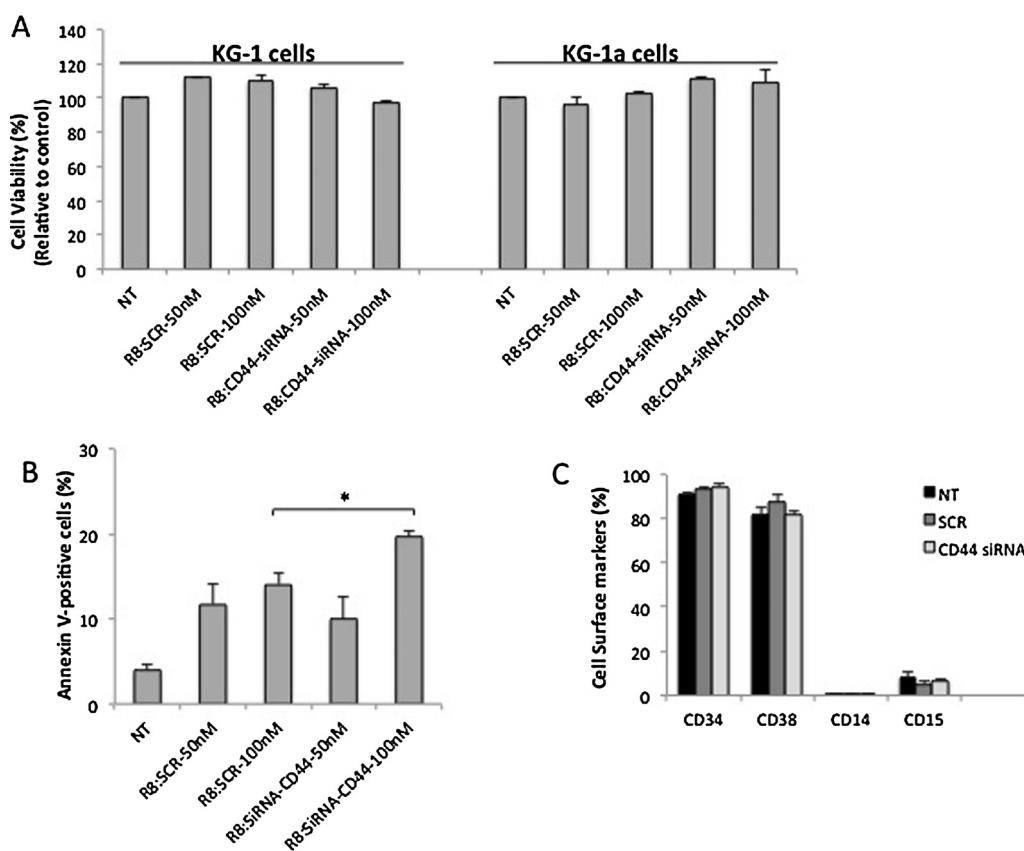


Fig. 4. Viability and differentiation of CD44-silenced cells. (A) Cell viability in KG-1 and KG-1a were expressed relative to no-treatment control at day 3 after siRNA treatment. (B) FITC-Annexin V staining of KG-1 cells exposed to polymeric nanoparticles for 3 days. Results represent the mean \pm SEM of 2 independent experiments. * $p \leq 0.05$ compared to SCR control treated cells (C) Percentage of CD34-, CD38-, CD14-, and CD15-positive KG-1 cells at day 3 after transfection. Results represent the mean \pm SEM of 3 independent experiments.

using the Annexin-V assay (Fig. 4B) while the cytotoxicity of polymer-SCR-siRNA complexes was less (~13%). Next, we analyzed the effect of CD44 silencing on differentiation properties of KG-1 cells by surface antigen expressions (LSPC markers: CD34 and CD38; monocyte/macrophage differentiation marker: CD15 and CD14) using flow cytometry. Expression levels of these surface antigens were unaffected by polymeric nanoparticle-mediated CD44 silencing in KG-1 cells, indicating that CD44-siRNA

treated KG-1 cells maintained their phenotype as in non-treated samples (Fig. 4C).

3.3. CD44 silencing efficiency by quantitative RT-PCR

CD44 silencing efficiency by polymeric nanoparticles in KG-1 and KG-1a cells was also evaluated at the gene level using quantitative RT-PCR on day 3. We found that the decrease of the surface

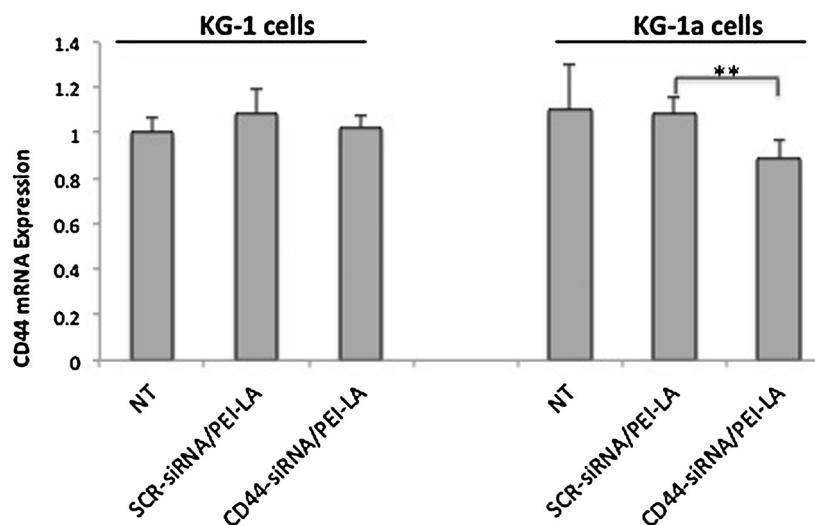


Fig. 5. CD44 mRNA levels in KG-1 and KG-1a cells as determined by real-time quantitative PCR at day 3. Human beta-actin was used the internal calibrator. A representative of two experiments yielding similar results was shown. ** $p < 0.01$ by Mann-Whitney U-test.

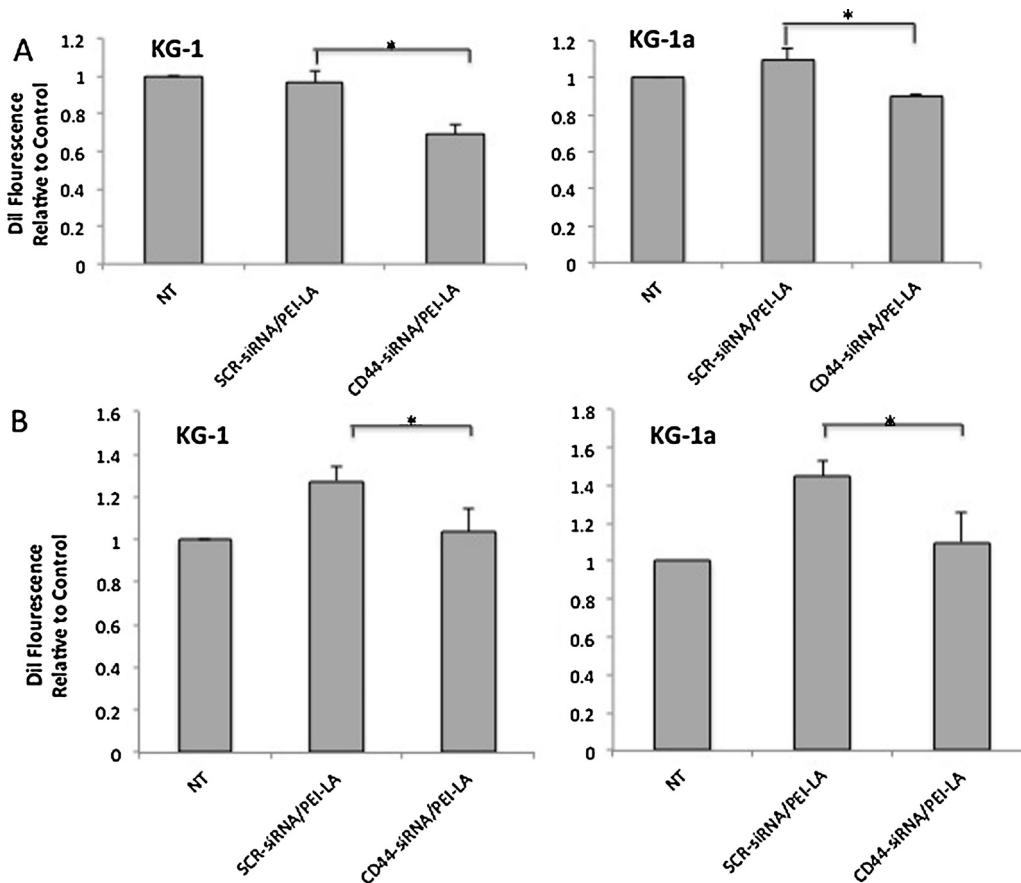


Fig. 6. Adhesion of CD44-silenced KG-1 and KG-1a cells to HA coated cell culture plates (A) and BM-MSC monolayer (B). Dil fluorescence values of stained cells were expressed relative to no-treatment control. Means and standard deviations were calculated from 2 (for KG-1a cells) or 3 (for KG-1 cells) independent experiments. * $p \leq 0.05$ compared to SCR control treated cells.

CD44 level on day 3 was not reflected at the mRNA level in KG-1 cells (Fig. 5), most likely because the changes at the gene level could have occurred at earlier time points in these cells. However, a ~20% decrease in the CD44 mRNA expression level was obtained in KG-1a cells on day 3 (Fig. 5). This result was consistent with prolonged silencing efficiency seen in the KG-1a cells compared to KG-1 cells.

3.4. Decreased adhesive properties of CD44-silenced KG-1 and KG-1a cells

To determine whether polymeric nanoparticle-mediated silencing of CD44 translates to a functional response, two adhesion assays were performed. We first evaluated *in vitro* adhesive ability of non-treated, SCR-siRNA and CD44-siRNA treated KG-1 and KG-1a cells to HA-coated plates. We observed that CD44 silencing resulted in as much as ~25% decrease in the capacity of both KG-1 and KG-1a cells to bind HA (Fig. 6A). The HA binding of non-treated and SCR-siRNA treated KG-1 cells were equivalent, whereas SCR-siRNA treated KG-1a cells were shown to bind to HA more compared to non-treated cells. This might be due to the adhesive nature of the polymeric complex. The binding capacities of these cells to BM-MSC were then assessed. The BM-MSC adhesion was decreased (by ~20%) in both CD44-silenced KG-1 and KG-1a cells in comparison to the SCR-siRNA treated cells (Fig. 6B), which is consistent with the achieved CD44 silencing efficiencies in these cells.

3.5. Polymeric nanoparticle-mediated CD44 silencing in primary AML cells

Since primary cells more accurately reflect the *in vivo* situation, we sought to determine CD44 silencing efficiency in highly

CD34⁺ positive primary AML cells. We evaluated cells from three AML patients with high levels of CD34⁺ blasts. The clinical characteristics of patients are summarized in Table 1. We analyzed surface CD44 level three days after siRNA delivery in primary AML cells and obtained CD44 silencing of ~16% for Pt#1 and #2 and ~20% for Pt#3, which was less than in KG-1 and KG-1a cell lines (Fig. 7A). The viability of cells was not affected. A functional BM-MSC adhesion assay using Pt#1 cells showed ~15% decrease in the binding capacity of cells to BM-MSC, reflecting a decrease in the cell surface CD44. Adhesion assays and silencing efficiency at the mRNA level were not performed on the other two AML patients due to insufficient numbers of cells recovered from frozen samples.

4. Discussion

The adhesion molecule CD44 has been identified as a therapeutic target in AML due to its altered properties in AML blasts and association with poor prognosis and high relapse rates in AML as well as many solid tumors [14,15,48–52]. Highly efficient blocking of CD44 was previously achieved using anti-CD44 moAb in AML and resulted in anti-tumor activity in leukemia xenografts [12]; however, the requirement of complex processes with high cost for therapeutic moAbs production and the possibility of adverse effects including immune reactions, infections, autoimmune diseases, cancer, platelet and thrombotic disorders, and cardiotoxicity by administration of moAbs are still major concerns [18–21]. In fact, clinical trials with CD44 neutralizing antibodies have been terminated due to unacceptable levels of toxicity [19]. In addition, there might be some disadvantages in using small molecule inhibitors

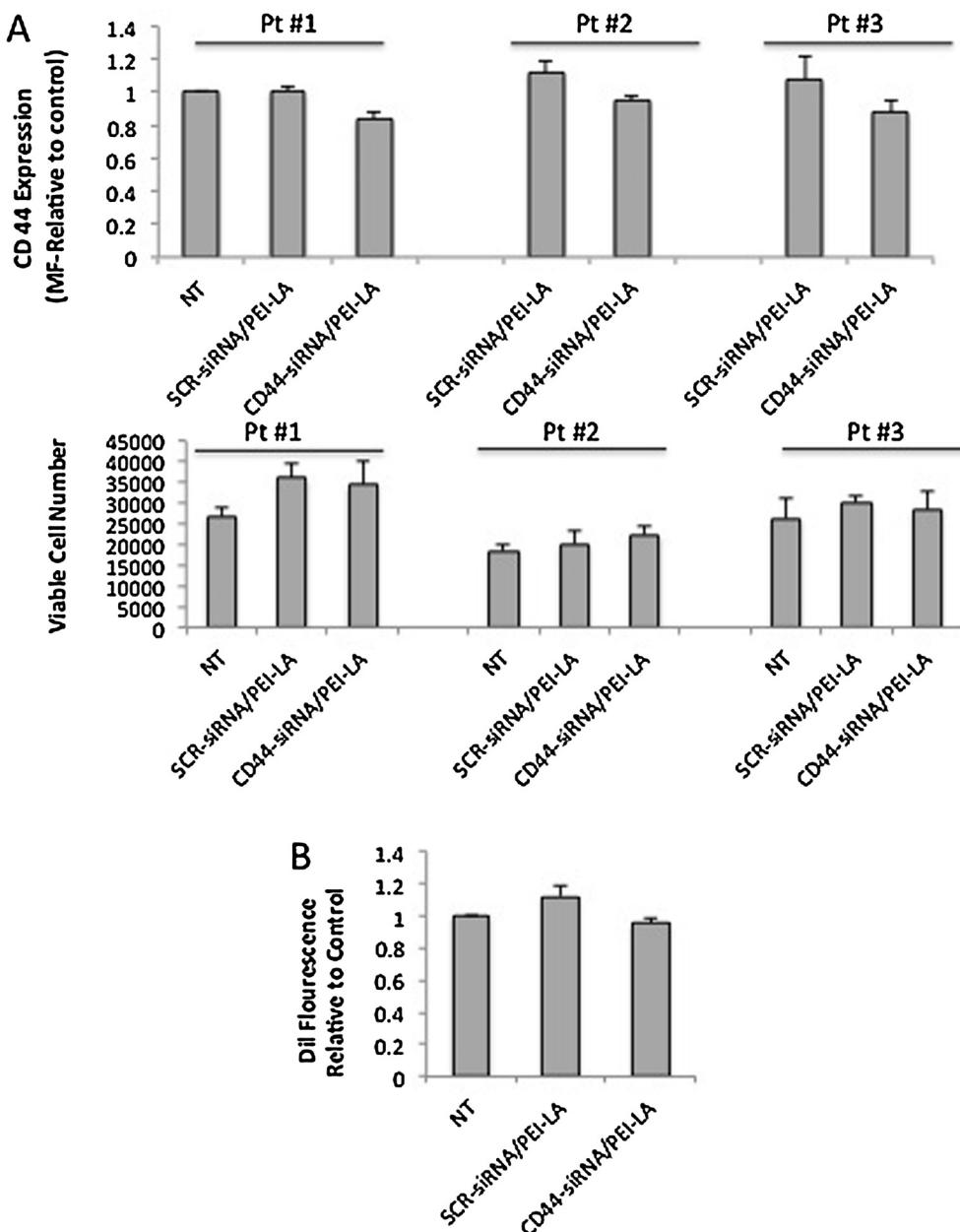


Fig. 7. CD44 silencing in highly CD34⁺ primary AML cells. (A) Surface CD44 levels (top) and viability (bottom) of CD44-silenced cells from 3 separate patients. (B) Adhesion of primary AML cells to BM-MSC after CD44 silencing.

against CD44 such as high toxicity, short-half-life, reduced efficacy due to resistance development as well as low specificity [17,20]. The siRNA-based therapies have been recognized as more specific approaches for treatment of leukemia, but there were no clinically-relevant means of delivering CD44-siRNA molecules into LSPC. In this study, we showed for the first time polymeric nanoparticle-mediated down-regulation (up to ~30%) of CD44 expression in CD34⁺ KG-1, KG-1a leukemic cell lines, as well as in CD34⁺ primary cells. Polymeric nanoparticle-mediated CD44 silencing has several potential advantages over the use of monoclonal antibodies or small molecule inhibitors for CD44 targeting including higher specificity, lower toxicity, and no induction of unwanted immune response or other adverse events due to immune stimulation. In addition, polymeric nanoparticles could be eliminated harmlessly from the body in a reasonable period of time after releasing siRNA (since building blocks are from small molecular species), and could be applied repeatedly if necessary [53]. In the present study,

PEI2-LA gave rise to the best silencing efficiency in these cells, supporting our previous finding that siRNA delivery was effective in KG-1 cells using PEI2-LA [33]. On the other hand, in our previous study using both PEI2-LA and PEI2-CA based delivery lower concentrations of siRNA (25–50 nM) were required to obtain GFP and CXCR4 (another surface receptor) silencing efficiency in a more differentiated AML cell line, THP-1 [34]. Furthermore, siRNA silencing was much more effective (~63%) in K562 cells (a CML cell line) than in THP-1 cells and in LSPC using PEI1.2-PA but not PEI2-LA [39], indicating that siRNA delivery in leukemic cells is dependent on the nature of the lipid substituent. The different efficiencies of siRNA silencing in leukemic cells could be explained by the different targets used, endocytic activities or endosomal processing pathway gene expression (Caveolin-1 and -2) in the target cells, as recently claimed [38]. Our CD44 silencing strategy using polymeric nanoparticles is more efficient compared to silencing of Raf-1 and Bcl-2 proteins in AML cells using the synthetic carrier Oligofectamine,

which required a very high siRNA concentration (400 nM) that is not clinically practical [32]. Moreover, it was recently reported that the same siRNA silencing efficiency in KG-1 cells was obtained with a 2.5-fold higher siRNA concentration using lipid nanoparticle-based delivery [38]. However, lipid nanoparticle-based siRNA silencing was highly efficient (~90%) at low siRNA concentration (50 nM) in other leukemic cell lines, including THP-1, HEL and K562 cells [38], indicating a higher efficiency for this system [34,39].

On the other hand, the maximum CD44 silencing efficiency obtained in our study is much lower (up to 30%) than reported for solid tumor cells (up to 78% *in vitro*) using lipid based gene silencing [28–30] because of the difficulty of siRNA delivery into suspended, slow-growing CD34⁺ AML cells in comparison to adherent tumor cells. Leukemic cells also have smaller size and lower endocytic activity [54] rendering them difficult to transfect. However, the efficiency of the polymeric nanoparticle-mediated silencing could be further enhanced by combinations of multiple siRNA against different CD44 isoforms and/or repeated delivery of siRNA in AML cells, and therefore further investigations are required. Importantly, our CD44 silencing was functional, and adhesion assays showed that adhesion of CD44-siRNA treated cells to both HA and BM-MSC was lower than those of control siRNA-treated cells (Fig. 6). The polymeric nanoparticle-mediated CD44 silencing also appeared to last longer, thereby being more effective in more primitive LSPC model KG-1a cells, which might be beneficial for targeting the leukemia-initiating compartment of CD34⁺/CD38⁻ LSPC. The difference could be explained by the lower growth rate and endocytic activity of KG-1a cells in comparison to KG-1 cells. Usually, optimal silencing effects last for 5–7 days using non-viral commercial reagents [55,56] and this appeared to be the case in this study as well. Furthermore, we did obtain a lower CD44 silencing efficiency in AML primary cells in comparison to AML cell lines most likely due to the presence of different CD44 variants in primary cells in addition to the targeted CD44 isoform. Hence, the usage of the combination of siRNA against different variants of CD44 might be essential for the efficient silencing of CD44 in AML primary cells.

Here, the CD44 expression and adhesion properties of AML LSPC were reduced while differentiation and inhibition of cell proliferation (as measured by the MTT assay) were not affected in these cells. However, silencing of CD44 led to apoptosis induction in KG-1 cells at the high (100 nM) concentration of siRNA used. Beyond its adhesive function, CD44 is known to be an important regulator of proliferation, homing, migration, apoptosis, angiogenesis and tumor metastasis [6,7,57]. In fact, the specific anti-CD44 mAb H90 was reported to inhibit the proliferation and promote the differentiation of the LSPC in engrafted mice because of the inhibition of the interaction of LSPC with the BM niche [12]. Furthermore, anti-CD44 mAbs induced inhibition of cell proliferation in KG-1a cells as well as more differentiated types of AML cells, including THP-1, HL-60 and NB4 [58]. However, these anti-CD44 mAbs did not overcome the differentiation block in KG-1a cells similar to our observation with siRNA delivery in KG-1 cells. In addition, silencing of CD44 in solid tumors has been reported to result in inhibition of growth and metastasis [28,29]. The reason for the lack of growth inhibition in this study might be due to the lower CD44 silencing efficiency, and the MTT assay used for growth inhibition might not be as sensitive as the annexin-based apoptosis assay, which better revealed the functional consequence of CD44 silencing. It is also possible that a growth inhibition effect could still manifest at a later point in time, and this will be investigated in future studies. Reduction of the adhesive properties of LSPC by polymeric nanoparticles might more clearly reveal the LSPC growth inhibition *in vivo* due to blocking the interaction with BM niche of these cells which needs to be further investigated by *in vitro* BM-MSC co-culture assays or in animal model.

In conclusion, this study showed that polymeric nanoparticle-mediated silencing of CD44 in CD34⁺ leukemic cell lines and primary AML blasts results in decreased adhesion properties to both HA-coated plates and a BM-MSC monolayer. This approach presents a safe, feasible strategy for dislodging LSPC from the protective BM microenvironment but needs to be explored further. Clinically, CD44 down-regulation in LSPC could be used to inhibit leukemia progression or enhance chemotherapeutic response, and undertaking this task with non-viral delivery of siRNA could provide a unique treatment strategy for AML in the future.

Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

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