



# Targeting CXCR4/SDF-1 axis by lipopolymer complexes of siRNA in acute myeloid leukemia

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## ABSTRACT

In spite of high complete remission rates in Acute Myeloid Leukemia (AML), little progress has been made in the long-term survival of relapsing AML patients, urging for the development of novel therapies. The CXCR4/SDF-1 axis is a potential therapeutic target in AML to reduce the enhanced survival and proliferation of leukemic cells, with current drug development efforts focusing on antagonists and blocking antibodies. The RNAi technology mediated by siRNA is a promising alternative; however, further development of clinically relevant siRNA carriers is needed since siRNA on its own is an incompetent silencing agent. Here, we report on lipid-substituted polymeric carriers for siRNA delivery to AML cells, specifically targeting CXCR4. Our results demonstrate an effective suppression of CXCR4 protein with the polymeric siRNA delivery in AML THP-1 cells. The suppression of CXCR4 as well as its ligand, SDF-1 (CXCL12), decreased THP-1 cell numbers due to reduced cell proliferation. The reduced proliferation was also observed in the presence of human bone marrow stromal cells (hBMSC), suggesting that our approach would be effective in the protective bone marrow microenvironment. The combination of CXCR4 silencing and cytarabine treatment resulted in more effective cytotoxicity when the cells were co-incubated with hBMSC. We observed a decrease in the toxicity of the lipopolymer/siRNA complexes when THP-1 cells were treated in the presence of hBMSC but this effect did not negatively affect CXCR4 silencing. In addition, siRNA delivery to mononuclear cells derived from AML patients led to significant CXCR4 silencing in 2 out of 5 samples, providing a proof-of-concept for clinical translation. We conclude that decreasing CXCR4 expression via lipopolymer/siRNA complexes is a promising option for AML therapy and could provide an effective alternative to current CXCR4 inhibition strategies.

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## 1. Introduction

Acute myeloid leukemia (AML) is a heterogeneous group of disorders characterized by abnormal proliferation of myeloid blasts with reduced capacity to differentiate into mature cells. Little has changed with AML treatment methods in the past decade and chemotherapy remains as the standard form of treatment, often using cytarabine in combination with an anthracycline [1,2]. Although conventional treatment yields high rates of complete remission, the majority of patients (more than 85%) eventually relapse due to proliferation of drug-resistant leukemic blasts in the bone marrow [3,4]. Besides high relapse rates,

current therapies display immediate toxic side-effects, patient incompatibility with high-dose treatments and undesirable long-term effects [1,2,5,6]. In addition, the five-year survival rates are only 31% in patients younger than 65 years of age, and a staggering 4% in patients above 65 years of age [7]. The development of alternative, novel therapies for AML is therefore urgently needed. As an alternative therapeutic modality for AML, siRNA therapy provides the flexibility of choosing different targets and/or combining multiple targets under the same therapeutic approach. The requirements for siRNA therapy include (i) an effective carrier to deliver the siRNA, and (ii) an effective therapeutic target protein for the siRNA.

Without the protection of a carrier, siRNA is readily degraded in the physiological milieu and is unable to enter the cell due to its relatively large size and negative charge. Furthermore, carrier-mediated siRNA delivery to cells that grow in suspension remains challenging and is

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therefore a major obstacle in the development of siRNA therapy for treatment of leukemic cells [8–10]. Much of the siRNA related work with leukemia has been performed with commercial carriers or by electroporation, both of which are not clinically applicable [8–10]. The difficulties of siRNA delivery to leukemic cells are not well understood but recent findings suggested that low expression of key proteins involved in caveolae-mediated endocytosis (Caveolin 1 and 2) [9] and/or limited presence of extracellular matrix attachment proteins [11] may be partially responsible. We are currently developing polymeric carriers, namely lipid substituted low molecular weight polyethylenimines (PEIs), to be used in cancer therapy. Using PEI as the backbone of siRNA carriers takes advantage of its well-known beneficial features, which include effective siRNA binding due to its high charge density, electrostatic interaction with plasma membranes needed for internalization, and endosomal escape mechanisms through a combination of buffering capacity and membrane interactions. Moreover, utilizing lower molecular weight PEI overcomes the disadvantageous features of high molecular weight PEIs which include high toxicity and limited biodegradability [12–14]. Without further modification, however, low molecular weight PEIs are not effective for siRNA delivery into cells, likely due to minimal charge of assembled complexes [15]. Therefore, we have utilized lipid substitution of 2 kDa PEI, in particular caprylic and linoleic acid, to enhance the interactions with cellular membranes. A library of lipopolymers was shown to efficiently bind to siRNA to form distinct complexes, provide efficient siRNA delivery (comparable to commercial carriers) as well as to effectively silence a model protein (Green Fluorescence Protein, GFP) in leukemic cells [8]. In order to validate the utility of the proposed lipopolymer/siRNA delivery system for clinical use, further analysis was required on its ability to target a therapeutically useful protein in AML disease.

In terms of promising therapeutic protein targets for AML treatment, we focus on the CXC chemokine receptor 4 (CXCR4)/stromal-cell derived factor-1 (SDF-1) axis. The CXCR4-expressing leukemic cells have been found to migrate to bone marrow microenvironment as a result of bone marrow stromal cells (BMSC) and endothelial cells releasing the chemo-attractant SDF-1. SDF-1 binds to cell surface located CXCR4, resulting in its activation through phosphorylation and endocytosis of surface-located CXCR4, followed either by ubiquitination and then degradation or surface re-location [16]. CXCR4 activation causes signaling through numerous pathways, including the Src family of tyrosine kinases, phospholipase C- $\beta$ , PI3K/Akt, JAK/STAT, MAPK and NF- $\kappa$ B, leading to enhanced survival, increased proliferation, drug resistance, degradation of extracellular matrix and angiogenesis [16]. High levels of CXCR4 expression at initial diagnosis and an increase in CXCR4 expression as a response to chemotherapy have both been demonstrated in many leukemias including AML [16,17]. Current strategies targeting CXCR4 include small molecular antagonists and blocking antibodies, [16] several of which are progressing through clinical trials [18]. Promising effects of the CXCR4 antagonists in AML cell lines as well as primary AML cells have been reported and include decreased adhesion to BMSC/SDF-1, decreased proliferation, increased apoptosis, decreased survival support and decreased resistance to chemotherapy drugs [18,19]. More importantly, in a phase I/II trial, CXCR4 antagonist AMD3100 (Plerixafor) was found to mobilize leukemia cells into the peripheral blood by 2-fold and provide chemosensitization with mitoxantrone, etoposide and cytarabine treatment [18]. In addition, AMD3100 and TN140, used without chemotherapy drugs, caused regression in high CXCR4-expressing leukemic patient cells in a mouse model, where increased apoptosis and increased mobilization of leukemic cells were specifically observed [20]. The effects of CXCR4 antagonist on AML cells have been attributed to two separate mechanisms; (i) physical disruption of cell adhesion to drug resistance-supporting bone marrow microenvironment and (ii) prevention of signaling through the CXCR4 pathway that includes the pro-survival pathways PI3K/AKT and MAPK [18].

Downregulating CXCR4 expression with an siRNA may provide a more beneficial therapeutic modality as compared to CXCR4 antibodies

and small molecular inhibitors. siRNA is a targeted technology, specific for the mRNA of interest that results in decreased protein formation. Meanwhile, the drawbacks of antibody therapies include complex and costly development, unpredictable toxicity, low efficacy/safety ratio and risk of immunogenicity [21,22], whereas the challenges of antagonists/inhibitors include lower specificity, short-half life, toxicity issues and varied treatment response due to target mutations and complicated mechanism(s) of action [19,21,23]. For instance, resistance to the antagonist AMD3100 can occur simply due to a specific single amino acid substitutions in a certain region of the CXCR4 protein [24]. In addition, through the CXCR4 antagonist binding mechanism, a signaling response through CXCR4/SDF-1 pathways can be activated [19,23]. Antagonists, such as AMD3100 and ALX40-4C, have been found to induce G protein signaling activation, as a result of being weak partial agonists, resulting in phosphorylation of some SDF-1/CXCR4 signaling molecules (MAPK p44/p42) [19,23].

In this study, we investigated the impact of silencing CXCR4 expression in AML cells with the lipopolymer-mediated siRNA delivery. We probed the effect of silencing with clinically relevant variables including the presence of human bone marrow stromal cells (hBMSC) and the chemotherapy drug, cytarabine. Using a cell model of AML (THP-1 cells), we show that silencing both CXCR4 and SDF-1 provide decreased leukemic cell survival and that CXCR4 silencing remains effective when leukemic cells were co-incubated with hBMSC. CXCR4 siRNA co-treatment with cytarabine provided an enhanced anti-survival effect on AML cells, which was especially evident in the presence of hBMSC. We additionally show that siRNA delivery to AML patient derived cells was effective with the chosen polymers and CXCR4 silencing was feasible in a subset of primary AML patient cells employed.

## 2. Material and methods

### 2.1. Materials

Two kDa polyethylenimine (PEI2;  $M_n$ : 1.8 kDa,  $M_w$ : 2 kDa), anhydrous dimethyl sulfoxide (DMSO), caprylic chloride (C8), 3-(4,5-dimethyl-2-thiazoyl)-2,5-diphenyl-2 H-tetrazolium bromide (MTT), trypan blue solution (0.4%), and Cytarabine (Cytosine  $\beta$ -D-arabinofuranoside; C1768-100MG) were purchased from Sigma-Aldrich (St. Louis, MO). Negative control siRNA (AM4635), FAM-labeled negative control siRNA (AM4620), Dil (Molecular Probes), and Hoechst (33,258; Molecular Probes) were purchased from Life Technologies (Carlsbad, CA). The CXCR4 siRNA used throughout this study (Cat No: HSC.RNAI.N001008540.12.1) and SDF-1 siRNA (Cat No: HSC.RNAI.N000609.12.1) were purchased from IDT Inc. (Coralville, IA). Two additional siRNAs (siRNA-2: Cat No: HSC.RNAI.N003467.12.1, and siRNA-3: Cat No: HSC.RNAI.N003467.12.2) used for the PCR study were also obtained from IDT. Hanks Balanced Salt Solution (HBSS), Dulbecco's Modified Eagle Medium (DMEM; low glucose with L-glutamine; 11,885), RPMI Medium 1640 with L-glutamine (11,835), IMDM (12,440) solution, penicillin/streptomycin solution (10,000 U/mL/10 mg/mL), Minimum Essential Media (MEM)  $\alpha$  medium, MEM non-essential amino acids (100 $\times$ ) and Trypsin EDTA Solution, 1 $\times$  Liquid 0.25% Trypsin/1 mM EDTA (25,200-056) were from Invitrogen (Grand Island, NY). Accutase (SCR005) was from Millipore (Billerica, MA). Fetal bovine serum (FBS; A15-751) was purchased from PAA Laboratories Inc. (Etobicoke, ON). The PE-labeled mouse anti-human CXCR4 (CD184) and mouse IgG isotype control antibody were from BD Pharmingen (Mississauga, ON). Ficoll-Paque™ PREMIUM was purchased from Fisher Scientific.

### 2.2. Cell model and culture

THP-1 cells (AML-M5; French-American-British (FAB) classification) were obtained from the American Type Culture Collection (Manassas, VA). THP-1 cells were maintained in RPMI medium containing 10%

FBS (heat inactivated at 56 °C for 30 min) and 1% penicillin/streptomycin under normal conditions (37 °C, 5% CO<sub>2</sub> under humidified atmosphere) in suspension flasks at concentrations between 1–10×10<sup>5</sup> cells/mL (monitored by hemocytometer cell counts) and passaged by dilution after reaching 10×10<sup>5</sup> cells/mL. GFP-expressing THP-1 cells (THP-GFP) were obtained through retroviral transfection of enhanced GFP cloned into pMSCVpuro (Invitrogen), as described previously [8], and were cultured as above. Human BMSC (hBMSC) (35 years, male; isolation previously described [25]; with informed consent and approval from the institutional health research ethics board) were maintained in αMEM with 1X non-essential amino acids, 10% FBS (heat inactivated at 56 °C for 30 min) and 1% penicillin/streptomycin under normal conditions (37 °C, 5% CO<sub>2</sub> under humidified atmosphere). The hBMSC were sub-cultured weekly at confluency (after trypsinization) by one-quarter dilution and used in the described experiments between the passages 3 and 7.

### 2.3. AML patient cell harvest and culture

Peripheral blood (PB) or bone marrow (BM) samples were obtained from AML patients with active disease at diagnosis at the University of Alberta Hospital. Patient characteristics are shown in Table 1. Written informed consent was obtained from patients according to the declaration of Helsinki. The project outlines and consent procedures were submitted and approved by the Ethic Committee of the University of Alberta Hospital (#Pro00043783). All specimens were collected prior to treatment. PB was collected in heparinized vacutainer tubes and BM aspirates were collected in heparinized syringes. Mononuclear cell (MNC) fractions were obtained by density gradient centrifugation using Ficoll-Paque™ PREMIUM. Briefly, the samples were diluted 2 to 4 times with PBS with 2% FBS and gently layered onto the Ficoll-Paque™ PREMIUM solution and then centrifuged for 40 min at 400 g at room temperature. The MNCs were carefully aspirated from the Ficoll-plasma interface and washed with PBS/2% FBS at 200 g for 10 min (×2) and resuspended in 50% RPMI/40% FBS/10% DMSO medium for freezing at the Canadian Biosample Repository (University of Alberta). To thaw the cells, MNCs were thawed quickly at 37 °C (water bath) and 125 µL of filtered DNase (1 mg/mL) was added directly to the cells. Cells were then immediately transferred to 10 mL of IMDM medium (20% FBS) and centrifuged at 300 g for 5 min. Supernatant was aspirated and 10 mL of IMDM medium (20% FBS) was added and then transferred to cell culture flask and incubated under normal conditions (37 °C, 5% CO<sub>2</sub> under humidified atmosphere). Trypan blue staining was used to determine cell viability after 24 h of seeding.

### 2.4. Preparation of lipopolymer carriers

The preparation of caprylic acid (CA) and linoleic acid (LA) substituted PEI2 with a range of substitution levels were described elsewhere [8,

26,27]. In summary, CA and LA were substituted onto the previously lyophilized PEI2 polymer by N-acylation of the amines. Caprylic or linoleoyl chloride (varying amounts in 5 mL dichloromethane) was drop-wise added to 100–400 mg of PEI2 in dichloromethane (15 mL; containing 50–200 µL triethylamine) for 24 h at ambient temperature under N<sub>2</sub> producing a range of lipid substitutions, which were dependent on the feed ratio of lipid:polymer. The polymers were then precipitated and washed with excess ethyl ether and dried under vacuum at ambient temperature overnight. The actual substitution ratios were determined by <sup>1</sup>H-NMR in D<sub>2</sub>O (Bruker 300 MHz; Billerica, MA). Here the characteristic proton shifts of lipids (0.8 ppm; –CH<sub>3</sub>) and PEI (2.5–2.8 ppm; NH–CH<sub>2</sub>–CH<sub>2</sub>–NH–) were integrated and normalized to the number of protons in each peak (summarized in Table S1). The numbers of lipid methylenes substituted in each polymer (lipids/PEI2) were calculated by multiplying the level of lipid substitution (from <sup>1</sup>H-NMR) with the number of methylenes in each lipid. Percent lipid substitution of amines (% amine substitution) was calculated by dividing the number of lipid substituted with the number of amines (44 amines/PEI2). Lipopolymer concentrations used in the experiments were determined by dissolving the freeze-dried polymers with RNASE free/DNASE free water and performing a copper (II)/PEI assay on the solutions [28,29]. The specific polymers used for most studies were PEI-CA5.4 (5.4 CA substitution per PEI2) or PEI-LA2.1 (2.1 LA substitution per PEI2) at indicated experiments. Complex characterization was carried out with a zetasizer (Malvern 3000) following the same polymer-siRNA complexation formation steps as described below (Section 2.5) but without use of NaCl in the complex solution, as described in [15].

### 2.5. Lipopolymer/siRNA complex and cytarabine treatments

Lipopolymer complexes of siRNA were formed immediately prior to addition to THP-1 cells. First, the required amount of siRNA (e.g., 0.35 µg to give 50 nM final siRNA concentration in wells) was added to 150 mM NaCl solution in a 1.5 mL microcentrifuge tube. The polymers (dissolved in ddH<sub>2</sub>O) were then added to the siRNA solutions at a 4:1 polymer:siRNA ratio (which corresponds to 31.6:1 N/P), and incubated, for 30 min (at room temperature) before addition, in triplicate, to the cells (15 µL/well containing 0.5 mL medium). In all cases, cells were seeded in the wells the day before the siRNA treatment, transfection was performed in the presence of serum (10% FBS) and the complexes remained in the solution for the duration of the experiment. The concentration of siRNA in the wells was 50 nM, unless otherwise noted. Lipopolymer complexes containing control siRNA were used in all incidences in order to rule out contributions due to any autofluorescence or physical effects caused by complex exposure to the cells. For the cytarabine treatment studies, cytarabine was prepared in HBSS at a stock concentration of 1 mg/mL before each experiment and stored at 4 °C for a maximum of 2 days.

**Table 1**  
Mononuclear cells isolated from untreated AML patients.

Patient	Age (yr.)	Sex	BM / PB	Prognosis Category	Cytogenetics	Mutation NPM1/FLT3-ITD	WBC	Blast %	Markers CD34/ CD38	Viability (%)	Cell Diameter* (a.u)
#1	59	M	BM	Better	–Y	+/-	30	>80	-/+	75/86	9.63
#2	77	M	PB	Poor	Normal	+/+	268	>90	-/dim	56/52	7.87
#3	45	F	BM	Poor	Normal	+/+	10	75	-/dim	48/56	8.42
#4	67	M	BM	Poor	+13,+19,+21	-/-	18	>80	+ /dim	72	7.50
#5	49	F	PB	Poor	del(3q),-7	-/-	107	78	NA/NA	54	11.88

Abbreviations: PB = peripheral blood; BM = bone marrow; NA = not available.

Prognosis category: *Better prognosis*: inv(16), t(16;16), t(8;21), t(15;17); *Normal cytogenetics* with NPM1 mutation or isolated CEBPA mutation, in the absence of FLT3-ITD. *Intermediate prognosis*: Normal cytogenetics, +8, t(9;11); other chromosomal abnormalities. *Poor prognosis*: –5, 5q–, –7, 7q–, 11q23 other than t(9;11), inv(3), t(3;3), t(6;9), t(9;22), complex findings (≥3 clonal chromosomal abnormalities), FLT3-ITD mutated (FLT3/ITD or FLT3/TKD). FAB and WHO classifications were not available. Viability was measured by trypan blue, 24 h after thawing cells. Where two viability values are reported for a single patient, the second values were measured separately during a CXCR4 silencing experiment performed with different sample vials. \* Cell diameters are the averaged values reported by flow cytometry during siRNA uptake studies where standard deviations were ±0.14 (a.u) or less. Diameter readings corresponded with visual size observations.



## 2.6. Detection of CXCR4 silencing

THP-1 cells were seeded in 24-well plates (0.50 mL fresh medium/well) and allowed to acclimatize for 24 h under normal maintenance conditions prior to addition of lipopolymer/siRNA complex solutions as described above. At indicated time points, after complex addition, (see figures), the cells were transferred to microcentrifuge tubes and centrifuged at 1600 rpm (240 g). The supernatant was removed and re-suspended cells were stained with 4  $\mu$ L of PE-labeled mouse anti-human CXCR4 (CD184) or mouse IgG isotype control antibody in 90  $\mu$ L of medium for 45 min at 4 °C. When silencing was performed in the presence of hBMSC, unattached THP-1 cells were first removed to the microcentrifuge tubes. The hBMSC and attached THP-1 cells were then washed with HBSS, the supernatant was added to tubes and the attached cells were then removed with Accutase (100  $\mu$ L/well) and added to the same tubes. Wells were rinsed with HBSS and the cells were centrifuged, stained with the labeled-antibodies at 4 °C, as described above.

After antibody staining, cells were re-suspended in HBSS and fixed with 2.0% formalin (final concentration of 1% formalin) and analyzed by flow cytometry (FL2 channel) with Cell Lab Quanta™ SC (Beckman Coulter). Mean fluorescence values per cell from the FL2 channel was used as a measure of bound antibody (i.e., CXCR4) levels. When GFP positive THP-1 cells were used in the experiments, LSR-Fortessa SORP (BD Biosciences) was used for simultaneous detection of PE antibodies (detection filter Ex/Em of 561 nm/586 nm) and GFP (detection filter Ex/Em of 488 nm/530 nm). GFP positive cells were used to clearly select for THP-1 population when grown in contact with hBMSC. Changes in mean CXCR4 levels (based on specific Ab fluorescence levels) and the CXCR4-positive cell population were calculated as a result of siRNA treatments. The cell population stained with non-specific antibody was used for flow cytometry calibration (i.e., designated as 1% CXCR4-positive population).

## 2.7. Droplet digital PCR for CXCR4 silencing

THP-1 cells were seeded in 6-well plates at 200,000 cells/well, and were reverse transfected with scrambled siRNA, and three different siRNAs (siRNA-1, siRNA-2 and siRNA-3) targeting CXCR4 (25 and 50 nM) at 1:8 siRNA:PEI-CA ratio. TRIzol reagent (Invitrogen, Carlsbad, CA) was employed to isolate total RNA from THP-1 cells after 48 h of siRNA treatment. One microgram of total RNA was converted into cDNA using oligo-dT, random primers and M-MLV reverse transcriptase (Invitrogen) according to manufacturer's instructions. The absolute quantity of CXCR4 mRNA transcripts was detected by ddPCR (QX100, Bio-Rad, Hercules, CA) using 2 ng of each cDNA sample and ddPCR supermix for the specific probes (Bio-Rad). The PrimeTime qPCR assays for CXCR4 (Assay ID, Hs.PT.58.22298491) and a reference gene, ACTB (Assay ID, Hs.PT.56a.19461448.g) were obtained from IDT (Coralville, IA). The ddPCR conditions comprised of an initial denaturation for 10 min at 95 °C followed by 45 cycles of denaturation for 30 s at 94 °C, and annealing and extension for 1 min at 60 °C, and the final extension for 10 min at 98 °C. No template control (NTC) was used as a negative control for ddPCR by omitting template cDNA from the reaction. The results of ddPCR were analyzed using the QuantaSoft Software (Bio-Rad), and the absolute concentration of CXCR4 transcripts determined by ddPCR was divided by the ACTB transcripts and presented as percentage based on untreated cells (taken as 100%).

## 2.8. Cell counts and viabilities after CXCR4 silencing

Relative cell concentrations were determined by counting in a flow cytometer (Cell Lab Quanta™ SC; Beckman Coulter). Samples were prepared by a single centrifugation (unless further processing was necessary as in antibody staining) at 1600 rpm (240 g). The cells were suspended in clear HBSS and fixed by adding formalin for a final concentration of 1% formalin. Cells were added to 96-well plate (200  $\mu$ L) for

automated processing by the flow cytometer. When GFP-positive THP-1 cells were used in contact with hBMSC, cell concentration reported was from the GFP positive cells within the cell population region.

To visualize and detect nucleus fragmentation, Hoechst staining (250 ng/mL) was performed after the cells were fixed with 1% formalin (25 min). Images were taken with a FSX100 Olympus Fluorescent Microscope using both the FITC filter for GFP and the DAPI filter for Hoechst. Composite images were created with ImageJ software (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, <http://imagej.nih.gov/ij/>, 1997–2012.) where GFP positive cells (changed to black on white background) and Hoechst stained nucleuses were combined. GFP-positive THP-1 nucleuses were then visually compared between the study groups.

## 2.9. BMSC adhesion assay

Cell adhesion to hBMSC was measured with both GFP-positive and Dil-stained THP-1 cells. The adherence assay was modified from the referenced procedure [30]. For studies with GFP-positive cells, cells were treated with lipopolymer complexes containing CXCR4 siRNA or control siRNA (50 nM) for 48 h in 24-well plates as described above. Cells were then plated (330  $\mu$ L of medium/well) on a hBMSC monolayer (which were seeded in 96-well plates the day before at 15,000 cells/well). Non-treated cells were also added to wells without hBMSC, for control purposes. Cells were then incubated under normal conditions for 2 h to permit adherence to hBMSC. The 96-well plate was subsequently turned over and incubated for 2 h to allow for non-adhered cells to gravitate away from hBMSC. Supernatants were then collected with a pipette, while the plate remained inverted, and placed in a separate 96-well plate and then processed for flow cytometry (fixed in 300  $\mu$ L of 1% formalin). The adhered THP-1 cells and hBMSC were also trypsinized and processed for flow cytometry (fixed in 300  $\mu$ L of 1% formalin). Cell concentrations of the GFP-positive cells were then determined by flow cytometry, as described above, for THP-1 cells from supernatant and the portion adhered to the hBMSC. The percent adhered cells (%) were calculated as =  $100 - ([\text{cell conc. measured from supernatant}] / [\text{combination of conc. measured from supernatant and adhered cells}] \times 100\%)$ . No treatment adhered cells from hBMSC monolayer wells was  $64.4 \pm 4.2\%$  where as adhered cells from wells without hBMSC was  $1.5 \pm 1.9\%$  (not shown).

When Dil-stained parental cells were used, Dil staining was performed after CXCR4 silencing prior to incubating the cells with the hBMSC. For Dil staining, wells from the same group were combined and cells were re-suspended in medium without serum with 1.25  $\mu$ g/mL of Dil for 30 min in normal growth conditions followed by 2X washing with HBSS and re-suspension in normal medium. The Dil-stained THP-1 cells were then seeded in triplicate onto the hBMSC (330  $\mu$ L medium), allowed to adhere to hBMSC, followed by the plate inversion, as described above. Non-attached cells were collected from the supernatant, when the plate was inverted. Fresh medium (330  $\mu$ L) was added to the adhered THP-1 cells and the hBMSC. Medium only wells were added for base-line fluorescence measurements. Relative cells numbers were then determined by Dil fluorescence by a fluorescence plate reader (Ex/Em of 536 nm/607 nm). After subtracting the base-line fluorescence (medium-only wells) from the readings, the percentage of adhered cells was calculated as described above.

## 2.10. Cell division assay

To determine changes in cell proliferation, THP-1 cells were stained with 0.45  $\mu$ M Cell Tracker™ Green CMFDA (Life Technologies) according to manufacturer's directions. Briefly, cells ( $12 \times 10^5$  cells/mL) were re-suspended in FBS medium without serum and Cell Tracker™ Green CMFDA (10 mM in DMSO) was added for a final concentration of 0.45  $\mu$ M. Cells were then incubated under growth conditions (37 °C, 5% CO<sub>2</sub> under humidified atmosphere) in suspension flask for 30 min.

Then, cells were centrifuged, medium was removed and fresh regular growth medium was added. Cells were then seeded in plates (with the pre-seeded hBMSC) at  $1 \times 10^5$  cells/ml and allowed to acclimatize for 24 h. Lipopolymer complexes containing CXCR4 or SDF-1 siRNA were added in the presence of hBMSC as described above (designated as Day 0). Cells were then fixed (1% formalin) and processed for flow cytometry (as described above) for each subsequent time-point. The mean CMFDA fluorescence of the CMFDA positive population was detected via FL1 channel, as described above. The CMFDA concentration was chosen after testing a range of staining concentrations (0.50–20  $\mu$ M). We determined that cell numbers over the time period of 0–4 days was negatively affected at dye concentrations of 10 and 20  $\mu$ M and proliferation (as seen by change in CMFDA fluorescence of Cell Tracker™ Green) was affected at the 20  $\mu$ M dye concentration (Fig. S1). We also ensured that detectability of fluorescence was achievable for 4 days (not shown).

### 2.11. Lipopolymer/siRNA complex treatment in patient cells

The cells were transfected with polymer-siRNA complexes prepared from control siRNA (C-siRNA), FAM-labeled siRNA (FAM-siRNA) and CXCR4 targeting siRNA (using siRNA-1). To prepare the complexes, the desired volume of siRNA was added to IMDM medium followed by the polymer to give a final volume of 300  $\mu$ L, vortexed and incubated for 30 min at room temperature, after which 100  $\mu$ L of complex solution was added to 48-well plates (in triplicate). The MNCs were then added to each well (300  $\mu$ L/well) at  $0.5\text{--}2 \times 10^6$  cells/mL. The details of final siRNA concentrations and polymer:siRNA weight ratios (typically 75 nM at 4:1, 8:1 and 12:1 ratios) are reported in the figure captions. In all cases, transfection was performed in the presence of serum, (final concentration of 15% FBS taking into account complex solution volume) and the complexes remained in the solution for the duration of the experiment.

To assess cell uptake with FAM-siRNA, cell suspensions were transferred to microcentrifuge tubes after 24 h and centrifuged at 300 g. Supernatant was removed, cells were re-suspended in HBSS, then fixed with 2.0% formalin (final concentration of 1% formalin) and analyzed by flow cytometry with Cell Lab Quanta™ SC (FL1 channel; Beckman Coulter). Flow cytometry laser settings were kept constant for all samples. The gating for FL1+ region was adjusted to 1% for non-treated patient cells. To account for the change in cell surface area between patient samples, the cell-associated mean fluorescence are normalized to cell surface area. Surface areas were calculated from the average diameter reported from the flow cytometer and assuming a spherical geometry ( $= 4\pi r^2$ ).

To assess CXCR4 Silencing, the MNCs were treated with complexes derived from CXCR4-siRNA and control siRNA. After incubation, cell suspensions were transferred to microcentrifuge tubes at indicated time points, and centrifuged at 300 g for 5 min. The supernatant was aspirated, cells were re-suspended, stained with antibodies (4  $\mu$ L of PE-labeled mouse anti-human CXCR4 (CD184) or mouse IgG isotype control antibody) in 90  $\mu$ L of medium for 45 min at 4 °C and then washed twice with HBSS. Finally, cells were re-suspended in HBSS and fixed with 2.0% formalin (final concentration of 1% formalin) and analyzed by flow cytometry (FL2 channel) with Cell Lab Quanta™ SC.

### 2.12. Statistics

All experiments were performed in triplicate with mean result displayed and error bars indicating the standard deviations. Statistical analysis was performed with GraphPad InStat v3.06 (GraphPad Software, San Diego, CA USA). One-way ANOVA with Bonferroni post-test was used to compare groups (unless described otherwise in the figure captions). Statistical significant difference when comparing to NT is indicated by +/+ +/+ +/+ +/+ and to lipopolymer complexes containing control siRNA is indicated by \*/\*\*/\*\* where +/\* indicates  $p < 0.05$ ,

+ +/\*\*  $p < 0.01$  and + + +/\*\*  $p < 0.001$ . Other details, as required, are described in further detail in relevant figures.

## 3. Results

### 3.1. CXCR4 silencing in THP-1 cells

We first performed siRNA-mediated CXCR4 silencing utilizing the lipopolymers in the well-established AML cell model, THP-1 cells, since they display high level of CXCR4 expression ( $>80\%$ ). We based the polymer:siRNA ratio in the formulations on the previously determined minimum ratio required for complete siRNA binding, which occurs at 1:1 [8], and the ratios that were effective for siRNA delivery and silencing, which were previously shown to be between 2:1 and 12:1 [8]. Further features of siRNA complexes are shown in Fig. S2, where the polymer hydrodynamic diameter ranged between 210 and 467 nm for PEI-CA (utilized in most of the following studies) and between 364 and 1036 nm for PEI-LA (utilized in the primary AML patient sample studies) depending on the polymer:siRNA ratio. The zeta-potential of the complexes was always positive and ranged between 16.0 and 30.5 mV for PEI-CA and between 10.0 and 19.5 mV for PEI-LA, depending on the polymer:siRNA ratio utilized.

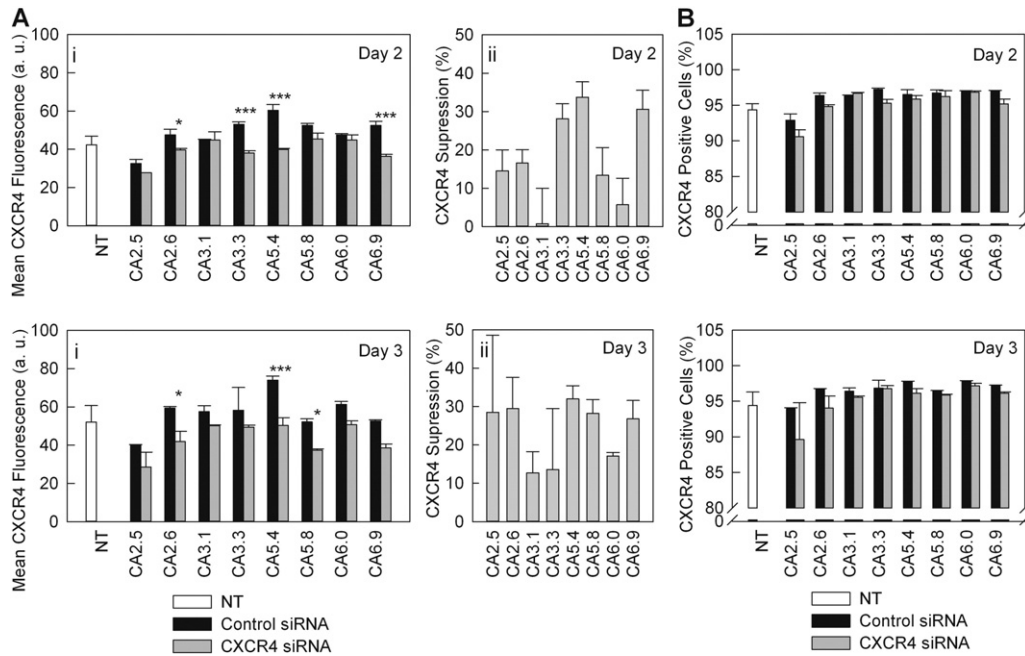
Our previous studies indicated CA-substituted polymers to be the most effective in THP-1 cells [8], so that we first assessed the ability of a PEI2 library, ranging in CA substitutions from 2.5 to 6.9 per PEI2, for down-regulating CXCR4. We investigated a range of CA modification levels to determine if a specific substitution provided an obvious improvement and if there was a correlation between the CA substitution and the silencing ability. With the prepared CA library, the maximal CXCR4 silencing achieved was up to 34% on day 2 and 32% on day 3 with the siRNA concentration of 50 nM (Fig. 1A). However, there was very little decrease in CXCR4 expressing population (Fig. 1B), suggesting that silencing was uniform among the cell population. The PEI2-CA5.4 (i.e., 5.4 CA substitution per PEI2) was chosen for further studies as it demonstrated the most significant and consistent silencing on both day 2 and day 3. The extent of CXCR4 silencing did not correlate with the level of CA substitution (Fig. S3). Perhaps a trend would have been evident if lower siRNA concentration was used, or higher CA substitutions were obtained from the polymer library.

We next investigated the duration of CXCR4 silencing after a single treatment with lipopolymer/CXCR4 siRNA complexes of PEI2-CA5.4 over 5 days (Fig. 2). Based on the mean CXCR4 levels, CXCR4 silencing was achieved from day 1 to day 3 but the silencing was lost by day 5 (Fig. 2A–B). A small decrease in the percentage of CXCR4-positive cells was observed on day 1, but not afterwards (Fig. 2B). Serendipitously, we noted a decrease in the concentration of THP-1 cells ( $\sim 30\%$ ) from day 1 to day 5 as a result of CXCR4 siRNA treatment (Fig. 2C).

The CXCR4 silencing was also investigated at the mRNA level after treatment of THP-1 cells with a control and three different CXCR4 specific siRNAs (Fig. 3). Using siRNA concentrations of 25 and 50 nM, significant reductions in mRNA levels were evident with all CXCR4 specific siRNAs, where the higher concentration of siRNA treatment gave more reduction in CXCR4 mRNA levels.

### 3.2. Effect of CXCR4 silencing on THP-1 cell numbers

In co-culture experiments with hBMSC, we utilized GFP-positive THP-1 cells in order to distinguish THP-1 population from the hBMSC. We therefore verified the ability of our polymers to silence CXCR4 in GFP-positive THP-1 cells as well (Fig. 4). As before, maximal silencing after lipopolymer/siRNA complex treatment was 28.5% on day 1 based on mean CXCR4 levels (Fig. 4Ai), but the duration of silencing was shorter since no silencing was observed by day 3. This was less effective than what we have previously seen in native THP-1 cells. A difference between the two cell types was also evident in the cell growth rates in regular culture passage, where the GFP-positive THP-1 cells appeared

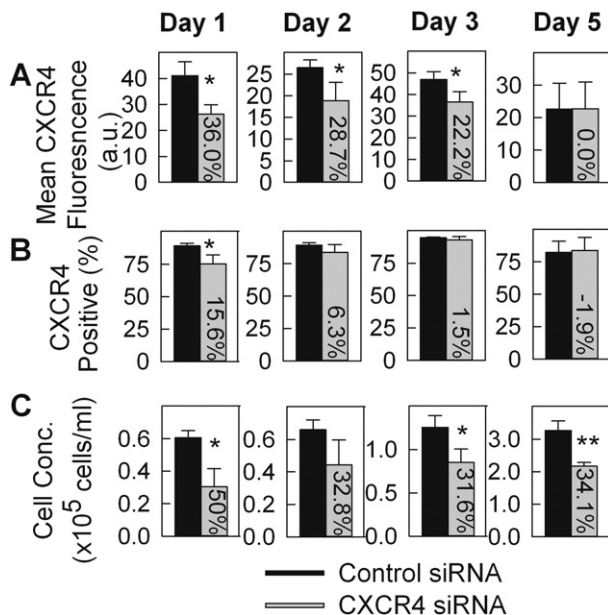


**Fig. 1.** Effect of CA substitution level on PEI2 on CXCR4 siRNA silencing ability of lipopolymer/siRNA complexes. (A) i. Mean CXCR4 levels based on antibody fluorescence (arbitrary units, a.u.) on day 2 and day 3. ii. Relative CXCR4 suppression levels with respect to control siRNA treated cells on day 2 and day 3 after lipopolymer/siRNA complex treatment. (B) CXCR4 positive cell population on Day 2 and Day 3 after lipopolymer/siRNA siRNA treatment. It was possible to obtain up to ~33% CXCR4 silencing (based on mean CXCR4 levels), without significant changes in the percentage of CXCR4-expressing cell population. NT: non-treated cells.

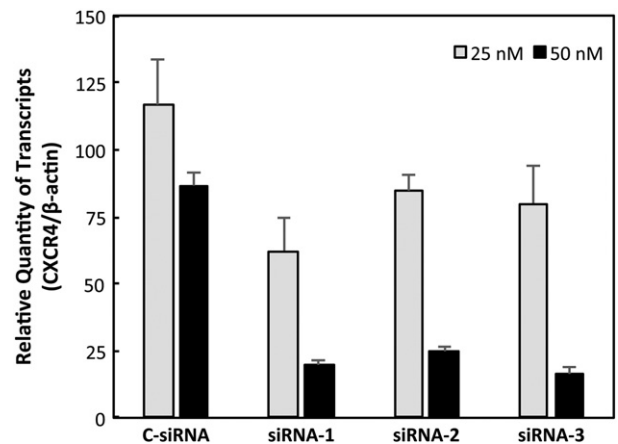
to have a faster proliferation rate than native THP-1 cells (visual observation). It was possible that the silencing effect lasted for a shorter duration as a result of faster proliferation of the cells. We then assessed the CXCR4 silencing in GFP-positive THP-1 cells in the presence of hBMSC. The extent of silencing was similar to the cells treated with CXCR4 siRNA but in the absence of hBMSC (compare Fig. 4Ai and Aii). However, when the GFP-positive THP-1 cells were silenced in the presence of hBMSC, the silencing duration was longer, since we were able to detect silencing up to day 3. Although we normalized each silencing

group with non-treated cells, we noted a significant increase in CXCR4 antibody staining when THP-1 cells were grown with hBMSC (1.7-fold higher on day 1 and 2.0-fold higher on day 2 and 3), suggesting an increase in CXCR4 levels when in contact with hBMSC.

The changes in cell number as a result of CXCR4 silencing with the lipopolymer/CXCR4-siRNA complexes are summarized in Fig. 4B for cells grown in the absence and presence of hBMSC. The toxicity of the C-siRNA was evident on THP-1 cells at 50 nM where CXCR4 silencing did not lead to a specific reduction in cell numbers (i.e., that of beyond control siRNA treatment). A significant effect of CXCR4 silencing however was evident at 25 nM siRNA given the minimal toxicity of control siRNA at this concentration. The lipopolymer complexes with control siRNA did not appear to be toxic on the cells when they are treated in the presence of hBMSC, and a more pronounced reduction in cell

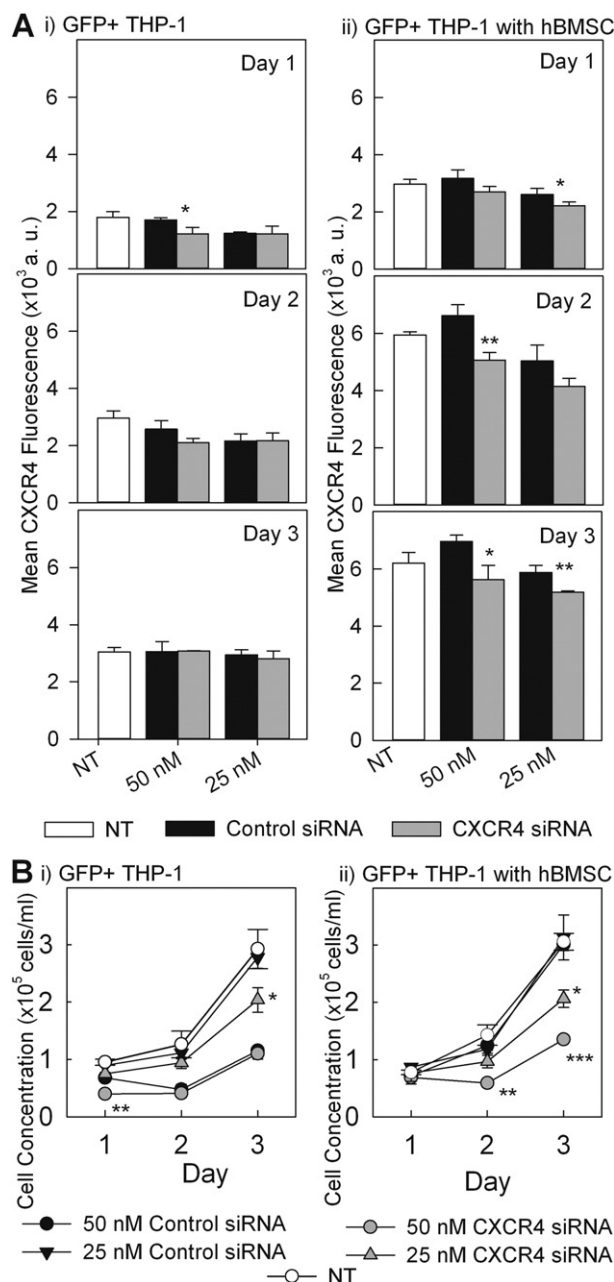


**Fig. 2.** Time course of CXCR4 silencing with lipopolymer/siRNA complex treatment. (A) Change in mean levels of CXCR4 over 5 days. (B) Change in CXCR4-positive cell population over 5 days. (C) Change in cell concentration over 5 days. A two-tailed unpaired t-test was used to determine statistical significance. One time treatment of the cells resulted in CXCR4 silencing for 3 days and ~30% reduction in cell numbers.



**Fig. 3.** PCR analysis of CXCR4 mRNA levels. The mRNA levels in THP-1 cells were investigated 48 h after treatment with control siRNA complexes and with 3 different CXCR4 siRNA complexes (25 and 50 nM). The level of CXCR4 mRNA was normalized with non-treated cells for each siRNA. Note the higher extent of silencing with the higher siRNA concentration.





**Fig. 4.** Lipopolymer/siRNA complex mediated CXCR4 silencing in GFP-positive THP-1 cells without and with co-incubation with hBMSC. The cells were either untreated or treated with lipopolymer complexes containing control or CXCR4 specific siRNA (25 and 50 nM). (A) Mean CXCR4 levels without hBMSC (i) and with hBMSC (ii) co-incubation from day 1 to day 3. (B) Changes in cell concentration from day 1 to day 3 without hBMSC (i) and with hBMSC co-incubation (ii). A two-tailed unpaired t-test was used to determine statistical significance.

numbers were observed after CXCR4 siRNA treatment. The GFP-positive THP-1 cells grown with hBMSC were stained with Hoechst for visualization of nuclear fragmentation as a sign of cellular apoptosis. There was no visual indication of increased apoptosis based on nuclear fragmentation after CXCR4 silencing (Fig. S4).

When comparing the effect of CXCR4 siRNA treatment on CXCR4 surface levels and cell concentration for THP-1 cells incubated with hBMSC (as a stronger response was seen with hBMSC as compared to without the BMSC), the CXCR4 suppression levels remained fairly constant between days 1 and 3, but the cell concentration was decreased over time to a maximum value of ~55% reduction for 50 nM CXCR4-siRNA and ~34% reduction for 25 nM CXCR4-siRNA.

### 3.3. CXCR4 silencing and cytarabine effect in THP-1 cells

We then investigated the effect of CXCR4 silencing with the siRNA complexes for 2, 3 and 4 days (50 nM) on subsequent cytarabine treatment. The effect of cytarabine on THP-1 cells (in the absence of siRNA addition) was the same whether the cells were cultured with or without hBMSC (Fig. 5A). When silencing CXCR4 in the absence of hBMSC (Fig. 5B), a significant decrease in cell concentration (toxicity) was evident with control siRNA treatment on days 2, 3 and 4 in the absence of cytarabine. Increasing concentrations of cytarabine further reduced the cell concentration as expected. With CXCR4-siRNA treatment, further decrease in cell concentrations was evident on day 2, but not on day 3 and day 4. In the presence of hBMSC, CXCR4 silencing again demonstrated a more robust reduction in cell numbers (Fig. 5C), partly due to reduced toxicity of the lipopolymer complexes containing control siRNA, which better revealed the specific effect of CXCR4 siRNA. When cytarabine was added after CXCR4 silencing, we observed a further decrease in cell concentration for all days and all cytarabine concentrations (except 5 µg/mL on day 2). The CXCR4 silencing sensitized the cells regardless of cytarabine concentrations (0.5–5 µg/mL) with a further 30–70% decrease in cell numbers compared to control siRNA treatment.

### 3.4. CXCR4 silencing and adhesive properties of THP-1 cells

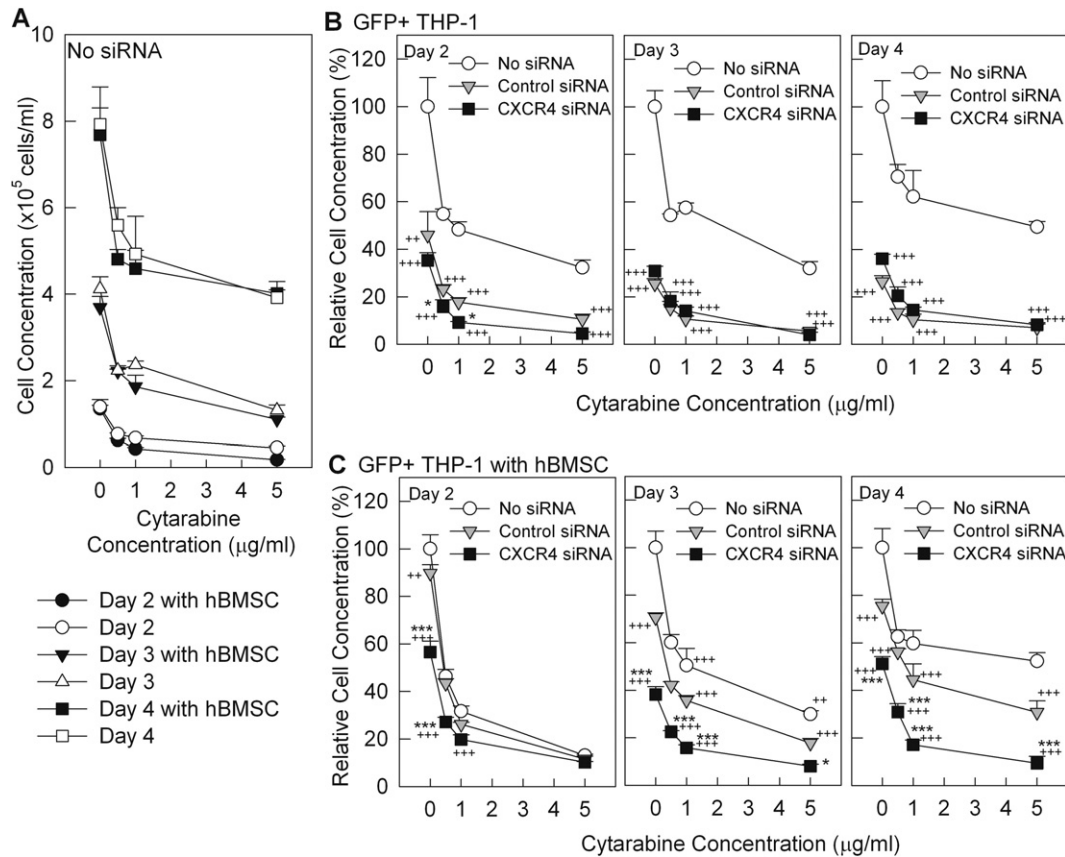
As CXCR4 binding to hBMSC via SDF-1 secretion is one mechanism of adhesion to hBMSC, we investigated the effect of CXCR4 silencing on the adhesion ability of THP-1 cells to hBMSC monolayers (Fig. 6). We found a slight but significant decrease in cell adhesion after CXCR4 silencing with both GFP-positive THP-1 cells (10.6% vs. control siRNA) (Fig. 6A) as well as Dil-stained THP-1 cells (13.7% vs. control siRNA) (Fig. 6B), as compared to control siRNA treated and non-treated cells.

### 3.5. Effect of SDF-1 silencing on THP-1 cells

We next determined the effect of silencing CXCR4 ligand SDF-1 (CXCL12) in conjunction with CXCR4 silencing with siRNAs targeting SDF-1 or CXCR4, (Fig. 7). Although SDF-1 is secreted by hBMSC, other cells including THP-1 cells were also shown to produce it [31,32]. If CXCR4 requires interaction with SDF-1 for increased proliferation, then this interaction could still occur without hBMSC. In the absence of hBMSC (Fig. 7A), silencing SDF-1 by itself appeared to give a similar decrease in cell concentration to that of silencing CXCR4 alone. There was no enhanced effect when cells were co-treated with SDF-1 and CXCR4 siRNAs at the same time, which suggested that the silencing effect observed on cell numbers was the result of inhibiting the same pathway. The results were similar when CXCR4 siRNA treatment was performed in the presence of hBMSC (Fig. 7B). It was possible that hBMSC produced SDF-1 was also decreased when silencing was performed in the presence of hBMSC, but this was not verified in this experiment. Again, as previously seen, the toxicity of the non-targeting control siRNA containing complexes was lower when they were grown with hBMSC, so that the effect of CXCR4 and SDF-1 silencing was more clearly revealed in the co-culture experiment.

### 3.6. Effect of silencing CXCR4 and SDF-1 on cell division

To investigate the mechanism behind the effect of CXCR4 silencing on decreased THP-1 numbers, we assessed cell proliferation by using an established dye-dilution assay with Cell Tracker™ Green CMFDA [33,34]. After Cell Tracker™ Green CMFDA diffuses into the cell, it is converted into a cell impermeable form intracellularly, which is then diluted through cell division onto daughter cells [34]. The THP-1 cells were initially labeled with an optimal concentration of Cell Tracker™ Green CMFDA prior to lipopolymer/siRNA complex treatments (Fig. S1). After silencing cells with the CXCR4 or SDF-1 siRNA with co-



**Fig. 5.** Effect of cytarabine treatment on GFP-positive THP-1 cell concentration after lipopolymer/siRNA complex mediated CXCR4 silencing. (A) Effect of cytarabine treatment on GFP-positive THP-1 cells concentration with and without hBMSC incubation. (B) Effect of cytarabine treatment after CXCR4 silencing without hBMSC. (C) Effect of cytarabine treatment after CXCR4 silencing with hBMSC co-incubation. Concentrations include both attached and unattached GFP-positive THP-1 cells. The lipopolymer/siRNA complex mediated treatment was performed at 50 nM for 48, 72 and 96 h with cytarabine treatment (at different concentrations) for the last 24 h of siRNA treatment. The resultant cell concentrations were expressed with respect to untreated cells (i.e., cells that received no siRNA or cytarabine).

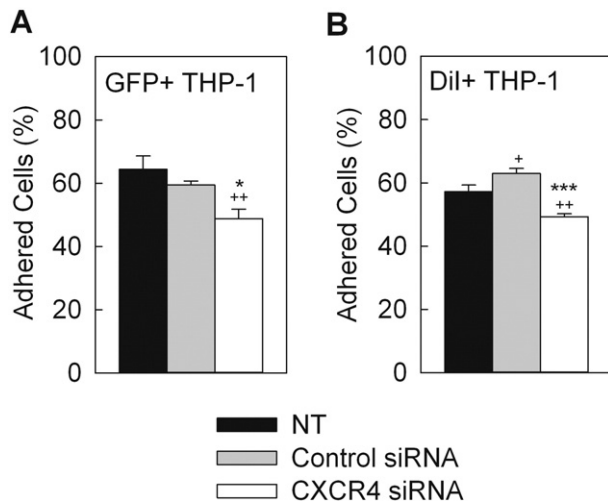
incubation with hBMSC, we observed a decline in fluorescence, providing a measure of cell division and subsequent CMFDA dilution (Fig. 8A). The CMFDA dilution was less with THP-1 cells treated with 25 nM and 50 nM SDF-1 siRNA and 25 nM CXCR4 siRNA, indicating a decrease in proliferation after silencing these targets (Fig. 8A). The decreased

proliferation rate of CXCR4 and SDF-1 silenced cells corresponded to the slower cell growth rates from direct cell counts (Fig. 8B). The SDF-1 siRNA provided more significant effects in this experiment with a marked decrease in proliferation rates measured by CMFDA and cell concentrations.

### 3.7. siRNA delivery to primary AML MNCs

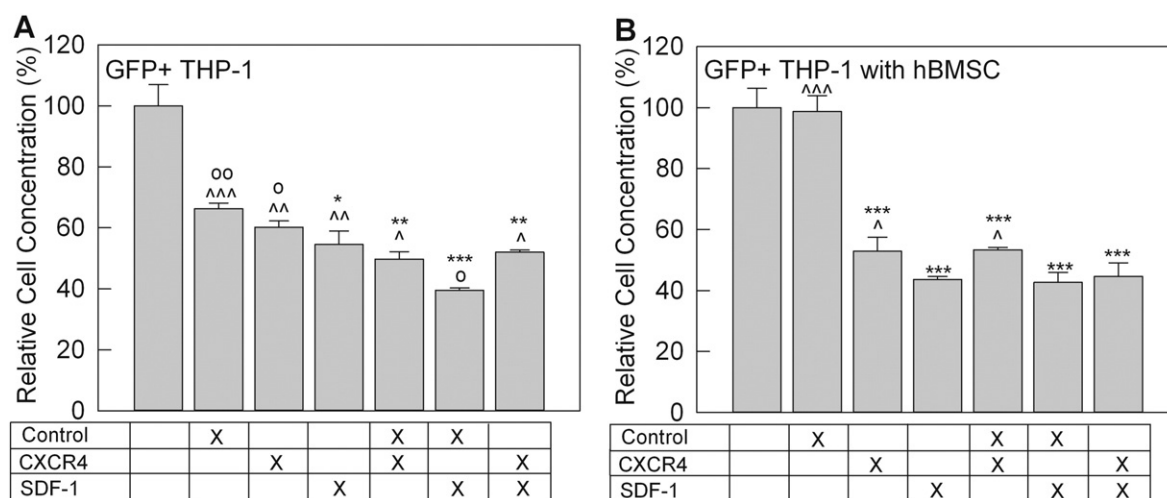
To explore siRNA delivery beyond the THP-1 cell model, we evaluated polymer-mediated FAM-siRNA delivery to primary MNCs from AML patients. Untreated AML samples ( $n = 5$ ; Table 1) were selected based on high blast percentage, ranging from 75% to >90%, ensuring that the majority of cell population was in fact leukemic cells. The patient samples vary by age (45–77 years), cytogenetics, mutations, white blood cell (WBC) counts and location of harvest (BP or BM). All AML patients were determined to have a poor prognosis based on their cytogenetics and mutations except Patient #1 (prognosis was determined as described in Table 1). The cell viabilities (measured after 24 h of thawing) ranged between 48 and 75%, which are low but typical for AML patient cell recovery after freeze-thaw. The siRNA delivery was investigated by the CA-substituted polymer (PEI2-CA5.4) used in THP-1 cells, as well as a LA-substituted polymer (PEI2-LA2.1) that was found effective in other cell types [15,26]. The latter polymer was also effective in silencing CXCR4 in THP-1 cells (Fig. S5).

The results are summarized in Fig. 9, where (i to v) indicate siRNA delivery to individual patient samples and (vi) the average for all patients. Based on the mean fluorescence (Fig. 9A), the delivery was higher with increased polymer:siRNA ratio, as expected. Generally, PEI2-LA2.1 provided higher delivery as compared to PEI2-CA5.4, except



**Fig. 6.** Effect of lipopolymer/siRNA complex mediated CXCR4 silencing on BMSC attachment. Cell attachment was assessed by (A) GFP-positive THP-1 cells and (B) Dil-stained THP-1 cells. The CXCR4 silencing causes a decrease in THP-1 hBMSC attachment as demonstrated by both methods.





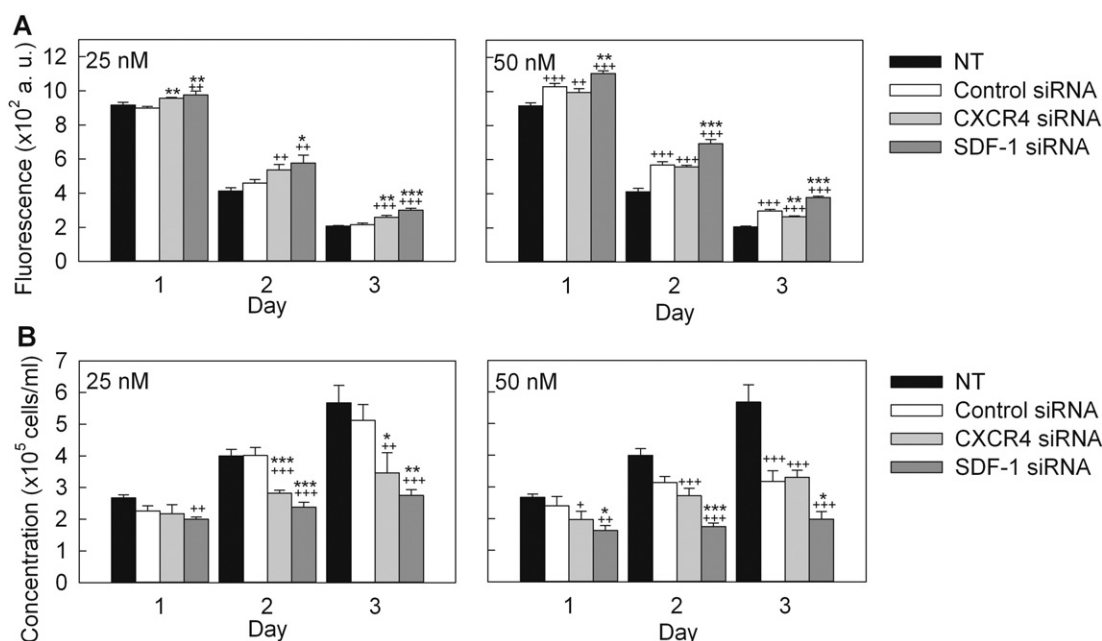
**Fig. 7.** Effect of SDF-1 silencing in conjunction with CXCR4 silencing with lipopolymer/siRNA complexes. Silencing performed with (A) GFP-positive THP-1 cells without hBMSC, and (B) GFP-positive THP-1 cells with hBMSC co-incubation. In all treatments, the total siRNA concentration was 50 nM siRNA. \*\*\* compares against control siRNA, ^^ compares against control + SDF-1 siRNA, 'o' compares against control + CXCR4 siRNA. While CXCR4 and SDF-1 silencing was separately effective in reducing cell numbers, silencing both CXCR4 and SDF-1 simultaneously did not enhance the observed decrease in cell concentration.

with one sample (Patient #2) where the reverse occurred. Much higher mean fluorescence levels with Patient #5 were evident with PEI2-LA2.1. Upon normalizing cell-associated fluorescence with average cell surface area (Fig. 9B), the mean fluorescence became more comparable among patients, especially evident when considering Patient #5. Normalization with cell volume instead of cell surface area provided similar results (not shown). The percentages of siRNA delivery among the cell population are summarized in Fig. 9C. The percent delivery remained more consistent regardless of MNC size. LA-substituted polymer gave a delivery percentage that ranged from 37.6% for the polymer:siRNA ratio of 4:1, to 55.5% for the ratio of 8:1 and to 64.2% for the ratio of 12:1. PEI2-CA5.4 delivery percentage was significantly lower with 20.6% for ratio of 4:1, 34.7% for ratio of 8:1 and 44.2% for ratio of 12:1. In Patient #2, where PEI2-CA5.4 had demonstrated better delivery, the percent delivery between the two polymers was more comparable.

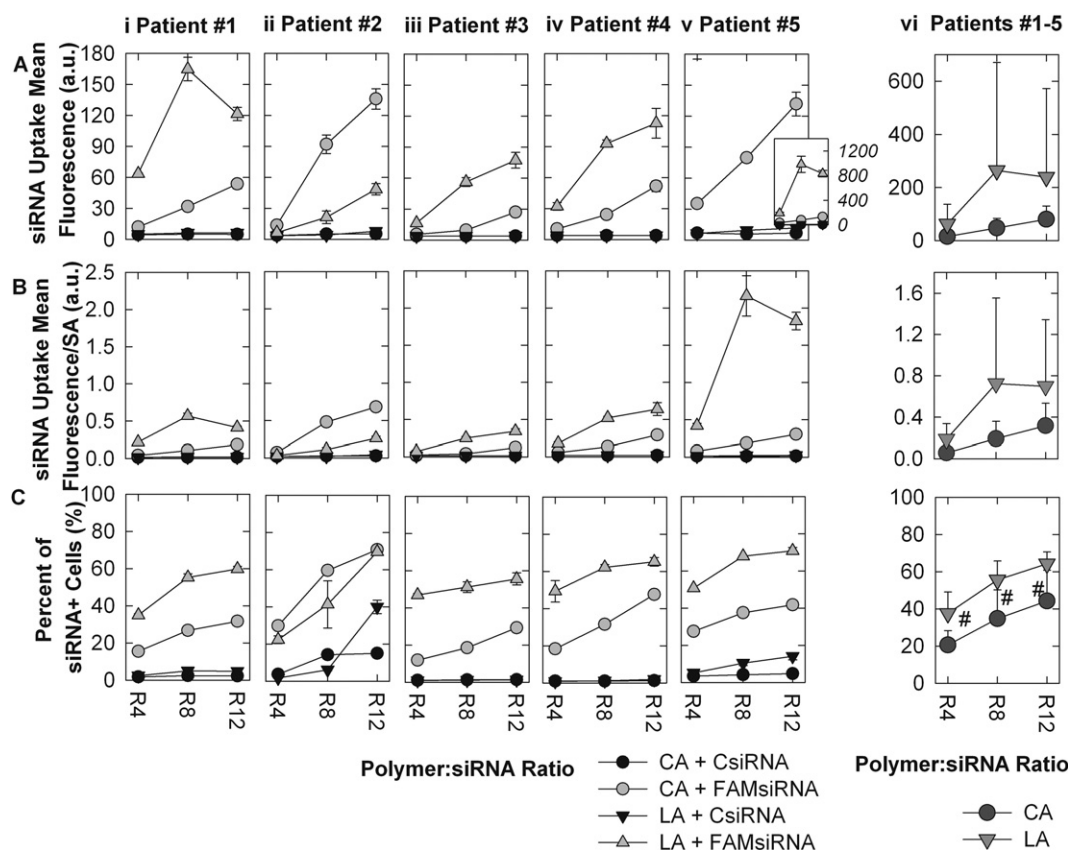
Interestingly, Patient #5 sample, with significantly larger cell size, displayed the most significant siRNA delivery.

### 3.8. CXCR4 siRNA silencing in primary MNCs

CXCR4 silencing was attempted in the same five patient samples. A single polymer:siRNA ratio of 8 was utilized due to limited cell numbers. In Patients #1 and #5, clear CXCR4 silencing was apparent with the use of CXCR4-specific siRNA (siRNA-1); Patient #1 demonstrated silencing with both polymers but only PEI2-CA5.4 demonstrated effective silencing in Patient #5, which was evident in both mean CXCR4 levels (Fig. 10i) as well as the percentage of CXCR4-positive cell population (Fig. 10ii). A decrease of up to 30.7% in cell numbers was also observed in Patient #5 as compared to control siRNA treated group (which was equivalent to no treatment group), but not in Patient #1 (Fig. 10iii). It was interesting to



**Fig. 8.** Effect of lipopolymer/siRNA complex mediated CXCR4 and SDF-1 silencing on cell proliferation with co-incubation of hBMSC. The cells were either untreated (NT) or treated with control, SDF-1 or CXCR4 specific siRNAs at 25 and 50 nM concentration. (A) Cell proliferation as measured by the loss of CMFDA dye through cell division. The results are summarized as the mean ( $\pm$  SD) cell-associated fluorescence for 3 days following siRNA treatment. (B) Changes in cell concentrations as a result of the siRNA treatment. A reduced proliferation was evident by increased intracellular fluorescence levels in A and decreased cell numbers in B.



**Fig. 9.** siRNA deliver to AML patient mononuclear cells. siRNA delivery in AML patient mononuclear cells on day 1. siRNA delivery is presented as (A) mean FAM-siRNA levels, (B) mean FAM-siRNA levels normalized to cell surface area (SA) (to provide better comparison between patient samples), and (C) percentage of FAM-siRNA positive cells. Delivery results are shown for individual patients or as an average siRNA uptake in patient samples ( $n = 5$ ). R4, R8 and R12 indicate the polymer:siRNA weight ratios used in complex formulations. siRNA concentration was 75 nM. A two-tailed unpaired t-test was used to determine statistical significance. Both CA and LA provided significant siRNA delivery in all AML cells. LA was able to deliver to a higher percentage of the AML cells than CA. Significant difference in siRNA delivery between CA and LA polymer is indicated by '#' ( $p < 0.05$ ). CA refers to PEI2-CA5.4 and LA refers to polymer PEI2-LA2.1.

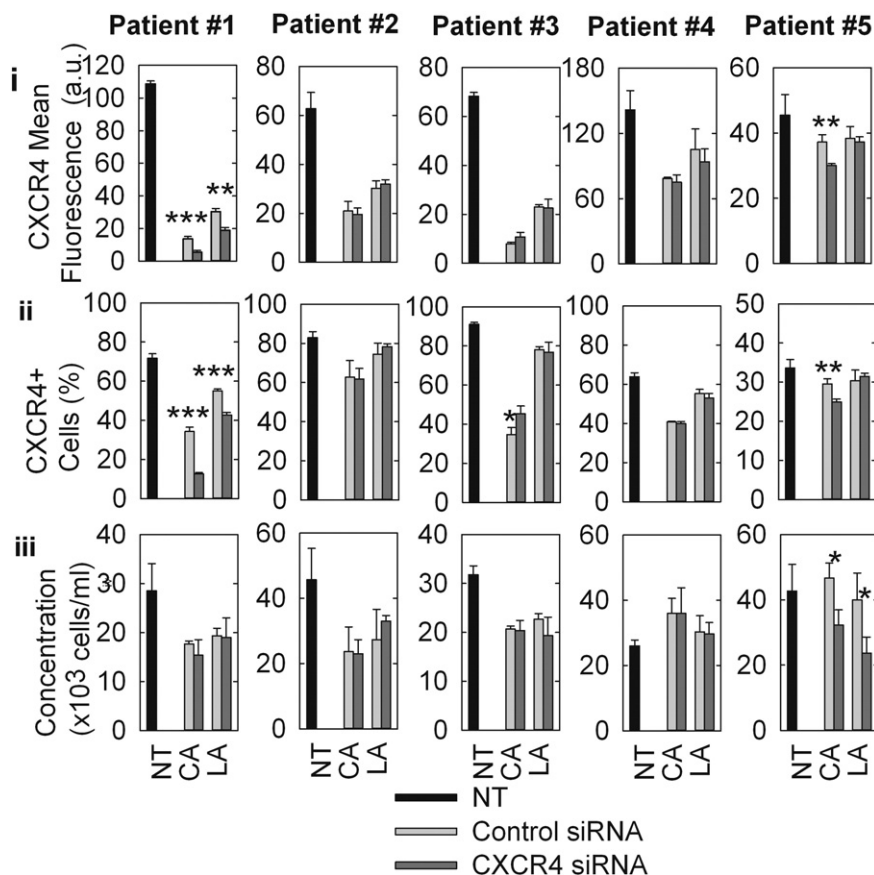
note that the CXCR4 expression levels in untreated cells were higher than polymer complex treated cells (utilizing control siRNA) to varying degrees. Overall, a varying range of responses (decrease in CXCR4 levels and cell concentrations) was observed depending on the specific patient samples.

#### 4. Discussion

Initial studies on the lipopolymers utilized in this paper focused on physicochemical features of polymers and lipopolymer/siRNA complexes that were critical for intracellular delivery and silencing a model target (GFP) in AML cells [8,15]. Lipopolymers are one class of carrier among other types of carriers (e.g., cell-penetrating cationic peptides, cationic lipids and liposomes) tested in leukemic cells [35]. In this paper, we focus on demonstrating the therapeutic potential of most promising lipopolymers in siRNA-mediated silencing of the CXCR4 receptor and its ligand SDF-1 (CXCL12) in AML. We demonstrated successful reduction of CXCR4 protein levels (by immunostaining) when siRNA delivery was undertaken to THP-1 cells with the polymer PEI2-CA. The suppression of CXCR4 resulted in a decrease in cell numbers, in part, due to a decrease in proliferation as demonstrated by the dye dilution method. The SDF-1 is predominantly expressed by BMSC and binds to AML cells through the CXCR4 receptor, however it is also expressed and released by AML cells and has been implicated in many roles besides chemotaxis [31,32]. We also demonstrated a decrease in cell number as result of SDF-1 siRNA mediated silencing with the lipopolymer/siRNA complexes. Kim et al. have recently investigated the effect of silencing SDF-1 by siRNA with the commercial HiPerFect

reagent in AML cells where suppressing SDF-1 resulted in decreased proliferation and decreased SDF-1 related signaling. All together their findings indicated a stimulatory (autocrine) role of SDF-1 in AML cells to enhance cellular proliferation [32]. We observed no enhanced effect when simultaneously silencing CXCR4 and SDF-1, suggesting that the proliferative effect is a result of the same pathway. Kim et al. similarly suggested that proliferative effects of CXCR4 and SDF-1 are a result of the same pathway with their observation that upregulation of cytoplasmic CXCR4 was observed as a result of SDF-1 silencing [32].

Besides CXCR4, other adhesion proteins have also been found to result in decreased proliferation when suppressed with RNAi. In a shRNA screen *in vivo*, integrin-beta-3, (ITGB3) was found to decrease homing and BMSC adhesion of leukemic cells, as well cause decrease proliferation and differentiation of MLL-AF9 oncogene transduced granulocyte-monocyte progenitor cells (transplantable MLL-AF9 AML model) [36]. Furthermore, suppression of integrin-alpha-V (ITGAV), which forms a dimer with ITGB3, and ITGAV pathway members Syk, Vav1, Rac2, RhoA and CD47 showed similar results [36]. Similarly, shRNA suppression or antibody treatment for integrin-alpha-6 (ITGA6), as well as ecotropic viral integration site-1 (EVI1) and integrin-beta-4 (ITGB4), have been found to decrease leukemic cell adhesion to BMSC environment and survival ability as well as to increase chemosensitivity in EVI1-high expressed AML [37]. siRNA suppression of insulin-like growth factor-binding protein 7 (IGFBP7), known as a tumor suppressor in solid tumors, was similarly found to decrease endothelial cell adhesion, migration, invasion as well as proliferation in U937 AML cells [38]. The involvement of proteins in both adhesion to bone marrow microenvironment as well as leukemia cell survival/proliferation was also



**Fig. 10.** siRNA mediated CXCR4 silencing in AML patient mononuclear cells. Effect of CXCR4 silencing on day 2 is indicated based on (i) mean CXCR4 antibody fluorescence levels, (ii) the CXCR4 positive cell population and (iii) the cell concentrations. A two-tailed unpaired t-test was used to determine statistical significance. R8 (ratio 8) with 75 nM siRNA was utilized for siRNA delivery. CA refers to PEI2-CA5.4 and LA refers to polymer PEI2-LA2.1. CXCR4 silencing was achieved in two out of the five AML patient samples.

observed in leukemic stem cell population (CD34+/CD38–), where suppression of CD82 (by siRNA and shRNA) was found to decrease adhesion as well as cell survival [39].

Determining potential targets in order to re-sensitize AML cells to cancer drugs is an important strategy for improved drug therapy. Previous siRNA screens to determine siRNA targets that sensitize cells to cytarabine included cell cycle check-point and DNA-damage and repair proteins [40,41]. Other effective RNAi targets for re-sensitization to various AML treatments include anti-apoptosis proteins (Bcl-210 [42], Bcl-2 [43,44], Mcl-1 [45–48], C-FLIP<sub>L</sub> [49]), epigenetic modifiers (LSD1 [50], HDACs 1 and 6 [51]), a protein involved in autophagy (S100A8 [52]), a molecular chaperone protein (NPM1 [53]), MEK/ERK signaling pathway proteins (MEK-1 [54], 4E-BP1 [48]), the oncogene Cot1 [55] and the kinases (Mnk1 and 2 [56]). The siRNA mediated CXCR4 suppression primed AML cells for cytarabine mediated cell death in the presence of hBMSC. The observed effect could be due to different mechanisms of cytarabine toxicity and anti-survival effect of CXCR4 suppression. Alternatively, CXCR4 pathways could mediate partial chemo-resistance to cytarabine exposure where silencing CXCR4 would then reduce cellular resistance to the drug [57–59]. CXCR4 activation by SDF-1 has been found to contribute to resistance to cytarabine through suppression of the microRNA let-7a, which activates Myc and Bcl-XL [57]. Secretion of unspecified soluble factor(s) from BMSCs, which could possibly include SDF-1, may additionally provide chemo-resistance to cytarabine (observed through decreased apoptosis) by causing decreased activity of drug transporters such as equilibrative nucleoside transporter 1 (ENT1) [58]. We did not however observe an increased resistance to cytarabine when AML cells were grown with hBMSC, suggesting that other BMSC secreted factor(s) might not be significant in our culture system.

The decrease in THP-1 cell numbers due to CXCR4/SDF-1 silencing was observed both in the presence and absence of hBMSC. The toxicity of the complexes was evident in the *in vitro* experiments with AML THP-1 cells in the absence of hBMSC. However the observed toxicity was not minimized by experimental strategies, such as removing the complexes after a short incubation period. While we generally attribute the observed toxicities to polymer/nanoparticle aspects of the delivery system, it is possible that the DICER-substrate siRNA used in this study might have also contributed to this by depleting the endogenous RNAi pathway components. *In vivo* studies are not expected to display as drastic of a toxicity response due to shorter exposure time and eventual clearance of the complexes. Co-incubation of cells with the hBMSC revealed more dramatic results of CXCR4 silencing. This was partly due to a decreased toxicity of the polymeric carrier system (i.e., control siRNA complexed with PEI2-CA) when THP-1 cells were treated in the presence of hBMSC, suggesting a protective role of the hBMSC on the THP-1 cells. The decrease in toxicity did not however negatively affect silencing of the CXCR4 or its anti-survival response. It was conceivable that some of the siRNA complexes could be consumed by the hBMSC and THP-1 cells could be exposed to lower dose of siRNA in this way. Although this was not directly determined, no impediment was seen in the functional response to CXCR4 siRNA treatment. As noted above, BMSC environment has been previously reported to provide protection against the drugs' toxic effects. This seems to be true for the cytotoxic effects of our PEI2-CA carrier system as well, but not for the specific effects of siRNA-mediated silencing.

The CXCR4 silencing demonstrated a significant although nondramatic decrease in THP-1 cell attachment to hBMSC. We also did not achieve full silencing with CXCR4 (only ~30% decrease, based on cell surface immunolabeling) so that the remaining CXCR4 could mediate



the observed binding to hBMSC. Additionally, CXCR4 expression levels range among AML cells (both in AML cell lines and primary cells) and THP-1 cells are among the high expressing cell types having >80% CXCR4-positive cell population. THP-1 cells do have other adhesion molecules mediating their adhesion to BMSC besides CXCR4, such as the CXCR7 and CD44, [31,60,61] which were not targeted in this study. Therefore, in order to completely prevent adhesion and dislodge leukemic cells from the protective bone marrow, multiple adhesion proteins may need to be targeted. Although displacing leukemic cell from the bone marrow environment is one of the main purposes of targeting CXCR4, inhibition of adhesion may not be required for disruption of activating signaling through the CXCR4 pathway and resulting survival pathways [18].

We additionally explored the feasibility of siRNA-mediated CXCR4 silencing in MNCs derived from AML patients that displayed a high blast percentage. *In vitro* CXCR4 silencing with novel carriers have been performed in AML cell lines but not in primary AML cells. In a SDF-1 siRNA target study (with a commercial reagent, HiPerFect), AML patient cells were tested for SDF-1 expression, but no attempts to silence SDF-1 in patient cells were made and cell lines were utilized instead for silencing experiments [32]. A modified siRNA, for TLR9 targeted delivery, was delivered in AML and multiple myeloma (MM) patient cells where most TLR9+ positive cells displayed uptake of FITC-siRNA, however again the silencing studies were limited to cell lines both in *in vitro* and in a xenograft model with these cell lines [62]. The fact that AML patient cells were used in some studies related to siRNA delivery, but not for silencing or for measuring siRNA-mediated therapeutic effects, suggests limitations of siRNA-mediated delivery and/or silencing with AML patient cells *in vitro* (i.e., it is likely that the outcomes were not successful and were not reported). AML patient cells are well known for difficulty for cultivating *in vitro*, requiring careful thawing process (if cryopreserved), use of deoxyribonuclease (DNase) and/or filtering to prevent clumping (as result of DNA released from dying cells), resulting in lower cell viabilities and usually restricting the studies to short-term culture (<1 week). We had previously demonstrated CD44 silencing in CD34+ AML patient samples (n = 3) [63] and to our knowledge this was the first time siRNA mediated silencing was demonstrated in AML primary cells *in vitro* with a non-viral polymer carrier. We now further show that effective siRNA delivery was achieved in all 5 patients tested with lipopolymers, but CXCR4 silencing was possible in only 2 out of 5 patient cells. It will be important to clarify the reasons behind the varying responses of siRNA-complexes (control siRNA) on the CXCR4 levels when compared to untreated patient samples. This could be a result of the non-specific effects of non-targeting control siRNA previously observed [64] or due to compounding effects of the lower viability of the patient samples and the added toxicity of the siRNA/polymer complexes. Although further improvements in silencing efficiency are desired, successful silencing of CXCR4 with lipopolymer/siRNA complexes in AML patient cells provide another step towards clinical AML therapy with the siRNA approach. With the limited pool of patient cells (n = 5), we have already observed variations in siRNA delivery, siRNA-mediated silencing of the chosen (CXCR4) target and anti-leukemic effects (i.e., decrease in cell number). It will be important to fully characterize such variations in larger studies with greater number of patient samples. Such studies will further include mRNA changes for targeted proteins, since the limited sample size did not allow us to characterize this response in this study.

The complexes effectiveness within *in vivo* systemic leukemic models remains to be determined. Previous work has shown successful silencing after intratumoral, subcutaneous (close to tumor site) and intraperitoneal injections of lipopolymer complexes in breast cancer cells with no signs of nephrotoxicity or hepatotoxicity [65,66]. Future work will need to demonstrate silencing, after systemic administration of the siRNA complexes, within the peripheral blood, spleen and bone marrow as well as biodistribution and pharmacokinetics of the complexes.

It is foreseeable that other cell types will experience CXCR4 silencing with systemic siRNA delivery and that they will respond to CXCR4 silencing differently. This might be reminiscent of the potential side-effects of classical CXCR4 antagonists, including possible effects on normal hematopoiesis [67,68]. If CXCR4 silencing did mobilize normal hematopoietic cells, they would be more susceptible to toxic effects of any co-treatments with chemotherapy drugs [67]. Disruption of CXCR4/SDF-1 mediated homing and trafficking of non-leukemic cells could negatively affect the immune system and hematopoietic functions, [68] especially in case of long-term or repeat siRNA therapy. Future work should further explore the effects of CXCR4/SDF-1 silencing in other hematopoietic cells as well as bone marrow cells. In some cases, such as the leukemic stem cell population, silencing CXCR4 would likely remain beneficial. To overcome off-target effects and improve efficacy of silencing, targeting to leukemic cells may need to be employed. Although very little work has been performed with leukemic cell-targeted siRNA nanoparticles, there are numerous potential ligand targets with varying degrees of specificity; from more general ligand targets, such as transferrin and folate, to more leukemic specific ligand targets (such as CD33 and CD34), to even more highly specific leukemic stem cell targets (such as CD32, CD44, CD47, CD96 and CLL1) [35]. An even more tailored approach could be applied where the nanoparticles target the same protein (surface located) as the siRNA it is carrying is meant to silence (a potential example being CXCR4) [35].

## 5. Conclusions

Development of siRNA carriers and siRNA-mediated silencing as a therapy in leukemia, and specifically in AML, has not been explored in detail at the present time. In contrast to numerous CXCR4/SDF-1 antagonist studies on leukemic cells ranging from *in vitro* to clinical trials, very few studies utilized the siRNA technology as a therapeutic option for leukemia. Despite a lesser degree of focus in leukemia, siRNA therapy has been progressing into clinical trials as a cancer therapy [69,70]. Additionally, the benefit of CXCR4 as a target for siRNA therapies has been realized with solid tumors [71–73]. Here, we demonstrated a significant decrease in proliferation of AML model THP-1 cells as a result of silencing CXCR4 expression utilizing lipopolymer/siRNA complexes. This study represents the first polymeric system used specifically for CXCR4 silencing in an AML model. Moreover, two out of five AML patient MNC samples were found to be responsive to siRNA delivery, resulting in significant reduction of CXCR4 levels in these patient cells. While additional studies are warranted to enhance the extent of CXCR4 silencing and better understand why some patient cells did not respond to siRNA therapy, we provide the proof-of-principle that decreasing CXCR4 expression via siRNA could be a promising therapy and an additional option from the antagonists and blocking antibodies already in pursuit for AML therapy.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.jconrel.2015.12.052>.

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